



Short communication

Interspecific competition by non-exoelectrogenic *Citrobacter freundii* An1 boosts bioelectricity generation of exoelectrogenic *Shewanella oneidensis* MR-1

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ABSTRACT

The performance of bioelectrochemical systems (BESs) is significantly influenced by metabolic interactions within a particular microbial community. Although some studies show that interspecific metabolic cooperation benefits BESs performance, the effect of interspecific substrate competition on BESs performance has not yet been discussed. Herein, the impact of interspecific competition is investigated by monitoring the extracellular electron transfer of exoelectrogenic *Shewanella oneidensis* MR-1 and non-exoelectrogenic *Citrobacter freundii* An1 alone and simultaneously. The bacterial consortia generate the highest current of 38.4 $\mu\text{A cm}^{-2}$, 6 times of that produced by the single strain *S. oneidensis* MR-1. Though *S. oneidensis* MR-1 loses out to *C. freundii* An1 in solution, the competition enhances the metabolic activity of *S. oneidensis* MR-1 on electrode, which facilitates the biofilm formation and therefore helps *S. oneidensis* MR-1 to gain a competitive advantage over *C. freundii* An1. Increased metabolic activity triggers more electrons generation and flavin secretion of *S. oneidensis* MR-1 which contributes to its excellent exoelectrogenic capacity. The proteomics analysis confirms that the expression of proteins related to lactate metabolism, biofilm formation, and outer membrane c-type cytochromes are significantly upregulated in *S. oneidensis* MR-1 from bacterial consortia.

1. Introduction

Bioelectrochemical systems (BESs) are electrochemical bioreactors employing microorganisms to simultaneously degrade organic matter and recover electricity, hydrogen, or high-value products from wastewater (Cheng and Logan, 2007; Liu et al., 2005; Nazari et al., 2020; Rabaey and Rozendal, 2010; Xiao et al., 2016). In BESs research, highly efficient electricity output is always one of the most important pursuits. Electricity generation and pollutant degradation is positively correlated with microbial activity. Because no single species has all the enzymes required for complete degradation of complex pollutants, metabolic cooperation between different species is essential for the mineralization

of complex pollutants. BESs with microbial consortia commonly produced higher electricity and disposed pollutants much more effectively than that with pure microbial species (Ishii et al., 2008).

As microbial consortia are intertwined by metabolic links (Ponomarova and Patil, 2015), a key challenge with BESs therefore is to understand the metabolic interactions within microbial consortia. Interspecific cooperation (such as mutualism, syntrophism, and commensalism) and competition are the most important microbial interactions affecting the performance of BESs communities. Previous studies mainly focused on metabolic cooperation and even proposed synthetic biology for the rational design of microbial consortia for energy and chemicals production (Brenner et al., 2008; Guzman et al.,

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2019; Jia et al., 2016; Lindemann et al., 2016; Liu et al., 2017; Minty et al., 2013). In fact, the effect of interspecific competition on the performance of BESs has barely investigated.

Interspecific competition for substrate is traditionally considered to restrain a system's performance (Conklin et al., 2006). While microbes inevitably have to scramble for limited substrate in a complex BES community, understanding the effect of interspecific substrate competition on the performance of BESs helps us manipulate the microbial consortia. Noticing that strains from genus *Shewanella* and species *Citrobacter freundii* have often been observed in the same bioreactor for dissimilatory reduction of Fe(III) (Sikora et al., 2011), we constructed a synthetic consortium in BESs with two bacterial strains, *S. oneidensis* MR-1 and *C. freundii* An1, both utilizing lactate as carbon source but not their metabolite of acetate (Fig. 1 A and S1), which built a model for investigating interspecific substrate competition. BESs inoculated with single *S. oneidensis* MR-1 or *C. freundii* An1 strains served as control.

2. Material and methods

2.1. Strains and culture medium

Strain *C. freundii* An1 was isolated from a microbial fuel cell and classified to species *C. freundii* by 16S rRNA gene sequencing (GenBank accession number of MW740336) (Fig. S2). *S. oneidensis* MR-1 and *C. freundii* An1 were cultured aerobically at 30 °C in medium which is a 50 mM phosphate buffer (pH 7.00) containing (g·L⁻¹) 0.560 of DL-lactate, 0.500 of tryptone and 0.250 of yeast extract.

2.2. Construction and operation of BESs

Three-electrode system was served as BES to evaluate the electricity generation and the lactate utilization by single strain and two-strain consortium. Each three-electrode system contained 3 working electrodes sharing the same counter and reference electrodes (Fig. S3). Carbon felt of 2 cm × 2.5 cm, graphite plate of 2.5 cm × 5 cm, and Ag/AgCl (KCl saturated) were used as working, counter, and reference electrode, respectively. The effective volume of each electrolytic cell was 76 mL, and about 70 mL sterilized culture medium was served as electrolyte and culture medium.

For each operation circle, three groups of three-electrode system were operated, inoculating with *C. freundii* An1 (1%, v/v, designated as An1), *S. oneidensis* MR-1 (1%, v/v, designated as MR-1), and both *C. freundii* An1 (0.5%, v/v) and *S. oneidensis* MR-1 (0.5%, v/v) (designated as Mix), respectively. The cultivation temperature was 28 ± 2 °C, and the potential of the working electrode was set at +0.2 V (vs. Ag/AgCl) by a CHI1000C potentiostat (CHI Inc., Shanghai, China). An1, MR-1, and Mix represent the BESs inoculated with strain An1, strain MR-1, and both strains An1 and MR-1, respectively.

2.3. Bacterial community analysis

Electrolyte samples were extracted from the three-electrode systems with *C. freundii* An1 and *S. oneidensis* MR-1 consortia, and suspending cells were collected by centrifugation for DNA extraction. The 16S rRNA gene sequencing was performed on Illumina platform by Biomarker Technologies Corporation (Beijing, China).

2.4. Electrochemical measurements

After being cultured aerobically at 30 °C for 18 h and sequentially washed twice with 50 mM phosphate buffer (pH 7.0), strain *C. freundii* An1 (Fig. S4) was subjected to electrochemical measurement of differential pulse voltammetry (Xiao et al., 2017). Glassy carbon, platinum wire and Ag/AgCl (KCl saturated) were used as working, counter, and reference electrodes, respectively.

2.5. Assay of protein concentration

The protein concentration of cell extracts was measured using a commercial kit, and biomass was represented by protein concentration. Culture medium or electrolyte of 1 mL in three-electrode systems was collected, then centrifuged at 13000 g for 5 min. The centrifuged bacteria were stored at -20 °C for more than 3 h. Adding 100 µL 0.1 M NaOH and then heating 90 °C for 15 min in a water bath when the sample is kept at room temperature. After centrifuged at 8000 g for 20 min, the supernatant was quantified with the BCA Protein Assay Kit (BOSTER, Wuhan, China).

2.6. Analysis of volatile fatty acids

Culture medium of 2 mL in three-electrode systems was collected. The liquid samples were centrifuged at 8000 g for 5 min, then filtered through 0.22 µm filter and acidified to pH lower than 2 prior to volatile fatty acids analysis. The composition and concentration of volatile fatty acids were detected by ion chromatography (ICS-3000, Dionex, USA) equipped with an anion exchange column (Dionex IonPac™ AS11-HC, 4 mm × 250 mm).

2.7. Analysis of flavins

Flavins, i.e., riboflavin, flavin adenine dinucleotide and flavin mononucleotide, are important electron shuttles for bioelectricity generation. Culture medium or electrolyte of 2 mL in three-electrode systems was collected, and the concentration was determined by high performance liquid chromatography following our previous report (You et al., 2018).

2.8. Label-free quantitative proteomic analysis

To examine the variations of protein level in the An1, Mix and MR-1, the greatest current difference of electrode microorganisms on the third day were selected as proteomics samples. Three biological replicates were performed separately. The proteomics samples were collected and performed on Label-free Quantitative Proteomic Analysis by Shanghai Applied Protein Technology Co. Ltd. (Shanghai, China).

The extraction and digestion of proteins were based on the published method (Gang et al., 2019). Each sample was injected for nanoLC-MS/MS analysis. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 120 min. The MS data were analyzed using MaxQuant software version 1.5.3.17 (Max Planck Institute of Biochemistry in Martinsried, Germany), and then the protein expression difference was analyzed by bioinformatics analysis.

3. Results and discussion

3.1. Bioelectricity generation

The bioelectricity generation is shown in Fig. 1B. The BESs inoculated with *S. oneidensis* MR-1 (designated as BESs-MR-1) produced peak current density of 6.4 µA cm⁻² at 48–72 h, which was consistent with previous reports (Bretschger et al., 2007; Luo et al., 2016; Wu et al., 2013). The BESs inoculated by single strain of *C. freundii* An1 (designated as BESs-An1) produced negligible electricity with peak current density below 0.2 µA cm⁻².

The electricity generation were boosted in BESs inoculated by both strains of *C. freundii* An1 and *S. oneidensis* MR-1 (designated as BESs-Mix) with the highest peak current density of about 38.4 µA cm⁻² at approximately 72 h (Fig. 1B), which was 6 times of that in BESs-MR-1. Furthermore, BESs-Mix collected an average electric quantity of 9.09 C cm⁻² whereas BESs-An1 and MR-1 collected electric quantity of 0.05

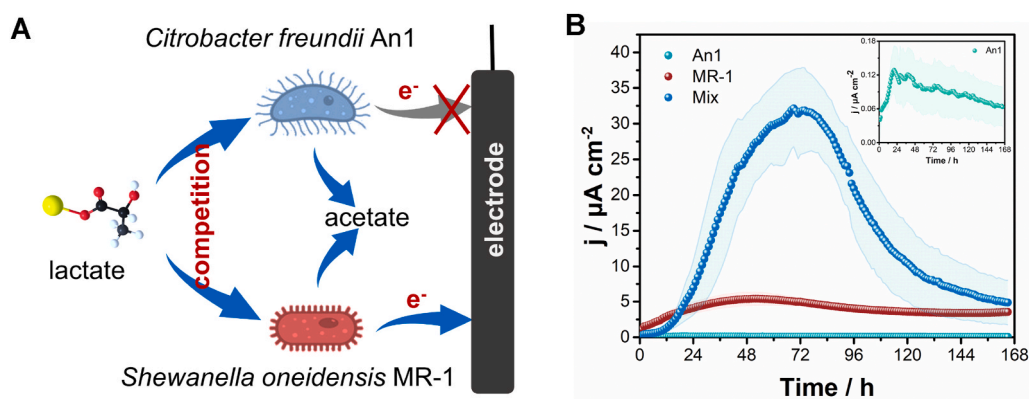


Fig. 1. (A) *S. oneidensis* MR-1 and *C. freundii* An1 strains were used to investigate the effects of substrate competition for lactate on EET. (B) current generation from the working electrodes of BESs inoculated with different bacteria. Inset shows the current production with strain An1 only.

and 2.41 C cm^{-2} , respectively.

Initially, we anticipated a smaller current from BESs-Mix compared to that from BESs-MR-1 as *C. freundii* An1 in BESs-Mix partly occupied the surface area of the working electrode and consumed part of the substrate and barely produced current. Moreover, the main metabolite of *C. freundii* An1 is acetate which does not support the growth of *S. oneidensis* MR-1 (Fig. S1), which may further decrease the extracellular electron transfer (EET) in BESs-Mix. To our surprise, the current generation of BESs-Mix was 6 times of that of BESs-MR-1. Hence, we decided to analyze the data step by step to discover the reasons behind these interesting results.

3.2. Impacts on the formation of electrode biofilm

Biomass is one of the most important factors affecting the bacterial performance, and protein quantity measurement is usually used to determine the biomass of bacteria (Wu et al., 2013). We quantified the protein on electrode (Fig. 2A) and in supernatant (Fig. S5) from different BESs at 24, 72, and 168 h which represented the rising, peak, and descending phase of electricity production (Fig. 1B and Fig. S6), respectively. The results showed that the biofilm in BESs-An1 and MR-1 could be stabilized with protein quantity of approximately 0.50 mg in 72 and 24 h, respectively. Proteins from the biofilm in BESs-Mix kept increasing from approximately 0.25 to 0.97 and then to 2.02 mg during the 168-h operation, indicating that the interspecific competition

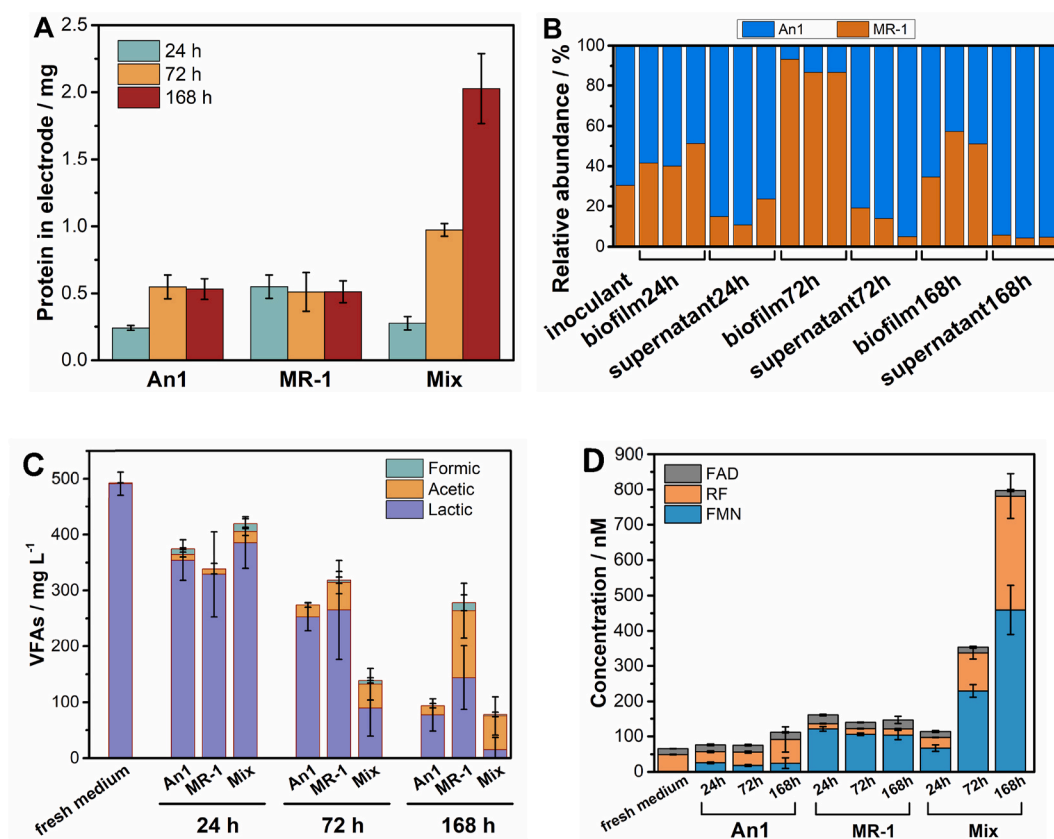


Fig. 2. (A) Protein-based biomass of the biofilm. (B) corresponding community of bacterial consortia in BESs-Mix as revealed by high-throughput sequencing of PCR amplicons of the 16S rRNA gene. (C) lactate consumption and its primary metabolites. (D) flavins in the supernatant at time 24, 72, and 168 h. RF, riboflavin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

enhanced biofilm formation on electrode.

We employed high-throughput sequencing to detect the diversity of bacterial consortia in BESs-Mix (Fig. 2B). While the abundance of *S. oneidensis* in inoculant was approximately 30%, it decreased to less than 20% at 24 and 72 h and even about 5% at 168 h in the supernatant. In contrast, the abundance of *S. oneidensis* in biofilm was approximately 45%, 90%, and 50% at 24, 72, and 168 h, respectively, meaning that forming biofilm on electrode could help the extracellular respiratory bacterium of *S. oneidensis* MR-1 to gain an advantage over *C. freundii* An1 in substrate competition.

The images of the electrode biofilm at 72 and 168 h obtained by scanning electron microscope showed more bacterial cells on the electrode from BESs-Mix than that from BESs-An1 and MR-1 (Fig. S7). These results further indicated that substrate competition was conducive to biofilm formation of MR-1 on electrode and sequentially enhanced the its EET and electricity generation in BESs-Mix.

3.3. Substrate consumption

We detected the consumption of substrate, i.e., lactate and its primary metabolites of acetic and formic in BESs (Fig. 2C). Lactate in BESs-MR-1 was consumed faster than that in the other BESs in the first 24 h, which was consistent with their highest protein quantity, ATP quantity, and electrical current among the three groups of BESs. Lactate of approximately 250 mg L⁻¹ was consumed in BESs-An1 and MR-1 at 72 h, whereas approximately 400 mg L⁻¹ was consumed in BESs-Mix. Because the relative abundance of *S. oneidensis* MR-1 in the electrode biofilm of BESs-Mix at 72 h was approximately 90% and the least lactate (approximately 100 mg L⁻¹) was consumed at the first 24 h, this result meant that substrate competition in BESs-Mix have significantly stimulated the metabolism of lactate in *S. oneidensis* MR-1 during the 24 h to 72-h periods. This result was also consistent with high ATP concentration in electrode biofilm, the highest protein quantity, and current generation at 72 h in BESs-Mix. At the end of the 168 h-operation, only lactate of approximately 15 mg L⁻¹ remained in BESs-Mix, whereas lactate of 360 mg L⁻¹ and 420 mg L⁻¹ was consumed in BESs-MR-1 and An1, respectively. It indicated that substrate competition in BESs-Mix forced both strains to utilize lactate as much as possible to survive.

Both strains yielded acetic and formic metabolites from lactate, and *C. freundii* An1 seemed to accumulate less acetic metabolite than *S. oneidensis* MR-1. Therefore, less acetic metabolite was accumulated in BESs-Mix compared to that of BESs-MR-1 at 168 h, which might be beneficial to *S. oneidensis* MR-1 for keeping a high activity of lactate metabolism. However, the concentration of either acetic or formic metabolite in BESs-Mix at 72 h was similar to that in BESs-MR-1 due to high abundance of *S. oneidensis* MR-1 in biofilm from BESs-Mix at 72 h, meaning that metabolites had little effect on the high electricity generation in BESs-Mix at 72 h.

3.4. Flavins secretion increased in microbial consortia

Flavins, including riboflavin, flavin adenine dinucleotide, and flavin mononucleotide, are recognized electron mediators for indirect EET production (Marsili et al., 2008; Zhang et al., 2020). Therefore, we measured the flavins at three time points of 24, 72, and 168 h (Fig. 2D) and found that the total flavin concentration was positively correlated with the protein-based biomass. Flavin concentrations in BESs-An1 and MR-1 were maintained at approximately 80 and 150 nM, respectively, during the 168-h operation, which is consistent with previous study (Delgado et al., 2019). BESs-MR-1 and An1 had similar biomass and ATP quantity in the electrode biofilm and similar flavin concentration in the supernatant at 72 h; however, BESs-MR-1 produced current 30 times higher than that of BESs-An1 (Fig. 1B). The results once again indicated that *C. freundii* An1 did not generate electricity in BESs.

Flavin concentration in BESs-Mix increased continuously from 110 nM at 24 h to 350 nM at 72 h, which was 0.7 and 2.5 times that in BESs-

MR-1 at the same time point, respectively. The increased concentration of flavins in BESs-Mix initially enhanced the indirect EET of *S. oneidensis* MR-1 in the electrode biofilm. Secondly, high flavin concentration in BESs-Mix at 72 h increased the concentration of hopping sites, thereby facilitating the EET via extracellular polymeric substances in biofilm (Xiao et al., 2017). Although flavin concentration was increased to 800 nM at 168 h in BESs-Mix, approximately two times that at 72 h, the current was reduced to one quarter of that at 72 h, which could be ascribed to the low concentration of lactate in the cells.

3.5. Expression of EET related proteins

c-Type cytochromes are primary transfers on cell outer membrane for transporting intracellular electrons outside to the electrode directly (Jing et al., 2020; Mitchell et al., 2012; Shi et al., 2016), to flavins (Okamoto et al., 2013), or to the hopping sites in extracellular polymeric substances in *S. oneidensis* MR-1 (Xiao et al., 2017). Therefore, we employed label-free proteomics to quantify and analyze the proteins expression of strains in the electrode biofilm at 72 h. Compared with *S. oneidensis* MR-1 in BESs-MR-1, the expression of 60 proteins expression was upregulated in *S. oneidensis* MR-1 from BESs-Mix whereas only the expression of 14 proteins was downregulated (Fig. S10). The result indicated that compared with those in BESs-MR-1, *S. oneidensis* in BESs-Mix expressed more proteins to compete with the other species of *C. freundii* An1.

We further observed that the primary upregulated proteins were mainly involved in transportation and metabolism of lactate, outer membrane cytochrome *c*, and biofilm formation (Fig. 3 and Table S2). For example, the upregulated protein of flagellum hook-associated protein 2 could facilitate the growth of flagella (Ikeda et al., 1993) which was beneficial to *S. oneidensis* MR-1 in acquiring nutrients and forming biofilm on the electrode (Qin et al., 2011). The result also accorded with our recent study where flagella-related proteins were upregulated in *S. oneidensis* MR-1 showing greater ability for reducing Cr (VI) (Gang et al., 2019). Furthermore, the upregulated proteins, e.g., L-lactate permease and dehydrogenase LldE, could facilitate the lactate uptake and metabolism, which was consistent with faster lactate consumption in BESs-Mix than that in BESs-MR-1. The most important thing was that, compared with *S. oneidensis* MR-1 in BESs-MR-1 at 72 h, the expression of outer membrane *c*-type cytochromes of OmcA, MtrB, and MtrC were 3.34–4.04 times upregulated in *S. oneidensis* MR-1 from BESs-Mix. The upregulated expression of *c*-type cytochromes directly facilitated *S. oneidensis* MR-1 to timely release electrons to the electrode, i.e. the EET, from fast lactate metabolism in BESs-Mix, which helped the strain to dominate the electrode biofilm and enhance the electricity generation.

Interspecific competition was traditionally thought to reduce the performance of engineering systems (Conklin et al., 2006). However, the present study showed an EET promotion and more efficient substrate consumption. Under the competition pressure from *C. freundii* An1, *S. oneidensis* MR-1 globally enhanced its intracellular lactate metabolism and the expression of outer membrane *c*-type cytochromes, then the enhanced metabolism of MR-1 increased the biomass in biofilm and the flavins in supernatant. The findings help us to understand the mechanism of the operation of complex communities and provide us with a new approach to manipulate microbial consortia in many engineering systems aiming for energy production and pollutants disposal.

4. Conclusions

In this work, the substrate competition between *S. oneidensis* MR-1 and *C. freundii* An1 boosts the electricity generation of BES-Mix, 6 times of that in BES-MR-1. The results showed that interspecific substrate competition promoted *S. oneidensis* MR-1 to form biofilm on electrode to gain a competitive advantage over *C. freundii* An1. Increased metabolic activity not only provides *S. oneidensis* MR-1 more

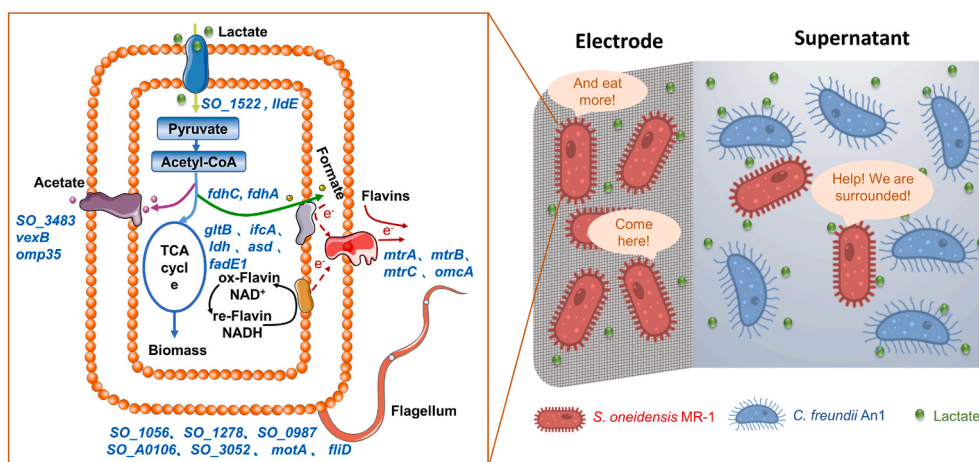


Fig. 3. Competition with *C. freundii* An1 for substrate forced *S. oneidensis* MR-1 to dominate in biofilm. The upregulated proteins were showed in blue font in the left half. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

electrons for EET but also yields more flavins facilitating the EET. This work provides new insights into the effects of interspecific competition on EET and perfects the impact of interspecific metabolic interaction on EET, which is conducive to manipulation of microbes for environmental applications. We will pay attention to whether such competitive promotion mechanism is prevalent in the interspecific competition of microorganisms. Moreover, how to use such competitive mechanism to control the EET of BES in practical engineering is also one of the focuses of our future work.

CRedit authorship contribution statement

Yong Xiao: Conceptualization, Methodology, Investigation, Visualization, Supervision, Writing – original draft, Writing – review & editing. **Geng Chen:** Methodology, Investigation, Visualization, Writing – review & editing. **Zheng Chen:** Investigation, Writing – review & editing. **Rui Bai:** Investigation, Writing – review & editing. **Biyi Zhao:** Investigation, Writing – review & editing. **Xiaochun Tian:** Investigation. **Yicheng Wu:** Investigation. **Xiao Zhou:** Investigation. **Feng Zhao:** Supervision, Writing – review & editing.

Declaration of competing interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2021.113614>.

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