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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SUMMARY

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.

64 **INTRODUCTION**

65

66 Microorganisms capable of extracellular respiration can alter the redox state of particulate metal
67 oxides in soils and sediments, controlling their solubility and bioavailability (Tadanier *et al.*,
68 2005; N'Guessan *et al.*, 2008; Toner *et al.*, 2009; Williams *et al.*, 2011; Yelton *et al.*, 2013;
69 Couture *et al.*, 2015). To respire extracellular metals, bacteria must transfer electrons from the
70 cell interior to outer surface redox proteins, requiring unique mechanisms compared to growth
71 with soluble electron acceptors. The requirement for surface exposed electron transfer proteins
72 also presents opportunities for transformation of heavy metals, biological nanoparticle synthesis,
73 and a new generation of microbially-powered electrochemical devices using bacteria grown on
74 electrodes (Bond *et al.*, 2002; Bond and Lovley, 2003; Holmes *et al.*, 2004; Ren *et al.*, 2008;
75 Logan and Rabaey, 2012; Schrader *et al.*, 2016; Schievano *et al.*, 2016).

76

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

90

91 A model organism widely studied for its ability to reduce a diversity of metals and electrodes is
92 the δ-Proteobacterium *Geobacter sulfurreducens*, and recent work suggests this organism can
93 adjust its electron transfer pathway depending on conditions. CbcL, a combination *c*- and *b*-type
94 inner membrane cytochrome (Zacharoff *et al.*, 2016), is only required when extracellular metals
95 and electrodes are below redox potentials of -0.1 V vs. SHE, while the inner membrane *c*-type
96 cytochrome ImcH (Levar *et al.*, 2014), is essential when acceptors are at higher redox potentials

97 (Levar *et al.*, 2017). Beyond the outer membrane, the secreted cytochrome OmcZ is needed
98 only during electrode growth, while the secreted cytochrome PgcA only enhances reduction of
99 Fe(III)-oxides (Nevin *et al.*, 2009; Leang *et al.*, 2010; Tremblay *et al.*, 2011; Qian *et al.*, 2011;
100 Smith *et al.*, 2014; Peng and Zhang, 2017). Separating the initial event of inner membrane
101 proton motive force generation from the extracellular protein-mineral interaction lies the outer
102 membrane, an insulating barrier which was recently found to also contain electron transfer
103 proteins of surprising complexity (Richardson *et al.*, 2012; Liu *et al.*, 2014).
104
105 A mechanism for electron transfer across the outer membrane is through a porin-cytochrome
106 electron conduit, consisting of an integral outer membrane β-barrel proposed to anchor a
107 periplasmic multiheme cytochrome to an outer surface lipoprotein cytochrome. By linking heme
108 cofactors through the membrane spanning complex, electron flow is permitted (Hartshorne *et*
109 *al.*, 2009; Richardson *et al.*, 2012). The first electron conduit described was the ~210 kDa
110 MtrCAB complex from *S. oneidensis*, which can catalyze electron transfer across membranes to
111 extracellular substrates when purified and placed in lipid vesicles (Wang *et al.*, 2008; Coursolle
112 and Gralnick, 2012; White *et al.*, 2013). The *mtrCAB* gene cluster is essential for reduction of all
113 tested soluble metals, electron shuttles, metal oxides, and electrodes by *S. oneidensis* (Baron *et*
114 *al.*, 2009; Coursolle *et al.*, 2010; Coursolle and Gralnick, 2012). Related porin-cytochrome
115 complexes capped with an extracellular DMSO reductase allow *Shewanella* to reduce DMSO on
116 the cell exterior, while similar conduits support electron uptake by Fe(II)-oxidizing
117 *Rhodopseudomonas* TIE-1 cells (Gralnick *et al.*, 2006; Jiao and Newman, 2007).
118
119 In *G. sulfurreducens*, a gene cluster encoding the periplasmic cytochrome OmbA, putative ß-
120 barrel OmaB, and lipoprotein cytochrome OmcB also forms a conduit functionally similar to
121 MtrCAB, though the two complexes lack any sequence similarity (Liu *et al.*, 2014). This '*ombB-*
122 *omaB-omcB'* gene cluster is duplicated immediately downstream in the *G. sulfurreducens*
123 genome as the near-identical '*ombC-omaC-omcC'*, together forming the '*omcBC*' cluster.
124 Antibiotic cassette insertions within *omcB,* as well as insertions deleting the entire '*ombB-omaB-*
125 *omcB'* conduit decrease growth with Fe(III) as an electron acceptor, but the impact differs
126 between reports and growth conditions (Leang *et al.*, 2003; Leang and Lovley, 2005; Liu *et al.*,
127 2015). This variability between studies could be due to polar effects from inserted cassettes,

128 partial complementation by duplicated components, or the presence of undiscovered alternative
129 pathways that also catalyze electron transfer across the outer membrane.
130
131 Recently, genome-wide transposon data found that insertions in *omcB* or *omcC* had no effect
132 on *G. sulfurreducens* growth with electrodes poised at -0.1 vs. SHE, a low potential chosen to
133 mimic the redox potential of Fe(III)-oxides (Chan *et al.*, 2017). However, transposon insertions
134 within an unstudied four-gene cluster with porin-cytochrome signatures caused significant
135 defects during growth on -0.1 V electrodes (Chan *et al.*, 2017). Deletion of this cluster, named
136 *extABCD*, severely affected growth on low-potential electrodes, while Δ*extABCD* mutants grew
137 similar to wild type with Fe(III)-oxides. In contrast, deletion of both conduits contained in the
138 *omcBC* cluster had little impact on low-potential electrode growth. These data suggested that
139 the outer membrane pathway used for electron transfer could vary depending on environmental
140 conditions, but also raised new questions; are different conduits required at higher redox
141 potentials, during growth with mineral forms such as Mn(VI), or when metals become soluble?
142
143 At least five electron conduits may be encoded in the genome of *G. sulfurreducens*. Using new
144 markerless deletion methods, this study constructed combinations of mutants lacking these
145 gene clusters, to simultaneously compare growth using Fe(III)- and Mn(IV)-oxides, poised
146 electrodes at two different redox potentials, and soluble Fe(III)-citrate as terminal electron
147 acceptors. We found that only strains lacking *extABCD* showed a growth defect when
148 electrodes were the electron acceptor, and this effect was similar at all redox potentials. A strain
149 containing *extABCD* but lacking all other conduit clusters grew faster and to a higher final
150 density on electrodes. Phenotypes were more complex during metal reduction. The largest
151 defects were in Δ*omcBC* strains, but deletion of the newly identified cluster *extEFG* in the
152 Δ*omcBC* background was needed to severely inhibit Fe(III)-reduction. Deletion of all five
153 clusters was necessary to eliminate reduction of soluble and insoluble metals tested. Strains
154 containing only a single cluster showed preferences for reduction of different metals, such as
155 the *extEFG*- and *extHIJKL*-only strains performing better with Mn(IV)-oxides than Fe(III)-oxides.
156 These data provide evidence that multiple conduit clusters in the *G. sulfurreducens* genome are
157 functional and are utilized during electron transfer in a substrate-dependent manner.

158 **RESULTS**

159

160 **Description of putative outer membrane electron conduits.** At least five gene clusters can
161 be identified in the *G. sulfurreducens* genome encoding putative porin-cytochrome electron
162 conduits, based on three key elements; (1) a multiheme periplasmic *c*-type cytochrome, (2) an
163 outer membrane ß-barrel protein, and (3) one or more outer membrane lipoproteins with redox
164 cofactors (Fig. 1A). Two of these clusters correspond to the well-studied OmcB-based (*omb*B-
165 *oma*B-*omc*B, GSU2739-2737) conduit and its near-identical duplicate OmcC-based cluster
166 immediately downstream (*omb*C-*oma*C-o*mc*C, GSU2733-2731). For clarity, and due to the fact
167 that *omaBC* and *ombBC* are identical, these are together referred to as the "*omcBC*" cluster.

168

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

180

181 A significant difference between *G. sulfurreducens* Ext clusters and the *S. oneidensis* Mtr
182 conduits (Hartshorne *et al.*, 2009), is that the porin-cytochrome conduits in *S. oneidensis* are
183 paralogs. The periplasmic MtrA and MtrD cytochromes share over 50% identity, are similar in
184 size and heme content, and can cross complement (Coursolle and Gralnick, 2010). The
185 lipoprotein outer surface cytochromes of *Shewanella* also demonstrate high sequence,
186 functional, and structural conservation (Coursolle and Gralnick, 2010; Clarke *et al.*, 2011;
187 Richardson *et al.*, 2012; Edwards *et al.*, 2012). In contrast, no component of the Ext or OmcBC
188 complexes share any homology. For example, the predicted periplasmic *c*-cytochromes ExtA,

189 ExtG, ExtK, and OmaB vary in size from 25 to 72 kDa, contain 5 to 15 hemes, and share 18%-
190 26% identity (Fig. 1B).
191
192 To test physiological roles of these loci, single cluster mutants were first constructed, comprising
193 Δ*extABCD, ΔextEFG,* Δ*extHIJKL,* and Δ*omb*B-*oma*B-*omc*B-*orfS*-*omb*C-*oma*C-o*mc*C
194 (abbreviated as the Δ*omcBC* cluster) mutants. As these mutant strains lack any antibiotic
195 cassettes, they were used as backgrounds for further deletions. Multiple cluster deletion
196 mutants leaving only one conduit cluster on the genome are referred to by their single remaining
197 cluster, e.g. "*extABCD*$^{+}$*" = ΔextEFG* Δ*extHIJKL* Δ*omcB*C*,* while the mutant lacking the *extABCD,*
198 *extEFG, extHIJKL*, *omcB*-based and *omcC*-based clusters is referred to as "Δ5". These strains
199 were tested under six different extracellular growth conditions varying in solubility, chemical
200 composition, and redox potential.
201
202 **Mutants lacking *extABCD* are defective in electrode growth at all redox potentials, while**
203 **mutants containing only *extABCD* outperform wild type.** When grown with electrodes
204 poised at high (0.24 V vs. SHE) or low (-0.1 V, (Chan *et al.*, 2017)) redox potentials, only
205 Δ*extABCD* mutants showed a defect in rate and extent of growth. Mutants lacking the *omcBC*
206 and *extEFG* clusters grew similar to wild type, while Δ*extHIJKL* demonstrated a lag before
207 growing with a similar doubling time as wild type to nearly wild type final current density (Fig.
208 2A). In all experiments, Δ*extABCD* grew slower than a 20 h doubling time, or over 3-fold slower
209 than wild type, and could only achieve 20% of wild type final current density, or 116 ± 33 μA/cm^2
210 vs. 557 ± 44 μA/cm^2 (n ≥ 5).
211
212 Mutants containing only one gene cluster (*extABCD*$^{+}$, *extEFG*$^{+}$, *extHIJKL*$^{+}$, *omcBC*$^{+}$) as well as
213 a mutant lacking all gene clusters (Δ5) were then analyzed for growth on electrodes. The Δ5
214 mutant grew at the low rate and extent of growth as the Δ*extABCD* single mutant at both redox
215 potentials, suggesting than none of the additional clusters were responsible for residual growth
216 originally seen in Δ*extABCD*. In contrast, *extABCD*$^{+}$ grew faster than wild type (4.5 ± 0.2 h vs.
217 6.5 ± 0.3 h doubling time, n ≥ 9) and reached a final current density 40% higher than wild type
218 (768 ± 52 μA/cm^2 vs. 557 ± 44μA/cm^2, n≥9). All other multiple-deletion strains containing only

219 one cluster grew as poorly as the Δ5 mutant, further indicating that under these conditions,
220 *extEFG*, *extHIJKL,* and *omcBC* did not contribute to electron transfer to electrodes (Fig. 2B).
221
222 **A 5-conduit deletion mutant expressing *extABCD* has a faster growth rate on electrodes**
223 **than wild type.** To further investigate the specific effect of *extABCD* on electrode growth,
224 *extABCD* was provided on a vector in the Δ5 strain. The 3-gene *omcB* conduit cluster (*omb*B-
225 *oma*B-*omc*B) was also placed in the Δ5 strain using the same vector, and both were compared
226 to wild type cells containing the empty vector. While the plasmid is stable for multiple
227 generations, routine vector maintenance requires growth with kanamycin, and kanamycin carry-
228 over into biofilm electrode experiments is reported to have deleterious effects on electrode
229 growth (Levar *et al.*, 2014; Chan *et al.*, 2015). Thus, we re-examined growth of the empty vector
230 strain. When selective levels of kanamycin (200 $\mu g \cdot ml^{-1}$) were present in electrode reactors,
231 colonization slowed and final current production decreased 74%. At levels resulting from carry-
232 over during passage of cells into the electrode reactor (5 $\mu g \cdot ml^{-1}$) growth rate was not affected,
233 but final current was decreased up to 30%, suggesting interference with biofilm thickness rather
234 than respiration (Fig. 3A). All subsequent complementation was performed in the presence of 5
235 $\mu g \cdot ml^{-1}$ residual kanamycin and compared to these controls.
236
237 Expressing the *omcB* conduit cluster in the Δ5 strain failed to increase growth with electrodes as
238 electron acceptors. These data were consistent with the lack of an effect seen in Δ*omcBC*
239 deletions, as well as the poor growth of *omcBC*$^{+}$ mutants containing both the OmcB and OmcC
240 clusters (Fig. 3B). However, when *extABCD* was expressed on the same vector in the Δ5
241 background, colonization was faster and cells reached a higher final current density compared
242 to wild type (421 ± 89 $\mu A/cm^2$ vs. 297 ± 11 $\mu A/cm^2$, n=3) (Fig. 3B). This enhancement was
243 similar to the positive effect observed in the *extABCD*$^{+}$ strain, and further supported the
244 hypothesis that *extABCD* played a central role during electron transfer to electrodes (Fig. 2B).
245
246 Growth of intermediate two-conduit deletion mutants were unchanged from single-cluster strains
247 (Fig. S1). Just as the mutant lacking *extABCD* produced the same phenotype as the Δ5 strain
248 (Fig. 2), deletion of second clusters from the Δ*extABCD* strain produced similar results as
249 Δ*extABCD,* and no other two-cluster combination of *omcBC*, *extEFG* or *extHIJKL* mutants

250 showed defects to suggest they were utilized or expressed during these electrode growth
251 conditions.
252
253 **Cells lacking single gene clusters have partial reduction defects with Fe(III)- and Mn(IV)-**
254 **oxides.** In contrast to the dominant effect of *extABCD* on electrode respiration, no single cluster
255 deletion eliminated the majority of growth with particulate Fe(III)- or Mn(IV)-oxides. The most
256 severe defect was observed in the Δ*omcBC* cluster mutant, which reduced 68% of Fe(III)-oxide
257 compared to wild type (Fig. 4A). Minor defects were observed for Δ*extEFG* and Δ*extHIJKL*,
258 while Δ*extACBD* reduced Fe(III)-oxide near wild-type levels. None of the single mutants
259 displayed defects with Mn(IV)-oxides (Fig. 4C). These results suggested that multiple clusters
260 were active during metal oxide reduction.
261
262 **Any one gene cluster is sufficient for partial Fe(III)- or Mn(IV)-oxide reduction, while**
263 **deletion of all 5 clusters eliminates electron transfer to these metal oxides.** Unlike
264 electrode respiration, deletion of the full suite of clusters eliminated all residual electron transfer
265 to Fe(III)-and Mn(IV)-oxides (Fig. 4B and D). When multiple-deletion strains containing only one
266 cluster were tested for Fe(III)-oxide reduction, results supported key roles for *omcBC* and
267 *extEFG* in metal oxide reduction, and little involvement by *extABCD*. For example, Fe(III)-oxide
268 reduction by *omcBC*$^+$ was nearly 80% of wild type, *extEFG*$^+$ was over 60%, but the *extABCD*$^+$
269 strain reduced less than 30% of wild type. The *omcBC*$^+$, *extEFG*$^+$, and *extHIJKL*$^+$ strains
270 achieved about 80% of wild type Mn(IV)-reduction at 80 hours, but the *extABCD*$^+$ strain again
271 displayed poor growth with Mn(IV)-oxide.
272
273 **Only strains lacking both *omcBC* and *extEFG* had a significant defect in Fe(III)- and**
274 **Mn(IV)-oxide reduction.** Because Δ*omcBC* demonstrated the largest defect in Fe(III)-oxide
275 reduction, additional deletions in this background were tested for Fe(III) and Mn(IV)-oxide
276 reduction (Fig. 5). Fe(III)-oxide reduction by Δ*omcBC* Δ*extEFG* was less than 25% of wild type,
277 while the Δ*omcBC* Δ*extACBD*, and Δ*omcBC* Δ*extHIJKL* strains still reduced Fe(III)-oxides
278 similar to the Δ*omcBC* strain. The Δ*omcBC* Δ*extEFG* strain also had a severe Mn(IV)-oxide
279 reduction defect. Unlike Fe(III)-oxide reduction, the Δ*omcBC* Δ*extABCD* and Δ*omcBC*

280 Δ*extHIJKL* strains had a modest Mn(IV) reduction defect, suggesting contributions of the
281 *extABCD* and *extHIJKL* clusters in the presence of Mn(IV) compared to Fe(III).
282
283 The poor growth of the Δ*omcBC* Δ*extEFG* mutant on insoluble metals was surprising since this
284 strain still contained *extHIJKL,* and the *extHIJKL*$^{+}$ strain reduced 50% of Fe(III)-oxide and 75%
285 of Mn(IV)-oxide compared to wild type (Fig. 4B and D; Table 2). This suggests *extHIJKL*
286 expression or function of its product could be negatively affecting the level or activity of other
287 clusters.
288
289 **Expression of single conduit clusters partially recovers Fe(III)- and Mn(IV)-oxide**
290 **reduction.** When compared to empty-vector controls with low (5 μg·ml^{-1}) levels of kanamycin
291 carryover, complementation of the Δ5 strain with single *omcB (*as *ombB-omaB-omcB*) or
292 *extABCD* clusters resulted in partial recovery (Fig. 6), consistent with the intermediate
293 phenotypes displayed by mutants retaining single clusters on the genome. Expression of the
294 *omcB* cluster reestablished Fe(III)-oxide reduction, although to a level less than that seen in the
295 *omcBC*$^{+}$ strain containing the full duplicated cluster (Fig. 4B). Expressing *extABCD* from a
296 plasmid restored Fe(III)-oxide reduction in the Δ5 strain near the low levels of the *extABCD*$^{+}$
297 strain. Reduction of Mn(IV)-oxides by *omcB* or *extABCD*-expressing strains was even lower.
298
299 **Only strains lacking both *omcBC* and *extABCD* had a significant defect in Fe(III)-citrate**
300 **reduction.** As with Fe(III)- and Mn(IV)-oxides, deletion of single conduit clusters in *G.*
301 *sulfurreducens* only had modest effects on Fe(III)-citrate reduction (Fig. 7A) and additional
302 conduit cluster deletions were needed to severely impact growth (Fig. 7B and C). The single
303 cluster deletion strains Δ*omcBC*, Δ*extEFG,* and Δ*extHIJKL* still reduced ~60% of soluble Fe(III).
304 However, in contrast to Fe(III)-oxides, the Δ*extABCD* strain showed near wild-type reduction.
305 The Δ5 strain lacking all *omcBC* and *ext* clusters failed to reduce Fe(III)-citrate (Fig. 7B). Also
306 unlike Fe(III)-oxide reduction, strains with only *omcBC*$^{+}$ or *extABCD*$^{+}$ clusters had near wild-type
307 Fe(III)-citrate reduction rate, while *extEFG*$^{+}$ and *extHIJKL*$^{+}$ reduced Fe(III)-citrate to just 20% of
308 wild type.
309
310 Since the Δ*omcBC* Δ*extEFG* strain showed the largest defect in Fe(III)-oxide reduction, this
311 strain was analyzed with Fe(III)-citrate as well. However, this double cluster deletion mutant
312 showed little difference compared to the parent Δ*omcBC* strain (Fig. 7C). In contrast to Fe(III)-

313 oxides, where deletion of *extABCD* had little effect, Δ*omcBC* Δ*extABCD* was the only conduit
314 deletion combination that severely affected growth with Fe(III)-citrate (Fig. 7C). Compared to
315 growth of *extEFG*$^+$ and *extHIJKL*$^+$ (Fig. 7B), the Δ*omcBC* Δ*extABCD* mutant (containing both
316 *extEFG* and *extHIJKL*) reduced Fe(III)-citrate to the same level (Fig. 7C). These data suggest
317 that when both *extEFG* and *extHIJKL* remained in the genome in the Δ*omcBC* Δ*extABCD*
318 mutant, their activity was not additive. Plasmids containing either *ombB-omaB-omcB* or
319 *extABCD* restored Fe(III)-citrate reduction in a Δ5 strain to levels within 90% of the respective
320 *omcBC*$^+$ and *extABCD*$^+$ strains (Fig. 7D).
321
322 Not shown in Fig 7 is metal reduction data for intermediate deletion mutants such as Δ*extEFG*
323 Δ*extHIJKL*. Screens performed after such double mutants were constructed revealed no
324 changes to phenotypes that deviated from wild type or their parent single-cluster deletions. Only
325 the intermediate strains with phenotypes, such as Δ*omcBC* background strains, are shown in
326 Fig 7.
327
328 **Proposed role(s) for the Omc and Ext electron conduits.** Table 2 summarizes all
329 extracellular reduction phenotypes of single cluster deletions and deletions leaving only one
330 conduit, adjusted to wild type performance. Many of the recently described *ext* gene clusters are
331 necessary for wild-type metal reduction, yet few are sufficient. For example, *extEFG* and
332 *extHIJKL* were necessary for Fe(III)-citrate reduction, as strains lacking these clusters only
333 reduced ~65% of wild type levels. But when only *extEFG* or *extHIJKL* was present, they were
334 not sufficient to reduce Fe(III)-citrate to more than 25% of wild type levels.
335
336 In contrast, the *omcBC* cluster or the *extABCD* cluster alone was sufficient for Fe(III)-citrate
337 reduction, and the *extABCD* cluster alone was sufficient for electrode growth. These phenotypes
338 could be due to electron acceptor preferences of each complex, or differential expression driven
339 by each electron acceptor, but in either case, each gene cluster was linked to phenotypes only
340 under specific conditions. Deletion of all five conduits resulted in complete elimination of metal
341 reduction abilities, while activity remained when the Δ5 strain was grown using electrodes as
342 terminal electron acceptor. This comparison shows each gene cluster can be functional, but

343 only under particular conditions, and provides evidence for additional undiscovered pathways
344 enabling transmembrane electron transfer.
345
346 **DISCUSSION**
347
348 Sequencing of the *G. sulfurreducens* genome revealed an unprecedented number of electron
349 transfer proteins, with twice as many genes dedicated to respiratory and redox reactions as
350 organisms with similarly-sized genomes (Methé *et al.*, 2003). Out of 111 *c*-type cytochromes, 43
351 had no known homolog, and many were predicted to reside in the outer membrane. The large
352 complement of outer membrane redox proteins in *G. sulfurreducens* became even more of an
353 anomaly as the electron transfer strategy of metal-reducing *S. oneidensis* emerged, where only
354 a single outer membrane conduit was used to reduce a multitude of substrates (Wang *et al.*,
355 2008; Baron *et al.*, 2009; Coursolle *et al.*, 2010; Coursolle and Gralnick, 2010).
356
357 Evidence that more than one *G. sulfurreducens* outer membrane pathway exists for reduction of
358 extracellular substrates has accumulated since the discovery of OmcB (Leang *et al.*, 2003).
359 Deletion of *omcB* impacted Fe(III)-reduction, but had little effect on U(IV) or Mn(IV)-oxide
360 reduction (Shelobolina *et al.*, 2007; Aklujkar *et al.*, 2013). A Δ*omcB* suppressor strain evolved
361 for improved Fe(III)-citrate growth still reduced Fe(III)-oxides poorly (Leang and Lovley, 2005).
362 Strains lacking *omcB* grew similar to wild type with electrodes in four different studies, (Holmes
363 *et al.*, 2006; Richter *et al.*, 2009; Nevin *et al.*, 2009; Peng and Zhang, 2017), and OmcB
364 abundance was lowest on cells near the electrode (Stephen *et al.*, 2014). An insertional mutant
365 lacking six secreted and outer membrane-associated cytochromes in addition to *omcB* still
366 demonstrated some Fe(III)-oxide reduction (Ueki *et al.*, 2017). After replacing the entire *omcBC*
367 region with an antibiotic cassette and also finding residual Fe(III)-reducing ability, Liu *et al.*
368 (2015) speculated that other porin-cytochrome-like clusters in the genome might be active. Most
369 recently, Tn-seq analysis of electrode-grown cells found little effect of *omcB* mutations, yet
370 noted significant defects from insertions in cytochromes with porin-cytochrome features (Chan
371 *et al.*, 2017). This evidence led us to study if alternative *ext*-family conduits are functional in *G.*
372 *sulfurreducens* under different conditions.
373
374 The genetic analysis presented here confirms a role for these unstudied conduits in extracellular
375 respiration. All mutants still containing at least one cluster retained at least partial activity

376 towards metals, and deletion of the *omcBC* region, plus all three *ext* clusters, finally was able to
377 eliminate metal reduction. This need to delete more than one conduit cluster helps explain prior
378 variability and rapid evolution of suppressors in Δ*omcB*-only mutants. In the case of electrodes
379 at both high and low potentials, only deletion of *extABCD* affected growth. Since residual
380 electron transfer to electrodes was still detected after deletion of all clusters, additional
381 mechanisms remain to be discovered. Overall, these data support the conclusion that for all
382 tested metal and electrode acceptors, more than one conduit is functional and capable of
383 participating in electron transfer. We found no pattern of specific gene clusters being required at
384 particular redox potentials, suggesting that periplasmic proteins act as a 'translator' to interface
385 the array of outer membrane complexes with the energy conserving inner membrane
386 cytochromes ImcH and CbcL.
387
388 More difficult to resolve is whether each putative conduit is designed for interaction with specific
389 extracellular substrates. The fact that single cluster mutants performed differently with each
390 substrate, along with evidence that *omcB* could not complement electrode growth while
391 *extABCD* could, supports the hypothesis of substrate specificity. Promoters more active in the
392 presence of Fe(III) vs. Mn(IV) could create some of these phenotypes, but differential
393 expression still suggests cells prefer to use each cluster under specific conditions. Some
394 complexes may preferentially interact with secreted extracellular proteins who carry electrons to
395 the final destination, and activity from a complex is masked in the absence of its partner protein.
396 While many extracellular proteins are known to be involved in electron transfer, such as OmcS,
397 OmcE, OmcZ, PgcA, and pili, a lack of secreted proteins encoded within *omcBC* or *ext* gene
398 clusters argues against co-evolution of dedicated partners. The availability of strains containing
399 only one gene cluster will enable easier purification, engineered changes in expression levels,
400 and protein-protein interaction studies to test these hypotheses.
401
402 The genetic context of *ext* genes may aid identification of similar clusters in genomes of other
403 organisms, and reveal clues to their intended function. None of the *ext* regions fits the *mtr* 3-
404 gene 'porin cytochrome' operon of one small (~40 kDa) periplasmic cytochrome, a β-barrel, and
405 one large (>90 kDa) lipoprotein cytochrome. For example, *extABCD* includes two small
406 lipoprotein cytochromes, *extEFG* is part of a hydrogenase-family transcriptional unit, and
407 *extHIJKL* contains a rhodanese-like lipoprotein instead of an extracellular cytochrome (Fig. 1).
408 The transcriptional unit beginning with *extEFG* includes a homolog of YedY-family periplasmic

409 protein repair systems described in *E. coli* (Gennaris *et al.*, 2015), followed by a NiFe
410 hydrogenase similar to bidirectional Hox hydrogenases used to recycle reducing equivalents in
411 Cyanobacteria (Appel *et al.*, 2000; Coppi, 2005; Qiu *et al.*, 2010). Rhodanase-like proteins
412 related to ExtH typically are involved in sulfur metabolism (Ravot *et al.*, 2005; Aussignargues *et*
413 *al.*, 2012; Prat *et al.*, 2012) and an outer surface rhodanese-like protein is linked to extracellular
414 oxidation of metal sulfides by *Acidithiobacillus ferrooxidans* (Ramírez *et al.*, 2002). Future
415 searches for electron conduit clusters should consider the possibility of non-cytochrome
416 components, and be aware that conduits might be part of larger complexes that could draw
417 electrons from pools other than periplasmic cytochromes.
418
419 Including genes from *ext* operons in searches of other genomes reveals an interesting pattern in
420 putative conduit regions throughout Desulfuromonadales strains isolated from freshwater,
421 saline, subsurface, and fuel cell environments (Fig. 8). In about 1/3 of cases, the entire cluster is
422 conserved intact, such as *extABCD* in *G. anodireducens, G. soli,* and *G. pickeringii* (Fig. 8B).
423 However, when differences exist, they are typically non-orthologous replacements of the outer
424 surface lipoprotein, such as where *extABC* is followed by a new cytochrome in *G.*
425 *metallireducens, Geoalkalibacter ferrihydriticus,* and *Desulfuromonas soudanensis.*
426 Conservation of the periplasmic cytochrome coupled to replacement of the outer surface redox
427 protein also occurs in the *omcB* and *extHIJKL* clusters (Fig 8A and D). For example, of 18
428 *extHIJKL* regions, 10 contain a different extracellular rhodanese-like protein upstream of
429 *extIJKL*, each with less than 40% identity to *extH*. This remarkable variability in extracellular
430 components, compared to conservation of periplasmic redox proteins, suggests constant lateral
431 gene transfer and selection of domains exposed to electron acceptors and the outside
432 environment.
433
434 The data presented here significantly expands the number of outer membrane redox proteins
435 contributing to electron transfer in *G. sulfurreducens*, and highlights a key difference in the
436 *Geobacter* electron transfer strategy compared to other model organisms. In general, the
437 pattern of multiple proteins with seemingly overlapping or redundant roles is less like respiratory
438 reductases, and more reminiscent of cellulolytic bacteria that produce numerous similar β-
439 glucosidases in response to a constantly changing polysaccharide substrate (Wang *et al.*, 2008;
440 Coursolle *et al.*, 2010; Nelson *et al.*, 2017). A need for multiple outer membrane strategies could
441 be a response to the complexity of metal oxides during reduction; minerals rapidly diversify to

442 become multiphase assemblages of more crystalline phases, the cell:metal interface can
443 become enriched in Fe(II), and organic materials can bind to alter the surface (Cutting *et al.*,
444 2009; Coker *et al.*, 2012; Eusterhues *et al.*, 2014). Expressing a complex array of electron
445 transfer pathways makes cells competitive at all stages with all electron acceptors, allowing
446 *Geobacter* to outgrow more specialized organisms during perturbations in the environment.
447
448
449
450 **EXPERIMENTAL PROCEDURES**
451
452 *Growth conditions.*
453 All experiments were performed with our laboratory strain of *Geobacter sulfurreducens* PCA
454 freshly streaked single colonies from freezer stocks. Anaerobic NB media (0.38 g/L KCl, 0.2 g/L
455 NH_4Cl, 0.069 g/L $NaH_2PO_4H_2O$, 0.04 g/L $CaCl_22H_2O$, 0.2 g/L $MgSO_47H_2O$, 1% v/v trace mineral
456 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
457 acetate as electron donor, 40 mM fumarate as electron acceptor was used to grow liquid
458 cultures from colony picks. For metal reduction assays, 20 mM acetate was added with either
459 55 mM Fe(III) citrate, ~20 mM birnessite (Mn(IV)-oxide), or ~70 mM Fe(III)-oxide freshly
460 precipitated from $FeCl_2$ by addition of NaOH and incubation at pH 7 for 1 h before washing in DI
461 water. All experiments were carried out at 30°C.
462
463 *Deletion and complementation construction*
464 Putative conduits were identified through a genomic search for gene clusters containing loci
465 predicted to encode a β-barrel using PRED-TMBB (Bagos *et al.*, 2004), contiguous to
466 periplasmic and extracellular multiheme *c*-cytochromes or other redox proteins. Localization
467 was predicted by comparing PSORT (Yu *et al.*, 2010) and the presence/absence of lipid
468 attachment sites (Juncker *et al.*, 2003). Constructs to delete each gene cluster were designed to
469 recombine to leave the site marker-free and also non-polar when located in larger transcriptional
470 units, with most primers and plasmids for the single deletions described in Chan *et al.*, 2017.
471 When genes were part of a larger transcriptional unit or contained an upstream promoter, it was
472 left intact. For example, in the case of the *omcBC* cluster the transcriptional regulator *orfR*

473 (GSU2741) was left intact, and in *extEFG* the promoter and untranslated region was left intact
474 so as to not disrupt the downstream loci.
475
476 For deletion mutant construction, the suicide vector pK18*mobsacB* (Simon *et al.*, 1983) with
477 ~750 bp flanking to the target region was used to induce homologous recombination as
478 previously described (Chan *et al.*, 2015). Briefly, two rounds of homologous recombination were
479 selected for. The first selection used kanamycin resistance to select for mutants with the
480 plasmid inserted into either up or downstream regions, and the second selection used sucrose
481 sensitivity to select for mutants that recombine the plasmid out of the chromosome, resulting in
482 either wild type or complete deletion mutants. Deletion mutants were identified using a
483 kanamycin sensitivity test and verified by PCR amplification targeting the region. Multiple PCR
484 amplifications with primers in different regions were used to confirm full deletion of each gene
485 cluster (Chan *et al.*, 2017 and Table S1).
486
487 During this work, we found that manipulations in the *omcBC* cluster, which contains large
488 regions containing 100% identical sequences, frequently underwent recombination into
489 unexpected hybrid mutants which could escape routine PCR verification. For example, when the
490 *omaB* and *omaC* genes recombined, a large hybrid operon containing *omaB*-linked to *ombC*-
491 *omc*C would result. Routine primer screening, especially targeting flanking regions, failed to
492 detect the large product. Only via multiple internal primers (Chan *et al.*, 2017 and Table S1), as
493 well as longer-read or single molecule sequencing, were we able to verify and isolate strains in
494 which complete loss of the *omcBC* cluster occurred, and dispose of hybrid mutants. Whole-
495 genome resequencing was also performed on strains containing only one cluster, such as the
496 strain containing only *extABCD*, especially since this strain has an unexpected phenotype
497 where it produced more current than wild type. Thorough verification by PCR and whole
498 genome sequencing are recommended to confirm deletions of large and repetitive regions such
499 as the *omcBC* cluster.
500
501 Mutants lacking a single gene region were used as parent strains to build additional mutations.
502 In this manner, six double gene-cluster deletion mutants, four triple-cluster deletion mutants and
503 one quintuple-cluster deletion mutant lacking up to nineteen genes were constructed (Fig. 1;
504 Table 1). For complementation strains, putative conduits were amplified using primers listed in
505 Table S1 and inserted into the *G. sulfurreducens* expression vector pRK2-Geo2 (Chan *et al.*,

506 2015), which contains a constitutive promoter P_{acpP}. The putative conduit *extABCD* was
507 assembled into a single transcriptional unit to ensure expression.
508
509 *Electrode reduction assays*
510 Sterile three-electrode conical reactors containing 15 mL of NB with 40 mM acetate as electron
511 donor and 50mM NaCl to equilibrate salt concentration were flushed with a mix of N_2-CO_2 gas
512 (80:20, v/v) until the O_2 concentration reached less than 2 ppm. Liquid cultures were prepared
513 by inoculating 1 ml liquid cultures from single colonies inside an anaerobic chamber. Once
514 these cultures reached late exponential to stationary phase, they were used to inoculate 10 ml
515 cultures with 10% v/v. Each reactor was then inoculated with 25% v/v from this liquid culture as
516 it approached acceptor limitation, at an OD_{600} between 0.48 and 0.52. Working electrodes were
517 set at either -0.1 V or +0.24 V vs SHE and average current density recorded every 12 seconds.
518 Each liquid culture propagated from an individual colony pick served no more than two reactors,
519 and at least three separate colonies were picked for all electrode reduction experiments for a
520 final $n \geq 3$.
521
522 *Metal reduction assays*
523 NB medium with 20 mM acetate as electron donor and either 55 mM Fe(III)-citrate, ~70 mM
524 Fe(III) oxide, or ~20 mM birnessite ($Mn(IV)O_2$) as electron acceptor was inoculated with a 0.1%
525 inoculum of early stationary phase fumarate limited cultures. Time points were taken as
526 necessary with anaerobic and sterile needles. These were diluted 1:10 into 0.5 N HCl for the
527 Fe(III) samples and into 2 N HCl, 4mM $FeSO_4$ for Mn(IV) samples. Samples were diluted once
528 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
529 HCl. Ferrozine^R reagent was then used to determine the Fe(II) concentration in each sample.
530 Original Fe(II) concentrations were calculated for Fe(III) reduction assays by accounting for
531 dilutions and original Mn(IV) concentrations were calculated by accounting for the concentration
532 of Fe(II) oxidized by Mn(IV) based on the following: Mn(IV) + 2Fe(II) = Mn(II) + 2Fe(III).
533
534 *Homolog search and alignment*
535 Homologs to each of the individual cytochrome conduit proteins were queried on 11-30-2016 in
536 the Integrated Microbial Genomes database (Markowitz *et al.*, 2012) with a cutoff on 75%
537 sequence length and 40% identity based on amino acid sequence within the
538 Desulfuromonadales. A higher percent identity was demanded in this search due to the high

539 heme binding site density with the invariable CXXCH sequence. Only ExtJ and ExtL were
540 excluded from the search and the OmcBC region was collapsed into a single cluster due to the
541 high identity shared between the two copies. The gene neighborhood around each homolog hit
542 was analyzed. With a few exceptions (see Table S2), all homologs were found to be conserved
543 in gene clusters predicted to encode cytochrome conduits and containing several additional
544 homologs to each corresponding *G. sulfurreducens* conduit. The proteins within each
545 homologous cytochrome conduit that did not fall within the set cutoff were aligned to the amino
546 acid sequence of the *G. sulfurreducens* component they replaced using ClustalΩ (Sievers *et al.*,
547 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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771 **Tables**:

772

773 **Table 1**

Strains and Plasmids	Description or relevant genotype	Reference
Geobacter sulfurreducens strains		
DB1279	ΔGSU2731-39 (Δ*omcBC*)	Chan *et al*., 2017
DB1280	ΔGSU2645-42 (Δ*extABCD*)	Chan *et al*., 2017
DB1281	ΔGSU2940-36 (Δ*extHIJKL*)	Chan *et al*., 2017
DB1282	ΔGSU2724-26 (Δ*extEFG*)	Chan *et al*., 2017
DB1487	ΔGSU2731-39 ΔGSU2645-42 (Δ*omcBC* Δ*extABCD*)	This study
DB1488	ΔGSU2731-39 ΔGSU2724-26 (Δ*omcBC* Δ*extEFG*)	This study
DB1289	ΔGSU2731-39 ΔGSU2940-36 (Δ*omcBC* Δ*extHIJKL*)	This study
DB1489	ΔGSU2645-42 ΔGSU2724-26 (Δ*extABCD* Δ*extEFG*)	This study
DB1490	ΔGSU2645-42 ΔGSU2940-36 (Δ*extABCD* Δ*extHIJKL*)	This study
DB1290	ΔGSU2731-39 ΔGSU2940-36 ΔGSU2724-26 (*extA*$^{+}$)	This study
DB1291	ΔGSU2731-39 ΔGSU2645-42 ΔGSU2936-2940 (*extEFG*$^{+}$)	This study
DB1491	ΔGSU2731-39 ΔGSU2645-42 ΔGSU2726-24 (*extHIJKL*$^{+}$)	This study
DB1492	ΔGSU2645-42 ΔGSU2726-24 Δ2940-36 (*omcBC*$^{+}$)	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		
pK18*mobsacB*		Simon *et al*., 1983
pRK2-Geo2		Chan *et al*., 2015
pD*omcBC*	Flanking regions of *omcBC* in pK18*mobsacB*	This study
pD*extABCD*	Flanking regions of *extABCD* in pK18*mobsacB*	This study
pD*extEFG*	Flanking regions of *extEFG* in pK18*mobsacB*	This study
pD*extHIJKL*	Flanking regions of *extHIJKL* in pK18*mobsacB*	This study
p*omcB*	*ombB-omaB-omcB* in pRK2-Geo2	This study
p*extABCD*	*extABCD* in pRK2-Geo2	This study

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777 **Table 1. Strains and plasmids used in this study.**

Table 2

	% of wild type								
Substrate	Δ*omcBC*	Δ*extABCD*	Δ*extEFG*	Δ*extHIJKL*	*omcBC*$^{+}$	*extABCD*$^{+}$	*extEFG*$^{+}$	*extHIJKL*$^{+}$	Δ5
Fe(III)-citrate	61.2	105	62.5	66.3	101.1	99.2	22.5	23.8	0.1
Fe(III)-oxide	68.9	83.3	87.5	95.8	78.8	29.2	60.4	52.1	0.1
Mn(IV)-oxide	94.5	95.1	99.6	97.9	83.3	26.7	86.8	75.6	1.7
Electrode	76.5	20.9	104.8	86.3	28.3	137.9	21.2	25.9	21.9

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 Legends

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 ß-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 Δ*omcBC* strain and additional deletions in an Δ*omcBC* background. All experiments were conducted in triplicate and curves are average ± SD of n ≥ 3 replicates.

Figure 6. Co-presence 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

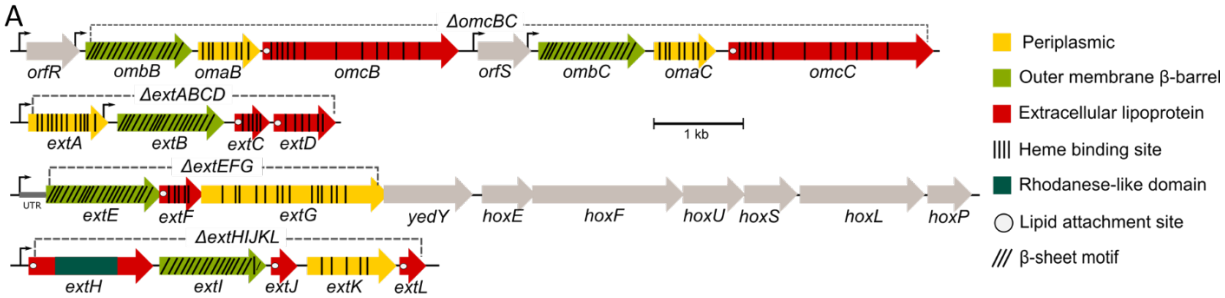
832 mutants, B) triple mutants lacking all but one cytochrome conduit, as well as the Δ5 strain
833 lacking all five cytochrome conduits, C) mutants in an Δ*omcBC* background strain, and D) Δ5
834 mutants expressing *omcB* or *extABCD* or carrying an empty expression vector as control. All
835 experiments were conducted in triplicate and curves are average ± SD of n ≥ 3 replicates.

836

837 **Figure 8. Cytochrome conduit conservation across the Order Desulfuromonodales**.
838 Schematic representation of cytochrome conduits from the Desulfuromonodales with homologs
839 to either A) OmcBC, B) ExtABCD, C) ExtEFG, or D) ExtHIJKL. Red arrows = putative outer
840 membrane products with a predicted lipid attachment site, yellow arrows = predicted periplasmic
841 components, and green arrows = predicted outer membrane anchor components. Complete
842 clusters with all components sharing >40% identity to the corresponding *G. sulfurreducens*
843 cytochrome conduit are represented in boxes to the left of each gene cluster. Clusters in which
844 one or more proteins are replaced by a new element with <40% identity are listed on the right
845 side of each gene cluster. Proteins with numbers indicate the % identity to the *G. sulfurreducens*
846 version. [a]OmcBC homologs in these gene clusters also encoding Hox hydrogenase complexes.
847 [b]Gene clusters have contiguous *extBCD* loci but *extA* is not in near vicinity, as *extA* were found
848 un-clustered in separate parts of the genome for some of those organisms (see Supplemental
849 Table S2). [c]Gene cluster has additional lipoprotein decaheme *c*-cytochrome upstream of *extE*.
850 [d]Lipid attachment sites corresponding to ExtJL could not be found but there is an additional
851 small lipoprotein encoded within the gene cluster. For ExtHIJKL encoding clusters, homologs
852 depicted above *extH* are found in gene clusters containing only *extI*, whereas homologs
853 depicted below *extH* are found in gene clusters containing full *extHIJKL* loci. Upstream and on
854 the opposite strand to all gene clusters homologous to *extHIJKL* there is a transcription
855 regulator of the LysR family, except [e], where there is no transcriptional regulator in that region,
856 and [f], where there are transcriptional regulators of the TetR family instead.

857

Figure 1.



B

Percent Identity based on Amino Acid Sequence

Periplasmic cytochromes

	ExtA	ExtG	ExtK	OmaB	OmaC
ExtA	100	25.9	17.9	23.9	23.9
ExtG		100	20.9	23.2	23.2
ExtK			100	19.7	19.7
OmaB				100	100
OmaC					100

β-barrels

	ExtB	ExtE	ExtI	OmbB	OmbC
ExtB	100	30.2	21.1	22	22
ExtE		100	20.2	20.4	20.4
ExtI			100	17.1	17.1
OmbB				100	100
OmbC					100

Lipoprotein cytochromes

	ExtC	ExtD	ExtF	OmcB	OmcC
ExtC	100	16.3	41.5	21.2	24.2
ExtD		100	31.3	26.9	28.5
ExtF			100	22.9	23
OmcB				100	70.8
OmcC					100

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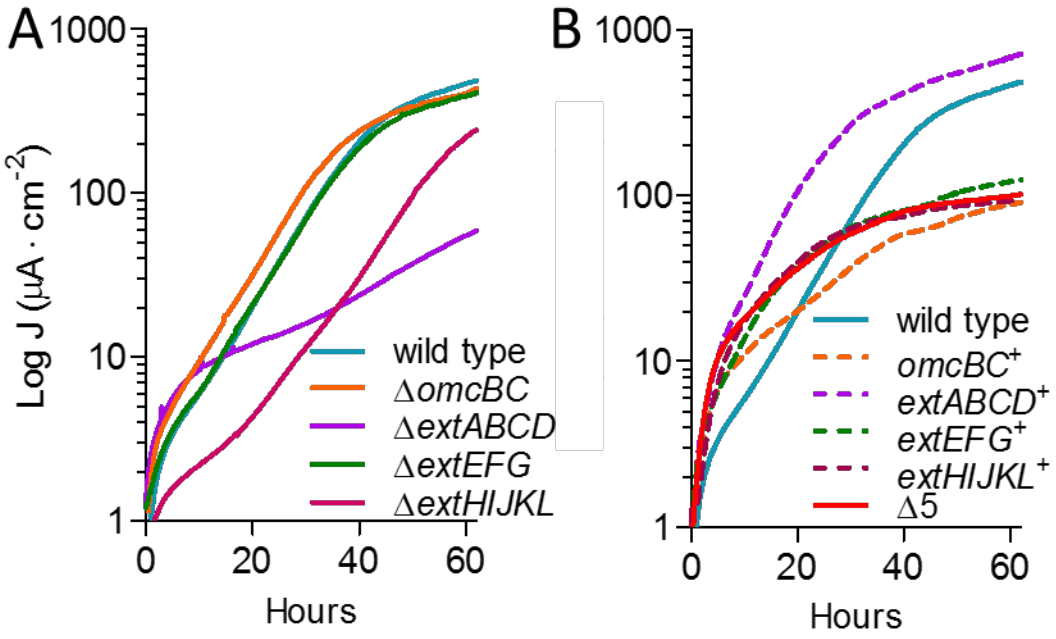

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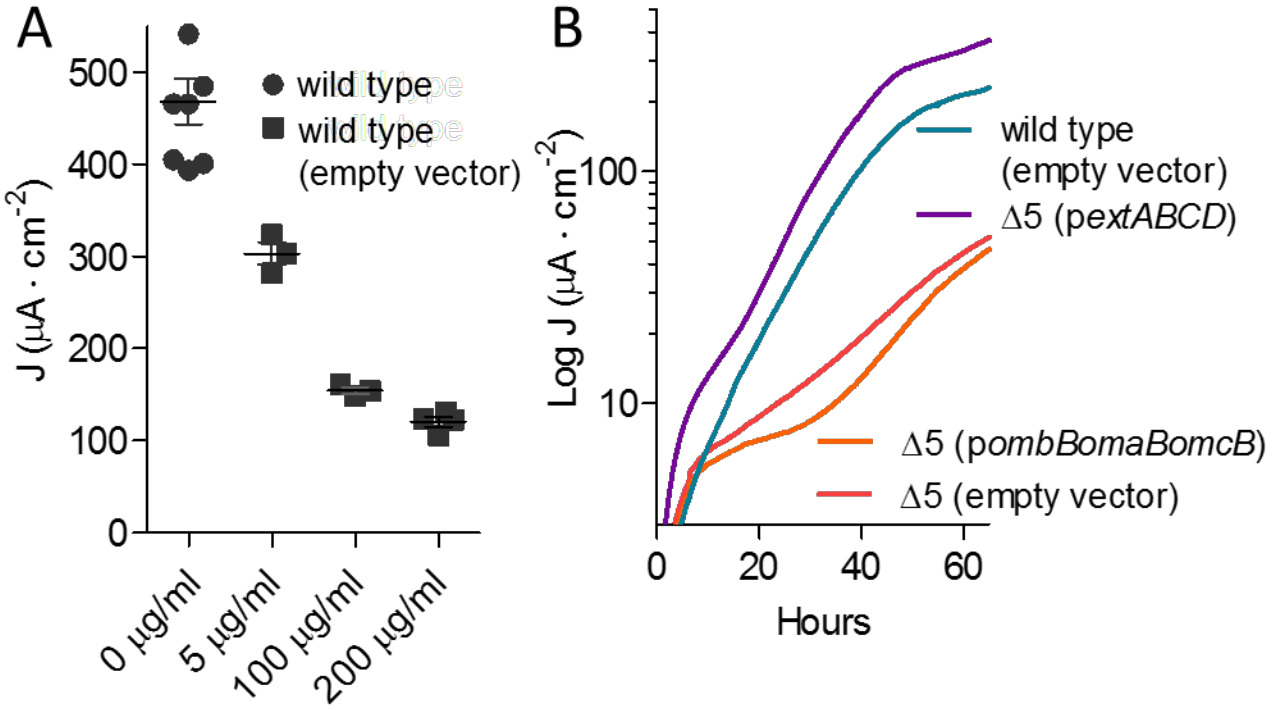

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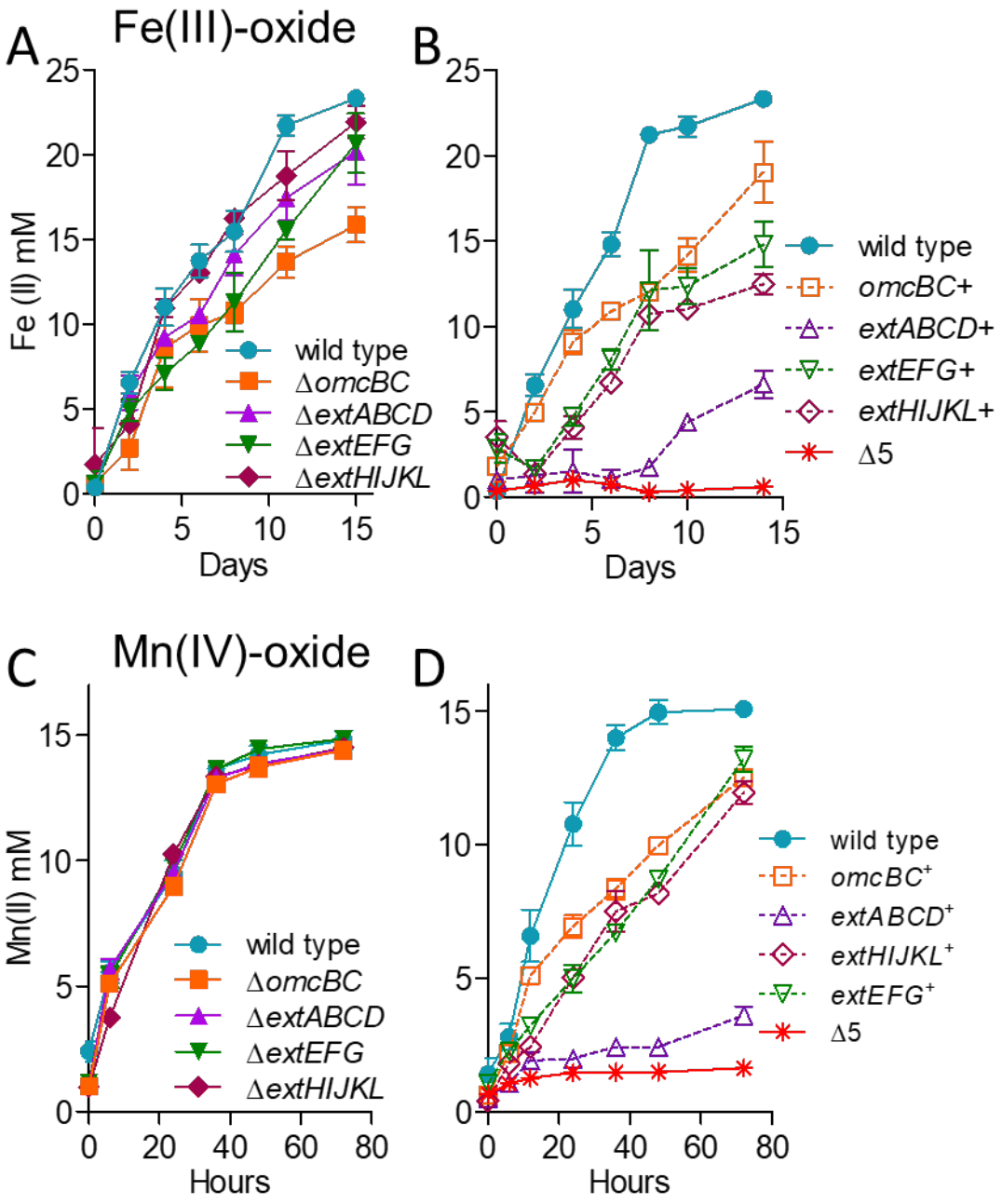

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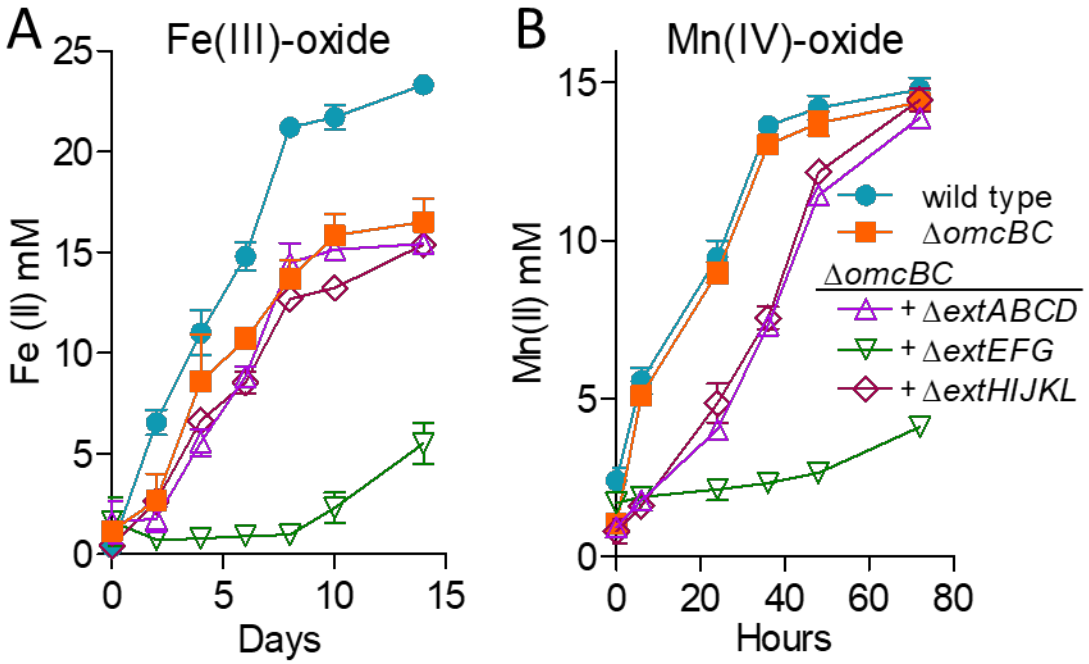

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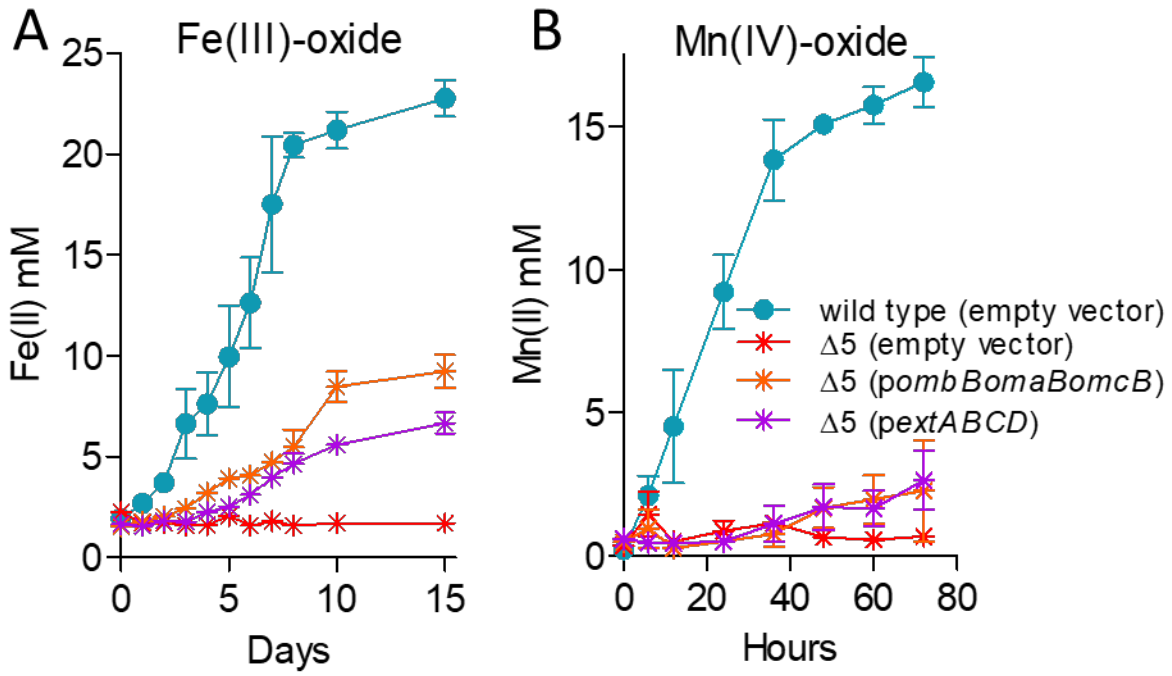

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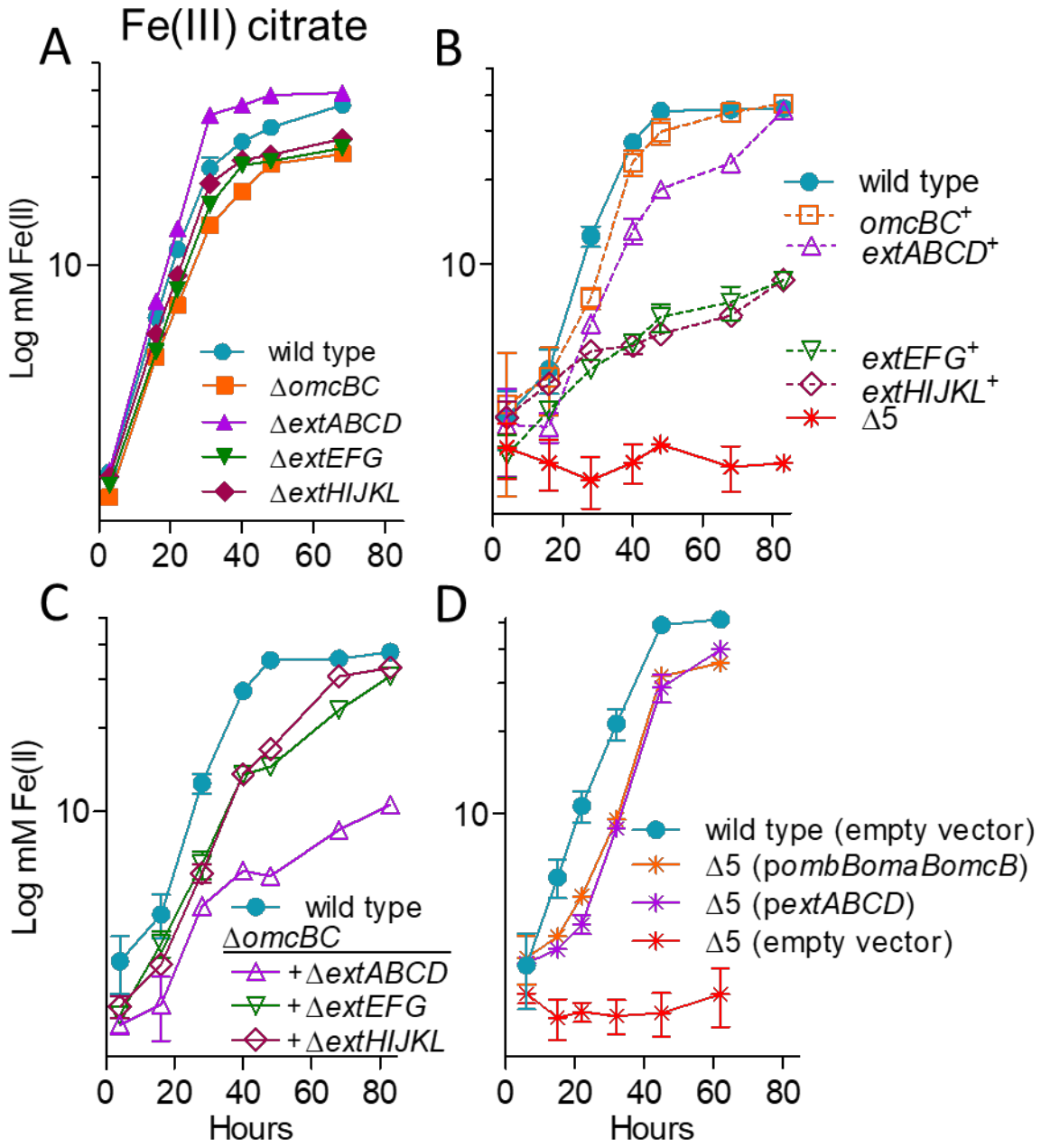

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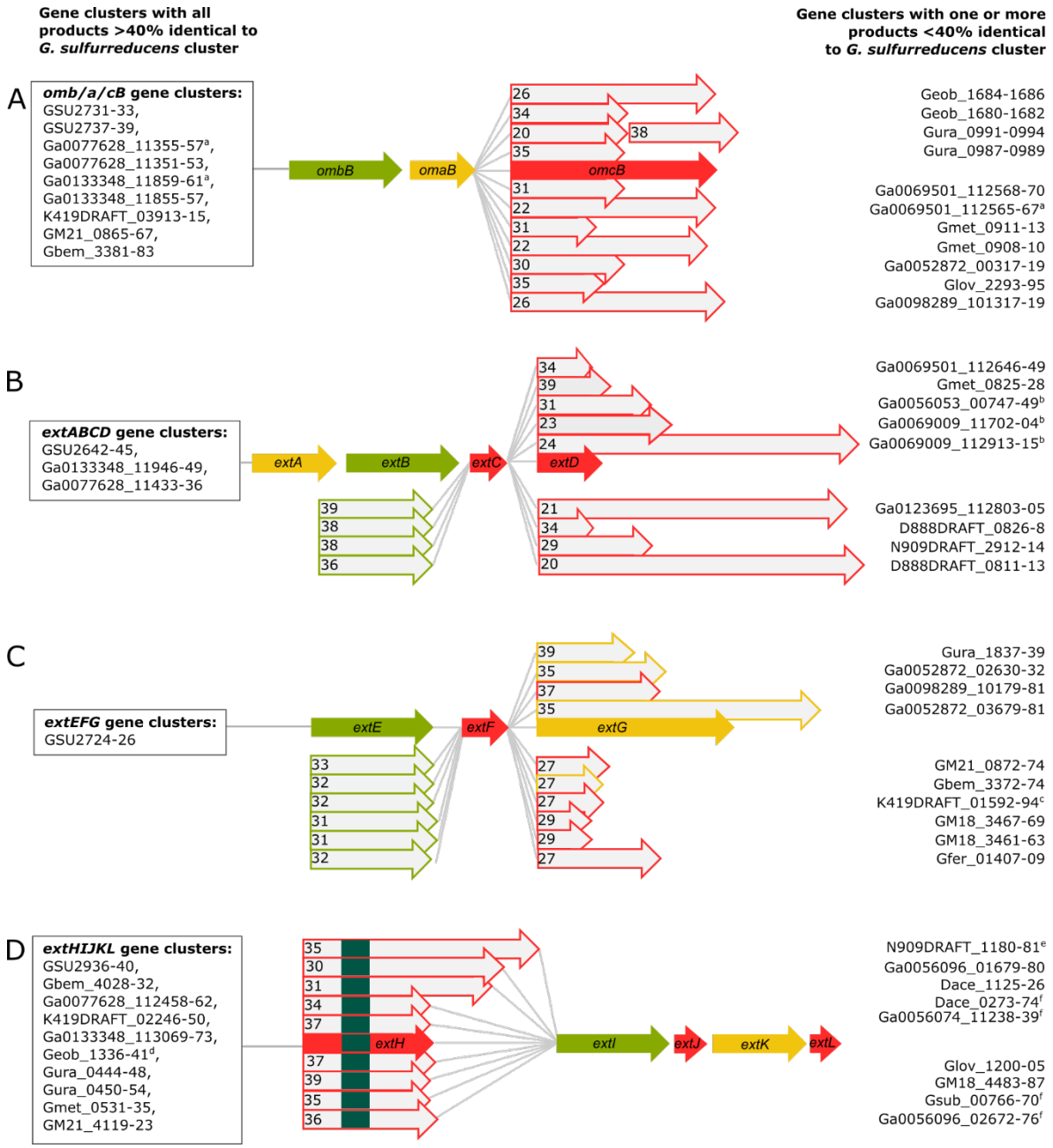

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