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Nontoxic colloidal particles impede antibiotic resistance of swarming bacteria by disrupting collective motion and speed

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A monolayer of swarming *B. subtilis* on semisolid agar is shown to display enhanced resistance against antibacterial drugs due to their collective behavior and motility. The dynamics of swarming motion, visualized in real time using time-lapse microscopy, prevents the bacteria from prolonged exposure to lethal drug concentrations. The elevated drug resistance is significantly reduced when the collective motion of bacteria is judiciously disrupted using nontoxic polystyrene colloidal particles immobilized on the agar surface. The colloidal particles block and hinder the motion of the cells, and force large swarming rafts to break up into smaller packs in order to maneuver across narrow spaces between densely packed particles. In this manner, cohesive rafts rapidly lose their collectivity, speed, and group dynamics, and the cells become vulnerable to the drugs. The antibiotic resistance capability of swarming *B. subtilis* is experimentally observed to be negatively correlated with the number density of colloidal particles on the engineered surface. This relationship is further tested using an improved self-propelled particle model that takes into account interparticle alignment and hard-core repulsion. This work has pertinent implications on the design of optimal methods to treat drug resistant bacteria commonly found in swarming colonies.

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I. INTRODUCTION

Collective motion is widely observed in nature and is known to affect population-wide social behavior (e.g., escape from predators in fish schools) [1–18]. Certain bacteria such as *Bacillus subtilis*, *Salmonella enterica*, and *Pseudomonas aeruginosa* are capable of undergoing cell differentiation to a swarming phenotype that performs rapid coordinated motion across a semisolid surface in multicellular rafts. The collective behavior of these swarming cells is known to aid in the rapid expansion of bacterial colonies [19–32] and is an integral part of pathogenesis [33–38]. Unfortunately, swarming bacteria have been demonstrated to show elevated resistance against several antibiotics when compared to their corresponding planktonic cells [33–38]. This gives rise to potential health problems since conventional antimicrobial drugs may not be able to effectively eliminate swarming bacteria in early stage infections.

The exact mechanism driving the elevated antibiotic resistance in swarming bacteria is still not completely known [37]. Kim *et al.* have suggested that the resistance is due to a physiology attribute [33,34], whereas Butler *et al.* have proposed that high cell density coupled with rapid motion of cells in multilayered colonies are important [35]. The dynamics of *B. subtilis* under sublethal drug concentration has recently been reported [39]. Important information regarding the real-time evolution of the collective behavior of swarming bacteria exposed to lethal amounts of antibiotics is still missing.

The collective dynamics of wild type *B. subtilis* has previously been examined and the factors governing their swarming behavior elucidated [19–29,40–43]. In this article, the two-dimensional (2D) rapid coordinated motion within

multicellular packs is visualized in real time and found to be instrumental in ensuring that bacteria located at the expanding monolayered colony front suffer minimal damages when in contact with lethal drug concentrations. We designed a simple method to impede drug resistance by judiciously disrupting swarming motion. Nontoxic polystyrene (PS) colloidal particles are immobilized in the pathways of motile *B. subtilis* such that the colloidal particles block and hinder the collective motion of cells without chemically impairing flagellar functions or cell activities (Fig. 1). In turn, the bacteria significantly reduce their resistance against tobramycin (Tob) and vancomycin (Van). To further investigate the dynamics of bacterial motion in the presence of colloidal particles, computational simulation is performed using an improved self-propelled Vicsek model where hard-core repulsion between motile and nonmotile particles and an alignment rule are systematically implemented [29].

In this work, wild type *B. subtilis* 3610 strain is used and PS particles are directly deposited onto the semisolid agar. The disk diffusion method is used to determine inhibition zone sizes (r) [36], and all swarming motions are recorded using time-lapse microscopy. Further experimental details are given in the Supplemental Material [44].

Rapid coordinated motion within multicellular packs is visualized in real time and found to be instrumental in ensuring that bacteria located at the expanding colony front suffer minimal damages when in contact with antibiotics, a defense mechanism that is absent in vulnerable planktonic cells. In this manner, we have designed a simple method to impede drug resistance by judiciously disrupting swarming motion. Nontoxic polystyrene (PS) colloidal particles are immobilized in the pathways of motile *B. subtilis* such that the colloidal particles block and hinder the motion of cells and hence reduce both their collectivity and speed without chemically impairing flagellar functions or cell activities (Fig. 1). In turn,

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FIG. 1. (Color online) Bacteria swarm in large rafts in the non-PS particle zone. The collectivity is disrupted in the presence of colloidal particles (in the PS particle zone) which results in more bacteria becoming vulnerable to the antibiotic.

the bacteria drastically lose their defense against tobramycin and vancomycin.

To further investigate the dynamics of bacterial motion in the presence of colloidal particles, computational simulation is performed using an improved self-propelled Vicsek model where hard-core repulsion between particles and an alignment rule are systematically implemented [13]. The latter takes into account the mutual alignment between particles and their weighted contribution to the overall direction of motion [29]. In a previous study, we have shown that high intrinsic noise mimics external stress capable of disrupting collectivity [29]. In this work, stationary hurdles such as PS particles are modeled as hard spheres and the relationship between motile particle collectivity and immobilized particle density is examined and compared with experimental observations.

II. RESULTS AND DISCUSSION

A. Resistance to antibiotics

Resistance of wild type swarming *B. subtilis* against tobramycin (Tob) and vancomycin (Van) is tested using the disk diffusion method. An inhibition zone is clearly seen at the top half of the semihard (0.5%) agar where an antibiotic disk is placed [Fig. 2(a-i) for Tob and Fig. S1 in Supplemental Material [44] for Van]. When a swarming colony expands, two layers are seen [36]—a multilayered colony that forms a primary front closer to the inoculum, and a monolayered colony that advances beyond the primary front to form a secondary front that faces the virgin agar [36]. The size of the inhibition zone (r) is determined to be 1.3 ± 0.6 mm and 2.5 ± 0.3 mm for Tob (750 $\mu\text{g}/\text{disk}$) and Van (30 $\mu\text{g}/\text{disk}$), respectively [Fig. 2(b)].

For planktonic cells on hard agar (i.e., 3%), nonmotile bacteria within an inhibition zone of $r = 8.2 \pm 0.2$ mm and 4.6 ± 0.2 mm for Tob and Van, respectively, are either killed or unable to proliferate (Fig. S2 [44]). To avoid an ambiguous comparison arising from the use of different concentrations of cells, both the densities of swarming and planktonic bacteria are maintained at ca. $0.05\text{--}0.06$ cells μm^{-2} . Since the size of

FIG. 2. (Color online) (a) Swarming colony morphology recorded at various times after inoculation t_i for tobramycin [Tob, (i) $t_i = 150$ min, (ii) $t_i = 210$ min, and (iii) $t_i = 240$ min]. The agar is inoculated at the center (y) and the drug disk x is placed at the top of the agar plate. The shortest distances between colony edge and drug disk are 9.4, 3.5, and 1.0 mm for (i), (ii), and (iii), respectively. (b) Inhibition zone sizes of planktonic and swarming cells in the absence and presence of colloidal particles for Tob and Van.

the inhibition zone is not dependent on agar concentrations [36], lethal amounts of drugs are found within 8.2 ± 0.2 mm and 4.6 ± 0.2 mm from the Tob and Van disks, respectively [Fig. 2(b)]. All inhibition zone sizes are measured between 4.5 and 5 h after incubation to ensure that drug diffusion is standardized in all experiments.

B. Collective behavior leads to drug resistance

In order to unravel the dynamics of collective motion of swarming bacteria as they move toward the antibiotic disk, real-time imaging of cells close to the secondary front (i.e., <200 μm from the edge) at various times after inoculation (t_i) is performed using time-lapse microscopy. At $t_i = 150$ min, the swarm colony has not encountered lethal drug concentration and continues to expand normally [Fig. 2(a-i)]. Movie S1 [44] shows the healthy bacteria moving cohesively with an average speed $V = 23.6 \pm 1.6$ $\mu\text{m s}^{-1}$, and the spatial correlation function of instantaneous velocity (C_v) yields a correlation length $L = 13.1$ μm (Fig. 3). Frontal cells at the colony edge are not considered when computing V and L . At $t_i = 210$ min, the swarm monolayer is exposed to Tob [Fig. 2(a-ii)]. The bacteria continue to move cohesively in rafts, albeit with reduced collectivity (i.e., $L = 10.0$ μm , Fig. 3) and significantly slower speed (i.e., $V = 15.8 \pm 0.4$ $\mu\text{m s}^{-1}$) (Movie S2 [44]). Compared to healthy *B. subtilis* at $t_i = 150$ min, the cell density of tobramycin-affected bacteria at $t_i = 210$ min increases sharply from $D = 0.048$ cells μm^{-2} to $D = 0.090$ cells μm^{-2} due to a relatively slower colony expansion rate across the agar (2.7 $\mu\text{m s}^{-1}$ at $t_i = 150$ min vs 0.40 $\mu\text{m s}^{-1}$ at $t_i = 210$ min). This indicates that Tob has an adverse effect on cell motion, causing both the stalled bacteria at the edge to pump water and surfactant outward less efficiently and motile bacteria to swarm in smaller packs and at slower speed. It is important to note that despite the effects of Tob on the swarming behavior of *B. subtilis*, the bacteria continue to survive and spread.

As the colony continues to expand closer to the antibiotic disk, a larger fraction of the cells experiences high dosages

FIG. 3. (Color online) The spatial correlation function of instantaneous velocity (C_v) vs distance for Tob at $t_i = 150$ min (red, $\circ$), 210 min (green, ∇), and 240 min (blue, $\diamond$). The inset shows the corresponding L and V .

of Tob. For example, at $t_i = 240$ min (Movie S3 [44]), the swarm stops advancing [Fig. 2(a-iii)]. Apart from a lack of sufficient surfactin secreted to induce proper swarm expansion, the slow moving bacteria behind the thick layer (three to four cell lengths) of stalled bacteria at the edge are unable to effectively collide and replace the jammed cells. In this case, the average speed and correlation length of the motile cells are reduced to $10.3 \pm 0.3 \mu\text{m s}^{-1}$ and $7.8 \mu\text{m}$, respectively (Fig. 3). After a certain period of drug exposure, cells close to the swarm edge suffer sufficient damage and stop moving (e.g., at 270 min, Movie S4 [44]). However, a large portion of cells located further away from the front remain mobile (e.g., $\sim 500 \mu\text{m}$; see Movie S5 [44]). About 90% of frontal cells are not killed by Tob as demonstrated by bacterial viability experiments (Fig. S3 [44]).

When the drug used is vancomycin, the correlation length and average speed of *B. subtilis* display similar qualitative behavior as observed for Tob (Fig. S1 [44]). Before encountering Van, the colony front advances rapidly as the bacteria mature. In this case, L and V decrease from $L = 14.4 \mu\text{m}$ and $V = 25.3 \pm 0.9 \mu\text{m s}^{-1}$ at $t_i = 150$ min to $L = 9.6 \mu\text{m}$ and $V = 12.8 \pm 0.3 \mu\text{m s}^{-1}$ at $t_i = 210$ min. Van-affected cells suffer a further drop in collectivity and speed with $L = 6.5 \mu\text{m}$ and $V = 8.7 \pm 0.3 \mu\text{m s}^{-1}$ at $t_i = 240$ min.

C. PS colloidal particles are nontoxic to bacteria

Polystyrene (PS) particles with a $1.1\text{-}\mu\text{m}$ diameter, approximately the width of the bacteria, are used in this study. Figure 4(a) shows the bacterial colony formed from an inoculum of *B. subtilis* and PS colloidal particles [1% (w/w)]. Both bacteria and PS particles were coincubated at the center of the agar for ~ 2.5 h before a swarming colony was developed. The colony pattern is identical to the pattern observed for an inoculum of *B. subtilis* without PS particles [e.g., Fig. 2(a-i)].

A cell viability assay was conducted to ascertain that the proliferation of planktonic *B. subtilis* is not affected by the presence of PS particles. In this case, two sets of bacterial

FIG. 4. (Color online) (a) Swarming colony of *B. subtilis* on a semisolid agar (0.5%). The cell culture used to inoculate the agar was first incubated with a PS colloidal suspension [1% (w/w)]. (b) The growth curves of bacterial culture with (black) and without (red) PS particles [0.0625% (w/w)] for different incubation times.

culture with the same cell concentrations were prepared; one without PS colloidal particles and the other with PS particles [0.0625% (w/w)]. Colony forming unit (CFU) assay was conducted for both cell cultures after incubation for 0, 1, 2, 3, and 4 h. Figure 4(b) shows the growth curves of the bacterial cultures in the absence and presence of PS colloidal particles for different incubation times. It is observed that there are no significant differences in the growth curves, demonstrating that the presence of the colloidal particles does not affect the viability and proliferation of the bacteria. Furthermore, swarming bacteria are observed in the monolayer leading to the colony front in Fig. 4(a) (Movie S6 [44]). The corresponding correlation length is $13.7 \mu\text{m}$ and average speed is $25.4 \pm 0.9 \mu\text{m s}^{-1}$ (close to the values obtained for colonies formed from inoculum without PS particles), indicating that the PS particles do not affect cell differentiation from planktonic to swarming phenotypes. Another important feature to note is that *B. subtilis* do not adhere to the surfaces of the inert colloidal particles.

D. PS colloidal particles do not hinder colony expansion

Figure 5(a) shows the colony patterns on the agar with PS particles (i.e., PS particle zone at the top half of the agar plate, particle density $C_p = 0.06$ particles μm^{-2}) and without

FIG. 5. (Color online) (a) Colony morphology in PS particle zone (top half) and non-PS particle zone (bottom half). (b) The distance from the colony front to the edge of inoculum in the center of the agar (X) for the colony in the non-PS particle zone ($\circ$) and PS particle zone ($\square$) at different times. The experiment for each condition was repeated three times.

PS particles (i.e., non-PS particle zone at the bottom half). Clearly, the colony in the PS particle zone grows normally and develops a similar pattern when compared to the non-PS particle zone, suggesting that colloidal particles do not hinder colony expansion.

Figure 5(b) shows the distances of the colony front from the edge of the inoculum in the center of the agar (X) at various times for both the colony in the PS particle ($0.06 \text{ particles } \mu\text{m}^{-2}$) and non-PS particle zones. It is observed that X does not vary significantly between the two different zones at shorter times (e.g., 120 and 150 min). Indeed, the colony expansion rates at 150 min are 2.7 and $2.9 \text{ } \mu\text{m s}^{-1}$ for the non-PS and PS particle zones, respectively. Interestingly, the colony expands at a slightly faster rate in the presence of colloidal particles at later times ($> 150 \text{ min}$).

For a swarming colony *without* PS particles, a layer of stalled bacteria is found at the edge of the colony as seen in Movie S1 [44]. As discussed by Darnton *et al.* [45], the expansion of swarming colonies is due to (i) surfactant pumped out to virgin agar by the jammed cells (facing inwards with flagella pointing outwards) and wetness at the edge, and (ii) an outward force arising from the collisions of motile cells (i.e., bacterial gas pressure) that pushes the layer of stalled cells outwards.

For a swarming colony in the PS particle zone, a layer of stalled bacteria is not formed because the colloidal particles break up the aggregation of the frontal cells (see Movie S7 [44]). Smaller packs and individual motile bacteria are seen at the colony front. We note from Movie S7 that the cells are moving *forward* relatively quickly. Apart from secreted surfactant and bacterial gas pressure (arising from the motion of smaller swarming rafts), other possible factors that may cause the cells to surge forward are more nutrients and oxygen in the virgin agar (i.e., chemotaxis) and the lack of resistance from the wall of stalled bacteria. Therefore, colony expansion and front propagation are not hindered even though swarm collectivity is perturbed by colloidal particles as discussed below.

E. PS colloidal particles disrupt collectivity and speed

The collective motion of the swarming bacteria is significantly perturbed when cells move from an area without surface PS particles (i.e., non-PS particle zone) into an area containing particles [i.e., PS particle zone, concentration of particle suspension used is 0.0625% (w/w)], as illustrated in Movie S8 [44]. The particle image velocimetry (PIV) image in Fig. 6(a) shows that in the non-PS particle zone (i.e., right of the green partition line), several meandering jets of collectively high velocities are seen [e.g., jet centered at $(x = 83 \text{ } \mu\text{m}, y = 39 \text{ } \mu\text{m})$]. On the other hand, there is a noticeable absence of rapidly moving large rafts in the PS particle zone (i.e., left of the green partition line). Apart from blocking the motion of swarming bacteria, PS particles reduce the amount of free space available for the cells to move. Large bacterial rafts entering the PS particle zone break up into smaller packs in order for the cells to maneuver across narrow spaces. Bacteria are forced to squeeze through the gaps between PS particles which leads to the observed diminished collectivity and speed.

FIG. 6. (Color online) (a) Instantaneous velocity field image of *B. subtilis* (Movie S8 [44]). The green line divides the PS particle zone (left of the dividing line) from the non-PS particle zone (right of the dividing line). (b) Spatial correlation function of instantaneous velocity for different PS particle densities: 0.06 (cyan, $\diamond$), 0.015 (blue, Δ), 0.0035 (green, $\circ$), and 0 (red, $\square$) particles μm^{-2} . The inset shows the corresponding L and V .

The number of colloidal particles immobilized on the agar surface affects both the collective behavior and speed of swarming bacteria. In this case, the concentration of the PS particle suspension used to prepare the sample is further diluted by $4\times$ and $16\times$. The resulting particle densities (C_p) on the agar surface are $0.06 \text{ particles } \mu\text{m}^{-2}$, $0.015 \text{ particles } \mu\text{m}^{-2}$, and $0.0035 \text{ particles } \mu\text{m}^{-2}$ for suspension concentrations of 0.0625% (w/w), 0.0156% (w/w), and 0.0039% (w/w), respectively (Fig. S4 [44]). Since the speed of healthy swarming bacteria increases with an increase in bacterial density D [45], a similar D ($\sim 0.07 \text{ cells } \mu\text{m}^{-2}$) is maintained throughout the experiment at various C_p . Figure 6(b) shows that when C_p increases, both the collectivity (L) and speed of swarming bacteria (V) decrease. The L (V) values for $C_p = 0.06, 0.015, 0.0035$, and $0 \text{ particles } \mu\text{m}^{-2}$ are $7.8 \text{ } \mu\text{m}$ ($11.7 \pm 0.4 \text{ } \mu\text{m s}^{-1}$), $12.9 \text{ } \mu\text{m}$ ($23.8 \pm 2.4 \text{ } \mu\text{m s}^{-1}$), $14.9 \text{ } \mu\text{m}$ ($34.1 \pm 2.9 \text{ } \mu\text{m s}^{-1}$), and $18.4 \text{ } \mu\text{m}$ ($42.7 \pm 6.9 \text{ } \mu\text{m s}^{-1}$), respectively. When the number of PS particles on the surface is reduced, the cells experience less hindrance to their motion, leading to smaller perturbation imposed on their collectivity and speed.

Computational modeling via a simplified self-propelled particle (SPP) model [12,29] is used to further quantify the role of colloidal particles and their densities in disrupting the collective motion of motile cells behind the colony edge. Therefore, only motile particles are considered (i.e., temporarily stalled cells at the colony edge are not taken into account in the simulation). Modeling the behavior of the colony front and its propagation requires a significantly more complex model and extensive modifications to the simplified SPP model must be made, which is beyond the scope of this study. For example, details such as extracellular fluid secretion, bacterial gas pressure, nutrient concentration and consumption, and models without explicit alignment rule must be taken into account in the simulation in order to properly address the behavior and propagation of the colony front [19,46].

Since the objective here is to understand the effects of stationary hurdles on the *collectivity* of motile particles, the speed of each moving particle (v) is assumed to remain invariant [29]. The direction of motion of particle i at time t is determined by the average motion of all its mobile neighboring

j particles located within an interaction active area of radius x [29]:

$$\theta_i(t + \Delta t) = \text{Arg} \left[\sum_{(i,j)} \beta e^{i\theta_j(t)} \right] + \eta \xi_i(t),$$

where $\eta \xi_i(t)$ is an intrinsic noise with intensity η and $\xi_i(t)$ is a δ -correlated white noise that is uniformly distributed between $-\pi$ and π [29]. The intrinsic noise is ascribed to the bacterium's intrinsic uncertainty in its motion after receiving an average "signal" from the neighboring j bacteria. The contribution of the alignment between any pair of i, j particles to the overall direction of motion is weighted by an alignment coefficient $\beta = \{1 + [(\vec{v}_i \cdot \vec{v}_j) / (|\vec{v}_i| |\vec{v}_j|)]\} / 2$, where $\beta = 1$ when the directions of both particles are perfectly parallel and in the same direction and $\beta = 0$ when they move in opposite directions [29]. Motile particles are reflected off the point of collision using a hard-core repulsion model that considers the incoming angle, and is implemented between all particles (i.e., motile and nonmotile). We have assumed that the intrinsic noise in the absence of colloidal particles is unchanged when particles are included. This is because the cells remain healthy in the presence of nontoxic PS particles and the intrinsic uncertainty in motion should not be affected by the presence of the particles. Instead, any changes in the collectivity and correlation length should be due to collision with and repulsion from the stationary hurdles. In the simulation, the density of moving particles is 0.07, $v = 0.1$, $\eta = 0.4$, and $x = 5$ [29]. The periodical boundary condition was applied, and the total number of time steps for each run is 15 000. Further details regarding the computational simulation are provided in the Experimental Section below.

The correlation length of the moving particles is drastically reduced from $L = 19.4$ in the absence of stationary particles to $L = 15.6$, 13.0, and 8.5 when the surface densities of immobile particles $C'_p = 0.0034$, 0.014, and 0.054 respectively, are used in the simulation (Fig. 7). The total free area available for motile particles to move is decreased significantly by 22% when $C'_p = 0.054$ as compared to 1.4% when $C'_p = 0.0034$. Furthermore, when hard-core repulsion between moving and stationary particles is considered, the average distance l between nearest neighbors of immobilized particles for which mobile particles can pass through is $l = 18.6$ (ca. 8–9 particles) for $C'_p = 0.0034$. This distance is reduced to $l = 4.7$ (ca. 1–2 particles) for $C'_p = 0.054$ (Fig. S5 [44]). Therefore, when C'_p is large (e.g., 0.054), the mobile particles have smaller amounts of unobstructed area to move. In addition, the swarming particles are forced to move through narrower gaps between densely packed stationary particles which break up large rafts into smaller ones. Therefore, the relationship between collectivity and particle density seen in the simulation study agrees with the experimental observation.

In terms of cluster size, it is noted that large clusters of mobile particles (size of ca. 20–30 particles per cluster) are seen in the absence of stationary particles (Fig. S6 [44]). However, a more dispersed distribution of moving particles and smaller clusters (size of ca. five to ten particles per cluster) is found in the presence of stationary particles (density = 0.054) (Fig. S6 [44]). This is in line with the experimentally observed cluster sizes: 10–30 cells per cluster in the absence of PS

FIG. 7. (Color online) The spatial correlation functions computed at the 15 000th time step in the absence of stationary particles (a) and presence of stationary particles with surface density = 0.0034 (b), 0.014 (c), and 0.054 (d).

particles and three to ten cells per cluster in the presence of PS particles.

F. PS colloidal particles impede antibiotic resistance

The inhibition zone size r of planktonic *B. subtilis* in the presence of PS particles ($C_p = 0.06$) are 8.2 ± 0.3 mm and 4.3 ± 0.2 mm for Tob and Van, respectively (Fig. S2 [44]), which are not significantly different from the values determined for planktonic cells in the absence of PS particles [Fig. 2(b)]. Therefore, colloidal particles do not affect the antibiotic distribution gradient on the agar nor do they alter the efficacies of the drugs on planktonic cells.

The effect of PS particles on the drug resistance properties of swarming bacteria is studied by placing an antibiotic disk at both ends of the agar plate equidistant from the point of inoculation (i.e., center of culture plate) (Figs. 1 and 8). Colloidal particles ($C_p = 0.06$) are immobilized at the top half of the agar surface while the bottom half is free from PS particles. Figure 8(d) shows the inhibition zones formed in both the PS particle zone (top half) and non-PS particle zone (bottom half) after the swarming colony has ceased expansion for Tob. In the presence of PS particles, the inhibition zone size is $r = 5.1 \pm 0.9$ mm which is significantly larger than $r = 1.3 \pm 0.6$ mm observed in the non-PS particle zone [Fig. 2(b)]. When the drug utilized is Van, $r = 4.1 \pm 0.7$ mm and 2.5 ± 0.3 mm for the PS particle zone and non-PS particle zone, respectively (Fig. S7(c) [44]). Similar qualitative behavior is observed when different drug concentrations are used (see Fig. S8 in the Supplemental Material [44] for Tob disk concentration of 150 $\mu\text{g}/\text{disk}$). This unambiguously demonstrates that for the same drug exposure time and concentration gradient, colloidal particles, at suitable concentrations, are capable of impeding drug resistance and colony expansion.

FIG. 8. (Color online) Colony morphology in PS particle zone (top half of each panel) and non-PS particle zone (bottom half of each panel) at different t_i [(a) $t_i = 150$ min, (b) $t_i = 210$ min, (c) $t_i = 240$ min, and (d) $t_i = 270$ min]. The agar is inoculated at the center (y). The Tob disks x are placed equidistant from y , and z is the rectangular area containing immobilized PS colloidal particles. The shortest distances between the colony edge and drug disk in the PS particle zone are 13.3, 6.2, 5.2, and 5.1 mm for (a–d), respectively.

On the other hand, when C_p is reduced to 0.015 and 0.0035, the PS particle zones r are 2.0 ± 0.1 and 1.4 ± 0.4 mm, respectively, for Tob (Fig. 9). For low C_p (e.g., 0.0035), the bacteria persist to display strong collectivity and the relatively intact group dynamics allows the cells to remain resilient against drugs, whereas for large C_p (e.g., 0.06), the disrupted cells quickly lose their strong multicellular group dynamics and are efficiently damaged by Tob. For the rest of this section, we will focus on Tob, noting that similar behavior is also observed when the antibiotic is replaced with Van.

A time-lapse microscopy study of the swarming behavior of the bacteria in the PS particle zone at different times is presented here. At $t_i = 150$ min, the advancing front has just entered the PS particle zone [$C_p = 0.06$, Fig. 8(a)] where a distinct edge of jammed bacteria is not observed (Movie S7 and Fig. S9 [44]). The colony has not encountered Tob; however, small packs and individual bacteria are seen to surge forward in a haphazard manner through the spaces between colloidal particles. When exposed to Tob between $t_i = 210$ min [Fig. 8(b)] and $t_i = 270$ min [Fig. 8(d)], the shortest distance from the colony edge to the drug disk is decreased by 16% and 73% for the PS particle zone and non-PS particle zone, respectively. Therefore, when the colony moves deeper into the PS particle zone towards the drug disk, the swarm spreads at a slower rate due to more efficient cell damage by Tob.

Particle tracking is performed to demonstrate the extent of transportation and exchange of cells in the monolayer. In this case, only discernible cells were tracked manually. Figure 10 shows typical trajectories of motion of single bacteria close to the colony front and initially away from the edges of the observation window (a) without colloidal particles and drugs, (b) with colloidal particles but in the absence of drugs, and (c) without colloidal particles but exposed to drugs. In the

FIG. 9. (Color online) (a) Colony morphology of swarming *B. subtilis* with different PS particle densities C_p : (i) 0.0035, (ii) 0.015, and (iii) 0.06 particles μm^{-2} . x is the Tob disk and y is the inoculum. Arrows mark the colony fronts in the PS particle zones. (b) The inhibition zone sizes in the absence and presence of different C_p .

absence of colloidal particles and drugs, there exist bacteria that are capable of moving long distances away from the colony edge and toward the colony interior [Fig. 10(a)]. In particular, bacteria 1, 2, and 3 in Fig. 10(a) move out of the observation window within the time studied. This is because the bacteria are transported more efficiently by large rafts in the absence of colloidal particles. Bacteria initially outside the window can also move inside the window to ensure that the cell density is kept relatively constant [e.g., see bacterium 6 in Fig. 10(a)]. We find that even when the bacteria are exposed to Tob (at 210 min), there exist bacteria close to the front that are capable of moving away from the edge and toward the colony interior [Fig. 10(c)]. Bacteria 1 and 4 are also seen to move out of the observation window. On the other hand, when colloidal particles are present, there are no obvious bacteria close to the colony front (and initially close to the center of the window) that are capable of moving long distances and out of the window within the observation time even when the bacteria are not exposed to Tob [Fig. 10(b)]. This is because the swarming motion is disrupted by the stationary hurdles leading to the inability of the cells to move over long distances.

We selected ten bacteria each from Movie S1 and S2 [44] that move efficiently away from the front to the colony

FIG. 10. (Color online) (a) Trajectories of motion of five bacteria close to the colony front on agar without both PS particles and drugs. S1, S2, S3, S4, S5 are the starting points of the five bacteria. The trajectory of motion of bacterium 6 is represented by the dotted line and moves from outside the observation window into the window and close to the colony front. (b) Trajectories of motion of five bacteria close to the colony front on agar with PS particles but in the absence of drugs. S1, S2, S3, S4, S5 are the starting points of the five bacteria. (c) Trajectories of motion of five bacteria close to the colony front on agar without PS particles but exposed to drugs. S1, S2, S3, S4, S5 are the starting points of the five bacteria. The trajectories were calculated using Movie S1 for (a), Movie S7 for (b), and Movie S2 for (c); the observation time is from 13.3 to 15 s.

interior and calculated their mean square displacement (MSD) to illustrate the effects of stationary hurdles. The MSD of bacterium i is calculated from

$$\text{MSD}(\tau) = \langle [R(t) - R(t + \tau)]^2 \rangle_t \quad t \in [T_s, T_e - \tau],$$

where τ is the lag time, t is a time between the starting T_s and ending T_e times, and $R(t)$ and $R(t + \tau)$ are the positions of the bacterium at times t and $t + \tau$, respectively. The root mean square displacement (RMSD) for $\tau = 1.0$ s is used. In this case, the average RMSDs for ten typical bacteria that move long distances in the absence of colloidal particles in Movies S1 and S2 [44] are 39 and 29 μm , respectively. On the other hand, the average RMSD for ten typical bacteria in the presence of colloidal particles (Movie S7 [44]) is 10 μm . Clearly, the RMSD without colloidal particles is larger than the RMSD with colloidal particles. In addition, 100 randomly selected particles from the computational simulation study were also examined, and the average RMSDs are 4.1 and 1.9 (lag time of 100 time steps) in the absence and presence of stationary particles, respectively. The average RMSD is 2.2 times larger in the absence of hurdles, which is in alignment with the experimental observations.

Based on the above observations, we propose a plausible reason to rationalize the decrease in resistance when colloidal particles are utilized. Motile bacteria close to the swarm edge are transported away from the front (i.e., relatively high drug concentration areas) toward the colony interior (i.e., lower drug concentration areas) and vice versa. Furthermore, close to the border between the primary and secondary fronts (ca. 8–10 mm away from the edge), a constant exchange between cells in the drug-exposed monolayer with cells in the more protected multilayered colony takes place (Movie S9 [44]). It is also observed that motile bacteria moving into the advancing front squeeze out stalled bacteria at the edge (Movie S10 and Fig. S10 [44]). The latter are subsequently recruited into neighboring swarming rafts, hence allowing them to resume their collective motion. Bacteria in the monolayer are therefore not continuously confined and exposed to areas of lethal Tob concentrations, allowing the colony to resist severe drug damage and to continue spreading [35].

In the absence of Tob, the cell density of bacteria close to the colony front remains relatively unchanged in the absence and presence of PS particles (e.g., ~ 0.05 cells μm^{-2} at $t_i = 150$ min). In the presence of Tob, the cell density is 0.09 cell μm^{-2} (at $t_i = 210$ min) in the absence of PS particles. When compared to healthy *B. subtilis*, the cell density of Tob-affected bacteria increases by about a factor of 2 due to a relatively slower colony expansion rate across the agar, as discussed above. This in turn causes the colony front to expand more slowly (rate = $0.4 \mu\text{m s}^{-1}$ as compared to $2.7 \mu\text{m s}^{-1}$ for a healthy colony). Therefore, the supply of cells from interior regions of the colony to areas adjacent to the relatively slower-moving swarm edge gives rise to the ca. $2\times$ increase in cell density when compared to the healthy colony.

In the case when PS particles are present, the sluggish behavior and lack of strong group dynamics (i.e., reduced collectivity and speed) of the frontal bacteria prevent an efficient relocation of frontal cells away from areas of lethal drug concentrations (Movie S7 [44] and Fig. 10), resulting in the cells being exposed to the drugs for longer duration and hence becoming less resilient against Tob. Continuous exposure of the colony eventually leads to sufficient damages on the bacteria so that a thin (ca. two to three cell lengths) but densely packed monolayer of cells (~ 0.17 cells μm^{-2}) with negligible motion followed by a multilayer of bacteria is observed (e.g., Movie S11 and Fig. S9(b) for $t_i = 240$ min [44]). The frontal cells rapidly suffer damages when exposed to Tob which significantly impedes colony expansion and cell exchange, leading to the buildup of sluggish and immotile cells close to the front and the larger inhibition ring size. This underscores the importance of collectivity and swarming dynamics that are sustained for a longer period of time in bacterial colony without colloidal particles.

III. EXPERIMENTAL SECTION

A. Bacterial strain and chemicals

Wild type *B. subtilis* 3610 strain (ATCC 6051) was purchased from ATCC. Vancomycin and tobramycin were purchased from Sigma-Aldrich and used as received. Polystyrene

(PS) beads, 10% (w/w) (1.1 μm in diameter, Sigma-Aldrich) suspended in water were diluted to the desired concentration using filter-sterile water.

B. Growth conditions

B. subtilis was cultured in LB broth (10 g/liter tryptone, 5 g/liter yeast extract, and 5 g/liter NaCl in water) at 37 °C until an OD_{600} of ~ 0.9 was achieved. LB broth solidified with agar (0.5% for swarming cells and 3% for planktonic cells) was then prepared in a 90-mm-diameter petri dish and dried for 1 h. To obtain swarming cells, 2 μl of the bacterial culture was inoculated at the center of the agar (0.5%), dried for 1 h, and then cultured at 37 °C (80% humidity). For experiments involving planktonic cells, 50 μl of diluted bacteria culture was spread evenly on the surface of hard agar (3%), dried for 1 h, and cultured at 37 °C (80% humidity) for 2 h.

Polystyrene colloidal particles were deposited by evenly spreading 50 μl of colloidal suspension [0.0625% (w/w), 0.0156% (w/w), and 0.0039% (w/w)] over a $2 \times 4 \text{ cm}^2$ rectangular area on the agar surface. The plate was subsequently dried until the PS colloidal particles remained immobilized on the agar surface.

C. Antimicrobial assays

The disk diffusion method was used to assess bacterial sensitivity to antibiotics (i.e., Tob or Van). The antibiotic disks were made from circular filter paper (6 mm diameter) and sterilized under a UV-ozone lamp for 30 min. Then 5 μl of freshly prepared 150 mg ml^{-1} tobramycin or 6 mg ml^{-1} vancomycin solution was added onto the paper disks and dried. The final drug concentrations were 750 μg tobramycin and 30 μg vancomycin per disk ($\mu\text{g}/\text{disk}$). One to 1.5 h after inoculating the center of the agar with bacteria, an antibiotic disk was placed 25 mm from the inoculum [Fig. 2(a)]. The inhibition zone size (r) was defined as the shortest distance from the edge of the antibiotic disk to the leading edge front of the bacterial colony after spreading has stopped, and was measured using a vernier caliper.

The inhibition zones for planktonic cells were determined following the method described by Butler and co-workers [35]. Briefly, 10 ml of bacterial culture was poured onto an agar (3%) plate and allowed to settle for 1 min. The liquid culture was drained and the agar dried before being incubated at 37 °C for 2.5 h to allow the surface bacteria density to reach ca. 0.05–0.06 cells per μm^{-2} (close to that obtained for swarming bacteria). Subsequently, a drug disk was placed at the center of the agar. The inhibition ring was observed after 4 h of incubation at 37 °C. In the case of planktonic cells in the presence of PS particles, the colloidal particles [400 μl of 0.0625% (w/w)] were first immobilized on the agar surface before the bacteria were inoculated.

LIVE/DEAD[®] BacLight[®] Bacterial Viability Kits staining was performed by slicing out the agar carrying the secondary front and placing it upside down on a glass cover slip containing 10 μl of the live and dead stain (BacLight, Invitrogen), and incubating it for 15 min. The fluorescent image was collected using a fluorescent microscope (Nikon Eclipse TE2000-E).

A viability assay for planktonic cells with and without PS particles was conducted. Overnight the bacterial culture was diluted 40 times with LB media with [0.0625% (w/w)] and without PS particles. The colony forming unit (CFU/ml) data were collected from the bacterial culture at 0, 1, 2, 3, and 4 h after dilution, and evenly applied on the surface of a LB-agar plate with an L-shaped spreader. The plate was incubated overnight, and the colony number was counted the next day.

D. Microscopy study

The microscopy setup consists of an inverted microscope (IX 71, Olympus) coupled to a highly sensitive CCD camera (CascadeII 512B, Photometrics). A 30 W halogen bulb was used to observe bacteria motion. The transmitted light from the sample was passed through an air objective lens (20 $\times$, NA = 0.40, Olympus) and magnified using a 3.3 $\times$ camera lens before detection. The dimension of an image frame was measured to be $120 \times 120 \mu\text{m}^{-2}$ (512×512 pixels) using a stage micrometer, and the rate of the CCD camera is 30 frames per second. Throughout the microscopy measurements, the sample was maintained at 30 °C–35 °C via a heating plate. The diffraction of light from colloidal particles was subtracted.

Bacterial motion was analyzed using particle image velocimetry (PIV) (PIVLAB1.31 software) with each moving bacterium acting as a tracer. The size of an interrogation window was $2.8 \times 2.8 \mu\text{m}^{-2}$ (12×12 pixels) and the separation between the centers of any two overlapping interrogation windows was 6 pixels (50% overlap between windows). From the recorded consecutive microscopy images, the velocity flow field of *B. subtilis* near the edge of a growing colony was constructed, and the average speed and spatial correlation function calculated from the velocity vectors. The average speed (V) for a particular area of interest is calculated from $V = \sum_i |\vec{v}_i|/n$, where $\vec{v}_i$ and n are the velocity vector of the i th interrogation window and the total number of interrogation windows, respectively. The spatial correlation function of instantaneous velocity (C_v) is computed:

$$C_v = \langle \vec{v}_i \cdot \vec{v}_j \rangle_{ij} / \langle \vec{v}_i \cdot \vec{v}_i \rangle_i,$$

where $\vec{v}_i$ and $\vec{v}_j$ are the velocity vectors of two interrogation windows with a center-to-center distance r , and $\langle \cdots \rangle_{ij}$ is the ensemble average over all possible i, j pairs. The dark images of colloidal particles captured in the movie were replaced by background intensity during the PIV analysis and are excluded when computing the average speed.

Each experiment was repeated at least three times. For the inhibition zone size experiment, we repeated the experiment at least five times. All experiments were highly reproducible (e.g., the error for the observed correlation lengths was $< \pm 0.5$).

E. Computational simulation

The self-propelled particle simulation is carried out in a two-dimensional square of size 120×120 . For the sake of simplicity and without losing generality, both motile (i.e., bacteria) and stationary (i.e., colloidal particles) particles are assumed to be circular with diameter r [29]. The moving particles are homogeneously distributed throughout the square.

To better represent the surface density of PS colloidal particles observed in the experiments, the square is further divided into (28×28) , (14×14) , and (7×7) smaller squares corresponding to $n = 784$, 196, and 49 stationary particles, respectively. The surface densities of the stationary particles $C'_p = 0.054$, 0.014, and 0.0034 correspond to $n = 784$, 196, and 49, respectively. A stationary particle is assigned to a random position in each of the smaller squares. A picture of the distribution of the different numbers of stationary particles is given in Fig. S5 [44]. We defined the nearest immobilized neighbors for each stationary particle to be the four particles located at its immediate left, right, top, and bottom. The static particles do not move during the simulation and are not considered in the computation of C_v and V . At any given time t , each particle is defined by its location and direction of motion specified by angle $\theta_i(t)$. At each time step $\Delta t (=1)$, a moving particle i is displaced by a magnitude of $v = 0.1$ (velocity) in the direction of $\theta_i(t + \Delta t)$.

Volume exclusion is considered by introducing a hard-core repulsion which is defined by a circular zone of radius $r = 2$, equivalent to the diameter of the particle, and centered at particle i . The simple hard-core repulsion model used here takes into account the “incoming” angle, which better describes the actual pathways of the particles after collision. Two orthogonal axes are defined—a parallel axis (y) that joins the centers of particles i and j , and a vertical axis (x) that corresponds to the common tangent at the point of contact between the particles (see Fig. S11 [44]). The incoming velocity of the mobile particle i is resolved into a vertical component (V_v) along x and a parallel component (V_p) along y . The vertical component of the outgoing velocity after repulsion remains as V_v . The parallel component of the outgoing velocity V'_p of particle i moves in the opposite direction of V_p when particle j is stationary. When particle j is mobile, V'_p is computed by $V'_p = V_p \text{Sgn}[V_p \cdot \mathbf{R}_{ji}]$ where $\mathbf{R}_{ji}$ is the vector from j to i along the line joining the two centers and $\text{Sgn}[V_p \cdot \mathbf{R}_{ji}]$ is the sign of $(V_p \cdot \mathbf{R}_{ji})$. The latter ensures that the particles do not experience back-to-back collisions with each other. The outgoing velocity is constructed from V_v and V'_p as illustrated in the figure below. Therefore, the incident angle and reflected angle are the same

with respect to the y axis. When particle i collides with N multiple particles simultaneously, $V'_p = V_p \text{Sgn}[V_p \cdot \mathbf{R}'_{ji}]$, where $\mathbf{R}'_{ji} = \sum_{j=1}^N \hat{\mathbf{R}}_{ji}$ and $\hat{\mathbf{R}}_{ji}$ is the normalized $\mathbf{R}_{ji}$ vector. At least three simulation runs were repeated for each condition and the results were reproducible (i.e., error for the calculated correlation length L was $< \pm 1.0$).

IV. CONCLUSION

The antibiotics employed in this study possess different mechanisms of action. Tobramycin is an aminoglycoside antibiotic that inhibits protein biosynthesis by irreversibly binding to a bacterial ribosome subunit. On the other hand, vancomycin is a glycopeptide antibiotic whose mode of action involves the inhibition of cell growth by selectively binding to the -D-Ala-D-Ala termini of the bacterial cell wall peptidoglycan. The results presented here are in agreement with a previous study that has shown that swarming *B. subtilis* are resistant against multiple antibiotics [36].

Despite a recent study that has looked at the effects of sublethal drug concentrations on the collective behavior of *B. subtilis* [39], there remains little insight into the effects of lethal drug dosages on the physical properties of swarming dynamics. An important outcome from our study is the decrease in both Tob and Van resistance when the swarming behavior of the bacteria is perturbed by PS particles. The surface modification of agar is easy to achieve, in contrast to more complex and expensive methods such as micropatterning. When the raft size and speed are significantly reduced, the bacteria become highly sensitive to the drugs regardless of the mechanism of action imposed by the antibiotics used. Another effective way of perturbing the collective dynamics of swarming cells is photodynamic therapy [29]. In this case, light irradiation of dye-stained bacteria produces toxic singlet oxygen capable of reducing both the swarm collectivity and speed.

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