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Characterization of Electrogenic Gut Bacteria

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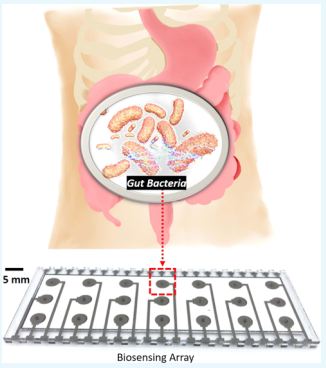
ABSTRACT: While electrogenic, or electricity-producing, Gram-negative bacteria predominantly found in anaerobic habitats have been intensively explored, the potential of Gram-positive microbial electrogenic capability residing in a similar anoxic environment has not been considered. Because Gram-positive bacteria contain a thick non-conductive cell wall, they were previously believed to be very weak exoelectrogens. However, with the recent discovery of electrogenicity by Gram-positive pathogens and elucidation of their electron-transfer pathways, significant and accelerated attention has been given to the discovery and characterization of these pathways in the members of gut microbiota. The discovery of electrogenic bacteria present in the human gut and the understanding of their electrogenic capacity opens up possibilities of bacterial powered implantable batteries and provide a novel biosensing platform to monitor human gastrointestinal health. In this work, we characterized microbial extracellular electron-transfer capabilities and capacities of five gut bacteria: *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus agalactiae*, *Lactobacillus reuteri*, and *Lactobacillus rhamnosus*. A 21-well paper-based microbial fuel cell array with enhanced sensitivity was developed as a powerful yet simple screening method to accurately and simultaneously characterize bacterial electrogenicity. *S. aureus*, *E. faecalis*, and *S. agalactiae* exhibited distinct electrogenic capabilities, and their power generations were comparable to that of the well-known Gram-negative exoelectrogen, *Shewanella oneidensis*. Importantly, this system was used to begin a large-scale transposon screen to examine the genes involved in electrogenicity by the human pathobiont *S. aureus*.



1. INTRODUCTION

Electrogenic bacteria, or exoelectrogens, are a group of microorganisms that, under anaerobic or microaerobic conditions, can transfer electrons extracellularly across the cell envelope to or from electron acceptors including electrodes, oxide minerals, and other bacteria.[1−3] Through extracellular electron transfer (EET), some microorganisms can acquire energy for their growth and reproduction or facilitate cell-to-cell communication between the cells for essential syntrophic interactions within microbial communities.[4,5] A detailed understanding of the regulation of these pathways and subsequent experimentation into the harnessing EETs could revolutionize new renewable energy and electro-synthetic technologies. In particular, the most widely applied technology of the bacterial EET is the microbial fuel cell (MFC), wherein the microorganisms generate power by using the electrons harvested from the EET.[1,3,4] Until recently, in-depth scientific studies of EET primarily examined a narrow range of electrogenic Gram-negative bacteria because Gram-positive bacteria were believed to be much less electrogenic because of their thick and potentially non-conductive cell envelopes.[6,7]

Reports published in 2018 demonstrated the electrogenic capability and the EET pathways of some Gram-positive bacteria, including *Listeria monocytogenes* and *Enterococcus faecalis*.[8−10] Since then, more attention has been paid to the potential of the electrogenicity of gut bacteria with a growing appreciation of their role in human health and disease.[11] A couple of Gram-positive gut bacteria has been further explored to see whether they exhibit EET.[12−14] In addition, the electrogenicity of the mouse gut microbiota cultured *in vitro* and directly *in vivo* was electrochemically demonstrated.[15,16]

The predominant bacterial groups in the human gastrointestinal tract are obligate and facultative anaerobes, comprising mainly Gram-positive Firmicutes and Gram-negative Bacteroidetes phyla. The groups function in the oxygen-poor but nutrient-rich environment that provides optimal conditions for the anaerobic microbial EET process.[7,8,17,18] An in-depth understanding of the electrogenic capacity of individual bacterial species and the current outputs generated from the syntrophic metabolism in gut microbial communities may provide a novel biosensing platform to electrically monitor human gastrointestinal health. This knowledge will open up possibilities of bacterial powered batteries in other fields of implantable health care research.

Thus far, only a handful of gut bacterial species have been studied by using a three-electrode electrochemical setup with a precisely controlled applied potential, which is mainly limited to

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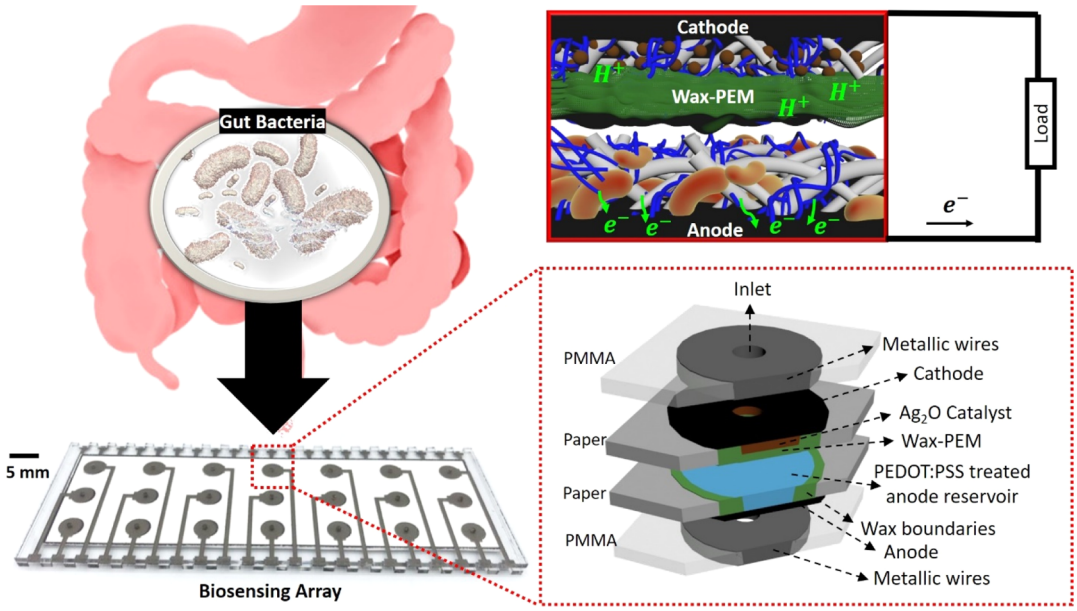

Figure 1. Conceptual image of the proposed work on the characterization of electrogenic gut bacteria (right, top). A photo of the paper-based MFC array (left) and schematic diagrams of an individual MFC sensing unit (right, bottom).

demonstrating whether and how individual species perform exoelectrogenic activities. Unfortunately, individual research focuses on a single species, and this fragmented approach supports unbalanced comparisons and views of gut bacterial electrogenicity. Furthermore, their capacity to produce electrical power outputs for practical use in two-electrode MFCs has never been demonstrated.

Here, by using a 21-well paper-based MFC array, we evaluated the electrogenic capabilities and capacities of five bacterial species, *Staphylococcus aureus*, *E. faecalis*, *Streptococcus agalactiae*, *Lactobacillus reuteri*, and *Lactobacillus rhamnosus*, in three different growth phases (lag, exponential, and stationary). All of these species are known to colonize human mucosal surfaces, including the gastrointestinal tract (Figure 1). Optimized microbial media for each species were used as the negative controls, while well-known electrogenic Gram-negative *Shewanella oneidensis* MR1 was tested as the positive control.

The high-performance paper-based MFC array was achieved by improving the bacterial electron exchange with the electrodes in an engineered conductive anodic reservoir and reducing cathodic overpotential by using silver oxide. The paper-based sensing array allowed a rapid, sensitive, and high-throughput evaluation of the bacterial electrogenicity and capacity from a single drop of bacterial culture. In the growth conditions tested, *L. rhamnosus* showed no electrogenicity, while *L. reuteri* produced very little current and power. *S. aureus*, *E. faecalis*, and *S. agalactiae* in the stationary growth phase showed distinct electrogenic capabilities compared to the negative control, and their power generations were comparable to that of Gram-negative *S. oneidensis*.

As significant electricity was produced by these organisms, we used this novel system to examine electrogenic pathways in *S. aureus* by screening transposon mutants for altered electrogenic potential. Numerous transposon mutants were found to have increased or decreased electrogenicity compared to a wildtype strain and an unrelated transposon mutant, indicating that this system can be used to help elucidate novel electrogenic pathways in important human pathobionts.

2. RESULTS AND DISCUSSION

2.1. Paper-Based MFC Array. A paper-based MFC array has been gradually recognized as an innovative sensing tool for reliable, fast, and high-throughput characterization of bacterial electrogenicity. The safe disposability of the paper-based devices by incineration prevents the potential risk of exposure to bacterial infections.[3] Furthermore, the capillary-based wicking capabilities of paper avoids energy- and labor-intensive fluidic pumping systems, leading to simple, easy, and cost-effective device operation.[19,20] Also, their rapid and sensitive power assessment of electrogenic bacteria even from a sample as small as a microliter is revolutionarily achieved by improving bacterial adhesion and current outputs with the development of a conductive polymeric anodic reservoir and a low overpotential cathode.[20−22] Our previous 48-, 64-, and 96-well MFC arrays were leveraged to create a simple and compact 21-well MFC array to be suitable for 21 test samples: 18 samples of *S. aureus*, *S. agalactiae*, *L. reuteri*, *L. rhamnosus*, *E. faecalis*, and *S. oneidensis* in three different growth phases, and three media samples of Todd-Hewitt broth (THY), De Man, Rogosa and Sharpe (MRS), and Luria Bertani-broth (LB) (Figure 1).[23−25] Five sets of the 21 samples were tested with different MFC arrays for assessing the repeatability and reproducibility of the data.

2.2. Bacteria Loading and Operating Principle. *S. aureus* is a major opportunistic human pathobiont, causing a large burden of morbidity and mortality.[26] *S. agalactiae* is a commensal of the human gastrointestinal and rectovaginal tracts and is present in 15−30% of healthy adults but can also be an invasive pathogen in infants and the elderly.[27] *E. faecalis* are also a subdominant group of the gastrointestinal microbiota, known as a prevalent multidrug-resistant nosocomial lactic acid pathogen.[6,28] On the contrary, *L. reuteri* and *L. rhamnosus* are well-known probiotic bacteria that can colonize the gastrointestinal tract, mainly providing antimicrobial activity and inhibiting colonization by pathogenic microorganisms or gastrointestinal disorders.[29,30] The electrogenicity and EET pathways of *E. faecalis* have been recently elucidated, demonstrating that other Gram-positive lactic acid bacteria

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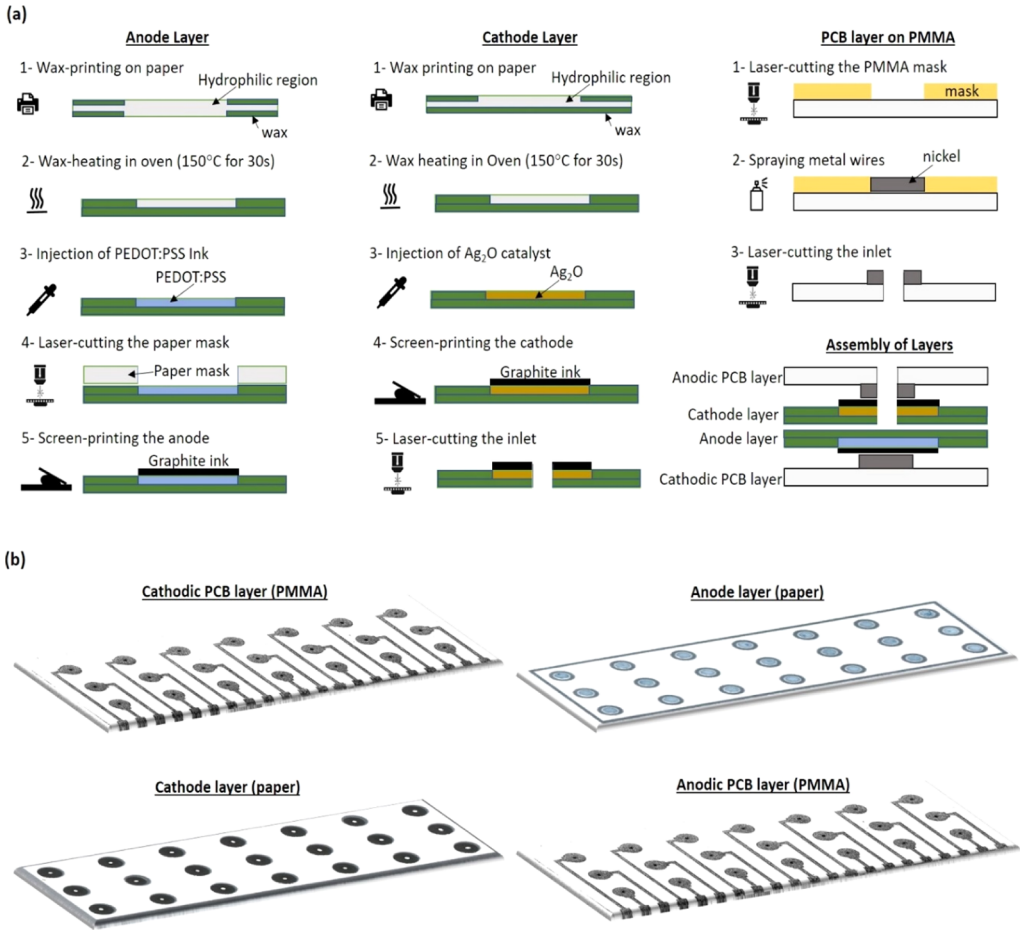

Figure 2. (a) Schematic illustrations of the fabrication processes used to create the MFC array integrating an anode layer, a cathode layer, and PCB layers. (b) Photos of the individual layers.

might also be electroactive.[9,10,31] Here, *E. faecalis* was included in the test set as a positive control electrogenic Gram-positive bacterial species. Electrogenic capability and capacity of other Gram-positive bacteria in the test set, including *S. aureus*, *S. agalactiae*, *L. reuteri*, and *L. rhamnosus*, have not been evaluated and no details about the EET processes are available. This work is to examine their potential for electrogenicity and measure their capacity quantitatively by the paper-based MFC array. Each of the 21 samples was added into a separate well of the MFC array (Figure 1). Identical batches were taken from each of the 21 samples and tested on five different MFC arrays to demonstrate the reproducibility, which is indicated with error bars in the data. Based on the growth curve of each bacterial strain, each sample was prepared in ∼10 μL volume in the lag, exponential, and stationary phases. After sample introduction onto the array, 30 min were allowed to pass for bacterial cells to become attached and acclimated to the conductive paper fibers. The hydrophilic and porous paper reservoir could allow sufficient nutrient exchange to support the growth and metabolism of the bacteria (Figures 1 and 2). Electrogenic bacteria can perform their respiration and acquire energy by transferring the electrons to the anode and further moving them to the cathode through the external circuit. At the cathode, Ag_2O can be reduced to Ag by the electrons that traveled from the anode. Therefore, measuring the current flow from the anode to the cathode can determine the bacterial electrogenicity and their power capacity.

2.3. Electrogenic Characterization. The polarization and power curves as a function of current (I) were acquired by varying the external resistance (R_{ext}) when the voltage output (V_{out}) was stable (Figure 3). The power (P) was calculated by the following equation

$$P = \frac{V_{out}^2}{R_{ext} I}$$

The power and current density were obtained by normalizing the power and current outputs to the anode surface area. Figure 3 shows the polarization curves and power outputs of six bacterial samples in three different growth phases and their specific medium control without bacteria. Figure 4 summarizes their open circuit voltage (OCV) values and maximum power densities extracted from Figure 3. Overall, *S. aureus*, *E. faecalis*, and *S. agalactiae* in their stationary phase exhibited increased electrogenic capability compared to their cell-free medium, generating 25.48, 17.12, and 13.25 $\mu W/cm^2$, respectively. Their capacity was comparable to that of the positive control, *S. oneidensis* (21.5 $\mu W/cm^2$). Surprisingly, the electrogenic performance of *S. aureus* was significantly larger than that of the well-known exoelectrogen, *S. oneidensis*. *L. rhamnosus* shows a small amount of electrogenic capability, while *L. reuteri* did not indicate any electrochemical activity in the conditions tested.

During the lag phase of the bacterial growth cycle, the cells synthesize proteins, enzymes, and other molecules necessary for cellular activity and replication while less cell division occurs.

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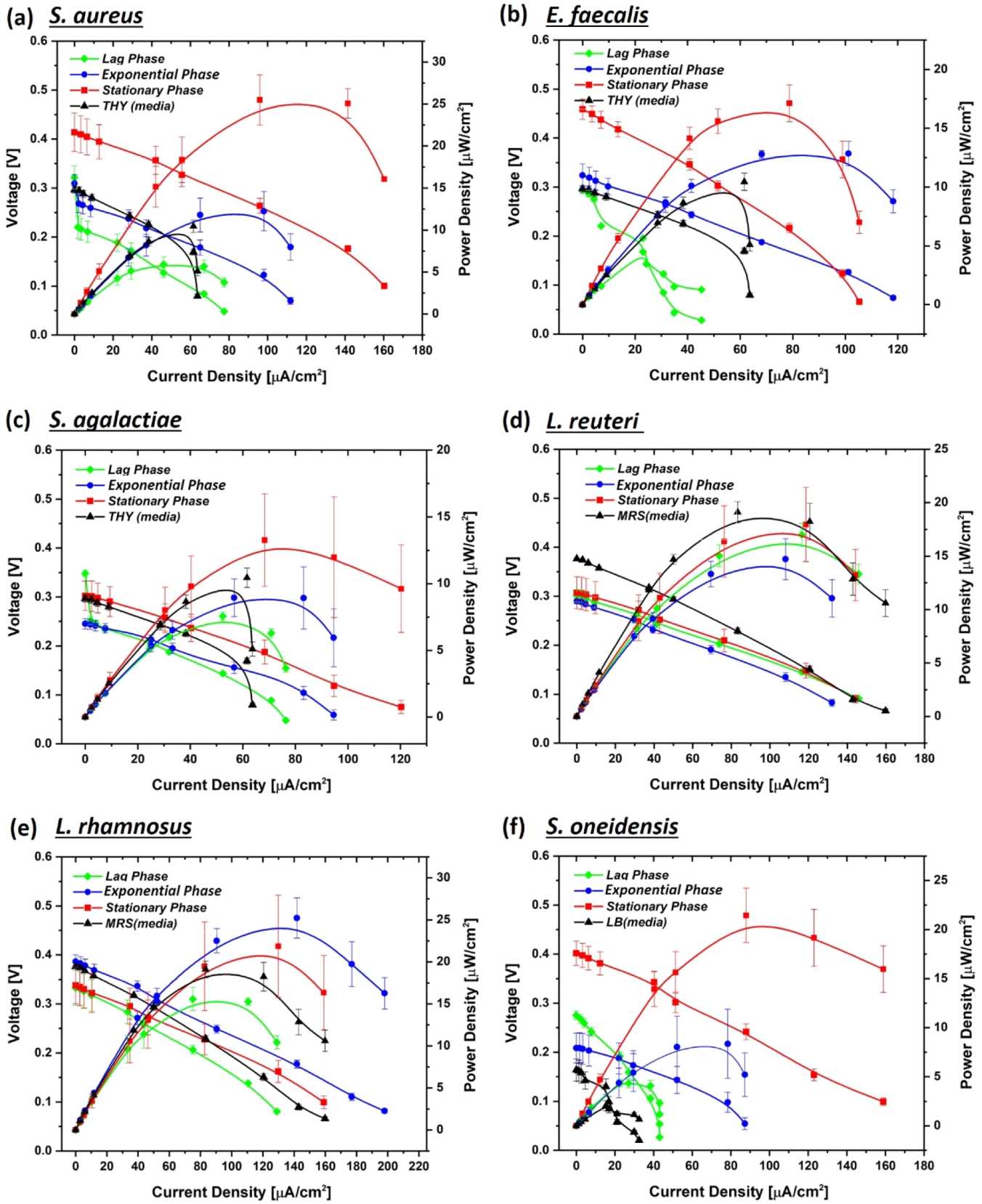

Figure 3. Polarization curves and power outputs of the five gut bacteria and the well-known exoelectrogen, *S. oneidensis* in three different growth phases: lag, exponential, and stationary phase. (a) *S. aureus*, (b) *E. faecalis*, (c) *S. agalactiae*, (d) *L. reuteri*, (e) *L. rhamnosus*, and (f) *S. oneidensis*.

Therefore, the OCV and maximum power density in this phase for the five gut bacteria are, unsurprisingly, comparable to or lower than those with medium only. The Gram-negative *S. oneidensis* in the lag phase shows a little bit stronger performance than the negative control. In the exponential phase, the cells are actively growing and dividing and their metabolic activity is

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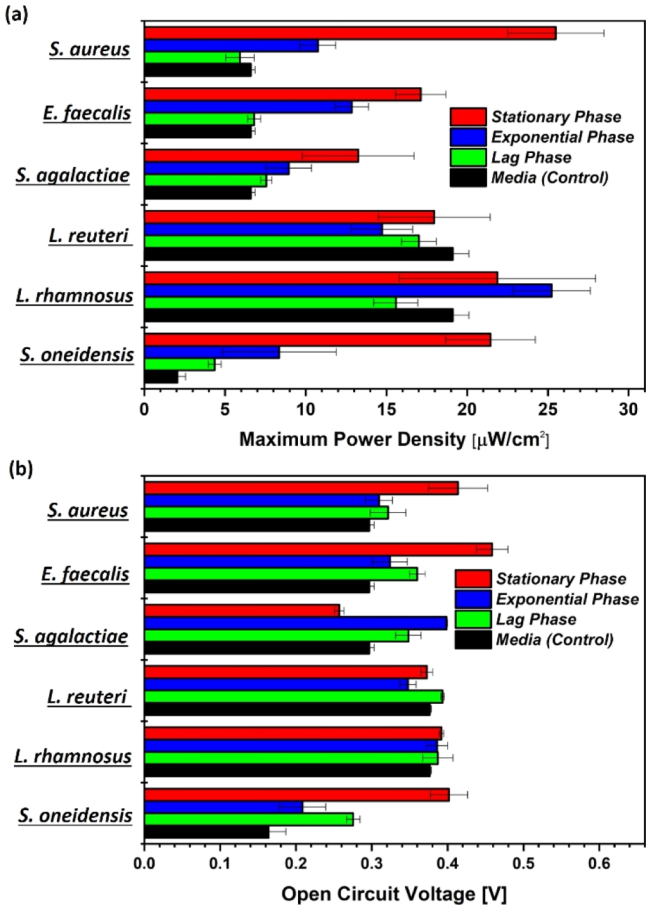

Figure 4. (a) Maximum power densities and (b) OCVs generated from the five gut bacteria and the well-known exoelectrogen, *S. oneidensis*.

highest. For the electrogenic Gram-positive and -negative bacteria (i.e., *S. aureus*, *E. faecalis*, *S. agalactiae*, *L. rhamnosus*, and *S. oneidensis*), the power generation increased from their lag phase as the cell number increased with its enhanced EET process. In the final stationary phase, bacteria achieved maximum growth numbers and reached a plateau. During this phase, the potential electron-shuttling compounds can accumulate, leading to the maximum power generation. Therefore, *S. aureus*, *E. faecalis*, *S. agalactiae*, and *S. oneidensis* in their stationary phases have a considerably better power performance than those in their other phases. One exception was shown in *L. rhamnosus*, demonstrating decreased power performance in stationary phase. Given that *L. rhamnosus* shows weak overall electrogenicity with a huge performance variation, it is hard to make conclusions on its electrogenic abilities. Unfortunately, the growth medium commonly used for Lactobacilli propagation, MRS, has a very high background power output, making it difficult to examine electrogenicity in these species. Further experimentation on growth requirements and electrogenicity would be required to determine whether these organisms actually have electrogenic potential.

The OCV is an important parameter of the MFC and a result of the electrochemical reactions determined by *Gibbs free energy*.[32] As shown in Figure 4b, THY and MRS generate greater OCV than LB, leading to a higher background current (Figure 4a). This is because THY and MRS media include more electroactive ions and carbon sources than LB. The OCVs of *L. reuteri* and *L. rhamnosus* do not generally vary by growth phase, while the others produced differing amounts of electric potential that was growth-phase dependent. With the changes in the number of the cells and their bacterial metabolism, the OCVs of the strongly electrogenic bacteria (i.e., *S. aureus*, *E. faecalis*, *S. agalactiae*, and *S. oneidensis*) in the MFC can vary. The OCV of *S. aureus*, *E. faecalis*, and *S. oneidensis* was significantly higher during stationary phase, while the value of *S. agalactiae* was smaller. Although further studies must be performed to understand EET pathways of these Gram-positive exoelectrogens, this work demonstrated that many Gram-positive bacteria could transfer electrons to the exterior of their cells, and this

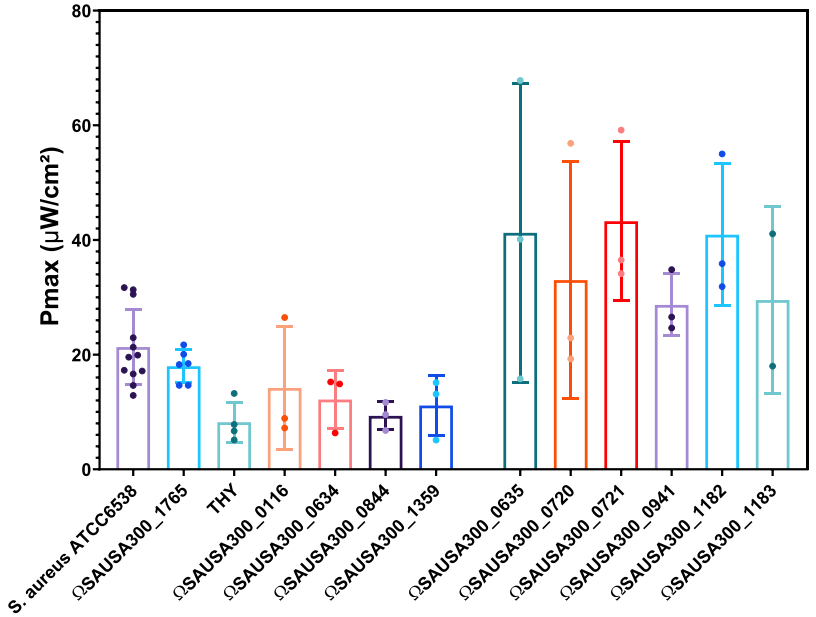

Figure 5. P_{max} output of selected transposon mutants of *S. aureus*. Power generation by *S. aureus* ATCC6538 and the unrelated *S. aureus* transposon mutant strain ΩSAUSA300_1765 as well as THY medium alone served as controls. Selected gene mutants are shown with decreased or increased power output compared to the controls. Each dot represents a biological replicate, all of which included 5 technical replicates.

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finding has enormous upside potential for the fields of bioenergy, biosensing, biocomputing, and biosynthesis. Moreover, our compact screening system is individually addressable for the identification and characterization of electrogenic bacteria and can pave the way to a new era of electromicrobiology.

2.4. Selected Hypothesis-Driven Genes in *S. aureus*. To determine whether this system could be used to elucidate the potential EET pathways in *S. aureus*, transposon mutants from the Nebraska Transposon Mutant Library (BEI Resources, ATCC) were selected based on hypothetical involvement in metal ion transport, the electron transport chain, and genetic proximity or predicted interactions with any potentially relevant genes. Transposon mutants were screened, and the power output of mutants was compared to both a wildtype strain of *S. aureus* (ATCC 6538) and a transposon mutant not predicted to be involved in EET in *S. aureus* USA300 FPR3757 (ΩSAUSA300_1765) which served as positive controls. THY medium without bacteria acted as a negative control for power generation. As seen in Figure 5, some of the transposon mutants tested displayed lower overall power output, while some mutants produced increased power compared to the positive control strains, although results were not significant via one-way ANOVA and more biological replicates may increase statistical significance.

One operon that showed lower EET when deleted was *sirABC* (SAUSA300_0115-0117) (Figure 5). This operon encodes an iron ABC transporter system with iron compound-binding protein SirA and permeases SirB and SirC. Only *sirB* is shown on this graph as this was the only gene in which three independent biological replicates were completed but *sirA* and *sirC* also showed decreased power output in initial trials (data not shown).

Deletion of SAUS300_0720/0721 also led to altered EET generation. These genes are part of an operon (SAUSA300_0718-0721) known as *sstBCDA*, another ABC iron transport system found in *S. aureus*.[33] Interestingly, deletion of *sstD* and *sstA* resulted in increased rather than decreased EET compared to that in controls (Figure 5). EET generation by *sstBC* mutants is underway.

The *sir* and *sst* operons are both regulated by cellular iron concentrations and are also both involved in iron acquisition in *S. aureus*. However, the iron acquisition mechanisms are somewhat different. Sir allows iron acquisition via transportation of the carboxylate-type siderophore staphyloferrin B, while the Sst system transports catechol siderophores.[34] Potentially the pathways they regulate act divergently on EET generation, although this could be directly or indirectly related to their roles in iron acquisition.

Because *S. aureus* is highly evolved to live in and on host surfaces, it has many, potentially redundant, iron acquisition systems. These systems allow for rapid adaptation of bacteria to low iron concentrations found in the host. Importantly, many of the mutants tested (including *sirABC* and *sstBCDA*) have different effects on the cell during growth in iron-replete and iron-poor media, demonstrating the need for further testing of power output in media with altered iron levels. This may show more significant changes in terms of EET generation than the single transposon mutant strains. In addition, redundancy in iron acquisition systems could lead to decreased changes in EET in single gene transposon knockout strains. To further examine the role of these systems in EET, we plan to examine strains in which multiple systems are deleted. Our research highlights the fact that the full relationship between iron acquisition and EET in *S. aureus* is very complicated and likely changes greatly depending on the availability of important cofactors such as iron.

3. CONCLUSIONS

In this work, we characterized the electrogenic capability of five Gram-positive bacteria including *S. aureus*, *E. faecalis*, *S. agalactiae*, *L. reuteri*, and *L. rhamnosus* and compared their power performances in the MFC to the well-known Gram-negative exoelectrogen, *S. oneidensis*. We also began the process of screening one pathogen, *S. aureus*, to determine the genetic pathways that could be involved in this electrogenic ability.

For rapid, sensitive, reliable, and high-throughput characterization, we developed a paper-based MFC array and enhanced its performance by integrating conductive and hydrophilic anodes and the low-overpotential solid Ag_2O cathodes. All bacterial electrogenic capabilities and capacities were examined in three different growth phases: log, exponential, and stationary. *S. aureus*, *E. faecalis*, and *S. agalactiae* showed distinct electrogenic capabilities, and their power capacities were comparable to that of Gram-negative *S. oneidensis*. We demonstrated that bacterial growth phase significantly affects the power performance of these Gram-positive exoelectrogens.

This work is significant because it will advance and expand understanding of the electrogenic potential of pathogens present in the human microbiome, which remains mostly uncharacterized. Direct correlations between the microbial community and complexity and the MFC electrical performance will eventually be explored and compared with bacterial 16s sequencing data to begin to establish an innovative biosensing method for real-time monitoring of gut microbiota.

4. EXPERIMENTAL SECTION

4.1. Materials. Whatman Grade 3MM chromatography paper was purchased from VWR International, LLC and sterilized by ultraviolet radiation before use. Silver oxide (Ag_2O), dimethyl sulfoxide, and 3-glycidoxypropy-trimethoxysilane were obtained from Sigma-Aldrich and used as received. Poly(3,4-ethylenedioxythiophene):polystyrene sulfonate (PEDOT:PSS) (Clevios PH1000) was purchased from Heraeus. Conductive graphite ink (NC1044060) was acquired from Fisher Scientific Company, LLC. A nickel conductive spray was purchased from MG Chemicals. Polymethyl methacrylate (PMMA) layers (1/16 in.) were acquired from McMaster-Carr.

4.2. Preparation of the Paper-Based MFC Array. The MFC array consisted of four functional layers: anode on paper, anodic PCB (printed circuit board) on PMMA, cathode on paper, and cathodic PCB on PMMA (Figures 2 and S1). Hydrophobic wax was first printed on paper by a solid-wax printer (Xerox Phaser8570dn) and heat-treated in an oven to define hydrophilic regions where the PEDOT:PSS and the Ag_2O ink were introduced to form an anodic reservoir and a cathodic catalytic region, respectively (Figure 2). The wax was also printed on the back side of the anodic and cathodic paper layers so that the wax-patterned region could act as an ion exchange membrane between the anode and the cathode when the layers were sandwiched to form an MFC (Figure 1a). Because the wax membrane is hydrophobic and can be controllably porous by regulating the heating temperature and time, the anodic and cathodic compartments can be physically and electrically separated without the leaking of the bacterial anolytes. The ions are transferrable through the wax region,[19,20] maintaining

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electroneutrality when electrons generated from bacterial metabolism in the anode flow to the cathode through an external circuit. Introduction of 1 wt % of PEDOT:PSS and 5 wt % of dimethyl sulfoxide followed by 2 wt % of 3-glycidoxypropyltrimethoxysilane onto the patterned hydrophilic regions formed conductive anodic reservoirs, while their hydrophilicity remained without any morphological changes of the paper fibers.[21,22] This engineered paper allowed for the same surface area as the bare paper, while the polymeric PEDOT:PSS enabled higher titers of bacterial cells to attach, thereby increasing the power density. The cathode was constructed by introducing 500 mg of Ag_2O in 10 mL of PEDOT:PSS ink. Compared to conventional MFC cathodes that use oxygen or chemical compounds as the electron acceptor, a solid-state Ag_2O cathode provides several advantages including low cathodic overpotential, versatility in device design, and limiting fouling by microbes.[22] Then, the graphite ink was screen-printed on the anodic PEDOT:PSS and cathodic catalyst regions through the pre-patterned paper masks. The cathodic layer was laser-cut to create an inlet for bacterial sample introduction. Metallic wires were spray-coated on PMMA through PMMA-based masks and the laser-cut for the inlet. Finally, all functional layers were well-aligned and assembled with spray adhesive glue (3M Super 77).

4.3. Preparation of the 5-Well Paper-Based MFC Array. The 5-well MFC array was also developed for a quick screening of temporal-sequentially obtained *S. aureus* mutants (Figure S2). All device materials and configurations except for the electrical connection with the copper tape were the same as the 21-well MFC array.

4.4. Bacterial Strains and Growth Conditions. For initial electrogenic studies, *S. aureus* (ATC 6538), *E. faecalis* (ATCC 19433), *S. agalactiae* (A909), *L. rhamnosus* (GG), and *L.*reuteri (ATCC 23272) were used. The Nebraska Transposon Mutant Library (strain USA300 FPR3757) was obtained from BEI Resources and contains >2000 individual mutants in non-essential genes. *S. aureus*, *E. faecalis*, and *S. agalactiae* cultured in Todd-Hewitt broth with 2% yeast extract [20 g of peptone, 3.1 g of beef heart (solids from infusion), 2.5 g of Na_2CO_3, 2 g of glucose, 2 g of NaCl, 0.4 g of Na_2HPO_4, and 20 g of yeast extract per liter of deionized (DI) water (THY)], while *L. reuteri* and *L. rhamnosus* were grown on MRS (10 g of peptone, 8 g of beef extract, 4 g of yeast extract, 20 g of glucose, 2 g of K_2HPO_4, 5 g of $C_2H_9NaO_5$, 2 g of $C_6H_{17}N_3O_7$, 0.2 g of $H_{14}MgO_{11}S$, and 0.05 g of H_8MnO_8S per liter of DI water (MRS))Broth. The well-known exoelectrogen, *S. oneidensis* MR1 was cultivated in a Luria Bertani-broth [10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter of DI water (LB)]. Bacteria cultured in these media progress through four phases of growth: lag, exponential, stationary, and death. Samples in the first three growth phases were obtained by measuring the optical density at 600 nm (OD_{600}). All samples were cultivated at 37 °C and acquired without centrifugation to retain endogenously produced redox mediators that act as electron shuttles for bacterial EET process. For transposon mutant screening, individual mutants were grown overnight at 37 °C. Cultures were then normalized to an OD600 of ∼2.0 prior to inoculation into the 5-well paper-based MFC arrays. 10 μL bacterial samples were applied to each MFC well. All experiments were performed in a candle jar to provide anaerobic conditions for efficient bacterial EET processes. All experiments were completed with at least three technical replicates as well as biological replicates.

4.5. Electrical Measurement Setup. All experiments were performed in the temperature-controlled laboratory at 25 °C. The electrical voltages generated from the MFC with different samples were measured with a data acquisition system (National Instruments, USB-6212) and recorded every 30 s via a customized LabView interface. The polarization curves and power outputs as a function of current were calculated by sequential connection with external resistors (1 MΩ, 0.5 MΩ, 248 kΩ, 68 kΩ, 47 kΩ, 22 kΩ, 10 kΩ, 5 kΩ, 3.3 kΩ, 2.2 kΩ, 1 kΩ, 0.5 kΩ, 330 Ω, and 220 Ω) between the anodes and cathodes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04362.

Designs and dimensions of the sensing array and experimental setups (PDF)

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Notes

The authors declare no competing financial interest.

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