Gene clusters encoding putative outer membrane electron conduits have specific roles during metal and electrode respiration in *Geobacter sulfurreducens*.

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**Short title:** Electron transfer across the outer membrane of *G. sulfurreducens*

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At least five gene clusters in the *Geobacter sulfurreducens* genome encode putative outer membrane 'electron conduits', which are redox active complexes containing a periplasmic multiheme c-cytochrome, integral outer membrane β-barrel, and outer membrane redox lipoprotein. Single gene-cluster deletions and all possible multiple deletion mutant combinations were constructed and grown with graphite electrodes poised at +0.24 V and -0.1 V vs. SHE, Fe(III)- and Mn(IV)-oxides, and soluble Fe(III)-citrate. Different gene clusters were necessary for reduction of each electron acceptor. For example, only the Δ*extABCD* cluster mutant had a severe growth defect on graphite electrodes at all redox potentials, but this mutation did not affect Fe(III)-oxide, Mn(IV)-oxide, or Fe(III)-citrate reduction. During metal oxide reduction, deletion of the previously described *omcBC* cluster caused defects, but deletion of additional components in the Δ*omcBC* background, such as *extEFG*, was necessary to produce defects greater than 50% compared to wild type. Deletion of all five gene clusters was required to abolish all metal reduction. Mutants containing only one cluster were able to reduce particular terminal electron acceptors better than wild type, suggesting routes for improvement by targeting substrate-specific electron transfer pathways. Our results show *G. sulfurreducens* utilizes different membrane conduits depending on the extracellular acceptor used.
INTRODUCTION

Microorganisms capable of extracellular respiration can alter the redox state of particulate metal oxides in soils and sediments, controlling their solubility and bioavailability (Tadanier et al., 2005; N’Guessan et al., 2008; Toner et al., 2009; Williams et al., 2011; Yelton et al., 2013; Couture et al., 2015). To respire extracellular metals, bacteria must transfer electrons from the cell interior to outer surface redox proteins, requiring unique mechanisms compared to growth with soluble electron acceptors. The requirement for surface exposed electron transfer proteins also presents opportunities for transformation of heavy metals, biological nanoparticle synthesis, and a new generation of microbially-powered electrochemical devices using bacteria grown on electrodes (Bond et al., 2002; Bond and Lovley, 2003; Holmes et al., 2004; Ren et al., 2008; Logan and Rabaey, 2012; Schrader et al., 2016; Schievano et al., 2016).

An extracellular electron transfer strategy must overcome several biological and inorganic issues. In Gram negative cells, a conductive pathway capable of crossing the inner membrane, periplasm, and outer membrane must first be constructed (Gralnick and Newman, 2007; Shi et al., 2016). Because metal oxides vary widely in chemistry, surface charge, redox state, and surface area, an additional diversity of proteins may be needed to link cell surfaces with different terminal minerals (Navrotsky et al., 2008; Majzlan, 2013; Levar et al., 2017). Many metal-reducing bacteria can also transfer electrons to extracellular electrodes (Bond and Lovley, 2003; Marsili et al., 2010; Snider et al., 2012; Robuschi et al., 2013). Unlike metal oxide particles, electrodes represent an unlimited electron acceptor where cells directly in contact with the inorganic surface can support more distant daughter cells linked by a conductive network of redox proteins that relay electrons to cells at the electrode. These biological and chemical variables raise the possibility that different electron transfer proteins may be needed to access each different kind of extracellular mineral, surface, or environment.

A model organism widely studied for its ability to reduce a diversity of metals and electrodes is the δ-Proteobacterium Geobacter sulfurreducens, and recent work suggests this organism can adjust its electron transfer pathway depending on conditions. CbcL, a combination c- and b-type inner membrane cytochrome (Zacharoff et al., 2016), is only required when extracellular metals and electrodes are below redox potentials of -0.1 V vs. SHE, while the inner membrane c-type cytochrome ImcH (Levar et al., 2014), is essential when acceptors are at higher redox potentials.
Beyond the outer membrane, the secreted cytochrome OmcZ is needed only during electrode growth, while the secreted cytochrome PgcA only enhances reduction of Fe(III)-oxides (Nevin et al., 2009; Leang et al., 2010; Tremblay et al., 2011; Qian et al., 2011; Smith et al., 2014; Peng and Zhang, 2017). Separating the initial event of inner membrane proton motive force generation from the extracellular protein-mineral interaction lies the outer membrane, an insulating barrier which was recently found to also contain electron transfer proteins of surprising complexity (Richardson et al., 2012; Liu et al., 2014).

A mechanism for electron transfer across the outer membrane is through a porin-cytochrome electron conduit, consisting of an integral outer membrane β-barrel proposed to anchor a periplasmic multiheme cytochrome to an outer surface lipoprotein cytochrome. By linking heme cofactors through the membrane spanning complex, electron flow is permitted (Hartshorne et al., 2009; Richardson et al., 2012). The first electron conduit described was the ~210 kDa MtrCAB complex from S. oneidensis, which can catalyze electron transfer across membranes to extracellular substrates when purified and placed in lipid vesicles (Wang et al., 2008; Coursolle and Gralnick, 2012; White et al., 2013). The mtrCAB gene cluster is essential for reduction of all tested soluble metals, electron shuttles, metal oxides, and electrodes by S. oneidensis (Baron et al., 2009; Coursolle et al., 2010; Coursolle and Gralnick, 2012). Related porin-cytochrome complexes capped with an extracellular DMSO reductase allow Shewanella to reduce DMSO on the cell exterior, while similar conduits support electron uptake by Fe(II)-oxidizing Rhodopseudomonas TIE-1 cells (Gralnick et al., 2006; Jiao and Newman, 2007).

In G. sulfurreducens, a gene cluster encoding the periplasmic cytochrome OmbA, putative β-barrel OmaB, and lipoprotein cytochrome OmcB also forms a conduit functionally similar to MtrCAB, though the two complexes lack any sequence similarity (Liu et al., 2014). This ‘ombB-omaB-omcB’ gene cluster is duplicated immediately downstream in the G. sulfurreducens genome as the near-identical ‘ombC-omaC-omcC’, together forming the ‘omcBC’ cluster. Antibiotic cassette insertions within omcB, as well as insertions deleting the entire ‘ombB-omaB-omcB’ conduit decrease growth with Fe(III) as an electron acceptor, but the impact differs between reports and growth conditions (Leang et al., 2003; Leang and Lovley, 2005; Liu et al., 2015). This variability between studies could be due to polar effects from inserted cassettes,
partial complementation by duplicated components, or the presence of undiscovered alternative pathways that also catalyze electron transfer across the outer membrane.

Recently, genome-wide transposon data found that insertions in omcB or omcC had no effect on *G. sulfurreducens* growth with electrodes poised at -0.1 vs. SHE, a low potential chosen to mimic the redox potential of Fe(III)-oxides (Chan et al., 2017). However, transposon insertions within an unstudied four-gene cluster with porin-cytochrome signatures caused significant defects during growth on -0.1 V electrodes (Chan et al., 2017). Deletion of this cluster, named *extABCD*, severely affected growth on low-potential electrodes, while Δ*extABCD* mutants grew similar to wild type with Fe(III)-oxides. In contrast, deletion of both conduits contained in the omcBC cluster had little impact on low-potential electrode growth. These data suggested that the outer membrane pathway used for electron transfer could vary depending on environmental conditions, but also raised new questions: are different conduits required at higher redox potentials, during growth with mineral forms such as Mn(VI), or when metals become soluble?

At least five electron conduits may be encoded in the genome of *G. sulfurreducens*. Using new markerless deletion methods, this study constructed combinations of mutants lacking these gene clusters, to simultaneously compare growth using Fe(III)- and Mn(IV)-oxides, poised electrodes at two different redox potentials, and soluble Fe(III)-citrate as terminal electron acceptors. We found that only strains lacking *extABCD* showed a growth defect when electrodes were the electron acceptor, and this effect was similar at all redox potentials. A strain containing *extABCD* but lacking all other conduit clusters grew faster and to a higher final density on electrodes. Phenotypes were more complex during metal reduction. The largest defects were in ΔomcBC strains, but deletion of the newly identified cluster *extEFG* in the ΔomcBC background was needed to severely inhibit Fe(III)-reduction. Deletion of all five clusters was necessary to eliminate reduction of soluble and insoluble metals tested. Strains containing only a single cluster showed preferences for reduction of different metals, such as the *extEFG*- and *extHIJKL*-only strains performing better with Mn(IV)-oxides than Fe(III)-oxides. These data provide evidence that multiple conduit clusters in the *G. sulfurreducens* genome are functional and are utilized during electron transfer in a substrate-dependent manner.
RESULTS

Description of putative outer membrane electron conduits. At least five gene clusters can be identified in the *G. sulfurreducens* genome encoding putative porin-cytochrome electron conduits, based on three key elements: (1) a multiheme periplasmic c-type cytochrome, (2) an outer membrane β-barrel protein, and (3) one or more outer membrane lipoproteins with redox cofactors (Fig. 1A). Two of these clusters correspond to the well-studied OmcB-based (*ombB-omaB-omcB*, GSU2739-2737) conduit and its near-identical duplicate OmcC-based cluster immediately downstream (*ombC-omaC-omcC*, GSU2733-2731). For clarity, and due to the fact that *omaBC* and *ombBC* are identical, these are together referred to as the “omcBC” cluster.

The *ext* genes comprise three new clusters, named for their putative roles in extracellular electron transfer (Chan *et al.*, 2017). The *extABCD* (GSU2645-2642) cluster encodes ExtA, a periplasmic dodecaheme c-cytochrome, ExtB, an outer membrane β-barrel with 18 trans-membrane domains, and ExtCD, two outer membrane lipoprotein c-cytochromes with 5 and 12 heme binding sites, respectively. The second cluster, *extEFG* (GSU2726-2724), encodes ExtE, an outer membrane β-barrel with 21 trans-membrane domains, ExtF, an outer membrane lipoprotein pentaheme c-cytochrome, and ExtG, a periplasmic dodecaheme c-cytochrome. The final cluster, *extHIJKL* (GSU2940-2936) lacks an outer membrane c-cytochrome, but encodes ExtH, a rhodanese-family lipoprotein, ExtI, a 21 trans-membrane domain outer membrane β-barrel, ExtK, a periplasmic pentaheme c-cytochrome, and ExtJL, two small outer membrane lipoproteins.

A significant difference between *G. sulfurreducens* Ext clusters and the *S. oneidensis* Mtr conduits (Hartshorne *et al.*, 2009), is that the porin-cytochrome conduits in *S. oneidensis* are paralogs. The periplasmic MtrA and MtrD cytochromes share over 50% identity, are similar in size and heme content, and can cross complement (Coursolle and Gralnick, 2010). The lipoprotein outer surface cytochromes of *Shewanella* also demonstrate high sequence, functional, and structural conservation (Coursolle and Gralnick, 2010; Clarke *et al.*, 2011; Richardson *et al.*, 2012; Edwards *et al.*, 2012). In contrast, no component of the Ext or OmcBC complexes share any homology. For example, the predicted periplasmic c-cytochromes ExtA,
ExtG, ExtK, and OmaB vary in size from 25 to 72 kDa, contain 5 to 15 hemes, and share 18%-26% identity (Fig. 1B).

To test physiological roles of these loci, single cluster mutants were first constructed, comprising \(\Delta\text{extABCD}, \Delta\text{extEFG}, \Delta\text{extHIJKL}\), and \(\Delta\text{ombB-omaB-omcB-orfS-omcB-omaC-omcC}\) (abbreviated as the \(\Delta\text{omcBC}\) cluster) mutants. As these mutant strains lack any antibiotic cassettes, they were used as backgrounds for further deletions. Multiple cluster deletion mutants leaving only one conduit cluster on the genome are referred to by their single remaining cluster, e.g. “\(\text{extABCD}^+\) = \(\Delta\text{extEFG} \Delta\text{extHIJKL} \Delta\text{omcBC}\)”, while the mutant lacking the \(\text{extABCD}\), \(\text{extEFG}\), \(\text{extHIJKL}\), \(\text{omcB}\)-based and \(\text{omcC}\)-based clusters is referred to as “\(\Delta5\)”. These strains were tested under six different extracellular growth conditions varying in solubility, chemical composition, and redox potential.

Mutants lacking \(\text{extABCD}\) are defective in electrode growth at all redox potentials, while mutants containing only \(\text{extABCD}\) outperform wild type. When grown with electrodes poised at high (0.24 V vs. SHE) or low (-0.1 V, (Chan et al., 2017)) redox potentials, only \(\Delta\text{extABCD}\) mutants showed a defect in rate and extent of growth. Mutants lacking the \(\text{omcBC}\) and \(\text{extEFG}\) clusters grew similar to wild type, while \(\Delta\text{extHIJKL}\) demonstrated a lag before growing with a similar doubling time as wild type to nearly wild type final current density (Fig. 2A). In all experiments, \(\Delta\text{extABCD}\) grew slower than a 20 h doubling time, or over 3-fold slower than wild type, and could only achieve 20% of wild type final current density, or 116 ± 33 \(\mu\)A/cm\(^2\) vs. 557 ± 44 \(\mu\)A/cm\(^2\) (n ≥ 5).

Mutants containing only one gene cluster (\(\text{extABCD}^+, \text{extEFG}^+, \text{extHIJKL}^+, \text{omcBC}^+\)) as well as a mutant lacking all gene clusters (\(\Delta5\)) were then analyzed for growth on electrodes. The \(\Delta5\) mutant grew at the low rate and extent of growth as the \(\Delta\text{extABCD}\) single mutant at both redox potentials, suggesting than none of the additional clusters were responsible for residual growth originally seen in \(\Delta\text{extABCD}\). In contrast, \(\text{extABCD}^+\) grew faster than wild type (4.5 ± 0.2 h vs. 6.5 ± 0.3 h doubling time, n ≥ 9) and reached a final current density 40% higher than wild type (768 ± 52 \(\mu\)A/cm\(^2\) vs. 557 ± 44\(\mu\)A/cm\(^2\), n≥9). All other multiple-deletion strains containing only
one cluster grew as poorly as the Δ5 mutant, further indicating that under these conditions, extEFG, extHIJKL, and omcBC did not contribute to electron transfer to electrodes (Fig. 2B).

A 5-conduit deletion mutant expressing extABCD has a faster growth rate on electrodes than wild type. To further investigate the specific effect of extABCD on electrode growth, extABCD was provided on a vector in the Δ5 strain. The 3-gene omcB conduit cluster (ombB-omaB-omcB) was also placed in the Δ5 strain using the same vector, and both were compared to wild type cells containing the empty vector. While the plasmid is stable for multiple generations, routine vector maintenance requires growth with kanamycin, and kanamycin carry-over into biofilm electrode experiments is reported to have deleterious effects on electrode growth (Levar et al., 2014; Chan et al., 2015). Thus, we re-examined growth of the empty vector strain. When selective levels of kanamycin (200 µg·ml⁻¹) were present in electrode reactors, colonization slowed and final current production decreased 74%. At levels resulting from carry-over during passage of cells into the electrode reactor (5 µg·ml⁻¹) growth rate was not affected, but final current was decreased up to 30%, suggesting interference with biofilm thickness rather than respiration (Fig. 3A). All subsequent complementation was performed in the presence of 5 µg·ml⁻¹ residual kanamycin and compared to these controls.

Expressing the omcB conduit cluster in the Δ5 strain failed to increase growth with electrodes as electron acceptors. These data were consistent with the lack of an effect seen in ΔomcBC deletions, as well as the poor growth of omcBC⁺ mutants containing both the OmcB and OmcC clusters (Fig. 3B). However, when extABCD was expressed on the same vector in the Δ5 background, colonization was faster and cells reached a higher final current density compared to wild type (421 ± 89 µA/cm² vs. 297 ± 11 µA/cm², n=3) (Fig. 3B). This enhancement was similar to the positive effect observed in the extABCD⁺ strain, and further supported the hypothesis that extABCD played a central role during electron transfer to electrodes (Fig. 2B).

Growth of intermediate two-conduit deletion mutants were unchanged from single-cluster strains (Fig. S1). Just as the mutant lacking extABCD produced the same phenotype as the Δ5 strain (Fig. 2), deletion of second clusters from the ΔextABCD strain produced similar results as ΔextABCD, and no other two-cluster combination of omcBC, extEFG or extHIJKL mutants
showed defects to suggest they were utilized or expressed during these electrode growth conditions.

**Cells lacking single gene clusters have partial reduction defects with Fe(III)- and Mn(IV)-oxides.** In contrast to the dominant effect of *extABCD* on electrode respiration, no single cluster deletion eliminated the majority of growth with particulate Fe(III)- or Mn(IV)-oxides. The most severe defect was observed in the ΔomcBC cluster mutant, which reduced 68% of Fe(III)-oxide compared to wild type (Fig. 4A). Minor defects were observed for ΔextEFG and ΔextHIJKL, while ΔextACBD reduced Fe(III)-oxide near wild-type levels. None of the single mutants displayed defects with Mn(IV)-oxides (Fig. 4C). These results suggested that multiple clusters were active during metal oxide reduction.

Any one gene cluster is sufficient for partial Fe(III)- or Mn(IV)-oxide reduction, while deletion of all 5 clusters eliminates electron transfer to these metal oxides. Unlike electrode respiration, deletion of the full suite of clusters eliminated all residual electron transfer to Fe(III)-and Mn(IV)-oxides (Fig. 4B and D). When multiple-deletion strains containing only one cluster were tested for Fe(III)-oxide reduction, results supported key roles for *omcBC* and *extEFG* in metal oxide reduction, and little involvement by *extABCD*. For example, Fe(III)-oxide reduction by *omcBC*+ was nearly 80% of wild type, *extEFG*+ was over 60%, but the *extABCD*+ strain reduced less than 30% of wild type. The *omcBC*+, *extEFG*+, and *extHIJKL*+ strains achieved about 80% of wild type Mn(IV)-reduction at 80 hours, but the *extABCD*+ strain again displayed poor growth with Mn(IV)-oxide.

**Only strains lacking both *omcBC* and *extEFG* had a significant defect in Fe(III)- and Mn(IV)-oxide reduction.** Because ΔomcBC demonstrated the largest defect in Fe(III)-oxide reduction, additional deletions in this background were tested for Fe(III) and Mn(IV)-oxide reduction (Fig. 5). Fe(III)-oxide reduction by ΔomcBC ΔextEFG was less than 25% of wild type, while the ΔomcBC ΔextACBD, and ΔomcBC ΔextHIJKL strains still reduced Fe(III)-oxides similar to the ΔomcBC strain. The ΔomcBC ΔextEFG strain also had a severe Mn(IV)-oxide reduction defect. Unlike Fe(III)-oxide reduction, the ΔomcBC ΔextABCD and ΔomcBC
\[ \text{extHIJKL} \] strains had a modest Mn(IV) reduction defect, suggesting contributions of the 
\[ \text{extABCD} \] and \[ \text{extHIJKL} \] clusters in the presence of Mn(IV) compared to Fe(III).

The poor growth of the \[ \Delta \text{omcBC} \Delta \text{extEFG} \] mutant on insoluble metals was surprising since this strain still contained \[ \text{extHIJKL} \], and the \[ \text{extHIJKL}^+ \] strain reduced 50% of Fe(III)-oxide and 75% of Mn(IV)-oxide compared to wild type (Fig. 4B and D; Table 2). This suggests \[ \text{extHIJKL} \] expression or function of its product could be negatively affecting the level or activity of other clusters.

**Expression of single conduit clusters partially recovers Fe(III)- and Mn(IV)-oxide reduction.** When compared to empty-vector controls with low (5 μg·ml\(^{-1}\)) levels of kanamycin carryover, complementation of the \[ \Delta 5 \] strain with single \[ \text{omcB} \] (as \[ \text{ombB-omaB-omcB} \]) or \[ \text{extABCD} \] clusters resulted in partial recovery (Fig. 6), consistent with the intermediate phenotypes displayed by mutants retaining single clusters on the genome. Expression of the \[ \text{omcB} \] cluster reestablished Fe(III)-oxide reduction, although to a level less than that seen in the \[ \text{omcBC}^+ \] strain containing the full duplicated cluster (Fig. 4B). Expressing \[ \text{extABCD} \] from a plasmid restored Fe(III)-oxide reduction in the \[ \Delta 5 \] strain near the low levels of the \[ \text{extABCD}^+ \] strain. Reduction of Mn(IV)-oxides by \[ \text{omcB} \] or \[ \text{extABCD} \]-expressing strains was even lower.

**Only strains lacking both \[ \text{omcBC} \] and \[ \text{extABCD} \] had a significant defect in Fe(III)-citrate reduction.** As with Fe(III)- and Mn(IV)-oxides, deletion of single conduit clusters in \[ \text{G. sulfurreducens} \] only had modest effects on Fe(III)-citrate reduction (Fig. 7A) and additional conduit cluster deletions were needed to severely impact growth (Fig. 7B and C). The single cluster deletion strains \[ \Delta \text{omcBC}, \Delta \text{extEFG}, \text{and } \Delta \text{extHIJKL} \] still reduced ~60% of soluble Fe(III). However, in contrast to Fe(III)-oxides, the \[ \Delta \text{extABCD} \] strain showed near wild-type reduction. The \[ \Delta 5 \] strain lacking all \[ \text{omcBC} \] and \[ \text{ext} \] clusters failed to reduce Fe(III)-citrate (Fig. 7B). Also unlike Fe(III)-oxide reduction, strains with only \[ \text{omcBC}^+ \] or \[ \text{extABCD}^+ \] clusters had near wild-type Fe(III)-citrate reduction rate, while \[ \text{extEFG}^+ \] and \[ \text{extHIJKL}^+ \] reduced Fe(III)-citrate to just 20% of wild type.

Since the \[ \Delta \text{omcBC} \Delta \text{extEFG} \] strain showed the largest defect in Fe(III)-oxide reduction, this strain was analyzed with Fe(III)-citrate as well. However, this double cluster deletion mutant showed little difference compared to the parent \[ \Delta \text{omcBC} \] strain (Fig. 7C). In contrast to Fe(III)-
oxides, where deletion of extABCD had little effect, ∆omcBC ∆extABCD was the only conduit deletion combination that severely affected growth with Fe(III)-citrate (Fig. 7C). Compared to growth of extEFG\(^+\) and extHIJKL\(^+\) (Fig. 7B), the ∆omcBC ∆extABCD mutant (containing both extEFG and extHIJKL) reduced Fe(III)-citrate to the same level (Fig. 7C). These data suggest that when both extEFG and extHIJKL remained in the genome in the ∆omcBC ∆extABCD mutant, their activity was not additive. Plasmids containing either ombB-omaB-omcB or extABCD restored Fe(III)-citrate reduction in a Δ5 strain to levels within 90% of the respective omcBC\(^+\) and extABCD\(^+\) strains (Fig. 7D).

Not shown in Fig 7 is metal reduction data for intermediate deletion mutants such as ∆extEFG ∆extHIJKL. Screens performed after such double mutants were constructed revealed no changes to phenotypes that deviated from wild type or their parent single-cluster deletions. Only the intermediate strains with phenotypes, such as ∆omcBC background strains, are shown in Fig 7.

**Proposed role(s) for the Omc and Ext electron conduits.** Table 2 summarizes all extracellular reduction phenotypes of single cluster deletions and deletions leaving only one conduit, adjusted to wild type performance. Many of the recently described ext gene clusters are necessary for wild-type metal reduction, yet few are sufficient. For example, extEFG and extHIJKL were necessary for Fe(III)-citrate reduction, as strains lacking these clusters only reduced ~65% of wild type levels. But when only extEFG or extHIJKL was present, they were not sufficient to reduce Fe(III)-citrate to more than 25% of wild type levels.

In contrast, the omcBC cluster or the extABCD cluster alone was sufficient for Fe(III)-citrate reduction, and the extABCD cluster alone was sufficient for electrode growth. These phenotypes could be due to electron acceptor preferences of each complex, or differential expression driven by each electron acceptor, but in either case, each gene cluster was linked to phenotypes only under specific conditions. Deletion of all five conduits resulted in complete elimination of metal reduction abilities, while activity remained when the Δ5 strain was grown using electrodes as terminal electron acceptor. This comparison shows each gene cluster can be functional, but
only under particular conditions, and provides evidence for additional undiscovered pathways enabling transmembrane electron transfer.

**DISCUSSION**

Sequencing of the *G. sulfurreducens* genome revealed an unprecedented number of electron transfer proteins, with twice as many genes dedicated to respiratory and redox reactions as organisms with similarly-sized genomes (Methé *et al.*, 2003). Out of 111 c-type cytochromes, 43 had no known homolog, and many were predicted to reside in the outer membrane. The large complement of outer membrane redox proteins in *G. sulfurreducens* became even more of an anomaly as the electron transfer strategy of metal-reducing *S. oneidensis* emerged, where only a single outer membrane conduit was used to reduce a multitude of substrates (Wang *et al.*, 2008; Baron *et al.*, 2009; Coursolle *et al.*, 2010; Coursolle and Gralnick, 2010).

Evidence that more than one *G. sulfurreducens* outer membrane pathway exists for reduction of extracellular substrates has accumulated since the discovery of OmcB (Leang *et al.*, 2003). Deletion of *omcB* impacted Fe(III)-reduction, but had little effect on U(IV) or Mn(IV)-oxide reduction (Shelobolina *et al.*, 2007; Aklujkar *et al.*, 2013). A ∆omcB suppressor strain evolved for improved Fe(III)-citrate growth still reduced Fe(III)-oxides poorly (Leang and Lovley, 2005).

Strains lacking *omcB* grew similar to wild type with electrodes in four different studies, (Holmes *et al.*, 2006; Richter *et al.*, 2009; Nevin *et al.*, 2009; Peng and Zhang, 2017), and OmcB abundance was lowest on cells near the electrode (Stephen *et al.*, 2014). An insertional mutant lacking six secreted and outer membrane-associated cytochromes in addition to *omcB* still demonstrated some Fe(III)-oxide reduction (Ueki *et al.*, 2017). After replacing the entire *omcBC* region with an antibiotic cassette and also finding residual Fe(III)-reducing ability, Liu *et al.* (2015) speculated that other porin-cytochrome-like clusters in the genome might be active. Most recently, Tn-seq analysis of electrode-grown cells found little effect of *omcB* mutations, yet noted significant defects from insertions in cytochromes with porin-cytochrome features (Chan *et al.*, 2017). This evidence led us to study if alternative ext-family conduits are functional in *G. sulfurreducens* under different conditions.

The genetic analysis presented here confirms a role for these unstudied conduits in extracellular respiration. All mutants still containing at least one cluster retained at least partial activity
towards metals, and deletion of the *omcBC* region, plus all three *ext* clusters, finally was able to eliminate metal reduction. This need to delete more than one conduit cluster helps explain prior variability and rapid evolution of suppressors in Δ*omcB*-only mutants. In the case of electrodes at both high and low potentials, only deletion of *extABCD* affected growth. Since residual electron transfer to electrodes was still detected after deletion of all clusters, additional mechanisms remain to be discovered. Overall, these data support the conclusion that for all tested metal and electrode acceptors, more than one conduit is functional and capable of participating in electron transfer. We found no pattern of specific gene clusters being required at particular redox potentials, suggesting that periplasmic proteins act as a ‘translator’ to interface the array of outer membrane complexes with the energy conserving inner membrane cytochromes ImcH and CbcL.

More difficult to resolve is whether each putative conduit is designed for interaction with specific extracellular substrates. The fact that single cluster mutants performed differently with each substrate, along with evidence that *omcB* could not complement electrode growth while *extABCD* could, supports the hypothesis of substrate specificity. Promoters more active in the presence of Fe(III) vs. Mn(IV) could create some of these phenotypes, but differential expression still suggests cells prefer to use each cluster under specific conditions. Some complexes may preferentially interact with secreted extracellular proteins who carry electrons to the final destination, and activity from a complex is masked in the absence of its partner protein. While many extracellular proteins are known to be involved in electron transfer, such as OmcS, OmcE, OmcZ, PgcA, and pili, a lack of secreted proteins encoded within *omcBC* or *ext* gene clusters argues against co-evolution of dedicated partners. The availability of strains containing only one gene cluster will enable easier purification, engineered changes in expression levels, and protein-protein interaction studies to test these hypotheses.

The genetic context of *ext* genes may aid identification of similar clusters in genomes of other organisms, and reveal clues to their intended function. None of the *ext* regions fits the *mtr* 3-gene ‘porin cytochrome’ operon of one small (~40 kDa) periplasmic cytochrome, a β-barrel, and one large (>90 kDa) lipoprotein cytochrome. For example, *extABCD* includes two small lipoprotein cytochromes, *extEFG* is part of a hydrogenase-family transcriptional unit, and *extHIJKL* contains a rhodanese-like lipoprotein instead of an extracellular cytochrome (Fig. 1). The transcriptional unit beginning with *extEFG* includes a homolog of YedY-family periplasmic
protein repair systems described in *E. coli* (Gennaris *et al.*, 2015), followed by a NiFe hydrogenase similar to bidirectional Hox hydrogenases used to recycle reducing equivalents in Cyanobacteria (Appel *et al.*, 2000; Coppi, 2005; Qiu *et al.*, 2010). Rhodanase-like proteins related to ExtH typically are involved in sulfur metabolism (Ravot *et al.*, 2005; Aussignargues *et al.*, 2012; Prat *et al.*, 2012) and an outer surface rhodanese-like protein is linked to extracellular oxidation of metal sulfides by *Acidithiobacillus ferrooxidans* (Ramírez *et al.*, 2002). Future searches for electron conduit clusters should consider the possibility of non-cytochrome components, and be aware that conduits might be part of larger complexes that could draw electrons from pools other than periplasmic cytochromes.

Including genes from *ext* operons in searches of other genomes reveals an interesting pattern in putative conduit regions throughout Desulfuromonadales strains isolated from freshwater, saline, subsurface, and fuel cell environments (Fig. 8). In about 1/3 of cases, the entire cluster is conserved intact, such as *extABCD* in *G. anodireducens*, *G. soli*, and *G. pickeringii* (Fig. 8B). However, when differences exist, they are typically non-orthologous replacements of the outer surface lipoprotein, such as where *extABC* is followed by a new cytochrome in *G. metallireducens*, *Geoalkalibacter ferrirydriticus*, and *Desulfuromonas soudanensis*. Conservation of the periplasmic cytochrome coupled to replacement of the outer surface redox protein also occurs in the *omcB* and *extHIJKL* clusters (Fig 8A and D). For example, of 18 *extHIJKL* regions, 10 contain a different extracellular rhodanese-like protein upstream of *extIJKL*, each with less than 40% identity to *extH*. This remarkable variability in extracellular components, compared to conservation of periplasmic redox proteins, suggests constant lateral gene transfer and selection of domains exposed to electron acceptors and the outside environment.

The data presented here significantly expands the number of outer membrane redox proteins contributing to electron transfer in *G. sulfurreducens*, and highlights a key difference in the *Geobacter* electron transfer strategy compared to other model organisms. In general, the pattern of multiple proteins with seemingly overlapping or redundant roles is less like respiratory reductases, and more reminiscent of cellulolytic bacteria that produce numerous similar β-glucosidases in response to a constantly changing polysaccharide substrate (Wang *et al.*, 2008; Coursolle *et al.*, 2010; Nelson *et al.*, 2017). A need for multiple outer membrane strategies could be a response to the complexity of metal oxides during reduction; minerals rapidly diversify to
become multiphase assemblages of more crystalline phases, the cell:metal interface can become enriched in Fe(II), and organic materials can bind to alter the surface (Cutting et al., 2009; Coker et al., 2012; Eusterhues et al., 2014). Expressing a complex array of electron transfer pathways makes cells competitive at all stages with all electron acceptors, allowing *Geobacter* to outgrow more specialized organisms during perturbations in the environment.

EXPERIMENTAL PROCEDURES

Growth conditions.

All experiments were performed with our laboratory strain of *Geobacter sulfurreducens* PCA freshly streaked single colonies from freezer stocks. Anaerobic NB media (0.38 g/L KCl, 0.2 g/L NH$_4$Cl, 0.069 g/L NaH$_2$PO$_4$H$_2$O, 0.04 g/L CaCl$_2$2H$_2$O, 0.2 g/L MgSO$_4$7H$_2$O, 1% v/v trace mineral mix, pH 6.8, buffered with 2 g/L NaHCO$_3$ and flushed with 20:80 N$_2$:CO$_2$ gas mix) with 20 mM acetate as electron donor, 40 mM fumarate as electron acceptor was used to grow liquid cultures from colony picks. For metal reduction assays, 20 mM acetate was added with either 55 mM Fe(III) citrate, ~20 mM birnessite (Mn(IV)-oxide), or ~70 mM Fe(III)-oxide freshly precipitated from FeCl$_2$ by addition of NaOH and incubation at pH 7 for 1 h before washing in DI water. All experiments were carried out at 30°C.

Deletion and complementation construction

Putative conduits were identified through a genomic search for gene clusters containing loci predicted to encode a β-barrel using PRED-TMBB (Bagos et al., 2004), contiguous to periplasmic and extracellular multiheme c-cytochromes or other redox proteins. Localization was predicted by comparing PSORT (Yu et al., 2010) and the presence/absence of lipid attachment sites (Juncker et al., 2003). Constructs to delete each gene cluster were designed to recombine to leave the site marker-free and also non-polar when located in larger transcriptional units, with most primers and plasmids for the single deletions described in Chan et al., 2017. When genes were part of a larger transcriptional unit or contained an upstream promoter, it was left intact. For example, in the case of the *omcBC* cluster the transcriptional regulator *orfR*
(GSU2741) was left intact, and in extEFG the promoter and untranslated region was left intact so as not to disrupt the downstream loci.

For deletion mutant construction, the suicide vector pK18mobsacB (Simon et al., 1983) with ~750 bp flanking to the target region was used to induce homologous recombination as previously described (Chan et al., 2015). Briefly, two rounds of homologous recombination were selected for. The first selection used kanamycin resistance to select for mutants with the plasmid inserted into either up or downstream regions, and the second selection used sucrose sensitivity to select for mutants that recombine the plasmid out of the chromosome, resulting in either wild type or complete deletion mutants. Deletion mutants were identified using a kanamycin sensitivity test and verified by PCR amplification targeting the region. Multiple PCR amplifications with primers in different regions were used to confirm full deletion of each gene cluster (Chan et al., 2017 and Table S1).

During this work, we found that manipulations in the omcBC cluster, which contains large regions containing 100% identical sequences, frequently underwent recombination into unexpected hybrid mutants which could escape routine PCR verification. For example, when the omaB and omaC genes recombined, a large hybrid operon containing omaB-linked to ombC-omcC would result. Routine primer screening, especially targeting flanking regions, failed to detect the large product. Only via multiple internal primers (Chan et al., 2017 and Table S1), as well as longer-read or single molecule sequencing, were we able to verify and isolate strains in which complete loss of the omcBC cluster occurred, and dispose of hybrid mutants. Whole-genome resequencing was also performed on strains containing only one cluster, such as the strain containing only extABCD, especially since this strain has an unexpected phenotype where it produced more current than wild type. Thorough verification by PCR and whole genome sequencing are recommended to confirm deletions of large and repetitive regions such as the omcBC cluster.

Mutants lacking a single gene region were used as parent strains to build additional mutations. In this manner, six double gene-cluster deletion mutants, four triple-cluster deletion mutants and one quintuple-cluster deletion mutant lacking up to nineteen genes were constructed (Fig. 1; Table 1). For complementation strains, putative conduits were amplified using primers listed in Table S1 and inserted into the G. sulfurreducens expression vector pRK2-Geo2 (Chan et al.,
2015), which contains a constitutive promoter $P_{acpP}$. The putative conduit $\text{extABCD}$ was assembled into a single transcriptional unit to ensure expression.

**Electrode reduction assays**

Sterile three-electrode conical reactors containing 15 mL of NB with 40 mM acetate as electron donor and 50mM NaCl to equilibrate salt concentration were flushed with a mix of N$_2$-CO$_2$ gas (80:20, v/v) until the O$_2$ concentration reached less than 2 ppm. Liquid cultures were prepared by inoculating 1 ml liquid cultures from single colonies inside an anaerobic chamber. Once these cultures reached late exponential to stationary phase, they were used to inoculate 10 ml cultures with 10% v/v. Each reactor was then inoculated with 25% v/v from this liquid culture as it approached acceptor limitation, at an OD$_{600}$ between 0.48 and 0.52. Working electrodes were set at either -0.1 V or +0.24 V vs SHE and average current density recorded every 12 seconds. Each liquid culture propagated from an individual colony pick served no more than two reactors, and at least three separate colonies were picked for all electrode reduction experiments for a final $n \geq 3$.

**Metal reduction assays**

NB medium with 20 mM acetate as electron donor and either 55 mM Fe(III)-citrate, ~70 mM Fe(III) oxide, or ~20 mM birnessite (Mn(IV)O$_2$) as electron acceptor was inoculated with a 0.1% inoculum of early stationary phase fumarate limited cultures. Time points were taken as necessary with anaerobic and sterile needles. These were diluted 1:10 into 0.5 N HCl for the Fe(III) samples and into 2 N HCl, 4mM FeSO$_4$ for Mn(IV) samples. Samples were diluted once more by 1:10 in the case of Fe(III) assays and by 1:5 in the case of Mn(IV) assays into 0.5 N HCl. Ferrozine$^R$ reagent was then used to determine the Fe(II) concentration in each sample. Original Fe(II) concentrations were calculated for Fe(III) reduction assays by accounting for dilutions and original Mn(IV) concentrations were calculated by accounting for the concentration of Fe(II) oxidized by Mn(IV) based on the following: $\text{Mn(IV)} + 2\text{Fe(II)} = \text{Mn(II)} + 2\text{Fe(III)}$.

**Homolog search and alignment**

Homologs to each of the individual cytochrome conduit proteins were queried on 11-30-2016 in the Integrated Microbial Genomes database (Markowitz *et al.*, 2012) with a cutoff on 75% sequence length and 40% identity based on amino acid sequence within the Desulfuromonadales. A higher percent identity was demanded in this search due to the high
heme binding site density with the invariable CXXCH sequence. Only ExtJ and ExtL were
excluded from the search and the OmcBC region was collapsed into a single cluster due to the
high identity shared between the two copies. The gene neighborhood around each homolog hit
was analyzed. With a few exceptions (see Table S2), all homologs were found to be conserved
in gene clusters predicted to encode cytochrome conduits and containing several additional
homologs to each corresponding *G. sulfurreducens* conduit. The proteins within each
homologous cytochrome conduit that did not fall within the set cutoff were aligned to the amino
acid sequence of the *G. sulfurreducens* component they replaced using ClustalΩ (Sievers *et al*.,
2011).
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AUTHOR CONTRIBUTIONS

FJO was responsible for the design of the study and the acquisition of data. Both FJO and DRB were responsible for the analysis and interpretation of the data; as well as writing of the manuscript.

ABBREVIATED SUMMARY

*Geobacter sulfurreducens* cells utilize electron conduits, or chains of redox proteins spanning the outer membrane, to transfer electrons to extracellular acceptors. Five different gene clusters encoding putative electron conduits were deleted in single- and multiple-gene-cluster markerless deletion strains. Mutants containing single conduit gene clusters each showed specific abilities to reduce Fe(III)- and Mn(IV)-oxides, Fe(III)-citrate and poised electrodes, and multiple conduits appeared to have overlapping roles during metal reduction. ExtABCD was the only electron conduit involved in electrode reduction.
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Table 1. Strains and plasmids used in this study.

| Strains and Plasmids | Description or relevant genotype | Reference |
|----------------------|----------------------------------|-----------|
| Geobacter sulfurreducens strains |
| DB1279               | ΔGSU2731-39 (ΔomcBC)             | Chan et al., 2017 |
| DB1280               | ΔGSU2645-42 (ΔextABCD)           | Chan et al., 2017 |
| DB1281               | ΔGSU2940-36 (ΔextHIJKL)          | Chan et al., 2017 |
| DB1282               | ΔGSU2724-26 (ΔextEFG)            | Chan et al., 2017 |
| DB1487               | ΔGSU2731-39 ΔGSU2645-42 (ΔomcBC ΔextABCD) | This study |
| DB1488               | ΔGSU2731-39 ΔGSU2724-26 (ΔomcBC ΔextEFG) | This study |
| DB1289               | ΔGSU2731-39 ΔGSU2940-36 (ΔomcBC ΔextHIJKL) | This study |
| DB1489               | ΔGSU2645-42 ΔGSU2724-26 (ΔextABCD ΔextEFG) | This study |
| DB1490               | ΔGSU2645-42 ΔGSU2940-36 (ΔextABCD ΔextHIJKL) | This study |
| DB1290               | ΔGSU2731-39 ΔGSU2940-36 ΔGSU2724-26 (extA<sup>+</sup>) | This study |
| DB1291               | ΔGSU2731-39 ΔGSU2645-42 ΔGSU2936-2940 (extEFG<sup>+</sup>) | This study |
| DB1491               | ΔGSU2731-39 ΔGSU2645-42 ΔGSU2726-24 (extHIJKL<sup>+</sup>) | This study |
| DB1492               | ΔGSU2645-42 ΔGSU2726-24 Δ2940-36 (omcBC<sup>+</sup>) | This study |
| DB1493               | ΔGSU2731-39 ΔGSU2645-42 ΔGSU2726-24 ΔGSU2940-36 (Δ5) | This study |

| Escherichia coli strains |
|--------------------------|-----------------|-------------|
| S17-1                    | recA pro hsdR RP4-2-Tc::Mu-Km::Tn7 | Simon et al., 1983 |

Plasmids

| Plasmids          | Description                                | Reference |
|-------------------|---------------------------------------------|-----------|
| pK18mobsacB       | Flanking regions of omcBC in pK18mobsacB    | Simon et al., 1983 |
| pRK2-Geo2         | Flanking regions of extABCD in pK18mobsacB  | Chan et al., 2015 |
| pDomcBC           | Flanking regions of extABCD in pK18mobsacB  | This study |
| pDextEFG          | Flanking regions of extEFG in pK18mobsacB   | This study |
| pDextHIJKL        | Flanking regions of extHIJKL in pK18mobsacB | This study |
| pombB             | Flanking regions of extABCD in pK18mobsacB  | This study |
| pextABCD          | extABCD in pRK2-Geo2                        | This study |
Table 2

| Substrate         | ΔomcBC | ΔextABCD | ΔextEFG | ΔextHIJKL | ΔomcBCΔextABCD | ΔextEFGΔextHIJKL | Δ5 |
|-------------------|--------|----------|---------|-----------|----------------|------------------|----|
| Fe(III)-citrate   | 61.2   | 105      | 62.5    | 66.3      | 101.1          | 99.2             | 22.5|
| Fe(III)-oxide     | 68.9   | 83.3     | 87.5    | 95.8      | 78.8           | 29.2             | 60.4|
| Mn(IV)-oxide      | 94.5   | 95.1     | 99.6    | 97.9      | 83.3           | 26.7             | 86.8|
| Electrode         | 76.5   | 20.9     | 104.8   | 86.3      | 28.3           | 137.9            | 21.2|

Table 2. Performance of *G. sulfurreducens* strains lacking one cluster, or containing only one cluster. Growth of single cytochrome conduit deletion mutants and mutants lacking all except one cytochrome conduit, averaged from all incubations and represented as the percent of wild type growth. Averages calculated from n ≥ 8 experiments.
Figure 1. The outer membrane electron conduit gene clusters of *G. sulfurreducens*. A) Genetic organization and predicted features of operons containing putative outer membrane conduits. Deletion constructs indicated by dashed line. B) Identity matrix from amino acid sequence alignment of each cytochrome or β-barrel component using ClustalΩ.

Figure 2. Only ExtABCD conduit is involved in electrode reduction. Current density produced by A) single and B) multiple-cluster deletion mutants on graphite electrodes poised at +0.24 V vs. SHE. All mutants were grown in at least two separate experiments, and curves are representative of n ≥ 3 independent replicates. Similar results were obtained at lower (-0.1 V vs. SHE) redox potentials.

Figure 3. Effect of kanamycin on final current density, and comparison of ExtABCD and OmcBC complementation. A) Final current density of wild type *G. sulfurreducens* compared to wild type carrying an empty vector in the presence of increasing kanamycin concentrations. B) Current density produced by Δ5 strain plus either extABCD or omcB cluster-containing vectors, in the presence of 5 μg/ml residual kanamycin. Wild type and Δ5 strains carrying the empty vector were used as controls. All experiments were conducted in duplicate and curves are representative of n ≥ 3 replicates.

Figure 4. No single outer membrane cluster is essential but all are functional for electron transfer to Fe(III)- and Mn(IV)-oxides. Growth of single cluster deletion mutants and triple mutants lacking all but one cytochrome conduit, as well as Δ5 mutant lacking all clusters utilizing A) 70 mM Fe(III)-oxide or B) 20 mM Mn(IV)-oxide as terminal electron acceptor. All experiments were conducted in triplicate and curves are average ± SD of n ≥ 3 replicates.

Figure 5. OmcBC and ExtEFG have dominant roles in Fe(III) and Mn(IV) oxide reduction. Reduction of A) 70 mM Fe(III)-oxide or B) 20 mM Mn(IV)-oxide by the ΔomcBC strain and additional deletions in an ΔomcBC background. All experiments were conducted in triplicate and curves are average ± SD of n ≥ 3 replicates.

Figure 6. Co-presentation of multiple conduit complexes is responsible for wild-type levels of metal oxide reduction. Reduction of A) 70 mM Fe(III)-oxide or B) 20 mM Mn(IV)-oxide by the Δ5 mutant expressing extABCD or the omcB cluster compared to the empty vector control. All experiments were conducted in triplicate and curves are average ± SD of n ≥ 3 replicates.

Figure 7. OmcBC and ExtABCD are the key cytochromes during Fe(III)-citrate reduction. Growth using 55 mM Fe(III)-citrate as an electron acceptor by A) single conduit cluster deletion
mutants, B) triple mutants lacking all but one cytochrome conduit, as well as the Δ5 strain lacking all five cytochrome conduits, C) mutants in an ΔomcBC background strain, and D) Δ5 mutants expressing omcB or extABCD or carrying an empty expression vector as control. All experiments were conducted in triplicate and curves are average ± SD of n ≥ 3 replicates.

**Figure 8. Cytochrome conduit conservation across the Order Desulfuromonadales.**

Schematic representation of cytochrome conduits from the Desulfuromonadales with homologs to either A) OmcBC, B) ExtABCD, C) ExtEFG, or D) ExtHIJKL. Red arrows = putative outer membrane products with a predicted lipid attachment site, yellow arrows = predicted periplasmic components, and green arrows = predicted outer membrane anchor components. Complete clusters with all components sharing >40% identity to the corresponding *G. sulfurreducens* cytochrome conduit are represented in boxes to the left of each gene cluster. Clusters in which one or more proteins are replaced by a new element with <40% identity are listed on the right side of each gene cluster. Proteins with numbers indicate the % identity to the *G. sulfurreducens* version. *a*OmcBC homologs in these gene clusters also encoding Hox hydrogenase complexes.

*b*Gene clusters have contiguous extBCD loci but extA is not in near vicinity, as extA were found un-clustered in separate parts of the genome for some of those organisms (see Supplemental Table S2). *c*Gene cluster has additional lipoprotein decaheme c-cytochrome upstream of extE.

*d*Lipid attachment sites corresponding to ExtJL could not be found but there is an additional small lipoprotein encoded within the gene cluster. For ExtHIJKL encoding clusters, homologs depicted above extH are found in gene clusters containing only extI, whereas homologs depicted below extH are found in gene clusters containing full extHIJKL loci. Upstream and on the opposite strand to all gene clusters homologous to extHIJKL there is a transcription regulator of the LysR family, except *e*, where there is no transcriptional regulator in that region, and *f*, where there are transcriptional regulators of the TetR family instead.
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