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Interspecies Electron Transfer via Hydrogen and Formate Rather than Direct Electrical Connections in Cocultures of *Pelobacter carbinolicus* and *Geobacter sulfurreducens*

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Direct interspecies electron transfer (DIET) is an alternative to interspecies H_2/formate transfer as a mechanism for microbial species to cooperatively exchange electrons during syntrophic metabolism. To understand what specific properties contribute to DIET, studies were conducted with *Pelobacter carbinolicus*, a close relative of *Geobacter metallireducens*, which is capable of DIET. *P. carbinolicus* grew in coculture with *Geobacter sulfurreducens* with ethanol as the electron donor and fumarate as the electron acceptor, conditions under which *G. sulfurreducens* formed direct electrical connections with *G. metallireducens*. In contrast to the cell aggregation associated with DIET, *P. carbinolicus* and *G. sulfurreducens* did not aggregate. Attempts to initiate cocultures with a genetically modified strain of *G. sulfurreducens* incapable of both H_2 and formate utilization were unsuccessful, whereas cocultures readily grew with mutant strains capable of formate but not H_2 uptake or vice versa. The hydrogenase mutant of *G. sulfurreducens* compensated, in cocultures, with significantly increased formate dehydrogenase gene expression. In contrast, the transcript abundance of a hydrogenase gene was comparable in cocultures with that for the formate dehydrogenase mutant of *G. sulfurreducens* or the wild type, suggesting that H_2 was the primary electron carrier in the wild-type cocultures. Cocultures were also initiated with strains of *G. sulfurreducens* that could not produce pili or OmcS, two essential components for DIET. The finding that *P. carbinolicus* exchanged electrons with *G. sulfurreducens* via interspecies transfer of H_2/formate rather than DIET demonstrates that not all microorganisms that can grow syntrophically are capable of DIET and that closely related microorganisms may use significantly different strategies for interspecies electron exchange.

Since the discovery of the "S organism" (6), microbiologists have tried to understand the mechanisms of electron exchange between microorganisms syntrophically degrading organic compounds under anaerobic conditions. For example *Pelobacter carbinolicus*, which is a modern-day analog for the S organism, can metabolize ethanol to acetate, H_2, and carbon dioxide only when an H_2-consuming partner, such as *Methanospirillum hungatei*, maintains low H_2 partial pressures (32). In some syntrophic cultures, formate may be the electron carrier between species (24, 33, 35). Previous studies provided evidence for H_2 and formate transfer by evaluating H_2- and/or formate-utilizing microorganisms as electron-accepting partners (24, 33, 35) and also by adding exogenous excess H_2 or formate to the cocultures to disrupt the syntrophic metabolism, decoupling methanogenesis from utilization of the substrate (1, 2, 40). Thermodynamic calculations have demonstrated that a small window of opportunity exists for the syntrophic partners, where the concentration of H_2 or formate provides optimum conditions for both partners (33, 36). Other electron carriers that facilitate electron exchange between syntrophic partners include the humic substance analog anthraquinone-2,6-disulfonate (19, 21) and cysteine (15). Direct interspecies electron transfer (DIET), could be an efficient alternative strategy for microorganisms to cooperate in the anaerobic degradation of organic substrates (20, 27, 37). DIET was discovered in cocultures of *Geobacter metallireducens* and *Geobacter sulfurreducens*, which grew with ethanol as the electron donor and fumarate as the electron acceptor (37). *G. sulfurreducens* cannot metabolize ethanol, whereas *G. metallireducens* cannot use fumarate as an electron acceptor. Adaptive evolution of the coculture for enhanced ethanol metabolism was associated with the formation of large aggregates of the two species. Although *G. sulfurreducens* is capable of utilizing either H_2 or formate as an electron donor for fumarate reduction when acetate is available as a carbon source (9), cells within the aggregates were not effective in H_2 or formate metabolism, and cocultures were readily initiated with a mutant strain of *G. sulfurreducens* that was unable to use H_2 as an electron donor (37). These results suggested that the coculture was functioning via an alternative to interspecies H_2 or formate transfer.

In the adapted cocultures, *G. sulfurreducens* produced large quantities of the multiheme *c*-type cytochrome OmcS (25, 37), which is localized (18) along the electrically conductive (23, 30) type IV pili of *G. sulfurreducens*. Increased OmcS expression was attributed to point mutations that accumulated in the gene for the transcriptional regulator PilR (37). Deleting *pilR* in *G. sulfurreducens* accelerated aggregate formation and adaption for rapid ethanol metabolism (37). Deletion of genes required for OmcS or pilus expression inhibited ethanol metabolism (37). Furthermore, the aggregates were electrically conductive, likely due to the pili, which have been shown to provide long-range conductivity in *G. sulfurreducens* biofilms (23, 24). These results suggested that electrons were directly transferred from *G. metallireducens* to *G. sulfurreducens*.

There was also substantial evidence for DIET within aggregates

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from an anaerobic digester converting brewery waste to methane, in which *Geobacter* was abundant (27). The mixed community aggregates exhibited metal-like conductivity (27) similar to that of *Geobacter* current-producing biofilms and the pili of *G. sulfurreducens* (23).

To better understand the mechanisms of DIET, it is important to determine if other microorganisms are capable of DIET and what features those microorganisms must have to enable DIET. The potential for *P. carbinolicus* to participate in DIET was evaluated because both *P. carbinolicus* and *G. metallireducens* appear to have evolved from a common ancestor capable of extracellular electron transfer (7), but the two differ significantly in several aspects of their basic physiology and mechanisms for extracellular electron transfer (7, 12, 31). Thus, it was unknown whether the absence of previous evidence for DIET with *P. carbinolicus* could be attributed to syntrophic growth being evaluated with an electron-accepting partner incapable of DIET or whether *P. carbinolicus* lacks key physiological features required for DIET. The results indicate that *P. carbinolicus* is not capable of DIET and must rely on interspecies transfer of H_2 or formate for electron exchange with *G. sulfurreducens*.

MATERIALS AND METHODS

Organisms, media, and growth conditions. All incubations of pure cultures and cocultures were performed under strict anaerobic culturing techniques as previously described (3). Cultures were incubated in 27-ml pressure tubes or 160-ml serum bottles sealed with butyl rubber stoppers and filled with 10 or 50 ml of medium. The increase in culture turbidity was monitored at 600 nm by placing the culture tubes into a Genesys 5 spectrophotometer (Spectronics Instruments) with a path length of 1.5 cm.

P. carbinolicus (DSM 2380) was regularly transferred under fermentative conditions with 10 mM acetoin as the substrate and 0.02 mM Na_2S as the reductant, as previously described (12). *G. sulfurreducens* PCA (ATCC 51573) and mutants of this microorganism which were tested for the study (Δ*hybL*, Δ*fdnG*, double Δ*hybL* Δ*fdnG*, Δ*omcS*, and Δ*pilA* mutants) were routinely cultured in freshwater medium containing 1 mM cysteine as the reductant, 10 or 15 mM acetate, and 40 mM fumarate as previously described (8). Newly constructed mutants of *G. sulfurreducens* were tested for growth with H_2 (138 KPa) or formate (40 mM and 10 mM) as the electron donor in freshwater medium in the presence of 1 mM acetate as the carbon source.

For cocultures of *P. carbinolicus* and *G. sulfurreducens*, 20 mM ethanol and 40 mM fumarate served as substrates for growth in a medium prepared as previously described (12). Cocultures of *G. metallireducens* and the *G. sulfurreducens* strain deficient in formate dehydrogenase and hydrogenase activities were initiated using a 2% inoculum of each syntrophic partner added to a freshwater medium prepared as previously described (37) with fumarate and ethanol as substrates.

All cocultures were regularly transferred (2% inocula) under strict anaerobic conditions at least six times prior to monitoring organic acids and ethanol over time. The only exception was a coculture of *P. carbinolicus* with the *G. sulfurreducens* double mutant incapable of H_2 and formate utilization. This coculture could not grow on ethanol and was therefore analyzed during the initial transfer.

Construction of *G. sulfurreducens* mutants. The *fdnG* gene (GSU0777) was replaced with a kanamycin resistance gene, such that the coding region for amino acid residues from 62Asp to 951Pro was deleted. Double-crossover homologous recombination was carried out by electroporation (8) with the linear DNA fragment consisting of the kanamycin resistance gene flanked by ~0.7-kbp DNA fragments containing the upstream and the downstream regions of *fdnG*. These flanking DNA fragments were amplified by PCR with primers *fdnG*-P1 (TCTCTAGAACGG CTTGGTGACGTAGTC; the XbaI site is underlined) and *fdnG*-P2 (TC GGATCCTTGGTATGGACGATCAG; the BamHI site is underlined) for the upstream region and *fdnG*-P3 (TCTAAGCTTCAACGTGCAGGGCA AGC; the HindIII site is underlined) and *fdnG*-P4 (TCTCTCGAGACCA CTTTCACGTAGCGGTC; the XhoI site is underlined) for the downstream region. The kanamycin resistance gene was amplified by PCR with Km-Fwd (GCATGAGAATTCCTGACGGAACAGCGGGAAGTCCAGC; the EcoRI site is underlined) and Km-Rev (GCTATGAAGCTTTCATAG AAGGCGGCGGTGGAATCGAA; the HindIII site is underlined) and using pBBR1MCS-2 (17) as the template. Gene replacement was confirmed by PCR analysis. The Δ*fdnG* Δ*hybL* double mutant was constructed in a similar manner by deleting the *fdnG* gene from a previously characterized Δ*hybL* uptake hydrogenase mutant (10).

Reverse transcription-quantitative PCR (RT-qPCR). To quantify the abundance of hydrogenase and formate dehydrogenase transcripts in cocultures of *P. carbinolicus* with the wild-type strain of *G. sulfurreducens*, the hydrogenase-deficient strain, and the formate dehydrogenase-deficient strain, four biological replicates of each late mid-exponential-phase coculture, 10 ml each, were treated with 2 ml RNA Later (Ambion), mixed well, and harvested at 4°C by centrifugation at 6,000 × *g* for 20 min. The tubes were opened, and cocultures were removed for further use for RNA extraction using TRIzol (Invitrogen) with a slight modification of the manufacturer's protocol. Briefly, the cell pellets were mixed homogenously with a 1-ml volume of TRIzol reagent. The mix was transferred to a 2-ml O-ring tube containing 0.5 g of 0.1-mm glass-zirconia beads and homogenized for 20 s on a FastPrep instrument (MoBio Laboratories) at 3 m/s. The tubes were then incubated at room temperature for 5 min before addition of 200 μl chloroform, vortexed for 15 s, and centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous layer was then used for the RNA isolation. The RNA thus obtained was purified using the MiniElute PCR purification kit (Qiagen) and further treated with rDNAse I (Ambion) to digest any traces of genomic DNA contamination. A final round of RNA purification was done with a MiniElute PCR purification kit (Qiagen) following the manufacturer's protocol. The quality and the quantity of pure RNA were assessed with the Experion RNA standard sensitivity kit (Bio-Rad). Furthermore, absence of genomic DNA contamination was verified by 16S rRNA gene PCR using 9F and 519R primer sets (34).

For whole-transcriptome amplification (WTA), about 300 ng of total RNA was converted into WTA cDNA libraries and amplified by WTA PCR using reagents and protocols supplied with or recommended by Sigma. Briefly, 300 ng of total RNA was mixed with 2.5 μl WTA library synthesis buffer and 2.5 μl WTA library stabilization solution, the total volume was adjusted to 24 μl using nuclease-free water, and the mixture was heated at 70°C for 5 min and immediately cooled. Library synthesis enzyme (1 μl) was added, and WTA cDNA libraries were synthesized using the following thermocycler program: 24°C for 15 min, 42°C for 2 h, and 95°C for 5 min. Aliquots were WTA PCR amplified using JumpStart *Taq* DNA polymerase (Sigma), WTA amplification master mix, and deoxynucleoside triphosphate (dNTP) mix following the manufacturer's protocol except that the total number of cycles was reduced to 15. The enriched product was then purified using a PCR purification kit (Qiagen) and used as a template in qPCR experiments.

Real-time PCR was carried out using an ABI prism 7900 (Applied Biosystems). Primers designed for *G. sulfurreducens* (26) were used to target *hybA*, *fdnG*, and the housekeeping gene *recA*: *fdnG*-F, 5′-ACTTCA CCAAGGACGTCACC-3′; *fdnG*-R, 5′-TCCCTTCGTTGGTGTAGGAG-3′; *hybA*-F, 5′-CTACGGCGAGAAGGAAGTTG-3′; *hybA*-R, 5′-CCCCT TGTAGATGGTGTGCT-3′; *recA*-F, 5′-CACCGGCATAATCTCCAAGT-3′; and *recA*-R, 5′-ATCTTGCGGATATCGAGACG-3′. Reactions were performed in triplicate for each gene tested in a final volume of 20 μl containing 10 μl of Power Sybr green PCR master mix, 0.6 mM reverse and forward primers were made, and 2 μl of enriched WTA product was added as the template. The real-time PCR was run for 50 cycles using 60°C as the annealing temperature with the absolute quantification option.

Microscopy. To resolve whether cells grew freely in the medium or whether they were associated in aggregate structures, cells were visualized by phase-contrast microscopy on a Nikon Eclipse E600 microscope.

To resolve the cell abundance and overall distribution of the two microorganisms in the cocultures, cells were fixed [2% paraformaldehyde and 0.5% glutaraldehyde in 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) at pH 7.2] for 1 h at room temperature, and a droplet was placed on a gelatin-coated slide, dried at 46°C for 5 min, and then dehydrated in 70% ethanol for 30 min at 4°C. Dehydrated samples were hybridized as described previously (29) using the probes PCARB1 (5′-Cy3-GCCTATTCGACCACGATA-3′), specific for *P. carbinolicus* (31), and GEO2 (5′-Cy5-GAAGACAGGAGGCCCGAAA-3′), specific for *G. sulfurreducens* (37). Samples were visualized on a Leica TCS SP5 confocal fluorescence microscope using consecutive line scanning to detect Cy3 and Cy5 fluorochromes.

Identification of OmcS cytochrome content in cocultures. OmcS abundance was determined in *P. carbinolicus-G. sulfurreducens* and *G. metallireducens-G. sulfurreducens* cocultures versus *G. sulfurreducens* cells grown on freshwater medium with 40 mM fumarate and 10 mM acetate as substrates (8). Cells were retrieved during the late stages of mid-exponential growth, and the whole-cell lysates obtained (5 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting and probing with an OmcS-specific antiserum as previously described (37).

Analytical techniques. For determination of substrate depletion and production of metabolic products, samples were withdrawn with hypodermic needles and syringes under strict anaerobic conditions and passed through 0.2-μm Acrodisc filters. A minimum of three biological replicates were analyzed for each coculture type. Volatile fatty acids were monitored by high-performance liquid chromatography (HPLC) as previously described (28). Changes in ethanol concentration over time were monitored by gas chromatography (GC) as previously described (27).

RESULTS AND DISCUSSION

Syntrophic growth on ethanol. When *P. carbinolicus* and *G. sulfurreducens* were simultaneously inoculated into a medium with ethanol as the electron donor and fumarate as the electron acceptor, the coculture grew with the metabolism of ethanol and the reduction of fumarate to succinate (Fig. 1 and 2a). In contrast to the previously described cocultures of *G. metallireducens* and *G. sulfurreducens*, which lagged for several weeks before utilizing significant ethanol (37), growth and metabolism of the *P. carbinolicus-G. sulfurreducens* cocultures typically began within a day (Fig. 1). Furthermore, the *P. carbinolicus-G. sulfurreducens* cocultures metabolized most of the ethanol provided in 3 days, whereas even after months of adaptation for syntrophic growth, the *G. metallireducens-G. sulfurreducens* cocultures still required 5 days to metabolize 70% of the added ethanol (37). Although *G. metallireducens-G. sulfurreducens* cocultures formed large (>1-mm) aggregates (37), the *P. carbinolicus-G. sulfurreducens* cocultures did not aggregate even after 400 consecutive transfers of the coculture. The cells did not appear to form physical associations, even at the level of individual cells (Fig. 3a). Contact between syntrophic partners is considered to be a requirement for DIET, and although it may also facilitate interspecies H_2 or formate transfer (5, 14, 39), long-term coculture studies demonstrated that contact is not necessary for the later (13). Examination of the coculture with fluorescent *in situ* hybridization (FISH) probes specific for the two species revealed that *G. sulfurreducens* was more abundant than *P. carbinolicus* (Fig. 3).

Interspecies electron transfer via H_2 or formate. In order to evaluate the possibility of interspecies H_2 or formate transfer, cocultures were initiated with one of the following strains of *G. sulfurreducens*: (i) a strain that could not metabolize H_2 because the gene for the large subunit of the uptake hydrogenase (*hybL*) was deleted (10), (ii) a strain that could not grow on formate because the gene for the catalytic subunit of formate dehydrogenase (*fdnG*) was deleted (Fig. 4b), or (iii) a strain that could not grow on H_2 or formate because both *hybL* and *fdnG* were deleted (Fig. 4c). Cocultures initiated with *G. sulfurreducens* strains that could metabolize only formate (Fig. 2b) or only H_2 (Fig. 2c) readily metabolized ethanol with the reduction of fumarate.

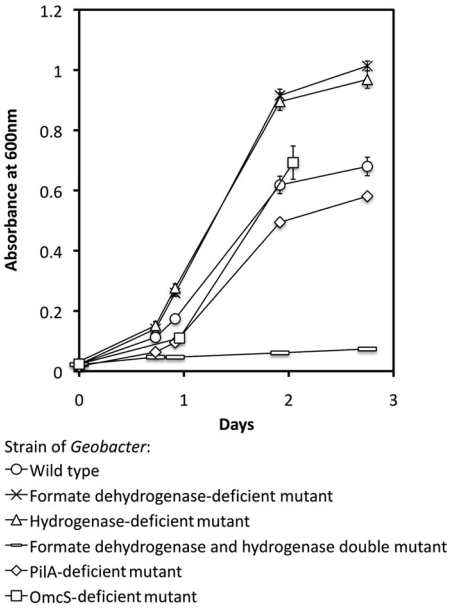

FIG 1 Initial growth of cocultures in ethanol-fumarate medium started with *P. carbinolicus* and different strains of *G. sulfurreducens*. The results are the means and standard deviations for triplicate cultures.

However, growth and ethanol metabolism did not proceed in cocultures initiated with a strain of *G. sulfurreducens* that could not metabolize either H_2 or formate (Fig. 2d). These results indicate that either H_2 or formate can serve as an electron carrier for interspecies electron transfer, and interspecies electron transfer via one of these two electron carriers was the only mechanism by which the coculture could function. In contrast, *G. metallireducens* formed well-functioning syntrophic cultures with the *G. sulfurreducens* strain that could not utilize H_2 and formate, consistent with the concept of DIET in that coculture system (see Fig. SM1 in the supplemental material).

In order to evaluate the potential contributions of H_2 and formate as electron carriers between *P. carbinolicus* and *G. sulfurreducens*, the transcript abundance of an uptake hydrogenase subunit (*hybA* product) and the large subunit of formate dehydrogenase (*fdnG* product) were monitored (Fig. 5a). When H_2 uptake was not possible, *G. sulfurreducens* adapted with increased expression of *fdnG* ($P = 0.009$). In contrast, when formate metabolism was inhibited, the transcript abundance of *hybA* was not significantly different ($P = 0.5$) from that in the wild type (Fig. 5a). These results, and the fact that *hybA* transcripts were much more abundant than *fdnG* transcripts in the wild type, suggest that although the cocultures could function via either interspecies H_2 or formate transfer, H_2 was the primary electron carrier between species in cocultures with wild-type *G. sulfurreducens*.

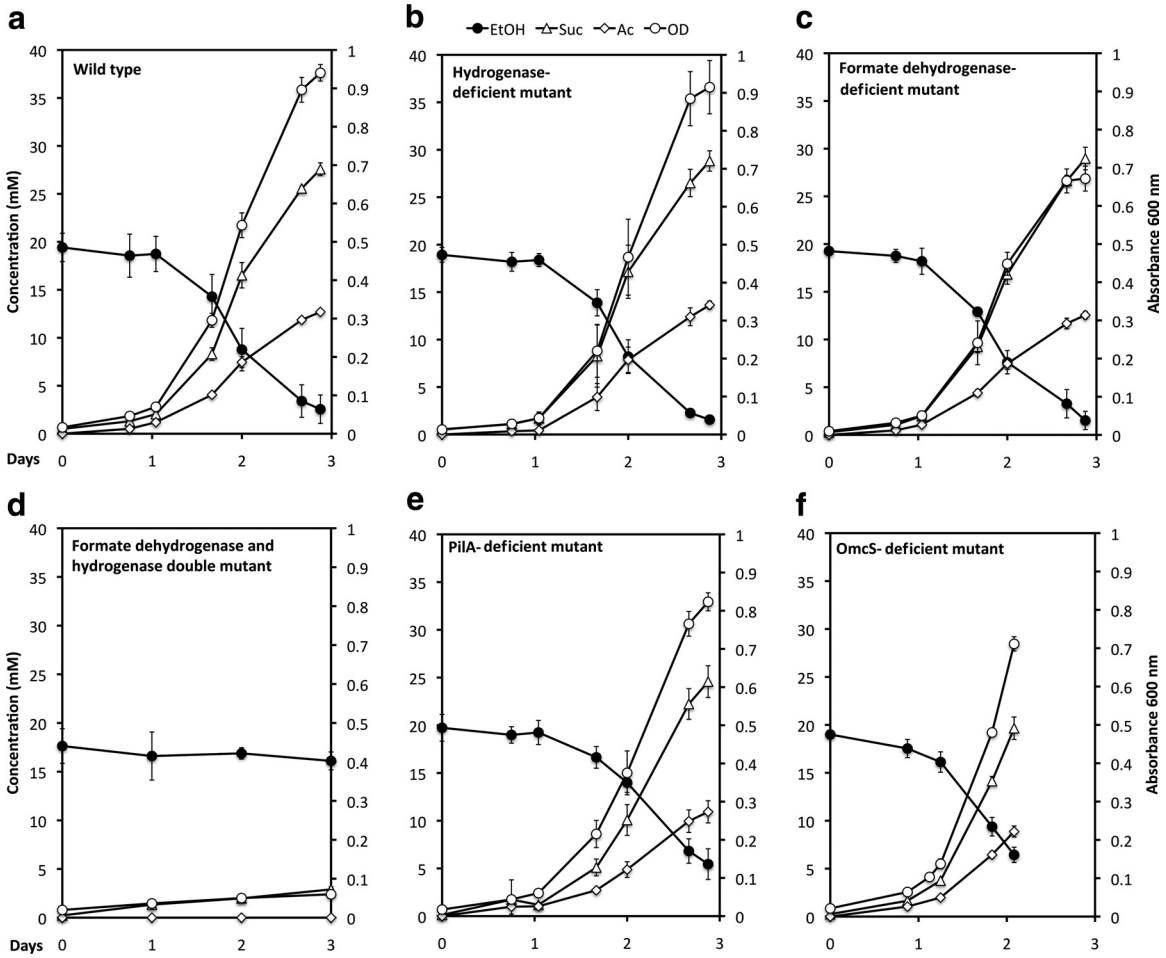

FIG 2 Growth (optical density [OD]), ethanol (EtOH) metabolism, acetate (Ac) accumulation, and succinate (Suc) production from fumarate reduction after more than five consecutive transfers of cocultures of *P. carbinolicus* with different strains of *G. sulfurreducens*. Also shown are the data from the initial attempt to start a coculture with a strain of *G. sulfurreducens* unable to utilize formate or H_2. The results are the means and standard deviations for triplicate cultures.

In contrast to the case for *G. metallireducens-G. sulfurreducens* cocultures (see Fig. SM1 in the supplemental material), acetate accumulated over time in *P. carbinolicus-G. sulfurreducens* cocultures (Fig. 1). The likely explanation for this difference is that the expression of citrate synthase in *G. sulfurreducens* is inhibited in the presence of H_2, preventing acetate metabolism (4, 38). Thus, the availability of H_2 in *P. carbinolicus-G. sulfurreducens* cocultures would be expected to limit acetate metabolism of *G. sulfurreducens*, whereas no such inhibition of acetate metabolism is expected in *G. metallireducens-G. sulfurreducens* cocultures because of the lack of H_2 production during DIET.

Pili and OmcS are not required during H_2/formate electron transfer. Deleting the gene for PilA or OmcS in *G. sulfurreducens* did not prevent *P. carbinolicus* from forming effective cocultures (Fig. 2e and 2f, respectively). This contrasts with the previous finding (37) that *G. metallireducens-G. sulfurreducens* cocultures could not be established if either *pilA* or *omcS* was deleted from *G. sulfurreducens* (37). As previously reported (37), *G. sulfurreducens* expressed OmcS at high levels in *G. metallireducens-G. sulfurreducens* cocultures, but OmcS was not detected in *P. carbinolicus-G. sulfurreducens* cocultures (Fig. 5b). These results suggest that the model for DIET between *G. metallireducens* and *G. sulfurreducens*, in which OmcS and pili are important components of the electrical connection between the two species (20, 37), does not apply to the *P. carbinolicus-G. sulfurreducens* coculture.

Implications. These findings demonstrate that not all microorganisms that can grow syntrophically via interspecies electron exchange are capable of DIET and that even closely related microorganisms may differ in their modes of syntrophic growth. The finding that *P. carbinolicus* was not able to directly transfer electrons to another species capable of DIET is consistent with previous findings which suggest that *P. carbinolicus* is poorly suited for direct electron transfer to insoluble extracellular electron acceptors, such as electrodes (31) and Fe(III) oxide (12). The ability to growth syntrophically via interspecies hydrogen/formate transfer but not DIET may be common in laboratory cocultures. For example a syntrophic coculture of *Desulfovibrio vulgaris* and *Methanococcus maripaludis* did not form aggregates even after 300 generations (13), suggesting a lack of DIET in that system as well.

Although there is evidence for DIET in microbial aggregates from methanogenic wastewater digesters (27) the prevalence of DIET in natural environments and the factors that might favor DIET over interspecies H_2 and formate transfer are unknown. It may be that *G. metallireducens* interacts with *G. sulfurreducens* via

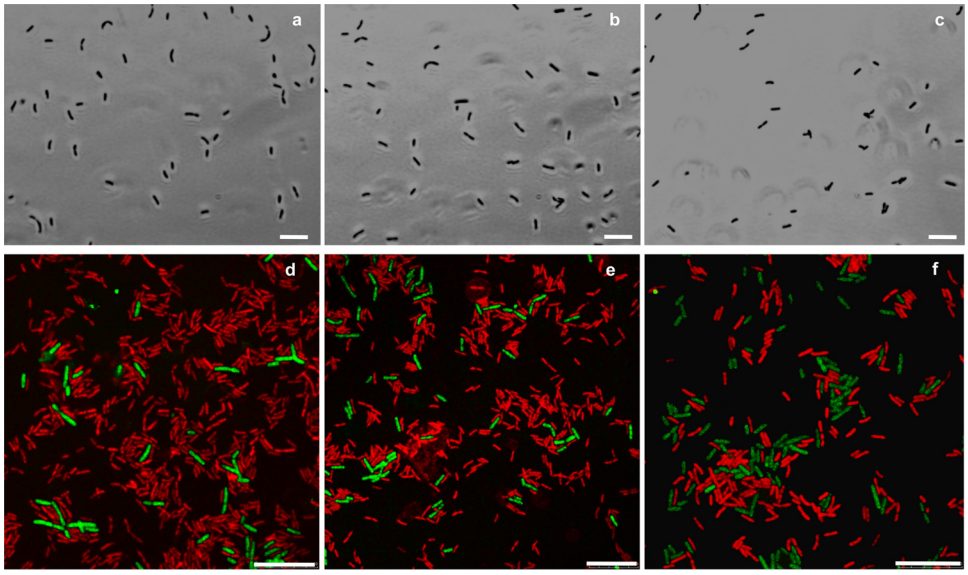

FIG 3 Phase-contrast (a, b, and c) and epifluorescence (d, e, and f) micrographs of *P. carbinolicus* cells in coculture with *G. sulfurreducens* wild-type cells (a and d), the hydrogenase-deficient *G. sulfurreducens* strain (b and e), or the strain deficient in formate dehydrogenase (c and f). Epifluorescence of *in situ* hybridized cells with *P. carbinolicus* is shown as green, and that with *G. sulfurreducens* is shown as red. Scale bars, 10 μm.

DIET because it is well suited for extracellular electron transfer (22) but has limited ability to produce H_2 (11).

Metabolizing substrates with the release of electrons as H_2 or formate requires less coordination with syntrophic partners than DIET and may account for the ability of the *P. carbinolicus-G. sulfurreducens* cocultures to initiate syntrophic growth much faster and to metabolize ethanol more rapidly than *G. metallireducens-G. sulfurreducens* cocultures. Another consideration is that consortia cooperating via DIET must bear the additional energetic investment of producing the proteins necessary to establish the electrical connections required for DIET. However, the high abundance of *Geobacter* species in electrically conductive aggregates from methanogenic digesters (27) and the finding that addition of conductive or semiconductive supplementary materials enhance DIET with increased rates of methanogenesis in sediments (16) and methanogenic digester aggregates (19) suggest that DIET can be more favorable than interspecies H_2/formate transfer in important methane-producing environments. Genome-scale metabolic modeling might offer an approach for calculating the cost/benefit relationships of the different strategies for interspecies electron transfer under diverse environmental conditions, as evidenced by the ability of this approach to effectively predict the outcome of microbial competition in different subsurface environments (41).

The physiological differences between microorganisms that are effective in DIET and those that rely on interspecies H_2/formate transfer are important considerations when attempting to

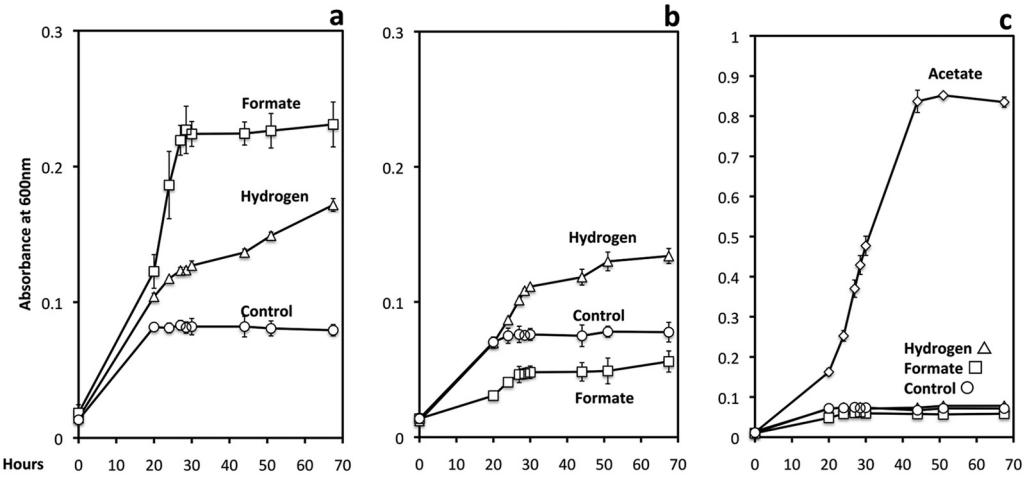

FIG 4 Growth on formate (10 mM) or H_2 (138 KPa) in the presence of 1 mM acetate for wild-type *G. sulfurreducens* (a), a strain deficient in a formate dehydrogenase subunit (b), or a strain deficient in both a formate dehydrogenase subunit and an uptake hydrogenase subunit (c). In controls without added hydrogen or formate, the acetate added as a carbon source could also serve as an electron donor to support growth. Growth of the double mutant growth on 15 mM acetate is also shown (c) to demonstrate that cells were viable yet unable to grow on formate or H_2. The results are the means and standard deviations for triplicate cultures.

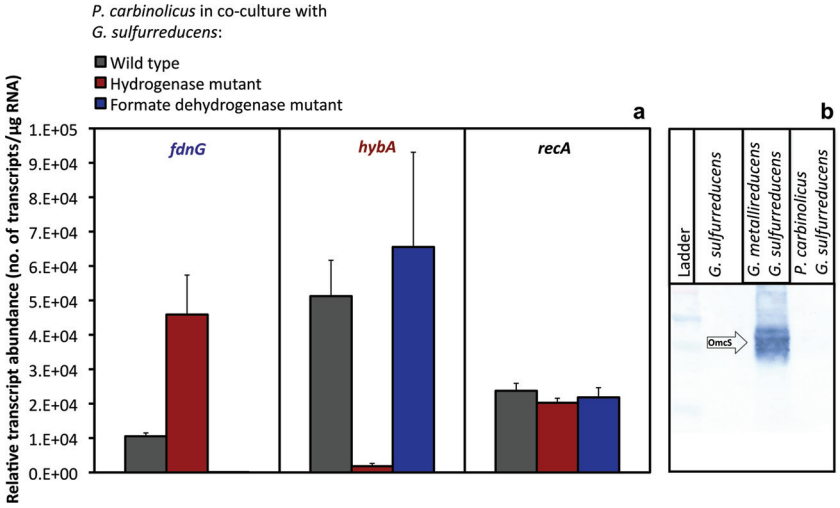

FIG 5 Molecular analysis of cocultures. (a) Relative transcript abundances of the formate dehydrogenase gene (*fdnG*), the hydrogenase gene (*hybA*), and the housekeeping gene *recA* in *P. carbinolicus-G. sulfurreducens* cocultures as determined by RT-qPCR. Results are the means and standard deviations for triplicate cultures. (b) Western blot analysis of OmcS in equivalent cell protein of *G. sulfurreducens* grown with fumarate as the electron acceptor or ethanol-fumarate cocultures of *P. carbinolicus-G. sulfurreducens* or *G. metallireducens-G. sulfurreducens* cocultures.

enrich and isolate syntrophic microorganisms capable of DIET. Common procedures for the isolation of syntrophic microorganisms, such as the use of fermentable substrates (33) or coculturing with an H_2-consuming partner (24), may fail to recover organisms that specialize in DIET. Thus, new approaches for the isolation and study of syntrophic interactions are required to better assess the diversity and environmental relevance of microorganisms capable of DIET.

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