Simultaneous Cellulose Degradation and Electricity Production by *Enterobacter cloacae* in a Microbial Fuel Cell[∇]

Farzaneh Rezaei,¹ Defeng Xing,² Rachel Wagner,² John M. Regan,²
Tom L. Richard,¹ and Bruce E. Logan^{2*}

Department of Agricultural and Biological Engineering¹ and Department of Civil and Environmental Engineering,²
The Pennsylvania State University, University Park, Pennsylvania 16802

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Electricity can be directly generated by bacteria in microbial fuel cells (MFCs) from many different biodegradable substrates. When cellulose is used as the substrate, electricity generation requires a microbial community with both cellulolytic and exoelectrogenic activities. Cellulose degradation with electricity production by a pure culture has not been previously demonstrated without addition of an exogenous mediator. Using a specially designed U-tube MFC, we enriched a consortium of exoelectrogenic bacteria capable of using cellulose as the sole electron donor. After 19 dilution-to-extinction serial transfers of the consortium, 16S rRNA gene-based community analysis using denaturing gradient gel electrophoresis and band sequencing revealed that the dominant bacterium was *Enterobacter cloacae*. An isolate designated *E. cloacae* FR from the enrichment was found to be 100% identical to *E. cloacae* ATCC 13047^T based on a partial 16S rRNA sequence. In polarization tests using the U-tube MFC and cellulose as a substrate, strain FR produced 4.9 ± 0.01 mW/m², compared to 5.4 ± 0.3 mW/m² for strain ATCC 13047^T. These results demonstrate for the first time that it is possible to generate electricity from cellulose using a single bacterial strain without exogenous mediators.

Exoelectrogenic microorganisms can release electrons to electron acceptors outside the cell, such as iron oxides or carbon anodes in microbial fuel cells (MFCs). Members of many genera, including *Rhodospirillum rubrum* (6), *Shewanella* (13, 14), *Pseudomonas* (29), *Aeromonas* (28), *Geobacter* (2), *Geopsychrobacter* (10), *Desulfuromonas* (1), *Desulfobulbus* (9), *Clostridium* (27), *Geothrix* (3), *Ochrobactrum* (40), and *Rhodopseudomonas* (38), have been shown to produce electricity in an MFC. These bacteria have been grown on simple soluble substrates, such as glucose or acetate, that can be directly taken into the cell and used for energy production.

Cellulose is the most abundant biopolymer in the world, and there is great interest in using this material as a substrate in an MFC. However, use of a particulate substrate in an MFC has not been well investigated. Cellulose must first be hydrolyzed to a soluble substrate that can be taken up by the cell. In previous MFC tests this has required the use of enzymes to hydrolyze the cellulose into sugars or the use of cocultures or mixed cultures (32, 33, 35). For example, Ren et al. (32) used a coculture of the cellulose fermentor *Clostridium cellulolyticum* and the exoelectrogen *Geobacter sulfurreducens* to generate electricity in an MFC fed with cellulose. Analysis of the anode microbial communities in other studies of cellulose-fed MFCs showed that *Clostridium* spp. (in a biofilm) and *Comamonadaceae* (in suspension) were predominant when rumen contents were used as an inoculum (35), while a rice paddy soil inoculum (12) converged to a *Rhizobiales*-dominated anode community (more than 30% of the population).

To date, it has not been demonstrated that a single microbe can both degrade cellulose and generate current.

Conventional methods of isolating exoelectrogenic microorganisms are based primarily on identifying microorganisms that can respire using soluble or insoluble metal oxides in agar plates (20–22). However, not all dissimilatory metal oxide-reducing bacteria are capable of producing electricity in an MFC, and not all bacteria that produce current in an MFC can grow using metal oxides (5, 34). Therefore, these methods may miss important electrochemically active strains of microorganisms. A new method to isolate exoelectrogenic microorganisms was recently developed (40); this method is based on dilution to extinction and a specially designed U-tube MFC that enriches exoelectrogenic bacteria on the anode. Using this method, a bacterium that could produce electricity in an MFC but not respire using iron was isolated (40).

The main objective of this study was to isolate a bacterium capable of producing current from particulate cellulose. A cellulose-degrading consortium was diluted and serially transferred into U-tube MFCs using cellulose as the sole electron donor. Community analysis demonstrated the predominance of a single bacterium, which was isolated and compared to a culture collection strain for generation of current in an MFC.

MATERIALS AND METHODS

MFC construction and operation. The U-tube MFCs had a 10-ml anode chamber and a 30-ml cathode chamber constructed from glass anaerobic culture tubes as described previously (40). The two chambers were separated by a cation-exchange membrane (CMI 7000; 1.77 cm²; Membranes International Inc., United States) and were held together by a C-type clamp. The anode was ammonia-treated carbon cloth (type A; E-Tek, United States) with a total surface area of 1.13 cm². The cathode was made of five tow strands of 15-cm-long carbon fiber (GRANOC; Nippon Graphite Fiber Corporation, Japan) that were joined together at the top using titanium wire. The anode solution (9 ml) consisted of 50 mM phosphate buffer (PB) (2.45 g/liter NaH₂PO₄ · H₂O and 4.576 g/liter Na₂HPO₄), 0.31 g/liter NH₄Cl, 0.13 g/liter KCl, and mineral (12.5

* Corresponding author. Mailing address: Department of Civil and Environmental Engineering, The Pennsylvania State University, University Park, PA 16802. Phone: (814) 863-7908. Fax: (814) 863-7304. E-mail: blogan@psu.edu.

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ml/liter) and vitamin (12.5 ml/liter) solutions (23). To provide a better nutritional medium for enrichment of cellulolytic bacteria, autoclaved rumen fluid (30%, vol/vol) was added to the anode solution for the first 15 cycles. Pure cellulose of plant origin (type 50-50; cotton linters; particle size, 50 μ m; SigmaCell; Sigma-Aldrich Co, United States) was the primary substrate (0.4%), and it consisted of 15% amorphous cellulose and 85% crystalline cellulose (7). This model substrate has the structure of natural cellulose but consists of particles that are a defined size and have a defined composition for study. The catholyte solution (29 ml) was 100 mM potassium ferricyanide [$K_3Fe(CN)_6$] in PB (100 mM). After the reactor was assembled, both chambers were sparged with N_2 gas, sealed with a rubber stopper and an aluminum crimp top, and autoclaved prior to use for each fed-batch cycle.

Enrichment procedure. Wastewater used for the initial inoculum was obtained from a paper recycling plant (American Eagle Paper Company, Tyrone, PA). A dilution-to-extinction method was used to enrich exoelectrogenic and cellulolytic bacteria. Experiments were repeated until the community (numbers and intensities of bands) did not change for at least two consecutive cycles. U-tubes were initially inoculated with wastewater diluted 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} with medium and were connected to the circuit with a fixed resistance (1,000 Ω). After each cycle, the anode chamber suspension and the anode were transferred from each MFC into a sterile 15-ml tube (Falcon, Becton Dickinson Labware, United States) containing sterilized glass beads and vortexed. In each subsequent transfer, the homogenized suspension from the reactor containing the most dilute culture that generated electricity was used to inoculate new batches. Samples were diluted 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} for transfers 2 through 15 and 10^{-2} , 10^{-4} , 10^{-6} , and 10^{-8} for transfers 16 to 19. An additional sterile reactor (not inoculated) was used to monitor possible contamination of the growth medium during each transfer. The remaining suspension in each tube was preserved at -20°C for further analysis.

DNA extraction, PCR, and DGGE. DNA was extracted from the preserved anode suspension from the reactor containing the most dilute culture that generated power in each cycle using a PowerSoil DNA isolation kit (MO BIO Laboratories, United States) according to the manufacturer's instructions. DNA integrity was verified using a 1% agarose gel. PCR was then performed using an iCycler iQ thermocycler (Bio-Rad Laboratories, United States) to amplify the V6-V8 region of the 16S rRNA gene using the following primers (37), which included a GC clamp in the forward primer for subsequent denaturing gradient gel electrophoresis (DGGE) analysis: GC968F (5'-CGCCCCGCCGCGCCCCGCGCCCCGCCGCCCCGCCCAACGCGAAGAACCTTA C-3') and 1401R (5'-CGGTGTGTACAAGACCC-3'). The PCR conditions used have been described previously (40). PCR products then were separated by DGGE using a DCode universal mutation detection system (Bio-Rad Laboratories, United States) as described previously (31, 40).

Serial transfer was performed until the DGGE gel contained five bands that were consistent for more than two transfers. Each of these bands was excised from the gel using a sterile pipette tip and transferred to a sterile microcentrifuge tube. DNA was eluted from the bands by adding 40 μ l deionized water, crushing the gel against the side of each tube using a pipette tip, and then incubating the tubes at 4°C overnight. DNA integrity was verified using a 1% agarose gel. Two sets of PCR were performed with the eluted DNA. The first PCR was used to check the purity of each band using the PCR and DGGE conditions described above (31). After the results confirmed that there was only one band, a second PCR was performed to reamplify each band for subsequent sequencing using the same PCR primers, except that the forward primer lacked the GC clamp (primer 968F [5'-AACGCGAAGAACCTTAC-3']), and the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 60°C for 30 s, and 72°C for 1.5 min and finally 72°C for 7 min. PCR products then were purified using a QIAquick PCR purification kit (Qiagen, United States) according to the manufacturer's instructions and were sequenced using an ABI 3730XL DNA sequencer (Applied Biosystems, United States).

Cloning and sequence analysis. In addition to the DGGE analysis, extracted DNA from the last cycle was amplified using the following PCR primers to amplify nearly complete 16S rRNA genes as described previously (31, 40): 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCC AGCC-3'). The PCR conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 60°C for 30 s, and 72°C for 1.5 min and finally 72°C for 7 min. The PCR products were then cloned using a TOPO TA cloning kit (Invitrogen, United States) according to the manufacturer's instructions. The plasmids of clones were extracted and purified using a QIAprep Spin miniprep kit (Qiagen, United States) and were sequenced in both directions using an ABI 3730XL DNA sequencer (Applied Biosystems, United States). Sequences were analyzed using the GenBank database and the BLAST program, and a neighbor-joining

phylogenetic tree was constructed using Kimura's two-parameter method with the Molecular Evolutionary Genetics Analysis package (MEGA, version 3) (15).

Isolation and characterization of bacteria. Once the dominant bacterium was determined based on the DGGE band's sequences and nearly complete cloned 16S rRNA gene sequences, the corresponding type strain, strain *Enterobacter cloacae* ATCC 13047, was purchased from American Type Culture Collection (ATCC) and grown based on instructions provided by the ATCC. At the same time, we tried to isolate the bacterium directly from a mixed culture from the last cycle by plating it using the nutrients suggested by ATCC and growing it overnight. Six colonies with the same colony morphology as the culture collection strain were selected and grown on nutrient broth overnight. To confirm the purity and the similarity of the selected colonies to the dominant bacterium, DNA was extracted from each overnight suspension, and nearly complete 16S rRNA genes were amplified and sequenced as described above.

Carbon utilization characteristics of *E. cloacae* ATCC 13047^T and the isolated bacterium were determined using BIOLOG GN2 MicroPlates according to the manufacturer's instructions. The ability of the isolated strain to reduce iron was determined using insoluble hydrous ferric oxide (HFO) (100 mM) (8) with 1 g/liter cellulose and 1 g/liter glucose in anaerobic tubes incubated for 7 days at 30°C (triplicate tests). Duplicate uninoculated tubes were used as controls for contamination. Reduction of Fe(III) was measured using a ferrozine colorimetric method as described previously (24).

Electricity generation and analyses. Current and power generation in the MFCs were determined by measuring the voltage (V) every 20 min across a fixed external resistance ($R = 1,000 \Omega$, unless noted otherwise) using a data acquisition system (model 2700; Keithley, United States). Current (I) was calculated by using $I = V/R$, and power (P) was calculated by using $P = IV$. Power density and current density were normalized to the projected area of the anode. Polarization curves were obtained by using a single resistor for two complete batch cycles (250 Ω to open circuit).

The cellulose concentration remaining at the end of each batch cycle was measured using a colorimetric method (32, 33). Volatile fatty acids (VFAs) were measured at the end of each cycle using gas chromatography (19). Coulombic efficiency (CE) (the ratio of the recovered electrons as current to the total available electrons from the substrate) was calculated at the end of a cycle based on cellulose removal as described previously (33).

E. cloacae ATCC 13047^T cultures grown overnight were also examined for electricity generation using different carbon sources in PB media (inoculant, 10% [vol/vol]). The carbon sources tested were cellulose, glucose, lactate, *N*-acetyl-D-glucosamine, glycerol, and sucrose. Two controls were included, an uninoculated reactor (using the same medium and 0.4% cellulose) to ensure there was no contamination and an inoculated reactor without substrate to monitor the possibility that electricity was generated by the working medium. All media were autoclaved prior to experiments.

Nucleotide sequence accession number. The *E. cloacae* FR nucleotide sequence has been deposited in the GenBank database under accession number EU849019.

RESULTS

Exoelectrogenic and cellulolytic enrichment. U-tube reactors were run for 19 cycles after wastewater inoculation until the community was stable for at least two consecutive cycles. For cycles 1 to 15, the 10^{-4} dilution (the most dilute solution) produced electricity each time, and therefore the MFC with this dilution was used to inoculate the next series of reactors (Fig. 1A). When higher dilutions were used (cycles 16 to 19), the highest dilution (10^{-8}) did not generate any power; therefore, the next most dilute solution (10^{-6}) was used to inoculate the subsequent batch (Fig. 1B). In all 19 cycles, no power was generated with reactors lacking an inoculum.

Phylogenetic analysis. After 16 cycles, the community composition as indicated by the number and intensity of bands in the DGGE gels was constant for the next three cycles (Fig. 2). Analysis of the sequences from each of the five bands from the last cycle indicated that the top three bands were derived from members of the family *Enterobacteriaceae*, while bands 4 and 5 exhibited 100% identity to *Stenotrophomonas* sp. and

FIG. 1. Power generation for (A) the first cycle in a U-tube using four different dilutions and (B) the last (19th) cycle in a U-tube using four different dilutions.

Exiguobacterium sp., respectively (Table 1 and Fig. 3). The first band exhibited 100% identity to a *Klebsiella pneumoniae* strain recently found to be exoelectrogenic (39). Bands 2 and 3 exhibited 99% and 100% similarity to *E. cloacae*, respectively (Fig. 3). Sequences from the clone library from the last cycle were also analyzed to further identify the dominant bacterium. Phylogenetic analysis of the clone library from cycle 19 showed that all of the cloned fragments analyzed belonged to *Enterobacter* species, with *E. cloacae* ATCC 13047^T (100% identity) the dominant bacterium (Fig. 3). An isolate obtained from the mixed culture using a suspension from the last cycle had a

TABLE 1. Closest reported strain in GenBank to the sequences obtained from bands in the last cycle

Band	BLAST result	% Identity
1	<i>Klebsiella pneumoniae</i>	100
2	<i>Enterobacter cloacae</i> strain E717	99
3	<i>Enterobacter cloacae</i> strain ATCC 13047 ^T	100
4	<i>Stenotrophomonas</i> sp. strain SWCH-5	100
5	<i>Exiguobacterium</i> sp. strain ZM-2	100

colony morphology similar to that observed for the culture collection strain *E. cloacae* ATCC 13047^T, and its nearly full-length 16S rRNA gene sequence was identical to that of *E. cloacae* ATCC 13047^T. This isolate was designated *E. cloacae* FR. **Biochemical, physiological, and electrochemical characteristics of *E. cloacae* ATCC 13047^T and *E. cloacae* FR.** *E. cloacae* ATCC 13047^T is a gram-negative, facultative anaerobic, rod-shaped bacterium that is motile by means of peritrichous flagella (4). The results of biochemical characterization of *E. cloacae* ATCC 13047^T and *E. cloacae* FR showed that they both utilized the same substrates (Table 2). Although *E. cloacae* ATCC 13047^T was electrochemically active in an MFC, it did not show Fe(III) reduction using insoluble iron (HFO) with either glucose or cellulose as the carbon source. Electricity was rapidly generated from cellulose in MFCs inoculated with *E. cloacae* ATCC 13047^T or *E. cloacae* FR. The maximum current density at a fixed resistance was 119 ± 2.2 mA/m² (1.6 ± 0.006 mW/m²; R = 1,000 Ω) for *E. cloacae* ATCC 13047^T, which was slightly less than that for *E. cloacae* FR (127 ± 14 mA/m²; 1.8 ± 0.02 mW/m²; R = 1,000 Ω). The current density for the mixed culture from the last cycle was 221 ± 16 mA/m² (5.5 ± 0.03 mW/m²), which was about twice that for either pure culture at the same resistance. Polarization curves showed that the maximum power densities produced by the two strains were similar (5.4 ± 0.3 mW/m² for *E. cloacae* ATCC 13047^T and 4.9 ± 0.01 mW/m² for *E. cloacae* FR). Both values were lower than the value for the mixed culture from the last cycle (18 ± 2.2 mW/m²) (Fig. 4). In

FIG. 2. DGGE bands for the 19 cycles for the most dilute U-tube that produced electricity. Bands 1 to 5 were extracted from the gel for sequencing.

FIG. 3. Phylogenetic tree for extracted bands for the last cycle of DGGE and closely related species based on the 16S rRNA gene sequence. The tree was constructed using the neighbor-joining method. The bootstrap values at the nodes were calculated using 1,000 replicates (only values greater than 50% are indicated). Scale bar = 2% divergence.

all cases, the maximum power density was produced using a 5,000- Ω resistor.

CE was calculated based on cellulose removal for *E. cloacae* ATCC 13047^T and the mixed culture from the last cycle. The cellulose concentration decreased from 4 g/liter to 2.8 g/liter for *E. cloacae* ATCC 13047^T, and the overall CE was 14%. VFA analyses indicated that acetic acid (119 ± 14 ppm) was the primary constituent of organic matter in solution at the end of the batch culture and that there were lower concentrations of ethanol (17 ± 1.1 ppm) and propanol (19 ± 3.5 ppm). For the mixed culture, 3.1 g/liter of cellulose remained at the end of cycle, and the CE was 26%.

TABLE 2. Biochemical characteristics of *E. cloacae* ATCC 13047^T and *E. cloacae* FR

Carbon source and electron donor	Utilization by:	
	<i>E. cloacae</i> ATCC 13047 ^T	Isolate FR
Dextrin	+	+
Glycogen	+ ^a	+ ^a
N-Acetyl-D-glucosamine	+	+
D-Cellobiose	+	+
L-Arabinose	+	+
Gentiobiose	+ ^a	+ ^a
D-Glucose	+ ^a	+ ^a
D-Lactose	+	+
Sucrose	+	+
Acetic acid	+ ^a	+ ^a
cis-Aconitic acid	+	+
Citric acid	+	+
Formic acid	+ ^a	+ ^a
Lactic acid	+	+
Glycerol	+	+

^a Weak.

FIG. 4. Polarization curves used to measure the maximum power density generated in reactors inoculated with pure *E. cloacae* ATCC 13047^T, *E. cloacae* FR, and the mixed culture from the last U-tube cycle. Error bars are $\pm$ SD, based on duplicate measurements in two complete batch cycles.

The ability of *E. cloacae* ATCC 13047^T to produce electricity using other carbon sources was monitored using an initial concentration of 4 g/liter for each substrate for three complete cycles. The maximum current density, 493 ± 0.8 mA/m², was obtained with sucrose. The maximum current densities were 486 ± 30 mA/m² for glycerol, 328 ± 47 mA/m² for glucose, and 307 ± 15 mA/m² for N-acetyl-D-glucosamine. These values were all greater than the value obtained with cellulose (119 ± 2.2 mA/m²), suggesting that cellulose hydrolysis rates limited power generation (Fig. 5). *E. cloacae* ATCC 13047^T generated

FIG. 5. Current densities produced by a pure culture of *E. cloacae* ATCC 13047^T in a U-tube with different carbon sources. Error bars are $\pm$ SD, based on duplicate measurements in three complete batch cycles.

a much lower current density with lactate (62 ± 12 mA/m²), and no current was produced with acetate. A low current density (18.5 mA/m²) was generated using only PB, likely due to microbial decay (11) (Fig. 5). No power was generated in any test with reactors that were not inoculated.

DISCUSSION

Previously, conversion of cellulose to electricity in MFCs has required mixed cultures or separate microorganisms to hydrolyze cellulose and generate electricity. It was demonstrated here for the first time that *E. cloacae* could be used as the sole microorganism to accomplish both cellulose degradation and electricity generation. *E. cloacae* has been found to have endo-1,4-β-D-glucanase activity, and therefore it is able to degrade cellulose (36). However, it was not previously known that *E. cloacae* could produce electricity in an MFC in the absence of exogenous mediators. *E. cloacae* FR, isolated from a cellulose-degrading MFC in this study, generated about the same amount of power as an authenticated strain (*E. cloacae* ATCC 13047^T). It was previously reported that *E. cloacae* II-BT 08 could produce power in an MFC with a complex medium (1% malt extract, 0.4% yeast extract, 1% glucose). However, the current produced by *E. cloacae* II-BT 08 was examined using only exogenous mediators (methylene blue and methylene viologen) (25). Thus, there was no evidence of current generation in a mediatorless MFC or growth on cellulose. Although *E. cloacae* strains ATCC 13047^T and FR produced similar power densities, isolates from MFCs do not always have the same properties as cultivated strains. For example, Xing et al. (38) found that *Rhodospseudomonas palustris* DX-1 isolated from an MFC generated high power densities in an MFC, but an ATCC strain, *R. palustris* ATCC 17001, could not do this.

E. cloacae ATCC 13047^T was found to be capable of generating power using two different sugars (glucose and sucrose), as well as glycerol. Furthermore, *E. cloacae* can produce hydrogen from fermentation of various substrates, including glucose, sucrose, and cellobiose (18). However, it was found using single substrates in U-tube MFC tests that strain ATCC 13047^T could not produce much current with a common fermentation end product (lactate), and it did not utilize acetate or butyrate. It was also observed that growth of pure cultures on cellulose resulted in accumulation of various VFAs and solvents, and acetate was the predominant product. Therefore, while this strain can both degrade cellulose and produce electricity, it cannot fully utilize some breakdown products for power generation. Complete utilization of the carbon sources in an MFC, therefore, would still require addition of other microbial strains to the culture or genetic modification of *E. cloacae* to use these substrates.

While *E. cloacae* ATCC 13047^T was able to generate electricity in an MFC, it was unable to reduce solid Fe(III) oxide (HFO), and therefore it is not a dissimilatory metal oxide-reducing bacterium (20–22). This is not the first observation of current generation by a bacterium that is incapable of dissimilatory iron reduction. For example, two mutants of *Shewanella oneidensis* MR-1 (SO4144 and SO4572) were shown to produce electricity in an MFC but were not able to reduce Fe(III) oxide (5). In another study, *Ochrobactrum anthropi* YZ-1 was shown to produce electricity in an MFC but lacked the ability

to respire using iron (40). These findings reinforce the need to identify important electricity-generating bacteria in MFCs by using techniques that isolate bacteria based on their ability to generate current and not just their dissimilatory iron reduction ability. Although *E. cloacae* could not reduce Fe(III), it has been reported that this strain can reduce soluble chromate ion to Cr(III) and selenate to selenium (16, 17).

The current densities produced by the mixed culture during the last three serial transfers was higher than that produced by either strain of *E. cloacae*. The reason for this is not known, but it is likely that other bacteria in the mixed community were able to use breakdown products produced by *E. cloacae* for power generation. For instance, *K. pneumoniae*, one of the bacteria present in the mixed culture, has recently been shown to produce electricity using starch or glucose (39). It is also possible that multiple bacteria had a synergistic effect on power generation. Pure cultures have been found to produce both more (26, 38) and less (29, 30, 40) power than the mixed cultures from which they were isolated, depending on the strain and the specific MFCs used (26). Additional research is needed to understand the effects of additional bacteria on production of power by certain strains of bacteria in MFCs.

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