

Preferential Use of an Anode as an Electron Acceptor by an Acidophilic Bacterium in the Presence of Oxygen[∇]

Moustafa Malki,^{1,2} Antonio L. De Lacey,^{1*} Nuria Rodríguez,³
Ricardo Amils,^{2,3} and Victor M. Fernández¹

Instituto de Catálisis, CSIC, Madrid, Spain¹; Centro de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain²; and Centro de Astrobiología, CSIC-INTA, Torrejón de Ardoz, Spain³

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Several anaerobic metal-reducing bacteria have been shown to be able to donate electrons directly to an electrode. This property is of great interest for microbial fuel cell development. To date, microbial fuel cell design requires avoiding O₂ diffusion from the cathodic compartment to the sensitive anodic compartment. Here, we show that *Acidiphilium* sp. strain 3.2 Sup 5 cells that were isolated from an extreme acidic environment are able to colonize graphite felt electrodes. These bacterial electrodes were able to produce high-density electrocatalytic currents, up to 3 A/m² at a poised potential of +0.15 V (compared to the value for the reference standard calomel electrode) in the absence of redox mediators, by oxidizing glucose even at saturating air concentrations and very low pHs.

Río Tinto, a river located in the Iberian Pyritic Belt (Spain), is an unusual ecosystem with a rather constant acidic pH (mean pH of 2.3), a high concentration of heavy metals (Fe, Cu, Zn, As, Mn, Cr, etc.), and a high level of microbial diversity, mainly eukaryotic (22, 23, 37). One important characteristic of Río Tinto is the high concentration of ferric iron and sulfates found in its waters, which are the products of the biooxidation of pyrite, the main mineral component of the system. Ferric iron is maintained in solution due to the acidic pH of the river and is responsible for the constant pH due to the buffer characteristics of this cation according to the following equilibrium: $\text{Fe}^{3+} + 3\text{H}_2\text{O} \rightleftharpoons \text{Fe}(\text{OH})_3 + 3\text{H}^+$.

The combined use of conventional microbial ecology methods and molecular ecology techniques has confirmed that 80% of water column prokaryotic diversity corresponds to three bacterial groups, *Leptospirillum* spp., *Acidithiobacillus ferrooxidans*, and *Acidiphilium* spp., with all of them being conspicuous members of the iron cycle (11). All *Leptospirillum* isolates from the Río Tinto are aerobic iron oxidizers. *A. ferrooxidans* can oxidize iron aerobically and reduce it anaerobically. All *Acidiphilium* isolates can use ferric iron as an electron acceptor and can use reduced organic compounds as electron donors. Although some other microorganisms able to oxidize (i.e., *Ferroplasma* spp.) or reduce iron (i.e., “*Ferrimicrobium*” spp.) have been detected in the River Tinto ecosystem, their low numbers suggest that they play a minor role in the function of this cycle, at least in the water column (26).

Members of the *Acidiphilium* genus are acidophilic, gram-negative, rod-shaped bacteria that originally were reported as obligate aerobes (13). However, more recent research has identified a significant number of *Acidiphilium* and *Acidiphilium*-related bacteria that carry out a dissimilatory reduction of

ferric iron under anoxic as well as microaerophilic conditions (15, 29).

Several metal-reducing bacteria have been shown to be able to donate electrons directly to the electrode without the need to add redox mediators to the solution (5, 8, 16). This functional property is of great interest for developing microbial fuel cells (25, 21). In this work, we studied the interaction of *Acidiphilium* cells with carbon electrodes and the influence of O₂ in the electrocatalytic properties of the bacterial electrodes.

MATERIALS AND METHODS

Isolation and growth of *Acidiphilium* spp. Samples were collected from the Tinto River in 1-liter bottles and kept on ice until they were inoculated. The solid medium that was used to isolate *Acidiphilium* contained the following components (per liter): 0.25 g K₂HPO₄, 0.25 g MgSO₄ · 7H₂O, 0.1 g KCl, 1 g glucose, 1 g yeast extract, and 1% agar, with the pH adjusted to 4.6 with 1 N H₂SO₄ before sterilization. Colonies were inoculated in liquid medium in aerobic conditions at 30°C in a chemically defined medium as reported by Hiraishi and Kitamura (13). The pH was adjusted to 2.5 with 1 N H₂SO₄ prior to sterilization at 120°C and 0.5 atm for 30 min.

Acidiphilium sp. strain 3.2 Sup 5 has been deposited in the Spanish Type Culture Collection (CECT 7285).

Acidiphilium SJH cells were kindly supplied by Barry Johnson.

Ferric iron reduction. Ferric iron reduction was measured in liquid medium containing 50 mM ferric sulfate, 10 mM glucose, and 0.025% tryptone soya broth-basal salts, pH 2.0 (Fe-TSB). Inoculated cultures were incubated (120 ml of medium in 250-ml Erlenmeyer flasks that were shaken at 120 rpm) under aerobic or anaerobic conditions (in universal tubes completely filled with pure N₂ and CO₂ and capped with gas-tight stoppers). Ferrous iron concentrations were measured as reported previously (27).

Total DNA extraction and phylogenetic analysis. DNA was extracted from cells using the FastDNA spin kit for soil according to the instructions supplied by the manufacturer. Bacterial 16S rRNA gene fragments were amplified by PCR using bacterial primers 341F and 907RM.

Sequences of 16S rRNA genes initially were compared to reference sequences contained in the EMBL nucleotide sequence database by using the BLAST program and were subsequently aligned with 16S rRNA reference sequences in the ARB package (<http://www.mikro.biologie.tu-muenchen.de>). A 16S rRNA gene sequence phylogenetic tree was obtained by using the DNAPARS parsimony tool included in the ARB software.

Electrochemical measurements. A sterilized electrochemical glass cell from Radiometer that had been thermostated at 30°C was used. The working electrodes were graphite felt (9-μm fiber diameter; 3,500 cm² g⁻¹ surface area; RVG

* Corresponding author. Mailing address: Instituto de Catálisis, CSIC, c/Marie Curie 2, Cantoblanco, 28049 Madrid, Spain. Phone: 34-915854928. Fax: 34-915854760. E-mail: alopez@icp.csic.es.

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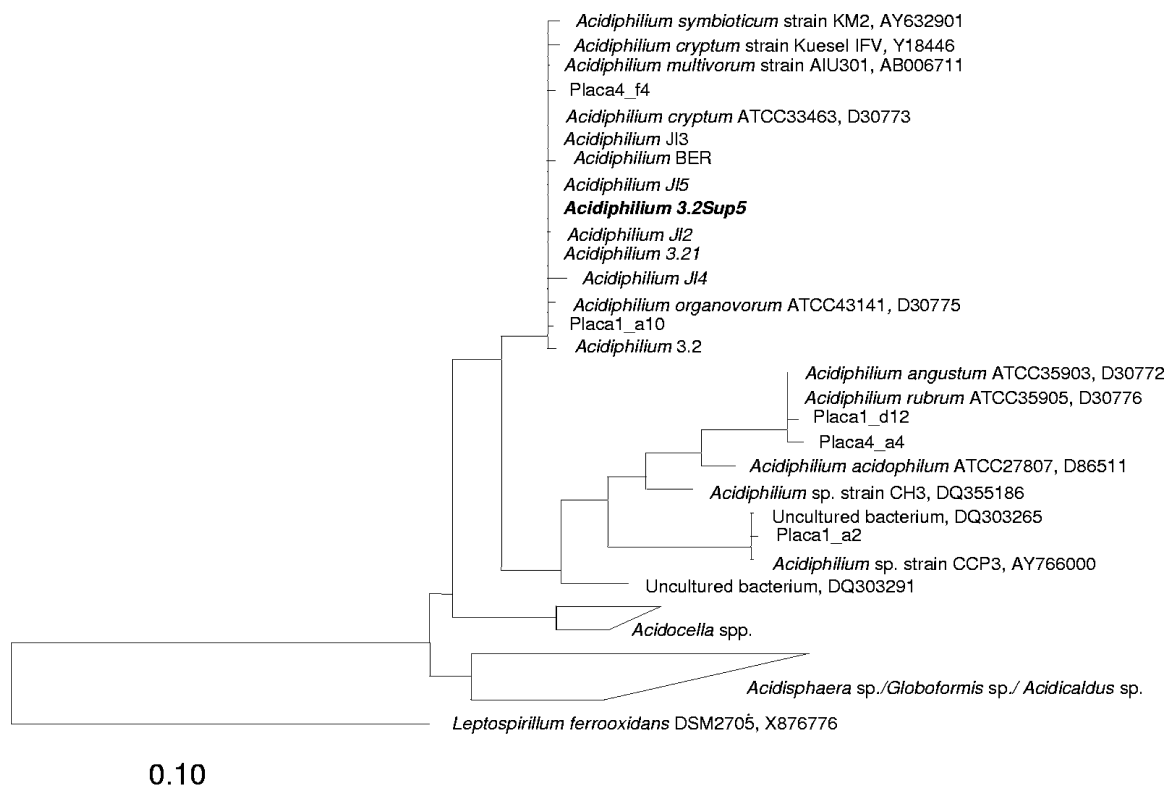


FIG. 1. Neighbor-joining phylogenetic tree based on almost-complete 16S rRNA gene sequences of *Acidiphilium* isolates from Río Tinto and *Acidiphilium* reference species. The strain used in this work, *Acidiphilium* sp. strain 3.2 Sup 5, is in boldface.

4000; Le Carbon Lorraine) disks of approximately 13 mm in diameter and 10 mm in thickness (6.5 cm² total geometric area). Before use, the disks were cleaned in 2 N H₂SO₄ and then sterilized at 120°C and 1 atm for 30 min. A standard calomel electrode (SCE) was used as the reference electrode, and the counter electrode was a platinum electrode; both were supplied by Radiometer. The electrochemical cell contained a pH 2.5 solution of 2.0 g/liter (NH₄)₂SO₄, 0.1 g/liter KCl, 0.25 g/liter K₂HPO₄, 0.25 g/liter MgSO₄ · 7H₂O, and 0.01 g/liter Ca(NO₃)₂. Glucose was added to a final concentration of 10 mM, *Acidiphilium* sp. was inoculated into the electrochemical cell, and the solution was stirred. Air was diffused into the cell through a permeable membrane. The oxygen concentration in the electrochemical cell was determined with a Syland Simplair F-15 oxymeter (Syland Scientific GmbH). Cyclic voltammetry experiments were performed with an Autolab PGSTAT 30 potentiostat (Eco-Chemie) at 50 mV/s in an air atmosphere, except when purging with N₂ was indicated.

Electron microscopy. The scanning electron microscopy (SEM) of modified electrodes was done according to a protocol described previously (1) with a JEOL-5600LV scanning electron microscope.

RESULTS

Isolation, identification, and phylogenetic analysis of *Acidiphilium* sp. strain 3.2 Sup 5. Different *Acidiphilium* isolates from Río Tinto were grown in the conditions described in Materials and Methods. Their purities were checked by pulsed-field gel electrophoresis and characterized by comparisons of 16S rRNA gene sequences (Fig. 1). The different species that belong to the *Acidiphilium* genus cluster in two groups. The sequence obtained from strain 3.2 Sup 5, the strain used in this work, clustered with those of “*Acidiphilium symbioticum*,” *Acidiphilium cryptum*, *Acidiphilium multivorum*, and *Acidiphilium organovorum*.

Ferric iron reduction. The ability of the *Acidiphilium* sp. strain 3.2 Sup 5 culture to reduce ferric iron using glucose as the energy source was tested with different concentrations of O₂ in the medium. The isolated culture was able to reduce a significant amount of ferric ion under all conditions, although under anaerobic conditions the rate was significantly slower (Fig. 2). These results are in agreement with those of previous reports (15, 29).

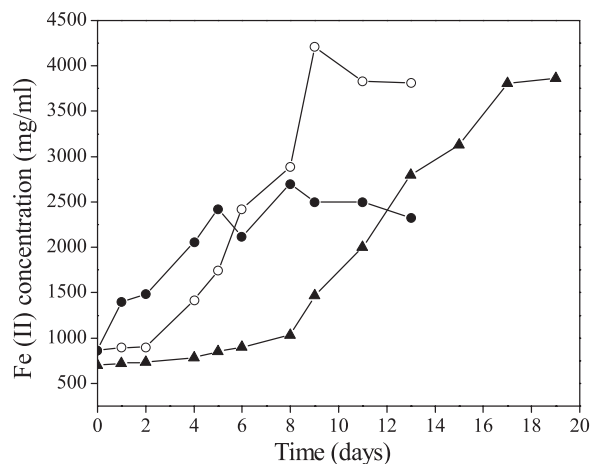


FIG. 2. Reduction of ferric ions by *Acidiphilium* sp. strain 3.2 Sup 5 cells incubated under aerobic (closed circles), microaerobic (open circles), and anaerobic conditions (closed triangles).

Growth of *Acidiphilium* spp. on graphite felt electrodes. The electrochemical cell containing 10 mM glucose as the energy source was inoculated with *Acidiphilium* sp. strain 3.2 Sup 5 culture. Additional glucose was added at intervals to keep its initial concentration constant. After 6 days of inoculation at 30°C and aerobic conditions, the number of cells in the solution had increased considerably: 4×10^9 cells ml⁻¹ were measured by microscopy observation and the counting of cells stained by 4',6'-diaminophenylindol. In parallel, cyclic voltammetry experiments were done in order to detect the electrocatalytic oxidation of glucose at the graphite felt electrode without purging the solution with N₂. Before the addition of the bacterial culture, only background current was observed in the cyclic voltammetry, because glucose does not oxidize directly on electrodes. The electrocatalytic oxidation of glucose was gradually detected after the inoculation of *Acidiphilium* sp. strain 3.2 Sup 5 in the electrochemical cells under aerobic conditions (Fig. 3A). This electrocatalytic effect is shown in the cyclic voltammograms (Fig. 3) as a positive current that begins at approximately 0 V and increases as the potential was scanned toward higher redox potentials, whereas in the reverse scan no negative currents due to a reduction process are observed. The formal redox potential of glucose at pH 2.5 is -0.136 V; thus, there is not a great overpotential for the electrocatalytic process. The maximum electrocatalytic effect was measured after 28 days of bacterial inoculation. Ten electrodes were prepared in this form (although in most cases the incubation period was less than 10 days), and the final electrocatalytic current measured at +0.15 V above the value for the reference SCE ranged between 2.0 and 3.0 A/m², considering the geometric area of the electrodes. When a carbon felt electrode modified with *Acidiphilium* sp. strain 3.2 Sup 5 cells was changed to a new glucose solution free of cells, a significant amount of the catalytic effect was observed by cyclic voltammetry (Fig. 3B). Different parts of the working electrode were cut and analyzed by SEM. The images show that the bacterial cells had colonized the graphite felt electrode, as individual cells clearly can be observed attached to the fibers of the electrode (Fig. 4).

Acidiphilium sp. strain SJH cells also were grown on a graphite felt electrode. In this case, the cyclic voltammogram measured after 20 days of inoculation was almost equal to the background one (not shown).

Figure 3C shows how the medium conditions within the electrochemical cell affected the electrocatalytic properties of the *Acidiphilium* sp. strain 3.2 Sup 5 electrode. A highly catalytic current of glucose oxidation is measured even if the O₂ concentration in solution was almost saturating. Sparging the cell solution with pure N₂ for 40 min in order to establish anaerobic conditions seldom affected the catalytic effect observed in the voltammogram. On the contrary, when 5% phenol, which is an inhibitor of *Acidiphilium* spp. cells, was added to the solution, the electrocatalytic current gradually decreased with time and after 6 h was almost suppressed.

DISCUSSION

In this work, we have shown that *Acidiphilium* sp. strain 3.2 Sup 5 cells colonized graphite felt disks when glucose is the energy source and O₂ is the only redox acceptor. Moreover, the

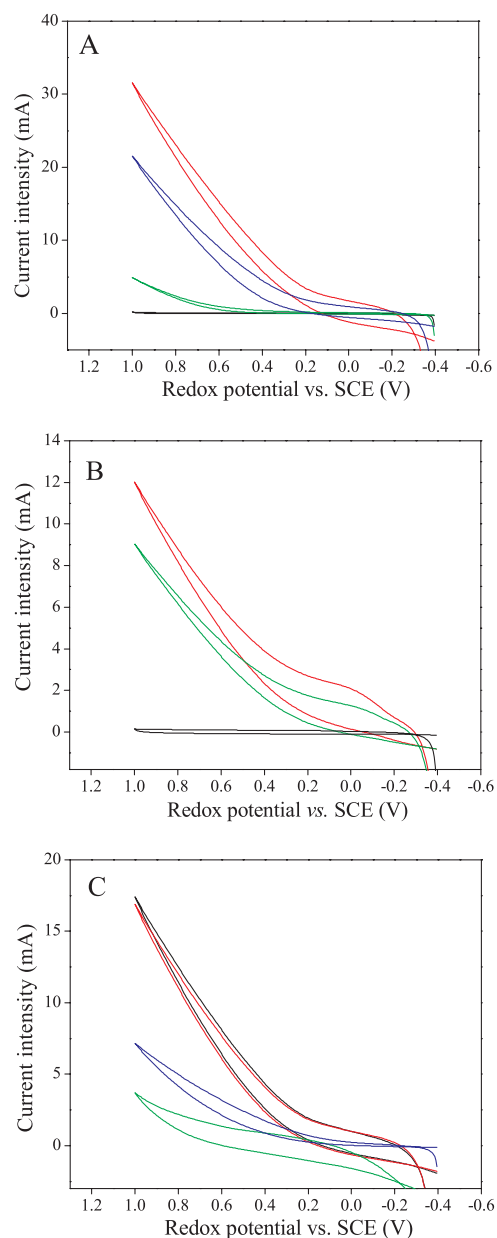


FIG. 3. Cyclic voltammograms of electrocatalytic oxidation of 10 mM glucose with different carbon felt electrodes (A) before (black) and 3 (green line), 13 (blue line), and 28 days (red line) after the inoculation of *Acidiphilium* sp. strain 3.2 Sup 5 into the electrochemical cell or (B) 23 days after the inoculation of *Acidiphilium* sp. strain 3.2 Sup 5 cells into the electrochemical cell (red line) and then put into a new solution without *Acidiphilium* sp. cells (green line). (C) Carbon felt electrode modified with *Acidiphilium* sp. strain 3.2 Sup 5 cells in the presence of 6.2 ppm O₂ in solution (black line), after 20 min of being bubbled with pure N₂ (red line), and 4 (blue line) or 6 h (green line) after the addition of 5% phenol to the electrochemical cell.

attached cells were able to establish electronic communication with the carbon support when they were used as a working electrode in an electrochemical cell configuration, as high catalytic current densities due to the oxidation of glucose were measured by cyclic voltammetry. This electrocatalytic phenomenon most probably is due to the cells of *Acidiphilium* spp. that

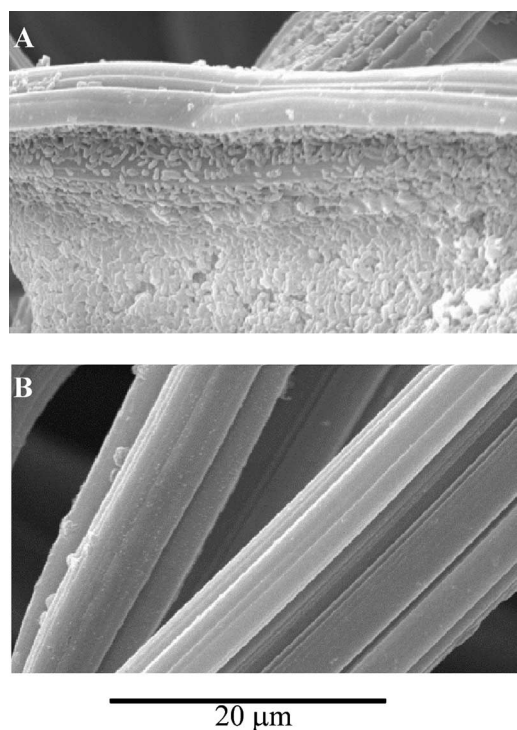


FIG. 4. Bacterial electrode characterization by SEM. (A) Image of a fiber of a carbon felt electrode 28 days after the inoculation of the electrochemical cell with *Acidiphilium* sp. strain 3.2 Sup 5. (B) Control image of a carbon felt electrode fiber before inoculation with *Acidiphilium* sp. strain 3.2 Sup 5 cells.

are attached to the electrode surface and not to planktonic cells, because when the carbon felt electrode modified with *Acidiphilium* spp. cells was changed to a new glucose solution free of cells, a significant amount of the catalytic effect was observed by cyclic voltammetry. The suppression of glucose electrocatalysis by the addition of phenol, which is a strong inhibitor of *Acidiphilium* spp., was a further indication of bacterial cells acting as the catalysts of the system. Because no redox mediators were added to the electrolyte solution or immobilized onto the electrode surface, we can conclude that bacterial cells were able to directly donate the electrons obtained from glucose metabolism to the electrode.

Direct electron exchange between microbial cells and a carbon electrode was first reported by Kim et al. in 1999 (16). Since then, several more microorganisms have been shown to be able to donate electrons directly to carbon electrodes (4, 5, 8, 17, 30). This functional property has spurred research in microbial fuel cell development, in which bacteria act as the electrocatalysts of the anodic compartment (7, 21, 25). A novel aspect of the bacterial anode of the present work is that it operates at low pH, whereas all other reported exoelectrogenic bacteria require nearly neutral pH conditions. This is of interest, because low proton concentrations can limit the overall reaction rate in microbial fuel cells (9).

The electrocatalytic currents measured with the bacterial electrode of this work are very high; a maximum density current of 3 A/m² was measured at a poised potential of +0.15 V compared to that for the reference SCE and taking into ac-

count the geometric area of the electrode. This value is approximately three times higher than the maximum value reported by Bond and Lovley for *Geobacter sulfurreducens* cells that were attached to graphite electrodes that were poised at the same redox potential under anaerobic conditions and with acetate as the electron donor (5). The high electrocatalytic current density of our system could be attributed to the highly electroactive surface area of the carbon felt electrode, but in spite of this, the high-density currents indicate an efficient electron transfer between the bacterial cells and the electrode. Three different routes for direct electron transfer between bacteria and electrodes have been proposed: (i) via redox proteins located on the bacterial membrane (5, 18); (ii) via redox compounds (i.e., phenazines or quinones) excreted from the bacteria (30, 31, 36); and (iii) via conductive nanowires that interconnect bacterial cells to the electrode surface (12, 32, 33). At this stage, we do not know which of these mechanisms efficiently transfers the electrons from the *Acidiphilium* sp. cells to the carbon electrode. However, the shape of the electrocatalytic cyclic voltammograms measured, in which the current increases greatly with the overpotential at the electrode without reaching a plateau (Fig. 3), suggests that interfacial electron transfer between the biocatalyst (*Acidiphilium* sp. cells in our case) and the electrode is the rate-limiting step (19, 28). Thus, routes (i) and (ii) for electron transfer are more probable in our system.

In microbial fuel cell designs, the anode compartment has to be separated from the cathode compartment, generally by a proton exchange membrane, in order to avoid the diffusion of O₂ to the anodic compartment, which either inhibits the growth of the microbial electrocatalyst (5, 8, 30, 35) or acts as a competitor to the anode by taking electrons from the bacteria, which decreases the current intensity measured in the biofuel cell (14, 20, 34). A remarkable property of the *Acidiphilium* sp. strain 3.2 Sup 5 electrode is that the electrocatalytic oxidation of glucose was not affected by the presence of O₂ in the electrolyte solution (Fig. 3C). This result has not been reported before for any microorganism. This behavior of the bacterial electrode concurs with the ability of *Acidiphilium* sp. cells to reduce the level of ferric ions in the presence of O₂ that was shown in this work and previously by other authors (15, 29). This physiological property is quite uncommon for metal-reducing bacteria, which generally are anaerobic organisms (3, 5, 10, 24). Nevertheless, this ability is a necessary condition, but is not sufficient, for the direct electrocatalytic oxidation of glucose in the presence of O₂, because *Acidiphilium* sp. strain SJH grown on the electrode gave negative results. Besides, a very recent article has shown that *Acidiphilium cryptum* cells were unable to transfer electrons directly to a graphite felt electrode (6). Both of these species are highly effective iron reducers. Therefore, we conclude that adequate electrical connectivity between the adhered bacterial cells and the electrode surface is an essential parameter for the preferential donation of electrons to the electrode instead of to O₂. Genetic differences between *Acidiphilium* spp. will be studied in order to explain why those isolated from Río Tinto have this special property.

In summary, this work shows that *Acidiphilium* sp. strain 3.2 Sup 5 cells, which were isolated from an extremely acidic environment, are able to donate electrons obtained from the

oxidation of an organic substrate directly to an electrode, producing high-density electrocatalytic currents in the presence of O₂, a property of interest for the further development of microbial fuel cells (2). Future work will involve studying the performance of this *Acidiphilium* sp. strain 3.2 Sup 5 electrode in a fuel cell configuration and the further characterization of the bacteria in order to understand better its noteworthy functional properties.

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