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A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the reduction of carbon dioxide to methane†

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Anaerobic conversion of organic wastes and biomass to methane is an important bioenergy strategy, which depends on poorly understood mechanisms of interspecies electron transfer to methanogenic microorganisms. Metatranscriptomic analysis of methanogenic aggregates from a brewery wastewater digester, coupled with fluorescence *in situ* hybridization with specific 16S rRNA probes, revealed that *Methanosaeta* species were the most abundant and metabolically active methanogens. Methanogens known to reduce carbon dioxide with H₂ or formate as the electron donor were rare. Although *Methanosaeta* have previously been thought to be restricted to acetate as a substrate for methane production, *Methanosaeta* in the aggregates had a complete complement of genes for the enzymes necessary for the reduction of carbon to methane, and transcript abundance for these genes was high. Furthermore, *Geobacter* species, the most abundant bacteria in the aggregates, highly expressed genes for ethanol metabolism and for extracellular electron transfer *via* electrically conductive pili, suggesting that *Geobacter* and *Methanosaeta* species were exchanging electrons *via* direct interspecies electron transfer (DIET). This possibility was further investigated in defined co-cultures of *Geobacter metallireducens* and *Methanosaeta harundinacea* which stoichiometrically converted ethanol to methane. Transcriptomic, radiotracer, and genetic analysis demonstrated that *M. harundinacea* accepted electrons *via* DIET for the reduction of carbon dioxide to methane. The discovery that *Methanosaeta* species, which are abundant in a wide diversity of methanogenic environments, are capable of DIET has important implications not only for the functioning of anaerobic digesters, but also for global methane production.

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Broader context

In this study we report a fundamentally new concept for the microbial ecology of anaerobic digestion, one of the oldest bioenergy strategies. The reliance of methanogenic communities on interspecies electron transfer has been recognized for over forty years, but it has been thought that only H₂ or formate served as the interspecies electron carriers. However, the finding that *Methanosaeta* species can make direct electrical connections with *Geobacter* species, accepting electrons for the reduction of carbon dioxide to methane, demonstrates that direct interspecies electron transfer (DIET) is an alternative to interspecies H₂/formate transfer. DIET appears to predominate over interspecies H₂/formate transfer in upflow anaerobic digesters converting brewery waste to methane, and the metatranscriptomic approach described here provides a tool to discriminate between pathways for interspecies electron transfer in other digester designs, treating other types of wastes or biomass. *Methanosaeta* species are also ubiquitous in methanogenic soils and sediments, suggesting that a substantial portion of global methane production could be derived from DIET.

Introduction

Anaerobic conversion of organic compounds to methane is one of the few proven, economical, large-scale bioenergy strategies. Methanogenic treatment of wastewaters is already a widespread practice and new approaches to reactor design are expected to further improve this technology and expand its application.^{1,2}

Nearly half a century ago, a major breakthrough in the understanding of the function of methanogenic microbial

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communities was made with the discovery of interspecies H_2 transfer.^{3–5} In interspecies H_2 transfer non-methanogenic microorganisms metabolize key fermentation products, such as ethanol and volatile fatty acids, to acetate, which methanogens then convert to methane. This acetate production also releases carbon dioxide and reduces electron carriers in the acetate-producing microbes. The reduced electron carriers are regenerated to the oxidized state *via* the reduction of protons to H_2 . Methanogens consume the H_2 with the reduction of carbon dioxide to methane. This syntrophic degradation of fermentation intermediates functions well as long as methanogens maintain the concentration of H_2 low enough that the production of H_2 is thermodynamically favourable. Formate can serve as a substitute for H_2 as an interspecies electron carrier.^{6,7} Interspecies H_2 /formate transfer has been documented in many defined co-cultures in which H_2 and/or formate-donating microorganisms were paired with H_2 and/or formate-consuming methanogens.^{7–10}

It has been assumed that the interspecies H_2 /formate transfer observed in laboratory co-cultures is also the primary mechanism for interspecies electron exchange in anaerobic digesters and other complex methanogenic environments, such as anaerobic soils and sediments. However, it has been difficult to determine the extent of interspecies H_2 /formate transfer in such environments because of the lack of methods for reliably measuring turnover rates of H_2 and formate. A potential alternative to interspecies H_2 /formate transfer is direct interspecies electron transfer (DIET), in which species exchange electrons through biological electrical connections.^{11–14}

DIET was first documented in co-cultures of *Geobacter metallireducens* and *Geobacter sulfurreducens* grown in a medium with ethanol as the electron donor and fumarate as the electron acceptor.¹¹ Interspecies electron exchange was required because *G. metallireducens* can metabolize ethanol, but cannot use fumarate as an electron acceptor, whereas *G. sulfurreducens* can reduce fumarate, but cannot metabolize ethanol. Studies with a diversity of mutant strains, deficient in key aspects of interspecies H_2 /formate transfer or DIET, as well as genome-wide transcriptomic analysis, demonstrated that H_2 or formate could not be the interspecies electron carrier.^{11,12,15} Instead, the co-cultures established electrical connections through the pili of the two *Geobacter* species, which are electrically conductive.^{16–18} Consistent with the DIET concept, the *Geobacter* co-cultures formed large (1–2 mm diameter), electrically conductive aggregates to promote interspecies electron exchange.¹¹

Methanogenic aggregates from a brewery wastewater digester were also electrically conductive, with a temperature dependence characteristic of the metal-like conductivity of *Geobacter* pili.¹⁹ *Geobacter* species were the dominant bacteria, accounting for *ca.* 25% of the bacterial 16S rRNA gene sequences recovered. A similar abundance of *Geobacter* species has been observed in many similar brewery waste digesters.²⁰

Methanosaeta species accounted for over 90% of the 16S rRNA sequences recovered that could be attributed to methanogens.¹⁹ *Methanosaeta* species can convert acetate to methane but cannot utilize H_2 or formate as an electron donor for the reduction of carbon dioxide to methane.²¹ In accordance with the low abundance of methanogens known to metabolize H_2 or

formate, the aggregates only slowly converted these compounds to methane.¹⁹ Based on these observations it was proposed that DIET, rather than interspecies H_2 /formate transfer, was the mechanism for interspecies electron exchange within the methanogenic digester aggregates.¹⁹ However, this was speculative because it had not been shown that *Methanosaeta* or any other methanogens were actually capable of accepting electrons *via* DIET. Furthermore, in order for *Methanosaeta* species to participate in DIET they would need to reduce carbon dioxide to methane, an unknown metabolic capability in these organisms.

Recent studies with co-cultures suggested that community gene expression patterns are different during DIET than interspecies H_2 /formate transfer.^{12,15} Therefore, in order to overcome the challenges of directly tracking the flow of H_2 or electrons between microorganisms in complex communities we used gene expression patterns as a diagnostic tool to elucidate mechanisms of interspecies electron exchange in the digester aggregates.

Results and discussion

Evidence for direct electron transfer in digester aggregates

Analysis of gene transcript abundance in the aggregates from the digesters treating simulated brewery wastewater revealed that *Methanosaeta* species were the predominant and active methanogens (Fig. 1a). Fluorescence *in situ* hybridization (FISH) confirmed the abundance of *Methanosaeta*, which were homogeneously distributed throughout the aggregates (Fig. 1b–e). Less than 0.6% of gene transcript reads could be ascribed to methanogens capable of metabolizing H_2 or formate, and FISH verified that such methanogens were rare (Fig. 1c–e).

The metabolism of ethanol, the primary waste in brewery digesters, produces acetate with the release of electrons:

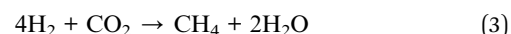


Therefore, it was not surprising that *Methanosaeta* genes encoding enzymes involved in converting acetate to methane were highly expressed in the reactor aggregates (Fig. 2). However, in order for ethanol to be completely metabolized, the electrons released during ethanol metabolism must be consumed.

In a methanogenic environment the most direct route for consumption of these electrons is the reduction of carbon dioxide to methane. During interspecies H_2 transfer the ethanol-metabolizing microorganisms produce H_2 :



and the H_2 serves as the electron donor for methane production:



The inability of *Methanosaeta* species to use H_2 , or the H_2 substitute formate,²¹ eliminated the possibility for this mode of electron exchange. However, genes for a complete pathway for carbon dioxide reduction were present and highly expressed in *Methanosaeta concilii*, the abundant species in the digester aggregates (Fig. 2). This suggested that *Methanosaeta* species

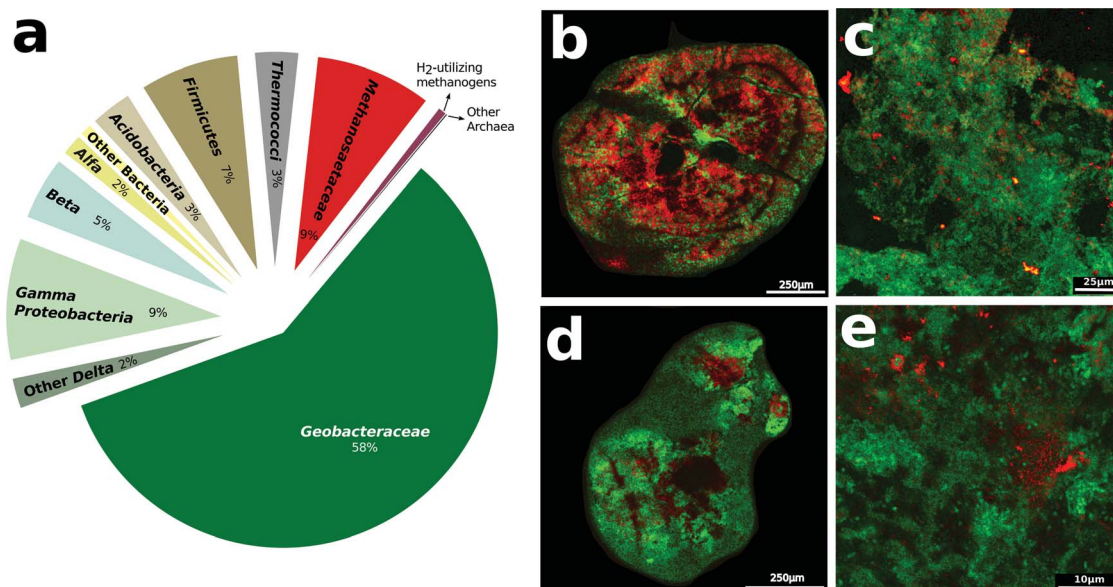


Fig. 1 Digester community. (a) Relative distribution of community BLASTX transcript reads in digesters. (b–e) FISH images of digester aggregates treated with probes for *Methanosaeta* species (green) or other organisms (red). Red staining in the four panels corresponds to (b) *Geobacter* species, or potential H_2 /formate utilizing methanogens such as (c) *Methanomicrobiales*, (d) *Methanobacteriaceae*, and (e) *Methanosarcina* species.

were actively reducing carbon dioxide to methane, possibly with electrons derived by a mechanism other than interspecies H_2 or formate transfer.

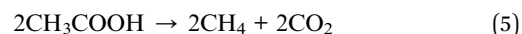
Previous studies demonstrated that *Geobacter* species were the most abundant bacteria in the digesters¹⁹ and in this study we observed that *Geobacter* species accounted for most of the gene transcripts recovered, demonstrating their high metabolic activity (Fig. 2, Table S2†). The *Geobacter* species highly expressed genes for ethanol metabolism (Fig. 2, Table S2†), suggesting that they played an important role in utilizing this primary substrate in the digesters. *Geobacter metallireducens* is known to transfer electrons derived from ethanol metabolism to *Geobacter sulfurreducens* via pili¹⁴ that are electrically conductive.^{16,17} The gene for Pila, the structural protein for electrically conductive pili, was highly expressed (Fig. 2, Table S3†), as expected^{12,15} if the abundant *Geobacter* species were metabolizing ethanol with direct electron transfer to *Methanosaeta*.

Direct electron transfer to *Methanosaeta* in defined co-cultures

In order to evaluate whether *Methanosaeta* was capable of functioning in the manner suggested by the metatranscriptomic analysis, *Methanosaeta harundinacea*, an isolate from another anaerobic digester treating brewery waste²² was co-cultured with *Geobacter metallireducens*, which served as a representative for the abundant *Geobacter* species in the digesters. Metabolic modelling has suggested that *G. metallireducens* is unable to conserve energy to support growth from syntrophic metabolism of ethanol with the production of H_2 or formate^{13,23} and the inability of *G. metallireducens* to grow via interspecies H_2 or formate transfer was further evident from its failure to generate functioning co-cultures with the H_2 - or

formate-utilizing methanogens *Methanospirillum hungatei* or *Methanobacterium formicicum* (Fig. 3). These results, coupled with the inability of *M. harundinacea* to metabolize H_2 or formate,^{22,24} ruled out the possibility of electron exchange via these indirect electron carriers.

Yet, *G. metallireducens* and *M. harundinacea* grew in co-culture converting ethanol to methane, forming aggregates in which the two species were in close physical proximity (Fig. 4). The amount of methane produced in the co-cultures was consistent with complete conversion of the added ethanol to methane based on the following reactions:



Each mole of ethanol yielded *ca.* 1.5 moles of methane (Fig. 4). This indicated that *M. harundinacea* was not only converting the acetate produced from ethanol to methane (reaction (5)), but was also utilizing the additional electrons available from the conversion of ethanol to acetate (reaction (4)) for methane production (reaction (6)). Metatranscriptomic analysis of the co-culture revealed that the genes for the putative carbon dioxide reduction pathway in *M. harundinacea* were highly expressed (Fig. 2, Table S3†), as expected if *M. harundinacea* was directly accepting electrons from *G. metallireducens* for the reduction of carbon dioxide to methane.

In order to further evaluate this possibility, co-cultures were transferred into fresh medium that was amended with

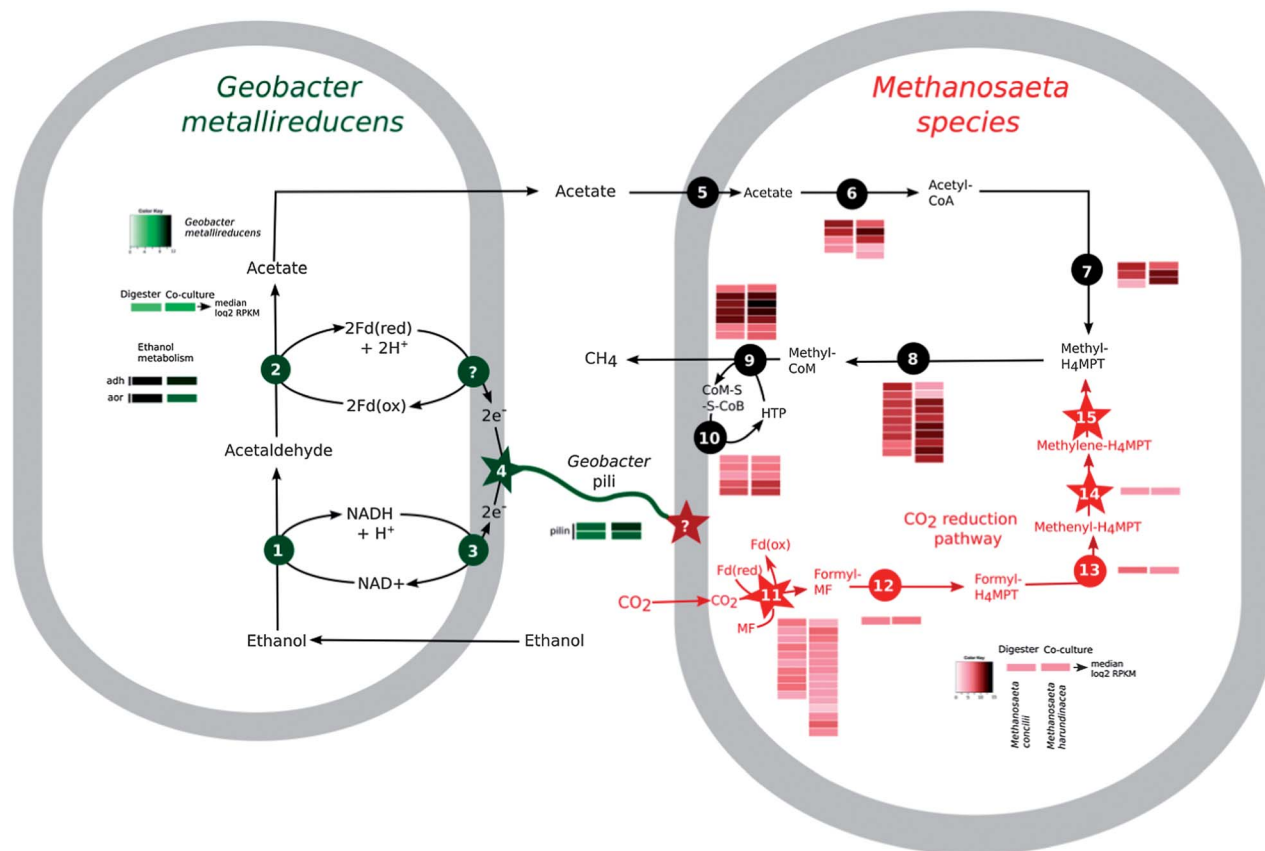


Fig. 2 DIET-based metabolism in digester samples and defined co-cultures of *Geobacter metallireducens* and *Methanosaeta harundinacea* as revealed by metatranscriptomics. The schematic depiction of the pathways in effect during syntrophic growth on ethanol. *Geobacter* genes involved in ethanol metabolism and DIET include those that encode for: (1) ethanol dehydrogenase (*adh*); (2) aldehyde-ferredoxin oxidoreductase (*aor*); (3) NADH dehydrogenase complex; and (4) the structural proteins for conductive pilin used during DIET. *Methanosaeta* genes involved in acetate metabolism and reduction of carbon dioxide to methane include those that encode for: (5) Na^+ /solute symporter; (6) acetyl-CoA synthetase; (7) CO dehydrogenase/acetyl-CoA synthase; (8) tetrahydromethanopterin (H_4MPT)-S-methyltransferase; (9) methyl-CoM reductase; (10) CoM-S-S-CoB heterodisulfide reductase; (11) formyl methanofuran dehydrogenase; (12) formyl methanofuran- H_4MPT formyl-transferase; (13) methenyl- H_4MPT cyclohydrolase; (14) F_{420} -dependent methylene- H_4MPT dehydrogenase; and (15) F_{420} -dependent methylene H_4MPT reductase. Starred reactions represent the steps where electrons are donated by *Geobacter* and could be received by the methanogen. Inset heat maps show the transcript abundance of genes encoding the above-mentioned proteins in *Geobacter* (green) and *Methanosaeta* (red) presented as \log_2 RPKM values (Reads Per Kilobase target per one Million mapped reads). Median \log_2 RPKM for *Methanosaeta* species in the digester and co-culture were 5.4 and 5.6, respectively, whereas the median \log_2 RPKM for *Geobacter metallireducens*, in the digester and co-cultures, were 4.2 and 6.3, respectively.

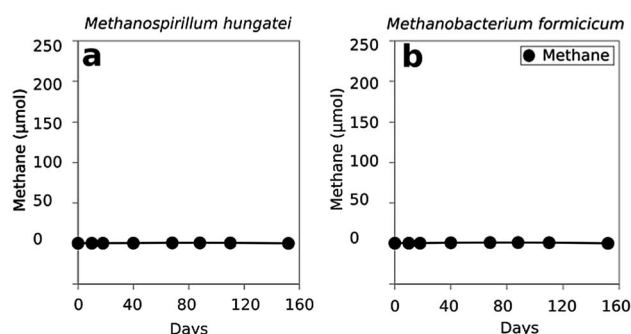


Fig. 3 Absence of methane production from ethanol when *G. metallireducens* was co-cultured with H_2 /formate utilizing methanogenic partners *Methanospirillum hungatei* (a) or *Methanobacterium formicicum* (b). The data represent averages of four replicate co-cultures for each strain.

$[^{14}\text{C}]$ -bicarbonate. The specific activity (^{14}C disintegrations per minute per mole compound) of the CH_4 that was produced was *ca.* $\frac{1}{3}$ of the specific activity measured for CO_2 (Fig. 5). This is the result expected according to reactions (4)–(7) in which $\frac{1}{3}$ of the methane produced should be derived from CO_2 (Fig. 5).

Geobacter species require their electrically conductive pili for extracellular electron transfer to insoluble electron acceptors, such as $\text{Fe}(\text{III})$ oxides,^{16,25} but not for reduction of soluble extracellular molecules that might function as electron shuttles between cells.¹⁶ *Geobacter* strains in which the gene for PilA was deleted, were incapable of DIET in *G. metallireducens*–*G. sulfurreducens* co-cultures.^{11,12} In this study, *G. metallireducens* highly expressed the PilA gene in co-culture with *M. harundinacea* (Fig. 2, Table S3†), and the PilA-deficient strain of *G. metallireducens* did not metabolize ethanol or produce methane in co-culture with *M. harundinacea* (Fig. 5). These results

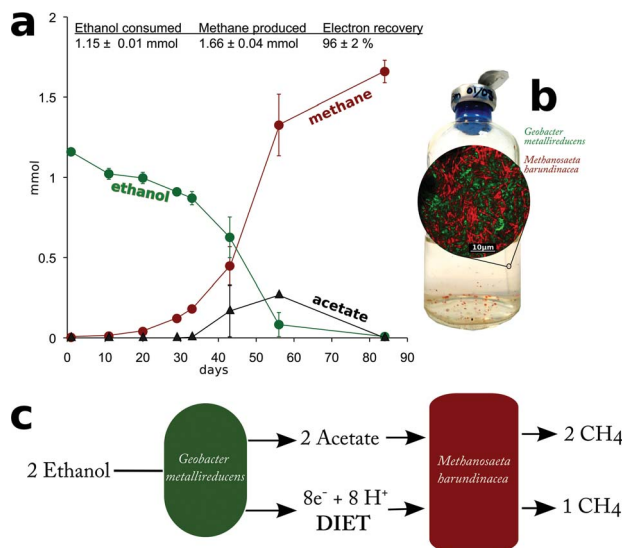


Fig. 4 Defined co-cultures of *Geobacter metallireducens* and *Methanosaeta harundinacea* converting ethanol to methane. (a) Time course and stoichiometry of ethanol conversion to methane. The data are representative of six replicate co-cultures. (b) Appearance of aggregates in co-culture and distribution of *Methanosaeta* (red) and *Geobacter* (green) as revealed by FISH (round inset). (c) Model of electron transfer in the co-culture.

indicate that pili are an important component for electron transfer between *G. metallireducens* and *M. harundinacea*.

The stoichiometric conversion of ethanol to methane coupled with the high expression of genes for carbon dioxide reduction in *M. harundinacea* and the reduction of $^{14}CO_2$ to $^{14}CH_4$ at the appropriate specific activity demonstrated that *M. harundinacea* was capable of accepting electrons from *G. metallireducens* for the reduction of carbon dioxide to methane. Although genes for carbon dioxide reduction were previously noted in genomes of *Methanosaeta* species,²⁴ our study is the first to document that

Methanosaeta is capable of producing methane from carbon dioxide. The fact that *M. harundinacea* reduced carbon dioxide under conditions in which interspecies H_2 /formate transfer was impossible, and that co-cultures could not be established with a strain of *G. metallireducens* that could not produce conductive pili, indicated that *G. metallireducens* and *M. harundinacea* exchanged electrons through a biological electrical connection. This is the first example, of a methanogen participating in DIET.

Methods

Laboratory scale digesters

Microbial aggregates were propagated in the laboratory in three replicate mesophilic ($37^\circ C$) laboratory-scale (0.9 liter) digesters.¹⁹

Strains, media, culturing conditions

Cultures were grown under strict anaerobic conditions in anaerobic pressure tubes or serum bottles sealed with thick butyl rubber stoppers. *Geobacter metallireducens*, wild type and the PilA-deficient strain, were routinely maintained on Fe(III)-citrate (FC) medium with 10 mM acetate as an electron donor.²⁵ Prior to co-cultivation, all *Geobacter* strains were adapted to growth on Fe(III) citrate medium with 20 mM ethanol as the substrate for more than three transfers until ethanol metabolism was synchronized in the wild type and the PilA-deficient strain. *Methanosaeta harundinacea* (JCM-13211) was purchased from the Japanese culture collection and *Methanospirillum hungatei* (DSM-13809), and *Methanobacterium formicicum* (DSM-1535) were purchased from the German culture collection DSMZ. The methanogens were grown under the conditions specified by the culture collections.

Co-cultures were initiated with 0.5 mL of *G. metallireducens*, and 1 mL of the methanogen cultures inoculated into 10 mL modified fresh water medium with 20 mM ethanol and carbon

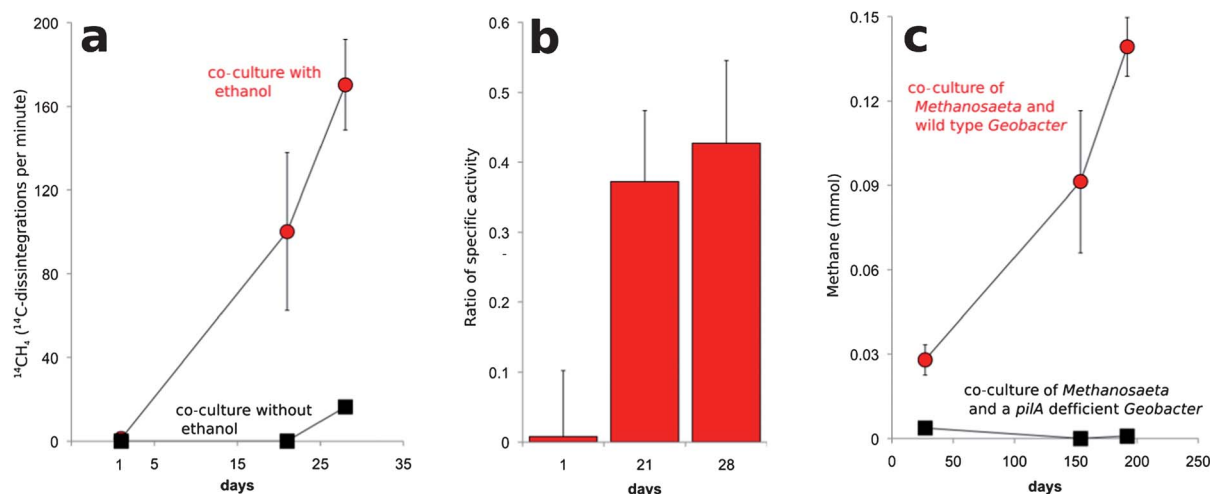


Fig. 5 Reduction of carbon dioxide to methane via DIET in co-cultures of *M. harundinacea* and *G. metallireducens*. (a) Production of $^{14}CH_4$ in co-cultures amended with $[^{14}C]$ -bicarbonate. (b) Ratio of the specific activity of $^{14}CH_4$ to the specific activity of $^{14}CO_2$. (c) Lack of methane production in co-cultures initiated with a strain of *G. metallireducens* that could not produce conductive pili. Data are results of four replicate co-cultures for radiolabel studies and three replicate co-cultures with the PilA-deficient mutant.

dioxide as the only electron acceptor. The medium was modified from a previously described fresh water medium.²⁶ Modifications consisted of boiling the medium to reduce O₂ solubility, then cooling under N₂ : CO₂ (80 : 20) for increased gas exchange. Prior to addition of cells the medium was amended from anaerobic sterile stocks with: 20 mM ethanol, vitamins,²⁶ and a premix of 1 mM cysteine, and 0.5 mM Na₂S·9H₂O. For studies on the stoichiometry of ethanol metabolism co-cultures were grown in 50 mL of medium in 160 mL serum bottles. Samples were withdrawn regularly with N₂ : CO₂ (80 : 20) degassed hypodermic syringes to monitor ethanol, acetate, and methane as previously described.^{15,19}

For radiotracer experiments, a sterile anaerobic solution of [¹⁴C]-bicarbonate (18.7 × 10⁴ Bq per 10 mL) was added to co-cultures to obtain a final concentration of 8.1 × 10⁴ Bq. ¹⁴CH₄ and ¹⁴CO₂ were monitored with a gas chromatograph gas proportional counter as previously described.²⁷

Fluorescent *in situ* hybridization (FISH)

The distribution of microorganisms within thin sections of digester or co-culture aggregates was examined with FISH probes as previously described.²⁸ The probes were: MX825 targeting *Methanosaeta*,²⁹ MB1174 (ref. 29) targeting *Methanobacteriaceae*; MS1414 (ref. 29) targeting *Methanosarcina*; GEO825 (ref. 30) specific for *Geobacter* species; and GEO1 (ref. 11) specific for *Geobacter metallireducens*. Samples were imaged with a Leica TCS SP5 microscope as previously described.¹⁵

mRNA extraction

Digester aggregates were sampled from two independent digesters, and were immediately mixed with RNAlater (Ambion) as described previously.¹² Samples of three replicate *Geobacter metallireducens* and *Methanosaeta harundinacea* co-cultures were harvested as previously described.¹² Samples were then processed immediately or stored at −80 °C.

RNA was extracted and mRNA was enriched as described previously.¹² The workflow for the metatranscriptomic analysis can be found in Fig. S1.†

Illumina sequencing

Digester mRNA was sequenced with paired end, strand specific RNA sequencing with the dUTP method as previously described^{23,31} on an Illumina Genome Analyzer II. For the co-cultures, directional libraries were prepared with the ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre) and single end sequencing was done with a Hi-Seq 2000 following the manufacturer's instructions.

Assembly of Illumina reads

All the raw sequencing data were quality checked by visualization of base quality scores and nucleotide distributions. Then the sequences were sorted by trimming of reads and read filtering based on the base quality score and sequence properties such as primer contaminations, N content and GC bias with PRINSEQ.³² We removed sequence reads matching 16S and 23S

rRNA genes using Ribopicker.³³ The remaining reads were then used for the BLASTX against the NR database at the "FutureGrid Portal (<https://portal.futuregrid.org/>)". The output text file of the BLASTX was imported into MEGAN,³⁴ to carry out phylogenetic, and KEGG analysis. The MEGAN phylogenetic output file was used to present the relative abundance of transcripts belonging to different bacterial and archaeal groups (Fig. 1).

Mapping mRNA reads

For analysis of gene expression in *Methanosaeta*, digester mRNA reads were mapped against the published genome of *Methanosaeta concilii* (NC_015416.1), the dominant *Methanosaeta* in the digesters.¹⁹ *Geobacter* gene expression was examined by mapping against the genome of *G. metallireducens* (NC_007517.1). Mapped reads were normalized with the RPKM (reads assigned per kilobase of target per million mapped reads) method³⁵ using ARRAY STAR. Co-culture sequence reads were filtered for mRNA sequences, which were then mapped against *G. metallireducens* (NC_007517.1) and *M. harundinacea* (NC_017527) genomes as described previously.¹² Reads from biological replicates were compared with each other graphically after mapping onto the template genomes (Table S1, Fig. S2†). Due to high reproducibility of data from biological replicates (Fig. S2†) the values were merged and averaged before further analysis (Tables S2 and S3†).

Accession number

Sequence reads have been submitted to the EMBL databases under accession no. ERP003805.

Conclusions

The results demonstrate that *Methanosaeta* species can directly accept electrons through biological electrical connections for the reduction of carbon dioxide to methane and that DIET can predominate over interspecies H₂/formate transfer during anaerobic digestion. These findings greatly expand the known metabolic capabilities of *Methanosaeta*, which are abundant not only in anaerobic digesters,^{19,20} but also in a diversity of methanogenic soils and sediments.^{36–39} *Methanosaeta* is considered to produce more methane on Earth than any other methanogen, due to its ubiquitous distribution and its high affinity for acetate, the precursor of more than half of the methane in most methanogenic environments.²¹ However, the energy yield from the conversion of acetate to methane is low (−75.7 kJ mol^{−1} methane) and the ability of *Methanosaeta* to also produce methane with electrons derived from DIET may add to their competitive advantage. The electron-accepting components that allow *Methanosaeta* species to participate in DIET are not known. However, the apparent ability of other methanogens to accept electrons from abiotic donors such as metallic iron^{40,41} or electrodes,⁴² as well as the enhancement of electron transfer between *Geobacter* and *Methanosarcina* species with conductive minerals⁴³ or granular activated carbon⁴⁴ is analogous to the biological connections proposed here. The inability of *Methanospirillum hungatei* or *Methanobacterium formicicum* to form

co-cultures with *G. metallireducens* suggests that not all methanogens are capable of DIET.

The results demonstrate that although *Geobacter* species are primarily known for their ability to grow with the reduction of extracellular electron acceptors, such as Fe(III) oxides, humic substances, and electrodes,⁴⁵ they are also effective syntrophs, essentially using other organisms as another extracellular electron sink. *Geobacter* species were the most metabolically active microorganisms in methanogenic rice paddy soils,⁴⁶ which suggests they may function as syntrophs in methanogenic environments other than anaerobic digesters. There is a wide diversity of organisms that are known to grow in co-culture with methanogens via interspecies H₂/formate transfer,^{4,8–10} which when grown with *Methanosaeta*, may also be found to be capable of DIET.

The importance of DIET in a diversity of methanogenic environments is as yet unknown. Analysis of twenty-four brewery waste digesters revealed that each produced electrically conductive aggregates in which *Geobacter* and *Methanosaeta* species were abundant, suggesting that DIET is common in such systems.²⁰ It should be possible to determine the relative importance of DIET in digesters treating more complex wastes as well as in methanogenic soils and sediments with the metatranscriptomic approach described here.

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