A Human Pathogen *Capnocytophaga Ochracea* Exhibits Current Producing Capability

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**ABSTRACT**

Microbial extracellular electron transfer (EET) in diverse environments has gained increasing attention. However, the EET capability of oral pathogens and associated mechanisms has been scarcely studied. Here, our results suggest that the *Capnocytophaga ochracea*, an etiological human pathogen showed current production and demonstrated a rate enhancement of electron transport at a high cell-density. *C. ochracea* produced ~10-fold more current at an OD<sub>600</sub> of 0.5 associated with twice a higher glucose consumption rate per cell, compared to 0.1, measured in a three-electrode electrochemical system by single-potential amperometry at +0.2 V (vs Ag/AgCl [sat. KCl]). During current production, the accumulation of the redox molecules on the electrode was observed at high OD<sub>600</sub> compared to low OD<sub>600</sub>. Apart from cell released redox active product, externally added redox active additives enhanced the electron transport, suggesting the EET capability of *C. ochracea* via electron mediator. A higher metabolic activity via single-cell assay (based on anabolic incorporation of ^15^NH<sub>4</sub>+) in cells that did not attach to the electrode strongly suggests the EET rate enhancement through an electron mediator. As bacterial populations play a role in the pathogenesis of human infections such as periodontitis, our results suggest that population-induced EET mechanisms may facilitate *in-vivo* colonization of *C. ochracea*.

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Keywords : extracellular electron transfer, oral pathogen, redox active molecules, electrochemical system

1. Introduction

Extracellular electron transfer (EET) mechanism has been well-studied in environmental microbes, such as *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA.1-4 Direct electron transport by outer-membrane cytochromes and long-range electron shuttling processes are known as major mechanisms of EET in iron-reducing strains. Further, nanowire or conductive biofilm matrices also mediate long-range electron transport, particularly in matured biofilms.5,6 Recently, distinct kinds of enzymes were identified in marine sulfate-reducing bacteria *Desulfovibrio ferrophilus* ISS<sup>5</sup> and food-borne pathogen *Listeria monocytogenes*.7 In particular, the identification of flavin-based enzymes in *L. monocytogenes*, a human pathogen, implies divergent EET mechanisms in the human microbiome. Further, *Enterococcus faecalis*<sup>8,10</sup> and two other isolates from the human gut share homology with *Klebsiella pneumoniae* and *Enterococcus avium*, with respect to the 16S rRNA gene sequence,<sup>11,12</sup> and are capable of EET. Their EET potential may facilitate their colonization in the host environment, similar to EET in the natural environment. Therefore, it is of great importance to understand the mechanism of EET at high cell-density.

Cell-density plays an important role in microbial physiology and ecology. This feature profoundly affects the overall function of microbial interactions in biofilms, such as metabolism, diversity, and pathogenesis.12,13 In the 1970s, it was determined that bacteria could sense the population-density of their and neighboring microorganisms and integrate this information into gene regulatory circuits via the production of diffusible signal molecules (autoinducers, i.e., AI-2), which is now commonly known as ‘quorum sensing’ (QS).<sup>14,15</sup> Bacteria in biofilms display a different phenotype through QS in response to fluctuations in cell population and ultimately many physiological traits.16 However, the role of QS in microbial EET, especially in bacterial pathogens, is not well understood.

To this end, we studied the bacterial species, *Capnocytophaga ochracea* (*C. ochracea*), an oral pathogen that causes periodontal disease and is also found in blood, contributing to opportunistic infections such as sepsis and brain abscesses in immunocompromised patients.<sup>17,18</sup> In the oral cavity, *C. ochracea* is prone to form biofilms with gliding motility and also known to possess a QS system.<sup>19</sup> In addition, dehydrogenases-cytochromes in the cellular membrane of *C. ochracea* were identified four decades ago,<sup>20</sup> however, the biological function of these redox enzymes and the potential EET capability of this microbial strain have yet to be studied. Therefore, we conducted a whole-cell electrochemical assay, single-potential amperometry, and differential pulse voltammetry to study the EET capability and the effect of cell-density dependency on current production. The microbial capability to use soluble electron shuttling molecules was also examined in detail combined with a cell-specific isolate, single-cell activity assay. The EET mechanisms were further examined using a mutant strain of...
2. Materials and Methods

2.1 Bacterial growth media and culture conditions

*Cupnocytophaga ochracea* ATCC 27872 was grown in 80 mL of DSMZ 340 medium supplied with 1 g/L sodium bicarbonate at 37°C. The medium excluding glucose and hemin was autoclaved for 30 min at 121°C before use. Glucose and hemin were separately prepared, and filter-sterilized prior to inoculation. To maintain the anaerobic growth condition, 0.2% of N2/CO2 (80:20 v/v) gas sparging was done prior to culture inoculation. In the electrochemical experiments, *C. ochracea* at the late exponential growth phase or early stationary phase was used. The luxS gene (*Coch.*_1216) deleted *C. ochracea* termed as LKT7 was previously constructed using a PCR-ligation-mutation strategy, kindly provided by the group of Professor Kazuyuki Ishihara from Tokyo Dental College and grown with the same conditions as wild-type for our experiments.

2.2 Electrochemical cell operation and measurements

Electrochemical experiments were established using a single-chamber three-electrode reactor in the anaerobic chamber (COY glovebox). Three electrodes include: 1- Working electrode, a glass substrate coated with tin-doped indium oxide (ITO) layer of 600 nm by spray pyrolysis deposition (SPD Laboratory, Inc., Japan); 2- Counter electrode, platinum wire of 0.1 mm thickness; and 3- Reference electrode, Ag/AgCl (sat. KCl). Transparent ITO electrode was used to confirm cellular attachment before the further analysis. As an electrolyte, a defined medium (DM) was prepared with following composition (1L): 2.5 g NaHCO3, 0.08 g CaCl2·2H2O, 1.0 g NH4Cl, 0.2 g MgCl2·6H2O, 10.0 g NaCl, 7.2 g HEPES, 0.5 g yeast extract and 10 mM glucose as the carbon source. The pH of the medium was adjusted to 8.0. The sterilized electrolyte (4.8 mL per reactor) was purged with N2 for 15 min to remove dissolved oxygen in the reactor. Active *Capnocytophaga* bacteria, collected from enrichment medium was washed twice with DM to remove nutrients and resuspended in DM. Concentrated cell culture was added into the electrochemical reactor with a final OD600 according to the requirement of the experiments. All electrochemical experiments were carried out at 37°C without any agitation in the reactors. Active *Capnocytophaga* and grown with the same conditions as wild type for our experiments. Glucose and hemin were separately filter-sterilized prior to inoculation. To maintain the anaerobic growth condition, 0.2% of N2/CO2 (80:20 v/v) gas sparging was done prior to culture inoculation. In the electrochemical experiments, *C. ochracea* at the late exponential growth phase or early stationary phase was used. The luxS gene (*Coch.*_1216) deleted *C. ochracea* termed as LKT7 was previously constructed using a PCR-ligation-mutation strategy, kindly provided by the group of Professor Kazuyuki Ishihara from Tokyo Dental College and grown with the same conditions as wild type for our experiments.

2.3 Scanning electron microscopy (SEM)

At the end of electrochemical experiments, the ITO electrodes were collected for SEM sample preparation. The ITO slides were first fixed with 2.5% (v/v) glutaraldehyde and followed by gradient dehydration. Briefly, fixed samples were washed three times by PBS buffer, followed by dehydration in (v/v) 25%, 50%, 75%, 90%, and 95% ethanol, and three times with t-butanol and then overnight freeze-dried under vacuum. The vacuum freeze-dried slides were coated with evaporated platinum and observed via a Keyence VE-9800 microscope.

2.4 Fluorescence spectroscopy

Flavin secretion by *C. ochracea* was tested by measuring the fluorescence intensity of cell-free supernatant in an anaerobic quartz cuvette. JASCO-FDT 538 (Jasco corporation, Japan) instrument was used for the fluorescence spectrometry measurements. Flavin was the target secretion molecule and therefore excitation wavelength pertaining to flavin, i.e., 450 nm was selected for fluorescence spectroscopy. Control analysis of de aerated 3 mL DM in the presence and absence of 5 µM riboflavin was subjected to fluorescence and emission spectrum of oxidized riboflavin was measured by excitation at 450 nm. Emission peak intensity at 530 nm was measured.

2.5 Sample preparation for nanoscale secondary ion mass spectroscopy (NanoSIMS)

Prior to NanoSIMS analysis, for the electrode non-attached cells, supernatant from the reactor was removed without disturbing the electrode attached cells, and the cells collected from the supernatant were placed on a new ITO electrode for NanoSIMS analysis. For electrode attached cells, the ITO surface was washed several times and only the firmly attached cells on ITO were analyzed by NanoSIMS. Samples of each condition were fixed in 2.5% (v/v) glutaraldehyde phosphate medium and dehydrated in an ethanol gradient and t-butanol as previously described. CAMECA NanoSIMS 50L system (CAMECA, Gennesvilliers, France) was used to analyze the biofilm sample. Briefly, a Cs+ beam approach sample and irradiated four secondary ions (12C++, 13C--, 12C14N--, and 13C15N--) emitted from the sample surface. Four secondary-ion images of each sample were recorded with a raster size of 25 x 25 µm2 and analyzed by Fiji (version 1.0) for the isotope assimilation calculation. Given C. ochracea cells tend to form web-like morphology, to fully recover the isotope signals, the signals for each NanoSIMS image were averaged. Using the plugin, Open MIMS Image, all signal regions, including clusters of cells were selected from each image (regions of interest, ROI) and their pixel values were quantified for isotopic ratio analysis. Average and standard deviation were calculated based on the number of ROIs. The ratio of 13C and 15N was calculated as follows:

\[
\frac{13C}{12C} = \frac{13C \text{pixel}}{12C \text{pixel}} \times 100\%
\]

\[
\frac{15N}{13C15N + 13C14N} = \frac{13C15N \text{pixel}}{13C14N \text{pixel}} \times 100\%
\]

3. Results and Discussion

3.1 Enhanced current production capability in *C. ochracea* at higher cell-density

To address the possibility of current production and to evaluate cell-density behavior during the current generation of *C. ochracea*, a three-electrode electrochemical system was used. Single-potential amperometry was performed at +0.2 V using the DM as an electrolyte in the presence of 10 mM glucose. Upon the addition of bacterial cells into the reactor to a final OD600 of 0.5, an immediate current increase was observed, reaching approximately 60 nA cm⁻² after 5 h (Fig. 1A and Fig. S1A). Such low current is also observed in other weak electricians. A significantly less extent, but a similar time course of current production was observed even in the absence of glucose, indicating that approximately 50% of current production was derived from glucose oxidation and the remaining current from nutrients such as yeast extract present in the medium (Fig. S1A). Accordingly, we confirmed a significant decrease in glucose concentration during current production (Fig. S1B). These results strongly suggest that *C. ochracea* is an EET-capable bacterium with current production associated with glucose oxidation.

We next examined the cell-density dependency on current production in *C. ochracea* at different OD600 from 0.1 to 1.0 under
the same electrochemical condition as mentioned above. At ODs of 0.1 and 0.2, current production did not significantly differ from the background current (Fig. 1A), while cellular attachment on the electrode surface was confirmed by SEM (Fig. S2A). In contrast, when the cell-density was at an OD$_{600}$ of 0.5, current production was ~10-fold higher than 0.1. Furthermore, although the cell-density was sufficient to cover the whole electrode surface at an OD$_{600}$ of 0.5 (Fig. S2B), a higher current was produced at an OD$_{600}$ of 1.0. Also, a significant increase in charge transfer to the anode at higher ODs was observed, which was calculated for 12 h by subtracting the charge generated in the absence of glucose from that generated in the presence of glucose for 12 hours. (C) Glucose consumption rate measured for anaerobic reactors at OD$_{600}$ of 0.1 and 0.5 with initial glucose concentration of 10 mM. Data values represent the mean ± standard deviations from two independent experiments. (D) Baseline subtracted differential pulse (DP) voltammogram of C. ochracea measured for anaerobic reactors at OD$_{600}$ of 0.1 and 0.5.

C. ochracea exhibited that the current production is not proportional to the cell concentration unlike Shewanella oneidensis MR1, a model EET-capable microbe in the environment. The current production of Shewanella came from the electrode-attached single cell using a direct electron transport mechanism via outer-membrane $c$-type cytochromes.\textsuperscript{25} However, electron transport of C. ochracea was enhanced at a certain threshold of cell-density, not following the same mechanism of direct electron transport to the electrode as in Shewanella. A possible explanation is that Shewanella uses non-fermentable carbon sources and so the metabolic activity of Shewanella might be low when it cannot use an electron acceptor. Therefore, cells that are not able to perform direct electron transport via outer membrane cytochromes to the electrode (or able to avail themselves of a mediator) would be expected to be relatively metabolically inactive. In contrast, C. ochracea can ferment glucose and so that the cells regardless of the direct electron transport mechanism to the electrode should also have considerable metabolic activity and impact the current production.

Glucose concentration was measured after 8 h for OD$_{600}$ of 0.1 and 0.5 during current production under the same condition as Fig. 1A. A 10-fold higher glucose consumption rate at OD$_{600}$ of 0.5 compared to 0.1 showed that the metabolism of C. ochracea was substantially activated at high cell-density (Fig. 1C). The electrochemically detected quantity of cell released reductive active product on the electrode by differential pulse voltammetry also showed a significant increase with OD (Fig. 1D and Fig. S3). However, the magnitude of difference in DP voltammogram peaks for low OD and high OD is not same as the current production and glucose consumptions, because DP voltammogram analysis reflects the information about reductive active molecules, either coming from bacterial surface electron transfer agent and/or soluble mediators. DP voltammogram measured at OD$_{600}$ of 0.1 and 0.5 showed a slight difference in the peak potential ($E_p$) shift approximately from $-0.35$ (OD$_{600}$ of 0.1) to $-0.30$ (OD$_{600}$ of 0.5) V (Fig. 1D). These data suggest that current enhancement at high cell-density results from both metabolic activation and the increase in cell released reductive active product having peak potential of approximately $-0.30$ V.

3.2 Population-induced EET mechanism via cell released reductive active product in C. ochracea

To examine whether C. ochracea could use the cell released reductive active product detected in DP voltammogram in its current production, we performed supernatant swapping with fresh medium in the reactor at an OD$_{600}$ of 0.5, after current production was
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Figure 2. Electron mediating capability of *C. ochracea* and cell released redox active product accumulation. (A) Supernatant replacement during the current production of *C. ochracea* at the poised potential of +0.2 V vs Ag/AgCl (sat. KCl). At indicated times, the medium was removed and replaced with fresh defined medium (DM) containing 10 mM glucose led to a decrease in current production. (B) Baseline subtracted differential pulse (DP) voltammogram showing the peak potential of cell-free supernatants collected from OD600 of 0.2 and OD600 of 0.5 reactors.

The removal of planktonic cells decreased the current production (Fig. 2A), suggesting that an electron mediator may contribute to the current production at the OD600 of 0.5. We confirmed the presence of cell released redox active product at low and high OD600 by voltammetry analysis of the cell-free supernatant (cells were removed by centrifugation) collected from the reactor. The DP voltammogram of the cell-free supernatant solution from OD600 of 0.5 showed a 7-times higher peak current (OD600 of 0.2) compared to OD600 of 0.2. Furthermore, while an OD600 of 0.2 showed a single peak at *E*<sub>p</sub> of −0.20 V, an oxidative signal in the case of OD600 at 0.5 contained multiple peaks at different *E*<sub>p</sub> values (Fig. 2B). These significant differences in oxidative signals suggest that the accumulated cell released redox active product at OD600 of 0.5 is critical for the current enhancement.

We next examined the effect of external redox active additives (which are also essential nutrients in many dietary products), such as menadione (Vitamin K3, VK3) and riboflavin (Vitamin B2, VB2), on current production. The addition of VK3 and VB2 to the reactor with high OD significantly increased current production over 10-fold (Fig. 3A) compared to the absence of redox active additives. In addition to *E*<sub>p</sub> of −0.20 V assignable to the cell surface protein, sharp oxidative peaks were observed with *E*<sub>p</sub> at −0.40 V and +0.30 V in the presence of VB2 and VK3 in DP voltammogram, respectively (Fig. 3B). Therefore, the population-induced EET mechanism was dominated by the electron mediator mechanism, and *C. ochracea* likely accumulated cell released redox active product as shown in the schematic (Fig. 4). Further, because *Capnocytophaga* is a flavobacterium that secretes flavins, we measured the fluorescence spectrum of the cell-free supernatant collected from the electrochemical reactor during EET to detect the presence of secreted flavins (Fig. S4). However, no difference was observed between fresh DM and cell-free supernatant, indicating that *C. ochracea* produced insignificant amounts of flavins in the electrochemical reactor, confirming that flavin was not involved in the current generation by *C. ochracea* and there is likely an involvement of other cell released redox active product.

The larger current production at high OD600 0.5 was associated with both higher glucose metabolic activity (Fig. 1C) and the higher concentration of cell released redox active product secretion compared to low OD (Fig. 1C, 2B). To this end, we analyzed the metabolic activity of bacterial cells in the electrochemical cell by nanoscale secondary ion mass spectrometry (NanoSIMS). NanoSIMS is a robust tool to visualize and quantify the incorporation of labeled substrates in a single cell. Therefore, we used 13C labeled glucose and 15N labeled ammonium chloride (15NH₄Cl) to investigate the anabolic activity coupled to glucose metabolism in *C. ochracea*. In the single-potential amperometry condition, *C. ochracea* harvested from the electrode surface presented less 13C and 15N anabolic activity than non-attached cells in the supernatant. Subsequently, the 13C/C<sub>total</sub> (%) and 15N/N<sub>total</sub> (%) in the non-electrode attached *C. ochracea* were compared in the presence of external redox active additives, VB2 and VK3 (Fig. 5A and B). It can be seen that high activity was more abundant in electrode non-attached cells in comparison to electrode attached cells, supporting the significance of non-attached cells on metabolic activity and ultimately enhanced the EET.

It is important to mention that electron mediators affect interactions with the bacterial surface and eventually enhance the
3.3 EET enhancement of *C. ochracea*

EET and QS is likely a strategy against environmental stresses by a mediator. Cell-released mediator attaches to the cell and transfers the electrons to ITO via reduction (Red) and oxidation (Ox) state cycling.

Figure 4. Schematic of possible electron transport mechanism by *C. Ochracea* cells. At low OD the EET is limited by the concentration of cell released redox active product, whereas at higher ODs the concentration is increased which acts as an electron mediator. Cell-released mediator attaches to the cell and transfer the electrons to ITO via reduction (Red) and oxidation (Ox) state cycling.

Electron transfer rate to maintain the intracellular redox environments, i.e., redox homeostasis. Metabolism-associated electron transfer of *C. ochracea* could act in concert to promote pathogenicity and biofilm formation. Moreover, EET linked to population-level phenotypes can be critical during microbial colonization and biofilm formation. Therefore, crosstalk between EET and QS is likely a strategy against environmental stresses by *C. ochracea*.

Figure 5. NanoSIMS analysis of *C. ochracea* in the presence and absence of redox active additives. NanoSIMS images of electrode attached cells (A) and non-attached cells (B) showing the 15N/Ntotal (%) assimilation. Arrows indicating the rod-shaped *C. ochracea* cells (scale bars: 5 µm). Warmer colored cells are more enriched in 15N/Ntotal (%), which corresponds with the higher levels of anabolic activity. (C) Bar graph showing the isotopic assimilation of 13C/Ctotal (%) and 15N/Ntotal (%) in electrode surface attached and non-attached cells analyzed via NanoSIMS analysis. Given *C. ochracea* cells tend to form web-like morphology, to fully recover the isotope signals, the signals for each NanoSIMS image were averaged. Data values representing the mean ± standard deviations from two independent experiments and a similar tendency was observed in more than four individual experiments.

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In oral microbiota, it has been reported that *Porphyromonas gingivalis* enhanced biofilm formation of *Fusobacterium nucleatum* by releasing diffusible molecules, although AI-2 was not involved. Moreover, the release of soluble molecules from *C. ochracea* played a significant role in biofilm formation by *F. nucleatum*. Further, induction of coaggregation between *F. nucleatum* and *C. ochracea* has been reported. However, the soluble molecules released by *C. ochracea* in our study have redox properties and may play a critical role both in oral polymicrobial biofilms and the association of other niche bacteria (independent of QS). The cell-density dependency for metabolic activation and cell released redox active product. There are many other types of QS mechanism have been identified in Gram-negative bacteria, such as *LasI/LuxR*, *LasI/LasR*, *RhfI/RhfR*, *PQS*, and quorum quenching (QQ) enzymes. Therefore, other types of QS mechanisms in enhancing EET at higher cell-density is also possible. Moreover, the pathogenesis of oral bacteria has been well studied; yet, their microbial electrical activities, in particular, the capability of extracellular electron transportation have not been reported regarding population-level phenotypes. Therefore, the activities of *C. ochracea* and other extracellularly electron-transferring organisms should be reexamined to develop pathogenicity control models.
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Conflict of Interests

Authors declared that there is no conflict of interest involved in this work.

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4. Conclusions

Our electrochemical analysis results suggested that C. ochracea is a human pathogen that has the EET capability with population-induced EET paradigm and metabolic activation. Interestingly, the cell-density dependent electron transport observed in this study is independent of the QS regulation luxS. Importantly, the EET rate was enhanced at higher cell densities and single-cell anabolic activity showed that electrode non-attached cells played a very vital role in EET enhancement. These distinct mechanisms are likely critical during microbial colonization and biofilm formation of C. ochracea in the human environment. Therefore, the present findings of cell-density dependent mechanisms may provide critical aspects for suppressing the microbial activity, e.g., medical application and drug design. Identification of cell released redox active product and proteins would be an interesting future study.

Supporting Information

The Supporting Information is available on the website at DOI: https://doi.org/10.5796/electrochemistry.20-00021.

Author Contributions

This work was conceived by S.Z., and A.O. S.Z., D.N., W.M., and S.G. performed the experiments and collected data. All authors contributed intellectually to the analysis and interpretation of the data. W.M., D.N., S.Z., and A.O wrote the manuscript.

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