Genetically engineering *Shewanella oneidensis* MR-1 to improve the performance of microbial fuel cells

Zhang, Lei

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**GENETICALLY ENGINEERING SHEWANELLA
ONEIDENSIS MR-1 TO IMPROVE THE
PERFORMANCE OF MICROBIAL FUEL CELLS**

ZHANG LEI

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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Abstract

The development of sustainable and renewable power supplies is one of the most pressing issues in modern society, due to the rapid depletion of fossil fuels. Microbial electrochemical techniques, such as microbial fuel cells (MFCs), offer an attractive solution because they can couple the production of bioelectricity with processes such as bioremediation or wastewater treatment. *Shewanella oneidensis* MR-1 is one of the most widely used microbial hosts in MFCs due to its versatile metabolic capabilities. It is capable of oxidizing different organic compounds and transferring intracellular electrons through its cell membrane to the anode of the MFC, thereby resulting in the flow of an electrical current. Despite their obvious potential, present-day MFCs have a limited range of industrial applications and are not widely adopted largely because they suffer from a low power output. In this PhD work, different strategies have been applied to genetically engineer *Shewanella oneidensis* MR-1 in order to enhance the bioelectricity production capability of MFCs.

Lactate is the primary substrate utilized by *Shewanella oneidensis* MR-1 to survive and thrive in natural environment. In MFCs operations, lactate is the ultimate electron donor for bioelectricity generation. However, *Shewanella oneidensis* MR-1 has low efficiency in consuming lactate under MFC operation condition. Thus, genes encoding lactate permease and lactate dehydrogenase have been introduced and overexpressed in MR-1 with the aim of enhancing the lactate consumption rate and eventually improve electricity output in MFCs operations. Strain SP-1 had the best performance among all the resultants and was selected to generate mutant libraries for further screening. A mutant strain 7M6-SH8 was discovered to have the highest maximum power density output of $118.58 \pm 2.67 \text{ mW/m}^2$, which was 2.8 times of that attained by the control strain ($42.03 \pm 6.59 \text{ mW/m}^2$). Moreover, MFCs inoculated with 7M6-SH8 achieved a Coulombic efficiency of 16.69%, which was ~ 2.2 times of that obtained by the control strain (7.77%). Catalytic current densities achieved from both mediated and direct electron transfer pathways by the mutant strain were significantly higher than the control strain with 3.1-fold and 2.7-fold

increases, respectively. Quantitative real-time reverse transcription PCR results also showed that genes encoding proteins in the electron generation and transport pathways have increased expressions, and SEM images showed that biofilm formation on the electrode surface was much thicker than the control strain. Overall, the engineered strain has improved the consumption and utilization of lactate, increased the efficiency of electron transfer in different pathways, and enhanced biofilm formation on the anode electrode surface.

Compared with extracellular electron transfer, the intracellular electron generation and transport has not drawn enough attention among studies of *Shewanella oneidensis* MR-1. In the search of relevant proteins in the intracellular electron transfer pathway, computational approaches were adopted to conduct genome-scale metabolic modeling of *Shewanella oneidensis* MR-1. The *hya* genes were shown to have the highest potential in enhancing electricity generation. NADH is the intracellular electron carrier. Thus, the *hya* genes and *ndh* encoding NADH dehydrogenase were selected for overexpression in MR-1. Results showed that the co-overexpression of *hyaA* and *ndh* in the strain *plac-hyaA-Ptac-MT12*(IPTG_1.0mM) achieved a maximum power density of $112.00 \pm 1.77 \text{ mW/m}^2$, which was 2.8 times of that attained by the control strain ($40.22 \pm 1.92 \text{ mW/m}^2$). Moreover, it achieved a Coulombic efficiency of 18.59%, which was 2.4 times of that obtained by the control strain (7.85%). Catalytic current density via direct electron transfer pathway was significantly increased with a 3.86-fold change.

Anode efficiency is one of the limiting factors in electricity output in MFCs. Improving the efficiency in the extracellular electron transfer (EET) is highly demanded for the further improvement in MFCs operations. In recent decades, the popularly studied pathway in *Shewanella oneidensis* MR-1 is the metal-reducing (Mtr) pathway with several important relevant *c*-type cytochromes. Herein, we have overexpressed the most studied *c*-type cytochromes in MR-1, and the resultant achieved a maximum power density of $88.41 \pm 1.09 \text{ mW/m}^2$, which was 2.14 times of that attained by the control strain ($41.30 \pm 1.23 \text{ mW/m}^2$). Moreover, it achieved a Coulombic efficiency of 15.48%, which was 1.94 times of that obtained

by the control strain (7.98%). Catalytic current density via direct electron transfer pathway was significantly increased with a 3.82-fold change.

In order to achieve scalable control of gene expressions in *Shewanella oneidensis* MR-1 and flexible operation in MFCs, a library of genetic parts that is usable for *Shewanella oneidensis* MR-1 is of high demand. We have generated mutagenesis library of a ribosome binding site, and characterized several terminators as well as constitutive and inducible promoters in MR-1 in order to initiate a library construction.

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CHAPTER 1 Background and Objectives

1.1 Background knowledge of microbial fuel cells

Though providing effective energy, the traditional chemical fuel systems have some disadvantages: 1) chemical fuel cells are not a renewable source of energy; 2) most of the chemical fuel cells require operation under extreme conditions, such as high temperature and ionic strength; 3) Chemical fuel systems are usually highly explosive and toxic, bringing safety concerns to transportation and toxic waste processing and control.

Due to the shortage of fossil fuels and the growing demand for alternative renewable energy in recent years, biomass energy has drawn increasing attentions in research. Among the many techniques in the area of biomass energy, microbial fuel cells (MFCs) have emerged as a new technology. Capable of coupling energy production with wastewater treatment, MFCs have made great contributions in recent decades.¹⁻³

However, the idea of using microorganisms to produce current was not a new concept. The first published study dates back to 1911, where the organic compound decomposition could combine with the release of bioelectricity was observed.⁴ Nearly a century has passed before this phenomenon has returned to the center focus when it was discovered that wastewater and renewable biomass could be a source of electricity generation.⁵ Since then, a variety of microbial species have been isolated and studied for the ability of producing electrical current, which includes acidobacteria, firmicutes, several yeast strains, and four out of five of the Proteobacteria classes.⁶⁻¹⁴ In principle, all MFCs systems should engage microbes that are capable of transferring electrons generated from cellular metabolism to solid electrode (anode) instead of the usual electron acceptors (O_2 , NO_3^- , etc.), also known as extracellular electron transfer (EET). EET can take place via membrane proteins (*c*-type cytochromes, conductive pili, and filaments, etc.) together with soluble electron mediators.^{6, 15-20} Afterwards, the electrons travel through until reaching the terminal electron acceptors in the cathode to complete the circuit. The

intercellular metabolism is separated with the terminal electron acceptor in MFCs, compared with the natural respiration pathway.

Compared to traditional fuel cell systems, the MFCs technology has a few merits. (1) MFCs are a clean and renewable way to produce energy, without generating pollutions. (2) MFCs can be operated at milder conditions (neutral pH and room temperature). (3) Instead of using costly catalysts in chemical fuel systems, MFCs utilize microbes to catalyze natural reactions. (4) MFCs have a broad range of fuel source, different organic matters, such as biomass, wastewater and sludge, can be utilized to harvest energy in MFCs. (5) Though MFCs were developed for wastewater treatment initially, technologies originated from MFCs have expanded the applications, for example, bioproduction, bioremediation, hydrogen production, toxicity sensor, as well as biochemical oxygen demand (BOD) sensors.^{3, 21, 22}

However, there still exists some bottlenecks restricting MFCs to be utilized in real-world applications. One of the main limitations is its low energy output compared with traditional fuel cells. In order to enhance electricity output, researchers have focused on improving the interaction between electricigen and electrode by engineering of materials to increase conductivity and expand surface area of the electrode.²³⁻²⁸ Hence the performance of MFCs can be improved by immobilizing the electricigen over the modified electrode. However, nanomaterials are of high cost and not economically sustainable in the vast production. The contribution of nanomaterials is limited against engineering the bacteria itself in terms of the performance improvement of MFCs.²⁸ To achieve a cost-efficient industrial application of MFCs, genetic engineering and modification of the biological processes of the electricigen should be a much more effective approach. In order to enhance the bioelectricity production capability of MFCs, researchers have genetically modified *Shewanella oneidensis* strains in different ways. One laboratory introduced key glycolysis enzymes absent from the *Shewanella* genome back into the MR-1 strain to enable the bacteria to metabolize glucose as the sole carbon source and to generate bioelectricity from glucose under anaerobic conditions²⁹, however the bioelectrical performance was not ideal compared with

utilizing lactate as the carbon source. Another laboratory heterologously overexpressed a light-dependent proton pump, proteorhodopsin, in MR-1, which led to light-induced changes in membrane potential and a concomitant increase in lactate uptake and bioelectricity production³⁰.

Figure 1.1 Schematic view of central metabolism and auxiliary pathways in *Shewanella oneidensis* MR-1. Reprinted with permission from³⁷, copyright Elsevier.

Since the actual energy that can be harvested from MFCs is quite low, and one of the main factors is the potential loss during the intracellular EET process. Thus, the EET pathways are targeted in this work to enhance the MFCs performance. In this thesis, we are focused on genetically engineering the pathways related to electron generation and transport in the intracellular metabolism of *Shewanella oneidensis* MR-1 (Figure 1.1), thus the increased electrons transported to the outer membrane could be efficiently utilized in MFCs operation for improved power output.

1.2 Objectives and organization of this thesis

This thesis aims at improving the power output of MFCs by genetically engineering electron-related pathways in *Shewanella oneidensis* MR-1. In chapter 2, background of respiration, basics of electron transfer and related proteins and microorganisms, and methodology of MFCs are reviewed. Chapter 3 provides information about chemicals, materials, computational simulations and characterization methods used in this thesis. Chapter 4 to Chapter 8 represent individual projects in engineering strain MR-1 to realize the goal of MFCs performance improvement (Figure 1.2 and Figure 1.3).

Chapter 4 explores the feasibility of engineering the lactate metabolic network to enhance bioelectricity production. Based on the L-lactate consumption pathway, we focused on testing genes encoding the lactate permease or the lactate dehydrogenase from different bacterial species to be heterogeneously expressed in the *Shewanella oneidensis* MR-1.

In Chapter 5, we continued with the finding in Chapter 4, and further explored whether the lactate consumption could be further improved. In order to achieve that, we have generated mutagenesis libraries for the screening of a better mutant. We have also investigated the mechanism of how the improved lactate consumed by MR-1 could enhance electricity generation and searched for explanations in why the mutations contributed to further increased electricity output.

In Chapter 6, we implemented genome-scale modeling of the metabolism of *Shewanella* in the search of possible reactions in increasing electron production and transport. From the resulted possible reactions, we focused on overexpressing the genes encoding the related proteins in those reactions in *Shewanella* MR-1 to verify our computational results.

In Chapter 7, we aimed at the *c*-type cytochromes related in extracellular electron transfer and biofilm formation in *Shewanella* MR-1 to enhance efficiency in transporting intracellular electrons to the electrode in MFCs to improve electricity generation.

Lastly, since MR-1 has become a new model strain for studies of its metabolism and different mechanism in electron generation and respiration, there is a need for modular transcriptional parts that be utilized in MR-1 to realize scalable control in protein expression, functional analysis as well as genetic circuit designs. In Chapter 8, we intended to characterize genetic parts generally used in synthetic biology to build a library of usable parts for *Shewanella oneidensis* MR-1.

Figure 1.2 Schematic view of the pathways engineered in this thesis.

Figure 1.3 Summary of the work in this thesis. Different screening strategies were utilized in characterizing engineered *Shewanella* strains, all with the same goal of improving MFCs performance.

CHAPTER 2 Literature Review

2.1 Dissimilatory metal-reducing bacteria and electron transfer

2.1.1 Background knowledge of respiration

Electron transfer is deemed to be a common phenomenon in our daily lives, most visibly in the electronic devices people use. In fact, electron transfer is one of the most fundamental and crucial factors in life sustainability.^{32, 33} Respiration is the oxidation of organic substrates coupled with the flow of electrons via membranes and proteins to some electron acceptor. Both electron donors and acceptors are required in respiration. Electrons flow from an electron donor with a more negative potential to an electron acceptor with a more positive potential.

In capable organisms, respiration is the substantial mean to produce biochemical energy for the synthesis of macromolecules, compounds and solute transport. The flow of electrons through living cells gives energy to organisms to maintain their functions.³⁴ The energy from this flow can be harvested in the form of a proton motive force using quinones, which are electron carrier proteins with coupling sites. This energy is used by cells to generate Adenosine Triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate.³⁴ ATP can be utilized for a variety of functions such as driving locomotion, facilitating transport and biosynthesis. Even though in some organisms (*Shewanella* included), there are alternative strategies to produce ATP in some organisms under certain conditions such as substrate level phosphorylation (SLP)³⁵, transferring electrons to electron acceptors still need to be continued for the balance of cellular redox reactions.

Although most organisms store energy in the form of ATP by using a certain type of electron transport from an electron donor to an electron acceptor, the specific pathways and the electron donor/acceptor used in the process may vary. Aerobic organisms generate electrons via oxidation of the organic substrate and transfer them to molecular oxygen in soluble form within the cell. Thus, molecular oxygen is required for the aerobic organisms to produce ATP and survive. On the other hand, anaerobic organisms are capable of utilizing alternate electron acceptors

instead of molecular oxygen to maintain the electron flow; some examples of alternate electron acceptors are fumarate, nitrate, as well as metals such as manganese, iron or uranium³⁶. For instance, some bacteria thrived on the planet long before the accumulation of molecular oxygen in the Earth atmosphere was enough to sustain aerobic life. Iron and other metals would have served as the terminal electron acceptors for microorganisms.³⁷ In order to reduce insoluble inorganic minerals, respiratory electrons are required to be transported outside of the cellular outer membrane to perform extracellular electron transfer (EET). Today there still exist species of microbes that own the capability of EET to survive in anaerobic environment, which are called dissimilatory metal-reducing bacteria (DMRBs).^{38, 39}

Redox active cofactors can mediate electron transfers. Flavoproteins are a type of proteins with either a flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) cofactor; the flavin cofactors are made from riboflavin (vitamin B₂) precursor⁴⁰ and an isoalloxazine ring that is shared by all three flavins. The flavin co-factor usually is located in the active site of the flavoprotein, whether covalently or non-covalently bound. A free flavin has a reduction potential of approximately -200 mV versus SHE (standard hydrogen electrode), while flavin cofactors depending on protein structure, ranging from -495 mV to +80 mV.⁴¹

Similarly, cytochromes can also transfer electrons. Unlike flavoproteins using flavin cofactors to carry electrons, cytochromes use hemes, of which the structure consists of an iron atom in the center coordinated by four nitrogen atoms in a porphyrin circle.⁴² The central iron atom can be reduced to ferrous form and oxidized back to ferric form, which is why hemes in cytochromes can only transfer single electrons at one time. Different cytochromes can contain various types of hemes, or associate with other proteins that transfers electrons, such as iron-sulfur proteins.⁴³ In an iron-sulfur protein, instead of porphyrin rings, sulfur atoms coordinate the redox active center. Same as cytochromes, it can only transfer one electron at one time.⁴⁴

Quinones are lipid-based hydrophobic molecules in the lipid phase of membranes that can carry electrons and protons.⁴⁵ All quinones have hydrophobic isoprenoid side chains, which contribute to the fact that quinones are mobile within membranes and can transport electrons and protons from protein to protein. Menaquinone and ubiquinone are two types of quinones commonly found in organisms. Menaquinone has more negative reduction potential than ubiquinone and is usually utilized with low potential electron acceptors in anaerobic respiration, while ubiquinone is used when electropositive electron acceptors (such as oxygen) are present.

2.1.2 Dissimilatory metal-reducing bacteria

Geobacter and *Shewanella* were the first DMRBs discovered in the late 1980s⁴⁶⁻⁴⁸, since then, the search for extracellular electron transfer mechanisms had begun to investigate the long-range electron transport that moves the electrons for intracellular space to the exterior for solid metal oxides reductions. It was reported that multiheme cytochromes are important factors enabling this process, within their structures are multiple electron carrier heme sites.⁴⁹⁻⁵³ These multiheme cytochromes have been the center of attention in the past two decades, owing to their substantial role in the EET.^{50, 52-57}

EET has gathered a wide range of interests in recent years due to its potential applications in sustainable energy conversion^{58, 59}, bioremediation^{60, 61}, and wastewater treatment⁶². DMRBs are not only able to reduce metal oxides in the environment, but also to transfer electrons to synthetic insoluble electrodes.⁵ By providing soluble electron donor, and tuning the flow of respiratory electrons and the redox potential of the electrode, the cellular growth and metabolism can be controlled. Therefore, electrons flowing at a steady pace can be pooled and turned into electricity from the microbe community in microbial fuel cells (MFCs).⁵⁸ Microbial electrosynthesis is an alternative application of EET, in which microbes perform a reverse procedure: the bacteria reduce soluble chemicals using electrons obtained from renewable energy sources, solar cells for instance, subsequently, biofuels can be synthesized.⁶³ DMRBs can also be used in bioremediation;

electrons can be used to convert soluble toxic chemicals (chromium, uranium, etc.) to insoluble form upon reduction, making it easier to remove from water.⁶⁰ Moreover, DMRBs can be utilized to combine wastewater treatment with electricity production, due to the high organic content in wastewaters.⁶²

Although a wide range of microbes has been discovered with the ability to reduce external electron acceptors, only a few have been clearly depicted for their EET mechanisms.^{21, 64} Among those microorganisms, *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1 are two model organisms that have been extensively studied, due to their ability to reduce extracellular insoluble electron acceptors, which is necessary for MFCs operations.

Geobacter species are all gram-negative anaerobes, generally more abundant than other Fe(III)-reducing microbes in areas where Fe(III) reduction occurs. Various electron acceptors can be utilized by *Geobacter* for respiration, such as elemental sulfur, Fe(III), Mn(IV), Np(V), U(VI), Co(III)-EDTA, and humic substances.^{61, 65-69} In addition, it was found that *Geobacter sulfurreducens* can utilize cytochromes in the outer membrane for extracellular electron transfer.⁷⁰⁻⁷⁷ In pure-culture MFCs operations, *Geobacter sulfurreducens* generally can produce the highest current densities. However, since *Geobacter sulfurreducens* is an obligate anaerobe, which is difficult to be manipulated, *Shewanella oneidensis* MR-1 was chosen for this PhD work.

Shewanella oneidensis is a gram-negative facultative anaerobe with a broad range of electron acceptors with fully sequenced genome.⁷⁸ Based on its annotated genome sequence, *Shewanella oneidensis* has 42 putative *c*-type cytochromes, and most of them are located in the outer membrane.⁷⁹ During the past decade, *Shewanella oneidensis* has been extensively studied for its ability to combine the intracellular oxidative metabolism with extracellular reduction of electrodes or metal oxides via the metal reducing pathway.^{58, 80-84}

2.1.3 Microbial Electron Transfer

Respiration is an energy transformation mechanism for microorganisms, in which the synthesis of ATP occurs, which is driven by a series of redox reactions known as oxidative phosphorylation. There are three components in heterotrophic respiration: glycolysis, the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC). Glucose is broken down to form pyruvate, ATP, and NADH in the glycolysis process via phosphorylation on substrate level. In the TCA cycle, pyruvate can be oxidized into reducing equivalents like NADH, ATP, FADH₂ as well as the regeneration of oxaloacetate.⁸⁵ NADH and FADH₂ can be oxidized during respiration and the electrons travel to electron acceptor through ETC, generating a proton gradient, which then drives the ATP synthase to produce ATP – an important energy source in cells.^{85, 86} ETC usually consists of a series of electron carriers or proteins which are associated with the periplasmic space or cell membranes.⁸⁶ Under aerobic conditions, oxygen is typically used as the terminal electron acceptor to provide microorganisms with high levels of energy gain. When oxygen or other soluble electron acceptors (nitrite, nitrate, sulfate, fumarate, iron, etc.⁸⁷) are absent, in order to respire anaerobically, microbes are forced to utilize alternative terminal electron acceptors, such as minerals (including manganese oxides, iron oxides, and polysulfides⁸⁷) or anodic electrodes in bio-electrochemical systems (BES)⁶³.

Distinguished from general respiration pathways, extracellular electron transfer (EET) specifically utilizes extracellular solid compounds (electrodes, manganese oxides, iron oxides, etc.) or soluble oxidizing agents as the final electron acceptors instead of intracellular electron acceptors.^{21, 88} A transport system is needed in order to overcome the physical barrier between the bacteria and the electron acceptors in the EET (Figure 2.1). There are two main EET mechanisms: direct electron transfer (DET) and mediated electron transfer (MET). In DET, electrons are transferred via membrane-bound cytochromes, conductive nanowires or pili, with the requirement of direct contact between the bacteria and insoluble electron acceptors.^{15, 89-92} While the alternative pathway MET is facilitated by redox mediators.^{19, 93-98}

Figure 2.1 The extracellular electron transfer pathway in *Shewanella oneidensis* MR-1. Respiratory electrons are transported to inner-membrane cytochrome CymA and subsequently transferred to the outer-membrane porin-cytochromes, where they can be shuttled to metals distanced from the cell surface via flavins (pathway on the left) or contact with insoluble metals directly (pathway on the right). Reprinted with permission from⁹⁹, copyright Nature Publishing Group.

The involvement of cytochromes in EET has been extensively studied in *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1.^{89, 91, 100, 101} In *Shewanella oneidensis* MR-1, outward current flows from intracellular space to the outer membrane via the metal-reducing (Mtr) pathway, which is comprised of five primary proteins: CymA (inner-membrane tetraheme c-cytochrome)¹⁰²⁻¹⁰⁵ MtrA (periplasmic decaheme c-cytochrome), MtrB (β-barrel trans-outer-membrane protein), OmcA and MtrC (two outer-membrane decaheme c-cytochromes)^{80, 82, 106}, and (Figure 2.1). Electrons (from the intracellular electron carrier NADH) flow through the menaquinol pool, CymA, MtrA, MtrB to MtrC and OmcA. The EET mechanisms between outer membrane and the electrode have three main interfacial ways: (1) DET via outer-membrane c-type cytochromes; (2) MET by electron shuttles; and (3) nanowires.

2.2 Basics and methodology of microbial fuel cells

Figure 2.2 shows a two-chamber MFC with organic compounds as the substrate in the anode chamber and oxygen as the terminal electron acceptor in the cathode chamber. The reactions are as follows:

Figure 2.2 Schematic presentation of a microbial fuel cell. The MFC is comprised of two chambers (anode and cathode) and a proton exchange membrane (PEM) separating the chambers. Oxidation of organic compounds is catalyzed by microbes, donating electrons to the anode electrode. The electrons are accepted in the cathode for reductive reactions.

In the anode chamber, the biomass degradation takes place and electrons are released to the anodic electrode. Protons produced from intracellular metabolism are released into the anode chamber and travel through the proton exchange membrane (PEM) to the cathode chamber. Specifically, for MFCs, in the anode, the microbes are responsible for oxidizing the substrates to produce electrons, acting as a role of a biocatalyst (not a real catalyst as there is energy benefit for the microbes in this step).

At least two metabolic pathways involve in the EET while NADH works as the source of electrons in EET.¹⁷ In theory, the minimum anode potential should be the standard potential of NADH (-0.32V vs. SHE), therefore, MFCs with the goals of power output and energy production should have a controlled anode potential higher than this value. The theoretical harvested energy achievable by MFCs is determined by the redox potential difference between the electron donor and the terminal electron acceptor (Figure 2.3), while the actual energy obtained is usually quite low, owing to potential loss during the EET process.

During the last decades, there has been an explosion of publications in MFCs development; however, there existed huge differences in terminology, technology as well as parameters. This created great difficulties in comparison among different works. Thus, it is of great importance to establish universal terminology and analysis parameters in the MFCs technology.^{2, 17, 107} The commonly used parameters in MFCs studies are listed below.

Figure 2.3 Redox potentials of reactions occur in the electron transport chains catalyzed by different bacteria. Reprinted with permission from⁷⁰, copyright ©2015 Kracke, Vassilev and Krömer.

2.2.1 Open circuit potential

Open circuit potential (OCP) is the potential difference between anode and cathode when there is no current in the external circuit. OCP stands for the maximum theoretical potential that can be obtained from MFCs. Most importantly, OCP can help provide instant observation of the system's performance, where it depends mainly on the choice of reactions occurring in the anode and cathode. The theoretical anode potential by substrate degradation cannot be reached experimentally because of anode overpotential.

2.2.2 Anode and cathode overpotential

When the electrons are being transferred through the circuit, the overpotentials in the anode and cathode are obvious. Polarization curves and cyclic voltammetry (CV) scanning can provide useful information on the potential loss in MFCs due to the overpotentials. Activation loss is always observed in the anode of an MFC.¹⁰⁸ When the reaction at the anode is not fast enough, overpotential is required to drive the MFCs to higher current output, resulting in high anodic current with continuous increase in anodic potential.¹⁰⁹ Improving electrode surface area and improve anode materials are good strategies to reduce anode overpotential. In the cathode, especially those in which oxygen is the ultimate electron acceptor, cathode overpotential is of great concern. To balance efficiency with economic cost is very important, thus suitable catalysts need to be used in the cathode. Chemical cathode such as hexacyanoferrate solutions could reduce the cathode potential, although it is not recommended for industrial applications.^{110, 111}

2.2.3 Internal resistance

Even though increasing electrode surface area and implementing novel electrode materials can help reduce anode and cathode overpotentials, the internal resistance is another potential loss factor in MFCs operations. internal resistance mostly is comprised of resistance of materials used in the MFCs, resistance of ion immigration in the chambers, and ion transfers across the PEM.^{21, 112} The potential loss caused by internal resistance becomes problematic when MFCs are scaled up from laboratory level (several to several hundred milliliters in volume) to industrial level (tens of liters to cubic meters in volume).

2.2.4 Coulombic efficiency

Coulombic efficiency is the ratio of the number of electrons transferred towards the anode to the maximum theoretical number of electrons generated from substrate degradation (substrate fully oxidized). When the MFCs are fed with single carbon source, the calculation is as follows:

$$\varepsilon_c = \frac{\int_0^t Idt}{FbV\Delta C}$$

I is measured current in the external circuit, F is the Faradic constant, b is the number of electrons transferred with one substrate molecule being fully oxidized, V is the anode volume and ΔC is the concentration change of substrate.²

Coulombic efficiency is one of the most important parameters in evaluating MFCs performance; it directly indicates how the EET contributes to microbial respiration. The ideal value is 100%, indicating respiration in the anode is the only process undergoing in the MFCs and the substrate is completely oxidized. Till now, only pure *Geobacter* and *Geothrix fermentans* inoculated MFCs with acetate as carbon source can approach to this ideal value.^{6, 89, 113} MFCs inoculated with pure *Shewanella* culture only have coulombic efficiency around 20% (if calculated from lactate to CO₂) using lactate as substrate. During MFCs operations, *Shewanella* can oxidize lactate partially to acetate (leaving 8 electrons in the form of acetate).¹¹⁴

2.2.5 Power density output

Power output is another important parameter in evaluating MFCs performance, which can be calculated by:

$$P = I \times E_l$$

Where I is the measured current in the external circuit, E_l is the voltage measured on the load. In experiments, the load is usually a constant resistor, as result, the power output can be calculated as:

$$P = \frac{E_l^2}{R_{ext}}$$

Where R_{ext} is the external resistor value.

To compare power output in different MFCs systems is difficult due to the differences in MFC configuration and material choices. A reasonable way to compare is to normalize the power output to the anode surface area or volume. The corresponding power density output should be expressed in the form of mW/m² and mW/m³, respectively.

Power density output highly depends on the external resistor; thus, the maximum power density output could represent the “real capacity” for the system. Polarization curves can help with finding out the limitation of power output. In most MFCs systems, polarization curves can be measure by changing the external resistor and recording the corresponding voltage on the load. Then a v-j curve can be obtained from the relation between MFCs loading voltages and output current densities. Thus, deriving from the v-j curve, the P-j curve can be obtained to depict the relation between power density output and the current density output, from which the maximum power density output can be found.

2.2.6 Cyclic voltammetry

As the most extensively utilized electrochemical technique, cyclic voltammetry (CV) provides useful information on the electron transfer process in MFCs operations. From the CV curves, useful parameters such as peak location, peak current density can be obtained. Detailed information can be depicted from those parameters, such as (1) standard potential of EET related electrochemical active species; (2) relative abundance of such species in the interfaces between biofilm and electrode; (3) diffusion type and electrochemical reversibility of those species. Specifically, at medium and high scanning rates (50mV/s and above), non-catalytic current mainly contributes to the total responsive current; while at low scanning rate (1mV/s and lower), catalytic current is dominant. By changing scanning rates, how various electrochemical active species contribute to the anode performance can be observed.

2.3 Developments in MFCs technologies

2.3.1 Anode developments in MFCs

Obviously, microorganisms that generate electrons play the most important role in anode chamber. Anaerobic anode compartment is one of the main parts in MFCs configurations. This chamber is filled with microorganism, substrate, mediator (optional) and the anode electrode. The electrode is a critical part in enhancing the efficiency in an MFC.¹¹⁵ An ideal electrode material should have the following

features: 1) low resistance and good conductivity; 2) strong biocompatibility; 3) large surface area; 4) suitable mechanical toughness and strength; 5) chemical stability and anticorrosion.¹¹⁶ The most frequently used materials in anode chamber are made of carbon materials for their stability in bacterial culture, large surface area as well as high conductivity.¹¹⁷

To improve the performance of MFCs, anode electrode modification using nano-engineering techniques has been extensively studied.^{118, 119} One feasible means to improve power output of MFCs is to use metal-based anode and modified carbon with conductive polymers.¹²⁰ Between different kinds of conductive polymers, polyaniline (PANI) has been studied most to modify the anode electrode.¹²¹ It has been reported that PANI/titanium dioxide composite¹²² and modified PANI polymers¹²⁰ indeed could result in improved current densities. CNT/polyaniline composite could also be used as anode material and enhance MFCs performance.^{118, 123} Polytetrafluoroethylene (PTFE) was chosen as electrode due to its hydrophobic nature and chemical stability. As reported, graphite/PTFE could be excellent for anode material selection and produced a power density of 760 mW/m² with *Escherichia coli* as the active biocatalyst.¹²³

2.3.2 Cathode developments in MFCs

Oxygen is used as a final electron acceptor in the cathode thanks to its intense oxidation potential, accessibility as well as being able to produce no poisonous end products.^{116, 121} However, the reduction kinetic of oxygen on a plain electrode surface is slow and need a catalyst for high potential. Thus, oxygen in the cathode of MFCs is also a restricting agent.¹²⁴ Therefore, potassium ferricyanide ($K_3[Fe(CN)_6]$) has been proposed in MFCs operations.^{116, 125} Chemical cathode represented by $K_3[Fe(CN)_6]$ have quite a small overpotential even under high anode current density. Thus, it is preferred for lab use.¹²⁶ However, $K_3[Fe(CN)_6]$ is not sustainable and have to be refilled upon depletion, making it unfavorable for industrial applications.¹²⁷ Similarly, the anaerobic condition in the anode can also be affected by $K_3[Fe(CN)_6]$ that can penetrate through the proton exchange membrane (PEM) into the anode chamber.¹²⁸ Alternatively, $K_3[Fe(CN)_6]$ still

remain a favorite due to its low over potential on plain electrodes. So far, the highest power densities reported for MFC systems have used $K_3[Fe(CN)_6]$ at the cathode.^{126, 129} While in terms of MFCs performance ferricyanide is a good choice for catholyte, it has to be regenerated chemically and it is not sustainable in industrial practice. Therefore, usage of ferricyanide is restricted to researches in lab scales.

Platinum is another frequently chosen catalyst regarding cathodic reaction. However, platinum is not an appropriate catalyst in MFCs because of its poisoning sensitivity on some substances in the solution.¹³⁰ Many researchers choose to add artificial redox mediators into the cathode to amplify MFCs performance.^{131, 132} Najafpour *et al.* demonstrated that low concentration of potassium permanganate resulted well to increase power output in MFCs as the oxidizing agent.¹³³

In recent years, biocathodes have been explored in order to overcome catalysis requirement by oxygen oxidation.^{134, 135} A biocathode is a cathode using microorganisms to catalyze cathodic reactions.^{121, 136} It can take the place of catalysts and artificial mediators and be adopted to enhance cathode performance using a variety of electron acceptors (nitrate, fumarate, perchlorate, Fe(III), Cr(VI), U(VI), Mn(IV), sulfate, etc.) to improve MFCs electricity production.^{133, 134, 137} Advantages of biocathodes include: 1) since expensive mediators and catalysts are not necessary, the configuration and expenses of MFCs can be reduced;¹²¹ 2) biocathodes can be used to remove by-products and generate practical products by using microbial metabolism;¹³⁸ 3) biocathodes can improve MFCs sustainability, since there is no need to consider problems such as sulfur poisoning of Pt chamber and electron mediators requirements.¹³⁸ However, oxygen solubility in biocathode is a severe problem for high current density, making aerostation a necessity, increasing the operation cost. Moreover, even though biocathode performance has been improved greatly in the past years, there is still a lack of fundamental knowledge on electron transfer mechanism and microorganism physiology, more works on biocathode are called for in the future.

CHAPTER 3 Experimental Approaches

This chapter includes basic information on culture media and experimental methods used in this Ph. D work.

3.1 Chemicals, strains and culture media

Table 3.1 Chemicals used in this thesis

Chemical	Product Number	Source
THIAMINE HYDROCHLORIDE	T4625-5G	Sigma-Aldrich
NICOTINIC ACID	N4126-5G	Sigma-Aldrich
D-PANTOTHENIC ACID CALCIUM SALT	21210-5G	Sigma-Aldrich
4-AMINOBENZOIC ACID	A9878-5G	Sigma-Aldrich
(1)-ALPHA-LIPOIC ACID	T5625-1G	Sigma-Aldrich
VITAMIN B12	V2876-100MG	Sigma-Aldrich
CHOLINE CHLORIDE	C7527-100G	Sigma-Aldrich
RIBOFLAVIN	R9504-25G	Sigma-Aldrich
BIOTIN	B4639-100MG	Sigma-Aldrich
FOLIC ACID	F8758-5G	Sigma-Aldrich
FERROUS SULFATE HEPTAHYDRATE	F8633-250G	Sigma-Aldrich
SODIUM MOLYBDATE	737860-5G	Sigma-Aldrich
BORIC ACID	202878-10G	Sigma-Aldrich
SODIUM SELENATE DECAHYDRATE	450294-5G	Sigma-Aldrich
SODIUM TUNGSTATE DIHYDRATE	379751-5G	Sigma-Aldrich
NITRILOTRIACETIC ACID	N9877-100G	Sigma-Aldrich
ALUMINUM POTASSIUM SULFATE DODECAHYDRATE	237086-100G	Sigma-Aldrich
CASEIN HYDROLYSATE	22090-100G	Sigma-Aldrich
FUMARIC ACID DISODIUM	F1506-500G	Sigma-Aldrich
L(+)-LACTIC ACID SODIUM	L7022-50G	Sigma-Aldrich
HEPES	H3784-100G	Sigma-Aldrich

GLUTARALDEHYDE 50% AQUEOUS SOLUTION	G6403-100ML	Sigma-Aldrich
POTASSIUM FERRICYANIDE(III)	244023-500G	Sigma-Aldrich
2,6-Diaminopimelic acid	33240-1G	Sigma-Aldrich
LB Broth Miller	BIO-4000-1kg	1st BASE Biochemicals

Table 3.2 Strains and plasmids used in this thesis

Strains	Description	Reference or source
<i>E. coli</i>		
DH5 α	Host for regular cloning	Lab stock
WM3064	Donor strain used for conjugation with <i>S. oneidensis</i> MR-1, $\Delta dapA$	
<i>S. oneidensis</i>		
MR-1	Wildtype	Lab stock
Plasmids		
pHG101	Promoterless vector derived from broad host range vector pBBR1MCS-2; Km ^r	¹³⁹
iGEM distribution kit	Over 1000 high quality dried DNA part samples	parts.igem.org

Proton exchange membrane Nafion 117 (PEM): Gas Hub, Singapore

Carbon cloth: Gas Hub, Singapore

Luria-Bertani (LB) medium containing (per liter):

NaCl	10 g
tryptone	10 g
yeast extract	5 g

Dissolved in DI water and sterilized for 20 min at 121 °C.

Basic salts solution containing (per liter of *Shewanella* basal medium):

NH ₄ Cl	0.46 g
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K ₂ HPO ₄	0.225 g
KH ₂ PO ₄	0.225 g
MgSO ₄ ·7H ₂ O	0.117 g
(NH ₄) ₂ SO ₄	0.225 g

Dissolved in DI water and sterilized for 20 min at 121 °C.

Mineral mix stock solution, pH=7.0 containing (per liter):

nitrilotriacetic acid	1.5 g
MnCl ₂ ·4H ₂ O	0.1 g
FeSO ₄ ·7H ₂ O	0.03 g
CoCl ₂ ·6H ₂ O	0.017 g
ZnCl ₂	0.1 g
CuSO ₄ ·5H ₂ O	0.04 g
AlK(SO ₄) ₂ ·12H ₂ O	0.005 g
H ₃ BO ₃	0.005 g
Na ₂ MoO ₄	0.09 g
NiCl ₂	0.12 g
NaWO ₄ ·2H ₂ O	0.02 g
Na ₂ SeO ₄	0.1 g

Dissolved in DI water and filtered with a 0.2 µm membrane.

Vitamin mix stock solution, pH=6.5, containing (per liter):

biotin	0.002 g
folic acid	0.002 g
pyridoxine HCl	0.02 g
thiamine	0.005 g
nicotinic acid	0.005 g
pantothenic acid	0.005 g
cyanocobalamine	0.0001 g
p-aminobenzoic acid	0.005 g
thioctic acid	0.005 g

Dissolved in DI water and filtered with a 0.2 µm membrane.

Amino acids stock solution, pH=6.2:

10% (w/v) casamino acids solution

Dissolved in DI water and sterilized for 20 min at 121 °C.

Shewanella basal medium (SBM) containing (per liter):

Basic salts solution

Mineral mix stock solution 5 ml

Vitamin mixture stock solution 5 ml

Amino acids stock solution 5 ml

HEPES (1M, pH=7.0) 10 mM for aerobic growth

100 mM for anaerobic growth

MFCs cathodic solution containing: 50mM $K_3[Fe(CN)_6]$, 50mM K_2HPO_4 , and 50mM KH_2PO_4 .

3.2 Mutagenesis library generation

In Chapter 4 and Chapter 5, error-prone PCR of target genes was performed with GeneMorph® II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Primers were designed to amplify target genes with biobrick restriction sites. PCR products were purified and digested with XbaI and PstI. The mutagenized fragments were cloned into the vector pHG101, with the lac promoter upstream and double terminators downstream.

In Chapter 6 and Chapter 7, error-prone PCR of target genes was performed with GeneMorph® II EZClone Domain Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Primers were designed to amplify target genes within the coding sequences.

3.3 Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was purified from bacterial cultures in mid-log phase using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Quantitative analyses of transcripts were carried out with iQ SYBR Green Supermix (Biorad) and CFX96 Real Time PCR Detection System (Biorad). Samples were analyzed in triplicates. Gene *gyrA* (SO_2411) was used for normalization. Data analysis was performed as previously described.¹⁴⁰

3.4 Scanning electron microscope (SEM) imaging of biofilm

To obtain an SEM image of the anode after operation, a small piece of the anodic carbon cloth was cut off and immersed in 1% glutaric dialdehyde solution overnight for bacteria fixation on the electrode surface. After stabilization, samples were rinsed 3 times using 0.9% NaCl solution and went through dehydration, which consists of immersion of the sample in 30%, 50%, 70%, 90% and 100% ethanol solution for 20 minutes each at room temperature. Samples were then air-dried and ready for imaging. The morphology and relative density of biofilm was qualitatively characterized by a JEOL field emission scanning electron microscope (FESEM, JSM-6700F-FE-SEM, Japan).

3.5 Microbial fuel cell characterization

3.5.1 Microbial fuel cell setup and operation

All experiments were performed using dual-chamber MFCs as shown in Figure 3.1. The working volume of each of the chambers is 150mL. A Nafion 117 membrane (Gashub, Singapore) was used to separate the anode and cathode chambers after it had been immersed overnight in 0.5M sulfuric acid and washed with DI water. Electrodes for both anode and cathode were made of carbon cloth (Gashub, Singapore) with a size of 2cm × 3cm. Cathodic solution consisted of 50mM K₃[Fe(CN)₆], 50mM K₂HPO₄, and 50mM KH₂PO₄.

Shewanella MR-1 strains were grown overnight in a 30°C incubator at a shaking rate of 200rpm. Cell suspensions were adjusted to an optimal density of 0.5 before adding into the anode chamber. The anodes were purged with nitrogen to remove oxygen. After inoculation, the circuit was connected by copper wire with a constant external resistance of 2k Ω . The MFCs were operated at room temperature in batch mode. The duration of a full cycle was determined by the consumption of substrate and voltage reduction from its peak value to 50mV.

Figure 3.1 Schematic presentation of MFCs setup in this thesis. The MFC is comprised of two chambers (anode and cathode) and a proton exchange membrane (PEM) separating the chambers. Carbon cloth was used as electrodes for both anode and cathode.

3.5.2 Data acquisition and calculations

The voltage output of each MFC was measured using a digital multimeter. To evaluate MFC performance, polarization curves were obtained by applying different loads or external resistances (R_{ext}) and then measuring the voltage (V) in order to calculate the current (I) by Ohm's Law ². By normalizing power based on

the surface area (A) of the anode electrode, power density (P) can be calculated by using the formula, $P = \frac{V \times I}{A}$.

3.5.3 Electrochemical analysis

Electrochemical measurements were performed using an electrochemical working station (CHI760D, CH Instruments Inc.) employing a three-electrode system. Saturated calomel electrode (SCE, -0.243V vs. SHE) was used as the reference electrode, with the anode as the working electrode and the cathode as the counter electrode. Cyclic voltammetry (CV) was carried out with a scanning range of -0.7 to 0.2V.

3.6 Genome-scale modeling of *Shewanella oneidensis* MR-1

3.6.1 Monod Model

The main objective is to propose an optimized model which couples the Flux Balance Analysis (FBA) algorithm with Monod equations, with the goal of accurately predicting *Shewanella oneidensis* MR-1 metabolism by observing four key factors of growth (biomass, acetate, lactate and pyruvate) and simultaneously optimizing intracellular electron transfer in *Shewanella oneidensis* MR-1 powered MFCs.

Proposition 1.1: Consider an initial 4 reactions that was developed to describe 4 main growth components in *Shewanella oneidensis* MR-1: cell growth, lactate uptake or production, acetate and pyruvate production or reuse,

$$\frac{dLACT}{dt} = \left(-X \cdot \frac{\mu_L}{Y_{X/L}} - r_{P,L} - r_{A,L} \right) \cdot S(t - t_L)$$

$$\frac{dACT}{dt} = (r_{A,L} + r_{A,P} - X \cdot \frac{\mu_A}{Y_{X/A}}) \cdot S(t - t_L)$$

$$\frac{dPYR}{dt} = \left(r_{P,L} - X \cdot \frac{\mu_P}{Y_{X/P}} - r_{A,P} \right) \cdot S(t - t_L)$$

$$\frac{dX}{dt} = X \cdot (\mu_A + \mu_P + \mu_L - k_e) \cdot S(t - t_L)$$

Where X is the biomass (in g DCW/L), $LACT, ACT, PYR$ are lactate, acetate and pyruvate concentrations respectively (in mmol/L), μ_L, μ_A, μ_P are the specific growth rates (in h^{-1}) on lactate, acetate and pyruvate respectively, k_e is the endogenous metabolism rate constant (in h^{-1}), $Y_{x/L}, Y_{x/A}, Y_{x/P}$ are biomass yield coefficients (in DCW/mol substrate) of lactate, acetate and pyruvate respectively, $r_{P,L}$ and $r_{A,L}$ are the production rates of pyruvate and acetate from lactate (in mmol/L/h) of acetate and pyruvate from lactate respectively, $r_{A,P}$ is the production rate of acetate from pyruvate, $S(t - t_L)$ is the dimensionless Heaviside unit-step time delay function to describe a lag phase L during cultivation before actual growth which was discovered in previous study¹⁴¹ such that

$$s(t - t_L) = \begin{cases} 0 & \text{if } t < t_L \\ 1 & \text{if } t = t_L \end{cases}$$

While the specific cell growth rate can be summarized in the next 3 Monod equations:

$$\mu_L = \frac{\mu_{max,L} \cdot LACT}{K_{s,l} + LACT}$$

$$\mu_A = \frac{\mu_{max,L} \cdot ACT}{K_{s,a} + ACT}$$

$$\mu_P = \frac{\mu_{max,P} \cdot PYR}{K_{s,p} + PYR}$$

Where μ_L, μ_A, μ_P are the maximum growth rates (in h^{-1}) on lactate, acetate and pyruvate respectively for full aerobic conditions, and $K_{s,l}, K_{s,a}$ and $K_{s,p}$ are Monod constants (mmol/L) for lactate, acetate and pyruvate respectively. The acetate and pyruvate production rates are assumed to be proportional to the biomass as expressed below by the last 3 equations:

$$r_{A,L} = k_{al} \cdot LACT \cdot X$$

$$r_{P,L} = k_{pl} \cdot LACT \cdot X$$

$$r_{A,P} = k_{ap} \cdot PYR \cdot X$$

Where k_{al} and k_{pl} are rate constants of acetate and pyruvate production from lactate respectively (in $L \cdot (h \cdot g DCW)^{-1}$) and k_{ap} are rate constants of acetate production from pyruvate (in $L \cdot (h \cdot g DCW)^{-1}$). In total, there are 14 kinetic parameters in 10 equations in the kinetic Monod model.

To estimate the values of the parameters from an initial range of values first determined for each parameter, we apply Monte Carlo method to randomly sample multiple sets of values for the parameters over their constrained range uniformly in order to construct the optimal set of values for the parameters. The optimal set of values for the parameters should produce a set of predicted results where its squared difference with the experimental results is minimized. This squared difference is calculated from the summation of all the squared differences between corresponding predicted and observed results for Biomass, Acetate, Lactate and Pyruvate uptake/production. Since the measurements results for Biomass, Acetate, Lactate and Pyruvate uptake/production have different scales, we normalize each difference with the maximum value of its observed result i.e. for all time points i over 3 replicates of n time points,

$$\sum_{i=1}^{3n} \left(\frac{X_{pred,i} - X_{obs,i}}{\max_i X_{obs,i}} \right)^2 + \left(\frac{ACT_{pred,i} - ACT_{obs,i}}{\max_i ACT_{obs,i}} \right)^2 + \left(\frac{LACT_{pred,i} - LACT_{obs,i}}{\max_i LACT_{obs,i}} \right)^2 + \left(\frac{PYR_{pred,i} - PYR_{obs,i}}{\max_i PYR_{obs,i}} \right)^2$$

From our simulation, the results of the estimated parameters for growth factors summarized in Table 3.1 suggest that growth rate dependent on lactate, denoted by $\mu_{max,L}$, is much higher than the growth rate dependent on pyruvate, denoted by $\mu_{max,P}$, or on acetate, denoted by $\mu_{max,A}$, indicating that lactate is the major carbon substrate for biomass growth. This result was confirmed in many other studies and simulations¹⁴¹⁻¹⁴³.

Table 3.3 Parameters estimated in optimized Monod Equations (with Monte Carlo)

Symbols	Notation	Unit	FBA
$\mu_{\max,L}$	Maximum specific growth rate using lactate	h^{-1}	0.57
$\mu_{\max,P}$	Maximum specific growth rate using pyruvate	h^{-1}	0.13
$\mu_{\max,A}$	Maximum specific growth rate using acetate	h^{-1}	0.13
$Y_{X/L}$	Apparent biomass yield coefficient from lactate	$\text{g} \cdot \text{DCW/mol lactate}$	17.0
$Y_{X/P}$	Apparent biomass yield coefficient from pyruvate	$\text{g} \cdot \text{DCW/mol pyruvate}$	16.9
$Y_{X/A}$	Apparent biomass yield coefficient from acetate	$\text{g} \cdot \text{DCW/mol acetate}$	10.6
$K_{s,l}$	Monod lactate saturation constant	mM	19.9
$K_{s,p}$	Monod pyruvate saturation constant	mM	20.0
$K_{s,a}$	Monod acetate saturation constant	mM	10.0
k_{al}	Acetate production coefficient from lactate	$\text{L} \cdot (\text{h} \cdot \text{g DCW})^{-1}$	0.70
k_{pl}	Pyruvate production coefficient from lactate	$\text{L} \cdot (\text{h} \cdot \text{g DCW})^{-1}$	0.44
k_{ap}	Acetate production coefficient from pyruvate	$\text{L} \cdot (\text{h} \cdot \text{g DCW})^{-1}$	0.96
k_e	Endogenous metabolism rate constant	h^{-1}	0.02
t_L	Lag time in growth	h	6.96

Since there are many proposed theoretical models in literature under both aerobic and anaerobic growth conditions, in our study we consider a theoretical possibility of our MFCs set-up to harness electricity from the electrons using a derivative of EET, the direct electron transfer (DET) method. Previous findings demonstrated that with a pure culture of *Shewanella oneidensis* MR-1, an MFC fed with a certain amount of oxygen could produce higher maximum current outputs^{142, 144, 145}. Subsequently, a recent study investigated the theoretical possibility of applying the metabolism of *Shewanella oneidensis* MR-1 under aerobic conditions to identify the maximum potential for electricity output in MFCs¹⁴⁶. Hence, our study continues this contribution by using our proposed framework. In order to understand the central metabolic pathways of MR-1 in MFCs setups, we consider measurements (growth rates, lactate metabolism and flux activities, etc.) in key reactions of the central metabolism under the theoretical possibility that MR-1 was allowed to grow under aerobic conditions with sufficient level of oxygen.

First, we consider the electron transfer to the anode to begin with *c*-type cytochromes denoted as CymA protein, which is the first electron conductor shuttling the electrons from the intracellular quinone pool to the next *c*-type cytochrome protein at MtrA. These multi-heme proteins, existing in the cell

membrane of *Shewanella oneidensis* MR-1, have also been shown to be responsible for reducing poisonous heavy metal ions through their carefully designed metabolism¹⁴⁷. MtrA is involved in the electron transfer to MtrC and OmcA by forming a stable configuration with β -barrel protein MtrB and OM bound *c*-type cytochromes MtrC on OM at a stoichiometric ratio of 1:1:1 in order to support the electron exchange through OM to the last OM-bound *c*-type cytochromes OmcA which then finally releases the electrons to the electrode^{53, 148, 149}.

In DET mode, because MFCs are designed to conduct electricity by continuously extracting electrons from all the participating membrane-bound proteins in Mtr pathway to the anode, extra metabolic pressure on specific cellular activities of MR-1 may exist in order to maintain the electricity. It is believed that MR-1 contains 42 different types of *c*-type cytochrome proteins possibly interacting with one another in its genome⁷⁹. A recent simulation suggested that the surplus flux of the reduced *c*-type cytochrome required in order to maintain that electricity in DET mode can be attributed to reaction 175 (ID: CYOO2) in iSO783¹⁴⁶. Past experimental results have suggested that electron transfer involved *c*-type cytochrome genes from CymA to MtrC involves reactions directed by either the small tetraheme cytochrome (STC) also known as CctA or the periplasmic fumarate reductase FccA^{150, 151}. Under anaerobic conditions in the presence of fumarate reductase, electron transfer can be directed by the FccA¹⁵². Furthermore, end products from lactate metabolism in MR-1 are also determined by the type of growth conditions, making it an important reference for our study. Under fully aerobic conditions, MR-1 utilizes the complete Tricarboxylic Acid (TCA) cycle and therefore is expected not to produce any metabolites from lactate other than CO_2 because the pyruvate produced from lactate should be completely oxidised into CO_2 . However under anaerobic conditions, MR-1 produces acetate as the main metabolite from lactate through the Pta-AckA pathway by oxidizing pyruvate to acetyl-CoA, CO_2 or Formate and then from Acetyl-CoA converted to Acetyl-Phosphate and to Acetate. Hence differences between aerobic and anaerobic

growth conditions can be studied by the different flux activity in Pta-AckA and TCA pathways.

3.6.2 Integrating Kinetic Model with FBA

Given the Monod equations are sufficiently reliable in simulating growth rate, we propose an optimized model which involves both FBA and the same set of Monod equations to predict accurately the metabolism of MR-1 by minimizing the errors between our predicted results (for Biomass, Acetate, Lactate and Pyruvate uptake/production) from the model and all the observed results from the 3 independent replicates of MR-1 growth culture earlier and then simultaneously optimizing a simulated study of electron transfer for MR-1 in DET mode for the design of MFCs. Previously, an integration of an optimized dual-objective Dynamic Flux Balance Analysis (dFBA) model with a set of Kinetic Monod equations to simulate an optimal growth rate with appropriate lactate, acetate and pyruvate consumption or production was proposed¹⁴¹. Here, we propose a method for triple-objective Monod-constrained dFBA (TOM-dFBA) model in order to accommodate a more complex optimization of three possibly competing objectives: growth rate, enzyme efficiency and electron transfer.

Given we have an initial value of growth rate determined with a set of Monod equations denoted as μ_{Monod} and a $M \times N$ stoichiometric matrix S which summarizes all N stoichiometric reactions of MR-1 with M metabolites, TOM-dFBA model is able to determine a new optimized growth rate μ_{FBA} , a new set of flux values $v = (v_1, \dots, v_i, \dots, v_N)$ (each in unit: mmol/g DCW/h) for all N reactions considered in the metabolism and $K \in \{1, \dots, N\}$ for some K th reaction such that,

$$\begin{aligned} & \min(\mu_{Monod} - \mu_{FBA})^2 \quad s.t. \\ & \min \left(w_1 \cdot \sum_i v_i^2 - (1 - w_1) \cdot c'v \right) \quad s.t. \\ & S \cdot v = 0, \end{aligned}$$

$$lb \leq v \leq ub \text{ and}$$

$$v_{lac_{inflow}}, v_{act_{outflow}}, v_{pyr_{outflow}} \text{ from Monod equations,}$$

$$0 \leq w \leq 1,$$

$$c' = (0, \dots, 0, w_2 \cdot g_K, 0, \dots, 0, 1 - w_2)$$

$$\Rightarrow c'v = (w_2 \cdot g_K)v_K + (1 - w_2)v_N.$$

The first minimization ensures that the differences between the predicted growth rates for both FBA and kinetic Monod models are kept minimal by a constraint. The second minimization problem ensures that there is minimal trade-off between three maximization objectives: growth rate, electron activity and enzyme efficiency. During the optimization, the reaction fluxes must satisfy the stoichiometric balance at $S \cdot v = 0$ with the reaction flux for lactate, acetate and pyruvate determined from the Monod equations. v_N is the growth reaction flux for MR-1 and v_K is the targeted flux for maximizing electron activity.

In the second optimization, we consider three objectives when determining an appropriate objective equation for it: (1) an objective representing the maximization of growth rate since micro-organism are experimentally proven to have evolved to optimize their metabolism to achieve maximal biomass production¹⁵³, (2) an objective representing the maximization of the production of an overflow flux of desirable reducing chemicals which can restore the redox balance of electron extraction process to support electricity activity in MFC and (3) an objective representing the maximization of enzyme efficiency by reducing the sum of all fluxes in the metabolic network so as to maintain minimal stress on the system¹⁴⁴. For enzyme efficiency to be minimized, TOM-dFBA minimizes the sum of squared of all reaction fluxes in the system for minimal system stress. Since previous simulation studies have shown that *c*-type cytochrome production shares a general inverse relationship with growth rate¹⁴⁶, this means that the triple-objective optimization would have to involve the use of a Pareto-optimal space to determine an appropriate solution balancing the three possibly conflicting objectives in the

modeling using weights¹⁵⁴. The Pareto-optimal space method ensures that there are no solutions other than its proposed solution, which can allow further optimization of an objective without making the other objectives worse off. Solutions from the Pareto-optimal space, are known to be as Pareto-efficient or Pareto-optimal. Given in Figure 3.2 where each point consists of 2 elements, one belonging to the f_1 and the other belonging to the f_2 coordinate, since point C is dominated by point A and point B, that is, point C is further from the nearest point A and point B to the f_1 and f_2 coordinate, it does not belong to the Pareto-optimal solution space. Clearly, point A and point B cannot dominate one another since both have at least one point which dominates over the other along either the f_1 and f_2 coordinate.

Figure 3.1 Illustration of set of determinable Pareto-optimal solutions. Blue boxes represent the Pareto-optimal solutions; Grey boxes represent solutions that are not Pareto-optimal solutions. All points illustrated here are solutions in $\mathbf{R}^2$ solution space with f_1 and f_2 axis.

3.6.3 Simulation of *Shewanella oneidensis* MR-1 growth

In our study, we consider V_N where $N = 775$ in our algorithm as the growth rate, v_N determined by our FBA model as μ_{FBA} and v_K as the targeted surplus flux for some reaction K in iSO783 which can produce the reducing compounds required to support maximum electron activity as μ_K . In DET, we consider our $K = 175$ which represents the flux for reaction CYOO2 involved in the reduction of a type of c -type cytochrome protein (denoted as Cco (SO2361 and SO2362 and SO2363 and SO2364) or Cyco (SO4606 and SO4607 and SO4609) in iSO783). CYOO2

contributes significantly to the predicted maximum *c*-type cytochrome production rate for electricity output through their FATMIN algorithm¹⁴⁶. g_K is included in the expression and is set as 0.8869g/mmol which is the molar mass of a *c*-type cytochrome protein in order to ensure that w_2 of $(w_2 \cdot g_K)v_K$ in the Pareto-optimal solution space is a unit-less relative contribution to the combined objective with respect to other competing objectives.

Following iSO783 for our dFBA model, our stoichiometric matrix S would have 774 stoichiometric reactions with $M = 634$ metabolites with an additional growth rate equation, making it $N = 775$ reactions. Furthermore, the optimization done on the 775 reactions would follow the same range of values constrained for each reaction implemented successfully in several other studies^{141, 146, 155} for iSO783 except with a few distinct modifications. Firstly, this optimization would be carried out over 408 time points spanning 34 hours in order to model transient states in MR-1 metabolism. Based on the SOA method, each time point is assumed to be an independent pseudo-steady state. Secondly, the maximum and minimum rate of lactate, acetate and pyruvate rates were determined from the Monod model to simulate the optimal growth of MR-1. In order to confirm the influence of an optimized CYOO2 reaction on the Mtr pathway in the simulation, we constructed simulations with different variations of unconstrained flow in all reactions directly involved in the protein components of the Mtr pathway from the iSO783 model. These protein components are CymA, MtrA, MtrB, MtrC and OmcA.

Under full aerobic growth we expect reaction 263, 264, 272, 273, 464, 465, 766 and 767 denoted in iSO783 with their corresponding reaction IDs: FER3, FER4, MNOR, MNOR2, URNR3 and URNR4 should still be involved in the Mtr pathway for electron transfer. Reaction CYOO2 is our target flux for the optimization electron activity for TOM-dFBA. For aerobic growth, reaction 272, 273 with IDs: FRD8 and FRD9 would be constrained to 0 in order to ensure that oxygen remains as the main electron acceptor in the TCA cycle since fumarate may replace oxygen as the main electron acceptor in anaerobic growth of MR-1¹⁵⁵ under the enzyme,

fumarate reductase in the same cycle. These reactions can be summarized as follows:

Table 3.4 Reactions involved in Mtr pathway (of iSO783)

No	ID	Stoichiometric Reaction
263	FER3	$\text{Fe3 [e]} + \text{mql7 [c]} \rightarrow 2\text{Fe2 [e]} + 2\text{H [e]} + \text{mqn7 [c]}$
264	FER4	$\text{Fe3 [e]} + \text{mmql7 [c]} \rightarrow 2\text{Fe2 [e]} + 2\text{H [e]} + \text{mmqn7 [c]}$
272	FRD8	$\text{fum [e]} + \text{mql7 [c]} \rightarrow \text{succ [e]} + \text{mqn7 [c]}$
273	FRD9	$\text{fum [e]} + \text{mmql7 [c]} \rightarrow \text{succ [e]} + \text{mmqn7 [c]}$
464	MNOR	$\text{Mn4o [e]} + \text{mql7 [c]} + 2\text{H [e]} \rightarrow \text{mn2 [e]} + \text{mqn7 [c]} + 2\text{h2o [e]}$
465	MNOR2	$\text{Mn4o [e]} + \text{mmql7 [c]} + 2\text{H [e]} \rightarrow \text{mn2 [e]} + \text{mmqn7 [c]} + 2\text{h2o [e]}$
766	URNR3	$\text{Urnyl [e]} + \text{mql7 [c]} \rightarrow \text{Urdio [e]} + \text{mqn7 [c]} + 2\text{H [e]}$
767	URNR4	$\text{Urnyl [e]} + \text{mmql7 [c]} \rightarrow \text{Urdio [e]} + \text{mmqn7 [c]} + 2\text{H [e]}$

Since these reactions are all forward and irreversible, we set their unconstrained flow as a range of [0, 100000]. Since TOM-dFBA requires the rate of Lactate, Acetate and Pyruvate from the Monod equations, the algorithm begins with optimizing the Monod equations for an optimal set of values for the parameters, which should produce a set of predicted results where its squared difference with the experimental results is minimized. From the optimal set of values for the parameters, TOM-dFBA applies the SOA method to divide the time period of 34 hours into 409 pseudo-steady states with increments of 1/12 in order to determine the transience flux of Lactate, Acetate and Pyruvate based on the Monod equations.

3.6.4 Methods of Assessment

One way to approach such a problem is to consider the behavior of flux with limited amount of resources, which tends to approach and maintain close proximity to the largest attainable flux until it dies down when the resources used to sustain the flux becomes depleted. The trend becomes a plateau over the time period when resources are only sufficiently enough to sustain the level of flux. By measuring the sum of distances between all points to the largest attainable flux value given by its optimization, it is expected that all other fluxes with at most such a sum of distances is highly possible to have the same trend and be optimized too. In this case, such a measurement is also not fully reliable since different fluxes have different maximum attainable values (based on same conditions, growth rate was measured highest at 0.5375 but reaction 175 has flux of up to 70) and the

magnitude of differences in one flux with a small maximum attainable value cannot be compared directly to the magnitude of differences in another flux with a huge maximum attainable value based on its raw difference from 0 or the highest attainable value. Hence normalizing the differences with an appropriate scale is important. Furthermore, since there are many optimizable reactions (in iSO783, there are 775 reactions), optimizing with a large number of fluxes individually or simultaneously to determine the reactions that can produce the greatest optimized flux is computationally exhausting with Pareto-optimal space method. Hence identifying at most 1-2 fluxes to be optimized is highly crucial.

For this purpose, we propose the ARC (Analysis of Rate change under Curve) framework which can be used to identify appropriate reactions that are potentially involved in the optimization. To determine the reactions that may produce the highest amount of electricity in MR-1, we propose the workflow as follows:

Optimize the entire Mtr pathway with all its 6 reactions involved. Determine the reactions with fluxes that have a larger sum of ratio of all points from the maximum flux value than at least one reaction from the Mtr pathway i.e. given flux B is the reaction to be tested, flux A is one of the reaction from the Mtr pathway, T is all the time points, and the denominator is the largest attainable flux value over all time points

$$\sum_{t \in T} \frac{f_A(t)}{\max_t f_A(t)} \leq \sum_{t \in T} \frac{f_B(t)}{\max_t f_B(t)}$$

A difficulty in optimization for MR-1 is to determine the least number of appropriate reactions to optimize with the weights from computationally intensive Pareto-efficient method. One approach to do so is to identify a group of related reactions such that the optimization of one could imply an optimization for others. Hence, this framework is motivated by the fact that given a reaction is optimized, then the flux of the reaction over time must have a sufficiently large number of points that are near to an unknown maximum attainable value of the optimized flux. Because the amount of resources is limited, the targeted flux may not always be

able to reach or stay at that unknown point throughout the experimental time period and so these points may not be able to reach or may tend to drop below this unknown point, resulting in a trend of a plateau. Thus, any other reactions that may have the same trend of plateau, given a reaction is optimized, must also be in some ways, optimized directly or indirectly by the influence of that optimized reaction. However, because highest value of that plateau may differ for different reactions due to different scale of flux, the comparison must be normalized. Each reaction is the sum of all ratios of points normalized by the highest obtainable value of its flux so that any reactions with a sum higher than any of the optimized reaction from the Mtr pathway are considered as potentially related reactions. Since every point is normalized by their highest obtainable value, the comparisons become relatively fair. Furthermore, this framework also identifies and rejects fluxes with unrealistically high values since they provide unrealistic stress on the system and metabolically inevitable in realistic settings.

CHAPTER 4 Engineering the lactate pathway in *Shewanella oneidensis* MR-1 for improved bioelectricity production

4.1 Introduction

The development of sustainable and renewable power supplies is one of the most pressing issues in modern society, due to the rapid depletion of fossil fuels. Microbial electrochemical techniques, such as microbial fuel cells (MFCs), offer an attractive solution because they can couple the production of bioelectricity with processes such as bioremediation or wastewater treatment ^{3, 156-160}. *Shewanella oneidensis* is one of the most widely used microbial hosts in MFCs due to its versatile metabolic capabilities ^{46, 58, 161, 162}. It is capable of oxidizing different organic compounds and transferring intracellular electrons through its cell membrane to the anode of the MFC, thereby resulting in the flow of an electrical current.

Despite their obvious potential, present-day MFCs have a limited range of industrial applications and are not widely adopted largely because they suffer from a low power output. In order to enhance the bioelectricity production capability of MFCs, researchers have genetically modified *Shewanella* bacteria in different ways. One laboratory introduced key glycolysis enzymes absent from the *Shewanella* genome back into the MR-1 strain to enable the bacteria to metabolize glucose as the sole carbon source and to generate bioelectricity from glucose under anaerobic conditions ²⁹. Another laboratory heterologously overexpressed a light-dependent proton pump, proteorhodopsin, in MR-1, which led to light-induced changes in membrane potential and a concomitant increase in lactate uptake and bioelectricity production ³⁰. We have also previously adopted various genetic engineering approaches to improve the performance of *Shewanella* in MFCs, including increasing the membrane permeability of electron shuttles by expressing a foreign porin gene ¹⁶³, enhancing biofilm formation on the anode by overexpressing a c-di-GMP synthase *ydeH* ¹⁶⁴, and enhancing extracellular electron transfer to the anode by reconstructing a synthetic flavin biosynthesis pathway in MR-1 ¹⁶⁵.

Figure 4.1 An overview of lactate metabolism in bacteria.

(A) Schematic depicting the passage of lactate through the cell membrane and its subsequent oxidation into pyruvate.

(B) The general lactate respiratory pathway in *S. oneidensis* MR-1. Under anaerobic conditions, the L-lactate substrate is oxidized to acetate and carbon dioxide, during which electrons are also released.

Shewanella bacteria demonstrate a strong preference for two- or three-carbon compounds, such as L-lactate, as energy sources. L-lactate crosses the outer membrane of *Shewanella* from extracellular space into the periplasm by passive diffusion (Figure 4.1A). Subsequently, it enters the cytoplasm through the lactate

permease (LldP) by facilitated diffusion. Afterwards, intracellular L-lactate is converted into pyruvate by the enzyme L-lactate dehydrogenase (Ldh) and then mainly converted into acetyl-CoA by the enzyme pyruvate dehydrogenase. In aerobic conditions, acetyl-CoA enters the TCA cycle to provide the cell with energy and biomass. However, in anaerobic conditions, it can be converted to acetate and ATP with the release of four electrons (Figure 4.1B) ¹⁶⁶. The electrons are delivered to and stored within the cytoplasmic membrane in a menaquinone pool and can be further transferred to external electron acceptors, such as electrode surfaces in MFCs, in which case bioelectricity will be generated ^{104, 167-171}.

In this work, we explored the feasibility of engineering the lactate metabolic network to enhance bioelectricity production. Based on the L-lactate consumption pathway, we focused on testing genes encoding the lactate permease or the lactate dehydrogenase from different bacterial species in the *Shewanella* strain MR-1.

4.2 Experiments

4.2.1 Bacterial strains, plasmids, and culture conditions

All strains and plasmids used in this study are given in Table 4.1. The *E. coli* and *S. oneidensis* MR-1 strains were routinely maintained and cultured in Luria-Bertani (LB) medium at 37°C and 30°C respectively. When necessary, the medium was supplemented with either kanamycin (50µg/ml) or ampicillin sodium (100µg/ml). In *E. coli* WM3064 culture, a final concentration of 0.3mM 2,6-Diaminopimelic acid (DPA) was added. The *S. oneidensis* MR-1 experiments were performed in *Shewanella* basal medium ¹⁰⁹ with 15mM L-lactate acting as the electron donor and carbon source. When needed, 40mM fumarate was supplemented as the electron acceptor.

Table 4.1 Bacterial strains and plasmids used in this chapter.

<i>Strains</i>	<i>Description</i>	<i>Reference or source</i>
<i>E. coli</i>		
<i>DH5α</i>	Host for regular cloning	Lab stock
<i>WM3064</i>	Donor strain used for conjugation with <i>S. oneidensis</i> MR-1, <i>AdapA</i>	Lab stock
<i>S. oneidensis</i>		

MR-1	Wildtype	Lab stock
pHG101	MR-1 harboring pHG101, used as control in this study; Km ^r	This study
SM-1	MR-1 harboring pM- <i>lldP-lldEFG</i>	This study
SM-2	MR-1 harboring pM- <i>lldEFG</i>	This study
SM-3	MR-1 harboring pM- <i>lldP</i>	This study
SE-1	MR-1 harboring pE- <i>lldP-lldD-ykgEFG</i>	This study
SE-2	MR-1 harboring pE- <i>lldD-ykgEFG</i>	This study
SE-3	MR-1 harboring pE- <i>lldP</i>	This study
SB-1	MR-1 harboring pB- <i>lctP-yvfV-yvfW-yvbY</i>	This study
SB-2	MR-1 harboring pB- <i>yvfV-yvfW-yvbY</i>	This study
SB-3	MR-1 harboring pB- <i>lctP</i>	This study
SP-1	MR-1 harboring pP- <i>lldP-lldD</i>	This study
SP-2	MR-1 harboring pP- <i>lldD</i>	This study
SP-3	MR-1 harboring pP- <i>lldP</i>	This study
<hr/>		
<i>Plasmids</i>		
pHG101	Promoterless vector derived from broad host range vector pBBR1MCS-2; Km ^r	¹³⁹
pSB1A2-Plac	Biobrick containing <i>lac</i> promoter (BBa_R0011, iGEM) in pSB1A2 plasmid backbone; Amp ^r	Lab stock
pSB1AK3-TT	Biobrick containing double terminators (BBa_B0015, iGEM) in pSB1AK3 plasmid backbone; Km ^r	Lab stock
SH1	Synthetic gene containing SO_1522 (<i>lldP</i>) in pEasyBlunt vector; Km ^r	This study
SH3	Synthetic gene containing SO_1520 (<i>lldE</i>) in pEasyBlunt vector; Km ^r	This study
SH4	Synthetic gene containing SO_1519 (<i>lldF</i>) in pEasyBlunt vector; Km ^r	This study
SH5	Synthetic gene containing SO_1518 (<i>lldG</i>) in pEasyBlunt vector; Km ^r	This study
SH7	Synthetic gene containing codon optimized PA4771 (<i>lldD</i>) in pEasyBlunt vector; Km ^r	This study
SH8	Synthetic gene containing codon optimized PA4770 (<i>lldP</i>) in pEasyBlunt vector; Km ^r	This study
SH10	Synthetic gene containing codon optimized Y75_p3569 (<i>lldD</i>) in pEasyBlunt vector; Km ^r	This study
SH11	Synthetic gene containing codon optimized Y75_p3571 (<i>lldP</i>) in pEasyBlunt vector; Km ^r	This study
SH12	Synthetic gene containing codon optimized Y75_p0297 (<i>ykgE</i>) in pEasyBlunt vector; Km ^r	This study
SH13	Synthetic gene containing codon optimized Y75_p0298 (<i>ykgF</i>) in pEasyBlunt vector; Km ^r	This study
SH14	Synthetic gene containing codon optimized Y75_p0299 (<i>ykgG</i>) in pEasyBlunt vector; Km ^r	This study
SH15	Synthetic gene containing codon optimized BSU03050 (<i>ldh</i>) in pEasyBlunt vector; Km ^r	This study
SH16	Synthetic gene containing codon optimized BSU03060 (<i>lctP</i>) in pEasyBlunt vector; Km ^r	This study
SH17	Synthetic gene containing codon optimized BSU34030 (<i>yvbY</i>) in pEasyBlunt vector; Km ^r	This study

SH18	Synthetic gene containing codon optimized BSU34050 (<i>yvfV</i>) in pEasyBlunt vector; Km ^r	This study
SH19	Synthetic gene containing codon optimized BSU34040 (<i>yvfW</i>) in pEasyBlunt vector; Km ^r	This study
pM- <i>lldP-lldEFG</i>	pHG101 harboring SO_1522 (<i>lldP</i>), SO_1520 (<i>lldE</i>), SO_1519 (<i>lldF</i>) and SO_1518 (<i>lldG</i>)	This study
pM- <i>lldEFG</i>	pHG101 harboring SO_1520 (<i>lldE</i>), SO_1519 (<i>lldF</i>) and SO_1518 (<i>lldG</i>)	This study
pM- <i>lldP</i>	pHG101 harboring SO_1522 (<i>lldP</i>)	This study
pE- <i>lldP-lldD-ykgEFG</i>	pHG101 harboring Y75_p3571 (<i>lldP</i>), Y75_p3569 (<i>lldD</i>), Y75_p0297 <i>ykgE</i> , Y75_p0298 <i>ykgF</i> and Y75_p0299 <i>ykgG</i>	This study
pE- <i>lldD-ykgEFG</i>	pHG101 harboring Y75_p3569 (<i>lldD</i>), Y75_p0297 (<i>ykgE</i>), Y75_p0298 (<i>ykgF</i>) and Y75_p0299 (<i>ykgG</i>)	This study
pE- <i>lldP</i>	pHG101 harboring Y75_p3571 (<i>lldP</i>)	This study
pB- <i>lctP-yvfV-yvfW-yvbY</i>	pHG101 harboring BSU03060 (<i>lctP</i>), BSU34050 (<i>yvfV</i>), BSU34040 (<i>yvfW</i>) and BSU34030 (<i>yvbY</i>)	This study
pB- <i>yvfV-yvfW-yvbY</i>	pHG101 harboring BSU34050 (<i>yvfV</i>), BSU34040 (<i>yvfW</i>) and BSU34030 (<i>yvbY</i>)	This study
pB- <i>lctP</i>	pHG101 harboring BSU03060 (<i>lctP</i>)	This study
pP- <i>lldP-lldD</i>	pHG101 harboring PA4770 (<i>lldP</i>) and PA4771 (<i>lldD</i>)	This study
pP- <i>lldD</i>	pHG101 harboring PA4771 (<i>lldD</i>)	This study
pP- <i>lldP</i>	pHG101 harboring PA4770 (<i>lldP</i>)	This study
pP- <i>lldD-lldP</i>	pHG101 harboring PA4771 (<i>lldD</i>) and PA4770 (<i>lldP</i>)	This study

4.2.2 Plasmid constructions

All genetic manipulation work was performed using standard protocols. Gene sequences encoding L-lactate permease and L-lactate dehydrogenase from *Pseudomonas aeruginosa*, *E. coli*, and *Bacillus subtilis* were obtained from KEGG¹⁷² and codon optimized for expression in *S. oneidensis* MR-1 by using JCat online codon adaptation tool (www.jcat.de)¹⁷³. Cleavage sites for the restriction enzymes EcoRI, XbaI, SpeI and PstI were avoided during this process. Each of the optimized sequences was designed as a biobrick¹⁷⁴, flanked by an upstream prefix containing EcoRI and XbaI and a downstream suffix containing SpeI and PstI. A ribosome binding site (BBa_B0034, iGEM) was also introduced 6 base pairs in front of the start codon of each optimized gene sequence. The designed gene sequences were synthesized *in vitro*, verified by Sanger sequencing, and inserted into the pEasyBlunt vector (Genewiz, China), as shown in Figure 4.2A. All synthesized sequences are given in Appendix A. The lactate pathway was then assembled through routine steps of biobrick ligation, inserting a *lac* promoter

(BBa_R0011, iGEM) in front and a double terminator (BBa_B0015, iGEM) at the back to regulate gene expression, forming a *Plac*-genes-TT construct in the vector pSB1AK3 (iGEM). Finally, by using the restriction enzymes EcoRI and SpeI, the *Plac*-genes-TT fragment was cut and ligated into the broad-host-range expression vector pHG101¹³⁹. The *E. coli* strain DH5 α was used to amplify each plasmid. Every plasmid was confirmed by sequencing. Each of the final recombinant pHG101-based plasmids was transformed into *E. coli* WM3064 and subsequently introduced into *S. oneidensis* MR-1 via conjugation. Successful conjugation was confirmed by colony PCR.

Figure 4.2 Construction of synthetic lactate metabolic pathways in *S. oneidensis*.
(A) Schematic representation of a plasmid with a synthesized functional gene fragment. The restriction sites EcoRI (E) and XbaI (X) together with a ribosome binding site (RBS) are located upstream of each codon-optimized gene sequence, while the restrictions SpeI (S) and PstI (P) are located downstream of the gene.
(B) Layout of the various constructs containing lactate pathway components tested in this study.

4.2.3 Lactate consumption assays of the engineered *S. oneidensis* MR-1 strains

At each time point, after removing the bacterial cells, we evaluated L-lactate consumption by measuring the amount of L-lactate remaining in the culture medium with the L-Lactate Assay Kit (AAT Bioquest, Sunnyvale, CA). Briefly, a 1% inoculum of an overnight stationary phase culture was used to start fresh cultures grown in *Shewanella* basal medium, supplemented with 15mM L-lactate and 40mM fumarate. A final concentration of 50 μ g/ml kanamycin was added if required. For each reading, a volume of 200 μ L culture was centrifuged to remove the insoluble contents. 50 μ L of the test sample was mixed with 50 μ L L-lactate assay mixture in a 96-well plate to form an L-lactate assay volume of 100 μ L/well. The reaction was maintained at room temperature for half an hour before absorbance ratio was measured at the wavelength of 575nm/605nm using a microplate reader.

4.2.4 Microbial fuel cell setup and operation

All experiments were performed using dual-chamber MFCs. The working volume of each of the chambers is 150mL. A Nafion 117 membrane (Gashub, Singapore) was used to separate the anode and cathode chambers after it had been immersed overnight in 0.5M sulfuric acid and washed with DI water. Electrodes for both anode and cathode were made of carbon cloth (Gashub, Singapore) with a size of 2cm $\times$ 3cm. Cathodic solution consisted of 50mM K₃[Fe(CN)₆], 50mM K₂HPO₄, and 50mM KH₂PO₄.

Shewanella strains were grown overnight in a 30°C incubator at a shaking rate of 200rpm. Cell suspensions were adjusted to an optimal density of 0.5 before adding into the anode chamber. The anodes were purged with nitrogen to remove oxygen. After inoculation, the circuit was connected by copper wire with a constant external resistance of 2k Ω . The MFCs were operated at room temperature in batch mode. The duration of a full cycle was determined by the consumption of substrate and voltage reduction from its peak value to 50mV.

4.2.5 Data acquisition and calculations

The voltage output of each MFC was measured using a digital multimeter. To evaluate MFC performance, polarization curves were obtained by applying different loads or external resistances (R_{ext}) and then measuring the voltage (V) in order to calculate the current (I) by Ohm's Law ². By normalizing power based on the surface area (A) of the anode electrode, power density (P) can be calculated by using the formula, $P = \frac{V \times I}{A}$.

4.2.6 Electrochemical analysis

Electrochemical measurements were performed using an electrochemical working station (CHI760D, CH Instruments Inc.) employing a three-electrode system. Saturated calomel electrode (SCE) was used as the reference electrode, with the anode as the working electrode and the cathode as the counter electrode. Cyclic voltammetry (CV) was carried out with a scanning range of -0.7 to 0.2V.

4.3 Results and discussion

4.3.1 Overexpression of *lldP* and *lldD* genes increased rate of lactate consumption

To perturb the lactate metabolic network, we focused on the upstream steps, whereby the carbon source L-lactate was transported across the cell membrane and converted into pyruvate by the lactate permease and lactate dehydrogenase respectively. The original genes from *S. oneidensis* MR-1 for L-lactate transport (SO_1522) and conversion (SO_1518-SO_1521) ¹⁷⁵ as well as genes encoding the permease and dehydrogenase from other species, including *Escherichia coli* (Y75_p3571, Y75_p3569, Y75_p0297-Y75_p0299), *Bacillus subtilis* (BU03050, BU03060, BU34030, BU34040, BU34050), and *Pseudomonas aeruginosa* (PA4770, PA4771), were selected for this study. We overexpressed in *S. oneidensis* MR-1 either the permease or dehydrogenase singly or both at once under the control of a *lac* promoter. In total, we constructed 12 distinct MR-1 strains for detailed characterization (Figure 4.2B, Table 4.3).

Table 4.2 Related genes in the lactate pathway used in this chapter.

Organism	Strain name	Gene	Description
<i>Shewanella oneidensis</i> MR-1	SH1	<i>lldP</i>	lactate transport
	SH2	<i>dld-II</i>	respiratory FAD-dependent D-lactate dehydrogenase Dld
	SH3	<i>lldE</i>	L-lactate dehydrogenase
	SH4	<i>lldF</i>	L-lactate dehydrogenase iron-sulfur cluster-binding protein LldF
	SH5	<i>lldG</i>	L-lactate dehydrogenase complex protein
<i>Pseudomonas aeruginosa</i> PAO-1	SH6	<i>ldhA</i>	D-lactate dehydrogenase
	SH7	<i>lldD</i>	L-lactate dehydrogenase
	SH8	<i>lldP</i>	L-lactate permease
<i>Escherichia coli</i> K-12 W3110	SH9	<i>dld</i>	D-lactate dehydrogenase
	SH10	<i>lldD</i>	L-lactate dehydrogenase
	SH11	<i>lldP</i>	L-lactate permease
	SH12	<i>ykgE</i>	oxidoreductase
	SH13	<i>ykgF</i>	amino acid dehydrogenase
	SH14	<i>ykgG</i>	transporter
<i>Bacillus subtilis</i> subsp. subtilis 168	SH15	<i>ldh</i>	L-lactate dehydrogenase
	SH16	<i>lctP</i>	L-lactate permease
	SH17	<i>yvbY</i>	lactate utilization protein C
	SH18	<i>yvfV</i>	lactate utilization protein A
	SH19	<i>yvfW</i>	lactate utilization protein B

Figure 4.3 Characterization of MR-1 strains overexpressing lactate permease or lactate dehydrogenase genes from different bacteria (n=3).

(A) Growth curve measurements of the strains under anaerobic conditions.

(B) Lactate consumption assays of the strains under anaerobic conditions

(C) Voltage outputs of MFCs inoculated with various strains in the anodic chamber.

We first checked the growth of the 12 engineered MR-1 strains relative to a control strain carrying an empty pHG101 plasmid in *Shewanella* basal medium (SBM). We observed that the growth of bacteria strains overexpressing both the lactate permease gene and the lactate dehydrogenase gene from *Pseudomonas* or *E. coli* (i.e. strains SP-1 and SE-1) are significantly improved compared to that of the control strain (Figure 4.3A). In contrast, the strains overexpressing only the lactase permease gene from *E. coli*, *Shewanella*, or *B. subtilis* (i.e. strains SE-3, SM-3 and SB-3) exhibited poorer growth than the control.

The improved growth may be attributed to an increased consumption of lactate as the carbon source. To verify the hypothesis, we examined the concentration of lactate in the culture media over time (Figure 4.3B). As expected, the amount of lactate remaining in the media decreased with time for all strains and the metabolite was essentially exhausted after 36 hours of incubation, confirming that MR-1 could consume lactate as the carbon source. More importantly, we found that the engineered strains, SP-1 and SE-1, showed the fastest rate of lactate consumption, whereas the strains SM-3 and SB-3 consumed lactate even more slowly than the control. Hence, the results from our lactate consumption assays are in good agreement with our growth curve measurements.

4.3.2 Enhanced lactate metabolism improved bioelectricity generation

Next, we sought to determine whether an increase in lactate consumption could lead to an enhancement of power output from MFCs. To this end, we set up MFCs with all the different inoculum strains and operated them in batch mode. Overall, the voltage output of all MFCs increased until a variable peak value and then decreased at a slower pace to background levels (Figure 4.3C). We observed that the voltage output peak values concur well with the growth curves (Figure 4.3A) and the lactate consumption rates (Figure 4.3B). The strains that overexpressed only the lactase permease gene, namely SP-3, SE-3, SM-3 and SB-3, performed worse than the control strain harboring an empty pHG101 vector. Notably, MFCs containing the SP-1 strain, which heterologously overexpressed both the permease

and dehydrogenase genes from *Pseudomonas*, produced a maximum voltage of $299 \pm 15 \text{ mV}$ at 88h, which was much higher than that of the control ($192 \pm 10 \text{ mV}$ at 160h). We calculated the total charge transferred by integrating the area enclosed by the V-t curve and then dividing it by the external resistance (Table 4.4). Coulombic efficiencies were also obtained as previously described^{2, 176}. Again, the MFCs with the SP-1 strain discharged the most electrons and exhibited the highest Coulombic efficiency. Hence, given the consistency in results among the growth curve measurements, lactate consumption assays, and MFC experiments, we focused our subsequent investigations on the SP-1 strain.

Table 4.3 The total charge transferred and columbic efficiency achieved in MFC anodes.

Anode	Electrons discharged (C)	Coulombic efficiency (%)
pHG101	67.45	7.77
SM-1	111.12	12.80
SM-2	106.03	12.22
SM-3	52.32	6.03
SE-1	116.31	13.36
SE-2	108.31	12.48
SE-3	58.00	6.68
SB-1	103.26	11.90
SB-2	100.21	11.54
SB-3	51.35	5.92
SP-1	129.03	14.87
SP-2	111.03	12.79
SP-3	88.62	10.21

Table 4.4 Mediated electron transfer-based (MET) and direct electron transfer-based (DET) catalytic current densities with different *S. oneidensis* strains.

Anode	MET (mA/m ²)	DET (mA/m ²)
pHG101	22.49	46.06
SP-1	65.06	124.00

A **B**

Figure 4.4 Electrochemical characterization of MFCs with inoculations of SP-1 or the control strain carrying an empty pHG101 vector at different scanning rates. **(A)** Representative CV curves of SP-1 and the control strain at a scan rate of 50mV/s. **(B)** CV curves of MFCs inoculated with SP-1 obtained under various scanning rates ranging from 1mV/s to 1000mV/s. **(C)** A linear relationship between peak current density and scanning rates.

To investigate the mechanism of enhanced extracellular electron transfer (EET), we utilized cyclic voltammetry (CV) to investigate the redox reaction on cell-electrode interfaces. At a scan rate of 50mV/s, one typical pair of oxidized and reduced peaks, centered at -0.3V vs. SCE, was identified under non-turnover condition for both SP-1 and the control strain (Figure 4.4A). These peaks corresponded to the outer membrane *c*-type cytochromes and their precise positions could vary depending on the microenvironment¹⁷⁷. Subsequently, we analyzed the peak current density with respect to the scanning rate for our engineered SP-1 strain (Figure 4.4B). The height of the peak current (centered at -0.3V vs. SCE) was linear with the scanning

rate ($R^2 = 0.999$) (Figure 4.4C), indicating the presence of outer membrane *c*-type cytochromes^{109, 178}. Additionally, the heights of the anodic peaks were lower than the cathodic peaks, which may be due to a partially irreversible redox reaction on the electrodes^{109, 179, 180}.

Various EET pathways can influence the electrochemical behavior of the *Shewanella* biofilm on the electrode. To dissect the effects of the different pathways on SP-1 and control biofilms, we conducted CV analysis at a low scan rate of 1mV/s under turnover condition (Figure 4.5A). For both strains, the anodic curve first rose above background at -0.59V vs. SCE due to the occurrence of shuttle-mediated electron transfer (MET) via flavins^{181, 182}. This shuttle-mediated catalytic current was 64.88mA/m² and 22.49mA/m² for SP-1 and the control strain respectively. Additionally, a second catalytic current arose at -0.36V vs. SCE for both strains due to the occurrence of contact-based direct electron transfer (DET) via the outer-membrane *c*-type cytochromes. Based on the first-derivative of the curves¹⁸³, we determined that this cytochrome-mediated current was 123.22mA/m² and 46.06mA/m² for SP-1 and the control strain respectively. Hence, the enhanced EET observed for our engineered SP-1 strain relative to control was caused by an increase in both MET and DET.

We obtained polarization curves at the peak output voltages to further characterize the performance of the MFCs (Figure 4.5B). The slope of SP-1's voltage-current plot was less steep than that of the control. From Ohm's law, this implied that there was a lower internal resistance in the MFCs with SP-1 in the anodic chamber, probably due to the enhanced electron transfer. Furthermore, based on the power density curves (Figure 4.5C), the short circuit current densities were 363.89mA/m² and 220.83mA/m² for SP-1 and the control strain respectively. Importantly, SP-1 achieved a maximum power density of 78.54±1.67mW/m², which was 2.1 times of that attained by the control strain (37.62±1.93mW/m²). Consistent with our earlier results, the highest currents were achieved by the strain SP-1 (Table 4.5). This strain gave a MET-based current and a DET-based current that were 189.3% and 169.2% higher than the control respectively. Taken together, our data highlight that

overexpression of the lactate permease and lactate dehydrogenase genes from *Pseudomonas* in *Shewanella* MR-1 could substantially enhance the performance of MFCs.

Figure 4.5 Detailed electrochemical characterization of MFCs with inoculations of SP-1 or the control strain at a slow scan rate of 1mV/s under turnover condition.

(A) Representative CV curves of SP-1 and the control strain.

(B) Output voltage vs. current density (V-j) curves of SP-1 and the control strain.

(C) Power density vs. current density (P-j) curves of SP-1 and the control strain.

4.4 Chapter summary

L-lactate is one of the primary carbon sources of *Shewanella* and an important electron donor in MFCs. A limited lactate consumption rate can be a major drawback in the usage of *Shewanella* in MFCs to generate bioelectricity. This work was initiated to improve the performance of the MR-1 strain in MFCs by enhancing the exoelectrogen's ability to metabolize lactate and eventually transfer more electrons to the electrode. To perturb the lactate metabolic network, we overexpressed either the lactate permease gene or the lactate dehydrogenase gene from different bacteria species and assayed the resulting strains for their growth rates, lactate consumption rates, and bioelectricity output. The best improvement was observed when we heterologously overexpressed both the *lldP* and the *lldD* genes from *Pseudomonas*, which led to a 2.1-fold increase in the power density of MFCs relative to control. Interestingly, we also found that overexpression of a

lactate permease gene alone frequently resulted in poorer growth, a slower rate of lactate consumption, and lower bioelectricity generation. This might be because although there is an increase in lactate entering the cell, this intracellular lactate could not be converted fast enough to pyruvate and hence is causing cell toxicity or is shunted off to other metabolic pathways, thereby resulting in flux imbalances.

CHAPTER 5 Generating mutagenesis libraries for the screening of positive *lldD* mutants

5.1 Introduction

In the work presented in Chapter 4, the SP-1 strain heterologously overexpressing both the *lldP* and the *lldD* genes from *Pseudomonas* showed the best improvement, which led to a 2.1-fold increase in the power density of MFCs relative to control. We asked whether the SP-1 strain could be optimized to generate even more bioelectricity. To this end, we randomly mutagenized the lactate dehydrogenase gene (*lldD* or PA4771) and the lactate permease gene (*lldP* or PA4770) from *Pseudomonas* by error-prone PCR. We varied the amount of PCR template and the number of PCR cycles to achieve different mutation frequencies. Out of all the individual clones that were fully sequenced, a total of 24 *lldD* mutants and 13 *lldP* mutants did not carry any frameshift mutations that might inactivate protein function (Table 5.1). Hence, we selected these mutants for further study.

We uncovered a set of mutations in the *lldD* gene that was able to boost the power density of MFCs to 2.8-times that of control cells. And we conducted a few approaches such as quantitative real-time reverse transcription PCR, SEM biofilm imaging as well as protein structure prediction analysis in order to further understand how the selected mutant strain could make more enhancement in the bioelectricity generation and MFCs performance. We have found that the improved lactate consumption in MR-1 could elevate the level of metabolism in a broader range, and increased expression levels of genes relevant to the electron production and electron transfer pathways. We have also discovered that the biofilm formation on the anode electrode surface had been significantly improved compared with the control strain, which further improved the extracellular electron transfer in MFCs operations.

Table 5.1 Mutations of PA4771 and PA4770 examined in this chapter. *

Name	Mutated residues															
PA4771 mutations																
MSH7-M-1	I62T	T85A	L90V	G121D	H122R	V255D	N261D									
MSH7-M-2	A103D	A107T	S119F	A185D	G252S	G368S										
MSH7-M-3	I117V															
MSH7-M-4	S6Y	R10P	Q14K	L60S	A95D	F126L	K132I	R138P	A177T	W193R	W233S	V271F	T311I			
MSH7-M-5	R310S															
MSH7-M-6	D167G	S208T	E354D													
MSH7-M-7	R10H	A31T	I113F	P159L	S170Y	K200M	Deletion on TAA to AA				Next stop 21 aa away					
MSH7-M-8	A12V	D194N	N213D	L280F	R310H											
MSH7-M-9	S170P	R180G	I240N	H275Q	P72Q	A76V										
MSH7-M-10	F103L	L198F	S231Y	A301V	M318V											
MSH7-M-11	F103L	L198F	S231Y	A302V	M318V											
MSH7-M-12	K53I	A119T	P214T													
MSH7-M-13	P19S	A31D	R36C	L64I	L128S	A146G	A292S	V333I	M358I							
MSH7-M-17	A13P	A29T	A93P	N139I												
MSH7-M-18	T151A	S231T	V271A													
MSH7-M-20	N38I	A95V	P118S	P230S	V344I											
MSH7-H-2	P18S	F154S	T187I	E218K	R310C	T311A										
MSH7-H-4	G27C	A74S	A76D	A85S	N139D	W223-	A323T	A372T								
MSH7-H-5	P18Q	R122P	R134H	S170F	A296D	G329C	A338T	R356G								
MSH7-H-11	G27C	A74S	A76D	A85S	N139D	W223-	A323T	A372T								
MSH7-H-12	P18Q	A168T	H169R	W234-	I270T	A338D	G343S									
MSH7-H-13	D8N	D26N	V51-	R94H	A96T	N139I	A226T	A267T	G308S	K353I	G363A					
MSH7-H-15	D26I	Q49R	T81I	G88A	K145I	D157Y	D203V	D306L	S307V	G308S	R310L	T311V	G312M			
	D314F	V316S	R317L	G322-	A323-	D324-	S325-	G329V	R330L	A331S	V333T	A339K	G340L			
	E341V	G343V	V344T	R345Y	L347S	L348K	E349K	L350K	E352V	K353L	E354L	R356F	V360N			
	G363-	G368L	E369I	S371F	D373N	S374V	V376X	R377-	E378-	L379-	G380-	A381-				
MSH7-H-16	E32H	Y33-	Q49L	R87C	A119T	K132I	G174A	L183S	L224S	A335T	V376D	L379-				
PA4770 mutations																
MSH8-L-1	A57T	G48C														
MSH8-L-2	I64M	S152N														
MSH8-L-3	A519V															
MSH8-L-4	M64V	P79S	F89I	G283S												
MSH8-L-6	A42D	T384I	A450T													
MSH8-L-7	T142N	Q198*														
MSH8-L-8	S17F	-21.5D	A50T	A289T												
MSH8-M-1	L105V	F120L	V169A	I190K	D183G											
MSH8-M-2	I88V	S136G	S201F	D231G	V279A	S290G	T337P	A339G	I484T							
MSH8-M-6	A180V	A235T	S298F	A401T	A542T											
MSH8-H-3	V95D	N243I	D251Y	Q355-	T382A	L459G	G460-	G289S	S510F	A517D						
MSH8-H-4	W78R	G112R	A163T	G219V	G290S	Q355-	T406N	E414K	G463D	A520T						
MSH8-H-11	L37F	A57T	L259F	I309V	T384S	A442S	G503A									

(*Note: X100- indicates that amino acid X at position 100 has been deleted. Similarly, -100.5X indicates that amino acid X has been inserted between the protein's 100th and 101st residues.)

5.2 Experiments

5.2.1 Bacterial strains, plasmids, and culture conditions

All strains and plasmids used in this study are given in Table 5.2. The *E. coli* and *S. oneidensis* MR-1 strains were routinely maintained and cultured in Luria-Bertani

(LB) medium at 37°C and 30°C respectively. When necessary, the medium was supplemented with either kanamycin (50µg/ml) or ampicillin sodium (100µg/ml). In *E. coli* WM3064 culture, a final concentration of 0.3mM 2,6-Diaminopimelic acid (DPA) was added. The *S. oneidensis* MR-1 experiments were performed in *Shewanella* basal medium¹⁰⁹ with 15mM L-lactate acting as the electron donor and carbon source. When needed, 40mM fumarate was supplemented as the electron acceptor.

Table 5.2 Bacterial strains and plasmids used in this chapter.

Strains	Description	Reference or source
<i>E. coli</i>		
DH5α	Host for regular cloning	Lab stock
WM3064	Donor strain used for conjugation with <i>S. oneidensis</i> MR-1, <i>AdapA</i>	Lab stock
<i>S. oneidensis</i>		
MR-1	Wildtype	Lab stock
pHG101	MR-1 harboring pHG101, used as control in this study; Km ^r	This study
SP-1	MR-1 harboring pP- <i>lldP-lldD</i>	This study
SP-2	MR-1 harboring pP- <i>lldD</i>	This study
SP-3	MR-1 harboring pP- <i>lldP</i>	This study
7M1~20	MR-1 harboring PA4771 mutants with medium mutagenesis efficiency in pHG101	This study
7H1~16	MR-1 harboring PA4771 mutants with high mutagenesis efficiency in pHG101	This study
7M1~20-SH8	MR-1 harboring PA4771 mutants with medium mutagenesis efficiency and wildtype PA4770 in pHG101	This study
7H1~16-SH8	MR-1 harboring PA4771 mutants with high mutagenesis efficiency and wildtype PA4770 in pHG101	This study
8L1~8	MR-1 harboring PA4770 mutants with low mutagenesis efficiency in pHG101	This study
8M1~6	MR-1 harboring PA4770 mutants with medium mutagenesis efficiency in pHG101	This study
8H1~11	MR-1 harboring PA4770 mutants with high mutagenesis efficiency in pHG101	This study
8L1~8-SH7	MR-1 harboring PA4770 mutants with low mutagenesis efficiency and wildtype PA4771 in pHG101	This study
8M1~6-SH7	MR-1 harboring PA4770 mutants with medium mutagenesis efficiency and wildtype PA4771 in pHG101	This study
8H1~11-SH7	MR-1 harboring PA4770 mutants with high mutagenesis efficiency and wildtype PA4771 in pHG101	This study
SH7(D167G)-SH8	MR-1 harboring pP- <i>lldD-lldP</i> with mutation site D167G in <i>lldD</i>	This study
SH7(S208T)-SH8	MR-1 harboring pP- <i>lldD-lldP</i> with mutation site S208T in <i>lldD</i>	This study
SH7(E354D)-SH8	MR-1 harboring pP- <i>lldD-lldP</i> with mutation site E354D in <i>lldD</i>	This study

SH7(S208T,E354D)-SH8	MR-1 harboring pP- <i>lldD</i> - <i>lldP</i> with mutation sites S208T and E354D in <i>lldD</i>	This study
<i>Plasmids</i>		
pHG101	Promoterless vector derived from broad host range vector pBBR1MCS-2; Km ^r	¹³⁹
pSB1A2-Plac	Biobrick containing <i>lac</i> promoter (BBa_R0011, iGEM) in pSB1A2 plasmid backbone; Amp ^r	Lab stock
pSB1AK3-TT	Biobrick containing double terminators (BBa_B0015, iGEM) in pSB1AK3 plasmid backbone; Km ^r	Lab stock
SH7	Synthetic gene containing codon optimized PA4771 (<i>lldD</i>) in pEasyBlunt vector; Km ^r	This study
SH8	Synthetic gene containing codon optimized PA4770 (<i>lldP</i>) in pEasyBlunt vector; Km ^r	This study
pP- <i>lldP</i> - <i>lldD</i>	pHG101 harboring PA4770 (<i>lldP</i>) and PA4771 (<i>lldD</i>)	This study
pP- <i>lldD</i>	pHG101 harboring PA4771 (<i>lldD</i>)	This study
pP- <i>lldP</i>	pHG101 harboring PA4770 (<i>lldP</i>)	This study
pP- <i>lldD</i> - <i>lldP</i>	pHG101 harboring PA4771 (<i>lldD</i>) and PA4770 (<i>lldP</i>)	This study

5.2.2 Mutagenesis of Lactate Dehydrogenase and Lactate Permease Genes.

Error-prone PCR of target genes was performed with GeneMorph[®] II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Primers were designed to amplify target genes with biobrick restriction sites. PCR products were purified and digested with XbaI and PstI. The mutagenized fragments were cloned into the vector pHG101, with the *lac* promoter upstream and double terminators downstream.

5.2.3 Lactate consumption assays of the engineered *S. oneidensis* MR-1 strains

At each time point, after removing the bacterial cells, we evaluated L-lactate consumption by measuring the amount of L-lactate remaining in the culture medium with the L-Lactate Assay Kit (AAT Bioquest, Sunnyvale, CA). Briefly, a 1% inoculum of an overnight stationary phase culture was used to start fresh cultures grown in *Shewanella* basal medium, supplemented with 15mM L-lactate and 40mM fumarate. A final concentration of 50µg/ml kanamycin was added if required. For each reading, a volume of 200µL culture was centrifuged to remove the insoluble contents. The L-lactate assay with a volume of 100µL/well was formed by mixing 50µL of the test sample with 50µL L-lactate assay mixture in a

96-well plate. The reaction was maintained at room temperature for half an hour before absorbance ratio was measured at the wavelength of 575nm/605nm using a microplate reader.

5.2.4 Microbial fuel cell setup and operation

All experiments were performed using dual-chamber MFCs. The working volume of each of the chambers is 150mL. A Nafion 117 membrane (Gashub, Singapore) was used to separate the anode and cathode chambers after it had been immersed overnight in 0.5M sulfuric acid and washed with DI water. Electrodes for both anode and cathode were made of carbon cloth (Gashub, Singapore) with a size of 2cm × 3cm. Cathodic solution consisted of 50mM K₃[Fe(CN)₆], 50mM K₂HPO₄, and 50mM KH₂PO₄.

Shewanella strains were grown overnight in a 30°C incubator at a shaking rate of 200rpm. Cell suspensions were adjusted to an optimal density of 0.5 before adding into the anode chamber. The anodes were purged with nitrogen to remove oxygen. After inoculation, the circuit was connected by copper wire with a constant external resistance of 2kΩ. The MFCs were operated at room temperature in batch mode. The duration of a full cycle was determined by the consumption of substrate and voltage reduction from its peak value to 50mV.

5.2.5 Data acquisition and calculations

The voltage output of each MFC was measured using a digital multimeter. To evaluate MFC performance, polarization curves were obtained by applying different loads or external resistances (R_{ext}) and then measuring the voltage (V) in order to calculate the current (I) by Ohm's Law ². By normalizing power based on the surface area (A) of the anode electrode, power density (P) can be calculated by using the formula, $P = \frac{V \times I}{A}$.

5.2.6 Electrochemical analysis

Electrochemical measurements were performed using an electrochemical working station (CHI760D, CH Instruments Inc.) employing a three-electrode system. Saturated calomel electrode (SCE) was used as the reference electrode, with the

anode as the working electrode and the cathode as the counter electrode. Cyclic voltammetry (CV) was carried out with a scanning range of -0.7 to 0.2V.

5.2.7 Computational Modeling of LDH Protein Structure

The amino acid sequence of lactate dehydrogenase was used to computationally model its three-dimensional structure. Models were built in Modeller¹⁸⁴ with four different templates (PDB IDs: 1HUV, 1AL7, 1GOX, and 2A7N) using standard single template modeling procedures. The models built with the different templates were nearly identical to one another with the secondary structures and overall domain orientations preserved. Hence, further predictions were performed based on the models obtained using 1HUV as the template. Images were made using VMD¹⁸⁵.

5.2.8 Scanning electron microscope (SEM) imaging of biofilm

To obtain an SEM image of the anode after operation, a small piece of the anodic carbon cloth was cut off and immersed in 1% glutaric dialdehyde solution overnight for bacteria fixation on the electrode surface. After stabilization, samples were rinsed 3 times using 0.9% NaCl solution and went through dehydration, which consists of immersion of the sample in 30%, 50%, 70%, 90% and 100% ethanol solution for 20 minutes each at room temperature. Samples were then air-dried and ready for imaging. The morphology and relative density of biofilm was qualitatively characterized by a JEOL field emission scanning electron microscope (FESEM, JSM-6700F-FE-SEM, Japan).

5.2.9 Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was purified from bacterial cultures in mid-log phase using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Quantitative analyses of transcripts were carried out with iQ SYBR Green Supermix (Biorad) and CFX96 Real Time PCR Detection System (Biorad). Samples were analyzed in triplicates. Gene *gyrA* was used for normalization. Samples were analyzed in triplicates using the listed primers (Table 5.3). Data analysis was performed as previously described.¹⁴⁰

Table 5.3 Oligonucleotide primers used in this chapter for qRT-PCR.

Primer	Sequence
omcA-q-F	GATACTCGCTACGCTTACATCC
omcA-q-R	TCCTTGTTATGGCAACCTGAAC
mtrA-q-F	CGGCACTTACCATCACAATG
mtrA-q-R	TAAGGTGGCTTCGACTTGCT
mtrB-q-F	CGGCTTAAAACAAGCCTCTG
mtrB-q-R	GAGTGCGGTAAACCAACGAT
mtrC-q-F	CGGCAATGATGGTAGTGATGG
mtrC-q-R	TGGCATGTCGGCTTCGTTA
cymA-q-F	GGTTGTTGGTATCGTGATTGGT
cymA-a-R	GCAGATGCCAGCACTTCATT
hyaA-q-F	ACTCGATATGTTCAACCGTCC
hyaA-q-R	TTCTTCAACGAACTCACCTGC
ndh-q-F	CTAAGTTACCCATTACGCCTCG
ndh-q-R	AATAAAGCACAATCCCCAATCG
ldh-q-F	ATGGTTTCGAGGTGATCTGC
ldh-q-R	ATCAGGGCCACAGTATGCTC
gyrA-q-F	CACGCAGCCCACTGATGCCT
gyrA-q-R	GTGGCCGAGCTGTCATGCGT

5.3 Results and discussion

5.3.1 A mutated lactate dehydrogenase further improved MFC power density

To determine whether any of the mutated genes could enable *Shewanella* to produce more bioelectricity than SP-1, we overexpressed each mutant in MR-1 and then examined the growth rate and MFC voltage output of the resulting strains. For the lactate permease gene *lldP*, every combination of mutations that we tested caused a reduction in bacterial growth and a lower voltage output in MFCs (Figure 5.1 and Figure 5.2). In contrast, we found one variant of the lactate dehydrogenase gene *lldD* whose overexpression consistently led to better growth and higher MFC voltage output than the original gene. Specifically, heterologous overexpression of this variant, termed 7M6, in MR-1 resulted in a slightly faster growth than the SP-2 strain (Figure 5.3A). The improvement was more pronounced when 7M6 was overexpressed together with a wildtype copy of the lactate permease gene *lldP* from *Pseudomonas* (Figure 5.3B). Similarly, MFCs containing the strain

overexpressing 7M6 alone produced a maximum voltage that was reproducibly higher than SP-2, although the increase was only marginal (Figure 5.4A). More importantly, MFCs powered by *Shewanella* cells that overexpressed 7M6 in combination with wildtype *lldP* were able to generate a voltage output that was 22.4% higher than MFCs containing SP-1, which overexpressed both wildtype *lldD* and wildtype *lldP* (Figure 5.4B). Hence, our random mutagenesis experiments yielded a *lldD* variant that improved MFC performance even further.

Figure 5.1 Characterization of *S. oneidensis* strains overexpressing different randomly mutated *lldP* gene variants.

(A) Growth curves of strains overexpressing *lldP* variants alone.

(B) Growth curves of strains overexpressing randomly mutated *lldP* genes together with wildtype *lldD*.

Figure 5.2 MFCs characterization of *S. oneidensis* strains overexpressing different randomly mutated *lldP* gene variants.

(A) Voltage outputs of strains overexpressing *lldP* variants alone.

(B) Voltage outputs of strains overexpressing randomly mutated *lldP* genes together with wildtype *lldD*.

Figure 5.3 Characterization of *S. oneidensis* strains overexpressing different randomly mutated *lldD* gene variants.

(A) Growth curves of strains overexpressing *lldD* variants alone.

(B) Growth curves of strains overexpressing randomly mutated *lldD* genes together with wildtype *lldP*.

Figure 5.4 MFCs characterization of *S. oneidensis* strains overexpressing different randomly mutated *lldD* gene variants.

(A) Voltage outputs of strains overexpressing *lldD* variants alone.

(B) Voltage outputs of strains overexpressing randomly mutated *lldD* genes together with wildtype *lldP*.

5.3.2 Multiple mutations in the *lldD* gene acted synergistically to enhance MFC performance

The 7M6 gene variant contained three recoding mutations, namely D167G, S208T, and E354D. To determine the relative contribution of each amino acid change to the improved MFC performance, we introduced the three mutations separately into the wildtype *lldD* gene from *Pseudomonas* (also named as SH7), so that we could evaluate them one at a time. Each of the *lldD* variant genes bearing only a single mutation was co-expressed with a wildtype copy of the *lldP* gene (also named as SH8) from pHG101. Surprisingly, our optical density (OD) measurements revealed that mutating SH7 at only a single amino acid residue reduced bacterial growth relative to SP-1 (Figure 5.5A). In fact, the D167G mutation appeared to be particularly detrimental, as MR-1 cells overexpressing SH7(D167G) and SH8 grew more slowly than our empty vector control. Subsequently, we generated a strain overexpressing wildtype *lldP* with a mutated *lldD* gene bearing the S208T and E354D mutations. However, while this strain exhibited a faster growth rate than SP-1, it still grew slower than the 7M6-overexpressing cells.

Figure 5.5 Comparative analysis of the effects of three mutations in the *lldD* gene (n=3).

- (A) Growth curves of various *S. oneidensis* strains under anaerobic conditions.
 (B) Lactate consumption assays of engineered strains under anaerobic conditions.
 (C) Voltage outputs of MFCs inoculated with different engineered strains.

To confirm the results from our cell proliferation experiments, we performed lactate consumption assays and also set up MFCs in batch mode to measure voltage outputs with different strains in the anodic chamber. Data from our lactate consumption assays largely corroborated our growth curve measurements (Figure 5.5B). In particular, the D167G mutation alone dramatically affected the bacteria's ability to metabolize lactate when compared to SP-1. Additionally, from our MFC experiments, we found that the best performing strain, which overexpressed 7M6 and SH8, produced a maximum voltage of $367 \pm 14 \text{ mV}$, which was 1.9-times that of the control strain ($191 \pm 12 \text{ mV}$) (Figure 5.5C). However, none of the *Shewanella* cells overexpressing SH8 with a *lldD* gene bearing only one or two mutations could produce as high a voltage as our optimal strain, which also gave the largest amount of transferred charges and the highest coulombic efficiency (Table 5.4).

Figure 5.6 Detailed electrochemical analysis of the effects of three mutations in lldD .

(A) Cyclic voltammograms at a scan rate of 1mV/s.

(B) Output voltage vs. current density (V-j) curves of various engineered strains.

(C) Power density vs. current density (P-j) curves of various engineered strains.

To further compare the EET of all the strains, we performed CV analysis at a slow scan rate of 1mV/s under turnover condition (Figure 5.6A). We observed that the MET-based catalytic current arose at -0.46V vs. SCE, while the DET-based catalytic current arose at -0.32V vs. SCE for all strains. Consistent with our earlier results, the highest currents were achieved by the strain that overexpressed 7M6 (with three amino acid changes) and SH8 (Table 5.5). This strain gave a MET-based current and a DET-based current that were 43.0% and 36.0% higher than SP-1 respectively. In comparison, the cells that overexpressed SH7 with only two mutations (S208T and E354D) and SH8 yielded a MET-based current and a DET-based current that were 35.4% and 33.8% higher than SP-1 respectively, indicating that the third mutation, D167G, though detrimental on its own, could further boost power output from the MFCs. These results were confirmed by polarization curves that were obtained at the peak output voltages. MFCs containing the 7M6-overexpressing strain had the lowest internal resistance (Figure 5.6B) and achieved the highest maximum power density (Figure 5.6C). Notably, our optimal strain produced a maximum power density of $118.58 \pm 2.67 \text{ mW/m}^2$, which was 2.8-fold higher than that attained by the control cells carrying an empty pHG101 plasmid ($42.03 \pm 6.59 \text{ mW/m}^2$) and also 1.4-fold higher than that achieved by SP-1 ($81.92 \pm 2.62 \text{ mW/m}^2$). The results achieved are comparable with other reported MFCs performances in similar configurations.^{164, 186-188} Again, the cells overexpressing SH7 with only two amino acid changes (S208T and E354D) generated a maximum power density of $96.14 \pm 2.58 \text{ mW/m}^2$, which was better than SP-1 but worse than the 7M6-overexpressing strain. Collectively, our results indicated that all three mutations contributed synergistically to the performance of the optimal strain.

To further understand how the overexpressed lactate permease and lactate dehydrogenase could affect the central metabolism in *Shewanella oneidensis* MR-1 and the electron generation and transport pathways, we used qRT-PCR to quantify cDNA abundance of several genes in the *Shewanella* metabolism pathways in 7M6-SH8 and the control strain, including: genes in the Mtr pathway (*omcA*, *mtrA*,

mtrB, *mtrC* and *cymA*), the *ldh* gene encoding lactate dehydrogenase, the *hyaA* gene encoding quinone reactive NiFe-dehydrogenase subunit, as well as *ndh* gene encoding NADH dehydrogenase.

The expression fold changes of respective genes in 7M6-SH8 relative to the control strain are all above 1 (Figure 5.7). Particularly, the expression of native lactate dehydrogenase also increased possibly due to the enhanced lactate transport into the intracellular space. The increased lactate consumption elevated the expression levels of NiFe dehydrogenase and NADH dehydrogenase which are key enzymes in catalysing NADH to produce electrons. The enhanced abundance in *c*-type cytochromes in the Mtr pathway, in turn, improved the efficiency of transporting the produced electrons to the outermembrane to generate electricity.

Figure 5.7 qRT-PCR analysis of 7M6-SH8 normalized to the control strain. Fold change represents expression in mutant normalized to that in the control strain (n=3). All data are shown as the mean $\pm$ SD. All *p*-values are based on *gyrA* versus selective gene. **p*<0.05, ***p*<0.01, ****p*<0.001.

Table 5.4 The total charge transferred and coulombic efficiency achieved in MFC anodes.

Anode	Electrons discharged (C)	Coulombic efficiency (%)
pHG101	67.45	7.77
SP-1	131.12	15.11
7M6-SH8	144.85	16.69
SH7(D167G)-SH8	59.06	6.80
SH7(S208T)-SH8	104.67	12.06
SH7(E354D)-SH8	85.24	9.82
SH7(S208T, E354D)-SH8	138.63	15.97

Table 5.5 Mediated electron transfer-based (MET) and direct electron transfer-based (DET) catalytic current densities with different *S. oneidensis* strains.

Anode	MET (mA/m ²)	DET (mA/m ²)
pHG101	22.49	46.06
SP-1	65.06	124.00
7M6-SH8	93.05	168.70
SH7(D167G)-SH8	20.05	44.11
SH7(S208T)-SH8	52.06	104.73
SH7(E354D)-SH8	43.04	88.11
SH7(S208T, E354D)-SH8	88.11	165.86

5.3.3 The expression of 7M6-SH8 enhanced various metabolism pathways in *Shewanella* and improved biofilm formation

We speculate that the enhanced lactate consumption could also elevate the formation of biofilm over the surface of electrode, to further increase direct electron transfer in MFCs operations. To confirm that, we have taken SEM images of the biofilms after 400 hours of MFCs operation (Figure 5.8). Biofilm of the control strain could barely cover the surface of the electrode (Figure 5.8A), while biofilm formed by 7M6-SH8 could cover up most of the electrode surface with a relatively thicker layer (Figure 5.8B).

Figure 5.8 Characterization of MFC's anodic biofilms after 400 hours of operation. (A) SEM image of the biofilm of the control strain. (B) SEM image of the biofilm of the 7M6-SH8 strain.

5.3.4 Protein structure modelling analysis

Protein structure modeling revealed that the three mutated residues appeared to lie far apart from one another (Figure 5.9A). Currently, it is unclear how these mutations function together to improve MFC performance. Based on our computational structure model, we did not detect significant changes in the number of hydrogen bonds formed by mutating these amino acids. Furthermore, the D167 residue lies adjacent to the putative catalytic site (Figure 5.9B), which may explain why the D167G mutation alone has a detrimental effect on bacterial growth, rate of lactate consumption, and bioelectricity generation (Figure 5.5 and Figure 5.6). We speculate that the three mutations may act synergistically to influence the binding of the LDH protein to its interacting partners. However, further work is needed to dissect the molecular mechanism. Additionally, our study raises the question of whether there are any other combinations of mutations that can improve bioelectricity generation besides D167G, S208T, and E354D. A high throughput screen or a directed evolution approach can be implemented in the future to address this issue.

Figure 5.9 Computational modeling of the LDH protein from *Pseudomonas*.

(A) Predicted structure of the lactate dehydrogenase from *Pseudomonas* in new cartoon representation (in gray). The three mutated residues are depicted in van der Waal's representation (D167 - orange, S208 - yellow, and E354 - green). The S208 residue appears to be in an exposed loop, while the D167 and E354 residues are located in α -helices.

(B) A different view of the lactate dehydrogenase to highlight the location of the putative active site residues in surface representation (in red). The D167 residue is located next to the active site.

5.4 Chapter summary

To further improve our engineered strain SP-1, we randomly mutagenized *lldP* and *lldD* by error-prone PCR and tested the mutant genes for an improvement in growth rate and MFC voltage output. We uncovered a set of mutations (D167G, S208T, and E354D) in the *lldD* gene that was able to boost the power density of MFCs to 2.8-times that of control cells.

In summary, we have constructed a synthetic L-lactate metabolic pathway in the *Shewanella* MR-1 strain to enhance MFC performance. Heterologous expression of the *lldP* lactate permease gene from *Pseudomonas* as well as a mutated variant of the *lldD* lactate dehydrogenase gene led to a sizeable improvement in power output. We have found that the improved lactate consumption in MR-1 could elevate the level of metabolism in a broader range, and increased expression levels of genes relevant to the electron production and electron transfer pathways. We have also discovered that the biofilm formation on the anode electrode surface had been

significantly improved compared with the control strain, which further improved the extracellular electron transfer in MFCs operations.

Together with other genetic engineering strategies, our work will help to expand the range of industrial applications of future MFCs and meet our society's energy needs.

CHAPTER 6 Engineering *hya* genes and *ndh* for the enhancement of bioelectricity generation

6.1 Introduction

Bioelectrochemical systems (BESs) are considered promising and sustainable platforms due to its capability of transforming chemical energy from wastewater or organic wastes into biological energy in the form of electricity via microbial fuel cells (MFCs).^{17, 189} BESs have been proved to have great potentials compared with traditional chemical fuel cells as it utilizes a wide range of substrates, works under relatively mild conditions, and usually does not require expensive catalysts such as precious metals.

A few of the dissimilatory metal-reducing bacteria (DMRB) species have the ability of generating bioelectrical currents in MFCs by reducing solid metal oxides as electron acceptors.^{89, 190-193} Even though mixed incubations are occasionally used in MFC experiments¹⁹⁴, owing to the complexity in the communities of microbes, in search for a mechanical interpretation of the electron transfer pathway to electrode surfaces generally requires targeting on pure cultures of such DMRB. In the recent decades, two DMRB models have been frequently used in most investigations, which are *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA.^{18, 58, 89, 114, 195}

As a metabolically diverse DMRB, *S. oneidensis* MR-1 has been extensively studied for its potential in bioremediations applications, due to its versatile respiratory abilities, and its genome sequence was the first to be fully sequenced and annotated in the *Shewanella* genus.^{78, 196} Furthermore, when *S. oneidensis* MR-1 was reported to be able to transfer electrons to external electrodes without the aid of exogenously added mediator¹⁹¹, it has become a model organism in exploring the mechanism of electron transfers in MFCs. By far, the most researched area has been the ability to reduce iron and manganese oxides and radionuclides^{99, 161, 197} while more recent studies focused on its application in MFCs^{114, 142, 198}.

A few genes encoding the *c*-type cytochromes have been extensively studied for their functions in the *S. oneidensis* MR-1 electron transfer pathways.

In search of relevant genes functioning in the electron transfer pathways, and in the aim of further improve the amount of electricity that can be generated by *S. oneidensis* MR-1 in MFCs operations, we applied systems-biological tools such as computational approaches to develop a deeper understanding of *S. oneidensis* MR-1 electron transfer mechanism in its metabolic pathways. The genome-scale metabolic model of *S. oneidensis* MR-1 (namely *i*SO783)¹⁵⁵ was considered a platform for computational predictions and validating those results with experimental data. It could offer understanding at system level of how this respiration versatile microbe regulates the flow of energy and substrates, and utilizes its electron-transport systems to reduce electron acceptors in different environments.

Based on the computational predictions, the *hya* genes and genes encoding NADH dehydrogenase serves as the best candidates in further enhancing the electron transfer in *S. oneidensis* MR-1. We further implemented experimental data to validate the results.

6.2 Experiments

6.2.1 Bacterial strains, plasmids, and culture conditions

All strains and plasmids used in this study are given in Table 6.1. The *E. coli* and *S. oneidensis* MR-1 strains were routinely maintained and cultured in Luria-Bertani (LB) medium at 37°C and 30°C respectively. When necessary, the medium was supplemented with either kanamycin (50µg/ml) or ampicillin sodium (100µg/ml). In *E. coli* WM3064 culture, a final concentration of 0.3mM 2,6-Diaminopimelic acid (DPA) was added. The *S. oneidensis* MR-1 experiments were performed in *Shewanella* basal medium¹⁰⁹ with 15mM L-lactate acting as the electron donor and carbon source. When needed, 40mM fumarate was supplemented as the electron acceptor.

Table 6.1 Bacterial strains and plasmids used in this chapter.

<i>Strains</i>	<i>Description</i>	<i>Reference or source</i>
<i>E. coli</i>		
DH5α	Host for regular cloning	Lab stock
WM3064	Donor strain used for conjugation with <i>S. oneidensis</i> MR-1, Δ <i>dapA</i>	Lab stock
<i>S. oneidensis</i>		
MR-1	Wildtype	Lab stock
pHG101	MR-1 harboring pHG101, used as control in this study; Km ^r	This study
<i>hyaA</i>	MR-1 harboring <i>plac-hyaA</i> -pHG101	This study
<i>hyaB</i>	MR-1 harboring <i>plac-hyaB</i> -pHG101	This study
<i>hyaC</i>	MR-1 harboring <i>plac-hyaC</i> -pHG101	This study
<i>hyaABC</i>	MR-1 harboring <i>plac-hyaABC</i> -pHG101	This study
<i>Ptac-MT12</i>	MR-1 harboring <i>Ptac-MT12</i> -pHG101	This study
<i>plac-hyaA-Ptac-MT12</i>	MR-1 harboring <i>plac-hyaA-Ptac-MT12</i> -pHG101	This study
<i>Plasmids</i>		
pHG101	Promoterless vector derived from broad host range vector pBBR1MCS-2; Km ^r	¹³⁹
pSB1A2-Plac	Biobrick containing <i>lac</i> promoter (BBa_R0011, iGEM) in pSB1A2 plasmid backbone; Amp ^r	Lab stock
pSB1AK3-TT	Biobrick containing double terminators (BBa_B0015, iGEM) in pSB1AK3 plasmid backbone; Km ^r	Lab stock
<i>plac-hyaA</i> -pHG101	pHG101 harboring SO_2099 (<i>hyaA</i>)	This study
<i>plac-hyaB</i> -pHG101	pHG101 harboring SO_2098 (<i>hyaB</i>)	This study
<i>plac-hyaC</i> -pHG101	pHG101 harboring SO_2097 (<i>hyaC</i>)	This study
<i>plac-hyaABC</i> -pHG101	pHG101 harboring SO_2099 (<i>hyaA</i>), SO_2098 (<i>hyaB</i>) and SO_2097 (<i>hyaC</i>)	
<i>Ptac-MT12</i> -pHG101	pHG101 harboring SO_3517 (<i>ndh</i>), expression controlled by inducible <i>tac</i> promoter	This study
<i>plac-hyaA-Ptac-MT12</i> -pHG101	pHG101 harboring SO_2099 (<i>hyaA</i>) and SO_3517 (<i>ndh</i>), expression controlled by constitutive <i>lac</i> promoter and inducible <i>tac</i> promoter, respectively	This study

6.2.2 Plasmid construction

All genetic manipulation work was performed using standard protocols. Gene sequences were obtained from KEGG ¹⁷² and amplified via standard PCR protocol. Each of the amplified gene sequence was designed as a biobrick ¹⁷⁴, flanked by an upstream prefix containing EcoRI and XbaI and a downstream suffix containing SpeI and PstI. The *hya* genes were then assembled through routine steps of biobrick ligation, inserting a *lac* promoter (BBa_R0011, iGEM) and a ribosome

binding site (BBa_B0034, iGEM) in front and a double terminator (BBa_B0015, iGEM) at the back to regulate gene expression, forming a *Plac*-RBS-genes-TT construct in the vector pSB1AK3 (iGEM). Finally, by using the restriction enzymes EcoRI and SpeI, the *Plac*-RBS-genes-TT fragment was cut and ligated into the broad-host-range expression vector pHG101¹³⁹. The *ndh* gene was designed to be regulated by an inducible tac promoter (BBa_K180000, iGEM) with IPTG acts as the inducer. The *E. coli* strain DH5 α was used to amplify each plasmid. Every plasmid was confirmed by sequencing. Each of the final recombinant pHG101-based plasmids was transformed into *E. coli* WM3064 and subsequently introduced into *S. oneidensis* MR-1 via conjugation. Successful conjugation was confirmed by colony PCR.

6.2.3 Microbial fuel cell setup and operation

All experiments were performed using dual-chamber MFCs. The working volume of each of the chambers is 150mL. A Nafion 117 membrane (Gashub, Singapore) was used to separate the anode and cathode chambers after it had been immersed overnight in 0.5M sulfuric acid and washed with DI water. Electrodes for both anode and cathode were made of carbon cloth (Gashub, Singapore) with a size of 2cm $\times$ 3cm. Cathodic solution consisted of 50mM K₃[Fe(CN)₆], 50mM K₂HPO₄, and 50mM KH₂PO₄.

Shewanella strains were grown overnight in a 30°C incubator at a shaking rate of 200rpm. Cell suspensions were adjusted to an optimal density of 0.5 before adding into the anode chamber. The anodes were purged with nitrogen to remove oxygen. After inoculation, the circuit was connected by copper wire with a constant external resistance of 2k Ω . The MFCs were operated at room temperature in batch mode. The duration of a full cycle was determined by the consumption of substrate and voltage reduction from its peak value to 50mV.

6.2.4 Data acquisition and calculations

Voltage output of each MFC was measured using a digital multimeter. To evaluate MFC performance, polarization curves were obtained by applying different loads or external resistances (R_{ext}) and then measuring the voltage (V) in order to

calculate the current (I) by Ohm's Law ². By normalizing power based on the surface area (A) of the anode electrode, power density (P) can be calculated by using the formula, $P = \frac{V \times I}{A}$.

6.2.5 Electrochemical analysis

Electrochemical measurements were performed using an electrochemical working station (CHI760D, CH Instruments Inc.) employing a three-electrode system. Saturated calomel electrode (SCE) was used as the reference electrode, with the anode as the working electrode and the cathode as the counter electrode. Cyclic voltammetry (CV) was carried out with a scanning range of -0.7 to 0.2V.

6.2.6 Mutagenesis of *hya* and *ndh* genes.

Error-prone PCR of target genes was performed with GeneMorph[®] II EZClone Domain Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Primers were designed to amplify target genes within the coding sequences. Primers used in this process are listed in Table 6.2.

Table 6.2 Primers used in this chapter for mutagenesis

Primer	Sequence
hyaA-EZ-F	ccgaccgtgtacccttaatt
hyaA-EZ-R	aatccatggagcagttgacg
MT12-EZ-F	TTTGGCGCTTGCCTCTAAGTTA
MT12-EZ-R	GCGCAATGCGATAGATAATTGC

6.3 Results and discussion

6.3.1 Genome-scale modeling to identify potential genes for optimization

Based on the modeling method mentioned in Chapter 3, by optimizing all reactions in the Mtr pathway, the ARC framework has chosen the reaction responsible for the maximum flux activity for the Mtr pathway (Table 6.3). During the MFCs operation under direct electron transfer via the Mtr pathway, electrons should be extracted continuously from the anode. The surplus flux required to support the electron transfer in the Mtr pathway must be obtained from other reactions. From

our results, there are 11 reactions potentially contributed in the process. The highest surplus fluxes are attributed to Reaction 384 and 385 (ID: HYD5i and HYD6i), involving the *hya* genes for electron transfer with methymenaquinol 7 or methylmenaquinone 7. The *hya* genes encodes a hydrogenase (HyaAB) enzyme involved in H₂ production by *Shewanella oneidensis* MR-1 in the pyruvate fermentation process.¹⁹⁹ However, independently optimizing either reaction would show an extremely huge flux. The next highest surplus flux reactions are Reaction 480 and 478, which involves genes encoding the proton-pumping NADH dehydrogenase for electron transfer via NADH with methymenaquinol 7 or methylmenaquinone 7.

Table 6.3 Possible Reactions for Optimization from iSO783 (based on ARC framework)

Reaction independently optimized	Constraints	Maximum possible MtrABC pathway flux, mmol	Maximum current output, A	Coulombic efficiency, %
46	Enzyme efficiency	136.8848	6.6995	69.29
122	Enzyme efficiency	125.7581	6.6312	87.46
386	Enzyme efficiency	131.4129	6.6312	76.8
424	Enzyme efficiency	67.2468	3.216	110.5
426	Enzyme efficiency	60.0006	3.216	112.34
478	Enzyme efficiency	165.8858	8.8447	122.89
480	Enzyme efficiency	178.1829	8.9888	124.39
597	Enzyme efficiency	125.196	5.7673	64.33
698	Enzyme efficiency	148.6433	5.7737	66.95
384	Enzyme efficiency	100170	5370	199580
385	Enzyme efficiency	100190	5370	187470

Directly optimizing Reaction 384 and 385 independently would result in the congregation of most of the system fluxes in a few reactions, truing off the other reactions in the system. In Table 6.4, Reaction 384 produces a maximum possible total flux of 202210, where 99.89% is contributed by the top 4 fluxes in its system (Reaction 263, 464, 766 and 384) (Table 6.5). Reaction 385 produces a maximum possible total flux of 200190, from which 99.97% is contributed by the top 4 fluxes in its system (Reaction 264, 465, 767 and 385) (Table 6.6). These results are in huge contrast with strict optimization of the growth rate (Reaction 775) or the

reduced *c*-type cytochrome flux (Reaction 175), under which condition the sum of fluxes in the system is smaller and well spread across all reactions (Table 6.4).

Table 6.4 Proportion of flux with optimization from iSO783

Reaction independently optimized	Constraints	Maximum possible total flux, mmol	Sum of top 4 flux, mmol	Total contribution of top 4 flux, %
384	Enzyme efficiency	200210	200000	99.89
385	Enzyme efficiency	200190	200130	99.97
775	Enzyme efficiency	884.05	86.60	0.433
175	Enzyme efficiency	1255.3	30.97	0.0155

Table 6.5 Reactions of top 4 flux in optimized reaction 384 and key proteins involved (of iSO783)

No	ID	Stoichiometric Reaction	proteinGeneAssociation
263	FER3	Fe3 [e] + mql7 [c] -> 2Fe2 [e] + 2H [e] + mqn7 [c]	CctA (SO2727), CymA (SO4591), MtrAso (SO1777) and MtrCso (SO1778)
464	MNOR	Mn4o [e] + mql7 [c] + 2H [e] -> mn2 [e] + mqn7 [c] + 2h2o [e]	(CymA (SO4591), MtrAso (SO1777, OmcA (SO1779)) or (CymA (SO4591), MtrAso (SO1777), MtrCso (SO1778))
766	URNR3	Urnyl [e] + mql7 [c] -> Urdio [e] + mqn7 [c] + 2H [e]	CymA (SO4591), MtrAso (SO1777), MtrCso (SO1778)
384	HYD5i	h2[e] + mqn7[c] --> mql7[c]	Hya (SO2097 and SO2098 and SO2099)

Table 6.6 Reactions of top 4 flux in optimized reaction 385 and key proteins involved (of iSO783)

No	ID	Stoichiometric Reaction	proteinGeneAssociation
264	FER4	Fe3 [e] + mmql7 [c] -> 2Fe2 [e] + 2H [e] + mmqn7 [c]	CctA (SO2727), CymA (SO4591), MtrAso (SO1777) and MtrCso (SO1778)
465	MNOR2	Mn4o [e] + mmql7 [c] + 2H [e] -> mn2 [e] + mmqn7 [c] + 2h2o [e]	(CymA (SO4591), MtrAso (SO1777, OmcA (SO1779)) or (CymA (SO4591), MtrAso (SO1777), MtrCso (SO1778))
767	URNR4	Urnyl [e] + mmql7 [c] -> Urdio [e] + mmqn7 [c] + 2H [e]	CymA (SO4591), MtrAso (SO1777), MtrCso (SO1778)
385	HYD6i	h2[e] + mmqn7[c] --> mmql7[c]	Hya (SO2097 and SO2098 and SO2099)

6.3.2 Overexpressing *hya* genes to improve electron production and transfer

From our genome-scale modeling results, the original genes SO_2099 (*hyaA*), SO_2098 (*hyaB*) and SO_2097 (*hyaC*) from *Shewanella oneidensis* MR-1 were selected for this study. We overexpressed in *Shewanella oneidensis* MR-1 either

individual genes singly or the *hya* operon (*hyaABC*) under the control of a *lac* promoter. In total we constructed 4 distinct MR-1 strains for detailed characterization.

We first checked the growth of the 4 engineered MR-1 strains relative to a control strain harboring an empty pHG101 vector in *Shewanella* basal medium (SBM). We observed that the growth of bacteria strain overexpressing *hyaA* is the only positive result while bacteria strains overexpressing *hyaB* and *hyaC* showed growth defect comparing with the control strain (Figure 6.1A). Specifically, strain *hyaB* and *hyaC* exhibited poorer growth than the control strain. Strain *hyaABC* showed slight poorer growth than the control strain, possible attributed to the detrimental effect of *hyaB* and *hyaC*, even though *hyaA* could help with elevating the growth rate.

The improved growth may be attributed to an improved electron transfer efficiency. To verify the hypothesis, we set up MFCs with all the different inoculum strains and operated them in batch mode (Figure 6.1B). Overall, the voltage output values concur well with the growth curves (Figure 6.1A). The strain overexpressing *hyaA* performed better than the control strain carrying an empty pHG101 plasmid while the rest performed worse. Notably, MFCs containing the *hyaA* strain produced a maximum voltage of $239 \pm 18 \text{ mV}$ at 176h, which was much higher than that of the control ($191 \pm 12 \text{ mV}$ at 140h). We calculated the total charge transferred by integrating the area enclosed by the V-t curve and then dividing it by the external resistance (Table 6.7). Coulombic efficiencies were also obtained as previously described^{2, 176}. Again, the MFCs with the *hyaA* strain discharged the most electrons and exhibited the highest Coulombic efficiency. Hence, given the consistency in results between the growth curve measurements and MFC experiments, we focused our subsequent investigations on the *hyaA* strain.

Table 6.7 The total charge transferred and coulombic efficiency achieved in MFC anodes.

Anode	Electrons discharged (C)	Coulombic efficiency (%)
pHG101	68.13	7.85
hyaA	113.81	13.11
hyaB	66.05	7.61
hyaC	54.27	6.25
hyaABC	66.89	7.71

Figure 6.1 Characterization of MR-1 strains overexpressing *hya* genes (n=3).
(A) Growth curve measurements of the strains under anaerobic conditions.
(B) Voltage outputs of MFCs inoculated with various strains in the anodic chamber.

Various EET pathways can influence the electrochemical behavior of the *Shewanella* biofilm on the electrode. To dissect the effects of the different pathways on *hyaA* and control biofilms, we conducted CV analysis at a low scan rate of 1mV/s under turnover condition (Figure 6.2A). For both strains, the anodic curve first rose above background at -0.59V vs. SCE due to the occurrence of shuttle-mediated electron transfer (MET) via flavins^{181, 182}. This shuttle-mediated catalytic current was 22.30mA/m² and 22.07mA/m² for *hyaA* and the control strain respectively, showing very little difference. Additionally, a second catalytic current arose at -0.45V vs. SCE for both strains due to the occurrence of contact-based direct electron transfer (DET) via the outer-membrane *c*-type cytochromes. Based on the first-derivative of the curves¹⁸³, we determined that this cytochrome-mediated current was 75.72mA/m² and 46.99mA/m² for *hyaA* and the control strain respectively (Table 6.7). Hence, the enhanced EET observed for our engineered *hyaA* strain relative to control was caused by a significant increase in DET.

We obtained polarization curves at the peak output voltages to further characterize the performance of the MFCs (Figure 6.2B). The slope of *hyaA*'s voltage-current plot was less steep than that of the control. From Ohm's law, this implied that there was a lower internal resistance in the MFCs with *hyaA* in the anodic chamber, probably due to the enhanced electron transfer. Furthermore, based on the power density curves (Figure 6.2C), the short circuit current densities were 250.00mA/m² and 141.66 mA/m² for *hyaA* and the control strain respectively. Importantly, *hyaA* achieved a maximum power density of 49.92±1.31mW/m², which was 1.24 times of that attained by the control strain (40.22±3.61mW/m²).

Taken together, our data highlight that overexpression of the *hyaA* gene in *Shewanella* MR-1 could enhance the performance of MFCs via improving the direct electron transfer efficiency. Since our experimental findings are incomparable to the modeling results and overexpressing *hyaA* did in fact result in the enhancement of direct electron transfer via *c*-type cytochromes, the next step is to further investigate the mechanism for a clearer explanation.

A **B**

Figure 6.2 Detailed electrochemical characterization of MFCs with inoculations of *hyaA* or the control strain.

(A) Cyclic voltammograms at a scan rate of 1mV/s under turnover condition.

(B) Output voltage vs. current density (V-j) curves of *hyaA* and the control strain.

(C) Power density vs. current density (P-j) curves of *hyaA* and the control strain.

6.3.3 Overexpressing NADH dehydrogenase to enhance electron production and transfer

According to our modeling results, Reaction 478 and Reaction 480 from iSO783¹⁵⁵ involving the proton-pumping NADH dehydrogenase are the next two highest surplus flux reactions. In addition, the intracellular NADH works as the electron source in the EET. Thus, the original gene SO_3517 (*ndh*) from *Shewanella oneidensis* MR-1 was selected for this study. We overexpressed *ndh* in *Shewanella oneidensis* MR-1 under the control of an inducible tac promoter, with induction under different IPTG concentrations for detailed characterization.

We first checked the growth of engineered MR-1 strain relative to a control strain harboring an empty pHG101 vector in *Shewanella* basal medium (SBM). We observed that the growth of bacteria strain with no induction shows similar growth patterns as the control strain (Figure 6.3A). When different concentrations of IPTG were added in the medium, the engineered strains show slight defect in growth. Specifically, the higher the concentration of inducers added, the worse the strain grew. We speculate that this is due to the overexpressed NADH dehydrogenase interrupts the normal metabolism in the cell, resulting in the decrease of bacterial growth rate.

To verify if the overexpressed NADH dehydrogenase could in fact enhance electricity generation, we set up MFCs with the engineered strain under different induction conditions and operated them in batch mode (Figure 6.3B). Overall, the voltage output values show the alternative results as the growth curves (Figure 6.3A). The strain overexpressing *ndh* performed better than the control strain carrying an empty pHG101 plasmid in general. Higher concentration of IPTG to induce the expression of *ndh* resulted in higher voltage output value. Notably, MFCs with 1.0mM IPTG induction (Ptac-MT12(IPTG_1.0mM)) produced a maximum voltage of 289 ± 22 mV at 118h, which was much higher than that of the control (191 ± 14 mV at 166h). We calculated the total charge transferred by integrating the area enclosed by the V-t curve and then dividing it by the external resistance (Table 6.6). Coulombic efficiencies were also obtained as previously described^{2, 176} (Table 6.6). Again, the MFCs with Ptac-MT12(IPTG_1.0mM) discharged the most electrons and exhibited the highest Coulombic efficiency.

Figure 6.3 Characterization of MR-1 strains overexpressing *ndh* gene under different IPTG concentrations (n=3).

(A) Growth curve measurements of the strains under anaerobic conditions.

(B) Voltage outputs of MFCs inoculated with various strains in the anodic chamber.

Various EET pathways can influence the electrochemical behavior of the *Shewanella* biofilm on the electrode. To dissect the effects of the different pathways on *ndh* and control biofilms, we conducted CV analysis at a low scan rate of 1mV/s under turnover condition (Figure 6.4A). For all strains, the anodic curve first rose above background at -0.59V vs. SCE due to the occurrence of shuttle-mediated electron transfer (MET) via flavins^{181, 182}. This shuttle-mediated catalytic current was 32.17mA/m² and 22.07mA/m² for Ptac-MT12(IPTG_1.0mM) and the control strain respectively, showing very little difference. Additionally, a second catalytic current arose at -0.45V vs. SCE for both strains due to the occurrence of contact-based direct electron transfer (DET) via the outer-membrane *c*-type cytochromes. Based on the first-derivative of the curves¹⁸³, we determined that this cytochrome-mediated current was 157.26mA/m² and 46.99mA/m² for Ptac-MT12(IPTG_1.0mM) and the control strain respectively (Table 6.7). Hence, the enhanced EET observed for our engineered Ptac-MT12(IPTG_1.0mM) strain relative to control was caused by a significant increase in DET.

We obtained polarization curves at the peak output voltages to further characterize the performance of the MFCs (Figure 6.4B). The slope of Ptac-MT12(IPTG_1.0mM)'s voltage-current plot was less steep than that of the control. From Ohm's law, this implied that there was a lower internal resistance in the MFCs with Ptac-MT12(IPTG_1.0mM) in the anodic chamber, probably due to the enhanced electron transfer. Furthermore, based on the power density curves (Figure 6.4C), the short circuit current densities were 341.66mA/m² and 183.33 mA/m² for Ptac-MT12(IPTG_1.0mM) and the control strain respectively. Importantly, Ptac-MT12(IPTG_1.0mM) achieved a maximum power density of 77.44±1.59mW/m², which was 1.88 times of that attained by the control strain (41.30±1.78mW/m²). Taken together, our data highlight that overexpression of the *ndh* gene in *Shewanella* MR-1 could enhance the performance of MFCs via improving the direct electron transfer efficiency.

A **B**

Figure 6.4 Detailed electrochemical characterization of MFCs with inoculations of engineered strains.

(A) Cyclic voltammograms at a scan rate of 1mV/s under turnover condition.

(B) Output voltage vs. current density (V-j) curves of Ptac-MT12 with 1mM IPTG and the control strain.

(C) Power density vs. current density (P-j) curves of Ptac-MT12 with 1mM IPTG and the control strain.

6.3.4 Couple *hyaA* and *ndh* for further improvement in electron production and transfer

We ask if combining genes *hyaA* and *ndh* could further improve electron transfer efficiency in MFCs performance. Therefore, in *Shewanella oneidensis* MR-1, we overexpressed *hyaA* under the control of a lac promoter together with *ndh* under the control of an inducible tac promoter, with induction under different IPTG concentrations for detailed characterization.

We first checked the growth of the engineered MR-1 strain relative to the control strain harboring an empty pHG101 vector in *Shewanella* basal medium (SBM). We

observed that the growth of bacteria strains *plac-hyaA* and *plac-hyaA-Ptac-MT12(IPTG_0mM)* grew better than the control strain (Figure 6.5A). *plac-hyaA-Ptac-MT12(IPTG_0mM)* grew slower than *plac-hyaA* possibly because of the leaky expression of NADH dehydrogenase has negative effect on the growth of *plac-hyaA-Ptac-MT12(IPTG_0mM)*. When different concentrations of IPTG were added in the medium, the engineered strains show slight defect in growth. Specifically, the higher the concentration of inducers added, the worse the strain grew with *plac-hyaA-Ptac-MT12(IPTG_1.0mM)* grew even poorer than the control strain.

To verify if the co-overexpressed *hyaA* and NADH dehydrogenase could in fact further enhance electricity generation, we set up MFCs with the engineered strain under different induction conditions and operated them in batch mode (Figure 6.5B). All strains performed better than the control strain carrying an empty pHG101 plasmid in general. Higher concentration of IPTG to induce the expression of *ndh* resulted in higher voltage output value. Notably, MFCs with 1.0mM IPTG induction (*plac-hyaA-Ptac-MT12(IPTG_1.0mM)*) produced a maximum voltage of $359 \pm 19 \text{mV}$ at 166h, which was much higher than that of the control ($191 \pm 14 \text{mV}$ at 166h). We calculated the total charge transferred by integrating the area enclosed by the V-t curve and then dividing it by the external resistance (Table 6.8). Coulombic efficiencies were also obtained as previously described^{2, 176} (Table 6.8). Again, the MFCs with *plac-hyaA-Ptac-MT12(IPTG_1.0mM)* discharged the most electrons and exhibited the highest Coulombic efficiency.

Figure 6.5 Characterization of MR-1 strains overexpressing *hyaA* and *ndh* genes under different IPTG concentrations (n=3).

(A) Growth curve measurements of the strains under anaerobic conditions.

(B) Voltage outputs of MFCs inoculated with various strains in the anodic chamber.

We also conducted CV analysis at a low scan rate of 1mV/s under turnover condition (Figure 6.6A). For all strains, the anodic curve first rose above background at -0.59V vs. SCE due to the occurrence of shuttle-mediated electron transfer (MET) via flavins^{181, 182}. This shuttle-mediated catalytic current was 33.88mA/m² and 22.07mA/m² for plac-hyaA-Ptac-MT12(IPTG_1.0mM) and the control strain respectively, showing very little difference. Additionally, a second catalytic current arose at -0.45V vs. SCE for both strains due to the occurrence of contact-based direct electron transfer (DET) via the outer-membrane *c*-type cytochromes. Based on the first-derivative of the curves¹⁸³, we determined that this cytochrome-mediated current was 181.40mA/m² and 46.99mA/m² for plac-hyaA-Ptac-MT12(IPTG_1.0mM) and the control strain respectively (Table 6.9). Hence, the enhanced EET observed for our engineered plac-hyaA-Ptac-MT12(IPTG_1.0mM) strain relative to control was caused by a significant increase in DET.

We obtained polarization curves at the peak output voltages to further characterize the performance of the MFCs (Figure 6.6B). The slope of plac-hyaA-Ptac-MT12(IPTG_1.0mM)'s voltage-current plot was less steep than that of the control. From Ohm's law, this implied that there was a lower internal resistance in the MFCs with plac-hyaA-Ptac-MT12(IPTG_1.0mM) in the anodic chamber, probably due to the enhanced electron transfer. Furthermore, based on the power density curves (Figure 6.6C), the short circuit current densities were 466.66mA/m² and 200.00mA/m² for plac-hyaA-Ptac-MT12(IPTG_1.0mM) and the control strain respectively. Importantly, plac-hyaA-Ptac-MT12(IPTG_1.0mM) achieved a maximum power density of 112.00±1.77mW/m², which was 2.8 times of that attained by the control strain (40.22±1.92mW/m²). The results achieved are comparable with other reported MFCs performances in similar configurations.^{164, 186-188} Taken together, our data highlight that co-overexpression of the *hyaA* and *ndh* gene in *Shewanella* MR-1 could further enhance the performance of MFCs via improving the direct electron transfer efficiency.

Table 6.8 The total charge transferred and coulombic efficiency achieved in MFC anodes.

Anode	Electrons discharged (C)	Coulombic efficiency (%)
pHG101	68.13	7.85
hyaA	113.81	13.11
hyaB	66.05	7.61
hyaC	54.27	6.25
hyaABC	66.89	7.71
Ptac-MT12 (IPTG_0Mm)	87.04	10.03
Ptac-MT12 (IPTG_0.3mM)	94.52	10.89
Ptac-MT12 (IPTG_0.5mM)	102.13	11.77
Ptac-MT12 (IPTG_0.7mM)	116.72	13.45
Ptac-MT12 (IPTG_1.0mM)	135.63	15.62
plac-hyaA-Ptac-MT12 (IPTG_0mM)	89.73	10.34
plac-hyaA-Ptac-MT12 (IPTG_0.3mM)	105.87	12.20
plac-hyaA-Ptac-MT12 (IPTG_0.5mM)	116.17	13.38
plac-hyaA-Ptac-MT12 (IPTG_0.7mM)	129.26	14.89
plac-hyaA-Ptac-MT12 (IPTG_1.0mM)	161.37	18.59

Table 6.9 Mediated electron transfer-based (MET) and direct electron transfer-based (DET) catalytic current densities with different *S. oneidensis* strains.

Anode	MET (mA/m ²)	DET (mA/m ²)
pHG101	22.07	46.99
hyaA	22.30	75.72
Ptac-MT12 (IPTG_0Mm)	22.63	54.16
Ptac-MT12 (IPTG_0.3mM)	22.93	99.40
Ptac-MT12 (IPTG_0.5mM)	27.93	120.70
Ptac-MT12 (IPTG_0.7mM)	29.76	150.39
Ptac-MT12 (IPTG_1.0mM)	32.17	157.26
plac-hyaA-Ptac-MT12 (IPTG_0mM)	24.02	96.35
plac-hyaA-Ptac-MT12 (IPTG_0.3mM)	24.02	113.80
plac-hyaA-Ptac-MT12 (IPTG_0.5mM)	29.28	142.12
plac-hyaA-Ptac-MT12 (IPTG_0.7mM)	30.90	152.85
plac-hyaA-Ptac-MT12 (IPTG_1.0mM)	33.88	181.40

A **B**

Figure 6.6 Detailed electrochemical characterization of MFCs with inoculations of engineered strains.

(A) Cyclic voltammograms at a scan rate of 1mV/s under turnover condition.

(B) Output voltage vs. current density (V-j) curves of various engineered strains.

(C) Power density vs. current density (P-j) curves of various engineered strains.

6.3.5 Generating mutagenesis libraries for future screening of better mutants

We asked whether the plac-hyaA-Ptac-MT12 strain could be optimized to generate even more bioelectricity. To this end, we randomly mutagenized the *hyaA* and *ndh* genes by error-prone PCR. Out of all the individual clones that were fully sequenced, a total of 16 *hyaA* mutants (Table 6.10) and 10 *ndh* mutants (Table 6.11) did not carry any frameshift mutations that might inactivate protein function. Hence, we will select these mutants for future study.

Table 6.10 Mutations of *hyaA* to be tested in this chapter.

hyaA variants		
A01V3	E128K	L165H
A01V8	E125D	L165H
A01V10	V142G	L165H
A01V11	S101T	E128G
A01V13	P147T	L165H
A01V14	R137L	L165H
A01V15	Y134D	A149T
A01V16	L109M	G164C
A01V17	L165H	P219A
A01V18	N98S	L165H
A01V19	E83G	A94V
A01V20	N98I	L140Q
A01V21	C84R	G86S
A01V22	G120S	L165H
A01V23	R93S	L165H
A01V25	G120R	L165H

Table 6.11 Mutations of *ndh* to be tested in this chapter.

MT12 variants						
MT12V1	K39R	W45R	S99R	G102E	E164*	
MT12V2	G70C					
MT12V3	A276G	C311Y	R325H	V383D		
MT12V6	L147I	T248A	W267C	G301A	V341E	F366Y
MT12V8	F381I					
MT12V9	H239L	Y337F				
MT12V10	L152P					
MT12V13	L219P	V341M	I393T			
MT12V14	G251A					
MT12V15	A225V					

6.4 Chapter summary

In this chapter, based on our genome-scale modeling results, we have overexpressed *hya* operon genes encoding quinone active NiFe dehydrogenase and *ndh* gene encoding the NADH dehydrogenase in *Shewanella oneidensis* MR-1 to improve electron yield and electron transfer efficiency in MFCs operation. The results showed that *hyaA* gene and *ndh* gene can increase electricity output in

MFCs while overexpressing either *hyaB*, *hyaC* or *hyaABC* could not generate positive results. We further combined the overexpression of *hyaA* with *ndh* to obtain better enhancement in MFCs performance, which resulted in 2.78-fold of increase in power output in MFCs inoculated with the engineered MR-1 strain.

We asked whether the improvement could be further elevated and generated mutagenesis libraries of respective genes, with the aim of screening for positive mutants that could achieve higher electron yield and electron transfer efficiency in MFCs operations.

CHAPTER 7 Engineering the Mtr pathway for the enhancement of bioelectricity generation

7.1 Introduction

As a structured microbial community, biofilm adhering to the anode surface of MFCs plays an important role in the EET by two types of mechanism: 1) direct electron transfer by outer membrane cytochromes or conductive pili; 2) mediated electron transfer by soluble mediators.^{6, 18, 89, 96, 200, 201} There are several reasons why biofilm can facilitate EET in MFCs, such as higher bacterial viability owing to anode respiration, and higher biomass density within the biofilm comparing with planktonic microbes in the medium.^{6, 202, 203} Nonetheless, natural biofilm in the anodes have limited thickness, which is mostly caused by limitations in nutrients diffusion and insufficient interaction of the microbial community to the anode electrode materials.^{113, 202, 204} Thus, optimizing thickness and morphology of the biofilm in anodes is a preferable strategy in improving the power output in MFCs. However, altering biofilm structures is not a simple task, since the underlying mechanisms of biofilm formation of many DMRBs remain ambiguous. Recently, efforts have been made in fabrication and functional decoration of the electrode materials in the anode to enhance the infinity between the biofilm and electrode.²⁰⁵⁻²⁰⁷ However, the limitations in the natural biofilm formation process could still be an obstacle in further enhancing the power output in MFCs using advanced electrode materials.

Shewanella oneidensis MR-1 is known for its distinguished extracellular electron transfer (EET) mechanisms. There are two main identified EET mechanisms that have been studied extensively: direct electron transfer (DET) by outer membrane *c*-type cytochromes and mediated electron transfer (MET) by soluble shuttles. In DET, five primary protein components have been identified: OmcA, MtrA, MtrB, MtrC and CymA, in which OmcA and MtrC (also named OmcB) are outer membrane *c*-type cytochromes contacting with electrode surface directly. Anode efficiency is one of the limiting factors in restricting electricity output in MFCs.^{97,}

²⁰⁸⁻²¹¹ Thus, improving EET efficiency is highly demanded for further enhancing MFCs performance.

In this chapter, we overexpressed genes related to the Mtr pathway to improve the electron transfer efficiency in MFCs inoculated with *Shewanella oneidensis* MR-1. Our results provided new insights in strategically improving MFCs performance via the means of genetic engineering.

7.2 Experiments

7.2.1 Bacterial strains, plasmids, and culture conditions

All strains and plasmids used in this study are given in Table 7.1. The *E. coli* and *S. oneidensis* MR-1 strains were routinely maintained and cultured in Luria-Bertani (LB) medium at 37°C and 30°C respectively. When necessary, the medium was supplemented with either kanamycin (50µg/ml) or ampicillin sodium (100µg/ml). In *E. coli* WM3064 culture, a final concentration of 0.3mM 2,6-Diaminopimelic acid (DPA) was added. The *S. oneidensis* MR-1 experiments were performed in *Shewanella* basal medium ¹⁰⁹ with 15mM L-lactate acting as the electron donor and carbon source. When needed, 40mM fumarate was supplemented as the electron acceptor.

Table 7.1 Bacterial strains and plasmids used in this chapter.

Strains	Description	Reference or source
<i>E. coli</i>		
DH5α	Host for regular cloning	Lab stock
WM3064	Donor strain used for conjugation with <i>S. oneidensis</i> MR-1, Δ <i>dapA</i>	Lab stock
<i>S. oneidensis</i>		
MR-1	Wildtype	Lab stock
pHG101	MR-1 harboring pHG101, used as control in this study; Km ^r	This study
MT19	MR-1 harboring MT19-pHG101	This study
MT20	MR-1 harboring MT20-pHG101	This study
MT21	MR-1 harboring MT21-pHG101	This study
MT22	MR-1 harboring MT22-pHG101	This study
MT23	MR-1 harboring MT23-pHG101	This study
mtrABC	MR-1 harboring mtrABC-pHG101	
Mtr operon	MR-1 harboring MtrOperon-pHG101	This study
Plasmids		

<i>pHG101</i>	Promoterless vector derived from broad host range vector ¹³⁹ <i>pBBR1MCS-2</i> ; <i>Km^r</i>	
<i>pSB1A2-Plac</i>	Biobrick containing <i>lac</i> promoter (<i>BBa_R0011</i> , iGEM) in <i>pSB1A2</i> plasmid backbone; <i>Amp^r</i>	Lab stock
<i>pSB1AK3-TT</i>	Biobrick containing double terminators (<i>BBa_B0015</i> , iGEM) in <i>pSB1AK3</i> plasmid backbone; <i>Km^r</i>	Lab stock
<i>MT19-pHG101</i>	<i>pHG101</i> harboring <i>SO_1779</i> (<i>omcA</i>)	This study
<i>MT20-pHG101</i>	<i>pHG101</i> harboring <i>SO_1777</i> (<i>mtrA</i>)	This study
<i>MT21-pHG101</i>	<i>pHG101</i> harboring <i>SO_1776</i> (<i>mtrB</i>)	This study
<i>MT22-pHG101</i>	<i>pHG101</i> harboring <i>SO_1778</i> (<i>mtrC</i>)	
<i>MT23-pHG101</i>	<i>pHG101</i> harboring <i>SO_4591</i> (<i>cymA</i>)	This study
<i>mtrABC- pHG101</i>	<i>pHG101</i> harboring <i>SO_1777</i> (<i>mtrA</i>), <i>SO_1776</i> (<i>mtrB</i>) and <i>SO_1778</i> (<i>mtrC</i>)	
<i>MtrOperon- pHG101</i>	<i>pHG101</i> harboring <i>SO_1779</i> (<i>omcA</i>), <i>SO_1777</i> (<i>mtrA</i>), <i>SO_1776</i> (<i>mtrB</i>), <i>SO_1778</i> (<i>mtrC</i>) and <i>SO_4591</i> (<i>cymA</i>)	This study

7.2.2 Plasmid construction

All genetic manipulation work was performed using standard protocols. Gene sequences were obtained from KEGG ¹⁷² and amplified via standard PCR protocol. Each of the amplified gene sequence was designed as a biobrick ¹⁷⁴, flanked by an upstream prefix containing EcoRI and XbaI and a downstream suffix containing SpeI and PstI. The target genes were then assembled through routine steps of biobrick ligation, inserting a *lac* promoter (*BBa_R0011*, iGEM) and a ribosome binding site (*BBa_B0034*, iGEM) in front and a double terminator (*BBa_B0015*, iGEM) at the back to regulate gene expression, forming a *Plac*-RBS-genes-TT construct in the vector *pSB1AK3* (iGEM). Finally, by using the restriction enzymes EcoRI and SpeI, the *Plac*-RBS-genes-TT fragment was cut and ligated into the broad-host-range expression vector *pHG101* ¹³⁹. The *E. coli* strain DH5α was used to amplify each plasmid. Every plasmid was confirmed by sequencing. Each of the final recombinant *pHG101*-based plasmids was transformed into *E. coli* WM3064 and subsequently introduced into *S. oneidensis* MR-1 via conjugation. Successful conjugation was confirmed by colony PCR.

7.2.3 Microbial fuel cell setup and operation

All experiments were performed using dual-chamber MFCs. The working volume of each of the chambers is 150mL. A Nafion 117 membrane (Gashub, Singapore) was used to separate the anode and cathode chambers after it had been immersed

overnight in 0.5M sulfuric acid and washed with DI water. Electrodes for both anode and cathode were made of carbon cloth (Gashub, Singapore) with a size of 2cm × 3cm. Cathodic solution consisted of 50mM K₃[Fe(CN)₆], 50mM K₂HPO₄, and 50mM KH₂PO₄.

Shewanella strains were grown overnight in a 30°C incubator at a shaking rate of 200rpm. Cell suspensions were adjusted to an optimal density of 0.5 before adding into the anode chamber. The anodes were purged with nitrogen to remove oxygen. After inoculation, the circuit was connected by copper wire with a constant external resistance of 2kΩ. The MFCs were operated at room temperature in batch mode. The duration of a full cycle was determined by the consumption of substrate and voltage reduction from its peak value to 50mV.

7.2.4 Data acquisition and calculations

Voltage output of each MFC was measured using a digital multimeter. To evaluate MFC performance, polarization curves were obtained by applying different loads or external resistances (R_{ext}) and then measuring the voltage (V) in order to calculate the current (I) by Ohm's Law². By normalizing power based on the surface area (A) of the anode electrode, power density (P) can be calculated by using the formula, $P = \frac{V \times I}{A}$.

7.2.5 Electrochemical analysis

Electrochemical measurements were performed using an electrochemical working station (CHI760D, CH Instruments Inc.) employing a three-electrode system. Saturated calomel electrode (SCE) was used as the reference electrode, with the anode as the working electrode and the cathode as the counter electrode. Cyclic voltammetry (CV) was carried out with a scanning range of -0.7 to 0.2V.

7.2.6 Scanning electron microscope (SEM) imaging of biofilm

To obtain an SEM image of the anode after operation, a small piece of the anodic carbon cloth was cut off and immersed in 1% glutaric dialdehyde solution overnight for bacteria fixation on the electrode surface. After stabilization, samples were rinsed 3 times using 0.9% NaCl solution and went through dehydration,

which consists of immersion of the sample in 30%, 50%, 70%, 90% and 100% ethanol solution for 20 minutes each at room temperature. Samples were then air-dried and ready for imaging. The morphology and relative density of biofilm was qualitatively characterized by a JEOL field emission scanning electron microscope (FESEM, JSM-6700F-FE-SEM, Japan).

7.2.7 Mutagenesis of the Mtr pathway genes.

Error-prone PCR of target genes was performed with GeneMorph[®] II EZClone Domain Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Primers were designed to amplify target genes within the coding sequences. Primers used in this process are listed in Table 7.2.

Table 7.2 Primers used in this chapter for mutagenesis

Primer	Sequence
MT19-EZ-F	TCATACCTTCGATGCTACCG
MT19-EZ-R	AATTCCTTGCCGATAGGTTG
MT22-EZ-F	TGCCGAAATGGTTGAAGATT
MT22-EZ-R	GGTTTTGGCTGTGTGTAAGT
MT23-EZ-F	GAGGAGAAATACTAGAATG
MT23-EZ-R	ACCACCTGCAGGTTA

7.3 Results and discussion

7.3.1 Overexpression of the Mtr pathway genes could enhance direct electron transfer

The original genes SO_1779 (*omcA*), SO_1777 (*mtrA*), SO_1776 (*mtrB*), SO_1778 (*mtrC*) and SO_4591 (*cymA*) from *Shewanella oneidensis* MR-1 were selected for this study. We overexpressed in *Shewanella oneidensis* MR-1 either individual genes singly, the *mtrABC* operon or all 5 genes together as the Mtr pathway under the control of a lac promoter. In total we constructed 7 distinct MR-1 strains for detailed characterization.

We first checked the growth of the 7 engineered MR-1 strains relative to a control strain harboring an empty pHG101 vector in *Shewanella* basal medium (SBM). We observed that the growths of all engineered strains have better growth comparing with the control strain (Figure 7.1A) with strain MT19 overexpressing *omcA* grew the best among all strains. Then we set up MFCs with all the different inoculum strains and operated them in batch mode (Figure 7.1B). Overall, the voltage output values show very different trends as the growth curves (Figure 7.1A). Specifically, the strain Mtr_pathway performed the best while the strain mtrABC overexpressing the mtr operon ran the second. In contrast, strain MT20 and MT21 overexpressing *mtrA* and *mtrB* respectively have the lowest voltage outputs, poorer than the control strain. Notably, even though overexpressing *mtrA* or *mtrB* decreased the voltage outputs, when co-overexpressed with other genes in strains mtrABC and Mtr_pathway, the voltage output values had significant increase. MFCs containing the strain Mtr_pathway produced a maximum voltage of 306 ± 21 mV at 122h, which was much higher than that of the control (191 ± 12 mV at 166h). We calculated the total charge transferred by integrating the area enclosed by the V-t curve and then dividing it by the external resistance (Table 7.3). Coulombic efficiencies were also obtained as previously described^{2, 176} (Table 7.3). Again, the MFCs with the Mtr_pathway strain discharged the most electrons and exhibited the highest Coulombic efficiency.

Table 7.3 The total charge transferred and columbic efficiency achieved in MFC anodes.

Anode	Electrons discharged (C)	Coulombic efficiency (%)
pHG101	69.31	7.98
MT19	124.79	14.38
MT20	60.13	6.92
MT21	65.79	7.57
MT22	118.16	13.61
MT23	113.85	13.12
mtrABC	128.99	14.86
Mtr_pathway	134.36	15.48

Figure 7.1 Characterization of MR-1 strains overexpressing genes in the mtr pathway (n=3).

(A) Growth curve measurements of the strains under anaerobic conditions.

(B) Voltage outputs of MFCs inoculated with various strains in the anodic chamber.

Next, we conducted CV analysis at a low scan rate of 1mV/s under turnover condition (Figure 7.2A). For all strains, the anodic curve first rose above background at -0.59V vs. SCE due to the occurrence of shuttle-mediated electron transfer (MET) via flavins^{181, 182}. This shuttle-mediated catalytic current was 31.66mA/m² and 22.61mA/m² for Mtr_pathway and the control strain respectively. Additionally, a second catalytic current arose at -0.45V vs. SCE for both strains due to the occurrence of contact-based direct electron transfer (DET) via the outer-membrane *c*-type cytochromes. Based on the first-derivative of the curves¹⁸³, we determined that this cytochrome-mediated current was 186.31mA/m² and 48.77mA/m² for Mtr_pathway and the control strain respectively (Table 7.4). Notably, for all engineered strains in Table 7.4, the shuttle-mediated catalytic current values were slight higher than the control strain, while the cytochrome-mediated current values were significantly increased. Hence, the enhanced EET observed for our engineered Mtr_pathway strain relative to control was caused by a significant increase in DET.

We obtained polarization curves at the peak output voltages to further characterize the performance of the MFCs (Figure 7.2B). The slopes of the listed engineered strains' voltage-current plots were less steep than that of the control. From Ohm's law, this implied that there were lower internal resistances in the MFCs with engineered strains in the anodic chamber, probably due to the enhanced electron transfer. Furthermore, based on the power density curves (Figure 7.2C), the short circuit current densities were 358.33mA/m² and 183.33mA/m² for Mtr_pathway and the control strain respectively. Importantly, Mtr_pathway achieved a maximum power density of 88.41±1.09mW/m², which was 2.14 times of that attained by the control strain (41.30±1.23mW/m²). The results achieved are comparable with other reported MFCs performances in similar configurations.^{164, 186-188} Taken together, our data highlight that overexpression of the Mtr pathway in *Shewanella* MR-1 could substantially enhance the performance of MFCs via improving the direct electron transfer efficiency.

A **B**

Figure 7.2 Detailed electrochemical characterization of MFCs with inoculations of engineered strains.

(A) Cyclic voltammograms at a scan rate of 1mV/s under turnover condition.

(B) Output voltage vs. current density (V-j) curves of various engineered strains.

(C) Power density vs. current density (P-j) curves of various engineered strains.

Table 7.4 Mediated electron transfer-based (MET) and direct electron transfer-based (DET) catalytic current densities with different *S. oneidensis* strains.

Anode	MET (mA/m ²)	DET (mA/m ²)
pHG101	22.61	48.77
MT19	29.88	121.74
MT22	27.61	116.69
MT23	24.89	131.13
mtrABC	30.65	151.90
Mtr_pathway	31.66	186.31

7.3.2 Overexpression of Mtr pathway genes could enhance biofilm formation

To confirm that the enhanced electricity generation is also improved by the enhancement of biofilm formation on the MFC anode's surface, we have taken SEM images of the biofilms after 400 hours of MFCs operation (Figure 7.3). Biofilm of the control strain could barely cover the surface of the electrode (Figure 7.3A), while biofilm formed by Mtr_pathway strain could cover up most of the electrode surface with a relatively thicker layer (Figure 7.3B).

Figure 7.3 Characterization of MFC's anodic biofilms after 400 hours of operation. (A) SEM image of the biofilm of the control strain. (B) SEM image of the biofilm of the Mtr_pathway strain.

7.3.3 Generating mutagenesis libraries for future screening of better mutants

We asked whether Mtr_pathway strain could be optimized to generate even more bioelectricity. To this end, we randomly mutagenized the *omcA*, *mtrC* and *cymA* genes by error-prone PCR. Out of all the individual clones that were fully sequenced, a total of 6 *omcA* mutants (Table 7.5), 10 *mtrC* mutants (Table 7.6) and 15 *cymA* mutants (Table 7.7) did not carry any frameshift mutations that might inactivate protein function. Hence, we will select these mutants for future study.

Table 7.5 Mutations of *omcA* to be tested in this chapter.

MT19 variants			
MT19V1	A381T	K389I	
MT19V3	C367Y	K398N	
MT19V4	H368Q		
MT19V5	H312R	A319T	D393N
MT19V6	C347R	H359R	
MT19V8	L395H		

Table 7.6 Mutations of *mtrC* to be tested in this chapter.

MT22 variants	
MT22V3	I289F
MT22V7	H233L
MT22V9	Q170H
MT22V10	C189Y
MT22V12	V232L
MT22V13	L267F
MT22V14	E263D
MT22V16	T278A
MT22V17	N231I
MT22V20	G217C
MT22V21	D290Y
MT22V22	F167S

Table 7.7 Mutations of *cymA* to be tested in this chapter.

MT23 variants				
MT23V1	Y30-	K96R		
MT23V2	W3S	D105E		
MT23V3	Q74-	K90-	N108S	
MT23V4	Y99N	K165R		
MT23V5	N131K			
MT23V6	G83V	V94L		
MT23V7	Y30N	C46S	S133-	K165N
MT23V8	Q35-	G83S		
MT23V13	H82L	C139F	C176R	
MT23V14	C46F	M47K	M151I	P167H
MT23V15	K152N			
MT23V17	S133P			
MT23V18	K165R			
MT23V19	Q35K	T72N	T109I	
MT23V20	A5V	T42I	E119K	N135I

7.4 Chapter summary

In this chapter, with the aim of increasing the efficiency of electron transfer in *Shewanella oneidensis* MR-1 during MFCs operations, we chose to overexpress genes in the Mtr pathway, which is the most important pathway for electron transport from the quinone/quinol pool to the outermembrane. We have found that the overexpression of all the genes involved in this pathway could significantly improve the direct electron transfer efficiency. And the resultant achieved a 2.14-fold increase in power density output in MFCs.

We also found that the resultant not only increased the Mtr pathway effectively, but also enhanced biofilm formation over the anode electrode surface, which is the most efficient path to transfer electrons from the bacteria community to the electrode via direct electron transfer.

In addition, we generated mutagenesis libraries for related genes in the Mtr pathway in the search of positive mutants to further improve the EET in MR-1 as well as speed up biofilm formation process, to eventually improve MFCs performance of *Shewanella oneidensis* MR-1.

CHAPTER 8 Characterization of genetic parts in *S. oneidensis* MR-1

8.1 Introduction

As *Shewanella oneidensis* MR-1 has become an important model organism for bioelectricity generation and MFCs applications, expanding genetic parts characterization library is highly demanded for improving modularity in genetically manipulating this microbe. In this chapter, we have made some preliminary characterization of ribosome binding site (RBS), a few constitutive and inducible promoters as well as terminators in *Shewanella oneidensis* MR-1.

8.2 Experiments

8.2.1 Bacterial strains, plasmids, and culture conditions

All strains and plasmids used in this study are given in Table 8.1. The *E. coli* and *S. oneidensis* MR-1 strains were routinely maintained and cultured in Luria-Bertani (LB) medium at 37°C and 30°C respectively. When necessary, the medium was supplemented with either kanamycin (50µg/ml) or ampicillin sodium (100µg/ml). In *E. coli* WM3064 culture, a final concentration of 0.3mM 2,6-Diaminopimelic acid (DPA) was added. The *S. oneidensis* MR-1 experiments were performed in *Shewanella* basal medium¹⁰⁹ with 15mM L-lactate acting as the electron donor and carbon source. When needed, 40mM fumarate was supplemented as the electron acceptor.

Table 8.1 Bacterial strains and plasmids used in this chapter.

Strains	Description	Reference or source
<i>E. coli</i>		
DH5α	Host for regular cloning	Lab stock
WM3064	Donor strain used for conjugation with <i>S. oneidensis</i> MR-1, <i>AdapA</i>	Lab stock
<i>S. oneidensis</i>		
MR-1	Wildtype	Lab stock
Plasmids	All plasmids were transformed into MR-1 for characterization	
pHG101	Promoterless vector derived from broad host range vector pBBR1MCS-2; Km ^r	¹³⁹
plac-RGT-pHG101	plac-RBS-GFP-TT fragment in pHG101 vector	This study
A1C	plac-RGT-pHG101 with the 1st base pair in RBS changed from A to C	This study
A1G	plac-RGT-pHG101 with the 1st base pair in RBS changed from A to G	This study

A1T	<i>plac-RGT-pHG101 with the 1st base pair in RBS changed from A to T</i>	<i>This study</i>
A2C	<i>plac-RGT-pHG101 with the 2nd base pair in RBS changed from A to C</i>	<i>This study</i>
A2G	<i>plac-RGT-pHG101 with the 2nd base pair in RBS changed from A to G</i>	<i>This study</i>
A2T	<i>plac-RGT-pHG101 with the 2nd base pair in RBS changed from A to T</i>	<i>This study</i>
A3C	<i>plac-RGT-pHG101 with the 3rd base pair in RBS changed from A to C</i>	<i>This study</i>
A3G	<i>plac-RGT-pHG101 with the 3rd base pair in RBS changed from A to G</i>	<i>This study</i>
A3T	<i>plac-RGT-pHG101 with the 3rd base pair in RBS changed from A to T</i>	<i>This study</i>
G4A	<i>plac-RGT-pHG101 with the 4th base pair in RBS changed from G to A</i>	<i>This study</i>
G4C	<i>plac-RGT-pHG101 with the 4th base pair in RBS changed from G to C</i>	<i>This study</i>
G4T	<i>plac-RGT-pHG101 with the 4th base pair in RBS changed from G to T</i>	<i>This study</i>
A5C	<i>plac-RGT-pHG101 with the 5th base pair in RBS changed from A to C</i>	<i>This study</i>
A5G	<i>plac-RGT-pHG101 with the 5th base pair in RBS changed from A to G</i>	<i>This study</i>
A5T	<i>plac-RGT-pHG101 with the 5th base pair in RBS changed from A to T</i>	<i>This study</i>
G6A	<i>plac-RGT-pHG101 with the 6th base pair in RBS changed from G to A</i>	<i>This study</i>
G6C	<i>plac-RGT-pHG101 with the 6th base pair in RBS changed from G to C</i>	<i>This study</i>
G6T	<i>plac-RGT-pHG101 with the 6th base pair in RBS changed from G to T</i>	<i>This study</i>
G7A	<i>plac-RGT-pHG101 with the 7th base pair in RBS changed from G to A</i>	<i>This study</i>
G7C	<i>plac-RGT-pHG101 with the 7th base pair in RBS changed from G to C</i>	<i>This study</i>
G7T	<i>plac-RGT-pHG101 with the 7th base pair in RBS changed from G to T</i>	<i>This study</i>
A8C	<i>plac-RGT-pHG101 with the 8th base pair in RBS changed from A to C</i>	<i>This study</i>
A8G	<i>plac-RGT-pHG101 with the 8th base pair in RBS changed from A to G</i>	<i>This study</i>
A8T	<i>plac-RGT-pHG101 with the 8th base pair in RBS changed from A to T</i>	<i>This study</i>
G9A	<i>plac-RGT-pHG101 with the 9th base pair in RBS changed from G to A</i>	<i>This study</i>
G9C	<i>plac-RGT-pHG101 with the 9th base pair in RBS changed from G to C</i>	<i>This study</i>
G9T	<i>plac-RGT-pHG101 with the 9th base pair in RBS changed from G to T</i>	<i>This study</i>
A10C	<i>plac-RGT-pHG101 with the 10th base pair in RBS changed from A to C</i>	<i>This study</i>
A10G	<i>plac-RGT-pHG101 with the 10th base pair in RBS changed from A to G</i>	<i>This study</i>
A10T	<i>plac-RGT-pHG101 with the 10th base pair in RBS changed from A to T</i>	<i>This study</i>
A11C	<i>plac-RGT-pHG101 with the 11th base pair in RBS changed from A to C</i>	<i>This study</i>
A11G	<i>plac-RGT-pHG101 with the 11th base pair in RBS changed from A to G</i>	<i>This study</i>
A11T	<i>plac-RGT-pHG101 with the 11th base pair in RBS changed from A to T</i>	<i>This study</i>
A12C	<i>plac-RGT-pHG101 with the 12th base pair in RBS changed from A to C</i>	<i>This study</i>
A12G	<i>plac-RGT-pHG101 with the 12th base pair in RBS changed from A to G</i>	<i>This study</i>
A12T	<i>plac-RGT-pHG101 with the 12th base pair in RBS changed from A to T</i>	<i>This study</i>
<i>plac-mCherry-GFP-pHG101</i>	<i>No terminator between mCherry and GFP as negative control</i>	<i>This study</i>
ECK120033736	<i>plac-mCherry-ECK120033736-GFP-pHG101</i>	<i>This study</i>
ECK120010818	<i>plac-mCherry-ECK120010818-GFP-Phg101</i>	<i>This study</i>
ECK120010799	<i>plac-mCherry-ECK120010799-GFP-pHG101</i>	<i>This study</i>
BBa_B0010	<i>plac-mCherry-BBa_B0010-GFP-pHG101</i>	<i>This study</i>
ECK120051401	<i>plac-mCherry-ECK120051401-GFP-pHG101</i>	<i>This study</i>
L3S2P21	<i>plac-mCherry-L3S2P21-GFP-pHG101</i>	<i>This study</i>
L3S3P21	<i>plac-mCherry-L3S3P21-GFP-pHG101</i>	<i>This study</i>

<i>L3S2P24</i>	<i>plac-mCherry-L3S2P24-GFP-pHG101</i>	<i>This study</i>
<i>L3S1P47</i>	<i>plac-mCherry-L3S1P47-GFP-pHG101</i>	<i>This study</i>
<i>L3S2P44</i>	<i>plac-mCherry-L3S2P44-GFP-pHG101</i>	<i>This study</i>

8.2.2 Plasmid construction

All genetic manipulation work was performed using standard protocols. Each of the amplified gene sequence was designed as a biobrick¹⁷⁴, flanked by an upstream prefix containing EcoRI and XbaI and a downstream suffix containing SpeI and PstI. The target genes were then assembled through routine steps of biobrick ligation. For RBS characterization, a *lac* promoter (BBa_R0011, iGEM) and a ribosome binding site (BBa_B0034, iGEM) were inserted in front of GFP (BBa_E0040, iGEM) and a double terminator (BBa_B0015, iGEM) at the back to regulate gene expression, forming a *Plac*-RBS-GFP-TT construct in the vector pSB1AK3 (iGEM). For terminator characterizations, an mCherry coding sequence was inserted in front of the GFP with each of the terminator sequence to be tested lying in between the two reporters, forming a *Plac*-RBS-mCherry-terminatorX-RBS-GFP-TT construct in the vector pSB1AK3. Finally, by using the restriction enzymes EcoRI and SpeI, the *Plac*-RBS-genes-TT and *Plac*-RBS-mCherry-terminatorX-RBS-GFP-TT fragments were cut and ligated into the broad-host-range expression vector pHG101¹³⁹. Figure 8.1 shows the schematic views of the constructed plasmids for this chapter. The *E. coli* strain DH5 α was used to amplify each plasmid. Every plasmid was confirmed by sequencing. Each of the final recombinant pHG101-based plasmids was transformed into *E. coli* WM3064 and subsequently introduced into *S. oneidensis* MR-1 via conjugation. Successful conjugation was confirmed by colony PCR.

Figure 8.1 Schematic illustration of constructed plasmids for genetic parts characterization in this chapter.

- (A) Plasmid constructed to test RBS mutants.
- (B) Plasmid constructed to test selected terminators.
- (C) Plasmid constructed to test selected promoters.

8.2.3 Generation of RBS mutants

RBS mutants were constructed by using the QuickChange lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Plasmid plac-RGT-pHG101 was used as the template for mutant generation. Primers used in this process are listed in Table 8.2.

Table 8.2 QuickChange primers used to generate RBS mutants in this chapter.

Sequence Name	Sequence
RBS_bp1_A2C_sense	GAT ACT GAG CAC ATA CTA GAG CAA GAG GAG AAA TAC TAG ATG C
RBS_bp1_A2C_anti	GCA TCT AGT ATT TCT CCT CTT GCT CTA GTA TGT GCT CAG TAT C
RBS_bp1_A2G_sense	GAT ACT GAG CAC ATA CTA GAG GAA GAG GAG AAA TAC TAG ATG C
RBS_bp1_A2G_anti	GCA TCT AGT ATT TCT CCT CTT CCT CTA GTA TGT GCT CAG TAT C

RBS_bp1_A2T_sense	GAT ACT GAG CAC ATA CTA GAG TAA GAG GAG AAA TAC TAG ATG C
RBS_bp1_A2T_anti	GCA TCT AGT ATT TCT CCT CTT ACT CTA GTA TGT GCT CAG TAT C
RBS_bp2_A2C_sense	ATA ACA AGA TAC TGA GCA CAT ACT AGA GAC AGA GGA GAA ATA CTA G
RBS_bp2_A2C_anti	CTA GTA TTT CTC CTC TGT CTC TAG TAT GTG CTC AGT ATC TTG TTA T
RBS_bp2_A2G_sense	ATA ACA AGA TAC TGA GCA CAT ACT AGA GAG AGA GGA GAA ATA CTA G
RBS_bp2_A2G_anti	CTA GTA TTT CTC CTC TCT CTC TAG TAT GTG CTC AGT ATC TTG TTA T
RBS_bp2_A2T_sense	ATA ACA AGA TAC TGA GCA CAT ACT AGA GAT AGA GGA GAA ATA CTA G
RBS_bp2_A2T_anti	CTA GTA TTT CTC CTC TAT CTC TAG TAT GTG CTC AGT ATC TTG TTA T
RBS_bp3_A2C_sense	ACA AGA TAC TGA GCA CAT ACT AGA GAA CGA GGA GAA ATA CTA GA
RBS_bp3_A2C_anti	TCT AGT ATT TCT CCT CGT TCT CTA GTA TGT GCT CAG TAT CTT GT
RBS_bp3_A2G_sense	ACA AGA TAC TGA GCA CAT ACT AGA GAA GGA GGA GAA ATA CTA GA
RBS_bp3_A2G_anti	TCT AGT ATT TCT CCT CCT TCT CTA GTA TGT GCT CAG TAT CTT GT
RBS_bp3_A2T_sense	ACA AGA TAC TGA GCA CAT ACT AGA GAA TGA GGA GAA ATA CTA GA
RBS_bp3_A2T_anti	TCT AGT ATT TCT CCT CAT TCT CTA GTA TGT GCT CAG TAT CTT GT
RBS_bp4_G2A_sense	ACA AGA TAC TGA GCA CAT ACT AGA GAA AAA GGA GAA ATA CTA GAT GC
RBS_bp4_G2A_anti	GCA TCT AGT ATT TCT CCT TTT TCT CTA GTA TGT GCT CAG TAT CTT GT
RBS_bp4_G2C_sense	AAG ATA CTG AGC ACA TAC TAG AGA AAC AGG AGA AAT ACT AGA TG
RBS_bp4_G2C_anti	CAT CTA GTA TTT CTC CTG TTT CTC TAG TAT GTG CTC AGT ATC TT
RBS_bp4_G2T_sense	ACA AGA TAC TGA GCA CAT ACT AGA GAA ATA GGA GAA ATA CTA GAT GC
RBS_bp4_G2T_anti	GCA TCT AGT ATT TCT CCT ATT TCT CTA GTA TGT GCT CAG TAT CTT GT
RBS_bp5_A2C_sense	GAG CAC ATA CTA GAG AAA GCG GAG AAA TAC TAG ATG CGT
RBS_bp5_A2C_anti	ACG CAT CTA GTA TTT CTC CGC TTT CTC TAG TAT GTG CTC
RBS_bp5_A2G_sense	GAG CAC ATA CTA GAG AAA GGG GAG AAA TAC TAG ATG CGT
RBS_bp5_A2G_anti	ACG CAT CTA GTA TTT CTC CCC TTT CTC TAG TAT GTG CTC
RBS_bp5_A2T_sense	GAG CAC ATA CTA GAG AAA GTG GAG AAA TAC TAG ATG CGT
RBS_bp5_A2T_anti	ACG CAT CTA GTA TTT CTC CAC TTT CTC TAG TAT GTG CTC
RBS_bp6_G2A_sense	CTG AGC ACA TAC TAG AGA AAG AAG AGA AAT ACT AGA TGC GTA AAG
RBS_bp6_G2A_anti	CTT TAC GCA TCT AGT ATT TCT CTT CTT TCT CTA GTA TGT GCT CAG
RBS_bp6_G2C_sense	GAG CAC ATA CTA GAG AAA GAC GAG AAA TAC TAG ATG CGT AA
RBS_bp6_G2C_anti	TTA CGC ATC TAG TAT TTC TCG TCT TTC TCT AGT ATG TGC TC
RBS_bp6_G2T_sense	CTG AGC ACA TAC TAG AGA AAG ATG AGA AAT ACT AGA TGC GTA AAG
RBS_bp6_G2T_anti	CTT TAC GCA TCT AGT ATT TCT CAT CTT TCT CTA GTA TGT GCT CAG
RBS_bp7_G2A_sense	GAG CAC ATA CTA GAG AAA GAG AAG AAA TAC TAG ATG CGT AAA G
RBS_bp7_G2A_anti	CTT TAC GCA TCT AGT ATT TCT TCT CTT TCT CTA GTA TGT GCT C
RBS_bp7_G2C_sense	GAG CAC ATA CTA GAG AAA GAG CAG AAA TAC TAG ATG CGT AAA G
RBS_bp7_G2C_anti	CTT TAC GCA TCT AGT ATT TCT GCT CTT TCT CTA GTA TGT GCT C
RBS_bp7_G2T_sense	GAG CAC ATA CTA GAG AAA GAG TAG AAA TAC TAG ATG CGT AAA G
RBS_bp7_G2T_anti	CTT TAC GCA TCT AGT ATT TCT ACT CTT TCT CTA GTA TGT GCT C
RBS_bp8_A2C_sense	GCA CAT ACT AGA GAA AGA GGC GAA ATA CTA GAT GCG TAA AG
RBS_bp8_A2C_anti	CTT TAC GCA TCT AGT ATT TCG CCT CTT TCT CTA GTA TGT GC
RBS_bp8_A2G_sense	GCA CAT ACT AGA GAA AGA GGG GAA ATA CTA GAT GCG TAA AG
RBS_bp8_A2G_anti	CTT TAC GCA TCT AGT ATT TCC CCT CTT TCT CTA GTA TGT GC
RBS_bp8_A2T_sense	GCA CAT ACT AGA GAA AGA GGT GAA ATA CTA GAT GCG TAA AG

RBS_bp8_A2T_anti	CTT TAC GCA TCT AGT ATT TCA CCT CTT TCT CTA GTA TGT GC
RBS_bp9_G2A_sense	GCA CAT ACT AGA GAA AGA GGA AAA ATA CTA GAT GCG TAA AGG A
RBS_bp9_G2A_anti	TCC TTT ACG CAT CTA GTA TTT TTC CTC TTT CTC TAG TAT GTG C
RBS_bp9_G2C_sense	CAC ATA CTA GAG AAA GAG GAC AAA TAC TAG ATG CGT AAA GG
RBS_bp9_G2C_anti	CCT TTA CGC ATC TAG TAT TTG TCC TCT TTC TCT AGT ATG TG
RBS_bp9_G2T_sense	GCA CAT ACT AGA GAA AGA GGA TAA ATA CTA GAT GCG TAA AGG A
RBS_bp9_G2T_anti	TCC TTT ACG CAT CTA GTA TTT ATC CTC TTT CTC TAG TAT GTG C
RBS_bp10_A2C_sense	CAC ATA CTA GAG AAA GAG GAG CAA TAC TAG ATG CGT AAA GGA G
RBS_bp10_A2C_anti	CTC CTT TAC GCA TCT AGT ATT GCT CCT CTT TCT CTA GTA TGT G
RBS_bp10_A2G_sense	CAC ATA CTA GAG AAA GAG GAG GAA TAC TAG ATG CGT AAA GGA G
RBS_bp10_A2G_anti	CTC CTT TAC GCA TCT AGT ATT CCT CCT CTT TCT CTA GTA TGT G
RBS_bp10_A2T_sense	CAC ATA CTA GAG AAA GAG GAG TAA TAC TAG ATG CGT AAA GGA G
RBS_bp10_A2T_anti	CTC CTT TAC GCA TCT AGT ATT ACT CCT CTT TCT CTA GTA TGT G
RBS_bp11_A2C_sense	CAT ACT AGA GAA AGA GGA GAC ATA CTA GAT GCG TAA AGG AG
RBS_bp11_A2C_anti	CTC CTT TAC GCA TCT AGT ATG TCT CCT CTT TCT CTA GTA TG
RBS_bp11_A2G_sense	CAT ACT AGA GAA AGA GGA GAG ATA CTA GAT GCG TAA AGG AG
RBS_bp11_A2G_anti	CTC CTT TAC GCA TCT AGT ATC TCT CCT CTT TCT CTA GTA TG
RBS_bp11_A2T_sense	CAT ACT AGA GAA AGA GGA GAT ATA CTA GAT GCG TAA AGG AG
RBS_bp11_A2T_anti	CTC CTT TAC GCA TCT AGT ATA TCT CCT CTT TCT CTA GTA TG
RBS_bp12_A2C_sense	ACA TAC TAG AGA AAG AGG AGA ACT ACT AGA TGC GTA AAG GAG
RBS_bp12_A2C_anti	CTC CTT TAC GCA TCT AGT AGT TCT CCT CTT TCT CTA GTA TGT
RBS_bp12_A2G_sense	ACA TAC TAG AGA AAG AGG AGA AGT ACT AGA TGC GTA AAG GAG
RBS_bp12_A2G_anti	CTC CTT TAC GCA TCT AGT ACT TCT CCT CTT TCT CTA GTA TGT
RBS_bp12_A2T_sense	ACA TAC TAG AGA AAG AGG AGA ATT ACT AGA TGC GTA AAG GAG
RBS_bp12_A2T_anti	CTC CTT TAC GCA TCT AGT AAT TCT CCT CTT TCT CTA GTA TGT

8.2.4 Fluorescence quantification

Overnight cultures were diluted in LB medium to $OD_{600nm} \sim 0.1$ for subcultural growth. When cell growth reached mid-log phase, induction was started by the addition of inducers. After 6 hours of induction, 200 μ L of cells were collected and washed with phosphate buffer solution (PBS) for three times. Then the cell pellets were re-suspended in 200 μ L PBS and allocated into a 96-well plate for microplate measurements. Fluorescent intensities (For GFP: excitation at 485 nm and emission at 520nm; for mCherry: excitation at 587 nm and emission at 610nm) and optical density (OD_{600nm}) were measured by a microplate reader (SpectraMax M5, Molecular Devices, USA). Three replicates were performed for each sample.

8.3 Results and discussion

8.3.1 Characterization of a ribosome binding site and its mutants in *Shewanella oneidensis* MR-1

We have generated a library of RBS mutants using a popular BBa_B0034 from the iGEM registry by substituting all three possible single base pair for each of the 12 nucleotides in the original sequence (Figure 8.2A). For example, switching the first base pair from A to C, G and T. By using a green fluorescent protein (GFP) (BBa_E0040) as a reporter, we characterized how strong each of the RBS mutants can initiate the GFP translation in MR-1 under a constitutive lac promoter (BBa_R0010).

The relative fluorescence of GFP among the mutants are exhibited in Figure 8.2B. Though for all mutants, the growth showed little difference from the control strain, fluorescent intensities vary among the mutants. It was found that substitutions occurring to positions ranging from the 5th to the 9th nucleotides within the BBa_R0034 sequence exhibited decreased GFP expression, while others showed enhanced GFP expression. Specifically, for mutations in the 7th nucleotide, all 3 mutants showed near to total-depression GFP expression.

A

Wildtype			AAAGAGGAGAAA		
No.	Name	Sequence	No.	Name	Sequence
1	A1C	<u>C</u> AAGAGGAGAAA	19	G7A	AAAGAG <u>A</u> AGAAA
2	A1G	<u>G</u> AAGAGGAGAAA	20	G7C	AAAGAG <u>C</u> AGAAA
3	A1T	<u>T</u> AAGAGGAGAAA	21	G7T	AAAGAG <u>T</u> AGAAA
4	A2C	A <u>C</u> AGAGGAGAAA	22	A8C	AAAGAGG <u>C</u> GAAA
5	A2G	A <u>G</u> AGAGGAGAAA	23	A8G	AAAGAGG <u>G</u> GAAA
6	A2T	A <u>T</u> AGAGGAGAAA	24	A8T	AAAGAGG <u>T</u> GAAA
7	A3C	AA <u>C</u> GAGGAGAAA	25	G9A	AAAGAGG <u>A</u> AAA
8	A3G	AA <u>G</u> GAGGAGAAA	26	G9C	AAAGAGG <u>C</u> AAA
9	A3T	AA <u>T</u> GAGGAGAAA	27	G9T	AAAGAGG <u>T</u> AAA
10	G4A	AAA <u>A</u> AGGAGAAA	28	A10C	AAAGAGGAG <u>C</u> AA
11	G4C	AAA <u>C</u> AGGAGAAA	29	A10G	AAAGAGGAG <u>G</u> AA
12	G4T	AAA <u>T</u> AGGAGAAA	30	A10T	AAAGAGGAG <u>T</u> AA
13	A5C	AAAG <u>C</u> GGAGAAA	31	A11C	AAAGAGGAG <u>C</u> A
14	A5G	AAAG <u>G</u> GGAGAAA	32	A11G	AAAGAGGAG <u>G</u> A
15	A5T	AAAG <u>T</u> GGAGAAA	33	A11T	AAAGAGGAG <u>T</u> A
16	G6A	AAAGA <u>A</u> GAGAAA	34	A12C	AAAGAGGAG <u>A</u> C
17	G6C	AAAGA <u>C</u> GAGAAA	35	A12G	AAAGAGGAG <u>A</u> G
18	G6T	AAAGA <u>T</u> GAGAAA	36	A12T	AAAGAGGAG <u>A</u> T

Figure 8.2 Characterization of RBS mutants in *Shewanella oneidensis* MR-1 (n=3).
(A) Sequences of 36 RBS mutants generated in this chapter.
(B) Normalized fluorescent intensities GFP measured in engineered MR-1 strains harboring different RBS mutants.

8.3.2 Characterization of terminators in *Shewanella oneidensis* MR-1

We have selected 10 terminators to characterize in *Shewanella oneidensis* MR-1. The selected terminators were respectively placed between two reporters, mCherry and GFP. Figure 8.3 showed fluorescent microscope images of all the resultants, while Figure 8.4 showed quantification results of the strength of the selected terminators. Terminators ECK120051401, ECK120010799, L3S2P21 and L3S2P44 exhibited weaker terminating strength while the rest were stronger in terminating the upstream mCherry transcriptions and reducing GFP expressions.

Figure 8.3 Characterization of selected terminators in *S. oneidensis* MR-1. Fluorescent microscope imaging of engineered MR-1 strains harboring different terminators.

Figure 4 Characterization of selected terminators in *S. oneidensis* MR-1 (n=3). Normalized fluorescent intensities of mCherry and GFP in engineered MR-1 strains harboring different terminators. All data are shown as the mean $\pm$ SD. All *p*-values are based on negative control versus terminator group. **p*<0.05, ***p*<0.01, ****p*<0.001.

8.3.3 Characterization of promoters in *Shewanella oneidensis* MR-1

To characterize promoters in *Shewanella oneidensis* MR-1, we have selected several constitutive promoters, including several Anderson promoters (BBa_J23101, BBa_J23102, BBa_J23104, BBa_J23105, BBa_J23108, BBa_J23109, BBa_J23110, BBa_J23115) and a lac promoter (BBa_R0010) from the parts.igem.org library. Figure 8.5 showed results of the tested promoters in controlling the expression of GFP, the fluorescent intensities were normalized by OD_{600nm}. For the measured Anderson promoters, BBa_J23101, BBa_J23102, BBa_J23104 and BBa_J23109 showed relative high strength, BBa_J23110 and BBa_J23115 showed medium strength, BBa_J23105 and BBa_J23108 showed low strength. However, none could compare with BBa_R0010, which is a super high strength constitutive promoter. These results have provided us a selection of constitutive promoters with different strengths to utilize in future circuit designs.

Figure 8.5 Characterization of selected constitutive promoters in *Shewanella oneidensis* MR-1 (n=3). All data are shown as the mean $\pm$ SD. All p -values are based on R0010 versus selective promoter. * p <0.05, ** p <0.01, *** p <0.001.

Figure 8.6 Characterization of inducible pBAD promoter with different amount of L-arabinose in *Shewanella oneidensis* MR-1 (n=3). Induction began after cells grown to mid-log phase. GFP fluorescence was corrected with OD_{600nm} value. All data are shown as the mean±SD. All *p*-values are based on negative control versus induction group. **p*<0.05, ***p*<0.01, ****p*<0.001.

Figure 8.7 Characterization of inducible tac promoter with different amount of IPTG in *Shewanella oneidensis* MR-1 (n=3). Induction began after cells grown to mid-log phase. GFP fluorescence was corrected with OD_{600nm} value. All data are shown as the mean $\pm$ SD. All *p*-values are based on negative control versus induction group. **p*<0.05, ***p*<0.01, ****p*<0.001.

We have also tested inducible promoters in *Shewanella oneidensis* MR-1, including an L-arabinose inducible pBAD promoter and an IPTG inducible tac promoter. Figure 8.6 showed the relative fluorescence of GFP controlled by the pBAD promoter, under the induction of L-arabinose at different concentrations. It was shown that the expression level of GFP increased with the amount of L-arabinose. GFP expression level reached the highest with the induction of 0.5% L-arabinose.

Figure 8.7 showed the GFP expression controlled by the tac promoter induced with IPTG at different concentrations. It showed that tac promoter is a relatively strong promoter comparing with pBAD promoter. And the higher the concentration of IPTG, the better the expression level of GFP.

All together, these results have provided selections of inducible promoters with different inducers for future applications in genetic circuit design.

8.4 Chapter summary

In this chapter, we have characterized ribosome binding sites, terminators as well as constitutive and inducible promoters in *Shewanella oneidensis* MR-1, resulting in a wide range of selection of genetic parts that could be utilized in *Shewanella oneidensis* MR-1 for applications in synthetic biology. The characterized parts are very helpful in designing functional logic gates, as well as scalable control of gene expressions.

CHAPTER 9 General conclusions and future perspectives

9.1 Conclusions

In this PhD work, our research focus was to genetically engineering *Shewanella oneidensis* MR-1 to improve its performance in electricity generation in MFCs operation. By adopting different strategies, we successfully increased lactate consumption rate and lactate utilization in *Shewanella oneidensis* MR-1, improved intracellular electron generation and transport, and enhanced extracellular electron transfer. Therefore, the power output in MFCs inoculated with pure *Shewanella oneidensis* MR-1 culture was improved.

In order to improve MFCs performance, better efficiency of utilizing lactate as the electron donor should be achieved. In Chapter 4 and Chapter 5, we overexpressed lactate permease and dehydrogenase from different organisms in MR-1, and generated mutagenesis library for further screening of better mutants. We have successfully identified a winning mutant with 3 mutational sites being analyzed, and improved not only lactate consumption and transport, but also important electron respiring pathways in the cells as well as extracellular electron transfer and biofilm formation. The MFCs power output was significantly improved.

In Chapter 6, computational modeling was adopted to search for useful potential proteins in improving electron transfer in *Shewanella oneidensis* MR-1. We validate the computational results with experiments and overexpressed relevant genes. As a result, the direct electron transfer was greatly improved in *Shewanella oneidensis* MR-1 and eventually the MFCs performance was enhanced. By implementing an inducible promoter in controlling gene expressions, we also realized a need for usable genetic parts in future for MFCs applications, which is addressed in Chapter 8.

To further improve the extracellular electron transfer and biofilm formation, in Chapter 7, we investigated several important *c*-type cytochromes in the enhancement of electricity generation. The Mtr pathway genes were overexpressed in *Shewanella oneidensis* MR-1 and resulted in drastically improved direct electron

transfer and biofilm formation, addressing the limiting factor of anode efficiency in MFCs performance.

Lastly in Chapter 8, we initiated the construction of a genetic part library for the model microorganism *Shewanella*. We generated mutants from a commonly used ribosome binding site and characterized terminators and promoters. These are useful modular tools for scalable control and flexible operation in MFCs applications in future.

The power outputs achieved in this Ph. D work are compared with several reported MFCs performances inoculated with genetically modified *S. oneidensis* MR-1 strains in similar configurations (Table 9.1), the works in this thesis showed comparable results with other studies.

Table 9.1 Summary of reported power output of MFCs with inoculation of biologically engineered *S. oneidensis* MR-1 with lactate as carbon source.

Reference	MFC configuration	Electrode material	Power density (mW/m ²)	Benefits of modification
¹⁸⁶	Single chamber, 450 mL	Graphite felt	~66	Enhanced Hydrophobic cell surface
¹⁸⁷	Single chamber, 450 mL	Graphite felt	~105	Enhanced cell-electrode interaction
¹⁸⁸	Single chamber, 450 mL	Graphite felt	~90	Enhanced hydrophobic cell surface
¹⁶⁴	Dual chamber, 50mL	Carbon cloth	167.6±3.6	Enhanced biofilm formation
Chapter 5	Dual chamber, 150mL	Carbon cloth	118.58±2.67	Enhanced lactate utilization pathway
Chapter 6	Dual chamber, 150mL	Carbon cloth	112.00±1.77	Enhanced intracellular electron transfer
Chapter 7	Dual chamber, 150mL	Carbon cloth	88.41±1.09	Enhanced Mtr pathway

9.2 Directions for future research

Though only a small portion in the field of MFCs technology, the works presented in this thesis bring new strategies to the development in MFCs performance – the electron pathways from substrate degradation, intracellular electron production, to the extracellular electron transfer were being carefully manipulated to achieve

improved electricity generation in MFCs. Mutagenesis technology is a promising means to engineer microorganisms and enhance the efficiency of microbial electrochemical systems. Computational modeling offers new perspectives in investigating molecular mechanism under complex conditions.

In some of the chapters, mutagenesis libraries were generated, and novel high throughput screening methods are demanded for more efficient filtering to obtain better resultants in future works. For example, a competition assay could be implemented for the screening of mutants in Chapter 5, since the mutated protein is related to substrate consumption and utilization. When lactate is being used as the sole nutrient source and the competing factor, pools of mutants can be generated and inoculated together to compete for the limited amount of lactate in the environment. The winner mutant strains should be equipped with the best positive mutations in order to utilize lactate faster than most of its competitor strains.

Since MFCs operations of a large number of mutants are time requiring, for the screening and characterization of mutants generated in Chapter 6 and Chapter 7, a new screening approach should be developed. The new approach should be able to filter a large number of mutants with shorter operation time and easy detecting method to determine the efficiencies of electron transfer from different mutant samples.

In Chapter 4 and Chapter 5, the enhanced lactate utilization pathway resulted in an improved consumption of the substrate and increased production of electrons. There are still more room for further increases in electron generation by silencing irrelevant non-fatal pathways inside the cell and direct more lactate into the sufficient pathways related to bioelectricity generation. In Chapter 6 and Chapter 7, modifications were done to increase electron production and transfer. More can be done to eliminate the flow paths of electrons that are not necessarily important in cellular survival and to guide the electron flow to the outer membrane to increase the efficiency in bioelectricity generation. Moreover, another future direction, is to combine all the engineered genes in this thesis together in a large circuit to enhance different pathways in *S. oneidensis* MR-1 simultaneously, with the expectation of

resultant with a more efficient and economical construct of modified pathways and a much more improved bioelectrical performance in MFCs operations.

Additionally, the genetic parts library should be enriched further with more usable parts, such as ribosome binding sites, promoters, terminators, degradation tags, etc. with different strength and efficiency. Moreover, in order to achieve modularity in designing and constructing genetic circuits, standardized building blocks should be generated and characterized for faster and more convenient assembly of genetic modules.

A. Synthesized gene sequences in Chapter 4.

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SH5	<p>CGaattcggcgccgttctagagAAAGAGGAGAAACTAGAAatgtctagtaagcacgaaattctcaatgcgctcaaactgtcggcttaacaacctcct atgccaaagcatcgatgtcggcccgagggtgaagatctggtggccagtttgaaccaatcttaaacctggcgggcacactgcaccgcgaaggcgccgttcggcgactgc aagccaaagtggatgagctgattgcccaaggcttacaagttatctccctcgttgaggcggtcacggcgaaccgtgatgtgccaccaacggctcacgagctcagggataatcgat tatgcggttattcctggggatgaggtgttgcgaaaaatgggtgccatttgggttaacaataagaatctcgccacagagtgcacacctttatctgtgaaaacctgatcttagccttac ccatccataaaatgtgccaaatgcatcaggccgcgaaagaagtgcacttagatgcaggcgagtttggcgtgttatcgaggccctcaaaaaccgcgatatcgagcaag ccctcgtcgtcggcgcccgacgggtccccaacttaaatgtgtatctgggttaatactagtagcgccgcctgcaggCG</p>
SH7	<p>CGaattcggcgccgttctagagAAAGAGGAGAAACTAGAAATGATCATCTCTGCTTCTACTGATTACCGTGCTGCTG CTCAACGTAAATTACCACCATTCTTATTCCACTACATCGATGGTGGTGCTTACGCTGAATACACTTTACGT CGTAACGTTGAAGATTTATCTGCTATCGCTTTACGTCAACGTGTTTTAAAAAATGTCTGAATTATCTTTA GAAACTCGTTTATTCGATGAACTTTAGCTATGCCAGTTGCTTTAGCTCCAGTTGGTTTAACTGGTATGTA CGCTCGTCGTGGTGAAGTTCAAGCTGCTCGTGCTGCTGCTGCTAAAGGTGTTCCATTCACTTTATCTACTGT TTCTGTTTGTCCAATCGAAGAAGTTGCTCCAGCTATCGATCGTCCAATGTGGTTCCAATTATACGTTTTAA AAGATCGTGGTTTCATGCGTAACGCTTTAGAACGTGCTAAAGCTGCTGGTGTACTACTTTAGTTTTACT GTTGATATGCCAGTTCAGGTGCTCGTTACCGTGATGCTCACTCTGGTATGTCTGGTCCATACGCTGCTCC ACGTCGTATCTTACAAGCTATGACTCACCCAGCTTGGGCTTGGGATGTTGGTTTATTAGGCAAGCCACACG ATCTCGGTAACATCTCGGCTTACCGTGGTAACCCAAGTGGTTTGAAGATTACATCGGTTGGTTAGGTGCT AACTTCGATCCATCTATCTTGGAAAGATTTAGAATGGATCCGTgagtttTGGGATGGTCCAATGGTTATCA AAGGTATCTTAGATCCAGAAGATGCTCGTGATGCTGTTAAATTCGGTGCTGATGGCATAGTTGTAAGCAA CCACGGTGGTCGTCAATTAGATGGTGTGTTTATCTTCTGCTCGTGCTTTACCAGCTATCGCTGATGCTGTTAA AGGTGAATTAGCTATCTTAGCTGATTCTGGTATCCGTAAGTGGTTTAGATGTTGTTTCGTATGATCGCTTTAGG TGCTGATTCTGTTTTATTAGGTCGTGCTTTCGTTTACGCTTTAGCTGCTGCTGGTGAAGCTGGTGTTCGTAA CTTATTAGAATTAATCGAAAAAGAAATGCGTGTGCTATGGTTTTAACTGGTGCTAAATCTATCGGTGAAA TCTCTGCTGATTCTTTAGTTCGTGAATTAGGTGCTTAAactagtagcgccgcctgcaggCG</p>

SH8	<p>CGgaattcggccgcttctagagAAAGAGGAGAAATCTAGTATGCAAGCTTGGCAACAATTATACACTCCATTAGGT TCTTTAGGTTTATCTGCTGCTGCTGCTGTTATCCCAATCGTTTTCTTCTTCTTAGCTTTAGCTGTTTTCCGTT TAAAAGGTCACATCGCTGGTCTATCACTTTAGCTTTAGCTATCGCTGTTGCTATCTTCGCTTCCACATGC CCGCTGACATGGCGTTCGCTGCTGCTGGCTACGGTTTCTTATACGGTTTATGGCCAATCGCTTGGATCATC GTTGCTGCTGTTTTCTTATACAAATTAAGTGTAAATCTGGTCAATTCGAAGTTATCCGTTCTTCTGTTTTA TCTATCACTGGTGATCAACGTTTACAAGTTTATTAATCGGTTTCTCTTCGGTGCTTTCTTAGAAGGGGCG GCTGGCTTCGGTGCTCCAGTTGCTATCACTGCTGCTTATTAGTTGGTTTAGGTTTCAACCCATTATACGCT GCTGGTTTTATGTTAATCGCTAACACTGCTCCAGTTGCTTTCGGTGCTTTAGGTATCCCAATCATCGTTGCT GGTCAAGTTACTGGCATAGATGCGTTCAAAATCGGTGCGATGACTGGTCGTCGAATTACCATTCTTATCTAT CTTAGTTCCATTCTGGTTAGTTTTCATGATGGATGGTTGGCGTGGTATCCGTGAAACTTGGCCAGCTGCTA TCGTTGCTGGTGGTTCTTTCGCTATCACTCAATACTTAACTTCTAACTTCATCGGTCCAGAATTACCAGATA TCACTTCTGCTTTAGTTTCTTTAGTTTCTTTAACTTTATTCTTAAAAAGTTTGGCAACCACAAGGTGCGTCGG AAGCTCTCGCTGCTGGTGGCGGTGCTGCTGTTGCTGTTGCTGGTGGTGGTTTCGGTGGTCAACGTTCTGTT GAACCATCTGCTTACTCTTTCGGTGAAATCTTAAAAAGCTTGGTCTCCATTCTTAGTTTTAACTGTTTTAGTT ACTATCTGGACTTTAAAACCATTCAAAGCTTTATTCGCTCCAGGTGGTGAATTATACTGGTTAGTTTTCAA CTTCGCTATCCACACTTAGATCAATTAGTTGTTAAAGGTGCTCCAATCGCTACTTCTGCTACTCCAATCGC TGCTGTTTTCAAATTCGATCCAATCTCTGCTACTGGTACTGCTATCTTCTTATCTGCTTTAGTTTCTATGTTT ATCTTACGTATCGGTGCTAAAATCGGTATCACTACTTTAAGGAAGTGTGGTTGAATTTAAATGGCCAAT CTTATCTATCGGTATGGTTTTAGCTTTCGCTTTCGTTACTAACTACTCTGGTATGTCTACTACTTTAGCTTTA GTTTTAGCTGGTACTGGTGCTGCTTTCCCATTTCTCTCTCCATTCTTAGGTTGGTTAGGTGTTTTCTTAACTG GTTCTGATACTTCTTCTAACGCTTTATTCTCTCTTTACAATCTACTACTGCTCATCAATAGGTGTAAGCG ATACTTTATTAGTTGCTGCTAACACTTCTGGTGGTGTACTGGTAAAATGATCTCTCCACAATCTATCGCTG TTGCTTGTGCTGCTACTGGTATGGTTGGTAAAGAATCTGATTTATCCGTTTCACTTTAAACACTCTTTAA TCTTCGCTGCTTTAGTTGGTTTAACTCACTTTAGCTCAAGCTTACTTATCACTGGTATGTTAGTTCACTAAac tagtagcgccgctgcaggCG</p>
SH10	<p>CGgaattcggccgcttctagagAAAGAGGAGAAAAGTAGAATGATCATCTCTGCTGCTTCTGATTACCGTGCTGCTG CTCAACGTATCTTACCACCATCTTATTCCTACTACATGGATGGTGGTGCTTACTCTGAATACACTTTACGTC GTAACGTTGAAGATTTATCTGAAGTTGCTTTACGTCAACGTATCTTAAAAAACATGTCTGATTTATCTTTA GAAACTACTTTATTCAACGAAAAATTATCTATGCCAGTTGCTTTAGCTCCAGTTGGTTTATGTGGTATGTA CGCTCGTCGTGGTGAAGTTCAAGCTGCTAAAGCTGCTGATGCTCACGGTATCCCATTCACCTTATCTACTG TTTCTGTTTGTCCAATCGAAGAAGTTGCTCCAGCTATCAAACGTCCAATGTGGTTCCAATTATACGTTTTAC GTGATCGTGGTTTATGCGTAACGCTTTAGAACGTGCTAAAGCTGCTGGTTGTTCTACTTTAGTTTTCACTG TTGATATGCCAACTCCAGGTGCTCGTTACCGTGATGCTCACTCTGGTATGTCTGGTCCAAACGCTGCTATG CGTCGTTACTTACAAGCTGTTACTCACCCACAATGGGCTTGGGATGTTGGTTTAAACGGTCGTCCACACGA TTTAGGTAACATCTCTGCTTACTTAGGTAAACCACTGGTTTAGAAGATTACATCGGTTGGTTAGGTAACA ACTTCGATCCATCTATCTCTTGAAAGATTTAGAATGGATCCGTGATTCTGGGATGGTCCAATGGTTATC AAAGGTATCTTAGATCCAGAAGATGCTCGTGATGCTGTTCTGTTTCGGTGCTGATGGCATAGTTGTAAGCAA CCACGGTGGTCGTCAATTAGATGGTGTCTTATCTTCTGCTCGTGCTTTACCAGCTATCGCTGATGCTGTTAA AGGTGATATCGCTATCTTAGCTGATTCTGGTATCCGTAAACGGTTTAGATGTTGTTCTGATGATCGCTTTAG GTGCTGATACTGTTTTATTAGGTCGTGCTTTCTTATACGCTTTAGCTACTGCTGGTCAAGCTGGTGTGCTA ACTTATTAACCTTAATCGAAAAAGAAATGAAAGTTGCTATGACTTTAACTGGTGCTAAATCTATCTCTGAA ATCACTCAAGATTCTTTAGTTCAAGGTTTAGGTAAAGAATTACCAGCTGCTTTAGCTCCAATGGCTAAAGG TAACGCTGCTTAAtactagtagcgccgctgcaggCG</p>

SH11	<p>CGgaattcgggccgcttctagagAAAGAGGAGAAATCTAGTATGAACTTATGGCAACAAAACCTACGATCCAGCTGGT AACATCTGGCTCTCGAGCTTAATCGCTTCTCTCCCAATCTTATTCTTCTTCTCGCTTAAATCAAATTA TAAAGGTTACGTTGCTGCTTCTTGGACTGTTGCTATCGCTTTAGCTGTTGCTTTATTATTCTACAAAATG CCAGTTGCTAACGCTTTAGCGTCGGTTGTATACGGGTTCTTCTACGGTTTATGGCCAATCGCTTGGATCAT CATCGCTGCTGTTTTCGTTTACAAAATCTCTGTTAAACTGGTCAATTCGATATCATCCGTTCTTCTATCTT ATCTATCACTCCAGATCAACGTTTACAAATGCTCATAGTTGGCTTCTGTTTCGGTGCCTTCTAGAAAGGGG CGGCTGGCTTCGGTGCTCCAGTTGCTATCACTGCTGCTTTATTAGTTGGTTTAGGTTTCAAACCATTATACG CTGCTGGTTTATGTTAATCGTTAACAACCTGCTCCAGTTGCTTTCGGTGCTATGGGTATCCCAATCTTAGTTG CTGGTCAAGTTACTGGTATCGATTCTTTCGAAATCGGTCAAAATGGTTGGTGCCTCAATTACCATTCATGACT ATCATCGTTTTATTCTGGATCATGGCTATCATGGATGGTTGGCGTGGTATCAAAGAACTTGGCCAGCTGT TGTTGTTGCTGGTGGTTCTTTCGCTATCGCTCAATACTTATCTTCTAACTTCATCGGTCCAGAATTACCAGA TATCATCTCTTCTTTAGTTTCTTTATTATGTTAACTTTATTCTTAAACGTTGGCAACCAGTTTCGTGTTTT CGTTTCGGTGATTTAGGTGCTTCTCAAGTTGATATGACTTTAGCTCACACTGGTTACACTGCTGGTCAAGTT TTACGTGCTTGGACTCCATTCTTATTCTTAACTGCTACTGTTACTTTATGGTCTATCCCACCATTCAAAGCT TTATTTCGTTCTGGTGGTGCTTTATACGAATGGGTTATCAACATCCCACTTCCATACTTAGATAAAATTAGTT GCTCGTATGCCACCAGTTGTTTCTGAAGCTACTGCTTACGCTGCTGTTTTCAAATTCGATTGGTTCTCTGCT ACTGGTACTGCTATCTTATTCGCTGCTTTATTATCTATCGTTTGGTTAAAAATGAAACCATCTGATGCTATC TCTACTTTCGGTCTACTTTAAAGAATTAGCTTTACCAATCTACTCTATCGGTATGGTTTTAGCTTTCGCT TTCATCTCTAACTACTCTGGTTTATCTTCTACTTTAGCTTTAGCTTTAGCTCACACTGGTCACGCTTTCACCT TCTTCTCTCCATTCTTAGGTTGGTTAGGTTGTTTCTTAACTGGTTCTGATACTTCTTCTAACGCTTTATTTCG TGCTTTACAAGCTACTGCTGCTCAACAAATCGGTGTTTCTGATTTATTATTAGTTGCTGCTAACACTACTGG TGGTGTACTGGTAAATGATCTCTCCACAATCTATCGCTATCGCTTGTGCTGCTGTTGGTTTAGTTGGTAA AGAATCTGATTTATTCCGTTTCACTGTAAACACTCTTAACTCTCACTTGTATCGTTGGTGTATCACTAC TTTACAAGCTTACGTTTTAACTTGGATGATCCCATAAactagtagcgccgctgcaggCG</p>
SH12	<p>CGgaattcgggccgcttctagagAAAGAGGAGAACTGCATGTGAACGTTAACTTCTTCGTTACTTGTATCGGCGACG CTCTCAAATCTCGTATGGCTCGTGATTCTGTTTTATTATTAGAAAAATTAGGTTGTCGTGTTAACTTCCCAG AAAAACAAGGTTGTTGTGGTCAACCAGCTATCAACTCTGGTTACATCAAAGAAGCTATCCCAGGTATGAA AACTTAATCGCTGCTTTAGAAGATAACGATGATCCAATCATCTCTCCAGCTGGTTCTTGTACTTACGCTG TTAAATCTTACCCAATTACTTAGCTGATGAACCAGAATGGGCTTCTCGTGCTGCTAAAGTTGCTGCTCGT ATGCAAGATTTAACTTCTTTCATCGTTAACAAATTAGGTGTTGTTGATGTTGGTGCTTCTTTACAAGGTCGT GCTGTTTACCACCCATCTTGTCTTTAGCTCGTAAATTAGGTGTTAAAGATGAACCATTAACCTTTATTA AAACGTTTCGTGGTTTGAATTTAACTTTCGCTGAACAAGATACTTGCTGTGGCTTCGGTGGTACTTTTTC TGTTAAATGGCTGAAATCTCTGGTGAAATGGTTAAAGAAAAAGTTGCTCACTTAATGGAAGTTTCGTCCA GAATACTTAATCGGTGCTGATGTTTCTGTTTATTAACATCTCTGGTCTTACAACGTGAAGGTCAAAA AGTTAAAGTTATGCACATCGCTGAAGTTTTAATGTCTCGTTAAactagtagcgccgctgcaggCG</p>

	<p>CGgaattcgggccgcttctagagAAAGAGGAGAAAAGCTAGAATGTCTATCAAAACTTCTAACACTGATTTCAAAACT CGTATCCGTCAACAAATCGAAGATCCAATCATGCGTAAAGCTGTTGCTAACGCTCAACAACGTATCGGTG CTAACCGTCAAAAAATGGTTGATGAATTAGGTCACCTGGGAAGAATGGCGTGATCGTGCTGCTCAAATCCG TGATCACGTTTTATCTAACTTAGATGCTTACTTATACCAATTATCTGAAAAAGTTACTCAAAACGGTGGTC ACGTTTACTTCGCTCGTACTAAAGAAGATGCTACTCGTTACATCTTACAAGTTGCTCAACGTAAAAACGCT CGTAAAGTTGTTAAATCTAAATCTATGGTTACTGAAGAAATCGGTGTTAACCACGTTTTACAAGATGCTGG TATCCAAGTTATCGAAACTGATTTAGGTGAATACATCTTACAATTAGATCAAGATCCACCATCTCACGTTG TTGTTCCAGCTATCCACAAAGATCGTCACCAAAATCCGTCGTGTTCTCCATGAACGTCTCGGTTACGAAGGC CCAGAAACTCCAGAAGCTATGACTTTATTCATCCGTCAAAAAATCCGTGAAGATTCTTATCTGCTGAAAT CGGTATCACTGGTTGTAACCTCGCTGTTGCTGAAACTGGTTCTGTTGTTTAGTTACTAACGAAGGTAACG SH13 CTCGTATGTGTACTACTTTACCAAAAAGCTACATCGCTGTTATGGGTATGGAACGTATCGCGCCAACATTC GCTGAAGTTGACGTTTTAATCACTATGTTAGCTCGTTCTGCTGTTGGTGCTCGTTAACTGGTTACAACACT TGGTTAACTGGTCCACGTGAAGCTGGTCACGTTGATGGTCCAGAAgagtttCACTTAGTTATCGTTGATAACG GTCGTTCTGAAGTTTTAGCTTCTgagtttCGTGATGTTTTACGTTGTATCCGTTGTGGTGCTTGTATGAACACTT GTCCAGCTTACCGTCACATCGGTGGTCACGGTTACGGTTCTATCTACCCAGGTCCAATCGGTGCTGTTATC TCTCCATTATTAGGTGGTTACAAAGATTTCAAAGATTTACCATACGCTTGTCTTTATGTACTGCTTGTGAT AACGTTTGTCCAGTTCGTATCCCATTTATCTAAATTAATCTTACGTCACCGTCGTGTTATGGCTGAAAAAGG TATCACTGCTAAAGCTGAACAACGTGCTATCAAAATGTTGCTTACGCTAACTCTCACCCAGGTTTATGGA AAGTTGGTATGATGGCTGGTGCTCACGCTGCTTCTGGTTCATCAACGGTGGTAAACTCCATTAATAATTC GGTGCTATCTCTGATTGGATGGAAGCTCGTGATTTACCAGAAGCTGATGGCGAGAGCTTCCGTTCTGTGGTT CAAAAAACACCAAGCTCAAGAAAAAAGAAACGGTTAAAtactagtagcgccgctgcaggCG</p>
	<p>CGgaattcgggccgcttctagagAAAGAGGAGAAATCTAGTATGGATAACCGTGTTgagtttTTAAACAACGTTGCTCAA GCTTTAGGTTCGTCCATTACGTTTAGAACCACAAGCTGAAGATGCTCCATTAAACAACACTACGCTAACGAAC GTTTAACTCAATTAACCAACAACAACGTTGTGATGCTTTCATCCAATTCGCTTCTGATGTTATGTAACTC GTTGTGAATTAACCTCTGAAGCTAAAGCTGCTGAAGCTGCTATCCGTTTATGTAAAGAATTAGGTGATCAA TCTGTTGTTATCTCTGGTGATACTCGTTTAGAAGAATTAGGTATCTCTGAACGTTTACAACAAGAATGTAA SH14 CGCTGTTGTTTGGGATCCAGCTAAAGGTGCTGAAAACATCTCTCAAGCTGAACAAGCTAAAGTTGGTGTT GTTTACGCTGAATACGGTTTAACTGAATCTGGTGGTGTTGTTTATTCTCTGCTGCTGAACGTGGCCGTAG CTTATCTCTCTTACCAGAATACTCTTATTCATCTTACGTAAATCTACTATCTTACCACGTGTTGCTCAATT AGCTGAAAAATTACCAAAAAAGCTCAAGCTGGTGAACGTATGCCATCTTGTATCAACATCATCTCTGGT CCATCTTCTACTGCTGATATCGAATTAATCAAAGTTGTTGGTGTTTACGGTCCAGTTAAAGCTGTTTACTTA ATCATCGAAGATTGTTAAAtactagtagcgccgctgcaggCG</p>

SH15	<p>CGaattcggcgccgttctagagAAAGAGGAGAACTGCATATGATGAACAAACACGTTAACAAAGTTGCTTTAATC GGTGCTGGTTTCGTTGGTTCTTCTTACGCTTTTCGCTTTAATCAACCAAGGTATCACTGATGAATTAGTTGTT ATCGATGTTAACAAAGAAAAAGCTATGGGTGATGTTATGGATTTAAACCACGGTAAAGCTTTCGCTCCAC AACCAGTTAAACTTCTTACGGTACTTACGAAGATTGTAAAGATGCTGATATCGTTTGTATCTGTGCTGGT GCTAACCAAAAACCAGGTGAAACTCGTTTAGAATTAGTTGAAAAAACTTAAAAATCTTCAAAGGTATCG TTTCTGAAGTTATGGCGTCGGGCTTCGATGGTATCTTTTTAGTTGCTACTAACCAGTTGATATCTTAACTT ACGCTACTTGAAATTCTCTGGTTTACCAAAAAGAACGTGTTATCGGTTCTGGTACTACTTTAGATTCTGCT CGTTTCCGTTTCATGTTATCTGAATACTTCGGTGCTGCTCCACAAAACGTTACGCTCACATCATCGGTGA ACACGGTGATACTGAGCTCCCAGTCTGGAGCCACGCTAACGTTGGTGGTGTTCAGTTTCTGAATTAGTTG AAAAAACGATGCTTACAAACAAGAAGAATTAGATCAAATCGTTGATGATGTTAAAAACGCTGCTTACCA CATCATCGAAAAAAGGTGCTACTTACTACGGTGTGCTATGCTTTAGCTCGTATCACTAAAGCTATCT TACACAACGAAAACTCTATCTTAACTGTTTCTACTTACTTAGATGGTCAATACGGTGCTGATGATGTTTAC ATCGGTGTTCCAGCTGTTGTTAACCGTGGTGGTATCGCTGGTATCACTGAATTAACCTTAAACGAAAAAG AAAAAGAACAATTCTTACACTCTGCTGGTGTTTAAAAAACATCTTAAACCACACTTCGCTGAACAAAA AGTTAACTAAactagtagcgccgcctgcaggCG</p>
SH16	<p>CGaattcggcgccgttctagagAAAGAGGAGAAACTAGAATGTGGGAACAATTATACGATCCATTCGGTAACGAA TATGTTTCTGCGTTAGTTGCTTTAACTCCAATCTTATTCTTCTTATTAGCTTTAACTGTTTTAAAAATGAAA GGTATCTTAGCTGCTTCTTAACTTTAGCTGTTTCTTCTTCGTTTCTGTTTGGGCTTCCACATGCCAGTTG AAAAAGCTATCTTCTGTTTTATTAGGTATCGGTTCTGGTTTATGGCCAATCGGTACATCGTTCTCATGG CTGTGTGGCTGTACAAAATCGCTGTAAAACTGGTAAATCACTATCATCCGTTCTTCTATCGCTGGTATCT CTCCAGATCAACGTTTACAATTATTATTAATCGGTTTCTGTTTCAACGCTTCTTAGAAGGGGCGGTGGC TTCGGTGTTCCAATAGCTATCTCTGCTGCTTTATTAGTTGAATTAGGTTTCAAACCATTAAGCTGCTGCT TTATGTTAATCGCTAACGCTGCTTCTGGTGCTTTCGGTGCTATCGGTATCCAGTTATCACTGGTGCTCAA ATCGGTGATTTATCTGCTTTAGAATTATCTCGTACTTTAATGTGGACTTTACCAATGATCTCTTCTTAAATC CCATTCTTATTAGTTTCTTATTAGATCGTATGAAAGGTATCAAACAACTTGGCCAGCTTTATTAGTTGTT TCTGGTGGTTACACTGCTGTTCAAACCTTAACTATGGCTGTTTTAGGTCCAGAATTAGCTAACATCCTCGC GGCGTTATTCTCGATGGGTGGTTAGCTTTATTCTTACGTAAATGGCAACCAAAAAGAAATCTACCGTGAAG AAGGTGCTGGTGATGCTGGTGAAAAAAGCTTACCGTGCTGCTGATATCGCTAAAGCTTGGTCTCCATT CTACATCTTAACTGCTGCTATCACTATATGGTCTCTCCAGCGTTCAAAGCTTTATTTCAAGAAGGTGGTTT ATTATACCAATCTACTTTATTATTCAAAATGCCATTCTTACACCAACAAATCATGAAAATGCCACCAATCG CTCCATCTGCTATGCCATTAGATGCTGTTTTCAAAGTTGATTTATTATCTGCTACTGGTACTGCTATCTTAG CTGCTGTTATCGTTACTGGTTTATTCTCTAAAAAATTCTTCTCTCGTGATGCTTTTGCGAGCTTAAAGAAA CTGGCAAAGAATTATGGGTTCCAATCATGACTATCTGTTTCGTTATGGGTTTCGCTAACTTAGCTAACTTC GCTGGTTTATCTTCTTCTATCGGTTTAGCTTTAGCTAAAACCTGGTGATTTATCCCATTGCTTCTCCAGTTT TAGGTTGGATCGGTGTTTTATCACTGGTTCTGTAGTATCTAACAACGCGTTATTCGGTCACCTCCAAGTT GTTACTGGTGCTCAAATCGGTGCTGGTTCTGATTTATTATTAGCTGCTAACACTGCTGGTGGTGTATGGCT AAATTAGTTTCTCCAAATCTATCGCTATCGCTGCTGCTGCTGTTGGTCAAACCTGGTAAAGAATCTAAATT ATTCAAACGTAAGTTGCTTACTCTTAACTTATTATTAATCATCTGTATCTGGACTTTCATCTTAGCTCGT TTAGGTGTTTAAactagtagcgccgcctgcaggCG</p>

	<p>CGaattcggggcgttctagagAAAGAGGAGAAAAGCTAGAATGACTAAAGGTACTATCCAAAACCAAGAATCTTTC</p> <p>TAAACCGTATCGCTTCTCTTTAGGTCGTGAACGTCGTACTGGTGGTGTGCTGTTCCAGAATGGGCTCA</p> <p>CCAACCACAATACAAAACCTTTAGAAGGTTACTCTGCTGATGATTAGTTACTGTTTTAAAAAACCACTGTG</p> <p>TAAAAATCCACACTGAATTAATCGAAACTGATTCTACTGGTTTATACGATGCTTTACGTGAACAAGTTTCT</p> <p>CGTTTCTCTGGTGGTCCAGTTATCATCCCAAGATCCACGTTTCGAAGAATACGGTTTAAAAATCTTTATT</p> <p>SH17 AACTAAAGATTGGCCATCTGAGGGTACTCCAGTCTGGGAATGGGACGCTGATAAAGGTGAAGAAAACAT</p> <p>CAAAAAAGCTGAACAAGCTAACGTTGGTATCACTTCTCTGAAATCACTTTAGCTGAATCTGGTACTGTTG</p> <p>TTTTATTCTCTTCTAAAGATATCGGTCGTTCTGTTTCTTTATTACCAACTACTTACATCGCTATCGTTCCAAA</p> <p>ATCTTCTATCGTTCCACGTATGACTCAAGCTTCTGATATCATCCGTCAAAACATCGCTAACGGTGTACTG</p> <p>TTCCATCTTGATCAACTACATCACTGGTCCATCTAACTCTGCTGATATCGAAATGGATTAGTTGTTGGTG</p> <p>TTACGGTCCAGTTAAAGCTGCTTACATCTTAGTTTCTGATCGTTAAactagtagcgggccgctgcaggCG</p>
	<p>CGaattcggggcgttctagagAAAGAGGAGAAAAGCTAGTATGAAAGTTTCTTTATTCTGTTACTGTGTTAGTTGATA</p> <p>TGTTCCAAACTAACGTTGGTAAAGCTACTGTTGAATTATTAGAACGTTTAGGTTGTGAAGTTGATTCCCA</p> <p>GAAGGTCAAATCTGTTGTGGTCAACCAGCTTACAACCTCTGGTTACGTTACGATGCTAAAAAAGCTATGA</p> <p>AACGTATGATCGAAACTTTCCAAGATTCTGAATACGTTGTTTCTCCATCTGGTTCTGTACTACTATGTTCC</p> <p>GTGAATACCCACACTTATTCCAAGATGATCCAAAATGGGCTGATAAAGCTAAAAAATTAGCTGATAAAAC</p> <p>SH18 TTACGAATTAAGTATTCATCGTTAACGTTTATAGGTGTTGAAGATGTTGGTGCTACTTTACACACTAAGG</p> <p>CTACTCTCCACACTTCTTGCCACATGACTCGTTTATTAGGTGTTGTAAGAACCAATGAAATTATTATCTC</p> <p>ACGTTAAAGGTTTACAATCACTGAATTACCAGGTAAACACAATTGCTGTGGCTTCGGTGGTACTTTTTCT</p> <p>GTTAAAAATGGCTCAAATCTCTGAACAAATGGTTGATGAAAAAGTTGAATGTGTTGAAGAACTGGTGCTG</p> <p>AAGTTTTAATCGGTGCTGATTGTGGTTGTTAATGAACATCGGTGGTCGTTAGGTGCTAAAGATAAAAAC</p> <p>GTTAAAGTTATGCACATCGCTGAAGTTTTAACTCTCGTTAAactagtagcgggccgctgcaggCG</p>
	<p>CGaattcggggcgttctagagAAAGAGGAGAAAAGCTGCATATGGCTATGAAAATCGGTACTGATGCTTTCAAAGAA</p> <p>CGTGTCTCAAGGTATCGATAACgagtttATGCGTGGTGCTGTTTCTGGTGCTCAAGAACGTTTACGTACTCG</p> <p>TCGTTTAGAAGCTGCTGAAGAATTAGGTAACGGGAAGAATGGCGTTCTTTATCTGAAGAAATCCGTCAA</p> <p>CACGTTTTAGAAAACCTAGATTCTACTTAGGTCAATTAGCTGAAAACGTTGCTAAAAGGGGTGGCCACG</p> <p>TGTACTTCGCGAAAACCTGCTGAAGAAGCTTCTTCTTACATCCGTGATGTTATCCAAAAAAGCGTAA</p> <p>AAAAATCGTTAAATCTAAATCTATGGTTACTGAAGAAATCAACTTAAACGAAGTTTTAGAAAAAGAAGGT</p> <p>TGTGAAGTTGTTGAAACTGATTAGGTGAATACATCTTACAAATCGATGATCACGATCCACCATCTCACAT</p> <p>CGTTGCGCCCGCTCTCCACAAAAACAAGGAACAAATCCGTGATGTTTTTAAGGAACGCTCTCGATTACCAA</p> <p>CATACTGAAAAACCAGAAGAATTAGTTATGCACGCTCGTGCTATCTTACGTAAAAAATCTTAGAAGCTG</p> <p>ATATCGGTATCACTGGTTGTAACCTCGCTATCGCTGATACTGGTTCTGTTTCTTTAGTTACTAACGAAGGTA</p> <p>SH19 ATGGCAGGTTAGTTTCTACTCTCCAAAAACCTCAAATCACTGTTATGGGTATGGAACGTATAGTACCAAGC</p> <p>TTCTCTgagtttGAGGTTTTAGTTTCTATGTTAACTCGTTCTGCTGTTGGTCAACGTTTAACTTCTTACATCACT</p> <p>GCTTTAACTGGTCCAAAATTAGAAGGTGAAGTTGATGGTCCAGAAgagtttCACTTAGTTATCGTTGATAACG</p> <p>GTCGTTCTAACATCTTAGGTACTgagtttCAATCTGTTTTACAATGTATCCGTTGTGCTGCTTGATCAACGTTT</p> <p>GTCCAGTTTACCGTCACGTTGGTGGTCACTCTTACGTTTCTATCTACTCTGGTCCAATCGGTGCTGTTTTAT</p> <p>CTCCATTATTAGGTGGTTACGATGATTACAAAGAATTACCATACGCTTCTTCTTTATGTGCTGCTGTTCTG</p> <p>AAGCTTGTCAGTTAAAAATCCATTACACGAATTATTATTAACACCGTCAAAACATCGTTGAAAAAGA</p> <p>AGGTCGTGCTCAATCTCTGAAAAATTAGCTATGAAAGCTTTCGGTTTAGGTGCTTCTCTTTATCTTTATA</p> <p>CAAAATGGGTTCTAAATGGGCTCCAGCTGCTATGACTCCATTCACTGAAGATGAAAAATCTCTAAAGGT</p> <p>CCAGGTCCATTAAAAAACTGGACTCAAATCCGTGATTTCCAGCTCCACACAAATCTCGTTTCCGTGATTG</p> <p>GTTGCTGATCGTGAAACTTCTGAACGTACTAAAGAAGAACATAAactagtagcgggccgctgcaggCG</p>

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