Title: Redox potential as a master variable controlling pathways of metal reduction by *Geobacter sulfurreducens*

Running Title: Fe(III) reduction by *G. sulfurreducens*

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Abstract:

Geobacter sulfurreducens uses at least two different pathways to transport electrons out of the inner membrane quinone pool before reducing acceptors beyond the outer membrane. When growing on electrodes poised at oxidizing potentials, the CbcL-dependent pathway operates at or below redox potentials of -0.10 V vs. the Standard Hydrogen Electrode (SHE), while the ImcH-dependent pathway operates only above this value. Here, we provide evidence that *G. sulfurreducens* also requires different electron transfer proteins for reduction of a wide range of Fe(III)- and Mn(IV)- (oxyhydr)oxides, and must transition from a high- to low-potential pathway during reduction of commonly studied minerals. Freshly precipitated Fe(III)-(oxyhydr)oxides could not be reduced by mutants lacking the high potential pathway. Aging these minerals by autoclaving did not change their powder X-ray diffraction pattern, but restored reduction by mutants lacking the high-potential pathway. Mutants lacking the low-potential, CbcL-dependent pathway had higher growth yields with Fe(III). Together, these data suggest that the ImcH-dependent pathway exists to harvest additional energy when conditions permit, and CbcL allows respiration closer to thermodynamic equilibrium conditions. With evidence of multiple pathways within a single organism, the study of extracellular respiration should consider not only the crystal structure or solubility of a mineral electron acceptor, but rather the redox potential, as this variable determines the energetic reward affecting reduction rates, extents, and final microbial growth yields in the environment.

45 **Introduction:**

46 Fe(III)-(oxyhydr)oxides can exist in at least 15 mineral forms that have variable physiochemical
47 properties and span a wide range of formal oxidation-reduction (redox) midpoint potentials (Majzlan,
48 Navrotsky, & Schwertmann, 2004; Majzlan, 2011, 2012; Nealson & Saffarini, 1994; Schwertmann &
49 Cornell, 2000; Thamdrup, 2000). The electron-accepting potential of any given Fe(III)-(oxyhydr)oxide
50 structure is not a fixed value, and becomes less favorable with increasing crystallinity, particle size, pH,
51 or ambient Fe(II) concentration (Majzlan, 2012; Navrotsky, Mazeina, & Majzlan, 2008; Sander,
52 Hofstetter, & Gorski, 2015; Thamdrup, 2000). Differences in effective redox potential alter the energy
53 available to be captured by bacteria able to couple the oxidation of an electron donor to reduction of
54 these minerals (Thauer, Jungermann, Decker, & Pi, 1977). Because of this structural and environmental
55 diversity, organisms able to reduce metals could sense the energy available in their electron acceptors
56 and utilize different electron transfer pathways, similar to how *E. coli* uses distinct terminal oxidases in
57 response to levels of available oxygen (Green & Paget, 2004; Russell & Cook, 1995).

58 One well-studied dissimilatory metal reducing organism, *Geobacter sulfurreducens*, can grow via
59 reduction of metal (oxyhydr)oxides ranging in predicted midpoint redox potential from +0.35 V vs. the
60 Standard Hydrogen Electrode (SHE)(e.g., birnessite, ca. $Na_xMn_{2-x}(IV)Mn(III)_xO_4$, x ~ 0.4) to -0.17 V vs. SHE
61 (e.g., goethite, α-FeOOH) (Caccavo et al., 1994; Majzlan et al., 2004; Majzlan, 2012; Post & Veblen, 1990;
62 Thamdrup, 2000). Recent work examining electron transfer from *G. sulfurreducens* to poised graphite
63 electrodes demonstrated that this organism uses at least two different inner membrane electron
64 transfer pathways, known as the CbcL- and ImcH-dependent pathways (Levar, et al., 2014; Zacharoff, et
65 al., 2016). The 'low' potential, CbcL-dependent pathway, is required for growth with electrodes at or
66 below potentials of -0.10 V vs. SHE, while the 'high' potential, ImcH-dependent pathway, is essential
67 when electrodes are poised at redox potentials above this value. As mutants lacking each pathway only

68 grow with electrodes poised above or below these thresholds, cells containing only CbcL or ImcH could
69 be used as 'sensors' to report the redox potential of an extracellular electron acceptor.

70 The discovery of multiple inner membrane pathways is based on work with electrodes held at
71 constant potentials, but poses many questions regarding *G. sulfurreducens'* interactions with more
72 complex mineral substrates. Does the organism transition from one pathway to the other as simple
73 environmental factors such as Fe(II):Fe(III) ratios alter redox potentials? Do manipulations known to
74 alter redox potential, such as pH or particle size, also influence the pathway utilized? As this +/- 0.5 V
75 redox potential range represents nearly 50 kJ per mol of electrons transferred, do different electron
76 transfer mechanisms allow higher yield of this microbe?

77 Here, we demonstrate that *G. sulfurreducens* requires both the CbcL- and the ImcH-dependent
78 electron transfer pathways for complete reduction of a variety of Fe and Mn minerals. By using mutants
79 only able to function at specific redox potentials, we show that minerals often used for study of
80 extracellular electron transfer begin as 'high' potential electron acceptors reducible by the ImcH-
81 dependent pathway, but transition during reduction to 'low' potential electron acceptors requiring the
82 CbcL-dependent pathway. Simple variations in mineral handling such as autoclaving, or 0.5 unit pH
83 changes that alter redox potential by 30 mV, can alter the electron transfer pathway required by the
84 organism, further showing that bacteria detect subtle changes in mineral redox potentials. These data
85 highlight the complexity of studying growth with minerals, and suggests that the proteins used for
86 electron flow out of *G. sulfurreducens* are more strongly influenced by redox potential than the crystal
87 structure, aggregation behavior, or even solubility of the terminal acceptor.

88

89 **Materials and methods:**

90 **Bacterial strains and culture conditions:** Strains of *Geobacter sulfurreducens* used in this study
91 are described in Table 1. All strains were routinely cultured from freezer stocks stored at -80 degrees
92 Celsius, and single colony picks from agar plates were used to initiate all experiments. Strains were
93 cultured in NB minimal medium composed of 0.38 g/L KCl, 0.2 g/L NH_4Cl, 0.069 g/L $NaH_2PO_4 \cdot H_2O$, 0.04
94 g/L $CaCl_2 \cdot 2H_2O$, and 0.2 g/L $MgSO_4 \cdot 7H_2O$. 10 ml/L of a chelated mineral mix containing 1.5g/L NTA, 0.1
95 g/L $MnCl_2 \cdot 4H_2O$, 0.3 g/L $FeSO_4 \cdot 7H_2O$, 0.17 g/L $CoCl_2 \cdot 6H2O$, 0.1 g/L $ZnCl_2$, 0.04 g/L $CuSO_4 \cdot 5H2O$, 0.005 g/L
96 $AlK(SO_4)_2 \cdot 12H_2O$, 0.005g/L H_3BO_3, 0.09 g/L Na_2MoO_4, 0.12 g/L $NiCl_2$, 0.02 g/L $NaWO_4 \cdot 2H2O$, and 0.1 g/L
97 Na_2SeO_4 was also added. Fumarate (40 mM) was used as an electron acceptor for growth of initial stocks
98 picked from colonies, and acetate (20mM) was used as the sole electron and carbon source. Unless
99 otherwise noted, the pH of the medium was adjusted to 6.8 and buffered with 2 g/L $NaHCO_3$, purged
100 with N_2:CO_2 gas (80%/20%) passed over a heated copper column to remove trace oxygen, and
101 autoclaved for 20 minutes at 121 degrees Celsius.

102 **Here Table 1**

103 **X-ray diffraction (XRD) measurements:** Approximately 0.2-0.5 g of untreated bulk mineral
104 sample or 2.5 ml of mineral suspension in medium were analyzed by powder XRD. Minerals in basal
105 media were separated from suspension by filtration (0.22 µm) in an anaerobic chamber (Coy Laboratory
106 Products) under a N_2:CO_2:H_2 (75%:20%:5%) atmosphere, and stored in sealed mylar bags at -20 degrees
107 Celsius prior to analysis. Diffractograms were measured from using 20° to 89° (2Θ) range, a step size of
108 0.02 2Θ, and a dwell of 0.5°/minute using a Rigaku MiniFlex ASC-6AM with a Co source. The resulting
109 diffractograms were background subtracted, and phases were identified using Jade v9.1 software (MDI,
110 Inc.).

111 **Preparation of Fe(III)-(oxyhydr)oxides:** "Poorly crystalline FeO(OH)" was produced after Lovley
112 and Phillips, 1986, by adding 25% NaOH dropwise over 90 minutes to a rapidly stirring 0.4 M solution of
113 $FeCl_3$ until the pH was 7.0. The solution was held at pH 7.0 for one hour, and the resulting suspension
114 was washed with one volume of de-ionized and distilled water and used immediately (≤24 after
115 synthesis) for XRD analysis and growth studies as rapid aging of such products to more crystalline
116 minerals (e.g., goethite) has been observed even when kept in the dark at 4 degrees Celsius (Raven, Jain,
117 & Loeppert, 1998). The untreated mineral was identified as akaganeite (β-FeOOH) by powder X-ray
118 diffraction. Washing the mineral sample with up to three volumes of de-ionized and distilled water did
119 not change the XRD pattern of the mineral or the reduction phenotypes observed.

120 Goethite and 2-line ferrihydrite (ca. $Fe_{10}O_{14}(OH)_2$) (Michel et al., 2007) were synthesized after
121 Schwertmann and Cornell, 2000, with some modifications. For goethite, a solution of $FeCl_3$ was
122 precipitated through neutralization with 5 N KOH, and the resulting alkaline mineral suspension was
123 aged at 70 degrees Celsius for 60 hours to facilitate goethite formation. The suspension was then
124 centrifuged, decanted, and washed with de-ionized and distilled water prior to freeze drying. For 2-line
125 ferrihydrite, a solution of $FeCl_3$ was neutralized with KOH until the pH was 7.0. Suspensions were rinsed
126 with de-ionized and distilled water and either freeze dried or centrifuged and suspended in a small
127 volume of de-ionized and distilled water to concentrate the final product. Freeze dried samples yielded
128 XRD patterns indicative of 2-line ferrihydrite, but suspension of hydrated samples in growth medium
129 had an altered XRD pattern more similar to that of akaganeite. Freeze dried samples were stored at -80
130 degrees Celsius prior to addition to growth medium, while hydrated samples were used within 24 hours
131 of synthesis.

132 Schwertmannite ($Fe_8O_8(OH)_6(SO_4)\cdot nH_2O$) was synthesized using a rapid precipitation method
133 involving the addition of a 30% solution of H_2O_2 to a solution of $FeSO_4$ (Regenspurg, Brand, & Peiffer,

134 2004). The mixture was stirred rapidly for at least 12 hours until the pH was stable, at ~2.35. The solids
135 were allowed to settle for 1 hour, and the supernatant carefully decanted. Solids were washed by
136 centrifuging at 3,700 x g and suspending in 1 volume of de-ionized and distilled water. The product was
137 concentrated by a final centrifugation at 3,700 x g for 5 minutes and resuspension in 1/20 the initial
138 volume. XRD identified this initial product as schwertmannite, though subsequent addition to media at
139 neutral pH values and autoclaving rapidly altered the mineral form.

140 **Iron reduction assays:** Basal media were prepared as above with some modifications. Fumarate
141 was omitted as the Fe(III)-(oxyhydr)oxides were desired as the sole electron acceptor. NTA free trace
142 mineral mix (in which all components were first dissolved in a small volume of HCl) was used in order to
143 eliminate exogenous chelating compounds from the media. All media were purged with N_2:CO_2 gas
144 (80%:20%) passed over a heated copper column to remove trace oxygen. Where indicated, media were
145 autoclaved for 20 minutes at 121 degrees Celsius on a gravity cycle, and were immediately removed to
146 cool at room temperature in the dark. As is standard for *G. sulfurreducens* medium containing Fe(III)-
147 (oxyhydr)oxide (Caccavo et al., 1994), additional $NaH_2PO_4 \cdot H_2O$ (to a final concentration of 0.69 g/L) was
148 added prior to autoclaving, as more recalcitrant and less reducible forms of Fe(III)-(oxyhydr)oxide will
149 form when autoclaved without additional phosphate. For freshly precipitated akaganeite and 2-line
150 ferrihydrite, 1 ml of the Fe-(oxyhydr)oxide suspension was added to 9 ml of basal medium. The final pH
151 was adjusted by altering the pH of the basal medium prior to mineral addition and by altering the
152 concentration of $NaHCO_3$ used prior to purging with anaerobic N_2:CO_2 gas (80%/20%). For goethite, an
153 88 g/L suspension was made in de-ionized and distilled water and 1 ml of this suspension was added to 9
154 ml of basal medium. The resulting medium had an effective Fe_{total} concentration of ~20 mM as
155 determined by fully reducing an acidified sample with hydroxylamine and measuring the resulting Fe(II)
156 using a modified Ferrozine assay (Lovley & Phillips, 1987). Because the synthesis of schwertmannite
157 results in an acidic product below the pH at which *G. sulfurreducens* grows optimally (Caccavo et al.,

158 1994; Regenspurg et al., 2004), the mineral suspension was added to the basal medium (1:9) and the pH
159 of the solution was adjusted with NaOH and bicarbonate to produce a final pH of 6.8. For freeze dried 2-
160 line ferrihydrite, 0.2 grams of freeze dried sample was added per 10 ml of basal medium.

161 In all cases, 100 µl of electron acceptor-limited stationary phase cells (OD_{600} = 0.55) grown from
162 single colony picks in NB basal medium containing acetate (20 mM) and fumarate (40 mM) were used to
163 inoculate the Fe(III)-(oxyhydr)oxide containing media, with acetate provided as the electron and carbon
164 donor and the Fe(III)-(oxyhydr)oxide as the sole electron acceptor.

165 For all Fe(III)-(oxyhydr)oxide incubations except those involving goethite, a small sample was
166 removed at regular intervals and dissolved in 0.5 N HCl for at least 24 hours at 23-25 degrees Celsius in
167 the dark before the acid extractable Fe(II) present in the sample was measured. For incubations with
168 goethite, samples were incubated in 1 N HCl at 65 degrees Celsius for at least 24 hours in the dark,
169 followed by centrifugation at 13,000 x g for 10 minutes to remove solids. Fe(II) in the supernatant was
170 then measured.

171 **Manganese reduction assays:** Birnessite ($Na_xMn_{2-x}(IV)Mn(III)_xO_4$, x ~ 0.4) was prepared using the
172 protocol described in Chan, et al, 2015. Briefly, a solution of $KMnO_4$ was added to an equal volume of
173 $MnCl_2 \cdot 4H_2O$ solution and mixed vigorously. Solids were allowed to settle, and the overlaying liquid was
174 decanted off. The resulting solid was washed in de-ionized and distilled water and concentrated by
175 centrifugation. This suspension was added (~20mM) to basal medium containing acetate (10 mM) as the
176 carbon and electron donor. Cells were inoculated to a calculated OD_{600} of 0.005 from electron acceptor-
177 limited stationary phase cells. Mn(II) was measured indirectly as previously described (Chan et al., 2015;
178 Levar et al., 2014). Briefly, samples were acidified in 2 N HCl with 4 mM $FeSO_4$ added and allowed to
179 dissolve overnight at 25 degrees Celsius in the dark. After all solids were dissolved, the Fe(II)

180 concentration was measured. Because the reduction of Mn(IV) by Fe(II) is thermodynamically favorable,
181 the measured Fe(II) concentration can be used to extrapolate the Mn(II) present a given sample.

182 **Results:**

183 The inner membrane multiheme cytochromes ImcH and CbcL are implicated in transfer of
184 electrons out of the quinone pool when electron acceptors fall within different redox potential windows
185 (Levar et al., 2014; Zacharoff et al., 2016). Mutants lacking *imcH* are only able to reduce poised
186 electrodes with 'low' redox potentials (≤ -0.10 V vs. SHE), and are deficient in reduction of electrodes at
187 redox potentials above this value (Levar et al., 2014). In contrast, *cbcL* mutants have the opposite
188 phenotype, reducing electrodes with relatively high redox potentials, and having a deficiency in
189 reduction of acceptors at or below -0.10 V vs. SHE (Zacharoff et al., 2016). These pathways are
190 constitutive (Zacharoff et al., 2016), and use of each pathway appears to be able to switch in a matter of
191 minutes during sweeps of increasing or decreasing potential known as catalytic cyclic voltammetry
192 (Marsili, Rollefson, Baron, Hozalski, & Bond, 2008; Yoho, Popat, & Torres, 2014; Zacharoff et al., 2016).

193 A simple experiment testing if these two pathways could also be essential for reduction of 'low'
194 and 'high' potential metal oxide acceptors is shown in Figure 1. When wild type *G. sulfurreducens* was
195 compared to markerless deletion mutants lacking each pathway, mutants lacking ImcH could not reduce
196 laboratory synthesized birnessite (a ~+0.35 V acceptor), while CbcL mutants could not reduce goethite (a
197 ~-0.17 V acceptor) (Majzlan, 2012; Nealson & Myers, 1992; Orsetti, Laskov, & Haderlein, 2013;
198 Thamdrup, 2000; Thauer et al., 1977) (Figure 1). These observations were consistent with redox
199 potential preferences first described with electrodes poised at +0.24 or -0.10 V vs. SHE, and show the
200 utility of using these strains to report the effective redox potentials of minerals (Chan et al., 2015; Levar
201 et al., 2014; Zacharoff et al., 2016).

202

Here Figure 1

Evidence that the electron transfer pathway changes even in the absence of XRD-pattern changes

Most Fe(III)-(oxyhydr)oxides do not exist at these high or low extremes, but possess electron accepting potentials predicted to lie near the -0.10 V redox potential threshold that requires a transition from ImcH-dependent to the CbcL-dependent reduction pathways in electrode-grown cells (Levar et al., 2014; Majzlan et al., 2004; Majzlan, 2012; Thamdrup, 2000; Zacharoff et al., 2016). While testing this with electrodes is straightforward, the redox potential of Fe(III)-(oxyhydr)oxides in this range is not easy to predict or measure (Sander et al., 2015). Aging (accelerated by autoclaving or freeze drying) can increase particle size and lower redox potential, or drive re-crystallization to lower redox potential forms (Navrotsky et al., 2008; Roden, Urrutia, & Mann, 2000; Roden & Zachara, 1996; Roden, 2006; Thamdrup, 2000). As Fe(III)-(oxyhydr)oxide reduction is a proton consuming reaction (Kostka & Nealson, 1995; Majzlan, 2012; Thamdrup, 2000), for each unit decrease in pH, the redox potential of the Fe(III)/Fe(II) redox couple increases by ~59 mV per the Nernst equation (Bonneville, Van Cappellen, & Behrends, 2004; Kostka & Nealson, 1995; Majzlan, 2012; Thamdrup, 2000).

To test the hypothesis that *G. sulfurreducens* also utilizes ImcH- or CbcL-dependent pathways to reduce minerals on the basis of redox potential, we prepared Fe(III)-(oxyhydr)oxides by slow precipitation of $FeCl_3$ with NaOH (Lovley & Phillips, 1986), and characterized minerals before and after addition to medium using XRD. While Fe(III)-(oxyhydr)oxides prepared in this manner are often referred to as “poorly crystalline” or “amorphous”, all XRD signatures of our preparations were consistent with akaganite (β-FeOOH) (Figure 2E). Because the β-FeOOH/Fe(II) redox couple has a midpoint potential near -0.10 V vs. SHE (Majzlan, 2012), a fresh, fully oxidized suspension of fresh akaganeite would initially have a redox potential above 0 V vs SHE, and theoretically require only the ‘high’ potential pathway of *G. sulfurreducens*.

226 When wild-type, Δ*imcH,* and Δ*cbcL* strains were incubated with freshly precipitated akaganeite,
227 wild-type cultures rapidly reduced the provided Fe(III)-(oxyhydr)oxide. The Δ*cbcL* cultures containing the
228 high-potential pathway initially reduced fresh akaganeite at rates similar to wild type, but reduction
229 slowed as Fe(II) accumulated, and the concentration of Fe(II) never reached the extent of wild type. In
230 contrast, Δ*imcH* cultures lacking the high potential pathway demonstrated a substantial lag, remaining
231 near background for 7 days (Figure 2A). Once metal reduction began, Δ*imcH* cultures were able to reach
232 the final extent observed in wild type. No reduction was observed in cell-free controls.

233 When medium containing akaganeite was autoclaved to lower redox potential due to
234 accelerated aging, both wild type and Δ*cbcL* performed similarly, but the lag observed with Δ*imcH* was
235 much shorter (Figure 2B). Despite the substantial change in the Δ*imcH* Fe(III)-(oxyhydr)oxide reduction
236 phenotype, the XRD pattern before and after autoclaving was similar (Figure 2E). Such a lack of XRD
237 alteration in response to autoclaving has been previously noted (Hansel et al., 2015). This suggests that
238 phenotypic changes were due to mineral alteration outcomes not detected by powder XRD, such as
239 short-range structural order or particle aggregation, that lowered effective redox potential and allowed
240 growth of the Δ*imcH* strain lacking the high-potential pathway.

241 **Reduction phenotypes also respond to changes in medium pH that alter redox potential**

242 The lag in extracellular electron transfer by Δ*imcH*, in contrast to immediate reduction by Δ*cbcL,*
243 suggests that fresh akaganeite has an initial redox potential greater than -0.10 V vs. SHE. Based on this
244 hypothesis, only after a small amount of Fe(II) accumulates does potential drop into a range reducible by
245 Δ*imcH*. Similarly, aging fresh akaganite by autoclaving shortened the lag in Figure 2B likely by lowering
246 redox potential. If this is true, then manipulations designed to raise redox potential should extend this
247 lag or prevent Δ*imcH* from reducing akaganeite altogether.

248 Because Fe(III)-(oxyhydr)oxide reduction is a proton consuming reaction, decreasing pH will
249 increase the redox potential, an effect that has been used to explain magnetite reduction by *Shewanella*
250 *oneidensis* (Kostka & Nealson, 1995; Majzlan, 2012; Thamdrup, 2000). When the pH of basal medium
251 containing akaganeite was adjusted to 6.3 (raising the redox potential ~30 mV), near complete inhibition
252 of reduction by Δ*imcH* was observed (Figure 2C). Autoclaving pH 6.3 akaganeite medium was not
253 sufficient to restore the reduction phenotype of Δ*imcH*, but did aid reduction by Δ*cbcL* (Figure 2D). As
254 with medium at pH 6.8, autoclaving at 6.3 did not substantially alter the XRD pattern of the mineral
255 provided (Figure 2E). Importantly, wild type, Δ*imcH,* and Δ*cbcL* grew without significant defects at pH
256 values of 6.3 or 6.8 in control experiments containing fumarate as the sole terminal electron acceptor
257 (Table 1). Based on these results, when akaganeite is autoclaved at pH 6.8, the initial redox potential is
258 lowered close to -0.10 V vs. SHE, permitting wild-type extents of reduction by cells containing the CbcL-
259 dependent, low-potential pathway. Fresh akaganeite, especially at pH 6.3, behaves consistent with a
260 redox potential of 0 V or greater, thus preventing reduction by cells containing only the low-potential
261 pathway.

262 **Here Figure 2**

263 These two experiments, using aging to reduce redox potential and pH to raise redox potential,
264 caused changes in Δ*imcH* phenotypes that provided evidence that *G. sulfurreducens* discriminates
265 between extracellular electron acceptors independent of the mineral properties accessed by powder
266 XRD. They also pointed to best practices for medium preparation in order to obtain consistent results if
267 the question involves the *initial* redox conditions created by a mineral, and the proteins needed for
268 electron transfer under those *initial* conditions. The amount of Fe(II) accumulated after 7 days provided
269 a repeatable readout of the ability of a *G. sulfurreducens* strain or mutant reduce a given Fe(III)-

270 (oxyhydr)oxide. According to this assay, the more Fe(II) produced in 7 days by an Δ*imcH* mutant, the
271 lower the initial redox potential of the mineral.

272 **Other mineral forms consistent with a multiple-pathway model**

273 A variety of Fe(III)-(oxyhydr)oxide minerals were then synthesized to test interactions of these
274 variables. When schwertmannite was synthesized using a rapid precipitation method (Regenspurg et al.,
275 2004), the mineral was stable at pH values below pH 4.0 and its structure could be confirmed by XRD.
276 However, abiotic transformation of schwertmannite to other mineral forms is accelerated at pH values
277 greater than 4.5 (Blodau & Knorr, 2006) and analysis of XRD patterns after schwertmannite was added
278 to *Geobacter* growth medium revealed rapid formation of XRD-amorphous minerals (Figure 3E).
279 Reduction phenotypes suggested this fresh mineral also produced a redox potential near 0 V vs. SHE, as
280 cells lacking the high potential pathway (Δ*imcH*) showed a lag at pH 6.8, which was shorter after the
281 mineral was autoclaved (Figure 3B). Reduction by Δ*imcH* was worst when the redox potential was
282 further raised by buffering to pH 6.3 (Figure 3C), while autoclaving pH 6.3 medium allowed a small
283 amount of Fe(II) accumulation that ended the lag after >8 days.

284 **Here Figure 3**

285 When Fe(III)-(oxyhrdr)oxide was prepared according to the 2-line ferrihydrite protocol of
286 Schwertmann and Cornell (2000), patterns were again similar to those observed with akaganite and
287 schwertmannite. Freshly prepared mineral was reduced by Δ*cbcL* to within 67% of wild type after seven
288 days. When this mineral was freeze dried (resulting in XRD-confirmed 2-line ferrihydrite), reduction by
289 Δ*cbcL* was less than 30% of wild type, suggesting that freeze drying lowered redox potential of the
290 mineral to a point where the low-potential pathway, encoded by *cbcL,* was more important. Changing
291 pH by 0.5 units was not sufficient to alter reduction phenotypes, consistent with the freeze drying
292 process lowering redox potential of ferrihydrite much more than 30 mV of pH change could overcome.

293 A general trend emerged during these experiments, consistent with autoclaving, freeze drying,
294 and pH influencing redox potential in similar ways, regardless of the mineral. When reduction by Δ*imcH*
295 was near wild-type levels, reduction by Δ*cbcL* was typically at its lowest. When reduction by Δ*cbcL* was
296 near wild-type levels, Δ*imcH* cultures failed to reduce the provided electron acceptor. Figure 4
297 represents all seven-day reduction data from 17 different XRD-characterized mineral forms incubated
298 with three different strains, ranked according to Δ*imcH* performance. This trend suggests the two
299 mutants act as an *in vivo* sensor of initial redox potential, across a suite of minerals and conditions.

300 For example, if initial conditions were well above 0 V vs. SHE, such as with freshly precipitated
301 minerals in medium at pH 6.3, Δ*imcH* mutants lacking the high potential pathway were unable to
302 respire. When exposed to the same minerals, Δ*cbcL* mutants containing only the high potential pathway
303 performed similar to wild type. As minerals were aged or manipulated to lower their initial redox
304 potential, the CbcL-dependent pathway became increasingly important to achieve wild-type extents of
305 reduction. These incubations highlighted the different contributions of ImcH and CbcL to the initial rate
306 vs. final extent of Fe(III) reduction, respectively.

307 Interestingly, some level of metal reduction was always observed for Δ*cbcL* mutants exposed to
308 minerals predicted to be very low potential acceptors. This result is similar to previous studies where
309 Δ*cbcL* still demonstrated a slow but detectable rate of respiration on electrodes poised at low redox
310 potentials (Zacharoff et al., 2016). This activity may reflect a yet-undiscovered pathway used under
311 thermodynamically challenging conditions, where neither ImcH- nor CbcL-dependent pathways can
312 function.

313 The results in Figure 4 represent a suite of minerals (2-line ferrihydrite, goethite, akaganeite,
314 schwertmannite, and birnessite), in which additional variability is introduced through freeze-drying,
315 autoclaving, and pH adjustments. These variables could alter mineral crystal structure, particle size,

316 degree of aggregation, surface area, and surface charge, and thus influence microbial attachment or
317 behavior in unpredictable ways (Cutting, et al., 2009; Majzlan et al., 2004; Navrotsky et al., 2008; Roden,
318 2006). However, all of these manipulations change redox potential in predictable ways. Further, these
319 phenotypes are caused by the deletion of genes encoding inner membrane proteins, far from the outer
320 surface reduction step(s) where metal charge or structure should play an important role. Based on the
321 overall pattern of responses, we propose that redox potential should be viewed as a master variable
322 affecting the pathway of extracellular electron acceptor reduction used by *G. sulfurreducens*.

323 **Here Figure 4**

324 **Cells lacking the CbcL-dependent pathway have increased growth yield.**

325 A key question stemming from this work relates to the evolutionary forces selecting for multiple
326 electron transfer pathways at the inner membrane. As Δ*imcH* cells are often able to completely reduce a
327 provided mineral after a small amount of Fe(II) accumulates, why not encode a single, CbcL-dependent
328 electron transfer pathway and allow it operate at all potentials? One hypothesis is that *G. sulfurreducens*
329 is able to take advantage of the different amounts of energy represented by different metal
330 (oxyhydr)oxides.

331 To test this hypothesis, the cell yield of *G. sulfurreducens* strains in which one pathway was
332 removed was measured. Because both Δ*imcH* and Δ*cbcL* can initially reduce akaganeite (albeit to
333 different extents), direct comparisons were possible when a low inoculum of each strain was added to
334 medium containing this mineral (autoclaved at pH 6.8, as in Figure 2B) and incubated. Samples were
335 removed for measurement of Fe(II) and direct plate counts on solid minimal medium. In all experiments,
336 Δ*cbcL* generated more colony forming units per mol Fe(II) produced than either wild type *G.*
337 *sulfurreducens* or Δ*imcH*. For each mM of Fe(III) reduced, Δ*cbcL* produced 2.5 ± 0.3 x 10^6 CFU, while wild
338 type and Δ*imcH* produced 0.8 ± 0.1 and 1.6 ± 0.1 x 10^6 CFU, respectively (n=5 for each strain). These

339 results indicated that when electron flow was forced through the ImcH-dependent pathway (as in
340 Δ*cbcL*), more cells were produced per Fe(III) reduced.

341 Cell attachment to mineral particles could confound plate counts in experiments with cells
342 grown on solid particles. When wild type *G. sulfurreducens* CFU were measured over the course of
343 akaganeite reduction, a decrease was seen for time points shortly after inoculation when Fe(II) begins to
344 rapidly accumulate, which may suggest an association of cells to akaganeite particles or aggregation of
345 cells to other cells. In a second series of experiments, basal medium containing acetate and fumarate
346 was inoculated with a 2 % v/v inoculum from akaganeite-grown cultures. Because all wild-type and
347 mutant cultures grow at similar rates with fumarate as the electron acceptor (see Table 1), the point at
348 which optical density is detectable in fumarate grown cultures is a function of the number of cells in the
349 initial inoculum. In all of these experiments, Δ*cbcL* again demonstrated significantly more cells per mol
350 Fe(II) than did wild type or Δ*imcH.*

351 These fumarate outgrowth experiments, which reflect the total number of cells in the mineral
352 containing medium at a given time point, agreed with the trend seen in direct plate counts from
353 akaganeite cultures. In both cases, Δ*cbcL* generated more cells per mol Fe(III) reduced than either wild
354 type or Δ*imcH*. When normalized to the amount of Fe(II) produced, Δ*cbcL* yield was 246.2% ± 15.11 of
355 that of wild-type, whereas Δ*imcH* yield was 105.2% ± 24.33 (n=3 in both cases). Combined with previous
356 data showing that the ImcH-dependent pathways support faster growth rates when electrodes poised at
357 +0.24 V vs. SHE (Zacharoff et al., 2016), this supports the hypothesis that *G. sulfurreducens* alters its
358 electron transfer pathway to use a higher yield pathway whenever possible. Somehow, these inner
359 membrane pathways are able to switch 'on' and 'off' in response to the redox potential of external
360 electron acceptors. This difference provides one explanation for maintaining the complexity and burden
361 associated with encoding multiple pathways of electron transfer across the inner membrane.

362 **Discussion:**

363 Fe(III)-(oxyhydr)oxide minerals in nature exist as a complex continuum of potential energies. As
364 such, it is unsurprising that bacteria would evolve equally complex mechanisms able to harness the
365 energy available during respiration to these metals. While a single pathway essential for reduction of all
366 metals emerged in the model metal-reducing *Shewanella spp.,* a similarly simple solution has remained
367 elusive in *Geobacter spp.*. The data reported here demonstrates that even under the most common
368 laboratory conditions where Fe(III)-(oxyhydr)oxide is precipitated and added to medium, *G.*
369 *sulfurreducens* will utilize multiple electron transfer pathways to accomplish what is considered to be
370 wild-type levels of Fe(III) reduction. For Fe(III)-(oxyhydr)oxides that are predicted to have redox
371 potentials around 0 V, such as schwertmannite, akaganeite, and ferrihydrite, the electron-accepting
372 potential decreases as Fe(II) accumulates, and triggers utilization of electron transfer pathways that
373 support lower cell yield and slower growth rate, but still allow some respiration. The ability of *G.*
374 *sulfurreducens* to respond to the changing redox potential of its environment likely allows this organism
375 to make the most efficient use of the provided mineral.

376 Based on proteomic studies, *G. sulfurreducens* expresses at least 78 expressed multiheme *c*-type
377 cytochromes, yet mutant analyses were initially unable to link particular cytochromes to reduction of
378 specific extracellular electron acceptors (Aklujkar et al., 2013; Leang et al., 2003, 2010; Rollefson et al.,
379 2009, 2011; Shi et al., 2007). One possible source of cytochrome diversity could be separate pathways
380 required for soluble Fe(III)-citrate, insoluble Fe(III)-(oxyhydr)oxides, and Mn(IV)-(oxyhydr)oxides.
381 However, despite their many differences, Fe(III)-citrate and Mn(IV)-(oxyhydr)oxides both represent high
382 potential acceptors, from the viewpoint of the inner membrane. As we detect evidence for at least one
383 other pathway in this work, and the *G. sulfurreducens* genome appears to encode at least 4 other inner
384 membrane quinone oxidoreductases, redox potential differences may help to explain additional
385 cytochrome complexity of *Geobacter.* Possessing protein machinery able extract the full advantage from

386 minerals as they descend through the redox tower also helps explain the dominance of these metal-
387 reducing organisms in contaminated subsurface environments undergoing rapid redox potential
388 changes.

389 In order to characterize and biochemically dissect extracellular electron transfer in dissimilatory
390 metal reducing organisms such as *G. sulfurreducens*, closer attention may need to be paid not just to
391 crystal structure, but the actual redox potential experienced by the organisms interacting with these
392 minerals. Commonly synthesized Fe-(oxyhydr)oxides prepared by precipitation of Fe(III) can produce
393 very different rates of reduction or mutant phenotypes depending on the age of the material and the
394 length of time one is willing to incubate cells. A distinction between initial rates of Fe(III)-(oxyhydr)oxide
395 reduction, where higher-potential conditions exist, and final extent of reduction achieved by lower
396 potential pathways may help separate some of these confounding effects. One would also expect
397 significant differences in washed cell suspensions, where the degree of respiratory coupling to growth is
398 not part of the assay, since electron flow through the ImcH- and CbcL-dependent pathways support
399 different growth rates and yields.

400 It now appears that in *G. sulfurreducens* a transition from an ImcH-dependent electron transfer
401 pathway to a CbcL-dependent pathway occurs both when electrodes and many commonly used Fe(III)-
402 (oxyhydr)oxides are provided as sole terminal electron acceptors. The initial rate and total extent of
403 Fe(III)-(oxyhydr)oxide reduction may also be the result of a series of redox potential dependent
404 processes in environmental samples, with each step supporting different cell yields. Competition in the
405 environment could occur along any part of this continuum, rewarding those capable of rapid growth
406 when the Fe(III)-(oxyhydr)oxide is high potential, or those able to survive with electron acceptors at
407 relatively low redox potentials. The prevalence of ImcH and CbcL homologues in other dissimilatory
408 metal reducing organisms and in metagenomics data from sites undergoing active metal reduction

409 (Levar et al., 2014; Zacharoff et al., 2016) suggests that this type of redox discrimination may also occur

410 in diverse organisms, and provides interesting opportunities for further exploration of redox potential

411 dependent respiration in these organisms and environments.

412

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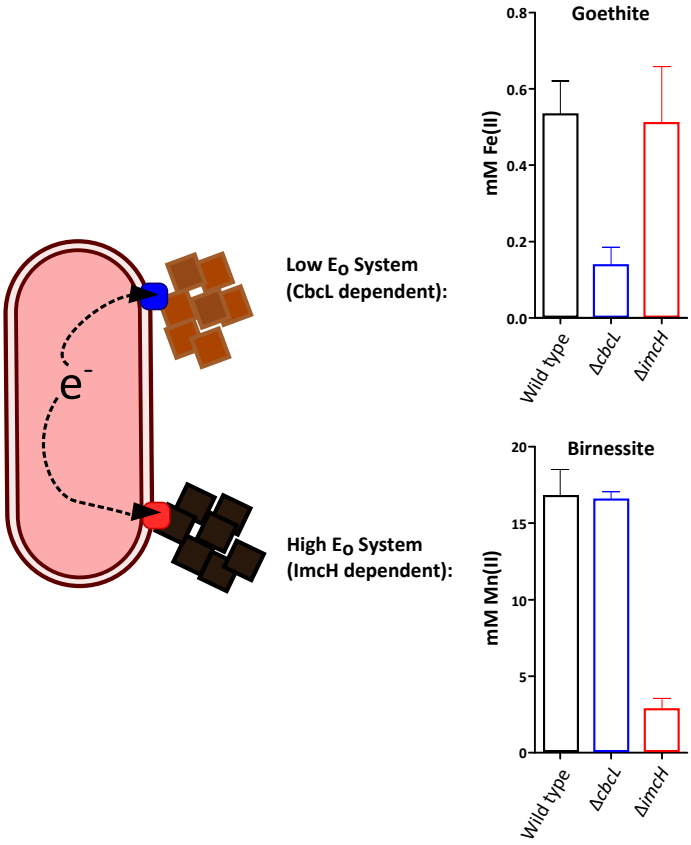
Table 1

Strain	Source	Doubling time in minimal medium at different pH (hours)*	
		6.3	6.8
Geobacter sulfurreducens			
Wild type (ATCC 51573)	Caccavo et al., 1994	6.5±0.2	5.6±0.2
Δ*cbcL*	Zacharoff et al., 2016	5.6±0.2	6.4±0.1
Δ*imcH*	Chi Ho Chan et al., 2015	5.7±0.1	6.0±0.2

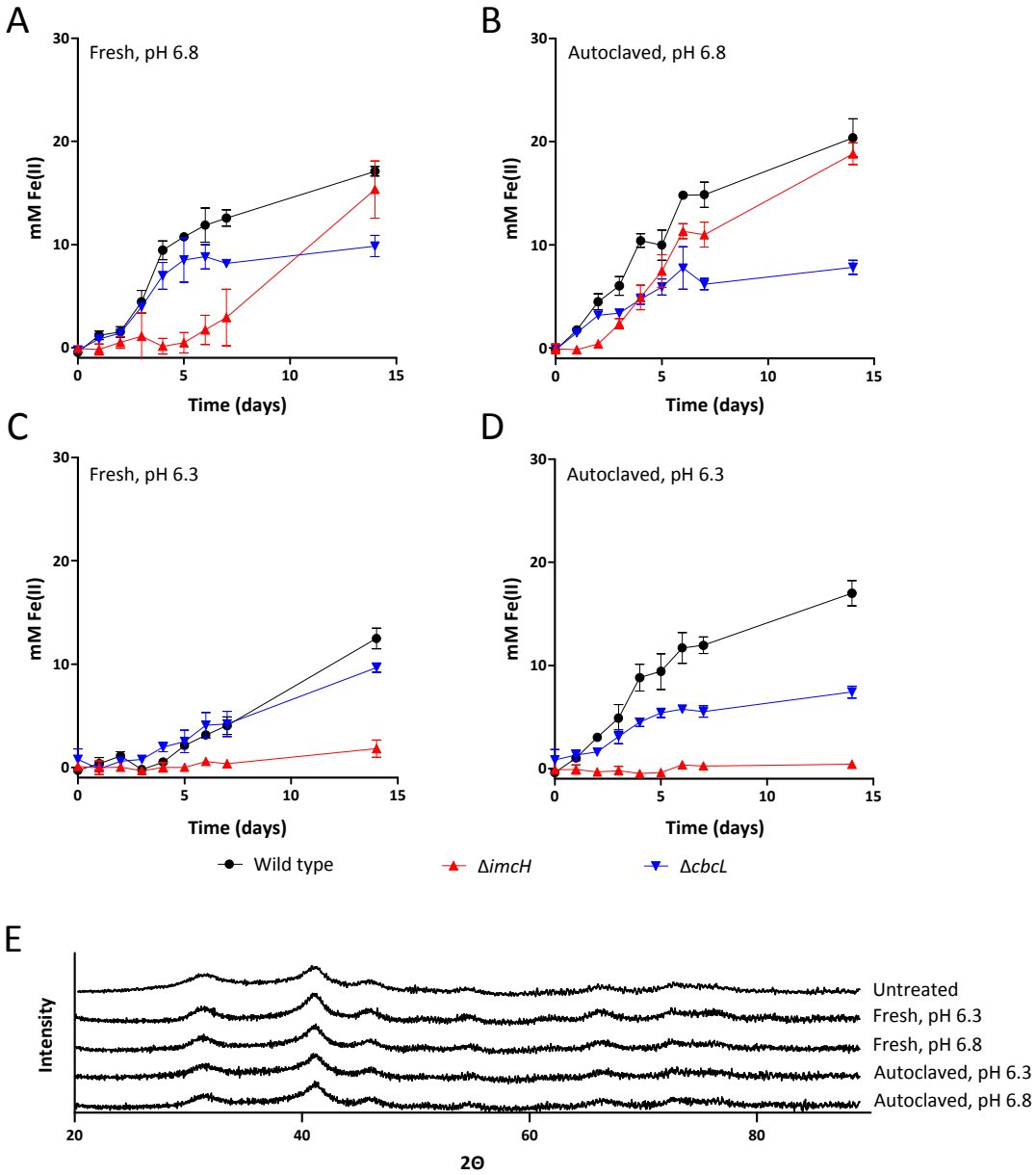
* Doubling times are the mean and standard deviation of at least two idependent experiments of triplicate cultures

Figure 1



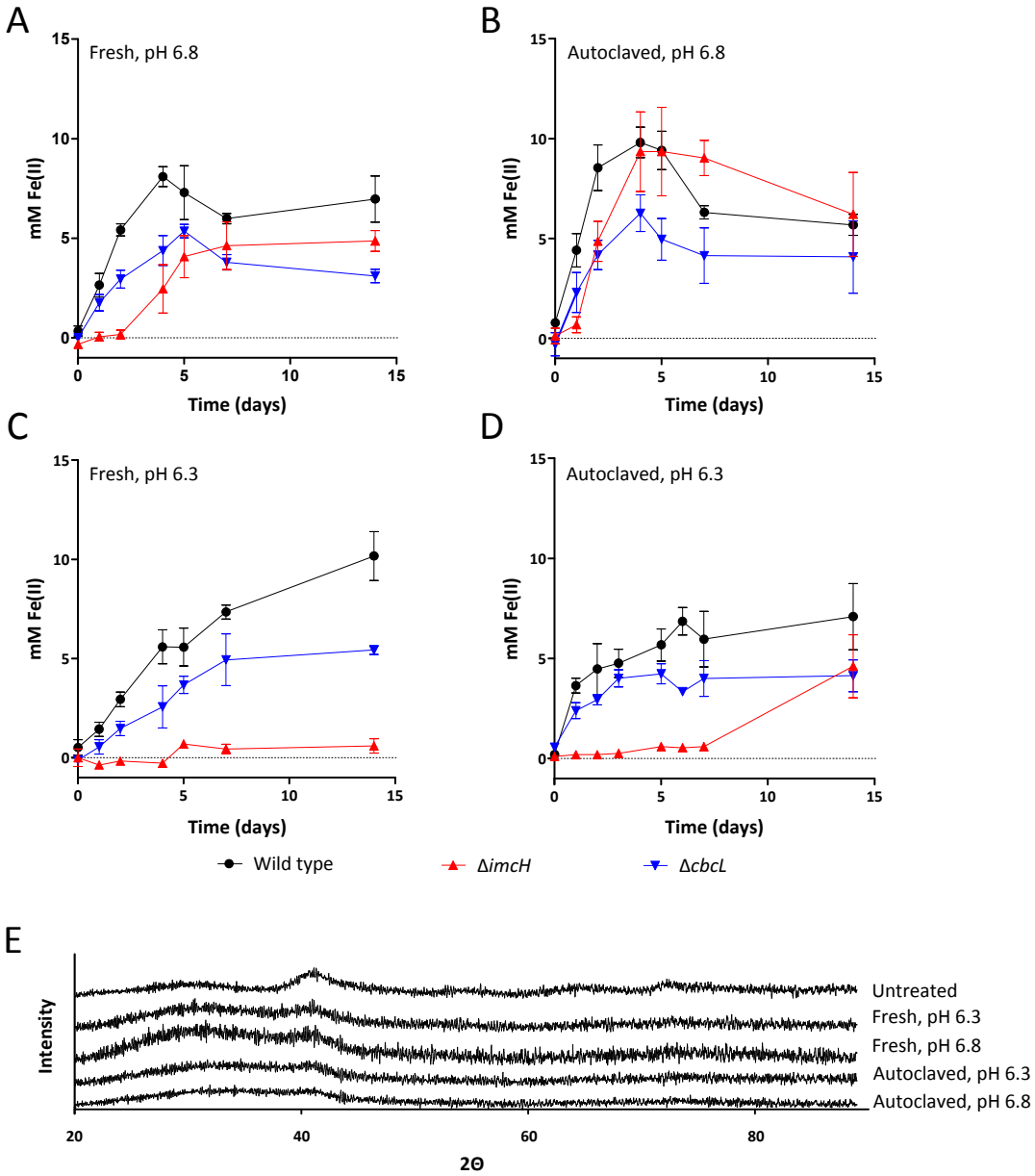
G. sulfurreducens *requires at least two distinct electron transfer pathways for the reduction of minerals*: Wild type *G. sulfurreducens* has the ability to reduce extracellular electron acceptors ranging from the low potential Fe(III)-(oxyhydr)oxide goethite (α-FeOOH) to the high potential Mn(IV)-oxide birnessite ($Na_xMn_{2-x}(IV)Mn(III)_xO_4$, x ~ 0.4). Mutants deficient in CbcL or ImcH show reduction phenotypes consistent with redox potential discrimination. Δ*cbcL* reduces birnessite as well as wild type, but fails to reduce goethite after 14 days of incubation. Δ*imcH* reduces goethite as well as wild type, but is unable to reduce birnessite. Data shown are the mean and standard deviation of triplicate cultures.

Figure 2



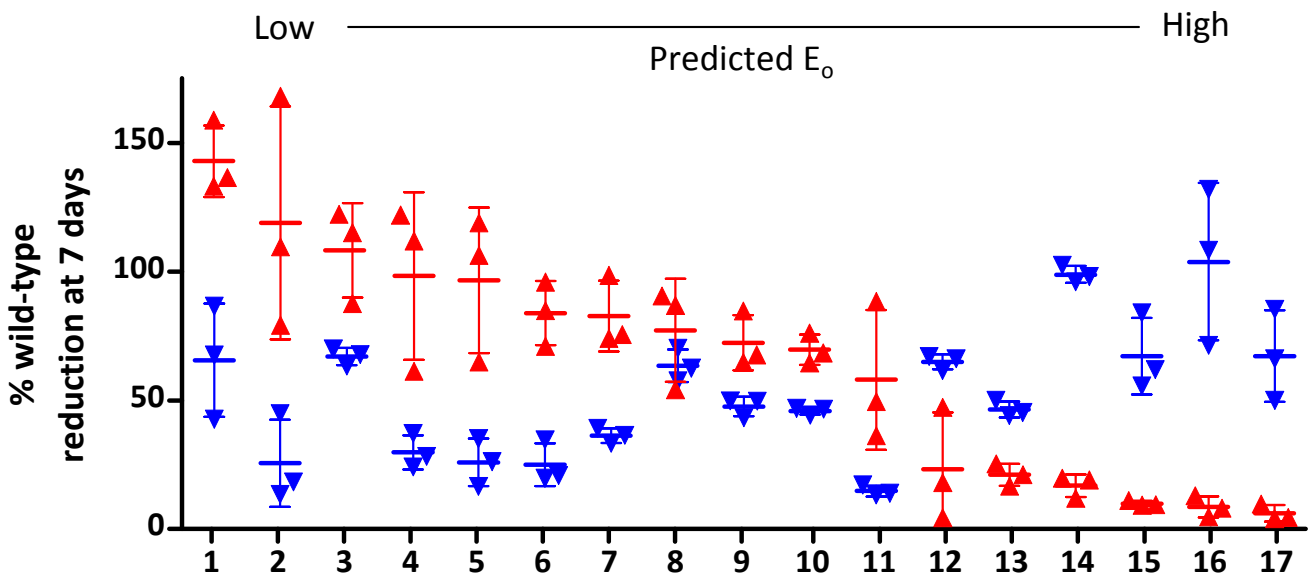
Autoclaving and changing pH alters mutant phenotypes. In all cases, wild type is represented by black circles, Δ*cbcL* by downward pointing blue triangles, and Δ*imcH* by upward pointing red triangles. (A) A long lag is observed for Δ*imcH* inoculated into medium with no autoclaving. (B) The lag observed for Δ*imcH* is decreased when the medium is aged through autoclaving. (C) Decreasing the pH by 0.5 units eliminates akaganeite reduction by Δ*imcH*. (D). Aging pH 6.3 medium through autoclaving is not sufficient to decrease the redox potential enough to allow for reduction by Δ*imcH*. All data shown are the mean and standard deviation for triplicate cultures, and are representative of at least duplicate incubations. (E) XRD patterns of the mineral used in panels A-D. The XRD pattern for the freshly precipitated mineral is also shown. No changes in XRD patterns were observed between multiple batches of synthesis and media preparation, suggesting that differences in Δ*imcH* behavior are due to changes in the initial redox potential of the medium.

Figure 3



Treatment of schwertmannite with growth medium at pH 6.3 or pH 6.8 alters mineralogy: Rapid precipitation of an Fe sulfide solution with hydrogen peroxide yields XRD-pure schwertmannite ("untreated", Panel E), but when this mineral suspension is added to growth medium and adjusted to circumneutral pH values, abiotic transformation to other mineral forms is observed. Autoclaving these media lead to further mineral transformation.

Figure 4



#	Starting mineral*	Freeze dried (Y/N)	Autoclaved (Y/N)	pH	Resulting mineral[†]
1	Schwertmannite	N	Y	6.8	XRD Amorphous
2	2-line Fh	Y	N	6.3	2-line Fh
3	2-line Fh	N	N	6.8	Akaganeite
4	Goethite	Y	N	6.8	Goethite
5	2-line Fh	Y	N	6.8	2-line Fh
6	2-line Fh	Y	Y	6.3	2-line Fh
7	Akaganeite	N	Y	6.8	Akaganeite
8	Schwertmannite	N	N	6.8	XRD Amorphous
9	Akaganeite	N	Y	6.6	Akaganeite
10	Akaganeite	N	Y	6.5	Akaganeite
11	2-line Fh	Y	Y	6.8	2-line Fh
12	Akaganeite	N	N	6.8	Akaganeite
13	Akaganeite	N	Y	6.3	Akaganeite
14	Birnessite	N	N	6.8	Birnessite
15	Schwertmannite	N	Y	6.3	XRD Amorphous
16	Akaganeite	N	N	6.3	Akaganeite
17	Schwertmannite	N	N	6.3	XRD Amorphous

*Listed mineral is mineral identity prior to treatment with media or autoclaving.

[†]Listed mineral is mineral identity after treatmeant with media or autoclaving and before innoculation with *G. sulfurreducens*

G. sulfurreducens *requires both ImcH and CbcL for complete reduction of a range of minerals:* Cultures of Δ*imcH* (red upward pointing triangles) and Δ*cbcL* (blue downward pointing triangles) were incubated with minerals as the sole terminal electron acceptor, and the extent of Fe(II) or Mn(II) accumulation after seven days of incubation were measured relative to that of wild type *G. sulfurreducens*. Conditions are ordered left to right in order of Δ*imcH* reduction, from best to worst. Akaganeite, β-FeOOH. Schwertmannite, $Fe_8O_8(OH)_6(SO_4)\cdot nH_2O$. 2-line Fh (Ferrihydrite), ca. $Fe_{10}O_{14}(OH)_2$. Birnessite $Na_xMn_{2-x}(IV)Mn(III)_xO_4$, x ~ 0.4, Goethite, α-FeOOH.