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Justin C. Biffinger
US Naval Research Laboratory, justin.biffinger@nrl.navy.mil

Ricky Ray
Naval Research Laboratory

Brenda J. Little
Naval Research Laboratory, blittle@nrlssc.navy.mil

Lisa A. Fitzgerald
Naval Research Laboratory, lisa.fitzgerald@nrl.navy.mil

Meghann Ribbens
University of Southern California

See next page for additional authors

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Authors

Justin C. Biffinger, Ricky Ray, Brenda J. Little, Lisa A. Fitzgerald, Meghann Ribbens, Steven E. Finkel, and Bradley R. Ringeisen

Simultaneous Analysis of Physiological and Electrical Output Changes in an Operating Microbial Fuel Cell With *Shewanella oneidensis*

Justin C. Biffinger,[1] Ricky Ray,[2] Brenda J. Little,[2] Lisa A. Fitzgerald,[1] Meghann Ribbens,[3] Steven E. Finkel,[3] Bradley R. Ringeisen[1]

[1]Chemistry Division, Naval Research Laboratory, 4555 Overlook Avenue, SW, Code 6113, Washington 20375, District of Columbia; telephone: 202-767-2398; fax: 202-404-8119; e-mail: justin.biffinger@nrl.navy.mil

[2]Oceanography Division, Naval Research Laboratory, Building 1009, John C. Stennis Space Center, Slidell, Mississippi

[3]Molecular and Computational Biology Section, Department of Biological Sciences, University of Southern California, Los Angeles, California

ABSTRACT: Changes in metabolism and cellular physiology of facultative anaerobes during oxygen exposure can be substantial, but little is known about how these changes connect with electrical current output from an operating microbial fuel cell (MFC). A high-throughput voltage based screening assay (VBSA) was used to correlate current output from a MFC containing *Shewanella oneidensis* MR-1 to carbon source (glucose or lactate) utilization, culture conditions, and biofilm coverage over 250 h. Lactate induced an immediate current response from *S. oneidensis* MR-1, with both air-exposed and anaerobic anodes throughout the duration of the experiments. Glucose was initially utilized for current output by MR-1 when cultured and maintained in the presence of air. However, after repeated additions of glucose, the current output from the MFC decreased substantially while viable planktonic cell counts and biofilm coverage remained constant suggesting that extracellular electron transfer pathways were being inhibited. *Shewanella* maintained under an anaerobic atmosphere did not utilize glucose consistent with literature precedents. Operation of the VBSA permitted data collection from nine simultaneous *S. oneidensis* MR-1 MFC experiments in which each experiment was able to demonstrate organic carbon source utilization and oxygen dependent biofilm formation on a carbon electrode. These data provide the first direct evidence of complex cellular responses to electron donor and oxygen tension by *Shewanella* in an operating MFC at select time points.
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Introduction

Microbial fuel cells (MFCs) are electrochemical devices capable of generating an electrical current directly from the oxidation of carbon electron sources using bacterial metabolic pathways. These devices are currently being developed for a variety of applications ranging from the generation of electricity using wastewater (Aelterman et al., 2006; Angenent et al., 2004) to autonomous power sources for sensors (Shantaram et al., 2005) and beacons (Tender et al., 2008). Different bacterial strains have evolved a variety of strategies for delivering electrons to solid electron accepting materials (metal oxides, carbon electrodes). For example, direct contact with the electrode surface is required for *Geobacter sulfurreducens* to generate current from a MFC (Reguera et al., 2006) while *Shewanella oneidensis* MR-1 can deliver reducing equivalents to electron accepting surfaces without direct contact (Lies et al., 2005) using redox mediators (Marsili et al., 2008; von Canstein et al., 2008). Several reviews have been published recently that summarize the significant progress in understanding electron transport pathways within electrochemically active bacteria (EAB) (Chang et al., 2006; Fredrickson et al., 2008; Hernandez and Newman, 2001; Lovley, 2008; Schröder, 2007). However, few studies have been published that address the complex

real-time cellular physiological changes that determine how EAB interact with anodes during MFC operation (Lanthier et al., 2008).

Both environmental bacterial consortia and single strain MFCs are reported in the literature (Logan et al., 2006). Working with single strains allows mechanistic and physiological details to be observed directly. Two bacterial families, Geobacteracea and Shewanellacea, are commonly used in pure culture MFC research. *S. oneidensis* MR-1 is a facultative, anaerobic γ-proteobacterium capable of dissimilatory metal reduction (Myers and Nealson, 1988) as well as generating current within MFCs (Bretschger et al., 2008; Kim et al., 2002; Ringeisen et al., 2006). *Shewanella* was chosen for this work because of its adaptability to aerobic and anaerobic environments.

Glycolytic aerobic metabolic pathways in *Shewanella* have been identified through genomic and proteomic studies (Beliaev et al., 2005; Driscoll et al., 2007; Fang et al., 2006; Leaphart et al., 2006; Serres and Riley, 2006; Wan et al., 2004) and experimental evidence linking current output with glucose metabolism was recently reported using aerobic cultures of *S. oneidensis* DSP10 in a miniature MFC (Biffinger et al., 2008). Prior to the aforementioned work, *S. oneidensis* was considered limited in the range of organic electron sources (e.g., formate, lactate, pyruvate, amino acids) that could be used for anaerobic metal reduction or current output from MFCs (Nealson et al., 2002). Bacterial metabolic pathways dictate how different types of organic electron sources (carbohydrates, linear carboxylic acids, polysaccharides) are utilized for current output from MFCs. Therefore, in situ monitoring of both cellular and culture environment conditions is important for improving the long-term survivability of MFC devices.

Since removing electrode samples from a continuously operating MFC is not practical and running multiple laboratory scale MFCs under identical conditions for sampling electrodes is unfeasible, no research has been reported on the study of microbial cellular changes within an operating MFC. Direct real time measurements of biofilm formation and coverage have been analyzed by nuclear magnetic resonance (NMR) (McLean et al., 2008) and confocal microscopy (Teal et al., 2006) on transparent supports. However experiments performed on non-conductive surfaces may not be germane to the conditions in an operating MFC (Lanthier et al., 2008). Indirect real time analysis of biofilm formation by electrochemical impedance spectroscopy (EIS) (Manohar et al., 2008; Ramasamy Ramaraja et al., 2008) or utilizing pre-formed biofilms on electrodes placed in MFCs (Venkata Mohan et al., 2008) have also been used to monitor biofilm dynamics but are difficult to relate to actual biofilm coverage in an operating MFC.

In this study, a voltage based screening assay (VBSA) was used to monitor voltage output from EAB under both closed and open circuit conditions (Biffinger et al., 2009). Additionally, the use of a high-throughput assay for monitoring current output from bacteria provided a pathway to correlate electrical current output with cellular and metabolic changes; factors that have not been studied within an operating MFC to date. The VBSA was used to monitor real-time current output as it relates to anaerobic and air-exposed cultures, planktonic cell concentration, and extent of biofilm formation on a carbon electrode at defined time points during the experiment. The combination of these data resulted in a physiological description of how *Shewanella* respond to glucose and lactate in the presence of oxygen in an operating MFC.

Materials and Methods

Solutions and Media

A stock solution of 1.95 M sodium lactate was adjusted to pH 7.0 and sterilized by autoclaving for 15 min at 121°C. A D-glucose (1 M) stock solution was sterilized with a 0.2 μm cellulose nitrate filter. Luria-Bertani (LB) Broth (Miller) and LB/agar (Difco LB-Agar, Miller) was used for liquid cultures and plates, respectively (Fisher Scientific, Inc, Pittsburgh, PA). The solvent for each solution was Millipore 18 MΩ water. Serial dilutions for viable planktonic cell concentration measurements were performed in phosphate buffered saline (pH 7.0) with 0.03% Triton-X100 (Sigma–Aldrich, Milwaukee, WI).

Cell Culture Conditions

S. oneidensis MR-1 (obtained from Dr. Kenneth Nealson (University of Southern California, Los Angeles, CA)) was grown from a single colony isolated from a LB/agar plate inoculated from a −80°C glycerol stock culture. A single colony was transferred to 50 mL of LB broth and incubated aerobically at 25°C with gentle shaking (100 rpm). Experimental cultures were sub-cultured after 20 h of growth three times before being used in VBSA experiments. Anaerobic *S. oneidensis* MR-1 cultures were created from a MR-1 culture, which was incubated aerobically for 48 h. These aerobic stationary phase MR-1 cultures were then degassed with a nitrogen purge and shaken (100 rpm) in an anaerobic chamber for 24 h at 25°C prior to MFC experimentation.

VBSA Construction and Data Acquisition

Dimensions and fabrication of the VBSA were published previously (Biffinger et al., 2009). The anodes were single-sided carbon-coated titanium flags and the cathode system was graphite paper in a 50 mM potassium ferricyanide (dissolved in 100 mM phosphate buffer at pH 7.0). Each experiment was completed in a nine-well VBSA apparatus depicted in Figure 1. Experiments with no addition of glucose or lactate were labeled as blank. Planktonic cell concentrations of each well were determined from serial dilution of aliquots in phosphate buffered saline with 0.03%
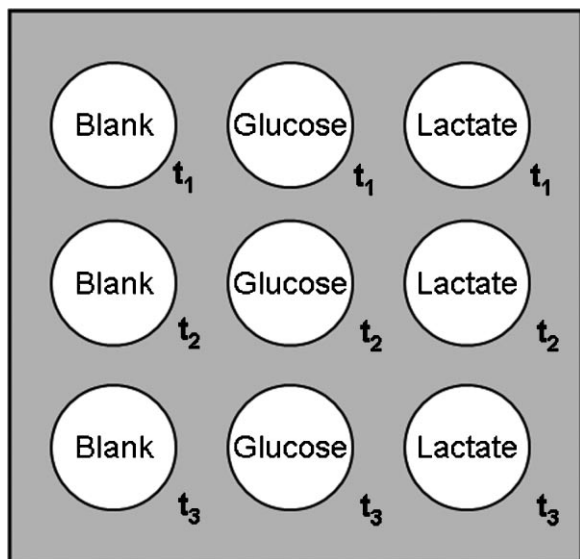
Figure 1. General diagram of the nine-well VBSA experimental setup for both aerobic and anaerobic studies. Electrodes were removed and chemically fixed at time 1 (t_1), time 2 (t_2), and time 3 (t_3) with the carbon electron source indicated in each well.

Trition-100 and plated onto LB/agar with average cell counts reported for glucose, lactate, and blank during the experiment. Once the electrode was removed for environmental scanning electron microscopy (ESEM) fixation, the well was no longer used for planktonic cell concentration determination. The voltages across a 100 kΩ resistor bank (in a custom nine-resistor bank made for simultaneous measurements) were recorded with a personal data acquisition device (I/O tech, personal daq/54) every 4 min. Ohm's law was used to convert voltage to current. Anaerobic (performed in a Coy instruments anaerobic chamber) and aerobic (or air-exposed) experiments were performed at 23°C.

Imaging *S. oneidensis* Biofilms

Environmental scanning electron microscopy (ESEM) of carbon surfaces on the titanium anodes was performed at the Naval Research Laboratory, Stennis Space Center, (NRLSSC). Unattached biomass was removed by washing each anode with three separate 1 mL aliquots of phosphate buffered saline solution at the Naval Research Laboratory, Washington, DC (NRLDC). Each anode was placed in 2 mL of 4% cacodylate buffered glutaraldehyde fixative (Ray et al., 1997) in water at NRLDC and fixed for at least 24 h at 4°C prior to shipment to NRLSSC without further manipulations. Anaerobic samples were fixed in the anaerobic hood using degassed 4% cacodylate buffered glutaraldehyde fixative. For collecting ESEM images, each anode was removed from the fixative and washed with 50 mL of

distilled water. After 2 min of gentle rinsing, each anode was placed on a mounting stub on the Peltier cooling device inside the ESEM chamber. The anodes were kept wet/moist by using the Peltier cooling device maintained at 4°C and a chamber water vapor pressure between 4.5 and 5.5 torr. Water vapor was allowed to condense on the cooled anodes to keep it moist while performing ESEM imaging. Liquid water was removed from the top layer, several microns thick, to view the biofilm on the carbon surface of each titanium anode. A gaseous secondary electron detector (GSED) was used to collect the ESEM images of the wet/moist sample surface.

Results and Discussion

The combination of time course results from ESEM images of electrode surfaces, viable planktonic cell densities, and electrical current output generates a broader understanding of how *S. oneidensis* interact with carbon electrode surfaces in an operating batch MFC. Correlating the three parameters mentioned previously was made possible by using a small modular array of identical MFCs operated in parallel. These data demonstrate distinct cellular differences
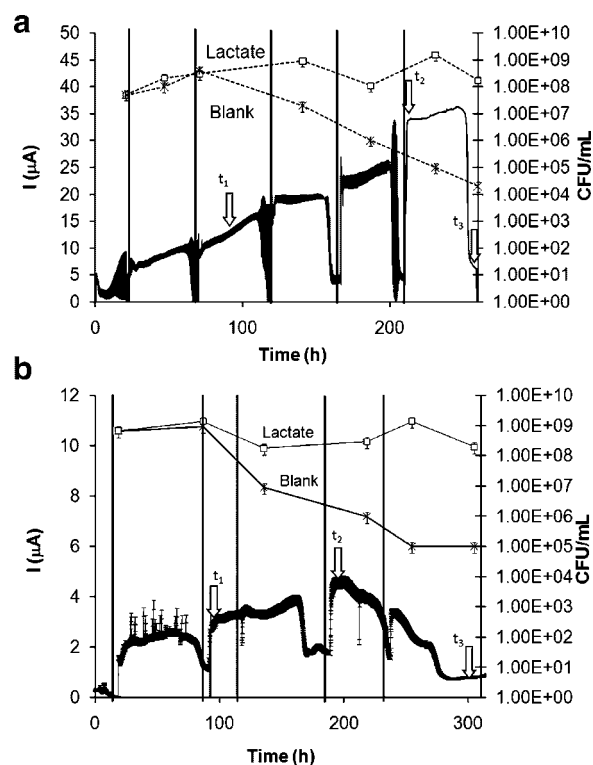

Figure 2. Average current output from *S. oneidensis* MR-1 containing VBSA exposed to (**a**) aerobic or (**b**) anaerobic atmospheres with 10 mM lactate as the sole electron carbon source with baseline correction. Secondary axis reports planktonic cell count with time in colony forming units (CFU)/mL. Solid vertical lines indicate when lactate was added and block arrows designate when anode was removed and chemically fixed for ESEM at t_1, t_2, and t_3.

with carbon source utilization and oxygen tension as well as providing insight into the role cellular physiology plays on current output from an operating *S. oneidensis* MFC.

Lactate Metabolism by *S. oneidensis*

Current output (Fig. 2) from *S. oneidensis* MR-1 was correlated to both planktonic cell density (Fig. 2) and biofilm formation (Fig. 3) with lactate as the sole electron source. Subsequent additions of lactate over the first 170 h for air exposed anodes resulted in a four-fold current increase (Fig. 2a). The remaining 100 h of the experiment resulted in a doubling of the current output. In general, the current output doubled from successive additions of lactate to air-exposed MR-1. The maximum current generated by anaerobic MR-1 with lactate (Fig. 2b) was eight-fold less than air-exposed MR-1 (Fig. 2a). However, there were rapid current responses (<4 min) from lactate additions for anaerobic cells.

The planktonic cell density remained constant for lactate (~8×10^8 CFU/mL) in both the presence and absence of air (Fig. 2a and b, respectively). Planktonic cell density in the blank anode decreased exponentially after 70 h, correlating with LB nutrient depletion. Biofilm formation was weak for all blank electrodes under aerobic (Fig. 3a–c) and anaerobic (Fig. 5a–c) atmospheres. Electrodes from air-exposed anode chambers (Fig. 3g–i) showed significant biofilm coverage with a complete lawn of MR-1 formed over the entire anode surface after 220 h of operation (Fig. 3h). Anaerobic MR-1 did not form a substantial biofilm in the presence of lactate (Fig. 3d–f).

The gradual increase in current with time (Fig. 2a) correlated with the formation of biofilm for MR-1 (Fig. 3g–i). This gradual current increase is typically described as a conditioning period where the bacteria modify the electrode surface for either bacterial attachment or mediator release. However, when using air-exposed anodes this gradual increase in current should also be attributed to a decrease in oxygen concentration at the electrode surface, which would
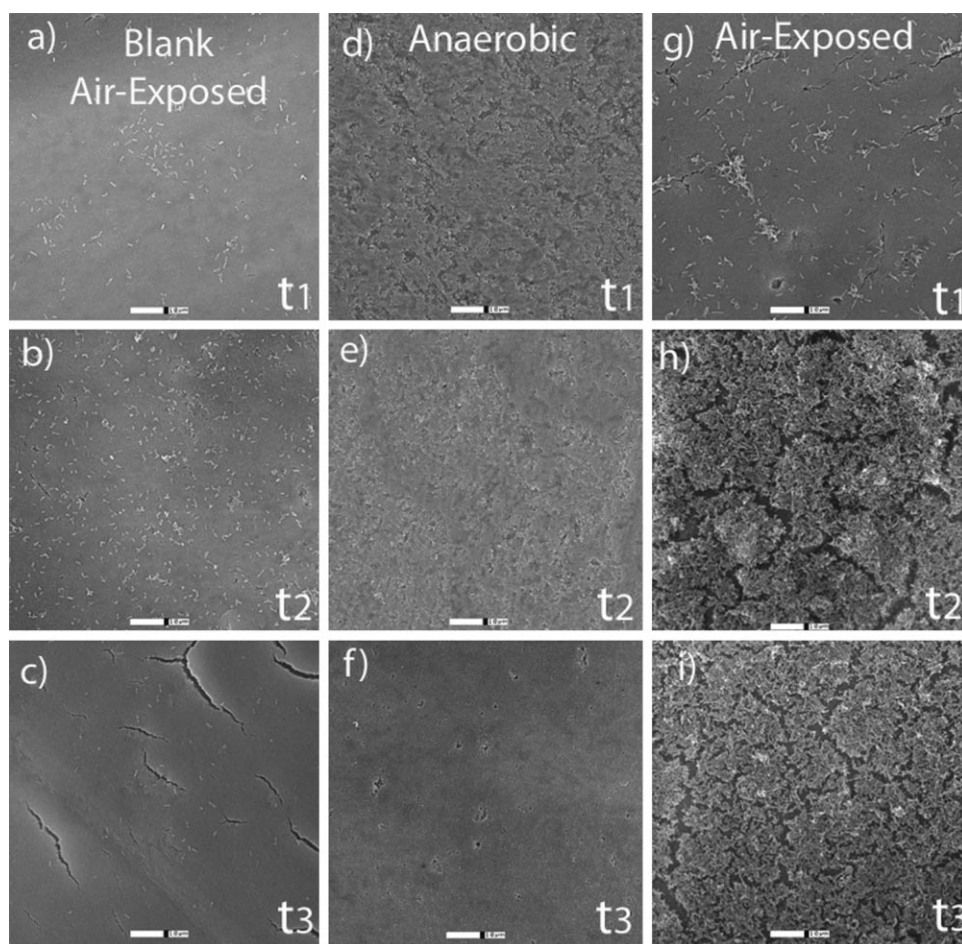

Figure 3. ESEM images of the chemically fixed carbon anode surfaces (designated by block arrows in Fig. 2) from acellular (**a–c**) and *S. oneidensis* MR-1 anaerobic (**d–f**) or air exposed (**g–i**) anode chambers with lactate as the sole carbon electron source. Scale bar is 10 μm.

eliminate the competitive oxygen reduction reaction and increase the Coulombic efficiency of the MFC. The Coulombic efficiency doubled as substantial biofilm was formed on the electrode surface (Fig. 2a). This concept of oxygen gradients in biofilms was first demonstrated using direct microelectrode measurements showing a decrease in oxygen concentration with increasing biofilm thickness (Rasmussen and Lewandowski, 1998) and is consistent with these results.

Since *S. oneidensis* does not need to be in contact with electrode surfaces to deliver electrons at a distance, planktonic cell density would impact current output significantly for a *Shewanella* containing MFC. The viable planktonic cell count remained essentially constant throughout the air-exposed experiment, and significant current was generated immediately, even with sparse biofilm formation over the first 100 h of operation. We observed little change in planktonic cell concentration with time (Fig. 2a), but found a significant increase in biofilm coverage on the anode (Fig. 3). This experiment demonstrated that the decrease in oxygen concentration at the anode and increased number of bacteria near the electrode surface is primarily responsible for the gradual increase in current from lactate. This colonization of the electrode is certainly enhanced in the presence of oxygen when comparing ESEM images from MR-1 exposed to air (Fig. 3g–i) and anaerobic experiments (Fig. 3d–f).

There has been only one other study that has monitored *Shewanella* growth and biofilm formation with an active MFC carbon electrode as the sole electron acceptor (Lanthier et al., 2008). Our results are consistent with their observation that planktonic biomass is primarily responsible for current output from anaerobic *S. oneidensis* containing MFCs but is not consistent for air-exposed cultures. It is clear from our results that substantial biofilms of *S. oneidensis* MR-1 are formed with air-exposed anodes and lactate (Fig. 3 g–i) in an operating batch MFC, while a significant biofilm is not formed under anaerobic conditions (Fig. 3 d–f). Therefore, a lack of biofilm formation yet a sustained planktonic cell concentration over time indicates *Shewanella* utilizes lactate as a food source and our observations under anaerobic conditions indicate that planktonic cells rather than direct cell-anode contact are primarily responsible for current output.

Glucose Metabolism by *S. oneidensis*

Until recently, only a limited range of organic electron sources were known which *S. oneidensis* could use for anaerobic metal reduction or current output in a MFC (Fredrickson et al., 2008). Lactate is one such electron source that has been utilized for studies of current production from a *Shewanella* MFC (Kim et al., 1999, 2002; Ringeisen et al., 2006) and also was shown in the previous section. However, the natural abundance of lactate is limited. Therefore, in order to use a *Shewanella*-containing MFC in a variety of applications, such as an autonomous power source for sensors, we must understand the physiological role naturally occurring electron sources might play on bacteria. The results presented here demonstrate a simultaneous time course analysis of the physiological and electrical output changes *S. oneidensis* undergoes in an operational MFC with glucose as the sole electron source.

S. oneidensis MR-1 cultured and exposed to air within a MFC (Fig. 4a) can utilize glucose as an electron source for current production. However, repeated additions of glucose resulted in a gradual increase in current over the first 150 h with a subsequent decrease in current after this time period (Fig. 4a). The addition of glucose did result in smaller current increases after 150 h for MR-1 but significantly less than the maximum current of 17 µA recorded in the first 150 h. This result is consistent with similar experiments using air-exposed *S. oneidensis* DSP10 cultures in a flowing miniature MFC (Biffinger et al., 2008) with glucose as the sole electron source. Significantly more of the electrode surface was covered by MR-1 using glucose with oxygen-exposure (Fig. 5g–i) than without (Fig. 5d–f). Planktonic cell density remained high for air-exposed cells indicating that glucose was being utilized by *Shewanella* during this experiment.
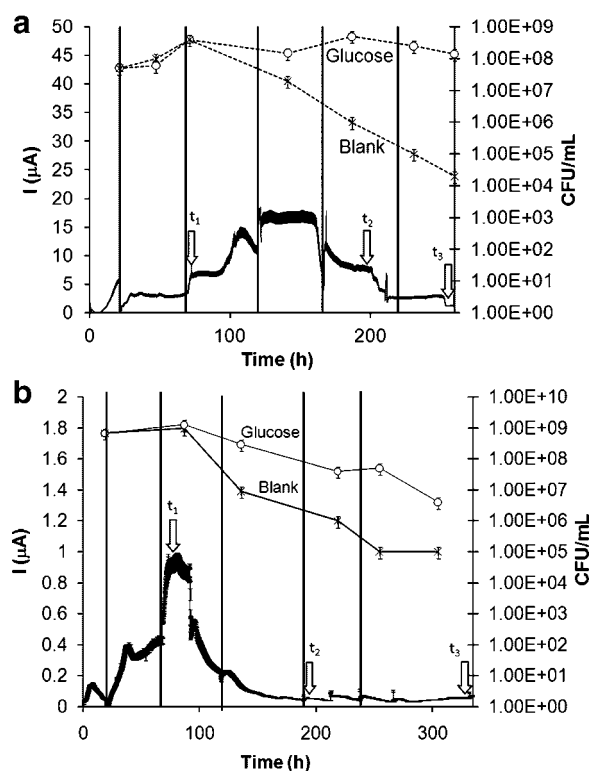

Figure 4. Average current output from *S. oneidensis* MR-1 containing VBSA exposed to (**a**) aerobic or (**b**) anaerobic atmospheres with 10 mM glucose as the sole electron carbon source with baseline correction. Secondary axis reports planktonic cell count with time in colony forming units (CFU)/mL. Solid vertical lines indicate when glucose was added and block arrows designate when anode was removed and chemically fixed for ESEM at t_1, t_2, and t_3.
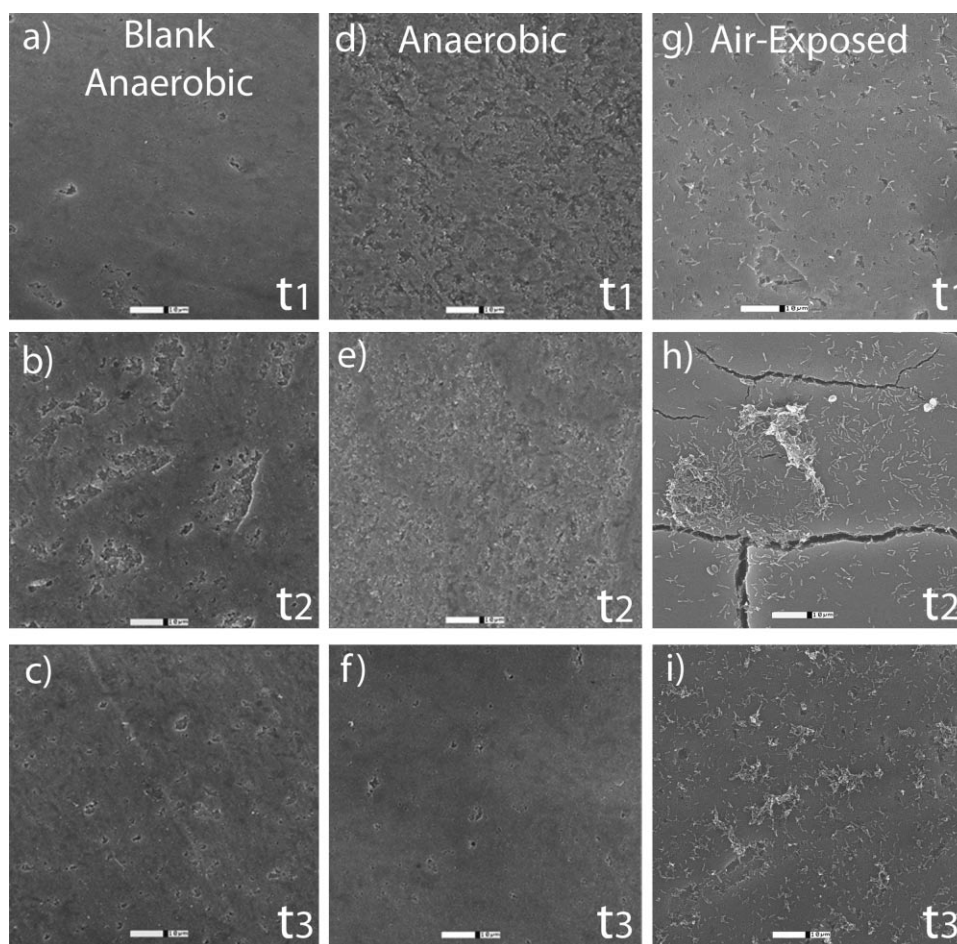
Figure 5. ESEM images of the chemically fixed carbon anode surfaces (designated by block arrows in Fig. 4) from acellular (**a–c**) and *S. oneidensis* MR-1 anaerobic (**d–f**) or air exposed (**g–i**) anode chambers with glucose as the sole carbon electron source. Scale bar is 10 μm.

Current output after addition of glucose for air-exposed MR-1 was initially weak (<5 μA) but over the next 150 h generated approximately 17 μA (Fig. 4a). Since improvements in current output correlate with biofilm formation with air exposed cultures, then maximizing biofilm formation is a major factor in optimizing *Shewanella* containing MFCs. In general, there were only sparse biofilms formed when glucose was the sole electron source under all conditions. Air-exposed MR-1 current vs. time data (Fig. 4a) is consistent with *Shewanella* utilizing glucose upon continued exposure to oxygen. The present experiments show distinct cellular physiological responses from repeated exposure to glucose as well as a gradual decrease in current after 170 h consistent with previous results (Biffinger et al., 2008). The viable planktonic cell concentration and biofilm coverage remained constant throughout the air-exposure experiments, suggesting that repeated additions of glucose eventually down-regulates extracellular electron transport pathways in favor of sustaining growth. The conservation of energy for growth rather than biofilm formation on an electrode by anaerobic *S. oneidensis* was reported recently,

although a complete picture of bacterial growth changes on the electrode was not provided (Lanthier et al., 2008).

S. oneidensis is capable of reducing a wide range of electron acceptors, but only a small number of electron donors have been utilized effectively in anaerobic environments (Fredrickson et al., 2008). The initial current response of *S. oneidensis* under anaerobic conditions to glucose is attributed to the transition of aerobically cultured cells to an anaerobic MFC environment. Since air exposed stationary phase MR-1 cultures were degassed for anaerobic experiments, the decrease in current with time after 170 h is consistent with the decreased expression of proteins in glycolytic pathways under low oxygen levels (Scott and Nealson, 1994). This conclusion is also supported by the repeated positive current responses to additions of glucose in the presence of oxygen (Fig. 4a), while anaerobic MR-1 cells did not generate any current response with repeated glucose additions (Fig. 4b). Planktonic cell densities also decreased with time under anaerobic conditions and glucose exposure, while air-exposed cells were able to maintain their cell density (Fig. 4). All of these data indicate that

glucose was metabolized when exposed to air and not utilized efficiently under anaerobic conditions in a single experiment.

Conclusions

The miniature modular design of the VBSA resulted in the first time-lapse analysis correlating cellular physiological responses to current output from an operating MFC. Large differences in current output and physiology were observed between MFCs utilizing air-exposed and anaerobic MR-1 cultures exposed to glucose and lactate. The reduced response in current generation from lactate-exposed anaerobic *S. oneidensis* MR-1 was fivefold greater than the current response from glucose-exposed anaerobic MR-1. However, the sustainability of aerobic *Shewanella* cultures in the presence of glucose, a naturally occurring electron source, is a promising result for developing long-term autonomous sensors. Nonetheless, the fact that sustained current production has not been demonstrated when glucose is the sole electron donor means that consortia will still be necessary to achieve efficient energy harvesting by MFCs. These results demonstrate, for the first time, the ability to correlate current output in relation to carbon source utilization, culture conditions, and biofilm coverage in an operational MFC.

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