**Sensitizing *Pseudomonas aeruginosa* to antibiotics by electrochemical disruption of
membrane functions**

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Short running title: Electrochemical disruption of *Pseudomonas aeruginosa* cell membranes

1. ABSTRACT

Recently, we reported synergistic effects between 70 $\mu\text{A}/\text{cm}^2$ direct current and tobramycin in killing *Pseudomonas aeruginosa* PAO1 persister cells, a phenomenon we named electrochemical control of persister cells (ECCP; Niepa et al. Biomaterials 33: 7356-7365, 2012). To understand the mechanism of ECCP, the effects of electrochemical treatments mediated via stainless steel 304 and carbon electrodes on *P. aeruginosa* PAO1 were systematically compared using complementary approaches in this study. Electron microscopic analyses revealed that $\mu\text{A}/\text{cm}^2$ level direct current (DC) caused substantial changes in the structure and membrane integrity of *P. aeruginosa* PAO1 cells. DC treatments using SS304 electrodes induced cell lysis, while the same level of DC generated using carbon electrodes led to aggregation of intracellular proteins and increased permeabilization of *P. aeruginosa* PAO1 cells to antibiotics. The profound effects of DC on the physiology of persister cells were corroborated with DNA microarray analysis, which revealed the induction of genes associated with pyocin production and SOS response in DC-treated persister cells. Interestingly, sequential treatment using DC mediated with carbon electrodes followed by tobramycin was found more effective than concurrent treatment; and total eradication of persister cells was achieved.

Keywords: Persister cells, membrane, gene expression, TEM, SEM-EDS, electrodes

2. INTRODUCTION

Each year, millions of medical devices with various complexities are temporarily or permanently implanted in humans to administer treatments or palliate failing body components. The implantation of biomaterials can lead to recurrent complications associated with the adherence of microbial pathogens on these devices, and the development of chronic infections in surrounding tissues. Such implant-associated infections account for about half of hospital-acquired infection cases (1); e.g., 5% of the 2 million individuals receiving fracture fixation in the U.S. each year are infected with bacteria adhering to the implants (2). The infection rate (40%) is higher for ventricle-assisted heart implants (2). In addition to high morbidity and mortality, implant-related infections are also responsible for a massive financial loss. According to the Centers for Disease Control and Prevention, between \$29 and \$45 billion is spent each year to cover the inpatient services associated with infections acquired in U.S. hospitals (3), and more than \$9 billion is attributed to central line-associated bloodstream infections, surgical site infections, *Clostridium difficile* infections, ventilator-associated pneumonia, and catheter-associated urinary tract infections (4). These complications put a high burden on our healthcare system and seriously impact the quality of life of affected patients (5).

Multiple mechanisms contribute to bacterial persistence and drug resistance. In addition to well-documented acquired mechanisms such as enzymatic degradation of antibiotics and modification of cellular targets, bacteria can tolerate a broad spectrum of antibiotics through intrinsic mechanisms (6). Because the interaction between antibiotics and the cellular targets primarily corrupt growth-related pathways, such as DNA replication and protein synthesis (7), dormant and slow-growing cells have an inherent advantage in survival under the attack of antibiotics compared to actively growing cells. Also, increasing evidence indicates that within a

1 given bacterial population, a small percentage (e.g., ~1% for *Pseudomonas aeruginosa* PAO1 (8))
2 of metabolically inactive phenotypic variants, known as persister cells, are extremely tolerant to
3 antibiotics. Bacterial persistence can seriously compromise the effects of antibiotics because
4 persister cells can wake up from their dormant state after antibiotic treatment, and regenerate a
5 new bacterial population with a similar percentage of persister cells (9, 10). Beyond these
6 intrinsic capabilities of individual persister cells to survive antibiotic treatments, bacteria can
7 also obtain tolerance to antibiotics by attaching to surfaces and forming biofilms, a condition that
8 is commonly found in chronic infections such as those associated with medical devices and lung
9 infection in cystic fibrosis patients (2, 11). In addition to harboring many slow-growing cells
10 including persister cells, biofilm cells secrete a protective polymeric matrix comprising
11 polysaccharides, proteins, extracellular DNA and other substances (12). Consequently, a large
12 number of biofilm cells can tolerate antibiotics at concentrations up to 1000 times higher than
13 those required to kill their planktonic (freely swimming) counterparts (13). These challenges
14 emphasize the need for novel techniques that can eradicate bacterial infections involving
15 persister cells and biofilms.

16 Novel approaches integrating physical, electrical, chemical and biological factors offer
17 promising solutions to the issues associated with bacterial persistence (14-18). Recently, we
18 introduced a new method for controlling bacterial persistence based on a phenomenon we named
19 electrochemical control of persister cells (ECCP). We reported that 98% of *P. aeruginosa* PAO1
20 persister cells can be killed by applying a low-level direct current ($70 \mu\text{A}/\text{cm}^2$ DC) generated
21 using stainless steel 304 (henceforth SS304) electrodes for 1 h. Also, DC generated using SS304
22 electrodes was found to enhance the killing activity of tobramycin, showing an interesting
23 synergy with a total of 5-log killing of *P. aeruginosa* PAO1 persister cells (8). In contrast, the

same level of DC generated with carbon electrodes was less effective in killing *P. aeruginosa* PAO1 persister cells and the concurrent treatment with carbon electrode mediated DC and tobramycin did not exhibit synergistic effects. We speculated that the electrode materials play an important role in ECCP, and the movement of the DC-generated electrochemical products (e.g. metal ions released from the anode and reactive oxygen species (ROS) and intermediates generated from the cathode) is essential for the synergistic killing effects. To decipher the mechanism of ECCP, complementary approaches were used in this study to systemically investigate the physiological response of *P. aeruginosa* cells to DC treatments. Specifically, we characterized DC-induced changes in the structure, mechanical properties and genetic response of *P. aeruginosa* cells. The effects of treatment time, electrode material and antibiotic on the cells were also investigated.

3. MATERIALS AND METHODS

3.1. Bacterial strains, growth media, and persister isolation

The wild-type *P. aeruginosa* PAO1 (henceforth PAO1) was cultured in Lysogeny broth (LB) medium (19) containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. The overnight cultures were incubated at 37°C with shaking at 200 rpm. Isogenic mutants of *P. aeruginosa* PAO1 were acquired from the PAO1 transposon mutant library (UW-Genome Sciences, University of Washington, WA, USA). The mutants PW4389 (PA1939-G06::ISlacZ/hah), PW10231 (PA5461-A02::ISlacZ/hah), PW2800 (*pqsB*-B05::ISlacZ/hah), PW8462 (PA4430-C09::ISphoA/hah), PW4830 (*pvcA*-F05::ISlacZ/hah), PW4567 (*fusA2*-F06::ISlacZ/hah), PW7877 (*oprG*-A04::ISphoA/hah), PW6348 (*glk*-C11::ISphoA/hah), PW3811 (*aer*-B08::ISlacZ/hah), and PW4324 (PA1897-B09::ISphoA/hah) (20) were grown in LB medium

supplemented with 10 µg/mL tetracycline (Tet) at 37°C with shaking at 200 rpm. The persister cells were isolated as described previously (8). Briefly, PAO1 cells were harvested from overnight cultures by centrifugation (Sorvall Legend RT, Thermo Scientific, Asheville, NC, USA) at 8000 rpm for 10 min, washed twice with 0.85% NaCl solution, and treated with 200 µg/mL ciprofloxacin (Cip) for 3.5 h at 37°C, with rotation at 200 rpm. Finally, the cells were washed three times with 0.85% NaCl to remove residual antibiotic and the debris of lysed cells.

3.2. Synergy between DC and antibiotics: sequential treatment

The harvested persister cells were resuspended in 0.85% NaCl solution to optical density at 600 nm (OD₆₀₀) of 0.9. Three mL of cell suspension was transferred to a polystyrene electrochemical cell to perform DC treatments as described previously (8). Briefly, two electrodes were inserted against the opposite walls of a cuvette with 1 cm distance between them; and an Ag/AgCl reference electrode was inserted in the middle. The 70 µA/cm² DC was delivered using a potentiostat (WaveNow, Pine Research Instrumentation, Raleigh, NC, USA) to treat PAO1 persister cells for 60 min. Immediately after, 1 mL of the suspension was collected to quantify the total number of viable cells by counting colony-forming units (CFUs) after a series of a 10× dilution with 0.85% NaCl solution. Then, 10 µL of each dilution was dropped on a LB agar plate (1.5% agar) and incubated at 37°C for 16 h to count the number of CFU. Each sample was tested with 5 replicates. The remaining DC-treated samples was transferred to microcentrifuge tubes and further treated with 200 µg/mL Cip for persister isolation as described above. Similarly, the total number of viable cells and the number of persister cells in DC-free samples were quantified as controls.

To understand if DC could sensitize persister cells to antibiotics in sequential treatments, PAO1 persister cells were resuspended in 0.85% NaCl solution to OD₆₀₀ of 0.6 and treated with

70 $\mu\text{A}/\text{cm}^2$ DC using carbon or SS304 electrodes for 1 h. Immediately after this step, the DC-treated persister cells were challenged with 1.5 $\mu\text{g}/\text{mL}$ tobramycin (Tob), 60 $\mu\text{g}/\text{mL}$ kanamycin (Kan), or 60 $\mu\text{g}/\text{mL}$ spectinomycin (Spec) for 1 h, and the viability of persister cells was assessed by counting CFU.

3.3. Membrane integrity of *P. aeruginosa* PAO1 cells

First, Propidium Iodide (PI), a membrane impermeable dye that stains cells through interaction with DNA, was used to evaluate membrane integrity. The stationary-phase PAO1 cells were resuspended in 0.85% NaCl solution to OD_{600} of 1.5 and treated with DC using carbon or SS304 electrodes as described above. Immediately after the DC treatment, the cells were stained with 1.5 μL PI (20 mM) for 15 min, and then washed with 0.85% NaCl solution to remove non-specifically bound dye. The absorbance of PI associated with the PAO1 cells was measured using a fluorescence plate-reader with excitation and emission at 530/25 and 590/35 nm, respectively (Synergy 2 Multi-Mode Microplate Reader, Biotek, VT, USA).

In a complementary analysis, transmission electron micrographs were obtained to examine the membrane integrity of DC-treated and untreated cells. One hundred μL of each sample was fixed in 400 μL of 2.5% glutaraldehyde solution in PBS for 1 h. The cells were attached to a carbon-coated copper grid (T400H-Cu, Electron Microscopy Science, PA, USA) and allowed to fully dry at room temperature. The samples were stained for 15 s with 2% uranyl acetate, washed three times with HPLC grade water, and the grid was dried at room temperature to examine the morphology of cells using TEM (JEOL 2000EX, JOEL, Japan). Further analyses were performed to investigate the structural changes in cell membrane after 20, 40 and 60 min of treatment with DC. To achieve this, PAO1 cells in stationary phase cultures were treated with DC using carbon or SS304 electrodes for 20, 40 and 60 min; and all the samples, including the

untreated cells, were collected by centrifugation at 5,000 g for 5 min. After each sample was fixed in 2.5% glutaraldehyde solution in PBS for 1 h, the cells were collected by centrifugation and treated with 2% osmium for 1 h to cross-link the lipid molecules in cell membrane. The cell pellets were then dehydrated with increasing concentration of ethanol (in the order of 50, 70, 80, 90 and 100%; dehydration in 100% ethanol was repeated three times). Then, the cell pellets were rinsed three times with a solution of propylene oxide (100%), and gradually exposed to increasing concentrations (50%, 75%, and 100%) of Araldite 502 epoxy resin (Electron Microscopy Sciences, PA, USA) prepared with propylene oxide. Once the cells were embedded in 100% epoxy resin, the samples were incubated overnight in a 60°C oven to allow polymerization of the resin. Using a MT2-8 ultra-microtome (Sorvall Porter-Blum), 80 nm thin transections of the samples were cut and stained for 30 s successively with 2% uranyl acetate and Sato's lead citrate (21) before imaging with TEM (JEOL JSM-2000EX) at the State University of New York College of Environmental Science and Forestry.

3.4. Membrane composition of *P. aeruginosa* PAO1 cells after DC treatment using carbon electrodes

To investigate the membrane composition, PAO1 cells were harvested from an overnight culture, washed twice with 0.85% NaCl solution, and resuspended in 0.85% NaCl solution to an OD₆₀₀ of 1.5. The cells were then transferred to an electrochemical cell as described previously (8), and treated with 70 $\mu\text{A}/\text{cm}^2$ DC for 1 h using carbon electrodes as described above. The DC-treated samples and untreated controls were deposited on gold-coated glass slides. The samples were placed in vacuum for 24 h before they were analyzed using SEM (JEOL 5600, JEOL, Japan) with energy dispersive spectroscopy (EDS)(Princeton Gamma Tech) to determine the relative intracellular concentration of C, P, N, O, Fe, Cr, Mn, and Ni.

3.5. DNA microarray and qPCR analyses

To understand the effects of DC treatments on gene expression in PAO1 persister cells, DNA microarrays were used to obtain transcriptional profiles of PAO1 persister cells after various levels of DC treatment. PAO1 persister cells were isolated from overnight cultures (50 mL each) as described above. After washing, the cells were resuspended in 3 mL 0.85% NaCl and treated with 70 $\mu\text{A}/\text{cm}^2$ DC using carbon or SS304 electrodes for two treatment durations (20 min and 40 min). DC-free samples were used as controls. Immediately after each treatment, the cell pellet was collected by centrifugation at 15,000 g for 2.5 min at 4°C, frozen in a dry-ice ethanol bath immediately and stored at -80°C until RNA extraction. The total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) including on-column DNase treatment as described previously (22), and sent to J. Craig Venter Institute for DNA microarray analysis.

To perform DNA microarray analysis, DNA probes were synthesized by adding 2 mg of total RNA in a solution containing 6 mg of random hexamers (Invitrogen, Grand Island, NY, USA), 0.01 M dithiothreitol, an aminoallyl-deoxynucleoside triphosphate mixture (25 mM each dATP, dCTP, and dGTP, 15 mM dTTP, and 10 mM amino-allyl-dUTP) (Sigma, St. Louis, MO, USA), reaction buffer, and 400 units of SuperScript III reverse transcriptase (Invitrogen). After incubation at 42°C overnight, the RNA template was hydrolyzed with 0.1 M sodium hydroxide and 0.2 M EDTA at 70°C for 15 min. After unincorporated aa-dUTP was removed with a Minelute column (Qiagen), the probe was eluted with 4 mM KPO_4 (in ultrapure water; pH 8.5), dried, and resuspended in sodium carbonate buffer (0.1 M; pH 9.0). Normal human serum-Cy3 or serum-Cy5 (GE Healthcare, Pittsburgh, PA, USA) was added and incubated at room

temperature for 1 h to couple the amino-allyl cDNA with fluorescent labels. Extra label was removed using a Minelute column (Qiagen).

Aminosilane-coated slides were pre-hybridized in 5x SSC (Invitrogen), 0.1% sodium dodecyl sulfate, and 1.0% bovine serum albumin at 42°C for 60 min. The slides were then washed at room temperature with DI water, dipped in isopropanol, and then allowed to dry. Cy3- and Cy5-labeled probes were combined in equal volume, dried, and then resuspended in a solution of 5xSSC, 40% formamide, and 0.1% dodecyl sulfate. For hybridization, resuspended probes were first heated to 95°C, and then added to the microarray slide to hybridize at 42°C overnight. Hybridized slides were washed with a series of solutions of 1xSSC-0.2% SDS, 0.1xSSC-0.2% SDS, and 0.1xSSC at room temperature, and then dried in air. DTT was supplemented to all wash buffers at 0.1 mM. TIFF images of slides were obtained using an Axon GenePix 4000 scanner and analyzed with TIGR Spotfinder (available at <http://pfgrc.jcvi.org/index.php/bioinformatics.html>). Microarray data were normalized by LOWESS with in-slide replicate analysis using the TM4 software MIDAS (available at <http://pfgrc.jcvi.org/index.php/bioinformatics.html>).

To validate the results of the microarray analysis, 10 representative genes including *PA0647*, *PA4430*, *PA4614*, *PA0166* (for DC treatment using SS304 electrodes), *PA1032*, *PA4067*, *PA0633*, *PA0640* (for DC treatment using carbon electrodes), *pscF* (for both DC conditions), and the housekeeping gene *pbpA* (penicillin-binding protein 2) were selected to perform quantitative PCR (qPCR). The primers (forward and reverse) designed for the selected genes are shown in Table S1 (Supplementary Information). The gene expression profiles of PAO1 persister cells treated with 70 $\mu\text{A}/\text{cm}^2$ DC for 20 min or 40 min using carbon or SS304 electrodes were compared with those of the untreated control samples. The iScript cDNA

1 synthesis kit (Bio-Rad, Hercules, CA, USA) was used to generate cDNA templates based on 200
2 ng of total RNA. Following the manufacturer's protocol, the primer mix (containing the forward
3 and reverse primers) and Syber green mix (including the Syber green dye and cDNA templates)
4 were prepared to quantify the expression ratio of the genes of interest by running qPCR using an
5 Eppendorf Realplex Mastercycler (Eppendorf, Westbury, NY, USA).

6 **3.6. Susceptibility of *P. aeruginosa* PAO1 and selected mutants to tobramycin**

7 To understand if any of the genes identified from microarray results are important to the
8 response of bacteria to DC, a total of 10 genes induced by the DC treatments using carbon and
9 SS304 electrodes were selected, and the corresponding mutants were acquired from the PAO1
10 transposon mutant library (UW-Genome Sciences, University of Washington, WA, USA) for
11 further investigation. The mutants of *PA1939*, *PA5461*, *pqsB*, *PA4430*, and *pvcA* were selected to
12 compare with the wild-type PAO1 for the effects of SS304-generated DC. Similarly, the mutants
13 of *fusA2*, *oprG*, *glk*, *aer*, and *PA1897* were chosen to compare the response to DC generated with
14 carbon electrodes. First, the susceptibility of the selected mutants to 1.5 µg/mL Tob was tested to
15 understand if any of these mutations affects cell growth. The wild-type PAO1 and selected
16 mutants were grown overnight, and then sub-cultured with an initial OD₆₀₀ of 0.05 in a 96 well-
17 plate (Costar ® No. 9017, Corning, NY, USA) containing 200 µL of LB medium in each well
18 supplemented with or without (as control) 1.5 µg/mL Tob. The samples were tested with 8
19 replicates and incubated at 37°C for 7 h. Growth curves were recorded by measuring OD₆₀₀
20 every hour using an ELx808 Absorbance Microplate Reader (Biotek, Winooski, VT, USA).

3.7. Persister formation and response of selected mutants to DC treatment

Our previous work (8) demonstrated that PAO1 persister cells can be isolated by killing the regular cells with 25-200 µg/mL Cip for 3.5 h, and 200 µg/mL was selected for persister isolation. To determine if 200 µg/mL Cip is also appropriate for persister isolation from mutant strains, the selected mutants were challenged with 0 - 200 µg/mL Cip for 3.5 h in duplicated experiments, and the killing activity of Cip was evaluated by counting CFU of surviving cells as described previously (8). To investigate the impact of gene deletion on the response of persister cells to DC treatments, the mutants were cultured overnight in LB medium supplemented with 10 µg/mL Tet. Then persister cells of the selected mutants were isolated with 200 µg/mL Cip and treated with 70 µA/cm² DC for 1 h, as described above. During DC treatments, 100 µL of sample was taken every 20 min to evaluate the viability of persister cells by counting CFU.

3.8. AFM-based indentation testing

To test the effects of DC on the mechanical properties of PAO1 cells, nanoindentations were performed on DC-treated (with carbon electrodes for 1 h) and untreated control cells. Before the test, the cells were fixed with 2.5% glutaraldehyde in PBS to preserve the integrity of the cells and prevent cell deflation due to drying, and their relative differences in membrane moduli were probed. Using AFM, the force curves of the cell membranes were recorded and the mechanical properties including stiffness (Young's modulus) and hardness of the cell membranes were derived from the slope of the linear portion of unload curve, based on the Hertz model (23). For our condition, the modulus was defined as:

$$\text{Modulus: } E = \frac{3 F (1-\nu^2)}{4 R^{0.5} I^{1.5}}, \text{ with Poisson ratio } \nu=0.5$$

$$\text{Hardness: } H = \frac{F_{\max}}{A_{\square}}$$

Where “F” represents the force applied for each indent. The radius of curvature “R” of the tip and the depth of the indent “I” (nm) performed on the sample were used to compute the area of indentation “Ai” (nm²), which is the contact area between the tip and the sample.

3.9. Statistical analysis.

SAS Software (SAS Institute, NC) was used to conduct t-test and one-way ANOVA followed by Tukey test. Differences with $p < 0.05$ were considered statistically significant.

4. RESULTS

4.1. DC treatment reduced persistence of PAO1 cells

Figure 1. DC treatment reduced the level of persistence. Late exponential phase cells of *P. aeruginosa* PAO1 (OD₆₀₀ of 0.9) were treated with 70 $\mu\text{A}/\text{cm}^2$ DC for 1 h using carbon or SS 304 electrodes. After the DC treatment, the cells were exposed to ciprofloxacin (Cip) for 3.5 h to count the number of persister cells. The CFU before Cip treatment was also counted to quantify the total number of cells. All conditions were tested with at least 2 replicates.

As shown in Fig. 1, the electrochemical treatments exhibited cidal effects on PAO1 and reduced

the level of persistence. For example, 70 $\mu\text{A}/\text{cm}^2$ DC delivered using carbon electrodes for 1h reduced the viability of the total PAO1 population ($\sim 5.10^8$ cells/mL) by 74.0 ± 3.1 %, and the persistence level by 92.5 ± 1.0 % (t-test, $p=0.003$) in comparison to the untreated control. The effects were even greater with DC treatment using SS304. Under this condition, 99.2 ± 0.1 % stationary phase cells were killed, and the level of persistence was reduced by 95.5 ± 0.6 % (t-test, $p=0.002$) compared to the untreated control. Overall, these results demonstrated that DC treatment can reduce bacterial persistence.

4.2. Sequential treatment with carbon electrode mediated DC followed by Tob led to complete eradication of persister cells.

Electron transfer across cell membrane and the associated membrane potential play vital roles in bacterial physiology. We hypothesized that DC may alter membrane structure and functions of PAO1 cells and therefore increase their susceptibility to antibiotics. To test this hypothesis and more specifically characterize the effects of DC, we treated PAO1 persister cells with 70 $\mu\text{A}/\text{cm}^2$ DC using SS304 or carbon electrodes for 1 h. Then, the tolerance of persister cells to the antibiotics Tob, Kan, and Spec was investigated immediately. According to the results shown in Fig. 2, pre-treatment of persister cells with 70 $\mu\text{A}/\text{cm}^2$ DC using carbon electrodes significantly enhanced the killing of PAO1 persister cells when followed by 1.5 $\mu\text{g}/\text{mL}$ Tob. In fact, no viable persister cells were found after the treatment, corresponding to a 7-log reduction in persistence (one-way ANOVA followed by Tukey test, $p=0.0004$). This is 2 logs more than the killing achieved by concurrent treatment with DC using SS304 and 1.5 $\mu\text{g}/\text{mL}$ Tob, and 6 logs more compared to the concurrent treatment with DC using carbon electrodes and 1.5 $\mu\text{g}/\text{mL}$ Tob (8). Interestingly, sequential treatment with DC (carbon electrodes) followed by Kan (one-way ANOVA, $p=0.08$) or Spec (one-way ANOVA, $p=0.6$) did not exhibit enhanced killing compared

to killing by DC alone (Fig. 2). Additionally, the treatment with 70 $\mu\text{A}/\text{cm}^2$ DC generated using SS304 electrodes did not enhance the killing by any of these antibiotics (one-way ANOVA followed by Tukey test, $p>0.05$ for all conditions tested, Fig. 2). For example, treatment with SS304-mediated DC followed by 1.5 $\mu\text{g}/\text{mL}$ Tob only showed killing by 2 logs, which is similar to the killing achieved with DC alone using SS304 electrodes, and lower than concurrent treatment with DC and 1.5 $\mu\text{g}/\text{mL}$ Tob (8).

Figure 2. Sequential treatment of *P. aeruginosa* PAO1 persister cells with 70 $\mu\text{A}/\text{cm}^2$ DC followed by antibiotics. The persister cells were treated for 1 h with DC using carbon or SS304 electrodes. Immediately after DC treatment, the cells were exposed to antibiotics for 1 h, including 1.5 $\mu\text{g}/\text{mL}$ Tob, 60 $\mu\text{g}/\text{mL}$ Kan, and 60 $\mu\text{g}/\text{mL}$ Spec. The number of viable cells was quantified by counting CFU. Three replicates were tested for each condition.

It is intriguing that carbon electrode-mediated DC can sensitize PAO1 persister cells to a very low concentration of Tob (1.5 $\mu\text{g}/\text{mL}$) leading to eradication of the entire persister population. To further understand this phenomenon, the DC-treated PAO1 persister cells were

transferred to LB medium and evaluated to see if the DC-treated cells can recover in the presence of nutrients. The density of cells (measured at OD₆₀₀) treated with carbon electrodes continuously declined during the 6 h incubation in the presence of nutrients regardless of presence or absence of antibiotics (Supplementary Fig. S1A). In comparison, the density of persister cells treated with SS304 electrodes remained relatively constant both in the presence and absence of antibiotics (Supplementary Fig. S1B). These findings suggest that electrode material plays an important role in ECCP and DC treatment using carbon electrodes can cause permanent damage to the cells.

4.3. DC treatment affected the membrane structure of *P. aeruginosa* PAO1 cells.

Figure 3. Permeability of *P. aeruginosa* PAO1 to Propidium Iodide (PI) following DC treatments. The cells were treated with 70 $\mu\text{A}/\text{cm}^2$ DC using carbon or SS304 electrodes for 1 h. Then, the DC treated cells and the untreated control cells were stained with PI for 15 min and the absorbance was measured. Excitation and emission wavelength were 530/25 and 590/35 nm, respectively.

As shown in Fig. 3, PAO1 cells were found to be more permeable to PI after DC treatment and the effects of carbon electrode-mediated DC were stronger than SS304-mediated DC. This

finding suggests that DC may affect membrane integrity of PAO1, which is consistent with the finding that the cells treated with carbon-mediated DC failed to recover over 6 h of incubation in LB medium, as shown in Fig. S1A&B (Supplementary Information).

Figure 4. Representative TEM images of untreated planktonic *P. aeruginosa* PAO1 cells (A) and the cells treated with 70 $\mu\text{A}/\text{cm}^2$ DC for 1 h (B&C). DC was generated with carbon (B) or SS304 (C) electrodes. The cells were stained with 2% uranyl acetate. Bar = 200 nm if not indicated separately.

The above findings were corroborated with TEM analysis. Significant differences in cell

morphology between the PAO1 cells treated with carbon and SS304 electrodes were observed in whole-cell images (Fig. 4). Compared to the untreated PAO1 cells (Fig. 4A), the cells treated with 70 $\mu\text{A}/\text{cm}^2$ DC mediated with carbon electrodes showed two major changes: deformed cell membrane suggesting a loss of integrity (Fig. 4B1), and dark areas near the poles of these treated cells (Fig. 4B2). In contrast, the cells treated using SS304 electrodes exhibited extensive membrane damage leading to cell lysis and release of cellular contents (Fig. 4C). Interestingly, it appears that DC treatment of PAO1 using SS304 electrodes for 1 h resulted in the release of materials with appearance of outer membrane vesicles (OMVs, Fig. 4C3) from the cells that have not been substantially lysed. These TEM observations are consistent with the DNA microarray results, which showed that 24 genes involved in OMV secretion were induced within 40 min of the DC treatment mediated with SS304 electrodes (see discussion below).

To further understand the nature of these structural changes and how they occurred, we conducted cross sectional TEM analysis and the results showed significant differences between treatments. Compared to the untreated control (Fig. 5A), compartmentalization of intracellular materials was visible after 20 min of DC treatment using carbon electrodes (Fig. 5B1). After 40 min of the treatment, the size of the compartments increased and the cells exhibited some morphological changes around the cell membrane (Fig. 5B2). The cell membrane deformation was more apparent after 60 min of the DC treatment using carbon electrodes, and the intracellular features were less visible (Fig. 5B3). The effects of SS304 -mediated DC on the membrane of PAO1 cells were different than those on the cells treated using carbon electrodes. As shown in Fig. 5C, cell lysis during treatment with SS304 occurred over time. After 40 min of the treatment, the outer membrane of cells treated using SS304 electrodes became loose (Fig. 5C2). The extent of membrane damage culminated after 60 min of the DC treatment with

1 apparent cell lysis, as shown in Fig. 5C3 (most cells were completely lysed).

2
3 **Figure 5.** Representative TEM images showing the cross sections of untreated planktonic *P.*
4 *aeruginosa* PAO1 cells (A), and the cells treated with DC mediated with either carbon electrodes
5 for 20 min (B1), 40 min (B2) and 60 min (B3), or with SS304 electrodes for 20 min (C1), 40 min
6 (C2) and 60 min (C3). The cells were embedded in resin, and stained with 2% uranyl acetate and
7 lead citrate. To perform TEM, the cells were sectioned to 80 nm thin slides. Bar = 200 nm.

8

4.4. DC treatment altered the mechanical properties of *P. aeruginosa* PAO1 cells.

Figure 6. Effects of DC treatment using carbon electrodes on cellular composition of *P. aeruginosa* PAO1 cells. (A) Stationary phase cells were treated with 70 $\mu\text{A}/\text{cm}^2$ DC using carbon electrodes for 1 h and SEM-EDS analysis was performed to quantify the relative cellular concentration of major elements. At least four replicates were tested for each condition. (B) SDS-PAGE of cell membrane (CM) and cytoplasmic (C) proteins isolated from DC-free control and DC-treated *P. aeruginosa* PAO1 cells. DC was delivered for 1 h at the level of 70 $\mu\text{A}/\text{cm}^2$ using carbon electrodes.

To determine the origin of polar compartmentalization (Fig. 4B2) in cells treated with carbon electrode-mediated DC and the impact of this treatment on the mechanical properties of cells, further study was conducted to compare the chemical composition of treated and untreated cells. To understand if the dark areas were provoked by the accumulation of particles and ions from the carbon electrodes within the cells, SEM-EDS was conducted, and the relative concentration of C, O, N, S and metal cations in samples was determined. As shown in Fig. 6A, no significant change in the chemical composition of PAO1 cells was found after DC treatment using carbon electrodes, except for carbon which was lower in the treated cells than the control cells. In

addition, the weight percentage of intracellular metal cations, including that of Fe, Ni, Mn and Cr, was negligible in both the carbon-treated PAO1 cells and the untreated control. This suggests that the dark areas shown in the TEM images of DC-treated cells (Fig. 4B & 5B) were primarily due to the localization of intracellular materials, instead of the accumulation of heavy metals or carbon particles released from the electrodes. Thus, we speculated that the aggregation of proteins might have occurred in the form of inclusion bodies due to the electrochemical stress from DC treatment using carbon electrodes. This was confirmed by SDS-PAGE analysis (24). As shown in Fig. 6B, the amount of membrane proteins of the DC-treated cells was less than that of the untreated control, but the amount of proteins associated with intracellular aggregates (dark areas in Fig. 4B & 5B) was more than the proteins contained in the cytosol of the control cells. This finding suggests that the DC treatment using carbon electrodes may have caused membrane damage and protein aggregation in PAO1 cells.

4.5. Nanoindentation of PAO1 persister cells

In previous studies, the localized accumulation or compartmentalization of DNA, nucleoids, and proteins such as stress-induced DNA-binding protein Dps, have been associated with increase in the stiffness of bacterial cell membrane (25, 26). Our results show that DC treatment using carbon electrodes resulted in statistically significant increase in the stiffness of PAO1 cells. Compared to the control cells, which had a Young's modulus of 0.39 ± 0.5 GPa (Table 1), the modulus of the PAO1 cells treated with DC using carbon electrodes increased to 3.0 ± 1.5 GPa (t-test, $p=0.001$). Thus, our results suggest that the cells treated using carbon electrodes were ~8 times stiffer than the control cells. Consistently, the DC treated cells also showed three times less surface deflection by the cantilever during indentation than the untreated control (1044.5 ± 46.5 nm² vs. 3536.5 ± 717.3 nm²). These findings indicate that DC generated

using carbon electrodes impacts the mechanical properties of PAO1 cells.

Table 1. Mechanical properties of stationary phase cells of *P. aeruginosa* PAO1 following treatment with 70 $\mu\text{A}/\text{cm}^2$ DC using carbon electrodes.

	Untreated PAO1 (control) Mean $\pm$ SD	DC treated PAO1 cells Mean $\pm$ SD
Maximum indent (nm)	28.9 $\pm$ 3.6	11.9 $\pm$ 0.5
Maximum load (nN)	398.9 $\pm$ 78.6	391.8 $\pm$ 16.6
Indentation Contact Area (nm ²)	3536.5 $\pm$ 717.3	1044.5 $\pm$ 46.5
Slope of linear range (nN/nm)	32.1 $\pm$ 1.1	135.8 $\pm$ 67.8
Modulus (GPa or nN/nm ²)	0.39 $\pm$ 0.05	3.0 $\pm$ 1.5**
Hardness (GPa or nN/nm ²)	0.11 $\pm$ 0.01	0.38 $\pm$ 0.0**

4.6. Effects of carbon electrode-mediated DC on gene expression in PAO1 persister cells

To understand the effects of DC at the genetic level, the transcriptional profiles of persister cells were assessed before and after DC treatment using DNA microarray analysis, and the results were confirmed with qPCR (Supplementary Table S1). Gene expression with a two-fold or higher change was considered significant. The overall analysis of the transcriptome of PAO1 persister cells showed moderate induction and repression of genes. For instance, the treatment with 70 $\mu\text{A}/\text{cm}^2$ DC using carbon electrodes for 20 min induced 51 genes and repressed 32 genes consistently in duplicated experiments (Fig. 7A); while 40 min treatment induced 139 genes and repressed 47 genes (Fig. 7B and Supplementary Tables S2&S3).

These results indicated that DC treatment using carbon electrodes might affect multi-drug resistance, pyocin synthesis and ribosomal biosynthesis in PAO1 persister cells. For example, *mexA*, *OprM*, *PAI032* and *PA4375*, which are involved in antibiotic resistance and bacterial efflux systems, were induced after 40 min of DC treatment using carbon electrodes. Also, 17 out of 18 phage-related genes that were induced after 40 min of DC treatment using carbon

electrodes are known to be involved in pyocin production, which include *PA0616*, *PA0617*, *PA0619*, *PA0622*, *PA0623*, *PA0624*, *PA0626*, *PA0627*, *PA0628*, *PA0629*, *PA0633*, *PA0636*, *PA0637*, *PA0638*, *PA0639*, *PA0640*, and *PA0644*. These results are consistent with previous finding that oxidative stress caused by hydrogen peroxide induced pyocin genes in *P. aeruginosa* cells (27).

Figure 7. Transcriptional profiles of *P. aeruginosa* PAO1 persister cells treated with $70 \mu\text{A}/\text{cm}^2$ DC using carbon or SS304 electrodes for 20 min (A) and 40 min (B), respectively.

4.7. Effects of SS304-mediated DC on gene expression in PAO1 persister cells

Compared to the treatment using carbon electrodes, DC treatment using SS304 electrodes affected gene expression in PAO1 persister cells to a greater extent. A total of 250 genes were

1 induced and 244 genes were repressed by 20 min-DC treatment using SS304 electrodes (Fig. 7A);
2 while 135 genes were induced and 95 genes were repressed by DC treatments using SS304 for
3 40 min (Fig. 7B and Supplementary Tables S4&S5). Overall, the response of persister cells to
4 the electrochemical treatment mediated via SS304 electrodes was characterized by functions
5 such as protein secretion and export, energy-related functions, SOS response, and antibiotic
6 susceptibility. For instance, genes involved in “transport of small molecules” (23 genes), “energy
7 metabolism” (14 genes), “transcriptional regulators” (13 genes), “biosynthesis of cofactors,
8 prosthetic groups and carriers” (12 genes) and “putative enzymes” (11 genes) are among the
9 most represented groups of genes induced by SS304 mediated DC for 20 min. The most
10 significant functions of the repressed genes are related to “transport of small molecules” (26
11 genes), “transcriptional regulators” (25 genes), and “putative enzymes” (24 genes). In addition,
12 antibiotic resistance genes such as *PA1129*, *PA1237*, and *PA2018* were repressed by SS304-
13 mediated DC treatment for 20 min.

14 Among the genes for transport of small molecules that were repressed by either 20 min or
15 40 min of DC treatment using SS304 electrodes, 4 (*PA0811*, *PA1108*, *PA2055*, *PA3336*) are
16 identified as probable major facilitator superfamily (MFS) genes and 9 (*PA0137*, *PA0206*,
17 *PA0313*, *PA0314*, *PA0860*, *PA1808*, *PA1964*, *PA4909*, *PA5376*) are related to the ATP-binding
18 cassette (ABC) transporters. These two major types of transporters regulate bacterial response to
19 chemiosmotic gradients and the cross-membrane transport of large molecules, including
20 antibiotics (28). In addition, 6 genes involved in the translation or the repair of DNA were also
21 found induced at 20 min or 40 min, including *ruvB* and *ruvC* (related to Holliday structures,
22 which are single stranded recombination intermediates holding together homologous duplex
23 DNA molecules), *uvrB* (encoding excinuclease ABC subunit B), *ihf* (encoding integration host

factor beta unit, a histon-like protein involved in high structural order of DNA (29)), *gyrA* (encoding DNA gyrase subunit A), and *PA5348* (encoding a probable DNA-binding protein).

Table 2. Expression ratios of genes associated with outer membrane vesicles after treatment with 70 $\mu\text{A}/\text{cm}^2$ DC using SS304 electrodes

Gene	Function	Fold change	
		20 min	40 min
<i>PA4623</i>	hypothetical protein	1.4	2.1
<i>PA1526</i>	probable transcriptional regulator	3.0	6.4
<i>PA0647</i>	hypothetical protein	3.2	2.3
<i>PA4638</i>	hypothetical protein	3.0	0.8
<i>PA2885</i>	probable transcriptional regulator	2.1	1.2
<i>PA4451</i>	hypothetical protein	2.4	3.9
<i>PA0631</i>	hypothetical protein	2.3	1.7
<i>PA0648</i>	hypothetical protein	1.2	2.3
<i>xylZ</i>	toluate 1,2-dioxygenase electron transfer component	1.4	2.3
<i>PA2479</i>	probable two-component response regulator	3.2	3.0
<i>PA2135</i>	probable transporter	2.5	5.1
<i>hepA</i>	ATP-dependent helicase HepA	3.0	2.4
<i>PA1895</i>	hypothetical protein	1.9	2.5
<i>PA3330</i>	probable short chain dehydrogenase	2.9	1.4
<i>PA0368</i>	hypothetical protein	3.8	2.0
<i>rpsI</i>	30S ribosomal protein S9	3.0	2.0
<i>PA4372</i>	hypothetical protein	2.3	2.1
<i>PA3517</i>	probable lyase	2.2	2.4
<i>PA0616</i>	hypothetical protein	2.2	2.0
<i>PA0206</i>	probable ATP-binding component of ABC transporter	3.0	1.8
<i>PA5380</i>	probable transcriptional regulator	3.4	1.1
<i>PA0779</i>	probable ATP-dependent protease	1.5	3.3
<i>PA1504</i>	probable transcriptional regulator	0.6	4.0
<i>PA0308</i>	hypothetical protein	2.0	1.8

DC treatment using SS304 also induced 24 genes involved in the synthesis of outer membrane vesicles (OMV) (Table 2), corroborating the TEM results in Fig. 4C and indicating potential changes in membrane integrity and functions. Membrane vesiculation is a common

feature of bacterial response to both internal and external stresses (30, 31). The up-regulation of ATP-related genes might suggest energy requirement for stress response such as OMV secretion under these conditions, or an attempt for cell membrane repair (32, 33).

4.8. Susceptibility of selected *P. aeruginosa* mutants to DC treatments

Table 3. List of mutants selected to study the gene functions under DC treatments.

Treatment Condition	Gene	Function	Fold change	
			20 min	40 min
DC using SS304 electrode		beta-keto-acyl-acyl-carrier protein		
	<i>pqsB</i>	synthase-like protein	7.0	2.1
	<i>pvcA</i>	pyoverdine biosynthesis protein PvcA	6.2	2.4
	<i>PA1939</i>	hypothetical protein	10.5	3.5
	<i>PA4430</i>	probable cytochrome b	3.1	16.9
	<i>PA5461</i>	hypothetical protein	9.7	12.5
DC using carbon electrode	<i>aer</i>	aerotaxis receptor Aer	1.5	4.0
	<i>glk</i>	glucokinase	3.2	8.5
	<i>fusA2</i>	elongation factor G	4.7	6.1
	<i>oprG</i>	Outer membrane protein OprG precursor	4.5	2.4
	<i>PA1897</i>	hypothetical protein	2.3	4.1

Our DNA microarray analysis revealed changes in the transcriptional profiles of PAO1 persister cells due to DC treatments. The mutants of *PA1939*, *PA5461*, *pqsB*, *PA4430*, *pvcA*, *fusA2*, *oprG*, *glk*, *aer* and *PA1897* were chosen to further study since these genes were induced by 4-17 fold in one of the DC conditions (Table 3). These mutations did not affect the susceptibility of the cells to Tob, because the mutants exhibited similar growth characteristics as the wild type PAO1 in LB medium and LB supplemented with 1.5 µg/mL Tob (Supplementary Fig. S2). We then confirmed that 200 µg/mL Cip is also appropriate for the isolation of persister cells from these mutant strains since no further killing can be achieved by further increasing the concentration of Cip (Supplementary Fig. S3). Interestingly, the mutant of *PA1897* had a fairly low level of persister cells, e.g. 0.01% of the total population, which is 100 fold lower than the wild-type

PAO1. To understand if any of these genes plays an important role in response to DC, the persister cells of selected mutants were isolated and treated with DC for 1 h. The mutants of *PA1939*, *PA5461*, *pqsB*, *PA4430*, and *pvcA* were treated with 70 $\mu\text{A}/\text{cm}^2$ DC using SS304 electrodes, while the remaining mutants (*fusA2*, *oprG*, *glk*, *aer*, and *PA1897*) were treated with 70 $\mu\text{A}/\text{cm}^2$ DC using carbon electrodes for 1 h based on the DNA microarray results. The mutants were found to respond to DC treatment differently. As shown in Fig. 8A, DC treatment using carbon electrodes resulted in more killing of the persister cells of *PA1897* mutant. Compared to the wild-type PAO1 (1.1 log of killing), the persister cells of *PA1897* mutant were killed by 1.7 log after 1 h DC treatment (t-test, $p < 0.0001$), suggesting that this mutation rendered *P. aeruginosa* cells more sensitive to DC treatment. In comparison, mutants of *oprG* (t-test, $p = 0.8$) and *aer* (t-test, $p = 0.5$) did not exhibit significant changes in susceptibility to DC. The mutants of *fusA2* (t-test, $p = 0.001$) and *glk* (t-test, $p = 0.02$) were less susceptible to DC treatment compared to the wild-type PAO1.

Similar results were found for DC treatment using SS304 electrodes (Fig. 8B). For instance, while the persister cells of wild-type PAO1 were killed by 1.6 log with 70 $\mu\text{A}/\text{cm}^2$ DC using SS304 electrodes for 1h, the persister cells collected from the mutants of *pqsB*, *pvcA* and *PA4430* were killed by 2.8, 2.8 and 2.1 logs (t-test, $p < 0.05$ for all), respectively. In comparison, mutation in *PA5461* did not change the susceptibility (t-test, $p = 0.6$), while the mutant of *PA1939* survived from DC better than the wild-type PAO1 (Fig. 8B). These findings suggest that the DC treatments have profound impact on *P. aeruginosa* cells and multiple pathways may be involved in response to DC treatments.

Figure 8. Effects of DC on the persister cells of *P. aeruginosa* PAO1 and its isogenic mutants.

The persister cells were treated with 70 $\mu\text{A}/\text{cm}^2$ DC for 1 h using carbon electrodes (A) or SS304 electrodes (B).

DISCUSSION

Recently, we reported that *P. aeruginosa* persister cells can be effectively killed by weak electric currents and the synergistic effects between DC and tobramycin, a phenomenon named ECCP (8). To understand the mechanisms of ECCP, we conducted the present study and investigated the effects of DC on the structure and electrophysiology of *P. aeruginosa* cells. It was found that DC alone could reduce persistence in PAO1 cultures; and total eradication of persister cells is possible using sequential treatments with carbon electrode-mediated DC followed by 1.5 $\mu\text{g}/\text{mL}$ Tob. Such effects by DC were not alleviated when the cells were cultured in LB medium to recover, indicating that DC generated with carbon electrodes could cause permanent damage to the structure and function of persister cells. To understand if DC treatments using carbon electrodes may increase the permeability of the persister cells and facilitate the transport of killing agents (e.g. reactive oxygen species and/or antibiotics) into the

cells, the intracellular contents of major elements released from the electrode materials were characterized. SEM-EDS analysis revealed no significant increase in the concentration of key elements in PAO1 cells treated with DC using carbon electrodes vs. the untreated cells. DC treatment using carbon electrodes did not result in the intracellular accumulation of carbon, but caused aggregation of intracellular proteins around the poles of the treated cells. We speculate that the electrical field generated by DC treatment may have altered the movement of charged molecules (intracellular proteins, RNA or DNA) inside the cells, which may subsequently affect the physiology and the viability of the treated cells (34). We also obtained evidence that the DC treatment impaired the membrane functions of the cells; e.g., the electrochemical currents generated via carbon electrodes caused increased PI permeability and antibiotic susceptibility.

DC generated using SS304 electrodes induced cell membrane damage as shown in the TEM results. During the treatments, the cells lost membrane integrity, exhibiting separation of the outer membrane from the inner membrane, and finally cell lysis. Consistently, these morphological changes corroborated with the effects of DC on the mechanical properties of the PAO1 cells. Compared to the cells that hardened under carbon-mediated DC treatment, PAO1 cells treated with DC using SS304 exhibited greater deformation with deeper indentation in the cell membrane with lower maximum load according to the AFM results. Such deformation is consistent with TEM analysis, which showed that DC generated using SS304 disrupted cell membrane. Similar effects of metal ions on the stiffness of bacterial cells were previously reported in a study by Kumar *et al.* (35), which showed that the membrane elasticity of Gram-positive *Brevibacterium casei*, initially represented by Young's modulus of 0.769 GPa, decreased to 0.171 GPa, and 0.082 GPa as a result of prolonged exposure (for 24 h and 48 h, respectively) to 1 mM cobalt acetate (Co^{2+}) solution. The effects were attributed to biochemical

1 interactions between cell membrane components and metal cations (35). We speculated that the
2 interaction between SS304-released metal cations (Fe, Cr, Mn, Ni in various oxidation states)
3 and PAO1 cells affected the membrane integrity of treated cells leading to lysis. Such change in
4 the membrane properties of the cells could also affect the physiological response of the persister
5 cells to DC and antibiotics.

6 To further understand the mechanism of ECCP, the differences in the
7 electrophysiological properties of the persister cells were investigated using DNA microarrays.
8 Only the treatments for 20 min and 40 min were analyzed, since strong killing effects observed
9 after 1 h treatment may affect the integrity of RNA profile due to short half-life of bacterial
10 mRNA (8). For the DC generated with carbon electrodes, the most striking finding was that the
11 DC treatment included a large number of pyocin genes, which are known to be induced under
12 stresses. This finding is intriguing and more studies are needed to reveal the physiological
13 relevance of such changes. However, the oxidative stress generated by the treatment using
14 carbon electrodes might have not been sufficient to cause DNA damage, since *PA2138*, *ogt* (at
15 20 min) and *recR* (at 40 min) involved in DNA repair, were repressed. In addition, genes related
16 to putative functions and oxido-reduction such as *PA0237*, *PA4621*, *PA5327* were repressed and
17 proton transport was down-regulated as indicated by the repression of genes related to energy
18 metabolism such as *cyoD*, *PA1600*, *PA0510* (at 20 min), *ccmH*, *nirJ*, *PA2716* (at 40 min).
19 However, the ribosomal genes *rpsB*, *rpsL*, *rpsO*, *rpsP*, *rpsS*, and the elongation factor genes
20 *fusA1*, and *fusA2* were induced, suggesting that DC treatment interrupted some physiological
21 functions of PAO1 cells.

22 For DC generated by SS304 electrodes, a number of differentially expressed genes
23 are involved in the transport of small molecules. The ABC transporters are required when energy

is needed to perform cellular functions such as DNA repair (36). Under DC treatments using SS304, it appears that the cells acquired energy by activating the proton-pump machinery or through anaerobic respiration. For example, 14 genes involved in energy metabolism were induced after 20 min of the DC treatment using SS304, suggesting anaerobic respiration through reduction of nitrogenous compounds (*nirS*, *norBC*, *napD*, *nosZ*) (37), and assembly of the NADH respiratory complex (*nuoDFIJ*) (38) or other cytochromes (*ccmG*, *PA4430*, *cc4*). Electrochemical treatments are known to generate reactive oxygen species (ROS) through redox or Fenton reactions, and the free oxygen radicals are known to cause DNA damage (39). As we previously reported, DC treatment using SS304 electrodes provides an exogenous source of Fe and Cr (8), which can increase the intracellular concentration of free metal cations, and result in oxidative stress (40). Our DNA microarray results suggest that SOS response due to oxidative stress may be more prevalent under DC treatment using SS304, since the SS304 mediated treatments induced 6 genes involved in DNA repair, while none was induced under DC generated with carbon electrodes. The 6-fold induction of *lexA* during DC treatments using SS304 for 40 min (Supplementary Table S4) indicated possible DNA damage. It is possible that electrochemical treatment and the accumulation of some mutagens and chemicals within the cells can provoke lesions in the DNA sequences, which require the activation of nucleotide excision repair mechanisms through the induction of genes such as *lexA*, *uvrB* and *ruvB* as found under our DC conditions (41). Transport of toxic materials across cell membrane requires the regulation of specific transport systems, which is consistent with the finding that 23 and 26 genes related to the transport of small molecules were induced and repressed, respectively.

While no synergistic killing activity was observed during the concurrent treatment of PAO1 persister cells with antibiotics and DC using carbon electrodes (8), synergy was achieved

1 during sequential treatment with DC generated with carbon electrodes for 1 h followed by 1.5
2 $\mu\text{g/mL}$ Tob for 1 h. Since DC was found to affect the integrity and membrane properties of
3 PAO1 cells, it is possible that DC in concurrent treatments using SS304 can enhance the
4 penetration of antibiotics leading to strong killing effects. Alternatively, DC may trigger efflux
5 mechanisms and thus alter the energy balance of the cells and the interaction between antibiotic
6 molecules and cellular targets. This is supported by the finding the several drug efflux genes
7 were repressed (*PA1129*, *PA1237*, *PA2018*) or induced (*mexA*, *oprM*, *mexW*, *oprM*) in response
8 to DC treatment using SS304 and/or carbon electrodes.

9 For treatment using carbon electrodes, there is no release of metal cations from the
10 anode. However, the reduction reactions do generate reactive oxygen species directly (i.e.,
11 intermediates during the generation of hydroxide radicals, hydrogen peroxide produced by the
12 cathode, etc.), which affect cell viability. It will be interesting to further characterize the
13 generation of related ROS species and their roles in the synergy during sequential treatment of
14 persister cells using carbon electrode mediated DC and tobramycin.

15 Many genes exhibited significant changes in response to electrochemical treatments, and
16 some mutants selected based on the microarray results were found to respond differently to DC
17 compared to the wild-type PAO1. For instance, mutation of *fusA* and *glk* genes that encode
18 elongation factor G and glucokinase, respectively, rendered the persister cells less susceptible to
19 DC treatments. In comparison, the mutant of *PA1897* exhibited increased sensitivity to DC.
20 Previous studies indicated that *PA1897* encodes a cytoplasmic membrane protein regulated by
21 quorum sensing genes such as *qscR* (42). *OspR*, a redox-active regulator involved in oxidative
22 stress response by *P. aeruginosa* has been found to control *PA1897* (43). It is intriguing to find
23 that the mutant of *PA1897* not only harbored a lower level of persister cells (0.01% of the cell

population), but also is more sensitive to the electrochemical stress generated by DC using carbon electrodes. Further studies on the regulation of these genes are necessary for better understanding of their roles in the response of persister cells to DC treatments.

Table 4. Summary of the effects of DC mediated with carbon and SS 304 electrodes.

	Electrode material	
	Carbon	SS 304
DC condition during 1 h	70 $\mu\text{A}/\text{cm}^2$ DC, (voltage from 0-800mV)	70 $\mu\text{A}/\text{cm}^2$ DC, (voltage from 200-50mV)
Persister killing effects	Synergy in sequential treatment (DC then 1.5 $\mu\text{g}/\text{mL}$ Tob); resulted in 7-log killing	Synergy in concurrent treatment with DC and 1.5 $\mu\text{g}/\text{mL}$ Tob; resulted in 5-log killing (8)
Cell structure and contents	No significant accumulation of carbon particles, but compartmentalization of intracellular materials with protein aggregates	Intracellular accumulation of metal cations
Membrane properties	Increased cell stiffness, Tob susceptibility, and PI permeability	Membrane damage and cell lysis
Gene expression	Induction of genes related to pyocin and antibiotic resistance	Repression of antibiotic resistance genes Induction of more genes related to transport of small molecules, metabolism, and protein secretion /export (OMVs)

6. CONCLUSIONS

As summarized in Table 4, the results from this study revealed that DC treatment disrupted the membrane integrity of *P. aeruginosa* and led to structural damage depending on treatment time and electrode material. The electrode material used to perform the treatment plays an important role in synergistic killing of persister cells with antibiotics. While SS304 mediated DC and Tob showed strong synergy in persister killing by concurrent treatment, the sequential treatment with

DC using carbon electrodes followed by Tob was found even more effective, leading to total eradication of PAO1 persister cells. The DNA microarray results revealed that genes related to pyocin production and SOS response were induced by DC treatments using carbon and SS304 electrodes, respectively. Overall, this study provided important new information for understanding the mechanism of ECCP and for designing more effective methods to eliminate bacterial persister cells.

ACKNOWLEDGEMENTS

The authors are grateful to Blue Highway Inc. and the U.S. National Science Foundation (EFRI-1137186) for partial support. We also thank Dr. Thomas K. Wood at Penn State University for sharing the strain *P. aeruginosa* PAO1, and Robert P. Smith at SUNY ESF for helping with the TEM analysis.

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