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Optimization of the biological component of a bioelectrochemical cell

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Abstract

The efficiency of electron transduction by *Shewanella oneidensis* MR-1 was investigated both in batch culture and in a dual-chambered electrochemical cell. Aerobically grown bacteria were inoculated into an insoluble FeOOH suspension in an anaerobic environment. As the bacteria reduced Fe(III) to Fe(II) there was a visible color change from red to bluish black; this simple color change assay proved to be a robust method for determining the electrochemical activity of *S. oneidensis* MR-1.

In an effort to improve electricity production by *S. oneidensis* MR-1, the performance of the electrochemical cell with lactate as a fuel was first optimized with respect to both poised potential and fuel concentration. Ultimately, electricity production proved to be proportional to both the poised potential and to fuel concentration. In particular, higher poised potentials increased charge production.

Finally, we attempted to optimize the bacteria themselves for the efficient transduction of reduced carbon sources into electricity. The batch culture underwent a series of serial dilutions; after the 4th dilution the microbe population exhibited a 30% increase in charge production. We are further exploring whether this increase was due to metabolic adaptations or to genetic mutations, and examining additional ways to evolve electrogenic organisms.

Keywords: Electrogenesis; Microbial *electrochemical* cell; Bacteria; *S. oneidensis* MR-1; Anaerobic

1. Introduction

Recently, a number of bacteria have been isolated based on their ability to use metal ions, including Fe(III), as electron acceptors. Iron-reducing microbes are found in the genera *Shewanella* [1,2], *Geobacter* [3,4], *Geovibroi* [5], *Disulfobulbus propionicus* [6], *Clostridium butyricum* [7,8], *Aeromonas hydrophila* [9], and *Rhodoferax ferrireducens* [10], and have shown to produce energy by coupling the oxidation of organic compounds to the reduction of Fe(III).

Among these metal-reducing bacteria, *Shewanella* are perhaps the most widely studied. Recent studies revealed that cytochrome c participates in the reduction of water-insoluble Fe(III) by physically and directly contacting Fe(III) on surfaces. Indeed, Myers et al. have shown that when *Shewanella putrefaciens* is grown anaerobically, 80% of its cytochromes are localized to the outer membrane [11]. Direct electron transfer by bacterial attachment to an electrode has also proven possible with *Geobacter sulferreducens* [12].

Microbial fuel cells are electrochemical cells that directly convert microbial redox metabolism into electricity [13,14]. Microbial fuel cells have recently received increased attention as a means to produce 'green' energy from organic waste or natural carbohydrate substrates. Members of the genera *Shewanella* have been used in electrochemical cells to produce electricity with lactate as an electron donor (or fuel) and an electrode as the sole electron acceptor, without soluble, organic mediators [1,2].

The efficiency of electricity production in a microbial fuel cell can be optimized by altering its physical or electrochemical components as reviewed by Angenent et al. and Rabaey et al. [15,16]. For example, Kim et al. have reported that Mn^{2+} modified graphite electrodes increased electrical current 10-fold compared to an unmodified graphite electrode. These authors also optimized the lactate and bacterial cell concentrations to enhance electrogenesis [8].

In contrast to the many studies that have focused on optimizing physical or electrochemical components of fuel cells, little has been reported on the optimization of the biological components. Recently, Rabaey et al. started with anaerobic sludge and enriched

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a mixed bacterial culture in the anode compartment of a biofuel cell via serial dilution. These authors demonstrated that power output (rate of electron transfer) improved by over 7-fold during the 71-day enrichment period [17,18].

In this paper, we not only investigated the impact of poised potential and fuel concentration on current generation under anaerobic conditions, but also attempted to improve the bacterial component under specifically defined conditions as well. Serial dilution of a single strain, *Shewanella oneidensis* MR-1, were carried out in a strict anaerobic batch culture system using lactate and fumarate as electron donor and acceptor, respectively, in order to enrich electrochemically active microbes. The electrogenic efficiency of the enriched population was compared with the efficiency of the parental microbes. We found that the bacteria adapted through 4 serial dilutions showed a 30% improvement in charge production.

Another potential difficulty with the bacterial components of fuel cells is that they may become inactivated upon exposure to air. The electrochemical activity of anaerobically grown cells exposed to air can be reactivated by lactate and/or poised potential even after it has been inactivated by exposure to air [19]. However, cyclic voltammetry studies have shown that while *Shewanella putrefaciens* grown under anaerobic conditions was electrochemically active with a redox potential of around −0.2 V against Ag/AgCl, no electrochemical activity was observed with cells grown under aerobic conditions [2]. We now show that even aerobically grown *Shewanella oneidensis* MR-1 can be activated by switching to an anaerobic environment. These results have important implications for the preparation of practical bacterial fuel cells.

2. Experimental

2.1. Bacterial growth conditions

Shewanella oneidensis MR-1 (ATCC 700550) was obtained from the American Type Culture Collection (Manassas, VA). Aerobic growth of cells was carried out in Luria–Bertani (LB) medium. Anaerobic growth was carried out in either LML medium (0.02% yeast, 0.01% peptone, 10 mM HEPES, pH 7.4) or growth medium (50 mM sodium phosphate buffer (pH 7.4), 0.1 M NaCl, 1% vitamin mix (ATCC, MD-SD, Manassas, VA), and 1% trace mineral mix (ATCC, MD-TMS, Manassas, VA)). Cells were grown at 30 °C.

2.2. Electrochemical activity assays

The electrochemical activity of *S. oneidensis* MR-1 was assayed by two methods. (1) A batch test was done with amophorous Fe(III) oxyhydroxide (FeOOH) prepared as described by Schwertmann and Cornell [20]. Sterilized serum bottles (60 ml; Wheaton Scientific, Millville, NJ) containing 20 ml of growth medium and 800 μl of saturated FeOOH suspension were prepared and sealed with butyl rubber stoppers and aluminum caps (Wheaton Scientific, Millville, NJ). The bottles were then purged with deoxygenated N_2 for 30 min; trace oxygen in the N_2 gas was removed using a furnace filled with pure copper and heated to 360 °C. An inoculum (1% v/v) of cells was prepared aerobically, then added to the serum bottle. (2) Cyclic voltammetry of anaerobic cell suspensions was measured using a potentiostat (CH Instrument 832, Austin, TX) at 25 °C with a scanning rate of 0.05 V s^{-1}. Bacterial cells grown anaerobically in

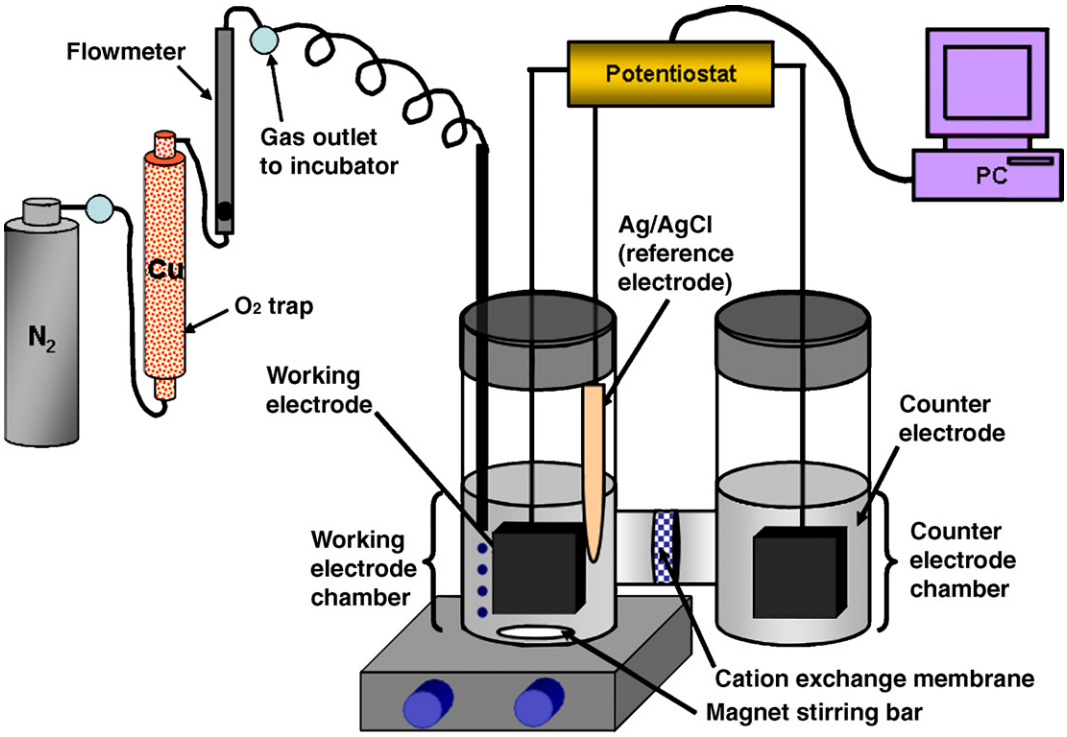

Fig. 1. Model of two-chambered electrochemical cell used for monitoring current generation and bacterial growth.

a serum bottle with LB were washed under strict anaerobic conditions and suspended in phosphate buffer (50 mM, pH 7.0) with 0.1 M NaCl. A 3 mm diameter glassy carbon disc working electrode (MF-2012, BAS, West Lafayette, IN), a platinum wire counter electrode (MW-4130, BAS), and an Ag/AgCl reference electrode (MF-2052, 3 M KCl, BAS) were used in the electrochemical cell with a working volume of 1 ml.

2.3. Electrochemical cell design

A dual-chambered electrochemical cell was constructed as shown in Fig. 1. The chambers were separated with a cation-selective membrane (Nafion 117, Ion Power, Inc., Bear, DE). The electrodes were graphite plates (grade G10, Graphite Engineering and Sales, Greenville, MI) with an area of 70 or 13 cm^2. Connections were made with threaded watertight connectors using no. 20 AWG marine-grade wire (Impulse, San Diego, CA) screwed into holes drilled directly into the graphite electrodes. Holes were filled with electronic insulating resin (3M Scotchcast 2130, flame retardant compound, Micro Parts and Supplies, Inc., Fox River Grove, IL). New electrodes were soaked in 1 N HCl for 1 day. After each use, the electrodes were washed in 1 N HCl, polished with sand paper (3M, Maplewood, MN), washed in 5% bleach, rinsed copiously with water and ethanol, and allowed to fully dry before placing them into the electrochemical cell chamber. The liquid volume in each chamber was approximately 150 ml (electrode area = 70 cm^2) or 20 ml (electrode area = 13 cm^2), respectively. The working electrode chamber was filled with sterilized growth medium and lactate. The counter electrode chamber was filled with growth medium and maintained in an aerobic environment. Bacteria were inoculated into the working electrode compartment after the working electrode chamber was purged with oxygen-free nitrogen for more than 18 h. The potential at the working electrode was maintained with potentiostat (Model 2053, AMEL Instruments, electrolytica, Amherst, NY) at a specific voltage against an Ag/AgCl reference electrode (c-905, electrolytica, Amherst, NY). Current was monitored through the potentiostat and data was logged with Chart 4.0 software (ADInstruments, Colorado Springs, CO). During bacterial growth, the cell density (OD at 600 nm) of aliquots was measured using a UV spectrometer (UV-1601, Shimadzu, Columbia, MD).

2.4. Anaerobic batch growth

Sterilized serum bottles containing 20 ml LML medium were sealed with butyl rubber stoppers and aluminum caps and purged with deoxygenated N_2 for 20 min prior to the inoculation of cells. For some experiments, lactate (0.2 M) and fumarate (10 mM) were added before sealing the bottle. The cells for inoculation were prepared from frozen cultures that were revived under aerobic conditions and were added to the serum bottle using a sterilized syringe. The OD was measured right after the inoculation and then 48 h after incubation. All experiments were performed in triplicate and the data shown are averages with standard errors.

2.5. Serial batch cultures

Sterilized serum bottles containing LML medium with 0.2 M lactate and 10 mM fumarate was prepared as described above. To initiate a serial transfer experiment, the first batch was inoculated with 100 μl of aerobically grown cells and incubated overnight. For the serial dilutions, a 10% (v/v) aliquot of a previous batch culture was inoculated into a freshly prepared serum bottle containing LML medium, 0.2 M lactate, and 10 mM fumarate. Frozen stocks of enriched cultures were made after every 5 batches (1st, 5th, 10th, 15th, and 20th), and the efficiency of electricity generation was assayed using the electrochemical cell described above.

3. Results and discussion

3.1. Electrochemical activity of S. oneidensis MR-1

In order to determine the basal electrochemical activity of *S. oneidensis* MR-1 grown in our system, cultures were prepared under both aerobic and anaerobic conditions. As shown in Fig. 2, the cyclic voltammograms (CV) of cell suspensions show that the cells grown in anaerobic conditions have a redox potential of around − 0.2 V against an Ag/AgCl reference electrode, whereas no redox potential was found with cells grown in aerobic conditions, as previously reported [19]. The ability to reduce insoluble Fe(III) to Fe(II) in the presence of the electron donor, lactate, was examined in liquid cultures supplemented with amophorous iron oxyhydroxide. Two samples were prepared as controls; one had no fuel, while the other had glucose as a fuel and anaerobically grown *E. coli* was inoculated into the media rather than *S. oneidensis* MR-1. The color of the FeOOH suspension was investigated after overnight incubation. FeOOH in a batch culture that contained *S. oneidensis* MR-1 and lactate turned to blue from red, due to the reduction of Fe(III) to Fe(II) as cells grew anaerobically (Fig. 3A). In contrast, the two controls (without lactate; Fig. 3B, or with *E. coli*

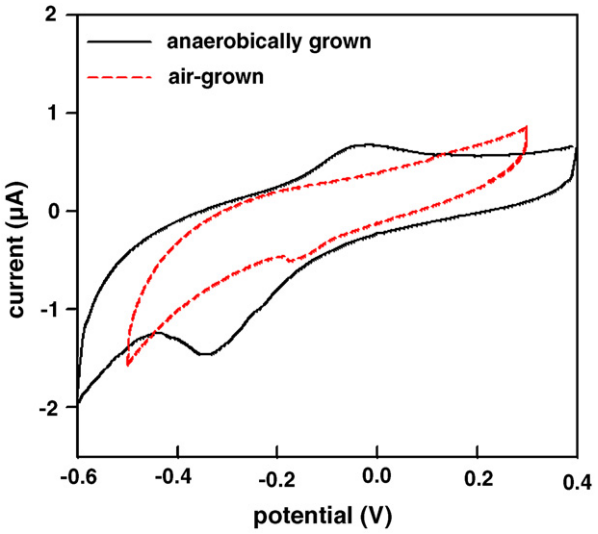

Fig. 2. Cyclic voltammograms of *S. oneidensis* MR-1 grown anaerobically and aerobically.

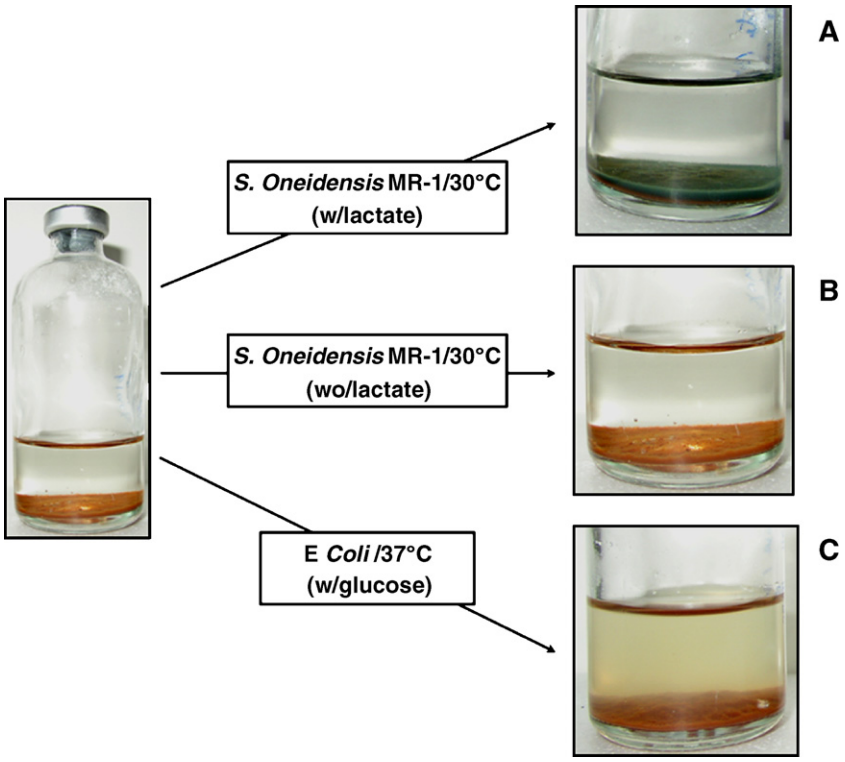

Fig. 3. Fe(III) reduction by *S. oneidensis* MR-1 and *E. coli* in an anaerobic batch culture system. (A) A batch culture containing FeOOH with lactate, inoculated with *S. oneidensis* MR-1 and incubated at 30 °C. (B) A control batch culture containing FeOOH without lactate, inoculated with *S. oneidensis* MR-1 and incubated at 30 °C. (C) A control batch culture containing FeOOH with glucose, inoculated with *E. coli* and incubated at 37 °C.

and glucose; Fig. 3C) did not change the color of FeOOH. Because *S. oneidensis* MR-1 does not grow without the addition of lactate, there was no electron production, and thus FeOOH (III) was not reduced (Fig. 3B). Similarly, although cell growth (increase in OD) was observed in the presence of *E. coli* with glucose, FeOOH did not change its color because *E. coli* is not capable of extra cellular electron transduction (Fig. 3C). This result is consistent with previous reports that *E. coli* requires soluble mediator compounds [21] or a conducting polymer-modified electrode [22,23] to shuttle electrons between cells and the electrode. These results confirm that electron exchange is occurring between anaerobically grown *S. oneidensis* MR-1 and the electrode surface, and also establish that the batch culture method can be readily used for the simple determination of the electrogenic activity of bacteria.

3.2. Optimization of reaction conditions for electrogenesis

Previously, Kim and co-workers demonstrated that *S. putrefaciens* grown on lactate under anaerobic conditions could generate electricity in a three-electrode electrochemical cell connected to a

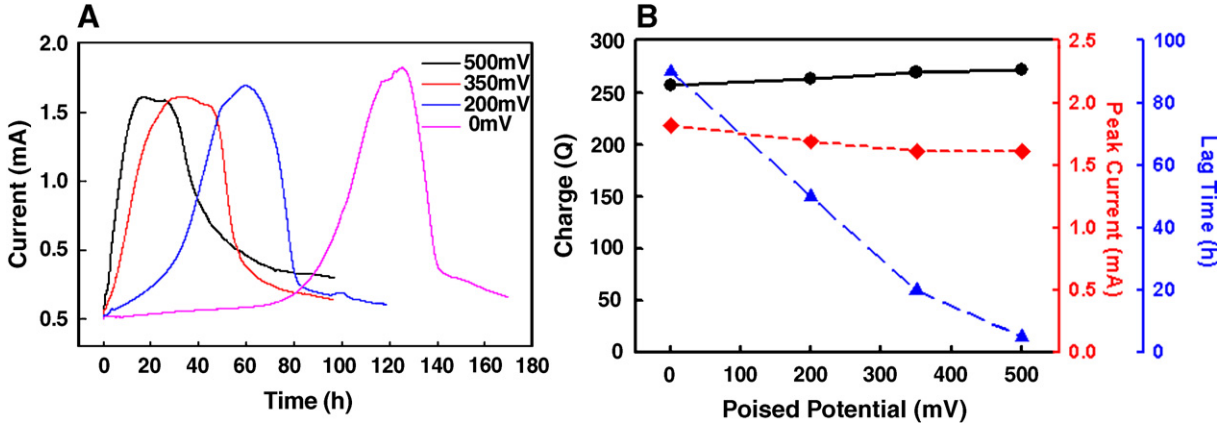

Fig. 4. Effect of poised potential on current generation by *S. oneidensis* MR-1 grown in 500 mM lactate. (A) Current profiles as a function of poised potential. (B) Charge, peak current, and lag time as a function of poised potential. (●) represents charge, (◆) represents peak current, and (▲) represents lag time.

potentiostat [1]. To gain a better understanding of electron transduction during anaerobic growth and to optimize physical components of the electrochemical cell prior to optimizing the bacterial component we carried out similar experiments with *S. oneidensis* MR-1 while varying poised potential and lactate concentrations. A similar electrochemical cell with graphite electrodes was used, in which the working electrode was poised at a constant potential against an Ag/AgCl reference electrode, as shown in Fig. 1. Once the background current of the electrochemical cell had reached to a steady value, an aliquot of aerobically grown *S. oneidensis* MR-1 (final concentration 0.7%, v/v) was inoculated into the anodic compartment of electrochemical cells and then the change in current was recorded.

3.2.1. Effect of poised potential on bacterial electrogenesis

Fig. 4A represents the current profile as a function of the poised potential applied to the working electrode. *S. oneidensis* MR-1 electrogenesis was examined in the presence of excess lactate (500 mM) to avoid electron donor limitation. Following inoculation, there was a variable lag period prior to the onset of electrogenesis. As the poised potential was increased, lag periods grew shorter (Fig. 4B), from 90 to 5 h. Cellular ODs, in general, decreased during the lag time and then increased by 50% along with the increase in current. It is suspected that since *S. oneidensis* MR-1 transfers electrons directly to the working electrode that it becomes enriched on the electrode surface, like *Geobacter sulferreducens* [12], rather than in solution.

We attribute the lag period to the fact that aerobically grown *S. oneidensis* MR-1 was not electrochemically active when it was first inoculated into the electrochemical cell, but that cellular metabolism altered in the anaerobic environment to allow electrogenesis, as previously reported by Kim et al. [19]. We now find that the activation process can be accelerated by increasing the poised potential to the working electrode. Interestingly, when even higher poised potentials (above 750 mV) were applied, cells either did not grow or current was not generated (data not shown). The maximum potential (and shortest lag time) at which *S. oneidensis* MR-1 could carry out productive electrogenesis was around 0.55 V, consistent with previous reports that measured the potential of a bacterial electrochemical cell in the absence of a poised potential [2]. These findings should prove useful in recruiting natural, electrocompetent bacteria to fuel cells newly introduced into the field. In particular, it may be possible to recruit different sets of bacteria with different poised potentials, and to build electrogenic ecology on an electrode by varying potential over time. In addition, this discovery may prove useful in waste water treatment; specific contaminants could be decomposed rapidly by applying the appropriate poised potential to an electrochemical cell.

After reaching a maximum value, the current fell to a basal value over time, probably due to bacterial growth-induced reduction in pH (the final pH of the media in the working electrode chamber was around 5.5 in all experiments). Park et al. have previously reported that the pH in bacterial fuel cells can decrease due to lactate fermentation [8].

Fig. 4B compares the effect of poised potential on overall current generation. In general, as the poised potential was increased from 0 to 500 mV the charge production (Q = current × time) also increased slightly (by 6%), but peak current decreased slightly (by 12%). These changes are only slightly higher than the variations in charge and peak current observed at 500 mV poised potential (0.7% and 7.5%, respectively). It might be expected that peak and charge should be constant in the presence of excess lactate. However, since increasing the poised potential was found to shorten lag times for electrogenesis, it is also possible that higher poised potential might change either bacterial adaptations or biofilm formation, and concomitantly change the efficiency of electron transduction.

3.2.2. Effect of fuel concentration

A similar electrochemical cell with a graphite electrode poised at a 500 mV against the Ag/AgCl reference electrode was used to investigate the effect of lactate on bacterial electrogenesis. Aerobically grown, stationary-phase cell cultures (final concentration 0.7%, v/v) were inoculated into the anodic compartment. Fig. 5A shows the current profile at

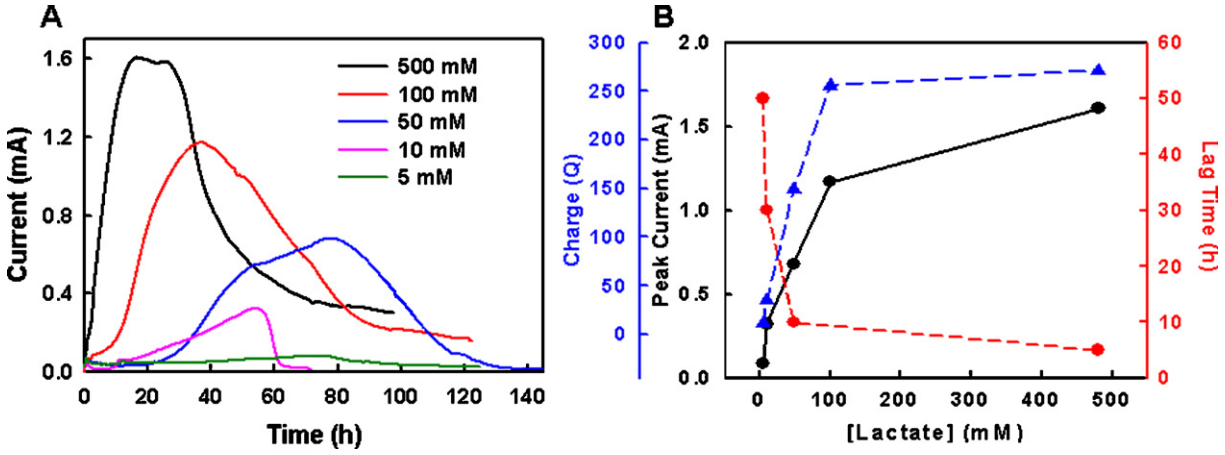

Fig. 5. Effect of lactate concentration on current generation by *S. oneidensis* MR-1 with the working electrode poised at 500 mV against Ag/AgCl reference electrode. (A) Current profiles as a function of lactate concentration. (B) Charge, peak current, and lag time as a function of lactate concentration. (▲) represents charge, (●) represents peak current, and (◆) represents lag time.

various concentration of lactate. In general, current production was faster (shorter lag time) and more efficient as the lactate concentration was increased. Fig. 5B summarizes the effect of fuel concentration on charge, peak current, and the lag time. As lactate concentration was increased from 5 to 500 mM, charge and peak current increased dramatically at values greater than 100 mM, up to ~270 C (22.6-fold above the value at 5 mM lactate) and ~1.6 mA (18.6-fold above the value at 5 mM lactate). The lag time decreased from 50 to 5 h. These results imply that *S. oneidensis* MR-1 electrogenesis is proportional to fuel concentration but can be saturated at 100 mM lactate. These results suggest that pre-concentration of fuel may allow more efficient electricity generation. In addition, the fact that the bacterial electron transfer properties can change dramatically as a function of fuel concentration may mean that the bacterial portion of a fuel cell can be optimized for specific situations by either adaptation or evolution.

3.3. Anaerobic batch growth

In preparation for serial transfer experiments, and to investigate the effect of an electron donor (lactate) and/or an acceptor (fumarate) on the anaerobic growth of *S. oneidensis* MR-1 in batch culture experiments, four different media with various compositions were prepared (LM1=LML medium with lactate and fumarate; LM2=LML medium with lactate; LM3=LML medium with fumarate; and LM4=LML medium without lactate and fumarate). Aerobically grown cells were inoculated to serum bottles containing the various media and OD was measured both right after inoculation and 48 h after incubation. The best cell growth was obtained in medium LM1 (2.6-fold above inoculation; data not shown). There was slight growth in medium LM3 (0.08×), but cells did not appear to grow in medium LM2 and LM4, which did not contain fumarate. These results confirmed that fumarate is necessary during anaerobic culture growth as an electron sink. Based on these results, we used medium LM1 for serial transfer experiments.

3.4. Biological optimization through serial transfer

It is plausible that the bacterial component of a fuel cell can be optimized by adaptation or evolution, as has also been demonstrated by Rabaey et al. [17] for a bacterial consortium. In order to determine to what extent a single, electrochemically active culture could be adapted or selected, we carried out a series of serial growth experiments in batch culture system under anaerobic conditions. The batch culture system was constructed with a serum bottle containing LML medium with lactate and fumarate, which was sealed and then purged for 30 min to remove any trace oxygen. Since lactate and fumarate function as an electron donor and an acceptor, respectively, this batch culture system mimicked a biofuel cell. The first batch growth was inoculated with 100 µl of aerobically grown cells and grown for 24 h. Serial transfer was initiated by anaerobic transferring 10% (v/v) of this batch to a new serum bottle and again incubating for 24 h. As summarized in Fig. 6A, cell growth was similar following each dilution, and the maximum OD after 24 h incubation was between 0.12 and 0.15. Frozen stocks of anaerobically-adapted cells were made approximately every 5 serial transfers (1st, 5th, 10th, 15th, and 20th batches) and, these generations were assayed for electrogenesis using the electrochemical cell system. In detail, frozen stocks from the archived generations were revived in LB medium, grown aerobically, and then inoculated into the anaerobic electrochemical cell at identical cell densities (OD) with 50 mM lactate as a fuel. We used 50 mM lactate rather than 100 mM so that fuel was not saturating and improvements in electrogenesis could be distinguished. As summarized in Fig. 6B, after the 4th serial transfer (5th batch), bacteria retained better charge production (1.3 times higher) with higher peak current (1.7 times).

While it seems unlikely that significant evolution could have occurred over only five generations, it is nonetheless quite plausible that the cells have physiologically adapted to the batch culture conditions and that this physiological change persists over at least one generation of aerobic growth. These results are also somewhat surprising given that fumarate, rather than an electrode

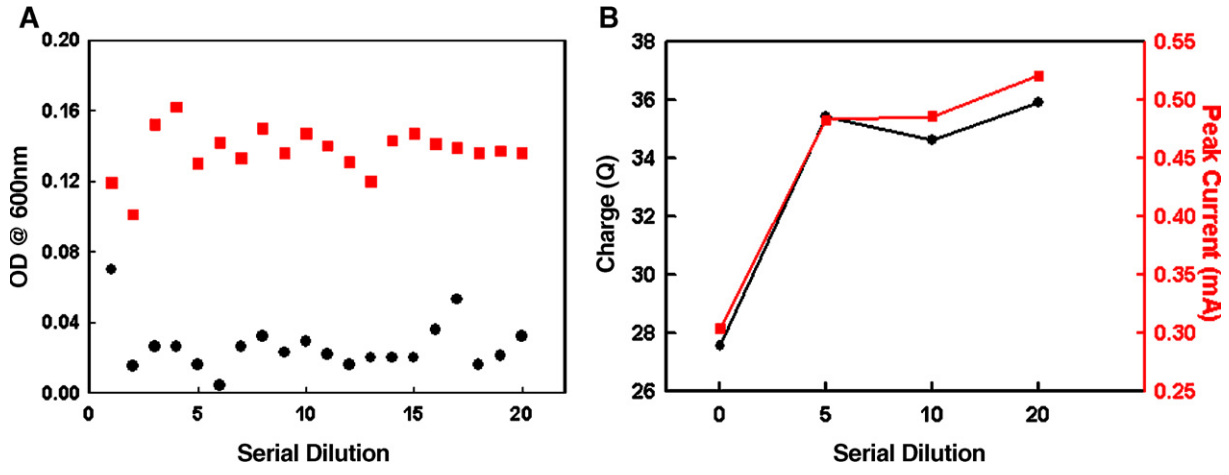

Fig. 6. Serial transfer of *S. oneidensis* MR-1 in a batch culture system. (A) OD as a function of serial dilution; (●) represents initial OD upon inoculation and (■) represents the final OD after 24 h incubation at 30 °C. (B) Efficiency of electricity generation as a function of serial dilution. (●) represents charge and (■) represents peak current.

surface, was the electron acceptor, and further emphasize the importance of anaerobic metabolism in fuel cell function. Given that cells are thought to attach themselves to the electrode surface and may undergo only limited growth and metabolic differentiation following attachment, these results provide significant insights into how to prepare bacterial cultures for fuel cells.

It is difficult to directly compare results from an electrochemical cell and a single bacterial species (this work) with results from a biofuel cell and a bacterial consortium (Rabaey et al.). Nonetheless, while the change in peak current we observed (1.7 fold improvement) is less than the improvement in power output (7-fold) observed by Rabaey et al., it should be noted that most of the change in the efficiency of the bacterial consortium likely resulted from changes in the relative proportions of individual species in the population, whereas our improvements were due solely to improvements in a single species. These results suggest that it may be possible to improve individual strains and then reintroduce them into a consortium, providing a further mechanism for increasing the efficiency of electrogenesis.

Moreover, the fact that physiological adaptations can be readily observed implies that it should also be possible to select for evolutionary changes as well. The simple serial transfer method described here can be used to further select microbes or microbe populations that are more efficient in electrogenesis.

4. Conclusion

The ability of *S. oneidensis* MR-1 to oxidize organic compounds with an electrode serving as the electron acceptor has implications for harvesting energy from waste matter, and may provide a means for bioremediation of subsurface environments contaminated with organic compounds.

We regulated the working electrode chamber in the electrochemical cell system in order to investigate the effect of poised potential and fuel concentration on the electrogenesis by *S. oneidensis* MR-1. Increasing poised potential resulted in shorter lag times until current generation was observed. Increasing lactate concentration to 100 mM also resulted in shorter lag times, and yielded 22.6 times more charge, 18.6 times higher peak current. These series of optimization experiments suggested that the electricity generation and microbe enrichment in an electrochemical cell can be optimized in part by changing poised potential and fuel concentration. In addition, regulation of the electrochemical cell allowed us to speed up the recovery of aerobically grown microbes.

The biological component of the electrochemical cell could also be optimized. Anaerobic, serial transfer experiments with *S. oneidensis* MR-1 were initiated with a soluble electron donor and acceptor in order to pre-adapt bacterial metabolism to an anaerobic environment. Compared with unadapted bacteria, the anaerobically adapted *S. oneidensis* MR-1 showed improved efficiency in electricity generation, even following re-exposure to air. These experiments should now serve as a starting point for the directed evolution of organismal electrogenesis. The optimization of electrochemical and biological components should help improve the sustainability of electrogenesis under conditions where fuel is continuously replenished.

Acknowledgments

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