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An expanding bacterial colony forms a depletion zone with growing droplets

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Abstract: Many species of bacteria have developed effective means to spread on solid surfaces. This study focuses on the expansion of *Pseudomonas aeruginosa* on an agar gel surface under conditions of minimal evaporation. We report the occurrence and spread of a depletion zone within an expanded colony, where the bacteria laden film becomes thinner. The depletion zone is colocalized with a higher concentration of rhamnolipids, the biosurfactants that are produced by the bacteria and accumulate in the older region of the colony. With continued growth in population, dense bacterial droplets occur and coalesce in the depletion zone, displaying remarkable fluid dynamic behavior. Whereas expansion of a central depletion zone requires activities of live bacteria, new zones can be seeded elsewhere by adding rhamnolipids. These depletion zones due to the added surfactants expand quickly, even on plates covered by bacteria that have been killed by ultraviolet light. We explain the observed properties based on considerations of bacterial growth and secretion, osmotic swelling, fluid volume expansion, interfacial fluid dynamics involving Marangoni and capillary flows, and cell-cell cohesion.

Key words: bacterial swarming, intercellular interaction, biofilms, Marangoni effect, capillary flow, droplet dynamics

Introduction

In addition to planktonic existence, many species of bacteria have acquired an ability to colonize and spread on moist surfaces. While expanding on agar gel, a permeable, nutrient containing, semisolid surface, neighboring bacteria often move collectively, in packs [1], rafts [2], or large swirls [3]. When not mediated by functional flagella, collective bacterial spreading is referred to as "rafting" [2] or "sliding" [4]. In cases when the collective bacterial motility is aided by flagellated propulsion, this mode of surface motility is commonly defined as swarming [2, 5]. A popular depiction of swarming shows bacteria lined side by side, each driven by multiple flagella [5]. However, certain strains of flagellated bacteria with deficient or no flagella can spread with similar speed to that of their swarming counterparts [6-8], suggesting that a broader mechanism other than flagellated motility, such as sliding [4, 5] or volumetric fluid expansion [9, 10], may be the root cause for colony expansion. The roles of active flagella may include facilitating collective motion or fluid flow [5], and arguably, pumping fluids out of the agar or overriding surface friction [11].

A growing number of studies have attempted to explain features of bacterial colony expansion and pattern dynamics based on physics concepts and principles [8-10, 12-17]. From a physics perspective, a bacterial colony/swarm is a complex fluid containing active particles that grow in number. Coordinated motion, such as neighboring cells moving in packs, might simply be the outcome of a large number of densely packed cells spreading on a surface, through volumetric

expansion driven by osmotic swelling [9, 13, 15], rather than by precisely controlled cell body alignment or direction of propulsion. Recent studies have provided measurements of cell density profiles [1, 12, 18] and fluid flow [19, 20] within large bacterial swarms, leading to an analytical model to account for the findings on these physical properties [17]. Another recent model based on the roles of surface forces predicts several different patterns that may emerge in spreading bacterial colonies [10, 16]. A physical picture is starting to emerge that the governing mechanism for the spread and pattern evolution of a bacterial colony or swarm is an interplay between growth and interfacial fluid dynamics, involving osmotic and thin film flows [8, 20], wetting [10, 16], contact line pinning [8], Marangoni flow [12], and evaporation [15, 21].

In the meantime, species-dependent features and patterns have been widely reported. *Proteus mirabilis*, in particular, forms spatially periodic structures as the edge of its colony expands circularly outwards [22]. *Salmonella* [11] and *E. coli* [1, 19] tend to expand on agar with a smooth front. *Paenibacillus dendritiformis* [7] form dendritic protrusions, which can develop into highly branched structures. *Bacillus subtilis* [17, 23] and *Pseudomonas* [15, 24-27] have been shown to expand in either smooth fronts or dendritic patterns dependent on various chemical and physical factors. We and others have shown, for instance, that by simply varying the agar concentrations [15, 26], adding external surfactants into the agar [15], or simply leaving the agar surface to dry for some time [15], the colonies or swarms of *Pseudomonas aeruginosa* can vary from expanding with smooth front to forming dendritic patterns. Given the vast variation among different species, and even for the same species, but under different conditions of experiments, bacterial patterns and forms remain a subject of ongoing study. The broader

research community has not reached consensus concerning the underlining mechanisms, including to what extent they are biological or physical in nature [14, 28, 29].

Pseudomonas aeruginosa is a human pathogen with a rich variety of motility modes. It can swim, driven by its flagellum with a rotary motor at its base [30, 31]. It can also drag itself over a solid surface by attachment and contraction of its Type IV pili [32-34]. When reaching high density on an agar surface, the flagellated bacteria display robust swarming motility [15, 25, 35]. Since mutants of *P. aeruginosa* with neither flagellum nor pili are still able to spread on agar [6], however, the species also manifests sliding motility [4]. In sessile state, *P. aeruginosa* is well known to form biofilms [36-40], thereby acquiring some essential biological functions such as mechanical resilience [41, 42] and antibiotic resistance [37, 43-47]. Studies on *P. aeruginosa* have also shown, however, that its swarming motility may directly contribute to antibiotic resistance [44, 48]. *P. aeruginosa* secretes biosurfactants and other extracellular polymeric substance (EPS) to facilitate cell-cell interactions, which are important for its swarming motility [25, 35, 40, 44, 49-51], as well as biofilm formation [37, 47, 52-54]. The biofilms of *P. aeruginosa* play essential roles on persistent infections [37, 47], which is a general issue on microbial infections [28, 55, 56]. The importance of *P. aeruginosa* as a pathogen, its different forms and numerous modes of motility account for the extensive study of this species of bacterium.

Here, we report new features observed on the largescale colony expansion of *Pseudomonas aeruginosa* on agar surface. With experiments performed under better humidity control, an important factor that caught our attention in recent studies [21, 57], we observe occurrence and

spread of a depletion zone at the center of a large colony of *P. aeruginosa*, followed by growth and coalescence of bacterial droplets that display fluid dynamic behavior. We explore these features and explain them with a simple model based on cell-cell cohesion and thin film fluid dynamics. Our findings and interpretation offer new insights on bacterial collective motility, pattern development, as well as early stage biofilm growth.

Experimental

Bacterial growth

Pseudomonas aeruginosa PAO1 (wild type) and its pilus-less mutant PAO1 ΔpilA, generously provided by Dr. Keiko Tarquinio of Emory University Medical School, were stored at -80^{o}C in frozen medium containing 25% glycerol as cryo-protectant. A tiny amount was taken by scraping a frozen stock to grow in Tryptic Soy Broth (TSB) solution overnight in a flask at 37^{o}C. We adopted a recipe from the Xavier lab [51] to prepare 0.5% agar plates. Specifically, the gel mixture containing 17 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.5% glucose, and 0.5% agar is autoclaved for 35 minutes, mixed with 5g/L casamino acids (Bacto), pre-cooled to about 55 ^{o}C, poured in 50 mL volumes to large petri dishes of 15 cm diameter and placed in a bio-safe cabinet with lids open for 30 minutes to form 0.5% agar gel at room temperature. Then, 2.5 μL of bacterial growth in Tryptic Soy Broth (TSB) was deposited at the center of the agar plate. The inoculated plate was placed inside a custom-built incubator with transparent top cover. The incubator maintains constant temperature at 37^{o}C with variation within 0.5^{o}C, controlled by a feedback sensor. Another sensor with a moisture release and a 3W mini-fan controls the humidity within the incubator to desired values between 20% and 80%. A

Canon 7D DSLR camera was mounted on a stand so that pictures can be taken above the incubator without altering the temperature and humidity within it.

Fluorescence Imaging

We designed and assembled a light box with illumination by LED lights on the sides (detailed in Electronic Supplementary Information) in order to detect the distribution of rhamnolipids secreted by *P. aeruginosa* on the large agar plate. We added into agar gel Nile red, a dye that becomes visible when bound to rhamnolipids [33, 58]. The design works as the dye molecules enter the swarm fluid due to osmotic flow that fuels the bacterial colony expansion. Nile red, purchased in powder form from Fisher Scientific, was dissolved in methanol to the concentration of 1mg/mL [33]. The dye solution was added into agar mix in 1:100 (by volume) and then poured to a petri dish (diameter 15 cm) to form agar containing the dye. This gel plate was inoculated with PAO1 (wild type) or its mutant strain, ΔpilA, and grown in a dark incubator at 37^{o}C and 60% humidity. A set of photos was taken each hour using a colored camera with RGB readings.

To obtain a radial fluorescence distribution plot, we split the round petri dish area in the photo to 1000 concentric annuli and calculated the average value of fluorescence intensity in each ring. The numbers of annuli from 1 to 1000 were scaled to radius of the petri dish from 0 to 7.0 cm. Then, fluorescence intensity-radius curves were plotted for photos shown at 3 time points.

Microscopy

An aliquot was taken from a dense bacterial droplet either by suction using a pipet or by scooping using a soft PDMS sheet. It was then pressed by a coverslip to a sample thickness of ~ 50 μm for observation under an Olympus CKX41 with a 20x phase objective. Images were taken using a monochrome CMOS camera (Thorlabs; Cat.# CS505MU; Newton, NJ).

Cell density measurement

A 5-10 μL aliquot was taken from a large bacterial droplet using a pipet. It was diluted 1,000 times and mixed by vertexing. Then, 10 μL of the diluted suspension was transferred on a glass slide. A coverslip was mounted, and the suspension was squeezed to expand an area ~ 2.0 cm x 5.0 cm. Dividing the known volume by the area, the thickness of the fluid sandwiched between the glass slide and the cover slip is about 10 μm. Under the microscope, all cells were counted over a region of 210 μm x 174 μm. Thus, the bacterial number density was calculated.

Results

A depletion zone occurs at inoculation site hours following colony expansion.

Following spot inoculation of wildtype *Pseudomonas aeruginosa* PAO1, the region containing the bacteria becomes visible in several hours. By 12 hours, the colony starts expanding notably beyond the initial area (Figure 1), with corresponding time lapse Movie S1 linked to Electronic Supplementary Information (ESI). A similar example is also shown there as Figure S1 in ESI, with corresponding time lapse Movie S2). By 20 hours, a region of depleted bacterial density appears at the center. In the next 10 hours, the depletion region spreads behind the colony edge with a few hours in lag time, until it covers the whole plate. While the depletion zone expands,

bacteria aggregate along its boundary, increasing in thickness of the bacterial film there. Meanwhile, droplets are shed when the depletion zone expands. Those droplets reside inside the depletion zone, migrating on the agar surface and coalescing with each other. Finally, the plate is covered by a film of seemingly dormant bacteria, with only a few large droplets stuck on the plate surface. Another example is shown in Figure S1 in the ESI with similar timeline and features. One notable difference between the two sequences is that the droplets appear later in the 2nd sequence, emerging from within the depletion zone.

We tested how robustly the depletion zone and droplet formation occur, by repeating the experiments using 0.4-1.0% agar plates and observing colony growth under 20-80% ambient humidity. The depletion zone is sensitive to both parameters, but it is reproducibly observed on 0.45-0.70% agar plates and in the range of 40-80% ambient humidity. Droplets occurred in the late stage of colony growth under all those conditions as a depletion zone occurs, plus a few tests on 0.70-0.75% agar when no depletion zone occurred. Whereas previous studies on swarming of *P. aeruginosa* have covered a wider range of agar percentage than this study [15, 26], we note here that reduction of evaporation by better humidity control plays a key role on occurrence of depletion zone and growth of large bacterial droplets.

To test whether the depletion zone only occurs to the wildtype strain, we performed the same experiment using a mutant strain of PAO1, called ΔpilA, which expresses no pili. Our previous study confirmed that this pilus-less mutant spreads even better on agar than the wildtype [15]. Figure S2 in ESI, with corresponding time lapse Movie S3), shows a recorded sequence of the mutant colony's spread, which features similarly prominent occurrence of a central depletion

zone. We conclude that the depletion zone can occur on an expanding bacterial swarm independent of pili. In subsequent experiments, we focus on the wildtype strain in search of a common mechanism instead of looking for differences that are notable between different strains.

To test whether a depletion zone might always occur at the central area in a large colony irrespective of growth history, we inoculated an agar plate with the bacteria around its edge so that the population filled the plate surface from the edge inwards. We found that at about the same time as the colony reached the central region, roughly 18 hours into the colony spread, several depletion regions occurred near the outside edge (Figure S3), where the colony was located several hours previously. This result indicates that indeed it is the growth history, not the exact geometry or shape of the colony that dictates where a depletion zone occurs. For convenience, all subsequent experiments were performed by point inoculation at the plate center.

The shape of a depletion zone tracks that of a growing colony with several hours of delay.

We noted that a depletion zone typically resembles the shape of a spreading colony. In Figure 2, for example, three pairs of dashed lines are drawn on corresponding pictures with consistent colors to match patterns between colony edges and depletion zones six hours later. This sequence of images shows the depletion zone tracking the colony edge with a constant time lag. In Figure 2f, the areas of the depletion zone and the colony are plotted versus time, showing essentially the same curve with a shift in time by about six hours. The traces of the depletion zone, viewed as the second wave of spreading, track that of the colony edge.

In cases when a colony spreads into irregular shapes, and often with dendritic protrusions (such as one shown in Figure S1, for instance), the depletion zone resembles the rough contour of the colony several hours back, but is devoid of fine features. This is not surprising since steep gradients of chemicals within a fluid layer are expected to be blunted by diffusion over several hours. We also performed a control experiment with no age variation within a large colony, by covering the plate surface with a thin layer of bacteria-containing medium. As expected, no depletion zone formed as the bacterial density increased uniformly over the entire plate (images not shown).

The depletion zone colocalizes with the region of higher concentration of rhamnolipids.

We hypothesize that rhamnolipids, the bio-surfactants secreted by *P. aeruginosa*, may be a key material that promotes the occurrence of depletion zone. Thus, we use Nile red, a fluorescent dye with high affinity to rhamnolipids [58], to visualize its spatial distribution in the colony. The agar with Nile red dissolved in it supplies the colony with the dye, along with nutrients and water. After rhamnolipids bind Nile red, they fluorescently emit red light when excited with green light. Figure 3 shows good overlap of a depletion zone with the area of enhanced fluorescence, suggesting that rhamnolipids indeed track the depletion zone. The more time bacteria occupied a location, the more rhamnolipids accumulated. The history of spot inoculation gives rise to the highest concentration of rhamnolipids at the center, decreasing radially outward.

Arrest of depletion zone by UV irradiation

We performed another experiment to test whether the expansion of the depletion zone requires a live bacterial population or whether the zone might continue to expand, once formed, due to

purely physical mechanisms. To do so, we placed a plate containing a developing depletion zone under UV illumination for 98 mins. It was compared with a control plate kept in the same incubator with the exception that its exposure to UV light was blocked by two layers of aluminum foil. The results show that UV exposure stopped expansion of the depletion zone (Figure 4), suggesting that continued expansion of the depletion zone requires live bacteria and/or their continued secretion of surface-active materials, such as rhamnolipids.

Effect of surfactants on depletion zone

We performed an additional experiment to test whether added surfactants could induce a depletion zone. The effect of added drops of liquids containing 0.5% rhamnolipids were compared to that of water as control. This was done on both a live colony and one inactivated by UV irradiation. The results show (Figure 5), surprisingly, that the added rhamnolipid drops germinate developing depletion zones under both conditions. In contrast, whereas water droplets containing no surfactant caused similar spots of notably diluted bacterial film initially, the spots did not expand over time. This result shows that a Marangoni flow due to surfactant density gradients on the colony surface is the likely cause of a depletion zone. In light of this finding, the observation shown in Figure 4 can be further interpreted to suggest that growth of a depletion zone depends on a high level of surfactants produced by live bacteria. Inactivated cells can no longer produce the high level of surfactants required to sustain the spread of the depletion zone. An alternative interpretation to Figure 4, that loss of motility or cell death might be causing the bacteria to form a solid jam, thereby freezing the depletion zone, is ruled out by the observation here, namely, depletion zones can be geminated by surfactant droplets and they expand even on a UV irradiated swarm.

Droplet dynamics

Returning to the large bacterial droplets, we note interesting behaviors in their growth, coalescence, and migration. These droplets may be shed from an expanding boundary of a depletion zone (Figure 1, Movie S1). They can also form deep within a depletion zone (Figure S1, Movie S2). In the latter case, nascent droplets first move towards each other to form bigger droplets. They then move slowly, either outward towards the plate boundary, or inward towards a giant island of accumulated bacteria at the central region. Tracks within depletion zones are sometimes visible to the naked eye. They are mostly radial. This may be due to radial expansion of the colony with tracks left by nascent droplets that moved outwards with the dense swarm population when the depletion zone expanded. The speeds of most migrating droplets increase steeply as they approach their destinations (Figure 6). The characteristic feature of the rapid droplet sliding, particularly near the end their journey, points to a mechanism of fluid dynamic nature. These droplets appear to be connected to the larger regions of their eventual destinations, via fluid channels. Thus, their migration may be driven by a Laplace pressure imbalance and capillary flow. This mechanism is essentially the same as what drove the earlier stage droplet coalescence. Chemotaxis did not seem to play a major rule in droplet motion or coalescence, judged by the large length scale and absence of elaborate concentration gradient of specific chemoattractant over the large plate surface. We do not know if flagellar motility plays a role in the formation and coalescence of the dense bacterial droplets, but the orders of magnitude difference in length scale would require a multiscale mechanism in order to establish a potential connection.

Collective motion and intercellular interaction

We performed microscopic observation with dilution experiment in order to probe collective bacterial motion and intercellular interactions. Unfortunately, the millimeter sized bacterial droplets of *P. aeruginosa* formed on the large agar plate is unsuitable for imaging and trajectory tracking at the microscopic scale. We took an alternative approach, instead, by aspirating fluid from a large bacterial droplet, and then applying it either directly, or after diluting with M8 medium, on a slide chamber for microscopic observation. We observed collective swarming (Figure 7). The collective swarming patterns persisted in samples up to 5x dilution in M8 medium and thorough mixing. In fact, we observed similar swirling patterns between samples without dilution and that of 5x dilution. Upon 20x and 100x dilutions, however, no collective motion was observed. Instead, we only saw dispersed cells.

We performed measurement of cell density in the large droplets as described under the "Experimental" section. Specifically, for a volume of 0.21 mm x 0.17 mm x 0.01 mm = 3.6 x 10^{-4} μL, we counted the total number of cells in 3 different regions. Averaging three numbers of 130, 86, and 147, for instance, we obtained an average cell density of the 1000x diluted suspension, which then converts to (2.4 ± 0.9) x 10^{11} cells/mL as the cell density in the undiluted droplets. Assuming that each bacterium occupies a 1μm x 1μm x 2μm space, or v=2.0x10^{-12} cm^3, our measured cell density translates to nearly 50-50 between the space occupied by the cells and the space between them, filled by fluid. Notably, this cell density is ~ 100x higher than that of live bacteria in an overnight bacterial culture, which is, for example, ~2.2 x 10^9 per mL [59]. In light of this measurement, we know that the motile bacteria form collective swarms in the volume fractions of 10-50%. The similar patterns observed between samples of five time

difference in bacterial density attest to the robust nature of the collective swarming motility. This result offers us a good perspective on why bacterial collective dynamics are seen in numerous species and among distinct genera and species.

The initial hypothesis that motivated the dilution experiment was that microscopic observation of bacterial droplet upon dilution may reveal evidence of cell-cell cohesive interaction. However, most cells appear to readily disperse following 20x and 100x dilution, suggesting that the cell-cell interaction may be either transient or weak enough, so that their individual motility causes them to separate and disperse from each other upon dilution. We performed an additional experiment by placing a droplet of bacteria next to M8 medium, and observe how dense bacteria disperse over time in the region of contact. We observed decreasing cell density extending to a large, millimeter sized region within minutes (Figure 8). Surprisingly, except for a small fraction of fast swimming cells, most cells display little individual motion. The motile bacteria swam vigorously, in speeds on the order of tens of micrometers per second. The properties manifested by on spot dilution are consistent with those observed collectively from the 5x, 20x, and 100x dilutions with thorough mixing. The results from both experiments indicate that robust swarming requires rigorous motility of only a small fraction of cells as long as the cell density is very high. The outcome of these dilution experiments also suggests that this technique may be further refined in order to probe bacterial intercellular interactions.

Discussion

Several physical conditions must be met for a bacterial colony to grow and spread over a nutrient rich agar gel. In order to take up nutrients from the agar for their growth, the bacteria must be

able to secrete osmolytes and thereby draw fluids out of the agar. The rate of the volume increase must surpass loss due to evaporation in order to allow volumetric expansion of the bacteria-laden fluid. The bacteria must be able to produce surfactants in order to reduce the surface tension at the air-liquid interface and overcome the contact line pinning [8, 60, 61], so that the bacteria-containing fluid can readily spread over the agar surface. The fluid expansion may be further facilitated by Marangoni flow due to a surface tension gradient, which can be caused by higher concentration of rhamnolipids accumulated in the older region of a colony. Factors opposing fluid expansion on agar surfaces include contact line pinning [8, 60], a rapid rise of viscosity due to excretion of extracellular matrix in some species [8], and evaporation [21, 57]. Below, we discuss a few key observations made in this study and then provide a simple model, which at least qualitatively accounts for the main findings in this report.

Control of evaporation is a crucial factor in reproducible experiments on agar plates.

Our recent experiments informed us that evaporation sensitively affects the bacterial colony expansion rate and the patterns formed. Although most microbiology experiments use covered agar plates and keep them in enclosed incubators or humidified chambers for the sake of minimizing evaporation, we recognize through recent studies that evaporation is always a factor while growing bacteria on agar plates [15, 21]. Most commercially available incubators are designed to allow enough air circulation to prevent condensation, which can easily form on the inside surface of a plate cover, for instance, due to even the slightest drop in temperature as soon as the plates are taken out for observation and/or imaging. Whereas the main function of the plate cover is suppressing evaporation by keeping the moisture inside, it is manufactured to have several bumps as spacers so that when it covers an agar plate, there remains a millimeter-thick

gap of air for venting. Thus, the humidity inside the covered plate never approaches 100%. This means evaporation is a constant factor during incubation or observation. In this study, by keeping our incubation chamber humidified, at 60% or even 80%, we found the rate of evaporation is significantly reduced as compared with placing plates in a commercial incubator. Note that the actual humidity inside the covered plate is much higher due to the large amount of agar inside, which contains over 97% water by volume. Since the venting only occurs around the thin gap on the edge, the actual humidity inside the large covered plate is also expected to have a gradient, reaching nearly 100% in the center, but gradually falling radially. The flanged plate cover ensures that the humidity inside is maintained at well over 90% even near the edge, evidenced by our observation that condensation frequently occurs on the inside cover if the outside humidity was increased to above 80%, as soon as the temperature fluctuates by 0.5^{o}C or more. Therefore, better calibration and selection among various commercial incubators (including their stated operation settings, such as gravity convection versus mechanical convection) must be made in order for different microbiology laboratories to yield consistent results on bacterial colony growth and pattern observation. We conclude discussing this technical aspect by noting that effective reduction of evaporation using an incubating chamber built in house has led us to discovering new phenomena and gaining new insights.

Fluidization and Marangoni flow jointly account for occurrence of a depletion zone. Our experimental findings show that a constant time lag exists between two spreading fronts, indicating the same spreading speed of the two fronts (Figure 2), and the region with higher rhamnolipid concentration coincides with the depletion zone (Figure 3). The key property of constant time lag suggests that the spreading speed of the second front is not determined by

dynamic parameters such as the local gradient of surface tension and viscosity. Instead, it is dictated by the altered rheological properties of the bacteria-laden film in a history dependent manner: after a period of time since the local settlement of bacteria, the concentrations of rhamnolipids and possibly other extracellular matrix polymers reach threshold values. Some of the excreted molecules act as osmolytes, to pump fluid out of the agar in order to fuel the bacterial growth. The increased concentration of rhamnolipids, in the meantime, may facilitate the flow of the bacteria-laden liquid outward due to the well-known Marangoni effect [12], which predicts a flow along the concentration gradient of surfactants at the air-liquid interface. If the Marangoni flow is sufficiently strong, then, the majority of bacteria in the fluidized region might be carried by the flow and accumulate at the solid-fluid boundary [62]. Thus, the growth of the depletion zone may be limited by that of the fluidized region, which accounts for our results of Figures 2 and 3. Further experiments are required to verify if the edge of the depletion indeed delineates a border between solid and fluid phases, as implied by the assessment above. For instance, a future study may be designed to directly measure locally the rheological properties in regions both inside and outside a depletion zone.

A simple model of droplet formation based on cell-cell cohesion

We propose a simple model that explicitly assumes bacterium-bacterium cohesion, which makes their aggregation into large droplets energetically favorable. The specific assumption is that the motile bacteria prefer energetically to gather and form densely packed droplets rather than to remain evenly dispersed in the fluid layer on agar. This may occur due to cohesive cell-cell interaction, which could be mediated by extracellular polymeric substances, including cell surface polymers [52, 54], or by surface layer proteins [63]. One can then envision a transition

from a film with bacteria uniformly dispersed to one with bacteria congregate into dense droplets, within which bacteria remain alive and motile, as shown in our microscopic observation. Such a transition may occur when congregation of bacteria into large and dense droplets lowers the total energy of the system more than the energy cost of budding droplets out of the flat surface of a uniform bacterial laden film.

Here is a simple analysis to predict and estimate the condition of droplet occurrence. One relevant term of energy is due to surface tension that inhibit droplet production. Specifically,

$$E_{surf} = \gamma(A - A_0) ,$$

where A is the area of the liquid droplet in the shape of a spherical cap, A_0 is the area of the flat film out of which the droplet protrudes, and γ is the surface tension of the liquid.

The second energy term, which is bulk energy due to cell-cell interactions, is

$$E_{bulk} = -Nnb = -\frac{V_{drop}}{v}nb,$$

where N is the number of bacteria packed in a droplet, V_{drop} is the droplet volume, v is the average volume occupied by each cell, n is the average number of the nearby neighbors each cell interacts with, and b is the bonding energy with the negative sign in the formula to indicate explicitly cohesive interaction.

Consider a nascent droplet to appear as a spherical cap of radius r and contact angle α. We performed a simple estimate based on geometric analysis, setting $E_{surf} + E_{bulk}$=0. The analysis (detailed in ESI) yields, for a millimeter sized droplet to occur, $E_{cell-cell}$ to be on the order of 1000 k_BT, where k_B is Boltzmann constant and T is temperature in Kelvin. This model, assuming

strong cell-cell cohesion, implies that the bacterial droplets would only appear in the later period of the colony expansion, when the areal cell density reaches a high enough threshold. Once nascent bacterial droplets occur, they will grow at the expense of overall film thickness and their size will increase indefinitely. Based on cohesion among motile bacteria, albeit transient and dynamic in nature, we find this simple model to adequately account for the experimentally observed features. In addition, since the key parameter of cell-cell cohesion is species and strain dependent, this simple model can also account for droplet occurrence in other species of strong cell-cell cohesion, for instance, in *Bacillus subtilis*, for which large bacteria droplets have been previously reported [64].

A potential link between colony spread and biofilm growth.

P. aeruginosa is known to form biofilms, which have been extensively characterized [36-40, 54, 58, 65]. At microscopic level, most of the published studies have shown images of the biofilms formed on glass or plastic surfaces, using mutant strains expressing green or cyan fluorescent proteins (GFP or CFP), which are amenable to confocal imaging (see, for instance, [38-40]). One study has shown that, intriguingly, biofilms formed by *P. aeruginosa* can detach upon aging or by raising the level of rhamnolipids, creating cavities on the scale of 100 μm [39].

The large depletion zone we observed, however, is different from the reported biofilm dispersal caused by rhamnolipids [39]. First, although the wildtype and ΔpilA strains are known to form biofilms, the time scale for that process is several days, much longer than the period for the features to occur in our study. Second, the central hollowing effect in aged biofilms, as reported by Boles and co-workers, occurs locally at numerous locations and on the length scale of 100 μm.

In contrast, the depletion zone we observed occur to the entire colony, typically several centimeters in size. Last but not least, unlike in the biofilm growth experiment, the depletion zone occurrence we observed does not depend on adhesion of the bacteria to a solid surface. Because our experiments were performed on a large agar plate not amenable to microscopic imaging, we do not know if cells actually adhere to the agar surface. The bacterial film in the depletion zone may still be rather thick based on the observation that bacteria-laden droplets continue to appear and grow in the region over time. Notably, our observations are on much larger sizes and thickness than the previous studies performed by microscopic imaging. In the future, we hope to be able to measure the thickness profile of the large bacterial colony spread over the agar plate, perhaps by adopting a method similar to that reported in [64], for instance.

Conclusion

Bacterial growth and pattern formation have strong bearing on their biological functions, such as their spread and accumulation, biofilm growth [41, 47, 66] & its roles on infection [28, 55, 56], particularly, antibiotic resistance [37, 43, 45]. *Pseudomonas aeruginosa*, in particular, is a human pathogen responsible for frequent infections in wounds, airways, and urinary tract, especially when involving the use of catheters. We report observation of a depletion zone in an expanding colony of *P. aeruginosa*, as well as occurrence and growth of bacteria-laden droplets. Our observation is explained by assessing bacterial growth, osmotically driven volume expansion, surface tension, interfacial fluid dynamics involving Marangoni and capillary flows, and cell-cell cohesion. This report opens the door to more experiments as well as comprehensive modeling in future work, taking into consideration multiple parameters. By recognizing the robust interfacial

fluid dynamics microbes must follow, new applications may be developed towards controlling, facilitating or inhibiting, when needed, the spread of microbes on various fluid-solid interfaces, which are highly relevant to biofilm growth and infection control. In addition, the new findings in this study suggest that better control of evaporation is required for consistency among common microbiology experiments using agar plates.

Conflicts of interest:

There are no conflicts of interest to declare.

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Author Contribution:

J.X.T., J.B., H.M., W.C. & S.M. designed the study. H.M., J.B., and W.C. performed the experiments and analyzed the images and results. J.X.T & H.M. wrote the paper with inputs from all coauthors.

Figures and Captions

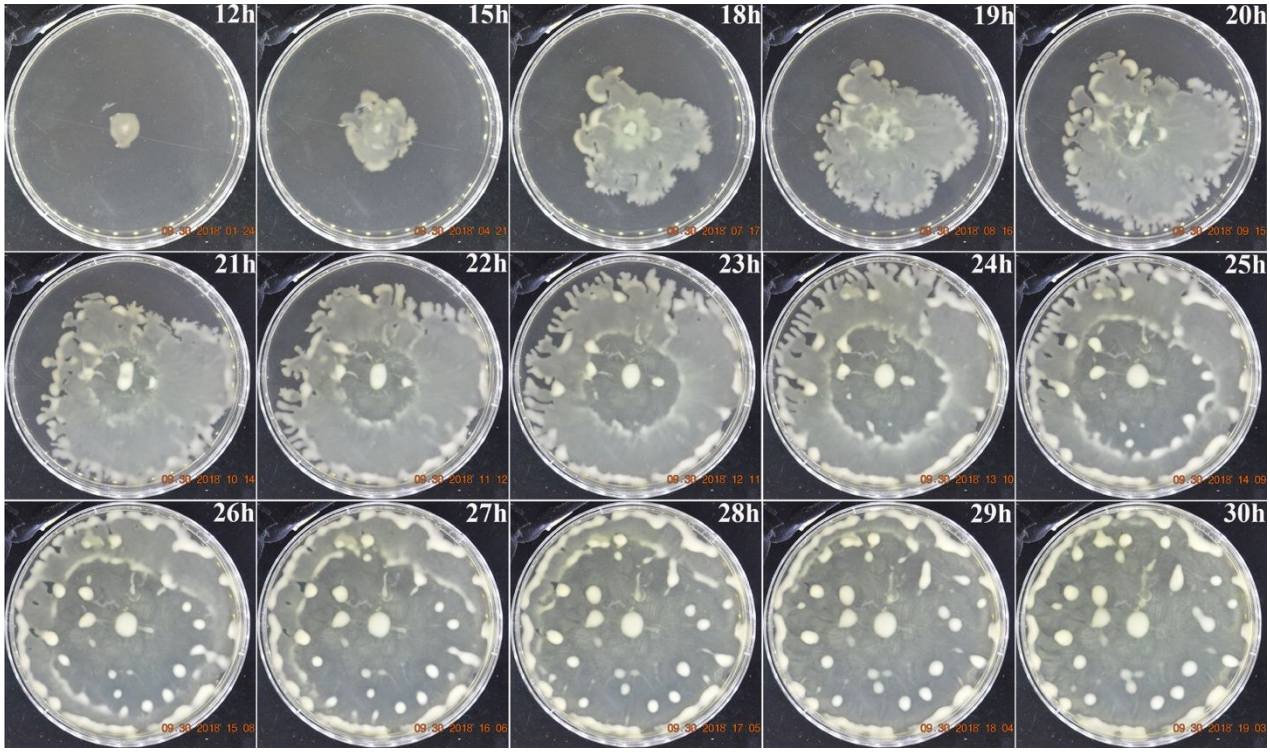

Figure 1. Expansion of *P. aeruginosa* colony over the course of 30 hours. The colony spreads over a large plate of 15 cm diameter in ~24 hours. A depletion zone occurs from the central region at ~20 hours and spreads over the entire plate in several hours. In the meantime, large droplets of bacteria form. They grow, migrate radially, and fuse with neighboring droplets. The growth took place on 0.5% agar on the covered plate, at 37^{o}C, under 60% ambient humidity.

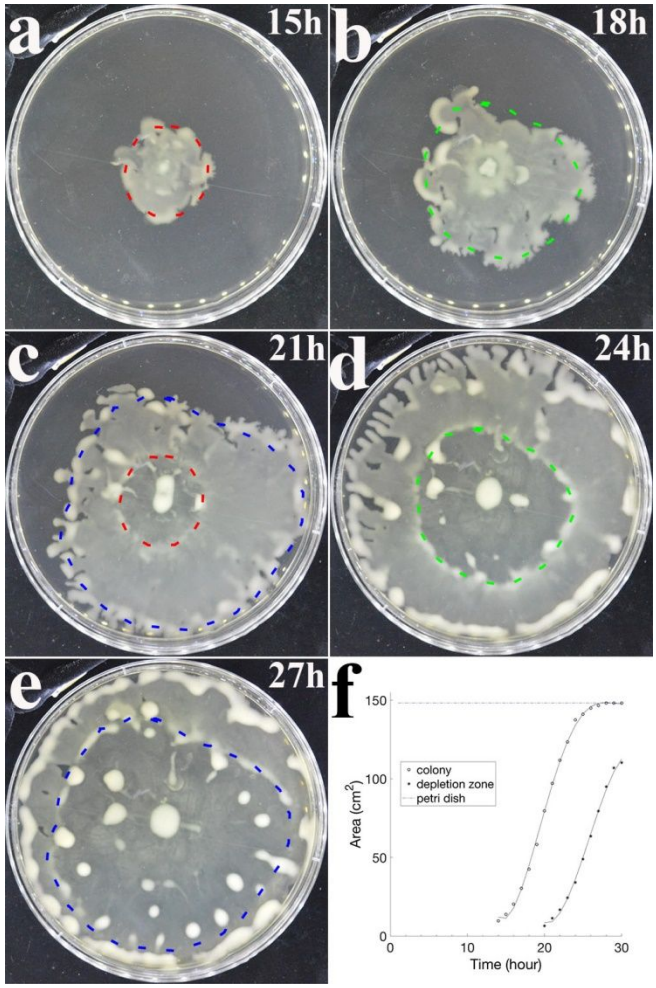

Figure 2. Occurrence and spread of a depletion zone at the central region of an expanding colony. The depletion zone, which was shown in Figure 1 to start about 20 hours after inoculation, tracked the colony covered area with about a 6-hour delay. Note the dotted contour lines in matching colors between vertically adjacent images. The tracking of the areas is most notable as they are plotted versus time in comparison at the bottom right. The plate diameter is 15 cm. The bacteria colony was inoculated on 0.5% agar gel, incubated at 37^{o}C and under 60% humidity surrounding the covered plate.

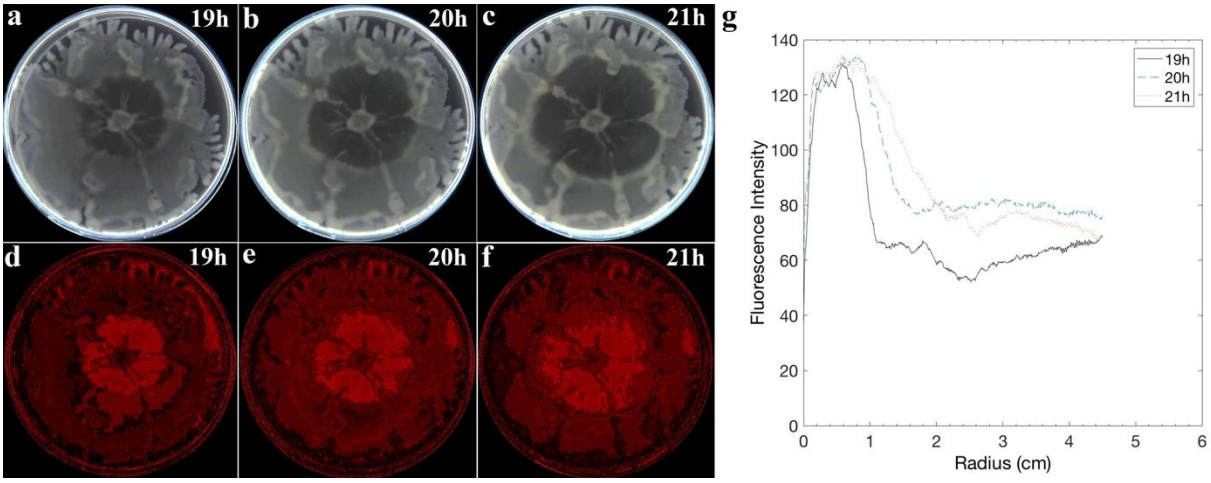

Figure 3. Imaging of rhamnolipid profile in a colony spread over an agar plate. a.-c. Photographic images of a large colony with an expanding depletion region. **d.-f.** Images of fluorescent emissions from Nile red illuminated by green LED light. The bright red region indicates high rhamnolipid expression. This area colocalizes well with the depletion region. **g.** profiles of radially averaged fluorescence intensity as functions of radius. The broadening peak tracks the growth of the depletion region over the 2 hour interval.

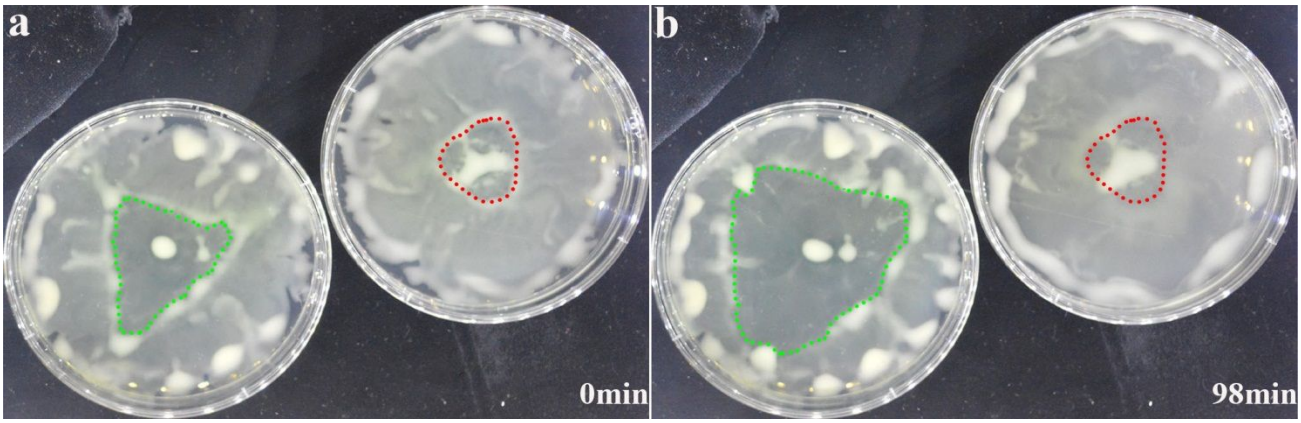

Figure 4. Arrest of a depletion zone by UV treatment. **a**. Depletion zones are noted on both plates fully covered by the bacteria grown over ~26 hours. **b**. The right plate was sterilized by UV light for 98 min while, as control, the left plate was blocked from UV damage. Both plates were kept in the same incubator. The dotted lines mark the boundaries of depletion zones formed in two plates. Note the depletion zone on the right plate stopped spreading due to UV sterilization, whereas on the control plate the zone continued to expand outward.

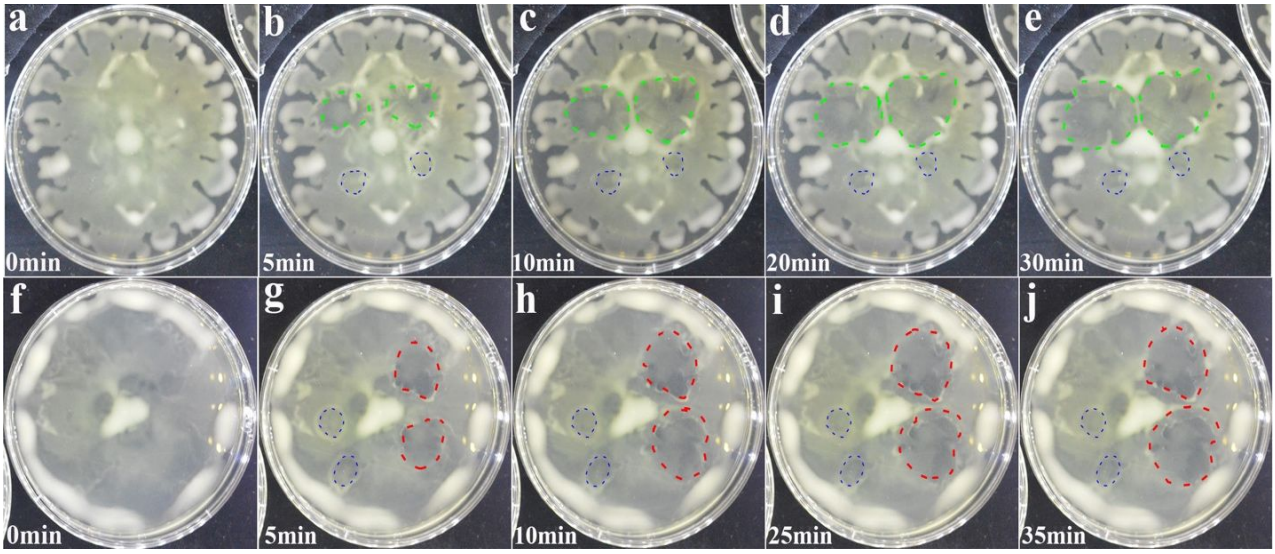

Figure 5. Comparison between plates covered by bacteria, either live or sterilized by UV after adding the liquid droplets. Bacteria are alive in the top row and sterilized in the bottom row. Two droplets each of water and rhamnolipids solution (10 μL) were added to the surface of each plate. Blue circles represent water droplets. Red and green circles represent rhamnolipids droplets (0.5%). Water droplets did not alter bacterial distribution in the area of addition, whereas rhamnolipids droplets induce small but expanding depletion zones on both plates.

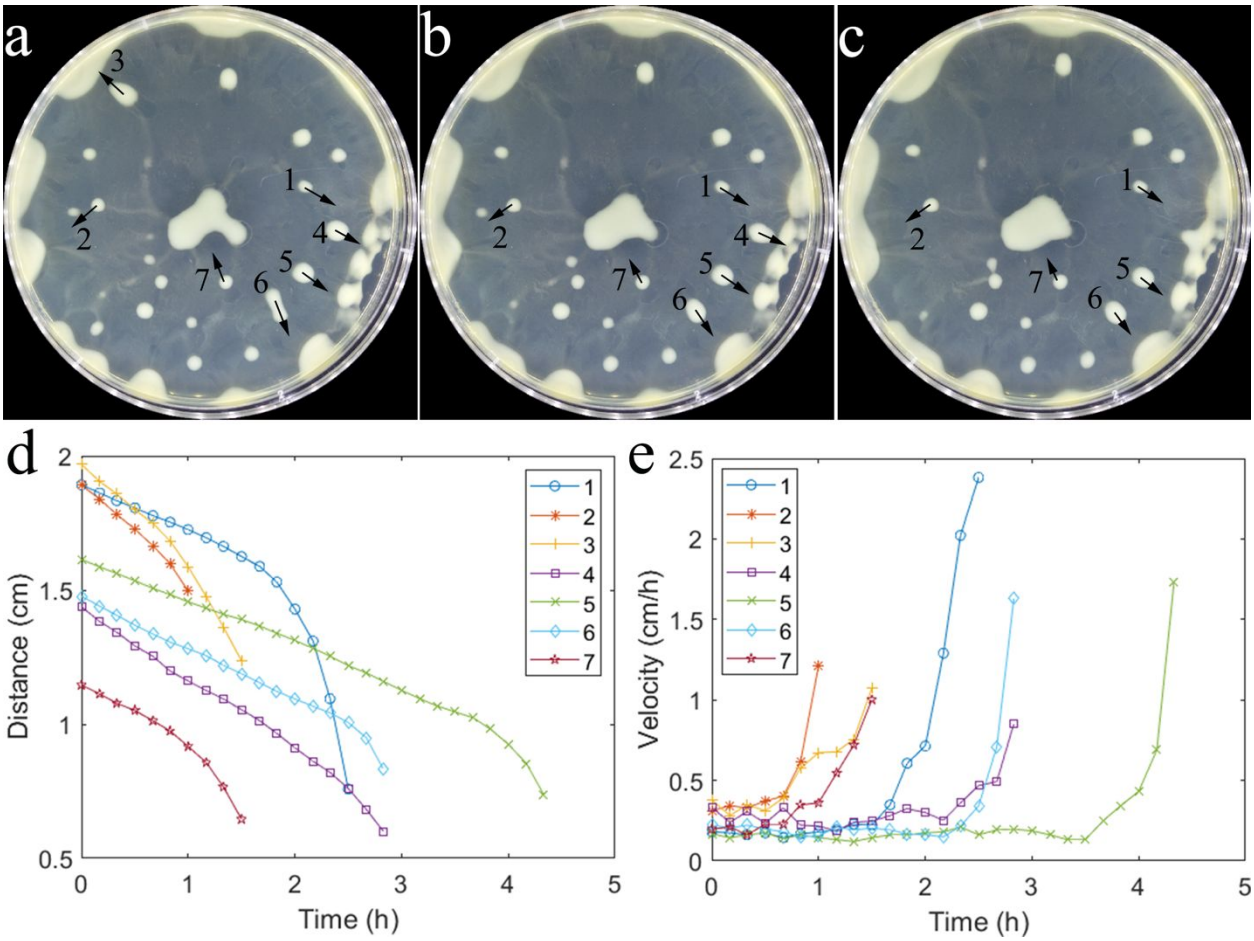

Figure 6. Speed of droplet migration. The positions of 7 droplets are tracked over time. Their distance from either the edge (1-6) or the center (7) of the plate is plotted, where ever is their destination. Also plotted is their velocity of migration, measured from the displacement between consecutive images taken 10 mins apart. Note the common behavior among all the droplets of slow but relatively constant speeds for some time ahead of steep rises near their destinations. The plate diameter is 15 cm. The bacteria colony was inoculated on 0.5% agar gel, incubated at 37^{o}C and under 60% humidity surrounding the covered plate.

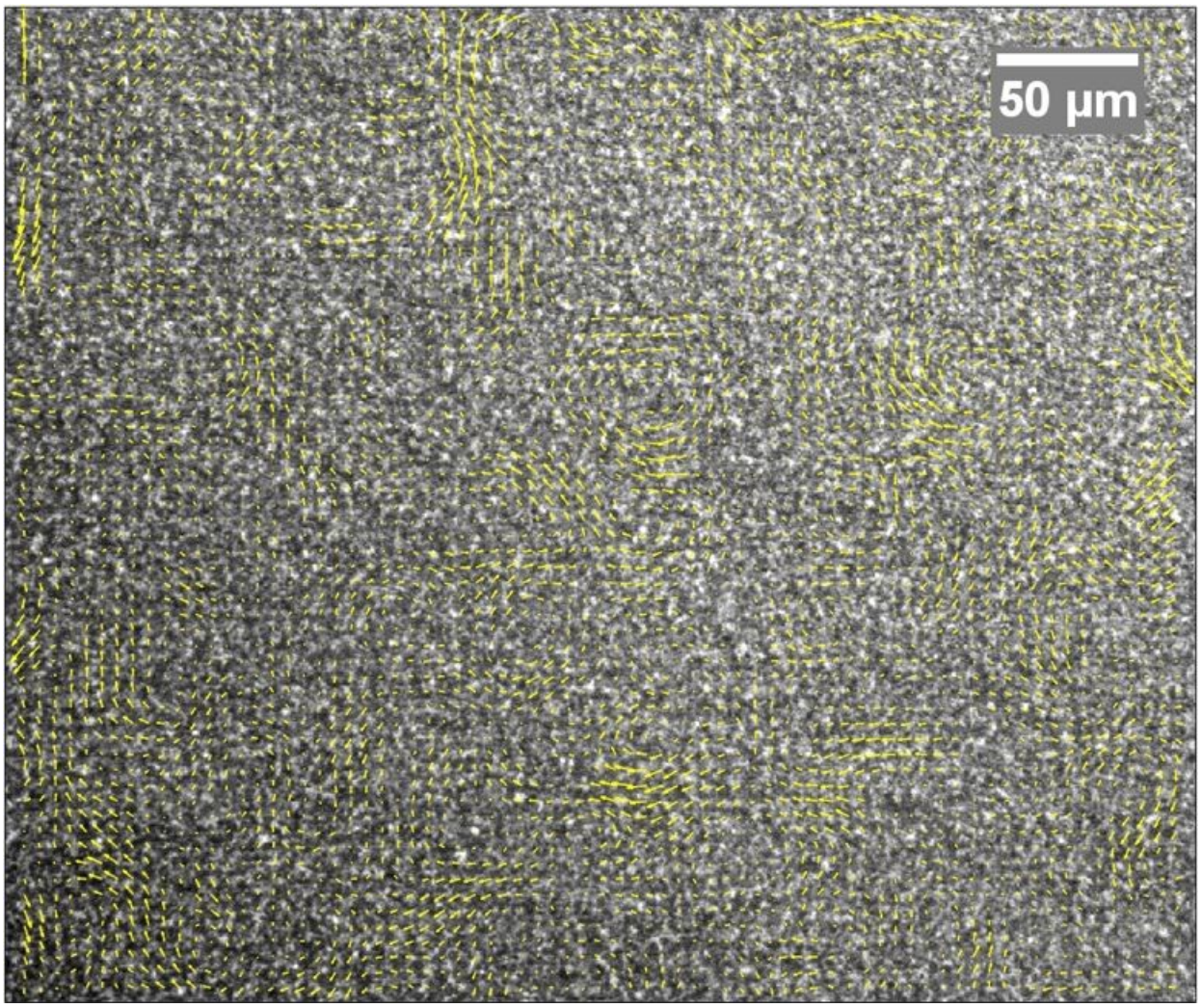

Figure 7. Collective motion in a dense swarms of *P. aeruginosa*. The thickness of the sample was ~50 µm. The images were taken using an Olympus CKX41 using a 20x phase objective. The arrows indicate local flow field. There is a movie link in the ESI to view the recording of the collective motion (Movie S5).

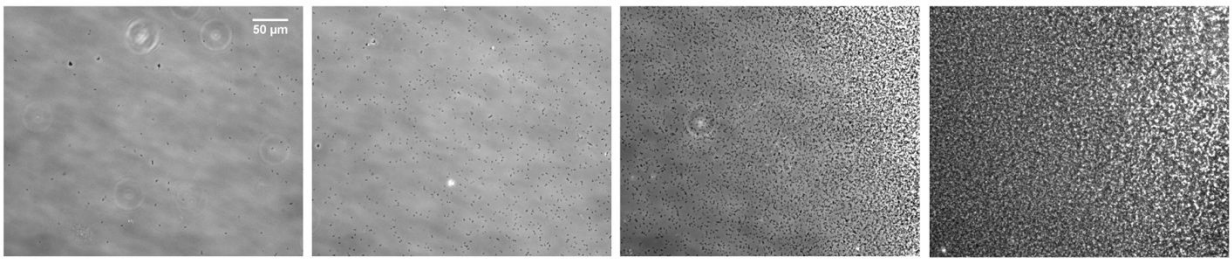

Figure 8. Dispersal of *P. aeruginosa* from a dense swarm. When an aliquot of a dense swarm (right) is placed next to M8 medium (left), motile bacteria disperse, forming a continuous gradient in cell density within minutes. Upon dilution, no large packs or aggregates of cells remain. The pictures were taken ~0.5 mm apart, collectively covering the region of dilution about 2mm wide. There is a movie link in the ESI to view the recording of the dispersal over time (Movie S6).

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