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

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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 that H_2 and formate are transferred between microbes or for quantifying interspecies electrical currents in complex

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

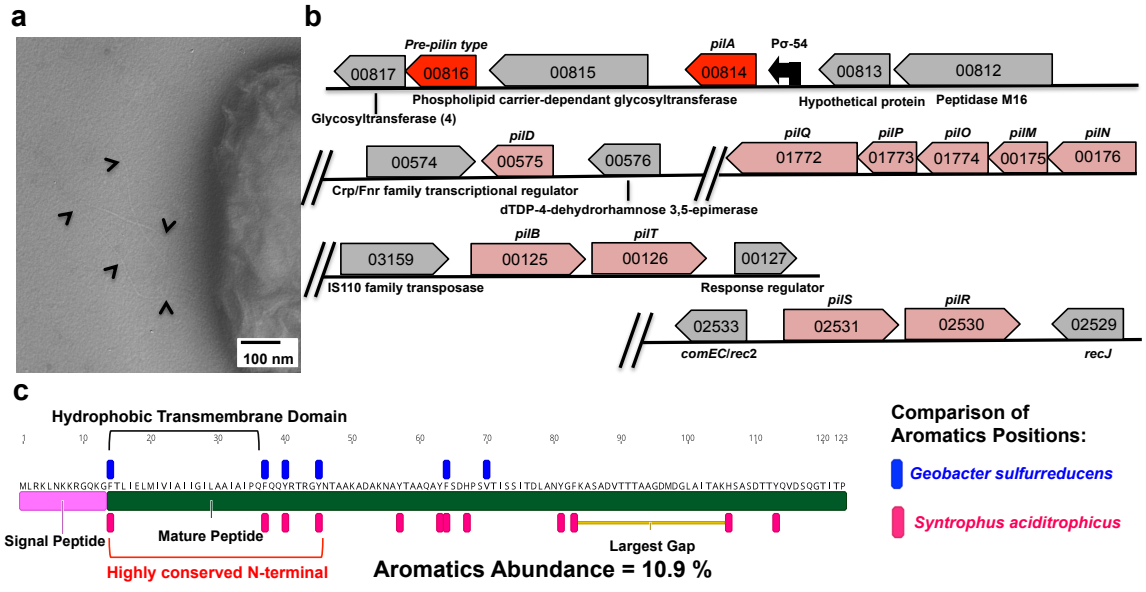
It has previously been considered that DIET is primarily restricted to environments in which *Geobacter* species are abundant [8-12], because *Geobacter* species are the only microbes in pure culture that have been definitively shown to function as electron-donating partners for DIET [9,13-17]. It is assumed that HFIT predominates where microorganisms closely related to traditional syntrophs known to grow via HFIT are most abundant. However, most cultures of electron-donating syntrophs were characterized prior to the concept of DIET. Their capacity for DIET has not been fully explored.

For example, *Syntrophus aciditrophicus* is one of the most intensively studied pure culture models for HFIT [18-21]. It was previously concluded that *S. aciditrophicus* would be unlikely to participate in DIET [21,22] because it lacks a gene homologous to the gene for the *Geobacter* pilin monomer that assembles into its electrically conductive pili (e-pili) [23], a conduit for extracellular electron transfer required for *Geobacter* species to function as electron-donating partners in DIET[17]. This gene would not be expected in *S. aciditrophicus* because *Geobacter* e-pili have evolved relatively recently and are primarily restricted to *Geobacter* species and close relatives [24]. However, it has recently been found that e-pili, phylogenetically distinct from *Geobacter* e-pili have independently evolved multiple times [25].

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

Results and Discussion

Transmission electron microscopy of *S. aciditrophicus* revealed filaments with a morphology typical of type IV pili (Fig. 1a). A complement of genes consistent with type IV pili assembly is present in the genome (Fig. 1b). One gene, SYN_00814 encodes a N-terminal domain characteristic of PilA, the pilin monomer for Type IVa pili (Fig. 1c). This includes a short signal peptide (13 amino acids) which is cleaved by PilD at the G|FTLIE recognition site and a highly conserved, hydrophobic, transmembrane domain. Additional genes for pilus assembly (*pilD, pilM, pilN, pilO, pilP, pilQ*) and transcriptional control of pilus expression (*pilS, pilR*) are also present in the genome (Figure 1b). The amino acid sequence of the putative PilA protein fits the empirical criteria [25] 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 % found to be necessary for high e-pili conductivity; and 3) no large gaps (> 35 amino acids) that lack aromatic amino acids (Fig. 1c).



78 **Fig.1. Pili expression and pili-related genes in *Syntrophus aciditrophicus.*** (**a**) Transmission
79 electron micrograph illustrating pili expression by *S. aciditrophicus* (pili highlighted with
80 arrows). (**b**) Arrangement of genes associated with pili expression. (**c**) Key characteristics of the
81 predicted amino acid sequence of the pilin monomer.
82
83 Low culture densities of *S. aciditrophicus* prevented harvesting sufficient quantities of
84 pili to measure pili conductance on the electrode arrays previously employed for the study of
85 other e-pili [25]. Therefore, the method initially employed to document the presence of e-pili in *G.*
86 *sulfurreducens* [26] was adapted [27] as an alternative approach. Cultures of *S. aciditrophicus* were
87 directly drop-cast on highly ordered pyrolytic graphite (HOPG), washed with deionized water,
88 and examined with atomic force microscopy. Pili visualized with topographic imaging, (Fig. 2a),
89 had a height/diameter of ca. 4 nm (Fig. 2b). Conductive imaging revealed that the pili were
90 electrically conductive (Fig. 2b, c). Point-mode current-voltage (I-V) spectroscopy yielded an
91 Ohmic-like response that was similar to *G. sulfurreducens* pili prepared in the same manner [27]
92 (Fig. 2d) and in other similar studies of *G. sulfurreducens* pili [26]. As previously reported [27], the
93 pili from *G. sulfurreducens* strain Aro-5, which lack key aromatic amino acids required for high
94 conductivity [28-31], had much lower conductance (Fig. 2d).

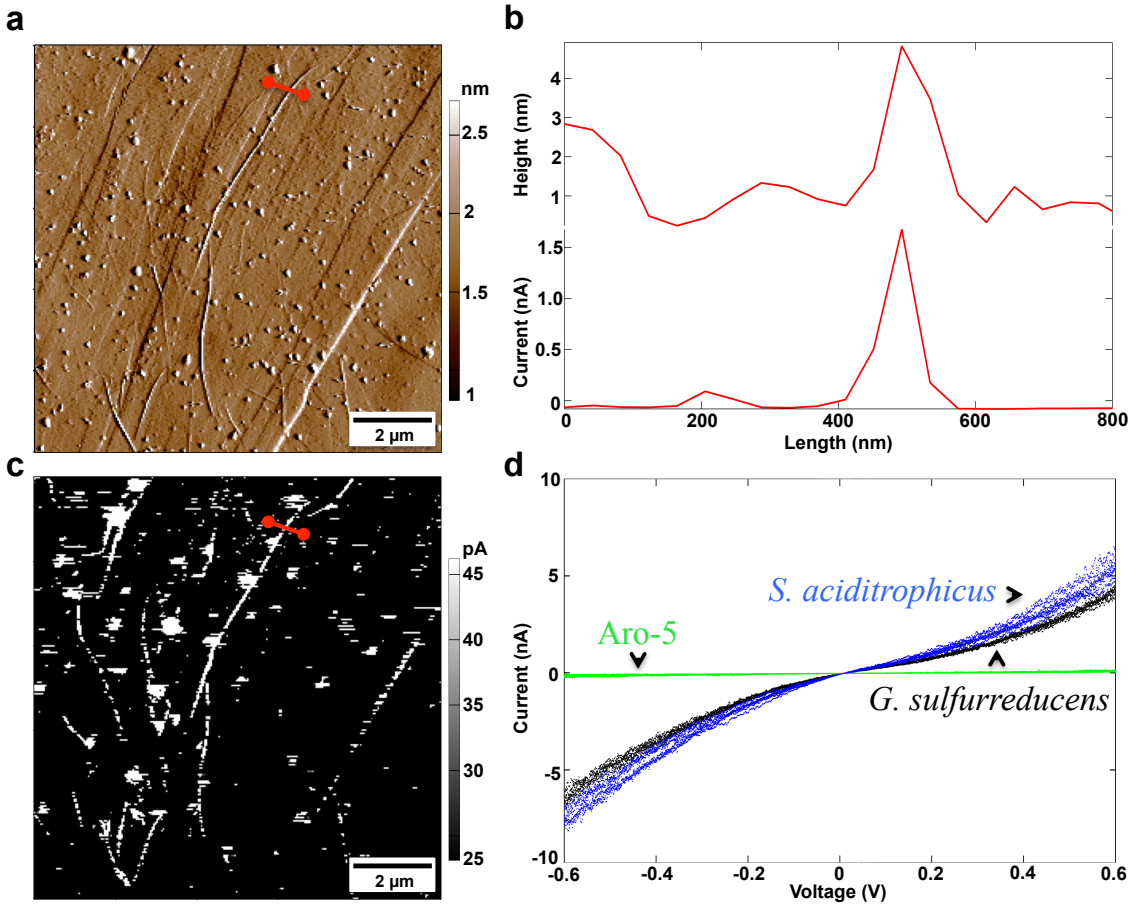
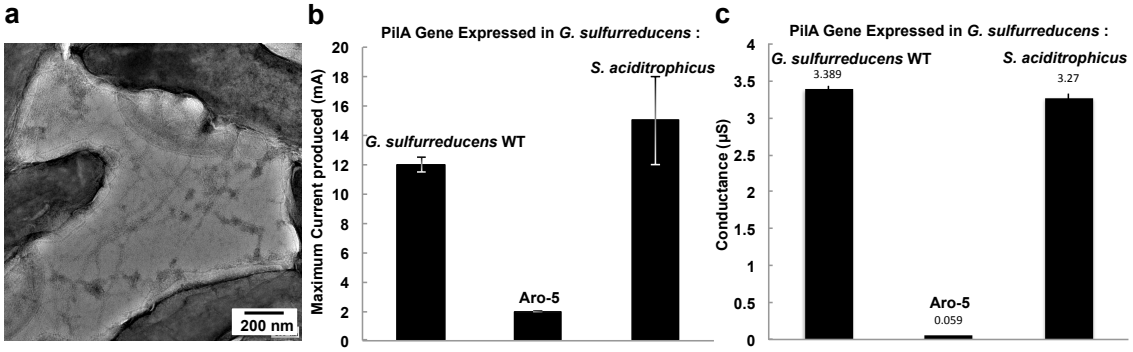


Fig. 2. Conducting tip atomic force microscopy demonstrated that *Syntrophus aciditrophicus* pili are electrically conductive. (**a**) Contact mode topographic imaging of pili on highly ordered pyrolytic (HOPG). Red line designates the cross section examined in (b). (**b**) Topographic analysis of the height/diameter of an individual pilus and corresponding current measurements (100 mV differential between the tip and the HOPG) across the same pilus cross section. (**c**) Current response of the pili shown in (a). (**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 scans are shown for one pilus of each type and are representative of analysis of 10 separate pili. *G. sulfurreducens* wild-type and strain Aro-5 data from reference 27.

106

107 The lack of tools for genetic manipulation of *S. aciditrophicus* limited further functional
108 analysis of the putative PilA gene predicted to yield its e-pili in the native organism. Therefore,
109 the gene was heterologously expressed in *G. sulfurreducens*, replacing the native *G.*
110 *sulfurreducens pilA* with an approach that has successfully yielded *G. sulfurreducens* strains that
111 express a diversity of both highly conductive and poorly conductive heterologous pili [25,32-35].
112 This new *G. sulfurreducens* strain, designated strain SP (for *Syntrophus* pili), expressed abundant
113 pili (Fig. 3a) and produced electrical current at densities comparable to the control strain
114 expressing the *G. sulfurreducens* wild-type *pilA* (Fig. 3b). Such high current densities are only
115 possible when *G. sulfurreducens* expresses e-pili [25,28]. As previously reported [25,28], *G.*
116 *sulfurreducens* strain Aro-5, with its poorly conductive pili, produced much lower currents (Fig.
117 3b). Networks of pili sheared from the electrode-grown biofilm of strain SP, purified, and drop
118 cast on electrode arrays as previously described [25], had a conductance comparable to *G.*
119 *sulfurreducens* wild-type pili (Fig. 3c). These results further demonstrated that the *S.*
120 *aciditrophicus* PilA gene yields a pilin monomer that can assemble into e-pili.



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122 **Fig. 3. Functional analysis of *Syntrophus aciditrophicus* PilA gene via heterologous**
123 **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*. (**b**) Current production by *G. sulfurreducens* expressing its wild-type *pilA*, the synthetic Aro-5 *pilA* designed to yield poorly conductive pili, or *S. aciditrophicus pilA*. (**c**) Conductance of films of pili from strains of *G. sulfurreducens* expressing different *pilA*s. Error bars represent the mean and standard deviation of triplicates.

The presence of e-pili in *S. aciditrophicus* suggested it might be capable of establishing an electrical connection for DIET. To evaluate this, *S. aciditrophicus* was grown in co-culture with *G. sulfurreducens*, the microbe most intensively studied as an electron-accepting partner for DIET [4]. *G. sulfurreducens* can also function as a H_2- and formate-consuming partner for HFIT, providing a positive control for establishing the *S. aciditrophicus/G. metallireducens* co-culture if DIET was not possible [36]. The electron donor was benzoate, a substrate that *S. aciditrophicus* can metabolize, but *G. sulfurreducens* cannot. The electron acceptor was fumarate, an electron acceptor only *G. sulfurreducens* can utilize. In the presence of a H_2/formate-consuming partner *S. aciditrophicus* metabolizes benzoate to acetate with the production of either H_2:

$$C_7H_5O_2^- + 7\ H_2O \rightarrow 3CH_3COO^- + HCO_3^- + 3\ H^+ + 3\ H_2$$

or formate:

$$C_7H_5O_2^- + 2\ HCO_3^- + 4\ H2O \rightarrow 3\ CH_3COO^- + 3\ H^+ + 3\ HCOO^-$$

With electron transfer via DIET the relevant reaction is:

$$C_7H_5O_2^- + 7\ H_2O \rightarrow 3CH_3COO^- + HCO_3^- + 9\ H^+ + 6\ e^-$$

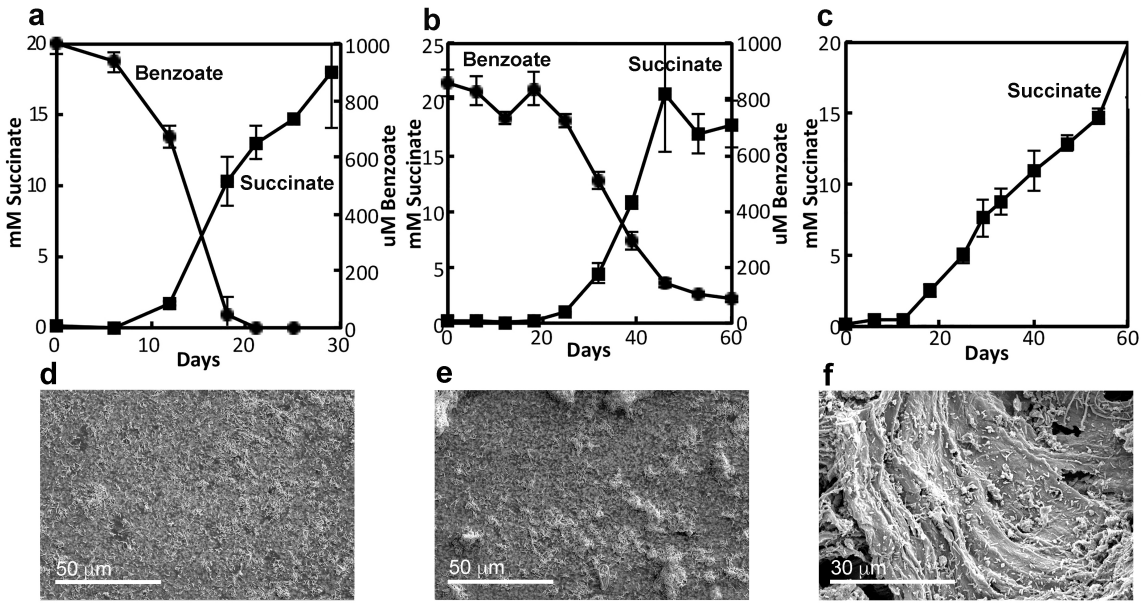
In addition to oxidizing H_2 and formate, or consuming electrons released during DIET, *G. sulfurreducens* can also metabolize acetate, making the overall reaction expected for the oxidation of benzoate with the reduction of fumarate to succinate in the co-culture:

147 $C_7H_5O_2^- + 15\ ^-OOC\text{-}CH{=}CH\text{-}COO^- + 19\ H_2O \rightarrow 7\ HCO_3^- + 15\ ^-OOC\text{-}CH_2\text{-}CH_2\text{-}COO^- + 6\ H^+$

148 *S. aciditrophicus/G. sulfurreducens* co-cultures grew with repeated sub-culturing and,
149 within the experimental error, exhibited the expected stoichiometry of benzoate consumption and
150 succinate production (Fig. 4a). In these co-cultures HFIT, DIET, or a combination of the two,
151 were feasible. Therefore, to eliminate the possibility of HFIT, *S. aciditrophicus* was co-cultured
152 with the previously described strain of *G. sulfurreducens* [37], designated here as *G.*
153 *sulfurreducens*$_{HF}$, that can not utilize H_2 or formate because the genes for the uptake hydrogenase
154 and formate dehydrogenase were deleted. The *S. aciditrophicus/ G. sulfurreducens*$_{HF}$ co-culture
155 had a longer initial lag period, but then metabolized benzoate with the reduction of fumarate
156 almost as fast as the co-culture with wild-type *G. sulfurreducens* (Fig. 4b). These results
157 demonstrate that *S. aciditrophicus* can grow via DIET.

158 Although some co-cultures form visible aggregates during growth via DIET [13], others
159 produce small, relatively fragile aggregates [14]. There were no visible aggregates in the *S.*
160 *aciditrophicus/G. sulfurreducens* co-cultures but it appeared in scanning electron micrographs of
161 cells collected on filters that co-cultures grown under conditions in which only DIET was
162 possible have a greater tendency to form more small clumps than co-cultures grown under
163 conditions in which HFIT was also an option (Fig. 4 d,e).

164 Granular activated carbon (GAC) can greatly reduce the initial lag time in establishing
165 DIET-based co-cultures because both partners attach to GAC, which functions as an electrically
166 conductive conduit [14,38]. GAC significantly reduced the lag time of the *S. aciditrophicus/ G.*
167 *sulfurreducens*$_{HF}$ co-cultures (Fig. 4c). As previously observed for other co-cultures in which
168 GAC promoted DIET, cells from the *S. aciditrophicus/ G. sulfurreducens*$_{HF}$ co-culture heavily
169 colonized the GAC (Fig. 4e), consistent with a GAC conduit for DIET.



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171

172 **Figure 4. Co-cultures of *Syntrophus aciditrophicus* and *Geobacter sulfurreducens* grow via**
173 **DIET.** Metabolism with *S. aciditrophicus* co-cultured with (**a**) wild-type *G. sulfurreducens*, (**b**)
174 *G. sulfurreducens* strain$_{HF}$, which is unable to use H_2 or formate, or (**c**) *G. sulfurreducens*
175 strain$_{HF}$ with granular activated carbon (GAC) amendment. No acetate was detected in any of the
176 co-cultures. GAC interfered with determination of benzoate, which is not shown for GAC-
177 amended cultures. Scanning electron micrographs (SEM) of cells collected on filters from co-
178 cultures with (**d**) *G. sulfurreducens* wild-type or (**e**) *G. sulfurreducens* strain$_{HF}$. (**f**) SEM of cells
179 on GAC from co-culture of *S. aciditrophicus* with *G. sulfurreducens* strain$_{HF}$. Circles-benzoate;
180 squares-succinate. Data are the mean and standard deviation of triplicate cultures.

181

182 In addition to e-pili, multi-heme outer-surface *c*-type cytochromes appear to play an
183 important role in DIET in *Geobacter* species [4,5]. However, the S. *aciditrophicus* genome encodes
184 only a few putative *c*-type cytochromes [21] and cytochromes were not readily apparent in heme-

stained preparations of cell protein (Supplementary Fig. 1). Not all microbes require cytochromes for effective electron transport to the outer cell surface [39]. More detailed examination of the role of e-pili and other *S. aciditrophicus* components in DIET will require the development of methods for genetic manipulation of this microorganism.

S. aciditrophicus is the first isolate outside the genus *Geobacter* found to grow via DIET and is the first syntroph shown to have the option to grow via HFIT or DIET. Other *Syntrophus* species also have pilin genes likely to yield e-pili, as do other diverse genera of syntrophic microorganisms known to grow via HFIT in defined co-cultures (Fig. 5). Establishing conditions that favor DIET often enrich for microbes in these genera [6]. For example, in enrichment cultures specifically designed to promote the metabolism of propionate or butyrate via DIET, *Smithella* (propionate enrichment) or *Syntrophomonas* (butyrate enrichment) species were the most abundant bacteria [40-42]. The greater energetic demands [43] required for synthesizing the abundant aromatic amino acids that are required for e-pili conductivity suggests that e-pili provide a strong selective advantage under some environmental conditions. Conferring the capacity for DIET is a likely explanation.

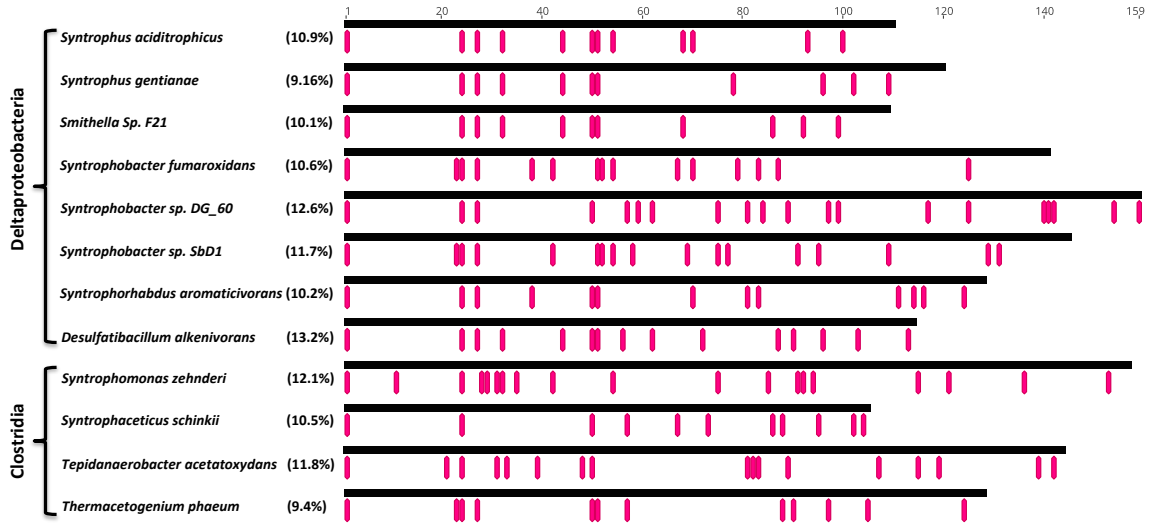

Figure 5. A diversity of syntrophs known to produce H_2 and/or formate as an interspecies electron carrier have pilin monomer genes likely to yield electrically conductive pili. The high abundance of aromatic amino acids as percentage of total amino acids is consistently greater than 9 % and the positioning of aromatic amino acids (red bars) are predictors of assembly into electrically conductive pili.

For decades, data on the functioning of complex anaerobic microbial communities has been interpreted through the lens of HFIT, but the available data does not rule out the possibility of DIET, which was not known to be an option at the time. For example, estimated H_2 turnover rates in anaerobic digesters, rice paddy soils, and aquatic sediments were consistently less than 10 % of the independently determined rate of methane production derived from the reduction of carbon dioxide to methane [44-46], a result that is consistent with DIET providing most of the electrons for carbon dioxide reduction. The mismatch between measured H_2 turnover rates and the concept of H_2 as a primary interspecies electron carrier was rationalized with the suggestion that there was a separate pool of H_2 within closely juxtapositioned assemblages of H_2 producers and H_2 consumers. However, the existence of two distinct pools of H_2 that did not equilibrate over time via diffusion, a seemingly physical impossibility, was never verified. Attempts to accurately measure formate fluxes have also been problematic [47] and there is no method for directly measuring DIET-based electron fluxes in complex communities.

A fresh perspective and new analytical tools will be required to resolve the relative importance of HFIT and DIET in diverse anaerobic microbial communities. Just as electron-accepting partners have different gene expression patterns depending on whether they are participating in HFIT or DIET [37,48], it may be possible to determine whether electron-donating

224 syntrophs are engaged in DIET or HFIT from metatranscriptomic analysis of anaerobic
225 communities.
226
227 **METHODS**
228 **Bacterial strains, plasmids and culture conditions**
229 *Syntrophus aciditrophicus, G. sulfurreducens* wild-type, *G. sulfurreducens* strain Aro-5,
230 and *G. sulfurreducens*$_{HF}$ were obtained from our laboratory culture collections. A strain of *G.*
231 *sulfurreducens* expressing the PilA gene of *S. aciditrophicus* instead of the native *G.*
232 *sulfurreducens* PilA gene was constructed as previously described [25]. *S. aciditrophicus* and *G.*
233 *sulfurreducens* strains were routinely grown under strict anaerobic conditions at 30 °C in the
234 previously described [49] defined, bicarbonate-buffered medium with N_2:CO_2 (80:20) as the gas
235 phase. For *S. aciditrophicus* the medium was amended with crotonate (20 mM) and for *G.*
236 *sulfurreducens* strains acetate (10 mM) was the electron donor and fumarate (40 mM) was the
237 electron acceptor. The presence of *c*-type cytochromes in whole cell lysates was evaluated with
238 heme-staining of proteins separated on denaturing gels as previously described [17]. *G.*
239 *sulfurreducens* strains were grown with a graphite electrode as the electron acceptor as
240 previously described [50].
241 Cocultures were established in 10 ml of culture medium [49] in anaerobic pressure tubes
242 with benzoate (1 mM) as the electron donor and fumarate (40 mM) as the electron acceptor with
243 cysteine (2 mM) and sulfide (1 mM) added as reducing agents. When noted cultures were
244 amended with granular activated carbon (0.25 g; 8-20 mesh). Benzoate, acetate, and succinate
245 were analyzed with high-performance liquid chromatography as previously described [16].

Previously describe methods were employed for transmission electron microscopy [21] and scanning electron microscopy [26].

Pili conductivity

A 100 µl sample of cultures was drop cast onto highly oriented pyrolytic graphite (HOPG). After 10 min the HOPG was washed twice with 100 µl of deionized water, blotted dry to remove excess water, and dried for 12 hours at 24 °C in a desiccator. Samples were equilibrated with atmospheric humidity for at least 2 hours and then examined with an Oxford Instruments Cypher ES Environmental AFM in ORCA electrical mode equipped with a Pt/Ir-coated Arrow-ContPT tip with a 0.2 N/m force constant (NanoWorld AG, Neuchâtel, Switzerland). Pili were located in contact mode, with a set point of 0.002 V and a scan rate of 1.5 Hz. For conductive imaging, the grounded tip, attached to a transimpedance amplifier, served as a translatable top electrode to locally detect the current response of the individual pili to a 100 mV bias applied to the HOPG substrate. Individual pili conductivity was further characterized by lightly pressing the AFM tip (set point 0.002 V) to the top of the pili and applying a quadruplicate amplitude of ±0.6 V voltage sweep at a frequency of 0.99 Hz, receiving ca. 8,000 points of reference per measurement. Pili expressed in *G. sulfurreducens* were further analyzed with four probe conductivity measurements on films of pili purified from the cells as previously described [25].

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