An Inner Membrane Cytochrome Required Only for Reduction of High Redox Potential Extracellular Electron Acceptors

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ABSTRACT Dissimilatory metal-reducing bacteria, such as Geobacter sulfurreducens, transfer electrons beyond their outer membranes to Fe(III) and Mn(IV) oxides, heavy metals, and electrodes in electrochemical devices. In the environment, metal acceptors exist in multiple chelated and insoluble forms that span a range of redox potentials and offer different amounts of available energy. Despite this, metal-reducing bacteria have not been shown to alter their electron transfer strategies to take advantage of these energy differences. Disruption of imcH, encoding an inner membrane c-type cytochrome, eliminated the ability of G. sulfurreducens to reduce Fe(III) citrate, Fe(III)-EDTA, and insoluble Mn(IV) oxides, electron acceptors with potentials greater than 0.1 V versus the standard hydrogen electrode (SHE), but the imcH mutant retained the ability to reduce Fe(III) oxides with potentials of ≤−0.1 V versus SHE. The imcH mutant failed to grow on electrodes poised at +0.24 V versus SHE, but switching electrodes to −0.1 V versus SHE triggered exponential growth. At potentials of ≤−0.1 V versus SHE, both the wild type and the imcH mutant doubled 60% slower than at higher potentials. Electrodes poised even 100 mV higher (0.0 V versus SHE) could not trigger imcH mutant growth. These results demonstrate that G. sulfurreducens possesses multiple respiratory pathways, that some of these pathways are in operation only after exposure to low redox potentials, and that electron flow can be coupled to generation of different amounts of energy for growth. The redox potentials that trigger these behaviors mirror those of metal acceptors common in subsurface environments where Geobacter is found.

IMPORTANCE Insoluble metal oxides in the environment represent a common and vast reservoir of energy for respiratory microbes capable of transferring electrons across their insulating membranes to external acceptors, a process termed extracellular electron transfer. Despite the global biogeochemical importance of metal cycling and the ability of such organisms to produce electricity at electrodes, fundamental gaps in the understanding of extracellular electron transfer biochemistry exist. Here, we describe a conserved inner membrane redox protein in Geobacter sulfurreducens which is required only for electron transfer to high-potential compounds, and we show that G. sulfurreducens has the ability to utilize different electron transfer pathways in response to the amount of energy available in a metal or electrode distant from the cell.

Geobacter sulfurreducens is a model dissimilatory metal-reducing anaerobe able to completely oxidize organic compounds inside the cell and transfer the resulting electrons to terminal acceptors beyond the outer membrane (1, 2). Extracellular electron acceptors utilized by G. sulfurreducens include chelated transition metals, particulate Fe(III) and Mn(IV) oxides (2), and electrodes poised at oxidizing redox potentials (3). Geobacter representatives are abundant in anoxic metal-reducing habitats, including aquatic sediments (2), Fe(III)-rich petroleum-contaminated sites (4), zones where U(VI) reduction is stimulated by organic acid addition (5), subsurface aquifers where Fe(III) reduction releases arsenic into drinking water (6, 7), and on electrodes used to produce electrical energy (8, 9). Despite their contribution to global biogeochemical processes and emerging biotechnological applications, the molecular mechanism for electron transfer across the inner membrane of Geobacter is not known, and there is no respiratory protein-based marker for monitoring the activity of these ubiquitous metal-reducing bacteria in their natural environment.

Part of the difficulty in studying Geobacter stems from the diversity of redox proteins potentially utilized by these organisms for respiration. Geobacter genomes typically encode 60 to 90 multiheme c-type cytochromes, few of which are conserved between all species (10). This is in stark contrast to another well-studied metal-reducing family, the Shewanellaceae. This group of facultative anaerobes encodes a single inner membrane NapC/NirT family tetraheme cytochrome (CymA) (11) that passes electrons to outer membrane conduits comprised of two decaheme c-type cytochromes (12–14). Synthesis of all proteins involved in the Shewanella pathway is simply induced by a shift to anaerobic conditions, rather than the presence of metals, and deletion of the CymA inner membrane cytochrome eliminates growth with all extracellular electron acceptors (11, 15). In contrast, G. sulfurreducens exhibits a complex transcriptional response to different...
extracellular electron acceptors (16–19), and no single deletion eliminates electron transfer to all electron acceptors (20, 21).

Despite this evidence for complexity, published models of the *Geobacter* electron transport chain invoke a single Shewanella-like route from the quinone pool to an array of outer surface proteins able to interact with soluble compounds, which then are suggested to pass electrons further to extracellular proteins interacting with larger acceptors (22–24). If such models are true, mutants defective in reduction of soluble metals, such as Fe(III) citrate, should also be defective in utilization of all insoluble acceptors, such as Fe(III) oxides. Such a hypothesis is challenged by directed mutant (20, 25) and transposon mutagenesis (21) studies that continue to find *G. sulfurreducens* mutants defective in electron transfer to only a subset of extracellular acceptors. The array of *Geobacter* mutant phenotypes argues against a simple single pathway, as well as models where different proteins are required based on solubility (chelates versus oxides) or metal content (Fe versus Mn) of the acceptor.

In this report, we describe how redox potential explains many of these discrepancies, through discovery of ImcH, an inner membrane c-type cytochrome in *G. sulfurreducens*. The *imcH* mutant reduced insoluble Fe(III) oxides yet could not reduce insoluble Mn(IV) oxides or chelated Fe(III). Because of the low electron-accepting potential of Fe(III) oxides relative to these other compounds, poised electrodes were used to investigate the role of redox potential. A switch from high to low potential induced respiration of the *imcH* mutant but only when electrodes were poised at a sufficiently low redox potential of \(-0.1\) V (versus the standard hydrogen electrode [SHE]). These experiments are consistent with different pathways being used by *G. sulfurreducens* for transfer of electrons out of the quinone pool to low- versus high-potential acceptors and suggests a mechanism for sensing the redox potential of extracellular objects. These findings also provide a molecular explanation for recent electrochemical evidence supporting at least two separate electron transfer pathways out of *Geobacter* electrode-grown cells (26). Since Fe(III) naturally occurs in up to 15 different oxide or oxyhydroxide forms (27), spanning more than half a volt of redox potential (28, 29), multiple electron transfer pathways could be utilized by *Geobacteraceae* in response to the energy available in environmentally relevant metals and provide an explanation for cytochrome diversity in these organisms.

**RESULTS**

**ImcH is an inner membrane multiheme c-type cytochrome.** In a modified transposon mutagenesis protocol (21), 18 independent mutants containing insertions in the locus GSU3259 which were unable to grow with soluble Fe(III) citrate as the electron acceptor were found (Fig. 1A). The protein disrupted in these mutants was predicted to contain up to three transmembrane helices (depending on processing of a putative signal-anchor), a region of NapC/NirT homology, and up to 7 c-type heme binding motifs (Fig. 1B). Since NapC/NirT-family proteins typically transfer electrons from the quinone pool to periplasmic acceptors (11, 30) and no such enzyme has been described for *Geobacter* spp., this protein was targeted for further study. By fusing a C-terminal polyhistidine tag to genomic GSU3259, we confirmed that the protein was expressed even during growth with the non-metal acceptor fumarate and colocализed with succinate dehydrogenase in membranes separated via sucrose gradient centrifugation (Fig. 1C). This was consistent with proteomic surveys which reported that the native protein is localized to the inner membrane (18), as well as software predictions (PSORTb, v 3.0.2) (31). Based on these results, GSU3259 was named *imcH* (inner membrane cytochrome H).

**Deletion of imcH eliminates electron transfer to soluble Fe(III).** When the monocistronic *imcH* gene was replaced with a kanamycin resistance cassette, growth with fumarate as the electron acceptor was unaffected (Fig. 2A). The Δ*imcH::Kan* mutant retained the ability to attach to polystyrene plates in crystal violet biofilm assays (92% ± 10.3% of the wild-type level [\(n = 4\)]) and fumarate-grown mutant cells did not show differences in major c-type cytochromes commonly visualized by staining with 3,3′,5,5′-tetramethylbenzidine.

Consistent with the transposon mutagenesis findings, the Δ*imcH::Kan* mutant was unable to grow with chelated Fe(III) as the electron acceptor (Fig. 2B) and could not reduce Fe(III) citrate in washed-cell assays (Fig. 2C). Complementation with *imcH* in trans restored the mutant’s ability to reduce Fe(III) citrate (Fig. 2C).

Experiments were performed to test if the inability to reduce Fe(III) citrate was due to toxicity, rather than a defect in electron

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**FIG 1** Domain structure and inner membrane localization of ImcH. (A) The monocistronic *imcH* gene was interrupted 18 times using transposon mutagenesis (black triangles). All transposon mutants in this gene failed to reduce soluble Fe(III) citrate. (B) ImcH has three putative transmembrane helices (gray bars), seven putative c-type heme motifs (black bars), and a region of homology to NapC/NirT-like quinone oxidoreductases (gray box). The first putative c-type heme motif is CXXCH. (C) Sucrose-gradient separated ImcH-His×6 membrane fractions, tested for succinate dehydrogenase (SDH) activity as a marker of inner membranes, and probed using an anti-6×His antibody. ImcH was detected only in fractions with SDH activity.
transferred. Wild-type and ΔimcH:Kan’ cells using fumarate as the electron acceptor grew at identical rates after addition of Fe(III) citrate. Mutants lacking imcH failed to reduce Fe(III) even after hours of incubation. Further assays demonstrated that ΔimcH:Kan’ mutants also could not reduce Fe(III)-EDTA, consistent with a defect in electron transfer rather than toxicity due to iron or a specific chelator.

Deletion of imcH eliminates electron transfer to extracellular Mn(IV) oxides but not Fe(III) oxides. In addition to their inability to transfer electrons to soluble acceptors, ΔimcH:Kan’ cells also could not reduce laboratory-synthesized Mn(IV) oxides (birnessite) (Fig. 3A). This lack of reduction was observed using Mn(IV) oxide concentrations between 5 and 50 mM, with inoculation sizes ranging from an optical density at 600 nm (OD600) of 0.005 to 0.05, suggesting that this was again related to electron transfer and not due to Mn(IV) toxicity or growth inhibition by the metal.

Based upon the general defect with chelated Fe(III) and insoluble Mn(IV), it was expected that the imcH mutant also would not reduce insoluble Fe(III) oxides. Unexpectedly, laboratory-synthesized Fe(III) oxides were reduced by the ΔimcH:Kan’ mutant (Fig. 3B). Using the freshly precipitated Fe(III) oxide ferrihydrite, a minor lag was observed in the first days of Fe(III) reduction by the mutant, but rates and extents converged within 1 week (n = 4). The same phenotype of wild-type Fe(III) reduction by ΔimcH:Kan’ cells was observed using goethite, a more crystalline form of Fe(III) oxide (Fig. 3C). The observation that deletion of a cytochrome prevented reduction of insoluble Mn(IV) oxides but not insoluble Fe(III) oxides contradicted a model where electron acceptors beyond the outer membrane are reduced by similar machinery. Since mutant cultures reduced some forms of Fe(III) (oxides) but not others (chelates), the phenotype also did not support a model where soluble metal reductions handed off electrons to more distant proteins able to interact with all insoluble metals.

In these experiments, a trend did emerge; the imcH mutant could not reduce electron acceptors with high redox potentials, such as Fe(III)-EDTA (+0.1 V), Fe(III) citrate (+0.38 V), and Mn(IV) oxides (> +0.4 V). The two Fe(III) oxides still reduced by the mutant represented much lower redox potentials, reported to be in the range of −0.2 to 0 V (28, 29, 32). With less energy available per electron to pump protons or drive reduction, we hypothesized that Fe(III) oxides might require a different electron transfer chain and that ImcH was not essential with these low-potential acceptors. The ability to alter electrode potentials in electrochemical reactors while other variables remain constant allowed for the direct testing of this hypothesis.

Mutants lacking imcH can transfer electrons only to electrodes poised to mimic low-potential extracellular electron acceptors. To examine the effect of redox potential on the ΔimcH:Kan’ mutant, poised electrodes were used as electron acceptors. The redox potential typically chosen for bioelectrochemical experiments is relatively high (+0.2 to +0.4 V) (33–35), since electrode-based studies are often aimed at providing an unlimited electron sink for bacteria (34, 36). The ΔimcH:Kan’ mutant was unable to transfer electrons to an electrode held at this standard potential (+0.24 V), while the wild type grew with a maximum doubling time of ~6 h to a value of 400 μA/cm² within 3 days (Fig. 4A).

In contrast, when electrodes were poised at a lower redox potential (− 0.1 V versus SHE), growth of the ΔimcH:Kan’ mutant occurred. Mutant cells grew at rates identical to that of wild type controls exposed to the same low redox potential. However, at this
lower potential, the exponential growth rates of both the mutant and the wild type were slower, with current doubling every ~9 h, and both cultures achieved a similar current density of ~150 μA/cm² within 3 days (Fig. 4B). When imcH mutants were exposed to low potentials to establish a biofilm capable of the slower growth rate and then switched to high potentials, the slow growth rate persisted (see Fig. S1 in the supplemental material).

Additional experiments provided evidence that only specific redox potentials triggered respiration. First, the precision of electrodes allowed testing of whether growth gradually improved as
potential was lowered or if there was a threshold required to induce growth of cells lacking imcH. External potentials of $V_1 = 0.24$ V, $V_2 = 0.1$ V, and 0 V did not support growth of imcH mutants (Fig. 4A and C). In contrast, lowering the redox potential by only 100 mV further, to $V_3 = 0.1$ V, triggered growth (Fig. 4C).

Failure to grow on electrodes could hypothetically be caused by an inability of cells to attach to surfaces (37). To test if attachment played a role, imcH mutants were incubated with high-potential ($V_1 = 0.24$ V) electrodes, and planktonic cells were washed from the chamber prior to a switch to lower potential ($V_2 = 0.1$ V). Growth of imcH mutants was again triggered after the potential switch and produced responses similar to data shown in Fig. 4C, showing that cells attached during the high-potential phase. Additionally, electrodes harvested after 12-h incubations had similar levels of attached protein at both permissive ($V_3 = 0.1$ V, $18.9 \pm 0.4$ μg/electrode; $n = 2$) and nonpermissive (0.0 and 0.1 V, $22.40 \pm 3.4$ and $15.11 \pm 3.9$ μg/electrode, respectively; $n = 2$) potentials. These data confirmed that the mutation did not affect binding of cells to electrodes and indicated that the redox potential of a solid electron acceptor could be the sole signal causing $G. sulfurreducens$ to alter its electron transfer pathway.

DISCUSSION

These results show that $G. sulfurreducens$ cannot transfer electrons to high redox potential electron acceptors without the inner membrane multiheme c-type cytochrome ImcH. However, after exposure to low redox potentials, either in the form of certain Fe(III) oxides or poised electrodes, imcH mutants are able to utilize an alternative pathway that enables respiration and growth. The growth rate of wild-type and mutant cultures at lower redox potentials is slower, supporting the hypothesis that these strategies are distinct and that cells generate less ATP per electron when using lower-energy strategies. Based on these data, $G. sulfurreducens$ has electron transfer pathways which can be differentially utilized in response to the redox potential of an extracellular electron acceptor, such as a metal oxide or an electrode. Since the potentials of many environmentally relevant metals lie on opposite sides of the threshold able to trigger this choice, these findings help explain conflicting $Geobacter$ mutant phenotypes, suggest that genetic markers may exist for monitoring subsurface redox conditions, and imply that bacteria can sense the redox potential of terminal electron acceptors.

With its transmembrane helices, N-terminal NapC/NirT homology, and c-type hemes, ImcH represents a new family of bacterial redox proteins implicated in extracellular electron transfer. Homologs of ImcH are present in all $Geobacteraceae$ isolated for a metal-reducing phenotype, as well as related metal-reducing $Anaeromyxobacter$ spp., but are notably absent in fermentative ($Pelobacter$ spp.) and chlororespiratory ($Geobacter lovleyi$) members of this cluster (Fig. 5). Homologs of imcH are found in the Acidobacteria and Planctomycyes-Verrumicrobia-Chlorobium phyla, which contain Fe(III)-reducing genera ($Geothrix$ and $Melioribacter$, respectively) (38, 39), suggesting that other relatives should be reinvestigated for their respiratory abilities (Fig. 5). With the increasing availability of single-cell and metagenomic sequences, imcH may serve as an aid for prediction of respiratory strategies, but discovery of what proteins interact with ImcH will greatly improve sequence-based predictions.

In the laboratory, electrochemical systems are routinely used to study electron transfer kinetics (3, 36), biofilm development (21, 37, 40), and long range electron flow between metal-reducing bacteria (40–42), with the justification that these insights help
explain reduction of environmentally relevant metal oxides. These data show that most electrodes are not poised at the proper potential to mimic electron transfer to poorly crystalline Fe(III) oxides, such as ferricyanide (Fig. 6), typically the largest reservoir of reducible Fe(III) in sediments and aquifers (27). Based on recent measurements, the redox potential of laboratory goethite is ~0.17 V versus SHE (43), and ferricyanide/Fe(II) mixtures begin at ~0.0 V versus SHE but decrease rapidly to ~0.1 V or lower as Fe(II) accumulates in the medium (28, 32, 44). The difference between high-potential laboratory electrode experiments and environmental Fe(III) oxide electron acceptors may explain how outer membrane cytochromes, such as OmcS or OmcB, can appear to be important to reduction of Fe(III) oxides but not electrodes and lead to confusion when proposing electron transfer models (33, 45, 46).

Looking forward, the observation that growth at low redox potential acceptors requires different proteins and induces a characteristically lower growth rate produces a number of hypotheses which can be tested in Geobacter. First, multiple inner membrane quinone oxidoreductases must exist in *G. sulfurreducens*, with at least one that is essential for low-potential respiration. These low-potential pathways should demonstrate a lower midpoint potential for electron transfer and result in a lower H+/e− stoichiometry than the ImcH-dependent pathway. At least five candidates for alternative quinone oxidoreductases exist in the *G. sulfurreducens* genome, most containing b-type cytochromes next to periplasmic c-type cytochromes. These "**c**bc family" proteins are among the most conserved within the metal-reducing *Geobacteraceae* (10). Unlike the case of ImcH, which proteomic studies show to be abundant under all conditions (17, 18), **c**bc family protein levels are often altered in the presence of different metals (20), and mutants lacking these proteins yield intermediate phenotypes depending on the metal acceptor (21). It is possible that studies attempting to uncover the role of **c**bc family proteins were confounded by the presence of other electron transfer pathways, such as the ImcH-dependent pathway, and the need to control redox potentials more precisely to observe phenotypes.

A second hypothesis is that each inner membrane cytochrome interacts with separate periplasmic and/or outer membrane redox proteins to create independent conduits out of the cell, based on external redox potential. This could explain why different acceptors induce differential expression of the five periplasmic trhene c-type cytochromes (PpcA to -E), why there are many outer membrane cytochromes of *G. sulfurreducens*, and why strains evolved to overcome some cytochrome deletions regain only 60% of the wild-type growth rate after mutations that lead to expression of repressed or cryptic cytochromes (46). With the identification of ImcH, it may be possible to construct strains lacking one or more routes out of the *Geobacter* cell and study electron transfer pathways independently of each other.

In nearly every microbial respiration, separate strategies are utilized in response to thermodynamic constraints. Oxygen is reduced by multiple terminal oxidases, depending on available concentrations (47). Nitrate reductases exist in the periplasm or cytoplasm, leading to differences in energy conservation (48). Different methanogens compete at distinct thermodynamic thresholds of hydrogen and acetate (49). Since their discovery, metal-reducing bacteria have been outliers, despite the fact that for every 0.5-V difference in redox potential [such as with Mn(IV) oxides versus Fe(III) oxides], enough energy is available to make at least one extra ATP/2e−. These new findings show that Geobacter also has a response to this thermodynamic challenge. By sensing and responding to the energy available in external surfaces, *Geobacter*’s respiratory strategy may provide a competitive advantage in subsurface and syntrophic habitats where redox potential, rather than substrate concentration, can be the dominant environmental variable.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *Geobacter sulfurreducens* PCA (ATCC 51573) was cultivated for each experiment from colony picks grown from freezer stocks in anaerobic minimal medium (NB salts as described in reference 21) with acetate as the electron donor, pH 6.8. When ferricyanide (~70 mM) or goethite (~20 mM) was added as the electron acceptor, 0.69 g/liter NaH₂PO₄·H₂O stabilized synthesized minerals. Birmesite (20 mM) was added after autoclaving. All medium was buffered with 2 g/liter NaHCO₃ made anaerobic by flushing with oxygen-free N₂-CO₂ (80:20, vol/vol), and sealed with butyl rubber stoppers. All strains used in this study can be found in Table 1.

**Deletion of imcH.** *imcH* (GSU3259) was replaced with a kanamycin resistance cassette (50) using the primers 3259KP1/3259KP2 and 3259KP3/3259KP4 to generate fragments with homology to regions up- and downstream of *imcH*. Primerless fusion with a kanamycin resistance cassette yielded a fragment that was electroporated into wild-type *G. sulfurreducens* (51). Integration was verified by PCR amplification across the region. All primers used in this study can be found in Table 1.

**Generation of imcH complement.** The primers 3259CompF and 3259CompR were used to amplify *imcH* from wild-type DNA. The resulting fragment was digested with XbaI plus BamHI, ligated into pSRKGm (52), and transformed into *Escherichia coli* DH5α. The purified plasmid
was used to transform *E. coli* WM3064 (53), which was mated on a 0.2-μm filter with the ΔimcH::Kanr mutant and recovered as previously described (37).

**Generation of chromosomal C-terminal polyhistidine-tagged ImcH (ImcH-His×6).** The primers 3259KHis1 and 3259KHis2 amplified an ~300-bp fragment of the 3' end of *imcH*. Primers 3259KHis5 and 3259KHis6 were used to amplify genomic DNA downstream of *imcH*, and 3259KHis3 and 3259KHis4 were used to amplify a kanamycin resistance cassette with ends homologous to the amplified products described above. Gel-purified products were fused using primerless PCR, and the linear fragment was electroporated into *G. sulfurreducens* cells (51). Integration of the fragment was assessed by PCR amplification across the region. Cells containing the genomic poly-His fusion were verified to retain the ability to reduce Fe(III) citrate using the methods described below.

### TABLE 1 Strains, plasmids, and primers used in this work

| Name                            | Relevant characteristic or sequence | Source or usage               |
|---------------------------------|------------------------------------|--------------------------------|
| **Geobacter sulfurreducens**    |                                    |                                |
| Wild type                       | ATCC 51573                         | Caccavo et al. (2)             |
| ΔimcH::Kanr strain              | Replacement of GSU3259 with a kanamycin resistance cassette | This study                     |
| imcH-His×6 strain               | C-terminally polyhistidine-tagged ImcH | This study                     |
| **Escherichia coli**            |                                    |                                |
| DH5α                            | Host for cloning                   | Invitrogen                     |
| WM3064                          | Donor strain for conjugation: thrB1004 pro thi rpsL lacD151 RP4-1360 Δ(araBAD)::ΔapA1341::erm pir(wh) ] | Saltikov and Newman (53) |
| **Plasmids**                    |                                    |                                |
| pSRKGm                          | Empty vector for complementation   | Khan et al. (52)               |
| pimcH                           | Complementation vector-*imcH* in pSRKGm | This study                     |
| pBBR1MCS-2                      | Source of kanamycin resistance cassette | Kovach et al. (50)             |
| **Primers**                     |                                    |                                |
| 3259K1                          | CGTCTGGGGGGTCGGGTTTCGC             | Upstream fragment for gene replacement |
| 3259K2                          | AGCTTGCAATTCCGGTTCGCTTTTCTCTCAATTTGAAAGCGC | Upstream fragment for gene replacement, homology to Kan in italics |
| 3259K3                          | GCCTTCTTGACGAGTTTCTCTGAAGCAGTCACGCGGCTCA | Downstream fragment for gene replacement, homology to Kan in italics |
| 3259K4                          | GTCCGACACACGGGGGGGACT              | Downstream fragment for gene replacement |
| KanF                            | AACCGGAAACCGGAATGCTGAGCT           | Kanamycin cassette from pBBR1MCS-2 |
| KanR                            | TCAGAAGAACTCGTCAAGAAGGCC           | Kanamycin cassette from pBBR1MCS-2 |
| 3259CompF                       | CGCGTTCTAGAGTTAAAAAAGGGTTTCAAAAGTGAGG | GSU3259 for complementation, restriction site underlined |
| 3259CompR                       | TAGACGGATCTACGTGTTGCCGGGGGC        | GSU3259 for complementation, restriction site underlined |
| 3259KHis1                       | CCCCGGATAGAGTCGTCACGTCT           | 3' end of GSU3259 for C-terminal polyhistidine tag |
| 3259KHis2                       | GCTCAATGTGATGTTGATGTTGATGTTGCCGGTCCGCTCGTCTGCTGCTGCTGG | 3' end of GSU3259 for C-terminal polyhistidine tag, polyhistidine tag in italics |
| 3259KHis3                       | CATCACATTCAATCACATGAGACGGAAGCCGAAACCGGAATGCCAGCTG | Kanamycin resistance cassette, polyhistidine tag in italics |
| 3259KHis4                       | CGGGGGGGTTTGTGCTGTCAGCCGGCTGTCAAGAAGACTCGTGAAGACGGC | Kanamycin resistance cassette |
| 3259KHis5                       | GCCCTTCTGACGAGTTCTCTGACAGCGGCCTGCAGCAAGGCAAGGGCG | Downstream region of GSU3259 for C-terminal polyhistidine tag |
| 3259KHis6                       | CGGAAGATCATCAGACACAAA              | Downstream region of GSU3259 for C-terminal polyhistidine tag |

*Primers were used to amplify the product listed, and were used as described in Materials and Methods.*
20 h. Fractions were diluted extensively and filter concentrated through centrifugation.

Protein was quantified using the bicinchoninic acid (BCA) assay (Thermo Scientific Pierce, Waltham, MA). Succinate dehydrogenase activity was assayed anaerobically in the presence of the phenazine methosulfate (PMS) (40 μM) and 2,6-dichlorophenolindophenol (DCPIP) (50 μM) by monitoring the absorbance at 600 nm, after addition of succinate. Protein fractions were resolved on 12% polyacrylamide gels and stained with Coomassie Brilliant Blue R-250 or gold-stained with silver nitrate.

**Reduction of Fe(III) and Mn(IV).** For soluble Fe(III) growth assays, late-log-phase cells were incubated with Fe(III) or Mn(IV) at 30°C using 10 mM acetate as the electron donor. Fe(II) and Mn(II) was determined using a colorimetric assay (54). For washed-cell assays, cells were washed 3× with centrifugation at 3,700 × g for 10 min using an anaerobic minimal medium lacking ammonium, phosphate, and sulfate, with 100 mM NaCl to maintain osmotic pressure. Wild-type and mutant cells were incubated at an OD₆₀₀ of 0.05 with Fe(III) citrate (50 mM) and 2,6-dichlorophenolindophenol (DCPIP) (Thermo Scientific Pierce, Waltham, MA).

**Electrochemical analysis.** Three-electrode bioreactors were prepared using working graphite electrodes polished with 1,500-grit wet/dry sandpaper (Ali Industries Inc., Fairborn, OH) and sonicated for 1 h in deionized (DI) water with multiple water exchanges to remove particulates. Reactors contained Pt counter electrodes and calibrated Calomel reference electrodes (34). Sterile anaerobic reactors were poised at the shown potentials using a VMP3 multichannel potentiostat, and a 50% (vol/vol) volume of DI water 0.50 cells entering acceptor limitation was used to initiate experiments with 20 mM acetate as the electron donor. Reactors were purged with humidified 80%/20% N₂/CO₂ passed over a heated copper column to remove trace oxygen.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02034-14/-/DCSupplemental.

**ACKNOWLEDGMENTS**

C.L.E., M.G.M-K., and D.R.B. were supported by Office of Science (BER), U.S. Department of Energy, DE-SC0006868. C.E.L. was supported by the Office of Naval Research, N000141210308.

Special thanks go to Evan Brutinel and Jeffrey Granick for aid in identifying initial transposon mutants.

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