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 *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 *Methanobacterium* and the two *Methanosarcinas* revealed a higher abundance of genes associated with extracellular electron transfer in *Methanosarcina* species. Between the two *Methanosarcina* we observed 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 (i.e. *Geobacter*), which can modulate its surface redox potentials by adjusting the expression of electroactive surface proteins.

Introduction

Methanogenesis or biological methane formation by methanogenic archaea is a vital process in the global carbon cycle (Falkowski et al., 2008). As such, the relatively recent discovery of direct external electron transfer in methanogens may have important implications. A few studies have shown that methanogens belonging to *Methanosarcinales* are able to accept electrons directly from an electrogenic partner by Direct Interspecies Electron Transfer (DIET) via conductive proteins located on the surface of the electrogen (Rotaru et al., 2014a, 2014b). The only methanogens shown to date to carry out DIET belong to *Methanosarcinales*– *Methanosarcina barkeri* (Rotaru et al., 2014a) and *Methanothrix harundinacea* (Rotaru et al., 2014b). Both methanogenic species formed aggregates when co-cultured with *Geobacter metallireducens*, emphasizing the need for physical contact. However, the mechanism

40 employed by DIET methanogens to retrieve electrons from the electrogen is poorly understood. A
41 glimpse at this mechanism was provided in a recent comparative transcriptomic study (Holmes et. al,
42 2018, submitted). In this study the transcriptomes of DIET co-cultures (*G. metallireducens* – *M.*
43 *barkeri*) were compared to those of co-cultures performing interspecies H_2-transfer (*Pelobacter*
44 *carbinolicus* – *M. barkeri*). During DIET, *M. barkeri* had higher expression of membrane bound redox
45 active proteins like cupredoxins, thioredoxins, pyrroloquinoline, and quinone- ,cytochrome- or Fe-S
46 containing proteins (Holmes et al., 2018, submitted). Still, the exact mechanism of electron uptake
47 during DIET has not been validated and warrants further investigation.

48 Moreover, DIET interactions between *G. metallireducens* and *M. barkeri* were stimulated at the
49 addition of conductive particles, such as GAC (Liu et al., 2012), carbon cloth (Chen et al., 2014a),
50 biochar (Chen et al., 2014b), or magnetite (Wang et al., 2018). On the other hand, the addition of non-
51 conductive particles like glass beads (Rotaru et al., 2018) or cotton cloth did not stimulate DIET
52 associations (Chen et al., 2014a).

53 Numerous mixed methanogenic communities degrading various substrates were also stimulated by
54 electrically conductive particles made of carbon (Lee et al., 2016; Lin et al., 2017b; Rotaru et al., 2018;
55 Zhang et al., 2017), iron-oxide minerals (Cruz Viggi et al., 2014; Jing et al., 2017; Ye et al., 2018;
56 Zheng et al., 2015; Zhuang et al., 2015), and also stainless steel (Li et al., 2017). In this paper we will
57 call this phenomenon mineral-syntrophy or MIET (conductive particle-Mediated Interspecies Electron
58 Transfer). The methanogens mostly enriched at the addition of electrically conductive particles were
59 DIET-related methanogens like *Methanosarcina* sp. (Dang et al., 2016; Lei et al., 2016; Zheng et al.,
60 2015) or *Methanosaeta* sp. (Li et al., 2017; Zhao et al., 2017). However, exceptions were observed
61 since sometimes electrically conductive particles enriched for hydrogenotrophic methanogens such as

62 *Methanospirillum* sp. (Lee et al., 2016) or *Methanobacterium* sp. (Lin et al., 2017a; Zhuang et al.,
63 2015).

64 A hydrogenotrophic species belonging to the genus *Methanobacterium*, *M. palustris,* was later
65 suggested to carry out electromethanogenesis as it produced CH_4 when subjected to a cathode potential
66 of – 500 mV versus the standard hydrogen electrode (SHE) (Cheng et al., 2009). Although H_2 can be
67 theoretically generated electrochemically at -500 mV (redox potential of H^+/H_2 couple at pH 7 is – 414
68 mV) (Buckel and Thauer, 2013), the authors suggested that this methanogen could directly receive
69 electrons from the cathode, based on low hydrogen concentrations and high current density (Cheng et
70 al., 2009).

71 Afterwards, other studies have used less negative potentials around - 400 mV (vs. SHE) where H_2 is
72 unlikely to be generated electrochemically with carbon-based electrodes (Batlle-Vilanova et al., 2014).
73 Under such conditions, both a mixed hydrogenotrophic methanogenic community (Fu et al., 2015) and
74 a pure culture of a H_2-utilizing methanogen (*Methanobacterium sp.* strain IM1) (Beese-Vasbender et
75 al., 2015) were suggested to reduce CO_2 using cathodic electrons directly. Later studies on
76 *Methanococcus maripaludis*, demonstrated that extracellular enzymes would generate H_2 and formate
77 enabling hydrogenotrophic methanogenesis on cathodes(Deutzmann et al., 2015; Lienemann et al.,
78 2018; Lohner et al., 2014).
79 Until now electromethanogenesis was only demonstrated for hydrogenotrophic methanogens. Yet, no
80 hydrogenotrophs were shown to be capable of DIET (Rotaru et al., 2014b). Conversely, none of the
81 two methanogens capable of DIET have been tested if they are capable of electron uptake from a
82 cathode. Here we investigate the ability to carry DIET and electromethanogenesis in three
83 methanogenic species, two *Methanosarcina* and a *Methanobacterium*. While both *Methanosarcina*

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

86 # Materials and Methods

87 ### Microorganism strains and cultivation conditions

88 *Methanosarcina barkeri* MS (DSM 800) and *Methanosarcina horonobensis* HB-1 (DSM 21571) were
89 purchased from the German culture collection DSMZ while *Methanobacterium formicicum* (NBRC
90 100475) was from the Japanese culture collection NBRC. *Geobacter metallireducens* GS-15 was sent
91 to us by Dr. Sabrina Beckmann from the University of New South Wales, Australia.

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

101 The co-cultures of *Geobacter* and methanogens were initiated with 0.5 mL of *G. metallireducens* and 1
102 mL of acetate-grown *Methanosarcina*-species or H_2-grown *M. formicicum* inoculated into 8.5 ml of the
103 media prepared as above. Incubations were carried out in 20 ml pressure vials. For the co-cultures
104 ethanol (10 mM) was added as 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., 2018).

Electrochemical set up and measurements

We used bioelectrochemical reactors with a standard dual chamber configuration as shown in **Fig. 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 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 bio-compatible 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. Because we noticed small gases like methane and hydrogen passed the cation permeable membrane between the chambers, headspace gas was sampled and measured from both the working and counter electrode chambers and the sum was considered as the total gas production. 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^{o}C, 70^{o}C and 200^{o}C respectively. The detection limit for CH_4 and H_2 was ca. 500 ppm for both. The concentration unit was converted to molarity by using the ideal gas law (pV = nRT) under standard conditions, where p = 1 atm, V is volume of the gaseous phase (L), n is amount of gas (mol), R is the gas constant (0.08205 atmL/molK) 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 mins at 60^{o}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^{o}C, 40^{o}C and 230^{o}C respectively. Short chained volatile fatty acids (VFA) were analysed 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 analysed 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. For searching the cytochrome motif (CxxCH), the 3of5

pattern matching application (Seiler et al., 2006) in addition to manual search was used to scan through all the genomes.

Results and Discussion

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

A strict hydrogenotroph, *Methanobacterium formicicum*, was unable to produce methane using extracellular electron uptake from cells or solid electron donors

M. formicicum was chosen as the representative hydrogenotrophic methanogen due to its low hydrogen uptake threshold (approximately 6 nM (Lovley, 1985)). Previously it was demonstrated that the electrogen *G. metallireducens* could not establish DIET co-cultures with the hydrogenotroph *M. formicicum* even after 6 months of incubation (Rotaru et al., 2014b). This result was anticipated because *G. metallireducens* is a respiratory organism without the genetic potential to produce H_2 (Aklujkar et al., 2009; Shrestha et al., 2013), which was also demonstrated in early physiological experiments (Cord-Ruwisch et al., 1998). However, it is unknown if conductive particles might enable

191 interactions between a strict hydrogenotroph like *M. formicicum* and *G. metallireducens*. This became
192 especially relevant because hydrogenotrophic methanogenesis by *M. formicicum* was enhanced by
193 conductive carbon nanotubes (Salvador et al., 2017). Thus in this study we tested if *M. formicicum* was
194 capable to establish syntrophic co-cultures with *G. metallireducens* via conductive GAC.

195 *M. formicicum* co-cultured with the exoelectrogen *G. metallireducens* did not generate methane even
196 after 120 days regardless of the presence or absence of conductive particles **(Fig. 1A & B)**. Conductive
197 GAC did not aid the abiotic oxidation of ethanol **(Fig. 1C)**. Control experiments with only ethanol-fed
198 *G. metallireducens* provided with GAC, revealed that *Geobacter* oxidized ethanol incompletely to 6.72
199 ± 0.02 mM acetate **(Fig. S2).** Incomplete oxidation of ethanol also happened in GAC amended co-
200 cultures of *Geobacter* and *M. formicicum* **(Fig. 1A)**. GAC was likely used as a poor electron acceptor
201 by *G. metallireducens.* Without GAC, *Geobacter* could not oxidize ethanol alone (Shrestha et al.,
202 2013). *M. formicicum* did not utilize ethanol alone in the presence or absence of conductive particles
203 (data not shown).

204 We further used *M. formicicum* to test whether cryptic electrogenic hydrogen could fuel
205 methanogenesis in our bioelectrochemical setup. It is well accepted that electrochemical hydrogen gas
206 evolution from a graphite electrode is unlikely at - 400 mV under physiological conditions due to high
207 overpotentials (Batlle-Vilanova et al., 2014; Beese-Vasbender et al., 2015). Indeed, hydrogen gas was
208 not detected in abiotic reactors at – 400 mV except at day 27, when it reached at most 0.16 ± 0.23 μM
209 **(Fig. 2A)**. Still, since the inoculum was a mid-exponential active culture, there was a possibility that
210 microbial enzymes attach onto the electrode to produce hydrogen or formate (Deutzmann et al., 2015).
211 *M. formicicum* is particularly well-suited for such a test because, the H_2 threshold of such a strict
212 hydrogenotroph is lower than that of *Methanosarcina*-methanogens (Thauer et al., 2008, 2010). For

instance, the dissolved H_2 threshold of the hydrogenotrophic *M. formicicum* (~ 6 nM) is ~60 times lower than that of *M. barkeri* (296 nM – 376 nM) (Kral et al., 1998; Lovley, 1985). In electrochemical reactors with *M. formicicum*, no hydrogen or methane was detected and no substantial current draw was observed at – 400 mV **(Fig. 2B).** Thus, *M. formicicum* could not carry electromethanogenesis neither via electrochemical nor enzyme-mediated H_2 in our bioelectrochemical set-up.

The non-hydrogenotrophic *Methanosarcina horonobensis* produced methane using extracellular electron uptake from *Geobacter* directly or via conductive particles

The non-hydrogenotrophic, *Methanosarcina horonobensis* paired syntrophically with *G. metallireducens* with or without conductive particles as electric conduit **(Fig 3A & B)**. This is the second *Methanosarcina* species shown to be capable of electron uptake from *Geobacter* (Rotaru et al., 2014a) and the second non-hydrogenotroph besides *Methanothrix harundinacea* capable of DIET (Rotaru et al., 2014b). Theoretically, *G. metallireducens* could oxidize ethanol to acetate only if they could use the methanogen as an electron acceptor (Eqn. 3 & 4). The acetate is then further disproportionated by the acetoclastic methanogens to produce methane and CO_2 (Eqn. 5). During DIET we expect a conversion of 1 mol ethanol to 1.5 mol methane according to equations 3 to 5. 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 syntroph or the methanogen alone **(Fig. 3C & S2)**.

234 $2C_2H_6O + 2H_2O \rightarrow 2C_2H_4O_2 + 8H^+ + 8e^-$ **Eqn. (3)**

235 $CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$ **Eqn. (4)**

236 $2C_2H_4O_2 \rightarrow 2CH_4 + 2CO_2$ **Eqn. (5)**

237 Co-cultures of *G. metallireducens* and *M. horonobensis* could not carry interspecies hydrogen transfer
238 1) because *G. metallireducens* is a strict respiratory microrganism which cannot ferment ethanol to
239 generate hydrogen (Shrestha et al., 2013) and 2) because *M. horonobensis* is unable to use H_2 or
240 formate as electron donors for their metabolism (Shimizu et al., 2010). Co-cultures of *G.*
241 *metallireducens* and *M. barkeri* with and without GAC were run in parallel and we found that the time
242 taken to establish these syntrophic interactions were similar among the two co-cultures **(Fig. S3)** as
243 well as to previous studies (Liu et al., 2012; Rotaru et al., 2014a)**.**

244 **Electroactivity of *Methanosarcina* species**

245 The ability of *Methanosarcina barkeri* and *Methanosarcina horonobensis* to grow by MIET or DIET
246 with *G. metallireducens* suggested that they are capable of extracellular electron uptake. We tested this
247 by providing the two species solely with an electrode poised at – 400 mV as electron donor. Only
248 *Methanosarcina barkeri* was capable of methanogenesis under these conditions. This is the first
249 demonstration of a *Methanosarcina* acting as an electrotroph when provided with a cathode as the sole
250 source of electrons.

251 *Methanosarcina barkeri* produced significantly more methane (approximately 2.2 ± 0.4 times) using an
252 electrode as sole electron donor in reactors poised at - 400 mV compared to open circuit control
253 reactors **(Fig. 4A)**. In all the triplicate reactors, the methane production was significantly higher

(P<0.001) from day 14 onwards until it plateaued. A similar trend was reflected in the current consumed. The hydrogen concentrations after day 7 remained very low (0.039 ± 0.008 mM). Methane production in the open-circuit control was attributed to substrates carried over in the inoculum. Since both control reactors and poised reactors had the same amount of carry-over substrates, circa 2.58 ± 0.3 mM of methane could be solely credited to the addition of electricity.

Surprisingly, *Methanosarcina horonobensis* which could grow by DIET and MIET was incapable of electromethanogenesis **(Fig. 4B)**. Similar to *M. barkeri* the hydrogen gas concentrations were low (0.042 ± 0.02 mM) and H_2 accumulated a week later than with *M. barkeri*. To further explain why these two *Methanosarcina* species which were similarly capable of DIET and MIET behaved dissimilarly on cathodes poised at - 400 mV, we compared their genomes.

Genotypic characteristics of the two *Methanosarcina* versus *M. formicicum*

While both *Methanosarcina* species were able to carry out DIET and MIET, only *M. barkeri* produced methane by electromethanogenesis at - 400 mV **(Table 1)**. As a first step towards determing what could lead to differences in their ability to carry cathodic methanogenesis, we compared genotypic differences between the two species and against the hydrogenotroph *M. formicicum* **(Table 2 & 3).**

Compared to *M. formicicum*, both *Methanosarcina* encode in their genomes thrice the amount of electron transport genes and twice the amount of genes for cell surface proteins and transport **(Table 2 & 3).** Representing cell surface proteins, S-layer proteins were only present in *Methanosarcina* species but not in *Methanobacterium*. S-layer proteins have been previously proposed to play a role in extracellular electron transfer (EET) in *Methanosarcina* relatives such as the anaerobic methanotrophic archaea (ANME-2) (McGlynn, 2017; McGlynn et al., 2015; Timmers et al., 2017). Transport proteins

(iron carriers or aminoacid transporters) as well as thioredoxins and ferredoxins were also better represented in *Methanosarcina* genomes compared to *Methanobacterium* **(Table 3)**. The abundance of EET genes and surface proteins asumed to be involed in EET indicates that extracellular electron transport was indeed more likely to occur in the two *Methanosarcinas* than in *M. formicicum*.

When contrasting the genomes of the two *Methanosarcina* species we observed 79% average nucleotide identity (ANI) including predicted differences regarding iron transport, electron transport and nitrogen fixation **(Table 2)**. *M. horonobensis* encoded for more 23% more iron carriers, 16% more electron transport proteins (including three multiheme c-type cytochromes), 185% more small molecule interaction proteins and 16 times more mobile elements than *M. barkeri* **(Table 2)**. On the other hand, *M. barkeri* encoded for more 86% more N_2-fixation proteins and 13% more heme-biosynthesis proteins.

Beside these differences, we also observed significant differences regarding energy metabolism **(Table 3)**. *M. barkeri* utilizes hydrogen-cycling for its energy metabolism employing the energy-converting hydrogenase (Ech) as proton pump (Kulkarni et al., 2018). The differences in energy conservation were obvious with *M. barkeri* using the energy-converting hydrogenase (Ech), while *M. horonobensis* only encoded the Rnf complex (Buckel and Thauer, 2013, 2018; Meuer et al., 2002). Ech-hydrogenases couple 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). In contrast, the Rnf-complex is thought to produce a Na^+ ion gradient with NAD^+ in the process (Buckel and Thauer, 2018). To our knowledge, apart from *M. horonobensis*, the Rnf complex is only described in three other *Methanosarcina* species, yet fully characherized solely in *M. acetivorans* (Li et al., 2006, 2007; Schlegel et al., 2012; Suharti et al., 2014). In the other two *Methanosarcinas, M. lacustris* and M.

297 *thermophila*, the Rnf-complex was only predicted via genome mining (Wang et al., 2011). The Rnf
298 complex was also described in the genomes of ANME-2 archaea – close relatives of
299 *Methanosarcinales* (Wang et al., 2014), which were foreseen to carry DIET with sulfate reducing
300 bacteria (McGlynn et al., 2015). These operational and energetic differences in the proteins used for
301 energy metabolism, suggests that *Methanosarcina* with variations in H+/Na+-pumping could carry
302 EET differently. Also the redox coupling of the Rnf and the Ech differs as one uses c-cytochromes and
303 the other Fe-S centers, which could impact the overall redox potential of the cell surface.

304 But why would differences in energy metabolism and cell surface matter for growth on electrodes but
305 not in co-culture with an electrogen? It is possible that *Geobacter* coordinates its cytochrome
306 expression depending on the DIET partner or conductive mineral. It has been shown that the *Geobacter*
307 were capable to modulate their redox potential by coordinated expresion of extracellular cytochromes
308 depending on the voltage applied or iron oxide (Chan et al., 2017; Ishii et al., 2018; Levar et al., 2017).
309 If *Geobacter* modulates the expression of extracellular cytochromes to match the redox active
310 molecules on the surface of the methanogen, the latter would not need to modulate its electron
311 accepting machinery during DIET/MIET. On the other hand, electromethanogenesis at a set potential (
312 – 400 mV in this study) is unlikely to match the redox requirements of each type of electroactive
313 *Methanosarcina*.

314 ## Implications

315 Our understanding of interspecies interactions impacts carbon cycling in both natural and man-made
316 environments. Recently environmentally relevant *Methanosarcinales* methanogens were shown to
317 interact via direct electron transfer with electrogenic *Geobacter* (Rotaru et al., 2014a, 2014b, 2015)

318 However, *Methanosarcina* have never been shown to carry electron uptake from an electrode. Here we
319 demonstrated that a *Methanosarcina* capable of DIET, *M. barkeri* could also retrieves electons from a
320 cathode, under non-hydrogenotrophic conditions.

321 The diversity of methanogens capable of DIET is poorly known, with only two species of
322 *Methanosarcinales* previously shown to do DIET (Rotaru et al., 2014a, 2014b). Here we demonstrated
323 that a third *Methanosarcina* species, the non-hydrogenotrophic *M. hornobensis* performed DIET with
324 *Geobacter*. However, *M. horonobensis* could not grow on a cathode with a fixed potential at – 400 mV.
325 Nevertheless, *M. horonobensis* is a better candidate for understanding electron uptake in DIET-fed
326 *Methanosarcina*. This is because *M. horonobensis* is fast growing as single cells rather than rossettes
327 on freshwater media, which makes it amenable for genetic studies. Besides, freshwater conditions are
328 compatible with downstream co-cultivation of the gene-deletion mutants together with *Geobacter*. This
329 is unlike *M. barkeri* which has to be grown on high salt media in order to obtain single cell colonies for
330 genetic studies (Kulkarni et al., 2009, 2018; Mand et al., 2018) and then adapted to freshwater
331 conditions prior to co-cultivation with *Geobacter*. This makes *M. horonobensis* a better candidate for
332 genetic studies to investigate in detail the mechanisms of electron uptake in *Methanosarcina* growing
333 by DIET or MIET.

334 ## Conclusion

335 Three methanogens were investigated for their ability to do DIET, MIET or electron uptake from
336 cathodes. Out of the two *Methanosarcina* tested, only the metabolically versatile *M. barkeri* was able
337 to carry out all three forms of electron uptake. To our knowledge, this is the first study to show a
338 *Methanosarcina* in pure culture performing electromethanogenesis. On the other hand, the strict

hydrogenotrophic *M. formicicum* was unable to receive electrons from any type of solid surfaces tested in this study. A closer look into their genomes showed fundamental differences; compared to *Methanobacterium*, the *Methanosarcina* appeared to be better equipped for extracellular electron transport encoding for S-layer proteins, but also more electron transport proteins and transport proteins in general. Between the two *Methanosarcina* we observed disparities in areas such as energy metabolism, extrachromosomal functions and ion (i.e. iron) transporters . While *M. horonobensis* participated only in DIET and MIET, the inability of electromethanogenesis is a matter requiring further exploration. Compared to *M. barkeri*, *M. horonobensis* is arguably a more suitable candidate as a methanogenic model organism for studying extracellular electron uptake mechanisms due to its amenable culture conditions for genetic studies. A detailed understanding of EET mechanisms in environmentally widespread *Methanosarcina*, will be important for our understanding of the global methane and carbon cycles as well as for in improving anaerobic digestion and biogas upgrading.

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Author contribution

MOY and AER conceived the study with support from BT and LDMO. MOY performed all experiments with support from OSW. MOY analysed the data with support from AER. MOY wrote the manuscript. All authors (MOY, AER, BT, OSW and LDMO) contributed to drafting and editing the manuscript.

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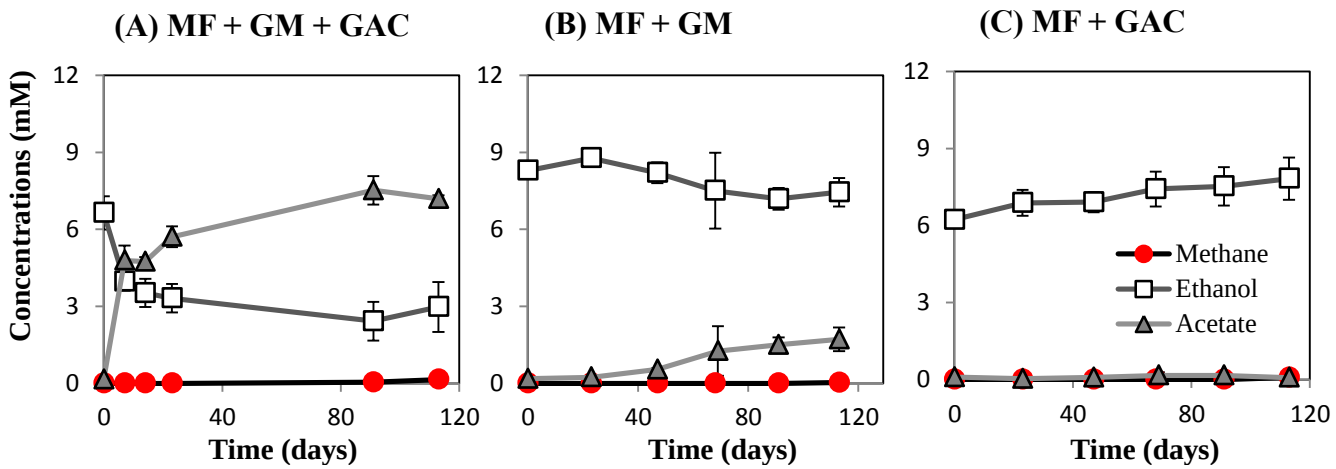
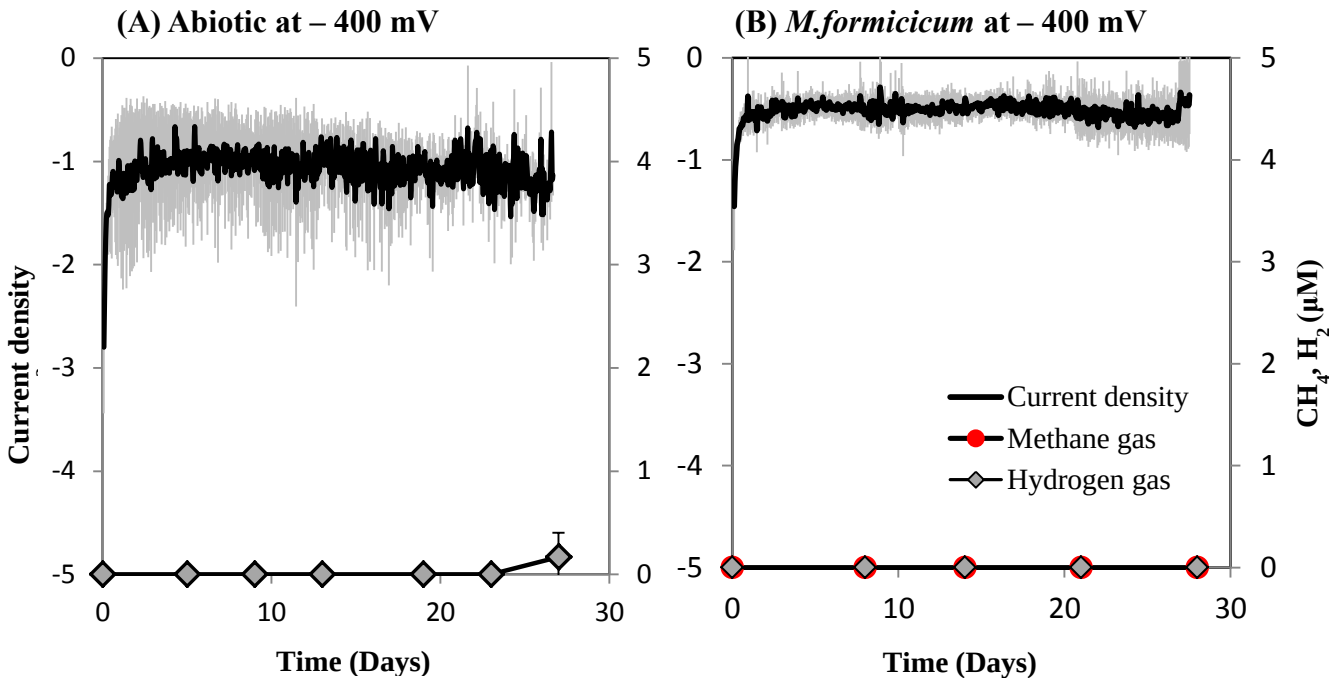
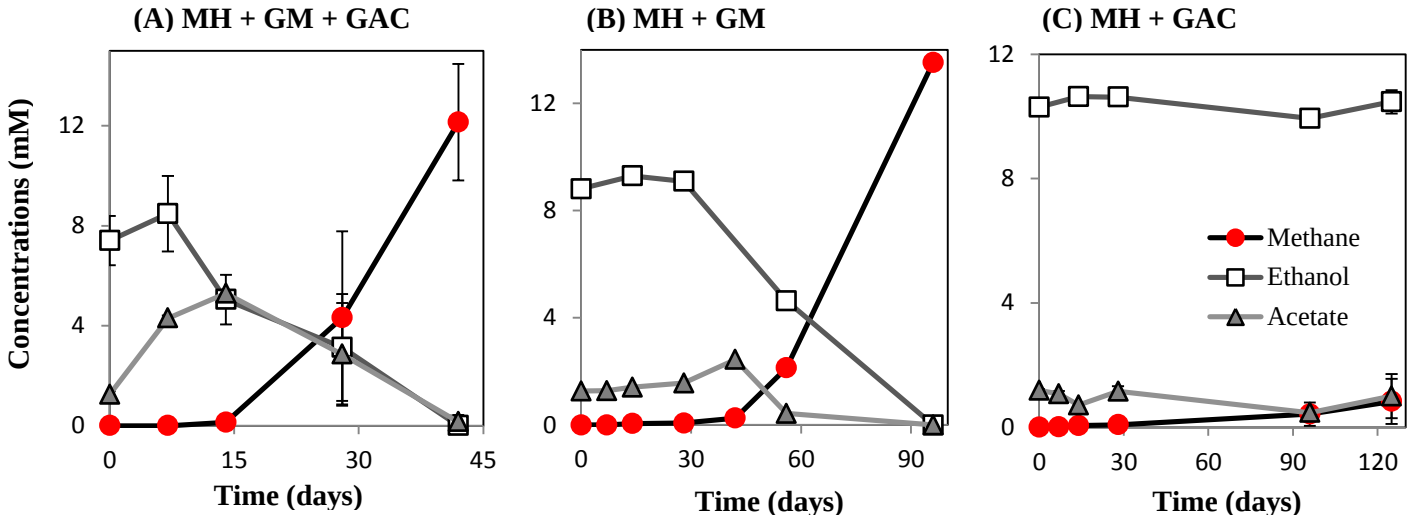


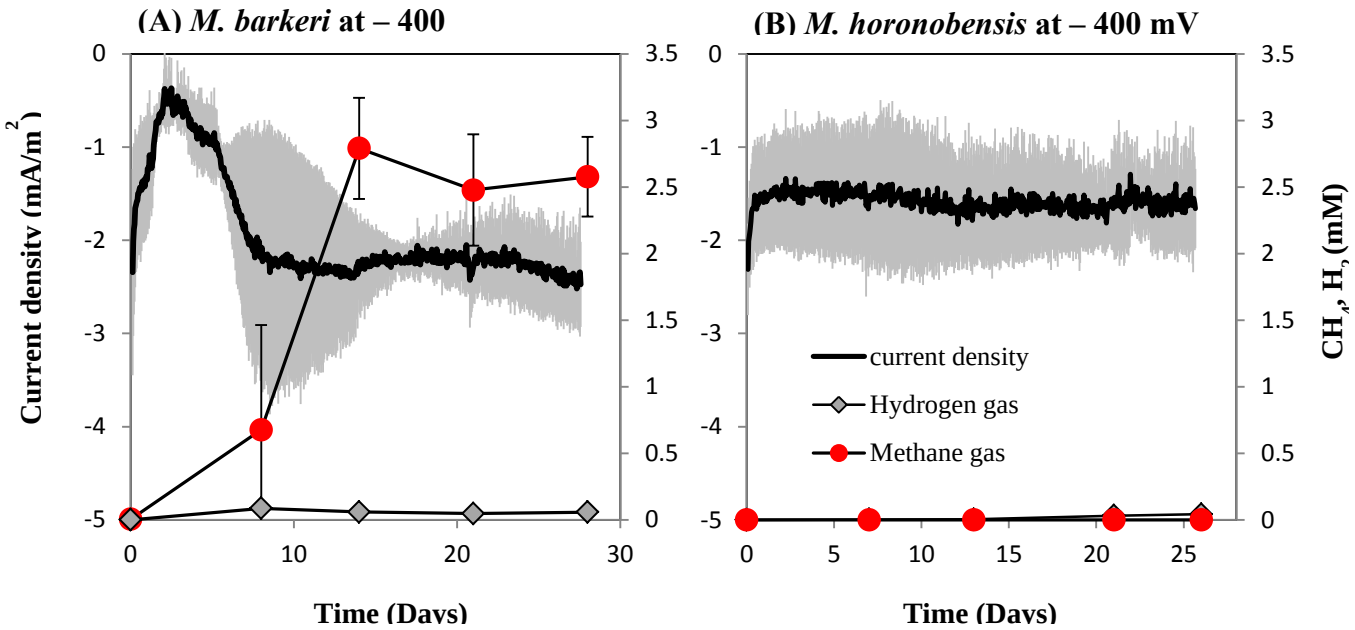
(Fig. 1) Co-cultures of *M. formicicum* with *G.metallireducens* feeding on ethanol with or without GAC. Error bars are based on standard deviation (n=3). MF; *Methanobacterium formicicum*, GM; *Geobacter metallireducens*, GAC; granular activated carbon, Circles; Methane, Squares; Ethanol, Triangles; Acetate .



(Fig. 2) Current consumption and gas production from (A) abiotic and (B) *M. formicicum* reactors at -400 mV (vs. SHE). Only the excess values of gases compared to that of the open circuit control are plotted here. Error bars are based on standard deviation (n=2 for abiotic, n=3 for *M.formicicum* reactors).



(Fig. 3) Co-cultures of *M. horonobensis* with *G. metallireducens* feeding on ethanol with or without GAC. MH; *Methanosarcina horonobensis*, GM; *Geobacter metallireducens*, GAC; granular activated carbon, Circles; Methane, Squares; Ethanol, Triangles; Acetate. The results are from triplicate samples except for (B) which is a representative sample for which the rest of the replicate data is presented in Fig S4.



(Fig. 4) Current consumption and gas production from (A) *M. barkeri* reactors and (B) *M. horonobensis* at - 400 mV (vs. SHE). Only the excess values of gases compared to that of the open circuit control are plotted here. Error bars are based on standard deviation (n=3).

602 **Table 1**. Phenotypic differences between the methanogens tested during this study

Species	Max. DIET methanogenesis rate	Max. MIET methanogenesis rate	Max. Electromethanogenesis at – 400 mV (vs. SHE)	Growth on H_2	Morphology
Methanosarcina barkeri MS	0.2 ± 0.06 mM/day CH_4 (lag-phase ca. 42 days)	0.98 ± 0.15 mM/day CH_4 (lag-phase ca. 7 days)	0.17 ± 0.03 mM/day CH_4	Yes	Cocci aggregated into rosettes (Bryant and Boone, 1987)
Methanosarcina horonobensis HB-1	0.29 ± 0.04 mM/day CH_4 (lag-phase ca. 42 days)	0.64 ± 0.24 mM/day CH_4 (lag-phase ca. 14 days)	No	No	Single irregular cocci (Shimizu et al., 2010)
Methanobacterium formicicum DSM1535	No	No	No	Yes	Rods (Ferry, 1993)

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608 **(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
Cell processes	26	18	22
Cell envelope	28	27	14
Purines, pyrimidines, nucleosides, and nucleotides	33	33	33
Mobile and extrachromosomal element functions	39	2	2
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
Regulatory functions	84	33	51
Protein synthesis	87	89	75
Energy metabolism	95	86	68
Transport and binding proteins	97	85	63
Unknown and hypothetical	119	78	92

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611 **Table 3**. Relevant genotypic differences between the methanogens tested during this study

Species	Energy conservation	S-layer proteins	Sum of electron transfer proteins	Predicted c-type cytochromes (CxxCH motif proteins)	Other cytochromes	Predicted Ferredoxins	Predicted thioredoxins
Methanosarcina barkeri MS	Ech-hydrogenase	8	37	20 (0/1 multiheme*)	3 (cyt b)	4	10
Methanosarcina horonobensis HB-1	Rnf-complex	9	47	30 (3 multiheme)	3 (cyt b)	6	8
Methanobacterium formicicum DSM1535	EhaA/EhbA hydrogenase	None	23	16 (None)	None	4	2

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

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