RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

INTERNATIONAL MICROBIOLOGY 20(2):55-64 (2017)
doi:10.2436/20.1501.01.285. ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

Electrochemical characterization of *Geobacter lovleyi* identifies limitations of microbial fuel cell performance in constructed wetlands

Clara Corbella,[1,2] Rebecca P. Steidl,[1] Jaume Puigagut,[2] Gemma Reguera[1]*

[1]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, USA.
[2]Department of Civil and Environmental Engineering, Technical University of Catalunya, Barcelona-Tech, Spain

Received 22 March 2017 · Accepted 15 April 2017

Summary. Power generation in microbial fuel cells implemented in constructed wetlands (CW-MFCs) is low despite the enrichment of anode electricigens most closely related to *Geobacter lovleyi*. Using the model representative *G. lovleyi* strain SZ, we show that acetate, but not formate or lactate, can be oxidized efficiently but growth is limited by the high sensitivity of the bacterium to oxygen. Acetate and highly reducing conditions also supported the growth of anode biofilms but only at optimal anode potentials (450 mV vs. standard hydrogen electrode). Still, electrode coverage was poor and current densities, low, consistent with the lack of key *c*-type cytochromes. The results suggest that the low oxygen tolerance of *G. lovleyi* and inability to efficiently colonize and form electroactive biofilms on the electrodes while oxidizing the range of electron donors available in constructed wetlands limits MFC performance. The implications of these findings for the optimization of CW-MFCs are discussed. [**Int Microbiol** 20(2):55-64 (2017)]

Keywords: microbial fuel cells · bioelectrochemical systems · constructed wetlands · extracellular electron transfer · electricigens

Introduction

Horizontal subsurface flow constructed wetlands (HSSF CW) are natural wastewater treatment systems where organic matter is oxidized by means of physical, chemical and biological processes under mainly anaerobic conditions [3]. During treatment, a redox gradient is naturally established between the top layer exposed to air and the deeper anaerobic areas of the treatment bed that can be exploited to harvest an electrical current with a sediment microbial fuel cell (MFC) [7]. Although MFCs operating in HSSF CW (CW-MFCs) increase organic matter removal efficiency, power density and coulombic efficiencies are generally low (below 50 mW/m^2 and 4%, respectively) [10]. As with other MFCs that process domestic wastewater, system performance is ultimately dependent on the syntrophic interactions among the organisms in the anode biofilm, which first hydrolyze the complex substrates and then ferment them to generate electron donors (e.g., acetate, formate, and lactate) for the electricigenic population [5,9].

Abbreviations:
CW Constructed Wetlands
MEC Microbial Electrolysis Cell
MFC Microbial Fuel Cell
CW-MFC Microbial Fuel Cells implemented in Constructed Wetlands
HSSF CW Horizontal subsurface flow constructed wetlands

*Corresponding author: Gemma Reguera
E-mail: reguera@msu.edu

Efficiency is also dependent on the ability of the electricigens to couple the oxidation of the available organic substrates to the electron transfer to the anode electrode [23]. Indeed, changes in the chemical composition of the wastewater in CW influence substrate availability, the type and relative abundance of bacteria that grow in the anode biofilms, and CW-MFC performance [8].

The fluctuating chemical and physical conditions that dominate HSSF CW promote the establishment of several pathways for organic matter degradation and influence the range of electron donors available to support the growth and activity of the electricigenic population [13]. Acetate, a preferred electron donor for efficient electricigens in the genus *Geobacter* [31], is the most common electron donor generated in these anaerobic pathways and often enriches for *Geobacter* species in the anode biofilms of bioelectrochemical systems fed with domestic wastewater [8,9,12].

More than half of the 16S rRNA sequences retrieved from anode biofilms grown in a single chamber microbial electrolysis cell (MEC) fed with domestic wastewater were, for example, most closely related to electricigenic species of *Geobacter* available in pure culture (*Geobacter metallireducens*, *Geobacter sulfurreducens*, *Geobacter lovleyi* and *Geobacter uraniireducens*) [9]. Acetate is also available as an electron donor in CW [2]. Consistent with this, Operational Taxonomic Units (OTUs) most closely related to members of the Geobacteraceae family were enriched in the anode biofilms of an active CW-MFC fed with the effluent of a hydrolytic upflow sludge blanket reactor, with the highest relative abundance (13–16 of the total OTUs) corresponding to *G. lovleyi* [8]. However, despite the enrichment of *Geobacter* electricigens in the anode biofilms, power densities in these systems were low (<40 mW/m^2) [8].

The fact that *G. lovleyi* OTUs dominate the electricigenic population in active CW-MFC ssuggests that further optimization must consider the factors that limit the growth and/or electrochemical activity of *G. lovleyi* in these systems. In contrast with the amount of information that is available for other model *Geobacter* representatives such as *G. sulfurreducens* and *G. metallireducens* (reviewed in [24]), little is known about the physiological constraints that may limit the growth and electrochemical activity of *G. lovleyi* under conditions relevant to CW. Evidence is indeed available that suggests that the physiology of *G. lovleyi* may be substantially different from previously investigated *Geobacter* species. The first strain of *G. lovleyi* recovered in pure culture, strain SZ, was isolated from non-contaminated creek sediment microcosms based on its ability to grow by coupling the oxidation of acetate to the reductive dechlorination of tetrachloroethene (PCE) to cis-1,2-dichloroethene (cis-DCE) [37]. Closely related 16S rRNA gene sequences have been retrieved from environments where dechlorination is an active process, placing *G. lovleyi*-like sequences in a distinct, dechlorinating clade within the metal-reducing *Geobacter* group, which model electricigens belong to [1,37]. Further, strain SZ retains the ability to reduce Fe(III), the major hallmark of the physiology of *Geobacter* electricigens [37]. However, its genome shows marked reductions in the number of *c*-type cytochrome genes required for metal reduction in other *Geobacter* species [41], which could negatively affect its ability to couple growth to the reduction of an anode electrode. Strain SZ is also capable of dissimilatory nitrate reduction to ammonium (DNRA), a process that is promoted with acetate availability [40] and could potentially divert respiratory electrons away from the anode electrode in CW-MFCs. Moreover, while it is possible to harvest some low levels of current from anode biofilms of strain SZ grown in acetate-fed MECs with fumarate supplementation [36], its ability to gain energy for growth using an electrode as sole electron acceptor has never been evaluated. Also relatively unexplored is the range of electron donors and carbon sources that support the growth of *G. lovleyi* biofilms on anode electrodes. For example, organic acids more reduced than acetate such as lactate and formate are produced in CWs [2], yet not all *Geobacter* electricigens can efficiently assimilate them for carbon and/or oxidize them in bioelectrochemical systems [6,31]. Based on these considerations, we investigated the electrochemical activity of the model representative *G. lovleyi* strain SZ with electron donors (acetate, lactate and formate) commonly found in CW. The results reveal substantial metabolic differences between *G. lovleyi* and other model *Geobacter* electricigens that limit the performance of bioelectrochemical systems driven by these organisms. The implications of these findings for the optimization of CW-MFCs are discussed.

Materials and methods

Bacterial strains and culture conditions. *Geobacter lovleyi* SZ (ATCC BAA-1151; DSM 17278), kindly provided by Dr. Dawn Holmes (Western New England University), was used throughout the study. The strain was routinely cultured anaerobically in DB medium, a medium opti-

mized for the growth of current-harvesting anode biofilms in MECs [31]. Unless otherwise indicated, the medium was supplemented with 2.5 mM cysteine HCl as a reducing agent and with sodium acetate (20 mM) and sodium fumarate (40 mM) as the electron donor and acceptor, respectively (DBAF medium). The growth medium was dispensed into tubes (10 ml) and serum bottles (50 ml) and sparged with an oxygen-free gas mix of N_2:CO_2 (80:20) [32] before sealing the vessels with rubber stoppers and autoclaving for 30 minutes.

When indicated, acetate, lactate and formate were provided to the medium at concentrations (9 mM sodium acetate, 6 mM D,L-lactate, or 36 mM sodium formate) that provided equimolar amounts of electrons (acetate, 8; lactate, 12; and formate, 2). Growth with each of these electron donors was studied using fumarate (40 mM) or Fe(III) citrate (80 mM) as an electron acceptor. For these experiments, cells were first grown to late exponential phase (OD600, ~0.4) in DB medium with the electron donor-acceptor pair to be tested, harvested by centrifugation (3200 ×*g*, 10 min, 30 °C), washed once in sterile medium, and suspended in 0.5 ml of the growth medium to prevent nutrient carry-over. In some experiments, the centrifugation and washing steps were omitted and the cultures were transferred three times in the same medium before calculating the growth rates for each electron donor-acceptor pair. All incubations were at 30 °C. Growth in fumarate cultures was monitored spectrophotometrically (OD_{600}) and in Fe(III) citrate cultures, as the amount of HCl-extractable Fe(II) resulting from the reduction of Fe(III) [25] measured using the ferrozine assay [34].

Analytical techniques. Organic acids in filtered (0.45 µm syringe Titan3™ filters, Thermo Scientific) culture supernatant fluids were identified and quantified by high-performance liquid chromatography (HPLC) (Waters, Milford, MA, USA) as reported elsewhere [31]. Cysteine was measured spectrophotometrically (OD_{412}) as freethiols with the Ellman's Reagent, as described previously [29], and in reference to l-cysteine hydrochloride standards. When indicated, the total cell protein content in the culture was estimated using Pierce BCA Protein Assay Kit (Thermo Scientific) using a previously published method [44].

MECs and confocal microscopy. MECs were the same dual-chambered, H-type fuel cells described previously [31]. Anode and cathode chambers were separated by means of a Nafion membrane (N117, Ion Power, Inc. New Castle, DE, USA). Each chamber contained 90 ml of DB-acetate (1 mM) medium and housed a graphite rod electrode (1.27 cm diameter, 99 % metal basis, 12 cm^2 anode surface area) similar to the graphite rods use to enrich for *G. lovelyi* OTUs in the anode biofilms of CW-MFCs [8]. The anode and cathode chambers were sparged with oxygen-free N_2:CO_2 (80:20) to ensure anaerobiosis and the anode potential was set with a potentiostat (VSP, BioLogic, Claix, France) at –0.179 V, 0.240 V or 0.561 V vs. a 3 M Ag/AgCl reference electrode (Bioanalytical Systems, Inc., West Lafayette, IN, USA). The anode chamber was then inoculated with cells of *G. lovleyi* or, when indicated, of *G. sulfurreducens* harvested by centrifugation (3200 rpm, 10 min, 30 °C) from stationary phase cultures grown with DBAF medium and suspended in 10 ml of DB medium with acetate. All experimental conditions were tested at least in duplicate MECs. Culture broth samples were periodically removed from the anode chamber and analyzed by HPLC. When indicated, anode biofilms were grown to the point of maximum current, stained with the BacLight viability kit (Molecular Probes), and examined by Confocal Laser Scanning Microscopy (CLSM) using a FluoView FV1000 inverted microscope system (Olympus, Center Valley, PA, USA), as described elsewhere [31].

Results and Discussion

Effect of the redox potential during the reduction of Fe(III) citrate and fumarate. Because oxygen intrusions are common in CW, which could inhibit the growth and electroactivity of electricigens, we investigated the influence of the medium redox potential in the growth of *G. lovleyi* strain SZ. Although the media preparation involved extensive sparging of the broth and head space with oxygen-free gases (N_2:CO_2) [32], growth was only observed in cultures supplemented with cysteine as a reducing agent (Fig. 1). Strain SZ coupled, for example, the oxidation of acetate (20 mM acetate or 160 mM electron equivalents) to the reduction of Fe(III) citrate (Fig. 1A) but only when the cultures were supplemented with cysteine (2.5 mM). Replacing the cysteine with a mild reducing agent such as $FeCl_2$ (2.5 mM) [35] did not support Fe(III) reduction (data not shown). Generation times in the cultures with cysteine were 18 ± 1 h (average and standard deviation of triplicate cultures) but were reduced to 8 h (± 0.1 h) after three direct transfers in the same medium that bypassed the centrifugation and washing steps of the cells prior to inoculation. These doubling times are within the ranges (~10 h) reported for *G. sulfurreducens* grown in the same DBA-Fe(III) citrate medium but without cysteine [31]. Furthermore, the total amount of Fe(II) (~50 mM) reduced by *G. lovleyi* was as in the *G. sulfurreducens* cultures [31], indicating that once the medium was pre-reduced optimally, strain SZ was able to couple the oxidation of acetate and Fe(III) as efficiently as *G. sulfurreducens*.

Addition of cysteine as a reducing agent was also required to support optimal growth in cultures with acetate and fumarate (Fig. 1B). We tested various concentrations of cysteine ranging from 0.1 to 10 mM and we consistently measured maxima growth yields and shortest generation times at cysteine concentrations between 2 and 3 mM. Cultures supplemented with 2.5 mM (Fig. 1B) yielded, for example, maxima cell biomass (measured as total cell protein at the point of maximum growth, when all the acetate was depleted) similar to that measured in the DBA-Fe(III) citrate cultures (~ 10 g protein per mol acetate consumed). Cysteine in these cultures was rapidly oxidized within the first 10 h of incubation (Fig. 1B) at rates comparable to the oxidation of cysteine in uninoculated controls (Fig. 1C), consistent with its abiotic oxidization to cystine dimers [17]. Exponential growth started after a short (2–3 h) *lag* phase, when approximately 1 mM of cysteine had been oxidized, and proceeded with doubling times of 6 ± 0.4 h (average and stan-

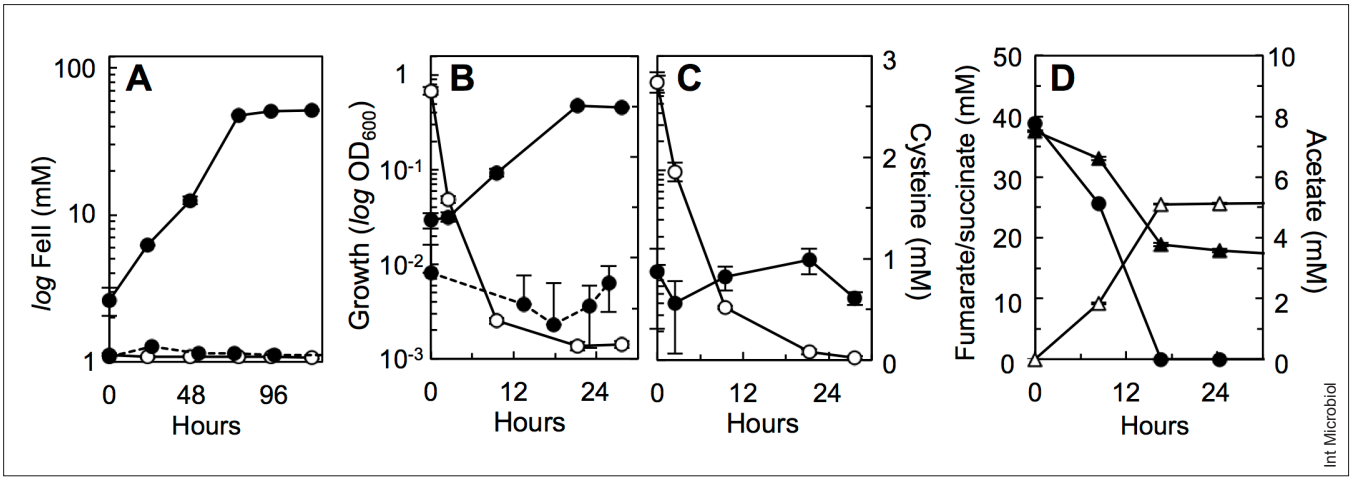

Fig. 1. Effect of cysteine on growth of *Geobacter lovleyi* with acetate and Fe(III) citrate (**A**) or fumarate (**B–D**). A. Growth (log of the acid-extractable Fe[II]) with 15 mM acetate and 80 mM Fe(III) citrate in the presence (solid symbols) or absence (open symbols) of 2.5 mM cysteine. An uninoculated control with cysteine is shown as well (dashed line). B-C Growth (OD_{600}, solid symbols) and cysteine concentration (mM, open symbols) in DBAF cultures with (solid line) or without (dashed line) 2.5 mM cysteine (B) in reference to uninoculated controls (C). D. Oxidation of acetate (solid circles) coupled to the reduction of fumarate (solid triangles) to succinate (open triangles) in the DBAF cultures supplemented with 2.5 mM cysteine shown in B. All of the data points in panels A-D are average and standard deviation of triplicate samples.

dard deviation of triplicate cultures) (Fig. 1B). But the *lag* phase was eliminated and doubling times were reduced (4.4 ± 0.1 h) when exponential phase cultures were sequentially transferred three times in the same medium with cysteine to avoid the cell centrifugation and washing steps prior to inoculation. These fast generation times are within the ranges we calculated (~ 4.6 ± 0.2 h) for the model electricigen *G. sulfurreducens* growing in the same DBAF medium but without cysteine [31]. Thus, the rates of acetate oxidation coupled to fumarate reduction are also comparable in the two strains, but growth by *G. lovleyi* requires the presence of sufficient concentrations of cysteine as a reducing agent.

The absolute requirement to pre-reduce the medium sufficiently in order to support cell growth is in accordance with genomic data, which indicates that *G. lovleyi* cannot respire oxygen and lacks several key genes involved in oxygen tolerance and detoxification of reactive oxygen species [41]. Indeed, field experiments show significant (up to 50%) decreases in the relative abundance of *G. lovleyi* like sequences in uranium-contaminated sediments following the intrusion of oxygenated ground water [39]. By contrast, *G. sulfurreducens* tolerates exposure to atmospheric oxygen for up to 24 h and can use oxygen as terminal electron acceptor for respiration under microaerophilic conditions, a metabolic capability that allows them to not only tolerate but also boost their growth in response to oxygen intrusions [21].

Metabolic constrains limiting growth with organic acids. Although *G. lovleyi* was able to double every 4–5 h, like *G. sulfureducens*, in acetate-fumarate cultures with cysteine, growth yields were significantly lower for *G. lovleyi* (OD_{600} max. ~0.5; Fig. 1B) than for *G. sulfurreducens* (OD_{600} max. ~0.8) [31]. Higher growth yields of *G. sulfurreducens* in cultures with acetate and fumarate have been attributed to the ability of this organism to assimilate some of the fumarate carbon, which diverts more of the acetate substrate (72.5%) for energy generation via respiration [43]. To investigate a similar metabolic capability in *G. lovleyi*, we monitored acetate and fumarate consumption and the production of succinate (from the reduction of fumarate) or malate (from the assimilation of fumarate carbon) in *G. lovleyi* DBAF cultures that contained growth-limiting concentrations of acetate (~9 mM or 72 mM electron equivalents) (Fig. 1D). Acetate was consumed during the first 16 h and, concomitantly, fumarate was reduced to succinate. We estimated that approximately 19 (±0.3) mM fumarate was consumed, or the equivalent of 60% of the electrons provided as acetate in the medium, suggesting that *G. lovleyi* diverted more acetate carbon (~40%) for biomass synthesis than *G. sulfurreducens* (27.5%) [43]. Although the assimilation of acetate carbon in the TCA cycle generates malate [30], we did not detect any malate in the acetate-fumarate cultures of *G. lovleyi* but measured an excess succinate (~7 mM) that could not be accounted for by the fumarate that

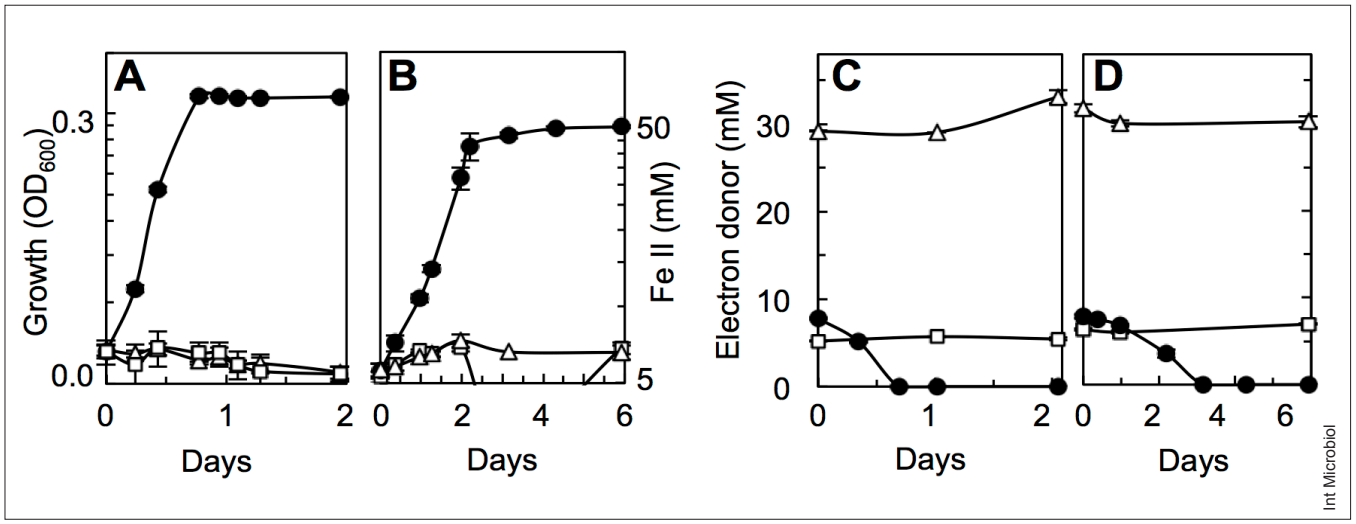

Fig. 2. Growth (**A-B**) and electron donor consumption (**C-D**) of *G. lovleyi* with fumarate (A and C) or Fe(III) citrate (B and D) as electron acceptors, respectively. The electron donors tested were acetate (solid circles), lactate (open squares), and formate (open triangles). Other organic acids such as fumarate, succinate, malate, and pyruvate were also monitored in the culture broths (C and D) but were not detected.

was reduced (Fig. 1D). As strain SZ can reduce malate to succinate [37], the excess succinate detected in the cultures likely resulted from the reduction of acetate-derived malate.

The finding that *G. lovleyi* diverts more acetate carbon for assimilation than *G. sulfurreducens* suggests that metabolic differences do exist between *G. loveyi* and model electricigens that could limit the performance of CW-MFCs despite the availability of acetate as an electron donor. Organic acids such as lactate and formate are also available as electron donors and carbon sources in CW [2]. Thus, we investigated the ability of *G. lovleyi* to grow with these two organic acids (Fig. 2) using the cultivation conditions with cysteine that supported optimal growth of *G. lovleyi* with acetate (Fig. 1). Although formate and lactate are more reduced than acetate and can, therefore, theoretically produce higher cell voltages (–0.403 and –0.325 V vs. standard hydrogen electrode [SHE], respectively), and more energy for growth, than acetate (–0.277 V vs. SHE) [18], neither electron donor supported the growth of *G. lovleyi* in cultures with fumarate or Fe(III) citrate serving as the electron acceptor and supplemented with 2.5 mM cysteine (Fig. 2A and B, respectively). Moreover, fumarate concentrations in the cultures with formate and lactate as electron donors remained relatively constant throughout the incubation period and the reduced product of the reaction, succinate, did not accumulate in the culture broth (Fig. 2C). Similarly, electron donor consumption was only detected in the positive controls that contained acetate as the electron donor (Fig. 2C and D).

To better understand the metabolic constrains that limited growth of strain SZ with formate and lactate, we used the BLAST engine (http://blast.ncbi.nlm.nih.gov/) to reconstruct the metabolism of these two organic acids in reference to the metabolism of the model electricigen *G. sulfurreducens*. As shown in Fig. 3, *G. sulfurreducens* oxidizes formate to carbon dioxide in a reaction catalyzed by the formate dehydrogenase (FDH) enzyme and assimilates formate carbon with acetyl-CoA in a separate reaction catalyzed by the pyruvate formate lyase (PFL) enzyme [31]. The genome of strain SZ contains two FDH homologues (Glov_1164 and Glov_0899) [41]. Furthermore, supplementing the formate cultures with 0.1 mM acetate to provide the acetyl-CoA substrate needed for formate carbon assimilation did not promote growth either, suggesting that formate cannot be oxidized with fumarate serving as electron acceptor, as observed during the reduction of PCE and Fe(III) [37]. Formate does not sustain the coupled oxidation of H_2 and reduction of PCE or Fe(III) either [37], suggesting it cannot assimilate formate carbon either. Indeed, we were unable to identify PFL-like sequences in the genome of *G. lovleyi*. Thus, even with active FDH enzymes for formate oxidation, the lack of a PFL enzyme would prevent the cells from assimilating formate carbon to generate pyruvate for gluconeogenesis, biomass synthesis, and other assimilatory reactions (Fig. 3).

As with the formate cultures, acetate additions to lactate cultures of *G. lovleyi* (Fig. 2) did not stimulate growth with either fumarate or Fe(III) citrate, indicating that lactate cannot be used as electron donor during the reduction of fumarate

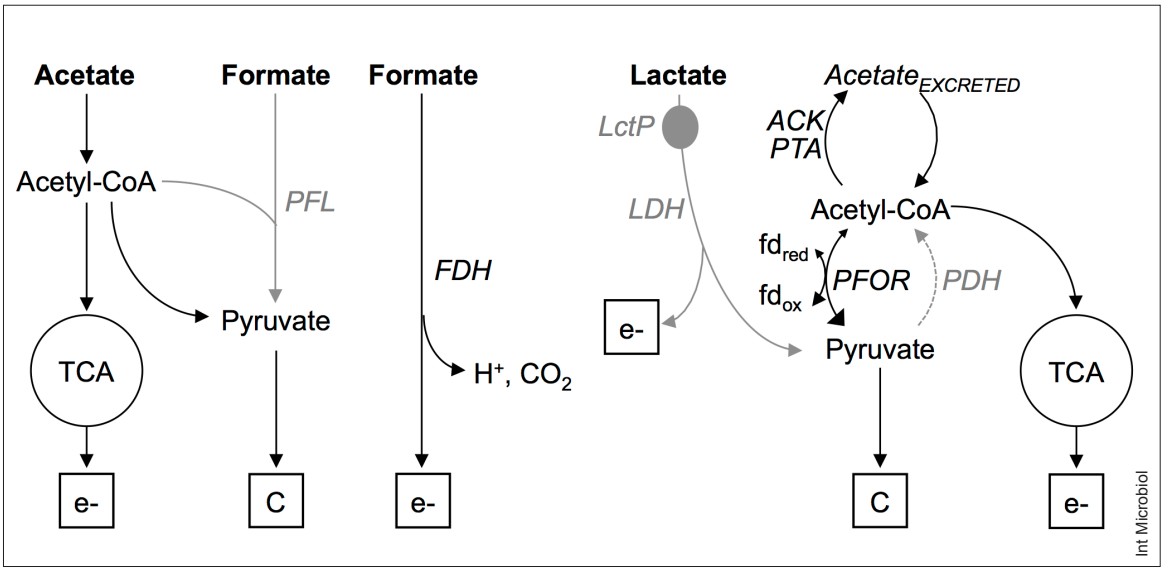

Fig. 3. Metabolic pathways used by *G. sulfurreducens* for the oxidation (e–) and carbon assimilation (C) of acetate, formate, and lactate substrates (in bold) and identification of reactions (in gray) absent in *G. lovleyi*. Dashed arrows indicate reactions that operate in cultures with fumarate. Enzyme abbreviations, from left to right: *PFL*, pyruvate formate lyase; *FDH*, formate dehydrogenase; *LctP*, lactate permease; *LDH*, lactate dehydrogenase; *ACK*, acetate kinase; *PTA*, phosphotransacetylase; *PFOR*, pyruvate ferredoxin oxidoreductase; *PDH*, pyruvate dehydrogenase.

and Fe(III), as also reported for PCE reduction [37]. Lactate carbon, however, has been reported to be assimilated during the reduction of PCE and Fe(III) with H_2 as electron donor [37]. However, a comparative search in the genome of *G. lovleyi* failed to identify any lactate permeases, including homologs of the two lactate transporters annotated in the genome of *G. sulfurreducens* (GSU1622 and GSU0226). Furthermore, the SZ genome does not contain any genes annotated as lactate dehydrogenases (LDH), which catalyze the partial oxidation of lactate to pyruvate (Fig. 3). Similarly, our search retrieved no significant matches for homologs of the two subunits of the glycolate oxidase (GO) enzyme of *G. sulfurreducens* (GSU1623 and GSU1624), which is structurally homologous to LDH but has a reduced LDH activity [31]. Also unclear is how any lactate-derived pyruvate could be fully oxidized in the TCA cycle by *G. lovelyi*. Also absents in the genome of strain Z were homologs of the subunit A of the pyruvate dehydrogenase E1 complex (PDH), which provides a route to convert pyruvate into acetyl-CoA substrate for the oxidative TCA cycle (Fig. 3). The genome of *G. lovleyi* does contain five gene clusters (one on plasmid pSZ77) encoding pyruvate ferredoxin/flavodoxin oxidoreductase (PFOR) complexes [41], which could catalyze the conversion of pyruvate into acetyl-CoA (Fig. 3). These PFOR complexes are homologous to the PFOR enzyme of *G. sulfurreducens* that has been proposed to preferentially work in the opposite direction to promote the flux of acetyl-CoA to gluconeogenetic pyruvate [43]. The preferential direction of the PFOR reactions towards pyruvate for gluconeogenesis in *G. lovleyi* is supported by the greater amounts of acetate carbon that we estimated to be assimilated by strain SZ (40%) compared to *G. sulfurreducens* (27.5%). Yet strain SZ does use pyruvate as sole electron donor [37], suggesting that one, if not more, of the PFOR enzymes may preferentially operate in the opposite direction to divert lactate-derived pyruvate to acetyl-CoA for its full oxidation in the TCA cycle (Fig. 3).

The genome of *G. lovleyi* also contains two genes (Glov_1754 and Glov_1210) annotated as acetate kinase (ACK) and phosphotransacetylase (PTA), respectively. *G. sulfurreducens* diverts pyruvate as acetyl-CoA substrate for the ACK/PTA pathway to excrete excess carbon as acetate in an ATP-yielding reaction (Fig. 3) [31]. This suggests that *G. lovleyi* could also partially oxidize lactate to acetate to balance excess fluxes of carbon while generating energy for growth. However, we did not measure any acetate in cultures with lactate (Fig. 2C and D). Thus, other rate-limiting steps such as inefficient lactate transport or oxidation to pyruvate likely prevented the oxidation and assimilation of lactate and prevented growth of *G. lovleyi* with lactate.

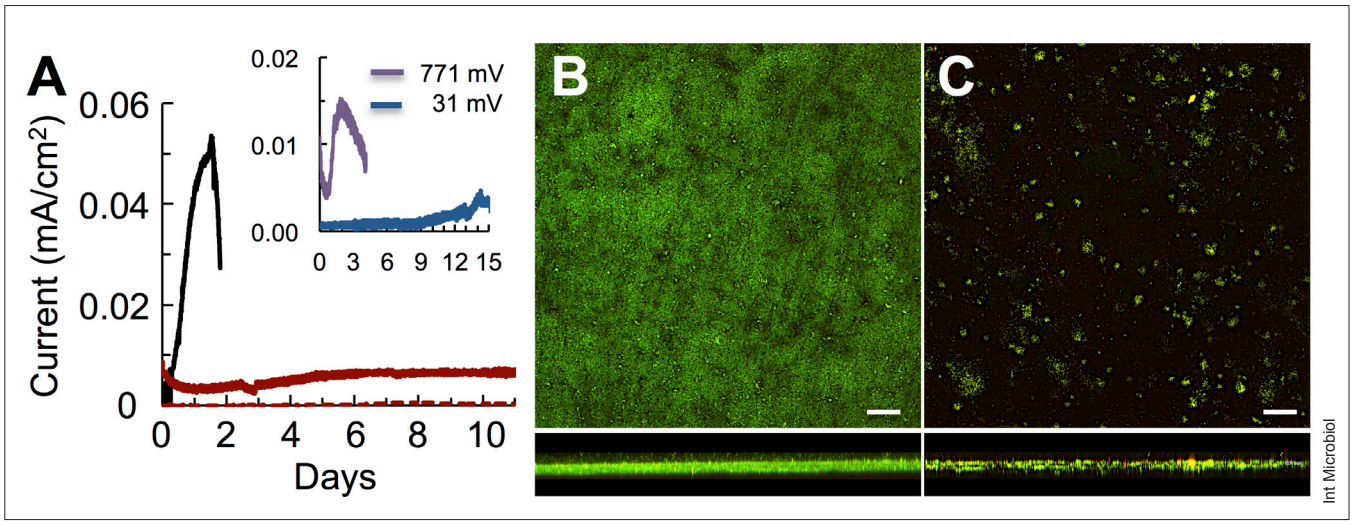

Fig. 4. Current density (**A**) and growth of anode biofilms (**B-C**) in MECs driven by *Geobacter lovleyi* or *G. sulfurreducens* fed with an initial concentration of 1 mM acetate. A. Current density (mA/cm^2) by *G. lovleyi* (maroon) and *G. sulfurreducens* (black) in MECs poised at an anode potential of 450 mV (vs. SHE) with (solid line) or without (dashed line) 2.5 mM cysteine. Inset, current density by G. lovleyi in MECs with cysteine as a function of the anode potential (771 or 31 mV vs. SHE). Axis units are as in panel A. B-C. Top and side views of CSLM projections of anode biofilms of *G. lovleyi* (B) and *G. sulfurreducens* (C) collected at the point of maximum current from MECs at 450 mV (vs. SHE) and supplemented with cysteine (A). The biofilms were stained with the BacLight viability dies (green, live; red, dead). Scale bar, 20 µm.

Reduced electrode colonization and biofilm electroactivity limit MEC performance. The growth and electrochemical activity of strain SZ was investigated in acetate-fed MECs with an anode electrode poised at a metabolically oxidizing potential to provide a terminal electron acceptor for growth of the anode biofilms (Fig. 4). MECs with anode electrodes poised at a potential (450 mV vs. SHE) that is optimal for the growth of anode biofilms of the model electricigen *G. sulfurreducens* [31] produced current soon after inoculation but required the pre-reduction of the medium with 2.5 mM cysteine (Fig. 4A). Under optimal reducing conditions, current production increased over the course of ten days until it reached maximum current (0.086 ± 0.004 mA, average and standard error of duplicate MECs); it was sustained for several more days until all of the acetate was depleted (Fig. 4A). By contrast, control MECs driven by *G. sulfurreducens* grown under the same conditions with cysteine reached a maximum current of 0.642 mA in less than 2 days. Moreover, coulombic efficiencies (CE) in MECs driven by *G. lovleyi* ranged from 30 to 40%, which is less than half of those estimated for *G. sulfurreducens* (~80%) [31].

Even when operated under optimal conditions, the growth and electrochemical activity of *G. lovleyi* was limited by poor electrode colonization and biofilm growth. Indeed, confocal laser scanning microscopy (CLSM) of anode biofilms from MECs with cysteine and with anodes poised at the optimal potential (450 mV vs. SHE) revealed poor electrode coverage by *G. lovleyi* cells, in contrast to the saturating biofilms formed by *G. sulfurreducens* (Fig. 4B and C, respectively). Though sparse, the anode microcolonies formed by strain SZ reached average thickness (9.1 ± 1.2 µm) similar to that of the saturating biofilm of *G. sulfurreducens* (10.8 ± 2.3 µm). To reach this thickness, *G. sulfurreducens* anode biofilms couple cell growth to electron transfer to the underlying electrode, a process that requires the combined redox activities of matrix-associated *c*-type cytochromes such as OmcZs and conductive pili [33]. The genome of *G. lovleyi* contains a homologue of the gene encoding the peptide subunit or pilin that polymerizes to make the conductive pili of *G. sulfurreducens* [41]. However, we were unable to identify a clear homolog of OmcZ (GSU2078), the precursor of the matrix-associated *c*-type cytochrome OmcZs [14] that concentrates near and on the electrode and is required for efficient electron transport to the anode surface [15]. Outer membrane cytochromes required for extracellular electron transfer to solid-phase electron acceptors such as OmcS [26] did not retrieve a clear homolog either. Also absent in the genome of strain SZ are *c*-type cytochromes with more than 12 hemes [41], which have been proposed to store electrons and continue energy generation through a proton motive force until the cell establishes elec-

tronic contact with the electron acceptor [11]. Indeed, the genome of *G. lovleyi* contains less cytochrome-encoding genes than any other sequenced *Geobacter* genome [41]. Thus, the inability of *G. lovleyi* to colonize and respire the electrode efficiently could result, at least partially, from the lack of *c*-type cytochromes needed for the cells to establish electronic contact with the electrode. Poising the anode potential at 31 mV (vs. SHE) to mimic the theoretically lower energy gain derived from the two-electron reduction of fumarate to succinate reduced MEC performance proportionally (Fig 4A, inset). In *G. sulfurreducens,* the ability of the cells to reduce low potential, solid-phase electron acceptors depends on the expression of the inner membrane cytochrome CbcL [45]. A search of the genome of *G. lovleyi* retrieved no clear homologs of CbcL, consistent with the inability of the anode biofilms to efficiently reduce the electrode when poised at low metabolically oxidizable potentials. By contrast, we identified a gene (Glov_2063) encoding a protein homologous (63.9% identity and 72.8% similarity) to ImcH, an inner membrane cytochrome of *G. sulfurreducens* that is required for extracellular electron transfer at higher (>240 mV vs. SHE) redox potentials [20]. We also set up MECs with anodes poised at the potential (771 mV vs. SHE) of the Fe(III)/Fe(II) pair but maximum current (0.21 ± 0.05 mA) and rates of current production (0.190 ± 0.071 mA/day) were still low in these MECs (Fig 4A, inset). Furthermore, acetate was not consumed in these MECs and confocal micrographs of the anode electrodes revealed very sparse cell colonization. Thus, current production in these MECs was not biological but rather mediated by redox interactions between the cysteine and the anode electrode, as previously observed in MFCs [22].

Implications for CW-MFC performance. The results presented herein demonstrate that the physiology of *G. lovleyi* is substantially different from previously studied *Geobacter* electricigens. Indeed, we identified several physiological constraints not reported for other model electricigens that are likely to limit the performance of CW-MFCs. Of special significance is the low oxygen tolerance of *G. lovleyi*, which requires the presence of oxygen scavengers to maintain the redox potential of the medium at a level sufficiently low to permit growth. This sensitivity contrasts with the oxygen tolerance and respiratory capacity of model electricigens such as *G. sulfurreducens*, a metabolic capacity that contributes to their survival and growth in oxic environments [21]. Thus, environmental surveys in CW cannot solely rely on the presence of *Geobacter*-like sequences as a proxy of electricigenic activity, and need to consider species-specific genes, particularly those encoding proteins involved in oxygen tolerance such as superoxide reductase [16], superoxide dismutase [27], and NADH oxidase [42]. This is of particular relevance to planted systems such as HSSF CW, which comprise a macrophytes root system that continuously releases oxygen in the surrounding medium [38]. Moreover, plant evapotranspiration, which is needed to supply oxygen to the cathode in CW-MFCs [10], causes daily fluctuations of the water level that favor oxygen intrusions in the gravel media and increase the redox potential in some areas of the treatment bed [28]. This is expected to reduce the representation in the anode biofilms and/or electrochemical activity of oxygen-sensitive electricigens, such as *G. lovleyi*, and reduce the performance of CW-MFCs.

Results from this study also suggest that power generation by *G. lovleyi* anode biofilms is limited by the inability of this organism to use reduced electron donors, such as lactate and formate, that are abundant in CW. Acetate is a key metabolic intermediate in anaerobic digestion and an abundant electron donor in HSSF CW [2], providing adequate conditions for the enrichment of *Geobacter* electricigens in CW-MFCs, including *G. lovleyi* [8]. However, the inability of *G. lovleyi* to oxidize and assimilate other abundant, and more reduced, organic acids such as formate and lactate limits the amount of power that can be harvested by anode biofilms during the degradation of organic matter in CW. We also show that optimal current harvesting from *G. lovleyi* anode biofilms required the anode to be poised at a sufficiently high potential (450 mV vs. SHE) (Fig. 4). Such operational parameters are difficult to implement in HSSF CW, where redox gradients fluctuate widely in response to external conditions [7]. Redox potentials in deep zones of planted HSSF CW can reach ca.–200 mV vs SHE, and generate a maximum voltage of 140 mV [7]. This suggests that the anode potential in CW-MFCs is negative and, thus, suboptimal for the growth and electroactivity of *G. lovleyi* on the anode electrode. Our MEC studies (Fig. 4) also revealed that, even under optimal conditions, the colonization of the anode electrode by *G. lovleyi* is sparse, in contrast to the saturating electrode coverage reported for the most efficient *Geobacter* electricigens [31]. The inability of *G. lovleyi* to grow saturating biofilms leaves areas of the anode electrode exposed and available for colonization by fastidious non-electricigens, whose growth on the electrode reduce the performance of MFC systems [5]. Indeed, the best performing

CW-MFCs enriched for not only *G. lovleyi* OTUs, but also for methanogens [8]. However, increasing the electrode surface area and controlling substrate loadings could be used to help minimize the growth of methanogens on the anode electrode and increase the performance of CW-MFCs, as reported for other MFC systems [4]. The presence of alternative electron acceptors, which can divert electrons away from the anode electrode, also deserves special attention. *G. lovleyi* can use nitrate as electron acceptor to produce ammonia using the DNRA pathway [37]. Furthermore, high acetate-to-nitrogen ratios such as those that prevail in CW promote the DNRA activities and enrich for *G. lovleyi*-like organisms [40]. Hence, improved CW-MFC performance also needs to consider pretreatment approaches that either maintain acetate:nitrate ratios favoring electricity generation by *G. lovleyi* over DNRA or that enrich for more efficient *Geobacter* electricigens.

In conclusion, we can say that the electrochemical characterization of the model representative *G. lovleyi* strain SZ identified critical parameters (low oxygen tolerance, limited range of oxidizable electron donors, requirement of sufficiently high anode potentials, and inefficient electrode colonization and reduction) limiting the growth and electroactivity of *G. lovleyi* anode biofilms in CW-MFCs. Further investigations are recommended that test the effectiveness of increases in anode electrode surface, controlled substrate loadings and pretreatments in the growth and electroactivity of *G. lovleyi* and perhaps other electricigens on the anode electrode so as to improve the performance of CW-MFCs.

Acknowledgements. This research was supported by funds from the AgBioResearch at Michigan State and the National Institute of Food and Agriculture (NIFA) of the US Department of Agriculture to G.R. and a predoctoral fellowship from the Generalitat de Catalunya (2014 FI_AGAUR) to C.C. We are grateful to Dawn Holmes (Western New England University) for providing a culture of *G. lovleyi*.

Competing interests. None declared.

References

1. Amos BK, et al. (2007) Detection and quantification of *Geobacter lovleyi* strain SZ: implications for bioremediation at tetrachloroethene- and uranium-impacted sites. Appl Environ Microbiol 73:6898-6904
2. Bagley DM, et al. (2000) Acclimation of anaerobic systems to biodegrade tetrachloroethene in the presence of carbon tetrachloride and chloroform. Water Res 34:171-178
3. Baptista JD, et al. (2003) Microbial mechanisms of carbon removal in subsurface flow wetlands. Water Sci Technol 48:127-34
4. Borole AP, et al. (2009) Improving power production in acetate-fed microbial fuel cells via enrichment of exoelectrogenic organisms in flow-through systems. Biochem Eng J 48:71-80
5. Borole AP, et al. (2011) Electroactive biofilms: Current status and future research needs. Energy Environ Sci 4:4813-4834
6. Call DF, Logan BE (2011) Lactate oxidation coupled to iron or electrode reduction by *Geobacter sulfurreducens* PCA. Appl Environ Microbiol 77:8791-8794
7. Corbella C, Garfi M, Puigagut J (2014) Vertical redox profiles in treatment wetlands as function of hydraulic regime and macrophytes presence: Surveying the optimal scenario for microbial fuel cell implementation. Sci Total Environ 470:754-758
8. Corbella C, et al. (2015) Operational, design and microbial aspects related to power production with microbial fuel cells implemented in constructed wetlands. Water Res 84:232-242
9. Cusick RD, Kiely PD, Logan BE (2010) A monetary comparison of energy recovered from microbial fuel cells and microbial electrolysis cells fed winery or domestic wastewaters. Int J Hydrogen Energy 35:8855-8861
10. Doherty L, et al. (1985) A review of a recently emerged technology: Constructed wetland--Microbial fuel cells. Water Res 85:38-45
11. Esteve-Nunez A, et al. (2008) Fluorescent properties of c-type cytochromes reveal their potential role as an extracytoplasmic electron sink in *Geobacter sulfurreducens*. Environ Microbiol 10:497-505
12. Fang Z, et al. (2013) Performance of microbial fuel cell coupled constructed wetland system for decolorization of azo dye and bioelectricity generation. Bioresour Technol 144:165-171
13. Faulwetter JL et al. (2009) Microbial processes influencing performance of treatment wetlands: A review. Ecol Eng 35:987-1004
14. Inoue K et Al. (2010) Purification and characterization of OmcZ, an outer-surface, octaheme c-type cytochrome essential for optimal current production by *Geobacter sulfurreducens*. Appl Environ Microbiol 76:3999-4007
15. Inoue K, et al. (2011) Specific localization of the *c*-type cytochrome OmcZ at the anode surface in current-producing biofilms of *Geobacter sulfurreducens*. Environ Microbiol Rep 3:211-217
16. Jenney FE Jr, et al. (1999) Anaerobic microbes: oxygen detoxification without superoxide dismutase. Science 286**:**306-309
17. Kaden J, Galushko SG, Schink B (2002) Cysteine-mediated electron transfer in syntrophic acetate oxidation by cocultures of *Geobacter sulfurreducens* and *Wolinella succinogenes*. Arch Microbiol 178:53-58
18. Kiely PD, et al. (2010) Anodic biofilms in microbial fuel cells harbor low numbers of higher-power-producing bacteria than abundant genera. Appl Microbiol Biotechnol 88:371-80
19. Kiely PD, Regan JM, Logan BE (2011) The electric picnic: synergistic requirements for exoelectrogenic microbial communities. Curr Opin Biotechnol 22:378-385
20. Levar CE, et al. (2014) An inner membrane cytochrome required only for reduction of high redox potential extracellular electron acceptors. MBio 2014. **5**:e02034
21. Lin WC, Coppi MV, Lovley DR (2004) *Geobacter sulfurreducens* can grow with oxygen as a terminal electron acceptor. Appl Environ Microbiol 70: 2525-2528
22. Logan BE, et al. (2005) Electricity generation from cysteine in a microbial fuel cell. Water Res 39: 942-952
23. Logan BE, RabaeyK (2012) Conversion of wastes into bioelectricity and chemicals by using microbial electrochemical technologies. Science 337:686-690
24. Lovley DR, et al. (2011) *Geobacter*: The microbe electric's physiol-

ogy, ecology, and practical applications, in Adv. Microb. Physiol., K.P. Robert, Editor. 2011, Academic Press. p. 1-100
25. Lovley DR, Phillips EJ (1986) Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl Environ Microbiol 51:683-689
26. Mehta T, et al. (2005) Outer membrane *c*-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. Appl Environ Microbiol 71:8634-8641
27. Nakanishi T, Inoue H, Kitamura M (2003) Cloning and expression of the superoxide dismutase gene from the obligate anaerobic bacterium *Desulfovibrio vulgaris* (Miyazaki F). J Biochem 133:387-393
28. Pedescoll A, et al. (2013) Evapotranspiration affecting redox conditions in horizontal constructed wetlands under Mediterranean climate: Influence of plant species. Ecol Eng 58:335-343
29. Robyt JF, White BJ (1987) Biochemical techniques: Theory and practice. Prospect Heights. Waveland Press, IL, USA
30. Segura D, et al. (2008) Computational and experimental analysis of redundancy in the central metabolism of *Geobacter sulfurreducens*. PLoS Comput Biol 4:e36
31. Speers AM, Reguera G (2012) Electron donors supporting growth and electroactivity of *Geobacter sulfurreducens* anode biofilms. Appl Environ Microbiol 78:437-444
32. Speers AM, Reguera G (2015) Theoretical and practical considerations for culturing *Geobacter* biofilms in microbial fuel cells and other bioelectrochemical systems. In: Beyenal H, Babauta J (eds), Biofilms in bioelectrochemical systems. John Wiley & Sons, pp 37-60
33. Steidl R, Lampa-Pastirk S, Reguera G (2016) Mechanistic stratification in electroactive biofilms of *Geobacter sulfurreducens* mediated by pilus nanowires. Nature Comm **7**:12217
34. Stookey LL (1970) Ferrozine: a new spectrophotometric reagent for iron. Anal Chem 42**:**779-781
35. Straub KL, Kappler A, Schink B (2005) Enrichment and isolation of ferric-iron- and humic-acid-reducing bacteria. In Mehods in enzymology. Leadbetter JR (ed) pp 58-77
36. Strycharz SM, et al. (2008) Graphite electrode as a sole electron donor for reductive dechlorination of tetrachlorethene by *Geobacter lovleyi*. Appl Environ Microbiol 74:5943-5947
37. Sung Y, et al. (2006) *Geobacter lovleyi* sp. nov. strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium. Appl Environ Microbiol 72:2775-2782
38. Tanner CC (2001) Plants as ecosystem engineers in subsurface-flow treatment wetlands. Water Sci Technol 44:9-17
39. Thomas SH, et al (2010) Unique ecophysiology among U(VI)-reducing bacteria as revealed by evaluation of oxygen metabolism in *Anaeromyxobacter dehalogenans* strain 2CP-C. Appl Environ Microbiol 76:176-183
40. van den Berg EM, et al. (2015) Enrichment of DNRA bacteria in a continuous culture. ISME J 9:2153-2161
41. Wagner DD et al. (2012) Genomic determinants of organohalide-respiration in *Geobacter lovleyi*, an unusual member of the Geobacteraceae. BMC Genomics 13
42. Ward DE, et al. (2001) The NADH oxidase from *Pyrococcus furiosus*. Implications for the protection of anaerobic hyperthermophiles against oxidative stress. Eur J Biochem 268:5816-5823
43. Yang TH, et al. (2010) Metabolic response of *Geobacter sulfurreducens* towards electron donor/acceptor variation. Microb Cell Fact 9:90
44. Young JM, Leschine SB, Reguera G (2011) Reversible control of biofilm formation by *Cellulomonas* spp. in response to nitrogen availability. Environ Microbiol. 14: 594-604
45. Zacharoff L, Chan CH, Bond DR (2016) Reduction of low potential electron acceptors requires the CbcL inner membrane cytochrome of *Geobacter sulfurreducens*. Bioelectrochemistry 107:7-13