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Extracellular Electron Transfer Mediated by Flavins in Gram-positive *Bacillus* sp. WS-XY1 and Yeast *Pichia stipitis*

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ABSTRACT

Extracellular electron transfer (EET) of microorganisms represents a communicative bridge between the interior and exterior of the cells. Most prior EET studies have focused on Gram-negative bacteria. However, fungi and Gram-positive bacteria, that contain dense cellular walls, have rarely been reported. Herein, two model dense cell wall microorganisms (*Bacillus* sp. WS-XY1 and the yeast *Pichia stipitis*) were identified to be electrochemically active. Further analysis indicated that the two microorganisms were able to secrete flavins to mediate their EET. The discovery, that dense cell wall containing microorganisms can undertake mediated EET, adds to the body of knowledge towards building a comprehensive understanding of biogeochemical and bioelectrical processes.

1. Introduction

Microbial extracellular electron transfer (EET) is a critical process occurring in the metal and carbon cycles of biogeochemical and bioenergy systems [1]. Most prior reports related to EET have focused on Gram-negative bacteria *e.g. Shewanella* sp. and *Geobacter* sp., whereas few Gram-positive bacteria and fungi have been reported to have EET capability [2]. Gram-positive bacteria and fungi are widespread in a broad range of aquatic and soil environments. They have been found to participate in extracellular reduction/oxidation of organic pollutants and insoluble minerals [3,4]. Compared to Gram-negative bacteria, Gram-positive bacteria and fungi are enveloped by thicker cell walls, which can increase the stress resistance of the cells by regulating their architecture and biophysical properties [5,6]. For example, Gram-positive bacteria have applications in high temperature and extreme pH environments [7–10]; whereas, studies into the EET mechanism that operates with such microorganisms have rarely reported in detail [11].

Flavins, a group of redox-active compounds, are found in most microorganisms [12]. The flavins present in *Shewanella oneidensis* account for 75% of the EET capacity [13]. Reduced flavins are able to dissolute the minerals goethite and hematite, which is useful for biogeochemical cycling of iron [14]. In addition, flavins can enhance hexavalent chromium reduction [15] and azo dye decolorization [16] in bioelectrochemical systems. Although flavins are secreted by many microorganisms, previous research concerning flavins mediated EET focused only on Gram-negative *Shewanella* spp. The mechanism of EET mediation with microorganisms that have dense cell walls has not been reported.

In this study, a yeast and an isolated Gram-positive bacterium were selected, as a model fungus and prokaryote respectively, to investigate their EET mechanism. Although both of these model microorganisms are enveloped by dense cell walls, they were identified to be electrochemically active and to mediate EET via self-secreted flavins. These results demonstrate that flavins are ubiquitous mediators for microorganism EET and will play an important role in the application of microorganisms in geochemical mineral cycling and bioelectrochemical systems.

2. Experimental

2.1. Bacteria isolation and identification

We constructed an MFC with a micro-aerobic anode to explore the functional bacteria in it. The micro-aerobic anode was fed

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with sodium acetate and showed stable operation for more than one year. Bacteria were isolated from it using traditional aerobic screening technology (rather than the more commonly applied anaerobic screening technologies). Hence, an O_2-tolerant *Bacillus* strain WS-XY1 was isolated. The dilution-to-extinction method was used and a single colony was isolated from a solid LB agar with a composition of: yeast extract (5 g L^{-1}, Sangon, China), NaCl (5 g L^{-1}), peptone (10 g L^{-1}, Sangon), and agar (20 g L^{-1}). Alternating liquid incubation and plate streaking procedures were repeated three times to obtain a pure isolate. 16S rRNA gene sequencing and phylogenetic analysis of the pure isolate were performed according to the method conducted by Yang [17]. Gram reaction was determined using a crystal violet and safranin stain. The stained cells were observed using a microscope (IX71, Olympus, Japan).

2.2. Microbial cultivation

Flavins are reported as components of yeast extract and mediate electron transfer between an electrode and bacteria [18]. We prepared two yeast-extract-free media in the following microbial cultivation experiments to exclude the interferences from exogenous flavins. LB(Y-) medium consisting of peptone (10 g L^{-1}) and NaCl (5 g L^{-1}) was used to cultivate the *Bacillus* sp. WS-XY1. YPD(Y-) medium consisting of peptone (20 g L^{-1}) and glucose (20 g L^{-1}) was used to cultivate the *Pichia stipitis*. Subsequent HPLC experiments showed that no flavins were detected in these media (before cultivation of the microorganisms). The microorganisms were incubated in bed temperature incubator (32 °C, 150 rpm).

2.3. Electrochemical measurements

A glassy carbon disk (∅ = 3 mm), a platinum wire, and a saturated Ag/AgCl (3 mol L^{-1} KCl) were used as the working electrode (WE), counter electrode and reference electrode (RE), respectively. All the potentials presented below are *vs.* the saturated Ag/AgCl. The electrolyte used was aqueous phosphate buffer solution (PBS, 50 mmol L^{-1}, pH = 7). Stationary stage cells were harvested and then washed three times with the PBS prior to conducting electrochemical measurements. To clarify if the redox peaks originated from the reaction of compounds in the supernatant, the culture supernatant was collected for subsequent differential pulse voltammetry (DPV). As a control experiment, pure riboflavin (Sinopharm, China) was dissolved in the PBS and studied using DPV. The cyclic voltammetry (CV) scan rate used was 10 mV s^{-1}. For DPV studying the oxidation reactions, the staircase was 0.004 V, the initial potential was -0.60 V, and the final potential was -0.20 V.

Chronoamperometry measurements was used to prove that the flavins are directly linked to the microbial metabolism [19]. In the three-electrode system, carbon felts were used as working and counter electrodes (surface area of 9 cm^2), saturated Ag/AgCl was used as RE. The three-electrode system was placed in a single chamber reactor. The reactor was sealed by rubber seal to prevent oxygen into the chamber. During the anaerobic testing, the WE was held at +0.30 V for WS-XY1 and +0.40 V for *P. stipitis*. In the control experiment, only glucose was added (to a final concentration of 10 mmol L^{-1} when testing WS-XY1 and 50 mmol L^{-1} for *P. stipitis*). Experiments where both glucose and riboflavin (to a final concentration of 100 μmol L^{-1}) were added were used to prove that the flavins can mediate between the respiratory metabolism of these microbes and the anode.

2.4. HPLC measurements

Riboflavin is the precursor of flavin coenzymes (*i.e.* flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD)) [20]. It can be secreted out of microbial cells [21,22]. Therefore, riboflavin was selected for the following analysis. Culture supernatant was collected and filtered through membrane filter of 0.22 μm for HPLC analysis (Agilent, USA) [23].

3. Results and Discussion

3.1. Gram-positive Bacillus *sp. WS-XY1 and Yeast* Pichia stipitis

The presence of a certain amount of oxygen in anode chamber was reported to enhance the performance of microbial fuel cells [24,25]. In this work, we isolated functional microbes from microaerobic anode chamber to elucidate their electron transfer mechanism. 16S rRNA gene sequencing indicated that the isolate phylogenetically belongs to the *Bacillus* genus (Fig. 1), which has been extensively studied as the important prokaryote. It showed 100% identity with 3 strains of *B. cereus* (LH1, S74, 43-3) and strain B62 of *B. thuringiensis*. Gram reaction showed WS-XY1 was Gram-positive in accordance with that of the *Bacillus* genus.

Yeast is environmentally ubiquitous and is widely applied for energy production and environmental bioremediation [3,26]. *P. stipitis* is a model ethanol-producing yeast and has been thoroughly studied at the genome and protein scales. Both WS-XY1 and *P. stipitis* are enveloped by dense cell walls making them ideal candidates with which to study the EET mechanisms in such species.

3.2. Characterization of electrochemical activity

The redox peaks in the CVs of the microorganisms (Fig. 2A) provide the first indications that both WS-XY1 and *P. stipitis* may have electrochemical activity. It is known that *S. oneidensis* MR-1 secretes flavins to mediate EET between the cells and an electrode: the formal potential of flavins is -0.41 V [27]. Abbas et al. summarized that both bacteria in the genus *Bacillus* and yeast in the genus *Pichia* have riboflavin excretion system [12]. Both WS-XY1 and *P. stipitis* yielded redox peaks at *ca.* -0.40 V (the formal potential for WS-XY1 is -0.40 ± 0.01 V and for *P. stipitis* is 0.39 ± 0.01 V) in Fig. 2A, although these peaks were not very pronounced. Compared to CV, DPV can detect lower concentrations of redox compounds by optimization of faradaic and capacitive currents (i.e. minimizing capacitive current) [28]. This was assisted in studying the weak redox peaks in Fig. 2A (insert).

In Fig. 2B, the supernatant (microbe-free) showed similar CV oxidation peaks at *ca.* -0.41 V (the oxidation potential for WS-XY1 is -0.41 ± 0.01 V and for *P. stipitis* is -0.42 ± 0.01 V): approximate to the redox potential of pure riboflavin (-0.41 ± 0.01). The small shifts in the peak potentials were originated from the small pH differences of the supernatant

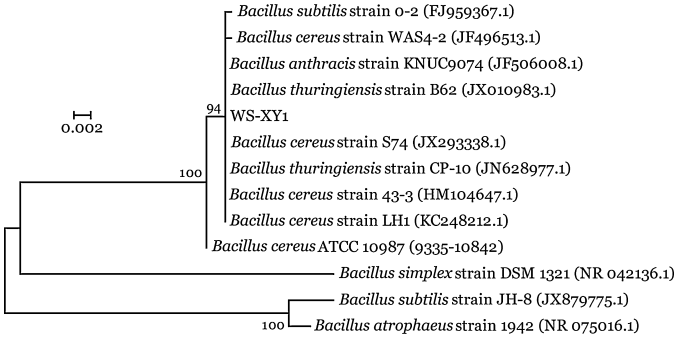

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of *Bacillus* sp. WS-XY1. The tree was constructed using neighbor-joining algorithm. The percentage showed at each branch is gained from 10000 bootstrap replications.

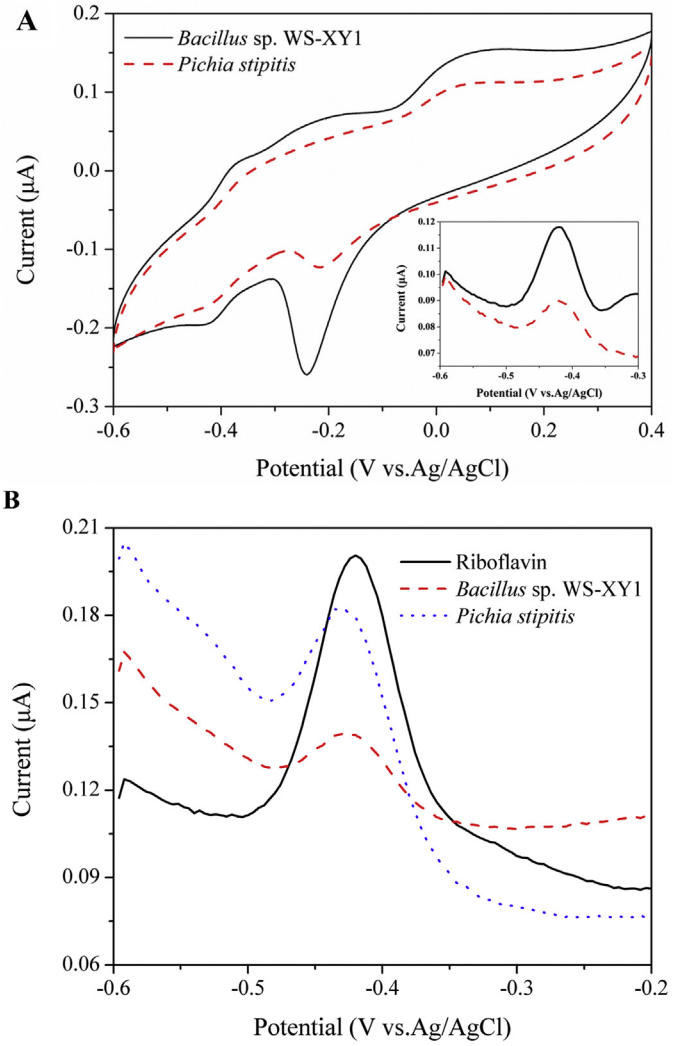

Fig. 2. (A) Cyclic voltammetry of *Bacillus* sp. WS-XY1 (solid line) and *P. stipitis* (dash line) biofilm-covered working electrodes [insert: DPV of WS-XY1 (solid line) and *P. stipitis* (dash line) biofilms]. (B) DPV recorded: when 1.5 μM riboflavin was added to PBS (pH = 7, microbe-free) and with the culture medium supernatants (microbe-free) of WS-XY1 (pH = 7) and *P. stipitis* (pH = 7.2). All these tests were repeated more than 3 times under a N_2 environment.

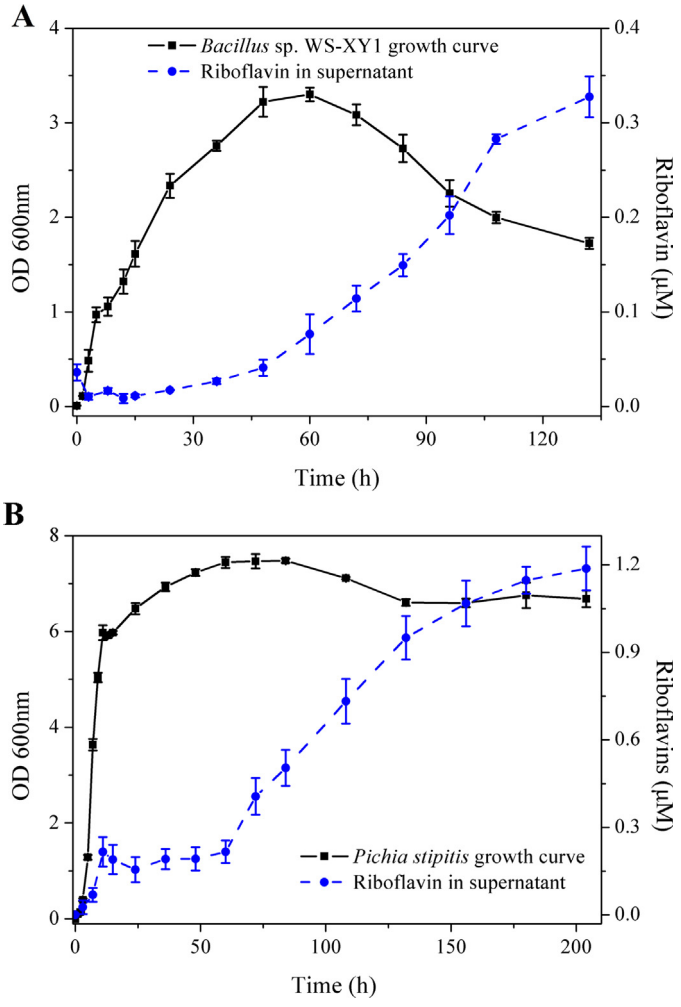

Fig. 3. The growth curve (solid line) of WS-XY1 (A) and *P. stipitis* (B) and the riboflavin concentrations detected in the respective microorganism-free culture supernatants (dash line). The data are averages from $n = 4$ cultures.

samples (Fig. 2B). Therefore, we hypothesize that the redox peaks at *ca.* -0.40 V in Fig. 2A are, not from membrane-bound protein, due to flavins that are absorbed to cells' surfaces and are not easily washed off by PBS [1].

Shewanella oneidensis MR-1 and *Geobacter sulfurreducens* utilize outer-membrane MtrC and OmcA, OmcE and OmcS to reduce extracellular electron acceptor, respectively. The species investigated in this work may possess homologues of cytochromes which *Shewanella* or *Geobacter* species have; i.e. the redox peaks observed between -0.30 V and 0.10 V may originate from some proteins associated with the cell walls [11,29]. In this communication, we focus on redox peaks at *ca.* -0.40 V, the mechanism study of other redox peaks via molecular biotechnology with electrochemical technologies will be addressed in further work.

3.3. Detection of secreted flavins

Riboflavin, FMN and FAD are the main representatives of flavins. Intracellular flavins are primarily represented by the FMN and FAD in most of microorganisms, while riboflavin is main form released out of the cells to environment [30]. To confirm the hypothesis in 3.2, HPLC was employed to detect riboflavin in the supernatants during microbial cultivation. The results verified that both of the two strains secrete riboflavin (Fig. 3). These results are in good accordance with prior studies on *Bacillus subtilis* and yeast *Candida famata*, which are the mostly used microorganisms for commercial synthesis of riboflavin [31].

3.4. Effect of flavins on EET

Although flavins are known electron shuttles in *Shewanella* sp., they can't mediate EET in *Escherichia coli* [18]. Chronoamperometry was therefore used to further evaluate the effect of flavins on the EET of WS-XY1 and *P. stipitis*. For WS-XY1, the peak current after adding flavins was 20.5 ± 4.3 μA (Fig. 4A), which was considerably higher than the control (6.3 ± 2.5 μA). In the case of *P. stipitis*, the peak current after adding flavins was 16.3 ± 2.5 (Fig. 4B), again higher compared to the control (4.9 ± 0.8 μA). These results indicated that the flavins contribute to the respiratory metabolism of these microbes via the anode.

4. Conclusions

The flavins mediated EET of Gram positive *Bacillus* sp. WS-XY1 and the yeast *P. stipitis* (both widespread in various aquatic and soil environments) were studied. Electrochemical

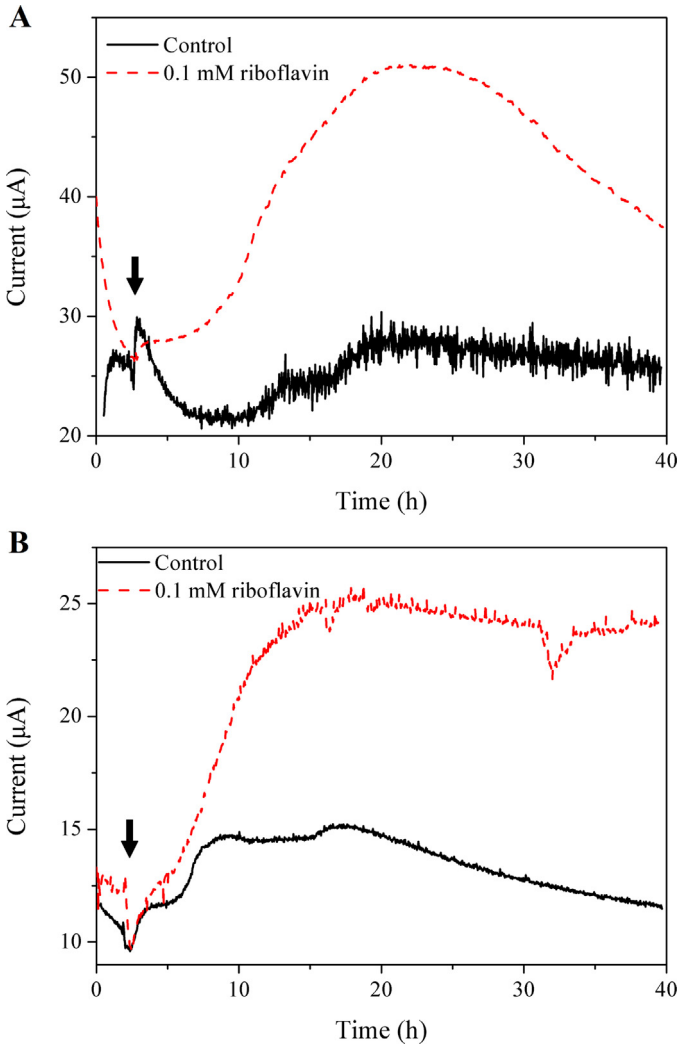

Fig. 4. Chronoamperometry of the WS-XY1 (A) and *P. stipitis* (B) biofilms. The arrows indicate the addition of glucose-only (solid line) or addition of both glucose and riboflavin (dash line).

results indicated that both microorganisms exhibit electrochemical activity despite being enveloped by dense cell walls. Subsequent HPLC and chronoamperometry experiments proved that flavins were secreted by, and contributed to the EET with, both microorganisms. Hence, flavins are ubiquitous mediators and play important roles in mediated EET. This research will promote the understanding of biogeochemical and bioelectrical processes.

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