Syntrophus Conductive Pili Demonstrate that Common Hydrogen-Donating Syntrophs can have a Direct Electron Transfer Option

David J.F. Walker[1,2], Kelly P. Nevin[1], Dawn E. Holmes[1,3], Amelia-Elena Rotaru[1,4], Joy E. Ward[1], Trevor L. Woodard[1], Jiaxin Zhu[5], Toshiyuki Ueki[1], Stephen S. Nonnenmann[2,5], Michael J. McInerney[6], and Derek R. Lovley[1,2*]

[1]Department of Microbiology, University of Massachusetts-Amherst, Amherst, MA, USA

[2]Institute for Applied Life Sciences, University of Massachusetts-Amherst

[3]Department of Physical and Biological Science, Western New England University, Springfield, MA, USA

[4]Department of Biology, University of Southern Denmark, Odense, Denmark

[5]Department of Mechanical and Industrial Engineering, University of Massachusetts-Amherst

[6]Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK, USA

*Corresponding author: dlovley@microbio.umass.edu

Abstract

Syntrophic interspecies electron exchange is essential for the stable functioning of diverse anaerobic microbial communities. Hydrogen/formate interspecies electron transfer (HFIT), in which H_2 and/or formate function as diffusible electron carriers, has been considered to be the primary mechanism for electron sharing because most common syntrophs were thought to lack biochemical components, such as electrically conductive pili (e-pili), necessary for direct interspecies electron transfer (DIET). Here we report that *Syntrophus aciditrophicus*, one of the most intensively studied microbial models for HFIT, produces e-pili and can grow via DIET. Pilin genes likely to yield e-pili were found in other genera of hydrogen/formate-producing syntrophs. The finding that DIET is a likely option for diverse syntrophs that are abundant in many anaerobic environments necessitates a reexamination of the paradigm that HFIT is the predominant mechanism for syntrophic electron exchange within anaerobic microbial communities of biogeochemical and practical significance.

Introduction

H_2/formate interspecies electron transfer (HFIT) and direct interspecies electron transfer (DIET) are mechanisms for the electron-donating partner in syntrophic consortia to dispose of electrons released from the oxidation of key intermediates (organic acids, alcohols, aromatics) during the anaerobic degradation of complex organic matter [1-5]. The relative proportion of electron flux through DIET or HFIT can influence the speed of interspecies electron transfer, the stability of anaerobic microbial communities, and their ability to adapt to environmental change [4,6,7]. However, there are no accurate methods for measuring the rates at which H_2 and formate are transferred between microbes or for quantifying interspecies electrical currents in complex

40 communities. Therefore, the importance of HFIT or DIET in microbial communities is typically
41 inferred from the composition of the microbial community.

42 It has previously been considered that DIET is primarily restricted to environments in
43 which *Geobacter* species are abundant [8-12], because *Geobacter* species are the only microbes in
44 pure culture that have been definitively shown to function as electron-donating partners for DIET
45 [9,13-17]. Studies with *Geobacter metallireducens* have suggested that electrically conductive pili
46 (e-pili) are an important conduit for electron transfer from the electron-donating partner [9,14,17,18].
47 It was initially considered that only short pilin monomers (ca. 60 amino acids), such as those
48 found in *G. metallireducens* and *G. sulfurreducens*, could assemble into e-pili [19]. However, it
49 was subsequently found that larger pilin monomers (> 100 amino aids), phylogenetically distinct
50 from *Geobacter* pilins, can yield e-pili and that e-pili have independently evolved multiple times
51 [20]. This raises the possibility that some microorganisms not previously known to be capable of
52 extracellular electron transfer may express e-pili to enable DIET.

53 For example, *Syntrophus aciditrophicus* is one of the most intensively studied pure
54 culture models for HFIT [21-24]. It was previously concluded that *S. aciditrophicus* would be
55 unlikely to participate in DIET [24,25] because it lacks a gene encoding a protein homologous to the
56 Geobacter pilin monomer (PilA) that assembles into electrically conductive pili (e-pili) [26].

57 Here we report that *S. aciditrophicus* expresses e-pili and is capable of growing via
58 DIET. These results, and analysis of the pilin genes of other common syntrophs, indicate that the
59 capacity for DIET should be considered as an option for microorganisms known to grow via
60 HFIT and suggest that DIET may be more widespread than previously considered.

61 **Results and Discussion**

Transmission electron microscopy of *S. aciditrophicus* revealed filaments with a morphology typical of type IVa pili (Fig. 1a). A complement of genes for protein components required for type IV pilus assembly (*pilA, pilD, pilM, pilN, pilO, pilP, pilQ*) and transcriptional control of pilus expression (*pilS, pilR*) are also present in the genome (Supplementary Fig. 1). One gene, SYN_00814 encodes a peptide with a N-terminal domain characteristic of PilA, the pilin monomer for Type IVa pili found in other microrganisms (Fig. 1b). This includes a short leader peptide (13 amino acids) that is cleaved by PilD at the G|FTLIE recognition site and a highly conserved, hydrophobic, pilin assembly interface that facilitates pilin polymerization into pili [27,28].

The amino acid sequence of the putative PilA fits the empirical criteria [20] for a pilin monomer likely to yield e-pili: 1) aromatic amino acids are located in the key positions required for conductivity in *G. sulfurreducens* e-pili; 2) the abundance of aromatic amino acids (10.9 % of amino acids) is above the minimum threshold of 9 % thought to be necessary for high e-pili conductivity; and 3) no large gaps (> 40 amino acids) that lack aromatic amino acids (Fig. 1b).

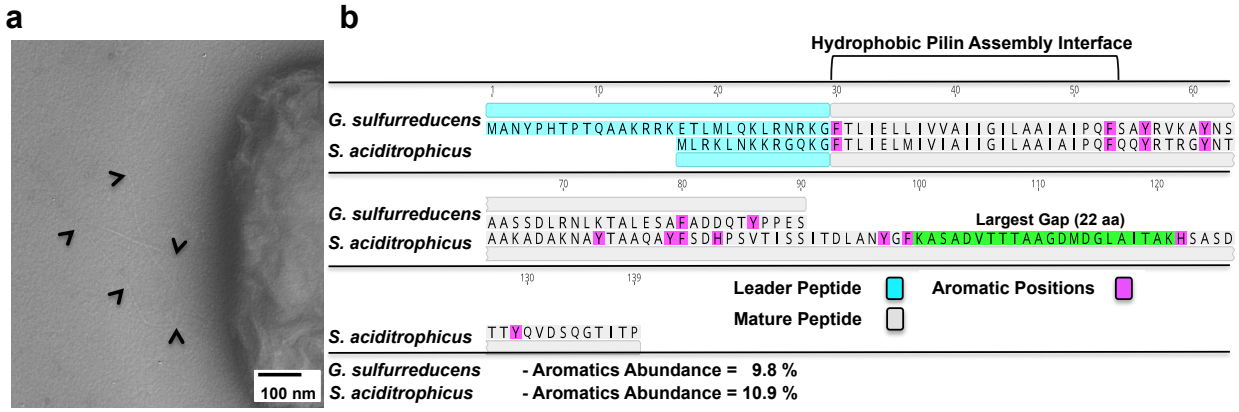


Fig.1. Pili protruding from *S. aciditrophicus* and the putative *S. aciditrophicus* PilA pilin monomer peptide identified from the genome sequence. (**a**) Transmission electron micrograph showing multiple pili protruding from *S. aciditrophicus* (pili highlighted with black

80 arrows). (**b**) Key characteristics of the predicted amino acid sequence of the *S. aciditrophicus*
81 pilin monomer. The PilA monomer of *G. sulfurreducens* that yields conductive pili is shown for
82 comparison. Aromatic amino acid abundance is calculated as a percentage of the mature peptide.
83
84 Low culture densities of *S. aciditrophicus* prevented harvesting sufficient quantities of
85 pili to measure pili conductance on the electrode arrays previously employed for the study of
86 other e-pili [20]. Therefore, the method initially employed to document the presence of e-pili in *G.*
87 *sulfurreducens* [29] was adapted [30] as an alternative approach. Cultures of *S. aciditrophicus* were
88 directly drop-cast on highly ordered pyrolytic graphite (HOPG), washed with deionized water,
89 and examined with atomic force microscopy. Topographic imaging, (Fig. 2a), indicated that the
90 distance from the surface of the HOPG to the top of the pili (Fig. 2b) was 4.0 $\pm$ 0.7 nm (n=27;
91 nine different locations on three separate pili). The 4 nm diameter of the *S. aciditrophicus* pili is
92 larger than the 3 nm reported for the e-pili of *Geobacter* species [31-33], but thinner than the ca. 5-6
93 nm diameter of the intensively studied type IVa pili of *Psedumonas* and *Neisseria* species [34-36].
94 Conductive imaging revealed that the pili were electrically conductive (Fig. 2b, c). Point-
95 mode current-voltage (I-V) spectroscopy (Fig. 2d; Supplementary Fig. 2) yielded an Ohmic-like
96 response with a conductance of 6.1 $\pm$ 1.2 nS (mean $\pm$ standard deviation; n=9) that was similar to
97 the previously reported [30] conductance of 4.5 $\pm$ 0.3 nS for wild-type *G. sulfurreducens* pili. As
98 previously reported [30], the pili from *G. sulfurreducens* strain Aro-5, which lack key aromatic
99 amino acids required for high conductivity [31,33,37,38], had much lower (0.004 $\pm$ 0.002 nS)
100 conductance (Fig. 2d).

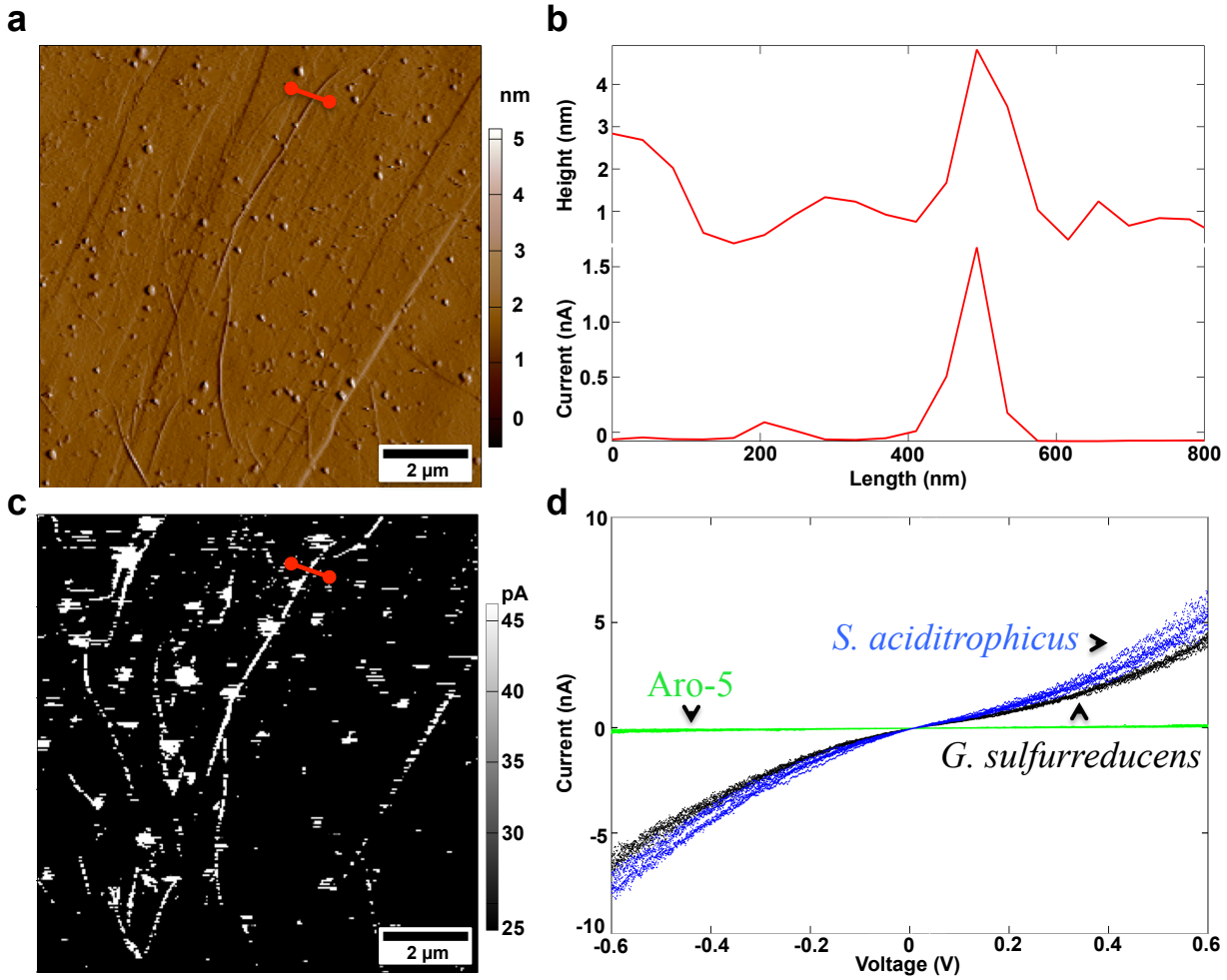


Fig. 2. Conducting tip atomic force microscopy demonstrated that *Syntrophus aciditrophicus* pili are electrically conductive. (**a**) Contact mode topographic imaging of pili on highly ordered pyrolytic (HOPG). Red line designates the cross section examined in (b). (**b**) Topographic analysis of the height/diameter of an individual pilus and corresponding current measurements (100 mV differential between the tip and the HOPG) across the pilus cross-section. (**c**) Current response of the pili shown in (a) with an applied 100 mV differential. (**d**) Current-voltage analysis of individual pili of *S. aciditrophicus* (blue data points), wild-type *G. sulfurreducens* (black data points) and the Aro-5 strain of *G. sulfurreducens* (green data points). Current-voltage spectroscopy is shown for one pilus of each type and are representative of

111 analysis of three distinct locations on three separate pili of each type. Additional scans of *S.*
112 *aciditrophicus* pili available in Supplementary Fig. 2. The *G. sulfurreducens* wild-type and
113 strain Aro-5 data are from reference [30].
114
115 The lack of tools for genetic manipulation of *S. aciditrophicus* limited further functional
116 analysis of the putative PilA gene predicted to yield its e-pili in the native organism. Therefore,
117 the gene was heterologously expressed in *G. sulfurreducens*, replacing the native *G.*
118 *sulfurreducens pilA* with an approach that has successfully yielded *G. sulfurreducens* strains that
119 express a diversity of both highly conductive and poorly conductive heterologous pili [20,32,39-41].
120 This new *G. sulfurreducens* strain, designated strain SP (for *Syntrophus* pili), expressed abundant
121 pili (Fig. 3a). The heterologously expressed pili had a diameter (4.1 $\pm$ 0.4 nm) and conductance
122 (5.9 $\pm$ 0.7 nS) nearly identical to the native pili expressed by *S. aciditrophicus* (Fig. 3b). *G.*
123 *sulfurreducens* strain SP produced electrical current densities comparable to the control strain
124 expressing the *G. sulfurreducens* wild-type *pilA* (Fig. 3c). Such high current densities are only
125 possible when *G. sulfurreducens* expresses e-pili [20,37]. As previously reported [20,37], *G.*
126 *sulfurreducens* strain Aro-5, with its poorly conductive pili, produced much lower current
127 densities (Fig. 3c). Networks of pili sheared from the electrode-grown biofilm of strain SP,
128 purified, and drop cast on electrode arrays as previously described [20], had a conductance
129 comparable to *G. sulfurreducens* wild-type pili networks (Fig. 3d). These results further
130 demonstrated that the *S. aciditrophicus* PilA gene yields a pilin monomer that can assemble into
131 e-pili.
132

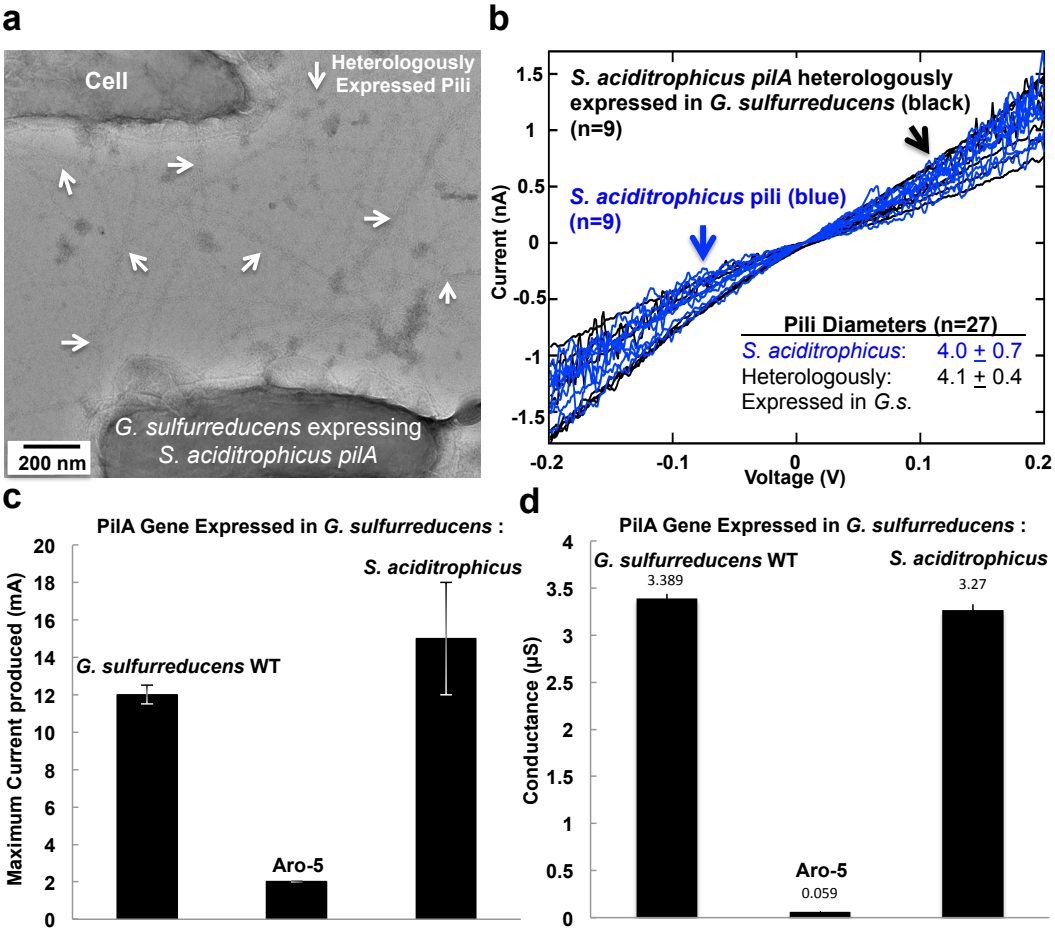


Fig. 3. Physical, electrical, and functional, analysis of the putative *Syntrophus aciditrophicus* PilA via heterologous expression in *Geobacter sulfurreducens*. (**a**) Transmission electron micrograph of pili expression in the strain of *G. sulfurreducens* in which the native *pilA* was replaced with the *S. aciditrophicus pilA*. Examples of the location of pili is designated with white arrows. (**b**) Current-voltage profile for three separate locations on three pili expressed by *S. aciditrophicus* pili (data aggregated from individual plots in Fig.1d and Supplementary Fig. S2) and for three separate locations on three pili expressed in the SP strain of *G. sulfurreducens* in which the wild-type *pilA* was replaced with the *S. aciditrophicus pilA* (individual plots shown in Supplentary Fig. 3). (**c**) Current production by *G. sulfurreducens* with wild-type PilA; the

143 synthetic Aro-5 PilA designed to yield poorly conductive pili; or *S. aciditrophicus* PilA. (**d**)
144 Four-probe conductance (mean ± standard deviation n=9) of films of purified pili from strains of
145 *G. sulfurreducens* expressing; *S. aciditrophicus* PilA compared with previously reported [20]
146 conductances of pili derived from wild-type and Aro-5 strains of *G. sulfurreducens*.
147
148 The presence of e-pili in *S. aciditrophicus* suggested it might be capable of establishing
149 an electrical connection for DIET. To evaluate this, *S. aciditrophicus* was grown in co-culture
150 with *G. sulfurreducens*, the microbe most intensively studied as an electron-accepting partner for
151 DIET [4]. *G. sulfurreducens* can also function as a H_2- and formate-consuming partner for HFIT [42].
152 Therefore, even if *S. aciditrophicus* was unable to grow via DIET it would be expected to grow
153 in co-culture with *G. sulfurreducens* via HFIT, providing a positive control to ensure that the
154 culture medium was appropriate for co-culture growth. The electron donor was benzoate, a
155 substrate that *S. aciditrophicus* can metabolize, but *G. sulfurreducens* cannot. The electron
156 acceptor was fumarate, an electron acceptor that only *G. sulfurreducens* can utilize. In the
157 presence of a H_2/formate-consuming partner, *S. aciditrophicus* metabolizes benzoate to acetate
158 with the production of either H_2:
159 $C_7H_5O_2^- + 7\ H_2O \rightarrow 3CH_3COO^- + HCO_3^- + 3\ H^+ + 3\ H_2$
160 or formate:
161 $C_7H_5O_2^- + 2\ HCO_3^- + 4\ H2O \rightarrow 3\ CH_3COO^- + 3\ H^+ + 3\ HCOO^-$
162 With electron transfer via DIET the relevant reaction is:
163 $C_7H_5O_2^- + 7\ H_2O \rightarrow 3CH_3COO^- + HCO_3^- + 9\ H^+ + 6\ e^-$

164 In addition to oxidizing H_2 and formate, or consuming electrons released during DIET, *G.*
165 *sulfurreducens* can also metabolize acetate, making the overall reaction expected for the
166 oxidation of benzoate with the reduction of fumarate to succinate in the co-culture:

167 $C_7H_5O_2^- + 15\ ^-OOC\text{-}CH{=}CH\text{-}COO^- + 19\ H_2O \rightarrow 7\ HCO_3^- + 15\ ^-OOC\text{-}CH_2\text{-}CH_2\text{-}COO^- + 6\ H^+$

168 *S. aciditrophicus/G. sulfurreducens* co-cultures grew with repeated sub-culturing and,
169 within the experimental error, exhibited the expected stoichiometry of benzoate consumption and
170 succinate production (Fig. 4a). In these co-cultures HFIT, DIET, or a combination of the two,
171 were feasible. Therefore, to eliminate the possibility of HFIT, *S. aciditrophicus* was co-cultured
172 with the previously described strain of *G. sulfurreducens* [18], designated here as *G.*
173 *sulfurreducens*$_{HF}$, that cannot utilize H_2 or formate because the genes for the uptake hydrogenase
174 and formate dehydrogenase were deleted. The *S. aciditrophicus/ G. sulfurreducens*$_{HF}$ co-culture
175 had a longer initial lag period, but then metabolized benzoate with the reduction of fumarate
176 almost as fast as the co-culture with wild-type *G. sulfurreducens* (Fig. 4b). These results
177 demonstrate that *S. aciditrophicus* can grow via DIET.

178 DIET requires physical electrical contacts between the electron-donating and electron-
179 accepting partner, whereas contact is not required for HFIT [4,5]. In some co-cultures the
180 requirement for contact manifests as visible aggregates [13], but other DIET co-cultures produce
181 small, relatively fragile aggregates [14]. There were no visible aggregates in the *S.*
182 *aciditrophicus/G. sulfurreducens* co-cultures. However, more small clumps were seen in
183 scanning electron micrographs of co-cultures in which *G. sulfurreducens*$_{HF}$ was the electron-
184 accepting partner than co-cultures with wild-type *G. sulfurreducens* provided as the electron-
185 accepting partner (Fig. 4 d,e). This observation is consistent with the need for contact between
186 electron-donating and electron-accepting partners participating in DIET. In contrast, HFIT was

187 an option for the co-cultures established with wild-type *G. sulfurreducens* and aggregation is not

188 required when *G. sulfurreducens* grows via HFIT [42].

189 Granular activated carbon (GAC) can greatly reduce the initial lag time in establishing

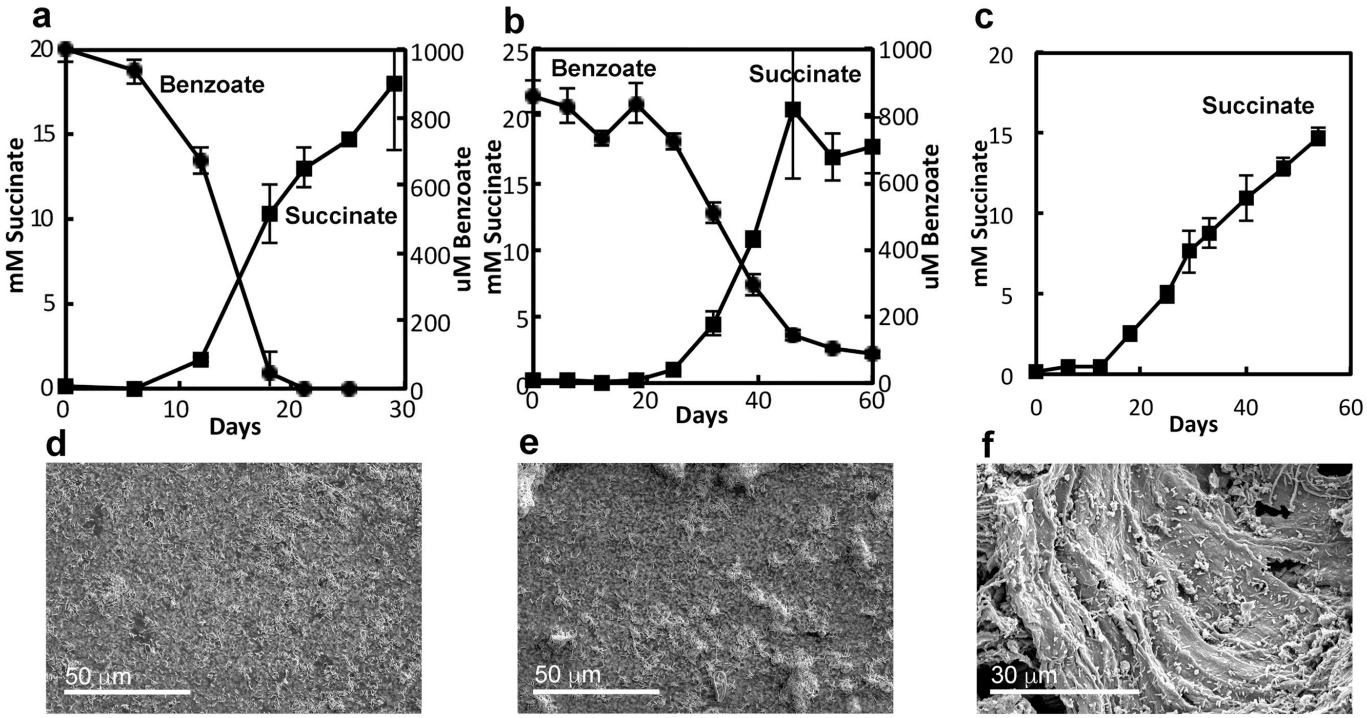
190 DIET-based co-cultures because both partners attach to GAC, which functions as an electrically

191 conductive conduit [14,43]. GAC substantially reduced the lag time of the *S. aciditrophicus/ G.*

192 *sulfurreducens*$_{HF}$ co-cultures (Fig. 4c). As previously observed for other co-cultures in which

193 GAC promoted DIET, cells from the *S. aciditrophicus/ G. sulfurreducens*$_{HF}$ co-culture heavily

194 colonized the GAC (Fig. 4e), consistent with a GAC conduit for DIET.



196 **Fig. 4. Co-cultures of *Syntrophus aciditrophicus* and *Geobacter sulfurreducens* grow via**

197 **DIET.** Metabolism with *S. aciditrophicus* co-cultured with (**a**) wild-type *G. sulfurreducens*, (**b**)

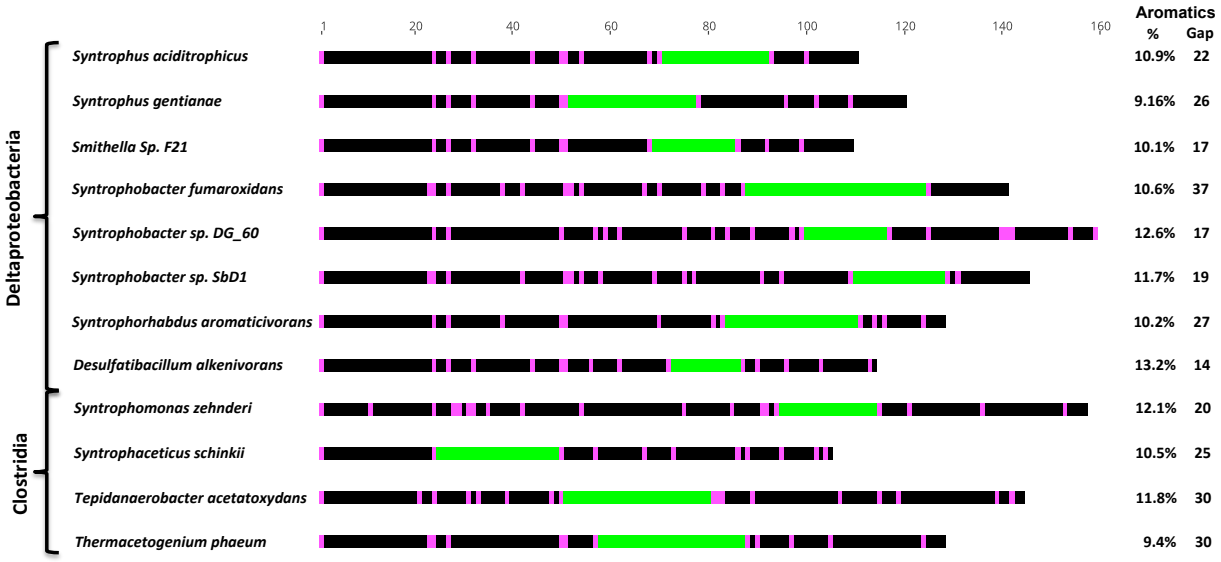
198 *G. sulfurreducens*$_{HF}$, which is unable to use H_2 or formate. (**c**) *G. sulfurreducens*$_{HF}$ with granular

199 activated carbon (GAC) amendment. No acetate or formate was detected in any of the co-

200 cultures. GAC interfered with determination of benzoate, which is not shown for GAC-amended

201 cultures. Scanning electron micrographs (SEM) of cells collected on filters from co-cultures with
202 (**d**) *G. sulfurreducens* wild-type or (**e**) *G. sulfurreducens* strain$_{HF}$ demonstrating greater
203 aggregation in co-cultures with *G. sulfurreducens*$_{HF}$ in which DIET was the only option for
204 interspecies electron exchange. (**f**) SEM of cells on GAC from co-culture of *S. aciditrophicus*
205 with *G. sulfurreducens* strain$_{HF}$. Circles-benzoate; squares-succinate. Data are the mean and
206 standard deviation of triplicate cultures.
207
208 In addition to e-pili, multi-heme outer-surface *c*-type cytochromes appear to play an
209 important role in DIET in *Geobacter* species [4,5]. However, the S. *aciditrophicus* genome encodes
210 only a few putative *c*-type cytochromes [24] and cytochromes were not readily apparent in heme-
211 stained preparations of cell protein (Supplementary Fig. 4). Not all microbes require
212 cytochromes for effective electron transport to the outer cell surface [44]. More detailed
213 examination of the role of e-pili and other *S. aciditrophicus* components in DIET will require the
214 development of methods for genetic manipulation of this microorganism.
215 *S. aciditrophicus* is the first isolate outside the genus *Geobacter* found to grow via DIET
216 and it is the first syntroph shown to have the option to grow via HFIT or DIET. Other
217 *Syntrophus* species also have pilin genes with the empirical criteria, detailed above, of aromatic
218 amino acid placement and abundance likely to yield e-pili (Fig. 5). The pilins of other diverse
219 genera of syntrophic microorganisms known to grow via HFIT in defined co-cultures also meet
220 these criteria (Fig. 5). Establishing conditions that favor DIET often enrich for microbes in these
221 genera [6]. For example, in enrichment cultures specifically designed to promote the metabolism of
222 propionate or butyrate via DIET, *Smithella* (propionate enrichment) or *Syntrophomonas*
223 (butyrate enrichment) species were the most abundant bacteria [45-47]. The greater energetic

224 demands [48] required for synthesizing the abundant aromatic amino acids that are required for e-
225 pili conductivity suggests that e-pili provide a strong selective advantage under some
226 environmental conditions. Conferring the capacity for DIET is a likely explanation.



227

228 **Fig. 5. A diversity of syntrophs known to produce H_2 and/or formate as an interspecies**
229 **electron carrier encode for pilin monomers likely to yield electrically conductive pili.**
230 Position of aromatic amino acids designated in magenta and the largest gap that lacks aromatic
231 amino acids designated in green. The full protein sequence alignment for each pilin can be found
232 in Supplementary Fig. 5. Each of the pilins meets the empirical criteria derived from previous
233 studies [20] of: 1) aromatic amino acids located in the key positions required for conductivity in *G.*
234 *sulfurreducens* e-pili; 2) the abundance of aromatic amino acids greater than 9%; and 3) no
235 aromatic-free gaps of greater than 40 amino acids.
236
237 For decades, interactions within complex anaerobic microbial communities have been
238 interpreted through the lens of HFIT, but the available data does not rule out the possibility of
239 DIET, which was not known to be an option at the time. For example, estimated H_2 turnover

240 rates in anaerobic digesters, rice paddy soils, and aquatic sediments were consistently less than
241 10 % of the independently determined rate of methane production derived from the reduction of
242 carbon dioxide to methane [49-51], a result that is consistent with DIET providing most of the
243 electrons for carbon dioxide reduction. The mismatch between measured H_2 turnover rates and
244 the concept of H_2 as a primary interspecies electron carrier was rationalized with the suggestion
245 that there was a separate pool of H_2 within closely juxtapositioned assemblages of H_2 producers
246 and H_2 consumers. However, the existence of two distinct pools of H_2 that did not equilibrate
247 over time via diffusion, a seemingly physical impossibility, was never verified. Attempts to
248 accurately measure formate fluxes have also been problematic [52] and there is no method for
249 directly measuring DIET-based electron fluxes in complex communities.
250 A fresh perspective and new analytical tools will be required to resolve the relative
251 importance of HFIT and DIET in diverse anaerobic microbial communities. Just as electron-
252 accepting partners have different gene expression patterns depending on whether they are
253 participating in HFIT or DIET [18,53], it may be possible to determine whether electron-donating
254 syntrophs are engaged in DIET or HFIT from metatranscriptomic analysis of anaerobic
255 communities.
256
257 **METHODS**
258 **Bacterial strains, plasmids and culture conditions**
259 *Syntrophus aciditrophicus, G. sulfurreducens* wild-type, *G. sulfurreducens* strain Aro-5,
260 and *G. sulfurreducens*$_{HF}$ were obtained from our laboratory culture collections. A strain of *G.*
261 *sulfurreducens* expressing *pilA* from *S. aciditrophicus* rather than native *G. sulfurreducens pilA*
262 was constructed as previously described [20]. *S. aciditrophicus* and *G. sulfurreducens* strains were

263 routinely grown under strict anaerobic conditions at 30 °C in previously described [54] defined,
264 bicarbonate-buffered medium with N_2:CO_2 (80:20) as the gas phase. For *S. aciditrophicus,* the
265 medium was amended with crotonate (20 mM) and for *G. sulfurreducens* strains, acetate (10
266 mM) was the electron donor and fumarate (40 mM) was the electron acceptor. The presence of *c*-
267 type cytochromes in whole cell lysates was evaluated with heme-staining of proteins separated
268 on denaturing polyacrylamide gels as previously described [17]. *G. sulfurreducens* strains were
269 grown with a graphite electrode as the electron acceptor as previously described [55].
270 Cocultures were established in 10 ml of culture medium [54] in anaerobic pressure tubes
271 with benzoate (1 mM) as the electron donor and fumarate (40 mM) as the electron acceptor with
272 cysteine (2 mM) and sulfide (1 mM) added as reducing agents. When noted, cultures were
273 amended with granular activated carbon (0.25 g; 8-20 mesh). Benzoate, acetate, and succinate
274 were analyzed with high-performance liquid chromatography as previously described [16].
275 Previously described methods were employed for transmission electron microscopy [21] and
276 scanning electron microscopy [26].
277 **Pili conductivity**
278 A 100 µl sample of cultures was drop cast onto highly oriented pyrolytic graphite
279 (HOPG). After 10 min, the HOPG was washed twice with 100 µl of deionized water, blotted dry
280 to remove excess water, and dried for 12 hours at 24 °C in a desiccator. Samples were
281 equilibrated with atmospheric humidity for at least 2 hours and then examined with an Oxford
282 Instruments Cypher ES Environmental AFM in ORCA electrical mode equipped with a Pt/Ir-
283 coated Arrow-ContPT tip with a 0.2 N/m force constant (NanoWorld AG, Neuchâtel,
284 Switzerland). Pili were located in contact mode, with a set point of 0.002 V and a scan rate of 1.5
285 Hz. For conductive imaging, the grounded tip, attached to a transimpedance amplifier, served as

286 a translatable top electrode to locally detect the current response of the individual pili to a 100
287 mV bias applied to the HOPG substrate. Individual pili conductivity was further characterized by
288 lightly pressing the AFM tip (set point 0.002 V) to the top of the pili and applying a
289 quadruplicate amplitude of ±0.6 V voltage sweep at a frequency of 0.99 Hz, receiving ca. 8,000
290 points of reference per measurement. Pili expressed in *G. sulfurreducens* were further analyzed
291 with four probe conductivity measurements on films of pili purified from cells as previously
292 described [20].

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