High-resolution electrochemistry of the extracellular electron transfer of *Escherichia coli*

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Abstract: *Escherichia coli* is one of the most important model bacteria in microorganism research and is broadly encountered in nature. In the present study, a wild-type *E. coli* strain K-12 was used for electrochemical investigations. Differential pulse voltammetry showed five pairs of redox peaks both for K-12 cells and the supernatant with potentials (anodic/cathodic) at -0.450/-0.378, -0.125/-0.105, -0.075/-0.055, +0.192/+0.264, and +0.300/+0.414 V (vs. Ag/AgCl), respectively. Chronoamperometry indicates that K-12 cells can produce immediate current by addition of glucose. The current production from K-12 can be 8-fold enhanced by 10.0 μ M exogenous vitamin K3, but addition of 10.0 μ M riboflavin did not enhance the current production. Medium replacement experiments show that 50 % of the K-12 biofilm current was produced via direct extracellular electron transfer pathways. The study provides new insight in the voltammetry of strain K-12 and confirms that *E. coli* is an electrochemically active bacterium. *E. coli* has the potential to serve as a model bacterium for studying microbial extracellular electron transfer mechanisms.

Keywords: *Escherichia coli*; Vitamins; Flavins; Extracellular electron transfer

25 Introduction

26 Bioelectrochemical systems combine biological and electrochemical processes to generate electricity,
 27 hydrogen or other useful chemicals from organic materials e.g. pollutants ¹. Microbial (EET), in which
 28 microorganisms exchange electrons with extracellular electron donors or acceptors, is here the central
 29 core process ²⁻⁴. A full understanding of EET mechanisms is therefore essential to improve the production
 30 of chemicals and energy as well as enhance bioremediation efficiency. Microbial EET mechanisms
 31 proposed are mainly based on studies of *Shewanella oneidensis* or *Geobacter sulfurreducens*. Redox
 32 proteins such as *c*-type cytochromes and other molecular electron shuttles, conductive pili/nanowires and
 33 extracellular polymeric substances are regarded as the core elements in extracellular transport of electrons
 34 ^{5,6}. However, it is not fully clear, how microorganisms conduct the EET process, due to the limitation of
 35 the model strains presently used: (i) *G. sulfurreducens* is an obligately anaerobic bacterium but not easy
 36 to culture, with few tools available for genetic manipulation; (ii) *S. oneidensis* is easy to culture, but
 37 genetic tools to manipulate the strain for in-depth investigations are lacking; (iii) neither of the bacteria
 38 dominate the natural environment, even in bioelectrochemical systems ⁷.

39 *Escherichia coli*, a facultative bacterium widely present in nature, is the most important model bacterium
 40 in microbiology and molecular biology research, with clear metabolism mechanisms mapped out and
 41 many available tools for genetic manipulation. However, there are many contradictory reports on EET
 42 ability of *E. coli*. Schröder *et al.* reported that *E. coli* K-12 produced a current of 19.5 mA through
 43 metabolic H₂ conversion, but the electrons involved are not directly from the cells and the modified anode
 44 played an important role in the bioelectricity production ⁸. Zhang *et al.* recorded an enhanced current
 45 production from electrochemically-evolved *E. coli* K-12 together with an optimized carbon/PTFE
 46 composite anode ⁹, but did not provide a pathway for electron transfer. *E. coli* cells evolved under
 47 electrochemical tension showed electrochemical behavior in a microbial fuel cell with hydroquinone
 48 assumed to be the electron mediator ¹⁰. However, whether the *E. coli* K-12 of wild type have the EET
 49 ability is still widely argued; some researchers claimed that *E. coli* is a non-exoelectrogenic bacterium

since the cells could not produce current in their MFCs^{11, 12}; a small residual current was ascribed to electrons and protons stored in the *E. coli* culture in a redox battery-like manner¹¹.

E. coli is widely abundant in soil, and understanding of *E. coli* EET mechanisms, if *E. coli* can be confirmed as an electrochemically active bacterium, can help us to illustrate and map EET processes and electrochemically active microorganisms broadly¹³. We noted that the previous studies usually rested on biofilms grown on electrodes with large surface area and high background current. Some weak but important electrochemical signals may therefore have been missed. Hence, we have undertaken the present study of the electrochemistry of *E. coli* using a high-resolution electrochemistry method, differential pulse voltammetry (DPV) and the wild-type model strain K-12 with the view of obtaining more details on the electrochemical profile and EET ability of *E. coli*.

Experimental Section

Bacterial strains and growth conditions

E. coli K-12 strain was bought from the German Collection of Microorganisms and Cell Cultures (DSMZ) (DSM number 498). M9 medium was used to culture *E. coli* K-12 cells. The M9 medium¹⁴ contains (g/L) glucose of 4.00, Na₂HPO₄ of 6.78, KH₂PO₄ of 3.00, NaCl of 5.00, NH₄Cl of 1.00, MgSO₄•7H₂O of 0.493, and CaCl₂ of 0.011. The initial pH of the M9 medium was adjusted to 7.00 using 1% HCl or 1 M NaOH. The strain was grown aerobically at 37 °C by shaking at 120 rpm. Glucose, Na₂HPO₄, KH₂PO₄, NaCl, NH₄Cl, MgSO₄•7H₂O, and CaCl₂ were analytically pure products from Sigma-Aldrich. Riboflavin and vitamin K3 were USP Grade from Sigma-Aldrich and from Coolaber Inc., China, respectively. *E. coli* K-12 cells were harvested by centrifugation (5000 g, 10 min, 4 °C) and washed twice with 0.05 M phosphate potassium buffer (pH 7.00) before use.

The *E. coli* K-12 strain grew fast aerobically in M9 medium by shaking at 125 rpm and reached the stationary phase in 12 h (Figure S1.A). Cells grown at 12-16 h with a length of around 2 μm (Figure S1.B

73 and C) were harvested from the M9 medium for voltammetric recording by DPV, CV, and
74 chronoamperometry.

75 **Electrochemical measurements on *E. coli* K-12 cells**

76 All electrochemical voltammetry experiments were carried out using a CHI832D or CHI732D
77 potentiostat (CHI, TX, USA) with a three-electrode chamber containing a glassy-carbon working
78 electrode (3 mm diameter), a platinum-wire counter electrode and a KCl saturated Ag/AgCl reference
79 electrode ^{6, 15}. All potentials reported refer to Ag/AgCl sat. KCl. Phosphate potassium buffer solution of
80 0.05 M (pH 7.00) was used as electrolyte. All experiments were conducted under a nitrogen atmosphere.
81 The parameters were: for cyclic voltammetry (CV), equilibrium time 5 s, scan rate 10 mV/s; for
82 differential pulse voltammetry (DPV), equilibrium time 5 s, increment potential 6 mV, amplitude 60 mV,
83 pulse width 0.2 s, pulse period 0.4 s; for *i-t* chronoamperometry, the working electrode was kept at +0.10,
84 +0.20, or +0.30 V as indicated in each figure legend.

85 ***E. coli* K-12 biofilm**

86 *E. coli* K-12 biofilm was anaerobically grown on carbon felt (area 1.0 cm²) which also served as the
87 working electrode for subsequent *i-t* chronoamperometry. Stainless steel mesh (area 9.0 cm²) and a KCl
88 saturated Ag/AgCl electrode served as counter and reference electrode, respectively. The electrodes were
89 connected to a CHI1000C potentiostat (CHI, TX, USA). The electrode system was inserted in a sealed
90 100 mL flask filled with oxygen-free medium during the cultivation. A polypropylene tube was connected
91 to the bottom of the flask when the medium was replaced. Magnetic stirring (50 rpm) was also used.

92 **Results and Discussion**

93 **Voltammetry and chronoamperometry of *E. coli* K-12**

94 Five pairs of DPV redox peaks of *E. coli* K-12 cells grown at 12-16 h i.e. the early stationary growth
95 phase (Figure 1A) were observed. The potentials of the anodic peaks were -0.450, -0.125, -0.075, +0.192,

and +0.300 V. The corresponding cathodic peak potentials were -0.378, -0.105, -0.055, +0.264, and +0.414 V. The redox peaks around -0.400 V, -0.200 to 0 and 0.200 to 0.400 V were grouped into the redox peak group I, II and III, respectively (Figure 1A). The potentials of the group II and III redox peaks were so close that they sometimes overlap into a single redox peak (Figure S2). Only one or two anodic peaks were observed in the voltammetry of biofilms on electrodes with large surface area, for example -0.100, +0.100 V¹⁶ or -0.070 V¹⁷. By conducting voltammetry on collected *E. coli* K-12 cells on a glassy carbon electrode, the data in the present study displayed, however, a notably more sensitive *E. coli* electrochemical redox profile than in previous reports^{16,17}.

Figure 1 Electrochemical patterns of *E. coli* K-12 cells grown at 16 h. (A) DPV of *E. coli* K-12 cells dropcast onto a glassy carbon electrode. Five redox peak pairs were found and grouped into three groups. While there is only one redox peak pair in group I, group II and III each contains two pairs of redox peaks. (B) Chronoamperometry confirms a current response to added glucose from *E. coli* K-12 cells when the working electrode was polarized at +0.10 V. 3.00 mL 40.0 g/L glucose solution was added to 27.00 mL electrolyte, making the final concentration 4.00 g/L glucose. No current was produced from bare glassy carbon electrode in 4.00 g/L glucose. Glassy carbon, Ag/AgCl, and platinum wire served as working, reference and counter electrode, respectively. In 0.05 M phosphate potassium buffer of pH 7.00.

All the five redox peak pairs observed for *E. coli* K-12 cells were also found in the CV profiles of the supernatant (Figure S3). As the scans continued, the group II current increased, whereas those in group

115 III decreased. No obvious change was observed in group I. These results indicate that the electroactive
116 molecules on the cell membrane are also excreted to the supernatant. Furthermore, it seems that the
117 molecules related to group II are easily adsorbed on glassy carbon while those related to group III are
118 very unstable, since the peak current of this group continued to decrease.

119 Chronoamperometry confirmed that *E. coli* K-12 cells can produce current and have EET ability using
120 glucose as electron donor at a polarization potential of +0.10 V, whereas glucose cannot be used for
121 current production without cells (Figure 1B). Compared to glucose, *E. coli* K-12 cells used lactose for a
122 smaller current production while they did not produce current from lactate and acetate 1 h following
123 substrate addition (Figure S4). Furthermore, biofilm formed anaerobically on carbon felt (1.0 cm²) can
124 produce a current increment of 0.20 μ A by supplying glucose at a polarization potential of +0.20 V
125 (Figure S5). These results confirm that *E. coli* is an electrochemically active bacterium, able to produce
126 electricity from appropriate organic substrate such as glucose, and redox peak groups I and II may both
127 contribute to the electricity production based on the hold potential of +0.20 V.

128 What is the role of riboflavin in EET?

129 The pair of redox peaks around -0.40 V i.e. group I is ascribed to flavins as the redox potentials are similar
130 to flavins potentials in many other microorganisms such as *Shewanella* spp.^{6, 18, 19}, *Bacillus* spp.^{20, 21},
131 *Listeria monocytogenes*²², *Pichia stipitis*²⁰, and *Pachysolen tannophilus*²³. Previous studies also reported
132 that *E. coli* can excrete flavins during growth with glucose²⁴. Besides, we found that the redox peaks are
133 strengthened by riboflavin addition (Figure S6), meaning that the two substances have similar redox
134 potentials.

Figure 2 Chronoamperometry of *E. coli* K-12 cells dropcast on glassy carbon electrode. This result shows a fast current response to each glucose addition from *E. coli* K-12 cells when the working electrode was polarized at +0.20 V. Each time glucose with a final concentration of 1.00 g/L increase was added. However, no current increment was observed after the addition of riboflavin (final 1.0 μ M). The Ag/AgCl and platinum wire served as reference and counter electrode, respectively. 0.05 M phosphate buffer of pH 7.0.

Flavins have been widely reported as ET mediators for microbial EET process ^{6, 20, 21, 23}, but low concentration of riboflavin cannot be used by *E. coli* K-12 cells to facilitate its EET as no current increment was observed after the addition of extra 10.0 μ M riboflavin (Figure 2). This result agrees well with a previous study which showed that *E. coli* secreted three types of flavins ¹⁹, but that *E. coli* did not utilize these molecules in EET ²⁵. The result further confirmed that the electric current produced by *E. coli* K-12 at polarization potentials +0.10 or +0.20 V are mainly contributed by the redox peak group II, but not by group I ascribing to flavins.

Effect of vitamin K3 on the *E. coli* EET

Previous reports suggested that *E. coli* may use menaquinone or related compounds as mediators for EET ¹⁷, and the bacterium can definitely produce vitamin K2, i.e. menaquinone ²⁶. However, vitamin K2 is

not soluble in water and it is not obvious, how *E. coli* can utilize this substance as an electron shuttle in solution. One possible explanation if the compound is truly a K2 molecule, is that this molecule may combine with another molecule or even proteins. In the present study, we added another soluble synthetic vitamin K3 (final concentration 10.0 μ M) with the same functional group *i.e.* the quinone group to test whether *E. coli* K-12 cells can use it as a mediator during the CV of *E. coli* K-12. The result shows that compared with the redox peak group II, vitamin K3 gave more negative redox peak potentials and definitely strengthened the oxidative current but weakened the reduction current of the *E. coli* K-12 strain (Figure 3A).

Figure 3 Effects of vitamin K3 on the EET of *E. coli* K-12. (A) CV of vitamin K3 and *E. coli* K-12 cells grown for 16 h with and without extra vitamin K3 added. 10.0 μ M vitamin K3 showed a pair of redox peaks around -0.200 V (red line). K3 (final 10.0 μ M) was added to the electrolyte at the beginning of cycle 7. The original CV pattern (blue dotted line, data from cycle 3) showed the three groups of redox peaks from *E. coli* K-12. The presence of K3 gradually strengthened the oxidative current beginning at -0.250 V (green dotted line and purple dashed line, data from cycle 8 and 10, respectively) as it diffused into the biofilm on the glassy carbon surface. Scan rate 10 mV/s. (B) Chronoamperometry shows that *E. coli* K-12 cells can use vitamin K3 but not riboflavin to enhance EET. Only a very small current increment from bare glassy carbon on addition of vitamin K3 was observed. Glassy carbon, Ag/AgCl and platinum wire served as working, reference and counter electrodes, respectively. 0.05 M potassium phosphate

171 buffer, pH 7.00. The working electrode potential was kept at +0.30 V. The final concentration of
172 riboflavin, glucose and vitamin K3 were 10.0 μ M, 4.00 g/L and 10.0 μ M, respectively.

173 We further evaluated the impact of vitamin K3 on the current production of *E. coli* K-12 from glucose
174 and compared with that of riboflavin by sequentially adding riboflavin, glucose and K3 into the electrolyte
175 (Figure 3B). Riboflavin apparently had very little positive effect on the EET of *E. coli* K-12 cells, and we
176 only observed a very small current increment (from 35.5 to 40.1 nA) caused by the addition of 10.0 μ M
177 riboflavin. The current increased (from 33.8 nA to a maximum of 69.8 nA) by sequential addition of 4.00
178 g/L glucose, which agreed well with the result in Figure 1B. As a contrast, vitamin K3 was added into the
179 electrolyte when the current was decreasing. The current continued to increase very fast from 46.8 nA to
180 more than 380.0 nA. A current increment of only about 1.0 nA was observed when the same amount of
181 glucose and vitamin K3 was added to a similar system but without *E. coli* K-12 cells on the working
182 electrode (Figure 3B).

183 Vitamin K3 can also boost current from *E. coli* K-12 biofilm formed on carbon felt (area about 1.0 cm²,
184 Figure S7). These results indicate that *E. coli* K-12 cells can use water soluble vitamin K3 as a mediator
185 or combine this molecule with something on the cell surface to promote significantly the EET process
186 and current production. However, whether *E. coli* can use vitamin K2 with the same quinone group as
187 that in vitamin K3 to facilitate the EET process is not clear.

188 **Direct or indirect mechanisms dominate the EET process of *E. coli* K-12 cells?**

189 Following a previous study on *S. oneidensis* MR-1¹⁸, in the present study, we have employed medium
190 replacement to identify which, direct or indirect pathway, dominates the EET process of *E. coli* K-12
191 cells. As shown in Figure S8, about 50% oxidative current remains after the original supernatant is
192 replaced by oxygen-free fresh M9 medium. These results were entirely reproducible both in 60 and 18 h-
193 old *E. coli* K-12 biofilms formed on carbon felt. The results indicate that direct and indirect electron
194 transfer mechanisms are of equal importance in the EET of *E. coli* cells. Since flavins do not contribute
195 to current production, the direct as well as the indirect electron transfer, of *E. coli* should be contributed

196 by the redox peak groups II and III. However, more in-depth work including molecular biology and
197 electrochemistry are needed to specify the contribution of each redox peak to direct or indirect electron
198 transfer.

199 **Conclusions**

200 By using small working electrodes and highly sensitive DPV measurements, high-resolution
201 voltammetric profiles of *E. coli* K-12 were acquired in the present study. Five pairs of DPV redox peaks
202 in the electrochemical window -0.600 to +0.400 V were disclosed. The redox peaks around -0.400 V (the
203 group I peaks) belong to flavins which could not be utilized by *E. coli* K-12 cells to facilitate their EET
204 processes. *E. coli* K-12 cells can instead use water soluble vitamin K3 to enhance the oxidative current
205 from glucose. Direct and indirect electron transfer mechanisms are of equal importance in the EET of *E.*
206 *coli* K-12 cells. Though wildtype *E. coli* K-12 produces relative small currents, this study confirms *E.*
207 *coli* as an electrochemically active bacterium. To fully illustrate the EET process of *E. coli*, future
208 combined work from electrochemistry and molecular biology, can be performed to elucidate the origins
209 of the other four pairs of redox peaks. Such information would also boost the application of *E. coli* in
210 bioelectrochemical systems and bioelectricity production.

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216 **Contributions**

217 Y.X., F.Z. and J.Z. designed the experiments. Y.X., Z.Z. and H.G. performed the experiments. Y.X.
218 drafted the manuscript. All the authors revised and approved the manuscript.

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