Bacterial Electron Transfer

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Electrochemical Characterization of a Single Electricity-Producing Bacterial Cell of *Shewanella* by Using Optical Tweezers**

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Members of the genus *Shewanella* are Gram-negative gamma-Proteobacteria that are widely distributed in nature and can utilize various types of electron acceptors for respiration. When soluble electron acceptors are sparse or not accessible in the environment, these microbes are capable of utilizing solid-state metal oxides as terminal electron acceptors. As a result of this property, *Shewanella* has attracted attention in the fields of bioremediation, biogeochemical circulation of minerals, and bioelectricity.[1] *Shewanella* is thought to mediate extracellular electron transfer (EET) to solid-state acceptors through the expression of abundant *c*-type cytochromes in the outer membrane (OMCs), although the precise mechanisms remain unclear. This EET ability can be exploited in microbial fuel cells by providing an electrode of an appropriate potential as the sole electron acceptor, which allows the capture of respiratory (metabolic) electrons and enables the detection of microbial respiration activity as an electric current. This electrochemical technique has been used to investigate electron transfer between microbes and electrodes,[2,3] and many research efforts aimed at revealing the mechanisms of respiratory EET have been conducted.[4–8]

To date, two different pathways have been proposed for EET in *Shewanella*: indirect electron transfer mediated by self-secreted flavins (mediator pathway) and direct electron transfer from OMCs to solid surfaces (direct pathway). Evidence for the mediator pathway has been provided by Nevin and Lovley[4] and Lies et al.,[5] who demonstrated that *Shewanella* can reduce Fe^{III} oxides at a distance without direct contact. Furthermore, Canstein et al.[6] and Marsili et al.[7] later proposed that *Shewanella* secretes flavins that mediate EET. On the contrary, conclusive experimental evidence for the direct electrical interaction between respiring microbes and solid electron acceptors has yet to be reported. However, previous studies have demonstrated that purified OMCs, such as OmcA and MtrC from *S. oneidensis* MR-1, display strong affinity for Fe^{III} oxides and are electrochemically active.[8] In addition, our group recently reported that *S. loihica* PV-4 shows clear electrochemical redox peaks in whole-cell cyclic voltammograms assignable to OMCs.[2] Although these studies suggest that *Shewanella* is capable of direct EET, a definitive conclusion has not been reached, in part because extracellular OMCs are also present in *Shewanella* biofilms[9] and in shed membrane vesicles.[10]

The main challenge for investigating the direct EET pathway arises from the difficulty of excluding the possible effects of biofilm, secreted mediators, and extracellular OMCs in population-level experiments. In the work presented herein, we therefore developed an optical tweezers technique to examine EET by attaching a single bacterial cell to a microelectrode. This approach is advantageous as it allows the direct characterization of the electrical interaction between a single microbe and an electrode under controlled conditions in the absence of biofilm, and also minimizes the effect of secreted materials.

To trap a single bacterial cell on a microelectrode, optical tweezers were used to manipulate the cells (see Figure 1 a). In this experimental system, optical traps of the microbes were formed by using a wavelength of 1064 nm generated with a continuous-wave Nd:YAG laser. The laser head and necessary optics were arranged on an inverted optical table beneath the microscope stage. Laser light with a power of 2 mW was focused through a 100× oil-immersion objective lens (numerical aperture: 1.4) to form an optical trap in the specimen plane. As the working electrode, a lithographically micropatterned tin-doped In_2O_3 (ITO)-coated cover glass with an electrically active area 2 μm in diameter was placed on the bottom of the reactor (Figure 1 b). The optically trapped microbe could be attached and detached from the working electrode by moving the objective lens vertically.

Prior to electrochemical characterization, the microbes were grown aerobically in marine broth (MB) for one day, and subsequently cultured in defined media (DM) for two days using lactate as a carbon and electron source. After collecting the cells by centrifugation, they were resuspended in deaerated DM and further cultured electrochemically at either +200 or −200 mV versus Ag/AgCl for one day. A small amount of these cells was gently taken from the electrode and introduced into a single-chamber, three-electrode system equipped with the optical tweezers instrument. Using optical

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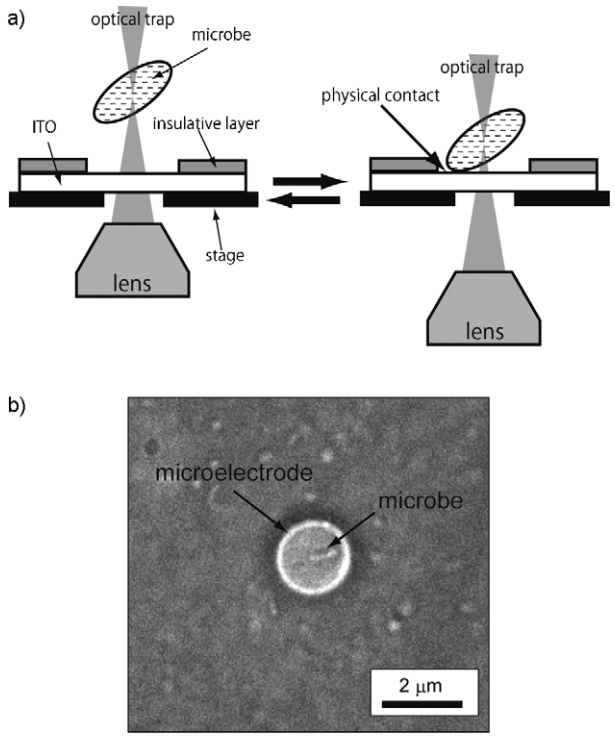

Figure 1. a) Use of optical tweezers to achieve the physical contact of a single microbe with a patterned ITO electrode. b) Top-view photograph of the micropatterned electrode and a single trapped bacterial cell.

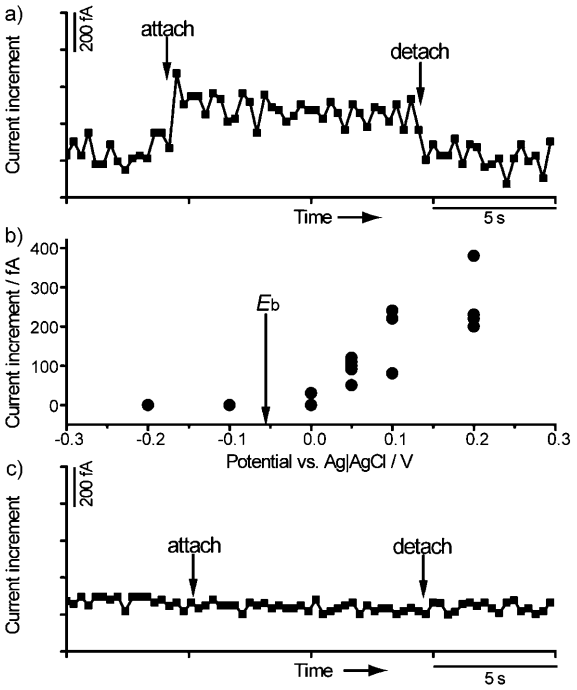

Figure 2. a) Representative current response from a single bacterial cell. An optically trapped microbe was attached and detached from the ITO electrode by using optical tweezers. Prior to the measurements, the microbe was cultured at +200 mV for 1 day. The electrode potential was set at +200 mV. b) Current responses of a single microbe by using electrodes poised at various potentials. Microbes cultured at +200 mV for 1 day were used for the analyses. The arrow indicates the potential (E_b) at which the relative amounts of oxidized and reduced forms of OMC were balanced under the operation conditions.[2a] c) Representative current response for a single cell precultured at −200 mV. The experiment was performed similarly to that described in (a).

tweezers, a single planktonic cell could be trapped and manipulated in the 3D space by moving the microscope stage and the vertical position of the lens. After positioning the cell over the microelectrode, electrochemical measurements were performed under strict anaerobic conditions. As the absolute value of the background current differs between experiments, we focused on the change of current at the precise moment the microbial cell physically contacted the ITO electrode.

A representative current response of a single bacterial cell precultured at +200 mV is shown in Figure 2a. The electrode potential was kept constant at +200 mV as this is more positive than the redox potential of the major OMCs (E_{OMC}). Under these conditions, the microbe produced a current of approximately 200 fA. Notably, the observed current showed a sudden increase and decrease at the precise moments when the microbe was physically attached and detached, respectively, from the microelectrode. Control experiments without a trapped cell did not display an electrochemical response. This result suggests that the observed current response was mainly triggered by a direct electrical connection between the microbe and ITO electrode.

To confirm whether the current observed during the physical contact was mediated by OMCs, we repeated the single-cell measurements at various potentials. In Figure 2b, the increments in current following physical contact with the electrode are plotted against the potential. In this experiment, different individual cells were used for each trial. When the electrode was poised at potentials of −200 and −100 mV, no response was observed in all trials. At a potential of 0 V, a current response of less than 50 fA was detected in only one of the four trials. However, at potentials greater than +50 mV, a current response was always observed at the moment the microbe was attached to the electrode. We have previously revealed by in vivo electronic absorption spectroscopy that the potential at which the amounts of oxidized and reduced forms of OMCs are balanced under operating conditions (E_b) is approximately −55 mV (±50) versus Ag/AgCl.[2a] The E_b value is more positive than the E_{OMC} estimated from whole-cell cyclic voltammetry (CV) and can be explained by considering that electrons are continuously supplied to OMCs in respiring cells.[2a] In the single-cell experiments, the E_b (arrow in Figure 2b) was close to the potential required for a current response. For EET through the direct pathway, oxidized OMCs are required as only the oxidized form can serve as a conduit to mediate electron transfer from metabolic pathways to the solid electrode. Thus, the fact that a current response was obtained at a potential more positive than E_b strongly suggests that the current observed during the physical contact between the microbe and electrode is because of direct electron transfer from OMCs to the electrode.

To examine the electrochemical properties of the *Shewanella* outer membrane, we also performed whole-cell CV using suspensions containing a high density of cells (Figure 3). For microbes cultured electrochemically at +200 mV, clear

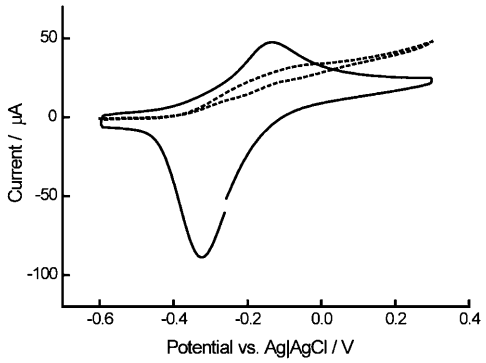

Figure 3. Whole-cell cyclic voltammograms of a microbial suspension cultured electrochemically at either +200 mV (——) or −200 mV (----) for 1 day prior to the measurement. Microbial cell suspensions with an optical cell density at 600 nm of 2.0 were used. The scan rate of the potential was 50 mV s^{-1}.

redox peaks in the CV were observed (solid line, Figure 3) that were assigned to the oxidation and reduction of OMCs, which is consistent with previous reports.[2] However, when the microbes were cultured at −200 mV, no clear redox peaks were observed (dashed line, Figure 3). This was not because of viability, as the microbes cultured in the electrochemical cell at −200 mV generated a microbial current. Taken together, these results indicate that the amount of OMC that can directly interact with electrodes is smaller for microbes cultured at −200 mV than for those cultured at +200 mV.

As these results indicate that *Shewanella* cells alter the outer membrane composition in response to the Fermi level of the terminal electron acceptor, we examined the electricity-producing performance of a single microbe cultured at −200 mV by the optical tweezers technique. In good agreement with the difference observed in the whole-cell CV (Figure 3), when a microbe precultured at −200 mV was attached to the microelectrode, a current was not generated, even at an applied electrode potential of +200 mV (Figure 2c). This result lends further support to the conclusion that the current response observed in Figure 2a and b was mediated by OMCs. The lack of current generation by microbes cultured at −200 mV may be because of a lack of OMCs that can electrically interact with the ITO electrode poised at −200 mV.

In summary, we have successfully characterized the direct electrical connection between a single *Shewanella* bacterial cell and a microelectrode by using an optical tweezers technique. The main advantage of this technique is the ability to place a single microbe on the microelectrode and exclude the possible effects of biofilm and secreted materials. This approach has enabled us to characterize the direct electrical interaction between a microbe and an electrode. Generally, analysis at the single-cell level can provide information that is extremely difficult to obtain in population-level experiments because of their increased complexity.[11] We anticipate that further studies of single cells with this optical tweezers technique will enable the identification of the individual components of the EET mechanism, and allow the complexities of population-level dynamics of electricity-generating bacteria to be unraveled in a bottom-up approach.

Experimental Section

Microbe preparation: A *Shewanella loihica* PV-4 mutant strain (Δ1348), which contained a gene deletion of orf 1348 (predicted to encode flagellar basal body FlaE domain protein; Oak Ridge National Laboratory annotation) and displayed reduced motility compared to wild-type PV-4, was used in this study. Strain Δ1348 was cultured aerobically in MB (10 mL, 20 g L^{-1}) at 30 °C for 24 h with shaking. The cells were collected by centrifugation, washed three times with DM ($NaHCO_3$ (2.5 g), $CaCl_2{\cdot}2H_2O$ (0.08 g), NH_4Cl (1.0 g), $MgCl_2{\cdot}6H_2O$ (0.2 g), NaCl (10 g), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 7.2 g) per liter), and then further cultivated in DM supplemented with lactate (15 mL, 10 mM) as a carbon source at 30 °C for 24 h with shaking.

Electrochemical characterization: A single-chamber, three-electrode system was used to monitor the electrochemical behavior of the microbe. Ag/AgCl (sat. KCl) and a platinum wire were used as the reference and counter electrodes, respectively. An ITO-coated thin glass with an electrically active area 2 μm in diameter was used as the working electrode. To prepare the electrode, a cover glass with a thickness of 0.12–0.17 mm was sequentially cleaned with detergent, pure water, and ethanol (70%) before use. The coating of the glass with ITO was carried out by the dip-coating method using a commercially available dip-coating precursor (ITO-05C, Kojundo Chemical Lab. Co., Ltd., Japan) for the In_2O_3–SnO_2 film. The cover glass was dipped into and vertically removed from the solution containing the precursor. The as-prepared film was sequentially calcined at 520 °C in the ambient atmosphere for 20 h and in a nitrogen atmosphere for 3 h. A micrometer-scale conductive pattern was prepared by a photolithography method in which a spin-coated polyimide was used as the insulating layer. DM containing lactate (10 mM) was used as electrolyte and was deaerated by bubbling with N_2 for 30 min prior to the measurements. Following deaeration, the remaining O_2 (ca. 0.1 ppm) was further removed by the addition of the reducing agent $(NH_4)_2SO_3$ before injection of the cell suspension into the reactor. The entire setup was placed on the stage of an inverse Olympus IX 71 microscope equipped with a 100× oil-immersion objective lens.

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