Chapitre 2

Single-cell analysis of flagellar filament re-growth after damage by laser ablation

2.1 Avant-propos

L'idée originale de ce projet fut élaborée quelques années avant mon arrivée dans le groupe de recherche par Dr. Kelly T. Hughes ainsi que Dr. Simon Rainville en collaboration avec Dr. Mathieu Gauthier qui était alors en cours de doctorat. Lors d'un séjour à l'université Laval du Dr. Hughes, un premier essai fut tenté avec le laser femtoseconde pour tenter de couper des filaments et de les revisiter par la suite. La courte durée du séjour de même que la grande difficulté technique de ce projet firent en sorte qu'aucun résultat ne fut obtenu. Durant la conférence BLAST de 2011 à Nouvelle-Orléans, nous avons rencontré le Dr. Marc Erhardt, ancien étudiant du Dr. Hughes. Le laboratoire du Dr. Erhardt avait développé auparavant une souche de *Salmonella enterica* ne produisant qu'un ou deux filaments. En utilisant cette souche, nous avons mis au point à l'Université Laval une nouvelle procédure expérimentale qui a mené aux résultats présentés dans ce chapitre sur le mécanisme de croissance du filament bactérien.

Ces travaux ont pour but de répondre à la question suivante : Un filament peut-il continuer de s'assembler après avoir été coupé ou cassé ? Cette question étant encore au coeur de débat au sein de la communauté, nous avons voulu amener une autre perspective en utilisant un faisceau laser à impulsions ultra courtes comme outil pour briser des filaments bactériens. Cette nouvelle méthode a permis d'obtenir des résultats intéressants allant à l'encontre de la littérature existante.

Ce manuscrit est donc la version initiale qui fut présenté à la revue *Scientific Reports*. Après certains commentaires des évaluateurs, des expériences supplémentaires ont été réalisées par

le laboratoire du Dr. Erhardt concernant principalement le *shearing*. D'autres expériences de marquage du *Cap* du filament à l'aide de nanoparticules d'or ont aussi été complétées. Des modifications ont donc été apportées au manuscrit pour ajouter ces éléments et l'article a été resoumis depuis. Dans le but de faire le point sur les expériences réalisées dans notre laboratoire, ce chapitre reproduit le manuscrit original comportant exclusivement le travail que j'ai réalisé au cours de ce doctorat. Ce chapitre est donc rédigé en anglais et sous format article avec le titre original qui a aussi été modifié. Le texte final révisé et comprenant les expériences de nos collaborateurs a été accepté pour publication dans *Scientific Reports* et se retrouve à l'annexe A. Cet article fut publié le 28 avril 2017[41].

2.2 Résumé

Plusieurs types de bactéries peuvent se déplacer dans leur milieu à l'aide de flagelles rotatifs. Les filaments flagellaires peuvent atteindre plusieurs fois la longueur du corps à l'extérieur de la cellule. Ceux-ci sont composés d'un assemblage de plusieurs milliers de protéines identiques appelées flagelline. Ces flagellines sont exportées dépliées dans un canal de sécrétion et sont assemblées à l'aide d'une protéine agissant comme bouchon au bout du filament. Nous répondons ici à la question suivante : Un filament peut-il former un nouveau bouchon (*cap*) et continuer de s'assembler après avoir été endommagé ? D'importants défis techniques ont dû être résolus dans le but de pouvoir conduire les expériences au niveau d'une cellule unique. Les filaments furent visualisés à l'aide de marquages fluorescent et furent individuellement coupés par ablation laser. Après une période de croissance, ces mêmes filaments furent marqués d'un second fluorophore de différente couleur et revisités pour tenter d'observer une reprise d'assemblage. Aucune croissance supplémentaire ne fut observée après l'ablation nous suggérant que l'assemblage fut arrêté de façon permanente. Nous concluons donc que les filaments flagellaires ne peuvent reprendre leur croissance suivant un dommage mécanique produit par ablation laser. Cette conclusion est en opposition avec l'observation qu'un filament peut reprendre sa croissance suite à un endommagement par cisaillement (*shearing*). La possibilité d'une reprise de croissance d'un filament dépend donc de la méthode utilisée pour le briser.

2.3 Abstract

Many bacteria swim by rotating long appendages called flagella. Flagellar filaments are assembled from thousands of subunits that are exported unfolded through a secretion channel and added at the tip of the filament with the help of a capping protein. The assembly of a flagellum uses a significant proportion of the biosynthetic capacities of the cell. Here, we address the simple yet significant question whether a flagellar filament can form a new cap and continue to grow after being damaged. Important technical challenges had to be overcome to test this on a single-cell level. Filaments were visualized by fluorescence microscopy and

cut individually using laser ablation. After a growth period, the exact same filaments were labeled again with a fluorophore of a different color and revisited to examine re-growth. No re-growth was observed on filaments that had been cut (independent on overexpression of the capping protein FliD), suggesting that the growth of filaments was permanently stopped. We thus conclude that flagellar filaments do not re-grow after mechanical damage using laser ablation. This result contrasts with the observation that filaments broken by mechanical shear do re-grow. Hence, whether a filament can re-grow or not depends on how it was broken.

2.4 Introduction

The bacterial flagellum of enteric bacteria consists of three main structural parts : (i) a basal body complex that spans the inner to the outer membrane and is embedded in the cell wall ; (ii) an external, flexible linking structure (the hook) and (iii) a rigid, helical filament made of several thousand flagellin subunits[16, 13]. The basal body complex harbors a flagellum-specific protein export machine [13]. This flagellar-specific type-III secretion system exports most extra-cytoplasmic building blocks of the flagellum in a proton motive force (PMF) dependent manner[14, 15]. Secreted flagellar substrates travel through a narrow channel to the tip of the growing flagellum where they self-assemble with the help of capping proteins[13, 42]. The flagellar filament is connected to the basal body and the hook structure via two hook-associated proteins (FlgK, FlgL or HAP1, HAP3) and polymerization of filament subunits (flagellin, FliC or FljB in *Salmonella enterica*) requires the filament cap protein (FliD or HAP2)[22, 23].

In *Salmonella enterica*, expression of flagellar genes is temporally coupled to the assembly state of the flagellum and ordered in a transcriptional hierarchy of three promoter classes[43, 44]. The flagellar master regulatory complex FlhDC is expressed from the Class 1 promoter and directs RNA polymerase together with σ^{70} to transcribe from Class 2 promoters. Gene products expressed from Class 2 promoters include the components of the hook basal body complex, as well as regulatory proteins, e.g. the flagellar-specific, alternative σ^{28} factor and its cognate anti-σ factor FlgM. The completion of the hook-basal-body complex results in a switch in secretion specificity within the type-III secretion apparatus from secretion of early (hook basal body-type) substrates to the secretion of late (filament-type) substrates. After the switch in secretion specificity, FlgM is secreted as a late substrate thus freeing σ^{28} to activate transcription from Class 3 promoters[18]. Class 3 gene products are needed for completion of the flagellum (e.g. the filament subunits, the filament cap, the motor-force generators) and the chemotaxis system. Thus, by secreting the FlgM protein after the secretion specificity switch, the cell ensures that genes needed after hook basal body completion are only expressed after a functional hook basal body complex has been assembled.

The flagellar filament consists of several thousand subunits of flagellin and grows to a length of 10 to 15 µm. It is presumed that shearing of flagellar filament frequently occurs in nature, however it is not clear if a sheared flagellum can re-grow[45]. A sheared flagellum would need to re-assemble the filament cap structure, as the original cap would have been removed by the shearing event. In *Salmonella enterica*, the filament cap gene is transcribed from both a Class 2 and a Class 3 promoter[46]. The presence of FliD cap protein prior to hook basal body completion would allow for an efficient transition to filament assembly after the switch in substrate specificity. FliD expressed from its Class 2 promoter would be secreted together with FlgM prior to flagellin gene expression, thereby circumventing competition with flagellin secretion. FliD might be expressed from its Class 3 promoter in case of a shearing event to allow the formation of a new cap, which would be a prerequisite for the regeneration of a sheared filament.

Rosu and Hughes have previously analyzed the dynamics of Class 3 gene expression after flagellar shearing in *S. enterica*[45]. FlgM is constantly secreted during flagellar growth and it was presumed that the rate of secretion decreases exponentially with length of the filament[47]. Thus, shearing of a filament would result in a sudden increase in the rate of FlgM secretion, which would result in a burst of Class 3 gene expression needed for filament regeneration. However, the levels of intracellular FlgM and Class 3 gene expression remained unchanged after flagellar shearing[45].

In a recent study, Turner and colleagues used fluorescent bi-color labeling of flagellar filaments to measure filament growth in *Escherichia coli* in a population-approach[48]. They used differential fluorescent labeling of flagellar filaments to distinguish new filament growth from old filament growth and concluded by measuring the length of hundreds of filaments that the growth of filaments is independent of their length. By comparing the length distributions of a population that had not been sheared with one that had been sheared, the authors concluded that flagellar filaments of *E. coli* that were broken by mechanical shearing by viscous forces continued to grow.

In this study, we examined whether filament re-growth occurs after mechanical damage on an individual cell basis. To meet this challenge, we first used a *S. enterica* mutant that assembled only a single filament. Then ultrashort laser pulses were used to cut individual filaments in a process called laser ablation. Finally, a motorized microscope stage allowed us to revisit the filaments that were damaged after a two-hour incubation period and a second fluorescent labeling of a different color. The experimental setup is shown in Figure 2.1.

This is yet another example of the enormously fruitful approach of working at the single cell level, which often reveals crucial aspects hidden in population observations. We envision that the specific methods developed here consisting of laser ablation to disrupt individual cellular systems coupled with time-lapse microscopic approaches will be applicable to a number of

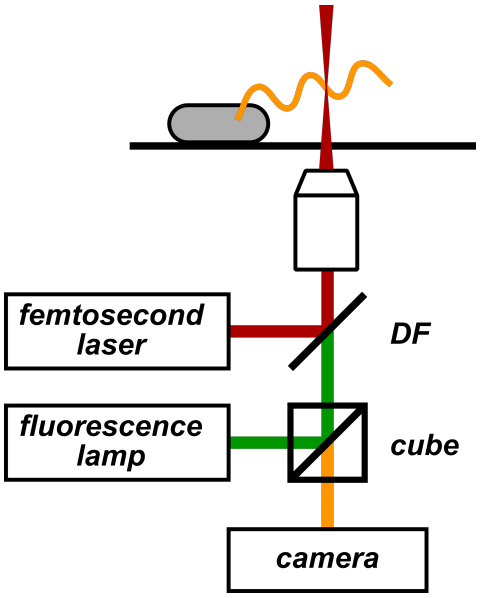

FIGURE 2.1 – Schematic of the experimental setup. The femtosecond laser is added to the optical axis through a dichroic filter (DF) and focused on the sample with a 100X 1.3 NA objective. The same objective is used for fluorescence imaging. The sample is illuminated with a broadband light source and a fluorescence cube selects the excitation and emission wavelengths. The bacterial filaments are then visualized on an EMCCD camera. Every figure in this chapter is taken from [41]

different single-cell applications in the study of microbial physiology.

2.5 Results

In order to unambiguously identify individual filaments on a microscope slide over multiple hours, we required the majority of cells to possess on average a single filament. A serendipitous discovery was made that a strain deleted for the *fliO* gene, harboring a P*flhD* P1 and P4 promoter up mutation[49] and in addition missing the anti-σ factor FlgM (termed Δ*fliO**) preferentially assembled only a single filament (Figure 2.2). The FliO component of the flagellar-specific type-III secretion apparatus is essential for export apparatus function and a Δ*fliO* strain is non-flagellated under normal export substrate conditions[50, 51]. However, it was recently found that the requirement for *fliO* could be bypassed by mutations in *fliP*[52]. We found that the Δ*fliO** strain retained slight motility in soft-agar plates (Figure 2.2A) and that more than half of the cells of the Δ*fliO** strain produced at least one flagellum (Figure 2.2 B and C).

We next introduced a single cysteine amino acid substitution (T237C) in the flagellin *fliC* into the Δ*fliO** strain to allow observation of flagellar filaments by fluorescent microscopy. Residue T237 in the variable loop of the FliC flagellin of *S. enterica* was chosen for cysteine substitution

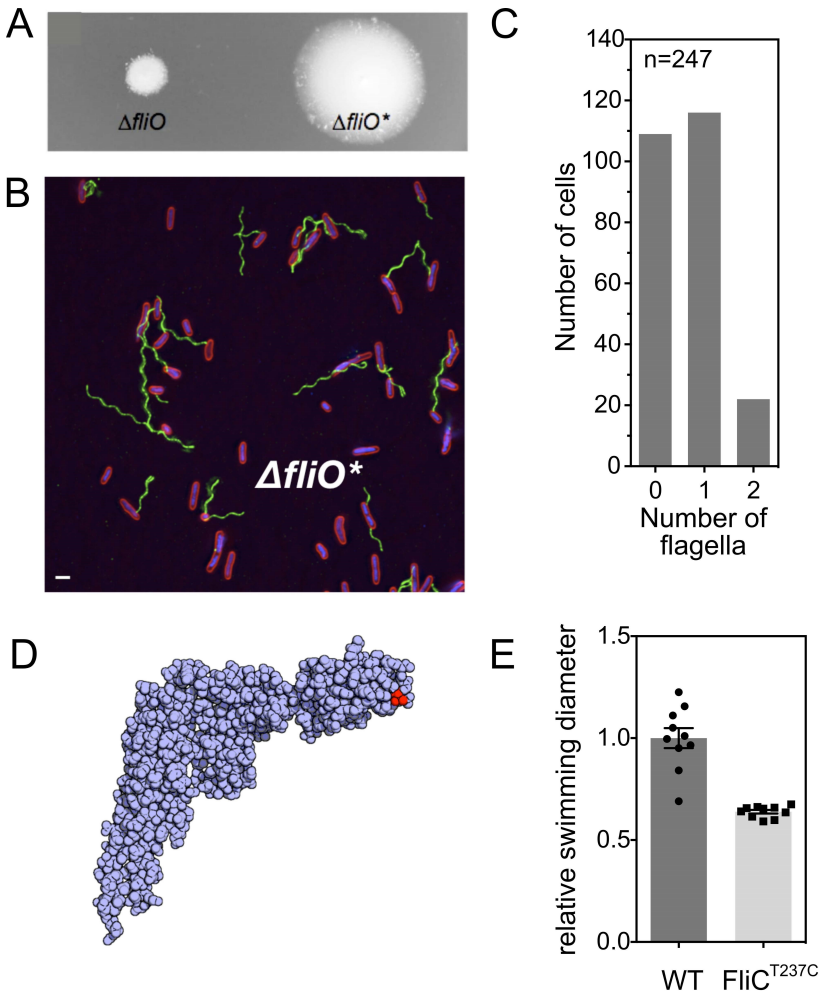

FIGURE 2.2 – (A) Enhanced motility of strain TH16123 that is deleted for *fliO* and that has increased flagellar gene expression resulting from deletion of the negative-regulator FlgM and a promoter-up mutation in the *flhDC* operon (Δ*fliO**). Motility plates were incubated overnight for 18 hours before imaging. The parental strain TH10548 deleted for *fliO* displays a non-motile phenotype. (B) Fluorescent microscopy analysis revealed the preferential formation of a single flagellum in the Δ*fliO** strain TH16123. Exemplary fluorescent microscopy image of the Δ*fliO** strain. Flagellin FliC was immunostained as described in Materials and Methods. Membranes were stained using FM-64 and DNA using DAPI. Scale bar 2 µm. (C) Quantification of numbers of flagella per cell of the Δ*fliO** strain by anti-FliC immunostaining. (D) Graphical visualization of the surface localization of the cysteine-substituted residue T237 (shown in red) using PDB no. 1IO1 of flagellin. (E) Relative swimming motility of strain TH9671 harboring a T237C substitution in the flagellin FliC compared to the wildtype control TH6232. Swimming motility was assayed using soft-agar swimming plates containing 0.3 % agar.

(Figure 2.2D). As shown in Figure 2.2E, the motility of otherwise wildtype *Salmonella* cells harboring the *fliC*(T237C) mutation was approximately 64% of the wildtype *fliC* allele.

As described in the Materials and Methods section, cells of the Δ*fliO** *fliC*(T237C) strain were immobilized in a custom-made flow chamber and labeled with Alexa-Fluor maleimide 546 dye after incubation. Only cells that were firmly attached to the coverslip and that had one single flagellum were selected for shearing of the filament by laser ablation. In order to ensure that the observed cell was alive and healthy, we considered only the rotating filaments. In addition, we selected filaments that were not only rotating on their axis, but also slowly gyrating (i.e. the filament axis itself was rotating around slowly). Indeed, initial trials showed that if the filament was not gyrating, the laser pulses cutting the filament often stopped the rotation of the motor. It is not exactly clear why that was the case, but presumably non gyrating filaments have a much stronger tendency to stick to the cell body or the coverslip upon laser ablation. The ideal candidate was therefore a rotating filament that was also gyrating in a somewhat uniform circular trajectory. The laser beam was then positioned in the vicinity of the bacterium so that its filament would cut itself on the train of ultrafast laser pulses (at 250 kHz repetition rate). A successful cutting operation was clearly identified by the acceleration of the filament gyration, and the cut portion was often seen diffusing away. To be sure that the laser did not damage the flagellar motor or compromised the cell membrane, we made sure that the filament was still rotating after ablation. Figure 2.3 shows the same bacterium before (panel A) and after (panel B) its filament was cut. The length of the cut filament was reduced from ∼3.5 µm to ∼2 µm.

A total of 82 individual bacterial filaments were cut using the femtosecond laser and observed after a two-hour incubation period. We never observed any filament re-growth on any of the filaments that had been cut. Statistically this observation allows us to conclude (with 95% confidence) that the proportion of filaments that can regrow after being cut by the laser is less than 4% (by the "rule of three", $3/n = 3/82 = 4\%$)[53]. Table 2.1 breaks down the number of observed filaments by strain and by their rotation status when we revisited them after the incubation period. The fact that a filament was still rotating after incubation demonstrates that this particular bacterium was alive and healthy (and therefore potentially able to re-grow filaments). However, if a filament was stopped, it was most likely because it simply stuck to the poly-L-lysine-coated coverslip surface. We considered it valid data because dead bacteria could easily be identified due to their cell bodies filling up with fluorophore. As a control, we observed many filaments that were left intact on the same coverslip. As shown in Figure 2.3C, the portion of the filament that grew during incubation is clearly visible as a green extension at the end of the orange filament (when images in both channels are combined digitally). To acquire such image, the filament's rotation had to be stopped either by exposing the bacterium to a large amount of blue-green light, or by punching a hole in the cell body with the laser. Overall, between 90% and 95% of the uncut filaments that were still turning

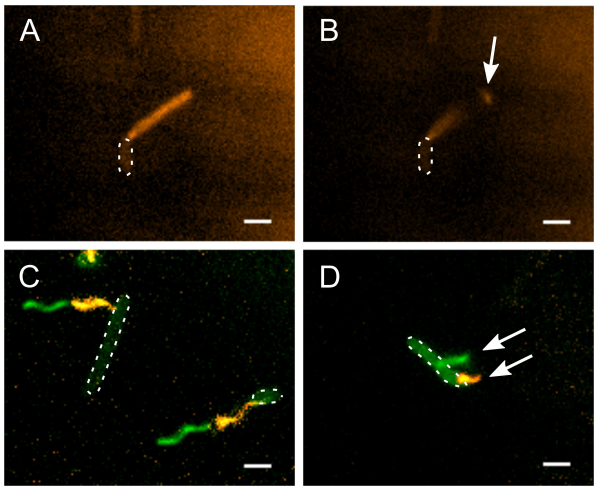

FIGURE 2.3 – Flagellar filament of strain EM800 (A) before and (B) after being cut by an ultrafast laser beam. The cell body is barely visible (highlighted with white dotted line) and the filament shows up large and fuzzy because it is rotating much faster than the image acquisition rate. The white arrow points to the cut portion of the filament drifting away and out of focus. Scale bars are 2 µm. (C) Control cells of strain EM800 whose filaments were left intact. The filaments were first labeled with an orange fluorophore and then, after a 2-hour incubation at 37°C in TB, labeled again with a green fluorophore. The portions of filaments that grew during incubation are clearly distinguishable. (D) Example of a bacterium (EM800) that grew a new flagellum during incubation. The top arrow points to the new filament that grew after the first labeling (in the 2 hour incubation). The filament is blurry since it was rotating during the exposition. The bottom arrow points to the cut filament (orange) that did not regrow. The continued rotation of the flagellar filament demonstrates that the cell was still alive and potentially able to re-synthesize a new filament.

after the incubation period showed a green "regrowth" portion.

On a few occasions, we observed cells that grew a second filament during the incubation period. Figure 2.3D shows a cell on which the new filament (green and fuzzy due to rotation) is seen besides the old cut filament (orange) that clearly did not regrow. Such cases with "built-in" control further support our conclusion that filaments do not regrow after being cut by laser ablation.

Strain	Total	Still turning	Stopped
EM800	64	17 (27%)	47
EM1283	18	8 (44%)	10

TABLE 2.1 – Number of filaments that were cut and observed after a 2-hour incubation for each strain used. The rotation status of the filaments when we revisited them is also detailed. None of these 82 filaments continued to grow after being cut.

The Δ*fliO** strain EM800 harbored a deletion of the anti-σ factor FlgM that ensures constant σ^{28}-dependent gene expression from Class 3 promoters. Accordingly, both flagellin subunits

and the filament cap FliD should be available for filament regrowth. In fact, as shown in Figure 2.3D, the cells were able to grow a new flagellum when the first filament was damaged by laser ablation. However, to provide an excess of cap protein, we additionally performed the laser shearing experiments with a strain that overexpressed *fliD* from an inducible arabinose promoter (EM1283, see Figure 2.5 in Supplementary Material). This enabled us to test the possibility that excess FliD in the cytoplasm could accelerate the formation of a new cap structure, and thereby allow filament growth after damage. We performed the same laser ablation manipulations with strain EM1283 and, as shown in Table 2.1, none of the 18 filaments that were cut grew back (44% were still turning after incubation), while undamaged two-color filaments were frequently observed. Supplementary Figure 2.5A shows complementation of a Δ*fliD* strain by overexpressed *fliD* in a motility plate assay. The same arabinose-inducible *fliD* construct did not affect motility of the Δ*fliO** strain EM1283.

2.5.1 Shearing

A surprising aspect of the results described above is that they differ from the conclusions of Turner et al. who found that mechanically sheared *E. coli* filaments can re-grow[48]. The principal difference between the experiments described in that paper and the ones reported here is the method used for breaking the filaments : mechanical shearing (with small syringe needle) in [48] versus ultrafast laser ablation here. That aspect will be discussed in detail below, but to test whether the different results could simply be explained by the different bacterial species (e.g. *Salmonella* versus *E. coli*), we mechanically sheared the filaments of the *Salmonella* strain used here. To this end, we constructed strain EM2046 that harbored the flagellar master operon *flhDC* under control of the tetracycline-inducible P_{tet} promoter[44]. Induction of flagellar synthesis by addition of tetracycline allowed us to synchronize production of basal bodies within the population of strain EM2046. In addition, this strain allowed us to stop the production of new basal bodies before the end of the incubation period by removal of the inducer tetracycline and this ensured that most filaments (∼90%) were longer than 2 µm before shearing. As described in the Methods section, the filaments of strain EM2046 were sheared using a 22-gauge needle. After comparing the distributions of filament lengths with and without shearing (shown in Figure 2.4), we concluded that the shearing is effective at shortening filaments (4.0 ± 1.6 µm (s.d.) on average for normal population vs 2.0 ± 1.1 µm on average for sheared population). The distribution of filaments that failed to produce a green segment (i.e. did not regrow) is shown by the white bars Figure 2.4B. To answer the question whether the mechanically sheared filaments regrow, filaments below 2 µm length were examined in detail.

The drawback of the two-color labeling approach after mechanical shearing is that we can only make statistical arguments. Indeed we can never be sure that a given filament observed to be two-colored (i.e., did grow after the shearing) was in fact broken by the initial shearing process.

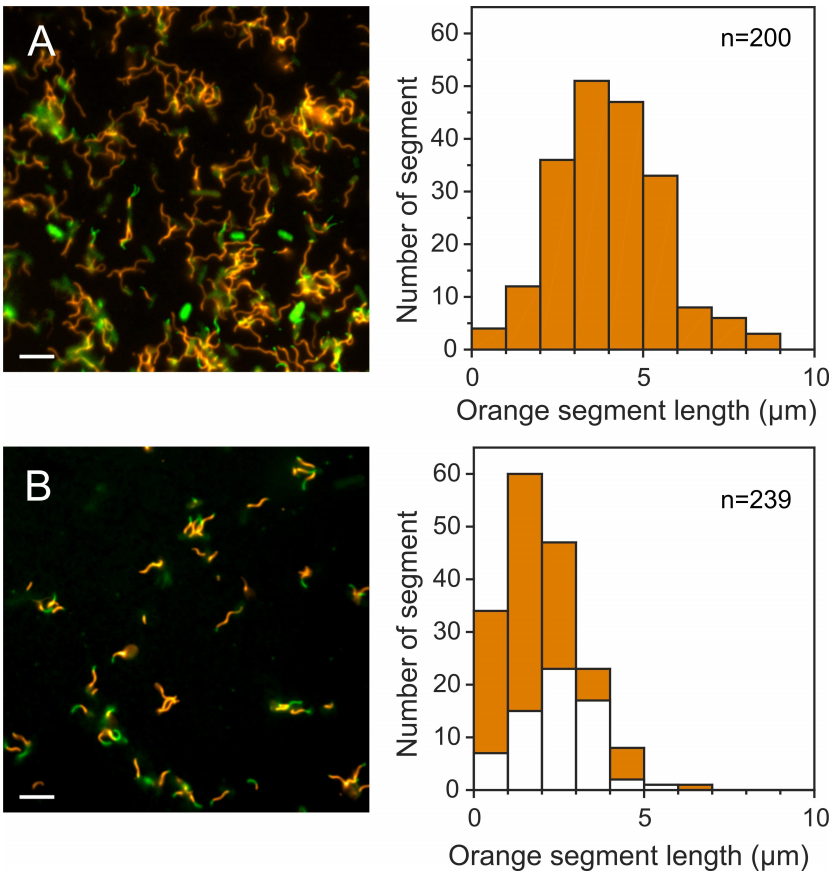

FIGURE 2.4 – Results of the shearing experiment. The two top panels (A) show the data without mechanical shearing between the two labeling of EM2046 bacteria and the two bottom panels show the results with mechanical shearing. The left panels are sample images of two-color fluorescent labeling of EM2046 bacteria (scale bar is 5 μm). The length of the first portion of the filament (near the cell body, labeled in orange) is generally shorter when the bacteria are sheared (in B). This is easily observable on the right panels, which show histograms of the filament length measurements in the two situations (200 and 239 filaments in total in the non-sheared and sheared population respectively). One also notices that most orange filaments have a green tip, which indicates that the filament grew back after shearing. The white bars (in front of the orange bars on the bottom right panel) indicate the number of filaments that did not regrow after the first labeling.

However, the use of the EM2046 strain increased the odds as it allowed us to synchronize filament assembly. As displayed in Figure 2.4A, only 8% of the filaments (16 out of a total of 200) are less than 2 µm long when the culture was not sheared, as opposed to 49% (116/239) after shearing. Therefore, we concluded that the shearing process was efficient. In the worst case scenario, if no filament below 2 µm could ever be broken by mechanical shearing, we would expect a maximum of about 8% of the sheared filaments to be intact and therefore continue to grow. Among the 116 short filaments that had been sheared, 22 did not show a green portion, which leaves 94 filaments that continued to grow (39% of the total number). That 39% is significantly higher than 8% ($p<0.0001$, or the difference is $31 \pm 8\%$ with 95% confidence – see details in Supplementary section 2.8.1), and we conclude that mechanically sheared filaments from *Salmonella* are able to re-grow, in contrast to the filaments broken by mechanical laser ablation. The observed filament growth after shearing also demonstrated that cells were alive and healthy after the mechanical shearing process.

2.6 Discussion

This work studied whether it is possible for a flagellar filament of *S. enterica* to continue to grow after being damaged by laser shearing. The combination of femtosecond laser ablation and the use of a single-filament bacterial strain enabled us to achieve the technical challenge of inducing specific damage to identified filaments and revisit each one individually after an incubation period. Our conclusion is unequivocal : the bacterial filaments do not re-grow under these conditions. In contrast, we observed re-growth of filaments after applying breaking forces by viscous shearing.

The process of flagellar assembly and its genetics has been extensively studied[16, 13, 24, 6]. A crucial structure in the specific question studied here is the filament cap, which is a protein complex that sits at the distal end of the flagellum[23, 54]. The principal role of the filament cap is to allow the flagellin proteins to polymerize at the tip. The flagellin proteins are synthesized in the cytoplasm of the cell and exported via the flagellum-specific type-III secretion system through the central channel of the flagellum. The cap structure is formed by a protein called the hook-associated protein FliD (or HAP2), and has been shown to be essential for the growth of filaments[20, 21]. In [24], the observation that hook-associated proteins are constantly secreted through the filament channel suggested that the cap could be replaced. On the other hand, Homma and Iino concluded that a different hook-associated protein, FlgL (HAP3), is essential to the attachment and assembly of the cap[19]. FlgL is located at the base of the flagellum between FlgK (HAP1) and the beginning of the filament. A filament that broke in the middle would therefore not provide the FlgL interface, and thus would simply let the constantly produced cap proteins leak out of the cell. Without a cap, the broken filament should not continue to elongate. This would be consistent with our observations that laser-cut filaments stopped growing. This hypothesis would also explain why overexpressed cap protein

(FliD) was unable to allow re-growth of broken filaments.

In contrast to our present findings, a previous study by Turner et al. concluded that mechanically sheared filaments of *E. coli* continued to grow[48]. In that experiment, filament lengths were measured before and after mechanical shearing (with a syringe needle). Since the authors did not monitor individual filaments before and after shearing, it was impossible to ensure that a given filament observed to have re-grown was actually sheared initially. However, their result is statistically significant and convincing due the large number of observations they made (about 5000 filaments). Another difference between their experiment and ours is that they left the sheared cells overnight at 4°C before the second fluorescent labeling, whereas our incubation period was 2 hours at 37°C. Leaving the cells in rich medium overnight was impractical for us since it dramatically reduced our ability to find the specific bacterium whose filament was cut because of cell division and movements (we typically found between 30% and 50% of these cells after the incubation period). However, since reported growth rates of flagella range from 0.12 to 0.55 µm/min[47, 55], we are confident that 2 hours is largely sufficient for a filament's regrowth to be measurable. The fact that we observed numerous "green" filaments (a few micrometers long) after the 2 h incubation period demonstrates that observable filament polymerization occurred between the first and second labeling (see Figure 2.3).

Another notable difference between our results and those of Turner et al.[48] concerns the use of the bacterial species for the shearing experiments (*E. coli* in case of Turner and al., whereas we worked with *S. enterica*). To test whether a flagellar regrowth mechanism differed between bacterial species, we performed the same mechanical shearing assay (by viscous forces) as in [48], but using a *Salmonella* strain. The results shown in Figure 2.4 suggest that mechanically sheared filaments in fact continue to grow, an observation that is in agreement with the conclusions of Turner et al. The total number of observed filaments, 463 and therefore the statistical significance of this result is lower here than in [48]. However, our goal was not to reproduce their study, but to confirm that our *Salmonella* strains behaved the same way as *E. coli*.

We are then naturally led to the interesting conclusion that the method used to shear filaments has an impact on whether the filaments can continue to grow or not : mechanically sheared filaments (with a small needle) continue to grow whereas filaments sheared with ultrashort laser pulses do not. Intuitively, one could be tempted to think that the heat deposited by the laser pulse "cauterize" the end of the filament, preventing further growth. We will argue here that this is not the correct picture.

Numerous studies have been performed on the subject of laser ablation of biological tissues with ultrashort pulses[27, 56, 34, 57]. In general, a wavelength between 700-1100 nm is used since water and most of biological tissues are transparent at those wavelengths[34]. In our

setup, the central wavelength is 790 nm which can be slightly tuned by ± 15 nm around it (This however reduced the output power.) For a pulsed-laser at 250 kHz repetition rate with 80 fs pulses at 790 nm, the heat diffusion time (∼98% of the heat is diffused after 1 µs) is much smaller than the time between two pulses[27, 34]. This prevents the accumulation of heat at the ablation site. Physical damage to the material outside the plasma volume is not thermal, but rather induced by the emission of a shock wave driven by the rapid expansion of a laser-induced plasma (a so-called Coulomb explosion)[58, 59]. In other words, the use of ultrashort pulses implies that very little energy is deposited (essentially no heat) and that the tissue is damaged by the creation of a violent but very localized shock wave. Proteins contained in the plasma volume are vaporized. The ablations were done using the minimum power required for the filament to be cut at 250 kHz. We also performed the same assay using 1kHz and 10 kHz repetition rates and the results were identical. Furthermore, we tried to vary the amount of power applied at the ablation site. However, when the power was higher than the minimum needed, the cells would often get loose from the cover slip making it impossible to go back to revisit later. We also couldn't assess the integrity of the motor as the filament would stop most of the time. We thus had to work within a very narrow window of power for our experiments. Finally, since the effect of the ablation is highly nonlinear, the result doesn't depend on the wavelength as ablation using regular impulsion would.

In our experiments the bacterial filaments slowly approach the focal point of the laser from which a shock wave is emitted every 4 µs. The energy contained in each laser pulse (about 5×10^{-10} J) is largely sufficient to break the hydrogen bonds between the protein subunits constituting the filament (∼20 kJ/mol = 3×10^{-20} J/bond), or even the peptide bonds inside those proteins (∼300 kJ/mol = 5×10^{-19} J/bond)[60]. It thus appears possible that laser ablation might damage and destroy individual flagellin proteins. One could argue that damaged or unfolded flagellin proteins left at the tip of the filament could interfere with the re-assembly of a new cap. However, a recent study[39] suggests that aqueous protein stay folded when vaporized by femtosecond laser. This implies that proteins left at the tip of the broken filament would stay folded with only the cap missing. Hence, the question that needs to be asked is whether it's even possible for a new cap to form.

However, since the binding energy between FliC proteins (hydrogen bonds) is lower than the energy of the peptide bonds within the protein (at least $1/10^{\text{th}}$) the possibility that intact FliC proteins would be left at end of the filament would also seem reasonable. If the laser does not "cauterize" the filament, what else could explain the difference between laser-shearing and mechanical shearing ? Even though this is highly speculative, we would like to propose the hypothesis that when a filament is mechanically sheared at the tip of a small syringe needle, mechanical forces are applied on it, which might induce polymorphic transitions just before it breaks (for example into a "straight" form). This could possibly lead to a "cleaner" tip on which a cap could reform, thereby enabling growth. The shock to the filament is certainly

more sudden and local in the case of laser-shearing and that seems to prevent the formation of a new cap. Adding too much energy to the system prevents the filament to continue to grow. This could explain why sheared filaments don't regrow 100% of the time.

In conclusion, we observed that the method used to break bacterial filaments has an impact on whether a broken filament can continue to grow or not. This is a surprising result that highlights once again the richness of biophysical studies probing the mechanical properties of protein assemblies and the impact of these properties on biological function. Our conclusion calls for further experimental work, for example examining in detail the mechanism of how filaments are broken by mechanical shearing.

2.7 Materials and Methods

2.7.1 Bacterial strains and growth conditions

The *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) strains used in this study are listed in table 2.2 in the Supplementary Material. The generalized transducing phage of *Salmonella* Typhimurium P22 *HT105/1 int-201* was used in all transductional crosses[61]. Strains were streaked for single colonies from frozen stock (-80°C) on bacterial plates (10 g Bacto tryptone, 15 g Bacto agar and 5 g NaCl per liter). For the laser ablation experiments, an isolated colony was inoculated in 10 ml TB broth (10 g Bacto tryptone and 5 g NaCl per liter) in a 125 ml Erlenmeyer flask and placed at 34°C for 15 hours with gyration at 200 rpm. A volume of 100 µl of the saturated culture was inoculated in 10 ml TB broth in a 125 ml Erlenmeyer flask and placed at 34°C for 4 hours with gyration at 200 rpm, until it reached an $OD_{600} \approx 0.45$ ($\sim 4 \times 10^8$ cells/ml). The culture was centrifuged for 5 minutes at 1,500×g and gently resuspended in 1 ml motility buffer (MB) (0.01 M potassium phosphate at pH 7.0, 10^{-4} M EDTA). For quantitative assessment of motility in soft-agar plates, single colonies grown overnight on a LB plate were inoculated in soft-agar motility plates (10 g Bacto tryptone, 3 g Bacto agar and 5 g NaCl per liter) for 4.5 hours at 37°C. The diameter of the motility swarms was measured using ImageJ[62], and the motility relative to a wildtype control was calculated.

2.7.2 Flagellin labeling and fluorescent microscopy

A custom-made flow-cell was fabricated using a standard microscope slides (25 × 75 mm) and a 18 × 18 mm coverslip (cleaned with 70% ethanol). Two stripes of Parafilm were placed ∼1 cm apart between the slide and coverslip and gently pressed after heating over a flame in order to form a tunnel. A drop of Poly-L-lysine 0,01% (Sigma) was left on the coverslip for 5 minutes and then rinsed before the flow-cell was fabricated. This tunnel was then filled with 50 µl of cell suspension and left 10 minutes upside-down for the cells to deposit and stick to the coverslip. Cells that were still in suspension were rinsed away with 200 µl of motility

buffer (MB). To label the filaments, the flow-cell was then filled with 50 µl of Alexa-Fluor maleimide 546 dye (A-10258, Life Technologies) at 1 mM concentration and the flow-cell was left in a dark humidity chamber for 1 hour at room temperature. The excess dye was washed by gently flowing 600 µl of MB, and the filaments could then be observed in fluorescence microscopy. After cutting filaments with the laser (as described below), a second labeling was performed with a different dye : Alexa-Fluor maleimide 488 (A-10254, Life Technologies). During that second labeling, the dye was diluted (again at 1 mM) in TB and the cell was left in the dark humidity chamber at 37°C for 2 hours. For overexpression of the FliD protein from the inducible arabinose promoter, arabinose was added to the TB broth during the second labeling to a final concentration of 0.2%. These observations were performed under an IX71 microscope from Olympus (100x 1.3NA objective) using an Excite light source (EXFO) and images were processed in Matlab (MathWorks, Natick, MA).

Alternatively, flagellar filaments of FliC-locked strains were labeled using anti-FliC immunostaining as described previously[63]. Images were collected using an inverted Applied Precision Deltavision microscope and assembled using ImageJ.

2.7.3 Laser Ablation

Between the two labeling with fluorophores, individual flagellar filaments were cut by laser ablation. The laser source used in these experiments was a RegA 9000 from Coherent providing ultrashort pulses (∼75 fs duration) centered on a wavelength of 780 nm at a repetition rate of 250 kHz. The energy of the pulses entering the microscope was controlled using a motorized rotating half wave retardation waveplate placed between two crossed polarizers. The optical power at the entrance of the microscope was estimated to be 120 µW (or 0.48 nJ/pulse). As can be seen on Figure 2.1, the laser was focused on the sample by the same high-numerical-aperture objective (Olympus, 100x, 1.3 NA) used for imaging.

After a filament was cut, the position of its bacterium was logged by noting the coordinates of the 3-axis micro manipulator (MP-285, Sutter Instruments) that holds the microscope slide. High-speed videos of the bacterium (1 second at 500 frames per second) were recorded both in fluorescence and in bright field microscopy with an EMCCD camera (iXon 888, Andor Technology). After about an hour of cutting filaments (generally ∼10 filaments), the slide was placed at 37°C for the "regrowth" period (and second labeling). To account for the small variations in the position of the slide on the holder, the position of two reference points were recorded at the beginning of the manipulations. The reference points were formed by the intersection of three lines (two vertical and one horizontal) drawn on the coverslip with a black marker. After the 2 hours incubation period, the coordinates of the two reference points were noted again and the new coordinates of the bacteria of interest were calculated. Each one was then revisited and videos with both fluorescence filter cubes (corresponding to the two fluorophores used for labeling) were recorded.

2.7.4 Shearing of flagellar filaments

For the shearing experiments, an isolated colony of strain EM2046 was inoculated in 10 ml TB broth (10 g Bacto tryptone and 5 g NaCl per liter) in 125 ml Erlenmeyer flask and placed at 37°C for 15 hours with gyration at 200 rpm. A volume of 100 µl of the saturated culture was inoculated in 10 ml TB broth in 125 ml Erlenmeyer flask and placed at 37°C for 2 h with gyration at 200 rpm. Then 15 µg/ml of tetracycline was added to trigger basal bodies production by inducing expression of the flagellar master operon *flhDC*. After 2 h, the culture was centrifuged for 5 minutes at 1,500 × g and gently resuspended in 10 ml of fresh TB without tetracycline to prevent further expression of flhDC and thus the production of a new round of basal bodies. The culture was returned back to 37°C for an additional 1.5 hours for the filaments to continue growing. The first labeling was done in a 13 ml tube. The culture was centrifuged for 5 minutes at 1,500 × g and gently resuspended in 100 µl/ml MB. 5 µl of Alexa-Fluor maleimide 546 dye at 10 mM concentration was added in the tube. The tube was shielded from light and left at room temperature for an hour. After washing twice with 10 ml MB, the culture was gently resuspended in 1 ml MB. 500 µl was put aside for non-sheared experiments and 500 µl was sheared by passing it 10 times in and out of a 1 ml syringe with a 22-gauge needle. A second labeling was performed in the flow-cell the same way as in the ablation experiments. To measure the length of the filament segments on the images, the NeuronJ plugins was used in ImageJ[64].

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Author contributions

Conceived and designed the experiments : GP KTH ME SR. Performed the experiments : GP ME. Analyzed the data : GP KTH ME SR. Contributed reagents/materials/analysis tools ME SR. Wrote the paper : GP KTH ME SR.

Additional Information

Supplementary information accompanies this paper at http ://www.nature.com/srep Competing financial interests : The authors declare no competing financial interests.

2.8 Supplementary Material

2.8.1 Can sheared filaments regrow

We can break the filaments of bacteria by mecanically shearing them with viscous forces, and we ask the question whether the filaments can regrow afterwards. In practice, the difficulty comes from the fact that shearing is not 100% effective, i.e., we cannot be sure that a given filament on which we observe a regrowth has really been sheared. To increase that probability, we use a bacterial strain that allows us to stop the production of new basal bodies before the end of the incubation period, so that most filaments (∼90%) are longer than 2 µm before shearing. Experimentally, we perform a two-color fluorescent labeling of the filament so that we can distinguish the portions of the filament that grew before (orange) and after (green) shearing. We then compare the proportion P_1 of filaments with a green section on top of a short (<2 µm) orange portion with the proportion P_2 of short filaments (<2 µm) in a culture that has not been sheared.

We have to decide between two hypotheses :

— $H_0 : P_1 = P_2$ (i.e. $P_1 - P_2 = 0$), which means that the filaments cannot regrow ; only the filaments that are not sheared can grow.

— $H_0 : P_1 - P_2 \neq 0$ and the filaments have some non-zero probability to regrow after being sheared.

Looking up "differences of proportions" in any Statistics textbooks, we find that

$$\sigma_{P_1-P_2} = \sqrt{p(1-p)\left(\frac{1}{N_1}+\frac{1}{N_2}\right)} \text{ where } p = \frac{N_1P_1 + N_2P_2}{N_1+N_2}$$

Using the experimental results $P_1 = \frac{16}{174}$, $N_1 = 174$, $P_2 = \frac{94}{239}$, $N_2 = 239$ we find

$$P_1 - P_2 \pm 1.96 \times \sigma_{P_1-P_2} = 31 \pm 8\%$$

. Thus we are 95% confident that the true difference in proportion is between 23% and 39%, and we therefore reject H_0 (p<0,0001). The sheared filaments do regrow.

2.8.2 Supplementary table and figure

Strain number	Relevant genotype	Reference
TH6232	Δ*hin-5717* ::FRT	Lab collection
TH9671	Δ*hin-5717* ::FRT *fliC6500*(T237C)	Lab collection
TH10548	Δ*fliO6708*(ΔAA6-121)	Lab collection
TH16123	*ΔflgM5628* ::FRT Δ*fliO6708* P*flhDC7460* Δ*hin-5717* ::FCF	This study
EM800	Δ*flgM5628* ::FRT Δ*fliO6708* P*flhDC7460* Δ*hin-5717* ::FCF *fliC6500*(T237C)	This study
EM808	Δ*araBAD1005* ::FRT	This study
EM1283	Δ*flgM5628* ::FRT Δ*fliO6708* Δ*hin-5717* ::FCF *fliC6500*(T237C) Δ*araBAD980* ::*fliD+*	This study
EM1730	Δ*flgM5628* ::FRT Δ*fliO6708* Δ*hin-5717* ::FCF *fliC6500*(T237C) Δ*araBAD1005* ::FRT	This study
EM1769	Δ*fliD5630* ::FRT Δ*araBAD980* ::*fliD+*	This study
EM1770	Δ*fliD5630* ::FRT Δ*araBAD1005* ::FRT	This study
EM2046	Δ*hin-5717* ::FRT *fliC6500*(T237C) P*flhDC5451* ::Tn*10d*Tc[*del-25*]	This study

Table 2.2 – *S. enterica* serovar Typhimurium LT2 strains used in this study

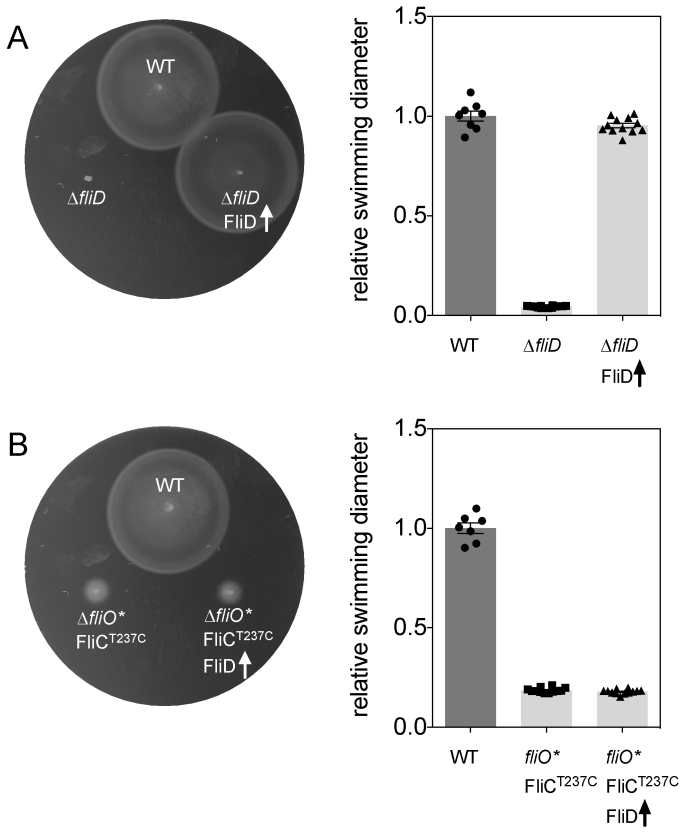

FIGURE 2.5 – Functional analysis of arabinose inducible fliD. (A) Left panel : Exemplary motility plate of strains EM808 (WT), EM1770 (Δ*fliD*) and EM1769 (Δ*fliD* P*araB-fliD*+) in the presence of 0.2% arabinose. Right panel : Quantification of motility of a Δ*fliD* complemented by arabinose-inducible *fliD*. (B) Overexpression of *fliD* does not affect motility of the Δ*fliO** strain. Left panel : Exemplary motility plate of strains EM808 (WT), EM1730 (Δ*fliO**) and EM1283 (Δ*fliO** P*araB-fliD*+) in the presence of 0.2% arabinose. Right panel : Quantification of motility of the Δ*fliO** strains after overproduction of *fliD*.