Membrane potentials, oxidative stress and the dispersal response of bacterial biofilms to 405 nm light

J A Blee1,2,3, I S Roberts1 and T A Waigh1,2,3

1 Division of Infection, Lydia Becker Institute of Immunology and Inflammation Immunity & Respiratory Medicine, Immunity & Respiratory Medicine, School of Biological Sciences, University of Manchester, Oxford Road, M13 9PL, United Kingdom
2 Biological Physics, Department of Physics and Astronomy, School of Natural Sciences, University of Manchester, Oxford Road, M13 9PL, United Kingdom
3 Photon Science Institute, Alan Turing Building, University of Manchester, Oxford Road, M13 9PL, United Kingdom

E-mail: i.s.roberts@manchester.ac.uk and t.a.waigh@manchester.ac.uk

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Abstract

The majority of chronic infections are caused by biofilms, which have higher levels of antibiotic resistance than planktonic growth. Violet-blue 405 nm light has recently emerged as a novel bactericide, but limited studies have been conducted on its effectiveness against biofilms. We found that in response to 405 nm light both Pseudomonas aeruginosa and Bacillus subtilis biofilms exhibited cell dispersal and membrane potential hyperpolarisations. The response to 405 nm light depended on the stage of biofilm growth. The use of reactive oxygen species scavengers reduced membrane hyperpolarisation and biofilm dispersal in response to 405 nm light. This is the first time that membrane potential hyperpolarisations have been linked with photooxidative stress in bacteria and with biofilm dispersal. These results provide a new insight into the role of membrane potentials in the bacterial stress response and could be used in the development of 405 nm light based biofilm treatments.

Introduction

Bacterial biofilms are communities of bacteria encased in a self-produced extracellular polymeric substance (EPS). The EPS makes up 75%–90% of the biofilm: giving it structure, contributing to the adhesive properties, influencing the genetic regulation and controlling the flow of nutrients and toxins through it [1, 2]. The protection provided by the EPS, combined with the collective, coordinated behaviour of cells, produces a prevalent and resilient form of growth [1–3]. It is estimated that approximately 65% of all microbial and 80% of chronic infections are associated with biofilms [4], while other phenomena, such as biofouling, are costly to industry [5]. Bacterial biofilms require antibiotics and biocides at levels 500–5000 times higher than planktonic cells [6]. This has led to a critical need for the development of new techniques to tackle biofilm growth.

In terms of studying B. subtilis and P. aeruginosa, biofilm growth has been divided into stages [7–10] (summarised in figure 1). This dissection of biofilm growth has facilitated our understanding of the biology of bacterial biofilms, but one must be cautious in extrapolating from such model organisms to all microbial biofilms.

One alternative approach to combat biofilm growth is antimicrobial photodynamic therapy (PDT), that has been extensively investigated as a technique for treating localized infections [11] and as a decontamination method for both industrial and clinical applications [12]. However, current PDT methods rely on the use of chemicals and/or ultraviolet light. This causes issues with the sub-optimal uptake of photosensitisers and a lack of selectivity for bacterial cells over host cells [13].

Recent studies have shown that 405 nm light may provide a superior alternative to current PDT methods, with a broad range of both Gram-positive and Gram-negative bacteria being inactivated by 405 nm light [14, 15]. The antimicrobial action of 405 nm light does not require additional photosensitisers and studies have found that the doses required to elicit a bactericidal response are not harmful to mammalian cells [16]. The cytotoxic response of bacteria to 405 nm light involves the photoexcitation of intracellular
porphyrin molecules, which cause the generation of reactive oxygen species (ROS) [16, 17]. During photosensitization of the cell, the porphyrin molecules are converted to their triplet state [18], which then facilitates the production of ROS via either the Type I or the Type II pathway. In the Type I pathway the porphyrin molecules react with the cellular components, producing free radicals which then cause further reactions. Whereas the Type II mechanism involves the excited photosensitisers reacting directly with molecular oxygen to produce singlet oxygen [19]. As a consequence, oxidative stress may lead to DNA or RNA damage, lipid peroxidation, protein oxidation, enzyme inhibition and the activation of programmed cell death [20–22].

Recent studies have shown that bacteria may regulate their membrane potentials in response to stress [23–25]. This led us to investigate the role of membrane potential in the oxidative response of bacterial biofilm cells to 405 nm light. We used two model organisms: P. aeruginosa and B. subtilis. P. aeruginosa is a Gram-negative bacterium that has been extensively used to study biofilms [26]. It causes difficult to treat, nosocomial infections, in particular topical skin infections, that might be targets for localised, 405 nm light treatment [27]. In addition, P. aeruginosa can cause biofouling on nano-filtration devices involved in seawater desalination systems [28]. B. subtilis is a Gram-positive spore forming bacterium that is ubiquitous in the environment [7] and that has been extensively studied as a model organism for many years [7]. It is reported to be resistant to UV-C [29]. In this paper we show for the first time that photooxidative stress leads to hyperpolarisation of the membrane potential, dispersal from biofilms and develop a Hodgkin–Huxley model for the behaviour of biofilm bacteria in response to 405 nm light at different stages of biofilm growth.

**Results**

**Physical response of P. aeruginosa cells to 405 nm light treatment**

Biofilms were grown to the five key stages (figure 1). A more in-depth description of these five stages can be found in the supplementary information (stacks.iop.org/PhysBio/17/036001/mmedia). A finely focused laser was used to provoke a response (see Materials and Methods for more details).

The physical response of cells to 405 nm light depended on the stage of biofilm growth. Figure 2(A) shows the cell dispersal dose response to 120 ± 4 μW cm⁻² of 405 nm light, at the five stages of biofilm growth. During Stage I and II of biofilm growth the cells left the surface in response to 405 nm laser illumination at 120 ± 4 μW cm⁻². Dispersed cells maintained a membrane potential demonstrating their viability. At Stage III of biofilm growth, most cells did not leave the surface in response to the laser. Initially 10% of the cells left the surface, but these cells were then replaced by other cells. After approximately 250 min of treatment at an irradiance of 120 ± 4 μW cm⁻², which is equivalent to a dose of 1.8 J cm⁻², these cells became physically altered by the laser treatment. Cells morphologically transformed from their traditional rod shapes to coccoids (supplementary figure 2). A similar morphological transformation has been observed in P. aeruginosa in response to antibiotic stress [30]. This
stress response has been linked to an increased antibiotic resistance, due to a change in the cells’ metabolic activity.

At Stage IV, some cells dispersed. However, most cells remained on the surface following treatment and were not visibly altered by treatment. Cell dispersal was specifically restricted to 405 nm light with no equivalent response observed to an equivalent dose of 488 nm wavelength light (supplementary figure 4).

At Stage V of biofilm growth almost all cells remained on the surface following treatment. Surface cells on the bottom layer of the biofilm stacks all remained after 6 h of illumination at 120 ± 4 μW cm⁻², which is equivalent to a dose of 2.6 J cm⁻². These cells showed no visible changes in response to the light.

The dispersal of bacteria at Stages I, II and IV of biofilm growth can be quantified using their residence probabilities, which are known as survival functions [31]. A survival function is defined as the probability that an event has not occurred by a certain time. Such functions are broadly used in survival analysis to investigate the probability of events occurring with time [32]. In order to avoid confusion with cell death, we call the survival functions depicted in figure 2(A) biofilm residence probabilities. These provide the probabilities that the cells remain on the surface at a given time. This statistical tool has not previously been used with bacteria but provides a robust description of their behaviour.

The Kaplan–Meier estimator is used to predict the survival function from experimental lifetime data [33]. An important advantage of the Kaplan–Meier method is that it can account for censored data, specifically right censoring, which may occur if a cell is lost during the tracking procedure. The biofilm residence probability was well estimated by the Kaplan–Meier function (figure 2(A)). The biofilm residence probability with time (t) given by the Kaplan–Meier estimator (S(t)) is defined as:

\[ S(t) = \prod_{i: t_i \leq t} \left(1 - \frac{d_i}{n_i}\right) \] (1)
where \( t_i \) is the time until at least one cell leaves the surface, \( d_i \) is the number of events at time \( t_i \) and \( n_i \) is the number that are known to remain on the surface or that have not been censored by time \( t_i \).

Hazard functions are used to determine which periods in time have the highest and lowest chance of an event happening [31, 34, 35]. The hazard function \( h(t) \) gives the instantaneous failure rate of an individual (rate of leaving in our case) conditioned on the fact that the individual survived until a given time:

\[
h(t) = \lim_{\Delta t \to 0} \frac{P(t \leq T < t + \Delta t \mid T \geq t)}{\Delta t}
\]

(2)

where \( P(t \leq T < t + \Delta t \mid T \geq t) \) is the probability of a single cell leaving the surface between \( t \) and \( t + \Delta t \).

We calculated the hazard function from the Kaplan–Meier function using the relation

\[
h(t) = -\frac{d}{dt} \ln(S(t)).
\]

(3)

Figure 2(B) shows the hazard rates for the biofilm residence probabilities described by \( S(t) \). The cumulative hazard rate \( H(t) \) was calculated using

\[
H(t) = -\ln(S(t)).
\]

(4)

Figures 2(B) and (C) show the hazard functions and cumulative hazard functions derived from the Kaplan–Meier estimate with corresponding fits. The hazard functions \( h(t) \) increased linearly with time at Stages I, II and IV of biofilm growth and were well described by

\[
h(t) = -a_1 + 2b_1 t
\]

(5)

where \( a_1 \) is the intercept constant and \( b_1 \) is the slope constant.

The cumulative hazard functions were defined as

\[
H(t) = -a_1 t + b_1 t^2.
\]

(6)

Mathematically this defines a highly non-Markovian statistical process. The increase in the hazard function implies an increase in the chance of a cell leaving the surface with time. Increases in survival hazard functions are commonly observed in radiation damage of tissue in medical physics, although their origin is contentious [36]. The ratio of two different biofilm residence hazard rates \( h(t)_1 \) and \( h(t)_2 \) was dependent on time,

\[
\frac{h(t)_1}{h(t)_2} = \frac{a_{11} + 2b_{11} t}{a_{12} + 2b_{12} t}
\]

(7)

where \( a_{11}, b_{11} \) and \( a_{12}, b_{12} \) were the constants of functions 1 and 2, respectively (equation (5)).

The non-proportionality of our hazard functions meant we could not directly compare different functions using the hazard ratio as a measure of survival. Instead, \( a_1 \) ratios were used to compare between initial surviving rates, and \( b_1 \) ratios were used to compare dispersal rates (figure 2(D)).

The \( a_1 \) ratio is 18.0 ± 0.4 between Stage I and Stage II of biofilm growth and 0.186 ± 0.009 between Stage I and Stage IV of biofilm growth. The \( b_1 \) ratio was found to be 41.075 ± 0.269 between Stage I and Stage II and 0.186 ± 0.002 between Stage I and Stage IV.

The increase in the hazard function constants \( a_1 \) and \( b_1 \) from Stage I to Stage II of biofilm growth implies a larger initial and faster overall event rate. Whereas the decrease in the hazard function constants \( a_1 \) and \( b_1 \) from Stage I to Stage IV of biofilm growth implies a lower initial and overall slower dispersal rate.

Overall, these results show that initially cells became more physically responsive to 405 nm light with biofilm growth, but that as the biofilm matured this responsiveness decreased, until in the mature biofilm no obvious physical response was observed.

Membrane potential changes for \( P. aeruginosa \) in response to 405 nm light stress

To understand the mechanisms involved in the response of bacteria to 405 nm light the membrane potential changes were monitored using the membrane potential indicator dye Thioflavin-T (ThT). Its suitability in the role as a membrane potential indicator in bacterial cells was established by Prindle et al [23], since then it has become widely used as an indicator of membrane potential in bacteria [37–39]. It is cheaper and for biofilm studies often more sensitive than other commonly used membrane potential indicators (such as DiSC3(5)). DiSC3(5) has also been shown to inhibit bacterial growth and so is inappropriate for long term measurements, whereas at a working concentration of 10 µM ThT does not inhibit growth (supplementary figure 6).

Across a range of different environmental conditions and physiological states the response of biofilm cells to 405 nm light was accompanied by an increase in ThT fluorescence. An example of a typical membrane potential hyperpolarisation of an aggregate of \( P. aeruginosa \) cells is shown in figure 3 and in supplementary video 1. More ThT is retained in a cell as it becomes more negatively charged and therefore the observed increases in ThT imply an opposite change in the cell’s membrane potential. To confirm our results we measured the membrane potential dose response in early stage biofilm cells using DiSC3(5). This produced equivalent results to ThT (supplementary figure 5), confirming the suitability of ThT as a membrane potential indicator.

During all stages of biofilm growth, surface adhered cells exhibited membrane potential hyperpolarisations in response to 405 nm light. Hyperpolarisations were observed in response to all irradiances in our experimental range (70–740 µW cm−2). This behaviour was specifically restricted to 405 nm light with no membrane potential changes observed in response to an equivalent dose of 488 nm light (supplementary figure 3) or 647 nm light (supplementary figure 5). Figure 4(A) shows five hyperpolarisation curves observed in response to an irradiance of 120 ± 4 µW cm−2 at the five previously defined stages of biofilm growth (figure 1). These hyperpolarisations can be described by Boltzmann sigmoidal curves of the form
\[ \text{ThT}(D) = \text{ThT}_0 + \frac{(\text{ThT}_{\text{max}} - \text{ThT}_0)}{1 + e^{(D - D_0)/x}} \] (8)

where \( \text{ThT}(D) \) is the fluorescence intensity as a function of dose \( D \), \( \text{ThT}_0 \) is the bottom plateau constant, \( \text{ThT}_{\text{max}} \) is the top plateau constant, \( D_0 \) is the half dose constant and \( x \) is the slope constant, which describes the steepness of the curve.

The only stage in biofilm growth which was not well described by a single sigmoidal function was Stage III. During this stage of growth, sigmoidal behaviour was followed by an exponential phase. In order to compare this stage of biofilm growth with other stages, we fitted the initial behaviour to equation (8) (shown in figure 4(A)).

We found that the parameters governing the sigmoidal fits of the membrane potential dose response varied with the stage of biofilm growth (figure 5). The slope constant (\( x \)), which is inversely proportional to the steepness of the response, decreased from Stages I through to III, as the cells responded faster to the laser light. There was a 67\% \pm 2\% decrease in \( x \) from Stage I to Stage II and a 96\% \pm 9\% decrease in \( x \) from Stage I to Stage III.

The cells responded slowest to the light during the late biofilm stages IV and V of growth. This is shown by an increase in \( x \), which was found to be 34\% \pm 2\% higher in stage IV than in Stage I and 291\% \pm 7\% times higher in Stage V than Stage I. The \( D_0 \) value, which defines the position of the half maximal membrane potential, followed a similar pattern, with the only difference being that \( D_0 \) was larger during Stage IV than during Stage V. Surface cells which remained on the surface depolarised back to their original values following hyperpolarisation at longer time scales (\( >500 \) min, figure 4(B)) demonstrating viability.

The dose response of cells depended on the laser irradiance (see S.I.5), however the change in steepness of the dose response due to biofilm growth was not dependent on the irradiance used to evoke the response. This meant that the steepness ratios of different biofilm growth dose responses obtained at \( 120 \pm 4 \mu W \mathrm{cm}^{-2} \) were representative of the ratios observed across the entire range of irradiances that we tested. The membrane potential dose response was 2.91 \pm 0.02 times less steep in the mature biofilm than in the initially adhered cells.

Figure 4 was obtained by averaging the ThT intensity of cells in the region of treatment and so is representative of the global behaviour. Figure 6(A) shows the heterogeneity in cell membrane potential response of Stage I biofilm growth cells in response to \( 120 \pm 4 \mu W \mathrm{cm}^{-2} \) light. The half maximal membrane potential (\( D_0 \)) varied between 82\,s and 422\,s (presented here in units of time rather than dose to allow comparison with the leaving time). The leaving time (time at which a cell leaves the surface) also varied (figure 2). Similar stochasticity is used as a strategy in gene regulation [40].

There was a positive correlation, at the 0.05 significance level, between individual cell half maximum membrane potential (\( D_0 \)) and leaving time (figure 6(B)). The difference in \( D_0 \) and leaving time of different cells had no statistically significant correlation with cell separation. This confirms that, we were not
observing a coordinated stress response across multiple cells (figures 6(C) and (D)).

**Response to 405 nm light in the presence of scavengers**

To confirm that the effect of exposure to 405 nm light was associated with the generation of ROS, the exposure of *P. aeruginosa* biofilms to 405 nm light in the presence of ROS scavengers was tested. Exposure of *P. aeruginosa* to 405 nm light in the presence of ROS scavengers altered the membrane potential and dispersal response. Figure 7 shows the response of *P. aeruginosa* to 405 nm light in the presence and absence of a scavenger mix consisting of 100 mM sodium pyruvate and 200 U ml$^{-1}$ catalase. The hyperpolarisation dose response was slower and delayed in the presence of ROS scavengers. There was a $74.1\% \pm 0.2\%$ increase in the half dose constant ($D_0$) as well as a $73.2\% \pm 0.8\%$ decrease in the steepness ($\alpha$) of the membrane potential dose response in the presence of scavengers. The dispersal response was

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**Figure 4.** (A) Average cell ThT intensity as a function of time (or equivalently dose) observed in response to 405 nm light at different stages of *P. aeruginosa* biofilm growth, in the same media, at a constant irradiance of $120 \pm 4 \text{ µW cm}^{-2}$ with corresponding sigmoidal fits to equation (8). (B) Average ThT fluorescence of mature *P. aeruginosa* biofilm cells as a function of time (or equivalently dose) in response to 405 nm light. Data was collected for a much longer time than that shown in (A) i.e. 900 min compared to 1500 s.
Figure 5. Boltzmann sigmoidal fit parameters (half maximal dose ($D_0$) and slope constant $x$ as given by equation (8)) which define the average hyperpolarisation of cells at the five stage of biofilm growth in response to 405 nm light 120 ± 4 $\mu$W cm$^{-2}$ of 405 nm light.

Figure 6. (A) Individual cell ThT intensity as a function of time (or equivalently dose) observed in response to 405 nm light at Stage I of P. aeruginosa biofilm growth, in response to 405 nm light constant irradiance of 120 ± 4 $\mu$W cm$^{-2}$. (B) Leaving time of individual cells from (A) as a function of half maximal time, with corresponding linear fit shown in red. (C) Average difference in leaving time of cells as a function of cell separation. (D) Average difference in half maximal time of cells as a function of cell separation.
also significantly reduced in the presence of ROS scavengers with a 46% ± 11% reduction in the number of cells that had dispersed in response to 85 mJ cm⁻² of 405 nm light.

Membrane potential response of fixed cells
To test what happened when cells were not free to leave the surface, we used an agarose microscope slide set-up (Materials and Methods) in which cells are contained within a narrow 2D slot between agarose and a cover slip with only room for a single layer of cells. Cells were spotted onto a semi-solid agarose medium. If left these cells grew and divided to form microcolonies. When treated with 405 nm light, the trapped bacterial cells became deformed and lysed. These cells hyperpolarised, as observed previously in the flow cell set-up, before depolarising as they were damaged/ killed by the 405 nm light (figure 8). Differences between the flow cell set-up and this set-up, such as flow, could affect the levels of ROS. However, the rate of hyperpolarisation of the initial cells in this set-up was comparable to the initially adhered cells in the flow cell set-up, which suggests that experimental differences did not have a significant effect on the membrane potential response.

We compared the response of these cells to mature biofilm cells in the flow cell (which also did not disperse; figure 4(B)). It was clear that, although the ThT curves were similar in shape (increase followed by decrease), the two responses differed significantly. The ThT pro-

Figure 7. (A) Average cell ThT intensity as a function of time (or equivalently dose) of Stage I P. aeruginosa cells with and without added scavengers (100 mM sodium pyruvate and 200 U ml⁻¹ catalase) exposed to 405 nm light at a constant irradiance of 120 ± 4 µW cm⁻² with corresponding sigmoidal fits to equation (8) shown in blue. (B) Residence probability (probability of surface cells remaining) of Stage I P. aeruginosa cells following 700 s (equivalent to 85 mJ cm⁻²) of 405 nm light with and without added scavengers (100 mM sodium pyruvate and 200 U ml⁻¹ catalase).
file of initial cells on the agarose slides was much faster (figure 8), with a peak at 1.33 ± 0.02 min, whereas for mature biofilm cells (figure 4(B)) the peak was at 102 ± 2 min. Even despite possible experimental differences, this suggests that the differences in the membrane potential response of mature biofilm cells was not caused solely by their inability to leave. The mature biofilm cells also returned to membrane potential values comparable with their starting rest potential values, indicating viability after treatment, whereas the initially adhered cells became damaged by treatment when they were not free to leave and depolarised to values lower than their original resting potentials.

Response of B. subtilis to 405 nm light
To extend our observations of bacteria in response to 405 nm light, we also studied the Gram-positive bacterium B. subtilis. The response of B. subtilis to 405 nm light treatment was also accompanied by membrane potential hyperpolarisations (figure 9 and supplementary video 2). Confirming that B. subtilis responds to 405 nm light, despite its established resistance to UV light [29]. These hyperpolarisations followed the same profile as those of P. aeruginosa and were well described by sigmoidal fits (defined by equation (8)). The addition of ROS scavengers (100 mM sodium pyruvate and 200 U ml⁻¹ of catalase) also altered the hyperpolarisation response of B. subtilis (figure 10). The half dose constant (D₀) increased by 65.7% ± 0.9%, analogous to P. aeruginosa, in contrast however the steepness of the response (ξ) was not greatly affected by the addition of scavengers in B. subtilis with only a 6.1% ± 0.1% decrease observed.

Early stage B. subtilis biofilm cells were seen to disperse or become deformed in response to 405 nm light. Dispersed cells maintained a membrane potential, signifying viability. The observed dispersal response of B. subtilis and P. aeruginosa differed. P. aeruginosa cells moved away from the surface gradually, whereas the clearance of B. subtilis cells was sudden. This was demonstrated by a sharper cumulative hazard function for B. subtilis (figure 11 inset) than was seen for P. aeruginosa (figure 2(C)). The cumulative hazard function of B. subtilis was initially flat, followed by a sharp increase, representing a sudden increase in the chance of cells leaving the surface. This is suggestive of a dispersal mechanism which is activated above a threshold concentration of ROS with a corresponding lag time e.g. it could be due to a coherence feed forward loop gene circuit [41]. In contrast, the P. aeruginosa hazard function increased from the outset of treatment and was less steep, this suggests that different mechanisms may govern the dispersal response of these two bacterial strains. However, due to differences in the experimental protocols used for the two species, we cannot rule out that differences were a consequence of different experimental conditions e.g. the availability of nutrients.

Hodgkin–Huxley model for the stress response
It is known that the oxidative stress response of cells may be affected by changes in the responsiveness of cells and due to differences caused by surrounding cells and EPS [42–44]. In order to test the hypothesis that changes in the production and loss of ROS at different stages of biofilm growth cause the differences
in the observed membrane potential dose responses, we created a Hodgkin–Huxley model for the single cell response \cite{45}. Our model is similar in style to that of Prindle et al \cite{23}, which is based on the response of potassium ion channels to nutrient starvation. We modelled the bacteria as excitable cells with a membrane potential \( V \) given by

\[
\frac{dV}{dt} = -g_m (V - V_I) - g_{\text{leak}} (V - V_{\text{leak}})
\]  

(9)

where we have assumed that the observed behaviour is described by one representative ion channel \( I \) and a leakage current \( \text{leak} \). \( g_I \) and \( g_{\text{leak}} \) are the respective channel conductances. \( V_I \) and \( V_{\text{leak}} \) are the ion and

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**Figure 9.** Representative hyperpolarisation of Stage II \emph{B. subtilis} cells in response to 120 ± 4 µW cm\(^{-2}\) of 405 nm light in an Ibidi shear flow cell. Average ThT intensity is shown as a function of time (or equivalently dose) with a corresponding sigmoidal fit (equation (8)).

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**Figure 10.** Average cell ThT intensity as a function of time (or equivalently dose) of Stage I \emph{B. subtilis} cells with and without added scavengers (100 mM sodium pyruvate and 200 U ml\(^{-1}\) catalase) exposed to 405 nm light at a constant irradiance of 120 ± 4 µW cm\(^{-2}\) with corresponding sigmoidal fits to equation (8) shown in blue.
Nernst potentials, respectively. $n$ is the ion activation constant and is given by

$$\frac{dn}{dt} = \alpha(S)(1 - n) - \beta(V)n$$

(10)

where the opening rate $\alpha(S)$ was taken to be dependent on the stress induced by ROS ($S$) following a Hill equation with the cooperativity parameter ($n$) set equal to 1,

$$\alpha(S) = \frac{\alpha_0S^n}{S_{th}^n + S^n}$$

(11)

where $\alpha_0$ is the maximal opening rate and $S_{th}$ is the threshold stress value.

Following Hodgkin–Huxley’s original work with squid axons, the ion channel closing rate $\beta(V)$ was assumed to be dependent on the voltage,

$$\beta(V) = \beta_0 e^{-V/\sigma_v}$$

(12)

where $\beta_0$ is the maximal closing rate and $\sigma_v$ is the coefficient of the closing rates’ voltage dependency. Both $P. aeruginosa$ and $B. subtilis$ contain voltage-gated ion channels [23, 46, 47].

We assumed that the stress induced by ROS was proportional to the difference between the production and loss of the ROS in the cell. Following dynamical equations of neuronal excitability [45, 48, 49] and the subsequent work of Prindle et al [23] we represent the production of ROS by a threshold-linear function of laser irradiance ($I$)

$$\frac{dS}{dt} = \frac{\alpha_s(I_{th} - I)}{\exp\left(\frac{I_{th} - I}{\sigma_s}\right) - 1} - \gamma_sS$$

(13)

where $\alpha_s$ is the production constant and $\gamma_s$ is the decay constant, both of which we assume depend on the stage of biofilm growth.

In our experiment the irradiance was constant so that

$$\frac{dI}{dt} = 0.$$  

(14)

Following Prindle et al [23] we assume the simplest dependence between ThT fluorescence ($F$) and level of membrane hyperpolarisation ($V_0 - V$),

$$\frac{dF}{dt} = \alpha_t(V_0 - V) - \gamma_t F$$

(15)

where $V_0$ is the resting membrane potential, $\alpha_t$ is the constant of proportionality between ThT fluorescence and the level of membrane potential hyperpolarisation and $\gamma_t$ is the ThT decay rate. The parameters which define this model are shown in supplementary table 1 and, where possible, were matched with previously known results, the rest were chosen by parameter fitting.

To test our model, we varied both the input irradiance ($I$) and the parameters $\alpha_s$ and $\gamma_s$. For input irradiances in the range 85–575 µW cm$^{-2}$ our model correctly produces an increase in the rate of the membrane potential response with increasing irradiance (figure 12(A)).

We assumed that larger ROS production rates ($\alpha_s$) were observed when cells were more metabolically active and that $\gamma_s$ increased with biofilm growth, so that from Stage I to Stage V: $\alpha_s = 0.001, 0.05, 4, 0.0008, 0.0005$ µM (min mV)$^{-1}$ and $\gamma_s = 0.001, 0.003, 0.005, 0.008, 0.1$ min$^{-1}$, respectively. Figure 12(B) shows that

![Figure 11](image)

Figure 11. Biofilm residence probability as a function of time (or equivalently dose) for a Stage II $B. subtilis$ biofilm exposed to 120 ± 4 µW cm$^{-2}$ of 405 nm light, the Kaplan–Meier estimate (equation (1)) is shown in red, with an inset of the corresponding cumulative hazard function (equation (4)). Averages were taken from at least 20 cells in the field of view.
these assumptions may be used to correctly model the differences in the membrane potential response at different stages of biofilm growth.

The differences in the changes to the membrane potential response caused by the addition of ROS scavengers, combined with the differences in the cumulative hazard function of \( P. \) aeruginosa and \( B. \) subtilis, suggest a different response mechanism to phototoxic oxidative stress. To test this hypothesis, we adapted our original model so that rather than a Hill dependency, the channel opening rate \( \alpha(S) \) was assumed to depend on the ROS stress following a unit step response,

\[
\alpha(S) = \alpha_0 \theta(S - S_{th}) \tag{16}
\]

where \( \theta = 0 \) when \( S < S_{th} \), \( \theta = 1 \) when \( S \geq S_{th} \) and the closing rate \( (\beta) \) was assumed to be constant. The lag time described by the step function represents a delay, for example in the internal gene circuit of the bacterium. The rest of the model and its assumptions remained the same. The parameters are shown in supplementary table 1.

ROS scavengers increase the decay rate of ROS, this translates directly to an increase in the decay constant \( (\gamma) \). Figure 12(C) shows the change in the membrane potential dose response predicted by our original model with corresponding sigmoidal fits (equation (8)) produced by an increase in the ROS decay rate. The model predicts an increase in the half maximal dose constant \( (D_0) \) and a corresponding decrease in the steepness of the response \( (1/x) \). This confirms that our original model and its assumptions may be used to explain the membrane potential hyperpolarisations of \( P. \) aeruginosa (figures 4 and 7). Figure 12(D) shows the change in the membrane potential dose response predicted by the adapted model due to addition of ROS scavengers with corresponding sigmoidal fits. This adapted model (including equation (16)) produced an increase in the half maximal dose constant, but with no corresponding decrease in the steepness of the response, successfully describing the observed membrane potential dose response of \( B. \) subtilis (figure 10).

**Discussion**

The response of both Gram-positive and Gram-negative bacteria to 405 nm light was accompanied by membrane potential hyperpolarisations. Hyperpolarisations were...
observed across a range of biofilm growth states. This suggests that this behaviour may be universal. The hyperpolarisation response was delayed in the presence of ROS scavengers. This implies a link between the hyperpolarisations and the ROS generated by 405 nm light.

Recent studies have revealed that 405 nm light induces photophysiological responses in a range of bacteria via different blue-light receptor classes (LOV, BLUF and PYP) [50, 51]. For example, 405 nm light has been shown to induce responses, such as biofilm formation and motility in *Acinetobacter baumannii* via the blue light using flavin (BLUF) protein BlsA [52] and in *Synechocystis sp. PCC 6803* via a cyclic adenosine monophosphate (cAMP) signal transduction system [53]. Blue light was shown to activate σ^H and the general stress response of *B. subtilis* [54], via the LOV protein, YtvA [55]. Providing a possible mechanism via which *B. subtilis* responds to blue light.

It has recently emerged that *P. aeruginosa* responds to light via the photoreceptor BphP [56]. It was found that biofilm formation and virulence were regulated by the phosphorylation and activation of AlgB, by BphP, in response to far-red light. The photosensory proteins LOV-HK and BphP1 form an integrated network that regulates swarming motility in *Pseudomonas syringae* in response to multiple light wavelengths [57]. While the motility of *Synechocystis sp. PCC 6803* is affected by 405 nm light via a cyclic diguanylate (c-di-GMP) signal transduction system [53].

As c-di-GMP/cAMP levels have been shown to regulate the stress response of *P. aeruginosa* [58], we suggest that a similar mechanism may be responsible for the observed 405 nm light induced dispersal. The majority of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) that have been identified in the genome of *P. aeruginosa* still remain uncharacterized [10]. One hypothesis is that 405 nm light stimulates the production of one or more of these PDEs, ultimately resulting in lower levels of c-di-GMP and cell dispersal.

Despite many recent advances, the role of most bacterial blue-light receptors remains elusive.

The observed correlation between membrane potential increases and the dispersal of bacterial cells from the surface is interesting because membrane potentials have been shown to be crucial in motility regulation [59], mechanosensation [60] and adhesion [61]. Recent studies have also linked stress induced cell dispersal with the membrane potential. It was shown that starvation-induced dispersal in *P. aeruginosa* operates through the intracellular secondary messenger cAMP and that dispersal is an active process, requiring a membrane potential [24].

Cell dispersal was observed in both *P. aeruginosa* and *B. subtilis* in response to 405 nm light. Despite the analogous responses of *P. aeruginosa* and *B. subtilis*, there were some differences observed between the two species. We used hazard functions to reveal a difference in the dispersal behaviour. The dispersal of *B. subtilis* was sudden, after a threshold dose of 405 nm light was reached, cells were very likely to leave the surface (figure 12). Whereas, the dispersal of *P. aeruginosa* was less drastic, with gradual increases in dispersal observed (figure 2). There was also a difference in hyperpolarisation changes of the two species upon the addition of ROS scavengers. The hyperpolarisation half dose constant increased in both species, but a large decrease in the steepness of the response was only observed in *P. aeruginosa*. Differences in the experimental protocols used for the two species prevented direct comparison, however, we demonstrated using a Hodgkin–Huxley model that the photooxidative stress dependency may explain these differences.

We found that both the photophysical and the membrane potential response to 405 nm light was dependent on biofilm growth. Intermediate stage biofilm cells (Stage II and Stage III) showed the fastest and most significant physical changes in response to 405 nm light. These cells also exhibited the fastest membrane potential changes. Mature biofilm cells (Stage V) showed no physical response to 405 nm light and exhibited the slowest membrane potential changes. This correlation between the physical and the membrane potential responsiveness is interesting because membrane potentials have been shown to be crucial in motility regulation [59], mechanosensation [60] and adhesion [61]. The changes in the sensitivity of cells with biofilm growth are likely due to differences in the cells’ metabolic states and due to the influence of the surrounding cells and the biofilm EPS. Our Hodgkin–Huxley style model demonstrates how these differences can explain the changes in the membrane potential response observed at different stages of biofilm growth.

Intracellular photosensitisers and antioxidant levels vary with the cells’ metabolic state and have been shown to alter the magnitude of the ROS response [62]. The initial colonising cells (Stage I) were at stationary growth phase following overnight planktonic growth. This may explain why they were found to be less sensitive to 405 nm light than the cells at Stage II and Stage III of biofilm growth. The steepness of the membrane potential dose response (β) increased by 67% ± 3% from Stage I to Stage II and the hazard slope constant (b_1) increased by 41.1% ± 0.3%. This implies that a cells’ metabolic state greatly affects its responsiveness to 405 nm light. During the Stage III of biofilm growth the cells continued to become increasingly more responsive. The combination of this, and larger adhesive forces preventing cell dispersal, may have caused the morphological changes (coccoid formation S1.2) observed exclusively at this stage of biofilm growth.

The membrane potential dose response was 2.91 ± 0.02 times less steep in mature biofilm cells (Stage V) than in initially adhered cells (Stage I). This, taken together with the lack of dispersal, suggests that biofilm growth may afford considerable protection for...
cells against 405 nm light. Initial cells that could not leave the surface hyperpolarised on a similar timescale to the initial cells in the flow cells that were free to leave, but following hyperpolarisation, these cells depolarised to levels below the initial resting potential and showed physical damage, suggestive of cell death. This further suggested that the differences in the responses of biofilm cells were due to additional protection afforded by biofilm growth. This is an important consideration for antimicrobial therapies especially when looking at mature biofilms. Biofilm growth is associated with changes in metabolic activity, motility and adhesion. Higher levels of catalase, which significantly enhance the protection against oxidative stress, have been detected in biofilm cells [43]. During biofilm growth the role of other external factors and surrounding cells also becomes increasingly important. It has been shown that alginate can protect P. aeruginosa biofilms by shielding them against UV [42]. The biofilm matrix polysaccharides cellulose and alginate have also been shown to protect against ROS generated under stress [44].

Studies have demonstrated the importance of membrane potentials in the stress response of P. aeruginosa and B. subtilis. Starvation-induced dispersal in P. aeruginosa operates through the intracellular second messenger cAMP and requires a membrane potential [24]. Studies have also found that B. subtilis biofilms cells may communicate nutrient stress via electrical signalling [23, 25]. Nutrient deprived inner biofilm cells were found to hyperpolarise in response to starvation via the opening of potassium channels. This nutrient stress was then transferred to outer cells via a potassium wave. Our results show that membrane potential changes may also play a role in the response of B. subtilis to photoinduced oxidative stress, although in our experimental geometry the main determinant was the total dosage of light received by each cell rather than communication between the cells.

An open question concerns additional experiments with the 405 nm laser that used pulsed illumination. There was a difference observed in the low dosage (subthreshold) response from the high dosage (superthreshold) response as expected for a Hodgkin Huxley like non-linear hyperpolarisation phenomenon. However, the data could not be collapsed onto a master curve when rescaled by total dosage, which implies an additional time dependent process is occurring that is unaccounted for in our current model e.g. an additional ion channel could be contributing to the process with a slower response compared with the K+ channel.

Furthermore, we hypothesize that in the future further evidence will emerge connecting a range of different bacterial stresses and responses with associated changes in membrane potential. It would also be interesting to consider a wider range of bacterial species with biofilms grown in a wider range of conditions e.g. at the air/liquid interface rather than just the solid/liquid interface.

**Conclusion**

Membrane potential hyperpolarisations were seen in response to 405 nm light in both the Gram-positive bacterium B. subtilis and the Gram-negative bacterium P. aeruginosa. This provides the first evidence that photooxidative stress in bacteria is correlated with increases in membrane potential. We found that the photophysical response of early stage biofilms to 405 nm light includes cell dispersal, which is problematic for some treatments seeking to implement this technique for widespread decontamination, but useful for others hoping to prevent biofilm surface formation. We also found that both the cell dispersal and the cell membrane potential dose response were dependant on the stage of biofilm growth. The membrane potential dose response was $2.91 \pm 0.02$ times less steep in mature biofilm cells than in initially adhered cells. This suggests that biofilm growth affords considerable protection for bacteria against 405 nm light. A Hodgkin–Huxley model was able to describe the variations in the experimental membrane potential dose response observed at different stages of biofilm growth and due to the addition of ROS scavengers. Residence probabilities provided a robust statistical tool to quantify the motile response of the bacteria to irradiation.

**Materials and methods**

**Cell preparation and biofilm growth**

*Pseudomonas aeruginosa*

Experiments were conducted using *P. aeruginosa* PA01. The cells were freshly streaked onto TSA (Tryptic Soy Agar) plates from glycerol stocks two days before the experiment and incubated at 37 °C overnight. The next day 10 ml of TSB (Tryptic Soy Broth) in a glass universal was inoculated with a single colony. The inoculum was then incubated and shaken at 200 rpm at 37 °C overnight or until O.D$_{600}$ ≈ 2. The cells were then diluted 1:2 with fresh TSB directly before injection into the flow cell.

*P. aeruginosa* biofilms were grown in Ibidi µ-slide VI$^{®}$ flow cells, with rectangular size chambers of size $17 \times 3.8$ mm and height 0.4 mm. This size allowed high shear stresses to be exerted on cells, while still allowing plenty of space for biofilm growth.

Media was supplied by the NE-1002X Programma-ble Microfluidics Syringe Pump and experiments were conducted at room temperature. The flow chamber was primed with media prior to the experiment. The cell culture was then injected into the flow cell which was mounted on an Olympus IX-71 inverted micro-
were then centrifuged at 2100 rcf for 2 min and then resuspended in phosphate buffered saline (PBS). Cells were centrifuged for 10 min at 3000 rcf and then resuspended in PBS.

Bacillus subtilis
Experiments were conducted using the B. subtilis strain NCIB 3610. The cells were freshly streaked onto LB agar plates from glycerol stocks on the day before the experiment and incubated at 37 °C overnight. The next day 3 ml of LB in a glass universal was inoculated with a single colony. The inoculum was then incubated and shaken at 200 rpm at 37 °C for approximately 3 h or until the cells reached an O.D 600 ≈ 0.6. The cells were then centrifuged at 2100 rcf for 2 min and then resuspended in a minimal MSgg medium to promote biofilm growth.

The MSgg medium was made from stock solutions on the day of the experiment and contained: 5 mM potassium phosphate buffer (pH 7.0), 100 mM MOPS buffer (pH 7.0 adjusted using NaOH), 2 mM MgCl2, 700 μM CaCl2, 50 μM MnCl2, 100 μM FeCl3, 1 μM ZnCl2, 2 μM thiamine HCl, 0.5% (v/v) glycerol and 0.5% (w/v) monosodium glutamate, as described by Branda et al [63].

DiSC3(5) experiments were conducted in PBS. Cells were centrifuged for 10 min at 3000 rcf and then resuspended in PBS.

B. subtilis biofilms were grown in Ibidi μ-slide III 3D perfusion flow cell slides, which are made of three channels, each containing two wells. We conducted experiments in the first of these two wells. The wells were 5.5 mm in diameter and 1.2 mm deep. The use of the wells allowed a constant supply of fresh nutrients, without a high shear stress. Media was supplied by the NE-1002X Programmable Microfluidics Syringe Pump and the experiments were conducted at 30 °C. The flow chamber was primed with media prior to the experiment. The resuspended cell culture was then injected into the flow cell which was mounted on the Olympus IX-71 inverted microscope. Cells were given an hour to adhere before the flow was initiated at 10 μl min⁻¹.

DiSC3(5) was made up in 3 mM stocks in Dimethyl sulfoxide prior to use and stored at −20 °C. DiSC3(5) reached mid-exponential phase (OD600 ≈ 0.5). This cell culture was then diluted to OD600 ≈ 0.05 using fresh 1% TSB. Microscope slides were prepared an hour in advance of the cells reaching mid-exponential phase, following the protocol of Jong et al [64].

ROS scavengers
Stocks of both sodium pyruvate and catalase were prepared before the experiments. Stocks were then added to the cell suspension and media half an hour before injection into the flow cell at concentrations of 100 mM sodium pyruvate and 200 U ml⁻¹ catalase. Experiments were all conducted at the same time after initial inoculation to prevent biofilm growth affecting the results.

Microscopy

Microscope set-up
The Ibidi flow cells were mounted on an Olympus IX-71 inverted microscope with an Olympus UPAON 100XOTIRFM (NA 1.49) immersion oil and TIRF objective lens.

Bright field images were acquired using an LED (525 nm) which was focused onto the sample using a condenser lens. For fluorescence imaging the sample was illuminated with an OBIS 405LX, OBIS 488LX or OBIS 647LX laser. Time lapse images were recorded using an ORCA-Flash4.0 LT PLUS Digital CMOS camera (C11440-22CU).

405 nm light treatment
An OBIS 405LX laser focused on the surface of the flow cell was used to treat cells. The irradiance was varied from 70 μW cm⁻² to 750 μW cm⁻². Time lapse images were acquired every 0.25–2 s depending on the responsiveness of the cells.

488 nm light treatment
An OBIS 488LX laser focused on the surface of the flow cell was used to treat cells. Treatment was performed at 480 ± 6 μW cm⁻². Bright field microscopy images were obtained to track the cell number. Fluorescence time lapse images were collected once every 10 s, using the 405 nm laser at an irradiance of 50 μW cm⁻², with an exposure time of 1 s. This resulted in a dose of 405 nm light low enough to prevent hyperpolarisation of the cells.

Membrane potential indicators
ThT is commonly used to stain amyloid fibres [65], however its positive charge allows it to also be used as a Nernstian voltage indicator [23, 37, 39]. ThT was supplied by Sigma-Aldrich. Fresh 2 mM stocks were made up on the day of the experiment and added to the cells and media at a final working concentration of 10 μM. ThT can be excited at 405 nm, allowing it to be used in conjunction with 405 nm light treatment.

DiSC3(5) was made up in 3 mM stocks in Dimethyl sulfoxide prior to use and stored at −20 °C. DiSC3(5) is excited at 488 nm but can be used in conjunction with 405 nm light treatment.
was added to the cell suspension immediately before injection into the flow cell at a final working concentration of 6 µM. *P. aeruginosa* cells were left to incubate and adhere for 20 min before time lapses were conducted. For *B. subtilis* the 20 min adherence and incubation was followed by addition of media containing DiSC3(5) at a final concentration of 9 µM and then another 20 min incubation period prior to imaging. An OBIS 647LX laser at an irradiance of 1.8 mW cm$^{-2}$ was used to excite DiSC3(5), time lapse images were recorded once every second.

Irradiance/dose measurements

The optical power was measured at the sample using a Thorlabs PM121D digital power meter. We found that the power across the area in which we made measurements could be assumed homogenous, so that the irradiance is

$$I = \frac{P}{A},$$  \hspace{1cm} (17)

where $I$ is the irradiance, $P$ is the power at the sample and $A$ is the illumination area. The dose is then given as

$$D = \frac{It}{1000},$$ \hspace{1cm} (18)

where $D$ is the dose in J/cm$^2$, $I$ is the irradiance in mW/cm$^2$ and $t$ is the time of illumination in seconds.

Data analysis

Image analysis was conducted using Image J (National Institutes of Health), MATLAB (MathWorks) and Origin (OriginLab Corporation). Background subtraction was done using the ImageJ ‘rolling ball’ background plugin with a radius of 4–8 µm depending on the experimental condition e.g media. To obtain ThT curves we measured the fluorescence using the ‘plot Z axis’ function on the ImageJ image analysis toolbox. ThT curves were averaged over all the cells in the region of treatment. Signal processing was performed in MATLAB, including signal smoothing using a cubic weighted Savitzky-Golay filter, which removed sinusoidal noise from the pump and instantaneous noise introduced when motile cells passed behind the field of view. The errors presented are standard errors.

In order to perform cell tracking, a bleach correction using a histogram matching method was performed on the time lapse images. Cells were tracked using the ImageJ plugin TrackMate and tracks were then exported to MATLAB where further analysis was performed. Survival analysis (the calculation of residence probabilities) was also performed in MATLAB using the Statistics and Machine Learning Toolbox. Curve fitting and fitting comparisons were performed in Origin.

Mathematical modelling

Our simple Hodgkin–Huxley [45] model consists of a set of coupled differential equations (equations (9)–(16)). Parameters for this model (supplementary table 1) were given physically relevant values where possible, otherwise parameter scanning within an expected range was implemented. Parameter scanning, simulations and plotting were all conducted using custom made MATLAB scripts.

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Author contributions

JB conducted experiments, analysed data, performed simulations and wrote the manuscript. TW and IR supervised the project and helped shape the research, analysis and manuscript.

Competing interests

The authors declare no competing interests.

Availability of materials and data

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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ORCID iDs

J A Blee https://orcid.org/0000-0001-6873-0841

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