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A water reservoir maintained by cell growth fuels the spreading of a bacterial swarm

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Abstract

Flagellated bacteria can swim across moist surfaces within a thin layer of fluid, a means for surface colonization known as swarming. This fluid spreads with the swarm, but how it does so is unclear. We used micron-sized air bubbles to study the motion of this fluid within swarms of *Escherichia coli*. The bubbles moved diffusively, with drift. Bubbles starting at the swarm edge drifted inwards for the first 5 s and then moved outwards. Bubbles starting 30 μm from the swarm edge moved inwards for the first 20 s, wandered around in place for the next 40 s, and then moved outwards. Bubbles starting at 200 or 300 μm from the edge moved outwards or wandered around in place, respectively. So the general trend was inwards near the outer edge of the swarm and outwards farther inside, with flows converging on a region about 100 μm from the swarm edge. We measured cellular metabolic activities with cells expressing a short-lived GFP and cell densities with cells labeled with a membrane fluorescent dye. The fluorescence plots were similar, with peaks about 80 μm from the swarm edge and slopes that mimicked the particle drift rates. This suggests that net fluid flow is driven by cell growth. Fluid depth is largest in the multilayered region between ~ 30 and ~ 200 μm from the swarm edge, where fluid agitation is more vigorous. This water reservoir travels with the swarm, fueling its spreading. Regulatory mechanisms are not required; cells need only grow.

Key words: biofilm, living fluids, spreading, microbubble, flagellar motility

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Introduction

When colonizing a moist nutrient-rich surface, such as agar, many flagellated bacteria elongate, secrete wetting agents, and swim across the surface in multicellular groups within a thin layer of fluid, a process known as swarming (1-3). Swarming provides a remarkable example of bacterial adaptation to diverse environments. It also provides a unique biophysical system for the study of active fluids (4) and self-propelled particles (5). Much has been learned about the genetics and biochemistry of bacterial swarming as well as its relevance to biofilm formation and pathogenic infections (1-3). More recent advances have been made at the single-cell level (6-9). However, relatively little is known about the thin layer of fluid that supports flagellar motility and allows swarm cells to maintain a distinct physiological state (10-11). Understanding the properties of swarm fluid is fundamental to a full understanding of bacterial swarming.

Our focus here is on the motion of swarm fluid, and a key question is how this fluid spreads. Some bacteria synthesize bio-surfactants (1). Marangoni flows driven by surface-tension gradients can account for the flagellar-independent colony expansion of some species, such as *Bacillus subtilis* that produces surfactin (12-14). However, this cannot be the general mechanism driving swarm fluid spreading, because most swarming is flagella-dependent and does not always require production of surfactants (1). Our organism of choice is the model bacterium *Escherichia coli*, which was shown to swarm on Eiken agar by Harshey & Matsuyama (15). Although surfactants play important roles in its behavior (16), *E. coli* is not known to secrete surfactants. Recently we developed a method for making micron-sized air bubbles that can serve as tracers of flow in thin fluid films, such as those found in *E. coli* swarms (17). Using this

technique, we found that the action of rotating flagella of cells transiently stuck to the substratum near the outer edge of a swarm generates a river running along the swarm edge. This river flows rapidly clockwise (when the swarm is viewed from above) and moves outwards as the swarm expands (17). So one mechanism driving swarm-fluid expansion involves the action of flagella that pump fluid outwards. But this mechanism does not explain how spreading is sustained: pumping by flagellar action would reduce the thickness of the fluid film near the swarm edge and eventually abolish flagellar motion. Fluid must move out of the underlying agar into the body of the swarm. Here we extended the application of the microbubble technique to map flow patterns at large spatial scales within *E. coli* swarms. We found that only the fluid in the outer ~300- μm wide rim of the swarm has net movement. Within this rim, the fluid drifts along the direction of swarm expansion, either inwards or outwards, depending upon the distance from the swarm edge. Fluid tends to flow towards a region ~100 μm from the swarm edge, a region that exhibits maximum metabolic activities and maximum cell density. Gradients in metabolic activities and cell density correlate with mean speeds of fluid drift, suggesting that this drift is caused by cell growth. A fluid balance model that takes into account the measured drifts predicts that most of the new swarm fluid comes out of the agar in a region ~70- μm wide near the edge of the swarm. As a result, an *E. coli* swarm maintains a water reservoir of greater fluid depth centered ~100 μm from the swarm edge. This reservoir fuels spreading and sustains colony expansion.

Results

Fluid flows in the interior of *E. coli* swarms exhibit complex drift

Microbubbles were formed following the explosive transformation of micron-sized droplets of the water-insoluble surfactant Span 83 that were placed on the agar surface a few cm in front of an advancing swarm (17); see Methods. Some of the bubbles remained stable for hours and were taken up by the advancing swarm. Many of these traveled within the river at the swarm edge; see, for example, the bubble indicated by the black arrow in Fig. 1A. Occasionally, bubbles moved into the swarm; see, for example, the bubble indicated by the white arrow in Fig. 1A. The motion of bubbles with a diameter of $2.1 \pm 0.4 \mu\text{m}$ (mean \pm s.d., $n = 51$) were tracked (Methods and Movie S1), allowing us to map the flow patterns in the interior of the swarm. These bubbles moved freely within the swarm, without sticking to cells or to fluid boundaries.

At the leading edge of an *E. coli* swarm there is a monolayer of cells spanning a width of $31 \pm 4 \mu\text{m}$ (mean \pm s.d., $n = 12$). Just behind this monolayer is a distinct multilayered region with a width that depends upon colony size, Fig. 1A. The multilayered region exhibits greater fluctuation in brightness compared to other regions of the swarm, as shown in the plot of Fig. 1B, allowing us to determine the boundaries of the multilayered region (see Methods). Under our experimental conditions, the width of the multilayered region spans $154 \pm 27 \mu\text{m}$ (mean \pm s.d., $n = 12$). The boundaries of the multilayered region remain nearly fixed relative to the swarm edge as the swarm expands.

Microbubbles displayed mostly random movement in the interior of the swarm (Movie S1), but when an ensemble of bubble trajectories was averaged, we found net radial displacements, i.e., drifts. The direction of drift was either inwards, i.e. toward the center of the swarm, or outwards, i.e., toward the edge of the swarm, depending upon the distance from that edge. As soon as bubbles entered the

cell monolayer at the edge of the swarm, they drifted inwards for ~ 5 s and then outwards for ~ 10 s until overtaken by the cells in the multilayered region. Fig. 2A is a plot of the mean radial displacement in the laboratory reference frame of 29 bubbles, shown as a function of time, $\langle r_x(t) \rangle$. The drift speeds, indicated by the initial and final slopes of this curve (v_x , dashed lines) were -1.1 ± 0.1 $\mu\text{m/s}$ and 1.3 ± 0.1 $\mu\text{m/s}$, respectively. These values were determined by the best linear fits of the $\langle r_x(t) \rangle$ plot. While the inwards drift could be an artifact due to the selection of inwards moving bubbles at the beginning of the experiment, the outwards drift is most likely generated by the action of rotating flagella of cells stuck at the swarm edge that tends to move fluid from thicker to thinner regions of the swarm (17). The outwards drift speed (1.3 ± 0.1 $\mu\text{m/s}$) is comparable to the average swarm expansion rate (1.7 ± 0.3 $\mu\text{m/s}$, $n = 12$).

Once the bubbles reached the multilayered region, Fig. 2B, $\langle r_x(t) \rangle$ exhibited a complex and unexpected pattern. The bubbles moved inwards for ~ 20 s ($v_x = -1.51 \pm 0.05$ $\mu\text{m/s}$), wandered around with a weakly outwards drift for ~ 40 s ($v_x = 0.05 \pm 0.04$ $\mu\text{m/s}$), and then moved outwards for the final 40 s of data acquisition ($v_x = 0.6 \pm 0.1$ $\mu\text{m/s}$). Defining the intersecting points of the linear fits in Fig. 2B as switching points, switching from inwards to outwards drift occurred at $t = 20 \pm 1.4$ s and $\langle r_x(t) \rangle = -62 \pm 2$ μm . Taking account of the distance the swarm has expanded, the switching point corresponds to a distance from the swarm edge of 96 ± 7 μm . At the end of the data acquisition, the bubbles were 208 ± 32 μm from the swarm edge. Thus, the data presented in Fig. 2B covers the entire multilayered region.

To probe the flows beyond the multilayered region, we tracked bubbles starting at distances of 200 and 300 μm from the swarm edge. Beginning at 200 μm ,

bubbles drifted outwards at $v_x = 0.34 \pm 0.01 \mu\text{m/s}$ (Fig. 2C). Taking account of the distance the swarm has expanded, these bubbles ended up at a distance of $292 \pm 23 \mu\text{m}$ from the swarm edge. Beginning at $300 \mu\text{m}$, the bubbles remained driftless, with $v_x = -0.01 \pm 0.03 \mu\text{m/s}$ (Fig. 2D). Figs. 2B-D suggest that the motion of swarm fluid displays outwards drift from ~ 170 to $\sim 300 \mu\text{m}$ from the swarm edge, and then remains stationary $\sim 300 \mu\text{m}$ from the swarm edge.

To convince ourselves that these patterns represented motion for the fluid as a whole, and not just for its uppermost layer, we repeated these measurements with a smaller number of polystyrene latex spheres ($1.4 \mu\text{m}$ dia.) or carboxylate-sulfate-modified polystyrene latex spheres ($1.0 \mu\text{m}$ dia.) or hydroxylate polystyrene latex spheres ($0.9 \mu\text{m}$ dia.) (Polysciences, Inc.). The polystyrene latex spheres have larger density than water and tend to sink, so their movement should reflect the motion of the lower portion of the swarm fluid. The spheres behaved in a similar way as microbubbles, drifting inwards and then outwards in the multilayered region (Movie S2). However, the latex spheres tended to move back and forth between the surface of the agar and the body of the swarm, sticking briefly, wandering freely for a time, and then sticking again; among the three types of latex spheres, the $0.9 \mu\text{m}$ dia. hydroxylate spheres appeared to have the greatest mobility.

Taken together, the drift patterns of bubble motion described in Fig. 2 reveal that only the outer $\sim 300\text{-}\mu\text{m}$ wide rim of the swarm fluid film spreads. The fluid in the outermost edge of this rim (i.e. in the swarm-cell monolayer) flows outwards, directly supporting swarm expansion. Remarkably, the swarm fluid further inside this rim flows (in the reference frame of the laboratory) towards a region $\sim 100 \mu\text{m}$ from the swarm edge, suggesting that the swarm fluid film in the multilayered

region has a greater depth above the agar. To support these flows, swarm fluid must be constantly supplied from the underlying agar.

Swarm fluid in the multilayered region is highly agitated

Individual microbubbles within swarms diffused with drift, and the diffusivity varied with the distance from the swarm edge. For the bubble trajectories reported in each panel of Fig. 2, we calculated the mean-squared displacement corrected for drift,

$$MSD(t) = \frac{1}{N} \sum_i^N \left\langle \left[\vec{r}_i'(\tau+t) - \vec{r}'(\tau) \right]^2 \right\rangle_{\tau} \text{ with } \vec{r}_i'(t) = \vec{r}_i(t) - \langle \vec{r}(t) \rangle,$$

where N is the number of bubble trajectories, i is trajectory index, \vec{r}_i is the displacement of the i -th trajectory and $\langle \vec{r}(t) \rangle$ is the mean displacement of N trajectories (i.e. the drift). The diffusion processes of bubbles in different regions within the swarm were then characterized by the effective self-diffusion coefficient (D_{eff}) and the anomalous diffusion exponent (α), which are defined in $MSD(t) = 4D_{eff} t^\alpha$. At short time scales (5 s), microbubbles within the swarm displayed super-diffusion, with $\alpha > 1$ (Fig. 3). The value for D_{eff} in the multilayered region ($64 \pm 2 \mu\text{m}^2/\text{s}$), was about twice as large as in other regions of the swarm ($\sim 30 \mu\text{m}^2/\text{s}$), reflecting greater agitation.

The diffusive flows described here are similar to the surface flows observed near “bacterial carpets” (18), but with a higher level of agitation. The 1- μm -diameter beads near bacterial carpets had an effective diffusion coefficient of $19 \pm 5 \mu\text{m}^2/\text{s}$, about 40 times larger than expected for such beads in bulk water, but only about 1/3 as large as found for the 2- μm bubbles in the multilayered region of the swarm. In this region of the swarm, bubbles are immersed in a bath of freely-swimming cells, which drift outwards with uniform radial speeds ($\sim 0.4 \mu\text{m}/\text{s}$), as determined by particle image velocimetry, Fig. 4. Surprisingly, cell

drifts and fluid drifts do not appear to be correlated. For other reports of enhanced tracer diffusion in concentrated bacterial suspensions, see (19) and (20).

Cell-density profiles in the multilayered region correlate with fluid drift patterns

Since cells are mostly water, bulk fluid flow could be driven by metabolic activities associated with cell growth. To verify this idea, we measured metabolic activities, utilizing a short-lived GFP, and cell number, utilizing a membrane-specific fluorescent dye, neither of which appeared to affect swarming (see Methods). We monitored the fluorescence of swarm cells of *E. coli* strain MG1655 expressing a short-lived derivative of GFP, ASV, which degrades with a half-life <1 h and is rapidly cleared from non-growing cells, thus reflecting the current rate of biosynthesis and serving as a reporter of cellular metabolic activities (21-22). The swarms were grown on agar supplemented with the dye FM 4-64, which fluoresces when absorbed by cell membranes (23), thus indicating cell number. The GFP was green and the FM 4-64 was red, so the two could be measured simultaneously using an FITC/Texas Red cube, as shown in Fig. S1. The GFP and the FM 4-64 fluorescence plots were similar, suggesting that cells near the swarm edge are metabolically active and have similar growth rates. For *E. coli* strain HCB1668, the FM 4-64 fluorescence plot shown in Fig. 5 revealed that the cell densities (hence growth activities) were higher in the multilayered region, peaking at a distance of 76 ± 11 μm (mean \pm s.d., $n=5$) from the swarm edge, close to the point where the swarm-fluid flows switch from inwards to outwards (Fig. 2B). The ratios of the slopes at 30-50 μm and 150-200 μm from the swarm edge (2.4 ± 0.1 , dashed lines in Fig. 5) coincide with the ratio between the drift speeds in these two regions (2.5 ± 0.4). These results support

the view that fluid drift rates in the multilayered region are caused by cellular metabolic activities.

Discussion

We have studied the motion of fluid near the outer edge (the rim) of *E. coli* swarms, using microbubbles as novel flow tracers. The flows exhibit complex drift patterns that differ in different regions of the swarm. The fluid beyond the swarm-edge monolayer in the multilayered region flows inwards and outwards towards a region $\sim 100\ \mu\text{m}$ from the swarm edge. These flows maintain a water reservoir of greater fluid depth extending between ~ 30 and $\sim 200\ \mu\text{m}$ from the swarm edge. In this reservoir, fluid flows are more highly agitated.

The cause of fluid drift

The flows within the multilayer region appear to be driven by cellular metabolic activities (Figs. 5, S1), not by cell motility (Fig. 4). Metabolic activities can affect water activity in at least two ways. One way is by the increase of cell number (cell volume) per unit area of agar surface. This requires water, because cells are $\sim 80\%$ water. The other way is by secreting osmolytes, as byproducts of metabolism, raising the osmolarity of the extracellular medium. A spatial gradient in metabolic activities will then be accompanied by a gradient in osmolarity, which will drive fluid up the gradient. The cell-density profile shown in Fig. 5 implies that cells will move fluid toward the center of the multilayered region, as evidenced by the drifts observed towards the region $\sim 100\ \mu\text{m}$ from the edge of the swarm. This fluid must be drawn from the underlying agar: the agar supplies the swarm with fluid that sustains its expansion.

Fluid balance near swarm edge

Because the height profile of a swarm remains constant as the swarm expands, we can derive a fluid balance equation for the fluid film near the edge of the swarm. Denoting the net height of swarm fluid at position x and at time t as $h(x,t)$ (excluding the volume occupied by cells), the change of $h(x,t)$ at x due to the drift to the right (at the speed of swarm expansion, v_s) of the entire height profile equals the change of fluid volume (area in the 2D representation of Figure S2) due to the fluid flows within the swarm ($v_f(x,t)$) plus the change in volume due to the flow from the agar substrate ($v_o(x,t)$) minus the change in volume due to fluid taken up by cells for volume increase and division. For convenience, we set $t=0$ and define $h(x,0)\equiv H(x)$. The following equation is obtained (see SI):

$$v_o(x) = rC(x) - v_s \frac{dH(x)}{dx} + \frac{d[v_f(x)H(x)]}{dx} \quad (1)$$

Here r is the growth rate of cells (chosen as $1/1200 \text{ s}^{-1}$; see SI) and $C(x)$ is the cell volume per unit area of agar surface. $C(x)$ is proportional to the normalized fluorescence intensity from FM 4-64 stained cells measured in Fig. 5. $H(x)$ can be inferred from $C(x)$, and $v_f(x)$ can be approximated by fitting the measured flow speeds in different regions of the swarm (Fig. 2); see Figs. S3 and S4. With $C(x)$, $H(x)$ and $v_f(x)$, Eq. (1) allows us to calculate $v_o(x)$ as a function of the distance from the swarm edge, as shown in Fig. 6A. The result suggests that water is drawn from the agar mostly within $\sim 70 \mu\text{m}$ from the swarm edge, with v_o greater than zero and peaking at $\sim 0.15 \mu\text{m/s}$ near $\sim 30 \mu\text{m}$ from the swarm edge (i.e. the boundary between the multilayered region and the swarm edge monolayer). In the region between ~ 70 and $\sim 200 \mu\text{m}$ from the swarm edge, surprisingly, the fluid balance requires that the agar absorbs water from the swarm ($v_o < 0$). This can be understood, if the osmolarity in the swarm fluid of this region is smaller than that in the agar underneath.

Proposed model of swarm expansion

To summarize, we suggest a model for *E. coli* swarm expansion shown in Fig. 6B. As cellular metabolic activities in the multilayered region draw water from the surroundings, a water reservoir is maintained near the swarm edge (the solid black profile in Fig. 6B). In the reference frame of the laboratory, the fluid in the inner half of the water reservoir (in between the inner edge of the water reservoir and the profile peak) flows outwards, decreasing the fluid depth in this region; the fluid in the outer half of the water reservoir (in between the outer edge of the water reservoir and the profile peak) flows inwards, with water supplied from the underlying agar, increasing the fluid depth in this region. Meanwhile, swarm fluid is pumped outwards by the action of flagella of cells stuck at the outer edge of the swarm monolayer, where the mean fluid depth is most likely less than the cell width. The outer region of the water reservoir provides the fluid source that sustains this pumping. Consequently, the profile of the entire swarm-fluid film shifts outwards (the dashed red profile in Fig. 6B). As the swarm fluid spreads, stuck cells are freed and the swarm expands. It is important to note that the swarm fluid does not have to flow outwards everywhere, because of movement of fluid in and out of the much larger reservoir contained in the underlying agar.

The above picture requires a sufficiently wettable surface, so that the flagella of cells near swarm edge can pump fluid outwards. If the surface were not wettable, water would tend to accumulate in droplets rather than flow outwards. In some cases, surfactants are required. For example, a mutant strain of *Bacillus subtilis* defective in surfactin biosynthesis (the *srfAA* mutant) cannot swarm on Difco agar; however, externally supplied surfactin restored its swarming capability (12). Strains of *E. coli* K12 fail to swarm on Difco agar because they

are missing the lipopolysaccharide O-antigen, but they do swarm on Eiken agar, which is more wettable (15). Cell surfaces need to be wettable, as well, so that cells can move into the advancing fluid film. There is a mutant of *Salmonella* missing a surface component, FlhE, that cannot swarm on Difco agar; an externally-supplied nonionic surfactant (Tween 80) restored its swarming capability (24).

This is a minimal model of swarm expansion, requiring only cell growth, functional flagella, and wettable surfaces. Only the rim of the swarm, which extends about 300 μm from the swarm edge, is involved in the expansion of swarm fluid. We expect that this model applies to the swarming of flagellated bacteria in general, as a simple but effective means for colonizing surfaces. Regulatory mechanisms are not required.

Materials and methods

Bacterial strains. The strain used for studies of fluid motion was *E. coli* HCB1668 (FliC S353C), an AW405 derivative that swarms well, developed for flagellar visualization (8). The strain used for studies of cell growth and cell density was *E. coli* MG1655-ASV (a gift from Kim Lewis), which expresses a short-lived derivative of GFP (ASV) under control of the ribosomal *rrnBP1* promoter, developed for studies of cell growth (21-22). Single-colony isolates were grown overnight in LB medium (1% Bacto tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.5) at 30°C to stationary phase. For *E. coli* HCB1668, kanamycin (50 $\mu\text{g/ml}$), chloramphenicol (34 $\mu\text{g/ml}$), and arabinose (0.5%) were added to the growth medium. For *E. coli* MG1655-ASV, ampicillin (50 $\mu\text{g/ml}$) and chloramphenicol (34 $\mu\text{g/ml}$) were added to the growth medium. These cultures were diluted 10^{-5} to provide cells for inoculation of swarm plates.

Swarm plates. Swarm agar was 0.6% (HCB1668) or 0.5% (MG1655-ASV) Eiken agar in 1% Bacto peptone, 0.3% beef extract, and 0.5% NaCl. At these agar concentrations, HCB1668 and MG1655-ASV swarmed at similar rates and exhibited similar morphologies near the swarm edge. The agar was autoclaved and stored at room temperature. Before use, it was melted in a microwave oven, cooled to ~60°C, and pipetted in 25 mL aliquots into 150 x 15 mm polystyrene petri plates (8). Antibiotics (for *E. coli* HCB1668 and MG1655-ASV) and arabinose (for *E. coli* HCB1668) were added to the liquefied swarm agar before pipetting at the concentrations used in liquid cultures. For surface cell density measurements with *E. coli* HCB1668 or MG1655-ASV, the dye FM 4-64 (Invitrogen-Molecular Probes) was dissolved in de-ionized water and added to the liquefied swarm agar before pipetting at a final concentration of 1 µg/ml. The agar plates were swirled gently to ensure complete wetting, and then cooled for 30 min without a lid inside a large Plexiglas box. Drops of diluted cell culture (2 µL, described above) were inoculated at a distance of 2-3 cm from the edges of the plates, and the plates were dried for another 30 min without a lid, covered, and incubated overnight at 30°C and ~100% relative humidity, until the swarms grew to a diameter of ~5 cm.

Microbubble fabrication. Suspensions of the surfactant Span 83 (Sorbitan sesquioleate, S3386, Sigma-Aldrich) were prepared in water at a wt/wt ratio of 0.03-0.04%, following the procedures described previously (17). When viewed with a phase-contrast microscope, the suspension appeared full of refractile droplets with diameters ranging from a fraction of a µm to a few µm. A 0.5 µL drop of this suspension was placed 3-4 cm in front of the *E. coli* HCB1668 swarms. As water in the drop was absorbed by the agar, Span 83 droplets transformed into

arrays of micron-sized bubbles (17). Some of these bubbles were stable enough to be engulfed by the advancing swarms.

Phase contrast and epifluorescence imaging. The motion of microbubbles in the interior of swarms was observed in phase contrast with a 10x objective and a 1x relay lens mounted on a Nikon Optiphot2 upright microscope maintained at 30°C. Recordings were made with a CCTV camera at 30 frames/s (model KPC-650BH, KT&C, Korea) and a digital tape recorder (model GV-D1000, Sony). The video sequences were transferred to a PC as “avi” files and uncompressed using the free software VirtualDub (<http://www.virtualdub.org/>) for further analysis. The epifluorescence of cells expressing GFP (ASV) and/or stained by FM 4-64 was observed with the same objective and relay lens, by illuminating the swarm with a mercury arc lamp via a FITC/Texas Red cube (51006, Chroma Technology Corp., Bellows Falls, VT, excitation 497/20 and 570/30 nm; emission 530/40 and 625/60 nm). The fluorescence was recorded with a Nikon D80 digital camera with 5 s exposure times in RGB color mode, utilizing the D80 Camera Control Pro 2 installed in a PC. Fluorescence from GFP (ASV) and FM 4-64 was recorded in the Green and Red channels of the RGB images, respectively.

Image analysis. Microbubbles were tracked in the phase-contrast video sequences either automatically with a program based on an open-source package (see http://www.rowland.harvard.edu/labs/bacteria/index_software.html) (16), or manually using the MTrackJ plugin (Erik Meijering, <http://www.imagescience.org/meijering/software/mtrackj/>) developed for ImageJ (<http://rsbweb.nih.gov/ij/>). The sizes of microbubbles were determined by doing Gaussian fits to the light-intensity profiles of lines crossing

bubble centers plotted in ImageJ. The width of Gaussian fits (2σ) was taken as the diameter of bubbles (17).

The boundaries of the multilayered region of swarms were determined by the brightness fluctuation across the swarm, defined as the normalized standard deviation of pixel values in images in the transverse direction as a function of the distance from the edge of the swarm. The brightness fluctuation is averaged over a given number of successive video frames and then normalized by the average standard deviation of pixel values at the outer boundary of the multilayered region (defined below). The brightness fluctuation provides a measure of the swarm porosity. Just behind the swarm edge monolayer, this function exhibits a local maximum, whose position was taken as the position of the outer boundary of the multilayered region (Fig 1B, at distance $\sim -30 \mu\text{m}$). This function also exhibits a ramp near the inner boundary of the multilayered region, the midpoint of which was taken as the position of the inner boundary of the multilayered region (Fig 1B, at distance $\sim -170 \mu\text{m}$).

Particle image velocimetry (PIV) was performed using the open-source package MatPIV 1.6.1 written by J. Kristian Sveen (<http://folk.uio.no/jks/matpiv/>). For each pair of consecutive images, the interrogation window size started at $32.9 \mu\text{m} \times 32.9 \mu\text{m}$ and ended at $4.1 \mu\text{m} \times 4.1 \mu\text{m}$ after 8 iterations. The grid size of the resulting velocity field was $2.05 \mu\text{m} \times 2.05 \mu\text{m}$. The average radial speed of cells at a certain distance from the swarm edge was then calculated by averaging the radial component of all the velocity vectors in the velocity field at that particular distance from the swarm edge.

Fluorescence images were analyzed with MatLab (The MathWorks, MA). After making background corrections, the image data extracted from the Green and the Red channels corresponds to the epifluorescence signal from GFP (ASV) and FM 4-64 stained cells, respectively. The peak location of a fluorescence intensity profile was determined as the maximum position of the best polynomial fit to the profile.

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References

1. Kearns DB (2010) A field guide to bacterial swarming motility. *Nat Rev Microbiol* 8:634-644.
2. Copeland MF & Weibel DB (2009) Bacterial swarming: a model system for studying dynamic self-assembly. *Soft Matter* 5:1174-1187.
3. Harshey RM (2003) Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol* 57:249-273.
4. Koch DL & Subramanian G (2010) Collective hydrodynamics of swimming microorganisms: living fluids. *Annual Review of Fluid Mechanics* 43(1):637-659.
5. Ramaswamy S (2010) The mechanics and statistics of active matter. *Annu Rev Condens Matter Phys* 1:323-345.

6. Copeland MF, Flickinger ST, Tuson HH, & Weibel DB (2010) Studying the dynamics of flagella in multicellular communities of *Escherichia coli* using biarsenical dyes. *Appl Environ Microbiol* 76:1241-1250.
7. Darnton NC, Turner L, Rojevsky S, & Berg HC (2010) Dynamics of bacterial swarming. *Biophys J* 98:2082-2090.
8. Turner L, Zhang R, Darnton NC, & Berg HC (2010) Visualization of flagella during bacterial swarming. *J Bacteriol* 192:3259-3267.
9. Zhang HP, Be'er A, Florin E-L, & Swinney HL (2010) Collective motion and density fluctuations in bacterial colonies. *Proc Natl Acad Sci USA* 107:13626-13630.
10. Wang Q, Frye JG, McClelland M, & Harshey RM (2004) Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. *Mol Microbiol* 52(1):169-187.
11. Kim W & Surette MG (2004) Metabolic differentiation in actively swarming *Salmonella*. *Mol Microbiol* 54(3):702-714.
12. Kinsinger RF, Shirk MC, Fall R (2003) Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. *J Bacteriol* 185(18):5627-31.
13. Fall R, Kearns DB, & Nguyen T (2006) A defined medium to investigate sliding motility in a *Bacillus subtilis* flagella-less mutant. *BMC Microbiol* 6:31.
14. Angelini TE, Roper M, Kolter R, Weitz DA, & Brenner MP (2009) *Bacillus subtilis* spreads by surfing on waves of surfactant. *Proc Natl Acad Sci U S A* 106(43):18109-18113.
15. Harshey RM & Matsuyama T (1994) Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. *Proc Natl Acad Sci U S A* 91(18):8631-8635.

16. Zhang R, Turner L, & Berg HC (2010) The upper surface of an *Escherichia coli* swarm is stationary. *Proc Natl Acad Sci USA* 107:288-290.
17. Wu Y, Hosu BG, & Berg HC (2011) Microbubbles reveal chiral fluid flows in bacterial swarms. *Proc Natl Acad Sci U S A* 108(10):4147-4151.
18. Darnton N, Turner, L., Breuer, K., Berg, H.C. (2004) Moving fluid with bacterial carpets. *Biophys J* 86(3):1863-1870.
19. Wu X-L & Libchaber A (2000) Particle diffusion in a quasi-two-dimensional bacterial bath. *Phys Rev Lett* 84:3017-3020.
20. Kim MJ & Breuer KS (2004) Enhanced diffusion due to motile bacteria. *Phys Fluids* 16:78-81.
21. Sternberg C, *et al.* (1999) Distribution of bacterial growth activity in flow-chamber biofilms. *Appl Environ Microbiol* 65(9):4108-4117.
22. Shah D, *et al.* (2006) Persisters: a distinct physiological state of *E. coli*. *BMC Microbiol* 6:53.
23. Pogliano J, *et al.* (1999) A vital stain for studying membrane dynamics in bacteria: a novel mechanism controlling septation during *Bacillus subtilis* sporulation. *Mol Microbiol* 31(4):1149-1159.
24. Stafford GP & Hughes C (2007) *Salmonella typhimurium flhE*, a conserved flagellar regulon gene required for swarming. *Microbiology* 153:541-547.

Figure legends

Fig. 1. (A) A phase-contrast image of the region near the edge of a typical *E. coli* swarm grown on 0.6% Eiken agar (Methods). Microbubbles appear as bright spots. Some move in the river that flows clockwise in front of the swarm, e.g., black arrow, and others move within the body of the swarm, e.g., white arrow. A monolayer of cells appears at the swarm edge (to the right of the second vertical dashed line). The area bounded by the first and second dashed lines looks more porous, but the cells are multilayered (stacked on top of one another). The swarm is expanding to the right, as shown by the arrow x. See Movie S1. (B) Normalized brightness fluctuation of pixels, P , in a direction parallel to the arrow y averaged over 900 consecutive frames of Movie S1, plotted as a function of the distance from the edge of the swarm. See Methods.

Fig. 2. Net radial displacement in the laboratory frame ($\langle r_x(t) \rangle$, black solid line) of bubble trajectories at different regions inside the swarm, shown as a function of time. Linear fits to $\langle r_x(t) \rangle$, indicating radial drift velocities, v_x , are shown by red dashed lines. The grey areas indicate standard errors in the mean. The insets show typical bubble tracks in the laboratory frame measured in μm , beginning at + and ending at x. Tracking began at distances from the swarm edge shown on the ordinates at $t=0$ and continued over the time span shown on the abscissas. The numbers of tracks analyzed were *A*, 29; *B*, 35; *C*, 43; and *D*, 22. Data analysis was continued until about half of the trajectories extended beyond the region of interest, e.g., the cell monolayer, *A*, the multilayered region, *B*, and the regions beyond the multilayered region, *C* and *D*.

Fig. 3. Mean-squared displacement of microbubbles (MSD) as a function of time, corrected for drift. Four data sets of bubble trajectories (the same as those used in the panels of Fig. 2, truncated at $t = 5$ s) were computed and plotted with different symbols. Each solid line is the best linear fit of $\log[MSD(t)]$ versus $\log(t)$, which yields the effective self-diffusion coefficient D_{eff} and the anomalous diffusion exponent α , as the y-intercept and the slope, respectively. The data sets are representative of the bubble motion in the following regions of a swarm: the swarm edge monolayer ($D_{eff} = 31 \pm 1 \mu\text{m}^2/\text{s}$, $\alpha = 1.11 \pm 0.04$, squares); the multilayered region ($D_{eff} = 64 \pm 2 \mu\text{m}^2/\text{s}$, $\alpha = 1.20 \pm 0.03$, circles); the region between ~ 200 and $\sim 300 \mu\text{m}$ from the swarm edge ($D_{eff} = 26 \pm 1 \mu\text{m}^2/\text{s}$, $\alpha = 1.27 \pm 0.03$, triangles); and the region between ~ 300 and $\sim 350 \mu\text{m}$ from the swarm edge ($D_{eff} = 26 \pm 1 \mu\text{m}^2/\text{s}$, $\alpha = 1.15 \pm 0.04$, upside-down triangles).

Fig. 4. Average radial cell speed as a function of the distance from the swarm edge. Particle image velocimetry was performed on a phase-contrast movie of a typical swarm lasting ~ 33 s, and the radial components of the velocity vectors in each velocity field were averaged (see Methods). The grey area indicates standard error of the mean.

Fig. 5. Cell density profiles of swarms of cells of *E. coli* strain HCB1668 shown as a function of the distance from the swarm edge. The agar contained a membrane-specific fluorescent dye, FM 4-64. The solid curve is the average fluorescence intensity profile ($n=5$), with the grey area indicating the standard error of the mean. The dashed lines are best linear fits of the black solid curve ranging from -200 to $-150 \mu\text{m}$ and from -50 to $-30 \mu\text{m}$, with slopes $0.0039 \pm 0.0001 \mu\text{m}^{-1}$ and $-0.0092 \pm 0.0004 \mu\text{m}^{-1}$, respectively.

Fig. 6. A model of *E. coli* swarm expansion. (A) The predicted flows in and out of the agar substrate (v_o) computed with Eq. (1). (B) An illustration of fluid balance of a swarm traveling from left to right. As the swarm fluid spreads, the height profile of swarm fluid shifts outwards (changing from the black solid line to the red dashed line). Along the direction of swarm expansion, successive grey dots at the swarm/agar interface denote distances from the swarm edge of 300, 200, 100, 30 and 0 μm , respectively. Note that the length scales in the horizontal and vertical directions are different. The observed drift of swarm fluid in different regions is depicted by the solid arrows, with the relative length of arrows roughly corresponding to the magnitude of the measured flow speeds (Fig. 2). The predicted flows in and out of the agar are illustrated by the open arrows with a dashed boundary, with the relative height of arrows roughly corresponding to the magnitude of the predicted flow speeds in A.











