

1 **Extracellular electron uptake by two *Methanosarcina* species**

2 **Authors:**

3 Mon Oo Yee¹, Oona Snoeyenbos-West^{1,2,a}, Bo Thamdrup¹, Lars DM Ottosen², Amelia-Elena Rotaru^{1*}

4 ¹ Nordcee, Department of Biology, University of Southern Denmark, Odense, Denmark

5 ²Department of Engineering, University of Aarhus, Aarhus, Denmark

6 ^a(Current address) Department of Microbiology & Molecular Genetics, Michigan State University, East
7 Lansing, USA

8 *Correspondence to: arotaru@biology.sdu.dk

9 **Abstract**

10 Direct electron uptake by prokaryotes is a recently described mechanism with a potential application
11 for energy and CO₂ storage into value added chemicals. Members of *Methanosarcinales*, an
12 environmentally and biotechnologically relevant group of methanogens, were previously shown to
13 retrieve electrons from an extracellular electrogenic partner performing Direct Interspecies Electron
14 Transfer (DIET) and were therefore proposed to be electroactive. However, their intrinsic
15 electroactivity has never been examined. In this study, we tested two methanogens belonging to
16 *Methanosarcina*, *M. barkeri* and *M. horonobensis*, regarding their ability to accept electrons directly
17 from insoluble electron donors like other cells, conductive particles and electrodes. Both methanogens
18 were able to retrieve electrons from *Geobacter metallireducens* via DIET. Furthermore, DIET was also

19 stimulated upon addition of electrically conductive granular activated carbon (GAC) when each was
20 co-cultured with *G. metallireducens*. However, when provided with a cathode poised at -400 mV (vs
21 SHE), only *M. barkeri* could perform electromethanogenesis. In contrast, the strict hydrogenotrophic
22 methanogen, *Methanobacterium formicicum*, did not produce methane regardless of the type of
23 insoluble electron donor provided (*Geobacter* cells, GAC or electrodes). A comparison of functional
24 gene categories between *Methanobacterium* and the two *Methanosarcinas* revealed a higher abundance
25 of genes associated with extracellular electron transfer in *Methanosarcina* species. Between the two
26 *Methanosarcina* we observed differences regarding energy metabolism, which could explain
27 dissimilarities concerning electromethanogenesis at fixed potentials. We suggest that these
28 dissimilarities are minimized in the presence of an electrogenic DIET partner (i.e. *Geobacter*), which
29 can modulate its surface redox potentials by adjusting the expression of electroactive surface proteins.

30 **Introduction**

31 Methanogenesis or biological methane formation by methanogenic archaea is a vital process in the
32 global carbon cycle (Falkowski et al., 2008). As such, the relatively recent discovery of direct external
33 electron transfer in methanogens may have important implications. A few studies have shown that
34 methanogens belonging to *Methanosarcinales* are able to accept electrons directly from an electrogenic
35 partner by Direct Interspecies Electron Transfer (DIET) via conductive proteins located on the surface
36 of the electrogen (Rotaru et al., 2014a, 2014b). The only methanogens shown to date to carry out DIET
37 belong to *Methanosarcinales*– *Methanosarcina barkeri* (Rotaru et al., 2014a) and *Methanotherix*
38 *harundinacea* (Rotaru et al., 2014b). Both methanogenic species formed aggregates when co-cultured
39 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 H₂-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 CH₄ when subjected to a cathode potential
66 of – 500 mV versus the standard hydrogen electrode (SHE) (Cheng et al., 2009). Although H₂ can be
67 theoretically generated electrochemically at -500 mV (redox potential of H⁺/H₂ 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 H₂ 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 H₂-utilizing methanogen (*Methanobacterium* sp. strain IM1) (Beese-Vasbender et
75 al., 2015) were suggested to reduce CO₂ using cathodic electrons directly. Later studies on
76 *Methanococcus maripaludis*, demonstrated that extracellular enzymes would generate H₂ 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°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 H₂:CO₂ (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 N₂:CO₂ (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 H₂-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 CO₂ was the sole electron acceptor. When noted,

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

107 **Electrochemical set up and measurements**

108 We used bioelectrochemical reactors with a standard dual chamber configuration as shown in **Fig. S1**.
109 Two-chamber glass bottles were purchased from Adams & Chittenden Scientific Glass (USA) with
110 side ports fitted with butyl septa to allow for medium transfer, sampling, and introduction of a
111 reference electrode. Each chamber of the reactors had a total volume of 650 ml with a flange diameter
112 of 40 mm and the chambers were separated by a Nafion™ N117 proton exchange membrane (Ion
113 Power) held by an O-ring seal with a knuckle clamp.

114 Both the working and counter electrodes were made of graphite (Mersen MI Corp., Greenville USA)
115 with dimensions of 2.5 cm x 7.5 cm x 1.2 cm thus a total projected surface area of 61.5 cm². The
116 working and counter electrodes were coupled to titanium wires, which pierced through rubber stoppers
117 fitted into the main opening of each chamber. A 2 cm deep and 2 mm wide hole was drilled on the
118 short side of the electrode and a 12.5 cm long, 2 mm wide titanium rod (Alfa-aesar, DE) was inserted
119 and sealed from the outside with bio-compatible non-conductive epoxy. Electrodes with a resistance of
120 less than 10 Ω were used to ensure proper electrical connections. The assembled electrodes were
121 introduced into the chamber via the main opening and 2 mm-wide holes were drilled in the black
122 rubber stopper to allow access of the titanium rod. After autoclaving the reactors, sterile medium was
123 transferred into the reactors anaerobically and aseptically. Sterile (bleach and ethanol series) reference
124 electrodes were lodged through a side port in the working electrode chamber at a distance of about 1
125 cm from the surface of the working electrode. After lodging the electrodes, degassing with N₂:CO₂

126 (80:20) for circa 30 minutes in each reactor chamber ensured anaerobic conditions. When the pre-
127 cultures were in mid exponential phase, they were inoculated (20%) into fresh medium in the cathodic
128 chamber following sterile anoxic techniques to a final volume of 550 ml leaving a headspace of
129 approximately 100 mL in each chamber. The approximate cell numbers at the time of inoculation into
130 the electrochemical reactors for *M. barkeri*, *M. formicicum* and *M. horonobensis* were 2.6×10^7
131 cells/mL, 8.2×10^7 cells/mL and 6.7×10^6 cells/mL respectively. Cell counts were done with
132 microscopic examination using DAPI (1 μ g/mL) stained cells.

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

141 **Analytical measurements and calculations**

142 Headspace samples for CH₄ and H₂ analysis were taken with hypodermic needles and kept in airtight
143 exetainers until measurement. Because we noticed small gases like methane and hydrogen passed the
144 cation permeable membrane between the chambers, headspace gas was sampled and measured from
145 both the working and counter electrode chambers and the sum was considered as the total gas
146 production. Methane (CH₄) and hydrogen gas (H₂) were measured on a Trace 1300 gas chromatograph

147 (Thermo-Scientific) with a TracePLOT™ TG-BOND Msieve 5A column and a thermal conductivity
148 detector (TCD). The carrier gas was argon at a flow rate of 25 mL/min. The injector, oven and detector
149 temperatures were 150°C, 70°C and 200°C respectively. The detection limit for CH₄ and H₂ was ca.
150 500 ppm for both. The concentration unit was converted to molarity by using the ideal gas law ($pV =$
151 nRT) under standard conditions, where $p = 1$ atm, V is volume of the gaseous phase (L), n is amount of
152 gas (mol), R is the gas constant (0.08205 atmL/molK) and $T = 298.15$ K. For ethanol detection, 0.5 mL
153 samples were filtered (0.2 µm pore size) into appropriate sampling vials and were heated for 5 mins at
154 60°C. The headspace gas was then pass through the Trace 1300 gas chromatograph (Thermo-Scientific)
155 with a TRACE™ TR-Wax column and detected by a flame ionization detector (FID). Nitrogen gas at a
156 flow of 1 mL/min was used as the carrier and the injector, oven and detectors were kept at 220°C, 40°C
157 and 230°C respectively. Short chained volatile fatty acids (VFA) were analysed with a Dionex™ ICS-
158 1500 Ion Chromatography system, using a Dionex™ IonPac™ AS22 IC Column and a mixture of 1.4
159 mM NaHCO₃ and 4.5 mM Na₂CO₃ as the eluent fitted with an electron capture detector (ECD) at 30
160 mA.

161 **Genome comparison**

162 Genomes for all tested microorganisms were available at the JGI integrated microbial genomes and
163 microbiomes. Functional category comparisons and pairwise average nucleotide identity (ANI) were
164 determined using the IMG/M- “Compare Genomes” tools. The IMG genome IDs of the studied *M.*
165 *barkeri*, *M. horonobensis* and *M. formicicum* used were 2630968729, 2627854269 and 2645727909
166 respectively. The gene functions were analysed from the annotated names of all the protein coding
167 genes retrieved from the National Center for Biotechnology Information (NCBI) database. The
168 accession numbers used were NZ_CP009528, NZ_CP009516 and NZ_LN515531 for *M. barkeri*, *M.*
169 *horonobensis* and *M. formicicum* respectively. For searching the cytochrome motif (CxxCH), the 3of5

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

172 **Results and Discussion**

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

182 **A strict hydrogenotroph, *Methanobacterium formicicum*, was unable to produce methane using** 183 **extracellular electron uptake from cells or solid electron donors**

184 *M. formicicum* was chosen as the representative hydrogenotrophic methanogen due to its low hydrogen
185 uptake threshold (approximately 6 nM (Lovley, 1985)). Previously it was demonstrated that the
186 electrogen *G. metallireducens* could not establish DIET co-cultures with the hydrogenotroph *M.*
187 *formicicum* even after 6 months of incubation (Rotaru et al., 2014b). This result was anticipated
188 because *G. metallireducens* is a respiratory organism without the genetic potential to produce H₂
189 (Aklujkar et al., 2009; Shrestha et al., 2013), which was also demonstrated in early physiological
190 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.

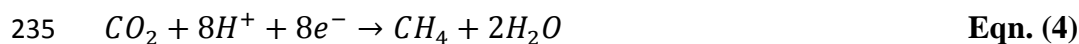
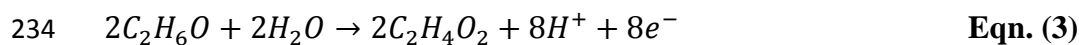
195 *M. formicicum* co-cultured with the exoelectrogen *G. metallireducens* did not generate methane even
196 after 120 days regardless of the presence or absence of conductive particles (**Fig. 1A & B**). Conductive
197 GAC did not aid the abiotic oxidation of ethanol (**Fig. 1C**). Control experiments 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 μ M
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

213 instance, the dissolved H₂ threshold of the hydrogenotrophic *M. formicicum* (~ 6 nM) is ~60 times
214 lower than that of *M. barkeri* (296 nM – 376 nM) (Kral et al., 1998; Lovley, 1985). In electrochemical
215 reactors with *M. formicicum*, no hydrogen or methane was detected and no substantial current draw
216 was observed at – 400 mV (**Fig. 2B**). Thus, *M. formicicum* could not carry electromethanogenesis
217 neither via electrochemical nor enzyme-mediated H₂ in our bioelectrochemical set-up.

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

220 The non-hydrogenotrophic, *Methanosarcina horonobensis* paired syntrophically with *G.*
221 *metallireducens* with or without conductive particles as electric conduit (**Fig 3A & B**). This is the
222 second *Methanosarcina* species shown to be capable of electron uptake from *Geobacter* (Rotaru et al.,
223 2014a) and the second non-hydrogenotroph besides *Methanotrix harundinacea* capable of DIET
224 (Rotaru et al., 2014b). Theoretically, *G. metallireducens* could oxidize ethanol to acetate only if they
225 could use the methanogen as an electron acceptor (Eqn. 3 & 4). The acetate is then further
226 disproportionated by the acetoclastic methanogens to produce methane and CO₂ (Eqn. 5). During DIET
227 we expect a conversion of 1 mol ethanol to 1.5 mol methane according to equations 3 to 5. As
228 predicted, in the *G. metallireducens* – *M. horonobensis* co-cultures, the syntroph oxidized 8.8 ± 0.4
229 mM ethanol providing the reducing equivalents (directly and via acetate) to generate 13.1 ± 0.8 mM
230 CH₄ by the methanogen. These co-cultures achieved stoichiometric recoveries of 98.5 ± 3.3 %. Similar
231 recoveries (109 ± 18.5 %) were also observed at the addition of conductive particles. Single species
232 controls with GAC showed that ethanol could not be converted to methane by the syntroph or the
233 methanogen alone (**Fig. 3C & S2**).



237 Co-cultures of *G. metallireducens* and *M. horonobensis* could not carry interspecies hydrogen transfer
238 1) because *G. metallireducens* is a strict respiratory microorganism which cannot ferment ethanol to
239 generate hydrogen (Shrestha et al., 2013) and 2) because *M. horonobensis* is unable to use H₂ 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

254 (P<0.001) from day 14 onwards until it plateaued. A similar trend was reflected in the current
255 consumed. The hydrogen concentrations after day 7 remained very low (0.039 ± 0.008 mM). Methane
256 production in the open-circuit control was attributed to substrates carried over in the inoculum. Since
257 both control reactors and poised reactors had the same amount of carry-over substrates, circa $2.58 \pm$
258 0.3 mM of methane could be solely credited to the addition of electricity.

259 Surprisingly, *Methanosarcina horonobensis* which could grow by DIET and MIET was incapable of
260 electromethanogenesis (**Fig. 4B**). Similar to *M. barkeri* the hydrogen gas concentrations were low
261 (0.042 ± 0.02 mM) and H₂ accumulated a week later than with *M. barkeri*. To further explain why these
262 two *Methanosarcina* species which were similarly capable of DIET and MIET behaved dissimilarly on
263 cathodes poised at - 400 mV, we compared their genomes.

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

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

269 Compared to *M. formicicum*, both *Methanosarcina* encode in their genomes thrice the amount of
270 electron transport genes and twice the amount of genes for cell surface proteins and transport (**Table 2**
271 **& 3**). Representing cell surface proteins, S-layer proteins were only present in *Methanosarcina* species
272 but not in *Methanobacterium*. S-layer proteins have been previously proposed to play a role in
273 extracellular electron transfer (EET) in *Methanosarcina* relatives such as the anaerobic methanotrophic
274 archaea (ANME-2) (McGlynn, 2017; McGlynn et al., 2015; Timmers et al., 2017). Transport proteins

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

279 When contrasting the genomes of the two *Methanosarcina* species we observed 79% average
280 nucleotide identity (ANI) including predicted differences regarding iron transport, electron transport
281 and nitrogen fixation (**Table 2**). *M. horonobensis* encoded for more 23% more iron carriers, 16% more
282 electron transport proteins (including three multiheme c-type cytochromes), 185% more small molecule
283 interaction proteins and 16 times more mobile elements than *M. barkeri* (**Table 2**). On the other hand,
284 *M. barkeri* encoded for more 86% more N₂-fixation proteins and 13% more heme-biosynthesis
285 proteins.

286 Beside these differences, we also observed significant differences regarding energy metabolism (**Table**
287 **3**). *M. barkeri* utilizes hydrogen-cycling for its energy metabolism employing the energy-converting
288 hydrogenase (Ech) as proton pump (Kulkarni et al., 2018). The differences in energy conservation were
289 obvious with *M. barkeri* using the energy-converting hydrogenase (Ech), while *M. horonobensis* only
290 encoded the Rnf complex (Buckel and Thauer, 2013, 2018; Meuer et al., 2002). Ech-hydrogenases
291 couple the reduction of protons with ferredoxin (Fdx⁻) to the production of a proton motive force
292 according to the reaction: $\text{Fdx}^- (\text{red}) + 2\text{H}^+ \rightleftharpoons \text{Fdx} (\text{ox}) + \text{H}_2 + \Delta\mu\text{H}^+ / \Delta\mu\text{Na}^+$ (Thauer et al., 2008). In
293 contrast, the Rnf-complex is thought to produce a Na⁺ ion gradient with NAD⁺ in the process (Buckel
294 and Thauer, 2018). To our knowledge, apart from *M. horonobensis*, the Rnf complex is only described
295 in three other *Methanosarcina* species, yet fully characterized solely in *M. acetivorans* (Li et al., 2006,
296 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 expression 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 retrieve electrons 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. hornobensis* could not grow on a cathode with a fixed potential at -400 mV.
325 Nevertheless, *M. hornobensis* is a better candidate for understanding electron uptake in DIET-fed
326 *Methanosarcina*. This is because *M. hornobensis* is fast growing as single cells rather than rosettes
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. hornobensis* 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

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

351 **Acknowledgements**

352 This work was funded by the Innovationsfond grant number: 4106-00017. We would like to thank
353 Lasse Ørum Smidt and Heidi Grøn Jensen for lab assistance.

354 **Author contribution**

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

359 **References**

- 360 Aklujkar, M., Krushkal, J., Dibartolo, G., Lapidus, A., Land, M. L., and Lovley, D. R. (2009). The
361 genome sequence of *Geobacter metallireducens*: Features of metabolism, physiology and
362 regulation common and dissimilar to *Geobacter sulfurreducens*. *BMC Microbiol.* 9, 1–22.
363 doi:10.1186/1471-2180-9-109.
- 364 Batlle-Vilanova, P., Puig, S., Gonzalez-Olmos, R., Vilajeliu-Pons, A., Bañeras, L., Balaguer, M. D., et
365 al. (2014). Assessment of biotic and abiotic graphite cathodes for hydrogen production in
366 microbial electrolysis cells. *Int. J. Hydrogen Energy* 39, 1297–1305.
367 doi:10.1016/j.ijhydene.2013.11.017.
- 368 Beese-Vasbender, P. F., Grote, J.-P., Garrelfs, J., Stratmann, M., and Mayrhofer, K. J. J. (2015).
369 Selective microbial electrosynthesis of methane by a pure culture of a marine lithoautotrophic
370 archaeon. *Bioelectrochemistry* 102, 50–55. doi:10.1016/j.bioelechem.2014.11.004.
- 371 Buckel, W., and Thauer, R. K. (2013). Energy conservation via electron bifurcating ferredoxin
372 reduction and proton/Na⁺ translocating ferredoxin oxidation. *Biochim. Biophys. Acta - Bioenerg.*
373 1827, 94–113. doi:10.1016/j.bbabi.2012.07.002.
- 374 Buckel, W., and Thauer, R. K. (2018). Flavin-based electron bifurcation, ferredoxin, flavodoxin, and
375 anaerobic respiration with protons (Ech) or NAD⁺(Rnf) as electron acceptors: A historical review.
376 *Front. Microbiol.* 9. doi:10.3389/fmicb.2018.00401.
- 377 Chan, C. H., Levar, C. E., Jiménez-Otero, F., and Bond, D. R. (2017). Genome scale mutational
378 analysis of *Geobacter sulfurreducens* reveals distinct molecular mechanisms for respiration and

- 379 sensing of poised electrodes versus Fe(III) oxides. *J. Bacteriol.* 199, 1–18. doi:10.1128/JB.00340-
380 17.
- 381 Chen, S., Rotaru, A.-E., Liu, F., Philips, J., Woodard, T. L., Nevin, K. P., et al. (2014a). Carbon cloth
382 stimulates direct interspecies electron transfer in syntrophic co-cultures. *Bioresour. Technol.* 173,
383 82–86. doi:10.1016/j.biortech.2014.09.009.
- 384 Chen, S., Rotaru, A.-E., Shrestha, P. M., Malvankar, N. S., Liu, F., Fan, W., et al. (2014b). Promoting
385 Interspecies Electron Transfer with Biochar. *Sci. Rep.* 4, 1–7. doi:10.1038/srep05019.
- 386 Cheng, S., Xing, D., Call, D. F., and Logan, B. E. (2009). Direct biological conversion of electrical
387 current into methane by electromethanogenesis. *Environ. Sci. Technol.* 43, 3953–3958.
388 doi:10.1021/es803531g.
- 389 Cord-Ruwisch, R., Lovley, D. R., and Schink, B. (1998). Growth of *Geobacter sulfurreducens* with
390 acetate in syntrophic cooperation with hydrogen-oxidizing anaerobic partners. *Appl. Environ.*
391 *Microbiol.* 64, 2232–2236. doi:0099-2240/98/\$04.00+0.
- 392 Cruz Viggi, C., Rossetti, S., Fazi, S., Paiano, P., Majone, M., and Aulenta, F. (2014). Magnetite
393 Particles Triggering a Faster and More Robust Syntrophic Pathway of Methanogenic Propionate
394 Degradation. *Environ. Sci. Technol.* 48, 7536–7543. doi:10.1021/es5016789.
- 395 Dang, Y., Holmes, D. E., Zhao, Z., Woodard, T. L., Zhang, Y., Sun, D., et al. (2016). Enhancing
396 anaerobic digestion of complex organic waste with carbon-based conductive materials. *Bioresour.*
397 *Technol.* 220, 516–522. doi:10.1016/j.biortech.2016.08.114.

- 398 Deutzmann, J. S., Sahin, M., and Spormann, A. M. (2015). Extracellular Enzymes Facilitate Electron
399 Uptake in Biocorrosion and bioelectrosynthesis. *MBio* 6, 1–8. doi:10.1128/mBio.00496-15.Editor.
- 400 Falkowski, P. G., Fenchel, T., and Delong, E. F. (2008). The Microbial Engines That Drive Earth 's
401 Biogeochemical Cycles. *Science* (80-.). 320, 1034–1039. doi:10.1126/science.1153213.
- 402 Fu, Q., Kuramochi, Y., Fukushima, N., Maeda, H., Sato, K., and Kobayashi, H. (2015).
403 Bioelectrochemical analyses of the development of a thermophilic biocathode catalyzing
404 electromethanogenesis. *Environ. Sci. Technol.* 49, 1225–32. doi:10.1021/es5052233.
- 405 Ishii, S., Suzuki, S., Tenney, A., Nealson, K. H., and Bretschger, O. (2018). Comparative
406 metatranscriptomics reveals extracellular electron transfer pathways conferring microbial
407 adaptivity to surface redox potential changes. *ISME J.* doi:10.1038/s41396-018-0238-2.
- 408 Jing, Y., Wan, J., Angelidaki, I., Zhang, S., and Luo, G. (2017). iTRAQ quantitative proteomic analysis
409 reveals the pathways for methanation of propionate facilitated by magnetite. *Water Res.* 108, 212–
410 221. doi:10.1016/j.watres.2016.10.077.
- 411 Kral, T. A., Brink, K. M., Miller, S. L., and McKay, C. P. (1998). Hydrogen consumptions by
412 methanogens on the early earth. *Orig. Life Evol. Biosph.* 28, 311–319. doi:Doi
413 10.1023/A:1006552412928.
- 414 Kulkarni, G., Kridelbaugh, D. M., Guss, A. M., and Metcalf, W. W. (2009). Hydrogen is a preferred
415 intermediate in the energy-conserving electron transport chain of *Methanosarcina barkeri*. *Proc.*
416 *Natl. Acad. Sci. U. S. A.* 106, 15915–20. doi:10.1073/pnas.0905914106.

- 417 Kulkarni, G., Mand, T. D., and Metcalf, W. W. (2018). Energy Conservation via Hydrogen Cycling in
418 the Methanogenic Archaeon *Methanosarcina barkeri*. *MBio* 9, 1–10. doi:10.1128/mBio.01256-18.
- 419 Lee, J. Y., Lee, S. H., and Park, H. D. (2016). Enrichment of specific electro-active microorganisms
420 and enhancement of methane production by adding granular activated carbon in anaerobic
421 reactors. *Bioresour. Technol.* 205, 205–212. doi:10.1016/j.biortech.2016.01.054.
- 422 Lei, Y., Sun, D., Dang, Y., Chen, H., Zhao, Z., Zhang, Y., et al. (2016). Stimulation of methanogenesis
423 in anaerobic digesters treating leachate from a municipal solid waste incineration plant with
424 carbon cloth. *Bioresour. Technol.* 222, 270–276. doi:10.1016/j.biortech.2016.10.007.
- 425 Levar, C. E., Hoffman, C. L., Dunshee, A. J., Toner, B. M., and Bond, D. R. (2017). Redox potential as
426 a master variable controlling pathways of metal reduction by *Geobacter sulfurreducens*. *ISME J.*
427 11, 741–752. doi:10.1038/ismej.2016.146.
- 428 Li, L., Li, Q., Rohlin, L., Kim, U., Salmon, K., Rejtar, T., et al. (2007). Quantitative Proteomic and
429 Microarray Analysis of the Archaeon *Methanosarcina Acetivorans* Grown with Acetate Versus
430 Methanol. *Proteins* 6, 759–771. doi:10.1021/pr060383l.Quantitative.
- 431 Li, Q., Li, L., Rejtar, T., Lessner, D. J., Karger, B. L., and Ferry, J. G. (2006). Electron Transport in the
432 Pathway of Acetate Conversion to Methane in the Marine Archaeon *Methanosarcina acetivorans*.
433 *J. Bacteriol.* 188, 702–710. doi:10.1128/JB.188.2.702–710.2006.
- 434 Li, Y., Zhang, Y., Yang, Y., Quan, X., and Zhao, Z. (2017). Potentially direct interspecies electron
435 transfer of methanogenesis for syntrophic metabolism under sulfate reducing conditions with
436 stainless steel. *Bioresour. Technol.* 234, 303–309. doi:10.1016/j.biortech.2017.03.054.

- 437 Lienemann, M., Deutzmann, J. S., Milton, R. D., Sahin, M., and Spormann, A. M. (2018). Mediator-
438 free enzymatic electrosynthesis of formate by the *Methanococcus maripaludis* heterodisulfide
439 reductase supercomplex. *Bioresour. Technol.* 254, 278–283. doi:10.1016/j.biortech.2018.01.036.
- 440 Lin, Q., Fang, X., Ho, A., Li, J., Yan, X., Tu, B., et al. (2017a). Different substrate regimes determine
441 transcriptional profiles and gene co-expression in *Methanosarcina barkeri* (DSM 800). *Appl.*
442 *Microbiol. Biotechnol.*, 1–14. doi:10.1007/s00253-017-8457-4.
- 443 Lin, R., Cheng, J., Zhang, J., Zhou, J., Cen, K., and Murphy, J. D. (2017b). Boosting biomethane yield
444 and production rate with graphene: The potential of direct interspecies electron transfer in
445 anaerobic digestion. *Bioresour. Technol.* 239, 345–352. doi:10.1016/j.biortech.2017.05.017.
- 446 Liu, F., Rotaru, A.-E., Shrestha, P. M., Malvankar, N. S., Nevin, K. P., and Lovley, D. R. (2012).
447 Promoting direct interspecies electron transfer with activated carbon. *Energy Environ. Sci.* 5,
448 8982. doi:10.1039/c2ee22459c.
- 449 Lohner, S. T., Deutzmann, J. S., Logan, B. E., Leigh, J., and Spormann, A. M. (2014). Hydrogenase-
450 independent uptake and metabolism of electrons by the archaeon *Methanococcus maripaludis*.
451 *ISME J.* 8, 1673–1681. doi:10.1038/ismej.2014.82.
- 452 Lovley, D. R. (1985). Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Appl.*
453 *Environ. Microbiol.* 49, 1530–1. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16346820>
454 [Accessed October 4, 2016].
- 455 Mand, T. D., Kulkarni, G., and Metcalf, W. W. (2018). Genetic, biochemical, and molecular
456 characterization of *Methanosarcina barkeri* 2 mutants lacking three distinct classes of

- 457 hydrogenase. *bioRxiv*. doi:10.1101/334656.
- 458 McGlynn, S. E. (2017). Energy Metabolism during Anaerobic Methane Oxidation in ANME Archaea.
459 *Microbes Environ.* 32, 5–13. doi:10.1264/jsme2.ME16166.
- 460 McGlynn, S. E., Chadwick, G. L., Kempes, C. P., and Orphan, V. J. (2015). Single cell activity reveals
461 direct electron transfer in methanotrophic consortia. *Nature* 526, 531–535.
462 doi:10.1038/nature15512.
- 463 Meuer, J., Kuettner, H. C., Zhang, J. K., Hedderich, R., and Metcalf, W. W. (2002). Genetic analysis of
464 the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and
465 ferredoxin in methanogenesis and carbon fixation. *Proc. Natl. Acad. Sci.* 99, 5632–5637.
466 doi:10.1073/pnas.072615499.
- 467 Rotaru, A.-E., Calabrese, F., Stryhanyuk, H., Musat, F., Shrestha, P. M., Weber, H. S., et al. (2018).
468 Conductive Particles Enable Syntrophic Acetate Oxidation between *Geobacter* and
469 *Methanosarcina* from Coastal Sediments. *MBio* 9, 1–14. doi:10.1128/mBio.00226-18.
- 470 Rotaru, A.-E., Shrestha, P. M., Liu, F., Markovaite, B., Chen, S., Nevin, K. P., et al. (2014a). Direct
471 Interspecies Electron Transfer between *Geobacter metallireducens* and *Methanosarcina barkeri*.
472 *Appl. Environ. Microbiol.* 80, 4599–4605. doi:10.1128/AEM.00895-14.
- 473 Rotaru, A.-E., Shrestha, P. M., Liu, F., Shrestha, M., Shrestha, D., Embree, M., et al. (2014b). A new
474 model for electron flow during anaerobic digestion: direct interspecies electron transfer to
475 *Methanosaeta* for the reduction of carbon dioxide to methane. *Energy Environ. Sci.* 7, 408–415.
476 doi:10.1039/C3EE42189A.

- 477 Rotaru, A.-E., Woodard, T. L., Nevin, K. P., and Lovley, D. R. (2015). Link between capacity for
478 current production and syntrophic growth in *Geobacter* species. *Front. Microbiol.* 6, 1–8.
479 doi:10.3389/fmicb.2015.00744.
- 480 Salvador, A. F., Martins, G., Melle-Franco, M., Serpa, R., Stams, A. J. M., Cavaleiro, A. J., et al.
481 (2017). Carbon nanotubes accelerate methane production in pure cultures of methanogens and in a
482 syntrophic coculture. *Environ. Microbiol.* 19, 2727–2739. doi:10.1111/1462-2920.13774.
- 483 Schlegel, K., Welte, C., Deppenmeier, U., and Müller, V. (2012). Electron transport during acetoclastic
484 methanogenesis by *Methanosarcina acetivorans* involves a sodium-translocating Rnf complex.
485 *FEBS J.* 279, 4444–4452. doi:10.1111/febs.12031.
- 486 Seiler, M., Mehrle, A., Poustka, A., and Wiemann, S. (2006). The 3of5 web application for complex
487 and comprehensive pattern matching in protein sequences. *BMC Bioinformatics* 7, 1–12.
488 doi:10.1186/1471-2105-7-144.
- 489 Shimizu, S., Upadhye, R., Ishijima, Y., and Naganuma, T. (2010). *Methanosarcina horonobensis* sp.
490 nov., a methanogenic archaeon isolated from a deep subsurface Miocene formation. *Int. J. Syst.*
491 *Evol. Microbiol.* 61, 2503–2507. doi:10.1099/ijs.0.028548-0.
- 492 Shrestha, P. M., Rotaru, A.-E., Aklujkar, M., Liu, F., Shrestha, M., Summers, Z. M., et al. (2013).
493 Syntrophic growth with direct interspecies electron transfer as the primary mechanism for energy
494 exchange. *Environ. Microbiol. Rep.* 5, 904–910. doi:10.1111/1758-2229.12093.
- 495 Smith, T. J., and Stevenson, K. J. (2007). ‘Reference electrodes’, in *Handbook of Electrochemistry*
496 (Elsevier B.V.), 73–110. doi:10.1016/B978-0-444-51958-0.50005-7.

- 497 Suharti, S., Wang, M., De Vries, S., and Ferry, J. G. (2014). Characterization of the RnfB and RnfG
498 subunits of the Rnf complex from the archaeon *Methanosarcina acetivorans*. *PLoS One* 9, 1–10.
499 doi:10.1371/journal.pone.0097966.
- 500 Thauer, R. K., Kaster, A.-K., Goenrich, M., Schick, M., Hiromoto, T., and Shima, S. (2010).
501 Hydrogenases from Methanogenic Archaea, Nickel, a Novel Cofactor, and H₂ Storage. *Annu.*
502 *Rev. Biochem.* 79, 507–536. doi:10.1146/annurev.biochem.030508.152103.
- 503 Thauer, R. K., Kaster, A.-K., Seedorf, H., Buckel, W., and Hedderich, R. (2008). Methanogenic
504 archaea: ecologically relevant differences in energy conservation. *Nat. Rev. Microbiol.* 6, 579–
505 591. doi:10.1038/nrmicro1931.
- 506 Timmers, P. H. A., Welte, C. U., Koehorst, J. J., Plugge, C. M., Jetten, M. S. M., and Stams, A. J. M.
507 (2017). Reverse Methanogenesis and Respiration in Methanotrophic Archaea. *Archaea* 2017.
508 doi:10.1155/2017/1654237.
- 509 Wang, F. P., Zhang, Y., Chen, Y., He, Y., Qi, J., Hinrichs, K.-U., et al. (2014). Methanotrophic archaea
510 possessing diverging methane-oxidizing and electron-Transporting pathways. *ISME J.* 8, 1069–
511 1078. doi:10.1038/ismej.2013.212.
- 512 Wang, M., Tomb, J.-F., and Ferry, J. G. (2011). Electron transport in acetate-grown *Methanosarcina*
513 *acetivorans*. *BMC Microbiol.* doi:10.1186/1471-2180-11-165.
- 514 Wang, O., Zheng, S., Wang, B., Wang, W., and Liu, F. (2018). Necessity of electrically conductive pili
515 for methanogenesis with magnetite stimulation. *PeerJ* 6, e4541. doi:10.7717/peerj.4541.

- 516 Ye, Q., Zhang, Z., Huang, Y., Fang, T., Cui, Q., He, C., et al. (2018). Enhancing electron transfer by
517 magnetite during phenanthrene anaerobic methanogenic degradation. *Int. Biodeterior. Biodegrad.*,
518 0–1. doi:10.1016/j.ibiod.2018.01.012.
- 519 Zhang, S., Chang, J., Lin, C., Pan, Y., Cui, K., Zhang, X., et al. (2017). Enhancement of
520 methanogenesis via direct interspecies electron transfer between Geobacteraceae and
521 Methanosaetaceae conducted by granular activated carbon. *Bioresour. Technol.* 245, 132–137.
522 doi:10.1016/j.biortech.2017.08.111.
- 523 Zhao, Z., Zhang, Y., Li, Y., Dang, Y., Zhu, T., and Quan, X. (2017). Potentially shifting from
524 interspecies hydrogen transfer to direct interspecies electron transfer for syntrophic metabolism to
525 resist acidic impact with conductive carbon cloth. *Chem. Eng. J.* 313, 10–18.
526 doi:10.1016/j.cej.2016.11.149.
- 527 Zheng, S., Zhang, H., Li, Y., Zhang, H., Wang, O., Zhang, J., et al. (2015). Co-occurrence of
528 *Methanosarcina mazei* and Geobacteraceae in an iron (III)-reducing enrichment culture. *Front.*
529 *Microbiol.* 6, 1–12. doi:10.3389/fmicb.2015.00941.
- 530 Zhuang, L., Tang, J., Wang, Y., Hu, M., and Zhou, S. (2015). Conductive iron oxide minerals
531 accelerate syntrophic cooperation in methanogenic benzoate degradation. *J. Hazard. Mater.* 293,
532 37–45. doi:10.1016/j.jhazmat.2015.03.039.

533

534

535

536

537

538

539

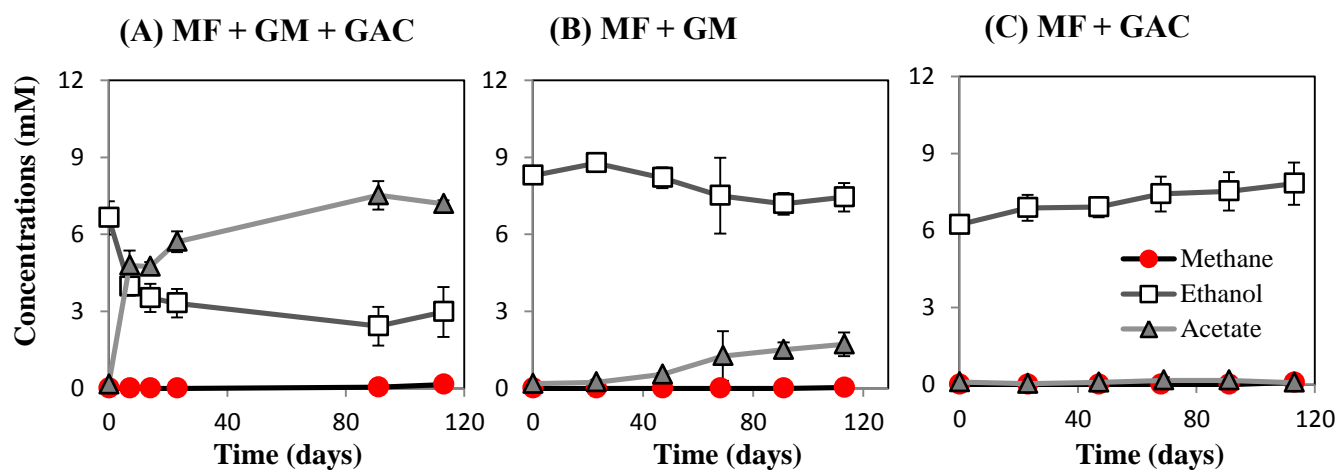
540

541

542

543

544



545 **(Fig. 1)** Co-cultures of *M. formicicum* with *G.metallireducens* feeding on ethanol with or without GAC.

546 Error bars are based on standard deviation (n=3). MF; *Methanobacterium formicicum*, GM; *Geobacter*

547 *metallireducens*, GAC; granular activated carbon, Circles; Methane, Squares; Ethanol, Triangles;

548 Acetate .

549

550

551

552

553

554

555

556

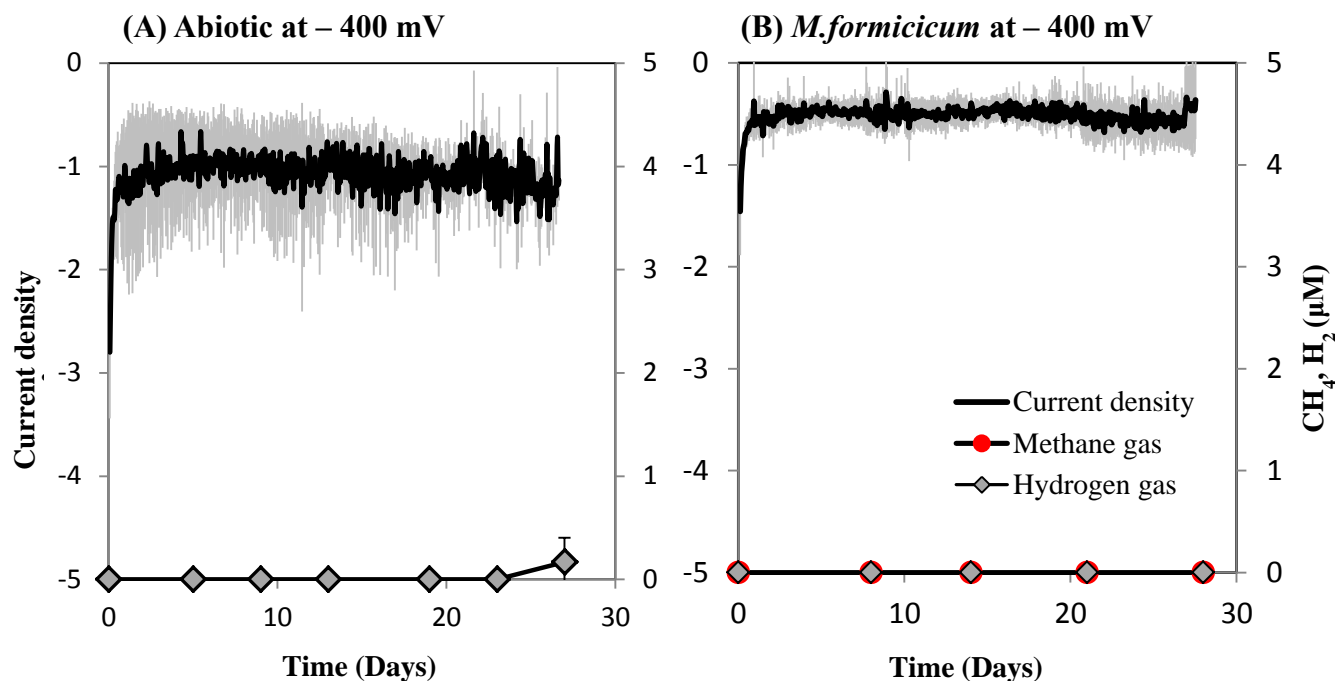
557

558

559

560

561



562 **(Fig. 2)** Current consumption and gas production from (A) abiotic and (B) *M. formicicum* reactors at -
563 400 mV (vs. SHE). Only the excess values of gases compared to that of the open circuit control are
564 plotted here. Error bars are based on standard deviation (n=2 for abiotic, n=3 for *M. formicicum*
565 reactors).

566

567

568

569

570

571

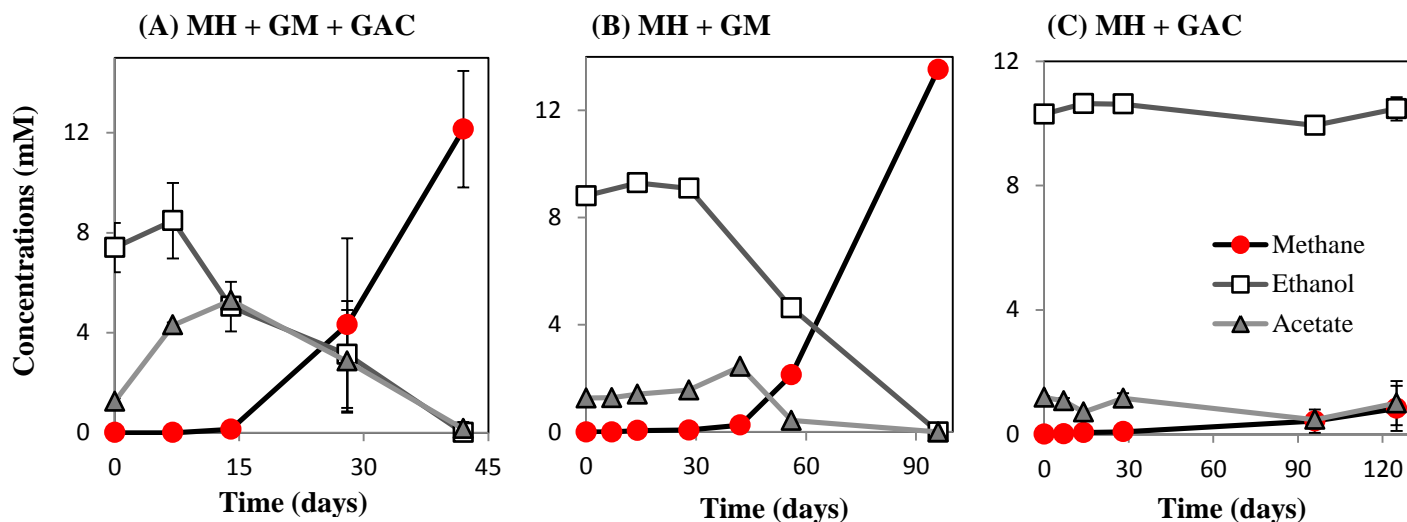
572

573

574

575

576



577 **(Fig. 3)** Co-cultures of *M. horonobensis* with *G. metallireducens* feeding on ethanol with or without

578 GAC. MH; *Methanosarcina horonobensis*, GM; *Geobacter metallireducens*, GAC; granular activated

579 carbon, Circles; Methane, Squares; Ethanol, Triangles; Acetate. The results are from triplicate samples

580 except for (B) which is a representative sample for which the rest of the replicate data is presented in

581 Fig S4.

582

583

584

585

586

587

588

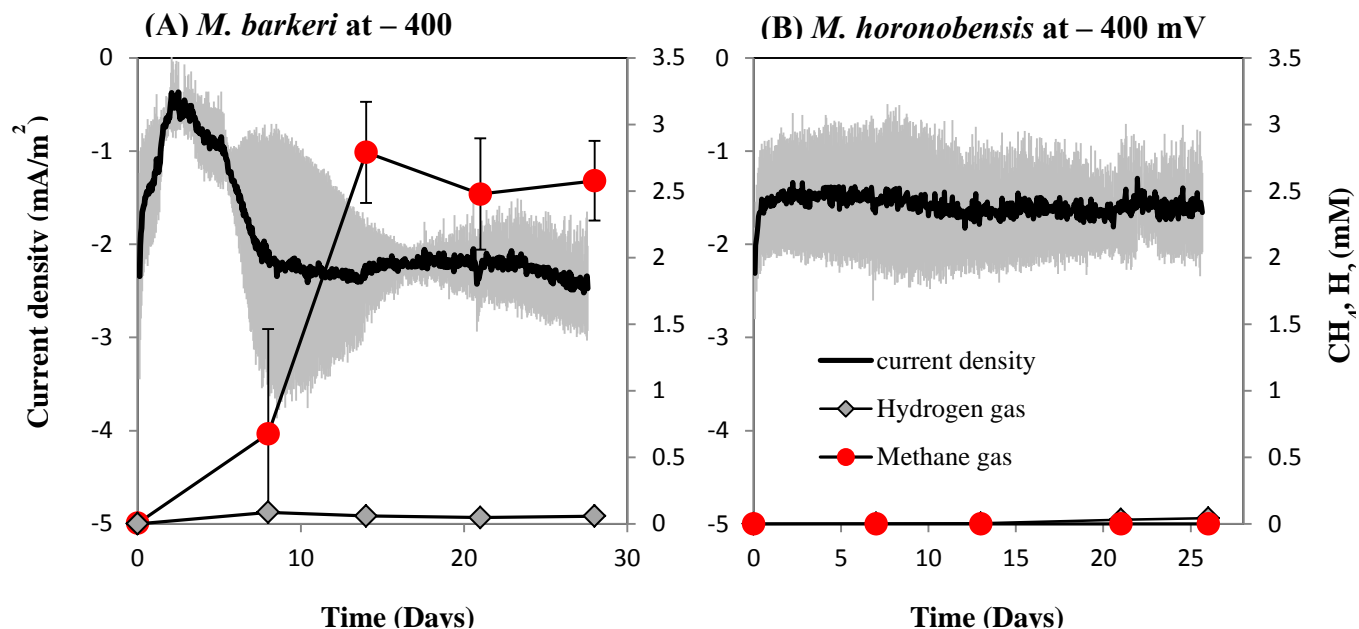
589

590

591

592

593



594

595

596

597

598

599

600

601

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
<i>Methanosarcina barkeri</i> 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)
<i>Methanosarcina horonobensis</i> 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)
<i>Methanobacterium formicicum</i> DSM1535	No	No	No	Yes	Rods (Ferry, 1993)

603

604

605

606

607

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

TIGRfam Categories	No. of genes associated within a TIGR family		
	<i>Methanosarcina horonobensis</i>	<i>Methanosarcina barkeri</i>	<i>Methanobacterium formicicum</i>
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

609

610

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
<i>Methanosarcina barkeri</i> MS	Ech-hydrogenase	8	37	20 (0/1 multiheme*)	3 (cyt b)	4	10
<i>Methanosarcina horonobensis</i> HB-1	Rnf-complex	9	47	30 (3 multiheme)	3 (cyt b)	6	8
<i>Methanobacterium formicicum</i> 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.

613

614