Taking Control of the Flagellar Motor

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ABSTRACT

Numerous types of bacteria swim in their environment by rotating long helical filaments. At the base of each filament is a tiny rotary motor called the bacterial flagellar motor. A lot is already known about the structure, assembly and function of this splendid molecular machine of nanoscopic dimensions. Nevertheless many fundamental questions remain open and the study of the flagellar motor is a very exciting area of current research. We are developing an *in vitro* assay to enable studies of the bacterial flagellar motor in precisely controlled conditions and to gain direct access to the inner components of the motor. We partly squeeze a filamentous *E. coli* bacterium inside a micropipette, leaving a working flagellar motor outside. We then punch a hole through the cell wall at the end of the bacterium located inside the micropipette using a brief train of ultrashort ($\sim$ 60 fs) laser pulses. This enables us to control the rotation of the motor with an external voltage (for at least 15 minutes). In parallel, new methods to monitor the speed of rotation of the motor in the low load (high speed) regime are being developed using various nanoparticules.

Keywords: motility, Escherichia coli, molecular motors, bacterial flagellum, flagellar motor, femtosecond laser ablation, laser nanosurgery, semiconductor quantum dots

1. INTRODUCTION

Motility, the ability to move around in one's environment, is critical for most living cells. Like most other swimming bacteria, *Escherichia coli* (*E. coli* in short) swims by rotating helical filaments that can be up to 10 µm long.[1] The body of *E. coli* (about 1 µm in diameter by 2 µm long) is randomly covered with many filaments (average of about 4),[2] and each one is driven at its base by a rotary motor called the bacterial flagellar motor (as illustrated in Fig.1). The bacterial flagellar motor is one of only a few biological motors that are known to be rotary engines, and it generates a force far greater than any other molecular motor.[1,2] With a diameter of less than 50 nm, the flagellar motor is a nanotechnological marvel spanning the entire membrane (inner and outer layers). This amazing machine is made of $\sim$20 different kinds of proteins, and it requires 40-50 genes for its expression, assembly and control.[3]

The energy source for this motor is the proton flux across the membrane (there is no direct ATP-involvement[4]). The work that can be done by a proton diffusing from the outside to inside of the cell is called the protonmotive force (the sum of the electrical and pH difference). *E. coli*'s flagellar motors can spin at a speed up to $\sim$ 350 Hz (depending on the load) in either the counterclockwise (CCW) or clockwise (CW) direction. This allows the cell to perform a random walk in three dimensions. When all the motors of a cell rotate CCW, all its filaments bundle together and push the cell in one direction at about 30 µm/s – the cell is said to run. When one or many motors reverse their direction of rotation for a small fraction of a second, their filaments leave the bundle and the cell moves erratically in place – it is said to tumble – and later swims steadily again in a new direction. In a process called chemotaxis, receptors on the surface of the cell detect the concentration of molecules of interest (sugars, amino acids, etc.) and, through a remarkably simple chain of biochemical components, control the probability that the cell tumbles and changes its trajectory.[5,6] If the cell is swimming towards a source of nutrients, the probability for a tumble is decreased, i.e., the length of that run is extended. By thus biasing its random walk, *E. coli* actively finds favorable regions in its environment.

A great deal is known about the bacterial flagellum.[3,7–9] Yet many important questions remain unanswered. The details of how the torque is generated are still unknown, and so is the mechanism by which the motor manages to shift abruptly from one direction of rotation to the other. This system is extremely rich, with new aspects continually being discovered, and its study is a very active area of current research.

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Photonics North 2008, Réal Vallée, Michel Piché, Peter Mascher, Pavel Cheben, Daniel Côté, Sophie LaRochelle, Henry P. Schriemer, Jacques Albert, Tsuneyuki Ozaki, Eds., Proc. of SPIE Vol. 7099, 70990F, (2008) · 0277-786X/08/$18 · doi: 10.1117/12.807183

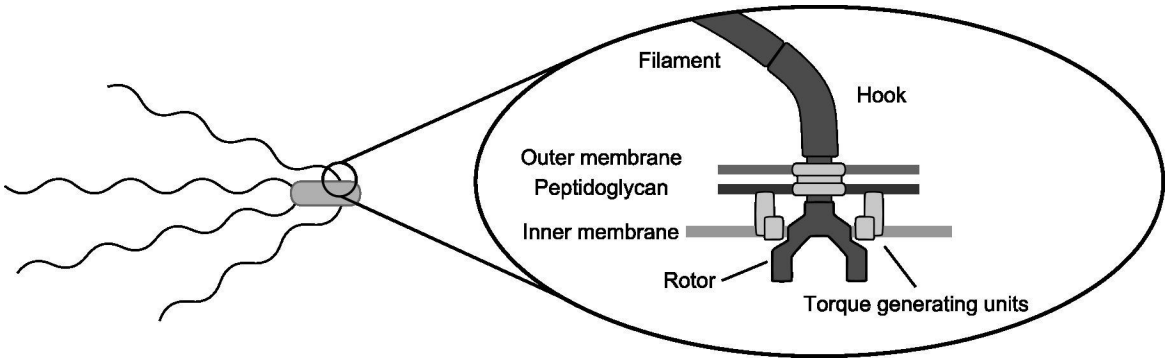

Figure 1. Schematic representation of the bacterial flagellar motor. The bacterium *E. coli* has many flagella randomly distributed on its body. At the base of each filament, a rotary motor of ~ 45 nm in diameter is embedded in the three layers of the bacterium membrane. The filament is linked to the motor by a flexible hook that allows the filament to rotate about an arbitrary axis. The moving parts of the flagellum (the rotor) are colored in dark grey. In light grey are the different rings that anchor the motor in the membrane and the torque generating units (the stator). Protons power the motor by diffusing through the torque generating units where their protonmotive force is converted into torque.

2. *IN VITRO* SYSTEM

2.1 Motivation and description

As described in section 1, the bacterial flagellar motor is a fairly complex machine. Furthermore, it needs to be embedded in the multiple layers of the bacterial membrane to assemble and function properly. That explains why, unlike many other molecular motors, it has not been studied *in vitro*, that is, in an artificial system outside of the living cell. As spectacular studies of linear motors have clearly demonstrated, an *in vitro* system provides the essential control over experimental parameters to achieve the precise study of the motors physical and chemical characteristics. For example, the stepping behavior of kinesin, myosin and dynein has been resolved with *in vitro* systems using optical traps, thus providing much information about these motors mechanochemical cycles and working mechanisms.[10–12] Our goal is therefore to develop an *in vitro* system to study the bacterial flagellar motor.

Our *in vitro* assay improves upon a previous attempt by Fung and Berg.[13] As illustrated in Fig. 2, the system consists of a filamentous *E. coli* bacterium (~ 75 μm long) partly pulled inside a glass micropipette. A constriction matching the cell diameter is made near the tip of the micropipette, thereby insuring a good electrical seal between the inside and the outside portions of the cell. Outside the micropipette, the rotation of one (or many) flagellar motors can be monitored. By compromising the cell membrane inside the micropipette we create the artificial assay we are looking for. In Ref.,[13] the ionophore gramicidin S was used to permeabilize the portion of the cell membrane inside the micropipette. The difficulty of this "chemical permeabilization" was that gramicidin S diffused either around the cell, through the periplasm or within the cytoplasm in a few minutes. This compromised the membrane outside of the micropipette, thereby destroying the electrical seal between the outer and inner parts. To improve on these results, we looked for a way to induce highly localized damage to the cell membrane. That is achieved using the process of laser ablation with ultrashort pulses, also called laser nanosurgery (see section 2.2 below).

Once the membrane inside the micropipette has been perforated, we have complete electrical and physical access to the inside of the cell. This opens the door to easily label components of the flagellar motor with fluorophores, or change the concentration of various molecules that affect the motor's behavior. Moreover the electrochemical potential across the membrane that powers the flagellar motor, can be controlled. By applying a voltage between the inside and outside of the micropipette, we should thus be able to externally modify the rotation speed of the motors.

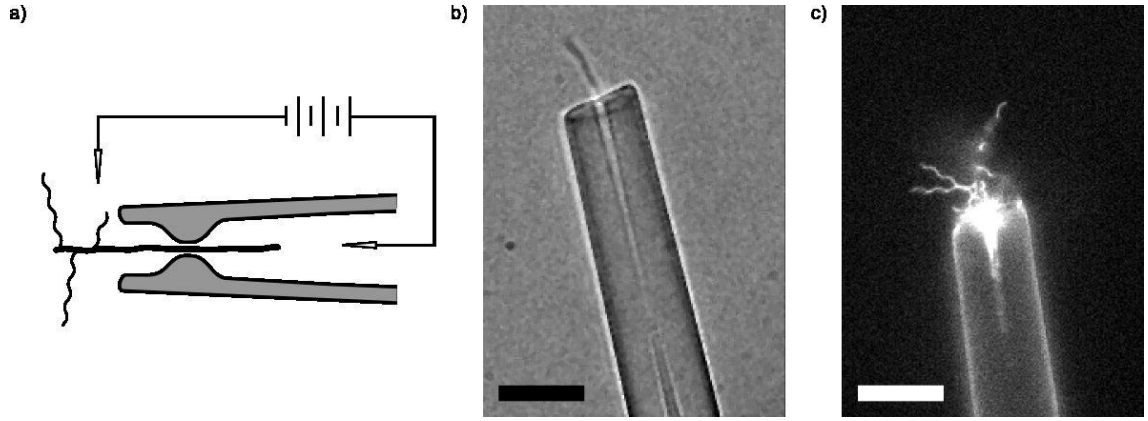

Figure 2. The *in vitro* assay. a) Diagram our *in vitro* assay showing the tip of the micropipette with a filamentous bacterium squeezed in the constriction. To artificially power the motor, an electrical voltage is applied between one electrode back-inserted in the micropipette and a second electrode placed in the bath. b) Brightfield image of a typical micropipette with a bacterium in the constriction. c) Still frame from a movie showing fluorescently labeled filaments whose rotation is under the control of an external voltage. The bright spot at the tip is from all the fluorescent filaments that were stripped as the cell was pulled into the micropipette. Scale bars are 10 μm

2.2 Laser ablation

When pulses from a femtosecond laser are tightly-focused on the part of the bacterium that is located inside the micropipette, a submicrometer-sized hole is vaporized in the wall of the bacterium. The ablation occurs when plasma is produced at the focal spot by a highly non-linear process which allows for an ablation diameter below the diffraction limit (< 300 nm).[14,15] The resulting damage to the biological tissues is a consequence of both the creation of the plasma and of the pressure waves it produces.[16,17] Very little energy is actually deposited in the medium and thus thermal damage to the surrounding biological structures is minimal. That is exactly what we need to preserve the integrity of the flagellar motors at the other end of the cell. We have experimentally characterized the hole made by the laser pulses by directly imaging pierced bacteria using a scanning electron microscope. Images taken both before and after fixation using glutaraldehyde show holes in the membrane of about 200 nm in diameter. In phase contrast microscopy the hole itself is not visible, but it manifests itself as an instantaneous loss of contrast of the cell body upon firing of the laser.

To achieve the desired ablation, we use around 100 pulses from an attenuated NIR femtosecond laser (RegA Coherent, 790 nm, 60 fs pulse duration, 10 kHz repetition rate). Highly localized ablation of biological tissue can be achieved by focusing the laser with a high NA objective (Olympus 100x, 1.3 NA). The energy per pulse before the laser enters the objective is around 10 nJ.

2.3 Experimental details

Our experimental setup, based on a modified Olympus IX71 inverted microscope, is shown schematically in Fig. 3. The femtosecond laser pulses used for ablation are inserted in the optical axis of the microscope with a dichroic mirror, and focused on the sample with the same objective that is used for imaging.

Micropipettes are fabricated using $1,5$ mm external diameter borosilicate glass capillary tubing. The tubes are pulled using a modified two-stage vertical pipette puller (P-10, Narishige) whose parameters are adjusted to achieve an external diameter at the tip around 10 μm. The micropipette is then positioned between two V-shaped, 50 μm diameter platinum wires in a home-made microforge. By slowly heating the micropipette, a constriction with an inner diameter of around 1 μm (matching the outer diameter of the bacteria we study) is produced near the tip. A good relative measure of the diameter is taken using the bubble number.[18] We discovered that the femtosecond laser used for piercing the bacteria (but with about 10 times more energy) can be focused inside of the micropipette to cleanly break it. This is used to shorten the distance between the constriction and the micropipette's tip, allowing us to use shorter bacteria.

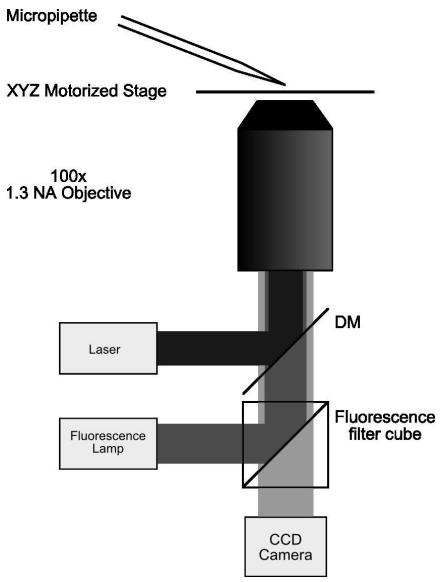

Figure 3. The experimental setup. The femtosecond laser pulses are introduced in the optical axis of our inverted microscope with a dichroic mirror (DM), and then focused on the sample with a 100x high NA objective. The same objective is used to image the specimen in bright field or epifluorescence microscopy onto a CCD camera.

For the experiments reported here, we have used the *E. coli* strain HCB1661 (provided by H.C. Berg). That strain contains a mutated filament protein FliC^{T236C} in which a cysteine was introduced by site directed mutagenesis. This allows specific labeling of the filaments with Alexa Fluor 546 C_5-maleimide (Invitrogen, A10258) that is used to visualize the rotation of the motor with fluorescence microscopy. Cells are grown to mid-exponential phase in Tryptone Broth containing (50 µg/ml) cefalexin, a β-lactam antibiotic that suppresses septation. After incubating with the fluorophores for a few hours, cells are washed and resuspended in motility buffer (10 mM potassium phosphate, pH 7.0, 0,1 mM EDTA, 10 mM lactic acid).

To control the electrical voltage across the bacterium membrane, we use a standard patch-clamp amplifier (Axopatch 200B). The first electrode Ag/Ag-Cl is back-inserted in the micropipette and the second electrode is put in the bath containing our bacteria (see 2a). The amplifier also measures the current flowing between the electrodes from which a measure of electrical resistance can be obtained. The seal between the bacterium's membrane and the glass of the pipette never reaches the giga Ohms (GΩ) that are common for regular patch-clamp experiments. As a quality measure, the electrical seal is continuously monitored and Table 1 shows the typical resistance measured at various stages of an experiment. The electrical seal between the cell and the micropipette is judged to be good when the resistance remains high after laser ablation (compared to when both ends of the cell are pierced).

Table 1. Electrical resistance between the electrode inside the micropipette and the one in the bath in various situations.

Micropipette alone	60 to 80 MΩ
Micropipette with intact cell	300 to 600 MΩ
Drop in resistance after ablation	10 to 40 %
Micropipette with cell pierced at both ends	$\sim$ 100 MΩ

A typical sequence of events for setting up an *in vitro* assay is as follows. A selected bacterium is partly sucked into the micropipette by applying negative pressure via a large syringe. Once in place, the rotation of the bacteriums filaments is confirmed using fluorescence imaging. Electrical measurements are taken to ensure that the seal between the bacteriums membrane and the micropipette is of good quality. A voltage of about -75 mV

is applied before (and during) the laser ablation to avoid defunctionalisation of some to the motors proteins.[19] A mechanical shutter is then opened for 10 ms to allow a burst of femtosecond laser pulses into the microscope and perform laser ablation of a small portion of the cell membrane inside the micropipette. Finally, we vary the applied voltage in order to confirm we have control over the proton-motive force that powers the motors.

3. EXPERIMENTAL RESULTS

Our experimental results using the *in vitro* assay are still preliminary, but show unambiguously that we can take control of the flagellar motor. We have recorded many sequences of images like the one shown in Fig.2c) while alternating the applied voltage between 0 and about -75 mV. The frame rate of our current camera (30 frames per second) is unfortunately not sufficient to provide us with a good measure of the rotation speed (which should be around 100 Hz), but we can identify whether the filament rotates or not. These movies clearly show that turning the applied voltage on and off results in restarting or stopping the flagellar motors. Because of photobleaching, we could not continually observe the system for more than a few minutes. By leaving the fluorescence illumination off for many minutes and then checking whether the rotation could be controlled, we found that the assay is stable for at least 15 minutes. Beyond that time, the electrical seal around the cell is decreasing (as observed in ref.[13]) and the results have not been as reproducible. These results also demonstrate that the hole pierced in the cell with laser ablation is stable and does not reseal, which is what we hoped for on the basis that all three layers of the cell wall (including the rigid peptidoglycan layer) are damaged by the laser.

4. WORK IN PROGRESS

To contribute to the understanding of the bacterial flagellar motor, our *in vitro* assay needs to become quantitative. For example, we want to precisely measure the rotation speed as a function of the applied voltage. In order to achieve this, we are first automating the system and buying a faster and more sensitive camera. Since photobleaching is a serious limitation in the current setup, novel methods to monitor the rotation of the motor are being implemented. We need an assay with a relatively high yield and also a method compatible with filamentous cells that are more fragile than normal bacteria. Our efforts are therefore concentrated on detecting the motion of various nanoparticles (quantum dots, gold nanoparticles, ...) specifically attached to the filament (or the hook). The small size of these probes will allow us to study the regime where the load on the motor is near zero, a regime that has been a lot less studies experimentally, and to investigate the stepping behavior of this motor.[20] To increase the electrical seal resistance and the "lifetime" of a cell preparation, we are trying to embed the tip of the micropipette (with the bacterium) in a partly cured RTV silicone bubble.[21]

5. CONCLUSION

In conclusion, we have recently made significant progress toward the development of an *in vitro* assay to study the bacterial flagellar motor. The system consists of a filamentous cell squeezed into a custom-made micropipette. A stable hole is punched in the cell membrane inside the micropipette using a burst of femtosecond laser pulses. By varying an external voltage applied between the inside and the outside of the micropipette, we have been able to stop and restart the flagellar motors located outside of the micropipette. For these preliminary results, the rotation of the motors was observed using video microscopy of fluorescently labeled filaments. By providing physical access to the inside of the cell and control of the motors' energy source, this assay opens the door to many new experiments. We are confident it will enable us to probe in more details the working mechanisms of the bacterial flagellar motor, and possibly other membrane-bound systems that are difficult to isolate.

ACKNOWLEDGMENTS

We are indebted to Howard C. Berg and members of his laboratory at Harvard University for their support (NIH grant number AI016478). This project was begun by S.R. as a postdoctoral fellow in his laboratory (in collaboration with Aravinthan Samuel). Funding for this work is provided by Natural Sciences and Engineering Research Council of Canada (NSERC) and Fond québécois de la recherche sur la nature et les technologies (FQRNT).

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