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Controlling *Pseudomonas aeruginosa* persister cells by weak electrochemical currents and synergistic effects with tobramycin

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ABSTRACT

It is well recognized that bacterial populations commonly contain a small percentage of phenotypic variants, known as persister cells, which are dormant and extremely tolerant to antibiotics. When the antibiotic treatment is stopped, surviving persister cells can regenerate the bacterial population with a similar percentage of persister cells. Such persistence presents a great challenge to curing chronic infections, such as those associated with implanted medical devices. In this study, we report that bacterial persister cells can be effectively eliminated by low-level direct currents (DCs); e.g. treatment with 70 μA/cm^2 DC for 1 h using stainless steel (SS) 304 reduced the number of viable planktonic persister cells of *Pseudomonas aeruginosa* PAO1 by 98% compared to the untreated control. In addition to persister killing by applying DC alone, synergistic effects were observed when treating persister cells with 70 μA/cm^2 DC and 1.5 μg/mL tobramycin together using SS304 electrodes. The same level of DC was also found to be cidal to biofilms-associated persister cells of *P. aeruginosa* PAO1. These results are helpful for developing more effective methods to control chronic infections associated with implanted medical devices.

1. Introduction

The rapid development and spreading of multidrug resistant bacteria are a major challenge to public health. Bacteria can become drug resistant through both intrinsic and acquired mechanisms. Intrinsic mechanisms are based on specific cellular structures and functions (e.g. drug extrusion by efflux pumps), which offer bacteria general protection from a variety of antibiotics and facilitate the further development of resistance through acquired mechanisms based on genetic mutations and horizontal gene transfer [1,2]. One of the major intrinsic mechanisms of resistance is biofilm formation, by which bacteria attach to surfaces and develop multicellular structures with cells embedded in an extracellular matrix [3–5]. With the high tolerance to antibiotics, biofilms cause serious medical problems. For example, biofilm formation on medical devices accounts for half of the cases of nosocomial infections in the U.S. and leads to repeated surgeries, and in more serious cases, amputations and deaths [6].

In addition to the formation of multicellular structures, it is well documented that bacterial cultures commonly contain a small fraction (normally no more than 0.1–1%) of dormant cells, known as persister cells [7–9]. Because most antibiotics are only effective against metabolically active cells, persister cells can survive the challenge of antibiotics at concentrations significantly higher than those required to kill regular cells of the same genotype [9–11]. When the antibiotic concentration drops under a threshold after treatment, the surviving persisters can revert to an active state and regenerate the bacterial population with a similar percentage of persister cells. Persister population increases significantly when bacteria form biofilms or when a bacterial culture enters stationary phase [10], rendering both states highly tolerant to antibiotic treatment [10,11]. In addition to dormancy, persisters embedded in biofilms are also protected from host immune systems by the polysaccharide matrix [12]. Thus, innovative technologies are needed to effectively eliminate persister cells, especially those in biofilms.

Due to the dormant nature of persister cells, antimicrobial approaches that work independently of bacterial growth phase

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have good potential to control bacterial persister cells. Rosenberg [13] reported that alternating electrical currents generated with platinum electrodes have bacteriostatic effects rendering *Escherichia coli* cells elongated. Later, direct currents (DCs) in the range of μA to mA/cm^2 and 100 kHz to 10 MHz alternating electric fields were reported for their cidal effects on bacteria after a relatively long period (several hours in most cases) of treatment [14–19]. Although the mechanism of these observations is not well understood, similar currents were also found to enhance the effects of antibiotics on biofilms, an interesting phenomenon known as bioelectric effect [18,20,21]. Consistently, a total current of 100 μA DC applied to surgical stainless steel pins has been found to prevent *Staphylococcus epidermidis* infection around percutaneous pins in goats [22].

The capability of controlling biofilm cells by DCs and alternating currents (ACs) led to our hypothesis that appropriate levels of electrochemical currents/potentials can also kill bacterial persister cells. To test this hypothesis, persister cells of the Gram-negative pathogen *Pseudomonas aeruginosa* PAO1 were challenged with 70 μA/cm^2 DC in this study. This strain was chosen because *P. aeruginosa* is one of the most important pathogens with clinical significance including medical device-associated infections [23–25]. The effects of DC on both planktonic persister cells and those in biofilms were studied. The possible mechanisms are discussed.

2. Materials and methods

2.1. Bacterial strain and growth media

The wild-type *P. aeruginosa* PAO1 was cultured in Luria–Bertani (LB) medium [26] containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. Each overnight culture was incubated at 37 °C with shaking at 200 rpm.

2.2. Colony forming units

The colony forming units (CFUs) were counted to quantify the number of viable *P. aeruginosa* PAO1 cells in planktonic cultures and in biofilms. For planktonic samples, 10 μL of each sample was diluted in a 10× series using 0.85% NaCl solution and spread on LB agar plates (1.5% agar). The CFUs were counted on the next day after incubation overnight at 37 °C. The biofilm cells were collected by sonication (Ultrasonic Cleaner Model No B200, Sinosonic Industrial Co., Ltd, Taipei Hsien, Taiwan) of the biofilm samples in 0.85% NaCl solution for 1 min first [27] and then well mixed and diluted with 0.85% NaCl solution for counting CFU as described above. No significant effects of sonication on the viability of *P. aeruginosa* PAO1 was found (viability was not changed after 2 min of sonication confirmed by counting CFU).

2.3. Isolation of persister cells

Previous work has demonstrated that *P. aeruginosa* persisters can be isolated by killing the regular cells with 25 μg/mL ciprofloxacin (Cip) for 3.5 h [9]. In order to determine the sufficient Cip concentration for our strain, *P. aeruginosa* PAO1 cells were challenged with various concentrations of Cip (0–200 μg/mL) for 3.5 h and the killing was evaluated by counting CFU of surviving cells. Briefly, *P. aeruginosa* PAO1 was cultured in LB medium overnight at 37 °C. Then the cells were collected by centrifugation (Sorvall Legend RT+, Thermo Scientific, Asheville, NC) at 8000 rpm for 10 min at room temperature, and washed twice with 0.85% NaCl solution before being treated with different concentrations of Cip for 3.5 h at 37 °C. After treatment, the cells were washed three times with 0.85% of NaCl solution to remove Cip and the debris of lysed cells. The number of viable persister cells was then quantified by counting CFU as described above. Five replicates were tested for each condition.

2.4. Growth rate measurement

Four antibiotics were tested including tobramycin (Tob), chloramphenicol (Cm), kanamycin (Kan) and spectinomycin (Spec). An overnight culture of *P. aeruginosa* PAO1 was used to prepare subcultures in 96 well plates (Costar® No. 9017, Corning Inc, Corning, NY) with a start OD_{600} of 0.05. Each well contained 200 μL of LB medium supplemented with one of the above antibiotics at different concentrations. The concentrations tested ranged from 0 to 10 μg/mL for Tob, 0–40 μg/mL for Cm, 0–90 μg/mL for Kan and 0–80 μg/mL for Spec. The OD_{600} of cultures was read every hour over a period of 6 h. The growth curves were obtained to calculate the specific growth rates and to identify the antibiotic concentrations that reduce the specific growth rate of *P. aeruginosa* PAO1 by around 50%. These reference concentrations were used to study the synergy between antibiotics and electrochemical currents (ECs).

2.5. Effects of DC on planktonic cells

The susceptibility of *P. aeruginosa* PAO1 to low-level DCs was investigated according to the following procedure. *P. aeruginosa* PAO1 cells in three different physiological stages were tested including persister cells isolated from stationary cultures, total cells in stationary cultures (with 99% as regular cells) and total cells in exponential cultures (with 99.9% as regular cells). The persister cells were isolated by treating the stationary phase cultures of *P. aeruginosa* PAO1 with 200 μg/mL Cip for 3.5 h as described above. To study the effects of DC on regular cells in stationary cultures, *P. aeruginosa* PAO1 grown overnight (99% as regular cells) were harvested by centrifugation at 8000 rpm for 10 min at room temperature. To estimate the effects of DC on regular cells in exponential phase, overnight cultures were used to inoculate LB medium to an OD_{600} of ~0.005 and the cells were harvested by centrifugation when OD_{600} reached 0.7 (99.9% as regular cells). The harvested cells, as described above, were washed twice with 0.85% NaCl solution, resuspended in 0.85% NaCl solution and treated with and without 70 μA/cm^2 DC. To deliver the DC, an electrochemical cell was constructed by inserting two SS304 electrodes along the opposite sides of a plastic cuvette (Thermo Fisher Scientific, Pittsburg, PA). A silver wire (0.015″ diameter, A-M Systems, Sequim, WA) was placed in bleach for 30 min to generate an Ag/AgCl reference electrode. The reference electrode was then placed in the cuvette. A 70 μA/cm^2 DC was generated using a potentiostat (Potentiostat WaveNow, Pine Research Instrumentation, Raleigh, NC) in a 3-electrodes' configuration as shown in Fig. S1. Using the AfterMath software (Potentiostat WaveNow, Pine Research Instrumentation, Raleigh, NC), a galvanostatic mode was selected to monitor the DC level and record the voltage across the system over an hour. During the treatment, 100 μL of sample was taken every 20 min to evaluate the viability of bacterial cells by counting CFU. Both SS304 and carbon electrodes were used to generate DCs to compare the effects of electrode materials. The same conditions were also tested in the presence of 1.5 μg/mL Tob to study the synergy between DC and antibiotics.

2.6. Effects of DC on biofilm-associated persister cells

P. aeruginosa PAO1 biofilms were formed on sterile SS304 coupons (3.5 cm × 0.95 cm × 0.05 cm). Briefly, an overnight culture of *P. aeruginosa* PAO1 was used to inoculate a petri dish containing 20 mL of LB medium and sterile coupons. The culture was incubated for 24 h at 37 °C without shaking to allow biofilms to form on the coupons. The coupons with attached biofilms were then removed from the petri dish using sterile forceps and washed gently three times with sterile 0.85% NaCl solution. The control coupons were transferred to a 15 mL polystyrene conical tube (BD Falcon, Becton Dickinson and Company, Franklin Lakes, NJ) containing 8 mL of 0.85% NaCl solution and the biofilm cells were released from the surface by sonication for 1 min to assess the total number of cells. Then the suspension was treated with 200 μg/mL Cip to count the CFU of viable persister cells. Other biofilm coupons were used either as working or counter electrodes. According to the setup, the oxidation of the SS304 occurred at the working electrode. Therefore, a biofilm coupon was referred to as anodic biofilm (AB) electrode when it was set as the working (positively charged) electrode of the potentiostat or as cathodic biofilm (CB) electrode when the biofilm coupon was placed as the counter (negatively charged) electrode in the electrochemical cell. In either case, the biofilm was treated galvanostatically with 70 μA/cm^2 DC for 30 min in the presence and absence of 15 μg/mL Tob. Higher Tob concentration was used for biofilms due to the intrinsic tolerance of biofilms. Each coupon was then transferred to a 15 mL polystyrene conical tube containing 8 mL of 0.85% NaCl solution and the cells were removed from the surface by sonication for 1 min after treatment. The number of viable cells detached from the biofilm during DC treatments (in the 0.85% NaCl solution) was also quantified by counting the CFU in the solution of the biofilm cultures before sonication. Each sample containing cells was also incubated with 200 μg/mL Cip at 37 °C for 3.5 h to isolate biofilms-associated persister cells. The total numbers of cells (without Cip treatment) and persisters (with Cip treatment) were quantified by counting CFU as described above.

2.7. Effects of SS304 electrolytes

To understand if the effects of DC on persister cells were due to the chemical species generated by the electrochemical reactions, 0.85% NaCl solution without bacterial cells was treated with the same level of DC (70 μA/cm^2 generated using SS304 electrodes) for 20, 40 and 60 min, respectively. Then the *P. aeruginosa* PAO1 persister cells were incubated in these pretreated 0.85% NaCl solutions for 1 h with and without 1.5 μg/mL Tob. The untreated control samples were exposed to 0.85% NaCl solution without an DC for 1 h in the presence or absence of 1.5 μg/mL Tob. The CFU of each sample was quantified as described above after treatment. To understand if the movement of ions or some short-life chemical species are important to the effects of DC, *P. aeruginosa* PAO1 persister cells were resuspended in a 0.85% NaCl