Bacterial Networks

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Self-Constructed Electrically Conductive Bacterial Networks**

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Microbial attachment to mineral surfaces is a fundamental process for initiating a broad range of biochemical and geological events in a natural environment.[1] The genus *Shewanella*,[2] which consists of dissimilatory metal-reducing bacteria often found in subsurface sediments, has the ability to recognize the surface of iron(III) oxides[3] and initiate extracellular electron transfer (ET)[4–8] to the attached iron oxides as a terminal process in its metabolism. This is an important process for its influence on the biogeochemical cycling of iron,[9] and it has also gained attention not only for a new aspect of the metabolic strategy of microorganisms,[5–8] but also for its applicability in microbial fuel cells.[10]

The outer-membrane (OM) redox proteins, *c*-type decaheme cytochromes (*c*-Cyt), play a crucial role in mediating ET from the cell to iron(III) oxides.[4–7,11] A great deal of research has been focused on the electrochemical and spectroscopic investigation of the purified OM proteins.[11] However, few studies have been performed by directly monitoring the ET process of intact cells, and therefore the mechanism of this process has largely remained unsolved. Herein, we report the ability of *S. loihica* PV-4 to self-assemble into an electrically conductive network in the presence of iron(III) oxides, and demonstrate the role of semiconductive nanominerals in promoting a long-distance extracellular ET process in the bacterial network.

To probe the extracellular ET of intact cells of *S. loihica* PV-4, we used a single-chamber, three-electrode system, with lactate as a carbon source and an electron donor. An optically transparent conductive-glass, tin-doped In_2O_3 (ITO) electrode, with a surface area of 1.8 cm^2, was used as the working electrode, and placed on the bottom surface of the reactor. A current was generated immediately after adding the cells into the reactor (Figure 1 a), and reached a constant value 0.4–0.6 μA. Current generation is a consequence of electrical

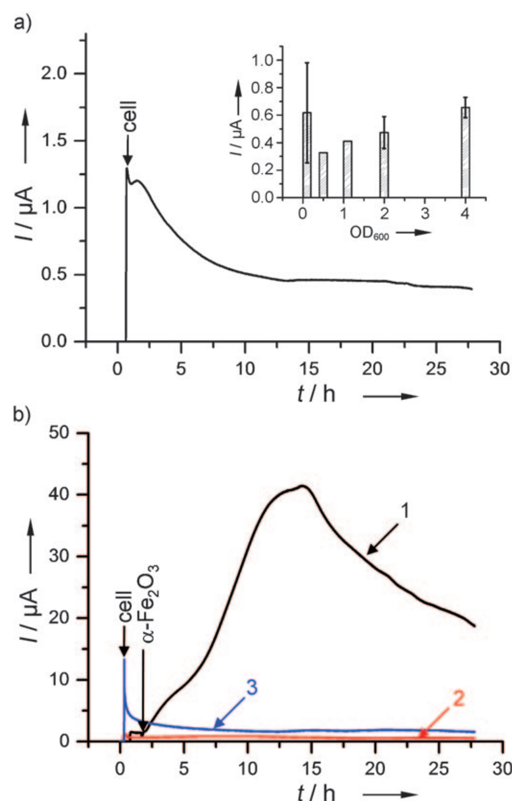

Figure 1. a) Current *I* versus time *t* measurements of microbial current generation for *S. loihica* on an ITO electrode. Inset: the dependence of the steady-state current on OD_{600}. b) *I* versus *t* curves in the presence (trace 1) and absence (trace 2) of α-Fe_2O_3 colloids (7.5 mM). The effect of iron citrate (7.5 mM) is shown in trace 3. No agitation was made during measurements.

connections from the cells to the electrode, followed by the injection of electrons from the OM *c*-Cyts to the ITO electrode, which is suggested by the absence of redox species in the cell-free supernatant solution. In addition, the current showed essentially no dependence on the optical cell density OD at 600 nm over the OD_{600} range of 0.1–4.0 (Figure 1 a, inset). This result implies that the current generation from *S. loihica* is dominated by the cells attached directly to the electrode surface. In other words, individual cells are electrically insulated from the others, and thus the long-distance ET process, even if it is present, is much less efficient than the *c*-Cyt-mediated ET to the electrode surface.

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Anaerobically cultivated *Shewanella* locates a significant content of multiheme *c*-Cyts to their OM compartment.[11c] OM *c*-Cyts have a high binding affinity for iron(III) oxide nanocolloids.[3] We therefore hypothesized that the α-Fe_2O_3 nanocolloids would force the cells to self-assemble into an interconnected bacterial network. In addition, among the naturally existing crystal forms of iron(III) oxide, α-Fe_2O_3 in particular is expected to serve as an electrical linkage owing to its *n*-type semiconductive properties.[12,13]

Figure 1b shows the effect of the addition of α-Fe_2O_3 colloids on current generation from *S. loihica*, in which the cells had been injected to give an OD_{600} of 2.0 inside the reactor. Upon adding the colloids, the current initially dropped to zero (Figure 1b, trace 1); however, after the colloids were completely precipitated on the ITO surface (after about 1 h), the current generation recovered and then showed a steep, fiftyfold increase until reaching the maximum value at approximately 15 h after cultivation. Thereafter, the current decreased in correlation with a decrease in the concentration of lactate. No current generation was observed when α-Fe_2O_3 colloids were added into the reactor lacking the cells, confirming that the current generation is a consequence of microbial activity of *S. loihi*ca. SEM images of the electrode surface revealed the formation of thick layers composed of cells and colloids (Figure 2 and Supporting Information, Figure S3), in which the outer surfaces of the cells were covered with the colloids, with the colloids serving to interconnect the cells. In contrast, the addition of iron citrate as a soluble redox mediator resulted in a much lower improvement of current generation (less than a threefold increase; Figure 1b, trace 3), demonstrating that the semiconductive property of the α-Fe_2O_3 colloids is responsible for the large increase in current generation.

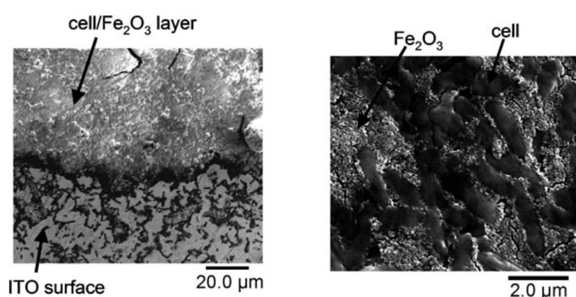

Figure 2. SEM images of the surface of the ITO electrode (seen from above) after 28 h of current generation in the presence of α-Fe_2O_3 colloids.

Direct evidence for the formation of a conductive bacterial network was given by whole-cell cyclic voltammetry (CV). As shown in Figure 3, trace 1, the intact cells grown on the electrode exhibited a clear redox wave with a midpoint potential $E_m = 20$ mV (vs. SHE). This value is in accordance with the reported E_m value of OM *c*-Cyts isolated from *Shewanella*,[11d–f] and thus demonstrates the *c*-Cyt-mediated electron-exchange process at the intact cell/electrode interface. The possibility that secreted redox mediators were the origin of the redox wave was excluded by protein unfolding experiments and mutation experiments on the OM *c*-Cyts. When the cells were inoculated in the presence of α-Fe_2O_3 colloids, E_m shifted to −15 mV and the peak current was enhanced more than a 300-fold (Figure 3, trace 2). Neither the colloids alone nor the cell-free supernatant gave the redox waves in this potential region (Figure 3, traces 3 and 4). Therefore, the drastic enhancement of the redox current demonstrates the formation of a long-distance ET conduit in the colloidal network, which enables the abundant cells located at a distance from the electrode to participate in the current generation.

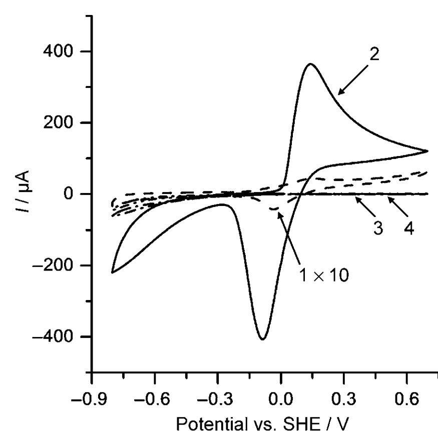

Figure 3. Whole-cell CVs (scan rate 10 mVs^{-1}) of *S. loihica* measured in the absence (trace 1) and presence (trace 2) of α-Fe_2O_3 colloids (7.5 mM). Trace 3: CV of the α-Fe_2O_3 colloidal solution lacking cells, trace 4: CV of the cell-free supernatant obtained after the CV measurement of *S. loihica* in the presence of colloids. The waveform in trace 1 was expanded in the *y*-axis direction by a factor of 10.

To investigate the role of α-Fe_2O_3, we photoirradiated intact cells grown on the electrode surface after adding α-Fe_2O_3 nanocolloids into the reactor. Photoexcitation of semiconductive α-Fe_2O_3 using light with a wavelength longer than 420 nm produces a conduction-band electron, which is also the same electron generated by the *c*-Cyt-mediated ET of *Shewanella*.[3,5–7] No photocurrent generation was observed in the early period (Figure 4a, up to about 2 h, at irradiation times indicated by filled circles). However the photocurrent appeared to increase with an increase in the microbial current, and showed exactly the same time profile as that for the microbial current generation (Figure 4a inset, and Figure 4b, trace 1). In contrast, no photocurrent was observed if photoirradiation of the colloidal solution lacking *S. loihica* was performed (Figure 4b, trace 2). It should be noted that photocurrent generation is a consequence of the collection of photogenerated conduction-band electrons at the electrode surface, which requires the efficient electron-hopping process from colloid to colloid. Accordingly, the increase in photocurrent observed in the presence of the cells demonstrates the process in which the cells work as an electrical linkage between the colloids and establish a long-distance ET conduit to transport the conduction-band electron to the electrode surface (Figure 5a).

The energy barrier for the electron-exchange process does not exist between the OM *c*-Cyts and α-Fe_2O_3, as indicated by

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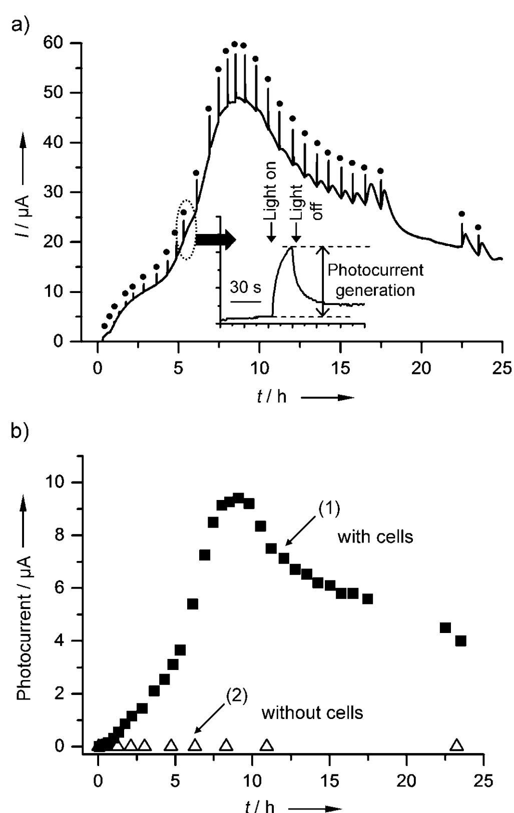

Figure 4. a) I versus t measurements of microbial current generation for *S. loihica* in the presence of α-Fe_2O_3 colloids (7.5 mM). Photoirradiation ($\lambda > 420$ nm) was conducted for 30 s at times indicated by the filled circles. b) Photocurrent versus time for the α-Fe_2O_3 colloids with *S. loihica* (trace 1). No photocurrent generation was observed for the α-Fe_2O_3 colloids lacking *S. loihica* (trace 2).

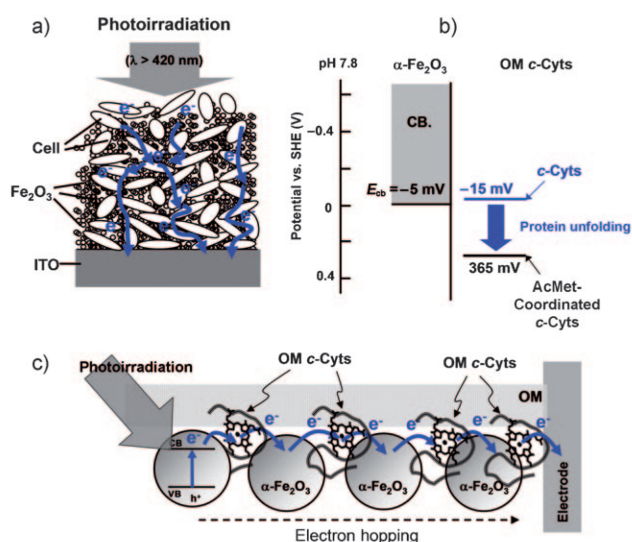

Figure 5. a) Photocurrent generation from the bacteria/colloid network. b) Energy levels of the outer-membrane *c*-Cyts in the presence of α-Fe_2O_3 colloids. E_{CB} is the energy level of the conduction band edge of α-Fe_2O_3. c) Proposed model for the bacterial long-distance ET process mediated by semiconductive α-Fe_2O_3.

the CVs of intact cells (Figure 5b). Therefore, it is possible to consider a semiconductor-mediated electron-hopping process through the bacterial network as a model for the long-distance extracellular ET (Figure 5c). The involvement of OM *c*-Cyts in the electron-hopping process was confirmed by the mutation experiment, in which the single deletion of the gene encoding MtrC homologue (Δ2525) resulted in a circa 50% decrease in the current from the colloidal network (Supporting Information, Figure S4). Also, further support for this was given by the protein-unfolding experiments. Lowering the E_m of the OM *c*-Cyts by the ligand coordination reaction of the *c*-hemes with acetylmethionine (AcMet) caused the formation of a large energy barrier for the electron-exchange process, resulting in complete suppression of a microbial current and photocurrent generation in the bacteria network (Figure 5b and Supporting Information, Figure S5).

Measuring and establishing the existence of a bacterial extracellular ET is a research subject that is not only relevant to the understanding of microbial activities in subsurface environments,[1–9] but also for designing and fabricating bioanode materials for microbial fuel cells.[10] The present findings provide experimental evidence of ET, and demonstrate the significant influence of semiconductive nanominerals on the electrochemical activities of the bacterial networks.

Experimental Section

α-Fe_2O_3 nanocolloids were synthesized according to the literature.[13] *S. loihica* PV-4 was grown aerobically in 10 mL of marine broth (20 gL^{-1}) at 30°C for 24 h. The marine broth was subsequently replaced with defined media (DM)[14] and the cell was further cultivated at 30°C for 2 days using lactate as a carbon source. The *S. loihica* PV-4 Δ2525 in-frame deletion mutant was generated using suicide vector pSMV10 and a two-step homologous recombination method.[15]

Microbial current measurements: The counter electrode used was Ag/AgCl (KCl sat.) and the reference electrode was platinum wire. DM (4.0 mL) containing lactate (10 mM) was deaerated by bubbling with N_2. After a dissolved O_2 concentration 0.1 ppm was reached, the freshly prepared cell suspension was injected into the reactor at a constant voltage of 0.2 V (25°C, pH 7.8). Photoirradiation was conducted at the bottom surface of the electrode using a 300 W xenon lamp as the light source. A cut-off ($\lambda > 420$ nm) filter was used to remove UV light from the light source.

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