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Bacterial motility on abiotic surfaces

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Bacterial motility on abiotic surfaces

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of the requirements for the degree
Doctor of Philosophy in Bioengineering

by

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Bacterial biofilms are structured microbial communities which are widespread both in nature and in clinical settings. When organized into a biofilm, bacteria are extremely resistant to many forms of stress, including a greatly heightened antibiotic resistance. In the early stages of biofilm formation on an abiotic surface, many bacteria make use of their motility to explore the surface, finding areas of high nutrition or other bacteria to form microcolonies. They use motility appendages, including flagella and type IV pili (TFP), to navigate the near-surface environment and to attach to the surface. Bacterial motility has previously been studied on a large scale, describing collective motility modes involving large aggregates of cells such as swarming and twitching. This dissertation provides an in-depth look at bacterial motility at the single-cell level, focusing on *Pseudomonas aeruginosa* and *Myxococcus xanthus*, two commonly-studied organisms; in addition, it describes particle tracking algorithms and methodology used to analyze single-bacterium behaviors from flow cell microscopy video. *P. aeruginosa* flagella are used in swimming but also in surface-bound spinning, which is a precursor to detachment; for *P. aeruginosa*, flagella and TFP work synergistically in a detachment sequence. *P. aeruginosa* TFP drive walking, a surface-bound exploratory motility mode in which the bacterium is oriented normal to the surface, as well as crawling, in which the bacterium is oriented parallel to the surface and moves
directionally. The transition between the two modes is affected by cyclic di-GMP, which *P. aeruginosa* uses as a global regulator of biofilm formation. *M. xanthus* does not swim or walk, but when stimulated to initiate social motility it exhibits a slow pili-driven crawling behavior. By imaging this motion at high frame rates, we see earthquake-like sticks and slips; these are well-described by crackling noise friction models, and are caused by the friction between the bacterial body, bacterial exopolysaccharides, and the surface. This crackling noise analysis reveals that *M. xanthus* EPS is an efficient lubricant as well as a glue, and facilitates the social motility of connected cells.
The dissertation of Maxsim L. Gibiansky is approved.

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2013
To everyone who supported me though my time in graduate school.
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\[ c(v_1, v_2) = \frac{(v_1 - \bar{v}_1)(v_2 - \bar{v}_2)}{|v_1 - \bar{v}_1||v_2 - \bar{v}_2|} \] .......................................................... 64

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(2011) *Stick-slip behavior of Myxococcus Xanthus S motility observed by high-framerate microscopy and particle tracking algorithms.* Maxsim Gibiansky, Wei Hu, Fan Jin, Kun Zhao, Wenyuan Shi, and Gerard C. L. Wong. UC Systemwide Bioengineering Symposium


CHAPTER 1

Introduction to Biofilms

1.1 What is a biofilm?

Bacteria have often been studied in liquid culture, where cells are able to grow and swim freely; sparsely distributed bacteria can be thought about individually, with the behavior of a large group of bacteria being similar to the behavior of the individual cells. However, in nature, bacteria often congregate on surfaces in aggregates called biofilms [1, 2]. These biofilms are multicellular surface-bound communities [1, 3, 4], and are macroscopically observable as layers of bacterial slime of various thicknesses. Both the colorful streaks in the hot springs of Yellowstone National Park and the thin film of brown sludge on an office water cooler are created by bacterial biofilms; they appear in a wide variety of environments, both natural and artificial [5, 6, 7, 8]. Organisms were conglomerating into biofilms long before true multicellularity evolved, as biofilms are an ancient prokaryotic adaptation and are found in the fossil record as far as 3 billion years ago [5]. Indeed, a biofilm can be considered as a prototype multicellular organism [9, 10, 11]. Cells in biofilms can differentiate into dormant “persister” cells which resist stress, dividing cells which are vulnerable to stress but reproduce rapidly, stationary cells which build up the biofilm matrix and motile cells which migrate to form channels, and even cells which die so that the biofilm as a whole can survive [12]. Nevertheless, biofilms are also hotbeds of competition. Even though laboratory investigations usually study one bacterial strain at a time, multi-species biofilms are common in nature [13]. Indeed, marine biofilms often grow from thin films of
bacteria to involve algae, protozoans, and larger organisms. Bacteria have evolved complex mechanisms for identifying whether their neighbors are their family or are enemy species, and have evolved a variety of ways for either cooperating with, attacking, or escaping from these other bacteria.

Bacterial biofilms are also found on artificial surfaces and impact many technological fields. Attempts to control or prevent biofouling of surfaces is a multi-billion dollar industry. Most applications which involve a liquid interface must contend with the formation of a biofilm at the interface. Biofilms form on the hulls of ships, degrading their hydrodynamic performance; biofilms form in pipes which deliver drinking water and on food preparation equipment, and are a major concern in water treatment plants. In addition, bacterial biofilms are of extreme importance in medical settings. Artificial implants often fail due to the formation of bacterial biofilms at the implant surface [14]. They are commonly found on catheters, where they form a bacterial reservoir that cannot be eradicated by the host immune system and which continually releases infectious bacteria into the blood. To make matters worse, bacteria in biofilms are extremely resistant to many forms of stress. Due to the physical protection of the biofilm matrix and the variety of expressed phenotypes, they have an antibiotic resistance which is heightened by up to a factor of 1000 relative to planktonic cells [15, 16, 17]. They are also more resistant to heat, starvation, radiation, and the human immune response.

The transition from free-swimming, planktonic cells to a surface-attached biofilm precipitates many changes to bacterial behavior. Even though cells in a biofilm are genetically identical to the free-swimming planktonic cells, they have significantly different gene expression, and a radically different phenotype. While a biofilm lifestyle can favor certain mutations over others, the transition from planktonic to sessile behavior does not require genomic changes - nevertheless, individual cells plucked out of a biofilm behave quite differently than cells which
have not been exposed to the biofilm environment.

1.2 Life cycle

The biofilm life cycle is often viewed as consisting of five broad stages: initial attachment, EPS production and irreversible attachment, microcolony formation, biofilm maturation, and biofilm dispersal [18]. Though there has been some debate over whether this is a true developmental program or a reversible sequence of events, it is nevertheless instructive to consider biofilm formation through this lens and treat it as a starting point for further understanding [19].

The first step in biofilm formation is initial attachment to the surface. Planktonic cells must come in contact with the surface, either via an active mechanism such as flagellum-driven swimming or via passive diffusion. They then attach to the surface, either passively by physical interactions or via active attachment by bacterial appendages. It is known that the efficacy of bacterial attachment is affected by the properties of the surface [20], the conditioning of the surface by other organic molecules [21], as well as the properties of the bacterium [22, 23]. In addition, attachment to the surface is affected by the nutrient conditions in the bulk [24]; depending on the bacterial species, either starvation conditions or the presence or absence of specific nutrients can trigger biofilm formation [25, 10, 26]. This can be viewed as a bacterium integrating information from its environment in order to make the decision of whether a biofilm state is more or less advantageous to it than remaining planktonic.

Following initial attachment, the bacteria begin production of EPS and transition to irreversible attachment. This process is still poorly understood; many necessary components and behaviors have been located, but how they fit together remains unclear [27]. In some species, such as P. aeruginosa, the transition from reversible to irreversible attachment occurs via a switch from polar to lateral sur-
face attachment. At this stage of biofilm formation, bacteria begin to express a variety of species-specific adhesion proteins, which anchor the bacteria to the surface. Adhesin-deficient mutants are unable to make the transition, and continue to transiently attach and detach when adhesin-competent cells remain on the surface and progress to the next stage of biofilm formation. Cyclic-di-GMP (3’-5’-cyclic di guanylic acid) is considered to be a master regulator for this transition in many bacterial species [28, 29, 30, 31]. It binds to a diverse range of targets and has direct affect on polysaccharide production, adhesion, and bacterial motility. High c-di-GMP levels lead to increased biofilm formation through a variety of distinct methods. Bacterial motility [22, 32, 33], exopolysaccharide (EPS) production [34, 35], and quorum sensing also contribute to irreversible attachment [36].

As the bacterial density on the surface increases, the cells self-organize into dense microcolonies [37, 33]. These microcolonies grow and mature into 3D structures composed of thousands of cells, held together by a mixture of extracellular DNA [38, 39, 40], secreted polysaccharides [41, 42], and a variety of other proteins, glycoproteins, and glycolipids [43, 44]. Common biofilm morphologies include flat biofilms, mushroom-shaped stalks separated by channels, and streamers in flow [45, 33, 18, 46, 47, 48].

Finally, the mature biofilm releases cells back into the medium. Like the earlier stages in biofilm formation, dispersal can also be triggered by changes in nutrient conditions, bacterial density, and interbacterial signaling [49, 50, 51, 52]. Dispersal can proceed gradually, or via a sudden “seeding” dispersal in which the biofilm undergoes internal disintegration and leaves behind hollow shells. [53, 54].
1.3 Bacterial motility

Biofilm formation, development, and growth depend critically on how planktonic bacteria adapt their motility mechanisms near a surface [55, 22, 56, 57, 33, 58, 59]. For P. aeruginosa, a commonly studied organism for biofilm formation [60], motility is driven by two types of appendages: a single polar flagellum and multiple type IV pili (TFP). Defects in either flagellum or TFP function result in biofilm formation defects and decreases in virulence.

The flagellum is a helical filament, approximately 5µm in length, which rotates and generates force via the hydrodynamic drag opposing its rotation [61]. It is driven by a 50-nm rotary motor embedded in the cell membrane. This motor is powered by protons moving down an electrochemical gradient, and not directly by ATP. Measurements on Streptococcus cells indicate that it takes approximately 1200 protons to carry out one full flagellar rotation [62], with typical rotation speeds being on the order of 100 Hz. Even though the typical picture of flagellar motility involves the flagellum oriented behind the bacterium and pushing it forward, it is also possible for a singly-flagellated cell, such as P. aeruginosa, to be pulled along behind its flagellum. In multi-flagellated cells such as E. coli, the flagella exhibit cooperative behavior. When all the flagella rotate in the same direction, they are pulled together and form a flagellar bundle that causes the cell to move forward. However, when one of the flagella reverses direction, the flagellar bundle breaks apart and the cell tumbles, ceasing its forward motion and rotating rapidly. This randomizes the cell’s direction of motion. This “run and tumble” behavior is used for chemotaxis, as bacteria can modulate their tumble rate based on environmental gradients [63, 61, 64, 65].

Type IV pili (TFP) are thin filaments, approximately 6 nm in diameter and between 1 and 5 µm long, which typically form at cell poles [57, 66, 67]. The pili are somewhat inflexible, with a persistence length of 5 µm. They extend to their
full length of 500-1000 pilin sub-units by a polymerization reaction inside the cell membrane. The tip of the extended pilus then attaches to the surface [68, 66]; the rest of the pilus does not display any adhesive properties. In *P. aeruginosa*, this binding is nonspecific, but for *M. xanthus* the pilus tip also functions as an exopolysaccharide sensor, and in *Neisseria* strains the pilus tip has specific interactions with human epithelial cells [69]. *V. cholerae* produces multiple types of pili for attachment to different surfaces. After attachment, the TFP retract via depolymerization, acting as linear actuators that pull the bacterium along a surface [70, 71]. Typical forces exerted by bacterial pili range from 10 pN to 200 pN, though multiple pili can cooperatively generate even higher forces [71, 66, 72, 73].

Bacteria can move collectively on surfaces using distinct appendage-specific motility modes [74]. Flagella are implicated in swarming, a motility mode used for colony expansion along a semisolid surface such as agar [75]. Though TFP also contribute to swarming, swarming bacteria are often hyperflagellated, expressing multiple flagella even in species such as *P. aeruginosa* which are typically singly flagellated [76, 75]. In a swarm, adjacent bacteria align into rafts and coordinate their flagellar action in order to create a tendril of bacteria which moves away from the center of the colony. Repeated swarming interrupted by growth and division can lead to a characteristic biofilm morphology composed of concentric terraces. Swarming cells secrete rhamnolipids which serve as a surfactant to promote colony migration and as a signal to prevent tendrils of migrating bacteria from crossing [77]. A variety of external signals, such as nutrient conditions and the viscosity of the medium, contribute to the transition to swarming behavior; investigators have identified additional factors that can influence collective motility, such as biosurfactants [58, 78, 79, 80] that play a key role in swarming.

TFP mediate twitching, a motility mode commonly observed in dense aggregates with cell-to-cell contact [57, 81, 82]. Unlike swarming, which uses both flag-
ella and TFP, twitching is solely pili-driven [83]. As with swarming, bacteria can accumulate into rafts, but can also move singly; the cells move in a characteristic “jerky” manner which gives twitching motility its name. The efficacy of twitching depends both on extrinsic factors such as the nutrient conditions, the viscosity of the medium, and the surface hydrophobicity, as well as on intrinsic factors such as the number of pili per cell and their retraction rates; typical twitching rates are on the order of 1mm per hour [84]. The individual pili on a bacterium can act both individually and cooperatively, though how cooperativity between pili is achieved during twitching is unknown. Twitching and swarming surface motility modes are both coupled to signaling networks and nutritional sources, and enable exploration of newly colonized surface environments [58, 85, 86].

Swarming and twitching are predominantly studied with the use of plate assays, in which an increase in agar concentration drives the transition from swarming to twitching [75, 86, 87, 88]. Cells are inoculated at the center of an agar plate, with carefully controlled agar and nutrient conditions, and colony expansion after some period of time, typically 12-72 hours, is recorded. This methodology can compare colony morphologies generated by different mutant strains in order to identify genes and proteins critical for motility, but does not access the microscopic behavior of individual cells. Such bulk assays are also ill-suited for observing the cooperation between distinct motility appendages that must occur when multiple appendages are available; they also do not allow investigation of the general conditions that promote the selection of specific motility modes.

At present, little is known about motility of individual cells in the initial stages of biofilm formation, during which bacteria transition from a free-swimming planktonic state to a surface-associated state and subsequently form microcolonies. Assays have shown that both flagella and TFP influence these developmental steps [22, 33, 89], as deleting or altering either appendage leads to variations or deficiencies in cell attachment and growth. To understand surface motility in the
low-density transition regime, it is necessary to correlate the spatiotemporally
resolved motion of individual bacteria to life-cycle events such as attachment, de-
tachment, and division. Genetic techniques can identify specific appendages that
mediate collective motility modes; however, these methods do not probe single-
bacterium behavior. Conversely, single-cell techniques can measure forces exerted
by motility appendages [59, 71, 72, 90] but do not probe collective behavior or
interbacterial interactions. To quantify the initial stages of biofilm formation, dur-
ing which individual bacteria may rapidly change their behavior in response to
their environment [33, 60], we develop new particle tracking techniques, similar to
those previously used in colloid physics, that can couple single-cell resolution with
large sample populations. These techniques allow us to simultaneously access the
trajectories and surface behaviors of many interacting bacteria over long periods
of time.
CHAPTER 2

Particle Tracking Algorithms

2.1 Motivation

The bacterial transition from the free-swimming planktonic state to a mature biofilm is a process which can take multiple days and which involves many changes in bacterial behavior [91, 92]. This is often studied with techniques with low resolution in both time and space, describing overall biofilm characteristics such as shape or total mass; the COMSTAT program is a commonly used tool for such analyses. [93, 94, 95]. However, there is a wealth of information that can be exploited if the biofilm is observed at a single-bacterium level with high time resolution. A single brightfield microscopy video taken at a time resolution of 20 frames per minute over a $100\mu m \times 100\mu m$ field of view for 12 hours will take up tens of gigabytes of space and have the full motility history of thousands of cells, which cannot be analyzed with global measurements but instead require specialized software. High-resolution measurements (400 frames per second for 5 minutes) lead to similar difficulties. In addition, the relevant metrics of cell motility vary greatly depending on the research problem; different measurements are necessary for every new investigation. To handle these issues, we developed two distinct tracking methodologies which are discussed below.
2.2 Minimum displacement tracking

We began by modifying particle-tracking algorithms previously used in colloid physics and written in IDL (ITT VIS, White Plains, NY) [96]. The first step in the tracking process is to filter the images to reduce the bacteria to bright features on a dark background. This is accomplished with a collection of image processing techniques, applied individually to each image in the movie, with parameters selected individually for each image type.

**Background subtraction.** For brightfield images, a background image is taken by refocusing the microscope into the coverslip, below the surface where the bacteria are, resulting in an image of all of the non-bacterial features in the image path - the overall brightness and brightness gradient of the image, and any contaminants in the light path or in the lenses. This background image is subtracted from each target image, resulting in a clean image of the bacteria. The background image may need to be scaled in brightness to match the target image if the image brightness changes over the course of the movie.

**Bandpass filter.** A 2D bandpass filter can be applied to the target image; the lower and upper limits of the bandpass are set to half the width of a bacterium and the length of a bacterium, respectively. This is most critical for fluorescence images where a background subtraction is not done; it is unnecessary for brightfield images where the bacteria are significantly brighter than the background.

**Backbone enhancement.** Pixels along the center of the bacterial body can be identified since they are local maxima along all directions except the direction of the bacterial body. These pixels, defined as those who are local maxima along three of four tested directions, are enhanced in brightness.
**Thresholding.** A threshold could be applied before or after bandpass filtering or background subtraction, to remove features which are too dim to be bacteria.

Since the bacteria are rod-shaped, they cannot be located by finding local maxima in the image [96]. Thresholding reduces the bacteria to bright spots on a dark background, and they are located by locating all bright connected features larger than a minimum length. The pixels comprising each bacterium are analyzed to determine single-bacterium properties. The position and orientation are calculated from the moments of the backbone distribution [97]. To determine the length and width of the bacterium, we rotate each feature by its orientation angle and then calculated the maximum x and y distances between the pixels comprising the bacterium. The positions of the two endpoints are calculated based on the length, centroid, and orientation. The aspect ratio is calculated by dividing the length by the width. We classified the bacteria as horizontal or vertical using a cutoff generated from the histogram of bacterial lengths; bacteria below the cutoff were considered vertical, whereas bacteria longer than the cutoff were considered horizontal.

Particles are linked into trajectories via a minimum squared displacement criterion. Given the positions for \(n\) particles at time \(i\), and \(m\) positions for particles at time \(i + 1\), the software examines all possible connections from \(n\) old particles to \(m\) new particles, constrained by the requirement that particles cannot move more than a preset distance \(D\) between frames and selects the set of connections that minimizes the total squared distance all bacteria travel. The preset distance \(D\) is selected based on the mean separation between particles - if between two frames, a bacterium moves more than its distance from its neighbors, then it cannot be tracked.

Dynamical properties are then calculated for each of the trajectories, now represented as a sequence of \(x\) and \(y\) positions.
Velocity. We calculate the velocity of bacterium $b$ at time $t$ as $v_x(t, b) = \frac{x(t+1, b) - x(t, b)}{2}$, $v_y(t, b) = \frac{y(t+1, b) - y(t, b)}{2}$, $v(t, b) = \sqrt{v_x(t, b)^2 + v_y(t, b)^2}$, where $x(i, b)$ is the $x$ coordinate of bacterium $b$ at time $i$, and $y(i, b)$ is the $y$ coordinate of bacterium $b$ at time $i$.

Angular velocity. We calculate the angular velocity as $v_\theta(t) = \frac{\theta(t+1) - \theta(t)}{2}$

Angle deviation. We calculate the angle deviation as the angle between the body axis of the bacterium and the velocity vector, limited to the range $0$ to $\frac{\pi}{2}$.

MSD. The mean-squared displacement (MSD) is $\Delta x^2(\Delta t) = \langle (x(t + \Delta t) - x(t))^2 \rangle$, where the angled brackets denote an average over all times $t$.

Directional persistence. The directional persistence length $L_p$ of a single bacterium track is the length scale over which correlation in the direction of motion is lost, and is defined via $\cos \theta_{ij} = e^{-L_{ij}/L_p}$, where $L_{ij}$ is the integrated track length between time $i$ and time $j$, and $\theta_{ij}$ is the angle between the velocity vectors at times $i$ and $j$.

This approach for bacterial tracking is most effective at the early stages of biofilm formation when bacteria are sparse. It allows for easy access to the trajectories and dynamic properties of individual bacteria, and is quite fast. However, it encounters difficulties as the bacteria increase in density. The speed of the algorithm is limited by the density of the densest portion of the image, leading to slow tracking when one microcolony forms. In addition, this tracking method is ineffective for tracking cell divisions, since the separation between bacterium identification and trajectory composition steps does not preserve the connection between daughter cells. For accurate tracking of dense coverages and division, we develop a novel tracking methodology in MATLAB.
2.3 Adjacency tree tracking

The adjacency tree tracking algorithm, rather than reducing each bacterium to a point particle with a set of numerical properties, maintains a list of all the pixels which comprise each bacterium. The image preprocessing step remains unchanged from the previously described minimum displacement tracking; background subtraction, an optional bandpass, and thresholding are used to reduce the image to a set of bright bacteria on a dark background. These bacteria are located, as before, by leveraging existing image processing tools to find connected components. This code was written in MATLAB and used the Image Processing toolbox for this task.

The same connected components methodology is used to identify connections between bacteria in different frames. The images are treated as a three-dimensional matrix, with the third dimension being the frame number; thus, a connected component which spans multiple frames represents a bacterium which is present for multiple frames. In this case, the tracking relies on the assumption that between frames, the bacteria move less than their average width. When a bacterium divides, its 3D trace will split from being a single 2D connected component to being multiple connected components; conversely, multiple bacteria coming together to form a cluster will be evident in the form of multiple 2D connected components which are connected to each other through a future frame.

To increase tracking accuracy, minimum squared displacement tracking is used to reconnect trajectories of bacteria which move further than their radius between frames, after all the overlap connections are made using the connected components method.

This data is stored as a set of binary trees; for convenience, the whole data structure is treated as a single tree with multiple roots. Each bacterium that attaches on to the surface from the medium is stored as a root. When the bacterium
divides, that event is labeled as a node of the tree, with two outgoing branches of the node representing the two daughter cells and the incoming branch of the tree representing the parent bacterium. When two bacteria come together to join into one cluster, that is likewise represented by a node with two incoming branches and one outgoing branch; and, lastly, when bacteria leave the surface, this event is represented by a leaf of the tree. The properties of the bacteria and of their trajectories can then be analyzed as before, via the sequence of positions of a bacterium; however, division and clustering events are detected directly, as nodes in the tree.

2.4 Algorithm comparison

It is instructive to compare the two approaches and compare their strengths and weaknesses.

2.4.1 Memory

When preprocessing the image, both approaches need to load an entire image into memory. The squared-displacement tracking method reduces each such image to a set of entries with the properties of the bacteria. Storing the processed data requires tens to hundreds of megabytes, and the entire data structure can be easily loaded into memory. The tree-tracking method retains all of the original pixels in the bacterium in the data structure; for a typical 1-2 day dataset, this will take up multiple gigabytes. As the whole tree must be loaded into memory for analysis, this is significantly more memory-intensive; the memory capabilities of a the computer set the limit to how large a dataset this algorithm can handle. For both algorithms, the memory usage scales with the total number of bacteria in the field of view.
2.4.2 Speed

As both approaches use similar image processing techniques, there is no difference in the image processing step. For squared-displacement tracking, the speed is dictated by the number of possible connections between frames that must be analyzed; this is proportional to $2^d$ where $d$ is the density of the bacteria. Crucially, since the relation is exponential, this is dominated by the highest-density region in the image; the algorithm slows down as soon as the first microcolony forms.

The tree-tracking method is unaffected by the density of cells, and its speed scales with the total number of cells, regardless of how they are distributed. The higher memory requirements for the tree-tracking technique also lead to a significant slowdown both during initial tracking and when loading, saving, or processing the data during future analysis.

2.4.3 Trajectory analysis

The squared-displacement tracking is more effective for analyzing trajectory properties due to the data structure it generates. The data is stored as a list of cell positions, requiring no additional processing to prepare for trajectory analysis. It is straightforward to link the dynamic properties of a trajectory with the instantaneous properties of a cell. The tree-tracking algorithm, on the other hand, stores the data in tree form; calculating trajectory properties requires first extracting the data in linear form from the tree, and being careful to handle division nodes and clustering nodes properly. This creates additional programming requirements for even simple measurements, and causes an extra speed bottleneck.

2.4.4 Social microbiology analysis

The tree-tracking methodology is more powerful for analyzing social events - division, clustering, and bacterial interactions. These events are stored natively as
nodes in the tree and can be easily located and accessed. Since the full pixel array for each bacterium remains stored in the data structure, it is possible to analyze a feature’s shape in the context of its motility history to locate clusters which have been treated as individual bacteria, and to analyze dividing cells.

The squared-displacement tracking, on the other hand, does not lend itself to this approach. The moment when a bacterium divides has to be located algorithmically; one of the daughter cells will be arbitrarily assigned to be the “same bacterium” as the mother cell, whereas the other one will be treated as a newly-attached cell. It is possible to locate divisions by analyzing the length of the bacteria and their behavior around the time of division, but this is often error-prone. Since the full bacterial image does not remain stored, it is not viable to check for overlap between daughter cells and mother cell directly, and the overlap has to be inferred from single-bacterium measurements.
CHAPTER 3

Flagella and Pili-Mediated Near-Surface Single-Cell Motility Mechanisms in P. aeruginosa

3.1 Overview

We extracted the motility histories of individual surface-associated P. aeruginosa cells by translating video microscopy movies into searchable databases of bacterial behavior using the tracking methods described in Chapter 2 [96, 97]. We designed automated searches of bacteria trajectories to identify life-cycle events (e.g., attachment, detachment, division) that were then correlated to patterns of surface motility. Using this search-engine strategy, we quantitatively characterized four fundamental appendage-specific surface motility mechanisms of P. aeruginosa that imply different strategies for surface exploration (Figure 3.1).

TFP mediate two surface motility mechanisms: crawling, by which the bacterium moves lengthwise with high directional persistence, and upright walking perpendicular to the surface, by which the bacterium moves with low directional persistence to rapidly explore microenvironments. The flagellum mediates two additional mechanisms: swimming and surface-anchored spinning, which often precedes detachment from a surface. Cooperation between different appendages influences motility after division and before detachment. These motility mechanisms have striking implications for early biofilm formation. We show that by
Figure 3.1: Images of surface motility mechanisms observed for *P. aeruginosa*. (A) TFP-driven vertical walking, in which the bacterial body (green) is oriented perpendicular to the surface (blue) and the pili (orange) pull the bacterium with low directional persistence. (B) TFP-driven crawling, in which the bacterium is oriented parallel to the surface and moves directionally. (C) Flagellum-driven near-surface swimming. (D) Flagellum-driven surface-attached spinning, in which the flagellum anchors to the surface and spins the bacterium, which may be tilted away from the surface.

enabling vertical orientations that facilitate detachment from a surface, TFP contribute to uniform biofilm morphologies.

These distinct motility mechanisms can be observed in *P. aeruginosa* shortly after attachment, before microcolony formation [98, 99]. Flagella-deficient Δ*fliM* mutants, whose movement is strictly TFP-dependent, “crawl” along their body axis with high directional persistence when oriented horizontally, parallel to the
Figure 3.2: MSD properties of $\Delta fliM$ bacteria. Walking bacteria (red) move with slope = 1.1, horizontal bacteria (blue) are subdivided into a superdiffusive (slope=1.4) population and a subdiffusive (slope=0.8) population. A dashed line of slope 1.0 is shown for comparison.

Each mechanism confers advantages for surface exploration [33]. From the ensemble-averaged mean-square displacement, it is evident that that vertical walking bacteria move nearly diffusively, whereas horizontal bacteria can be divided into crawling superdiffusive and surface-anchored subdiffusive subpopulations (Figure 3.2). Walking bacteria moved with slope = 1.1, but with the highest instantaneous velocity. Horizontal bacteria can be subdivided into a superdiffusive subpopulation which moved with slope 1.4 and a subdiffusive, surface-anchored subpopulation which moved with slope 0.8 and low instantaneous velocity. Slopes of 1.0 and 2.0 represent random diffusive motion and geometrically linear motion, respectively.

The average persistence length, $L_p$, over which trajectories appear straight is shorter for walking bacteria (2 $\mu$m) than for crawling bacteria (6 $\mu$m); the former is similar to extension distances of TFP [66], suggesting that walking is caused by pulls of splayed TFP. Walking bacteria exhibit a higher instantaneous velocity [mean 71 ± 2 nm/s versus 41 ± 2 nm/s], but crawling bacteria move further on long time scales because of the $L_p$. Crawling enables directional motion; walking enables rapid local exploration. These trends are preserved in wild-type (WT)
bacteria, though they are easier to observe in the flagellum-deficient strains.

Bacterial orientation plays a key role in life cycle events. In 99% of 214 WT division events, one daughter cell remained attached horizontally; the majority (67%) of the other daughters left the division site by detaching, walking, or crawling. TFP governed this motility; TFP-deficient bacteria did not move apart after division. Examining all all detachment events, we observed that detaching bacteria were overwhelmingly oriented out of plane (Figure 3.3). TFP facilitate detachment by allowing bacteria to tilt from horizontal to vertical orientations.

TFP-deficient ΔpilA bacteria were defective in making this transition. This suggests a physical onset of biofilm formation, mediated by the transition from reversible polar attachment to irreversible longitudinal attachment [24].

Bacteria lacking TFP neither crawled nor achieved vertical orientations for walking and detachment. ΔpilA biofilms contained heterogeneous bacterial clusters [33] whose positions were determined by initial attachment sites (Figure 3.4). Bacterial divisions ($N_d = 79$) outnumbered attachments ($N_a = 18$) during 1 hour of cluster formation, indicating that clusters primarily grew via division. TFP-competent WT could actively walk, crawl, redistribute, and detach; despite a similar number of divisions ($N_d = 95$), the WT biofilm did not contain clusters, indicating the ΔpilA biofilm morphology is caused by motility defects rather than adhesion defects.
Figure 3.4: Δ\textit{pilA} biofilms exhibit large clusters of bacteria separated by sparsely populated areas. By contrast, WT biofilms were more uniform, as bacteria were able to move around and detach.

### 3.2 Materials and Methods

\textit{P. aeruginosa} strain ATCC 15692 wild-type (WT) 1C and Δ\textit{pilA} or Δ\textit{fliM} isogenic mutants of this strain were used for all experiments [86]. The Δ\textit{pilA} strain did not produce type IV pili and thus moved only using its flagellum, whereas the Δ\textit{fliM} strain lacked a flagellum and moved solely using its TFP. The motility of surface-attached cells was monitored in sterilized flow cells containing FAB medium [94, 100, 101] with 0.6 mM glucose, glutamate, or succinate as the sole carbon source. The medium flow rate was \(~\)3.75 mL/hr. Both higher and lower flow rates inhibited bacterial attachment and biofilm formation; at low flow rates, the bacteria did not have access to sufficient nutrients and grew slowly, whereas at
high flow rates they were swept away into the waste container instead of attaching to the surface.

Bacteria were prepared by streaking onto an LB agar plate for 16 hrs; a single colony from the LB plate was transferred to a tube containing FAB medium with 30 mM carbon with shaking at 30°C. This culture was grown overnight to stationary phase. The stationary phase culture was diluted 100x and allowed to grow into log phase to OD600 ≈ 0.3. We diluted the cultures by adding 50 μL of the bacterial suspension into 950 μL of sterilized FAB (1:20). Flow cells were constructed by attaching a glass coverslip to the PMMA base (DTU-Biosys) with a silicone sealant. They were sterilized via 8 hrs of 3% hydrogen peroxide solution and cleaned with 12 hrs of Millipore water flow. All strains contained mini-Tn7 chromosomal, constitutive, GFP-expressing insertions that allowed the cells to be visualized by fluorescence microscopy [102]. Long time-lapse movies containing 750-2000 images of fluorescent cells were collected at 12 frames per minute with EZC1 software (Nikon, Tokyo, Japan) on a Nikon C1 confocal laser microscope equipped with a 60x objective. Brightfield movies containing up to 20,000 images were collected using an Olympus (Tokyo, Japan) microscope equipped with a 100x objective at 10 frames per second (short-time acquisitions) or 20 frames per minute (long-time acquisitions).

3.3 TFP-driven motility

3.3.1 Pili drive vertical orientation in P. aeruginosa

We begin by examining the post-division motility of surface-bound WT bacteria immediately after attachment to the surface. We algorithmically identify dividing cells by observing the gradual growth of a cell followed by the sharp drop as the daughter cell pinched off; it is not always possible to locate both daughter cells on the surface after division. Surprisingly, the two sibling daughter cells exhibit
a marked asymmetry in motility after division. In over 99% of 214 WT division events, at least one sibling remains horizontally attached to the substrate. However, the other sibling may detach, move away horizontally, or exhibit a “walking” motility mechanism whereby it tilts away from the surface and then walks upright away from the division site on one pole (Figure 3.5).

To identify the motility appendage responsible for walking, we compare the post-division motility of surface-associated WT bacteria with that of TFP-deficient (ΔpilA) and flagellum-deficient (ΔfliM) mutants. In WT, 40% of daughter cells move after division and 60% remain stationary (Figure 3.6).

No ΔpilA daughter cells move after division, indicating that the observed
post-division surface motility mechanisms must depend on TFP, though this does not rule out flagellar involvement. \( \Delta fliM \) bacteria are more likely to move away after division than the WT (60% motile), consistent with reports indicating that flagella are implicated in surface attachment [56].

3.3.2 Pili govern distinct walking and crawling surface motility mechanisms

To investigate TFP-dependent motility quantitatively, we first examine \( \Delta fliM \) mutants immediately after surface attachment occurs and then compare the results with those obtained in the WT and \( \Delta pilA \) strains. These bacteria, whose movement is strictly TFP-driven, exhibit two distinct orientations: horizontal, in which bacteria are oriented parallel to the surface, and vertical, in which bacteria attached to the surface at one end are oriented normal to the surface [98]. The two orientations can be distinguished quantitatively by examining the histogram of bacterial lengths or bacterial aspect ratios. The histogram of bacterial lengths projected onto the surface is bimodal, with a peak at short lengths (~ 1.5 \( \mu m \), approximately the width of a bacterium) corresponding to vertically oriented bacteria and a wider peak at long lengths (~ 3 \( \mu m \)) corresponding to horizontally oriented bacteria (Figure 3.7). The two peaks have some overlap; bacteria can tilt upwards into an intermediate state which is neither horizontal nor vertical. However, for the purposes of this study, we use a length cutoff of 1.75 \( \mu m \) to classify all bacteria as either horizontal or vertical. In Figure 3.7, the section of the histogram corresponding to horizontal bacteria is colored blue, whereas the section of the histogram corresponding to vertical bacteria is colored red.

Next, we examine the probability distribution of time spent vertical. A bacterium which is always smaller than the length cutoff is considered 100% vertical; a bacterium which is always longer than the cutoff is considered 100% horizontal. Since bacteria are able to tilt, lie down, or stand up, some bacteria exhibit a mix
of both behaviors. The probability distribution of time spent vertical exhibits local maxima at 0 and 1, indicating that the system is bistable, i.e., the bacteria prefer to remain either horizontal or vertical (Figure 3.8).

However, bacteria are able to change orientations, and have been observed doing so as frequently as once every 10 s. Several cells were observed to cartwheel by rapidly changing from vertical to horizontal to vertical, switching the end adhered to the surface. Because $\Delta fliM$ bacteria do not possess flagella, TFP can thus mediate attachment at nonflagellated poles. Most $\Delta fliM$ bacteria spend time in both orientations, indicating that they can readily switch between TFP-driven mechanisms.

We find that switching from a horizontal to a vertical orientation does not depend on the direction of flow relative to the bacterium, and occurs in the absence of flow. This suggests that motility switching is actively driven and is not due to interactions with flow, though it may be the case that the hydrodynamic environment contributes in some way. $\Delta pilA$ mutants do not exhibit this switching, and therefore TFP are necessary for active switching. Related pili-driven...
switching was previously observed in *M. xanthus*, which exhibits a slow, vertical, pili-driven jiggling motion before it transitions to a horizontal orientation for conventional crawling lateral motion [68]. By contrast, *P. aeruginosa* can undergo lateral motion while oriented either horizontally or vertically.

The trajectories of vertical and horizontal ∆*fliM* bacteria exhibit distinct morphological and dynamical signatures. Visually, the tracks of horizontal bacteria appear smoother and straighter than those of vertical bacteria (Figure 3.9), and a close examination of individual trajectories suggests that crawling bacteria appear to move along their body axis (Figure 3.10). The tracks of vertical bacteria appear jagged, and do not correspond to the bacterial body direction.

![Figure 3.9: The trajectories of all walking cells (red, left) compared to the trajectories of all crawling cells (blue, right). By visual inspection, walking trajectories appear more jagged and crawling trajectories appear more straight.](image)

To quantify this difference, we measure the average directional persistence length of the trajectories $L_p$, which measures the average length over which trajectories appear straight. $L_p$ is longer for tracks of horizontal bacteria ($L_p \approx 6\mu m$) than for those of vertical bacteria ($L_p \approx 2\mu m$). The typical $L_p$ of tracks of verti-
Figure 3.10: Representative trajectories of walking and crawling cells. The crawling bacterium moves along its body axis, whereas the walking bacterium changes direction rapidly.

cal bacteria is similar to the extension distance of a single TFP [66], suggesting that sequential steps in these tracks are caused by multiple splayed TFP pulling the bacterium in different, uncorrelated directions. By contrast, because TFP are predominantly located at the poles of the bacterium, horizontal pulling can result in significantly more directional persistence.

The morphological differences can be examined by looking at the relation of dynamic trajectory properties to the angle deviation, the angle between the bacterial body and the direction of motion. We find that horizontal bacteria are more likely to move along their body axis (low angle deviation) than perpendicular to it (high angle deviation). In addition, horizontal bacteria move at a higher instantaneous velocity when moving along their body axis; this leads to long and straight trajectories. By contrast, horizontal bacteria exhibit no change in velocity with respect to angle deviation, and are equally likely to move at any angle deviation (Figure 3.11). It is noteworthy that walking bacteria are able to move faster than crawling bacteria, even those moving along their body axis.

These differences indicate that TFP mediate two distinct motility mechanisms:

- vertically oriented walking with low directional persistence;
- horizontally oriented crawling with high directional persistence.

We investigate differences in the area- and distance-covering properties of walking and crawling by calculating the ensemble-averaged mean squared displacement (MSD) as a function of time. The MSD is a measure of how far, on average,
a bacterium moves in a given time interval, and the slope of the MSD on a logarithmic scale is a measure of the shape of the trajectories. Motion in a straight line at constant velocity results in an MSD slope of 2.0, whereas random diffusive motion gives a slope of 1.0, and a caged trajectory would approach a slope of 0.0 at long time intervals.

MSD slopes for different bacterial behaviors in the ΔfliM strain are shown in Figure 3.12. Walking bacteria traverse linear distances slightly more efficiently than diffusion (slope = 1.1). Crawling bacteria can be separated into two sub-populations: one with nearly straight, superdiffusive motion (slope = 1.4) and one with subdiffusive motion (slope = 0.8). Walking bacteria exhibit the highest instantaneous velocity, whereas superdiffusive crawling bacteria exhibit a lower instantaneous velocity but move further and more efficiently on long timescales due to the longer $L_p$. Subdiffusive crawling bacteria have both the lowest instantaneous velocity and a low displacement on long timescales, and often appear locally trapped on length scales (1-2 μm) comparable to those of motility appendages. Each motility mechanism thus confers specific advantages for surface exploration [22, 33, 103]: crawling enables directional motion for efficient coverage of distance, whereas walking enables rapid local exploration of the area. At later times after surface attachment, the magnitude of the MSD (and, thus, the

Figure 3.11: Motility characteristics of the ΔfliM strain. Number and mean speed of walking (red triangles) and crawling (blue circles) bacteria versus angle deviation for ΔfliM bacteria (N = 70,073). Error bars indicate 1 standard deviation.
bacterial velocity) decreases for each type of motion, as shown for acquisitions lasting 1, 4, and 7 h after initial attachment (Figure 3.12) as the bacteria transition to a sessile morphology. However, the slopes of the MSD are nearly constant, indicating that the characteristic dynamical properties of each mechanism remain the same even though both the density of bacteria and the biofilm characteristics change dramatically over time.

To determine how bacteria use pili-driven motility mechanisms in the presence of flagella, we characterize the motility of WT bacteria using the same metrics. In WT bacteria, which possess both flagella and TFP, the distribution of projected lengths is again bimodal (Figure 3.13), with distinct populations of vertical and horizontal bacteria. There are fewer vertical bacteria and more horizontal bacteria than in the ∆fliM strain, indicating that the flagellum plays a role in promoting the horizontal and suppressing the vertical orientations. The distribution of
Figure 3.13: Histogram of projected length for WT bacteria (N = 170,073 individual bacteria images). “Walking” peak is shorter and “crawling” peak is larger than for the \(\Delta fliM\) strain.

time spent vertical again exhibits local maxima at 0 and 1, with a high number of bacteria staying horizontal for the duration of the experiment (Figure 3.14). Nevertheless, the vertical mode is still present and clearly detectable.

WT bacteria also exhibit walking, superdiffusive crawling, and subdiffusive trapped behavior as distinguished by their characteristic signatures (Figure 3.15). Walking bacteria move at the same velocity at any angle deviation, and are equally likely to move at any angle deviation; they exhibit a slightly superdiffusive MSD slope of 1.2. Crawling bacteria are more likely to move along their body axis, at low angle deviation, and move faster when doing so - at low angle deviation, the instantaneous velocity is three times the instantaneous velocity at high angle deviation. This leads to a higher MSD slope of 1.4. The subdiffusive population moves with slope 0.7, indicating that there are bacteria stuck to the surface.

However, the prevalence of walking decreases from 36% in \(\Delta fliM\) to 16% in WT; moreover, the number of vertically oriented bacteria and the percentage of
time spent vertical also decrease in WT (Figures 3.13, 3.14). These quantitative differences reflect the availability of additional surface motility mechanisms driven by flagella. Conversely, organisms that do not possess flagella, such as *Neisseria gonorrhoeae* [104], *N. meningitidis* [105], *M. xanthus* [106, 107], and *Synechocystis* species [108], should rely even more heavily on TFP-mediated motility mechanisms. This work will discuss the surface motility of *M. xanthus*, a flagellum-deficient bacterium, in the next chapter.

### 3.4 Flagella mediate swimming and spinning mechanisms

To elucidate the role of flagella in near-surface motility, we examine the Δ*pilA* strain, which lacks TFP and whose motility is strictly flagellum-driven, in com-
parison with the WT and Δ*fliM*. Neither walking nor crawling is present in Δ*pilA*, confirming that they are governed by TFP. In contrast to Δ*fliM* and WT, the distribution of projected lengths appears trimodal: the first peak indicating vertical bacteria and second peak indicating horizontal bacteria are present as in the WT, but there is an extra third peak at the longest lengths (∼4µm) which represents pairs of bacteria that have not moved appreciably after dividing and are thus difficult to distinguish from single long bacteria (Figure 3.16).

![Figure 3.16: Histogram of projected length for Δ*pilA* bacteria (N = 17,437 individual bacteria images). The first peak (vertical bacteria) is small, and there is an additional peak at ∼4µm for bacteria which have not separated after division.](image)

Both vertical and horizontal surface-attached bacteria remain immobile over long periods of time. The slope of the MSD is nearly zero, indicating that Δ*pilA* bacteria move even less than the trapped subdiffusive bacteria in TFP-competent strains (Figure 3.17). Neither he walking nor the crawling behaviors are evident in the MSD plots; the angle deviation behavior is not shown, as no meaningful angle deviation can be measured for a stationary bacterium. Visual observation confirms that all bacteria that attach to the surface do not move away from their attachment position except by growth, division, or detachment.

However, the flagella drive two distinct motility modes - swimming and spinning - whose characteristic trajectories are readily distinguished via other metrics. A sequence of images of a spinning bacterium and a swimming bacterium are
shown in Figure 3.18. We find that *P. aeruginosa*, like other flagellated species such as *Escherichia coli*, *Vibrio alginolyticus*, and *Caulobacter crescentus*, can swim at high speeds approximately parallel to a surface for long distances as a result of hydrodynamic surface coupling that generates curved trajectories [109]. The typical swimming speed (∼60 µm/s) and curvature (∼0.2) for WT are similar to those measured for other species [110, 111, 112, 113] (Figure 3.19). This speed is high enough that swimming bacteria are not followed by the tracking software when surface-attached bacteria are present, since they can cross most of the field of view in one frame; the curvatures were measured in a separate, higher frame rate experiment without long-term tracking of surface-attached bacteria.

In addition, both WT and Δ*pilA* bacteria can spontaneously anchor one pole to the surface by the flagellum and spin either clockwise or counterclockwise about an axis perpendicular to the surface while oriented slightly out of plane. Attach-
ment for flagellum-driven spinning can only occur at the flagellar pole, in contrast to the bipolar attachment seen for TFP-driven walking. The attachment pole can also be visualized via a pilU-YFP fusion protein, which labels the flagellar pole with YFP [88, 114]; we confirm that the surface-attached pole in this behavior is flagellated.

The typical angular velocity for both directions of rotation in WT (\( \sim 5 \text{ rad/s} \)) is comparable to that measured for artificially tethered species [115, 116], indicating that the motor speed of the *P. aeruginosa* flagellum is similar to that in *E. coli* (Figure 3.20). We confirm that diguanylate cyclase mutants such as the \( \Delta \text{sadC} \) strain, which is known to be a hyperswarmer [117], change their spinning behavior relative to the WT.

Figure 3.19: Two-dimensional histogram of trajectory curvature and instantaneous velocity for swimming WT bacteria. Curvatures are calculated from three consecutive points in each trajectory.

Figure 3.20: Histogram of angular velocity for a representative spinning WT bacterium; positive angular velocity indicates clockwise motion.
3.5 Bacteria can switch between motility mechanisms mediated by different appendages

Using single-particle tracking techniques, we identified four near-surface single-bacterium motility mechanisms, with each motility appendage driving two mechanisms: TFP drive walking and crawling, whereas flagella drive spinning and swimming. Experiments on *N. gonorrheae* have shown that bacteria can cooperatively deploy multiple pili to generate lateral crawling motion [118, 59], and we also show here (Figure 3.8) that pili-competent bacteria can switch between walking and crawling. However, to fully exploit the advantages of each motility mechanism, bacteria must switch between motility mechanisms driven by different appendages. In addition, we expect that this switching must happen based on the fact that neither the Δ*pilA* nor the Δ*fliM* strains are able to form WT-like biofilms, indicating that both flagella and pili are used over the course of biofilm formation. The post-division asymmetry in motility (Figure 3.5) provides one such example: the daughter cell lacking a fully developed flagellum [119, 120] is more likely to adopt a TFP-driven mechanism, whereas the flagellum-competent daughter cell is more likely to remain stationary. Instances of motility appendage switching and synergy also occur during cell detachment. A typical example is shown in Figure 3.21. Initially a surface-bound WT bacterium spins around a fixed center. It then ceases to rotate, after which it tilts up from the surface on one pole and detaches. The pole which remains attached to the surface during the tilt process is the pole nearest the center of rotation (Figure 3.21).

Spinning bacteria are observed in WT and Δ*pilA* but not in Δ*fliM*, consistent with a flagellum-driven mechanism. However, this full launch sequence is only observed for WT; many Δ*pilA* cells spin, but we do not observe any cells tilting upwards after spinning. This suggests that the TFP can facilitate detachment of spinning bacteria by interrupting their rotation and changing their orientation to
Figure 3.21: Vertical orientation and appendage cooperation facilitate detachment, as shown in this Representative image series of a spinning WT bacterium detaching from the surface. Dots indicate the original center of rotation, dashed lines indicate the initial radius of the trajectory, solid lines indicate the bacterial backbone, and arrows indicate the direction and magnitude of rotation between consecutive images. Images inside the box outline (2.4-3.3 s) are those in which the bacterium has tilted off the surface. The bacterium rotates (0-0.9 s), slows (1.2-2.1 s), tilts away from the surface (2.4 s), and then detaches (3.3 s), using both flagella and TFP.

out-of-plane. Indeed, ΔpilA bacteria maintain an angle of < 30° relative to the surface while spinning, whereas WT bacteria spin at angles of up to 70°. Although both spinning and post-detachment swimming are driven by the flagellum, the transition between them is mediated by the transient attachment of TFP and
the intermediate vertically oriented state. Ultimately, both the flagellum and the TFP must detach from the surface to allow the bacterium to swim away.

To estimate the force exerted by TFP to change bacterial orientation, we equate the torque required to effect rotation at an angular velocity $\omega$, $\tau = b\omega$, where $b = 16\pi \eta a^2 L/3$ is the rotational drag on a bacterium of half-width $a$ and length $L$ in a medium with viscosity $\eta$ [65], to $\tau = rF \sin \theta$, where $\theta$ is the angle between a lever arm of length $r$ and an applied force of magnitude $F$. In this simple estimate, we ignore the effects of adhesins that are present on the bacterial surface and may increase the adhesive force. Although the bacterium tilts 0.86 radians away from the surface in 1.8 s, approximately half of this angular change (0.42 rad) occurs in $< 0.1$ s, when the initial angle between the bacterium and the surface is $\theta = 0.31$ rad. We thus estimate the maximum force exerted by TFP during the tilting process in water as $F = b\omega/r \sin \theta \approx 0.2$ pN. Because this value is significantly smaller than the stall force of a single TFP ($\sim 70$ pN [72]), even a single pilus can exert sufficient force to change a bacterium’s orientation.

We further test the relationship between vertical orientation and detachment by identifying the orientation and motility mechanism of detaching WT bacteria. We classify spinning and walking as out-of-plane motility mechanisms, and crawling and subdiffusive stuck cells as in-plane motility mechanisms. We find that detaching bacteria are overwhelmingly more likely to exhibit the two out-of-plane motility mechanisms, and rarely crawl before detachment. As a result, the detachment probability of vertical bacteria is roughly twice that of horizontal bacteria (Figure 3.22).

To quantify the effects of appendage cooperativity on detachment, we calculate the detachment probabilities for the $\Delta fliM$ and $\Delta pilA$ strains. As noted previously, for all strains, the detachment probability of vertical bacteria is higher than the detachment probability of horizontal bacteria. Moreover, once bacteria are vertical, there is very little difference in their detachment probability; how-
Figure 3.22: Left: Percentage of detaching WT bacteria that exhibit out-of-plane (red bar: walking, spinning) and in-plane (blue bar: crawling, stationary) motility mechanisms. Right: Detachment probabilities for total, horizontal, and vertical bacteria as a function of strain (ΔfliM (N = 70,073), ΔpilA (N = 17,437), and WT (N = 170,073)). The WT consistently exhibit higher detachment probabilities, showing that both flagella and TFP facilitate detachment. Error bars indicate 1 SD. The difference is not statistically significant for the bacteria which are already vertical, indicating that the ability for bacteria to become vertical is the critical step.

However, for bacteria which are horizontal, the difference in detachment probability between strains is high. The difference between the horizontal and vertical detachment probabilities is least pronounced in the WT strain; moreover, the total detachment probability for ΔfliM and ΔpilA is significantly smaller than that of WT, indicating that both TFP deficiency and flagellum deficiency decreases detachment. This decrease results primarily from the pronounced decrease in detachment probability for horizontal bacteria; once the bacteria are vertical, the detachment probabilities are similar. This supports our observation that both
flagella and TFP facilitate surface detachment via a launch sequence that includes a change from a horizontal to a vertical orientation; furthermore, this launch sequence requires the TFP to pull horizontal cells to a vertical orientation. TFP-deficient bacteria are unable to tilt upwards to detach. Together, these observations suggest a physical mechanism for the onset of biofilm formation that is signaled by the transition from reversible polar (vertical) attachment to irreversible longitudinal (horizontal) attachment [24].

The motility defects that reduce bacteria’s ability to detach strongly influence biofilm morphology [121]. In the first 6 h of \( \Delta pilA \) biofilm formation, the proportion of spinning bacteria increases dramatically with time (Figure 3.23).

![Figure 3.23: Percentage of bacteria that exhibit the spinning motility mechanism or vertical orientation for \( \Delta pilA \) (left, \( N = 376 \) and 270 at 0 h and 5.5 h) and WT (right, \( N = 355 \) and 257 at 0 h and 5.5 h) bacteria. Error bars indicate 1 SD. The percentage of spinning bacteria increases with time for \( \Delta pilA \) because they cannot tilt up and detach.]

However, these bacteria lack TFP to achieve the near-vertical orientations that facilitate detachment. Consequently, the launch sequence is impaired and these
cells detach less frequently than WT cells. The spinning cells continue to spin and remain trapped in the intermediate stages of the launch sequence. The resultant ΔpilA biofilm contains a heterogeneous distribution of bacterial clusters [103], whose positions are governed by the sites of initial attachment, because the bacteria do not walk or crawl away from the attachment sites. Division events \(N_d = 79\) significantly outnumber attachment events \(N_a = 18\) during a 1-h period of cluster formation, indicating that clusters grow primarily via division. This can be confirmed by examining individual clusters and tracing the division history of the cells back to the first parent cell which initiated the cluster. By contrast, TFP-competent WT cells actively redistribute and detach, and the numbers of spinning and vertical bacteria decrease over time. Despite a similar number of division events \(N_d = 95\) and a similar surface cell density, the WT biofilm does not contain clusters (Figure 3.4), indicating that the ΔpilA biofilm morphology is caused by the motility defects in the ΔpilA strain that impede cluster dispersion via pili-driven motility behaviors such as detaching, walking, and crawling. This is consistent with the strong observed likelihood that a daughter cell will deploy TFP to walk or detach after division occurs (Figure 3.6). The active regulation of cell density via TFP in WT results in a uniform initial surface coverage that precedes WT biofilm formation. The morphology of a ΔfliM biofilm is less uniform than that of WT, as the bacteria still have a small detachment defect (Figure 3.22); however, the ΔfliM biofilm does not exhibit the large aggregates seen in the ΔpilA biofilm.

### 3.5.1 Aside: Vibrio cholerae near-surface swimming

Multi-appendage synergy is also present in *V. cholerae* near-surface swimming. *V. cholerae* is an aquatic bacterium which is the causative agent of the disease cholera [122]. Like *P. aeruginosa*, *V. cholerae* swims using a single polar flagellum, which also aids in attachment [123, 124, 125]. It is known that attachment to
surfaces is mediated by several different types of pili, with the mannose-sensitive hemagglutinin (MSHA) pilus used for attachment to abiotic surfaces and the toxin-coregulated pilus (TCP) to attach to biological surfaces [125]. Unlike *P. aeruginosa*, *V. cholerae* does not use its pili for surface motility; surface-attached cells do not twitch, walk, or crawl. However, we find that the presence of the MSHA pili has a significant effect on the flagellum-driven near-surface swimming motility mode.

Near a surface, *V. cholerae* can swim in small circles using its flagellum (Figure 3.24). We characterize these trajectories by examining their radius of curvature (Figure 3.25). In the flagellum-deficient Δ*flaA* strain, the bacteria are unable to swim - this is exhibited as a radius of curvature of near zero. In the wild-type strain, cells can be stationary or can swim with a radius of curvature of approximately 1.5µm - a distinct peak in the curvature histogram. However, in the Δ*mshA* strain, which lacks the MSHA pili, the cells lose this characteristic curvature - in addition to being attachment-deficient, they move at a wide range of curvatures. This indicates that the near-surface pre-attachment swimming motility of *V. cholerae* involves both pili and flagella. We hypothesize that the pili anchor the bacterium to the surface while the flagellum continues to rotate.

Figure 3.24: An example trajectory of a *V. cholerae* cell, exhibiting the flagellum-driven near-surface circular swimming prior to attachment.
prior to irreversible surface attachment.

Figure 3.25: Curvatures of near-surface swimming of *V. cholerae*. For the Δ*flaA* strain (left), bacteria do not exhibit swimming, indicated by a curvature near zero. For the WT (middle), stationary and circular swimming is evident, with the swimming trajectories exhibiting an average radius of curvature near 1.5µm. Δ*mshA* bacteria are unable to attach and so do not have a curvature peak at zero, and do not exhibit the characteristic circular swimming of radius 1.5µm, instead swimming in much straighter (larger radius of curvature) trajectories.

### 3.6 Conclusion

By using an efficient search-engine-based approach to analyze bacterial motility, we were able to identify four fundamental near-surface motility mechanisms in *P. aeruginosa*. The flagellum mediates near-surface swimming and surface-bound spinning. TFP mediate crawling, the higher directional persistence of which enables efficient directional motion, and walking, the lower directional persistence of which enables efficient local exploration. Tracking the motility of thousands of bacteria over many hours allows us to identify specific mechanisms that make up the TFP-driven motility modes investigated in earlier studies [103, 126]. Moreover, the improved spatiotemporal resolution of our study enables fundamentally new investigations of the cooperative deployment of motility appendages. We have shown that appendage switching and synergy allow bacteria to exploit the advantages conferred by different motility mechanisms. For example, we have
demonstrated how motility and detachment defects in the ΔpilA strain engender a biofilm morphology distinct from that of WT. Our technique of total analysis, in which every cell in a movie is automatically and individually examined, can be applied to a broad range of microorganisms to quantitatively characterize motility mechanisms that are inaccessible by traditional microscopy methods. In the next chapter, we discuss the application of these techniques to *M. xanthus* pili-driven social motility.
CHAPTER 4

Earthquake-like dynamics in *Myxococcus xanthus* social motility

4.1 Introduction to *M. xanthus*

The soil bacterium *Myxococcus xanthus* is an advanced Gram-negative bacterium that is capable of highly organized social behavior [51]. For example, *M. xanthus* can form predatory “wolf packs” to prey on other species, and under starvation conditions, they can self-organize into macroscopic fruiting bodies to ensure community survival [127]. This rudimentary social behavior [128] relies on the gliding ability to move in the direction of the cell’s long axis on solid surfaces, which is regulated by the adventurous (“A”)- and social (“S”)-motility systems [129]. As two distinct motility systems, A-motility allows movement of individual and isolated cells, whereas S-motility controls the coordinated motility of large numbers of cells. S- and A-motility convey survival advantages on different types of surfaces, with A-motility being more important on hard surfaces and S-motility playing a bigger role on soft surfaces. Both A- and S-motility are quite slow, propelling the bacterium at speeds of roughly 2-4 µm per minute, significantly slower than swimming flagellated cells which can move at 30-80 µm per second.

The mechanism of adventurous gliding motility in *M. xanthus* is the subject of considerable debate [130], as it does not involve any visible external appendages [131]. A recent model proposes a helical structure, homologous to the flagellar stator of other bacteria, which attaches to the surface via focal adhesions and
pulls the bacterium via rotation, driven by the proton motive force caused by a pH gradient [132, 133, 134]. Alternative models have proposed that \textit{M. xanthus} glides via propulsion by slime secretion at the trailing pole [135, 136].

S-motility in \textit{M. xanthus} is better understood, as it is mechanistically equivalent to the twitching motilities in \textit{P. aeruginosa} and \textit{N. gonorrheae} [57], which are all driven by type IV pili [106]. TFP are located at the two bacterial poles and propel the cell by cycles of extension, attachment, and retraction [68, 137]. Recently it has been shown that TFP are used to sense the exopolysaccharides (EPS) that are secreted by other cells onto the cell body or the surface [138, 139], which enables \textit{M. xanthus} to coordinate movement along EPS tracks. Although only one pole is piliated at a time, bacteria are known to reverse direction by disassembling the TFP apparatus on the pole and reassembling it at the other one [140]. This reversal behavior, which is driven by the \textit{frZ} chemotaxis system, allows cells to retrace their paths, locate other cells, and form fruiting bodies [141, 142], which incorporate significant concentrations of EPS within their structures [143, 144]. Although it is clear that this coupling between TFP and EPS is critical to the formation of structured fruiting bodies by \textit{M. xanthus}, it is not known how \textit{M. xanthus} TFP-EPS technology allows it to achieve its unique coordinated multicellular movements [144, 145], given that TFP and EPS exist in a variety of bacterial species [42] that do not exhibit S-motility.

Because \textit{M. xanthus} S-motility involves TFP pulling the bacterium along surfaces with heterogeneous coverages of EPS, friction is expected to play an important role. Recently, theoretical “crackling noise” models for frictional motion on surfaces have been shown to be widely applicable to slowly sheared materials, ranging from the deformation of slowly sheared nanocrystals to the slip dynamics along geological faults via earthquakes [146, 147, 148, 149, 150, 151, 152, 153, 154, 155]. These systems exhibit “stick-slip” movement with broadly distributed slip sizes, involving long pauses separated by rapid transitions to a new state. Crackling
noise models show that the observed slip statistics can be used to obtain information about the system. In this work, we first compare the observed slip statistics to crackling noise model predictions and then find a description of the system that is consistent with them. We validate the model by comparing to previously described crackling noise systems. We then use the slip statistics to extract information about the EPS and other properties of the system [156]. S-motility of M. xanthus is known to be slow compared with the motility of other bacterial species and is usually studied using time-lapse microscopy. To get the required data for theoretical comparisons, we instead do the opposite and investigate the S-motility of M. xanthus on a surface at 250-ms resolution with particle-tracking algorithms [99, 98, 157].

4.2 Materials and Methods

4.2.1 M. xanthus culture and imaging

M. xanthus cells were grown overnight in casitone yeast extract (CYE) medium. They were transferred onto a glass slide and overlaid with Mops buffer containing 1% methylcellulose. They were recorded and recorded at 30°C [68]; these conditions suppress adventurous motility and promote TFP-driven S-motility even in isolated cells and not just aggregates. The cells were given 30-60 min to attach to the surface and initiate S-motility. Thirty-second recordings with a wide field of view (>100 visible bacteria) were taken to identify cells that were motile and not stationary; then, longer movies (2-10 min) of individual motile cells were recorded. Movies were acquired at 400 frames per second, using a Phantom V12.1 camera (Vision Research) on an Olympus microscope with a 60x objective, resulting in images with a resolution of 0.2 µm per pixel.
4.2.2 Image processing and tracking

Each image was smoothed by applying a spatial bandpass filter with background subtraction, reducing the bacteria to bright objects on a black background; then a brightness threshold criterion was used to identify them. Since each movie contained only a single bacterium and the recordings were short, the parameters were hand-picked for each movie for optimal tracking. The centroid position and orientation of each bacterium were calculated from the moments of the backbone distribution [97, 99, 98, 99], and tracking was done using a minimum squared-displacement criterion [96]. As *M. xanthus* moves and changes direction slowly, the leading and lagging poles of the bacterium exhibited the same dynamics as the centroid; the position of the centroid was used as the bacterial position for subsequent analysis.

The noise level of the system was determined by examining an immobile bacterium; position measurements of an immobile bacterium varied by \( \sim 0.2 \mu m \), the width of one pixel. This determined a rough lower limit for locating slips. Averaging the position over multiple frames (boxcar smoothing) was used to improve spatial accuracy, at the cost of giving up fast-timescale information. To eliminate high-frequency noise in the velocity and position measurements, the trajectory was first smoothed with a boxcar average of width 0.05 s. At each point in the trajectory, the velocity was calculated as \( v = \frac{x(t+\Delta t) - x(t-\Delta t)}{2\Delta t} \), using \( \Delta t = 0.05 \) s. Slips were identified by their peak velocity; sections of the trajectory where the smoothed velocity remained below the noise threshold (0.1 \( \mu m/s \)) were labeled as plateaus, whereas sections of the trajectory where the velocity remained higher than the noise threshold were labeled as slips. To calculate the power spectrum of the velocity, segments of the trajectory identified as plateaus were excised; the magnitude of the velocity was calculated at each remaining trajectory point, using the unsmoothed data and a \( \Delta t = 0.0025 \) s. The fast Fourier transform of this signal was squared to give the power spectrum and then binned logarithmically,
and the slope was calculated using a least-squares fit.

For each slip, we calculated the displacement using the smoothed data as $\Delta x = x_f - x_i$, where $x_i$ and $x_f$ are the positions before and after the slip, respectively; likewise, we calculated the duration as $\Delta t = t_f - t_i$, where $t_i$ and $t_f$ are the start and end times of the slip. The distributions of slip displacements and durations were binned logarithmically for plotting. The power law exponents for these quantities were calculated directly without fitting via $\alpha = 1 + n\left[\sum_{i=1}^{n} \frac{x_i}{x_{\text{min}}}\right]^{-1}$, where the $x_i$ are the data points within the power law region [158].

The above calculations were repeated with different parameters to ensure that observed trends were due to physical effects and not analysis artifacts due to parameter selection; changing the smoothing widths to $\Delta t = 0.1s$ or $\Delta t = 0.2s$ as well as velocity thresholds to 0.075 $\mu$m/s or 0.125 $\mu$m/s affected the calculated exponents by 0.1 or less and did not disrupt the power law scaling.

It is worthwhile to point out that a “backward” movement can be discerned after some of the slips. We hypothesize that these movements indicate the existence of a finite a relaxation time in the EPS on the surface and suggest that we may be able to extract more information on the polymeric nature of EPS by using secretion mutants. For the purposes of the present work, we repeated the slip distribution analysis with the post-slip backward motion counted as part of the slip and alternately as part of the plateau. We found that the resultant effects on the slip duration and displacement distributions are not large enough to affect the measured exponents.

We calculate the Lyapunov exponent from the sequences of bacterial velocities as follows [159]. The program keeps track of an initial point $p(i)$, which is initialized to be the first point on the first bacterial trajectory. Then, the program finds a second point $p(j)$, which may be on the trajectory of a different bacterium, with a similar velocity, differing from the velocity at $p(i)$ by 0.05$\mu$m/s; it looks ahead by $\Delta t = 0.25s$, comparing the magnitude of the final velocity difference between
the two trajectories $\Delta v_f$ to the initial velocity difference $\Delta v_i$ and updating the running average of $\lambda = \frac{\ln \frac{v_f}{v_i}}{\Delta t}$. The next $p(i)$ is selected to be similar, in velocity and direction, to $p(i + \Delta t)$, but differing by at least 0.05$\mu$m/s, and the procedure is repeated. This converges to the maximal Lyapunov exponent of the system. This is a measure of the degree of chaos in the system and indicates how fast similar trajectories diverge from each other.

4.2.3 *M. xanthus* EPS Purification and Analysis

Wild-type *M. xanthus* (DK1622) EPS was isolated and purified following a protocol modified from two different sources [160, 161]. After overnight incubation to log phase in casitone yeast extract (CYE) liquid medium [162] the bacteria were spread onto five large CYE plates ($\sim 1.24 \times 10^8$cells per plate) and allowed to grow for 3 days at 32°C. The cells were then scraped off the plates into 0.9% NaCl solution ($\sim 10$ mL per plate). Phenol was added to a final concentration of 5% (vol/vol) and stirred for 5 hours at 4°C. The cells and debris were removed by centrifugation and the EPS was then precipitated from the supernatant with 4 vol of isopropanol. After centrifugation, the pellet was redissolved in water. The precipitation and resuspension were repeated three times. The resulting product was dialyzed first against 0.1 M NaCl and then against water. The EPS solution was lyophilized.

Glycosyl analysis of lyophilized EPS was performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis [163]. For determination of glycosyl linkages, the sample was permethylated, depolymerized, reduced, and acetylated; and the resultant partially methylated alditol acetates (PMAAs) were analyzed by GC/MS.
4.3 Results and Discussion

4.3.1 Visual inspection of *M. xanthus* trajectories

From a visual examination of motile *M. xanthus* bacteria undergoing TFP-dependent single-cell S-motility, which has been reviewed extensively [51, 106, 68, 138, 139, 142, 164, 165], we see that their trajectories are not smooth, but are characterized by intermittent, aperiodic stick-slip motion; the bacteria stay in one position and appear stationary, before rapidly moving to a new state. Similar “burst” behavior has been observed in other biological systems [166, 167]. In a sequence of images evenly spaced in time, the motion appears uneven, even though on longer timescales the velocity appears constant (Figure 4.1).

![Figure 4.1: Sequence of snapshots of a moving *M. xanthus* cell. (Scale bar: 1µm.) The images are equally spaced in time, taken 2.5 s apart, but show uneven movement of the front of the bacterial cell.](image)

Plotting the position of the centroid of such a bacterium, we see that the
example plot of the bacterial displacement is composed of distinct “slips”, where the bacterium moves rapidly, separated by plateaus where the bacterium “sticks” to the surface and appears stationary (Figure 4.2). A small backwards motion is evident just after a slip.

Figure 4.2: The trajectory of the centroid of an individual WT cell (top). The section in the blue box is expanded below; at a small scale, the trajectory can be broken down into distinct plateaus (blue) and slips (red). These are small compared with the bacterial size. A small backward motion can be discerned at the transition from a slip to a plateau (blue to red) in some of the slips.

Unlike \textit{P. aeruginosa}, which exhibits different dynamics at the leading and the lagging pole [157], moving \textit{M. xanthus} bacteria move and rotate slowly and show little asymmetry between their leading and lagging poles. We can thus analyze the trajectory of the bacterial midpoint and treat it as representative. In a single slip, the bacterium can move up to several microns; short slips of just above the detection limit (\(\sim 0.1\mu m\)) are also observed. The observed duration of individual slips varies from \(\sim 0.01\) s to several seconds. Interestingly, the motion is not unidirectional; after a slip, a bacterium may move backward, indicating that there is viscoelastic relaxation in the system. The stick-slip motion found in the wild-type (WT) DK1622 [106] is also observed in the DK3088 (\textit{stk}) strain that
overproduces EPS by a factor of 2 [168], even though the magnitude of motions is much smaller in the latter case (Figure 4.3).

Figure 4.3: The stk mutant that overproduces EPS moves at a lower average velocity; an example trajectory is shown (top). The section in the blue box is expanded below; at a small scale, the trajectory can be broken down into distinct plateaus (blue) and slips (red). The small backward motion at the transition from a slip to a plateau (blue to red) is still present in some of the slips.

However, stick-slip motion is suppressed in the SW810 (ΔepsA) strain (Figure 4.4), which underproduces EPS [138]. This strain is still able to both move and be stationary on the surface, but does not produce a distinct separation between plateaus and slips. We find that the slips in the WT and stk strains are analogous to the avalanches observed in other crackling noise systems [152]. As the TFP exert a force on the bacterium, the trajectory is broken into distinct slips by the friction-like interaction between the cell surface and the substrate, mediated by an EPS layer which is absent in the ΔepsA strain.

4.3.2 Crackling noise model for M. xanthus trajectories

To quantitatively compare the crackling noise model to the M. xanthus system, we algorithmically separate the trajectories into distinct plateaus and slips and exam-
Figure 4.4: The trajectory of the $\Delta epsA$ mutant that underproduces EPS has a different appearance on a small scale, with distinct plateaus and slips appearing less frequently (top). The section in the blue box is expanded below; there is no distinct separation of the trajectory into plateaus and slips.

...ine the distributions of displacements and durations of these slips, as well as the velocities during a slip. The slips are analogous to crackling noise “avalanches.”

Many experimental crackling noise systems, especially small systems that exhibit avalanche behavior, exhibit only 1-1.5 decades of power law scaling due to their finite size [169, 170, 171]. Moreover, we expect any manifestation of crackling noise power laws to be strongly modified by the inherent complexity of a biological system, such as the influence of signaling, or possible variable output of the TFP molecular motor. Surprisingly, power law scaling survives in the $M. xanthus$ system and is observed in the slip duration and slip displacement distributions (Figure 4.5).

However, the apparent slip duration power law breaks down above a cutoff value. Likewise, the power spectrum of the velocity also breaks down above a cutoff frequency. As a useful consistency check, it is interesting to note that these cutoff values correspond closely to typical TFP pull durations ($\sim 4$ s) and TFP
Figure 4.5: Power law scaling of slips is evident in *M. xanthus* motion. (left) The distribution of slip durations can be modeled as a power law, with an exponent of 2.3. This breaks down at low durations due to the displacement of a single short slip being below the imaging resolution. (middle) The distribution of slip displacements follows a power law, with an exponent of 2.0. (right) The power spectrum of the velocity has an exponent of 1.7, with a plateau at high frequency (>1 Hz).

lengths (∼5µm) [106, 71]. The lower limits on the power laws are given by the spatial resolution of the image acquisition, approximately 0.1µm.

Given that the number of decades of observable power law behavior is necessarily small, we subject the measured slip distributions to a series of quantitative tests, based on comparisons to a mean field theory of friction. Qualitatively, the contact area between a bacterium and the substrate is modeled as an array of patches, each of which has an individual random slip threshold, taken from a distribution of such thresholds. It can be formally shown that the exact shape and width of the random distribution are not important for the slip statistics on long length scales [150, 152, 172]. As the pili retract, the force on each of these patches increases until eventually a weak patch on the contact surface slips. Elastic interactions between the patches on the surface then lead to an increase in the force on the other patches, which can be so large that it causes other patches to slip as well. By the same mechanism, these patches trigger additional slips, resulting in a slip avalanche, which we observe as a single slip of the whole system. This simple model can be solved analytically in mean field theory, using the approximation
Table 4.1: Comparison of exponents observed in *M. xanthus* to other systems: exponents are consistent with mean field theoretical values and with exponents previously observed in other crackling noise systems. The exponent relation between the slip displacement, slip duration, and power spectrum of the velocity $|\tau + \sigma v_z - 1)/(\sigma v_z) - \alpha|$ provides a consistency check.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Crystal deformation</th>
<th>Earthquake dynamics</th>
<th>M. xanthus behavior</th>
<th>Mean field value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slip displacement, $\tau$</td>
<td>1.4-1.6</td>
<td>1.3-2.5</td>
<td>1.9-2.2</td>
<td>$3/2$</td>
</tr>
<tr>
<td>Slip duration, $\alpha$</td>
<td>-</td>
<td>$\sim2$</td>
<td>2.2-2.6</td>
<td>2</td>
</tr>
<tr>
<td>Velocity power, $\frac{1}{\sigma v_z}$</td>
<td>2</td>
<td>1.8-3.4</td>
<td>1.5-1.8</td>
<td>2</td>
</tr>
<tr>
<td>$</td>
<td>\tau + \sigma v_z - 1)/(\sigma v_z) - \alpha</td>
<td>$</td>
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</tr>
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</table>

that every patch interacts equally strongly with every other patch in the system. Mean field theories have been shown to correctly predict the correct avalanche size distributions, duration distributions, and exponent relations for slip statistics on surfaces coupled by full 3D elasticity, as well as the power spectra of the velocity time trace and average temporal avalanche profile, as a function of parameters such as forcing rate and substrate properties [150, 172]. The model predicts values of the exponents for the displacement, duration, and power spectrum of velocity and a quantitative relation between these exponents (Table 4.1), which can be tested against experimental results.

The measured power law exponents for displacement, duration, and power spectrum of velocity compare well with theoretical crackling noise predictions from mean field models, such as those used to describe friction between two sheared surfaces, and the exponents observed for other crackling noise systems; here, we compare to a crystal deformation model and to earthquake dynamics [151, 152, 173, 174, 175, 176, 177] (Table 4.1). We observe slip displacement distribution exponents between 1.9 and 2.2, within the 1.3-2.5 range previously observed for
earthquakes and similar to the mean field predictions of $3/2$ [or 2, depending on the details of the model [146]]. The slip duration distribution exponent is 2.2-2.6, similar to the mean field prediction of 2. The decay exponent of the power spectrum of the velocity is 1.5-1.8, again similar to the mean field prediction of 2 [151]. These measurements provide an excellent test of current theoretical descriptions for crackling noise and allow a variety of exponents to be calculated.

Importantly, we calculate a general scaling relation between exponents (Table 4.1). This relation demonstrates self-consistency between the measured quantities. The agreement of multiple observed exponents with the crackling noise model and their internal consistency provides strong support for treating the data as a crackling noise system over the observed range and validates the treatment of the trajectory as a sequence of plateaus and slips. The broad agreement between our experiments and the crackling noise model suggests that *M. xanthus* motions are derived from a constant TFP-generated force acting against friction. The agreement also implies that the frictional interaction in *M. xanthus* must take place over the two-dimensional interface between the bacterium and the surface and not just at the leading or lagging pole as in the case of *P. aeruginosa* [157].

### 4.3.3 Knockout strains

To elucidate the source of the crackling noise behavior, we compare the power law exponents observed for WT *M. xanthus* with the exponents observed for several mutant strains (Table 4.2). The mutant strains SW2070 ($\Delta frzD$) and SW2071 ($\Delta frzE$), which have previously observed defects in chemotaxis-driven reversal behavior [142, 178], have exponents quite similar to those of the WT; moreover, their exponents are the same despite the mutations having opposite effects on reversal behavior, with the $\Delta frzD$ strain being a hyperreverser that changes direction every 2.2 min and the $\Delta frzE$ strain being reversal deficient and reversing once every 2 h [142]. This indicates that the power law exponents do not depend
Slip displacement 1.9-2.2 2.0-2.2 1.9-2.3 1.8-2.0 1.3-1.7
Slip duration 2.2-2.6 2.3-2.5 2.3-2.6 2.0-2.4 1.4-1.8
Velocity power spectrum 1.5-1.8 1.7-2.2 1.6-2.2 1.3-1.7 0.9-1.2

Table 4.2: Comparison of exponents observed in different M. xanthus mutant strains:

The EPS overproducer (stk) and the chemotaxis mutants (ΔfrzD, ΔfrzE) all have power law exponents similar to that of the WT. All three indicator exponents for the EPS-deficient strain (ΔepsA) are significantly different from the values for the EPS-competent strains, indicating that the presence of EPS produced by M. xanthus is critical for the crackling noise scaling, whereas chemotaxis is not.

on the chemotactic behavior of M. xanthus and are not influenced by changes on the bacterial reversal timescale of minutes.

To test whether EPS was implicated in the observed exponents, we examined the ΔepsA strain, which is defective in EPS production [138], and the stk strain, which overproduces EPS [168]. The EPS overproducer has a lower average velocity than the WT, and individual slips are shorter (Figures 4.2, 4.3). However, it exhibits very similar crackling noise exponents to the WT, indicating that the frictional interaction is preserved. In contrast, the observed exponent signature of plateaus and slips is suppressed for the EPS underproducer, although its average velocity is similar to the WT (Figure 4.4). Its stops and starts do not exhibit the characteristic crackling noise exponents observed in the other strains (Table 4.2). Note that, as previously observed in Figure 4.4, the ΔepsA mutant trajectory cannot be easily subdivided into plateaus and slips; the exponents presented here use the same parameters to attempt the subdivision as are used for the stk and WT strains, and serve as a consistency check for the previous visual analysis.

Thus, the crackling noise power laws result from the interaction between the bacterial body and the EPS-covered surface as the bacterium is pulled along by its
TP. This is consistent with crackling noise models in general, in which stick-slip movements are generated by the competition between long-range elastic interactions on the surface, which favors long slips, and heterogeneity in surface contacts, which favors short slips.

4.3.4 EPS as a lubricant

Interestingly, we find that the EPS secreted by *M. xanthus* acts as much like a lubricant as it does like a glue; TFP-driven *M. xanthus* movement on EPS-covered glass involves the shearing of two smooth surfaces separated by a lubricant, differing from other crackling noise systems like earthquakes. Previously, thin lubricant films of branched hydrocarbons sheared between smooth surfaces have been shown to exhibit chaotic stick-slip behavior, as well as smooth sliding and periodic stick-slip regimes [179]. Experiments with hydrocarbon lubricants of linear architecture or those with no lubricants (dry friction) exhibit only periodic stick-slip and smooth sliding. The chaotic stick-slip regime is characterized by irregular, aperiodic velocity oscillations and has been associated with the presence of large, multimolecular domains in an architecturally complex lubricant having a broad band of relaxation times. The TFP-driven stick-slip motions that we observe in *M. xanthus* span \(\sim 1.5\) orders of magnitude in dynamic range before being interrupted by the motility cycle. To determine whether the system exhibits chaotic behavior, we calculate a Lyapunov exponent from these bacterial trajectories [159]. We find that the maximal Lyapunov exponent is \(\sim 2.2\), indicating that the behavior is indeed chaotic because the exponent is positive and greater than 1 (Figure 4.6).

The presence of this chaotic regime suggests that *M. xanthus* EPS behaves like highly branched molecules, similar to the branched hydrocarbon lubricants that exhibit chaotic stick-slip behavior between smooth surfaces; simpler lubricants do not exhibit this regime. Highly branched molecules are known to be the best low-friction lubricant fluids [180], and it is believed that the reason for this is their
Figure 4.6: A selection of *M. xanthus* slips that start with similar initial velocities and their accelerations quickly diverge due to the chaotic nature of the system; the degree of chaos is quantified by the Lyapunov exponent, which we calculate to be 2.2.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>mol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>1.4</td>
</tr>
<tr>
<td>Xylose</td>
<td>4.7</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>21.9</td>
</tr>
<tr>
<td>Mannose</td>
<td>16</td>
</tr>
<tr>
<td>Galactose</td>
<td>4.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>34.1</td>
</tr>
<tr>
<td><em>N</em>-acetylglucosamine</td>
<td>12</td>
</tr>
<tr>
<td><em>N</em>-acetylmannosamine</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Table 4.3: Analysis of the glycosyl composition of *M. xanthus* EPS.

inability to pack efficiently at a molecular scale, which gives them a tendency to remain liquid-like instead of “freezing” into solid-like ordered arrangements when confined between two shearing microscopically smooth surfaces [181, 182, 183]. However, although characterization of bacterial EPS distributions is complex, it is known that highly branched EPS is rare in bacteria [184].

To support these dynamical measurements, we perform a glycosyl composition analysis of purified *M. xanthus* EPS (Table 4.3). The four primary sugars found in the sample were glucose (34.1%), rhamnose (21.9%), mannose (16.0%), and *N*-acetyl glucosamine (12.0%). Additionally, arabinose, xylose, galactose, and *N*-acetyl mannosamine were identified but in amounts less than 10%. The analy-
<table>
<thead>
<tr>
<th>Glycosyl residue</th>
<th>% total*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chain end</strong></td>
<td></td>
</tr>
<tr>
<td>Terminal arabinopyranosyl residue (t-ara)</td>
<td>4.0</td>
</tr>
<tr>
<td>Terminal glucopyranosyl residue (t-glc)</td>
<td>8.1</td>
</tr>
<tr>
<td>Terminal rhamnopyranosyl residue (t-rha)</td>
<td>5.8</td>
</tr>
<tr>
<td>Terminal xylopyranosyl residues (t-xyl)</td>
<td>4.3</td>
</tr>
<tr>
<td><strong>Linkages within chains</strong></td>
<td></td>
</tr>
<tr>
<td>3-Linked galactopyranosyl residue (3-gal)</td>
<td>5.5</td>
</tr>
<tr>
<td>3-Linked glucopyranosyl residue (3-glc)</td>
<td>5.4</td>
</tr>
<tr>
<td>4-Linked glucopyranosyl residue (4-Glc)</td>
<td>4.6</td>
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<tr>
<td>6-Linked glucopyranosyl residue (6-Glc)</td>
<td>12.3</td>
</tr>
<tr>
<td>2-Linked mannopyranosyl residue (2-man)</td>
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<td>3-Linked mannopyranosyl residue (3-man)</td>
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<td>2-Linked rhamnopyranosyl residue (2-rha)</td>
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<td>4-Linked xylopyranosyl residues (4-xyl)</td>
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<tr>
<td><strong>Branching residues</strong></td>
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</tr>
<tr>
<td>3,4-Linked mannopyranosyl residue (3,4-man)</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Table 4.4: Glycosyl linkages in *M. xanthus* EPS.

* % total has an estimated relative error of 10%
sis also reveals a complex molecule containing a variety of linkages and indicating that the polysaccharide has a number of branches (Table 4.4). These data suggest that the \textit{M. xanthus} EPS is a carbohydrate with at least three different types of branches and four different terminating residues. This is consistent with our dynamical findings that also suggested a complex, branched structure for \textit{M. xanthus} EPS.

4.3.5 Deviations from crackling noise model

The \textit{M. xanthus} system exhibits several interesting deviations from theoretical crackling noise predictions. A plot of the slip duration against the slip displacement (Figure 4.7) has a slope which is near 1, significantly different from the theoretical value of 2 [185]. In fact, a slope of 1 indicates that there is no power law at all, only a linear relation.

Figure 4.7: The slip duration vs. displacement plot has an exponent of 1.0; this is a linear relation and not a power law, different from the predicted power law with exponent 2.

Additionally, the characteristic velocity profiles for the wide range of observed slips deserve comment. Mean field theory predicts that this velocity profile should be scale invariant and therefore maintain the same shape for all slips. We calculate this profile by rescaling all slips and calculating the average velocity at different time points along the slip and find a subtle difference from the expected behavior. We find instead two characteristic shapes for the large number of slips observed, one for short and one for long slips (Figure 4.8) [152, 170, 171, 186, 187].

Slips that are short (<1 s) exhibit a slightly asymmetric shape; although the
Figure 4.8: Short slips (<1 s, red) have a slightly left-skewed shape and long slips (>1 s, blue) are more uniform. These observations can be explained by the TFP reaching a maximum retraction velocity during individual slips.

mean field model predicts a symmetric shape, similar asymmetric avalanche shapes have also been observed in other crackling noise systems [185, 188, 152] However, long slips (>1 s) have a significantly more uniform velocity profile [170]. Both of these deviations can be caused either by short slips overlapping in time and merging into long slips, such as due to excessive force generation, or by the slips reaching a peak velocity. We distinguish between these explanations by examining a histogram of the velocities over individual slips (Figure 4.9), which has a distinct peak and does not exhibit power law scaling as predicted by mean field theories, even in the high-velocity tail of the histogram, despite the power law scaling of the displacement and duration individually.

Figure 4.9: The histogram of slip velocities exhibits a peak at 0.4 μm/s, consistent with previously measured retraction velocities of individual TFP.

The average slip velocity is 0.4 μm/s; this is similar to the retraction velocity of individual *M. xanthus* TFP, which has been measured as 0.7 μm/s when exerting a force of 60 pN and as 0.2 μm/s when exerting a force of 150 pN [71]. We hypothesize that the peak velocity over an individual slip may be limited by the retraction velocity of the TFP motor in *M. xanthus*, where it exerts a force of 60-150 pN. That slips exhibit self-similar behavior in their velocity profiles, however,
is a striking and stringent indication of crackling noise behavior.

### 4.3.6 Multi-bacterial interactions

The social behavior of *M. xanthus* can be examined in the context of this observed stick-slip motion, which is exquisitely sensitive to coordination between cells. We imaged bacteria at cell densities where a range of surface-to-surface distances between bacteria are observed, from cell-cell contact to distances comparable to the bacterial length. We located paired bacteria by visually finding cells that maintained a low end-to-end separation (< 4µm) while moving over a long distance (> 25µm). Pairs of bacteria are able to attach end-to-end and move together, with an interbacterial separation of less than 0.2 µm (Figure 4.10). In contrast with this strong coupling, we also observed pairs of bacteria moving along similar trajectories, with interbacterial surface-to-surface separations of 1-4 µm (Figure 4.10).

![Figure 4.10: A pair of bacteria can move together when attached end-to-end (left). In this configuration, there is no visible distance between the end of one bacterium and the start of the next, and they appear to move as one body. Alternatively, bacteria can follow each other at a small distance (right). They are within TFP sensing range of each other, and the lagging cell may be following the EPS trail of the leading cell.](image)

To quantify the spatiotemporal signatures of how pairs of bacteria follow one another during the initiation of social interactions, we calculate the frequency-dependent velocity-velocity correlation between the motions of two paired bacteria. Bacterial motion was first projected onto a straight line. For each frequency
bin, we apply a bandpass filter to remove the contributions from all other frequencies; then, the correlation between the velocities of the two bacteria could be calculated directly for each frequency bin as \( c(v_1, v_2) = \frac{(v_1 - \bar{v}_1)(v_2 - \bar{v}_2)}{|v_1 - \bar{v}_1||v_2 - \bar{v}_2|} \), where \( v_1 \) and \( v_2 \) are the frequency-filtered velocity sequences. For bacteria that are in end-to-end contact, there is strong correlation at low frequencies (<1 Hz) that progressively decays at higher frequencies (Figure 4.11). It is interesting to note that for bacteria that are within \( \sim 4 \mu m \), velocity correlations qualitatively similar to those in the case of end-to-end contact are observed, although they are significantly weaker (Figure 4.11).

Figure 4.11: The velocities of paired bacteria are correlated in a frequency-dependent manner, with the correlation lasting up to a frequency of 1 Hz (left). Bacteria which follow each other at a distance have a lower but detectable frequency-dependent velocity correlation. \( c(v_1, v_2) = \frac{(v_1 - \bar{v}_1)(v_2 - \bar{v}_2)}{|v_1 - \bar{v}_1||v_2 - \bar{v}_2|} \)

We hypothesize that some of the interactions that act at cell-cell contact already exist at such distances. A fraction of these cell pairs move beyond this initial exploratory engagement and eventually transition to cell-cell contact, so that \( M. \text{xanthus} \) social interactions occur in a series of “handshaking” steps that involve different mechanical coupling at different distances, progressing from cells that simply follow the same EPS-directed path to those that progressively link and eventually “dock”.

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There has been significant effort aimed at understanding whether the two coupled cells contribute equally to motility and how the dynamics of the coupled cells are coordinated. The stick-slip behavior allows us to access this directly. We calculate the correlation between slip times of the leading and lagging bacterium as $c(b_1, b_2)$, where $b_1$ and $b_2$ are sequences that are equal to 1 when the bacterium is in a slip and 0 otherwise. We extend this to a time-dependent correlation by shifting $b_1$ in time (Figure 4.12).

Figure 4.12: For attached bacteria, there is a strong correlation in slip times (blue); unattached bacteria show no such correlation in slip times (red). The peak correlation for attached bacteria is at a delay of 50 ms (upper inset), indicating that, on average, the leading bacterium has its slips 0.05 s before the lagging bacterium.

For bacteria in end-to-end contact, this correlation reaches a peak of 0.4; for unattached bacteria, this stays less than 0.1. Importantly, we find that the correlation peak is not centered at zero, but at a lag time of $\sim 50$ ms. By a Granger causality analysis [189], we confirm that for bacteria in end-to-end contact, the motion of the leading cell Granger causes the motion of the lagging cell ($P =$
0.00003, the P value of the statistical test); for unattached bacteria, Granger causality is not detectable (P = 0.47). These observations show unambiguously that not only do the coupled bacteria move with coordinated slips, but also there is a “locomotive” cell that leads a trailing cell, with the lead cell initiating the motion and the trailing cell being pulled along slightly afterwards. This is direct evidence indicating that the TFP of the lead cell pull both cells and determine the trajectory of the pair. The force generation requirements on the locomotive cell become increasingly stringent for the multiply entrained cells observed in late-stage *M. xanthus* S-motility. This suggests that our observation of lubricating behavior in *M. xanthus* EPS is an enabling factor in S-motility. That some *M. xanthus* EPS can have a lubricating function explains the puzzling observation that more EPS often lead to more motility, rather than less motility, as would be the case if EPS were to function only as a “glue”. For example, the highly motile cells that actively drive fruiting body formation move in the presence of high concentrations of EPS [144, 145]. *M. xanthus* alone among bacteria is capable of S-motility. This observation is consistent with the empirical fact that highly branched EPS is rare for bacteria and indicates that small changes in TFP-EPS technology can lead to qualitative differences in bacterial social organization.

### 4.3.7 Summary

By examining the dynamics of *M. xanthus* cells at fast timescales, we are able to observe the aperiodic stick-slip movements that are caused by the TFP-generated force acting against EPS-derived friction. At timescales shorter than the motility cycle (the time required for typical TFP pulls), the movements of *M. xanthus* cells are consistent with a crackling noise model, which has been previously applied to the dynamics of earthquakes [151] and a variety of other nonbiological systems [152, 153]. We measure critical exponents for the *M. xanthus* system and find that they are consistent with mean field theoretical models of crackling noise and with
other crackling noise systems [154, 155]; additionally, by examining the $\Delta frzD$ and $\Delta frzE$ mutants, which have defects in reversal behavior, we confirm that the sticks and slips are unaffected by the chemotactic decisions of the bacterium. Interestingly, although EPS is usually thought of as a molecular glue, we find that EPS of *M. xanthus* can both promote surface adhesion and also function as a lubricant. To analyze the lubricating properties of EPS we estimate the degree of chaos in the system by calculating a Lyapunov exponent and find that it suggests the existence of highly branched EPS, similar to artificial lubricants but different from the EPS of most other bacteria. We hypothesize that this is necessary for S-motility: We show that the TFP of leading locomotive cells initiate the collective motion of follower cells, whose motions lag behind those of the lead cell by $\sim 50$ ms. The unique lubricating properties of *M. xanthus* EPS may alleviate the force generation requirements on the lead cell and thus make coordinated social motility possible.
CHAPTER 5

Outlook

5.1 C-di-GMP signaling in P. aeruginosa

In previous chapters, this work has discussed the mechanisms by which bacteria move along surfaces - *P. aeruginosa* flagellar-driven spinning and swimming, TFP-driven walking and crawling, and detachment using both motility appendages, and *M. xanthus* TFP-driven social motility. As an extension of this work, it is natural to look at the biochemical triggers which lead bacteria to adopt these motility modes. This can also be examined with particle tracking analysis.

A secondary messenger which induces biofilm formation in *P. aeruginosa* and many other bacteria is cyclic-di-GMP (3’-5’-cyclic diguanylic acid, c-di-GMP) [28, 29, 30, 31]. C-di-GMP is synthesized by a large number of diguanylate cyclases (DGCs) with a characteristic GGDEF domain [190]. Many such DGCs have been identified via genomic analysis [191, 192]. Mutations in individual DGCs result in many distinct phenotypes, indicating that the formation of c-di-GMP is highly localized and linked to particular targets. C-di-GMP is degraded by phosphodiesterases (PDEs) with HD-GYP or EAL domains [30, 193]. As with DGCs, the many PDEs each have specific functions, and can be activated by environmental triggers; some control bacterium-wide c-di-GMP levels, whereas others appear to have spatially localized effects [194, 195, 31].

It is known that low c-di-GMP levels inhibit biofilm formation and can even trigger biofilm dispersal [196]. C-di-GMP levels have traditionally been measured
with chromatography or mass spectrometry performed on cell extracts. However, this global measurement is insufficient to elucidate the details of the c-di-GMP signaling process due to the wide range of behaviors that c-di-GMP influences and the variety of timescales involved. C-di-GMP stimulates the production of adhesins and exopolysaccharide matrix components, controls motility by down-regulating flagellar synthesis, assembly, or motor function, upregulates production of fimbriae, may induce or suppress virulence in pathogenic bacteria, and controls progression through the cell cycle [197, 28, 29, 198, 30, 199].

5.2 c-di-GMP reporter strains and imaging

In order to investigate c-di-GMP dependent behavior, we use a collection of \textit{P. aeruginosa} mutants with a \textit{pCdrA::gfp} fusion [200]. The \textit{cdrA} protein is an adhesin which is believed to crosslink Psl polysaccharide polymers or link them to the cell for increased biofilm structural stability; it is directly regulated by c-di-GMP [201, 202]. The \textit{cdrA} promoter was inserted into a plasmid along with sequences coding for antibiotic resistance and stable GFP and unstable GFP (\textit{gfp ASV}). The resulting plasmid is thus a reporter plasmid for c-di-GMP: a \textit{P. aeruginosa} cell expressing this plasmid produces GFP when activated by c-di-GMP, and ceases GFP production when c-di-GMP levels are low.

In order to measure the cellular expression of c-di-GMP over the early stages of biofilm formation, we record cells with both fluorescence and brightfield imaging. Brightfield images are taken at 1 frame every 3 seconds; fluorescence images are taken at 1 frame every 15 minutes. At this acquisition rate, photobleaching was not a problem and cells did not experience any adverse effects from the fluorescence acquisitions. For the two brightfield images adjacent to a fluorescence image, the GFP intensity of all of the cells could be measured by overlaying the fluorescence image with the located positions of the bacteria. Particle tracking was done using
the brightfield images as before; the fluorescence intensities, and by extension the c-di-GMP levels of the cells, were interpolated for all time based on the levels in the available fluorescence images.

The measured c-di-GMP levels for \textit{P. aeruginosa} PA14 \textit{pCdrA::gfp} cells fall into three categories (Figure 5.1). Some cells attach and detach between fluorescence acquisitions, and so their c-di-GMP levels are not known; they are the thin black bar at the left of the histogram, with a nonphysical intensity of zero. There is a population of cells whose fluorescence is indistinguishable from the autofluorescence levels of WT cells; these bacteria may still fluoresce, but are not producing high levels of c-di-GMP. These cells comprise the gray and white sections in the histogram; the cells with intensities indistinguishable from background are counted in the gray area, and the cells with brightnesses indistinguishable from autofluorescence are in the white area. Lastly, the cells whose fluorescence is brighter than the WT autofluorescence are the cells with high levels of c-di-GMP. These comprise the green area in the histogram. There is no sharp cutoff between cells which are or are not producing GFP when the reporter plasmid is present, since cells are able to increase their levels of c-di-GMP gradually. The cutoff between the cells classified as c-di-GMP high and c-di-GMP low is calculated from the brightnesses of cells which do not have the \textit{pCdrA::gfp} plasmid. The cutoff is set to the maximum level of autofluorescence observed in those cells.

We then look at how the proportion of c-di-GMP producing cells in the WT changes over time in the early stages of biofilm formation. Some cells are fluorescent (producing c-di-GMP) even at initial attachment. The proportion of cells high in c-di-GMP increases over time, from 3\% at the start of the recording to 24\% eight hours later (Figure 5.2). We contrast this with the behavior of two mutant strains, the \textit{\Delta sadC \Delta roeA} and \textit{\Delta bifA} strains. The \textit{\Delta sadC \Delta roeA} strain is deficient in two diguanylate cyclases, \textit{sadC} and \textit{roeA}, and is known to have low c-di-GMP levels. By contrast, the \textit{\Delta bifA} strain is deficient in the \textit{bifA} phospho-
diesterase and has high c-di-GMP levels. We can see that in those strains, the proportion of high c-di-GMP cells does not increase over time. In the ∆sadC ∆roeA strain, the proportion of high c-di-GMP cells stays low, at just a few percent; in the ∆bifA strain, the proportion of high c-di-GMP cells starts high and even decreases, though this may be due to photobleaching of cells that remain on the surface for long times. This is consistent with global assays, which indicate that the ∆sadC ∆roeA strain has low c-di-GMP levels, whereas the ∆bifA strain has high c-di-GMP levels.

Using these metrics, we consider the effect of c-di-GMP on individual cells within a genomically uniform population. We find that in the WT, cells producing c-di-GMP are less likely to be oriented vertically than cells low in c-di-GMP (Figure 5.3). The aspect ratio histograms have a clearly visible distinction between vertical bacteria (aspect ratio ~1, colored red) and horizontal bacteria (aspect ratio ~4, colored blue). The c-di-GMP high cells are 30% less likely to be vertical than the c-di-GMP low cells, indicating that c-di-GMP is associated
Figure 5.2: Fraction of cells lit up in *P. aeruginosa* strains over time. Left: wild-type strain increases c-di-GMP production over time. Middle: ∆sadC ∆roeA strain does not increase in c-di-GMP production. Right: ∆bifA strain has high c-di-GMP levels even at early times.

with the transition from reversible attachment (vertical) to irreversible attachment (horizontal).

Figure 5.3: Cyclic di-GMP production is correlated with bacteria transitioning from vertical to horizontal orientation. Left: aspect ratio histogram of WT cells which are high in c-di-GMP. Peak labeled in red indicates vertical cells, peak colored in blue indicates horizontal cells. Right: aspect ratio histogram of WT cells which are low in c-di-GMP. The vertical peak is higher.
CHAPTER 6

Conclusions

This work has discussed a variety of motility mechanisms that bacteria use in the early stages of biofilm formation. Specifically, we discuss the motility that bacteria exhibit immediately after and just before attachment. In this stage of biofilm formation, the cells transition from the planktonic state to the surface-associated state, and begin to form the foundations for later collective action and biofilm development.

The near-surface swimming we observe in *P. aeruginosa* and *V. cholerae* allows bacteria to explore both the surface and the bulk medium effectively, in order to “decide” whether active attachment to the surface is advantageous or not. *P. aeruginosa* and *V. cholerae* swimming is primarily flagellum-driven; the flagellum-deficient *M. xanthus* does not swim. In addition, we find that *V. cholerae* uses its pili to anchor itself to the surface to transition from bulk swimming to near-surface circular orbits.

On the surface, the bacteria we observe move using their pili. *P. aeruginosa* can stand up vertically to walk on the surface, moving with high velocity in an undirected manner; this motility mode is advantageous for rapid surface exploration. When it orients itself parallel to the surface, *P. aeruginosa* can crawl with high directional persistence, using alternating pili pulls and releases [157]. *M. xanthus*, on the other hand, crawls slowly, with the motion dominated not by the dynamic of pili pulls and releases but by the friction interaction between the bacterial body and the surface, mediated by the secreted EPS layer. Its EPS is
composed of complex branched molecules, similar to artificial lubricants, which allow \textit{M. xanthus} cells to exhibit collective social motility. \textit{V. cholerae} uses its pili for attachment but does not exhibit pili-driven surface motility.

\textit{P. aeruginosa} detachment from the surface is also an active process, driven by both flagella and TFP. The detachment sequence begins with bacteria spinning, attached to the surface by their flagellar pole; the pili then tilt the bacterium to a vertical orientation to facilitate detachment. Detachment without vertical orientation is rare. We find that the cyclic di-GMP levels of \textit{P. aeruginosa} individual cells can determine whether those cells are likely to be vertical or horizontal, and thus whether they stay on the surface or detach.

These single-bacterium motility modes are complementary to the often-studied collective motility modes such as twitching and swarming. We show how for \textit{M. xanthus}, the motility of individual bacteria can support the collective behavior of bacterial groups - the lubricant-like EPS alleviates the force generation requirements on the leading cell in a pack. For \textit{P. aeruginosa}, the uniform biofilm morphology of a WT biofilm relies on bacterial detachment and redistribution, as exhibited by individual cells.

It is as of yet unclear how the collective behaviors of swarming and twitching arise from the motility of individual cells. Even though swarming is typically viewed as flagellum-driven and twitching is typically considered pili-driven, it is reasonable to expect cooperativity between appendages in both of those motility modes. Indeed, assays have shown that pili-deficient bacteria are also defective in swarming. Further research will focus on how global biofilm properties can be understood in terms of the behavior of the individual biofilm cells.
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