Dynamic clustering in suspension of motile bacteria

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Abstract – Bacteria suspension exhibits a wide range of collective phenomena, arising from interactions between individual cells. Here we show Serratia marcescens cells near an air-liquid interface spontaneously aggregate into dynamic clusters through surface-mediated hydrodynamic interactions. These long-lived clusters translate randomly and rotate in the counterclockwise direction; they continuously evolve, merge with others and split into smaller ones. Measurements indicate that long-ranged hydrodynamic interactions have strong influences on cluster properties. Bacterial clusters change material and fluid transport near the interface and hence may have environmental and biological consequences.

Active systems are composed of self-propelled particles that can produce motion by taking in and dissipating energy [1–4]. Examples exist at different length scales, from bacteria suspension [5–10] to flocks of birds [11–13]. Being far from thermal equilibrium, active systems are not subject to thermodynamic constraints, such as detailed balance or fluctuation-dissipation theorem [14–16]. This renders the physics of active systems much richer than that of thermal systems. For example, collective motion with extended spatio-temporal coherence has been reported in many active systems [5–9,11–13,17–19]. Such coherent motion can arise from local interactions that align a particle’s motion with its neighbors through biological coordination [11,12] or physical interactions [17,19].

Active systems without alignment interactions also exhibit interesting collective behavior. Theoretical models have shown that systems with a density-dependent motility phase separate into dense dynamic clusters and a dilute gas phase [14,15,20]. Numerical simulations of repulsive self-propelled disks confirmed the theoretical prediction of phase separation [21,22]. Effects of motility, attractive interaction, and hydrodynamic forces have been extensively explored in simulations [23–26]. On the experimental side, dynamic clusters have been observed in Janus particles (platinum-coated [27] and carbon-coated [28]) and colloidal particles with an embedded hematic cube [29]. Schwarz-Linek et al. observed clusters of motile bacteria when they added polymers to bacteria suspension to induce depletion attraction between bacteria [30]. In a very recent paper [31], Petroff et al. reported that Thiouvalum majus bacteria form two-dimensional crystals near a liquid-solid interface. Understanding the origins and properties of these dynamic clusters may provide new insights into emergent behaviors of active matters and open up possibilities to build novel materials [15].

In this letter, we report experimental results for a new type of bacterial clusters formed near an air-liquid interface in a pure suspension without depletant agents. Fluid dynamic calculation and flow visualization are used to show surface-mediated hydrodynamic interactions can explain the formation of these clusters. We further quantify the statistical and dynamic properties of bacterial clusters and show long-ranged hydrodynamic forces have important influences on cluster properties. We conclude with discussions on related research and on possible technological and environmental implications of our work.

Experiments. – Our experiments are carried out in drops of wild-type Serratia marcescens (ATCC 274) bacteria, which are propelled by a bundle of a few rotating

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flagella [32]. For cultivation, a small amount of bacteria from a frozen stock is put in 4 ml of Luria Broth (LB) growth medium consisting of 0.5% yeast extract (Sangon GO961), 1% tryptone (Sangon TN5250), and 1% NaCl (Sigma). Bacteria are incubated for 13 hours to a stationary phase in a shaking incubator which operates at 30°C and 200 rpm shaking speed. We then extract 1 ml bacteria solution and re-grow bacteria in 10 ml fresh LB growth medium supplemented with 5 μg/ml of A22 for 4 hours at 33°C and 200 rpm. A22 is a small molecular inhibitor of MreB, a protein needed to maintain the rod-like shape of many bacteria [33]. *S. marcescens* cells growing in a media with A22 are short ellipses with a minor axis of 0.8 μm; the ratio of the major to minor axes has a mean of 1.2 and standard deviation of 0.12. The final bacteria solution is further diluted in distilled water to generate samples with various bacteria densities. To control bacterial motility, 1 μg/ml photosensitizer FM 4-64 is added to the bacterial suspension [34]. In the presence of FM 4-64, the bacteria will be temporally paralyzed upon exposure to strong light. A 100 W mercury lamp (Nikon C-SHG1) is used to activate photodynamic effects.

Sample is enclosed in a sealed chamber (cf. fig. 1(f)) which consists of a plastic spacer, a silicone well, and two cover slips. Cover slips and plastic spacer are 0.13 mm and 2.2 mm in thickness, respectively; the well has a diameter of 0.8 cm and a height of 0.1 cm. In some experiments, 1 μm super-paramagnetic tracer beads are added for flow visualization. Beads are confined to the interface by a permanent magnet and the strength of confinement can be tuned by moving the magnet relative to the sample. Two-dimensional bacterial or tracer motion in the trapping plane is imaged through a 60× phase-contrast objective (Nikon ELWD ADL 60×C) and recorded by a camera (Basler acA2040-180km). We use a holographic microscope to measure three-dimensional motion of tracer particles and bacteria. A red LED is used for illumination and recorded holograms are analyzed by the Rayleigh–Sommerfeld back-propagation method to extract the spatial coordinates of the scatter [35,36].

**Bacteria clusters.** – *S. marcescens* is known to adhere strongly to the air-liquid interface possibly due to hydrophobic interactions [37-39]. We use a holographic microscope to record such attaching events. Supplementary movie Movie1.mp41 shows two typical events: bacteria swim from the bulk towards the interface and get trapped near the interface. Trapped bacteria can move freely in the trapping (xy) plane; their centers of mass are tracked to quantify translational motion. Rotation motion is quantified by following the principal axes of the elliptical bacteria. The main control parameter in experiments is bacteria density which is quantified by the number of bacteria per unit area on the trapping plane and denoted as $\phi$.

As shown in fig. 1(a), (b), trapped bacteria aggregate to form dynamic clusters which are identified through Voronoi analysis of bacterial positions (see supplementary text ST.pdf2 for details). Clustering formation is a robust phenomenon and occurs under a wide range of bacteria densities and in various liquid environments including LB media, motility buffer, and water. However, formation of dynamic clusters requires bacterial motility. As shown in Movie2.mp43, clusters dissolve immediately after we reduce the motility by using strong light [34]. Clusters

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1The movie Movie1.mp4 shows the bacterium trajectory as a blue line and the raw hologram as a black-and-white image. A black dot marks the instantaneous position of the bacterium. The objective is focused on the interface.

2ST.pdf contains information of data analysis procedures and fluid dynamic analysis. It is available at http://ins.sjtu.edu.cn/people/hpzsang/EPL/ST.pdf.

3The movie Movie2.mp4 shows identified clusters in colors on phase-contrast images. Light irradiation starts at 16.8 s in the video.
reappear when bacteria motility is partially recovered after the light irradiation is stopped. Heavy metal ions (CuSO$_4$) are also used to reduce motility [40]; similar results are obtained.

To understand the connection between motility and cluster formation, we zoom-in and investigate how bacteria move in a cluster. We mark a bacterium in a cluster with a red ellipse in fig. 1(c), (d) (cf. Movie3.mp4); the marked bacterium rotates its body in the trapping plane with an angular velocity of $\Omega_z = -20$ rad/s. In contrast, a bacterium outside cluster (marked in green) shows little change in its body orientation. In fig. 1(e) we plot the probability distribution functions of angular velocity, $P(\Omega_z)$, for bacteria in and outside clusters. While peaking around zero for bacteria outside clusters, $P(\Omega_z)$ shows a second peak at $-23$ rad/s for bacteria in clusters.

**Hydrodynamic interactions.** — *S. marcescens* bacteria swim by rotating their flagellar bundles. When viewed from the front of a bacterium, the bundle rotates clockwise and the cell body rotates in the opposite direction to achieve hydrodynamic torque balance. Fast body rotation suggests the bacteria in clusters orient their bundles perpendicular to the interface$^5$. Bacteria in such a configuration can generate fluid flow that leads to cluster formation [31,41,42]. To illustrate the mechanism, we numerically compute fluid flow around a bacterial model that is oriented perpendicular to the interface. As shown in fig. 2(a), the model has a 1-μm-diameter spherical body whose center is located at $(0, 0, 0.54 \mu m)$ and is driven by a rotating single flagellum. The translational degrees of freedom are frozen for the model. The model bacterium exerts a positive force in the $z$-direction on the fluid, draws in fluid along the interface (at $z = 0$) and pushes fluid to the bulk. Rotation of the flagellum and cell body also produces a tangential flow component that can be seen in the bulk. Rotation of the flagellum and cell body also generates inward radial flow that attracts a passive tracer confined to the interface$^5$. Such a mechanism only works in the background, cell orientations are marked by arrows, and ellipses are used to highlight two bacteria.

Important flow features in fig. 2 are qualitatively confirmed in experiments with two types of tracer beads. The first kind is strongly confined in the $z$-direction and only probes fluid flow in the trapping plane. A typical result is shown in fig. 3(a)–(d) and in Movie4.mp4$^4$. A tracer bead is driven by a rotating bacterium from $t = 0$ to $t = 0.5$ s, which demonstrates the inward radial flow. While the bead and bacterium are bound, they rotate around each other counterclockwise, which is a manifestation of the tangential flow in fig. 2(b). At $t = 7$ s, the bacterium stops rotating and swims away from the bead. In the second experiment, a tracer bead is subject to weak confinement and can be advected also in the $z$-direction. As shown in fig. 2(e)–(h) and Movie5.mp4, the bead is drawn towards the bacterium in the trapping plane until $t = 3.5$ s, when the separation between the bead and bacterium is about 1 μm. The bead is then advected quickly into the bulk, which demonstrates strong flow into the bulk around the bacterium. Tracer particles are also used to visualize flow around bacteria clusters. Movie6.mp4$^6$ shows that clusters attract tracers and advect them into the bulk, mimicking results in figs. 2 and 3. This supports the following picture: bacteria in clusters orient their flagella perpendicular to the interface; they generate inward radial flow that attracts...

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$^4$In the movie Movie3.mp4, phase-contrast images are shown in the background, cell orientations are marked by arrows, and ellipses are used to highlight two bacteria.

$^5$A hydrodynamic mechanism was proposed to explain perpendicular orientation of *T. majus* cells [31]. Such a mechanism only works at a no-slip boundary and does not apply in the case of *S. marcescens* near a liquid-air interface.

$^6$In the movie Movie4.mp4, a passive tracer confined to the interface visualizes attractive flow around a rotating bacterium.

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$^7$The movie Movie5.mp4 shows three-dimensional motion of a passive tracer around a rotating bacterium.

$^8$Movie6.mp4 shows three-dimensional motion of a passive tracer around bacterial clusters. A 1 μm tracer bead (marked by red +) is drawn towards a bacterial cluster during the first 1.7 s, and then advected quickly into the bulk, as shown by the enlarging interference rings in the hologram.

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neighbors to form clusters and counterclockwise tangential flow that drives clusters into rotation.

**Cluster properties.** Bacteria clusters observed in experiments are highly dynamic; they constantly evolve and change their sizes. We defined cluster size as the number of the constituent bacteria, \( n \). Probability distributions for finding a cluster of a given size at six bacteria densities \( \phi \) are shown in fig. 4(a). As the density increases, probability to find large clusters increases. Probability distribution function decays exponentially for large \( n \). Similar exponential distributions have been observed in many previous studies [8,9] and may be modeled by fusion-fission processes [46].

We compute the following quantities to quantify translation and rotation of the \( I \)-th cluster which contains the \( i \)-th bacterium at a location \( \vec{r}_{i,I} \) and with a velocity \( \vec{v}_{i,I} \). The center of mass of the \( I \)-th cluster is located at \( \vec{R}_I = \langle \vec{r}_{i,I} \rangle_I \), where \( \langle \cdot \rangle_I \) denotes an average over all \( n_I \) bacteria in the \( I \)-th cluster. The speed of the center of mass is \( V_I = |\vec{V}_I| = |\langle \vec{v}_{i,I} \rangle_I| \). The angular speed of the \( I \)-th cluster is defined as \( \omega_I = \frac{\langle (\vec{v}_{i,I} - \vec{R}_I) \times (\vec{v}_{j,I} - \vec{R}_I) \rangle_I}{\langle (\vec{R}_{I,1} - \vec{R}_{I}) \times (\vec{R}_{I,1} - \vec{R}_{I}) \rangle_I} \), whose inversion is the rotation period: \( T_I = \frac{1}{\omega_I} \). Averaging \( V_I \) and \( T_I \) over all clusters of size \( n \), we have mean translation speed \( V(n) = \langle V_I \rangle_{n_I=n} \) and mean rotation period \( T(n) = \langle T_I \rangle_{n_I=n} \).

Results in fig. 4(b), (c) show that, for a given \( \phi \), larger clusters translate and rotate more slowly than smaller ones. This dependence is qualitatively consistent with a simple model of active clusters made of self-propelled particles [31]. Each particle is driven by a propulsive force \( F_{sp} \) in the \( \vec{v} \)-direction and has a drag coefficient of \( \gamma \); they interact through pairwise forces in the radial and tangential directions. If \( n \) particles in a cluster are randomly oriented, total propulsive force on the cluster is \( \sum F_{sp} \bar{e}_I = F_{sp} n^{0.5} \). The total friction coefficient is the sum of all particles: \( n \gamma \). The average velocity of the center of mass is the ratio of the total force to the total friction coefficient and scales as \( V \propto n^{-0.5} \). Using a similar torque-balance argument [31,47], we can get a scaling law for rotation period: \( T \propto n \). These two scalings are shown in fig. 4(b) and (c) as black lines which clearly deviate from experimental results.

First, we notice that cluster motion in experiments depends strongly on global densities, \( \phi \). Figure 4(b), (c) show that a cluster of a given size translates faster and rotates more slowly in a system with a higher density. The dependence on system density likely arises from long-ranged hydrodynamic interactions which enable fluid disturbances to propagate far and couple different clusters over large distances. Second, fig. 4(b) shows that cluster velocity \( V(n) \) scales as \( V \propto n^{-0.25} \) rather than \( V \propto n^{-0.5} \). This is also possibly related to hydrodynamic interactions which determine how cluster friction and net propulsive force scale with cluster size. In the limit of Stokes drag, cluster friction scales as \( \sqrt{n} [48] \) in two dimensions, which suggests that net propulsive force of clusters may scale as \( n^{0.25} \). Data in fig. 4(b), (c) indicate that hydrodynamic interactions in our experiments are too complex to be represented by simplified pairwise forces.

**Discussion.** Inward radial flow in fig. 2(a) is similar to feeding flow that many micro-organisms use to gather food from the fluid environment near an interface [41,42]. The same flow pattern is also used to explain the formation of bounded Volvox pair [49] and to explain the attractive force between thermophoretic colloids [50–52]. Such an attractive boundary flow should exist near any low-Reynolds-number swimmer that is oriented perpendicular to a fluid or solid boundary and swims...
into the boundary; consequently, these oriented swimmers experience effective attraction and can be hydrodynamically assembled into clusters. This provides a new mechanism, besides phoretic [29], depletion [30] interactions and self-trapping effects [28], to generate clusters of active particles.

Petroff et al. [31] recently reported that T. majus bacteria form two-dimensional crystals near a liquid-solid interface through a mechanism similar to that in S. marcescens clusters. However, T. majus and S. marcescens systems are significantly different in at least two aspects. First, the strength of attractive interaction is different. Petroff et al. used the product of propulsive force and swimmer size to estimate the energy scale (denoted as $E$) for attraction between cells. T. majus have an average diameter of 8.5 μm and swim at a speed of 600 μm/s; it was found that the attractive energy is much larger than thermal energy: $E \sim 10^4k_BT$. Consequently, T. majus crystals are very stable and can contain up to a thousand cells. On the other hand, S. marcescens, like many other commonly studied bacteria [7,18,30] and artificial swimmers [28,29], are approximately ten times smaller in both size and speed than T. majus; the attractive energy scale is 1000 times smaller and is on the order of 10$k_BT$. Therefore, thermal fluctuations play a more important role in the S. marcescens system and render S. marcescens clusters dynamic and constantly evolving. Thermal fluctuations may also be able to destabilize large clusters and prevent the appearance of giant clusters of system size [22,53]. Second, T. majus crystals and S. marcescens clusters form near different hydrodynamic boundaries. A no-slip boundary makes the hydrodynamic attraction, $f$, between two T. majus cells decays rapidly as their separation, $r$, increases: $f \sim r^{-4}$; for S. marcescens clusters near a free-slip boundary, we have $f \sim r^{-2}$. Consequently, while hydrodynamic interaction between T. majus cells are severely screened, fig. 4 shows that long-ranged hydrodynamic interactions influence S. marcescens clusters properties.

**Conclusion.** In summary, we have investigated dynamic clusters of S. marcescens bacteria near an air-liquid interface. Fluid dynamic calculation and flow visualization suggest that the constituent bacteria of these clusters orient their flagella perpendicular to the interface. Bacteria in such a configuration generate radial flow that attracts neighbors to form clusters and tangential flow that sets clusters into counterclockwise rotation. We measured statistical properties of bacteria clusters and showed that cluster properties are affected by long-ranged hydrodynamic interactions. S. marcescens clusters efficiently change material and fluid transport near the air-liquid interface; they may have environmental and biological consequences [6].

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