Electricity Generation by *Rhodopseudomonas palustris* DX-1

DEFENG XING,[†,‡] YI ZUO,[†] SHAOAN CHENG,[†] JOHN M. REGAN,[†] AND BRUCE E. LOGAN*[,†]

Engineering Environmental Institute, and Department of Civil and Environmental Engineering, 212 Sackett Building, The Pennsylvania State University, University Park, Pennsylvania 16802, and School of Municipal and Environmental Engineering, Harbin Institute of Technology, Harbin 150090, China

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Bacteria able to generate electricity in microbial fuel cells (MFCs) are of great interest, but there are few strains capable of high power production in these systems. Here we report that the phototrophic purple nonsulfur bacterium *Rhodopseudomonas palustris* DX-1, isolated from an MFC, produced electricity at higher power densities (2720 ± 60 mW/m^2) than mixed cultures in the same device. While *Rhodopseudomonas* species are known for their ability to generate hydrogen, they have not previously been shown to generate power in an MFC, and current was generated without the need for light or hydrogen production. Strain DX-1 utilizes a wide variety of substrates (volatile acids, yeast extract, and thiosulfate) for power production in different metabolic modes, making it highly useful for studying power generation in MFCs and generating power from a range of simple and complex sources of organic matter. These results demonstrate that a phototrophic purple nonsulfur bacterium can efficiently generate electricity by direct electron transfer in MFCs, providing another model microorganism for MFC investigations.

Introduction

In a microbial fuel cell (MFC) exoelectrogenic bacteria oxidize organic matter and transfer electrons to the anode, where they flow to the counter electrode (cathode) and react with protons and oxygen to form water (*1*). Bacteria isolated for their ability to respire using solid metal oxides have been shown to be capable of electricity generation (*2–4*), although there have been recent reports of dissimilatory metal-reducing bacteria (DMRB) unable to generate electricity (*5, 6*). *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* PCA are two DMRB capable of electricity generation that are being studied to better understand the mechanisms of exocellular electron transfer. Known mechanisms for electron transfer that do not require exogenous mediators include self-produced mediators (*7, 8*), direct electron transfer via membrane-bound cytochromes (*9*), and nanowires (*10, 11*).

DMRB being examined for power production are capable of chemoheterotrophic metabolism limited to a small range of substrates, with some strains not able to fully oxidize certain chemicals. For example, *S. oneidensis* oxidizes lactate to acetate under anaerobic conditions, while *G. metallireducens* oxidizes acetate but not glucose (*12*). *Rhodoferax ferrireducens* can oxidize acetate, lactate, and glucose, but does not degrade ethanol, another common fermentation end product (*13*). It is thought that MFCs will be used to generate electricity from waste biomass having a complex composition, i.e., consisting of a variety of different types of organic matter such as combinations of volatile acids (*14*) or different types of wastewaters (*15*). Few isolates have been obtained from MFCs, and maximum power densities obtained for these isolates have generally been low (*3, 8, 16–20*), due in part to the high internal resistance of the systems used in these studies (*1, 4*). There are many reports of high power production by mixed cultures, but side-by-side tests of isolates and mixed cultures are usually not performed. Here we show for the first time power production by a newly isolated strain of *Rhodopseudomonas palustris*, and we compare power production by this isolate to a wastewater inoculum in the same device under the same solution conditions.

Materials and Methods

Isolation. Strain DX-1 was isolated from an air-cathode MFC fed with acetate operated in the normal laboratory light over a period of three months. Cells were extracted by shaking a portion of the carbon paper anode (2 cm^2) in an anaerobic culture tube (18 mm × 150 mm, Bellco Glass, Inc., Vineland, NJ) containing 10 mL of sterile anaerobic 1% NaCl and glass beads (2 mm diameter). Isolation was performed by serial dilution using the Hungate roll-tube technique (*21*) with medium containing 20 mM sodium acetate, Wolfe's trace mineral (12.5 mL/L), and vitamins (5 mL/L) (*22*). The electron acceptor was 10 mM amorphous iron (III). Red single colonies were picked and transferred to the same broth and incubated at 30 °C. The roll-tube procedure was repeated several times until a pure culture was obtained. DX medium [1 g/L of sodium acetate, 1 g/L of yeast extract, 10 mM NTA-Fe, Wolfe's trace minerals (12.5 mL/L), and vitamins (5 mL/L)] was used for routine cultivations. Physiological and biochemical traits were determined by conventional methods and using Biolog GN2 MicroPlates for examining substrate utilization (Biolog, Inc., Hayward, CA). All tests were performed in duplicate. *Rhodopseudomonas palustris* ATCC 17001 was obtained from The American Type Culture Collection.

16S rRNA Gene Sequencing and Phylogenetic Analysis. Genomic DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using a pair of universal primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′), corresponding to base positions 8−27 and 1525−1541 of the 16S rRNA gene of *Escherichia coli*, respectively (*23*). PCR solutions contained 2 × GoTaq Green Master Mix (Promega, Madison, WI), 1 μM of each primer, 0.5 μg of DNA template, and sterile deionized water to make up the total volume of 50 μL. PCR amplification was carried out in an iCycler iQ (Bio-Rad Laboratories, Hercules, CA) with an initial denaturation of DNA for 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 30 s at 57 °C, and 1.5 min at 72 °C, and then final extension for 7 min at 72 °C.

PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), ligated into vector pCR2.1 using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and cloned into chemically competent One Shot *E. coli* cells

* Corresponding author e-mail: blogan@psu.edu; phone: (814) 863-7908.
[†] The Pennsylvania State University.
[‡] Harbin Institute of Technology.

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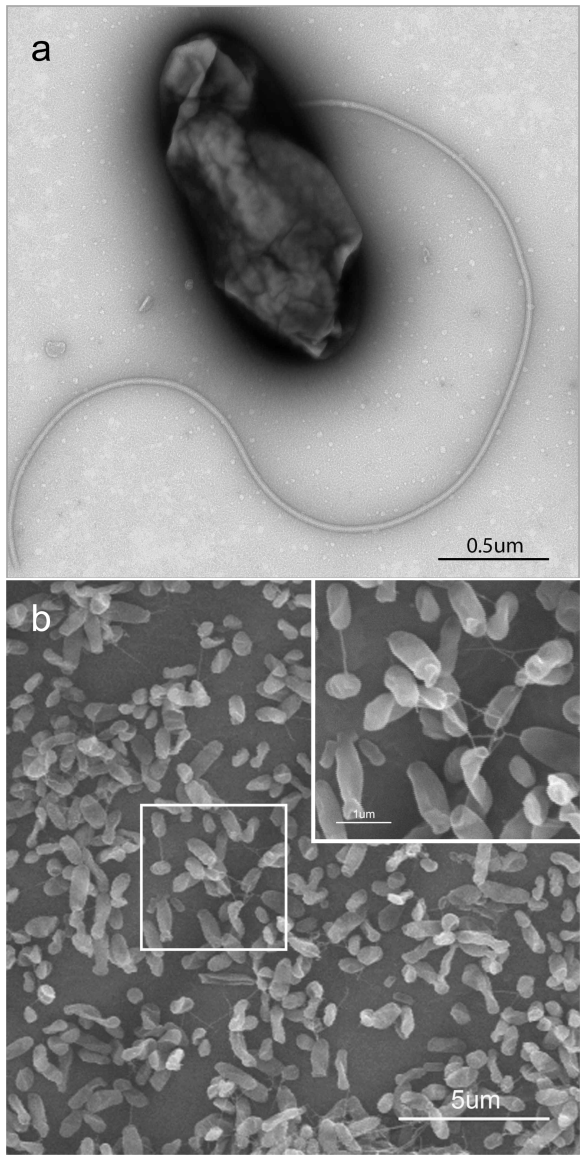

FIGURE 1. Micrographs of strain DX-1: (a) transmission electron micrograph of strain DX-1 on medium with 10 mM chelated iron(III) nitrilotriacetic acid (NTA) as electron acceptor and 20 mM acetate as electron donor; (b) scanning electron micrograph of the surface of carbon paper anode in a cubic MFC following growth of strain DX-1 with acetate as an electron donor (1 g/L). The inset shows a magnified image of the framed part.

provided with the cloning kit, as recommended by the manufacturer's instructions. Plasmids were isolated from clone colonies that were randomly selected to form a clone library with the QIAprep Spin Miniprep Kit (Qiagen), and 15 plasmid inserts were sequenced (ABI 3730XL DNA sequencer, Applied Biosystems, Foster, CA).

The 16S rRNA gene sequences were queried against the GenBank and Ribosomal Database Project databases using the BLAST and SEQMATCH algorithms. Phylogenetic relationships were analyzed by the evolutionary distance matrix calculated using the neighbor-joining method with Kimura's two-parameter method (*24*). A neighbor-joining tree was constructed with the program MEGA4 (*25*). Confidence estimates of branching order were determined by bootstrap resampling analysis with 1000 replicates.

Electron Microscopy. A 5 μL cell suspension of DX-1 was placed on 200 mesh Formvar carbon-coated copper grid and wicked off after 3 min. The sample was soaked in 5 μL of uranyl acetate (2%) for 30 s, then drained and air-dried and examined using a transmission electron microscope (JEM 1200 EXII, JEOL) at an accelerating voltage of 80 kV. A 1 cm^2 piece of the carbon paper anode was fixed in 1.5% glutaraldehyde for 1 h, washed three times using 0.1 M cacodylate buffer, soaked in 1% OsO_4 for 30 min, and washed again 3 times using buffer. After dehydration by a graded ethanol series, the sample was dried with liquid CO_2, mounted on an aluminum stub, sputter-coated with Au/Pd, and examined with a scanning electron microscope (JSM 5400, JEOL) at an accelerating voltage of 20 kV.

MFC Construction. Anodes were carbon paper (7 cm^2, nonwet proofed, type A, E-TEK) or a graphite fiber brush (*26*) of 2.5 cm diameter and 2.5 cm length (PANEX33 160K, ZOLTEK) treated with a high-temperature ammonia gas process (*27*). Cathodes contained 0.5 mg/cm^2 Pt and four diffusion layers on 30 wt % wet-proofed carbon cloth (7 cm^2, type B-1B, E-TEK) (*28*). A 0.22-μm-pore diameter filter (Fluoropore membrane, Millipore, Billerica, MA) was attached on the outer surface of the air cathode to prevent reactor contamination. The MFC was constructed as previously described with a chamber liquid volume of 24 mL (flat carbon paper anode) or 22 mL (graphite brush anode) (*26, 29*).

MFC Operation. The wastewater inoculum was collected from the primary clarifier of the Pennsylvania State University Wastewater Treatment Plant. This wastewater inoculum was used to develop a fully enriched mixed culture, where power output was stable and reproducible over many cycles, for power output comparisons to isolates. Stationary phase cultures of strain DX-1 ($5.0 \pm 0.3 \times 10^8$ CFU, 10/100 v/v) were inoculated and operated in the light (1000 ± 50 lx except as noted; light meter model 21800-0124, Control Inc., Friendswood, TX) or in the dark using GM medium containing the indicated electron donor at a concentration of 1 g/L and a 50 mM or 200 mM phosphate buffer (PBS) (*26*). Solutions were replaced in a laminar flow hood using a 25 mL sterile pipet when the voltage was <20 mV. All tests were conducted at ambient temperatures (23 ± 3 °C). All reactors and media were sterilized before use, and reactors were inoculated using sterile techniques. MFCs without inocula were used as controls. The purity of the cells on the anode and in suspension were evaluated at the conclusion of an experiment using restriction fragment length polymorphism (RFLP) and clone libraries of the PCR-amplified 16S rRNA gene as described above. PCR products (5 μL) were digested with restriction endonucleases *Hha*I, *Hae*III, and *Msp*I in a 10-μL final volume following the manufacturer's instructions (Promega, Madison, WI). Fragments were separated in 3% (w/v) agarose gels and visualized by staining with SYBR Safe. Pure cultures were used as a positive control.

Cell voltages (V) were recorded across a fixed external resistance (1000 Ω except as noted) using a multimeter with a data acquisition system model 2700 (Keithley Instruments, Cleveland, OH). Current (I), power ($P = IV$), and Coulombic efficiency (CE) were calculated as previously described (*1*), and normalized by the projected surface area of one side of the cathode.

Electrochemical Analysis. Electrochemical measures were performed with a potentiostat (PC 4/750, Gamry Instrument Inc., PA). Internal resistances of the MFCs were determined using electrochemical impedance spectroscopy (EIS) with the anode chamber filled with 50 mM PBS. Cyclic voltammetry (CV) was conducted at scan rate of 10 mV/s, in the potential range from −0.8 to 0.2 V, starting at the open circuit potential (OCP). A sterile Ag/AgCl reference electrode was used as a standard constant potential. For CV scans of strain DX-1, the solution in an MFC was replaced with 50 mM PBS when the sodium acetate (1 g/L) was completely consumed. This PBS solution was then replaced with fresh medium containing 1 g/L of sodium acetate, and CVs were

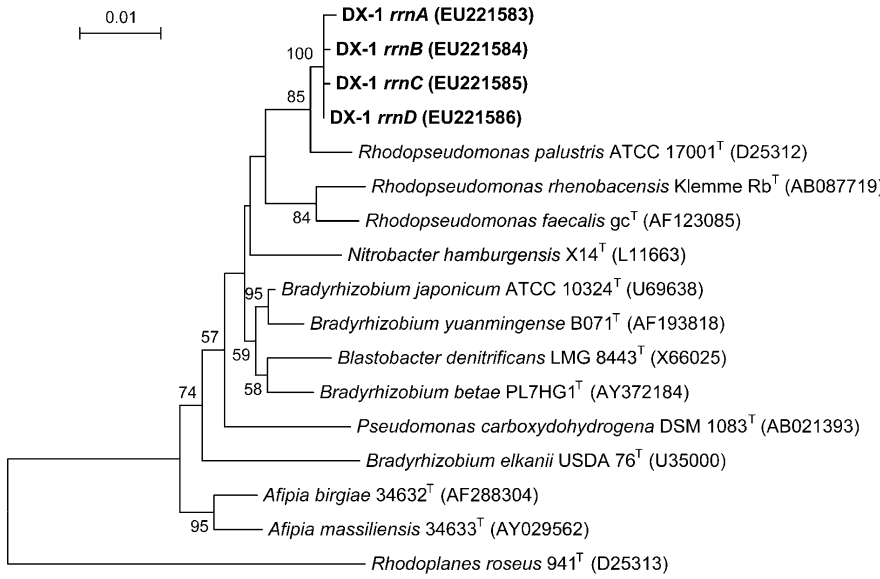

FIGURE 2. Phylogenetic tree showing the relationships between strain DX-1 and related species based on 16S rRNA gene sequence. The sequence of strain *Rhodoplanes roseus* 941^T (D25313) served as an outgroup sequence. The tree was constructed using the neighbor-joining method. Bootstrap values at nodes were calculated using 1000 replicates (only values >50% are indicated). Bar, 1% sequence divergence.

performed again. All electrochemical analyses were performed at 30 °C.

Results

Isolate Identification. Through exoelectrogen enrichment in MFCs and serial transfer by plating, we obtained an isolate (strain DX-1) that produced high power densities in an MFC with low internal resistance (~8 Ω). DX-1 is a gram-negative rod 0.5−0.8 μm wide and 1.5−2.0 μm long that is motile using a polar flagellum (Figure 1). The cells multiply by budding and form rosette-like clusters in old suspended cultures. A phylogenetic analysis based on 16S rRNA gene sequencing indicated that strain DX-1 had a closest match to *Rhodopseudomonas palustris* ATCC 17001^T (99%) (Figure 2). Strain DX-1 demonstrated characteristics of *R. palustris* (Table S1, Supporting Information). For example, cultures were dark red when grown anaerobically in the light and colorless when grown in the dark. Absorption maxima of photosynthetically grown cells indicated the presence of bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series.

Power Generation from MFCs with Carbon Paper Anodes. Strain DX-1 produced power densities similar to those of a mixed culture enrichment (wastewater inoculum) in single chamber air-cathode cubic MFCs (flat anode) fed a medium containing acetate (1 g/L) at a fixed resistance of 1000 Ω in normal laboratory light (1000 ± 50 lx) or the dark. Reactors were operated for at least 10 cycles at ambient temperature of 23 ± 3 °C (50 mM PBS), and contained anodes treated by an ammonia gas process shown to increase power densities (Figure 3a). The voltage produced in these MFCs increased over the first 5−8 cycles, and over the next cycle strain DX-1 produced maximum voltages of 0.54−0.57 V, while the wastewater inoculum produced slightly lower voltages (0.52−0.53 V). In contrast, *R. palustris* ATCC 17001 did not produce a voltage over a 500 h period. When operated in the dark, maximum voltages produced by strain DX-1 developed more slowly over the first 8 cycles, but then reached maximum voltages comparable to those produced under normal light conditions (0.54−0.55 V). Strain DX-1 formed thin biofilms but showed good coverage of the anode surface (Figure 1b).

Power density and polarization curves were obtained to determine the maximum possible power densities using this flat-anode architecture. Previous reports have shown that power densities can be increased using solutions with higher conductivities (*27*), therefore tests were also conducted with a higher buffer concentration (200 mM PBS). Strain DX-1 produced a maximum power density of 1170 ± 30 mW/m^2 (34 ± 1 W/m^3, 0.33 mA/cm^2) in the light, which was similar to that obtained with reactors kept dark (1130 ± 50 mW/m^2) with the higher buffer concentration (Figure 3b). The maximum power density of the wastewater inoculum was slightly lower (1100 ± 40 mW/m^2) in the same buffer, and in all cases power densities were reduced when using the lower (50 mM) buffer concentration. The Coulombic efficiencies (CEs) of all reactors increased with current densities, reaching a maximum of 40% in the light and 50% in the dark with strain DX-1, compared to only 30% with the wastewater inoculum (Figure 3c). The CEs are lower than reported in some studies when oxygen is not used as the catholyte (i.e., when the anode is set at a potential, or ferricyanide is used as a catholyte) due to aerobic degradation of substrate sustained by diffusion of oxygen into anode chamber (*30*). RFLP and clone libraries of the PCR-amplified 16S rRNA gene indicated that cells on the anode and in suspension in MFCs were not contaminated during these tests.

Power Generation from MFCs with Brush Anodes. Increasing the surface area of the anode has been shown to increase power production in other studies (*16, 26*). Using graphite-fiber-brush electrodes with an estimated surface area of 2235 cm^2 (95% porosity), voltages reacted a maximum of 0.55−0.57 V within four fed-batch cycles (1000 Ω resistor, 50 mM PBS, data not shown). A maximum power density of 2720 ± 60 mW/m^2 (0.99 mA/cm^2, 86.6 ± 1.8 W/m^3) was produced by strain DX-1 in the light, and 2640 ± 60 mW/m^2 in the dark (Figure 4). Power was immediately generated when the medium was changed, indicating that self-produced soluble mediators were not needed for electron transfer. These power and current densities are the highest yet achieved in any MFC with a pure culture under conditions where the anode potential is not set using a potentiostat. The wastewater inoculum with the same medium produced only 1740 ± 60 mW/m^2, compared to 2400 mW/m^2 (73 W/m^3) found in previous tests under optimal conditions using this type of MFC (*26*). The CEs for strain DX-1 reached 60% in the dark and 50% in the light, compared to 40% for the

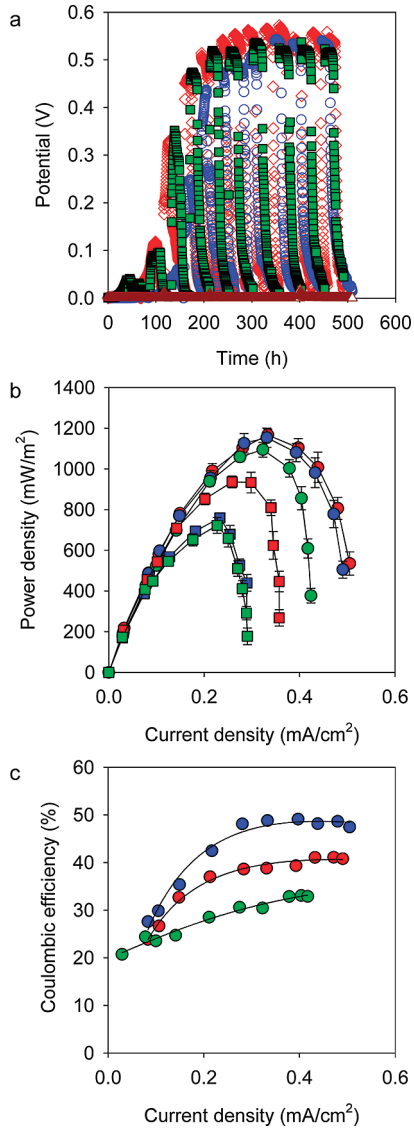

FIGURE 3. Power generation of strain DX-1 at 23 ± 3 °C in single chamber air-cathode MFCs with carbon paper anodes. (a) Potential curve (1000 Ω resistor) of strain DX-1 in the light (red) or dark (blue), *R. palustris* ATCC 17001 (brown), and wastewater inoculum (green). Each spike in potential curve is followed by refueling of the reactor with 1 g/L acetate (50 mM PBS), resulting in the next cycle of power generation. (b) Power density curve for strain DX-1 in the light (red) and dark (blue), and wastewater inoculum (green) in 50 mM PBS (squares) and 200 mM PBS (circles). Error bars are ± SD based on data collected in triplicate, from two or more separate batch experiments. (c) Coulombic efficiency for strain DX-1 in the light (red) and dark (blue), and wastewater inoculum (green) in 200 mM PBS.

wastewater (Figure 5). These CEs are approximately 10% higher than those produced in the flat-anode reactor, perhaps as a result of more bacteria attached on the anode in this system which reduces the cycle time of the reactor, and thus the mass of oxygen that can diffuse into the reactor over a fed-batch cycle.

Power Generation with Different Substrates. Strain DX-1 produced power from a wide variety of organic compounds (Figure 6), consistent with our expectations for a great metabolic diversity of substrates utilized by *R. palustris*.

Repeatable and stable cycles of power were obtained using DX-1 in MFCs having flat carbon anodes (1000 Ω; 50 mM PBS), with power densities for these substrates of 360−450 mW/m^2 using acetate, lactate, and ethanol; 50−200 mW/m^2

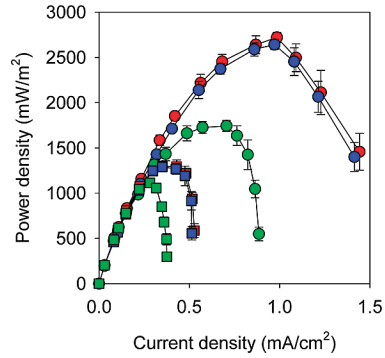

FIGURE 4. Power densities of MFCs with brush anode for strain DX-1 in the light (red) and dark (blue), and wastewater inoculum (green) in 50 mM PBS (squares) and 200 mM PBS (circles) with acetate as the electron donor. Error bars are ± SD based on data collected in triplicate, from two or more separate batch experiments.

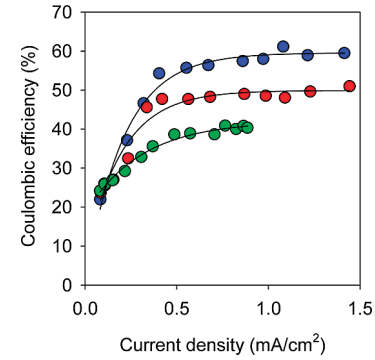

FIGURE 5. Coulombic efficiencies in MFCs using brush anodes for strain DX-1 in the light (red) and dark (blue) and wastewater inoculum (green) with acetate (1 g/L) in 200 mM PBS.

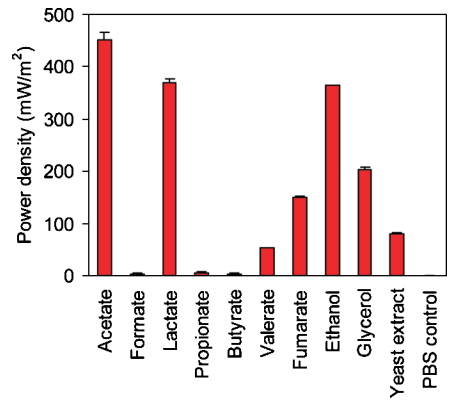

FIGURE 6. Power densities in MFCs using carbon paper anodes for strain DX-1 with different electron donors (50 mM PBS, 1000 Ω resistor). The control lacks an electron donor (PBS control). Error bars are SD based on data collected in triplicate, from two or more separate batch experiments.

with yeast extract, valerate, fumarate, and glycerol; and 3−6 mW/m^2 using formate, butyrate, and propionate (Figure 6). Power densities have been observed to vary with the type of substrate using mixed cultures in low internal resistance MFCs such as the one used here (*29*), but not in high resistance systems (*14, 20*). Strain DX-1 also utilized the inorganic electron donor thiosulfate (2 g/L), producing 0.8−5.0 mW/m^2 under the same reactor conditions (Figure 7). In contrast, a wastewater inoculum did not produce electricity under the same conditions.

Analysis of Electron Transfer Using CV. To further investigate the mechanism of exocellular electron transfer

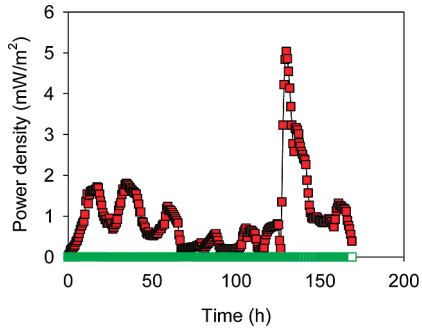

FIGURE 7. Power densities in MFCs with carbon paper anodes using thiosulfate (2 g/L) and strain DX-1 (red), compared to a wastewater inoculum (green) (25 °C, 50 mM PBS, 1000 Ω resistor).

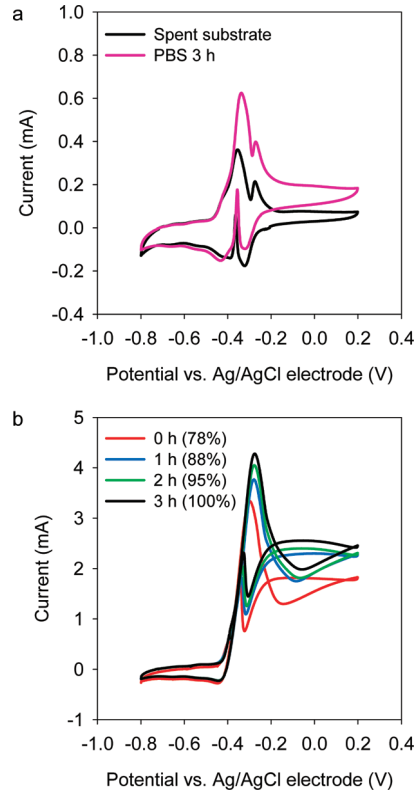

FIGURE 8. CV analysis of strain DX-1 in MFCs with carbon paper anodes: (a) CV after a complete cycle and then with fresh medium without substrate; (b) CV after the solution is replaced with fresh medium containing substrate (1 g/L acetate). The numbers in parentheses represent the percent of current recovery after the medium was changed.

of strain DX-1, the electrochemical activity of the anode biofilm in MFCs with carbon paper anodes was measured after a complete cycle in fresh medium lacking substrate and in medium with substrate (Figure 8). At the end of a batch cycle (when the substrate was fully consumed), two oxidization peaks at −0.35 and −0.27 V (vs Ag/AgCl electrode) and two reduction peaks at −0.44 and −0.32 V were observed (Figure 8a). The presences of these peaks could indicate the presence of a mediator in the MFC. However, when the solution was replaced with 50 mM PBS lacking substrate the oxidization peak height was still present, indicating that if mediators were present they were not washed out with the solution. The peak increased slightly over time likely as the bacteria adapted to the fresh solution without substrate (Figure 8a). Thus, an established biofilm was the key factor for exocellular electron transfer of strain DX-1, not mediators in solution.

When the PBS solution was replaced with fresh medium containing acetate the oxidization current immediately reached a much greater peak height of 4.28 mA at −0.28 V (vs Ag/AgCl electrode), supporting direct electron transfer as the main mechanism for current generation (Figure 8b). The oxidization current recovery was 78% at 0 h (3.34 mA), and 95% at 2 h (4.05 mA). An increase in the oxidization peak height would not be immediately observed if water-soluble mediators were needed for transfer of electrons from the cell to the surface. We conclude from these CV scans that electron transfer did not depend on the production of soluble mediators as these chemicals would have required substantial time for production, excretion, and accumulation to levels needed to produce peaks in CVs and affect power generation. Although the evidence supports direct electron transfer, we cannot rule out a possible role for water-insoluble mediators within the biofilm playing a role in power generation by strain DX-1. Further research is needed into the biophysical mechanisms of electron transfer by strain DX-1.

Discussion

These results demonstrate for the first time that a photosynthetic purple nonsulfur bacterium can transfer electrons to an electrode in an MFC and produce very high current and power densities. Strain DX-1 produced 2720 ± 60 mW/m^2 and 0.99 mA/cm^2 in a brush anode MFC, which is higher than that produced by mixed cultures in the same device. Although it has been demonstrated that another purple nonsulfur bacterium (*Rhodobacter capsulatus*) can reduce Fe(III) (*31*), this does not prove the capacity for current generation in an MFC. *Pelobacter carbinolicus*, for example, reduces iron but does not generate current (*5*). *Rhodobacter sphaeroides* produces current in an MFC but only by producing H_2 that reacts at the anode with a Pt catalyst (*32*). There was no current generation in the absence of the catalyst. Tests here with an ATCC strain of *R. palustris* failed to generate power. The finding that *R. palustris* strain DX-1 generates current in an MFC increases the diversity of high power producing exoelectrogenic bacteria to include *Alphaproteobacteria*, and provides a new strain for fundamental studies of electron transfer mechanisms to surfaces in addition to existing strains (notably, *Gamma-* and *Deltaproteobacteria* such as *Shewanella* and *Geobacter* species) (*4*).

Based on our knowledge of *R. palustris*, our findings have widespread implications for the study of exoelectrogenic bacteria and power generation in MFCs. *R. palustris* is widely distributed in soil and sediments (*33*), and metabolically a quite versatile gram-negative species. *Rhodopseudomonas* spp. are abundant in anaerobic wastewaters exposed to light (*34*), and thus they may play a dominant role in some microbial communities. It was also shown that electricity can be produced by strain DX-1 using the same wide variety of compounds that other *Rhodopseudomonas* spp. degrade (*33*) and that are typically found in many domestic and industrial wastewaters.

R. palustris has recently received much attention as a microorganism useful for bioenergy production due to its ability to produce hydrogen gas (*35*). Hydrogen production was not the reason for electricity production here, however, as no metal catalyst was used on the anode (*32*). Our finding that an *R. palustris* strain can also directly produce current is further evidence of the versatility of the microorganism, and a property that may allow high yields of hydrogen production in the absence of sunlight. It was recently shown using mixed cultures that hydrogen could be produced in microbial electrolysis cells from acetate at yields approaching 99%, and energy efficiencies of 288% based on electricity applied or 82% when the substrate was included in the energy balance (*36*). Thus, the further study of this microorganism

will be useful not only for increasing our understanding of electron transport, but also for the production of bioenergy directly as electricity or hydrogen from waste biomass.

Acknowledgments

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Note Added after ASAP Publication

There was an error in Figure 2 in the version published ASAP April 25, 2008; the corrected version was published ASAP April 30, 2008.

Supporting Information Available

Differential characteristics of strain DX-1, *R. palustris*, *R. rhenobacensis*, and *R. faecalis* shown in Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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