Interspecies electron transfer via H₂ and formate rather than direct electrical connections in co-cultures of Pelobacter carbinolicus and Geobacter sulfurreducens

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Abstract

Direct interspecies electron transfer (DIET) is an alternative to interspecies H\textsubscript{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 co-culture with *Geobacter sulfurreducens* with ethanol as electron donor and fumarate as 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 co-cultures with a genetically modified strain of *G. sulfurreducens* incapable of both H\textsubscript{2} and formate utilization were unsuccessful, whereas co-cultures readily grew with mutant strains capable of formate but not H\textsubscript{2} uptake, or vice-versa. The hydrogenase mutant of *G. sulfurreducens* compensated, in co-cultures, with significantly increased formate-dehydrogenase gene expression. In contrast, the transcript abundance of a hydrogenase gene was comparable in co-cultures with the formate dehydrogenase mutant of *G. sulfurreducens* or wild-type, suggesting that H\textsubscript{2} was the primary electron carrier in the wild-type co-cultures. Co-cultures 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\textsubscript{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.

Introduction
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 a 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 as well by adding exogenous excess H$_2$ or formate to the co-cultures 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 substances 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 co-cultures of *G. metallireducens* and *G. sulfurreducens*, which grew with ethanol as the electron donor and fumarate as the electron acceptor (37). *G. sulfurreducens* can not metabolize ethanol, whereas *G. metallireducens* can not use fumarate as an electron acceptor. Adaptive evolution of the co-culture 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 H₂ or formate metabolism and co-cultures were readily initiated with a mutant strain of *G. sulfurreducens* that was unable to use H₂ as an electron donor (37). These results suggested that the co-culture was functioning via an alternative to interspecies H₂ or formate transfer.

In the adapted co-cultures, *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 pili expression inhibited ethanol metabolism (37). Furthermore, the aggregates were electrically conductive, likely due to the pili that 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 from an anaerobic digester converting brewery waste to methane, in which *Geobacter* were abundant (27). The mixed community aggregates exhibited metallic-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 H₂ or formate for electron exchange with *G. sulfurreducens*.

**Materials and Methods**

**Organisms, media and growth conditions**

All incubations of pure cultures and co-cultures 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. Increase in cultures 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 substrate, and 0.02 mM Na₂S as reductant, as previously described (12). *G. sulfurreducens* PCA (ATCC 51573) and mutants of this microorganism which were tested for the study (ΔhybL, ΔfdnG, a double mutant ΔhybL-ΔfdnG, ΔomcS, ΔpilA) were routinely cultured in freshwater medium containing 1 mM cysteine as 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 H₂ (20 psi) or formate (40 mM and 10 mM) as the electron donor in freshwater medium in the presence of 1 mM acetate as carbon source.
For co-cultures 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). Co-cultures of *G. metallireducens* and the *G. sulfurreducens* strain deficient in formate dehydrogenase and hydrogenase activity were initiated using 2% inocula of each syntrophic partner added to a freshwater medium prepared as previously described (37) with fumarate and ethanol as substrates.

All co-cultures 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 co-culture of *P. carbinolicus* with the *G. sulfurreducens* double mutant incapable of H₂ and formate utilization. This co-culture 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 kilobase pairs (kbp) DNA fragments containing the upstream and the downstream regions of *fdnG*. These flanking DNA fragments were amplified by PCR with primers *fdnG*-P1 (TCTCTAGAACGGCTTGGTGACGTAGTC, the *Xba*I site is underlined) and *fdnG*-P2 (TCGGATCCCTGGTTATGGACGATCAG, the *BamH*I site is underlined) for the upstream region and *fdnG*-P3 (TCTAAGCTTCAACGTGCAGGGCAAGC, the *HindIII* site is underlined) and *fdnG*-P4 (TCTCTCGAGACCACTTTCACGTAGCGTGC, the *Xho*I site is underlined) for the downstream region. The kanamycin resistance gene was amplified by
PCR with Km-Fwd (GCATGAGAATTCTGACGGAACAGCGGGAAGTCCAGC, EcoRI site is underlined) and Km-Rev (GCTATGAAAGCTTCATAGAAGGCAGCGGTGGAATCGAA, the HindIII site is underlined), and using pBBR1MCS-2 (17) as template. Gene replacement was confirmed by PCR analysis. The ΔfdnG-ΔhybL double mutant was constructed in a similar manner by deleting the fdnG gene from a previously characterized uptake hydrogenase mutant, ΔhybL (10).

Reverse transcription quantitative PCR

To quantify the abundance of hydrogenase and formate dehydrogenase transcripts in co-cultures 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 co-culture, 10 mL each, were treated with 2 mL RNA later (Ambion), mixed well and harvested at 4°C by centrifugation at 6000×g for 20 min. Tubes were opened and co-cultures were removed for further use for RNA extraction using Trizol (Invitrogen) with slight modification of manufacturers’ protocol. Briefly, the cell pellets were mixed with 1 ml volume of TRIzol reagent and mixed homogenously. 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 sec 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, vortexted for 15 sec and centrifuged at 12000 × g for 15 min at 4° C. The aqueous layer was then used for the RNA isolation. The RNA thus obtained was purified using MiniElute PCR Purification Kit (Qiagen) and further treated with rDNAse I (Ambion) to digest any traces of genomic DNA contamination. Final round of RNA purification was done on a MiniElute PCR Purification Kit (Qiagen) following the manufacturer’s protocol. The quality and the quantity of pure RNA were accessed 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 were 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 and the total volume was adjusted to 24 µL using nuclease-free water, the mixture was heated at 70°C for 5 min and immediately cooled. Library synthesis enzyme (1µL) was added, and WTA cDNA libraries were synthesized using the following thermocycler program: 24°C for 15 minutes, 42°C for 2 hours, and 95°C for 5 minutes. Aliquots were WTA PCR-amplified using JumpStart™ Taq DNA Polymerase (Sigma), WTA Amplification Master Mix and dNTP Mix following the manufacturers’ protocol except total cycle was reduced to 15 cycles. The enriched product was then purified using PCR purification kit (Qiagen) and used as a template in qPCR experiment.

Real time PCR was carried out using ABI prism 7900 (Applied Biosystem). Primers designed for *G. sulfurreducens* (26) were used to target the *hybA*, *fdnG*, and the housekeeping gene *recA*: 

- *fdnG*-F: 5’-ACTTCACCAAGGACGTCACC-3’, *fdnG*-R: 5’-
- TCCCTTCGTGGTGTAGGAG-3’, *hybA*-F: 5’-CTACGGCGAGAAGGAAGTTG-3’, *hybA*-R: 5’-CCCCTTGTAGATGGTGTGCT-3’, *recA*-F: 5’-CACCGGCATAATCTCCAAGT-3’ and 
- *recA*-R: 5’-ATCTTGCAGATATCGAGACG-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 of reverse and forward primers were made and 2 µl of enriched WTA product was added as template. The real time PCR was run for 50 cycles using 60°C as the annealing temperature using absolute quantification option.
Microscopy

To resolve if cells grew freely in the medium or if 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 co-cultures, cells were fixed (2% paraformaldehyde and 0.5% glutaraldehyde in 50 mM PIPES at pH 7.2) for one hour at room temperature, a droplet was placed on a gelatin-coated slide and dried at 46°C for 5 min, and was then dehydrated in 70% ethanol for 30 min at 4°C. Dehydrated samples were hybridized as described (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 co-cultures

OmcS abundance was determined in P.carbinolicus/G.sulfurreducens and G.metallireducens/G.sulfurreducens co-cultures versus G. sulfurreducens cells were grown on fresh-water 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 cells lysates obtained (5µg), were separated by sodium dodecyl sulfate-polyacrylamide 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 was analyzed for each co-culture type. Volatile fatty acids were monitored by high performance liquid chromatography as previously described (28). Changes in ethanol concentration over time was monitored by gas chromatography 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 co-culture grew with the metabolism of ethanol and the reduction of fumarate to succinate (Fig. 1; Fig. 2a). In contrast to the previously described co-cultures 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* co-cultures typically began within a day (Fig. 1). Furthermore, the *P. carbinolicus* - *G. sulfurreducens* co-cultures metabolized most of the ethanol provided in three days whereas even after months of adaptation for syntrophic growth, the *G. metallireducens*/*G. sulfurreducens* co-cultures still required five days to metabolize 70% of the added ethanol (37). Although *G. metallireducens*/*G. sulfurreducens* co-cultures formed large (> 1 mm) aggregates (37), the *P. carbinolicus*/*G. sulfurreducens* co-cultures did not aggregate even after 400 consecutive transfers of the co-culture. 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 H₂ or formate transfer (5, 14, 39), long-term co-culture studies demonstrated that contact is not necessary for the later (13). Examination of the co-culture with FISH probes specific for the two species revealed that *G. sulfurreducens* was more abundant than *P. carbinolicus* (Fig. 3).

Interspecies electron transfer via H₂ or formate

In order to evaluate the possibility of interspecies H₂ or formate transfer, co-cultures were initiated with one of the following strains of *G. sulfurreducens*: 1) a strain that could not...
metabolize H₂ because the gene for the large subunit of the uptake hydrogenase (hybL) was deleted (10); 2) a strain that could not grow on formate because the gene for the catalytic subunit of formate dehydrogenase (fdnG) was deleted (Fig. 4b); or 3) a strain that could not grow on H₂ or formate because both hybL and fdnG were deleted (Fig. 4c). Co-cultures initiated with *G. sulfurreducens* strains that could metabolize only formate (Fig. 2b) or only H₂ (Fig. 2c) readily metabolized ethanol with the reduction of fumarate.

However, growth and ethanol metabolism did not proceed in co-cultures initiated with a strain of *G. sulfurreducens* that could not metabolize either H₂ or formate (Fig. 2d). These results indicate that either H₂ or formate can serve as electron carriers for interspecies electron transfer, and interspecies electron transfer via one of these two electron carriers was the only mechanism by which the co-culture could function. In contrast, *G. metallireducens* formed well-functioning syntrophic cultures with the *G. sulfurreducens* strain that could not utilize H₂ and formate, consistent with the concept of DIET in that co-culture system (Fig. 1SM).

In order to evaluate the potential contributions of H₂ and formate as electron carriers between *P. carbinolicus* and *G. sulfurreducens* the transcript abundance of an uptake hydrogenase subunit (hybA) and the large subunit of formate dehydrogenase (fdnG) were monitored (Fig. 5a). When H₂ uptake was not possible, *G. sulfurreducens* adapted with increased expression of fdnG (P=0.009). In contrast, when formate metabolism was inhibited, transcript abundance of hybA was not significantly different (P=0.5) than the wild type (Fig 5a). These results, and the fact that hybA transcripts were much more abundant than fdnG transcripts in wild-type, suggest that although the co-cultures could function via either interspecies H₂ or formate transfer, H₂ was the primary electron carrier between species in co-cultures with wild-type *G. sulfurreducens*. 


In contrast to *G. metallireducens/G. sulfurreducens* co-cultures (Fig. S1), acetate accumulated over time in *P. carbinolicus/G. sulfurreducens* co-cultures (Fig. 1). The likely explanation for this difference is that the expression of citrate synthase in *G. sulfurreducens* is inhibited in the presence of H₂, preventing acetate metabolism (4, 38). Thus, the availability of H₂ in *P. carbinolicus/G. sulfurreducens* co-cultures would be expected to limit acetate metabolism of *G. sulfurreducens*, whereas no such inhibition of acetate metabolism is expected in *G. metallireducens/G. sulfurreducens* co-cultures because of the lack of H₂ production during DIET.

**Pili and OmcS not required during H₂/formate electron transfer**

Deleting the gene for PilA or OmcS in *G. sulfurreducens* did not prevent *P. carbinolicus* from forming effective co-cultures (Fig. 2e and 2f, respectively). This contrasts with the previous finding (37) that *G. metallireducens/G. sulfurreducens* co-cultures 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* co-cultures, but OmcS was not detected in *P. carbinolicus/G. sulfurreducens* co-cultures (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* co-culture.

**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 mode 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 co-cultures. For example a syntrophic co-culture 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 H₂ and formate transfer are unknown. It may be that *G. metallireducens* interacts with *G. sulfurreducens* via DIET because it is well suited for extracellular electron transfer (22), but has limited ability to produce H₂ (11).

Metabolizing substrates with the release of electrons as H₂ or formate requires less coordination with syntrophic partners than DIET and may account for the ability of the *P. carbinolicus/G. sulfurreducens* co-cultures to initiate syntrophic growth much faster and to metabolize ethanol more rapidly than *G. metallireducens/G. sulfurreducens* co-cultures. 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/(semi)-

conductive 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 H₂/formate transfer in important methane-producing environments.
Genome-scale metabolic modeling might offer an approach for calculating the cost/benefit 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 versus
those that rely on interspecies H₂/formate transfer are important considerations when attempting
to 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 co-
culturing with a H₂-consuming partner (24), may fail to recover organisms that specialize in
DIET. Thus, new approaches for 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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References


32. Schink, B. 1984. Fermentation of 2,3-butanediol by Pelobacter carbinolicus sp. nov. and Pelobacter propionicus sp. nov., and evidence for propionate formation from C2 compounds. Archives of Microbiology 137:33-41.


**Figure legends**

**Figure 1.** Initial growth of co-cultures in ethanol-fumarate medium started with *P. carbinolicus* and different strains of *G. sulfurreducens*. The results are the mean and standard deviation of triplicate cultures.

**Figure 2.** Growth, ethanol metabolism, acetate accumulation, and succinate production from fumarate reduction after more than five consecutive transfers of co-cultures of *P. carbinolicus* with different strains of *G. sulfurreducens*. Also shown is the data from the initial attempt to start a co-culture with a strain of *G. sulfurreducens* unable to utilize formate or H₂. The results are the mean and standard deviation of triplicate cultures.

**Figure 3**

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

**Figure 4**

Growth on formate or H₂ in the presence of 1 mM acetate for *G. sulfurreducens* wild type (a), a strain deficient in a formate dehydrogenase subunit (b) or a strain deficient in both a formate dehydrogenase and an uptake hydrogenase subunit (c). In controls without added hydrogen or formate the acetate added as a carbon source could also serve as 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 mean and standard deviation of triplicate cultures.

**Figure 5**

Molecular analysis of co-cultures. (a) Relative transcript abundance of formate dehydrogenase ($fdnG$), hydrogenase ($hybA$) and the housekeeping gene, $RecA$, in $P$. carbinolicus/$G. sulfurreducens$ co-cultures as determined by RT-qPCR. Results are the mean and standard deviation for triplicate cultures. (b) Western blot analysis of OmcS in equivalent cell protein of $G. sulfureducens$ grown with fumarate as the electron acceptor or ethanol-fumarate co-cultures of $P. carbinolicus/G. sulfurreducens$ or $G. metallireducens/G. sulfurreducens$ co-cultures.
Strain of *Geobacter*:
- ○ Wild type
- × Formate dehydrogenase-deficient mutant
- ▲ Hydrogenase-deficient mutant
- ← Formate dehydrogenase and hydrogenase double mutant
- ◇ PilA-deficient mutant
- □ Omcs-deficient mutant
P. carbinolicus in co-culture with G. sulfurreducens:

- Wild type
- Hydrogenase mutant
- Formate dehydrogenase mutant

Graph showing relative transcript abundance (µg transcript/µg RNA) for fdnG, hybA, and recA.