

Dynamic velocity response of *E. coli* powered by proteorhodopsin

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ABSTRACT *Escherichia coli* swimming motility is powered by the flagellar motor, a rotary nanomachine driven by inward proton flux through its torque-generating stators. How these proton currents arise from proton motive force is often described using a simple circuit model, in which the membrane acts as a capacitor discharging through the flagellar motors and other resistive proton channels. By monitoring the swimming activity of *E. coli* expressing a light-driven outward proton pump, we probe the dynamical response of the system under tunable optical driving and test the limits of simplified circuit-based description. Our results show that the flagellar motors are not the main sink for proton motive force discharge. Instead, other membrane channels carry a larger proton current and exhibit a nonlinear resistive behavior. Using the same experimental approach, we directly quantify proteorhodopsin pumping activity as a function of illumination wavelength and compare it with previously reported absorption spectra.

WHY IT MATTERS Proteorhodopsin, a well-known light-activated proton pump, enables precise regulation of bacterial motility. Here, we present a systematic and quantitative analysis of light-driven *E. coli* propulsion, examining how response time and amplitude depend on illumination intensity and wavelength. We use our measurements to test an electrical circuit model for the proton motive force, providing insights into proton fluxes through flagellar motors while simultaneously revealing the model's limitations.

INTRODUCTION

Proteorhodopsin is a light-driven outward proton pump that has been found in marine microorganisms (1), and it is believed to be responsible for most of the total energy harvest from light (2). Its presence becomes vital in energy-starving bacteria (3,4) where it can boost growth (5,6) or reduce the consumption of internal resources (7). If the flagellar motors are driven by an inward flow of protons, as in the case of *Escherichia coli*, the proteorhodopsin creates a proton electrochemical gradient that can be readily used.

E. coli does not produce proteorhodopsin endogenously, but it can be easily expressed via plasmid. In a medium rich in oxygen and sugar, cell metabolism provides a large proton motive force (PMF) so that

proteorhodopsin has no significant effect on swimming speed. In the absence of carbon sources (such as glucose) in the surrounding medium, the cell is still able to swim by switching to endogenous metabolism (8–10), albeit at a reduced speed. Endogenous metabolism is aerobic, so it is halted by adding a respiratory poison (3) or by oxygen depletion (11,12). In both cases, the main effect is the inhibition of cytochrome oxidase, an enzyme involved in the respiratory electron transport chain and responsible for the extrusion of protons during respiration (13). When endogenous metabolism is suppressed, the PMF is mainly generated by proteorhodopsin, providing an ideal situation to study the dynamics of PMF response to time-varying light stimuli. In bead assay experiments, motor speed is usually considered as a proxy for PMF (14–17). Although it has recently been reported that motor speed saturates at large PMF when a high load is applied (18), the same work suggests that free swimming occurs at the limit of linear regime. Swimming speed is proportional to

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flagellum rotation frequency as predicted by low Reynolds number hydrodynamics (19) and is experimentally confirmed (20,21). This makes swimming speed a valuable alternative proxy for the PMF, as it enables access to a large cell population within a single field of view, ensuring robust statistical analysis. By tracking individual cells, we achieve a much higher temporal resolution compared with differential dynamic microscopy (11,22,23), which provides velocity measurements averaged over a large population but suffers from poor time resolution.

Here, we investigate the temporal dynamics of the swimming speed response in proteorhodopsin-expressing bacteria exposed to light pulses of varying intensities. We find that when light is rapidly turned on and off, the swimming speed relaxes with characteristic times that are strongly influenced by the instantaneous level of the PMF. This suggests that PMF dynamics cannot be completely described by an electric circuit model where currents and voltages are coupled by linear differential equations with constant coefficients (3,24–27). This phenomenon had not been reported previously, likely because prior studies either focused on small-amplitude PMF modulations (26), examined large-amplitude modulations with limited statistical data (3), or suffered from the limited temporal resolution (11). In addition, we observe no difference in the characteristic time of PMF discharge between swimming cells with partially disassembled motors and those with fully assembled motors, indicating that the motors are not the primary consumers of PMF. Finally, we characterize the cells' velocity response as a function of illumination wavelength.

MATERIAL AND METHODS

Sample preparation

E. coli AB1157 is a motile K-12 derivative strain (28). This strain was engineered to be a smooth swimmer by *cheY* deletion, and ATP synthase was removed by deletion of the *unc* operon. Both deletions were obtained through λ -Red recombination, as described respectively in (29) and (30). All strains were equipped with SAR86 γ -proteorhodopsin, expressed using the pBAD-His C expression system (4). Bacteria from frozen glycerol stock were streaked on a Petri dish containing 1.5% agar, lysogeny broth (LB: 1% tryptone, 0.5% yeast extract and 0.5% NaCl), and antibiotic. A single colony was inoculated into LB plus ampicillin antibiotic (100 μ g/mL) and grown overnight in a shaking incubator at 30°C and 200 rpm. The overnight culture is diluted 100-fold into 5 mL of LB plus antibiotic, grown at 30°C, 200 rpm. 5 mM arabinose and 20 μ M retinal are added once OD $\approx$ 0.3, keeping the culture in the dark to avoid retinal degradation. At OD $\approx$ 0.6, cells are collected by centrifugation (1300 rcf, 5'). The resulting pellet is washed twice by centrifugation (1300 rcf, 5') with purified water. The bacterial concentration is eventually adjusted to OD $\approx$ 3; 0.02% of Tween-20 was also added to the suspension to minimize the cell adhesion to the cover glass. The

sample was prepared by filling a 50- μ m-thick chamber made by a glass slide and a cover glass glued together with a UV photore-sist (Norland Optical Adhesive 81). After loading the chamber with bacteria, we seal it using the same UV photore-sist. Before filling and sealing the chamber to isolate the sample from air, we measure the suspension's pH, which is typically between 6 and 7. At our cells' concentrations, after about 20 minutes (31), bacteria deplete the oxygen in the sample and do not swim unless the sample is illuminated with visible light.

Experimental setup/cell tracking

Cells are observed using a custom-made dark-field microscope equipped with a monochrome camera (Basler avA1000-100gm Nikon, 5.5 μ m pixel size) and a Nikon Plan Fluor 20 $\times$ (f_o = 10 mm, NA = 0.5) objective. Considering the microscope magnification (M = 20), the pixel pitch is 5.5 μ m / M = 275 nm. The light source for dark-field microscopy is an LED emitting at 730 nm, a wavelength that falls outside the absorption band of proteorhodopsin. To track the cells, we apply a threshold to segment the image and then extract the center of mass of all the detected objects. Velocity is obtained by comparing two subsequent frames. Given the low density of cells in our sample, each object is matched with the closest one in the next frame. Fig. 1 b shows a typical image acquired on our dark-field microscope. The focal plane is close to the cover glass plane. The persistence in the swimming direction and the hydrodynamic interactions result in the accumulation of the cells on bounding surfaces (32,33). Most of the cells swim while aligned with the cover glass plane, so the out-of-plane component of their velocity is negligible. Using a medium with low ion concentration and adding Tween-20 results in only a small fraction of the cells adhering to the cover glass.

The response curves in Fig. 2 were obtained by stimulating proteorhodopsin with a fiber-coupled green LED (Prizmatix UHP-T-LED-520, peak wavelength 520 nm), which was coupled to the microscope objective through a 50:50 beam splitter. The corresponding fitted parameters are shown in Fig. 3. The LED fiber could be easily switched to illuminate the same sample with a red LED (Prizmatix UHP-T-LED-630) or a blue LED (Prizmatix UHP-T-LED-460). The spectra of the three LEDs are shown in Fig. 5 d. For spectral response measurements, we used a white LED (Thorlabs MWWHL2P). The LED light passes through a monochromator (Acton SP-2300), which outputs a dispersed image of the LED's active area. This image is then refocused by a tube lens (f = 150 mm) and the microscope objective onto the sample plane. The dispersion is such that, in the observed field of view, the wavelength resolution is approximately 3 nm.

When the cells are completely de-energized, they are subject to thermal diffusion only. We estimate the diffusion coefficients by computing the displacement distributions along x and y separately and fitting them to a Gaussian. The standard deviation of these Gaussians is expected to satisfy $\sigma^2(t) = 2Dt$, where t is the time interval between the two frames. We repeat the procedure for $t = 0.04, 0.08, 0.16, 0.2$ s and fit $\sigma(t)^2$ to obtain $D = 0.10 \pm 0.01$ μ m²/s, a value comparable to what is expected for freely diffusing flagellated *E. coli* cells (34).

RESULTS

Linear circuit model

The proton electrochemical gradient across the membrane represents one of the major energy

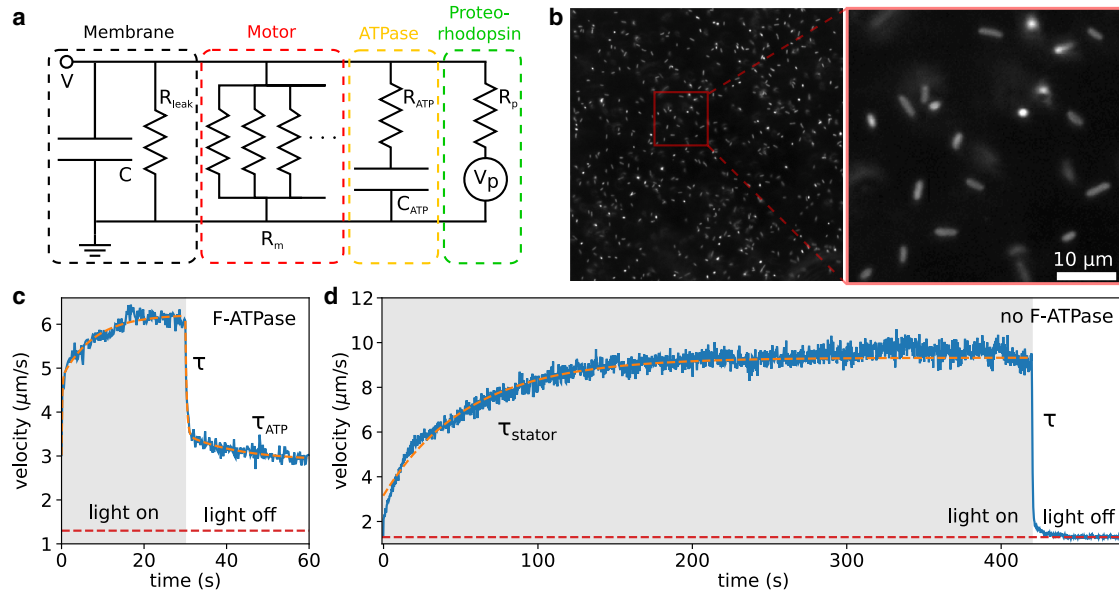


FIGURE 1 Equivalent circuit model and velocity response timescales. (a) An equivalent circuit model representing our cells under conditions of halted metabolism. (b) Cells imaged with dark-field microscopy. (c) Mean velocity of cells expressing ATP synthase. The two timescales τ and τ_{ATP} are associated to the PMF discharge across the membrane and to the consumption of ATP reservoir used to build the proton gradient. The shaded area indicates the period during which green light is on (intensity 23 mW/mm²). (d) Velocity of cells lacking ATP synthase. Cells were kept in the dark for several minutes, leading to the disassembly of their stator units from the flagellar motors. Upon exposure to green light (intensity 70 mW/mm²), the stator units reassemble, resulting in a recovery of velocity on a timescale of approximately 10² s. The red horizontal line indicates the expected apparent velocity due to Brownian diffusion. Orange dashed lines are fits.

sources of the cell, along with ATP. Inward proton currents correspond to an expenditure of this energy, for example, to generate ATP through F-ATPase, propel the cell through flagellar motors, or to actively

transport molecules across the membrane. At the same time, respiration, proton pumps, and F-ATPase itself produce outward currents that recharge the electrochemical gradient. These

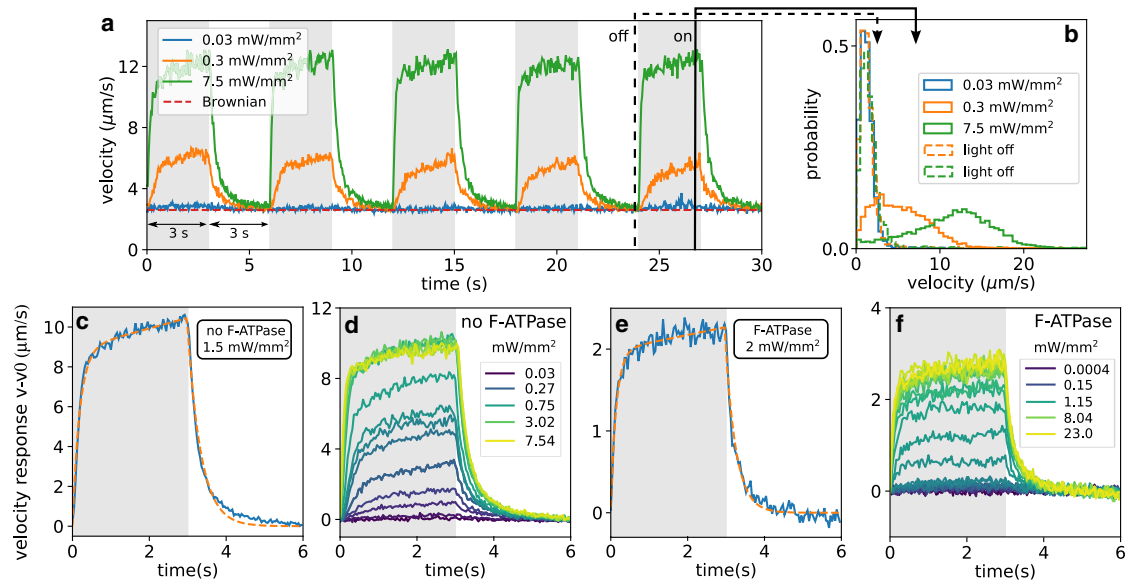


FIGURE 2 Velocity time response. (a) Time traces of the population mean velocity for cells lacking F-ATPase, plotted against time for three different green light intensities. (b) Population velocity distribution corresponding to the curves in (a) at the time instants indicated by black dashed vertical (end of light off sequence) and by black solid line (end of light on sequence). (c and e) Average response curve for cells without and with ATP synthase. In both cases, to obtain the response, the velocity when green light is off is subtracted. Orange line indicates a fit to Eqs. 4 and 5. (d and f) Response curves for cell without and with ATP synthase at various intensities.

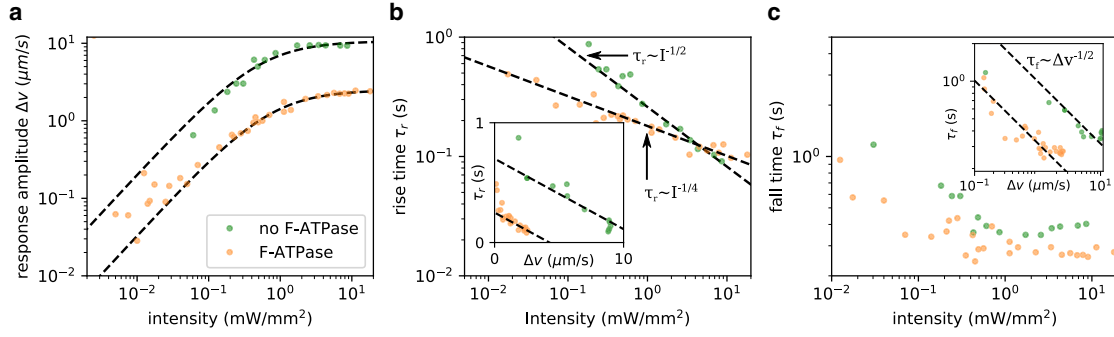


FIGURE 3 Fitted parameters as a function of the green intensity I . (a) Response amplitude Δv , as defined in Eq. 4. (b and c) Rise and fall time as a function of I . Insets in (b) and (c) plot, respectively, the rise and the fall time as a function of Δv . When light is turned off the fall time scales as $\Delta v^{-1/2}$. Dashed lines in (a) are fits to the function $\Delta v(I) = \alpha I / (I + I_{1/2})$, whereas dashed lines in (b) and (c) are guides to the eye.

processes can be modeled using an electrical circuit analogy as shown in Fig. 1 a. The key biological components of the PMF generation/consumption system are represented using linear electrical elements such as resistors and capacitors (3,24–26). For simplicity, we retain only the electric component V from the full expression of the PMF, $V + k_B T \Delta p H / \log(10)$, as the entropic contribution is either negligible or results in only a moderate correction (25). Protons are the charge carrier in this circuit where the largely impermeable dielectric membrane functions as a capacitor C interposed between the cytoplasm and the periplasmic space. The flagellar motors act as a passive resistive element R_m , allowing a proton current to flow in proportion to the voltage V (which represents the PMF) across its ends. In Fig. 1 a, R_m is represented as a set of parallel resistors, each corresponding to a torque-generating unit. Assuming a tight coupling between proton flux and motor speed, the current through R_m serves as the system's readout. To account for the presence of any trans-membrane proteins involved in proton transport and residual membrane permeability, we also include a leakage resistance R_{leak} . The F_0 motor of ATP synthase, like the flagellar motor, uses the PMF to drive rotation, whereas the F_1 motor rotates in the opposite direction via ATP hydrolysis. When the PMF is strong, F_0 dominates and drives ATP synthesis. If the proton gradient is weak, F_1 takes over and hydrolyzes ATP to pump out protons. An electric analog of this reversible component is the R_{ATP} , C_{ATP} series where the current can flow in both directions depending on the balance between V and $V_{\text{ATP}} = Q_{\text{ATP}} / C_{\text{ATP}}$. Although it is ATP that is actually stored, we measure it in proton currency Q_{ATP} , which is the proton charge that can be pumped out by consuming stored ATP. When present, metabolism can be modeled by adding an imperfect battery to the circuit (24,25). In our case, where metabolism is halted, this element

can be omitted, as shown in the scheme in Fig. 1 a. Finally, we have proteorhodopsin, a light-driven current source that can be modeled as a voltage generator V_p with an internal resistor R_p , both in principle controlled by light.

Within this model, we assume that swimming speed v is proportional to motor speed, which in turn is proportional to motor current, leading to $v \propto V / R_m$. In the steady-state $V = V_p R / (R + R_p)$ with R being the parallel between the motor and leak resistances $R = (1/R_{\text{leak}} + 1/R_m)^{-1}$, and thus the speed is

$$v \propto \frac{V_p}{R_m} \frac{R}{R + R_p} \quad (\text{Eq. 1})$$

When all the resistive and capacitive elements in the circuit are constant, changes in the input voltage V_p , whether stepping up or down, will always result in a motor current response that relaxes with a double exponential form. If $C_{\text{ATP}} \gg C$, we have two well-separated timescales, a fast one (τ) and a slow one (τ_{ATP}):

$$\tau = C \left(\frac{1}{R} + \frac{1}{R_p} + \frac{1}{R_{\text{ATP}}} \right)^{-1}, \quad (\text{Eq. 2})$$

$$\tau_{\text{ATP}} = R_{\text{ATP}} C_{\text{ATP}} \left(1 + \frac{R'}{R_{\text{ATP}}} \right), \quad (\text{Eq. 3})$$

where $R' = (1/R + 1/R_{\text{ATP}})^{-1}$. The assumption $C_{\text{ATP}} \gg C$ can be justified considering that the cytoplasmic ATP concentration is of the order of 1–10 mM (35–37), corresponding to an estimated minimum of 2×10^5 molecules, for a cell approximated as a $1 \mu\text{m} \times 1 \mu\text{m} \times 2 \mu\text{m}$ parallelepiped. Each ATP molecule can be used to pump four protons (38), so the ATP reservoir corresponds to 1.2×10^6 protons. Thus, for a fully charged cell, we have $C_{\text{ATP}} = Q_{\text{ATP}} / V \approx 1.2 \times 10^6 \times e / (150 \text{ mV}) \approx 1 \text{ pF}$, a value that is much larger than the membrane capacitance

$C \approx 0.1$ pF. The latter can be estimated by multiplying the capacitance per unit area, which is about $1 \mu\text{Fcm}^{-2}$ (39,40), by the cell membrane area, which is $10 \mu\text{m}^2$.

The double exponential form predicted by the circuit is visible experimentally as shown in Fig. 1 c. When a green LED is turned on and illuminates the cells, the mean velocity increases rapidly, followed by a slower rise. Upon turning off the green light, both a fast and a slow timescale are again apparent. During both the charging and discharging phases, the velocity response can be fitted with the sum of two exponentials: $v(t) = c_0 + c_1 e^{-t/\tau} + c_2 e^{-t/\tau_{ATP}}$. The two fits are reported as a dashed line in Fig. 1 c. Fitted values of τ_{ATP} are 7.8 s and 13 s during charge and discharge, respectively; the fast timescale τ is of the order of 0.1 s and will be discussed in detail in the next section.

An additional response on the timescale of 100 s, due to the stator dynamics, is also present. Torque-generating units, also called stators, can bind to or unbind from the motor (41) with a rate that depends on how much torque they generate (42). As a result, a sudden change in the PMF is followed by a change in the number of stators that occurs on a timescale of about 1 min (4). As described in (42), the relaxation rate k is determined by the sum of the binding and unbinding rates, which for this is $k \approx 0.004 \text{ s}^{-1}$. Consistently, a rate $k = 0.009 \pm 0.006 \text{ s}^{-1}$ was directly measured for swimming cells (43). In both cases, this corresponds to a relaxation time for stator dynamics of about 100 s, which is significantly longer than the period of light modulation and the fast components associated with PMF charge and discharge. Fig. 1 d shows the velocity of cells lacking F-ATPase, which were kept in the dark for several minutes, allowing most of their stator units to disassemble. Upon illumination, the PMF is restored, leading to only a tiny, sharp increase in velocity. Subsequently, the stator units reassemble, resulting in a gradual velocity recovery that is fitted by an exponential function with a time constant of $\tau_{\text{stator}} = 71$ s. In the absence of F-ATPase, once stators are assembled, the short-term velocity response exhibits a larger amplitude. When the green light is turned off, the velocity drops rapidly (on a timescale associated with membrane discharge) to a background apparent velocity v_0 , which arises from Brownian motion. This apparent velocity is expected to be approximately $\sqrt{4D/\delta t}$, where $\delta t = 0.2$ s is the inverse of the frame rate used for both Fig. 1 c and d. Using a diffusion coefficient of $D \approx 0.1 \mu\text{m}^2/\text{s}$, this yields an apparent velocity of $1.3 \mu\text{m}/\text{s}$, consistent with the observed value of v_0 .

Swimming cell velocity response

In the previous section, we discussed that the slow components of the speed response arise from stator dynamics and from the F-ATPase, which can both consume ATP to generate PMF and operate in reverse (38,44). Here, we focus on the short-time response using light modulations faster than the characteristic timescales of stator dynamics and F-ATPase activity. To further remove slow dynamics components in the PMF response, we primarily use a strain in which the F-ATPase has been deleted. Under fast light modulation conditions, the number of engaged stators remains effectively constant, and the motor speed is expected to be proportional to the PMF (14,16,17). In this regime, proteorhodopsin serves as a controllable input that sets the PMF, whereas the swimming speed of the cells provides a direct and accessible output. We first illuminate the sample for a few minutes with a high light intensity, as in Fig. 1 d, to ensure that the cells start from a state in which the number of engaged stator units is at saturation. During acquisition, a green LED illuminates the sample for 3 s and then turns off for 3 s. This on-off sequence is repeated five times. We then repeat the measurement across a range of different LED powers, which are controlled by pulse width modulation. The initial condition is reset by illuminating the sample at full intensity (100% duty cycle in pulse width modulation) for 30 s before switching to a new light intensity to avoid significant changes in stator number during the entire light intensity scan. Throughout transients, the velocity response may not be proportional to the motors speed and thus to PMF, as the dynamics of bundle formation may also play a role. However, direct visualization of fluorescently stained flagella in cells (21) reveals a linear relationship, provided that the light is turned off for a few seconds, a duration too short for thermal fluctuations to cause each flagellum to adopt a random orientation relative to the cell body (21).

Fig. 2 a shows the mean velocity modulus as a function of time for three different green LED intensities in cells lacking F-ATPase. The corresponding velocity distributions are plotted in Fig. 2 b. For all subsequent measurements investigating the short-time response, the frame rate is consistently 25 fps. This results in a background velocity for nonswimming cells of approximately $2.8 \mu\text{m}/\text{s}$ (calculated using $D \approx 0.1 \mu\text{m}^2/\text{s}$ and $\delta t = 0.04$ s). The velocity curve in Fig. 2 a is smoothed by taking the mean over the five repetitions. Also, we subtract to velocity curve the background velocity v_0 and thus obtain the velocity response $v(t) - v_0$ curve shown in Fig. 2 c. A similar curve is plotted in Fig. 2 e for a strain in which ATP

synthase has not been removed. In this case, v_0 is not solely due to Brownian motion; instead, there is a residual motility of a few micrometers per second supported by the presence of ATP. After light is turned on, both curves display a fast rise, but instead of reaching a stable plateau, there is a slow increase in time. We fit the response to the rising/falling step to the following functions:

$$(\text{rise}) \ v(t) = \Delta v [1 - e^{-t/\tau_r}] + \beta_r t + v_0 \quad (\text{Eq. 4})$$

$$(\text{fall}) \ v(t) = [v(0) - v_0] e^{-t/\tau_f} - \beta_f t + v_0 \quad (\text{Eq. 5})$$

The drifts $\beta_r t$ and $\beta_f t$ account for the slower response, which, in the absence of ATP synthase, is due to the dynamics of MotAB stator units, a phenomenon observed at the single-motor level when the PMF is removed and restored (4). At the population level, the average number of stators, and thus the speed, follows an exponential trend, with a characteristic time $\tau \approx 100$ s (43). As discussed in the previous section, when F-ATPase is present, the slow response results from ATP synthesis/consumption that also leads to an exponential response with $\tau \approx 10$ s. On the timescale of Fig. 2, these exponentials are approximated by the linear drifts in Eqs. 4 and 5.

Fig. 2 d and f shows the velocity response curves at various intensities for strains without and with ATP synthase. For each illumination intensity, we fit the response curves and plot in Fig. 3 the parameters Δv , τ_r and τ_f as green dots and orange dots for the cells without and with F-ATPase respectively. When F-ATPase is absent, at steady state, the PMF remains constant and is supplied by the proton-pumping proteorhodopsin. This is reasonably proportional to the light intensity I , for low I , but it saturates at large intensities. A simple function that interpolates between these two regimes is $I/(I_{1/2} + I)$, where $I_{1/2}$ is the intensity at which the PMF reaches half of its maximum value. It then follows that the cell velocity is $\Delta v = \alpha I/(I_{1/2} + I)$, with α being a constant. This behavior well reproduces the experimental green points of Fig. 3 a. The same trend is observed when F-ATPase is present. The fitted $I_{1/2}$ values, i.e., the intensity for which the velocity is the half of the maximum, are 0.62 mW/mm² and 0.42 mW/mm² for the cells with and without F-ATPase, respectively. The rise time τ_r and fall time τ_f are plotted as a function of the illumination intensity I in Fig. 3 b and c. The rise time is strongly dependent on illumination, with $\tau_r \sim I^{1/2}$ when F-ATPase is absent, whereas $\tau_r \sim I^{1/4}$ when F-ATPase is present. The fall time also decreases sensibly as the illumination intensity increases with a limit value reached for $I \gg I_{1/2}$

where the velocity response is saturated. Reasonably, the fall time does not depend directly on the light intensity during the charge but rather on the state of the cell at the moment the light is turned off. A simple behavior is visible in the inset in Fig. 3 c that shows τ_f plotted as a function of the velocity Δv . In both cases the points follow roughly the trend $\tau_f \sim \Delta v^{-1/2}$. Using differential dynamic microscopy, it was reported that the charge/discharge times are of the same order and independent on the light intensity (11). Subsequently, due to the better time resolution provided by the microsphere assay, it was reported that the discharge time is slightly longer than the charge time (26). The latter experiment, however, only probed a small-amplitude modulation, and variation of response time with light intensity is not reported.

Let us consider Eq. 2 and try to interpret the above measurements within the framework of the equivalent circuit. As the light intensity approaches zero, we must send R_p to infinite to prevent current from flowing through the proteorhodopsin branch. This results in $\tau_f > \tau_r$, as already observed in (26) and confirmed here in Fig. 3. Additionally, when ATP synthase is removed ($R_{ATP} \rightarrow \infty$) we should observe an increase in both the rise and fall times. A prediction that is also supported by the data shown in Fig. 3. The total motor resistance R_m can be estimated as follows. Each single motor applies a torque of 0.4 pN μ m to a flagellum that rotates at 100 Hz (21) resulting in a mechanical power $P = 2\pi \times 100 \text{ Hz} \times 0.4 \text{ pN}\mu\text{m}$. Assuming a motor efficiency close to 1, the mechanical power $6P$ generated by an average number of six flagella will be equal to the electric power V^2/R_m so that $R_m = V^2/6P = 1.5 \times 10^{13} \Omega$, where we assumed $V \simeq 150$ mV; this value is line with the estimate given in (11). When the F-ATPase is absent, if one assumes $R_{\text{leak}} \gg R_m$, the fall time would be $\tau_f = CR_m \sim 1.5$ s, a value lying at the higher edge of the range observed in Fig. 3 c. This suggests that the motor is not the main proton sink during discharge. Indeed, in the next section, we show that the consumption of the PMF by the motors is only marginal if not negligible. In circuit-model terms, this corresponds to $R_{\text{leak}} < R_m$.

Thus far, we have shown how the linear circuit model can be used to interpret several key features of the complex speed response. When the F-ATPase is absent, during discharge the current can only flow through R_m and R_{leak} so that $\tau_f = CR$. Both C and R cannot depend directly upon the light intensity. Moreover, in a linear model, these values are fixed coefficients not depending on the instantaneous value of the voltage. As a consequence, PMF should relax

with a fixed τ_f . In contrast, we observe that τ_f has a marked dependence on Δv , which is a proxy for cellular PMF Fig. 3 c.

Discharge time is weakly dependent on stator numbers

Fig. 4 a shows the velocity of cells lacking F-ATPase. Similarly to Fig. 1 d, we begin with a sample that has been kept in the dark for several minutes, resulting in cells with disassembled motors. We then illuminate the cells with a dim intensity of $I = 0.15 \text{ mW/mm}^2$ (this value can be compared with the knee intensity $I_{1/2} = 0.62 \text{ mW/mm}^2$ obtained from the response amplitude $\Delta v(I)$ shown in Fig. 3 a), which leads to a slow exponential recovery of the velocity as the motors partially reassemble. When the green light intensity is subsequently increased, we observe both a rapid rise in velocity due to the sharp increase in PMF and a slower exponential increase indicating that the number of bound stators is converging to a new steady-state value. Fig. 4 b shows a similar experiment in which, in the final step, the intensity is decreased back to 0.15 mW/mm^2 . In this case, we observe the opposite behavior: a sharp drop in velocity due to PMF loss, followed by a slower exponential decrease reflecting partial motor disassembly. Both for upward and downward intensity steps, the velocity can be fitted to the function $v(t) = c_0 [1 - c_1 e^{-t/\tau}] [1 - c_2 e^{-t/\tau_{\text{stator}}}] + c_3$ plotted as orange dashed lines. Since v is proportional to the PMF, we interpret the first exponential term in the parentheses as the PMF response, whereas the second represents a phenomenological contribution associated with the fraction of assembled stator

units. The two terms appear multiplicatively because the velocity is zero if either the PMF is absent or no stators are bound to the motor. For assembling motors (upward intensity steps), we obtain a characteristic timescale of $\tau_{\text{stator}} \approx 90 \text{ s}$, whereas for motors that are partially disassembling, as in Fig. 4 b, we find $\tau_{\text{stator}} \approx 50 \text{ s}$. Thus, the assembly and disassembly time constants differ substantially, even at the same light intensity of 0.15 mW/mm^2 . Modeling stator dynamics remains an open problem. Although several insightful studies have appeared in recent years (4,42,45–49), a coherent understanding is still lacking.

In Fig. 3 c, we showed that τ_f depends on the initial charge. Here, we aim to determine how τ_f changes as the number of stators bound to the motor varies. To do so, we first illuminate the cells with high intensity to maximize the number of active stator units. We then switch off the light, allowing the stators to begin unbinding from the motor. At intervals of 6 s, the light is turned on for 1 s to fully recharge the PMF. As shown in Fig. 4 c, each time the green light is turned on, the velocity reaches a progressively lower value because the number of active stators decreases. The gray dashed line in Fig. 4 c serves as a guide to the eye.

For each iteration, we fit the discharge to Eq. 5 and extract τ_f . Fig. 4 d plots the PMF discharge time as a function of the velocity Δv immediately before the light is turned off. Each color corresponds to data from an independent experiment similar to the one shown in Fig. 4 c, whereas the black line represents the mean value of τ_f for points within a given Δv interval. With the exception of the leftmost points, the discharge time τ_f remains constant over nearly one

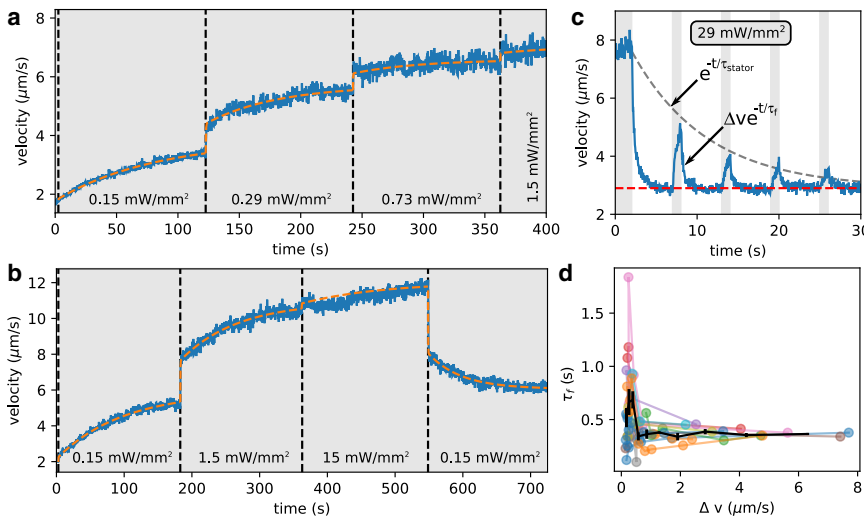


FIGURE 4 Velocity response and motor assembly/disassembly. (a and b) Velocity of cells lacking F-ATPase. The vertical dashed line marks the moment when the green light intensity is switched. The sharp changes in velocity reflect variations in PMF, whereas the slower exponential relaxations are attributed to stator dynamics. (c) Motor disassembly when the light is turned off (*unshaded regions*) is probed by briefly illuminating the cells for 1 s at 29 mW/mm^2 (*shaded regions*). The red horizontal line indicates the apparent velocity expected from Brownian diffusion. The gray dashed line is a guide to the eye and corresponds to an exponential fit with $\tau_{\text{stator}} = 9.8 \text{ s}$. (d) Fitted fall times τ_f as a function of Δv obtained from discharges with a decreasing number of bound stator units. Each color corresponds to data from an experiment of the type shown in (c). The black line and error bars represent the mean and standard error of τ_f taken over small intervals in Δv .

decade in Δv . Since Δv here is modulated only by the number of active staters, we conclude that PMF consumption by the motors is marginal compared with that through other channels. By averaging the plateau in Fig. 4 d, we obtain $\tau_f = 0.37$ s. From this value, we compute $R = \tau_f / C = 3.7 \times 10^{12} \Omega$, where R is the effective parallel resistance of R_{leak} and R_m . To keep τ_f constant as the motors disassemble, R_{leak} must dominate the parallel ($R_{\text{leak}} \ll R_m$), which implies $R_{\text{leak}} \approx R$.

Spectral response

For the strain lacking ATP synthase, we measure the response using also a blue (450 nm) and a red (630 nm) LED to excite the proteorhodopsin. The three LEDs could be mounted rapidly so that the responses are measured on the same sample. The three LED spectra are plotted in Fig. 5 d along with the absorption spectrum for the purified Bac31A8 proteorhodopsin as reported in (1). Given a certain intensity I , to obtain the number of absorbed photons, we multiply it by a dimensional factor O that takes into account the overlap between the spectra. Starting from the LED spectrum $S(\lambda)$, we compute the number of photons $N(\lambda)$ contained in a wavelength interval $d\lambda$ by dividing $S(\lambda)$ by the photon energy hc/λ and then normalize it to one. Calling $A(\lambda)$ the proteorhodopsin

absorption spectrum, we compute the prefactor $O = \int d\lambda N(\lambda) A(\lambda)$ that, once multiplied by the intensity, gives us a quantity proportional to the number of absorbed photons per unit time. In the case of the green LED the intensity is multiplied by $O^{\text{green}} = 0.895$. The velocity response Δv and the fall/rise time $\tau_{f,r}$ with blue and red illumination are plotted in Fig. 5 a–c along with the ones measured using the green LED. Green and blue data points in Fig. 5 a–c follow the same curve so that we can conclude that proteorhodopsin makes no difference between an absorbed blue or green photon. Differently, data relative to the red LED do not follow the trend in Fig. 5 a–c so that O^{red} appears to be overestimated approximately by a factor 10. A similar discrepancy persists even when O^{red} is calculated using the spectra reported in (50). It has been measured that the absorption spectrum shifts toward blue as the environment becomes more basic (51,52). Therefore a possible explanation for our overestimate of the absorption in the red tail could be due to the cytoplasmic pH, which could be more alkaline with respect to that of the purified protein solution used in (1,50).

To measure the effective spectrum, we measure the amplitude of the response Δv as a function of the wavelength. We use a monochromator to separate the wavelength components of a white LED. In the

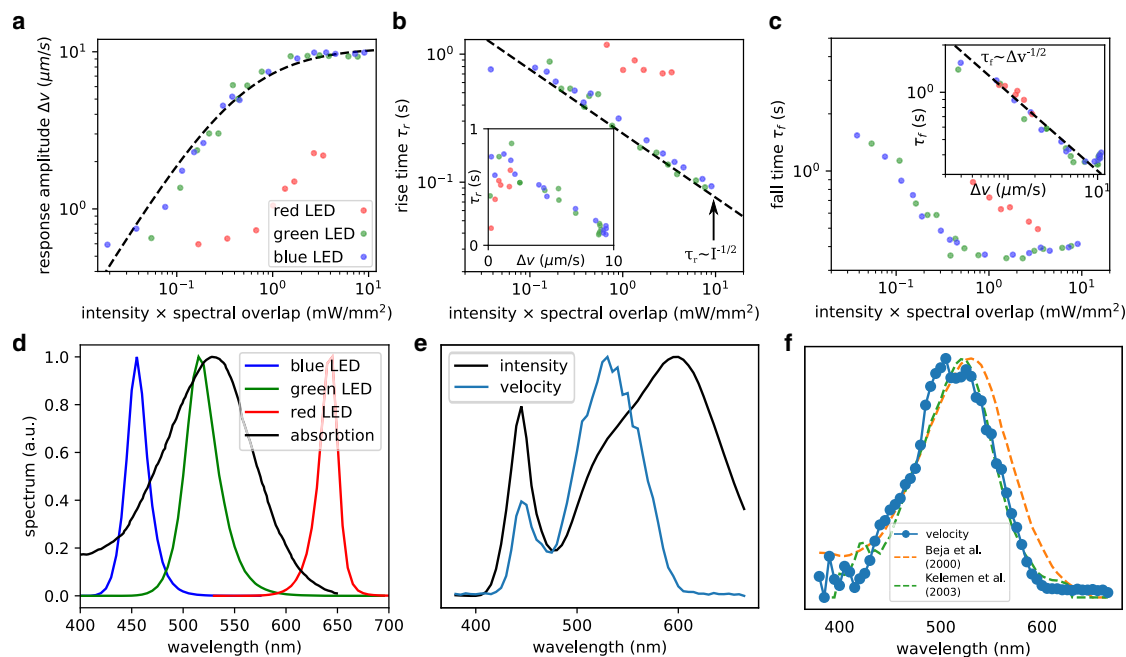


FIGURE 5 Spectral response. (a–c) Fitted parameters as a function of intensity for cells lacking ATP synthase, stimulated with red, green, and blue light. The intensity I is multiplied by the overlap O between the LED emission and the absorption spectrum, so that the x-axis is proportional to the number of absorbed photons. (d) Blue, green, and red LED spectra used to obtain the curves in (a)–(c) along with the proteorhodopsin absorption spectrum (1). (e) Black line plots the light intensity $I(\lambda)$ of a white LED as a function of the wavelength selected by a monochromator. Cell velocity response $\Delta v(\lambda)$ as a function of the illumination wavelength is plotted by blue line. (f) Velocity response per unit intensity $\Delta v(\lambda)/I(\lambda)$ plotted as blue data points. Dashed orange and green lines plot the absorption spectrum of the same proteorhodopsin (Bac31A8) as reported in (1) and (50).

observed field of view the wavelength dispersion is about 3 nm (the latter estimated given the grating dispersion and slit aperture). Fig. 5 e plots the intensity $I(\lambda)$ on the sample at a given wavelength. The corresponding response $\Delta v(\lambda)$ is also plotted in the same figure. Given the low intensity values of $I(\lambda)$, we can safely assume to be in the range where $\Delta v \propto I$. Therefore, we can obtain effective spectral response by simply computing $\Delta v(\lambda)/I(\lambda)$ as plotted in blue dots in Fig. 5 f. Our response spectrum peaks at 517 nm, which is compatible with that reported in (50).

CONCLUSIONS

We characterized the velocity response of *E. coli* cells powered by light through proteorhodopsin. The equivalent circuit model, commonly used to describe membrane charging and discharging in bacterial motility (3,24–27), provides a useful qualitative framework for interpreting our observations. By observing a constant discharge time at fixed PMF while the number of stators bound to the motor decreases, we conclude that the motor is not the primary sink for protons ($R_{\text{leak}} \ll R_m$). When the number of active stators is held constant, the linear circuit model fails to capture the observed behavior: although the model predicts a constant $\tau_f \approx CR_{\text{leak}}$, we observe that τ_f decreases as cells are increasingly charged, suggesting that the current flowing through R_{leak} depends nonlinearly on the PMF. Finally, by measuring the response as a function of illumination wavelength, we quantify the proteorhodopsin pumping activity, which is in good agreement with previously reported absorption spectra (1,50).

DATA AND CODE AVAILABILITY

The data sets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

S.B. and R.D.L. designed the research. S.B. carried out experiments. S.B. and G.D. analyzed the data. S.B., G.D. and R.D.L. wrote the manuscript. G.F. and M.C.C. constructed the bacterial strains.

DECLARATION OF INTERESTS

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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