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Engineering *S. oneidensis* for Performance Improvement of Microbial Fuel Cell—a Mini Review

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Abstract
Microbial fuel cell (MFC) is a promising technology that utilizes exoelectrogens cultivated in the form of biofilm to generate power from various types of sources supplied. A metal-reducing pathway is utilized by these organisms to transfer electrons obtained from the metabolism of substrate from anaerobic respiration extracellularly. A widely established model organism that is capable of extracellular electron transfer (EET) is *Shewanella oneidensis*. This review highlights the strategies used in the transformation of *S. oneidensis* and the recent development of MFC in terms of intervention through genetic modifications. *S. oneidensis* was genetically engineered for several aims including the study on the underlying mechanisms of EET, and the enhancement of power generation and wastewater treating potential when used in an MFC. Through engineering *S. oneidensis*, genes responsible for EET are identified and strategies on enhancing the EET efficiency are studied. Overexpressing genes related to EET to enhance biofilm formation, mediator biosynthesis, and respiration appears as one of the common approaches.

Keywords MFC · *Shewanella* · Microbial fuel cell · EET · Bioelectricity · Overexpressing

Introduction

Research are being focused on microbial fuel cell (MFC) technology in recent years due to its potential in generating electricity from organic matters such as wastewater, as an alternative strategy for sustainable energy production. An MFC consists of an anode chamber and a cathode chamber, separated by a proton-exchange membrane (PEM). Microbes grow on the

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electrode surface in the anode chamber which is kept in anaerobic condition and consume substrates such as glucose via oxidation, producing carbon dioxide, protons, and electrons. The electrons are channeled to the cathode chamber, which is usually kept in aerobic condition, via an external circuit, and protons diffuse through the PEM to combine with the final electron acceptor such as molecular oxygen to form water (reduction process). The redox reaction thus generates electricity. MFC uses microorganisms in the form of biofilm that is capable of transferring electrons produced during anaerobic respiration extracellularly to an anode, known as extracellular electron transfer (EET). Due to the ability to transfer electrons, these microorganisms are termed as exoelectrogens.

Exoelectrogens that are widely studied with established genome database are *Shewanella* spp. and *Geobacter* spp., more specifically *Shewanella oneidensis* and *Geobacter sulfurreducens* [1]. The former is well known for its bidirectional flow of electrons across the cellular membranes [2–6], while the latter exchanges electrons through conductive nanowires to various electron acceptors [7]. Different substrates can be utilized depending on the strain of microorganisms used, where lactate, acetate, and glucose are the typical substrates used by exoelectrogens for anaerobic respiration [1]. *Shewanella oneidensis* was also found to be a potential exoelectrogen for generating bioelectricity from MFCs by degrading chitin and biomass hydrolysate, thus providing a new opportunity for the food waste and biomass industries [8, 9]. Additionally, more organisms that confer exoelectrogenic capabilities are being identified [10, 11]. Recent studies discovered that different exoelectrogens could be used to liberate electrons from cellulose, which is a complex polymer [12]. This process is achieved through a syntrophic consortium where interspecies communication leads to decomposition of cellulose into volatile acids through metabolic cooperation. Recently, Ueoka et al. [13] demonstrated an electrode plate-culture (EPC) method that allowed selective isolation of exoelectrogens specifically through utilizing an electrode plate covered with medium as a sole electron acceptor. The isolation of exoelectrogens in a complex microbiome was solely based on their capabilities to undergo EET, and not on their specific nutrient requirement commonly practiced at present. This method could thus potentially lead to more different exoelectrogens being isolated and subsequently identified.

Along with the refinement of techniques in molecular biology and the rapid advancement of biotechnology, it is only a matter of time where the metabolic pathways of various organisms are identified and cloned into a strain where it could be ubiquitously utilized as an exoelectrogen for efficient power generation in an MFC. At the current stage of research, the relationship and interactions among various exoelectrogens in a mixed-cultured biofilm are still not well defined. This signifies the importance to look into the metabolic pathway of various exoelectrogens at the DNA level and to also further establish the biological interactions between different species in a biofilm and how each of them plays a role in substrate oxidation. In this review, research studies on *S. oneidensis* leading to the discovery of the respective gene functions for EET are introduced. Comparison on overexpression of these genes for increased efficiency when applied in MFC is also conducted. Coupled with foreign gene conferring additional utilities such as substrate metabolism and mediator regeneration, these approaches had allowed manipulations of *S. oneidensis* from the biological aspects. These experimental findings when complemented with electrochemical manipulations will potentially allow the MFC technology to be applicable in an actual treatment plant for sustainable energy production.

Biofilm Formation and Metal-Reducing Pathway of *S. oneidensis*

Biofilms are referred to as a complex of embedded microorganisms that could be of a pure or mixed culture within an extracellular matrix composed of self-produced extracellular polymeric substances [14, 15]. Electroactive biofilms are imperative for exoelectrogens to efficiently carry out EET for bioelectricity generation. Bioelectricity generation is directly proportional to the biofilm and type of electrode surface where negatively charged and hydrophobic anode surfaces are less favorable for electroactive biofilm formation. An optimum thickness is preferable to achieve substantial current densities as very thick deposition of biofilm was shown to limit electron flow [15, 16]. The electron transfer processes are mediated by the c-type cytochromes (*c*-Cyts), also known as the redox-active proteins [17, 18]. Microbial proteins (such as type IV pili) and electron shuttles (such as flavins and pyocyanin) also enhance the conductivity of the biofilm, thus increasing electron transfer [15, 19, 20].

Electron transfer of *S. oneidensis* biofilm is based on both direct electron transfer (DET) and mediated electron transfer (MET). Marsili et al. [21] showed that the removal of electron shuttle flavin resulted in more than 70% loss in electron transfer in the biofilm. Three factors, including electrode material [22, 23], environmental parameters [23, 24], and biofilm thickness [25], affect the characteristics of biofilms formed by *S. oneidensis*. The genes *msh* (encodes mannose-sensitive hemagglutinin)/*pilT* and *mxd* (encodes a putative carbohydrate containing cell-associated component) are involved in surface attachment and biofilm formation [26, 27]. A cluster of chemotaxis genes allow the strain to sense the electron acceptors. Genes within the clusters which are essential for chemotactic response to electron acceptor during anaerobic respiration are *cheA-3* and *cheA-1*, where *CheA-3* histidine protein kinase will be expressed when the electron acceptors are present [28, 29]. The downregulation of motility protein flagellin and proteins associated with oxidative stress, along with the upregulation of quorum sensing proteins, riboflavin synthesizing proteins, heme, and transporter components ABC, causes metabolic shifts in *S. oneidensis* biofilms [30].

For *S. oneidensis*, a metal-reducing (mtr) conduit is responsible for the flow of current from the interior of cells to the outer membrane and subsequently to the extracellular anodes, and to minerals such as Fe(III), Mn(III), or Mn(IV) [31] via the outer membrane *c*-type cytochromes (OMCs) [32]. A six multi-haem *c*-Cyts consist of (i) CymA (inner membrane tetraheme *c*-Cyts), (ii) Fcc_3, (iii) MtrA (periplasmic decaheme *c*-Cyts), (iv) MtrC, (v) OmcA and a small tetrahaem cytochrome (STC), and lastly (vi) porin-like outer membrane protein MtrB [33–37]. Electrons from the organic matters and the electron donor, typically lactate in the case of *S. oneidensis*, go through an intracellular electron transfer pathway, from NADH through the quinol pool in the inner membrane, subsequently through CymA where quinol is oxidized and the electrons are transferred to STC and Fcc_3 [38–41], then to a terminal reductase complex consisting of MtrA, MtrB, and MtrC. While the exact pathway of the electrons being transferred from STC and Fcc_3 is unsure, a study involving mutant STC and Fcc_3 showed reduced efficiency in Fe(III) oxides or oxyhydroxides-reducing activities, indicating that they may play a role in the transportation of electrons from CymA to MtrA [35, 42]. This complex then transfers electrons from the periplasmic proteins to the surface of bacteria [43–46], where physical interactions of MtrC and OmcA enable electron transfer directly to Fe(III)-containing minerals (Fig. 1). To transport electrons exogenously to the surface of electrodes, *S. oneidensis* secretes co-factors to OMC, riboflavins (RF) for OmcA, and flavin mononucleotide (FMN) for MtrC, illustrated by the exhibition of complementary binding sites for these molecules on the cytochromes [17, 18, 48]. These flavins also act as an "electron shuttle" that mediates the

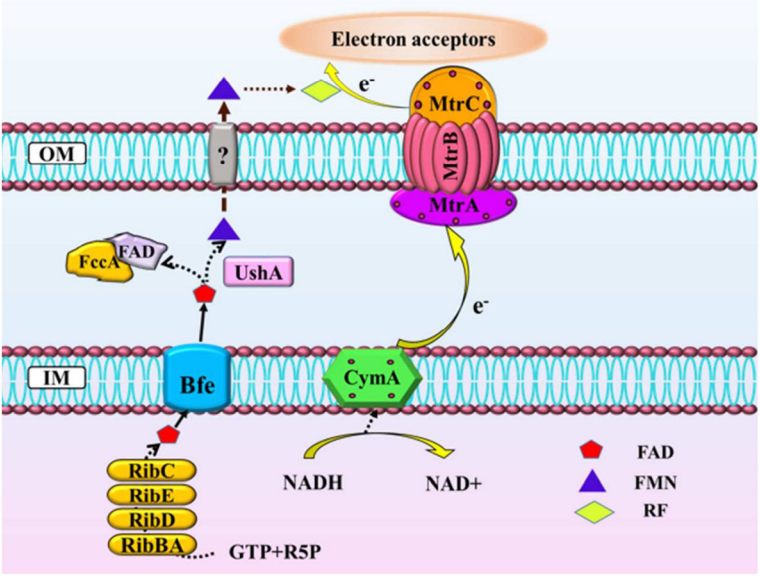

Fig. 1 Electrons produced from the oxidation of substrate catalyzed by CymA are transported to the MtrA-MtrB-MtrC complex, then to the surface of the terminal electron acceptor extracellularly, and subsequently for power generation. Reprinted with permission from Min et al. [47]. Copyright (2017) American Chemical Society

transfer of electrons between the cells and the electrode. Recent studies showed that adding these electron shuttles exogenously or through amplifying their expression in *S. oneidensis* improved power generation [17, 18, 49].

Transformation of *S. oneidensis*

Transformation of *S. oneidensis* is mainly carried out to study the genes essential for EET by gene repression or overexpression, with the aim of enhancing the overall power generation capabilities and wastewater treating potential of MFC. The important factors in a typical gene transformation experiment on *S. oneidensis* include promoter, plasmid, and transformation method. In most studies, researchers tend to use an isopropyl β-d-1-thiogalactopyranoside (IPTG)–inducible promoter element, P*lacIq-lacIq*-P*tac*, to induce overexpression of inserted gene in the plasmid vector. Table 1 summarizes the recent approaches in engineering *S. oneidensis* for improved power generation in an MFC. West et al. [61] demonstrated the utilization of trimethylamine N-oxide (TMAO) as an inducer for EET by replacing the native *mtrCAB* promoter, which controls the expression of metal-reduction pathway responsible for EET with PtorF. PtorF regulates the expression of operon to encode the TMAO respiratory system in *S. oneidensis*. This transformation enables the overexpression mtr pathway in *S. oneidensis* to be induced by a range of TMAO concentrations, albeit with comparatively lower current produced than that of the WT strain.

Cao et al. [51] developed a synthetic plasmid toolkit for gene transformation experiments designed for *S. oneidensis*. In the study, different elements within the plasmid vector such as promoters, antibiotic resistant cassette, and replication origins which are typically used for a gene transformation procedure in *S. oneidensis* were

Table 1 List of experiments performed to engineer *S. oneidensis* for performance improvement of MFC

Elements	Remarks	Power density (mW/m^2)		Improved %	Ref.
		Initial	Final		
Biofilm formation					
speF	Gene repression by CRIPSRi	24.4	36.8	51	[50]
uvrY	Gene repression by CRIPSRi	24.4	30.5	25	[50]
speF-uvrY	Gene repression by CRISPRi	24.4	40.3	65	[50]
Metal-reducing pathway					
mtrCAB	Overexpression through a medium-strength promoter	3.8	17	347	[51]
oprF	Porin gene from *P. aeruginosa*. Mutant *S. oneidensis* is inoculated into MFC together with *S. cerevisiae*	71.5	123.4	73	[52]
*mtr*C-*mtr*A-*mtr*B	Genes cluster for metal-reducing pathway amplified from *S. oneidensis* and overexpressed	10	20	100	[47]
*mtr*C-*mtr*A-*mtr*B	Genes cluster for metal-reducing pathway amplified from *S. oneidensis* and overexpressed, with the addition of Ptac	10	37	270	[47]
c-type cytochromes					
cymA	Gene encoding c-cytochromes in *S. oneidensis*	55	65	18	[53]
Flavin biosynthesis					
*rib*ADEHC	Overexpression of flavin biosynthetic pathway from *B. subtilis*	16.4	233	1321	[54]
*rib*D-*rib*C-*rib*BA-*rib*E	Gene cluster for flavin biosynthesis amplified from *S. oneidensis* and overexpressed	10	15	50	[47]
*rib*ADEHC	Overexpression of flavin biosynthetic pathway from *B. subtilis* in *S. oneidensis*, co-cultured with genetically modified *Klebsiella pneumoniae*	6	19	217	[55]
NADH regeneration					
pflB-fdh-gapA2-mdh*	Plasmid constructed with both *pflB* and *fdh** operating under one module and *gapA2* and *mdh* under another module, both with individual p*lacI*	26.2	105.8	304	[56]
ycel-pncB	Genes encoding biosynthetic pathway facilitating utilization of nicotinic acid and nicotinamide	30.3	70.8	134	[57]
ycel-pncB-nadM-nadD--nadE**	Genes cluster introduced homogenously and heterogeneously responsible for enhancement of NAD^+ biosynthesis	30.3	162.8	437	[57]
Xylose utilization					
xylT-xylA-xylB	*xylT* encodes D-xylose-proton symporter from *Clostridium acetobutylicum*, *xylA* encodes xylose isomerase, and *xylB* encodes xylulokinase, both from *E. coli*	-	0.6	-	[58]
Gxf1-xylA-xylB	*Gxf1* encodes glucose/xylose facilitator 1 from *Candida intermedia*	-	0.9	-	[58]
xylT-XYL1-XYL2-XKS1	*XYL1* encodes D-xylose reductase, *XYL2* encodes xylitol dehydrogenase, *XKS1* encodes D-xylulokinase, both from *Scheffersomyces stipites*	-	1.4	-	[58]

Table 1 (continued)

Elements	Remarks	Power density (mW/m^2)		Improved %	Ref.
		Initial	Final		
Gxf1-XYL1-XYL2-XKS2	Genes expressed as a module	-	2.1	-	[58]
Co-cultivation					
Klebsiella pneumoniae	Mixed-culture MFC reactor fed with glycerol	0.62	2.15	247	[59]
Streptococcus bovis	Mixed-culture MFC reactor fed with starch	-	50	-	[60]

evaluated and characterized using green fluorescent protein (GFP) as a reporter. This was further fine-tuned through the investigation by overexpressing the *mtrCAB* gene cluster using promoters of different strengths. Expression of *mtrCAB* at a moderate level showed 134% improvement in the maximum power density of an MFC, demonstrating the convenience of the toolkit for fine-tuning of gene expressions.

Similarly, Ng et al. [62] evaluated the performance of various plasmids and promoters through the expression of GFP proteins, and subsequently mtr pathway genes, in recombinant *S. oneidensis*. They reported that the replication origin *rep*B along with pLacI promoter was able to drive expression of inserted genes with higher enzymatic activity as compared with the controlled strain. Plasmid DNA is delivered into cells most commonly through conjugation from *Escherichia coli* or electroporation. The latter is more convenient for transfer of both linear and circular DNA. Gene editing tools are recombineering (Lambda-red, Cre/*lox*) and via clusters of regularly interspaced short palindromic repeats (CRISPR). Lambda-red protein is a protein derived from phage lambda and consists of three parts: Exo (lambda exonuclease), Gam, and Beta. Exo degrades dsDNA from 5′ to 3′ ends, leaving a ssDNA at the recessed region; Gam prevents nucleases produced by *E. coli* from degrading linear dsDNA; and Beta binds to ssDNA regions produced by Exo and facilitates the recombination process by promoting the incorporation of product to target site (Fig. 2) [63].

While this recombineering technique is generally employed in programming *E. coli*, Corts et al. [64] established the optimum conditions for making electrocompetent cells and demonstrated the application of Lambda-red recombineering in *S. oneidensis* by expressing recombinases under an arabinose inducible promoter pBAD and targeted the integrated *lacZ* gene from *E. coli* in the chromosome of *S. oneidensis*, then subsequently the native *rpsL* gene. The Cre/*lox* system is a site-specific recombinase containing the *Cre* protein originated from P1 bacteriophage which catalyzes the recombination at the *loxP* sequence [65]. So far, no research has utilized the Cre/*lox* system for programming *S. oneidensis*. However, Enyeart et al. [66] established the system of genome editing via targetrons and recombinases (GETR) where mobile group II introns, termed "targetrons," worked in tandem with Cre/*lox* recombination in *S. oneidensis*. Retargetable mobile group II introns have been developed recently as a tool that can be designed to be inserted into a given DNA site at high efficiency. Intron-encoded proteins (IEP) are involved in self-splicing and in the process of

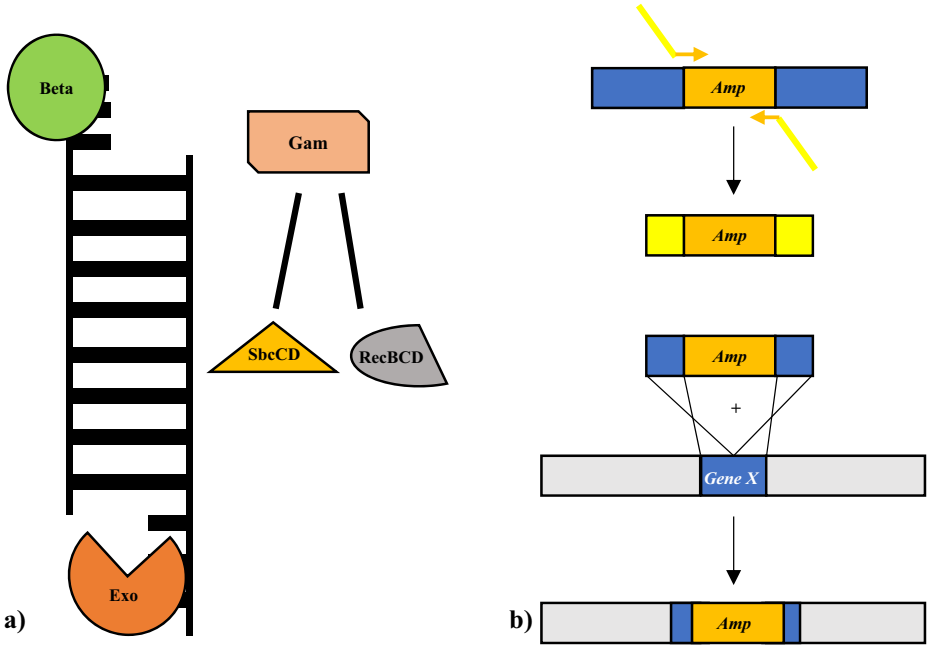

Fig. 2 **a** The three components of Lambda-red recombineering system with Exo, Beta, and Gam. Gam prevents digestion of linear DNA by endogenous RecBCD and SbcCD, Exo degrades linear dsDNA from 5′, and Beta promotes annealing of ssDNA created by Exo to a complementary target in the cell. **b** An overview of using this system to knockout a gene of interest with an ampicillin-resistant cassette

"retrohoming" where the intron site reverses splices into DNA specifically [66]. Enyeart et al. [66] designed a targetron containing a *loxP* site that allowed gene insertion into ribosomal *rrs* in *S. oneidensis*. It was reported that the insertion was found in most copies of *rrs* gene in the genome, showing that the system could be potentially utilized for reprogramming *S. oneidensis*.

The CRISPR system allows targeted genome editing in a range of different organisms (Fig. 3) [68]. The first usage of the CRISPRi system was reported to repress mtr and biofilm formation genes, *speF* and *uvrY*, in order to establish a CRISPRi-sRNA method that allowed transcriptional and translational gene regulation in *S. oneidensis* [50]. Through CRISPRi, the repression of *speF* (encodes a putrescine biosynthesis gene that produces putrescene as a strategy employed by prokaryotes for active cell dispersal from biofilms) and *uvrY*, which is related to synthesis of surface polysaccharide that deters formation of biofilms, enhanced power generation of MFC by 65% when compared with the control strain. Li et al. [69] then further developed a similar system on gene interference based on CRISPR in which several genes within *S. oneidensis* were repressed and subsequently resulted in enhanced EET. This system assumed that electrons flux would split to diverse terminal electron acceptors during energy metabolism. Thus, not all electron flux would contribute to the extracellular electron acceptor for EET. By targeting gene sequences related to these competitive electron transfer pathways for interference, it allows the electron flux to be redirected to the desired EET pathway which can elevate the efficiency of EET. However, this synthetic *S. oneidensis* was not tested in MFC for power generation. These various tools in engineering *S. oneidensis* could be further exploited for more discoveries on EET-related genes, and manipulations could be

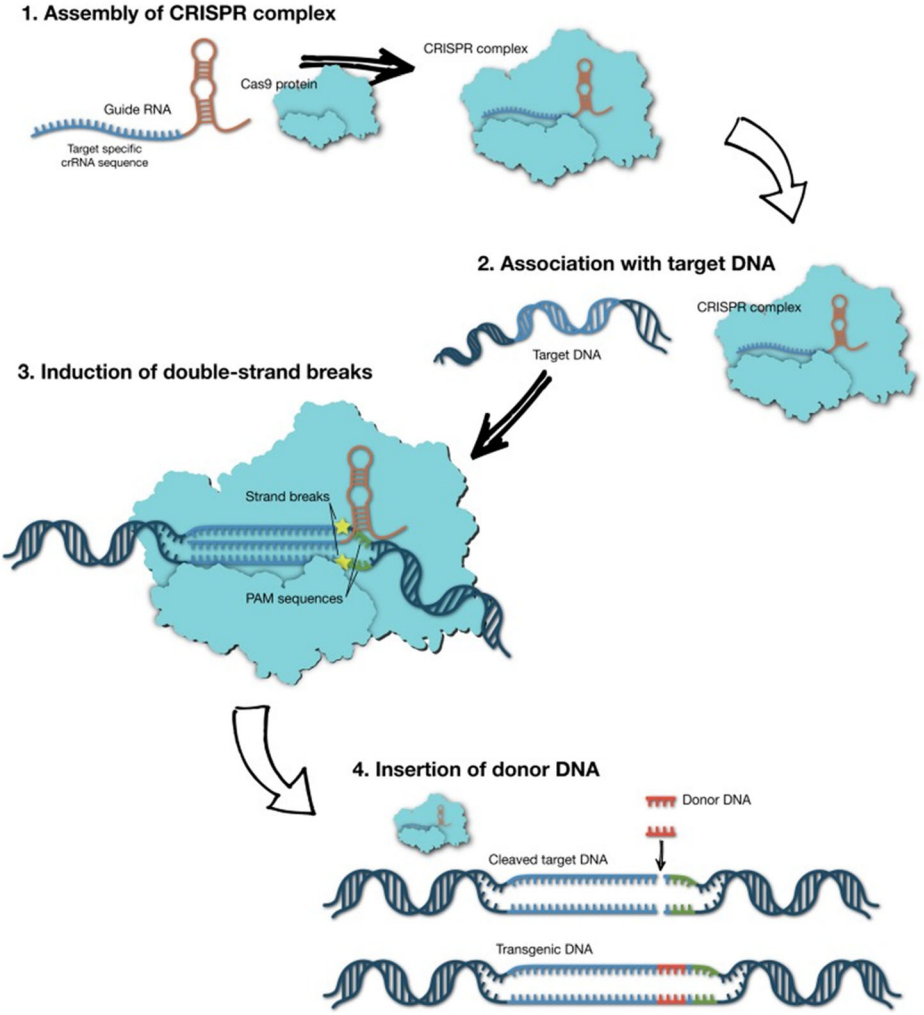

Fig. 3 Mechanism of genome editing using the CRISPR/Cas9 system adopted from [67]. This editing system requires a single-guide RNA (sgRNA) that directs the Cas9 endonuclease to the specific site of interest in the genomic DNA, resulting in a double-strand break. With the presence of a donor DNA in trans, a transgenic DNA can be synthesized, whereas with the absence of a donor DNA, double-strand break will be repaired by the host cell, resulting in an insertion or deletion that will disrupt the ORF of a gene

performed to enhance the effect of the said genes with the objectives of increasing the overall potential of an MFC.

Enhancing MFC Performance Through Engineering Biofilm Formation

One of the strategies in enhancing MFC performance is to promote biofilm formation in exoelectrogens through biotechnology. Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) was reported in multiple literatures that they play a central role in bacterial biofilm formation by promoting expression of adhesive matrix components which in turn facilitate

formation of bacterial biofilm [70]. Liu et al. [70] demonstrated an enhancement in power generation of MFC by *S. oneidensis* via the expression of heterologous YdeH, which is a diguanylate cyclase encoded by *ydeH* in *E. coli*, to catalyze the biosynthesis of c-di-GMP. It was reported that the YdeH-engineered strain with a constitutive promoter was able to produce maximum power density 2.8 times higher than that of the controlled strain [70].

Lin et al. [52] showed that the expression of porin gene, *oprF* from *Pseudomonas aeruginosa* in *S. oneidensis*, coupled with the addition of recombinant *Saccharomyces cerevisiae* that produces lactate with ethanol synthesis pathway knocked-out, was able to enhance power generation of MFC by up to 73% compared with the control. These studies showed that biofilm formation affects MFC performance, and heterologous expression of genes related to biofilm formation could be one of the potential strategies in enhancing its performance. One of the major bottlenecks in MFC technology remains to be the long-term robustness and efficiency of the biofilms. Ding et al. [71] demonstrated that the disruption of the putrescine biosynthesis gene, *speF*, allowed *S. oneidensis* to form highly cohesive biofilms. Studies on the detachment of *S. oneidensis* found out that in-frame deletions of the global transcriptional regulators ArcA and CRP resulted in severe impacts on the detachment of the mutants in *arcA* and *crp*. This suggested a role for the genes to play in the formation of biofilm [25]. These mutants were not tested on an MFC and could be a potential subject for consideration in future experimental design. Quorum sensing (QS) is a process which is often characterized alongside biofilm formation processes. As of now, QS in *S. oneidensis* is not fully characterized yet. This could be a topic to be further explored by transcriptomic profiling as it could serve as a potential process to be exploited to enhance biofilm formation.

Enhancing MFC Performance Through Engineering EET Pathway

It was previously described that *c*-Cyts CymA mediates EET and acts as a redox-active protein. Overexpression of the *cymA* gene in *S. oneidensis* MR-1 showed higher maximum power generated in MFC and higher growth rate of the synthetic MR-1 in the anodic chamber compared with wild type MR-1 [53]. The overexpression of *cymA* facilitates more electrons generated from anaerobic respiration to be transported extracellularly to the electron acceptor. Cyclic adenosine 3′,5′-monophosphate (cAMP) plays a very important role in a multitude of biological processes. Additionally, Cheng et al. [72] also reported that cyclic adenosine 3′,5′-monophosphate receptor protein, cAMP-CRP complex, regulates EET through upregulation of expression levels of *c*-Cyts and flavin synthetic pathway–related genes. EET performance was enhanced via elevating the cAMP level in synthetic *S. oneidensis* performed through the expression of an exogenous gene encoding adenylate cyclase from *Beggiatoa* sp. However, these strains were not tested in an MFC reactor.

The expression of the EET mediators, flavins, is able to increase the performance of MFC. As previously stated, flavins act as an electron shuttle for EET where *S. oneidensis* secretes electrons as a product of anaerobic respiration. These flavins facilitate the transportation of these electrons to the electrode. Riboflavin secretion by *S. oneidensis* was proposed to aid in taxis of the organisms towards insoluble electron acceptor through sensing the redox gradient in the environment [73]. Yang et al. [54] showed that flavin synthetic pathway from *Bacillus subtilis*, *rib*ADEHC, expressed heterologously in *S. oneidensis* resulted in 13.2 times increase in power generation compared with WT. Min et al. [47] reported an increase in power generation of approximately 1.1 times when using expression of *S. oneidensis* in which both the flavin synthetic gene cluster, *rib*D-*rib*C-*rib*BA-*rib*E, and the mtr pathway gene cluster,

*mtr*C-*mtr*A-*mtr*B, were co-expressed in *S. oneidensis*. Detachment of *S. oneidensis* cells from biofilms was also studied. It was reported that rapid decrease in the concentration of oxygen stimulated cell detachment. In-frame deletions of individual genes *arcA*, *crp*, and *etrA* were reported to allow the transformed *S. oneidensis* to be defective in the detachment response, where most profound effect was observed in the transformed cell with *crp* deleted [25].

Coupling both genetic modification with mixed microbial community was shown to further enhance MFC performance through mutualistic relationship. In [55], glycerol was firstly converted to lactate by *Klebsiella pneumoniae* and subsequently fed to *S. oneidensis* as the carbon source for power generation. Alcohol dehydrogenase gene *adhE* in *K. pneumoniae* was knocked out, and foreign lactate dehydrogenase gene (*lhdD*) and lactate transporter gene (*lldP*) gene were introduced. This modified *K. pneumoniae* was then co-cultured with modified *S. oneidensis* expressing a foreign gene (*rib*ADEHC) from *B. subtilis* which encodes a biosynthetic flavin pathway. This combination of organisms when inoculated into MFC resulted in 217% improvement in power density as compared with the wild type consortium [55].

Enhancing MFC Performance Through Engineering Respiratory Mechanisms of *S. oneidensis*—NAD^+

Sources of electrons transported through the mtr pathway are derived from dehydrogenases such as lactate dehydrogenase, or NAD(H/$^+$) dehydrogenases [74]. In the past, the role of NADH in EET and under anoxic conditions was not well characterized. Recent research found out that *S. oneidensis* utilizes Nuo or Nqr1, which are NADH dehydrogenases, for growth and EET via anaerobic respiration of lactate in high-potential electron acceptor [74, 75].

Li et al. [56] recently demonstrated that modifications in expression of NADH enhanced power generation capabilities of MFC. This is due to the role of NADH in respiration and as an intracellular electron carrier and source for EET in *S. oneidensis*. NADH is regenerated by essential redox enzymes, such as GapA2, to serve as an electron acceptor in glycolysis. In Barrangou et al. [68], several genes were identified from carbon metabolism pathways and were overexpressed together, resulting in an overall enhancement in power generated from MFC and also increased regeneration of NADH in the mutant *S. oneidensis*. These genes were namely *gapA2* and *pflB* from glycolysis, and *mdh* from TCA cycle. *pflB* encodes enzyme pyruvate–formate lyase which catalyzes the conversion of acetyl-CoA and formate from pyruvate. As formate is a reduced metabolite, it can be utilized as an electron donor to *S. oneidensis* for power generation purposes. To enhance the release of stored electrons from formate to anode, *fdh** from *Candida boidinii*, which encodes a NAD^+-dependent formate dehydrogenase, was also expressed exogenously, to facilitate the conversion of NADH and CO_2 from formate. TCA cycle, also known as the Krebs cycle, is essential for ATP production in most organisms. The overexpression of the native gene *mdh* in *S. oneidensis* that encodes a NAD^+-dependent malate dehydrogenase to convert oxaloacetate from malate showed promising results in enhancing power generating ability of an MFC. Overexpressing each of these genes individually in *S. oneidensis* was able to increase voltage output significantly when compared with a wild type strain by 26%, 28%, 9%, and 29% for *gapA2*, *pflB*, *fdh**, and *mdh* respectively. Li et al. [56] then generated a mutant *S. oneidensis* with all these genes identified for the performance improvement of MFC. This mutant strain was reported to be able to enhance power generation by three times compared with the controlled WT *S. oneidensis*.

In a separate study, Li et al. [57] identified genes in the NAD^+ biosynthetic pathway and subsequently overexpressed them to increase power generated from MFC through increasing NADH synthesized. Engineered genes were mainly identified from the salvage pathway through assimilating Nm, utilizing nicotinic acid (Na) as a precursor, and the universal biosynthesis pathway synthesizing NAD^+. *S. oneidensis* was unable to utilize Na as a precursor due to the absence of Na transporter and nicotinate phosphoribosyltransferase that converts nicotinic acid mononucleotide (NaMN) from Na. Hence, Na assimilation pathway was reconstructed through *ycel* from *B. subtilis* that encodes a bifunctional niaP transporter for Na and Nm. Endogenous Nm metabolic pathway was constructed through gene *pncB* which encodes the nicotinate phosphoribosyltransferase from *Salmonella typhimurium* for the conversion of Na to NaMN and was introduced to create a mutant *S. oneidensis*. It was reported that these mutants harboring both *ycel* and *pncB* were able to generate voltage higher than that of the WT strain, showing increased intracellular NADH, NAD^+, and NAD(H/+) levels at 78%, 44%, and 54% times, respectively. Li et al. [57] then subsequently overexpressed endogenous gene *nadV* (which encodes a Nm phosphoribosyltransferase) and *pnc* (which encodes the Nm mononucleotide deaminase) in the Nm utilization pathway. It was reported that both mutants harboring *ycel*, and with the genes *ycel*, *nadV*, and *pncC*, showed superior power outputs. For the universal biosynthesis pathway, *nadE** and *nadD** from *E. coli* were expressed heterogeneously along with native *nadM* in *S. oneidensis*. All genes identified from both pathways were assembled, resulting in mutant *S. oneidensis* strains that were able to produce maximum power density 4.4 times more as compared with the control strain. Additionally, intracellular NAD(H/+) pool size of the mutant *S. oneidensis* was increased by 2.1 times when compared with the WT strain [57].

Engineering *S. oneidensis* for Enhanced Utilities

For the enhancement of wastewater treating capabilities in MFC, several studies were conducted to engineer *S. oneidensis*, including the incorporation of foreign metabolic pathway to allow the organism to metabolize substrates that they normally do not utilize. An example of such cases would be the incorporation of xylose metabolic pathway into *S. oneidensis* which allowed xylose to be used as a substrate in an MFC, resulting in an improved power generation up to 2.1 mW/m^2 [58]. This demonstrated the potential in reprogramming *S. oneidensis* with foreign metabolic pathways for utilization of different kinds of substrates commonly found in wastewater.

Microbial communities exhibit mutualistic properties in which the presence of mixed culture enhances the growth of biofilm in MFC [52, 55, 59]. This leads to the studies of interactions between different organisms and subsequently the development of engineering approaches to allow enhanced performance, or utilization of substrate for power generation that would otherwise not be possible. Parallel fermentation was carried out in which the co-cultivation of *Streptococcus bovis* with *S. oneidensis* allowed simultaneous fermentation of lactic acid from starch by *S. bovis*, followed by power generation by *S. oneidensis* up to 50 mW/m^2 [60]. These experimental findings indicate that the combination of different organisms, coupled with genetic modifications in an MFC, can be further exploited to enhance the mutualistic interactions for improved power generation.

Computational Modeling

Computational modeling has been utilized to facilitate understanding of metabolism by *S. oneidensis* at the system level over the past decade. Pinchuk et al. [76] developed a genome-scale constraint-based model (referred as *i*SO783) for metabolism in *S. oneidensis* MR-1. This model investigated MR-1's capabilities and metabolic behavior in the presence of oxygen and estimated ATP requirements in both growth and non-growth phases. This study integrated both computational and experimental approaches to determine that lactate-grown *S. oneidensis* aerobically used less efficient enzymes to stimulate electron flux for the generation of proton motive force. The modeling approach demonstrated the predictions of cell behavior in response to genetic or external environmental interventions, and enhanced the current understanding of microbial metabolism by integrating experimental data to refine metabolic pathways reconstructed from a genome annotation [76]. Ong et al. [77] then expanded the model based on updated genome annotation. They developed a core model for *Shewanella* sp. (referred as Core) using genome annotations of the 21 sequenced strains which represented the conserved metabolic functionalities of all strains of *Shewanella*. Besides, the study utilized a computational algorithm, Comparison of Networks by Gene Alignment (CONGA), to identify the functional difference in developed metabolic networks in order to reveal unique genetic and metabolic differences among different *Shewanella* strains. Recently, Gaffney et al. [78] published a thorough review on the exploration of computational approaches on microbial electrochemical cells, allowing a deeper understanding of the complex EET mechanisms.

Conclusions

The advancement in the field of molecular biology has enabled rapid development of strategies in engineering *S. oneidensis* which is important for the future development of MFC. There are several obstacles that need to be addressed from the biological, electrochemical, and physical aspects of the MFC technology. Coupling modifications on the electrochemical parameters with genetic manipulations and computational modeling can be the potential aspects where future research can venture into. The reviewed improvement technologies would certainly be beneficial to the evaluation studies, development, and integration of microbial fuel cells to the real industries for sustainable energy generation.

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