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ORIGINAL ARTICLE

Hydrogenase-independent uptake and metabolism of electrons by the archaeon *Methanococcus maripaludis*

Svenja T Lohner[1,5], Jörg S Deutzmann[1,5], Bruce E Logan[2], John Leigh[3] and Alfred M Spormann[1,4]

[1]*Department of Civil and Environmental Engineering, Stanford University, Stanford, CA, USA;* [2]*Department of Civil and Environmental Engineering, Pennsylvania State University, University Park, PA, USA;* [3]*Department of Microbiology, University of Washington, Seattle, WA, USA and* [4]*Department of Chemical Engineering, Stanford University, Stanford, CA, USA*

Direct, shuttle-free uptake of extracellular, cathode-derived electrons has been postulated as a novel mechanism of electron metabolism in some prokaryotes that may also be involved in syntrophic electron transport between two microorganisms. Experimental proof for direct uptake of cathodic electrons has been mostly indirect and has been based on the absence of detectable concentrations of molecular hydrogen. However, hydrogen can be formed as a transient intermediate abiotically at low cathodic potentials (< -414 mV) under conditions of electromethanogenesis. Here we provide genetic evidence for hydrogen-independent uptake of extracellular electrons. Methane formation from cathodic electrons was observed in a wild-type strain of the methanogenic archaeon *Methanococcus maripaludis* as well as in a hydrogenase-deletion mutant lacking all catabolic hydrogenases, indicating the presence of a hydrogenase-independent mechanism of electron catabolism. In addition, we discovered a new route for hydrogen or formate production from cathodic electrons: Upon chemical inhibition of methanogenesis with 2-bromo-ethane sulfonate, hydrogen or formate accumulated in the bioelectrochemical cells instead of methane. These results have implications for our understanding on the diversity of microbial electron uptake and metabolism.

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Introduction

Direct uptake of free, extracellular electrons from an abiotic source into cellular metabolism has been postulated to occur in several natural microbial systems. For example, some minerals, such as pyrite, are electrically conductive and enable a flow of electrons in the mineral phase towards more positive potentials (Sato and Mooney, 1960). Kato *et al.* (2012) showed that in deep sea hydrothermal vents, the hot reducing geothermal fluid can abiotically reduce pyrite of the chimney wall whereas in the oxic 4 °C sea water outside the chimney such electrons can be metabolized by a microbial community. Sulfate-reducing bacteria can mediate corrosion of elemental iron and mild steel by direct uptake of electrons and by the formation of an electrically conductive iron-sulfur-carbonate mineral, in addition to the accelerated corrosion caused by hydrogen uptake and sulfide production (Dinh *et al.*, 2004; Enning *et al.*, 2012). In the same context, methanogens have been suggested to facilitate iron corrosion in a more direct manner than via hydrogen consumption (Dinh *et al.*, 2004). In marine sediments it has been shown that filamentous bacteria can transport electrons over centimeter distances within conductive cables (Pfeffer *et al.*, 2012). It was also reported that some bacteria can relay electrons via conductive pili (Reguera *et al.*, 2005) and outer membrane cytochromes (Rabaey *et al.*, 2007), and are capable of direct electron transfer in a syntrophic association (Rotaru *et al.*, 2013). The mechanism(s) of such electron uptake are largely unknown.

Much controversy remains about whether microbes are capable of direct uptake of extracellular electrons, and therefore, whether the observed

Correspondence: AM Spormann, Department of Civil and Environmental Engineering, Stanford University, 318 Clark Center, Campus Drive, Stanford, CA 94305, USA.
E-mail: spormann@stanford.edu
[5]These authors contributed equally to this work.
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uptake could be indirect. For the context of our research presented here, we provide a molecular perspective to distinguish between 'direct' and 'indirect' electron transfer between a surface and to and from a cell. We define 'direct' electron uptake as a process where electrons are transferred between a cell and a surface by a cell-associated, redox-active component, for example, via MtrC in *Shewanella oneidensis* and OmcC in *Geobacter* sp. (Ross *et al.*, 2011), in the absence of a diffusible mediator. In contrast, indirect electron transfer does not rely on cellular contact to a surface, and can involve organic as well as inorganic compounds that operationally function as diffusible electron carriers between a cell and a surface. Indirect electron uptake relies on a reaction cycle between surface-derived electrons and a redox-active compound, which is abiotically reduced and subsequently microbially oxidized. Some complex soluble electron shuttle molecules such as flavins are released by some microorganisms (Stams *et al.*, 2006; Kotloski and Gralnick, 2013), although these extracellular electron shuttling molecules can be used by microorganisms incapable of their synthesis.

Molecular hydrogen has been recognized as a key shuttling molecule (Stams *et al.*, 2006). Consequently, investigations on an indirect electron uptake have been focused primarily on H_2, as H_2 can be formed abiotically from low potential electrons (< -414 mV) and protons, which are ubiquitous in aqueous nonalkaline environments. It is therefore possible that hydrogen represents the ultimate source of electrons metabolized in microbial systems where a 'direct' mechanism has been postulated (Dinh *et al.*, 2004; Villano *et al.*, 2010). Many anaerobic microorganisms, including methanogenic archaea, utilize hydrogen via hydrogenases as a primary source of catabolic electrons (Thauer *et al.*, 2010). Several studies eliminated hydrogen as an intermediate of free electron metabolism via kinetic arguments as well as based on the absence of detectable H_2 levels (Nevin *et al.*, 2010; Villano *et al.*, 2010; Nevin *et al.*, 2011). The same general challenge of distinguishing between direct and indirect electron uptake arises when electron transfer between different microorganisms is investigated. An undetectable or low steady-state concentration of a potential metabolic intermediate such as hydrogen does not exclude a rapid cycling of these compounds as electron carrier. On the basis of the absence of appropriate enzymes and of detectable levels of redox-active small molecules, a direct biological interspecies electron transfer in cocultures of acetoclastic *Methanosaeta harundinacea* and the exoelectrogen *Geobacter metallireducens* has recently been suggested as a mechanism to enable more complete conversion of ethanol to methane, as *M. harundinacea* cannot use hydrogen directly as a donor for CO_2 reduction (Rotaru *et al.*, 2013). Although evidence for the existence of a direct electron transfer between two prokaryotic cells, for example, via conductive pili, cables or other cellular structures becomes more apparent (Reguera *et al.*, 2005; Rabaey *et al.*, 2007; Morita *et al.*, 2011; Liu *et al.*, 2012; Pfeffer *et al.*, 2012), the direct uptake of electrons from abiotic surfaces such as minerals or electrodes is not well understood and may be mechanistically different from direct interspecies electron transfer (Lovley, 2012).

In this study we investigated the uptake of cathodic electrons from a graphite electrode by the methanogenic archaeon *Methanococcus maripaludis* as a well-defined model system. The main focus was to investigate whether hydrogen is an obligatory intermediate for electron uptake during electromethanogenesis in this system or whether cells harbor an alternative mechanism for external electron transfer.

Materials and methods

Growth of M. maripaludis *wild type (wt) and MM1284 hydrogenase mutant*

M. maripaludis wild type (wt, strain MM901; Costa *et al.*, 2010) and the Δ6 mutant strain (MM1284) were routinely cultured in a modified DSMZ mineral medium 141 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium141.pdf) omitting Na-acetate, yeast extract and trypticase. The growth medium also contained 200 mM sodium formate and 200 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 6.8), and H_2/CO_2 (80:20) was added to the culture headspace to provide H_2 needed for anabolic reactions (Costa *et al.*, 2013a, b). Cultures were inoculated with 2% (v/v) of a stationary phase culture and grown at 37 °C in a water bath shaker. Cells were harvested for bioelectrochemical experiments in early stationary phase at an optical density of 600 nm (OD_{600}) of ~0.4 when formate was completely consumed. The bioelectrochemical reactors were exclusively inoculated with harvested planktonic cells as no biofilm formation was observed in the growth vessels under these culture conditions.

Setup of bioelectrochemical systems

The bioelectrochemical reactor consisted of a two chambered borosilicate gastight H-cell, in which the 150 ml anode and cathode chambers were separated by a Nafion 117 proton-exchange membrane (Fuel Cell Store Inc., College Station, TX, USA, surface area 7.5 cm^2). In between experiments, the membrane was regenerated by boiling in H_2O_2 (3% v/v), DI water, 0.5 M H_2SO_4 and DI water each for 1 h and stored in DI water. Graphite rods (McMaster-Carr Atlanta, GA, USA, 8 cm^2 surface exposed to the reactor medium) were used as anode and cathode material, and titanium wire (McMaster-Carr, 0.032 in diameter) was used together with a nylon connector to connect the electrodes to the circuit. Only electrodes with a resistance $< 5\,\Omega$ were used

for experiments to prevent a significant voltage drop between potentiostat and electrode. In between experiments, electrodes were either rinsed overnight in 1 M HCl, and subsequently in 1 M NaOH, and brushed with sandpaper, or fresh electrodes were used. A NaCl saturated Ag/AgCl reference electrode (RE-5B, BASI) (+201 mV versus standard hydrogen electrode) was placed in the cathode chamber to set constant cathode potentials using a multichannel potentiostat (VMP3, Bio-logic, Claix, France) that was also used to monitor current. All potentials indicated in this article are relative to standard hydrogen electrode (SHE).

Bioelectrochemical experiments
Bioelectrochemical reactors were set up, autoclaved and then flushed with a N_2/CO_2 (80:20) gas mix at least for 15 min to achieve an oxygen-free headspace. The anode and cathode compartments were filled with 90 ml sterilized and anoxic DSMZ 141 medium buffered with 200 mM MOPS (pH 6.8). The reactor medium contained no carbon source and lacked resazurin and cysteine. After filling the reactors, they were again flushed for at least 5 min with N_2/CO_2 (80:20) and put on a magnetic stir plate at 30 °C. Approximately 10% of freshly harvested early stationary cultures of *M. maripaludis* wt or MM1284 Δ6 mutant were introduced into the cathode chamber to a maximum OD_{600} of 0.03–0.04. The bioelectrochemical reactor was connected to the potentiostat, and a constant cathode potential was set between 1–12 h after cells were introduced. In experiments with 2-bromoethanesulfonic acid (BES) inhibition, 7 mM BES was added aseptically and anaerobically to the cathode chamber once bioelectrochemical methane formation was confirmed. Each experiment was performed in duplicates or triplicates and lasted for 1–2 weeks.

Cumulative electric charge, that is, the amount of electrons consumed, was calculated by integration of the current profile logged by the potentiostat, omitting the data of the first minutes to account for the initially high current during capacitive charging of the system upon application of the potential. Obvious perturbations that were caused by handling of the reactors (that is, high current spikes caused by touching the leads or electrode contacts during sampling for very short periods of time (milliseconds to few seconds)) were corrected manually by omitting current data for this short time interval. Current efficiencies were calculated as the ratio of electrons theoretically needed to form the measured products (eight e^- for CH_4 from CO_2 and two e^- for H_2 from H^+) and the cumulative electric charge measured as the current by the potentiostat.

Electrochemical measurements
Polarization curves were performed at the end of the bioelectrochemical experiments to compare the abiotic and biotic cathodes by means of chronoamperometry. Using the potentiostat, the cathodic potential was slowly stepped down from −0.35 to −0.7 mV in 25 or 50 mV increments. At each potential, the current was logged continuously for 1 h and the average current over the last 45 min was plotted in the polarization curve.

Analytical methods
Hydrogen, methane and formate were monitored throughout each experiment to assess the potential of *M. maripaludis* to utilize cathodic electrons. Gaseous headspace samples were taken in regular intervals from the cathode chamber with a gastight syringe (VICI). Samples from the headspace (200 μl) were injected into a gas chromatograph equipped with a reducing compound photometer (Peak Performer 1, Peak Laboratories, Mountain View, CA, USA) for hydrogen analysis using nitrogen (99.999%) as carrier gas and a mercury bed temperature of 210 °C. The column was heated to 100 °C. Hydrogen calculations were based on a multi-point external calibration curve and three hydrogen standards were measured before each sampling series.

Analysis of methane was made using 300 μl from a headspace sample with a gas chromatograph (Agilent 6890N, Agilent, Santa Clara, CA, USA) equipped with flame ionization detector. Separation was accomplished in a GS-Q capillary column (30 m length, 0.530 μM ID) and helium was used as carrier gas at a flow rate of 7.4 ml min^{-1}. The injector (split ratio 0.1:1) and flame ionization detector temperatures were 250 °C, and analyses were isothermal at 100 °C column temperature.

Liquid samples (1 ml) were withdrawn from the cathode chamber and filtered using syringe filters (0.2 μm diameter pore size) into HPLC sampling vials. Analysis of 50 μl sample volume was performed at 20 °C using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) with 5 mM H_2SO_4 as the eluent, at a flow rate of 0.4 ml min^{-1}. Formate was identified by comparison to known standards according to retention time, using a variable wavelength detector detector (210 nm) (35 °C) with a detection limit of 5 μM.

Results

Cultures grown in mineral medium on formate, harvested in early stationary phase and introduced into the anoxic, bicarbonate-containing MOPS buffer immediately formed methane at a rate of approximately 0.38 μmol h^{-1} when the cathode was set at a potential of −600 mV (versus a standard hydrogen electrode) (Figure 1a). Based on the current and methane recovery, the coulombic efficiency was 70–80% (Supplementary Figure S1a). This activity was observed for at least 7 days (Figure 1). Methane formation was not detected in

controls without cells (abiotic control; Figure 1a) or in the absence of a cathodic potential (data not shown). At a potential of −600 mV, molecular hydrogen was formed abiotically at a rate of 0.04 μmol h^{-1} (Figure 1b). The concentration of hydrogen did not increase in the presence of *M. maripaludis* wt cells, but reached a low steady-state concentration during the experiment.

When the cathode potential was lowered to −700 mV, a transient increase in H_2 concentration coupled to an increased rate of methane formation was observed (Figure 1). As the cathodic potential affects the rate of abiotic hydrogen release, the lower potential led to a faster production of hydrogen, which was presumably consumed subsequently by the cells, indicative of an indirect electron uptake via hydrogen as an intermediate (Figure 1b). Consequently, the rate of methane formation increased to about 1 μmol h^{-1} at −700 mV (Figure 1a).

To investigate whether an H^+/H_2 cycling between cathode and this archaeon might be involved in the apparent direct cathodic electron uptake, we used *M. maripaludis* mutant MM1284, which carries markerless in-frame deletions of all five catabolic hydrogenase genes, *fru*, *frc*, *hmd*, *vhu* and *vhc*, plus a deletion in the anabolic *ehb* hydrogenase (Costa *et al.*, 2013a, b). These deletions rendered MM1284 strain defective in methane formation from H_2 and CO_2, and unable to grow by this catabolic reaction. The only hydrogenase present, the energy conserving hydrogenase Eha that is needed to reduce ferredoxin for anabolic reactions under the consumption of proton motive force, does not enable growth or methane formation from H_2/CO_2 (Lie *et al.*, 2012; Costa *et al.*, 2013a, data not shown). When cells of strain MM1284 were tested in the bioelectrochemical reactor with a cathode potential at −600 mV, methane was formed at a

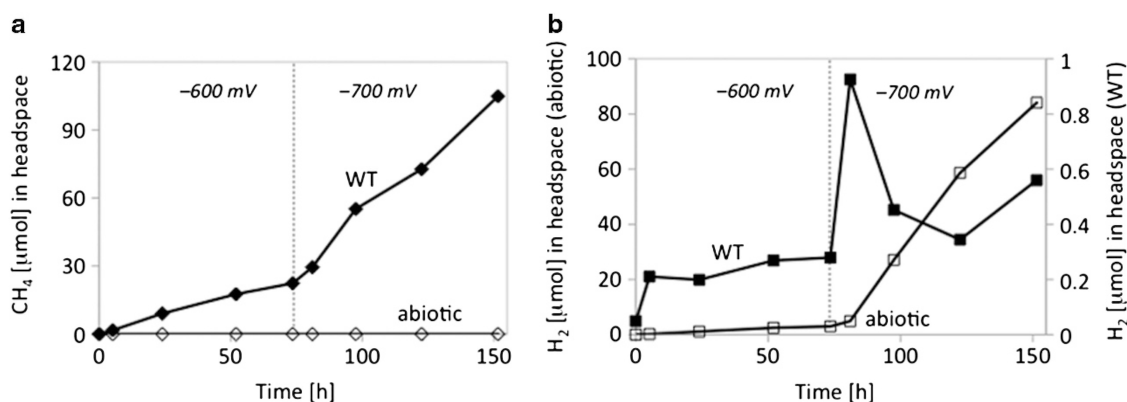

Figure 1 Bioelectrochemical methane formation in *M. maripaludis* wild-type cells. (**a**) Potential dependent methane formation was observed in the wt strain (◆) but not in the abiotic control (◇) when tested in bioelectrochemical reactors with cathode potentials of −600 and −700 mV. (**b**) Hydrogen concentrations in the abiotic control (□) were much higher and potential dependent compared with the wt strain (■). Results shown are a representative example of replicate experiments ($n = 4$). The potential was decreased from −600 mV to −700 mV at $t = 73$ h. Electron recovery in the form of methane and hydrogen, that is, the coulombic efficiency under those conditions was in the range of 70–80% (Supplementary Figure S1).

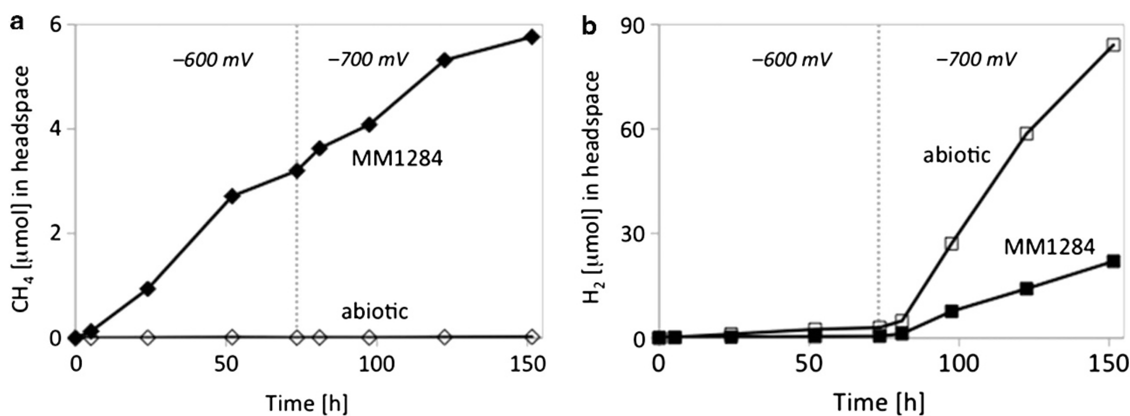

Figure 2 Hydrogen-independent methane formation in the *M. maripaludis* strain MM1284. (**a**) Bioelectrochemical methane formation was observed in mutant strain MM1284 (◆) but not in the abiotic control (◇), and it was independent of the set cathode potential in bioelectrochemical reactors at cathode potentials of −600 and −700 mV. (**b**) Hydrogen did accumulate in tests with either strain MM1284 (■) or the abiotic control (□), and in both the cases formation rates were dependent on the set cathode potential. The potential was decreased from −600 mV to −700 mV at $t = 73$ h. Results shown are a representative example of replicate experiments ($n = 2$).

rate of 0.04 μmol h^{-1}, which was about 1/10 of the rate of methane formation in wt at a coulombic efficiency of 50–60% (Figure 2a, Supplementary Figure S1c). The onset of methanogenesis was immediate after setting this potential. When the cells of the mutant strain in the electrochemical reactor were subjected to the same downshift in cathodic potential to −700 mV, no change in the rate of methanogenesis was observed, in contrast to the increase in methanogenesis rates observed for wt cells. A concurrent steady increase in H_2 was found, as expected from an abiotic reaction at the cathode, which is consistent with the inability of strain MM1284 to consume hydrogen (Figure 2b).

To test whether metabolism of cathode-derived electrons in *M. maripaludis* is impeded when the central catabolic pathway and main electron sink (reduction of CO_2 to CH_4) is inhibited, we treated wt and MM1284 mutant cells with 7 mM BES, a specific inhibitor of methylcoenzyme M reductase, the key enzyme in the last step in methanogenesis (Smith, 1983). As expected, in both wt as well as the MM1284 mutant experiments, methane formation ceased upon introduction of BES (Figures 3a and b). Bioelectrochemical reactors carrying wt cells previously grown on formate were found to accumulate both H_2 and formate, whereas the reactors with MM1284 mutant cells accumulated formate only (Figures 3a and b). When wt cells, previously grown on H_2 and CO_2 without formate, were tested in the electrochemical reactor under the same conditions, no formate but hydrogen accumulation was detected (data not shown).

As close contact of cells with the cathodic surface is a prerequisite for a direct electron uptake, we investigated whether the observed microbial activity was directly associated with electrode contact. We removed all planktonic cells from the cathodic chamber after a week of active electromethanogenesis, and the chamber was rinsed twice and subsequently refilled with fresh anoxic sterile medium. The subsequent rate of methane formation observed at a cathode potential of −600 mV was in the same range as the rate in the reactor before rinsing and even increased to a small extent for wt cells (Figure 4), indicating that most of the electromethanogenic activity was cathode associated.

We also found that the presence of *M. maripaludis* wt cells effectively lowered the cathodic overpotential for hydrogen evolution. Reactors containing wt cells started consuming current at more positive potentials (−400 mV to −450 mV) than the abiotic control where significant current consumption started at −600 mV (Figure 5). This ability of cells to form methane at a more positive cathode potential than that needed for H_2 production is also reflected by a H_2 production rate that is twice the abiotic rate in BES-inhibited wt cells on the cathode at −600 mV. When the hydrogenase-deletion strain MM1284 was used in the reactors, the H_2 formation rate was lower than that in the abiotic control. This reduction in the H_2 evolution rate in MM1284 cells

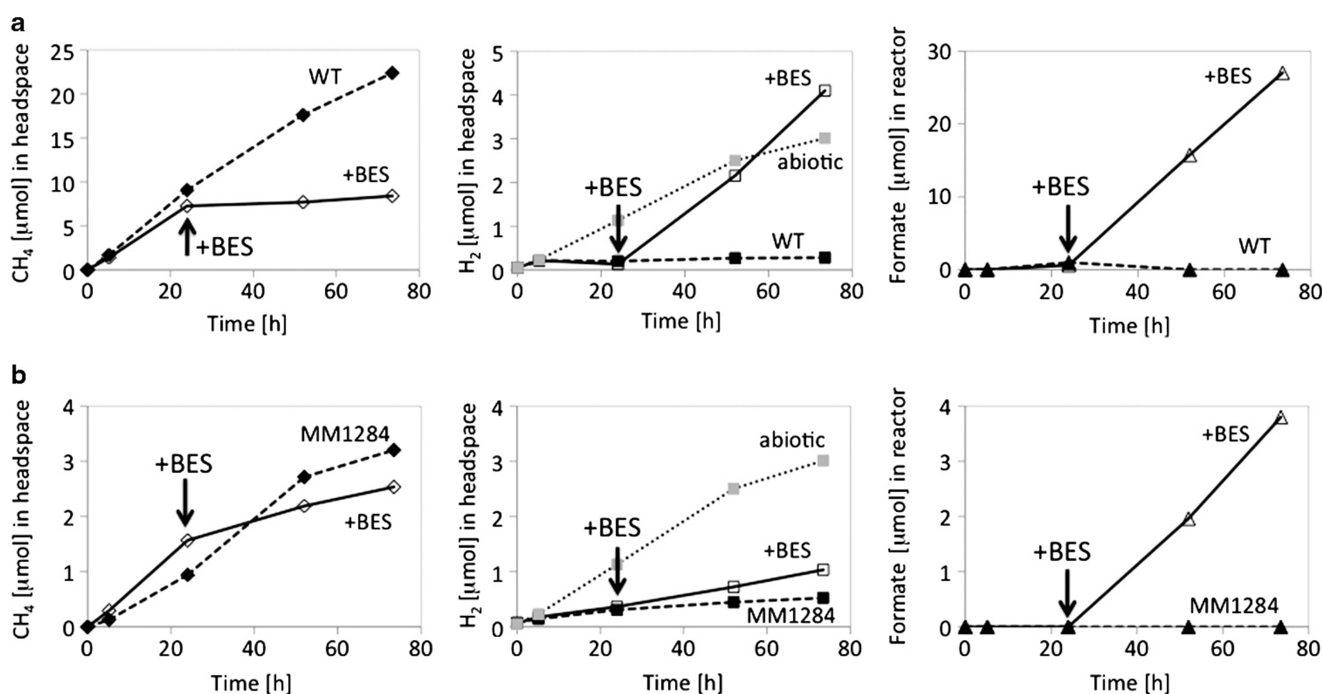

Figure 3 Inhibiting electron flow towards methanogenesis at a cathodic potential of −600 mV results in the formation of other reduced compounds. (**a**) When inhibited with 7 mM BES (solid line), wt *M. maripaludis* ceased forming methane (◇), increased hydrogen formation (□) compared with the abiotic control (▲), and produced formate (△). (**b**) Mutant strain MM1284 also showed inhibition of methane formation (◇) but no significant change in the rate of hydrogen production (□). Formate was observed when cells were inhibited with 7 mM BES (△). Black filled symbols indicate the respective non-inhibited controls results shown are a representative example of replicate experiments ($n = 2$)

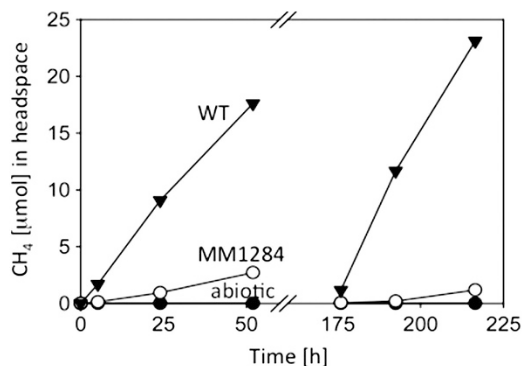

Figure 4 Bioelectrochemical methane formation seems to be linked to cells associated with the cathode rather than planktonic cells. Methane production rates at −600 mV are not affected significantly by replacing the entire medium and washing planktonic cells out of the cathode chamber (after 170 h). Wild-type reactors continued to produce methane at almost unchanged rates (▼) and strain MM1284 still produced methane at 50% of its previous rate (○). In the abiotic control, methane was not detected (●).

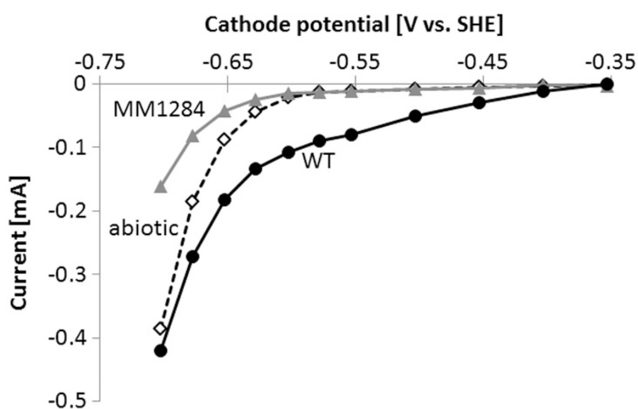

Figure 5 Biocathode with wt *M. maripaludis* cells exhibits a lower overpotential for cathodic reactions. The wt biocathode (●) outperformed the abiotic (◇) and MM1284 cathode (▲) with respect to current consumption over the complete measured voltage range of −350 to −700 mV. SHE, standard hydrogen electrode.

shows that the presence of such cells on the surface reduces, rather than increases, the rate of abiotic electron release as H_2. Thus, microbial biomass may ‘passivate’ a cathodic graphite surface.

Discussion

Our studies provide evidence for a hydrogenase-independent uptake of cathodic electrons by the methanogenic archaeon *M. maripaludis*. Interestingly, the rate of methane formation in the hydrogenase-deficient MM1284 strain was about 1/10 of the rate of methane formation of wt cells. This suggests that in wt, most of the cathodic electrons utilized for methane formation were transferred to the cells by an indirect mechanism involving hydrogen as an intermediate. However, we also demonstrated that an alternative, hydrogen-independent pathway exists. Such hydrogenase-independent methane formation could only be observed in the MM1284 strain, which is devoid of all catabolic and anabolic hydrogenases.

A direct uptake of electrons has been postulated for various microorganisms that also use hydrogen as electron donor including homoacetogens (Nevin *et al.*, 2011), sulfate reducers (Dinh *et al.*, 2004; Enning *et al.*, 2012) and methanogens (Dinh *et al.*, 2004; Uchiyama *et al.*, 2010). In these studies hydrogen was excluded as an intermediate based on indirect evidence. By showing that the strain MM1284 is able to perform electromethanogenesis in the absence of hydrogenases, we provide direct conclusive evidence for the existence of a hydrogen-independent electron uptake mechanism. In fact, methane formation from cathodic electrons was not only observed in *M. maripaludis*, but also in *Methanococcus thermolithotrophicus* and *Methanosarcina barkeri* (Supplementary Figures S2 and S3). Methane formation from cathodic electrons has been reported also for several undefined enrichment cultures (Cheng *et al.*, 2009; Villano *et al.*, 2010, 2011). Therefore, the involvement of a hydrogen-independent electron uptake in these strains deserves further research and might have profound impact in geochemical environments as well as in the biotechnological use of electromethanogenesis.

At this point it is unclear whether *M. mariplaudis* cells are able to grow during hydrogenase-independent electromethanogenesis as we did not find any evidence for the growth of *M. mariplaudis* in the bioelectrochemical reactors. No measurable change in optical density throughout the experiments was observed. Assuming a coupling of growth to methane formation, as observed in cells grown on formate or H_2/CO_2, the observed amount of methane formed after 150 h (~100 μM) in the reactors harboring wt cells would have resulted in a maximal theoretical change in optical density (OD_{600}) of only 0.015 (assuming a cell mass of 200 mg dry weight per liter culture of $OD_{600} = 1$; yield = 3 g mol^{-1} methane formed). Thus, for MM1284 cells, the increase in OD_{600} would be lower by a factor of 10. We could not reliably quantify this increase in biomass, as the change is too small compared with the cell mass introduced into the reactor ($OD_{600} = 0.03$–0.04). Also a fraction of the cells may have attached to the electrode over time, and sulfide precipitates interfered with optical cell density measurements as well as protein quantification. Attempts to enumerate biomass on the cathode surface were inconclusive although single attached cells but no dense biofilm were observed microscopically (data not shown). Therefore, consistent with the linear rather than exponential rate of methane formation in the electrochemical reactor, we have currently no evidence for microbial growth in our experimental system. Moreover, in the absence of further physiological experiments, we also have no evidence that *M. maripaludis* conserved energy during hydrogenase-independent methanogenesis. We assume that cells in the

bioelectrochemical reactor are limited by their energy substrate, because addition of H_2 gas to the cathode chamber resulted in rapid methane formation and visible planktonic growth of the wt (data not shown).

These experiments did not enable us to determine what fraction of the total cells in the cathodic reactor was active in methanogenesis. Therefore, it is not possible to compare specific rates of methanogenesis between the cells in the bioelectrochemical reactors and the cells growing planktonically on H_2/CO_2 or formate. Further evidence for hydrogen-independent methanogenesis being limited by the cathode surface arises from the experiments where the medium was replaced. After the medium was exchanged with fresh medium, methanogenesis rates with MM1284 reached levels similar to those with the original medium containing planktonic cells, and methane production rates slightly increased in experiments with the wt. We attribute the slight increase in the methane formation rate of wt to the supply of fresh buffer solution to the cathode (there is a small pH difference (up to 0.5 pH units) building up with time between the anode and cathode chamber) in combination with the removal of reaction products. In addition, more cells could be attached to the cathode surface after prolonged incubation.

Currently, the molecular mechanism of hydrogen-independent uptake of cathodic electron is unknown and the existence of multiple modes cannot be ruled out. Because we excluded H_2 as an intermediate for methanogenesis in the MM1284 mutant due to its inability to use H_2, we searched for other, primarily organic compounds that may have been formed abiotically at significant rates at the cathode and could have served as a diffusible mediator for indirect electron transfer. Carbon monoxide was formed at low rates (0.2–7 $nmol\,h^{-1}$) in all reactors (data not shown), but the accumulation in all the biotic treatments was similar or faster than in the abiotic controls, indicating that a fast CO cycling as electron source for methanogenesis was highly unlikely. Notably, CO did not serve as sole electron donor in *M. maripaludis* (Costa *et al.*, 2013a). Also, no formate was found in the reactors containing MM1284 mutant cells during electromethanogenesis, and no detectable amounts of formate accumulated in the abiotic controls ($<5\,\mu M$, detection limit). Thus, it appears that the observed uptake of cathodic electrons might occur by a direct mechanism in *M. maripaludis*. However, we cannot rule out that cycling of some organic or inorganic, redox-active molecule may mediate the net current consumption. As we have shown for H_2 in wt, the absence of detectable levels of such molecules does not exclude a rapid cycling. However, the observation that a lowering of cathodic potential in the MM1284 hydrogenase mutant did not increase the rate of methanogenesis, whereas it did in wt, as well as the concurrent transient H_2 formation are consistent with a direct uptake mechanism that does not involve abiotic formation of small molecules.

The observed current efficiencies in this study are not sufficiently high to be explained by diffusive or sampling losses of the formed gases. Thus, we have to assume that some other compounds are generating the background current. Possible current consuming reactions in our system are reactions of reduced compounds present in the cathode chamber (for example, sulfides, Fe^{2+}) with oxidants generated in the anode chamber (for example, O_2, H_2O_2 or Cl_2). As the Nafion membrane is not impermeable to neutral and positively charged small molecules, some of these molecules could be re-oxidized or re-reduced at the anode or cathode directly, or react in solution and generate intermediates that are subsequently re-oxidized or re-reduced at the anode or cathode, respectively.

The presence of a direct uptake of cathodic electrons by *M. maripaludis* would be supported by the recent finding of direct interspecies electron transfer between *Geobacter metallireducens* and another methanogenic archaeon, *Methanosaeta harundinacea* (Rotaru *et al.*, 2013). In that study, *M. harundinacea* was postulated to take up electrons provided by the well-studied exoelectrogen *G. metallireducens* via an unknown, but hydrogen-independent mechanism. In conjunction with the genetic evidence for a hydrogen-independent electron uptake presented here, it seems likely that this yet unknown mechanism of electron uptake might be widespread in methanogens. Our study also showed that formate is produced by an archaeon when methanogenesis is inhibited (Figure 3). There are at least two hypotheses explaining the formation of this one carbon compound: (1) the catabolic electrons, taken up by an unknown mechanism, are diverted in the cell to reduce CO_2 to compounds other than CH_4 (such as formate and hydrogen) or (2) intermediates are formed abiotically outside the cell at the cathode and accumulate because they are not metabolized by the cells due to the inhibition of cellular metabolism. As we did not observe an accumulation of intermediates in reactors without cells, we consider the first explanation as more likely.

The decrease in overpotential when wt cells are present on a cathode is also consistent with cells facilitating the electron uptake from the cathode, thereby resulting in higher currents and, concomitantly, enhanced rate of hydrogen formation. This apparent microbe-dependent decrease in the cathodic overpotential might also operate in the other microbial bioelectric systems where no or little H_2 formation is observed in abiotic controls, and where significant microbial formation (and consumption) of H_2 is possible. This property of *M. maripaludis* enables efficient production of H_2 or formate by the biocathode at more positive potentials compared with the abiotic graphite surface. While not optimized, this activity might provide an opportunity for electrosynthesis using Archaea in addition to

those based on direct biological electron transfer in bacteria (Nevin *et al.*, 2011) to produce formate and other small, reduced molecules.

Bearing in mind the environmental, economical and geopolitical concerns about the use of and access to fossil carbon and the associated net increase in atmospheric carbon dioxide, alternative technologies are needed to produce fuels or chemical compounds renewably from CO_2 (Köpke *et al.*, 2010). In particular, microbial electrosynthesis was proposed as a highly promising new technology (Nevin *et al.*, 2010; Rabaey and Rozendal, 2010). Here we presented one example of how biological processes provide an attractive platform to do exactly that: *M. maripaludis* can fix CO_2 and produce different simple organic molecules (methane and formate) at high specificity. To our knowledge this is the first study showing that methanogenic archaea can be used to produce small organic molecules from CO_2 and cathode-derived electrons in a hydrogen-independent manner; a biotechnological field restricted to the domain of bacteria until now.

In conclusion, our results provide novel insights into the diversity of extracellular electron transport processes and demonstrate that methanogenesis is possible without hydrogen as an essential intermediate during cathodic electron uptake. These findings are of special interest not only for biotechnological purposes but also to better understand geochemical microbial systems and microbe–mineral interactions. Direct and indirect electron transfer as well as electron transport across long distances are emerging as a new, quantitatively important concept shaping and connecting (bio)geochemical processes and element cycles (Pfeffer *et al.*, 2012). Thus, further research about microbial uptake of free electrons is needed to complete our understanding about global biogeochemical cycles.

Conflict of Interest

The authors declare no conflict of interest.

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