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Activation Enhancement of Citric Acid Cycle to Promote Bioelectrocatalytic Activity of *arcA* Knockout *Escherichia coli* Toward High-Performance Microbial Fuel Cell

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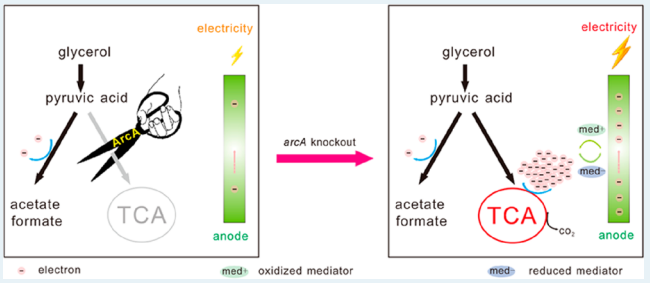
Supporting Information

ABSTRACT: The bioelectrocatalysis in microbial fuel cells (MFCs) relies on both electrochemistry and metabolism of microbes. We discovered that under MFC microaerobic condition, an *arcA* knockout mutant *Escherichia coli* (arcA$^-$) shows enhanced activation of the citric acid cycle (TCA cycle) for glycerol oxidation, as indicated by the increased key enzymes' activity in the TCA cycle. Meanwhile, a diffusive electron mediator (hydroxyl quinone derivative) is excreted by the genetically engineered arcA$^-$, resulting in a much higher power density than its parental strain toward glycerol oxidation. This work demonstrates that metabolic engineering is a feasible approach to construct efficient bioelectrocatalysts for high-performance MFCs.



KEYWORDS: *microbial fuel cell, bioelectrocatalysis, citric acid cycle, arcA knockout, extracellular electron transfer, glycerol*

Microbial fuel cells (MFCs) are a green energy source that convert organic wastes into electricity catalyzed by microorganisms.[1−4] In the anodic chamber of MFCs, organic substrates are oxidized by microbes anaerobically or microaerobically, and the electrons generated in such microbial metabolism are subsequently transferred to the extracellular anodes, then to the cathode through the external circuit.[2,5,6]

Although many electricigenic bacteria, such as *Geobacter*, are capable of completely oxidizing organic substrates such as acetate, lactate, and glucose to generate and transfer electrons to the MFCs' electrodes via extracellular electron transfer (EET) pathways,[7−10] there are few studies on using glycerol, a widely presented "waste stream" in the environment as the byproduct of biodiesel,[11] as the carbon source by electricigens in MFCs.[11,12] To use glycerol as the carbon source and electron donor in MFCs, we here select *Escherichia coli* (*E. coli*), a readily available and popular biocatalyst,[13,14] as the biocatalyst to degrade glycerol.

Under anaerobic condition in MFCs, bacteria such as *Geobacter sulfurreducens* are commonly found to respire to the anode through the citric acid cycle (TCA cycle) of their metabolism for oxidizing a carbon source such as acetate. However, the TCA limitation could occur when using *E. coli* in a MFC, since it is not an effective anode respirer.[5,15] The low activation of the TCA cycle could cause a considerable portion of the electrons in organic substrates to be stored in reduced metabolites such as acetate, ethanol, and formate, decreasing the releasable intracellular electrons (Supporting Information Figure S1). According to the availability of oxygen, the TCA cycle in *E. coli* is elaborately regulated by several sensing/regulating systems, including the two-component Arc system under microaerobic conditions.[16,17] Upon stimulation by the lack of oxygen, ArcA represses the syntheses of most enzymes involved in the TCA cycle, leading to the blockage of the aerobic respiration and production of many reduced fermentation metabolites.[17,18] We thus intend to reactivate the TCA cycle under microaerobic conditions of the MFC via knocking out the repressive *arcA* gene of *E. coli* so as to increase the releasable intracellular electrons and improve the

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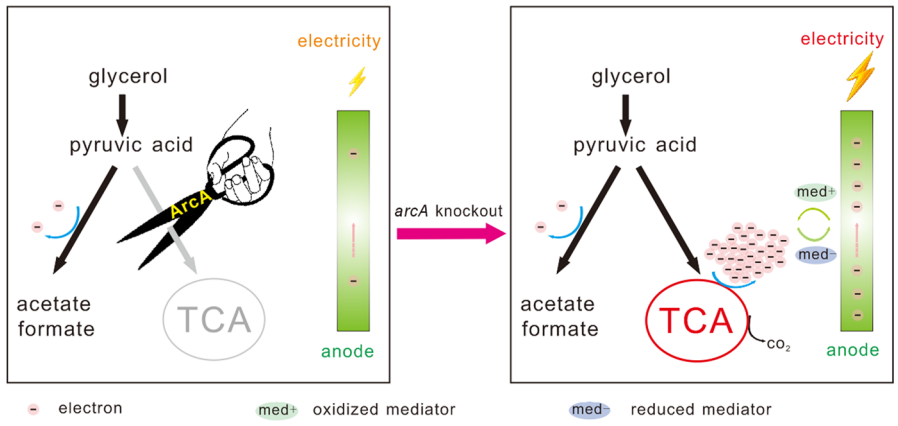

Figure 1. Schematic of the glycerol metabolism of *E. coli* in microaerobic MFC chamber and the genetic manipulating strategy of this study.

biocatalytic performance and electricity power output of MFCs (Figure 1).[13]

The gene *arcA* encoding a redox regulator ArcA in the *E. coli* strain BL21 was disrupted by the one-step gene inactivation method (see details in Supporting Information, and Figure S2). As a result, the *arcA* fragment in the genomic DNA was replaced by a kanamycin resistance cassette (*kan*, a genetic marker for the mutant selection). As evidenced by the DNA electrophoresis analysis of PCR products, the band corresponding to the ~0.6 kb *arcA* gene fragment is observed in the BL21 strain, but not in the arcA$^-$ strain (left panel, Figure 2a), which indicates the absence of the intact *arcA* gene in the genome of the arcA$^-$ strain. Furthermore, the replacement of the ~0.6 kb *arcA* fragment by the ~1.4 kb *kan* in the genome of the arcA$^-$ strain is evidenced by a ~0.8 kb shift of the PCR product for the arcA$^-$ strain (right panel, Figure 2a). These results substantiate that the redox regulator gene *arcA* is eliminated in the genome of the arcA$^-$ strain.

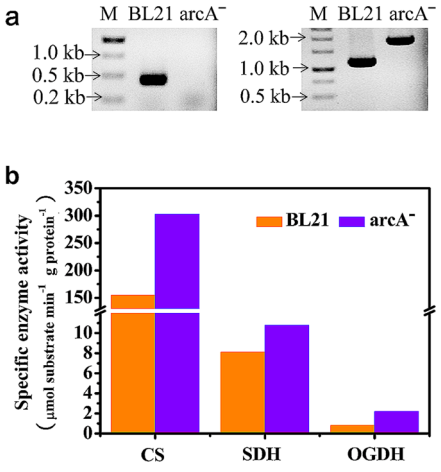

Figure 2. Confirmation of arcA knockout and reactivation of the TCA cycle in the *E. coli* strain arcA$^-$. (a) DNA electrophoresis analysis of the PCR products of the two strains (BL21 and arcA$^-$) using primer pairs of P1−P2 (left panel) and P3−P4 (right panel), respectively. The primers P1 and P2 are to testify to the existence of the *arcA* gene fragment in strain BL21; the primers P3 and P4 are to validate the replacement of *arcA* by *kan* in the strain arcA$^-$ (see details in Supporting Information Figure S2). The sequences of the primers P1−P4 are listed in Supporting Information Table S1. M indicates the DNA standard molecular weight marker. (b) Specific activities of the key enzymes in the TCA cycle of the two strains in the MFC. CS, citrate synthase; OGDH, 2-oxoglutarate dehydrogenase; SDH, succinate dehydrogenase.

Upon inoculation in the microaerobic anodic chamber of MFCs,[19,20] the cell density was monitored as function of time (Supporting Information Figure S3). For the parental BL21 strain, the turbidity decreases at ~45 h after inoculation, probably due to its insufficient anode respiration in MFCs, whereas the arcA$^-$ strain can survive for a long time (~800 h) without further addition of glycerol. We next investigated whether the TCA cycle was activated in the arcA$^-$ strain in the microaerobic anodic chamber of MFC by measuring the specific enzymatic activities of several key enzymes involved in the TCA cycle, including citrate synthase, 2-oxoglutarate dehydrogenase, and succinate dehydrogenase. The specific activities of the three enzymes in the arcA$^-$ strain are all up-regulated in comparison with its parental strain BL21 (Figure 2b), which indicates that the activity of the TCA cycle of *E. coli* cells in the MFC is enhanced through the *arcA* knockout. The enhanced activation of the TCA cycle may result in more efficient oxidation of glycerol and release of more electrons, potentially enabling more efficient anode respiration and production of higher electricity power (right panel, Figure 1).

To this end, the electrochemical behaviors and the anodic respiration processes of the two *E. coli* strains (BL21 and arcA$^-$) were further investigated. Figure 3a shows a pair of well-defined redox peaks (at −0.54 V) in the cyclic voltammogram (CV) of the arcA$^-$ strain in the substrate-free medium, but no redox peak is observed for the BL21 strain (Figure 3a) or in the culture medium without bacteria (data not shown). This indicates that the *arcA* knockout increases the electrochemical activity of the *E. coli* strain, converting an electrochemically inactive strain (BL21) to an electrochemically active strain (arcA$^-$). The linear relationship between the peak current density and the square root of the scan rate indicates that the bioelectrocatalytic process of the arcA$^-$ strain is a diffusion-controlled process (Supporting Information Figure S4).[13,21] In addition, the supernatant of the arcA$^-$ culture also exhibits the redox peaks; however, the bacteria resuspended in phosphate-buffered saline do not show any redox peaks (data not shown), indicating that the bioelectrocatalysis of the arcA$^-$ strain is enabled by a diffusive self-excreting redox species. This endogenous shuttle is identified as a hydroxyl quinone derivative, which is revealed by the UV−vis and FTIR spectra analysis (find details in Supporting Information Figure S5).

The functional TCA cycle involves a number of dehydrogenases, some of which, such as membrane-associated malate/

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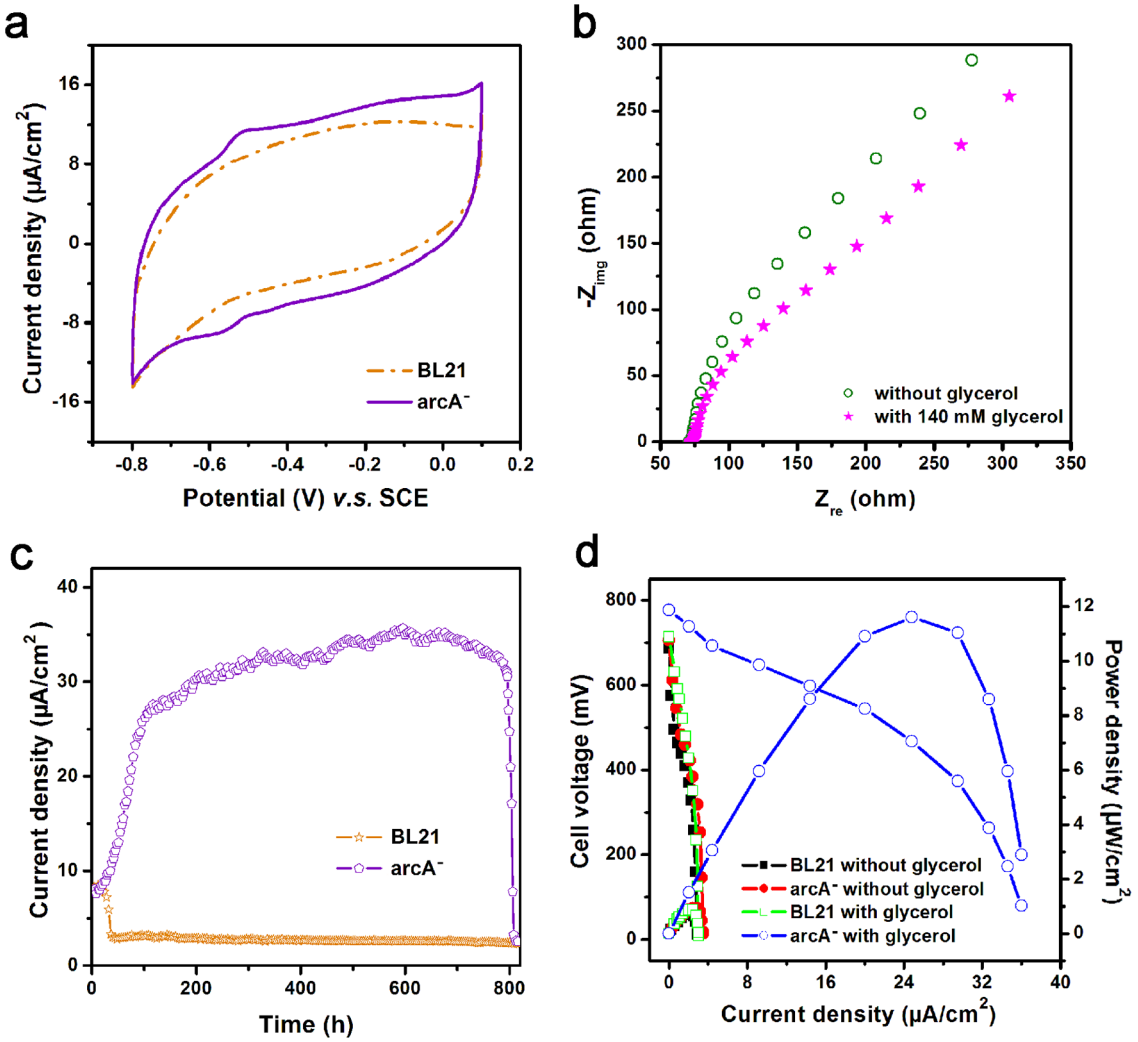

Figure 3. Bioelectrocatalytic behaviors and MFC performance of the strains of BL21 and arcA⁻. (a) CVs of the strains of BL21 and arcA⁻ in a substrate-free medium. (b) EIS of the arcA⁻ mutant in the culturing medium without and with 140 mM glycerol, respectively. (c) Discharge performance in dual-chamber MFCs inoculated with the strains of BL21 and arcA⁻, respectively. (d) Polarization and power density curves of the arcA⁻ mutant strain-catalyzed MFC.

quinone-oxidoreductase, are associated with the biosynthesis of quinone and its derivatives as coenzymes.[22,23] These quinone-derivative molecules can easily diffuse across a cell membrane and play fulfill the function of superior electronic shuttles between microbes and the anode. Electrochemical impedance spectra (EIS) were measured to evaluate the charge transfer resistance (R_{ct}) of these strains on anodes. The arcA⁻ cell suspension without glycerol illustrates a well-defined semicircle, followed by a straight line, whereas the parental BL21 strain does not show any well-defined semicircle (Supporting Information Figure S6). Since the semicircles of the EIS are not well-defined, the impedance data were fitted with the Randle equivalent circuit (Supporting Information Figure S6) to obtain accurate results,[24] The R_{ct} of the arcA⁻ strain is 134 Ω, which is much smaller than that (700 Ω) of the BL21 strain, clearly indicating that the EET rate of the arcA⁻ strain is significantly increased. Thus, the endogenous mediator can efficiently facilitate EET in the arcA⁻ strain. With the addition of glycerol in the arcA⁻ cell suspension, a significantly increased oxidative current is observed at the same potential (−0.54 V) in CV curves (Supporting Information Figure S7). Meanwhile, an improved EET rate is reflected in the EIS (Figure 3b), indicating an effective biocatalytic behavior of glycerol oxidation by the arcA⁻ strain.

To evaluate the performance of the arcA⁻ strain in the MFC, the output current was recorded with a constant load resistance of 19.6 kΩ (shown in Figure 3c). The current density of the arcA⁻ strain increases slowly during the first 100 h as the initialization stage of the MFC, and then it reaches and is maintained at a plateau of 35 μA/cm^2 for 700 h without nutrition refreshment, finally followed by a quick extinguishment due to the exhaustion of glycerol. However, the strain BL21 does not display a significant output current (only 3.5 μA/cm^2).

To investigate the effect of the mediator produced by the arcA⁻ strain on the current output of the BL21 strain, discharge experiments of the BL21 strain resuspended in the supernatant of arcA⁻ were carried out (shown in Supporting Information Figure S8). Upon inoculation into the supernatant of arcA⁻, the BL21 strain could deliver a steady current of about 14 μA/cm^2, which is higher than that (3.5 μA/cm^2) of the original BL21, but still much lower than that (35 μA/cm^2) of arcA⁻. This finding indicates that the mediators produced by arcA⁻ could increase the current output of BL21 to a certain extent, but the

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metabolic activity of the microorganism plays a crucial role in the electron recovery. Further, the steady current density in BL21 with added mediators could maintain for only 60 h (Supporting Information Figure S8), which is much shorter than that (700 h) in arcA$^-$. This very likely indicates that arcA$^-$ could continuously produce the mediators to maintain the bioelectrocatalytic activity for a much longer discharge time. On the basis of these results, the superior bioelectrocatalytic performance of the arcA$^-$ strain should be due to the enhancement of the TCA cycle in the microaerobic MFC, which leads to the production of an endogenous redox mediator to shuttle excessive electrons to the anode. This facilitated EET dramatically enhances electricity power output in the MFC (Figure 3c). The Coulombic efficiency of arcA$^-$-catalyzed MFC calculated from the discharge curve is 3.5%, which is much higher than that (0.03%) of the BL21-catalyzed MFC and higher than that of MFC also using glycerol as the carbon source with mixed culture as inoculums (0.5−2%).[11] The polarization and power output curves were further obtained by varying load resistances (Figure 3d). The open circuit potential of the arcA$^-$-strain-catalyzed MFC is 778 mV, and the maximum power density is 11.6 μW/cm^2, which is ∼2 times the reported maximum power density of MFC using glycerol as the substrate (6 μW/cm^2).[25] The arcA$^-$ strain constructed in this work significantly increases the power density of the MFC and brings the MFC with glycerol as a substrate closer to practical applications.

In summary, the TCA cycle of *E. coli* in the microaerobic MFCs is enhanced by the knockout of the *arcA* gene, resulting in a significant increase in the current density and energy conversion efficiency over its parental strain. This study provides an efficient approach to tune the bioelectrocatalysis and performance of MFCs by the genetic manipulation of microbial metabolic pathways.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details of bacterial culture, genetic construction, MFC setup, enzyme activities measurements, electrochemical characterization; primer sequences, gene knockout strategy, proliferation curves of bacteria, UV−vis and FTIR spectra, electrochemical results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

□J. Liu and Y. C. Yong contributed equally to this work.

Notes

The authors declare no competing financial interest.

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