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ATR-SEIRAs characterization of surface redox processes in *G. sulfurreducens*

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ABSTRACT

In this work we report on the occurrence of at least two different redox pairs on the cell surface of the electrogenic bacteria *Geobacter sulfurreducens* adsorbed on gold that are expressed in response to the polarization potential. As previously reported on graphite (Environ. Sci. Technol. 42 (2008) 2445) a typical low potential redox pair is found centered at around −0.06 V when cells are polarized for a few hours at 0.2 V, while a new pair centered at around 0.38 V is expressed upon polarization at 0.6 V. Reversible changes in the IR band pattern of whole cells where obtained by Attenuated Total Reflection-Surface Enhanced Infrared Absorption Spectroscopy (ATR-SEIRAS) upon potential cycling around both redox pairs. Changes clearly resemble the electrochemical turnover of oxidized/reduced states in c-type cytochromes, thus evidencing the nature of the involved molecules. The expression of external cytochromes in response to the potential of the electron acceptor suggests the existence of alternative pathways of electron transport with different energy yield, though it remains to be demonstrated.

1. Introduction

Electrode-reducing bacteria are able to produce electricity by channeling their respiratory flux of electrons to an electrode. This ability is now being exploited for the construction of microbial fuel cells (MFCs), to generate a considerable amount of power from organic waste [1,2]. Although microbiological research has led to the identification of some molecules participating in the electron transport pathway to the cell exterior [3], the arrangement of the pathway(s) and importantly, the identification of final reductases wiring cells to electrodes are yet a matter of discussion.

Main tasks in the development of bioelectrochemical devices exploiting electrode-reducing bacteria are to define the nature of molecules involved in the direct electron transport to electrodes and to determine their redox properties. To these ends, the use of well defined electrode materials and the application of advanced (spectro)electrochemical techniques can provide valuable information.

Carbon is the most commonly used material for the implementation of MFCs, mainly because it has a relatively low cost and an acceptable performance. Nevertheless, its surface properties are poorly defined and its use in fine electrochemical techniques is limited, as is its use for spectro-electrochemistry. In order to overcome this technical limitation it is necessary to gain information into the applicability of well defined electrode materials as gold or platinum in microbial electrochemistry. In this direction, it has been previously demonstrated that gold can work as an electron acceptor for *G. sulfurreducens* [4,5] and importantly that surface redox processes at the surface of cells can be studied by spectroscopic methods with an unprecedented level of resolution [5].

In this work we extend the analysis of the electrochemical features of *G. sulfurreducens* at gold electrodes in order to compare results with those previously obtained using graphite. Having confirmed the suitability of gold for microbial electrochemistry we used ATR-SEIRAS to perform the spectro-electrochemical analysis of redox pairs at the cell surface. Previous and present results are interpreted here to propose the existence of inducible cytochromes in *G. sulfurreducens* that can act as the final reductase depending on the potential of the acceptor to reduce. Indeed, we discuss the significance of results in the structuring of the external respiratory chain in *Geobacter*.

2. Experimental

2.1. Culture of microorganisms and electrochemical conditioning

Geobacter sulfurreducens was anaerobically cultured in chemostats as previously described [6]. Acetate was used as the carbon source and electron donor under conditions in which the electron acceptor fumarate was the growth-limiting factor. Steady-state cells were harvested by centrifugation at 6000 rpm during 10 min, washed and concentrated by a factor of 10 in an anoxic solution containing 30 mM KCl, 30 mM $NaHCO_3$ and 5 mM acetate. 5 ml of the bacterial suspension were anaerobically transferred to the ATR cell described below containing 10 ml of the same electrolyte equilibrated at pH 7 under a N_2:CO_2 (80:20) atmosphere (L'Air liquide). The working

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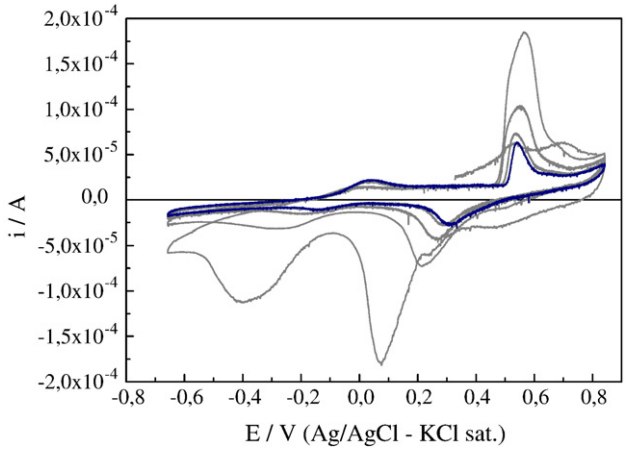

Fig. 1. Cyclic voltammograms of *G. sulfurreducens* whole cells on a thin-film gold electrode after the exposure by 8 h under continuous polarization at 0.6 V. Grey lines: cycles one to four. Blue line: fifth cycle.

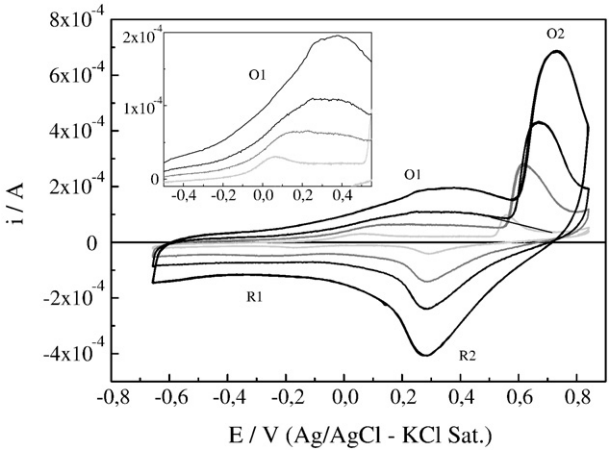

Fig. 2. Cyclic voltammograms of *G. sulfurreducens* whole cells on a thin-film gold electrode after the exposure by 8 h under continuous polarization at 0.6 V. Scan rate: (▁) 0.005, (▂) 0.010, (▃) 0.050 and (—) 0.100 V s^{-1}. O: oxidation and R: reduction process. Inset: scale magnification to show the O1 process in detail.

electrode was polarized to the selected potential (typically 0.2 or 0.6 V) for 4 to 8 h before performing the SEIRAS analysis. To ensure that cells were using the gold electrode as the only electron acceptor, no other acceptor was added to the solution.

2.2. ATR-SEIRAS

Spectro-electrochemical experiments were carried out in a glass cell at room temperature (around 20 °C) as described previously [7]. 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.

2.3. 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–3 M NaCl electrode. The potential was scanned between −0.7 and 0.7 V starting positively from 0.4 V. Scan rates of 0.005, 0.010, 0.050 and 0.100 V s^{-1} where used. All the experiments were performed in a 30 mM sodium bicarbonate solution supplemented with 30 mM KCl and 5 mM acetate as the electrolyte. Potentials are reported against the Ag/AgCl–KCl sat. electrode ($E = 0.220$ V NHE) for clarity.

3. Results and discussion

3.1. Whole cell voltammetry on gold

In order to determine if gold can be a suitable electrode material for the electrochemical analysis of *G. sulfurreducens*, whole cells were polarized at 0.2 and 0.6 V reproducing the experiments previously performed on graphite [8].

After several hours of polarization the voltammetric features of adsorbed bacterial cells nearly resemble those previously reported [8] at both adsorption potentials. After polarization to 0.2 V the voltammogram included a single redox process centered at about −0.06 V [5] (results not shown), while after polarization to 0.6 V an additional high potential redox pair centered at about 0.38 V was detected (Fig. 1). The similarity in the response confirms that gold is able to induce the expression of electron transport pathways in these bacteria, as previously shown for graphite [8]. Voltammograms evolved with the cycles until reaching a well defined profile, a behaviour that has been interpreted as related to the change in position of the electron transport protein at the bacteria/electrode interface due to the potential cycling [8]. Two oxidation peaks, at 0.50 and 0.67 V, and at least four reduction signals at 0.43, 0.20, 0.03 and −0.43 V were clearly observed in the first cycle of the voltammetric sweep (Fig. 1), which are indicative of a complex process involving probably more than one single-site redox molecules or a multi-site molecule. The low potential redox pair remained visible after polarization to 0.60 V, which is believed to be a consequence of the shorter time of conditioning used in the present case, as compared to previous experiments using graphite [8]. After several cycles, the high potential contribution remains centered around 0.42 V and the low potential couple stays around −0.10 V.

To further explore the behavior of the remaining redox processes, voltammetry was performed at various potential swept rates and the results are reported in Fig. 2. Peak potentials for each redox process or its components are also indicated in Table 1 for clarity.

The potential difference between peaks (ΔE_p) in the upper redox pair was observed to increase with the scan rate (Table 1). It was mainly due to the shift of the oxidation process to higher potentials (Fig. 2). The reduction process on the other hand, was surprisingly fast and remained at 0.24 V, independently of the sweep rate (Table 1). A quasi-reversible behavior has already been reported for reduction of Cyt-c [9], but it is

Table 1
Peak potentials determined from the voltammetric analysis in Fig. 2 for the oxidation and reduction processes detected at the surface of *G. sulfurreducens*.

Scan rate (mV s^{-1})	Peak potential (V)			
	O1	R1	O2	R2
5	0.06	−0.17	0.55	0.29
10	~0.15	−0.23	0.61	0.28
	~0.22			
	~0.37			
50	~0.15	>−0.56	0.67	0.28
	~0.27			
	~0.37			
100	~0.27	>−0.66	0.73	0.28
	~0.37			

important to note that during faster scans in Fig. 2 (i.e. at 0.050 and 0.100 V s^{-1}), the available redox sites were only partially oxidized in the positive-going sweep and further oxidation could take place after the reversal of the sweep direction. If this is the case, distortion of the reduction wave or severe shifts in its position can take place.

The lower potential redox pair showed a much more irreversible behavior (Fig. 2). The oxidation wave becomes broader with the increase of the sweep rate, evidencing the presence of at least two electron transfer reactions with a different degree of irreversibility (inset Fig. 2). The broad reduction peak clearly visible at the lower scan rate (i.e. 0.005 V s^{-1}) becomes hardly detectable at 0.010 V s^{-1}, while it moved out of the potential window at higher scan rates. This fact can also modify the redox processes observed at higher potentials, especially if they are dealing with the same, or a closer, electroactive group and this may be a simple result of a ligand exchange that leads to a more (or less) stable complex with a particular cation.

In any case, to get information about the relevant electroactive species, it is necessary to identify what are the chemical bonds involved in the electron transfer. As this assignment can not be made univocally from pure electrochemical data, in situ spectroelectrochemical techniques are required. In the following we would report experimental information in this direction.

3.2. ATR-SEIRAS of redox pairs

It has been recently demonstrated that spectroelectrochemical techniques as Attenuated Total Reflection-Surface Enhanced InfraRed Absorption Spectroscopy (ATR-SEIRAS), Substractively Normalized Interfacial Fourier Transform InfraRed Spectroscopy (SNIFTIRS), Surface Enhanced Raman Spectroscopy (SERS) and UV/Vis spectroscopy can provide valuable information about the activity of bacteria at interfaces [5,7,10,11]. In particular, infrared techniques allowed the recognition of *Geobacter* external C-type cytochromes as the electrode-reductases during electricity production [5]. The interaction was demonstrated through the differential analysis of IR absorption spectra of oxidized and reduced protein states upon electrochemical conversion [5]. Following the same protocol, redox pairs in Figs. 1 and 2 were analyzed by ATR-SEIRAS. Spectra were collected during a slow potential scan at 5 mV s^{-1} starting at 0.25 V and going up to 0.80 V, then down to −0.70 V and finally again up to 0.25 V. One spectrum was acquired every 0.05 V during this slow scan. Difference spectra were calculated taking the first single beam spectrum collected at 0.25 V as reference. Through this analysis oxidized-minus-reduced and reduced-minus-oxidized spectra were obtained from a single voltammetric cycle from the upper and lower redox pairs, respectively. This strategy allows the direct comparison of data.

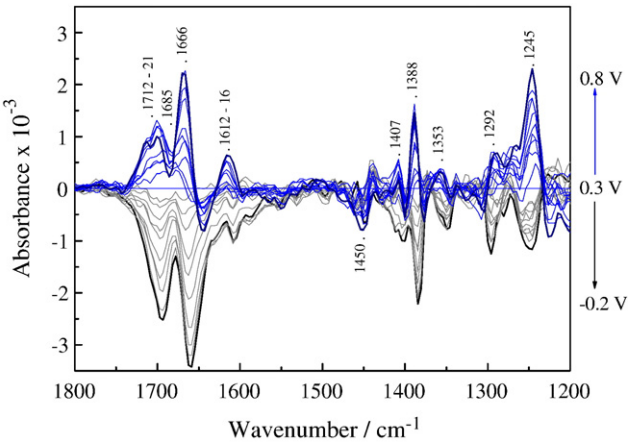

Fig. 3. ATR-SEIRAS spectra of *G. sulfurreducens* acquired during the second cycle of the voltammetric analysis shown in Fig. 1. Differential spectra were obtained at 0.05 V stepped potentials using as a reference single beam data obtained at 0.3 V. Blue lines: oxidized-reduced spectra showing the oxidation of a high potential redox pair; Black lines: reduced-oxidized spectra showing the oxidation of a low potential redox pair.

As it can be observed in Fig. 3, signals corresponding to the reduced form of the lower redox pair perfectly reproduce previously reported data [5].

Signals are due to the participation of external cytochromes in the electron transfer to the electrode. Notably, the oxidized state of the upper redox pair produced differential spectra (blue lines in Fig. 3) with variations in the same wavenumbers that those from the lower pair (black lines in Fig. 3), clearly pointing to the participation of cytochromes in the two processes. Main signals correspond to the amide I mode (1685; 1666 cm^{-1}), which arises mainly from the C=O stretching vibration of the amide group in the peptide chains and there are some minor contributions from the out-of-phase CN stretching and CCN deformation and in-plane NH bending [12]. The frequency for amide I bands can be related to the secondary structure of the protein and therefore, in this way, the higher frequency band (1685 cm^{-1}) is normally designated as amide I β-turn type III and the lower (1666 cm^{-1}) as amide I β-turn type II [12]. Other features can be related to the vibrational modes of the heme ring (1612-16; 1407; 1388; 1245) [13] and some others that remain unassigned (Fig. 3) are the same as those previously defined through a more precise analysis by SNIFTIRS [5]. Amplitude and minor distribution differences between the IR signals from both redox processes are not surprising since they depend on structural changes during the conformational transition of different cytochromes (Fig. 3).

3.3. Significance for the structure of the electron transport chain

Within the 111 different cytochromes coded in the genome of *G. sulfurreducens*, more than 30 are located in the outer sphere of the cell. Indeed, aminoacid sequence indicates that most of them posses several CXXCH motifs for binding heme groups. It constitutes a complex scenario for the interpretation of electrochemical and spectro-electrochemical data presented in Figs. 1–3. Voltammetric signal evidence multiple and overlapped processes of oxidation and reduction (Figs. 1 and 2) that would involve the conformational transition of c-type cytochromes (Fig. 3), but observed currents may also come from the cooperative and sequential electrochemical transition of several cytochromes (i.e. a putative enzymatic complex), or from single multi-heme cytochromes. OmcS, a molecule that probably acts as an iron and manganese oxide reductase [14], is predicted to be a hexaheme cytochrome that can easily be sheared from intact cells together with OmcE (a predicted tetraheme molecule). Their outermost location points to them as potential candidates for being involved in the heterogeneous electron transport processes reported here. Indeed, the lower potential process in Figs. 1 and 2 presents an appropriate half cell potential for Fe (III) reduction [8]. Having the opportunity to work with strains overexpressing OmcS, as the OmcF-deficient mutant [15], could give some insight into this point.

As discussed by Lovley [16] *Geobacter* render comparable cell yield growing on soluble and insoluble forms of Fe(III), in spite of the difference in potential of these electron acceptors. It supports the reasoning that external electron acceptors merely function to oxidize the electron transporters earlier in the electron transport chain, allowing the continuous production of energy in the inner membrane [16,17]. An implicit assumption in this argument is that electrons are transported from the inner membrane to the cell exterior (i.e. to the periplasm) through a single gate, thus yielding the same amount of energy in spite of the final exocellular acceptor used.

Results from our previous [8] and present work on the other hand, let us to propose that the potential of the electron acceptor is the

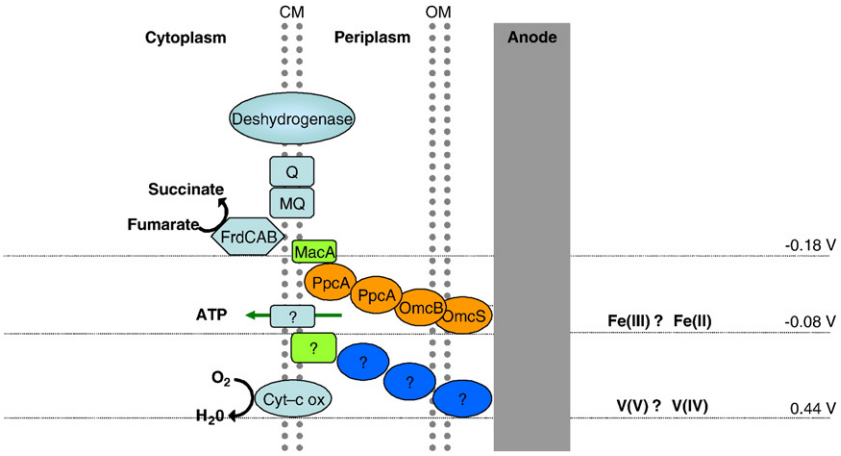

Fig. 4. Schematical model of the *G. sulfurreducens* electron transport to electrodes. Alternative pathways to exocellular electron acceptors are indicated. Orange and blue symbols indicate the pathways to metal oxides and to high potential acceptors, respectively. Green symbols indicate the gate molecules driving electrons to the cell exterior. Details are given in the text. CM, cytoplasmic membrane; Cyt-c ox, predicted cytochrome oxidase; FrdCAB, fumarate deshydrogenase; MacA, cytoplasmic membrane-bound cytochrome; MQ, menaquinone; OM, outer membrane; Omc, outer-membrane-bound cytochromes B and S partially exposed on the cell surface; PpcA, a tri-heme periplasmic c-type cytochrome; Q, quinone. Potentials are expressed taken against the Ag/AgCl–KCl sat. reference electrode. Those of external reductases were measured by chronopotentiometry in Busalmen et al. [5]. Modified from Di Gristina (2005), Weber (2006) and Lovley [16,17].

signal that triggers the use of a particular electron transport pathway. For this proposal to be true, a benefit must be obtained by bacteria growing on a higher potential acceptor like electrodes poised at 0.6 V. Moreover, it would demand both, the existence of different gates conducting to alternative pathways to external acceptors (Fig. 4) and their appropriate location as related to energy coupling sites in the inner membrane. Unfortunately, the difficulty of obtaining accurate measurements for cell and mass balances using electrode-reducing bacteria does not allow us to confirm this hypothesis yet. In spite of this limitation, it seems reasonable to think that having a wider profile of high potential cytochromes on its surface will allow *Geobacter* to outcompete for the resources of exocelullar acceptors. So, we believe that to extend the survival is a reason powerful enough to justify the energy cost involved in producing a high potential pathway.

As previously pointed out [18], electrode-reducing microorganisms have not evolved to produce electricity, but to use a variety of electron acceptors covering a wide range on potentials. Electrodes on the other hand, has been proposed to mimic the presence of natural electron acceptors if poised at the appropriate potential, thus triggering the specific response to it [8]. Interestingly, in the natural environment of *Geobacter* we can found electron acceptors like vanadate with a redox potential as high as 1.1 V (Ag/AgCl–KCl sat.) that seem to be exocellularly reduced by *Geobacter* [19]. So, the induction of citochrome(s) at about 0.6 V (Figs. 1 and 2) could be interpreted as a vanadate-like response, since vanadate is the only known exocellular electron acceptor reduced by *Geobacter*, with a redox potential high enough to produce this particular cellular response.

Two major differences between mitochondrial and bacterial electron transport chains are that (a) bacterial chains are branched and that (b) many bacteria can alter their chains depending on growth conditions [20]. Indeed, different branches are not equally efficient in generating a potential differences (Δp) or making ATP [20]. Little is known about the branching of the external chain in *Geobacter* or the location of coupling sites, but the available data (including those presented here) allow the recognition of at least three different electron transport pathways of increasing potential (Fig. 4): the reduction of fumarate (−0.18 V Ag/AgCl–KCl sat.) by the fumarate reductase (FrdCAB) [21], the reduction of iron and metal oxides (−0.10 to +0.10 V Ag/AgCl–KCl sat.) through the pathway composed by PpcA, OmcB and probably OmcS (orange pathway in Fig. 4) and finally the reduction of high potential acceptors (≥0.6 V Ag/AgCl–KCl sat.) including vanadates [22] and oxygen [23] (blue pathway in Fig. 4). Interestingly, the cell yield of *G. metallirreducens* growing on vanadium (V) has been shown to be 2.4 times higher than that on iron in the same medium [19], which supports the existence of the more energetic pathway conducting to higher potential exocellular acceptors.

Finally, a novel role for multiheme cytochromes as electron storage has been recently shown in *Geobacter*. The network of hemes would act for accepting electrons from oxidative metabolism in the absence of suitable electron acceptors. Those so-called “iron-lungs” would confer this bacterium the ability of conserve energy for certain time even in the absence of respiratory substrates [9,11]. The production of high potential cytochromes located at the cell surface can clearly increase the electron accepting capacity of the cell beyond the cytoplasm and the membrane, extending the time that bacteria satisfy their maintenance energy requirements till a new electron acceptor resource is found.

4. Concluding remarks

Elucidation of the mechanism(s) by which bacteria can electrically communicate to a solid electrode can deeply impact on present and future biotechnological applications of electrode-reducing bacteria, by leading to the development of novel and more efficient bacteria–electrode interfaces. In this context, it is crucial to identify the molecules involved in the direct electron transport to electrodes. The spectro-electrochemical recognition of cytochromes as the electrode-reductases give an insight into this complex task and provide a unique opportunity to explore the process of electron exchange at the interface. Refined electrochemical and spectro-electrochemical techniques require the use of well-characterized electrode materials as gold or platinum. In this direction, we have shown here that gold is a suitable material for the examination of *Geobacter*, opening the avenue for more detailed studies.

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