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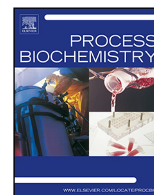

Characterization of a new electrochemically active bacterium, *Lysinibacillus sphaericus* D-8, isolated with a WO_3 nanocluster probe

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ABSTRACT

Microorganisms capable of extracellular electron transfer play important roles in biogeochemical redox processes and have been of great interest in the fields of energy recovery, waste treatment, and environmental remediation. In this study, a new electrochemically active bacterium was identified with a high-throughput method using WO_3 nanoclusters as probes. The 16S rRNA gene sequence designated the strain as *Lysinibacillus sphaericus* D-8, a Gram-positive bacterium. Its electrochemical activity was characterized in a two-chamber microbial fuel cell and a three-electrode electrochemical cell. Strain D-8 produced 92 mW/m^2 of power using lactate as the electron donor. The electrochemical impedance spectroscopy results confirmed the electrochemical activity of this strain. Cyclic voltammetry analysis indicated that the presence of soluble redox active compounds might play an important role in the extracellular electron transfer by *L. sphaericus* D-8. This work might be the first report that demonstrates the electrochemical activity of *Lysinibacillus* species.

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1. Introduction

In recent years, there has been growing interest in microbial fuel cells (MFCs), an emerging technology that utilizes the metabolic and extracellular electron transfer activities of some bacteria [1]. However, most bacteria do not have extracellular electron transfer ability, which is essential for electricity generation by an MFC and in biogeochemical redox processes. Over the past 10 years, approximately 30 electrochemically active bacteria (EAB), which are defined bacteria capable of current production in mediatorless MFCs [2], have been isolated, covering a wide range of genetic groups, with *Proteobacteria* being the most abundant phylum [3,4]. More EAB species are expected to be discovered.

Different methods have been utilized to isolate EAB, such as U-tube MFCs [5], serial dilution using Hungate roll tubes [6], and dilution to extinction methods [3]. In some cases, EAB were identified directly based on their electricity-producing ability in MFCs. Although MFCs have been used as an effective tool for EAB identification, they are time consuming and specific MFC reactors are usually needed. Metal oxide-reducing capability has also been used as an alternative screening technique. However, exceptions have been found, and some bacteria strains that lack electricity production ability exhibit Fe(III) oxide reduction capacity [7]. Recently, a novel screening method has been developed by our group [8]. Using nanosized tungsten trioxide (WO_3), which changes color from white to blue with electron intercalation, EAB can be identified visually within several minutes, and the coloration density can be utilized to quantitatively evaluate extracellular electron transfer ability. Meanwhile, the use of a 96-well microplate enables high-throughput screening of different microbes. Sediments harbor enormous microbial communities with diverse metabolic potentials. Some bacterial consortia can perform dissimilatory metal reduction using organic or inorganic energy sources [9]. This implies that new EAB from sedimentary environments can be found using the WO_3 nanocluster probe.

In this study, we screened chromatic changes of the WO_3 nanocluster probe, which were used as a signal to evaluate the electrochemical activities of microbial strains that were isolated from sediment. A new EAB strain was identified and designated *Lysinibacillus sphaericus*. To further characterize its electrochemical activity, two-chamber MFCs and electrochemical cells were used to examine the current generation by this strain. Preliminary characterization with cyclic voltammetry (CV) and electrochemical impedance spectra (EIS) revealed that the presence of soluble redox active compounds might play an important role in the extracellular electron transfer by *L. sphaericus* D-8.

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2. Materials and methods

2.1. WO_3 nanocluster synthesis and EAB isolation

Crystalline WO_3 nanoclusters were synthesized using a hydrothermal process method described by Yuan et al. [8]. After the Teflon digestion tubes were cooled down to room temperature, the white WO_3 nanocluster powder was collected by filtering through a 0.45-μm membrane, washing three times with deionized water, and drying at 40 °C in an oven. The morphology of the material was confirmed using a scanning electronic microscope (SEM; JSM-6700F, JEOL Co, Japan).

The initial inoculum was obtained from the sediment samples collected from Shiwuli River located near Hefei City, China. Pure cultures of bacterial strains were acquired by restreaking multiple times on LB agar plates. Liquid inoculums were prepared by growing bacteria overnight to the mid-log phase in LB broth, and the bacteria were harvested by centrifugation at 8200 × *g* for 10 min, washed three times and re-suspended in sodium lactate mineral salt medium [10] to obtain an inoculum density of approximately 5×10^8 CFU/mL. The microbial electrochemical activity was screened based on WO_3 nanocluster coloration as described by Yuan et al. [8] using a 96-well plate (CellstarH, Greiner Bio-One Co., Germany). The plate was incubated at 30 °C, and color development was assessed. Triplicate plates were cultivated in parallel.

2.2. DNA extraction, PCR, and sequence analysis

Genomic DNA of the identified EAB was extracted using a 3S DNA isolation kit (Shenergy Biocolor Co., China) according to the manufacturer's instructions. PCR was performed using universal primers for bacteria 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [11]. The PCR products were then purified and sequenced (Invitrogen Co., China). The 16S rRNA gene sequence was compared to the GenBank database using the BLAST program, and the *Lysinibacillus sphaericus* D-8 sequence has been deposited in the GenBank database under the accession number KC691284. A neighbor-joining phylogenetic tree was constructed using the program MEGA5 with Kimura's two-parameter method [12] and bootstrapping with 1000 repetitions.

2.3. Physiological and biochemical characterization

Well-isolated colonies were obtained on LB agar plates using the streak plate method for morphology observation. Gram staining properties were observed with an optical microscope (Olympus BX 51, Japan). For TEM imaging, a 10 μL of bacterial suspension was dropped onto a carbon-coated copper grid and then negatively stained using 2% phosphotungstic acid (pH 6.5). The grid was air dried and examined with a transmission electron microscope (Tecnai G^2 F20, FEI Co.) at an accelerating voltage of 200 kV.

Oxidase and phenylalanine deaminase tests were performed using two commercially available isolation media (Sigma–Aldrich, St. Louis, USA), and color changes were observed. A slide drop method was used for the catalase test. The ability to use glucose was studied using M9 minimal medium with glucose (2 g/L) by measuring cell growth spectroscopically.

2.4. MFC construction and operation

Two-chamber MFCs with a working volume of 125 mL for each chamber (Supplementary material, Fig. S1) were sterilized by autoclaving at 121 °C for 20 min. A proton exchange membrane (GEFC-10N, GEFC Co., China) was used to separate the two chambers and was pretreated using a method that was modified from Chae et al. [13] by soaking in H_2O_2 (3%, v/v) and 1 M HNO_3, each at 80 °C for 1 h. The membrane was then washed three times with sterile deionized water and then stored in sterile deionized water until MFC construction. The anode chamber was filled with 100 mL of basal medium with mineral and vitamin mix as described by Baron et al. [10]. Lactate (2 mM) was added as the sole carbon source. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 10 mM) was added to buffer against pH changes. The medium was sparged with N_2 gas to remove oxygen. The cathode chamber was filled with 100 mM $Fe(CN)_6^{3-}$ in 50 mM phosphorus buffer solution. Carbon felt (3 cm × 3 cm, 190 μm thick, Toray Co., Japan) was used as the electrodes. The external resistor was 1000 Ω, and the concentration of strain D-8 in the anode chamber was adjusted to an OD_{600} of 0.4. An MFC without bacterial inoculation was used as an abiotic control. During MFC operation at 30 °C, the substrate was renewed when the cell voltage dropped to below 35 mV.

2.5. Electrochemical analysis

MFC voltages were collected every 10 min using an Agilent 34970A Data Acquisition/Switch Unit. Polarization curves were obtained using linear sweep voltammetry conducted on a CHI660C electrochemical workstation (Chenhua Instrument Co., China) scanning at 0.2 mV/s. The power and current density were normalized to the anode area. When the current generation stabilized at the maximum level, CV and EIS measurements were conducted in single-chamber electrochemical cells with a graphite sheet (2 cm^2), a platinum wire and an Ag/AgCl (KCl sat.) electrode as the working, counter and reference electrodes, respectively. To investigate the electrochemical characteristics of the washed biofilm, planktonic phase and supernatant, CV analysis was performed from −0.6 to 0.4 V (vs. Ag/AgCl) at a scan rate of 2 mV/s, followed by a reverse scan to the original value of −0.6 V. The working electrode with an attached biofilm was rinsed gently with fresh medium and transferred into a new electrochemical cell filled with fresh medium for CV analysis. The planktonic phase was retained, and a new working electrode was added for the CV analysis. The supernatant was obtained by plankton phase centrifugation from another electrochemical cell. The supernatant was added into an electrochemical cell with new electrodes for CV assessments.

For potentiostatic EIS, the working electrode was poised at +0.15 V (vs. Ag/AgCl) until the current generation stabilized at the maximum level. Then, EIS measurements were performed in a frequency range of 100 kHz to 100 mHz with an AC amplitude of 5 mV. All of the electrochemical cells were sparged with sterile high-purity N_2 for 15 min to remove oxygen before analysis.

3. Results and discussion

3.1. Isolation of EAB using WO_3 nanoprobes

WO_3 nanomaterials have shown great potential as chemical sensors and for use in energy conversion [14,15]. By utilizing the unique colorimetric properties of WO_3 nanomaterials, our previous work [16] demonstrated that WO_3 nanoprobes can provide a rapid, inexpensive, visible and high-throughput approach for EAB identification that may be used for quantitative evaluation of their extracellular electron transfer abilities. With this electrochromic high-throughput method, we evaluated the extracellular electron transfer abilities of bacteria from a collection of more than 8000 microbes that were isolated from Shiwuli River sediment samples. As shown in Fig. 1, the WO_3 nanoclusters in each well displayed various chroma after incubation with different bacterial isolates. The color varied from white to blue, which could be easily distinguished visually, thus facilitating EAB detection in a short time.

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