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Direct Electrode Reaction of Fe(III)-Reducing Bacterium, *Shewanella putrefaciens*

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Abstract Anaerobically grown cells of an Fe(III)-reducing bacterium, *Shewanella putrefaciens* IR-1, were electrochemically active with an apparent reduction potential of about 0.15 V against a saturated calomel electrode in the cyclic voltammetry. The bacterium did not grow fermentatively on lactate, but grew in an anode compartment of a three-electrode electrochemical cell using lactate as an electron donor and the electrode as the electron acceptor. This property was shared by a large number of Fe(III)-reducing bacterial isolates. This is the first observation of a direct electrochemical reaction by an intact bacterial cell, which is believed to be possible due to the electron carrier(s) located at the cell surface involved in the reduction of the natural water insoluble electron acceptor, Fe(III).

Key words: Fe(III)-reducing bacteria, cyclic voltammetry, biofuel cell, bioelectrochemistry, *Shewanella putrefaciens*

Recently, a number of bacteria have been isolated based on their characteristics to use metal ions including Fe(III) as their electron acceptors [10, 16]. Fe(III), used as an electron acceptor by the iron-reducing bacteria, is essentially insoluble in water at neutral pH. Therefore, physical contact between the bacterial cell and Fe(III) minerals is necessary for the reduction of the water insoluble electron acceptor [11]. Cytochromes are localized to the outer membrane of a gram negative Fe(III)-reducing bacterium, *S. putrefaciens* MR-1 [14]. The outer membrane cytochromes are believed to be involved in the reduction of water insoluble Fe(III). Four distinct cytochromes have been identified in the outer membrane fraction of the bacterium [15]. The reduced forms of these cytochromes were rapidly oxidized by Fe(III). The specific content of 83 kDa *c*-type cytochromes, one of the four outer membrane cytochromes, was lower in the cells grown under aerobic conditions than that of anaerobically grown cells. Fe(III) reduction-deficient mutants of *S. putrefaciens* (*mtrB*) did not produce an outer membrane cytochrome *c* [3]. In spite of extensive studies on the outer membrane cytochromes, little is known about electron transfer reactions of the outer membrane cytochromes. Electrochemical techniques are the methods of choice to study the electron transfer reactions in the bacterium, which has electrochemically active cytochromes on the cell surface to react with the water insoluble electron acceptor, Fe(III).

Several electrochemical techniques have been used in various fields of biology to characterize redox proteins including cytochromes [6]. A large number of redox proteins are electrochemically active. However, it is known that a direct electron transfer between a redox protein and an electrode is hindered by the peptide chain adjoining the active redox center of the protein. For this reason, the electrochemical reaction of a protein is generally enhanced by the modification of electrodes or the protein to direct the active center of the protein toward the electrode surface [18]. Additionally, intact bacterial cells containing electrochemically active proteins are also electrochemically inactive since they are enclosed by an electrically non-conductive cell wall and other surface structures. For these reasons, various electrochemical mediators have been applied in the electrochemical studies of intact bacterial cells [7, 8]. In this case, the mediators carry electrons between bacterial cells and an electrode. As an alternative method, bacterial cells have been modified by hydrophobic conducting polymers to increase the electrochemical activity [17].

Cyclic voltammetry was employed to test if the cell suspension of an Fe(III)-reducing bacterium is electrochemically active, and a fuel cell-type electrochemical cell was used to cultivate the bacterium on lactate, which cannot support anaerobic fermentative growth without an appropriate electron acceptor. To the authors' knowledge, this is the first report on the direct electrochemical activity of an intact bacterial cell.

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MATERIALS AND METHODS

Bacterial Strain and Culture Media

An Fe(III) reducing bacterium, *S. putrefaciens* IR-1, was used throughout the study. The bacterium was isolated from a rice paddy field and characterized in this laboratory. The identification was made based on biochemical, physiological, and morphological characteristics as well as 16s rRNA gene sequence analysis (manuscript in preparation). LB broth (peptone 10 g, yeast extract 5 g, and NaCl 5 g in 1 l distilled water) was used to cultivate the bacterium in aerobic conditions. Anaerobic growth was made using phosphate buffered basal medium (PBBM) containing 35 mM lactate as the electron donor and 10 g/l FeOOH as the electron acceptor [4]. The bacterium was cultivated for 72 h at 30°C, and harvested and washed three times using anaerobic 50 mM phosphate buffer containing 0.1 M NaCl. Strict anaerobic culture techniques were employed for anaerobic cultivations [13].

Cyclic Voltammetry

For cyclic voltammetry, a conventional three-electrode electrochemical cell was used. The electrochemical cell was constructed of glass (0.5 ml of working volume), and fitted with a water jacket for the temperature control of the cell contents. A cyclic voltammogram (CV) of the bacterial cell suspension was obtained using a potentiostat (CV-50W, BAS, West Lafayette, U.S.A.) interfaced to a personal computer with software supplied by the manufacturer. A 2 mm diameter glassy carbon working electrode, a platinum counter electrode, and a saturated calomel reference electrode (SCE) were used in the electrochemical cell [18]. Measurements were carried out at 25°C in anaerobic conditions and the scanning rate for the CV of the cell was 0.1 Vs^{-1}.

Three-Electrode Electrochemical Cell

A separate three-electrode electrochemical cell was constructed as shown in Fig. 1 to cultivate the bacterium. The electrodes used were a graphite felt anodic electrode (surface area: 1400 cm^2, Electrosynthesis, East Amherst, U.S.A.), an Ag/AgCl reference electrode, and a platinum counter electrode (surface area: 140 cm^2). The counter electrode was enclosed by a dialysis membrane (molecular weight cut-off range 12,000- 14,000, Sigma Chemical Co., St. Louis, U.S.A.) to separate it from the working anodic compartment. Working volumes of the anodic and the cathodic compartments were 500 ml and 40 ml, respectively. Both compartments were filled with sterilized PBBM containing 50 mM lactate and 0.1 M KCl, and the cell was incubated at 30°C. A potentiostat (E-series, Thompson Electrochemistry, UK) was used to maintain a constant poised potential to the working electrode at 1.0 V against the Ag/AgCl reference electrode. The bacterial growth was initiated by an inoculation of the bacterium via the inoculation port. During the cultivation, changes of optical density (at 660 nm) of the anode solution and changes of current between working and counter electrodes were monitored.

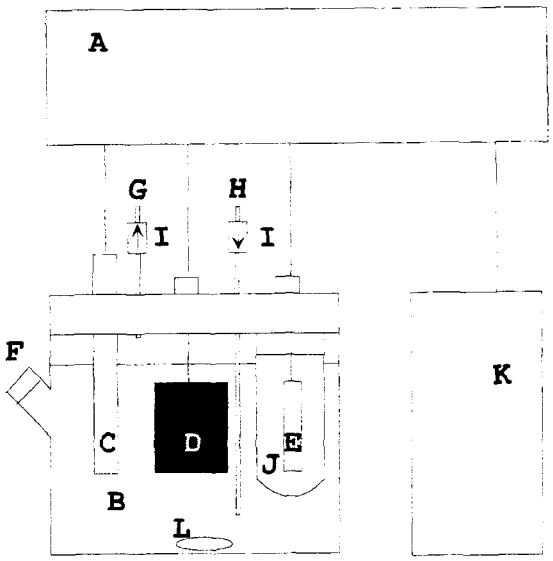

Fig. 1. Schematic diagram of the three-electrode electrochemical cell for the growth of *S. putrefaciens* IR-1.
(A) Potentiostat. (B) Electrochemical cell (anode compartment). (C) Ag/AgCl reference electrode. (D) Working electrode (anode). (E) Counter electrode (cathode). (F) Sampling and inoculation port (butyl rubber gasket). (G) N_2 outlet. (H) N_2 inlet (flow rate 20 ml min^{-1}). (I) Check valve (arrows indicate direction of flow). (J) Cathode compartment (separated from anode by dialysis membrane). (K) Recording system. (L) Magnetic stirrer bar.

High Performance Liquid Chromatography

Lactate and acetate were quantified by HPLC [4]. An integrated HPLC system (Model M910, Young-In Science, Korea) equipped with a sulfonated divinyl benzene-styrene copolymer column (300×7.8 mm, Aminex HPX-87H, BioRad, U.S.A.) and a photometric detector (216 nm) was used. H_2SO_4 (0.01 N) solution was used as the mobile phase at the flow rate of 0.6 ml per min.

RESULTS AND DISCUSSION

Direct Electron Transfer of *S. putrefaciens* IR-1 to Electrode

Cell suspensions (0.36 g/l) were prepared from cultures made under aerobic and anaerobic conditions. Figure 2 shows the CVs of *S. putrefaciens* IR-1 cell suspensions grown aerobically and anaerobically. Anaerobically grown cell suspension produced a quasi-reversible cyclic voltammogram with a reductive peak at -0.32 V and an oxidative peak at 0.03 V against an SCE. The apparent reduction potential of the anaerobically grown cell suspension was about -0.15 V against the SCE, which is about 0.05 V against a natural

hydrogen electrode. No peaks were observed in the CV of the aerobically grown cells. Neither phosphate buffer nor FeOOH suspension gave any peak in the CVs obtained in the same conditions (data not shown). Results obtained over a 2-year period were consistent and reproducible.

These results show that electron exchange is possible between the electrode surface and the anaerobically grown bacterial cells under the given conditions. The electrochemical activity of the bacterial cell suspension is possible due to the presence of electrochemically active compounds on the cell surface. The outer membrane cytochromes might be the electrochemically active compounds on the cell surface. It is known that the bacterium does not use Fe(III) as an electron acceptor under aerobic conditions [2], and that the reduced outer membrane cytochromes are rapidly oxidized by Fe(III) [15]. The bacterium cannot use the water-insoluble electron acceptor under aerobic conditions probably because the cytochromes are not localized to the outer membrane. The bacterium has been reported to synthesize a c-type outer membrane cytochrome under aerobic conditions at a reduced amount [15]. The c-type outer membrane cytochrome of the aerobically grown cells might not be able to reduce Fe(III) since the electrochemically active reaction center is not exposed to the cell surface as shown by the CV of the aerobically grown cell suspension. For this reason, it can be said that cyclic voltammetry is a better and simpler method than biochemical methods [1, 2, 15] to study regulation in the electron metabolism of this bacterium.

There are contradicting reports on the regulation in Fe(III)-reducing activity by alternative electron acceptors such as oxygen and nitrate. Cells of *S. putrefaciens* strain 200 grown at high oxygen tension showed *in vitro* Fe(III) reductase activity, although Fe(III) reduction was active only in the absence of oxygen, and it is not clear if nitrate inhibits Fe(III) reduction [1, 2]. Cyclic voltammetry can also be used to solve these problems.

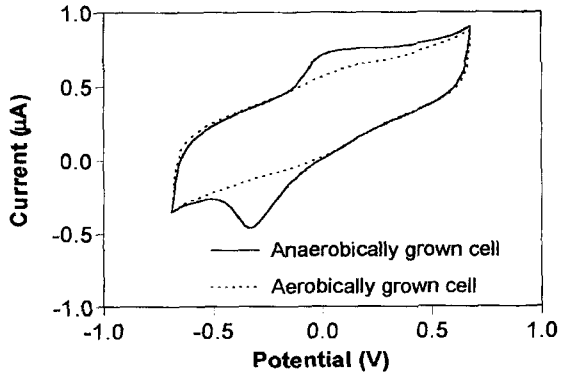

Fig. 2. Cyclic voltammograms of *S. putrefaciens* IR-1 grown aerobically and anaerobically.
The cell suspensions (dry cell weight of 0.356 g/l) were prepared under anaerobic conditions.

Bacterial Growth in an Electrochemical Cell

A three-electrode electrochemical cell connected to a potentiostat was used to investigate if *S. putrefaciens* can grow on lactate by transferring electrons to the electrode in the absence of natural electron acceptors under anaerobic conditions. After the background current of the electrochemical cell had reached to a steady value, *S. putrefaciens* IR-1 was inoculated into the anodic compartment of the electrochemical cell, and the change in current was recorded. Samples were taken at the predetermined time intervals to measure the bacterial growth, and to analyze lactate and acetate by HPLC. A control experiment was run with a similar electrochemical cell where the electrodes were disconnected from the potentiostat. Figure 3 illustrates the changes in current, bacterial cell density, and lactate and acetate concentrations in the anodic compartment. This figure was prepared from the data averaged from three separate experiments. The bacterial growth and the substrate consumption patterns in the electrochemical cell were very similar to a typical batch growth of the organism at a reduced rate. A long lag period preceded the rapid increases in the optical density and the current output. The current increased up to 70 h of cultivation, and decreased probably due to the limitation in the electron

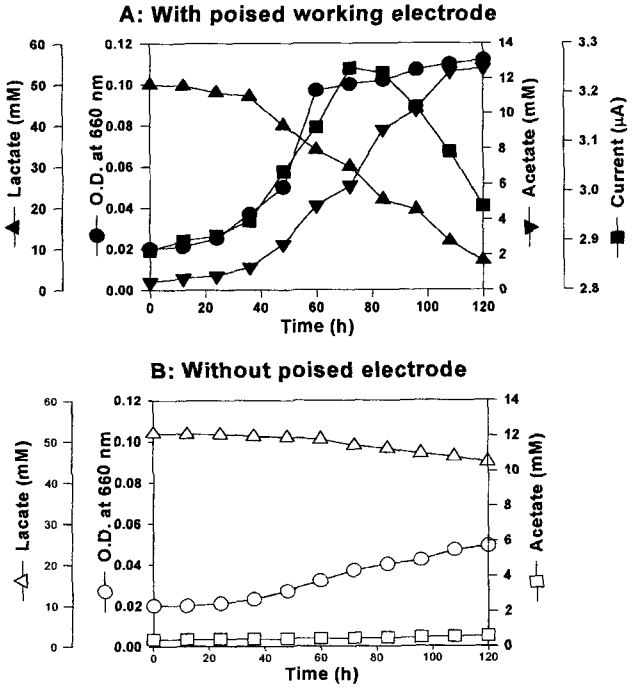

Fig. 3. Growth of *S. putrefaciens* IR-1 on lactate in the anodic compartment of a three-electrode electrochemical cell. *S. putrefaciens* IR-1 was inoculated into the anodic compartment which contained basal medium with lactate.
(A) The three-electrode electrochemical cell was connected to a potentiostat to poise a potential of 1.0 V against the Ag/AgCl reference electrode. (B) A control culture was made in a similar electrochemical cell where the electrodes were not connected to the potentiostat.

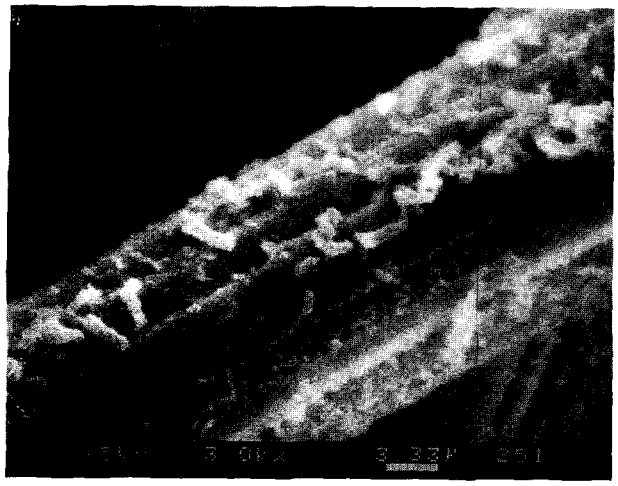

Fig. 4. Scanning electron micrograph of the electrode (anode) in the three-electrode bacteria cultivation system.

donor. At the end of the experiment, heavy bacterial growth on the surface of the anode was observed by electron microscopy (Fig. 4). The low growth rate was probably due to the limitation in electron transfer to the electrode. The growth rate on the insoluble electron acceptor, FeOOH, was slower than on soluble ferric citrate. The surface properties of the electrode might be different from those of the FeOOH, which will influence the electron transfer from the cell to the electrode. KCl at the concentration of 0.1 M was used in the experiment as the electrolyte. The high osmotic pressure caused by KCl might be another reason for the slow growth. A small increase in the optical density was observed with a small consumption of lactate in the control experiments without a poised working electrode (Fig. 3b). Yeast extract used in the medium supported the fermentative growth of the bacterium with lactate as the carbon source. These results show that although the bacterium cannot grow on lactate fermentatively without an electron acceptor, the bacterial growth was possible in the anode compartment since the anode connected to the potentiostat functioned as an electron acceptor or an electron sink. When lactate was added to the system after the current output was reduced to less than 2.9 μA, an increase in the current output was observed (data not shown).

Application of Electrochemical Techniques for Investigation of Metal Reducing Bacteria

A cyclic voltammetric technique was used to measure electrochemical activities of Fe(III)-reducing isolates enriched and isolated in this lab using glucose, fructose, peptone, acetate, and lactate as the electron donors. More than 80% of over 200 isolates were electrochemically active (unpublished data). The three-electrode electrochemical cell could be used to enrich electrochemically active microorganisms, which were mostly Fe(III)-reducers from environmental samples [5]. These results indicate that the electrochemical activity is not a unique property of *S. putrefaciens* IR-1, but is widely spread in Fe(III)-reducers. This is the first report on the direct electrode reaction of an intact bacterial cell. This finding can be employed in various fields of pure and applied microbiology. As mentioned above, the electrochemical activity of a bacterium can be used to study the regulation of bacterial electron metabolism. A biofuel cell can be constructed to generate electricity from substrates that may serve as electron donors for electrochemically active bacteria [9]. This will lead to the development of not only a novel wastewater treatment process but also a method to generate electricity. The biofuel cell can also be used as a simple biosensor to measure the concentration of electron donors. Excess reducing-equivalent can be also removed electrochemically from a bioremediation process using Fe(III)-reducers which can metabolize wide range of organic contaminants [12].

Acknowledgments

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