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Electrically induced bacterial membrane-potential dynamics correspond to cellular proliferation capacity

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Membrane-potential dynamics mediate bacterial electrical signaling at both intra- and intercellular levels. Membrane potential is also central to cellular proliferation. It is unclear whether the cellular response to external electrical stimuli is influenced by the cellular proliferative capacity. A new strategy enabling electrical stimulation of bacteria with simultaneous monitoring of single-cell membrane-potential dynamics would allow bridging this knowledge gap and further extend electrophysiological studies into the field of microbiology. Here we report that an identical electrical stimulus can cause opposite polarization dynamics depending on cellular proliferation capacity. This was demonstrated using two model organisms, namely *Bacillus subtilis* and *Escherichia coli*, and by developing an apparatus enabling exogenous electrical stimulation and single-cell time-lapse microscopy. Using this bespoke apparatus, we show that a 2.5-second electrical stimulation causes hyperpolarization in unperturbed cells. Measurements of intracellular K^+ and the deletion of the K^+ channel suggested that the hyperpolarization response is caused by the K^+ efflux through the channel. When cells are preexposed to 400 ± 8 nm wavelength light, the same electrical stimulation depolarizes cells instead of causing hyperpolarization. A mathematical model extended from the FitzHugh–Nagumo neuron model suggested that the opposite response dynamics are due to the shift in resting membrane potential. As predicted by the model, electrical stimulation only induced depolarization when cells are treated with antibiotics, protonophore, or alcohol. Therefore, electrically induced membrane-potential dynamics offer a reliable approach for rapid detection of proliferative bacteria and determination of their sensitivity to antimicrobial agents at the single-cell level.

bacterial electrophysiology | bioelectricity | cell biophysics | rapid bacterial detection | electrical signaling

Compared with animal bioelectrical signaling, bacterial electrical signaling is understudied and only recently were the excitation dynamics of membrane potential shown to mediate the intra- and intercellular signaling which regulates important physiological processes, namely mechanosensation, spore formation, and biofilm dynamics (1–4). In animal bioelectrical signaling, externally applied electrical stimuli and measurements of cellular electrical properties have been the principle methodology (5–8). This approach has led to many key discoveries regarding the roles of animal bioelectrical signaling [e.g., early tissue development (9, 10), regeneration (11), and carcinogenesis (12–14)] and has fostered the development of real-world applications such as for tissue engineering (15–17), wound healing (6, 18), and electroceuticals (19). Utilizing exogenous stimuli is an important step forward toward understanding bacterial electrical signaling and development of applications based on bacterial electrophysiology. In the past, applications of electric currents to bacteria were used for sanitization (20), electroporation (21), and most recently redox synthetic biology (22). However, due to the only recent discovery of bacterial membrane-potential excitation dynamics, use of external electrical signals in the context of bacterial electrophysiology has been left largely unexplored.

An external electrical stimulus alters cellular membrane potential according to the Schwan equation: $\Delta\psi_{max} = \Delta\psi_{max} = 1.5aE(1 + (2\pi f\tau)^2)^{-\frac{1}{2}}$, where $\Delta\psi_{max}$ is the induced membrane potential, a is the cell radius, E is the applied field strength, f is the AC field frequency, and τ is the relaxation time of the membrane (23). This equation, derived from the electromagnetic theory (24), expresses that the maximum change in the membrane potential of a cell caused by an electrical stimulus is proportional to the applied field strength. Theoretically, when an electrical stimulus is applied to proliferative bacterial cells, it should lead to opening of voltage-gated K^+ channels (Kv) and consequent hyperpolarization due to K^+ efflux. Substituting the typical values of bacterial resting potential [$-140 \sim -75$ mV (25, 26)] and threshold potential for Kv [~ -50 mV (27)] to the equation, one can expect that the depolarization by an electrical stimulus with the field strength of $+35 \sim 120$ mV/ μ m should open voltage-gated K^+ channels on bacteria.

In addition to its role in bioelectrical signaling, membrane potential is central to cellular proliferation; it provides the essential driving force for ATP synthesis (28) and is crucial for cell division (29). A quantitative estimation based on the measured

Significance

Transmembrane voltage in bacteria plays a central role both in electrical signaling and cell proliferation. However, whether proliferation state influences the response to electrical signals was unknown. Combining fluorescence time-lapse microscopy with a bespoke device and mathematical modeling, we show that proliferative and inhibited cells respond in opposite directions to an identical electrical signal. The response differentiation can be seen within a minute after stimulation. Therefore, our findings offer an approach for rapid detection of proliferative bacteria at the single-cell level.

Author contributions: J.P.S. and M.A. designed research; J.P.S., C.L.A.E., M.J.G., D.M., M.A.D., Y.H., and M.A. performed research; J.P.S., C.L.A.E., M.A.D., Y.H., and M.A. contributed new reagents/analytic tools; C.L.A.E., M.J.G., D.M., and M.A. analyzed data; and M.A. wrote the paper.

Conflict of interest statement: Based on the findings of this work, a UK patent application for the approach of rapidly detecting proliferative bacteria using electricity has been filed and a spin-out company (Cytecom Ltd.) was founded with the support from the University of Warwick. The spin-out company and the corresponding author (M.A.) were awarded the funding for the commercialization of the technology from United Kingdom Research and Innovation (UKRI), the UK national funding agency. The authors clarify that the scientific conclusions that are presented in this study are not influenced by the activity of the spin-out company.

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energy consumption of *Escherichia coli* suggests that the maintenance of membrane potential accounts for about half of the total energy consumption (30), and thus it is inherently linked to the proliferative capacity, here defined as the capacity to stay out of thermodynamic equilibrium. The proliferative capacity is commonly determined either by direct time-lapse observation of individual cells or by probing the intracellular state using membrane potential. The latter could be achieved using fluorescent indicators for membrane potential such as thioflavin T (ThT), DiOC₂ (3), and rhodamine 123 (2, 31–34). However, the use of such indicators for determining the proliferative capacity is known to be difficult because membrane potential can be affected by many physiological states and environmental conditions (35–37). Due to the baseline fluorescence being affected by a variety of conditions, comparisons between individual cells, populations of cells, and different species are technically complex. This means that meticulous and tedious calibrations are required for species, strains, media, and detection systems. The difficulties associated with these calibrations often preclude the broad use of these agents for the detection of proliferative bacteria. Nevertheless, these fluorescent indicators provide a useful qualitative measure of intracellular physiological state for live-cell imaging.

The dual roles of membrane potential in both signaling and proliferation prompt the question regarding the interplay between these two roles of membrane potential. In particular, could the electrical response of cells be affected by the proliferative capacity of individual cells? This is an important question for understanding bacterial electrical signaling because the input–output (I/O) relations are fundamental to any “signaling” (38). However, whether cellular responses to an electrical stimulus differ depending on their proliferative capacity remains unclear. If it does, one may expect that an identical signal input produces different outputs depending on their proliferative capacities.

In this study, we utilized an exogenous electrical stimulus to investigate the impact of proliferative capacity on electrical signal response. By experimentally testing a prediction from a mathematical model, we showed that an exogenous electrical stimulus induces hyperpolarization in unperturbed cells while inducing depolarization in inhibited cells. This finding offers an application to use bacterial electrophysiological dynamics for rapid detection of proliferative cells and differentiation of proliferative and non-proliferative cells within a minute after electrical stimulation.

Results

Development of an Apparatus That Enables the Monitoring of Membrane-Potential Response to an Exogenous Electrical Stimulus.

To investigate the potential impacts of proliferative capacity on electrical signaling responses, direct observation of cell proliferation, membrane potential, and its response to electrical stimuli at the individual-cell level is needed. However, the commercially available apparatus for neural electrophysiology were unsuitable due to the small size of bacterial cells; i.e., $\sim 1.4 \mu\text{m}^3$ for *Bacillus subtilis* and *E. coli* (39). To overcome this technical challenge, we designed and developed a tool for bacterial electrophysiology. The tool consists of an electrical relay circuit with an open-source I/O board, Arduino UNO, and a bespoke electrode-coated glass-bottom dish (Fig. 1A and SI Appendix, Figs. S1–S3, see Materials and Methods for details). Bacterial cells were inoculated on agarose pads and placed on the electrode surface. Importantly, this setup enables the monitoring of cells at single-cell resolution using phase contrast and the fluorescence membrane-potential indicator, ThT (Fig. 1B).

Electrical Stimulation Causes Hyperpolarization of Cells via K⁺ Efflux.

To examine whether an externally applied electrical stimulus is capable of opening K⁺ channels on bacterial membranes, we applied an exogenous electrical stimulus (60 mVpp/ μm AC 0.1 kHz for 2.5 s) to *B. subtilis* cells placed between the 50- μm

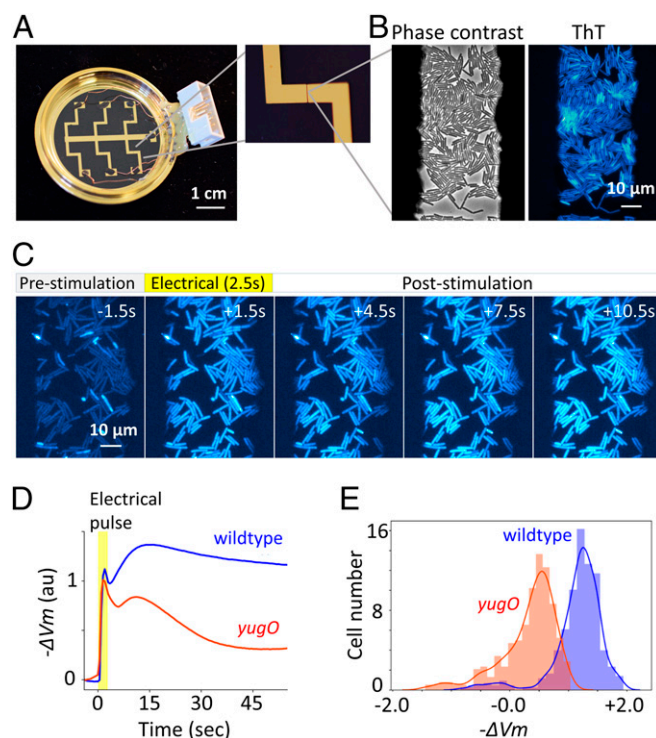


Fig. 1. An apparatus enabling concurrent single-cell microscopy and stimulation with exogenous electrical signal revealed hyperpolarization response to an electrical stimulus. (A) Bespoke glass-bottom dish coated with gold-titanium electrodes. Zoomed image on the Right shows 50- μm gap between electrodes. Dish is connected to relay circuit to apply electrical stimulation to bacterial cells (see SI Appendix, Figs. S1–S3 for details). (B) *B. subtilis* cells within the 50- μm electrode gap are visible in phase-contrast and ThT fluorescence images. (C) Film-strip images of ThT fluorescence of *B. subtilis* before, during, and after electrical stimulation. Increase in ThT fluorescence indicates hyperpolarization response to an electrical stimulus. (D) Mean $-\Delta V_m$ over time for *B. subtilis* wild-type and *yugO* strains. $-\Delta V_m$ was calculated by $\log(F_{ThT}/F_{ThT,R})$, where F_{ThT} is ThT fluorescence and $F_{ThT,R}$ is ThT fluorescence at resting state (SI Appendix). Time traces of individual cells are shown in SI Appendix, Fig. S4 (WT, $n = 321$; *yugO*, $n = 308$). Images were taken at 2 fps. (E) Histogram of $-\Delta V_m$ at 30 s after electrical stimulation. The distributions of WT and *yugO* are clearly distinguishable.

electrode gap (Fig. 1B). Upon electrical stimulation, the intensity of ThT fluorescence increased, indicating a hyperpolarization response (Fig. 1C). Single-cell analysis of the fluorescence dynamics revealed that most cells exhibited the hyperpolarization of membrane potential (V_m), while a small subpopulation of cells depolarizes upon stimulation (SI Appendix, Fig. S4A). Intriguingly, the cell elongation rate of these depolarizing cells was found to be much lower compared with other cells (SI Appendix, Fig. S5). No significant change in ThT intensity was observed with the absence of electrical stimulus (SI Appendix, Fig. S6) or mild change in pH (SI Appendix, Fig. S7), indicating that the observed dynamics are induced by the electrical stimulus. The hyperpolarization response suggests that electrical stimulation causes the efflux of cations such as K⁺, the dominant intracellular cation. It has been shown that chemical depolarization opens the YugO potassium channels (2). The Schwan equation predicts that external electrical stimuli can depolarize bacterial cellular membranes (40). Therefore, we hypothesized that depolarization by the electrical stimulation opens YugO channels, resulting in membrane hyperpolarization by K⁺ efflux.

To test this hypothesis, the same experiment was conducted with a mutant strain lacking the gene encoding the YugO potassium channel. Although the strain still showed an initial hyperpolarization

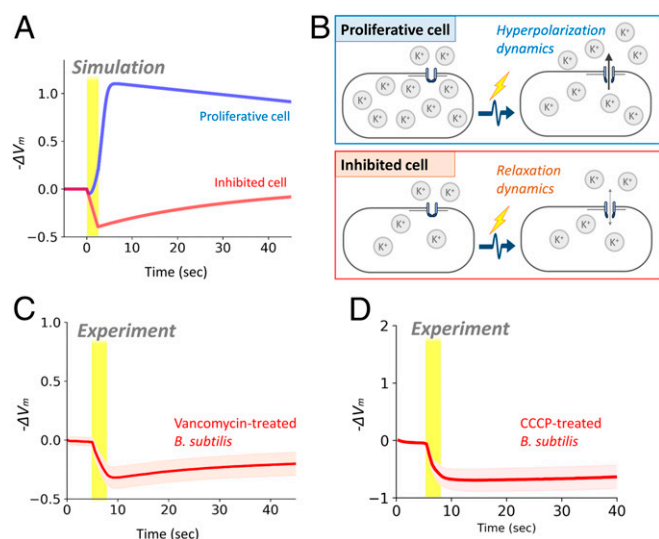


Fig. 3. Shift in resting-state membrane potential is sufficient to describe the distinct responses between proliferative and inhibited cells. (A) Numerical simulation of the FHN bacteria model, corresponding to Fig. 2C. Despite being stimulated by an identical electrical stimulus, proliferative cells (blue) hyperpolarize and inhibited cells (red) depolarize. See *SI Appendix* for model details. (B) Illustration of the biological mechanism of the response differentiation between proliferative and inhibited cells. (C and D) Time trace of membrane potential change ($-\Delta V_m$) with *B. subtilis* cells exposed to (C) vancomycin or (D) CCCP. Shading shows SD.

cells exhibited a clear depolarization in response to an exogenous electrical stimulus (Fig. 3C), as opposed to the hyperpolarization response seen in unperturbed cells (Fig. 1C). Experimental tests with the cells treated with ethanol or CCCP also showed depolarization response (Fig. 3D and *SI Appendix*, Fig. S13). These results strongly support the conclusion drawn from the FHN model that general stress treatments alter the cellular response to an electrical stimulus. Conversely, this finding suggests that the proliferative and inhibited cells can be distinguished based on their response to an exogenous electrical stimulus.

Selective Antibiotics Enable Classification of Coliforms in a Mixed Culture. The above observations and understanding raised the possibility of using the electrically induced membrane-potential dynamics for rapid detection of proliferative cells in a mixed-species culture. Such application would be significant to industries and medical sectors as it can offer rapid single-cell level detection of proliferative cells of biological samples. For proof of concept, we explored this possibility using a coculture of *E. coli* (gram-negative) and *B. subtilis* (gram-positive). Our hypothesis was that the above approach of measuring electrically induced membrane-potential dynamics combined with exposure to vancomycin allows for the rapid differentiation of *E. coli* and *B. subtilis*. This is based on the fact that vancomycin inhibits the cell-wall synthesis of gram-positives but is largely inactive to gram-negatives due to their outer membrane barrier (47).

To test this hypothesis, we cocultured fluorescently labeled strains of *E. coli* and *B. subtilis* expressing mCherry and YFP under IPTG-inducible promoters for *E. coli* and *B. subtilis*, respectively. Multichannel fluorescence imaging exhibited distinct signals for *E. coli* and *B. subtilis* (Fig. 4A). The fluorescence of ThT showed lower intensity with *E. coli* compared with *B. subtilis* (Fig. 4B). Although the exact reason for this is unclear it may suggest that there is a difference in stability or fluorescence yield of ThT between these two species. This difference in intensity highlights an advantage of our approach focusing on the response dynamics rather than being reliant on the initial affinity of

the cells for the dye. After an hour-long exposure to vancomycin, the coculture was treated with an external electrical stimulus. The result revealed distinct patterns of membrane-potential dynamics for *E. coli* and *B. subtilis* cells treated with vancomycin. Specifically, the electrical stimulation causes *E. coli* to become hyperpolarized, while depolarizing vancomycin-treated *B. subtilis* (Fig. 4C and D and *SI Appendix*, Fig. S14). Similar patterns were observed with monocultures of *E. coli* or *B. subtilis* (*SI Appendix*, Fig. S15). We also confirmed that vancomycin is active on *B. subtilis*, but not on *E. coli* (*SI Appendix*, Fig. S16). The histogram of individual-cell response revealed distinct distributions between *E. coli* and *B. subtilis* (Fig. 4E). These results thus demonstrate that our approach can be combined with selective culture methods that are commonly used for species differentiation.

Discussion

In this study, we developed and demonstrated an experimental tool for studying the input–output relation of bacterial electrical signaling. Using this tool combined with time-lapse microscopy, we showed that proliferative and inhibited cells respond to an electrical stimulation in apparent opposite directions. A mathematical model suggested that the apparent opposite responses can arise from diverse antibacterial stresses. We experimentally confirmed this using UV-V light, antibiotics, ethanol, and CCCP. The distinct response dynamics between proliferative and inhibited cells offer a rapid single-cell detection of proliferative cells, which can be combined with differentiation techniques using selective growth media.

Bacterial electrical signaling mediated by membrane-potential dynamics regulates various physiological processes, such as metabolic coordination in biofilms and mechanosensation (4, 48). However, it remains unclear how cells decode various information from the received electrical signal. We demonstrated that proliferation capacity adds to the complexity of bacterial electrical signaling, which presents a clue for elucidating the signal-decoding mechanism. Recent study showed that only a subpopulation of cells contributes to the biofilm electrical signaling, which optimizes the cost-benefit tradeoff of signal transmission (49). Cell-to-cell heterogeneity in electrical signaling is also observed in mechanosensation (4), sporulation (3), and antibiotics stress response (50). These heterogeneities are important for understanding how cells decode electrical signal

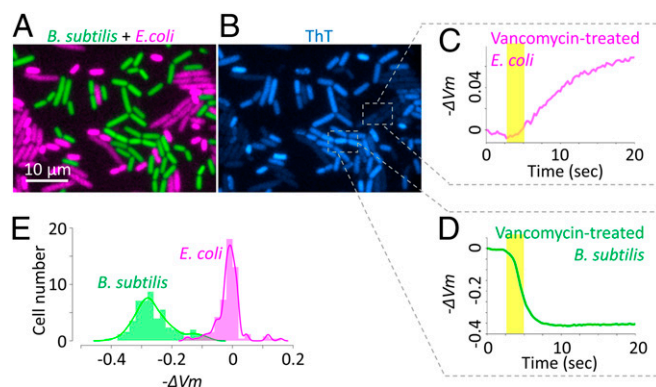


Fig. 4. When treated with vancomycin, *B. subtilis* and *E. coli* cells can be differentiated based on their response to an electrical stimulus. (A) Microscopy image showing the coculture of fluorescently labeled *E. coli* (magenta) and *B. subtilis* (green) cells treated with vancomycin. (B) ThT fluorescence image at the corresponding region. (C and D) Time trace of $-\Delta V_m$, calculated from ThT fluorescence intensity, of the regions defined in B. (E) Histogram of $-\Delta V_m$ at 10 s after electrical stimulation of *B. subtilis* and *E. coli* shows clear differentiation of peaks with the presence of vancomycin.

using a filter set consisting of Ex 554/23, Em 609/54, and dichroic mirror 573 (Semrock). The exposure time for the imaging of both YFP and mCherry was 300 ms.

For irradiation to UV violet light, cells were irradiated by UV-Violet light for 30 s using the inverted microscope DMI8 (Leica Microsystems) and the LED light source, SOLA SM II Light Engine (Lumencor), with an excitation filter 400/16. Field diaphragm of the microscope was used to irradiate only a specific region of the field of view. Before electrical stimulation, a 1-h growth period was allowed during which cells were observed using phase-contrast microscopy to ascertain the effects of UV-V light. For ethanol experiments, MSgg containing 1% (vol/vol) ethanol was used instead of MSgg.

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