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A paper-based microbial fuel cell array for rapid and high-throughput screening of electricity-producing bacteria

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There is a large global effort to improve microbial fuel cell (MFC) techniques and advance their translational potential toward practical, real-world applications. Significant boosts in MFC performance can be achieved with the development of new techniques in synthetic biology that regulate microbial metabolic pathways or control their gene expression. For this new direction, a high-throughput and rapid screening tool for microbial biopower production is needed. In this work, a 48-well, paper-based sensing platform was developed for high-throughput and rapid characterization of microbial electricity-producing capability. Spatially distinct 48 wells of the sensor array were prepared by patterning 48 hydrophilic reservoirs in paper with hydrophobic wax boundaries. The paper-based platform exploited the ability of paper to quickly wick fluid and promote bacterial attachment to the anode pads, resulting in instant current generation upon loading of the bacterial inoculum. We validated the utility of our MFC array by studying how strategic genetic modifications impact the electrochemical activity of various *Pseudomonas aeruginosa* mutant strains. Within just 20 minutes, we successfully determined the electricity generation capacity of eight isogenic mutants in *P. aeruginosa*. These efforts demonstrate that our MFC array displays highly comparable performance characteristics and identify genes in *P. aeruginosa* that trigger higher power density.

1. Introduction

A microbial fuel cells (MFCs) is a promising green energy technology that utilizes microbial consumption of organic materials.[1] As such, MFCs have attracted considerable interest in numerous applications, including wastewater treatment,[2] environmental power sources,[3] desalination,[4] biogas generation,[5] and bioremediation.[6] During the last decade, a great deal of work has been done to improve MFC performance: exploring carbon sources for metabolism, optimizing operating conditions, modifying device configurations, and engineering electrodes.[7-10] Although many researchers successfully demonstrated significant increases in power density and energy efficiency, MFC performance is still insufficient for the prevailing potential applications.[2, 11] In their current form, MFCs are not a good substitute to conventional energy technologies.

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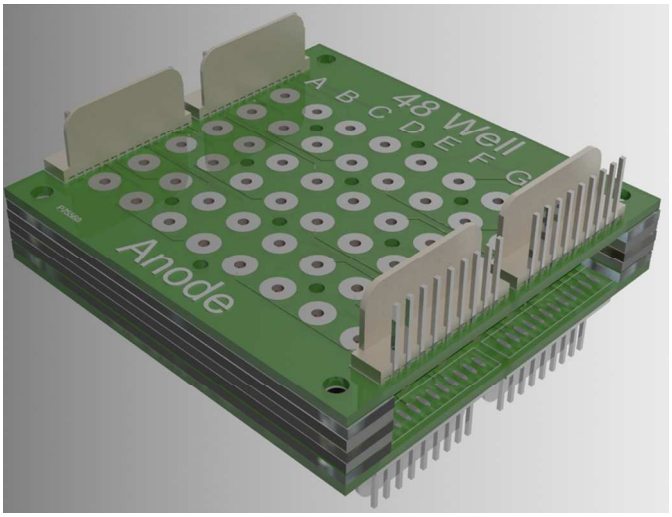

Figure 1. Schematic diagram of the assembled 48-well MFC sensor array. The customized PCB boards have direct contacts with the anode and cathode layers of the paper-based MFC array.

Significant performance breakthroughs cannot be achieved through naturally existing species without the power of genetic engineering techniques, either maximizing a specific organism's electrogenic potential, stitching together genes involved in electrogenesis from various organisms or a bacterial electrogenic consortium.[12-14] Although substantial research has been conducted on genetic engineering of microbial metabolic pathways for biofuel generation,[15-17] the genetic approaches for their higher electricity generation is quite limited to date. This is mainly due to the limitations in current screening methods for the bacterial electrical properties, while microbial biofuel-producing capacity can be readily performed by using well-established microarray techniques,[18, 19] which are widely used to monitor gene expression under different cell growth conditions and detect specific mutations in DNA sequences.[20] Microbial screening arrays for bioelectricity generation require much more complicated device configurations and fabrications, which include an active feeding system and dual chambers separated by a proton exchange membrane,[21-24] compared to the general microbial microarray including only one chamber without any electrical measurements.[25] Recently developed MFC arrays have complex MFC architectures with many tubings/channels that operate with external pumps, constraining the number of distinct wells on the array only to 24.[26] If a 24-well MFC array was designed to have a two-chambered configuration requiring individual anolyte/catholyte inlets and outlets, 96 tubing ports and fluidic pathways would have to be implemented and operated by several multichannel syringe or peristaltic pumps. The electrical contacts for electrical characterization of the MFC units may increase the complexity of the device architecture. Furthermore, each MFC unit requires long start-up times for bacterial accumulation and acclimation as biofilms adhere to the anode.[22] These limitations have motivated us to develop a new conceptual MFC array, such that the high-throughput and rapid power assessment can be significantly improved with a compact and simple device design.

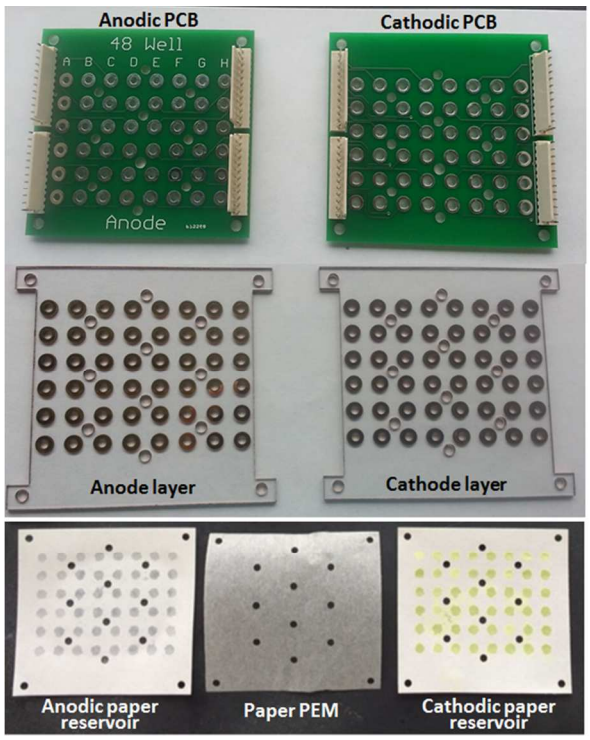

Figure 2. Photo-images of individual layers of the 48-well sensor array

In this work, we introduce a paper-based microbial sensor array as a high-throughput, rapid screening tool for microbial electricity generation study. A 48-well MFC array was fabricated on paper substrates, providing 48 high-throughput measurements and highly comparable performance characteristics in a reliable and reproducible manner. The MFC array was developed by simply patterning hydrophilic reservoirs in a paper substrate with hydrophobic wax boundaries. Within just 20 minutes, the electricity generation capacity of the selected bacterial electrogens was successfully characterized. This paper-based 48-well MFC array did not require external pumps/tubings and represents the most rapid and the highest throughput test platform for electrogenic bacterial screening. This work will expose the potential realization of a practical tool for efficient high-throughput bacterial screening and fundamental bacterial electrogenic understanding.

2. Materials and methods

2.1 Device fabrication

Fig. 1 shows the assembled 48-well paper-based MFC array. Spatially-distinct 48 wells of the MFC array consisted of seven functional layers: anodic/cathodic printed circuit boards (PCBs), anode/cathode layer (Au/Cr/Cu on polymethyl methacrylate (PMMA)), anodic/cathodic paper reservoir layers, and a paper-based proton exchange membrane (PEM) (Fig. 2 & Fig. 3). All layers were carefully aligned and clamped with 4 large screws at the corners and 10 smaller screws in the middle (Fig. 4a). We

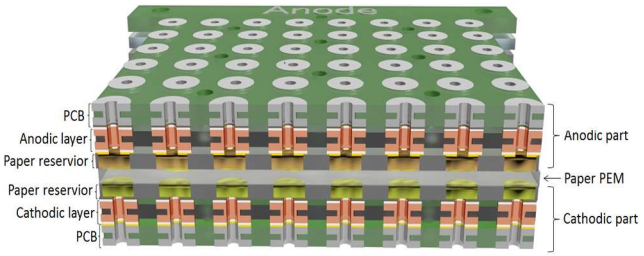

Figure 3. Schematic diagram of a cross section of the array showing individual layers

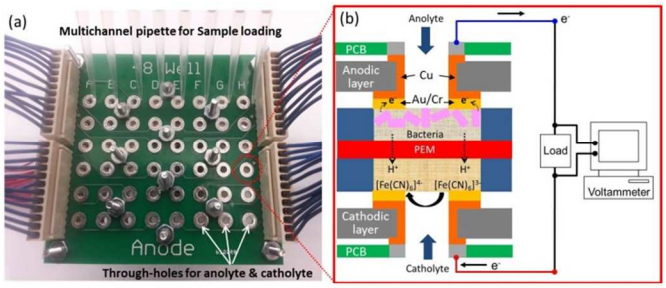

Figure 4. (a) Photo-image of the assembled sensor array and (b) schematic diagram of individual MFC unit. Voltage recordings from all 48 wells were conducted by connecting 120Ω load resistor to each well through customized LabView interface.

used a commercial solid wax printer (Xerox® ColorQube 8570DN-37) to rapidly deposit hydrophobic wax on paper (Whatman #1 filter paper). The paper was then heated to re-melt the wax that penetrates through the paper to generate complete hydrophobic barriers.[27-29] The customized PCB board (80 x 85.68 mm) was designed to simplify the electrical contacts from 48 MFC units and measure electricity generation of each well in the array. The anodic/cathodic PCB boards had 1.63 mm-wide metal pads with 48 through-holes in the center to introduce anolyte and catholyte. The metal pads had direct contacts with the anode/cathode layer (Fig. 3). There were 24-pin holes on each side of the board for four 12-pin wire connectors (Fig. 1 & Fig. 4a). PMMA substrates for anode and cathode layers were initially patterned by micromachining (Universal Laser System VLS 3.5). By utilizing magnetron sputtering, 500 nm-thick Cu was deposited on both the front and back sides of the anode/cathode layers to cover the side-walls of the 48 holes (Fig. 4b). Finally, 100 nm gold was deposited with 20 nm Cr as an adhesion layer over the back side of Cu layer by E-beam evaporation. Gold has been widely used as a potential MFC anode material because it is biocompatible, highly conductive, and compatible with conventional microfabrication techniques.[21] A commercially available parchment paper was used as a PEM because of its unique advantages: (i) the paper PEM is cheap, thin, and easy to handle, (ii) it is hydrophobic enough to physically separate the anolyte and catholyte, and (iii) its porous structure allows for proton transportation across the paper.[30]

2.2 Anolyte and catholyte

Eight isogenic mutants of *P. aeruginosa* were prepared and their electricity generations were compared with two known electrogens, wild-type *P. aeruginosa* and wild-type *Shewanella oneidensis*. Two negative controls (water and media) were also included for the performance comparison. The eight isogenic mutants were (i) *pmpR* (encoding a negative regulator of the *Pseudomonas* quinolone signal (PQS) involved in the process of quorum sensing), (ii) *rpoS* (encoding the stationary phase sigma factor, RpoS),[31] (iii) *lasR* (encoding LasR, the master regulator of quorum sensing),[32] (iv) *pilT* (encoding PilT, that, when absent, allows for overproduction of type IV pili that are incapable of retraction and thus are always fully extended, thereby enhancing mediatorless electrogensis),[33] (v) *bdlA*, (encoding BdlA, protein involved in biofilm dispersion),[34] (vi) *katA*, (encoding KatA, the major catalase, that functions to detoxify H_2O_2,[35] and buffer anaerobically produced nitric oxide (NO)),[36] (vii) *phzS* (a strain that overproduces the electrogenic mediator, pyorubrin), and (viii) *fliC pilA* (a strain that lacks the surface appendages, flagellum and type IV pili).[37] The mutants were generated using classical allelic replacement techniques with sucrose counter-selection as described by Hoang et al.[38] While *S. oneidensis* can theoretically conduct extracellular electron transfer via three mechanisms; (i) direct electron transfer, (ii) shuttle transfer, and (iii) nanowire transfer,[39] *P. aeruginosa* produce phenazine electron shuttles such as pyocyanin and pyorubrin that can aid in electron transfer to electrodes.[40] Normally, bacteria that rely only on chemical shuttles for their electron transfer (e.g., *P. aeruginosa*) cannot produce high current/power density because the diffusion rates of the shuttles significantly limit the rate of the electron transfer.[39, 41] However, *P. aeruginosa*, a Gram-negative bacterium that is virtually ubiquitous in nature, is metabolically voracious relative to the other electrogenic organisms. The rationale for using *P. aeruginosa* is that it is one of the world's most metabolically versatile organisms, able to utilize well over 300 different carbon sources for growth. Also, it is one of the leading organisms studied by researchers world-wide in the important field of biofilm research (a necessary component for mediatorless electrogenesis) and the genes necessary for biofilm (1) attachment, (2) development, (3) maturation, (4) matrix formation, (5) dispersion are becoming well understood.[42, 43] The physiological characteristics of the organism are well established, in particular its electron transport system and its abilities to grow anaerobically, such that the organism can be engineered for optimal generation of electrical power. One of our hypotheses is that we can engineer *P. aeruginosa* so that it generates significantly higher power density than their genetically unaltered counterparts. All species were grown in L-broth medium (10.0 g tryptone, 5.0 g yeast extract and 5.0 g NaCl per liter). we used a common rich medium known as Luria-Bertani (LB) broth. This medium allowed for robust growth of all bacteria used in our study. In fact, the bacteria can divide as rapidly as once every 30 minutes under optimal conditions. LB media includes tryptone, an assortment of peptides generated by the digestion of casein by trypsin and yeast extract. In this case, especially given the overall complexity of this medium, it is difficult to show the

electrochemical reactions in the anodic chamber. Each sample was loaded on four anodic chambers for generating error bars. The catholyte was 50 mM ferricyanide in a 100 mM phosphate buffer in which the pH was adjusted to 7.5 ± 0.2 with 0.1 M NaOH. Protons released by bacteria catabolism travel through the PEM towards the cathode. At the cathode, ferricyanide, $[Fe(CN)_6]^{3-}$, captures the electrons (Eq. (1)) and the cycle is completed.

$$[Fe(CN)_6]^{3-} + e^- \rightarrow [Fe(CN)_6]^{4-} \quad \text{-------- (1)}$$

The cathodic potential mainly depends on $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ ratios.

2.3 Measurement setup

We measured the potentials between the anodes and the cathodes with a data acquisition system (National instrument, USB-6212), and recorded the readings every 30 sec via a customized LabView interface (Fig. 4b). An external resistor (120 Ω) was connected between the anode and cathode to obtain the current flow through the resistor by Ohm's law (I = V/R). Anodic inocula (30 µl) were injected by an 8-channel pipette and quickly absorbed in the hydrophilic region of the paper reservoir (Fig. 4a). The device was operated under 40% relative humidity and 30℃.

3. Results and discussion

3.1 MFCs on paper

We begin the discussion of our previous reports with an overview of the fundamental principles behind a paper-based MFC.[30, 44-46] Therefore, a basic understanding of MFCs is first required. MFCs are typically comprised of anodic and cathodic chambers separated by a PEM so that protons can pass from the anode to the cathode. A conductive load connects the anode and cathode to complete the external circuit. Microorganisms oxidize organic materials in the anodic chamber, interrupting respiration by transferring electrons to the anode. Typically, the MFCs are prepared on solid-state substrates such as glass, plastic, or silicon wafers with commercial PEMs.[47, 48] Recently, we found that using a paper anode/cathode reservoir instead of the usual rigid materials allows for rapid adsorption of bacteria-containing liquid.[30, 44-46] This adsorption immediately promotes bacteria cell attachment to the electrode, where bacterial respiration can then transfer electrons from the organic liquid to the electrode. The paper-based MFCs can therefore show a very short start-up time relative to conventional MFCs, since paper substrates eliminate the time that traditional MFCs require to accumulate and acclimate bacteria on the anode. Moreover, existing MFCs have a relatively large anode chamber depth ranging from several millimeters to hundreds of centimeters. Paper-based MFCs, by contrast, use hundreds-of-micrometer-thick filter paper as a reservoir (anodic chamber). In these applications, paper is used primarily as a reservoir for holding the anolyte or catholyte, or as a microfluidic channel for transporting those chemicals and bacterial cells.

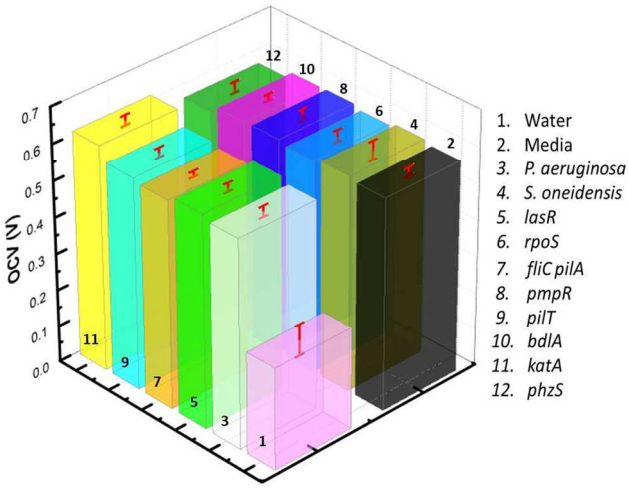

Figure 5. Open circuit voltages (OCVs) of ten bacterial species with two negative controls.

3.2 Open circuit voltages (OCVs)

The open circuit voltages (OCVs) from each MFC unit in the array were first compared. No bacteria were in the anode reservoirs for these experiments, in which the cathode potentials were controlled by ferricyanide at ~300 mV. The OCVs of the 48 MFCs increased and reached a value of approximately 540 mV with less than 2.5% variation, which is far less than that of other MFC array (25%).[22] After we confirmed that our device had such a low percent deviation, we began our experiment with anodic samples. Twelve samples were prepared including (1) water, (2) LB media, (3) *P. aeruginosa*, (4) *S. oneidensis*, (5) *lasR*, (6) *rpoS*, (7) *fliC pilA*, (8) *pmpR*, (9) *pilT*, (10) *bdlA*, (11) *katA*, and (12) *phzS*. The OCVs were first recorded for 3 minutes (Fig. 5). Each sample was loaded on four MFC units in the array and the experiment was repeated twice. Although the OCV should have the overall cell electromotive force (emf), the OCV is substantially lower than the cell emf because of energy loss of the MFC system. In this work, the OCVs ranged from 570 to 618 mV while the MFC unit with water as an anolyte showed very low OCV. The measured voltages varied slightly between the MFC units, which clearly indicates that energy losses of the MFC units according to the bacterial species are consistent. It should be noted that the initial OCV value of the #2 media sample without bacteria is comparable to those of other samples with bacteria, suggesting that the initial OCVs are independent of bacterial strains in our experiments.

3.3 Bacterial bioelectricity generation

Figure 6 shows current generation after the anodic samples and catholyte were loaded on to the MFC array. After measuring the OCVs under no-load conditions for 3 minutes, the 120 Ω resistors were connected to enable current production and the voltage differences under the resistor were recorded. The current with the external resistor gradually decreased for 20 minutes due to the depletion of the solution. The comparison of the current generations from each sample was made two times, the first after 1 minute of operation and the second at 20 minutes (Fig. 6). The current generated from negative controls

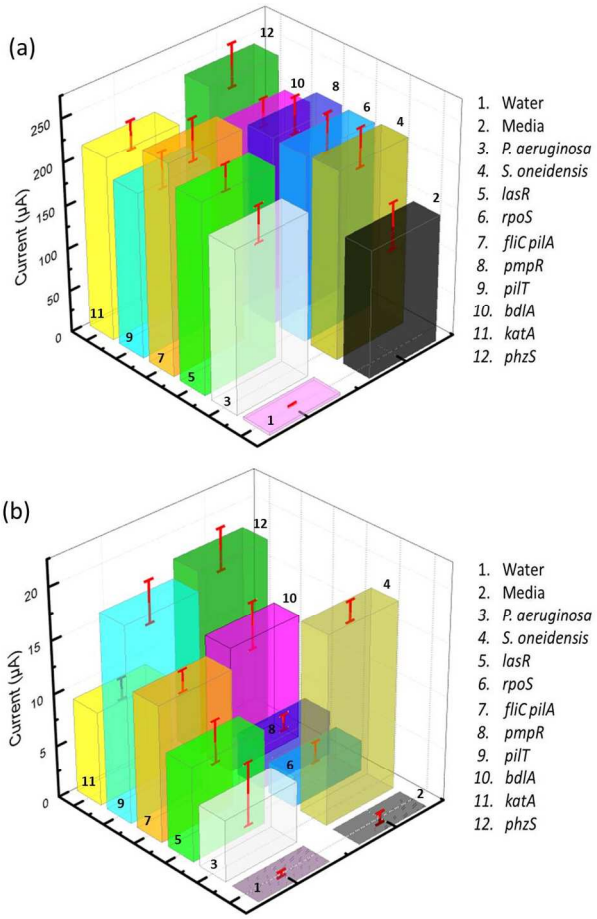

Figure 6. Currents calculated from voltage measurements in (a) 1 min. and (b) 20 min. At 1 min, the *fliC pilA* mutant has higher current generation followed by *phzS*. At 20 min, however, the *pilT* mutant showed the highest current generation.

(#1 & #2 in Fig. 6) showed distinct differences from bacterial samples, indicating that the current was generated by bacterial metabolism. The media-only sample produced a certain amount of current at 1 minute (Fig. 6a, #2) because chemical ions present in the media contributed to the current generation. However, the value shortly reached zero due to their depletion.

At 1 minute, the *fliC pilA* and *phzS* mutants generated higher electric currents while the wild-type *P. aeruginosa* and the *pilT* yielded a lower current. However, most samples have small differences between the various species used in terms of current generation, leading to difficulties in determining which bacterial species are superior with respect to their current generation. This might be due to the fact that instant current generation in Fig. 6a is attributed to the mixture of ionic current from the media and initial bacterial electron transfer.

At 20 minutes, Fig. 6 (b) clearly shows significant differences between MFC units due to the fact that the current generated from the chemical ions in media-only sample was limited. The output data demonstrated the differences in the electricity generation capabilities of each microorganism, showing that our device proves useful for bacterial screening and

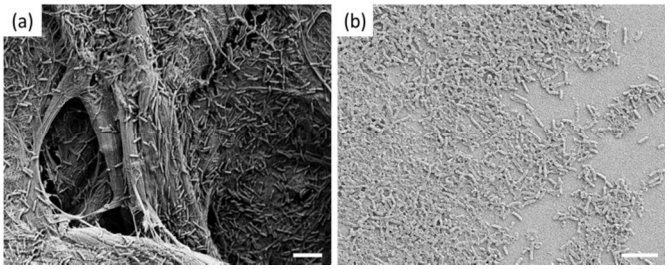

Figure 7. SEM images of the (a) paper reservoir and (b) gold anode used in the paper-based MFC array after current generation with *S. oneidensis*. Scale bar is 5 μm. A large number of bacteria cells were found on both layers.

characterization even after this relatively short operation time compared to previous MFC array techniques which require longer periods of time (several days to weeks).[1]

3.4 Characterization of 8 isogenic mutants

The *pilT* mutant of *P. aeruginosa* displayed the highest current generation, even higher than that of *S. oneidensis*. This finding is quite interesting because reducing twitching motility increases biofilm formation via hyperpiliation, thereby increasing the current generation due to overproduction of electron-conductive pili that are incapable of retraction. This is in good agreement with our previous report.[22] Also, the *phzS* mutant produced much higher current than that of the wild-type bacteria. The *phzS* mutant is known to generate the merlot-colored redox cycling agent pyorubrin at very high levels.[49, 50] The higher than wild-type production of current by *lasR*, *fliC pilA*, and *phzS* mutants may be explained as follows. First, the *lasR* and *fliC pilA* mutants are known to have enhanced production of the mediator pyocyanin. In contrast, the *phzS* mutant cannot produce pyocyanin but it overproduces pyorubrin. This work validates the utility of our MFC array by studying how strategic genetic modifications impact the electrochemical activity of bacteria.

The SEM images of the paper reservoir and gold anode layer with sample #4, *S. oneidensis*, were obtained (Fig. 7). A large amount of bacterial cells were observed both on the paper reservoir and on the gold anodic layer. Since we used a large pore-sized paper (Whatman #1; ~10 μm) to allow bacterial cells (1~3 μm in length and ~1 μm in width) to be transported through the paper matrix, high titers of the bacteria could reach the anode. The strong capillary force of the paper allowed for rapid adsorption of bacterial samples and promoted aggregates of bacterial cells in the paper matrix, enabling many cells to attach to the gold anode. More efficient bacterial movement within a paper matrix can be controlled by the paper's pore size and its hydrophilic area. A better understanding of bacterial cell movement through the paper layer will be important for achieving a high performance paper-based MFC array, because the more bacterial cells that attach to the surface, the higher energy can be produced by the MFC unit.

4. Conclusion

We created a 48-well paper-based MFC array for studies of selected hypothesis-driven genes in *P. aeruginosa*. We rapidly determined eight isogenic mutants' electricity-generating capabilities. The device was successively fabricated on paper and operated without using external pumps and tubing. The use of paper decreased the operating time considerably, and reduced total cost. This paper-based MFC array can be easily directed toward the development of a much higher throughput array by simply patterning hydrophilic reservoirs in paper with hydrophobic wax boundaries. The array will promote and accelerate the discovery and characterization of electrogenically active microbes, which will help us to translate MFC technology from the bench-top setting to practical applications that demand and produce high performance.

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