Plugging in or going wireless: strategies for interspecies electron transfer

Pravin Malla Shrestha1,2* and Amelia-Elena Rotaru3

1 Department of Microbiology, University of Massachusetts, Amherst, MA, USA
2 Energy Biosciences Institute, University of California, Berkeley, CA, USA
3 Nordic Center for Earth Evolution, University of Southern Denmark, Odense, Denmark

INTRODUCTION

Interspecies electron exchange plays a key role in the functioning of methane-producing microbial communities, which have a significant impact on the global carbon cycle (Stams and Plugge, 2009; Sieber et al., 2012). Organic matter mineralization to methane by microbial processes contributes to 69% of the atmospheric CH4 (Conrad, 2009) and it involves four major steps (Figure 1A):

1. Hydrolytic bacteria break down complex compounds such as polysaccharides, proteins, nucleic acids, and lipids to monomeric substances (Schink and Stams, 2013), (2) Primary fermenters convert monomeric substances to H2/formate, CO2 and small organic molecules such as lactate, succinate, fatty acids, and acetate (Morris et al., 2013; Schink and Stams, 2013), (3) Syntrophic bacteria carry out secondary fermentation of small organic molecules to produce acetate, formate, H2 and CO2 (Morris et al., 2013; Schink and Stams, 2013), or releases electrons for direct electric connections (Summers et al., 2010; Rotaru et al., 2014), (4) Methanogenic Archaea uses electrons from H2/formate/shuttles or directly to reduce CO2 to CH4 (Morris et al., 2013; Rotaru et al., 2014; Sieber et al., 2014).

Interspecies electron transfer via H2/formate has been extensively reviewed in recent years (Morris et al., 2013; Schink and Stams, 2013; Sieber et al., 2014). Besides, H2/formate, there are many important mechanisms of interspecies electron transfer reported, which include but are not limited to pili mediated direct interspecies electron transfer (DIET; Summers et al., 2010; Morita et al., 2011; Nagarajan et al., 2013; Shrestha et al., 2013a;b; Rotaru et al., 2014) and mineral mediated direct interspecies electron transfer (Kato et al., 2012a;b; Liu et al., 2012, 2014; Chen et al., 2014), or by shuttle molecules like cysteine (Kaden et al., 2002), sulfur compounds (Biebl and Pfennig, 1978; Milucka et al., 2012), and humics (Lovley et al., 1999; Liu et al., 2012). This review discusses recent findings on interspecies electron transfer during syntrophic interactions, with the main focus on DIET mechanisms.

H2 AND FORMATE AS ELECTRON TRANSFER MOLECULES

H2 and formate are important electron transfer molecules that are reported in various methanogenic environments (Schink and Stams, 2006, 2013; Stams and Plugge, 2009), and these are described briefly under separate headings below:

H2 AS ELECTRON TRANSFER MOLECULE

Interspecies electron transfer via H2 was first demonstrated almost four decades ago in a defined co-culture (Bryant et al., 1967) of the “S organism,” which converted ethanol to acetate and H2, only in the presence of Methanobacterium ruminantium, which consumed H2 for the reduction of CO2 to CH4 (Bryant et al., 1967). H2 is a very powerful electron donor under anoxic conditions and must be continuously removed by partner organism in order for the syntrophic interaction to take place (Nedwell and Banat, 1981; Lovley and Ferry, 1985; Kleerebezem et al., 1999; Wintemute and Silver, 2010). The generation of H2 is energetically unfavorable at H2 partial pressures above 10^-3 bar (Schink and Stams, 2013), however, syntrophic microorganisms bypass this energetic barrier by coupling the unfavorable H2 production with
the energetically favorable oxidation of a reduced compound like ferrodoxin (Figure 1B), a process known as electron confurcation (Schut and Adams, 2009; Sieber et al., 2010, 2012). Confurcating hydrogenases are found in the genomes of all H2 generating syntrophs described to date (Sieber et al., 2010, 2012).

**FORMATE AS ELECTRON TRANSFER MOLECULE**

Formate is an alternative to H2 and could also act as an electron carrier between syntrophic partners (Thiele and Zeikus, 1988; Boone et al., 1989; Hattori et al., 2001; de Bok et al., 2004; Stams et al., 2006; Stams and Plugge, 2009). The use of formate as an electron transfer molecule has been noticed especially in co-cultures thriving on proteins (Zindel et al., 1988) or fatty acids like propionate and butyrate (de Bok et al., 2004; Sousa et al., 2007). Certain communities might favor formate transfer because formate has ca. three times higher diffusion coefficient as compared to H2, and allows larger mass transfer to methanogens (Boone et al., 1989). It has been also reported that some syntrophic interactions uses both formate and H2 to transfer electrons between species (Boone et al., 1989; Dong and Stams, 1995; Stams et al., 2006; Rotaru et al., 2012). This dual mechanism of electron...
**FIGURE 2 | Examples of mechanisms of electron transfer.**

H₂ transfer between *P. carbinolicus* and *G. sulfurreducens* (A), bDIET between *G. metallireducens* and *G. sulfurreducens* (B), mineral mediated mDIET between *G. metallireducens* and *G. sulfurreducens* with nano-sized minerals (D) or GAC (E) in the presence of ethanol as the electron donor and fumarate as the electron acceptor. DIET in a co-culture of *G. metallireducens* and *Methanosaeta harudinacea* where ethanol was used as electron donor and CO₂ is reduced to CH₄ by *Methanosaeta* using electrons received directly from *G. metallireducens* via bDIET (C).
transfer using H₂ and formate (Figure 2A) has been studied in detail using deletion mutants, in a co-culture of Pelobacter carbinolicus and Geobacter sulfurreducens (Rotaru et al., 2012). For example, when a co-culture was established with a hydrogenase mutant (hybL) of G. sulfurreducens, the formate dehydrogenase (fdnG) gene of G. sulfurreducens was over-expressed (Rotaru et al., 2012).

**ELECTRON TRANSFER VIA SHUTTLE MOLECULES**

Electron shuttles are chemical compounds that facilitates the transfer of electrons to and from bacteria these may include sulfur compounds (Bielbl and Pfennig, 1978), humic substances (Lovley et al., 1996, 1998, 1999; Newman and Kolter, 2000), and flavins (Marsili et al., 2008; von Canstein et al., 2008; Brulinel and Granlinc, 2012), etc.

**SULFUR COMPOUNDS AS MEDIATORS FOR INTERSPECIES ELECTRON TRANSFER**

Sulfur compounds as shuttle were first discovered between green sulfur bacteria and sulfate-reducing bacteria (SRB; Bielbl and Pfennig, 1978), S(0) is converted to sulfide by a sulfate reducing bacteria and then recycled back to S(0) by a photosynthetic green-sulfur bacteria creating an interspecies S-cycle (Bielbl and Pfennig, 1978). The second discovered S-based interspecies interaction used cysteine as electron shuttle between G. sulfurreducens and Wolinella succinogenes, growing with acetate as electron donor and nitrate as electron acceptor (Raden et al., 2002). S-compounds were also found responsible for electron transfer between anaerobic methane oxidizing Archaea (ANME) and sulfate reducing bacteria (Boetius et al., 2000), which oxidizes methane with sulfate, one of the most studied, yet least understood interactions. The members of the anaerobic oxidation of methane consortia were initially thought to exchange electrons via methyl-sulfides (Moran et al., 2008), however, more recently the electron carrier within the consortium was revealed to be polysulfides (Milucka et al., 2012).

**HUMICS AND HUMICS EQUIVALENTS AS ELECTRON SHUTTLES**

Humic substances are ubiquitous in nature (Lovley et al., 1996; Bittner et al., 2007). The humic substance analog, anthraquinone disulphonate (AQDS) serves as an electron shuttle between G. metallireducens and G. sulfurreducens (Liu et al., 2012), or between G. metallireducens and W. succinogenes (Lovley et al., 1999). This came as no surprise because it is known that certain microorganisms can use AH₂QDS as electron donor (Lovley et al., 1999), while others use AQDS as electron acceptor (Lovley et al., 1996). However, AQDS cannot mediate electron transfer in G. metallireducens and M. barkeri co-cultures, likely because of the redox potential of the AQDS couple is too high to reduce carbon (E0' = −184 mV) to reduce carbon dioxide to methane (E0' = −240 mV; Liu et al., 2012).

**FLAVINS AS ELECTRON SHUTTLES**

Flavins were also noted to improve electron transfer to electrodes in Shewanella biofilms (Marsili et al., 2008; von Canstein et al., 2008; Brulinel and Granlinc, 2012) yet their impact on interspecies interactions remains to be reported.

**DIRECT INTERSPECIES ELECTRON TRANSFER**

To clearly distinguish between conductive mineral mediated DIET and direct cell contact DIET, we have subcategorized the pili mediated electron transfer, as biological DIET (bDIET), and the conductive mineral mediated DIET, as mineral DIET (mDIET).

**BIOLOGICAL DIET**

Biological DIET (Figures 2B,C) was first described in G. metallireducens and G. sulfurreducens co-cultures, growing in a defined minimal medium with ethanol as electron donor and fumarate as electron acceptor (Summers et al., 2010). Tightly associated aggregates were consistently noticed in co-cultures growing via bDIET (Summers et al., 2010; Shrestha et al., 2013a; Rotaru et al., 2014) but not during growth via H₂/formate electron transfer (Rotaru et al., 2012). The mechanism for bDIET in Geobacter co-cultures was intensely studied during the past few years, combining phenotypic, genetic, transcriptomics, proteomics analysis (Summers et al., 2010; Shrestha et al., 2013a,b). bDIET might be favored over H₂ or formate transfer under certain conditions (Lovley, 2011) as demonstrated using genome-scale models including genomic, transcriptomic and physiological data (Nagarajan et al., 2013). The absence of H₂/formate mediated electron transfer in the co-culture was best shown by the ability of G. metallireducens to generate successful syntrophic co-cultures with a double mutant of G. sulfurreducens (ΔhybLΔfdnG) incapable of H₂ or formate uptake (Rotaru et al., 2012). Furthermore, bDIET is seemingly capable to produce successful co-cultures in the absence of acetate transfer as supportive mechanism of electron exchange as revealed in a recent study (Shrestha et al., 2013a) in co-cultures of G. metallireducens with strain of G. sulfurreducens depleted in acetate utilization capacity, a citrate synthase mutant (ΔgltA; Ueki and Lovley, 2010). This study clearly revealed that bDIET alone is sufficient for energy conservation in syntrophic co-cultures.

Biological DIET interactions with fumarate as terminal electron acceptors are probably not ecologically relevant, but more recently bDIET was discovered in co-cultures of G. metallireducens with Methanoseta haradinaeae (Rotaru et al., 2014). These two genera of methanogens are responsible for the majority of methane emission in environments such as paddy soils (Grosskopf et al., 1998; Feng et al., 2013) or anaerobic digesters (Vavilin et al., 2008; Morita et al., 2011; Rotaru et al., 2014; Ying et al., 2014). Only these acetoclastic methanogens were capable of bDIET-interactions with G. metallireducens, whereas hydrogenotrophic methanogens were not (Rotaru et al., 2014). Methanoseta was shown to use electrons directly for the reduction of CO₂ to methane because the methanogen converted 1/3 of the 14C-bicarbonate to 14C methane (Rotaru et al., 2014). Other shuttles were excluded as electron transferring mechanisms because a pili-deficient G. metallireducens could not produce successful co-cultures with Methanoseta or Methanosarcina (Rotaru et al., 2014).

**Role of pili in bDIET**

Pili are known to have an important role in biofilm formation (Moreira et al., 2006; Reguera et al., 2007; Oxaran et al., 2011; Snider et al., 2012), but also for the conductive properties of Geobacter biofilms (Summers et al., 2010; Malvankar et al., 2012; Summers et al., 2010; Brutinel and Granlinc, 2012), etc.
et al., 2011; Malvankar and Lovley, 2012, 2014; Vargas et al., 2013). Co-cultures do not grow when initiated with a strain of either G. metallireducens (Summers et al., 2010) or G. sulfurreducens (Rotaru et al., 2014) in which the gene for PilA is deleted, confirming the importance of conductive pili (Reguera et al., 2005, 2006; Lovley, 2011; Malvankar et al., 2011) networks for bDIET. It has been proposed that the stacking of π–π orbitals of five aromatic amino-acids in the carboxyl-terminus of PilA, the pilin monomer, contribute to the metallic-like conductivity similar to that of conductive organic polymers (Vargas et al., 2013). A G. sulfurreducens strain deficient in the five aromatic amino acids (AROS), the pili were still produced with properly localized OmcS and yet the biofilms of ARO5 showed greatly diminished conductivity (Vargas et al., 2013). In another study, the gene for conductive pili in G. sulfurreducens was replaced with the non-conductive pilA gene of Pseudomonas aeruginosa PAO1 (Liu et al., 2013) generating a mutant strain PAO1, which can express properly assembled P. aeruginosa pili ornamented by outer surface c-type cytochromes. However, PAO1 biofilms had significantly lower conductivity than wild type G. sulfurreducens and was unable to reduce Fe^{3+}-oxides or produce current (Liu et al., 2013). The lack of conductivity in PAO1 biofilms indicates that three out of five aromatic amino acids at the C-terminus domain are necessary for conductivity (Liu et al., 2013). These findings validated that OmcS alone on scaffold-pili is insufficient to confer conductivity to Geobacter biofilms, in contrast to a recent hypothesis, which suggested that conductivity is the result of electron-hopping via cytochromes aligned on the pili of G. sulfurreducens (Strycharz-Glaven et al., 2011).

Role of cytochromes in bDIET

Geobacter sulfurreducens was used as model organism for the study of extracellular electron transfer, and several studies revealed that besides pili, G. sulfurreducens require a multitude of extracellular and periplasmic cytochromes for insoluble Fe^{3+} oxide reduction (Lloyd et al., 2003; Butler et al., 2004; Qian et al., 2007, 2011; Aklujkar et al., 2009; Lovley et al., 2011; Lovley, 2012), current production (Nevin et al., 2009; Inoue et al., 2010), or current uptake on electrodes (Holmes et al., 2006; Strycharz et al., 2011). However, there are slight differences in the type of cytochromes expressed during growth in electron-donating and electron up-taking modes (Strycharz et al., 2011).

Geobacter sulfurreducens growing via bDIET with G. metallireducens highly expresses an extracellular c-type cytochrome, OmcS (Summers et al., 2010; Shrestha et al., 2013a,b). OmcS decorates the pili of G. sulfurreducens (Leang et al., 2010; Summers et al., 2010) and is required for bDIET and Fe^{3+} reduction (Mehta et al., 2005; Ding et al., 2008; Qian et al., 2011) but not for current production (Nevin et al., 2009). OmcS is not necessary while growing via H_{2} interspecies transfer with P. carbinolicus (Rotaru et al., 2012).

Another extracellular cytochrome OmcZ, which helps G. sulfurreducens achieve high current densities in single species biofilms (Nevin et al., 2009; Richter et al., 2009), was not required for bDIET in G. sulfurreducens – G. metallireducens co-cultures (Shrestha et al., 2013b) or during iron oxide reduction (Nevin et al., 2009).

There is no correspondence between the well studied extracellular cytochromes in G. sulfurreducens and G. metallireducens, and today we have yet no clear understanding, about the exact role of each cytochrome in G. metallireducens during extracellular electron transfer processes. And yet it must be noted that extracellular cytochrome like OmcS in the electron acceptor strain, G. sulfurreducens were highly relevant for the interspecies association. How exactly they aid the electron transfer process is yet to be uncovered.

bDIET in environmental communities

The possible existence of bDIET in the natural ecosystem was first reported by Morita et al. (2011), while studying the mechanism of interspecies electron exchange in the natural methanogenic communities that formed conductive aggregates in a simulated anaerobic wastewater digester converting brewery wastes to methane. The microbial community structure in up-flow anaerobic sludge blanket digester aggregates showed the predominance of Geobacter spp. (Morita et al., 2011; Rotaru et al., 2014). It is interesting to note that in most of the methanogenic environments where bDIET is reported, Geobacter spp. are abundant (Kato et al., 2012a; Aulenta et al., 2013; Zhou et al., 2013a; Rotaru et al., 2014), which is probably because Geobacter spp. form conductive networks using pili (Malvankar et al., 2011; Malvankar and Lovley, 2012) and transfer electrons to methanogens such as Methanosaeta (Morita et al., 2011; Rotaru et al., 2014). Similar species abundance has also been reported in enrichment culture converting coal to methane, where Geobacter and Methanosaeta were the dominant genera (Jones et al., 2010) possibly using coal as an electron donor and an electron transfer mediator.

MINERAL MEDIATED DIET (mDIET)

The need to produce biological conductive molecular networks can be averted by the addition of conductive minerals (Liu et al., 2012, 2014). mDIET could take place via non-biological conductive networks of semi-conductive minerals (Figures 2D,E) like nano-magnetite (Kato et al., 2012a,b; Liu et al., 2014), granulated activated carbon (GAC; Liu et al., 2012) or biochar (Chen et al., 2014) in the absence of molecular conduits.

For example, electrically conductive magnetite nano-particles facilitate mDIET from G. sulfurreducens to Thiobacillus denitrificans, accomplishing acetate oxidation coupled to nitrate reduction (Kato et al., 2012b). Recently, magnetite nano-particles were shown to compensate for the absence of OmcS on the pili of a deficient G. sulfurreducens co-cultured with G. metallireducens in the presence of ethanol and fumarate (Liu et al., 2014; Figure 2D). Another conductive material, GAC promotes mDIET, bypassing biologically produced electrical conduits (Liu et al., 2012), as evident from the ability to restore syntrophic metabolism in co-cultures deficient in pili or cytochromes (Liu et al., 2012).

mDIET in environmental communities

Although extracellular appendages are required for the respiration of extracellular electron acceptors (Reguera et al., 2005; Tremblay et al., 2012), they can be replaced with conductive materials which can mediate electron transfer between cells during mDIET. Naturally occurring minerals could offer ecological advantages.
because of their abundance in natural ecosystems (Kato et al., 2012b), where they could aid mDIET in the absence of pre-evolved molecular conductors. Iron is one of the most ubiquitous metals in Earth’s crust (Braunschweig et al., 2013) and could act as conductive mediator for mDIET, demanding less energetic investment from the species exchanging electrons because there would be no need to produce extracellular components for biological electrical connections (Kato et al., 2012b). For example, magnetite, a conductive iron (III&III)-oxide, stimulated methane production in rice paddy soils and enriched for Geobacter and Methanosarcina species, which likely exchanged electrons via magnetite minerals (Kato et al., 2012a; Zhou et al., 2013b). Electrically conductive magnetite (Fe3O4) nano-particles could also enhance reductive dechlorination of trichloroethene, an ubiquitous groundwater pollutant, by allowing electrons to be transferred extracellular from acetate oxidizing microorganisms to trichloroethene dechlorinating microorganisms (Aulenta et al., 2013). In this study the abundant microorganisms were also Geobacter spp., which accounted for 50% of the total bacterial population (Aulenta et al., 2013).

Similarly, it has been reported that poorly crystalline akaganeite (β-polymer of FeOOH) enhanced mDIET to methanogens in slurries from river sediments (Jiang et al., 2013). In such slurries, Clostridium coupled Fe3+-akaganeite reduction to Fe2+ with acetate oxidation. Partly, electrons from Fe2+ were used by the methanogen to convert bicarbonate to methane. Partly, Fe2+ ions were re-adsorbed onto akaganeite nano-rods, followed by re-precipitation as structural Fe3+ with the simultaneous formation of goethite (α-polymer of FeOOH) nanofibers (Jiang et al., 2013).

Anthraquinone disulphonate was also suggested to facilitate mDIET between Geobacter spp. and Methanosarcina spp. in rice paddies (Zhou et al., 2013b). The impact of AQDS on methanogenesis is in contrast with studies in defined co-cultures of Geobacter and Methanosarcina (Liu et al., 2012). However, soils are not well-defined systems, and it is possible that in soil other interactions happen between humics and soil components, which should be further investigated.

**IMPLICATIONS**

The electron exchange between syntrophic partners growing together by bDIET requires cells to develop efficient conductive biological contacts via pili and cytochromes in the absence of conductive mediators (mDIET). However, little is known about the importance of bDIET/mDIET-based interactions in the environment or in man-made systems. A better understanding could help devise better strategies for wastewater digestion, or to control methane emission in environments where such emission are high, like landfills, or rice paddies.

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Interspecies electron transfer

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