In-vivo identification of direct electron transfer from *Shewanella oneidensis* MR-1 to electrodes via outer-membrane OmcA–MtrCAB protein complexes

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**ABSTRACT**

The direct electron-transfer (DET) property of *Shewanella* bacteria has not been resolved in detail due to the complexity of in vivo electrochemistry in whole-cell systems. Here, we report the in vivo assignment of the redox signal indicative of the DET property in biofilms of *Shewanella oneidensis* MR-1 by cyclic voltammetry (CV) with a series of mutants and a chemical marking technique. The CV measurements of monolayer biofilms formed by deletion mutants of *c*-type cytochromes (ΔmtrA, ΔmtrB, ΔmtrC/ΔomcA, and ΔcymA), and pilin (ΔpilD), capsular polysaccharide (ΔSO3177) and menaquinone (ΔmenD) biosynthetic proteins demonstrated that the electrochemical redox signal with a midpoint potential at 50 mV (vs. SHE) was due to an outer-membrane-bound OmcA–MtrCAB protein complex of decaheme cytochromes, and did not involve either inner-membrane-bound CymA protein or secreted menaquinone. Using the specific binding affinity of nitric monoxide for the heme groups of c-type cytochromes, we further confirmed this conclusion. The heterogeneous standard rate constant for the DET process was estimated to be 300 ± 10 s\(^{-1}\), which was two orders of magnitude higher than that previously reported for the electron shuffling process via riboflavin. Experiments using a mutant unable to produce capsular polysaccharide (ΔSO3177) revealed that the DET property of the OmcA–MtrCAB complex was not influenced by insulating and hydrophilic extracellular polysaccharide. Accordingly, under physiological conditions, *S. oneidensis* MR-1 utilizes a high density of outer-membrane-bound OmcA–MtrCAB complexes as terminal reductases for the DET electrode-respiring process.

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**1. Introduction**

Dissimilatory metal-reducing bacteria (DIRB), such as members of the Gram-negative bacterial genus *Shewanella*, are model organisms to study electrochemistry associated with respiratory electron transfer [1–7]. The mechanism of which has been examined extensively for application in biological fuel cells [8–14]. DIRB are capable of reducing solid-phase minerals, such as Fe(III) and Mn(IV) oxides, as the terminal step in the respiratory process [5]. Since such minerals are insoluble in water at neutral conditions and are unable to enter cells, the electrons generated by the TCA cycle need to be delivered to the mineral electron acceptors located in the extracellular space. One of the proposed mechanisms for extracellular electron transfer (EET) in *Shewanella oneidensis* strain MR-1 is the Mtr respiratory pathway, in which a series of multiheme *c*-type cytochromes (*c*-CysTs) transports electrons from the inner membrane (IM) to the outer membrane (OM) (Scheme 1) [15,16].

Electrons finally reach the OM-bound cytochromes (OMCs) OmcA and MtrC, resulting in enzymatic reduction of Fe(III) and Mn(IV) oxides.

It has been demonstrated using protein film voltammetry (PFV) that purified OmcA and MtrC are able to mediate direct ET (DET) to graphite and Fe(III)-oxide electrodes [17,18]. Kinetic analyses revealed that ET occurs within millisecond orders at the protein/electrode interface [18]. Moreover, protein–protein interaction to facilitate DET to a graphite electrode has been recently demonstrated using the MtrCAB protein complex [19]. Although the electrochemical properties of protein-mediated EET have become clearer through studies utilizing PFV, controversy exits concerning the roles of OMCs in EET, and the DET process has not been resolved in detail due to the complexity of in vivo electrochemistry in whole-cell systems.

To examine the EET properties of OMCs in vivo, our group has developed a chemical marking technique for the detection of OMCs attached directly on an electrode surface [4]. Using the specific affinity of nitrogen monoxide (NO) for heme groups of c-CysTs, the redox signal assigned to OMCs responsible for DET in *Shewanella loihica* PV-4 cells was identified under physiological conditions. Fast-scan voltammetry revealed that the ET process was medi-
2.1. Cell preparation

*S. oneidensis* MR-1 was grown aerobically in 10 ml Luria–Bertani (LB) medium (20 g l\(^{-1}\)) at 30 °C for 16 h. Subsequently, the culture was centrifuged at 6000 rpm for 10 min, and LB was replaced with defined medium (DM), which contained 10 mM lactate as the sole carbon source, as described by Roh et al. [20]. The cells were further cultivated aerobically at 30 °C for approximately 10 h in DM containing lactate (DM-L). The cell culture (OD\(_{600} = 1.6\)) was then centrifuged at 6000 rpm for 10 min, and the resultant cell pellet was washed twice with DM prior to being used for electrochemical experiments. For confocal fluorescence microscopic measurements, *S. oneidensis* MR-1 was transformed to constitutively express the gene for green fluorescent protein (GFP) to facilitate visualization of individual cells on the ITO electrode. Briefly, the gfp gene from the vector pZSGreen (Clontech) was subcloned into plasmid pBBR1-MSC2 (kanamycin resistance), and the resulting plasmid was transformed into *S. oneidensis* MR-1 using competent cells of *Escherichia coli* strain WM6026. Transformants were selected on LB agar containing with 20 μg/ml kanamycin. All culturing and electrochemical experiments with LB or DM-L were also conducted using medium supplemented with 20 μg/ml kanamycin, unless otherwise noted.

2.2. Mutant bacteria and cultivation

Mutant MR-1 strains lacking MtrC/OmcA, MtrB, MtrA, CymA, PilD, MenD and SO3177 were constructed as described previously [6,21–24]. The culture procedure for those mutants was identical to that used for wild-type (WT) cells, with the exception of ΔmenD, which was cultivated in medium supplemented with 25 μg/ml kanamycin.

2.3. Electrochemical measurements

A single-chamber three-electrode system was used for the electrochemical measurements of intact cells [4,6]. Tin-doped indium oxide (SPD Laboratory, Inc.) (ITO) grown on a glass substrate by spray pyrolysis deposition was used as the working electrode (resistance, 8 Ω (square; thickness, 1.0 mm surface area, 3.1 cm\(^2\)), while the reference and counter electrodes were Ag/AgCl (KCl sat.) and platinum wire, respectively. The cyclic voltammetry measurements were conducted using automatic polarization system (HZ-5000; Hokuto Denko). Five milliliters DM containing 10 mM lactate was added into the electrochemical cell as an electrolyte, and the solution was then deaerated by bubbling with N\(_2\) for approximately 30–45 min. After reaching 0.1 ppm of dissolved O\(_2\), as measured using a Microx TX3 trace instrument (PreSens), 0.3 ml freshly prepared cell suspension was injected into the electrochemical cell under the potentiostatic condition of 0.2 V (vs. Ag/AgCl KCl sat.). The reactor temperature was maintained at 30 °C and no agitation was made during the measurements. Charging current subtraction for cyclic voltammograms (CVs) was conducted using analysis software (HZ-5000ANA; Hokuto Denko), and the charging current was approximated with a linear function.

2.4. Confocal fluorescence microscopy

Microscopic observations of biofilms were performed on the stage of an inverse Olympus IX 71 microscope equipped with a 60× oil-immersion objective lens. For in situ imaging with electrochemical measurements, the system and procedures were identical as those described above, except that thinner ITO glass (thickness: 150 μm) was used as the working electrode to achieve high resolution imaging. An indium gallium nitride laser (λ = 405 nm) was used in conjunction with a long-pass filter (λ = 415–505 nm). Plane-view-transmission sections with side profile slices were generated using Andor iQ software (version 1.9; Andor Technology).

2.5. Scanning electron microscopy

The ITO electrode with attached cells was carefully removed from the electrochemical reactor and washed with HEPES buffer. The sample was then dried under vacuum at 25 °C, coated with evaporated platinum, and viewed using a YE-9800 electron microscope (Keyence) at an operating voltage of 2 kV.

![Scheme 1](image-url)
Fig. 1. (A) Confocal fluorescent images of biofilm of MR-1 cells expressing green fluorescent protein on an ITO electrode after 25 h of electrochemical cultivation at +200 mV vs. Ag/AgCl KCl sat. (B) A cross-sectional image of the biofilm (vertical length is 6.0 μm). For each image, 64 pictures (captured every 25 ms) were averaged. (C) An SEM image of the ITO electrode surface after 25 h electrochemical cultivation.

2.6. Protein content assay

To determine total protein content, the ITO electrodes with attached cells were washed once with an aqueous buffer solution consisting of 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄ (pH 7.5), placed in a 2-ml tube with 300 μl phosphate buffered saline (PBS), and then vortexed for 5 min. The protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce) according to the manufacturer’s instructions.

3. Results and discussion

3.1. Electrochemical signals from WT cells

Biofilms of S. oneidensis MR-1 cells were fabricated on an ITO electrode by inoculating an electrochemical reactor poised at +0.2 V (vs. Ag/AgCl KCl sat.) with a cell suspension of OD₆₀₀ 0.1 in medium with lactate as the carbon and electron source. Within 1 h of adding the cells into the reactor, a current of ~2 μA cm⁻² was observed, which reflects the cellular oxidation of lactate and EET to the ITO electrode. By in situ monitoring of the cell growth on the ITO electrode using fluorescence confocal microscopy (Fig. 1A and B), a monolayer biofilm with a cell density of ~7 × 10⁷ cell cm⁻² (~75% coverage) was obtained after 25 h of electrochemical cultivation. An SEM image of the electrode surface obtained after rigorous washing indicated the robustness of the attached biofilm (Fig. 1C).

To determine the feasibility of detecting DET from whole cells in vivo, we next obtained a baseline-subtracted cyclic voltammogram (CV) for the electrode with attached cells at a scan rate of 100 V s⁻¹ (Fig. 2A). The midpoint potential (Eₘ), peak current, and half-bandwidth of the redox signal were similar with those obtained for a monolayer biofilm of strain PV-4 [4]. Although the CV measurement was conducted in the presence of lactate, the lack of a catalytic current, which typically appears at a potential more positive than ~0.3 V (vs. SHE), indicates that a scan rate of 100 V s⁻¹ is sufficiently fast to eliminate the effects of the cellular oxidation of lactate, which would lead to the injection of electrons to OMCs, from the EET process to electrodes. The plot of the anodic and cathodic peak currents, IₚA and IₚC, respectively, as a function of scan rate displayed a linear relationship (Fig. S1). Such
a relationship is characteristic for electron-exchange processes of immobilized species lacking mass-transfer limitations, confirming the occurrence of the DET reaction from cells to electrodes.

The $k_0$ of DET was estimated to be $300 \pm 10 \text{s}^{-1}$ by Trumpet plot analysis [30]. This value is the same order of magnitude with the $k_0$ previously determined for OMCs in strain PV-4 biofilms ($k_0 = 150 \text{s}^{-1}$) [4] and those reported for purified MtrC and the MtrCAB protein complex immobilized on a basal-plane graphite electrode (220 and 195 s$^{-1}$, respectively) [19]. Notably, the $k_0$ value for MR-1 cells is two orders of magnitude higher than that for riboflavin immobilized on an electrode surface ($k_0 < 0.7 \text{s}^{-1}$) [4,26], which is reported to be an important electron shuttle produced by Shewanella cells for EET [26].

3.2. ΔomcA/ΔmtrC and ΔpilD mutants

To confirm the involvement of the OMC proteins of *S. oneidensis* cells in the observed DET reaction, we first examined a mutant that lacked the genes encoding OmcA and MtrC (ΔomcA/ΔmtrC). Following the introduction of the mutant into the electrochemical reaction, the double deletion of OmcA and MtrC initially impaired cellular respiratory current; however, the current had recovered to 25% of the WT value after 25 h. At the end of the cultivation period, a monolayer biofilm similar to that formed by WT cells was observed on the electrode. As shown in Fig. 2A, ΔomcA/ΔmtrC decreased the $I_{pa}$ and $I_{pc}$ by 68% and 67%, respectively, with a shift of $E_m$ from 50 to 135 mV relative to those observed in WT. When the $I_{pa}$ was normalized by total protein content in the biofilms formed by WT and ΔomcA/ΔmtrC cells, the coulomb value of ΔomcA/ΔmtrC showed a 78% decrease compared to WT (Fig. 2B). We confirmed that ΔpilD also exhibited a comparable reduction with ΔomcA/ΔmtrC (Fig. 2B). The PilD gene has been demonstrated by western blot analysis of cell extracts to process type IV, Msh, and T2S pilin proteins, and strain ΔpilD is also reported to lack OMCs, including OmcA and MtrC [27]. Our electrochemical comparisons among WT, ΔomcA/ΔmtrC, and ΔpilD cells clearly indicate that OmcA and MtrC function as the major reductases for DET from attached cells to electrode surfaces.

3.3. ΔmtrA, ΔmtrB, and ΔcymA mutants

In *S. oneidensis* MR-1, OmcA and MtrC proteins are part of a protein complex that delivers respiratory electrons over an approximately 10 nm distance, from the periplasm to the outer cell surface [28,29], as depicted in Scheme 1B. MtrB protein was identified as a β-barrel porin of the MtrCAB complex structure and serves as an electron conduit from MtrA to MtrC [19]. MtrA is predicted to be located on the inner side of the OM, while CymA is an IM-bound tetraheme c-Cyt that acts as a menaquinone oxidase [18,19]. Here, we subjected in-frame deletion mutants that lacked either MtrA, MtrB, or CymA to electrochemical analysis of the MtrCAB protein complex. The effects of each gene deletion on DET were compared using the $E_m$ value, as changes in the $E_m$ would sensitively reflect any conformational changes in the protein complex. As shown in Fig. 3, the $E_m$ of ΔmtrA and ΔmtrB exhibited a significant shift relative to that of WT, whereas the deletion of the CymA gene caused only a subtle change in $E_m$. As MtrA and MtrB are directly associated with OmcA and MtrC, but not CymA, the comparison of the $E_m$ shown in Fig. 3 indicates that the most plausible assignment for the redox signal for monolayer biofilms is the OM-bound complex composed of OmcA–MtrCAB proteins.

3.4. Chemical marking of c-Cyts and the ΔmenD mutant

It is worth noting here that in addition to OMCs, *S. oneidensis* synthesizes flavin and quinone derivatives that function as electron shuttles, a few of which strongly bind to outer cell surfaces and provide the redox signals characteristic of immobilized electroactive species [4,25,26]. Among the produced derivatives, the $E_m$ of menaquinone locates in the range of 0 to −0.1 V (vs. SHE), which is within the potential region of the broad redox signal resolved here and by other groups for *Shewanella* biofilms [2]. Thus, several reports suggest the possibility that cell-attached menaquinone represents the origin of the redox signal of *Shewanella* at an $E_m$ of approximately 50 mV [30,31].

To examine the role of menaquinone in DET, we prepared MR-1 cells whose c-Cyts contained heme groups ligated with an NO molecule [4]. The NO-ligated hemes were present in the c-Cyts located in both the OM and periplasm regions, as confirmed by UV/vis evanescent wave experiments (data not shown) [4]. In the CV obtained at a scan rate of 10 mV s$^{-1}$ for NO-MR-1 cells, the redox wave observed at 50 mV for untreated cells completely disappeared, and a new redox wave appeared at 650 mV (Fig. 4).

![Fig. 3. Midpoint potentials ($E_m$) of the redox signal for a monolayer biofilm of WT, ΔmtrA, ΔmtrB, ΔmtrC/ΔomcA, ΔcymA, ΔmenD, and ΔSO3177. Error bars indicate the standard error of the means calculated with data obtained from greater than three individual experiments.](image)

![Fig. 4. Cyclic voltammograms of strain MR-1 at a scan rate of 10 mV s$^{-1}$ (a) before and (b) after an NO coordination reaction. The NO ligation to hemes of strain MR-1 was performed by exposing the bacterial suspension to NO for 10 min under anaerobic conditions. The suspension was then centrifuged and the supernatant was replaced with fresh DM-L to remove the excess dissolved NO. The resultant cell suspension was injected into the reactor to give an OD$_{600}$ of 2.0, and was then subjected to cyclic voltammogram measurements after 30 min of electrochemical cultivation.](image)
Since menaquinone possesses no specific affinity for NO, the results exclude the contribution of menaquinone in the redox signal at 50 mV.

It was further confirmed that deletion of the ΔmenD gene, which encodes a menaquinone biosynthetic protein, caused a subtle change in the half-bandwidth and redox potential of the S. oneidensis electrode biofilm (Figs. 3 and 5). These results demonstrate that secreted menaquinone is not involved in the DET process at cell/electrode interfaces. Collectively, the CV experiments with both WT and mutant biofilms, combined with the examination of NO-ligated MR-1 cells, has verified the ability of strain MR-1 to perform DET via the OmcA–MtrCAB complex of proteins (Scheme 1B). This conclusion follows the assumption that the redox signal observed in our previous study with strain PV-4 was due to homologs of OmcA, MtrC, MtrA, and MtrB [4].

The diameter of the decaheme c-Cyts MtrC and OmcA adsorbed on solid electrodes is reported to be 5 and 8 nm, respectively, based on STM measurements [32]. Therefore, the coverage of decaheme c-Cyts adsorbed on the ITO electrode surface was estimated to be approximately 20% (the amount of electrode-attached c-Cyts was 0.83 pmol cm⁻²), as determined from the coulombic area of the redox signal [33]. The accuracy of this estimate is supported by the values determined by antibody recognition force microscopy experiments, in which the surface coverage of decaheme c-Cyts on MR-1 cells was estimated to range from 8% to 34% [34]. The reasonable accordance of the estimated surface coverage of OMCs with that determined for WT biofilms. In addition, both the Eₘ shift and increase in the amount of electroactive OMCs was not prominent, as shown in Figs. 2B and 3. The DET reaction between OMCs and electrodes requires a distance of less than 10 Å between the heme groups and electrode if electrons are transferred to the electrode by through-space ET, as reported for a small tetraheme Cyt isolated from strain MR-1 [37]. Accordingly, the experiments with ΔSO3177 indicate that under physiological conditions, most OmcA–MtrCAB protein complexes are in direct contact with the electrode surface, and extracellular polysaccharides do not have a significant influence on this interaction and the DET process.

In summary, we have demonstrated for the first time that OmcA–MtrCAB protein complexes mediate DET from MR-1 cells to electrode under physiological conditions. We also identified that nearly all of the OmcA–MtrCAB complexes exposed at a cell/electrode interface directly contact the electrode surface and are only subtly influenced by the presence of insulating and hydrophilic capsular polysaccharide, as illustrated in Scheme 1B. Based on the present in vivo assignment of a DET pathway in MR-1, a further study is underway to elucidate the role of self-secreted flavin on the DET process, which has been recently proposed to be a critical cofactor for EET from OMCs to electrodes and Fe(III) oxides [13,26].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.electacta.2011.03.076.

References

[33] The amount of electroactive OM c-Cyts was estimated from the redox peak area in the cyclic voltammogram with the assumption that all the OM Cyts are composed of decaheme.