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# Extracellular Electron Uptake by Two *Methanosarcina* Species

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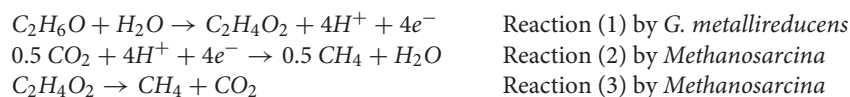
Direct electron uptake by prokaryotes is a recently described mechanism with a potential application for energy and CO<sub>2</sub> storage into value added chemicals. Members of Methanosarcinales, an environmentally and biotechnologically relevant group of methanogens, were previously shown to retrieve 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.

**Keywords:** extracellular electron uptake, methanogen, *Methanosarcina*, direct interspecies electron transfer (DIET), electromethanogenesis, GAC (Granular Activated Carbon), *Geobacter*

## 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-transferable molecules. Moreover, syntrophy requires a partner methanogen to scavenge the transferable molecules. For decades, we have assumed that interspecies-transferable molecules were either H<sub>2</sub> 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,b) (Figure 1).



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) (Shrestha et al., 2013; Holmes et al., 2018b). *Geobacter's* requirement for outer-surface proteins during DIET was confirmed earlier with gene-deletion studies (Rotaru et al., 2014a,b). 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 L. et al., 2018a). In these cases, the addition of conductive particles enriched for DIET-related *Methanosarcinales* (Zheng et al., 2015; Dang et al., 2016; Holmes et al., 2018b; Rotaru et al., 2018a). However, exceptions were observed since occasionally conductive particles enriched for  $H_2$ -utilizing

methanogens of the genus *Methanospirillum* (Lee et al., 2016) or *Methanobacterium* (Zhuang et al., 2015; Lin et al., 2017).

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* performed methanogenesis 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 formicum* (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  $37^\circ C$ . All the microorganisms had been adapted to grow in DSMZ medium 120c with the following modifications: 1 g/L NaCl, 0.5 g/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. formicum* 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. formicum* inoculated into 8.5 ml of the media prepared as above. The starting cell numbers for the methanogens in co-cultures were approximately

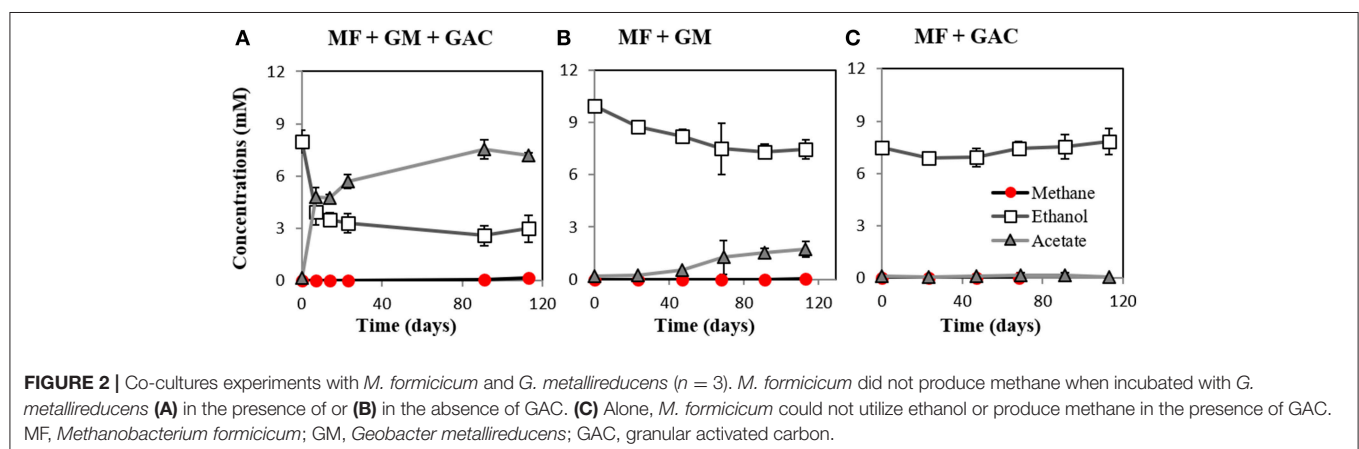
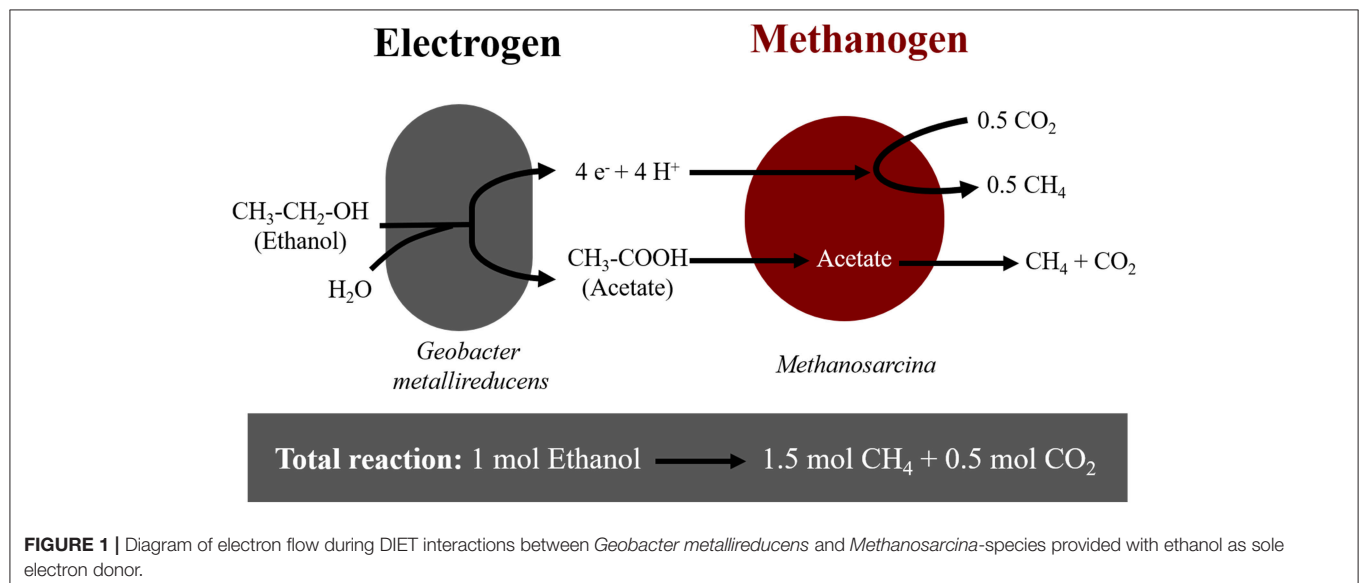
$2.6 \times 10^6$  cells/mL,  $8.2 \times 10^6$  cells/mL and  $6.7 \times 10^5$  cells/mL for *M. barkeri*, *M. formicicum*, and *M. horonobensis*, respectively. The starting cell numbers for *G. metallireducens* in co-cultures were circa  $1.5 \times 10^5$ . Incubations were carried out in 20 ml pressure vials. For the co-cultures ethanol (10 mM) was added as an electron donor and  $\text{CO}_2$  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  $2.5 \times 7.5 \times 1.2$  cm thus a total projected surface area of  $61.5 \text{ cm}^2$ . 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  $<10 \Omega$  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)



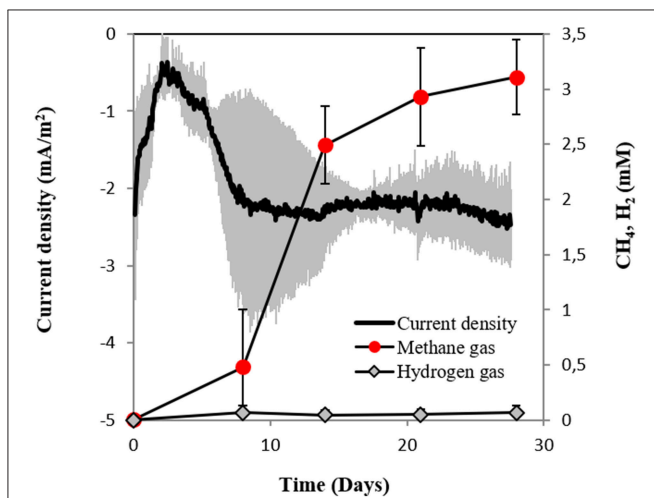
reference electrodes were lodged through a side port in the working electrode chamber at a distance of about 1 cm from the surface of the working electrode. After lodging the electrodes, degassing with N<sub>2</sub>: CO<sub>2</sub> (80:20) for circa 30 min in each reactor chamber ensured anaerobic conditions. 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  $2.6 \times 10^7$  cells/mL,  $8.2 \times 10^7$  cells/mL, and  $6.7 \times 10^6$  cells/mL respectively. Cell counts were done with microscopic examination using DAPI (1  $\mu$ g/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)

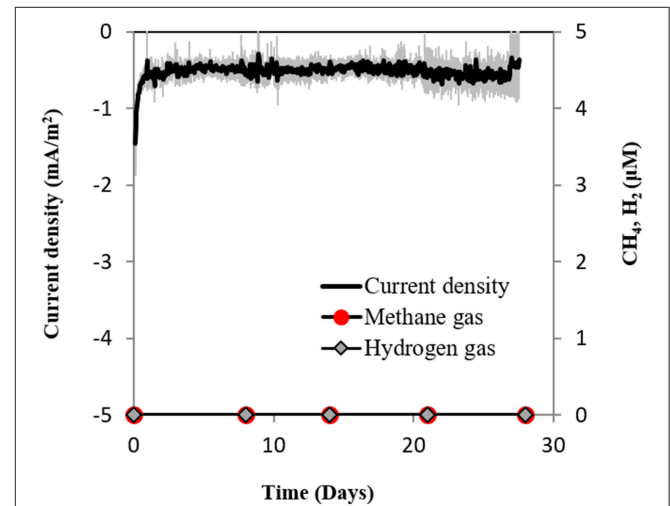
according to the manufacturer and our own measurements against a Hydroflex<sup>®</sup> reference electrode used as NHE (normal hydrogen electrode). The difference between NHE and SHE is experimentally negligible (Smith and Stevenson, 2007). All potentials in this paper from here onwards are reported vs. SHE by adjusting accordingly from the Ag/AgCl reference electrodes values. Cathode poisoning and electrochemical measurements were carried with a multichannel potentiostat (MultiEmstat, Palmsens, NL) operated by the Multitrace software (Palmsens, NL).

## Analytical Measurements and Calculations

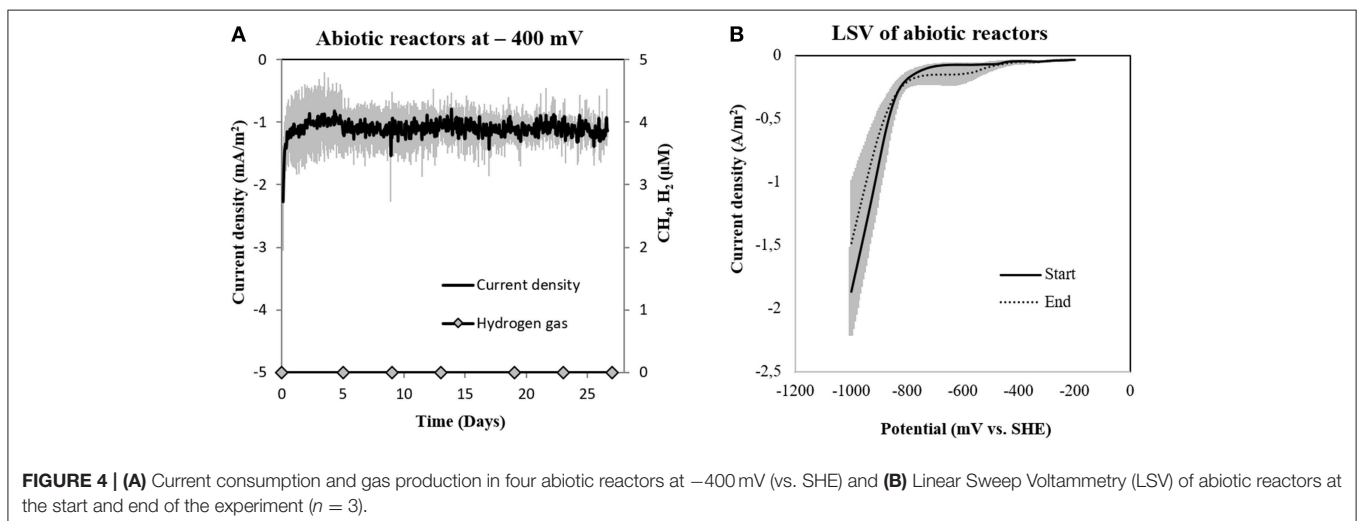
Headspace samples for CH<sub>4</sub> and H<sub>2</sub> analysis were taken with hypodermic needles and kept in airtight exetainers until measurement. Methane (CH<sub>4</sub>) and hydrogen gas (H<sub>2</sub>) were measured on a Trace 1300 gas chromatograph (Thermo-Scientific) with a TracePLOT<sup>™</sup> TG-BOND Msieve 5A column and a thermal conductivity detector (TCD). The carrier gas was



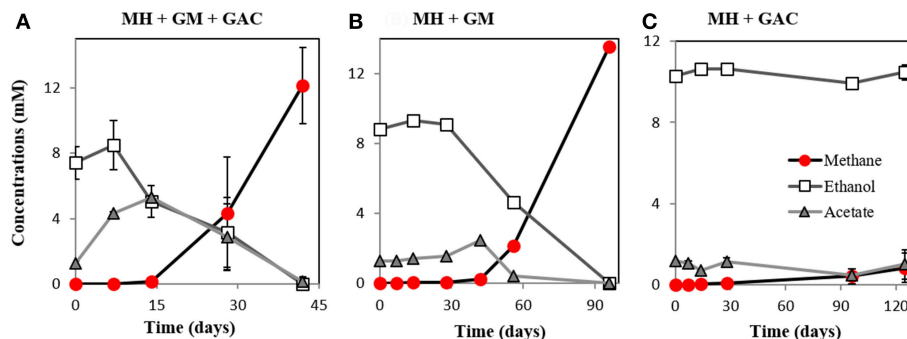
**FIGURE 3** | Current consumption and gas production in triplicate *M. barkeri* cultures provided with a poised cathode at  $-400$  mV (vs. SHE) as sole electron donor.



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



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

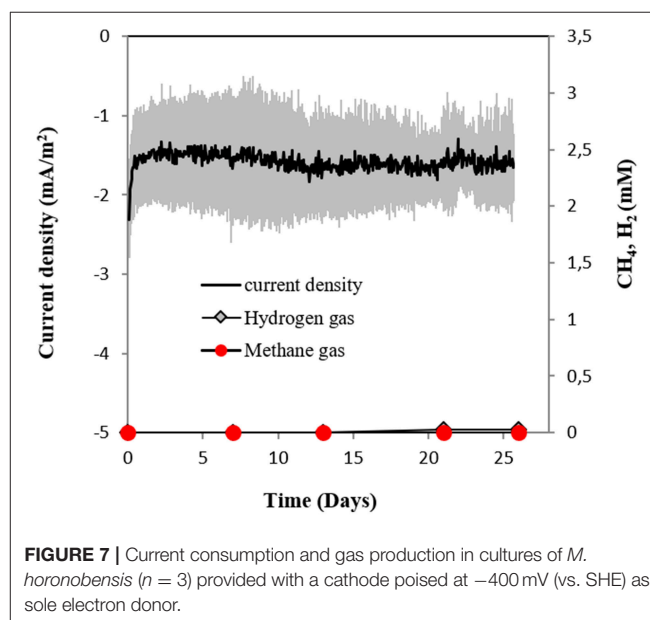


**FIGURE 6** | Co-culture experiments with *M. horonobensis* and *G. metallireducens*. *M. horonobensis* established successful co-cultures with *G. metallireducens* as apparent from ethanol utilization and methane production in the presence (A,  $n = 3$ ) or absence of GAC (B,  $n = 1$ ; see replication in Figure 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.

argon at a flow rate of 25 mL/min. The injector, oven and detector temperatures were 150, 70, and 200°C respectively. The detection limit for CH<sub>4</sub> and H<sub>2</sub> 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 × L / mol × 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 passed 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, 40, 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<sub>3</sub> and 4.5 mM Na<sub>2</sub>CO<sub>3</sub> 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.



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

## RESULTS AND DISCUSSION

It was previously shown that two Methanosarcinales-methanogens, *Methanosarcina barkeri* and *Methanotherix harundinacea* grew via DIET whereas strict H<sub>2</sub>-utilizing methanogens did not (Rotaru et al., 2014a,b). Here we show that *M. barkeri* could retrieve electrons not only from an exoelectrogen but also from an electrode poised at  $-400$  mV (non-H<sub>2</sub> generating conditions) to carry electromethanogenesis. As expected, the H<sub>2</sub>-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, **Figure S2**). This was anticipated because *G. metallireducens*, a respiratory organism, is incapable of substrate fermentation and consequent H<sub>2</sub> production according to previous physiological tests (Cord-Ruwisch et al., 1998) and genetic investigations (Aklujkar et al., 2009). Since H<sub>2</sub> could not be generated by *G. metallireducens*, a strict H<sub>2</sub>-utilizer like *M. formicicum* was rendered incapable of an interspecies association based on H<sub>2</sub>-transfer with this bacterium (Rotaru et al., 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 (**Figure 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 ( $4.4 \pm 0.33$  mM;  $p < 0.001$ ) (**Figure 3**) when provided with an applied potential at the cathode, in contrast to open circuit controls without an applied potential ( $1.3 \pm 0.33$  mM) (**Figure 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 ( $3.1 \pm 0.34$  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* (**Figure 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<sub>2</sub> generated electrochemically at the cathode, or

2. It retrieves electrons directly via an unknown mechanism

To determine abiotic electrochemical H<sub>2</sub> evolution we (i) verified for H<sub>2</sub> accumulation over a month of incubation and (ii) verified the threshold for H<sub>2</sub>-evolution by linear sweep voltammetry at the beginning and the end of the incubation. H<sub>2</sub> did not accumulate over a month of incubation in six independent abiotic controls (**Figure 4A**). Linear sweep voltammetry profiles indicated that in our media the threshold for H<sub>2</sub>-evolution was below  $-700$  mV (**Figure 4B**). This was in agreement with previous studies determining electrochemical H<sub>2</sub>-evolution under physiological conditions on a graphite electrode, which was below  $-400$  mV due to high overpotentials (Cheng et al., 2009; Mitov et al., 2012; Battle-Vilanova et al., 2014; Beese-Vasbender et al., 2015).

On the other hand, in reactors inoculated with *M. barkeri*, the detected H<sub>2</sub> stabilized at  $0.065 \pm 0.02$  mM, similar to concentrations observed for co-cultures of *M. barkeri* with (0.077  $\pm$  0.03 mM) or without conductive particles (0.076  $\pm$  0.06 mM) and in pure culture (0.068 mM). This is supported by previous research, which demonstrated H<sub>2</sub>-cycling (H<sub>2</sub>-production and H<sub>2</sub>-uptake) in *M. barkeri* (Kulkarni et al., 2009, 2018; Mand et al., 2018). The cellular-evolved H<sub>2</sub> is well above the H<sub>2</sub>-uptake threshold for *M. barkeri* (296–376 nM) (Lovley, 1985; Kral et al., 1998) possibly because in these cultures there is an alternative, competitive electron donor.

Secondly, if H<sub>2</sub> 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<sub>2</sub>, draw current and produce methane. To test this hypothesis we used a highly effective H<sub>2</sub>-utilizing methanogen—*M. formicicum*, which has a low H<sub>2</sub> uptake threshold of approximately 6 nM (Lovley, 1985). However, when *M. formicicum* was incubated in electrochemical reactors, neither H<sub>2</sub>, methane nor current draw was observed at  $-400$  mV (**Figure 5**) indicating that methanogenesis from H<sub>2</sub> 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<sub>2</sub> as substrate. *M. formicicum* was unaffected by a poised electrode since it produced methane from the extrinsic H<sub>2</sub> in an electrochemical setup (**Figure S4**).

As electrochemical H<sub>2</sub> was unlikely in our electrochemical setup, according to cumulative gas-detection analyses,

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

electrochemical tests, and tests with a highly effective H<sub>2</sub>-utilizer, we confer that *M. barkeri* is likely to retrieve electrons directly from the electrode.

### *Methanosarcina horonobensis*

The distribution of extracellular electron uptake to other species of the order Methanosarcinales has not been explored, with the exception of *M. barkeri* and *M. harundinacea*. *M. barkeri* and *M. harundinacea* species have been isolated from and associated with anaerobic wastewater treatment (Bryant and Boone, 1987; Ma et al., 2006; De Vrieze et al., 2012). 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 (Shimizu et al., 2010; Holmes et al., 2018a).

Here we showed that *M. horonobensis* did establish successful syntrophic associations with *G. metallireducens* with or without conductive particles as an electrical conduit (Figure 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<sub>2</sub> (Reactions 2, 3). During DIET we expect the conversion of 1 mol ethanol to 1.5 mol methane according to Reactions 1–3 (Figure 1). As predicted, in the *G. metallireducens*–*M. horonobensis* co-cultures, the syntroph oxidized  $8.8 \pm 0.4$  mM ethanol providing the reducing equivalents (directly and via acetate) to generate  $13.1 \pm 0.8$  mM CH<sub>4</sub> by the methanogen. These co-cultures achieved stoichiometric recoveries of  $98.5 \pm 3.3$  %. Similar recoveries ( $109 \pm 18.5$  %) 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 (Figure 6C and Figure S5). However, similar to previous reports (Zhang P. et al., 2018b), *Geobacter* could partially convert ethanol to acetate using GAC as insoluble electron acceptor (Figure S5; Van Der Zee et al., 2003; Zhang P. et al., 2018b), 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<sub>2</sub> transfer because *G. metallireducens* is a strict respiratory microorganism which cannot ferment ethanol to generate H<sub>2</sub> (Shrestha et al., 2013) and because *M. horonobensis* is unable to use H<sub>2</sub> as electron donor for their metabolism (Shimizu et al., 2010).

Surprisingly, *M. horonobensis*, which could grow by DIET, was incapable of electromethanogenesis (Figure 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 –400 mV.

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<sup>–</sup>) to the production of a proton motive force according to the reaction: Fdx<sup>–</sup> (red) + 2H<sup>+</sup> → Fdx (ox) + H<sub>2</sub> + ΔμH<sup>+</sup>/ ΔμNa<sup>+</sup> (Thauer et al., 2008). *M. horonobensis* does not have the Ech (Table 1). An alternative to Ech is the Na<sup>+</sup>-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).

**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 formicum</i>
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, propherin, 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



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 et al., 2015; McGlynn, 2017; 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,

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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  $-400$  mV, 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. horonobensis* 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.

## DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

## AUTHOR CONTRIBUTIONS

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

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## SUPPLEMENTARY MATERIAL

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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