

1 **Detrimental Impact of a Type VI Secretion System on Direct Interspecies Electron**
2 **Transfer**

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24 **ABSTRACT**

25 Direct interspecies electron transfer (DIET) is important in anaerobic
26 communities of environmental and practical significance. Other than the need for close
27 physical contact for electrical connections, the interactions of DIET partners are poorly
28 understood. Type VI secretion systems (T6SSs) typically kill competitive microbes.
29 Surprisingly, *Geobacter metallireducens* highly expressed T6SS genes when DIET-based
30 co-cultures were initiated with *Geobacter sulfurreducens*. T6SS gene expression was
31 lower when the electron shuttle anthraquinone-2,6-disulfonate was added to alleviate the
32 need for interspecies contact. Disruption of *hcp*, the *G. metallireducens* gene for the main
33 T6SS needle-tube protein subunit, and the most highly upregulated gene in DIET-grown
34 cells, eliminated the long lag periods required for the initiation of DIET. The mutation
35 did not aid DIET in the presence of granular activated carbon, consistent with the fact
36 that DIET partners do not make physical contact when electrically connected through
37 conductive materials. The *hcp*-deficient mutant also established DIET quicker with
38 *Methanosarcina barkeri*. However, the mutant also reduced Fe(III) oxide faster than the
39 wild-type strain, a phenotype not expected from the loss of the T6SS. Quantitative PCR
40 revealed greater gene transcript abundance for key components of extracellular electron
41 transfer in the *hcp*-deficient mutant versus the wild-type strain, potentially accounting for
42 the faster Fe(III) oxide reduction and impact on DIET. The results highlight that
43 interspecies interactions beyond electrical connections may influence DIET effectiveness.
44 The unexpected increase in the expression of genes for extracellular electron transport
45 components when *hcp* was deleted emphasize the complexities in evaluating the
46 electromicrobiology of highly adaptable *Geobacter* species.

47 **IMPORTANCE**

48 Direct interspecies electron transfer (DIET) is an alternative to the much more
49 intensively studied process of interspecies H₂ transfer as a mechanism for microbes to
50 share electrons during the cooperative metabolism of energy sources. DIET is an
51 important process in anaerobic soils and sediments generating methane, a significant
52 greenhouse gas. Facilitating DIET can accelerate and stabilize the conversion of organic
53 wastes to methane biofuel in anaerobic digesters. Therefore, a better understanding of the
54 factors controlling how fast DIET partnerships are established is expected to lead to new
55 strategies for promoting this bioenergy process. The finding that when co-cultured with
56 *G. sulfurreducens*, *G. metallireducens* initially expressed a type VI secretion system, a
57 behavior not conducive to interspecies cooperation, illustrates the complexity in
58 establishing syntrophic relationships.
59

60 INTRODUCTION

61 A better understanding of the physiological characteristics of microbes that
62 participate in direct interspecies electron transfer (DIET) is required in order to determine
63 how both natural and engineered anoxic environments function (1-3). For example, DIET
64 appears to be the primary route for electron exchange between electron-donating bacteria
65 and electron-accepting partners in some types of anaerobic digesters (4, 5). In digesters in
66 which interspecies H₂ transfer predominates, modifying operating conditions to enhance
67 DIET can accelerate and stabilize the conversion of organic wastes to methane, a needed
68 improvement to this important bioenergy strategy (3, 6). Molecular studies have
69 demonstrated that DIET may be a major process in terrestrial methanogenic
70 environments that are significant sources of atmospheric methane (7), a conclusion that is
71 further supported by the reinterpretation of data on H₂ fluxes in these environments (8).

72 Most of the initial research following the discovery of DIET (9) focused on
73 identifying which microbes have the potential to participate in DIET and the organic
74 substrates that can support DIET (5, 8, 10-21). Study of the expression of genes and
75 proteins that enhance electron exchange between species has also been emphasized (5, 9,
76 10, 13, 22-25). However, other adaptations that promote the switch from a free-living
77 existence to living in close physical association, as is necessary to establish electrical
78 connections for DIET, seem likely.

79 The expression of type VI secretion systems (T6SSs) is expected to be antithetical
80 to interspecies cooperation. Approximately 25% of Gram-negative bacteria have T6SSs
81 that form contractile nanomachines that inject toxins directly into other microbes to
82 eliminate their competition (26-36). T6SSs are important in such polymicrobial

83 environments as the human colon (37-41), cow rumen (42, 43), the plant rhizosphere (44,
84 45), the light organ of the bobtail squid (46), and soil (47-49). In some instances, T6SSs
85 can also be involved in such non-antagonistic behaviors as the modulation of quorum
86 sensing and stress response (50), self-recognition (51-53), and the acquisition of various
87 metals such as zinc, copper, manganese, or iron (48-51, 54-57).

88 Molecular analyses have demonstrated that *Geobacter* species are important
89 electron-donating partners for DIET in natural environments, such as subsurface
90 terrestrial soils (7) as well as in some anaerobic digesters (4, 5). The often-observed
91 enrichment of *Geobacter* when methane production is stimulated with the addition of
92 conductive materials provides further circumstantial evidence for a role of *Geobacter* in
93 DIET (3, 6, 58). The availability of pure cultures of genetically tractable *Geobacter*
94 species that can participate in DIET in defined co-cultures has enabled elucidation of
95 important electrical contacts for DIET, such as multi-heme *c*-type cytochromes and
96 electrically conductive pili (5, 9, 10, 23, 25, 59), as well as strategies for enhancing DIET
97 with electrically conductive minerals and carbon materials (60-64).

98 However, *Geobacter* species are also often found to be free-living in anaerobic
99 soils and sediments, typically transferring electrons to extracellular electron acceptors
100 such as Fe(III) oxides and humic substances (65). T6SS genes are present in some but not
101 all *Geobacter* genomes (66, 67) (Table S1; Fig. S1). It might be expected that T6SSs
102 could be beneficial to free-living *Geobacter* species competing against other microbes for
103 resources, but not for developing syntrophic cooperation. Here we report that *Geobacter*
104 *metallireducens* highly expresses genes coding for its T6SS in the initial stages of
105 establishing a DIET-based co-culture with *Geobacter sulfurreducens*, a factor possibly

106 lengthening the adaptation period required for DIET-based growth of the co-culture and
107 accounting for the ability of conductive materials to accelerate DIET.

108

109 **Materials and Methods**

110 *Laboratory strains and culture conditions*

111 *Geobacter* cultures were obtained from our laboratory culture collection and
112 routinely cultured under strict anaerobic conditions, as previously described (68). *G.*
113 *metallireducens* was grown in Fe(III) citrate (FC) medium (69) with 20 mM ethanol
114 provided as the sole electron donor and 56 mM Fe(III) citrate as the sole electron
115 acceptor, or with 20 mM acetate as the donor and 50 mM Fe(III) oxide as the acceptor. *G.*
116 *sulfurreducens* was grown in medium with 10 mM acetate provided as the sole electron
117 donor and 40 mM fumarate as the sole electron acceptor (NBAF medium) (68). Co-
118 cultures were initiated with equal amounts of both organisms in anaerobic pressure tubes
119 containing 10 mL of NBF medium (acetate-free NBAF), with 10 mM ethanol provided as
120 the sole electron donor and 40 mM fumarate as the electron acceptor. When noted,
121 additions of anthraquinone-2,6, -disulfonate (AQDS) were made from a concentrated
122 stock to provide a final concentration of 50 μ M. In some instances, granular activated
123 carbon (GAC; 8-20 mesh (Sigma-Aldrich)) was added at 0.1g/ 10 ml.

124 *Methanosarcina barkeri* was obtained from our laboratory culture collection and
125 grown in co-culture with *G. metallireducens* with ethanol as the electron donor, as
126 previously described (59).

127

128

129 ***Analytical techniques***

130 Organic acids were monitored with high performance liquid chromatography
131 (HPLC), as previously described (70). Changes in ethanol concentration were monitored
132 with gas chromatography, as previously described (4). Methane was monitored in the
133 headspace by gas chromatography with a flame ionization detector (SHIMADZU, GC-
134 8A), as previously described (71). Fe(II) concentrations were determined by first
135 incubating samples for 1 hour in 0.5N HCl and then measuring Fe(II) with a ferrozine
136 assay at an absorbance of 562 nm (72).

137 ***Illumina sequencing and data analysis***

138 For all experimental conditions, total RNA was extracted from triplicate samples
139 at mid-log phase growth when succinate concentrations reached approximately 25 mM
140 using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.
141 Samples were treated with Turbo DNA-free DNase (Ambion, Austin, TX), and the RNA
142 samples were tested for genomic DNA (gDNA) contamination by PCR amplification of
143 the 16S rRNA gene. mRNA was enriched using the MICROBExpress kit (Ambion),
144 according to the manufacturer's instructions.

145 Directional libraries were prepared with the ScriptSeq™ v2 RNA-Seq Library
146 Preparation Kit (Epicentre) and single end sequencing was performed on a Hi-Seq 2000
147 platform at the Deep Sequencing Core Facility at the University of Massachusetts
148 Medical School in Worcester, Massachusetts. The program FASTQC
149 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used to visualize and
150 quality check all raw data. Initial raw non-filtered libraries contained an average of
151 13175155.5 +/- 1758892 and 10227370.2 +/- 1558219.6 reads in the DIET and quinone-

152 mediated interspecies electron transfer (QUIET) libraries that were ~100 base pairs long.
153 Sequences from all of these libraries were trimmed and filtered with Trimmomatic
154 (Bolger 2014) yielding an average of 9286241 +/- 1665081.9 and 12393095 +/-
155 1719373.9 reads for the DIET and QUIET libraries.

156 ***Mapping of mRNA reads***

157 Trimmed and filtered mRNA reads from triplicate samples for the two different
158 co-culture conditions (DIET and QUIET) were mapped against the *G. metallireducens*
159 strain GS-15 genome (NC_007517) and the *G. sulfurreducens* strain PCA genome
160 (NC_002939) downloaded from IMG/MER (img.jgi.doe.gov) using ArrayStar software
161 (DNASTar). Common dispersion (Disp) and biological coefficient of variation (BCV)
162 values between DIET and QUIET replicates were calculated with the edgeR package in
163 Bioconductor (73). Common dispersion and BCV values for DIET and QUIET libraries
164 were Disp=0.01953 and BCV=0.1398 and Disp=0.11752 and BCV=0.3428, respectively.
165 A multidimensional scaling (MDS) plot was also generated with edgeR software and
166 showed that replicates from the DIET and QUIET libraries clustered together but
167 separately from each other (Fig. S2).

168 Once the quality of RNAseq libraries was determined, differential expression
169 studies were done with the edgeR package in Bioconductor (73). Genes with p-values \leq
170 0.05 and fold changes ≥ 2 were considered differentially expressed. Using these criteria,
171 945 *G. metallireducens* genes and 967 *G. sulfurreducens* genes were up-regulated in
172 DIET-grown co-cultures and 603 *G. metallireducens* genes and 848 *G. sulfurreducens*
173 genes were up-regulated in QUIET-grown co-cultures (Table S2).

174

175 ***Quantitative RT-PCR***

176 Quantitative RT-PCR was conducted with mRNA extracted from triplicate
177 cultures of *G. metallireducens* wild-type and Δhcp (Gmet_0280) strains grown by Fe(III)
178 oxide respiration, in co-culture with *M. barkeri*, or in co-culture with *G. sulfurreducens*.
179 Cells were harvested during the mid-logarithmic phase by centrifugation at 4,000 rpm for
180 15 min at 4°C. After centrifugation, the pellets were frozen in liquid nitrogen and stored
181 at -80°C until RNA extraction procedures were performed. Total RNA from sample
182 pellets was extracted as previously described (74). Complementary DNA (cDNA) was
183 generated from mRNA using the Invitrogen SuperScript IV First Strand Synthesis
184 System (ThermoFisher Sci).

185 Primer pairs used for qRT-PCR are provided in Table S3. Three different
186 housekeeping genes were used as external controls; *recA* which codes for recombinase A,
187 *proC* which codes for pyrroline-5-carboxylate reductase, and *rpoB* which codes for the
188 beta subunit of RNA polymerase. Power SYBR green PCR master mix (Applied
189 Biosystems, Foster City, CA) and an ABI 7500 real-time PCR system were used to
190 amplify and to quantify all PCR products. Each reaction mixture consisted of forward and
191 reverse primers at a final concentration of 200 nM, 5 ng of gDNA, and 12.5 μ l of Power
192 SYBR green PCR master mix (Applied Biosystems). Relative levels of expression of the
193 studied genes were calculated by the $2^{-\Delta\Delta CT}$ threshold cycle (CT) method (75).

194 ***Mutant construction***

195 Primers used for construction of gene replacement mutants and complement
196 strains are listed in Table S4. Deletion mutants were made by replacing the gene of
197 interest with a spectinomycin antibiotic resistance cassette (76). All restriction digestions

198 were carried out according to manufacturer's instructions. PCRs were done using the
199 JumpStart Taq DNA polymerase (Sigma-Aldrich). Primer pairs were used to amplify by
200 PCR flanking regions of approximately 500 bp downstream and upstream of the target
201 genes using the appropriate genomic DNA as a template. PCR products were digested
202 with the *AvrII* (CCTAGG) (NEB, Beverly, MA) restriction endonuclease, ethanol
203 precipitated, and ligated with T4 DNA ligase (NEB). The ligation reaction was loaded
204 onto a 1% agarose gel, and a 1 kb band was purified using the Qiaquick Gel Extraction
205 Kit (Qiagen) and cloned into pCR2.1 TOPO cloning vector. Sequences of the cloned
206 products were verified by Sanger sequencing. The spectinomycin cassette was digested
207 with *XbaI* (TCTAGA) (NEB) from pUC19-*Spr^r loxP* (76), and the recombinant plasmid
208 was digested with *AvrII*. The spectinomycin resistance cassette was cloned into the
209 plasmid to complete the construction of the mutant alleles. Plasmids bearing mutant
210 alleles were linearized and concentrated by ethanol precipitation. The linearized
211 plasmids were electroporated as described previously (76). Antibiotics were added for
212 selection purposes only. Replacement of wild type alleles by mutant alleles was verified
213 by PCR and Sanger sequencing.

214 Gmet_0280 was complemented *in trans* by amplifying the gene with its native
215 ribosome binding site (RBS) using *G. metallireducens* genomic DNA as a template. The
216 resulting PCR product was then digested and cloned under control of a constitutive lac
217 promoter into pCM66 (77) and electroporated into the Gmet_0280-deficient strain, as
218 previously described (76).

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221 **Data availability**

222 Illumina sequence reads have been submitted to the SRA NCBI database under
223 BioProject PRJNA722959 and Biosample SAMN18796025.

224 **Results and discussion**

225 **Increased expression of *G. metallireducens* T6SS genes during DIET**

226 Comparing gene expression patterns in defined co-cultures growing via DIET
227 versus co-cultures growing with the exchange of diffusible electron shuttles has proven to
228 be an effective strategy for identifying mechanisms for electron transfer during DIET (23,
229 24). Therefore, co-cultures of *G. metallireducens* and *G. sulfurreducens* were grown in
230 medium with ethanol as the electron donor and fumarate as the electron acceptor as
231 previously described (9, 78). The two species must cooperate to share electrons in this
232 medium because only *G. metallireducens* can utilize ethanol as an electron donor and
233 only *G. sulfurreducens* uses fumarate as electron acceptor (9). AQDS (50 μ M) was added
234 to one set of co-cultures to promote quinone-mediated interspecies electron transfer
235 (QUIET) in which the two species remain free-living and AQDS serves as an electron
236 shuttle between them (78). However, in the absence of AQDS the two species must form
237 tight physical association for direct electron transfer from *G. metallireducens* to *G.*
238 *sulfurreducens* because *G. metallireducens* cannot produce H₂ or formate as electrons
239 shuttles when metabolizing ethanol (22, 23).

240 The initial AQDS-amended and unamended co-cultures were sampled for gene
241 expression analysis after they had reduced ca. 25 mM of the 40 mM fumarate available.
242 DIET-grown *G. sulfurreducens* had higher transcript abundances for genes previously
243 found to be important in DIET (Table S2c). These included genes for over 25 *c*-type

244 cytochromes, including *omcS*, *omcB*, *omcX*, and *omcI*, which encode multi-heme, outer-
245 surface *c*-type cytochromes. The gene coding for the pilin monomer, PilA, which is
246 assembled into electrically conductive pili (79) was also 63-times more highly expressed
247 in DIET-grown cells.

248 Transcripts for 19 different *c*-type cytochrome genes were also ≥ 2 -fold more
249 abundant in DIET-grown *G. metallireducens* (Table S2a). These included Gmet_0930,
250 which codes for an octaheme outer membrane *c*-type cytochrome and Gmet_0910, the
251 gene for the outer membrane *c*-type cytochrome, OmcF, from the PccF porin-cytochrome
252 complex (59). Both Gmet_0930 and Gmet_0910 are important for Fe(III) oxide reduction
253 and DIET-based growth (17, 59, 80). Gmet_2029, which codes for a lipopolysaccharide
254 protein likely to be involved in biofilm formation and required for Fe(III) oxide reduction
255 (80), was expressed more than 3 fold higher in DIET-grown cells, but *pilA* was not more
256 highly expressed by DIET-grown *G. metallireducens* cells (Table S2a).

257 Other genes that would not be expected to be involved in electron transfer were
258 also more highly expressed in *G. metallireducens* cells growing by DIET (Table 1; Table
259 S2a). The two genes with the greatest increase in abundance of transcripts in DIET-
260 versus QUIET-grown cells were Gmet_2080 and Gmet_2078, annotated as ‘T6SS needle
261 tube protein TssD’ and ‘T6SS protein ImpB’. Transcripts for other T6SS proteins were
262 also much more abundant in DIET- versus QUIET-grown *G. metallireducens* (Table 1).
263 This included 13 of the genes needed to construct the T6SS nanomachine in other
264 microbes (26, 31, 81). Furthermore, genes coding for all putative T6SS effectors,
265 immunity proteins, and effector chaperones in *G. metallireducens*, with the exception of
266 Gmet_0291 which codes for a putative chaperone protein, were at least 5-times more

267 highly expressed in DIET-grown *G. metallireducens* cells (Table 1). Only some of the *G.*
268 *sulfurreducens* T6SS-related genes were highly expressed in DIET- versus QUIET-
269 grown cells (Table S5).

270 **Disrupting the *hcp* gene in *G. metallireducens* accelerates adaption to DIET**

271 The high expression of T6SS genes in *G. metallireducens* during growth via
272 DIET was surprising because a primary function of the T6SS is elimination of competing
273 species (31, 32, 34, 82-84). To determine whether expression of the T6SS by *G.*
274 *metallireducens* impacted DIET, the gene for the Hcp needle-tube protein (Gmet_0280),
275 the most highly differentially expressed gene in DIET- versus QUIET-grown cells (Table
276 S2A), was disrupted by replacing the gene with a spectinomycin resistance cassette.

277 As previously described (9), co-cultures established in ethanol-fumarate medium
278 with wild-type *G. metallireducens* required over 25 days to begin DIET, monitored as the
279 accumulation of succinate from fumarate reduction (Fig. 1a). In contrast, there was a
280 shorter lag in adaption to DIET in co-cultures initiated with the Hcp-deficient strain of *G.*
281 *metallireducens* (Fig. 1a). Large aggregates (1-2 mm diameter) were visibly apparent in
282 the co-cultures with the Hcp-deficient strain of *G. metallireducens* even when the co-
283 cultures were first established. In contrast, as previously reported (9), in co-cultures
284 established with wild-type *G. metallireducens* large aggregates only appeared after
285 multiple successive transfers of the co-cultures. The Hcp-deficient strain also produced
286 visible aggregations in Fe(III) citrate medium, which was not observed in the wild-type
287 strain (Fig. S3).

288 The Hcp-deficient *G. metallireducens* strain did not have a substantial advantage
289 over wild-type cells if granular activated carbon (GAC) was added to the co-cultures

290 (Fig. 1b). In the presence of GAC, which is electrically conductive, *G. metallireducens*
291 and *G. sulfurreducens* attach to the GAC surface rather than producing dual-species
292 aggregates and the cells are not close enough for DIET via electrically conductive pili or
293 *c*-type cytochromes (60). Deletion of the genes for these biological electrical connections
294 does not inhibit DIET in the presence of GAC, suggesting that GAC is a highly effective
295 conduit for DIET (60). Co-cultures established with the Hcp-deficient *G. metallireducens*
296 or wild-type *G. metallireducens* grew at the same rate when AQDS was provided as an
297 electron shuttle for QUIET (Fig. 1c). Like GAC, AQDS also eliminates the need for
298 direct cell-to-cell contacts for interspecies electron transfer (78).

299 Disrupting the gene for Hcp from *G. sulfurreducens* (Δ G_{SU3174}) did not
300 substantially decrease the lag time required for initiation of co-culture metabolism under
301 conditions that require DIET for growth (Fig. 1d). This is consistent with the observation
302 that *G. sulfurreducens* did not increase expression of genes for most T6SS components in
303 DIET- versus QUIET-grown cells (Table S5).

304 To determine the potential impact of T6SS expression on *Geobacter* interactions
305 with methanogens, co-cultures of *G. metallireducens* and *M. barkeri* were initiated as
306 previously described (10) in medium with ethanol as the electron donor. As previously
307 described (10), there was a lag period of more than 30 days in co-cultures initiated with
308 wild-type *G. metallireducens* (Fig. 2). In contrast, there was very little lag in co-cultures
309 initiated with the Hcp-deficient *G. metallireducens* strain. While studies have focused on
310 T6SSs targeting bacterial and eukaryotic cells (85, 86), the effect of T6SSs on archaeal
311 cells requires further study (87).

312 **Disrupting the *hcp* gene in *G. metallireducens* has unexpected impact on**
313 **extracellular electron transfer**

314 The impact of the *hcp* deletion on extracellular electron transfer was evaluated.
315 The Hcp-deficient strain grew faster than wild-type *G. metallireducens* when insoluble
316 Fe(III) oxide was provided as the electron acceptor (Fig. 3). A complement strain
317 containing the *hcp* gene *in trans* grew at rates similar to the wild-type strain,
318 demonstrating that elimination of the T6SS impacted extracellular electron transfer
319 capabilities.

320 Quantitative RT-PCR (Fig. 4) revealed that, compared to the wild-type strain, the
321 Hcp-deficient strain of *G. metallireducens* more highly expressed genes for key outer-
322 surface components previously shown to be important in extracellular electron transfer
323 (Fig. 4; Table S6). These included genes for PilA, the monomer that is assembled into
324 electrically conductive pili that are required for Fe(III) oxide reduction and DIET (25), as
325 well as genes for the outer surface *c*-type cytochromes (Gmet_0930 and Gmet_0910) and
326 a lipopolysaccharide protein (Gmet_2029) that are required for Fe(III) oxide reduction
327 and expected to play an important role in DIET (23, 59, 80).

328 Increased expression of these genes is a likely explanation for the accelerated
329 Fe(III) oxide reduction and may also have contributed to accelerated DIET. The
330 biosynthetic and energetic costs of deploying T6SS machinery is high (88). Therefore, it
331 may be that eliminating some of this cost by deleting *hcp* enabled a greater investment in
332 expression of outer-surface proteins important for extracellular electron transfer. Notably,
333 T6SS genes were not up-regulated in *G. metallireducens*/*G. sulfurreducens* co-cultures
334 that had undergone long-term adaptation to growth via DIET (23), suggesting that

335 lowering expression of T6SS genes is part of *G. metallireducens*' adaptive response to
336 DIET-based growth.

337 **Implications**

338 The results demonstrate that the expression of a T6SS can be detrimental for the
339 establishment of DIET consortia and offer a new insight into the mechanisms by which
340 conductive materials might facilitate DIET. It has previously been considered that
341 conductive materials that are larger than cells, such as GAC, biochar, or carbon cloth
342 accelerate the initiation of DIET because: 1) expression of electrically conductive pili and
343 some outer-surface cytochromes is no longer necessary, conserving energy; and 2) it is
344 easier for an electroactive microbe to establish electrical contact with a large conductive
345 surface than small, disperse electrical contacts on another microbial cell (1, 89). The
346 results presented here suggest that another benefit, in some instances, may be that
347 conductive surfaces alleviate the need for close physical contact between DIET partners
348 (60-62). Thus, DIET partners can 'socially distance' to avoid the possible negative
349 impact of close physical associations as electrons zoom through the conductive material
350 enabling the cells to connect remotely.

351 *G. metallireducens*' high expression of its T6SS is clearly not in its best interest in
352 the context of DIET in a defined laboratory co-culture, but *G. metallireducens* seems
353 unlikely to exemplify the *Geobacter* species that participate in DIET in natural
354 communities. *G. metallireducens* was recovered from an enrichment culture that selected
355 for microbes rapidly growing via Fe(III) oxide reduction (69, 90), conditions likely to
356 favor interspecies competition, not cooperation. Not all *Geobacter* species possess a
357 T6SS (Table S1). Many other microbes that participate in DIET lack a T6SS, including

358 *Prosthecochloris aestaurii* (16), *Syntrophus aciditrophicus*, (8), *Rhodoferax*
359 *ferrireducens* (13), *Desulfovibrio* sp. JY (15), and *Rhodopseudomonas palustris* (18, 20)
360 (Table S1b).

361 The finding that deletion of a major gene necessary for T6SS function in *G.*
362 *metallireducens* unexpectedly increased expression of key components for extracellular
363 electron transfer emphasizes a frequent problem in studies of *Geobacter*
364 electromicrobiology. Adaption to gene deletions often result in changes in the mutant's
365 physiology beyond the direct function of the missing protein (65, 91, 92). Thus, multiple
366 experimental approaches are warranted when developing models for *Geobacter*
367 extracellular electron exchange.

368 **References**

- 369 1. Lovley DR. 2017. Syntrophy Goes Electric: Direct Interspecies Electron Transfer.
370 *Annu Rev Microbiol* 71:643-664.
- 371 2. Van Steendam C, Smets I, Skerlos S, Raksin L. 2019. Improving anaerobic digestion
372 via direct interspecies electron transfer requires development of suitable
373 characterization methods. *Curr Opin Biotechnol* 57:183-190.
- 374 3. Zhao Z, Li Y, Zhang Y, Lovley DR. 2020. Sparking anaerobic digestion: promoting
375 direct interspecies electron transfer to enhance methane production. *iScience*
376 23:101794.
- 377 4. Morita M, Malvankar NS, Franks AE, Summers ZM, Giloteaux L, Rotaru AE, Rotaru C,
378 Lovley DR. 2011. Potential for direct interspecies electron transfer in methanogenic
379 wastewater digester aggregates. *MBio* 2:e00159-11.
- 380 5. Rotaru AE, Shrestha PM, Liu FH, Shrestha M, Shrestha D, Embree M, Zengler K,
381 Wardman C, Nevin KP, Lovley DR. 2014. A new model for electron flow during

- 382 anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the
383 reduction of carbon dioxide to methane. *Energy Environ Sci* 7:408-415.
- 384 6. Park J-H, Kang H-J, Park K-H, Park H-D. 2018. Direct interspecies electron transfer
385 via conductive materials: A perspective for anaerobic digestion applications.
386 *Bioresource Technol* 254:300-311.
- 387 7. Holmes DE, Shrestha PM, Walker DJF, Dang Y, Nevin KP, Woodard TL, Lovley DR.
388 2017. Metatranscriptomic Evidence for Direct Interspecies Electron Transfer
389 between *Geobacter* and *Methanothrix* Species in Methanogenic Rice Paddy Soils.
390 *Appl Environ Microbiol* 83.
- 391 8. Walker DJF, Nevin KP, Holmes DE, Rotaru AE, Ward JE, Woodard TL, Zhu J, Ueki T,
392 Nonnenmann SS, McInerney MJ, Lovley DR. 2020. *Syntrophus* conductive pili
393 demonstrate that common hydrogen-donating syntrophs can have a direct electron
394 transfer option. *ISME J* 14:837-846.
- 395 9. Summers ZM, Fogarty HE, Leang C, Franks AE, Malvankar NS, Lovley DR. 2010.
396 Direct exchange of electrons within aggregates of an evolved syntrophic coculture of
397 anaerobic bacteria. *Science* 330:1413-5.
- 398 10. Rotaru AE, Shrestha PM, Liu F, Markovaite B, Chen S, Nevin KP, Lovley DR. 2014.
399 Direct interspecies electron transfer between *Geobacter metallireducens* and
400 *Methanosarcina barkeri*. *Appl Environ Microbiol* 80:4599-605.
- 401 11. Wang L-Y, Nevin KP, Woodard TL, Mu B-Z, Lovley DR. 2016. Expanding the diet for
402 DIET: electron donors supporting direct interspecies electron transfer (DIET) in
403 defined co-cultures. *Frontiers in Microbiol* 7:236.
- 404 12. Walker DJF, Adhikari RY, Holmes DE, Ward JE, Woodard TL, Nevin KP, Lovley DR.
405 2018. Electrically conductive pili from genes of phylogenetically diverse
406 microorganisms. *ISME J* 12:48-58.

- 407 13. Yee M, Rotaru A. 2020. Extracellular electron uptake in *Methanosarcinales* is
408 independent of multiheme *c*-type cytochromes. *Sci Rep* 10:372.
- 409 14. Yee MO, Snoeyenbos-West O, Thamdrup B, Ottosen L, Rotaru A-E. 2019.
410 Extracellular electron uptake by two *Methanosarcina* species. *Front Energy Research*
411 7:29.
- 412 15. Zheng S, Li M, Liu Y, Liu F. 2021. *Desulfovibrio* feeding *Methanobacterium* with
413 electrons in conductive methanogenic aggregates from coastal zones. *Water*
414 *Research* 202:117490.
- 415 16. Ha PT, Lindemann SR, Shi L, Dohnalkova AC, Fredrickson JK, Madigan MT, Beyenal
416 H. 2017. Syntrophic anaerobic photosynthesis via direct interspecies electron
417 transfer. *Nat Commun* 8:13924.
- 418 17. Zhou J, Smith JA, Li M, Holmes DE. 2023. Methane production by *Methanothrix*
419 *thermoacetophila* via direct interspecies electron transfer with *Geobacter*
420 *metallireducens*. *bioRxiv* doi:10.1101/2023.02.13.528421:2023.02.13.528421.
- 421 18. Huang L, Liu X, Zhang Z, Ye J, Rensing C, Zhou S, Nealson KH. 2022. Light-driven
422 carbon dioxide reduction to methane by *Methanosarcina barkeri* in an electric
423 syntrophic coculture. *ISME J* 16:370-377.
- 424 19. Zhou J, Holmes DE, Tang HY, Lovley DR. 2021. Correlation of Key Physiological
425 Properties of *Methanosarcina* Isolates with Environment of Origin. *Appl Environ*
426 *Microbiol* 87:e0073121.
- 427 20. Liu X, Huang L, Rensing C, Ye J, Nealson KH, Zhou S. 2021. Syntrophic interspecies
428 electron transfer drives carbon fixation and growth by *Rhodopseudomonas palustris*
429 under dark, anoxic conditions. *Sci Adv* 7.
- 430 21. Zheng S, Liu F, Wang B, Zhang Y, Lovley DR. 2020. A *Methanobacterium* capable of
431 direct interspecies electron transfer. *Env Sci and Technol* 54:15347-15354.

- 432 22. Shrestha P, Rotaru A, Aklujkar M, Liu F, Shrestha M, Summers Z, Malvankar NS,
433 Flores D, Lovley DR. 2013. Syntrophic growth with direct interspecies electron
434 transfer as the primary mechanism for energy exchange. *Env Microbiol Rep* 5:904-
435 910.
- 436 23. Shrestha PM, Rotaru AE, Summers ZM, Shrestha M, Liu F, Lovley DR. 2013.
437 Transcriptomic and genetic analysis of direct interspecies electron transfer. *Appl*
438 *Environ Microbiol* 79:2397-404.
- 439 24. Holmes DE, Rotaru AE, Ueki T, Shrestha PM, Ferry JG, Lovley DR. 2018. Electron and
440 Proton Flux for Carbon Dioxide Reduction in *Methanosarcina barkeri* During Direct
441 Interspecies Electron Transfer. *Front Microbiol* 9:3109.
- 442 25. Ueki T, Nevin K, Rotaru A, Wang L, Woodard T, Lovley DR. 2018. *Geobacter* strains
443 expressing poorly conductive pili reveal constraints on direct interspecies electron
444 transfer mechanisms. *mBio* 9:e01273-18.
- 445 26. Gallique M, Bouteiller M, Merieau A. 2017. The Type VI Secretion System: A Dynamic
446 System for Bacterial Communication? *Front Microbiol* 8:1454.
- 447 27. Cianfanelli FR, Monlezun L, Coulthurst SJ. 2016. Aim, Load, Fire: The Type VI
448 Secretion System, a Bacterial Nanoweapon. *Trends Microbiol* 24:51-62.
- 449 28. Wang J, Brodmann M, Basler M. 2019. Assembly and Subcellular Localization of
450 Bacterial Type VI Secretion Systems. *Annu Rev Microbiol* 73:621-638.
- 451 29. Silverman JM, Brunet YR, Cascales E, Mougous JD. 2012. Structure and regulation of
452 the type VI secretion system. *Annu Rev Microbiol* 66:453-72.
- 453 30. Schwarz S, Hood RD, Mougous JD. 2010. What is type VI secretion doing in all those
454 bugs? *Trends in Microbiol* 18:531-537.
- 455 31. Coulthurst S. 2019. The Type VI secretion system: a versatile bacterial weapon.
456 *Microbiol* 165:503-515.

- 457 32. Hood RD, Singh P, Hsu F, Guvener T, Carl MA, Trinidad RR, Silverman JM, Ohlson BB,
458 Hicks KG, Plemel RL, Li M, Schwarz S, Wang WY, Merz AJ, Goodlett DR, Mougous JD.
459 2010. A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to
460 bacteria. *Cell Host Microbe* 7:25-37.
- 461 33. MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S. 2010. The *Vibrio cholerae* type VI
462 secretion system displays antimicrobial properties. *Proc Natl Acad Sci USA*
463 107:19520-4.
- 464 34. Murdoch SL, Trunk K, English G, Fritsch MJ, Pourkarimi E, Coulthurst SJ. 2011. The
465 Opportunistic Pathogen *Serratia marcescens* Utilizes Type VI Secretion To Target
466 Bacterial Competitors. *J Bacteriol* 193:6057-6069.
- 467 35. Russell AB, Peterson SB, Mougous JD. 2014. Type VI secretion system effectors:
468 poisons with a purpose. *Nat Rev Microbiol* 12:137-48.
- 469 36. Jani AJ, Cotter PA. 2010. Type VI secretion: not just for pathogenesis anymore. *Cell*
470 *Host Microbe* 8:2-6.
- 471 37. Russell AB, Wexler AG, Harding BN, Whitney JC, Bohn AJ, Goo YA, Tran BQ, Barry NA,
472 Zheng H, Peterson SB, Chou S, Gonen T, Goodlett DR, Goodman AL, Mougous JD.
473 2014. A type VI secretion-related pathway in *Bacteroidetes* mediates interbacterial
474 antagonism. *Cell Host Microbe* 16:227-236.
- 475 38. Sana TG, Flaugnatti N, Lugo KA, Lam LH, Jacobson A, Baylot V, Durand E, Journet L,
476 Cascales E, Monack DM. 2016. *Salmonella Typhimurium* utilizes a T6SS-mediated
477 antibacterial weapon to establish in the host gut. *Proc Natl Acad Sci USA* 113:E5044-
478 51.
- 479 39. Roelofs KG, Coyne MJ, Gentyala RR, Chatzidaki-Livanis M, Comstock LE. 2016.
480 Bacteroidales Secreted Antimicrobial Proteins Target Surface Molecules Necessary
481 for Gut Colonization and Mediate Competition In Vivo. *mBio* 7.

- 482 40. Zhao W, Caro F, Robins W, Mekalanos JJ. 2018. Antagonism toward the intestinal
483 microbiota and its effect on *Vibrio cholerae* virulence. *Science* 359:210-213.
- 484 41. Logan SL, Thomas J, Yan J, Baker RP, Shields DS, Xavier JB, Hammer BK,
485 Parthasarathy R. 2018. The *Vibrio cholerae* type VI secretion system can modulate
486 host intestinal mechanics to displace gut bacterial symbionts. *Proc Natl Acad Sci USA*
487 115:E3779-E3787.
- 488 42. Wan B, Zhang Q, Ni J, Li S, Wen D, Li J, Xiao H, He P, Ou HY, Tao J, Teng Q, Lu J, Wu W,
489 Yao YF. 2017. Type VI secretion system contributes to Enterohemorrhagic
490 *Escherichia coli* virulence by secreting catalase against host reactive oxygen species
491 (ROS). *PLoS Pathog* 13:e1006246.
- 492 43. Auffret MD, Dewhurst RJ, Duthie CA, Rooke JA, John Wallace R, Freeman TC, Stewart
493 R, Watson M, Roehe R. 2017. The rumen microbiome as a reservoir of antimicrobial
494 resistance and pathogenicity genes is directly affected by diet in beef cattle.
495 *Microbiome* 5:159.
- 496 44. Bernal P, Llamas MA, Filloux A. 2018. Type VI secretion systems in plant-associated
497 bacteria. *Environ Microbiol* 20:1-15.
- 498 45. Records AR. 2011. The type VI secretion system: a multipurpose delivery system
499 with a phage-like machinery. *Mol Plant Microbe Interact* 24:751-7.
- 500 46. Speare L, Cecere AG, Guckes KR, Smith S, Wollenberg MS, Mandel MJ, Miyashiro T,
501 Septer AN. 2018. Bacterial symbionts use a type VI secretion system to eliminate
502 competitors in their natural host. *Proc Natl Acad Sci USA* 115:E8528-E8537.
- 503 47. Ma LS, Hachani A, Lin JS, Filloux A, Lai EM. 2014. *Agrobacterium tumefaciens* deploys
504 a superfamily of type VI secretion DNase effectors as weapons for interbacterial
505 competition in planta. *Cell Host Microbe* 16:94-104.

- 506 48. Troselj V, Treuner-Lange A, Sogaard-Andersen L, Wall D. 2018. Physiological
507 Heterogeneity Triggers Sibling Conflict Mediated by the Type VI Secretion System in
508 an Aggregative Multicellular Bacterium. *mBio* 9.
- 509 49. Thiery S, Kaimer C. 2020. The Predation Strategy of *Myxococcus xanthus*. *Front*
510 *Microbiol* 11:2.
- 511 50. Weber B, Hasic M, Chen C, Wai SN, Milton DL. 2009. Type VI secretion modulates
512 quorum sensing and stress response in *Vibrio anguillarum*. *Environ Microbiol*
513 11:3018-28.
- 514 51. Alteri CJ, Himpsl SD, Pickens SR, Lindner JR, Zora JS, Miller JE, Arno PD, Straight SW,
515 Mobley HL. 2013. Multicellular bacteria deploy the type VI secretion system to
516 preemptively strike neighboring cells. *PLoS Pathog* 9:e1003608.
- 517 52. Wenren LM, Sullivan NL, Cardarelli L, Septer AN, Gibbs KA. 2013. Two independent
518 pathways for self-recognition in *Proteus mirabilis* are linked by type VI-dependent
519 export. *mBio* 4.
- 520 53. Konovalova A, Petters T, Sogaard-Andersen L. 2010. Extracellular biology of
521 *Myxococcus xanthus*. *FEMS Microbiol Rev* 34:89-106.
- 522 54. Chen WJ, Kuo TY, Hsieh FC, Chen PY, Wang CS, Shih YL, Lai YM, Liu JR, Yang YL, Shih
523 MC. 2016. Involvement of type VI secretion system in secretion of iron chelator
524 pyoverdine in *Pseudomonas taiwanensis*. *Sci Rep* 6:32950.
- 525 55. Wang T, Si M, Song Y, Zhu W, Gao F, Wang Y, Zhang L, Zhang W, Wei G, Luo ZQ, Shen
526 X. 2015. Type VI Secretion System Transports Zn²⁺ to Combat Multiple Stresses and
527 Host Immunity. *PLoS Pathog* 11:e1005020.
- 528 56. Han Y, Wang T, Chen G, Pu Q, Liu Q, Zhang Y, Xu L, Wu M, Liang H. 2019. A
529 *Pseudomonas aeruginosa* type VI secretion system regulated by CueR facilitates
530 copper acquisition. *PLoS Pathog* 15:e1008198.

- 531 57. Lin J, Zhang W, Cheng J, Yang X, Zhu K, Wang Y, Wei G, Qian PY, Luo ZQ, Shen X. 2017.
532 A *Pseudomonas* T6SS effector recruits PQS-containing outer membrane vesicles for
533 iron acquisition. *Nat Commun* 8:14888.
- 534 58. Martins G, Salvador AF, Pereira L, Alves MM. 2018. Methane production and
535 conductive materials: a critical review. *Environ Sci Technol* 52:10241-10253.
- 536 59. Holmes DE, Zhou J, Smith JA, Wang C, Liu X, Lovley DR. 2022. Different outer
537 membrane *c*-type cytochromes are involved in direct interspecies electron transfer
538 to *Geobacter* or *Methanosarcina* species. *mLife* 1:272-286.
- 539 60. Liu F, Rotaru A, Shrestha P, Malvankar N, Nevin K, Lovley D. 2012. Promoting direct
540 interspecies electron transfer with activated carbon. *Energy Environ Sci* doi:DOI:
541 10.1039/c2ee22459c.
- 542 61. Chen S, Rotaru A-E, Liu F, Philips J, Woodard TL, Nevin KP, Lovley DR. 2014. Carbon
543 cloth stimulates direct interspecies electron transfer in syntrophic co-cultures.
544 *Bioresource Tech* 173:82-86.
- 545 62. Chen S, Rotaru A-E, Shrestha PM, Malvankar NS, Liu F, Fan W, Nevin KP, Lovley DR.
546 2014. Promoting interspecies electron transfer with biochar. *Sci Rep* 4:5019.
- 547 63. Kato S, Hashimoto K, Watanabe K. 2012. Methanogenesis facilitated by electric
548 syntrophy via (semi)conductive iron-oxide minerals. *Environ Microbiol* 14:1646-
549 1654.
- 550 64. Liu F, Rotaru A-E, Shrestha PM, Malvankar NS, Nevin KP, Lovley DR. 2015. Magnetite
551 compensates for the lack of a pilin-associated *c*-type cytochrome in extracellular
552 electron exchange. *Environ Microbiol* 17:648-655.
- 553 65. Lovley DR, Ueki T, Zhang T, Malvankar NS, Shrestha PM, Flanagan KA, Aklujkar M,
554 Butler JE, Giloteaux L, Rotaru AE, Holmes DE, Franks AE, Orellana R, Risso C, Nevin

- 555 KP. 2011. *Geobacter*: the microbe electric's physiology, ecology, and practical
556 applications. *Adv Microb Physiol* 59:1-100.
- 557 66. Russell AB, Singh P, Brittnacher M, Bui NK, Hood RD, Carl MA, Agnello DM, Schwarz
558 S, Goodlett DR, Vollmer W, Mougous JD. 2012. A widespread bacterial type VI
559 secretion effector superfamily identified using a heuristic approach. *Cell Host*
560 *Microbe* 11:538-49.
- 561 67. Boyer F, Fichant G, Berthod J, Vandembrouck Y, Attree I. 2009. Dissecting the
562 bacterial type VI secretion system by a genome wide in silico analysis: what can be
563 learned from available microbial genomic resources? *BMC Genomics* 10:104.
- 564 68. Coppi MV, Leang C, Sandler SJ, Lovley DR. 2001. Development of a genetic system for
565 *Geobacter sulfurreducens*. *Appl Environ Microbiol* 67:3180-7.
- 566 69. Lovley DR, Giovannoni SJ, White DC, Champine JE, Phillips EJ, Gorby YA, Goodwin S.
567 1993. *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of
568 coupling the complete oxidation of organic compounds to the reduction of iron and
569 other metals. *Arch Microbiol* 159:336-44.
- 570 70. Nevin KP, Richter H, Covalla SF, Johnson JP, Woodard TL, Orloff AL, Jia H, Zhang M,
571 Lovley DR. 2008. Power output and columbic efficiencies from biofilms of *Geobacter*
572 *sulfurreducens* comparable to mixed community microbial fuel cells. *Environ*
573 *Microbiol* 10:2505-14.
- 574 71. Holmes DE, Giloteaux L, Orellana R, Williams KH, Robbins MJ, Lovley DR. 2014.
575 Methane production from protozoan endosymbionts following stimulation of
576 microbial metabolism within subsurface sediments. *Frontiers in Microbiol* 5.
- 577 72. Anderson RT, Lovley DR. 1999. Naphthalene and Benzene Degradation under
578 Fe(III)-Reducing Conditions in Petroleum-Contaminated Aquifers. *Bioremediation J*
579 3:121-135.

- 580 73. Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for
581 differential expression analysis of digital gene expression data. *Bioinformatics*
582 26:139-40.
- 583 74. Holmes DE, Risso C, Smith JA, Lovley DR. 2012. Genome-scale analysis of anaerobic
584 benzoate and phenol metabolism in the hyperthermophilic archaeon *Ferroglobus*
585 *placidus*. *ISME J* 6:146-57.
- 586 75. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-
587 time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-8.
- 588 76. Tremblay PL, Aklujkar M, Leang C, Nevin KP, Lovley D. 2012. A genetic system for
589 *Geobacter metallireducens*: role of the flagellin and pilin in the reduction of Fe(III)
590 oxide. *Environ Microbiol Rep* 4:82-8.
- 591 77. Marx CJ, Lidstrom ME. 2001. Development of improved versatile broad-host-range
592 vectors for use in methylotrophs and other Gram-negative bacteria. *Microbiol*
593 147:2065-2075.
- 594 78. Smith JA, Nevin KP, Lovley DR. 2015. Syntrophic growth via quinone-mediated
595 interspecies electron transfer. *Front Microbiol* 6:121.
- 596 79. Lovley DR, Holmes DE. 2020. Protein Nanowires: The Electrification of the Microbial
597 World and Maybe Our Own. *J Bacteriol* 202:e00331-20.
- 598 80. Smith JA, Lovley DR, Tremblay PL. 2013. Outer cell surface components essential for
599 Fe(III) oxide reduction by *Geobacter metallireducens*. *Appl Environ Microbiol* 79:901-
600 7.
- 601 81. Navarro-Garcia F, Ruiz-Perez F, Cataldi A, Larzabal M. 2019. Type VI Secretion
602 System in Pathogenic *Escherichia coli*: Structure, Role in Virulence, and Acquisition.
603 *Front Microbiol* 10:1965.

- 604 82. LeRoux M, De Leon JA, Kuwada NJ, Russell AB, Pinto-Santini D, Hood RD, Agnello
605 DM, Robertson SM, Wiggins PA, Mougous JD. 2012. Quantitative single-cell
606 characterization of bacterial interactions reveals type VI secretion is a double-edged
607 sword. *Proc Natl Acad Sci USA* 109:19804-19809.
- 608 83. Basler M, Ho BT, Mekalanos JJ. 2013. Tit-for-tat: type VI secretion system
609 counterattack during bacterial cell-cell interactions. *Cell* 152:884-894.
- 610 84. Carruthers MD, Nicholson PA, Tracy EN, Munson RS, Jr. 2013. *Acinetobacter*
611 *baumannii* utilizes a type VI secretion system for bacterial competition. *PLoS One*
612 8:e59388.
- 613 85. Hernandez RE, Gallegos-Monterrosa R, Coulthurst SJ. 2020. Type VI secretion
614 system effector proteins: Effective weapons for bacterial competitiveness. *Cellular*
615 *Microbiol* 22:e13241.
- 616 86. Monjarás Feria J, Valvano MA. 2020. An Overview of Anti-Eukaryotic T6SS Effectors.
617 *Frontiers in Cellular and Infection Microbiol* 10.
- 618 87. Gallegos-Monterrosa R, Coulthurst SJ. 2021. The ecological impact of a bacterial
619 weapon: microbial interactions and the Type VI secretion system. *FEMS Microbiol*
620 *Rev* 45.
- 621 88. Basler M. 2015. Type VI secretion system: secretion by a contractile nanomachine.
622 *Philos Trans R Soc Lond B Biol Sci* 370.
- 623 89. Lovley D. 2017. Happy together: microbial communities that hook up to swap
624 electrons. *ISME J* 11:327-336.
- 625 90. Lovley DR, Phillips EJP. 1988. Novel mode of microbial energy metabolism: organic
626 carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl*
627 *Environ Microbiol* 54:1472-1480.

- 628 91. Lovley DR, Holmes DE. 2022. Electromicrobiology: the ecophysiology of
629 phylogenetically diverse electroactive microorganisms. *Nat Rev Microbiol* 20:5-19.
- 630 92. Smith JA, Tremblay PL, Shrestha PM, Snoeyenbos-West OL, Franks AE, Nevin KP,
631 Lovley DR. 2014. Going wireless: Fe(III) oxide reduction without pili by *Geobacter*
632 *sulfurreducens* strain JS-1. *Appl Environ Microbiol* 80:4331-40.

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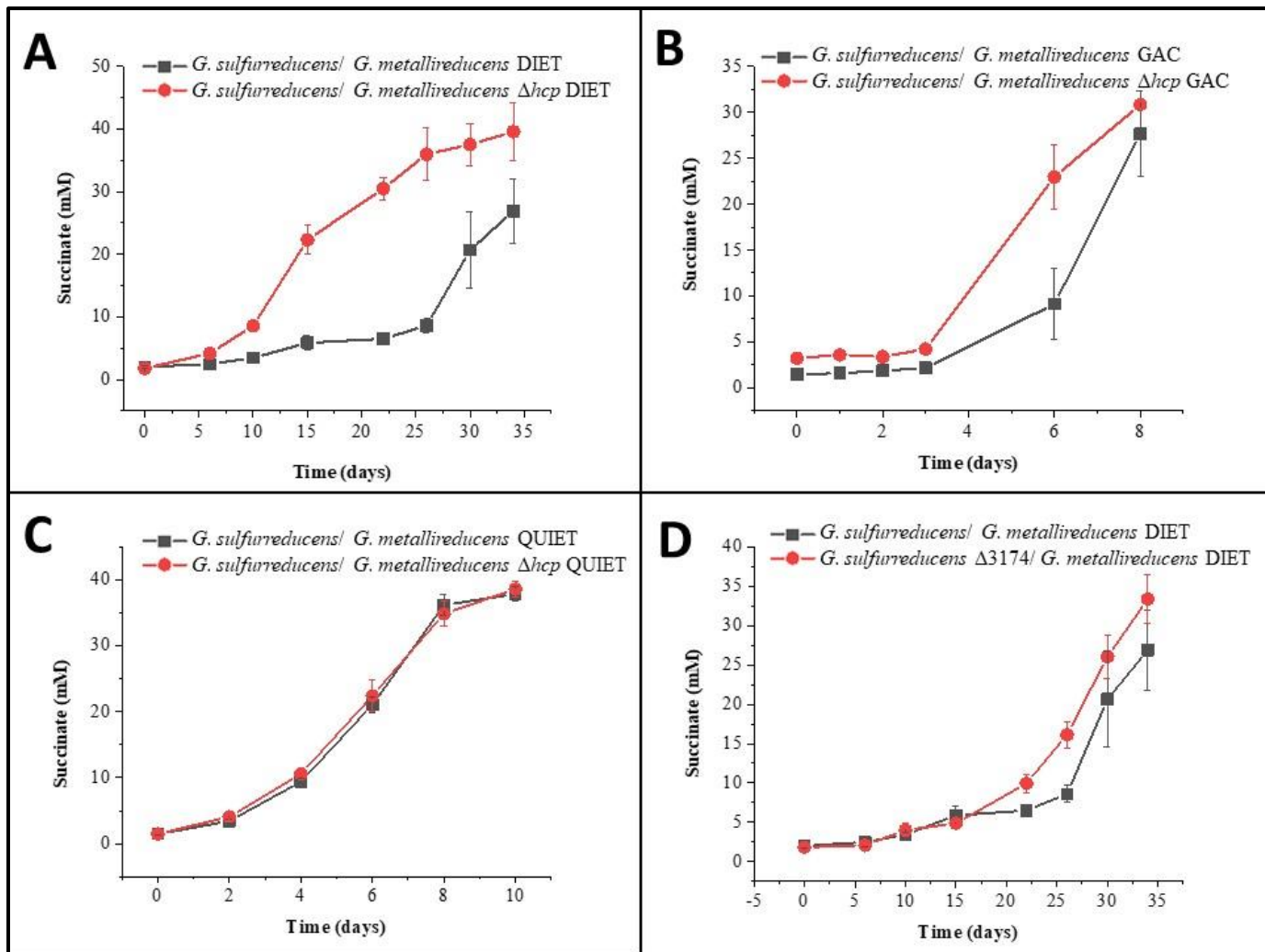
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652 **Figures and Figure Legends**

653 **Figure 1.** The impact of deletion of a key T6SS component on DIET. Metabolism of (A)
654 DIET co-cultures initiated with the wild-type or *hcp*-deficient strain of *G.*
655 *metallireducens* and wild-type *G. sulfurreducens*, (B) GAC-supplemented co-cultures
656 initiated with the wild-type or *hcp*-deficient strain of *G. metallireducens* and wild-type *G.*
657 *sulfurreducens*, (C) QUIET co-cultures initiated with the wild-type or *hcp*-deficient strain
658 of *G. metallireducens* and wild-type *G. sulfurreducens*, and (D) DIET co-cultures
659 initiated with the wild-type or GSU3174-deficient strain of *G. sulfurreducens* and wild-
660 type *G. metallireducens*. Each point and error bars represent the average and standard
661 deviation of triplicate measurements.



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663 **Figure 2.** Methane production from DIET co-cultures initiated with the wild-type or *hcp*-
664 deficient strain of *G. metallireducens* and wild-type *M. barkeri* during the first transfer.
665 Each point and error bars represent the average and standard deviation of triplicate
666 measurements.

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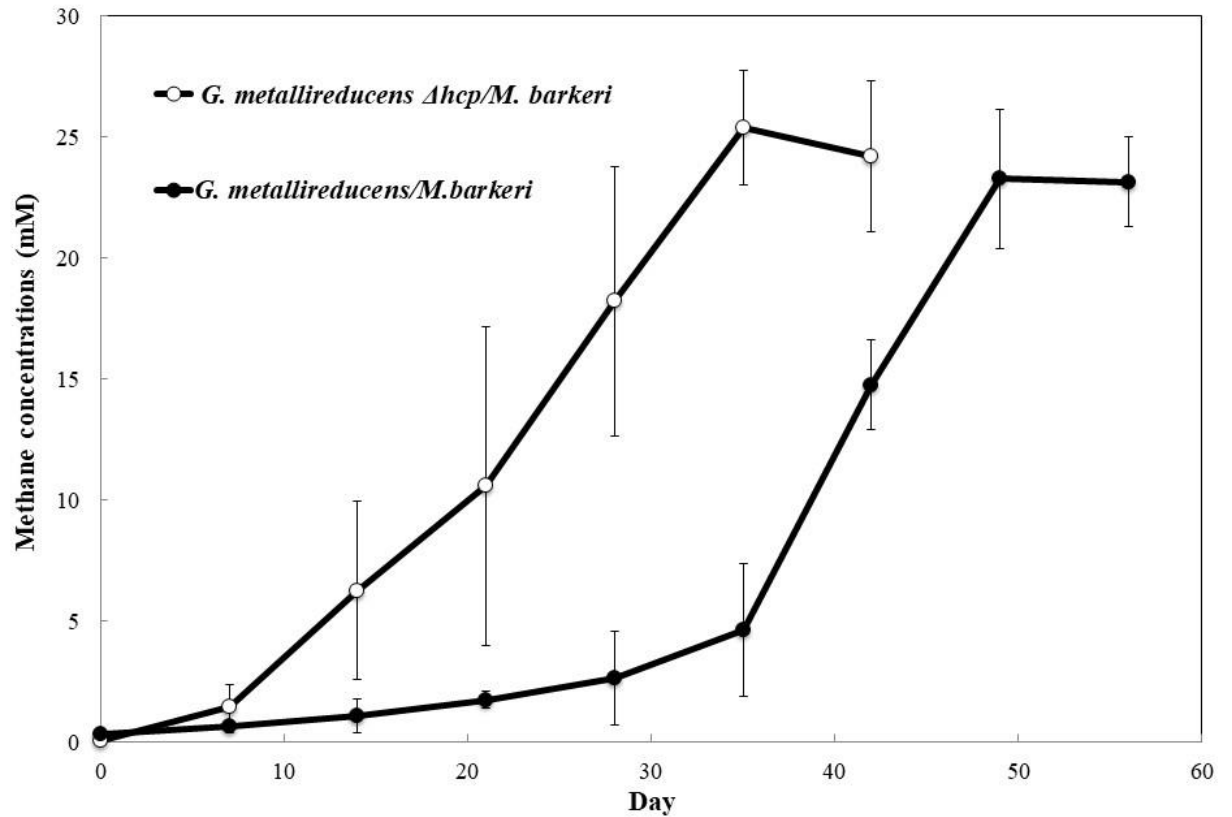
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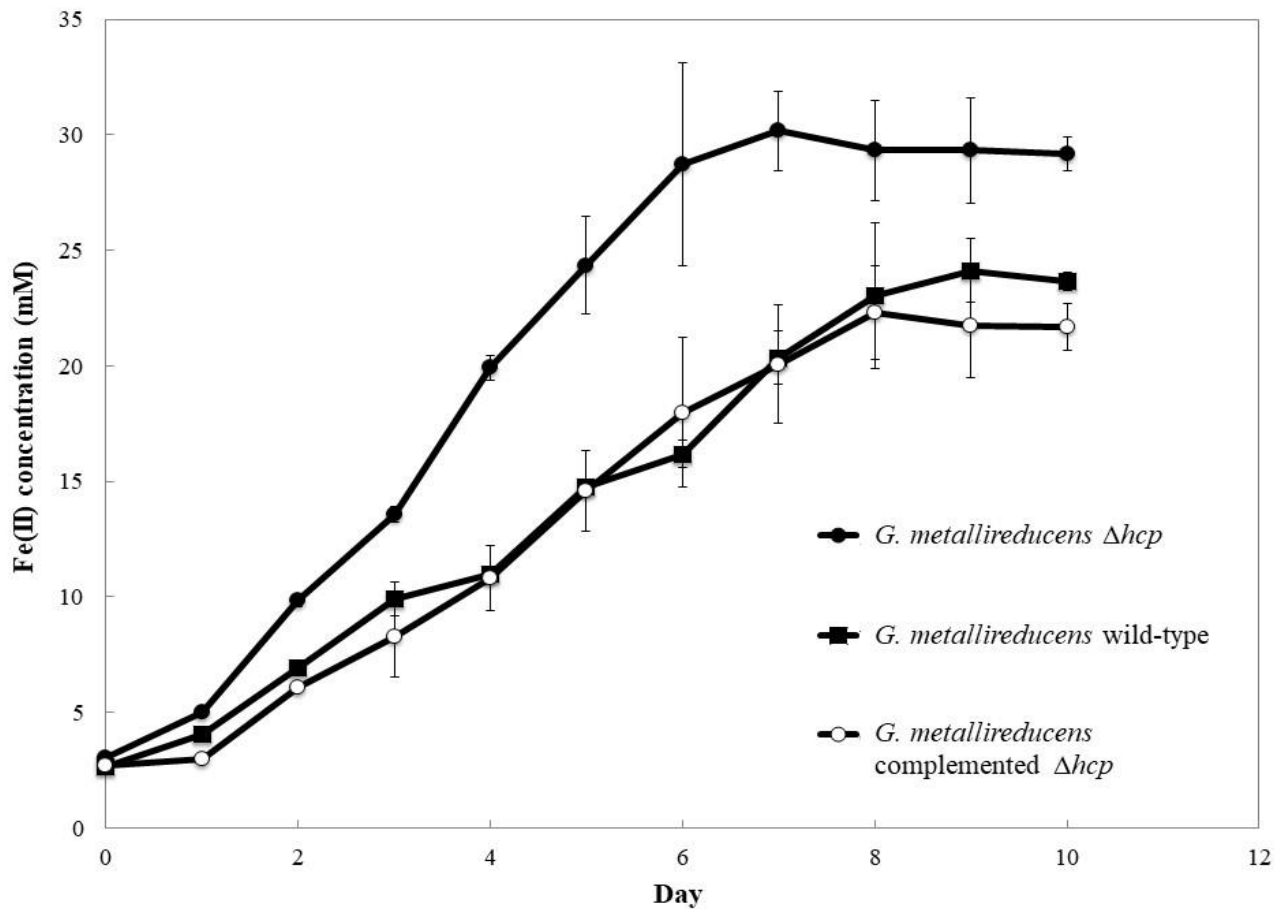
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677 **Figure 3.** Fe(II) production by wild-type *G. metallireducens*, Δhcp , and complemented
678 Δhcp strains during growth with acetate (20 mM) provided as the electron donor and
679 Fe(III) oxide (50 mM) provided as the electron acceptor. Results and error bars represent
680 triplicate cultures. The growth rate for Δhcp was 1.5 (p-value=0.002) and 1.4 (p-
681 value=0.02) times greater than wild-type or complemented Δhcp strains.



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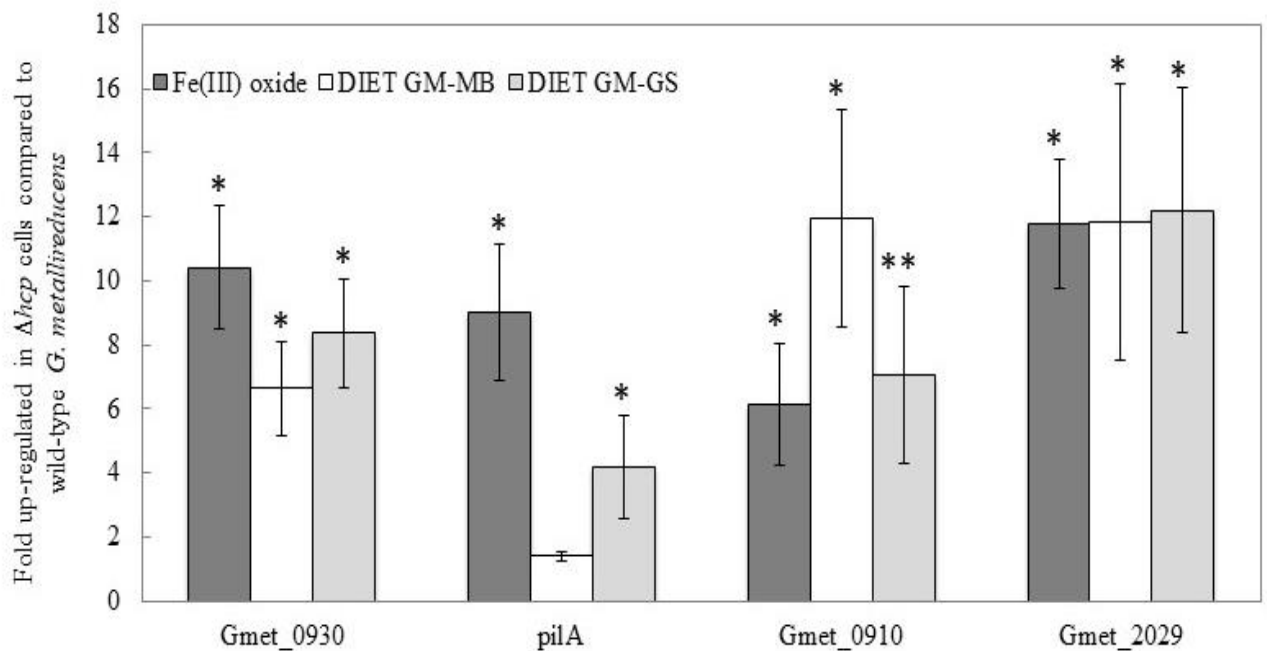
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687 **Figure 4.** Results from quantitative RT-PCR using primers targeting various genes that
688 code for proteins shown to be involved in extracellular electron transfer. mRNA used as
689 template was extracted from cultures of *G. metallireducens* growing by Fe(III) respiration
690 with acetate (20 mM) as the electron donor and Fe(III) oxide (50 mM) as the electron
691 acceptor (Fe(III)-oxide), growing in co-culture with *M. barkeri* (DIET GM-MB), or
692 growing in co-culture with *G. sulfurreducens* (DIET GM-GS). Results were calculated
693 from triplicate biological and technical replicates using three different housekeeping
694 genes as references (*proC*, *recA*, and *rpoB*).
695 *represents p-values < 0.05; ** represents p-values < 0.01. Further details regarding qRT-
696 PCR results and p-values are available in Table S6.



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Table 1 Differences in transcript abundance for all 13 core T6SS genes in *G. metallireducens* grown via DIET compared to QUIET and for genes coding for putative T6SS effector and associated immunity proteins (P-value cutoff ≤ 0.05).

ND: no difference

tse: T6SS effector protein; tsi: T6SS immunity protein

Gene	Name	Annotation	Fold-change DIET vs. QUIET	p-value
Core T6SS Genes				
Gmet_0273	<i>tssL</i>	membrane core complex protein	129.56	1.38×10^{-6}
Gmet_0274	<i>tssM</i>	membrane core complex protein	47.98	8.99×10^{-26}
Gmet_0275	<i>tssA</i>	baseplate complex protein	91.34	2.71×10^{-23}
Gmet_0278	<i>tssB</i>	Sheath protein	703.55	1.71×10^{-21}
Gmet_0279	<i>tssC</i>	Sheath protein	44.18	4.29×10^{-27}
Gmet_0280	<i>hcp</i>	inner tube protein	1397.32	1.74×10^{-30}
Gmet_0286	<i>vrgG</i>	puncturing device	44.88	9.12×10^{-15}
Gmet_3310	<i>tssJ</i>	membrane core complex protein	27.08	5.50×10^{-10}
Gmet_3311	<i>tssK</i>	baseplate complex protein	5.69	4.86×10^{-8}
Gmet_3312	<i>tssE</i>	baseplate complex protein	44.53	3.93×10^{-3}
Gmet_3313	<i>tssF</i>	baseplate complex protein	136.45	7.68×10^{-7}
Gmet_3314	<i>tssG</i>	baseplate complex protein	52.36	1.78×10^{-3}
Gmet_3315	<i>tssH</i>	ClpV1 protease involved in sheath recycling	11.47	5.50×10^{-11}
Effector/ Immunity Genes				
Gmet_0284	<i>tsi1</i>	NTF-domain protein (immunity protein)	14.01	1.02×10^{-8}
Gmet_0285	<i>tse1</i>	peptidoglycan-binding D-alanyl-D-alanine carboxypeptidase	15.92	9.05×10^{-10}
Gmet_0287	<i>tse2</i>	fatty acid metabolism protein	5.16	7.36×10^{-5}
Gmet_0288		PAAR-like DUF4150 domain protein	126.78	1.76×10^{-6}
Gmet_0290	<i>tse3</i>	PGAP1 domain protein; phospholipase	7.71	1.31×10^{-8}
Gmet_0291		DUF2169 domain chaperone protein	ND	