Electrochemical Study on the Extracellular Electron Transfer Pathway from *Shewanella* Strain Hac319 to Electrodes

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Shewanella can transfer electrons to various extracellular electron acceptors. We electrochemically investigated the pathway of extracellular electron transfer from *Shewanella* strain Hac319 to electrodes. A resting cell suspension of *Shewanella* strain Hac319 containing lactate produced a steady-state sigmoidal wave in the presence of flavin mononucleotide (FMN) in cyclic voltammetry, but not in the absence of FMN. A harvested cell suspension without cell-washing also produced a similar catalytic wave without any external addition of free FMN. The midpoint potentials of the two sigmoidal waves were identical to the redox potential of free FMN. The data indicate that FMN secreted from the *Shewanella* strain Hac319 works as an electron-transfer mediator from the cell to electrodes. An addition of cyanide to a resting cell suspension of *Shewanella* strain Hac319 increased the rate of the FMN reduction in the presence of lactate, while it decreased the respiration rate. By considering the fact that cyanide is coordinated to the heme moiety of hemoproteins and shifts the redox potential to the negative potential side, the data indicate that the electron derived from lactate is predominantly transferred in a down-hill mode from an electron donor with a redox potential more negative than that of FMN without going through outer membrane cytochromes *c* molecules.

Keywords *Shewanella*, extracellular electron transfer, flavin mononucleotide, cytochrome *c*, cyanide

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Introduction

Electron-transfer reactions form the basis of biological energy production, such as the glycolysis process, tricarboxylic cycle, respiratory chain, and photosynthetic process.^{1,2} The genera *Shewanella* and *Geobacter* are able to respire several substrates as electron acceptors, including not only soluble compounds but metallic oxides and electrodes.^{3,4} The catabolic strategies of *Shewanella* and *Geobacter* (reduction of extracellular substrates) are called bacterial extracellular electron-transfer (EET). The EET is important for the elimination of excess electrons generated during the microbial oxidation of organic and inorganic compounds, and for biogeochemical cycling of metals.⁵ The EET has also been used in bacterial leaching and microorganism fuel cells.⁶⁻⁸

Although it is known that the genus *Shewanella* can pass electrons to electrodes,⁴ several proposed mechanisms of the EET in *Shewanella* are controversial, even when the electron acceptor is restricted to an electrode. One of the candidates involved in the EET is flavin mononucleotide (FMN). It was reported that free FMN accelerated the electron transfer from *Shewanella* to iron oxides and electrodes, and *Shewanella* secreted FMN to the supernatant of its culture.⁹⁻¹¹ Knockout of the genes concerning the secretion system and removal of the supernatants resulted in a low output in a microbial current production in the oxidation of substrates.^{10,11} Secreted FMN

may enable one to mediate the electron transfer from *Shewanella* to electrodes. However, the mechanism of the EET remains to be elucidated; FMN may work as a free EET mediator between an internal(s) of the cell and an electrode; or FMN may pass electrons to electron acceptors only inside the cell,¹⁰ or FMN works as an electron carrier for proteins bound on the outer membrane.¹²

On the other hand, series of redox proteins (CymA, MtrA, MtrC and OmcA) are also considered to participate in the EET of *Shewanella*.¹³ These proteins and the electron-transfer pathway through these proteins are called metal-reducing (Mtr) proteins and Mtr pathway, respectively. All Mtr proteins are multiheme *c*-type cytochromes (cyt). CymA is anchoring to the inner membrane of *Shewanella* and the other Mtr proteins are anchoring to the outer membrane (OM). In the Mtr pathway, it is expected that electrons from the oxidation of carbon sources are transferred *via* the menaquinone pool to CymA in the inner membrane. These electrons are transferred through MtrA, and eventually to MtrC and OmcA. MtrC and OmcA are considered to reduce various substrates directly, including organic compounds, metallic oxides, and electrodes.¹⁴⁻¹⁶ It was also reported that MtrC and OmcA existed on the outer membrane extension,¹⁷ called “nanowire”, and transferred electrons directly to an electrode.^{18,19} FMN-reducing activities of the wild type of *Shewanella* were higher than those of various mutant strains lacking the Mtr pathway components.^{20,21} Purified MtrC can transfer electrons through flavins²² and a detailed crystal analysis of MtrC suggested that flavin can access the heme centers.²³ Additionally, other OM cyt *c* molecules, MtrF, and UndF are also considered to be candidates for the EET.^{24,25}

However, it seems to be difficult from the thermodynamic and

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kinetic viewpoints to explain the involvement of the Mtr pathway in the EET. The biological standard redox potentials (E°) of menaquinone and hemes in CymA and MtrA of *Shewanella oneidensis* MR-1 were estimated to be -70 mV, -110 to -265 mV, and -200 to -375 mV vs. SHE.^{26–28} It was proposed that in the Mtr pathway, the electrons derived from carbon sources were transferred to menaquinone with E° of a positive value in the Mtr pathway, and then from menaquinone to CymA with E° of a middle value, and to MtrA with an E° of a negative value. This means that the Mtr pathway from menaquinone to MtrA consists of heavy up-hill electron-transfer reactions, unlike major non-circulating down-hill catabolic systems. Furthermore, the E° values of FMN and hemes of MtrC are located at -220 and $+100$ to -400 mV, respectively.^{29,30} Therefore, when MtrC transfers electrons to FMN, the process is also up-hill from MtrA to FMN via MtrC. Although it was proposed that a positive Gibbs energy in the electron transfer from MtrC to FMN would be decreased by the shift in E° on the binding of FMN to MtrC,¹² the Mtr pathway has still several up-hill processes. As a similar situation, it was reported that riboflavin-bound OmcA also played the role of an EET pathway,³¹ although the electron transfer from OmcA to riboflavin is an up-hill process.

It is reported that ligands, such as cyanide, nitric oxide, and carbon monoxide, are coordinated to the heme moiety, and change the E° of the proteins.^{32–34} For example, the E° of horse heart cyt *c* changes from 260 to 220 mV vs. SHE by cyanide coordination.³⁵ In addition, it is confirmed that nitric oxide is coordinated to the OM cyt *c* of *Shewanella*.³⁶ If cyanide is coordinated to the OM cyt *c* in the Mtr pathway, the coordination will shift the E° of the OM cyt *c* molecules to the negative-potential side, and then it will be expected that the coordination drastically decreases the rate of the up-hill processes in the Mtr pathways.

In this study, we examined the participation of FMN in the EET from *Shewanella* strain Hac319 cells to a glassy carbon (GC) electrode, and also the effects of cyanide addition on the EET using electrochemical methods. The *Shewanella* strain Hac319 relates to *Shewanella oneidensis* MR-1 in 99% similarity over 1454 bases of 16S rRNA gene sequence and produces larger amounts of external FMN than *Shewanella oneidensis* MR-1.⁹ Therefore, this strain is suitable for investigating the influence of FMN secretion on the EET pathway of *Shewanella*. To clarify the participation of FMN, we attempted to observe a *Shewanella*-catalyzed oxidation wave of lactate with artificially added FMN and with FMN secreted from *Shewanella* strain Hac319. In addition, both reaction rates of the FMN-reduction and the respiration by *Shewanella* strain Hac319 were measured in both the presence and absence of cyanide to discuss the electron-transfer pathway of the EET.

Experimental

Materials

The bacterial strain, *Shewanella* strain Hac319, was isolated from dye-works wastewater by growth on a Luria-Bertani (LB) solid medium, and identified by 16S rRNA gene sequencing.⁹

Hipolypepton was purchased from Nihon Pharmaceutical Company (Japan). Bacto® yeast extract was purchased from Becton, Dickinson and Company (USA). All other chemicals were of analytical grade and all solutions were prepared with distilled water.

Growth conditions and preparation of cell suspensions

The *Shewanella* strain Hac319 was grown on a LB liquid medium at 28°C under vigorous shaking conditions. After cultivation for 18 h, the cells were collected by low-speed centrifugation ($3000 \times g$, 15 min, 4°C) and used without washing as harvested cells. In order to remove any secreted FMN remaining in the cell culture media, the harvested cells were washed twice with a phosphate buffered saline (PBS, pH 7.4) and used as resting cells. The cells were collected by low-speed centrifugation ($3,000 \times g$) and re-suspended with the PBS at an optical density at 600 nm (OD_{600}) of 1×10^2 .

Electrochemical measurements

Cyclic voltammetry and chronoamperometry were performed on an electrochemical analyzer, BAS CV-50W. The experiments were conducted under anaerobic conditions with Ar flow to suppress aerobic respiration and autoxidation of the reduced FMN.³⁷ The working electrode was a GC electrode. The reference and counter electrodes were a handmade Ag/AgCl/sat. KCl electrode and a Pt wire, respectively. All of the potentials reported here are referred to the standard hydrogen electrode (SHE). In rotating disk electrode measurements, the GC electrode was attached to the shaft of an electrode rotator BAS RDE-1. Chronoamperometry with the rotating disk GC electrode was carried out at 2000 rpm under anaerobic conditions with Ar flow. The dissolved O_2 concentration was measured with a Clark-type O_2 electrode (Optoscience, Japan) under stirring. All measurements were carried out in cell suspensions of *Shewanella* at an OD_{600} of 1×10^2 and at 25°C using a water-jacket cell under conditions of blocking out light to avoid photo-decomposition of FMN.³⁸

UV-Vis spectrometry

UV-Vis spectrophotometry was carried out on a UV-2550 UV-Vis spectrophotometer (Shimadzu, Japan). Scattering UV-Vis spectroscopy was carried out with an ISP-2200 integral sphere accessory device. Measurements were performed in cell suspensions of *Shewanella* at room temperature.

Results and Discussion

Electrochemical confirmation of FMN participation in the EET

We electrochemically examined the participation of FMN in the EET of *Shewanella* strain Hac319 at pH 7.4. Cyclic voltammograms (CVs) of a concentrated resting cell suspension of *Shewanella* at an $\text{OD}_{600} = 1.0 \times 10^2$ did not show any detectable faradaic current in the presence of 50 mM sodium lactate ($M = \text{mol dm}^{-3}$); but did show a clear sigmoidal steady-state catalytic wave of lactate oxidation in the presence of 20 μM free FMN (Fig. 1). The half-wave potential ($E_{1/2}$) of the sigmoidal wave is -210 mV and is coincident with the E° of free (or dissolved) FMN, -219 mV (pH 7.0).²⁹ Therefore, the wave is ascribed to a *Shewanella*-catalyzed and FMN-mediated oxidation of lactate.^{39,40}

It is well known that *Shewanella* synthesizes FMN and secretes the FMN out of the cell.^{9–11} When we used a harvested cell suspension as a catalyst instead of the resting cell suspension, a similar sigmoidal catalytic wave was observed in the presence of lactate in cyclic voltammetry without an external addition of free FMN (Fig. 1B). The $E_{1/2}$ of the sigmoidal wave is identical with that shown in the resting cell suspension with free FMN (Fig. 1A). These results indicate that FMN secreted from *Shewanella* strain Hac319 participates in the EET from the cell to the GC electrode as an electron-transfer mediator.

Fig. 1 (A) CVs in a resting cell suspension of *Shewanella* strain Hac319 ($OD_{600} = 1.0 \times 10^2$) containing 50 mM sodium lactate under anaerobic conditions in the presence (black solid line) and absence (gray dashed line) of FMN (20 μ M). (B) CVs in resting cell (gray dashed line) and harvested cell (black solid line) suspensions of *Shewanella* ($OD_{600} = 1.0 \times 10^2$) containing 50 mM sodium lactate under anaerobic conditions before at 25°C. The CVs were recorded at a scan rate of 5 mV s⁻¹.

It was reported that FMN binds with MtrC of *Shewanella oneidensis* MR-1 and the binding shifts $E^{\circ'}$ of FMN by +115 mV ($E_b^{\circ'} - E^{\circ'} = 0.115$ V, $E_b^{\circ'}$ being the formal potential of the bound state),¹² indicating an extremely strong attractive interaction of the reduced FMN compared with the oxidized one,

$$K_R/K_O = \exp\{nF(E_b^{\circ'} - E^{\circ'})/RT\} = 8.01 \times 10^3, \quad (1)$$

where K_R and K_O are the binding constants of the reduced and oxidized forms, respectively; n is the number of electrons ($n = 2$ in this case); F is the Faraday constant; R is the gas constant; and T is the absolute temperature. However, our results show no change in $E^{\circ'}$ of FMN between in the free state and in the EET process. It can be concluded that the bound FMN (if any) hardly contributes to the EET process of *Shewanella* strain Hac319 examined under our experimental conditions.

Probability of up-hill electron transfers

If Mtr proteins (for example, MtrC) transfer electrons to FMN, up-hill reactions must proceed between the Mtr proteins, or between the Mtr protein and FMN. Here, we discuss the probability of such up-hill electron transfers as catabolic processes of *Shewanella*. From the transition-state theory and the Marcus theory, the electron transfer rate constant (k_{et}) can be given as follows:

$$\begin{aligned} k_{et} &= \frac{\kappa k_B T}{h} \exp\{-(\Delta_r G^{\circ'} - \lambda)^2/4\lambda RT\} \\ &= Z \exp\{-(\Delta_r G^{\circ'} - \lambda)^2/4\lambda RT\}. \end{aligned} \quad (2)$$

Here, κ is the transition coefficient; k_B is the Boltzmann's constant; $\Delta_r G^{\circ'}$ is the standard Gibbs energy of the electron transfer; λ is the reorganization energy; and h is the Planck constant. From Eq. (2), the self-exchange rate constant of the donor and acceptor (k_{DD} and k_{AA}) can be given as follows, since $\Delta_r G^{\circ'} = 0$ ($\Delta E^{\circ'} = 0$) in the self-exchange electron transfer reaction:

$$k_{DD(AA)} = Z_{DD(AA)} \exp\{-\lambda_{DD(AA)}/4RT\}. \quad (3)$$

When, $|\Delta_r G^{\circ'}| \ll \lambda$, $\kappa = \sqrt{\kappa_{DD}\kappa_{AA}}$ and $\lambda = (\lambda_{DD} + \lambda_{AA})/2$, the Marcus cross-relation can be given as:

$$k_{et} = \sqrt{k_{DD}k_{AA}} \exp(-\Delta_r G^{\circ'}/RT), \quad (4)$$

where λ is derived from the influences of solvents neighboring the redox species.

The self-exchange rate constants of redox proteins range from 10^2 M⁻¹ s⁻¹ to 10^7 M⁻¹ s⁻¹.⁴¹ These values include the factors concerning the diffusion step, the structural factor, and the long-distance electron transfer between the active sites surrounded by polypeptides. Long distance intramolecular electron transfer of one protein can get faster than the above-measured values (equal or larger than 10^8 M⁻¹ s⁻¹).⁴² Therefore, the intramolecular electron transfers in proteins are hardly rate-determining steps. The diffusion-limited process gives a rate constant equal to or smaller than 10^8 M⁻¹ s⁻¹ for redox reactions between free proteins and dissolved redox species under quiescent conditions.

The self-exchange rate constant of FMN (k_{AA}) in water is estimated to be 2.0×10^2 M⁻¹ s⁻¹.⁴⁴ When the self-exchange rate constant from the heme of an Mtr protein to FMN (k_{DD}) is assumed to be 1.0×10^8 M⁻¹ s⁻¹, k_{et} is estimated to be 1.4×10^5 M⁻¹ s⁻¹, even at $\Delta_r G^{\circ'} = 0$ ($\Delta E^{\circ'} = 0$) from Eq. (4). This value is too small as a process in the natural catabolic pathway. Therefore, we can safely conclude that the electron transfer from a protein to a solvated FMN should proceed under down-hill conditions ($\Delta_r G^{\circ'} \ll 0$ or $\Delta E^{\circ'} \gg 0$). An advanced discussion concerning the up-hill electron transfer requires an accurate determination of the standard redox potential of Mtr proteins.

Effects of cyanide on the EET

As described in Introduction, it is well known that cyanide inhibits the respiratory chain due to the coordination to heme proteins. Cyanide is also coordinated to the OM cyt *c* molecules, and shifts the $E^{\circ'}$ of the heme proteins to the negative potential side. This situation must cause a drastic decrease in the rate constant of the up-hill processes of in the EET pathways, especially that in the OM. Therefore, we investigated the effects of cyanide on the EET.

First, we attempted to confirm the state of heme *c* by scattering UV/Vis spectroscopy (Fig. 2). Scattering UV/Vis spectra are ascribed to the absorption of the heme *c* in the microbes. The spectrum of the resting cell suspension of *Shewanella* strain Hac319 showed only a peak at 409 nm. This is a γ peak (Soret band) of cyt *c*. After the addition of dithionite, the γ peak shifted to 413 nm, and two new peaks appeared at 520 and 550 nm as α and β peaks of the reduced cyt *c* molecules. Thus, we can spectroscopically confirm the complete reduction of cyt *c* molecules in *Shewanella* strain Hac319 by dithionite.

Figure 3 shows scattering UV-Vis absorption spectra in the

Fig. 2 Scattering UV-Vis absorption spectra of a resting cell suspension of *Shewanella* strain Hac319 ($OD_{600} = 1.0$) in the absence (gray dotted line) and presence (black solid line) of dithionite (25 mM) under aerobic conditions.

Fig. 4 Continuous measurements of the dissolved O_2 concentration in an aerobic PBS in the absence (gray dotted line) and presence (black solid line) of a resting cell suspension of *Shewanella* strain Hac319 at $OD_{600} = 1.0$. A concentrated cell suspension of *Shewanella* was added to the PBS at a point of at 100 s. CAs were recorded with the Clark-type O_2 electrode at -400 mV.

Fig. 3 Scattering UV-Vis absorption spectra of the resting cell suspension of *Shewanella* strain Hac319 ($OD_{600} = 1.0$) in the absence (gray solid line) and presence of (dotted line) 0.1 mM and (black solid line) 1 mM KCN. Panel A is the original spectrum from 350 to 650 nm. Panels B and C are enlarged views of panel A.

absence and presence of KCN. After the addition of 1 mM KCN, the γ peak shifted to the long-wavelength side and small α and β peaks appeared. The phenomena may not simply be explained by the cyanide coordination to the oxidized form of the OM cyt *c* molecules, but some reductions of the OM cyt *c* are suggested. Because cyanide is not able to directly reduce heme *c*,³⁷ but inhibits aerobic respiration, inhibition due to the cyanide coordination seems to induce a reduction of the OM cyt *c* molecules. The electron donor here may be some contaminants in the resting cell suspension.

In order to confirm the above points, we measured O_2 consumption by the Clark-type O_2 electrode in the resting cell suspension of *Shewanella* strain Hac319 in the absence of externally added lactate. Figure 4 shows a chronoamperogram (CA) of the O_2 reduction; the consumption of dissolved O_2 was observed in the presence of the resting cell suspension of *Shewanella*. This O_2 consumption is due to the aerobic respiration of *Shewanella*. The result means that small amounts of electron donors remained in the resting cell suspension in this study. The possible origins of the electron donors are some organic compounds in the microbes and/or the culture media, but we could not identify them. As described in the next paragraphs, it is found that cyanide decreases the respiration rate of *Shewanella* cells in the presence of lactate. The coordination of cyanide to heme proteins induces a negative shift in E° of the heme proteins, which seems to be responsible for the inhibition of the respiration. The electrons generated in the metabolism of contaminants seem to be transferred to the OM cyt *c* molecules as evidenced in the scattering UV-Vis spectral change. However, the reduction does not seem to be completed, as compared with the scattering UV-Vis spectral change on the full reduction with dithionite (Fig. 2). Most probably, the reduction process is up-hill and different from the EET process.

We also measured the effects of cyanide on the EET rate of *Shewanella* strain Hac319 in the presence of lactate and FMN. Figure 5 shows CA in the resting cell suspension of *Shewanella*. The oxidation current was measured at 0 mV with the rotating GC electrode, and was proportional to the concentration of the reduced FMN in the cell suspension. When FMN was added to the suspension at a point of 80 s in Fig. 5, the steady-state oxidation current increased with time. Such an oxidation current was not observed in the absence of *Shewanella*.

Fig. 5 CA in a resting cell suspension of *Shewanella* strain Hac319 ($OD_{600} = 1.0 \times 10^2$) containing 50 mM sodium lactate under anaerobic conditions. FMN was added to the cell suspension at an analytical concentration of 200 μM at a point of 80 s. CA was recorded with the rotating disk GC electrode at 2000 rpm and 0 mV.

Fig. 6 KCN concentration (c_{KCN}) dependence of the FMN-reducing rate (square) and the respiration rate (triangle) of the resting *Shewanella* strain Hac319 cells. The rates of the FMN reduction by *Shewanella* were evaluated from the slope of the tangent of CAs after the addition of an FMN stock solution into the resting cell suspension of *Shewanella* ($OD_{600} = 1.0 \times 10^2$) containing 50 mM sodium lactate under the anaerobic condition. The rates of the O_2 consumption by *Shewanella* were evaluated with the Clark-type O_2 electrode from the slope of the tangent of CA s after the addition of *Shewanella* cells into the PBS at $OD_{600} = 1.0 \times 10^2$. The rates of the FMN reduction and the O_2 consumption are normalized by the rates at $c_{\text{KCN}} = 0$ M.

These results indicate that an increase in the oxidation current is ascribed to *Shewanella*-catalyzed FMN reduction in which lactate is an electron donor. We evaluated the FMN-reducing rate by *Shewanella* from the initial slope in CA immediately after the addition of FMN. Using the same method, we also evaluated the FMN-reducing rates at several KCN concentrations. As shown in Fig. 6, KCN increased the rate of *Shewanella*-catalyzed FMN reduction. From the initial slope of the O_2 consumption, we also examined the effects of cyanide on the respiration rate by the O_2 electrode. As shown Fig. 6, KCN decreased the respiration rate.

Cyanide is coordinated to cyt *c* oxidase and inhibits the respiration of microorganisms.⁴⁵ In fact, the respiration rate decreases with KCN, as shown in Fig. 6. Cyanide must also be coordinated to the OM cyt *c* to induce a negative shift in E° of the OM cyt *c* molecules. The situation seems to drastically decrease the rate constant of the Mtr pathway. However, the rate of *Shewanella*-catalyzed FMN reduction increases with an addition of KCN. The increase in the FMN reduction seems to

be due to an inhibition of the respiratory chain. The results clearly indicate that the electron transfer from menaquinone to FMN via the OM cyt *c* molecules is not the major process in the EET pathway. Electrons generated in the catabolic pathway are predominantly transferred from an electron donor with a more negative E° , such as NADH to FMN as a main EET pathway.

Conclusions

Participation of FMN in the EET of *Shewanella* strain Hac319 has been confirmed in the view of thermodynamics and kinetics mainly based on electrochemical methods. We compared the effects on the EET of externally added FMN with those of the FMN secreted from *Shewanella*. Because similar sigmoidal waves were obtained in both cases, we have concluded that secreted FMN is involved in the EET. The E° of FMN involved in the EET is almost identical with that of free FMN. We cannot find any evidence concerning FMN binding to the OM cyt *c* molecules in the *Shewanella* strain Hac319. Secreted FMN seems to penetrate into the cell to accept electrons. We also investigated the effect of cyanide on the EET. Cyanide is coordinated to the OM cyt *c* molecules and other heme proteins including cyt *c* oxidase and causes a negative shift in E° of the heme proteins. It has been found that the cyanide coordination enhances the EET rate and suppresses the respiratory rate. Since the negative shifts in E° of the OM cyt *c* molecules must drastically inhibit the up-hill electron transfer from menaquinone to FMN via the OM cyt *c* molecules (if any), the electrons for the reduction of FMN is predominantly supplied from an electron donor with a negative E° (such as NADH) to realize a down-hill electron transfer without going through the OM cyt *c* molecules. This work of the EET mechanism is useful not only for understanding the biogeochemical iron cycle in subsurface environments, but also for improving systems for bacterial leaching and anode construction for microorganism fuel cells.

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