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Spectroelectrochemical Investigation on Biological Electron Transfer Associated with Anode Performance in Microbial Fuel Cells

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1. Introduction

Microbial fuel cells (MFCs) are considered to be an attractive future option for the treatment of organic wastes and recovery of bioenergy from renewable biomass resources (Kim et al., 1999; Schroder et al., 2003; Lovley 2006; Bretschger et al., 2007; Logan et al., 2010). MFCs are devices that generate electricity from organic matter by exploiting the catabolic activities of microbes. Although MFCs have operational and functional advantages over the current technologies used for generating energy from organic matter, the full potential and application of these devices remains unclear, primarily because it is an emerging technology that requires further technical development. Another limitation of MFCs is their relatively low process performance in comparison with competing technologies. Therefore, improvement of the efficiency of electricity generation in MFCs will require both biological and engineering research approaches (Schroder et al., 2003; Zhao et al., 2010).

Recently, electrochemists have focused on the mechanisms of microbial current generation in an attempt to improve the anode performance in MFCs (Reguera et al., 2006; Torres et al., 2010). In particular, a metal-reducing bacterial species of the genus *Shewanella*, *S. oneidensis* MR-1, is one of the most extensively studied organisms for microbial current generation (Heidelberg et al., 2002; Bretschger et al., 2007; Newton et al., 2009). This microorganism has a significant quantity of redox proteins, namely *c*-type cytochromes (*c*-Cyts), in the outer membrane (OM) (Myers & Myers 1992; Heidelberg et al., 2002) that are proposed to transport electrons generated by the intracellular metabolic oxidation of organic matter to extracellular electrodes as a terminal process for anaerobic respiration (Myers & Myers 1997; Shi et al., 2009; Gralnick et al., 2010). To date, however, the basic electrochemical analyses on the extracellular electron transfer (EET) process has been strictly limited to studies with purified OM *c*-Cyts or synthesized model compounds immobilized on graphite electrodes (Hartshorne et al., 2007; Wigginton et al., 2007; Marsili et al., 2008). Thus, the processes by which living microorganisms deliver electrons to extracellular solid electrodes remain largely unknown. The main obstacle to determining the underlying mechanisms of electron transfer (ET) is the shortage of *in-vivo* spectroscopic and electrochemical techniques to

directly differentiate OM *c*-Cyts from the numerous uncharacterized biological molecules, such as self-secreted redox shuttles, present in bacterial cells. Furthermore, the highly dynamic nature of *in-vivo* ET resulting from protein-protein and protein-membrane interactions impedes our ability to utilize knowledge and methodologies developed in studies with purified proteins.

Herein, we report the *in-vivo* spectroscopic and electrochemical techniques recently developed in our laboratory for the study of microbial EET. The following three techniques are introduced in this chapter:

- Whole-microorganism electrochemistry with a three-electrode system to examine EET pathways and dynamics under physiological conditions.
- UV-vis evanescent wave (EW) spectroscopy for monitoring the electronic state of *c*-Cyts located at the cellular membrane/electrode interface in the course of microbial current generation.
- In-frame deletion mutants of OM and periplasmic *c*-Cyts to determine the specific location of proteins responsible for the respiratory EET chain.

The application of these techniques with intact *Shewanella* cells allowed us to reveal the thermodynamic properties of OM *c*-Cyts under *in-vivo* conditions, which significantly differed from those previously found *in vitro*. Together with previous biochemical investigations of purified *c*-Cyts, the techniques introduced in this chapter will aid basic electrochemists to perform experiments using living microorganisms as the subject of electrochemical and spectroscopic studies. We speculate that such studies will be an important precondition to cultivate and advance the emerging technology of MFCs as a feasible option for the treatment and recovery of bioenergy from organic wastes and renewable biomass.

2. Whole-microorganism electrochemistry

2.1 The role of OM *c*-Cyts for EET processes under *in-vivo* conditions

Using protein film voltammetry (PFV), several purified decaheme OM *c*-Cyts of *S. oneidensis* MR-1, particularly OmcA and MtrC, have been demonstrated to mediate direct electron transfer (DET) to graphite electrodes (Hartshorne et al., 2007; Firer-Sherwood et al., 2008). Kinetic analyses revealed that ET at the protein/electrode interface occurs within the order of milliseconds (Firer-Sherwood et al., 2008). Moreover, the importance of protein-protein interactions to facilitate DET to a graphite electrode has been demonstrated using the MtrCAB protein complex (see Fig. 1) (Hartshorne et al., 2009). Although the fundamental properties of protein-mediated EET have become clearer through studies involving PFV, the mechanisms by which living organisms deliver electrons to extracellular electrodes under physiological conditions are largely unknown, and a number of discrepant results exist concerning the kinetics and energetics of bacterial EET.

To confirm the existence of EET pathways mediated by OM *c*-Cyts under physiological conditions (Fig. 1), we have developed a highly sensitive, whole-cell voltammetry technique. Electrochemical measurements with *S. oneidensis* MR-1 cells were conducted using an in-house constructed, vacuum-tight, single-chamber, three-electrode reactor (Fig. 2). A flat, uniform layer of tin-doped In₂O₃ (ITO) grown on a glass substrate by r.f. magnetron sputtering (8 Ω/square resistance, 1.0-mm glass thickness, 3.1-cm² surface area; SPD

Fig. 1. A schematic illustration of the EET process of *S. oneidensis* MR-1 cells under anaerobic conditions. Respiratory electrons are continuously generated by the metabolic oxidation of lactate to pyruvate and acetyl-CoA, and are transferred to the extracellular electrode via the electron transport system consisting of the NADH dehydrogenase and menaquinone redox couple, followed by the protein network of *c*-Cyts (CymA and OmcA-MtrCAB protein complexes) spanning the inner and outer membranes.

Fig. 2. (a) Photograph and (b) schematic illustration of the electrochemical reactor used for whole-microorganism electrochemistry.

Laboratory, Inc.,) was used as the working electrode, while Ag/AgCl ($\text{KCl}_{\text{sat.}}$) and a platinum wire were used as the reference and counter electrodes, respectively. The reactor temperature was maintained at 303 K using an external water circulation system (Fig. 2 (b)). It is important to note that the ITO electrode was placed on the bottom surface of the reactor, allowing the injected cells to statically settle on the electrode surface within a few minutes. This design improves the sensitivity of current detection, as it permits the cells to form a uniform electroactive biofilm on the electrode with high surface coverage.

2.2 EET from living microbes to ITO electrodes

To investigate EET *in vivo*, electrochemical experiments were performed under conditions of static potential at 0.4 V (vs SHE). As an electrolyte solution, 4.7-mL of defined medium (DM) containing lactate (10 mM) and yeast extract (0.5 g per liter) was added into the electrochemical reactor (Roh et al. 2006), which was subsequently deaerated by bubbling with dry N_2 (99.999% purity). After a dissolved O_2 concentration of 0.1 ppm was reached (PreSens, Microx TX3 trace), 0.3 mL of a freshly prepared cell suspension with an optical density at 600 nm (OD_{600}) of 1.6 was injected into the electrochemical reactor to give a final OD_{600} inside the reactor of 0.1.

As shown in Fig. 3 trace (a), upon adding the cell suspension to the reactor, an anodic current was immediately generated and reached $1.0 \mu\text{A cm}^{-2}$ after 2 h of cultivation. The current continued to increase with time, reaching approximately $2.0 \mu\text{A cm}^{-2}$ after 12 h. In contrast, when medium lacking lactate was used as an electrolyte solution, the microbial current was strongly impaired (Fig. 3 trace (b)). In this system, a maximum density of less than $0.3 \mu\text{A cm}^{-2}$ was attained, with the small amount of current generation likely attributable to the trace amounts of organic compounds present in the yeast extract. These results demonstrate that the observed current is due to the metabolic oxidation of lactate, followed by EET from the cell surfaces to the ITO electrode. Given that the anaerobic lactate oxidation pathway in strain MR-1 has been elucidated (Scott & Nealson 1994), as schematically shown in Fig. 1, we can conclude that the generated current is associated with the production of NADH via the conversion of lactate to pyruvate and acetyl-CoA. Subsequently, menaquinone and *c*-Cyts located in the inner and outer membranes, respectively, sequentially transport the electrons to the electrode surface.

Fig. 3. Microbial current generation versus time for *S. oneidensis* MR-1 cells inoculated in electrochemical reactors containing medium with (a) and without (b) 10 mM lactate.

2.3 Whole-microorganism voltammetry

To identify the redox molecules responsible for the EET, we conducted whole-cell voltammetry measurements of the cell suspension of MR-1 strain. As shown in the cyclic voltammogram (CV) in Fig. 4(a), the electrode with attached MR-1 cells exhibited a single redox wave with a midpoint potential (E_m) of 50 mV (vs SHE). The waveform remained stable even after hundreds of repeated potential scans from 0.7 to -0.8 V at scan rates ranging from 0.01 to 200 V s⁻¹. As the plot of the peak current as a function of scan rate gave a linear relationship, the redox wave at 50 mV was attributed to an electron-exchange process mediated by adsorbed species. In other words, the bacterial cells localized abundant and robust redox species at the cell membrane/electrode interface.

Following the CV measurements, the reactor electrode was washed with standard HEPES buffer and subjected to scanning electron microscopy (SEM). The SEM image shown in Fig. 4 (b) revealed that rod-shaped cells of approximately 2 μ m in length and 0.5 μ m in width were immobilized on the ITO electrode surface, with most cells orientated horizontally to the electrode surface, rather than via the apex of their long axis. As MR-1 cells possess a high quantity of *c*-Cyts on the OM, we can reasonably assign the observed redox signal with an E_m of 50 mV in Fig. 4 (a) to the redox reaction of the major OmcA-MtrCAB protein complex with the ITO electrode (Fig. 1).

The kinetic analysis of the electron exchange reaction at the cell-electrode interface also supports the assignment of the redox species as the OmcA-MtrCAB complex. The heterogeneous standard rate constant (k_0) for intact cells was estimated to be 300 ± 10 s⁻¹ at 303 K by Trumpet plot analysis (Laviron 1979). This value is the same order of magnitude with the k_0 values previously determined for purified MtrC and the MtrCAB protein complex immobilized on a basal-plane graphite electrode of 220 and 195 s⁻¹, respectively (Hartshorne et al., 2009). Note that these k_0 values are two orders of magnitude higher than that for riboflavin immobilized on an electrode surface ($k_0 < 0.7$ s⁻¹) (Okamoto et al., 2009), which is reported to be a major electron shuttle secreted by *Shewanella* cells for EET (Marsili et al., 2008).

Fig. 4. (a) Cyclic voltammogram of *S. oneidnesis* MR-1 cells at a scan rate of 50 mV s⁻¹. (b) SEM image of the ITO electrode surface (top view) after 25 h of current generation in the presence of lactate.

It should be noted that the E_m of 50 mV detected for intact MR-1 cells is a markedly more positive potential compared to those reported for purified OM *c*-Cyts (−170 mV for MtrC and −210 mV for OmcA) (Eggleston et al., 2008; Firer-Sherwood et al., 2008; Hartshorne et al., 2009). In addition to OM *c*-Cyts, MR-1 cells are known to synthesize several flavin and quinone derivatives that function as electron shuttles, a few of which strongly bind to the outer cell surface and provide redox signals characteristic of immobilized electroactive species (Saffarini et al., 2002; Okamoto et al., 2009; Bouhenni et al., 2010). Therefore, the large difference in E_m between purified OM *c*-Cyts and intact cells has led many researchers to speculate that the observed redox wave at 50 mV is attributable to cell-attached menaquinone, whose midpoint potential is approximately 80 mV (Li et al., 2010; Zhao et al., 2010), rather than OM *c*-Cyts.

In the following sections, we will demonstrate based on the results of spectroscopic and molecular biological techniques that the redox wave at +50 mV is indeed assignable to the OmcA-MtrCAB protein complex of *S. oneidensis* MR-1. The definitive assignment of this peak will also highlight the importance of *in-vivo* electrochemistry to verify the true energetics and kinetics of EET in living microbes.

3. *In-vivo* EW spectroscopy

3.1 EW spectroscopy for selective monitoring of the electronic state of OM *c*-Cyts

UV-vis EW spectroscopy is a powerful technique that allows the selective monitoring of molecules adsorbed on an electrode surface (Fig. 5) and has been used to investigate the EET mechanisms of *Shewanella* cells under physiological conditions. The *c*-Cyts located in the cytoplasmic (inner) membrane can be spectroscopically detected owing to both their large quantity and the intense visible absorption band of heme groups present in the proteins. To obtain conclusive evidence for the assignment of the redox signal at +50 mV to OM *c*-Cyt, we subjected an ITO electrode with attached *Shewanella* cells to EW spectroscopy and monitored changes in the electronic state of heme in OM *c*-Cyts by measuring electrode potentials.

Fig. 5. Schematic illustration of EW spectroscopy with an optical waveguide.

As a potential approach to differentiate between the redox signal derived from OM *c*-Cyts and that of menaquinone or other uncharacterized redox shuttles, we noted that the hemes of *c*-Cyts have the ability to bind small molecules such as O₂, NO, CO, and imidazole,

because of the existence of an open coordination site on the centered heme iron (Andrew et al., 2002; Fornarini et al., 2008). The ligation reaction of heme is an established method for analyzing the redox and electronic states of purified proteins (Hoshino et al., 1993). Previous *in-vitro* studies showed that the NO ligation of heme caused a large positive shift (~ 600 mV) in the E_m due to the strong back donation of NO to the centered iron (Chiavarino et al., 2008). More importantly, NO has a high binding affinity to metal centers, particularly for the hemes of *c*-Cyts (the equilibrium binding constant of NO to a ferrous heme is $\sim 10^5$ M $^{-1}$ (Hoshino et al., 1993)).

We first performed NO ligation of the hemes of strain MR-1 by exposing a bacterial suspension to NO for 10 min under anaerobic conditions. The suspension was centrifuged and the supernatant was replaced with fresh medium to remove the excess dissolved NO. The *in-vivo* optical absorption spectra of the bacterial suspension before and after the NO ligation were measured in diffuse-transmission mode. Prior to the NO ligation, strain MR-1 exhibited an intense band at 419 nm and weak bands at 522 and 552 nm which originated from the Soret and Q bands, respectively, of the reduced ferrous hemes in *c*-Cyts (Fig. 6, trace (a)) (Nakamura et al., 2009). The obtained spectral position and shape are consistent with those reported for purified OmcA and MtrC of MR-1 cells (Shi et al., 2006; Hartshorne et al., 2007; Wigginton et al., 2009). Following NO ligation, however, the spectrum showed a distinct blue shift of the Soret band to 408 nm, concomitantly with the loss of Q band absorption (Fig. 6, trace (b)). This change in the spectrum agreed well with those reported for nitrosyl adduct formation in purified proteins and synthetic hemes (de Groot et al., 2007).

Fig. 6. Diffuse-transmission UV-vis absorption spectra of a cell suspension of *S. oneidensis* MR-1 (a) before and (b) after NO exposure.

Second, to confirm if NO-ligated hemes were present in the *c*-Cyts located in both the OM and periplasmic regions, UV-vis EW experiments were conducted (Fig. 7 (a)). The UV-vis EW spectra of intact MR-1 cells were measured using a SIS-5000 Surface and Interface Spectrometer System (System Instruments) equipped with a 150-W xenon lamp. The angle of incident light was 8° relative to the surface of the optical waveguide (OWG) substrate (reflective index of 1.89). The bacterial suspension casted on a quartz OWG showed a blue shift of the Soret band from ~ 419 to ~ 408 nm after the NO ligation of ferrous hemes,

consistent with the results obtained by diffuse-transmission UV-Vis spectroscopy (Fig. 7 (b)). Because EW is only generated in the vicinity of the substrate surface and has a penetration depth of less than 30 nm (Nakamura et al., 2009), these results confirm that the nitrosyl adducts are located at the cell-substrate interface, as the thicknesses of the OM, periplasm, and inner membrane of *Shewanella* are ~15, ~10, and ~8 nm, respectively.

Fig. 7. (a) Schematic illustration of the NO coordination reaction to heme molecules in OM *c*-Cyts at the cell/electrode interface detected by UV-vis EW spectroscopy. (b) UV-vis EW spectra of whole cells of *S. oneidensis* MR-1 before (1) and after NO exposure (2).

The NO-ligated cells were next subjected to CV experiments to examine the effects of NO ligation of hemes on the redox properties of MR-1 cells. As expected, the NO-ligated cells were found to have an open circuit potential of 270 mV, which was 470 mV more positive than that for cells not exposed to NO. Furthermore, in the CV of NO-MR-1 cells obtained at a scan rate of 10 mV s⁻¹, the redox wave observed at 50 mV for untreated cells completely disappeared, and a new redox wave appeared at 650 mV (Okamoto et al., 2010). A similarly large positive shift in potential was also reported following the NO ligation of purified *c*-Cyts and is consistent with the strong back-donation effect of NO ligands (de Groot et al., 2007). Accordingly, we can conclude that the bacterial redox wave at 50 mV is attributable to *c*-Cyts localized at the cell membrane/electrode interface. In addition, it should be noted that menaquinone possesses no specific affinity for NO, excluding the contribution of this mediator to the redox signal at 50 mV.

3.3 EW spectroscopy combined with whole-microorganism electrochemistry

Based on the aforementioned UV-vis EW experiments, we further investigated the role of OM *c*-Cyts in the movement of metabolically generated electrons by monitoring the redox state of hemes under potential-controlled conditions. In this experiment, a quartz OWG coated with an ITO film was placed on the bottom surface of the electrochemical reactor to serve as an internal reflection element for the generation of an EW at the interface between the electrode and electrolyte solution (Fig. 8). A single-chamber electrochemical reactor consisting of Ag/AgCl (KCl_{sat.}) and platinum wire as the counter and reference electrodes, respectively, was mounted onto the ITO substrate and then sealed with a silicon rubber O-ring (Fig. 8).

Fig. 8. Photographs of the evanescent wave electroabsorption spectroscopy system used for *in-situ* electrochemistry with an ITO-coated optical waveguide (OWG).

In the course of microbial current generation with an electrode potential of 320 mV (vs SHE), the UV-vis EW spectrum exhibited a Soret absorption band at ~410 nm, characteristic of the oxidized form of *c*-Cyts (Fe^{3+}) (Richardson et al., 2000; Hartshorne et al., 2007). The penetration depth at which the EW field (reflective index of 1.47) decayed to $1/e$ was estimated to be ~110 nm at a wavelength of 400 nm. Accordingly, the appearance of the Soret peak at ~410 nm demonstrates that the oxidized *c*-Cyts are adjacent to the electrode surface during the respiratory ET reaction. However, upon lowering the electrode potential from 320 to -180 mV, a rapid red shift (419 nm) of the Soret band was observed, which was due to the reduced form of *c*-Cyts (Fe^{2+}). The spectral changes were fully reversible for both the positive- and negative-direction scans. The plot of the peak intensity (at 419 nm) against electrode potential shows that the *c*-Cyts of strain MR-1 have a potential distribution ranging approximately 300 mV, from fully oxidized (270 mV) to fully reduced (20 mV) (Fig. 9). The E_m of the *c*-Cyts under operating conditions of the reactor was spectroscopically estimated to be 145 ± 50 mV, which is shifted by +100 mV from the E_m electrochemically determined for purified OM *c*-Cyts. In contrast to purified proteins, electrons in living cells are continuously supplied to the heme groups of OM *c*-Cyts through the oxidation reaction of lactate. The constant influx of electrons results in a displacement of the equilibrium and production of the reduced form of OM *c*-Cyts, which is likely reflected in the positive shift of the E_m in the living system. The occurrence of the E_m shift further demonstrates that hemes localized at the cellular membrane are reduced by metabolically generated electrons. Namely, the microbial current generation originates from the electrons supplied from NADH, which is continuously generated by microbial metabolic processes, such as the conversion of lactate $\rightarrow$ pyruvate $\rightarrow$ acetyl-CoA, or an incomplete TCA cycle, to the electrode via the electron transport system consisting of quinol derivatives and *c*-Cyts in the inner and outer membranes, respectively (see Fig. 1).

4. In-frame deletion mutants of OM *c*-Cyts

The experimental data presented in the above sections clearly demonstrates that the redox wave at 50 mV is attributable to the OM *c*-Cyts of strain MR-1. To identify the specific location of the heme molecules involved in the observed redox reaction, we employed

Fig. 9. Plot of absorbance at 419 nm as a function of electrode potential (black squares) and a cyclic voltammogram of a whole cell of strain MR-1 (scan rate, 10 mV s⁻¹).

several mutant MR-1 strains of membrane-bound *c*-Cyt proteins (OmcA, MtrA, MtrB, MtrC, and CymA). The location and function of these five proteins in MR-1 have been identified using genome sequence analyses (Heidelberg et al., 2002) in conjunction with the biochemical analyses of purified membrane fractions (Myers & Myers 1992). As schematically shown in Fig. 1, OmcA and MtrC are integral OM proteins that are exposed to the extracellular space, and are proposed to mediate DET to solid substrates. These two proteins are a part of the OmcA-MtrCAB a protein complex, which delivers respiratory electrons over a distance of approximately 10 nm, spanning the periplasm to the outer cell surface (Myers & Myers 1997; Pitts et al., 2003). MtrB is a β -barrel porin of the MtrCAB complex and serves as an electron conduit between MtrA to MtrC (Hartshorne et al., 2009), while MtrA is predicted to be located on the periplasmic side of the OM. CymA is an inner-membrane-bound tetraheme *c*-Cyt that acts as a menaquinone oxidase (Firer-Sherwood et al., 2008; Hartshorne et al., 2009). To confirm the involvement of heme molecules in the OM *c*-Cyts proteins of MR-1 cells in the observed EET reaction between bacteria and electrodes, we examined mutant strains with in-frame deletions of the genes encoding MtrA, MtrB, MtrC, OmcA, and CymA. Each mutant was constructed by allele replacement using a two-step homologous recombination method, as previously reported (Saltikov & Newman 2003; Kouzuma et al., 2010).

We first introduced a double-deletion mutant lacking the genes encoding OmcA and MtrC (Δ omcA/ Δ mtrC) into the electrochemical reactor and found that the respiratory current generation ability of the mutant was impaired by 25% relative to wild-type (WT) cells at 400 mV (vs SHE). After the mutant cells were immobilized onto the electrode surface, whole-microorganism voltammetry was conducted. The deletion of the omcA and mtrC genes decreased the anodic (I_{pa}) and cathodic peak currents (I_{pc}) by 68% and 67%, respectively, relative to those observed in WT, with a corresponding shift of E_m from 50 to 135 mV. When the I_{PA} was normalized by total protein content in the biofilms formed by WT and Δ omcA/ Δ mtrC cells, the coulomb value of Δ omcA/ Δ mtrC displayed a 78% decrease compared to WT (Fig. 10). We confirmed that deletion of the gene encoding PilD (Δ pilD), a predicted prepilin peptidase, also resulted in a comparable reduction with Δ omcA/ Δ mtrC (Fig. 10). Western blot

analysis of cell extracts of strain $\Delta pilD$ has demonstrated that PilD is involved in the processing of type IV, Msh, and T2S prepilin proteins, and this strain is also reported to lack OMCs, including OmcA and MtrC (Bouhenni et al., 2010). Our electrochemical comparisons among WT, $\Delta omcA/\Delta mtrC$, and $\Delta pilD$ cells clearly indicate that OmcA and MtrC function as the major reductases for EET from attached cells to electrode surfaces (*i.e.*, DET reaction).

Fig. 10. Coulomb area of the redox peak signals for wild-type (WT) *S. oneidensis* MR-1 and the $\Delta omcA/\Delta mtrC$ and $\Delta pilD$ mutants normalized by the total protein content of cells attached on the electrode surface (QPC). Error bars indicate the standard error of the means calculated with data obtained from greater than three individual experiments.

Fig. 11. Midpoint potentials (E_m) of the redox signal for wild-type (WT) *S. oneidensis* MR-1 and the $\Delta mtrA$, $\Delta mtrB$, $\Delta mtrC/\Delta omcA$, and $\Delta cymA$ mutant strains. Error bars indicate the standard error of the means calculated with data obtained from greater than three individual experiments.

To confirm that the observed redox reaction involves the OmcA-MtrCAB protein complex, the in-frame deletion mutants of MtrA, MtrB, or CymA were further subjected to CV measurements. The effects of each gene deletion on the redox wave of whole cells were compared using E_m values, as changes in the E_m would sensitively reflect conformational and functional changes in the protein complex. The E_m of the $\Delta mtrA$ and $\Delta mtrB$ mutants exhibited a significant E_m shift relative to WT, whereas deletion of the CymA gene caused only a subtle change in the E_m (Fig. 11). As only MtrA and MtrB are directly associated with OmcA and MtrC, comparison of the E_m values among the mutant strains indicates that the most plausible assignment for the redox signal at 50 mV is the OM-bound complex composed of OmcA-MtrCAB.

5. *In-vivo* vs *in-vitro* study of bacterial EET

As can be seen from the energy diagram of bacterial EET presented in Fig. 12, the midpoint potential of OM *c*-Cyts (50 mV) is close to that of menaquinone (80 mV), which is localized to the inner membrane. This result is thermodynamically consistent with the reported ET pathway for *S. oneidensis* MR-1 (Fig. 12) (Shi et al., 2007). However, our studies have revealed that the redox properties of OM *c*-Cyts under *in-vivo* conditions significantly differ from those under *in-vitro* conditions. Specifically, purified OM *c*-Cyts have an E_m that is 200 mV more negative than that observed under *in-vivo* conditions. This finding indicates that the heme environment *in vivo* significantly differs from that *in vitro*, as the redox potential of heme molecules is strongly affected by ligation and hydration (Sola et al., 2002). Therefore, the large potential difference for OM *c*-Cyts under these two conditions highlights the limitation of *in-vitro* studies for understanding the *in-vivo* energetics and dynamics of bacterial EET.

Fig. 12. Energy diagram for the DET pathway from inside to outside cells. Electrons generated by bacterial metabolic processes, such as the conversion of lactate to acetate, are transferred to an extracellular electrode through the redox reaction of menaquinone in the inner membrane and OM *c*-Cyts. The E_m region of purified OM *c*-Cyts is represented as a dashed-line box.

The redox potential of OM *c*-Cyts determined under *in-vivo* conditions severely complicates the current understanding of the flavin-mediated EET pathway, which is considered the primary EET path for microbial current production by *Shewanella* species (Marsili et al., 2008). In this pathway, flavin is proposed to function as a redox mediator that receives two electrons from the reduced form of OM *c*-Cyts (Fe^{2+}) and subsequently delivers the electrons to an extracellular acceptor, such as an electrode surface ($\text{FMN} + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{FMNH}_2$, $E_m = -210$ mV) (Marsili et al., 2008; Gralnick et al., 2010). However, the E_m of FMNH_2 (-210 mV) is approximately 260 mV more negative than that of OM *c*-Cyts determined under *in-vivo* conditions (Fig. 12). As a 260-mV energy barrier would strongly inhibit ET from OM *c*-Cyts to flavin, a different mechanism is likely operating in cells that accounts for the flavin reduction reaction by OM *c*-Cyts. We are currently conducting investigations on the *in-vivo* EET process to resolve this apparent conflict in energetics. Recently, our group has elucidated a novel mechanism underlying the flavin-mediated EET pathway, which will be described in the near future, by noting the specific affinity of flavin for OM *c*-Cyts.

6. Conclusion

We have introduced three techniques to tackle the study of *in-vivo* EET mediated by OM *c*-Cyts: whole-microorganism electrochemistry, UV-vis electrochemical EW spectroscopy, and the in-frame deletion of OM *c*-Cyts. The EET process of *S. oneidensis* MR-1 to an ITO electrode was confirmed by monitoring metabolic current using a single-chamber, three-electrode system. The application of an NO chemical labeling technique indicated that the redox wave detected at +50 mV was assignable to *c*-Cyts localized at the cellular membrane, and EW electrospectroscopy confirmed that the ET process was coupled with metabolic lactate oxidation. Furthermore, using the mutant MR-1 strains, the specific *c*-Cyts responsible for EET were determined to be members of the OmcA-MtrCAB protein complex. Together, these findings represent the first *in-vivo* evidence for the involvement of OM *c*-Cyts in direct EET by *S. oneidensis* MR-1, in which the metabolic electrons generated by intracellular lactate oxidation are delivered to extracellular electrode surfaces as a terminal step for anaerobic respiration.

The results of the new spectroscopic and electrochemical techniques presented in this chapter emphasize the importance of *in-vivo* bioelectrochemistry for the chemical and physical elucidation of the EET process.

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