NANOSCALE ELECTROCHEMISTRY AT LIQUID/LIQUID INTERFACE FOR BIOMEDICAL AND ENERGY APPLICATIONS

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NANOSCALE ELECTROCHEMISTRY AT LIQUID/LIQUID INTERFACE FOR BIOMEDICAL AND ENERGY APPLICATIONS

BY
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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN CHEMISTRY

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ABSTRACT

The charge transfer across the liquid/liquid interface has been playing a crucial role in many biological and energy-related systems such as drug permeation through-bacterial membrane, ion or electron transfer through metal-reducing bacteria in microbial fuel cells (MFCs), and metabolic interaction in commensal bacterial species related to human health. Primarily, direct probing of these phenomena in situ is needed for more realistic fundamental analysis, thereby solving the problem related to public health and renewable energy generation. The interfacial CT in these systems mainly involves redox-inactive species that cannot be detected with conventional metal electrodes. Herein, the liquid/liquid interface is applied as an analytical tool to probe (1) antimicrobial permeation through the membrane, (2) microbial fuel oxidation, a core step of extracellular electron transfer pathways in MFCs, and (3) metabolic interaction between oral commensal bacteria relevant to human health. In my first project, we studied the transport kinetics of pristine antimicrobials, e.g., quinolones, and sulfonamides using a nanopipet-supported liquid/liquid interface as a mimic of a biological membrane as well as a probe. Finite element analysis of experimental voltammograms revealed a relationship between the structure of hydrophobic drug ions and their permeation across the interface. While the metal-reducing bacteria produces CO$_3^{2-}$ and electrons during organic fuel oxidation, a novel probe is highly demanded to assess this mechanism in real time to quantitatively elucidate extracellular electron transfer in this bacterial system. In my second project, we developed nanoscale CO$_3^{2-}$ amperometric ion-selective electrodes (ISEs) based on ion-
ionophore recognition using Simon’s ionophore, and fundamentally investigated hidden barriers to the development of this nanoscale ISE. The experimentally and theoretically proved fundamental understanding enabled us to mechanistically and kinetically evaluate the interfacial CO$_3^{2-}$ ion transfer reaction facilitated by ionophore as well. The analytic utility of our probe was further verified by quantifying CO$_3^{2-}$ produced by metal-reducing bacteria. In the third project, we applied a nanopiet-supported liquid/liquid interface as a probe and nanoscale scanning electrochemical microscopy (SECM) to *in situ* investigate metabolic interaction between oral commensal bacterial species related to human health. Herein, we could real-time visualize and quantitatively assess the metabolite exchange between two bacterial species via lactate production and consumption rate at a single-cell level. These findings will help to monitor potential disease risks in humans by probing multi-species metabolic interaction. We will further employ this nanoscale probe and nanoscale SECM to study drug resistance in real bacteria, and extracellular electron transfer pathways in MFC at a single-cell level.
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This thesis is presented in manuscript format according to the guidelines of the graduate school of the University of Rhode Island.

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CHAPTER 1
INTRODUCTION

Charge transfers (CTs)\textsuperscript{1–3} across the liquid/liquid interface is a ubiquitous process in various biological\textsuperscript{4–8} and energy-related systems\textsuperscript{9,10} such as drug permeation through the infectious-bacterial membrane\textsuperscript{11–13}, ion or electron transfer through metal-reducing bacteria used for microbial fuel cells (MFCs),\textsuperscript{10,14,15} and metabolic interaction between commensal bacteria in human body.\textsuperscript{16,17} In fact, these systems are closely related to the worldwide problems mankind confronting in public health and energy crisis. Accordingly, the direct probing of these CTs \textit{in situ} is critical to elucidate the fundamental understanding in given systems and get a clue to solve the problems. The majority of these CT processes, however involves redox-inactive species that cannot be monitored by the conventional metal probes.\textsuperscript{18–21} Herein, we employed a nanopipet-supported interface\textsuperscript{22–26} between two immiscible electrolytes solutions (ITIES) as a probe, and directly scrutinized interfacial CTs of drug ions and various metabolites to fundamentally study (1) bacterial drug resistance,\textsuperscript{11} (2) extracellular electron transfer pathways in MFCs,\textsuperscript{27} and (3) cross-feeding between oral commensal bacteria relevant to human health. Throughout these nanoelectrochemical studies at the various stages, we could obtain deeper fundamental understanding, which could not be unequivocally addressed by other conventional approaches. First, as a preliminary work, we investigated the interfacial ion transfer kinetics of pristine antimicrobials with a nanopipet-supported ITIES and nanopipet voltammetry. The liquid/liquid interface at a nanopipet was utilized not only as a probe to
electrochemically sense pristine drug ions, but also as lieu of a biological membrane\textsuperscript{27} determine the permeability of drug ions through an artificial membrane. We revealed that hydrophobic drugs have surprisingly low permeability due to the strong interaction between localized charges on drug and water molecules across the liquid/liquid interface, thereby possessing high hydrophilic property during interfacial CTs.\textsuperscript{11} Newly elucidated relationship between drug structure and its permeability will be further correlated to real bacterial system to investigate bacterial drug resistance with an aid of nanoscale scanning electrochemical microscopy (SECM).\textsuperscript{28–32} In MFCs, microbial fuel oxidation is the crucial step to generate electrons during extracellular electron transfers, which produces CO$_3^{2–}$ as a metabolic waste. For \textit{in situ} study of microbial fuel oxidation, a proper probe is needed to real-time sense and monitor local CO$_3^{2–}$ at metal-reducing bacteria. Herein, we demonstrated hidden puzzles to develop nanoscale amperometric CO$_3^{2–}$ ion-selective electrodes (ISEs) based on nanopipet-supported ITIES and broadly available Simon’s ionophore\textsuperscript{34–38} such as slow dissolution and peculiar solubility of ionophores at the nanoscale interface, ionophore activation, and cleanness at the nanoscale interface, which were theoretically and experimentally proved. Reliable and reproducible responses of CO$_3^{2–}$ ion transfer in nanopipet voltammetry enabled us to mechanistically and kinetically assess facilitated CO$_3^{2–}$ ion transfer reactions,\textsuperscript{7,39–41} and unequivocally clarify the one-step electrochemical interfacial reaction mechanism. As a proof of concept, we could determine CO$_3^{2–}$ concentration produced by \textit{Shewanella oneidensis} as a result of microbial fuel oxidation. The developed probes combined with SECM will be employed to study metal-reducing bacteria at a single cell level, and identify rate-determining step in multiple electron pathways, which is pivotal to improve the efficiency in MFCs. Lastly, we
investigated metabolic interactions between oral commensal bacteria in a symbiotic relationship with the human host. Two highly abundant species in the human supragingival plaque, *Streptococcus mitis* and *Corynebacterium matruchotii*\(^{32,43}\) were studied to elucidate their real-time chemical communication in commensal harmony. We applied nanoscale SECM using a submicropipet-supported ITIES as an SECM probe to real-time visualize the metabolic interaction between two individual microbes via lactate production/consumption, and quantitatively assessed the metabolic relationship. Significantly, this study, for the first time describes a mechanism of *in situ* metabolic interaction between oral commensals at the single cell level, supporting the observed *in vivo* spatial arrangements of these microbes. Our successful application of SECM towards polymicrobial interactions opens up a new route to investigate multi-species cooperation and competition via real-time probing of metabolites *in situ*, and provides a new diagnostic tool to determine whether changes in the oral microbiome precede clinical signs of disease

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CHAPTER 2

Kinetics of Antimicrobial Drug Ion Transfer at a Water/Oil Interface Studied by Nanopipet Voltammetry

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Kinetics of Antimicrobial Drug Ion Transfer at a Water/Oil Interface Studied by Nanopipet Voltammetry

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Abstract

Effective delivery and accumulation of antimicrobial agents into the microbial organism is essential for the treatment of bacterial infections. Transports of hydrophilic drug molecules, however encounter a robust barrier of hydrophobic double membrane cell envelope, thus leading to drug-resistance in Gram-negative bacteria. Accordingly, deeper understanding about a transit of charged molecules through bacterial membrane is needed to remediate the antibacterial resistance. Herein, we apply a steady-state voltammetry using nanopipet-supported interfaces between two immiscible electrolyte solutions (ITIES) to quantitatively study transport-kinetics of antimicrobial drug-ions (quinolones and sulfonamides) at a water/oil interface. Importantly, ITIES can mimic cellular membrane system, thus being employed as insightful surrogates for kinetic study of drug entry through bacterial cytoplasmic membranes. This approach enables us to voltammetrically and amperometrically detect redox-inactive drug-ions as pristine under physiological conditions. Considerably slow kinetics of drug-ion transports are successfully measured by nanopipet voltammetry and theoretically analyzed. This analysis reveals that the drug-ion transport is ~3 orders of magnitude slower than tetrabutylammonium ion transport. In addition, the extreme hydrophilicity of drug-ions in comparison to ClO$_4^-$ is quantitatively assessed from half-wave potentials of obtained voltammograms. The high
hydrophilicity exclusively attributed to localized negative charges on carboxylate or amide group of deprotonated quinolone or sulfonamide, respectively may play a dominant role in sluggish kinetics due to the increase in energy barriers upon interfacial ion transfer. Notably, this study using nanopipet voltammetry provides physicochemical insights on the correlation between structural properties of pristine drug-ions and their transfer-kinetics at a water/oil interface in lieu of biological membranes.

Keywords: Nanopipet voltammetry, ITIES, Antimicrobial ions, Ion transfer kinetics, Effective hydrophilicity.

Introduction

Bacterial drug resistance has been a global health issue. A major challenge we are facing in Gram negative (GN) antibacterial research, is to thoroughly understand and control membrane permeation of antibiotics in clinically relevant microbial organism. In fact, penetrating the membrane barrier to reach a critical concentration inside microbial organism is a essential step for most antibiotics. GN bacteria, however have a hydrophobic double-membrane cell envelope, serving as a major barrier for hydrophilic molecules to enter the cell. Accordingly, in a fight with resistant GN organisms, primary obstacle confronting the discovery and development of new antibiotics is the lack of a rational strategy to understand the transit of charged molecules through bacterial membrane, and equip antimicrobial agents with requisite properties to permeate bacterial membrane. Hence it is crucial to understand antibiotic permeation and accumulation in the cell, and a better method to quantify antibiotic concentration and kinetics of its permeation through the cell membrane is urgently required. If these measurements are correlated with an intrinsic structural property of respective antibiotics, a deeper and more
relevant insight about the physicochemical behavior can be achieved for the battle with bacterial drug resistance.

In this regard, ion transfer processes across a polarized interface between two immiscible electrolyte solutions (ITIES) can be a powerful analytical tool for this demand compared to other types of either electrochemical, or no electrochemical approaches. Specifically, ITIES supported by pipet can selectively and directly probe charged molecules transferring across the various types of liquid/liquid interface. The interfacial ion transfer studied by amperometry and (or) voltammetry gives an electrical current signal, enabling to quantify the concentration and transfer kinetics of respective ions. Importantly, ITIES can mimic a cell membrane system, thus being an insightful surrogate for kinetic study of drug entry through the bacterial cytoplasmic membrane. Moreover, this analysis can be used as an excellent alternative for understanding lipophilicity (or, hydrophilicity) of transferred ions. Considering these aspects, the ITIES would be a proper model system to (1) mimic a cell membrane, (2) study the transfer process of differently charged drug molecules at different pH values, and (3) provide insightful information related to physicochemical behavior and drug delivery, thus assisting drug design and synthesis.

Herein, we apply a steady state voltammetry using an ITIES to sense antibacterial drugs and determine their ion transfer kinetics under a physiological condition. Specifically, we employ nanopipet based ITIES as an artificial membrane system and quantitatively assess the ion transfer kinetics of common antibacterial agents, quinolones and sulfonamides. At ~pH 8.00, all quinolones and sulfonamides undergo deprotonation with a net charge, $-1$, thus being detectable as pristine with ITIES (Figure 1). In comparison to metal electrodes,
a nanopipet supported ITIES not only allows to sensitively detect redox inactive ions\textsuperscript{23, 26-28} but also provides a selectivity for diverse ions\textsuperscript{29-30} physical robustness,\textsuperscript{31} and an extremely small size of active area for a high spatial resolution measurement.\textsuperscript{32} In this study, a nanopipet supported ITIES is formed at the 70~120 nm radius pipet tip filled with a water-immiscible 1,2-dichloroethane (DCE) solution to voltammetrically and amperometrically detect an aqueous drug ion (Figure 2-2). As a potential is applied to the organic phase against aqueous reference electrode, a target ion is driven to transfer from water to organic phase across the interface, giving electrical currents. Importantly, this study reveals that the kinetics of interfacial ion transfer for both quinolone and sulfonamides significantly slow down compared to a typical hydrophobic cation, TBA\textsuperscript{+}. Also, a considerably high hydrophilicity of drug ions is evaluated quantitatively against a hydrophobic anion, ClO\textsubscript{4}\textsuperscript{-}. We attribute the observed sluggish kinetics exclusively to a high hydrophilicity of respective drug ions (see Result and Discussions). In fact, this high hydrophilicity is likely ascribed to unshielded, localized negative charges on the carboxylate or amide group for quinolones or sulfonamides respectively. Here, we argue that the presence of these localized negative charges could play a dominant role on increasing a hidden barrier relevant to formation/breaking of a water-finger upon interfacial ion transfer. Hence, this structural property could mainly induce a retarded rate of interfacial ion transfer. It should be noted that this study suggests a close correlation between transfer-kinetics and the effective hydrophilicity related to the molecular structure of drug ions during ion transfer, thereby leading to a great insight into the physicochemical behavior of drug ions upon the permeation through an artificial membrane system.
Experimental Section

Chemicals. The following chemicals were used as received: Boric acid, N, N-dimethyltrimethylsilylamine (≥ 99%), tetrabutylammonium (TBA⁺) chloride, Nalidixic acid (NA), Flumequine (FMQ), Sulfadimethoxine (SDM), Sulfamethazine (SMT), Sulfamerazine (SMR), and Difloxacin (DFX), sodium perchlorate, 1,2-dichloroethane (DCE), and hydrochloric acid from sigma aldrich. The tetrakis(pentafluorophenyl)borate salt of tetradodecylammonium (TDDA⁺·TFAB⁻) was obtained by metathesis and used as

\[
\text{(A) Sulfonamides} \\
\begin{array}{c}
\text{NH}_3 \\
\text{SO}_2-\text{N}R \\
\end{array}
\rightleftharpoons
\begin{array}{c}
\text{NH}_2 \\
\text{SO}_2-\text{N}R \\
\end{array}
\rightleftharpoons
\begin{array}{c}
\text{NH}_3 \\
\text{SO}_2-\text{N}R \cdot \text{H}^+ \\
\end{array}
\]

\[
\text{(B) Quinolones} \\
\begin{array}{c}
\text{COOH} \\
\text{R}_1 \text{R}_2 \text{R}_3 \text{R}_4 \\
\end{array}
\rightleftharpoons
\begin{array}{c}
\text{COO}^- \\
\text{R}_1 \text{R}_2 \text{R}_3 \text{R}_4 \\
\end{array}
\]

Figure 2-1. Acid dissociation equilibria of (A) sulfonamides (SDM, SMR, SMT) (B) acidic quinolones (top, NA, FMQ) and piperazinyl quinolones (bottom, DFX). The negatively charged group after deprotonation is highlighted. Detailed structures of each drug molecules a molecules are shown in Figure 2-S1.
organic supporting electrolytes. Deionized nanopure water (18.2 MΩ·cm, TOC 2 ppb; Milli-Q Integral 4-System, Millipore) was used to prepare all the aqueous electrolyte solution.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nalidixic acid (NA)</td>
<td>5.95</td>
</tr>
<tr>
<td>Difloxacin (DFX)</td>
<td>5.66, 7.24</td>
</tr>
<tr>
<td>Flumequine (FMQ)</td>
<td>6.35</td>
</tr>
<tr>
<td>Sulfadimethoxine (SDM)</td>
<td>2.13, 6.08</td>
</tr>
<tr>
<td>Sulfamerazine (SMR)</td>
<td>2.22, 6.80</td>
</tr>
<tr>
<td>Sulfamethazine (SMT)</td>
<td>2.37, 7.49</td>
</tr>
</tbody>
</table>

Table 2-1. pK<sub>a</sub> values for the quinolones and sulfonamides. Detailed structures of each drug molecules are shown in Figure S1.

Figure 2-2. (A) Schematic illustration of an ion transfer of target drug ions at a water/1,2-DCE interface measured by nanopipet voltammetry. (B) SEM image of an orifice of a silanized nanopipet with 3 nm thick Au layer coated by Au sputter coater.

Nanopipet Preparation and Characterization by SEM. Tapered nanopipets with an inner tip radius of 70~120 nm were obtained by pulling 10 cm long quartz capillaries (outer/inner diameter ratio of 1.0/0.7; Sutter Instrument Co., Novato, CA) using a CO<sub>2</sub> laser capillary puller (model P-2000, Sutter Instrument). In details, A quartz capillary was cleaned by compressed-air blowing and pulled in CO<sub>2</sub> laser puller with pulling parameters.
(heat= 710, filament= 4, velocity= 30, delay= 130 and pull= 130). The pipets were then cleaned with UV plasma cleaner for 3 min under pursing with Ar gas before the silanization. The pulled nanopipets were dried for 1.5 hours under vacuum (~70 mTorr) in a desiccator (Mini-vacuum desiccator, Bel-Art Products, Pequannock, NJ) and then silanized by introducing 50 μL of N, N-dimethyltrimethylsilylamine into round bottom flask connected to the desiccator. Silanization was performed for 40 min under a constant relative humidity 16 % at 20 °C controlled in a glove bag purged with N₂. After silanization, the desiccator was vacuumed for 10 min followed by purging with N₂ for 5 min to remove extra silanization reagent. The silanized pipets were further filled with 10 μL of a 1,2-DCE solution containing 0.1 M of the organic supporting electrolytes (TDDA⁺·TFAB⁻) right before electrochemical measurements.

The silanized nanopipets were characterized by a field emission SEM (SIGMA VP Field Emission-Scanning Electron Microscope, Zeiss) after coating 3~4 nm thick Au layer by Au sputter coater (108 auto sputter coater with thickness controller MTM-20, Cressington). A nanopipet was vertically observed, and its orifice was visualized with 5 kV accelerating voltage (Figure 2-2B).

**Solution Preparation for the Electrochemistry.** 10 mM boric acid solution with 50 mL was prepared with the nanopure water. By adding ~0.5 mL of 0.1 M NaOH with drop by drop, a pH of boric acid buffer was adjusted to pH 8.20 ~ 8.40 using pH meter. Using this boric acid buffer solution, 5 mM of drug molecule solution with 10 mL was prepared, of which the final pH was adjusted to pH 8.30 ± 0.1. 10 mM TBA⁺ solution with 10 mL using the boric acid buffer solution was separately prepared as well.
**Electrochemical Measurements.** All electrochemical experiments were carried out at room temperature (20 °C) in a two electrode cell: The silanized nanopipets were filled with organic solution from the rear using a 10 µL syringe. Cyclic voltammograms (CVs) were obtained with a bipotentiostat (CHI 760E, CH Instrument, Austin, TX). The voltage was applied between Ag/AgCl reference electrode in the boric acid buffer solution and an electrochemically etched Pt wire as an electrode in the nanopipet. For the voltammetry measurements, sequential experiments were performed in (1) aqueous boric acid buffer only, (2) with adding drug molecules, and finally (3) adding TBA$^+$ in the solution. Briefly, first, a voltammogram was obtained in 9 mL of an aqueous boric acid buffer solution. Subsequently, 2 mL of 5 mM drug ion solution containing respective quinolones or sulfonamides was added into the aqueous buffer solution, and the voltammogram of a target drug ion was recorded with the final concentration, 0.9 mM of drug ions. Finally, 1mL of solution was taken out, and 1mL of 10 mM TBA$^+$ solution was further added. A voltammogram of TBA$^+$ ion transfer with final concentration, 0.9 mM was monitored to characterize the pipet size. This sequential measurement of voltammeteric response clarifies a well-resolved ion transfer of a target drug ion as well as an accurate pipet characterization. All voltammograms were recorded with a scan rate at 25 mV/s.

cell 1 (buffer only in aqueous phase):

Pt | 0.1 M TDDATFAB + 1,2-DCE || 10 mM boric acid || 1 M KCl | AgCl | Ag

cell 2 (buffer + drug ions in aqueous phase):

Pt | 0.1 M TDDATFAB + 1,2-DCE || 0.9 mM drug ions + 10 mM boric acid || 1 M KCl | AgCl | Ag
Results and Discussion

Drug Ion Transfer at a Water/1,2-DCE Interface and Kinetic Analysis of Nanopipet Voltammograms. A boric acid buffer at pH 8.30 was particularly used, where a neutral acid form is dominant (pKa 9.24),\textsuperscript{33} thus minimizing interference from ionic species in the aqueous bulk solution, and better resolving a response of a target drug ion transfer during the voltammetric measurements. Particularly, pH 8.30 is within a physiological pH range in human digestive track e.g. duodenum (pH 7.0 ~ 8.5).\textsuperscript{34} More importantly, bacteria have shown the highest activity at pH 8.0~9.0 range.\textsuperscript{35} Given these two factors, all the electrolytes with drug ions were prepared at pH 8.30 ± 0.10. At pH 8.30, drug molecules are deprotonated, and negatively charged on the carboxylate or amide group of quinolones (NA\textsuperscript{−}, DFX\textsuperscript{−} and FMQ\textsuperscript{−}) or sulfonamide derivatives (SMR\textsuperscript{−}, SMT\textsuperscript{−}, SDM\textsuperscript{−}), respectively (Figure 1), which enables to be sensed as pristine by ITIES.

To clarify the ion transfer of a target drug as well as a characterization of the corresponding nanopipet geometry simultaneously, a series of electrochemical measurements were sequentially performed. First, we recorded a voltammetric response of the bulk solution and confirmed the available potential window. Once we defined an available potential window in aqueous buffer solutions, a stock solution of drug molecules (5 mM) was further added in the bulk solution to make a final concentration 0.9 mM. Then, voltammetry was carried out in the presence of drug ions in the bulk solution within the potential window.
determined by the above bulk experiment. Any voltammetric current in this potential range is solely attributed to a drug ion transfer across an interface.

Figure 2-3. Steady-state voltammograms of (A) TBA⁺ (B) NA⁻ (C) FMQ⁻ (D) SDM⁻ (E) SMR⁻ (F) SMT⁻ transfers across the DCE/water interface obtained with nanopipets. Inset shows a molecular structure of each ion. The best theoretical fitting (red open circles)

After either well or poorly resolved voltammograms of a drug ion transfer were obtained, 1 mL of 10 mM TBA⁺ solution was finally added, thus being a final concentration 0.9 mM TBA⁺ used to characterize a nanopipet size and the intactness of ITIES. In this case, the direction of a potential scan is opposite to the case of anionic drug molecules, i.e. from
positive to negative polarity, thereby the obtained current from the TBA\(^+\) ion transfer has an opposite sign as well, showing a positive limiting current (Figure 2-4).

Experimental voltammograms of TBA\(^+\) and drug ions (SDM\(^-\), SMR\(^-\), SMT\(^-\), NA\(^-\), FMQ\(^-\), and DFX\(^-\)) are shown in Figure 2-4 a-f and Figure 2-S4. The ion transfer of tested drug-ions gave well-resolved voltammetric responses against a response from the bulk solution in a given potential window. In addition, nearly well-defined voltammograms for SDM\(^-\), SMR\(^-\), SMT\(^-\), NA\(^-\) and FMQ\(^-\) could be obtained with distinct inflection points, whereas DFX\(^-\) showed a poorly defined voltammogram without any noticeable inflection point (see Figure 2-S5 in supporting information). Although most of recorded voltammograms were fairly well defined, we could not obtain a clear/stable steady-state diffusion limiting current (\(i_{\infty, i}\)). It is because a drug ion transfer occurred in highly positive potential window, thereby not only partially overlapping with a bulk solution response, but also increasing a resulting current due to a deformed geometry of an interface induced by extreme potential.\(^{36}\) Except DFX\(^-\), the inflection points observed from other drug ion transfers were distinct enough to allow for extrapolation of voltammograms to estimate a steady state limiting current as explained below. A steady-state diffusion limiting current and mass transfer coefficient (\(m_i\)) based on the ion transport are given by\(^{19,37}\)

\[
i_{\infty, i} = 4xz_iFD_iC_i\pi a \quad (1)
\]

\[
m_i = \frac{4xD_i}{\pi a} \quad (2)
\]

where \(F\) is the Faraday constant (96485 C/mol), and \(z_i, D_i,\) and \(C_i\) are the charge (−1) of the transferred drug ion \(i\), its diffusion coefficient, and bulk concentration (0.9 mM) in the aqueous boric acid buffer solution, respectively, and \(x\) is a function of outer radius (rg) /
inner radius \((a)\) of a nanopipet. Silanized pipet with \(rg/a = 1.4\) determined by SEM measurements has \(x = 1.18\).\(^{38}\) \(a\) was determined from the limiting current of a TBA\(^+\) transfer using eq 1 with \(z_{\text{TBA}^+} = 1\), \(C_{\text{TBA}^+} = 0.9\) mM, and \(D_{\text{TDTBA}^+} = 6.0 \times 10^{-6}\) cm\(^2\)/s.\(^{23}\) The inner radii from this electrochemical characterization and SEM measurements are nearly identical indicating an inlaid-disk shaped geometry of ITIES owing to a well-controlled silanization of a nanopipet.

<table>
<thead>
<tr>
<th>ions</th>
<th>pipe radius, (a) (nm)</th>
<th>(D) in aq. phase (cm(^2)/s)</th>
<th>(m) (cm/s)</th>
<th>(k^0) (cm/s)</th>
<th>(\varphi_0^i) (V)</th>
<th>(\alpha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBA(^+)</td>
<td>75</td>
<td>(6.0 \times 10^{-6})</td>
<td>1.20</td>
<td>3.6</td>
<td>0.0675</td>
<td>0.45</td>
</tr>
<tr>
<td>NA(^-)</td>
<td>100</td>
<td>(7.0 \times 10^{-6})</td>
<td>1.05</td>
<td>0.0011</td>
<td>0.110</td>
<td>0.36</td>
</tr>
<tr>
<td>FMQ(^-)</td>
<td>121</td>
<td>(1.3 \times 10^{-6})</td>
<td>1.69</td>
<td>0.0034</td>
<td>0.190</td>
<td>0.45</td>
</tr>
<tr>
<td>SMT(^-)</td>
<td>68</td>
<td>(1.5 \times 10^{-6})</td>
<td>3.22</td>
<td>0.0016</td>
<td>(-0.060)</td>
<td>0.37</td>
</tr>
<tr>
<td>SMR(^-)</td>
<td>77</td>
<td>(8.0 \times 10^{-6})</td>
<td>1.50</td>
<td>0.0030</td>
<td>0.070</td>
<td>0.40</td>
</tr>
<tr>
<td>SDM(^-)</td>
<td>89</td>
<td>(8.5 \times 10^{-6})</td>
<td>1.42</td>
<td>0.0028</td>
<td>0.001</td>
<td>0.40</td>
</tr>
<tr>
<td>DFX(^-)</td>
<td>77</td>
<td>(1.1 \times 10^{-5})</td>
<td>2.15</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 2-2. Geometric, transport and kinetic parameters determined from nanopipet voltammograms with target drug ions and TBA\(^+\). Note that a pipet radius, \(a\) for all the drug ions was determined by subsequently measuring a limiting current of TBA\(^+\) ion transfer. More details about determining the above parameters are given in SI.

With using parameters, a nanopipet radius (a) determined from TBA\(^+\) voltammogram and an extrapolated steady state limiting current from drug ion voltammogram, a diffusion coefficient of each drug ion could be calculated (see in Table 2-1). Herein, In order to not only extrapolate the limiting current, but also theoretically analyze experimental voltammograms for extracting the kinetic information, we performed the finite element analysis using COMSOL Multiphysics version 5.3a (COMSOL, Inc., Burlington, MA). In this analysis, we assume Butler-Volmer model with the heterogeneous rate constants, \(k_f\) and \(k_b\) given by\(^{37}\)
\[ k_f = k^0 \exp \left[ \frac{-az_iF(\Delta\phi - \Delta\phi^0_i)}{RT} \right] \] (3)

\[ k_b = k^0 \exp \left[ \frac{(1-\alpha)z_iF(\Delta\phi - \Delta\phi^0_i)}{RT} \right] \] (4)

where \( \phi_i^0 \) a formal transfer potential of ion \( i \), \( \alpha \) is a transfer coefficient, \( R \) is a gas constant, \( k^0 \) is a standard heterogeneous ion transfer rate constant, and \( \Delta\phi \) is the Galvani potential difference between the aqueous and DCE phases.

Simply, the corresponding ion transfer with a charge of \( z_i \) is defined as below

\[ i^{z_i} \text{(aqueous)} \xrightleftharpoons[k_b]{k_f} i^{z_i} \text{(1,2-DCE)} \] (5)

As a result, theoretical currents of an interfacial ion transfer were simulated by solving diffusion problems for ion transfer voltammetry with COMSOL Multiphysics, and plotted together with the experimental voltammograms (Figure 2-4). For the precision of kinetic analysis, background currents measured in aqueous buffer solution only (grey solid curves in Figure 2-4) were numerically subtracted from the experimental voltammograms of respective drug ions. The forward waves of each experimental voltammogram were fitted with theoretical curves. From these fittings, we could assess the kinetic parameters for both the drug ions and TBA\(^+\) transfer. In Table 2-1, we summarize all the geometric and kinetic parameters determined from nanopipet voltammograms with drug ions and TBA\(^+\). Notably, we could determine \( k^0 = 3.61 \pm 0.02 \) cm/s and \( \alpha = 0.45 \) for the interfacial transfer of TBA\(^+\), showing a quasi-reversible behavior. These obtained values are nearly close to \( k^0 = 6.1 \pm 0.9 \) cm/s and \( \alpha = 0.49 \pm 0.09 \) reported for the transfer of tetraethylammonium (TEA\(^+\)) at the water/DCE interface using nanopipet voltammetry.\(^1\) This result implies a good quality of the experimental conditions for our nanopipet voltammetry, thus guaranteeing a reliability of our kinetic study with drug ion transfer under the given system.
In this analysis, the determined $k^0$ values of the ion transfer for target drugs are markedly lower than the rate constant of TBA$^+$ ion transfer. For instance, they range from 0.0011 to 0.0034 cm/s, thus being ~3 orders of magnitude smaller compared to the TBA$^+$ ion transfer at a water/1,2-DCE interface, and indicating an obvious kinetic limitation. This clear kinetic effect on the drug ion transfer could be ascribed to structurally relevant properties such as a hydrophilicity of the drug molecules. We will discuss more details about this kinetic issue related to the hydrophilicity of drugs in the following section. The values of $\alpha = 0.41 \pm 0.04$-Showed a good agreement with the experimental voltammograms, nearly close to 0.5. $\alpha = 0.5$ has been predicted in the ion transfer models considering either the slow diffusion of the transferred species through the mixed interfacial layer$^{39,40}$ or assuming an activation barrier.$^{41,42}$

![Figure 2-4](image)

**Figure 2-4.** Normalized voltammograms of various ions transferred across a water/1,2-DCE interface supported at a nanopipet. Reference electrode is Ag/AgCl in the aqueous phase. Potential sweep rate, 25 mV/s.

**Effective Hydrophilicity of Drug Ions.** Herein, we quantitatively assess the effective hydrophilicity of tested drug molecules in comparison to a typical hydrophobic anion,
ClO$_4^-$). For the comparison among the different ions, the experimental voltammograms were normalized against the theoretically predicted or experimentally obtained limiting currents, $i_{T,\infty}$ for drug ions or TBA$^+$, respectively, and plotted together with respect to Ag/AgCl reference electrode in the aqueous phase. The voltammogram of a more hydrophilic anion appears at the more positive potentials, showing an order of FMQ$^- >$ NA$^- >$ SMR$^- >$ SMT$^- >$ SDM$^- >$ ClO$_4^-$. 

From the half-wave potentials of the experimental voltammograms, the effective hydrophilicity of FMQ$^-$, NA$^-$, SMR$^-$, SMT$^-$ and SDM$^-$ was quantitatively evaluated given by$^{23,24}$

<table>
<thead>
<tr>
<th>Ions</th>
<th>$\Delta E_{1/2}$ (mV)</th>
<th>Hydrophilicity factor ($P_i/P_{ClO4^-}$) ($10^x$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDM$^-$</td>
<td>108</td>
<td>1.8</td>
</tr>
<tr>
<td>SMT$^-$</td>
<td>126</td>
<td>2.1</td>
</tr>
<tr>
<td>SMR$^-$</td>
<td>184</td>
<td>3.1</td>
</tr>
<tr>
<td>NA$^-$</td>
<td>218</td>
<td>3.7</td>
</tr>
<tr>
<td>FMQ$^-$</td>
<td>336</td>
<td>5.7</td>
</tr>
</tbody>
</table>

**Table 2-3.** Estimated effective hydrophilicity of drug ions against a typical hydrophobic anion, ClO$_4^-$ determined from Figure 2-4. $\Delta E_{1/2}$ is a difference in half-wave potentials between corresponding drug ions and ClO$_4^-$. DFX$^-$ was not applicable in this analysis.

$$\frac{P_i}{P_{ClO4^-}} = \exp \left[ - \frac{zF(E_{i,1/2} - E_{ClO4^-,1/2})}{RT} \right]$$  \hspace{1cm} (6)

where $P_i$ and $P_{ClO4^-}$ are the partition coefficients of drug ion, $i$ and ClO$_4^-$ between the aqueous buffer and 1,2-DCE phases, $z$ is a net charge ($z = -1$), and $E_{i,1/2}$ and $E_{ClO4^-,1/2}$ are
the half-wave potentials of the corresponding ions as determined from the experimental voltammograms in Figure 2-5.

For instance, FMQ$^-$ is approximately 105 times more hydrophilic than ClO$_4^-$ with a difference of $\sim$300 mV between their half-wave potentials. Likewise, SMT$^-$ showed $\sim$120 mV difference in half-wave potentials, thereby showing $\sim$10$^2$ times higher hydrophilicity than ClO$_4^-$. Quantitatively assessed effective hydrophilicity for all other drug ions with respect to the corresponding half-wave potential differences is summarized in Table 2-2. Notably, the resulting hydrophilicity of deprotonated drug ions are extremely high with about 2 $\sim$ 5 orders of magnitude compared to a hydrophobic anion ClO$_4^-$. 

From the given structure of drug molecules (see insets in Figure 2-4 and 2-S1), one could intuitively expect a high lipophilicity because of the presence of aromatic ring with delocalized $\pi$-electrons. These drugs, however, have isolated negative charges on the carboxylate or amide group once deprotonated, which are unshielded in their molecular structures as well.$^{43-46}$ Such localized negative charges could dominate the effective hydrophilicity of the drug ions, especially when they transfer across a water/DCE interface. In fact, nanopipet voltammetry in this study revealed an extremely high value of effective hydrophilicity for deprotonated quinolone and sulfonamide ions. So then, how does the high hydrophilicity of drug ions affect the kinetics of ion transfer? Recently, a hidden barrier of ion passage has been proposed during ion transfer across the interface between a water/dichloromethane (DCM) by the study of the molecular dynamic simulation.$^{47}$ In this theoretical approach, a barrier accompanied with a formation/breaking of water-finger explicitly elucidates the retarded rate of interfacial ion transfer. For example, upon the formation of water-finger structure, the excessive free energy barrier of $\Delta G^\ddagger = 4$ kcal/mol
is estimated. According to Arrhenius equation \( k = A \exp(-\Delta G^*/RT) \), this energy barrier leads to decrease in transfer kinetics with three orders of magnitude, which is consistent with our observation of ~3 orders of magnitude slower kinetics of drug molecules in interfacial transfer than typical hydrophobic cation, TBA\(^+\) interfacial transfer. In fact, higher charge densities localized on the carboxylate or amide group could cause stronger interactions between the ion and water, thereby increasing an energy barrier upon the water-finger formation and breaking during the interfacial ion transport. Correspondingly, a higher barrier leads to slow kinetics of drug ion transfer across the interface.

**Stability in Amperometric Response of Drug Ion Transport.** Amperometry was also performed to probe a stability of steady-state current of drug ion transfer over time. A constant potential at which a maximum steady state current was obtained, was applied, and the resulting currents were monitored over 800~1000 s. Stable currents over the measurement time have been observed for all the drug ions maintaining 80~100 % of initial currents (Figure 2-S5 in supporting information). Notably, DFX\(^-\) also showed remarkably stable currents with time, whereas an accurate kinetic analysis was hampered by a poorly defined voltammogram. These results imply that nanopipet supported ITIES can be employed as a nanoprobe to sense drug ions for the high spatial steady state measurement, such as scanning electrochemical microscopy (SECM). For instance, a sufficiently stable steady-state current over ~1000 s promises a reliable mapping of local drug-permeation across biological membranes of microbial organisms at a high spatial resolution.
Conclusions

This work successfully employed nanopipet supported ITIES in lieu of a biological system for studying the ion transfer of antimicrobial drugs, quinolones and sulfonamides as pristine. The high selectivity of nanopipet tips enabled us to quantitatively monitor drug ion transports across the interface under a physiological condition. Using theoretical analysis, we quantitatively evaluated kinetics of interfacial ion transfer of various target drugs and revealed ~3 orders of magnitude slower kinetics compared to a typical hydrophobic cation, TBA$^{+}$. In addition, extremely high effective hydrophilicity of target drug ions was quantitatively assessed from the nanopipet voltammograms. Notably, we could estimate about 2 ~ 5 orders of magnitude higher effective hydrophilicity of deprotonated drug ions than a typical hydrophobic anion, ClO$_4^−$. Herein, we ascribed such a high hydrophilicity exclusively to negative charges localized on the carboxylate or amide group of deprotonated quinolones or sulfonamides, respectively. Moreover, this localized charge density may play a dominant role in an increase of the energy barrier upon interfacial ion transfers, since stronger interaction between a high density of negative charges and water is expected during a water-finger formation/breakage near the interface, thereby leading to sluggish transfer kinetics. Our finding based on the study of nanopipet voltammetry gave an insight on the physicochemical behavior of pristine drug ions in the permeation through artificial cell membranes. Finally, a high stability of amperometric current response over time promises an application of nanopipet to a nanoprobe of high spatial steady-state measurements, e.g. SECM to spatially resolve a drug-permeation in the biological system.
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All authors have given approval to the final version of the manuscript

Notes
The authors declare no competing financial interest

Supporting Information

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References


CHAPTER 3

Nanoscale Carbonate Ion-Selective Amperometric/Voltammetric Probes Based on Ion-Ionophore Recognition at Organic/Water Interface: Hidden Pieces of the Puzzle in the Nanoscale Phase

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Nanoscale Carbonate Ion-Selective Amperometric/Voltammetric Probes Based on Ion-Ionophore Recognition at Organic/Water Interface: Hidden Pieces of the Puzzle in the Nanoscale Phase

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Abstract
Here, we report on the successful demonstration and application of carbonate (CO$_3^{2-}$) ion selective amperometric/voltammetric nanoprobes based on facilitated ion transfer (IT) at the nanoscale interface between two immiscible electrolyte solutions (ITIES). This electrochemical study reveals critical factors to govern CO$_3^{2-}$ selective nanoprobes using broadly available Simon-type ionophores forming a covalent bond with CO$_3^{2-}$, i.e., slow dissolution of lipophilic ionophores in the organic phase, activation for hydrated ionophores, peculiar solubility of hydrated ion-ionophore complex near the interface, and cleanness at the nanoscale interface. These factors are experimentally confirmed by nanopipet voltammetry, where a facilitated CO$_3^{2-}$ IT is studied with a nanopipet filled with organic phase containing trifluoroacetophenone derivative CO$_3^{2-}$ ionophore (CO$_3^{2-}$-ionophore VII) by voltammetrically and amperometrically sensing CO$_3^{2-}$ in water. Theoretical assessments of reproducible voltammetric data confirm that the dynamics of
$\text{CO}_3^{2-}$ ionophore VII-facilitated ITs (FITs) follows the one-step electrochemical (E) mechanism controlled by both water-finger formation/dissociation and ion-ionophore complexation/dissociation during interfacial ITs. The yielded rate constant, $k^0=0.048 \text{ cm/s}$ is very similar to reported values of other FIT reactions using ionophores forming non-covalent bonds with ions, implying that a weak binding between $\text{CO}_3^{2-}$ ion-ionophore enables to observe FITs in the fast nanopipet voltammetry regardless of the nature of bondings between ion and ionophore. The analytical utility of $\text{CO}_3^{2-}$ selective amperometric nanoprobe is further demonstrated by measuring a $\text{CO}_3^{2-}$ concentration produced by metal-reducing bacteria, Shewanella oneidensis MR-1 as a result of organic fuel oxidation in bacterial growth media in the presence of various interferents such as $\text{H}_2\text{PO}_4^-\cdot\text{Cl}^-$, and $\text{SO}_4^{2-}$.

**Introduction**

Selective ion-transfer (IT) at liquid/liquid interface has been extensively applied for electrochemical sensing of redox-inactive ions or ionized biomolecules critical for clinical,\(^1\) physiological,\(^2\) industrial,\(^3\) and environmental analysis.\(^4\) While potentiometric ion-selective electrodes (ISEs) have been the most common approach with measuring a change in phase boundary potential upon equilibrated analyte partitioning between two liquid phases,\(^5\)-\(^8\) amperometric/voltammetric ion-selective electrodes have steadily emerged owing to the high sensitivity.\(^9,10\) In fact, current responses of amperometric ISEs directly depend on analyte concentration, whereas potential responses of potentiometric ISEs are varied with the logarithm of the concentration.\(^5,6,11\) This high sensitivity of amperometric/voltammetric approach is more suitable to study heterogeneously distributed
analyte ions in the system by catalytic production, consumption, or other chemical reactions than potentiometric ISEs.\textsuperscript{12-14}

A glass pipet-supported interface between two immiscible electrolyte solutions (ITIES) has served a critical role in studying IT dynamics at liquid/liquid interfaces for both simple and facilitated ITs (FITs). For FITs, ion-selective ionophores existing inside or outside of a pipet facilitate the thermodynamically unfavorable transfer of hydrophilic ions from aqueous solution to organic phase.\textsuperscript{15-16} As pipets are readily pulled to micro-nanometer sizes, ion-selective amperometric/voltammetric probes could be easily miniaturized up to nanometer scale. Especially, a small current across a pipet-supported interface enables not only reliable study of IT dynamics due to a negligibly small ohmic potential drop.\textsuperscript{17} but also spatially resolved study of IT with a combination of scanning electrochemical microscopy (SECM).\textsuperscript{18} Accordingly, the anticipated advantages from a nanoscopic interface using a nanopipet are obvious, which enables determination of large IT rate constants owing to fast mass transfer\textsuperscript{19-22} and mapping heterogeneous distribution of analyte ions with SECM at high spatial resolution of nanoscale.\textsuperscript{23-24,5} So far, most studies in FITs have been focused on the microscale interface using micro-sized pipets, whereas a very few innovative works using nanopipets were reported.\textsuperscript{19-25} Especially, nanoscopic study of a FIT using nanopipets has been rare with broadly available ionophores a.k.a., Simon’s ionophores developed by Wilhelm Simon,\textsuperscript{26} having large molecular weight (~1000 or larger) and high lipophilicity. We suspect that reproducibility and stability could be main challenges in this nanoscale amperometric/voltammetric study. Since the active area of nanopipet interface is small, it is susceptible to adventitious contaminants or precipitate, thus being easily blocked. Accordingly, the stability and reproducibility of
current responses could rely on the control of these two factors, contaminants and precipitate at the nanoscale interface.

The visual characterization of an intact nanopipet interface related to contaminant and precipitate issue is technically limited due to the small size and the liquid phase interface, which makes empirical control of these factors complicated and even puzzled. We investigate CO$_3^{2-}$ selective IT system as a model, and study FITs using nanopipet-supported ITIES with voltammetry technique to demonstrate the origins of hampering factors in this amperometric nano-ISE. In this system, interfacial CO$_3^{2-}$ IT across the water/organic interface supported by a nanopipet is facilitated by CO$_3^{2-}$-selective ionophore dissolved in organic phase inside a pipet (Figure 3-1). A molecular tweezer-type ionophore,N,N-dioctyl-3α,12α-bis(4-trifluoroacetylbenezoyloxy)-5βcholan-24-amide (a.k.a., CO$_3^{2-}$ ionophore VII) has two trifluoroacetophenone groups interacting selectively with CO$_3^{2-}$ to form 1:1 complex with a mixture of covalent and hydrogen bonding (see Figure 3-1a and 3-S1).$^{27,28}$ In the presence of excess ionophore in the inner solution, conventional steady-state voltammograms can be observed for this FIT, where mass transfer is controlled by hemispherical diffusion of CO$_3^{2-}$ ion in the outer aqueous solution.$^{29}$ Herein, our nanoelectrochemical study focuses on four critical factors such as slow dissolution of bulky lipophilic ionophore in the organic phase, activation for hydrated ionophores, peculiar solubility of lipophilic ionophore and ion-ionophore complex in the organic phase near the interface, and cleanness at the nanoscale interface. All factors are directly related with the blockage of the nanoscale liquid/liquid interface, thus determining stability and reproducibility of current responses during FITs, i.e., the performance of amperometric nano-ISEs. Accordingly, resulting steady-state voltammograms should be
evaluated for continuity and stability of current responses, reproducibility during repetitive measurements, and the validity of FIT kinetics and mechanism.

CO$_3^{2-}$ sensing by amperometric nano-ISEs can be broadly applied to study not only total

![Figure 3-1](image)

**Figure 3-1.** Schematic view of a facilitated CO$_3^{2-}$ ion transfer at a water/1,2-DCE interface measured by nanopipet voltammetry/amperometry. (b) An SEM image of an orifice of a silanized nanopipet with ca. 3 nm thick Au layer coated by Au sputter coater. SEM image was obtained using an accelerating voltage 5 kV to minimize charging-up.

CO$_2$ in physiological fluids, oceanic carbonate system, industrial samples but also renewable energy systems such as organic fuel oxidation by dissimilatory metal-reducing bacteria (MRB) used for microbial fuel cells (MFCs), and electrocatalytic reduction of CO$_2$ to organic fuel carried by carbonate solution. Pioneering works on CO$_3^{2-}$ selective potentiometric sensing using CO$_3^{2-}$ ionophore VII have been reported by Bakker group and Nam group to determine CO$_2$ (or, CO$_3^{2-}$) concentration in aqueous/gaseous samples and oceanic carbon dioxide in seawater, which are suitable to investigate bulk samples from physiological fluids, fresh water system, or oceanic carbonate system. However, the study of organic fuel oxidation by MRB at an individual cell or electrochemical
reduction of CO$_2$ at an individual heterogeneous nanocatalyst requires both high sensitivity and miniaturization to nanoscale of CO$_3^{2-}$-ISEs. For example, the quantitative measurement of how fast organic fuel is oxidized at a single MRB is a crucial part to fundamentally understand the rate determining step in electron transport pathway of MRB, which determines the overall efficiency of MFCs.$^{39,40}$ This can be accomplished by real time measuring CO$_3^{2-}$ production over a single bacterium using amperometric nano-ISEs. Also, the catalytic activity of individual heterogeneous nanocatalyst for CO$_2$ reduction can be evaluated by measuring how fast and efficiently CO$_3^{2-}$ is consumed and converted over each discrete catalyst. Particularly, high spatial resolution as well as intrinsically high sensitivity of amperometric nano-ISEs can be combined with SECM to reveal active sites in heterogeneous target samples and establish the relationship between the structure and reactivity of a single object. Despite these analytical potentials, none of studies about amperometric nano- CO$_3^{2-}$-ISEs has been reported so far.

Here, we report on the successful demonstration and application of amperometric CO$_3^{2-}$-ISEs based on FIT at the nanopipet supported ITIES using “Simon” type ionophore (CO$_3^{2-}$ ionophore VII) forming a covalent bond with CO$_3^{2-}$. Nanopipet voltammetry is performed to clarify hampering factors in realizing nanoscale CO$_3^{2-}$-ISEs. Reproducible voltammetric data obtained are theoretically assessed to verify the dynamics and mechanism of the CO$_3^{2-}$-ionophore-FIT. The quantitative analysis yields a normal $\alpha$ value of 0.45 and a rate constant, $k^0$ 0.048 cm/s in one-step electrochemical (E) mechanism controlled by both water-finger formation/dissociation and ion-ionophore complexation/dissociation during interfacial IT.$^{41-43}$ This $k^0$ based on E mechanism is more reasonable than unrealistically fast interfacial IT rate constant, $k^0 = 35$ cm/s and an association rate constant, $k_a = 3.3 \times 10^{20}$
M⁻¹S⁻¹ exceeding diffusion limits in two step-electrochemical-chemical (EC) mechanism based on the simple interfacial transfer of CO₃²⁻ followed by complexation in the organic phase, thereby excluding the EC mechanism for our quasi-reversible FIT at nano ITIES (Table 3-S7 in SI). Notably, the obtained k⁰ in E mechanism is very similar to reported values of other FITs at microscale ITIES using non-covalent bond-forming ionophores,¹⁶ implying that a weak binding of CO₃²⁻ ion-ionophore enables to observe FITs in fast nanopipet-voltammetric measurements regardless of the nature of bonding. We further employed a fundamentally proved nanoscale CO₃²⁻-ISE to sense and quantify CO₃²⁻ produced by MRB, Shewanella oneidensis MR-1 as a result of organic fuel oxidation, thus demonstrating its analytical applicability as well Shewanella oneidensis MR-1 as a result of organic fuel oxidation, thus demonstrating its analytical applicability as well.

Experimental Section
Chemicals. Ammonium carbonate ((NH₄)₂CO₃), tetrabutylammonium chloride (TBA⁺Cl⁻), ammonium phosphate monobasic ((NH₄)H₂PO₄), ammonium phosphate dibasic ((NH₄)₂HPO₄), N,N-dimethyltrimethylsilylamine, 1,2-dichloroethane (DCE), 1,2-dichloromethane (DCM), isopropyl alcohol, tetrabdecylammonium bromide(TDDABr), acetic acid and nitric acid were purchased from Sigma Aldrich, and used as obtained. Potassium tetrakis(pentafluorophenyl)- borate (KTFPB) was purchased from Boulder Scientific. Ionic liquid supporting electrolytes (TDDA⁺TFPB⁻, or TDDATFPB) are prepared by metathesis of TDDABr and KTFPB. More details about the metathesis is in supporting information (SI). N,N-Dioctyl-3α,12α-bis(4-trifluoroacetylbenzoyloxy)-5β-cholan-24-amide (CO₃²⁻ ionophore VII) was used as purchased from Sigma Aldrich.
Modified M-1 medium for Shewanella federation was prepared and provided by Pellock group, and the recipe of modified M-1 medium is stated in detail in SI.

**Fabrication of Nanopipet Electrodes.** Nanopipets were pulled from a quartz capillary (outer diameter = 1 mm, inner diameter = 0.7 mm, length = 10 cm, FG-GQ100-70-10, Sutter Instrument Co, Novato, CA) using CO\(_2\)–laser puller (P-2000, Sutter Instrument Co., Novato, CA). As-pulled nanopipets were further silanized for electrochemical measurements. DCE solution containing 30 mM CO\(_3^{2-}\) ionophore VII and 0.1 M TDDATFPB is prepared by vortexing for 15 min up to 3 hrs for thorough dissolution. (There was no difference between 15 min and 3 hr vortex, data not shown). The silanized pipet is filled with either freshly prepared DCE solution or a premade one aged for ~12 hrs. An electrochemically etched Ni/Cu wire as an inner reference electrode is inserted from the back opening of a nanopipet, and immobilized at the position of c.a. 200 μm from the tapered tip end (details about silanization setup and procedure, and fabrication of inner reference electrodes are in SI)

**Nanopipet Characterization by Scanning Electron Microscopy.**

As-pulled quartz nanopipets were characterized by scanning electron microscopy (SEM) (SIGMA VP Field Emission-Scanning Electron Microscope, Zeiss)). Before SEM measurements, the pipet surface was sputter-coated with c.a. 3 nm thick Au layer to suppress the charging-up (Cressington Au sputter coater 108 auto equipped with Cressington thickness monitor mtm10, Ted Pella INC, Redding, CA, USA). The sputtered Au thickness can be monitored in situ with an aid of the equipped thickness monitor (Cressington thickness monitor mtm10), thereby optimizing the sputtering condition with
20 mA current, 10 s sputtering time and resulting thickness of 3.4~3.7 nm. The orifice of nanopipets was observed using e-beam with 5 kV accelerate voltage (Figure 3-1b).

**NMR Experiments**

All NMR experiments were performed on a Bruker Ascend 400 MHz spectrometer at 298 K. A capillary of 0.010 M hexafluorobenzene (HFB) in DMSO-d6 was prepared as an internal standard. 1H spectra were referenced to the solvent residual signal and collected using standard acquisition parameters. 19F spectra were referenced to HFB and collected using an acquisition delay of 30 seconds. Samples prepared for analysis included 30 mM ionophore VII in DCE, 0.100 M ionic liquid in DCE, and a combination of 30 mM ionophore and 0.100 M ionic liquid in DCE.

**Electrochemical Measurements.** Electrochemical workstations (CHI 8022D and CHI 760E, CH Instruments, Austin, TX) were used for voltammetric and amperometric measurements. Nanopipet voltammetry employed two-electrode cells using a Pt-wire (0.5 mm, hard, 99.95%, Alfa Aesar) as a quasi-reference electrode (QRE) as represented by, Pt QRE | CO$_3^{2−}$ ion and TBA$^+$ ion with supporting electrolytes in 40 mM phosphate buffer or M-1 medium (w) | 30 mM CO$_3^{2−}$ ionophore VII and 0.1 M TDDATFPB (DCE) | Ni/Cu (more details are in SI.)

**Standard Addition Method for Quantification of CO$_3^{2−}$ Level Produced by Shewanella oneidensis.** To quantify the CO$_3^{2−}$ concentration produced by S. oneidensis as a result of oxidation of various carbon sources, we performed the standard addition method as well as nanopipet voltammetry. 30 mM of CO$_3^{2−}$ stock solution prepared with fresh modified M-1 medium was added sequentially as spikes into the M-1 medium used for
bacterial growth, which contained 30 mM lactate or 30 mM acetate with or without fumarate, respectively. The subsequent voltammetry was conducted to measure ionic currents caused by CO$_3^{2-}$ FIT. A calibration plot of limiting currents obtained from background subtracted voltammograms vs. added CO$_3^{2-}$ concentrations was constructed, which resulted in a linear curve. Particularly, the x-intercept of this resulting plot directly corresponds to the original concentration of CO$_3^{2-}$ intrinsically produced by S. oneidensis. More details are in SI.

**Molecular Dynamics Simulations.** The molecular structures of CO$_3^{2-}$ ion, CO$_3^{2-}$ ionophore VII, water and CHCl$_3$ were designed, and each conformation with minimum energy was also found using the MOE (MOE2020, Molecular Operation Environment) software under the program of Merc-molecular force field (MMFF). More details are in SI.

**Results and Discussion**

I. Slow Dissolution of Bulky Ionophore in the Organic Phase and Peculiar Solubility Origin at the Nanoscale Interface

Homogeneous DCE solution containing 30 mM CO$_3^{2-}$ ionophore was prepared with vortex for 15 min prior to usage, which showed neither precipitation nor a separated layer. With this fresh DCE inner filling solution, a nanopipet electrode was fabricated by inserting an electrochemically etched Pt wire as an inner reference electrode. Subsequently, nanopipet voltammetry was performed in the aqueous bulk solution containing TBA$^+$ and CO$_3^{2-}$, where a simple TBA$^+$ IT was monitored in the negative potential scans to characterize a nanopipet, and a CO$_3^{2-}$ FIT was studied in the positive potential scans. Within 5 runs, a nanopipet was totally blocked, thus ionic current responses from both TBA$^+$ and CO$_3^{2-}$ ITs disappeared (grey curves, Figure 3-2a). These sudden blockages of pipets were observed
reproducibly during repetitive voltammetric measurements (100 % failure with n > 50, data not shown). Notably, when the ionophore concentration was reduced to 5 mM, the blockage of nanopipets filled with freshly prepared ionophore solution was mitigated with showing nearly reproducible TBA\(^+\) ITs and yet hardly resolved CO\(_3^{2−}\) FITs (Figure 3-S5). These results clearly imply that a solubility of ionophore in DCE solution would be a critical factor in the observed FITs.

In fact, dissolution is not an instantaneous process. Under the diffusion limiting process, the dissolution rate of a solid solute is dependent on its surface area and the diffusion coefficient as well as concentration gradient between the solid surface (or saturated), and bulk solution as described by Noyes-Whitney equation.\(^{46}\)

\[
\frac{dC}{dt} = \frac{D_s}{d} \left( C_s - C_b \right)
\]  

\((1)\)
where \( \frac{dC}{dt} \) is dissolution rate, \( t \) is time, \( D \) is diffusion coefficient of solute in solution, \( s \) is the surface area of dissolving solute exposed in solvent, \( d \) is the thickness of the diffusion layer \( i.e., \) the thickness of the boundary layer of solvent between the surface of the dissolving substance and bulk, \( C_s \) is a concentration of solute at the solid surface (or saturated), and \( C_b \) is a concentration of the solute in the bulk solution. Especially for a bulky solute like \( \text{CO}_3^{2-} \) ionophore VII dissolved in fairly viscous DCE solution, a small magnitude of \( D \) is anticipated by the Stokes-Einstein equation below, \(^{47}\)

\[
D = \frac{kT}{6\pi\eta r}
\]  

(2)

where \( k \) is Boltzmann constant, \( T \) is the absolute temperature, \( \eta \) is viscosity of solution, and \( r \) is the radius of solute molecule. Hence, small magnitudes of \( D \) and solute surface area, \( s \) of a bulky ionophore would lead to decrease in the overall dissolution rate (eq 1). This intuitive speculation, however, is not trivial, since an amperometric nano-ISE with highly lipophilic Simon type ionophores has been often fouled unknowingly, and its utilization has been rare to happen. \(^{48}\)

30 mM \( \text{CO}_3^{2-} \) ionophore VII in DCE is obviously excessive compared to the level of \( \text{CO}_3^{2-} \) studied, necessary for steady-state measurements. As \( \text{CO}_3^{2-} \) ionophore VII is highly lipophilic, an intrinsically high solubility in DCE solution is anticipated. In MD simulation, the solvation of ionophore in the organic phase has a large negative value of free energy, thus being thermodynamically favorable (Table 3-S5 and 3-S6).

However, our repetitive failures in realizing amperometric nano-ISEs with nanopipet voltammetry call attention to a need of better understanding distinctive solubility of lipophilic ionophore and its ion-complex in the organic phase. Subsequently, the solubility...
origin at the nanoscale in nonpolar environments was reconsidered. Earlier, Fileti and coworker performed classical MD simulations, and predicted the decrease in solvation of arbitrary nanostructures due to not favorable enthalpic but unfavorable entropic contributions as their dimension becomes c.a. 2 nm or smaller. A sharp positive increase in solvation free energy from c.a. 2 nm to 0.2 nm of nanostructures is predicted due to lower conformational diversity of solvent-solute interactions in the solution, thereby decreasing the solvation entropy. This theoretical speculation is consistent with our experimental observation of blocked current responses from nanopipets filled with DCE solution containing neutral lipophilic CO$_3^{2-}$ ionophore VII smaller than 2 nm. Hence, we suspected a decrease in the solubility of nanosized ionophores near the interface, which could transiently cause a local precipitation and the subsequent blockage of a nanopipet interface.

Moreover, the solubility of ion-ionophore complex near the organic/water interface at nanoscale should be differentiated from that in bulk organic phase. A FIT occurs across the interface from water to the organic phase, where a hydrated ion forms adduct with water molecules, so called a water-finger. The water-finger formation/dissociation during interfacial ITs has been suggested by various groups based on theoretical and experimental approaches. The breakage of water-finger leaves a partially hydrated ion surrounded by averagely 10 water molecules in the organic phase, which further undergoes a complexation with ionophore.

To elucidate the effect of partial hydration on the solubility of an ion-ionophore near the interface, we performed MD simulation using the Molecular Operation Environment (MOE) software under the program of Merc-molecular force field (MMFF). The solvation
free energies of a complex between CO$_3^{2−}$ ionophore VII and CO$_3^{2−}$ in organic mono-phase (CHCl$_3$) were calculated, where both free CO$_3^{2−}$ and partially hydrated CO$_3^{2−}$ with 10 water molecules were studied. Compared to a complex of CO$_3^{2−}$ ionophore VII and free CO$_3^{2−}$, the free energy of a complex of CO$_3^{2−}$ ionophore VII with partially hydrated CO$_3^{2−}$ merely increases to positive, c.a. 1.6 % (Figure 3-3b and Table 3-S5 in SI). Within the diphase system, however, the free energy of CO$_3^{2−}$ ionophore VII complexed with partially hydrated CO$_3^{2−}$ dissolved in the CHCl$_3$ showed c.a. 8.5 % positive increase compared to that of free CO$_3^{2−}$ ionophore VII dissolved in the CHCl$_3$ (Figure 3-3c and Table 3-S5 in SI). Because of an exponential dependence of solubility on the free energy, i.e., $K \sim \exp(-\Delta G)$, even a moderate increase of the free energy leads to a significant decrease in solubility.$^{49}$ Resultantly, the decrease in the solubility of an ion-ionophore complex near the organic/water interface, could hamper our voltammetric study of a FIT across the nanoscale interface.

Based on the speculation on the slow dissolution and the distinct solubility at nanoscale interface, sufficient time for CO$_3^{2−}$ ionophore VII solvation in DCE was given by preparing the DCE solution up to 15 min vortex and storing on the laboratory bench for 9~12 hrs before usage. In nanopipet voltammetry, nanopipet electrodes filled with this premade ionophore solution successfully showed current responses for the CO$_3^{2−}$ FIT in the positive potential scan as well as a simple TBA$^+$ IT occurred in the negative potential scan (Figure 3-2b). Conspicuously, each corresponding IT reaction was driven reproducibly without notable blockage of nanopipet electrodes in repetitive measurements (c.a. 80 % success rate with n > 50, data not shown). These reproducible voltammetric data validate our
theoretical speculations on both the slow dissolution rate of lipophilic bulky ionophore and the peculiar solubility of ion-ionophore complex at the nanoscale interface.

**Activation of Ionophore with Hydrated Carbonyl Centers in Trifluoroacetyl Group**

The reduced concentration of CO$_3^{2-}$ ionophore in DCE solution clearly mitigates the blockage of nanopipet interface during nanopipet voltammetry, thus proving an effect of ionophore solubility on the performance of nano-ISEs. However, nanopipets filled with a freshly prepared ionophore solution could not effectively drive FITs regardless of the

![Figure 3-3](image.png)

**Figure 3-3.** Snapshots of (a) a complex of CO$_3^{2-}$ ionophore VII and partially hydrated CO$_3^{2-}$ ion surrounded by 10 water molecules. The red circle represents 10 water molecules surrounding CO$_3^{2-}$ ion. (b) a complex of CO$_3^{2-}$ ionophore VII with CO$_3^{2-}$ surrounded by 10 water molecules solvated in CHCl$_3$ organic monophase. A CO$_3^{2-}$ ionophore VII is depicted with magenta balls, and the chloride atoms of CHCl$_3$ are shown in green. (c) a complex of CO$_3^{2-}$ ionophore VII with CO$_3^{2-}$ surrounded by 10 water molecules is solvated in CHCl$_3$ organic phase near water/ CHCl$_3$ interface. A CO$_3^{2-}$ ionophore VII is depicted with magenta balls. The oxygen atoms of water are shown in red, and the chloride atoms are shown in green. (d) Hydration of trifluoroacetyl ketone of CO$_3^{2-}$ ionophore VII.
concentration of CO$_3^{2-}$ ionophore in DCE solution, whereas the use of premade ionophore solution enabled the CO$_3^{2-}$ FITs (Figure 3-S5). This behavior cannot be explained by either solubility or dissolution rate. Indeed, the aging of DCE solution for 12 hrs seems to be critical to make CO$_3^{2-}$ FITs possible. Herein, we hypothesized that the aging of carbonate ionophore solution forms a hydrated ionophore, in which a hydrated carbonyl center of trifluoroacetyl group is an active form to readily bind with CO$_3^{2-}$ during interfacial ion transfer (Figure 3-3d and 3-S1). Earlier, the hydrated form of carbonate ionophore VII before complexing with CO$_3^{2-}$ has been reported by Nam group with their fast atom bombardment mass spectroscopic study.$^{27}$

We conducted $^{19}$F-NMR and $^1$H-NMR with a freshly prepared ionophore solution and monitored the spectra changes with time to determine if hydration occurs in this mixture. There was inappreciable moisture present in the original NMR sample, so we performed a time study on both the ionic liquid and the ionophore together (i.e., a similar composition to a DCE filling solution), to observe if hydration occurs in this mixture. There was no appreciable change over time (Figure 3-S6a and b). One small, but notable feature of the $^1$H spectrum of the freshly prepared ionophore solution is the appearance of a secondary, equivalent, resonance within the aromatic region with a lower intensity. This resonance appearing at 7.7-8.0 ppm expected from the gem-diol product is substantially upfield from the predominant species of ketone seen from 8.1-8.4 ppm. There is a similar low-intensity but substantially upfield resonance at 85 ppm present in the $^{19}$F spectrum of the freshly prepared ionophore solution. Comparing our spectra to others of compounds synthesized bearing similar gem-diol moieties, we see that the low-intensity resonance at 85 ppm on our $^{19}$F has a similar chemical shift to the reported one.$^{56,57}$ To further confirm if these low-
intensity resonances are the gem-diol, we spiked the solution with excess water (1-2 water drops added to a 500 μL NMR sample containing 30 mM CO$_3^{2-}$ ionophore in DCE). Though it is not as apparent in the $^1$H, the low-intensity resonance at 85 ppm in the 19F dramatically increases in intensity upon addition of water (Figure 3-S6c and d). Comparing this spectrum to that of the freshly prepared ionophore solution, we estimate that the gem-diol has converted to about 20% (from integrated peak area), where the freshly prepared solution had gem-diol converted to approximately 6% (Figure 3-S6e and f). The appearance of the gem-diol even in the freshly prepared sample occurs due to an equilibrium between the ketone and the hydrated form, where the addition of excess water to the sample pushes the equilibrium towards the formation of more gem-diol. Overall, our NMR data and additional voltammetric studies confirmed that the activation of ionophore with forming hydrated carbonyl centers in trifluoroacetyl group during aging could be a crucial factor to steadily drive facilitated-interfacial ion transfers.

**III. Cleanness at the Organic/Water Nanoscale Interface**

Despite the adequate control of dissolution, solubility, and preactivation of CO$_3^{2-}$ ionophores in the DCE solution, we sometimes struggled with reproducibility in electrochemical measurements of CO$_3^{2-}$ FITs. As an active area of a nanopipet electrode is a nanoscale interface, the cleanness at the interface is critical to accurately probe IT reactions, which can be another key factor to manage the overall reproducibility in measurements. Two major sources of adventitious contamination on the interface are further considered such as an inner reference electrode inserted through the back of a nanopipet, and particulate contaminants from the ambient air outside of a nanopipet.
Quartz nanopipets pulled by CO₂-laser puller have a long taper, thereby an etched Pt wire is used as an inner reference electrode to be brought to c.a. 200 μm from the nanopipet interface with tens of nanometer inner radii. The shorter distance between a Pt inner reference electrode and the interface, the smaller resistance of a nanopipet electrode is obtained. A 1 cm long etched Pt is attached to a long Ni/Cu (or tungsten) wire glued with Ag epoxy, and this total composite is inserted into a nanopipet and located near the interface to drive an interfacial potential against an outer reference electrode. Here, unwanted contamination at the interface can happen from nanoscale fractures or debris of cured Ag epoxy, which can partially or totally block the interface and affect the resulting interfacial ITs. Accordingly, a Pt/Ni-Cu/Ag composited electrode was replaced with Ni/Cu alloy wire after direct electrochemical etching in concentrated nitric acid/acetic acid etchant (see SI). Ni/Cu alloy is chemically and electrochemically stable, thus being utilized as an electrode. The etched Ni/Cu electrode has a similar geometry of a long and lean conical shape with smooth surface to an etched Pt wire, thus being located within 200 μm from a nanopipet-interface and retaining clean liquid/liquid interface free from nanoscale debris.

Further, particulate contaminants from the ambient air could be controlled to keep a nanopipet interface clean by employing the laminar flow, which predictably sweeps particles in a uniform direction from the cleanest area under the hood (i.e., the filter face) to the exit area (i.e., the front opening) (Figure 3-S3). Particularly, we introduced a vertical laminar flow cabinet equipped with HEPA filter above the standard bench top, where room air enters the system from above the HEPA filter and particle-free air is forced downward toward the work surface. All nanopipet electrodes were handled under the vertical laminar
flow, which could be more advantageous than horizontal one, as it reinforces the effect of gravity and efficiently sweeps particles out of the enclosure generally through a front access area.61

In Figure 3-4, voltammograms with both contaminated and clean nanopipets are shown, where discontinuous, capacitive, and almost blocked current responses are observed from contaminated nanopipets (grey and black solid lines). These contaminated nanopipets were prepared in the ambient air with an etched Pt wire as an inner reference electrode and a premade CO$_3^{2-}$ ionophore solution. Since the management of contamination degree was out of control, we observed variably blocked currents, which are defined either partially or totally contaminated status at the nanopipet interface based on the continuity and magnitude of ionic current responses. For example, totally contaminated nanopipet could drive neither a simple TBA$^+$ IT nor a CO$_3^{2-}$ FIT across DCE/water interface due to the complete blockage (black solid curves during forward scans in Figure 3-4a), whereas partially contaminated one showed discontinuous current responses for both TBA$^+$ and CO$_3^{2-}$ ITs indicating a partial blockage (grey solid lines during forward scans in Figure 3-4). Only, nanopipets prepared in the laminar flow cabinet with an etched Ni/Cu electrode and a premade ionophore solution could exclude two sources of adventitious contaminations and acquire retraceable quasi-reversible voltammograms for both CO$_3^{2-}$ FIT and simple TBA$^+$ IT (red solid curves, background subtracted in Figure 3-4). The reproducibility of nanopipet electrodes for both CO$_3^{2-}$ and TBA$^+$ ITs reached nearly 100% \((n > 60, \text{ data shown in SI, Figure 3-S6, 3-S7, 3-S9})\). As a control, we tested nanopipets, which were fabricated under the clean system with an etched Ni/Cu electrode and filled with freshly prepared ionophore solution. In this case, we reproducibly observed
discontinuous or blocked current responses, *i.e.*, 100 % failures in CO$_3^{2−}$ FITs due to the blockage of nanopipet interface (Figure 3-4b). Markedly, the inherent cleanliness of nanopipets was controlled as (1) quartz capillaries were blown with compressed air before pulling, (2) the subsequent as-pulled pipets were cleaned with plasma cleaner prior to the silanization process, and (3) silanized pipets were stored under the N$_2$ gas until their usage.

**IV. Electrochemical Mechanism and Kinetics of a Facilitated CO$_3^{2−}$ Ion Transfer**

We quantitatively evaluated experimental voltammograms of a CO$_3^{2−}$ FIT by the finite element analysis to verify FIT kinetics and mechanism. For the interfacial ion transfer, we can consider two mechanisms, electrochemical ion transfer followed by complexation between ion and ionophore (*i.e.*, EC mechanism) and concerted electrochemical ion transfer (*i.e.*, E mechanism). As we numerically analyzed the experimental voltammograms with theoretical simulation based on EC mechanism as below,

**Figure 3-4.** (a) CO$_3^{2−}$ selective nanopipet voltammograms with various contamination degree at the nanopipet interface from clean (red solid line) to fully contaminated (grey solid line). TBA$^+$ IT studied in the negative potential scan was used to characterize and confirm the nanopipet performance, while CO$_3^{2−}$ IT was monitored in the positive potential scan for the study of a FIT. Corresponding pipets were filled with *premade* 30 mM ionophore solution. (b) A control experiment with a clean nanopipet (laminar flow and Ni/Cu inner RE) filled with *freshly prepared* 30 mM ionophore solution. Within 5$^{th}$ running, a nanopipet is blocked. Scan rate is 25 mV/s. All ionophore solutions were vortexed for 15 min.
\[
\begin{align*}
\text{CO}_3^{2-}(w) & \xrightleftharpoons[k_{i,b}]{k_{i,f}} \text{CO}_3^{2-} (\text{or } g) \quad (3) \\
\text{CO}_3^{2-} (\text{or } g) + L(\text{or } g) & \xrightarrow[k_d]{k_a} \text{CO}_3^{2-} \cdot L (\text{or } g) \quad (4)
\end{align*}
\]

where L is CO\(_3^{2-}\) ionophore, which interacts selectively with CO\(_3^{2-}\) to form a 1:1 complex\(^{27}\), \(k_{i,f}\) and \(k_{i,b}\) are first-order heterogeneous rate constants for forward and reverse simple interfacial-ion transfers, respectively. The rate constants are given by Butler-Volmer relations as\(^{61,62}\)

\[
\begin{align*}
k_{i,f} &= k_i^0 \exp \left[ \frac{-\alpha z F (E-E_i^{0'})}{RT} \right] \quad (5) \\
k_{i,b} &= k_i^0 \exp \left[ \frac{(1-a)z F (E-E_i^{0'})}{RT} \right] \quad (6)
\end{align*}
\]

Also, \(k_a\) and \(k_d\) are association and dissociation rate constants, respectively. In the presence of the excess amount of ionophore (30 mM), the homogeneous rate constants are related to as below,

\[
\beta_n = \frac{L_T^0 k_a}{k_d} = \frac{k_a'}{k_d} \quad (7)
\]

where \(k_a'\) is defined as an pseudo-first order rate constant.

Especially, the thermodynamic parameter, \(\beta_n\) is directly related with electrochemical parameter, \(i.e.,\) potentials as below\(^{16}\),

\[
E_i^{0'} = E_i^{0'} + \frac{RT}{z_F} \ln \beta_n L_T^0 \quad (8)
\]

where \(E_i^{0'}\) is the formal potential of simple IT.

With a reported formation constant of the TFAB derivatives and carbonate ion, \(\log \beta_n = 14.4 \pm 0.2\),\(^{64}\) we could obtain the rate constant of ion-ionophore association, \(k_a = 3.33 \times\)
and the standard rate constant of simple carbonate ion transfer, $k^0 = 35 \text{ cm/s}$ (Figure S11 and Table 3-S7, see in SI). Notably, the obtained $k_a$ is extremely large compared to the diffusion limited value of $k_{a,d} = \sim 10^{10} \text{ M}^{-1}\text{s}^{-1}$ (see SI, eq s30). Also, the estimated $k^0 = 35 \text{ cm/s}$ of simple carbonate ion transfer is unrealistically high compared to simple tetrabutylammonium ion ($\text{TBA}^+$) transfer rate, $k^0 = 3.6 \text{ cm/s}$ determined by nanopipet voltammetry. These two facts enable us to exclude the possibility of EC mechanism.

Accordingly, we mainly consider E mechanism for given $\text{CO}_3^{2-}$ FIT, where interfacial $\text{CO}_3^{2-}$ ion transfer is accompanied with complexation of ionophore as well as water-finger formation/dissociation as well,

$$\text{CO}_3^{2-}(w) + L(\text{org}) \rightleftharpoons \text{CO}_3^{2-} \cdot L(\text{org}) \quad (9)$$

In the presence of excess amount of ionophores, a FIT can be further treated as a pseudo first-order process as,

$$\text{CO}_3^{2-}(w) \overset{k_f}{\rightleftharpoons} \text{CO}_3^{2-} \cdot L(\text{org}) \quad (10)$$

where $k_f$ and $k_b$ are first-order heterogeneous rate constants for forward and reverse transfers, respectively. The rate constants are given by Butler-Volmer relations as,$^{62,63}$

$$k_f = k^0 \exp \left[ -\frac{a z F (E - E_{0}')}{RT} \right] \quad (11)$$

$$k_b = k^0 \exp \left[ \frac{(1-a) z F (E - E_{0}')}{RT} \right] \quad (12)$$

where $E$ is the potential applied to a nanopipet electrode against an outer reference electrode, the formal potential, $E_{0}'$, was the potential at $k_f = k_b$, $a$ is a transfer coefficient, $z$ is a charge of transferred ion, $F$ is the Faraday constant, and $k^0$ is the standard rate constant.
We could obtain the standard rate constant of CO$_3^{2-}$ FIT, $k^0 = 0.048$ cm/s with $\alpha$ and $E^{0^*}$. The experimental voltammogram (black solid curves, background subtracted) fits well with simulated quasi-reversible voltammogram (red open circles), yielding $k^0 = 0.048$ cm/s, $\alpha = 0.45$, $z = -2$, $E^{0^*} = 0.365$ V vs. Pt QRE (or, 0.232 V vs $E_{1/2}$, TBA), and $\gamma$ (i.e., $D_{\text{complex}, \text{DCE/DCO}_3^{2-}, \text{aq}} = 0.68$ (Figure 5). Notably, $\alpha = 0.45$ is close to a normal value of 0.5, indicating one-step mechanism of the electrochemical interfacial IT reaction.

More interestingly, our obtained $k^0$ value with a given $\beta = 10^{14}$ is similar to rate constants obtained with non-covalent bond forming ionophores (e.g., for Ag$^+$, $k^0 = 0.026$ cm/s, $\beta = 2.5 \times 10^{12}$; and for K$^+$, $k^0 = 0.011$ cm/s, $\beta = 6.3 \times 10^{14}$) measured by micropipet voltammetry. Previously, Nam, Meyerhoff, and Simon reported the covalent bond formation between CO$_3^{2-}$ and ionophore of trifluoroacetophenone derivatives. If this covalent bond formation is strong, the ion-ionophore association/dissociation rate would become significantly slow, thereby the interfacial ion transfer would not be observable in the fast nanopipet voltammetry. In fact, our nanopipet voltammetry work is the first successful case with covalent bond-forming ionophore for FITs. Herein, the similarity of $k^0$ and $\beta$ values between non-covalent bond-forming ionophore and CO$_3^{2-}$ ionophore VII with forming covalent bonds implies that weak binding of CO$_3^{2-}$ionophore and CO$_3^{2-}$ results in fairly fast association/dissociation of ion-ionophore complex, thereby enabling the facilitated ion transfer within the reasonable potential window in the fast nanopipet voltammetry. In that sense, instead of the nature of bonding between ion and ionophore, the strength of the bonding could determine the feasibility and applicability of ionophore in the FIT study by nanopipet voltammetry.
V. Quantification of $\text{CO}_3^{2-}$ Produced by *Shewanella oneidensis* MR-1

We further demonstrate the analytical utility of amperometric nano-$\text{CO}_3^{2-}$ ISEs by in situ measuring the actual level of $\text{CO}_3^{2-}$ produced by MRB as a result of organic fuel oxidation. Dissimilar MRB such as *Shewanell oneidensis* MR-1 use organic waste molecules, e.g., acetate or lactate as food, oxidize them and produce $\text{CO}_3^{2-}$ and electrons as metabolic wastes.\(^{30}\) Accordingly, sensing and quantification of total $\text{CO}_3^{2-}$ amount enables to monitor the performance of MRB with respect to organic fuel oxidation, *i.e.*, production of electrons as a promising anode material in microbial fuel cells.\(^{39,40}\)

*Carbon sources* $\rightarrow$ $\text{CO}_2(aq) + H_2O \rightleftharpoons \text{HCO}_3^{-}(aq) + H^+ \rightleftharpoons \text{CO}_3^{2-}(aq) + 2H^+$ \hspace{1cm} (13)

In the practical application of nano- $\text{CO}_3^{2-}$ ISEs to biological system, a critical factor is biofouling. We conducted voltammetry and amperometry in bacterial growth media, modified M-1 medium prepared with piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES)
buffer containing 30 mM of acetate or lactate as a carbon source and other growth nutrients. None of noticeable signs of biofouling was seen for either TBA$^+$ or CO$_3^{2−}$ IT reactions in the supernatant of growth media used for bacterial incubation. Neither decrease in the current nor blockage of nanopipet responses was observed during measurements (Figure 3-S7).

In order to quantify the intrinsic CO$_3^{2−}$ present in the supernatant of bacterial growth media, concentration of CO$_3^{2−}$ is directly estimated from the current in the obtained voltammogram. Without background subtraction, a direct current at $E − E_{1/2, \text{TBA}} = 0.3$ V in a pristine voltammogram shows negligible contribution of background, which corresponds to $\sim 73$ % of an anticipated limiting current (at $E − E_{1/2, \text{TBA}} = 0.35$ V) in a background-subtracted voltammogram (Figure 3-5 and 3-6a). So, we selected a direct current, 5.0 ± 0.3 pA at $E − E_{1/2, \text{TBA}} = 0.3$ V, and multiplied 1.36 factor to compensate a deviation from 100 % of a limiting current. Subsequently, using a limiting current equation, we could directly estimate a concentration of CO$_3^{2−}$ produced by bacteria. A limiting current ($i_{\text{lim}}$) is linearly proportional to the concentration of CO$_3^{2−}$ as below,

$$i_{\text{lim}} = 4xzFDCr$$  \hspace{1cm} (14)

here $x$ is a RG factor (a ratio of outer/inner radii of nanopipet, $x = 1.15$ for RG 1.6), $z$ is a unit charge of analyte ion ($z = −2$), $F$ is the Faraday constant (96485 C/mol), $D$ and $C$ are diffusion coefficient and concentration of analyte ion, respectively, and $r$ is the inner radius of a nanopipet opening. The direct quantification of CO$_3^{2−}$ concentration gives 0.70 ± 0.04 mM from the pristine voltammogram without background subtraction. Further, this CO$_3^{2−}$
concentration is validated by standard addition method, where known amount of \( \text{CO}_3^{2-} \) spikes was sequentially added, and voltammetry was subsequently performed (Figure 3-6b). Particularly, standard addition method is useful to consider a matrix effect in our measurement in the presence of various anion interferents in growth media, e.g., \( \text{H}_2\text{PO}_4^- \), \( \text{Cl}^- \), and \( \text{SO}_4^{2-} \) (more details are in SI). Herein, limiting currents from background subtracted were used to plot a calibration curve of standard additions (figure 3-6b).

**Figure 3-6.** Steady-state voltammogram of \( \text{CO}_3^{2-} \) FITs across the DCE/water interface obtained with a nanopipet filled with *premade* 30 mM ionophore solution. Ionophore solutions was vortexed for 15 min. The best theoretical fitting (red open circles) to the experimental curves (black solid curves, background subtracted forward wave) was calculated from simulations with COMSOL Multiphysics using parameters in SI. Scan rate is 25 mV/s.

As shown in Figure 3-6c, a linear curve of \( i_{\text{lim}} \) vs. concentration of \( \text{CO}_3^{2-} \), \( C_{\text{CO}_3^{2-}} \) is obtained, where a slope contains information of \( D_{\text{CO}_3^{2-}} \) \( \text{aq} \), and x-intercept gives the original concentration of \( \text{CO}_3^{2-} \) in growth media produced by *S. oneidensis*. In the presence of each carbon source of acetate and lactate, the resulting concentration of \( \text{CO}_3^{2-} \)
are 0.675 ± 0.03 mM and 0.70 ± 0.04 mM (data not shown), respectively. Also, in modified M-1 media containing lactate as a carbon source and fumarate for anaerobic condition, the determined CO$_3^{2-}$ was 0.75 ± 0.03 mM (Figure 3-S5b). Diffusion coefficients of CO$_3^{2-}$ in M-1 media containing acetate or lactate are 1.83 × 10$^{-6}$ cm$^2$/s, or 1.70 × 10$^{-6}$ cm$^2$/s, respectively. Earlier, DiChristina and coworkers reported maximum 0.20 mM of total dissolved inorganic carbon (DIC, sum of aqueous species of CO$_2$, HCO$_3^-$, and CO$_3^{2-}$) produced from $^{13}$C-acetate during acetate oxidation by Shewanella strain, MN01 measured by liquid chromatography-isotope ratio mass spectrometry. Our in situ estimation of CO$_3^{2-}$ concentration shows a similar order of magnitude, while a small difference would be due to different amount of bacteria. Notably, our amperometric nano-CO$_3^{2-}$ ISEs will open up the applicability to nanoelectrochemical approach such as nanoscale SECM to directly probe CO$_3^{2-}$ as a result of an organic fuel oxidation by a single living bacterium. Also, this nanopipet electrode will be broadly useful for the study of electrocatalytic and photocatalytic reactions that produce or consume carbon dioxide in water.

**Conclusions**

In this work, we showed the successful demonstration and application of CO$_3^{2-}$-selective amperometric/voltammetric probes using nanopipet-supported ITIES and broadly available Simon-type ionophore. Especially, this is the first successful study of nanopipet voltammetry using an ionophore forming covalent bonding with ion. We revealed critical factors governing the overall performance of CO$_3^{2-}$-nano-ISE such as slow dissolution of bulky ionophores in the organic phase, activation for hydrated ionophores, distinct solubility of ion-ionophore complex in the organic phase near the interface, and the cleanness at the nanoscale interface. The adequate experimental control of these factors
leads to reproducible voltammogram data and reliable theoretical assessment, confirming one step E mechanism, where an interfacial ion transfer is concerted with a complexation of ion-ionophore. Notably, the obtained rate constant, $k^0 = 0.048 \text{ cm/s}$ is very similar to rates of other FIT reactions using ionophores forming non-covalent bonds with ions. This result clearly implies that a weak binding between $\text{CO}_3^{2-}$ ion-ionophore enables to observe FITs in the fast nanopipet voltammetry regardless of the nature of bonding between ion and ionophore. The analytical utility of amperometric nano-$\text{CO}_3^{2-}$ ISEs is further demonstrated by in situ measuring $\text{CO}_3^{2-}$ produced by S. oneidensis MR-1 via organic fuel oxidation. We will further employ a newly demonstrated amperometric nano-$\text{CO}_3^{2-}$ ISE with SECM to study organic fuel oxidation at a single bacterial cell with high spatial and temporal resolution. Importantly, our finding can be broadly applied to develop various types of amperometric/voltammetric nanoprobes utilizing ion-ionophore recognition for clinical, physiological, industrial, and environmental analysis as well.

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S. Elangovan and S. Puri contributed to this work equally.

J. Kim designed the experiments and interpreted all the data. S. Puri and S. Elangovan conducted electrochemical experiments and analyzed the data. H. Madawala performed MD simulation. J. Pantano and M. Kiesewetter performed NMR measurements and interpreted the NMR results. B. Pellock provided bacterial samples and discussed electrochemical results with J. Kim. S. Elangovan has contributed to the initial draft preparation but has been unreachable and did not participate in peer review of this manuscript.

All authors except S. Elangovan have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Supporting Information

Modified M-1 medium: Shewanella Federation recipe, Structure of Carbonate Ionophore VII and its complex with CO$_3^{2-}$, Experimental Section, Picture of Vertical Laminar Flow
Clean Cabinet, Cyclic Voltammograms with and without CO$_3^{2−}$ ionophore VII, Nanopipet Voltammetry with Nanopipets Filled with 5 mM CO$_3^{2−}$ ionophore VII Solutions, NMR Study to Confirm Hydration of Trifluoroacetyl Carbon Centers in CO$_3^{2−}$ ionophore VII for Activation, Amperometric $i$-$t$ Curves for Fouling Test of Nanopipet Electrodes in M-1 Medium used for Bacterial Incubation, and a Calibration Curve for Standard Addition Method, MOE Simulation Report (MD Simulation), Selectivity Tests, Reversibility Tests, Fine Element Simulation by COMSOL Multiphysics (I. E mechanism, II. EC mechanism), COMSOL Model Report.

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CHAPTER 4

Mechanistic Assessment of Metabolic Interaction between Single Oral Commensal Cells by Scanning Electrochemical Microscopy
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Mechanistic Assessment of Metabolic Interaction between Single Oral Commensal Cells by Scanning Electrochemical Microscopy

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Abstract
The human oral microbiome heavily influences the status of oral and systemic diseases through different microbial compositions and complex signaling between microbes. Recent evidence suggests that investigation of interactions between oral microbes can be utilized to understand how stable communities are maintained and how they may preserve health. Herein, we investigate two highly abundant species in the human supragingival plaque, Streptococcus mitis and Corynebacterium matruchotii to elucidate their real-time chemical communication in commensal harmony. Specifically, we apply nanoscale scanning electrochemical microscopy (SECM) using a submicropipet-supported interface between two immiscible electrolyte solutions as an SECM probe not only to image the permeability of S. mitis and C. matruchotii membranes to tetraethylammonium (TEA⁺) probe ions, but also to real-time visualize the metabolic interaction between two microbes via lactate production/consumption at a single cell level. Their metabolic relationship is quantitatively assessed by determining the passive permeability of both bacterial membranes of $2.4 \times 10^{-4} \text{ cm/s}$ to the free diffusion of TEA⁺, 0.5 mM of the lactate concentration produced by a single S. mitis at a rate of $2.7 \times 10^{-4} \text{ cm/s}$, and a lactate oxidation rate $\geq 5.0 \times 10^6 \text{ s}^{-1}$ at an individual C. matruchotii. Significantly, this study, for the first time describes a mechanism
of in situ metabolic interaction between oral commensals at the single cell level, which supports the observed in vivo spatial arrangements of these microbes.

**Introduction**

Among various human habitats, the oral cavity retains a rich and complex ecosystem accommodating different microbes, i.e., the oral microbiome. These microorganisms thrive in the dynamic oral environment in a symbiotic relationship with the human host. The unique microbial composition, however, is significantly affected by interspecies and host-microbial interactions, which can subsequently impact the health and disease status of the host. Moreover, the destruction of the oral microbial community is related to systemic diseases such as cancers, cardiovascular diseases, Alzheimer’s disease, and diabetes. Accordingly, mechanistic interaction data between species is urgently needed to understand how changes in oral microbiome composition precede clinical signs of disease, thereby preventing the development of disease with preserving the healthy community.

Regardless of health status, a robust polymicrobial biofilm has been found in supragingival plaque (SUPP), where two highly abundant species, Corynebacterium matruchotii and Streptococcus mitis, coexist. Recent microscopy studies discovered the unique spatial organization between these two species forming a “corncob” structure in vivo, where the long filamentous *C. matruchotii* is surrounded by *S. mitis*. This distinctive morphology is ascribed to *C. matruchotii*’s role in tethering and bridging colonizers e.g., *S. mitis* within plaque. *S. mitis* are Gram-positive oral microbes that ferment glucose to lactate, and produce hydrogen peroxide in aerobic conditions. The production of H₂O₂ enables beneficial oral streptococci to compete in the oral environment and can be a source of stress upon adjacent species. In a parallel study, we have identified how *C. matruchotii*
responds to *S. mitis* H$_2$O$_2$ dependent stress as well as lactate production. While the spatial organization / biogeography of these species has been well described, the exact nature of molecular interactions between these species, especially between individual cells, is relatively unknown. Accordingly, we seek to study these metabolic interactions by directly probing for local metabolites, *i.e.*, pristine lactate near individual bacterial cells to reveal mechanistic insight on how these interactions contribute to *in vivo* association.

Herein, we employ nanoscale scanning electrochemical microscopy (NanoSECM) to address the mechanistic understanding of metabolic cooperation between commensal bacteria at a single cell level. Recently, SECM has been applied to study metabolic interactions in various microbial system, including biofilms and small aggregates owing to its unique capability of studying real-time metabolic exchange between relevant species in a controlled spatial proximity. In fact, real-time SECM imaging of interspecies biofilms or aggregates not only quantified the local concentration of metabolites, but also visually confirmed metabolic cross-talking between interspecies. However, a few ~ tens of micrometer-sized probes with the corresponding instrumentation provides limited spatial and temporal resolutions, thus remaining a major challenge in grasping detailed mechanistic perceptions. In contrast, NanoSECM features precise tip-positioning capability at nm scale and nanometer-sized probes offering high spatial resolution at nanometer scale and rapid temporal resolution at micro-second scales. Accordingly, NanoSECM is suitable to study a single bacterial cell, and map/quantify the local concentration of metabolites produced or consumed in situ between single bacterial cells, thereby revealing/quantitatively assessing mechanistic interaction between species at single cell resolution in real time.
In this work, we investigated two highly abundant species in the human SUPP, *S. mitis* and *C. matruchotii* to elucidate their real-time chemical communication approximating their healthy commensal status. We resolved each single cell of *C. matruchotii* and *S. mitis* in coculture, and real-time image in situ metabolic interactions between these species as well as a topography of single cells by employing SECM tips based on submicro-pipet supported interfaces between two immiscible electrolyte solutions (ITIES) (Fig. 4-1A). With this pipet tip, a Ni/Cu electrode in the internal organic electrolyte applies a potential bias across the liquid/liquid interface respect to a Pt quasi reference electrode in the aqueous solution to yield the amperometric tip current based on the selective interfacial transfer of either a small probe ion, TEA$^+$ or a small metabolite ion, *i.e.*, lactate$^-$. Measurements of the SECM approach curve and the real-time SECM imaging of coculture based on TEA$^+$ ion transfer (IT) allowed us to determine the intrinsic permeability of both bacterial membranes (BMs) and topography of bacterial cells, respectively. Subsequently, SECM imaging based on lactate IT was studied to map the local concentration of lactate near individual *S. mitis* and *C. matruchotii* in coculture, and probe their metabolic interaction via metabolic exchange, *i.e.*, in situ production and consumption of lactate, respectively. Importantly, the quantitative analysis of SECM data using finite element simulations provides mechanistic assessment of in situ metabolic interactions between commensal bacteria at a single cell level, where the level of lactate concentration produced by a single *S. mitis*, the lactate production rate at a single *S. mitis*, and the consumption rate of lactate by a single *C. matruchotii* could be defined for the first time. This study confirms the relevance of *in vivo* spatial arrangements and co-occurrence between two commensals in healthy oral biofilms as studied by optical and fluorescence microscopy.
Experimental Section

**Strains and media.** *C. matruchotii* (ATCC 14266) and *S. mitis* (ATCC 49456) were grown on Brain Heart Infusion media supplemented with 0.5 % yeast extract (BHI-YE) at 37 °C in a static incubator with 5 % CO₂ or in 5% H₂, 10 % CO₂ and 85 % N₂ in anaerobic conditions. More details about growth of coculture and monoculture are explained in supporting information (SI).

**SECM sample preparation:**

Bacteria were grown overnight in BHI-YE and then washed by centrifugation in defined medium, which is an amended version of Teknova EZ RICH (Teknova, M2105). The medium was prepared as described in the manufacturers’ instructions with the addition of vitamin solution, lipoic acid, folic acid, riboflavin, NAD⁺ and nucleotides to final concentrations from that of an oral complete defined medium previously described and glucose at 10 mM. Bacteria were grown to an OD 600 of 0.3-0.5 and then diluted to final concentrations of 1.2 × 10⁷ and 6.0 × 10⁶ CFU of *C. matruchotii* and *S. mitis* respectively in 200 µL of defined medium. This was then incubated at 37 °C with 5 % CO₂ for 1 h. 10 µL of this solution was then added to a poly-lysine coated glass slide and incubated at 37 °C for 15 minutes after which medium was removed by micropipette to remove planktonic cells and ensure only attached cells remained. An additional 10 uL of pre-warmed defined medium was then added to a poly-L lysine coated glass slide (Glass Microscope Slides Rite-On®) with 15 mm × 9 mm size. The glass slide was rinsed by buffer media to remove loosely bound bacteria on the glass surface, and then assembled in SECM cell for further analysis.

1 mM of tetraethylammonium chloride (TEA⁺Cl⁻, Sigma Aldrich) solution was made in freshly prepared oral bacterial growth media at ~pH 7.5, where TEA⁺ was used as a probe
The solution was then filtered with a syringe filter (0.1 μm filter unit, SLVV033RS, Duropore PVDF membrane, MILLEX VV), and further filled the SECM cell containing a bacterial sample. TEA\(^+\) is particularly chosen, which does not affect the growth rate of each bacteria cell, thus maintaining the same physiological activity (data not shown).

**Fabrication of submicro-pipet electrodes as SECM tips.**

Fabrication of submicrosized pipet electrodes is reported in elsewhere.\(^{21}\) Briefly, a quartz capillary (outer diameter 1 mm, inner diameter 0.7mm, length 10cm, Sutter instrument) was cleaned by blowing with compressed air, and pulled with CO\(_2\) laser puller (Model P 2000-2035, Sutter Instrument) with parameters (heat = 720, filament = 4, velocity = 30, delay = 130 and pull = 110) giving c.a. 800 nm inner diameter. As-pulled pipets were cleaned by UV plasma cleaner (PDC-32G, Harrick Plasma). Further, cleaned pipets were silanized by chemical vapor deposition method using 50 μL of N, N-dimethyltrimethylsilylamine inside the vacuumed desiccator for 50 min under a constant relative humidity of 16 % at 20 °C. Resulting silanized pipets were then filled with 10 μL of 1,2-dichloroethane containing 0.1 M of tetrakis(pentafluorophenyl) borate–tetradodecylammonium\(^+\) (TFPB\(^-\) TDDA\(^+\)) as supporting electrolytes.\(^{21}\) An electrochemically etched Ni/Cu wire was inserted inside a pipet as an inner reference electrode, and was immobilized for electrochemical measurements. Potential was applied between a submicropipet tip (a Ni/Cu inner reference electrode) and a Pt quasi reference electrode by a potentiostat (CHI 8022D, CH Instruments, TX, Austin) during SECM measurements.
Nanoscale SECM measurements.

A home-built nanoSECM equipped in the isothermal chamber is employed to measure approach curves and image single bacterial cells at ~30 °C. In bulk solution containing TEA+, cyclic voltammetry was performed to estimate a steady state current of a pipet tip ($i_{T,\infty}$) for electrochemical characterization (eq 1). Then, the pipet tip was vertically approached over a glass substrate by SECM until it showed a sharp decrease in current, i.e., feedback current response. Once we observed a foot of feedback current, the pipet tip was withdrawn 6 μm above, and approached at a slow rate of 10 nm/s (1 nm step size /100 ms incremental time) until tip currents decreased to c.a. 93 – 90 % of $i_{T,\infty}$, corresponding to a distance from the substrate equivalent to a tip diameter. Subsequently, a tip was further withdrawn ~2 μm to prevent a tip crash with possible clumps of bacteria in coculture or monoculture during raster scanning. SECM imaging was obtained with the constant height mode. For SECM imaging, a pipet tip monitoring TEA+ IT was raster scanned along x- and y-axis at 1 μm/s (i.e., 100 nm step size /100 ms incremental time) in the region of 25 μm × 25 μm to get a topographical image of bacterial cells. Then, a pipet tip was brought to the original starting point. A pipet tip potential was switched to a potential to sense lactate IT (~0.42 V more positive than $E_{1/2}$ of TEA+ IT, shown in Fig. 4-1C). Continuously, a tip was raster scanned in the same region of 25 μm × 25 μm to real-time monitor lactate production/consumption by bacteria in situ. The permeability of bacterial membrane was studied by measuring SECM approach curves over a center of a bacterial cell. Numerical simulations using finite element methods with COMSOL Multiphysics are explained in the SI.
Results and Discussions
Electrochemical Sensing of Probe Ions, TEA\(^+\) and Metabolite Ions, Lactate.

We employed a submicropipet-supported ITIES as a probe to in situ investigate the topography of bacterial cells, and real-time map the metabolites, \textit{i.e.}, lactate surrounding an individual bacterium. With this submicro-tip filled with an electrolyte solution of 1,2-dichloroethane (DCE), an amperometric tip current is selectively monitored based on interfacial ITs of TEA\(^+\) as a probe ion, or lactate as a metabolite produced or consumed by bacteria, respectively (Fig. 4-1A and 4-1B). Herein, TEA\(^+\) IT is monitored at negative potential to study topography of bacterial cells without affecting their physiological activity, while lactate is sensed at positive potential to in situ monitor the chemical interaction between bacteria (Fig. 4-1C). A coculture of \textit{C. matruchottii} and \textit{S. mitis} at low optical density (OD 0.3~0.5) is immobilized over a poly L-lysine coated slide glass plate and studied by raster scanning or vertically approaching a \textasciitilde{}400 nm-inner radius pipet tip over individual bacterial cells (Fig. 4-1D). Under negative potential applied to a submicro-tip, the current response for TEA\(^+\)IT is lowered as the tip moves laterally toward the bacteria with a submicrometer gap between the tip and bacterium, where bacterial cells hinder the diffusion of TEA\(^+\) to the pipet tip. Subsequently, the tip current is recovered over the glass substrate, thereby yielding topographical information of the bacterial sample.

Once the tip potential is switched to more positive than TEA\(^+\) IT (0.41~0.42 V more positive than \(E_{1/2}\) of TEA\(^+\) IT) and induces lactate IT, the tip scans the same region as topography study to directly sense lactate near bacteria, thus mapping the local concentration of lactate as a result of in situ metabolic activity. Notably, a submicro-tip with 400 nm inner radius is sufficiently small enough to spatially resolve each single bacterial cell, and real-time probe chemical interactions between bacteria at a single cell
level, where *S. mitis* is characteristically round-to-ovoid coccus with 0.6~1.0 μm in diameter, and *C. matruchotii* has cell dimension of c.a. 0.8 μm in diameter and 1.5 to 8.0 μm in length. In fact, even smaller tips could enhance the spatial resolution in the SECM measurements. However, irregular and various sizes of bacteria in coculture readily induce tip crashes during raster scanning a small tip within a nanometer gap above bacteria. Accordingly, a ~400 nm radius tip was chosen to avoid tip crashes without compromising spatial resolution in our SECM study.

**Determination of Bacterial Membrane Permeability to Passive Diffusion of TEA⁺ Studied by SECM and Finite Element Simulation.**

The inherent low permeability of the Gram-positive bacterial membrane (BM) was investigated by vertically approaching a submicro-tip above a single bacterial cell with SECM, where tip currents were monitored with varying a distance between a tip and BM, a.k.a. an SECM approach curve. SECM approach curves were measured right above *S. mitis* and *C. matruchotii*, respectively to characterize the passive permeability of each BM to a small probe ion, TEA⁺.

When the tip was positioned far from the BM, the stable amperometric current based on the diffusion-limited IT of TEA⁺ was obtained as given below

\[
I_{T,\infty} = 4\delta zFD_w c^* a
\]  

where \(I_{T,\infty}\) is a current in bulk, \(\delta\) is the function of RG ratio (RG is the ratio of outer and inner diameters of a glass pipet, \(\delta = 1.16\) for a RG 1.5 tip), \(z\) is a charge of TEA⁺, \(F\) is Faraday constant (96485 C/mol), \(D_w\) is the diffusion coefficient \((6 \times 10^{-6} \text{ cm}^2/\text{s})\), \(c^*\) is a bulk concentration of TEA⁺ \((1.0 \text{ mM})\), and \(a\) is the inner radius of a pipet tip \((430 \text{ nm})\). The
tip radius was estimated from \( i_{T,\infty} \) using RG value determined by the SEM image (Fig 4-1B).

**Figure 4-1.** (A) Illustrated scheme of a submicropipet-supported ITIES to directly probe TEA\(^+\) IT or lactate IT, (B) an SEM image of a submicrometer sized pipet, (C) Cyclic voltammograms of TEA\(^+\) and lactate ITs in buffer solution containing 1 mM TEA\(^+\) and 2 mM lactate (background subtracted, scan rate = 25 mV/s), (D) Schematic of the SECM setup with living coculture bacterial cells immobilized on poly-L-lysine coated slide glass, where constant-height SECM imaging or SECM approach curve is measured over *S. mitis* and *C. matruchotii* coculture using a submicropipet tip, respectively.

Well-defined approach curves were obtained for *C. matruchotii* and *S. mitis*, respectively (Fig. 4-2A and 4-2B). The amperometric tip current, \( i_T \), begun decreasing significantly ~2.0 μm above BMs because the nonspecific diffusional flux of TEA\(^+\) induced by its IT at the tip was mediated only through porin-channels and was blocked by the rest of lipid bilayer membrane. In Figure 4 2, SECM approach curves are constructed by normalizing tip
displacements (d) with respect to the tip inner radius (a), i.e., d/a, where zero in d/a denotes the zero distance between the BM and the tip (blue solid curves in Fig. 4-2A and 4-2B).

SECM approach curves over a flat, smooth, and insulating glass substrate were separately measured, which show a decrease in currents over a glass substrate due to the hindered diffusion of TEA\(^+\) to the tip, i.e., negative feedback effect, and a sharp spike at ~30 nm above a glass substrate corresponding to a contact between a glass substrate and a glass sheath surrounding the tip (grey solid curves in Fig. 4-2A and 4-2B). SECM approach curves measured over a bacterial cell are overlaid with negative feedback approach curves to consistently set the contact point between the BM and the tip. Importantly, the close contact distance is required to minimize the uncertainty in determining permeability of the BMs.

The low permeability of both *S. mitis* and *C. matruchotii* BMs to TEA\(^+\) was determined by fitting experimental approach curves to theoretical curves. These theoretical curves were obtained by the finite element simulation of a two-phase SECM diffusion problem. The tip current was calculated from concentration profile of TEA\(^+\) in 2D simulation at various tip-BM distances, d (Fig. 4-2C). In this simulation, the BM was treated as a homogeneous membrane with inherent permeability, \(k_1\), as given by,

\[
\text{TEA}^+(\text{outer solution}) \rightleftharpoons \frac{k_1}{k_2} \text{TEA}^+(\text{inside bacterial cell})
\]

(2)

where the permeability of \(k_1\) and \(k_2\) for passive influx and efflux of TEA\(^+\), respectively, corresponds to the equilibrium concentration of TEA\(^+\) in the bulk outer solution and the bacterial cell. The equilibrium concentrations of small probe ions in the outer solution and in the bacteria, \(c_{10}\) and \(c_{20}\), respectively are related to the partitioning equilibrium constant, \(K_{e}\), and assumed to be ~0.9 for monovalent ion, \(^{24}\)

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\[ K_e = \frac{c_o}{c_i} = \frac{k_1}{k_2} \]  

(3)

Since small and hydrophilic TEA\(^+\) ions freely diffuse through porins, a kinetic effect on an approach curve is exerted from the impermeable region of the BM. This kinetic effect can be seen in the discontinuous concentration profile of TEA\(^+\) across the BM under the tip (Fig. 4-2C).

Experimental approach curves agreed well with simulated approach curves to yield a passive BM permeability, \( k_1 \) of \( 2.4 (\pm 0.1) \times 10^{-4} \) cm/s, identical for both \( C. \ matruchotii \) and \( S. \ mitis \) (Fig. 4-2A and 4-2B, blue open circles, see SI). The numerical analysis confirms the contact of the tip with the BM occurred at \( \sim 30 - 40 \) nm. This nanoscale contact distance is critical to unequivocally resolve the approach curves as obtained over bacterial cell and over a glass substrate. In this distance range, the tip current decreased rapidly and distinctly as the tip approached the BM. Noticeably, the BM permeability determined from approach curves contains an uncertainty of less than 5 % owing to the short nonzero distances at the tip–BM contact. Also, a mass transfer coefficient at this nanogap (\( d = 30 \) nm), \( i.e., D_w /d \) of \( \sim 2.0 \) cm/s across the tip–BM gap was 4 orders of magnitude higher than the \( k_1 \) values, thereby confirming the reliability of the determined permeability values.

Notably, the determined permeability of Gram-positive BMs is 10 –100 fold lower than other biological membranes such as algal protoplast cell membranes and the nuclear envelope, respectively.\(^{24,26}\) Herein, TEA\(^+\) was used in lieu of lactate to determine BM permeability for two reasons \( i.e., \) the same charge amount (\( \pm 1 \)) and similar diffusion coefficient of \( \sim 6 \times 10^{-6} \) cm\(^2\)/s in water.
Real-Time Electrochemical Mapping of Local Lactate Reveals in situ Metabolic Interaction between *S. mitis* and *C. matruchotii* via Simultaneous Lactate Production and Consumption.

To image cocultures of *S. mitis* and *C. matruchotii* for topography and metabolic activity (Fig. 4-3A), the constant-height mode of nanoscale SECM was employed. The submicro-tip enabled SECM images with the high spatial resolution to resolve pristine living coculture of *S. mitis* and *C. matruchotii* immobilized on poly-Lysine coated glass. For the topographical study, the pipet tip monitoring currents for TEA$^+$ IT approached a glass substrate until the tip current decreased to $\sim$90 % of a diffusion limited current response in the bulk solution, $i_{T,\infty}$, which is equivalent to the tip-glass substrate distance, $d$, of 0.85 μm with the tip radius, $a$, of 430 nm. Further, a tip was withdrawn 1.75~2.00 μm higher, and scanned laterally at the fixed height while the tip current was monitored to obtain an SECM image. Constant-height image of coculture bacteria was obtained with the gap between the tip and bacteria, $d = 0.75$ μm, i.e., 1.75 $d/a$. Low tip currents of $\sim 89$ % of $i_{T,\infty}$ above

![Figure 4-2. SECM approach curves based on TEA$^+$ IT](image-url)

**Figure 4-2.** SECM approach curves based on TEA$^+$ IT (A) over *C. matruchotii* (blue solid curves) and an insulating glass substrate (grey solid curves), and (B) over *S. mitis* (blue solid curves) and an insulating glass substrate (grey solid curves). Experimental curves (blue solid curves) are compared with theoretically simulated curves (blue open circles) with permeability, $k_1 = 2.4 (\pm 0.1) \times 10^{-4}$ cm/s. Theoretically simulated negative feedback approach curves are shown as black open circles. (C) Concentration profile of TEA$^+$ ions near *C. matruchotii* and a pipet tip.
bacteria is obtained due to hindered diffusion of TEA\(^+\) by bacteria with merely permeable membrane to TEA\(^+\) (Fig. 4-3B). In fact, individual bacterial cells tangled each other could not be differentiated in this topographical SECM image, where a lump appeared in 25 \(\mu\)m \(\times\) 16 \(\mu\)m image. As shown in the chronoamperometric responses \(i.e.,\) cross sections of the SECM image in Fig. 4-3B, currents were monotonically decreasing in 1450–1650 s (positive polarity for cationic currents) due to the hindered diffusion of TEA\(^+\) to the pipet tip right above bacteria (shown as raw data in Fig. 4-3D).

The same area was imaged by switching a tip potential to induce lactate IT, and raster scanning a tip at the gap between the tip and bacteria, \(d = 0.51\ \mu\)m (1.20 \(d/a\)) (Fig. 4-3C).

An initial current of \(~30\ \text{pA}\) above a glass substrate in the SECM image is nearly the same as \(i_{T,\infty}\) of lactate IT, and corresponds to \(~0.26\ \text{mM}\) of lactate determined by eq 1. As there was no exogenous lactate added to our medium, this amount of lactate is produced by \(S.\ mitis\), and diffuses into bulk solution surrounding the immobilized bacterial cells. In this metabolite-mapping image, individual \(S.\ mitis\) and \(C.\ matruchotii\) were successfully resolved in respect to both morphological and functional differences. Specifically, high tip currents of \(50\ \text{pA}\) corresponding to \(167\ %\) of \(i_{T,\infty}\) are yielded over a single spherical object, whereas dramatically low tip currents of \(~14\ \text{pA}, \ i.e.,\ \text{47}\ %\) of \(i_{T,\infty}\) are obtained over a long filamentous feature in contact with a spherical one, and tip currents reach \(22 \sim 24\ \text{pA}, \ i.e.,\ \text{75} \sim \text{80}\ %\) of \(i_{T,\infty}\) above the rest of a filamentous object far apart from \(S.\ mitis\) (Fig 4-3C and 3E). These unique morphologies identified in the lactate-mapping SECM image are consistent with optical microscopy, where spherical \(S.\ mitis\) surround a long filamentous \(C.\ matruchotii\) (white arrows for \(S.\ mitis\) and blue for \(C.\ matruchotii\) in Fig. 4-3A).
High tip currents above *S. mitis* are mainly due to the steady excretion of lactate as a fermentation product. By contrast, low tip currents above *C. matruchotii* are attributed to swift in situ consumption of lactate by *C. matruchotii* as well as the hindered diffusional flux of lactate to the tip by the BM of *C. matruchotii*. If the tip current over *C. matruchotii*

![Figure 4 - 2. A) Optical microscopic image of *C. matruchotii* (blue arrow) and *S. mitis* (white arrows) coculture. Constant-height SECM images based on (B) TEA\(^+\) IT (obtained with a gap between the tip and bacteria of 1.75 d/a) and (C) lactate IT (obtained at the gap of 1.20 d/a), tip scan rate during SECM imaging at 100 nm/100 ms. Chronoamperometric responses based on (D) TEA\(^+\) IT and (E) lactate IT (raw data, cross sections of SECM images in (B) and (C), respectively). The current polarity is set to positive for cationic current responses (D) and negative for anionic current responses (E).](image)

is only governed by inherent BM permeability to lactate, the resulting tip current at the gap of 1.20 d/a between the tip and a bacterium would be \(\sim 80\% \) of \(i_{T,\infty}\), i.e., \(\sim 24\) pA as obtained in SECM approach curve (Fig. 4-2A). In fact, 22–24 pA along a filamentous *C. matruchotii* far apart from *S. mitis* was observed, which is close to the tip currents determined by BM
permeability. However, the ~14 pA current above *C. matruchotii* contacting with *S. mitis* (red circle in Fig. 4-3E) is much lower than the tip current expected from SECM approach curves in (Fig. 4-2A). Interestingly, this lower tip current is not due to the taller height of the bacterial cell, thus a smaller gap between the tip and a bacterium. It is confirmed by monotonic decreases in tip currents along the scanning axis and time in topographical SECM image and chronoamperometric responses (Fig. 4-3B and 3D), respectively, which indicates no apparent protrusion on *C. matruchotii* in close proximity to *S. mitis*. Accordingly, the lower tip current results from local depletion of lactate by *C. matruchotii* via lactate catabolism. Non-uniform consumption of lactate along the length of *C. matruchotii* can be related to proximity with *S. mitis*. As a result, the dramatic contrast of tip currents above two different bacteria in the lactate-mapping SECM image visually confirms the real-time chemical exchange between *S. mitis* and *C. matruchotii* through simultaneous lactate production and its consumption, respectively. Each monoculture was separately studied by SECM to confirm that lactate is only produced by *S. mitis* and not by *C. matruchotii* (see SI). Markedly, this is the first successful case of real-time visualization of in situ metabolic interaction at a single cell level between two abundant oral bacterial cells and reveal their commensal relationship, which cannot be elucidated by optical microscopic imaging.

Earlier, the Koley group studied real-time metabolic interactions between dual species biofilms of lactate-producing Streptococcus mutans and *H₂O₂*-producing Streptococcus gordonii by constructing a pH profile with utilizing a carbon-based potentiometric pH microsensor as an SECM probe.¹⁷ While a pH microsensor responds to lactate produced by *S. mutans*,¹⁷ it cannot differentiate various acidic end-products, e.g., formate, lactate and
acetate. Also, the logarithmic dependence of potential response on the acidic product concentration limits sensitivity in metabolite measurements. In contrast, our submicropipet-supported ITIES tip amperometrically senses pristine lactate by inducing lactate ITs, where the resulting current is directly proportional to lactate concentration, thereby enabling highly sensitive measurements in this single cell study.

Quantitative Assessment of Mechanistic Interactions between Individual Commensal Bacteria by Finite Element Simulation.

In situ metabolic interaction between two commensals via steady production of lactate by S. mitis and its rapid oxidation by adjacent C. matruchotii is visually confirmed by real-time SECM imaging for the first time at a single cell level. Further, this commensal relationship is quantitatively assessed using finite element simulation (details are shown in SI).

In this simulation, lactate produced by S. mitis is released to aqueous bulk solution at a rate determined by the inherent BM permeability for efflux (eq 4), and diffuses near C. matruchotii, which readily oxidizes it (eq 5), while a pipet tip is sensing local lactate in aqueous solution by inducing lactate IT under diffusion limited condition (eq 6) as below.

\[
(S.\text{mitis}) \quad L_{S.\text{mitis}}(\text{bacteria}) \xrightleftharpoons[k_{2}]{k_{1}} L_{w}(aq,\text{outer solution}) \tag{4}
\]

\[
(C.\text{matruchotii}) \quad L_{w}(aq,\text{outer solution}) \xrightleftharpoons[k_{2}]{k_{1}} L_{c}(\text{bacteria}) \xrightarrow[k_{c,f}]{k_{c,b}} P(\text{bacteria}) \tag{5}
\]

\[
(\text{pipet}) \quad L_{w}(aq,\text{outer solution}) \xrightarrow{k_{1}} L_{o}(\text{org}) \tag{6}
\]

where \(L_{S.\text{mitis}}\) is a lactate inside \(S.\text{mitis}\), \(L_{w}\) is a lactate in outer aqueous solution, \(L_{c}\) is a lactate permeated in \(C.\text{matruchotii}\), \(L_{o}\) is a lactate in the internal organic solution of a
pipet tip, and P is a product of lactate oxidation. $k_2$ and $k_1$ are the first-order heterogeneous rate constants of forward and backward reactions, respectively in eq 4, where $k_1$ and $k_2$ are same values determined in eq 3, i.e., $k_1 = 2.4 \pm 0.1 \times 10^{-4}$ cm/s and $k_2 = k_1/K_e$. The partitioning equilibrium constant of lactate between in the bacteria and the outer solution, i.e., $c_2^0/c_1^0 (= k_1/k_2)$ is considered equivalently to the partitioning equilibrium constant, $K_e$ of small probe ions, TEA$^+$ in eq 3, thereby being ~0.9 for monovalent ion. Also, $k_1$ and $k_2$ are the first-order heterogeneous rate constants of forward and backward reactions, respectively in eq 5, where $k_1$ is the same value as BM permeability, i.e., $2.4 \pm 0.1 \times 10^{-4}$ cm/s determined in eq 3. Further, $k_{c,f}$ and $k_{c,b}$ are the first-order homogeneous rate constants of forward and backward reactions in eq 5, i.e., the oxidation of permeated lactate by *C. matruchotii*. Assuming a constant concentration of lactate dehydrogenase in *C. matruchotii*, a pseudo first-order homogeneous reaction is considered for lactate oxidation in eq 5.

A two-phase SECM diffusion problem was defined and solved in a 2D cylindrical model, where a pipet tip was located above the center of a single bacterium of *S. mitis* or *C. matruchotii* (Fig. 4-4D or 4-4E, respectively), and theoretical curves based on lactate IT were obtained (Fig. 4-4B and 4-4C, see SI). In this work, the BMs of both bacteria were treated as a laterally homogeneous membrane with unique permeability, $k_1$ determined by eq 2. Herein, the amperometric current response above the bacteria is a function of the tip-bacteria distance and permeability as well as the concentration of lactate. First, a theoretical approach curve over *S. mitis* producing lactate is simulated, where steadily produced lactate ions are released at a rate of $k_2 (= k_1/K_e$ in eq 3), $2.7 \pm 0.1 \times 10^{-4}$ cm/s from *S. mitis* in the presence of 0.26 mM lactate in aqueous bulk solution (Fig. 4-4B). In this theoretical curve,
~1.67 fold increase in amperometric currents from $i_{T,\infty}$ is obtained at 1.2 $d/a$, i.e., at the gap between the tip and bacteria, $d = 0.51 \ \mu m$. Notably, this increased current above S. mitis is consistent with the enhanced currents above S. mitis in a lactate-mapping SECM image (Fig. 4-3C and 4-3E). The resulting concentration profile of lactate near S. mitis and a pipet tip is depicted in Fig. 4-4D, where the lactate concentration right above the BM of a single S. mitis is estimated as 0.5 mM. This concentration, notably, is similar to the lactate concentration of saliva in healthy humans with a range between 0 and 3.5 mM.

Another theoretical curve over C. matruchotii is simulated, where lactate diffusing from adjacent S. mitis permeates the C. matruchotii BM, and is readily oxidized by C. matruchotii. In this case, the rapid consumption of lactate appreciably decreases lactate concentration near C. matruchotii, thus resulting in much smaller tip currents than currents only determined by BM permeability without lactate consumption (Fig. 4-4C, red closed circles and blue open circles, respectively). Under this condition, ~47 % of $i_{T,\infty}$ can be theoretically predicted at 1.2 $d/a$, while ~83 % of $i_{T,\infty}$ can be anticipated if no lactate consumption occurs by C. matruchotii. In fact, SECM image and chronoamperometric responses in Fig. 4-3C and 4-3E yielded tip currents of ~14 pA at the gap, $d = 0.51 \ \mu m$ between the tip and C. matruchotii adjacent to S. mitis, which is ~47 % of $i_{T,\infty}$ (~30 pA) as predicted in this theoretical simulation. This good agreement between the theoretical prediction and experimental currents validates the lactate consumption rate of C. matruchotii as $k_{c,f} \geq 5 \times 10^6 \ \text{s}^{-1}$ with thermodynamically favorable lactate oxidation in aerobic condition,$^{13,35}$ i.e., an equilibrium constant, $K_{eq} =$
The simulated concentration profile of lactate near \( C. \text{matruchotii} \) and a pipet tip is depicted in Fig. 4-4E, where the lactate concentration right
above the BM of a single *C. matruchotii* is estimated as 0.1 mM. Noticeably, the determined $k_{c,t}$ is in reasonable range with considering the theoretical association constant of lactate substrate and lactate dehydrogenase based on diffusion limit estimated by,

$$k_{a,d} = 4\pi N_A (D_E + D_L) d$$

(7)

where $N_A$ is Avogadro’s number, $d (= 1.5 \times 10^{-7}$ cm) is the enzyme-substrate separation at their collision, and $D_L$ and $D_E (= \sim 5 \times 10^{-6}$ cm$^2$/s) are diffusion coefficients of lactate and free enzyme in the cell. For our given system, $k_{a,d} = \sim 10^{10} \text{M}^{-1}\text{s}^{-1}$ can be estimated.

In Fig. 4-3C and 3E, tip currents above *C. matruchotii* far from *S. mitis* reach 22 ~24 pA of ~80 % of $i_{T,\infty}$, which corresponds to current values in theoretical curves governed by intrinsic BM permeability (blue open circles in Fig. 4-4C). This result clearly indicates almost negligible oxidation of lactate by *C. matruchotii* far from the source of lactate supply. Thereby, the high spatial resolution of SECM measurements unexpectedly reveals a non-uniform efficiency of lactate oxidation along the length of the filamentous *C. matruchotii* cell. More efficient depletion of local lactate is facilitated by lactate oxidation of *C. matruchotii* adjacent to *S. mitis* when producing lactate, which is a key mechanism supporting the previously described “corncob” spatial arrangement between these species *in vivo* in aerobic conditions.$^{9,10}$ Resultantly, the in situ metabolic interactions between two oral bacteria in close proximity explain the fitness benefit provided by *C. matruchotii* to *S. mitis* helping preserve their relationship in the oral cavity.

Significantly, our emphasis is that in situ study of SECM imaging enabled us to unequivocally demonstrate in situ metabolic cooperation between individual bacteria via concurrent lactate production and consumption at a single cell level for the first time, and quantitatively reveal their mechanistic relationship.
Traditional approaches to study interaction in polymicrobial communities are typically based on microscopy observations as well as genomics-based approaches (RNASEq, etc.). While powerful, these methods can only generate hypotheses regarding biochemical mechanisms of interaction. Biochemical studies employed to further investigate have been suitable to test large scale populations, but do not fully mimic the intimate cell-cell interactions observed in \textit{in vivo} biofilm structures. In that sense, our successful application of SECM towards polymicrobial interactions opens up a new route to investigate multi-species cooperation and competition via real-time probing of metabolites in situ and provides a new diagnostic tool to determine whether changes in the oral microbiome precede clinical signs of disease, thus enabling the use of the oral microbiome in the prediction of future disease risk.

\textbf{Conclusions} \\
In this work, we applied nanoscale SECM with submicropipet-supported ITIES probes to not only image the permeability of BMs, but also visualize chemical communications between two highly abundant species, \textit{S. mitis} and \textit{C. matruchotii} in SUPP at single cell resolution in real-time. The results from SECM experiments revealed their commensal relationship via metabolic synergy that cannot be observed by either optical imaging or other conventional assays. Specifically, permeability imaging by SECM verified that the Gram-positive BM is two orders of magnitude less permeable to small probe ions, TEA$^+$ than eukaryotic membranes such as the nuclear envelope or algal protoplast cell membranes. Subsequently, the electrochemical mapping of lactate around an \textit{S. mitis} and \textit{C. matruchotii} coculture by SECM enabled us to probe the local concentration of lactate produced by \textit{S. mitis} and simultaneously oxidized by \textit{C. matruchotii} in situ, thereby
visually confirming previously hypothesized metabolite exchange\textsuperscript{13} at the single cell level supporting their observed \textit{in vivo} spatial organization. Using the finite element analysis, important phenomenological parameters in the coculture system could be quantitatively determined, where a passive permeability of both BMs is $2.4 \times 10^{-4}$ cm/s corresponding to the free diffusion of TEA$^+$, 0.5 mM of lactate is produced by a single \textit{S. mitis} at a rate of $2.7 \times 10^{-4}$ cm/s, and a lactate consumption rate at an individual \textit{C. matruchotii} is higher than $5.0 \times 10^6$ s$^{-1}$. Considering the role of lactate as an immunomodulatory molecule as well as a microbial metabolic product, our unique approach for real-time monitoring of lactate between oral commensal bacteria and the quantitative assessment of their metabolic communication can provide a new diagnostic methodology to evaluate human health status and future disease risk.

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†S. Puri and E. Almeida contributed to this work equally.
J. Kim and M. Ramsey designed the experiments, and interpreted the data. S. Puri and S. Elangovan conducted SECM experiments, and analyzed the data. E. Almeida, A. Labossiere, and C. Collins managed bacterial samples and performed optical microscopic measurements. S. Elangovan has contributed to the initial draft preparation, but has been unreachable and did not participate in peer review of this manuscript.

All authors except S. Elangovan have given approval to the manuscript.

Notes

The authors declare no competing financial interest.

Supporting Information


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References


60. Horizontal or Vertical Laminar Flow Clean Bench | Air Science


CHAPTER 5

Corynebacterium matruchotii fitness enhancement of adjacent streptococci by multiple mechanisms
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Corynebacterium matruchotii fitness enhancement of adjacent streptococci by multiple mechanisms

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Abstract
Polymicrobial biofilms are present in many environments particularly in the human oral cavity where they can prevent or facilitate the onset of disease. While recent advances have provided a clear picture of both the constituents and their biogeographical arrangement, it is still unclear what mechanisms of interaction occur between individual species in close proximity within these communities. In this study we investigated two mechanisms of interaction between the highly abundant supragingival plaque (SUPP) commensal
Corynebacterium matruchotii and Streptococcus mitis which are directly adjacent *in vivo*. We discovered that *C. matruchotii* enhanced the fitness of streptococci dependent on its ability to detoxify streptococcal-produced hydrogen peroxide and its ability to oxidize lactate also produced by streptococci. We demonstrate that the fitness of adjacent streptococci was linked to that of *C. matruchotii* and that these mechanisms support the previously described “corncob” arrangement between these species but that this is favorable only in aerobic conditions. Further we utilized scanning electrochemical microscopy (SECM) to quantify lactate production and consumption between individual bacterial cells for the 1st time, revealing that lactate oxidation provides a fitness benefit to *S. mitis* not due to pH mitigation. This study describes mechanistic interactions between two highly abundant human commensals that can explain their observed *in vivo* spatial arrangements and suggest a way by which they may help preserve a healthy oral bacterial community.

**Importance** - As the microbiome era matures, the need for mechanistic interaction data between species is crucial to understand how stable microbiomes are preserved, especially in healthy conditions where they could help resist opportunistic or exogenous pathogens. Here we reveal multiple mechanisms of interaction between two commensals that dictate their biogeographical relationship to each other in previously described structures in human supragingival plaque. Using a novel variation for chemical detection, we observed metabolite exchange between individual bacterial cells in real-time validating the ability of these organisms to carry out metabolic crossfeeding at distal and temporal scales observed *in vivo*. These findings reveal one way by which these interactions are both favorable to the interacting commensals and potentially the host.
Main Text

Introduction

Over the past decades our knowledge of the human oral microbiome has increased drastically, revealing a robust polymicrobial biofilm in supragingival plaque (SUPP) that is present in healthy as well as diseased conditions. While we know a great deal about what bacteria reside in SUPP (1–4), we know very little about the interactions between taxa especially in healthy conditions relative to disease. Given that dysbiosis of the healthy microbiota is often a prelude to oral disease (5–7), we wish to study interactions within the healthy community to potentially reveal any community members that might help preserve stable community structure and constituency, potentially preventing the onset of disease.

Previous studies have shown the importance of attachment to the development of the oral biofilm (8) and new data has identified and refined the spatial organization / “biogeography” of abundant commensal organisms found in SUPP (9). Human microbiome project (HMP) data and recent microscopy of healthy individuals has revealed that one of the most abundant and prevalent species in SUPP is Corynebacterium matruchotii (1,4,9). It has been correlated with good dental health and hypothesized to be important in the organization of some plaque biofilm structures particularly due to its ability to adhere to Streptococcus species forming a structure referred to as a “corncob” with the Corynebacterium filament being surrounded by streptococci (9), a role typically ascribed to Fusobacterium (8,10). It has also been shown to help facilitate dental calculus formation via calcification (11). Previous studies have not likely appreciated the role C. matruchotii plays in bridging early and late colonizers within the plaque (8,10) and its importance in the structuring of the plaque community as it is presumed to bind to an existing biofilm of Streptococcus and
Actinomyces cells\(^{(9,12)}\). The spatial organization of microbes in these SUPP biofilm structures has been characterized in the ‘hedgehog’ model\(^{(9)}\) which visualizes *C. matruchotii* and its proximity to adjacent Streptococcus species, such as *S. mitis* at the SUPP perimeter\(^{(9,13)}\).

Streptococcus species, such as *S. mitis*, are one of the most abundant species in the oral microbiome\(^{(2)}\) and are known for their ability to compete in their environment by producing antimicrobial metabolites like \(\text{H}_2\text{O}_2\)\(^{(14)}\) via the spxB gene product, pyruvate oxidase\(^{(15)}\). Fermentation by streptococci (primarily lactate production) can decrease local pH which can select for other microbes including Streptococcus mutans which can thrive in the community causing caries\(^{(16–18)}\). Streptococci can also co-aggregate with other species to benefit from their catalase activity\(^{(19)}\) and we have previously shown that crossfeeding on Streptococcus-produced lactate by adjacent microbes increases their growth yields\(^{(20)}\) and that co-proximity results in a catalase-dependent removal of \(\text{H}_2\text{O}_2\)\(^{(21)}\). *C. matruchotii*, in close proximity with *S. mitis*, must survive in the presence of these same metabolites and how it does so is unknown. If their interaction were to result in Corynebacterium detoxification of streptococcal metabolites, this could help stabilize a diverse bacterial biofilm community which may in turn enhance colonization resistance, *i.e.*, the ability of these biofilms to limit growth of opportunistic or exogenous pathogens.

We employed a reductionist approach to investigate the relationship between *C. matruchotii* and *S. mitis* discovering that *S. mitis* has a considerable increase in growth yield with *C. matruchotii* aerobically but not anaerobically where *C. matruchotii* growth is also inhibited by *S. mitis*. We also observed that *C. matruchotii* upregulated lactate catabolism genes in coculture with *S. mitis* and surprisingly observed that oxidation of
lactate by *C. matruchotii* was a contributor to *S. mitis* growth enhancement and was pH independent. We then utilized scanning electrochemical microscopy to demonstrate that *C. matruchotii* lactate catabolism can deplete local concentrations of this organic acid swiftly in real-time at sub-micron scales, implying that metabolite consumption in coculture can occur in the observed *in vivo* arrangements between these organisms. These data reveal mechanisms of interaction that support the *in vivo* co-occurrence and biogeography between these species in healthy oral biofilms.

**Results**

*C. matruchotii enhances the growth of *S. mitis* in aerobic conditions.*

We performed pairwise coculture experiments aerobically and anaerobically with a solid medium colony biofilm model(22) to quantify growth yield between mono- and cocultures of *S. mitis* with *C. matruchotii* (Fig. 5-1) observing a 954-fold growth yield enhancement of *S. mitis* in coculture. Unexpectedly, *C. matruchotii* had no significant difference in growth yield with *S. mitis* (Fig. 5-1A). While previous studies have hypothesized that *C. matruchotii*–Streptococcus interactions occur in aerobic microenvironments within SUPP(9,13), we also performed the same experiment in anaerobic conditions as a comparison (Fig. 5 1B). Interestingly, the coculture growth benefit for *S. mitis* was lost while *C. matruchotii* yield decreased ~130-fold. To investigate how *C. matruchotii* enhances *S. mitis* growth yield in coculture we performed RNAseq to compare mono- vs coculture transcriptome data.
*C. matruchotii* upregulates genes necessary for L-lactate catabolism and oxidative stress response

*C. matruchotii* differentially expressed only 22 genes (greater than 2-fold) in aerobic coculture with *S. mitis* (Table 4-S2). Interestingly, *C. matruchotii* upregulated the lutABC operon (lutA, 4.37-fold; lutB, 3.76-fold; lutC, 3.20-fold), whose gene products in Bacillus subtilis catabolize L-lactate\(^{(23)}\) using oxygen as a terminal electron acceptor; therefore, in the absence of oxygen, *C. matruchotii* is no longer able to catabolize L-lactate, as previously shown\(^{(24)}\). *C. matruchotii* also significantly upregulates a bacterial non-heme ferritin-encoding gene (2.39-fold) in coculture. This protein has been characterized in Mycobacterium smegmatis to sequester ferrous ions as part of the oxidative stress response\(^{(25)}\). Given the coculture growth and transcriptome results, we broadly hypothesized that *C. matruchotii* crossfeeds on *S. mitis*-produced lactate while detoxifying *S. mitis*-produced \(\text{H}_2\text{O}_2\)-similar to other microbes in the oral cavity\(^{(19,20)}\). Given the fact that *C. matruchotii* cannot utilize L-lactate anaerobically and *S. mitis* is only provided a growth benefit in the presence of oxygen, we believe these data suggest one mechanism by which the biogeography of these species *in vivo* could be influenced by their metabolic interactions.

**Lactate utilization by *C. matruchotii* influences *S. mitis* growth yield**

The growth enhancement of *S. mitis* in coculture with *C. matruchotii* is likely due to several factors including \(\text{H}_2\text{O}_2\) decomposition and lactate catabolism. It is unclear if the removal of lactate itself or the removal of lactate and subsequent increase in pH is responsible for *S. mitis* growth yield enhancement. We 1st tested the impact of pH by performing growth experiments in the same medium with increased buffer capacity by adding 50 mM MOPS. Qualitatively, we observed that *S. mitis* monoculture colonies no longer produced yellow
coloration in buffered medium containing the pH indicator dye phenol red (i.e. no longer acidified the environment) compared to the original medium (data not shown). Quantitatively, we observed that S. mitis growth yield had no significant change in monoculture with additional MOPS (Fig. 5-S1) indicating that pH was not responsible for S. mitis growth yield increases in coculture.

To determine if removal of lactate by C. matruchotii via catabolism was enhancing streptococcal fitness we constructed a lutA gene deletion mutant (ΔlutA) since each gene within the lutABC operon had been described to be essential for L-lactate catabolism (23). The ΔlutA strain was significantly impaired in L-lactate utilization showing a diminished growth rate (doubling times of 17.9h for the wt and 27.4h for ΔlutA) and yield aerobically with L-lactate as the sole carbon source (Fig. 5-S2). C. matruchotii possesses two additional annotated L-lactate dehydrogenases which may function bidirectionally allowing it to slowly oxidize L-lactate without a functional lutABC system. A full lutABC operon deletion strain was also created and showed identical results (data not shown).

We next tested the ΔlutABC mutant in mono vs coculture with S. mitis to determine if impaired lactate utilization led to a decrease in S. mitis yield in coculture with C. matruchotii. Using defined medium in glucose-limited conditions to force competition for limited glucose and/or promote cross-feeding on streptococcal-produced lactate, we observed that both S. mitis and C. matruchotii ΔlutABC fitness were significantly decreased in coculture (Fig. 5-2). C. matruchotii ΔlutABC can only poorly catabolize L-lactate and thus poorly cross-feed on S. mitis-produced L-lactate compared to the wildtype. As ΔlutABC and S. mitis are now forced to compete for limited glucose, both exhibit a decreased growth yield. This is in agreement with previous data anaerobically (Fig. 5-1B),
where oxidation of lactate by *C. matruchotii* does not occur. The growth yield increase of *S. mitis* in coculture is diminished when *C. matruchotii* cannot oxidize lactate but this does not fully explain the total growth benefit provided, suggesting another mechanism(s) at work.

**Catalase abundance leads to enhanced streptococcal growth yields**

Given that lactate oxidation by *C. matruchotii* provides only a portion of the fitness benefit in coculture to *S. mitis* we next investigated if H$_2$O$_2$ detoxification by *C. matruchotii* also contributed. Surprisingly, in coculture with *S. mitis*, *C. matruchotii* did not upregulate expression of the single catalase (katA) encoded on its chromosome. We observed that catalase was maximally expressed aerobically and not expressed anaerobically (data not shown). To test if catalase-dependent H$_2$O$_2$ detoxification was important for *C. matruchotii* fitness in coculture, and subsequent *S. mitis* growth yield enhancement, we generated the catalase gene deletion mutant, *C. matruchotii* ΔkatA. Interestingly, this mutant had to be generated entirely under anaerobic conditions and does not survive incubation in aerobic or microaerophilic conditions, making it impossible to test this mutant in aerobic coculture with *S. mitis*. Instead, we determined the contribution of catalase to the growth of these species by adding it exogenously in growth medium amended with 100U/mL of bovine catalase. Previous studies$^{(19,26,27)}$ have indicated that streptococcal-produced H$_2$O$_2$ is capable of limiting their own growth. We observed that adding exogenous catalase elevated the monoculture growth yield of *S. mitis* 6.42-fold (Fig. 5-3A). This self-limitation by H$_2$O$_2$ production was also observed when comparing monoculture fold changes of *S. mitis* to the non H$_2$O$_2$–producing ΔspxB mutant (Fig. 5-3). Interestingly, the growth benefit of *S. mitis* in coculture with *C. matruchotii* dropped from 954-fold to 148-fold when amended with
Catalase.

**C. matruchotii requires a functional oxidative stress response to be fit to interact with S. mitis**

In coculture with *S. mitis*, *C. matruchotii* significantly upregulated a gene encoding ferritin, a bacterial non-heme protein involved in oxidative stress response\(^{(25)}\). We hypothesized that ferritin was needed for *C. matruchotii* fitness with H\(_2\)O\(_2\) -producing streptococci. To test this, we deleted the ferritin encoding gene generating *C. matruchotii* Δftn and performed cocultures with WT *S. mitis* and *S. mitis* ΔspxB (which is unable to produce H\(_2\)O\(_2\)) \(^{(14)}\). In coculture with WT *S. mitis*, we observed that the Δftn mutant fitness decreased 7.35-fold (Fig. 5-4A) and this decrease was not observed in coculture with the *S. mitis* ΔspxB strain. *S. mitis* WT had a 4.6-fold significant decrease in growth yield with *C. matruchotii* Δftn compared to *C. matruchotii* WT whereas there was no change in growth yield with *S. mitis* ΔspxB with either *C. matruchotii* strain. This shows that *C. matruchotii* needs a functional oxidative stress response to be fit in interactions with WT *S. mitis*. These data indicate that H\(_2\)O\(_2\) detoxification is the largest contributor to enhanced *S. mitis* fitness in coculture but also that other mechanisms, *i.e.* *C. matruchotii* lactate oxidation, further enhance fitness.

**Scanning electrochemical microscopy (SECM) reveals oxidation of *S. mitis*-produced lactate by adjacent *C. matruchotii* at sub-micron scale**

To investigate lactate production and consumption in situ by bacteria as well as the topography of bacterial cells, a submicropipet-supported interface between two immiscible electrolyte solutions (ITIES) was employed (Fig. 4-S3A)\(^{(28)}\). The full methodology and
findings of this work are part of a co-submitted manuscript. With this submicrotip, an etched Ni/Cu electrode in the internal organic electrolyte exerts a bias across the submicroscale liquid/liquid interface against an electrode in the aqueous solution to yield the amperometric tip current based on the selective interfacial transfer of a small probe ion \(^{(28)}\). The coculture of \textit{C. matruchotii} and \textit{S. mitis} was immobilized over a poly L-lysine coated glass slide, and studied by scanning or approaching an 800 nm-diameter pipet tip over the bacteria. Further detail is provided in the supplemental materials (Fig. 5-S3C and D).

We employed the constant-height mode of submicroscale SECM to successfully image single bacterial cells in coculture (Fig. 5-5). The high spatial resolution was obtained by using submicropipet tips, which were characterized by cyclic voltammetry for tetraethylammonium (TEA\(^+\)) ion transfer (IT) in situ to obtain a diffusion limited current in the bulk solution, \(i_{T,\infty}\) (Fig. 4-S3B). Constant-height imaging of cocultured bacteria for the probe ion TEA was obtained with the gap between the tip and bacteria, \(d_c = 0.75 \ \mu m\), \(i.e., 1.80 \ \text{normalized distance to tip radius, } (d/a)\). This SECM image could not resolve each individual bacterial cell. For instance, a lump was identified in \(25 \ \mu m \times 20 \ \mu m\) image based on TEA\(^+\) IT, which did not resolve any difference between bacterial cells (Fig. 5-5B). Low tip currents of \(\sim 80\% \ of \ i_{T,\infty}\) for TEA\(^+\) above these bacteria was obtained due to hindered diffusion of TEA\(^-\) by adjacent bacteria with partially permeable membranes to this probe ion.

The same area in \(25 \ \mu m \times 20 \ \mu m\) was imaged based on lactate IT with the gap between the tip and bacteria, \(d_c = 0.50 \ \mu m\) (1.20 \(d/a\)), which could resolve individual \textit{S. mitis} and \textit{C. matruchotii} clearly (Fig. 5-5C). An initial current, \(\sim 30 \ \mu A\) above a glass substrate
corresponds to c.a. 0.26 mM of lactate produced by ensemble of *S. mitis* and diffused to bulk solution near bacteria according to eq 1 below.

\[
i_{T,\infty} = 4xzFDCa
\]

(eq 1)

where \(i_{T,\infty}\) is a current in bulk, \(x\) is the function of RG ratio (RG is the ratio of outer and inner diameters of a glass pipet, \(x = 1.16\) for a RG 1.5 tip), \(z\) is charge of lactate, \(F\) is Faraday constant (96485 C/mol), \(D\) is the diffusion coefficient (\(6 \times 10^{-6}\) cm\(^2\)/s), \(C\) is a concentration of lactate (0.26 mM), and \(a\) is the inner radius of a pipet tip (430 nm).

In this SECM image, higher tip currents than \(i_{T,\infty}\) for lactate are obtained above spherical *S. mitis*, while lower tip currents than \(i_{T,\infty}\) are observed above filamentous *C. matruchotii*. Not only distinctive morphologies are clearly distinguished between two different bacteria as shown in an optical microscopic image (Fig. 5-5A), but also the production and consumption of lactate between them are visually confirmed in situ, where currents were dramatically transposed from an enhanced response over *S. mitis* to reduced ones over *C. matruchotii*, implying local increase in lactate produced by *S. mitis* and local depletion of lactate consumed by *C. matruchotii*. Notably, this SECM image successfully visualized the chemical interaction between two commensal oral microbes in real time and is the 1st SECM study to our knowledge that measures metabolite exchange between two individual bacterial cells. Specifically, *S. mitis* produces c.a. 0.50 mM lactate locally, which is efficiently depleted by *C. matruchotii* (Figs. 5-5C, S3E) thus verifying a standing question about their commensal relationship that cannot be answered only by optical microscopic imaging. Quantitative analysis of the permeability of the bacterial membrane and the local concentration of lactate produced by *S. mitis* are discussed in the supplemental materials and companion manuscript detailing this methodology.\(^{47}\)
Discussion
Interactions between commensal bacteria in the human microbiome are quite understudied. This is especially the case within healthy supragingival plaque (SUPP) where they likely have a role in maintaining plaque homeostasis and host health compared to subgingival plaque and oral disease\(^8,10,29\). While the organisms in plaque biofilms are in close proximity and capable of physical and biochemical interaction, the involved mechanisms are largely hypothetical\(^9\). Characterizing the behavior of abundant SUPP commensal organisms can help reveal necessary interactions that could maintain a healthy microbiome as well as explain their biogeographical arrangements. One set of interactions are those between Corynebacterium matruchotii, and Streptococcus spp in previously described ‘hedgehog’ structures\(^{9,13}\) where they occur at the presumed aerobic biofilm / saliva margin. This study investigates these interactions and provides novel data on metabolite exchange between individual cells that has broad implications on polymicrobial biofilms beyond the human oral cavity.

Our data indicates that *S. mitis* had a significant growth yield increase when cocultured with *C. matruchotii* (Fig. 5-1A) and this growth benefit was lost anaerobically (Fig. 5-1B) which can partly explain their proximal association only at the aerobic margin of hedgehog structures\(^{9,13}\). \(\text{H}_2\text{O}_2\)-producing Streptococcus and adjacent commensal species have been shown to coexist despite ROS production\(^{19}\). *C. matruchotii* is uninhibited when cocultured with *S. mitis* aerobically likely due to its catalase production. While a *C. matruchotii* \(\Delta\text{katA}\) mutant could not grow aerobically, we were able to demonstrate that addition of exogenous catalase to wild-type monoculture resulted in enhancing *S. mitis* yield like that of the non-peroxide producing *S. mitis* \(\text{spxB}\) mutant but not to the same extent observed in coculture with *C. matruchotii* with or without exogenous catalase (Fig. 5-3). These data suggest that
catalase can enhance *S. mitis* growth, even if produced by adjacent *C. matruchotii*, similar to observations we have made previously\(^{21}\), but also suggest that additional benefits to *S. mitis* exist due to coculture. Interestingly we observed coculture upregulation in *C. matruchotii* of a gene encoding a protein that has 82% identity to ferritin from Corynebacterium mustelae. Ferritins have been shown to protect from ROS by sequestering iron and binding to DNA\(^{25}\) to prevent the production of hydroxyl radicals\(^{30}\). A *C. matruchotii* \(\Delta ftn\) mutant showed a significant yield decrease in coculture with *S. mitis* but decreases were not observed with *S. mitis* \(\Delta spxB\) (Fig. 5-4). We observed that any decreases in *C. matruchotii* yield were mirrored by decreases in *S. mitis* yield as well, linking streptococcal fitness to that of *C. matruchotii*. We hypothesize that this should also be true for any other adjacent \(\text{H}_2\text{O}_2\) producing streptococcal species. Transcriptional responses of *S. mitis* to *C. matruchotii* are part of a separate ongoing study and are not reflected here.

Of the 22 genes that *C. matruchotii* differentially expresses with *S. mitis* aerobically, three belong to the lactate oxidization encoding lutABC operon\(^{23}\). *C. matruchotii* can only oxidize lactate aerobically and will not oxidize lactate anaerobically (24) which can explain its inability to grow with *S. mitis* in anaerobic coculture (Fig. 5-1B). Initially, we hypothesized that *C. matruchotii* oxidation of streptococci-produced lactate could provide a growth benefit to adjacent streptococci by elevating the local pH. However, no amount of additional MOPS buffer was sufficient to increase *S. mitis* monoculture yields (Fig. 5-S1), despite local acidification no longer being detectable via a phenol red indicator dye. Thus, we hypothesized that the removal of lactate itself (and elimination of feedback inhibition of this fermentation product) would benefit *S. mitis* in a pH-independent fashion.
by allowing it to ferment more carbohydrate. To test this, we deleted the lut operon in \textit{C. matruchotii} and competed this strain in a glucose-limited coculture where \textit{C. matruchotii} should rely on cross feeding of streptococci-produced lactate. \textit{C. matruchotii} ΔlutABC yields were significantly decreased in coculture with \textit{S. mitis} in limiting glucose when compared to monoculture with a similar decrease in \textit{S. mitis} yield (Fig. 5-2). While these changes were significant, they were modest, which is due to the ability of \textit{C. matruchotii} ΔlutABC strains to still oxidize lactate to a minor extent, likely due to the presence of at least 2 potentially reversible lactate dehydrogenase encoding genes (Fig. 5-S2). Even with only partial loss of function, these results indicate that \textit{C. matruchotii} cannot compete for glucose with \textit{S. mitis} and likely depends on cross-feeding of lactate when they are in direct proximity.

By utilizing scanning electrochemical microscopy (SECM) we were able to directly quantify lactate production by \textit{S. mitis} and its oxidation by adjacent \textit{C. matruchotii} in real time (Fig. 5-5)\textsuperscript{47} indicating a sharp decrease in lactate concentration between individual cells. To the best of our knowledge this is the first observation of metabolite exchange between individual bacterial cells by SECM. Based on these data, we believe that existing ‘corncob’ configurations observed \textit{in vivo}\textsuperscript{(9,13)} should readily be able to consistently remove lactate from their immediate area. Interestingly, we also observed in our companion study\textsuperscript{47} that lactate utilization in an individual \textit{C. matruchotii} filament was localized nearest to streptococcal cells which also fits well with their observed \textit{in vivo} arrangement towards the aerobic-oriented pole of the corncob structure\textsuperscript{(9,13)}. This would allow streptococcal metabolism to continue without inhibition while eliminating a source of acid stress to the host and other adjacent microbiota which contrasts well with the dense clustering
biogeography of S. mutans observed on enamel during caries formation\textsuperscript{(17)} This observation supports a mechanism whereby the interaction between these two commensals may contribute to the lack of cariogenic activity in a healthy oral biofilm. Previous studies by Frenkel and Ribbeck\textsuperscript{(31,32)} have demonstrated that physical separation of streptococcal aggregates via mucins are sufficient to enhance growth of competing species and limit damage to enamel. This is reminiscent of distal separation of streptococci bound to C. matruchotii ‘corncobs’ yet these can be further enhanced by their ability to also remove lactate actively from the coculture environment with spatial heterogeneity that favors the observed biogeographical heterogeneity\textsuperscript{(9,13)}.

This study has described two mechanisms of interaction between bacteria that exist in direct contact \textit{in vivo}. Using a reductionist approach, we were able to ascertain how each mechanism contributed to fitness of both organisms. Advantages provided to each species when these mechanisms are intact also explain the positional / biogeographical arrangement of these species \textit{in vivo}. Additionally, we were able to demonstrate real-time metabolite exchange between these species at sub-micron distances, indicating that crossfeeding between these organisms is likely occurring between them \textit{in vivo}. These interactions reveal how structural orientation and species composition between commensals may contribute to host health and potentially be one way by which a healthy biofilm composition is maintained \textit{in vivo} and answer hypotheses about mechanisms of interaction between these organisms that were hypothesized several years previously\textsuperscript{(9)}.

\textbf{Materials and Methods}
Strains and media. Strains and plasmids used in this study are listed in Table 5-S1. C. matruchotii (ATCC 14266) and S. mitis (ATCC 49456) were grown on Brain Heart
Infusion media supplemented with 0.5% yeast extract (BHI-YE) at 37°C in a static incubator with 5% CO₂ or in 5% H₂, 10% CO₂ and 85% N₂ in anaerobic conditions. E. coli was grown at 37 °C in standard atmospheric conditions with liquid cultures shaken at 200 RPM. Antibiotics were used at the following concentrations: kanamycin 40 µg/ml for E. coli and 10 µg/ml for C. matruchotii.

Colony biofilm coculture/ buffered coculture/ catalase coculture. Overnight cultures of C. matruchotii and S. mitis species were grown in Brain Heart Infusion media supplemented with 0.5% yeast extract (BHI-YE) at 37°C in a static incubator with 5% CO₂ or in 5% H₂, 10% CO₂ and 85% N₂ for anaerobic conditions. Colony biofilm assays were carried out as described previously\(^{22}\). Briefly, A semi permeable 0.22 µm polycarbonate membrane filter\(^{33}\) was placed on solid BHI-YE media (supplemented with 1.6% agar). Ten µl of each culture were spotted on the membrane filters and monocultures were spot with 10µL of BHI-YE. The cultures incubated for 48 h and the membranes were placed in a microcentrifuge tube with 1mL of BHI-YE. The tubes were vortexed to resuspend into media and serially diluted and track plated\(^{34}\) to count colony forming units per mL (CFU/mL). S. mitis was counted by using BHI-YE plates and C. matruchotii on BHI-YE plates supplemented with 100 µg/ml fosfomycin. Buffered and pH indicator cocultures were carried out with 50 mM MOPS and 18 mg/mL of phenol red added to BHI-YE. Catalase cocultures were carried out with 100 U/mL of catalase added to BHI-YE.

RNAseq experiment and analysis. Mono and cocultures were prepared similar to the colony biofilm coculture with the exception that culture membranes were incubated for 24hr and moved to fresh media for an additional 4hr. Membranes were then removed from solid agar and immediately placed into RNALater (Ambion) where cells were removed by agitation.
and pelleted by centrifugation. Cell pellets were stored in Trizol reagent at -80°C. Experiments were carried out in biological duplicates. RNA extraction, library preparation and sequencing were then carried out by the Microbial ‘Omics Core facility at the Broad Institute. RNASeq libraries were generated using previously described methods\cite{35}. Sequence data was aligned using Bowtie2\cite{36} and read counts per coding sequence were called using HTSeq-Count\cite{37}. Statistical analysis was carried out via DESeq2\cite{38} to determine differentially expressed genes. Scripts of this pipeline can be found at https://github.com/dasithperera-hub/RNASeq-analysis-toolkit. Sequence libraries are available through the NCBI short read archive (SRA) under bioproject number PRJNA832032.

Gene deletions. All *C. matruchotii* gene deletions were carried out with sucrose counter selection using a suicide vector derived from pMRKO\cite{20}, pEAKO2 which contains sacB from pK19mobsacB\cite{39}. Approximately 1000 bp up and downstream flanking regions for each gene were used for homologous recombination and fragments were cloned into pEAKO2 via Gibson Assembly\cite{40}. *C. matruchotii* cells will be made as previously described\cite{41}. Transformations were carried out with 50µL of competent cells and 1 µg of DNA electroporated with 0.2 cm gap cuvettes at 2.5 kV voltage, 400 Ω resistance, and 25 µF capacitance. After electroporation, 950 mL of prewarmed BHI-YE will be added to the cuvette and the mixture will be moved to a 46 °C heat block for 6 minutes. After heat shock, transformations will be shaken at 250 RPM at 37 °C for 4 h. Transformations were plated on BHI-YE Kan10 plates and incubated for 4 days at 37 °C. Mutants were verified through PCR.

Limiting glucose coculture. Cultures were prepared like colony biofilm coculture described
above except for being inoculated into 2mL of liquid defined medium. Modified RPMI medium (Gibco) was used as a base and supplemented with 8 mM glucose. Cocultures were inoculated for 48 h and track plated to determine CFU/mL.

**Scanning electrochemical microscopy sample preparation.** Bacterial sample preparation was performed using a previously described defined medium (42) and glucose at 10 mM. Further details are provided in the Supplemental Materials.

**Scanning electrochemical microscopy acquisition.** Scanning parameters and nano-probe design are similar to methods described previously. (43–47) A full description of SECM calibration, sample acquisition and metabolite quantification are provided in the Supplemental Materials and in a companion manuscript. (47)

**Acknowledgments**

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Data Sharing Plan

Scripts utilized for RNASeq analysis can be found at https://github.com/dasithperera-hub/RNASeq-analysis-toolkit. Sequence libraries are available through the NCBI short read archive (SRA) under bioproject number PRJNA832032.

References


Figures and Tables

**Figure 5-1.** Growth yield measurements of mono vs coculture biofilms. Aerobic (A) and anaerobic (B) CFU counts of *C. matruchotii* (*Cm*) and *S. mitis* (*Sm*) in mono and cocultures. Data are mean CFU counts for n ≥ 3 and error bars represent 1 standard deviation. * denotes p < 0.05 using a Student's *t*-test.

**Figure 5-2.** Limiting glucose colony biofilm cocultures. CFU counts of *C. matruchotii* (*Cm*), *C. matruchotii ΔlutA*, and *S. mitis* (*Sm*) in mono and cocultures. Data are mean CFU counts for n ≥ 3 and error bars represent 1 standard deviation. * denotes p < 0.05 using a Student's *t*-test.
**Figure 5.3.** *S. mitis* monoculture enhanced by exogenous catalase. A) CFU counts of *S. mitis* WT (*Sm*) in mono and coculture with *C. matruchotii* (*Cm*) on media containing 100 U/mL of catalase vs without. B) CFU counts of *S. mitis* ΔspxB in mono and coculture with *Cm* on media containing 100U/mL of catalase vs without. * denotes p < 0.05 using a Student's *t*-test.

**Figure 5.4.** *C. matruchotii* ferritin knockout inhibited when cocultured with *S. mitis*. A) Aerobic CFU counts of *C. matruchotii* WT (*Cm*) and ferritin knockout (Δftn) in mono and coculture with *S. mitis* WT and strain lacking ability to create H₂O₂ (ΔspxB). B) CFU counts of *Sm* and ΔspxB in mono and coculture with *Cm* and Δftn. Data are mean CFU counts with error bars indicating standard deviation for n ≥ 3. * denotes p < 0.05 using a Student's *t*-test compared to monoculture.
Figure 5-5. SECM detection of *C. matruchotii* oxidation of *S. mitis*-produced lactate. (A) Representative micrograph of *C. matruchotii* (red filament) and *S. mitis* (chained spheres) coculture. (B) Topographical SECM image based on TEA$^+$ IT (obtained with a gap between the tip and bacteria, $d_c = 1.8 \ d/a$). Monotonic current decrease over bacterial “lump” is observed due to hindered diffusions of TEA$^+$ through the impermeable parts of bacterial membrane. (C) Lactate-mapping SECM image based on lactate IT (obtained at $d_c = 1.2 \ d/a$). Sharp current transition between *S. mitis* and *C. matruchotii* is observed corresponding to simultaneous lactate production and consumption, respectively. SECM images were obtained in constant-height mode.
APPENDIX 1: CHAPTER 2-SUPPORTING INFORMATION

Kinetics of Antimicrobial Drug Ion Transfer at a Water/Oil Interface Studied by Nanopipet Voltammetry
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1. Structure of Antibacterial Drug Molecules

![A] nalidixic acid (NA), (B) flumequine (FMQ), (C) sulfadimethoxine (SDM), (D) sulfamethazine (SMT), and (E) sulfamerazine (SMR).

Figure 2-S1. Structure of antibacterial drug molecules (quinolones and sulfonamides); (A) nalidixic acid (NA), (B) flumequine (FMQ), (C) sulfadimethoxine (SDM), (D) sulfamethazine (SMT), and (E) sulfamerazine (SMR).

2. Fine Element Simulation by COMSOL Multiphysics: Time-dependent diffusion problem for voltammetry

The Nanopipet voltammetry diffusion problem was solved with differential equations in cylindrical coordinates:

\[
i^n (\text{aqueous}) \xrightarrow{k_f} i^n (1,2 - DCE) \]

\[
\frac{\partial c_1}{\partial t} = D_1 \left( \frac{\partial^2 c_1}{\partial r^2} + \frac{1}{r} \frac{\partial c_1}{\partial r} + \frac{\partial^2 c_1}{\partial z^2} \right) \]

\[
\frac{\partial c_2}{\partial t} = D_2 \left( \frac{\partial^2 c_2}{\partial r^2} + \frac{1}{r} \frac{\partial c_2}{\partial r} + \frac{\partial^2 c_2}{\partial z^2} \right)
\]

where \( t \) is time, \( r \) and \( z \) are coordinates in parallel and normal directions to the nanopipet interface, respectively. \( c_1 (r, z, t) \) and \( c_2 (r, z, t) \) are the concentrations of ions in aqueous and organic phase, respectively. The variables are introduced as follows (Figure 2-S2):
Radius = pipet inner radius at the tip

RG = pipet outer radius at the tip

RGP = pipet inner radius at 10μm region

RGP2 = pipet outer radius at 10μm region

$D_1$ = diffusion coefficient of drug ions in aqueous phase

$D_2$ = diffusion coefficient of drug ions in organic phase

Width = simulation space limit in the radial direction

Height = simulation space limit in the vertical direction

$C_1(r, z, \tau) = \text{the concentrations of ions in aqueous phase}$

$C_2(r, z, \tau) = \text{the concentrations of ions in organic phase}$

$n = \text{charges of ion} ( = z \text{ in eq 3, 4})$

$E_{ini} = \text{initial potential in voltammetry}$

$E_{fin} = \text{switching potential in voltammetry}$

$E^0 = \text{the formal transfer potential of ion} (\quad = \varphi^0_l \text{ in eq 3, 4})$

$k^0 = \text{standard heterogeneous ion transfer rate constant}$

$al = \text{the transfer coefficient} (\alpha)$

$v = \text{scan rate (V/s)}$

$\tau = (E_{fin} - E_{ini})/v$

$f = F/RT, 38.92 \text{ V}^{-1}$

The boundary condition at the liquid/liquid interface is given by

$$D_1 \left[ \frac{\partial c_1(r,z,t)}{\partial z} \right]_{z=0} = D_2 \left[ \frac{\partial c_2(r,z,t)}{\partial z} \right]_{z=0} = -k_f c_1(r,0,t) + k_p c_2(r,0,t) \quad (s4)$$
where \( k_f \) and \( k_b \) are the heterogeneous ion transfer rate constants for forward and reverse transfer, respectively (see eq S1).

The rate constants are given by the Butler-Volmer type relation as,

\[
    k_f = k^0 \exp \left[ \frac{(1-\alpha) n F (E - E^0)}{RT} \right] \\
    k_b = k^0 \exp \left[ -\alpha n F (E - E^0) \right]
\]

where \( k^0 \) is the standard heterogeneous ion transfer rate constant, \( \alpha \) is the transfer coefficient, \( E \) is the Galvani potential difference between the aqueous and organic phase, and \( E^0^* \) is the formal ion transfer potential. In voltammetry, the potential is swept linearly at the rate of \( v \) from the initial potential, \( E_{ini} \) to final potential, \( E_{fin} \), and the sweep direction is reversed at \( E_{fin} \). The potential wave is expressed as,

\[
    E = E_{ini} + \frac{2(E_{fin} - E_{ini})}{\pi} \sin^{-1} \left\{ \sin \left[ \frac{\pi v t}{2(E_{fin} - E_{ini})} \right] \right\}
\]

The initial conditions in the bulk are given by

\[
    c_1(r, z, 0) = 0.9 \text{ mM} \\
    c_2(r, z, 0) = 0 \text{ mM}
\]

The initial conditions inside the pipet are given by

\[
    c_1(r, z, 0) = 0 \text{ mM} \\
    c_2(r, z, 0) = 0 \text{ mM}
\]

The other boundary conditions are defined in the section 6 of COMSOL model report.

Herein, \( D_1 \) was determined by the extrapolated steady state current of a voltammogram with respective drug ions using the eq 1, where the concentration of drug ions is known as
0.9 mM and the radius of nanopipets is experimentally determined by a steady state current in the voltammogram with TBA$^+$ transfer. D was determined by Stokes-Einstein relation

\[ D = \frac{k_B T}{6 \pi \eta r} \]  

(s12)

where \( k_B \) is Boltzmann constant, \( T \) is the temperature, \( \eta \) is the viscosity of solvent (0.89 mPa·s for water and 0.83 mP·s for 1,2-DCE) and \( r \) is a radius of molecule.

The kinetic parameters, \( k^0 \), \( \alpha \), and the formal transfer potential of ion (\( \varphi_i^{0r} \)), denoted as \( E^{0r} \) in COMSOL simulation) in Table 1 were determined via the theoretical simulation. Since \( k^0 \) determines the overall shape and steepness of voltammograms and \( \varphi_i^{0r} \) controls the position of voltammograms, these two values are determined first. Subsequently, we determined \( \alpha \) by deviating it from 0.5 to finely fit the experimental voltammograms. The values of \( \alpha = 0.41 \pm 0.04 \) showed a good agreement with the experimental voltammograms, which are nearly close to 0.5 predicted based on the ion transfer models either based on the slow diffusion of the transferred species through the mixed interfacial layers$^{s1,s2}$ or assuming an activation barrier.$^{s3,s4}$ We further confirmed the \( \alpha \) values by plotting a Tafel plot, where a plot of log \( \left[ \frac{i}{1 - e^{\eta_{op}}} \right] \) vs. \( \eta_{op} \) (overpotential) yields a slope of \(-\alpha F/2.3RT\) under low overpotentials. For example, in Figure 2-S3, a Tafel plot for SMR is shown, and the resulting \( \alpha \) from the slope is 0.42 close to 0.40 determined by COMSOL Multiphysics. \( \alpha \) values for other drug molecules evaluated by COMSOL simulations are agreed with those from Tafel plots within 5 % variation (data not shown).
Figure 2-S2. Structure of antibacterial drug molecules (quinolones and sulfonamides); (A) nalidixic acid (NA), (B) flumequine (FMQ), (C) sulfadimethoxine (SDM), (D) sulfamethazine (SMT), and (E) sulfamerazine (SMR).

Figure 2-S3. A Tafel plot for the SMR ion transfer at nano ITIES. The dashed line corresponds to $\alpha = 0.42$. 

\[
y = -7.4316x - 13.442 \\
R^2 = 0.982
\]
3. Nanopipet Voltammograms of All Drug Ions compared with TBA$^+$ Transfer.

Herein, nanopipet voltammograms shown in Figure 4 are re-illustrated with their corresponding TBA$^+$ transfer. It should be noted that the $E_{1/2}$ from voltammograms of drug ion transfer is varied while nearly identical $E_{1/2}$ from voltammograms of TBA$^+$ transfer is observed for each measurement.

**Figure 2-S4.** Steady-state voltammograms (black lines for experimental, red/blue open circles for simulation) of (A) SMR, (B) NA, (C) SDM, (D) FMQ, (E) SMT, and (F) DFX with subsequently measured each voltammogram of TBA$^+$ transfers (dotted lines). Each corresponding background response from blank-buffer solution has been plotted together (grey lines). Same voltammograms for respective drug ions are shown in Figure 4.
4. Nanopipet Voltammogram of DFX$^-$

In Figure 2-S5, nanopipet voltammogram of DFX$^-$ is shown. Note that due to a lack of a clear inflection point, an accurate analysis with theoretical simulation was hampered.

![Diagram of Nanopipet Voltammogram](image)

**Figure 2-S5.** Steady-state voltammograms of DFX$^-$ transfers across the DCE/water interface obtained with nanopipets. Inset shows a molecular structure of DFX$^-$ ion. The theoretical fitting (blue open circles) to the experimental curves (black solid curves) were calculated from simulations with COMSOL Multiphysics using parameters in Table 1. Scan rate is 25 mV/s. The voltammogram of blank buffer solution (grey solid curves) was plotted together.

5. Amperometrical $i$-$t$ Curves for Drug Ions

We applied a constant potential where the maximum steady state current was obtained, and monitored a current over 800~1000 s. Here, all the drug molecules maintains c.a. 80~100 % of the initial current response, implying a negligible fouling behavior of a nanopipet supported interface between two immiscible electrolyte solutions. In Figure 2-S6, we normalized all the amperometric $i$-$t$ curves against respective initial currents to plot together. Stepwise currents as well as spiked responses could be due to a slight potential shift or fluctuation during the amperometric measurements.
Figure 2-S6. Normalized amperometric $i$-$t$ curves of various ions transferred across a water/1,2-DCE interface supported at a nanopipet. Reference electrode is Ag/AgCl in the aqueous phase.

References


APPENDIX 2: CHAPTER 3-SUPPORTING INFORMATION

Nanoscale Carbonate Ion-Selective Amperometric/Voltammetric Probes Based on Ion-Ionophore Recognition at Organic/Water Interface: Hidden Pieces of the Puzzle in Nanoscale Phase

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1. Modified M-1 medium: *Shewanella* Federation recipe.

For 1 L of Modified M-1 with 30mM lactate as electron donor:

- 5ml 200× vitamin stock solution
- 5ml 200× mineral stock solution
- 5ml 200× amino acid stock solution
- 50ml 20× buffer/salt solution
- 4.28 ml 7 M (60% w/w) sodium lactate syrup – 30 mM lactate
- H$_2$O to 1 L, then filter sterilize.

pH should be 7.0.

**Table 3-S1.** Buffer / salt solution (pH to 7.0 with NaOH, filter sterilize)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>g/L 20× stock</th>
<th>g/L 10× stock</th>
<th>FW</th>
<th>Concentration in 1× medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIPES buffer</td>
<td>18.14 g</td>
<td>9.07 g</td>
<td>302.4</td>
<td>3 mM</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>30.0 g</td>
<td>15.0 g</td>
<td>53.49</td>
<td>28 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.0 g</td>
<td>1.0 g</td>
<td>74.55</td>
<td>1.34 mM</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>10.56 g</td>
<td>5.28 g</td>
<td>120</td>
<td>4.4 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>146.1 g</td>
<td>73.05 g</td>
<td>58.44</td>
<td>125 mM</td>
</tr>
<tr>
<td>Sodium fumarate †</td>
<td>48.0 g</td>
<td>48.0 g</td>
<td>160.04</td>
<td>30 mM</td>
</tr>
</tbody>
</table>

† for anaerobic growth only

**Table 3-S2.** Table S2. 200× vitamin stock solution (filter sterilize)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>g/L 200× stock</th>
<th>FW</th>
<th>Concentration in 1× medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-biotin (B$_7$)*</td>
<td>0.004 g</td>
<td>244.31</td>
<td>81.8 nM</td>
</tr>
<tr>
<td>folic acid (B$_9$)*</td>
<td>0.004 g</td>
<td>441.4</td>
<td>45.3 nM</td>
</tr>
<tr>
<td>pyridoxine HCl (B$_6$)†</td>
<td>0.02 g</td>
<td>205.6</td>
<td>486.4 nM</td>
</tr>
<tr>
<td>riboflavin (B$_2$)*</td>
<td>0.010 g</td>
<td>376.37</td>
<td>132.8 nM</td>
</tr>
<tr>
<td>thiamine HCl (B$_1$)†</td>
<td>0.009 g</td>
<td>337.27</td>
<td>133.6 nM</td>
</tr>
<tr>
<td>nicotinic acid (B$_3$)*</td>
<td>0.010 g</td>
<td>123.11</td>
<td>406.2 nM</td>
</tr>
<tr>
<td>D-pantothenic acid, hemi-Ca (B$_5$)†</td>
<td>0.010 g</td>
<td>238.3</td>
<td>209.8 nM</td>
</tr>
<tr>
<td>vitamin B$_{12}$†</td>
<td>0.0002 g</td>
<td>1355</td>
<td>0.74 nM</td>
</tr>
<tr>
<td>p-aminobenzoic acid#</td>
<td>0.010 g</td>
<td>137.14</td>
<td>364.6 nM</td>
</tr>
<tr>
<td>lipoic acid (thioctic acid)#</td>
<td>0.010 g</td>
<td>206.3</td>
<td>242.4 nM</td>
</tr>
</tbody>
</table>

† solubilize in water; * add a few drops of NaOH to solubilize; # solubilize in 50% EtOH
**Table 3-S3.** 200× mineral stock solution (filter sterilize)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>g/L 200× stock</th>
<th>FW</th>
<th>Concentration in 1× medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>nitriloacetic acid (trisodium salt)</td>
<td>4.04 g</td>
<td>257</td>
<td>78.5 µM</td>
</tr>
<tr>
<td>MgSO₄ • 7 H₂O</td>
<td>12.128 g</td>
<td>246.48</td>
<td>249.1 µM</td>
</tr>
<tr>
<td>MnSO₄ • 1 H₂O</td>
<td>1.0 g</td>
<td>169.02</td>
<td>29.6 µM</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.0 g</td>
<td>58.44</td>
<td>171.1 µM</td>
</tr>
<tr>
<td>FeSO₄ • 7 H₂O</td>
<td>0.2 g</td>
<td>277.91</td>
<td>3.6 µM</td>
</tr>
<tr>
<td>CaCl₂ • 2 H₂O</td>
<td>0.2 g</td>
<td>147.02</td>
<td>6.8 µM</td>
</tr>
<tr>
<td>CoCl₂ • 6 H₂O</td>
<td>0.2 g</td>
<td>237.93</td>
<td>4.2 µM</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.26 g</td>
<td>136.3</td>
<td>9.54 µM</td>
</tr>
<tr>
<td>CuSO₄ • 5 H₂O</td>
<td>0.02 g</td>
<td>249.68</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>AlK(SO₄)₂ • 12 H₂O</td>
<td>0.02 g</td>
<td>474.39</td>
<td>0.21 µM</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.02 g</td>
<td>61.83</td>
<td>1.61 µM</td>
</tr>
<tr>
<td>Na₃MoO₄ • 2 H₂O</td>
<td>0.06 g</td>
<td>241.95</td>
<td>1.24 µM</td>
</tr>
<tr>
<td>NiCl₂ • 6 H₂O</td>
<td>0.048 g</td>
<td>237.71</td>
<td>1.01 µM</td>
</tr>
<tr>
<td>Na₂WO₄ • 2 H₂O</td>
<td>0.05 g</td>
<td>329.86</td>
<td>0.76 µM</td>
</tr>
</tbody>
</table>

**Table 3-S4.** Table S4. 200× amino acid stock solution (filter sterilize)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>g/L 200× stock</th>
<th>FW</th>
<th>Concentration in 1× medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamic acid</td>
<td>4 g</td>
<td>147.13</td>
<td>135.9 µM</td>
</tr>
<tr>
<td>L-arginine</td>
<td>4 g</td>
<td>174.2</td>
<td>114.8 µM</td>
</tr>
<tr>
<td>DL-serine</td>
<td>4 g</td>
<td>105.09</td>
<td>190.3 µM</td>
</tr>
</tbody>
</table>
2. Structure of Carbonate Ionophore VII and its Complex with CO$_3^{2-}$

![Structure of Carbonate Ionophore VII and its Complex with CO$_3^{2-}$]

Figure 3-S1. Illustration of interaction between the CO$_3^{2-}$ selective ionophore VII and CO$_3^{2-}$ ion. L is CO$_3^{2-}$ ionophore VII, which interacts selectively with CO$_3^{2-}$ to form a 1:1 complex.

3. Experimental Section

**Preparing cultures of *Shewanella oneidensis***

Aerobic cultures of *S. oneidensis* strain MR1 were grown in modified M-1 medium supplemented with either 30mM sodium lactate or 30 mM sodium acetate as the electron donor. Anaerobic cultures were further supplemented with 30 mM sodium fumarate as the terminal electron acceptor. All cultures were grown at 30 °C. Starter cultures (ABS$_{600}$ ~1.5) grown aerobically in M-1 medium supplemented with 30 mM lactate were diluted to an ABS$_{600}$ of 0.025 in fresh medium. Aerobic cultures were grown with agitation until stationary phase (24 hours). Anaerobic cultures were supplemented with Oxyrase for Broth reagent (Oxyrase, Inc., [www.oxyrase.com](http://www.oxyrase.com)) to maintain consistent anaerobic conditions in the culture and were grown without agitation in sealed flasks until stationary phase (48 hours). Cell-free culture supernatant was prepared by first pelleting cells via centrifugation then passing the culture supernatant through a 0.2 µm filter to remove any remaining cells.
Synthesis of Ionic liquid Supporting Electrolytes (TDDA⁺·TFPB⁻) by Metathesis.

Ionic liquid supporting electrolyte (TDDA⁺·TFPB⁻) was prepared by metathesis. 0.7713 g (1 mM) of tetradodecylammonium bromide (TDDABr) and 0.7718 g (1 mM) of potassium tetrakis(pentafluorophenyl) borate (KTFPB) were dissolved in 20 ml of dichloromethane and 1:1 mixture of methanol and water, respectively. Both of homogenous solutions were mixed in a 125 ml of Erlenmeyer flask, and stirred overnight to exchange each counter ion. The resulting immiscible products (TDDA⁺·TFPB⁻) were extracted with 15 ml of 1:1 methanol and water using a separatory funnel. This extraction process was repeated three times to separate the remaining aqueous constituents (KBr). Any remaining KBr in the ionic liquid product was further separated and removed by liquid chromatography, where a column filled with silica slurry was loaded with the ionic liquid product, and washed with

Figure 3-S2. The silanization set up with a glove bag (B) filled with dry N₂ gas to maintain a relative humidity less than 16 % at 20 °C during the silanization process. Inside a glove bag, a plastic mini-desiccator (C) containing as pulled pipets is connected to a vacuum pump (D) to remove moisture and humid air in one side as well as to the round bottom flask (A) to introduce N,N-dimethyltrimethylsilylamine into a mini-desiccator without vacuum. Any contamination by back flow from a vacuum pump is caught by a cold trap (E).
an eluent of a 1:1 mixture of DCM and hexane. Finally, the eluent containing the final ionic liquid product was evaporated with a rotary evaporator at 30 °C and 60 rpm, and dried with a vacuum for two days before usage.

**Fabrication of Nanopipet Electrodes.** Nanopipets were pulled from a quartz capillary (outer diameter = 1 mm, inner diameter = 0.7 mm, length = 10 cm, FG-GQ100-70-10, Sutter Instrument Co, Novato, CA) using CO₂–laser puller (P-2000, Sutter Instrument Co., Novato, CA). 100~120 nm inner diameter of paired nanopipets were obtained with a pulling program (1st line: heat = 715, filament = 4, velocity = 20, delay = 140, pull = 80, 2nd line: heat = 715, filament = 4, velocity = 38, delay = 135, pull = 115). As-pulled nanopipets were subsequently cleaned in a plasma cleaner for 3 minutes with Ar gas as a source followed by degassing for 10 minutes. Then, nanopipets were dried under vacuum for 1.5 hrs in a mini-desiccator (Mini-vacuum desiccator, Bel-Art Products, Pequannock, NJ), which was placed inside a glove bag purged with N₂ gas to maintain a relative humidity lower than ~16 % at 20 °C (Figure 3-S2). After drying process, pipets were silanized for 50 minutes with introducing 50µL of N,N-dimethyltrimethylsilylamine into a mini-desiccator without vacuum. Finally, vacuum was reapplied for 10 minutes to remove extra silanizing reagents. The proper control of humidity during silanization optimized our silanization process, thus leading to nearly 100 % success rate regardless of season or weather condition. A freshly silanized nanopipet was filled with 10 μL of 1,2-DCE solution containing 0.1 M organic supporting electrolytes (TDDA⁺·TFPB⁻) and 30 mM CO₃²⁻ ionophore VII. An electrochemically etched Pt or nickel-copper (Ni/Cu) wire was inserted from the back of a nanopipet under the vertical laminar flow clean cabinet (PURAIR-FLOW-48 Series Laminar Flow Cabinets Class 100, Air Science), and fixed with a clip.
**Electrochemical Etching of Nickel-Copper Wire.** In case of a Pt inner reference electrode, an etched platinum wire connected to Ni/Cu alloy wire is used as an inner reference electrode inserted inside nanopipets. Silver epoxy is often used for a connection between two wires, which could fall off and cause blockage or contamination of a pipet interface. Instead of an etched Pt wire composite, we electrochemically etched Ni/Cu wire as an inner reference electrode. For etching, a Ni/Cu wire (length: 10 cm, diameter: 0.13 mm, Ni : Cu = 45:55 wt %, Alfa Aesar) was immersed ca. 5 mm deeply in an etchant solution composed of acetic acid, nitric acid and water with a volume ratio of 1 : 2 : 6, and a DC voltage at 1.4 V was applied between a Ni-Cu wire and a graphite rod as a reference electrode. A wire was etched until the tip diameter of a wire is ca. 200~500 nm, thin enough compared to the inner diameter of an as-pulled nanopipet. The etching process takes less than a minute. After etching, a wire was thoroughly cleaned with nanopure water (18.2 MΩ·cm, TOC 2 ppb; Milli-Q Integral 4-System, Millipore) to remove the residual impurities and remaining etchant, and subsequently rinsed with isopropyl alcohol and DCM before insertion into nanopipets.

**Electrochemical Measurements.** Electrochemical workstations (CHI 8022D and CHI 760E, CH Instruments, Austin, TX) were used for voltammetric and amperometric measurements. Nanopipet voltammetry employed two-electrode cells using a Pt-wire (0.5 mm, hard, 99.95 %, Alfa Aesar) as a quasi-reference electrode (QRE) as represented by,

\[
\text{Pt QRE} \mid \text{CO}_3^{2-} \text{ion and TBA ion with supporting electrolytes in 40 mM phosphate buffer or M-1 medium (w)} \mid 30 \text{ mM CO}_3^{2-} \text{ ionophore VII and 0.1 M TDDATFPB (DCE)} \mid \text{Ni/Cu}
\]

For aqueous buffer solution, we used freshly prepared modified M-1 medium solution or M-1 solution used for incubating *S. oneidensis*. Both solutions are filtered with syringe
filter (0.1 μm filter unit, SLVV033RS, Duroapore PVDF membrane, MILLEX VV), and
adjusted to pH at 8.50. For DCE solution containing 30 mM CO$_3^{2-}$ ionophore VII and 0.1 M TDDATFPB, we vortexed the mixture for 1~3 hrs, and left on the laboratory bench for
at least 9~12 hrs before the usage. This premade DCE solution was brought to the back of
a silanized nanopipet using Hamilton Syringe followed by gentle tapping under the vertical
laminar flow clean cabinet. Subsequently, an etched Ni/Cu wire was inserted into the
nanopipet and fixed with a clip. This prepared nanopipet electrode was immersed in the
aqueous buffer solution, and the potential was applied between the inner Ni/Cu wire and
the outer Pt QRE. TBA IT studied in the negative potential scan was used to characterize
and confirm the nanopipet performance, while CO$_3^{2-}$ IT was monitored in the positive
potential scan for the study of a facilitated IT.

4. **Picture of Vertical Laminar Flow Clean Cabinet**

![Figure 3-S3](image)

*Figure 3-S3.* The benchtop clean hood equipped with HEPA filter to protect
prepared nanoscale interfaces from airborne adventitious contaminations.
5. Cyclic Voltammograms with and without CO$_3^{2-}$ ionophore VII

![Cyclic Voltammograms](image)

**Figure 3-S4.** Background subtracted cyclic voltammograms with and without CO$_3^{2-}$ ionophore VII. No appreciable ionic currents are observed within the potential window without CO$_3^{2-}$ ionophore VII. *Premade* 30 mM ionophore was used, which was vortexed for 15 min.

6. Nanopipet Voltammetry with Nanopipets Filled with 5 mM CO$_3^{2-}$ ionophore VII Solution

We tested 5 mM ionophore for CO$_3^{2-}$ FITs by nanopipet voltammetry in the presence of 0.45 mM carbonate ions. We didn’t observe a blockage of pipet interface during voltammetric study with either freshly prepared or premade ionophore solutions containing 5 mM ionophore. Thus, the solubility issue has been mitigated at lower concentration of ionophore. Interestingly (Figure 3-S5), only the pipets filled with premade carbonate ionophore solution still showed well-resolved CO$_3^{2-}$ FIT voltammograms (Figure 3-S5c and e), whereas pipets filled with a freshly prepared solution didn’t show any appreciable ionic currents corresponding to CO$_3^{2-}$ FITs (Figure 3-S5b).

7. NMR Study to Confirm Hydration of Trifluoroacetyl Carbon Centers in CO$_3^{2-}$ ionophore VII for Activation

We conducted $^{19}$F-NMR and $^1$H-NMR with a freshly prepared ionophore solution, and monitored the spectra changes with time to determine if hydration occurs in this mixture. There was inappreciable moisture present in the original NMR sample, so we performed a time study on both the ionic and the ionophore together (*i.e.*, a similar composition to a DCE filling solution), to observe if hydration occurs in this mixture.
Figure 3-S5. Background subtracted voltammograms with nanopipets filled with freshly prepared ionophore solution containing 5 mM CO$_3^{2-}$ ionophore VII for (a) TBA simple ion transfer in 1 mM TBA, (b) CO$_3^{2-}$ facilitated ion transfer in 0.45 mM CO$_3^{2-}$. Background subtracted voltammograms with nanopipets filled with premade ionophore solution containing 5 mM CO$_3^{2-}$ ionophore VII for (c) TBA simple ion transfer in 1 mM TBA, (d) CO$_3^{2-}$ facilitated ion transfer in 0.45 mM CO$_3^{2-}$, (e) voltammograms of CO$_3^{2-}$ facilitated ion transfer with successive addition of CO$_3^{2-}$ in aqueous buffer solution. The scan rate is 25 mV/s, and aqueous solution contains 40 mM phosphate buffer at pH 8.55. All ionophore solutions were vortexed for 15 min.
8. Amperometric i-t Curves for Fouling Tests of Nanopipet Electrodes in M-1 Medium used for Bacterial Incubation, and a Calibration Curve with Standard Addition Method.

For the fouling test of amperometric nano-ISEs, we performed amperometric $i$-$t$ technique to monitor the currents over time in M-1 medium used for bacterial incubation. A constant
potential of 0.35 V (vs $E_{1/2,TBA}$) was applied against a Pt quasi reference electrode in the aqueous phase, and currents were observed for 1200 sec in both absence and presence of bacteria (Figure 3-S7a and b). To avoid the stochastic collision of swimming bacteria onto a nanopipet tip, bacteria has been immobilized on the poly-Lysine coated glass. With our repetitive measurements, very stable tip currents were obtained without any notable sign of tip fouling.

Also, a standard addition method was conducted to validate the directly estimated carbonate concentration from a raw data of voltammograms (Figure 3-6a). Herein, an additional merit would be expected from the standard addition method due to three reasons. First, for the accuracy in the measurements, a background voltammogram in the blank solution needs to be subtracted from cyclic voltammogram in the analyte containing solution, which enables us to estimate limiting currents accurately. In this case, if a pipet interface is removed from the solution, exposed in the air, and immersed back to the solution, the nanopipet interface cannot be guaranteed to maintain the same condition as before, thus affecting the accuracy and reproducibility in our measurements. Accordingly, all our voltammetric and amperometric measurements (see Figure 3-2, 3-4, and 3-S4) were performed in blank solutions first, and then a known amount of carbonate stock solution was added to the blank to clearly observe the current responses from CO$_3^{2-}$ facilitated IT without exposure of a nanopipet interface to the air. For analyte samples of the bacterial growth media containing CO$_3^{2-}$, the standard addition method allows us to keep pipet electrodes in the solution during the entire measurements by sequentially adding a known amount of CO$_3^{2-}$ spikes, thereby securing the accuracy in this analysis with maintaining the same condition of a nanopipet interface. Second, the multipoint measurement provides
higher precision than one point measurement. The standard addition method enables to in situ construct a linear calibration curve and estimate the original CO$_3^{2-}$ concentration in analyte samples from the x-intercept of a linear calibration curve, thereby promising higher precision in the analysis compared to the direct analysis with one point measurement. Lastly, the matrix effect is considered in this analysis with the standard addition method, especially in the presence of various anion interferents in the growth media, e.g., H$_2$PO$_4^-$.
Cl⁻, and SO₄²⁻ where sequential carbonate spikes show good linear relationship with reasonable current magnitudes in the presence of many different ions in the bacterial growth media. A good linearity with high reproducibility clearly implies high precision in our measurement as well (Figure 3-6c and 3-S7c).

As long as a pipet is immersed in the solution, it can measure CO₃²⁻ facilitated IT reproducibly for 2 days (Figure 3-S7d). We haven’t tried longer usage than 2 days. This is good enough for long term stability required for a probe of scanning electrochemical microscopy. Since each pipet electrode is readily prepared by laser pulling followed by silanization surface modification, our ion selective amperometric nanoprobe is considered as consumable one for one-time usages instead of reuse.

9. MOE Simulation Report (MD Simulation)
Molecular structures of carbonate (II) ion, carbonate ionophore VII, carbonate ionophore IV, water and CHCl₃ were designed, and their conformations with minimum energy were also found using the MOE (MOE2020, Molecular Operation Environment) software under the program of Merc-molecular force field (MMFF). Because MOE doesn’t provide a selection of organic solvent of DCE, CHCl₃ is selected as a solvent for organic phase, which has similar dielectric constant to that of DCE. Geometry stabilization was done using Semi Empirical PM3 method, while thermodynamic energy values were obtained using DFT method. All basis sets and preceding calculations were done with the Slater type and B3LYP 631G functions as stated in Table 3-S5 and 3-S6. Development of the diphase system with two different solvents i.e., water and CHCl₃ was done after minimizing each solvated cube of molecules separately and merging them by enabling the periodic system option. The chosen cell size for the cube of molecules was 40 Å × 80 Å × 40 Å. The
monophase system was equilibrated in a cube of cell size 40 Å × 40 Å × 40 Å for Carbonate ionophore VII system, and a cube of cell size 64.75 Å × 64.75 Å × 64.75 Å for monophase of Carbonate ionophore IV system.

Table 3-S5. Solvation free energies dissolved in CHCl$_3$ estimated by MD simulation within monophase of CHCl$_3$, or diphase of CHCl$_3$/water.

<table>
<thead>
<tr>
<th>Molecules (Carbonate ionophore VII : carbonate ion = 1:1)</th>
<th>Gibb’s free Energy / GJ mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monophase (CHCl$_3$)</strong></td>
<td></td>
</tr>
<tr>
<td>Product 6 = Carbonate ion-ionophore complex dissolved in CHCl$_3$</td>
<td>−14.14</td>
</tr>
<tr>
<td>Product 8 = Carbonate ion-ionophore complex partially hydrated with 10 water molecules dissolved in CHCl$_3$</td>
<td>−13.91</td>
</tr>
<tr>
<td><strong>Diphase (CHCl$_3$ /water)</strong></td>
<td></td>
</tr>
<tr>
<td>Product 3 = Carbonate ionophore partially hydrated with 10 water molecules dissolved in CHCl$_3$</td>
<td>−36.57</td>
</tr>
<tr>
<td>Product 9 = Carbonate ion-ionophore complex partially hydrated with 10 water molecules dissolved in CHCl$_3$</td>
<td>−33.72</td>
</tr>
</tbody>
</table>
Table 3-S6. Solvation free energies dissolved in CHCl₃ estimated by MD simulation within monophase of CHCl₃, or diphase of CHCl₃/water.

<table>
<thead>
<tr>
<th>Molecules (Carbonate ionophore IV : carbonate ion = 3:1)</th>
<th>Gibb’s free Energy / GJ mol⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monophase (CHCl₃)</strong></td>
<td></td>
</tr>
<tr>
<td>Product 6 = Carbonate ion-ionophore complex dissolved in CHCl₃</td>
<td>−1.06</td>
</tr>
<tr>
<td>Product 8 = Carbonate ion-ionophore complex partially hydrated with 10 water molecules dissolved in CHCl₃</td>
<td>−0.73</td>
</tr>
<tr>
<td><strong>Diphase (CHCl₃/water)</strong></td>
<td></td>
</tr>
<tr>
<td>Product 3 = Carbonate ionophore partially hydrated with 10 water molecules dissolved in CHCl₃</td>
<td>−256.76</td>
</tr>
<tr>
<td>Product 9 = Carbonate ion-ionophore complex partially hydrated with 10 water molecules dissolved in CHCl₃</td>
<td>−226.39</td>
</tr>
</tbody>
</table>

9. Selectivity Tests

We conducted selectivity tests with possible interferents, H₂PO₄⁻, Cl⁻, and SO₄²⁻ present in the M-1 bacterial growth media. Accordingly, nanopipet voltammetry was performed with nanopipet electrodes filled with premade ionophore solution containing 5 mM Carbonate ionophore VII. 0.45–3 mM of H₂PO₄⁻, Cl⁻, and SO₄²⁻ was successively added to aqueous buffer solutions. None of anions were shown any appreciate ionic current as a result of facilitated ion transfers (Figure 3-S8).
11. Reversibility Tests

We conducted reversibility test with amperometric CO$_3^{2-}$ nano-ISE. Once a nanopipet sensed 0.45 mM CO$_3^{2-}$ with addition of CO$_3^{2-}$ stock solution (black solid curves in Figure 3-S9), an equivalent volume of a buffer solution to the original solution was added to dilute CO$_3^{2-}$ concentration to 0.22 mM, which caused a decrease in ionic current close to half of the original limiting current (blue solid curves in Figure 3-S9). Amperometric CO$_3^{2-}$ nano-ISEs are not only sensitively responding to the CO$_3^{2-}$ concentration in the aqueous solution, but also retaining the reversibility.
The Nanopipet voltammetry diffusion problem was solved with differential equations in cylindrical coordinates:

\[ i^n (\text{aqueous}) \xrightarrow{k_f} k_o \xrightarrow{i^n \cdot L (1,2 - DCE)} \]

\[ \frac{\partial c_1}{\partial t} = D_1 \left( \frac{\partial^2 c_1}{\partial r^2} + \frac{1}{r} \frac{\partial c_1}{\partial r} + \frac{\partial^2 c_1}{\partial z^2} \right) \]  \hspace{1cm} (s1)

\[ \frac{\partial c_2}{\partial t} = D_2 \left( \frac{\partial^2 c_2}{\partial r^2} + \frac{1}{r} \frac{\partial c_2}{\partial r} + \frac{\partial^2 c_2}{\partial z^2} \right) \]  \hspace{1cm} (s2)

\[ \frac{\partial c_3}{\partial t} = D_3 \left( \frac{\partial^2 c_3}{\partial r^2} + \frac{1}{r} \frac{\partial c_3}{\partial r} + \frac{\partial^2 c_3}{\partial z^2} \right) \]  \hspace{1cm} (s3)

**Figure 3-S9.** Background subtracted voltammograms with nanopipets filled with premade ionophore solution containing 5 mM CO$_3^{2-}$ ionophore VII for reversibility tests. After sensing 0.5 mM of CO$_3^{2-}$, an equivalent volume of the original solution was added to dilute to a half of concentration, resulting in nearly a half of the original limiting current. The background voltammogram is subtracted from another repetitively measured voltammogram of aqueous blank solution. The scan rate was 25 mV/s. Ionophore solutions were vortexed for 15 min.


I. E mechanism
where $t$ is time, $r$ and $z$ are coordinates in parallel and normal directions to the nanopipet interface, respectively. $c_1(r, z, t)$ and $c_2(r, z, t)$ are the concentrations of ions in aqueous and organic phase, respectively. The variables are introduced as follows (see Figure 3-S10):

Radius = pipet inner radius at the tip

RG = pipet outer radius at the tip

RGP = pipet inner radius at 10μm region

RGP2 = pipet outer radius at 10μm region

$D_1 = \text{diffusion coefficient of CO}_3^{2-} \text{ in aqueous phase}$

$D_2 = \text{diffusion coefficient of CO}_3^{2-} \text{ in organic phase}$

Width = simulation space limit in the radial direction

Height = simulation space limit in the vertical direction

$C_1(r, z, \tau) = \text{the concentrations of CO}_3^{2-} \text{ ions in aqueous phase}$

$C_2(r, z, \tau) = \text{the concentrations of CO}_3^{2-} \text{ ions in organic phase}$

$n = \text{charges of CO}_3^{2-} \text{ ion} (= z \text{ in eq s5, s6})$

$E_{\text{ini}} = \text{initial potential in voltammetry}$

$E_{\text{fin}} = \text{switching potential in voltammetry}$

$E^0 = \text{the formal transfer potential of CO}_3^{2-} \text{ ion} (= E^0_{\text{eq s5, s6}})$

$k^0 = \text{standard heterogeneous ion transfer rate constant}$
\( al = \) the transfer coefficient (\( \alpha \))

\( v = \) scan rate (V/s)

\( \tau = \frac{(E_{\text{fin}} - E_{\text{ini}})}{v} \)

\( f = \frac{F}{RT}, \text{ 38.92 V}^{-1} \)

The boundary condition at the liquid/liquid interface is given by

\[
D_1 \left[ \frac{\partial c_1(r,z,t)}{\partial z} \right]_{z=0} = D_2 \left[ \frac{\partial c_2(r,z,t)}{\partial z} \right]_{z=0} = -k_f c_1(r,0,t) + c_2(r,0,t)
\]

(s4)

where \( k_f \) and \( k_b \) are the heterogeneous ion transfer rate constants for forward and reverse transfer, respectively (see eq S1).

**Figure 3-S10.** Schematic view of geometry in finite element simulation with COMSOL Multiphysics.

The rate constants are given by the Butler-Volmer type relation as,
\[ k_f = k^0 \exp \left[ -\frac{\alpha z F (E - E^0)}{RT} \right] \] (s5)

\[ k_b = k^0 \exp \left[ \frac{(1-\alpha) z F (E - E^0)}{RT} \right] \] (s6)

where \( k^0 \) is the standard heterogeneous ion transfer rate constant, \( \alpha \) is the transfer coefficient, \( E \) is the Galvani potential difference between the aqueous and organic phase, and \( E^0 \) is the formal ion transfer potential. In voltammetry, the potential is swept linearly at the rate of \( v \) from the initial potential, \( E_{\text{ini}} \) to final potential, \( E_{\text{fin}} \), and the sweep direction is reversed at \( E_{\text{fin}} \). The potential wave is expressed as,

\[ E = E_{\text{ini}} + \frac{2(E_{\text{fin}} - E_{\text{ini}})}{\pi} \sin^{-1} \left\{ \sin \left[ \frac{\pi v t}{2(E_{\text{fin}} - E_{\text{ini}})} \right] \right\} \] (s7)

The initial conditions in the bulk are given by

\[ c_1(r, z, 0) = 2.1 \, \text{mM} \] (s8)

\[ c_2(r, z, 0) = 0 \, \text{mM} \] (s9)

The initial conditions inside the pipet are given by

\[ c_1(r, z, 0) = 0 \, \text{mM} \] (s10)

\[ c_2(r, z, 0) = 0 \, \text{mM} \] (s11)

The other boundary conditions are defined in the section 9 of COMSOL model report.

Herein, \( D_1 \) was determined by the extrapolated steady state current of a voltammogram with \( \text{CO}_3^{2-} \) ions using the eq 8 (Figure 3-6a), where the concentration of \( \text{CO}_3^{2-} \) is known as 2.1 mM and the radius of nanopipets is experimentally determined by a steady state current in the voltammogram with \( \text{TBA}^+ \) transfer. This value is consistent with
the one determined by the standard addition method (Figure 3-6b). $D_2$ was determined by Stokes-Einstein relation,

\[
D = \frac{k_B T}{6\pi \eta r}
\]

where $k_B$ is Boltzmann constant, $T$ is the temperature, $\eta$ is the viscosity of solvent (1.00 mPa·s for water and 0.83 mP·s for 1,2-DCE) and $r$ is a radius of ionophore molecule.

The kinetic parameters, $k^0$, $\alpha$, and the formal transfer potential of ion (denoted as $E^{0'}$ in COMSOL simulation) were determined via the theoretical simulation. Since $k^0$ determines the overall shape and steepness of voltammograms and $E^{0'}$ controls the position of voltammograms, these two values are determined first. Subsequently, we determined $\alpha$ by deviating it from 0.5 to finely fit the experimental voltammograms. The values of $\alpha = 0.43 \pm 0.02$ showed a good agreement with the experimental voltammograms, which are nearly close to 0.5, indicating the one-step mechanism of the facilitated CO$_3^{2−}$ ion transfer reaction based on the Butler-Volmer kinetics. Also, $\alpha = 0.5$ is predicted based on the ion transfer models either based on the slow diffusion of the transferred species through the mixed interfacial layer$^{1,82}$ or assuming an activation barrier$^{3,84}$.

II. EC mechanism

The Nanopipet voltammetry diffusion problem was solved with differential equations in cylindrical coordinates:

\[
i^n (\text{aqueous}) \xrightleftharpoons[k_{i,f}]{k_{i,b}} i^n (1,2 – DCE)
\]

\[
i^n (1,2 – DCE) + nL(1,2 – DCE) \xrightleftharpoons[k_{a}]{k_{d}} iL^n(1,2 – DCE)
\]
In the presence of the excess amount of ionophore, the diffusion of the free ion in the organic phase is given by,

\[
\frac{\partial c_i}{\partial t} = D_i \left( \frac{\partial^2 c_i}{\partial r^2} + \frac{1}{r} \frac{\partial c_i}{\partial r} + \frac{\partial^2 c_i}{\partial z^2} \right) - k'_a c_i + k_d c_c \tag{s15}
\]

where \( c_i \) and \( c_c \) are local concentration of free ion and its ionophore complex, respectively.

The diffusion of the complex in the organic phase is expressed by,

\[
\frac{\partial c_c}{\partial t} = D_c \left( \frac{\partial^2 c_c}{\partial r^2} + \frac{1}{r} \frac{\partial c_c}{\partial r} + \frac{\partial^2 c_c}{\partial z^2} \right) + k'_a c_i - k_d c_c \tag{s16}
\]

The diffusion of \( \text{CO}_3^{2-} \) ions in the aqueous phase is defined by,

\[
\frac{\partial c_w}{\partial t} = D_w \left( \frac{\partial^2 c_w}{\partial r^2} + \frac{1}{r} \frac{\partial c_w}{\partial r} + \frac{\partial^2 c_w}{\partial z^2} \right) \tag{s17}
\]

where \( c_w \) is the local concentration of transferring ion.

The boundary condition at the liquid/liquid interface is given by

\[
D_l \left[ \frac{\partial c_i(r,z,t)}{\partial z} \right]_{z=0} = D_w \left[ \frac{\partial c_w(r,z,t)}{\partial z} \right]_{z=0} = -k_{i,f} c_w(r,0,t) + k_{i,b} c_i(r,0,t) \tag{s18}
\]

where \( t \) is time, \( r \) and \( z \) are coordinates in parallel and normal directions to the nanopipet interface, respectively. \( c_i(r, z, t) \) and \( c_w(r, z, t) \) are the concentrations of ions in organic and aqueous phase, respectively. Also, \( k_{i,f} \) and \( k_{i,b} \) are the heterogeneous ion transfer rate constants for forward and reverse transfer, respectively (see eq S1).

The rate constants are given by the Butler-Volmer type relation as,

\[
k_{i,f} = k_i^0 \exp \left[ -\frac{\alpha zF(E-E_i^0)}{RT} \right] \tag{s19}
\]
\[ k_{l,b} = k_0^l \exp \left[ \frac{(1-\alpha)ZF(\bar{E}-E^0)}{RT} \right] \]  

(s20)

The variables are introduced as follows (see Figure S6):

Radius = pipet inner radius at the tip

RG = pipet outer radius at the tip

RGP = pipet inner radius at 10μm region

RGP2 = pipet outer radius at 10μm region

\( D_1 = \) diffusion coefficient of CO\(_{3}^{2-}\) in aqueous phase

\( D_2 = \) diffusion coefficient of CO\(_{3}^{2-}\) in organic phase

\( D_3 = \) diffusion coefficient of CO\(_{3}^{2-}\) ion-ionophore complex in organic phase

Width = simulation space limit in the radial direction

Height = simulation space limit in the vertical direction

\( C_1(r, z, \tau) = \) the concentrations of CO\(_{3}^{2-}\) ions in aqueous phase

\( C_2(r, z, \tau) = \) the concentrations of CO\(_{3}^{2-}\) ions in organic phase

\( C_3(r, z, \tau) = \) the concentrations of CO\(_{3}^{2-}\) ion-ionophore complex in org. phase

\( n = \) charges of CO\(_{3}^{2-}\) ion (= z in eq s19, s20)

\( E_{\text{ini}} = \) initial potential in voltammetry

\( E_{\text{fin}} = \) switching potential in voltammetry

\( E^0 = \) the formal transfer potential of CO\(_{3}^{2-}\) ion (= \( E^{0'} \) in eq s19, s20)
$k^0$ = standard heterogeneous ion transfer rate constant

$\alpha l$ = the transfer coefficient ($\alpha$)

$k_a$ = pseudo first order association constant ($s^{-1}$)

$k_d$ = dissociation constant ($s^{-1}$)

$\beta$ = formation constant ($\beta$)

$v$ = scan rate ($V/s$)

$\tau = (E_{\text{fin}} - E_{\text{ini}})/v$

$f = F/RT$, 38.92 $V^{-1}$

where $k^0$ is the standard heterogeneous ion transfer rate constant, $\alpha$ is the transfer coefficient, $E$ is the Galvani potential difference between the aqueous and organic phase, and $E^0$ is the formal ion transfer potential of the simple IT. In voltammetry, the potential is swept linearly at the rate of $v$ from the initial potential, $E_{\text{ini}}$ to final potential, $E_{\text{fin}}$, and the sweep direction is reversed at $E_{\text{fin}}$. The potential wave is expressed as,

$$E = E_{\text{ini}} + \frac{2(E_{\text{fin}}-E_{\text{ini}})}{\pi} \sin^{-1} \left\{ \sin \left[ \frac{\pi vt}{2(E_{\text{fin}}-E_{\text{ini}})} \right] \right\}$$

(s7)

The initial conditions in the bulk are given by

$$c_w(r,z,0) = 2.4 \text{ mM} \quad \text{(s21)}$$

$$c_l(r,z,0) = 0 \text{ mM} \quad \text{(s22)}$$

The initial conditions inside the pipet are given by

$$c_l(r,z,0) = 0 \text{ mM} \quad \text{(s23)}$$
\[ c_c(r, z, 0) = 0 \text{ mM} \quad (s24) \]

The other boundary conditions are defined in the COMSOL model report.


Nam and coworkers reported that 1:1 complexation between carbonate and trifluoroacetobenzoyl (TFAB) group is a mixture of covalent bonding and hydrogen bonding.\textsuperscript{55} With a reported formation constant of the TFAB derivatives and carbonate ion, \( \log \beta_n = 14.4 \pm 0.2, \textsuperscript{56} \) we performed additional numerical simulation for EC mechanism, where a facilitated ion transfer is divided into two steps, \textit{i.e.}, heterogeneous ion transfer (E) and homogeneous ion-ionophore complexation (C).

Specifically, simple IT is defined as below,

\[
\begin{align*}
\text{CO}_3^{2-}(w) & \rightleftharpoons k_f \quad \text{CO}_3^{2-}(\text{org}) \\
\text{CO}_3^{2-}(\text{org}) + L(\text{org}) & \rightleftharpoons k_a \quad \text{CO}_3^{2-} \cdot L(\text{org})
\end{align*}
\]

where \( k_f \) and \( k_b \) are first-order heterogeneous rate constants for forward and reverse transfers, respectively. The rate constants are given by Butler-Volmer relations as,\textsuperscript{89,90}

\[
\begin{align*}
k_{i,f} &= k_i^0 \exp \left[ -\frac{\alpha_x F (E - E_i^0)}{RT} \right] \quad (s26) \\
k_{i,b} &= k_i^0 \exp \left[ \frac{(1-\alpha_x) F (E - E_i^0)}{RT} \right] \quad (s27)
\end{align*}
\]

Ion-ionophore complexation in the organic phase is expressed as

\[
\begin{align*}
\text{CO}_3^{2-}(\text{org}) + L(\text{org}) & \rightleftharpoons k_a \quad \text{CO}_3^{2-} \cdot L(\text{org})
\end{align*}
\]

where \( k_a \) and \( k_d \) are association and dissociation rate constants, respectively. In the presence of the excess amount of ionophore (30 mM), the homogeneous rate constants are related to as below,
\[ \beta_n = (L_T^n k_a)/k_d = (k_a')/k_d \]  
\[(s29)\]

where \( k_a' \) is defined as an pseudo-first order rate constant.

Particularly, the theoretical ion-ionophore association constant, \( k_{a,d} \) based on diffusion limit can be estimated by, S8

\[ k_{(a,d)} = 4\pi N_A (D_L + D_i) d \]  
\[(s30)\]

where \( N_A \) is Avogadro’s number, \( d = 1.5 \times 10^{-7} \text{ cm} \) is the ionophore-ion separation at their collision, \( D_L \) and \( D_i \) \( (= 5 \times 10^{-6} \text{ cm}^2/\text{s}) \) are diffusion coefficients of free ionophore and free ion in DCE solution. For our given system, \( k_{a,d} \approx 10^{10} \text{ M}^{-1}\text{s}^{-1} \) can be estimated.

The thermodynamic parameter, \( \beta_n \) is directly related with electrochemical parameter, \( i.e., \) potentials as below, S11

\[ E_i^{0*} = E_i^{0*} + RT/zF \ln\beta_n L_T^n \]  
\[(s31)\]

where \( E_i^{0*} \) is the formal potential of simple IT. The simulation result based on EC reaction showed a nice fit with the background subtracted voltammogram (Figure 3-S11) with having \( \beta_n = 10^{14} \), an association constant, \( k_a \ (\text{M}^{-1}\text{s}^{-1}) = 3.33 \times 10^{20} \), a dissociation constant, \( k_d \ (\text{s}^{-1}) = 10^5 \), a standard rate constant, \( k_0 = 35 \text{ cm/s} \), and a transfer coefficient, \( \alpha = 0.45 \) (Table
3-S3). Notably, the obtained $k_a$ is extremely large compared to the diffusion limited value of $k_{a,d}$. Also, the estimated $k^0 = 35$ cm/s of simple carbonate ion transfer is unrealistically high compared to simple tetrabutylammonium ion (TBA$^+$) transfer rate, $k^0 = 3.6$ cm/s determined by nanopipet voltammetry. These two facts enable us to exclude the possibility of EC mechanism.

![Figure 3-S11. Schematic view of geometry in finite element simulation with COMSOL Multiphysics.](image)

<table>
<thead>
<tr>
<th>$\beta_n (n)$</th>
<th>$E^0 - E^{0,1}$ / V</th>
<th>$k_0^1$ / cm s$^{-1}$</th>
<th>$\alpha$</th>
<th>$k_a$ / M$^{-1}$s$^{-1}$</th>
<th>$k_d$ / s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{14} (1)$</td>
<td>$-0.375$</td>
<td>35</td>
<td>0.45</td>
<td>$3.33 \times 10^{20}$</td>
<td>$10^5$</td>
</tr>
</tbody>
</table>
References


Mechanistic Assessment of Metabolic Interaction between Single Oral Commensal Cells by Scanning Electrochemical Microscopy

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1. Supplemental Methods

**Chemicals** Tetraethylammonium (TEA\(^+\)) chloride, 1,2-dichloroethane (DCE), N, N-dimethyl—trimethylsilylamine, tetrakis(pentafluorophenyl)borate, tetradodecylammonium bromide and sodium lactate were used as obtained from Sigma Aldrich. The tetrakis(pentafluorophenyl)borate salt of tetradodecylammonium (TDDA\(^+\)-TFAB\(^-\)) was obtained by metathesis and used as organic supporting electrolytes. Deionized nanopure water (18.2 MΩ·cm, TOC 2 ppb; Milli-Q Integral 4-System, Millipore) was used to prepare all the aqueous electrolyte solutions.

**Growth of coculture or monoculture.** Overnight cultures of *C. matruchotii* and (or) *S. mitis* species were grown in Brain Heart Infusion media supplemented with 0.5 % yeast extract (BHI-YE) at 37 °C in a static incubator with 5 % CO\(_2\) or in 5 % H\(_2\), 10 % CO\(_2\) and 85% N\(_2\) for anaerobic conditions. A semi permeable 0.22 µm polycarbonate membrane filter (32) was placed on solid BHI-YE media (supplemented with 1.6% agar). Ten µl of each culture were spotted on the membrane filters and monocultures were spot with 10 µL of BHI-YE. The cultures incubated for 48 h and the membranes were placed in a microcentrifuge tube with 1mL of BHI-YE. The tubes were vortexed to resuspend into media and serially diluted and track plated (33) to count colony forming units per mL (CFU/mL). *S. mitis* was counted by using BHI-YE plates and *C. matruchotii* on BHI-YE plates supplemented with 100 µg/ml fosfomycin.

2. Optical Microscopic Image of Coculture Bacterial Sample.
Diluted bacterial samples with OD 600 at 0.3 were immobilized on a poly-L lysine coated slide glass. The image of this sample was obtained with an optical microscope.
(BX53MTRF, Olympus America Inc. Waltham, MA) and a digital camera (Lumenera 2-1RC CCD camera, Teledyne Lumenera, Ottawa, ON, Canada). In Figure S1, *C. matruchotii* appear with a filamentous feature, while spherical *S. mitis* exist in either scattered or aggregated manner surrounding *C. matruchotii*.

**Figure 4-S1.** An optical microscopic image of coculture bacterial sample with OD 600 of 0.3 immobilized on a poly-L lysine coated slide glass.

3. **SECM Imaging of *S. mitis* Monoculture.**

To image monoculture of *S. mitis* for topography and metabolic activity, the constant-height mode of nanoscale SECM was employed. As shown in Figure 4-S2A, topography of *S. mitis* monoculture was studied with monitoring currents for TEA⁺ IT in the same manner as coculture study shown in Fig. 4-3B. Two separated single *S. mitis* were resolved in this topographical SECM image with scanning a tip at the gap between the tip and *S. mitis*, \( d = 140 \text{ nm} \) (\(~0.35 \text{ ~} d/a\) with a tip radius 420 nm), where a tip current decreased up to \(~50 \text{ pA} \) above the apex of *S. mitis*. This tip current is consistent with the predicted value in simulated SECM approach curves governed by BM permeability (Fig. 4-2B). Subsequently, the same area was imaged to map the local lactate by switching a tip
potential to induce lactate IT, and raster scanned a tip at the gap between the tip and S. mitis, \( d = 250 \) nm (\( \sim 0.60 \) \( d/a \) with a tip radius 420 nm). In Figure 4-S2B and S2C, background currents of \( \sim 30 \) pA was yielded over a glass substrate, which indicates the presence of \( \sim 0.26 \) mM lactate near the glass substrate (eq 1). Notably, no exogenous lactate was added to the bulk solution of SECM cells, implying that the ensemble sample of S. mitis monoculture produced lactate at the similar level to the coculture bacterial sample studied in Fig. 4-3C and 4-3E. Higher tip current over a single S. mitis cell was obtained than the background currents, which visually confirmed \textit{in situ} production of lactate by a single S. mitis. Moreover, the highest tip current, \( \sim 55 \) pA over a S. mitis corresponded to \( \sim 1.83 \) fold enhancement compared to background currents, which agreed well with the theoretically predicted tip currents over a single S. mitis producing lactate at a rate of \( 2.7 (\pm 0.1) \times 10^{-4} \) cm/s in Fig. 4-4B. Resultantly, we found that monoculture S. mitis produces lactate at the system.

**Figure 4-S2.** SECM images of S. mitis monoculture based on (A) TEA\(^+\) IT for topography and (B) lactate IT for lactate mapping. A tip scan rate at 100 nm/100 ms during SECM imaging. (C) chronoamperometric responses based on lactate IT (raw data, cross sections of SECM images in (B)). The current polarity is set to negative for anionic current responses.
4. SECM Imaging of *C. matruchotii* Monoculture.

To image monoculture of *C. matruchotii* for topography and metabolic activity, the constant-height mode of nanoscale SECM was employed. As shown in Figure 4-S3A, topography of *C. matruchotii* monoculture was studied with monitoring currents for TEA\(^+\) IT in the same manner as coculture study shown in Fig. 4-3B. One isolated *C. matruchotii* with filamentous feature was resolved in this topographical SECM image with scanning a tip at the gap between the tip and *C. matruchotii*, \(d = 1.27 \mu\text{m} \approx 2.6 d/a\) with a tip radius 480 nm). Subsequently, the same area was imaged to map the local lactate by switching a tip potential to induce lactate IT, and raster scanned a tip at the gap between the tip and *C. matruchotii*, \(d = 290 \text{ nm} \approx 0.60 d/a\) with a tip radius 480 nm). In Figure 4-S3B, nearly zero background currents were yielded over a glass substrate, implying no lactate near the glass substrate. In fact, this result about no production of lactate by *C. matruchotii* is consistent with previous biochemical studies. Moreover, no current changes appeared over a single *C. matruchotii*, since the tip cannot sense the presence of a bacterial cell without lactate ions. Accordingly, zero currents were obtained all over the scanned area in lactate-mapping.
SECM image. As a result, we confirm that monoculture *C. matruchotii* does not produce lactate as a metabolite.

5. Fine Element Simulation by COMSOL Multiphysics

5.1 Finite Element Simulation for BM permeability

For the BM permeability determination, the two-dimensional SECM diffusion problem was solved using COMSOL Multiphysics finite element package (version 5.3a, COMSOL, Burlington, MA). In this simulation, the finite element simulation employed absolute parameters. When probe ions, X is partitioned across the BM between the outer buffer solution and the bacteria inside,

\[
X(\text{outer solution}) \xrightleftharpoons[k_1]{k_2} X(\text{inside bacterial cell})
\]  

where the permeability of \( k_1 \) and \( k_2 \) for passive influx and efflux of X, respectively, corresponds to the equilibrium concentration of X in the bulk outer solution and the bacterial cell. The equilibrium concentrations of small probe ions in the outer solution and in the bacteria, \( c_{10} \) and \( c_{20} \), respectively are related to the partitioning equilibrium constant, \( K_e \), and assumed to be \( \sim 0.9 \) for monovalent ion, as shown in eq 3.

The SECM problem was defined in cylindrical coordinates, where \( r \) and \( z \) are the coordinates in directions parallel and normal to the interface, respectively. The diffusion of ions in the outer solution of the BM can be expressed as

\[
\frac{\partial c_1(r,z,t)}{\partial t} = D_1 \left( \frac{\partial^2 c_1(r,z,t)}{\partial r^2} + \frac{1}{r} \frac{\partial c_1(r,z,t)}{\partial r} + \frac{\partial^2 c_1(r,z,t)}{\partial z^2} \right)
\]  

(s2)
where \( t \) is time, \( c_1(r, z, t) \) and \( D_1 \) are the local concentration and the diffusion coefficient of X ion in the outer solution, respectively. Also, diffusion of X ion in the bacteria can be expressed as

\[
\frac{\partial c_2(r,z,t)}{\partial t} = D_2 \left( \frac{\partial^2 c_2(r,z,t)}{\partial r^2} + \frac{1}{r} \frac{\partial c_2(r,z,t)}{\partial r} + \frac{\partial^2 c_2(r,z,t)}{\partial z^2} \right) \tag{s3}
\]

When the BM transport is kinetically limited, the boundary conditions at the BM are given for the outer solution side and the bacteria inside of the BM as

\[
D_1 \left[ \frac{\partial c_1(r,z,t)}{\partial z} \right]_{z=0} = D_2 \left[ \frac{\partial c_2(r,z,t)}{\partial z} \right]_{z=0} = -k_1 c_1(r,0,t) + k_2 c_2(r,0,t) \tag{s4}
\]
where \( z = 0 \) is the BM surface. When the BM membrane is freely permeable, partitioning of X ion at the BM is always in local equilibrium, thereby the boundary condition is equivalent to

\[
K_0 = \frac{c_2(r, 0, t)}{c_1(r, 0, t)} \tag{s5}
\]

The variables are introduced as follows (see Figure 4- S4.):

- **Height** = simulation space limit in the vertical direction
- **Radius** = pipet inner radius at the tip
- **RG** = pipet outer radius at the tip
- **Width** = simulation space limit in the radial direction
- **Length** = pipet length in the vertical direction
- \( a \) = longer radius of bacterial cell
- \( b \) = shorter radius of bacterial cell
- \( d \) = distance between pipet tip interface and bacterial membrane
- \( C1(r, z, t) \) = concentration of X ions in aqueous outer solution phase
- \( C2(r, z, t) \) = concentration of X ions in bacterial cell
- \( C1ini \) = initial concentration of X ions in aqueous outer solution phase
- \( C2ini \) = initial concentration of X ions in bacterial cell
- \( n \) = charge
- \( F \) = Faraday constant, 96485 [C/mol]
\[ D1 = \text{diffusion coefficient of X ion in aqueous phase} \]

\[ Iss = \text{limiting current} \]

\[ K = \text{permeability of bacterial membrane (} = k_1 \text{ in eq S1)} \]

\[ Ke = \text{the partitioning equilibrium constant, 0.9} \]

\[ Gamma = \text{Diffusion coefficient ratio of X ions between aqueous phase and bacterial cell, 0.94} \]

The initial condition in the aqueous bulk solution is given by

\[ C1 (r, z, 0) = 1.0 \text{ mM} \quad (s6) \]

The initial condition in bacterial cell is given by

\[ C2 (r, z, 0) = C1 (r, z, 0) \times Ke \quad (s7) \]

Since ion transfers at the pipet interface occur under diffusion limited condition, the boundary condition at the pipet interface is given as constant concentration,

\[ C1 (r, d, t) = 0 \text{ mM} \quad (s8) \]

The other boundary conditions are defined in the COMSOL model report.

### 5.2 Finite Element Simulation for Lactate production by \( S. mitis \)

For the determination of lactate concentration and lactate production rate at \( S. mitis \), the two-dimensional SECM diffusion problem was solved using COMSOL Multiphysics finite element method. In this simulation, the finite element simulation employed absolute parameters. When lactate is produced by \( S. mitis \) and released through the BM of \( S. mitis \) to aqueous outer solution,
\[ \text{L (inside bacterial cell)} \xrightarrow{k_2} \text{L (aq, outer solution)} \quad \text{(s9)} \]

where \( k_2 \) and \( k_1 \) are the first-order heterogeneous rate constants for passive efflux and influx of lactate, L, respectively. \( k_2 \) is considered as lactate production rate as lactate is produced by \( S. \text{mitis} \) and diffuses out to aqueous outer solution. Also, \( k_1 \) can be considered as the same BM permeability determined from the section 5.1.

The SECM problem was defined in cylindrical coordinates, where \( r \) and \( z \) are the coordinates in directions parallel and normal to the interface, respectively. The diffusion of lactate ion in the bacteria can be expressed as

\[ \frac{\partial c_2(r,z,t)}{\partial t} = D_2 \left( \frac{\partial^2 c_2(r,z,t)}{\partial r^2} + \frac{1}{r} \frac{\partial c_2(r,z,t)}{\partial r} + \frac{\partial^2 c_2(r,z,t)}{\partial z^2} \right) \quad \text{(s10)} \]

Also, the diffusion of lactate in the outer solution of the BM can be expressed as

\[ \frac{\partial c_1(r,z,t)}{\partial t} = D_1 \left( \frac{\partial^2 c_1(r,z,t)}{\partial r^2} + \frac{1}{r} \frac{\partial c_1(r,z,t)}{\partial r} + \frac{\partial^2 c_1(r,z,t)}{\partial z^2} \right) \quad \text{(s11)} \]

where \( t \) is time, \( c_1(r, z, t) \) and \( D_1 \) are the local concentration and the diffusion concentration of lactate ion in the outer solution, respectively.

When the BM transport of lactate is kinetically limited, the boundary conditions at the BM are given for the outer solution side and the bacteria inside of the BM as

\[ D_1 \left[ \frac{\partial c_1(r,z,t)}{\partial z} \right]_{z=0} = D_2 \left[ \frac{\partial c_2(r,z,t)}{\partial z} \right]_{z=0} = k_1 c_1(r,0,t) - k_2 c_2(r,0,t) \quad \text{(s12)} \]
were \( z = 0 \) is the BM surface of \( S. \textit{mitis} \). When the BM membrane is freely permeable, partitioning of lactate ion at the BM is always in local equilibrium, thereby the boundary condition is equivalent to the same \( K_e \) in eq s5.

**Figure 4-S5.** Cross section of the SECM geometry. Each boundary condition is defined in the simulation space. The boundary condition at bacterial cell membrane is given by eq s12. The boundary condition at the pipet tip is diffusion limited ITs of \( \text{Lactate}^- \). There is no normal flux across the axis symmetry, impermeable glass pipet wall and the surface of glass substrate.

The variables are introduced as follows (see Figure 4-S5.):

- **Height** = simulation space limit in the vertical direction
- **Radius** = pipet inner radius at the tip
- **RG** = pipet outer radius at the tip
- **Width** = simulation space limit in the radial direction
- **Length** = pipet length in the vertical direction
- \( a \) = radius of bacterial cell
\( b \) = radius of bacterial cell

\( d \) = distance between pipet tip interface and bacterial membrane

\( C_1(r, z, t) \) = concentration of lactate in aqueous outer solution phase

\( C_2(r, z, t) \) = concentration of lactate in bacterial cell

\( C_{1ini} \) = initial concentration of lactate in aqueous outer solution phase

\( C_{2ini} \) = initial concentration of lactate in bacterial cell

\( n \) = charge

\( F \) = Faraday constant, 96485 [C/mol]

\( D_1 \) = diffusion coefficient of lactate in aqueous phase

\( I_{ss} \) = limiting current

\( K \) = permeability of bacterial membrane (= \( k_1 \) in eq S1)

\( K_e \) = the partitioning equilibrium constant, 0.9

\( \Gamma \) = Diffusion coefficient ratio of lactate between aqueous phase and bacterial cell, 0.94

The initial conditions in the aqueous bulk solution are given by.

\[ C_1(r,z,0) = 0.26 \text{ mM} \] (s13)

The initial condition in bacterial cell is given by

\[ C_2(r,z,0) = 1.3 \text{ mM} \] (s14)
Since ion transfers at the pipet interface occur under diffusion limited condition, the boundary condition at the pipet interface is given as constant concentration,

\[ C_1(r,d,t) = 0 \text{ mM} \quad (s15) \]

The other boundary conditions are defined in the COMSOL model report.

### 5.3 Finite Element Simulation for Lactate consumption by *C. matruchotii*

For the determination of lactate concentration and lactate consumption rate at *C. matruchotii*, the two-dimensional SECM diffusion problem was solved using COMSOL Multiphysics finite element method. In this simulation, the finite element simulation employed absolute parameters. When lactate produced by *S. mitis* diffuses near *C. matruchotii*, and readily undergoes oxidation by *C. matruchotii*,

\[ L_w(\text{aq, outer solution}) \xrightarrow{k_f} L_c \text{ (inside bacterial cell)} \xrightarrow{k_{c,f}} P \text{ (inside bacterial cell)} \quad (s16) \]

\( k_f \) and \( k_2 \) are the first-order heterogeneous rate constants of forward and backward reactions, respectively in eq s16, where \( k_f \) is the same value as BM permeability determined in section 5.1. Further, \( k_{c,f} \) and \( k_{c,b} \) are the first-order homogeneous rate constants of forward and backward reactions in eq s16, *i.e.*, the oxidation of permeated lactate by *C. matruchotii*. Assuming a constant concentration of lactate dehydrogenase in *C. matruchotii*, pseudo first-order homogeneous reaction is considered for lactate oxidation in eq s16.

The diffusion of lactate in the outer solution of the BM of *C. matruchotii* can be expressed as

\[
\frac{\partial c_z(r,z,t)}{\partial t} = D_1 \left( \frac{\partial^2 c_z(r,z,t)}{\partial r^2} + \frac{1}{r} \frac{\partial c_z(r,z,t)}{\partial r} + \frac{\partial^2 c_z(r,z,t)}{\partial z^2} \right) \quad (s17)
\]
where $t$ is time, $c_1(r, z, t)$ and $D_1$ are the local concentration and the diffusion coefficient of lactate in the outer solution, respectively. Also, diffusion of lactate in the bacteria can be expressed as

$$\frac{\partial c_2(r,z,t)}{\partial t} = D_2 \left( \frac{\partial^2 c_2(r,z,t)}{\partial r^2} + \frac{1}{r} \frac{\partial c_2(r,z,t)}{\partial r} + \frac{\partial^2 c_2(r,z,t)}{\partial z^2} \right) - k_{c,f} C_2 + k_{c,b} C_3 \quad (s18)$$

where $c_2$ and $c_3$ are local concentration of lactate and oxidized product of lactate (P in eq s16) in the bacterial cell, respectively.

The diffusion of the oxidized product of lactate in the bacterial cell is expressed by,

$$\frac{\partial c_3(r,z,t)}{\partial t} = D_3 \left( \frac{\partial^2 c_3(r,z,t)}{\partial r^2} + \frac{1}{r} \frac{\partial c_3(r,z,t)}{\partial r} + \frac{\partial^2 c_3(r,z,t)}{\partial z^2} \right) + k_{c,f} C_2 - k_{c,b} C_3 \quad (s19)$$

The variables are introduced as follows (see Figure 4-S4):

- **Height** = simulation space limit in the vertical direction
- **Radius** = pipet inner radius at the tip
- **RG** = pipet outer radius at the tip
- **Width** = simulation space limit in the radial direction
- **Length** = pipet length in the vertical direction
- $a$ = longer radius of bacterial cell
- $b$ = shorter radius of bacterial cell
- $d$ = distance between pipet tip interface and bacterial membrane

$C1(r, z, t)$ = concentration of lactate in aqueous outer solution phase

$C2(r, z, t)$ = concentration of lactate in bacterial cell
$C_3(r, z, t) =$ concentration of oxidized product of lactate in bacterial cell

$C_{1ini} =$ initial concentration of lactate in aqueous outer solution phase

$C_{2ini} =$ initial concentration of lactate in bacterial cell

$C_{3ini} =$ initial concentration of oxidized product of lactate in bacterial cell

$n =$ charge

$F =$ Faraday constant, 96485 [C/mol]

$D_1 =$ diffusion coefficient of lactate in aqueous phase

$I_{ss} =$ limiting current

$k_{cf} =$ forward rate constant of homogeneous reaction of lactate oxidation

$k_{cb} =$ backward rate constant of homogeneous reaction of lactate oxidation

$K_{eq} =$ equilibrium constant ($k_{cf} / k_{cb}$)

$K =$ permeability of bacterial membrane ($= k_1$ in eq S1)

$K_e =$ the partitioning equilibrium constant, 0.9

$\Gamma =$ Diffusion coefficient ratio of X ions between aqueous phase and bacterial cell, 0.94

The initial conditions in the aqueous bulk solution are given by

$C_1(r, z, 0) = 0.26$ mM \hspace{1cm} (s20)

The initial condition in bacterial cell is given by

$C_2(r, z, 0) = 0.0$ mM \hspace{1cm} (s21)
\[ C_3(r, z, \theta) = 0.0 \text{ mM} \quad (s22) \]

The other boundary conditions are defined in the COMSOL model

**References**

APPENDIX 4: CHAPTER 5-SUPPORTING INFORMATION

*Corynebacterium matruchotii* fitness enhancement of adjacent streptococci by multiple mechanisms

Authors: ¹Eric Almeida*, ²Surendra Puri*, ²Subhashini Elangovan, ²Jiyeon Kim, ¹Matthew Ramsey**

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Supplemental Methods

Growth curves. Five milliliter cultures of *C. matruchotii* were grown in BHI-YE for 48hr and back diluted in modified RPMI media supplemented with 40mM lactate at an OD\text{600} of 0.025. Cultures were incubated statically at 37°C with 5% CO\text{2}. Optical density readings were taken every 4 h over a 72 h period.

Note. Additional SECM methods including tip design, development and acquisition parameters are part of a companion manuscript.³ Methods below highlight the most relevant aspects of SECM utilized to quantify coculture metabolite exchange of lactate between *S. mitis* and *C. matruchotii*.

Scanning electrochemical microscopy sample preparation. Bacteria were grown overnight in BHI-YE and then washed by centrifugation in defined medium. The defined medium used was an amended version of Teknova EZ RICH (Teknova, M2105). We prepared the medium as described in the manufacturers instructions with the addition of vitamin solution, lipoic acid, folic acid, riboflavin, NAD\textsuperscript{+} and nucleotides to final concentrations from that of an oral complete defined medium previously described \(^1\) and glucose at 10 mM. Bacteria were grown to an OD\text{600} of 0.3-0.5 and then diluted to final concentrations of 1.2x10\textsuperscript{7} and 6.0x10\textsuperscript{6} CFU of *C. matruchotii* and *S. mitis* respectively in 200 μL of defined medium. This was then incubated at 37 °C with 5 % CO\text{2} for 1 h. 10 μL of this solution was then added to a poly-lysine coated glass slide and incubated at 37 °C for 15 minutes after which medium was removed by micropipette to remove planktonic cells and ensure only attached cells remained. An additional 10 μL of pre-warmed defined
medium was then added to the slide. Samples were then transferred to the SECM instrument for further analysis.

**Solution preparation for SECM measurements**

Tetraethylammonium chloride (TEA⁺Cl⁻, Sigma Aldrich) solution of 1mM concentration was made in freshly prepared oral bacterial growth media of ~pH 7.5, where TEA⁺ was used as a probe ion. The solution was then filtered with a syringe filter (0.1 μm filter unit, SLVV033RS, Duropore PVDF membrane, MILLEX VV). The SECM cell made with Teflon and glass was cleaned in piranha solution, and thoroughly rinsed with nanopure water (18.2 MΩ·cm, TOC 2 ppb; Milli-Q Integral 4-System, Millipore).

**NanoSECM measurements**

Here, we used our home-built nanoSECM for measuring approach curves and imaging at single bacterial cells. Before approaching a submicrosized pipet tip to the bacteria, we measured cyclic voltammograms with tetraethylammonium (TEA⁺, Sigma Aldrich) ion and estimate a steady state current in bulk solution ($i_{T,∞}$) to confirm that nanopipet is working with a reasonable size. Then, our pipet was held c.a. 100 – 200 μm above the glass substrate with the help of a video microscope (Caltex VZM 400 lens with INFINITY2-1R 1.4 Megapixel USB 2.0 Microscopy Camera CCD) and a lockable micropositioner (DM-25L, Newport). We approached our pipet tip to the bacterial sample with z-piezo by measuring the steady-state current as a function of the distance between a pipet tip and glass substrate, and recorded an SECM approach curve. When the z-piezo moved 50 μm (maximum travel distance for piezo, P-620.ZCL, PI (Physik Instrumente), German) down at 50 nm/s, it was completely withdrawn and manually approached 50 μm with a lockable micropositioner. Then, the pipet tip was approached with z-piezo until it showed a sharp
decrease in current, \textit{i.e.}, feedback current response. Once we observed a foot of feedback current response, the pipet tip was withdrawn 6 μm above, and approached at a slower rate \textit{i.e.}, 10 nm/s (1 nm step size with 100 ms incremental time) until our steady-state current decreased to c.a. 93 – 90 % of the $i_{T,\infty}$, which corresponds to a distance of c.a. 2 times of tip radius from the substrate. Subsequently, a tip was further withdrawn 2 μm considering a height of bacteria. Here we approached only 93 % of $i_{T,\infty}$ to prevent the crash with possible clumps of bacteria in coculture. Here, SECM imaging was done at the constant height mode. A pipet tip was raster scanned along $x$- and $y$-axis at 1 μm/s (\textit{i.e.}, 100 nm/100 ms) in the region of 25 μm × 25 μm to get a topographical image of the bacteria. After topographical imaging of the bacteria with respect to TEA$^+$, a pipet tip was brought to the original starting point. We again withdrew $z$-piezo 6 μm above, approached again at 10 nm/s (\textit{i.e.}, 1 nm/100 ms) until we reached c.a. 90 % of $i_{T,\infty}$, and withdrew 2 μm just right before imaging similar to previous topographic imaging step. A pipet tip potential is switched to a potential to sense lactate ion transfer (0.46 V more positive than diffusion-limiting potential of TEA$^+$ ion transfer). Continuously, a tip was raster scanned in the same region of 25 μm × 25 μm to real time monitor lactate production/consumption by bacteria in situ.

**Electrochemical detection of TEA$^+$ and Lactate ion transfer by a submicropipet-supported interface between two immiscible electrolyte solutions (ITIES)**

To investigate lactate production and consumption by bacteria as well as the topography of bacterial cells, a submicropipet-supported ITIES was employed. With this submicrotip, an etched Ni/Cu electrode in the internal organic electrolyte exerts a bias across the submicroscale liquid/liquid interface against an electrode in the aqueous solution (Fig. 5-S3A and B) to yield the amperometric tip current based on the selective interfacial transfer
of a small probe ion (2). A quartz submicropipet is filled with the electrolyte solution of 1,2-dichloroethane (DCE) to detect tetraethylammonium (TEA\textsuperscript{+}) as a probe ion, and lactate as a metabolite ion produced or consumed by bacteria. Using this pipet electrode, TEA\textsuperscript{+} ion transfer (IT) is induced by applying negative potential, which can probe topography of bacterial sample, while lactate is sensed by switching a potential to positive to in situ monitor the chemical interaction between bacteria. The coculture of \textit{C. matruchotii} and \textit{S. mitis} is immobilized over a poly L-lysine coated slide glass plate, and studied by scanning or approaching a 800 nm-diameter pipet tip over the bacteria (Fig. 5-S3C and D). More detailed information about the electrochemical sensing of each ion species at the selective interfacial potentials should be found in the companion manuscript.\textsuperscript{3} Notably, a submicrometer-sized pipet tip enables the real time study of chemical interactions between bacteria as well as their topography at a single cell level.

Supplemental Results

Quantitative analysis of the permeability of bacterial membranes, and local lactate concentration produced by \textit{S. mitis} and consumed by \textit{C. matruchotii}

After obtaining SECM images, SECM approach curves were measured directly above \textit{S. mitis} and \textit{C. matruchotii}. The tip approached from the bulk solution while recording the steady-state tip current response for TEA\textsuperscript{+} IT until contacting the surface of bacterial cell. This resulted in decrease in current as a function of normalized distance to the tip radius \((d/a)\) with a sudden spike as a sign of contact between the tip and bacterial membrane (black and grey solid curves in Fig. 5-S3E). The intrinsic permeability of both \textit{S. mitis} and \textit{C. matruchotii} membrane to TEA\textsuperscript{+} \((k = 2.4 \pm 0.1) \times 10^{-4} \text{ cm/s}\) was determined by fitting experimental approach curves with a finite element simulation of a two phase SECM
problem as described in detail elsewhere (black open circles in Fig. 5-S3E)\textsuperscript{(2,3)}. TEA\textsuperscript{+} was used in lieu of lactate for determining permeability for two reasons \textit{i.e.}, the same charge amount (± 1) and similar diffusion coefficients of \( \sim 6 \times 10^{-6} \text{ cm}^2/\text{s} \). The approach curves over bacteria are nearly identical to the negative feedback approach curves obtained over an insulating glass substrate (black and grey solid curves in Fig. 5-S3E), indicating that both bacterial membranes are almost impermeable to TEA\textsuperscript{+}.

With a determined permeability, \( k \) of \textit{C. matruchotii} and \textit{S. mitis} membranes, an SECM approach curve over \textit{S. mitis} was further simulated using a two phase SECM problem as well, where tip current responses for lactate IT become higher over \textit{S. mitis} due to \textit{in situ} generation of lactate. Herein, enhanced tip currents to 167 % of \( i_{T,\infty} \) for lactate IT obtained at 1.20 d/a during SECM imaging (in Fig. 5-5C) are used as a criterion to verify the theoretically simulated approach curve. The resulting approach curve is depicted in Fig. S3E, where tip currents increase as the tip approaches over \textit{S. mitis}, and reach 1.66 fold of \( i_{T,\infty} \) at 1.2 d/a (red open circles in Fig. 5-S3E). The corresponding concentration profile of lactate near \textit{S. mitis} and a pipet tip is shown in Fig. 5-S3F, where the concentration of lactate produced by \textit{S. mitis} is estimated as 0.50 mM very near the bacterial surface. This localized concentration of lactate is two times higher than that in bulk solutions resulting from the diffusion of lactate produced by other ensemble of \textit{S. mitis} cells.

Notably, in approach curves over \textit{C. matruchotii} (blue open circles in Fig. 5-S3E), 48 % of \( i_{T,\infty} \) at 1.20 d/a is consistent with tip current responses over \textit{C. matruchotii} during constant-height SECM imaging based on lactate IT (\sim 48 % current responses of \( i_{T,\infty} \) over \textit{C. matruchotii} in Fig. 5-5C), which is much smaller than 85 % current responses of \( i_{T,\infty} \) based on simple-topographical response (black open circles in Fig. 5-S3E). This result
indicates that *C. matruchotii* efficiently depletes localized lactate produced by *S. mitis* in its proximity, thus tip current responses above *C. matruchotii* are not only affected by its intrinsic membrane permeability but also controlled by its characteristic consumption rate, \( \geq 5 \times 10^6 \text{ s}^{-1} \). Accordingly, tip current response based on lactate IT is lowered as the tip moves laterally toward *C. matruchotii*. More detailed information about the theoretical simulation and the quantitative analysis should be found in the companion manuscript.3

In summary, this work demonstrates the high significance and power of SECM, which enabled us not only to *in situ* monitor the chemical communication between two commensal bacteria, *S. mitis* and *C. matruchotii* by imaging, but also to quantify the lactate production by *S. mitis* and consumption by *C. matruchotii* in real time at submicron resolution.

**Figure 5-S1.** *S. mitis (Sm)* monoculture is unaffected by the addition of 50mM MOPS to BHI-YE. *S. mitis* had a starting 1.4×10^5. Data are mean CFU counts with error bars indicating standard deviation for \( n \geq 3 \).
Figure 5-S2. Growth curve of *C. matruchotii* and *C. matruchotii ΔlutA* grown on defined media containing lactate as the sole carbon source. Data are OD$_{600}$ readings for $n = 1$. 
Figure 5-S3. (A) Illustrated scheme of a submicropipet-supported ITIES to directly probe TEA⁺ or lactate IT, (B) Cyclic voltammograms of TEA⁺ and lactate ITs in buffer solution containing 1 mM TEA⁺ and 2 mM lactate (background subtracted, scan rate = 25 mV/s), (C) Schematic of the SECM setup with living coculture bacterial samples immobilized on poly-L lysine coated slide glass, (D) Constant-height SECM imaging (black arrow) or SECM approach curve (red arrow) by a submicropipet tip scanned or vertically approached over S. mitis and C. matruchotii coculture, respectively. (E) SECM approach curves based on TEA⁺ IT over C. matruchotii (black solid line), over S. mitis (grey solid line) and a theoretically simulated curve (black open circles). Experimental curves (black and grey solid lines) are compared with theoretically simulated curves (black open circles) with permeability, $k = 2.4 \pm 0.1 \times 10^{-4}$ cm/s. Simulated SECM approach curves based on lactate IT over S. mitis with considering determined $k$ (red open circles) and over C. matruchotii with lactate consumption rate $\geq 5 \times 10^6$ s⁻¹. (F) Concentration profile of lactate
ions near *S. mitis*, which produces 0.50 mM lactate (Figure 4-S3A, B, E and F are adapted from ref. 5-3).

Table 5-S1. Lists of plasmids and strains used in study.

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Table 5-S2. *C. matruchotii* differentially expressed genes when in coculture with *S. mitis* aerobically.

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Table 5-S3. *S. mitis* differentially expressed genes when in coculture with *C. matruchotii* aerobically.

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