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Flavins mediate extracellular electron transfer in Gram-positive *Bacillus megaterium* strain LLD-1

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ABSTRACT

The extracellular electron transfer (EET) mechanism of an isolated Gram-positive *Bacillus megaterium* strain (LLD-1), identified by 16S rRNA gene sequencing and physiological analysis, was investigated in the present study. The electrochemical activity of strain LLD-1 was confirmed by electrochemical *E-t* and amperometric *I-t* tests. Flavins in culture suspension from strain LLD-1 were further proved to be able to act as electron shuttles, strengthening the electron transfer from LLD-1 to the electrode. The output voltage and current output were increased 2.8 times and 3.7 times, respectively, by adding 100 nM exogenetic flavins into microbial fuel cells inoculated with LLD-1. Electricity generation by LLD-1 from different carbon sources can be enhanced by adding 100 nM exogenetic flavins. This study indicated that flavins were essential to the EET process of the Gram-positive strain LLD-1. Furthermore, a putative EET model for *B. megaterium* strain LLD-1 and even for Gram-positive bacteria was proposed.

1. Introduction

Electrochemically active microorganisms (EAMs) are species that can transport electrons from cells to extracellular electron acceptors such as minerals, contaminants and electrodes, and the electron transfer processes are defined as extracellular electron transfer (EET) [1]. Therefore, investigations into EAMs and their EET mechanisms are of vital importance to understanding various biogeochemical processes and to developing technologies for contaminant degradation coupled with energy production (such as bioelectrochemical systems) [2].

In recent years, more than 100 EAMs have been isolated or identified, covering a wide range of genetic groups, and Gram-negative *Proteobacteria* is the most abundant phylum [3,4]. However, there is still limited knowledge on the EET mechanisms, as the proposed EET pathways are mainly based on studies targeting Gram-negative *Shewanella* spp. and *Geobacter* spp. So far, two EET mechanisms have been proposed for EAMs, a direct electron transfer pathway via outer membrane *c*-type cytochrome proteins [5–7] or extended conductive pili/nanowires [8,9] and an indirect electron transfer mode via electron shuttles such as flavins [10,11].

The electron-shuttling compounds, either self-produced by EAMs [11,12] or exogenetic, can assist in microbial energy metabolism by facilitating EET processes. These water soluble electron shuttles can also enhance the bioremediation of contaminated environments by facilitating microbial contaminant transformations that are physically/spatially unavailable to microbes [13]. The cell walls of Gram-negative bacteria, i.e., the outer membranes, are much thinner (typically 10 nm) than those of Gram-positive bacteria (typically 30 to 80 nm). It has been noted that the structure and thickness of the cell membrane/wall are very important to microbial EET processes [14–16]. However, few studies have focused on gram-positive bacteria even though they spread widely in various environments [14–23]. These studies have focused on their current production in MFCs by direct electron transfer [20,21] or through reducing exogenetic electron mediators (humic acids, anthraquinone-2,6-disulfonate, etc.) to generate current indirectly [18] and their ability to reduce Fe(III) oxides [15,22]. In other words, endogenic mediators have rarely been reported/mentioned in proposed EET mechanisms for Gram-positive bacteria. S-layer associated *c*-type cytochromes (SLC) have been found on the cell wall surface of Gram-positive bacteria by Chang et al. [15]. Whether and how endogenic mediators work on SLC are still unsolved questions, even if that endogenic mediators are proved to be secreted by Gram-positive bacteria.

Bacillus megaterium (*B. megaterium*), one kind of Gram-positive industrial bacterium, is used extensively for producing polyhydroxybutyric acid, which is an organic polymer with potential

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commercial applications as a biodegradable thermo-plastic biomaterial [24]. The species is also a potential agent for biocontrol of diseases in tea plants and can be used for effective degradation of organophosphorus pesticides [25], dichloroanilines, monosultap and other hazardous cargos [26]. Cheung and Gu found that *B. megaterium* is able to reduce the toxicity of some pollutants, e.g., transforming Cr(VI) to Cr(III) [27]. However, the mechanisms by which *B. megaterium* interacts with and transforms heavy metallic salts or other contaminants are still unknown. Coman et al. reported that the electrochemical communication between Gram-positive *Bacillus* spp. strains and electrodes can be enhanced by osmium redox polymer electrodes [28]. Furthermore, long-range electron transfer via mediators through mass diffusion is beneficial to degrading pollutants in wastewater. Thus, investigation into EET mechanisms via the endogenic mediator of *B. megaterium* is essential for understanding the interactions at the SLC/electrode interface.

In our previous work, riboflavin was found in gram-positive *Bacillus* sp. WS-XY1 and yeast *Pichia stipites* [29]. In the present study, we report a new EAM, *B. megaterium* strain LLD-1, which was isolated from the anode of a microbial fuel cell (MFC) and identified by 16S rRNA gene sequencing technique coupled with physiological/biochemical identification methods. All soluble flavins related to EET mechanisms were measured based on cyclic voltammetry (CV), differential pulse voltammetry (DPV), chronoamperometric tests and high-performance liquid chromatography (HPLC). Moreover, an electron-transfer model for *B. megaterium* strain LLD-1 and gram-positive bacteria were comprehensively discussed and proposed.

2. Experimental section

2.1. Bacterial isolation and culture

A MFC was constructed as shown in Scheme S1. The anode chamber was inoculated with activated sludge from JiMei wastewater treatment plant in Xiamen City and fed with sodium acetate (analytical reagent) of 8.93 mM (i.e., 1 $g \cdot L^{-1}$) and 0.10 M phosphate buffer solution (PBS, KH_2PO_4 of 0.05 M, K_2HPO_4 of 0.05 M, pH 7.0, analytical reagent). After the MFC can stably output current of approximately 0.5 mA with 1000 Ω external resistance, biofilm was washed down from one piece of the anode (1 × 1 cm) and dispersed in 50 mL sterilized water. Then, bacteria were isolated following the method described in our previous study [30].

All experimental supplies were sterilized at 121 °C for 20 min before inoculation. Here, *B. megaterium* strain LLD-1 (indexed as LLD-1) was cultured in fresh sterilized peptone-glucose medium (20 g L^{-1} peptone, 20 g L^{-1} glucose, pH 7.0, Biological reagents) and grown aerobically with a slight shake of 150 rpm at 32 °C. The physiological and biochemical tests were performed when the optical density of the cell suspension at 600 nm (OD_{600nm}) reached 1.0.

2.2. DNA extraction, the 16S rRNA gene sequencing and physiological and biochemical test

Total genomic DNA was extracted from bacterial cells grown in YPD medium (20 g L^{-1} peptone, 20 g L^{-1} glucose, 10 g L^{-1} yeast extract, pH 7.0) using a previously reported method PL [31]. A 1.5-kb fragment of the 16S rRNA gene was amplified by PCR on a MasterCycler gradient PCR apparatus (Eppendorf, Germany). The primer sequences used for the amplification were 27F 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1492R 5′-TAC GGC TAC CTT GTT ACG ACT T-3′ for forward and reverse primer, respectively. After purification the PCR product was ligated into a pMD19-T vector (Takara, China) and then transformed into *Escherichia coli* competent cells to isolate plasmids containing the insert for sequencing (Shanghai Majorbio Bio-Pharm Technology Co., Ltd., China). The homology of the 16S rRNA partial gene sequence was compared with the existing sequences available in the data bank using a BLAST search. The sequence alignment was carried out using Clustal W (version 1.83) of NCBI and the phylogenetic tree was constructed by MEGA software (version 5.2) using the Neighbour Joining method.

Biochemical tests based on the API 50 CHB gallery (Biomerieux, France) were used to investigate the fermentation of 49 sugars (Biological reagents) according to the manufacturer's instructions, including D-cellobiose, glycerol, D-maltose, erythritol, D-lactose, D-arabinose, D-melibiose, L-arabinose, D-sucrose, D-ribose, D-trehalose, D-xylose, inulin, L-xylose, D-melezitose, D-adonitol, D-raffinose, D-galactose, Methyl-βD-mannopyranoside, starch, glycogen, D-glucose, xylitol, D-fructose, gentiobiose, D-mannose, D-turanose, L-sorbose, D-lyxose, L-rhamnose, D-tagatose, dulcitol, D-fucose, inositol, L-fucose, D-mannitol, D-arabitol, D-sorbitol, L-arabitol, methyl-αD-mannopyranoside, 5 kg potassium gluconate, methyl-αD-glucopyranoside, 2 kg potassium gluconate, N-Acetyl-glucosamine, esculinferric citrate, amygdaline, salicin, arbutin and gluconate. Other physiological and biochemical tests were performed according to Bergey's Manual of Determinative Bacteriology.

2.3. Morphological characterization

For scanning electron microscopy (SEM) imaging, bacterial cells were carefully collected by centrifugation at 5000 rpm for 5 min, washed three times with 0.10 M PBS (pH 7.4) and then immersed in 2.5% (v/v) glutaraldehyde with 0.10 M PBS for 4 h at 4 °C [18]. The cells subsequently were incubated in 0.1 M PBS for 1 h and rinsed with distilled water to remove and dissolve salts, followed by dehydration with 50%, 75%, 90%, 95%, and 100% (v/v) ethanol solution each for 5 min. The cells were finally dried in a drying oven at 60 °C for 12 h. The surface morphology of the bacteria was observed by S-4800 SEM (Hitachi, Tokyo, Japan, operated at 5 kV).

2.4. Electrochemical characterization

CV and DPV (potential increment: 4 mV; amplitude: 50 mV; pulse width: 0.06 s; pulse period: 0.5 s) experiments were carried out in a three-electrode system to investigate the electrochemical redox activity of strain LLD-1 by an electrochemical workstation (CHI660D, China). A glassy carbon (GC) electrode (3 mm diameter) was used as the working electrode while a platinum wire and Ag/AgCl (sat. KCl) served as counter and reference electrode, respectively. Bacterial cells were harvested with an optical density at OD_{600nm} of 1.0 in stationary phase. Cells were collected by centrifugation at 5000 rpm for 5 min and then washed three times with 0.10 M PBS (pH 7.0). Precipitated cells (5 μL) were directly transferred onto the GC electrode surface for electrochemical measurements referred to a bare GC without cells.

Amperometric *I-t* curves and *E-t* curves were performed in 125 mL two-chamber MFCs to evaluate the contribution of 100 nM flavins (33.3 nM each for FAD, FMN and RF) to EET efficiency as indicated by current generation and output voltage. Glucose culture medium (20 g L^{-1}) with or without cells was set as controls. Strain LLD-1 at OD_{600nm} of 1.0 was cultivated in peptone-glucose medium (20 g L^{-1} peptone, 20 g L^{-1} glucose, pH 7.0) on a GC electrode poised at +400 mV (vs. Ag/AgCl in sat. KCl) with CHI1000B (China) in a constant temperature incubator. All the solutions were deoxygenated by purging with nitrogen gas for 20 min before the test and maintained under nitrogen atmosphere during the electrochemical measurements [32]. All the electrochemical tests were in triplicate.

2.5. Quantitative analysis of flavins by HPLC

Commercially available FAD (Sigma), FMN (Sigma) and RF (Sigma), serving as reference standards in the HPLC analysis, were dissolved in water. The excitation/emission fluorescence spectra of these flavins were collected to optimize the detection condition of HPLC shown in Fig. S1. The characteristic fluorescence absorptive bands of flavins (RF-551, Shimadzu, Duisburg, Germany) indicated that the fluorescence