



Effect of impaired twitching motility and biofilm dispersion on performance of *Pseudomonas aeruginosa*-powered microbial fuel cells

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Abstract

Pseudomonas aeruginosa is a metabolically voracious bacterium that is easily manipulated genetically. We have previously shown that the organism is also highly electrogenic in microbial fuel cells (MFCs). Polarization studies were performed in MFCs with wild-type strain PAO1 and three mutant strains (*pilT*, *bdlA* and *pilT bdlA*). The *pilT* mutant was hyperpiliated, while the *bdlA* mutant was suppressed in biofilm dispersion chemotaxis. The double *pilT bdlA* mutant was expected to have properties of both mutations. Polarization data indicate that the *pilT* mutant showed 5.0- and 3.2-fold increases in peak power compared to the wild type and the *pilT bdlA* mutant, respectively. The performance of the *bdlA* mutant was surprisingly the lowest, while the *pilT bdlA* electrogenic performance fell between the *pilT* mutant and wild-type bacteria. Measurements of biofilm thickness and bacterial viability showed equal viability among the different strains. The thickness of the *bdlA* mutant, however, was twice that of wild-type strain PAO1. This observation implicates the presence of dead or dormant bacteria in the *bdlA* mutant MFCs, which increases biofilm internal resistance as confirmed by electrochemical measurements.

Keywords Microbial fuel cell · *Pseudomonas aeruginosa* · Biofilm dispersion · Twitching motility · Power density

Introduction

It is well established that environmental and pathogenic bacteria are capable of growing as complex, surface-attached communities enmeshed in extracellular polymeric substances (EPS) known as biofilms [1]. In microbial fuel cells (MFCs), electrogenic (power-generating) bacteria that utilize multiple carbon sources can generate direct current

electricity in planktonic (free-swimming or floating) and especially biofilm (surface-attached) cultures [2]. An ideal MFC biofilm occurs when bacteria bind tenaciously to an MFC electrode (either anode or cathode) at high densities in an open, porous structure that allows for nutrient/waste exchange by biofilm bacteria. The microbes should also be highly electroactive (capable of rapidly transporting electrons between cells and the electrode) either directly via direct surface attachment (e.g., mediatorless) or via soluble redox-active mediators [3, 4]. The anodic biofilm bacteria should ideally be able to oxidize a myriad of organic molecules. Thus, the Gram-negative rod, *Pseudomonas aeruginosa* (*PA*), is an ideal organism that fulfills these requirements, being highly competitive with other organisms in both soil and aquatic environments. *PA* forms robust biofilms on multiple surfaces including the anode of MFCs [5–7] and is highly electrogenic using both pilus-mediated (mediatorless) and phenazine-based (mediator) conductance [8, 9].

In *PA*, the surface appendages (flagella and type IV pili) are required for optimal biofilm formation [10]. However, there are hundreds of genes that affect various aspects of biofilm formation, including attachment, cell division, micro-

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macro-colony formation, matrix biosynthesis, and dispersion (active release of biofilm bacteria back to the free-swimming planktonic phase) [11]. One gene, *pilT*, encodes an ATPase, which, when absent, increases the number of type IV pili (polar, retractable, conductive appendages that enhance electron transfer to the anode [6]). In addition, a *pilT* mutant is incapable of a surface mode of movement known as twitching motility. Chiang and Burrows previously reported that suppression of twitching motility enhances biofilm formation by promoting surface attachment and cell-to-cell adhesion (for model, see Fig. 1, [12]). We recently demonstrated that a *PA pilT* mutant generates nearly three times the direct current electricity of wild-type bacteria using urine as a carbon source [5]. The *pilT* mutant also has been shown to be impaired in virulence when compared with wild-type bacteria [13].

Another gene discovered in 2006, coined *bdIA* (for biofilm dispersion locus), is critical for bacterial dispersion from mature biofilms [14]. The *pilT bdIA* double mutant has all the properties of a *pilT* mutant mentioned above, but the *bdIA* chemotaxis gene is inactivated. Based on previous studies using *PA* [14, 15], the process of chemotaxis generally is vital for the bacteria to disperse from a biofilm when the carbon sources are scarce or even in abrupt abundance. The *bdIA* mutation can impede the dispersion process in bacteria enmeshed in biofilms even upon an immediate tenfold increase in the concentration of glutamate in the medium

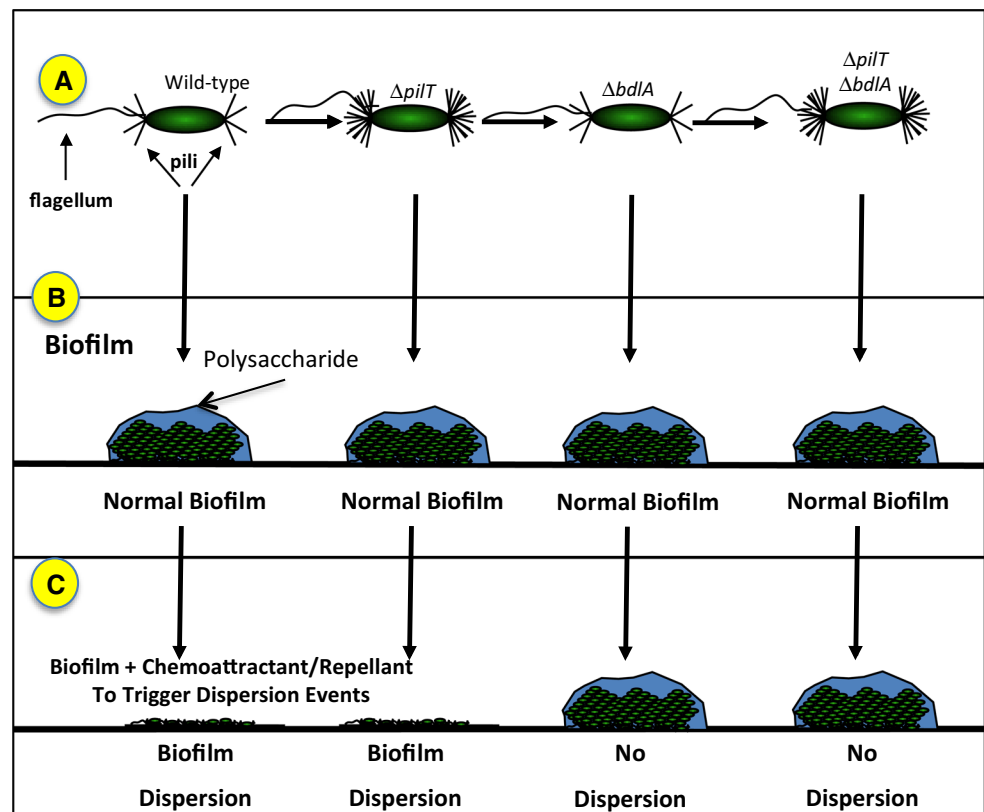
[14]. As the suppression of dispersion could lead to thicker biofilms, we postulated that the *pilT bdIA* double mutant would enhance the electrogenic potential when compared to wild-type and the *pilT* mutant organisms. The *pilT* mutant alone has been shown to generate substantially higher power than wild-type bacteria [5]. Figure 1 depicts the bacterial structure (A), predicted biofilm structure (B) and how the strains could potentially react to the sudden addition of a chemoattractant or chemorepellent (C). This work explores the electrogenic potential of three mutants of *PA*, namely a hyperpilated *pilT* mutant, a chemotaxis-suppressed *bdIA* mutant and a *pilT bdIA* double mutant.

Methods

Experimental setup

Cylindrical MFC reactors (110 ml anode/cathode chambers) were constructed using (poly (methyl methacrylate), PMMA) as previously described [5]. Graphite felt (GF, 4 cm × 0.8 cm × 0.6 cm, Bay Composites, MI) served as the anode, while Pt/carbon cloth (3 cm × 0.8 cm, Fuel Cell Store, Texas) served as the cathode. A Nafion 117 (Fuel Cell Store) proton exchange membrane divided the anode and cathode chambers. All materials were sterilized and pre-treated as discussed in our previous work [5]. The

Fig. 1 **a** Predicted bacterial biofilm structure upon *pilT*, *bdIA* or *pilT bdIA* mutations. **b** Predicted biofilm structure of each strain. The green color represents the bacteria, while the light blue color represents the biofilm matrix. **c** Predicted dispersion patterns in response to chemoattractants or chemorepellents in a flow from left to right. The conditions depicted are meant to demonstrate the response of biofilm bacteria, not necessarily the conditions in the MFCs used in these studies



catholyte was 50 mM potassium ferricyanide (pH 7.0), while the anolyte was Luria (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter). Rich media such as LB broth or nutrient broth are used frequently in MFC research to foster not only robust bacterial growth, but also biofilm formation and, ultimately, enhanced power generation [5, 16].

Inoculation of MFCs

PA wild-type strain PAO1, a wound isolate and annotated sequenced strain (www.pseudomonas.com) [17], was maintained indefinitely as a frozen stock at -80°C in 1 ml of stationary phase bacteria grown in LB medium and 1 ml of 45% sterile glycerol until use. Isogenic *pilT*, *bdlA* and *pilT bdlA* mutants were constructed by insertional mutagenesis using a gentamicin and/or tetracycline resistance cassettes followed by sucrose counter-selection to resolve co-integrates as previously described [18]. All strains from frozen stocks were first grown on LB agar plates under aerobic conditions for 24 h at 37°C . An isolated colony was inoculated into 5 ml of LB broth and incubated with continuous shaking at 37°C for 12–16 h. One ml of the stationary phase suspension was then used to inoculate 110 ml of fresh LB broth in the MFC anode. MFCs were maintained at room temperature ($\sim 21\text{--}23^{\circ}\text{C}$) after inoculation and all the electrochemical studies were performed between days 1–5 post-inoculation.

Data procurement

Electrochemical data (Fig. 2) were collected using a Gamry potentiostat (PCI4300-32034) as previously described [5]. Voltage and the current measurements were used to determine the MFC power density curves (Fig. 2a)

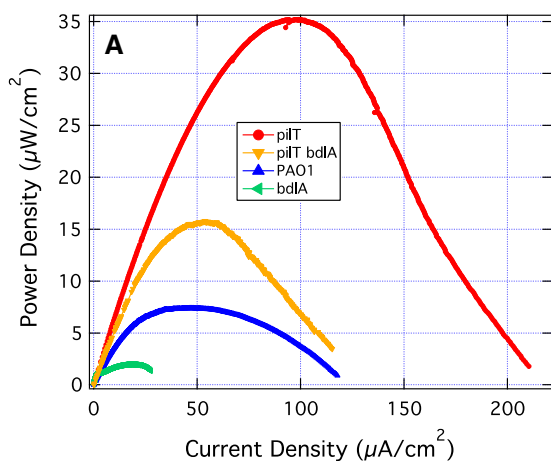


Fig. 2 Electrochemical data for *pilT* (red), *pilT bdlA* (orange), wild type PAO1 (blue), and *bdlA* (green): **a** full-cell power curves using graphite felt (12 cm^2) anode and a Pt/carbon cathode. **b** Polarization

and electrochemical impedance spectroscopy (EIS) was also performed (Fig. 3). Under ideal circumstances, EIS is used to elucidate the observations made through polarization studies and calculate the total resistance of the full cell. EIS was performed at the open circuit voltage with a frequency range varying from 0.01 to 100 kHz. The amplitude of the used AC voltage was 10 mV. All EIS and polarization were performed at room temperature ($21\text{--}23^{\circ}\text{C}$).

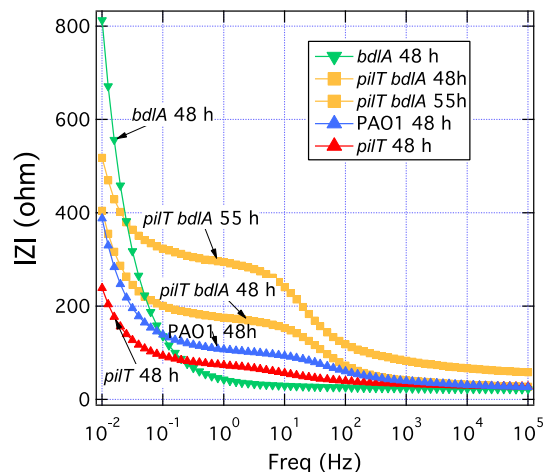
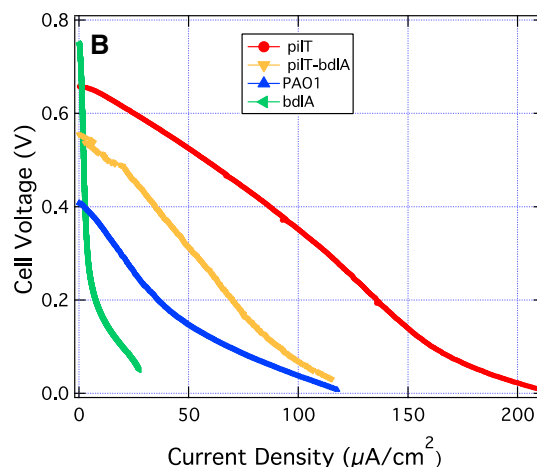


Fig. 3 Bode plot comparing the electrogenic performance of wild-type PAO1 and mutant strains. The Bode plot implies that the intermediate performance of the *pilT bdlA* mutant is not the result of averaging *pilT* and *bdlA* electrochemical responses. Rather, the *bdlA* mutant displays a qualitatively different electrochemical equivalent circuit reflected by the absence of an RC circuit element with a characteristic frequency ($= 1/RC$) of about 30 Hz



data. The poor performance of the *bdlA* MFC is due to high internal resistance (large initial slope)

Twitching motility assays

Twitching motility is a form of surface-associated movement by bacteria such as *PA* that is based upon extension and retraction of polar type IV pili [19]. This process is directly linked to the ability of *PA* to form robust biofilms, as strains lacking type IV pili form little or no in vitro-cultured biofilms [10, 20]. Overnight bacterial plate-grown cultures of *PA* strains PAO1, *pilT*, *bdlA* or *pilT bdlA* were stab-inoculated through 1% LB agar (3 mm thickness) to the bottom of the Petri dishes. The plates were then incubated for 2 days at 37°C, followed by incubation at room temperature until the agar was less than 1 mm thick. The agar was removed with tweezers and the twitching motility zone on the surface of the Petri dish was visualized by staining with 0.1% Coomassie Brilliant Blue R-250.

Crystal violet staining assay and colony forming unit (CFU) assessment of bacterial biofilm formation

Overnight bacterial cultures were diluted evenly in 200 µl of LB broth in 96-well polystyrene microtiter dishes and incubated at 37 °C for 24 h. Each plate containing all four bacteria biofilm cultures (*PA*, *bdlA*, *pilT* and *pilT bdlA*) was removed from the incubator daily from days 1–5 to investigate the amount of attached biofilm bacteria using the crystal violet (CV) staining assay developed by O’Toole and Kolter [10]. Briefly, the bacterial biofilms were washed twice with phosphate-buffered saline (PBS) after the unattached bacteria were decanted. For crystal violet staining, 200 µl of 0.1% crystal violet was added to each well and incubated at room temperature for 20 min. The crystal violet was removed and the adherent biofilms washed twice with water. Finally, 200 µl of 95% ethanol was added to each well to dissolve the crystal violet bound to the peptidoglycan layer of biofilm bacteria. After incubation for 30 min at room temperature, the level of purple color in each plate was determined by spectrophotometric analysis at O.D.₅₉₅ using a microplate reader. For CFU counts, PBS-washed and serially diluted samples were diluted tenfold and 10 µl of each dilution was pipetted on LB agar plates and CFU was recorded after 18 h incubation at 37 °C.

Results and discussion

Electrogenic performance evaluation of LB-fed MFCs

To investigate the electrogenic performance of the four selected bacterial strains, full-cell polarization analyses were performed. The cell voltage was decreased from open circuit voltage (OCV) to near zero. Figure 2a shows full-cell

power curves comparing wild-type PAO1 to the three mutant strains. The power curves are calculated from the measured current–voltage curves in Fig. 2b. Table 1 captures the performance parameters: peak power density, short-circuit current density and fill factor. The fill factor is the ratio of maximum power output of the cell to the theoretical maximum power output of the cell (product of OCV and short circuit current). The fill factor can also be directly correlated to the performance of the mutant. The error bars on the *bdlA* and *pilT bdlA* mutants are based on multiple measurements. For strain PAO1 and the *pilT* mutant, we used the standard deviation observed in our previous work [5].

The *pilT* mutant outperformed wild-type strain PAO1, while the *bdlA* mutant unexpectedly underperformed based on our initial hypothesis that the double *pilT bdlA* mutant would be the most electrogenic of the four strains. The electrogenic output of the *pilT bdlA* fell between the *bdlA* and *pilT* mutants, but still outperformed strain PAO1. Figure 2b indicates that the poor performance of the *bdlA* MFC is due to high internal resistance (refer to the initial slope). Although the *bdlA* cell produced the highest voltage at open circuit, the voltage dropped precipitously as the bacteria began to produce current.

Based on the aforementioned results, understanding the differences between the electrogenic performance of the *pilT* versus the *bdlA* and *pilT bdlA* mutants emerged as a critical feature of this study. The *pilT* mutant overexpresses type IV pili on the poles of the bacterium, which enhances electron transfer to the anode [5]. In addition, the *pilT* and *bdlA* mutants are incapable of twitching motility [12, 14], a form of surface-associated motility known to enhance biofilm formation by promoting surface attachment and cell-to-cell adhesion [12]. It was initially hypothesized that construction of another mutation within the *PA bdlA* gene in the *pilT* mutant background would further improve the power output, the logic being that suppressed chemotactic movement would impede dispersion from the already hyper-electrogenic *pilT* biofilm, thereby enhancing the electrogenic properties of the double *pilT bdlA* mutant. However, we observed that the performance of the *bdlA* mutant was below the performance of the wild-type strain PAO1. The *pilT bdlA* mutant performance fell between *pilT* and *bdlA*, but was still greater than that of PAO1.

Table 1 MCF performance parameters

Strain	Peak power density (µA/cm ²)	Short-circuit current (A/cm ²)	Fill factor
<i>pilT</i>	35 ± 5	210	0.26
<i>pilT bdlA</i>	11 ± 6	123	0.23
PAO1	7 ± 2	124	0.16
<i>bdlA</i>	2.0 ± 0.8	30	0.07

Table 2 Solution, anodic and total resistance 48 h after inoculation as calculated from Fig. 3

Bacterial strain	R_s (10^5 Hz) (ohms)	$ Z $ (0.01 Hz) (ohms)
PAO1	26 ± 3	390 ± 20
<i>pilT bdlA</i>	27 ± 3	400 ± 25
<i>pilT</i>	27 ± 3	240 ± 20
<i>bdIA</i>	22 ± 3	815 ± 50

EIS measurements

This section examines the electrochemical impedance spectroscopy (EIS) data from each of the four strains. The measured values of the imaginary impedance ($Z_{\text{imaginary}}(f)$) and real impedance ($Z_{\text{real}}(f)$) were used to calculate the magnitude of the impedance ($|Z|$) at different frequencies, f . The Bode plot in Fig. 3 displays the frequency dependence of $|Z|$. Several observations emerged from the Bode plot. We attribute the impedance at highest frequency (10^5 Hz) to solution resistance (R_s) and the impedance at zero frequency (0 Hz) to total resistance (R_T). R_T is considered to be the sum of all types of resistances in a full-cell (i.e., anodic, cathodic) solution and the biofilm resistance. [5] Since the data only extended to 0.01 Hz, we can only state that $R_T > |Z|(0.01 \text{ Hz})$. $|Z|$ at 0.01 Hz and the solution resistance (R_s) are listed in Table 2.

Several observations ensued from the EIS data. The larger $|Z|$ of the *bdIA* mutant confirms the observation made from Fig. 2b. High internal resistance has been observed to deleteriously affect the performance of MFCs [5]. The Bode plot also implies that the intermediate performance of the *pilT bdlA* mutant is not the result of averaging *pilT* and *bdIA* electrochemical responses. Rather, the *bdIA* mutant displayed a qualitatively different electrochemical equivalent circuit reflected by the absence of an RC circuit element with a characteristic frequency ($= 1/RC$) of about 30 Hz. Although the origin of this circuit element is not understood, the resulting EIS profile may be a diagnostic tool to probe the impact of genetic modification on microbial electrochemistry. In the double *pilT bdlA* mutant, for example, the resistance associated with the 30-Hz element was enhanced compared to all other strains. This resistance contributes to R_T and thus reduces the performance. Finally, after 48 h, the peak power of the *pilT bdlA* mutant dropped rapidly: at 55 h the peak power is comparable to wild-type strain PAO1 (not shown). As the peak power dropped, the resistances of all the EIS circuit elements increased (compare 48 and 55 h data in Fig. 3). This change could also indicate increased levels of dead or metabolically inactive bacteria.

Twitching motility and biofilm formation

To better comprehend the differences in the power output of each strain, twitching motility patterns as well as overall biofilm formation were examined. The primary reason behind the lower power output of the *bdIA* mutant was hypothesized to be a higher total MFC internal resistance. The higher internal resistance could be due to thicker biofilms with metabolically inactive/dormant bacteria or defective twitching motility. To test this hypothesis, we first measured twitching motility in each of the four strains to confirm that the three mutant strains are defective in twitching motility (Fig. 4). The *pilT*, *bdIA* and *pilT bdlA* mutants were all defective. The absence of twitching motility therefore does not differentiate the strains, since the *pilT* mutant generated a higher power density than wild-type bacteria [5] whereas the *bdIA* generates the lowest.

Thicker biofilms with inactive bacteria could potentially impede the transfer of electrons through the biofilms leading to a drop in the power output. To examine this possibility, bacterial viability was determined by measuring colony forming units (CFU) counts in biofilms cultured by each organism. Our results showed no difference in viable bacteria (Fig. 5a). Finally, to measure biofilm density, the crystal violet biofilm assay was again utilized [10]. This assay is commonly used to measure biofilm formation because of the direct relationship between O.D.₅₉₅ and surface-attached bacterial titers. Figure 5B indicates that the *bdIA* biofilm is almost twice as thick as that of strain PAO1. Since bacterial viability from our CFU count was similar in the two biofilms, it is likely that the *bdIA* biofilm harbors dormant or dead bacteria at the electrode surface, which explains the lower performance of this strain when compared with the other strains. Film thickness, however, is not sufficient to explain all the observations. On the basis of thickness alone the *pilT* and *pilT bdlA* mutants should perform similarly, whereas Fig. 2 shows that the *pilT* mutant is a substantially better electrogen. Further work is required to understand

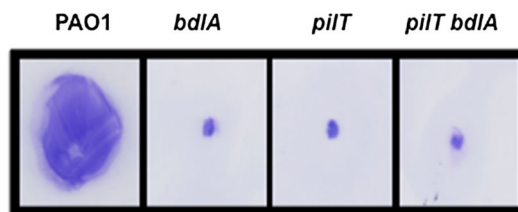
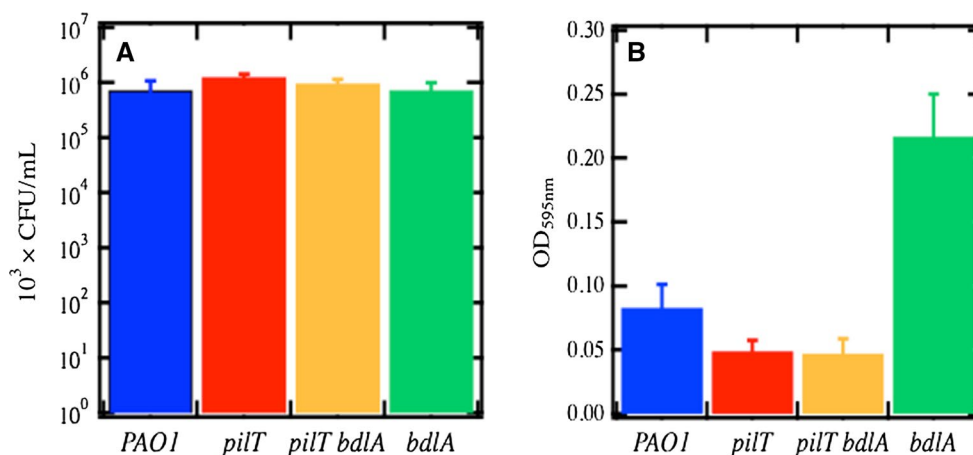


Fig. 4 Twitching motility by various PA strains. The four PA strains were stabbed to the base of 1% LB agar medium and the twitching motility zone allowed to develop for about 5 days. The twitching zone was amplified by staining of the plates with 0.1% Coomassie Brilliant Blue R-250

Fig. 5 CFU count and crystal violet assay of bacteria biofilms in MFC. **a** CFU count of 3-day-old bacteria biofilm in 96-well plates grown at room temperature (mean \pm SE of three replicates). **b** Crystal violet (0.1%) was used to stain 3-day-old bacterial biofilm in 96-well plates grown at room temperature and the O.D. at 595 nm recorded using a microplate reader (mean \pm SE of three replicates)



other factors contributing to the deteriorating performance of the *bdlA* mutant.

Conclusions

We observed that a *pilT* mutant of *PA* yielded a power output of 5.0 times that of the wild-type strain PAO1 and 3.2 times the *pilT bdlA* mutant (*pilT* > *pilT bdlA* > PAO1 > *bdlA*). The enhanced output of the *pilT* mutant was due to reduced twitching motility and overexpression of polar conductive pili relative to wild-type bacteria. The *bdlA* mutation shows increased biofilm thickness in addition to reduced twitching motility. Since all four strains showed similar CFU counts, the increased thickness of the *bdlA* mutant was correlated with an increased population of dead or dormant bacteria. Such bacteria increase biofilm resistance, especially if concentrated near the electrode. The reduced power output and the increased total resistance confirmed the presence of a “dead cell layer”.

Industrial relevance

Downstream industrial studies, although highly desirable, are currently not feasible due to a variety of issues affecting the overall performance of MFCs. These include but are not limited to (1) non-optimized electrogenic bacteria, (2) low power output, (3) non-optimal fuel cell design, (4) internal resistance, (5) biofouling and (6) scalability issues. Here, we offer a very promising solution, at least from the improvement of bacterial electrogen perspective. Using synthetic biology (SynBio) techniques, which marry electrical Boolean logic gates with bacterial genetic logic gates, we argue that MFC researchers can dramatically improve the overall power density of future MFCs. Our research team is currently using SynBio technology to construct highly controlled gene circuits designed to (1) optimize carbon

skeleton utilization, (2) enhance biofilm formation while limiting nutrient-impermeable exopolysaccharide production, and (3) optimize electron transfer from conductive pilus “nanowires” and from overproduction of redox-active mediators, pyocyanin and pyorubrin. Another issue for the use of *PA* in applied MFCs is the potential to cause human, animal or plant disease. In this case, we would create strains that are avirulent (unable to cause disease). Many genes in *PA* are required for full virulence in various animal models. Constructing mutations in genes such as the global regulator *gacA* [21] and the *suhB* gene [22] would eliminate any concerns for spread of disease from applied MFCs.

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