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Correlation between circuital current, Cu(II) reduction and cellular electron transfer in EAB isolated from Cu(II)-reduced biocathodes of microbial fuel cells

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Abstract
The performance of four indigenous electrochemically active bacteria (EAB) (Stenotrophomonas maltophilia JY1, Citrobacter sp. JY3, Pseudomonas aeruginosa JY5 and Stenotrophomonas sp. JY6) was evaluated for Cu(II) reduction on the cathodes of microbial fuel cells (MFCs). These EAB were isolated from well adapted mixed cultures on the MFC cathodes operated for Cu(II) reduction. The relationship between circuital current, Cu(II) reduction rate, and cellular electron transfer processes was investigated from a mechanistic point of view using X-ray photoelectron spectroscopy, scanning electronic microscopy coupled with energy dispersive X-ray spectrometry, linear sweep voltammetry and cyclic voltammetry. JY1 and JY5 exhibited a weak correlation between circuital current and Cu(II) reduction. A much stronger correlation was observed for JY3 followed by JY6, demonstrating the relationship between circuital current and Cu(II) reduction for these species. In the presence of electron transfer inhibitors (2,4-dinitrophenol or rotenone), significant inhibition on JY6 activity and a weak effect on JY1, JY3 and JY5 was observed, confirming a strong correlation between cellular electron transfer processes and either Cu(II) reduction or circuital current. This study provides evidence of the diverse functions played by these EAB, and adds to a deeper understanding of the capabilities exerted by diverse EAB associated with Cu(II) reduction.

Keywords: biocathode; microbial fuel cell; electrochemically active bacteria; Cu(II) reduction; electron transfer inhibitor
1 Introduction

Microbial fuel cells (MFCs) are emerging as new, sustainable and effective technologies for the recovery of heavy metals from waste and wastewater [1]. Among diverse heavy metals, Cu(II), which is common in the electroplating and mining industries [2-3], has attracted significant attention due to the potential for its recovery and simultaneous wastewater detoxification. The recovery of Cu(II) with abiotic cathode MFCs has been demonstrated over a wide range of operating conditions and cell architectures [4-9]. However, abiotic cathodes often require the use of costly noble or non-noble co-catalysts in addition to an acidic medium. Therefore, the development of biocathodic MFCs, which are based on the catalysis of self-regenerating electrochemically active bacteria (EAB) under a neutral environment, may provide a sustainable and green alternative to abiotic systems. Biocathodic MFCs avoid the use of expensive materials and toxic organic reagents, and reduce the consumption of energy and acids [10-14]. Biocathodes are also able to reduce electrode overpotentials, the production of sludge, and the overall cost and maintenance of MFCs. MFCs, utilizing mixed cultures, have been shown to be efficient in the recovery of Cu(II) from mixed metal influent and also a promising system for the synthesis of copper [15]. In contrast, MFCs with biocathodes operated with pure cultures have been used to study specific EAB and their electrochemical performance [10-14]. The exogenous EAB of Shewanella is one of the few examples which is able to reduce a metal (Cr(VI)) in MFCs [16-17].

Microorganisms possess endurance to aqueous Cu(II) through a variety of mechanisms due to their intrinsic abilities and habitat sites [18-20]. Therefore, well adapted microorganisms could provide new insights with regards to heavy metal reduction in MFCs. A limited number of microorganisms exhibiting efficient rates of Cu(II) reduction have been cultivated under Cu(II)-adaptive conditions [20-24]. Similar considerations, in principle, hold true for their use in cathodic reductive environments in MFCs, under which the activities of different indigenous EAB may exhibit various Cu(II) reduction rates. In parallel, EAB immobilized on the surface of cathodes may also be able to utilize cathodic electrons for their internal metabolism [11,25]. The operation of MFCs at different concentrations of Cu(II) in the catholyte is expected to clarify the correlation between the circuital current and the rate of Cu(II) reduction associated with the use of isolated EAB.

The bacteriological reduction of Cu(II), in the absence of a circuital current, is believed to be associated with cellular electron transfer processes through the cytoplasmic membrane and with the flux of protons through ATP-synthase [26-27]. Such mechanisms can be unraveled using cellular electron transfer inhibitors, such as 2,4-dinitrophenol (DNP) and rotenone (C23H22O6). DNP dissipates the proton motive force associated with cellular electron transfer processes, and decreases hydrogen production by inhibiting the ATP synthesis by photophosphorylation, in the absence of a circuital current [28]. In contrast, rotenone inhibits the activity of NADH-dehydrogenase and blocks the reduction of U(VI) by facultative anaerobic bacteria in the absence of a circuital current [29]. The utilization of rotenone and DNP, in the presence and absence of a circuital current passing through the EAB
cathodes of MFCs is thus expected to establish whether the circuitual current and Cu(II) reduction are associated with cellular electron transfer processes in the EAB species.

In this study, we elucidate from a mechanistic point of view the performance and impact of four indigenous different EAB on Cu(II) reduction in MFCs. The EAB tentatively identified as Stenotrophomonas maltophilia JY1, Citrobacter sp. JY3, Pseudomonas aeruginosa JY5 and Stenotrophomonas sp. JY6, were isolated from well adapted mixed cultures grown on the surface of MFC cathodes operated for Cu(II) reduction [15]. X-ray photoelectron spectroscopy (XPS), scanning electronic microscopy (SEM) coupled with energy dispersive X-ray spectrometry (EDS), linear sweep voltammetry (LSV) and cyclic voltammetry (CV) were used to investigate the effect of circuitual current on the speciation of the deposited copper, the morphologies of the cathodes and the cathodic redox reactions for each EAB species. The relationship between circuitual current, the rate of Cu(II) reduction and the cellular electron transfer processes associated with each of the four EAB were clarified through the system response to a step change of Cu(II) concentration in the catholyte and reaction mechanisms elucidated in the presence or absence of DNP or rotenone electron transfer inhibitors.

2 Materials and Methods

2.1 EAB isolation, incubation and identification

Bacterial isolates were obtained from mixed culture cultivated in the Cu(II)-reduced biocathodes of MFCs [15]. The isolation and incubation processes are detailed in the Supplementary Material (SM). The DNA of these EAB isolates was extracted using a Qubit2.0 DNA kit (Sangon Biotech (Shanghai) Co. Ltd., China) according to the manufacturer’s procedure. The 16S rRNA gene was amplified by PCR using universal primers 518 F (5’ CAGAGTTTGATCCTGGCT3’) and 1540R (5’AGGAGGTGATCCAGCCGCA3’), as described in SI. The sequence data were compared with the GenBank database using the Blast server at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) to accurately identify the bacterial strains. All tests were performed in duplicate.

2.2 MFC reactors setup and operation

Identical two-chamber MFCs with cylindrical chambers 4.0 cm long by 3.0 cm in diameter were used in all experiments. The anodic and cathodic chambers were separated by a cation exchange membrane (CEM) (CMI-7000 Membrane International, Glen Rock, NJ) with a projected surface area of 7.1 cm². Both the anode and cathode were filled with graphite felt (1.0 cm × 1.0 cm × 0.5 cm, 8 pieces, Sanye Co., Beijing, China) and carbon rods were used as current collectors in both anode and cathode. For each of the duplicate reactors three replicate experiments were performed. The MFCs were operated at a fixed external resistance of 510 Ω.

The anodes were inoculated with suspended bacteria collected from a previous acetate-fed MFC reactor and an equivalent volume of nutrient solution containing acetate (1.0 g/L) was added [30-31]. The cathodes were fed using the same medium,
except acetate was replaced by NaHCO₃ (10 mg/L), with further addition of Cu(II) (5 mg/L) to the cathodic chambers. Cathodes were quantitatively inoculated with the isolates with a total of 3 × 10⁸ colony forming unit. The anolyte and catholyte were sparged with N₂ gas for 15 min prior to adding the solutions into the electrode chambers. No measurable Cu(II) in the anolyte and acetate in the catholyte were observed during each cycle operation, excluding the possibility of Cu(II) and acetate diffusion between the chambers, although the retention of Cu(II) on the ion exchange membrane could not be precluded [15]. Other catholyte and operational conditions are described in SM.

The performance of the biocathodes was evaluated against four control experiments, including (i) the operation of the reactors under the open circuit condition (OCC), (ii) the operation with a closed circuit but without the inoculation of the EAB (abiotic control), (iii) the cathodes covered with the EAB but in the absence of Cu(II), and (iv) the cathodes tested in the absence of both EAB and Cu(II). The last two control experiments were used to evaluate the CVs performance of the cell.

2.3 Measurement, analysis and calculation

The circuitial current, dissolved oxygen, biomass, organics and Cu(II) concentration were determined according to the methodology reported in SM. The rate of Cu(II) removal and charge distribution were calculated as detailed in SM. Maximum power was obtained by running LSV at a scan rate of 0.1 mV/s [30-31]. Power and current densities were normalized to the projected surface area of the membrane. EAB cathode redox behavior was studied using CV (CHI 650, Chenhua, Shanghai). The potential was scanned between –0.36 V and +0.46 V (vs SHE) at a scan rate of 1.0 mV/s using a standard three-electrode arrangement with the biocathode as the working electrode, platinum plate as the counter electrode, and Ag/AgCl as the reference electrode. One-way ANOVA in SPSS 19.0 was used to analyze the statistical variation of the data, and all of the data indicated significance levels of p < 0.05.

The surfaces of the Cu-laden biomass were analyzed by X-ray photoelectron spectroscopy (XPS, Thermo Fisher Scientific, ESCALAB 250, US) with a Mono Al Kα X-ray source (1486.6 eV of photons). The X-ray source was run at a reduced power of 150 W. The morphology of the electrodes after Cu(II) reduction were examined with a SEM (QUANTA450, FEI company, USA) equipped with an EDS (X-MAX 20 mm²/50 mm², Oxford Instruments, UK) according to the method described previously [15,30].

3 Results and Discussion

3.1 EAB Isolation

Four EAB were successfully isolated from well adapted mixed cultures grown on the surface of MFC cathodes operated for Cu(II) reduction [15] (Table S1). All bacteria were gram-negative and major opportunistic to facultative or survived from an anaerobic environment. JY1 matching Stenotrophomonas maltophilia, is known to
convert Cu(II) into Cu(0) on the cell surface, in the absence of cathodic electrons
[22-24]. *S. maltophilia* has been isolated previously from a copper polluted area [22],
and has never been reported in MFCs. JY3 closely related to *Citrobacter* sp., is able to
remove Cu(II) in a medium of SO₄²⁻ through the formation of CuS precipitate in the
absence of cathodic electrons [20]. It has been isolated previously from anodic MFC
biofilms in the absence of Cu(II) [32]. JY5 matching *Pseudomonas aeruginosa*, is
able to remove Cu(II) in the absence of cathodic electrons [21]. It has been reported in
either denitrification autotrophic biocathodes of MFCs [33], or in MFCs operated for
the degradation of phenol and glycerol through mediated electron transfer [34-37].
Finally, JY6 corresponding to *Stenotrophomonas* sp., can tolerate high concentrations
of metals including Ag, Cu, Cd, Hg and Mn [38-39] and has been isolated previously
from a wide range of environments.

3.2 EAB activity assessment

**Here Fig. 1**

All four EAB played a significant role in the reduction of Cu(II) in the MFCs and
displayed rates of Cu(II) reduction higher than those found in both OCC and abiotic
CCC controls (Fig. 1A). Previous studies with mixed culture biocathodes using
nitrobenzene, pentachlorophenol, Cr(VI), Co(II) or Cu(II) as an electron acceptor
[31,40-44] support these findings. The Cu(II) reduction rates in the range (0.82 ± 0.05
– 1.11 ± 0.01 mg/L/h) were close to those found in mixed cultures (1.07 ± 0.01
mg/L/h) at the same Cu(II) concentration (5 mg/L) [15], reflecting the robust capacity
of these isolates for reducing Cu(II).

**Here Fig. 2**

The results in Figs. 1 and 2 show that the EAB catalytic process produced higher
circuital currents (Fig. 1B), open circuit potentials (Fig. 2A and B) and maximum
power production (Fig. 2C and D) in comparison with the values under abiotic control,
irrespective of the Cu(II) concentrations of 5 mg/L (Figs. 1, 2A and 2C) and 20 mg/L
(Figs. 1, 2B and 2D). The background circuital current, voltage output, power
production and electrode potential in the absence of Cu(II) (Figs. 1B, 2A and 2C, and
Fig. S1) were attributed to the reduction of residual dissolved oxygen [15,43].

Higher circuital currents and rates of Cu(II) reduction were observed by
increasing the concentration of Cu(II) in the cathodic chamber from 5 mg/L to 20
mg/L (Fig. 1). This effect was more significant for JY3 and JY6, demonstrating the
significant impact of circuital current on the rate of Cu(II) reduction for these two
species. Conversely, the circuital currents observed with JY1 and JY5 were
insignificant (Fig. 1B), in relation to the significant increase observed in the rate of
Cu(II) reduction (Fig. 1A). The reduction of Cu(II) can therefore be ascribed to the
effect of the abiotic cathodes only and to the effect of the individual JY1 or JY5
bacteria in response to the increase in Cu(II) concentration (Fig. 1A). These results
illustrate the weak correlation between circuital current and the rate of Cu(II)
reduction for both JY1 and JY5, a result that will be further supported by the XPS
The majority of the EAB cathodes displayed significant overshoots in voltage output and power density in comparison with the abiotic controls, at a Cu(II) concentration of 20 mg/L (Fig. 2B and D). This suggests that the demand for electrons in these experiments exceeded the rate of electrons supplied by microbial activity, and this resulted in the depletion of electrons and ions in the catholyte. Such observations have also been reported in other MFC studies using O₂ as electron acceptor [45-47]. The cathode with bacterial JY3 displayed the highest performance with the highest circuitual current (Fig. 1B) and most importantly without displaying an overshoot in power density, indicating that the microbial activity with this species was significant.

The cathodic potentials of all EAB were found to vary much more than the anodic potentials, over the current density range investigated (Fig. S2A, initial Cu(II) of 5 mg/L; Fig. S2B, initial Cu(II) of 20 mg/L). In consequence, the performance of the MFCs was controlled by the reduction of copper at the biocathode, rather than the microbial phenomena that occurred at the anode. The presence of the EAB at the cathode, therefore, had a significant impact on the propensity of the cathodes for electricity generation and voltage output.

Increasing the concentration of Cu(II) in the catholyte diverted a larger fraction of electrons from the anodic oxidation of organics towards the reduction of Cu(II), with JY6 showing the highest value among the different EAB (11.2 ± 0.1% from a total of 2.9 C and at a Cu(II) of 20 mg/L). The remaining 0.4 ± 0.0% was used for oxygen reduction, 52.3 ± 0.0% for organics production, and 33.5 ± 0.0 for biomass growth (Table S2). A large fraction of the cathodic electrons from 2.6 ± 0.1% for JY6 up to 43.2 ± 0.4 for JY3 were involved in other parallel reactions or unknown processes.

The abiotic cathodes displayed reductive peak potentials of 0.033 V at a Cu(II) of 5 mg/L (Fig. S3A and Table S3) and 0.037 V at 20 mg/L (Fig. S3B and Table S3), reflecting the positive shift of reductive peak potential at a higher Cu(II) concentration. Reductive onset potentials for all EAB cathodes were more positive than the abiotic controls (0.272 – 0.297 V vs. 0.264 V at a Cu(II) of 5 mg/L and 0.278 – 0.328 V vs. 0.267 V at a Cu(II) of 20 mg/L) (Table S3) and the shift was more significant for the reductive peak potentials of all EAB cathodes with respect to the abiotic controls (0.040 – 0.063 V vs. 0.033 V at a Cu(II) of 5 mg/L, 0.043 – 0.079 V vs. 0.037 V at a Cu(II) of 20 mg/L). These results, in combination, demonstrate the varying degree of influence exerted by each EAB on the catalytic activity toward Cu(II) reduction, which is consistent with the effect of EAB on the reduction of Co(II) and chloramphenicol on mixed culture cathodes [43,48]. The JY1 and JY5 bacteria exhibited much lower reductive onset potentials than JY3 and JY6, implying a weaker interaction between cathodic electrons and the reduction of Cu(II) through the mediation of these two species.

The reductive peak currents, at a Cu(II) concentration of 5 mg/L, observed on the cathodes covered by the EAB were lower than values registered with the abiotic cathode (0.973 mA) with the exception of JY6 (Table S3), suggesting some degree of mass transfer inhibition for Cu(II) due to the contact between the EAB and the
electrode surface [47]. At a higher concentration of Cu(II) (20 mg/L), the mass
transfer inhibition became less significant, as expected. Similar observations have
been reported for the reduction of other electron acceptors such as Cr(VI) and Co(II)
in mixed culture cathodes [41,43].

The lack of a significant difference observed between CVs of the abiotic controls
and the biotic cathodes in the absence of Cu(II) (Fig. S4), in concert, reflected the
importance of Cu(II) ions on the occurrence of reduction reactions on the EAB,
despite the expected variability due to the redox species present on the surfaces of
these EAB cells (Insets in Fig. S4).

3.3 Electrode morphology and product analysis

SEM examination showed that the EAB covered the surface of the cathodes only
sparsely (Fig. S5A, D, G, and J). EDS analysis confirmed the presence ofCu
precipitates on the surfaces of EAB cells (Fig. S5B, E, H and K) while no Cu, or very
little Cu precipitate, was observed on the bare surface of the electrodes (Fig. S5C, F, I
and L). EDS detection of carbon, oxygen, sodium and phosphorus was attributed to
the cellular components of EAB on the electrodes, while gold was associated with the
sample pretreatment.

XPS analyses (Fig. S6) indicated that the whole Cu2p region comprises 2p1/2
and 2p3/2 peaks, while only the Cu2p3/2 peaks could be used for the assignment of
copper chemical states [49]. The abiotic control reported the exclusive presence of
Cu(0) (Fig. S6A), shown with its characteristic peak at the Cu2p3/2 region of 932.4
eV [49], while under OCC only adsorbed Cu(II) was observed (Fig. S6B), as expected.
This result clearly demonstrates the importance of the effect of circuital current on the
reduction of Cu(II) to Cu(0) with the abiotic cathodes.

Here Fig. 3

The XPS analysis on the EAB cathodes in the presence of a circuital current (Fig.
3), reported the exclusive presence of Cu(0), while under OCCs, both Cu(0) and Cu(II)
were observed for JY1 (Fig. 4A) and JY5 (Fig. 4C), and only Cu(II) for both JY3 (Fig.
4B) and JY6 (Fig. 4D). JY5 had a net Cu(0) production of 1.81 ± 0.11 mg and JY1
exhibited a 1.45 ± 0.05 mg, both of which were nearly equivalent to the sum of 1.17 ±
0.07 mg in the abiotic controls and 0.70 ± 0.09 mg (JY5) or 0.44 ± 0.03 mg (JY1) in
the absence of a circuital current (Table S4). These results in concert confirm the
weak correlation between circuital current and the rate of Cu(II) reduction for both
JY1 and JY5, which is consistent with the results shown in Fig. 1. The observation of
Cu(0) in JY1 in the absence of a circuital current is consistent with previous studies,
where S. maltophilia converted Cu(II) into Cu(0) under facultative conditions [22-24].

Here Fig. 4

3.4 Effect of electron transfer inhibitors (rotenone and DNP)

Here Fig. 5
In the presence of either rotenone or DNP, the rate of Cu(II) reduction (Fig. 5A) or the circuital current (Fig. 5B) registered in the MFCs with JY1 or JY5 EAB, changed little, suggesting a weak correlation between the cellular electron transfer processes and either Cu(II) reduction or the circuital current. Conversely, the cathode with the JY6 species, returned an appreciable decrease in both the rate of Cu(II) reduction and circuital current, suggesting a strong interaction between the cellular electron transfer processes in JY6 and either Cu(II) reduction or circuital current. This strong interaction observed with JY6 rather than with JY1, JY3 and JY5, was also supported by the apparent decrease in cathodic electrons used for Cu(II) reduction in the former, in the presence of rotenone or DNP, while little change in was observed with the other three EAB (Table S2). The negative effects of rotenone or DNP electron transfer inhibitors on JY6 was also reflected by a decrease in the voltage output (Fig. S7A and B), maximum power density (Fig. S7C and D) and cathode potential (Fig. S7E and F), in addition to the negative shifts observed in reductive onset potential, reductive peak potential and reductive peak current (Table S3; Fig. S8). In contrast, little change on the above characterization parameters was observed for JY1, JY3 and JY5. These results further support the weak dependence between the cellular electron transfer processes and either Cu(II) reduction or circuital current for JY1, JY3 and JY5, and confirm the significant correlation observed between the cellular electron transfer processes and either Cu(II) reduction or circuital current for JY6. The findings of this study are summarized graphically in Fig. 6.

**4 Conclusions**

Metal reduction in MFCs is known to be dependent upon the availability of cathodic electrons and upon the composition of bacterial communities on the biocathodes [11,15,40]. On the basis of the results obtained with indigenous EAB JY1, JY3, JY5 and JY6 isolated from well adapted mixed cultures grown on the surface of MFC cathodes used for Cu(II) reduction, this study demonstrates the close correlation among circuital current, the rate of Cu(II) reduction and the cellular electron transfer processes for JY6, and the weak dependence of these for JY1 and JY5 (Fig. 6). We therefore suggest the possibility of a mechanism involving direct electron transfer from the surfaces of the cathodes to JY6, followed by the subsequent cellular electron transfer to Cu(II) for its reduction. This mechanism is not reflected in JY3, which only exhibited a close correlation between circuital current and the rate of Cu(II) reduction (Fig. 6). In summary, this study provides an evidence of the diverse functions played by these EAB species in the mixed cultures and the corresponding effect on MFC characterization parameters. The results in this study add to a deeper understanding of the capabilities exerted by diverse EAB associated with Cu(II) reduction, and provide new insights into the potential application of metallurgical biocathode MFCs for Cu(II) recovery at industrial scale.

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Figure captions

**Fig. 1** Comparison of Cu(II) removal rate (A) and circuitual current (B) with various EAB at an initial Cu(II) of either 5 mg/L or 20 mg/L.

**Fig. 2** Voltage output (A and B), power density (C and D) as a function of current density with various EAB at an initial Cu(II) of 5 mg/L (A and C) or 20 mg/L (B and D).

**Fig. 3** XPS spectra of Cu precipitates on the cathodes catalyzed by EAB of JY1 (A), JY3 (B), JY5 (C) or JY6 (D) (initial Cu(II): 20 mg/L; 20 batch cycles).

**Fig. 4** XPS spectra of Cu precipitates on the cathodes catalyzed by EAB of JY1 (A), JY3 (B), JY5 (C) or JY6 (D) in the absence of circuitual current (initial Cu(II): 20 mg/L; 20 batch cycles).

**Fig. 5** Comparison of Cu(II) removal rate (A) and circuitual current (B) in response to the inhibitor of rotenone or DNP (initial Cu(II): 20 mg/L).

**Fig. 6** Summary of correlation between circuitual current, Cu(II) reduction and cellular electron transfer in EAB of JY1, JY3, JY5 and JY6.
Figure 1
Figure 2
Figure 3
Figure 4

[Bar chart showing the Cu(II) removal rate (mg L$^{-1}$ hr$^{-1}$) with different treatments: OCCs, DNP-OCCs, biotic-CCCks, rotenone, and DNP.]

Figure 5

[Bar chart showing the circuit current (mA) with different treatments: JY1, JY3, JY5, JY6, and abiotic.]

Figure 6