論 文 の 内 容 の 要 旨

論文題目　**Fermentation-associated extracellular electron transport mechanism in human pathogens**

(ヒト病原細菌による発酵代謝と共役した細胞外電子移動機構の研究)

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Microbial communities have a critical impact on human health by causing various diseases with major energy mechanism of fermentation. The microbially generated fermentative metabolites such as hydrogen hamper the re-oxidation of pyridine nucleotides (NADH), hence individual cells encounter the challenge of sustaining the redox homeostasis to maximize energy production. Hydrogen consumption by niche-specific symbiotic bacteria has been considered as an alternative for balancing the intracellular energy, rather than the capability of individual bacteria. While, in environmental microbes, electron transfer is used for the regeneration of NAD^+ by a phenomenon called extracellular electron transfer (EET) through redox enzymes, where metabolically generated electron are transferred to an external electron acceptor. However, the ability of electron transfer to balance intracellular potential has not been widely investigated in fermentative bacteria as bacterial

fermentation doesn't require electron acceptor. In this study, we hypothesized that exporting reductive energy via electron transfer can be energetically conservative for the fermentative bacteria to sustain the redox balance. However, the capability of EET in human environmental oral pathogens and the physiological significance of such electron export in biofilm was not explored before.

We tested our hypothesis in an enormously studied fermentative pathogen of the oral environment *Streptococcus mutans* (*S. mutans*), with the application of bioelectrochemical and biochemical methods. The capability of EET by *S. mutans* and its associated mechanism along with its physiological role of EET in redox homeostasis was identified. Known, *S. mutans* has low-pH tolerance associated virulence properties and survives in acidic redox conditions by causing chronic periodontic diseases, yet its survival mechanism is ambiguous. Thus, we specifically examined the pH dependence on the electron transfer capability which could give insights into the mechanism of this acid-tolerant pathogen.

Understanding the implication of extracellular electron transport in oral biofilms is critical, where, exists diverse oxygen gradients creating anaerobic, anoxic and aerobic interfaces in which bacteria are spanned over a range of more than 200-micrometer length on the tooth surface. The oral biofilms were found to be arranged in an architectured manner, where anaerobes exits in deep anaerobic spaces, facultative anaerobes in anoxic and aero tolerants near the surface where plenty of oxygen available . Biofilms extracted from a patient having a bisphosphonate-related osteonecrosis of the jaw, were found to have electrically conductive nanowires connecting the bacteria. The existence of oxygen gradients and electric conductive nature of biofilm suggesting the possibility of long-range extracellular electron transport in oral biofilms, where anaerobes in the depth of biofilm can transfer electrons to the aerobic space to utilize oxygen as an electron acceptor. To this end, we tested the generality of EET capability on other predominant oral pathogens, such as *capnocytophaga ochracea, Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* to understand the ecophysiological role and associated energetics of EET in human pathogenic biofilms. such EET capability was used as a tool to scrutinize and study the pathogens of other human environments, for example, the human gut by integrating the electrochemical analysis. Furthermore, by using the electrochemical systems EET capable bacteria were enriched and isolated from a microbial community from human gut samples.

The structure of the present thesis is as follows.

In Chapter 1, General introduction on the history and importance of bacterial electron transfer processes are introduced. Types of metabolisms such as fermentation, respiration and the recent discovery of metabolism associated with the unique anaerobic respiration phenomenon called extracellular electron transfer (EET) were introduced. The mechanisms for direct and indirect extracellular electron mechanisms identified in model EET capable bacteria are briefly discussed followed by the importance of understanding the microorganism's electron transfer processes which is a focal point to identify the key mechanisms helping these microbe's survival in nature especially in human environments. Because bacteria co-exist with human environments in polymicrobial communities, the possibility of EET in human environmental oral biofilm pathogens and the physiological significance of such electron export in biofilm was introduced. Predominant oral pathogens of oral plaque and their cultivation methods, the description of the experimental details that were commonly used in this thesis for the oral pathogens such as single potential amperometry, differential pulse (DP) and Cyclic voltammograms (CV) combined with metal enzyme reactive chemical staining and electron microscopy techniques like scanning electron and transmission electron microscope analyses were given.

In Chapter 2, General description of a human oral Gram-positive model fermentative pathogen, *Streptococcus mutans*, and demonstration of our discovery of *S. mutans* shift in fermentation pattern via a mechanism dependent on both a very low level of electron flux via EET and a functional redox regulator (Rex), found in many Gram-positive bacteria. Single-cell mass spectrometry analysis for cell-specific stable isotope fixation was conducted to analyze the anabolic activity of cells on the electrode signifying the physiological importance of low EET in fermentative EET capable pathogen. The generality of Rex-EET coupling via amino acid alignment of the Rossman-like folding motif in the NAD-binding site of Rex among pathogens with EET capability. Support of EET in sensing the presence of other cells that affect redox environments in addition to chemical sensing mechanism and a greater prevalence of highly sensitive capabilities for redox compounds in pathogens that reside in reductive environments such as oral plaque was discussed.

In Chapter 3, The impact of well-known acid tolerance capability of *S. mutans* was analyzed on its EET capability on an electrode surface. Bioelectrochemical analyses were performed to test the pH

dependency on EET capability and checked the presence of the cell-surface redox enzymes by preculturing at acidic and neutral pHs. The significantly higher current production and presence of cell-surface redox enzymes in low-pH grown cells condition in comparison to neutral pH was demonstrated, which is crucial for the direct electron transfer mechanism, indicates that acid stress has an impact on the membrane topology. Further, a single-cell metabolic activity assay and the addition of antibiotics was performed to understand the impact of EET in cellular activity.

In Chapter 4, Extracellular electron transport- mechanism and energetics in oral pathogens were analyzed and the possibility of long-range electron transport in oral biofilm was discussed.
Brief introduction followed by the EET capability of the predominantly studied oral pathogens such as *Capnocytophaga ochracea*, *Aggregatibacter*, *Porphyromonas gingivalis* along the *Streptococcus mutans* was experimentally analyzed. Based on the electrogenic activity analyses, *Capnocytophaga ochracea* displayed a cell density induced EET capability via the secretion of outer membrane vesicles (OMVs), concentrated MVs isolated from large C. ochracea culture were added low cell density electrochemical system to examine the impact on the current generation, a large increase i.e., approximately five times higher current than the original was observed elucidating the collaboration of MVs with cells at electrode surface for enhanced electron transfer rate. Whereas, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* showed current production with the electrode. DPV and CV based kinetic analysis supported by transmission electron microscopy of the cells stained for transition-metal suggest a potential EET mechanism associated with the presence of redox-enzymes on the cell membrane. Tested the cell wall and metabolic inhibitors which significantly reduced the current production, demonstrating that current production reflects the cellular activity in these pathogens. Based on the energy levels i.e. redox potential of cell surface enzymes and redox molecules involved in their electron export observed in the differential pulse voltammograms of oral plaque pathogens, the energy level was determined. Oral microbes have displayed EET pathways associated with fermentation metabolism via direct/mediated electron transport mechanism, whose redox potentials are found to be by their natural habitat arrangement in the oral environment suggest the possibility of electron transport from anaerobic space to aerobic environment or can transfer electrons to surrounding microbes in the niche.

In chapter 5, The EET capability of two human pathogens habitat to the gut environment by using

in-vivo electrochemistry was tested and presented. Enterococcus avium and Klebsiella pneumoniae are two human gut pathogens that were electrochemically enriched from the mixed human gut samples with electron donors acetate and lactate respectively. The EET capability can be a characteristic of pathogens that can help the microbes redox balance and survival in polymicrobial biofilms was discussed. This understanding of the underlying electron transfer mechanism of these pathogens would pave new pathways for the development of disease control treatments.

In chapter 6, the general conclusion of the work and the future prospects are given.

Our study has identified the novel energy conservative mechanism of fermentation associated extracellular electron transport (EET) in the human pathogens. This study describes the critical impact of small electron export on the metabolic activity of these pathogens, because even a low level of electrical conduction by the biofilm appears to be beneficial for these bacteria. As EET would play a vital role in enhancing microbial activity in biofilms, specifically the Rex coupled acidic-pH dependent EET capability of *S. mutans* and the EET capability detected in other pathogens such as *Capnocytophaga ochracea, Aggregatibacter, Porphyromonas gingivalis* explains the survival mechanism of these pathogens in highly reductive environments of oral plaque. We hope this study on electroactive oral and gut pathogens would pave paths for new horizons by reevaluating the disease study models from pathogens' electroactive nature point of view and design the drug targets, disease control treatment methods.