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Combined spectroelectrochemical and proteomic characterizations of bidirectional *Alcaligenes faecalis*-electrode electron transfer

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ABSTRACT

Bioelectrochemical systems use microbes as catalysts for current production or consumption. Up to now only a few microbes have been demonstrated to be capable of both outward and inward extracellular electron transfer (EET) (i.e. bidirectional electron transfer). However, the mechanisms of electron exchange between microbes and extracellular solids remain uncertain. Here, we showed that *Alcaligenes faecalis* catalyzed an outward EET and generated electricity at a poised potential of +0.3 V vs. SHE, whereas it conducted an inward EET for autotrophic denitrification at −0.5 V vs. SHE. Both cyclic voltammetry and in situ electrochemical FTIR spectroscopy revealed that different redox components were utilized during the outward and inward EET. Electron transport inhibitor experiments indicated for the first time that complex I, II, III, and the quinone pool on the plasma membrane were involved in the bidirectional EET. Comparative proteomics showed that the protein expression profile of outward-EET biofilms differed greatly from those of inward-EET biofilms, implying that the pili and outer membrane proteins might be responsible for the interfacial outward and inward EET, respectively. These results suggest different electron transport conduits of *A. faecalis* biofilms could be used for bidirectional EET.

1. Introduction

Electrochemically active bacteria (EAB) can gain energy for growth by donating electrons to or accepting electrons from redox-active solids via a process termed extracellular electron transfer (EET) (Shi et al., 2016). The most intensively studied redox-active solids for EET are the anode and cathode of the bioelectrochemical system (BES), which includes microbial fuel cell (MFC), microbial electrosynthesis (MES) and microbial electrolysis cell (MEC), etc (Saratale et al., 2017; Wang and Ren, 2013). EET between microbes and electrodes can occur bidirectionally. For an outward EET, electrons flow from the inside of EAB to anodes, whereas for the inward EET electrons flow across cell membranes into the interior of cells. Such outward and inward EET (i.e. bidirectional EET) have shown potential applications in electricity generation, organic contaminant degradation, biofuel electrosynthesis and so on (Yuan et al., 2016; Tremblay and Zhang, 2015). Up to date, hundreds of EABs have been isolated from various environments. Most of them are solely capable of outward EET (current production) or solely capable of inward EET (current consumption). However, only a few EABs (e.g. *Shewanella oneidensis* MR-1 (Ross et al., 2011), *Geobacter sulfurreducens* (Strycharz et al., 2011), *Klebsiella pneumoniae* (Zhang et al., 2008; Harrington et al., 2015), *Escherichia coli* (Harrington et al., 2015; Park and Zeikus, 2000), *Comamonas testosterone* (Yu et al., 2015a, 2015b), *Geobacter soli* (Yang et al., 2017a)) were reported to catalyze bidirectional EET.

Interfacial electron exchange between EABs and extracellular solids follow two major pathways: (1) direct EET via bacterial outer membrane (OM) proteins or conductive nanowires (i.e. pili or pilus-like structures) (Yang et al., 2017b; Reguera and Mccarthy, 2005); (2) indirect EET via endogenous or exogenous electron shuttles (Wu et al., 2014). Electron flow across cell membrane typically requires the participation of periplasmic or membranous redox proteins such as c-type cytochromes (Kumar et al., 2017). For example, *S. oneidensis* MR-1 utilizes a so-called metal-reducing (Mtr) pathway for outward EET (Coursolle and Gralnick, 2010a). In the Mtr pathway, *S. oneidensis* MR-1 first produces intracellular reducing power (i.e. NADH) via anaerobic respiration of organics. Then electrons flow orderly from NADH to menaquinone pool in the plasma membrane, CymA (inner membrane tetrahaem c-type cytochrome), MtrA, MtrB (trans-OM protein), and finally to MtrC and OmcA (two c-type cytochromes) on the OM. MtrA, MtrB and MtrC form an OM complex (MtrABC) that carries electrons through OM to extracellular solids (e.g. anode) directly or via the

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mediation of flavins (Coursolle et al., 2010b). For the inward EET, the Mtr pathway can be reversed by *S. oneidensis* MR-1 to accept electron directly from extracellular solids (e.g. cathode) (Ross et al., 2011; Okamoto et al., 2014). In comparison, *G. sulfurreducens* is more branched in its outward EET pathways than is *S. oneidensis* MR-1 (Sydow et al., 2014). Moreover, the inward EET mechanism of *G. sulfurreducens* differs dramatically from its own outward EET mechanism because no shared EET components have been found between outward and inward EET (Strycharz et al., 2011; Sydow et al., 2014). For example, cytochrome proteins OmcZ, OmcS, and PilA of *G. sulfurreducens* that are essential for outward EET are not required for its inward EET (Strycharz et al., 2011). PccH of *G. sulfurreducens* is thus far the only identified protein involved in its inward EET, yet other inward EET components remain unknown (Dantas et al., 2015). The diversity of EET pathways seem to be widely represented among EABs because of the diverse EET components. However, most current studies on bidirectional EET are limited to *S. oneidensis* MR-1 and *G. sulfurreducens*, whereas knowledge on the bidirectional EET mechanisms of other EABs is still lacking.

Alcaligenes faecalis is a facultative anaerobic bacterium commonly found in the environment. It has a versatile carbon and nitrogen metabolism such as ammonium oxidation, nitrogen fixation and denitrification, which make it wide application in wastewater treatment for nitrogen removal and organics synthesis (e.g. penicillin) in pharmaceuticals industry. Meanwhile, *A. faecalis* was previously found to be electro-active in the anode of MFC and accept cathodic electrons for nitrate reduction (Rabaey et al., 2004; Wang et al., 2015). Thus, *A. faecalis* harbors the dual functions of electricity generation and denitrification via bidirectional EET. On this basis, *A. faecalis* was selected in this study to elucidate the bidirectional EET pathways. The EET components of *A. faecalis* were identified by a combination of in situ electrochemical FTIR spectroscopy (in-situ EC-FTIRS), electron transport inhibitors and comparative proteomics. The outward and inward EET features of *A. faecalis* were compared to give insights into the diversity of EET pathways among EABs.

2. Materials and methods

2.1. Microorganism and cultivation

A. faecalis (DSM 30030) was purchased from the Chinese General Microbiological Culture Collection Center (Institute of Microbiology, Chinese Academy of Sciences, Beijing). To prepare the inoculum, *A. faecalis* was cultured in Luria-Bertani (LB) medium at 30 °C (Kalyaeva et al., 2002).

2.2. BES construction and experimental setup

H-shaped two-chamber bioelectrochemical reactors with a liquid volume of 110 mL and a headspace volume of 50 mL for each chamber were constructed as previously described (Siegert et al., 2014). Carbon cloth (7 cm × 7 cm), carbon felt (3 cm × 5 cm) and saturated calomel reference electrodes (SCE) were used as the working, counter and reference electrode, respectively. The reactors were connected to a multi-potentiostat (CHI1040, Chenhua Co., Ltd., Shanghai, China). All of the potentials reported in this study were relative to standard hydrogen electrode (SHE) unless otherwise noted. For the outward EET, the working electrodes were poised at +0.3 V vs. SHE and an open circuit potential reactor (designated as OCP2) was used as a control. The medium composition in the working electrode chamber was (per L): CH_3COONa, 8.20 g; K_2HPO_4, 9.28 g; KH_2PO_4, 1.81 g; $Na_2MoO_4{\cdot}2H_2O$, 24.2 mg; $FeSO_4{\cdot}7H_2O$, 5.6 mg; $CaCl_2{\cdot}2H_2O$, 51.5 mg; $MgSO_4{\cdot}7H_2O$, 0.2 g (pH = 7.0). The counter chambers were filled with 110 mL of 0.1 M potassium phosphate buffer solution (PBS, pH 7.0 with 0.1 M KCl). For the inward EET, the working electrodes were poised at −0.5 V and an open circuit potential reactor (designated as OCP1) was used as a control. The cathodic electrolytes in the treatments of −0.5 V and OCP1 were the same as the medium described above except that the electrolytes were complemented with $NaHCO_3$ (2.0 g/L) and KNO_3 (0.50 g/L) whereas acetate was omitted. Each treatment (−0.5 V, OCP1, +0.3 V and OCP2) was performed in triplicate at 30 °C. All of the reactors and electrolytes were autoclaved and purged with CO_2 to remove oxygen prior to the experiments, and then sealed with rubber stoppers. Pure cultures of *A. faecalis* at the logarithmic phase were collected and washed for several times, and then were inoculated into the working electrode chambers (OD_{600} = 0.6). Currents were recorded every 60 s by a computer connected with the multi-potentiostat. When the experiments were completed, CV scans of the intact reactors were performed in a potential range of −1.0 to +0.5 V at a scan rate of 1 mV/s.

2.3. In situ electrochemical FTIR spectroscopy

In situ electrochemical FTIR spectroscopy (Nicolet, Nexus 870 spectrometer) was carried out at room temperature (around 22 °C) by following the same method as previously reported (You et al., 2015). A glassy carbon electrode (5 mm in diameter) served as the working electrode, and SCE and Pt foil were the reference electrode and counter electrode, respectively. *A. faecalis* in the BES reactors that were poised at −0.5 V and +0.3 V for 12 d were collected, and dripped onto the CaF_2 window to form a thin bacterial film. Reference potential was set at +0.54 V and sample potential increased from −0.7 V to +0.5 V with an interval of 0.2 V. A total scan of 200 at a resolution of 8 cm^{-1} was recorded for each potential. All spectral experiments were performed in PBS buffer (0.1 M, pH 7.0) under continuous purge of N_2.

2.4. Electron transport inhibition

The electron transport inhibitors dicumarol, quinacrine, dicyclohexylcarbodiimide (DCCD), rotenone and antimycin A were used to investigate the electron transport components of bidirectional EET. When the anodic (+0.3 V vs. SHE) or cathodic (−0.5 V vs. SHE) current reached stable states, the deoxygenated solutions of dicumarol (in 10% (v: v) acetone), quinacrine (in water), DCCD (in 10% (v: v) ethanol), rotenone (in 10% (v: v) acetone) and antimycin A (in 10% (v: v) ethanol) were spiked into the reactors (Yu et al., 2015a, 2015b). Control experiments with equivalent amounts of solvents showed no significant effects on the current generation.

2.5. Protein extraction and proteomic quantification

Carbon cloth bioanodes (+0.3 V) and biocathodes (−0.5 V) were collected, washed with distilled water, and then cut into pieces, respectively. Total proteins of the biofilms on the carbon cloths were extracted using a One Step Bacterial Active Protein Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instruction. The protein concentration was measured using the Bradford method. Sequential window acquisition of all theoretical spectra combined with LC-MS/MS (SWATH-MS) was used to identify and quantify each protein. The detailed information on proteomic quantification was provided in the Supplementary material.

2.6. Analytic techniques

Nitrate (NO_3^-−N) and nitrite (NO_2^-−N) in the cathodic chambers were determined by ion chromatography (ICS-90, DIONEX, USA) as previously described (Zhang et al., 2012). N_2O in the headspace was analyzed using a gas chromatograph equipped with an electron capture detector (ECD) (GC7900, Tianmei Scientific Instruments Inc., China). Measurements of ammonium (NH_4^+−N) and sample preparations for SEM (S-4800 FESEM, Hitachi Inc., Japan) were performed as previously described (Su et al., 2012; Zhang et al., 2013). The protocols of genomic DNA extraction and sequencing were provided in the supplementary