



# Electrical current generation in microbial electrolysis cells by hyperthermophilic archaea *Ferroglobus placidus* and *Geoglobus ahangari*

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## ABSTRACT

Few microorganisms have been examined for current generation under thermophilic (40–65 °C) or hyperthermophilic temperatures ( $\geq 80$  °C) in microbial electrochemical systems. Two iron-reducing archaea from the family Archaeoglobaceae, *Ferroglobus placidus* and *Geoglobus ahangari*, showed electro-active behavior leading to current generation at hyperthermophilic temperatures in single-chamber microbial electrolysis cells (MECs). A current density ( $j$ ) of  $0.68 \pm 0.11$  A/m<sup>2</sup> was attained in *F. placidus* MECs at 85 °C, and  $0.57 \pm 0.10$  A/m<sup>2</sup> in *G. ahangari* MECs at 80 °C, with an applied voltage of 0.7 V. Cyclic voltammetry (CV) showed that both strains produced a sigmoidal catalytic wave, with a mid-point potential of  $-0.39$  V (vs. Ag/AgCl) for *F. placidus* and  $-0.37$  V for *G. ahangari*. The comparison of CVs using spent medium and turnover CVs, coupled with the detection of peaks at the same potentials in both turnover and non-turnover conditions, suggested that mediators were not used for electron transfer and that both archaea produced current through direct contact with the electrode. These two archaeal species, and other hyperthermophilic exoelectrogens, have the potential to broaden the applications of microbial electrochemical technologies for producing biofuels and other bioelectrochemical products under extreme environmental conditions.

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## 1. Introduction

Microbial electrochemical technologies (METs), such as microbial fuel cells (MFCs) and microbial electrolysis cells (MECs), are extensively recognized for their potential for their ability to convert energy in organic matter into other useful products, using electro-active microorganisms growing on an anode or cathode [1,2]. Current generated by the action of exoelectrogenic microorganisms growing on the anode in an MFC can be harvested directly as electrical power [3], or the potential they generate can be used to produce biofuels and chemicals on the cathode such as hydrogen (H<sub>2</sub>) [4] in MECs. Exoelectrogenic microorganisms have been found in a diversity of environments, such as freshwater sediments [5], salt marshes, mangrove swamp sediments [5,6], and wastewater treatment plants [7,8]. Most exoelectrogens associated with these environments are iron respiring bacteria from the genera *Desulfobulbus* [9], *Geobacter* [10], *Desulfuromonas* [11], and *Shewanella* [12]. With the exception of a few species, such as *Pelobacter carbinolicus*

and *Geobacter bemidjensis* [13], most bacteria capable of insoluble Fe(III) reduction are exoelectrogens [1,14,15] as similar mechanisms are used for dissimilatory metal oxide reduction and transfer of electrons to an electrode in many of these species. These mechanisms include direct contact with the insoluble electron acceptor, utilization of electrically conductive pili, and utilization of soluble electron shuttles such as flavins [16]. In all of these situations, c-type cytochromes are involved in exogenous transport of electrons released by oxidation of organic or inorganic compounds to either solid iron oxides or to anodes in METs [16–18]. The genomes of the most extensively studied exoelectrogens, *Geobacter sulfurreducens* and *Shewanella oneidensis*, have numerous c-type cytochromes (111 putative genes in *G. sulfurreducens*, and 42 genes in *S. oneidensis*) [18–20].

Most identified exoelectrogenic microbes have been isolated at mesophilic temperatures [21]. Only a few thermophilic bacterial genera, including *Thermincola* [21,22] and *Thermoanaerobacter* [23] (optimal growth temperatures of 60 °C), and *Calditerrivibrio nitroreducens* [24] (grown at 55 °C) have been evaluated for their potential for current production in MECs or MFCs. Similar to mesophilic bacterial exoelectrogens, these thermophiles are capable of dissimilatory iron reduction and have numerous multi-heme c-type cytochromes that are likely to be involved in electron transfer to insoluble electron acceptors [25,26].

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The operation of METs under high temperature conditions is likely to enhance the bioavailability of poorly degradable and insoluble materials, increase microbial activity and mass transfer rates, and decrease the risk of contamination, all factors that should increase MET performance [27,28]. Evidence that higher temperatures can increase MET efficiencies is provided by a study that showed that marine sediment fuel cells operated at 60 °C generated 10 times more current than those operated at 22 °C with the same sediment [29]. In addition, MFCs treating alcohol distillery wastewater under thermophilic conditions achieved higher power output and coulombic efficiencies than mesophilic MFCs [30]. Biofuel production by METs may also be improved by operation at high temperatures, as studies have shown that thermophilic microorganisms can convert carbohydrate-rich substrates into H<sub>2</sub> gas by dark fermentation at significantly higher rates than mesophiles [28,31,32]. Hyperthermophiles, which grow at temperatures of 80–110 °C, have also been used for efficient conversion of various organic materials to H<sub>2</sub> and have been shown to produce the highest H<sub>2</sub> yields from degradation of such complex organics as wastewater biosolids of any organisms studied to date [33]. Another potential application of high temperature MFCs is for treatment of hot wastewaters like those associated with alcohol distilleries where the waste stream is 70 to 80 °C [30].

While there are obvious advantages to operation of METs at high temperatures, few studies have examined the ability for hyperthermophiles to function as exoelectrogens. Fu et al. [34] recently demonstrated that an MFC inoculated with subsurface microorganisms from a petroleum reservoir and operated at 80–95 °C produced a maximum power density of 165 mW/m<sup>2</sup>. The anodic microbial community that developed on the anode showed limited phylogenetic diversity compared to those in mesophilic or thermophilic MFCs, consisting primarily of hyperthermophilic bacteria closely related to the fermentative bacterium *Caldanaerobacter subterraneus* and the dissimilatory metal-reducing bacterium *Thermodesulfobacterium commune*; no archaeal sequences were detected [34,35]. *Pyrococcus furiosus* was the first hyperthermophilic archaeon examined in pure culture for current generation, producing 2 A/m<sup>2</sup> and a peak power density of 225 mW/m<sup>2</sup> in an MFC operated at 90 °C, using a medium containing maltose, cysteine and sodium sulfide [36]. Both cysteine/cystine and sulfur species such as sulfide have been shown to serve as electron shuttles [37,38] which can contribute to current generation in an MFC. Therefore, it is difficult to determine what proportion of current could be attributed to direct electron transport (DET) by *P. furiosus* to the anode surface in that study. In order to study mechanisms involved in DET by microorganisms, it is necessary to eliminate potential shuttles from the medium.

Certain hyperthermophilic archaea can couple the oxidation of organic compounds and/or H<sub>2</sub> with DET to insoluble Fe(III) oxides [39], and possess large multi-heme c-type cytochromes involved in DET [17,40]. Two hyperthermophiles, *Ferroglobus placidus* and *Geoglobus ahangari*, were examined here for current production in MECs as they both grow on acetate, are iron respiring archaea from the same family (Archaeoglobaceae), and their genomes contain multiple genes coding for putative c-type cytochrome proteins (30 for *F. placidus*, and 21 for *G. ahangari*) [17,41]. Owing to their Fe(III) oxide reduction capacity, *F. placidus* and *G. ahangari* were previously suggested to be capable of current generation in MFCs [14,42]. However, the capacity for Fe(III) oxide reduction does not necessarily confer electron transfer ability to anodes and the capacity for current generation must be evidenced experimentally [14,15]. The common physiological feature of these two species to use acetate as a source of carbon and electrons is a rare trait among hyperthermophilic archaea [43]. The utilization of acetate is of particular significance for conversion of the products of cellulosic biomass fermentation into H<sub>2</sub> gas. In order to establish the relative ability of these two archaea to produce current under hyperthermophilic conditions, current densities obtained in these MECs were compared to those produced by the model mesophilic exoelectrogen *Geobacter sulfurreducens* strain PCA.

## 2. Materials and methods

### 2.1. Organisms, media, and growth conditions

*Ferroglobus placidus* strain AEDII12DO<sup>T</sup> (DSM10642) and *Geoglobus ahangari* strain 234<sup>T</sup> (DSM27542) were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. *F. placidus* was originally isolated from a shallow submarine hydrothermal system located in Vulcano, Italy [44]. *G. ahangari* was isolated from the Guaymas Basin hydrothermal system at a depth of 2000 m [45]. Hyperthermophilic cells were initially grown with acetate (10 mM) as an electron donor and Fe(III)-citrate (56 mM) as an electron acceptor and pressurized to 100 kPa with a sterile H<sub>2</sub>:CO<sub>2</sub> (80%:20%) gas mixture. Strict anaerobic culturing and sampling techniques were used throughout the experiment. Hyperthermophilic growth medium included the following (per liter): MgCl<sub>2</sub>·6H<sub>2</sub>O, 4.3 g; NaCl, 18 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.14 g; KCl, 0.34 g; KH<sub>2</sub>PO<sub>4</sub>, 0.4 g; NH<sub>4</sub>Cl, 0.24 g; NaHCO<sub>3</sub>, 5 g; vitamin solution, 10 mL; and trace element solution, 10 mL. The trace element solution contained (g/L): nitrilotriacetic acid, 1.5; MgSO<sub>4</sub>·7H<sub>2</sub>O, 3; NaCl, 1; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.5; NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.2; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.1; CoCl<sub>2</sub>, 0.1; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.1; ZnSO<sub>4</sub>, 0.1; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.01; AlK(SO<sub>4</sub>)<sub>2</sub>, 0.01; H<sub>3</sub>BO<sub>3</sub>, 0.01; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.01; Na<sub>2</sub>SeO<sub>3</sub>, 0.01 and Na<sub>2</sub>WO<sub>4</sub>, 0.01. The medium was prepared anaerobically under an N<sub>2</sub>:CO<sub>2</sub> (80%:20%) atmosphere. After autoclaving, the vitamin solution [46], trace element solution, buffer (sodium bicarbonate) and electron donor (sodium acetate) were added from sterile anoxic stock solutions. All chemicals were purchased from VWR (Radnor, PA, USA) or Sigma-Aldrich (St. Louis, MO, USA) in the highest available purity. The pH of the growth medium ranged from 6.8 to 7.0 (at room temperature). Cultures were grown in the dark at 85 °C (*F. placidus*) or 80 °C (*G. ahangari*).

*Geobacter sulfurreducens* strain PCA (ATCC 51573) was initially grown from a freezer stock stored at –80 °C in ATCC medium 1957 (30 mM bicarbonate buffered medium with 10 mM sodium acetate provided as the electron donor, and 50 mM sodium fumarate as the electron acceptor). All media were sparged and maintained under a N<sub>2</sub>:CO<sub>2</sub> (80%:20%) atmosphere, and cells were grown at 30 °C in the dark [47].

### 2.2. MEC construction and operation

Small volume, single-chamber MECs, referred to as mini MECs, were constructed with 5 mL clear glass serum bottles as previously described and used to examine current production [48]. As the two hyperthermophiles examined here were obligate anaerobes, and could not tolerate even low concentrations of dissolved oxygen, MECs were used to avoid substantial oxygen crossover that occurs through the cathode in air-cathode MFCs, or small amounts of oxygen transfer through the membrane in two chamber MFCs. In addition, the cathode can limit power production when using dissolved oxygen as the electron acceptor for the cathode, and the solubility of oxygen is substantially decreased with increased temperature. Therefore, the use of MECs avoided the adverse impact of oxygen on the cultures during cultivation, and simplified the experiments as air did not need to be dissolved into water while the reactors were placed in ovens. Mini-MECs were used as they are inexpensive to construct, easy to operate and easy to sterilize, they do not require a membrane, they consume only minimal amounts of sterilized media per cycle, and they take up little room in the oven. MECs have previously been used for isolation and cultivation of exoelectrogenic mixed and pure cultures, and they are routinely used for analysis of exoelectrogenic microorganisms. The anode was a graphite plate 1 cm × 1.5 cm (GM-10, 0.32 cm thick, anode specific surface area of A<sub>A</sub> = 92 m<sup>2</sup>/m<sup>3</sup>) polished with sandpaper (grit type 400), sonicated to remove debris, soaked in 1 N HCl overnight, and rinsed three times in Milli-Q water. The cathode was a piece of stainless steel (SS) mesh (Type 304, mesh size 90 × 90, cathode specific surface area of A<sub>C</sub> = 86 m<sup>2</sup>/m<sup>3</sup>). The gap between the anode and cathode was

~1 cm, and an Ag/AgCl reference electrode was placed between the electrodes. Bottles were sealed with a thick butyl rubber stopper (20 mm diameter) and crimped with an aluminum cap. Electrode wires for the anode (Ti), cathode (SS) and reference electrode were passed through the rubber stopper.

Empty MECs were filled with sterile N<sub>2</sub>:CO<sub>2</sub> (80%:20%) gas, and then autoclaved prior to operation. *Geobacter* MECs (triplicate reactors) were filled with 4 mL of ATCC 1957 growth medium lacking sodium fumarate, inoculated with 1 mL of actively growing *Geobacter* cells and incubated in a temperature-controlled room (at 30 °C). Reactors were operated in fed-batch mode by replacing the media between batch cycles. The solution in the *Geobacter* MECs was replaced with 5 mL fresh, sterile, anaerobic growth medium every day by piercing the butyl stopper with a sterile needle and syringe, and purging the reactor headspace with a sterile N<sub>2</sub>:CO<sub>2</sub> gas mixture after media replacement. Hyperthermophilic MECs were filled with 4 mL of hyperthermophilic growth medium lacking ferric citrate (pH of 6.9–7.0 and conductivity of 30 mS/cm at room temperature) inside an anaerobic chamber and inoculated with 1 mL of actively growing cells. *Geoglobus* MECs were grown at 80 °C, and *Ferroglobus* MECs were grown at 85 °C. A new batch cycle was started when current dropped below 15% of maximum current densities, and all hyperthermophilic MEC solutions were replaced inside an anaerobic chamber (La-petite Glove box, Plas Labs, MI) containing an N<sub>2</sub> atmosphere. Reactor headspace was purged with sterile N<sub>2</sub>:CO<sub>2</sub> (80%:20%) gas before being returned to high temperature conditions. The hyperthermophilic MECs (duplicate reactors) provided with acetate (10 mM) as an electron donor were operated for nine cycles. Cyclic voltammetry was conducted at the end of the 8th cycle and protein concentrations were estimated at the end of the 9th cycle for both *F. placidus* and *G. ahangari* MECs.

All MECs were operated at an applied voltage of 0.7 V. This applied voltage was chosen based on previous tests with *Geobacter* sp. in the same reactor set-up [49]. MECs were operated using a power supply (model 3645A; Circuit Specialists Inc.) with a 10 Ω resistor connected in series for recording voltage used to calculate current [49]. A multimeter (model 2700; Keithley Instruments Inc.) was used to record voltage at 10 min intervals, and current was calculated using Ohm's law. Biofilms of *F. placidus*, *G. ahangari*, and *G. sulfurreducens* PCA were established on graphite plate anodes (anode specific surface area [ $A_A$ ] = 92 m<sup>2</sup>/m<sup>3</sup>). The current density per area ( $J_A$ ; A/m<sup>2</sup>) was calculated by normalizing current against anode surface area, and current density per volume ( $J_V$ ; A/m<sup>3</sup>) was calculated by normalizing current against reactor volume. Coulombic efficiency (%) was calculated from the total charge passed during a single batch, divided by the theoretical amount of charge available from complete substrate oxidation [50].

In order to ensure that the major source of electrons in the hyperthermophilic MECs was acetate and not produced hydrogen, a second set of hyperthermophilic MECs were started as described previously. After these reactors were operated for five cycles with acetate provided as the electron donor and current had stabilized (data not shown), acetate was replaced with hydrogen as the sole electron donor, and current production was monitored. MECs were filled with the same medium without acetate and the headspace was purged with a H<sub>2</sub>:CO<sub>2</sub> (80%:20%) gas at 200 kPa. In successive cycles the electron donor was switched between acetate and hydrogen. Because current production was more variable in original tests with *F. placidus* compared to *G. ahangari* in the second round of tests, *F. placidus* MECs were operated in triplicate while *G. ahangari* reactors were operated in duplicate.

*G. sulfurreducens* was used in MECs as a comparison because a considerable amount of data concerning growth of this organism in MECs is available and this organism is considered the model exoelectrogen [1,5]. As outlined above, all three organisms were grown within their optimal temperature ranges and with identical MEC parameters. Therefore, all data collected from both the mesophilic and hyperthermophilic reactors is comparable.

### 2.3. Cyclic voltammetry

Cyclic voltammetry (CV) was conducted on anodes with a cathode used as the counter electrode, and an Ag/AgCl reference electrode (3 M NaCl). All potentials were reported here versus Ag/AgCl (+165 mV vs. a standard hydrogen electrode, SHE at 80 °C and +161 mV at 85 °C) [51]. New reference electrodes were placed between the anode and cathode prior to each CV scan inside a sterile anaerobic chamber flushed with N<sub>2</sub>:CO<sub>2</sub> (80%:20%) gas to avoid long-term storage of reference electrodes at high temperatures (80–85 °C). Scans were conducted over a range of −0.6 V vs. Ag/AgCl to +0.2 V vs. Ag/AgCl at a rate of 1 mV/s. CV analyses were performed using biotic anodes under both turnover conditions (in the presence of substrate) and non-turnover conditions. Non-turnover CV scans were performed with the same medium used for reactor operation without substrate (acetate) [52]. Additionally, CV scans were conducted with bare abiotic anodes and cathodes with sterile medium (abiotic control) and spent medium (reactor supernatant) in the presence and absence of substrate. For spent medium tests, the liquid removed from the MECs at the end of the cycle was placed into a new MEC containing bare abiotic anodes and cathodes inside a sterile anaerobic chamber gassed out with N<sub>2</sub>:CO<sub>2</sub> (80%:20%).

Comparisons made between CV curves generated by spent medium (reactor supernatant) and anode biofilms under turnover and non-turnover conditions (with or without acetate) can help determine how much of the current produced by an MEC can be attributed to attached cells [53]. Therefore, CV scans of spent medium were conducted in addition to turnover and non-turnover CV scans. First derivative CVs (DCVs) were obtained from the CV data by plotting the slope of each CV point against the anode potential, and the slope was calculated by a central difference quotient ( $J' = dJ/dE$ ). The open-source software QSOAS was used for baseline (capacitive current) correction for non-turnover (acetate deprived) conditions [54].

### 2.4. Protein measurements

Biomass of attached and planktonic cells was determined with protein measurements at the end of nine cycles for both *F. placidus* and *G. ahangari*. Proteins were extracted from cells associated with anode biofilms or in suspension with the sodium hydroxide and freeze-thaw [10] procedure, and measured by the modified Lowry method (DC Protein Assay Kit, Bio-Rad, CA) using bovine serum albumin standards in 0.2 N NaOH. In order to extract proteins from the biofilm, the graphite block was placed into a 15-mL tube, 2 mL NaOH solution (0.2 N) was flushed over the anode surface 8 times over a 30-minute period, and then the tube was briefly vortexed. Cells associated with planktonic biomass were pelleted by centrifugation at 4500 × g for 15 min. The supernatant was discarded and the pellet was resuspended in 2 mL NaOH solution (0.2 N). Biofilm and planktonic suspensions were then incubated at −20 °C overnight, thawed at room temperature, and immediately incubated at 100 °C for 10 min.

### 2.5. Scanning electron microscopy

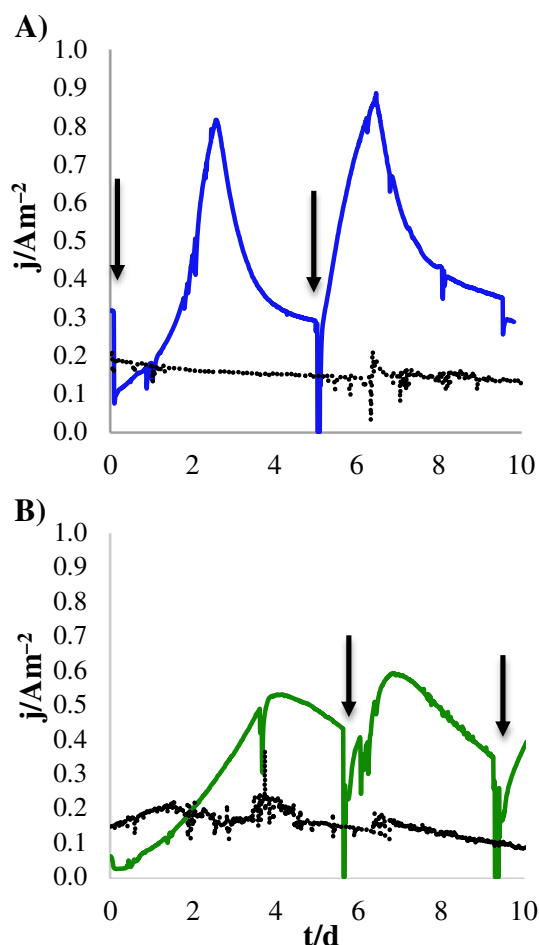
Scanning electron microscopy (SEM) analyses were performed at the Penn State Microscopy and Cytometry Facility. For SEM analyses, anodes were pulled from both *F. placidus* and *G. ahangari* MECs after nine cycles of operation. Anodes were removed from the mini MECs, rinsed with sterile medium, and immersed in 2% glutaraldehyde overnight. Electrodes were washed with Phosphate buffer saline (PBS) solution, and a secondary fixation was done with 1% osmium tetroxide (aqueous solution) followed by three more rinses with PBS buffer. The anodes were dehydrated by a graded ethanol series (25, 50, 70, 85, 95 and 100%; 5 min each stage with the last cycle repeated three times), and transferred to critical point drying (CPD) carriers. Samples were then mounted onto aluminum stubs before being sputter coated with

10 nm Au/Pd. Micrographs were obtained by SEM (ZEISS Sigma VP-FESEM, Jena Germany) with an accelerating voltage of 10 kV.

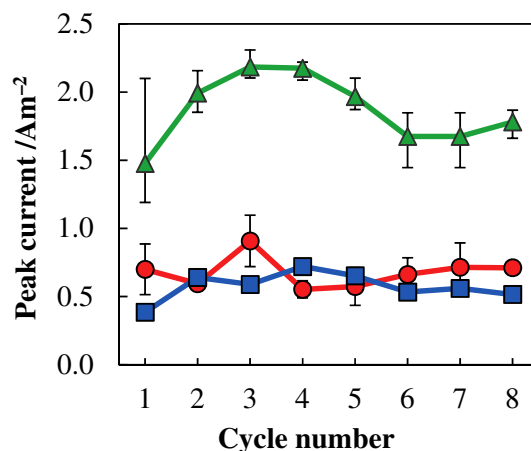
### 3. Results and discussion

#### 3.1. Current production

With an applied voltage of 0.7 V, MECs with both *F. placidus* or *G. ahangari* exhibited cycles of current production (Fig. 1), with about two days required to reach the maximum current densities (Fig. 1). Repeated cycles of current were sustained over several successive cycles (Fig. 2). Maximum current densities in the MECs averaged  $0.68 \pm 0.11 \text{ A/m}^2$  ( $J_V$ :  $62 \pm 10 \text{ A/m}^3$ ) over 8 fed-batch cycles for *F. placidus* at 85 °C, and  $0.57 \pm 0.10 \text{ A/m}^2$  ( $J_V$ :  $53 \pm 9 \text{ A/m}^3$ ) for *G. ahangari* at 80 °C. These current densities were not significantly different from each other ( $t$ -test  $P = 0.15$ ). Average coulombic efficiencies (CE) over 8 cycles were  $76.4 \pm 42.5\%$  and  $70.9 \pm 21.3\%$  for *F. placidus* and *G. ahangari* MECs. This variation in CEs might be attributed to relatively longer batch cycles at the start-up of the MECs. Repeated cycles of current were maintained over a period of three months with successive exchanges of the medium. Current densities obtained in hyperthermophilic MECs were higher than those reported for the thermophilic exoelectrogen *Thermincola ferriacetica* in a single-chamber MFC ( $0.5 \text{ A/m}^2$  at 60 °C) [55], but lower than densities obtained by



**Fig. 1.** Exemplary cycles of current production with fed-batch operation of (A) *F. placidus* MECs when acetate was provided as the sole electron donor (solid blue line shows *F. placidus* MEC, dotted black line shows abiotic control, arrows show medium replacement) (B) *G. ahangari* MECs when acetate was provided as the sole electron donor (solid green line shows *G. ahangari* MEC, dotted black line shows abiotic control, arrows show medium replacement). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Current densities generated by *G. sulfurreducens* (triangles), *F. placidus* (circles) and *G. ahangari* (squares) cells in mini MECs over multiple fed-batch cycles when acetate was provided as the sole electron donor. *F. placidus* and *G. ahangari* reactors were operated in duplicate at  $E_{AP} = 0.7 \text{ V}$  and *Geobacter sulfurreducens* reactors were operated in quadruplicate at  $E_{AP} = 0.7 \text{ V}$ . (Symbol size may be larger than the error bars).

this same organism in dual-chamber MECs ( $7\text{--}8 \text{ A/m}^2$ ) with a set anode potential of  $-0.06 \text{ V}$  vs. SHE [22]. These densities were also slightly higher than a mixed culture hyperthermophilic MFC that reached a current density of  $0.45 \text{ A/m}^2$  at 80 °C [34], although direct comparisons to current densities here is not possible due to differences in reactor operating conditions [56,57].

To relate current densities attained by these hyperthermophiles to other well-studied exoelectrogens, tests in the same MECs were conducted with *G. sulfurreducens* as reactor architecture (type and electrode materials) and operational conditions (added voltage or set potential) can impact performance [57]. Previous investigations reported that the mesophilic bacterium *G. sulfurreducens* was negatively impacted by high salinity conditions, as shown by a significant reduction in its ability to reduce Fe(III) in saline medium (20 g/L NaCl and 10 g/L  $\text{MgCl}_2$ ) [58]. Therefore, instead of using the same hyperthermophilic growth medium (18 g/L NaCl and 4.3  $\text{MgCl}_2$ ), the medium (ATCC 1957 Medium) used to grow *G. sulfurreducens* in MECs at 30 °C contained very little sodium or magnesium chloride salts (only 0.001 g/L NaCl).

The current produced by *G. sulfurreducens* ( $J_A$ :  $1.9 \pm 0.3 \text{ A/m}^2$  and  $J_V$ :  $175 \pm 22 \text{ A/m}^3$ ) under mesophilic conditions was significantly higher ( $t$ -test  $P < 0.001$ ) than those produced by either hyperthermophile (Fig. 1). Current densities obtained by *G. sulfurreducens* were similar to a previously reported current density of  $1.6 \text{ A/m}^2$  when *G. sulfurreducens* was grown in the same single-chamber MECs with acetate (10 mM) provided as the electron donor [49]. The slightly higher current density attained in this study may be due to the higher conductivity of the ATCC 1957 medium compared to the freshwater medium used for previous operation of the *Geobacter* MECs [49]. These current densities are lower than those previously reported for *G. sulfurreducens* in MECs with a set anode potential, where current densities reached  $9.2 \text{ A/m}^2$ , demonstrating the importance of comparisons based on the same reactor and operating conditions [59].

A possible explanation for the low current generated by *F. placidus* and *G. ahangari* compared to *G. sulfurreducens* may be that both hyperthermophiles lack some of the stress-related enzymes that were significantly expressed in *G. sulfurreducens* during growth on current-harvesting anodes [41,60]. Specifically, the gene coding for superoxide dismutase (SodA) is not present in either of these hyperthermophilic genomes, but was highly transcribed in *G. sulfurreducens* cells growing on current-harvesting anode surfaces [61]. While SodA is involved in oxidative stress response in *G. sulfurreducens* and other organisms, it is also highly expressed in *G. sulfurreducens* cells exposed to other

non oxygen related stresses [62]. The anode surface has the potential to be a stressful environment, as studies have shown that protons accumulate in *G. sulfurreducens* biofilms and can drop the pH near the anode surface to levels as low as 6.1 [63]. It has been shown that this drop in pH corresponded with increased transcription of *sodA* transcripts by cells in close proximity to the anode surface [61]. This lack of SodA activity may make it difficult for *F. placidus* and *G. ahangari* cells to tolerate such environmental stresses as proton accumulation encountered on the anode surface. Future studies done to optimize growth of both hyperthermophiles in MECs should focus on ways to minimize such stresses as drops in pH.

Protein measurements were done to compare the amount of biomass attached to the anode surface to that in solution. An average of 0.62 mg of cell protein (0.135 mg/cm<sup>2</sup> of electrode surface area,  $n = 2$ ) was extracted from electrodes in *F. placidus* MECs and ~0.64 mg cell protein (0.139 mg/cm<sup>2</sup> of electrode surface area,  $n = 2$ ) was extracted from electrodes in *G. ahangari* MECs, with no significant difference in the total extracted protein by these two strains ( $t$ -test,  $P = 0.74$ ).

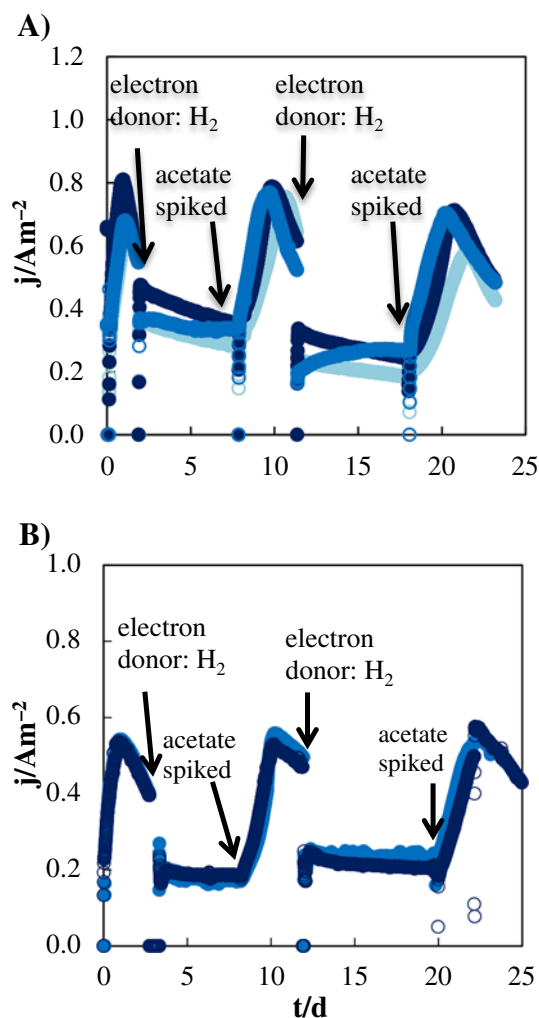
Protein concentrations were 3.0–3.2 times higher in biomass extracted from the anode surface than surrounding medium, suggesting that current production could primarily be attributed to attached biomass. SEM images also showed that cells were attached to the anode surface during operation of the MEC (Supplementary Information, Figs. S1 and S2). These results are consistent with the finding that both of these hyperthermophiles require direct contact with Fe(III) oxides to reduce them [17,40].

### 3.2. Optimal conditions required for current production

While the optimum growth temperature of *G. ahangari* was previously reported to be 88 °C [45], and it is commonly cultured at 85 °C for dissimilatory iron reduction [40,43], when MECs inoculated with *G. ahangari* were incubated at 85 °C, current production was erratic and ceased after only two fed-batch cycles (data not shown). However, current production was stable when MECs with *G. ahangari* were operated at 80 °C. Abiotic control reactors, which contained medium lacking ferric citrate, did not produce any current, and abiotic controls with ferric citrate (10% v/v) and other medium components produced minimal background current of <0.2 A/m<sup>2</sup> (Fig. 1).

*G. ahangari* has previously been shown to grow autotrophically with H<sub>2</sub> gas as the sole electron donor when Fe(III) is provided as the electron acceptor [45]. To evaluate current production by *G. ahangari* being fed H<sub>2</sub>, MECs were filled with the same medium without acetate, and the headspace was purged with a H<sub>2</sub>:CO<sub>2</sub> (80%:20%) gas mixture instead of N<sub>2</sub>:CO<sub>2</sub> and overpressurized to 200 kPa. *G. ahangari* was unable to generate electrical current when H<sub>2</sub> was provided as the sole electron donor at rates similar to those produced when provided with acetate (Fig. 3B), confirming that the major source of electrons was acetate for stable current production over multiple cycles (Fig. 2). However, upon addition of acetate, current production resumed and reached rates similar to those observed in previous cycles with acetate.

*F. placidus* can also use H<sub>2</sub> as an electron donor for growth with nitrate (NO<sub>3</sub><sup>-</sup>), thiosulfate (S<sub>2</sub>O<sub>3</sub><sup>2-</sup>), and Fe(III) provided as electron acceptors [44]. Similar to *G. ahangari*, providing H<sub>2</sub> as the sole electron donor did not enable production of electrical current by *F. placidus* MECs at rates similar to those observed with acetate (Fig. 3A). In contrast, *G. sulfurreducens* can use H<sub>2</sub> as the sole electron donor and produce current at similar rates to acetate in bioelectrochemical reactors [10]. Utilization of H<sub>2</sub> can cause H<sub>2</sub>-cycling in single-chamber MECs, where H<sub>2</sub> produced at the cathode is used by bacteria on the anode to produce current, thereby increasing apparent coulombic efficiencies of these electrochemical systems [10,49]. Current densities generated by abiotic control reactors (Fig. 1) and MECs without electron donor (i.e. acetate or H<sub>2</sub>) were similar to those generated by hyperthermophilic reactors fed



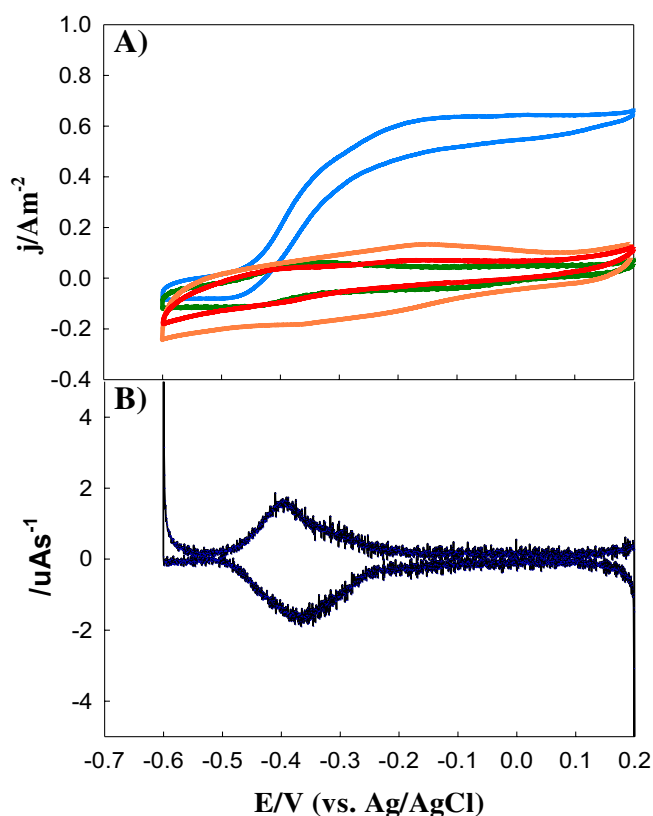
**Fig. 3.** Test for hydrogen-dependent current production by A) *F. placidus* and B) *G. ahangari* biofilms in MECs containing graphite anodes. During the fifth cycle (days 0–2/3), cells were grown with acetate (10 mM) provided as the electron donor. At the beginning of cycles 6 and 8 (days 2/3 and 11), MECs were replaced with acetate-free medium and hydrogen in a H<sub>2</sub>:CO<sub>2</sub> gas mixture (80%:20%) was provided as the only source of electrons for respiration. Acetate was then spiked into reactors as indicated (days 7/8 and 18/19). (x-axis shows the start of fifth cycle as day zero, replica reactors are shown with different shades of blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with H<sub>2</sub> (Fig. 3). Therefore, if H<sub>2</sub>-cycling was occurring in these MECs, it was not significant.

### 3.3. Cyclic voltammetry evaluation of *F. placidus* and *G. ahangari* biofilms

MECs with an established *F. placidus* biofilm (generating current for at least 30 days) displayed a sigmoidal (S-shaped) catalytic wave in CV tests, with an onset potential of  $-0.48$  V vs. Ag/AgCl and a maximum current of ca. 300  $\mu$ A above  $-0.2$  V vs. Ag/AgCl where a plateau was reached (Fig. 4A). Based on DCV analyses under turnover conditions, the mid-point potential of the current was around  $-0.39$  V vs. Ag/AgCl (Fig. 4B). The catalytic current under turnover conditions was significantly higher and more distinct than those from sterile medium (abiotic control) or spent medium (reactor supernatant), suggesting that neither the high salinity medium, planktonic cells, nor self-produced mediators were responsible for current production (Fig. 4A).

CV scans of spent medium only and spent medium plus acetate were identical. Therefore, only one trace is shown in Fig. 4A. The non-turnover CV of *F. placidus* biofilm and baseline subtracted curves are shown in Fig. 6A. The peaks shown in the baseline-subtracted curve

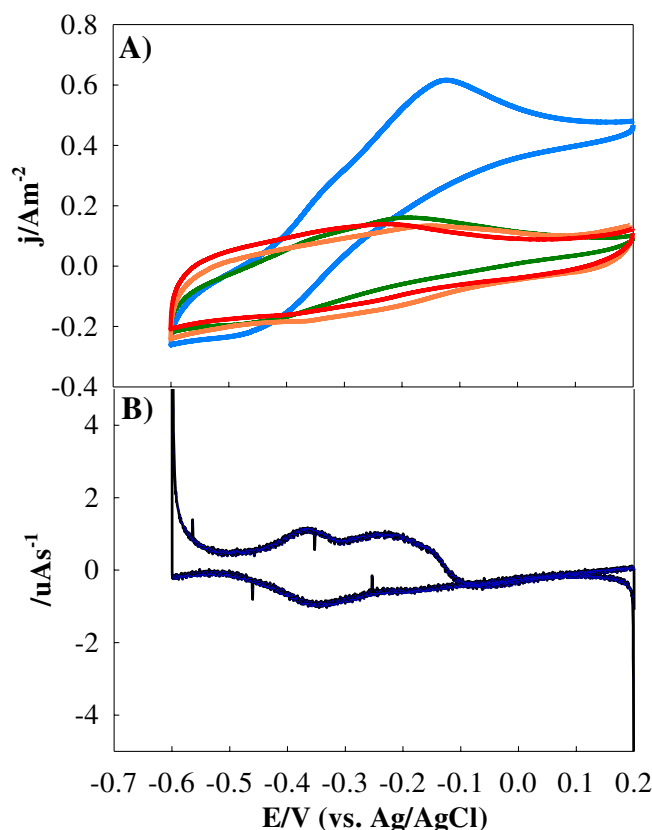


**Fig. 4.** A) Cyclic voltammogram of *F. placidus* MECs: under turnover conditions (biofilm + substrate, at the end of cycle 8), blue line; under non-turnover conditions (biofilm – substrate, at the end of cycle 8), green line; using sterile medium with abiotic anode and cathode (abiotic control), orange line; using the spent medium (at the end of cycle 8), red line. B) First derivative CV scan under turnover conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

appear to be the same potential range as the first derivative of turnover CV, indicating that the current generated in turnover CV was due to the formed biofilm. Clearly, the turnover CV signal obtained resembles the CV signals from exoelectrogenic bacteria capable of DET via outer membrane cytochromes. Previously, it has been shown that the inflection points of CV signals of bacteria that are able to perform DET have a very broad potential window of  $-0.42$  to  $-0.22$  V vs. Ag/AgCl ( $-0.26$  to  $-0.06$  vs. SHE) [64] and the mid-point potential of  $-0.39$  V for the *F. placidus* biofilm is within this bracket. The CV signals obtained here are indicative of biofilm-associated, DET by *F. placidus* to the electrode and this was also supported by the protein and SEM data.

Turnover CVs with *G. ahangari* biofilms (generating current for at least 30 days) had a similar onset potential of  $-0.48$  V vs. Ag/AgCl and only a slightly lower maximum current of ca.  $280 \mu\text{A}$  at  $-0.12$  V vs. Ag/AgCl (Fig. 5A). Unlike *F. placidus* biofilms, however, at potentials greater than  $-0.12$  V vs. Ag/AgCl current decreased by 20%, or from  $280 \mu\text{A}$  to  $220 \mu\text{A}$  at ca.  $0.1$  V vs. Ag/AgCl. This decline in current at higher potentials may be attributed to stress responses related to local pH changes or to increases in oxidizing capacities of the anode at higher potentials.

As shown in Fig. 5B, there are two peaks in the first derivative curve; one at  $-0.38$  V vs. Ag/AgCl, and a second one at  $-0.22$  V vs. Ag/AgCl, which were also present in the baseline-subtracted non-turnover CV (Fig. 6B). Two peaks in the first derivative curve have also been observed with *Geobacter* species and have been interpreted as the presence of a young biofilm [65]. CV scans of *G. ahangari* biofilms conducted at the end of the 8th fed-batch cycle did not show a corresponding reduction peak, and therefore the nature and role of the second peak (at  $-0.22$  V vs. Ag/AgCl) was not clear. There was an

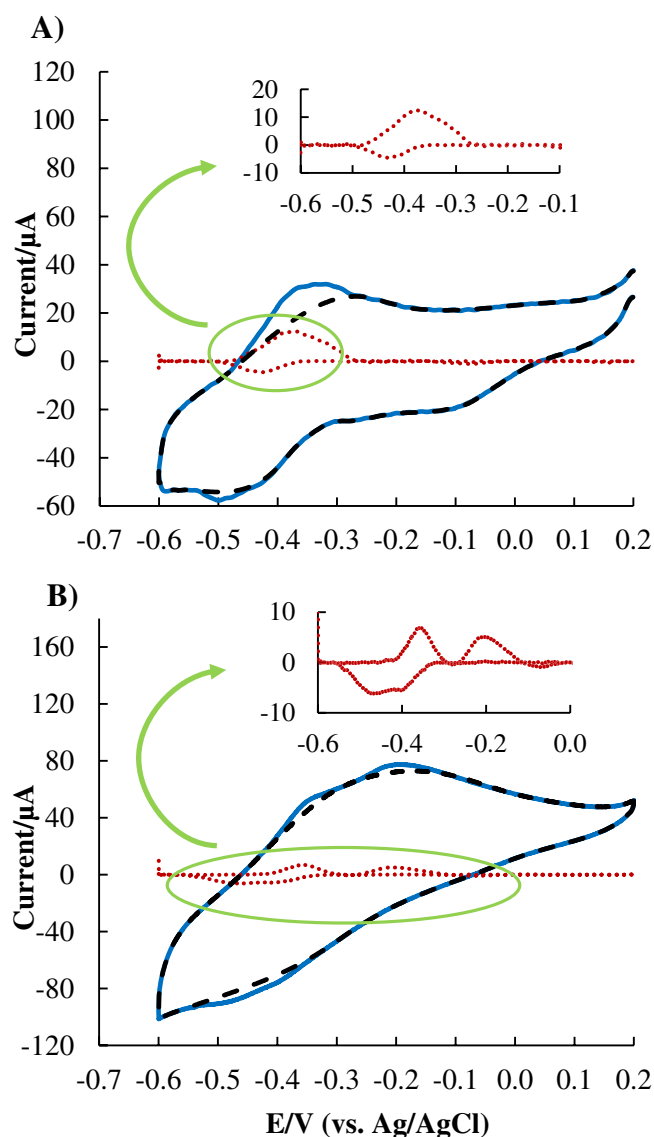


**Fig. 5.** A) Cyclic voltammogram of *G. ahangari* MECs under turnover conditions (biofilm + substrate, at the end of cycle 8), blue line; under non-turnover conditions (biofilm – substrate, at the end of cycle 8), green line; using sterile medium with abiotic anode and cathode (abiotic control), orange line; using the spent medium, red line. B) First derivative CV scan under turnover conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

appreciable difference between the turnover (Fig. 5A) and sterile medium (abiotic control) CV signal. Also, notable differences were observed between signals obtained from spent medium (reactor supernatant) (Fig. 5A) and the turnover CV. Scans of spent medium in the presence and absence of acetate were identical, so only one trace is shown (spent medium, Fig. 5). The significant differences in CV signals - i.e. the lack of significant peaks or S-shaped curves in CV signals of abiotic controls or spent medium coupled with three times higher cell protein of biofilm with respect to reactor supernatant (planktonic cells) - were indicative of biofilm-associated direct electron transfer.

#### 4. Conclusions

Current production was obtained in MECs with both *F. placidus* and *G. ahangari*. As previously reported, both species possess large multi-heme c-type cytochromes that are involved in respiration of insoluble Fe(III) oxides [17,40]. Therefore, it is likely that c-type cytochromes are involved in direct electron transfer to the anodes, although it is not known whether the same cytochromes are used for extracellular electron transfer to both acceptors. The identification and further examination of the ETCs is beyond the objective of this initial study, but this topic requires further research. Previous studies have shown that many exoelectrogenic bacteria are also able to grow syntrophically with other microorganisms via direct interspecies electron transfer (DIET) [15,66,67]. Effective interspecies electron transfer is key to the successful operation of anaerobic digesters and it has been shown that promotion of DIET in these systems improves digester efficiencies and biofuel production rates [67,68]. Therefore, the potential for electron transfer by these hyperthermophilic archaea to other species in the



**Fig. 6.** A) Non-turnover CV of *F. placidus* MECs. B) Non-turnover CV of *G. ahangari* MECs. (A solid line shows the non-turnover CV, the dashed line is the baseline, and the dotted line shows the respective baseline-subtracted curve. The baseline is obtained using QSOAS. Insets show zoomed in versions of the baseline-subtracted curves.)

environment should also be explored. In addition, *F. placidus* has been reported to couple Fe(III) oxide reduction with anaerobic degradation of aromatic compounds such as benzene, phenylalanine, benzoate and phenol [69–71], and this capacity might be useful in development of METs for removal of aromatic compounds from such waste streams as those produced by petroleum refineries and other industries [72,73] with simultaneous electricity production.

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