Microampere electric current caused bacterial membrane damage and two-way leakage in short time

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Abstract

Physical agents such as low electric voltage and current have recently gained attention for antimicrobial treatment due to their bactericidal capability. Although microampere electric current was shown to suppress the growth of bacteria, it remains unclear to what extent the microampere current damaged the bacterial membrane. Here, we investigated the membrane damage and two-way leakage caused by microampere electric current (≤ 100 μA) with a short exposure time (30 min). Based on MitoTracker staining, propidium iodide staining, filtration assays, and quantitative single-molecule localization microscopy, we observed significant membrane damage, which allowed two-way leakage of ions, small molecules, and proteins. This study paves the way to new development and antimicrobial applications of ultra-low electric voltage and current.

Importance

Although electric voltage and current have been studied for a long time in terms of their ability to suppress the growth of bacteria and kill bacteria, increasing interest has been aroused more recently due to the prevalence of antibiotic resistance of microbes in the past decades. Toward understanding the antimicrobial mechanism of low electric voltage and current, previous studies showed that treating bacteria with milliampere electric currents (≥ 5 mA) for ≥ 72 hours led to significant damage of the bacterial membrane, which likely resulted in leakage of cellular contents and influx of toxic substances through the damaged membrane. However, it remains unclear to what extent membrane damage and two-way (i.e. inward and outward) leakage are caused by lower (i.e., microampere) electric current in a shorter time frame. In this work, we set out to answer this question. We observed that the membrane damage was caused by microampere electric current in half an hour, which allowed two-way leakage of ions, small molecules, and proteins.

Keywords: Ultra-low electric current, super-resolution fluorescence microscopy, single-molecule localization microscopy, membrane damage, leakage

Introduction

As antibiotic resistance of bacteria has become one of the biggest threats to public health (1), alternatives to antibiotics have been attracting broad interest and attention (2, 3). Physical methods, such as sunlight/UV, fire, drying, high temperature, and high pressure, have played important roles in sterilization and disinfection from very early times of human history (4). Since the 1960s, electric voltage, current, and field have been explored as physical means for suppressing the growth of, and/or killing, bacteria. While most of the early studies focused on the bactericidal effects of high electric voltage and current (5–8), it was found, more recently, that low electric voltage and current can also effectively kill bacteria and biofilms (9–27). For example, Pareillexu and Sicard investigated the effects of low electric current ranging from 10 to 200 milliampere (mA) on the viability of *Escherichia coli* (*E. coli*) bacteria and found that current as low as 25 mA could kill the bacteria (27). The lethal activity of mA current was confirmed by other reports (9, 17–19, 25, 26). In addition, many studies showed that ultra-low electric current at microampere (μA) was also bactericidal (10, 13, 18, 20, 21, 24); even electric field (without current) can inhibit the growth of planktonic bacteria (16). Furthermore, electric current at μA and/or mA have been shown to be effective for treating biofilms (11, 12, 14, 15, 18, 22–24, 28).

Efforts have been made toward understanding the antimicrobial mechanisms of low electric voltage and current. Possible mechanisms include membrane damage and disruption, reduction in ATP production and enzymatic activities (17, 26), and generation of reactive oxygen species (ROS) (13–15). Membrane damage caused by high electric voltage has been well known for a long time. For example, electroporation is a commonly used microbiological technique to deliver DNA and proteins into bacteria and cells by applying high electric voltage at kilovolts (kV) (29–31). More recently, transmission electron microscopy showed that treating bacteria at low electric current of 5 mA for ≥ 72 hours led to significant damage, based on which leakage of cellular contents and influx of toxic substances through the damaged membrane were suggested (17). An interesting follow-up question is to what extent membrane damage and two-way (i.e. inward and outward) leakage are caused by microampere electric current in a shorter time frame. Although previous results showed that microampere electric current were effective for suppressing the growth of bacteria, this question remains unclear.

In this work, we set out to answer this question by investigating possible membrane damage of bacteria after subjecting them to microampere electric current of ≤ 100 μA

(corresponding to voltage ≤ 2.5 V) for 30 min. The effect of the electric current on the bacterial membrane was first examined by MitoTracker staining and fluorescence microscopy. In addition, propidium iodide (PI) staining and filtration assays were carried out to assess the two-way leakage of ions, small molecules, and proteins due to the membrane damage caused by the microampere electric current. Furthermore, quantitative single-molecule localization microscopy, which allowed us to localize, and count the number of, histone-like nucleoid structuring (H-NS) proteins inside individual bacteria, was performed to evaluate the changes in the organization and clustering of the H-NS proteins and to quantify the leakage of the H-NS proteins out of the bacteria caused by microampere electric current.

Materials and Methods

Bacterial strain and growth

Two K-12 derived *E. coli* strains were used in this study: the first strain is MG1655 (from the Yale Coli Genetic Stock Center) (32, 33), and the second strain has the *hns* gene on the bacterial DNA fused to the gene for mEos3.2 fluorescent protein, which was derived from the EosFP fluorescent protein from the stony coral *Lobophyllia hemprichii* (34, 35). The second strain expresses fluorescent H-NS-mEos3.2 fusion proteins, allowing us to perform fluorescence-based filtration assays and quantitative single-molecule localization microscopy (34, 36–39), as described below. The bacteria stocks were stored at -80ºC as glycerol stocks following standard protocols (40, 41). The purity of the stocks was regularly monitored by phenotypic characterizations based on colony morphology and growth curve. The bacterial morphology was also regularly monitored under a brightfield or phase contrast microscope. In addition, for the *E. coli* strain expressing fluorescent proteins, the fluorescence of the bacteria and the emission of individual fluorescent proteins were regularly monitored.

The bacteria were grown at 37°C overnight with orbital rotation at 250 rpm in defined M9 minimal medium (autoclave-sterilized), supplemented with 1% glucose (filter-sterilized using a 0.22 μm filter), 0.1% casamino acids (filter-sterilized using a 0.22 μm filter), 0.01% thiamine (filter-sterilized using a 0.22 μm filter) and appropriate antibiotics (none for MG1655; chloramphenicol at a final concentration of 34 μg/mL for the strain expressing H-NS-mEos3.2 proteins) (36–38, 42). On the second day, the overnight culture was diluted by 50 – 100 times into fresh medium so that the OD_{600} was 0.05 (36–38, 42). The

fresh cultures were grown again at 37°C in culture tubes equipped with two sterile aluminum electrodes with orbital rotation at 250 rpm without electric voltage or current (i.e., the wires were shorted, Fig.1A). The resistance of the bacterial cultures ($\approx$ 32 kΩ) was measured using a multimeter. When the fresh bacterial culture reached $OD_{600} \approx 0.3$, low DC voltage was applied to the culture for 30 min (0V – untreated negative control, 0.5, 1, 1.5, 2, and 2.5 V, Fig. 1B). The corresponding currents were 0, 16, 31, 47, 63, and 78 μA, respectively. After the 30 min treatment, the bacteria were quantified as described below.

Colony-forming-unit (CFU) assay

For the CFU assays, the untreated and treated bacteria were diluted by a factor of 4×10^5 or 4×10^6 in fresh LB medium and plated on agar plates. The plates were incubated at 37ºC overnight, followed by imaging the plates with a gel documentation system (Analytik Jena US LLC, Upland, CA) and manually counting the number of colonies on each plate. Four replicates were performed for each sample.

MitoTracker staining and quantification

To stain the bacterial membrane, MitoTracker Green FM dye (Thermo Fisher Scientific, Waltham, MA) was added to the untreated and treated bacteria at a final concentration of 300 nM, and incubated in a shaking incubator (250 rpm, 37°C) for 30 min (43). 8 μL of the stained bacteria were transferred to 5mm X 5mm agarose pads (3% in 1X PBS, ~1 mm thick) for mounting. The agarose pads with bacteria were flipped and attached to clean coverslips (cleaned by sonication sequentially in 1 M NaOH, 100% ethanol, and ultra-pure water). Chambers were then constructed by sandwiching rubber o-rings between the coverslips and cleaned microscope slides. The chambers were sealed using epoxy glue and then mounted on a microscope for fluorescence imaging (excitation = 488 nm). From the acquired images, the average fluorescence intensities of 100 bacteria treated at each voltage were measured using ImageJ (44–46).

Propidium iodide (PI) staining and quantification

To measure possible inward leakage due to membrane damage, the untreated and treated bacteria were first fixed by 3.7% formaldehyde (Sigma-Aldrich, St. Louis, MO) and stained with PI (G-Biosciences, St. Louis, MO) (47, 48). The PI-stained bacteria were mounted on agarose pads and prepared for fluorescence imaging similar to the

MitoTracker experiments, except that the excitation wavelength was 532 nm. The stained cells were counted, and their percentages were calculated based on the acquired fluorescence images.

Filtration assays

Filtration assays to assess the leakage of cellular contents due to membrane damage were performed similar to that of Chen and others (2013) (49). Briefly, the untreated and treated bacteria (the strain expressing H-NS-mEos3.2 fusion proteins) were filtered by 0.2 μm filters (VWR International LLC, Radnor, PA), resulting in filtrates containing leaked cellular contents if membrane damage occurred, in addition to proteins and molecules from the culture media. For each sample, the filtrate was aliquoted into 96-well plate (200 μL per well), followed by measuring the absorbance at 280 nm and fluorescence at 525 nm (excitation = 498 nm) on a multi-mode microplate reader (Synergy H1, BioTek, Winooski, VT). The relative change in the absorbance was estimated by $\delta A_{280} = \left(\frac{A_{280}^{+}}{A_{280}^{-}} - 1\right) \times 100\%$, where A_{280}^{-} and A_{280}^{+} were the absorbances at 280 nm of the filtrates for bacteria before and after microampere current treatment, respectively. Similarly, the relative change in the fluorescence intensities of the filtrates was estimated by $\delta F = \left(\frac{F^{+}}{F^{-}} - 1\right) \times 100\%$, where F^{-} and F^{+} were the fluorescence of the filtrates for samples before and after microampere current treatment, respectively.

Quantitative single-molecule localization microscopic assay

Quantitative single-molecule localization microscopy (50) was performed on the bacteria subjected to microampere electric currents, following our previous work (36–38, 42). Briefly, the untreated and treated bacteria were first fixed by 3.7% formaldehyde for 30 min at room temperature and harvested by centrifugation (1000 g for 10 min). The harvested cells were resuspended in 1X phosphate-buffered saline (PBS) buffer and washed three times in 1X PBS buffer. The prepared bacteria were then mounted on agarose pads for imaging. The single-molecule localization microscope was home-built on an Olympus IX-73 inverted microscope with an Olympus TIRF 100X N.A.=1.49 oil immersion objective. The microscope and data acquisition were controlled by Micro-Manager (51, 52). A 405 nm laser and a 532 nm laser from a multi-laser system (iChrome MLE, TOPTICA Photonics, Farmington, NY) were used to “activate” and excite the H-NS-mEos3.2 fusion proteins in the bacteria. Emissions from the fluorescent proteins were collected by the objective and imaged on an EMCCD camera (Andor Technology Ltd –

Oxford Instruments, Concord, MA) with an exposure time of 30 ms (the actual time interval between frames was 45 ms). The effective pixel size of acquired images was 160 nm, while the field of view was 256 X 256 pixels.

The resulted movies (20,000 frames) were analyzed with RapidStorm (53), generating x/y positions, x/y widths, intensity, and background for each detected fluorescent spot. If the intensity of a spot was below 4000 (i.e., too dim) or above 50000 (i.e., too bright), or if the width of a spot was below 200 nm (i.e., too narrow) or above 600 nm (i.e., too wide), the spot was rejected and excluded from later analysis (54). The positions of the spots surviving the rejection criteria were further corrected for sample-drift using a mean cross-correlation algorithm (55). The localizations that appeared in adjacent frames and within 10 nm to each other were regrouped as a single molecule (56). The resultant localizations were used for reconstruction of super-resolved images and for further quantitative analysis.

The localizations of H-NS molecules from each acquisition were first manually segmented into individual bacterial cells using custom MATLAB programs (36). Analysis and quantification of the spatial organization of H-NS proteins were performed on individual cells. Following the segmentation of cells, the number of localizations per bacterium N_p was counted (36). In addition, Voronoi-based quantification and clustering analysis were performed using custom MATLAB programs following Levet et al. (57), from which we estimated the molecular density of H-NS proteins ρ, the mean inter-neighbor distances Δ_{ave}, the number of clusters per bacterium N_{cl}, the number of proteins per cluster N_{pc}, the area of clusters of H-NS proteins A_{cl}, and the fraction of proteins forming clusters ϕ_{cl} (36, 57).

Results

Membrane damage caused by microampere electric currents

Microampere electric current was applied to the bacterial culture by applying low electric DC voltage. As the resistance (R) of the bacterial culture was measured to ≈ 32 kΩ, DC voltage (V) in the range of 0 – 2.5 V resulted in current (I) of 0 – 78 μA (I = V / R). After treatment for 30 min, we performed CFU assays and found that the number of live cells decreased after subjecting the bacteria to low electric voltage/current (Fig. 2A), confirming the antimicrobial effect of low electric voltage/current. In addition, we stained the bacterial

membrane by MitoTracker Green FM dye (43). As shown in Figs. 2B and 2C, the intensity of the bacterial membrane became higher after subjecting the bacteria to the microampere electric current. To quantify this observation, we estimated the mean intensities of the bacteria and examined the dependence of the mean intensity on the voltage (Fig. 2D). The intensity increased slightly at 0.5 V or 16 μA, indicating changes in the bacterial membrane. The average intensity increased ≥ 4-fold when cells were exposed to voltage ≥ 1.5 V (or current ≥ 47 μA), and many bacterial cells showed extremely high brightness (up to 19 times brighter) compared to the negative control, suggesting that many of the treated cells had significant changes in their membrane. Although controversy exists (58, 59), previous studies showed that the fluorescence of membrane-incorporated MitoTracker Green dye depends on the membrane potential (60, 61); it is possible that the microampere electric current altered the bacterial membrane potential.

Inward leakage due to membrane damage caused by microampere electric currents

It was previously proposed that membrane damage caused by low electric current allowed the influx of toxicants (17). To test this possibility with microampere electric current, propidium iodide (PI) (47, 48) was used to stain the DNA of the bacteria after treatment by microampere electric current. The rationale being that electrically induced membrane damage would allow PI to enter the bacteria to stain DNA, influx of ions and other small organic molecules would also be possible. Representative images of untreated (0 V or 0 μA) and treated (0.5 – 2.5 V or 16 – 78 μA) bacteria are shown in Fig. 3A, where the fluorescent images from PI staining (red) are superposed on the inverted phase contrast images (blue). It was observed that more bacteria were stained by PI at higher voltages and currents. Quantifying the percentage of PI-stained bacteria (P_{pi}) showed that the percentage of membrane-damaged bacteria increased quickly from 20% to ≈ 80% at 1.5 – 2 V or 47 – 63 μA (Fig. 3B). This observation confirmed that the membrane damage caused by microampere electric current were significant enough to enable inward leakage of at least ions and small molecules with a size of ≤ 1 nm.

Outward leakage due to membrane damage caused by microampere electric currents

Our PI-staining result showed that influx of ions and small organic molecules (≤ 1 nm) was possible due to membrane damage caused by microampere electric current, which

also implied that small cellular contents, including amino acids, could leak out of the bacteria. Another question is whether biological macromolecules, such as proteins and nucleic acids, could also leak out. To answer this question, we adopted a filtration assay that was used to study the membrane damage caused by carbon nanotubes (49). From the relative changes in the absorbance (δA_{280}) of the filtrates for the bacteria before and after microampere current treatment, we observed a slight increase, ~1% (Fig. 4A), providing weak evidence of possible outward leakage of macromolecules. Because the "background" from the culture media could contribute significantly to the absorbance, it is likely that the measured increase in δA_{280} was underestimated.

To lower the "background" due to the culture media, a modified filtration assay based on fluorescence was performed using an *E. coli* strain that expresses histone-like nucleoid structuring (H-NS) proteins fused to mEos3.2 fluorescent proteins (34). By quantifying the relative changes in the fluorescence intensities (δF) of the filtrates, we observed an increase of ~10% for the bacteria treated at 2.5 V or 78 μA. This observation suggested that the membrane damage caused by the microampere electric currents were significant enough to allow proteins to leak out of the bacteria.

Quantification of protein leakage by single-molecule localization microscopy

The filtration assays suggested that proteins leaked out of bacteria after treating the bacteria by ≤ 100 μA DC current. To further confirm such protein leakage, we measured the number of the histone-like nucleoid structuring (H-NS) proteins inside individual bacteria before and after microampere current treatment for 30 min using single-molecule localization microscopy (SMLM) (50, 54, 62, 63). SMLM is one type of super-resolution fluorescence microscopy that localizes individual molecules of interest with a precision of ≤10 nm (64); therefore, it not only produces super-resolved images with high spatial resolution, but also provides a convenient way to count the number of molecules. Without applying sophisticated algorithms (65–67), the number of molecules of interest is on average proportional to the intensity in the super-resolved images or the number of localizations obtained by SMLM (42).

We performed single-molecule localization microscopy on untreated and treated bacteria, using the *E. coli* strain expressing H-NS-mEos3.2 fusion proteins, as mEos3.2 fluorescent proteins are photoactivable and allow super-resolution imaging (34, 36). Representative images of H-NS proteins in untreated and treated bacteria are shown in Fig. 5A. For the

untreated negative control, H-NS proteins were organized as clusters inside the bacteria (first column in Fig. 5A and 5B), consistent with previously reported results (36, 37, 39). After subjecting the bacteria to DC voltage of 1 – 2.5 V (or current of 31 – 78 μA), the intensities on the super-resolved images became significantly dimmer (columns 3–6 in Fig. 5A and 5B). As the intensity in super-resolved images correlates with the probability of localizing the molecules of interest, this result indicates that less H-NS proteins were present in the bacteria after treating the bacteria with microampere electric current.

To quantify this result, we segmented the bacteria (36) and counted the number of localizations of H-NS proteins in each cell, N_p. Due to the stochastic nature in the activation and detection of the mEos3.2 fluorescent proteins, N_p is expected to be proportional to, on average, the number of H-NS proteins in the bacteria (36, 42). The histograms of N_p are shown in the inset of Fig. 5C, where the baselines of the histograms were vertically shifted for better visualization of the differences. It is clear that the peaks of the N_p distributions translated to the left as the applied voltage increased, suggesting that the number of H-NS proteins per bacterial cell decreased when subjecting the bacteria to low DC voltage and current. We also calculated the mean of N_p and standard error of the mean (SEM) for each sample. Note that, as the distributions of N_p were bell-shaped in the linear-log scale (inset of Fig. 5C), we estimated the mean of $log(N_p)$ and then calculated the mean of N_p from $N_p = exp(log(N_p))$. As shown in Fig. 5C, the average number of H-NS proteins per cell decreased nearly linearly. It is also noted that the observed decrease in the number of H-NS proteins per bacterial cell is unlikely due to cellular responses (e.g., lower expression level of H-NS proteins) for several reasons. First, the treatment time (30 min) is relatively short, comparing to the degradation time of proteins in bacteria (~ hours) (68). Second, our previous work on H-NS proteins in bacteria treated by antimicrobial silver ions and silver nanoparticles showed that the number of H-NS proteins did not decrease within several hours (36). Therefore, the microscopic data on individual bacteria directly suggested that H-NS proteins leaked out of the bacteria after microampere electric current treatment.

We examined how the spatial organization of the H-NS proteins was affected by the electric treatment based on Voronoi diagram tessellation (36, 57). We computed the molecular density (ρ) of the H-NS proteins and observed that the distribution of ρ shifted to the left, indicating the density decreased as the applied voltage increased (inset of Fig. 5D), which is expected as a result of the decrease of the number of H-NS proteins per

cell. Interestingly, the mean of the molecular density started to decrease at 1.5 – 2 V (or 47 – 63 μA) as shown in Fig. 5D, which is different from the dependence of the number of H-NS proteins per cell on the applied electric voltage and current (Fig. 5C). We also observed a shift to the right in the distribution of the mean inter-neighbor distances Δ_{ave} (inset of Fig. 5E), suggesting that the inter-molecular distances for some H-NS proteins became larger when bacteria were treated by low DC voltage and current, consistent to the result of the molecular density. In addition, we identified the clusters of H-NS proteins based on Voronoi diagrams (36, 57) and counted the number of clusters per bacterial cell (N_{cl}) and the number of proteins per cluster (N_{pc}). We found that both N_{cl} and N_{pc} decreased after treating the bacteria with DC voltages and currents (Fig. 5F). Furthermore, by quantifying the area of clusters of H-NS proteins, we observed that the H-NS clusters became smaller (Fig. 5G). Lastly, we examined the fraction of H-NS proteins forming clusters in individual bacteria, $\phi_c = N_{cp}/N_p$, where N_p is the number of localizations (i.e., detected proteins) per cell, and N_{cp} the number of proteins that form clusters in the cell. We observed that the clustering fraction decreased from 10% for the negative control to 0% at 2.5 V or 78 μA (Fig. 5H).

Conclusions and Discussions

To summarize, we investigated the membrane damage of bacteria and the two-way leakage caused by microampere electric current. We observed that bacteria subjected to ≥ 47 μA current for 30 min showed much higher intensities with MitoTracker staining, suggesting that the bacterial membrane was altered by the microampere electric current. The membrane damage caused by microampere current was large enough to allow PI molecules to enter the bacteria, suggesting that inward leakage of ions and small molecules was possible. In addition, we found based on filtration assays and super-resolution fluorescence microscopy that the membrane damage was significant enough to allow proteins to leak out of the cells. More importantly, using histone-like nucleoid structuring (H-NS) proteins as an example, we quantified the decrease in the number of H-NS proteins per bacterial cell and characterized the changes in the spatial organization of the H-NS proteins caused by the electric treatment. This study highlights that treating bacteria with electric current at ≤ 100 μA for 30 min caused significant membrane damage and led to two-way leakages of ions, small molecules and proteins.

It was noted previously that the bactericidal effects of electric voltage and current are complex, and involve various interactions between the bacteria, electricity, electrode

materials and medium (17). For example, in addition to the electrical effects, metal ions released from the electrodes into the medium are likely to affect the growth of bacteria, which has been shown in the literature (69). In addition, ROS generated by the electric voltage and current could be another significant contributor (13–15). The current study did not aim to distinguish the contributions of these different effects, while these are interesting questions worth pursuing in future investigations. On the other hand, we argue that heating by the electric voltage/current is unlikely to cause damage to the bacteria in this study, because the used electric power is very low. Considering the resistance of the bacterial culture was ≈ 32 kΩ, the electric power was below 200 μW; at this power, it would take more than one day to heat up the bacterial culture (5 mL) by one degree.

It is worthwhile to highlight that the electric power leading to serious membrane damage of bacteria is very low, which is expected to facilitate the use of microampere electric current (and low electric voltage) for antimicrobial applications. For example, commonly used household batteries can provide the needed voltages to kill bacteria. More importantly, solar panels have an output power of ~10 mW per cm_2 (70); therefore, a solar panel of 1 cm_2 can easily generate the needed electric power for damaging bacterial membranes, suppressing the growth of bacteria and/or killing bacteria. We anticipate that this study intrigues new development and antibiotic applications of low electric current.

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