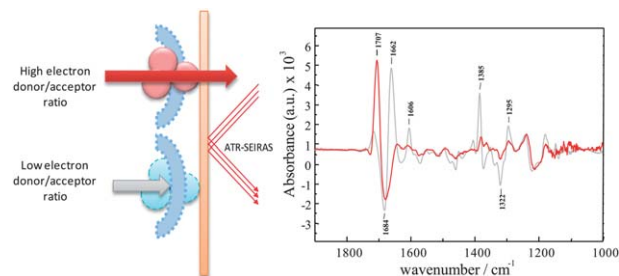


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Opportunities behind the unusual ability of *geobacter sulfurreducens* for exocellular respiration and electricity production

Abraham Esteve-Núñez,* Juan Pablo Busalmen,*
Antonio Berná, Cristina Gutiérrez-Garrán
and Juan Miguel Feliu

Bacteria can be induced to produce cytochromes profusely to connect to electrodes more efficiently.



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Opportunities behind the unusual ability of *Geobacter sulfurreducens* for exocellular respiration and electricity production†

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The possibility to improve the connection of cells to the electrode is significant for microbial fuel cell technology. In this communication we demonstrate that an improved connection can be made by controlling the physiological state of electricity-harvesting bacteria as *Geobacter sulfurreducens*.

The original interest of exploiting bacterial metabolic activity for electricity production^{1,2} has been greatly renewed by the discovery of bacterial ability to respire electrodes.³ Model organisms such as *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 have been intensively studied and the details of their electron transport mechanisms to electrodes are being elucidated.⁴⁻⁹

Species belonging to the *Geobacter* genus are ubiquitous in anaerobic sediments and usually dominate the communities enriched on polarized electrodes in those environments. Indeed, new data appearing on the community composition of electrogenic biofilms producing electricity from wastewater also reveal their dominance in these man-made environments.¹⁰ Thus, it is important to gain information about the physiology of *Geobacter* under growth conditions that could be relevant for these situations.

In this work we take advantage of the possibility to culture *Geobacter sulfurreducens* under different physiological conditions^{11,12} to

gain information about the unprecedented versatility of this bacterium to select and control its respiratory pathway, in order to adapt to external constrains. Results presented here provide new information that supports the success of *Geobacter* species in electrogenic biofilms.

Of main importance, c-type cytochromes located at the bacterial surface are the molecules responsible for electron transport to solid acceptors in *G. sulfurreducens*.^{4,5} This has been clearly shown using surface enhanced IR spectroscopy during electricity production.^{4,5} In those works the enhanced sensitivity of the SEIRA effect allowed the identification of proteins as the electrode-contacting molecules, while a differential analysis of oxidized minus reduced spectra revealed that electron transport to the electrode is directly related to the conformational change of external c-type cytochromes upon redox transition. A more detailed analysis using SNIFTIRS modulation allowed the recognition of the IR fingerprint of the *Geobacter* surface.⁵

The same combination of techniques is used in this work to show that growth under nutrient-limiting conditions can induce *Geobacter* to change the expression of external c-type cytochromes, according to different physiological strategies adopted by cells at each situation.

Growing *Geobacter* in continuous culture under selected nutrient limitations may produce phenotypes that can closely resemble those found in the environment.¹² For example, growing cells under electron donor limitation mimics the slow release of electron donors from complex organic matter in sedimentary environments. As in the case of previously reported results,¹² when *G. sulfurreducens* was grown under continuous culture with acetate as limiting substrate (5.5 mM) and fumarate (30 mM) as the electron acceptor (dilution rate 0.05 h⁻¹), biomass production was consistent with classical Monod-type growth. In this situation electrons recovered *via* fumarate reduction compared well with the number of electrons provided by acetate oxidation, confirming the use of fumarate as the unique electron acceptor. Oppositely, when cultures were grown under fumarate limitation (10 mM) with acetate in excess (10 mM), growth did not

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† Electronic Supplementary Information (ESI) available: Chronoamperometric, voltammetric and spectrometric data of bacterial layer are shown together with the fluorimetric analysis of cell cultures. See DOI: 10.1039/b000000x/

Broader context

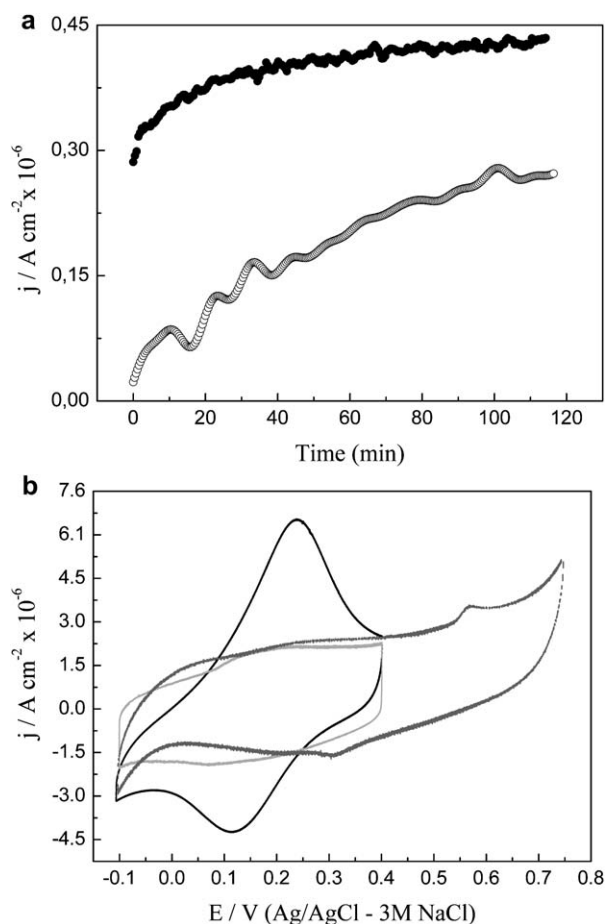
To wire or not to wire. Bacteria that are able to produce electricity by exchanging electrons with solid electrodes are studied by electrochemical and infrared techniques under controlled physiology. The approach identifies conditions under which the connection is improved, signaling the way to more efficient applications including microbial fuel cells and whole cell biosensors.

1 follow the Monod kinetics, the conversion of acetate into biomass
was lower and the specific respiration rate for fumarate was about
10% higher than that of acetate-limited cells.¹²

5 These results are relevant to understand the physiological response
of *Geobacter* in biotechnological applications based on acetate
amendments, like the reductive precipitation of uranium,¹³ or that
observed in electrogenic waste treatment applications were acetate
may be present in excess as a product of the acetogenic microbial
populations.¹⁴

10 Differences in respiration rates raised the question about the ability
of cells in both nutrient-limiting conditions to respire electrodes. In
order to gain a first picture about it, acetate-limited cells were
analyzed by ATR-SEIRAS in conjunction with classical voltammetry,
aiming to compare the results to those previously obtained with
15 fumarate-limited cells.⁵ In addition, cell cytochrome content was
estimated measuring the autofluorescence of intact cells under
reducing conditions.¹⁵

20 Current production during adsorption of cells to an electrode
polarized to 0.2 V (Ag/AgCl – KCl sat.) was markedly higher for



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Fig. 1 (a) Electricity production by fumarate-limited (filled symbols) and acetate limited (open symbols) *G. sulfurreducens* during the adsorption process of cells on a thin-film gold electrode. The electrode was initially polarized to 0.2 V in the electrolyte containing acetate or fumarate, respectively under a N₂:CO₂ (80 : 20) atmosphere. (b) voltammetry of (—) fumarate-limited and (---) acetate-limited adsorbed cells. The voltammetry of the gold film in the absence of bacteria is included as a control (grey continuous line).

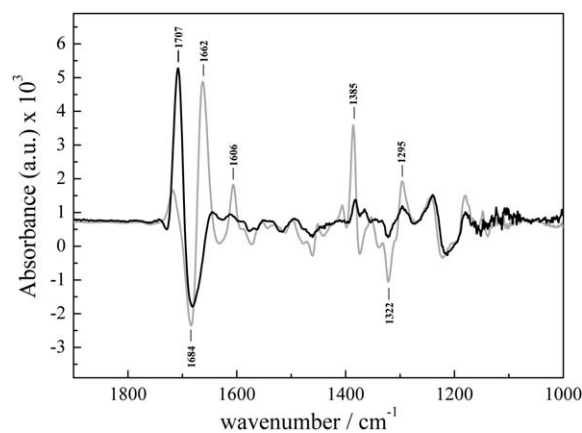
1 notably, these cells were able to
increase the current production as soon as they contacted the
electrode.

5 Acetate-limited cells on the other hand, produced a very low initial
current and showed an increasing trend in current production before
reaching the steady state value. Since cell suspensions were prepared
exceeding the amount of bacteria required for surface adsorption
saturation,¹⁶ the observed differences are interpreted as an evidence of
the higher aptitude of fumarate-limited cells for external electron
10 transport. The interpretation is supported by cyclic voltammetry
results that demonstrate the negligible capacity of acetate-limited cells
for electrons exchange with the electrode. Only a very low amplitude
signal probably related to a high redox potential pair previously
found in *Geobacter*^{4,17} was detected (Fig. 1(b)). It contrasted with the
15 well defined redox process found in fumarate-limited cells.

The spectral fingerprint region as obtained during adsorption of
acetate-limited cells at the thin film of gold in ART-SEIRAS exper-
iments also differ from that of fumarate-limited cells (Fig. S1†).

20 It becomes rapidly dominated by both, a sharp peak at 1230 cm⁻¹
assigned to phosphate/phosphonate groups in the bacterial surface¹⁸
and a complex band that expanded in the range from 1000 to 1100
cm⁻¹, with a maximum at 1103 cm⁻¹ and shoulders at 1049, 1122,
1149 and 1176 cm⁻¹ (Fig. S1†), evidencing the presence of carbohy-
25 drate molecules at the interface and suggesting that the adsorption
of acetate-limited cells is mediated by external lipopolysaccharides
(LPSs).^{19,20} The relative size of amide bands on the other hand, lower
than in fumarate-limited cells, points out the secondary role played by
proteins in the adhesion process.

30 Since current production is known to be mediated by external
redox proteins, a low content of them may also be responsible for
results in Fig. 1(a) and (b). The possibility was further explored by
SNIFTIRS. In this technique the differential absorption spectra are
calculated by subtraction of those obtained under oxidizing and
reducing potentials (0.4 and –0.1 V, respectively). The analysis of the
35 results shown in Fig. 2 provides evidence of conformational changes
associated to the redox transition of molecules at the cell surface upon



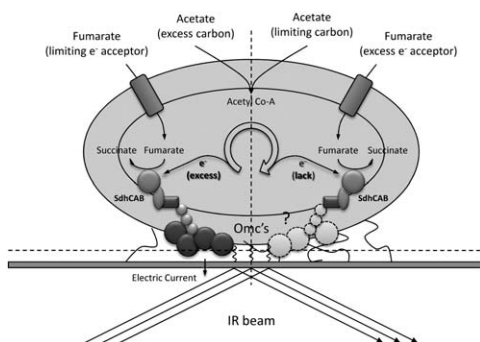
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Fig. 2 SNIFTIRS spectra of fumarate-limited (grey line) and acetate-limited (black line) cells of *G. sulfurreducens* on a thin-film gold electrode. The spectra represent the fully oxidized state of cells and is the result of five consecutive cycles of 100 interferograms at both the sample (0.4 V) and reference (–0.1 V) potentials. The resolution of the measurement is 2 cm⁻¹. Indicated wavenumbers are those previously related to cytochrome c or microperoxidase 8 in the literature.⁶

heterogeneous electron transfer. As previously reported, the fingerprint region of fumarate-limited cells clearly fits with that obtained from cytochrome C (C-Cyt) of different organisms,⁵ with major signals at 1684 (–), 1660(+), 1606(+), 1385(+) and 1322(–) cm⁻¹ (Fig. 1).⁵ The one of acetate-limited cells on the other hand, showed only minimal changes upon potential change, indicating a low amount of redox elements at the cell surface. Only a bipolar signal was observed at the position of the Amide I band (1707 (+), 1684 (–) cm⁻¹) (Fig. 1), probably involved in the electron transfer of the low current step observed in Fig. 1(a), or to a reversible conformational change induced by polarization in a non-electroactive surface protein.

Results presented above indicate that fumarate-limited cells have a higher amount of cytochromes at the cell surface, which improves the connection to the electrode. Considering that the expression of genes encoding for outer membrane cytochromes has been observed to change in relation to the kind and availability of the electron acceptor,¹¹ differences shown in Fig. 1 and 2 and the results in figure S1† are thought to be the consequence of a specific bacterial response to the electron acceptor limitation. Cells seem to exploit the availability of carbon and electrons provided by the excess of acetate to overproduce cytochromes, increasing their respiratory versatility, as represented in Scheme 1. In this way, they are ready to respire any electron acceptor (including electrodes) as soon as it is made available. To experimentally gain information to test this hypothesis, we analyzed the c-type cytochrome content and found that the amount was 5-fold higher in fumarate-limited cells as compared to those in acetate-limited ones (Fig. S2†). Importantly, overexpressed ones include outer membrane cytochromes such as OmcS (A. Esteve-Núñez, in preparation) and omcB,¹¹ in accordance to the detection of external cytochromes by ATR-SEIRAS.

This ability to improve the connection between cells and the electrode is especially important for microbial fuel cell technology. This is because it can help reduce the start-up time for current production. These results reinforce the importance of gaining physiological information under conditions representative of the intended cell application.

A complete analysis of *Geobacter* cytochromes expression under physiological conditions used here has already been performed and is currently under study (A. Esteve-Núñez *et al.*, in preparation).



Scheme 1 Schematic representation of respiratory strategies in *G. sulfurreducens* grown under acceptor or donor limitation. The presence of over expressed cytochromes is shown, including those external ones contacting the electrode to produce current during a typical ATR-SEIRAS experiment. SdhCAB: succinate dehydrogenase; Omc's: outer membrane cytochromes.

Findings reported here are also significant to interpret the physiological versatility of *Geobacter*, since they seem to support the proposal of external cytochromes functioning as iron lungs.²¹ In the lack of any acceptor, electrons produced from the excess of acetate can be funneled to cytochromes in the cell exterior, allowing the production of metabolic energy to continue until the lung is completely reduced. It is interesting to note that in this situation, charged cells should be ready to transfer electrons to any external acceptor when available, a hypothesis that is supported by results presented in Fig. 1(a), in which current is immediately produced upon contact to the polarized electrode.

Overproduction of external cytochromes in the event of an electron donor excess have been called to explain the predominance of *Geobacter* species in subsurface environments;^{12,22} in the same line of thinking the strategy may be linked to the success of *Geobacteraceae* enriched on anodes of microbial fuel cells producing electricity from waste.¹⁰ Volatile fatty acids (VFAs) such as acetate, butyrate and propionate are usually produced by the acetogenic community during the anaerobic treatment of waste, to be subsequently converted to methane by the methanogenic microorganisms. Alternatively, they can be consumed by *Geobacteraceae* for electricity production.¹⁰ Typical steady-state concentrations of VFAs in anaerobic treatments are in the millimolar range,^{23,24} while depending on the waste source and the imposed anode potential, alternate electron acceptors may be at extremely low concentrations,²⁵ conditions that can drive *Geobacteraceae* to over-express cytochromes, thus improving the connection to the electrodes.

Experimental section

ATR-SEIRAS

Spectro-electrochemical experiments were carried out in a glass cell at room temperature (around 20 °C) as described elsewhere.¹⁹ Spectra were collected with p-polarized light with a resolution of 4 cm⁻¹ (unless otherwise indicated) and are presented as the ratio $-\log(R2/R1)$, where R2 and R1 are the reflectance values corresponding to single beam spectra at the sample and reference condition indicated in the text for each experiment, respectively. Interferograms were acquired every 1 s to calculate each one of these single beam spectra.

Culture of microorganisms and spectroscopic analysis

Geobacter sulfurreducens were anaerobically cultured in chemostats as previously described.¹² Acetate was used as the carbon source and electron donor while fumarate was the electron acceptor. Electron donor limited cells were grown on 5.5 mM acetate using 30 mM fumarate as the electron acceptor. Electron acceptor limited cells on the other hand were grown on 10 mM acetate and 10 mM fumarate. Steady-state cells were harvested by centrifugation at 6000 rpm during 10 min, washed with (and concentrated by a factor of 10 in) an anoxic solution containing 30 mM KCl, 30 mM NaHCO₃ and 5 mM acetate. The bacterial suspension was anaerobically transferred to the ATR cell to perform the spectro-electrochemical analysis. To ensure that cells were using the gold electrode as the electron acceptor, no other acceptor was added to the solution.

Cyclic voltammetry (CV)

Cyclic voltammetry was performed using a three electrodes configuration for the electrochemical cell. Experiments were developed using an EA-161 potentiostat controlled by a universal programmer (model 175 from Princeton Applied Research) and connected to a PC through an e-corder 401 unit (E-DAQ Pty Ltd.). The counter electrode was a coiled gold wire and the reference was an Ag/AgCl – 3M NaCl electrode. The potential was scanned between –0.1 and 0.4 V starting positively from –0.1 V. The scan rate was 0.005V s⁻¹. All the experiments were performed using a 30mM sodium bicarbonate solution supplemented with 30 mM KCl and 5 mM acetate as the electrolyte. It was buffered at pH 7 under a N₂:CO₂ (80 : 20) atmosphere (L'Air liquide).

Cytochromes quantification

Cytochromes content in *Geobacter* was analyzed by measuring its autofluorescence under reducing conditions using a spectrofluoremeter (RF-1501 SHIMADZU).¹⁵ Intact cells were excited at 350 nm and fluorescence at 437 nm was monitored.

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