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Electrochemical reduction of oxygen catalyzed by a wide range of bacteria including Gram-positive

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A B S T R A C T

Most bacteria known to be electrochemically active have been harvested in the anodic compartments of microbial fuel cells (MFCs) and are able to use electrodes as electron acceptors. The reverse phenomenon, i.e. using solid electrodes as electron donors, is not so widely studied. To our knowledge, most of the electrochemically active bacteria are Gram-negative. The present study implements a transitory electrochemical technique (cyclic voltammetry) to study the microbial catalysis of the electrochemical reduction of oxygen. It is demonstrated that a wide range of aerobic and facultative anaerobic bacteria are able to catalyze oxygen reduction. Among these electroactive bacteria, several were Gram-positive. The transfer of electrons was direct since no activity was obtained with the filtrate. These findings, showing a widespread property among bacteria including Gram-positive ones, open new and interesting routes in the field of electroactive bacteria research.

1. Introduction

Electroactive bacteria are able to exchange electrons with conducting materials [1]. Therefore, these bacteria can produce current in microbial fuel cells by converting chemical energy from organic substrates into electrical energy.

Today, various bacteria have already been shown to be electroactive, to exchange electrons directly (without the addition of any exogenous mediators) with electrodes [2]. *Geobacter sulfurreducens* is certainly the most thoroughly studied of these microorganisms [3–5]. *Shewanella putrefaciens* also presents this characteristic [6], as do many others like *Pseudomonas aeruginosa* [7], *Escherichia coli* [8], etc. All these microorganisms were harvested in environmental backgrounds like compost, sea water, and sea sediments. The mechanisms of their electron transfers have been widely studied. Several bacteria use their own mediators [8–10], others are able to transfer electrons to the electrodes by direct contact via membrane-bound redox compounds [11] or via conducting nanowires produced by the cell [12,13].

Most of the research on electroactive bacteria has been done on Gram-negative bacteria [2]. The idea that Gram-positive bacteria can also exchange electrons with electrodes is still debated. Gram-positive bacteria possess a cell wall that could potentially reduce electron exchange by direct contact. However, a few recent

studies have shown the electroactivity of Gram-positive bacteria. The vast majority of them describe indirect transfer of electrons between the electrode and the cells. It has been shown, for instance, that *Brevibacillus* sp., is able to use metabolites produced by a Gram-negative bacteria, *Pseudomonas* sp., to achieve extracellular electron transfer [14], *Desulfitobacterium hafniense* is able to use humic acids as electron shuttles [15], and *Bacillus subtilis* and *Corynebacterium* sp. use their own mediators to reduce the electrode [16,17]. To our knowledge, only two genus of Gram-positive bacteria have already been shown to exchange electrons directly with electrodes: *Thermincola* [18] and *Clostridium* [19,20].

These various studies have been performed for anodic processes except the one done on *Clostridium isatidis* that showed a reversible electroactivity of the strain on graphite electrodes [20]. Not so many bacteria are known to be able to perform the opposite process, i.e. to microbially catalyze cathodic reductions. However, microbial catalysis of oxygen reduction remains an important challenge in the fields of biocorrosion [21] and MFC cathodes [22]. *Enterobacter* sp. E1, isolated from an electroactive biofilm formed in compost, was found to be able to catalyze the electrochemical reduction of oxygen when adsorbed on a carbon electrode [23]. This study used cyclic voltammetry to detect the catalytic effect, which implied a prolonged contact between bacteria and the working electrode. To our knowledge, only Gram-negative bacteria have been shown to be able to catalyze the electrochemical reduction of oxygen.

The purpose of the present work was to test a wide range of aerobes and facultative anaerobes, including Gram-positive bacteria,

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Table 1
Electroactivity of Gram-positive and Gram-negative bacteria tested by cyclic voltammetry. CVs were performed in phosphate buffer (0.1 M, pH 7) on 0.07 cm² glassy carbon rods (Glassy carbon V25, 3 × 150 mm, Carbone Lorraine, Gennevilliers, France) at 100 mV/s. The oxygen reduction started at the potential E_{start} and reached its maximum of current (I_{peak}) at E_{peak} . These three parameters, E_{start} , I_{peak} , and E_{peak} , characterized the catalyzed oxygen reduction. The detection time corresponded to the contact duration between electrodes and bacteria. Average values and standard deviations were calculated from three independent experiments for each strain.

Bacteria	Reference	Phenotype Cat, catalase Ox, oxidase	Catalyzed oxygen reduction peak			
			Detection time	E_{start} (V/SCE)	I_{peak} (μA)	E_{peak} (V/SCE)
<i>Pseudomonas aeruginosa</i>	PA01	Gram–Cat+Ox+	1 h	–0.19 ± 0.03	–11.71 ± 0.19	–0.45 ± 0.05
<i>Pseudomonas fluorescens</i>	Clinical isolate	Gram–Cat+Ox+	1 h	–0.18 ± 0.01	–10.01 ± 0.08	–0.45 ± 0.01
<i>Brevundimonas diminuta</i>	CIP 103020	Gram–Cat+Ox+	1 h	–0.13 ± 0.02	–14.21 ± 3.97	–0.33 ± 0.01
<i>Burkholderia cepacia</i>	CIP 80.24	Gram–Cat+Ox+	1 h	–0.14 ± 0.03	–17.14 ± 2.75	–0.36 ± 0.05
<i>Branhamella catarrhalis</i>	CIP 73.21	Gram–Cat+Ox+	1 h	–0.18 ± 0.01	–13.43 ± 0.13	–0.44 ± 0.00
<i>Enterobacter cloacae</i>	Clinical isolate	Gram–Cat+Ox–	6 h	–0.19 ± 0.01	–10.33 ± 1.02	–0.48 ± 0.01
<i>Escherichia coli</i>	K 12	Gram–Cat+Ox–	3 h	–0.21 ± 0.02	–10.25 ± 1.37	–0.49 ± 0.01
<i>Shigella flexneri</i>	CIP 82.48	Gram–Cat+Ox–	3 h	–0.21 ± 0.01	–11.54 ± 0.71	–0.51 ± 0.00
<i>Acinetobacter</i> sp.	Clinical isolate	Gram–Cat+Ox–	1 h	–0.16 ± 0.01	–10.14 ± 0.68	–0.41 ± 0.05
<i>Kingella kingae</i>	CIP 80.16	Gram–Cat–Ox–	3 h	–0.22 ± 0.01	–9.59 ± 0.99	–0.49 ± 0.01
<i>Kingella denitrificans</i>	CIP 103473	Gram–Cat–Ox–	1 h	–0.21 ± 0.01	–12.05 ± 0.21	–0.49 ± 0.01
<i>Micrococcus luteus</i>	CIP 53.45	Gram+Cat+Ox+	1 h	–0.19 ± 0.01	–13.48 ± 0.21	–0.46 ± 0.03
<i>Bacillus subtilis</i>	CIP 52.62	Gram+Cat+Ox+	1 h	–0.18 ± 0.01	–11.57 ± 0.81	–0.49 ± 0.02
<i>Staphylococcus carnosus</i>	CIP 103274	Gram+Cat+Ox–	1 h	–0.22 ± 0.01	–9.54 ± 0.49	–0.52 ± 0.00
<i>Staphylococcus aureus</i>	CIP 4.83	Gram+Cat+Ox–	No catalysis ^a			
<i>Staphylococcus epidermidis</i>	CIP 68.21	Gram+Cat+Ox–	No catalysis ^a			
<i>Enterococcus faecalis</i>	Clinical isolate	Gram+Cat–Ox–	No catalysis ^a			
<i>Enterococcus hirae</i>	CIP 58.55	Gram+Cat–Ox–	No catalysis ^a			
<i>Lactobacillus farciminis</i>	CIP 103136	Gram+Cat–Ox–	No catalysis ^a			
<i>Streptococcus mutans</i>	CIP 103220	Gram+Cat–Ox–	No catalysis ^a			

^a No catalysis was observed even after a 24-h immersion of the electrode in the cell suspension.

for their possible ability to catalyze oxygen reduction on a carbon electrode. Cyclic voltammetry was used as a fast method to test many different strains in order to determine whether such electroactivity may be widespread among aerobic bacteria or correspond to a particular distribution.

2. Experimental

2.1. Bacterial strains, culture conditions and chemicals

All the reference strains were provided by Institut Pasteur (Paris, France). Clinical isolates were obtained from the Hospital Bacteriology and Hygiene Laboratory in Toulouse (France). The strains are described in Table 1. They were maintained on Trypcase Soy agar (Biomérieux, France) under aerobic conditions except for *S. mutans* and *Kingella* sp., which were maintained on Columbia blood agar (Biomérieux, France), and *L. farciminis*, on M.R.S. agar (AES, France). Before each experiment, the strains were grown overnight in 20 mL Trypcase Soy broth (Biomérieux, France) at 37 °C under gentle stirring except for *S. mutans* and *L. farciminis*, which were not stirred. Bacterial suspensions were then centrifuged (10 min, room temperature, 3000g), washed twice in phosphate buffer (K₂HPO₄/KH₂PO₄, 0.1 M, pH 7), and re-suspended in the same buffer. All experiments were performed in the same buffer at room temperature (22 ± 2 °C).

Catalase and oxidase characterization were performed using the ID color catalase test (Biomérieux, France) and oxidase reagent test (Biomérieux, France).

2.2. Cyclic voltammetry (CV)

CVs were performed with 0.07 cm² glassy carbon rods (Glassy Carbon V25, 3 × 150 mm, Carbone Lorraine, Gennevilliers, France) used as working electrodes and with a platinum wire used as a counter electrode. Potentials were monitored with respect to a saturated calomel reference electrode (SCE) and CVs were performed at a scan rate of 100 mV/s with a Princeton Applied potentiostat. Voltammograms started at 0.1 V/SCE to 0.7 V/SCE and continued

with 3 cycles from 0.7 V/SCE to –1 V/SCE. In the figure, only the first cycle is shown, to make the curves clearer.

Experiments were conducted in a 40 mL cell. A first CV was run in 20 mL buffer to control the quality of the clean working electrode surface. The cell suspension was added to obtain a cell density of around 10⁸ bacteria/mL measured by the optical density at 640 nm. A second voltammogram was performed immediately after the addition of the microorganisms into the cell. Then the suspension was stirred for 1 h or more, depending on the strains (Table 1), to maintain a homogeneous oxygen concentration and CV was performed again. The filtrate of *M. luteus* was also tested. After 1 h of stirring, 20 mL of bacterial suspension was filtered through a 0.45 μm sterile filter then through a 0.20 μm one. The CV test was done on the resulting filtrate immediately and after one hour of stirring. Measurements under anaerobic conditions were carried out after removing oxygen from the solution by 20 min of nitrogen bubbling. There was no stirring or gas bubbling during current recording. Each CV experiment was performed three times, with clean working electrodes each time.

3. Results and discussion

The control voltammograms performed on phosphate buffer alone (Fig. 1A) showed a reproducible wave starting at –0.37 ± 0.01 V/SCE (seven independent experiments). Initial CVs were strictly identical to the CVs recorded after 1 h of stirring (Fig. 1A, lines a and b). This wave corresponded to the electrochemical reduction of dissolved oxygen since it disappeared after oxygen was removed by nitrogen bubbling (Fig. 1A, line c).

After 1 h of stirring in presence of *Micrococcus luteus*, the wave corresponding to the electrochemical reduction of oxygen started at higher potential and higher currents were obtained (Fig. 1B, line b). The oxygen reduction started at the potential E_{start} = –0.19 ± 0.01 V/SCE and reached its maximum of current at E_{peak} = –0.46 ± 0.03 V/SCE with I_{peak} = –13.48 ± 0.21 μA (average values of three independent experiments). These three parameters, E_{start} , I_{peak} , and E_{peak} , characterized the oxygen reduction reaction. Occurrence of a catalytic effect was identified by a shift of E_{start}

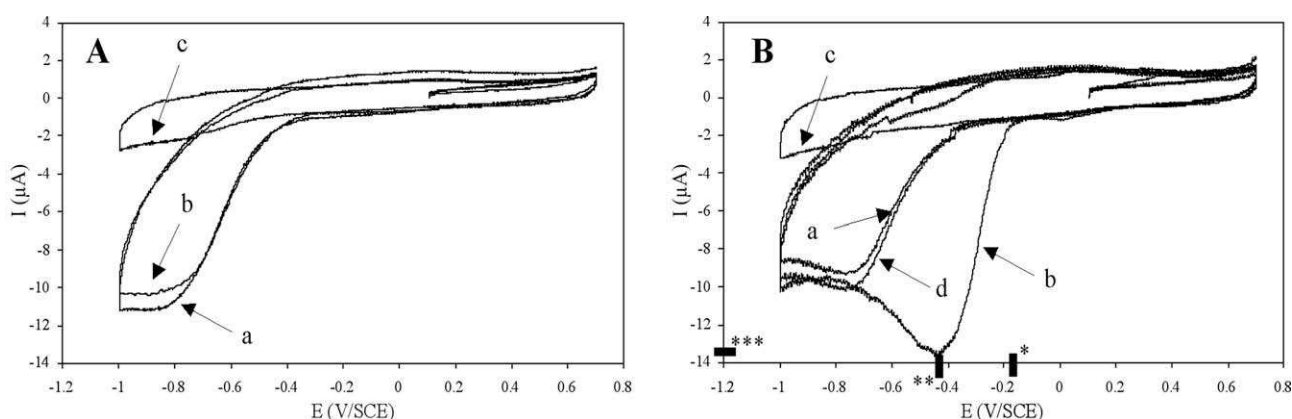


Fig. 1. Cyclic voltammograms of (A) phosphate buffer alone and (B) *M. luteus* in phosphate buffer and *M. luteus* filtrate. Initial CV (line a), after 1 h of gentle stirring (line b) and after oxygen removal by nitrogen bubbling (line c). Line d represents the voltammogram for *M. luteus* filtrate after 1 h of stirring. Solid lines on axes represent the characteristic values of the catalyzed peak: * E_{start} , ** E_{peak} , and *** I_{peak} .

towards positive potential and/or an increase in I_{peak} with respect to the control experiment (Fig. 1A). I_{peak} was four times the intensity obtained with the control at the same potential. After 1 h of stirring in presence of bacteria and 20 min of nitrogen bubbling, the CV was identical to the one obtained in phosphate buffer alone after nitrogen bubbling (Fig. 1B, line c). After one hour of stirring, the bacteria were filtered out of the suspension, and clean electrodes were plunged into the filtrate. The voltammogram did not reveal any catalysis of oxygen reduction for the filtrate, even after one hour of stirring (Fig. 1B, line d). Consequently, no soluble mediator was involved in the electron transfer pathway. This is the first time to our knowledge that a Gram-positive bacterium has been shown to be electroactive in cathodic reactions in presence of oxygen with neither addition of exogenous mediators nor involvement of an endogenous mediator.

Nineteen other bacteria were tested using the same protocol (Table 1). Each strain was tested 3 times independently. All the Gram-negative strains tested were able to catalyze the electrochemical reduction of oxygen and one-third of the tested Gram-positive bacteria were electroactive. The electrocatalytic capacity does not seem to be related to the genus since one of the three tested species of *Staphylococcus* was able to catalyze the reduction of oxygen, while two were not (Table 1). Statistical analyses were carried out on the data that characterized the catalyzed peak: I_{peak} , E_{peak} , and E_{start} using the Student's *t* test. Fluctuations were observed but no significant difference appeared among any of the positive strains. This indicates that the catalysis observed with all the bacteria was probably due to the same kind of mechanism. This phenomenon seems widespread among bacteria.

Using the filtrate from *M. luteus* (Fig. 1B) demonstrated that the catalytic mechanism did not involve diffusible mediators. Thus, membrane-bound compounds may be suspected of being involved. The electrochemistry of several enzymes has already been studied and, among them, catalase adsorbed on glassy carbon electrodes gave an oxygen reduction peak very close to the one observed in the present study [24]. However, *Kingella* species that did not present any catalase also induced a catalytic effect, showing that the phenomenon cannot be attributed to the presence of catalase or, at least, not only to the presence of catalase. No correlation was observed with oxidase activity either. Catalase and oxidase do not seem necessary for this catalysis.

Each aerobic strain tested was able to catalyze the electrochemical reduction of oxygen. Among the facultative anaerobes tested, Gram-negative bacteria, especially Enterobacteriaceae, were electroactive. A delay in the catalysis of oxygen reduction was noted for *E. coli*, *E. cloacae*, and *S. flexneri*, for which a contact time greater

than 1 h was required before a significant catalytic effect was observed (Table 1). The majority of the active strains were ubiquitous and possessed a large capacity of adaptation. The bacteria that did not reveal activity were Gram-positive facultative anaerobic or aerotolerant strains, some of them having higher cultural requirements (*S. mutans* and *L. farciminis*). The difficulty in detecting these bacteria might simply be due to the fact that the assay conditions impaired their physiological activity. However, CFU counting showed that there was no bacterial death after 1 h in this buffer (data not shown).

4. Conclusion

For the first time, *M. luteus*, a Gram-positive bacterium, has been shown to be able to catalyze the electrochemical reduction of oxygen on a carbon electrode. The electron transfer was not supported by exogenous or endogenous mediators. A wide range of aerobic and facultative anaerobic bacteria, including several Gram-positive bacteria presented the same electrochemical property. Catalase and oxidase, which have already been shown to affect oxygen reduction in similar ways, were not responsible for the phenomenon observed here. However, we still suspect membrane-bound redox compounds. The present work shows that the electrochemical property of bacteria is not restricted to species harvested in MFCs. All bacteria, including Gram-positive ones, should now be considered as potentially electroactive.

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