Extracellular Electron Uptake by Two *Methanosarcina* Species

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Abstract

Direct electron uptake by prokaryotes is a recently described mechanism with a potential application for energy and CO_2 storage into value added chemicals. Members of Methanosarcinales, an environmentally and biotechnologically relevant group of methanogens, were previously shown to retrieve electrons from an extracellular electrogenic partner performing Direct Interspecies Electron Transfer (DIET) and were therefore proposed to be electroactive. However, their intrinsic electroactivity has never been examined. In this study, we tested two methanogens belonging to the genus *Methanosarcina, M. barkeri* and *M. horonobensis,* regarding their ability to accept electrons directly from insoluble electron donors like other cells, conductive particles and electrodes. Both methanogens were able to retrieve electrons from *Geobacter metallireducens* via DIET. Furthermore, DIET was also stimulated upon addition of electrically conductive granular activated carbon (GAC) when each was co-cultured with *G. metallireducens.* However, when provided with a cathode poised at -400 mV (vs. SHE), only *M. barkeri* could perform electromethanogenesis. In contrast, the strict hydrogenotrophic methanogen, *Methanobacterium formicicum*, did not produce methane regardless of the type of insoluble electron donor provided (*Geobacter* cells, GAC or electrodes). A comparison of functional gene categories between the two *Methanosarcina* showed differences regarding energy

metabolism, which could explain dissimilarities concerning electromethanogenesis at fixed potentials. We suggest that these dissimilarities are minimized in the presence of an electrogenic DIET partner (e.g. *Geobacter*), which can modulate its surface redox potentials by adjusting the expression of electroactive surface proteins.

Introduction

Extracellular electron uptake by methanogens may impact carbon turnover in electron-acceptor limited environments (Morris et al., 2013). In these environments, thermodynamically challenging processes become possible due to syntrophic interactions between bacteria and archaea. A syntrophic interaction requires a bacterium, which oxidizes organics to interspecies-transferrable molecules. Moreover, syntrophy requires a partner methanogen to scavenge the transferable molecules. For decades, we have assumed that interspecies-transferrable molecules were either H_2 or formate (Stams and Plugge, 2009). We now know that some species can also transfer electrons directly (Lovley, 2017). During direct interspecies electron transfer (DIET), species like *Geobacter* oxidize ethanol according to reaction (1), only in the presence of methanogens like *Methanosarcina*, which scavenge reducing equivalents (H^+ and e^-) and acetate (Rotaru et al., 2014a, 2014b) **(Fig. 1)**.

$C_2H_6O + H_2O \rightarrow C_2H_4O_2 + 4H^+ + 4e^-$ Reaction (1) by *G. metallireducens*

$0.5\ CO_2 + 4H^+ + 4e^- \rightarrow 0.5\ CH_4 + H_2O$ Reaction (2) by *Methanosarcina*

$C_2H_4O_2 \rightarrow CH_4 + CO_2$ Reaction (3) by *Methanosarcina*

In DIET co-cultures, only those *Geobacter* species producing high current densities, met the energetic needs of their DIET partners (Rotaru et al., 2015). For this purpose, *Geobacter* up-regulates the expression of redox active and conductive proteins (outer membrane *c*-type cytochromes and pili) (Holmes et al., 2018b; Shrestha et al., 2013). *Geobacter*'s requirement for outer-surface proteins during DIET was confirmed earlier with gene-deletion studies (Rotaru et al., 2014a, 2014b). Thus, if *Geobacter* lacked the ability to produce e.g. pili it was incapable of DIET.

Although we understand how *Geobacter* releases electrons outside their cells during DIET, the way Methanosarcinales retrieve DIET-electrons is poorly understood. A glimpse at this mechanism was

provided in a recent comparative transcriptomic study (Holmes et al., 2018b). In this study, the transcriptomes of DIET co-cultures (*G. metallireducens – Methanosarcina barkeri*) were compared to those of co-cultures performing interspecies H_2-transfer (*Pelobacter carbinolicus – M. barkeri*). During DIET, *M. barkeri* had higher expression of membrane-bound redox-active proteins like cupredoxins, thioredoxins, pyrroloquinoline, and quinone-, cytochrome- or Fe-S containing proteins (Holmes et al., 2018b). Still, the exact mechanism of electron uptake by *Methanosarcina* has not been validated and warrants further investigation.

Moreover, *Methanosarcina* can also retrieve electrons from electrically conductive particles charged by *Geobacter* oxidizing organics (Shrestha and Rotaru, 2014). In effect, DIET is accelerated by electrically conductive particles/minerals perhaps because they replace conductive and redox active surface proteins diminishing cellular energy expenditure required to overexpress such surface constituents (Liu et al., 2012). For instance, co-cultures of *G. metallireducens* and *M. barkeri* were stimulated at the addition of conductive particles, such as GAC (Liu et al., 2012), carbon cloth (Chen et al., 2014a), biochar (Chen et al., 2014b), or magnetite (Wang et al., 2018). On the other hand, the addition of non-conductive materials did not stimulate DIET (Chen et al., 2014a; Rotaru et al., 2018a). In addition, conductive particles appear to play a significant role in interspecies interactions from natural and artificial ecosystems such as sediments, soils, rice paddies or anaerobic digesters (Holmes et al., 2017a; Rotaru et al., 2018b; Ye et al., 2018; Zhang et al., 2018). In these cases, the addition of conductive particles enriched for DIET-related Methanosarcinales (Dang et al., 2016; Holmes et al., 2017b; Rotaru et al., 2018a; Zheng et al., 2015). However, exceptions were observed since occasionally conductive particles enriched for H_2-utilizing methanogens of the genus *Methanospirillum* (Lee et al., 2016) or *Methanobacterium* (Lin et al., 2017; Zhuang et al., 2015).

Since methanogens retrieve extracellular electrons from cells or conductive particles to reduce CO_2 to methane, it was expected that they could also retrieve electrons from a poised electrode via electromethanogenesis. Nevertheless, electromethanogenesis was only verified with H_2-utilizing methanogens like *Methanobacterium palustre* (Cheng et al., 2009). Yet, H_2-utilizers were incapable of DIET (Rotaru et al., 2014b). Conversely, it is unknown if Methanosarcinales, which are capable of DIET, are also capable of electron uptake from a cathode. Our objective was to investigate the ability to carry electromethanogenesis and DIET in two *Methanosarcina* species. We have shown that both

Methanosarcina species grew by DIET, however only *M. barkeri* grew on the cathode at - 400 mV vs. SHE. This indicates that extracellular electron-uptake routes from cathodes or other cells might differ between *Methanosarcina* species.

Materials and Methods

Microorganism strains and cultivation conditions

Methanosarcina barkeri MS (DSM 800) and *Methanosarcina horonobensis* HB-1 (DSM 21571) were purchased from the German culture collection DSMZ while *Methanobacterium formicicum* (NBRC 100475) was from the Japanese culture collection NBRC. *Geobacter metallireducens* GS-15 was sent to us by Dr. Sabrina Beckmann from the University of New South Wales, Australia.

Routine cultivation was performed under strictly anaerobic conditions in serum bottles sealed with butyl rubber stoppers and incubated statically at 37^{o}C. All the microorganisms had been adapted to grow in DSMZ medium 120c with the following modifications: 1 g/L NaCl, 0.5g/L yeast, and no tryptone (Rotaru et al., 2014a). During incubations in co-cultures or for electrochemical experiments, sulfide and yeast extract was omitted. When grown in pure cultures, *Methanosarcina* species were provided with 30 mM acetate and 20 mM methanol as methanogenic substrates, while *M. formicicum* was provided with 150 kPa of H_2: CO_2 (80:20) in the headspace. *G. metallireducens* was routinely grown with 20 mM ethanol and 56 mM ferric citrate. All media and cultures were prepared and kept under an N_2: CO_2 (80:20) atmosphere.

The co-cultures of *Geobacter* and methanogens were initiated with 0.5 mL of *G. metallireducens* and 1 mL of acetate-grown *Methanosarcina*-species or H_2-grown *M. formicicum* inoculated into 8.5 ml of the media prepared as above. The starting cell numbers for the methanogens in co-cultures were approximately 2.6 x 10^6 cells/mL, 8.2 x 10^6 cells/mL and 6.7 x 10^5 cells/mL for *M. barkeri, M. formicicum* and *M. horonobensis*, respectively. The starting cell numbers for *G. metallireducens* in co-cultures were circa 1.5 x 10^5. Incubations were carried out in 20 ml pressure vials. For the co-cultures ethanol (10 mM) was added as an electron donor and CO_2 was the sole electron acceptor. When noted,

sterile granular activated carbon (GAC) was added at a concentration of 25 g/L and prepared as described before (Rotaru et al., 2018a).

Electrochemical setup and measurements

All bioelectrochemical incubations were carried with a modified DSMZ 120c medium (see above) in the absence of sulfide and yeast extract. The pH of this medium in the bioelectrochemical reactors was set to 6.5. We used bioelectrochemical reactors with a standard dual chamber configuration as shown in **Figure S1**. Two-chamber glass bottles were purchased from Adams & Chittenden Scientific Glass (USA) with side ports fitted with butyl septa to allow for medium transfer, sampling, and the introduction of a reference electrode. Each chamber of the reactors had a total volume of 650 ml with a flange diameter of 40 mm and the chambers were separated by a Nafion™ N117 proton exchange membrane (Ion Power) held by an O-ring seal with a knuckle clamp.

Both the working and counter electrodes were made of graphite (Mersen MI Corp., Greenville USA) with dimensions of 2.5 cm x 7.5 cm x 1.2 cm thus a total projected surface area of 61.5 cm^2. The working and counter electrodes were coupled to titanium wires, which pierced through rubber stoppers fitted into the main opening of each chamber. A 2 cm deep and 2 mm wide hole was drilled on the short side of the electrode and a 12.5 cm long; 2 mm wide titanium rod (Alfa-Aesar, DE) was inserted and sealed from the outside with biocompatible non-conductive epoxy. Electrodes with a resistance of less than 10 Ω were used to ensure proper electrical connections. The assembled electrodes were introduced into the chamber via the main opening and 2 mm-wide holes were drilled in the black rubber stopper to allow access of the titanium rod. After autoclaving the reactors, sterile medium was transferred into the reactors anaerobically and aseptically. Sterile (bleach and ethanol series) reference electrodes were lodged through a side port in the working electrode chamber at a distance of about 1 cm from the surface of the working electrode. After lodging the electrodes, degassing with N_2: CO_2 (80:20) for circa 30 minutes in each reactor chamber ensured anaerobic conditions. When the pre-cultures were in mid-exponential phase, they were inoculated (20%) into fresh medium in the cathodic chamber following sterile anoxic techniques to a final volume of 550 ml leaving a headspace of approximately 100 mL in each chamber. The approximate cell numbers at the time of inoculation into the electrochemical reactors for *M. barkeri, M. formicicum* and *M. horonobensis* were 2.6 x 10^7 cells/mL, 8.2 x 10^7 cells/mL

and 6.7 x 10^6 cells/mL respectively. Cell counts were done with microscopic examination using DAPI (1 µg/mL) stained cells.

The reference electrodes used were leak-free Ag/AgCl reference electrodes (3.4M KCl) (CMA Microdialysis, Sweden), which are 242 mV above the standard hydrogen electrode (SHE) according to the manufacturer and our own measurements against a Hydroflex® reference electrode used as NHE (normal hydrogen electrode). The difference between NHE and SHE is experimentally negligible (Smith and Stevenson, 2007). All potentials in this paper from here onwards are reported vs. SHE by adjusting accordingly from the Ag/AgCl reference electrodes values. Cathode poising and electrochemical measurements were carried with a multichannel potentiostat (MultiEmstat, Palmsens, NL) operated by the Multitrace software (Palmsens, NL).

Analytical measurements and calculations

Headspace samples for CH_4 and H_2 analysis were taken with hypodermic needles and kept in airtight exetainers until measurement. Methane (CH_4) and hydrogen gas (H_2) were measured on a Trace 1300 gas chromatograph (Thermo-Scientific) with a TracePLOT™ TG-BOND Msieve 5A column and a thermal conductivity detector (TCD). The carrier gas was argon at a flow rate of 25 mL/min. The injector, oven and detector temperatures were 150°C, 70°C, and 200°C respectively. The detection limit for CH_4 and H_2 was ca. 5 µM for both. The concentration unit was converted to molarity by using the ideal gas law ($p \times V = n \times R \times T$) under standard conditions, where p = 1 atm, V is the volume of the gaseous phase (L), n is amount of gas (mol), R is the gas constant (0.08205 $atm \times L / mol \times K$) and T = 298.15 K. For ethanol detection, 0.5 mL samples were filtered (0.2 µm pore size) into appropriate sampling vials and were heated for 5 min. at 60°C. The headspace gas was then pass through the Trace 1300 gas chromatograph (Thermo-Scientific) with a TRACE™ TR-Wax column and detected by a flame ionization detector (FID). Nitrogen gas at a flow of 1 mL/min was used as the carrier and the injector, oven, and detectors were kept at 220°C, 40°C and 230°C respectively. Short-chained volatile fatty acids (VFA) were analyzed with a Dionex™ ICS-1500 Ion Chromatography system, using a Dionex™ IonPac™ AS22 IC Column and a mixture of 1.4 mM $NaHCO_3$ and 4.5 mM Na_2CO_3 as the eluent fitted with an electron capture detector (ECD) at 30 mA.

Genome comparison

Genomes for all tested microorganisms were available at the JGI integrated microbial genomes and microbiomes. Functional category comparisons and pairwise average nucleotide identity (ANI) were determined using the IMG/M- "Compare Genomes" tools. The IMG genome IDs of the studied *M. barkeri, M. horonobensis* and *M. formicicum* used were 2630968729, 2627854269 and 2645727909 respectively. The gene functions were analyzed from the annotated names of all the protein-coding genes retrieved from the National Center for Biotechnology Information (NCBI) database. The accession numbers used were NZ_CP009528, NZ_CP009516, and NZ_LN515531 for *M. barkeri, M. horonobensis* and *M. formicicum* respectively. To scan for the cytochrome motif (CxxCH) through all the genomes, we used a pattern-matching Web-application (Seiler et al., 2006) in addition to manual search

Results and Discussion

It was previously shown that two Methanosarcinales-methanogens, *Methanosarcina barkeri* and *Methanothrix harundinacea* grew via DIET whereas strict H_2-utilizing methanogens did not (Rotaru et al., 2014a, 2014b). Here we show that indeed *M. barkeri* could retrieve electrons not only from an exoelectrogen but also from an electrode poised at - 400 mV (non-H_2 generating conditions) to carry electromethanogenesis. As expected, the H_2-utilizing methanogen *M. formicicum* did not carry electromethanogenesis under this condition. We tested an environmentally relevant *Methanosarcina*, *M. horonobensis* for extracellular electron uptake from cells and electrodes, and we observed that it could only retrieve electrons from exoelectrogenic *Geobacter* and from granular activated carbon but not from electrodes.

Methanosarcina barkeri

M. barkeri grows in co-culture with *G. metallireducens* via DIET, and the interaction could be accelerated by electrically conductive particles (Rotaru et al., 2014a, **Fig. S2**). This was anticipated because *G. metallireducens,* a respiratory organism, is incapable of substrate fermentation and consequent H_2 production according to previous physiological tests (Cord-Ruwisch et al., 1998) and genetic investigations (Aklujkar et al., 2009). Since H_2 could not be generated by *G. metallireducens*, a strict H_2-utilizer like *M. formicicum* was rendered incapable of an interspecies association based on H_2-

transfer with this bacterium (Rotaru 2014b). However, more recent studies indicated that conductive carbon nanotubes stimulated methanogenesis by *M. formicicum* (Salvador et al., 2017). This implied that *M. formicicum* might be encouraged by the presence of conductive particle to interact syntrophically with *Geobacter*. Therefore, we tested if conductive GAC aids *M. formicicum* to establish a syntrophic association with *G. metallireducens*. This was not the case since co-cultures of *M. formicicum* and *G. metallireducens* did not generate methane regardless of the presence or absence of conductive particles, over the course of 120 days **(Fig. 2)**.

During DIET, the extracellular electron transfer machinery of *G. metallireducens* plays a crucial role in *Geobacter-Methanosarcina* interactions, indicating that *Geobacter* releases extracellular electrons for the methanogen to use. Therefore, we suspected that *Methanosarcina* might also be able to directly retrieve extracellular electrons from electrodes to do electromethanogenesis.

In this study, we tested for the first time if *M. barkeri* could retrieve electrons directly from an electrode poised at – 400 mV. Indeed, *M. barkeri* produced significantly more methane (4.4 ± 0.33 mM; $p<0.001$) **(Fig. 3)** when provided with an applied potential at the cathode, in contrast to open circuit controls without an applied potential (1.3 ± 0.33 mM) **(Fig. S3)**. The background methane in control reactors resulted from carry-over substrates, once this was subtracted, the additional methane produced by *M. barkeri* in poised reactors (3.1 ± 0.34 mM) could be solely credited to electricity. Moreover, the highest rate of methane production was observed when current density profiles indicated the highest current draw by *M. barkeri* (Fig. 3).

There are two possible scenarios for *M. barkeri* growing successfully at a cathode poised at – 400 mV:

1. It may use low concentrations of H_2 generated electrochemically at the cathode, or
2. It retrieves electrons directly via an unknown mechanism

To determine abiotic electrochemical H_2 evolution we i) verified for H_2 accumulation over a month of incubation and ii) verified the threshold for H_2-evolution by linear sweep voltammetry at the beginning and the end of the incubation. H_2 did not accumulate over a month of incubation in six independent abiotic controls **(Fig. 4A)**. Linear sweep voltammetry profiles indicated that in our media the threshold for H_2-evolution was below –700 mV **(Fig. 4B)**. This was in agreement with previous studies

determining electrochemical H_2-evolution under physiological conditions on a graphite electrode, which was below -400 mV due to high overpotentials (Batlle-Vilanova et al., 2014; Beese-Vasbender et al., 2015; Cheng et al., 2009; Mitov et al., 2012).

On the other hand, in reactors inoculated with *M. barkeri*, the detected H_2 stabilized at 0.065 ± 0.02 mM, similar to concentrations observed for co-cultures of *M. barkeri* with (0.077 ± 0.03 mM) or without conductive particles (0.076 ± 0.06 mM) and in pure culture (0.068 mM). This is supported by previous research, which demonstrated H_2-cycling (H_2-production and H_2-uptake) in *M. barkeri* (Kulkarni et al., 2009, 2018; Mand et al., 2018). The cellular evolved H_2 is well above the H_2-uptake threshold for *M. barkeri* (296 nM - 376 nM) (Kral et al., 1998; Lovley, 1985) possibly because in these cultures there is an alternative, competitive electron donor.

Secondly, if H_2 evolved electrochemically to concentrations under the detection limit (which was not the case, see above), we anticipated that a sensitive hydrogenotrophic methanogen could effectively reclaim low concentrations of electrochemical H_2, draw current and produce methane. To test this hypothesis we used a highly effective H_2-utilizing methanogen – *M. formicicum,* which has a low H_2 uptake threshold of approximately 6 nM (Lovley, 1985). However, when *M. formicicum* was incubated in electrochemical reactors, neither H_2, methane nor current draw was observed at – 400 mV **(Fig. 5)** indicating that methanogenesis from H_2 could not occur at this potential. In addition, to ensure that the growth of *M. formicicum* was unrestrained by the poised electrode, we carried control incubations at – 400 mV with extrinsic H_2 as substrate. *M. formicicum* was unaffected by a poised electrode since it produced methane from the extrinsic H_2 in an electrochemical setup **(Fig. S4)**.

As electrochemical H_2 was unlikely in our electrochemical setup, according to cumulative gas-detection analyses, electrochemical tests, and tests with a highly effective H_2-utilizer, we confer that *M. barkeri* is likely to retrieve electrons directly from the electrode.

Methanosarcina horonobensis

Except for *M. barkeri* and *M. harundinacea*, the distribution of extracellular electron uptake to other species of the order Methanosarcinales has not been explored. *M. barkeri* and *M. harundinacea* species have been isolated from and associated with anaerobic wastewater treatment (Bryant and Boone, 1987;

De Vrieze et al., 2012; Ma et al., 2006). We were interested to see if other environmentally relevant *Methanosarcina* species had similar electron-uptake properties. We focused on *Methanosarcina horonobensis* because of its provenience and consistent association with deep aquifers (Holmes et al., 2018a; Shimizu et al., 2010).

M. horonobensis did establish successful syntrophic associations with *G. metallireducens* with or without conductive particles as an electrical conduit **(Fig. 6)**. Theoretically, *G. metallireducens* oxidizes ethanol to acetate only if they could use the methanogen as an electron acceptor (Reaction 1). The acetate is then further disproportionated by the acetoclastic methanogen to produce methane and CO_2 (Reaction 2 & 3). During DIET we expect the conversion of 1 mol ethanol to 1.5 mol methane according to Reactions 1 to 3 (Fig. 1). As predicted, in the *G. metallireducens – M. horonobensis* co-cultures, the syntroph oxidized 8.8 ± 0.4 mM ethanol providing the reducing equivalents (directly and via acetate) to generate 13.1 ± 0.8 mM CH_4 by the methanogen. These co-cultures achieved stoichiometric recoveries of 98.5 ± 3.3 %. Similar recoveries (109 ± 18.5 %) were also observed at the addition of conductive particles. Single species controls with GAC showed that ethanol could not be converted to methane by the methanogen or the syntroph alone **(Fig. 6C & S5).** However, similar to previous reports (Zhang et al., 2018), *Geobacter* could partially convert ethanol to acetate using GAC as insoluble electron acceptor (Fig. S5; Van Der Zee et al., 2003; Zhang et al., 2018), likely until it reaches its maximum capacitance of 40 F/g (Zhang et al., 2009). Co-cultures of *G. metallireducens* and *M. horonobensis* could not carry interspecies H_2 transfer because *G. metallireducens* is a strict respiratory microorganism which cannot ferment ethanol to generate H_2 (Shrestha et al., 2013) and because *M. horonobensis* is unable to use H_2 as electron donor for their metabolism (Shimizu et al., 2010).

Surprisingly, *M. horonobensis,* which could grow by DIET, was incapable of electromethanogenesis **(Fig. 7)**. Thus we compared the genomes of the two *Methanosarcina, M. horonobensis* and *M. barkeri* to further explain why they were both capable of DIET, but showed dissimilar activities on cathodes at -400mV.

The main difference between the genomes of *M. barkeri* and *M. horonobensis*, was related to their energy metabolism **(Table 1)**. *M. barkeri* utilizes an energy-converting hydrogenase (Ech) (Kulkarni et al., 2018), which couples the reduction of protons with ferredoxin (Fdx^-) to the production of a proton

motive force according to the reaction: Fdx^- (red) + $2H^+$ → Fdx (ox) + H_2 + $\Delta\mu H^+$/ $\Delta\mu Na^+$ (Thauer et al., 2008). *M. horonobensis* does not have the Ech (Table 1). An alternative to Ech is the Na^+-pumping Rnf complex described biochemically in *M. acetivorans* (Schlegel et al., 2012; Suharti et al., 2014), and predicted via genome mining in *M. thermophila* (Wang et al., 2011) and ANME-2 archaea (Wang et al., 2014). Since we could not find any Ech in the genome of *M. horonobensis,* we screened for the genes encoding an Rnf-complex. In *M. horonobensis*, we found all eight representative Rnf-genes (including the cytochrome subunit and Rnf A to G; MSHOH_3554 to 3561), which showed 65-91% protein identity to their *M. acetivorans* counterparts (MA_0658 to 0665).

Both Ech and Rnf contain Fe-S centers (Welte and Deppenmeier, 2014), however, the Rnf complex has an accompanying *c*-type cytochrome (Suharti et al., 2014) possibly influencing the overall redox-chemistry on the cell surface. We presume that differences in surface redox chemistry will impact how different *Methanosarcina* interact with extracellular electron donors. Thus, electromethanogenesis at a set potential of – 400 mV is unlikely to match the redox requirements of each type of *Methanosarcina*. On the other hand, in co-cultures, *Geobacter* may coordinate its cytochrome expression to match the redox potential of the partner methanogen, who plays the role of a terminal electron acceptor. This is supported by previous studies showing *Geobacter* modulates their cell-surface proteins to match the electron acceptor provided (Ishii et al., 2018; Otero et al., 2018).

When contrasting the genomes of the two *Methanosarcina* species we also observed significant differences regarding nitrogen fixation, mobile elements, and sensing/chemotaxis proteins **(Table 2)**. As such, compared to *M. horonobensis, M. barkeri* encodes for more N_2-fixation proteins (86%). Compared to *M. barkeri, M. horonobensis* encodes for more small-molecule-interaction proteins such as redox-sensing and chemotaxis proteins (185%) and mobile elements than *M. barkeri* (16 fold increase) **(Table 2)**. The exact role of these proteins in extracellular electron uptake by these *Methanosarcinas* is unknown and warrants further investigation.

Furthermore, to determine why *Methanosarcina* could do DIET, but not *Methanobacterium,* we compared the genomes of the two *Methanosarcina* species with that of *M. formicicum* **(Table 2).** In contrast to the *Methanobacterium,* both *Methanosarcina* species encode in their genomes three times the amount of genes for electron transport proteins and circa 50% more genes for cell surface and

transport proteins **(Table 2)**. Especially, outer surface S-layer proteins were only present in the two *Methanosarcina* (Table 2). S-layer proteins were previously suggested to play a role in extracellular electron transfer in *Methanosarcina* related ANME-2, which carry anaerobic methane oxidation syntrophically (McGlynn, 2017; McGlynn et al., 2015; Timmers et al., 2017). Future gene-expression and deletion studies could shed light on the possible role of S-layer proteins in DIET-interactions.

Conclusion

Three methanogens were investigated for their ability to do extracellular electron uptake from (1) a cathode at -400 mV, (2) directly from an electrogenic-DIET partner or (3) from a DIET-partner, but mediated by conductive particles. Only *M. barkeri* was able to carry out all three forms of extracellular electron uptake, making this the first observation of a *Methanosarcina* in pure culture performing electromethanogenesis. The conditions in our abiotic electrochemical controls did not lead to H_2-evolution at -400mV, according to electrochemical and analytical tests. Therefore, under these conditions, it was impossible to sustain a methanogen with high H_2-affinity, like *M. formicicum.* Besides *M. formicicum* was incapable to retrieve electrons directly from the electrode or from a DIET partner (direct or via conductive particles). In this study, we also demonstrated that another *Methanosarcina*, *M. hornobensis* performed DIET with *Geobacter* (direct or via conductive particles). However, surprisingly, *M. horonobensis* was incapable of electromethanogenesis. We screened the genomes of the two *Methanosarcina* and identified differences (e.g. energy metabolism), which could lead to phenotypic variability and thus contrasting electromethanogenesis-ability. Compared to *M. barkeri*, *M. horonobensis* is a better candidate for understanding electron uptake from a DIET syntrophic partner. This is because unlike *M. barkeri, M. horonobensis* does not utilize H_2, and it grows as single cells on freshwater media, which is ideal for genetic studies.

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Author contribution

MY and A-ER conceived the study with support from BT and LO. MY performed all experiments with support from OS. MY analyzed the data with support from A-ER. MY wrote the manuscript with help from A-ER. All authors (MY, A-ER, BT, OS and LO) contributed to drafting and editing the manuscript.

References

Aklujkar, M., Krushkal, J., Dibartolo, G., Lapidus, A., Land, M. L., and Lovley, D. R. (2009). The genome sequence of Geobacter metallireducens: Features of metabolism, physiology and regulation common and dissimilar to Geobacter sulfurreducens. *BMC Microbiol.* 9, 1–22. doi:10.1186/1471-2180-9-109.

Batlle-Vilanova, P., Puig, S., Gonzalez-Olmos, R., Vilajeliu-Pons, A., Bañeras, L., Balaguer, M. D., et al. (2014). Assessment of biotic and abiotic graphite cathodes for hydrogen production in microbial electrolysis cells. *Int. J. Hydrogen Energy* 39, 1297–1305. doi:10.1016/j.ijhydene.2013.11.017.

Beese-Vasbender, P. F., Grote, J.-P., Garrelfs, J., Stratmann, M., and Mayrhofer, K. J. J. (2015). Selective microbial electrosynthesis of methane by a pure culture of a marine lithoautotrophic archaeon. *Bioelectrochemistry* 102, 50–55. doi:10.1016/j.bioelechem.2014.11.004.

Bryant, M. P., and Boone, D. R. (1987). Emended Description of Strain MST(DSM 800T), the Type Strain of Methanosarcina barkeri. *Int. J. Syst. Bacteriol.* 37, 169–170. doi:10.1099/00207713-37-2-169.

Buckel, W., and Thauer, R. K. (2013). Energy conservation via electron bifurcating ferredoxin reduction and proton/Na+ translocating ferredoxin oxidation. *Biochim. Biophys. Acta - Bioenerg.* 1827, 94–113. doi:10.1016/j.bbabio.2012.07.002.

Buckel, W., and Thauer, R. K. (2018). Flavin-based electron bifurcation, ferredoxin, flavodoxin, and anaerobic respiration with protons (Ech) or NAD+(Rnf) as electron acceptors: A historical review. *Front. Microbiol.* 9. doi:10.3389/fmicb.2018.00401.

Chen, S., Rotaru, A.-E., Liu, F., Philips, J., Woodard, T. L., Nevin, K. P., et al. (2014a). Carbon cloth stimulates direct interspecies electron transfer in syntrophic co-cultures. *Bioresour. Technol.* 173, 82–86. doi:10.1016/j.biortech.2014.09.009.

Chen, S., Rotaru, A.-E., Shrestha, P. M., Malvankar, N. S., Liu, F., Fan, W., et al. (2014b). Promoting Interspecies Electron Transfer with Biochar. *Sci. Rep.* 4, 1–7. doi:10.1038/srep05019.

Cheng, S., Xing, D., Call, D. F., and Logan, B. E. (2009). Direct biological conversion of electrical current into methane by electromethanogenesis. *Environ. Sci. Technol.* 43, 3953–3958. doi:10.1021/es803531g.

Cord-Ruwisch, R., Lovley, D. R., & Schink, B. (1998). Growth of Geobacter sulfurreducens with acetate in syntrophic cooperation with hydrogen-oxidizing anaerobic partners. *Appl. Environ. Microbiol.*, 64(6), 2232–2236.

Dang, Y., Holmes, D. E., Zhao, Z., Woodard, T. L., Zhang, Y., Sun, D., et al. (2016). Enhancing anaerobic digestion of complex organic waste with carbon-based conductive materials. *Bioresour. Technol.* 220, 516–522. doi:10.1016/j.biortech.2016.08.114.

De Vrieze, J., Hennebel, T., Boon, N., and Verstraete, W. (2012). Methanosarcina: the rediscovered methanogen for heavy duty biomethanation. *Bioresour. Technol.* 112, 1–9. doi:10.1016/j.biortech.2012.02.079.

Holmes, D. E., Orelana, R., Giloteaux, L., Wang, L.-Y., Shrestha, P. M., Williams, K., et al. (2018a). Potential for Methanosarcina to Contribute to Uranium Reduction during Acetate-Promoted Groundwater Bioremediation. *Microb. Ecol.*, 1–8. doi:10.1007/s00248-018-1165-5.

Holmes, D. E., Rotaru, A.-E., Ueki, T., Shrestha, P. M., Ferry, J. G., and Lovley, D. R. (2018b). Electron and Proton Flux for Carbon Dioxide Reduction in Methanosarcina barkeri During Direct Interspecies Electron Transfer. *Front. Microbiol.* 9, 3109. doi:10.3389/fmicb.2018.03109.

Holmes, D. E., Shrestha, P. M., Walker, D. J. F., Dang, Y., Nevin, K. P., Woodard, T. L., et al. (2017a). Metatranscriptomic Evidence for Direct Interspecies Electron Transfer Between Geobacter and

376 Methanothrix Species in Methanogenic Rice Paddy Soils. *Appl. Environ. Microbiol.*, AEM.00223-
377 17. doi:10.1128/AEM.00223-17.

378 Holmes, D. E., Shrestha, P. M., Walker, D. J. F., Dang, Y., Nevin, K. P., Woodard, T. L., et al. (2017b).
379 Metatranscriptomic Evidence for Direct Interspecies Electron Transfer between Geobacter and
380 Methanothrix Species in Methanogenic Rice Paddy Soils. *Appl. Environ. Microbiol.* 83, e00223-
381 17. doi:10.1128/AEM.00223-17.

382 Ishii, S., Suzuki, S., Tenney, A., Nealson, K. H., and Bretschger, O. (2018). Comparative
383 metatranscriptomics reveals extracellular electron transfer pathways conferring microbial
384 adaptivity to surface redox potential changes. *ISME J.* doi:10.1038/s41396-018-0238-2.

385 Kral, T. A., Brink, K. M., Miller, S. L., and McKay, C. P. (1998). Hydrogen consumptions by
386 methanogens on the early earth. *Orig. Life Evol. Biosph.* 28, 311–319. doi:Doi
387 10.1023/A:1006552412928.

388 Kulkarni, G., Kridelbaugh, D. M., Guss, A. M., and Metcalf, W. W. (2009). Hydrogen is a preferred
389 intermediate in the energy-conserving electron transport chain of Methanosarcina barkeri. *Proc.*
390 *Natl. Acad. Sci. U. S. A.* 106, 15915–20. doi:10.1073/pnas.0905914106.

391 Kulkarni, G., Mand, T. D., and Metcalf, W. W. (2018). Energy Conservation via Hydrogen Cycling in
392 the Methanogenic Archaeon Methanosarcina barkeri. *MBio* 9, 1–10. doi:10.1128/mBio.01256-18.

393 Lee, J. Y., Lee, S. H., and Park, H. D. (2016). Enrichment of specific electro-active microorganisms and
394 enhancement of methane production by adding granular activated carbon in anaerobic reactors.
395 *Bioresour. Technol.* 205, 205–212. doi:10.1016/j.biortech.2016.01.054.

396 Lin, Q., Fang, X., Ho, A., Li, J., Yan, X., Tu, B., et al. (2017). Different substrate regimes determine
397 transcriptional profiles and gene co-expression in Methanosarcina barkeri (DSM 800). *Appl.*
398 *Microbiol. Biotechnol.*, 1–14. doi:10.1007/s00253-017-8457-4.

399 Liu, F., Rotaru, A.-E., Shrestha, P. M., Malvankar, N. S., Nevin, K. P., and Lovley, D. R. (2012).
400 Promoting direct interspecies electron transfer with activated carbon. *Energy Environ. Sci.* 5, 8982.

401 doi:10.1039/c2ee22459c.

402 Lovley, D. R. (1985). Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Appl.*
403 *Environ. Microbiol.* 49, 1530–1.

404 Lovley, D. R. (2017). Syntrophy Goes Electric: Direct Interspecies Electron Transfer. *Annu. Rev.*
405 *Microbiol.* 71, annurev-micro-030117-020420. doi:10.1146/annurev-micro-030117-020420.

406 Lovley, D. R., Giovannoni, S. J., White, D. C., Champine, J. E., Phillips, E. J. P., Gorby, Y. A., et al.
407 (1993). Geobacter metallireducens gen. nov. sp. nov., a microorganism capable of coupling the
408 complete oxidation of organic compounds to the reduction of iron and other metals. *Arch*
409 *Microbiol* 159, 336–344.

410 Ma, K., Liu, X., and Dong, X. (2006). Methanosaeta harundinacea sp. nov., a novel acetate-scavenging
411 methanogen isolated from a UASB reactor. *Int. J. Syst. Evol. Microbiol.* 56, 127–131.
412 doi:10.1099/ijs.0.63887-0.

413 Mand, T. D., Kulkarni, G., and Metcalf, W. W. (2018). Genetic, Biochemical, and Molecular
414 Characterization of Methanosarcina barkeri Mutants Lacking Three Distinct Classes of
415 Hydrogenase. *J. Bacteriol.* 200, e00342-18. doi:10.1128/JB.00342-18.

416 McGlynn, S. E. (2017). Energy Metabolism during Anaerobic Methane Oxidation in ANME Archaea.
417 *Microbes Environ.* 32, 5–13. doi:10.1264/jsme2.ME16166.

418 McGlynn, S. E., Chadwick, G. L., Kempes, C. P., and Orphan, V. J. (2015). Single cell activity reveals
419 direct electron transfer in methanotrophic consortia. *Nature* 526, 531–535.
420 doi:10.1038/nature15512.

421 Meuer, J., Kuettner, H. C., Zhang, J. K., Hedderich, R., and Metcalf, W. W. (2002). Genetic analysis of
422 the archaeon Methanosarcina barkeri Fusaro reveals a central role for Ech hydrogenase and
423 ferredoxin in methanogenesis and carbon fixation. *Proc. Natl. Acad. Sci.* 99, 5632–5637.
424 doi:10.1073/pnas.072615499.

425 Mitov, M., Chorbadzhiyska, E., Rashkov, R., and Hubenova, Y. (2012). Novel nanostructured

electrocatalysts for hydrogen evolution reaction in neutral and weak acidic solutions. *Int. J. Hydrogen Energy* 37, 16522–16526. doi:10.1016/J.IJHYDENE.2012.02.102.

Morris, B. E. L., Henneberger, R., Huber, H., and Moissl-Eichinger, C. (2013). Microbial syntrophy: interaction for the common good. *FEMS Microbiol. Rev.* 37, 384–406. doi:10.1111/1574-6976.12019.

Otero, F. J., Chan, C. H., and Bond, D. R. (2018). Identification of Different Putative Outer Membrane Electron Conduits Necessary for Fe(III) Citrate, Fe(III) Oxide, Mn(IV) Oxide, or Electrode Reduction by Geobacter sulfurreducens Downloaded from. *J. Bacteriol.* 200, 347–365. doi:10.1128/JB.00347-18.

Rotaru, A.-E., Calabrese, F., Stryhanyuk, H., Musat, F., Shrestha, P. M., Weber, H. S., et al. (2018a). Conductive Particles Enable Syntrophic Acetate Oxidation between Geobacter and Methanosarcina from Coastal Sediments. *MBio* 9, 1–14. doi:10.1128/mBio.00226-18.

Rotaru, A.-E., Posth, N. R., Miracle, M. R., Vicente, E., Cox, R. P., Thompson, J., et al. (2018b). Interspecies interactions mediated by conductive minerals in the sediments of the ferruginous Lake La Cruz, Spain. *Limnetica*, 1–30.

Rotaru, A.-E., Shrestha, P. M., Liu, F., Markovaite, B., Chen, S., Nevin, K. P., et al. (2014a). Direct Interspecies Electron Transfer between Geobacter metallireducens and Methanosarcina barkeri. *Appl. Environ. Microbiol.* 80, 4599–4605. doi:10.1128/AEM.00895-14.

Rotaru, A.-E., Shrestha, P. M., Liu, F., Shrestha, M., Shrestha, D., Embree, M., et al. (2014b). A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to Methanosaeta for the reduction of carbon dioxide to methane. *Energy Environ. Sci.* 7, 408–415. doi:10.1039/C3EE42189A.

Rotaru, A.-E., Woodard, T. L., Nevin, K. P., and Lovley, D. R. (2015). Link between capacity for current production and syntrophic growth in Geobacter species. *Front. Microbiol.* 6, 1–8. doi:10.3389/fmicb.2015.00744.

Salvador, A. F., Martins, G., Melle-Franco, M., Serpa, R., Stams, A. J. M., Cavaleiro, A. J., et al. (2017). Carbon nanotubes accelerate methane production in pure cultures of methanogens and in a syntrophic coculture. *Environ. Microbiol.* 19, 2727–2739. doi:10.1111/1462-2920.13774.

Schlegel, K., Welte, C., Deppenmeier, U., and Müller, V. (2012). Electron transport during aceticlastic methanogenesis by Methanosarcina acetivorans involves a sodium-translocating Rnf complex. *FEBS J.* 279, 4444–4452. doi:10.1111/febs.12031.

Seiler, M., Mehrle, A., Poustka, A., and Wiemann, S. (2006). The 3of5 web application for complex and comprehensive pattern matching in protein sequences. *BMC Bioinformatics* 7, 1–12. doi:10.1186/1471-2105-7-144.

Shimizu, S., Upadhye, R., Ishijima, Y., and Naganuma, T. (2010). Methanosarcina horonobensis sp. nov., a methanogenic archaeon isolated from a deep subsurface Miocene formation. *Int. J. Syst. Evol. Microbiol.* 61, 2503–2507. doi:10.1099/ijs.0.028548-0.

Shrestha, P. M., and Rotaru, A.-E. (2014). Plugging in or going wireless: strategies for interspecies electron transfer. *Front. Microbiol.* 5, 1–8. doi:10.3389/fmicb.2014.00237.

Shrestha, P. M., Rotaru, A.-E., Aklujkar, M., Liu, F., Shrestha, M., Summers, Z. M., et al. (2013). Syntrophic growth with direct interspecies electron transfer as the primary mechanism for energy exchange. *Environ. Microbiol. Rep.* 5, 904–910. doi:10.1111/1758-2229.12093.

Smith, T. J., and Stevenson, K. J. (2007). “Reference electrodes,” in *Handbook of Electrochemistry* (Elsevier B.V.), 73–110. doi:10.1016/B978-0-444-51958-0.50005-7.

Stams, A. J. M., and Plugge, C. M. (2009). Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat. Rev. Microbiol.* 7, 568–577. doi:10.1038/nrmicro2166.

Suharti, S., Wang, M., De Vries, S., and Ferry, J. G. (2014). Characterization of the RnfB and RnfG subunits of the Rnf complex from the archaeon Methanosarcina acetivorans. *PLoS One* 9, 1–10. doi:10.1371/journal.pone.0097966.

Thauer, R. K., Kaster, A.-K., Seedorf, H., Buckel, W., and Hedderich, R. (2008). Methanogenic

476 archaea: ecologically relevant differences in energy conservation. *Nat. Rev. Microbiol.* 6, 579–591.
477 doi:10.1038/nrmicro1931.

478 Timmers, P. H. A., Welte, C. U., Koehorst, J. J., Plugge, C. M., Jetten, M. S. M., and Stams, A. J. M.
479 (2017). Reverse Methanogenesis and Respiration in Methanotrophic Archaea. *Archaea* 2017.
480 doi:10.1155/2017/1654237.

481 Van Der Zee, F. P., Bisschops, I. A. E., Lettinga, G., & Field, J. A. (2003). Activated carbon as an
482 electron acceptor and redox mediator during the anaerobic biotransformation of azo dyes. *Environ.*
483 *Sci. Technol.*, 37(2), 402–408. https://doi.org/10.1021/es025885o

484 Wang, F. P., Zhang, Y., Chen, Y., He, Y., Qi, J., Hinrichs, K.-U., et al. (2014). Methanotrophic archaea
485 possessing diverging methane-oxidizing and electron-Transporting pathways. *ISME J.* 8, 1069–
486 1078. doi:10.1038/ismej.2013.212.

487 Wang, M., Tomb, J.-F., and Ferry, J. G. (2011). Electron transport in acetate-grown Methanosarcina
488 acetivorans. *BMC Microbiol.* 11. doi:10.1186/1471-2180-11-165.

489 Wang, O., Zheng, S., Wang, B., Wang, W., and Liu, F. (2018). Necessity of electrically conductive pili
490 for methanogenesis with magnetite stimulation. *PeerJ* 6, e4541. doi:10.7717/peerj.4541.

491 Welte, C. U., and Deppenmeier, U. (2014). Bioenergetics and anaerobic respiratory chains of
492 aceticlastic methanogens. *Biochim. Biophys. Acta - Bioenerg.* 1837, 1130–1147.
493 doi:10.1016/j.bbabio.2013.12.002.

494 Ye, Q., Zhang, Z., Huang, Y., Fang, T., Cui, Q., He, C., et al. (2018). Enhancing electron transfer by
495 magnetite during phenanthrene anaerobic methanogenic degradation. *Int. Biodeterior. Biodegrad.*,
496 0–1. doi:10.1016/j.ibiod.2018.01.012.

497 Zhang, L., Zhang, J., and Loh, K. C. (2018). Activated carbon enhanced anaerobic digestion of food
498 waste – Laboratory-scale and Pilot-scale operation. *Waste Manag.* 75, 270–279.
499 doi:10.1016/j.wasman.2018.02.020.

500 Zhang, P., Zheng, S., Liu, J., Wang, B., Liu, F., & Feng, Y. (2018). Surface properties of activated

sludge-derived biochar determine the facilitating effects on Geobacter co-cultures. *Water Res.*, 142, 441–451. https://doi.org/10.1016/j.watres.2018.05.058

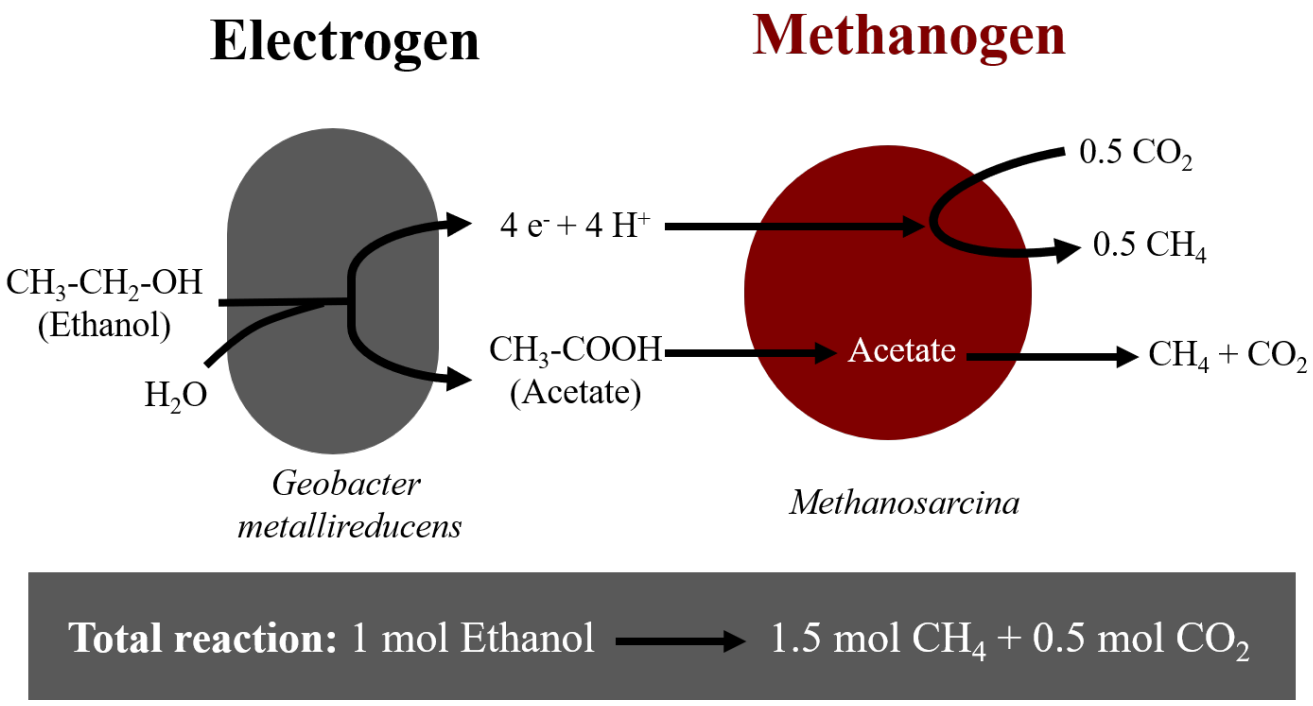
Zhang, Y., Feng, H., Wu, X., Wang, L., Zhang, A., Xia, T., … Zhang, L. (2009). Progress of electrochemical capacitor electrode materials: A review. *Int. J. Hydrogen Energy*, 34(11), 4889–4899. https://doi.org/10.1016/j.ijhydene.2009.04.005

Zheng, S., Zhang, H., Li, Y., Zhang, H., Wang, O., Zhang, J., et al. (2015). Co-occurrence of Methanosarcina mazei and Geobacteraceae in an iron (III)-reducing enrichment culture. *Front. Microbiol.* 6, 1–12. doi:10.3389/fmicb.2015.00941.

Zhuang, L., Tang, J., Wang, Y., Hu, M., and Zhou, S. (2015). Conductive iron oxide minerals accelerate syntrophic cooperation in methanogenic benzoate degradation. *J. Hazard. Mater.* 293, 37–45. doi:10.1016/j.jhazmat.2015.03.039.

513 **Figures**

514 (Fig. 1) Diagram of electron flow during DIET interactions between *Geobacter metallireducens* and
515 *Methanosarcina*-species provided with ethanol as sole electron donor.



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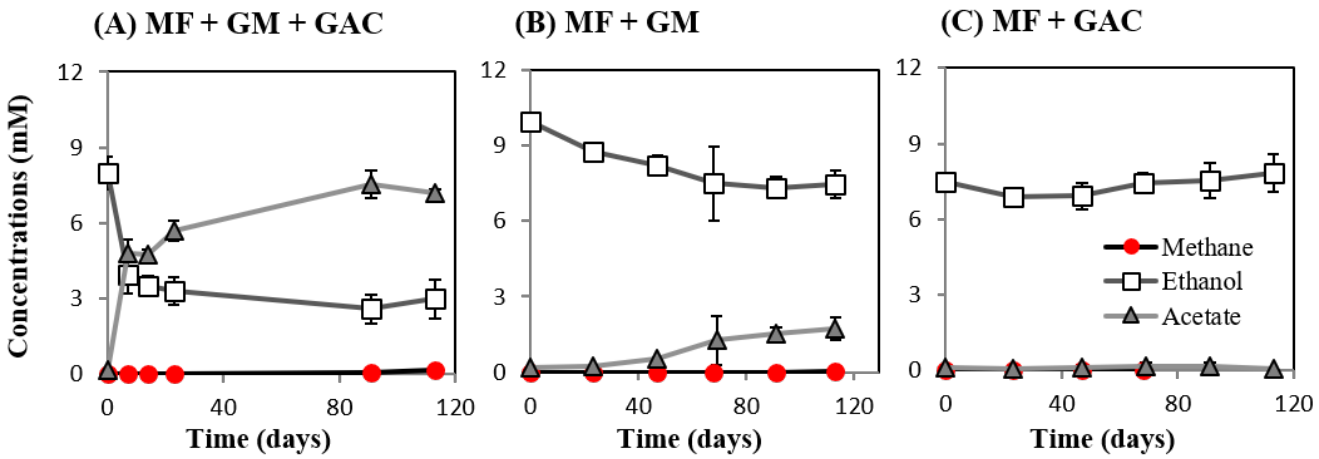
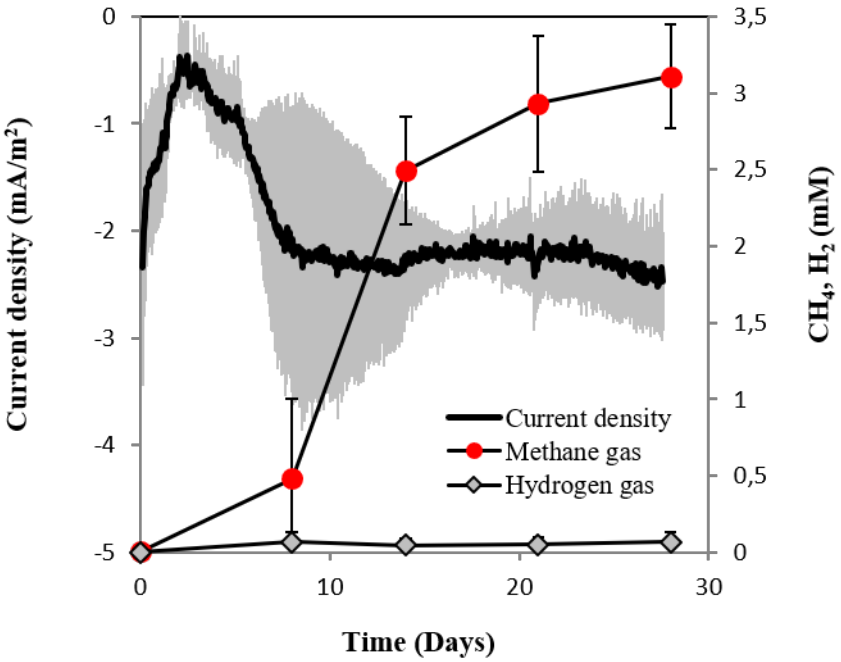
519 (Fig. 2) Co-cultures experiments with *M. formicicum* and *G.metallireducens* (n=3). *M. formicicum* did
520 not produce methane when incubated with *G. metallireducens* in the presence (A) or absence of GAC
521 (B). Alone, *M. formicicum* could not utilize ethanol or produce methane in the presence of GAC. MF -
522 *Methanobacterium formicicum*, GM - *Geobacter metallireducens*, GAC - granular activated carbon.



523

524 (Fig. 3) Current consumption and gas production in triplicate *M. barkeri* cultures provided with a poised
525 cathode at - 400 mV (vs. SHE) as sole electron donor.



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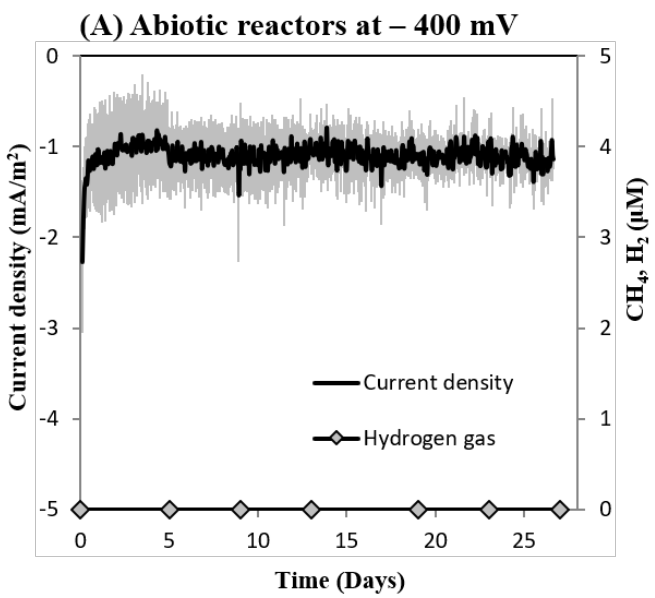
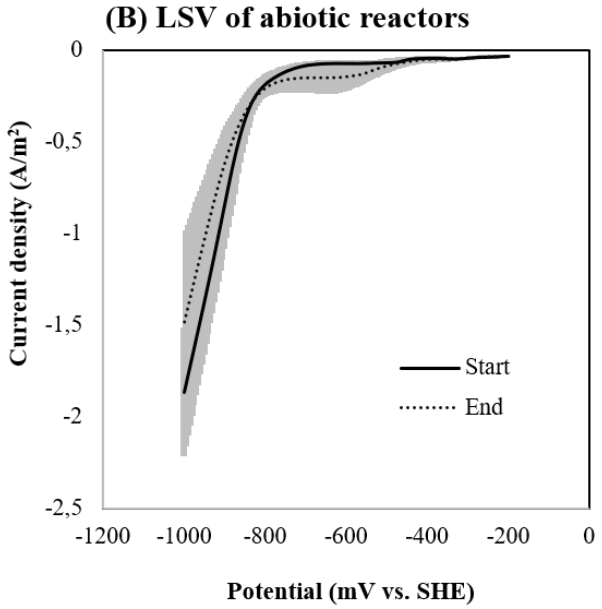
527 (Fig. 4) (A) Current consumption and gas production in four abiotic reactors at -400 mV (vs. SHE) and
528 (B) Linear Sweep Voltammetry (LSV) of abiotic reactors at the start and end of the experiment (n=3).





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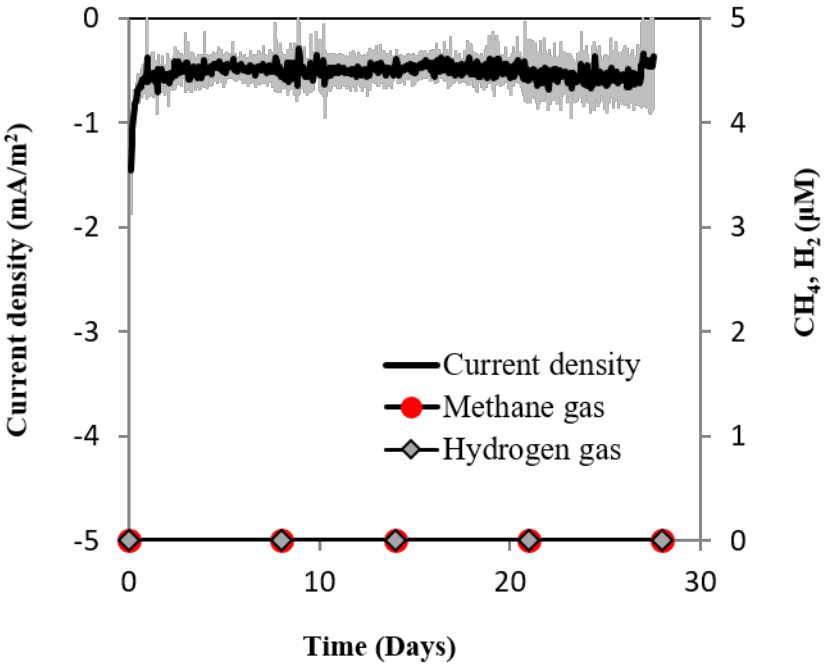
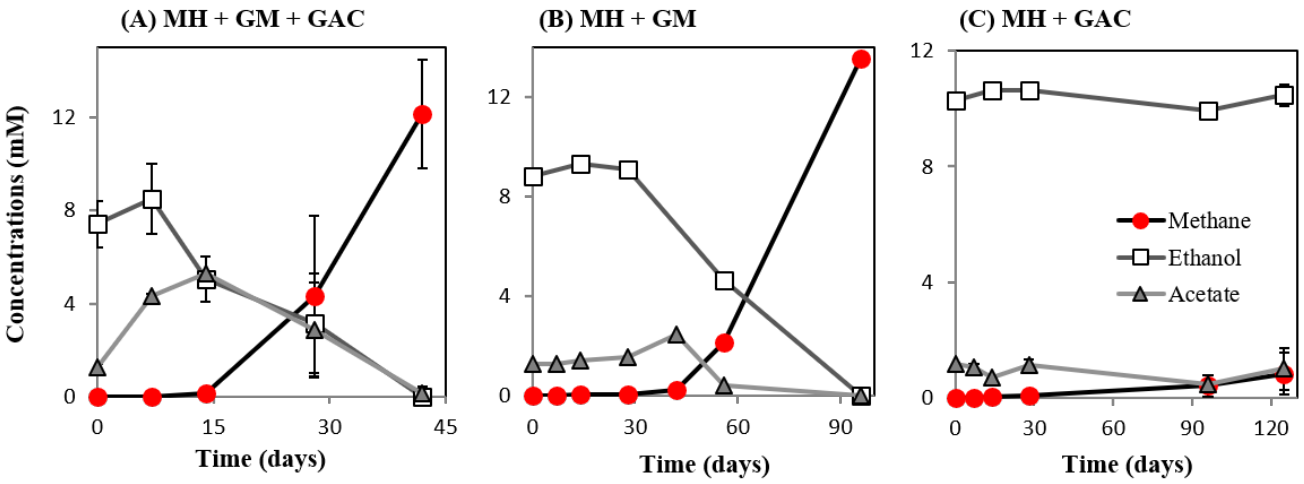
531 (Fig. 5) Current consumption and gas production in triplicate *M. formicicum* cultures provided with a
532 cathode poised at - 400 mV (vs. SHE) as sole electron donor.

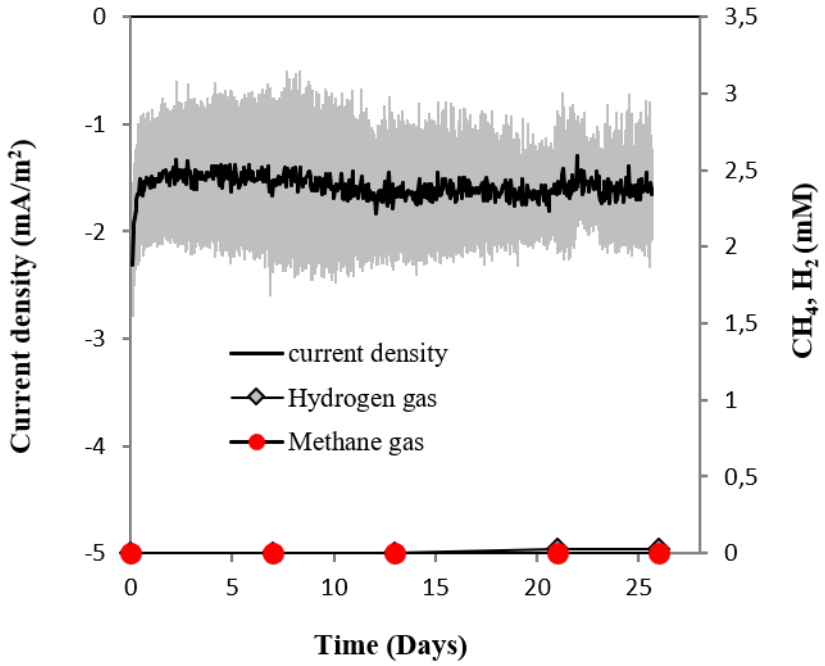


533

(Fig. 6) Co-culture experiments with *M. horonobensis* and *G. metallireducens*. *M. horonobensis* established successful co-cultures with G. metallireducens as apparent from ethanol utilization and methane production in the presence (A, n=3) or absence of GAC (B, n=1; see replication in Fig. S6). Alone, *M. horonobensis* could not utilize ethanol or produce methane in the presence of GAC (C, n=3). MH - *Methanosarcina horonobensis*, GM - *Geobacter metallireducens*, GAC - granular activated carbon.



541 (Fig. 7) Current consumption and gas production in cultures of *M. horonobensis* (n=3) provided with a
542 cathode poised at - 400 mV (vs. SHE) as sole electron donor.



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545 **Tables**

546 (Table 1) Relevant genotypic differences between the methanogens tested during this study

Species	Energy conservation	S-layer proteins	Predicted c-type cytochromes (CxxCH motif proteins)	Other cytochromes	Predicted Ferredoxins	Predicted thioredoxins
Methanosarcina barkeri MS	Ech-hydrogenase	8	20 (0/1 multiheme*)	3 (cyt b)	4	10
Methanosarcina horonobensis HB-1	Rnf-complex	9	30 (3 multiheme)	3 (cyt b)	6	8
Methanobacterium formicicum DSM1535	EhaA/EhbA hydrogenase	None	16 (None)	None	4	2

*The predicted multiheme cytochrome in *M. barkeri* strain MS had one standard CxxCH and one CxCH motif.

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549 (Table 2) Genomic comparison of three methanogens based on TIGR family protein categories.

TIGRfam Categories	No. of genes associated within a TIGR family		
	Methanosarcina horonobensis	***Methanosarcina barkeri***	***Methanobacterium formicicum***
Fatty acid and phospholipid metabolism	3	4	3
Transcription	13	12	13
Central intermediary metabolism	21	27	21
- Nitrogen fixation	7	13	7
Cell processes	26	18	22
Cell envelope	28	27	14
- Surface structures (S-layer)	9	8	0
Purines, pyrimidines, nucleosides, and nucleotides	33	33	33
Mobile and extrachromosomal element functions	39	2	2
- Transposons	32	2	0
DNA metabolism	43	38	27
Protein fate	48	44	33
Amino acid biosynthesis	56	57	57
Biosynthesis of cofactors, prosthetic groups, and carriers	60	61	65
- Heme, prophirin, cobalamin	22	25	19
Regulatory functions	84	33	51
- Small molecule interactions	77	27	40
Protein synthesis	87	89	75
Energy metabolism	95	86	64
- Electron transport proteins	21	17	7
Transport and binding proteins	97	85	63
- Iron carrying compounds	51	44	29
- Aminoacids and amines	17	12	0
Unknown and hypothetical	119	78	92

550