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Syntrophy Goes Electric: Direct Interspecies Electron Transfer

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Abstract

Direct interspecies electron transfer (DIET) has biogeochemical significance, and practical applications that rely on DIET or DIET-based aspects of microbial physiology are growing. Mechanisms for DIET have primarily been studied in defined cocultures in which *Geobacter* species are one of the DIET partners. Electrically conductive pili (e-pili) can be an important electrical conduit for DIET. However, there may be instances in which electrical contacts are made between electron transport proteins associated with the outer membranes of the partners. Alternatively, DIET partners can plug into conductive carbon materials, such as granular activated carbon, carbon cloth, and biochar, for long-range electron exchange without the need for e-pili. Magnetite promotes DIET, possibly by acting as a substitute for outer-surface *c*-type cytochromes. DIET is the primary mode of interspecies electron exchange in some anaerobic digesters converting wastes to methane. Promoting DIET with conductive materials shows promise for stabilizing and accelerating methane production in digesters, permitting higher organic loading rates. Various lines of evidence suggest that DIET is important in terrestrial wetlands, which are an important source of atmospheric methane. DIET may also have a role in anaerobic methane oxidation coupled to sulfate reduction, an important control on methane releases. The finding that DIET can serve as the source of electrons for anaerobic photosynthesis further broadens its potential environmental significance. Microorganisms capable of DIET are good catalysts for several bioelectrochemical technologies and e-pili are a promising renewable source of electronic materials. The study of DIET is in its early stages, and additional investigation is required to better understand the diversity of microorganisms that are capable of DIET, the importance of DIET to carbon and electron flow in anaerobic environments, and the biochemistry and physiology of DIET.

Contents

INTRODUCTION

Electrically connected microbial communities (e-communities) that participate in direct interspecies electron transfer (DIET) are increasingly being recognized as important in diverse environments. Since the initial discovery of DIET (109), there has been a growing appreciation that those microorganisms able to establish biological electrical contacts with partners have unique advantages under some environmental conditions and that the biogeochemical impact of DIET may be substantial. DIET has now been detected in some methanogenic environments (28, 79, 94), has been proposed for anaerobic environments in which methane is oxidized (75, 123), and could be an important source of electrons supporting anaerobic photosynthesis (26). Furthermore, microorganisms that participate in DIET (DIETers) have served as the foundation for a number of novel biotechnologies.

Direct interspecies electron transfer (DIET): cell-to-cell transfer of electrons between species through shared physical electrical connections

Anaerobic photosynthesis: light-dependent carbon dioxide fixation in which oxygen is not an end product

DIETer: a microorganism capable of direct interspecies electron transfer; analogous to common terms such as sulfate reducer and iron reducer

DIET is an alternative to mediated interspecies electron transfer (MIET) (16), in which soluble electron carriers shuttle electrons between electron-donating and -accepting partners. The best-known form of MIET is interspecies hydrogen transfer (11), a concept that revolutionized the understanding of how methanogenic microbial communities function. Numerous syntrophic associations that rely on the transfer of hydrogen (or the hydrogen surrogate formate) have been identified (76, 106). Other soluble electron shuttles, such as cystine/cysteine and $S^{\circ}/S^{=}$ (33), or quinone/hydroquinone moieties (103), can support interspecies electron transfer in laboratory cultures, but the significance of these shuttles for MIET in more complex environments has yet to be demonstrated. For example, the moderate stimulation of propionate conversion to methane when cysteine was added to enrichment cultures initiated with rice paddy soil was suggested to provide evidence for cystine/cysteine functioning as an electron shuttle to methanogens (132). However, there were no studies with defined cocultures to validate this claim and cysteine can promote growth in enrichment cultures by other mechanisms, such as serving as a reductant.

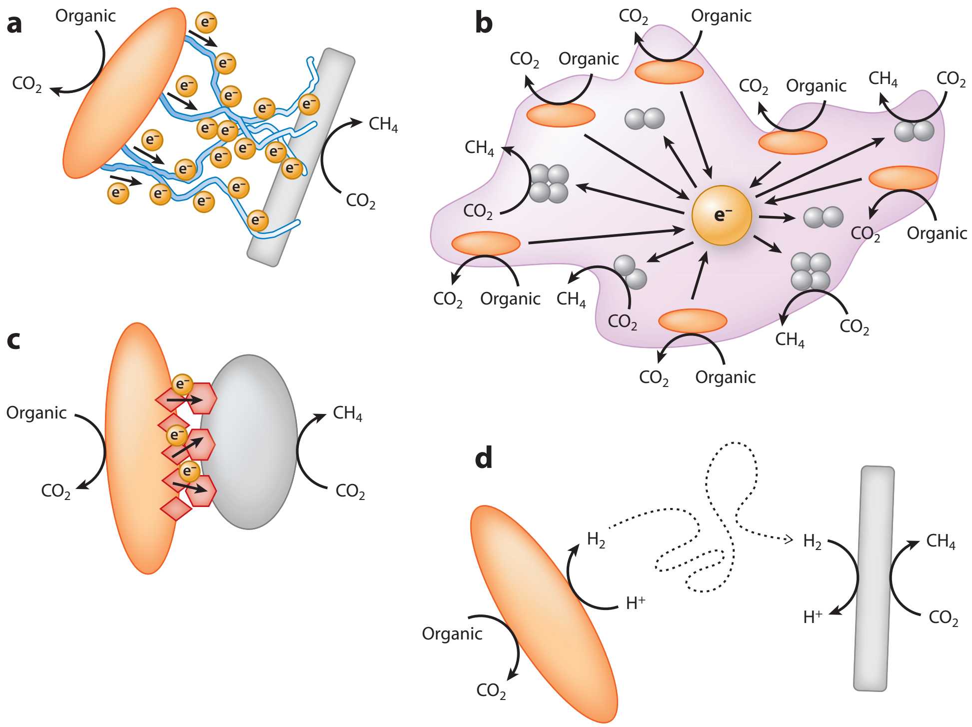

Figure 1

Mechanisms for interspecies electron transfer with emphasis on DIET. Potential DIET mechanisms include (*a*) electron transfer through electrically conductive pili (*blue*), (*b*) electron transfer through electrically conductive materials (*purple*), and (*c*) electron transfer between electron transport proteins (*red*) associated with outer cell surfaces. (*d*) DIET contrasts with the diffusive exchange of electrons between species through soluble electron shuttles such as H_2. In these examples the electron-accepting microbe is a methanogen.

It is equally important that claims for DIET, in either laboratory cultures or complex environments, be based on direct experimental evidence. Three potential strategies for DIET have been reported to date (**Figure 1**). As more is learned about the mechanisms for DIET, the microorganisms capable of DIET, and the gene expression patterns in e-communities, it may be possible to more readily detect DIET with metagenomic and metatranscriptomic analyses of natural communities. More information is also likely to lead to improved methods for recovering microorganisms capable of DIET in culture for more detailed investigations. The purpose of this review is to summarize the current understanding of DIET and to serve as a guide for such future studies.

Mediated interspecies electron transfer (MIET): a soluble molecule shuttles electrons between electron-donating and electron-accepting partners

DISCOVERY OF DIET

DIET has been definitively demonstrated only in defined cocultures in which one of the partners was a *Geobacter* species. The strongest evidence for DIET is available from cocultures of *G. metallireducens* and *G. sulfurreducens*. In initial studies, the *G. metallireducens/G. sulfurreducens* cocultures oxidized ethanol with the reduction of fumarate to succinate (109). The possibility of interspecies H_2 and formate was eliminated because cocultures initiated with mutant strains of *G.*

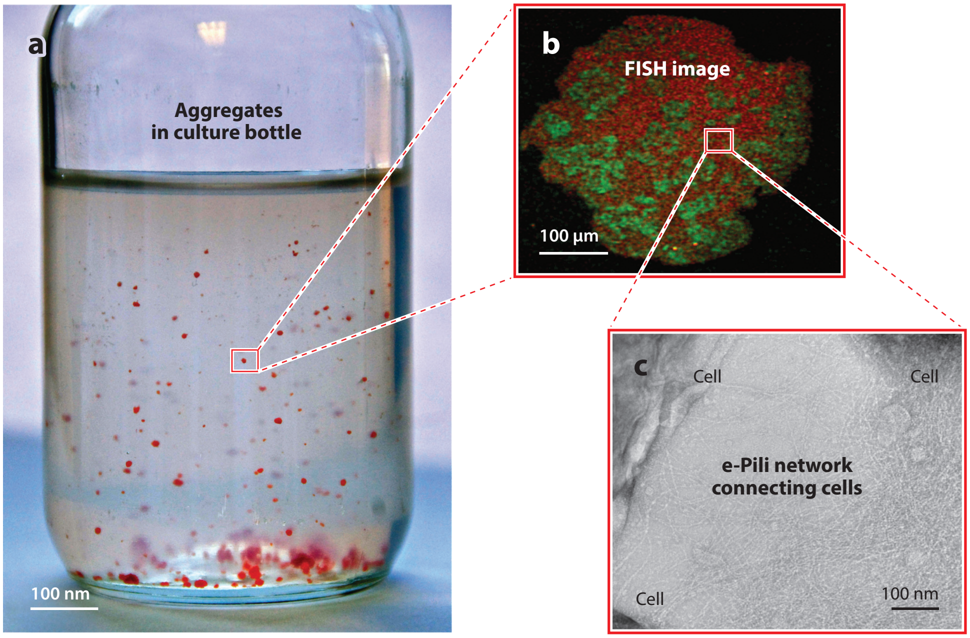

Figure 2

Aggregates formed in *Geobacter metallireducens/Geobacter sulfurreducens* cocultures growing via DIET. (*a*) Photograph of aggregates in culture; (*b*) confocal laser scanning micrograph of fluorescent in situ hybridization (FISH) of aggregate, with *G. metallireducens* stained green and *G. sulfurreducens* stained red; and (*c*) transmission electron micrograph of a portion of an aggregate. Panels *a* and *b* are from Reference 109. Panel *c* courtesy of Joy Ward, University of Massachusetts.

sulfurreducens that could not metabolize H_2 or formate continued to exchange electrons (92, 109). Furthermore, *G. metallireducens* is unable to conserve energy to support growth via H_2 production from ethanol (99), which also rules out H_2 as a potential electron carrier. Gene expression patterns in *G. sulfurreducens* grown with *G. metallireducens* were consistent with cells involved in extracellular electron exchange and inconsistent with growth on H_2 or formate (100).

Electrically conductive pili (e-pili): pili that have sufficient electrical conductivity to support physiologically relevant rates of extracellular electron exchange

Cocultures could not be established with mutant strains of *G. sulfurreducens* or *G. metallireducens* that lacked the ability to express electrically conductive pili (e-pili) or key outer-surface *c*-type cytochromes known to be required for extracellular electron transfer (100, 109). The requirement for e-pili ruled out electron transfer with other potential soluble electron shuttles because e-pili are not required for electron transfer to soluble extracellular electron acceptors (88, 103). For example, a *G. sulfurreducens* strain deficient in e-pili grew well in coculture with *Pelobacter carbinolicus*, a closely related microorganism that can participate in interspecies H_2 or formate transfer but not DIET (92). *G. metallireducens/G. sulfurreducens* cocultures formed large (1–2 mm) aggregates (**Figure 2**) with conductivities comparable to those in *G. sulfurreducens* biofilms that promote long-range electron transfer through thick (>50 μm) biofilms on electrodes (99, 109). The conductivity measurements provided a direct measurement of the potential for long-range interspecies electron exchange. These multiple lines of evidence ruled out soluble electron shuttles as interspecies electron carriers and are consistent with electron transfer between *G. metallireducens* and *G. sulfurreducens* through an electrically conductive matrix.

PROPOSED STRATEGIES FOR DIET

None of the three types of electrical connections that have been proposed for DIET (**Figure 1**) have been studied in great detail and mechanistic questions remain. It also seems likely that other DIET strategies remain to be discovered.

Pilin: the monomer protein that assembles into pili filaments in bacteria

Metallic-like conductivity: electron transfer through delocalized electrons rather than the electron hopping/tunneling typical of most biological electron transport

Electrically Conductive Pili

e-Pili are a requirement for DIET in several defined cocultures that include *G. sulfurreducens* and/or *G. metallireducens* (93, 94, 100, 109). Conductivity measured along the length of individual e-pili appears to be sufficient to account for observed rates of extracellular electron transfer (2, 111–113). Thick networks of e-pili connecting cells are apparent in *Geobacter* cocultures (**Figure 2**) and the cocultures could not be established with *Geobacter* strains that could not produce e-pili (93, 94, 100, 109). The flexibility and length of e-pili provide substantial opportunity for interspecies electrical connections. As detailed below, it seems likely that phylogenetically diverse microbes other than *Geobacter* species may produce e-pili, but their conductivity and/or role in DIET has yet to be verified.

The e-pili of *Geobacter sulfurreducens* are composed of a pilin monomer with only approximately a third of the amino acids found in the pilin proteins that are the building blocks for most type IV pili (27, 88). Models of the *G. sulfurreducens* pilus structure suggest that the truncation of the *G. sulfurreducens* pilin permits tighter packing of the monomers, bringing aromatic amino acids in close proximity (74, 124). These aromatic amino acids are essential for e-pili conductivity (2, 118). The influence of temperature and pH on conductivity (2, 66), as well as charge propagation along the pili (73), suggests that the *G. sulfurreducens* e-pili have a metallic-like conductivity that can be attributed to π-π stacking of the aromatic amino acids. The π-π stacking of the aromatics has been detected in structural studies, with a correlation between the degree of π-π stacking and conductivity of the pili (66, 73, 74). There is also a strong correlation between the abundance of ring structures in the e-pili of different *Geobacter* species and e-pili conductivity (113). Among the *Geobacter* species examined to date, there is nearly a million-fold range in pili conductivity (113), with some too poorly conductive to support DIET (112). These results emphasize the need for direct measurement of the conductivity of any pili before suggesting that pili observed in microbial communities are capable of supporting long-range electron transport.

Most research on e-pili function has focused on their role in long-range electron transfer through current-producing biofilms and in Fe(III) oxide reduction. These topics have been previously reviewed (62, 72) and will not be discussed in detail here. Alternative hypotheses have been proposed for e-pili conductivity (as discussed in 62, 72), but they lack supporting experimental evidence and will not be further detailed here other than to mention that the most recent studies of individual e-pili conductivity (2, 113) have further ruled out the possibility that cytochromes attached to the pili (45) play an important role in conductivity along the length of e-pili.

However, OmcS, the multiheme *c*-type cytochrome that is localized on *G. sulfurreducens* e-pili (45) (**Figure 3**), was required for DIET (109) as well as Fe(III) oxide reduction (77) and is thought to facilitate electron transfer between e-pili and other electron carriers (58, 60, 126). Growth via DIET selected for a mutation in a regulatory gene in *G. sulfurreducens* that resulted in much higher expression of OmcS (109). Initiating *G. metallireducens*/*G. sulfurreducens* cocultures with a strain of *G. sulfurreducens* with this mutation accelerated the initial growth of the cocultures (109). In a similar manner, cytochromes may be associated with *G. metallireducens* e-pili (102) and could be important for DIET (100).

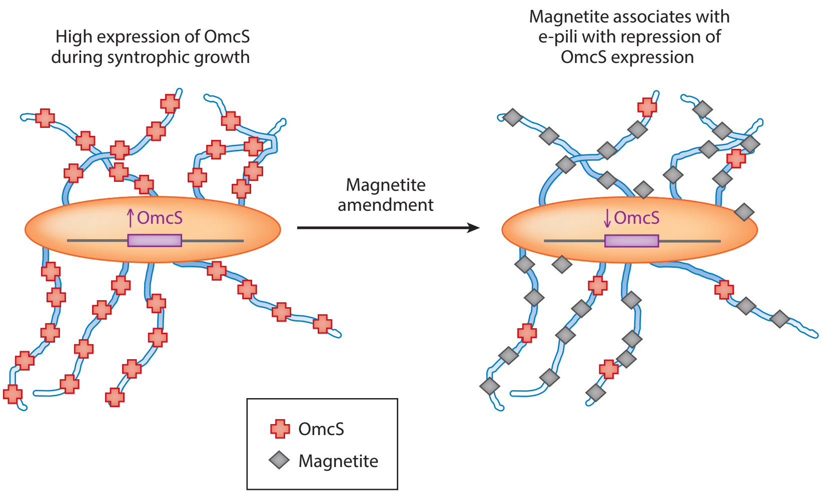

Figure 3

Differences in expression of OmcS and magnetite localization when magnetite is added to cocultures of *Geobacter metallireducens* and *Geobacter sulfurreducens* growing via DIET. Abbreviation: e-pili, electrically conductive pili.

Abiotic Conductive Conduits

A range of electrically conductive materials can either substitute for e-pili as the electrical connection between species or enhance the effectiveness of e-pili for interspecies electrical connections.

Magnetite. Magnetite stimulated methane production in enrichment cultures initiated with rice paddy soil (36). The enhanced methane production was associated with a greater abundance of *Geobacter* species that were proposed to use magnetite as a conduit for DIET to the *Methanosarcina* species that were also abundant (36). Subsequently defined coculture studies with *G. metallireducens* and *M. barkeri* confirmed that magnetite promotes DIET (114). Magnetite also stimulated DIET in defined cocultures of *G. sulfurreducens* and *Thiobacillus denitrificans* oxidizing acetate with the reduction of nitrate (35) and in *G. metallireducens/G. sulfurreducens* cocultures oxidizing ethanol with the reduction of fumarate (54).

Magnetite appears to function as a surrogate for the pili-associated cytochrome OmcS in *G. sulfurreducens* (**Figure 3**). Magnetite was preferentially associated with the e-pili, and *G. sulfurreducens* downregulated the expression of the OmcS gene in the presence of magnetite (54). A strain of *G. sulfurreducens* in which the OmcS gene was deleted was effective in DIET in the presence of magnetite (54) but not in its absence (109). In a similar manner, the OmcS-deficient strain was incapable of Fe(III) oxide reduction (77) but reduced Fe(III) oxide when magnetite was added to the culture (54). These results and a consideration of the electron transfer capabilities of magnetite suggested that magnetite was serving as a substitute for the OmcS cytochrome (54).

However, the more common illustration in papers discussing magnetite-mediated DIET is a chain of magnetite linking the DIET partners. There does not appear to be any direct evidence for such magnetite chains. Magnetite did not rescue a *G. metallireducens* strain that could not produce e-pili for growth in coculture with *G. sulfurreducens* (54), suggesting that the concept that magnetite can substitute for pili (35) may not be correct. Thus, the possibility of magnetite chains between cells facilitating DIET warrants further investigation.

Conductive carbon materials. Whereas magnetite particles are smaller than microbial cells and thus attach to individual cells, multiple partner cells attach to conductive carbon materials shown to facilitate DIET, such as granular activated carbon (53), biochar (15), and carbon cloth (14). There is no need for the microbial partners to be in close physical association when they are attached to these materials, as the connection with the conductive material is sufficient (**Figure 1*b***). Strains that could not express e-pili functioned in DIET as well as wild-type strains once they were in contact with the conductive carbon materials. Presumably, electrical contact between cytochromes associated with the outer cell surface and the conductive materials was sufficient for the interspecies electron exchange. Further investigation into which outer-surface electron transport proteins directly interact with the conductive materials is warranted.

Biological Electrical Connections at the Outer Cell Surface

The need for e-pili or abiotic conductive materials to serve as interspecies electrical connectors may be alleviated if cells can form tight connections between their outer surfaces (**Figures 1*c*, 4**). This possibility is evident in cocultures of *Prosthecochloris aestuarii* and *G. sulfurreducens* (26). *P. aestuarii*, an anaerobic phototroph, directly accepted electrons from electrodes or DIET to support photosynthesis (26). *P. aestuarii* and *G. sulfurreducens* formed tight associations with the two species in intimate contact (26). This is unlike cocultures of *G. metallireducens* with *G. sulfurreducens* (109), *Methanothrix harundinacea* (94), or *Methanosarcina barkeri* (93) as the electron-accepting partner, in which clusters of the two species are often segregated within separate zones of the aggregates. Large aggregates of *P. aestuarii*/*G. sulfurreducens* were not reported (26). Deletion of *G. sulfurreducens* genes for outer membrane porin-cytochrome systems required for extracellular electron transfer (97, 98) prevented growth of the coculture, consistent with DIET. These results do not necessarily rule out a role for e-pili, because extracellular electron transfer mediated by e-pili (88) also requires the porin-cytochrome network (44). Furthermore, pili that stained positive for heme appeared to be connecting some of the cells in the coculture (26). However, *G. sulfurreducens* closely associated with electrodes can perform cytochrome-dependent electron transfer to electrodes in the absence of e-pili (9, 55, 82, 89, 112, 118) and a similar type of electron transfer may take place in the *P. aestuarii*/*G. sulfurreducens* coculture. The multiheme outer-surface cytochrome OmcZ (31, 82) localizes at the cell-anode interface (30) and is essential for effective electron transfer to electrodes (90). Therefore, it will be of interest to determine whether OmcZ, which does not appear to be important for DIET in *G. sulfurreducens*/*G. metallireducens* cocultures (100), is an important component for DIET between *G. sulfurreducens* and *P. aestuarii*.

In some images, the cells of *P. aestuarii* and *G. sulfurreducens* appear to have merged, potentially through membrane fusions (26), which is another potential strategy for DIET (63). For example, *Desulfovibrio vulgaris* and *Clostridium acetobutylicum* growing in coculture fused to promote interspecies protein exchange, and it was hypothesized that an exchange of electron carrier proteins, such as ferredoxin, might facilitate DIET (7).

The abundance of genes encoding *c*-type cytochromes in the metagenome of anaerobic methane-oxidizing archaeal group 1 (ANME-1) methanotrophic archaea suggested that anaerobic methane oxidation coupled to sulfate reduction might proceed via DIET (78). A tight physical association of cells with dense heme-staining regions within consortia containing an ANME-2 anaerobic methane oxidizer and its sulfate-reducing partner was also suggestive of possible cytochrome-based electron transfer at the outer cell surface (75). Studies to evaluate the actual role of cytochromes in extracellular electron transfer to adjacent cells may soon be possible, if the ANME organisms can be cultured with alternative electron acceptors (23, 96, 115).

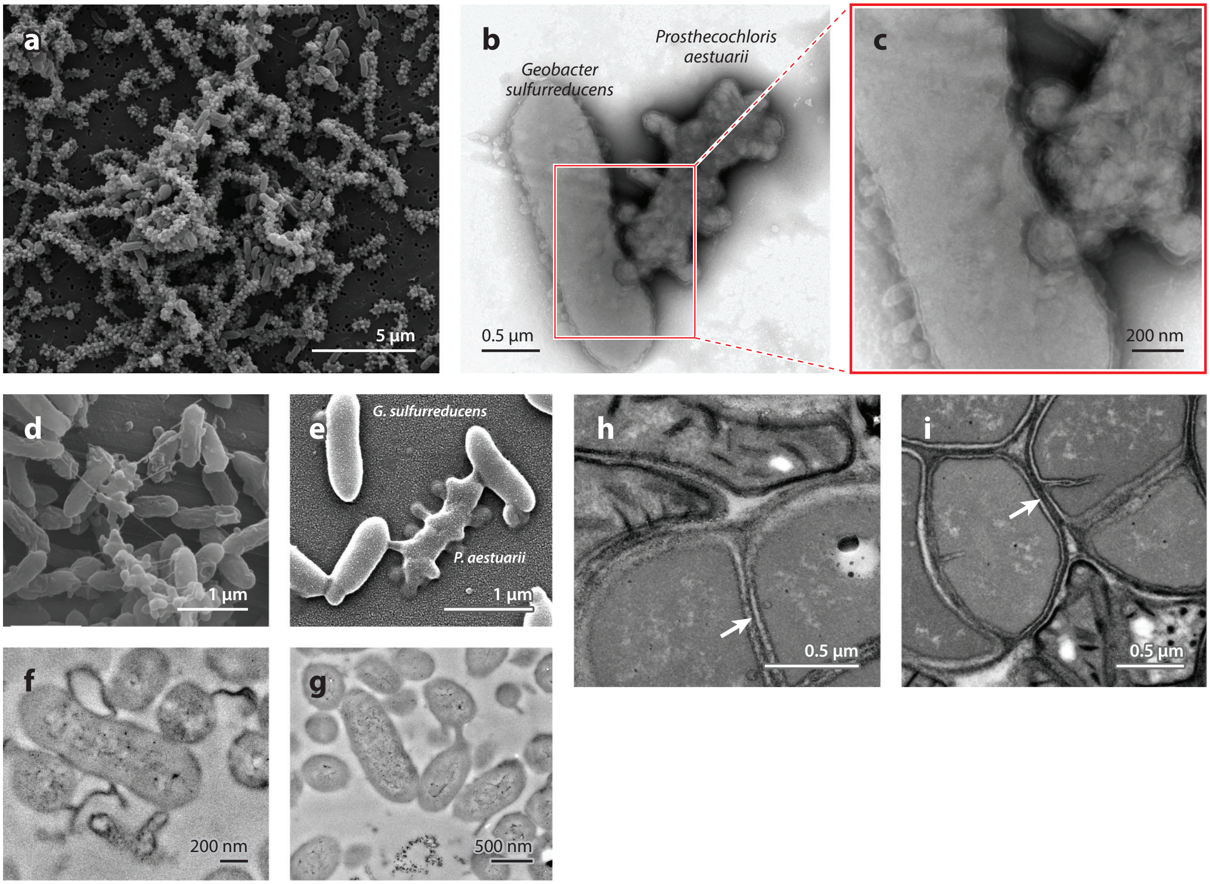

Figure 4

Examples of cells sharing electrons at the outer cell surfaces. Association of *Geobacter sulfurreducens* and *Prosthecochloris aestuarii* growing via light-dependent DIET seen in (*a*) a scanning electron micrograph and (*b*,*c*) transmission electron micrographs of the coculture. (*d*,*e*) Scanning electron micrographs demonstrating interconnections between the two species. Transmission electron micrographs of (*f*) heme-stained cells, revealing heme-containing pili, and (*g*) the control preparation without heme staining. (*h*,*i*) Transmission electron micrographs of heme-stained, anaerobic, methane-oxidizing consortia with arrows marking cell interfaces. Panels *a*–*g* are from Reference 26 and panels *h* and *i* from Reference 75.

DIET-BASED MICROBIAL COMMUNITIES

The identification of DIET-based microbial communities has not only provided an indication of the environmental significance of DIET but also suggested additional microbial systems for studying mechanisms for DIET. The number of DIET-based communities identified is growing steadily.

Anaerobic Digestion

The conversion of organic wastes to methane is an effective waste treatment method that yields the valuable product methane. The abundance of H_2-utilizing methanogens in many anaerobic digesters (38) is a strong indication of the importance of H_2 as an electron carrier in such systems. However, in some instances, methanogens that participate in DIET predominate (38, 79). Furthermore, DIET can be enhanced in digesters with the addition of conductive materials.

Upflow anaerobic sludge blanket (UASB) digester: a methanogenic system in which the waste influent enters the bottom of the digester

DIET without additives. The first indication of a DIET-based community outside the laboratory was in upflow anaerobic sludge blanket (UASB) digesters treating brewery waste in which ethanol was the primary substrate (79, 94, 101). Conditions in UASB reactors promote the growth of mixed-species cellular aggregates that share a gross morphology similar to that of the aggregates that form in *G. sulfurreducens/G. metallireducens* cocultures. Aggregates from the brewery digester were conductive, and in some instances more conductive than *G. sulfurreducens/G. metallireducens* aggregates (79, 101). The temperature dependence of the aggregate conductivity was consistent with metallic-like conductivity (79), similar to that found in *G. sulfurreducens* e-pili and biofilms (66). Analysis of multiple reactors at different times demonstrated a moderate correlation between aggregate conductivity and the abundance of *Geobacter* species in the aggregates, as determined with 16S rRNA gene analysis (101). There is a much stronger correlation between aggregate conductivity and the abundance of genes encoding e-pili (D.E. Holmes, personal communication).

More detailed studies were conducted on laboratory-scale digesters treating simulated brewery waste. Molecular analysis of the microbial community, first from 16S rRNA sequences (79) and then from the metagenome and metatranscriptome (94), revealed that *Geobacter* species were the most abundant bacteria and highly metabolically active. *Methanothrix* [formerly *Methanosaeta* (83)] species accounted for over 90% of the methanogens, and the aggregates had limited capacity to consume H_2 or formate, consistent with the inability of *Methanothrix* species to utilize these electron donors (79). However, *Methanothrix* species contain genes encoding enzymes for the reduction of carbon dioxide to methane and these genes were among the most highly expressed *Methanothrix* genes within the digesters (94). The potential for *Methanothrix* species to consume electrons derived from DIET with the reduction of carbon dioxide provided a possible explanation for how the community could consume the electrons released from ethanol metabolism (94). Studies with a defined coculture with *G. metallireducens* as a representative of an ethanol-metabolizing *Geobacter* species and *M. harundinacea*, a digester isolate, confirmed that *M. harundinacea* can reduce carbon dioxide to methane with electrons received from *G. metallireducens* via DIET (94). The e-pili of *G. metallireducens* were necessary for coculture growth. The electrical connection on *M. harundinacea* is yet to be determined.

M. barkeri, which is often an important methanogen in digesters (19), is also capable of accepting electrons via DIET from *G. metallireducens* (93). The availability of tools for genetic manipulation of *Methanosarcina* species (40) may make them the methanogens of choice for studies on the mechanisms by which methanogens can make electrical connections for DIET and conserve energy from carbon dioxide reduction with the electrons received via DIET.

A strategy for promoting DIET-based metabolism in digesters may be to initially feed digesters with ethanol to rapidly establish electrically connected communities (130). Reactors initiated with ethanol formed highly conductive aggregates and metabolized propionate and butyrate more rapidly than reactors not initially fed ethanol.

DIET promoted with conductive materials. Many studies have suggested that the addition of either conductive carbon materials or magnetite to promote DIET can stimulate and stabilize methane production in anaerobic digesters (4, 18, 48, 51, 64, 65, 120, 125, 128, 129). Magnetite also stimulated methane production in enrichment cultures initiated with anaerobic soils or sediments with butyrate (50, 127) or benzoate (131), substrates that are syntrophically metabolized in methanogenic digesters.

The strongest evidence for DIET in some of these studies has been a specific enrichment of *Geobacter* species or methanogens known to have the capability of DIET, in which these microorganisms were often specifically attached to the conductive materials (4, 5, 48, 64, 65, 120, 129). In other instances in which there has been no analysis of the microbial community, the inference of DIET is more speculative. In some studies, specific enrichment of bacteria or methanogens not previously shown to be capable of DIET, such as *Syntrophomonas*, *Thauera*, and *Methanospirillum* species, has been taken as evidence that species within these genera could be DIETers, but more detailed studies with defined cultures are warranted to verify this possibility (32, 49).

Although most DIET studies have focused on the metabolism of alcohols and short-chain fatty acids, it is possible that in the presence of conductive materials, some fermentative microorganisms also directly transfer electrons to methanogens. This would be an alternative to releasing H_2 from fermentation. For example, carbon cloth helped to stabilize methane production from complex organic waste at high loading rates, with a change in the composition of the methanogen community suggesting that a significant decrease in H_2 production and an increase in DIET had occurred (18). This shift in the methanogen communities was associated with a substantial enrichment of *Sporanaerobacter* and *Enterococcus* species on the carbon cloth that was not observed on nonconductive polyester cloth in control reactors. These findings, and previous studies demonstrating that representative species of these genera are capable of extracellular electron transfer, suggest that *Sporanaerobacter* and *Enterococcus* species coupled the metabolism of fermentable substrates with DIET to methanogens through the carbon cloth (18). If so, then the potential importance of DIET may extend beyond the metabolism of simple alcohols and fatty acids documented with *Geobacter* species (122).

An analysis of multiple studies suggested that conductive materials stimulated anaerobic digestion less than would be expected from the results of studies with defined cocultures (16). This finding emphasizes the need for more study of the environmental factors that might limit DIET in complex environments. However, results to date do suggest that redesigning anaerobic reactors to include conductive material may accelerate anaerobic digestion and make systems more resilient to upsets due to high organic loading rates or toxic components. Although most studies have focused on the addition of conductive particles, incorporation of permanent conductive materials, such as carbon cloth, may be more economical and effective because it alleviates the need for continual additions of materials and anchors the attached DIET community within the digesters. A better understanding of DIET mechanisms can be expected to lead to enhanced reactor designs that favor DIET and further stabilize anaerobic digestion at high organic loading rates or in the presence of toxic components.

Methanogenic Soils

Geobacter species are among the most metabolically active bacteria in methanogenic rice paddy soils (29, 37), even though Fe(III) reduction can only be a minor pathway for electron flow in sediments in which methane production predominates. Metagenomics and metatranscriptomics were conducted in order to evaluate whether the *Geobacter* species might be surviving through DIET (28). *Geobacter* genes encoding e-pili and pili-localized cytochromes were abundant and actively transcribed. Abundant transcripts of *Geobacter* genes involved in central metabolism suggested that the *Geobacter* species were metabolically active (28), as were *Methanothrix* species, which are only known to reduce carbon dioxide to methane with electrons derived from DIET (94). *Methanothrix* genes encoding enzymes in the pathway for carbon dioxide reduction to methane were highly expressed, suggesting that the *Methanothrix* species were receiving electrons via DIET (28). These results suggest that *Geobacter* species remain active in Fe(III)-depleted rice paddy soils by metabolizing substrates with DIET to *Methanothrix* species and possibly other methanogens. *Methanothrix* species in arctic peat soils highly expressed the *mer* gene, which encodes a key enzyme in the carbon dioxide reduction pathway (117), suggesting that *Methanothrix* species might be participating in DIET in yet another type of important methane-producing terrestrial environment.

The ability of *Methanothrix* species to effectively utilize acetate and their abundance in many terrestrial methanogenic environments suggest that they may be responsible for more methane production than any other genera on Earth (104). Additional methane production from carbon dioxide reduction further increases the global methane output of *Methanothrix* species. Low-potential electrons derived from DIET may improve growth of *Methanothrix* species beyond that possible with acetate as the sole substrate (122), further contributing to *Methanothrix* activity in soils and sediments.

The same gene expression approach used to evaluate DIET in *Methanothrix* species could not confidently be applied to *Methanosarcina* species, the only other methanogens known to participate in DIET, because some species of *Methanosarcina* also have the ability to use H_2 as an electron donor. Additional studies are required to identify key genes involved in DIET to develop a transcriptional approach to diagnose DIET in the genus *Methanosarcina*. Although less abundant than *Geobacter* species, other microorganisms such as *Desulfobacterium*, *Deferribacter*, *Geoalkalibacter*, and *Desulfobacula* species also expressed genes for e-pili in the rice paddy sediments (28). The possibility that that these microorganisms might contribute to DIET warrants further investigation.

Anaerobic Methane Oxidation

It has been reported that two communities involved in anaerobic methane oxidation exchange electrons via DIET (75, 123). The proposed mechanisms for DIET in the two communities were fundamentally different. One investigation was on an enrichment of thermophiles operating at 60°C that was primarily composed of an ANME-1 methane-oxidizing archaeon, and its sulfate-reducing bacterial partner "*Candidatus* Desulfofervidus auxilii" (41, 123). Interspecies H_2 transfer was ruled out because the ANME-1 genome did not contain identifiable hydrogenases and the consortium produced H_2 only at very low rates (123). When anaerobic methane oxidation was inhibited with the addition of H_2, both the ANME-1 and "*Ca*. D. auxilii" downregulated the expression of genes for multiheme cytochromes, and "*Ca*. D. auxilii" also repressed expression of the gene for PilA, the type IV pilus monomer. Filaments resembling pili were abundant in the enrichment during active methane oxidation, and it was proposed that "*Ca*. D. auxilii" pili were the interspecies electrical connection. A similar method for DIET was proposed for an enrichment of anaerobically oxidizing butane (43).

Although it was suggested that the PilA sequence of "*Ca*. D. auxilii" was similar to the PilAs of *Geobacter* species that form e-pili (123), the "*Ca*. D. auxilii" sequence is in fact predicted to yield a much longer pilin protein. When the PilA of "*Ca*. D. auxilii" was heterologously expressed in *G. sulfurreducens*, it yielded pili that were poorly conductive (121). Thus, the hypothesis that "*Ca*. D. auxilii" produces conductive filaments should be revisited. Ideally, the conductivity of pili from the enrichment culture should be measured directly.

Close examination of the filament images in the thermophilic methane-oxidizing consortia (123) suggests that the filaments may be emanating from the ANME-1 cells. The archaealla of archaea have homology to type IV pili, and the structure of the archaealla of *Methanospirillum hungatei* suggests tight π-π packing of aromatic amino acids (87). This might yield a conductive filament. Thus, an alternative to be investigated is that the ANME-1 actually produces conductive filaments.

In contrast to the DIET through e-pili proposed for the thermophilic enrichment, a consortium dominated by an ANME-2 methane oxidizer and its sulfate-reducing partner was proposed to carry out the long-range exchange of electrons through a conductive matrix of *c*-type cytochromes (75). As noted above, the cells and outer surface cytochromes were physically arranged for potential cytochrome-based electron transfer between adjacent cells. However, even longer-range cytochrome-based electron transfer to nonadjacent cells was proposed, because the metabolic activity of the sulfate-reducing cells was independent of proximity to the ANME-2 cells (75). If it is assumed that the activity of the sulfate-reducing partner was limited by electron donor availability, then this observation is not consistent with a diffusible interspecies electron shuttle, because electron-accepting cells closer to the electron-donating cells should receive electrons faster.

An approach to examine the cytochrome-based conductive-matrix hypothesis might be to simply measure the conductivity of the aggregates, as has been done in studies documenting DIET in anaerobic digesters (79, 101) and defined cocultures (99, 109). This approach stems from previous studies on the conductivity of *G. sulfurreducens* and mixed-species biofilms (66, 67). Although indirect analysis of electron transport mechanisms with cyclic voltammetry initially suggested that long-range electron transport through such biofilms might be mediated by cytochromes (90, 108), subsequent studies demonstrated that there were too few cytochromes to form a conductive matrix and that parameters employed in modeling the cytochrome-based electron transfer were unrealistic (68, 70, 71). Therefore, documenting that microbial consortia can in fact produce a conductive cytochrome matrix would be an important advance in expanding the known mechanisms for DIET.

Anoxic Photosynthesis

The finding that *P. aestuarii*, a phototroph known for deriving electrons from reduced sulfur compounds to support photosynthesis, can also obtain electrons via DIET greatly expands the potential environmental significance of DIET (26). Light-dependent DIET may be particularly important in low-sulfur environments, such as freshwater lakes (26). Other anaerobic phototrophs can also accept electrons from electrodes (10, 12) and some cyanobacteria may express electrically conductive filaments (110), further broadening the possibility of phototrophs participating in DIET.

EXPANDING THE SEARCH FOR DIET

The number of microorganisms conclusively shown to function as DIETers without conductive materials as a mediator is quite limited: *G. sulfurreducens* (109), *G. metallireducens* (109), *Geobacter hydrogenophilus* (95), *M. harundinacea* (94), *M. barkeri* (93), and *P. aestuarii* (26). *T. denitrificans* was a DIETer in the presence of magnetite (35). The diversity of DIETers is likely to be much greater than this short list would suggest.

DIETers may not be readily recovered in culture with standard enrichment and isolation techniques, which typically favor the most rapidly growing members of microbial communities. During coenriching for both partners, the need to establish physical electrical connections for DIET adds a constraint that may limit fast initial growth, permitting consortia that rely on MIET to outcompete DIETers. Conditions that simulate UASB reactors by providing a low but constant source of electron donor and retaining aggregates while washing out free cells might provide one strategy for recovering consortia that contain microorganisms not yet recognized as being capable of DIET. Other approaches to recovery, based on the characteristics of known DIETers, are described below.

Enrichment on Electrodes or Minerals

Any microorganism that has the ability to interact with extracellular electron donors or acceptors theoretically has the potential to exchange electrons with other cells. Thus, screening culture collections of microorganisms known to have a capacity for extracellular electron transfer for their capacity for DIET is likely to uncover more DIETers. An excellent example was the observed progression from enrichment of *P. aestuarii* on electrodes inserted in a photosynthetic mat, to isolation, demonstration of the isolate's ability to accept electrons from an electrode, and then successful coculture of the *P. aestuarii* isolate in coculture with *G. sulfurreducens* (26).

Many microorganisms have been identified that can electrically interact with electrodes (39) and the conductivity of electrode biofilms composed of complex communities (42, 47, 67) suggests that there is a diversity of microorganisms capable of long-range electron transport. Acetate is commonly added as an electron donor to enrich for current-producing microbes, but electron donors such as ethanol, butyrate, or propionate, which are typical substrates for syntrophic metabolism, might better select for DIETers. A broader range of media compositions and electrochemical conditions may expand the diversity of known electroactive microorganisms recovered (20).

Identifying Microorganisms with Electrically Conductive Pili

The importance of e-pili for DIET involving *Geobacter* species suggests that other microorganisms with e-pili may also be capable of DIET. Other microorganisms, some phylogenetically distinct from *Geobacter* species, possess pilin genes with high homology to the pilin genes that yield e-pili in *Geobacter* species (27). Many of these microorganisms have already been shown to be capable of extracellular electron transfer to Fe(III) or electrodes and are prime candidates to be DIETers.

Other types of pili might also be electrically conductive. A strategy to rapidly screen for the potential of pilin monomer genes to yield conductive e-pili is to replace the native pilin gene in *G. sulfurreducens* with the pilin gene of interest and determine whether the heterologously expressed gene confers the capacity for *G. sulfurreducens* to produce high current densities (113, 121). This is a potential method for evaluating whether as-yet-uncultured or difficult-to-culture microorganisms have the potential to produce e-pili. Although the presence of e-pili is not a guarantee that a microbe is a DIETer, it does point to microorganisms potentially worthy of further investigation.

Direct evaluation of pili conductivity is another option. It has been suggested that several microorganisms, including *Aeromonas hydrophila* (13), *Acidithiobacillus ferrooxidans* (52), *Desulfovibrio desulfuricans* (21), and *Rhodopseudomonas palustris* (119), express electrically conductive filaments, based on measurements of conductivity either across the diameter or along the filament length. It does not appear that the ability of any of these organisms to exchange electrons with other species via DIET has been investigated. There is a wide diversity of other filaments in the microbial world that might be conductive (110, 121) and warrant investigation for their potential role in DIET.

Physical Association

Microorganisms involved in DIET are, by necessity, physically associated with their partners. For example, bacteria attached to *Methanothrix* or *Methanosarcina* species have a higher probability of being DIETers than other bacteria in the same environment. Methods that can recover physically associated partners, such as magneto–fluorescent in situ hybridization (FISH) (84, 116), with probes designed to retrieve known DIETer methanogens might reveal previously unrecognized bacterial DIETers. In a similar manner, probes that hybridize with bacteria known to be DIETers, such as *Geobacter* species, might recover previously unrecognized DIETer methanogens. When DIET is promoted with magnetite, magnetic separation of the microbial community associated with the magnetite (105) might provide a specific selection for the organisms involved in magnetite-mediated DIET. Other strategies for physically sorting out aggregates from environments in which DIET is possible, such as density gradient centrifugation or size-selective filters, might also be effective.

Bioelectrochemical technology: a process in which microorganisms electrically interact with an electrode for a practical application

DIET VERSUS MIET

The examples above demonstrate that there are some instances in which environmental conditions favor the development of e-communities. However, the extent of DIET in many environments is not known, because full information on which microorganisms may be capable of DIET and tools to measure rates of electron flux via DIET in complex environments are lacking. The factors that favor DIET versus MIET are also poorly understood. It is expected that once electrical connections between two species are established, DIET is a faster and more efficient route for electron transfer than MIET (57). The observation of DIET in brewery UASB reactors and in methane seeps suggests that the initial investment in producing e-pili and other biological electrical connections may be best rewarded in stable environments in which there is a steady supply of a narrow range of substrate(s) requiring syntrophic metabolism. DIET is unlikely to be found in environments with a boom and then bust in availability of organic substrates. Under these conditions, rapid evolution of H_2 may be the best strategy for a potential electron-donating partner to generate ATP. This is apparent in defined laboratory batch cultures fed ethanol. In cocultures with *G. sulfurreducens* as the electron-accepting partner, *P. carbinolicus*, which relies on interspecies H_2 transfer, grows much faster than *G. metallireducens*, which exchanges electrons through DIET (92). *P. carbinolicus* metabolizes ethanol to acetate so rapidly that *G. sulfurreducens* cannot keep up and acetate accumulates (92). In contrast, metabolism is more balanced between the partners in the slower *G. metallireducens/G. sulfurreducens* cocultures, thereby avoiding large acetate accumulations (99).

Several different strategies for modeling DIET have been developed as a step toward predicting DIET prevalence under different conditions (56, 81, 107, 120). However, a poor understanding of the mechanisms for DIET, especially how electrons are transferred into electron-accepting microorganisms, is currently limiting model effectiveness. Basic comparative data, such as the relative cell yields for DIET versus MIET, are not available and strategies for obtaining essential data on some important parameters have yet to be developed (16).

PRACTICAL APPLICATIONS DERIVED FROM DIET

In addition to the practical application of enhancing DIET-based communities to accelerate and stabilize anaerobic digestion described above, special capabilities of DIETers, and their cellular components, are being developed for new technologies. For example, numerous bioelectrochemical technologies are emerging from the ability of microorganisms to directly exchange electrons with electrodes (6, 61, 80, 91). Applications actively being investigated include the harvesting of electrical current from waste organic matter, bioremediation, microbial reduction of carbon dioxide to organic commodities with electrons derived from electrodes, sensors, and biological computing. Most of these technologies are still in the early stages of development and face substantial challenges for commercial application. Although it is often pointed out that the study of microbial interactions with electrodes dates back over 100 years (85, 86), most bioelectrochemical technologies rely on direct electron transfer between electrodes and microbes, a much more recent discovery (8, 9, 24). A number of environmental selective pressures may have conferred properties that favor interaction with electrodes (59), but adaption for DIET might be the best explanation (95).

Thus, understanding DIET mechanisms can lead to improvements in bioelectrochemical technologies. For example, genetically modifying *G. sulfurreducens* to produce more e-pili led to anode biofilms that were more conductive and produced higher current (46). Designing electrodes that function more like the outer surface of DIETers, such as incorporating e-pili analogs, might further improve microbe-electrode transfer (34).

e-Pili show promise as sustainable electronic materials. With modifications of e-pili amino acid composition, their conductivities can be tuned over a wide range, with the highest conductivities rivaling that of carbon nanotubes of similar diameter (2, 111, 113, 118). e-Pili have some advantages over carbon nanotubes and other synthetic nanowires, including simple, energy-efficient mass production; no harsh chemical synthesis; no rare metal requirement; no toxic components in the final product; and production from inexpensive, renewable feedstocks, such as acetate. Although e-pili are composed of protein, they are very robust and stable over a wide pH range in water as well as in a diversity of organic solvents. An advantage of e-pili is that functionalization to provide binding sites for metals, chemicals, or biologics is readily feasible with genetic modification of the pilus monomer. e-Pili have transistor properties (1), which suggests that they may be ideal for nanowire sensor development. It is also likely that e-pili can be incorporated into polymers to provide novel electronic functional materials. Understanding the key features of e-pili conductivity may aid in the design of biomimetic peptide materials that assemble in vitro (3, 17, 25).

Conductive biofilms derived from DIETers may also be useful materials. For example, *G. sulfurreducens* biofilms function as supercapacitors, due to their high cytochrome content (22, 69).

FUTURE DIRECTIONS

The study of DIET is in its infancy. The possibility of DIET or its significance to carbon and electron flow has as yet to be investigated in many environments in which DIET may be important. Few pure culture isolates have been evaluated to determine whether they might be DIETers and no studies have been reported in which microorganisms have been recovered in culture with DIET as the enrichment/isolation procedure. Only a few details are known about potential electron transport components for DIET, and although several studies have demonstrated that growth is possible with electrons from DIET as the sole electron donor (99, 122), the mechanisms for energy conservation have yet to be explored.

Further research on DIET is warranted not only to understand the physiology and ecology of this process and its biogeochemical impact but also because of the potential practical spin-offs. Elucidating the mechanisms for cell-to-cell electron transfer is likely to lead to new electrode designs that provide better electrical contacts for microbe-electrode electron exchange, aiding the development of bioelectrochemical technologies. As already apparent from the initial studies of *Geobacter* e-pili, biological components involved in DIET may provide new electronic materials or new concepts for the design of biomimetic materials.

In short, the research possibilities are enormous. The study of DIET is likely to be a central component of investigations in the field of electromicrobiology for some time to come.

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