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, whereas 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 soluble and insoluble metal electron acceptors. 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 both soluble and insoluble Fe(III). Together, these data suggest that the ImcH-dependent pathway exists to harvest additional energy when conditions permit, and CbcL switches on to allow 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.

The ISME Journal (2017) 11, 741–752; doi:10.1038/ismej.2016.146; published online 3 January 2017

Introduction

Fe(III)-(oxyhydr)oxides can exist in at least 15 mineral forms with variable physiochemical properties that span a wide range of formal oxidation-reduction (redox) midpoint potentials (Nealson and Saffarini, 1994; Schwertmann and Cornell, 2000; Thamdrup, 2000; Majzlan et al., 2004; Majzlan, 2011, 2012). The electron-accepting potential of any given Fe(III)-(oxyhydr)oxides structure is not a fixed value, and becomes less favorable with increasing crystallinity, particle size, pH or ambient Fe(II) concentration (Thamdrup, 2000; Navrotsky et al., 2008; Majzlan, 2012; Sander et al., 2015). Differences in effective redox potential alters the energy available to be captured by bacteria able to couple the oxidation of an electron donor to reduction of these minerals (Thauer et al., 1977; Flynn et al., 2014). As a result of this structural and environmental diversity, organisms able to reduce metals could sense the energy available in their electron acceptors and utilize different electron transfer pathways, similar to how Escherichia coli uses distinct terminal oxidases in response to levels of available oxygen (Russell and Cook, 1995; Green and Paget, 2004).

One well-studied dissimilatory metal-reducing organism, Geobacter sulfurreducens, can grow via reduction of metal (oxyhydr)oxides ranging in predicted midpoint redox potential from +0.35 V vs the standard hydrogen electrode (SHE; for example, birnessite, ca Na,Mn$_{3.x}$(IV)Mn(III),O$_4$. x ~ 0.4) to −0.17 V vs SHE (for example, goethite, α-FeOOH) (Post and Veblen, 1990; Nealson and Myers, 1992; Caccavo et al., 1994; Thamdrup, 2000; Majzlan et al., 2004; Majzlan, 2012; Orsetti et al., 2013). Recent work examining electron transfer from G. sulfurreducens to poised graphite electrodes demonstrated that this organism uses at
least two different inner membrane electron transfer pathways, known as the CbcL- and ImcH-dependent pathways (Levar, et al., 2014; Zacharoff, et al., 2016). The ‘low’ potential, CbcL-dependent pathway, is required for growth with electrodes at or below potentials of ~0.10 V vs SHE, whereas the ‘high’ potential, ImcH-dependent pathway, is essential when electrodes are poised at redox potentials above this value. As mutants deficient in either pathway only grow with electrodes poised above or below these thresholds, cells lacking CbcL or ImcH may also be able to be used as ‘sensors’ to report the redox potential of an extracellular electron acceptor such as a metal (oxyhydr)oxide. As it is difficult to determine the effective redox potential of these minerals (Sander et al., 2015), such information could aid laboratory characterization, and provide evidence that bacteria use different pathways for different metals in the environment.

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

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

Materials and methods

Bacterial strains and culture conditions

Strains of G. sulfurreducens used in this study are described in Table 1. All strains were routinely cultured from freezer stocks stored at −80 °C.

Table 1 Strains used in this study

| Strain | Source | Doubling time (h)* |
|--------|--------|--------------------|
| Geobacter sulfurreducens | Wild-type (ATCCZ51573) Caccavo et al., 1994 | 6.5 ± 0.2 5.6 ± 0.2 |
| | Δcbl | Zacharoff et al., 2016 | 5.6 ± 0.2 6.4 ± 0.1 |
| | ΔimcH | Chi Ho Chan et al., 2015 | 5.7 ± 0.1 6.0 ± 0.2 |

*Doubling times are the mean and s.d. of at least two independent experiments of triplicate cultures.

Growth of G. sulfurreducens with Fe(III)-citrate and continuous redox potential monitoring

Minimal medium containing 20 mM acetate as the carbon and electron donor and ~ 80 mM Fe(III)-citrate as the electron acceptor was added to sterile bioreactors constructed as previously described, using bare platinum wire approximately 2 cm in length for both the working and counter electrodes. The headspace of each reactor was purged with anaerobic and humidified N₂/CO₂ gas (80%/20%) passed over a heated copper column to remove trace oxygen, and autoclaved for 20 min at 121 °C.

Growth of G. sulfurreducens with Fe(III)-citrate and continuous redox potential monitoring

Minimal medium containing 20 mM acetate as the carbon and electron donor and ~ 80 mM Fe(III)-citrate as the electron acceptor was added to sterile bioreactors constructed as previously described, using bare platinum wire approximately 2 cm in length for both the working and counter electrodes. The headspace of each reactor was purged with anaerobic and humidified N₂/CO₂ gas (80%/20%) passed over a heated copper column to remove trace oxygen, and autoclaved for 20 min at 121 °C.

Materials and methods

Bacterial strains and culture conditions

Strains of G. sulfurreducens used in this study are described in Table 1. All strains were routinely cultured from freezer stocks stored at −80 °C.
least 5 h, stationary phase cells were added to each bioreactor at a 1:100 v/v inoculum size and the change in redox potential was measured over time. When co-cultures were used, an equal volume of cells was added for each strain (that is, 1:100 each of ΔcbCL and ΔimcH was added after the voltage was stable).

X-ray diffraction (XRD) measurements
Approximately 0.2–0.5 g of untreated bulk mineral sample or 2.5 ml of mineral suspension in medium were analyzed by powder XRD. Minerals in basal media were separated from suspension by filtration (0.22 μm) in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) under a N2:CO2:H2 (75%:20%:5%) atmosphere, and stored in sealed mylar bags at 80 °C before analysis. Diffractograms were measured from using 20° to 89° (2θ) range, a step size of 0.02°, and a dwell of 0.5°/min using a Rigaku MiniFlex ASC-6AM with a Co source. The resulting diffractograms were background subtracted, and phases were identified using Jade v9.1 software (MDI Inc., Livermore, CA, USA).

Preparation of Fe(III)-(oxyhydr)oxides
‘Poorly crystalline Fe(OH)’ was produced after Lovley and Phillips (1986), by adding 25% NaOH dropwise over 90 min to a rapidly stirring 0.4 M solution of FeCl3 until the pH was 7.0. The solution was held at pH 7.0 for 1 h, and the resulting suspension was washed with one volume of de-ionized and distilled water and used immediately (<24 after synthesis) for XRD analysis and growth studies as rapid aging of such products to more crystalline minerals (for example, goethite) has been observed even when kept in the dark at 4 °C (Raven, Jain). The resulting mineral before its addition to medium and autoclaving (‘untreated mineral’) was identified as akaganeite (β-FeOOH) by powder XRD. Washing this ‘untreated mineral’ samples with up to three additional volumes of de-ionized and distilled water did not change the XRD pattern of the mineral or the reduction phenotypes observed. For all minerals, ‘untreated’ refers to the mineral suspension in MilliQ water before addition to medium and autoclaving, ‘fresh’ refers to the mineral suspension in medium, and ‘autoclaved’ samples result from adding the ‘untreated’ minerals to medium and autoclaving (that is, autoclaving the ‘fresh’ samples).

Goethite and two-line ferrihydrite (ca Fe10O14(OH)3; Michel et al., 2007) were synthesized after Schwertmann and Cornell (2000), with some modifications. For goethite, a solution of FeCl3 was precipitated through neutralization with 5 N KOH, and the resulting alkaline mineral suspension was aged at 70 °C for 60 h to facilitate goethite formation. The suspension was then centrifuged, decanted, and washed with de-ionized and distilled water before freeze drying. For two-line ferrihydrite, a solution of FeCl3 was neutralized with KOH until the pH was 7.0. Suspensions were rinsed with de-ionized and distilled water and either freeze dried or centrifuged and suspended in a small volume of de-ionized and distilled water to concentrate the final product. Freeze-dried samples yielded XRD patterns indicative of two-line ferrihydrite, but suspension of hydrated samples in growth medium had an altered XRD pattern more similar to that of akaganeite. Freeze-dried samples were stored at −80 °C before addition to growth medium, whereas hydrated samples were used within 24 h of synthesis.

Schwertmannite (Fe₈O₉(OH)₆(SO₄)·nH₂O) was synthesized using a rapid precipitation method involving the addition of a 30% solution of H₂O₂ to a solution of FeSO₄ (Regenspurg et al., 2004). The mixture was stirred rapidly for at least 12 h until the pH was stable, at ~2.35. The solids were allowed to settle for 1 h, and the supernatant carefully decanted. Solids were washed by centrifuging at 3700 × g and suspending in one volume of de-ionized and distilled water. The product was concentrated by a final centrifugation at 3700 × g for 5 min and resuspension in 1/20 the initial volume. XRD identified this initial product as schwertmannite, although subsequent addition to media at neutral pH values and autoclaving rapidly altered the mineral form.

Iron reduction assays
Basal media were prepared as above with some modifications. Fumarate was omitted as the Fe(III)-(oxyhydr)oxides were desired as the sole electron acceptor. Nitriilotriacetic acid-free trace mineral mix (in which all components were first dissolved in a small volume of HCl) was used in order to eliminate exogenous chelating compounds from the media. All media were purged in N2:CO2 gas (80%/20%) passed over a heated copper column to remove trace oxygen. Where indicated, media were autoclaved for 20 min at 121 °C on a gravity cycle, and were immediately removed to cool at room temperature in the dark. As is standard for G. sulfurreducens medium containing Fe(III)-(oxyhydr)oxide (Caccavo et al., 1994), additional NaH₂PO₄·H₂O (to a final concentration of 0.69 g l⁻¹) was added before autoclaving, as more recalcitrant and less reducible forms of Fe(III)-(oxyhydr)oxide will form when autoclaved without additional phosphate. For fresh akaganeite and two-line ferrihydrite, 1 ml of the Fe-(oxyhydr) oxide suspension was added to 9 ml of basal medium. The final pH was adjusted by altering the pH of the basal medium before mineral addition and by altering the concentration of NaHCO₃ used before purging with anaerobic N₂:CO₂ gas (80%/20%). For goethite, an 88 g l⁻¹ suspension was made in de-ionized and distilled water and 1 ml of this suspension was added to 9 ml of basal medium. The resulting medium had an effective Fe total concentration of ~20 mM as determined by fully reducing an acidified sample with hydroxylamine and measuring the resulting Fe(II) using a modified Ferrozine assay.  

---

Fe(III) reduction by G. sulfurreducens
CE Levar et al
(Lovley and Phillips, 1987). As the synthesis of schwertmannite results in an acidic product below the pH at which G. sulfurreducens grows optimally (Caccavo et al., 1994; Regenspurg et al., 2004), the mineral suspension was added to the basal medium (1:9) and the pH of the solution was adjusted with NaOH and bicarbonate to produce a final pH of 6.8. For freeze-dried two-line ferrihydrite, 0.2 g of freeze-dried sample was added per 10 ml of basal medium.

In all cases, 100 μl of electron acceptor-limited stationary phase cells (OD600 = 0.55) grown from single colony picks in NB basal medium containing acetate (20 mM) and fumarate (40 mM) were used to inoculate the Fe(III)-(oxyhydr)oxide containing media, with acetate provided as the electron and carbon donor and the Fe(III)-(oxyhydr)oxide as the sole electron acceptor.

For all Fe(III)-(oxyhydr)oxide incubations except those involving goethite, a small sample was removed at regular intervals and dissolved in 0.5 N HCl for at least 24 h at 23–25 °C in the dark before the acid extractable Fe(II) present in the sample was measured. For incubations with goethite, samples were incubated in 1 N HCl at 65 °C for at least 24 h in the dark, followed by centrifugation at 13,000 × g for 10 min to remove solids. Fe(II) in the supernatant was then measured.

Manganese reduction assays
Birnessite (Na0.6Mn2-x(IV)Mn(x)O4, x ~ 0.4) was prepared using the protocol described in Chan et al. (2015). Briefly, a solution of KMnO4 was added to an equal volume of MnCl2·4H2O solution and mixed vigorously. Solids were allowed to settle, and the overlying liquid was decanted off. The resulting solid was washed in de-ionized and distilled water and concentrated by centrifugation. This suspension was added (~20 mM) to basal medium containing acetate (10 mM) as the carbon and electron donor. Cells were inoculated to a calculated OD600 of 0.005 from electron acceptor-limited stationary phase cells. Mn(II) was measured indirectly as previously described (Levar et al., 2014; Chan et al., 2015). Briefly, samples were acidified in 2 N HCl with 4 mM FeSO4 added and allowed to dissolve overnight at 25 °C in the dark. After all solids were dissolved, the Fe(II) concentration was measured. As the reduction of Mn(IV) by Fe(II) is thermodynamically favorable, the measured Fe(II) concentration can be used to extrapolate the Mn(II) present a given sample. When measured Fe(II) concentrations are high, this implies that Mn(IV) concentrations are low (that is, Mn(IV) has been reduced by the bacterial culture).

Results
The inner membrane multiheme cytochromes ImcH and CbcL are implicated in transfer of electrons out of the quinone pool when electron acceptors fall within different redox potential windows (Levar et al., 2014; Zacharoff et al., 2016), although it is important to note that direct roles for ImcH and CbcL in the movement of electrons out of the quinone pool have not been demonstrated. Mutants lacking imcH are only able to reduce poised electrodes with ‘low’ redox potentials (≤−0.10 V vs SHE), and are deficient in reduction of electrodes at redox potentials above this value (Levar et al., 2014). In contrast, cbcL mutants have the opposite phenotype, reducing electrodes with relatively high electrode redox potentials, and having a deficiency in reduction of electrodes at or below −0.10 V vs SHE (Zacharoff et al., 2016). The genes for ImcH and CbcL are constitutively expressed (Zacharoff et al., 2016), yet use of each pathway appears to switch on or off in a matter of minutes during sweeps of increasing or decreasing potential known as catalytic cyclic voltammetry (Marsili et al., 2008; Yoho et al., 2014; Zacharoff et al., 2016). Genetic complementation of each pathway has been demonstrated using a variety of extracellular electron acceptors (Levar et al., 2014; Chan et al., 2015; Zacharoff et al., 2016).

As it is difficult to measure the effective redox potential of metal (oxyhydr)oxides (Sander et al., 2015), the redox potential of cultures undergoing active reduction of soluble Fe(III)-citrate was first monitored for each strain (Figure 1). Although the wild-type reduced Fe(III) completely to Fe(II), achieving a redox potential of −0.255 V vs SHE, the ΔcbcL mutant strain (lacking the low-potential system) was unable to reduce all Fe(III), and the redox potential only lowered to −0.15 V. The ΔimcH mutant strain (lacking the high-potential system) was unable to reduce Fe(III) when inoculated into the high-potential environment. When both ΔcbcL and ΔimcH mutant strains were inoculated into the same reactor, redox potential decreased with similar kinetics as the ΔcbcL strain, but continued on to a final value similar to wild-type. This was consistent with the ΔcbcL mutant strain first reducing Fe(III), until the redox potential was low enough to be usable by the ΔimcH mutant. This hard transition from where the ΔcbcL and ΔimcH mutant strains could actively reduce Fe(III) occurred in a range (between 0 and −0.1 V) was similar to the theoretical potentials of many Fe(III)-(oxyhydr)oxides. With the observation that these mutants behaved with Fe(III) citrate in a manner comparable to electrodes, we hypothesized that mutants lacking the ImcH- and CbcL-dependent electron transfer pathways would respond similarly when exposed to commonly used metal (oxyhydr)oxides.

Evidence that the electron transfer pathway changes even in the absence of XRD pattern changes
Most Fe(III)-(oxyhydr)oxides do not exist at the high or low extremes represented by electrodes or fresh Fe(III) citrate, 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- and Fe(III)-citrate grown cells
(Thamdrup, 2000; Majzlan et al., 2004; Majzlan, 2012; Levar et al., 2014; Zacharoff et al., 2016). Although testing this with electrodes or soluble electron acceptors 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 (Roden and Zachara, 1996; Roden et al. 2000; Thamdrup, 2000; Roden, 2006; Navrotzky et al., 2008). As Fe(III)-(oxyhydr)oxide reduction is a proton-consuming reaction (Kostka and Nealson, 1995; Thamdrup, 2000; Majzlan, 2012), 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 (Kostka and Nealson, 1995; Thamdrup, 2000; Bonneville et al. 2004; Majzlan, 2012).

To test the hypothesis that G. sulfurreducens also utilizes ImcH- vs CbcL-dependent pathways to reduce minerals on the basis of redox potential, we prepared Fe(III)-(oxyhydr)oxides by slow precipitation of FeCl3 with NaOH (Lovley and Phillips, 1986), prepared Fe(III)-(oxyhydr)oxides by slow precipitation of FeCl3 with NaOH (Lovley and Phillips, 1986), and characterized minerals before and after addition to medium using powder XRD. Although Fe(III)-(oxyhydr)oxides prepared in this manner are often poorly crystalline or amorphous, all XRD signatures of our slow precipitation preparations were consistent with akaganite (β-FeOOH) (Figure 2e). As the β-FeOOH/Fe(II) redox couple has a formal midpoint potential near ~0.10 V vs SHE (Majzlan, 2012), a fully oxidized suspension of akaganite would initially have a redox potential well above 0 V vs SHE, and theoretically require only the ‘high’ potential pathway of G. sulfurreducens.

When wild-type, ΔimcH and ΔcbcL strains were incubated with akaganite that had not been autoclaved (labeled as ‘Fresh’), wild-type cultures reduced the provided Fe(III)-(oxyhydr)oxide over the course of 15 days. The ΔcbcL cultures containing the high-potential pathway initially reduced fresh akaganite at rates similar to wild-type, but reduction slowed as Fe(II) accumulated, and the concentration of Fe(II) never reached the extent of wild-type. In contrast, ΔimcH cultures lacking the high-potential pathway demonstrated a substantial lag, remaining near background for 7 days (Figure 2a). Once metal reduction began, ΔimcH cultures were able to reach the final extent observed in wild-type. Fourteen-day cell-free controls did not exhibit any observable Fe(III)-(oxyhydr)oxide reduction.

Medium containing akaganite was then autoclaved, a treatment predicted to lower redox potential because of accelerated aging. Both wild-type and ΔcbcL performed similar to the fresh, un-autoclaved sample, but the lag observed with ΔimcH was now much shorter, consistent with a lower redox potential (Figure 2b). Despite the change in the ΔimcH Fe(III)-(oxyhydr)oxide reduction phenotype, the XRD pattern before and after autoclaving was similar (Figure 2e). Such a lack of XRD alteration in response to autoclaving has been previously noted (Hansel et al., 2015). This suggests that phenotypic changes were due to mineral alteration outcomes not detected by powder XRD, such as short-range structural order or particle aggregation, which lowered effective redox potential and allowed growth of the ΔimcH strain lacking the high-potential pathway.

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

The lag in extracellular electron transfer by ΔimcH, in contrast to immediate reduction by ΔcbcL, reduced the provided Fe(III)-(oxyhydr)oxide over the course of 15 days. The ΔcbcL cultures containing the high-potential pathway initially reduced fresh akaganite at rates similar to wild-type, but reduction slowed as Fe(II) accumulated, and the concentration of Fe(II) never reached the extent of wild-type. In contrast, ΔimcH cultures lacking the high-potential pathway demonstrated a substantial lag, remaining near background for 7 days (Figure 2a). Once metal reduction began, ΔimcH cultures were able to reach the final extent observed in wild-type. Fourteen-day cell-free controls did not exhibit any observable Fe(III)-(oxyhydr)oxide reduction.

Medium containing akaganite was then autoclaved, a treatment predicted to lower redox potential because of accelerated aging. Both wild-type and ΔcbcL performed similar to the fresh, un-autoclaved sample, but the lag observed with ΔimcH was now much shorter, consistent with a lower redox potential (Figure 2b). Despite the change in the ΔimcH Fe(III)-(oxyhydr)oxide reduction phenotype, the XRD pattern before and after autoclaving was similar (Figure 2e). Such a lack of XRD alteration in response to autoclaving has been previously noted (Hansel et al., 2015). This suggests that phenotypic changes were due to mineral alteration outcomes not detected by powder XRD, such as short-range structural order or particle aggregation, which lowered effective redox potential and allowed growth of the ΔimcH strain lacking the high-potential pathway.

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

The lag in extracellular electron transfer by ΔimcH, in contrast to immediate reduction by ΔcbcL,
suggests that fresh akaganeite has an initial redox potential greater than −0.10 V vs SHE. Based on this hypothesis, after many days, a small amount of Fe(II) accumulates, and potential drops into a range reducible by ΔimcH. If aging fresh akaganite by autoclaving shortened the lag in Figure 2b by lowering redox potential, then manipulations designed to raise redox potential should extend this lag or prevent ΔimcH from reducing akaganeite altogether.

When the pH of basal medium containing akaganeite was adjusted to 6.3 (raising the redox potential ~30 mV; Kostka and Nealson, 1995; Majzlan, 2012; Thamdrup, 2000), near complete inhibition of reduction by ΔimcH was observed (Figure 2c). Autoclaving pH 6.3 medium through autoclaving is not sufficient to decrease the redox potential enough to allow for reduction by ΔimcH. All data shown are the mean and s.d. 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 mineral before addition to medium and autoclaving (‘Untreated’) is also shown.
Based on these results, when akaganeite is autoclaved, the initial redox potential is likely lowered closer to $-0.10 \text{ V vs SHE}$, permitting operation of the CbcL-dependent, low-potential pathway. Fresh akaganeite, especially at pH 6.3, behaves consistent with a redox potential of 0 V or greater, thus preventing reduction by cells containing only the low-potential pathway.

These two experiments, using aging to reduce redox potential and pH to raise redox potential, caused changes in $\Delta\text{imcH}$ phenotypes that provided new evidence that $G. \text{sulfurreducens}$ discriminates between extracellular electron acceptors independent of the mineral properties accessed by powder XRD. They also pointed to best practices for medium preparation in order to obtain consistent results if the phenotype is sensitive to the redox potential of the environment. The amount of Fe(II) accumulated after 7 days provided a repeatable readout of the ability of a $G. \text{sulfurreducens}$ strain or mutant to reduce a given Fe(III)-(oxyhydr)oxide. According to this assay, the more Fe(II) produced in 7 days by an $\Delta\text{imcH}$ mutant, the lower the initial redox potential of the mineral likely was.

Figure 3 Addition of schwertmannite to growth medium at pH 6.3 or pH 6.8 alters mineralogy, and mutant behavior: 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, abiotic transformation is observed (as used in panels a and c). Autoclaving these media lead to further mineral transformation (as used in panels b and d). Treatments aimed at raising and lowering redox potential affected reduction by $\Delta\text{imcH}$ mutants similar to results seen in Figure 2. For example, autoclaving the medium at pH 6.8 shortened the lag time for the strain lacking ImcH (panels a and b), and raising redox potential by lowering pH extended the lag time for the strain lacking ImcH (panels b and d).
Other mineral forms consistent with a multiple-pathway model

Additional Fe(III)-(oxyhydr)oxide minerals were then synthesized to test interactions of these variables. When schwertmannite was synthesized using a rapid precipitation method (Regenspurg et al., 2004), the mineral was stable at pH values below pH 4.0 and its structure could be confirmed by XRD. However, abiotic transformation of schwertmannite to other mineral forms is accelerated at pH values > 4.5 (Blodau and Knorr, 2006) and analysis of XRD patterns after schwertmannite was added to Geobacter growth medium revealed rapid formation of XRD-amorphous minerals (Figure 3e), although structural information may be elucidated with more powerful techniques such as synchrotron EXAFS. Reduction phenotypes suggested this fresh mineral also produced a redox potential near 0 V vs SHE, as cells lacking the high-potential pathway (ΔimcH) showed a lag at pH 6.8, which was shorter after the mineral was autoclaved (Figure 3b). Reduction by ΔimcH was worst when the redox potential was further raised by buffering to pH 6.3 (Figure 3c), whereas autoclaving pH 6.3 medium allowed a small amount of Fe(II) accumulation that ended the lag after > 8 days. These patterns with schwertmannite-based minerals were similar to those observed with akaganite.

When Fe(III)-(oxyhydr)oxide was prepared according to the two-line ferrihydrite protocol of Schwertmann and Cornell (2000), wild-type and mutant phenotypes were again as what was observed with akaganite and schwertmannite. Freshly prepared mineral was reduced by ΔcblL to within 67% of wild-type after 7 days. When this mineral was aged by freeze drying (resulting in XRD-confirmed two-line ferrihydrite), reduction by ΔcblL was only 30% of wild-type, suggesting that freeze drying lowered redox potential of the mineral to a point where the low-potential pathway became more important. Changing pH by 0.5 units was not sufficient to further alter reduction phenotypes, suggesting that the freeze-drying-aging process lowered the redox potential of ferrihydrite more than 30 mV of pH change could overcome.

A general trend emerged during these experiments, consistent with autoclaving, freeze drying, and pH influencing redox potential in similar ways, regardless of the mineral. When reduction by ΔimcH was near wild-type levels, reduction by ΔcblL was typically at its lowest. When reduction by ΔcblL was near wild-type levels, ΔimcH cultures failed to reduce the provided electron acceptor. This trend suggests that (1) the two mutants act as an in vivo sensor of initial redox potential, across a suite of minerals, metals and conditions, and (2) that both systems are employed by wild-type bacteria encountering these minerals under normal conditions.

Figure 4 represents all reduction data after 7 days of incubation, from 17 different XRD-characterized mineral forms performed in triplicate with three different strains, ranked according to ΔimcH performance. As redox potentials of these minerals cannot directly be measured the order shown in Figure 4 cannot be directly related to a quantitative measure of actual redox potential. However, the data provide insight into how simple laboratory manipulations can affect experimental outcomes, and how these are linked to redox potential changes.

For example, when ΔimcH mutants lacking the high-potential pathway were unable to respire, initial conditions were likely well above 0 V vs SHE, such as with freshly precipitated minerals in medium at pH 6.3. When exposed to the same high-potential minerals, ΔcblL mutants containing only the high-potential pathway were able to perform similar to wild-type. As minerals were aged or manipulated to lower their initial redox potential, the CbcL-dependent pathway became increasingly important to achieve wild-type extents of reduction. These incubations highlighted the different contributions of ImcH and CbcL to the initial rate vs final extent of Fe(III) reduction, respectively.

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

The results in Figure 4 represent a suite of well-understood minerals (two-line ferrihydrite, goethite, akaganite, schwertmannite and birnessite), in which variability is typically introduced through freeze drying, autoclaving and pH adjustments. These variables alter mineral crystal structure, particle size, degree of aggregation, surface area and surface charge, and thus influence microbial attachment or behavior in unpredictable ways (Majzlan et al., 2004; Roden, 2006; Navrotsky et al., 2009; Cutting, et al., 2009). However, these manipulations change redox potential in predictable ways. Based on the overall pattern of responses, we propose that redox potential is a master variable affecting the inner membrane proteins used by G. sulfurreducens.

Cells lacking the CbcL-dependent pathway have increased growth yield

A key question stemming from this work relates to the evolutionary forces selecting for multiple electron transfer pathways at the inner membrane. As cells lacking the ImcH-dependent pathway are often able to completely reduce a provided mineral after a small amount of Fe(II) accumulates, why not encode a single electron transfer pathway and allow it operate at all potentials, thus eliminating the need to encode
pathways controlling respiration across multiple redox potential windows? One hypothesis is that the multiple pathways encoded *G. sulfurreducens* are able to take advantage of the different amounts of energy represented by the spectrum of metal (oxyhydr)oxides, by more efficiently conserving these energy differences.

To test this hypothesis, the cell yield of *G. sulfurreducens* strains in which one pathway was removed was measured. As ΔcbcL can initially reduce akaganeite, direct comparisons were possible when a low inoculum of each strain was added to medium containing this mineral (autoclaved at pH 6.8, as in Figure 2b) and incubated. In all experiments, ΔcbcL generated more colony-forming units (CFUs) per mol Fe(II) produced than wild-type *G. sulfurreducens*. For each mM of Fe(III) reduced, ΔcbcL produced $2.5 \pm 0.3 \times 10^6$ CFU, whereas wild-type produced $0.8 \pm 0.1$ and $1.6 \pm 0.1 \times 10^6$ CFU, respectively ($n=5$ for each strain; Figure 5a). These results indicated that when electron flow was forced through the ImcH-dependent pathway (as in ΔcbcL), more cells were produced per Fe(III) reduced.

Cell attachment to mineral particles could confound plate counts in experiments with cells grown on solid particles. In a second series of experiments, medium containing acetate and fumarate was inoculated with cultures at the same stage of akaganeite reduction. As all wild-type and mutant cultures grow at similar rates with fumarate as the electron acceptor (see Table 1), the point at which optical density is detectable is a function of the number of cells in the inoculum. In all of these experiments, ΔcbcL again demonstrated significantly more cells per mol Fe(III) reduced.

Yield was also measured after a small inoculum of wild-type or ΔcbcL cells were allowed to reduce Fe(III)-citrate, and cells were harvested and washed free of soluble iron (which can affect the BCA assay). The ΔcbcL cultures again had a significantly increased protein yield (Figure 5b). When standardized to the amount of Fe(II), the yield of cells forced to use only the ImcH pathway was 170% of the wild-type using both pathways during complete reduction of Fe(III).

Combined with previous data showing that cells using ImcH-dependent pathways support faster reduction of minerals, these results suggest that the multiple pathways encoded by *G. sulfurreducens* enable a more efficient conservation of energy by allowing cells to utilize the energy differences associated with the spectrum of metal (oxyhydr)oxides.
growth rates (Zacharoff et al., 2016), this supports the hypothesis that *G. sulfurreducens* alters its electron transfer pathway to obtain a higher yield per electron transferred. These data indicate that these electron transfer pathways are able to switch ‘on’ and ‘off’ in response to changing redox potential of external electron acceptors. This provides one possible explanation for the complexity and burden associated with encoding multiple pathways of electron transfer across the inner membrane.

**Discussion**

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

Based on proteomic studies, *G. sulfurreducens* expresses at least 78 multiheme c-type cytochromes under different laboratory conditions, yet mutant analyses were initially unable to link particular cytochromes to reduction of specific extracellular electron acceptors (Leang et al., 2003, 2010; Shi et al., 2009, 2011; Aklujkar et al., 2013). One hypothesis for cytochrome diversity that drives such -omic comparisons is that separate pathways will exist based on solubility (chelated vs oxide) or metal (Fe vs Mn). However, despite their many physiochemical differences, Fe (III)-citrate and Mn(IV)-(oxyhydr)oxides both represent high-potential acceptors, from the viewpoint of the inner membrane, and our data suggest that this variable influences at least one portion of the electron transfer pathway. As we detect evidence for at least one other pathway in this work, and the *G. sulfurreducens* genome appears to encode at least four other inner membrane quinone oxidoreductases, redox potential differences may help to explain additional complexity of *Geobacter*. Possessing protein machinery able to extract the full advantage from minerals as they descend through the redox tower also helps explain the dominance of these metal-reducing organisms in contaminated subsurface environments undergoing rapid redox potential changes.

In order to characterize and biochemically dissect extracellular electron transfer in dissimilatory metal-reducing organisms such as *G. sulfurreducens*, closer attention may be needed to be paid not just to crystal structure, but the actual redox potential experienced by the organisms. Commonly synthesized Fe-(oxyhydr)oxides prepared by precipitation of Fe(III) can produce very different rates of reduction or mutant phenotypes depending on the age of the material and the length of time one is willing to incubate cells. A distinction between initial rates of Fe(III)-(oxyhydr)oxide reduction, where higher-potential conditions exist, and final extent of reduction achieved by slower but lower potential pathways may help separate these confounding effects. As Fe(II) begins to accumulate during the course of Fe(III)-(oxyhydr)oxide reduction by metal-reducing microorganisms, the redox potential will change as the ratio of Fe(II)/Fe(III) changes and as mineral structure is altered in response in increased Fe(II) concentration. One would also expect significant differences in washed cell suspensions, where the degree of respiratory coupling to growth is not part of the assay, as electron flow through the ImcH- and CbcL-dependent pathways support different growth rates and yields.

It now appears that in *G. sulfurreducens* a transition from an ImcH-dependent electron transfer pathway to a CbcL-dependent pathway occurs both when electrodes and commonly used Fe(III)-(oxyhydr)oxides are provided as sole terminal electron acceptors. The initial rate and total extent of Fe(III)-(oxyhydr)oxide reduction may also be the result of a series of redox potential dependent processes in environmental samples, with each step supporting different cell yields. Competition in the environment could occur along any part of this continuum, rewarding those capable of rapid growth when the Fe(III)-(oxyhydr)oxide is high potential, or those able to survive with electron acceptors at relatively low redox potentials. The prevalence of ImcH and CbcL homologs in other dissimilatory metal-reducing organisms and in metageneomics data from sites undergoing active metal reduction (Levar et al., 2014; Zacharoff et al., 2016) suggests that this type of redox discrimination could also occur in diverse organisms, and provides opportunities for further exploration of redox potential dependent respiration in these organisms and their environments.

**Conflict of Interest**

The authors declare no conflict of interest.
Acknowledgements

CEL was supported by the State of Minnesota’s MNDrive program, DRB was partially supported by the Office of Naval Research grant N000141210308, AJD was supported by UMN UROP, and CLH and BMT were supported by the State of Minnesota’s LCCMR program.

References

Aklujkar MA, Coppi MV, Leang C, Kim BC, Chavan MA, Perpetua LA et al. (2013). Proteins involved in electron transfer to Fe(III) and Mn(IV) oxides by Geobacter sulfurreducens and Geobacter uranireducens. Microbiology 159: 515–535.

Bledau C, Knorr KH. (2006). Experimental inflow of groundwater induces a ‘biogeochemical regime shift’ in iron-rich and acidic sediments. J Geophys Res 111: 1–12.

Bonneville S, Van Cappellen P, Behrends T. (2004). ‘

Bonneville S, Van Cappellen P, Behrends T. (2004). ‘

Flynn TM, O’Loughlin EJ, Mishra B, DiChristina TJ, Kenmer KM. (2014). Sulfur-mediated electron shuttling during bacterial iron reduction. Science 344: 1039–1042.

Cutting RS, Coker VS, Fellowes JW, Lloyd JR, Vaughan DJ. (2009). Mineralogical and morphological constraints on the reduction of Fe(III) minerals by Geobacter sulfurreducens. Geochim Cosmochim Acta 73: 4004–4022.

Green J, Paget MS. (2004). Bacterial redox sensors. Nat Rev Microbiol 2: 954–966.

Hansel CM, Lentini CJ, Tang Y, Johnston DT, Wankel SD, Jardine PM. (2015). Dominance of sulfate-fueled iron oxide reduction in low-sulfate freshwater sediments. ISME J 9: 2400–2412.

Kostka JE, Nealson KH. (1995). Dissolution and reduction of magnetite by bacteria. Environ Sci Technol 29: 2535–2540.

Leang C, Coppi MV, Lovley DR. (2003). OmcB, a c-type polyheme cytochrome, involved in Fe(III) reduction in Geobacter sulfurreducens. J Bacteriol 185: 2096–2103.

Leang C, Qian X, Mester T, Lovley DR. (2010). Alignment of the c-type cytochrome OmcC along pili of Geobacter sulfurreducens. Appl Environ Microbiol 76: 4080–4084.

Levar CE, Chan CH, Mehta-Kolte MG, Bond DR. (2014). An inner membrane cytochrome required only for reduction of high redox potential extracellular electron acceptors. mBio 5: e02034–14.

Lovley DR, Phillips EJ. (1986). Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl Environ Microbiol 51: 683–689.

Lovley DR, Phillips EJ. (1987). Rapid assay for microbially reducible ferric iron in aquatic sediments. Appl Environ Microbiol 53: 1536–1540.

Majzlan J. (2011). Thermodynamic stabilization of hydrous ferric oxide by adsorption of phosphate and arsenate. Environ Sci Technol 45: 4726–4732.

Majzlan J. (2012). Minerals and aqueous species of iron and manganese as reactants and products of microbial metal respiration. In: Gescher J, Kappler A (eds), Microbial Metal Respiration; From Geochemistry to Potential Applications. 1st edn. Springer: New York, NY, USA; pp 1–28.

Majzlan J, Navrotsky A, Schwertmann U. (2004). Thermodynamics of iron oxides: part III. Enthalpies of formation and stability of ferrihydrite (~Fe(OH)₃), schwertmannite (~Fe₃O₄), and e-Fe₂O₃. Geochim Cosmochim Acta 68: 1049–1059.

Marsili E, Rolleffson JB, Baron DB, Hozalski RM, Bond DR. (2008). Microbial biofilm voltammetry: direct electrochemical characterization of catalytic electrode-attached biofilms. Appl Environ Microbiol 74: 7329–7337.

Michel FM, Ehm L, Antao SM, Lee PL, Chuapas PJ, Liu G et al. (2007). The structure of ferrihydrite, a nanocrystalline material. Science 316: 1726–1729.

Navrotsky A, Mazeina L, Majzlan J. (2008). Size-driven structural and thermodynamic complexity in iron oxides. Science 319: 1635–1638.

Nealson KH, Myers CR. (1992). Microbial reduction of manganese and iron: new approaches to carbon cycling. Appl Environ Microbiol 58: 439–443.

Nealson KH, Saffarini D. (1994). Iron and manganese in anaerobic respiration: environmental significance, physiology, and regulation. Annu Rev Microbiol 48: 311–343.

Norset S, Laskov C, Haderlein SB. (2013). Electron transfer between iron minerals and quinones: estimating the reduction potential of the Fe(III)-goethite surface from AQDS speciation. Environ Sci Technol 47: 14161–14168.

Post JE, Veihlen DR. (1990). Crystal structure determinations of synthetic sodium, magnesium, and potassium birnessite using TEM and the Rietveld method. Am Mineral 75: 477–489.

Raven KP, Jain A, Loeppert RH. (1998). Arsenite and arsenate adsorption on ferrihydrite: kinetics, equilibrium, and adsorption envelopes. Environ Sci Technol 32: 344–349.

Regenspurg S, Brand A, Peiffer S. (2004). Formation and stability of schwertmannite in acidic mining lakes. Geochim Cosmochim Acta 68: 1185–1197.

Rodon EE. (2006). Geochemical and microbiological controls on dissimilatory iron reduction. Comptes Rendus Geosci 338: 456–467.

Rodon EE, Urrutia MM, Mann CJ. (2000). Bacterial reductive dissolution of crystalline Fe (III) oxide in continuous-flow column reactors. Appl Environ Microbiol 66: 1062–1065.

Rodon EE, Zachara JM. (1996). Microbial reduction of crystalline Iron(III) oxides: influence of oxide surface area and potential for cell growth. Environ Sci Technol 30: 1618–1628.

Rolleffson JB, Levar CE, Bond DR. (2009). Identification of genes involved in biofilm formation and respiration via mini-Himar transposon mutagenesis of Geobacter sulfurreducens. J Bacteriol 191: 4207–4217.

Rolleffson JB, Stephen CS, Tien M, Bond DR. (2011). Identification of an extracellular polysaccharide network essential for cytochrome anchoring and biofilm formation in Geobacter sulfurreducens. J Bacteriol 193: 1023–1033.

Russel JB, Cook GM. (1995). Energities of bacterial growth: balance of anabolic and catabolic reactions. Microbiol Rev 59: 48–62.
Sander M, Hofstetter TB, Gorski CA. (2015). Electrochemical analyses of redox-active iron minerals: a review of non-mediated and mediated approaches. *Environ Sci Technol* **49**: 5862–5878.

Schwertmann U, Cornell RM. (2000). *Iron Oxides in the Laboratory: Preparation and Characterization* 2nd edn. Wiley-VCH Verlag GmbH: New York, NY, USA.

Shi L, Squier TC, Zachara JM, Fredrickson JK. (2007). Respiration of metal (hydr)oxides by *Shewanella* and *Geobacter*: a key role for multihaem c-type cytochromes. *Mol Microbiol* **65**: 12–20.

Thamdrup B. (2000). Bacterial manganese and iron reduction in aquatic sediments. In: Schink B (Ed), *Advances in Microbial Ecology*. 16th edn. Springer: New York, NY, USA; pp 41–84.

Thauer RK, Jungermann K, Decker K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* **41**: 100–180.

Yoho RA, Popat SC, Torres CI. (2014). Dynamic potential-dependent electron transport pathway shifts in anode biofilms of *Geobacter sulfurreducens*. *ChemSusChem* **7**: 3413–3419.

Zacharoff L, Chan CH, Bond DR. (2016). Reduction of low potential electron acceptors requires the CbcL inner membrane cytochrome of *Geobacter sulfurreducens*. *Bioelectrochemistry* **107**: 7–13.

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/4.0/