TAKING CONTROL OF THE FLAGELLAR MOTOR

BY MATHIEU GAUTHIER, DANY TRUCHON AND SIMON RAINVILLE

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 1µm in diameter by 2µm long) is randomly covered with an average of about 4 filaments [2]. Each one is driven at its base by a rotary motor called the bacterial flagellar motor (as illustrated in Fig. 1). The energy source for this amazing machine of nanoscopic dimensions is the proton flux across the membrane [3]. The work that can be done by a proton diffusing from the outside to the inside of the cell is called the protonmotive force (a combination of the electrical potential and pH difference across the membrane). *E. coli's* flagellar motors can spin at a speed of up to ~350Hz in either the counterclockwise or the clockwise direction. This allows the cell to perform a random walk in three dimensions. 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, they control the probability that a motor reverses its direction of rotation and that the cell changes its trajectory [4,5]. If the cell is swimming towards a source of nutrients, the probability for a change of trajectory is decreased. By biasing its random walk, *E. coli* actively finds favourable regions in its environment.

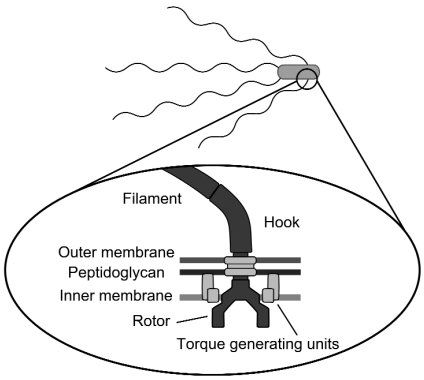

Fig. 1 Schematic representation of the bacterial flagellar motor. The bacterium *E. coli* has many flagella randomly distributed on its body. At the base of each filament, a rotary motor of ~ 45nm in diameter is imbedded in the three layers of the bacterium's membrane. The filament is linked to the motor 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. The different rings that anchor the motor in the membrane and the torque generating units (the stator) are in light grey. Protons power the motor by diffusing through the torque generating units where their protonmotive force is converted into torque.

SUMMARY

Progress towards the development of an *in vitro* system to study the bacterial flagellar motor using an adaptation of the patch-clamp technique and laser nanosurgery.

A great deal is known about the bacterial flagellum [6-9]. 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.

MOTIVATION AND EXPERIMENTAL SETUP

The bacterial flagellar motor is a fairly complex machine; it is made of ~20 different kinds of proteins, and it requires 40-50 genes for its expression, assembly and control [8]. 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 motor's physical and chemical characteristics. For example, the stepping behavior of kinesin, myosin and dynein has been resolved *in vitro* 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 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 (~75µm long) partly squeezed inside a glass micropipette. The micropipette is fabricated so that a constriction with an inner diameter of around 1µm (matching the outer diameter of the bacteria we study) is present near its tip. Outside the micropipette, the rotation of one (or many) flagellar

Mathieu Gauthier <mathieu.gauthier.5@ulaval.ca>, Dany Truchon <dany.truchon.1@ulaval.ca>, Simon Rainville <simon.rainville@phy.ulaval.ca>, Center for Optics, Photonics and Lasers; Department of Physics, Engineering Physics and Optics, Université Laval, QC, Canada, G1V 0A6

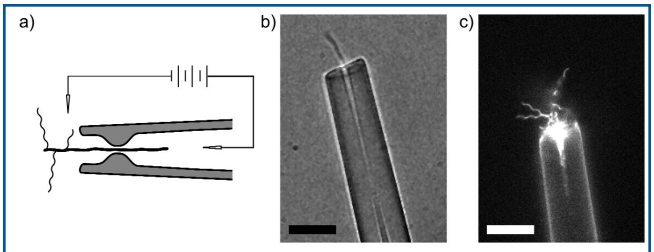

Fig. 2 The *in vitro* assay. a) Diagram of our *in vitro* assay showing the tip of the micropipette with a filamentous bacterium squeezed in the constriction. To artificially power the motor, an electrical voltage is applied between one electrode back-inserted in the micropipette and a second electrode 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 labelled filaments whose rotation is under the control of an external voltage. Scale bars are 10µm.

motors can be monitored. Compromising the cell membrane inside the micropipette creates the artificial assay we are looking for by giving us complete electrical and physical access to the inside of the cell. This opens the door to easy labelling of internal components of the flagellar motor and allows us to expose the motor to known concentrations of various molecules that affect the motor's behaviour. Moreover, the application of a voltage between the inside and outside of the micropipette changes the electrical potential that powers the motor, thereby modifying its rotation speed.

To achieve the localized damage to the cell membrane that our *in vitro* assay requires, we use the process of laser ablation with ultrashort laser pulses. When laser 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 (< 300nm) [14-17]. Very little energy is actually deposited in the medium and, thus, thermal damage to the surrounding biological structures is minimal. In practice, to achieve the desired ablation, we use around 100 pulses from an attenuated NIR femtosecond laser (790nm, 60fs pulse duration, 10kHz repetition rate, ~10nJ per pulse) focused with an 1.3 NA objective. We have experimentally characterized the hole made by the laser pulses by directly imaging pierced bacteria using a scanning electron microscope. Holes in the membrane of about 200nm in diameter were observed.

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. 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 bacterium's filaments is confirmed using fluorescence imaging. A standard patch-clamp amplifier is used to control the electrical voltage across the bacterium's membrane and to measure the current flowing between the electrodes (providing a measure of electrical resistance). A voltage of about -75mV is applied before (and during) the laser ablation to avoid defunctionalisation of some to the motors' proteins [18]. A mechanical shutter is then opened for 10ms 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.

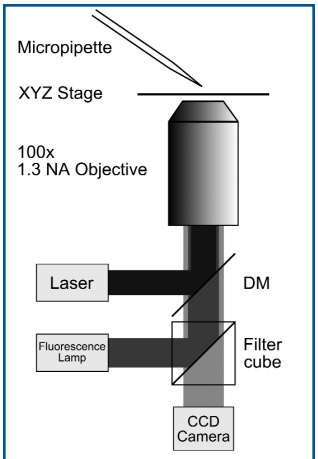

Fig. 3 The experimental setup. The femtosecond laser pulses are introduced in the optical axis of our inverted microscope with a dichroic mirror (DM) and then focused on the sample with a 100x high NA objective. The same objective is used to image the specimen in bright field or epifluorescence microscopy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

As a quality measure, the electrical resistance between the bacterium's membrane and the glass of the micropipette 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 TWO ELECTRODES 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	~ 100 MΩ

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 labelling 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 50µg/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 (10mM potassium phosphate, 0,1mM EDTA, 10mM lactic acid, pH 7,0).

EXPERIMENTAL RESULTS AND WORK IN PROGRESS

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. 3c) while alternating the applied voltage between 0 and about -75mV. These movies clearly show that turning the applied voltage on and off results in restarting or stopping the flagellar motors. 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 [13]) and this compromises our control over the flagellar motors. 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 [19].

To provide us with a quantitative measure of the filaments' rotation speed (which should be around 100Hz), we are buying a faster and more sensitive camera. Another serious limitation of the current setup is photobleaching of our fluorescent probe which limits us to a few minutes of continuous observation. To circumvent this problem, novel methods to monitor the rotation of the motor are being implemented (use of quantum dots, gold nanoparticles, etc.). 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 studied experimentally, and to investigate the stepping behaviour of this motor [20].

CONCLUSION

In summary, we have recently made significant progress towards 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 labelled 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 that 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).

REFERENCES

1. H.C. Berg, "The rotary motor of bacterial flagella", *Annu. Rev. Biochem* **72**, 19 (2003).
2. L. Turner, W.S. Ryu, H.C. Berg, "Real-time imaging of fluorescent flagellar filaments", *J. Bacteriol*. **182**, 2793 (2000).
3. M.D. Manson, P. Tedesco, H.C. Berg, F.M. Harold, C. Vanderdrift, "Protonmotive Force Drives Bacterial Flagella", *PNAS* **74**, 3060 (1977).
4. M. Eisenbach, "A hitchhiker's guide through advances and conceptual changes in chemotaxis", *J. Cell. Physiol*. **213**, 574 (2007).
5. R.B. Bourret, A.M. Stock, "Molecular Information Processing: Lessons from Bacterial Chemotaxis", *J. Biol. Chem*. **277**, 9625 (2002).
6. H.C. Berg, *E coli in Motion*, Springer-Verlag (2004).
7. R.M. Macnab, "How bacteria assemble flagella", *Annu. Rev. Microbiol*. **57**, 77 (2003).
8. F.F.V. Chevance, K.T. Hughes, "Coordinating assembly of a bacterial macromolecular machine", *Nat Rev Micro* **6**, 455 (2008).
9. D.F. Blair, "Flagellar movement driven by proton translocation", *FEBS Lett.* **545**, 86 (2003).
10. K. Visscher, M.J. Schnitzer, S.M. Block, "Single kinesin molecules studied with a molecular force clamp", *Nature* **400**, 184 (1999).
11. A.D. Mehta, R.S. Rock, M. Rief, J.A. Spudich, M.S. Mooseker, R.E. Cheney, "Myosin-V is a processive actin-based motor", *Nature* **400**, 590 (1999).
12. R. Mallik, B.C. Carter, S.A. Lex, S.J. King, S.P. Gross, "Cytoplasmic dynein functions as a gear in response to load", *Nature* **427**, 649 (2004).
13. D.C. Fung, H.C. Berg, "Powering the flagellar motor of Escherichia coli with an external voltage source", *Nature* **375**, 809 (1995).
14. A. Vogel, V. Venugopalan, "Mechanisms of Pulsed Laser Ablation of Biological Tissues", *Chem. Rev*. **103**, 577 (2003).
15. A. Vogel, J. Noack, G. Huttman, G. Paltauf, "Mechanisms of femtosecond laser nanosurgery of cells and tissues", *ApPhB* **V81**, 1015 (2005).
16. A. Heisterkamp, I.Z. Maxwell, E. Mazur, J.M. Underwood, J.A. Nickerson, S. Kumar, D.E. Ingber, "Pulse energy dependence of subcellular dissection by femtosecond laser pulses", *Opt. Express* **13**, 3690 (2005).
17. S. Kumar, I.Z. Maxwell, A. Heisterkamp, T.R. Polte, T.P. Lele, M. Salanga, E. Mazur, D.E. Ingber, "Viscoelastic Retraction of Single Living Stress Fibers and Its Impact on Cell Shape, Cytoskeletal Organization, and Extracellular Matrix Mechanics", *Biophys. J*. **90**, 3762 (2006).
18. D.F. Blair, H.C. Berg, "Restoration of torque in defective flagellar motors", *Sci* **242**, 1678 (1988).
19. C. Gabel, *Ph.D. thesis*, Harvard University (2003).
20. Y. Sowa, A.D. Rowe, M.C. Leake, T. Yakushi, M. Homma, A. Ishijima, R.M. Berry, "Direct observation of steps in rotation of the bacterial flagellar motor", *Nature* **437**, 916 (2005).