Long-range electron conduction of *Shewanella* biofilms mediated by outer membrane C-type cytochromes

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**Article info

**Abstract**

We investigated the role of c-type cytochromes (c-Cyts) in electron conduction across biofilms of *Shewanella oneidensis* MR-1 and the relevance of the electron conductivity for biological current generation. Following the formation of monolayer and multilayer biofilms on indium-tin oxide electrodes, we quantified the c-Cyts that were electrically wired with the electrode surface using whole-cell voltammetry. A multilayer biofilm with a thickness of 16 μm exhibited a redox peak with an 8-fold larger coulombic area than that of a monolayer biofilm (about 0.5-μm thickness), indicating an abundance of c-Cyts that are able to perform redox-cycling reactions with the distant electrode surface. To determine if this electron conduit of c-Cyts participated in biological current generation, we conducted slow-scan voltammetry for multilayer biofilms. A large anodic current of c-Cyts caused by microbial lactate oxidation was observed during the slow-potential scans, demonstrating the transport of respiratory electrons via the sequential redox cycling of c-Cyts. Experiments with deletion mutants deficient in outer-membrane (OM) c-Cyts (ΔmtrC/ΔomcA, Δ pilD), and the biosynthetic protein of capsular polysaccharide (ΔS03177) suggested that cell-surface-bound c-Cyts, but those located on pili or extracellular polymeric substrates, play a predominant role in the long-range electron conduction in the biofilm of *S. oneidensis* MR-1.

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1. Introduction

Dissimilatory metal-reducing bacteria (DIRB), such as members of the bacterial genera *Shewanella* and *Geobacter*, are capable of directly transporting respiratory electrons to solid electrodes without the use of exogenous redox mediators [1-4]. This type of electron transfer, termed extracellular electron transfer (EET), has attracted considerable recent attention because of its relevance in the development of bioelectrochemical systems, such as microbial fuel cells (MFCs) [5-8]. It has been experimentally demonstrated that DIRBs establish direct and indirect EET conduits to electrode surfaces using outer-membrane c-type cytochromes (OM c-Cyts) and self-secreted soluble redox mediators, respectively [9-13]. In addition to these EET pathways, the importance of electron transport through solid conductive biofilms for high performance anodes in MFCs has also been demonstrated [14]. Compared with a diffusion-controlled electron shuttling process mediated by redox mediators, the electron conduction ability of microbial biofilms is advantageous for the efficient collection of metabolically generated electrons from cells located at a distance from electrode surfaces [14].

To explain the electron conduction mechanism in biofilms, several models have been proposed based on the redox properties of bacterial c-Cyts. For example, Lovely et al. [15,16] reported in *Geobacter* that c-Cyts which are loosely bound to the matrices of extracellular polymeric substrates primarily mediate the transfer of metabolically generated electrons to distant electrode surfaces. Moreover, pilus-like appendages, termed bacterial nanowires, which also contain surface-located c-Cyts have been identified in both *Shewanella* and *Geobacter*, and are postulated to transport electrons from distant cells to electrodes [17-19]. However, these studies are based on analyses performed under in-vitro or non-catalytic conditions, and thus, the validity of these electron conduction mechanisms remains ambiguous. Therefore, more concrete evidence for the role of c-Cyts located at a distance from electrode surfaces in both electron conduction and electrical current generation is needed.

We recently identified the voltammetry signals of OM c-Cyts in monolayer biofilms of *S. oneidensis* [12,20], indicating that bacterial c-Cyts electrically linked with an electrode surface can be specifically quantified under in-vivo conditions. Here, we fabricated both monolayer and multilayer biofilms of *S. oneidensis* on tin-doped indium oxide (ITO) electrodes and compared the respective amounts of electroactive c-Cyts using the voltammetry techniques. The scan-rate dependence of cyclic voltammograms (CVs) was used to investigate the role of c-Cyts in the transfer of respiratory electrons to distant ITO electrodes. We also investigated the electron conduction mechanisms
of the two types of Shewanella biofilms using several mutant strains that were deficient in OM c-Cyts and a biosynthetic protein of capsular polysaccharide.

2. Materials and methods

2.1. Strains and culture conditions

S. oneidensis MR-1 was grown aerobically in 10 mL Luria–Bertani (LB) medium (20 g L$^{-1}$) at 30 °C for 16 h. The culture was then centrifuged at 6000 × g for 10 min, and the resultant cell pellets were resuspended in 10 mL of 10 mM defined medium (DM; NaHCO$_3$ (2.5 g), CaCl$_2$·2H$_2$O (0.08 g), NH$_4$Cl (1.0 g), MgCl$_2$·6H$_2$O (0.2 g), NaCl (10 g), and (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES; 7.2 g) (per liter)) [20] supplemented with 10 mM lactate as the sole carbon source (DM-L). The cells were further cultivated aerobically at 30 °C for 2 days, centrifuged for 10 min, and the resultant cell pellet was washed three times with DM prior to being used for electrochemical experiments. Mutant strains deficient in the genes encoding MtrC/OmcA, PilD, and SO3177 were previously constructed by allele replacement using a two-step homologous recombination method [21,22]. For cells labeled with green fluorescent protein (GFP), all culturing and electrochemical experiments were performed with LB or DM-L supplemented with 20 μg/mL kanamycin to select for the GFP-expressing plasmid.

2.2. Electrochemical measurements and biofilm preparation

A single-chamber, three-electrode system for whole-cell electrochemistry was constructed as described previously [23]. An ITO substrate (surface area: 3.1 cm$^2$) placed at the bottom of the reactor was used as the working electrode, and Ag/AgCl (sat. KCl) and a platinum wire were used as the reference and counter electrodes, respectively. Five mL of DM-L was added into the electrochemical cell as an electrolyte and was deaerated by bubbling with N$_2$ for more than 30 min. Cell suspensions with an optical density at 600 nm (OD$_{600}$) of 5.0 and 0.1 were inoculated into the reactor, which was maintained at 30 °C with the electrode poised at a potential of +0.2 V (vs. Ag/AgCl KCl sat.) for 25 h without agitation for the generation of multilayer and monolayer biofilms, respectively. Cyclic voltammetry measurements were conducted using an automatic polarization system (HZ-5000; Hokuto Denko). Charging current was subtracted using analysis software (HZ-5000ANA; Hokuto Denko) which fits a linear function to the baseline in regions sufficiently far from the peak, assuming continuation of a similar and smooth charging current throughout the peak region.

2.3. Confocal fluorescence microscopy

The microscopic observations of biofilms formed on the ITO electrodes were conducted using an inverse Olympus IX 71 microscope equipped with a 60× oil-immersion objective lens. For in-situ imaging during electrochemical measurements, S. oneidensis MR-1 was transformed to constitutively express the GFP gene to facilitate the visualization of individual cells on the ITO electrode [20]. The electrochemical system was identical as that described above, except that thinner ITO glass (thickness: 150 μm) was used as the working electrode to allow high-resolution imaging. Imaging was performed using the semiconductor laser (λ = 405 nm) of indium gallium nitrite (InGaN) 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).

3. Results and discussion

3.1. Electrochemical signals from multilayer and monolayer biofilms

To fabricate S. oneidensis MR-1 biofilms of different thickness, GFP-labeled cells were inoculated into an electrochemical reactor to give an initial OD$_{600}$ of either 5.0 or 0.1. After 25 h of cultivation in the reactor equipped with an ITO electrode poised at 200 mV (vs. Ag/AgCl KCl sat.), the biofilm structures that had developed on the electrodes were visualized using several mutant strains that were deficient in OM c-Cyts and a biosynthetic protein of capsular polysaccharide.

![Image](image_url)

**Fig. 1.** Three-dimensional confocal fluorescent images of biofilms of gfp-MR1 cells formed on an ITO electrode after 25 h of electrochemical cultivation at +200 mV (vs. Ag/AgCl) with an initial OD$_{600}$ of 5.0 (a) and 0.1 (b). Cross-sectional images of the biofilms in the cells of OD$_{600}$ 5.0 and 0.1 are presented in panels (c), and (d), respectively. Schematic image of biofilms grown in the reactors with an initial OD$_{600}$ of 5.0 and 0.1 are shown in panels (e) and (f), respectively.
was inspected by confocal fluorescence microscopy (Fig. 1). Fig. 1(a) shows the three-dimensional image of the electrode from the reactor inoculated with cells at an initial OD_{600} of 5.0. In this reactor, GFP-fluorescent cells covered the electrode surface heterogeneously (Fig. 1a). The cross-section image revealed the formation of a multilayer biofilm with a thickness of approximately 16 μm (Fig. 1c). For the electrochemical reactor with an initial OD_{600} of 0.1, a monolayer biofilm of a 1–2-μm thickness with a surface coverage of approximately 75% was obtained on the ITO electrode (Fig. 1b and d).

We estimated the amount of electrically active c-Cyts present in both the multilayer and monolayer biofilms using whole-cell voltammetry (Fig. 2). CVs were taken in the presence of electrolyte containing lactate, which provided a carbon and electron source to maintain cellular function. For the measurements of the CVs, a high scan rate of 100 Vs⁻¹ was applied to eliminate metabolic current generation from the redox reaction of c-Cyts [12]. As shown in Fig. 2, the CV for the multilayer biofilm exhibited a redox wave with a midpoint potential of 50 mV, which was identical to that of the monolayer biofilm. Notably, however, the CV for the multilayer biofilm displayed a large increase in both the anodic and cathodic currents. The redox waves at 50 mV were assignable to c-Cyts [12,20], and the concentration of the electrically active c-Cyts for the multilayer biofilm was estimated to 6.7 pmol cm⁻² with the assumption that all the Cyts are composed of decaheme. Since the diameter of Shewanella's decaheme Cyts is in the range of 5–8 nm, this value corresponds to a coverage of the electrode surface by c-Cyts of approximately 160% [12,24,25]. This redox charge is not limited by the diffusion process, as the peak current showed a linear increase against scan rates in the range from 100 mVs⁻¹ to 180 Vs⁻¹ (Fig. 2S). Thus, these results indicate the existence of an abundance of c-Cyts that can participate in redox-cycling reactions with a distant electrode surface in a diffusionless electron-exchange manner. This electron conduction property is consistent with that identified in previous electrochemical kinetic analysis of mixed-culture biofilms [14].

3.2. Redox current distortion in slow-potential scan voltammetry

We next investigated the contribution of the observed electron conduction property of Shewanella biofilms mediated by c-Cyts to microbial current generation. More specifically, to determine if respiratory electrons generated by microbial metabolism are transported to distant electrodes via the redox cycling of c-Cyts, multilayer biofilms were subjected to slow-scan voltammetry in the presence and the absence of lactate. During slow anodic potential scans, bacterial cells are expected to regenerate the ferrous (Fe²⁺) ion of c-Cyt heme groups by the oxidation of lactate, which follows the supply of additional electrons to electrodes in the oxidation cycle of c-Cyts. In our analysis using a scan rate of 1 mVs⁻¹ for the biofilm in the presence of lactate, a distorted redox wave was observed, in which the columbic area of anodic current (Qₐ) was 4-fold larger than that of the cathodic current (Qₖ) (Fig. 3a). In contrast, the cell culture lacking lactate had a markedly decreased Qₖ, and thus, the CV impaired the imbalance between the anodic and cathodic cellular areas (Fig. 3b). Taken together, these observations indicate that the large anodic current was a consequence of the metabolic activity of microbes.

We plotted the coulomb balance, defined as Qₐ/Qₖ, for the multilayer biofilm at several scan rates, and compared it to that of the monolayer biofilm (Fig. 3c). As seen in Fig. 3c, the Qₐ/Qₖ remained nearly constant at 1 for scan rates between 100 mVs⁻¹ and

Fig. 2. Baseline-subtracted cyclic voltammograms of multilayer (solid line) and monolayer (dotted line) biofilms of strain MR-1. The scan rate was 100 V s⁻¹. The raw data for these voltammograms are shown in the supporting information.

Fig. 3. Baseline-subtracted cyclic voltammograms for a multilayer biofilm of strain MR-1 obtained using a scan rate (ν) of 1.0 mVs⁻¹ in reactors (a) with and (b) without lactate. (c) Redox peak balance (anodic peak area over cathodic peak area) was plotted against the logarithm of ν (mVs⁻¹). Each plot was generated from the CVs of the multilayer (open circle) and monolayer biofilms (closed circle). The standard deviation for these data points is less than 5%, estimated from greater than three individual experiments.
100 V s\(^{-1}\); however, it largely exceeded 1 at scan rates slower than 10 mV s\(^{-1}\). In contrast, the monolayer biofilm displayed a \(Q_e/Q_a\) of approximately 1 for all of the examined scan rates. From these results, we can conclude that the respiratory electrons generated by microbial metabolism are transported via the sequential redox cycling of c-Cyts to distant electrodes.

3.3. Effects of gene-deletion mutants on electrochemical signals of biofilms

To identify the specific location of c-Cyts within biofilms responsible for the observed electron conduction, we employed three mutant strains, \(\Delta\text{mtrC}/\Delta\text{omcA}, \Delta\text{pilD}, \) and \(\Delta\text{SO3177}.\) The role of OM c-Cyts for the electron conduction was examined using \(\Delta\text{mtrC}/\Delta\text{omcA}\) and \(\Delta\text{pilD},\) which lack two major c-Cyts of \(\text{Shewanella}\) and the type II secretion system responsible for the proper localization of Cyts on cell surfaces, respectively [26]. We also examined mutant strain \(\Delta\text{SO3177},\) which is unable to produce the biosynthetic protein of capsular polysaccharide (CP) [21], as CP has been proposed to serve as a percolation condition by increasing the apparent cell-surface occupancy of c-Cyts [14,16]. In this set of experiments, wild-type (WT) strain MR-1 biofilms of monolayer thickness were first generated on ITO electrodes, as before, and cell suspensions (OD\(_{600}\) = 2.0) of \(\Delta\text{mtrC}/\Delta\text{omcA}, \Delta\text{pilD},\) or \(\Delta\text{SO3177}\) were then added to the reactor. CVs were taken after 30 min at a scan rate 100 V s\(^{-1}\) to examine the influence of the added cells on the redox current of c-Cyts relative to the reactor containing only the monolayer biofilm (Fig. 5).

Fig. 4 shows the changes in \(Q_e (\Delta Q_e)\) resulting from the addition of \(\Delta\text{mtrC}/\Delta\text{omcA}, \Delta\text{SO3177}, \Delta\text{pilD},\) and WT (control) cells to the reactors. The \(\Delta\text{mtrC}/\Delta\text{omcA}\) and \(\Delta\text{pilD}\) strains provided ~40% smaller \(\Delta Q_e\) compared with WT, suggesting that CP does not serve as a matrix to absorb secreted c-Cyts, but would electrically insulate a part of cell-surface-bound c-Cyts. Thus, the \(\Delta Q_e\) of WT observed under the experimental conditions in Fig. 4 is considered to be predominantly mediated by cell-surface-bound c-Cyts, but not by other forms of Cyts, such as those located on pili or extracellular polymeric substrates.

3.4. Proposed model for electron conduction in multilayer biofilms of strain MR-1

Based on the high density of cell-surface-exposed c-Cyts (10%-30% coverage [27]) in \(\text{Shewanella}\) and \(\text{Geobacter}\) species, it has been speculated that electron exchange reactions between OM c-Cyts contribute to the electron conduction properties of bacterial biofilms [14,16]. Using an optical tweezers technique, we have previously demonstrated that \(\text{Shewanella}\) cells can accomplish the EET reaction to a solid electron acceptor via OM c-Cyts even momentum contact for only approximately 300 ms [28]. Therefore, it is most plausible that the redox cycling reactions of c-Cyts in multilayer biofilms experimentally identified in this work is a consequence of sequential electron exchange via physical contact between OM c-Cyts (Fig. 5a). As shown in the proposed model, if electrons generated by the metabolic oxidation of lactate are conducted along the cell surfaces and delivered to a distant electrode, the surface occupancy of OM c-Cyts should fulfill the percolation threshold, which is estimated to be 50% in two dimensions by a resistor network model [29]. We speculate that the motility of cells present in the biofilm assist to establish the percolation condition by increasing the apparent cell-surface occupancy of c-Cyts in the biofilm matrix, resulting in electron conduction across the multilayer biofilm (Fig. 5b).

In conclusion, we have demonstrated that the electron conduction property of multilayer biofilms of \(\text{S. oneidensis}\) MR-1 was mediated by the sequential redox cycling of OM c-Cyts under normal physiological conditions. Furthermore, we revealed that the electron transport conduit composed of OM c-Cyts across biofilms contributed to the anodic current generation. Although the requirement for biofilm conduction to achieve high anodic performance in MFCs has been recognized in prior studies [14-19], its mechanism was not experimentally examined under in-vivo conditions. Present study further emphasizes the importance of forming thick and highly conductive biofilms on the electrode surface for improving MFC anode performance, and provides a new framework for the understanding of long-range EET mechanisms [10,14-16].

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![Fig. 4](image-url) Changes in the anodic peak current \((\Delta Q_e)\) of reactors containing a monolayer biofilm of strain MR-1 following the addition of cell suspensions of wild-type (WT), \(\Delta\text{SO3177}, \Delta\text{pilD},\) and \(\Delta\text{mtrC}/\Delta\text{omcA}\) MR-1 strains. The scan rate was 100 V s\(^{-1}\).

![Fig. 5](image-url) (a) Schematic illustrations for electron conduction in a multilayer biofilm of MR-1 cells mediated via OM c-Cyts. Metabolically generated electrons are transferred from the upper and middle parts of the biofilm to a distant electrode. (b) Speculated model for biofilm-mediated electron conduction. Electron exchange along the cell-surface c-Cyts developed a percolation pathway of electrons across multilayer biofilm by the assistance of cell motility to increase the apparent surface coverage of OM c-Cyts.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bioelechem.2011.12.003.

References


[25] The amount of c-Cyts was estimated from the cyclic voltammogram with the assumption that all the OM Cyts are composed of decachrome. The diameter of Shewanella's decachrome Cyts is in the range of 5 nm.


