Electronic Supplementary Information (ESI)

An expanding bacterial colony forms a depletion zone with growing droplets

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1. A different example of depletion zone growth and droplet dynamics

Here we show another example of swarm growth with slightly different features. First, the colony front appears highly dendritic, which is a fairly common occurrence and has been extensively reported in previous studies (see refs on dendritic growth in the main text). Second, although the depletion zone occurred at about the same time as the example shown in Figure 1 of the main paper, the depletion zone in the case shown here appears nonuniform. It consisted of bacteria dense channels, where droplets germinated. The droplets then grew, migrated radially, and fused with neighboring droplets until only a few remained on the aged plate. These droplets clearly displayed liquidlike properties, notable from their fusion and shape changes.

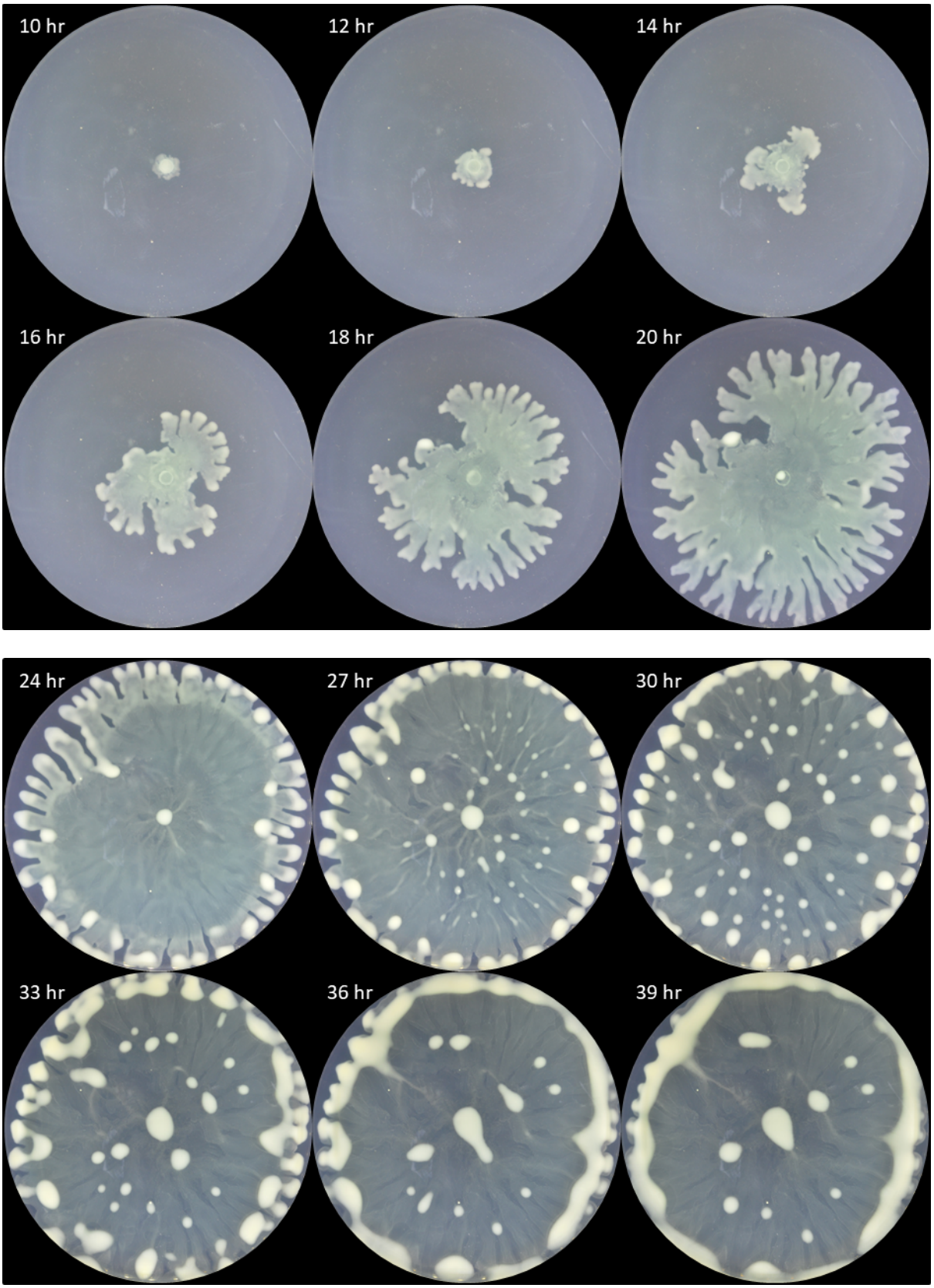
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Figure S1. Expansion of a wildtype *P. aeruginosa* colony over the course of 39 hours. The colony spreads over a large plate of 15 cm diameter in ~24 hours. A depletion zone occurs from the central region at ~18 hours and spreads over the plate in several hours. In this sample, droplets of bacteria formed in the depletion zone, which appeared nonuniform and consisted of bacteria dense channels. The droplets grew, migrated radially, and fused with neighboring droplets until only a few remained on the aged plate. The growth took place on 0.5% agar in the covered plate, at 37^{o}C, under 60% ambient humidity.

2. A similar depletion zone growth and droplet dynamics formed by a pilusless strain

Here is an example of colony growth of a pilusless strain of PAO1, called ΔpilA, showing a similar occurrence of depletion zone. In this case, the colony front appears more rounded, forming separate domains of protrusion in the later stage of colony expansion. The depletion zone occurred at about the same time as observed for the wildtype, except that the droplets appeared earlier and outside the depletion region. We also noted that several regions near the edge became thinner hours after the entire plate was covered, which can be marked as secondary depletion regions. These regions, along with the central depletion zone, pushed continually growing bacterial droplets into a chain of islands.

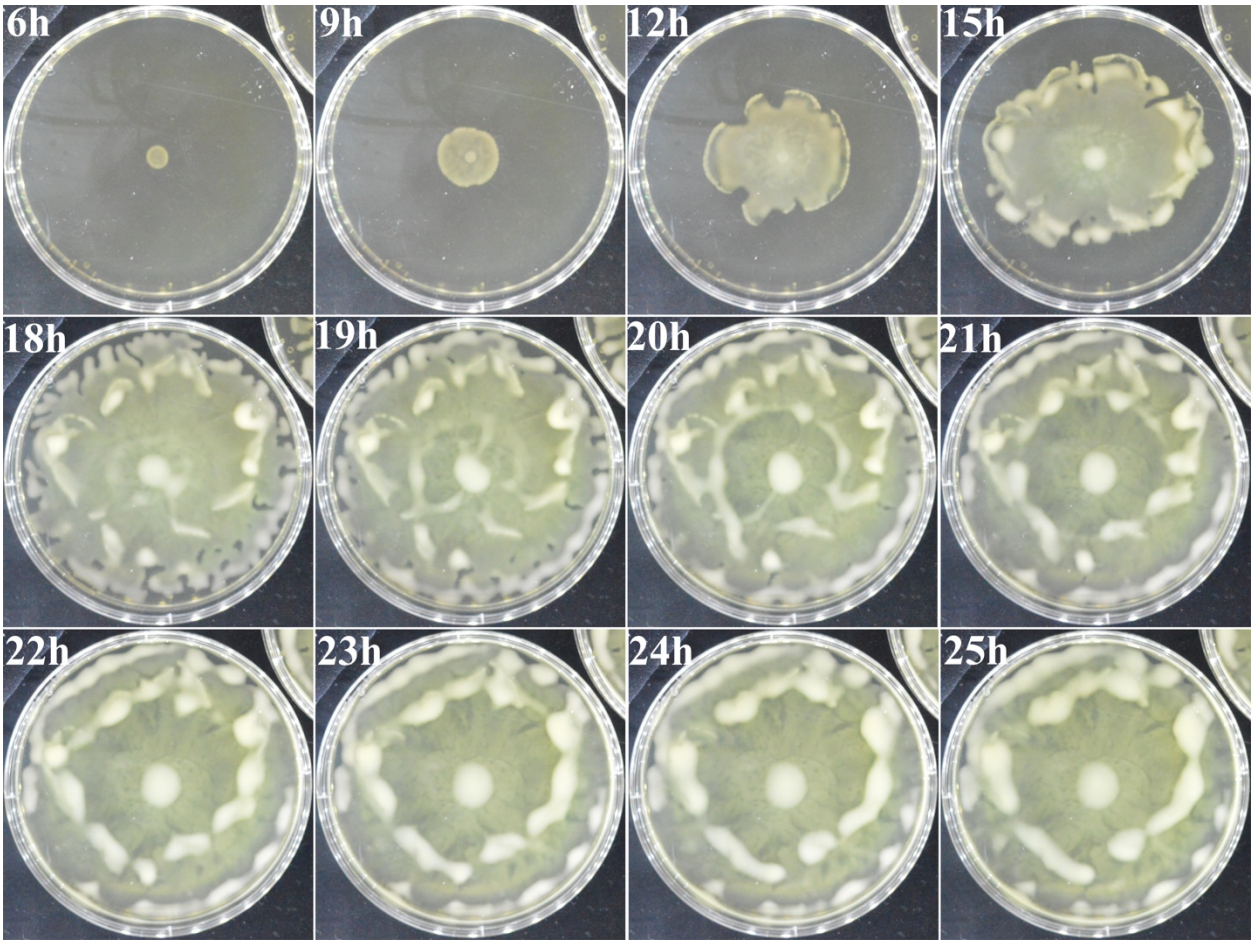

Figure S2. Expansion of a pilusless strain of *P. aeruginosa* over the course of 25 hours. The colony spread over a large plate of 15 cm diameter in about 18 hours. A depletion zone occurred from the central region at about the same time as the plate became fully covered. The depletion zone spread over the plate in several hours. In this sample, droplets of bacteria formed prior to occurrence of the depletion zone. They were later pushed towards the border of the depletion zone while secondary regions of depletion appeared near the edge of the plate. The growth took place on 0.5% agar in the covered plate, at 37^{o}C, under 60% ambient humidity.

3. **A different spread pattern following inoculating an agar plate from its outside edge.**

In order to gain insight whether a circular region of inoculation is essential for depletion zone formation, we performed an experiment to inoculate a large agar plate from the outside edge. Here we note occurrence of multiple depletion zones from near the outside edge of the plate, where the population of bacteria initially expanded to. This result indicates that depletion zones track the history of colony expansion, a behavior attributable to the requirement of surfactant

accumulation. Note also multiple droplets, which occur on the surface covered by a film of bacteria.

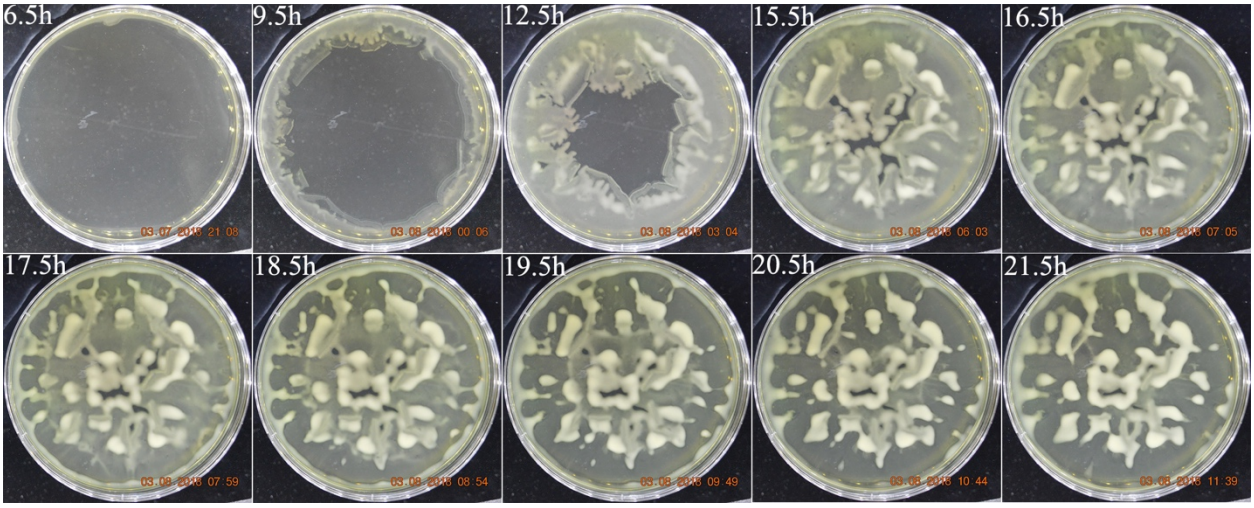

Figure S3. Inward spreading of a wildtype *P. aeruginosa* colony. The large agar plate was inoculated by spreading a bacterial broth along its edge using a pipette. The bacterial population spread inward to fill the plate in about 16 hours. In the meantime, several depletion zones appeared near the outside edge and expanded, pushing numerous bacterial droplets into islands. The growth took place on 0.5% agar in the covered plate, at 37°C, under 60% ambient humidity.

4. Supporting Movies:

Movie S1. Time lapse movie showing growth of a *P. aeruginosa* colony over 30 hours. The plate was kept at 37°C under 60% ambient humidity. A photograph was taken each 30 min. Selected images are shown in Figure 1 of the paper.

Movie S2. Time lapse movie showing growth of a *P. aeruginosa* colony over 72 hours. The plate was kept at 37°C under 60% ambient humidity. A photograph was taken each 30 min. Selected images are shown in Figure S1.

Movie S3. Time lapse movie showing colony spread of a pilusless strain. The plate was kept at 37°C under 60% ambient humidity. A photograph was taken each 30 min. Selected images are shown in Figure S2.

Movie S4. Time lapse movie showing an inward spread of *P. aeruginosa*. The plate was kept at 37°C under 60% ambient humidity. A photograph was taken each 30 min. Selected images are shown in Figure S3.

Movie S5. Live movie showing swarming motion of *P. aeruginosa* within a concentrated bacterial droplet. The sample thickness is ~ 50 μm. Images were acquired using a 20x phase objective on an

Olympus CKX41 microscope mounted to a monochrome CMOS camera (Thorlabs; Cat.# CS505MU; Newton, NJ). The first frame of the movie is shown as Figure 7 of the paper, with local flow field indicated by arrows.

Movie S6. Live movie showing motion of *P. aeruginosa* upon dilution. A droplet of the dense swarm (on the right side) is diluted by being placed in physical contact with liquid medium (left side). Images were acquired using a 20x phase objective on an Olympus CKX41 microscope mounted to a monochrome CMOS camera (Thorlabs; Cat.# CS505MU; Newton, NJ). A collection of 4 images from regions of the dilution are shown in Figure 8 of the paper.

5. Fluorescence imaging and processing

Here is the schematics of a lightbox setup we used for rhamnolipids detection (Figure S4). LED lights were placed on the sides around a petri dish in order to provide side illumination. Fractions of reflection and fluorescence lights travel upward to a color camera.

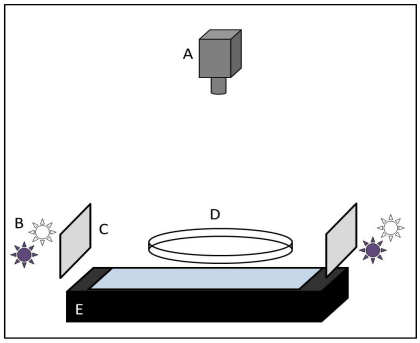

Figure S4: Schematics of a lightbox setup to measure the fluorescence of a bacterial swarm. A - camera, B - LED lights, C - Diffusing panes, D - Petri dish, E – heated platform. Images of a typical swarm plate can be taken by scattered light or fluorescence through illumination by LEDs of various wavelengths.

The combination of lipids and the Nile red dye emits red fluorescence under the excitation of green light, provided by LED lights. Yet, a significant amount of light signal in all colors came from reflection in the custom-built LED light box. We split the RGB photos to red (Figure S5b), green (Figure S5c) and blue channels, named r, g and b. Using MATLAB to extract fluorescence signal, each image channel was represented by a matrix. In order to normalize the matrixes, the top 1% largest elements of r and g were averaged and named as R and G, respectively. These R and G values come from the bright edge of the plate due to strong

reflections. They have nothing to do with fluorescence, but they serve as generic background intensities for normalization. To extract the fluorescence emission (in red) while removing background signals, the equation below is applied to obtain a new intensity matrix X,

$$X=|r/R-g/G|\cdot R$$

where, r/R and g/G are roughly equal other than random noise in the absence of fluorescence excitation and emission. Subtraction of the two thereby gives rise to the net gain in red signal due to fluorescence emission. The absolute values are taken, with the net ratio differences multiplied by the upper bound value R, so that the matrix X produces positive values in the proper range for image display (Figure S5d). In short, the visualized X shows only fluorescence, with background reflection removed.

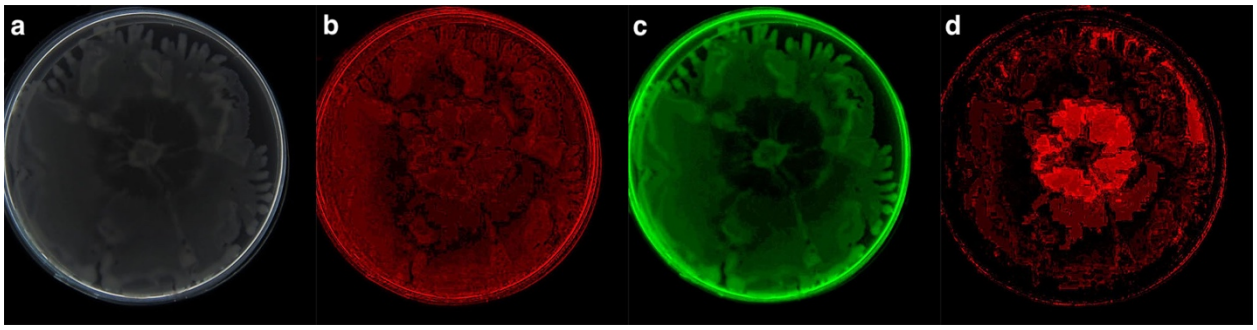

Figure S5. Imaging of rhamnolipid profile in a colony spread over an agar plate containing Nile red. a. Image of a large colony illuminated by room light, showing a depletion zone in the central region. **b.** The same plate illuminated by green LED light, but only red emission and reflection lights are detected. **c.** The same plate illuminated by green LED light and the reflected green light is detected. **d.** subtraction of c from b, showing primarily fluorescence emissions from Nile red. Thus, the bright red region in d shows the region of higher rhamnolipid expression. This area colocalizes well with the depletion region.

6. A prediction of threshold size for droplet occurrence

We hereby make an energetic analysis in order to predict a threshold size for droplet occurrence by comparing the surface energy cost against the gain due to cell-cell cohesive

interaction as they aggregate into large droplets. Assuming that the bacterial laden film protrudes to form a spherical cap, as illustrated in Figure S6, we estimate the surface and bulk energies below with the aid of geometric analysis.

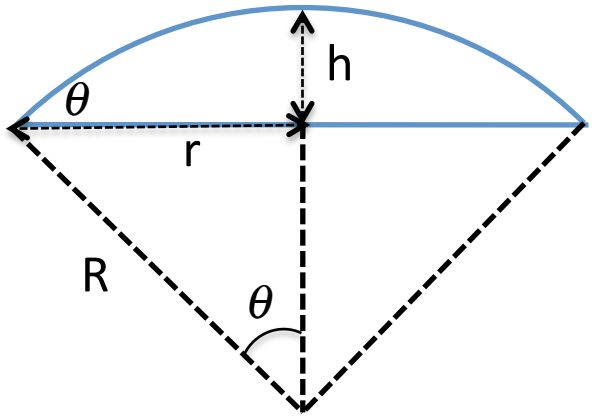

Figure S6. Illustration of geometric parameters of a spherical cap. R is the radius of the sphere, r is the radius of the cap. H is the height of the cap. θ is the contact angle at the edge of the cap. It is geometrically equal to that indicated within the triangle.

The area of the spherical cap

$$A = 2\pi Rh \,, \tag{1}$$

where R is the radius of the sphere, and h is the height of the cap.

The extra surface energy for protruding a spherical cap out of a flat film is,

$$E_{surf} = \gamma(A - A_0) \tag{2}$$

where γ is the surface tension, $A_0 = \pi r^2$ is the area out of which the droplet protrudes.

Noting that $r = R\sin\theta$, where θ is the contact angle of the droplet on agar surface. With manipulation of trigonometric functions, one yields,

$$E_{surf} = \gamma(A - A_0) = \pi r^2 \gamma \frac{(1-\cos\theta)}{(1+\cos\theta)} \,. \tag{3}$$

The volume of the spherical cap is

$$V_{cap} = \frac{AR}{3} - \frac{1}{3}\pi r^2(R-h) = \frac{\pi r^3}{3\,(\sin\theta)^3}(2+\cos\theta)(1-\cos\theta)^2. \quad (4)$$

The bulk energy gained in the spherical cap from cell-cell cohesion is

$$E_{bulk} = -Nnb = -\frac{V_{drop}}{v}nb = -\frac{\pi r^3 nb}{3\,v(\sin\theta)^3}(2+\cos\theta)(1-\cos\theta)^2, \quad (5)$$

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.

If one sets the criterion for droplet occurrence as $E_{surf} + E_{bulk} \leq 0$, combining equations (2) and (5) yields

$$r \geq \frac{\gamma v}{nb}\frac{3\sin\theta}{(2+\cos\theta)}\,. \quad (6)$$

We use 30 mPa.m, or $3\text{x}10^{-2}$ Pa.m as the surface tension, which is lower than that of pure water due to presence of surfactants on the bacterial film. For a crude estimate, we assume that each bacterium occupies a 1μm x 1μm x 2μm space, or v=$2.0\text{x}10^{-18}$ m^3. The other parameters used are n=3, θ=10°, and b=1000 K_BT, which yields $r \geq 0.9\ mm$. This crude estimate suggests that a rather large bulk energy due to cell-cell interaction, on the order of thousands of thermal energy units per cell, is required for the droplet to occur. This is not so surprising given the strong effects of surface energy that must be overcome for the droplets to form. This requirement can only be met involving hundreds or even thousands of molecular interactions between neighboring bacteria.