Quantifying Spatiotemporal Patterns in the Expansion of Twitching Bacterial Colonies

by

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ABSTRACT

QUANTIFYING SPATIOTEMPORAL PATTERNS IN THE EXPANSION OF TWITCHING BACTERIAL COLONIES

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Type IV pili (T4P) are protein filaments used by certain Gram-negative bacteria for a mode of surface motility called twitching. We have developed experimental and data analysis techniques to quantify the expansion of twitching P. aeruginosa colonies at an agar-glass interface as a function of agar concentration $C$. The advancing front consists of finger-like protrusions of bacteria, with cells in the expanding colony arranged in a lattice-like pattern. We find that the average finger width increases linearly with $C$, whereas the average finger speed is independent of $C$. This latter result, coupled with the observed transition from monolayer to multilayer coverage within fingers, suggests a critical number of T4P are required to break through the agar-glass interface. In addition, we have calculated line profiles across fingers, quantifying their average speed and bacterial orientation, and performed high spatial and temporal resolution studies of the fingers.
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Chapter 1

Introduction

In this Chapter, I will first introduce the Type IV pilus (T4P) system, and the bacterial strain Pseudomonas aeruginosa that is used in this study. This will be followed by a discussion of the three forms of motility for P. aeruginosa, swimming, swarming, and twitching motility, as well as collective motion of bacteria. Finally, I will describe the scope of this thesis.

1.1 Type IV Pili

Type IV pili (T4P) are protein filaments that are helical polymers consisting of pilin A subunits [1]. The filaments are typically 6 nm in diameter and several micrometres in length and are assembled and extended, and disassembled and retracted, through the cell wall by the collective action of a large number of proteins. The disassembly of pilin A subunits is mediated by the protein PilT. After a pilus extends, it can adhere to a surface and, when it retracts, the cell is pulled towards the point of attachment. T4P are able to adhere to many different surfaces, including glass, eukaryotic cells, and other bacterial cells [1-4]. Recently, AFM pulling
measurements on *P. aeruginosa* PAO1 by our research group have provided direct evidence for a high degree of flexibility of T4P, their tendency to form close-packed bundles, and their ability to adsorb onto both hydrophilic and hydrophobic surfaces over extended portions of their length rather than just at the distal end of the pilus [5]. This combination of T4P properties allows the bacteria to establish contact with and colonize a broad range of surfaces.

Optical tweezer studies have determined that in *Neisseria gonorrhoeae* the PilT motor can generate forces as great as 100 pN during the retraction of a single pilus [6, 7]. Additionally, the retraction rate of the pilus was measured to be 1.2 μm/s. Using total internal reflectance fluorescence (TIRF) microscopy, Skerker and Berg observed the extension and retraction of Cy3 fluorescently labeled *P. aeruginosa* T4P, and measured a pilus retraction rate of 0.5 μm/s [8]. The authors also observed that newly formed pili fluoresced with comparable intensity to those pili that were already extended. This implied that pili are at least partially formed from recycled subunits [8].

In cells with multiple pili, each pilus is capable of extending and retracting independently of the others. Micropillar assay studies by Biais *et al.* demonstrated that *N. gonorrhoeae* can form bundles of 8-10 pili which can retract collectively and exert nanonewton scale forces [9]. In addition to their function in surface motility, T4P also serve a role in DNA uptake and exchange, secretion of exoproteins, and modulation of biofilm architecture [2].

### 1.2 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* PAO1 is a rod-shaped, Gram-negative bacterium, typically 2 μm in length and 0.5 μm in diameter [10]. It possesses a single polar flagellum and multiple polar T4P
that it uses to achieve motility in both bulk liquid and across solid surfaces. *P. aeruginosa* PAO1 is classified as an opportunistic pathogen due to its ability to infect immunocompromised patients, such as those with cystic fibrosis, where it forms antibiotic resistant biofilms within the lungs. Both the flagella and T4P are necessary for biofilm development in *P. aeruginosa* PAO1 [10, 11]. *P. aeruginosa* is also known to secrete an exopolysaccharide Psl, which assists in surface adhesion and plays an integral role in biofilm formation [12]. An AFM image of a hyperpiliated *P. aeruginosa* PilT mutant cell is shown in Figure 1.1.

![Atomic force microscope image](image)

Figure 1.1: Atomic force microscope image of a hyperpiliated *Pseudomonas aeruginosa* PilT mutant cell dried onto a mica surface. This is a vertical deflection image collected in contact
mode in air. The red arrow indicates the flagellum, and the blue arrow indicates a Type IV pilus. Image courtesy of Josh Mogyoros.

1.3 Bacterial Motility

Bacteria have evolved multiple modes of motility, and are capable of colonizing surfaces in many different environments. Although many different forms of bacterial motility have been identified, we will describe the three forms employed by *P. aeruginosa*: swimming, swarming, and twitching motility [13].

1.3.1 Swimming Motility

Swimming is a form of motility many bacteria use to travel through bulk media. Swimming is mediated by flagella; helical protein filaments are typically 5-10 μm in length, and 20 nm in diameter [14]. Flagellar filaments are driven by a rotary motor embedded in the cell envelope, and are capable of reaching frequencies of several hundred Hz. Bacteria typically possess many peritrichous flagella (randomly oriented about the cell body), with a notable exception being *P. aeruginosa*, which possesses a single polar flagellum [13,14].

In several species, including *Escherichia coli*, counter-clockwise (CCW) rotation of the motors causes the flagella to bundle and orient along the long axis of the cell [14]. In *P. aeruginosa*, bundling does not occur as it has a single polar flagellum. CCW rotation allows the bacteria to swim at speeds up to 40 μm/s in smooth trajectories called ‘runs’. An abrupt transition to clockwise rotation disentangles the flagella, causing the bacteria to cease moving and
randomly reorient (tumble). Running and tumbling is controlled through the chemosensing system that biases its random walk to more favourable conditions for growth [14].

1.3.2 Swarming Motility

Swarming is a flagella-mediated motility in which cells rapidly move in a coordinated and collective manner on semi-solid surfaces [15]. In many species swarmer cells undergo morphological differentiation and become elongated and hyper-flagellated [16]. *P. aeruginosa* is a unique swarmer in that it requires both T4P and flagella to swarm [17]. Swarming typically occurs on 0.4 % - 0.7 % agar, and is highly dependent on cell density. Bacterial swarming is often associated with the production of surfactants, which promote colony expansion by reducing the surface tension between the cell and surface. Swarming cells can reach speeds of several μm/s, and form aligned groups of cells called rafts. In the extensively studied *Proteus mirabilis*, the flagella of cells within rafts bundle together and rotate in a coordinated manner [15, 16, 18].

1.3.3 Twitching Motility

Bacterial twitching is a form of surface motility that occurs on solid surfaces, and is mediated through the extension and retraction of type IV pili [19]. Mertz et al. provided evidence that the motion of T4P drives twitching motility through optical tweezers experiments on *N. gonorrhoeae* [7]. *N. gonorrhoeae* cells were observed to twitch on a glass surface at a rate of 1 μm/s, and aggregate into microcolonies. Cells immobilised in an optical trap, when constrained to within 1-2 μm, were observed to be pulled towards microcolonies on glass coverslips (by their pili). Bacteria unable to retract their pili (PilT mutants) did not display either behaviour [7].
Twitching typically occurs on agar surfaces with concentrations between 0.7 and 2.0 % (w/v) agar. The motion of individual twitching bacteria is flagella independent, and is characterized by jerky and intermittent movements along the surface [19]. Jin et al. characterized the motion of individual twitching *P. aeruginosa* cells in a flow cell [20]. They first identified a slow crawl, in which multiple pili are attached to the surface under tension, and the retraction of the pili moves the cell at a constant velocity for periods of time between 0.3 - 10 s. They also observed fast events (~100 ms) in which the cell both translates and rotates. The authors suggested that fast motion may be due to a ‘slingshot’ mechanism, in which one of the many attached pili is rapidly released [20].

Twitching colonies are often studied at an agar – glass interface that is created by point inoculating an agar disk with bacteria, and placing a glass coverslip atop the agar surface [4]. The agar – glass interface provides a smooth surface that has been shown to be necessary for optimal twitching behaviour, and allows the advancing colony to be easily visualised with optical microscopy [4]. A twitching colony at an agar – glass interface typically contains a dense central area in which bacteria are moving independently, with an advancing front in which cells have a tendency to align side by side along the long axis of the cell, in close contact with each other [1, 4]. The front contains rafts of aligned bacteria 5 to 30 cells across, which move radially outward across the surface. Although the average motion is radially outward, cells within rafts may reverse direction. The rafts appear as finger-like protrusions that can branch and combine with other rafts as they move, and may contain regions in which cells are multilayered. The leading rafts are followed by a lower density of aggregated cells that follow paths that are similar to those at the front, resembling a lattice- like network [1, 4]. These initial studies focused on qualitative observations of the colony morphology. In the present study, we focus on developing
procedures to quantify the morphology and motion of the twitching bacterial cells. An optical microscopy image of the leading edge of a twitching *P. aeruginosa* bacterial colony is shown in Figure 1.2.

Figure 1.2: An optical microscopy image of the leading edge of a twitching *P. aeruginosa* colony at an agar (1.0 %)-glass interface. The red arrow indicates a finger, and the blue area indicates the lattice network. The width of the image corresponds to 192 micrometers. Image was taken in the Dutcher lab.

**1.4 Bacterial Collective Motion**
Collective motion is found in a diverse array of systems, such as flocks of birds, motor proteins, and bacterial suspensions [21]. Collective motion is characterized by extended spatiotemporal coherence of self-propelled particles. Many studies have demonstrated that bacteria can act as a model system for studying collective motion [22]. Bacterial collective motion is often studied using sophisticated particle tracking algorithms, which can identify the orientation and velocity of large numbers of bacteria.

Concentrated suspensions of swimming and swarming bacteria display collective motion when the cell density is sufficiently high, and bacteria are locally aligned through hydrodynamic and excluded-volume interactions, and through the physical entwining of flagella [23]. The authors demonstrated that when confined to two dimensions, swimming *Bacillus subtilis* cells form dynamic clusters of up to 200 cells, and that bacterial motion within clusters is highly correlated, with bacterial speed increasing with increasing cluster size [23]. The motion of these clusters also causes large density fluctuations that scale differently from fluctuations for a population in thermal equilibrium [23].

Studies of swarming *B. subtilis* clusters showed that correlations of velocity, speed, motion direction and orientation are scale invariant (the range of correlations is only set by the size of the cluster), and long range [22]. These long-range, scale invariant correlations are similar to those observed in flocks of birds, despite large differences in length scale, and qualitative differences in the interactions between the objects [22]. Recently, Rabani *et al.* observed that spherical *Serratia marcescens* cells exhibit collective motion when confined in a two-dimensional monolayer, demonstrating that a rod-like shape is not necessary for bacterial collective motion [24].
Studies of collective motion in *P. aeruginosa* twitching interstitial biofilms by Gloag *et al.* showed that cells within leading rafts (fingers) show high orientation and velocity coherence [25]. By tracking individual bacteria within colonies, they showed that bacteria in the lattice network behind the leading edge have reduced orientation and velocity coherence with their neighbours, and show frequent directional changes. The authors also demonstrated that during colony expansion, the leading cells create a network of furrows between the agar substrate and glass, and trailing cells travel along this network. In addition, they showed that extracellular DNA (eDNA) enhanced local cell alignment, allowed twitching cells to efficiently travel through the furrow network, and increased coherence in the leading rafts [25].

The exopolysaccharide Psl was also shown to aid in the self-organization of surface attached *P. aeruginosa* cells [12]. Simulations and cell tracking of *P. aeruginosa* cells suggested that, when exploring a surface, these bacteria preferentially associated with regions of high Psl. This enabled the cells to organize into microcolonies, which are precursors to biofilms [12].

### 1.5 Scope of Thesis

The goal of this thesis is to characterize the morphology and motion of expanding twitching colonies over a range of agar concentrations in a well-controlled experimental geometry, and to observe twitching cells at high spatial and temporal resolution. This was achieved by performing digital Particle Image Velocimetry (PIV) on brightfield microscopy videos of colonies twitching at an agar-glass interface. In Chapter 2, we describe the details of the biological protocols and experimental setup. In Chapter 3, we describe the image processing techniques that were created and implemented for identifying the twitching colony edge, and identifying a line profile across a
finger. We also describe the procedure for processing and analyzing a time series of images with digital PIV and bacterial orientation analysis. In Chapter 4, we discuss the results of our analysis of the motion of twitching colonies over a range of agar concentrations. In Chapter 5, we conclude and summarize the results of this thesis and provide suggestions for future work.
Chapter 2

Materials and Methods

The current experimental procedure introduces several improvements that allow for the creation of a pristine agar-glass interface. Previous measurements of twitching motility at an agar-glass interface involved the manual transplanting of a glass coverslip onto an agar disk [4]. By directly pouring the agar into the aluminum cell, the agar-glass interface is free of contamination and is highly reproducible. This method also allows for precise control of agar concentration and nutrient level.

2.1 Bacterial Growth Procedure

Pseudomonas aeruginosa PAO1 was used as the wild type strain in all experiments. The P. aeruginosa mutant fliC was used for control experiments to ensure that bacterial motility was due to T4P alone, as fliC lacks flagella but produces type IV pili (T4P). Bacterial strains were stored as frozen stock cultures at -80°C, and transferred to 1.5% (w/v) agar plates for growth at 37°C until multiple single colonies were observed. A single colony was then extracted using a
sterile loop from the agar plate and grown at 150 rpm in a shaker (Fisher #SHKA4000) for 16 to 18 h at 37°C in lysogeny broth (LB) nutrient medium (Difco #244620).

2.2 Environmental Chamber

Samples were prepared using a custom built aluminum environmental chamber that was designed to fit the translation stage of an Olympus IX71 inverted optical microscope and consisted of two removable parts: a cell and a base. Photographs of the environmental chamber are shown in Figure 2.1. The centre of the base contained a rectangular groove designed to hold a 24 x 60 mm glass coverslip, on top of which the cell was placed and secured by screws. Two heating strips (Omega, KH-110/5-P) were attached to the base and used with a type-J thermocouple and a proportional-integrative-derivative (PID) temperature controller (Eurotherm 808). This allowed heating and control of the sample temperature to within ±0.05°C. In each experiment six replica samples were produced, each of which contained a central well that could hold 2 mL of liquid agar, as well as smaller 0.5 mL side wells that were filled with Milli-Q water to prevent drying of the agar during the experiments.
Figure 2.1: Images of the environmental chamber. A) The base with top and bottom heating strips. B) The base and cell connected by screws. C) A top down view of the chamber cell with the 2 mL central well and 0.5 mL side wells. D) The underside of the cell with glass coverslip. The red arrow indicates the Parafilm wax, and the blue arrow indicates the agar.

2.3 Sample Preparation

2.3.1 Agar Substrate

For experiments in which the agar concentration was varied, the agar gel was prepared in concentrations (w/v) of 1.0 -1.5% in intervals of 0.1% by mixing granulated agar (Difco #214530) with 20 mL of LB medium. This was autoclaved for 30 min at 121 °C at 15 psi, then
immediately transferred to a heat bath heated to approximately 80 °C to prevent gelling of the agar.

### 2.3.2 Bacterial Suspension

After growth of the bacterial cells in LB medium as discussed above (section 2.1), two 1 mL volumes of bacterial suspension were removed and centrifuged at 6600 rpm (Fisher #05-090-100) for 3 min. The supernatant was removed, and 1 mL of LB broth or LB/Milli-Q mixture was added. Bacteria were then re-suspended through agitation for 20 s and centrifuged for an additional 3 min.

### 2.3.3 Chamber Cell Preparation

To prepare a pristine agar-glass interface for bacterial twitching studies, a glass coverslip was quickly waved through the flame of a Bunsen burner and placed within the central groove of the base of the environmental chamber. A piece of Parafilm wax was cut to the dimensions of the coverslip and a central section was removed to fit the bottom hole of the chamber cell; this was then rinsed in ethanol and placed onto the coverslip after drying in air. The chamber cell was stored at 37°C after cleaning with ethanol, and then placed on top of the Parafilm and coverslip. 2 mL of liquid agar was prepared as described above (section 2.3.1), and pipetted into the central well of the chamber cell held at room temperature. The gel was allowed to solidify, and the cell was transferred to a sterile Petri dish containing a small reservoir of approximately 1 mL of water, sealed with Parafilm wax. After 1 h, a small vertical cylindrical hole was formed in the centre of the agar using a 20 μL pipette, and 2 μL of bacterial suspension, prepared as previously described (section 2.3.2), was transferred to the vertical cylindrical hole in the agar. A 24 x 40
mm coverslip coated with silicon vacuum grease (Dow Corning) was placed over the chamber cell, and the cell was transferred to the Petri dish and incubated at 37°C for 4 to 6 h. The incubation time was chosen to be the time required for colony expansion of ~ 100 μm from the inoculation point.

2.4 Light Microscopy

The sample was prepared as in section 2.3.3 and after incubation was transferred to the environmental chamber. Optical microscopy measurements were performed in an inverted optical microscope (Olympus IX71) using a 40x or 100x objective lens such that part of the leading edge of the bacterial colony was in the field of view. The sample temperature was fixed at 37°C. Microscopy images were collected using an Orca-Flash4.0 V2 sCMOS camera (4.0 Megapixel, Hamamatsu) and HC Image software (Hamamatsu). In studies using the 40x objective, experiments were performed on agar concentrations (w/v) of 1.0 -1.5%, and a time series of images was collected for 20 min at a rate of 1 frame every 5 s. Two or three 20 min microscopy videos from separate colonies were collected at each concentration. In studies using the 100x objective, agar concentrations (w/v) of 1.0 % and 1.1 % were used to better visualise the twitching of individual cells. In these experiments, a time series of images was collected for 2 min at 10 frames per second, and for 10 s at 100 frames per second.
Chapter 3

Image Analysis

Microscopy images of *P. aeruginosa* twitching at an agar–glass interface typically contain many densely packed cells collectively twitching outwards from the inoculation point. At the edge of the advancing front, the bacteria form finger-like protrusions. Within the growing bacterial colony, cells twitch individually and form a lattice-like network of cells. To quantify the motion of the bacterial colonies, we have chosen to use digital Particle Image Velocimetry (PIV) to analyze the motion of the bacterial colony edge, including the motion and orientation of cells within individual fingers. In this chapter, we describe the methods that we have implemented to process and analyze time series of microscopy images.

To perform the image analysis, we have chosen to use Fiji, which is an extension of the ImageJ software for biological applications, for the initial processing of our image sequences. Fiji is in the public domain and allows for visual representation of the processing of large data sets. We used MATLAB® 7.18.0.347 (R2009a) as the programming language for the analysis of the motion of the bacterial colonies. We have chosen PIVlab, an open source MATLAB-based extension, for the implementation of PIV.
3.1 Image Pre-Processing

Our microscopy images are stored as 16-bit TIFF stacks, consisting of three-dimensional matrices of intensity values. Images were converted to 8 bit grayscale TIFF images within Fiji, and the contrast was enhanced to improve the visibility of the advancing front of the bacterial colony. Often the intensity of illumination during an experiment lasting many hours was not constant, and it was necessary to remove background artefacts in the images produced by the time varying illumination. This was achieved by applying a Gaussian blur filter of radius of 20-35 pixels to the image sequence, and dividing the original images by their corresponding blurred versions. These corrected images were used as the input for PIV, edge detection, and line profile analysis.

3.2 Particle Image Velocimetry

For a typical image sequence of a twitching bacterial colony, particle tracking of each individual bacterium is unfeasible due to the high density and close packing of cells within the colony. For high cell densities, the motion of the cells within an image sequence can be determined using a technique called Particle Image Velocimetry (PIV). PIV uses the cross correlation of pairs of images to determine the average two-dimensional displacement field of areas of tracer particles (bacterial cells) between frames. This displacement field can be easily converted to a velocity vector field using the known time between images [26].
3.2.1 Cross Correlation Method

For our image sequence we can consider each consecutive image pair in a time series to consist of an input image $I$ and output image $I'$. The transfer function $H$ of the system (the ratio of the input and output functions) transforms $I$ to $I'$ and consists of a displacement function $d$, as well as some noise. The goal is to determine the displacement field, which is an estimate of the displacement of each particle in going from the input image to the output image.

PIVlab uses an FFT-based cross correlation method to determine the displacement field [27]. The cross correlation $C_{fg}$ is defined as the integral of the product of two functions, $f(x)$ and $g(x)$, where the latter has been shifted by a distance $\Delta x$.

$$f \ast g = C_{fg}(\Delta x) = \int_{-\infty}^{\infty} f(x) g(x + \Delta x) dx$$

(3.1)

In the case of an image pair, the cross correlation is computed in two dimensions over a region $W$ called the interrogation area, and functions $f$ and $g$ represent the intensity of $I$ and $I'$. In two dimensions, we can write:

$$C_{fg}(\Delta x, \Delta y) = \iint_{-W/2}^{W/2} f(x, y) g(x + \Delta x, y + \Delta y) dxdy$$

(3.2)

The maximum of $C_{fg}$ occurs for the shift in the function $g$ that corresponds to the displacement field in $W$. For functions consisting of $N$ points, in two dimensions the evaluation of the cross correlation scales as $N^4$. To reduce computation time, PIVlab takes advantage of the cross correlation theorem, which relates the cross correlation to the Fourier transforms of the input and output functions.
\[ C_{fg}(\Delta x) = F^{-1}[F^*(k)G(k)] \]  \hspace{1cm} (3.3)

As image intensity signals are discrete, we use fast Fourier transforms (FFT’s) to compute the two-dimensional Fourier transform of \( I \) and \( I' \) within \( W \). The cross correlation between the two images is calculated in the wavenumber \((k)\) domain through complex conjugate multiplication, and the inverse Fourier transform of this quantity corresponds to \( C_{fg}(\Delta x) \). A peak search on the two-dimensional cross correlation yields the two-dimensional displacement for \( W \). Employing this method reduces computation time to \( O(2N^2 \log_2 N) \) \cite{28}.

To calculate displacement field values for many particles in the image pair, both input and output images are divided into a square grid of interrogation areas (Figure 3.1).

![Figure 3.1](image.png)  

Figure 3.1: An image pair in a time sequence of images divided into a square grid of interrogation areas (blue dashed lines). The particles are indicated as black dots.
To achieve optimal resolution, the interrogation area size was progressively reduced in successive implementations (passes) of the algorithm. For our microscopy image sequences, three passes were used, and the interrogation areas were chosen to be 128, 64, and 32 pixels for the first, second and third passes. This resulted in displacement vectors on a 16 by 16 pixel (2.56 μm by 2.56 μm) grid that is set by the size of the third pass, and corresponds to the average motion of the particles within the interrogation area, and not the motion of individual bacteria. In PIV, the accuracy of displacement vectors is optimal when the distance travelled by the tracer particles is approximately half of the width of the smallest interrogation area [27]. To achieve this with a 16 by 16 grid, it was necessary to use microscopy images which were taken 10 seconds apart (half the time resolution of the original image sequence).

### 3.2.2 Window Deformation

A considerable challenge to implementing PIV with fixed interrogation areas is the loss of pairs of particles between images as particles pass out of the interrogation area, as this increases noise in the cross correlation and reduces the accuracy of the peak search. The existence of particles at the boundaries of interrogation areas can also create difficulties in FFT-based evaluation methods as they impose periodic boundary conditions. To overcome this, many PIV packages use a technique called window deformation to adjust the location and shape of the interrogation area in the second image so that all pairs are maintained and no particles reside at the edges [29]. Many different algorithms for window deformation have been developed, with the earliest and simplest being the linear deformation technique [30]. PIVlab implements
bilinear or spline deformations; we have chosen spline window deformation, as it has been shown to have superior performance to linear deformation for both uniform and shear flow [29].

Figure 3.2: An example of a linear deformation of an interrogation area W. The original interrogation area (f) has been shifted and stretched to form the interrogation area g.

### 3.2.3 Vector Post Processing

The output displacement fields from PIVlab were post processed to remove small displacement vectors produced by noise using median and standard deviation filters. Median filtering replaces the selected displacement value with the median of all neighbouring values. PIVlab contains an adjustable threshold value for the displacement magnitude above which the median filter will be applied. Typically median thresholds were chosen to contain a neighbourhood of 3 or 4 pixels. Standard deviation filtering removes all displacement values
outside a stated range of standard deviations from the mean; this was chosen to be 3.5 deviations from the mean ($\sigma$).

### 3.3 Bacterial Orientations

We have developed a Fourier transform-based analysis procedure to determine the average bacterial orientation in a high-density environment. Our method utilises the convolution theorem, which can be stated as follows: convolution in the spatial domain corresponds to filtering in the frequency domain.

\[
f(x, y) \ast h(x, y) \iff F(u, v)H(u, v) \tag{3.4}\]

By filtering the images in the frequency domain, i.e. multiplying the FFT of the image by an appropriate filter, we can obtain information about directional orientations that are present in an image.

In our procedure, the intensity of an input image is inverted (such that the bacteria are represented by a ‘high’ signal) and its Fourier transform is calculated. The Fourier transform image is then multiplied by a first order Butterworth filter that is oriented with the filter axis defined by a filter angle $\phi$ with respect to a reference direction (in this case the reference is the x-axis). A Butterworth filter is a low pass filter with the transfer function of equation 3.5:

\[
H(u, v) = \frac{1}{1+[D(u,v)/D_0]^{2n}} \tag{3.5}
\]
where $n$ is the order of the filter, $D_0$ is the distance from the origin (cutoff frequency), and $D(u,v)$ is:

$$D(u,v) = \sqrt{(u - P/2) + (v - L/2)^2}$$

(3.6)

$P$ and $L$ are the length and width of the input image respectively. We have chosen a first order Butterworth filter as it has a smooth transition between low and high frequencies, and does not introduce ringing artefacts [31].

This procedure is performed for filter angles between 0 and $180^\circ$ in steps of $4^\circ$. The inverse Fourier transforms of these resultant images are divided into the same 16 by 16 pixel grid that is used for PIV analysis. For each grid region, we select the IFFT image with the highest mean intensity (inside the grid region) to obtain the optimal angle of the filter. The overall preferred direction of the bacterial cells is perpendicular to this filter angle. An example of the steps of this procedure is shown in Figure 3.3.
Figure 3.3: A) An inverted microscopy image. B) The Fourier transform of image A. C) A Butterworth filter -24° from the x-axis. D) Inverse Fourier transform of the multiplication of images B and C. Regions of high intensity correspond to a preferred orientation along a direction perpendicular to the filter angle $\phi$. The width of each image corresponds to 54.4 micrometers.

The mean pixel intensity as a function of filter angle for the entire image D of Figure 3.3 is shown in Figure 3.4, with the maximum intensity occurring at $\phi = 24^\circ$. 
Figure 3.4: Mean pixel intensity of the entire IFFT image as a function of filter angle. The maximum intensity occurs at 24°.

### 3.4 Identification of Colony Edge

To analyse the overall motion of the expanding colony, we have chosen to calculate the average motion of the leading edge of the colony over time. The leading edge of the bacterial colony was calculated for each image by using a custom, automated sequence of steps in both Fiji and MATLAB. It was necessary to implement some variations in the procedure described below as colony features for each microscopy image time series could differ significantly, but the general strategy is robust.

In the procedure, an image sequence was loaded into Fiji, and a standard deviation filter of 15 pixels (2.4 μm) was applied, in which each pixel intensity value was replaced with the standard
deviation of all intensity values of pixels within the chosen radius. The radius determines the size of the image features that will be identified by the filter. An intensity threshold was then chosen such that for each pixel value in the filtered grayscale image \( g(x, y) \), the corresponding value in the binary image \( b(x, y) \) would be:

\[
b(x, y) = \begin{cases} 
0, & \text{if } g(x, y) < t \\
1, & \text{if } g(x, y) \geq t
\end{cases}
\]  

(3.7)

The threshold was set such that the area of the images corresponding to the bacterial colony was above the threshold and the remainder of the image was below this value. The image sequence was then imported into MATLAB.

The implementation of the standard deviation filter produced two unwanted results: features of the bacterial colony in these binary images were slightly larger than those of the original images, and the binary images also contained ‘holes’ (areas of missing intensity within otherwise continuous features). These issues were corrected using two built-in MATLAB functions: the hole filling operation \texttt{imfill()}, which fills (sets to 1) all dark pixels that cannot be reached by filling in dark pixels from the edge of the image, and the erosion filter \texttt{imerode()}. \texttt{imerode()} requires a structuring element to be chosen for the erosion procedure, for which we used a disk structure of radius 1-3 pixels, with larger features within images requiring larger disks (up to 3 pixels). This function places the centre of the structuring element at each foreground pixel location, and if the structuring element overlaps with any background pixel, the centre pixel is deleted. The erosion procedure was repeated 1-4 times until a visual inspection of the eroded images confirmed that the size and shape of features were consistent with those in the original image sequence. Finally, the Canny edge detection algorithm in the \texttt{edge()} function of MATLAB
was applied to determine the edges of the bacterial colonies. The output of the colony edge
detection algorithm is a matrix in which the coordinates of all pixels that have been identified as
edges are set to a value of 1. A step-by-step example of this procedure is shown in Figure 3.5.
Figure 3.5: Example of the edge detection process to determine the leading edge of a bacterial colony. A) A cropped microscopy image after image pre-processing. B) Image after applying a standard deviation filter of 15 pixel radius. C) Image after applying a binary threshold and hole filling. D) The multiplication of images A and C. E) Image after a series of erosions. F) Image after edge detection. The width of each image corresponds to 72 micrometers.

To determine the PIV displacement value that corresponds to each pixel, the edge matrix of the image was multiplied with the corresponding PIV data matrix. The resultant matrix contains the PIV displacement values only at the locations identified as edges. To store the edge PIV data in a more compact way, a find() operation was performed on the resultant matrix, which produced a list of all PIV displacement values with their pixel coordinates for each frame.

3.5 Line Profiles of Fingers

The finger-like protrusions at the leading edge of the expanding bacterial colonies are a distinctive feature produced by the twitching motility. To further investigate these intriguing features, we calculated line profiles of the PIV velocity and bacterial orientation across individual fingers. It was first necessary to crop each microscopy image to isolate the finger of interest. The cropped time series was processed using Fiji and MATLAB as described in section 3.4 (without edge detection) using a 4 pixel standard deviation filter. Small objects produced by the filtering procedure were removed by applying an area opening operation using the bwareaopen() command in MATLAB. This command generates a binary image in which all objects are removed that have a total number of pixels than is less than a threshold number specified by the user. We chose a 70-pixel threshold value, corresponding to objects with areas
less than 1.3 μm². Next, any irregularities introduced by applying the area opening operation were removed by applying a two-dimensional Gaussian smoothing kernel with a standard deviation of 4, using the MATLAB command imgaussfilt(). The Gaussian kernel is a two-dimensional discrete approximation to a Gaussian distribution, which is convolved with the original image to smooth features in the image. In many cases, several fingers were present in the cropped times series, and it was necessary to remove all objects which were not the finger of interest. To achieve this, we used the MATLAB command bwlabel() to label all objects within the images, and removed all those but the desired object.

To determine the coordinates of a line that spans the width of the finger and is perpendicular to its direction of motion, we rotated the image so that the direction of motion of the finger corresponded to the x-axis. For each image, the MATLAB command regionprops() was used to identify the angle of orientation of the finger, which was the angle \( \theta \) between the x-axis and the major axis of the ellipse that has the same second-moment as the finger. We then used the MATLAB command imrotate() to rotate the images by the corresponding angle of orientation. The leading edge of the finger was identified by locating the last foreground pixel along the finger axis. The location of the lateral profile across each finger was chosen to be 40 pixels (corresponding to 6.4 μm) from the leading edge of the finger.

Since we are interested in describing features in the reference frame of the original image, we converted the coordinates of our line scan in the rotated reference frame \((x_r, y_r)\) to those in the original reference frame \((x_o, y_o)\). By using the centre of the image \((x_c, y_c)\) as the new origin, a rotation of \((-\theta)\) provided the corresponding coordinates in the reference frame of the original image:
\[
\begin{pmatrix}
    x_o \\
    y_o
\end{pmatrix} =
\begin{pmatrix}
    \cos(-\theta) & \sin(-\theta) \\
    -\sin(-\theta) & \cos(-\theta)
\end{pmatrix} \left\{ \begin{pmatrix} x_r \\ y_r \end{pmatrix} - \begin{pmatrix} x_c \\ y_c \end{pmatrix} \right\} + \begin{pmatrix} x_c \\ y_c \end{pmatrix} 
\] (3.8)

Using the coordinates of the line scan in the reference frame of the original image, the PIV displacement and orientation values along the line scan were identified from the previous analysis. To allow comparison of fingers of different widths, each finger was divided into five lateral segments and the average PIV displacement and orientation values within each segment were calculated.
Figure 3.6: Example of the procedure used to calculate the line profile of a finger. A) Image of the finger after pre-processing. B) Image after area opening and Gaussian filtering. C) Image after rotation corresponding to the finger orientation along the horizontal direction (red dashed line). The line profile is indicated as a blue rectangle. D) Image after rotation back to the original frame of reference. The line profile has been divided into five equal segments for calculation of the average PIV displacement and orientation values. The width of each image corresponds to 72 micrometers.
Chapter 4

Results and Discussion

4.1 General Description of Morphology of Expanding Bacterial Colony

Microscopy images of *P. aeruginosa* twitching at an agar–glass interface typically contain many densely packed cells collectively twitching outwards from the inoculation point. Within the growing bacterial colony, cells twitch individually and form a lattice-like network of cells. At the edge of the advancing front, the bacteria form finger-like protrusions that can contain cells in either a monolayer or multilayer, with bacteria approximately aligned with one another along the long axis of the cells. Fingers can be characterized by several parameters within a time series of images, such as average width, average speed, monolayer versus multilayer coverage, and the orientation of bacterial cells within the finger. This quantification of the finger morphology and motion at the edge of the bacterial colony is useful in characterizing the collective motion of the bacterial cells, and is distinct from the characterization of correlations between individual cells in the study by Gloag *et al.* [25].
4.2 Morphology and Edge Speed Dependence on Agar Concentration

To characterize the morphology and motion of twitching bacterial colonies at the interface between a glass slide and agar of different stiffnesses, we performed colony expansion experiments as described in Chapter 2 in which the observation time was 20 min, with 2-3 repetitions for each agar concentration ranging from 1.0 % to 1.5 %. The stiffness of agar has been measured in AFM nanoindentation experiments performed on agar gels in water using indentation speeds ranging from 0.5 to 4.0 µm/s [32]. The elastic (Young’s) modulus ($E$) of agar, as determined using a conical Hertzian-based contact model, was independent of the indentation speed and increased from $57 \pm 1$ kPa at 1.0 %, to $222 \pm 27$ kPa at 1.5 % [32], corresponding to a four-fold increase in agar stiffness.

4.2.1 Maximum Finger Width

For each time series of optical microscopy images collected during an experiment, protrusions (fingers) at the edge of the expanding colonies were identified. The length of the fingers was measured every 50 frames using the length measurement tool in Fiji. To be included in the analysis, we required that fingers contained at least 8 bacteria, while remaining separated from the rest of the colony and fully within each frame within the time series. Using these selection criteria, we identified 100-150 fingers for each agar concentration, involving multiple experiments performed under the same conditions. A box plot of the distributions of the maximum finger width as a function of agar concentration is shown in Figure 4.1. In this plot,
the central horizontal line represents the median value, and the surrounding box contains all data between the 25th and 75th percentiles. The whiskers extend to the extrema of the data, which are considered to be within 1.5×IQR (inter-quartile range). Data outside 1.5×IQR are considered to be outliers, and are represented by open circles. Solid circles represent those outliers that are outside 3.0×IQR.

Figure 4.1: Finger width as a function of agar concentration. The data shown for each agar concentration contains measurements on 100-150 fingers.

It can be seen in Figure 4.1 that the average finger width increases approximately linearly from 6 μm to 12 μm as the agar concentration is increased from 1.0 % to 1.5 %. This corresponds to an increase in width from approximately 10 to 30 cells.
4.2.2 Multilayer Coverage

We observed that fingers consisted either of a monolayer of cells for soft agar, or multilayers consisting of multiple layers of bacteria for stiff agar. The multilayer areas within fingers could be identified because they had a lower intensity (they appeared darker) than the surrounding cells within the finger. During the formation of fingers on stiff agar, the multilayer regions emerged from the stacking of one or two cells at the centre of the leading edge of a finger. The upper layer of cells then expanded to span several μm along the finger within several minutes. Optical microscopy images of representative fingers on 1.0 % and 1.5 % agar are shown in Figure 4.2.

![Figure 4.2](image1.png)

Figure 4.2: A) Optical microscopy image of fingers for an expanding colony on 1.0 % agar with monolayer coverage. B) Optical microscopy image of fingers for an expanding colony on 1.5 % agar with multilayers of bacteria localized along the centre of the length of the finger. The area of lower intensity within the blue rectangle indicates a region of multilayer coverage. The width of the images corresponds to 80 μm.
4.2.3 Average Speed of Colony Edge

For each time series of optical microscopy images, particle image velocimetry (PIV) and colony edge detection was performed as in Chapter 3, and the magnitudes of the displacement vectors were used to calculate the average speed of the colony edge for each frame. This procedure yielded 50 colony speed values during the course of each experiment. Upper and lower thresholds were implemented during the averaging procedure, such that speed values outside the bounds of 0.2 – 10 pixels per time step were discarded. The lower bound was chosen such that the speed values were distinguishable from any background noise in the PIV vector field. The upper bound was chosen to prevent the possible contribution of errant vectors not removed from the vector post processing procedure. Two or three experiments were performed for each agar concentration. A box plot of the distributions of colony edge speeds as a function of agar concentration is shown in Figure 4.3.
We observed an average colony edge speed of approximately 0.016 μm/s that was independent of agar concentration. The constant value of the speed for different agar concentrations is surprising, but perhaps it can be understood in terms of the force that must be exerted by the bacteria at the leading edge of the fingers to break through the agar-glass contact.

In the case of the less stiff 1.0 % agar, a small number of bacteria in a monolayer finger are sufficient to provide the force to break through the agar-glass interface. For larger agar stiffnesses, more bacteria are required to provide the necessary force to break through the interface. To achieve this large force, the bacteria accumulate in multilayers within larger fingers, providing more pili that exert a correspondingly larger force. Figure 4.4 shows a
schematic diagram of the vertical profiles of the bacteria within the fingers for small and large agar stiffnesses.

Figure 4.4: A) Schematic diagram of a vertical cross-section through a finger at the edge of an expanding bacterial colony for small agar stiffness, indicating monolayer coverage. B) Schematic diagram of a vertical cross-section through a finger at the edge of an expanding bacterial colony for large agar stiffness, indicating multilayer coverage.

We can obtain an order-of-magnitude estimate of the force required by a single bacterium to deform the 1% agar matrix using the relationship between stress, strain and elastic modulus (neglecting the force of adhesion between the glass and the agar). If we imagine the bacterial cell moving at the agar-glass interface as displacing the agar by the width ($\delta L \sim 0.5 \mu m$) of the bacterial cell, and that the strain propagates into the agar a length comparable to the length of the bacterial cell ($L \sim 2 \mu m$), we can write the stress experienced by the bacterium as:

$$\sigma = \frac{F}{A} = E \epsilon \sim E \frac{\delta L}{L} \sim E \left( \frac{1}{L} \right)$$
where $\varepsilon$ is the strain, and $L$ is the length of the bacterial cell. For the 1.0% agar concentration ($E = 57$ kPa as measured using AFM [32]), the value of the force corresponds to

$$F \sim \frac{1}{4} EA \sim 12 \text{ nN}$$

This value of the force is approximately two orders of magnitude higher than measurements of the retraction force attributed to a single pilus [6]. A possible reason for the discrepancy is that the elastic modulus $E$ of the agar [32] was measured for AFM cantilever loading rates ranging from 0.5 to 4.0 $\mu$m/s, which is at least a factor of 30 larger than the average speed of the bacteria in a finger (0.016 $\mu$m/s). For a viscoelastic material such as agar, the modulus at smaller speeds could be considerably smaller. Despite the uncertainty in the agar modulus value at speeds comparable to the speed of the bacteria, this simple calculation suggests that the collective action of many bacteria with multiple pili in a finger-like protrusion is necessary to generate sufficient force for forward motion of the finger.

### 4.3 Line Profiles Across Fingers

We calculated line profiles of 12 fingers in time series of optical microscopy images measured in several experiments on 1.5% agar. The relatively large fingers in these high agar concentration colonies are more amenable to our analysis procedure (Chapter 3) for both PIV and bacterial orientation. The number of fingers used in this analysis was small because of the relatively small number of fingers within the field of view, as well as the unsuitability of many fingers because of their participation in collisions with other fingers, or their movement out of the field of view during the time series of images.
For all line profile analyses, we distinguished between straight and curved finger trajectories: straight portions of the trajectories were considered to be those in which the finger deviated by less than 30° from the original finger axis during the course of the experiment. We calculated the line profiles of both curved and straight trajectories over the entire experiment. Each complete experiment yielded 50 profiles for each finger over the course of each experiment.

Because of the small number of fingers included in the analysis, we also calculated the line profiles for straight sections of otherwise curved trajectories. In these cases, we limited the range of images included in the analysis to include only the motion for which the path direction deviated by less than 30°. The finger axis was then calculated for the partial time series of images, and a visual inspection was carried out to ensure that all images in the partial time series satisfied this criterion. The partial trajectories yielded 15 - 40 profiles over the course of the experiment. Of the 12 fingers included in our analysis, 10 were limited to partial trajectories.

### 4.3.1 Average Speed Line Profiles

To characterize the motion of the fingers, we calculated the magnitude of the velocities across each line profile using the PIV analysis data. We observed that, for fingers with straight trajectories over the entire experiment, the average speed was largest along the centre of the finger, reminiscent of the flow of a liquid in a pipe. A box plot of the average speed line profiles for a representative finger with a straight trajectory is shown in Figure 4.5.
Figure 4.5: Average speed line profile distribution across a representative finger moving along a straight trajectory. Within each of the five sections (quintiles) across the finger, there are 50 data points corresponding to the evaluation of the velocity magnitude at different times during the experiment. The rather wide speed distributions result from variability in finger speed over the time scale of the experiment.

Qualitatively, we observed that, for fingers travelling along curved trajectories, the average speed line profiles were asymmetric. A representative example of the speed line profile for such a finger is shown in Figure 4.6.
Figure 4.6: Velocity magnitude line profile distribution across a representative finger that executed a clockwise curved trajectory. Within each of the five sections (quintiles) across the finger, there are 20 data points corresponding to the evaluation of the velocity magnitude at different times during the experiment. The maximum average speed occurred in the rightmost quintile. The average speed of the finger was comparable to that of a finger travelling along a straight trajectory.

Combining the speed line profiles from all straight trajectories (complete and partial) into the box plot shown in Figure 4.7, we can see that there is a slight increase of the average speed in the central quintile relative to that in the edge quintiles, but the lateral variation is smaller than that shown for a single finger with a straight trajectory in Figure 4.5.
Figure 4.7: Average speed line profile distributions for all trajectories (complete and partial) included in the line profile analysis. Each quintile contains at least 150 data values.

The smaller variation of the average speed for the combined line profiles shown in Figure 4.7 is most likely due to slight curvatures that are present in the finger trajectories, as well as variation in the average speed between different fingers. We note that the average speed in each quintile in Figure 4.7 is comparable to the colony edge speed of 0.016 μm/s (Figure 4.3).

Our line profile analysis indicates for curved finger paths, the quintile of maximum speed often occurs on the inner curve of the finger path, i.e. a finger with maximum average speed in the right edge quintile rotates clockwise. Additional experiments are required to verify this preliminary result with a statistically significant number of curved finger paths.
4.3.2 Orientation Line Profiles

In addition to the average speed line profiles, it is also possible to calculate the average orientation line profiles for the same set of fingers traveling in straight trajectories, as described in section 3.5. As with the average speed line profiles, 15-50 lateral profiles were obtained for each complete trajectory for each experiment. For each lateral profile, the orientation angle is calculated as the angular difference from the direction of the finger axis. The orientation lateral profile for a representative finger with a straight trajectory (the same finger as in Figure 4.5) is shown in Figure 4.8.

**Figure 4.8:** Average orientation angle distribution across a representative finger moving along a straight trajectory (the same finger as in Figure 4.5). Within each of the five sections (quintiles) across the finger, there are 50 data points corresponding to the evaluation of the orientation angle.
at different times during the experiment. Each orientation angle is measured relative to the direction of the finger axis.

Qualitatively, we observed that for fingers travelling in straight trajectories, the average orientation angle was independent of position along the finger, and in each quintile 50-100% of the orientation angles were within $20^\circ$ of the finger axis.

Combining the orientation angle line profiles from all straight trajectories (complete and partial) into the box plot shown in Figure 4.9, we can see that average orientation angle for all fingers is independent of position across the finger.

Figure 4.9: Average orientation line profile distributions for all trajectories (complete and partial) included in the line profile analysis. Each quintile consists of at least 150 data values.
The average orientation angle for each quintile in Figure 4.9 is approximately centred about 0, with more than 50% of measured angles within 20° of the finger axis, indicating that the bacteria within a finger are predominately aligned along the direction of the motion of the finger.

### 4.4 High Resolution Studies

To characterize the motion of isolated twitching bacteria, and obtain high temporal and spatial information about individual fingers, we performed 6 experiments using a 100x objective and an image collection speed of 10 or 100 frames per second.

#### 4.4.1 Single Cell Observations

In all experiments, we observed a small number of bacteria in the lattice network that traveled approximately 1 μm in less than one tenth of a second. An example of such a bacterium is shown in Figure 4.10.
Figure 4.10: A) Optical microscopy image of a small area within the lattice network of a bacterial colony expanding on 1.1% agar. The bacterium of interest is the rightmost bacterium within the blue box. B) Optical microscopy image collected 0.05 s after image A. Within this short time, the bacterium of interest has moved ~ 1 μm. The width of the images corresponds to 22 μm.

The speed of such an event is typically 15-20 μm/s, which is considerably larger than the 0.5 μm/s of Skerker and Berg’s measurements of \(P. \text{ aeruginosa}\) pilus retraction speed. As we cannot currently visualise the pili in these experiments, we are unable to determine if these events are the result of the retraction of a single pilus or if this corresponds to a more complex, collective motion of pili.

In several cases, bacteria within the fingers were observed to reverse their direction multiple times, suggesting the possibility of a “battering ram” mechanism for the bacteria to break through the agar-glass interface.

### 4.5 Control Experiments

To ensure that the observed motion of bacterial cells at the agar-glass interface was solely due to twitching motility, control experiments were performed under the same conditions as described in Chapter 2 on 1.1% agar with the \(Pseudomonas \text{ aeruginosa}\) mutant \(fliC\), a flagellum-deficient strain. The morphology and speed of the advancing colony of the \(fliC\) mutant were
consistent with those observed on 1.1% agar with the PAO1 strain. Representative images of the colony edges for both strains are shown in Figure 4.11.

Figure 4.11: A) A representative optical microscopy image of a twitching PAO1 colony on 1.1% agar. B) A representative optical microscopy image of a twitching \textit{fliC} colony on 1.1% agar. The width of the images corresponds to 128 µm.
Chapter 5

Summary and Conclusions

5.1 Summary of Results

We have developed an experimental procedure that allows for the creation of a pristine agar-glass interface for bacterial motility experiments. We have observed the collective motion of twitching colonies on agar substrates of varying stiffness, and performed high resolution microscopy studies of these colonies.

We have developed a variety of image processing procedures to characterize the morphology of the expanding bacterial colonies utilizing Particle Image Velocimetry (PIV) and Fourier transform techniques. We find that the edge of the colonies consists of finger-like protrusions (fingers), with a lattice-like arrangement of cells within the colonies, consistent with previous observations of twitching colony morphology [1, 4]. In the analysis, the edge of the twitching colony was determined, and line profiles of the average speed and average bacterial orientation across the fingers were calculated. These properties were characterized for agar concentrations ranging from 1.0 % w/v to 1.5 % w/v, for which the stiffness of the agar increases by a factor of
four. We find that the average finger width increases approximately linearly with agar concentration, whereas the average speed of the fingers is essentially independent of agar concentration. This latter result is coupled with our observation of a transition from monolayer coverage to multilayer coverage within the fingers as the agar stiffness is increased, and suggests that a critical number of T4P are required for the bacteria to break through the pristine agar–glass interface. Within the line profiles of fingers traveling in straight trajectories, we find that the maximum finger speed occurred in the centre of the finger, and that the bacteria cells were highly aligned along the finger axis.

In high resolution studies, we observed motion of individual bacterial cells within the lattice-like network in which cells occasionally moved at speeds that were considerably larger than the pilus retraction speed measured previously. This may be due to a pilus slingshot mechanism suggested by Jin et al.. Although bacteria typically moved along the direction of the finger motion, some cells were observed to reverse their direction of motion within a finger multiple times, suggesting the possibility of a “battering ram” mechanism for breaking through the agar-glass interface.

### 5.2 Future Work

The results of this thesis have led to the development of robust experimental and image analysis techniques, and this has allowed the observation of some interesting results that should be investigated further. Further experiments on 1.5% agar should be performed so as to provide a more statistically significant data set for the line profile analysis of fingers.
It would be useful to develop a procedure to quantify the cell reversals within fingers at high time resolution, as analysis of this motion could lead to a better understanding of the mechanism by which the cells collectively break through the agar-glass interface. This could be achieved by possibly adapting a particle tracking algorithm to track the motion of each cell within the fingers. Recent work by Vallotton et al., has shown that it is possible to segment and track individual bacterial cells in a dense environment [33]. Additional experiments could be performed to observe these interesting bacterial reversals in fingers over longer times.

It would also be beneficial to quantify the motion of the cells within the lattice network in the high resolution microscopy videos. This could also perhaps be achieved with particle tracking. The individual twitching behaviour of these cells could be compared with the observations of Jin et al., who provided evidence for both a slow crawling motion and a fast slingshot motion [20].
Bibliography


   2002.

[5] Shun Lu, Maximiliano Giuliani, Hanjeong Harvey, Lori L. Burrows, Robert A. Wickham and 
   John R. Dutcher. Nanoscale Pulling of Type IV Pili Reveals Their Flexibility and Adhesion to 


