Microbial electrocatalysis with *Geobacter sulfurreducens* biofilm on stainless steel cathodes

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Abstract

Stainless steel and graphite electrodes were individually addressed and polarized at −0.60 V vs. Ag/AgCl in reactors filled with a growth medium that contained 25 mM fumarate as the electron acceptor and no electron donor, in order to force the microbial cells to use the electrode as electron source. When the reactor was inoculated with *Geobacter sulfurreducens*, the current increased and stabilized at average values around 0.75 A m^{-2} for graphite and 20.5 A m^{-2} for stainless steel. Cyclic voltammetry performed at the end of the experiment indicated that the reduction started at around −0.30 V vs. Ag/AgCl on stainless steel. Removing the biofilm formed on the electrode surface made the current totally disappear, confirming that the *G.sulfurreducens* biofilm was fully responsible for the electrocatalysis of fumarate reduction. Similar current densities were recorded when the electrodes were polarized after being kept in open circuit for several days. The reasons for the bacteria presence and survival on non-connected stainless steel coupons were discussed. Chronoamperometry experiments performed at different potential values suggested that the biofilm-driven catalysis was controlled by electrochemical kinetics. The high current density obtained, quite close to the redox potential of the fumarate/succinate couple, presents stainless steel as a remarkable material to support biocathodes.

Keywords: *Geobacter sulfurreducens*; Biofilm; Biocathodes; Stainless steel; Microbial fuel cell

1. Introduction

Research on microbial fuel cells (MFCs) has concentrated mainly on two aspects: deciphering the mechanisms of electron transfer from the bacteria to the electrode [1–3] and improving cell design and electrode materials [4]. Most studies have focused on the anode compartment and papers dealing with microbial cathodes are less numerous [5]. Aerobic microbial cathodes have been devised by mimicking the natural processes identified in aerobic biocorrosion. Following this strategy, microorganisms have been utilized to oxidize soluble metal compounds, such as manganese [6] or iron ions [7], and the metal oxides or hydroxides that are formed are subsequently reduced on the cathode. In these cases, the global process is obviously microbially controlled, but the purely electrochemical step remains abiotic. Also derived from biocorrosion studies, marine biofilms have proved to be able to catalyse oxygen reduction on stainless steel cathodes: implementing the biofilm-catalysed cathode in a hydrogen PEM fuel cell resulted in an increase of power density from 1.4 mW m^{-2} with a clean stainless steel cathode to 41 mW m^{-2} with the biofilm-covered cathode [8]. Several anaerobic biocathodes have been proposed, using different compounds as the final electron acceptor, such as nitrate [9–10], sulfate, carbon dioxide [11] or fumarate [9]. Gregory et al. have demonstrated that biofilms formed in pure culture of *Geobacter metallireducens* and *Geobacter sulfurreducens* are able to directly use graphite electrodes polarized at −0.50 V vs. Ag/AgCl as the electron source to achieve the reduction of nitrate to nitrite or fumarate to succinate [9].

Our previous studies devoted to the catalysis of oxygen reduction by marine biofilms have highlighted the efficiency of stainless steel to support electrochemically active biofilms [8]. Are the properties of stainless steels linked to marine biofilms only, or would stainless steels offer effective electrocatalytic properties for other kinds of biocathodes? The aim of the present study was to contribute new elements to the discussion of this issue. The two electrode materials were compared using operating conditions very close to the procedure described previously by Gregory et al. [9]. The electrochemical reactors were filled with a culture medium that contained fumarate as

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electron acceptor but no soluble electron donor. When inoculated with cultures of *Geobacter sulfurreducens*, the microbial species must develop on the cathode surface, the sole source of electrons. The biofilm thus catalysed the electron transfer from the cathode to fumarate, which was reduced to succinate.

2. Experimental

2.1. Media and growth conditions

G. sulfurreducens strain PCA (ATCC 51573) was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). The growth medium contained (per litre): 0.1 g KCl, 1.5 g NH_4Cl, 2.5 g $NaHCO_3$, 0.6 g NaH_2PO_4, 0.82 g acetate, 10 ml vitamin mix (ATCC MD-VS), and 10 ml trace mineral mix (ATCC MD-TMS). The medium was autoclaved and completed with a filtered (0.2 μm) solution of sodium fumarate for a final concentration of 8 g L^{-1}. Incubations were done at 30 °C during 5 days in the growth medium. The number of planktonic cells was evaluated through the absorbance at 620 nm. Absorbance was transformed into cell forming units per millilitre (CFU mL^{-1}) using the calibration formula:

$$[\text{CFU mL}^{-1}] = \text{OD}_{620\,\text{nm}} \times 472\,067$$

established with measurements in Petri dishes under a N_2/CO_2 atmosphere.

The reactor medium was the same as the growth medium except that it lacked sodium acetate and contained sodium fumarate at a final concentration of 4 g L^{-1} (25 mM). The reactor was filled with the reactor medium and flushed with N_2–CO_2 (80–20%) for at least 1 h. Bubbling was maintained at a lower flow rate during the experiments. Cells (5%, v/v) were then injected into the electrochemical reactor when the optical density at 620 nm in the growth medium was around 0.3 (i.e. around 142,000 CFU mL^{-1}).

2.2. Electrodes and electrochemical reactor

The electrochemical reactor contained 2 L reactor medium with 0.5 L headspace. Each electrode was drilled, tapped and had a titanium wire screwed onto it. The electrodes were 1 cm × 2.5 cm × 0.1 cm plates of stainless steel (SS) UNS S31254[1] (Outokumpu) or 5 cm × 2.5 cm × 0.5 cm plates of graphite (Goodfellow). Before the experiments, the stainless steel coupons were cleaned with 50–50% ethanol/acetone to dissolve organic adsorbed species, and then with a 2–20% fluoridric/nitric acid solution to remove the oxide layer. The graphite electrodes were cleaned with 1 N HCl and then 1 N NaOH to remove possible biomass contamination.

One to four working electrodes (graphite or SS) were set up in each reactor and connected to the same auxiliary (platinum grid, 0.5 mm wires) and silver/silver chloride reference electrodes (Ag/AgCl) through a multi-potentiostat (model VMP1 or VMP2, software EC-Lab v.8.3, Bio-Logic, SA). Each working electrode was individually monitored by a N-STAT device (Bio-Logic, SA). The potential of the reference electrode in the reactor medium was around $E_{(\text{Ag/AgCl})} = 0.31$ V vs. SHE (standard hydrogen electrode).

2.3. Microscopy methods

At the end of the experiment, the electrodes were stained with a solution of 0.03% orange acridine (A6014, Sigma) for 10 min, then rinsed with distilled water and air dried. Pictures were taken using a Carl Zeiss Axiotech 100 microscope equipped for epifluorescence with an HBO 50/ac mercury light source and Zeiss 09 filter (excitor AP 450–490, reflector FT 510, barrier filter LP 520) and a monochrome digital camera (Evolution VF). Images were processed with the Image-Pro Plus v.5 software.

The average surface roughness (Ra) of the cleaned electrodes was characterized using a white light interferometer Zygo New View 100 OMP-0348K.

3. Results

3.1. Comparison between graphite and stainless steel electrodes (reactors 1 & 2)

Two stainless steel (SS) and two graphite (GR) electrodes were put in a single electrochemical reactor (reactor 1). The reactor medium was the same as the growth medium, except that it did not contain any electron donor in order to oblige the cells to use the electrodes as the sole possible electron donor. The electron acceptor was fumarate 25 mM. SS1.1 and GR1.1 electrodes were polarized at −0.60 V vs. Ag/AgCl from the beginning of the experiment, while SS1.2 and GR1.2 were initially kept in open circuit and only polarized from day 2.6 (Table 1). No current was detected before bacteria injection, showing that no electrochemical reduction of fumarate occurred in the absence of cells (Fig. 1A). As soon as bacteria were injected, at day 0.6, current densities of 0.006 and 1.35 A m^{-2} were measured on the graphite and stainless steel electrodes, respectively. Current increased rapidly on day 4.8 to maximal values in the range of 0.28 ± 0.05 A m^{-2} for GR1.1 and 14.8 ± 2.5 A m^{-2} for SS1.1, and then stabilized. Electrodes GR1.2 and SS1.2, for which polarization only started 2 days after the injection, immediately gave a current equivalent to that of the electrodes connected from the beginning, with a low current density until day 4.8 and then a fast increase. Fig. 1B presents the cyclic voltammograms recorded at the end of the experiment with the stainless steel electrode SS1.1, which sustained 17.3 A m^{-2}. The reduction process started around −0.30 V vs. Ag/AgCl and gave a current of 5.7 mA (i.e. 22.8 A m^{-2}) at −0.60 V vs. Ag/AgCl. These values were consistent with the values obtained under chronoamperometry. A low oxidation reaction also appeared above −0.30 V vs. Ag/AgCl. The biofilm formed on the electrode surface was then removed by rubbing with wet paper, and the cleaned electrode was put back into the reactor. Cyclic voltammetry was performed again after the reactor had been deoxygenated by N_2/CO_2 bubbling. No redox reaction was observed with the

[1] Composition Cr: 19.9%, Ni: 17.8%, Mo: 6.0%, N: 0.2%, C : 0.01%, Fe: complement.

Table 1
Operating parameters and main results of experiments performed with *G.sulfurreducens* on graphite and stainless steel electrodes in five different reactors NP: Non-polarized

Reactor	Name	Imposed potential (V vs. Ag/AgCl)	Day of polarization	Maximum current density (A m^{-2}) *Abs. value*	Day of maximum current density
	SS1.1	−0.60	0	17.3	
1	SS1.2		2.6	12.3	6.6
	GR1.1		0	0.33	
	GR1.2		2.6	0.23	
	SS2.1	−0.60	0	16.8	
2	SS2.2		4.0	24.2	13.3
	GR2.1		0	0.66	
	GR2.2		4.0	0.83	
	SS3.1	−0.60	0	14.1	11.2
	SS3.2		7.7	15.2	
	SS3.3		11.5	18.2	14.4
3	SS3.4	−0.50	15.1	10.1	–
		−0.40	16.2	5.2	–
		−0.20	20.0	0	
		0.00	20.1	0	–
4	SS4	NP	–	–	–
5	SS5	−0.60	1.7	11.8 ± 2.5	14.2

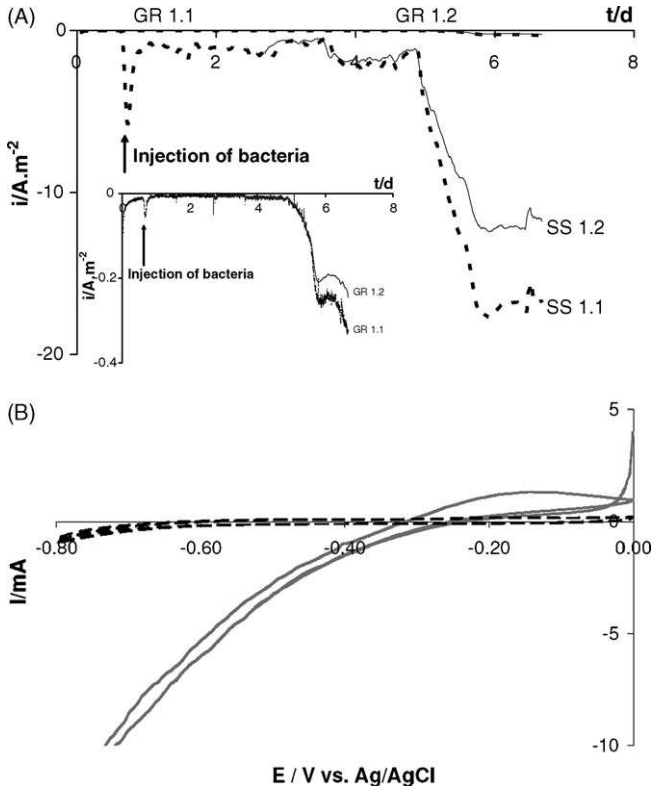

Fig. 1. Graphite (12.5 cm^2) and stainless steel (2.5 cm^2) electrodes exposed to a culture of *G.sulfurreducens* containing fumarate as electron acceptor (25 mM) in reactor 1. (A) Variation of current density with time on electrodes polarized at −0.60 V vs. Ag/AgCl: SS1.1 & GR1.1 from the beginning, SS1.2 & GR1.2 connected from day 2.6. The insert represents a zoom on the current density variation on graphite electrodes. (B) Cyclic voltammograms at 2 mV s^{-1} on stainless steel electrode SS1.1, with a biofilm sustaining 17.3 A m^{-2} (▩▩▩) and after biofilm removal (- - -).

cleaned electrode, confirming that neither the cells present in solution nor the metabolites produced were involved in the reduction process. The biofilm was required for the catalysis of fumarate reduction to occur.

The same variation of the current density with time was confirmed by repeating the chronoamperometry experiment in reactor 2 (Fig. 2A and Table 1). In reactors 1 and 2, whatever the reactor, the maximal current densities on day 7 were very close and in the range of 15.2 ± 2.9 A m^{-2} for SS electrodes and 0.32 ± 0.08 A m^{-2} for graphite, showing good reproducibility of the results. Fig. 2B presents the cyclic voltammograms recorded at day 13.3 on SS2.1 and GR2.1, which sustained 24.2, and 0.83 A m^{-2}, respectively. The reduction process started around −0.30 V vs. Ag/AgCl for stainless steel and −0.40 V vs. Ag/AgCl for graphite. Fig. 3 shows the epifluorescence microscopy pictures that were taken on stainless steel electrodes at the end of this last experiment. The two pictures were representative of the two kinds of morphology observed on the numerous zones examined. Single cells were distinguishable, but bacteria were preponderantly gathered in small (Fig. 3A) or quite large (Fig. 3B) clusters.

To study the influence of the potential, four stainless steel electrodes were placed in reactor 3. Three were polarized at −0.60 V vs. Ag/AgCl: the first electrode from the beginning, the second from day 7.7 and the third from day 11.5. For these three electrodes, the current density stabilized at very similar values in the range of 16.1 ± 2.0 A m^{-2} (Table 1). The fourth electrode was kept in open circuit until day 15.1, after which it was consecutively polarized at −0.50, −0.40, −0.20 and 0.00 V vs. Ag/AgCl (Table 1 and Fig. 4). The current densities stabilized at 10.1 and 5.2 A m^{-2} for −0.50 and −0.40 V vs. Ag/AgCl, respectively. At −0.20 and 0.00 V vs. Ag/AgCl, only residual current was detected (less than 0.003 A m^{-2}). These results were consistent with the cyclic voltammetries (Figs. 1 and 2), which

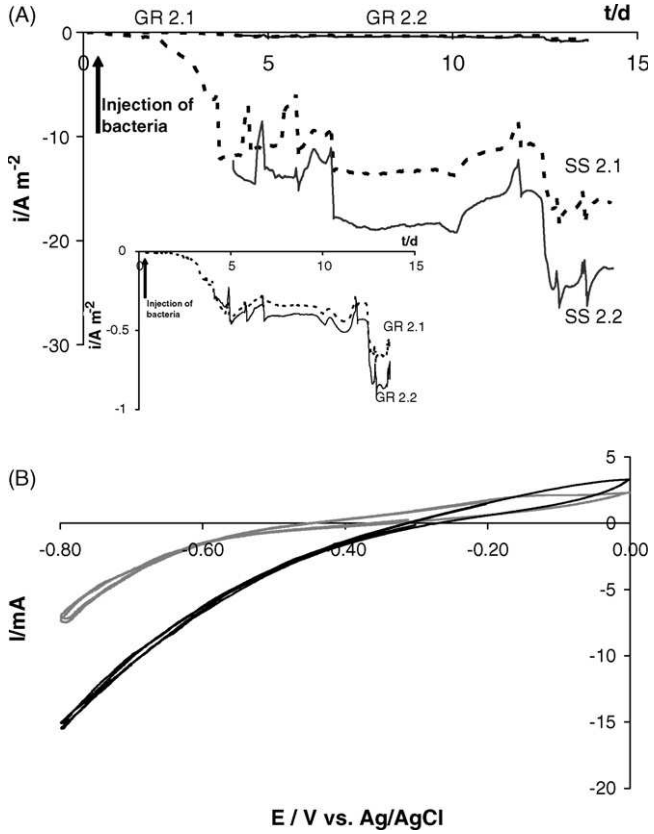

Fig. 2. Graphite (12.5 cm^2) and stainless steel (2.5 cm^2) electrodes exposed to a culture of *G.sulfurreducens* containing fumarate as electron acceptor (25 mM) in reactor 2. (A) Variation of current density with time on electrodes polarized at −0.60 V vs. Ag/AgCl: SS2.1 & GR2.1 from the beginning, SS2.2 & GR2.2 connected from day 4. The insert represents a zoom on the current density variation on graphite electrodes. (B) Cyclic voltammograms at 2 mV s^{-1} on day 13.3 on stainless steel electrode SS2.1(▬) with a biofilm sustaining 24.2 A m^{-2} and on graphite electrode GR1.2 with a biofilm sustaining 0.83 A m^{-2} (▬ ▬).

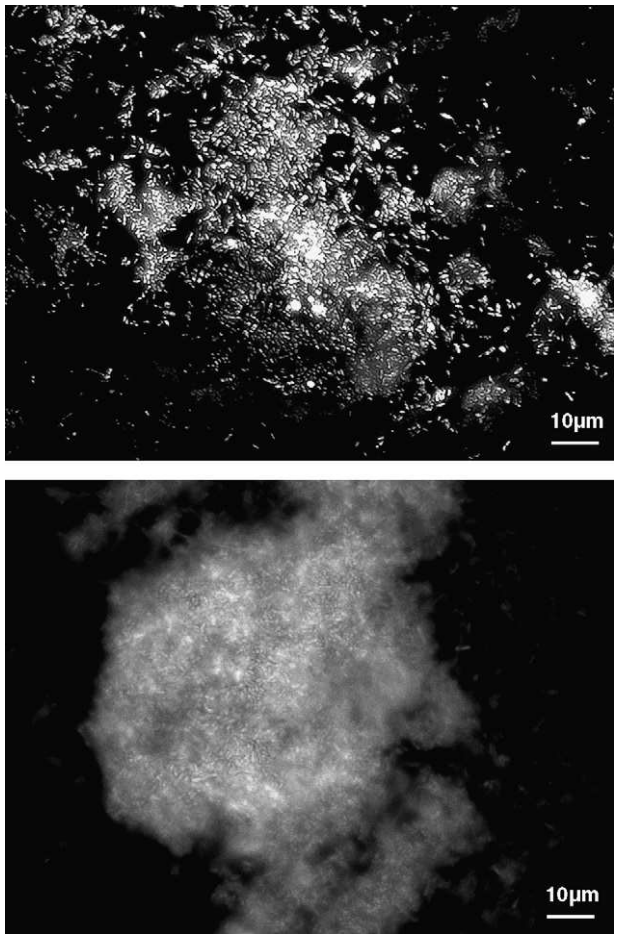

Fig. 3. Epifluorescence microscopy pictures of *G.sulfurreducens* biofilm on stainless steel electrode polarized at −0.60 V vs. Ag/AgCl from reactor 2 (magnification 500×), A: SS2.1; B: SS2.2.

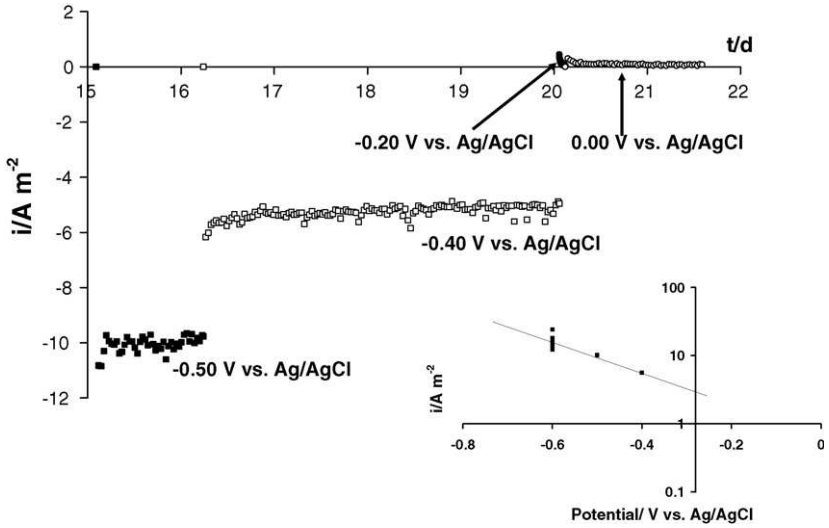

Fig. 4. Variation of the current density on electrode SS3.4 from reactor 3, previously kept in open circuit for 15 days and then consecutively polarized at ■−0.50 V □−0.40 V ●−0.20 V ○ 0.00 V vs. Ag/AgCl. Inset: current density obtained during the different electrolyses performed with stainless steel electrodes (values in Table 1) in logarithmic scale, as a function of potential.

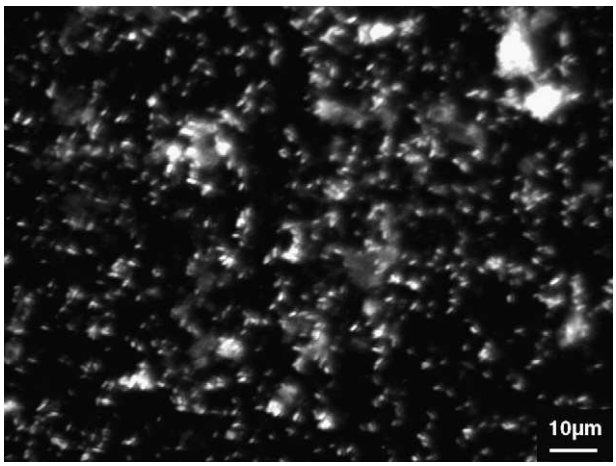

Fig. 5. Epifluorescence microscopy picture of *G.sulfurreducens* biofilm on a non-polarized stainless steel electrode SS4, placed in reactor 4 (magnification 500×).

showed the reduction of fumarate starting on a biofilm-covered stainless steel electrode below −0.30 V vs. Ag/AgCl.

Two other experiments were conducted in parallel, in two different reactors (4 and 5), at the same time and with the same *G.sulfurreducens* culture as the inoculum. Each reactor was equipped with only one stainless steel electrode, which was kept in open circuit in reactor 4 and polarized at −0.60 V vs. Ag/AgCl in reactor 5. Comparing the optical density variation at 620 nm on the solution samples taken from both reactors revealed a basal growth rate that, at day 13, led to around 19 800 CFU mL^{-1} in reactor 4 and 32,500 CFU mL^{-1} in reactor 5. As the electrode in reactor 4 was not polarized, the basal growth could only be due to the presence of acetate remaining from the inoculum that was an electron donor for the planktonic cells. The additional growth in reactor 5 may have been due to bacteria that grew on the surface of the polarized electrode (Fig. 3) and were then detached.

The biofilm observed on the non-connected coupons (Fig. 5) presented clusters that were rather smaller than on the polarized electrode (Fig. 3).

4. Discussion

The current densities reported here with graphite electrodes (maximum values of 0.75 ± 0.08 A m^{-2} in reactor 2) were of the same order of magnitude as, though slightly higher than, the ones reported previously under similar operating conditions [9]. This previous study, which was to our knowledge the first demonstration of the capability of *G.sulfurreducens* to use solid electrodes as electron donors, reported current densities of 0.4 A m^{-2} after successive additions of fumarate (total concentration 40 mM), but at −0.50 V vs. Ag/AgCl instead of the −0.60 V vs. Ag/AgCl used here. The influence of the potential value was clearly demonstrated here by cyclic voltammetry (reactors 1 and 2) and mainly by the successive electrolyses achieved at different potentials (reactor 3).

In the present experiment, the current was stable for more than 2 days at high current densities whereas, in the previous study, it decreased after around 1 day. The ratio between the total electrode surface areas (where the substrate was consumed) and the medium volume (containing the substrate) was around 15 cm^2 L^{-1} here for reactors 1 and 2, whereas it was more than 77 cm^2 L^{-1} in the previous study. This difference in the surface/volume ratios can explain the longer stability of the current in the experiments reported here, due to a slower consumption of the substrate. Assuming, as previously demonstrated [9], the transformation of fumarate to succinate with 2 electrons exchanged:

$$\text{Fumarate} + 2H^+ + 2e^- \rightarrow \text{Succinate}$$

the current was integrated from the beginning to the end of experiments 1 and 2. The charges transferred corresponded to 15% and 65% of the initial amount of fumarate in reactors 1 and 2, respectively.

The stainless steel electrodes gave current densities 25 times higher than graphite on the average. Measurements of the surface roughness gave average roughness (Ra) of 5.6 μm for graphite and 0.29 μm for stainless steel. It is generally agreed that higher values of surface roughness favour bacterial settlement, mainly when the roughness values are of the same order of magnitude than the size of microbial cells. Here the roughness should favour biofilm formation on graphite rather than stainless steel. The higher current density obtained on stainless steel can therefore not been explained by difference in the surface area available for biofilm formation. Moreover, as both electrode types (GR and SS) were set up in the same reactor, this difference can only be attributed to the intrinsic properties of the material. It should be concluded that stainless steel has better electrokinetic properties than graphite to support biofilm-driven reduction reactions.

The reduction of fumarate, which started on stainless steel around −0.30 V vs. Ag/AgCl (0.01 V vs. SHE), is quite close to the redox potential of the fumarate/succinate couple in the same conditions, i.e. −0.28 V vs. Ag/AgCl in the culture medium ($E_0 = 0.033$ V vs. SHE at pH 7.0). This phenomenon was observed on graphite at lower potentials around −0.40 V vs. Ag/AgCl (−0.09 V vs. SHE), confirming the efficiency of the stainless steel/*G.sulfurreducens* system. In the inset of Fig. 4, the current density values obtained with stainless steel at the stable plateau of the electrolyses were plotted as a function of the applied potential in logarithmic scale. A Tafel's approach may be suggested. Placing the *X*-axis at the redox potential of the fumarate/succinate couple (−0.28 V vs. Ag/AgCl) resulted in an exchange current density around 2.6 A m^{-2}. The slope of the curve gave a charge transfer coefficient α around 0.03 (with $n = 2$). This low value of α can be attributed to the presence of the space charge layer due to the metal oxides that constitute the surface of stainless steels. It may be suggested that, in this case, the current supplied by the microbial electro-catalysis looks like it follows a traditional exponential kinetics. The microbial process would thus be fully controlled by the electrochemical conditions.

The low current that was observed during the first few days after inoculation may be attributed to the initial amount of cells that adsorbed on the electrode surface. The subsequent exponential increase was certainly caused by the development of the catalytic biofilm of *G.sulfurreducens* on the electrode surface,

as shown by the microscope pictures. Electrodes polarized late gave current as soon as they were connected. As the reactor medium did not contain any electron donors, it is surprising that bacteria managed to survive without being in contact with a polarized electrode. Three hypotheses can be advanced. First, a basal growth occurred thanks to the acetate (electron donor in the growth medium) that remained in the inoculum volume, as demonstrated in reactor 4. Secondly, the succinate resulting from the fumarate reduction on the polarized electrodes may have been used as an electron donor by the bacteria in suspension and the ones adhering to non-connected coupons. A basic current due to the oxidation of succinate has already been brought to light for a medium poor in acetate [12]. Thirdly, cells may have been released from the biofilm formed on the polarized electrodes into the solution and then have colonized the surface of the non-connected electrodes. These two last phenomena should explain the higher concentration of free cells observed in reactor 5, which contained a polarized electrode, than in reactor 4, which only contained a non-connected coupon. Nevertheless, it must be recalled that, as already demonstrated for graphite electrodes [9], the biofilm was responsible for whole current produced by the catalysis of fumarate reduction, as removing the biofilm from the electrode surface and putting the cleaned electrode back into the reactor made the current disappear completely.

Extracellular electron transfer like what occurred at the interface bacteria/electrode has been widely studied in microorganisms that transfer electron to insoluble Fe(III) or Mn(IV) [13–15]. Outer membrane cytochrome (Omc) within the *c*-type family (particularly the *c*-type cytochrome OmcS) or outer membrane protein (Omp) were proven to be involved in the reduction of metals [16–17]. To our knowledge, no investigation has been carried out on the midpoint redox potential of OmcS. However, information was available on the midpoint potential of a periplasmic and extracellular cytochrome *c* involved in Fe(III) reduction, which was found to be around −0.167 V vs. SHE [18]. The studies on cytochromes of microorganisms grown on electrode as electron donor, like it is the case in our study, are rarer. The mechanisms of such bacterial driven transfer from a cathode have not been yet elucidated. The positive potential value (+0.01 V vs. SHE), observed on stainless steel in this study, is far from the low redox potentials of cytochrome *c* previously reported which suggested that cytochromes involved here may be different. Indeed, the genome of *G.sulfurreducens* contains 111 genes for *c*-type cytochromes, substantially more than what was found in other organisms whose sequence is available, including the intensively studied Fe(III) reducer *Shewanella oneidensis* [16].

This study confirmed that stainless steel has excellent electrokinetic properties to support biofilm-driven reductions. Such a capacity has already been observed with marine natural biofilms that catalysed efficiently oxygen reduction from potential values around +0.3 V/SCE [8]. Obviously, the fumarate/succinate couple has a too high redox potential value to be used for the cathode reaction of fuel cells. It was chosen here only has a model reaction with the objective to compare the electrochemical properties of different electrode materials. As this microbial system has been demonstrated to be fully mastered in a previous study [9], and it revealed here high differences with respect to the electrode material, it may become a kind of standard model. Moreover, the values of potential checked here may have direct application in bioremediation processes. *G.sulfurreducens* biofilm have demonstrated to catalyse efficiently the reduction of the soluble uranium (VI) to uranium (IV) that precipitated, at potential values of −0.50 V vs. Ag/AgCl [19]. It may be suspected that the current around 0.4 A m^{-2} that have been reached on graphite may be significantly increased on stainless steel. Similarly, *G.metalliruducens*, which is close to *G.sulfurreducens,* has been demonstrated to be effective in the electrocatalysis of nitrate to nitrite [9]. It might be hoped that using stainless steel cathode may improve the process effectiveness.

5. Conclusions

A large number of recent studies have made remarkably fast advances concerning the anodes of microbial fuel cells but only a few papers have dealt with biocathodes. Designing new materials or devising new microbial systems for cathodes remains an open challenge in the field of fuel cells or other biofilm-driven processes such as electrochemically assisted bioremediation. Stainless steel was tested here following a procedure already described in the literature, based on the catalysis of fumarate reduction by biofilms formed in pure culture of *G.sulfurreducens*. The current densities obtained were 25 times higher on average than those provided by graphite in the same conditions. Maximal current densities higher than 20 A m^{-2} were reached, and the reduction started at potential values quite close to the redox thermodynamic potential. Stainless steel is an industrial material with good mechanical properties, available in a wide variety of forms (grids, meshes, expanded material. . .), which can be easily packed to increase the active surface area, and would allow easy scaling up to large-scale pilots. Moreover, the potential values used in cathode processes protect the stainless steel against corrosion, so that quite cheap types of stainless steels can be used as electrode. Stainless steels should now be considered as very promising materials to support biofilm-catalysed cathodes.

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