Graphical Abstract

Improving Anaerobic Digestion via Direct Interspecies Electron Transfer Requires Development of Suitable Characterization Methods

Highlights

- Many recent AD studies mistakenly link performance improvements exclusively to DIET.
- Unequivocally proving the occurrence of DIET requires a complex set of methods.
- This paper groups DIET characterization methods in four categories and discusses their strengths and weaknesses.
- This paper presents promising DIET detection approaches that merit further exploration.

Improving Anaerobic Digestion via Direct Interspecies

Electron Transfer Requires Development of Suitable

Characterization Methods

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Abstract

Recent anaerobic digestion studies commonly attribute performance improvements (e.g., increased methane production, enhanced process stability, reduced startup times) to direct interspecies electron transfer (DIET), even though only indirect evidence of DIET is available and DIET alone does not explain enhanced performance in many cases. This review evaluates methods believed to confirm the occurrence of DIET in anaerobic systems. 16S rRNA gene

sequencing and meta-omics approaches are necessary to further DIET knowledge but are limited in their ability to confirm the occurrence of DIET. *In situ* use of cyclic voltammetry should be explored further, as well as microscopy and image analysis procedures to quantify stained cytochromes. Furthermore, linking interspecies distance, interspecies mixing, and cellular activity to a DIET-based electron transfer model is promising but needs further validation for anaerobic digestion systems. In short, a combination of methods is necessary to confirm the occurrence and expand our knowledge of DIET.

Introduction

Shortly after the suggestion of the importance of direct interspecies electron transfer (DIET) in anaerobic microbial communities [1,2] and its discovery in a co-culture in 2010 [3], researchers started to evaluate strategies to promote DIET with the aim of enhancing methane production and promoting stability in anaerobic digestion (AD) systems. During AD, complex communities of microorganisms mediate a series of biochemical reactions (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) to convert organic compounds in waste streams to biogas (a renewable energy source). Before the discovery of DIET, acetogens and methanogens were thought to transfer reducing equivalents through chemical intermediates such as hydrogen (H_2) and formate (recently referred to as mediated interspecies electron transfer, MIET [4]). It is now clear that some microorganisms, when in direct contact with each other, can exchange electrons using electron transport proteins such as c-type cytochromes located in their outer cell surface [5,6]. By producing electrically conductive pili [7,8] or through the use of abiotic conductive materials, some microorganisms also perform extracellular electron transfer (EET) over distances on the centimeter scale [7].

Recent AD studies intended to exploit the enhanced electron exchange associated with DIET for the treatment of organic waste streams and have reported improved startup times, performance stability, and methane production rates when promoting DIET [9-11]. While many studies attribute improvements in methane production to DIET, often only indirect evidence of DIET is available [12,13] and DIET alone does not explain enhanced AD performance in many cases [14]. Since it is challenging to unequivocally evaluate whether DIET takes place in AD systems [12,15], it is critical to develop and use appropriate monitoring methods to advance the field. Although several recent reviews have focused on the mechanisms behind DIET

[4,8,12,16-18], no clear method or combination of methods has been presented to directly evaluate whether DIET takes place in real AD systems.

This review presents and critically assesses methods believed to be able to confirm the occurrence of DIET. Methods are classified according to the DIET characteristics they aim to observe: species, genes, transcripts, or proteins; electrical properties; morphological characteristics and spatial distribution; and metabolic pathways. Relevant DIET principles are only discussed as they relate to the methods reviewed since in-depth discussions of different DIET mechanisms are available in several recent reviews [4,8,12,18]. Our focus on monitoring methods is motivated by the urgent need to carefully evaluate the potential of DIET enhancement strategies in real AD systems to help inform researchers and practitioners when to exploit DIET to boost AD.

Detection of species, genes, transcripts, or proteins

Some studies solely rely on species identification through 16S rRNA gene sequencing to suggest DIET takes place in anaerobic digesters [19,20]. However, current DIET knowledge is too limited to suggest that the co-occurrence of certain populations in one system indicates DIET takes place. As opposed to microorganisms in co-cultures that have unequivocally been demonstrated to be involved in DIET through the use of gene deletion and substrate elimination experiments [3,5,21], most microorganisms linked to DIET (e.g., listed in [18,22]) are also able to perform MIET. Additionally, partial 16S rRNA gene sequencing often does not provide the resolution needed to identify populations below the taxonomic order or family levels and is thus limited in suggesting metabolic function. For instance, phylogenetically related species within the order *Desulfuromonadales* utilize different electron exchange pathways [23]. Similarly, archaeal community structure determined by 16S rRNA gene sequencing has been found to not

always change upon DIET promotion [24]. Finally, it is unlikely that all microorganisms capable of DIET have been identified. Until 2018, *Geobacter* spp. were the only bacteria demonstrated to participate in DIET with methanogens. However, several anaerobic digesters utilizing conductive media to promote DIET primarily contained bacteria other than *Geobacter* spp. [19,20,25-27]. Furthermore, heterologous expression of pili genes from other bacteria into *Geobacter sulfurreducens* yielded conductive pili [28]. While some studies utilized preferential growth on conductive materials to suggest microorganisms likely participated in DIET (e.g., *Sporanaerobacter* [19] and *Syntrophomonas* [20]), the only bacterium outside of the *Geobacter* genus proven to participate in DIET through co-culture experiments is *Syntrophus aciditrophicus* [29]. The expectation that DIET will be found within other phylogenetic groups [28,29] is consistent with the recently discovered widespread occurrence of EET in a variety of environments (e.g., freshwater lakes [30], mammalian gut [31], marine sediments [32], and others [33,34]).

Detection of functional genes, transcripts, or proteins through (meta-)omic approaches can potentially identify whether DIET pathways are employed. A first strategy involves targeting DIET associated genes/transcripts/proteins (Table 1). Several studies identified genes essential for DIET in co-cultures by evaluating the ability of the co-cultures to still grow after gene deletion, including the deletion of *PilA* [3,5,6,11], Gmet_2896 [5], Gmet_1868 [6], and *OmcS* [3] genes. Shrestha et al. [5] observed upregulation of the *OmcT* gene in a DIET-performing co-culture relative to a MIET-performing co-culture. Other genes/transcripts/proteins in Table 1 have been linked to DIET by monitoring their up/down regulation or expression upon DIET promotion [13,35,36]. However, some of the genes/transcripts/proteins listed in Table 1 can be involved with other cell functions [37], precluding them as unique identifiers for DIET. Additionally, some genes/transcripts/proteins are only associated with specific DIET pathways

and the absence of such genes/transcripts/proteins does not imply an absence of DIET itself. For example, it has been hypothesized that genes associated with pili formation (e.g., *PilA* and *OmcS*) are downregulated when conductive materials are provided by allowing DIET over long(er) distances without the need for pili [13,36]. Furthermore, our knowledge regarding DIET mechanisms is still rapidly expanding [38-40]. An early study already noted minimal differences between both *PilA* gene expression and protein abundance in co-cultures interacting through DIET and MIET [5], and it has recently been demonstrated that pilus-free *Geobacter metallireducens* and *G. sulfurreducens* can still perform DIET [39], hypothetically using cytochromes alone. Conversely, the same pilus-free microorganisms were not able to grow syntrophically in another study but only grew in the presence of granular activated carbon (though not with magnetite) [40], suggesting that cytochromes are not always sufficient for DIET. Furthermore, not all cytochromes are essential to DIET, as the expressions of several outer surface cytochromes (e.g., *OmcZ*) were downregulated in a DIET-performing co-culture [5].

A second strategy involves detection of carbon dioxide (CO₂) reduction genes in *Methanothrix* spp. (formerly *Methanosaeta*). The full set of genes necessary for CO₂ reduction was found in several *Methanothrix* spp. even though *Methanothrix* spp. are acetoclastic methanogens and cannot utilize H₂ or formate as electron donors (typical electron donors for CO₂ reduction during hydrogenotrophic methanogenesis). Using a *G. metallireducens*-*Methanothrix harundinaceae* co-culture, Rotaru et al. [11] demonstrated that *M. harundinaceae* reduces CO₂ by accepting electrons through DIET. Thus, *Methanothrix* spp. are thought to only reduce CO₂ through DIET and the transcription of CO₂ reduction genes in *Methanothrix* spp. is used as direct proof that DIET is occurring [36,41]. Since *Methanothrix* is not consistently present as the dominant methanogen in AD systems [24,27], the applicability of this DIET-detection strategy is limited.

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132 A third strategy targets the detection of MIET genes/transcripts/proteins to infer DIET is not
133 taking place. Semenec et al. [21], for instance, noticed upregulation of HybA (uptake
134 hydrogenase protein critical to interspecies H₂ transfer) and downregulation of c-type
135 cytochrome proteins (OmcC, MacA, OmcS, PgcA) in subsequent co-culture transfers as
136 microbial communities adapted to syntrophic growth. They concluded that DIET was
137 particularly important during the initial transitory phase from pure culture growth to co-culture
138 growth. Similarly, Shrestha et al. [5] observed reduced expression of genes associated with the
139 use of H₂ and formate as electron donors in a DIET-performing co-culture relative to a MIET-
140 performing co-culture. Nevertheless, microorganisms that exhibit expression of H₂ transfer
141 genes/transcripts/proteins might still participate in DIET through the CO₂ reduction pathway.
142 If *ex situ* substrate assays indicate a microbial community is not exhibiting H₂-based
143 methanogenesis but meta-omics methods suggest H₂ transfer genes/transcripts/proteins are
144 expressed, it is possible that methanogens are involved in DIET via the CO₂ reduction pathway
145 [35].

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Table 1: Genes (*italicized*), transcripts (*in bold*), and proteins (*underlined*) associated with DIET and MIET. Only *Geobacter* spp. and *Syntrophus aciditrophicus* have been demonstrated to participate in DIET, so all other electron donating microorganisms listed are putatively involved.

Associated with DIET				
Function	<i>Genes/Transcripts/Proteins</i>	Electron donating microorganisms	Electron accepting microorganisms	System for each reference
E-pili structural protein	<i>PilA</i> [3,5,6,36] PilA [5,11,13] <i>PilA</i> [5]	<i>Geobacter</i> spp. [36] <i>Geobacter metallireducens</i> [5,6,11] <i>Thauera</i> spp. [36] <i>Syntrophus</i> spp. [36] <i>Pseudomonas</i> spp. [36]	<i>G. sulfurreducens</i> [3,5]	[36]: Mixed AD culture [6]: <i>G. metallireducens</i> – <i>Methanosarcina barkeri</i> co-culture [3]: <i>G. metallireducens</i> - <i>G. sulfurreducens</i> co-culture with and without gene-deletion (Δ OmcS, Δ PilA, Δ hyb) [11]: Mixed AD culture and <i>G. metallireducens</i> – <i>Methanoxanthus harundinacea</i> co-culture [41]: Mixed rice paddy soil culture [5]: <i>G. metallireducens</i> - <i>G. sulfurreducens</i> and <i>P. carbinolicus</i> - <i>G. sulfurreducens</i> co-cultures with and without gene-deletion (Δ PilA, Δ Gmet 2896) [35]: Mixed AD culture [21]: <i>G. sulfurreducens</i> – <i>Pseudomonas aeruginosa</i> co-cultures with and without gene-deletion (Δ phz, Δ OmcZ, Δ OmcS) [13]: Mixed AD culture
		Unassigned [13,41]		
E-pili accessory proteins	pilB, pilC, pilM, pilQ [13,41]	<i>Geobacter</i> spp. [41], Unassigned [13]		
	pilA-C, pilD, pile, pilN, pilO, pilR, pilS, pilT, pilV, pilW, pilY	<i>Geobacter</i> spp. [41]		
	Unspecified <i>genes</i> and transcripts		<i>Desulfobacula</i> , <i>Desulfobacterium</i> , <i>Deferribacter</i> , <i>Geoalkalibacter</i> [41]	
c-type cytochromes	<i>OmcS</i> [3], OmcS [5,41], <i>OmcS</i> [3,21] <i>OmcZ</i> [21] <i>Gmet</i> 2896 [5] <i>Gmet</i> 1868 [6] <i>OmcT</i> [5] <i>OmcC</i> , <i>MacA</i> , <i>PgcA</i> [21] Unspecified transcripts [13]	<i>G. metallireducens</i> [5,6]	<i>G. sulfurreducens</i> [3,5,21,41]	
		Unassigned [13,36]		
CO ₂ reduction pathway	<i>Pathway genes</i> [36] Pathway transcripts [11,13] fmd, ftr, mch, mtd, mer, frh [41]		<i>Methanoxanthus</i> spp. [11,36,41]	
		Unassigned [13]		
Acetate decarboxylation pathway	<i>Pathway genes</i> [36] acs, cdh [41] Pathway transcripts [13]		<i>Methanoxanthus</i> spp. [36,41]	
		Unassigned [13]		
Associated with MIET				
H ₂ transfer	<i>hyb</i> [3] hyb, hyp, hya, hox, mvh, hdr, and ehr [5]		<i>G. sulfurreducens</i> [3,5] <i>Methanosarcina</i> spp. [35]	
Formate transfer	Formate dehydrogenase transcripts		<i>G. sulfurreducens</i> [5]	

While meta-omics methods are necessary to identify DIET target genes, it is unclear if such genes will be found. The transcriptome of *Methanosarcina barkeri* was recently shown to differ during DIET versus MIET growth [42], which aligns with the expectation that cells produce specific cytochromes/proteins to allow electrons to traverse impermeable and electrically nonconductive cell envelopes [17]. However, universal DIET redox-active proteins may not exist. For instance, some microorganisms are known to use different sets of c-type cytochromes for EET [16], and various *Geobacter* spp. have been found to utilize different cytochromes [43].

Detection of electrical properties

Another set of methods aims to detect EET: cyclic voltammetry (CV) detects electron exchange between microorganisms and conductive materials, while conductivity measurements characterize the ability of biomass to conduct electrons. CV characterizes electron exchange between microorganisms and electrodes by determining the standard EET rate constant (k_{app}) [24,35,44] or the type of redox reactions occurring through electron transfer (with acetate oxidation and CO₂ reduction peaks at -0.35 V [45] and -0.32 V [46,47], respectively). The specific pathway through which microorganisms participate in DIET can differ from the one used in EET between cells and electrodes (e.g., in *G. sulfurreducens* different genes were found for cell-electrode and cell-mineral EET [48]). However, bacteria most commonly described to participate in DIET (i.e., *Geobacter* spp.) also perform EET with minerals and no microorganism capable of EET with electrodes but incapable of DIET has been identified to date. As such, an increased electron transfer rate upon DIET promotion signifies an increase in electro-active microorganisms likely capable of DIET. So far, CV has only been applied *ex situ*. Specifically, biomass from a system to be investigated for the occurrence of DIET is transferred into an electrochemical cell to allow biofilm formation on the electrode. A caveat is that such biofilm growth likely results in a shift in community structure, which thus may not be representative of the biomass in the system under investigation. In principle, CV could be

applied *in situ* by connecting the electrode with conductive materials often present in systems in which DIET is being promoted. Since CV cannot assess the effect of non-conductive media (necessary as controls when studying the effect of conductive materials [14,49]), electron transfer rates need to be evaluated as a function of time and directly linked with DIET-associated performance characteristics such as CH₄ production rates or system resilience upon disturbances. Yin et al. [35], for instance, correlated the difference in CH₄ production rates between sequencing batch reactors with and without conductive materials to the difference in k_{app} evaluated *ex situ* for biomass collected from both reactors. While the close correlation between performance and k_{app} over time is no definite proof of DIET, it supports causality. Since continuously polarized electrodes are hypothesized to promote DIET in bioelectrochemical cells [50,51], the effect of brief electric fields generated by using CV *in situ* should be characterized before applying this method.

Another increasingly common method involves characterizing biomass conductivity (or conductance). While biofilms typically act as insulators, biofilms involved in DIET can be conductive [7,15]. Bulk biomass conductivity in anaerobic digesters amended with conductive attachment media such as carbon cloth and granular activated carbon is often higher than the bulk biomass conductivity in the corresponding control reactors (9.77 $\mu\text{S}/\text{cm}$ versus 5.47 $\mu\text{S}/\text{cm}$ [52], $498.8 \pm 75.2 \mu\text{S}/\text{cm}$ versus $6.3 \pm 0.2 \mu\text{S}/\text{cm}$ [53], up to 27x increase [54]). The hypothesis that higher biomass conductivity is due to DIET was originally supported by a strong correlation between conductivity and pili protein abundance [7] or pilA expression [15]. However, biomass conductivity also was observed to increase upon the addition of non-conductive plastic materials [55], which would not promote DIET. Furthermore, biomass conductivity does not always increase upon DIET promotion [56]. Finally, biomass

conductivity depends on the applied redox potential [7,57], which should therefore be recorded and reported, though this is not standard practice.

Detection of cellular characteristics and spatial distribution

A third set of methods employs microscopy to visually assess characteristics and spatial distributions associated with DIET. As a first approach, components associated with DIET, such as pili [7], cytochromes [3,58-60], and redox mediators [61,62], can be visualized. While the visualization of pili was valuable when initially confirming the existence of DIET, recent discoveries show that the presence of pili is neither necessary [13,36,39] nor sufficient [5] for DIET. Alternatively, visualizing cytochromes in and around cells can provide insight in EET pathways [3,58-60]. It is no longer useful to solely stain specific c-type cytochromes (e.g., OmcS using antibodies [3]) as none have been conclusively linked with DIET. Instead, all cytochromes can be visualized using a 3,3'-diaminobenzidine stain, which reacts with redox active transition metal ions bound to heme groups [59]. This technique was used early on to associate the presence of heme groups with an EPS-bound mineral (UO₂-EPS) with which *Shewanella oneidensis* was extracellularly exchanging electrons [60]. More recent studies have used this technique to visualize heme-groups in cellular membranes [59], intracellular membrane invaginations [59], extracellular space [59], and filamentous structures bridging cells [58]. Since cytochromes can perform functions other than DIET and are often redundantly present [63], it is not sufficient to qualitatively evaluate the presence of cytochromes to confirm DIET. Rather, a robust quantitative image analysis procedure needs to be developed to confirm that (membrane-bound or extracellular) cytochrome concentrations increase upon DIET promotion. Furthermore, future research should include testing such cytochrome visualization methods in AD consortia since such applications have not been reported thus far. Scanning electrochemical microscopy is another visualization strategy, which not only focuses on

cytochromes but also on other electrochemical compounds, such as peptides, protein complexes, DNA, and enzymes by recording the electrical current response on conductive surfaces [61,62]. For example, Ren et al. [62] measured higher current peaks in cathode biofilms of a graphite-amended electrochemical cell than in those of non-graphite amended controls, suggesting higher concentrations of redox mediators or conductive small molecules, components essential to the occurrence of DIET. Given the complexity of current scanning electrochemical microscopy setups, this technology is currently only used *ex situ*.

A second microscopy strategy involves studying cellular spatial arrangements. For instance, transmission electron microscopy (TEM) [58] and fluorescence *in situ* hybridization (FISH) [64,65] are used to visualize juxtapositioning of syntrophic partners. While TEM relies on cell morphology for identification, FISH can identify syntrophic partners using oligonucleotide probes designed to target unique sequences in rRNA molecules. A related strategy involves using microscopy (e.g., scanning electron microscopy [9,10,66,67] or FISH [68]) to observe how closely cells are positioned to each other or to attachment media. The hypothesis here is that the observation of isolated cell growth on conductive attachment media indicates cell-media-cell DIET. Furthermore, scanning electron microscopy combined with energy dispersive X-ray analysis can ascertain if conductive nanoparticles (e.g., magnetite) are incorporated into cell aggregates, indicating the possibility of DIET via such conductive particles [13,69,70]. In addition to providing general spatial observations of microbial cells, microscopy can characterize distances between specific groups of microorganisms using techniques such as FISH [59,71]. McGlynn et al. [59] combined FISH with nanoscale secondary ion mass spectrometry and stable isotope probing and observed single cell metabolic activity to be independent of species intermixing or distance between syntrophic partners. The authors theorize that such a distance-independent trend of syntrophic partners is more likely to

correspond with DIET than diffusion-based syntrophy since the global electric potential for each consortium is highest upon spatial segregation of the participating cells, as confirmed by cellular activity models based on DIET. Since this study did not include a non-DIET control, further research is necessary to confirm that the same activity/distance trend is not observed for other syntrophic pathways. For instance, Felchner-Zwirello et al. [71] observed a random distribution of their syntrophic partners in an anaerobic digester in which DIET was not promoted (nor investigated). However, the (uncharacterized) cellular activity could still have been dependent on interspecies distance. Interestingly, Felchner-Zwirello et al. [71] observed the average interspecies distance to decrease with time, which, according to the polar charge separation theory, could correspond with a decreased occurrence of DIET. Such a time-dependent trend has been confirmed by proteomics in a syntrophic co-culture of *G. sulfurreducens* and *Pseudomonas aeruginosa* [21].

Metabolism evaluation

A final set of methods evaluates methanogenic metabolisms through substrate assays [72], carbon isotope labeling [13,73], and specific inhibition experiments [64,74]. Substrate assays aid the identification of active methanogenic pathways by performing *ex situ* batch experiments and monitoring acetate or H₂ consumption and methane production. Similar to metatranscriptomics based techniques [41], substrate assays denoting CO₂ reduction as the main methanogenic pathway can be used to infer DIET when *Methanothrix* is the primary active methanogen [72]. More broadly applicable information can be obtained from substrate assays when combined with carbon isotope labeling (i.e., monitoring the biogas isotope ratio of CH₄ and CO₂). When isotope labeling demonstrates increased CO₂ reduction while substrate assays do not show intensified H₂ consumption, the enhanced CO₂ reduction pathway is likely a result of DIET [13]. In the absence of such substrate assays, the use of isotope labeling can confirm

CO₂ reduction [73], which supports but does not prove DIET. A final set of methods determines how AD performance is affected when specific metabolisms are inhibited (e.g., acetoclastic [64], hydrogenotrophic [74], or overall methanogenesis [75]). For example, when AD is not affected by increasing H₂ partial pressures [74,76], it is likely to be occurring through DIET.

Conclusions and Future Work

Over the last decade, many AD studies have aimed to enhance methane production and promote performance stability through the stimulation of DIET. It is now apparent that performance improvements reported in these studies are not solely due to DIET. A complex set of methods is necessary to evaluate the potential of DIET enhancement strategies in real AD systems and not only suggest, but also confirm, the occurrence of DIET (Table 2). For instance, microbial community characterization through 16S rRNA gene sequencing cannot independently confirm DIET, but remains attractive to further our knowledge regarding DIET-performing microorganisms. Furthermore, the detection of genes/transcripts/proteins associated with DIET (e.g., H₂ and formate transfer) versus DIET (e.g., utilization of the CO₂ reduction pathway for *Methanothrix* spp.) using meta-omics approaches is helpful, but is best accompanied by other methods. For example, *ex situ* batch experiments can confirm that upregulation of H₂ transfer genes does not coincide with a concomitant increase in H₂ consumption. Another promising method entails the *in situ* use of CV to directly link performance characteristics with electron transfer rates over time. When performing CV, however, the possible effect of electric fields on DIET needs to be studied. Microscopy can provide conclusive proof of DIET in a few situations. Monitoring the abundance of cytochromes across TEM images is one such method, though it requires quantitative image analysis. The 3,30-diaminobenzidine stain, furthermore, is known to bind to cytochromes but has not yet been validated for AD systems. Additionally, scanning electrochemical microscopy can characterize biomass electric current responses, however,

presently only *ex situ*. The combination of FISH with nanoscale secondary ion mass spectrometry and stable isotope probing, on the other hand, can determine if the correlation between interspecies distance, interspecies mixing, and cellular activity corresponds to a DIET model versus a diffusion based MIET model. While this strategy has provided promising results, further research is necessary to confirm the previously identified distance-independent trend over time in DIET versus non-DIET promoted systems.

Table 2: DIET characterization methods organized according to their ability to detect DIET. The superscripts indicate the DIET characteristic they aim to detect. 1: species, genes, transcripts, and proteins, 2: electrical properties, 3: morphology and spatial distribution, 4: metabolic pathways. SECM = scanning electrochemical microscopy, FISH-nanoSIMS = fluorescence in situ hybridization nanoscale secondary ion mass spectrometry, TEM = transmission electron microscopy.

Methods requiring complementary tests	Methods that could detect DIET provided future development	Methods that provide inconclusive proof of DIET occurrence
Substrate assays ⁴	FISH-nanoSIMS combined with stable isotope labeling ³	16S rRNA gene sequencing ¹
Carbon isotopes ⁴	Cytochrome staining ³	Conductivity measurements ²
Inhibition tests ⁴	Cyclic voltammetry ²	Pili staining ³
Meta-omics approaches ¹	SECM ³	Standalone FISH or TEM ³

Acknowledgements

This work was supported by the U.S. National Science Foundation (Project CBET 1604069). CVS was supported by a University of Michigan (UM) Predoctoral Fellowship from the Rackham Graduate School and an Integrated Training in Microbial Systems (ITiMS) Fellowship. The ITiMS program “Instructional Program Unifying Population and Laboratory Based Sciences” at UM was funded by the Burroughs Wellcome Fund. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the funding sources.

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