THE PHYSICS OF BACTERIAL COLLECTIVE MOTION ON A SURFACE: THE (IR)RELEVANCE OF RUN AND TUMBLE CHEMOTAXIS TO RAPID GROUP MOTILITY AND EVIDENCE OF AN ABIOTIC JAMMING TRANSITION AS A PRIMARY CONTROL PARAMETER OF A SPREADING BACTERIAL SUSPENSION

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DISSERTATION ABSTRACT

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Microbes routinely face the challenge of acquiring territory and resources on wet surfaces. Cells move in large groups inside thin, surface bound water layers, often achieving speeds of 30 μm/s within this environment, where viscous forces dominate over inertial forces (low Reynolds number). The canonical Gram-positive bacterium *Bacillus subtilis* is a model organism for the study of directed, collective migration over surfaces with groups exhibiting motility on length scales three orders of magnitude larger than themselves within a few doubling times.

Genetic and chemical studies clearly show that the secretion of endogenous surfactants and availability of free surface water are required for this 'ultrafast' group motility. However, the relative importance of individual motility, chemosensing, and the presence of exogenous nutrient gradients in precipitating group surface motility are largely unknown. Here I use novel experiments to strengthen the case that (i) *B. subtilis* does not rely on chemotaxis to determine group motility direction, to establish that (ii) the rate of dendritic expansion has only a weak dependence on motility and that rapid dendritic group motility is possible even with non-motile cells, and demonstrate for the first time that (iii) water availability is likely a sensitive control parameter modulating an abiotic jamming transition that determines whether the group remains fluidized and therefore collectively motile.

These data suggest that rapid surface motility does not result from individual motility and chemotaxis properties of the bacteria, but rather that a combination of

biologically generated surface tension gradients and abiotic granular jamming regulate this ubiquitous ecological process.

This dissertation includes previously published co-authored material.

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And to all those who wonder, and seek to understand because of it.

And to all the teachers who have been and will be, for their patience and love is the greatest ratchet for knowledge.

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CHAPTER I – AWAY FROM REDUCTIONISM: BACTERIA AS A GROUP

1.1– Introduction, Emergent Phenomena

Reductionism grants certain insights about the Universe, but leaves many proverbial (and literal) stones unturned. Diving deep into the ever smaller and more precise measurements and models of matter's individual constituents yields understanding of exactly that: ever more complicated inner workings and interactions of these tiny particles (Frenkel 1944). Those new data and models, in turn expand our understanding of the building blocks for the Universe, indeed, even space and time itself. However, those data and models have limited ability to inform our experiences in the world we regularly interact with. Similarly, zooming out to the scale where: planets orbit stars, stars progress around galactic centers, which in turn remain gravitationally bound to their neighboring galaxies, simple rules seem to capture much of the relevant dynamics (ignoring a confusing and possibly depressing expansion of space and The Universe itself). And yet, when three or more bodies are gathered in the name of gravity (or any $1/r^2$ potential) things become very tricky–chaotic, in fact. Aside from a few special cases, the precise orbits become analytically incalculable, and their dynamics unpredictable beyond a relatively short timescale. If we zoom out a little though, we can effectively draw a bag around the system and know a few mathematical truths: the angular momentum of the system will remain constant and the energy of the system will remain constant (barring radiation in any form) (Noether 1971). Again, if we look back to the incredibly small scale, we find that although a few atoms' trajectories quickly get out of hand, macroscopic states of these systems, such as temperature, pressure, and entropy,

can be predicted as the number of particles grows toward molar levels. In this way, statistics weds with the study of physics to grant a more intimate and tangible understanding of "the water we swim in" than may otherwise be obvious (Wallace, 2009).

Statistical physics seeks to understand systems whose potentially complicated macroscopic behavior arises from comparatively simple rules governing underlying microscopic dynamics. Flocking birds are a pertinent biological example, where no central agent delivers instruction, yet large groups of birds move coherently and in dazzling ways. In flocking birds, and other interacting collectives, precise knowledge of any individual's behavior has, at best, limited ability to predict the behavior of the group as a whole (Toner, Tu, and Ramaswamy 2005).

This chapter provides an introduction and outline for our quest to understand the emergent properties and behaviors of expanding bacterial colonies, primarily in the canonical species *Bacillus subtilis.* Like many species of bacteria, *Bacillus* are rod shaped bacteria about a micron wide and three microns long, they swim using helical flagella and respond to chemical cues in their environment via run-and-tumble chemotaxis (Adler 1966; Garrity and Ordal 1995; Rao, Glekas, and Ordal 2008; Bischoff and Ordal 1992; Purcell, 1977). The individual behaviors of chemical sensing, flagellar propulsion, and random reorientation via tumbling constitute a suite of modulatable behaviors that I will use as tools to interrogate collective migration of bacteria over surfaces.

Chapter II provides background information and details initial experiments that explored rapid bacterial surface translocation and began to reveal key regulators of the collective motility on surfaces. Chapter III is an adaptation of previously published co-

authored work with Tristan Ursell, and describes evidence for the role of an abiotic jamming transition as a regulator of flow and consequently expansion of these surface bound colonies. Chapter IV focuses on future work and provides concluding remarks.

1.2 Mapping the Bacterial Expansion Landscape

Bacteria are the second largest component of the Earth's biomass at ~70 gigatons of carbon (GtC)(humans make up just 0.06 GtC) and they play crucial biochemical roles across all ecosystems (Bar-On, Phillips, & Milo, 2018). Across those ecosystems, they face complex environments that present them with other competing organisms, chemical and nutrient gradients, and a wide array of varied physical conditions, influenced by morphology, fluid properties, flow and temperature, to name a few [refs?]. How best to exploit or respond to these surroundings for the replication of one's individual DNA is the basis of genetic selection, natural or otherwise (Avery, MacLeod, & McCarty, 1944). Dispersal of genetic information by translocating DNA across an environment can provide a selective advantage by increasing access to and reducing local competition for new resources, and as a bet-hedging strategy against environmental fluctuations.

In contrast to the way that bacteria are commonly analyzed in the laboratory, in natural contexts bacteria exist primarily in groups. Communities are more robust to threats, are able to share resources and metabolic loads, and even specialize genetically and phenotypically to better exploit their environments (Damore & Gore, 2012; Hamilton, 1964). Deepening our understanding of how constituent microbes within a community cooperate and communicate has indeed been one of the great advances of the

application of systems biology, physics, and computation to the question of bacteria's (co)existence (Fletcher & Zwick, 2006; Nowak, 2006; Page & Nowak, 2002).

Although much is known about the physiology of group survival, and still more is known about the proteomic, genomic, and general growth programs of individual bacteria (something of a parts list), the spreading translocation of these colonies in their early stages as they first settle new territory still remains largely unexplored. As cartographers of this foreign landscape, we've sought to map out the physical processes and rules by which surface bound bacterial suspensions expand to settle new domains.

CHAPTER 2 – BACTERIAL SURFACE TRANSLOCATION, SETTLERS IN STRANGE LAND

2.1 – Introduction

Bacteria live in microscopic environments whose physical properties and challenges are distinct from those faced by organisms at the macroscopic scale. Among those unique challenges bacteria must propel themselves through highly viscous, chemically complex fluids at low Reynolds number in search of chemical resources using only their flagella and a rudimentary sense of smell (Purcell, 1977). Their chemical pursuit algorithm is called 'chemotaxis', and it requires an ability to move semi-ballistically through the fluid, to sense external chemicals, remember past measurements of their concentrations, and to respond to changes in those concentrations by deciding whether to continue swimming or reorient randomly (Berg and Brown 1972; Mesibov and Adler 1972; Bren and Eisenbach 2000). Bacterial cells use an array of surface sensor proteins to take periodic measurements of the concentration of relevant molecules (Bren and Eisenbach 2000; Garrity and Ordal 1995). A small internal memory consisting of a combination of methylation and phosphorylation adapts over a short (seconds) timescale to local receptor binding of chemical concentrations. Receptor binding readouts that differ from the state of the memory 'inform' the cell as to whether those measurements indicate an increase or decrease in the concentration of relevant molecules (Rao, Kirby, and Arkin 2004; Rao, Glekas, and Ordal 2008). If the change in nutrient concentration is positive, cells bias towards 'running' in the same direction, whereas if the nutrient concentration decreases, cells execute a 'tumble' -- a roughly random reorientation in

three dimensions. This simple program is a surprisingly robust and scale-invariant gradient ascent (or descent if considering repellents) algorithm.

Therefore it is clear that chemotaxis forms a crucial component of the bacterial survival toolkit, but it is not always clear in which contexts it is being employed or is capable of being employed. For instance, detecting beneficial or harmful molecules is only half the story -- bacteria must propel themselves in their viscous environment, which is a non-trivial task that requires symmetry breaking dynamics (Purcell, 1977). Helical flagella distributed across the bacterial surface spin counterclockwise, entraining fluid and transferring momentum to produce movement. While the flagella spin counterclockwise, the helicity of flagella ensures that 'tangles' propagate away from the molecular motors that power the flagella, leading to consistent forward motion called a 'run'. The aforementioned decision process (run and tumble chemotaxis) manifests as a reversal in the direction of rotation (to clockwise), which 'tangles' the flagella, resulting in random reorientation (Bischoff and Ordal 1992). Decades of excellent work has illuminated at high detail how this process happens genetically and molecularly, and it is known that this is an efficient algorithm for gradient ascent in open, isotropic fluids (Berg and Brown 1972; Lauffenburger and Zigmond 1981).

However, bacteria frequently reside on two dimensional surfaces whose chemical and physical properties differ significantly from those of bulk fluids (Henrichsen 1972). As compared to the consistent run-and-tumble swimming behavior in bulk fluids, the mechanisms and types of surface translocation are, arguably, more varied and complex. For instance, bacteria use a number of flagella-based motility mechanisms to colonize surface environments, including swimming in bulk fluids, and swarming and sliding

across surfaces (reviewed in Harshey 2003). When I inoculated wild type *B. subtilis* (NCIB 3610) on a soft agarose surface with rich defined media, they spread in coordinated tendrils, exploring in streams to the edges of a 5 cm plate within 4 hours (Fig. 1). Those tendrils subsequently expand to cover the entire plate within a total of ~10 hrs. This is a feat of coordination and material transport whose physical underpinnings present salient questions: How do these groups choose a direction? How does the group generate the force necessary to move the collective? Which biological and/or physical factors contribute to regulating this behavior? Should a colony be considered a multicellular organism? How does such a coordinated effort arise with no central regulator or guide?

Examining the development of these surface-bound communities of order 10^{10} cells in the time domain has revealed that dense bacterial colonies spread in a coordinated manner. Colonies form expansive patterns with length scales many orders of magnitude larger than their constituents and cover areas several orders of magnitude larger in size than that of individuals over a few doubling periods (Henrichsen 1972; Harshey 1994; Kearns and Losick 2003). The behaviors of individuals give rise to colony scale emergent behaviors, wherein new or irreducible properties arise from a collection of smaller components.

Understanding the translocation mechanisms of bacterial groups provides crucial insights into physical – rather than solely chemical – methods by which colonies move, acquire new territory, spatially spread their genomes, and avoid localized threats (chemical or otherwise). Where chemical antibiotic interventions fail due to rapid mutation rates of bacteria (by developing enzymes to metabolize compound A, or

preventing import of compound B), interruptions of a physical nature promise more permanent or lasting solutions to inhibit colonization and pathogenesis (Dakal et al. 2016; Butler, Wang, and Harshey 2010).

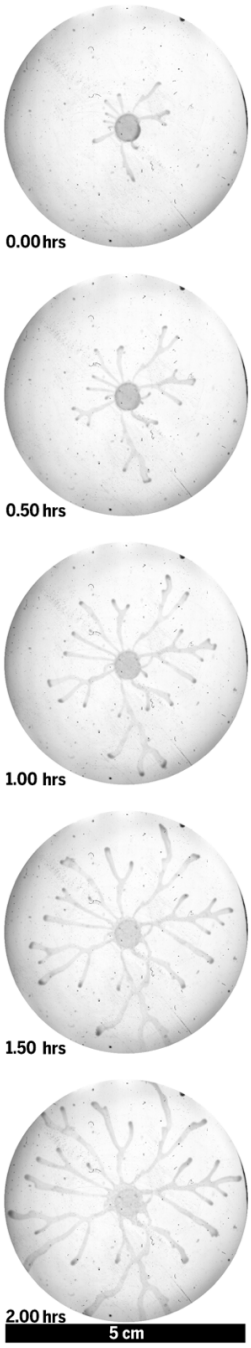

Figure 1. A *Bacillus subtilis* colony grows from an initial dense inoculum, reaching the edge of a 5cm soft agar plate in ~2 hours, exhibiting colony-scale collective motion at a rate of ~3.5 μm/s.

In *B. subtilis,* there exist a wide variety of colony surface spreading morphologies, each depending on subtle changes in environment or phenotypic expression even within a single genotype (Kearns and Losick 2003; Kearns 2010). Types of motility and dispersal are described using evocative terms, such as swimming, swarming, sliding, gliding, twitching, and dendritic expansion (Henrichsen 1972; Harshey 2003; Kearns 2010). Swimming refers to sparse, free-moving bacteria in liquid media, commonly a much larger volume than single cells (Henrichsen 1972). Swimming also occurs in spatially anisotropic environments such as low-percentage polymer ($\lesssim$.3% w/v) hydrogels, which act as mazes that slow down expansion without significantly altering typical behavior or nutrient availability (Henrichsen 1972). Swarming, sliding, and dendritic expansion require that the movement be across stiffer hydrogel surfaces ($\gtrsim$0.45% w/v, often >0.7% w/v), effectively constrained to move in 2D (Henrichsen 1972; Kearns 2010).

Swarming, sliding, and dendritic expansion can be difficult to differentiate and are not necessarily exclusive labels. Some definitions of swarming necessarily invoke groups moving collectively (Kearns 2010). Such motion entails 'packs' of bacteria, either lightly bound by entangled flagella or temporarily moving as a group due to steric nematic ordering, hydrodynamic coupling, and/or other local physical effects (Sinibaldi, Iebba, and Chinappi 2018; Copeland and Weibel 2009; Kearns 2010). Swarming *B. subtilis* are defined primarily by their rate of expansion on dried 0.7% w/v agarose plates, where swarm radius reaches ~ 30mm after 5 hours, compared with non-swarmers which progress < 2 mm over the same duration (Kearns 2010; Fall, Kearns, and Nguyen 2006). Much of what is known was learned from initial and final timepoints (24 hrs) of the

swarming process, with little attention given to multiple time scales of dynamics during the intervening hours.

I sought to understand whether the individual behaviors or decisions of the constituent bacteria were the source of force and direction determination in the outward expansion, and to what extent the bulk characteristics of the growth substrate determined their colony's expansion success and its shape. To understand the surface spreading bacteria I developed an experimental system in which a defined medium was chemically and physically modulated to cover a range of environmental conditions. I then inoculated the different media gel surfaces with bacteria and imaged the systems in high time resolution (up to 20 images per hour). To characterize how metabolic and mechanical features of the surface affected colony morphology and dynamics, I independently varied (1) glucose concentration to assess the effects of simple carbon source availability, and thus energy expenditure, on coordinated colonization and (2) the agarose (polymer) concentration to study the effects of gel stiffness on the ability of colonies to spread. Further, I employed multiple knockout strains to assess the role that different types of individual motility and limitations in chemosensation response might play in group motility.

2.2 Materials and Methods

For scanner-based experiments, dense cell cultures were deposited at the center of 5 cm petri dishes (Falcon) using the strains and methods described below, largely adapted from (Kearns 2010).

B. subtilis culture storage and preparation

Culture aliquots were liquid-nitrogen snap-frozen at mid-log growth phase in 50% glycerol, then stored at -80° C. The morning of an experiment, samples were thawed, then 200 µl of the cultures were added to 10 ml of standard Luria Broth (LB) and allowed to grow for ~4 - 6 hours in a shaking incubator at 37 C. During the plate preparation stage, while the agarose cooled and stiffened, the 10 ml cultures were concentrated by centrifugation at ~4000 g for ~8 - 10 minutes, and subsequently the supernatant was poured off. The concentrated cells were then resuspended with ~200 µl of fresh, room temperature Luria Broth (LB), then 1.5 µl of the resulting dense suspension was spotted onto a plate, usually in the center. The inoculum was left open to air under a flame and visually observed as the fluid suspension dried by a combination of evaporation and absorption into the gel (usually ~10 minutes). As soon as no excess liquid was present in the inoculum, the plates were then closed, sealed with parafilm, kept level, and taken to be imaged.

B. subtilis plate preparation

A standard recipe for 100 ml of Teknova EZ Rich Defined Media (EZRDM) was prepared according to manufacturer details by pipetting into a flask: 10 ml 10X MOPS buffer, 1 ml 0.132 M K2HPO4, 10 ml 10X ACGU Solution, 20 ml 5X Supplement EZ, 58 ml deionized H20, and 1 ml 20% glucose solution. Then 0.5 g powdered Optimized Grade Agarose (Research Products International) was weighed and added to the solution to reach 0.5% weight per volume (%w/v). The solution was then autoclaved according to volumetric guidelines and placed in a 50 C warming incubator until it was time to pour

plates (when cultures reached the appropriate log-growth phase). The solution was then poured into standard petri dishes of varying sizes until the bottom of the plate was evenly covered, then left on a bench fully open to air under a flame until solidified (~10 minutes), at which point concentrated colonies were inoculated onto the surface then sealed, as described above

Plates were always prepared the day of the experiments, usually in synchronization with culture preparation. Media could be prepared in replicate batches then stored in a refrigerator before autoclaving for up to one week, but unused samples older than one week were never used for manuscript-grade data collection, and instead became materials to be used for exploratory methods.

High-throughput screen media preparation

To sample across relevant ranges in agarose and glucose percentages, arrays of up to 32 different media recipes were prepared on the mornings of experiments beginning with the base EZRDM recipe described above, then modified as follows. For glucose modulation, a percentage of the prescribed volume of 20% glucose solution was added, and the difference in final volume from the original recipe was made up for by modifying H_2O volume added to reach the appropriate final volume of media; typical ranges for glucose started at 2% of the prescribed amount, increasing in powers of 2 to 256%. For agarose modulation, the weight of agarose added to the media was simply changed to match the experimental needs (i.e. 0.45 g to reach 0.45% w/v or 0.6 g for 0.6% w/v). All bottles were autoclaved together, then rested at 50° C until time to pour, at which point 2 people working rapidly (to avoid premature gel hardening) pipetted 7 ml of the media +

agarose into several 5 cm plates. Those plates were allowed to dry and harden ~10 minutes in a laminar flow hood before being covered with their lids and moved to the lab where they were inoculated as described above, then sealed. Because of the difficulty of consistent plate preparation and inoculation, extra plates were prepared for each experimental condition and only the 4 most consistent samples of each type (as judged visually) were used for data collection. Visual inspection for consistency involved avoiding plates with excessive bubbles, irregular menisci near their edges, and damaged gels from mistakes during inoculation.

Scanner experiment apparatus

Two consumer photo scanners (Epson V800) were controlled by a single Linux server running on consumer PC desktop hardware.The server stored image capture schedules in a MySQL database and executed captures in parallel across both scanners then stored the data locally for later use. To achieve stable and optimal growth temperature, scanner surfaces were heated with an 8.5 X 11" ITO coated PET sheet (Sigma Aldrich) laid between the imaging glass surface and the array of 16 plates being imaged. The two ITO sheets were connected via copper tape to independent turnkey PID devices (Inkbird) and shared a single universal power source, along with a thermocouple probe (Inkbird) which measured the temperature of the scanning surface and provided feedback to the PID thermostats. Temperature stability at 37 C was confirmed by substituting an un-inoculated agarose plate with an embedded thermocouple into the scanner -- observation of probe temperature on numerous occasions confirmed adequate (+/- 0.1 C) temperature stability. The time it takes the scanner to acquire images depends

on the size and aspect ratio of the image — generally, columns 1 plate wide and 4 plates tall scanned fastest. To optimize image acquisition rates, I used freely available software (The Lifespan Machine, Stroustrup et al. 2013) to select specific scanner regions and schedule them for serial acquisition. Columns were scanned successively, with the first column scanned at time *t* and *t*+6 minutes; the second at *t* + 1.5 and *t* + 7.5; and so on. In this way, all plates were scanned at 10 timepoints per hour. All parts used are listed in the appendix in Table A1.

B. subtilis strain construction

All strains used in this study were graciously provided by Prof. Dan Kearns, Indiana University. Constructs were introduced into the DS2569 strain background using DNA transformation, then transferred to the wild-type (WT) strain 3610 using SPP1-mediated generalized phage transduction (Yasbin and Young 1974; Mukherjee and Kearns 2014; Calvo and Kearns 2015; R. Chen et al. 2009). All strains are in the 3610 background and are listed in Table 1.

Strain	Genotype	Phenotype	Ref
3610	Wild type	Wild type background strain	(Branda et al. 2001)
DS6870	ΔcheY	Constitutively tumbles	(Calvo and Kearns 2015)
DS7306	ΔcheB	Constitutively runs	(Calvo and Kearns 2015)
DS1677	Δhag	Abolishes flagellar assembly	(Mukherjee and Kearns 2014)
DS1122	*srfAC*::Tn*10*	Abolishes surfactin production	(R. Chen et al. 2009)

Table 1. Strains used.

2.3 Results

To investigate which characteristics and environmental factors contribute to rapid surface colonization of agarose plates, Petri dishes of independently varying agarose concentrations and glucose concentrations in a background of rich defined media were inoculated and then imaged over the subsequent 24 hours at a rate of 10 frames per hour (Fig. 2). Agarose concentrations are commonly reported precisely as the percentage of weight of powdered agarose that has been added per volume of the growth media to which it is added (% w/v). Concentrations are described in qualitative terms of mechanical stiffness, where higher agarose %w/v leads to a stiffer gel and lower agarose percentages form a softer gel, in a generally linear fashion (Tuson et al. 2012). Agarose gel concentrations below a certain threshold (~0.3 - 0.4% w/v) allow motile bacteria to swim through the porous polymer network of the agarose and are thus called 'swim plates', whereas higher concentration gels (above ~0.45% w/v) yet below stiffnesses commonly used for DNA electrophoresis assays (~1.5-2% w/v) are called 'swarm plates', as the volume between polymers remains inaccessible to colonizing cells, constraining them to live on the surface (Adler 1966; Kearns and Losick 2003). Glucose was identified as a physiological target to modulate energy availability to spreading *B. subtilis* colonies because, like many other bacteria, it is a readily usable carbon source (Fisher and Sonenshein 1991).

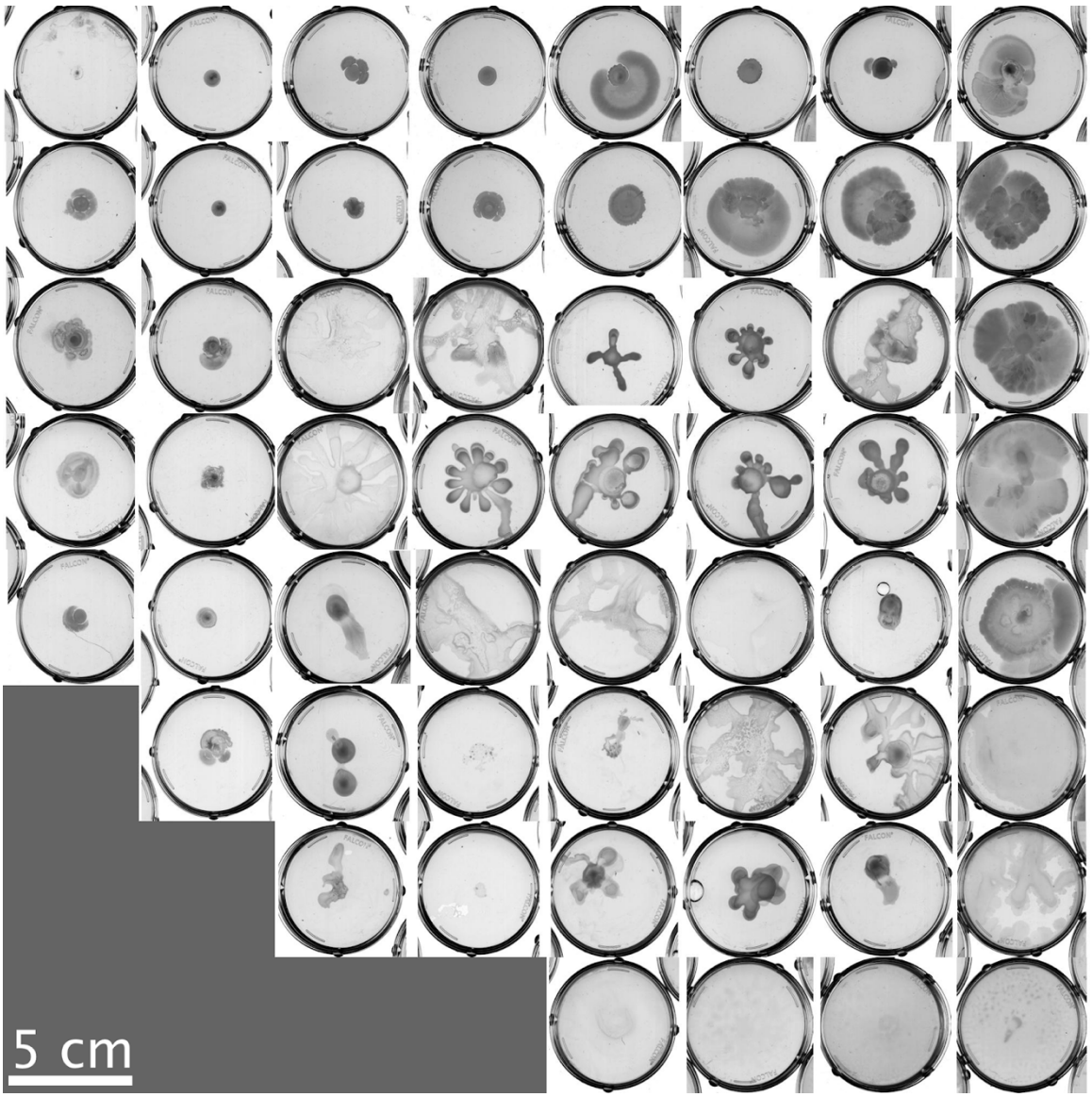

Figure 2. Agarose stiffness is a potent modulator of spreading rate and morphology. Samples of *B. subtilis* grown on soft agarose gels whose properties ranged from 0.35% to 0.7% w/v agarose in increments of 0.05% from bottom to top, and from 2% to 256% of the standard glucose ratio in the recipe for EZRDM in powers of 2 from left to right. In the regions of lowest agarose concentration, colonies form bulbous puddles of fluid, whereas at high agarose (0.7% w/v and above), they remain bound to grow by steric expansion, and hence spread roughly two orders of magnitude more slowly. Near the middle of the parameter space, with standard recipe EZRDM and 0.5% agarose, all surfactin-producing strains consistently expanded to cover their plate in branching dendritic morphologies within 6 to 12 hours.

In general, colonies on plates of agarose concentrations near the middle of the range tested (~0.45-0.6% w/v) began with a lag phase, where little growth happened, followed by a rapid dendritic growth phase, where branching structures of width ~1-2 mm grew outwards away from the colony until they reached the edge of the plate within a few hours. The colony dendrites then swelled to fill in the spaces between dendrites over the remainder of the experiments. On the lowest agarose concentration plates (<0.45% w/v), colonies either displayed other growth patterns which I won't address further due to shortcomings of the imaging setup for these sorts of growth, or failed to grow at all. At high agarose concentrations (>0.6% w/v) the colonies grew sterically with every dividing cell pushing on its neighbors to extend the colony's reach. These colonies displayed classic sectoring growth patterns that result from chance mutations leading to competitive advantages near the growing front.

To better understand how the microscopic behaviors of individual bacteria influence the macroscopic behavior of the colony, the scanner setup described above was used to image the surface colonization characteristics of a variety of strains. All strains were constructed in the NCIB 3610 wild-type (WT) background strain (Branda et al. 2001). To explore the effects of individual motility phenotypes on group translocation, I examined a strain that constitutively tumbles (Δ*cheY*) and a strain that constitutively runs (Δ*cheB*)(Calvo and Kearns 2015). This is accomplished by knocking out key genes for regulating switching between the running and tumbling states. Constitutive tumblers are unable to rotate their flagella in the direction that generates propulsive force and hence persistent runs, while the constitutive runners are unable to rotate their flagella backwards to change direction, and so move only in persistent runs. While WT cells and

constitutive runners colonized most rapidly (~1 - 5 μm/s), tumblers were also able to explore plates about an order of magnitude more slowly (~0.5 - 1 μm/s), but still far more rapidly than those achieved solely by steric growth (< 0.2 μm/s). To investigate whether flagella are required for rapid translocation, mutants deficient in the *hag* gene (Δ*hag*) were used, in which flagellar assembly is abolished, resulting in *B. subtilis* bacteria which contain and build all of the flagellar motor basal bodies but lack any form of flagellar motility (Mukherjee and Kearns 2014). On soft agarose surfaces, these colonies expanded in similar dendritic patterns to those with flagella, and at rates (~0.2 - 1 μm/s) that still exceeded steric colony growth.

Finally, under limited conditions, it was known that secretion of the endogenous surfactant *surfactin* was required for rapid colony translocation (Kinsinger, Shirk, and Fall 2003). Secretion of this compound decreases local surface tension, and I wondered if loss of group motility in its absence could be restored under different nutrient or agarose conditions. To examine the potential interplay between *surfactin* secretion, plate properties, and group translocation, I employed a *srf* gene deficient mutant (Δ*srf*), in which *surfactin* production was abolished(R. Chen et al. 2009). Across all of the strains and agarose stiffnesses tested Δ*srf* mutants spread the slowest, even though they were motile and chemotactic, suggesting that modulation of surface tension by *surfactin* was a necessary component of collective bacterial surface translocation.

Another benefit of the Δ*cheY* and Δ*cheB* motility mutants is that, in distinct ways, they lack the ability to perform run and tumble chemotaxis, in which a complex series of ligand binding, methylation, and phosphorylation enables an algorithm for chemical gradient ascent or descent. This spatial search algorithm requires controlled switching

between the two states of flagellar rotation to bias a random walk up or down chemical gradients (Rao, Kirby, and Arkin 2004; Rao, Glekas, and Ordal 2008). These mutants allowed me to determine if individual responses to chemical gradients were required for rapid surface translocation. The data did not demonstrate discernible modification to the dendritic colony morphologies, suggesting that directionally biased individual motility is not required for rapid colony translocation across soft agarose surfaces.

Although most colony strains exhibited dendritic expansion over some range of the tested agarose concentrations, none of the strains showed significant morphological differences across the range of glucose tested, for their respective agarose stiffnesses (Fig. 2). In contrast, all but the surfactant deficient strain (Δ*srf*) showed robust and consistent sensitivity to agarose percentages, displaying a range of different morphologies for each strain at different agarose percentage levels, with some consistencies between strains. For instance, regardless of strain type, colonies spread more rapidly near the low end of the agarose percentage range tested (0.4% w/v) and slowed with increasing agarose concentration, with all strains being restricted to apparent steric expansion in the highest concentrations (>0.6% w/v). This strong dependence on agarose concentration supports a model in which water availability plays a significant role in the surface-bound colony's ability to expand. Not only was there a strong dependence on agarose concentration, but a sharp transition between qualitatively distinct macroscopic growth morphologies occurred over a narrow range of this important control parameter.

2.4 Discussion

I imaged the growth of *B. subtilis* colonies on agarose surfaces spanning a range of conditions in glucose concentration and agarose concentration. Multiple genotypically and phenotypically distinct strains were used to explore the sensitivity of colony morphology to individual differences in motility, including WT, run only, tumble only, and flagella deficient mutants. A *surfactin* deficient mutant was also studied to determine if water availability (agarose %) could modulate group motility in this strain. These data laid the groundwork for studying a wide range of *B. subtilis* collective surface motility and confirmed the sensitivity of surface bound bacterial translocation to varying agar concentration (Fall, Kearns, and Nguyen 2006).

A glimpse into the complexity of bacterial metabolism is demonstrated by the spreading colonies' insensitivity to varying levels of glucose in otherwise rich media. I expected that the removal of a primary energy source (glucose) from the media would significantly reduce spreading group's velocities, but its elimination yielded little discernible morphological variation over the conditions tested. However, glucose metabolism is far more complex than a single, monotonic control over (e.g.) swim speed or colony growth rate. For instance, at least three separate mechanisms complicate the effects of glucose on metabolism. First, in the presence of specific nutrients, the expression of glucose metabolizing enzymes is repressed (Bren et al. 2016). Second, when in an excess energy state, a number of critical ion import and export channels become overwhelmed, which ultimately leads to slower growth when glucose is overabundant (Fisher and Sonenshein 1991). Lastly, minimal glucose availability can actually enhance biomass growth rates in colonies with other balanced abundances due to

improved efficiency of allosteric enzymes as well as increases in efficiency from enzymes that require the input of ATP to function (so called futile pathways) (Bren et al. 2016). Still, my data demonstrate that rapid surface colonization occurs independently of a wide range of glucose availability, which suggests that surfactin-mediated surface motility may serve as a viable strategy for nutrient deprived cells to colonize new environments.

Dendritic surface expansion by B. subtilis did not appear to be precipitated by local nutrient depletion and subsequent gradient ascent via chemotaxis, as demonstrated by the motile but chemotaxis deficient mutants Δ*cheB* and Δ*cheY* still exhibiting robust dendritic expansion. Outward dendritic expansion in the absence of individual chemotaxis demonstrates that other mechanisms must be responsible for determining the expansion direction and generating the force needed to advance the colony front. Most models and many of the explanations of the way these expanding bacterial colonies 'choose' the outward direction involve some form of chemical detection and behavioral response on the individual level (Kaiser 2007; Partridge and Harshey 2013; Tamar, Koler, and Vaknin 2016; Witten and Sander 1981). With some overlap, other models describe the bacteria as a fluid which generates a surface tension gradient by excreting biosurfactant molecules, which the colony then exploits and rides as a form of Marangoni forces (Kinsinger, Shirk, and Fall 2003; Trinschek, John, and Thiele 2018).

The extreme sensitivity of the system to agarose stiffness or brief evaporation events suggests that these colonies exist in a fragile state, where even slight changes in extracted, suspending fluid volume strongly modulate group motility. A sharp transition from a rapidly advancing fluidized bacterial suspension to an immotile sessile growth

state over a narrow reduction in confining volume is suggestive of a jamming transition. In jamming transitions, through an increase in density, athermal mesoscopic components (particles, grains, etc.) become sufficiently crowded so as to achieve bulk rigidity(Cates et al. 1998; Liu and Nagel 1998). Our data is consistent with a model in which the volume of the confining fluid of a spreading colony determines the cellular packing fraction, such that a decrease in confining volume increases cellular density and causes the cells to become jammed. More experiments which explore this mode of colony halting are discussed in Chapter III.

An implicated feature of such dense hydrogels hearkens to hydrology, where the movement of fluids through porous soils and rocks bears resemblance to a puzzle at hand: how much trouble is it for these cultures to extract the fluid they'll be swarming within from their substrate? The very fact that surface tension and hydrophilic interactions govern evaporation rates from fluid surfaces provides evidence that stiffer gels (with their more dense arrangement of agar polymers) will hold onto their fluid more strongly than lower density gels.

There are a number of arguments to unpack here. Surface tension works by minimizing surface area exposed to by polar molecules to other substances (air, for instance). Within the fluid, the polar molecules have no (or little) preferred orientation, but at an interface they tend to line up in polarity to minimize disorder energetics and contact with the neighboring phase. B. subtilis extracts fluid from its hydrogel substrate by excreting a combination of surfactants to lower surface tension, and exopolysaccharides, which increase local osmolarity and thus increase osmotic pressure across the interface, leading to net fluid flow out of the gel. Because the gel consists of a

highly circuitous and elaborate network, the water moving through the system experiences a situation very similar to resistance in electrical currents. In fact, hydrological systems are frequently modeled to first order using similar the similar relation of Darcy's law, which relates the permeability (κ) of the medium and viscosity of medium moving through it (μ) to provide a linear relation between fluid flow (q) and the pressure gradient (∇p):

$$q = \frac{\kappa}{\mu} \nabla p$$

This demonstrates that the less permeable (more dense) the porous media, the more resistance to extraction one should expect (Licata et al. 2016). This establishes the basis for the observation that higher concentration (%w/v) agarose gels prevent colonies from extracting enough fluid from the substrate to generate flow.

On the length and energy scale of the bacteria themselves, surface tension plays a major role. Pure water has a surface tension of 72 mN/m, or on the lengthscale of bacteria, 7.2×10^{-8} N/μm (Yeh et al., 2005). In comparison, the estimated thrust of a flagellar bundle in *B. subtilis* is 5.7×10^{-13} N (Chattopadhyay et al. 2006). The bacterially secreted biosurfactant *surfactin* can lower surface tension, but only by a factor of ⅓, which still leaves a 5 decade difference in force scales from bacterial propulsion. This further explains the lack of influence of individual motility behaviors on the direction and expansion of the colony edges.

To understand the differences in force scale here, one can calculate the force on a bacterium due to surface tension and compare it to the force generated by flagellar

propulsion (Fig. 3). Doing so helps put into perspective how futile it would be for a bacterium to press against the interface between their suspending fluid and air, from the inside. Consider the force which must be overcome for a rod-shaped bacterium to penetrate out (positive $\hat{z}$) of a surface with surface tension γ. The bacterium may be approximated by a hemispherical cap of radius R on a cylinder of the same radius. As the bacterium pushes through the surface a distance h the surface line tension F_T acts tangent to the surface and along the line of contact: the circumference ℓ of a spherical cap of radius a (Note: surface tension has units of force per length, $[F/L]$.

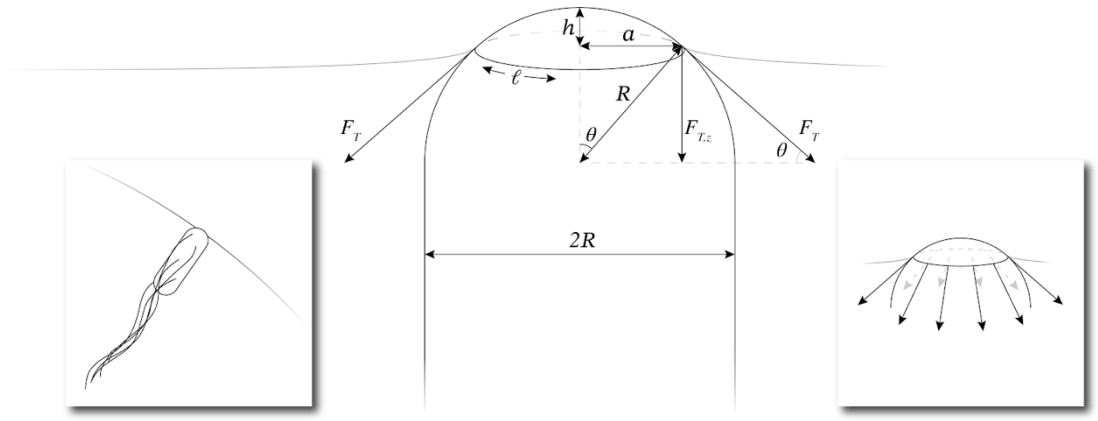

Figure 3. Diagram of a rounded rod penetrating out of a fluid against the force of surface tension. Inset left is a schematic zoomed out to aid in understanding the situation being modeled. Inset right demonstrates the forces distributed along the circumference of the penetrating sphere.

$$F_T = -\ell \cdot \gamma$$

is the force due to surface tension over the whole circumference, with

$$F_{T,z} = F_T \cdot \sin(\theta)$$

being the component in the inward direction. Given the circumference

$$\ell = 2\pi a$$

the total force acting on against the bacterium is

$$F_{T,z} = -\gamma \cdot 2\pi a \sin(\theta)$$

It can be seen that

$$\sin(\theta) = \frac{a}{R}$$

which gives

$$F_{T,z} = -\gamma 2\pi \frac{a^2}{R}$$

Now, rearranging the geometric relation

$$R = \frac{a^2 + h^2}{2h}$$

one finds

$$a^2 = 2Rh - h^2$$

which gives $F_{T,z}$ in terms of h:

$$F_{T,z} = -\gamma 2\pi \frac{2Rh - h^2}{R} = -\gamma\, 2\pi\, R \cdot \left(2\frac{h}{R} - \left(\frac{h}{R}\right)^2\right)$$

If we want to understand how far a bacterium could penetrate, this force could be compared with the force exerted by a *B. subtilis* flagellar bundle: $F_p = 5.7 \times 10^{-13}$N. Equating the two then solving for a grants:

$$a = \sqrt{\frac{F_p R}{\gamma 2\pi}}$$

Using the surface tension of water at room temperature $\gamma = 7.2 \times 10^{-8}\text{N}/\mu m$ and an approximation that $R = 0.45\mu m$ (Weart et al. 2007), we find $a = 0.75nm$, meaning a surface interaction area smaller than the diameter of a DNA double helix (~2 nm).

Given that $h = R - R\cos(\sin^{-1}\left(\frac{a}{R}\right)) = R - R\sqrt{1 - \left(\frac{a}{R}\right)^2}$, we find that the distance penetrated by the bacterium is $6.3 \times 10^{-7}\mu m$.

We can also see that a maximum force magnitude is reached when $h = R$, or

$$F_{T,z} = -\gamma 2\pi R \cdot \left(2\frac{R}{R} - \left(\frac{R}{R}\right)^2\right) = -\gamma\pi R \cdot (2-1) = -\gamma 2\pi R$$

If we instead want to consider the problem in terms of surface energy, we find that the surface area of a spherical cap of height h and radius R is

$$A_{cap} = 2\pi Rh$$

Now, given surface energy $\gamma = 7.2 \times 10^{-14}\ J/\mu m^2$ we have a surface energy of

$$E = \gamma \cdot A_{cap} = \gamma \cdot 2\pi Rh$$

We can take a derivative of E with respect to h to find a force F_A:

$$\frac{dE}{dh} = -F_A = \gamma\, 2\pi R$$

which yields the force relation in agreement with our above calculation:

$$F_A = -\gamma 2\pi R$$

Consequently this is the same force that would be generated by the areal increase of an additional cylindrical protrusion of the same radius $\left(A_{cylindrical\ segment} = 2\pi Rh\right)$ meaning there would be a constant force of $-\gamma 2\pi R$ for the bacterium's extrusion. Substituting in the approximations and values above, we find a maximum force of:

$$F_A = 2.0 \times 10^{-7}\text{N} \gg F_{propulsion} = 5.7 \times 10^{-13}\text{N}$$

To check this result, we can simplify even further, and consider the 2D case, in which a cross section of a bacterium tip, modeled as a rectangle with a semicircular cap

of radius $R = 0.45\ \mu m$ penetrates out a distance h of a 1D surface with line tension $\gamma = 7.3 \times 10^{-8}\mathrm{N}/\mu m$. For the portion of the bacterium where $0 \leq h < R$ (Fig. 4a), the area (A) extended is that of a circular segment of height h and radius R:

$$A = R^2\cos^{-1}\left(\frac{R-h}{R}\right) - (R-h)\sqrt{2rh-h^2}$$

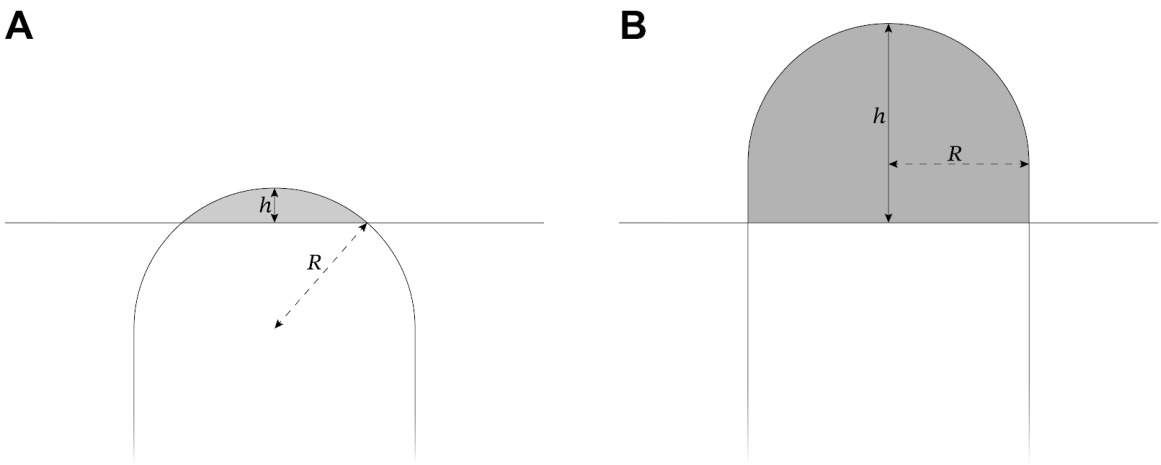

Figure 4. A simplified, 2D model of surface tension force due to rod penetration. Shaded regions represent the area subjected to surface energy. (A) the phase where $0 \leq h < R$, and (B) after the cap has passed.

We can non-dimensionalize this in terms of scaled height $h^\star = \frac{h}{R}$:

$$\frac{A}{R^2} = A^\star = \cos(1-h^\star) - (1-h^\star)\sqrt{2h^\star - h^{\star 2}}$$

After the cap is passed (Fig. 4b), the area extruded per height simply becomes:

$$A = 2Rh \text{ or } A^\star = 2h^\star$$

The surface energy added as a function of R and h is:

$$E = \begin{cases} \gamma R^2 \cos^{-1}\left(\frac{R-h}{R}\right) - (R-h)\sqrt{2Rh-h^2} : 0 \leq h < R \\ \gamma 2Rh \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad : R \leq h \end{cases}$$

To find the opposing force, we again take a negative derivative with respect to h:

$$\frac{dE}{dh} = -F$$

$$F = \begin{cases} -\gamma 2\sqrt{2Rh - h^2} : 0 \leq h < R \\ -\gamma 2R \qquad\qquad\quad : R \leq h \end{cases}$$

Plugging in our known values we find a maximum force of:

$$F_{max} = \ 6.5 \times 10^{-8}\ \mathrm{N}$$

These calculations, previous work, as well as my observations with Δ*srf* consistently show that surface tension and its gradients play a crucial role in this group motility, and that surface tension forces dominate individual bacterial propulsive forces in this environment (B. G. Chen, Turner, and Berg 2007; Fall, Kearns, and Nguyen 2006; Kearns and Losick 2003). The bacteria, once they reach a certain threshold density and population, excrete their surface tension lowering biomolecule, in addition to exopolysaccharides (Kearns and Losick 2003). Together, these exported molecules lower the energetic barrier of fluid extraction and produce an osmotic pressure difference which serves to pull fluid from the hydrogel. While stiff, dense gels hold onto their fluids more strongly, an apparent benefit of softer gels is to allow more fluid to be extracted by the colonies, and thus to increase the bacteria's local suspension volume and access to nutrients.

Surfactin excretion lowers local surface tension and generates a gradient of surface tension between two regions, which generates so called Marangoni forces (Velarde, Wilson, and Helliwell 1998). Marangoni forces occur when two adjacent regions of a fluid or film with different surface tensions are in contact. In such a system, the region of higher tension generates a net attractive force on the region of lower

tension. This force is used by bacterial cultures, which excrete surface lowering molecules and generate fluid flows outward (Trinschek, John, and Thiele 2018; Fall, Kearns, and Nguyen 2006; Angelini et al. 2009). However, the extent to which the Marangoni effect provides the force of expansion or provides a preferred direction to some other force of expansion remains to be determined.

CHAPTER III: THEY'VE BEEN JAMMIN

This chapter contains previously published co-authored material; it has been adapted from Ben Rhodeland and Tristan Ursell, "Rapid and directed group motility in B. subtilis does not rely on individual motility or chemotaxis" bioRxiv 719245; doi: https://doi.org/10.1101/719245 (2019). In this work, I contributed to designing the research, performing the research, analyzing the data, and writing the paper.

3.1 – Introduction

In their search for resources microbes contend with physically distinct environments ranging from soft surfaces to bulk Newtonian fluids and complex fluids like mucus. In bulk fluid environments, canonical microbes like Escherichia coli (Mesibov and Adler 1972) and Bacillus subtilis (Garrity and Ordal 1995; Bischoff and Ordal 1992) ascend favorable chemical gradients via run-and-tumble motility (Berg and Brown 1972). This mechanism of gradient ascent requires both flagellar-mediated motility and a complex system of phosphorylation-memory and chemical sensors on the bacterial surface, that together regulate the run-tumble transition frequency (Rao, Glekas, and Ordal 2008). In contrast, bacterial surface motility has different requirements and inputs, and different species have distinct modalities of surface transport. For instance, in the predatory species Myxococcus xanthus, individual cells move in back-and-forth motions via so-called 'twitching' motility and assemble into larger motile groups that traverse surfaces as monolayers (Balagam and Igoshin 2015). Many other species, including the opportunistic pathogens Serratia marcescens (Patteson, Arratia, and Gopinath 2018; Rabani, Ariel, and Be'er 2013) and Proteus mirabilis (Matsuyama et al.

2000), also form large groups of motile cells that are capable of rapidly expanding over surfaces, in some cases even against bulk fluid flow (Jones 2005). Similarly, when present in sufficient numbers Paenibacillus dendritiformis exhibits intricate fractal-like pattern formation on soft agar surfaces in response to lateral chemical gradients (Ben-Jacob, Cohen, and Levine 2000; Ben-Jacob 1997). Other cells respond to external gradients in non-chemical fields; for instance the cyanobacterium Synechocystis is phototactic, responding to incident light by asymmetrically extending and retracting pili from its surface to create a biased random walk toward a light source (Ursell et al. 2013). Crucial to its motion, Synechocystis modifies the local surface environment by secreting exopolysaccharides, and only when enough cells have participated in such surface modification can the group move toward the light source. These examples demonstrate that in response to various gradients, microbes have evolved distinct sensing capabilities and modalities of motion to acquire resources and respond to selective pressures on surfaces. Despite their differences, surface motility in all of these species appears to be a collective phenomenon, requiring the motion of and/or surface modifications by large numbers of cells. Therefore, understanding the physical forces that produce and guide microbial group motion on surfaces is integral to our understanding of microbial ecology in natural environments and will expand the suite of design tools for engineering microbial systems.

Efforts to model these systems (notably P. dendritiformis) have focused on reaction-diffusion partial differential equations, which reproduce, with high fidelity, many of the classes of bacterial surface patterning observed in experiments (Caiazza, Shanks, and O'Toole 2005; Ingham and Jacob 2008; Kaiser 2007; Parrish and Edelstein-

Keshet 1999; Steager, Kim, and Kim 2008; Marrocco et al. 2010). These models rely on bacteria using chemotaxis to follow nutrient gradients that the colonies themselves produce through local consumption of nutrients (Giverso, Verani, and Ciarletta 2015). Growth and spreading of cells in dendritic patterns are modeled as a diffusion-limited conversion of nutrients into biomass at the growing tips of the dendrite; such models do not incorporate advective flow of cells nor material within the dendrite. Within a dense, actively swarming group whose velocity correlations rapidly decay on the length scale of a few cells (Be'er and Ariel 2019), it is unclear how individual cells would be able to effectively modulate their tumble frequency and accumulate sufficiently long persistent runs to deliberately bias their random walks and hence execute run-and-tumble chemotaxis. Likewise, it is unclear how the chemotaxis of individual cells in a swarm could guide a group in the ascent of a nutrient gradient. Thus, the ability of large bacterial groups to traverse surfaces appears to involve the salient trio of motility, chemotaxis, and cell density, but the role that cell density plays and whether motility and chemotaxis are necessary features for surface motility are unknown.

In this work we begin to clarify the relative contributions of motility, chemotaxis and cell density by examining the surface motility of the extensively-studied Gram-positive bacterium B. subitilis (Rao, Glekas, and Ordal 2008; Ward et al. 2018; Srinivasan, Kaplan, and Mahadevan 2018; Kirby et al. 2000). From a small central inoculum, wild-type cells can rapidly colonize an entire wet 10 cm agar plate in 1 - 2 hours via apparent swarming motility (Kearns 2010). Group motility over soft agar surfaces has been shown to depend on the secretion of 'surfactin', a bacterially produced bio-surfactant and wetting agent (Giverso, Verani, and Ciarletta 2015; Schwartz and Roy

2001; Trinschek, John, and Thiele 2018; Kearns and Losick 2003; Kinsinger, Shirk, and Fall 2003). Functional knockouts for surfactin production (Δ*srf*) result in a phenotype where individual cells are still motile and chemotactic in bulk fluid, but bacterial groups cannot move across surfaces (Kearns and Losick 2003). Localized secretion of surfactin is thought to generate a gradient in surface tension, and thus produce motion via the Marangoni force (Srinivasan, Kaplan, and Mahadevan 2018; Trinschek, John, and Thiele 2018). Despite the recognized role of surfactin, the contributions of individual motility, chemotaxis, and surface density of cells toward group surface motility remain poorly understood. Thus, we employed a combination of high-resolution imaging and computational image processing, as well as genetic manipulations to determine to what extent motility and chemotaxis of individual cells are required for group level motility on surfaces. Surprisingly, we found that the movement of *B. subtilis* over soft agar surfaces in the direction of 'fresh' territory showed only weak dependence on local gradients in nutrient concentration, and that neither motility nor chemotaxis of individual cells was necessary for group motility over surfaces. Even in the extreme case where mutant *B. subtilis* were devoid of flagella (Δ*hag*) groups of cells traversed the agar surface with patterns and rates similar to wild-type cells. Finally, our image analysis suggests that at high cell density, groups of cells are subject to a jamming-fluidization transition, that is, dense groups of cells behave like a two-dimensional, shear-thinning fluid (Lopez and Lauga 2014). These data suggest that individual motility and group swarming have less to do with generating force for motion, and that rather, individual motility and subsequent swarming are mechanisms that maintain a fluidized state on which surface-tension gradient forces (Maragoni forces) can act to precipitate group motion over a surface.

Thus, a jamming-like transition, like those found in other macroscopic granular systems (Burel, Martin, and Bonnefoy 2017; Woldhuis et al. 2015; Cates et al. 1998), may be a key regulator of bacterial surface transport.

3.2 - Results

Bacillus subtilis is a model motile Gram-positive bacterium, capable of sensing and responding to its chemical environment via run-and-tumble chemotaxis (Rao, Glekas, and Ordal 2008). On wet surfaces wild-type *B. subtilis* rapidly move out from a central inoculum, apparently via collective swarming motility (Kearns 2010) that is known to require secretion of the endogenous bio-surfactant 'surfactin'. Mutants that lack the ability to produce surfactin (Δ*srf*) do not expand from their central inoculum. We inoculated small, dense droplets of *B. subtilis* on soft (~0.5% w/v agarose) nutrient-rich agar surfaces. After a brief quiescent phase, groups of cells rapidly expanded over the surface in a dendritic pattern that reached the edge of the plate (~5 cm of travel) in less than 6 hours. Dendrites robustly moved outward away from the original point of inoculation into fresh territory, at an average group motility rate of ~10 μm/s and up to ~30 μm/s (Fig. 5). Cells within the dendrites were highly motile, exhibiting swarming motility (Kearns and Losick 2003) with individual cells moving at rates of ~30 um/s but in highly circuitous paths. Notably, dendrites composed of individuals swimming in highly circuitous paths move directionally outward at peak rates comparable to what one would expect if all cells simply swam directly outward. Thus, we wanted to test the hypothesis that this rapid and directed movement is both related to the underlying motility of the bacteria and uses their innate chemotactic abilities to sense and direct

movement along the outward-facing nutrient gradients created by the bacteria's own consumption of metabolites in the agar.

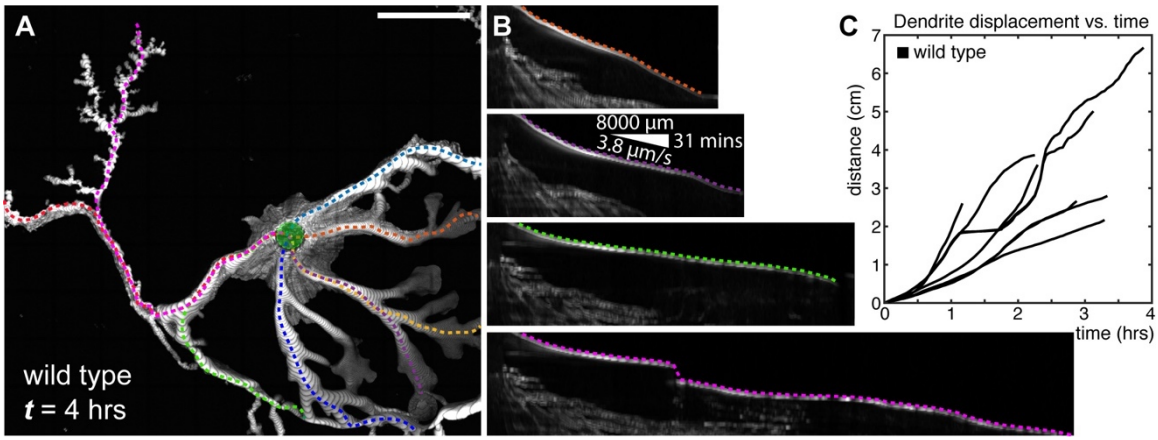

Figure 5. Expansion dynamics of wild-type *Bacillus subtilis.* **(A)** A maximum intensity projection of *B. subtilis 3610* (wt) spreading across nutrient rich plate with 0.5% agarose w/v. Colored contours (dashed lines) are drawn along selected dendrites. Original colony inoculation is shown as green circle. Scale bar is 1 cm. **(B)** Kymographs constructed from imaging data along specific contours (colors matched with (A)). The vertical dimension is time and the horizontal dimension is arc-length of the path of the dendrite. **(C)** Plot of dendrite displacement vs. time, showing rapid surface motility ranging from ~4 µm/s up to ~10 µm/s.

Surface motility does not require chemotaxis.

First, we wondered whether the surface motility of fully chemotactic wild-type cells followed local chemical gradients. We created agar plates with two distinct halves – one half with agar containing a rich defined medium (RDM), the other half containing NaCl buffer with agar, osmotically matched to the RDM to maintain the same osmotic potential (see Methods). An impermeable barrier separated the two halves, initially keeping the nutrients on one side. Once the agar set, we poured a thin (~ 1 mm) layer of osmotically matched NaCl buffer with agar, and thus linked the two regions into a contiguous surface that allowed cells to move freely between them. The nutrient-rich half also contained a red fluorescent tracer dye that allowed us to visualize chemical diffusion

over the barrier between the two halves, as an approximate reporter of nutrient diffusion over the barrier. With or without cells, these plates showed diffusion of the dye from the nutrient rich side into the side devoid of nutrients, meaning that there was a negative concentration gradient on the surface from the nutrient rich to the nutrient poor side. We reasoned that if wild-type cells were responding to the outward-facing chemical gradients on a standard plate (i.e. isotropic with nutrients), they would sense the reverse nutrient gradient on these split plates and either avoid the nutrient poor region or show reduced motility toward it. Remarkably, wild-type cells inoculated on the nutrient rich side moved roughly evenly in all directions, moving down the concentration gradient into the zero-nutrient side of the plate at rates comparable to colony mates moving up the nutrient gradient on the other side of the inoculation point (Fig. 6). On the same plate a control wild-type inoculum was deposited onto the zero-nutrient region. In contrast to the nutrient-rich inoculum, these cells did not exhibit collective motility of any kind. These data suggest that while the directionality of motile groups is not particularly sensitive to nutrient gradients, cells need nutrients (at least initially) to be collectively motile, and that cells provided with nutrients initially can traverse large stretches of nutrient barren territory, while cells devoid of nutrients cannot.

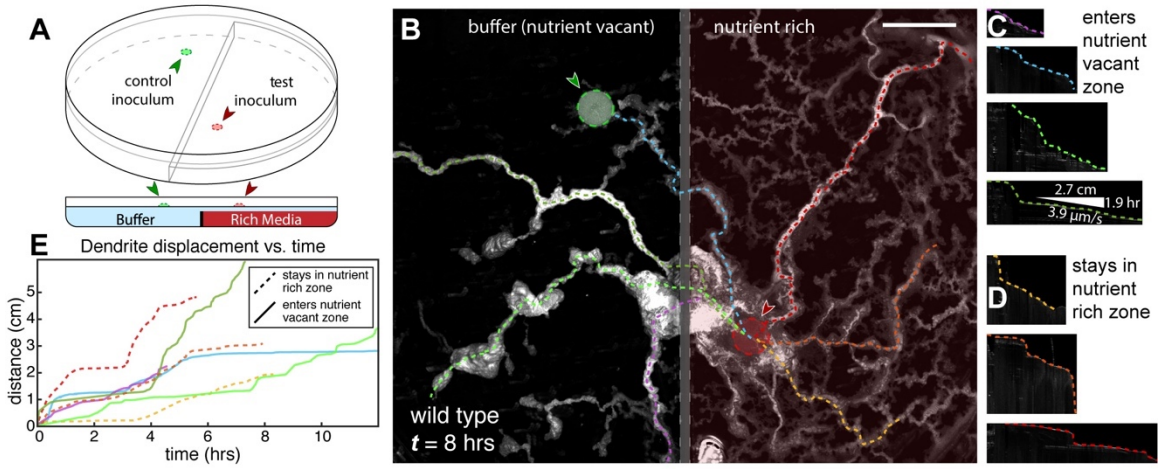

Figure 6. Surface motility in the presence of an exogenous nutrient gradient. **(A)** Low agarose plates were poured with a barrier between nutrient-rich media doped with a fluorescent tracer dye (rhodamine) on the right half of the plate and osmotically balanced zero-nutrient buffer on the left, with a top layer of balanced zero-nutrient buffer to create a contiguous surface. The spatial distribution of the dye is a proxy for nutrient diffusion, and demonstrates that the barrier limits diffusion into the zero-nutrient region. Two dense colonies (OD 5, 1.5µl) were deposited on either side of the barrier. The colony on the zero-nutrient side acts as a no-nutrient motility control. **(B)** Wild type *B. subtilis* inoculated on the nutrient-rich region (right, red dashed circle) spreads dendritically across the divided plate into the zero-nutrient region, effectively traversing along a negative nutrient gradient. An identical inoculum on the zero-nutrient region (green dashed circle) exhibits no collective motility. Scale bar is 1 cm. **(C,D)** Kymographs were generated along curves that trace the extending dendrites. **(C)** dendrite paths that cross the barrier into the zero-nutrient region. **(D)** dendrite paths that remain in the nutrient-rich region. Both types of dendrites exhibit similar rates and behaviors. **(E)** Dendrites extend across the plate from the colony on the nutrient rich side, both into the nutrient-rich region and across the barrier and down the steep negative nutrient gradient. Dashed lines correspond to dendrites which never encounter the barrie– and solid lines correspond to those that cross the boundary.

To determine whether chemotaxis of individual bacteria is required for groups of *B. subtilis* to traverse a soft agar surface we disrupted chemotaxis in two distinct ways,

reasoning that each method may produce different outcomes in terms of group surface motility. First, we examined the surface motility of run-only (Δ*CheB*) mutants that were motile but lacked the ability to reorient via tumbling, and hence cannot perform run-and-tumble chemotaxis, regardless of which surface chemoreceptors were expressed. Second, we examined the surface motility of tumble-only (Δ*CheY*) mutants that have all of the flagellar machinery to be motile, but are permanently in the tumble state, and hence are both (effectively) non-motile and non-chemotactic (Kearns and Losick 2003). Despite that both strains cannot follow a chemical gradient of any kind, surprisingly, both strains exhibited dendritic expansion over agar surfaces with morphologies and speeds similar to the expansion of wild-type cells (Fig. 7A-B).

Together, both the split plate data and the run-only / tumble-only mutant data strongly suggest that group surface motility of *B. subtilis* neither requires chemotaxis nor relies on chemical gradients as a determinant of expansion direction.

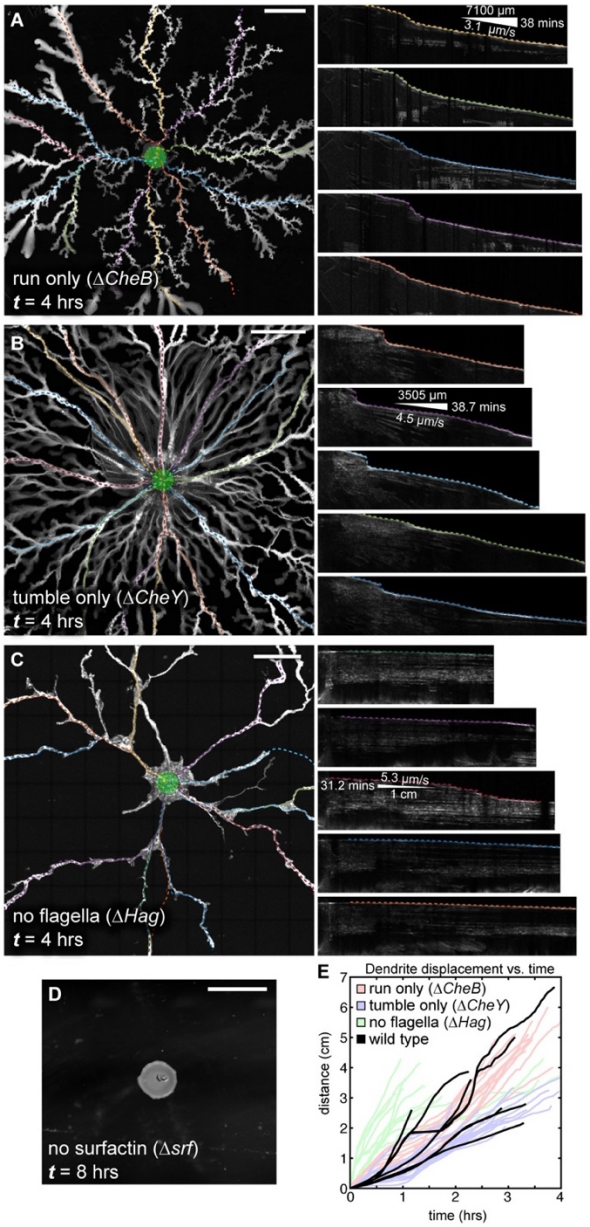

Figure 7. *B.*subtilis genotypes that lack the ability to chemotax or self-propel can still exhibit collective motility. Maximum intensity projections of dendrite dynamics for **(A)** run-only (Δ*CheB*), **(B)** tumble-only (Δ*CheY*), and **(C)** flagellar hook-deficient (Δ*Hag*) genotypes, with contours (dashed lines) overlaid on dendrites and associated kymographs (right) with leading edges indicated (dashed lines). **(D)** Surfactin deficient (Δ*srf*) mutants are not collectively motile. **(E)** Each of the motility mutants (A-C) exhibit similar dendritic morphologies and speeds of advancing dendrites, as illustrated in this plot of dendrite tip displacement vs. time. Note that some dendrites of the non-motile Δ*Hag* genotype outpace all other motile genotypes. These samples represent typical variation of advancing dendrites for their respective genotypes. All scale bars are 600µm.

Individual motility is not required for group motility

Given that tumble-only mutants, despite being motility deficient, exhibit surface expansion rates and morphologies similar to wild-type, we wondered if individual motility was in any way required for group surface motility. To be clear, we wanted to know if cells lacking the ability to move at all could expand across a plate at rates similar to wild-type. This is in contrast to expansion across plates through the replication of sessile cells at the leading edge of growth, as seen in *P. dentritiformis* (dendrite-like) or *E. coli* (growing circular colonies), both of which expand at much slower rates (Jauffred et al. 2017; Beloin, Roux, and Ghigo 2008; Tamar, Koler, and Vaknin 2016).

To assess surface motility in the absence of individual motility, we examined *B. subtilis* mutants lacking flagellar filaments (Δ*hag)* (Kearns and Losick 2003). These cells have intact chemotaxis machinery and sensors, and retain intact flagellar basal bodies (motors), but do not have flagella and hence cannot run, tumble, chemotax, or move in any way that requires the use of flagella. Even in the absence of any mechanism of individual propulsion, colonies inoculated at the center of a soft agar, nutrient-rich plate still expanded outward in a dendritic pattern (Fig. 6C). These data demonstrate that individual motility is not required for group motility, rather mechanisms other than individual motility and / or chemotaxis precipitate the emergent, colony-scale dendritic self-organization of *B. subtilis* on soft nutrient-rich surfaces.

Granular jamming transition may be a key regulator of surface motility

While the surface motility of *B. subtilis* was robust to genetic manipulations of chemotaxis and motility, it was very sensitive to agar gel stiffness as measured by

percentage of agarose. Below ~ 0.45% agarose by weight, the gel is sufficiently porous that bacteria can penetrate and swim within it, akin to canonical swim plates (Kearns 2010; Wolfe and Berg 1989), and above ~0.6% agarose, limited water availability hinders surface motility (Kearns 2010). Thus, across a relatively narrow range of agarose percentages, groups of *B. subtilis* exhibit three qualitatively distinct behaviors: swimming through agarose (below ~0.45%), rapid surface motility (between ~0.45% and ~0.6%), and slow growth by replication (above ~0.6%). Why does the transition from rapid surface motility to slow edge growth occur over such a narrow range of agarose stiffness, and correspondingly, water availability?

Past studies of *B. subtilis* characterized spreading and any attendant morphological behaviors at the length-scale of colonies (~1 cm) and on the timescale of bacterial replication, imaging colony morphology and spreading with minutes or hours between frames (Caiazza, Shanks, and O'Toole 2005; Kaiser 2007; Kearns 2010; Kearns and Losick 2003). Indeed, much of what is known about spreading phenotypes and their genetic mechanisms was discovered through imaging at these scales. However, after determining that chemotaxis and individual motility were not required for group surface motility, we decided to perform high temporal and spatial resolution imaging of spreading colonies to illuminate processes that potentiate spreading. Specifically, we captured images at 30 or 60 frames per second with spatial resolution of 5 μm/pixel or 15 μm/pixel, respectively, both of which enabled us to see intensity variations produced by the movements of individual cells within the swarm (see SI movies). We wrote a custom image analysis script that examines the magnitude of swarm motion (i.e. a scalar measure of motion) as a function of position through time. Briefly, the algorithm characterizes the

local intensity fluctuations at a position across a set of *N* (usually 5 – 7) frames and thus reports on the level of motile activity at each position through time. With this 'activity' filter, we were able to visualize which parts of the colony were actively swarming and in which parts cells were stationary (Fig. 8A-B).

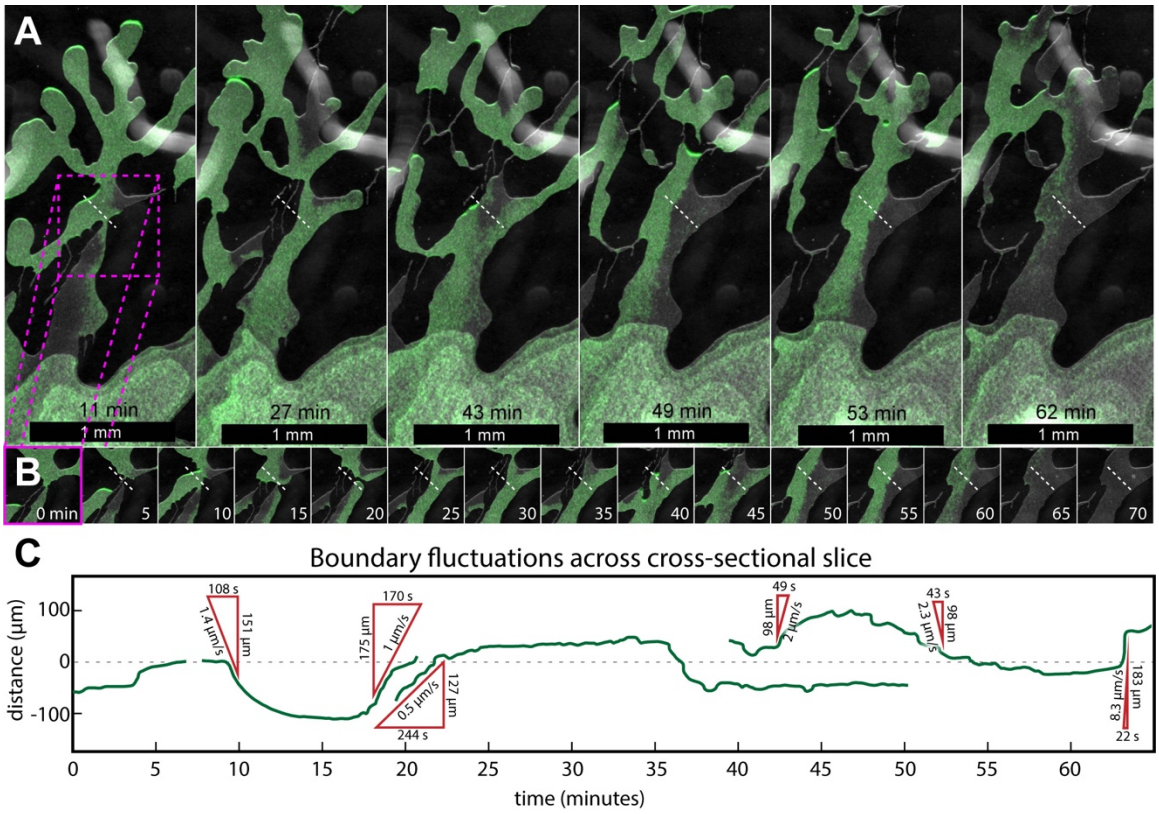

Figure 8. Cellular monolayers exist in fluidized, motile states and jammed, stationary states. **(A-B)** Time-lapse imaging of wild type *B. subtilis* spreading dendritically on a nutrient-rich low agarose surface. We applied a computational image filter that measures local intensity fluctuations over time, and then colors regions of high motile activity (green) and leaves stationary regions uncolored (gray). Transient active regions and fluctuating boundaries between active and stationary regions can be seen emerging and dissipating on timescales shorter than (potential) phenotypic switching (see SI video). These data are consistent with bacterial groups undergoing a dynamic jamming transition that halts collective motion and dendrite expansion. **(C)** A plot of the boundary positions between fluidized and jammed regions over time (across the white dashed line in (A)). Scale triangles indicate rates of boundary movement, with several boundary fluctuations moving on the order of 1 - 10 µm/s.

This analysis revealed a number of salient features of surface motility. We observed that the rapid movement of dendrites and cellular groups was highly correlated with significant increases in the quantitative measure of activity from our image analysis, indicating that movement of individuals positively correlates with movement of the group. Conversely, regions whose constituent cells were stationary (low measure of activity) did not move or flow on the surface. However, the most striking feature was that we observed the formation of stark boundaries in activity level within contiguous regions of cells, indicating that within a genotypically and phenotypically identical subpopulation, the group could adopt two qualitatively distinct states of motion.

We interpret the high activity state as the fluidized state in which cells are swarming, and the low activity state as the 'jammed' state where cells are stationary due to their packing density. Further, the phase boundary between these regions actively fluctuated in time, translating microns per second, which is faster than (potential) phenotypic switching in the local chemical environment (Fig. 8C).

Next, we performed high resolution imaging of expanding tips that were subjected to evaporation, and hence reduced water availability, which increased cellular packing fraction. These expanding tips showed a rapid and dynamic jamming transition as the meniscal boundary between the agar and the cellular suspension retracted (Fig. 9A-B). Then, to examine the reversibility of this transition, we took the same plates and expanding tips, resealed them to halt evaporation, and continued imaging while water from the gel rehydrated the cells. This allowed the colony to 'thaw' into domains of high activity cells, which then coalesced until the entire tip region regained fluidity and continued to expand. This strongly indicates that re-wetting and the corresponding

reduction in packing fraction 'reverses' the jamming transition back to a fluidized and collectively motile state (Fig. 9C-D).

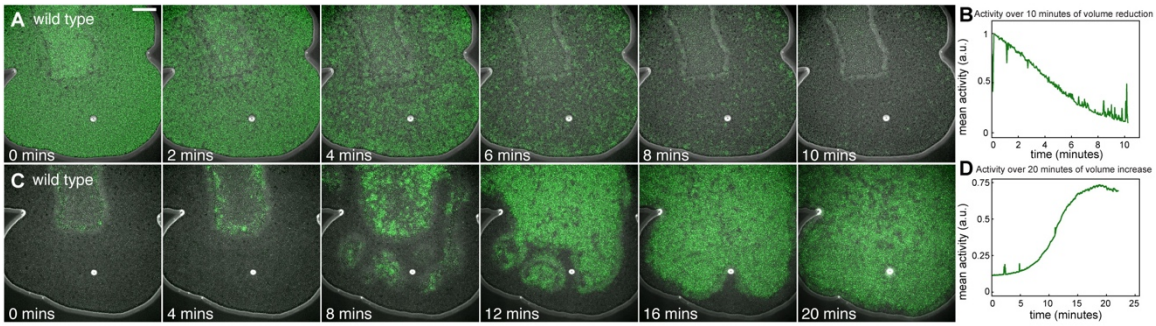

Figure 9. Water availability and cellular packing fraction control the jamming transition. **(A)** Time-lapse imaging of the tip of a wild type *B. subtilis* dendrite on a nutrient-rich low agarose plate. At $t = 0$, the plate was unsealed and its surface was exposed to air and imaged while being allowed to evaporate for 10 minutes. The overall effect was less water available at the agarose/air interface, and thus a slight, transient increase in cellular packing fraction that caused both collective motion (green color) and the computational measure of mean motile activity **(B)** to drop sharply as the cellular group entered the jammed state. Across the entire dendrite tip collective motility halted. **(C - D)** The same plate was then immediately resealed and imaged over 20 minutes. The sharp drop in evaporation rate allowed cells to retain more water from the gel and thus decreased their packing fraction. This ultimately allowed fluidized domains of motile cells to emerge and coalesce until the entire dendrite was, once again, collectively motile and had resumed expansion. Scale bar is 100μm.

Thus our data are consistent with a model in which the dense packing of surface bacteria act like a shear-thinning fluid that exhibits a jammed state if the non-uniform velocity field (internal shear) caused by motility decreases, and/or if water availability decreases due to either a stiffening of the agar surface or evaporation. In this model non-motile genotypes (such as Δ*hag*) can also exist in a surface-motile state if there is sufficient water availability to maintain a fluidized state, with the force and direction of group motility being produced by surface-tension gradients that result from the secretion and spreading of endogenous surfactin. Thus, an abiotic granular phase transition

between qualitatively distinct jammed and fluidized states clarifies the interacting roles of individual bacterial motility, swarming motility, and water availability in the rapid group surface expansion of wild-type *B. subtilis.*

3.3 - Methods

Isogenic cultures of *B. subtilis* strains at 0.5 OD were mixed 50% in glycerol aliquots and individually snap frozen and stored at -80 C. For each experiment, 200 μL aliquots were removed and thawed, then diluted in 10 ml LB and grown in a 37 C shaking incubator for 4 hours until mid-log phase, then pelleted. The supernatent was removed, and the bacterial pellets were then resuspended in 200 μL LB, yielding a culture with an approximate OD of 10, then 1.5 μL of that culture was deposited on plates of Teknova EZ-RDM and 0.5% agarose, then dried 10 mins and sealed with parafilm. Depending on the type of experiment, plates were then either incubated for 1 hr then imaged or imaged immediately at 37 C.

Plates were created by mixing 100 mL Teknova EZ-RDM and 0.5% agarose by weight, autoclaving for 15 minutes, allowing the media to cool to 50 C in an incubator before being poured into 4 plates (25ml each) and cooled under flame in open air for 10, 15, or 30 minutes before immediate inoculation, creating a range of initial gel hydrations to examine the effects of water availability on colony morphology.

Brightfield microscopy was performed using a Nikon SMZ-25 stereo zoom microscope, with a P2-SHR Plan Apo 1x objective, and a Prior ES111 OptiScan stage. High speed images were taken using an Andor Zyla 5.5 CMOS camera. Phase contrast microscopy was performed using a Nikon Eclipse Ti-E inverted microscope, with a S

Plan Fluor ELWD 20x Ph1 ADM objective. High speed images were taken using an Andor iXon Ultra 888 EMCCD camera.

Activity overlays were generated using custom Matlab scripts which calculated the per-pixel sum over the differences between 5 or 7 consecutive frames. Kymographs were generated using custom Matlab scripts to identify and interpolate contours, then sum over cross-sectional slices of intensities along the contours over time. All scripts and Matlab code are available upon request.

3.4 - Discussion

Our data support a model of bacterial surface motility in which run-and-tumble chemotaxis is not the mechanism that determines the direction of migration, and individual motility does not play a crucial role in whether cells are collectively motile. Indeed, we found that cells that cannot move at all (Δ*hag*) still exhibit group surface motility. Rather, our data suggest that water availability – which can be viewed as a proxy for bacterial packing fraction – is a sensitive control parameter for an abiotic jamming transition in the granular material that is densely packed, expanding bacterial populations. We hypothesize that motility plays a contributory role, encouraging a fluidized state which is able to flow over the surface when driven by surface-tension gradients (Marangoni forces). Thus individual motility is not required for surface motility, but contributes to shear-thinning of the bacterial suspension, and expands the range of water availability / packing fractions over which bacterial groups can move via surface tension gradients.

The fact that bacterial groups rapidly expand over surfaces but do not exhibit bulk chemotaxis may have multiple underpinnings. First, it may be due to the infeasibility of individual chemotaxis in a dense, swarming mass of cells. Regardless of any local chemical signals, it is unclear how cells in such dense, turbulent swarms could generate persistent runs with punctuated random reorientations (tumbles), when their available rotations are highly constrained by steric hindrance of neighboring cells and any persistent runs are reoriented by frequent collisions. Second, the magnitude of the nutrient gradient that cells create via consumption is reduced by the speed at which cells traverse the surface. At the leading edge, their rapid and persistent lateral motion into fresh territory might not significantly deplete the rich store of nutrients in the agar below. It remains an open question whether individual chemotactic activity – or other sets of individual motility rules – when enacted in a dense swarm could provide an emergent mechanism by which active, nematic actors influence their bulk directional flow. Other environmental contexts or species of bacteria may exhibit bulk chemotaxis, but in those situations it seems unlikely that the quintessential run-and-tumble algorithm would be effective for guidance.

The potential existence of an abiotic jamming transition that regulates group motility through the dense packing of nematic actors unifies a number of observed phenomena, including: (i) the fact that increasing agarose gel stiffness switches groups from rapid surface migration to slow steric growth, (ii) the necessity of secreting both surfactant and osmolytes to extract fluid from the substrate (Kearns 2010; Kearns and Losick 2003), and (iii) the augmentation of rapid colony expansion by unbiased swarming motility or agitation via individual motility. We hypothesize that all of these

reflect the relatively sharp transition between jammed states of densely packed bacteria that have low-shear properties akin to a 2D granular solid, and fluidized states that can flow over the surface. Colonies that are not able to achieve the fluidized state – for any of the above stated reasons – are constrained to expand on the order of 100 times slower over surfaces using the forces generated by cell wall growth and cell division (i.e. the mode of sessile growth one typically associates with single colonies growing on a petri dish).

Within the framework of an abiotic jamming transition, our data support multiple roles for surfactants (and specifically *surfactin*): (i) they reduce surface energy of the confining fluid envelope which permits a fixed osmolyte concentration to pull more fluid from the substrate, (ii) they reduce local surface tension and hence allow the 2D bacterial swarm to more easily extend its boundary, (iii) their localized secretion creates the surface energy gradient that drives group motion (independent of individual motility), and (iv) that same gradient points away from sources of secretion and therefore may be the director of motion away from previously colonized areas (as opposed to sensing and behavioral reactions to chemical gradients).

This framework also provides a self-consistent explanation for the effects of exogenous surfactant addition. Experiments that added small concentrations of detergent to the gel (Yang et al. 2017; B. G. Chen, Turner, and Berg 2007) showed increased surface coverage, presumably due to boundaries that expand more easily, and had less defined dendrites, presumably because there was more water available, less boundary energy, and less of a directed outward surface-energy gradient. In contrast, we hypothesize that the surface energy of aqueous media without surfactant provides enough

downward force to pin individual cells against the soft gel, whereas groups of cells secreting surfactants would only feel such downward forces at their meniscal boundaries.

Finally, this framework also suggests that the transition to rapid surface motility is not directly related to the mechanical stiffness of the substrate (e.g. bulk modulus), rather stiffer polymer gels increase the osmo-mechanical work required to extract a unit volume of water from the gel. This also potentially explains why surface spreading assays are so sensitive to local humidity and gel drying times – all of these variables modulate water availability and hence modulate the effective state-variable that regulates the jamming transition.

Whereas individual cells propel themselves and direct their motion according to the algorithms of chemotaxis, these data and previous work strongly suggest a fundamentally different set of rules governing group surface motility. Specifically, that collective secretion of surfactants drives and directs motion, while bacterial packing fraction – modulated through multiple mechanisms – is a sensitive control parameter that regulates transitions between fluidized (collectively motile) and jammed (immotile) states of the group. As the fields of bacterial ecology and engineering progress, we should thoughtfully consider how population dynamics are affected by mechanisms of group behavior, and their interactions with the physical environment, that are not fully described by genetics and biochemistry.

CHAPTER IV – DISCUSSION AND FUTURE WORK

4.1 – Introduction

In the preceding chapters I described completed experiments and conclusions which set the stage for promising future work. In this chapter, I will explore a number of open questions, hoping to provide insight toward closing a subset of them, and along the way I will describe new experiments and courses of study which may further our understanding of rapid bacterial surface translocation.

In Chapter II, I described a high-throughput scanning apparatus with which I investigated characteristics and environmental factors that contribute to rapid surface colonization of agarose plates. By independently varying agarose and glucose concentrations in a background of rich defined media I found that *B. subtilis* spreads robustly and with minimal morphological variation across a wide range of glucose concentrations, but undergoes a stark transition in colony shape and spreading speed over a narrow range of agarose gel concentrations. Further, I found that in the range of agarose concentrations permissive of rapid colony expansion, motility mutants Δ*cheY* and Δ*cheB*, as well as a flagellar knock-out mutant, Δ*hag*, all displayed similar rates of expansion and colony morphologies. Surfactin production knock-out mutants, Δ*srf*, on the other hand, were never able to colonize much more than their original inoculation radius, presumably due to their inability to lower the surface tension of their local suspension.

In Chapter III, we measured the expansion rates of dendritically expanding *B. subtilis* with high spatial and temporal resolution and characterized actively moving regions using a custom computational 'activity' filter. By monitoring the local activity of

expanding dendrites, we revealed rapidly fluctuating boundaries between actively motile and inactive regions, which are suggestive of jamming-fluidization transitions as a regulator of colony expansion. We then measured activity in colonies whose surface evaporation rates were in excess of their substrate fluid extraction rates, resulting in a decrease of surface-bound bacterial-suspension fluid. This reduction in available fluid volume precipitated an abiotic physical jam as the packing fraction of bacteria increased (in their confining volume). The same colonies were then re-sealed and the balance restored to favor fluid extraction from the gel substrate, allowing the previously jammed bacterial colonies to re-fluidize and continue their colony-scale expansion.

To our knowledge, these experiments are the first evidence of an abiotic jamming transition in surface bound, rapid group motility. Such a transition serves to unify a number of observed phenomena, explaining the sensitivity of swarming and sliding assays to agarose gel concentration, plate drying times, local humidity, endogenous osmolyte export, and surface tension lowering agents. All of these point to bacterial packing fraction as a sensitive control parameter that regulates transitions between fluidized and jammed states of the group.

4.2 – Remaining Questions

What evidence is there that colony-scale growth isn't the result of replication-based cell expansion?

In the literature, sliding motility is loosely defined as a passive form of surface spreading that does not require an active motor (Kearns 2010; Henrichsen 1972), but relies on surfactants to reduce surface tension, ultimately enabling the colony to spread

by other forces. Most references surveyed go further and assume that the spreading is "driven by the outward pressure of cell growth" (Kearns 2010). In essence, the driving force in sliding motility is posited to be the collective steric forces produced by the growth and division of individual cells. While it is clear that cell growth is occurring during collective expansion (however at a reduced rate), in this system it is also clear that collective movement is not reliant on such growth, as we frequently observed isolated groups of cells that break away from their parent dendrites and move outward at rates slightly in excess of the trailing tendrils. Without a connection back to the mother colony, any pressure due to cell growth is lost.

Can runs and tumbles be executed within a dense, active colony?

In short, it's probably the case that cells are at times engaging both directions of flagellar rotation (and thus executing runs and tumbles as far as they 'know'), but it's unlikely to meaningfully change an individual's path in pursuit of a destination. Swarming colonies exhibit bacterial turbulence (Dunkel et al. 2013), with velocity correlations rapidly decaying on the length scale of a few cells (Be'er and Ariel 2019). In an environment where the trajectories of individual bacteria are constantly buffeted by impacts with their neighbors it is unlikely a single cell could accomplish the sufficiently long, persistent runs necessary to deliberately bias their random walks and thereby execute run-and-tumble chemotaxis.

Can a single bacterium on a surface detect and respond to a negative nutrient gradient (as in the split-plate experiments from Chapter III)?

Isolated bacteria on soft agar surfaces are unable to move, pinned by surface tension. In groups of cells, the downward forces of surface tension are felt primarily a the meniscal boundaries, freeing those bacteria within the bulk of the fluid suspension to move relatively unencumbered within the thin film volume. If, instead, the gel is low enough in concentration for the bacteria to burrow into the polymer maze (as in swim plates), individual bacteria do show avoidance of nutrient deficient regions by biasing their tumble frequency.

Do expanding dendrites carry nutrients with them?

Very likely, and it would be an excellent future course of study to measure this transport. It has been shown before in other species that in genotypically heterogeneous mixtures, one subset of highly active cells can carry along another set of immotile cells as cargo, who is otherwise critical to the colony's survival (by providing antibiotic resistance, for instance) (Finkelshtein et al. 2015).In the colonies that expand across the boundary from the nutrient to the zero-nutrient side, rafts of cellular clumps can be seen flowing along the tendrils from the mother colony. It would be surprising if these rafts and the rest of the flows did not bring along some amount of entrained fluid and nutrients. Further, I found flow within the tendrils by doping the colonies with fluorescent beads (Fig. 11), and there is no obvious mechanism why such flow would not carry along nutrients, waste products, and other metabolites and chemical signals.

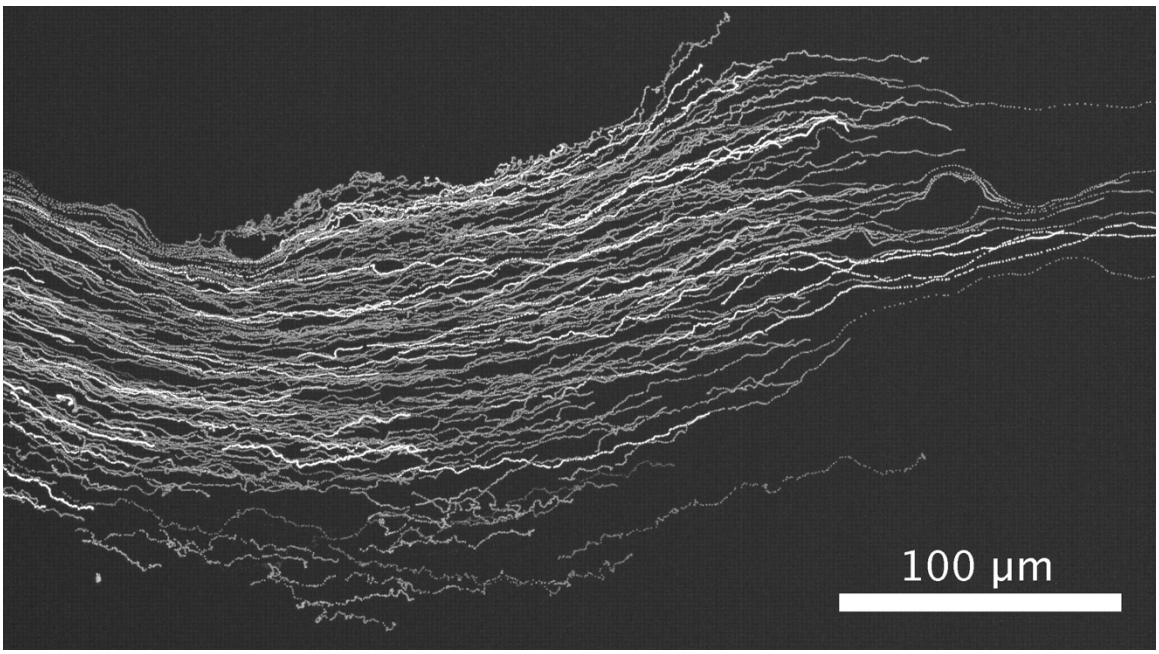

Figure 10. Flow in a dendrite is seen in this maximum intensity projection of fluorescent tracer beads (0.52 µm diameter) which are carried along in an expanding *B. subtilis* colony. Traces span 40 seconds of flow, taken at 10 fps.

4.3 – Future Directions In Nutrient Transport

When colonies expand to reach the edges of their plates within a few hours the spatial source of their nutrients remains an open question: do expanding groups of cells extract the majority of their fuel from the space directly under the mother colony, or do they extract fluid from the gel beneath the tendrils as they grow out, and if so, to what extent? A number of reaction-diffusion based models rely on local nutrient depletion under the tendrils, which then creates an outward-facing nutrient gradient (Giverso, Verani, and Ciarletta 2015; Marrocco et al. 2010; Golding et al. 1998) (Fig. 12). In my experiments, I observed flow by doping expanding colonies with fluorescent tracer beads, demonstrating non-zero material transport from the mother colony outwards.

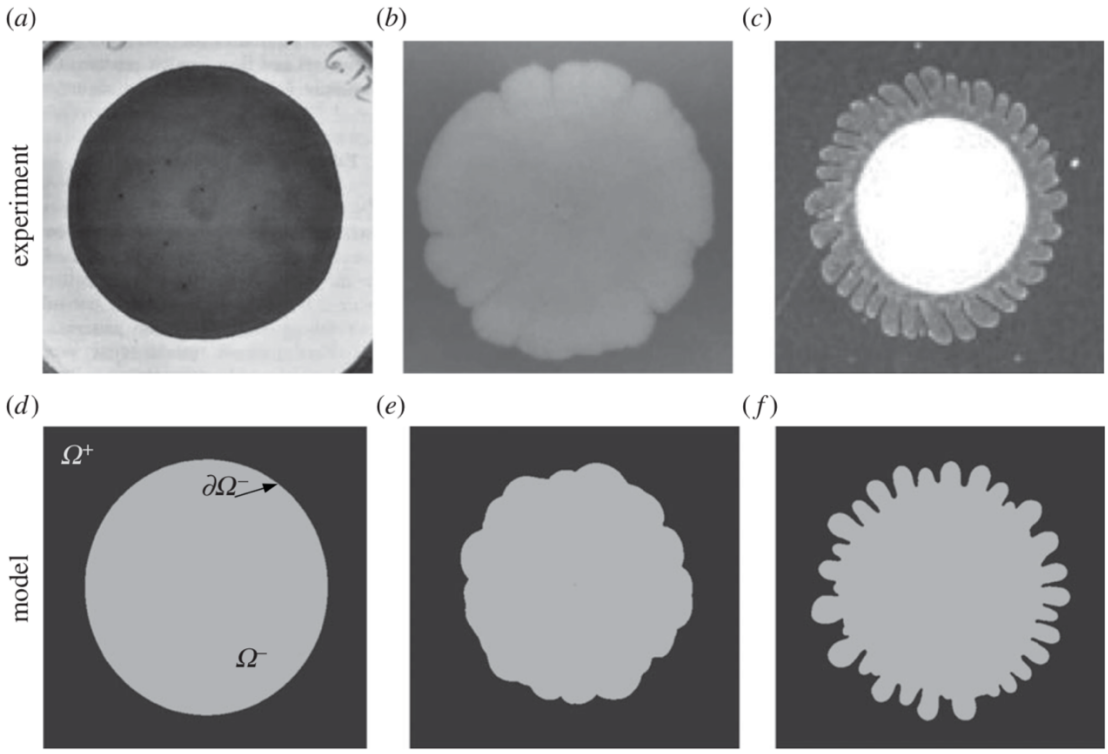

Figure 11. Adapted from Giverso et al. 2015. Comparison between some bacterial morphologies observed in biological experiments and the results obtained through the numerical simulations of the proposed mechanical model.

Careful characterization of the flow within our array of motility mutants (e.g. with passive tracer beads) could elucidate the extent of nutrient and material transport within these expanding groups. While using a tracer dye may provide a similar read-out, dyes present a number of difficulties that beads avoid: (i) dye diffuses into the gel and thus signal is confounded, (ii) dye diffuses away from the tendrils, and (iii) when dye is photobleached, the characterization of its concentration in space becomes more complicated than simply looking for whether a bead is present at all. Beads present their own challenges -- primarily sedimentation and adhesion to surfaces -- but these are alleviated by pre-treating the beads with bovine serum albumin to prevent adhesion, and simply depositing more beads.

There are straightforward ways to determine the source of the nutrients being consumed by the group. The first would be to use mutants with inducible fluorescence (say, with IPTG *lac* operon switches) and create plates where tendrils will pass over regions of the inducer. One could then observe whether they fluoresce and to what extent, to deduce the extent to which material is extracted from the hydrogel along the way (as opposed to being drawn along from the mother colony). A second, similar approach is to use *lacZ* transformed strains of *B. subtilis* on plates with sub-regions doped with X-gal, an analog of lactose, which when cleaved by ß-galactosidase (coded by *lacZ*) yields galactose (an unremarkable nutrient byproduct) and an intensely blue product which is insoluble, and could be used to trace the origin of consumed nutrients (El-Helow, Ghanem, and Mohamad 2001). Both of these methods would enable one to distinguish whether, when, and where colonies extract nutrients and consume them, even going so far as to use vertically stratified layers to tell how deep within the gel nutrients are drawn from.

4.4 – Closing Discussion

Here I presented a variety of novel experiments that together provide evidence for a previously unidentified abiotic jamming transition as a key regulator of fluidized, rapidly expanding, surface bound bacterial colonies. This physical transition between the fluidized and jammed states unifies several phenomena and explains the connection between gel hydration and rapid surface motility. Understanding the physical limitations that bacterial groups face as they spread over surfaces will enrich our understanding of life at the micro scale, will contribute to the modulation and engineering of bacterial

environments that achieve desired outcomes, and will inform our understanding of the principles that govern and constrain the motion and behaviors of interacting collectives.

APPENDIX – PARTS

Item Name	Company	Part no.
Agarose – Optimized Grade	RPI	A20090-100
EZ Rich Defined Media	Teknova	M2105
LB Broth Granulated	RPI	L240066-1000
Petri Dish – 100x15mm	Falcon	351029
Petri Dish – 50x15mm	Nunc	Cat No. 150288
15 mL Polypropylene conical tube	Falcon	352196
Indium tin oxide coated PET	Sigma Aldrich	639303
Epson V800 scanner	Epson	V800
Universal Dual Digital PID Temperature Controller	Inkbird	ITC-100VH
SSR Solid State Relay	Inkbird	SSR-25DA
K-type thermocouple	Inkbird	-

Table A1. Parts used

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