Physical Ecology of Marine Microbes

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Abstract

Marine microbes play a fundamental role in driving ocean ecosystem dynamics and biogeochemistry. While their importance is global in scale, microbial processes unfold at the level of single cells and are intimately dependent on interactions between microorganisms, their neighbors, and the surrounding physical and chemical environment. Furthermore, traditional imaging techniques often provide frozen snapshots of the marine microbial world, yet microbial interactions are inherently dynamic, as for example in the case of motility, chemotaxis, and the encounter of microbes with viruses and animal hosts. These biological processes are frequently driven by physical mechanisms, and our understanding of them can benefit from a focus on the physical ecology of marine microbes. This is the approach pursued in this thesis, by directly applying dynamic imaging and microfluidics, which offer powerful new opportunities to study microbial processes in a time resolved manner and with exquisite environmental control. Through single-cell, live imaging of three fundamental marine microbial processes – motility, chemotaxis and viral adsorption – we demonstrate how capturing previously unseen biophysical processes in microbial ecology at their natural timescales can both shed light on unexplained mechanisms and provide robust quantifications of interaction rates.

We first study a newly discovered nanoscale motility adaptation in the marine bacterium *Vibrio alginolyticus* using high-speed imaging. We found that marine bacteria can exploit a buckling instability of their flagellum to change direction during swimming, achieving the same functionality as multi-flagellated cells, but with the cost of synthesizing and operating only one flagellum. This finding not only reveals a new role of flexibility in prokaryotic flagella, but also highlights the exquisite motility adaptations of marine microbes to the resource-poor environment of the ocean.

We then determine how this motility adaptation affects the cells’ ability to climb chemical gradients (‘chemotaxis’). We found that, counter to current models, chemotaxis in *V. alginolyticus* is speed-dependent. Faster cells exhibited not only faster chemotactic migration, but also tighter accumulation around the resource peak. This result adds a new dimension to our
understanding of bacterial chemotaxis pathways, by demonstrating that swimming speed can be an important and counter-intuitive control parameter in how marine microbes encounter and exploit chemical resources.

Finally, we consider an encounter process that is motility-independent – that between a non-motile host and a virus. Using the globally abundant marine cyanobacterium *Prochlorococcus* and a cyanobacterial virus ("cyanophage") as a model system, we directly imaged the encounter and adsorption dynamics of the virus and the host at the level of single cells, using dual-wavelength epifluorescent microscopy. By applying this non-invasive approach to quantify thousands of encounter events using automated image acquisition and analysis, we directly measured the rate at which viruses encounter and adsorb to hosts. We found that the probability of adsorption is considerably lower than was obtained with traditional, bulk measurement approaches, suggesting the need for a revision of viral infection dynamics in marine ecosystem models and opening the door for studies of microbial individuality in the context of viral infection.

In summary, this thesis demonstrates that physical processes in microbial ecology, studied by means of new approaches including microfluidics and dynamic imaging at the single-cell scale, can contribute fundamental new insights into the ecology of marine microbes.

Thesis Supervisor: Roman Stocker
Title: Associate Professor
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INTRODUCTION

Many microbial processes are inherently dynamic, from the physics of cell motility, to behavioral responses toward chemical and physical stimuli, to host-virus interactions in microbial suspensions. The ocean is one of the largest microbial habitats where these events take place, and marine microbes play a fundamental role in driving ocean ecosystem dynamics and biogeochemistry. While their importance is global in scale, how microbes physically interact with the surrounding biotic and abiotic microenvironment was difficult to decipher first due to their miniscule size, and second due to highly dynamic and heterogeneous nature of the ocean even at the scale of a single microbe. In this thesis, we take a peek at the lives of marine microbes using videomicroscopy and microfluidics with the ultimate aim of understanding their physical ecology.

Many microorganisms are motile, a characteristic that is intimately associated with a multitude of behaviors including nutrient uptake, attachment to surfaces and the infection of hosts. Most knowledge of bacterial motility comes from the study of the run-and-tumble swimming of *Escherichia coli*, which has four to eight helical flagella emerging from random points on the cell body. Each flagellum is driven by a rotary motor, powered by a proton gradient across the cell membrane, and when all motors spin counterclockwise (as seen from behind), the flagella form a bundle that allows the cell to swim in nearly straight ‘runs’ at 10–30 μm/s. Cells reorient in a nearly random direction (‘tumbles’) when one or more motors switch direction due to the torque induced by the flagellar unbundling. The ability to transition between runs and tumbles is essential, for example, to climb chemical gradients in search of nutrients or surfaces for attachment.

The swimming strategies of bacteria living in marine habitats can be radically different from that of *E. coli*’s enteric habitat. It has been estimated that 90% of motile bacterial species from the ocean are single-flagellated and thus can not reorient using *E. coli*’s tumbling strategy. How they reorient has long remained unclear until recently, when the marine bacterium *Vibrio alginolyticus* was shown to turn or ‘flick’ by an off-axis motion of its flagellum. We experimentally demonstrate the underlying mechanism responsible for the flick in Chapter 2 of this thesis.

Motility is an essential component of one of the key microbial life strategy, ‘chemotaxis’, which allows cells to sense chemical gradients in the environment and to bias their motion toward favorable conditions. Bacterial chemotaxis is one of the most extensively studied biological sensory systems and plays fundamental roles in a broad range of ecological processes in natural microbial habitats. For instance, in the ocean chemotaxis enables bacteria to exploit the plethora of resource hotspots and organic matter gradients that characterize marine microenvironments, originating from phytoplankton exudation and lysis, sloppy feeding and excretions by larger
organisms, and sinking particles. Evidence that marine bacteria swim and chemotax considerably faster than the classic model organism, *E. coli*, has recently been augmented by the finding that their run-reverse-flick motility is also strongly differs from *E. coli*'s run-and-tumble. To gain a quantitative understanding of the biophysical origin of the high chemotactic performance of marine bacteria compared to *E. coli*, in Chapter 3 of this thesis, we study chemotaxis of the marine bacterium *V. alginolyticus* in well-defined chemical gradients in microfluidic devices at spatiotemporal scales and chemical concentration conditions relevant to marine microbes.

Microbial interactions in the ocean can also be driven purely by physical mechanisms, unlike the active sensing process in chemotaxis. One of the most important ecological interactions in this category is the encounter between hosts and viruses, which is the first step leading to viral infection. In the ocean, viral infection is a significant source of bacterial mortality and an important driver of microbial community dynamics with significant consequences for the biogeochemical processes that microorganisms mediate.

Encounters between non-motile bacterial hosts and viruses are determined purely by their random, Brownian motion. Upon encounter, the virus can irreversibly attach ('adsorb') to the host, if it binds to cell membrane receptors. Subsequently, the virus will infect the host by injecting its DNA, which commences to replicate. Adsorption is a critical yet poorly quantified step in this process: the rate of adsorption can represent a bottleneck in infection dynamics and the currently poor quantification of this process through bulk measurement suggests the need for a more robust experimental quantification. In this thesis, we directly quantify viral adsorption rates through imaging in microfluidic devices, using a model system in marine microbial ecology, the abundant marine cyanobacterium *Prochlorococcus* and one of its virus cyanophages.

These microbial processes occurring in the ocean are dynamic and span a broad range of ecological timescales. We combine dynamic imaging and microfluidics to interrogate these microbial processes with ecologically-relevant spatiotemporal resolution in the context of controlled microenvironments. This allowed us to overcome common limitations in most traditional ensemble-averaged methods because of two reasons: first, dynamic imaging is the most intuitive and often the most robust approach to understanding natural processes; second, microfluidics provides unprecedented control over multiple facets of the natural environment, including the chemical environment (*e.g.*, generating ephemeral resource pulses or precise gradients), the physical environment (*e.g.*, generating controlled fluid flows and shear), and the biological environment (*e.g.*, controlling the relative positioning of cells). Thus, the high-precision environmental control in conjunction with dynamic imaging enables the observation of microbes and viruses in controlled settings that mimic salient features of their natural microbial habitats.
In this thesis, we capture the dynamic nature of these biological processes using live imaging and microfluidics, to investigate the physical ecology of marine microbes that drive three fundamental microbial processes in the ocean: cellular motility, chemotaxis, and bacterial host-virus interactions. We demonstrate how understanding the physical principles behind these processes can provide the basis for new ecological frameworks of marine microbes and the effects they can have on ocean ecosystem dynamics.
CHAPTER 1

Dynamic imaging and microfluidics in microbial ecology*

* In Chapter 1, we provide a review of selected contributions that have leveraged dynamic imaging and microfluidic technology to advance our understanding of fundamental processes in microbial ecology spanning a wide range of microbial habitats including the marine ecosystem. This includes cell motility and flow fields, chemotaxis, microbial interactions with flow and surfaces, biofilm and streamer formation, and single-cell growth dynamics. We focus on the dynamic nature of these microbial processes and highlight the breadth of timescales in microbial ecology amenable to this approach. This chapter serves also as an introduction to the methods of microfluidics and dynamic microscale imaging used throughout this thesis.

A version of this chapter is currently under review for publication as a paper in Nature Reviews Microbiology, as:

Son, K., Brumley, D. B. & Stocker, R. Live from under the lens: Dynamic imaging and microfluidics in microbial ecology, Nature Reviews Microbiology, under revision.

I was responsible for a considerable fraction of the research and writing for this review article, which was put together in close collaboration with Douglas Brumley (postdoc in the Stocker lab) and Roman Stocker.
ABSTRACT

Microbial processes are inherently dynamic, unfold at the level of single cells and are intimately dependent on interactions between microorganisms, their neighbors, and the surrounding physical and chemical environment. Dynamic imaging and microfluidics offer powerful new opportunities to study microbial ecology in a temporally explicit manner and with exquisite control of the physicochemical microenvironment. In this Chapter, we outline how these advances have provided fundamental insights into microbial ecology by revealing previously unseen dynamics pertaining to cell motility, chemotaxis, flow-mediated transport, surface interactions, and single-cell growth, spanning timescales from milliseconds to days.

1.1 INTRODUCTION

Many processes in microbial ecology are inherently dynamic, from the physics of microbial motility, to behavioral responses toward chemical and physical stimuli, to cell-cell interactions in microbial suspensions and biofilms. Dynamic microbial processes span a broad range of ecological timescales, from mechanical instabilities in bacterial flagella that unfold over milliseconds, to biofilm formation and growth that occur over tens of minutes to days. Most traditional ensemble-averaged methods, such as population-scale measurements of motility, chemotaxis and growth, are ill suited to capturing the dynamic nature of microbial processes, and technological limitations have largely precluded the interrogation of microbial interactions with appropriate spatiotemporal resolution in the context of controlled microenvironments.

Dynamic imaging is the most intuitive and often the most robust approach to understanding natural processes, and is widely used in macro-ecology. Advances in experimental technologies and quantitative analysis methods are now bringing new dynamic imaging capabilities to the scales relevant to microbial ecology (see section 1.2.1). Optical microscopy has long been an essential tool for microbiologists, but the ongoing technological developments in digital imaging and image analysis have greatly expanded the opportunities for adding a dynamic dimension to observations, revealing hidden mechanisms and allowing for dynamic quantifications of microbial processes. Advances in time-lapse video microscopy achieved by the seamless integration of high-quality optical microscopes with fast and sensitive digital cameras enable the imaging of microbial processes that occur at timescales ranging from milliseconds to days, with exquisite precision and high signal-to-noise ratio. Dramatic increases in computational resources enable rapid processing of very large imaging datasets to automatically analyze microbial behaviors and interactions at a wide range of spatial scales, from the nanoscale mechanics of flagella or pili to the microscale tracking of individual surface-attached bacteria that self-organize into millimeter-scale biofilms.
This new level of dynamic imaging realizes its full potential when paired with the ability to precisely control the microbial environment using microfluidic technology (see section 1.2.2). Modern microfluidics, which is based on the soft lithography of inexpensive, biocompatible, transparent polymers, is becoming an increasingly enabling approach in the toolbox of microbial ecologists. The reasons for this success are twofold. First, microfluidics allows one to flexibly fabricate environmental arenas that are fully compatible with dynamic, microscale imaging of microbial processes. Second, microfluidics provides unprecedented control over multiple facets of a microorganism’s environment, including the chemical environment (e.g., generating ephemeral resource pulses or precise gradients), the physical environment (e.g., generating controlled fluid flows and shear), and the biological environment (e.g., controlling the relative positioning of cells). This high-precision spatiotemporal control in conjunction with dynamic imaging is having a major impact on microbial ecology by enabling the observation of microorganisms in controlled settings that mimic salient features of their natural habitats.

Figure 1.1 Microfluidics and dynamic imaging have recently shed new light on a broad range of microbial processes. (a) Microfluidics is uniquely suited to studying microbial ecology with exquisite user control of microenvironmental conditions, enabling direct, dynamic imaging of microbial processes. Selected examples include (b) microbial motility mechanics, (c) the flow signatures of swimming microbes, (d) microbial interactions in dense suspensions, (e)
microbial chemotaxis, (f) microbial interactions with flow and surfaces, (g) biofilm and streamer formation, and (h) single-cell growth.

In this Chapter, we describe selected contributions that have leveraged dynamic imaging and microfluidic technology to advance our understanding of fundamental processes in microbial ecology (Fig. 1.1). This includes cell motility and flow fields, chemotaxis, microbial interactions with flow and surfaces, biofilm and streamer formation, and single-cell growth dynamics. We will focus on the dynamic nature of these microbial processes and highlight the breadth of timescales in microbial ecology amenable to this approach by organizing the discussion from millisecond to multi-hour dynamics.

1.2 METHODS

1.2.1 DYNAMIC MICROBIAL IMAGING

Microscopic visualization and image analysis are powerful tools to study dynamic microbial processes. Each image acquired from a digital camera is a two-dimensional array of intensity values (a), where each value is proportional to the number of photons that hit the corresponding camera pixel. For dynamic imaging, one acquires a sequence of images (i.e., a video) ranging approximately from 1 frame/millisecond to 1 frame/hour, as required by the timescale of the process. Here we provide basic information on the use of image analysis for the quantitative interrogation of microbial processes.

Figure 1.2 Single-cell identification by image analysis. The images in part a-f are reproduced from REF. 64. The image in part g is reproduced from REF. 63.
The image of a microbe can be brighter or darker than the background, depending on the microscopy configuration. A simple image processing technique for a rapid first analysis, for example in real time during acquisition, is to take for each pixel the maximum (or minimum) intensity value of that pixel over the entire video. This yields a time-integrated view of the distribution and movement of microbes in a population, somewhat analogous to a photograph taken with a long exposure time.

The full power of image analysis resides in the automated digital identification of individual microbes in each frame and the reconstruction of cell trajectories. The first step often hinges on image segmentation, whereby every group of pixels (a) satisfying prescribed attributes (e.g., their intensity or intensity gradient exceeding a threshold value; their size and/or shape falling within given ranges) is identified as a microbe\(^6\) (b–f) [or, e.g., a flagellum\(^5\,\,7\,4\)]. From the group of pixels, one can then compute the microbe’s position (e.g., the group’s centroid), orientation (e.g., by fitting an ellipse), and size (e.g., the midline length (d)) and use this information for example to quantify growth\(^6\,\,3\,\,4\) (g).

Cell trajectories are reconstructed from individual cell positions in each frame through automated particle tracking algorithms. Tracking routines can be complex, but the fundamental approach is to identify the same microbe in two consecutive frames (e.g., by finding the nearest neighbor in the previous frame, when the rate of imaging is sufficiently high). Repeating this across frames, until a microbe is ‘lost’ (e.g., because it swims off the field of view or out of focus), yields its trajectory, from which one can compute swimming statistics including speed, direction, and turning rate.

In selected cases, three-dimensional (3D) tracking has been applied to microbes. The operating principle of 3D tracking microscopes is based on automatic motion of the microscope stage in 3D through a feedback control loop that maintains a single microbe in focus. This technique provided early data that were key to understand Escherichia coli’s motility\(^7\,\,5\) and was recently used to capture both the orientation and 3D position of individual Caulobacter crescentus cells\(^7\,\,6\). Powerful recent alternatives for 3D tracking are digital holographic microscopy (DHM)\(^4\,\,5\) and defocussed microscopy\(^\,7\,\,7\), which can capture the positions of hundreds of microbes simultaneously without movement of the microscope stage.

To quantify the motion of the fluid, the most frequently used tool is microscale Particle Image Velocimetry (microPIV), which involves seeding the fluid with small (often 0.2–1.0 µm diameter) tracer particles and imaging their motion as they are passively transported by the flow. Each frame is subdivided into rectangular boxes, as small as possible but large enough to contain several tracer particles. Correlation techniques are used to determine the mean displacement of the particles in a given box among consecutive frames, yielding the local fluid velocity. Both particle tracking and PIV algorithms are nowadays widely available in commercial and free
software packages (e.g., ImageJ, MatPIV), making cell tracking and fluid flow measurements in microbial systems broadly accessible.

1.2.2 MICROFLUIDICS FOR THE CONTROL OF MICROBIAL ENVIRONMENTS

Fabrication of microfluidic devices has now become commonplace at most research institutions and is also commercially available, making it widely accessible to microbial ecologists. A range of fabrication methods and materials exist, yet soft lithography and the use of the elastomeric polymer polydimethylsiloxane (PDMS) remain most common due to several advantages: PDMS is optically transparent, biocompatible, chemically inert, gas-permeable, flexible, and inexpensive. Here we illustrate key features of microfluidics that allow precise physical and chemical control of microbial microenvironments.

Controlling fluid flow. Microfluidic channels can be used to generate carefully controlled fluid flows, thereby mimicking salient features of the physical environment of microbial habitats. Flow inside microfluidic channels is typically laminar. The velocity inside the channel is non-uniform: it is zero at the top, bottom and sidewalls ('no-slip' condition) and maximum at the center. For a microfluidic channel with a high aspect ratio cross-section, the velocity profile is parabolic across the smallest dimension (often the microchannel’s depth; z-direction in panel a), and almost uniform ‘plug-flow’ across the widest dimension (y-direction in panel a). Cells in suspension are transported by the flow, while surface-attached cells experience flow as a drag force.

The non-uniformity of the velocity profile implies the existence of velocity gradients – or ‘shear’ – which and are strongest at the channel sidewalls, where fluid is at rest. Cells in suspension are continuously rotated by shear, while surface-attached cells experience shear as a torque that tends to bend them.

![Image](image_url)

Figure 1.3 Microfluidics allow generation of controlled fluid flow and chemical gradients. The images in part a is reproduced from REF. 81. The image in part b is reproduced from REF. 32.
Controlling chemical gradients. Microfluidic devices are ideally suited to create a wide range of chemical gradients (e.g., steady or unsteady, linear or nonlinear, of small molecules or gases) at the scale relevant to the chemical ecology of microbes. For example, unsteady chemical gradients designed to mimic ephemeral resource hotspots can be generated by creating a microscale solute band that subsequently spreads by molecular diffusion to become homogeneously distributed.

The integration of porous materials (hydrogels or membranes) within microfluidic devices enables the generation of steady chemical gradients in flow-free conditions. A prototypical case consists of a ‘test’ channel containing microbes, separated by thin hydrogel walls from a ‘source’ channel carrying chemoattractant on one side, and a ‘sink’ channel carrying buffer on the other side. By diffusing from the source channel through to the sink channel, the chemoattractant establishes a steady uniform gradient in the test channel, allowing the study of microbial behavior or physiology in a controlled chemical environment over extended timescales. Many variants of this concept exist, with other implementations enabling the creation of multiple, simultaneous chemical gradients, in parallel or in opposition; the generation of temporally alternating gradients; and the simultaneous quantification of chemotactic migration and surface attachment.

Finally, surface chemistry can also be controlled in microfluidic devices, for example by pretreatments that induce (e.g., Poly-L-Lysine) or prevent (e.g., bovine serum albumin) cell attachment, or change surface hydrophobicity (e.g., Octadecyltrichlorosilane, OTS).

1.3 CELL MOTILITY

Many microorganisms are motile. Cell motility is intimately associated with a multitude of behaviors including nutrient uptake, attachment to surfaces and the infection of hosts. Direct visualization of microbial locomotion has recently revealed the rich biomechanics underlying new motility adaptations of microorganisms (Fig. 1.1b), as well as the microscale fluid flow that swimming microorganisms produce in their immediate surroundings (Fig. 1.1c). The ability to resolve these flow fields has provided fundamental insights into microbial propulsion mechanisms and the collective motions of dense suspensions of bacteria (Fig. 1.1d).

1.3.1 Microbial motility mechanics

Fast imaging of microorganisms at scales as small as their nanometer-wide propulsion appendages – flagella – has revealed new motility adaptations in bacteria and fostered their mechanistic understanding. Most knowledge of bacterial motility comes from the study of the run-and-tumble swimming of *Escherichia coli*, which has four to eight flagella emerging from
random points on the cell body. Each flagellum is driven by a rotary motor, powered by a proton gradient across the cell membrane, and the resulting rotation of the helical flagella causes them to ‘push off’ the surrounding water and generate propulsion. Direct visualization of flagella revealed that when all motors spin counterclockwise (as seen from behind), the flagella form a bundle that allows the cell to swim in nearly straight ‘runs’ at 10–30 μm/s. The bundle comes apart when one or more motors switch direction, causing the cell to reorient in a nearly random direction (‘tumble’). The transition between ‘running’ and ‘tumbling’ is the key to performing chemotaxis and is common among peritrichous bacteria, including the pathogen *Salmonella typhimurium* and the soil-dwelling *Bacillus subtilis*.

**Figure 1.4** Microbial motility and microbial flow fields. a-c The marine bacterium *Vibrio alginolyticus* reorients via a ‘flick’, an off-axis deformation of the flagellum that allows bacteria with a single flagellum to change their direction of swimming (a). The image sequence, captured with high-intensity dark-field microscopy, shows the kinematics of the flagellum (magenta) during a flick (b). The flick occurs ~10 ms after the transition from backward (green) to forward (red) swimming and results from a buckling instability of the hook, which is compressed by the drag on the cell head and the propulsion force from the flagellum (c). d The flow field produced by a swimming *Escherichia coli* cell. *E. coli* cells are pushers, with the flagella at the back pushing the cell head, resulting in fluid to move away from the cell along the swimming direction and toward the cell from the sides (black streamlines). The red arrows show the forces exerted by the bacterium on the fluid. e A dense suspension of *Bacillus subtilis* cells exhibits turbulent-like collective motion, which can result in enhancement of mixing and solute...
transport. The images in part b and c are reproduced from REF. 5. The image in part d is reproduced from REF. 6. The image in part e is reproduced from REF. 8.

The swimming strategies of bacteria living in other habitats can be radically different from that of E. coli. It has been estimated that 90% of motile marine bacteria are monotrichous, i.e., they have only a single flagellum – and can thus not reorient using E. coli’s tumbling strategy. Instead of tumbling, monotrichous marine bacteria including Vibrio alginolyticus, Shewanella putrefaciens, Pseudoalteromonas haloplanktis, and Deleya marina reverse swimming direction by reversing the direction of rotation of their single motor. Unlike E. coli, which always swims forward with its flagella in the rear (called a “pusher”), these bacteria alternate forward swimming (flagellum pushing the cell head) and backward swimming (flagellum pulling the cell head) (Fig. 1.4a,b). In this ‘run-and-reverse’ swimming pattern, the ability to change swimming direction (as opposed to just orientation) remained difficult to explain.

Recently, this conundrum has been solved with the observation of a new motility adaptation – the ‘flick’ – that is prevalent among marine bacteria. The flick consists of a large, off-axis deformation of the flagellum that results in a 90° (on average) reorientation in the swimming direction (Fig. 1.4a,b). High-speed imaging of the 20 nm-thick flagellum of V. alginolyticus showed that the flick occurs approximately 10 ms after the onset of a forward run (Fig. 1.4b). At that instant, the drag force on the cell head and the propulsion force from the flagellum exert a compressive force on the ~100 nm long ‘hook’, connecting the flagellar filament with its motor, that is sufficient to make it buckle and causes the flagellum to deform off axis (Fig. 1.4c). This observation demonstrates that flexibility can be important in the functionality of prokaryotic and not only eukaryotic flagella.

There is strong evidence that the ‘run-reverse-flick’ motility strategy is used by other cultured isolates (P. haloplanktis, Vibrio corallilyticus) and widely among natural communities of coastal bacteria. The flick allows cells to effectively reorient with only one flagellum, saving on the cost of building and operating multiple flagella in the often nutrient-scarce ocean. Methodologically, the flick’s recent discovery exemplifies the power of dynamic imaging at extreme length- and time-scales in understanding the exquisite motility adaptations of microorganisms.

1.3.2 The flow signature of individual bacteria and dense suspensions

The hydrodynamic signature of a swimming microorganism impacts how the cell interacts with its physical and chemical microenvironments, yet has remained elusive due to its fast timescale and minuscule length scale. The propulsion of a swimming microorganism inevitably results in a disturbance to the surrounding fluid. This disturbance can affect the transport of chemicals, the
physical interaction of a cell with surfaces or with conspecifics, and may represent a hydrodynamic cue revealing the presence of a microorganism to its predators.

Dynamic visualization of a microbe’s fluid disturbance, or ‘flow field’, is challenging because of the small scale of the flow, the cells’ rapid translation and rotation, and the influence of thermal fluctuations. Despite these challenges, the flow field produced by individual swimming *E. coli* cells has recently been measured with exquisite precision \(^6\) (Fig. 1.4d) by tracking small ‘tracer’ particles in the cell’s vicinity (see section 1.3.1), where >5 billion tracer velocity vectors were necessary to resolve the flow field owing to the importance of thermal fluctuations (*i.e.*, Brownian motion) at this scale \(^6\).

The resulting flow field confirms long-standing theoretical predictions for *E. coli* both in terms of the nature and magnitude of the flow \(^7\). The observed flow is characteristic of ‘pusher’ microorganisms, those who use their flagella to push through the fluid (Fig. 1.4d). As a result, fluid is pushed away from the cell in the front and the back, and pulled towards the cell from the sides (Fig. 1.4d). These hydrodynamic signatures can affect the coupled motion of two bacteria in close proximity, but the rapid spatial decay of the flow field means that beyond a few micrometers, the effects of the flow field are drowned out by stochastic swimming motion and rotational diffusion.

Bacteria can also be ‘pullers’, when flagella pull the cells through the fluid (Fig. 1.4a,b). In this case, the flow field will be opposite in sign to that observed for *E. coli*. Many bacteria, in particular marine bacteria, alternate between pushing and pulling, as evidenced in the ‘run-reverse-flick’ motility pattern \(^4\) \(^5\) (see section 1.3.1). One potential consequence of alternating between the two modes of swimming is to maximize chemotactic performance compared to run-and-tumble \(^4\), but the determination of the optimal movement pattern for chemotaxis remains an open topic of investigation.

The flow field produced by an individual bacterium may be responsible for the striking, turbulent-like collective motions observed in dense suspensions of bacteria, when cell concentrations reach \(10^{10}-10^{11}\) ml (Fig. 1.4e). These collective motions have been studied extensively over the past decade exploiting the ability of microscale devices to control, confine, and visualize dense bacterial suspensions. They take the form of vortices and jets with dimensions (~50-100 µm) and speeds (50–200 µm/s) considerably greater \(^8\) \(^9\) than those of individual bacteria (~2 µm; 10–50 µm/s).

The origin of these emergent collective behaviors is in the physical interactions among densely packed cells \(^10\) \(^-\) \(^12\), though it remains unclear whether through hydrodynamic interactions or physical contact \(^13\). These collective motions may enhance the dispersion of microbes, accelerate the mixing of nutrients and oxygen \(^14\) \(^15\), and afford bacteria collective resistance to antibiotics \(^16\).
Whereas the full ecological implications of this physical process remain unexplored, we propose that it holds promise as a framework for microbial dispersion, chemical transport and physical interactions for microbial environments with densely packed bacterial populations, such as the human gut\textsuperscript{17}, where motility at the individual scale may thus impact environmental conditions at the population scale.

1.4 MICROBIAL INTERACTIONS WITH THE CHEMICAL MICROENVIRONMENT

Many microbial life strategies are based on sensing chemical gradients in the environment and biasing motility toward favorable conditions through a process called chemotaxis (Fig. 1.1e). Chemotaxis is one of the most extensively studied biological sensory systems and plays fundamental roles in a broad range of ecological processes, including nutrient consumption and cycling, pathogenesis, and surface colonization. Recently, the ability afforded by microfluidics to create chemical gradients at the spatial and temporal scales relevant to microbes (see section 1.2.2) has provided an important new tool for the study of chemotaxis. This has enabled the discovery and investigation of previously unknown principles in the bacterial chemosensory system, as well as the prediction of potential impacts of chemotaxis in the environment, including chemical cycling in the ocean.

1.4.1 Chemotaxis in \textit{Escherichia coli}

Chemotaxis hinges on the ability to sense chemical concentrations using trans-membrane chemoreceptors, to process this information via intracellular signal transduction systems, and finally to bias motility towards better environmental conditions. The unprecedented degree of control over chemical gradients in microfluidic devices and the ability to track chemotaxis by dynamically imaging bacteria at the single-cell level has furthered our understanding of how the model organism \textit{E. coli} modulates chemotaxis in different environments.

Imaging of hundreds of bacteria in steady gradients revealed that \textit{E. coli} exhibits a chemotactic response to amino acids (\(\alpha\)-methyl-DL-aspartate and L-Serine) that has approximately the same strength over a wide range of concentrations and gradients. \textit{E. coli} achieves this by biasing its swimming not in response to the concentration gradient \textit{per se}, but to the gradient normalized by the mean concentration. This property is called logarithmic sensing\textsuperscript{18}, and ensures a response-rescaling so that the cell retains high sensitivity under a broad range of environmental conditions, akin to what occurs in human vision and hearing.

Direct imaging of chemotactic migration in microfluidic devices together with fluorescence resonance energy transfer (FRET) measurements of intracellular signaling showed that \textit{E. coli}'s
chemotactic response has an even stronger rescaling property – fold-change detection (FCD)\(^9\) – whereby the full time course of the response remains unchanged if the chemical field is rescaled by a constant factor. Thus, cells respond to the temporal dynamics of the chemical signal, irrespective of its absolute intensity, demonstrating an exquisite ability to follow chemical cues under a broad range of conditions that is likely an adaptation to the diversity of chemical conditions cells encounter in the environment.

Microfluidic approaches are well suited to studying microbial responses to chemical landscapes that begin to capture the complexity of natural environments, where microorganisms often experience temporal fluctuations in the chemical landscape or multiple chemical gradients simultaneously. Imaging the response of an *E. coli* population to a gradient of L-aspartate that was periodically reversed revealed that cells were able to track the time-varying gradient when the flipping was not too rapid (period > 200 s), owing to the time required for adaptation\(^20\). This demonstrates that the internal adaptation rate, controlled by the chemoreceptor methylation level, sets a fundamental biophysical limit to the frequency of environmental fluctuations that *E. coli* can track by chemotaxis.

Exposing *E. coli* cells to simultaneous, opposing gradients of two different amino acids (\(\alpha\)-methyl-DL-aspartate and L-serine) in a microfluidic device showed that the cell’s relative preference for each depended on the relative number of Tar to Tsr receptors\(^21\). The receptor expression level was in turn determined by the density of cells in the population, demonstrating that the cellular environment influences the decision-making process of bacteria exposed to multiple chemical cues and opening the door to the investigation of more complex chemical landscape with microfluidics.

### 1.4.2 Chemotaxis in marine bacteria

Recent research has demonstrated the prevalence and ecological importance of chemotaxis among marine bacteria. Chemotaxis enables bacteria in the ocean to exploit the plethora of resource hotspots and organic matter gradients that characterize marine microenvironments, originating from phytoplankton exudation and lysis, sloppy feeding and excretions by larger organisms, and sinking particles. Imaging has revealed the ability of natural communities of marine bacteria to chemotactically cluster around particles and in the organic-matter rich ‘phycosphere’ surrounding stressed or dying phytoplankton cells\(^22,23,24\) (Fig. 1.5a). Imaging coupled with cell tracking has shown how exquisite the ability of marine bacteria to track phytoplankton is: in one case, the marine bacterium *Pseudoalteromonas haloplanktis* made up to 12 correct consecutive turns in chasing the swimming phytoplankton *Pavlova lutheri*\(^25\), tracking its chemical wake by chemotaxis. These close spatial associations between primary producers and bacterial consumers can provide a fitness advantage to motile bacteria and potentially shape the timescale and modes of transformation of dissolved organic matter (DOM) in the ocean.
Figure 1.5 Microbial chemotaxis. a | Snapshot of the phycosphere, the organic-matter-rich microzone surrounding individual phytoplankton cells. The maximum intensity projection image (see section 1.2.1) shows trajectories of natural marine bacteria (blue) strongly accumulating around a lysing *Chaetoceros* diatom by chemotaxis. b | Schematic of the microfluidic 'microinjector' used to study microbial behavioral responses to ephemeral resource hotspots, primarily among marine microorganisms. The resource is ephemeral because the initial chemoattractant band diffuses outwards (in the x-direction). Bacterial locations and trajectories can be captured by time-lapse imaging (see section 1.2.1). c | Bacterial pathogens can detect their coral hosts by chemotaxis. Motility is prevalent among putative coral pathogens and microfluidic experiments showed that *Vibrio coralliilyticus* exhibits strong chemotaxis towards coral mucus (yellow shading), which diffuses from the coral surface. This response is likely a mechanism for the pathogen to locate its coral host and is exacerbated under warming conditions. d | Model for the coexistence of closely related populations of marine bacteria based on tradeoffs in their spatial behaviors. Dynamic imaging in microfluidic devices showed that both populations of *Vibrio cyclitrophicus* employ chemotaxis to migrate towards particles, but only one population attaches and forms biofilms on particles (red cells). The other population (blue cells) hovers near the particle and retains the flexibility of rapidly migrating to new, more nutrient-rich particles. This represents the first microbial example of a competition-dispersal tradeoff. The image in part c is reproduced from REF. 31.

Microfluidics has provided a platform for the controlled production of microscale hotspots and the detailed study of the microbial response to them. Chemotaxis of marine bacteria to ephemeral, microscale DOM pulses has been studied with a microinjector device, in which a precisely controlled band of DOM with size commensurate with that of microscale hotspots in the ocean (≈300 μm) is generated and then allowed to freely diffuse (Fig. 1.5b). Imaging bacteria responding to the band revealed that *P. haloplanktis* exhibits a chemotactic response to these ephemeral DOM pulses that considerably exceeds the fastest chemotactic responses known for *E. coli*, resulting in more than an order of magnitude enhancement in the nutrient exposure.
Subsequent applications of the microinjector broadened this finding by demonstrating strong chemotaxis of marine bacteria towards a range of important chemical compounds, including DOM exuded from the highly abundant cyanobacteria *Synechococcus* and *Prochlorococcus*\(^\text{28}\), the exudates of the harmful-algal-bloom producing phytoplankton *Heterosigma akashiwo*\(^\text{29}\), and the sulfur compound dimethylsulfoniopropionate (DMSP), whose microbial breakdown can produce the climatically active dimethylsulfide\(^\text{30}\). These microfluidic observations demonstrate and quantify the pervasive behavioral responses of marine bacteria and provide the basis for new ecological frameworks of marine microbes and their biogeochemical roles that takes explicitly into account microscale heterogeneity.

Chemotaxis can also favor the association between marine bacterial pathogens and their animal hosts. In a further application of the microinjector device, the coral pathogen *Vibrio coralliilyticus* was observed to chemotax with striking speed and directionality towards a layer of the mucus from its coral host, *Pocillopora damicornis*\(^\text{31}\) (Fig. 1.5c). This response was driven primarily by DMSP present in the mucus. Tracking instantaneous cell responses showed that, in addition to chemotaxis (directional swimming in response to a gradient), *V. coralliilyticus* heightened its response to mucus by chemokinesis (the modulation of swimming speed in response to increased concentrations)\(^\text{31}\). Chemokinesis is typically absent in *E. coli* and its observation in this host-pathogen interaction is illustrative of the arsenal of behavioral responses that bacteria utilize in the ocean. Repeated experiments with mucus from heat-stress experiments revealed that higher temperatures further favor the pathogen, by causing a greater release of DMSP by the coral and consequently enhancing the chemotactic response of *V. coralliilyticus*.

In the water column, an important driver of chemotaxis is the opportunity to encounter and exploit particles, often rich in organic matter. Successful chemotaxis towards the particle surface can be ensued by significantly different behaviors, even among closely related bacteria. This was demonstrated in a recent microfluidic study, in which attachment and chemotaxis were assayed in two recently speciated, sympatric populations of the marine bacterium *Vibrio cyclitrophicus* to provide insights into the mechanism of their coexistence in nature\(^\text{32}\). By creating microenvironments mimicking both the particle surface and the chemical gradient near it, this study revealed that one population specialized to colonize particles by attachment and biofilm formation, whereas the other specialized to disperse among particles by chemotaxis to take advantage of the occurrence of fresh particles (Fig. 1.5d). These phenotypic differences were paralleled by corresponding genotypic differences related to surface attachment and biofilm formation. This is the first microbial example of a ‘competition-dispersal’ trade-off, developed and traditionally applied at the macroscale, and illustrates how differences in spatial behavior can drive coexistence in the environment.
1.5 MICROBIAL INTERACTIONS WITH THE PHYSICAL MICROENVIRONMENT

The combination of microfluidic technology and novel imaging techniques has resulted in powerful new approaches to study the interactions between microorganisms and their physical environment, from fluid flow to surfaces (Fig. 1.1f). High-speed tracking of individual bacteria in precisely controlled fluid flows has shown how flow exerts torques on microbes that bias their swimming and can impact pervasive microbial processes including chemotaxis and surface attachment. Tracking individual bacteria on surfaces has revealed a range of surface motility adaptations and yielded a new understanding of the initial stages of biofilm formation. Finally, observing surface-attached microbes under controlled physical conditions has shed light on the social dynamics within biofilms, including new solutions to the public goods dilemma, and how fluid flow and complex topography can lead to the formation of semi-suspended biofilms called streamers (Fig. 1.1g).

1.5.1 Fluid flow effects on microbial transport

Microbes often live in dynamic fluid environments, yet tools to study and quantify the effects of fluid flow on microbial processes have traditionally been lacking. In the last few years, microfluidics coupled with dynamic imaging has provided an ideal approach to study flow-microbe interactions, through precise tracking of microorganisms in exquisitely controlled flows (see section 1.2.2). This approach has already revealed important consequences of the coupling between flow and motility, including strong spatial heterogeneity and preferential migration of microbes in flow.

In a fluid flow, non-motile microbes travel faithfully with the flow, their small size precluding any deviations from fluid streamlines. However, inevitable fluid velocity gradients (‘shear’; see section 1.2.2) exert torques on a microbe that result in its periodic rotation, called a ‘Jeffery orbit’ (Fig. 1.6a). If the microbe is motile, this rotation impacts its swimming direction and thus where it ends up in the flow. Many bacteria are highly elongated, particularly if they possess flagella: for example, the hydrodynamic aspect ratio of B. subtilis is ~10. The rotation rate in the Jeffery orbit is faster when the cell is oriented transverse to the flow and slower when it is aligned with the flow (Fig. 1.6a,b). Consequently, elongated microbes spend most time aligned with the flow, and their ability to migrate across streamlines is hampered.

Fast imaging of B. subtilis cells swimming in a laminar flow revealed that Jeffery orbits can strongly alter the spatial distribution of motile bacteria (Fig. 1.6c). Shear varies linearly across a microfluidic device, from high near the sidewalls where fluid velocity is lowest, to low in the center where fluid velocity is highest (but mostly uniform) (Fig. 1.6b). Consequently, as a result of Jeffery orbits, bacteria are free to swim in all directions equally near the channel’s center,
where shear is low, but become trapped and accumulate near the channel’s sidewalls, where shear is high (Fig. 1.6b). This effect caused a >70% depletion of cells from the channel’s center (Fig. 1.6c) and this depletion occurred very rapidly, unfolding over only a few seconds. Strong heterogeneity in the population distribution was observed for both the tumbling, wild-type \textit{B. subtilis} and a smooth-swimming mutant, as well as for monotrichous \textit{Pseudomonas aeruginosa} \textsuperscript{35}. This strong redistribution in the positions of cells originates from the competition between the shear-induced alignment of the bacteria and the random reorientations due to active tumbling and passive Brownian rotational diffusion. Additional microfluidic observations revealed that this ‘shear-trapping’ can have direct consequences on major microbial phenotypes, including chemotaxis and surface attachment \textsuperscript{35}. Given that essentially all motile microbes have high effective aspect ratios and that in most flows shear is spatially non-uniform, shear-trapping is expected to apply to a broad range of bacteria in a wide array of habitats characterized by strong shear, including soil and other porous media, the coastal ocean, and biomedical devices such as catheters.

\textbf{Figure 1.6 Microbial interactions with flow.} \textit{a} | Elongated particles or microorganisms exposed to fluid velocity gradients (‘shear’) undergo periodic rotation, or ‘Jeffery orbits’ \textsuperscript{40}. The angular velocity varies with orientation relative to the flow, faster when the cell is oriented transverse to the flow and slower when it is aligned with the flow. Jeffery orbits can considerably affect the transport of microbes in the environment. \textit{b-c} | Fluid flow biases the motility of swimming bacteria. Like many natural flows, the parabolic flow profile in a microfluidic channel has non-uniform shear: bacteria are free to swim in all directions equally in the central, low-shear region, but preferentially align and become trapped in high-shear regions near the sides \textsuperscript{35} (\textit{b}). Thus, motile bacteria are depleted in regions of low shear and accumulate in regions of high shear, resulting in strong spatial heterogeneity in the bacterial distribution \textsuperscript{35} (\textit{c}). This ‘shear-
trapping’ increases surface attachment and hampers chemotaxis\cite{35}. Fluid flow causes upstream swimming of *E. coli*\cite{36,37} near a surface (d) and upstream twitching of *Pseudomonas aeruginosa*\cite{38} on a surface (e). The torque induced by the shear at the surface orients cells to point upstream in both cases. For twitching cells, the pili’s periodic extension and retraction pulls the cell upstream\cite{38}. Upstream motility can affect transport of bacteria in biomedical settings including the urinary tract, catheters or blood vessels. Part c is adapted from REF. 35. The images in parts d and e are adapted from REF. 37 and 38, respectively.

Beyond free-swimming cells, live imaging revealed that fluid flow can have important and counterintuitive consequences on bacteria moving on surfaces. Microfluidic experiments have shown that *E. coli*\cite{36,37} swimming near a surface (Fig. 1.6d) as well as *Xylella fastidiosa*\cite{41} and *P. aeruginosa*\cite{38} twitching on a surface migrate upstream (Fig. 1.6e) in the presence of flow. This upstream migration results from the shear at the surface exerting a torque on the cells that orients them to point upstream. Therefore, even fast flows cannot be assumed to simply wash out bacteria, with potential consequences on infection processes in the urinary tract, catheters or blood vessels where upstream migration may cause bacterial transport into unexpected regions of the flow.

### 1.5.2 Surface approach

Surfaces are ubiquitous in microbial habitats and the surface-attached lifestyle is dominant among many microbial species (Fig. 1.7). By providing the opportunity to observe microbes near and on surfaces under highly controlled conditions (see section 1.2.2), microfluidic platforms are ideal for imaging and understanding how microorganisms approach, interact with, disperse on and attach to surfaces.

Microbes swimming near surfaces display two characteristic features. First, microbes often become effectively trapped near the no-slip boundary\cite{42}, even when they are several body lengths from the surface. This trapping originates from the interaction between the shape of the microbe’s flow field and the surface: for *E. coli* this interaction leads to a reorientation of the cell parallel to the surface\cite{43}. Second, microbes often swim in circular trajectories near a surface (e.g., clockwise for *E. coli* when viewed from above), due to the different torques induced by the presence of the surface on the flagellum and the cell head\cite{44} (Fig. 1.7a). These phenomena affect a microbe’s approach to and residence time near a surface, and can thus influence the rate of attachment and biofilm formation. Furthermore, these processes highlight the importance of a microbe’s flow signature (see section 1.3.2), with different propulsion strategies resulting in different forces and interactions between microbes and surfaces\cite{43}.
Figure 1.7 Microbial interactions with surfaces. a | Bacteria near a surface often swim in circles\textsuperscript{44}. The rotation of the flagellar bundle near the surface (black solid arrow) induces a net reaction force on the flagellar bundle (blue arrow) from the surface; the cell head rotates in the opposite direction and experiences an opposite force (red arrow). Circular swimming results from the torque (green arrow) induced by these two forces\textsuperscript{42,44}. The inset shows observed trajectories of a smooth swimming mutant of \textit{E. coli}\textsuperscript{44}. b-d | Live imaging of cells on a surface revealed distinct modes of surface motility in \textit{P. aeruginosa}\textsuperscript{47,48}. Cells can either stand up on the
surface and ‘walk’ (b) or lie on the surface and ‘crawl’ (c). Walking results in jagged trajectories that are better for local exploration, whereas crawling has high directional persistence and allows cells to more effectively cover distance\(^47\). Simultaneous pulling of multiple type-IV pili that results in steady crawling motility (c) is interrupted by the rapid (100 ms), ‘slingshot’ release of a single pilus that causes an impulsive forward motion coupled with a change in direction\(^49\) (d). e-f Long-term imaging in a microchannel revealed the effect of *Caulobacter crescentus*’s curved shape in surface colonization under flow\(^51\) (e). Curved WT cells (green) colonize the surface more successfully than a straight mutant (red, creS) (f), because fluid flow more effectively bends the dividing cell towards the surface, conferring a higher sticking probability to the daughter cell\(^51\) (e). The inset image in part a is reproduced from REF. 44. The images in parts b and c are reproduced from REF. 47. The image in part d is adapted from REF. 49. The images in parts e and f are adapted and reproduced, respectively, from REF. 51.

The presence of a nearby surface can also affect the reorientation dynamics of bacteria, as revealed by tracking thousands of *E. coli* cells in three dimensions using digital holographic microscopy (see section 1.2.1). A ~50% reduction in tumbling frequency was observed within 20 \(\mu\)m of the surface, compared to locomotion in the bulk, resulting from the surface hindering the unbundling of flagella necessary for tumbling\(^45\). Furthermore, tumbles largely reoriented cells in surface-parallel directions that contributed to trap them near the surface\(^45\). This finding suggests that the flow field and run-and-tumble motility pattern of *E. coli* is either not ideal for escaping surfaces, or conversely is well suited for ensuring that cells retain position near surfaces, potentially to enhance attachment rates or benefit from surface-derived nutrients.

1.5.3 Surface contact

Appendages play an important role in the surface interaction of many microbes, and the initial contact with a surface can be strongly dependent on the behavior of a microbe’s flagella and pili. *Vibrio cholerae* cells swimming near a surface use their MSHA pili to mechanically scan the physical properties of the surface before attaching\(^46\). Rotation of the cell body during propulsion causes periodic contact of the MSHA pili with the surface: depending on the magnitude of the frictional forces between pili and the surface, the cells exhibit either ‘roaming’ motion, characterized by meandering trajectories with low frictional interaction, or ‘orbiting’ motion, characterized by high-curvature trajectories with strong pili–surface interactions. The distinction between these two modes is important in determining surface colonization, because only orbiting cells can attach irreversibly and form microcolonies\(^46\).

Microbes display several distinct surface-motility and chemical-tracking adaptations after landing on surfaces to transition from the planktonic to the surface-associated state and ultimately initiate biofilm formation. *P. aeruginosa* cells can repeatedly attach and detach their

37
type IV pili to either ‘walk’ on a surface in a vertical orientation (Fig. 1.7b) or ‘crawl’ on it in a horizontal orientation\(^47,48\) (Fig. 1.7c). Walking cells exhibit a higher instantaneous velocity (~70 nm/s) than crawling cells (~40 nm/s), but their trajectories on the surface are diffusive, allowing for rapid local exploration of the surface, whereas crawling trajectories are straighter, allowing for more effective large-scale exploration.

Especially during crawling, \(P.\ aeruginosa\)’s pili mediate two distinct actions: multiple pili can pull a cell at a constant velocity or the ‘slingshot’ release of a single pilus can transiently propel the cell 20 times faster while also rotating it\(^49\) (Fig. 1.7d). The 100 ms long slingshot motion may assist in propulsion through viscoelastic fluids, such as the polymeric matrix of biofilms, whose viscosity is reduced significantly at the large shear rates produced by this rapid action\(^49\).

How cells crawl on surfaces can have direct implications for the formation of biofilms. Tracking \(P.\ aeruginosa\) cells crawling on a surface and quantifying the cells’ visit frequency to each location on the surface revealed the mechanism by which cells begin to build biofilms\(^50\). After landing on a clean surface, cells begin to deposit a trail of the exopolysaccharide Psl as they crawl on the surface over many hours. This chemical trail influences the motility of bacteria that subsequently encounter the trail, creating a positive feedback loop where regions of high Psl concentration result in more Psl deposition, and thereby become nucleation points for microcolony formation\(^50\).

Beyond the role of cell appendages, the shape of the cell body can be an important phenotype for surface colonization, particularly in the presence of flow\(^51\) (Fig. 1.7e,f). In microfluidic experiments, shear was found to cause individual crescent-shaped \(Caulobacter\ crescentus\) bacteria to bend towards the surface, in the direction of the flow (Fig. 1.7e). During cell division, the natural curvature of \(C.\ crescentus\) aids in orienting the adhesive pili located at the daughter cell’s pole towards the surface. This mechanism promotes the surface attachment of the daughter cell, and in the presence of flow enhances surface colonization compared to straight mutants (Fig. 1.7f).

**1.5.4 Physical interactions in biofilms**

Biofilms represent a pervasive life strategy of bacteria in a broad range of environments\(^52\) (Fig. 1.8), where bacteria bunker down on surfaces by encasing themselves within a self-secreted matrix of extracellular polymeric substances (EPS) that confers them increased resistance against antibiotic insults and mechanical stress\(^53\). The principles underlying biofilm formation have largely remained elusive, in part because of the complexity of the physical interactions between cells, the substrate and the ambient fluid flow.
Figure 1.8 Biofilm and streamer formation. a-b | Microfluidic experiments recently revealed two solutions of the public goods dilemma in \textit{Vibrio cholerae} biofilms: cells can either produce thick biofilms that confine the goods to producers, or rely on fluid flow to wash away the goods and deny access to non-producing cheaters\textsuperscript{54}. Confocal image shows a population of chitinase-producing \textit{V. cholerae} (yellow) outcompeting a non-producing mutant (red) on chitin particles (blue)\textsuperscript{54}. c-e | The flexibility in the design of microfluidic channels has yielded new insights on the formation of biofilm streamers, tethered filamentous aggregations of bacteria held together by extracellular polymeric substances (EPS)\textsuperscript{55-57}. \textit{P. aeruginosa} cells are shown in red, EPS is visualized with green fluorescent dyes (yellow results from the superposition of green and red channels)\textsuperscript{57} (c). Biofilm streamers form in the presence of flow around a curved geometry, which can cause catastrophic disruption of flow by clogging\textsuperscript{57} (d,e) and affect the performance of environmental and medical systems. The images in parts a and b are reproduced REF. 54. The images in parts c-e are reproduced from REF. 57.

By providing a considerably greater degree of microenvironmental control compared to traditional flow chambers, including precise manipulation of flow for supply and removal of
chemicals (see section 1.2.2), microfluidics has enabled new ecological insights into biofilm processes. A recent example is the solution of a public goods dilemma in *V. cholerae* biofilms. A ‘producer’ population secretes the extracellular enzyme chitinase to forage on chitin particles, but the secreted enzyme can also be exploited by a non-producing, ‘cheater’ population (Fig. 1.8a). Confocal imaging combined with microfluidics revealed two different solutions to the dilemma. Firstly, some bacteria can produce thick biofilms that confine the enzyme molecules to the vicinity of the producers. Secondly, the public goods dilemma can also be solved by the presence of an ambient flow, in which soluble nutrients are washed away, thereby preventing their use by cheaters (Fig. 1.8b). Although the latter strategy also compromises the producer’s efforts to utilize chitin, the hindrance is more pronounced for the cheaters, resulting in a competitive advantage for the producers. These findings show that the physical environment, here in the form of a protective matrix or fluid flow, can crucially affect the social dynamics of populations of microbes.

In topographically complex environments exposed to fluid flow, biofilms can exist in the form of streamers, conglomerates of cells and cell-secreted polymeric substances (EPS) attached by one end to a surface and otherwise suspended in the flow. The development of *P. aeruginosa* streamers in curved channels, containing corners and bends, as featured in natural microbial habitats such as soils and medical devices, revealed the ubiquitous presence of biofilm streamers in regions where the substrate abruptly changes direction. Separate visualization of cells and EPS via differential fluorescent staining further revealed that EPS initially forms on the surfaces bounding the flow, creating a sieve-like network that captures floating bacteria and growing to straddle the width of the channel (Fig. 1.8d). By protruding into the flow, streamers can thus be a particularly deleterious form of biofilm, because of the catastrophic clogging they induce. Microfluidic approaches have enabled first steps, and are poised to reveal many more details, in understanding the conditions under which streamers form, and to develop approaches to prevent them.

### 1.6 SINGLE-CELL GROWTH

Bacterial growth has for a long time been studied at the population level, yet recently a much deeper understanding of growth is emerging from experiments at the single-cell level. These experiments are frequently based on the integration of microfluidic devices to control environmental conditions over long times (see section 1.2.2), including the supply of nutrients and the removal of waste, and accurate, time-resolved quantification of growth of single cells, for myriads of cells at a time (see section 1.2.1). These advances are providing new insights on the relationship between growth and death, on the principles behind cell-size maintenance and cell-wall expansion, and on the dependence of growth on environmental factors.
The mother of high-throughput microfluidic devices for bacterial growth studies is ‘the mother machine’\textsuperscript{58}, which allows the immobilization of large numbers of single bacteria (‘mother cells’) in an array of narrow, dead-end channels, one bacterium per channel. Growth and division are then monitored by time-lapse microscopy in each channel, where the tight cross section of the channel ensures that subsequent generations align in a single file. This approach was first applied to \textit{E. coli}, revealing the existence of a physiological steady state directly from single-cell observations, whereby the growth rate remained nearly constant over 200 generations, whereas the death rate of the mother cells increased exponentially with increasing replicative age. The death rate however remained constant when the SOS response was suppressed, suggesting that the death of mother cells results from the accumulation of growth-independent factors that trigger DNA damage.

One of the fundamental open questions in cell growth is how cells maintain size homeostasis. This question has been historically discussed in the context of two major paradigms, inferred from population-level and limited single-cell-level studies\textsuperscript{60}: the ‘sizer principle’, which posits that a bacterium divides when it reaches a critical size, and the ‘timer principle’, which posits that a bacterium grows for a prescribed amount of time before division. Recently, both of these hypotheses were overturned when the mother machine was applied to monitor hundreds of thousands of Gram-negative \textit{E. coli} and Gram-positive \textit{B. subtilis} cells under a wide range of steady external growth conditions\textsuperscript{61}. These species as well as \textit{C. crescentus} were found to grow by the same amount on average between divisions, irrespective of cell length at birth, suggesting that growth follows an ‘adder principle’\textsuperscript{61,62}. This mechanism ensures cell size homeostasis, because the addition of a constant biomass brings the cell size back to its average value over multiple generations. The exceedingly large data set required to resolve the ‘adder’ principle provides one of the best examples of the power of high-throughput microfluidic imaging. While the underlying molecular mechanism is yet to be determined, the adder principle provides new insights on how bacteria regulate their size in steady environments over multiple generations, and represents a strong mechanistic basis to test how cells regulate their size in more complex environments.

Use of a microfluidic platform revealed time-resolved correlations between growth and stochastic fluctuations in metabolic enzyme abundance\textsuperscript{63}. Growth rate fluctuations, which are omnipresent, drive changes in the overall protein reservoir, and conversely, growth fluctuations are partially caused by stochastic fluctuations in the expression of specific limiting metabolic enzymes. This is the first study to demonstrate these correlations and shows that cellular growth and metabolism are inherently dynamic, providing a source of phenotypic heterogeneity\textsuperscript{63}. It also suggests that due to continuous stochastic fluctuations of growth and metabolism, interactions of individual cells with their environment are never constant. On the other hand, growth can be stable overall in the face of certain environmental fluctuations. For example, monitoring the elongation of single \textit{E. coli} cells exposed to rapid changes in osmolarity within microfluidic
devices showed that – contrary to a long-standing hypothesis – turgor pressure is not essential for cell-wall expansion in *E. coli* and growth is robust to fluctuations of the intracellular hydrostatic pressure\(^\text{64}\). Sub-micrometer wide microfluidic channels have enabled the imaging of growth and division under stringent confinement\(^\text{65,66}\) in Gram-negative *E. coli* and Gram-positive *B. subtilis*, characterized by different turgor pressures and cell wall rigidity. Observations revealed that, as a result of their growth and division, *E. coli* cells are capable of squeezing in and traversing channels as narrow as half the cell width\(^\text{65,66}\). The shape of cells exiting the channels at the distal end after multiple generations was significantly different from *E. coli*’s canonical rod-like shape. *E. coli* cells eventually reverted to the original rod-like shape after several generations\(^\text{65}\), suggesting that irregular cell shape is inconsequential for accurate cell division. These studies demonstrate that Gram-negative bacteria can exhibit remarkable morphological plasticity and the ability to disperse even in tightly constrained environments, including soil ecosystems, biofilms and sterilization filters frequently used in clinical settings. These capabilities appear to be less pronounced in Gram-positive bacteria, which have thicker cell walls, as shown with *B. subtilis*\(^\text{65}\).

### 1.7 SUMMARY AND OUTLOOK

The combination of dynamic imaging and precise environmental control afforded by microfluidics represents a uniquely powerful approach to capture the fundamental nature of many microbial processes, which are often dynamic, unfold at the level of single cells, and depend intimately on the chemical and physical microenvironment.

The work reviewed here confirms the power of this approach in understanding biotic-biotic and biotic-abiotic interactions in microbial ecology (Fig. 1.1). Resolving microbes’ flagellar dynamics and flow signature at the single-cell level has yielded new insights on cell motility and propulsion strategies (Fig. 1.4), and on the consequences of motility on chemotaxis, pathogen-host interactions, transport in flow and surface attachment. Monitoring behavioral responses to precisely controlled chemical gradients has advanced the understanding of the chemosensory system in the model organism *E. coli*, as well as the prevalence and ecological consequences of chemotaxis in natural microbial habitats such as the ocean (Fig. 1.5). Microscale studies of microbial interactions with the physical environment, in particular fluid flow (Fig. 1.6) and surfaces (Fig. 1.7), has led to the discovery of biases on microbial migration induced by hydrodynamic or surface-induced forces, including shear-trapping and upstream swimming, which in turn affect the transport of bacteria and their initial surface colonization leading to biofilm formation (Fig. 1.8).
Despite this wealth of new insights, the full potential of systems integrating microfluidics and dynamic imaging has only begun to be tapped, and microbial ecology stands to gain much more from these techniques. Single-cell imaging can be fruitfully extended to other important microbial groups, for example archaea and viruses. Microfluidic devices can represent ideal arenas to study the interaction between different microbial trophic levels, from predator-prey interactions at the level of the dynamics of individual predation events, to the establishment of symbiotic cell consortia\textsuperscript{67}, to chemotactic aggregations of microbes around larger organisms, to competition among different microbial species. At the same time, the young field of microfluidics continues to produce advances relevant for microbial ecology at a fast pace. This will provide unprecedented opportunities to mimic increasingly realistic features of natural environments – such as temporal fluctuations, spatial heterogeneity, and chemical complexity – and to deploy microfluidic devices directly in the natural environment to measure microbial processes, from motility to growth to gene expression.

Reinforcing these opportunities are additional approaches for dynamically monitoring single-cell processes in controlled microenvironments with technologies that can be considered ‘cousins’ of imaging and can reveal important complementary information. For example, atomic force microscopy (AFM) has been used to ‘image’ the 3D structure of cells with nanometer precision\textsuperscript{68}, a feature that – if made dynamic – would profoundly enhance single-cell growth studies by providing quantitative information on cell volume, shape and surface properties. AFM extended to environmental samples demonstrated that individual microbes are often physically attached to other microbes, which suggests that symbiotic relationships among pelagic marine bacteria may be considerably more frequent than previously believed\textsuperscript{67}. When these symbioses occur between autotrophs and heterotrophs, they have the potential to affect the ocean’s carbon cycling by short-circuiting the flux of carbon from primary producers to consumers\textsuperscript{67}. Advances in chemical measurements, for example non-destructively through Raman microspectroscopy or destructively through nanoscale secondary-ion mass spectrometry (nanoSIMS), can fill a major gap in current studies of microbial ecology at the microscale by revealing the chemical landscape in which microbes interact. In particular, nanoSIMS measurements provide spatially explicit, nanometer-resolution maps of the chemical composition of individual cells\textsuperscript{69-71}. Whereas nanoSIMS is a destructive approach and thus not amenable to a dynamic implementation on the same cell, one can envisage that the coupling with microfluidic devices may open the door to dynamic nanoSIMS measurements based on single cells from a clonal population. Inertial mass sensing is a microfluidic technology that weighs single cells with femtogram precision and is already used to measure growth and monitor cellular responses to drugs and other stimuli over time\textsuperscript{72,73}. This high-precision platform can be integrated with emerging microtechnologies for the dynamic measurement of mass, volume and density of individual cells exposed to controlled and user-manipulated environmental conditions.
Imaging and in particular dynamic imaging appeals uniquely to our understanding of microbial ecology because it enables the direct visualization of processes for which we have poor intuition, and whose mechanisms may otherwise be difficult to decipher. The handshake between powerful imaging approaches and the growing field of microfluidics and microtechnology has still much to give to microbial ecology, because of the unique capability of controlling and seeing the world of microbes, at the scale of microbes.
REFERENCES (CHAPTER 1)


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CHAPTER 2

Bacteria can exploit a flagellar buckling instability to change direction*

* In Chapter 2, I focused on the motility of marine microbes. I studied the biomechanics of a newly discovered motility adaptation in marine bacteria using high-speed imaging at the submicrometer scale. The swimming strategy of bacteria living in the ocean is radically different from the well-studied enteric bacterium *E. coli*'s run-and-tumble motility. This is exemplified by the marine bacterium *Vibrio alginolyticus* reorienting by a 'flick' - a large, off-axis deformation of their single flagellum. I report on the mechanism underlying the flick, which my collaborators and I discovered to be the mechanical buckling instability of the ~100 nm long 'hook' connecting the flagellar filament with the rotary motor, during the onset of forward swimming, when the flagellum pushes the cell head and the hook is under compression.

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I have been responsible for all experiments, all of the data analysis, most of the theoretical analysis, and a significant fraction of the writing in this work and have benefitted from guidance from both Jeffrey Guasto (then a postdoc in the Stocker lab) and Roman Stocker.
ABSTRACT

Bacteria swim by rotating rigid helical flagella and periodically reorienting to follow environmental cues. Despite the crucial role of reorientations, their underlying mechanism has remained unknown for most uni-flagellated bacteria. Here, we report that uni-flagellated bacteria turn by exploiting a finely-tuned buckling instability of their hook, the 100 nm long structure at the base of their flagellar filament. Combining high-speed video microscopy and mechanical stability theory, we demonstrate that reorientations occur 10 ms after the onset of forward swimming, when the hook undergoes compression, and that the associated hydrodynamic load triggers the buckling of the hook. Reducing the load on the hook below the buckling threshold by decreasing the swimming speed results in the suppression of reorientations, consistent with the critical nature of buckling. The mechanism of turning by buckling represents one of the smallest examples in nature of a biological function stemming from controlled mechanical failure and reveals a new role of flexibility in biological materials, which may inspire novel microrobotic solutions in medicine and engineering.

2.1 INTRODUCTION

Flexibility is woven into every facet of living materials. At the cellular level, flexibility allows red blood cells to squeeze through capillaries and DNA to stretch and twist to compensate for variability in binding site length. At the organismal level, flexibility enhances structural performance, for example by enabling animal bones and plant branches to absorb mechanical energy. Flexibility also underpins a host of dynamic life functions, including locomotion, reproduction and predation, by enabling the storage and swift release of elastic energy, a mechanism used by jumping froggers to escape predators, by plants to catapult seeds for dispersal, and by aquatic invertebrates to suck in prey. An extreme consequence of flexibility is the occurrence of mechanical instabilities, such as buckling and fluttering, which in engineered systems are synonymous with failure, but in natural systems can serve functional purpose. The biomechanical repertoire of organisms includes mechanical instabilities over a wide range of timescales, from the millisecond snap-buckling instabilities that allow Venus flytraps and hummingbirds to capture insects to the gradual buckling responsible for the wavy edges of leaves and flowers.

Flexible appendages are widely used by organisms for locomotion in fluids, from the flapping of bird and bat wings, to the actuation of fish fins, to the bending of sperm flagella. Flexibility also plays a subtle role in the locomotion of bacteria with multiple flagella ('peritrichous'), such as Escherichia coli, which bundles its flagella together for propulsion (a 'run') by exploiting the compliance of the flagellum's base. When one or more flagella leave the bundle following a
change in the direction of rotation of their motor, the torque resulting from the unbundling, or from the associated polymorphic transformation of the flagellar filament, reorients the cell (a ‘tumble’\textsuperscript{1,2}). This ability to reorient is essential, for example, to climb chemical gradients in search of nutrients or to escape toxins\textsuperscript{2}. Yet, many bacteria have only a single flagellum (‘monotrichous’), including 90\% of motile marine bacteria\textsuperscript{3}, and how they reorient has long remained unclear. Only recently has the monotrichous marine bacterium \textit{Vibrio alginolyticus} been shown to exhibit the ability to turn or ‘flick’\textsuperscript{4} by a seemingly impossible off-axis motion of its flagellum. We discovered and experimentally demonstrated that the mechanism responsible for the flick is a buckling instability of the hook, thereby revealing a new, striking role of flexibility in bacterial locomotion and one the smallest example in nature of controlled mechanical failure used for biological function.

### 2.2 METHODS

#### 2.2.1 Cell culturing

##### 2.2.1.1 \textit{V. alginolyticus}

\textit{V. alginolyticus} YM4 was cultured overnight in VC medium [0.5\% (w/v) polypeptone, 0.5\% yeast extract, 0.4\% K$_2$HPO$_4$, 3\% NaCl, 0.2\% glucose], diluted 1:100 into VPG medium (1\% polypeptone, 0.4\% K$_2$HPO$_4$, 3\% NaCl, 0.5\% glycerol)\textsuperscript{4} and grown to late exponential phase (OD$_{600}$ = 0.5). To change the sodium concentration, [Na$^+$], cells were washed and resuspended in TMN motility medium [50 mM Tris-HCl (pH 7.5), 5 mM MgCl$_2$, 5 mM glucose, 300 mM NaCl + KCl]. The difference in [Na$^+$] was replaced with potassium to maintain osmolarity, a common approach devoid of negative physiological consequences\textsuperscript{24}.

##### 2.2.1.2 Lab cultures

The monotrichous marine bacteria \textit{Pseudoalteromonas haloplanktis} (ATCC 700530), which exhibits rapid chemotaxis towards algal exudates\textsuperscript{29}, and \textit{Vibrio coraliilyticus} (BAA-450), a coral pathogen\textsuperscript{30}, were both grown overnight in 2216 marine broth (Difco, Franklin Lakes, NJ, USA) at 30°C in a shaking incubator.

##### 2.2.1.3 Enriched natural bacterial community

A coastal seawater sample was collected at Revere Beach (MA, USA) on Aug 10, 2012, near the surface (depth < 1 m) via a net tow (63 $\mu$m mesh size) to concentrate planktonic particles. The concentrated particles and entrained seawater were incubated for 6 h at 22°C to stimulate
motility\textsuperscript{55,56} (this enrichment likely selected for motile taxa\textsuperscript{57}, but we consider this bias acceptable because of our focus on motile bacteria).

2.2.2 Imaging

Flagellar dynamics were visualized in a 25 μm deep chamber at 420 frames/s using high-intensity dark-field microscopy (Nikon Ti-E microscope; 40×, 0.6 NA objective; 1.2 NA oil condenser; mercury lamp illumination) and an Andor Neo camera (6.5 μm/pixel). To measure cell head dynamics, bacteria were imaged at mid depth in a 120 μm deep polydimethylsiloxane (PDMS) microchannel at up to 1,000 frames/s (cell position measurement precision ~20 nm) using Photron SA-5 (20 μm/pixel) or SA-3 (17 μm/pixel) high-speed cameras and phase contrast microscopy (100×, 1.4 NA objective). For the analysis of the probability of flicking and the experiments with varying sodium concentrations, cells were imaged at 30 frames/s by phase contrast microscopy (20×, 0.45 NA objective) using the Andor Neo camera.

2.2.3 Cell tracking and trajectory analysis

All analyses were performed in Matlab (The Mathworks) using in-house, automated software to track cells, measure cell size and shape, and identify flagellar filaments. Cell trajectories were smoothed using a second order Savitzky-Golay filter (window sizes: 133 ms for 30 frames/s, 9.5 ms for 420 frames/s, 4 ms for 1,000 frames/s), and reorientation events were identified using two criteria, a high rate of change of direction and a low instantaneous swimming speed (see section 2.2.4). Reorientation events were then classified as flicks or reversals based on the absolute reorientation angle, Δθ, defined as the angle between the swimming directions before and after a reorientation (see sections 2.3.4 & 2.2.5). Each computer-identified reorientation event was verified manually for 17,061 trajectories before performing statistical analysis.

2.2.4 Identification of reorientation events

The trajectories of \textit{V. alginolyticus} acquired using high-speed video microscopy at 420 and 1,000 frames/s reveal that their swimming pattern is composed of elements repeated in the following order: forward run, reversal, backward run, reversal, brief forward run, and flick. Whereas the brief forward run preceding the flick requires high-speed imaging to resolve, the statistical analysis of the reorientation events (reversals and flicks) only necessitates data acquisition at low temporal resolution (e.g., 30 frames/s), as done previously\textsuperscript{4}. At this lower temporal resolution, the sequence of a reversal, a brief forward run and a flick are captured as a single, rapid reorientation in the cell’s trajectory (as in Xie et al.\textsuperscript{4}). Thus, we first identified as reorientation events (either flicks or reversals) all sharp changes in direction or ‘kinks’ in a swimming trajectory accompanied by a brief reduction in speed. However, these reorientations have some
associated noise, resulting for example from Brownian motion, requiring a set of carefully
defined criteria for their identification, which we describe here.

To identify all reorientations from the trajectories that we acquired using video microscopy at 30
frames/s, we first smoothed each trajectory using a second-order Savitzky-Golay filter\textsuperscript{41} with a
133 ms window. The instantaneous speed, the average speed of the trajectory (computed over the
entire trajectory), and the direction of motion were evaluated on the smoothed trajectories. We
then located, within each trajectory, abrupt changes in direction (first criterion) or swimming
speed (second criterion) or both, similar to published methods for identifying tumbles in
\textit{Escherichia coli}\textsuperscript{42}, with specific criteria defined as follows.

A first criterion was aimed at identifying changes in the swimming direction. At every point
along a trajectory we calculated the dot product of the swimming directions before and after that
point (directional cosine). The two swimming directions were obtained
by fitting a parametric
linear polynomial to the cell trajectory over a window of 133-200 ms (4-6 data points). The
duration of the fitting window was varied according to the average speed of the trajectory, with
longer windows used for slower cells (specifically, 200 ms for \( V \leq 20 \, \mu m/s \); 167 ms for \( 20 < V \leq 30 \, \mu m/s \); 133 ms for \( V > 30 \, \mu m/s \)). We then identified as reorientation events (based on this first
criterion) all local minima in the directional cosine (equivalent to local maxima in the
instantaneous angular speed) falling below a threshold value of 0.985, corresponding to a
directional change of at least 10°. We found that an angular threshold of 10° provided robust
event detection, and was sufficient to avoid misidentification due to angular changes resulting
from Brownian rotational diffusion, wiggling trajectories\textsuperscript{43}, and our finite cell tracking resolution.

To identify reorientation events having a change in angle below the 10° detection threshold, we
also used a second criterion, based on swimming speed, because any reorientation is expected to
be accompanied by a brief reduction in swimming speed. Based on this second criterion, we
identified all local minima in the instantaneous swimming speed that are below 50% of the mean
speed of the trajectory. Then, if the cell was decelerating before and accelerating after the local
speed minimum over a 133-200 ms window (defined as above), we considered the event a
reorientation (based on this second criterion). These criteria were verified manually over a large
number of randomly chosen trajectories to confirm that they give sensible results, in agreement
with visual inspection.

Reorientation events were defined as those satisfying both criteria simultaneously. Then these
criteria were automated in Matlab and applied to a total of 79,273 trajectories at various sodium
concentrations ([Na\textsuperscript{+}]=3–513 mM). Because we limited our analysis to cells that swam within
the imaging volume for a time sufficient to exhibit at least one reorientation event, we discarded
trajectories of cells that swam out of the focal plane or out of the field of view before undergoing
a reorientation. After running the reorientation identification routine, trajectories devoid of
identified reorientation events (i.e., pure runs) were discarded, whereas those with at least one reorientation ($n \geq 1$) were stored (see section 2.2.5). To avoid the potential for spurious results from the automated routine, each single reorientation event was manually verified and trajectories that contained any falsely identified reorientation events were discarded. Finally, a total of 17,061 trajectories recorded over different sodium concentrations, along with the angular change in the swimming direction, $\Delta \theta$ (Fig. 2.1), of each identified reorientation were stored for further analysis.

**Figure 2.1 | Definition of the reorientation angle, $\Delta \theta$.** a, $\Delta \theta$ is defined as the angle between the swimming directions preceding and following a reorientation event, following the previously established convention. b-c, Examples illustrating reorientation angles (b) during a flick, and (c) during a reversal. Note that actual reorientation angles exhibit a distribution of values for both flicks and reversals (see Fig. 2.2).

### 2.2.5 Classification of reorientations and quantification of the probability of flicking, $P_F$

To determine the relative incidence of flicks, as observable evidence of the relative incidence of buckling, we needed to classify each reorientation event within our collection of swimming trajectories (see section 2.2.4) as either a reversal or a flick. However, reorientations have associated noise, resulting for example from Brownian motion and natural variability in the flick dynamics, causing potential ambiguity in classifying certain reorientations as either flicks or reversals (e.g., because a reorientation with $\Delta \theta \approx 180^\circ$ could in principle be either a flick or a reversal). Therefore, we adopted a statistical approach to determine the probability of flicking during a swimming cycle, $P_F$, that is the probability of observing one flick during a swimming cycle.

We obtained the relative occurrence of flicks and reversals from the probability density $p(\Delta \theta)$ of all reorientation angles, $\Delta \theta$, described in section 2.3.4 (Fig. 2.3, blue open circles). At high swimming speeds, $p(\Delta \theta)$ exhibits two distinct peaks resulting from two underlying distributions: one relatively sharp peak at $\Delta \theta = 180^\circ$ corresponding to reversals and one broad peak centered about $\Delta \theta = 90^\circ$ and corresponding to flicks. The overlap of the two distributions is the cause of the potential ambiguity (Fig. 2.2) in exactly discerning flicks from reversals. To overcome this
difficulty and unambiguously obtain the relative occurrence of flicks and reversals, we used a combination of two methods, described below.

Figure 2.2 | Reorientation dynamics depend on swimming speed. Probability density of the reorientation angle, $\Delta \theta$, (defined in Fig. 2.1) for four swimming speed ranges (see colored bars in Fig. 2.8b), showing narrow peaks at $\Delta \theta \approx 180^\circ$ (reversals) and broad peaks at $\Delta \theta \approx 90^\circ$ (flicks). The reduced occurrence of flicks at lower speeds is evident by the diminished peak at $\Delta \theta \approx 90^\circ$. The shaded red band corresponds to the range of threshold angles used to classify flicks and reversals (see section 2.2.5).

The first method consists of selecting a threshold for $\Delta \theta$ and classifying all reorientations with $\Delta \theta$ greater than the threshold as reversals, and those events below the threshold as flicks. The obvious choice for the threshold value is the local minimum in $p(\Delta \theta)$ occurring between the maxima of the probability density located at $\Delta \theta \approx 90^\circ$ and $\Delta \theta \approx 180^\circ$ (Fig. 2.3). The downsides of this simple method are that (i) it misclassifies flicks having a large reorientation angle and reversals having a relatively small reorientation angle; and (ii) for slowly swimming cells, which exhibit infrequent flicks (Fig. 2.2, red distribution), the local minimum may be ill-defined or entirely absent.

A second, improved classification method consists in viewing $p(\Delta \theta)$ as the sum of two distributions of known shape, representing the flicks and reversals, respectively. The broad $\Delta \theta$ distribution associated with flicks was previously shown to be well represented by a Gaussian distribution\(^4\). We found empirically that the $\Delta \theta$ distribution associated with reversals is well
represented by an exponential distribution. We thus parameterize \( p(\Delta \theta) \) by the sum of a Gaussian distribution (Fig. 2.3, red) and an exponential distribution (Fig. 2.3, green), as:

\[
p(\Delta \theta) = \gamma \frac{1}{\sigma_f \sqrt{2\pi}} e^{-\frac{(\Delta \theta - \mu_f)^2}{2\sigma_f^2}} + (1 - \gamma) \frac{1}{2\sigma_r} e^{\frac{\left|\Delta \theta - 180^\circ\right|}{\sigma_r}},
\]

where \( \mu_f \) and \( \sigma_f \) are the mean and standard deviation of the Gaussian distribution, \( \sigma_r \) is the standard deviation of the exponential distribution, and \( \gamma \) is the parameter that quantifies the relative contribution of the Gaussian distribution to the overall distribution (the contribution of the exponential distribution is thus \( 1 - \gamma \)). Values of the parameters are found by non-linear least squares fitting in Matlab. Compared to the first method, this method more accurately captures the overlapping features of the \( \Delta \theta \) distribution for high swimming speeds, when both maxima are prominent. However, the error still increases at low swimming speeds, when the Gaussian distribution is less prominent (Fig. 2.2).

![Figure 2.3](image)

**Figure 2.3 | Method used to classify reorientations and thus quantify the probability of flicking.** A Gaussian (red) and an exponential (green) fitting functions (Eq. 2.1) are used to fit (black) the observed distribution of reorientation angles \( \Delta \theta \) (blue circles). The threshold angle to identify flicks is selected as the intersection point (black dotted arrow) between the Gaussian and exponential fitting curves.

To maximize accuracy in determining the relative occurrence of flicks and reversals, we implemented a combination of the two methods: the second method was used to determine the
intersection point between the two distributions (Fig. 2.3, black dotted arrow) and the value of \( \Delta \theta \) at the intersection point was then used as the threshold angle to identify flicks, as in the first method. With this approach, the threshold angles were in the limited range 123° to 150° (Fig. 2.2, shaded box), demonstrating the robustness of the threshold identification technique.

We then computed the probability of flicking per swimming cycle, \( P_F \), as two times the relative occurrence of flicks among all reorientations. The factor of two was included so that a cell that flicks once per swimming cycle (i.e., a cell that always alternates a flick and a reversal) has \( P_F = 100\% \). This value of \( P_F \) is plotted in Fig. 2.9 as a function of cell swimming speed, where symbols correspond to \( P_F \) values identified by the combined method. The vertical error bar in Fig. 2.9 represents the sensitivity to the choice of the threshold angle of reorientation and was computed by separately applying classification methods 1 and 2. As expected, the vertical error bar is largest at the lowest swimming speed (\( P_F = 0.08 \pm 0.13 \)), when bimodal peaks are ill-defined (Fig. 2.2), and minimal at the highest speed (\( P_F = 0.78 \pm 0.01 \)), when both peaks are prominent (Fig. 2.2).

In the above analysis, we considered trajectories containing at least one reorientation event (i.e., either a flick or reversal). Restricting the analysis to trajectories containing at least two reorientation events caused a relative change in \( P_F \) of <8.5%, whereas the sigmoidal shape and sharp transition of \( P_F \) as a function of swimming speed (Fig. 2.9) were entirely conserved, showing that the criticality of the underlying process is independent of the specific set of trajectories chosen for processing.

2.2.6 Geometrical and dynamical parameters

![Diagram](image)

**Figure 2.4 | Geometrical and dynamical parameters of Vibrio alginolyticus.** All the parameters are defined in Tables 2.1-2.3 (see below) and used in the analysis throughout this chapter.
## Table 2.1 | *Vibrio alginolyticus* head dimensions and swimming parameters. Values in the present study are mean ± standard deviation, whereas values in ref. (36) are mean ± standard error of the mean. Parameters are defined in Fig. 2.4.

<table>
<thead>
<tr>
<th>Swimming speed</th>
<th>Head length</th>
<th>Head width</th>
<th>Head rotational speed</th>
<th>Flagellar rotational speed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V$ (µm/s)</td>
<td>$2a$ (µm)</td>
<td>$2b$ (µm)</td>
<td>$\psi_r/2\pi$ (Hz)</td>
<td>$\psi_l/2\pi$ (Hz)</td>
<td>Present study</td>
</tr>
<tr>
<td>47.1 ± 8.8</td>
<td>3.2 ± 0.5</td>
<td>1.2 ± 0.1</td>
<td></td>
<td></td>
<td>(36)</td>
</tr>
<tr>
<td>26 ± 1</td>
<td>571 ± 12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.2 | Dimensions and mechanical properties of the hook of various bacteria. Values in the present study are mean ± standard deviation. Parameters are defined in Fig. 2.4.

<table>
<thead>
<tr>
<th>Hook length</th>
<th>Outer radius</th>
<th>Inner radius</th>
<th>Bacterial strain</th>
<th>Hook shape</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$L$ (nm)</td>
<td>$r_o$ (nm)</td>
<td>$r_i$ (nm)</td>
<td><em>Vibrio alginolyticus</em></td>
<td>Straight</td>
<td>(5)</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td></td>
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<td>Straight</td>
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</tr>
<tr>
<td>55 - 70</td>
<td></td>
<td></td>
<td><em>Salmonella typhimurium</em></td>
<td>Curved</td>
<td>(46, 59)</td>
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<tr>
<td>59 - 80</td>
<td></td>
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<td>(25, 26, 46)</td>
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<tr>
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<td></td>
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<td>(46)</td>
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<tr>
<td>102</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>9</td>
<td>1.5</td>
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<td><em>Salmonella typhimurium</em></td>
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<td>$GJ$ (N m$^2$/rad$^2$)</td>
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<td></td>
<td><em>Rhodobacter sphaeroides</em></td>
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### Table 2.3 | Dimensions and mechanical properties of the flagellar filament of various bacteria.

Values in ref. (4) and (36) are mean ± standard error of the mean, whereas values in ref. (34) are mean ± standard deviation. Parameters are defined in Fig. 2.4.

#### 2.3 RESULTS

#### 2.3.1 High-speed imaging

Detailed observations using high-speed video microscopy revealed a new component of *V. alginolyticus*’ swimming pattern (Fig. 2.5). *V. alginolyticus* swims in a cyclic pattern, alternating between forward runs, when the flagellum pushes the cell head, and backward runs, when the flagellum pulls the head. At the end of a forward run, the cell reverses, changing its swimming direction by Δθ=180°. In contrast, cells have recently been reported to reorient by an average angle Δθ=90° (a ‘flick’4) at the end of a backward run, based on imaging at 30 frames/s4. By imaging at up to 1,000 frames/s (Fig. 2.5a,b), we discovered instead that the flick does not occur at the end of a backward run, but rather after the start of the subsequent forward run. These dynamics are revealed by the alignment, q, between the swimming direction and the cell head orientation (Fig. 2.5c). Following a backward run (q=-1), the swimming speed drops to zero (Fig. 2.5d) and within <1 ms the cell switches to a forward run (q=1; Δθ=180°; Fig. 2.5c), which lasts 10.2±5.7 ms (mean±std; n=52; Fig. 2.6). Only then, the flick begins, lasting ~60 ms and characterized by a departure of q from 1.
Figure 2.5 | High-speed video microscopy of *V. alginolyticus* reveals that flicks occur after the onset of forward swimming. a, Image sequence captured with high-intensity dark-field microscopy at 420 frames/s, showing the kinematics of the cell head and polar flagellum (tracked and colored magenta; see section 2.2.2) just prior to and during a flick. The dashed orange line provides a reference for cell position. b, Cell trajectory containing a flick, captured with high-
speed imaging (1,000 frames/s, phase contrast microscopy). Cell head positions are shown by circular markers at 1 ms intervals. A schematic of the head orientation at selected times is overlaid (not to scale). The inset shows the entire trajectory subsampled at a conventional frame rate of 30 frames/s (open circles). c, The alignment, \( q \) (directional cosine; see section 2.2.4), between cell head and swimming direction, for the trajectory in panel b, reveals the distinct elements of \( V. \text{alginolyticus} \)'s swimming cycle, and in particular the short forward swimming segment (here, 18 ms long; red) prior to the 60 ms-long flick (blue). The inset in panel b demonstrates that high-speed imaging is necessary to capture the short delay in the flick. d, Swimming speed during a flick (same color scheme as in panel c). e, Transmission electron microscopy (TEM) image of \( V. \text{alginolyticus} \), showing the single polar flagellum (mean head length \( 3.2 \mu m \), mean flagellar contour length \( 4.6 \mu m \); Table 2.1), which has a sheath that covers it and prevents polymorphic transformations. f-g, Schematics (not to scale) of the flagellar filament, hook, and rotary motor (f) during backward swimming, when the hook is in tension, and (g) during forward swimming, when the hook is in compression.

![Figure 2.6](image)

**Figure 2.6 | Distribution of the durations of the short forward runs that immediately precede flicks.** The forward run time (i.e., the time between a backward-to-forward reversal and the subsequent flick) was measured from 52 high-speed movies, captured with phase contrast microscopy at 1,000 frames/s (100×, 1.4 NA objective). The exact instants of the reversal and the flick are identified from the time series of the alignment, \( q \), between the head orientation and the swimming direction (as in Fig. 2.5c).
2.3.2 Mechanical analysis

The discovery of this ~10 ms delay is essential to correctly assess the state of stress of the cell. During backward swimming, the thrust from the flagellum and the drag on the head are equal in magnitude and directed away from one another\textsuperscript{21}, and thus the cell is under tension (Fig. 2.5f). Conversely, during forward swimming it is under compression (Fig. 2.5g). Because structures under compression can lose stability, the occurrence of flicks exclusively during forward swimming led us to hypothesize that their origin lies in a buckling instability of the flagellum, and thus depends on the flagellum’s material properties. In contrast to recent simulations\textsuperscript{23}, which focused on the stability of the flagellar filament and predicted buckling during backward swimming, we focus on the mechanical stability of the hook, the structure at the base of the flagellum that connects to the motor, because its bending stiffness $EI$ ($E$ is Young’s modulus; $I$ is the area moment of inertia) is 100–1,000 times smaller than that of the flagellar filament (Tables 2.2 and 2.3), and because the majority of the deformation during a flick is confined to the base of the flagellum\textsuperscript{4} (Fig. 2.5a).

The hook of \textit{V. alginolyticus} is a straight, hollow, slender rod [length $L$=100 nm (ref.5), slenderness ratio $\sim$20; see section 2.3.2.2], subject to a typical axial force $F=0.6$ pN from the thrust of the flagellum and a typical torque $T=554$ pN nm from the motor (see section 2.3.2.1). $F$ and $T$ stem from the drag-based thrust of the cell (see section 2.3.2.1) and are referred to here as viscous loads, because inertial forces are negligible at the bacterial scale\textsuperscript{21}. Euler beam theory\textsuperscript{14} predicts that a slender structure buckles above a critical load under compression when $F/F_{CR} = \pi^2 EI/L^2$ or under torsion when $T/T_{CR} = 2\pi EI/L$. Under simultaneous loading, buckling occurs when $F/F_{CR} + (T/T_{CR})^2 > 1$ (ref. 14). \textit{V. alginolyticus} exceeds this threshold when its swimming speed surpasses $V=51$ \textmu m/s, which is comparable to the mean swimming speed, $V=47$ \textmu m/s, at representative marine sodium concentrations ([Na$^+$]=513 mM; Fig. 2.8a), indicating that typical swimming loads are sufficient to cause the hook to buckle.

2.3.2.1 Viscous loads on the hook

Prior to flicking, bacteria swim forward (\textit{i.e.}, the rotating helical flagellar filament pushes the cell head through the fluid), placing the hook in a state of compression between the drag experienced by the cell head and the thrust provided by the flagellar filament (Fig. 2.5g). In addition, the hook is also under torsional load, due to the counter-rotating cell head and flagellar filament\textsuperscript{21}. In the low Reynolds number regime appropriate for swimming bacteria, the force $F_c$ and torque $T_c$ exerted on the cell head are completely specified by the geometry and the motion of the cell head, which we obtain through video microscopy and cell tracking (see section 2.2.3). These hydrodynamic loads on the cell head are equal and opposite to those on the flagellar filament ($F_f$ and $T_f$) and are transmitted from the head to the flagellar filament through the hook\textsuperscript{34,35}. Thus, the viscous force $F$ and torque $T$ on the hook are
\[ F = F_c = F_f, \]  
\[ T = T_c = T_f. \]

Figure 2.7 | Hydrodynamics of the cell head and the flagellar filament of a bacterium during steady swimming (i.e., translation along the major head axis). The dynamics of four elements are coupled: the cell head, the motor (green box), the hook (red rod), and the flagellar filament. All torques and forces are defined to be positive (see the arrows). All symbols are defined in the section 2.3.2.1 except \( \psi_c, \psi_r, \) and \( \psi_f, \) the angles by which the cell head, the rotor (located inside the motor) and the flagellar filament rotate, respectively (see section 2.3.5.2 for details).

Following previous work\(^{4,21,34,35}\), we provide a summary of the equations used in our analysis to estimate the force and torque on a cell, which we define to be positive values (see the arrows in Fig. 2.7). We make two approximations: (i) we ignore the hydrodynamic interaction between the head and the flagellar filament, based on theoretical and experimental results\(^{36}\) demonstrating that this interaction accounts for only \(~5\%\) of the swimming speed (a value comparable to our experimental measurement error); (ii) we use known average dimensions of the flagellar filament (Table 2.3) to calculate the drag coefficients of the flagellar filament because the flagellar filament is undetectable through phase contrast microscopy measurements (see also section 2.2.4; this approach is supported by the previously demonstrated lack of correlation between head size and flagellar length\(^{34}\)). Furthermore, we assume the mean flagellar geometry (Table 2.3) and use the measured cell head geometry, and the translational dynamics of the cell head to estimate the torque \( T \) on the cell (alternatively, the torque could be obtained from the rotational motion of the head or flagellar filament about the swimming direction of the cell, but these are difficult to measure accurately).
We first consider the cell head and the flagellar filament to be aligned and translating along the major axis of the head (Fig. 2.7). The drag force $F_c$ and torque $T_c$ acting on the cell head are

$$F_c = \alpha_c V,$$

$$T_c = \beta_c \dot{\psi}_c,$$  \hspace{1cm} (2.4) (2.5)

where $V$ is the swimming speed, $\dot{\psi}_c$ is the rotational rate of the cell head, and $\alpha_c$ and $\beta_c$ are translational and rotational drag coefficients, given by

$$\alpha_c = 6\pi\eta b \left[ 1 - \frac{1}{5} \left( 1 - \frac{a}{b} \right) \right],$$  \hspace{1cm} (2.6)

$$\beta_c = 8\pi\eta b^3 \left[ 1 - \frac{3}{5} \left( 1 - \frac{a}{b} \right) \right].$$  \hspace{1cm} (2.7)

by approximating the cell head as a prolate ellipsoid of major axis $2a$ and minor axis $2b$ swimming in a liquid of viscosity $\eta$.

Due to the chiral shape of the flagellar filament, its translation and rotation are coupled. Here we focus on motions about the long axis of the flagellar filament, which are relevant to the typical forward and backward swimming of these cells. The force $F_f$ and torque $T_f$ on a simultaneously translating and rotating helical filament are

$$F_f = \gamma_f \dot{\psi}_f - \alpha_f V,$$

$$T_f = \beta_f \dot{\psi}_f - \gamma_f V,$$  \hspace{1cm} (2.8) (2.9)

where $\dot{\psi}_f$ is the rotation rate of the flagellar filament, $\alpha_f$ and $\beta_f$ are approximated translational and rotational drag coefficients of the flagellar filament, and $\gamma_f$ is the ratio between the propulsive force of the rotating flagellar filament and its rotation rate. These coefficients are given by

$$\alpha_f = \frac{2\pi\eta l}{\ln(2p/r) - 1/2} \frac{1}{(4\pi^2 R^2 + p^2)} (8\pi^2 R^2 + p^2),$$  \hspace{1cm} (2.10)

$$\beta_f = \frac{2\pi\eta l}{\ln(2p/r) - 1/2} \frac{1}{(4\pi^2 R^2 + p^2)} (4\pi^2 R^2 + 2p^2) R^2,$$  \hspace{1cm} (2.11)

$$\gamma_f = \frac{2\pi\eta l}{\ln(2p/r) - 1/2} \frac{1}{(4\pi^2 R^2 + p^2)} (2\pi R^2 p).$$  \hspace{1cm} (2.12)
Here, $R$, $p$, $r$ and $l$ are the radius of the helical flagellar filament, its pitch, its cross-sectional radius and its contour length, respectively (Table 2.3).

For the analysis of the hook’s stability, we are primarily concerned with the forces and torques on the hook (Eqs. 2.2 and 2.3). As demonstrated in Fig. 2.5, just prior to flicking the cell swims forward, a configuration in which the long axis of its head and of the flagellar filament are aligned (Fig. 2.7). From the cell-tracking and image analysis, we measured the swimming speed, $V$, and the geometry of the head ($a, b$) of individual cells. However, the rotational speeds of the cell head ($\dot{\psi}_c$) and the flagellar filament ($\dot{\psi}_f$) of each cell required to calculate the torque $T$ (Eqs. 2.5 and 2.9) are more difficult to measure. Therefore, we seek an expression for $T$ as a function of the swimming speed, $V$. To do this, we first solve for the rotational speed of the flagellar filament, $\dot{\psi}_f$, by equating Eqs. 2.4 and 2.8, then substitute the resulting expression into Eq. 2.9. Then, balancing the torque on the cell head, $T_c$ (Eq. 2.5), with the torque on the flagellar filament, $T_f$ (Eq. 2.9), we find an expression for the rotational speed of the cell head,

$$\dot{\psi}_c = \frac{a_c \beta_f + \alpha_f \beta_f - \gamma_f^2}{\beta_c \gamma_f} V,$$  \hspace{1cm} (2.13)

which in turn is substituted into Eq. 2.5 to yield an expression for $T$ as a function $V$. In summary, we can compute the viscous loads on the hook of individual cells, both force $F$ and torque $T$, using only the measured cell head geometry and the swimming speed, as follows:

$$F = \alpha_c V,$$  \hspace{1cm} (2.14)

$$T = \frac{a_c \beta_f + \alpha_f \beta_f - \gamma_f^2}{\gamma_f} V.$$  \hspace{1cm} (2.15)

These two expressions are used to compute $F$ and $T$ in the stability analysis.

2.3.2.2 Buckling analysis

The calculated viscous loads ($F$ and $T$; see section 2.3.2.1) on individual cell are compared with the critical buckling loads of the hook. In our analysis, we consider the flagellar filament to be rigid because (i) its bending stiffness $EI$ is ~2-3 orders of magnitude larger than the bending stiffness of the hook (Tables 2.2-2.3) and (ii) it is known that the flagellar filament of V. alginolyticus does not undergo polymorphic transformations\(^{33}\), probably due to a sheath covering the flagellar filament\(^{37}\), unlike the flagellar filaments of peritrichous bacteria that change shape.
during different stages of swimming\textsuperscript{38,39}. Based on this, we focus on the stability of the hook (Fig. 2.12).

The ratio of the length of hook, $L$, to the radius of gyration of its hollow cross-section, $r_G$, is called the slenderness ratio,

$$S_R = \frac{L}{r_G},$$

where $I$ is the area moment of inertia and $A$ is the cross-sectional area of the hook (see Table 2.2; ref.14). From the geometry of \textit{V. alginolyticus}' hook (Table 2.2), we find that its radius of gyration is 5 nm. This yields a large slenderness ratio, $S_R \approx 20$, which justifies the use of Euler beam theory to analyze the stability of the hook\textsuperscript{14}.

The known geometry and mechanical properties of \textit{V. alginolyticus}' hook indicate that the hook can be well approximated as a straight, hollow, cylindrical rod\textsuperscript{5}, which we postulate buckles under the combined action of the viscous force $F$ and the viscous torque $T$. The presence of a torque implies that the buckling analysis must be conducted in three-dimensional space and the deflection of the hook is characterized by two displacements in the $y$ and $z$ directions, where $x$ is the direction along the hook’s major axis. Following Timoshenko\textsuperscript{4}, the change in torque about the deflected rod axis due to the displacements in the $y$ and $z$ directions are of second order and are thus neglected here. For this reason, the torsional stiffness $GJ$ of the rod is irrelevant for buckling and only the bending stiffness $EI$ matters for determining the critical force and torque, which are $F_{CR} = mn^2 EI/L^2$ and $T_{CR} = n\pi EI/L$, respectively\textsuperscript{14}, where $m$ and $n$ are constants that depend on the boundary conditions. We assumed that both ends of the hook are pinned to their respective supports, \textit{i.e.} the cell head and the flagellar filament, by ideal spherical hinges around which they are free to rotate, corresponding to $m=1$ and $n=2$. We measured the bending stiffness $EI$ of the hook (see section 2.3.7; Table 2.2) and with the known length of the hook, $L$ (ref. 5; Table 2.2), we computed the critical loads to be $F_{CR} = 9.9 \text{ pN}$ and $T_{CR} = 628 \text{ pN nm}$ for an average-sized cell swimming at the mean swimming speed ($V = 47 \mu\text{m/s}$; Fig. 2.9). Loss of stability was then determined as the viscous loads (computed in section 2.3.2.1) breaking the stability condition for the hook under simultaneous axial force and torque loading, \textit{i.e.}\textsuperscript{14}

$$\frac{F}{F_{CR}} + \left(\frac{T}{T_{CR}}\right)^2 < 1.$$ (2.18)
Figure 2.8 | Reorientation dynamics depend on swimming speed. a, The mean swimming speed of *V. alginolyticus* decreases with the ambient sodium concentration, [Na⁺], allowing for a controlled reduction of the viscous load on cells, which depends linearly on swimming speed²¹. For each sodium concentration, >2,000 trajectories were analyzed (error bars are standard deviations). Insets show typical trajectories at high ([Na⁺]=100 mM, aqua) and low ([Na⁺]=3 mM, magenta) sodium concentrations, illustrating the suppression of flicks at low swimming speeds. b, Probability density function of the swimming speed *V* (left axis), showing the broad distribution of speeds in a population for any given sodium concentration. The gray histogram denotes the total number of trajectories analyzed for each swimming speed (right axis), captured over a range of sodium concentrations ([Na⁺]=3–513 mM). Colored rectangles on the top axis represent speed ranges used in Fig. 2.3 and include >1,000 trajectories each.
2.3.3 Experimental verification of buckling-induced flicking

To demonstrate that cells flick because the viscous load exceeds the hook's buckling threshold, we systematically reduced the load on the cells by decreasing their swimming speed. This was achieved through a reduction in the sodium concentration of the suspending medium, [Na⁺] (Fig. 2.8a; see section 2.2.1), exploiting the fact that V. alginolyticus' motor is driven by transmembrane sodium gradients²⁴. We note that we chose not to modulate the load by changing the medium's viscosity, in order to avoid ambiguities in the interpretation of hydrodynamic forces on bacteria and their flagellar filaments associated with the use of media with enhanced viscosity⁴⁰.

At [Na⁺]=100 mM cells swam at 40 µm/s on average and regularly alternated between flicks and reversals (Fig. 2.8a). In contrast, at [Na⁺]=3 mM their mean speed fell to 12 µm/s and flicks nearly disappeared (Fig. 2.8a). To quantify the dependence of flicking on the swimming speed, and thus on the viscous load, we identified all reorientation events (flicks and reversals) from 17,061 trajectories (see section 2.2.4) recorded over a range of sodium concentrations (Fig. 2.8b) and computed the probability of flicking during a swimming cycle, PF (see sections 2.2.5 and 2.3.4), as a function of swimming speed, V (Fig. 2.9a). We found that PF plummeted from 80% at speeds V>47 µm/s to 10% at speeds V<25 µm/s. The steep decrease in PF when the swimming speed drops below 47 µm/s is consistent with the critical nature of buckling and with the predicted buckling load of the hook.

![Figure 2.9](image)

**Figure 2.9** | The probability of flicking shows a sharp increase with increasing load on the hook. The probability PF that a cell flicks during a swimming cycle (the sequence of two runs
and the two intervening reorientations) increases sharply with swimming speed $V$ (open squares). Cell trajectories for all sodium concentrations, $[Na^+]$, from Fig. 2.8b are included and binned by swimming speed, with colors corresponding to the speed ranges in Fig. 2.8b. Cells that flick after each start of a forward run have $P_F=100\%$, whereas cells that never flick have $P_F=0\%$. The dashed magenta line indicates the mean swimming speed, $V=47\mu m/s$, for cells swimming at $[Na^+]=513\ mM$, representative of natural marine conditions. The gray curve is a logistic fit, while horizontal and vertical error bars denote the standard deviation in cell speed and the error in classifying reorientations, respectively (see section 2.2.5). The dependence of $P_F$ on $V$ was confirmed by measuring $P_F$ for each individual $[Na^+]$ tested and binning each set by swimming speed (filled gray symbols, >300 trajectories each) by exploiting the natural variability in cell speed within a population. This rules out physiological effects associated with changes in $[Na^+]$ as a possible cause of the suppression of flicks.

2.3.4 The cell reorientation angle

2.3.4.1 Probability density of the cell reorientation angle at different swimming speeds

Using all 17,061 trajectories recorded over a wide range of sodium concentrations, $[Na^+]$ (Fig. 2.8b), we first segmented trajectories by swimming speed (Fig. 2.8b, color bars), because speed is proportional to the hydrodynamic load on the cell (Eq. 2.4 and 2.5), and then computed the probability density, $p(\Delta \theta )$, of the reorientation angle, $\Delta \theta$, for each range of swimming speeds (Fig. 2.2). At high swimming speeds (purple curve), $p(\Delta \theta)$ is bimodal, with a sharp peak at $\Delta \theta=180^\circ$ (indicating reversals) and a broad peak centered about $\Delta \theta=90^\circ$ (indicating flicks), as also reported before\(^4\). However, as swimming speed decreases the peak at $\Delta \theta=90^\circ$ decays, disappearing almost entirely for speeds $V<25\mu m/s$ (Fig. 2.2, red curve) and signifying a nearly complete suppression of flicks.

![Figure 2.10](image)

**Figure 2.10 | Hydrodynamics of the cell head and the flagellar filament during a flick. a,** Schematic of the motion during a flick, when the flagellar filament first rotates about the hook and then returns to be coaxial with the major axis of the cell head. Here, $\phi_c$ and $\phi_f$ are the
angular rate of rotation of the cell head and flagellar filament about the hook, respectively. The rotational drag coefficients of the cell head $\beta_{rh}$ and flagellar filament $\beta_{rf}$ are defined in section 2.3.4 and 2.3.5, respectively. The hydrodynamics of the flagellar rotation is explained in section 2.3.5. b, Schematic illustrating the effect of the cell head size on the reorientation angle during a flick. The cell configuration before the flick is shown in gray, after the flick in blue, green and red for a long, intermediate and short cell, respectively (see also Fig. 2.11). Note that larger cells rotate by a smaller amount, which (given the definition of $\Delta \theta$) results in a larger value of the reorientation angle $\Delta \theta$.

2.3.4.2 Effect of cell head size on the reorientation angle during a flick

The angular distribution of flicks exhibits the shape of a broad Gaussian centered about $\Delta \theta \approx 90^\circ$ (Fig. 2.2; ref. 4) for a population of cells at a given growth stage. Here we delve deeper into the reorientation angle during a flick by considering the effect of the cell head size. During a flick, the bending hook exerts a torque on the cell head, which is resisted by the viscous torque on the head. If we consider the motion of the cell head pivoted at the hook, rotating about an axis perpendicular to the major axis of the cell head (Fig. 2.10), the rotational drag coefficient of the cell head is

$$\beta_{rh} = \frac{16\pi(16a^4 - b^4)}{9[(8a^2 - b^2)Q - 4a]},$$

(2.19)

$$Q = 2(4a^2 - b^2)^{-1/2} \ln \left[ \frac{2a + (4a^2 - b^2)^{1/2}}{b} \right],$$

(2.20)

where $2a$ and $2b$ are the head’s long and short axes, respectively. The viscous torque on the cell head is thus, $\beta_{rh}\dot{\phi}_c$, where $\dot{\phi}_c$ is the angular rate of rotation of the cell head about the attachment point of the hook to the head (Fig. 2.10), which has a strong dependence on the length of the cell head, scaling as $a^3$, in the limit when $a/b \gg 1$ (Ref. 1). Thus, we expect that cells having different head size, due to natural variability within a population even at a same growth stage (here, late exponential phase, OD_{600}=0.5), have a different distribution of reorientation angles during a flick. In particular, we expect that larger cells on average turn less during a flick. In the previously established convention (Fig. 2.1; ref. 4), this corresponds to a larger mean value of $\Delta \theta$ (Fig. 2.10b). We note that we neglected the effect of the length of the flagellar filament, because for *V. alginolyticus* it is independent of the size of the head.
Figure 2.11 | Effect of the cell head length on the distribution of reorientation angles during a flick. The probability density of the reorientation angle, $\Delta \theta$, is shown for a total of 5,954 cells, binned by cell head length. The range of head lengths associated with each of the red, green and blue probability densities (symbols) is shown by the color bar in the inset [red ($2.0 < 2a \leq 2.9 \ \mu m$); green ($2.9 < 2a \leq 3.6 \ \mu m$); blue ($3.6 < 2a \leq 5.0 \ \mu m$)]. The gray histogram in the inset is the probability density of the cell head length, $2a$. The sodium concentration was $[\text{Na}^+] = 513 \ \text{mM}$ and solid curves are fits as described in section 2.2.5.

To test this, we recorded 5,954 trajectories of *V. alginolyticus* from a single culture (at a given sodium concentration, $[\text{Na}^+] = 513 \ \text{mM}$) and simultaneously measured the head size and shape for each cell via image analysis. We found a moderate variation in the head’s length ($2a = 3.2 \pm 0.5 \ \mu m$; Fig. 2.11 inset) and a minor variation in its width ($2b = 1.2 \pm 0.1 \ \mu m$). We binned trajectories by head length and computed the distribution of reorientation angles for the collection of trajectories in each bin. This calculation confirmed that the reorientation angle associated with a flick on average increases with increasing head size (Fig. 2.11). For example, the most probable flicking angle increases from $\Delta \theta \approx 80^\circ$ for short cells ($2.0 < 2a \leq 2.9 \ \mu m$; Fig. 2.11, red curve), to $\Delta \theta \approx 109^\circ$ for cells of intermediate length ($2.9 < 2a \leq 3.6 \ \mu m$; Fig. 2.11, green curve), to $\Delta \theta \approx 138^\circ$ for long cells ($3.6 < 2a \leq 5.0 \ \mu m$; Fig. 2.11, blue curve). This analysis also showed that cells with average head length have a most probable flicking angle of $\Delta \theta \approx 90^\circ$. The considerable increase in the flick angle with cell length is due to the decreased rotational hydrodynamic mobility of the cell head and supports the fact that the torque generated at the hook bending is responsible for the process of flicking.
2.3.5 Additional aspects of the hook dynamics and of the flicking process

In this section, we present additional experimental results, theoretical analyses and scaling arguments that provide further insight into the dynamics of the cell flicking process and further support the conclusion that a buckling instability of the hook is at the origin of the flick.

2.3.5.1 Maximum twist angle of the hook

Based on previous measurements of tethered cells made with optical tweezers\textsuperscript{25,26}, we expect that during steady swimming the torque generated by the rotary motor twists the elastic hook. Here, we estimate the maximum twist angle of the hook under the torque produced by the rotary motor of \textit{V. alginolyticus}. We hypothesized that it is this twisting that is responsible for the increased bending stiffness of the hook demonstrated in section 2.3.7. For a motor torque $T_M$ and a twisting spring constant of the hook $k_\psi$, the twist angle is $\psi_h = T_M / k_\psi$. For $T_M$ we use the maximum (stall) torque of the cell’s rotary motor. The only available information on the hook’s torsional stiffness comes from optical tweezer experiments for \textit{E. coli} ($k_\psi = 400$ pN nm/rad)\textsuperscript{25,26} and \textit{Rhodobacter sphaeroides} ($k_\psi = 3,800$ pN nm/rad)\textsuperscript{51}, the latter being a monotrichous bacterium with a straight hook, similar to \textit{V. alginolyticus}. When applied to \textit{E. coli}, for which $T_M = 1,260$ pN nm, this estimate yields $\psi_h = 180.5^\circ$, in good agreement with the maximum twist angle measured for \textit{E. coli}’s hook ($180^\circ$–$270^\circ$) using optical tweezers\textsuperscript{25,26}. Because the torsional hook stiffness of \textit{V. alginolyticus} is unknown, we use the two values above as lower and upper bounds. Further using the known stall torque of \textit{V. alginolyticus}’ sodium motor\textsuperscript{52}, $T_M = 4,000$ pN nm, we find that the maximum twist angle of \textit{V. alginolyticus}’ hook during steady swimming is in the range $\psi_h = 60^\circ$–$570^\circ$. These large angular deformations have the potential to significantly alter the hook’s microstructure, which in turn has been postulated to increase the torsional stiffness and the structural stability of the hook\textsuperscript{27,28}, a mechanism that could account for the observed increase in bending stiffness during forward swimming compared to flicking.

2.3.5.2 Unwinding time of the hook

To further support the hypothesis that buckling occurs when the hook unwinds and the bending stiffness $EI$ is reduced, we estimated the relaxation (or unwinding) time of the hook upon reversal of the rotary motor, \textit{i.e.}, from the torsionally loaded state during steady swimming to a relaxed state (see section 2.3.7). This analysis, described in detail below, shows that the unwinding time is comparable to the delay in the flick after motor reversal, measured experimentally to be $\sim$10 ms using high-speed imaging at 1,000 frames/s (Fig. 2.6; see also Fig. 2.5c).
To compute the unwinding time, we modeled the dynamics of the cell’s head-motor-hook-flagellar filament system (Fig. 2.7). Like any rotary motor, the bacterial flagellar motor consists of a rotor and a stator, and the rotor spins relative to the cell and is attached to the helical filament by the hook, whereas the stator is anchored to the cell wall\(^5\). A torque balance on each of the elements of the cell results in the following set of first-order, coupled differential equations:

**Cell head:**

\[
\beta_c \dot{\psi}_c = c(\psi_c - \psi_r). \tag{2.21}
\]

- Viscous drag torque on the cell head
- Coupled motor torque

**Rotor:**

\[
\beta_r (\dot{\psi}_r - \psi_c) = -c(\psi_c - \dot{\psi}_r) - k_\psi (\psi_r - \psi_f). \tag{2.22}
\]

- Viscous drag torque on the rotor
- Coupled motor torque
- Elastic coupling torque of the hook

**Flagellar filament:**

\[
\beta_f \dot{\psi}_f = k_\psi (\psi_r - \psi_f). \tag{2.23}
\]

- Viscous drag torque on the flagellar filament
- Elastic coupling torque of the hook

Here, \(\beta_c, \beta_r\) and \(\beta_f\) are the rotational drag coefficients for the cell head, rotor and flagellar filament (see section 2.3.2.1), respectively, \(k_\psi\) is the torsional spring constant of the hook\(^{25,26,51}\), and \(c = 0.196 \text{ pN nm s/rad}\) is the proportionality constant\(^5\) from the torque-speed curve of the sodium motor of \(V.\ alginolyticus\). Because of the lack of external loads, the cell head is torque-free\(^5\) (Eq. 2.21). The rotor-stator system has internal viscous dissipation (\(\beta_r = 0.002 \text{ pN nm s/rad}\))\(^\text{53}\) and the torque exerted on the head and flagellar filament are dissipated by viscous torques from the external fluid (see section 2.3.2.1). The hook is modeled as a torsional elastic element connecting the rotor and the flagellar filament and is assumed to incur negligible viscous drag due to its miniscule length compared to the flagellar filament\(^49\). The torque generated by the motor in a swimming cell is linearly proportional to the angular speed difference between the stator and the rotor\(^5\) (Eq. 2.22), which are fixed to the head and to the proximal end of the hook, respectively. For simplicity, here, we assume that the rotor-stator interaction in the motor is ideal, and that the cell head is rigidly attached to the stator\(^\text{53}\). Torsional Brownian forces are negligible compared to the motor torque and are thus not included.

Solving the coupled differential equations results in exponentially decaying twist angles of the head, rotor and flagellar filament,

\[
\psi_{c,r,f} = \psi_1 \exp\left(-\frac{t}{\tau}\right) + \psi_2, \tag{2.24}
\]
characterized by a single relaxation timescale,

\[
\tau = \frac{\beta_c \beta_f \beta_r - c\beta_c \beta_f}{k(\beta_c + c\beta_f - \beta_c \beta_f - \beta_c \beta_r)}, \tag{2.25}
\]

where \(1/\tau\) is computed as the (unique) eigenvalue of the coupled system of equations, and coefficients \(\psi_1\) and \(\psi_2\) vary among head, rotor and flagellar filament and depend on the initial twist angle of each. The twist angle of the hook, \(\psi_h = \psi_f - \psi_r\), is the difference in twist angles of flagellar filament and rotor. Using parameter values computed based on the known geometry of the cell (Tables 2.1-2.3; see sections 2.3.2.1, 2.3.5.1 and 2.3.5.2) and the range of \(k\) values described above (400 - 3,800 pN nm/rad; see section 2.3.5.1), Eq. 2.25 yields an unwinding time scale \(\tau = 0.5 - 4.4\) ms. Despite the simplified analysis, this timescale is of the same order of the observed elapsed time between the reversal and the initiation of the flick (10.2 ± 5.7 ms; Fig. 2.6), supporting the hypothesis that the hook buckles in the relaxed state, when the hook unwinds, and where its measured bending stiffness \(EI\) is minimal.

### 2.3.5.3 Flagellar realignment after buckling

When the hook buckles, the flagellar filament goes off axis and the cell turns\(^4\) (Fig. 2.5a). To resume forward swimming, the flagellar filament must return to its original position, in a coaxial alignment with the cell head. Here we examine a possible flagellar realignment mechanism associated with the stiffening of the hook as it twists.

The viscous torque involved in the realignment of the flagellar filament with the cell head axis (Fig. 2.10) is obtained by integrating the tangential and normal components of the viscous torque along the length of the helix. The rotational resistance of the flagellar filament\(^4\) is

\[
\beta_{rf} = \frac{1}{3} K_n l^3 \cos^2 \zeta \left[ 1 - \frac{1}{2} (1 - \cos^2 \zeta)(1 - \frac{K_t}{K_n}) \right], \tag{2.26}
\]

where \(K_t = \frac{2\pi \eta}{\ln(2p/r) - 0.5}\) and \(K_n = 1.5K_t\) are the tangential and normal resistance coefficients, respectively, the latter chosen in accordance with the range of values proposed in the literature for low Reynolds number flow \((K_n/K_t = 1.4 - 1.7)^5\), and \(\zeta\) is the pitch angle of the flagellar filament (Table 2.3). The viscous torque on the flagellar filament is thus \(\beta_{rf} \phi_f\), where \(\phi_f\) is the angular rate of rotation of the flagellar filament about the hook (Fig. 2.10).

When the hook buckles, the motor continues to turn in the CCW direction. As a result, the hook is torsionally loaded, and we propose that its bending stiffness increases as a result of the hook
twisting (see section 2.3.7.2). Again, approximating the off-axis bending of the hook as a torsional spring with constant \( k_{sp} \approx EI_{\text{LOADED}}/L \) (Eq. 2.33), the dynamics of the flagellar realignment process (Fig. 2.10) can be described by solving for the torque balance on cell head and flagellar filament, \( i.e. \)

\[
\begin{align*}
\text{Cell head:} & \quad \beta_{rh} \dot{\phi}_c = k_{sp}(\phi_c - \phi_f) \\
\text{Flagellar filament:} & \quad \beta_{rf} \dot{\phi}_f = -k_{sp}(\phi_c - \phi_f),
\end{align*}
\]

where \( \beta_{rh} \) (Eq. 2.19 and 2.20) and \( \dot{\phi}_c \) are the rotational drag coefficients of the cell head and the angular rate of rotation of the head about the hook, respectively (Fig. 2.10). We assumed for simplicity that the flagellar filament does not rotate about its own axis. Solving the coupled differential equations results in exponentially decaying rotation angles of the head, \( \phi_c \), and flagellar filament, \( \phi_f \). The inverse of the (unique) eigenvalue of the coupled system of Eqs. 2.22 and 2.23 is the characteristic timescale,

\[
\tau = \frac{\beta_{rh} \beta_{rf}}{k_{sp}(\beta_{rh} + \beta_{rf})},
\]

which we compute to be \( \tau = 42.2 \text{ ms} \) using values of the parameters in Eqs. 2.19, 2.26 and 2.33 computed from the known geometry of the cell (Tables 2.1-2.3) and the measured bending stiffness of the hook in a torsionally loaded state (see section 2.3.7.2). This estimate is comparable to the observed flicking time (\( \sim 60 \text{ ms} \); Fig. 2.5c), indicating that an increase in bending stiffness of the hook driven by motor rotation can potentially exert the torque necessary to restore the flagellar filament’s alignment with the cell head axis after each flick, allowing the cell to resume steady forward swimming.

2.3.6 Ruling out other potential buckling mechanisms

In assessing which mechanism is responsible for the buckling of the hook and the ensuing, striking reorientation observed in \( V. \text{ alginolyticus} \), we considered a range of candidate processes. Here we briefly summarize these and demonstrate that none of them represent a viable mechanism to explain the occurrence of the flick nor its characteristics.
2.3.6.1 A burst in swimming speed

Because the viscous (compressive and torsional) loads exerted on the cell increase linearly with swimming speed (see section 2.3.2.1), it is conceivable for a cell to induce buckling of its hook by briefly increasing its swimming speed beyond a critical value, e.g. after a backward-to-forward reversal (Fig. 2.5b, short red segment after reversal) due to a release of stored elastic energy from overcoiling during backward swimming. This mechanism would also explain why buckling does not occur during steady swimming, but it was ruled out through high-speed imaging. The trajectories of 135 cells were captured using at 1000 frames/s with phase-contrast microscopy (100x objective, 1.4 NA) to quantify swimming speed throughout the swimming cycle. The mean speeds were all in the range 58≤V≤69 μm/s and there was no consistent speed increase immediately preceding the flick.

2.3.6.2 Dynamic buckling

The very rapid (~10 ms) transition from a tensile to a compressive load possibly due to stored elastic energy that the hook undergoes upon reversal of the rotary motor led us to analyze the role of unsteadiness in the buckling process and to consider whether the impulse loading associated with the flick (but not with steady swimming) may contribute to the buckling of the hook. Assuming again Euler beam theory to govern the hook dynamics, the equation for the deflection of the hook, w, perpendicular to the major axis of the head, x, is

\[
EI \frac{\partial^4 w}{\partial x^4} + \rho A \frac{\partial^2 w}{\partial t^2} - \xi_\perp \frac{\partial w}{\partial t} = f(x, t),
\]  

(2.30)

where t is time and f is an applied forcing. The first term represents the contribution of bending, where the measured bending stiffness of the hook is on the order of \(EI = 10^{-26} - 10^{-25} \text{ N m}^2\) (Table 2.2), depending on the amount of twist (see section 2.3.7). The second term is the inertia of the hook, where A is the cross-sectional area of the hook and the hook density, \(\rho\), is assumed to be that of the surrounding medium (\(\rho \approx 1000 \text{ kg/m}^3\)). The third term is viscous damping, where \(\xi_\perp = 4\pi \eta /[\ln(2L/r_0) - 1/2]\) is the hydrodynamic drag coefficient per unit length of the hook for motions perpendicular to the hook axis, L is the length of the hook, \(r_0\) is the outer radius of its cross section, and \(\eta = 10^{-3} \text{ kg/(m s)}\) is the dynamic viscosity of water. In its unloaded configuration, the hook is a hollow straight rod with geometric parameters tabulated in Table 2.2. Comparing the order of magnitude of the inertial term and the bending term, we find

\[
\frac{\text{inertia}}{\text{bending}} \sim \left( \frac{\rho AL}{\tau^2} \right) \left( \frac{EI}{L^3} \right)^{-1} = \left( \frac{\rho \tau^2 L^4}{\tau^2 EI} \right) \sim 10^{-12} - 10^{-11},
\]  

(2.31)
where we take the timescale $\tau \sim 10\text{ ms}$ to be the duration of the forward swimming segment immediately preceding the flick (see also Fig. 2.6). Thus, the inertial term is of negligible importance in the buckling dynamics, even though the load is applied over millisecond timescales. Finally, inclusion of the viscous drag term in Eq. 2.30 over-damps the system, as seen by comparing the inertial and viscous terms:

$$\frac{\text{inertia}}{\text{viscosity}} \approx \left( \frac{p \Delta L}{\tau^2} \right) \left( \frac{\delta_{\perp}^L}{\tau} \right)^{-1} \approx \left( \frac{p \Delta^2}{\tau \eta} \right) \sim 10^{-14}. \quad (2.32)$$

The minute magnitude of this ratio further supports the negligible role of dynamic buckling and confirms the validity of a static analysis of buckling for the hook during the flick.

### 2.3.7 Measurement of the hook’s bending stiffness

If flicks are caused by buckling, how can bacteria achieve steady forward swimming after a flick and suppress further buckling? Their swimming speed, and hence the load, is not significantly different just prior to a flick compared to a steady run (Fig. 2.5d; see section 2.3.6.1). Rather, we find through direct measurements of the flagellar motion that a dynamic stiffening of the hook occurs during swimming (see section 2.3.7). The hook’s bending stiffness increases six-fold under the load of steady swimming ($EI_{\text{LOADED}}=2.2\pm0.4\times10^{-25} \text{ N m}^2$) compared to the unloaded state ($EI_{\text{RELAXED}}=3.6\pm0.4\times10^{-26} \text{ N m}^2$). This stiffening averts buckling during steady swimming and is likely caused by twisting of the hook, which is known to occur under the motor’s torsional load. We infer that, upon motor reversal, the hook temporarily unwinds, losing bending stiffness and becoming susceptible to buckling (see section 2.3.5). Stiffening of the hook under applied load is in line with crystallographic and electron cryomicroscopy studies in *Salmonella typhimurium*, which suggest that the hook’s interlocking protein microstructure increases its structural stability under torsion. Furthermore, optical tweezer measurements of tethered *E. coli* and *Streptococcus* show a torsionally soft phase up to $\sim180^\circ$ followed by a torsionally rigid phase for larger deformations, when the hook stiffens torsionally upon twisting. Ultimately, however, understanding the precise origin of the enhanced stiffness during steady swimming will likely require detailed molecular dynamics simulations of *V. alginolyticus*’ hook.

Buckling during a flick and the subsequent stability during steady forward swimming can both be understood in terms of the hook’s stability diagram. The marginal stability condition, $F/F_{CR}+(T/T_{CR})^2=1$, defines a parabolic separatrix in the space of the normalized force, $F/F_{CR}$, and torque, $T/T_{CR}$ (Fig. 2.12). Inside the parabola, the hook is stable and functional for swimming, whereas outside it buckles and triggers a reorientation. During steady forward and backward swimming, the normalized loads fall within the stability region due to the higher bending stiffness (higher $F_{CR}$ and $T_{CR}$). In contrast, just after the onset of forward swimming, immediately following a reversal, the normalized loads fall mostly outside the separatrix due to the lower bending stiffness, corresponding to buckling and resulting in flicks.
**Figure 2.12 | Stability diagram of the hook under combined axial and torsional loads.** The hook is stable when the normalized viscous force ($F/F_{CR}$) and torque ($T/T_{CR}$) fall underneath the stability boundary (black curve) and is predicted to buckle otherwise. Symbols represent measurements for steady backward swimming (green diamonds), forward swimming (blue triangles), and short forward runs prior to flicks (red circles) from 135 trajectories recorded at 1,000 frames/s at a sodium concentration of $[\text{Na}^+] = 513$ mM (see section 2.3.6.1). Yellow filled symbols represent averages of the three cases and error bars denote standard deviations. Critical loads were computed based on the bending stiffness for the loaded hook during steady swimming and for the relaxed hook during a flick (see section 2.3.7). Open squares extend the same analysis to the data set from Fig. 2.9, demonstrating the loss of stability as swimming speed increases.

### 2.3.7.1 Measurement of the hook's bending stiffness in the relaxed state

We directly measured the hook bending stiffness, $EI$, for non-motile *V. alginolyticus* cells whose head was immobilized on a glass substrate, for which the hook was in a relaxed (unstressed) state. Due to their small size, the flagellar filaments were susceptible to thermal Brownian fluctuations, which we used to compute the $EI$ of the hook. The large persistence length of the flagellar filament ($2.5 \text{ mm}$) compared to its length ($\approx 5 \mu\text{m}$) indicates that the flagellar filament is effectively rigid under thermal forcing. In contrast, the length $L=100 \text{ nm}$ of the hook$^5$ is comparable to its persistence length ($\approx 100 \text{ nm}$)$^46$, indicating that the hook is flexible when exposed to thermal forcing. Thus, any fluctuations of the flagellum (flagellar filament plus hook)
under thermal forcing are to be entirely ascribed to the deformation of the hook. The hook is also much shorter than the flagellar filament and the cell head, suggesting that, in terms of bending, it may be modeled as a torsional spring with spring constant

\[
k_{sp} \approx \frac{EI}{L},
\]

where \( EI \) is the hook’s bending stiffness. Thus, we approximate the cell-hook-filament complex as two rigid links, representing the head and filament, connected by a torsional spring, representing the hook (Fig. 2.13a). Assuming the spring to be linear with the head-filament orientation angle \( \phi \) (Fig. 2.13a), the elastic energy \( U \) of the discrete torsional spring system is

\[
U = \frac{1}{2} k_{sp} \phi^2.
\]  

Because cells are non-motile in this case (see below), any fluctuations of the flagellum are driven only by the thermal energy of the surrounding medium and thus the probability \( p(\phi) \) of occupying a given energy state with angle \( \phi \) is given by the Boltzmann relation

\[
p(\phi) = p_0 e^{-\frac{k_{sp} \phi^2}{2k_B T_A}},
\]

where \( k_B \) is Boltzmann's constant, \( T_A \) is the absolute temperature, and \( p_0 \) is a normalization constant. The variance of the orientation angle, \( \langle \phi^2 \rangle \), is related to the spring constant \( k_{sp} \) through the variance of the Boltzmann distribution by

\[
\langle \phi^2 \rangle = k_B T_A / k_{sp},
\]

which yields an expression for the bending stiffness,

\[
EI = L \frac{k_B T_A}{\langle \phi^2 \rangle},
\]

in terms of \( \langle \phi^2 \rangle \), which we measure experimentally.

To measure the angular distribution of the flagellar orientation, \( \phi \), a dilute suspension of \( V. \alginolyticus \) was observed at the bottom surface of a 25 \( \mu \)m deep glass chamber using dark-field microscopy to visualize the 30 nm diameter flagellar filament (Table 2.3). Randomly selected, non-motile cells that naturally adhered to the glass substrate via non-specific binding were imaged using a 40\( \times \) objective (0.6 NA), 1.2 NA oil condenser and an Andor Neo sCMOS camera (6.5 \( \mu \)m/pixel) for 500 s each at 10 frames/s. Through direct visualization of the flagellar filament, we verified that the helical flagellum was not rotating on these surface-attached cells, indicating that their rotary motors were inactive and, thus, the cells were non-motile. The cell head and the flagellar filament were identified and tracked using custom image analysis routines implemented in Matlab to measure their relative angular orientation, \( \phi \), in time (Fig. 2.13a,b).
From the variance of the flagellar orientation angle, \( \langle \phi^2 \rangle \), computed from the resulting distribution of angular orientations (Fig. 2.13c), the bending stiffness was calculated using Eq. 2.36. This yielded a value of the bending stiffness in the relaxed state of \( EI_{\text{RELAXED}} = 3.6 \pm 0.4 \times 10^{-26} \text{ N m}^2 \) (N=3 cells). For comparison, we computed the bending stiffness from the swimming speed \( (V = 47 \mu \text{m/s}) \) at which the sharp drop in the probability of flicking, \( P_F \), occurs (Fig. 2.9). By rearranging the marginal stability condition (Eq. 2.18), we solved for the bending stiffness as a function of the viscous loads, obtaining

\[
EI = \frac{FL^2 + \sqrt{F^2L^4 + \pi^2 T^2 L^2}}{2\pi^2},
\]  

(2.37)

then used the relation between viscous loads and swimming speeds (Eq. 2.14 and 2.15). For \( V = 47 \mu \text{m/s} \), this yielded \( EI = 1.3 \times 10^{-26} \text{ N m}^2 \), which is of the same order as the value computed from the thermal fluctuation experiments above.

Figure 2.13 | Thermal fluctuations used to compute the hook’s bending stiffness in the relaxed state. a, The bending stiffness of the hook is measured from the thermal fluctuations of the flagellar orientation, \( \phi \), of cells whose head is immobilized on a glass slide. The flagellar filament and the cell head are approximated as rigid links and the hook (red) as a torsional spring. b, Time series of the fluctuations of the flagellar orientation angle \( \phi \) arising from the thermal fluctuations of the hook. c, Probability density \( p(\phi) \) of the flagellar orientation angle \( \phi \) for a single cell recorded over 500 s at 10 frames/s.

We sought to compare these estimates of the hook bending stiffness under relaxed conditions with literature values, but limited information is available. To the best of our knowledge, only a single experimental study, by Sen and coauthors\(^{46} \), reported \( EI \) values for bacterial hooks. Those authors computed the hook’s bending stiffness for \( E. \text{coli} \), \( S. \text{typhimurium} \), \( V. \text{cholerae} \) and \( V. \text{parahaemolyticus} \) from the hook’s geometry measured via electron micrographs\(^{46} \). Their analysis showed that hooks from peritrichous and monotrichous bacteria have similar bending stiffness, but the values reported were \( \sim 100 \) fold smaller than our
measurement (Table 2.2). While the discrepancy can ultimately only be reconciled with further, independent data (e.g., using optical tweezers), we note that our approach is a rather direct method for the estimation of EI that involves only minimal manipulation of the cells, whereas Sen and coauthors\textsuperscript{46} recognize that their preparation steps (which involved air-drying and negatively-staining samples for electron microscopy and attaching them to carbon-coated grids) could have affected their measurements, particularly because they would have flattened and stretched the hook. Most importantly, the bending stiffnesses reported by Sen and coauthors\textsuperscript{46}, for example for Vibrio parahaemolyticus, could support a swimming speed of just $V\sim0.5$ μm/s before succumbing to buckling under viscous swimming loads, as compared to the known swimming speed of $V\sim50$ μm/s, providing support for the larger EI values measured in the current study (Table 2.2).

2.3.7.2 Measurement of the hook’s bending stiffness in the loaded state

Here, we present measurements demonstrating that during steady forward swimming (we use ‘steady’ to distinguish this from the ~10 ms forward run just before the flick) the hook has a higher bending stiffness compared to the bending stiffness in the relaxed state. We estimate the hook’s bending stiffness in swimming cells based on the relative orientation between the cell head and the flagellar filament under the natural axial and torsional loads experienced during steady swimming.

Figure 2.14 | Wobbling dynamics of V. alginolyticus used to determine the hook’s bending stiffness in the forward swimming state. a-b, Approximation of a swimming bacterium as two rigid links (the cell head and the flagellar filament) connected by a torsional spring (the hook). c, Time series of the projection angle $\phi'$ between the head axis and the flagellar axis during steady forward swimming, resulting from cell tracking using high-speed dark-field microscopy at 420 frames/s. $\phi$ is the mean amplitude of $\phi'$. d, Time series of the projection angle $\beta'$ between the
head axis and the swimming direction. $\beta$ is the mean amplitude of $\beta'$. In panels c and d, the black solid lines indicate the reference angles used to quantify the fluctuation amplitude.

High-speed imaging using high-intensity dark-field microscopy (40x, 0.6 NA objective; 1.2 NA oil condenser; mercury lamp illumination) revealed that the cell head and the flagellar filament 'wobble' during steady swimming and that the magnitude of the wobble is larger during forward swimming compared to backward swimming. We assume that the difference in the wobble between forward and backward swimming for a given cell arises due to the flexibility of the hook, as it switches from compressive (forward swimming) to tensile (backward swimming) axial load. As the hook bends, it forms an angle $\phi$ between the cell head and the flagellar filament. As the cell swims and its head rotates, the projection of this angle onto the plane of observation, $\phi'$, appears as a sinusoidal fluctuation and the angle $\phi$ between the cell head and the flagellar filament is the amplitude of this fluctuation (Fig. 2.14c). The cell head and the flagellar filament were tracked with an in-house routine implemented in Matlab. During steady forward swimming, the drag and thrust exert a compressional force on the hook, accentuating its bending, and we measured a mean wobbling angle of $\phi_F=24^\circ \pm 6^\circ$. In contrast, during steady backward swimming, drag and thrust exert a tensile force on the hook, reducing its bending, and we measured a smaller mean wobbling angle, $\phi_B=11^\circ \pm 5^\circ$.

To estimate the hook bending stiffness, we again modeled the head and the flagellar filament as rigid links and the hook as a torsional spring (Fig. 2.14a,b). Assuming that the head is precessing about the mean swimming direction at an angle $\phi$ (Fig. 2.14a,d) with a fixed head-flagellar filament angle $\phi_{0}$, the viscous drag on the head is

$$F = V(A_1 \sin^2 \beta + A_2 \cos^2 \beta),$$

where $A_1 = 32 \pi \eta a e^3 / [(3e^2 - 1)D + 2e]$, $A_2 = 16 \pi \eta a e^3 / [(1 + e^2)D - 2e]$, $D = \ln[(1 + e)/(1 - e)]$, and $e = \sqrt{(a^2 - b^2)/a}$ is the eccentricity. The thrust from the flagellar filament balances the drag force along the swimming direction and together they act to create a bending moment on the hook during forward (subscript F) and backward (subscript B) swimming,

$$M_F = \delta_F \times F_F = k_{sp}(\phi_F - \phi_0),$$

$$M_B = \delta_B \times F_B = k_{sp}(\phi_B - \phi_0),$$

where $\delta = a \sin \beta$ is the moment arm, $k_{sp}$ is the torsional spring constant used to estimate the bending stiffness $EI$ from Eq. 2.33, and $\phi_0$ denotes the attachment angle between head and flagellar filament. The small but finite angle between head and flagellar filament observed during backward swimming, $\phi_B=11^\circ$, is likely due to the curved shape of the *Vibrio* and/or a finite
angle between the hook and the head due to the exact position of the motor relative to the cell’s pole. By subtracting the relations above (Eq. 2.39 and 2.40), we find \((\delta_F \times F_F) - (\delta_B \times F_B) = k_s (\phi_F - \phi_B)\), which we solve for \(k_s\) to compute the bending stiffness in the loaded state from Eq. 2.33, finally finding \(EI_{LOADED} = 2.2 \pm 0.4 \times 10^{-25}\) N m² (N=3 cells). This value is a factor of 6.1 larger than the bending stiffness in the relaxed state, \(EI_{RELAXED} = 3.6 \pm 0.4 \times 10^{-26}\) N m², demonstrating that the hook is considerably stiffer during steady swimming and suggesting that this is the reason for the absence of buckling during steady forward swimming.

Figure 2.15 | Turning by buckling is widespread among uni-flagellated bacteria. Swimming trajectories of (a) *Pseudoalteromonas haloplanktis*, (b) *Vibrio corallilyticus*, and (c) a natural bacterial community enriched from a seawater sample and exposed to a dead copepod (see
2.3.8 Evidence for turning by buckling in other marine bacteria

Turning by buckling is a fundamental component of the motility strategy of *V. alginolyticus*, where it occurs in ~80% of swimming cycles for mean swimming speeds at natural sodium concentrations (Fig. 2.9). The observation that 90% of motile marine bacteria are monotrichous suggests that the same motility pattern, and potentially the same material properties of the hook, may be widespread among marine bacteria. This hypothesis is supported by our observations, which revealed the same alternation of reversals and flicks in the fast swimming marine bacterium *Pseudoalteromonas haloplanktis* (PF=40%; Fig. 2.15a; ref.4), in the coral pathogen *Vibrio coralliilyticus* (PF=80%; Fig. 2.15b), and, most strikingly, in a mixed seawater community (PF=60%; Fig. 2.15c) [see section 2.3.8].

2.3.8.1 *Pseudoalteromonas haloplanktis* and *Vibrio coralliilyticus*

We found that the trajectories of both *P. haloplanktis* and *V. alginolyticus* (Fig. 2.15a,b) exhibit the same alternation between ~90° flicks and 180° reversals as *V. alginolyticus*. *P. haloplanktis* has comparable head size and morphology to *V. alginolyticus* (Table 2.1), but a faster mean swimming speed (68 µm/s; ref. 28), and it exhibits a similar swimming pattern to *V. alginolyticus* as observed with conventional video microscopy (30 frames/s), suggestive of flick reorientations. Here, we recorded *P. haloplanktis* cells swimming with high-speed video microscopy (1,000 frames/s), which confirms that upon reversal from backward to forward swimming, *P. haloplanktis* also swims forward for a short period (~4 ms) before flicking (Fig. 2.16; red shaded region), as *V. alginolyticus* (Fig. 2.5). This confirms that hook of *P. haloplanktis* is in the same state of stress as *V. alginolyticus* just prior to a flick, suggesting that the same buckling mechanism enables their reorientation strategy.

To quantify the probability of flicking, 2,702 trajectories of *P. haloplanktis* and 3,960 trajectories of *V. coralliilyticus* were recorded at a sodium concentration representative of marine conditions ([Na⁺] = 513 mM), using phase-contrast microscopy at 30 frames/s, and analyzed as described in §2 and §4. The probability of flicking, PF, was 71% for *P. haloplanktis* cells swimming at 74±7 µm/s and decayed to 21% for swimming speeds of 26±7 µms. For *V. coralliilyticus* we found PF = 90% at 65±7 µm/s, which decreased to 58% at 30±7 µm/s. We note that in the experiments, we did not access the lower value of swimming speeds because, experiments were performed only at
natural sodium concentrations. In summary, flicking was prevalent in the swimming pattern of both species and the probability of flicking was reduced at slower swimming speeds, consistent with buckling.

![Diagram](image)

Figure 2.16 | The short forward run preceding a flick also occurs in *Pseudoalteromonas haloplanktis*. Time series of the alignment, \( q \), between the cell head (black arrow) and the swimming direction (green or red arrows) for a *P. haloplanktis* cell, measured using high-speed phase-contrast microscopy (1,000 frames/s; 100×, 1.4 NA objective). The alignment reveals the same four elements in the motility pattern of this bacterium as found for *V. alginolyticus* (Fig. 2.5). The red shaded region indicates the short (here, 4 ms long) period of forward swimming that immediately precedes the flick.

2.3.8.2 Enriched natural bacterial community

It has been reported that, out of 600 motile isolates from the sea, 90% are monotrichous\(^3\). This striking predominance led us to hypothesize that the buckling-based reorientation exhibited by *V. alginolyticus* is widespread in the ocean. To partially test this hypothesis, we studied the motility patterns of cells from an enriched natural bacterial community. We recorded swimming trajectories in the absence of chemical stimuli (Fig. 2.17a) as well as in the vicinity of individual copepods (Fig. 2.15c) using phase-contrast microscopy at 30 frames/s (20×, 0.45 NA objective).

In both cases, the alternation between reversals and flicks was commonly observed. From the trajectories we measured the distribution of reorientation angles (e.g., Fig. 2.17b), as described in §4, and computed a probability of flicking of \( P_F = 60\% \) in the absence of chemical stimuli. Taken together with the predominance of single polar flagellation among motile marine bacteria,
these results suggest that the alternation of flicks and reversals, and thus, potentially the mechanism of turning by buckling, might be a widespread behavior among bacteria in the ocean.

Figure 2.17 | **Flicks are common among marine bacteria.** **a,** A sample of bacterial trajectories from an enriched seawater sample showing flicks (open squares) and reversals (full circles), similar to the swimming pattern of *V. alginolyticus* (Fig. 2.8a, aqua inset). Different colors denote trajectories of different cells. Crosses denote starting positions, and cell head positions are shown by circular markers at 33 ms intervals. **b,** The probability density of reorientation angles for 5,362 trajectories acquired from an enriched seawater bacterial community. The inset shows the distribution of swimming speeds.

**2.4 DISCUSSION**

Other monotrichous motility strategies notwithstanding (e.g., the run-and-stop swimming of *Rhodobacter sphaeroides*), turning by buckling may represent a prevalent reorientation mechanism among monotrichous bacteria and a widespread counterpart to the classic tumbling of peritrichous bacteria. Whereas multiple flagella may be justified in nutrient-rich environments to generate the necessary torque to ‘drill’ through very viscous media, a single flagellum may embody a motility adaptation to oligotrophic environments, such as the ocean, by minimizing the costs of flagellar biosynthesis and actuation. However, this cost-saving strategy introduces the problem of reorientation, for which turning by buckling provides an effective, minimalistic solution.

These findings reveal a new role of flexibility in bacterial locomotion. The highly compliant hook is seemingly the Achilles heel of *V. alginolyticus*, teetering on the brink of mechanical failure. However, this vulnerability is only engaged when advantageous for the cell to reorient and represents one of the smallest examples in nature of how operating at the boundary of mechanical stability generates new functional solutions. Turning by buckling is an elegant,
under-actuated reorientation mechanism that highlights the value of incorporating flexibility and controlled mechanical failure into engineered systems⁶, and enriches the array of biological systems whose extreme mechanics can inspire novel robotic solutions in medicine and engineering⁷.
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CHAPTER 3

Speed-dependent chemotaxis in marine bacteria*

* One of the most important functions of motility is chemotaxis, which underpins important ecological processes among microbes and in particular in the ocean. In Chapter 3, I studied the biophysics of chemotaxis in the marine bacterium *Vibrio alginolyticus* by taking advantage of the findings from Chapter 2 on the reorientation behavior of this bacterium. Together with my collaborators, I found that chemotaxis in *V. alginolyticus* is swimming-speed dependent. This finding is at odds with canonical chemotaxis in *Escherichia coli* and existing mathematical models of chemotaxis, whereby faster cells are better at achieving tight accumulations at the peak of resource gradients. A detailed analysis of the population-scale swimming statistics and the single-cell swimming kinematics revealed that the mechanism at the origin of this unexpected result is the speed-dependent modulation of reorientation and flicking frequency.

This chapter is in the final stages of preparation for a journal submission in the coming weeks as:

Son, K., Menolascina, F. & Stocker, R. Speed-dependent chemotaxis in marine bacteria.

I have collaborated closely with Filippo Menolascina (a postdoc in the Stocker lab) on the design, fabrication and optimization of the new microfluidic device used in experiments; I have been responsible for the majority of data acquisition and analysis; Filippo has been responsible for the chemotaxis model. All coauthors have shared the writing of the paper.
ABSTRACT

Chemotaxis underpins important ecological processes in marine bacteria, from the association with primary producers to the colonization of particles and the infection of animal hosts. Marine bacteria often swim at high speeds with a single flagellum and exhibit a motility pattern composed of reversals and ‘flicks’, the latter resulting from a buckling instability of the flagellum. Both adaptations set marine bacteria apart from the model organism for bacterial motility, Escherichia coli, yet it has remained unclear how they result in the strong chemotaxis that characterizes marine bacteria. Here we elucidate the relationship between fast swimming, run-reverse-flick motility and high chemotactic performance in marine bacteria by tracking thousands of Vibrio alginolyticus cells in microfluidic gradients. At odds with current chemotaxis models, we find that chemotaxis is swimming-speed-dependent, with faster cells accumulating more tightly at the peak of the resource gradient. This behavior confers fast swimmers an advantage in the steady exploitation of the resource, which is additional to the advantage of more rapidly swimming up the gradient. Trajectory analysis shows that this behavior results from a speed dependence of the reorientation frequency: faster cells reorient more frequently. Swimming speed, in turn, is modulated by the resource concentration, a behavior called chemokinesis. To predict how these diverse motility adaptations interact to produce strong chemotaxis, we develop an agent-based model of V. alginolyticus and show that it quantitatively predicts the bacterium’s observed chemotactic performance. These findings indicate that, contrary to what occurs in E. coli, swimming speed can be a fundamental determinant of the gradient-seeking capabilities of marine bacteria, and may help explain the high swimming speeds often measured for bacteria from the ocean.

3.1 INTRODUCTION

Motility is an essential component of chemotaxis, the ability of organisms to sense chemical gradients and swim towards more favorable conditions, for example to find dissolved or particulate nutrients, colonize and infect hosts, or evade noxious substances\(^1,2\). Most of our knowledge of bacterial chemotaxis comes from the study of Escherichia coli, a bacterium that often inhabits the lower intestine of warm-blooded animals\(^3,4\) and uses multiple (4–10) flagella emerging from different points on its body (‘petritrichous’ flagellation) to explore the surrounding environment. Each one of E. coli’s flagella is a propeller powered by a proton motor\(^5\). Counter-clockwise (CCW) rotation of all motors causes the flagella to bundle and to propel the cell into a nearly straight ‘run’ at 10–30 µm/s. Changes in swimming direction are achieved when one or more motors switch to clockwise (CW) rotation, disrupting the flagellar bundle and leading to a nearly random reorientation or ‘tumble’\(^5\). The key to success in E. coli’s
chemotaxis strategy is the bacterium’s ability to control the switching frequency between CCW and CW flagellar rotation, giving rise to the well-known run-and-tumble swimming pattern.

Growing evidence indicates that marine bacteria often exhibit a higher chemotactic performance than *E. coli*, making them valuable model systems to understand the limits of behavioral responses in microorganisms and to determine optimal motion planning strategies for bioinspired robotic systems. For example, the marine bacterium *Pseudoalteromonas haloplanktis* responds up to 10-fold more rapidly than *E. coli* to resource patches, swimming on average at 68 μm/s with burst speeds of up to 445 μm/s (>100 times their body length in a second) when tracking nutrient sources, while the coastal marine bacterium *Vibrio alginolyticus* clusters 7-fold more tightly toward 500 μM of serine than *E. coli*. This elevated chemotactic performances appear to be mediated by more than just the higher swimming speeds that characterize many marine bacteria and suggest that marine bacteria may have evolved either more effective swimming patterns or better sensing capabilities.

The majority of motile marine bacterial species have a single flagellum, emerging from one pole of the cell head (‘monotrichous’ flagellation). A survey of 600 motile marine isolates found that 90% are monotrichous. Furthermore, the swimming patterns of marine bacteria have long been known to differ from *E. coli*’s run-and-tumble swimming by not displaying the same “tumbles” by not displaying the same “tumbles” by not displaying the same “tumbles”. Yet, the motility pattern and the underlying biomechanics of marine bacteria have been resolved only recently using *V. alginolyticus* as a model system, with strong indications that the same motility strategy is used by other cultured isolates (e.g., *P. haloplanktis*) and by the majority of motile cells within natural communities of coastal bacteria. Specifically, *V. alginolyticus* alternates between forward and backward runs by switching the direction of rotation of its sodium-driven motor between CCW and CW. In a forward run, the flagellum pushes the cells head (making the cell a ‘pusher’), whereas in a backward run it pulls it (making the cell a ‘puller’). At the end of a forward run, a cell executes a ‘reversal’ – a change of direction tightly distributed around 180° – and swims backwards. A second reversal separates the end of a backward run from the start of a forward run, yet in this case the reversal is rapidly followed (after ~10 ms) by a reorientation of the swimming direction that is normally distributed around a 90° angle from the previous direction. This reorientation has been recently discovered by Xie and coworkers, who termed it a ‘flick’ because of the large, off-axis deformation of the flagellum that causes it. We have shown that the origin of the flick resides in the buckling of the hook, a ~100 nm long, flexible structure that connects the flagellar filament to the rotary motor, demonstrating a previously unrecognized role of flexibility in the functionality of prokaryotic flagella.

The probability of a flick occurring is swimming-speed dependent. This has been demonstrated both by measurements of the flicking probability as a function of the swimming speed, exploiting the natural variation in speeds among a monoclonal population of bacteria, and by
experiments in which the swimming speed of a population was modulated through a variation of
the sodium concentration in the medium, exploiting the fact that the motor of *V. alginolyticus* is
driven by sodium gradients\(^8\,22\). Both approaches showed that the probability that the onset of a
forward run is followed by a flick – or probability of flicking, \( P_F \) – varies from \(-10\%\) at low
speeds to \(~80\%\) at high speeds, with a sharp rise \(20\text{–}50\ \mu\text{m/s}\). The speed at which this transition
occurs is consistent with a propulsive load that compresses the hook to the point of making it
buckle, and the sharp nature of the transition is consistent with the criticality of a buckling
instability\(^8\). At sodium concentrations typical of the ocean (> 500 mM), *V. alginolyticus* swims
at \(V_0 = 47.5 \pm 8.8\ \mu\text{m/s} \) (mean ± standard deviation)\(^18\), indicating that up to \(~30\%\) of forward runs
do not result in a flick\(^18\).

Here we study the motility pattern and chemotactic performance of *V. alginolyticus* as a function
of the cells’ swimming speed. An important difference between the marine bacterium *V.
alginolyticus* and *E. coli* is that speed-dependent swimming is absent in the ‘run-and-tumble’
motility. The swimming speed remains largely unchanged in this process, and, despite the
bacterium’s ability to sense mechanical stimuli\(^6\), it is generally held that its chemotaxis depends
only on the sensing of chemical stimuli in *E. coli*. Consequently, the swimming speed has not
been considered to affect the ability of cells to retain position in favorable regions of a gradient
and does not enter into models of *E. coli*’s chemotaxis pathway, beyond simply rescaling time
(*i.e.*, allowing proportionately faster climbing of a gradient)\(^7\,8\). In contrast to chemotaxis in *E.
coli* and to current mathematical predictions, we find that not only are faster cells in a population
of *V. alginolyticus* better at rapidly climbing gradients, they also accumulate tighter at the peak
of a gradient compare to slower bacteria. These results reveal that swimming speed can play a
strong and unexpected role in determining the chemotactic response of marine bacteria, and
therefore a new role of swimming speed in the intracellular pathways responsible for the high-
performance chemotaxis of these bacteria.

A second important difference between the motility of some species of marine bacteria and that
of *E. coli* is that the former often exhibit chemokinesis, the ability to modulate the swimming
speed in response to the concentration of a chemical in the environment. *E. coli* is generally
believed to not display chemokinesis\(^28\) (though some recent findings have questioned this
model\(^36\)), whereas some marine bacteria do, enhancing their swimming speed by up to 60%
when the local concentration of a chemical exceeds a threshold\(^23\,24\). For example, *P. haloplanktis*
increases its speed by \(23\%\) in response to algal exudates\(^23\), the coral pathogen *Vibrio
coralliilyticus*\(^24\) increases its speed by up to \(48\%\) when exposed to coral mucus, and *Shewanella
putrefaciens*, *Deleya marina*, as well as an enriched assemblage of marine bacteria increased
their speed in response to various amino acids\(^12\). Chemokinesis can enhance chemotaxis by
increasing the swimming speed of the cells and thus the rate at which they climb chemical
gradients, as demonstrated by a mathematical model based on observations of *V. coralliilyticus*\(^24\).
Here we hypothesize and demonstrate that chemokinesis represents a mechanism to shift the
distribution of swimming speeds within a population into a regime where flicks are predominant. To rationalize these findings, we propose a model that for the first time incorporates swimming speed as a variable in the intracellular pathway of chemotaxis and paves the way for an extension of our understanding, in particular, a rethinking of the largely overlooked role of swimming speed in microbial chemotaxis in the ocean. Taken together, our findings suggest that the chemotactic response of marine bacteria relies in the exquisite integration of different motility adaptations, possibly in response the nutrient-scarce conditions prevalent in the ocean, where motility can be costly relative to resources available\textsuperscript{25,26}.

### 3.2 METHODS

#### 3.2.1 Cell culturing

*V. alginolyticus* YM4 strain was cultured overnight in VC medium [0.5% (w/v) polypeptone, 0.5% yeast extract, 0.4% K\textsubscript{2}HPO\textsubscript{4}, 3% NaCl, 0.2% glucose], diluted 1:100 into VPG medium (1% polypeptone, 0.4% K\textsubscript{2}HPO\textsubscript{4}, 3% NaCl, 0.5% glycerol)\textsuperscript{13} and grown to late exponential phase (OD\textsubscript{600} = 0.5). Cells were then washed and resuspended in TMN motility medium [50 mM Tris-HCl (pH 7.5), 5 mM MgCl\textsubscript{2}, 5 mM glucose, 300 mM NaCl + KCl] to change the sodium concentration, [Na\textsuperscript{+}], from 3-600 mM. The difference in [Na\textsuperscript{+}] was replaced with potassium to maintain osmolarity, a common approach devoid of negative physiological consequences\textsuperscript{18,22}.

#### 3.2.2 Hydrogel-based microchannel fabrication.

Both chemotaxis and chemokinesis experiments were performed in a hydrogel-based microfluidic device (Fig. 3.1), which was fabricated in two steps: (1) Polydimethylsiloxane (PDMS) microchannel fabrication, and (2) hydrogel injection.

*Step 1.* Microchannels were fabricated using standard soft lithography techniques\textsuperscript{42} with a mold prepared by depositing SU-8 photoresist (MicroChem Corp., Newton, MA) on silicon wafers and patterning channel reliefs via photolithography. The mold was silanized to prevent PDMS (Sylgard 184 Silicone Elastomer Kit, Dow Corning, Midland, MI) from adhering to the master (especially between the hydrogel-trapping pillars) and to ease the demolding process. PDMS prepolymer (10:1 mixture of base and curing agent of Sylgard by Dow Corning) was cured by baking at 65°C for >1 hr. PDMS microchannels were then cut and bonded to a clean glass slide via plasma treatment and incubated on a hot-plate at 100°C for 1 hr.
Figure 3.1 | Hydrogel-based microfluidic channel is used to generate a linear steady chemical gradient. Planar layout of a microfluidic channel used in the chemotaxis experiment with three parallel 600 µm wide, 100 µm deep channels separated by 200 µm wide hydrogel-trapping sections, which consist of array of PDMS pillars. Agarose in liquid-phase is injected through the hydrogel injection port (C1) prior to experiments and fills the hydrogel-trapping sections before it solidifies (see section 3.2.2). Bacteria are injected in the test channel, and both inlet (D1) and outlet (D2) are sealed with glass coverslip to create a flow-free condition. Constant concentration boundary conditions in the source channel (flowing chemoattractant, 500 nM serine, from A1 to A2) and sink channel (flowing buffer, seawater with 0 nM serine, from B1 to B2) generate a linear steady gradient (400 to 100 nM in x-direction), C(x), in the test channel by diffusion through the agarose walls (see also Fig. 3.2). The imaging window covers the whole width of the test channel (x) and chemotactic responses are captured at multiple locations along the length of the channel.
Step 2. We used commonly used hydrogel agarose at 2% (w/v) to create a diffusion layer in the hydrogel-trapping sections in between the microfluidic channels. The key to this step was to inject agarose in between the arrays of trapping pillars (Fig. 3.1). Two features connected to the hydrogel-injection port (C1), serpentine and reservoir, were two visual indicators in the device utilized to maximize success rate of agarose injection. Liquid-phase agarose injection was conducted on a temperature control stage to prevent agarose from solidifying. Agarose was injected through the inlet port (C1) using a syringe pump to ensure constant injection pressure, and the injection process was observed using a standard microscope to follow the front end of the agarose meniscus. Agarose got injected sequentially through the serpentine, reservoir, dividing channel, PDMS pillar section, and to the outlets (C2). After completing the agarose injection step, miliQ water was injected inside all the microchannels to prevent trapping of air pocket in between the pillars before usage.
3.2.3 Imaging, cell tracking and trajectory analysis

Swimming bacteria were imaged at channel mid-depth at 22 frames/s by phase contrast microscopy (Nikon Ti-E microscope; 20×, 0.45 NA objective) using a digital camera (Andor Zyla, 6.5 μm/pixel). All analyses were performed in Matlab (The Mathworks) using in-house, automated software to track cells and reconstructed trajectories were smoothed using a second order Savitzky-Golay filter (window size = 182 ms). Reorientation events were identified using two criteria, a high rate of change of direction and a low instantaneous swimming speed, as described in detail before\textsuperscript{18}, and they were classified as flicks or reversals based on the absolute reorientation angle, Δθ, defined as the angle between the swimming directions before and after a reorientation\textsuperscript{18}. The probability of flicking was quantified as the relative occurrence of flicks among all the reorientations, multiplied by two to account for the fact that flicks can only occur after a backward run\textsuperscript{18}. The mean angle change of reorientation and reorientation frequency were quantified based on the identified reorientation events following the same criteria described before\textsuperscript{18}.

3.2.4 Computing nutrient exposure and accumulation time

\( \Delta N(V) \) is the cells’ resource exposure (here, the serine concentration experienced by a cell) of the subpopulation swimming within a certain speed range, relative to a hypothetical non-motile population that is uniformly distributed, \( \Delta N(V) = \int_0^W B(x) \cdot C(x) dx - N_0 \). Here, \( B(x) \) is the normalized bacterial distribution at a given speed, \( C(x) \) is the serine concentration field, \( W \) is the width of the test channel, and \( N_0 \) is the nutrient exposure of the non-motile population. \( \Delta N(V) \) was subsequently averaged over the entire population with and without the chemokinetic speed enhancement, and the total nutrient exposure was computed, \( N_T = \int_0^{V_{\text{max}}} \Delta N(V) \cdot B(V) dV \), where \( B(V) \) is the swimming speed distribution, and \( V_{\text{max}} \) is the maximum swimming speed observed in the experiments. Note that the computed total nutrient exposure, \( N_T \), is an instantaneous metric, whereby the population-averaged benefit increases with accumulation time of a population around a nutrient hotspot. In the simulation, the population-level nutrient exposure is computed from the CMC, \( N_T = \int_0^{V_{\text{max}}} \text{CMC}(V) \cdot B(V) dV \).

Similarly, \( T_A \) is speed-dependent and the population-averaged accumulation timescale, \( \langle T_A \rangle \) is again computed by weighting \( T_A \) by the speed distribution \( B(V) \), \( \langle T_A \rangle = \int_0^{V_{\text{max}}} T_A(V) \cdot B(V) dV \).

3.2.5 Computational model of chemotaxis in Vibrio alginolyticus

In order to capture the chemotactic behavior of \( V.\text{alginolyticus} \) we used a modeling framework based on cellular automata. In particular, in absence of more detailed information about the molecular mechanisms of signaling events governing chemotaxis in \( V.\text{alginolyticus} \), we adapted
the model described in Jackson\textsuperscript{8} to reflect distinct behavioral traits in our model organism. We modeled our agents to proceed in one of two modes: either “forward” or “backward” runs, meant to mimic a counterclockwise (CCW) and clockwise (CW) rotation of the polar flagellum respectively. We assume forward and backward, are formally indistinguishable and give rise to the same observation (the bacterium proceeding in a given direction, still being affected by rotational diffusion). The findings reported in Xie et al.\textsuperscript{13} (i.e., correlation between forward and backward swimming times) indicate this approach finds ground in experimental observations. Here we also assume that reorientations in between runs are instantaneous, a cell either reverses by simply inverting the swimming direction (corresponding to a 180° rotation) or flicks by turning 90°\textsuperscript{13,18}. Based on these considerations we modeled \textit{V. alginolyticus}' forward and backward swimming as \textit{Escherichia coli}'s runs and \textit{V. alginolyticus}' reorientations (reversals or flicks) as \textit{E. coli}'s tumbles.

In particular, in agreement with the previous model\textsuperscript{24}, we set the probability that at a generic time $t$ a cell will experience a reorientation within a time interval $\Delta t$ as:

$$ P_t = \frac{\Delta t}{\tau} \quad (3.1) $$

where, the mean run time, can be expressed as:

$$ \tau = \tau_0 e^{\alpha \frac{dP_b}{dt}} \quad (3.2) $$

with:

$$ \frac{dP_b}{dt} = \frac{1}{T_m} \int_{-\infty}^{t} \frac{dP_b}{dt} e^{\frac{t' - t}{T_m}} dt' \quad (3.3) $$

and:

$$ \frac{dP_b}{dt} = \frac{K_D}{(K_D + C)^2} \frac{dC}{dt} \quad (3.4) $$

where $C$ is the chemoattractant concentration in the extracellular environment, $T_m$, is a time constant of the bacterial system, $\tau_0$ is the mean run time in the absence of concentration gradients, i.e., average unbiased run time, $\alpha$ is a constant of the system, $P_b$ is the fraction of a cellular protein surface receptor bound by the substrate, $\frac{dP_b}{dt}$ is a weighted rate of time change of $P_b$, and $K_D$ is the half-saturation constant of the surface receptor binding to serine.
**Parameter identification.** As most of the other parameters are unknown for our combination of model system and the chemotattractant (serine), we will discuss parameter identification in the following (a-c).

**a. Reorientation frequency.** Not only do we have a direct quantification of reorientation frequency, $f$, as a function of the speed, this also gives us a simple yet effective way to implement the speed-dependent reorientation frequency. As a matter of fact by fitting a phenomenological model of the form:

$$f(v) = \left( \frac{\eta}{1 + e^{\zeta(v-v_t)}} + \theta \right)^{-1}$$  (3.5)

with $\eta = -0.3942$ s/μm, $\zeta = -0.2019$ s/μm, $v_t = 18.88$ μm/s and $\theta = 0.8452$ s/μm to capture the speed-dependent expression of $f$.

**b. Flicks.** In order to implement flagellar flicks, we assume we can neglect the short time interval between the reversal and the flick (~10 ms)\textsuperscript{18} and approximate the new swimming angle as a ±90° (the two directions are chosen with equal probability) deviation from the direction preceding the flicking event. Consistent with the biophysical nature of the phenomenon\textsuperscript{18}, we allow flicks to happen only after a backward-to-forward run transition. In order to determine whether to flick or not, the realization of a uniformly distributed random variable is compared with the probability of flicking $P_F$ which is approximated by a logistic function as in Son et al.\textsuperscript{18}:

$$P_F = 0.055 + 0.72/[1 + e^{-0.25(v-36)}].$$

**c. Chemokinesis.** As for what concerns chemokinesis, the chemoattractant-based speed modulation, we estimated an instantaneous speed enhancement of 30% as a result of increased chemoattractant availability, also following the previous mathematical model\textsuperscript{24}. Each agent has, at each time point, a speed that depends on the local concentration of chemoattractant and determined by the rule below:

$$v(x, y, t) = \begin{cases} 
  v_i \cdot 1.3 & \text{if } c(x, y) \geq c_T \\
  v_i & \text{if } c(x, y) < c_T 
\end{cases}$$

where $c_T$ (50 nM) is a concentration threshold, $v(x, y, t)$ is the speed of a generic cell sensing the chemoattractant concentration $c(x, y)$ at time $t$, and $v_i$ is the speed each agent is initialized to at the beginning of the simulation. This is meant to reflect an experimental observation we made both in presence and absence of a chemoeffect gradient. Cells in the simulation all experienced chemokinetic speed enhancement according to the criteria above.

With all the characteristics of _V. alginolyticus_ swimming and reorientation pattern implemented, we can focus on the parameters of the sensing model\textsuperscript{8,24}. Here our ability to determine $\alpha, T_m$ and $K_D$ is mainly limited by the lack of detail in the description of molecular events governing chemotaxis in _V. alginolyticus_. In order to overcome this issue we adopted an approach based on parameter space exploration and subjected the unknown parameters to an
empirical optimization routine. The choice of parameter value $K_D$ is close to the previous model\textsuperscript{24}, and the system time constant $T$ follows the experimentally observed run time ($= 1/f$), which is typically in the order of $\sim 0.1$ s. This approach allowed us to identify a set of values [$\alpha (= 30\,\text{s})$, $T_m (= 0.1\,\text{s})$ and $K_D (= 10\,\text{µM})$] that maximize the agreement between experimental quantifications and model predictions.

**Rotational diffusion.** Cells were constantly affected by rotational diffusion, responsible for a random reorientation component in the swimming trajectories. The value of the rotational diffusion coefficient during runs, $D_R = 0.035\,\text{rad}^2\,\text{s}^{-1}$, was based on a resistive force model that accounted for both the cell body (3.2 µm long and 1.2 µm wide) and the helical flagellum (contour length 4.6 µm, pitch 1.5 µm) of the bacterium\textsuperscript{18,24}.

**Simulation details.** With the chosen parameters, the model numerically simulated individual bacteria swimming in a linear steady chemoattractant gradient as in the microfluidic setup (Fig. 3.1). For each case, 3,000 cells were initially distributed uniformly in the 600 µm wide channel with random initial orientation. To maintain equal number of trajectories at each speed bin, the initial swimming speed of each bacterium was chosen randomly from the range of speeds observed in the absence of a gradient. Cells in the simulations did not interact with each other and they reflected randomly upon hitting the hard boundaries –meant to mimic agarose/PDMS walls in our device. Cells were constantly affected by rotational diffusion, responsible for a random reorientation component in the swimming trajectories, and they were allowed enough time (physical time of 7,000 s) to explore the gradient and to reach a steady spatial distribution. Then the analysis of the computational data followed the same steps as the analysis of the experimental data: we binned the bacterial distributions along the gradient by swimming speed, and for each distribution $B(x)$, we computed the CMC.
3.3 RESULTS

3.3.1 Dependence of chemotaxis on swimming speed

Steady linear concentration profiles (‘linear gradients’) of the amino acid serine, a chemoattractant for *V. alginolyticus*¹³, were generated in a purposely engineered microfluidic device (Figs. 3.1 and 3.2; see section 3.2.2). Serine concentrations were in the range of hundreds of nanomolar (100–400 nM; Fig. 3.1), corresponding to conditions representative of dissolved free amino acids in the ocean²⁷. We tracked >55,000 individual cells via video microscopy and determined the chemotactic response of a population by quantifying the steady-state chemotactic distribution of cells along the steady serine gradient. We computed the average swimming speed *V* of each cell by averaging its instantaneous speed over the duration of its trajectory. This allowed us to bin cells based on their swimming speed⁸ and thus to separately consider the chemotactic distribution of cells with different speeds (Fig. 3.3). In particular, we binned cells in 12 speed bins, ranging from 8.4 μm/s to 54.1 μm: in the following, we refer to cells belonging to different speed bins as sub-populations. To increase the dynamic range of swimming speeds, we carried out experiments over a range of sodium concentrations in the solution, [Na⁺] = 3–600 mM, exploiting the fact that the motor of *V. alginolyticus* is driven by trans-membrane sodium gradients⁸,²², so that the cells’ swimming speed (averaged over the population) increases with the sodium concentration, according to *V* = *V*₀ [Na⁺] / (14.9 + [Na⁺]) (refs. 18, ocean conditions typically correspond to [Na⁺] > 500 mM¹⁸).

Observations revealed a stark difference of the steady-state accumulation in the higher chemoattractant concentration region between cells swimming at different speeds. Faster cells accumulated more tightly than slower cells, as demonstrated for example by a collection of trajectories of two subpopulations corresponding to swimming speeds of *V* = 8.4±1.2 μm/s and *V* = 38.9±1.3 μm/s (Fig. 3.3a). To quantitatively determine the dependence of the strength of accumulation on the swimming speed, we computed the bacterial distribution along the chemoattractant gradient, *B*(x), separately for each speed bin (Fig. 3.3b). The 12 distributions clearly demonstrate that cells with higher speeds are more tightly accumulated in the high-serine region.

The speed-dependence of chemotaxis in *V. alginolyticus* is quantified by two metrics of the accumulation strength. First, each profile *B*(x) was fitted by an exponential, *B*(x) = *B*₀ exp(-x/L), where *x* is the direction along the chemoattractant gradient, *L* is the exponential decay length and the constant *B*₀ was chosen so that all profiles have the same area under the curve (Fig. 3.3b and inset). The choice of an exponential form is justified by the fact that such a distribution is the prototypical steady-state solution of classic formulations of the equation for bacterial transport (e.g., Keller-Segel’s formulation), under suitable simplifying assumptions²⁸. We found that an exponential distribution is a very good fit for all 12 profiles (*R*² > 0.95 for all cases; Fig. 3.3b,
inner inset). Thus, the exponential decay length scale, $L$, provides a reliable metric for the strength of the chemotactic accumulation: the smaller $L$, the tighter the accumulation. For *V. alginolyticus*, $L$ decreased from 186.6 µm at $V = 8.4 \pm 1.2$ µm/s to 55.9 µm at $V = 24.4 \pm 1.0$ µm/s, indicating a tighter accumulation with increasing speed in this range, and remained nearly constant for higher swimming speeds (Fig. 3.3c).

**Figure 3.3** | The steady-state chemotactic accumulation increases with swimming speed in *Vibrio alginolyticus*. (a) Single-cell trajectories at steady-state accumulation for two population fractions, one swimming at low speed ($V = 8.4 \pm 1.2$ µm/s; teal) and one swimming at high speed ($V = 38.9 \pm 1.3$ µm/s; magenta) in a linear concentration profile of serine (400 to 100 nM from left to right; see Fig. 3.1) within a microfluidic channel (error are standard deviations). Note the considerably tighter accumulation of the faster cells in the region of high serine concentration. (b) Steady-state bacterial concentration profiles, $B(x)$, for cells swimming at different speeds (see inset for speed color-coding) in the same serine gradient as in a. Data were collected over a range of sodium concentrations (3 – 600 mM) and binned by swimming speed. Each of the 12 bins is
2.5~10.0 μm/s apart and all contains the same number of trajectories (4,643). Each bacterial distribution was normalized to a mean of 1. (b, inset) Bacterial distributions are well fitted by an exponential function, as illustrated for the two speed bins shown in a. (b, inset’s inset) The fit is good for all speed bins, as demonstrated by R-squared values consistently close to 1. Horizontal error bars denote standard deviations of each speed bin. Where not visible, horizontal error bars are smaller than symbols. (c) Accumulation length scale, $L$, from the exponential fits to the bacterial distributions shown in b. (d) The chemotactic migration coefficient (CMC), a measure of the tightness of the steady-state accumulation, for the bacterial distributions in b. Gray symbols represent the same data, where speed-binning was performed separately for each sodium concentration, $[\text{Na}^+]$. The CMC values for *Escherichia coli* swimming in a gradient of α-methylaspartate (80 – 20 μM from left to right in the same device) are shown for comparison, parsed out into 5 speed bins (black open triangles).

![Figure 3.4](image)

**Figure 3.4** | Observed evolution of the CMC over time at the transient (<350 s) and steady (>500 s) states for cells swimming at different swimming speeds in the same hydrogel-based microfluidic device (Fig. 3.1).

As a second metric of the speed-dependence of chemotaxis, we computed the chemotactic migration coefficient$^{29}$, $\text{CMC} = (\langle x \rangle - W/2) / (W/2)$, a widely used parameter in the chemotaxis literature$^{29}$ that measures the displacement of a subpopulation’s center of mass, $\langle x \rangle = \int x B(x) dx$, from the center of the channel, where $W = 600$ μm is the channel’s width. The CMC increased with swimming speed up to $V \sim 30$ μm/s and saturated for higher speeds (Fig. 3.3d), confirming the trend revealed by the accumulation length scale, $L$ (Fig. 3.3c). We then use the CMC as a metric to divide swimming speeds for further analysis into ones leading to high chemotactic performance ($V > 30$ μm/s) and ones leading to lower chemotactic performance ($V < 30$ μm/s). We could rule out the hypothesis that the change in accumulation strength with speed was due to physiological changes arising from different sodium concentrations in the medium, as the same
speed dependence of the CMC was recovered when data were binned by speed for each sodium concentration separately (Fig. 3.3d, gray symbols).

Whereas it is intuitive that chemotactic cells gain a benefit from swimming faster because they can climb resource gradients more rapidly (as shown in Fig. 3.4), the tighter steady-state accumulation of faster cells at the peak of a resource gradient is unexpected and represents an additional benefit of enhanced speed for chemotaxis in marine bacteria. This speed dependence is absent in *E. coli*, as we confirmed by repeating experiments with this bacterium exposed to a gradient of 20–80 μM α-methylaspartate (Fig. 3.3d, black symbols), a commonly used chemoattractant for *E. coli*, in a range of concentrations known to elicit strong chemotaxis. Although *E. coli* displayed a smaller dynamic range of swimming speeds (and does not allow for simple speed modulation since its motor is proton-driven), their CMC value was constant across all speeds (CMC = 0.42±0.02). That *E. coli*’s maximum CMC was on the lower end of *V. alginolyticus*’ CMC, while serine concentrations eliciting the marine bacterium’s response were in the nanomolar range, is in line with recent evidence that the chemotactic performance of marine bacteria is typically greater than that of *E. coli*.11,13

3.3.2 Speed-dependent reorientation frequency as the origin of speed-dependent chemotaxis

The motility characteristics leading to the observed speed-dependence of chemotaxis were analyzed by computing both population-level statistics (random diffusivity and chemotactic velocity) and single-cell-level swimming statistics (reorientation frequency and probability of flicking) [Figs. 3.5 and 3.6].

3.3.2.1 Population-level swimming statistics.

The ability of a population of microorganisms to accumulate at the peak of a resource gradient is in general determined by the competition between two features of their motility pattern: the chemotactic velocity, \( V_c \), measuring the net speed at which cells on average move up the gradient, and the translational diffusivity, \( D \), determined by the intrinsic randomness of the swimming pattern and limiting the level of accumulation a population can achieve. A simple continuum advection-diffusion model of bacterial transport predicts that in a linear chemoattractant gradient the bacterial distribution at steady-state is exponential, \( B(x) = B_0 \exp(-xV_c/D) \), with a characteristic length scale, \( L = D/V_c \). To interpret the observed speed-dependent chemotactic accumulation (Fig. 3.3), we thus quantified \( D \) and \( V_c \) from our dataset (Fig. 3.5).

We computed \( D \) for each subpopulation based on a theoretical formulation recently derived for the run-reverse-flick motility pattern, \( D = \frac{(v^2/6)(f+4D_R)(f+2D_R)}{(f+4D_R)(f+2D_R)^2} \). The reorientation frequency \( f \) was obtained from individual bacterial trajectories (Fig. 3.6a; see section 3.3.2.2).
The rotational diffusivity $D_R$, measuring the gradual changes in orientation during runs due to Brownian rotation and off-axis propulsion, was computed from a resistive force model, accounting for the cell head and the helical flagellum (see section 3.2.5). We found that at large swimming speeds ($V > 30 \mu$m/s; high-chemotactic-performance speeds) $D$ increases quadratically with speed, $D \sim V^2$, as predicted by recent mathematical models of run-reverse-flick motility. In contrast, below this speed threshold our data yield a linear increase of $D$ with speed, $D \sim V$ (Fig. 3.5a, inset; Table 3.1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Units</th>
<th>Scaling at low speed ($V&lt;30\mu$m/s)</th>
<th>Scaling at high speed ($V&gt;30\mu$m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accumulation length</td>
<td>$L$</td>
<td>pm</td>
<td>$-V^{-1}$</td>
<td>$-\text{constant}$</td>
</tr>
<tr>
<td>Chemotactic velocity</td>
<td>$V_C$</td>
<td>$\mu$m/s</td>
<td>$-V^2$</td>
<td>$-V^2$</td>
</tr>
<tr>
<td>Diffusivity</td>
<td>$D$</td>
<td>$\mu$m$^2$/s</td>
<td>$-V$</td>
<td>$-V^2$</td>
</tr>
<tr>
<td>Reorientation frequency</td>
<td>$f$</td>
<td>1/s</td>
<td>$-V$</td>
<td>$-\text{constant}$</td>
</tr>
</tbody>
</table>

Table 3.1 | Swimming statistics and their dependence on swimming speed, $V$, in the two speed regimes.
Figure 3.5 | The random and directional components of swimming in *V. alginolyticus*. (a) The random diffusivity, $D$, and (b) the chemotactic drift velocity, $V_c$, of *V. alginolyticus* as a function of swimming speed, $V$. Data were collected over a range of sodium concentrations (3 – 600 mM) and binned by swimming speed (see Fig. 3.3). The random diffusivity was computed from the analytical formulation derived recently by measuring reorientation frequencies and speeds directly from trajectories. The chemotactic drift velocity was computed as $V_c = D/L$ by assuming that the measured accumulation length scale (Fig. 3.3c) is well described by $L = D/V_c^{28}$. Insets show the same data in log-log plots with speed-scaling lines for guidance (see also Table 3.1).

The run-reverse-flick motility pattern enables *V. alginolyticus* to significantly contain the random component of its motility, as revealed by a comparison of its diffusivity with that of *E. coli*. Although *V. alginolyticus* under natural sodium conditions swam 2.4 times faster than *E. coli* ($V_0 = 47.5$ μm/s vs. $V_{E.coli} = 19.7$ μm/s), in agreement with recent reports, we found that the random diffusivity of *V. alginolyticus* ($D_{V.algi} = 160.8$ μm$^2$/s) was 17% smaller than that of *E. coli* ($D_{E.coli} = 193.1$ μm$^2$/s). The difference is dramatic when the comparison is carried out for the same swimming speed, to single out the role of the different motility patterns of the two species, as the *V. alginolyticus* subpopulation swimming at $V = 19.2$ μm/s (comparable to the average speed of *E. coli*; Fig. 3.5a) had a diffusivity ($D_{V.algi} = 44.5$ μm$^2$/s) 72% smaller than *E. coli*’s. This substantial reduction in the value of $D$ reflects the backtracking in the trajectories of *V. alginolyticus*, caused by the ~180° reversals that occur after every forward run and after the backward runs not followed by a flick. This difference is key to understanding the much stronger chemotactic accumulation achieved by *V. alginolyticus* compared to *E. coli*, both in natural conditions (CMC$_{V.algi}$ = 0.79 and CMC$_{E.coli}$ = 0.42, respectively, for $V_0 = 47.5$ μm/s and $V_{E.coli} = 19.7$ μm/s; Fig. 3.3d) as well as when the comparison is carried out at the same swimming speed (CMC$_{V.algi}$ = 0.66 at $V = 19.2$ μm/s; Fig. 3.3d).

The chemotactic velocity, $V_c$, was computed as $V_c = D/L$ using the value of $L$ determined from the exponential fit to the population accumulation profile (Fig. 3.3c). This revealed a quadratic dependence of $V_c$ on the swimming speed, $V_c \sim V^2$, over the entire range of swimming speeds (Fig. 3.5b, inset). This result is in line with predictions from recent models and to the best of our knowledge represents the first experimental quantification of the dependence of the chemotactic velocity on the swimming speed. The measured chemotactic velocities ranged from 0.2 to 3.5 μm/s (Fig. 3.5b): this corresponds to a relative chemotactic velocity $V_c/V$ of <10% over the entire range of swimming speeds. The ratio $V_c/V$ is a measure of the directionality of the response: less than 10% of the swimming speed of *V. alginolyticus* is on average directed up the gradient at steady state. This level of directionality is on par with that of *E. coli*, which often is in the range of 5–10%. Although the relative chemotactic velocity can be higher for particular gradients and types of chemoattractants (values up to 35% have been measured), our
observations in linear chemoattractant profiles indicate that *V. alginolyticus*’ higher performance in accumulating at resource peaks is not due to a greater directionality in their gradient response but rather to a lower random diffusivity (Fig. 3.5a) associated with the run-reverse-flick motility. In summary, the different functional dependence of $D$ and $V_C$ on the swimming speed $V$ (insets of Fig. 3.5a,b; Table 3.1) is at the origin of the speed-dependence of the steady-state chemotactic accumulation of *V. alginolyticus* (Fig. 3.3). For high-chemotactic-performance speeds ($V > 30 \mu m/s$), both $D$ and $V_C$ scale quadratically with $V$ and thus the tightness of the chemotactic accumulation, measured by $L = D/V_C$ (Fig. 3.3c), is speed-independent. In contrast, for low-chemotactic-performance speeds ($V < 30 \mu m/s$), $V_C$ again scales quadratically but $D$ scales linearly with $V$, hence $L = D/V_C \sim 1/V$ decreases with increasing speed (Fig. 3.3c), resulting in stronger chemotactic accumulations with increasing swimming speed.

![Figure 3.6](image)

**Figure 3.6** | *V. alginolyticus* displays two different swimming modes below and above a speed threshold of $V = 30 \mu m/s$. (a) The CMC (black circles), the probability of flicking, $P_F$ (blue upright triangles), the reorientation frequency, $f$ (red squares), and the flicking frequency, $f_F$...
(brown inverted triangle), as a function of swimming speed in a linear concentration profile of serine (400 – 100 nM). The green dashed line marks the observed speed threshold, \( V = 30 \mu \text{m/s} \).

(b,c) The reorientation frequency, \( f \), as a function of the swimming speed in the (b) presence and (c) absence of a serine gradient. Red symbols include data at all sodium concentrations, grey symbols refer to data for individual sodium concentrations (see legend).

### 3.3.2.2 Single-cell level swimming statistics.

To understand the origin of the changeover between a quadratic and a linear dependence of diffusivity on swimming speed, we used single-cell trajectories to quantify the dependence on the swimming speed of the reorientation frequency, \( f \), which includes both reversals and flicks. We found that \( f \) increasing linearly with \( V \) for \( V < 30 \mu \text{m/s} \) and is independent of \( V \) for \( V > 30 \mu \text{m/s} \) (Fig. 3.6a). The speed-dependence of the reorientation frequency is thus responsible for the linear scaling of diffusivity with speed, \( D \sim V \), for \( V < 30 \mu \text{m/s} \) (Fig. 3.5a, inset).

The observed speed-dependence of the reorientation frequency led us to analyze more specifically the frequency of flicks, and we found them to also be markedly affected by swimming speed. It has been previously established that flicks occur prevalently above a threshold swimming speed of 20–50\(^{18}\), but only the probability of flicking \( P_F \) (the probability that the onset of a forward run is followed by a flick) and not the frequency of flicks has been quantified. Following the approach used previously\(^{18}\), we detected flicks from single-cell trajectories (see section 3.2.3) and computed both the probability of flicking, \( P_F \), and the flicking frequency, \( f_f \) (number of flicks per unit time), which is half of the product of the reorientation frequency and the probability of flicking, \( f_f = f/2 \times P_F \), because flicks can only occur after the onset of a forward run\(^{18}\). The change in the flicking frequency with swimming speed was dramatic, with a ~8-fold increase from \( V = 13.3 \mu \text{m/s} \) (\( f_f = 0.1 \) flicks/s) to \( V = 47.5 \mu \text{m/s} \) (\( f_f = 0.8 \) flicks/s). The increase in the flicking frequency with speed was due in part to the change in the overall reorientation frequency, \( f \), which increased ~2-fold (from 1.1/s to 2.3/s) over the same range of speeds, and mostly to the change in the probability of flicking, \( P_F \), which increased ~4-fold (from 18% to 68%) over that speed range (Fig. 3.6a). The functional dependence of both \( P_F \) and \( f_f \) on swimming speed, \( V \), follows the same dependence of the CMC and of the reorientation frequency, \( f \) (Fig. 3.6a), further highlighting the connection between the rate and nature of reorientations and the chemotactic accumulation.

Our data suggest that the dependence of reorientation frequency, probability of flicking and consequently flicking frequency on the swimming speed is independent of gradient sensing. For the probability of flicking, \( P_F \), this hypothesis is supported by comparison with previous experiments in the absence of chemical gradients\(^{18}\), which also showed a similar increase of \( P_F \) in the critical speed as observed here in a serine gradient. For the reorientation frequency, \( f \), the
hypothesis is supported by the comparison of observations in the presence (Fig. 3.6c) and absence (Fig. 3.6b) of a serine gradient: even in homogeneous conditions, \( f \) depends strongly on \( V \) (Fig. 3.6c) and, small differences notwithstanding, this dependence is similar with and without a gradient. Binning the data by sodium concentration allowed us to exclude the potential for this observation to originate from physiological effects of different sodium concentrations\(^\text{22}\) (Fig. 3.6b,c, grey symbols). This observation supports the conclusion that swimming speed itself is an important determinant of the reorientation frequency and flicking probability, irrespective of the presence or absence of a chemoattractant gradient. Because the modulation of the reorientation frequency is the basis of bacterial chemotaxis\(^\text{2,7,8}\), this finding suggests that swimming speed plays a key role in the chemotaxis of \( V. \) alginolyticus and possibly of other marine bacteria, complementing and extending the current paradigm based on chemical sensing alone.

![Figure 3.7](image)

**Figure 3.7 | Chemokinesis shifts the population's swimming speed towards the high-chemotactic-performance regime.** (a) The steady-state probability density of the swimming speed, \( p(V) \), in the absence (cyan) and presence (blue) of 500 nM serine. The green dashed line marks the speed threshold \( V = 30 \mu m/s \). Experiments were performed at a sodium concentration of \([Na^+] = 600 \text{ mM}\), typical of natural ocean conditions. Each probability density curves contains

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information from at least 2,380 trajectories. (a, inset) Instantaneous population-averaged relative nutrient exposure, expressed as a percentage of the 500 nM serine added condition (see section 3.3.3). (b) Temporal evolution of the probability density of the swimming speed, $p(V)$, at different times after the uniform addition of 5 μM glucose both in the source and sink channels (see Fig. 3.1). Different shades of magenta correspond to different times (see inset for time color-coding). The green dashed line marks the speed threshold $V = 30 \mu$m/s. Experiments were also performed at a sodium concentration of $[Na^+] = 600$ mM. Each temporal bins contain information from at least 2,340 trajectories. (b, inset) Population-averaged change in speed following the uniform addition of 5 μM glucose, expressed as a percentage of the initial speed in the first time point.

![Graph showing chemokinetic mean speed enhancement at steady-state with respect to the pre-addition (natural) condition compared over various serine and sodium conditions. Mean speed enhancement at natural sodium condition (600 mM) toward serine, glucose and their non-metabolizable analogs at 5,000 nM are shown in circular symbols with different colors.]

Figure 3.8 | Chemokinetic mean speed enhancement at steady-state with respect to the pre-addition (natural) condition compared over various serine and sodium conditions (red symbols). Mean speed enhancement at natural sodium condition (600 mM) toward serine, glucose and their non-metabolizable analogs at 5,000 nM are shown in circular symbols with different colors.

3.3.3 Chemokinetic speed enhancement

The motility repertoire of *V. alginolyticus* includes a further, important adaptation: chemokinesis. Chemokinesis is the modulation (typically, the increase) of swimming speed based on the local
chemical concentration. Chemokinesis is intertwined with chemotaxis because in general it can accelerate gradient climbing\textsuperscript{24}, and specifically for \textit{V. alginolyticus} it can affect all of the speed-dependent swimming kinematics by modulating swimming speed. The quantification of the distribution of swimming speeds within a population of \textit{V. alginolyticus} from single-cell trajectories revealed an overall shift towards higher speeds upon the spatially uniform addition of serine (Figs. 3.7a and 3.8), with a 33\% increase in the population-averaged speed upon addition of 500 nM serine (Fig. 3.7a) and a 24\% increase upon addition of 50 nM serine (Fig. 3.8). These speed increases are comparable to those reported in other marine bacteria\textsuperscript{12,23,24} and, furthermore, occurred over a range of sodium concentrations (Fig. 3.8). The temporal dynamics of the distribution of speeds after the addition of 5 \textmu M glucose (Fig. 3.7b) or 5 \textmu M serine (Fig. 3.9) showed that the timescale for chemokinetic speed increase is in the order of 226 s and 127 s, respectively.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure39.png}
\caption{Temporal evolution of the probability density of the swimming speed, $p(V)$, at different times after the addition of 5 \textmu M serine both in the source and sink channels (Fig. 3.1). Different shades of blue correspond to different times (see inset for time color-coding). The green dashed line marks the speed threshold $V = 30$ \textmu m/s. Experiments were also performed at a sodium concentration of $[Na^+] = 600$ mM. Each temporal bin contain information from at least 570 trajectories. (inset) Population-averaged change in speed following the uniform addition of 5 \textmu M serine, expressed as a percentage of the initial time point.}
\end{figure}

The observed chemokinetic speed increases are sufficient to push a sizeable fraction of the population from the low ($V < 30$ \textmu m/s) to the high-chemotactic performance speed regime ($V > 30$ \textmu m/s) (Fig. 3.7, green dashed lines). The addition of 500 nM serine increased the fraction of
cells in the population swimming at \( V > 30 \, \mu m/s \) from 39% to 70%. Chemokinesis is thus implicated in the strong chemotactic response of *V. alginolyticus*, since faster swimming results in tighter chemotactic accumulation (Fig. 3.3b-d). A model of resource exposure (see section 3.2.4), defined here as the mean serine concentration experienced by the population and a determinant of uptake, indicates that by enhancing the chemotactic accumulation, chemokinesis contributes a 12.5% increase in the resource exposure of the population (Fig. 3.7a, inset). The total nutrient exposure increased over time with the gradual speed enhancement for cells exposed to 5 \( \mu M \) glucose (Fig. 3.7b, inset). Therefore, not only does chemokinesis boost chemotaxis because of the quadratic dependence of the chemotactic velocity \( V_c \) on the swimming speed \( V \) (Fig. 3.5b), as previously found for example in *V. coralliilyticus*\(^{24}\), but chemokinesis in *V. alginolyticus* also lifts a considerable fraction of cells from the low-chemotactic-performance to the high-chemotactic-performance regime (Fig. 3.3c,d), ultimately owing to the dependence of the reorientation kinematics on the swimming speed (Fig. 3.6).

### 3.3.4 Model of speed-dependent chemotaxis

In order to understand how the different elements of *V. alginolyticus*’s motility combine to shape its chemotactic response, we developed an agent-based model that incorporates all the experimental observables (Figs. 3.6 and 3.7) as inputs (see section 3.2.5). In the absence of species-specific molecular information on gradient-sensing in *V. alginolyticus*, we used a general model of chemotaxis\(^8\) as our starting point, in which each bacterium swims in a 2-dimensional landscape containing a spatially heterogeneous chemoattractant concentration. In classic models of chemotaxis, mainly developed for *E. coli*, the ligand concentration \( L_c \) measured over time is the only control parameter of the chemotaxis pathway, and is processed by a ‘reorientation frequency block’ (R) that yields the reorientation (or ‘tumbling’) frequency, \( f \), as the pathway’s output (Fig. 3.10a: \( L_c \rightarrow R \rightarrow f \)).

The experimental data we collected on *V. alginolyticus*, instead, led us to augment this model by including the swimming speed, \( V \), as both an input and an output parameter in the chemotaxis model (Fig. 3.10b; see section 3.2.5). The model accounts for (i) the chemokinetic behavior, by including a modulation of the swimming speed based on the resource concentration (‘chemokinesis block’, \( C \), in Fig. 3.10b: \( L_c \rightarrow C \rightarrow V \)); (ii) the variable reorientation frequency, which depends not only on the sensed ligand concentration (Fig. 3.10b: \( L_c \rightarrow R \rightarrow f \)), as usual, but also on the swimming speed (‘reorientation frequency block’, \( R \), in Fig. 3.10b: \( V \rightarrow R \rightarrow f \)); and (iii) the linear dependence on swimming speed of the propulsive load, which determines the buckling of the flagellar hook\(^8\) and thus the second output of the system, the probability of flicking (‘flick block’, \( F \), in Fig. 3.10b: \( V \rightarrow F \rightarrow F_T \)) . The model thus implements the run-reverse-flick motility pattern, when one also imposes that all reorientations that are not flicks are reversals, as observed experimentally\(^{13,18}\).
Figure 3.10 | Model for speed-dependent chemotaxis. (a,b) System view of chemotaxis shown as block diagrams for (a) *E. coli* and (b) *V. alginolyticus*. Unlike *E. coli*, where the reorientation frequency \( f \) enabling gradient-climbing is determined solely by sensing of the ligand concentration \( L_C \) (block R; red), our observations suggest the presence of additional feedback in *V. alginolyticus*, including the ligand-dependent modulation of the swimming speed \( V \) (i.e., chemokinesis; block C; blue), the speed-dependent load on the flagellar hook (block F; green) that governs the probability of flicking \( P_F \), and the speed-dependent modulation of the reorientation frequency (integrated in block R). Each of these additional elements involves the swimming speed \( V \). (c) Model prediction of the steady-state chemotactic migration coefficient (CMC) for the bacterial distributions in the inset (see speed color-coding). (c, inset) Normalized steady-state bacterial distribution, \( B(x) \), from the model for cells swimming at 10 different speeds (300 trajectories each) averaged over the last 35 s in the 7,000 s long simulation (see section 3.2.5; see also Fig. 3.11). Horizontal error bars masked by the symbols are standard deviations. All 10 distributions are well fitted by exponentials, as in the experiment (see Fig. 3.3b). (c, inset’s inset) The fit is good for all speed bins, as demonstrated by R-squared values consistently close to 1.
The proposed model successfully captured the observed speed-dependent chemotaxis of *V. alginolyticus* (Figs. 3.10c and 3.11). We ran the model for 3000 bacteria swimming in a linear chemoattractant field, directly mimicking the microfluidic setup used in experiments (Fig. 3.1; see section 3.2.5). The unknown chemotaxis parameters of *V. alginolyticus* were chosen so as to produce CMC values of comparable magnitude to experimental observations (Figs. 3.3d and 3.10c) (see section 3.2.5). The modeling predictions capture the fundamental result from the experimental observations: the chemotactic accumulation was pronounced (high CMC) for swimming speeds above $V = 30 \, \mu$m/s, and degraded rapidly with decreasing speed below this threshold (Figs. 3.10c and 3.11). Furthermore, the predicted distributions $B(x)$ were accurately fitted by exponentials, as in the experiments (Fig. 3.10c, insets).

Figure 3.11 | Time evolution of the CMC values for cells swimming at 10 different speeds (300 cells at each speeds) from the mathematical model (see also Fig. 3.10c). Note that cells from the slowest speed bin reached a steady-state at >6,000 s.

Using the model, we quantified the contributions of the different components in the proposed chemotaxis pathway of *V. alginolyticus* (Fig. 3.10b), by selectively turning off individual functions (chemokinesis, flicking or reorientation frequency modulation, *i.e.*, blocks C, F or R in Fig. 3.10b, respectively). Removing the modulation of the reorientation frequency ($\Delta R$) resulted in a nearly constant value of CMC over the entire range of speeds (Fig. 3.12a, red symbols). Removing the ‘chemokinesis’ block ($\Delta C$) constrained the CMC values toward lower speeds (Fig. 3.12a, blue), which resulted in 5.9% decrease in the population-level nutrient exposure. Removing the ‘flick’ block ($\Delta F$) impacted the chemotactic performance, differently for different swimming speeds. If the ‘flick’ block (F) was turned off and all reorientations were reversals, the CMC values decreased mostly in the high-chemotactic-performance regime ($V > 30 \, \mu$m/s) where
flicking was originally dominant (Fig. 3.6a). This led to 7.8% decrease in the population-level nutrient exposure (Fig. 3.12a, green). Lastly, lack of multiple functions (e.g., both C and F blocks) led to an even higher, 9.7% decrease in nutrient exposure (Fig. 3.12a, magenta), which implies that both functions are important for the chemotactic performance of *V. alginolyticus*.

**Figure 3.12 | Individual functions in the chemotactic pathway are essential component of enhanced chemotaxis in *V. alginolyticus*.** (a) Model prediction of the chemotactic migration coefficient (CMC) for *in silico* mutant lacking ability to modulate reorientation frequency (block R; red), chemokinesis (block C; blue), flick (block F; green) and both chemokinesis and flick (blocks C and F; magenta), plotted together with cells with full functionality (black; same data as in Fig. 3.10c). For direct comparison, all the CMC values are calculated from the simulations of cells swimming at 10 different speeds (300 trajectories each) averaged over the last 35 s in the 7,000 s long simulation. The CMC value at the lowest speed bin has negligible impact on the population-averaged statistics when weighted by the experimental speed distributions (Fig. 3.7a). Red triangular symbol at the lowest speed bin connected by a dashed line is a modeling result of 200 cells in the 30,000 s long simulation, which demonstrates that cells in the lowest speed bin approaching the CMC value comparable those at higher speeds. (a, inset) Instantaneous population-averaged relative nutrient exposure, expressed as a percentage with respect to cells
with full functionality (black). (b) Model prediction of the accumulation time, $T_A$, for the same *in silico* mutants. $T_A$ is defined as time elapsed to reach 50% of the CMC values of cells with full functionality (a, black). (b, inset) Instantaneous population-averaged relative time delay with respect to cells without any loss in their chemotaxis function (black).

Chemokinesis and flicking are key motility elements also during the transient phase of chemotaxis (Fig. 3.12b), which is relevant for the bacteria’s ability to rapidly exploit ephemeral resource gradients, prevalent in the ocean. The transient chemotactic performance was quantified in terms of an accumulation time, $T_A$, the time taken by a subpopulation to reach 50% of its steady-state CMC value computed with the full model (Fig. 3.12a, black circles). As expected, $T_A$ is speed-dependent (Fig. 3.12b). Lack of chemokinesis ($\Delta C$), flicking ($\Delta F$) or both ($\Delta CF$) considerably slowed down the transient chemotactic response, resulting in a 4.3-fold, 2.7-fold and 5.1-fold increase, respectively, in the population-averaged accumulation time, $<T_A>$ (see section 3.2.4), over the full model ($<T_A>$ = 96 s). Chemokinesis favors transient chemotaxis by accelerating the gradient climb and flicking ensures more effective space exploration and gradient sampling over reversals alone. In contrast, suppressing the modulation of the reorientation frequency ($\Delta R$) had negligible impact on the transient response.

### 3.4 DISCUSSION

Our results suggest that, contrary to what happens in the enteric model bacterium *E. coli*, swimming speed and its modulation are important determinants of the chemotactic response in the marine bacterium *V. alginolyticus*. We identified two chemotactic regimes based on swimming speed. At low speeds ($V < 30 \, \mu m/s$), the bacteria’s diffusivity $D$ scaled linearly with $V$ and their chemotactic velocity $V_C$ scaled quadratically with $V$, so that the accumulation length $L = D/V_C \sim 1/V$ decreased with increasing speed (Fig. 3.3c; Table 3.1). In this low-chemotactic-performance regime, cells displayed constant run lengths due to the increase in the reorientation frequency with speed (Fig. 3.6) and their ability to accumulate tightly around a resource peak increased with speed, as measured by the increase in CMC (Fig. 3.3d). In contrast, at high speeds ($V > 30 \, \mu m/s$), both $D$ and $V_C$ scaled quadratically with $V$, so that $L = D/V_C$ remained constant with speed (Fig. 3.3c; Table 3.1). In this high-chemotactic-performance regime, run lengths increased with speed while the reorientation frequency was speed-independent (Fig. 3.6), and the cells’ ability to accumulate tightly around a resource peak saturated, as indicated by the constancy of the CMC (Fig. 3.3d).

Intriguingly, the distribution of swimming speeds in a *V. alginolyticus* population under natural sodium conditions and in the absence of chemoattractant gradients sits astride of the $V = 30 \, \mu m/s$ speed threshold separating the low- from the high-chemotactic-performance regimes (Fig. 3.7a,
cyan). This observation suggests that the speed distribution and its modulation in the presence of gradients may result from a trade-off between the uptake benefits from chemotaxis and the energetic cost of locomotion, a framework previously suggested for chemotaxis towards resource patches in turbulent flow\textsuperscript{25}. Because uptake will increase with residence time in resource-rich regions, by being able to better localize with the resource peak, fast cells will have a fitness benefit over slow cells. At the same time, the energetic cost of swimming increases quadratically with the swimming speed\textsuperscript{25}, making fast swimming very costly relative to the potential benefits of chemotaxis in the often resource-poor marine environment\textsuperscript{25}. In this context, the cells' active ability to increase speed in favorable resource conditions – \textit{i.e.}, chemokinesis (Fig. 3.7) – represents a desirable strategy to explore the environment with reduced motility cost in the absence of gradients, while activating the motility pattern that yields the strongest chemotactic performance when a favorable chemical environment is sensed. Our observation supports this speculation, for two reasons. First, the observed chemokinetic speed enhancement shifts a considerable fraction of the population from below to above the speed threshold (Fig. 3.7), resulting not only in a faster transient response to gradients (Figs. 3.7b and 3.9) but also in a tighter steady-state accumulation of cells at the resource peak (Fig. 3.2c,d). Second, the observed speed modulation is rapid, with increases of 50% in the average swimming speed of a population occurring over 2–4 min (Figs. 3.7b and 3.9). Although some nutrient hotspots in the ocean are briefer than this, many last in the order of 10 minutes or more\textsuperscript{11,25,26}, indicating that chemokinesis can be advantageous also at the single-resource-patch.

Our observations on the speed dependence of the swimming statistics (\textit{e.g.}, reorientation frequency) raise the question of whether and how bacteria sense their swimming speed. A likely candidate mechanism is mechanosensing, the ability to sense mechanical signals such as forces and torques. It has been recently shown that, in \textit{E. coli}, the mechanical load on the motor, sensed at the level of the stator, can contribute to regulate the tumbling rate\textsuperscript{6,38}. Mechanosensing has also been reported in \textit{Vibrios}\textsuperscript{39}, where inhibition of the rotation of the polar flagellum due to increased viscous resistance – for example near surfaces or under increased fluid viscosity – leads to the formation of myriad of lateral flagella for swarming motility through induction of the lateral flagellar gene (laf) expression. An alternative to sensing force is sensing swimming speed directly, since speed is linearly proportional to motor rotation rate\textsuperscript{40}. Irrespective of the origin of speed sensing, we propose that \textit{V. alginolyticus} integrates information on its speed in its chemotaxis pathway as an additional system input to regulate reorientation frequency (Fig. 3.10b). The success of our model in capturing the experimental observations (Fig. 3.10c) lends support to the hypothesis that speed is both a system input and output of the chemotaxis pathway (Fig. 3.10b), and highlights the need to better understand, at the molecular level, both gradient-sensing and speed-sensing in \textit{Vibrios}.

The results presented here reshape our understanding of bacterial motility in the ocean by demonstrating the role of swimming speed, its effect on reorientation frequency and its
modulation through chemokinesis, in determining the rate and strength of chemotaxis. The role of these motility adaptations on the ability of bacteria to exploit gradients has been poorly studied, largely owing to their absence in E. coli chemotaxis. The frequent occurrence of these chemotaxis elements among marine bacteria then suggests that speed-dependent chemotaxis may not be limited to V. alginolyticus, but might be prevalent among sea microbes. For example, chemokinesis has also been demonstrated in P. haloplanktis\textsuperscript{23} and V. corallilyticus\textsuperscript{24}, among others\textsuperscript{12}, and these same two species, as well as natural communities of marine bacteria, turn by flicking\textsuperscript{18}. Moreover, the motile marine bacterial species\textsuperscript{17}. This evidence suggests that this augmented form of chemotaxis, in which cellular decision-making is based on information not only regarding the chemical environment but also regarding the cell’s swimming speed, might be pervasive among marine bacteria. We surmise that the observed speed-dependence of gradient utilization among marine bacteria is related to the defining features of the marine resource landscape at the microscale, which is characterized by small, often ephemeral patches, pulses and gradients of chemicals\textsuperscript{37}, as well as ubiquitous fluid flow that both stirs chemical resources\textsuperscript{25} and influences bacterial motility\textsuperscript{41}. A quantitative link between the specific behavioral adaptations reported here and the features of the marine resource landscape remains to be established, and points more in general at the need for the development of an optimal foraging theory for bacteria. The observation that marine bacteria employ a form of chemotaxis that is speed-dependent demonstrates a new, potentially widespread element of bacterial chemotaxis, highlights the rich adaptations in the spatial behaviors of marine bacteria, and invites a better understanding of the ecosystem consequences of these refined behaviors.
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CHAPTER 4

Live imaging of host-virus encounter dynamics*

* In Chapter 4, I studied microbe-virus encounter dynamics driven by passive Brownian motion, focusing on a bottleneck in the infection process, i.e., the adsorption of the virus to the host. Together with my collaborators, I worked with a model system composed of the non-motile marine cyanobacterium Prochlorococcus and one of its cyanophages by visualizing both hosts and viruses, simultaneously, live. I found that this approach could successfully be used to quantify the encounter and adsorption rates from single-cell data, and this led to an adsorption rate that is significantly lower than that obtained with traditional bulk adsorption kinetics assays.

This chapter is in preparation for a journal submission as:


I have been responsible for all the microscale experiments and associated data analysis, constituting the vast majority of experiments in this chapter, with help from Andres Cubillos-Ruiz and Katya Frois-Moniz (Chisholm group) on Prochlorococcus husbandry and macroscale viral adsorption assays. I have led the effort of writing the paper, with input from all coauthors.
ABSTRACT

Viral infection is a significant source of bacterial mortality and impacts trophic and biogeochemical dynamics in natural ecosystems\(^1\). Viral adsorption, the adhesion of a virus to a host cell upon encounter\(^2,3\), is a fundamental step in the infection process. However, the probability of adsorption upon encounter or ‘adsorption efficiency’\(^2,3\), \(\alpha\), has proven difficult to quantify\(^4\) reliably, and current estimates often approach or even paradoxically exceed \(\alpha = 100\%\)\(^1,5-7\). Consequently, input parameters for viral adsorption even in the recent microbial ecosystem models typically span 2–3 orders of magnitude\(^8\). Here, we present a direct imaging approach to quantitatively study host-virus encounter dynamics and adsorption kinetics from thousands of individual host-virus interactions. Using a model system in marine microbial ecology, the abundant marine cyanobacterium Prochlorococcus\(^9,10\) and its cyanophage, we find that the adsorption efficiency noninvasively measured with this microscale imaging approach (\(\alpha = 2.2 \pm 0.7\%\)) is one order of magnitude lower than the value obtained with the macroscale\(^10,11\) classic adsorption kinetics assay (\(\alpha = 20.7 \pm 5.0\%\)). In light of the paradoxically high adsorption efficiencies often measured in both marine and enteric systems\(^1,5-7\), this result suggests that a revision of viral adsorption rates may be necessary in marine ecosystem models\(^1,8,13\) and possibly other host-virus systems.

4.1 INTRODUCTION

Viral infection is an important driver of microbial community dynamics, throughout the biosphere, with significant consequences for many biogeochemical processes that microorganisms mediate. In the ocean, viruses outnumber bacteria by tenfold, and with an estimated \(10^{23}\) viral infections per second\(^1\), are a significant cause of bacterial mortality\(^4\), in some cases even surpassing grazing\(^4\). Our understanding of viruses’ impact on the population dynamics and ecosystem consequences is limited primarily by two bottlenecks: first, mapping the complex and evolving landscape of host-virus specificity, where recent progress was triggered by high-throughput screening methods and network analysis\(^14,15\); second, obtaining a more robust and quantitative understanding of the infection process, particularly through a focus on events at the single host-virus pair level\(^9\).

Here, we contribute to this second direction by presenting a microscale, imaging-based approach for the quantification of rates of viral encounter and adsorption in the marine cyanobacterium Prochlorococcus MED4 (hereafter ‘MED4’) and the cyanophage myovirus P-HM2 (hereafter ‘P-HM2’\(^\text{16,17}\). Prochlorococcus is selected as a model system because of its ubiquity throughout the ocean’s nutrient-poor central gyres with the annual mean global abundance of \(\sim 10^{27}\) cells, which produces up to 10% of the annual input of oxygen into the atmosphere and represents a
significant fraction of global primary production\textsuperscript{9,10,18}. Viruses, or cyanophages, play a key role in the ecology of \textit{Prochlorococcus}\textsuperscript{10}, and cyanomyovirus is chosen because they often dominate the most commonly isolated phages from marine viral communities\textsuperscript{1}.

Adsorption, or irreversible attachment to cells, is a critical yet poorly quantified step in the infection process. The process begins when a virus encounters a host, which for a small, non-motile host like \textit{Prochlorococcus} occurs at a rate determined by the Brownian motion of both host and virus\textsuperscript{19}; stirring by ocean turbulence has only a minor effect on the encounter rates (as does swimming, among motile cells)\textsuperscript{19,20}. Thereupon, the virus adsorb to the host when it binds to cell membrane receptors\textsuperscript{2,5} and subsequently injects its DNA (or RNA) and commences replication (lytic infection) or integrates into the host’s genome (lysogeny). Adsorption efficiency, \(a\), is determined by the success of the virus in finding and binding to a host receptor, which in turn depends on the number and distribution of receptors, receptor binding kinetics, and the relative orientation of the binding site and the virus’s tail fibers, affected by the virus’ rapid spinning due to Brownian rotational diffusion. The number and complexity of these parameters as well as a multitude of bacteriophage resistance mechanisms\textsuperscript{21} makes theoretical prediction of adsorption efficiency exceedingly difficult and raises the need for its robust experimental quantification.

The prevalent method for the quantification of viral adsorption is Delbruck’s adsorption kinetics assay at the bulk scale\textsuperscript{8,9}. Although multiple variants exist, the assay fundamentally consists of adding viruses to host cultures, sampling from the mixture over time, and counting the unadsorbed (‘free’) viruses – after the removal of host cells by centrifugation or filtration\textsuperscript{12}. This assay, while useful for determining relative adsorption rates, can be problematic for determining absolute adsorption rates: first, it does not permit direct visualization of encounter and adsorption dynamics; second, the invasive steps (filtration, centrifugation) used in many implementations are a possible source of background viral losses\textsuperscript{15,16}, which are generally assumed to be adsorbed to the host, and potentially contributing to overestimates of \(a\)\textsuperscript{1,7-9}.

\section*{4.2 METHODS}

\subsection*{4.2.1 Biological sample preparation}

\subsubsection*{4.2.1.1 Host and control cell preparation}

Axenic \textit{Prochlorococcus} strains (MED4 and MIT9312) were grown in Pro99 medium at 24\degree C under a 12 h light/dark cycle (synchronized cells\textsuperscript{33}), or under 30–40 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) of continuous, cool white light (unsynchronized cells). Cells from late logarithmic cultures (~10\(^8\) cells ml\(^{-1}\)) were used for the experiments without further dilution or concentration.
4.2.1.2 Virus propagation and purification

Cyanophage P-HM2 was propagated using exponentially growing cultures of *Prochlorococcus* MED4 as a host. After completing the infection cycle, the viral lysates were pelleted by centrifugation and filtered using a 0.2 μm Supor filter (Pall, PN 4612).

4.2.1.3 Virus preparation for imaging

For fluorescence imaging, viruses were fluorescently stained (=labeled) using SYBR-Gold (Invitrogen S11494), which is a bright, stable nucleic acid stain with the least amount of background fluorescence compared to other common nucleic acid stains. It is frequently used for counting viruses and bacteria from aquatic systems. In comparison, purified viruses were used without any further treatment (=unlabeled) for nanoparticle light scattering imaging (see section 4.2.3).

The viral staining and washing procedure followed a protocol recently optimized for viral tagging\textsuperscript{14,15}. Briefly, purified viruses were stained by mixing SYBR-Gold into the viral suspension to a final concentration of 1x, diluted from the initial dye stock of 10,000x. Samples were mixed vigorously for 10 s, incubated in the dark at room temperature for 30 min, then washed six times using a 100-kDa ultrafiltration centrifuge tube (Nanosep, Pall OD100C33) to remove unincorporated dye molecules in the background. To avoid structural damage to the viruses, washing was done by gentle centrifugation at 1,000g and 10°C with MTN buffer (0.6M NaCl, 0.1M Tris-Cl pH 7.5, 0.1M MgCl\textsubscript{2}) for 30-40 min per washing cycle. The six washes resulted in a final concentration of unincorporated SYBR-Gold dye of 0.006x, which was found to be sufficiently low to prevent staining of the host cellular DNA upon mixing the viral and host suspensions. Especially, SYBR-Gold staining did not interfere with infectivity, as observed by the absence of any change in the number of plaques compared to unlabeled viruses, in line with similar findings in other virus-host systems including *E. coli*\textsuperscript{23} and 23 strains of cyanobacteria\textsuperscript{14,15}.

4.2.2 Biological sample enumeration

4.2.2.1 Host cell enumeration

For flow cytometric enumeration, 1 mL samples were fixed with 25% glutaraldehyde (final concentration of 0.125%; Sigma Aldrich). The strips were incubated at 4°C in the dark for at least 15 min, then flash frozen in liquid nitrogen. Samples were analyzed on an InFlux flow cytometer (BD Cytopeia) with laser excitation at 488nm (blue), using the BD FACS Sortware v1.0 package (BD Biosciences).
4.2.2.2 Virus enumeration

From the harvested phage lysate, whole phage particles were enumerated by epi-fluorescence microscopy, as previously described with the following specifications: samples were filtered in triplicate onto 0.02 μm Anodisc filters (Whatman, Cat. No. 6809-6002) at ~8" Hg vacuum pressure, stained with SYBR Gold (Invitrogen, S11494) at 50x for ~15 min in the dark, vacuum-dried, and mounted with 0.1% p-phenylenediamine (Sigma) in 50%/50% PBS/glycerol. Slides were stored at -20°C in the dark and were analyzed using an X-Cite 120Q mercury arc lamp (Lumen Dynamics). Ten fields of view, yielding a minimum of 250 particles, were counted per slide.

Infective titers were determined by the most probable number (MPN) approach (n=30-36) in 96-well plates (BD Falcon Clear Microtest Plates; #353072). Clearing was determined by visual inspection and chlorophyll fluorescence measurements over ~2 weeks in a BioTek Synergy 2 microplate reader (excitation 440/30nm, emission 680/30nm). The MPN was calculated with “MPN Calculator” (M. Curiale), available at http://www.i2workout.com/mcuriale/mpn/.

4.2.3 Microfluidics and optical set up

4.2.3.1 Microfluidics

Experiments were conducted in a microfluidic channel to provide a quiescent, carefully controlled fluid environment. Microchannels were fabricated using standard soft lithography techniques. Briefly, a mold was prepared by depositing SU-8 photoresist (MicroChem Corp.) on silicon wafers and patterning channel reliefs via photolithography. Polydimethylsiloxane (PDMS; Sylgard 184 Silicone Elastomer Kit, Dow Corning) was prepared with 10:1 weight ratio between PDMS and a cross-linker and cast on the molds. After curing at 65°C for at least 2 h, PDMS microchannels were bonded to a clean coverslip via plasma treatment and incubated on a hot-plate at 100°C for 1 h. Finally, to minimize non-specific surface attachment of viruses and host cells, 1% bovine serum albumin (BSA, Sigma) was injected inside the microchannels and incubated for 30 min before it was rinsed thoroughly with 18.2 MΩ cm-1 water (Milli-Q; Millipore) 2–3 times.

4.2.3.2 Fluorescence imaging

A dual-channel fluorescence microscopy system for simultaneous imaging of hosts and viruses was built on an inverted microscope (Nikon Ti-E), using blue LED light (470 nm; M470L3-C5, Thorlabs) for excitation and exploiting the well separated emission spectra between the SYBR-Gold stained viruses (green; maximum emission ~537 nm) and Prochlorococcus photosynthetic
chlorophyll auto-fluorescence (red; >590 nm emission). The dual wavelength images were separated using an image splitter [cutoff dichroic 565 nm (Chroma)] and projected side-by-side onto the two halves of an EM-CCD camera (Andor iXon; 1004 x 1002 pixels; 8.0 μm per pixel) to capture images of hosts and viruses, simultaneously, at up to 32.2 frames s⁻¹. The optically split images were transformed (polynomial) using Matlab (The Mathworks) according to a calibrated reference image via image registration to correct for minor alignment errors and optical distortions. We accounted for photobleaching of cells’ autofluorescence during image acquisition (Fig. 4.4; see section 4.2.6.2), and avoided using anti-photobleaching measures to eliminate any potential chemical interference with viral adsorption.

4.2.3.3 Fluorescence host/virus tracking

Individual viruses and hosts were tracked and virus-host interactions were identified from the obtained trajectories, using automated Matlab software developed in-house. For encounter and adsorption experiments, we mixed 2~5 μl of host and virus suspensions. Both the virus and host concentrations (>10⁷ ml⁻¹) were higher than typical ocean values to allow the acquisition of statistically robust encounter and adsorption statistics over manageable observation times, while ensuring that individual hosts did not interfere with each other. The host-virus suspension was immediately injected into a 7 μm deep polydimethylsiloxane (PDMS) microfluidic channel, which provided a controlled, quiescent environment for imaging. Imaging with a 60× objective (NA = 1.4) yielded a 133.9 μm x 267.2 μm field of view and 0.515 μm thin depth of field that allowed accurate encounter identification between viruses and hosts. Imaging at channel mid-depth ensured that virus-host interactions were not affected by the channel’s top and bottom surfaces. To capture sufficient virus-host encounters despite the exceedingly small imaging volume, long observation times were necessary: the cumulative recording time was 565 min. All analyses were performed in Matlab (The Mathworks) using in-house, automated software for tracking, and virus-host interactions were quantified from the reconstructed trajectories.

4.2.3.4 Nanoparticle light scattering imaging

Unlabeled viruses were visualized by Nanoparticle Tracking Analysis using a NanoSight LM10HS instrument (NanoSight Ltd.), equipped with a blue laser for illumination and a microscope. The technique allows small particles (10~1,000 nm) in liquid suspension moving under Brownian motion to be visualized and counted on a particle-by-particle from the light they scatter. All data files for a given experiment were captured and processed using identical acquisition and analysis settings, respectively. The sample chamber was thoroughly flushed with 18.2 MΩ cm⁻¹ water (Milli-Q; Millipore) before experiments, and visually examined to ensure that no particles were carried over during sample preparation.

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4.2.4 Population-scale adsorption kinetics assays

Adsorption kinetics of viruses to hosts is quantified by the depletion of unadsorbed (free) virus in a virus-host mixture. The adsorption process follows first-order kinetics, \(-dV/dt = AHV\), where \(V\) is the number of viruses remaining unadsorbed after a time \(t\), \(H\) is the concentration of host cells and \(A\) is the adsorption rate. Integration of this equation gives \(\ln (V/V_0) = -AHt\), predicting that the logarithm of the number of unadsorbed viruses relative to their initial number, \(V_0\), decreases linearly with time (Fig. 4.13a). In practice, the adsorption rate, \(A\), is quantified from the slope of the linear fit and the measured host concentration, \(H\). We quantified the adsorption rate between P-HM2 and MED4 using two different approaches: (1) non-invasive imaging-based measurement at the level of single viruses (‘microscale’; Fig. 4.13a, blue), as well as (2) the widely-used, traditional bulk assay by quantifying the viral decay in a suspension after separating viruses from host cells (‘macroscale’; Fig. 4.13b, red).

4.2.4.1 Microscale adsorption kinetic assay

(i) Measurement based on fluorescence imaging.

To perform direct imaging measurements of the rate of adsorption of freely-diffusing viruses to freely-diffusing hosts, labeled viruses were mixed separately with both host and non-host strains of Prochlorococcus cells (MED4 and MIT9312) to a total volume of 10 μL and vortexed briefly to mix. Subsamples from the mixture (2 μL) were taken every hour and injected into a microchannel for visualization (see section 4.2.3) because the small sample volumes and subsequent drying issue prevented continuous data acquisition with the same sample over the whole 4 h time interval. Videos were captured using epi-fluorescence microscopy for 40 min, including immediately after mixing at time zero (Fig. 4.13d; cyan ‘▲’). The adsorption rate was quantified from the number of unadsorbed viruses without any further treatment to the sample (e.g., filtration or centrifugation; see section 4.3.5). During the 4 h long experiment, the remaining virus-host mixture was kept in the dark at room temperature in a separate container to minimize photo-bleaching.

However, this method (unlike the single-cell tracking of adsorption events) inevitable caused small background loss of viruses due to non-specific binding to surfaces. This was evidenced in the control experiments with the non-host strain MIT9312, which showed slight decay in the number of unabsorbed viruses according to this method (\(\alpha <0.4\%\)). We confirmed in a separate quantification that there was no significant viral adsorption occurring on the cell side (Fig. 4.10b). Therefore, the adsorption efficiency (\(\alpha\)) measured at the population-level using fluorescence imaging inside a microfluidic channel was intentionally conservative and provides an upper limit of \(\alpha\) between the host cell MED4 and the virus P-HM2.
(ii) Measurement based on nanoparticle light scattering imaging.

Comparison experiment of adsorption kinetics between the labeled (stained and washed) and unlabeled (directly from the original stock) viruses was conducted using nanoparticle light scattering imaging (see section 4.2.3.4) at room temperature. For the adsorption kinetics experiment, P-HM2 was added to both the synchronized and unsynchronized MED4 cultures (6.4 x 10⁷ cells mL⁻¹) grown to exponential phase (see section 4.2.1.1), or fresh media (no-cell control), to a total volume of 500 μL, and vortexed briefly to mix. After injecting 300 μL of virus-host mixture to the sample chamber, microscale population-level adsorption kinetics were measured by capturing three 1-min videos every 5 min for up to 4 h. The number of unadsorbed viruses within the field of view were noninvasively counted over time (see Fig. 4.13a) using nanoparticle light scattering imaging without further sample manipulation.

4.2.4.2 Macroscale adsorption kinetic assay

In parallel to the quantification of adsorption efficiency using two imaging-based microscale quantifications as illustrated above, here we provide the experimental details of the bulk adsorption kinetics used in this study. We minimized any potential source of error in our macroscale quantifications as follows: first, background loss from non-specific bindings were minimized by using glass containers; second, aliquots were subsampled at a large volume (200 μl as opposed to a typical volume of ~10 μl) to overcome insufficient sample mixing issue; third, aliquots were analyzed with qPCR (quantitative polymerase chain reaction) to separate viral adsorption from infection (e.g., only infective titers are detected in plaque assays). By minimizing these potential sources of error, our macroscale measurement provides the most conservative, lower limit, value of the adsorption efficiency between the host cell MED4 and the virus P-HM2 using the bulk adsorption kinetics assay.

(i) Mixing and sampling.

P-HM2 was added to exponential-phase MED4 cultures (6.4x10⁷ cells ml⁻¹) or fresh media (no-cell controls), in triplicate, at high (= 6) and low (0.5) viruses per cell ratios, to a total volume of 9 ml, and vortexed briefly to mix. Treatments were sampled for 4 h at 10, 30 or 60 min time intervals (Fig. 4.13b). At each time point, 200 μl aliquots of the mixture were sampled and filtered at ~8” Hg through 96-well MultiScreen-HTS filter plates with 0.22 μm Durapore filters (Millipore, MSGVS2210), using a MultiScreen-HTS Vacuum Manifold (Millipore). The filtrate was immediately diluted 20-fold in nuclease-free water, and aliquoted (10 μl) into duplicate PCR reaction plates (BioRad, MLL9651). Plates were sealed with sterile adhesive foil and kept on ice until filled (<3 h) and then transferred to -20°C for storage until analyzed by qPCR (see below).

(ii) Quantitative polymerase chain reaction (qPCR) conditions.
Frozen, prepared templates (see above) were thawed on ice and briefly spun down before the addition of reagents and primers. 25 µL qPCR reactions were prepared with the QuantiTect SYBR Green PCR kit (Qiagen), with primers at 0.4 µM. Primers were specific to P-HM2 g20 (F: 5'-CGT AGA GAA GGT GGC AGA GG-3', R: 5'-GAC CTT CCG ATG TTA AAT TGC-3'). qPCR was performed on a Bio-Rad CFX96 C1000 Thermal Cycler, using the following amplification conditions: 15 min at 95°C; 40-50 cycles of denaturation (94°C, 15 s), annealing (55°C, 30 s), elongation (72°C, 30 s), and determination of SYBR incorporation into dsDNA; a final extension step at 72°C for 5 min; and melt curve analysis (50°C-90°C; read every 0.5°C, hold 5 s). Standards consisted of serial dilutions of fresh P-HM2 that were enumerated by epi-fluorescence microscopy (see section 4.2.2.2).

4.2.5 Direct virus-host encounter identification

The ability to reliably identify physical encounters between viruses and host cells occurring in three dimensions from two-dimensional image data was critical for accurate quantification of the encounter and adsorption rates. Ambiguity in identifying the exact proximity of viruses relative to host cells stems from the finite depth of field (DOF) of the imaging system along the optical axis (z-direction). We provide the information of the depth of field (section 1) and calculate the accuracy of encounter identification between a virus and a host in our optical setup (section 2).

4.2.5.1 Depth of field

We used a high-numerical-aperture, high-magnification oil-immersion objective (NA = 1.4, M=60x) in order to restrict the DOF (515 nm) below the size of the host (2RH = 800 nm) and the encounter zone (700 nm). Here, the DOF is primarily determined by the numerical aperture35, which is given by

$$\text{DOF} = \frac{\lambda_0 n}{\text{NA}^2} + \frac{n}{M \cdot \text{NA}} e, \quad (4.1)$$

where $n = 1.515$ is the refractive index of the immersion medium (oil), $\lambda_0$ is the illumination emission wavelength, and $e$ is the smallest distance that can be resolved by a detector that is placed in the image plane of the microscope.

Because of the relatively large size of the virus and host (2RH =800 nm) used in the experiments compared to the DOF, the distance between the in-focus virus and host in the imaging plane is a good indicator of a virus-host encounter with 84.7% accuracy (see below).
Figure 4.1 | Cross-sectional schematic view of the host cell geometry, located at an arbitrary position along the optical axis and within the depth of field (D). h denotes host distance with respect to the lower edge of the DOF, \( R_H \) is the host radius, \( R \) is the encounter zone size, and areas shaded in blue and red denote true and false encounter zones, respectively. Viruses located within the red result in false encounter identification: i.e., the projected host-virus separation distance in the image plane is within \( R \), but their Eulerian distance in three-dimensional space is not.

4.2.5.2 Encounter identification accuracy

In order to quantify the experimental encounter rate from the acquired virus-host trajectories, below we calculate the accuracy of encounter identification set by the DOF (D) and the virus-host geometries. For an in-focus host, a virus and a host are considered encountered when the centroid of a virus is located within the imaging volume (Fig. 4.1). However, this quantification includes false encounter events, i.e., when the center of a virus is located at the cell periphery shaded in red (Fig. 4.1). If we assume randomly distribution of viruses within the imaging volume around a host, the probability of identifying a true encounter is proportional to the ratio of the focal plane volume occupied by the spherical encounter zone (Fig. 4.1, blue zone) relative to the whole imaging volume (Fig. 4.1, sum of blue and red zones). The encounter identification accuracy is calculated below by averaging this volume fraction over all possible configurations of the host relative to the focal plane (e.g., Fig. 4.2a-c).
Figure 4.2 | Cross-sectional schematic view of different host configurations along the optical axis with respect to the depth of field \((D)\), showing change in the encounter zone (blue). The false encounter zone decreases as the host cell becomes centered in the focal plane (from configurations a to c) and reaches minimum when it is centered in the focal plane (configuration c). The encounter identification accuracies for these two extreme cases are 79.4% (a) and 94% (c).

Following the notations illustrated in Figure 4.1, we calculate the volume of the focal plane occupied by the host cell (here we refer to ‘inner segment’ 2-3-11-10-2) and the encounter zone (‘outer segment’ 1-4-12-9-1) and subtract them from the cylindrical volume \((\pi R^2 D)\) to calculate the total (true + false; blue and red) and false imaging (red) volume, respectively. The volume of an outer spherical segment (Fig. 4.1, segment 1-4-12-9-1) divided at the center of the sphere is

\[
V_{\text{OT}} = \pi (D - h) \left( R^2 - \frac{(D - h)^2}{3} \right),
\]

\[
V_{\text{OB}} = \pi h \left( R^2 - \frac{h^2}{3} \right),
\]

where \(D\) is the DOF, \(h\) is distance between the sphere center and the bottom plane, and \(V_{\text{OT}}\) (segment 1-4-5-8-1), \(V_{\text{OB}}\) (segment 8-5-12-9-8) refer to outer-top and outer-bottom, respectively. Here \(h\) is set between \(0 \leq h < D/2\) because it is symmetric on the other half. Average size of the outer spherical segment (1-4-12-9-1) over all possible configurations is then

\[
\bar{V}_O = \frac{1}{(D/2)} \int_0^{D/2} (V_{\text{OT}} + V_{\text{OB}}) \, dh \quad (4.4)
\]

\[
= \pi D(6R^2 - D^2)/6.
\]

For an inner spherical segment, the top half can completely lie within the DOF, forming a hemisphere (e.g., Fig. 4.2a). Thus the inner volume is calculated along two vertical segments between (a) \(0 \leq h < D - R_H\),

\[
V_{\text{ITa}} = \frac{2\pi R_H^2}{3},
\]

\[
V_{\text{IBa}} = \pi h \left( R_H^2 - \frac{h^2}{3} \right),
\]

\[
= \pi D(6R^2 - D^2)/6.
\]
and (b) \( D - R_H \leq h < \frac{D}{2} \),

\[
V_{ITb} = \pi(D - h)(R_H^2 - \frac{(D - h)^2}{3}), \tag{4.7}
\]

\[
V_{IBb} = \pi h \left( R_H^2 - \frac{h^2}{3} \right), \tag{4.8}
\]

where \( V_{IT} \) (segment 2-3-6-7-2), \( V_{IB} \) (segment 7-6-11-10-7) refer to inner-top and inner-bottom, respectively. Average size of the inner spherical segment (2-3-11-10-2) over all possible configurations is then

\[
\overline{V}_1 = \frac{1}{(D - R_H)} \int_0^{D - R_H} (V_{ITa} + V_{IBa}) \, dh
\]

\[
+ \frac{1}{\left[ \frac{D}{2} - (D - R_H) \right]} \int_0^{\frac{D}{2} - (D - R_H)} (V_{ITb} + V_{IBb}) \, dh
\]

\[
= \pi(13D^2R_H + 11DR_H^2 + 3R_H^3 - 9D^3)/12.
\]

Finally, subtracting the calculated inner and outer spherical segments from the cylindrical tube (volume, \( \pi R^2D \)) gives the total (true + false; blue and red zones) and false (red zone) imaging volume around a host cell.

\[
IM_T = \pi R^2D - \overline{V}_1 \tag{4.10}
\]

\[
IM_F = \pi R^2D - \overline{V}_0 \tag{4.11}
\]

and the accuracy of encounter identification is then

\[
P_{CI} = \frac{IM_T - IM_F}{IM_T} \tag{4.12}
\]

\[
= \frac{7D^3 + 12DR^2 - 13D^2R_H - 11DR_H^2 - 3R_H^3}{9D^3 + 12DR^2 - 13D^2R_H - 11DR_H^2 - 3R_H^3}
\]

\[
= 84.7\%
\]

with the DOF, \( D = 515 \text{ nm} \), encounter zone size \( R_V + R_H = 700 \text{ nm} \), and host size \( R_H = 400 \text{ nm} \).
4.2.6 Identifying virus-and-host within the DOF

One of the most critical steps for accurate encounter identification is to ensure both viruses and hosts are located within the focal plane. Here, we provide the experimental framework used to calibrate attenuated signals from 'out-of-focused' viruses and hosts (section 1), while accounting for the effect of photo-bleaching (section 2). Finally, we make direct comparison between our experimental quantification with the theoretical prediction (section 3).

**Figure 4.3** | Variation of host and virus fluorescence intensity with distance from the objective focal plane for surface-immobilized MED4 hosts (a) and P-HM2 viruses (b). Measurements were performed at the glass surface of a microchannel via z-stack image acquisition. Dotted symbols denote individual particle measurements and open circular symbols denote the average intensity. Green dashed lines indicate intensity threshold values for host and virus encounter identification. Note that setting the intensity threshold values constrain the effective depth of field (magenta lines and arrows) close to the theoretically predicted value of DOF = 515 nm.

4.2.6.1 Z-depth calibration

In practice, the image intensities of hosts and viruses decrease with their increasing distance from the focal plane of the objective. However, hosts and viruses having sufficiently high image intensity may be inadvertently detected outside of the DOF (Fig. 4.3). To further restrict our optical detection of hosts and viruses to within the DOF, we calibrate the observed intensity of viruses and hosts to their z-location relative to the focal plane (Fig. 4.3). To accomplish this, we immobilized hosts and viruses via non-specific attachment, to the glass surface of a microchannel. Surface-immobilized hosts and viruses were imaged by moving the focal plane.
along the optical axis in 50 nm increments (Fig. 4.3) to determine their intensity variation with distance from the focal plane along the optical axis (z). Hosts and viruses centered at the focal plane exhibited the smallest image radius and highest image intensity. The image radius increased and the intensity decreased with increasing distance from either side of the focal plane\(^{36}\). These measurements were repeated multiple times to account for natural variability in the image intensities of hosts and viruses as shown by the data scatter (Fig. 4.3, dotted markers). Finally, the average of the scatter (open circular symbols) was taken as a calibration curve. These curves were used to identify hosts and viruses that were in focus during experimental observation when both were freely diffusing.

4.2.6.2 Photo-bleaching

The initial intensity threshold values were set to the flat peaked region of the curve \((I_0 = 0.21\) for hosts and \(I_0 = 0.015\) for viruses; Fig. 4.3, green dashed lines). Hosts and viruses with intensity values higher than these threshold values were only considered for encounter identification, as they are located at the same focal plane, in addition to satisfying the first criterion \((r < R_H + R_V = 0.7 \, \mu m;\) Fig. 4.10a). However, the auto-fluorescence emission intensity of both host and control cells decreases with prolonged exposure to the illumination light due to photo-bleaching. While chemical treatments exist to quench photo-bleaching through oxygen scavenging, all experiments were carried out without any chemical additives to avoid potential consequences on host viability and viral adsorption. Instead, photo-bleaching was accounted for by careful calibration of the intensity threshold value based on the measured mean fluorescence intensity decay during video acquisition, which appeared to be approximately linear (Fig. 4.4). We calibrated fluorescence intensity threshold values with the measured intensity decay of the host and control cells, by fitting a line through the data points. In contrast, SYBR-gold stained viruses were less prone to photo-bleaching under the optimized illumination condition (Fig. 4.4) during video acquisition, and only a constant intensity threshold was applied over the course of the experiments.

![Figure 4.4](image-url)  
**Figure 4.4** | Fluorescence intensity decay over time due to photo-bleaching for the host (MED4, blue), non-host (MIT9312, green) strains of *Prochlorococcus* and the virus (P-HM2, red), which
are normalized with respect to the initial intensity. Both strains of *Prochlorococcus* show similar decay in their chlorophyll auto-fluorescence intensity level. The decay curves were used to calibrate intensity threshold values (Fig. 4.3) over time. Viruses (P-HM2) stained with SYBR-gold show no significant fluorescence intensity decay during the observation time.

### 4.2.6.3 Encounter identification and robustness

We eliminated false encounter events – instances in which a host and a virus were sufficiently close in the image \((x \text{ and } y)\) plane to indicate an encounter, but too distant from one another along the optical axis in \(z\) (e.g., Fig. 4.5) – by restricting the analysis to host cells and viruses located within 515 nm of the image plane (Fig. 4.3-4.5). The robustness of the resulting approach for the identification of host-virus encounters is further supported by comparing the measured encounter rate with the encounter rate predicted by theory (Fig. 4.10c). The latter is based on diffusive encounter theory, which predicts the number of encounters between two randomly diffusing particles based on their geometries and diffusivities. The close agreement between the predicted and measured encounter rates lends support to the accuracy of the image analysis.

![Sample trajectory of host-virus separation distance demonstrating the two encounter identification criteria](image)

**Figure 4.5** | Sample trajectory of host-virus separation distance demonstrating the two encounter identification criteria: (1) filled markers (both yellow and blue) represent virus and host within the encounter zone \((r \leq 0.7 \, \mu m)\), which occurs when the projected host-virus separation distance in the image plane \((x \text{ and } y)\) falls within the encounter zone; (2) by incorporating their separation along the optical axis in \(z\), only markers in blue are true encounters. Note that all virus-host interactions in the study took \(z\)-information into account for encounter identification as shown in this example.
4.2.7 Sensitivity analysis

Each step in the image analysis process was accompanied by a careful sensitivity analysis to ensure that the small, measured adsorption efficiencies were robust and significant in the face of the complexities associated with dynamic imaging and tracking at the small scales (see section 4.2.3.2, 4.2.3.3 and 4.2.5, 4.2.6). As shown in Figure 4.12, the ratio between the number of escape and adsorption trajectories is a good indicator of the adsorption efficiency, \( \alpha \). Here, we quantified the number of escape and adsorption trajectories using a range of different analysis parameters. We examined the sensitivity of our results to the (i) encounter zone size, (ii) analysis zone size, (iii) initial host intensity threshold value, and made direct comparison with the adsorption efficiency quantified from the macroscale adsorption kinetics assay (\( \alpha = 20.7\% \)). Below, parameters chosen for single-cell level data analysis are shown in bold. Each analysis parameters were varied individually for the sensitivity analysis, while keeping two other parameters constant.

(i) The encounter zone (\( r \leq 0.7 \mu m \)) is set by the geometries of viruses and hosts. In order to account for natural variability in size, we tested different encounter zone sizes ranging from 0.5 ~ 0.9 \( \mu m \).

<table>
<thead>
<tr>
<th>Encounter zone (( \mu m ))</th>
<th>0.5</th>
<th>0.6</th>
<th>0.7</th>
<th>0.8</th>
<th>0.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of escapes</td>
<td>148</td>
<td>201</td>
<td>273</td>
<td>355</td>
<td>440</td>
</tr>
<tr>
<td>Number of adsorptions</td>
<td>1</td>
<td>4</td>
<td>13</td>
<td>22</td>
<td>32</td>
</tr>
<tr>
<td>Percentage of adsorptions (%)</td>
<td>0.7</td>
<td>2.0</td>
<td>4.6</td>
<td>5.8</td>
<td>6.8</td>
</tr>
</tbody>
</table>

(ii) The analysis zone is set to 1.0 \( \mu m \) away from an encounter between viruses and hosts (1.7 \( \mu m = 0.7 \mu m + 1.0 \mu m \)). Here we tested different analysis zone sizes ranging from 1.2 ~ 2.2 \( \mu m \).

<table>
<thead>
<tr>
<th>Analysis zone (( \mu m ))</th>
<th>1.2</th>
<th>1.5</th>
<th>1.7</th>
<th>2.0</th>
<th>2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of escapes</td>
<td>581</td>
<td>365</td>
<td>273</td>
<td>166</td>
<td>118</td>
</tr>
<tr>
<td>Number of adsorptions</td>
<td>25</td>
<td>15</td>
<td>13</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Percentage of adsorptions (%)</td>
<td>4.1</td>
<td>4.0</td>
<td>4.6</td>
<td>6.2</td>
<td>7.8</td>
</tr>
</tbody>
</table>

(iii) The host intensity threshold value was experimentally determined from the intensity variation along the optical axis (see section 4.2.6.1; Figs. 4.3 and 4.4). In order to account for natural variability in the host intensity, we tested different initial host intensity threshold values ranging from 0.07 ~ 0.28 before photo-bleaching.

<table>
<thead>
<tr>
<th>Host intensity (a.u.)</th>
<th>0.07</th>
<th>0.14</th>
<th>0.21</th>
<th>0.25</th>
<th>0.28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of escapes</td>
<td>676</td>
<td>440</td>
<td>273</td>
<td>167</td>
<td>73</td>
</tr>
<tr>
<td>Number of adsorptions</td>
<td>62</td>
<td>32</td>
<td>13</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Percentage of adsorptions (%)</td>
<td>8.4</td>
<td>6.8</td>
<td>4.6</td>
<td>3.5</td>
<td>1.4</td>
</tr>
</tbody>
</table>

As shown above, absolute number of trajectories changes with the choice of analysis parameters but the percent ratio of adsorptions among all the trajectories remains relatively constant (<10%) in all cases, which were all lower than the macroscale quantification.

4.2.8 Theoretical encounter rate correction

Encounter rate theory predicts that two populations of freely diffusing particles (P-HM2 and MED4) with radii \( R_V \) and \( R_H \), diffusivities \( D_V \) and \( D_H \), and concentrations \( C_V \) and \( C_H \), encounter each other at a rate \( E_{TH} = \frac{N}{C_{V}C_{H}} \) (number of encounters per unit time per unit volume)\(^{20}\). The encounter kernel (unit: volume per unit time) in three-dimensional space is defined as \( E_p = 4n(D_V+D_H)(R_V+R_H) \). However, in practice, encounters occurring only within the image focal depth (DOF = 515 nm; see section 4.2.5.1) are captured experimentally due to the small DOF compare to the average host diameter (\( 2R_H = 800 \) nm) [Fig. 4.6]. The small DOF ensures high encounter identification accuracy (84.7%; see section 4.2.5.2) but inevitably leads to limited encounter detection, not capturing encounters that occur above or below a host cell (Fig. 4.6).

![Cross-sectional schematic view of different host configurations along the optical axis with respect to the depth of field (D, green dashed lines). Encounters are only captured experimentally when a virus-host encounter occurs within the DOF (surface area shaded in red). The blue circular region denotes the encounter zone. Correction for the theoretical encounter rate is calculated based the effective surface area fraction of a host cell, which depends on different host configurations along the optical axis with respect to the depth of field (D) [e.g., a: 50%, b: 64%, c: 64%].](image.png)

To account for two-dimensional effects in our experimental measurements (see Fig. 4.10c), we calculate the theoretical encounter rate correction based on the effective surface area of a host.
cell within the image focal depth, which varies with the host configuration along the optical axis (Fig. 4.6). If we assume random distribution of viruses in space, the probability of capturing an encounter in two-dimensional imaging volume is proportional to the ratio of a host cell surface within the DOF (Fig. 4.6; shaded zone in red) relative to its whole surface area, \( A_T = 4\pi R_h^2 \), which assumes encounters occurring in all three-dimensions.

We only select host cells having 50% or more of their volume coincident with the DOF based on the image intensity thresholding (see section 4.2.6.1; extreme examples shown in Fig. 4.6a). The surface area of a spherical cap is calculated as \( A_C = 2\pi R_h h \), where \( R_h \) is the sphere radius and \( h \) is the height of the cap. Depending on the relative position of host with respect to the DOF, the effective surface area changes from \( A_{CL} = 2\pi R_h^2 \) (see Fig. 4.6a) to \( A_{CH} = 4\pi R_h^2 - 2\pi R_h (2R_h - D) \) [see Fig. 4.6b], and remains constant in any configuration between Fig. 4.6b and Fig. 4.6c. The range of encounter rate correction, which takes limited DOF into account, thus varies between \( C_F = \frac{A_{CL}}{A_T} \sim \frac{A_{CH}}{A_T} = 50.0\% - 64.1\% \).

Figure 4.7 | Cross-sectional schematic view of the host cell geometry, located at an arbitrary position along the optical axis and within the depth of field (D). \( h \) denotes host distance with respect to the lower edge of the DOF (green dashed line), \( R \) is the encounter zone size. The area shaded in red denotes the experimentally identifiable encounter surface in the extreme case when both the host and the virus are assumed to be spheres with radii \( R_h \) and \( R_v \), respectively. \( \alpha \) and \( \beta \) are angles between the host center with the top and bottom planes of the DOF intersecting the encounter zone of radius \( R \).

Another extreme case is to approximate both viruses and hosts as spheres with radii \( R_v \) and \( R_h \), respectively, which further limits the effective encounter area to a band (Fig. 4.7; shaded zone in red). We calculate this area based on the encounter zone (\( R = R_v + R_h = 700 \text{ nm} \)) and the DOF (\( D = 515 \text{ nm} \)) integrated over all possible configurations. As shown in Fig. 4.7, surface area of a band on a sphere (representing the host) is calculated in spherical coordinate \((r, \theta, \phi)\):
\[ A_S = \int_{\pi/2}^{\pi} \int_0^{2\pi} R_H \sin \theta \, d\phi \cdot R_H \, d\theta \]

\[ = 2\pi R_H^2 \frac{D}{R_H + R_V}, \]

with

\[ \sin \alpha = \frac{D - h}{R_H + R_V}, \] \hspace{1cm} (4.14)

\[ \sin \beta = \frac{h}{R_H + R_V}. \] \hspace{1cm} (4.15)

Surface area of the encounter band, \( A_S \), is independent of \( h \) value and the dimensionality correction factor for the theoretical encounter rate under this extreme assumption (assuming both hosts and viruses as spheres) is

\[ C_F = \frac{A_S}{A_T} = \frac{D}{2(R_H + R_V)} = 36.8\%, \] \hspace{1cm} (4.16)

which results in a lower bound of the encounter rate correction (see Fig. 4.10c).

### 4.3 RESULTS

#### 4.3.1 Imaging host-virus interactions

As an alternative to the classic, macroscale adsorption assay, we used a non-invasive technique to directly quantify host-virus encounter and adsorption dynamics from their trajectories and abundances using real-time imaging (Fig. 4.8d,e) of a *Prochlorococcus*-cyanophage system: MED4 (Fig. 4.8a; 800 nm diameter) and P-HM2 (Fig. 4.8b; 60 nm diameter icosahedral capsid, 180 nm long rigid tail). P-HM2 infects several *Prochlorococcus* strains, including MED4, and has infection kinetics and productivity typical of *Prochlorococcus* cyanomyoviruses. This model system allowed us to simultaneously image host and viruses by two-channel-epifluorescent-microscopy, as both are excited by blue-light and their emission spectra are easily distinguished: red-auto-fluorescence from the host chlorophyll, green-fluorescence from the SYBR-Gold stain used to label the virus (see section 4.2.1). Experiments were conducted in microfluidic devices to attain precise control over the relative location of host and virus, which were directly imaged over seconds to minutes and simultaneously tracked through automated image analysis as they
diffused freely by Brownian-motion (see section 4.3.2). This approach captures the full encounter-and-adsorption or encounter-and-escape dynamics from thousands of interaction events, thus enabling the quantification of population-level adsorption efficiency directly from scaling up interactions occurring at the single host-virus level.

Figure 4.8 | Dynamic imaging of host-virus encounter and adsorption between MED4 and P-HM2. a,b, Transmission electron micrographs of the host cell Prochlorococcus MED4 (a) and
the cyanophage myovirus P-HM2 (b). c, Host-virus distance, $r$, for a host and a virus that are co-diffusing both translationally and rotationally after permanent adsorption. The encounter zone ($r \leq 0.7 \mu m$) is shaded gray. (Inset) The host and virus trajectories for this case. Colors denote time (host in orange $\rightarrow$ red; virus in green $\rightarrow$ blue), as indicated by the color bars above the main panel. Circular markers represent host and virus positions at 31 ms intervals. Black open squares denote trajectory starting positions and open circles denote encounter (see section 4.2.5). d, Time-lapse image sequence showing encounter and adsorption between host and virus. Host and virus are not to scale, as image intensities were adjusted for optimal contrast. Trajectories are color-coded by time and the same color-coding is used in panel e. The white scale bar is 1.0 $\mu m$. e, Host and virus trajectories showing Brownian motion and an encounter, for the same case as panel d. Colors represent time (host in orange $\rightarrow$ red; virus in green $\rightarrow$ blue; see panel d). Symbols as in panel c. f, Host-virus distance, $r$, for the same case shown in panels d-e. This trajectory pair contained two encounter events (open circles).

4.3.2 Translational and rotational diffusion coefficients

4.3.2.1 Measured translational diffusivity

The translational diffusion coefficients of the hosts and viruses were measured directly from the mean-square-displacement (MSD = $<\Delta x^2>$) [Fig. 4.9]. Videos of freely diffusing hosts and viruses were imaged separately in quiescent environments and ensemble averaged over >1,500 trajectories to calculate their diffusivities. Both exhibited diffusive behavior ($<\Delta x^2>$ $\sim$ $t$), and the corresponding translational diffusion coefficients [$D = <\Delta x^2>/(2t)$] were calculated from linear fits of the MSD versus time (Fig. 4.9), which resulted in $D_{MED4} = 0.56 \pm 0.01 \mu m^2 s^{-1}$, $D_{MIT9312} = 0.53 \pm 0.02 \mu m^2 s^{-1}$ and $D_{PHM2} = 1.36 \pm 0.15 \mu m^2 s^{-1}$. The equivalent radius of an spherically-shaped host cell ($r_H = 0.40 \mu m$) calculated from the Stokes-Einstein relation, $D_{MED4} = k_B T/6 \pi \eta r_H$, matched with the transmission electron micrographs of the host cell Prochlorococcus MED4 (Fig. 4.8a), where $k_B$ is Boltzmann’s constant, $T$ is the absolute temperature, and $\eta$ is viscosity of a medium (seawater). For the non-spherical myovirus P-HM2, the effective virus radius was $r_{VE} = 0.15 \mu m$, consistent with the encounter zone ($r \leq 0.7 \mu m$) set in the study: $r_{H} = 0.40 \mu m$, $r_{VE} = 0.15 \mu m$ and the addition of a typical length of viral tail fibers $= 0.14 \mu m$.

4.3.2.2 Theoretical rotational diffusivity

Rotational diffusivities of viruses and hosts are non-trivial to measure due to the diffraction limited size of the viruses and rotational symmetry of the hosts. Thus, the rotational diffusivities were calculated theoretically from the Einstein relation, $D_R = k_B T/f_R$, where $D_R$ is the rotational diffusion coefficient, and $f_R$ is the rotational frictional drag coefficient. For a spherically-shaped host cell MED4 and control cell MIT9312, the rotational frictional drag coefficients are isotropic.
and simply given as, \( f_R = 8\pi\eta r^3 \). A myovirus P-HM2 has an icosahedral capsid and a relatively long rigid tail with multiple tail fibers attached at the base. The rotational frictional drag coefficient of a virus is calculated from resistive-force theory by approximating it as an ellipsoid (length \( L_t = 300 \) nm; radius \( r_t = 40 \) nm). A virus can rotate in all three directions, along two directions perpendicular to the major axis (\( \perp \)) and one parallel to the major axis (\( \parallel \), along the major axis). The rotational frictional drag coefficients are given as

\[
\begin{align*}
    f_{RV\perp} &= \frac{8\pi\eta r_t L_t^2}{3}, \\
    f_{RV\parallel} &= \frac{\pi\eta L_t^3}{3 \left[ \ln \left( \frac{L_t}{r_t} \right) - 0.5 \right]}.
\end{align*}
\]

The mean rotational frictional drag coefficient for a virus \(^{37}\) is given as

\[
    f_{RV} = \left[ \left( f_{RV\parallel}^{-1} + f_{RV\perp}^{-1} + f_{RV\perp}^{-1} \right) / 3 \right]^{-1},
\]

From the relations above, the theoretical rotational diffusion coefficients are \( D_{RH} = 2.57 \text{ rad}^2 \text{ s}^{-1} \) and \( D_{RV} = 491.1 \text{ rad}^2 \text{ s}^{-1} \), which implies that the host, control and virus rotate approximately 0.36 and 5.0 revolutions per second.

![Figure 4.9](image-url)  

**Figure 4.9** | Mean-square displacements (MSD) of freely diffusing viruses (P-HM2; red) and hosts (MED4; blue) for >1,500 trajectories each. The black line has a slope of 1.0 for reference, which indicates diffusive behavior.
4.3.3 Encounter dynamics

Encounters were determined from trajectories of individual hosts and viruses freely diffusing by Brownian motion, with diffusivity $D_H = 0.56 \pm 0.01 \, \mu m^2 \, s^{-1}$ and $D_V = 1.36 \pm 0.15 \, \mu m^2 \, s^{-1}$, respectively, as measured from mean-square-displacements (Fig. 4.9). From trajectories, we quantified the time course of the center-to-center distance, $r$, between individual hosts and viruses (Fig. 4.8f). An encounter was scored when two criteria are simultaneously satisfied (see section 4.2.5): firstly, when $r < R_H + R_V = 0.7 \, \mu m$ (the ‘encounter zone’; Fig. 4.8f), where $R_H = 0.4 \, \mu m$ is the radius of MED4 (Fig. 4.8a) and $R_V = 0.3 \, \mu m$ is the effective rotational radius of P-HM2 (inclusive of the 0.14 µm long tail fibers\(^{22}\)) (Fig. 4.8b), and secondly when both MED4 and P-HM2 are located at the same focal plane, within the small depth of field (515 nm) [see section 4.2.5]. Especially, the choice of 0.7 µm as the size of the encounter zone was also supported by the observation that the probability density of the host-virus distance, pdf($r$), shows a gradual increase with time in the fraction of viruses within $r = 0.7 \, \mu m$ of a MED4 host (Fig. 4.10a), which we interpret as adsorbed viruses. Another Prochlorococcus strain, MIT9312 (hereafter ‘MIT9312’), which is similarly sized to MED4 but is not susceptible to infection by P-HM2 was used as a biological control. No temporal changes in pdf($r$) are observed for the control strain MIT9312 (Fig. 4.10b), confirming the host specificity of the adsorption.

Encounter rate theory predicts that viruses in concentration $V$ encounter hosts in concentration $H$ at a rate $E_V H$ (number of encounters per unit time per unit volume), where $E_T = 4\pi(D_V+D_H)(R_V+R_H)$ is the encounter kernel\(^{20}\) (presented as a unit volume per time, typically ml/min). For our system, the theoretical prediction of the encounter kernel is $E_T = 10.1 \pm 1.2 \times 10^{-10}$ ml/min based on the measured diffusivities ($D_V+D_H$) and the encounter distance ($R_V+R_H$), which ranges between $E_P = (3.7-6.5) \times 10^{-10}$ ml/min (Fig. 4.10c; see section 4.2.8) after taking into account the effect of small depth of field (515 nm) compared to the encounter distance (700 nm). Our observations of host and virus trajectories enabled us to also quantify the encounter kernel directly from experiments, by dividing the total number of encounters detected in our video recordings by the recording time (565 min), the imaging volume (3.9 picoliter), and the concentrations $V$ and $H$. This yields a value for the observed encounter kernel $E_O = 4.5 \pm 1.7 \times 10^{-10}$ ml/min (Fig. 4.10c). The good agreement between predicted ($E_P$) and observed ($E_O$) encounter rates (Fig. 4.10c) provides strong validation of our imaging and image analysis method, in particular of the encounter detection criteria (see section 4.2.5). Control experiments with P-HM2 and the non-host MIT9312 yielded an encounter rate ($E_{NH} = 4.5 \pm 1.8 \times 10^{-10}$ ml/min) close to that of MED4 (Fig. 4.10c), as expected based on the purely physical nature of the encounter process, which is agnostic of infectivity.
Figure 4.10  |  Encounters between MED4 and P-HM2 are driven by Brownian motion and host-agnostic, whereas adsorption is host-specific.  

**a,b,** The probability density of the host-virus distance, \( r \), at different time intervals after mixing P-HM2 with MED4, shows progressively increasing adsorption (a). The same quantification for the non-host strain MIT9312 shows no adsorption (b). The probability densities are calculated by normalizing the distribution of host-virus distance, \( r \), with the area of each radial bin. Curve are flat for a uniform distribution.
c. The host-virus encounter rate, $E$, predicted from theory$^{19,20}$ (brown; see section 4.2.8) and measured in experiments with host MED4 (cyan) and non-host MIT9312 (green). Error bars are standard deviations among three biological replicates.

### 4.3.4 Adsorption kinetics

#### 4.3.4.1 Adsorptions and escapes

Live imaging revealed that an encounter between a host and a virus was rarely followed by adsorption of the virus to the host and mostly resulted in an ‘escape’ (Fig. 4.11). We analyzed all viral approaches to within 1.0 μm from encountering a host ($r = 1.7$ μm; the ‘analysis zone’; Figs. 4.8g, 4.11c) to capture the sequence of events from free diffusion to encounter leading to either adsorption or escape, while saving analysis time on viruses that never approached a host. Among viruses venturing into the analysis zone, 4.7% encountered a MED4 host (Fig. 4.12a, left). For the non-host MIT9312, this value was 4.1% (Fig. 4.12b, left).

**Figure 4.11** | **Not all encounters between MED4 and P-HM2 lead to adsorption.** a, Time-lapse image sequence showing a virus escape from a host after multiple encounters. Host and virus are not to scale, as image intensities were adjusted for optimal contrast. Trajectories are color-coded by time. The white scale bar is 1.0 μm. b, Planar view of the same escape event. Circular markers represent host and virus positions at 31 ms intervals. Black open squares denote
trajectory starting positions. Colors represent time (host in orange→red; virus in green→blue; see panel a) and open circles denote encounter (see section 4.2.5). c, Host-virus distance, \( r \), for the same escape event. This trajectory pair contained two encounter events (open circles). The encounter zone \( (r \leq 0.7 \, \mu m) \) is shaded gray.

We classified encounters as adsorption events (Fig. 4.8d-f) or escapes (Fig. 4.11a-c). The criterion for an adsorption was that the encounter lasted until the end of the recording of that host-virus pair and more than a threshold time, taken to be the sum of mean and a standard deviations of the average encounter time (= 138.9 ms), to eliminate any false identification of adsorption events. This criterion provides an upper bound on the identification of adsorption events, because some adsorptions may have ended in escapes after the end of the recording, and validated by applying the same criterion to the control system (P-HM2 and non-host MIT9312), which resulted in no adsorption events (Fig. 4.12b, right). The criterion for an escape was that the host-virus distance increases to \( r \geq 1.7 \, \mu m \) (Fig. 4.11a-c), which was separated far enough so that the probability of a virus reentering the analysis zone for another encounter on the same host was low (only 0.2%), thereby considering each host-virus interactions as independent events. Viral approaches into the analysis zone that did not meet either criterion (e.g., due to diffusion out of the focal plane) were discarded. Strikingly, only 4.5% of encounters resulted in adsorption, with the remaining 95.5% being escapes (Fig. 4.12a, right). A sensitivity analysis showed that the predominance of escapes over adsorption events was robust to changes in the specific choice of the analysis parameters (see section 4.2.7).

**Figure 4.12 | Adsorptions are rare in encounters between MED4 and P-HM2.** a, Percentage (and number) of the viruses entering the analysis zone (distance \( r = 1.7 \, \mu m \) from a host) that resulted in no encounter between P-HM2 and MED4 (grey), escape of the virus (cyan), and
adsorption of the virus onto the host (pink), respectively. b, The same results for the non-host MIT9312.

Figure 4.13 | Imaging-based microscale quantification of host-virus adsorption reveals low adsorption efficiency of P-HM2 to MED4. a,b, Temporal decay in the concentration of unadsorbed viruses, \( V \), normalized by its initial value, \( V_0 \), measured at the microscale (a), by imaging-based viral counts, and the macroscale (b), by qPCR-based viral counts (see section
4.2.2. Time zero corresponds to when viruses and hosts were mixed. Symbols correspond to different host growth condition, and colors correspond to different virus treatments, as defined in panel d where ‘labeled’ refers to SYBR-gold stained and washed viruses (see section 4.2.1), ‘synchronized’ and ‘unsynchronized’ refer to the host growth conditions (see section 4.2.1), ‘MOI’ is ‘multiplicity of infection’, and ‘control’ experiments were performed only with viruses, without host cells. Solid lines are least-square fits. Because decay kinetics were linear only in the microscale quantifications, for the macroscale quantifications the fits were performed independently over two time intervals with respect to the inflection point (see section 4.2.4; see also panel c). Horizontal and vertical error bars denote time spans and standard deviations of multiple replicate measurements, respectively. The black dashed reference line denotes \( V/V_0 = 100\% \). 

c, Logarithm of the unadsorbed virus concentration, \( \ln(V/V_0) \), normalized by the host concentrations, \( H \), for comparison between different biological treatments (symbols and colors as in panel a and b; see panel d for legend). Curves are offset vertically for clarity. The slope of the lines is the adsorption rate, \( A \). For the macroscale quantifications, the initial time points from time zero before the inflection were used to compute \( A \) (see section 4.2.4)\(^{12} \). 

d, Adsorption efficiency, \( \alpha \), quantified at the microscale [single-cell level (green) and population-level (blue) scales], and macroscale (red). In the microscale quantifications, the first two bars were measured with fluorescence imaging (green and cyan triangles) and the rest were from nanoparticle light scattering imaging (see section 4.2.3). Symbols correspond to different host and virus treatments, as defined in the legend. Error bars are standard deviations among three biological replicates (open bar) and the fits (filled bars), respectively. Dashed lines and shadings denote averages and standard deviations computed over the 5 different quantifications at the microscale (blue) and the 2 treatments quantified at the macroscale (red), respectively.

**4.3.4.2 Microscale adsorption efficiency quantification (using labeled viruses)**

The adsorption efficiency, \( \alpha \), was directly measured from the video recordings using two independent approaches (Fig. 4.13). In the first approach, we computed \( \alpha \) directly from single-cell trajectories by computing the number of adsorption events among all the encounters that started off outside the analysis zone \( (r \geq 1.7 \mu m) \), which yielded \( \alpha = 2.6\pm1.0\% \) (Fig. 4.13d; green ‘A’). In the second approach, at the basis of the traditional adsorption kinetics assay\(^{12} \), we first obtained the adsorption rate, \( A \), from the rate of decay of the concentration of unadsorbed \((i.e.,\) freely diffusing\()\) viruses, \( V \), as \(\frac{dV}{dt} = -AHV\). From the integrated version of this equation, \(\ln(V/V_0) = -AHt\), one can determine \( A \) as the slope of \(\ln(V/V_0)\) versus time, divided by the host concentration \( H \) (Fig. 4.13c), where \( V_0 \) is the initial virus concentration. We applied this second approach to our microscale setup by quantifying \( V(t) \) and \( H \) through imaging via fluorescence microscopy (see section 4.2.3). Observations confirm the predicted linear decay over time of \(\ln(V/V_0)\) and yielded an adsorption rate \( A_p = 1.3\pm0.2 \times 10^{-11} \text{ ml/min} \), and \( \alpha = A_p / E_T = 1.3\pm0.3\% \) (Fig. 4.13; cyan ‘\(\Delta\)’). The gradual decay in the concentration of unadsorbed viruses is consistent
with the increase in the concentration of viruses within the encounter zone \((r \leq 0.7 \, \mu m)\) observed in the same experiments (Fig. 4.10a).

### 4.3.4.3 Microscale adsorption efficiency quantification (using unlabeled viruses)

We confirmed that neither the fluorescent staining of viruses with the nucleic acid dye SYBR-gold nor the growth conditions of the host had a major effect on the measured value of the adsorption efficiency. These were independently tested by repeating the population-scale quantification in a different set up, using nanoparticle light scattering (see section 4.2.3), instead of fluorescence microscopy, taking advantage of the imaging capability to visualize the unlabeled (untreated) viruses. SYBR-gold staining had a negligible impact (Fig. 4.13c,d; ‘cyan’ vs ‘blue’), in line with previous observations that this stain does not interfere with phage infectivity in both *E. coli* and other cyanobacteria. Host growth conditions also had a negligible effect (Fig. 4.13c,d; ‘△’ vs ‘n’ within microscale), showing no significant difference between the synchronized and unsynchronized MED4 cells. No-cell control experiments using the same setup showed no appreciable decrease in the number of unadsorbed viruses over the measurement time (Fig. 4.13a; ‘o’).

The microscopically measured adsorption efficiency of P-HM2 to MED4 was substantially lower than 100% reported in other cyanobacteria. This was true for both the single-cell approach based on the identification of encounter and adsorption events from trajectories, which provided the most direct experimental evaluation, yielding \(\alpha = 2.6 \pm 1.0\%\) (Fig. 4.13d; ‘green’), and for the population-scale approach based on the quantification of unadsorbed viruses over time using fluorescence or nanoparticle light scattering imaging, which yielded \(\alpha = 2.1 \pm 0.8\%\) (Fig. 4.13d; ‘cyan’ and ‘blue’). The complementarity of a single-cell approach with a population-scale approach that does not require any analysis parameters (e.g., the analysis and encounter zones, encounter time), as well as the independence of the assumptions and criteria used in each, yield confidence in the estimate of the adsorption efficiency.

### 4.3.4.4 Macroscale adsorption efficiency quantification (using unlabeled viruses)

The value of the adsorption efficiency obtained with the imaging-based, microscale approaches \((\alpha = 2.2 \pm 0.7\%\); Fig. 4.13d; ‘blue’ dashed line) was more than one order of magnitude lower than the value obtained for the same host-virus system with the traditional, macroscale adsorption kinetics assay (Fig. 4.13d; ‘blue’ vs ‘red’). The latter is also based on the quantification of the decrease in unadsorbed viruses, obtained by separating hosts from viruses, rather than by non-invasively imaging the host-virus mixture, which yielded \(\alpha = 20.7 \pm 5.0\%\) (Fig. 4.13d; ‘red’ dashed line). The temporal decay in the concentration of unadsorbed viruses was steeper than that of the microscale assay and non-linear, irrespective of the multiplicities of infection (MOI).
used (Fig. 4.13c,d; ‘pink’ vs ‘red’), denoting a higher adsorption rate and deviation from first-order kinetics\(^2\), respectively. We hypothesize that the 9.5-fold higher value of \(a\) in the macroscale approach compared to the microscale approach originates from background losses in the former\(^15,16\), where viruses can adsorb to filters and vessels (see section 4.3.5), particularly during frequently used filtration and centrifugation steps used to separate hosts from viruses. This hypothesis is supported by the observation that macroscale no-cell control experiments also exhibit considerable and rapid decay in unadsorbed viruses (>20%), despite the absence of hosts (Fig. 4.13b; ‘o’), whereas microscale no-cell control experiment does not (Fig. 4.13a; ‘o’). The non-invasive nature of imaging therefore appears crucial in obtaining a more accurate quantification of the adsorption efficiency.

### 4.3.5 Control experiment for macroscale adsorption kinetics assay

Classical adsorption kinetics assays over-predict the adsorption efficiency of virus-host encounters by an order of magnitude (Fig. 4.13). We demonstrate below that this discrepancy can arise from loss of viruses during the virus-host separation stage prior to quantification of unadsorbed (freely diffusing) viruses over different time points. The control experiments, even in the absence of any host cells, clearly show a dramatic decay in the viral counts under two different experimental conditions (Fig. 4.13b; also plotted in linear scale, Fig. 4.14). Here, we demonstrate that non-specific virus attachment to filters, glass and plastic surfaces can lead to over prediction of the adsorption efficiency.

![Figure 4.14](image_url)

**Figure 4.14** | Decay of unadsorbed virus ratio \((V/V_0)\) over time at the macroscale (same data as Fig. 4.13b), re-plotted here on a linear scale. Error bars are standard deviations.
4.3.5.1 Filtering losses

Macroscale adsorption kinetics assays require the physical separation of viruses from hosts in order to determine the adsorption rate. The change in the number of unadsorbed viruses remaining in the medium over time is presumed to be solely from viral adsorption to host cells.

![Graph showing viral decay](image)

**Figure 4.15** | Decay of viral counts in a suspension after sequential filtering, normalized by the initial value. The amount of viral loss was insensitive to the filter condition: blue symbol denotes result using a new filter; red symbols denote result from repetitively using the same filter (red). Error bars are standard deviations among trials.

To determine the viral loss due to filtration, we prepared a purified viral sample in the absence of host cells and quantified the number of viral particles in the suspension after serial filtering using a 0.2 μm Supor filter (Pall, PN 4612). This control experiment showed that each filtration stage resulted in ~20% loss in the viral count (Fig. 4.15). Such a significant loss from filtration can be larger than the decay rate of unadsorbed viruses, for instance due to adsorption on the host cells, and eventually leads to significant overestimation of the adsorption rate.

4.3.5.2 Surface attachment losses

Sample transfer and loading can also lead to loss of viruses due to non-specific surface attachments on to glass (Fig. 4.16) or plastic substrates, especially during centrifugation in the macroscale adsorption kinetics assays. Attachment to plastic substrates, such as pipet tips, syringes and sample containers, has been reported much more significant compare to glass substrates.
Figure 4.16 | Over-layered dual channel fluorescence image of immobilized hosts (red) and viruses (green), which were attached to the untreated glass surface of a microchannel (i.e., no BSA pre-coating) via non-specific binding.

4.4 DISCUSSION

This work demonstrates that host-virus interactions captured within a picoliter of imaging volume can be a starting point to better understand the ecological consequences of $10^5$ encounters between Prochlorococcus hosts ($10^5$/ml) and cyanophages ($10^6$/ml) in every milliliter of seawater each day. Moving one step further, the controlled environment afforded by microfluidic devices is also suitable to tease apart the reported dependence of viral infection on environmental and biological conditions, such as light level or host physiology.

The combination of imaging and microfluidic approaches has the potential to become the method of choice for the study of viral infection, not only for marine systems as demonstrated here, but for a wide range of host-virus systems in different natural and clinical contexts based on two complementary, microscale approaches used in this study: one approach based on viral staining and single-cell tracking, which is more laborious but provides a single-cell view of mechanisms, and a second approach based on imaging-based counts of unadsorbed viruses, which is more readily applicable without the need for fluorescent labeling and allows for faster data collection while also being non-invasive. When taken together with previous imaging approaches, our work increases the fraction of the viral infection process that can now be studied by imaging approaches at the scale of viruses that seamlessly scales to thousands of interactions, and thus yields population-level rates required for example in viral infection models. Further progress in expanding the use of live imaging, potentially to cover the full infection dynamics in a single experimental setup is an exciting prospect that our work suggests is now largely within reach. This imaging-based approach represents both a methodological and a conceptual shift in the study of viruses, from a view based mostly on indirect, often invasive, population-averaged statistics to one founded on non-invasive, imaging-based quantification and a direct visualization of processes and thereby a better understanding of mechanisms.
REFERENCES (CHAPTER 4)

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SUMMARY AND CONCLUSIONS

The combination of live imaging and precise environmental control afforded by microfluidics represents a uniquely powerful approach to understand the physical ecology of marine microbes based on capturing the fundamental nature of many microbial processes, which are often dynamic and unfold at the level of single cells.

In Chapter one, we reviewed the power of this approach in understanding biotic-biotic and biotic-abiotic interactions in microbial ecology. Resolving microbes’ flagellar dynamics and flow signature at the single-cell level has yielded new insights on cell motility and propulsion strategies, and on the consequences of motility on chemotaxis, pathogen-host interactions, transport in flow and surface attachment. Monitoring behavioral responses to precisely controlled chemical gradients has advanced the understanding of the chemosensory system in the model organism *E. coli*, as well as the prevalence and ecological consequences of chemotaxis in natural microbial habitats such as the ocean. Microscale studies of microbial interactions with the physical environment, in particular fluid flow and surfaces, has led to the discovery of biases on microbial migration induced by hydrodynamic or surface-induced forces, including shear-trapping and upstream swimming, which in turn affect the transport of bacteria and their initial surface colonization leading to biofilm formation.

Despite this wealth of new insights, the full potential of systems integrating microfluidics and dynamic imaging has only begun to be tapped, and microbial ecology stands to gain much more from these techniques. Single-cell imaging can be fruitfully extended to other important microbial groups, for example archaea and viruses. Microfluidic devices can represent ideal arenas to study the interaction between different microbial trophic levels, from predator-prey interactions at the level of the dynamics of individual predation events, to the establishment of symbiotic cell consortia, to chemotactic aggregations of microbes around larger organisms, to competition among different microbial species. In this thesis, then, we have contributed to harnessing this potential by using microfluidics and live imaging to study the physical ecology of microbial interactions, with an emphasis on motility, chemotaxis and host-virus encounter rates.

In Chapter two, we found that single-flagellated marine bacteria turn by exploiting a finely tuned buckling instability of their hook. Combining high-speed video microscopy and mechanical stability theory, we demonstrated that flick-reorientations occur 10 ms after the onset of forward swimming, when the hook undergoes compression. The mechanical origin of the flick was supported by experiments where the cells’ swimming speed and thus the compressive force was changed, resulting in a sharp decrease in flicking below a threshold speed that is consistent with the critical nature of buckling. The mechanism of turning by buckling represents one of the smallest examples in nature of a biological function stemming from controlled mechanical failure. This reorientation mechanism may enhance chemotaxis, is widespread in nature and its
discovery exemplifies the power of dynamic imaging at extreme length- and time-scales in understanding the exquisite motility adaptations of microorganisms.

This work revealed a new role of flexibility in prokaryotic flagella, or more specifically the hook, and extends the functionality of the hook beyond a simple universal joint mechanism. One potential research avenue is to directly modify the mechanical properties of the hook and study how flexibility can directly impact motility and chemotaxis. Existing genetical modification tools can be used to tune the hook's stiffness by changing the geometry (Chapter 2, ref. 25 and 26), or the material property, for instance, by binding streptavidin to biotinylated monomers (Chapter 2, ref. 22). Starting from the enteric model *E. coli* and marine model *V. alginolyticus* with distinctly different morphologies and motility patterns, this approach can be an experimental basis for molecular structural simulations of complex biomaterials such as the hook (Ch. 2, ref. 27 and 28), and furthermore provide us with insights on how flexibility sets specific motility adaptations of cells living in diverse environmental conditions.

**In Chapter three**, we found that the chemotaxis of the marine bacterium *V. alginolyticus* is strongly dependent on swimming speed. Using new microfluidic experiments and accurate single-cell tracking, we quantitatively assessed the chemotactic accumulation of cells exposed to chemical gradients as a function of their swimming speed. Faster cells had two advantages, exhibiting not only faster chemotactic migration during the transient response to a gradient, but also tighter chemotactic accumulation at the resource peak at steady state. Marine bacteria therefore stretch the boundary of the chemotactic abilities of microorganisms and represent an intriguing new model system for microbial motility studies.

The speed-dependent chemotaxis in marine bacteria opens two research avenues, one at the ecological and one at the intracellular level. First, a quantitative link between the speed-dependent behavioral adaptations and the features of the microscale marine resource landscape remains to be established, and points more in general at the need for the development of an optimal foraging theory in marine bacteria. Furthermore, the presence of an optimal swimming speed for chemotaxis may be a consequence of a trade-off between uptake benefits from enhanced chemotaxis and energetic costs of fast swimming. Second, at the intracellular level, how cells sense and control their swimming speed in order to maximize their chemotactic efficiency highlights the need to better understand the role of speed sensing in the context of the chemotactic signal transduction pathway.

**In Chapter four**, we presented a direct imaging approach to quantitatively study host-virus encounter dynamics and adsorption kinetics from thousands of individual host-virus interactions in the marine cyanobacterium *Prochlorococcus* and one of its cyanophages. We found that this direct approach towards visualizing host-virus interactions is successful at capturing individual encounters and at robustly quantifying encounter and adsorption rates. This approach also
eliminates invasive steps of classic adsorption kinetics assays, providing a more robust estimate of the adsorption efficiency. In light of the paradoxically high adsorption efficiencies often measured in marine systems, the low value of the adsorption efficiency recovered with this direct approach suggests a need to reconsider models of viral infection rates in the ocean. Furthermore, the ability to track individual hosts opens the door to understanding microbial individuality from the point of view of susceptibility to viral adsorption and infection.

The combination of different imaging technologies has the potential to become the method of choice for the study of viral infection, not only for marine systems as demonstrated here, but for a wide range of host-virus systems in different natural and clinical settings. As an example, recent advances in three-dimensional viral tracking and super-resolution microscopy enabled capturing the dynamics of viral entry, trafficking of animal viruses within live cells, and viral receptor searching on host surfaces. Further progress in expanding the use of live imaging, potentially to cover the full infection dynamics in a single experimental setup, is an exciting prospect that our work suggests is now largely within reach.

As evidenced in this thesis, imaging and in particular dynamic imaging is a uniquely powerful approach to understand the physical ecology of marine microbes and because it enables the direct visualization of processes for which we have poor intuition, and whose mechanisms may otherwise be difficult to decipher. The handshake between powerful imaging approaches and the growing field of microfluidics has great and growing potential to help explore other fundamental marine microbial processes, because of the unique capability of controlling and seeing their microscopic world, at the scale of microbes.

Finally, the physical mechanisms pertaining to motility and chemotaxis in the motile heterotrophic bacterium *V. alginolyticus*, and Brownian-motion driven encounter dynamics between viruses and the non-motile cyanobacterium *Prochlorococcus* studied in this thesis are potentially widespread among other microbes in the ocean. Revealing unexplained mechanisms and robustly quantifying interactions rates within a single-cell window can thus be a stepping-stone towards understanding the physical ecology of marine microbes, which drive marine ecosystems dynamics that sustain all life in the ocean.
APPENDIX 1

A bacterial pathogen uses dimethylsuloniopropionate as a cue to target heat-stressed corals*

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* In Appendix 1, we studied the ecological mechanism behind the association between a bacterial pathogen and its coral host, with the aim of contributing to understand the mechanisms leading to the worldwide decline of coral reefs. The ‘run-reverse-flick’ swimming strategy and high chemotactic performance characterizing V. alginolyticus were also evidenced in the coral pathogen Vibrio coralliilyticus. We measured the cells’ host seeking performance by capturing thousands of single-cell level trajectories in a microfluidic device using videomicroscopy, and quantified their chemotactic response toward diffusing coral mucus. We found that V. coralliilyticus targets a layer of the mucus from its coral host Pocillopora damicornis with striking speed and directionality.

This chapter has been published as:


I contributed to the microfluidic experiments and mathematical modeling used to quantify the chemotactic and chemokinetic responses of the coral pathogen.
ABSTRACT

Diseases are an emerging threat to ocean ecosystems. Coral reefs, in particular, are experiencing worldwide decline due to disease and bleaching, which have been exacerbated by rising seawater temperatures. Yet, the ecological mechanisms behind most coral diseases remain unidentified. Here we demonstrate that a coral pathogen, *Vibrio coralliilyticus*, uses chemotaxis and chemokinesis to target the mucus of its coral host, *Pocillopora damicornis*. A primary driver of this response is the host metabolite dimethylsulfoniopropionate (DMSP), a key element in the global sulfur cycle and a potent foraging cue throughout the marine food web. Coral mucus is rich in DMSP and we found that DMSP alone elicits chemotactic responses of comparable intensity to whole mucus. Furthermore, in heat-stressed coral fragments, DMSP concentrations increased five-fold and the pathogen’s chemotactic response was correspondingly enhanced. Intriguingly, despite being a rich source of carbon and sulfur, DMSP is not metabolized by the pathogen, suggesting that it is used purely as an infochemical for host location. These results reveal a new role for DMSP in coral disease, demonstrate the importance of chemical signaling and swimming behavior in the recruitment of pathogens to corals, and highlight the impact of increased seawater temperatures on disease pathways.

INTRODUCTION

The globally distributed marine bacterium *Vibrio coralliilyticus* (Pollock et al. 2010) causes bleaching and tissue loss in reef-building corals (Ben-Haim et al. 2003). Despite the widespread loss of corals to diseases (Harvell et al. 2009), little is known about their onset, and fundamental questions, such as how a pathogen finds its host, have remained largely unanswered (Bourne et al. 2009). Among human enteric pathogens, the ability to swim (motility) and guide movement in response to chemical gradients (chemotaxis) is a common phenotype in the infection process (Boin et al. 2004; Croxen et al. 2006). In the ocean, we found that motility is universal among putative coral pathogens (Table S1). This prevalence, together with the presence of strong chemical gradients that can extend over 2 mm from the coral surface (Kuhl et al. 1995; Mass et al. 2010), suggests that motile responses to chemical cues may be a pervasive mechanism for coral pathogens to locate and colonize their hosts. Yet, beyond evidence that motility and chemotaxis are involved in *Vibrio*-induced bleaching (Banin et al. 2001; Meron et al. 2009), there has been no direct, real-time observation of the motile behavior of pathogens, nor any insight into the specific chemical triggers of chemotaxis or its dependence on the host’s physiological state. By integrating microfluidic experiments with the collection of coral exudates, we found that *Vibrio coralliilyticus* (Pollock et al. 2010) dramatically changes its motility behavior in response to the mucus of its host, *Pocillopora damicornis*, to rapidly target the source of the cue.
The surface of a coral is lined with mucus, of variable viscosity, which is continuously excreted for cleansing, feeding, and defense (Brown & Bythell 2005). This mucus contains a broad range of chemicals, including water-soluble glycoproteins, amino acids, and metabolites (Brown & Bythell 2005). In the mucus of many coral species, the sulfur compound DMSP reaches concentrations (1-62 μM) orders of magnitude higher than in the surrounding seawater (6-11 nM) (Broadbent & Jones 2004; Van Alstyne et al. 2006). For corals, this molecule might act as an antioxidant (Sunda et al. 2002) or as an overflow system for the symbiotic zooxanthellae to excrete excess sulfur (Stefels 2000). DMSP has also been shown to be a potent chemoattractant for several marine micro- and macro-organisms (Debose & Nevitt 2007; Seymour et al. 2010). Here we show that DMSP is a primary chemical cue for V. coralliilyticus' behavioral responses to the mucus of P. damicornis and that its increased production under heat stress enhances the attraction of the pathogen.

MATERIALS AND METHODS

Organism growth conditions and laboratory mucus collection
All experiments were conducted using Vibrio coralliilyticus, strain BAA-450, acquired from the American Type Culture Collection (www.atcc.org, Manassas, Virginia, USA). Small colonies of the coral Pocillopora damicornis (from the Birch Aquarium at Scripps, La Jolla, CA, USA) were cultured 25°C in artificial seawater (Instant Ocean, Spectrum Brands Company, Cincinnati, OH) on a 12h light-dark cycle. Mucus was collected from the colonies by exposing them to air for 3 minutes. Due to volume requirements of the microfluidic assays, the mucus was then diluted 1:2 in FASW and vortexed for 10 s to mix thoroughly.

Mucus collection on Davis Reef (Great Barrier Reef)
Small colonies of the coral Pocillopora damicornis and Acropora millepora were collected from Davis Reef, Great Barrier Reef (GBR), Australia (18°05′ S/147°39′ E) and transferred to the outdoor aquarium facility of the Australian Institute of Marine Science (Townsville, Australia). Mucus was collected from the colonies by removing them from the water, shaking off excess water for 10 s, and then holding them upside down collecting dripping mucus with a syringe. Freshly collected mucus was then homogenized and divided in two: one half was flash-frozen in liquid nitrogen; the second half was directly extracted with 40 ml of HPLC-grade methanol (MeOH) for dimethylsulfoniopropionate (DMSP) quantification. The frozen portion was later used in chemotaxis assays.

Metabolism and DMSP measurements
i. DMSP metabolism
Two different basal media were used to determine the DMSP metabolic capabilities of V. coralliilyticus: a modified marine ammonium salt medium (MAMS) (Raina et al. 2009) lacking
any carbon source, and a modified basal salt medium (MBSM) lacking any sulfur source (Fuse et al. 2000). DMSP was added to both media (1 mM final concentration) and acted either as sole carbon source or as sole sulfur source. pH was adjusted to 8.2. To account for potential co-metabolism of DMSP with other compounds present in coral mucus, mucus was collected as described above, homogenized, filtered twice (0.2 μm), and sonicated for 10 minutes. Five ml of either MAMS, MBSM or sterile mucus were inoculated in triplicate from single V. coralliilyticus colonies and incubated at 28°C for between 1 and 6 days with shaking in gas-tight vials. Control bottles containing only the basal media and DMSP were used to account for possible chemical breakdown of DMSP. Results from these experiments were confirmed using an alternative V. coralliilyticus strain, LMG 23696 (Sussman et al. 2008). Bottles inoculated with Pseudovibrio sp. P12 (an alphaproteobacterium isolated from healthy P. damicornis) grown under identical conditions acted as the positive control.

ii. Acrylate metabolism
MAMS medium lacking carbon was used to investigate the ability of strain BAA-450 to degrade acrylate (1 mM, final concentration). Five milliliters of MAMS were inoculated in triplicate from single BAA-450 colonies and incubated at 28°C between 1 and 6 days with shaking. Control bottles containing only the basal medium and acrylate were set up, along with the ones inoculated with BAA-450, to account for its possible chemical breakdown. Bottles inoculated with Pseudovibrio sp. P12 grown under identical conditions served as a positive control.

iii. NMR measurements
DMSP metabolism assays and DMSP quantification were performed by 1H NMR (Tapiolas et al. 2013). Briefly, the headspace of each gas-tight vial was first sampled with a syringe. Methanol (MeOH; 40 ml) was then added to each culture tube to extract DMSP and acrylate, and the mixtures were subsequently dried in vacuo using a rotary evaporator (Buchi). The dried extracts were resuspended in a mixture of deuterated methanol (CD3OD, D 99.8%, 750 μl) and deuterium oxide (D2O, D 99.8%, 250 μl) (Cambridge Isotope Laboratories, Andover, MA, USA). A 750 μl aliquot of the particulate free extract was transferred into a 5 mm Norell tube (Norell Inc., Landisville, NJ, USA) and analyzed immediately by 1H NMR. Spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer with a TXI 5 mm probe and quantification was performed using the ERETIC method (Tapiolas et al. 2013). No DMSP degradation, acrylate by-products, or DMS smell were present in the DMSP medium experiments for V. coralliilyticus or the negative control, while all were present in the Pseudovibrio positive control. In the acrylate medium experiments, acrylate was degraded by both V. coralliilyticus and the Pseudovibrio positive control, but not the no-bacteria negative control. The same NMR protocol was also used to quantify the amount of DMSP present in coral mucus from P. damicornis and A. millepora.

Chemotactic index (Ic)
We quantified the magnitude of the chemotactic response using a chemotactic index, $I_c$, which measures the enhancement in the cell concentration within the region initially occupied by the mucus (i.e., the central band in Fig. S1), relative to the cell concentration outside that area, minus 1. $I_c = 0$ thus corresponds to a uniform cell distribution (i.e., no chemotaxis). See ref. 4 for more details. For each experiment, triplicate 0.2 μm-filtered autoclaved seawater (FASW) control trials were run first, whereby the same FASW used to grow the cells and to make the DMSP dilutions was injected into the microfluidic device in lieu of an attractant. All $I_c$ curves for a given attractant were normalized to their FASW control by subtracting the mean chemotactic index among the three FASW trials.

To compare the $I_c$ values observed in this study with values observed by Stocker et al. (Stocker et al. 2008) for E. coli and P. haloplanktis in a similar (but not identical) experimental setup, data were extrapolated from Fig. 2B of that manuscript and converted from the hot spot index ($H$) to the chemotactic index ($I_c$). The hotspot index was defined by Stocker et al. as the mean concentration of bacteria within the central, $W_c = 300$ μm wide region of the microchannel relative to the mean concentration over the entire channel width, $W = 1200$ μm. The data were converted to $I_c$ using the following conversion formula: $I_c = \{(W-W_c) / ((W/H) - W_c)\} - 1$.

**Diffusive gradient microfluidic experiments**

i. **Microinjector device for chemotaxis assays**

A 2.8 mm wide microchannel with a 400 μm wide injector (Fig. S1) was fabricated using soft lithography techniques described previously (Seymour et al. 2008) to establish diffusive gradients for chemotaxis assays. Briefly, the attractant was injected into the microchannel (Fig. S1; inlet B) as a 400 μm wide band equidistant from the channel side walls, while the cells were injected in the channel on either side of the band (Fig. S1; inlet A). The cells and attractant were flowed into the channel and then flow was stopped to allow the attractant to diffuse laterally and the cells to respond to the gradient. Dimethylsulfoniopropionate (DMSP-HCl; C5H10SO2-HCl; TCI) was freshly prepared with FASW to make 15 μM, 45 μM, and 61 μM working solutions that closely corresponded to the amount of DMSP measured in the P. damicornis and A. millepora mucus samples. A. millepora was chosen as a second species to test because V. coralliilyticus is known to infect it as well (Sussman et al. 2009). These freshly prepared DMSP solutions as well as P. damicornis mucus collected from Davies Reef (GBR; preserved at -80°C as described in section III and thawed on ice directly before experimental use; measured to contain 12-15 μM DMSP) and from corals maintained in the laboratory at MIT, A. millepora mucus from the GBR (containing 45-62 μM DMSP), and a FASW control were tested against overnight cultures of V. coralliilyticus.

The channel was loaded at moderate flow rates (2 μl per minute) to establish an initial experimental condition where the cells and the attractant were in discrete bands (Fig. S1B). To begin the experiment, the flow was stopped and the channel was imaged directly downstream of
the end of the microinjector using phase-contrast video microscopy on a Nikon Ti microscope equipped with an Andor Neo CCD camera (6.5 μm/pixel), at 1 frame per second for 6 minutes. Five replicates of each experiment were conducted and the microchannel was flushed for 30 s with fresh cells and attractant between replicates. Flushing with FASW lasted 2 minutes in between different attractants. Each video was analyzed for cell positions using automated image segmentation software developed in house with MATLAB (MathWorks, Natick, MA). Background subtraction and cross-correlation functions were used to detect non-motile cells or other particles from the mucus, which were excluded from the cumulative cell distribution across the channel.—Resulting time series of cell distributions are presented in the main text for *P. damicornis* (Fig. 1) and for *A. millepora* (Fig. S2).

ii. Temperature stress experiment on Heron Island
To test the response of *V. corallilyticus* to mucus from corals under high-temperature stress, a field experiment was carried out on Heron Island, Great Barrier Reef, Australia (23° 26' 37" S/151° 54' 44" E). Three colonies of *P. damicornis* were collected from the reef flat in front of Heron Island Research Station, fragmented into 48 nubbins, and allowed to recover and acclimate in a flow-through seawater tank pulling water from the reef flat for 8 days. Fragments were then distributed evenly into 6 tanks with 3 fragments from each donor colony in each tank. A randomized sample design for both fragment placement within the tanks and treatment assignment to each tank was employed. Three tanks were maintained at ambient seawater temperature (22°C) for the duration of the experiment and the other three tanks began at ambient temperature and then were slowly ramped by 1.5°C per day for 7 days. All fragments were sampled for mucus by air exposure as described above at the initial time and after 7 days, when the temperature-treated tanks reached 31°C. One third of the mucus samples were preserved for DMSP measurements (described above) by adding 600 μl of methanol and freezing at -20°C. Clonal replication is essential for comparing responses, because DMSP concentration can vary with irradiance, zooxanthellae density and seawater temperature (Sunda *et al.* 2002; Van Alstyne *et al.* 2006). The rest of the samples were immediately frozen at -80°C unaltered, shipped to MIT, where they were used in microfluidic chemotaxis experiments with the microinjector setup (Fig. S1). Replicate mucus samples from the heat-stress experiment were tested on three different days in the lab with freshly grown *V. corallilyticus* cells. All trials yielded comparable results to those shown in the main text (Fig. 2b; Fig. S6).

Mathematical model of simultaneous chemotaxis and chemokinesis
We modeled the chemotaxis and chemokinesis of *V. corallilyticus* by using an existing modeling framework for bacterial chemotaxis (Brown & Berg 1974; Jackson 1987; Kiorboe & Jackson 2001), augmented by a concentration-dependent swimming speed that was based on our experimental observations (Fig. 3A; SI). A new component of this model compared to previous implementations is the addition of chemokinesis, modeled as a concentration-dependent swimming speed. For simplicity, and based on results from Fig. 3A, we modeled chemokinesis
as a 24% increase in swimming speed, from 66 µm s⁻¹ at chemoattractant concentrations $C \leq 20\%$ to 82 µm s⁻¹ for $C > 20\%$. From the spatial distribution of the 3,000 bacteria across the channel at each time point, we computed the time series of the chemotactic index (as detailed in section V). This was done twice: once in the presence of chemokinesis, and once in the absence of chemokinesis, in which case the swimming speed was uniformly equal to 66 µm s⁻¹. Results are presented in Fig. 3B.

RESULTS AND DISCUSSION

To examine the ecological mechanism behind coral infection by *V. coralliilyticus* we performed chemotaxis experiments using a microfluidic assay. *V. coralliilyticus* responded to coral mucus with remarkable speed and directionality. A microfluidic device was used to create a 400-µm thick layer of mucus adjacent to a 1 mm thick seawater suspension of *V. coralliilyticus*, and we imaged the spatial distribution of the pathogen population with high-temporal-resolution (15 frames/s) video microscopy (Fig. 1; Fig. S1). Within 10 s of exposure to the mucus, bacteria began swimming up the associated chemical gradient. Within 60 s, >50% of cells had migrated into the 400-µm layer of mucus (Fig. 1; movie S1). We quantified the magnitude of the pathogen’s chemotactic response with a chemotactic index, $I_C$ (Seymour *et al.* 2010), which measures the enhancement in cell concentration within the initial mucus layer relative to the cell concentration outside of that layer ($I_C = 0$ corresponds to no chemotaxis). *V. coralliilyticus* reached $I_C > 14$ within 3 minutes of being exposed to mucus (Fig. 2A), a much more intense response than previously observed for either enteric or marine bacteria (Fig. 2A). The strength of this response was confirmed by tracking individual bacteria and quantifying their mean chemotactic velocity (Figs. S3-S5), which reached 36% of the average swimming speed, considerably higher than the 5-15% typical of the model organism for bacterial chemotaxis, *Escherichia coli* (Ahmed & Stocker 2008).

To determine the chemical signal responsible for this response, we analyzed coral mucus using quantitative nuclear magnetic resonance (qNMR (Tapiolas *et al.* 2013)). The DMSP concentrations in mucus from healthy colonies collected on Davies Reef, Australia, were high, ranging from 11.9 to 14.8 (±1.2) µM for *P. damicornis* and up to 62.2 (±2.0) µM for *Acropora millepora*, another coral species susceptible to *V. coralliilyticus* infection (Sussman *et al.* 2009). Additional chemotaxis experiments revealed that DMSP (15 µM), when used as the sole attractant, elicited a chemotactic response of comparable magnitude to *P. damicornis* mucus ($I_{C, \text{MAX}} > 14$; Fig. 2A). The pathogen’s response varied somewhat from colony to colony, as is expected owing to the natural variability in mucus composition among colonies. This variability notwithstanding, all responses observed were consistent with DMSP at comparable concentrations to those found in coral mucus being a primary driver of the chemotaxis. Additionally, all responses observed were substantially faster and stronger than previously
observed chemotactic responses of the enteric bacterium *Escherichia coli* and the marine bacterium *Pseudoalteromonas haloplanktis* to their preferred attractants (Fig. 2A). The same was true for *A. millepora* mucus when chemotactic responses to DMSP alone in mucus-equivalent concentrations were compared with responses to whole mucus (Fig. S2). These results demonstrate that DMSP within coral mucus is a major driver of the pathogen’s behavior.

Figure 1. *V. coralliilyticus* is strongly attracted to coral mucus. (A) Positions and (B) trajectories of individual *V. coralliilyticus* cells exposed to a diffusing coral mucus gradient in a microfluidic channel (SI; Fig. S1). A 400 µm thick layer of mucus, harvested from laboratory-cultured *P. damicornis* corals, was created in a microchannel (half of the layer is shown) and left to diffuse. The scale bars are 200 µm. In (A), cell positions at the start of the experiment and after two minutes are colored teal and red, respectively, and overlaid. In (B), trajectories acquired between 100 and 115 s after the start of the experiment are shown. The two panels show the strong shift in the cells’ position and their intense accumulation into the mucus layer. Also see movie S1. (C) The full time series of the spatial distribution of the pathogen population across the width of the microfluidic channel. Color and height both measure the local, instantaneous concentration of bacteria, normalized to a mean of 1. Note the intense wave of bacteria actively migrating into the mucus layer.

Chemotaxis by marine bacteria towards DMSP has previously been ascribed to DMSP’s value as a rich carbon and sulfur source, because bacteria may obtain up to 15% of their carbon and most of their sulfur from DMSP (Zubkov et al. 2001). Intriguingly, despite its vigorous swimming response towards DMSP, *V. coralliilyticus* does not detectably metabolize the compound. NMR analysis following a 6-day incubation of the pathogen in minimal media, where DMSP was either the sole carbon or the sole sulfur source, showed no degradation of DMSP by *V.*
*V. coralliilyticus* (Fig. S7). The bacterium similarly did not degrade DMSP within whole coral mucus (Fig. 2B), confirming that it cannot co-metabolize DMSP with other mucus-derived molecules. *V. coralliilyticus*’ inability to metabolize DMSP, combined with its lack of genes homologous to any known DMSP degradation gene (Table S2), indicates that DMSP is used by this pathogen purely as an infochemical, a function that DMSP and its derivatives also serve among pelagic reef fish (Debose & Nevitt 2007) and marine protists (Seymour et al. 2010; Garces et al. 2013). By demonstrating that the origin of the DMSP cue for *V. coralliilyticus* is its host, these results provide the first evidence that DMSP plays a signaling role in the onset of a bacterial disease by aiding in the detection of a suitable host.

![Diagram](image)

**Figure 2. The pathogen’s chemotaxis is primarily triggered by DMSP and is enhanced by heat stress of the host.** (A) Time series of the chemotactic index, $I_C$ (a measure of the strength of cell accumulation; SI), of *V. coralliilyticus* in response to a 400 µm layer (Fig. S1) of coral mucus (Colonies 1–3) or 15 µM DMSP (green line). Solid lines and shading represent the mean and standard error of three replicate experiments. Mucus was collected from three different colonies of *P. damicornis* on Heron Island and contained 11.9–14.8 µM DMSP (SI). The pathogen responds with comparable intensity to DMSP and mucus. Shown for reference are also the maximum chemotactic indices (suitably converted from ref. (Stocker et al. 2008); SI) attained over 15 min by *E. coli* responding to a mixture of two of its most potent chemoattractants at near-optimal concentrations (serine and aspartate, 10 µM each; grey triangle)
and by Pseudoalteromonas haloplanktis responding to algal exudates (purple triangle). All data were normalized against the respective no-attractant controls (SI). (B) Profiles from qNMR profiles (SI) of freshly collected P. damicornis mucus from Heron Island (initial mucus, purple) reveal distinct peaks for DMSP (grey boxes). Twenty-four hours of incubation of whole mucus with V. coralliilyticus (red) resulted in no measurable DMSP degradation, akin to the no-bacteria control (blue), whereas the positive control strain Pseudovibrio spp. (isolated from P. damicornis) degraded DMSP entirely, as evidenced by the disappearance of the DMSP peaks (black). (C) Time series of V. coralliilyticus' chemotactic index, $I_c$, in response to coral mucus from a clonally replicated temperature-stress experiment (maximum = 31°C) performed on Heron Island. Chemotaxis was twice as strong towards mucus from stressed coral fragments (red) as compared to fragments from the same colony maintained at ambient temperature (blue). Thin lines show each of three individual colonies (c1-c3), bold lines show their mean and shading represents the standard error. All curves were normalized against seawater controls (SI).

The importance of DMSP as a signaling cue, together with evidence that stressed corals are more susceptible to bacterial disease (Bruno et al. 2007), prompted us to examine whether the mucus of stressed corals contains more DMSP and elicits stronger responses in V. coralliilyticus compared to unstressed controls. We performed a clonally replicated high-temperature stress experiment (temperature raised 1.5°C/day over 6 days; SI) on Heron Island, Australia. Analysis by qNMR demonstrated a five-fold increase in DMSP concentration in the mucus of heat-stressed P. damicornis fragments (31°C) compared to control fragments from the same colonies (maintained at 22°C). No other molecule was substantially enriched in the stressed coral mucus compared with the controls with the exception of DMSP’s main degradation product, dimethylsulfide (DMS), toward which V. coralliilyticus chemotaxes only very weakly (Fig. S8). This result shows that DMSP may therefore be a strong cue for V. coralliilyticus to detect stressed or susceptible coral hosts. This latter hypothesis was supported by further chemotaxis experiments, which yielded a two-fold higher chemotactic index in response to mucus from stressed fragments ($I_{c,MAX}=8$) relative to mucus from control fragments ($I_{c,MAX}=4$) (Fig. 2C; Fig. S6). The stress-induced enhancement of host-released cues and the simultaneous intensification of the pathogen’s response strongly suggest that chemical signaling and active behavior represent important components in the pathway to disease. Furthermore, the widespread increase in susceptibility to bacterial infection of stressed animals (Mydlarz et al. 2006; Verbrugghe et al. 2012) suggests that chemical interactions such as these may be a recurring element in other marine diseases.

The behavioral response of V. coralliilyticus to coral mucus and DMSP is not limited to chemotaxis, but includes a second, powerful behavioral adaptation: chemokinesis. Whereas chemotaxis is the ability to bias swimming direction in response to a chemical gradient, chemokinesis is the ability to change swimming speed in response to a change in chemical
concentration. By analyzing trajectories of individual swimming cells (Fig. S3), we found that *V. coralliilyticus* exhibits a strong chemokinetic response to coral mucus (Figs. S4,S9). *V. coralliilyticus* increases its mean swimming speed by 24% (from 66 µm s\(^{-1}\) to 82 µm s\(^{-1}\)) in regions with \(C>20\%\) (where \(C\) is the local mucus concentration as a percentage of full mucus) and by up to 48% (98 µm s\(^{-1}\)) where \(C\approx60\%\) (Fig. 3A). At even higher concentrations, cells slowed down (70 µm s\(^{-1}\)), possibly to retain position near the source.

**Figure 3.** *V. coralliilyticus* exhibits chemokinesis, which dramatically increases the strength and speed of its response to coral mucus. (A) Swimming speed enhancement as a function of the mucus concentration instantaneously experienced by cells (SI; Fig S3). The enhancement is relative to the mean swimming speed in the absence of mucus (66 µm s\(^{-1}\)) and is expressed as both a speed difference (left axis) and a percent difference (right axis). Mucus concentrations, predicted from solution of the diffusion equation (SI), are expressed relative to full mucus (i.e., the initial mucus concentration in the microchannel). Error bars represent the standard error. (B) A mathematical model of bacterial motility (SI) shows that the maximum chemotactic index, \(I_{C,MAX}\), is 50% higher when chemokinesis in included (cyan) compared to the case of chemotaxis alone (gray), and that \(I_{C,MAX}\) without chemokinesis is reached in <50% of the time when chemokinesis is present. Chemokinesis was modeled as a 24% enhancement of the mean swimming speed in regions with mucus concentrations greater than 20% of full mucus, based on the observed speed-concentration relationship (panel A).

The rare ability of *V. coralliilyticus* to simultaneously employ chemotaxis and chemokinesis represents a powerful adaptation for responding to the chemical signals emanating from its coral host. Chemokinesis alone would result in the dispersion of cells away from regions of high chemical concentration because faster swimming cells have higher dispersal rates (Schnitzer 1990). However, a mathematical model (SI) reveals that, when paired with the ability to sense gradients, chemokinesis can substantially increase the chemotactic velocity and therefore reduce
the time required to traverse a chemical gradient. A modest concentration-dependent increase in swimming speed (24%) nearly halved the timescale of the response to mucus (55 s vs. 105 s to reach $I_C=10$) and increased the peak response intensity by 50% ($I_{C,MAX}$ of 15 vs. 10; Fig. 3B; SI). Although chemokinesis has been observed in a range of bacteria (Barbara & Mitchell 2003; Seymour et al. 2010), neither its disproportionate contribution to a bacterium’s ability to climb chemical gradients nor its potential importance in an infection process have been previously reported. This observation indicates that chemokinesis is a powerful behavioral adaptation in V. coralliilyticus’s response to host-derived chemical signals and that bacterial infection of corals may be driven by considerably more specific adaptations than a binary presence or absence of motility (Mcron et al. 2009).

If DMSP represents a steady signal that seemingly provides ample time to locate the coral surface, why does V. coralliilyticus exhibit multiple, energy-intensive motility (Taylor & Stocker 2012) adaptations of such magnitude? We hypothesize that the reason lies in the highly dynamic environment at the coral surface, where resident microbes contend for the best niches (Ritchie 2006), mucus is periodically shed (Garren & Azam 2012), surface cilia deter the attachment of fouling organisms (Wahl et al. 1998), and external flows on the order of millimeters per second sweep over the colony (Lesser et al. 1994). The residence time next to the surface is likely to be short, offering the pathogen only limited windows of opportunity for colonization of the host. In this complex environment, the ability to swim with high directionality (chemotaxis) and accelerate when a gradient is detected (chemokinesis) could aid V. coralliilyticus substantially in reaching the coral mucus.

Once a cell is within the mucus layer, it is only a short distance away from the coral tissue, and mucus itself is unlikely to slow its progress toward the tissue. This is supported by mathematical models (Spagnolie et al. 2013), which predict that even when the medium is viscoelastic and has twice the viscosity of seawater, a bacterium with a single polar flagellum, such as V. coralliilyticus, will actually experience a minor increase (<5%) in swimming speed. The mucus layer is furthermore often very thin (a few hundred micrometers; Jatkar et al. 2009), including for P. damicornis (Garren & Azam 2012), requiring only seconds to traverse at typical swimming speeds. Finally, our experiments on chemokinesis, conducted with a 333 μm thick layer of whole mucus, showed no evidence for any decrease in motility. Rather these experiments demonstrated an up to 48% increase in speed at intermediate mucus concentrations. As a result, we expect that bacterial pathogens will have no physical difficulty penetrating the mucus layer.
Figure 4. A new model for host detection by coral pathogens. The coral surface represents an intense source of molecules, such as DMSP, that diffuse (yellow gradient) away from the surface, through the mucus layer and out into the surrounding water, thereby establishing chemical gradients that motile bacterial pathogens (not to scale) can use to navigate toward their host. The striking prevalence of motility among putative coral pathogens (Table S1), together with the strength of the chemical signals at the coral surface relative to typical signals in the water column, indicate that the advanced motility adaptations described here could be a widespread phenotype associated with disease in the marine environment.

Our findings reveal a previously unrecognized role of DMSP in the pathway to coral disease. The discovery that DMSP is involved in a pathogen's response to its host broadens the diversity of roles that this molecule plays in the ocean (Kirst 1989; Kirst et al. 1991; Stefels 2000; Sunda et al. 2002; Debose & Nevitt 2007; Simó et al. 2009; Seymour et al. 2010) to include that of a kairomone (a chemical that benefits the receiving but not the producing organism) and, together with the recent observation of a marine parasitoid using dimethylsulfide to locate its dinoflagellate host (Garces et al. 2013), suggests that sulfur compounds may be involved in host
recognition in a broader class of marine infections. The surprising observation that *V. coralliilyticus* was unable to degrade DMSP, which is otherwise a rich carbon and sulfur source for bacteria (Zubkov *et al.* 2001), suggests that the pathogen either has an unknown pathway for utilizing DMSP at exceedingly slow rates or it uses DMSP solely as a strong infochemical: our metabolic analyses point towards the latter explanation. The finding that hosts under heat stress exude mucus that is richer in DMSP and triggers heightened pathogen responses indicates that *V. coralliilyticus* may use this chemical cue to target stressed hosts. This emphasizes the risks posed to corals by warming waters and contributes to understanding the mechanisms underlying disease outbreaks associated with increasing seawater temperatures. Taken together, these observations unveil a strong role of microscale chemical ecology and microbial behavior in coral disease (Fig. 4), emphasizing that the mechanistic drivers of many ecosystem processes may best be understood at the microscale.
REFERENCES (APPENDIX 1)


SUPPLEMENTAL MATERIAL (APPENDIX 1)

I. Review of motility among coral pathogens

A review of current literature indicates that all known putative bacterial pathogens of reef-building corals are motile (Table S1). The commonality of this trait suggests that it may play an important role in the disease process.

Table S1. Overview of currently known putative coral pathogens.

<table>
<thead>
<tr>
<th>Disease (Location)</th>
<th>Bacterium</th>
<th>Coral species infected</th>
<th>Motile</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleaching (Mediterranean)</td>
<td><em>Vibrio shilon</em></td>
<td><em>Occlina patagonica</em></td>
<td>yes</td>
<td>(Rosenberg &amp; Falkovitz 2004)</td>
</tr>
<tr>
<td>Bleaching/lysis (Indian Ocean, Red Sea)</td>
<td><em>Vibrio corallilyticus</em></td>
<td><em>Pocillopora damicornis</em></td>
<td>yes</td>
<td>(Meron et al. 2009)</td>
</tr>
<tr>
<td>White pox/White patch disease (Caribbean)</td>
<td><em>Serratia marcescens</em></td>
<td><em>Acropora sp.</em></td>
<td>yes</td>
<td>(Patterson et al. 2002)</td>
</tr>
<tr>
<td>Montipora white syndrome (Hawaii)</td>
<td><em>Vibrio owensii</em></td>
<td><em>Montipora capitata</em></td>
<td>yes</td>
<td>(Ushijima et al. 2012)</td>
</tr>
<tr>
<td>White plague (Red Sea)</td>
<td><em>Thalassomonas loyana</em></td>
<td>Multiple</td>
<td>yes</td>
<td>(Thompson et al. 2006)</td>
</tr>
<tr>
<td>White syndrome, Yellow Band (Caribbean)</td>
<td><em>Vibrio carchariae</em> (synonym for <em>V. harvey</em>)</td>
<td><em>Acropora sp.</em></td>
<td>yes</td>
<td>(Luna et al. 2010)</td>
</tr>
<tr>
<td>Yellow blotch/band (Caribbean)</td>
<td><em>Vibrio alginolyticus</em> (+3 other <em>Vibrio</em> spp.)</td>
<td><em>Montastraea sp.</em></td>
<td>yes (all 4)</td>
<td>(Cervino et al. 2004)</td>
</tr>
<tr>
<td>Black Band (widespread)</td>
<td>Consortium including <em>Phormidium corallyticum</em> and <em>Beggiatoa</em></td>
<td>Multiple</td>
<td>yes</td>
<td>(Richardson 1996)</td>
</tr>
</tbody>
</table>

II. Microfluidic experiments

i. Microinjector device for diffusive gradient chemotaxis assays
A 2.8 mm wide microchannel with a 400 μm wide injector (Fig. S1) was fabricated using soft lithography techniques described previously (Seymour et al. 2008) to establish diffusive gradients for chemotaxis assays. Briefly, the attractant was injected into the microchannel (Fig. S1; inlet B) as a 400 μm wide band equidistant from the channel side walls, while the cells were injected in the channel on either side of the band (Fig. S1; inlet A). The cells and attractant were flowed into the channel and then flow was stopped to allow the attractant to diffuse laterally and the cells to respond to the gradient. Dimethylsulfoniopropionate (DMSP-HCl; C₅H₁₀SO₂-HCl; TCI) was freshly prepared with FASW to make 15 μM, 45 μM, and 61 μM working solutions that closely corresponded to the amount of DMSP measured in the P. damicornis and A. millepora mucus samples. A. millepora was chosen as a second coral species to test because V. coralliilyticus is known to infect it as well (Sussman et al. 2009). These freshly prepared DMSP solutions, as well as P. damicornis mucus collected from Davies Reef (GBR; preserved at -80°C as described in section III and thawed on ice directly before experimental use; measured to contain 12-15 μM DMSP) and from corals maintained in the laboratory at MIT, A. millepora mucus from the GBR (containing 45-62 μM DMSP), and a FASW control were tested against overnight cultures of V. coralliilyticus.

The channel was loaded at moderate flow rates (2 μl per minute) to establish an initial experimental condition where the cells and the attractant were in discrete bands (Fig. S1B). To begin the experiment, the flow was stopped and the channel was imaged directly downstream of the end of the microinjector using phase-contrast video microscopy on a Nikon Ti microscope equipped with an Andor Neo CCD camera (6.5 μm/pixel), at 1 frame per second for 6 minutes. Five replicates of each experiment were conducted and the microchannel was flushed for 30 s with fresh cells and attractant between replicates. Flushing with FASW lasted 2 minutes in between different attractants. Each video was analyzed for cell positions using automated image segmentation software developed in house with MATLAB (MathWorks, Natick, MA). Background subtraction and cross-correlation functions were used to detect non-motile cells or other particles from the mucus, which were excluded from the cumulative cell distribution across the channel. Resulting time series of cell distributions are presented in the main text for P. damicornis (Fig. 1) and for A. millepora (Fig. S2).
Figure S1. The microinjector chemotaxis assay. (A) A schematic of the entire channel where A is the inlet used to inject cells and B is the entrance point for the attractant into the microinjector (elongated structure on the right of B). C is the exit point of the channel. The dashed box shows where the camera field of view is focused during experiments to image half of the channel width. The channel is 100 μm deep. (B) A close-up picture of the microinjector (whose boundaries were marked by white lines for clarity) and of the central band it creates in the microchannel, visualized by addition of 100 μM fluorescein.

Figure S2. The pathogen’s chemotaxis is primarily triggered by DMSP in coral mucus. The chemotactic response of *V. coralliilyticus* to a second coral species susceptible to infection, *A. millepora* (compare with Fig. 1 in the main text for *P. damicornis*). The mucus samples (blue lines) were run simultaneously with pure DMSP at the same concentration measured in each sample (green lines). All curves have been normalized by subtracting the mean value of the chemotactic index $I_c$ over three FASW control runs, at each time point. The solid lines are the averages from three runs with each attractant and the shading denotes the standard error of the mean. Note how response levels were similar for whole coral mucus and DMSP alone, as found also for *P. damicornis*. (A) Colony 1 was measured to contain $45.3 \pm 0.2$ μM DMSP, and thus 45 μM DMSP (final concentration) diluted in FASW was run in parallel. (B) Colonies 2 and 3 were measured to contain $60.7 \pm 0.9$ μM and $62.4 \pm 2.0$ μM DMSP, respectively, and were thus tested in parallel with 61 μM DMSP.

ii. 3-inlet device for single-cell tracking
To explore both the chemotactic and chemokinetic behaviors of *V. coralliilyticus* at the single cell scale, a 1 mm wide, 3-inlet channel (Fig. S3) was used to establish a transient diffusive gradient for long trajectory tracking. This channel configuration allowed a layer of FASW to be placed in between the cells and the attractant, where the latter was mucus, 100 μM DMSP or FASW (control). Compared to the microinjector (Fig. S1), the 3-inlet channel has the advantage
of extending the duration of the gradient with the added layer of seawater between the cells and the attractant; thus, a longer window in which to visualize the dynamics of a single cell’s behavioral response to the gradient is achieved. A 1:50 dilution of the overnight culture in FASW was injected in the upper inlet, FASW in the middle inlet, and the test attractant in the lower inlet. The channel was loaded at moderate flow rates (10 µl min⁻¹) to establish an initial condition where the three solutions were in three equal and discrete bands. To begin the experiment, the flow was stopped instantly by applying a pulse of negative pressure. Consequently, test attractants diffused across the width of the channel and the channel was imaged directly downstream of the inlet for 3 minutes in order to acquire long trajectories of individual bacteria. Darkfield video microscopy (15× objective, 60 frames/s) on a Nikon Ti microscope equipped with a Photron high-speed camera (SA-3, 17 µm/pixel) was used for video acquisition. Five replicates of each experiment were conducted and the microchannel was flushed for 30 s with fresh cells, FASW, and attractant between replicates. Flushing with FASW lasted for 2 minutes in between different attractants.

**Figure S3.** This 3-inlet channel is 100 µm deep and has three 333 µm wide inlets that converge to form a 1 mm wide test channel with a single outlet at the end. The three inlets are used to create parallel bands of different solutions, as indicated in the figure. Bacteria responding to the diffusive gradients of the attractant are imaged immediately downstream of the junction between the three inlet microchannels.

A minimum of 50 trajectories were recorded per experiment and Fig. S4 shows 30 randomly selected trajectories from each test attractant. In the presence of diffusing DMSP or mucus exudates, cells swam towards the region of higher attractant concentration and the average swimming speed of the population, calculated from trajectories of individual bacteria and averaged over the entire width of the channel and over ~30 s after start of the experiments, increased 17% and 19% in the presence of DMSP and mucus exudates, respectively (Fig. S4). This increase in speed is a result of chemokinesis – the process by which an organism changes its swimming speed in response to a change in the chemical concentration in its immediate environment.
Figure S4. Trajectories of individual bacteria obtained in the 3-inlet microchannel (Fig. S3) in response to a diffusing, initially 333 μm wide layer (initial position identified by the gray bar along the y axis) of (A) FASW (control); (B) 100 μM DMSP; and (C) freshly collected coral mucus. The entire width of the channel (1 mm) is captured in the field of view. Open circle indicates starting points of each trajectory. Cells exposed to seawater maintain a relatively
uniform, low speed, whereas cells exposed to mucus and DMSP considerably accelerate, a behavior indicative of chemokinesis. Note that trajectories were acquired at different times: because the initial attractant layer diffused, local instantaneous concentrations varied for different trajectories. This time component is removed from the analysis, truly demonstrating chemokinesis, in an analysis of the swimming speed as a function of the instantaneous local concentration, shown in Fig. 3A of the main text. In all panels, x and y are the coordinates across and along the microchannel, respectively.

However, this result does not reveal the full magnitude of the chemokinetic response because the change in speed was computed as an average over space and time, and cells experience different attractant concentrations at different positions and different times (since the gradient diffuses). To better identify the chemokinetic response, we sought to identify the relationship between the swimming speed and the mucus concentration, C (Fig. 3A in main text). To do so, we first computed the concentration of mucus exudates at each point across the channel for the entire time covered in the experiments by numerically solving the one-dimensional diffusion equation across the width of the microchannel in a domain having the same geometry as the experimental setup (Fig. S3) and with a diffusion coefficient $D_c$. To the best of our knowledge, the diffusion coefficient of DMSP is unknown. Therefore, we computed it using a correlation formula for the diffusivity of small molecules in water based on their molar volume (Wilke & Chang 1955), obtaining a value ($D_c = 10^{-9}$ m$^2$ s$^{-1}$) that is consistent with what has been used previously (Breckels et al. 2010). Trajectories were then used to compute instantaneous values of the swimming speed of single cells, $V_s$, and for each value of $V_s$ we determined the instantaneous mucus concentration, $C$. This allowed us to bin instantaneous swimming speeds according to instantaneous concentrations (Fig. 3A in the main text), revealing the actual magnitude of the chemokinetic response (see main text).

III. Chemotactic velocity ($V_C$)

The chemotactic velocity, $V_C$, is the mean drift velocity of a cell population in the direction of a gradient. In the microinjector experiments the chemoattractant gradient varied in space and time (Seymour et al. 2008), thus $V_C$ also varied spatially and temporally (Fig. S5). We calculated $V_C$ from the cell concentration profiles by numerically solving the bacterial transport equation (Keller & Segel 1971; Ahmed & Stocker 2008) and finding the parameters (chemotactic sensitivity and mean swimming speed) that best fitted the experimental cell distribution profiles, using a least squares method. The diffusivity $D_B$ of the bacterial population was calculated from the dispersion of the cell distribution in the control experiments and was found to be $2 \times 10^{-9}$ m$^2$ s$^{-1}$. This value is consistent with the random walk behavior of the bacteria, which leads to a diffusivity $D_B = V_s^2 \tau_0/3$ (Berg 1993), which would give $V_s \sim 77$ µm s$^{-1}$ with $\tau_0 \sim 1$ s (see section X).
Figure S5. Chemotactic velocity, $V_c$, of *V. coralliilyticus*, computed from the microinjector chemotaxis experiment shown in Fig. 1 (see device in Fig. S1). (A) The control experiment where FASW was injected into the channel showed no chemotaxis. (B) Coral mucus injected into the channel as a band initially spanning $x = 0$ to 200 μm, injected at time zero, elicited strong chemotaxis. The vertical axis and the color bar both denote speed.
Figure S6. Stressed corals elicit stronger chemotactic responses. Results of individual chemotaxis assays performed in the microinjector channel (Fig. S1) for mucus collected from the Heron Island temperature-stress experiment. For each, the full time series of the spatial distribution of the pathogen population (vertical axis) across the width of the microfluidic channel, x, is shown (the experimental layout is the same as in Fig. 1C). Color and height both measure the local, instantaneous concentration of bacteria, normalized to a mean of 1, and the color bar is the same for all panels. ‘Stressed’ samples are from the final sampling time point where the fragments were at 31°C, whereas the control, ‘non-stressed’ samples are from the same donor colony but had been maintained at 22°C. Note how, despite some variability, stressed corals consistently induced a stronger chemotactic response compared to non-stressed corals.

Figure S7. DMSP is not degraded by the pathogen during 6 day incubations. NMR traces after 6-day incubations in minimal media in which DMSP was (A) the sole carbon source and (B) the sole sulfur source. The “Control” traces refer to the growth medium where no bacteria were inoculated. The red and black traces show the results from inoculation with *V. coralliilyticus* and *Pseudovibrio* sp. (positive control), respectively. In both A and B, the DMSP peaks (black boxes) have the same intensity in the *V. coralliilyticus* treatment and in the no-bacteria, control, indicating that *V. coralliilyticus* did not consume DMSP, whereas they are depleted in the positive control. In the case of DMSP as a sole sulfur source (B), *Pseudovibrio* consumed the DMSP and other carbon sources in the medium and produced secondary metabolites (appearance of new peaks).
Figure S8. DMSP and DMS are the sole chemicals whose production greatly increased during heat stress. (A) Representative NMR spectra of *P. damicornis* mucus exposed to two different temperatures: 22°C (control) and 31°C (thermal stress). The chemical composition of coral mucus changed under thermal stress, with a dramatic increase in DMSP and its breakdown product DMS. The other natural constituents of coral mucus are largely unaffected by the rise in temperature. (B) Results from chemotactic assays demonstrating that *V. coralliilyticus* is only very weakly attracted to DMS in comparison to whole mucus. For reference, I_c = 0 corresponds to no chemotaxis and these experiments

IV. Steady gradient experiments in agarose microchannel

To test the chemokinetic response of *V. coralliilyticus* in a steady gradient, as a point of comparison to the unsteady, diffusive gradient experiments presented in Fig. 3A (see devices in Figs. S3, S4), a steady linear gradient of mucus solutes was created using a 2 mm wide agarose microchannel (Fig. S7A), fabricated by integrating a layer of agarose into a PDMS device as described previously (Ahmed et al. 2010). The agarose layer was made with 1.5% agarose in FASW and the overnight culture was diluted 1:3 in FASW to achieve an appropriate cell density for accurately tracking individual cells. Mucus solutes diffused through the agarose underneath the microchannel (Fig. S7B) and the gradient was allowed to establish in the channel by diffusion for a time of \( L^2/(2D_C) \sim 33 \text{ min} \) (Ahmed et al. 2010) before cells were added to the test channel (here, \( L \) is the distance between the source and sink channels, \( D \sim 10^{-9} \text{ m}^2 \text{ s}^{-1} \) is the diffusion coefficient of DMSP, and \( L^2/(2D_C) \) is the characteristic diffusion time of the system). The entire width of the channel (Fig. S7a, x-direction) was imaged using a mosaic of 5 adjacent videos, each having a field of view of 296 μm × 395 μm, lasting 5 s and acquired at 10 frames/s. This was repeated for 20 different positions along the length of the channel (Fig. S7a, y-direction). From the 100 videos acquired, cell abundance and swimming speed were averaged over 150 μm wide bins across the width of the channel (see Fig. S7c), where the highest
concentration of mucus is in the $x = 1800-1950 \, \mu m$ bin and the lowest is in the $x = 0-150 \, \mu m$ bin. The same experiment was repeated with a seawater control, using FASW in lieu of the mucus exudates.

Figure S9. (A) Schematic planar view of the diffusion-based agarose microfluidic device showing source, sink and test channels. The space between the channels is 200 $\mu m$. (B) Schematic vertical cross section of the same device. By diffusing through the agarose and into the sink channel, mucus exudates flowing in the source channel form a linear gradient in the agarose slab, which in turn is transferred passively by diffusion into the test channel, where cells are contained. See ref. (Ahmed et al. 2010) for details on the layout, fabrication and operation of this microdevice. (C) Tracking of cells in a steady linear gradient of mucus solutes (illustrated schematically by the green shading) shows that pathogen accumulation (blue squares) in the region of highest mucus concentration is accompanied by an increase in the population-averaged swimming speed (red circles) with increasing mucus concentration. This is a result of chemokinesis. The relative swimming speed measures the increase in the average population swimming speed over the swimming speed in the lowest concentration region ($x = 10 \, \mu m$). The speed enhancement observed in this device (up to 120%) is greater than for the single-cell
tracking experiment (Fig. 3A in the main text) because here the mean population speed was reduced by cells interacting with the agarose channel bottom, making percentage speed enhancements appear larger. Both experiments demonstrate that *V. coralliilyticus* exhibits strong chemokinesis.

**V. Mathematical model of simultaneous chemotaxis and chemokinesis**

We modeled the chemotaxis and chemokinesis of *V. coralliilyticus* by using an existing modeling framework for bacterial chemotaxis (BROWN & BERG 1974; Jackson 1987; Kiorboe & Jackson 2001), augmented by a concentration-dependent swimming speed that was based on our experimental observations (Fig. 3A). The model integrates the trajectories of individual bacteria as they swim in a chemoattractant gradient, within a domain that has the same width and boundary conditions as in the microfluidic setup used to quantify the chemotactic index of *V. coralliilyticus* responding to mucus or DMSP (Fig. S1). In this setup, the attractant gradient is transient, following the ‘release’ of an initial band of attractants. Thus, the spatiotemporal evolution of the attractant concentration at every position in the channel and every point in time was obtained by integrating the one-dimensional diffusion equation using MatLab (Mathworks, Natick, MA). The choice of the diffusivity, $D_C = 10^{-9} \text{ m}^2 \text{s}^{-1}$, was based on the diffusion coefficient of DMSP as described in section VI.ii. The model, largely based on *Escherichia coli*, as customary due to the lack of specific information for other species of bacteria, assumes that bacterial motion can be divided into runs (nearly straight swimming segments) and tumbles (reorientations in the swimming trajectory). When the bacterium experiences increasing attractant concentration, the probability of tumbling decreases (and vice versa), leading to positive chemotaxis.

The model is described in detail in ref. (Jackson 1987). Briefly, for each case we simulated 3,000 bacteria, initially distributed uniformly in the region of the channel not occupied by the central, 400-μm wide chemoattractant layer (Fig. S1). Bacteria in the simulations did not interact with each other. During a run a bacterium swims straight at a constant speed of 66 μm s$^{-1}$ (the mean speed of a population measured in the absence of chemoattractants; see below for a modification to this rule in the case of chemokinesis), except for the effect of rotational diffusion, arising due to random collisions with water molecules and responsible for a random reorientation component. The value of the rotational diffusion coefficient, $D_R = 0.035 \text{ rad}^2 \text{s}^{-1}$, was based on a resistive force model that accounted for both the cell body (3.2 μm long and 1.2 μm wide) and the helical flagellum (contour length 4.6 μm, pitch 1.5 μm) of the bacteria, as done in ref. (Marcos et al. 2012). During every time step of the integration, Δt, a new swimming direction was chosen at random (tumble) if a randomly generated number (between 0 and 1) was smaller than the probability of tumbling $P_r$, given by
At $P_t = -$Here, $\tau$ is the mean run time, given by

$$\ln \tau = \ln \tau_0 + \alpha \frac{dP_b}{dt},$$

$$\frac{dP_b}{dt} = \frac{1}{T_m} \int_{-\infty}^{t} \frac{dP_b}{dt'} \exp \left[ \frac{t-t'}{T_m} \right] dt',$$

$$\frac{dP_b}{dt} = \frac{K_D}{(K_D + C)^2} \frac{dC}{dt},$$

where $T_m = 1$ s is a time constant of the bacterial system (a memory term), $\tau_0 = 1$ s is the mean run time (tumbling interval) in the absence of concentration gradients, $C$ is the mucus concentration relative to the maximum mucus concentration initially released in the microchannel, $\alpha = 660$ s is a system time constant (amplification factor), $P_b$ is the fraction of surface receptors bound by the substrate, $dP_b/dt$ is a weighted rate of change of $P_b$, and $K_D = 20$ is the half-saturation constant of the surface receptor binding to the attractant (also relative to the maximum initial attractant concentration). The choice of parameter values is in line with previous models (Jackson 1987; Kiorboe & Jackson 2001), with the exception of $K_D$, for which a value was chosen that produced a chemotactic index of comparable magnitude to the experimentally observed one (Fig. 2A in the main text). The model, then, allowed for the comparison of the chemotactic index in the presence and absence of chemokinesis.

VI. Blast search for DMSP degradation genes

Given our finding that DMSP is a strong chemoattractant for $V. coralliilyticus$ even though the pathogen was unable to metabolize it, we further investigated the presence of genes involved in DMSP transformation in the $V. coralliilyticus$ genome. While uptake of DMSP is known to be mediated by transporters in the BCCT and ABC families (for example C9NQ29_9VIBR and C9NQK8_9VIBR in $V. coralliilyticus$; (Sun et al. 2012)), two main biochemical routes have been found in bacteria degrading DMSP (Moran et al. 2012): (a) the $dmdA$ gene allows DMSP demethylation and (b) $ddd$ genes mediate the conversion of DMSP to dimethylsulfide (DMS). To cover a broader range of possibilities, we performed a blast search for DMSP degradation genes where we considered all of the confirmed sequences available in the literature for each gene involved in DMSP degradation (Howard et al. 2006; Todd et al. 2007; Howard et al. 2008; Todd et al. 2009; Curson et al. 2011; Todd et al. 2011; Todd et al. 2012). Table S2 lists, for each gene, the species whose genomic sequences have been considered in this analysis.
In order to assess the potential ability of *V. coralliilyticus* to metabolize DMSP we looked for statistically significant alignments (blast v. 2.2.27+) of all the sequences for each gene in Table S2 to any genome available for *V. coralliilyticus* in NCBI’s nucleotide collection database (*V. coralliilyticus* P1, *V. coralliilyticus* YB1, *V. coralliilyticus* LMG 20984, *V. coralliilyticus* ATCC BAA-450, *V. coralliilyticus* CAIM616). In line with our results from the metabolic experiments, none of these queries returned alignments that would suggest any of the probed genes were present in *V. coralliilyticus* (best matches had <1% coverage and alignment scores <40). Similar results were obtained by extending the same alignment procedure to all species in the genus *Vibrio*. Together these results suggest that *V. coralliilyticus* may not have evolved mechanisms to metabolize DMSP. Therefore, chemotaxis towards this compound has most likely evolved for purposes other than metabolism in this organism.

Table S2. DMSP degradation genes blasted against *V. coralliilyticus* genome.

<table>
<thead>
<tr>
<th>DMSP degradation gene</th>
<th>Sequence source</th>
</tr>
</thead>
</table>
| **dddD**              | *Marinomonas* sp. MWYL1  
*Burkholderia cepacia* AMMD  
*Rhizobium* sp. NGR234  
*Marinomonas* sp. MED121  
*Marinobacter* sp. ELB17  
Marine gammaproteobacterium HTCC2207  
*Hoeflea phototrophica* DFL-43  
*Sagittula stellata* E-37  
*Burkholderia phymatum* STM815  
*Burkholderia ambifaria* MC40-6  
*Dinoroseobacter shibae* DFL 12  
*Ruegeria pomeroyi* DSS-3 |
| **dddL**              | *Dinoroseobacter shibae* DFL 12  
*Fulvimarina pelagi* HTCC2506  
*Loktanella vestfoldensis* SKA53  
*Oceanicola batsensis* HTCC2597  
*Rhodobacter sphaeroides*  
*Rhodobacterales bacterium* HTCC2654  
*Stappia aggregata* IAM 12614  
*Sulfitobacter* sp. EE-36 |
| **dddP**              | *Aspergillus oryzae* RIB40  
*Ruegeria pomeroyi* DSS-3 |
| **Roseobacter litoralis** Och 149 |
| **Oceanicola granulosus** HTCC2516 |
| **Phaeobacter gallaeciensis** 2.10 |
| Roseovarius sp. TM1035 |
| Rhodobacterales bacterium HTCC2255 |
| **Roseobacter denitrificans** OCh 114 |
| Roseovarius sp. 217 |
| Jannaschia sp. CCS1 |
| **Phaeobacter gallaeciensis** BS107 |
| Rhodobacterales bacterium HTCC2150 |
| Roseobacter sp. SK209-2-6 |
| Roseovarius nubinhibens ISM |
| Silicibacter sp. TM1040 |

| dddQ | **Ruegeria pomeroyi** DSS-3 |
| Roseovarius nubinhibens ISM |

| dddW | **Ruegeria pomeroyi** DSS-3 |
| Roseobacter sp. MED193 |

| dddY | **Shewanella baltica** OS155 |
| **Shewanella piezotolerans** WP3 |

| dmdA | **Roseovarius nubinhibens** ISM |
| **Dinoroseobacter shibae** DFL 12 |
| Jannaschia sp. CCS1 |
| Silicibacter sp. TM1040 |
| Alphaproteobacterium BAL199 |
| Rhodobacterales bacterium HTCC2150 |
| **Pelagibacter ubique** HTCC1062 |
| Roseovarius sp. TM1035 |
| Marine gammaproteobacterium HTCC2080 |
| Roseobacter sp. MED193 |

**Supplementary references**


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In Appendix 2, we studied the impact of increased seawater temperatures on the chemical interactions between bacterial pathogens and coral hosts. Using dynamic imaging and videomicroscopy, we captured the swimming behavior and chemotactic performance of thousands of individual pathogen cells at different temperatures. We found that increasing water temperatures in the range of typical natural conditions induce two temperature-dependent behavioral switches in the coral pathogen: a boost in chemotactic performance occurs when seawater temperatures rise above 23°C and a further swimming speed enhancement occurs above 27°C.

This chapter is in the final stages of preparation for a journal submission as:


In this work, I contributed the quantitative cell trajectory analysis to assess the impact of seawater temperature on the chemotactic responses of the coral pathogen.
ABSTRACT

Reef ecosystems are important sources of revenue, sustenance, and biodiversity that are currently in decline due to the confluence of many stressors, including warming seawater temperatures and disease. Bacterial pathogens have been shown to deploy exquisite behavioral adaptations based on chemical sensing and motility to locate coral hosts. Yet, while there is evidence that increasing seawater temperatures increase the occurrence and severity of coral disease, little is known about the effect of warming temperatures on the bacterial behaviors involved in colonization and infection. Here we present data demonstrating that increasing water temperatures induce two behavioral switches in the coral pathogen *Vibrio corallilyticus* that considerably augment the bacterium’s performance in tracking the chemical signals of its coral host, *Pocillopora damicornis*. Coupling field-based heat-stress manipulations with laboratory-based observations in microfluidic devices, we recorded the swimming behavior of thousands of individual pathogen cells at different temperatures, covering the range of typical natural conditions. Analysis of the swimming statistics revealed two distinct behavioral switches, in the form of major improvements in motility performance above specific temperature thresholds. The pathogen’s chemotactic ability increased by $\geq 60\%$ when temperature exceeded 23°C, while chemokinesis increased by $>40\%$ when temperature exceeded 27°C. Thus, the pathogen’s host-seeking performance initially increases when seawater temperatures rise above 23°C and a second time when they rise above 27°C. This work demonstrates the potential increasing seawater temperatures have for improving the efficiency of the microscale host-seeking behaviors that drive a marine pathogen’s success. Moreover, our observations of temperature-dependent chemokinesis presents an entirely new view of bacterial behavioral dynamics that diverges from the common models used to study bacterial motility, such as *E. coli* and *B. subtilis*.

INTRODUCTION

Although disease is a natural process in any ecosystem, the influence of a changing climate on host-pathogen interactions has the potential to dramatically shift the incidence of disease and thus alter ecosystem structure and functions in unpredictable ways (Burge et al 2014). There is growing evidence that marine diseases are most prominent during the warmest months of the year (Burge et al 2014, Case et al 2011, Heron et al 2010) and the cooler months serve as a respite from disease progression for the host animals. Furthermore, evidence is mounting that as mean winter temperatures rise, some critical ecosystem engineers, such as reef-building corals in the Caribbean, are losing their seasonal reprieve from disease advancement (Burge et al 2014, Weil et al 2009). Coral reefs are important reservoirs of marine biodiversity and provide many ecosystem services that benefit coastal communities (Hoegh-Guldberg 2004), and thus
understanding how warming seawater temperatures will affect their health is an important component of successfully managing these marine resources. Coral reefs around the globe are declining due to a confluence of factors, including increased disease and rising ocean temperatures (Hoegh-Guldberg 2004, Pandolfi et al 2003, Ruiz-Morenol et al 2012). While it is generally held that warming favors disease (Burge et al 2014, Cervino et al 2004, Jones et al 2004) and destabilizes the coral-algal symbiosis that is critical for coral survival (Baker et al 2008), the mechanisms through which warmer waters favor disease have remained elusive. Here we test the hypothesis that warmer waters may directly favor bacterial pathogens by enhancing their host-seeking abilities.

Several bacterial diseases of corals are linked specifically to increases in seawater temperature. For example, the chemotactic bacterium Vibrio shiloi causes slow partial bleaching at 23°C and rapid severe bleaching at 29°C in the Mediterranean coral Oculina patagonica (Banin et al 2001, Toren et al 1998). A dramatic increase in the growth rate of Caribbean Yellow Band Disease (CYBD) lesions during the winter months between 1998 and 2010 has been associated with an increase in winter seawater temperatures exceeding 27°C (Burge et al 2014, Weil et al 2009). The globally distributed bacterial pathogen of corals (Pollock et al 2010), Vibrio coralliilyticus, causes rapid tissue lysis of its coral host, Pocillopora damicornis, when temperatures exceed 26°C (Ben-Haim and Rosenberg 2002, Ben-Haim et al 2003). Recently, we demonstrated that V. coralliilyticus uses two specific behaviors, the ability to swim up chemical gradients (chemotaxis) and the ability to increase swimming speed in the presence of coral mucus (chemokinesis), to navigate toward its host, and that the concentration of an important chemical signal in that process, the sulfur compound dimethylsulfiniopropionate, is augmented when the coral hosts are heat-stressed (Garren et al 2014).

Increases in seawater temperatures can, in general, affect both the host and the pathogen. However, the effect of temperature on the pathogen’s host-sensing behaviors have not been tested. While warmer temperatures might increase the chance of infection in several ways, such as increasing bacterial growth rates or virulence (Kimes et al 2012), all putative bacterial pathogens of corals that have been identified thus far are motile cells (Garren et al 2014) that likely use their motility during the infection process. Increases in temperature have been previously reported to increase the swimming speeds of the enteric model organism Escherichia coli (Maeda et al 1976) and at least two marine Vibrio species, Vibrio alginolyticus (Magariyama et al 1995) and Vibrio anguillarum (Larsen et al 2004). The chemotactic responses measured using capillary assays in those studies showed that the maximum responses were highly variable with respect to different temperatures among the different organisms, indicating that temperature may influence behaviors among different bacteria in strain-specific ways. Thus, quantifying the effect of temperature specifically on the navigational performance of the pathogen V. coralliilyticus toward its coral host (or the mucus) is a key step in predicting the larger-scale effects of warming seawater temperature on coral disease outbreaks. Here we use
video microscopy and microfluidic technologies under carefully controlled temperature conditions to test the hypothesis that rising seawater temperatures, in addition to the known effect of making the host more susceptible, simultaneously enhance the pathogen’s motility and host-seeking capabilities.

MATERIALS AND METHODS

Two sets of experiments were conducted to (i) isolate the effect of temperature on pathogen behavior, and (ii) test the simultaneous and combined effect of temperature on host and pathogen. The first set of experiments was conducted by growing the pathogen, *Vibrio coralliilyticus*, at four temperatures (20°C, 23°C, 27°C, and 30°C) that span the range of seasonal mean temperatures experienced by this host-pathogen pair and include two temperatures (23°C and 27°C) that straddle the previously determined 26°C trigger point for increased virulence (Ben-Haim et al 2003, Kimes et al 2012). We explored the bacterium’s chemotactic and chemokinetic responses at these four temperatures by using a single, homogenized pool of coral mucus collected from laboratory-cultured corals growing at a moderate temperature (25°C). The second set of experiments coupled pathogen growth at ambient and high temperatures (22°C and 30°C) with mucus from field-collected corals before (22°C) and after a heat-stress (31°C) treatment to test the simultaneous effect of temperature on the host-pathogen interactions.

Organism growth conditions

Small colonies of the coral *Pocillopora damicornis* were cultured in the laboratory at 25 ± 1°C in artificial seawater (Instant Ocean, Spectrum Brands Company, Cincinnati, OH) on a 12 h light-dark cycle. All experiments were conducted using the bacterium *Vibrio coralliilyticus*, strain BAA-450, acquired from the American Type Culture Collection (www.atcc.org, Manassas, Virginia, USA). In the first set of experiment, cells were grown for 16–18 h, shaking (300 rpm), in 0.2 μm-filtered, autoclaved seawater (FASW) with 1% marine broth (2216; BD Difco) at each of four temperatures: 20°C, 23°C, 27°C, and 30°C. For growth below room temperature (20°C and 23°C), a chilling incubator (Multi-Therm, Denville Scientific, South Plainfield, NJ) was used. Given the availability of only one chilling incubator, experiments were run back to back on subsequent days with 30°C treatments repeated on each day as a standard: all replicates for 20°C, 27°C and 30°C were performed on one day and all replicates for 23°C and 30°C were performed the next day. Data were then normalized to the 30°C experiment from each given day. Three replicate cultures were grown at each temperature each day, all from the same three starting colonies streaked from glycerol stocks onto marine broth agar plates. The same starting colonies for a given experiment were used on both days to ensure that every temperature had identical biological replicates. In the second set of experiments using mucus collected in the field (see below), bacteria were grown at temperatures corresponding to the ambient (22°C) and heat-
stressed (30°C) coral conditions, again using the same 3 starting colonies to initiate the biological replicates for both temperatures.

Mucus collection
For the first set of experiments, mucus was collected in the laboratory from coral colonies cultured at 25°C by exposing them to air for 3 min over a sterile 50 ml tube (Falcon, Corning Life Sciences, Tewksbury, MA). Due to the volume requirements for completing all microfluidic experiments with a homogeneous pool of mucus, collection was carried out on each of 4 colonies once per day for 5 days, and stored at -80°C. Directly before the experiment, a single homogenized pool of mucus was created by thawing all of the frozen mucus (all colonies, all days), pooling samples together in a single 50 ml tube, vortexing for 2 min and subsequently using the pooled sample in experiments.

In the second set of experiment, to determine the effects of temperature on *V. corallilyticus* and its coral host *P. damicornis*, we ran a set of microfluidic experiments testing chemotaxis (see below) of cells grown at one of two temperatures (22°C and 30°C) to mucus from coral fragments before (T₀ = 22°C) and after (Tᵣ = 31°C) high-temperature stress conditions. The field experiment carried out on Heron Island, Great Barrier Reef, Australia (23° 26' 37" S/151° 54' 44" E) to obtain these mucus samples was described previously (Garren et al 2014). Briefly, clonally replicated heat-stress experiments were performed using *P. damicornis* over the course of one week. Mucus was collected from each fragment at the beginning (temperature T₀) and end (temperature Tᵣ) of the experiment by air exposure, as described above. Samples were immediately frozen at -80°C, shipped to MIT on dry ice, and subsequently used in microfluidic experiments.

Microfluidic chemotaxis experiments
To test the chemotactic response of *V. corallilyticus*, we used a 600 μm wide, 100 μm deep, channel with three inlets (Fig. 1a) to establish a transient chemical gradient by molecular diffusion. The device was fabricated using soft lithography techniques described previously (Seymour et al 2008, Weibel et al 2007) bonded to a microscope slide, and mounted onto a Nikon Ti microscope (Tokyo, Japan). All experiments were conducted at the same temperature at which the cells were grown, using a temperature controlled stage insert (Warner Instruments, Hamden, CT). Cell filtrate was used both as the control attractant and as the buffer in the center inlet, to create a band separating the cells and the mucus in the microchannel (see below). Cell filtrate was obtained by passing 1.5 ml of each cell culture through a 0.2 μm syringe filter into a sterile 1.5 ml tube (Eppendorf, Hamburg, Germany). The filtrate from each of three cultures (biological replicates) at each temperature was prepared, and then the three filtrates for a given temperature were pooled to create one homogenous filtrate for each temperature.
Figure 1. Schematics of the microfluidic devices used to study the temperature dependence of coral pathogen motility behaviors. (a) Planar view of the three-inlet channel used for the chemotaxis experiments. Three inlets converge into a 600 μm wide, 100 μm deep channel, creating three, 200 μm streams of cells, seawater and attractant, respectively. The imaging window is denoted by the red box. (Inset) Diffusion of the attractant across the channel (x direction) creates chemical gradients to which bacteria can respond by chemotaxis. (b) Parallel holding chambers, 100 μm in depth, used for the chemokinesis experiments. Bacteria injected through the inlet were observed in the test chamber and the 8 chambers on a chip allowed for parallel experiments. Scale bar = 500 μm.

The microfluidic devices were loaded using 23 gauge blunt-end needles (Grainger, Lake Forest, IL) attached to 1 ml gastight luer tip syringes (Hamilton, Reno, Nevada) and infused from a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA). Three syringes were filled with cells, filtrate, and attractant, respectively, and connected by Tygon tubing (0.02” ID, 0.06” OD; Cole-Parmer, Vernon Hills, IL) to the three inlets of the channel. Syringe contents were flowed into the channel at a moderate flow rate (2 μL min⁻¹) to establish an initial experimental condition where cells, filtrate, and attractant (i.e., mucus) – in this order – formed three adjacent 200 μm wide bands (Fig. 1a). The flow was then stopped, resulting in the mucus chemicals diffusing across the width of the microchannel and forming a gradient along x-direction. The cell behavior in response to this gradient was recorded for 165 s with video microscopy directly downstream of the convergence point of the three inlets (Fig. 1a). Negative control experiments
were run by replacing the attractant with filtrate. Syringes were washed with 70% ethanol and DI water between experiments.

Three technical replicates in the same microfluidic channel were carried out for each biological replicate. The microchannel was freshly loaded with the three bands from the three syringe reservoirs in between each technical replicate, by flushing for 1 min at 50 μL min⁻¹ and then slowing the flow to 2 μL min⁻¹ before beginning a new replicate run.

Microfluidic chemokinesis experiments
Chemokinesis is defined as a change in swimming speed induced by a change in the chemical concentration in the surrounding medium, and is different from chemotaxis because it does not relate to a bias in the swimming direction, but rather to a local change in speed. To isolate and quantify the temperature dependence of chemokinesis of *V. coralliilyticus* in response to mucus, we used a second microfluidic device, equipped with a small (5 mm diameter, 100 μm height) holding chamber for visualization (Fig. 1b). A single device contained eight individual chambers in parallel so that multiple samples could be imaged in rapid sequence, repeatedly over time, using computer-controlled motion of the microscope stage. The mucus treatment was comprised of a 1:1 mix of cells and the same mucus pools used in the chemotaxis experiments. To allow the cells enough time to respond to the chemical environment before imaging, all treatments were incubated for 8 min in 1.5 ml sterile tubes with or without mucus at their appropriate temperature (matching cell growth temperature). Thereafter, for each biological replicate at a given temperature we loaded 20 μL of the mucus-added treatment in one chamber and 20 μL of the control in the neighboring chamber, and immediately began acquisition of a 30 s video at 30 frames s⁻¹ of each treatment. We repeated the process to image a total of three technical replicates, using fresh cells each time, for each treatment, at each temperature, for all three biological replicates. The videos were processed as described below to obtain swimming speeds from cell trajectories.

Microscopy and image analysis
All images were acquired using phase-contrast video microscopy with a 20× objective (0.45 NA) and an Andor Neo camera (6.5 μm/pixel; Andor, Belfast, Northern Ireland). The focal plane was always at channel mid-depth to avoid wall effects. All videos were acquired at 30 frames s⁻¹ to robustly capture sharp directional changes in the swimming trajectories and detect reorientations. Videos were exported in tagged image file format (TIFF) for analysis in MATLAB (MathWorks, Natick, MA) using in-house, automated image segmentation and trajectory reconstruction software. Background subtraction and cross-correlation routines were used to detect any residual flow as well as non-motile cells or other particles from the mucus, which were excluded from the analysis. Individual trajectories were reconstructed from identified cell positions through subsequent frames using particle tracking routines. Cells tracked for less than three consecutive
frames were excluded from the analysis, as were trajectories with average velocity <10 μm s⁻¹ (considered non-motile).

Swimming and reorientation statistics
From the reconstructed trajectories, detailed swimming statistics such as the swimming speed \(V\), the reorientation frequency \(f\), and the chemotactic drift velocity \(V_D\) (the component of the swimming velocity directed along the chemical gradient) were calculated. The average swimming speed of each cell was calculated by averaging the instantaneous speed over the duration of that cell’s trajectory. The mean speed of the population, \(V\), was quantified by averaging over all trajectories at each temperature. Reorientations were identified as sharp changes in direction or ‘kinks’ in the trajectories (Fig. 2a-d). The swimming patterns of marine bacterial species including \(V. \) coralliilyticus differ substantially from \(E. \) coli’s run-and-tumble strategy (Son et al 2013, Xie et al 2011). \(V. \) coralliilyticus alternates between forward and backward swimming with a change in swimming direction distributed around 180°, called a ‘reversal’, or 90°, called ‘flick’, resulting in ‘run-reverse-flick’ motility pattern (Son et al 2013). Two criteria were used to identify reorientations following the previous study by Son et al (2013): (i) a high rate of change of swimming direction and (ii) a low instantaneous swimming speed. First, to identify changes in the swimming direction, at every point along a trajectory we calculated the dot product of the swimming directions before and after that point (directional cosine). We then identified as reorientation events all local minima in the directional cosine (equivalent to local maxima in the instantaneous angular speed) falling below a threshold value of 0.985, corresponding to a directional change of at least 10° (Son et al 2013). Second, to identify reorientation events having a change in angle below the 10° detection threshold, we used swimming speed information, because any reorientation is expected to be accompanied by a brief reduction in swimming speed. Based on this second criterion, we identified all local minima in the instantaneous swimming speed that are below 50% of the mean speed of the trajectory. The absolute reorientation angle \(\theta\), defined as the angle between the swimming directions before and after a reorientation, and the reorientation frequency, \(f\), were then quantified from all trajectories containing at least one (\(\theta\)) and two (\(f\)) reorientations, respectively.

The chemotactic drift velocity \(V_D\) for the run-reverse-flick motility pattern appropriate for \(V. \) coralliilyticus (Son et al 2013) was calculated based on a recent theoretical formulation (Taktikos et al 2013),

\[
V_D = \left| \nabla c \right| w V^2 f \frac{16 D_R^2 (2 - \alpha) + 4 f D_R^2 (22 - 5 \alpha) + 2 f^2 D_R (38 + 5 \alpha) + f^3 (20 + 11 \alpha)}{192 (f + D_R)^2 (f + 2 D_R)^2},
\]

(Eq. 1)

where \(\nabla c\) is the chemical (here, time-varying mucus) gradient (Fig. 1a), \(D_R\) is the rotational diffusion constant, \(\alpha = \langle \cos \theta \rangle\) is the persistence parameter, computed as the mean cosine of the absolute reorientation angle \(\theta\), and \(w\) is a normalization constant. Here, we quantified \(V, f\) and \(\theta\) (and thus \(\alpha\)) from experimental cell trajectories to directly compute the percent \(V_D\) enhancement, \(\Delta V_D\), over the low temperature (22°C) no mucus control (Fig. 6, left axis). Computing the
absolute magnitude of the chemotactic drift velocity, however, requires two sets of additional information on $V_c$ and $w$. $V_c$ varies over time in our experimental set up (Fig. 1a), and in the absence of full information on the specific values of certain parameters for *V. coralliilyticus*, we adopted values reported for a different Vibrio strain, *Vibrio alginolyticus* ($V_c = 1 \ \mu m^4$; $D_R = 0.2 \ rad^2 s^{-1}$; $w = 0.0458 \ \mu m^3$; (Taktikos et al 2013)) to compute the chemotactic drift velocity (Fig. 6, right axis). We applied these parameters equally in all cases to make direct comparison between different temperature and mucus conditions (Fig. 6).

**RESULTS AND DISCUSSION**

Previously, we reported that *V. coralliilyticus* grown at 30°C employs a combination of strong chemotactic and chemokinetic behaviors in response to coral mucus (Garren et al 2014). Here, we focused on the influence of temperature on the bacterium’s behavioral responses to whole coral mucus and found that chemotaxis and chemokinesis are both positively, but differentially, impacted by rising seawater temperatures.

**Pathogen behavior at 20°C, 23°C, 27°C, and 30°C**

The chemotaxis experiments revealed that the ability of *V. coralliilyticus* cells to migrate up a gradient of coral mucus was substantially impaired at the lowest temperature (20°C; Fig 2a,e). Only a negligible accumulation of cells in the regions of highest mucus concentration occurred at 20°C compared with temperatures ≥23°C where the cell trajectories (Fig. 2a-d) and spatial distribution of cells (Fig. 2e-h) differed substantially. This weaker accumulation at 20°C was not associated with a reduction in swimming speed, which was nearly constant over the temperatures tested (see Fig. 4c), suggesting that the bacterium’s sensing system, rather than its propulsion system, was impaired under the coolest conditions tested.

The magnitude of the chemotactic response and its temporal evolution were quantified and compared using the chemotactic index, $I_c$ (Seymour et al 2010), which measures the relative difference in cell concentration between the 200 µm thick layer initially occupied by mucus and the 400 µm wide region outside of that layer. Absence of chemotaxis corresponds to $I_c = 0$. Some variability in the magnitude of the response among biological replicates notwithstanding, the response of cells grown at ≥23°C was always strong, with Raw $I_{c,MAX}$ ranging between 8 - 26, in contrast to cells grown at 20°C where the Raw $I_{c,MAX} < 5$ (Fig. 3). The chemotactic response of cells at 20°C was also slower, reaching $I_{c,MAX}$ in >50 s (Fig. 3; ~70 s and ~60 s for panels a and b, respectively). In contrast, cells at warmer temperatures all began accumulating rapidly in the mucus region and surpassed the maximum level of accumulation achieved by 20°C cells (Fig. 3, $I_c > 0$) within the first ~25 s of the experiment (Fig. 3 see also Fig. 2e-h). Together these data demonstrate that, at temperatures ≥23°C, the chemotactic response of *V. coralliilyticus* increases by ≥60% when measured as the average increase in $I_{c,MAX}$ at 23°C and 27°C over 20°C.
Figure 2. The strength of chemotaxis towards coral mucus by the pathogen *Vibrio coralliilyticus* is strongly temperature dependent. (a-d) Individual cell trajectories across the width of the three-inlet microchannel (Fig. 1a), showing accumulation towards the side where mucus was initially injected (400 μm < x < 600 μm; see Fig. 1a). The trajectories shown here were acquired from 43 s to 50 s after mucus injection. (e-h) Normalized cell concentration across the channel at different times after mucus injection: 10 s (dash-dotted line), 50 s (solid line), and 100 s (dashed line). In all panels (a-h) accumulation towards the right denotes positive chemotaxis to coral mucus. Panels correspond to different temperatures at which cells were grown and assayed: 20°C (a, e; blue), 23°C (b, f; cyan), 27°C (c, g; pink), and 30°C (d, h; red).
Figure 3. Time course of the chemotactic response of *V. coralliilyticus* towards coral mucus at different temperatures. Curves represent the temporal evolution of the chemotactic index, $I_c$, measured in the three-inlet channel (Fig. 1a). The two panels (a, b) show two independent biological replicates of *V. coralliilyticus* grown at each of four temperatures: 20°C (blue), 23°C (cyan), 27°C (pink), and 30°C (red). $I_c$ represents the concentration of bacteria in the 'mucus layer' (400 μm < x < 600 μm; see Fig. 1a) relative to the concentration of bacteria outside that layer (0 μm < x < 400 μm). The chemotactic index for each experiment was normalized by the value that the chemotactic index reached over the 165 s of observation, averaged over all biological replicates, for the 30°C experiment run on the same day (see Methods).

To isolate chemokinesis from chemotaxis at each temperature, we quantified the pathogen’s response to a uniform mucus addition in the absence of a gradient. We used this approach because both chemotaxis and chemokinesis can occur simultaneously, the former allowing cells to climb the gradient, the latter resulting in increases in swimming speed above a threshold mucus concentration that are thus folded into the quantification of chemotaxis. Experiments in isotropic chemical environments allow for the measurement of chemokinesis alone, by quantifying the absolute change in swimming speed ($\Delta V$) and the percentage change in the mean swimming speed ($\Delta V_{\%}$) after the uniform addition of mucus. These experiments revealed that the swimming speeds of *V. coralliilyticus* were not appreciably affected by the addition of mucus for
cells grown at 20°C ($\Delta V = 2 \pm 4 \text{ m s}^{-1}; \Delta V_m = 6\%$) or 23°C ($\Delta V = 2 \pm 9 \text{ m s}^{-1}; \Delta V_m = 5\%$) (Fig. 4). In contrast, swimming speeds were substantially enhanced by mucus addition in cells grown at 27°C ($\Delta V = 10 \pm 15 \text{ m s}^{-1}; \Delta V_m = 42\%$) or 30°C ($\Delta V = 23 \pm 7 \text{ m s}^{-1}; \Delta V_m = 64\%$) (Fig. 3b,d). These swimming speed enhancements occurred against the backdrop of an otherwise temperature-independent swimming speed, which in the absence of mucus was consistently in the range $V \approx 30-40 \text{ m s}^{-1}$ at the four temperatures tested (Fig. 4c). These experiments demonstrate that, in addition to the advantage in gradient-sensing (chemotaxis) that the pathogen experiences at and above 23°C, there is a second motility enhancement in the form of increased swimming speeds (chemokinesis) as sea temperatures warm further.

![Graphs showing swimming speed distributions and means](image)

**Figure 4.** The strength of chemokinesis of *V. coraliilyticus* in a uniform concentration of coral mucus is strongly temperature dependent. (a-b) The distribution of swimming speeds within the pathogen population. Curves correspond to different temperatures at which cells were grown and assayed: 20°C (a, blue), 23°C (a, cyan), 27°C (b, pink), 30°C (b, red). Solid lines are treatments with mucus and dashed lines are controls with cell filtrate. (c) The mean swimming speed, $V$, for cells with (solid bars) and without (open bars, controls) a uniform concentration of mucus added. Cells were grown and assayed at four temperatures, indicated on the axis and by the color-coding. (d) The enhancement in the mean swimming speed of a population in the presence of coral mucus over the no-mucus control. Data in c and d are means of >2,000
individual tracks for each case and the error bars denote standard errors. Experiments were performed in the circular microfluidic holding chambers (Fig. 1b).

**Figure 5.** Chemokinetic response of *V. corallilyticus* to mucus collected from control and heat-stressed corals. (a) The distribution of swimming speeds within the pathogen population. Pathogens grown at 22°C were presented with uniform mucus from healthy corals grown at the ambient temperature (22°C; solid blue line), whereas pathogens grown at 30°C were presented with uniform mucus from heat-stressed corals (solid red line). Dashed lines represent no-mucus controls for each case. (b) The mean swimming speed (left axis) for the four cases in panel a. The data are also expressed in terms of the percent speed enhancement over the 22°C experiment with no mucus (right axis). Error bars denote standard errors. Experiments were performed in the circular microfluidic holding chambers (Fig. 1b).
Combined effect of temperature on host and pathogen

The experiments described thus far focused on the effect of temperature on the bacterial pathogen, and the mucus we used came from a uniform pool collected from coral colonies grown at 25°C. In the natural environment, however, bacterial pathogens and corals will experience the same temperature conditions. In previous work, we have shown that the composition of coral mucus is altered when corals are subjected to elevated temperatures (Garren et al 2014). In particular, an increase in temperature from 22°C to 31°C resulted in a five-fold increase in dimethylsulfoniopropionate (DMSP) in the coral mucus, which in turn triggered a two-fold stronger chemotactic response of the pathogen when compared to mucus from un-stressed corals (Garren et al 2014). When taken together with the results presented here, these previous findings suggest a compounding effect of temperature, which increases both the motility performance of the pathogen and the production of the chemical signals in the mucus that attracts the pathogens.

To specifically test this combined effect of temperature on the pathogen and on the chemical composition of mucus, we carried out an additional set of chemotaxis and chemokinesis experiments where the pathogen was grown at similar temperatures to those in which the corals were living when their mucus was collected. We focused on two temperatures: 22°C, the ambient seawater temperature at the field site, and 30-31°C for the heat-stressed mucus.

The temperature-paired pathogen-mucus experiments confirmed that chemokinesis only occurs at elevated temperatures. When ‘ambient mucus’ (i.e., mucus from corals living at 22°C when mucus was harvested) was added to ‘ambient cells’ (i.e., cells grown at 22°C), the swimming speed of the cells remained unchanged (ΔV< 2 ± 3 μm s⁻¹; ΔV₉₉ = ±2%; Fig. 5). In contrast, when heat-stressed mucus was added to heat-grown cells (i.e., cells grown at 30°C), the swimming speed increased by >30% (ΔV = 21 ± 2 μm s⁻¹; ΔV₉₉ = 36%; Fig. 5) and was >35% higher than the ambient temperature case (Fig. 5). Notably, in the absence of mucus, the speed of ambient cells and heat-grown cells were within 10% of each other (Fig. 5b). These results are consistent with the previous set of experiments testing temperature’s effect on the pathogen alone, which also showed a ~20 μm s⁻¹ increase in the swimming speed of 30°C cells in the presence of mucus (Fig. 4c,d). This suggests that the chemical composition of heat-stressed mucus only influences chemotaxis (Garren et al 2014) and does not influence the strength of chemokinesis.

The most remarkable feature of *V. coralliilyticus*’ chemotactic behavior in the presence of heat-stressed mucus was the dramatic improvement in its chemotactic drift velocity, which quantifies the ability to swim directionally up the mucus gradient (Fig. 6). The addition of ‘ambient mucus’ to ‘ambient cells’ showed no enhancement in the chemotactic drift velocity (ΔV₉₉ = -1.8%; V_D = 5.6 μm s⁻¹; V_D/V = 8.2%; Eq. 1; Fig. 6) compared with in the absence of mucus (V_D = 5.7 μm s⁻¹; V_D/V = 8.1%). However, warmer growth temperatures alone, in the absence of mucus, resulted in >10% enhancement in the chemotactic drift velocity (ΔV₉₉ = 10.2%; V_D = 6.3 μm s⁻¹; V_D/V = 8.3%) for heat-grown cells compared to ‘ambient cells’. This enhancement increased to >45% (V_D = 8.4 μm s⁻¹; V_D/V = 8.6%) when the heat-stressed mucus was added to those heat-grown...
cells (Fig. 6). This dramatic increase in chemotactic velocity was due to the simultaneous increase in the swimming speed $V$ (+38%) and the reorientation frequency $f$ (+24%), both of which quadratically increase $V_D$ ($V_D \sim V^2 f$, Eq. 1).

![Diagram illustrating chemotactic drift velocity in V. coralliilyticus exposed to a gradient of mucus collected from ambient and heat-stressed corals. The four cases shown correspond to the same cases as in Fig. 5. Both the absolute value of the chemotactic drift velocity $V_D$ (left axis) and the percent speed enhancement over the 22°C experiment with no mucus $\Delta V_D$ (right axis) are shown. Experiments were performed in the three-inlet channel (Fig. 1a).](image)

The cumulative advantage that warmer waters confer to the pathogen by simultaneously improving its motility – both speed ($V$) and directionality ($V_D$) – and influencing the chemical composition of coral mucus is clearly evidenced by the higher chemotactic indices reached by heat-grown cells exposed to heat-stressed mucus compared with ambient cells exposed to ambient mucus (Fig. 7). Although the pathogen does not exhibit chemokinesis at either 22°C (Fig. 5b) or 23°C (Fig. 4d), relatively strong chemotaxis occurs at both of these temperatures (with mean $I_{C,MAX}$ values of 0.8 and 0.9, respectively; Figs. 3, 7). However, unlike the nearly identical chemotactic responses observed in cells grown at 23°C (mean $I_{C,MAX} = 0.9$) and 30°C (mean $I_{C,MAX} = 1.0$) in response to 25°C mucus (Fig. 3), cells grown at 30°C accumulated much more strongly ($I_{C,MAX} > 1.3$; Fig. 7) when responding to heat-stressed mucus than cells grown at 22°C and exposed to ambient (22°C) mucus ($I_{C,MAX} = 0.8$; Fig. 7). Not only would this improved chemotactic ability potentially give the bacterium an advantage in finding its host, but it could also improve cell growth by lengthening residence times in nutrient-rich (i.e., mucus-rich) zones.
 Figure 7. Chemotactic response of *V. corallililyticus* demonstrating the combined effect of temperature on pathogen motility and host physiology. Shown is the temporal evolution of the chemotactic index, $I_C$, for experiments in which the pathogen was grown at temperatures corresponding to that at which the corals were living in when their mucus was collected: 22°C (blue) and 30°C (red). The chemotactic index was defined as in Fig. 2, except that here normalization occurred relative to the maximum value in the 22°C experiments. Solid lines are means of three biological and three technical replicates. Shadings correspond to the standard error. Experiments were performed in the three-inlet channel (Fig. 1a).

CONCLUSIONS

Taken together, these data suggest that as the oceans continue to warm, *V. corallililyticus* will exhibit two independent behavioral switches: a dramatic improvement in chemotactic abilities at moderate temperatures followed by the induction of chemokinesis at warmer temperatures. These temperature-induced alterations in behavior combined with the changes in chemical ecology elicited in corals by heat stress (Garren et al 2014) will strongly favor the pathogen in reaching its targeted host. The cellular mechanisms underlying the switch from poor to acute chemotactic sensing and the induction of chemokinesis remain an open question. The answers will likely require a combined approach that couples genetic and transcriptomic approaches with direct observation and quantification of pathogen behaviors.

As new tools continually improve our ability to observe bacterial behaviors, it is becoming evermore clear that bacterial motility and chemotaxis are widespread in many marine habitats (Stocker and Seymour 2012). The marine environment is a heterogeneous landscape at the
microscale and a bacterium’s ability to accurately navigate gradients of nutrients and infochemicals may provide it with a competitive advantage (Taylor and Stocker 2012). Marine examples of chemotaxis are found governing bacteria-phytoplankton interactions (Bell and Mitchell 1972, Blackburn et al 1998, Stocker and Seymour 2012), facilitating symbioses such as that between *Vibrio fischeri* and squid (Mandel et al 2012), and being implicated in the onset of disease (Larsen et al 2004, Meron et al 2009, Otoole et al 1996, Rosenberg and Falkovitz 2004). In this context, reef-building corals are becoming a model system for studying the role of motility in bacteria-host interactions in the ocean. They have the advantage as a lab model of facilitating *in situ* visualization because they host an important portion of their microbiome on their external surface (Garren and Azam 2012). It is also a timely choice of model system from an ecological perspective because disease has been identified as an under-appreciated driver of the future state of reefs (Maynard et al 2015). If fact, under business as usual projections for CO₂ emissions, >40% of reefs world-wide are predicted to experience a doubling by 2030 in the number of months per year in which increased temperatures (mean monthly maximum >27°C) can trigger virulence in *V. coralliilyticus* (Maynard et al 2015). Taking all of the evidence together, it is becoming clear that motility adaptations under future climate scenarios have the potential to give bacterial pathogens the upper hand over their coral hosts (Maynard et al 2015; Garren et al 2014).

The goal is to ultimately gain a predictive understanding of coral disease that will aid management decisions and community planning where people are dependent on reef resources. Pathogen motility is one element of many that may affect the outcomes of host-pathogen interactions, and disease remains one of many stressors actively deteriorating reefs globally. While the overarching picture remains complex, the ever improving techniques available to observe the mechanistic underpinnings of such processes at the most pertinent scales are opening the door to understand the microscale mechanisms driving ecosystem-wide patterns. In this respect, the results presented here contribute to our understanding of the potential impacts of warming seawater temperatures on bacterial behaviors that influence coral disease.
REFERENCES (APPENDIX 2)


Coral reefs in a century of rapid environmental change.


Jeffrey Maynard, Ruben van Hooidonk, C. Mark Eakin, Marjetta Puotinen, Scott F. Heron, Melissa Garren, Joleah Lamb, Gareth Williams, Ernesto Weil, Bette Willis, C. Drew Harvell (2015) Climate projections of conditions that increase coral disease susceptibility and pathogen virulence. Nature Climate Change in press


