Real-time microscopic study and modeling of the disruption of bacterial swarming motion

Lu, Shengtao

2016

Lu, S. (2015). Real-time microscopic study and modeling of the disruption of bacterial swarming motion. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/65868>

<https://doi.org/10.32657/10356/65868>

Real-time Microscopic Study and Modeling of the Disruption of Bacterial Swarming Motion

SHENGTAO LU

School of Physical & Mathematical Sciences

A thesis submitted to the Nanyang Technological University

in fulfillment of the requirement for the degree of

Doctor of Philosophy in Chemistry

2015

ACKNOWLEDGEMENT

Firstly, I would like to express the most sincere thanks to my supervisor, Prof. Edwin Yeow, for his patient guidance and selfless help. Throughout the past four years of my graduate studies, I have received countless advice and encouragement from Prof. Yeow. Without his comprehensive instruction, this thesis would never have been completed.

I am grateful to all faculty and staff in the Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, for offering me the opportunity to study in such an excellent environment.

I would like to thank Prof. Bengang Xing for his help in biological experimental designs, Dr. Fang Liu for his advices on bio-techniques and Ms. Hwee Leng Tan (Analytical Technologies Pte. Ltd.) and Mr. Wee Lee Tok (EINST Technology Pte. Ltd.) for their technical support.

I am grateful to my group members, Dr. Xiangyang Wu, Dr. Wuguo Bi, Dr. Jefri Sanusi Teguh, Ms. Wang Jin, Ms. Shue Mei Ng and Mr. Cao Hien Le, for their contributions to build a friendly and helpful working atmosphere. I would like to thank all my friends for their friendship as well.

Last but not least, I would like to express my gratitude to my parents for raising me up and continuously supporting me. I would never have achieved all that I have today without their support and understanding throughout these years.

TABLE OF CONTENTS

ACKNOWLEDGEMENT	I
TABLE OF CONTENTS	II
LIST OF ABBREVIATIONS	VI
SUMMARY	1
Chapter 1	3
1.1 Collective motion.....	4
1.1.1 Statistical evaluations of collectivity	5
1.1.2 Collective motion in nature.....	8
1.1.3 Modeling of collective motion systems	9
1.2 Swarming	17
1.2.1 Conditions needed for swarming	17
1.2.2 Swarming behaviors.....	26
1.2.3 Swarming induced antimicrobial resistance	27
1.2.4 Methods of studying swarming bacteria	34
1.3 Photodynamic therapy (PDT) as a treatment of bacterial infections	37
1.3.1 History.....	37
1.3.2 Principles of PDT.....	37
1.3.3 Applying PDT as an antibacterial strategy.....	40
1.4 Aim.....	44
1.5 Bibliography	45
Chapter 2.....	58

2.1 Abstract	59
2.2 Introduction.....	60
2.3 Experimental	61
2.3.1 Culture Conditions and Preparation of Bacteria	61
2.3.2 Microscopy Setup and Data Analysis	62
2.3.3 Survival Assay for Swarming <i>B. subtilis</i> under Photodynamic Action	63
2.3.4 Photodynamic inhibition mechanism study	64
2.3.5 Modeling	65
2.4 Results and discussion	67
2.4.1 Bacterial uptake of PpIX.....	67
2.4.2 Photodynamic inhibition activity disrupts bacterial swarming.....	69
2.4.3 Recovery of collective motion after photodynamic inhibition	76
2.4.4 Simulation	79
2.4.5 Mechanism study of photodynamic inhibition	84
2.5 Conclusion	89
2.6 Movie captions.....	90
2.7 Bibliography	91
Chapter 3	96
3.1 Abstract	97
3.2 Introduction.....	98
3.3 Experimental	101
3.3.1 Bacterial Strain and Chemicals	101

3.3.2 Growth Conditions.....	101
3.3.3 Antimicrobial Assays	102
3.3.4 Microscopy Study	103
3.3.5 Computational Simulation	105
3.4 Results and discussion	109
3.4.1 Swarming motility protect single-layered bacteria in lethal concentrations of drugs	109
3.4.2 Polystyrene particles on the surface disrupt swarming motion	118
3.4.3 Simulation	126
3.4.4 Disruption of swarming motion leads to reduced antibiotic resistance for swarming colony	128
3.4.5 Effective cell translocation and exchange by collective raft motion increases antibiotic resistance in single-layered bacterial colony.....	134
3.5 Conclusion	139
3.6 Movie captions.....	140
3.7 Bibliography	142
Chapter 4	148
4.1 Abstract	149
4.2 Introduction.....	150
4.3 Experimental	152
4.3.1 Model description	152
4.3.2 Characterization of the PCH-SPP model	154

4.3.3 2-D information propagation experiment	155
4.3.4 2-D collective turning simulation	157
4.4 Results and discussion	157
4.4.1 Characterization of PCH-SPP	157
4.4.2 Information propagation	158
4.4.3 Influence of weightage parameter ε on C_s	162
4.4.4 Simulating collective turning in 2-D.....	163
4.5 Conclusion	166
4.6 Movie captions.....	167
4.7 Bibliography	168
4.8 Perspective	171

LIST OF ABBREVIATIONS

2-D	2-dimensional
CCW	Counterclockwise
CFU	Colony forming units
Cmf	Colony migration factor
CW	Clockwise
Cys	Cysteine
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
HOMO	Highest occupied molecular orbital
LB media	Luria-Bertani media
LPS	Lipopolysaccharides
LUMO	Lowest unoccupied molecular orbital
Lys	Lysine
MIC	Minimum inhibitory concentration
PCH-SPP	Position-correlated hierarchical self-propelled particle
PDT	Photodynamic therapy
PIV	Particle imaging velocimetry
PS	polystyrene
ROS	Reactive oxygen species
SPP	Self-propelled particle
Tob	Tobramycin
UV	Ultraviolet
Van	Vancomycin
VM	Vicsek model

SUMMARY

The collective motion of *Bacillus subtilis* in the presence of a photosensitizer is disrupted by reactive oxygen species when exposed to light of sufficient dosages and is partially recovered when light irradiation is suspended. The transition from a highly collective to a more random motion is modeled using an improved self-propelled model with alignment rule. The increment in noise level describes the enhanced uncertainty in the motion of swarming bacteria under stress as observed experimentally.

Monolayer of swarming *Bacillus subtilis* on semi-solid agar display elevated resistance against antibiotics due to their collective behavior and motility. The drug resistance is impeded when the collective motion of bacteria is judiciously disrupted using non-toxic polystyrene (PS) colloidal particles immobilized on the agar surface. The colloidal particles block and hinder the motion of the cells, causing cohesive rafts of bacteria to lose their collectivity and speed. In this case, the lack of strong group dynamics results in the bacteria becoming vulnerable to antibiotics. The negative correlation between the degree of collectivity and PS particle density is examined using an improved self-propelled model that takes in to account inter-particle alignment and hard-core repulsion.

Position-correlated hierarchy is introduced by adding a weightage parameter to Vicsek model (VM) to simulate collective motion systems. The new position-correlated hierarchical self-propelled particle (PCH-SPP) model displays a

rapid, linear-styled transfer of motion information during group turning: a phenomenon not observed in simulations by the VM. With the PCH-SPP model, the collective turning of bird flocks is reproduced by simulation. The model overcomes the inability of VM in simulating collective turning phenomenon by enhancing the propagation of information without having significant difference in the steady-state phase behavior of the VM simulations.

Chapter 1

General Introduction

1.1 Collective motion

For a system consisting of many moving individuals, when each individual's motion is influenced by other individuals so that the system shows coherency in motion, the motion is called collective motion. Unlike the manner in a random system, where all individuals move on their own, in collective motion system, individuals move in an ordered manner and interactions between individuals are present [1-3].

FIG. 1.1 Images illustrating collective motions in nature: (a) bird flock; (b) fish school; (c) locust swarm; (d) surface swarming bacteria. (a), (b), and (c) are taken from the internet.

Collective motion is widely observed in the biology world such as bird flocks or swarming bacterial colonies *etc.* (Fig. 1.1) [1-16]. Group motion in biology is achieved by individuals sharing and receiving signals such as vision or sound. The transport of information signal is carried out by interactions between neighboring individuals (localized interaction) [15]. One important feature that distinguishes the interaction in biological system from collision in classical statistical physics is that the former does not preserve momentum (*i.e.* the total momentum of interacting individuals does not have to be the same before and after interaction) [2]. Thus an ordered and non-equilibrium system could emerge (if the initial state is chaotic) after sufficient interactions throughout the system [1-4, 17].

1.1.1 Statistical evaluations of collectivity

Ordered motion shows coherency in direction of motion. Depending on the level of order, the group shows different organization patterns. This resembles equilibrium state phase transition behavior in physics [17]. Thus, some of the physical parameters could be adapted to evaluate the collective properties of group motion.

1.1.1.1 Order parameter

Group motion coherency can be evaluated by the overall alignment of each individual's direction of motion. The alignment is calculated using the instantaneous

order parameter $\varphi(t)$ [17-19]:

$$\varphi(t) = \frac{1}{N} \left| \sum_{j=1}^N e^{i\theta_j(t)} \right| \quad (1)$$

where N is the number of particles and j denotes each particle in the system. Eq. 1 defines $\varphi(t)$ as the absolute value of average normalized velocity at time t . The order parameter indicates the degree of symmetry, which is a characteristic property of a phase. In perfectly aligned systems (*i.e.* highly symmetric phase), where all particles move in exactly the same direction, $\varphi = 1$, while in totally chaotic systems (*i.e.* asymmetric phase), all particles move in random directions ($\varphi = 0$).

During the assembly of group motion, order-disorder transition occurs. When order parameter changes discontinuously, the transition is a first-order transition. When the order parameter changes continuously, the transition is a second-order transition.

1.1.1.2 Correlation function

For some collective motions, the local order parameter of part of the system is very high. However, the overall order parameter is low. For example, in a vortex motion, the streams of individuals within part of the vortex have very high motion coherency. But the overall order parameter is very low as the velocities of individuals in the circular motion cancels out each other in the calculation. In such cases, the correlation function could be a very useful indicator of local coherency [17, 20-22].

The correlation function is used to compare similarity of two sets of data. Several

functions are frequently used in collective motion evaluations:

The pair correlation function:

$$C_p(|\vec{r}|) = \frac{1}{4\pi|\vec{r}|^2\rho} \left\langle \sum_{j \neq i} \delta(|\vec{r}| - |\vec{r}_i - \vec{r}_j|) \right\rangle_i \quad (2)$$

where ρ denotes the particle number density, $|\vec{r}|$ is the distance, $\vec{r}_i$ and $\vec{r}_j$ are the positions of particle i and j respectively, and δ is the Dirac function. The pair correlation function is used to study the local spatial ordering in the distribution of particles [21].

The spatial velocity correlation function:

$$C_v(|\vec{r}|) = \frac{\langle \vec{v}_i \cdot \vec{v}_j \cdot \delta(|\vec{r}| - |\vec{r}_i - \vec{r}_j|) \rangle_{i,j}}{\langle \vec{v}_i \cdot \vec{v}_i \rangle_i} \quad (3)$$

Here $\vec{v}_i$ and $\vec{v}_j$ represent the velocity of particles i and j respectively, and $|\vec{r}|$ is the separation between i and j (controlled by the Dirac function $\delta(|\vec{r}| - |\vec{r}_i - \vec{r}_j|)$). This function is used to study the average similarity between the velocity of any pair of particles i and j separated by the distance of $|\vec{r}|$. This correlation function is very useful in evaluating local order of particle velocity. Short range order usually shows a decay of C_v as $|\vec{r}|$ increases [20-23].

Temporal velocity autocorrelation function:

$$C(\tau) = \frac{\langle \vec{v}(t) \cdot \vec{v}(t + \tau) \rangle - \langle \vec{v}(t) \rangle^2}{\langle \vec{v}(t)^2 \rangle - \langle \vec{v}(t) \rangle^2} \quad (4)$$

This function is used to study the velocity autocorrelation of one particle or one

position (in particle image velocimetry study). It shows the degree of similarity between the velocities at time t and time $t + \tau$. This provides information on how long in time the local order persists. For example, when studying clustered particle motions, the larger the cluster size the longer the time it needs to have significant velocity changes at a certain position [24].

1.1.2 Collective motion in nature

In the biological world, there is a large number of examples of collective motion. Many species develop into communities that show social behavior: individuals move and acting in a highly coherent manner. It is commonly assumed that the benefit of staying together and maintaining motion coherency are to improve group decision making, facilitate efficient hunting or seeking of resources, and enhance defense or evasion in the presence of predators [1, 2, 13, 15, 25-28]. The form of collective motion is highly diverse: from large groups of migrating mammals to temporary clusters formed in bacterial motion. Different unique collective patterns can be observed in each of these motions. However, it is still possible to find some commonalities in these motion patterns based on numerous observations. For example, individuals tend to align their movements with other individuals close to them in order to maintain motion coherency. The assumption that such localized alignment effort eventually cause large scale system motion collectivity is the foundation of many mathematical models used to describe collective motion [4, 17, 29, 30]. Some

of the most commonly studied collective motion systems include flying bird flocks [13, 31-35], swimming fish schools [12, 36-39], migrating insects [9, 10, 40, 41], migrating cells [8, 16, 42-44] and bacteria colonies [4, 5, 7, 19-23, 28, 45-59].

1.1.3 Modeling of collective motion systems

In many collective motion systems, the individuals can be viewed as identical particles that propel by themselves. Models that use particles that maintain motional speed to represent actual individuals are called self-propelled particles (SPP) based models [2, 17, 18]. In 1982, Aoki carried out a simulation that introduced certain rules that are now used in modern SPP models [60]. In his work to simulate collective motion of fish, three rules were used to achieve systematic motion coherence: i) when getting too close, particles “repel” each other to avoid collision; ii) particle adopts a parallel orientation to its neighboring particles; iii) when particle gets too far away from others it move towards the other particles. These rules do not include a leader, and in the simulation each particle does not have information of systematic orientation. This work shows that localized interaction with alignment rule alone is sufficient to reproduce collective motion.

1.1.3.1 Basic SPP model: the Vicsek model (VM)

In 1995 Vicsek *et al.* introduced statistical physics into the modeling of collective motion [17]. This "minimal model" is referred to as the Vicsek model (VM). VM is one of the most widely used SPP models so far.

In VM, particles undergo stepwise movement:

$$R_i(t+1) = R_i(t) + v_i(t) \quad (5)$$

where $R_i(t)$ and $v_i(t)$ are the position and velocity of particle i at step t respectively. All particles are moving at the same speed v_0 . Each particle i 's direction of motion in the next time step is determined by the average direction of motion of its neighboring particles:

$$\theta_i(t+1) = \text{Arg} \left[\sum_{\langle i,j \rangle} e^{i\theta_j(t)} \right] + \text{noise} \quad (6)$$

where $\theta_i(t)$ is particle i 's angle of motion at step t , and $v_i(t)$ can be presented as the vector: $(v_0 \cos(\theta_i(t)), v_0 \sin(\theta_i(t)))$. The interaction radius x defines the neighboring area of particle i (*i.e.* any particle j that falls within the circle centered at R_i with the radius x is considered as i 's neighboring particle and included in the calculation in Eq (6)). This localized alignment rule leads to system-wide coherent motion at high particle density and low noise.

In this alignment rule, the other parameter that controls the system coherency is the noise. There are two ways in which the noise can be applied.

In the original work by Vicsek *et al.*, the noise is applied after the calculation of the average direction of motion of all neighboring particles, which is known as the intrinsic noise:

$$\theta_i(t+1) = \text{Arg} \left[\sum_{\langle i,j \rangle} e^{i\theta_j(t)} \right] + \eta \xi_i(t) \quad (7)$$

The intrinsic noise $\eta\xi_i(t)$ could be interpreted as the uncertainty of motion after a particle receives velocity information from its neighboring particles.

In 2004 Gréoire *et al.* introduced a new way of applying noise to the alignment rule [18]. The noise is applied to the influence of every neighbor of particle i before it is averaged with others. This noise is referred to as the extrinsic noise:

$$\theta_i(t+1) = \text{Arg} \left[\sum_{\langle i,j \rangle} \left(e^{i\theta_j(t)} + \eta e^{i\xi_j(t)} \right) \right] \quad (8)$$

The extrinsic noise represents the uncertainty in the transfer of velocity information from each neighboring particle j to i .

In both Eq.(7) and Eq.(8), ξ is a δ -correlated white noise uniformly dispersed on $[-\pi, \pi]$, and η controls the intensity of noise. The larger η is, the more chaotic the whole system becomes. For example when the noise is maximum (*i.e.* $\eta = 1$), all particles will perform totally random walk. When the noise is absent (*i.e.* $\eta = 0$), the whole system (all particles) will have identical velocity if given enough time for the system to reach a steady-state condition.

VM successfully applies the idea from statistical physics into the simulation of collective motion. VM simulations have shown that collectivity changes with density and noise. The change of collectivity resembles the pattern of phase transition. It is worth mention that changing the intrinsic noise will cause continuous change in the order parameter. This indicates a second order phase transition. When extrinsic noise is applied, the order parameter changes discontinuously, which indicates first order

phase transition [18, 19] (Fig. 1.2).

FIG. 1.2 The order parameter change in a 2-D system with intrinsic noise (a) and extrinsic noise (b) applied separately. The system with intrinsic noise shows continuous change in order parameter (second order phase transition). The system with extrinsic noise show discontinuous change in the order parameter (first order phase transition). Other conditions of (a) and (b) are the same: 1000 particles were simulated in a 120×120 square with periodical boundary. The interaction radius is 5.

1.1.3.2 Variations of SPP model

Based on the basic Vicsek model, a series of variations of other SPP models are created. The rules mentioned above are adapted to fit into specific systems and conditions. Some notable adaptations are given as follow:

i) Adding repulsion/attraction between particles

In 2003 Grégoire *et al.* constructed a model based on the VM that incorporates the consideration of attractive/repulsive forces between particles [61]:

$$\theta_i(t+1) = \text{Arg} \left[\alpha \sum_{(i,j)} \hat{v}_j(t) + \beta \sum_{(i,j)} \vec{f}_{ij} \right] + \eta \xi_i(t) \quad (9)$$

Here α and β control the weight of two components (*i.e.* relative importance of the two). $\vec{f}_{ij}$ is a vector used to represent the repulsive/attractive forces:

$$\vec{f}_{ij} = \vec{e}_{ij} \begin{cases} -\infty & \text{if } r_{ij} > r_c, \\ \frac{1}{4} \frac{r_{ij} - r_e}{r_a - r_e} & \text{if } r_c < r_{ij} < r_a, \\ 1 & \text{if } r_a < r_{ij} < r_0. \end{cases} \quad (10)$$

where r_{ij} is the distance between i and j . $\vec{e}_{ij}$ is the unit vector along the segment going from i to j . r_0 is the interaction radius, r_a denotes attraction range, r_e is the preferred distance where the particles will not attract or repel each other, and r_c is the repulsion radius. Obviously, $r_0 > r_a > r_e > r_c$. When particle gets closer than r_c , they repel each other and ignore all other alignment rules. When particle distance is not the equilibrium distance r_e , they mildly repel or attract each other to adjust the distance to r_e . When particle distance exceeds r_a , the pair of particles attract each other with a

constant force.

Through the change of α and β they are able to reproduce various phases transitions that resemble cohesive flock. The repulsion prevents collision and attraction prevents "evaporation" of individuals from flock. By adding attraction and repulsion the model becomes more suitable for simulating real-life situations.

ii) Changing the way of alignment

In 2010, Ginelli *et al.* introduced the idea of "nematic collision" (Fig. 1-3) into the alignment rule [62]:

$$\theta_i(t+1) = \text{Arg} \left[\sum_{\langle i,j \rangle} \text{SIGN}[\cos(\theta_i(t) - \theta_j(t))] e^{i\theta_j(t)} \right] + \eta \xi_i(t) \quad (11)$$

Here nematic collision is carried out by using the sign function (SIGN): if the angle difference between the angular orientations of i and j is smaller than 90° , j influences i normally with its motion direction and if the angle difference between the angular orientations of i and j is larger than 90° , j influences i with the opposite direction of its motion. In their simulation, four phases could be observed with the increase of noise: Phase I is homogeneous and ordered, with all particles moving in either parallel or anti-parallel directions. In phase II, a low density region of chaotic moving particles occurs and as noise increases this chaotic region expands. The ordered portion, which resembles phase I, is compressed and finally become a band of particles. In phase III, though the segregated bands still exist, they become fragile and

unstable. These bands constantly change shape by breaking, merging, bending and reforming. Phase IV represents the most chaotic state where all bands disappear and the system becomes homogeneous again. All particles move in random directions. Ginelli's model describes a nematically ordered system without chemical signaling and adhesion. The phase III in their simulation resembles the aggregation behavior of myxobacteria. They suggest that their model can be used to simulate bacterial surface gliding or motion in biofilms.

FIG. 1.3 The idea of “nematic collision” is to keep alignment along the axis of motion, regardless of motion direction. Thus when two particles with similar motion direction interact they take parallel velocities (a) and when two particles with opposite velocity interact they take anti-parallel velocities and slide through each other (b).

1.1.3.3 Continuum model

Despite the models based on VM, another model built on continuum dynamics was created by Toner *et al.* in 1995 [63]. The aim of this model is to obtain the velocity vector field $\vec{v}$ and density ρ by solving the general continuum equations

with symmetries and conservation laws instead of "coarse graining" the hydrodynamics in particle interaction.

The starting continuum equation is:

$$\frac{\partial \vec{v}}{\partial t} + (\vec{v} \cdot \nabla) \vec{v} = \alpha \vec{v} - \beta |\vec{v}|^2 \vec{v} - \nabla P + D_L \nabla (\nabla \cdot \vec{v}) + D_1 \nabla^2 \vec{v} + D_2 (\vec{v} \cdot \nabla)^2 \vec{v} + \vec{\xi} \quad (12)$$

$$\frac{\partial \rho}{\partial t} + \nabla (\rho \vec{v}) = 0 \quad (13)$$

Eq. (13) is the conservation law of mass. In Eq. (12) D_L , D_1 and D_2 are diffusion coefficients related to actual conditions. $\alpha, \beta > 0$ in the ordered state, thus $\vec{v}$ has a non zero magnitude ($\sqrt{\alpha/\beta}$). $\vec{\xi}$ is a Gaussian random noise and the pressure P is determined by density ρ :

$$P = P(\rho) = \sum_{n=1}^{\infty} \sigma_n (\rho - \rho_0)^n \quad (14)$$

where σ_n is the coefficient of pressure expansion, and ρ_0 is the mean of local number density. The result shows an ordered state in 2-D. An interesting conclusion from the results is that the convective term, which stands for the intrinsically non-linear and non-equilibrium feature facilitates the stabilization of the ordered phase. The continuum approach enables the introduction of several parameters concerning microscopic dynamics. These parameters could be determined according to the real-world system being studied. Recently the phase diagram concerning motion speed and particle density was constructed.

1.2 Swarming

Swarming is a special collective motion observed in bacteria colonies growing on semi-solid surfaces. It is defined as a rapid multicellular motion of bacteria across the surface [20, 22, 47, 49]. The swarming bacteria are propelled by rotating flagella, like swimming [64-68]. Multicellularity is a very important feature that distinguishes swarming from other types of bacterial motion such as swimming, twitching, gliding or sliding (Fig. 1.4) [69]. Swarming bacteria will form clusters consisting of multiple cells, moving side-by-side with high velocity coherency (*i.e.* each member of the cluster has similar velocity).

1.2.1 Conditions needed for swarming

Surface bacteria usually have two ways of social organization: biofilm formation and swarming [59]. Biofilm formation involves bacterial attachment on a biotic or non-biotic surface and differentiating into a complex society of sub-populations with different functions. It is relevant in chronic infections in human body and shows enhanced resistance to antibiotics. Besides the biofilm, when under proper conditions, bacteria can differentiate into a more motile phenotype to colonize the surface. These bacteria, which are capable of swarming, are called swimmers. Swarming is the fastest known way for bacteria to travel across a surface. Based on a large number of observations, a series of conditions are found to be necessary to determine whether or not the bacteria can swarm on the surface [5, 20, 22, 46-49, 55].

FIG. 1.4 Bacterial motion patterns. (a) Swarming: multicellular motion across the surface by rotating flagella. (b) Swimming: single cell motion in bulk liquids by rotating flagella. (c) Twitching: slow cell movement on the surface by extension and retraction of Type IV pili. (d) Cell surface motion using the focal-adhesion complexes that bind to the surface. (e) Sliding: passive motion powered by the push of cell growth and facilitated by surfactant.

1.2.1.1 Bacterial strain

Swarming phenotype is more often observed in the wild types of certain bacterial species of *Bacillus*, *Clostridium*, *Escherichia*, *Salmonella*, *Pseudomonas*, *Proteus*, *Vibrio* [47]. On the other hand, typical lab strains (domesticated strains) usually do not show swarming motility. As surface motility is not an advantage in selection when bacteria are cultured in liquid media, the ability to swarm will disappear after generations of domestication [48, 70]. This is due to the loss of genes associated with swarming motility during lab-condition selection. Thus lab observations of swarming behavior are mostly carried out using wild type strains or their mutants.

1.2.1.2 Surface

So far, the exact physical or chemical properties of a surface that trigger bacterial transformation into swimmers are still unknown. It is generally believed that a wet, energy-rich hydrogel surface is needed for bacterial swarming [20, 46, 47, 49]. The content of water is crucial to swarming motility as bacteria moves in a 2-D layer of water extracted from the hydrogel by bacteria. Dryness of the surface will result in planktonic cells instead of swimmers. However, too much surface fluid will allow swimming motility and the cells may detach from the surface and enter the fluid body instead of undergoing surface motility. Therefore, appropriate medium wetness is needed for swarming [46].

In lab conditions, agar is often used as the hydrogel to sustain bacterial surface growth.

While the normal hard agar surface contains 1.5-2% of agar for most surface bacterial incubation, the ideal agar gel concentration for promoting swarming for most bacterial species is the semi-hard agar with 0.4-0.8% of overall agar content [57, 71]. For most swarming species, surface with > 1% agar will inhibit swarming motility and result in colonies grown by planktonic bacteria. Some exceptions exist such as *Proteus mirabilis* which can swarm on surfaces with up to 1.5-2% agar content [56]. If the surface content of agar is lower than 0.3%, too much surface water will enable swimming motion instead of swarming. The size of water-filled pores inside agar gel are larger, which enable the bacteria to swim inside the gel body so that they no longer stay on the surface.

1.2.1.3 Flagella

Flagella are helical, filamentous structures on the peripheral of a bacterium. Both swimming and swarming are powered by rotating flagella. When rotating, the helix structure of a flagellum acts like a screw propeller [64-67]. The powerful propelling of flagella is essential for swarming motility. It is not only needed for powering the motion but also reported to help maintain bacterial local alignment [64]. Generally, swarming bacteria will up regulate the genes controlling flagella synthesis and become hyperflagellated (*i.e.* a swarming bacterium grows more flagella than its planktonic phenotype). The structure and function will be introduced in details.

i) Structure and function

A flagellum consists of 3 structure regions according to their positions: the basal body anchored in the membrane, the helical filament outside of the cell and the hook that links the two parts [65, 67](Fig. 1.5).

FIG. 1.5 The flagellum have 3 structure domains: the filament, the hook and the basal body. The basal body consists of the anchor rings and a rotary motor that powers the rotation of filament.

A flagellum is anchored on the surface of a bacterium through its basal body. Besides the function as a structure anchor, the basal body includes a series of synthesis platforms that synthesize and secrete the protein monomers used to build up

the filament and hook. The rotary motor, which generates torque and rotate the filament, also belongs to the basal body. It consists of a rod-like rotor and a ring-like stator closely surrounding the rotor. The motor is powered by electrochemical gradient instead of ATP. When proton or sodium cations flow through the stator, conformational change in the stator will be induced. This will generate torque between the stator and rotor. As the stator is firmly anchored on the membrane, the rotor will be rotated [66, 67, 72].

The filament is a helical-shaped polymer of protein monomers called flagellin. The length of a filament could be as long as 15 μm with a width of 12-25 nm. It is connected to the rotor of the basal body by hook, which is a curved hollow cylindrical structure. The protein monomers that constitute the hook tolerate both expansion and compression at the same time, so that the hook is easily bent. This enables the hook to alter the torque orientation when transferring torque from the motor to the filament. This is important in keeping filaments at different sides of the cell coordinated during propulsion [66, 67].

During propulsion, the flagella rotate counterclockwise (CCW) to drive the bacterium forward along its long axis direction. All the left-handed helical filaments will bundle together and generate a strong propelling force. The period when a bacterium rotate the bundled flagella in a CCW direction and move forward is called a "run". On the contrary, when the flagella are rotated clockwise (CW), they are unbundled and the propulsion is disrupted. This motion is called a "tumble". The integration of "run" and "tumble" regulates bacterial motion. For example, in

chemotaxis, where bacteria move along or against a chemical gradient, there will be more runs when moving toward favorable environment (*e.g.* higher concentration of nutrient), and more tumbles when moving toward harsh environment (*e.g.* lower concentration of nutrient). Thus the overall movement will be toward a better environment [72].

ii) Role in swarming

In most cases when bacteria transform from planktonic to swarming phenotype, the number of flagella increases. Many swarming bacteria species have peritrichous flagella on the cell surface even if some of these bacteria only have one or few flagella in their planktonic form [73-76]. The increased flagella provide stronger propelling force for bacteria to overcome higher friction in viscous surface liquid [46, 47, 49].

In swarming bacteria, the flagella bundle and function similar to that of the swimming phenotype, despite the increased collisions in a more densely packed population. However, chemotaxis is reported to be not essential in swarming motility for most species. Also, at the swarming colony edge, the bacterial stack into a very densely packed wall. All the cells trapped at the wall propel the flagella to pump out surfactant and water out of the colony. This facilitates the wetting of virgin agar and reduce the friction of bacteria spreading out. Besides the above functions, the adjacent moving cells are found to form transient inter-cellular bundle of flagella as means for cellular communication and coherency maintenance. The bundling also provides

greater propulsion force for bacteria to overcome friction [20].

1.2.1.4 Surface modifier

An important criteria for swarming bacteria spreading is maintaining surface moisture. As introduced previously, bacterial surface motion is supported by the rotation of flagella in surface liquid. In a well-prepared agar surface there is little free water, so bacteria have to extract water from the hydrogel [46, 49]. A very common way to attract water to the surface is by secreting osmotic agents to the surface [49]. For example, swarming *Proteus mirabilis* secretes extracellular matrix (ECM), which contains polysaccharides and glycine betaine, and colony migration factor (Cmf) that is an acidic polysaccharide. These contents act as osmolytes that facilitate surface hydration [56, 77, 78]. For other Gram-negative bacteria, glutamate, proline and some lipopolyssacharides (LPS) can also act as the osmolytes [79-81]. The osmolytes for Gram-positive bacteria have been hypothesized but the exact content has not been fully elucidated. To date, the exact mechanism of osmolyte synthesis upregulation remains unknown. It is widely believed the ability to “sense” the surface triggers the upregulation in osmolyte synthesis related gene expression [81].

Surface tension is another hurdle that has to be overcome for surface motion. The origin of surface tension is that the molecular attraction within the liquid is stronger than the attraction between liquid molecule and the surface. High surface tension will cause the retraction of liquid body and makes it difficult for micro-swimmers to break through the liquid boundary. Thus a lower surface tension will benefit swarming.

Swarming bacteria were found to be able to produce wetting agents to reduce the surface tension of liquids [47, 49]. Though some species do not secrete specialized wetting agents (and LPS is assumed to take the role), many species produced surfactants such as lipopeptides, surfactins, or rhamnolipids *etc.* to facilitate swarming motion (Fig. 1.6) [82-85]. Unlike the osmolytes, the secretion of these surfactants is controlled by quorum sensing. The uneven surfactant distribution, which leads to a surfactant concentration gradient from the colony center to colony edge, has also been reported to be responsible for the fast outward expansion of swarming [86]. When a counter gradient of the same surfactant was created, the swarming expansion was arrested.

FIG. 1.6 Chemical structures of some surfactants secreted by swarming bacteria. (a): Serrawettin W2 (secreted by *Serratia liquefaciens*); (b): Rhamnolipid (secreted by *Pseudomonas aeruginosa*); (c): Surfactin (secreted by *Bacillus subtilis*).

1.2.2 Swarming behaviors

1.2.2.1 Lag time

After inoculating planktonic bacteria on the surface of swarming agar, the bacteria do not immediately start swarming. They remain immotile for a certain period of time. This time is called the lag time of swarming. During the lag time, planktonic bacteria gradually transform themselves into a swarming phenotype. This process includes the synthesis and secretion of osmolytes and surfactants, the accumulation of cell population, synthesis of more flagella and cell elongation [48, 87, 88].

It is commonly believed that swarming bacteria suppress cell division, which results in elongated filamentous cells. A filamentous cell is formed when bacteria finish replication but do not divide. Many swarming bacteria are found to have enriched subpopulations of elongated cells at the colony front. Some cells were found to be filamentous with multiple nucleoids within one elongated cell [57, 71, 89].

During the lag time, the bacteria transforms to swarming phenotype and cells continue to reproduce. When the cells sense the surface, a series of swarming-related gene expressions are upregulated, (*e.g.* the *swrA* gene in *Bacillus subtilis*, which controls flagellar synthesis) [87, 90]. The surfaces of bacteria become more flagellated, while some bacteria such as *Escherichia coli* and *Proteus mirabilis* will secrete extracellular matrix to prepare for the swarming motion. Osmolytes and surfactants are secreted and accumulate at the extracellular environment. Water inside

the hydrogel is extracted to the surface to sustain cell motion. The lag time ends when the colony is ready to swarm [48, 87, 88].

1.2.2.2 Rafting

Unlike swimming, swarming bacteria spontaneously assemble and move side-by-side in a group. The group of bacteria is called a raft. The individual motion within a raft is highly ordered while between different rafts there is little sign of collectivity. Thus the overall system order (in terms of order parameter) is very low. A dynamic balance of assembly and disassembly is observed in rafts where cells (especially those at the edge of the raft) drop out and at the same time new members are recruited into the raft [20, 48, 64, 89, 91]. The stabilization of raft relies on the orchestration of coherent motion of each member. The communication needed for such high coherent motion is not yet known clearly. Some evidence shows that flagella bundling may be the mechanism of well organized motion [91, 92].

1.2.3 Swarming induced antimicrobial resistance

Swarming motion benefits the colony not only by promoting rapid colony expansion, but also by increasing the resistance against multiple lethal antimicrobial agents. It was first reported by Kim *et al.* in 2003 that swarming *Salmonella enterica* show elevated resistance against distinct classes of antimicrobials [26, 27]. Recently more reports have shown that swarming bacteria have increased MIC (minimum

inhibitory concentration) compared to planktonic bacteria [25, 28].

1.2.3.1 Methods to measure surface bacteria sensitivity to drugs

Unlike the case of planktonic bacteria, whose vulnerability is easily measured in liquid culture with a serial gradient of drugs, the sensitivity to drugs of swarming bacteria is not easy to determine as controlling drug concentration on solid surface is non-trivial. However, several methods have been developed to measure the drug sensitivity of surface bacteria.

The most straightforward method is to use agar plates which are premixed with certain amount of drug [25, 93]. The drug concentration is known and could be adjusted in the premixing process before bacterial inoculation. Then by observing the growth of inoculated bacterial colony the MIC is determined. This method, though simple, requires complex preparations of agar plates with different drug concentrations. Furthermore, the quantification is very coarse.

A more convenient method is called the surface diffusion method. Instead of using plates with various drug concentrations, this method uses drug gradient on one plate to determine bacterial sensitivity to drugs [26-28]. The drug gradient is generated by placing a small paper disc, soaked with a fixed amount of drug, on the agar surface. As the drug diffuses from the disc to the plate, a stable gradient is generated and maintained. According to the principles of diffusion, on agar surface close to the drug disc the concentration of drug will be high. When growing or expanding bacterial colony encounters lethal drug concentrations, the bacteria will be

killed, thus forming a circular zone devoid of living bacteria around the drug disc. This zone is called an inhibition zone. By comparing the inhibition zone size (radius of inhibition zone minus radius of drug disc) the sensitivity is evaluated. The more sensitive the bacteria are to the drug the larger the inhibition zone size. When the bacteria are insensitive or highly resistant to the drug, there will be no inhibition zone. The disc diffusion method is a fast and convenient method to compare bacterial sensitivity to drug, especially in occasions where comparison between swarming and non-swarming phenotypes of the same strain is required. However, this is a semi-quantitative method. As there is no defined diffusive profile, the inhibition zone size cannot be directly linked to the MIC value.

An efficient method to determine MIC value is to use E-test based on the principles of the disc-diffusion method [25-28]. The E-test is a strip soaked with a serial gradient of drug from one end to the other end. Thus the drug concentration at different positions of the strip will be different after placing the strip on an agar surface. Currently, commercially available E-test strips have predefined diffusion profile that determines the drug concentrations at the edge of the strip. When drug sensitive bacteria are cultured in the presence of an E-test strip on the agar plate, at the end with higher drug concentration the inhibition zone size will be very large. The drug concentration decreases from one end to the other end of the strip. Thus the inhibition zone size shrinks from one end to the other. The position where the edge of inhibition zone meets the edge of the strip has the drug concentration equivalent to the MIC. Thus the MIC value is readily acquired by reading the mark of the concentration

at this position. The limitation of E-test is that the concentration range is predefined on the strips and is not possible to test bacteria with MIC outside this range.

1.2.3.2 Mechanisms of swarming-induced multi-drug resistance

Typical multi-drug resistance mechanisms involve reducing bacterial surface permeability (*e.g.* LPS layer outside Gram-negative bacteria) combined with efficient drug efflux systems that pumps out toxic molecules within the cell [94]. Though it was found that some virulence-related gene expressions are up-regulated in swarming phenotypes, according to recent research studies, it is widely believed that the resistance induced by swarming is most likely attributed to increased cell density and swarming motion dynamics.

By monitoring luciferase activity in their study on *Salmonella enterica*, Kim *et al.* found that during swarming, the LPS (lipopolysaccharide) related gene *pmrHFIJKLM* expression was upregulated and this resulted in a more positive charged LPS core [26]. This leads to higher resistance to cationic antibiotics such as polymixin. However this is not sufficient to explain multidrug resistance. Lai *et al.* reported the enhanced resistance of different swarming bacteria to a wide spectrum of toxins using the surface diffusion method [28]. In their study on *Pseudomonas aeruginosa* they found that the elevated resistance of swarming bacteria is transient and constrained to swarming condition only: as soon as swarming bacteria de-differentiate into planktonic phenotype the enhancement of resistance is lost. They also demonstrated

that the resistance increase is not a result of selection by showing that swarming colony front pre-exposed to drug and swarming front fresh to drug behave the same. Furthermore, they excluded the efflux mechanism as swarming mutants with different drug efflux ability show similar resistance to drugs.

It is worth mentioning that in both Kim and Lai's works, multiple fronts were observed despite the fact that different bacteria were used [26, 28]. In surface diffusion method, as the drug gradient gets higher, the colony front will stop expanding at the position where the drug concentration equals the MIC. Unlike the planktonic bacterial colony, where only one neat colony front is found at the edge of the inhibition zone, the swarming colony shows distinct waves of fronts. The front at the position of planktonic bacterial inhibition zone can be observed. This front is referred to as the primary front. Besides the primary front, swarming bacteria form another advancing front that continue advancing into areas with relatively high drug concentrations. This advancing front is called the secondary front (Fig. 1.7). Secondary front usually appear thinner than the primary front. Lai *et al.* in their experiment with *Bacillus subtilis*, *Bacillus thailandensis* and *Escherichia coli*, found that the cells harvested from the primary and secondary front show similar resistance levels when cultured in liquid medium [28]. This indicates that swarming-induced resistance is transient, reversible and related to surface motion.

FIG. 1.7 Multiple fronts observed in swarming *Bacillus subtilis* colony exposed to vancomycin. Arrow A denotes the primary front and arrow B denotes the secondary front. x is the paper plate infused with 30 μg vancomycin. The colony spreads from bottom to top. This figure is adapted from Chapter 3.

A study examining the relationship between swarming motion dynamics and antimicrobial resistance was carried out by Butler *et al.* in 2010 [25]. Their study on the drug resistance of swarming *Salmonella enterica* provided evidence that swarming motion dynamics affects drug resistance. They found that the bacterial density is higher at a swarming colony edge compared to that at a swimming edge. The high cell density at the colony front is one feature of swarming motion and it increases the resistance of the colony to drugs. They also noticed that when advancing towards area

with higher antibiotic concentrations, the swarming colony sustain death of some members. The swarming front in their experiment was found to be multilayered, where some bacteria “sacrifice” themselves by staying at the bottom layer and being continuously exposed to lethal amounts of antibiotics to shelter the bacteria swarming at upper layers from direct drug exposure. At the same time, the cells swarming in upper layers keeps circulating to minimize exposure to antibiotics. Proficient swarming ensures efficient translocation of cells from high drug containing areas to low drug containing areas. Another evidence which indicates cell motility plays an important role in overcoming areas with lethal drug concentrations is that at higher temperature, when bacteria swarming motion and colony expansion are faster, the colony tolerates higher drug concentration. They propose that the multilayered structure at swarming edge, together with the rapid swarming motion, protect the cells at swarming front and enable the colony to expand to areas with lethal drug concentrations.

The connection between the collective motion dynamics and antimicrobial resistance enhancement of swarming bacteria suggests that by disturbing swarming motility we may be able to achieve higher antimicrobial killing efficacy. Furthermore, the study of bacterial motion dynamics under disturbance (*e.g.* antibiotics or photodynamic oxidation) is relevant to the treatment and prevention of surface bacterial infection. Exploiting the response of swarming bacteria to disturbance may lead to more effective methods to control bacterial infection at the early stage.

1.2.4 Methods of studying swarming bacteria

1.2.4.1 Observation

The main task in observing bacterial swarming is to record a continuous video of bacterial motion for trajectory tracking and analysis. This task requires the combination of a microscope with a high speed scientific camera. As swarming bacteria are able to move at an average speed of $40\text{-}50\ \mu\text{m s}^{-1}$ [20, 21], camera with low shutter speed will not be able to resolve a single bacterium from frame to frame in swarming motion, especially when bacterial density is high. Differential interference contrast (DIC) microscopy or phase contrast microscopy is often used to enhance image contrast so that the trajectory tracking is more precise [20, 21].

1.2.4.2 Tracking methods

The most straightforward way of study bacterial motion is to track the motion of every single bacterium. The fast motion and the high density of swarming bacteria makes single bacterium tracking non-trivial. Zhang *et al.* in their work have shown a good example on how to track single bacterial motion in a 2-D monolayer of swarming cells using *Matlab* functions [21]. However, tracking single bacterium in a swarm requires good resolution in the movie and 2-D monolayer of bacteria. The strict condition limits the study of dynamics of multilayered swarming.

An alternative way to track swarming motion is particle imaging velocimetry (PIV) [22, 23, 95]. When using PIV to study fluid, the fluid is first seeded with

colored or fluorescent particles that flows with the fluid. Then the passive motion of these particles is recorded in movies. By tracking the particle motion in these movies the fluid motion could be known. The feature of PIV is that it tracks the average motion of all particles in one small area fraction (namely the interrogation window) instead of the motion of each particle. Each frame is divided into an array of interrogation windows (usually a rectangular array with square fractions) and the velocities of each interrogation window are calculated using signal processing and cross-correlation technologies. The resulting matrix of velocity vector reveals the details in the motion dynamics (Fig. 1.8). In swarming bacterial tracking, the bacteria act as intrinsic seeding particles in PIV. The advantage of PIV tracking is that slight defocus or blurring in the image of an individual bacterium does not significantly interfere with the overall tracking result. In bacterial swarming study this technique is very handy and lower requirements for equipments and controlled conditions.

FIG. 1.8 A matrix of velocity vector constructed from a bacterial swarming video by PIV. The arrows denotes the velocity of the fraction at its position. Large velocity fluctuations is shown in the matrix and closely packed parallel vectors indicates the existence of multicellular rafts. The background is a colormap where red color denotes high velocity regions and blue color denotes low velocity regions. This figure is adapted from Chapter 3

1.3 Photodynamic therapy (PDT) as a treatment of bacterial infections

1.3.1 History

Photodynamic therapy (PDT) utilizes a photosensitizer, which is usually a fluorophore, to absorb light and transfer the energy to create reactive oxygen species (ROS) in the presence of oxygen [96-102]. When this process is happening inside or very close to a living organism (*e.g.* a cell or a bacterium), the toxic ROS will damage and eventually kill it. This phenomenon was first discovered more than 100 years ago on dye-stained protozoans [103, 104]. In the 1970s PDT was first realized to be an efficient method to selectively kill malignant tumor [105]. The success in killing tumor cells suggests PDT's potential as a cure for diseases caused by other bioorganisms, such as bacteria. It was in the mid 1990s that the effective eradication of bacterial infections using PDT came into reality [96, 106].

1.3.2 Principles of PDT

1.3.2.1 Photophysics

Photodynamic therapy is conducted by shining light on the photosensitizer in the presence of oxygen [99-102, 107]. After absorption of a photon of light, the electron in the highest occupied molecular orbital (HOMO) of photosensitizer can be promoted to the lowest unoccupied molecular orbital (LUMO). The photosensitizer enters a short-lived excited singlet state. The excited singlet state is not stable and is rapidly followed by several processes (Fig. 1.9(a)). The process that is most important

for PDT is the inter-system crossing where the excited electron reverses its spin. The photosensitizer relaxes from the singlet state to a triplet state. The triplet state has a much longer lifetime, which makes it more likely to react with other species. According to selection rules, triplet-singlet interaction is spin forbidden and triplet-triplet interaction is spin allowed. The excited state triplet photosensitizer can react with the ground state triplet oxygen to create ROS.

FIG. 1.9 The physical and chemical processes in PDT. The photophysical processes of photosensitization are shown in (a). The pathways of ROS generation are shown in (b). PS refers to the photosensitizer.

1.3.2.2 Reactions that generate ROS

The interaction of triplet photosensitizer and oxygen has two pathways, namely the Type I process and the Type II process (Fig. 1.9(b)) [102, 108]. The Type I process involves the transfer of charge between the photosensitizer and a quencher (substrate or solvent molecule) where the photosensitizer mostly acts as the oxidant. In the presence of oxygen, the oxidation of bio-molecules is carried out and during this process highly toxic radical ROS are generated (*e.g.* $\cdot\text{OH}$ *etc.*). Type II process involves energy transfer where a triplet-triplet interaction occurs between ground state triplet oxygen and excited state triplet photosensitizer. This process generates excited state singlet oxygen and ground state singlet oxygen. The excited state singlet oxygen is extremely unstable and short-lived so that it does not have sufficient time to react with other species. Thus the main species that represents the reactivity of singlet oxygen is the ground state singlet oxygen, which is denoted as $^1\text{O}_2$ or $\text{O}_2(^1\Delta_g)$ (Fig. 1.10). In some instances, electron transfer is also involved in the Type II process generating superoxide O_2^- .

FIG. 1.10 The molecular orbital of different oxygen species: (a) ground state triplet oxygen; (b) ground state singlet oxygen (the normally referred “singlet oxygen”); (c) excited state singlet oxygen (very short-lived). Only the valence orbitals are shown.

1.3.2.3 Killing mechanism of ROS

The ROS generated can react with many bio-molecules, such as a protein's amino acid residues, many of which have reductive groups such as Cys (-SH) or Lys (-NH₂), the double bond on unsaturated lipids, or the base residues of single- or double- stranded DNA, especially guanine residues. The interaction in specific cases depends on: i) the location of photosensitizer, ii) the abundance of substrate and iii) the acting species of ROS [102].

Two possible mechanisms have been proposed to cause the killing of bacterial cells. There are many reports on PDT leading to DNA damage, including DNA breaks and loss of super-coiled fraction [109, 110]. Recently more and more evidences are provided indicating that damage to the membrane or cell wall may be the main cause of cell death, even though DNA damage occurs at the same time. The break of membrane or destruction of cell wall will lead to the leakage of cytoplasm and loss of functional ions [111, 112].

1.3.3 Applying PDT as an antibacterial strategy

PDT is a potential antibacterial treatment for the post-antibiotics era. It has many unique strengths in the treatment of pathogenic bacteria, such as a broad action spectrum, immunity to traditional antimicrobial resistance mechanisms, inability to induce resistant mutants, and a localized light application that enables a more precise dual-selection of killing bacterial cells.

1.3.3.1 Pathways for photosensitizer binding to cells

As the generated ROS is reactive and short-lived, it is not able to travel long distances in solution. Thus the photosensitizer must bind to or be uptaken by the cells to effectively work [107]. Though the cell wall on the surfaces of Gram-positive bacteria is very thick, the structure of peptidoglycan that constitutes the cell wall is very porous and highly permeable to large molecules such as polysaccharides with molecular weight as high as 60000 [113]. The permeability has largely exceeded the molecular weight of usual photosensitizer, which is mostly below 2000 [94]. Thus, Gram-positive bacteria does not have any permeability issue for photosensitizer. However the surface of the cell wall of Gram-negative bacteria is surrounded by an impermeable layer of lipopolysaccharide (LPS) that only allow hydrophilic molecules with molecular weight lower than 700 to pass through the porin channels. Thus enhancement of permeability is needed. Two methods are developed to solve this problem: by adding a polymeric cationic polymixin B or metal chelator to remove Ca^{2+} or Mg^{2+} cations from surface so that the electrostatic balance of the LPS is disturbed. Large portion of destabilized LPS layer will break and dissolve, leading to a larger permeability [106, 114]. The two different pathways for photosensitizer to enter Gram-positive and Gram-negative bacteria is compared and shown in Fig. 1.11.

FIG. 1.11 The scheme of pathways of photosensitizer entering the bacteria and the following damaging process. Pathway I is usual in the inhibition of Gram-positive bacteria and Pathway II is usual in the inhibition of Gram-negative bacteria. PS refers to the photosensitizer.

1.3.3.2 Photosensitizers used in antibacterial PDT

Photosensitizers used in PDT are usually large aromatic ring with branches surrounding the central chromophore. Traditional photosensitizers used in anticancer therapies, which are mainly based on tetrapyrrole rings such as porphyrins, are not able to diffuse into Gram-negative bacteria, though they are very effective against Gram-positive bacteria [98, 100, 101, 107]. In clinical applications, introducing permeability enhancers adds to inconvenience and limitations to the application. Thus

different frameworks of photosensitizers are needed. Cationic photosensitizers are used and found to be taken up by bacteria through a self-promoted uptake pathway. Cationic photosensitizer such as methylene blue or toluidine blue have achieved effective PDT killing on both Gram-positive and Gram-negative bacteria. As suggested by structure activity relationship studies, the amphiphilic derivatives of photosensitizer such as cationic porphyrins or phthalocyanines have larger affinity for Gram-positive bacteria, while hydrophilic derivatives have greater affinity for Gram-negative bacteria [107]. The structure of some common photosensitizers are presented in Fig. 1.12.

FIG. 1.12 Chemical structure of some photosensitizers used for PDT treatment for bacteria.

1.4 Aims

The previous studies on swarming dynamics focus on the motion of healthy and undisturbed bacteria. However, the studies on swarming bacterial response to drugs did not have an in-depth quantitative analysis of the swarming dynamics. The information on swarming dynamics of bacteria under disruption remains a missing piece in this field. Chapter 2 & 3 describes pioneering works aimed to illustrate the motion dynamics of damaged or disturbed swarming bacteria (*i.e.* bacterial swarming in the presence of photo-oxidative stress or static hurdle) and to provide useful information on the response of swarming bacteria to stress. Chapter 3 also describes a successful attempt to solve the swarming-induced antibiotic resistant problem by utilizing a novel method that physically disrupt swarming motion pattern. Furthermore, a revised Vicsek model is developed in Chapter 2 to better describe bacterial swarming motion under different circumstances and to provide theoretical support for experimental observations in both Chapter 2 & Chapter 3. Chapter 4 describes a PCH-SPP model that overcomes the shortcoming of Vicsek model in simulating the collective turning of bird flocks.

1.5 Bibliography

1. Deutsch, A., G. Theraulaz, and T. Vicsek, *Collective motion in biological systems*. Interface Focus, 2012. **2**(6): p. 689-692.
2. Vicsek, T. and A. Zafeiris, *Collective motion*. Physics Reports-Review Section of Physics Letters, 2012. **517**(3-4): p. 71-140.
3. Vicsek, T., *A question of scale*. Nature, 2001. **411**(6836): p. 421-421.
4. Czirok, A., et al., *Formation of complex bacterial colonies via self-generated vortices*. Physical Review E, 1996. **54**(2): p. 1791-1801.
5. Czirok, A., M. Matsushita, and T. Vicsek, *Theory of periodic swarming of bacteria: Application to Proteus mirabilis*. Physical Review E, 2001. **63**(3): p. 031915.
6. Dworkin, M., et al., *Microbiology departments: Lost in the shuffle?* Asm News, 1997. **63**(9): p. 459-459.
7. Sokolov, A., et al., *Concentration dependence of the collective dynamics of swimming bacteria*. Physical Review Letters, 2007. **98**(15): p. 158102.
8. Arboleda-Estudillo, Y., et al., *Movement Directionality in Collective Migration of Germ Layer Progenitors*. Current Biology, 2010. **20**(2): p. 161-169.
9. Couzin, I.D. and N.R. Franks, *Self-organized lane formation and optimized traffic flow in army ants*. Proceedings of the Royal Society B-Biological Sciences, 2003. **270**(1511): p. 139-146.
10. Buhl, J., et al., *From disorder to order in marching locusts*. Science, 2006. **312**(5778): p. 1402-1406.

11. Szabo, B., et al., *Phase transition in the collective migration of tissue cells: Experiment and model*. Physical Review E, 2006. **74**(6): p. 061908.
12. Ward, A.J.W., et al., *Quorum decision-making facilitates information transfer in fish shoals*. Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(19): p. 6948-6953.
13. Bajec, I.L. and F.H. Heppner, *Organized flight in birds*. Animal Behaviour, 2009. **78**(4): p. 777-789.
14. Attanasi, A., et al., *Information transfer and behavioural inertia in starling flocks*. Nature Physics, 2014. **10**(9): p. 692-697.
15. Couzin, I., *Collective minds*. Nature, 2007. **445**(7129): p. 715-715.
16. Friedl, P. and D. Gilmour, *Collective cell migration in morphogenesis, regeneration and cancer*. Nature Reviews Molecular Cell Biology, 2009. **10**(7): p. 445-457.
17. Vicsek, T., et al., *Novel Type of Phase-Transition in a System of Self-Driven Particles*. Physical Review Letters, 1995. **75**(6): p. 1226-1229.
18. Gregoire, G. and H. Chate, *Onset of collective and cohesive motion*. Physical Review Letters, 2004. **92**(2): p. 025702.
19. Pimentel, J.A., et al., *Intrinsic and extrinsic noise effects on phase transitions of network models with applications to swarming systems*. Physical Review E, 2008. **77**(6): p. 061138.
20. Darnton, N.C., et al., *Dynamics of Bacterial Swarming*. Biophysical Journal, 2010. **98**(10): p. 2082-2090.

21. Zhang, H.P., et al., *Collective motion and density fluctuations in bacterial colonies*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(31): p. 13626-13630.
22. Zhang, H.P., et al., *Swarming dynamics in bacterial colonies*. Epl, 2009. **87**(4): p. 48011.
23. Lu, S.T., et al., *Loss of Collective Motion in Swarming Bacteria Undergoing Stress*. Physical Review Letters, 2013. **111**(20): p. 208101
24. Schaller, V., et al., *Polar patterns of driven filaments*. Nature, 2010. **467**(7311): p. 73-77.
25. Butler, M.T., Q.F. Wang, and R.M. Harshey, *Cell density and mobility protect swarming bacteria against antibiotics*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(8): p. 3776-3781.
26. Kim, W., et al., *Swarm-cell differentiation in Salmonella enterica serovar typhimurium results in elevated resistance to multiple antibiotics*. Journal of Bacteriology, 2003. **185**(10): p. 3111-3117.
27. Kim, W. and M.G. Surette, *Swarming populations of Salmonella represent a unique physiological state coupled to multiple mechanisms of antibiotic resistance*. Biol Proced Online, 2003. **5**: p. 189-196.
28. Lai, S., J. Tremblay, and E. Deziel, *Swarming motility: a multicellular behaviour conferring antimicrobial resistance*. Environmental Microbiology, 2009. **11**(1): p. 126-136.
29. Huepe, C. and M. Aldana, *Intermittency and clustering in a system of*

- self-driven particles*. Physical Review Letters, 2004. **92**(16): p. 168701.
30. Baglietto, G. and E.V. Albano, *Nature of the order-disorder transition in the Vicsek model for the collective motion of self-propelled particles*. Physical Review E, 2009. **80**(5): p. 050103.
 31. Nagy, M., et al., *Hierarchical group dynamics in pigeon flocks*. Nature, 2010. **464**(7290): p. 890-U99.
 32. Ballerini, M., et al., *Empirical investigation of starling flocks: a benchmark study in collective animal behaviour*. Animal Behaviour, 2008. **76**: p. 201-215.
 33. Bode, N.W.F., D.W. Franks, and A.J. Wood, *Limited interactions in flocks: relating model simulations to empirical data*. Journal of the Royal Society Interface, 2011. **8**(55): p. 301-304.
 34. Cavagna, A., et al., *Scale-free correlations in starling flocks*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(26): p. 11865-11870.
 35. Cavagna, A., et al., *Flocking and Turning: a New Model for Self-organized Collective Motion*. Journal of Statistical Physics, 2015. **158**(3): p. 601-627.
 36. Becco, C., et al., *Experimental evidences of a structural and dynamical transition in fish school*. Physica a-Statistical Mechanics and Its Applications, 2006. **367**: p. 487-493.
 37. Katz, Y., et al., *Inferring the structure and dynamics of interactions in schooling fish*. Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(46): p. 18720-18725.

38. Makris, N.C., et al., *Critical Population Density Triggers Rapid Formation of Vast Oceanic Fish Shoals*. Science, 2009. **323**(5922): p. 1734-1737.
39. Makris, N.C., et al., *Fish population and behavior revealed by instantaneous continental shelf-scale imaging*. Science, 2006. **311**(5761): p. 660-663.
40. Bazazi, S., et al., *Collective motion and cannibalism in locust migratory bands*. Current Biology, 2008. **18**(10): p. 735-739.
41. Simpson, S.J., et al., *Cannibal crickets on a forced march for protein and salt*. Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(11): p. 4152-4156.
42. Deisboeck, T.S. and I.D. Couzin, *Collective behavior in cancer cell populations*. Bioessays, 2009. **31**(2): p. 190-197.
43. Czirok, A., et al., *Multicellular sprouting during vasculogenesis*. Multiscale Modeling of Developmental Systems, 2008. **81**: p. 269.
44. McCann, C.P., et al., *Cell speed, persistence and information transmission during signal relay and collective migration*. Journal of Cell Science, 2010. **123**(10): p. 1724-1731.
45. Wu, Y.L., et al., *Periodic reversal of direction allows Myxobacteria to swarm*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(4): p. 1222-1227.
46. Copeland, M.F. and D.B. Weibel, *Bacterial swarming: a model system for studying dynamic self-assembly*. Soft Matter, 2009. **5**(6): p. 1174-1187.
47. Kearns, D.B., *A field guide to bacterial swarming motility*. Nature Reviews

- Microbiology, 2010. **8**(9): p. 634-644.
48. Kearns, D.B. and R. Losick, *Swarming motility in undomesticated Bacillus subtilis*. Molecular Microbiology, 2003. **49**(3): p. 581-590.
 49. Partridge, J.D. and R.M. Harshey, *Swarming: Flexible Roaming Plans*. Journal of Bacteriology, 2013. **195**(5): p. 909-918.
 50. Aldana, M., H. Larralde, and B. Vazquez, *On the Emergence of Collective Order in Swarming Systems: A Recent Debate*. International Journal of Modern Physics B, 2009. **23**(18): p. 3459-3483.
 51. Benisty, S., et al., *Antibiotic-Induced Anomalous Statistics of Collective Bacterial Swarming*. Physical Review Letters, 2015. **114**(1): p. 018105.
 52. Hamze, K., et al., *Single-cell analysis in situ in a Bacillus subtilis swarming community identifies distinct spatially separated subpopulations differentially expressing hag (flagellin), including specialized swarmers*. Microbiology-Sgm, 2011. **157**: p. 2456-2469.
 53. Huepe, C. and M. Aldana, *New tools for characterizing swarming systems: A comparison of minimal models*. Physica a-Statistical Mechanics and Its Applications, 2008. **387**(12): p. 2809-2822.
 54. Kearns, D.B., et al., *Genes governing swarming in Bacillus subtilis and evidence for a phase variation mechanism controlling surface motility*. Molecular Microbiology, 2004. **52**(2): p. 357-369.
 55. Morin, A., et al., *Collective motion with anticipation: Flocking, spinning, and swarming*. Physical Review E, 2015. **91**(1): p. 012134 .

56. Rauprich, O., et al., *Periodic phenomena in Proteus mirabilis swarm colony development*. Journal of Bacteriology, 1996. **178**(22): p. 6525-6538.
57. Senesi, S., et al., *Swarming motility in Bacillus cereus and characterization of a fliY mutant impaired in swarm cell differentiation*. Microbiology-Sgm, 2002. **148**: p. 1785-1794.
58. Steager, E.B., C.B. Kim, and M.J. Kim, *Dynamics of pattern formation in bacterial swarms*. Physics of Fluids, 2008. **20**(7): p. 073601.
59. Verstraeten, N., et al., *Living on a surface: swarming and biofilm formation*. Trends in Microbiology, 2008. **16**(10): p. 496-506.
60. Aoki, I., *A Simulation Study on the Schooling Mechanism in Fish*. Bulletin of the Japanese Society of Scientific Fisheries, 1982. **48**(8): p. 1081-1088.
61. Gregoire, G., H. Chate, and Y.H. Tu, *Moving and staying together without a leader*. Physica D-Nonlinear Phenomena, 2003. **181**(3-4): p. 157-170.
62. Ginelli, F., et al., *Large-Scale Collective Properties of Self-Propelled Rods*. Physical Review Letters, 2010. **104**(18): p. 184502.
63. Toner, J. and Y.H. Tu, *Long-Range Order in a 2-Dimensional Dynamical Xy Model - How Birds Fly Together*. Physical Review Letters, 1995. **75**(23): p. 4326-4329.
64. Copeland, M.F., et al., *Studying the Dynamics of Flagella in Multicellular Communities of Escherichia coli by Using Biarsenical Dyes*. Applied and Environmental Microbiology, 2010. **76**(4): p. 1241-1250.
65. Berg, H.C. and R.A. Anderson, *Bacteria swim by rotating their flagellar*

- filaments*. Nature, 1973. **245**(5425): p. 380-2.
66. Berg, H.C. and L. Turner, *Torque Generated by the Flagellar Motor of Escherichia-Coli*. Biophysical Journal, 1993. **65**(5): p. 2201-2216.
 67. Mukherjee, S. and D.B. Kearns, *The structure and regulation of flagella in Bacillus subtilis*. Annu Rev Genet, 2014. **48**: p. 319-40.
 68. Patrick, J.E. and D.B. Kearns, *Swarming motility and the control of master regulators of flagellar biosynthesis*. Mol Microbiol, 2012. **83**(1): p. 14-23.
 69. Jarrell, K.F. and M.J. McBride, *The surprisingly diverse ways that prokaryotes move*. Nature Reviews Microbiology, 2008. **6**(6): p. 466-476.
 70. Patrick, J.E. and D.B. Kearns, *Laboratory Strains of Bacillus subtilis Do Not Exhibit Swarming Motility*. Journal of Bacteriology, 2009. **191**(22): p. 7129-7133.
 71. Harshey, R.M. and T. Matsuyama, *Dimorphic Transition in Escherichia-Coli and Salmonella-Typhimurium - Surface-Induced Differentiation into Hyperflagellate Swarmer Cells*. Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(18): p. 8631-8635.
 72. Berg, H.C., *The rotary motor of bacterial flagella*. Annual Review of Biochemistry, 2003. **72**: p. 19-54.
 73. Merino, S., J.G. Shaw, and J.M. Tomas, *Bacterial lateral flagella: an inducible flagella system*. Fems Microbiology Letters, 2006. **263**(2): p. 127-135.
 74. Ragatz, L., et al., *Macroscopic Phototactic Behavior of the Purple*

- Photosynthetic Bacterium Rhodospirillum-Centenum*. Archives of Microbiology, 1995. **163**(1): p. 1-6.
75. Gavin, R., et al., *Lateral flagella of Aeromonas species are essential for epithelial cell adherence and biofilm formation*. Molecular Microbiology, 2002. **43**(2): p. 383-397.
 76. Kirov, S.M., et al., *Lateral flagella and swarming motility in Aeromonas species*. Journal of Bacteriology, 2002. **184**(2): p. 547-555.
 77. Stahl, S.J., K.R. Stewart, and F.D. Williams, *Extracellular Slime Associated with Proteus-Mirabilis during Swarming*. Journal of Bacteriology, 1983. **154**(2): p. 930-937.
 78. Gygi, D., et al., *A Cell-Surface Polysaccharide That Facilitates Rapid Population Migration by Differentiated Swarm Cells of Proteus-Mirabilis*. Molecular Microbiology, 1995. **17**(6): p. 1167-1175.
 79. Wang, Q.F., et al., *Gene expression patterns during swarming in Salmonella typhimurium: genes specific to surface growth and putative new motility and pathogenicity genes*. Molecular Microbiology, 2004. **52**(1): p. 169-187.
 80. Inoue, T., et al., *Genome-wide screening of genes required for swarming motility in Escherichia coli K-12*. Journal of Bacteriology, 2007. **189**(3): p. 950-957.
 81. Toguchi, A., et al., *Genetics of swarming motility in Salmonella enterica serovar Typhimurium: Critical role for lipopolysaccharide*. Journal of Bacteriology, 2000. **182**(22): p. 6308-6321.

82. Hazelbauer, G.L., J.J. Falke, and J.S. Parkinson, *Bacterial chemoreceptors: high-performance signaling in networked arrays*. Trends in Biochemical Sciences, 2008. **33**(1): p. 9-19.
83. Lindum, P.W., et al., *N-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of Serratia liquefaciens MG1*. J Bacteriol, 1998. **180**(23): p. 6384-8.
84. Peypoux, F., J.M. Bonmatin, and J. Wallach, *Recent trends in the biochemistry of surfactin*. Appl Microbiol Biotechnol, 1999. **51**(5): p. 553-63.
85. Arima, K., A. Kakinuma, and G. Tamura, *Surfactin, a crystalline peptidelipid surfactant produced by Bacillus subtilis: isolation, characterization and its inhibition of fibrin clot formation*. Biochem Biophys Res Commun, 1968. **31**(3): p. 488-94.
86. Fauvart, M., et al., *Surface tension gradient control of bacterial swarming in colonies of Pseudomonas aeruginosa*. Soft Matter, 2012. **8**(1): p. 70-76.
87. Belas, R., R. Schneider, and M. Melch, *Characterization of Proteus mirabilis precocious swarming mutants: identification of rsbA, encoding a regulator of swarming behavior*. J Bacteriol, 1998. **180**(23): p. 6126-39.
88. Kearns, D.B. and R. Losick, *Cell population heterogeneity during growth of Bacillus subtilis*. Genes Dev, 2005. **19**(24): p. 3083-94.
89. Alberti, L. and R.M. Harshey, *Differentiation of Serratia-Marcescens 274 into Swimmer and Swarmer Cells*. Journal of Bacteriology, 1990. **172**(8): p.

4322-4328.

90. Ghelardi, E., et al., *Contribution of Surfactin and SwrA to Flagellin Expression, Swimming, and Surface Motility in Bacillus subtilis*. Applied and Environmental Microbiology, 2012. **78**(18): p. 6540-6544.
91. Jones, B.V., et al., *Ultrastructure of Proteus mirabilis swarmer cell rafts and role of swarming in catheter-associated urinary tract infection*. Infection and Immunity, 2004. **72**(7): p. 3941-3950.
92. Stickler, D.J. and R.C. Feneley, *The encrustation and blockage of long-term indwelling bladder catheters: a way forward in prevention and control*. Spinal Cord, 2010. **48**(11): p. 784-90.
93. Finkelshtein, A., et al., *Bacterial swarms recruit cargo bacteria to pave the way in toxic environments*. MBio, 2015. **6**(3): p. e00074-15.
94. Nikaido, H., *Prevention of Drug Access to Bacterial Targets - Permeability Barriers and Active Efflux*. Science, 1994. **264**(5157): p. 382-388.
95. Steager, E.B., C.B. Kim, and M.J. Kim, *Dynamics of pattern formation in bacterial swarms*. Physics of Fluids, 2008. **20**(7): p. 073601.
96. O'Rourke, J.F. and B.C. Dowds, *Dye-mediated photodynamic inactivation of Bacillus subtilis*. Biochemical Society Transactions, 1992. **20**: p. 76S.
97. Jori, G. and M.R. Hamblin, *Photodynamic Inactivation of Microbial Pathogens: Medical and Environmental Applications*. Royal Society of Chemistry, London, 2011: p. 1-18.
98. Ziegelhoffer, E.C. and T.J. Donohue, *Bacterial responses to photo-oxidative*

- stress*. Nature Reviews Microbiology, 2009. **7**(12): p. 856-863.
99. Ergaieg, K., et al., *Involvement of both Type I and Type II mechanisms in Gram-positive and Gram-negative bacteria photosensitization by a meso-substituted cationic porphyrin*. Solar Energy, 2008. **82**(12): p. 1107-1117.
 100. Wainwright, M., *Photodynamic antimicrobial chemotherapy (PACT)*. Journal of Antimicrobial Chemotherapy, 1998. **42**(1): p. 13-28.
 101. Hamblin, M.R. and T. Hasan, *Photodynamic therapy: a new antimicrobial approach to infectious disease?* Photochemical & Photobiological Sciences, 2004. **3**(5): p. 436-450.
 102. Vatansever, F., et al., *Antimicrobial strategies centered around reactive oxygen species - bactericidal antibiotics, photodynamic therapy, and beyond*. Fems Microbiology Reviews, 2013. **37**(6): p. 955-989.
 103. von Tappeiner, H., *Information on light effect (fluorescent) substances*. Deutsche Medizinische Wochenschrift, 1904. **30**: p. 579-580.
 104. Raab, O., *Effect of fluorescent substances on Infusoria*. Zeitschrift Fur Biologie, 1900. **39**: p. 524-546.
 105. Mitton, D. and R. Ackroyd, *A brief overview of photodynamic therapy in Europe*. Photodiagnosis and Photodynamic Therapy, 2008. **5**(2): p. 103-111.
 106. Nitzan, Y., et al., *Inactivation of Gram-Negative Bacteria by Photosensitized Porphyrins*. Photochemistry and Photobiology, 1992. **55**(1): p. 89-96.
 107. Jori, G., et al., *Photodynamic therapy in the treatment of microbial infections:*

- Basic principles and perspective applications. Lasers in Surgery and Medicine*, 2006. **38**(5): p. 468-481.
108. Foote, C.S., *Definition of Type-I and Type-II Photosensitized Oxidation*. Photochemistry and Photobiology, 1991. **54**(5): p. 659-659.
 109. Capella, M., A.M. Coelho, and S. Menezes, *Effect of glucose on photodynamic action of methylene blue in Escherichia coli cells*. Photochemistry and Photobiology, 1996. **64**(1): p. 205-210.
 110. Bertoloni, G., et al., *Photosensitizing activity of hematoporphyrin on Staphylococcus aureus cells*. Biochimica Et Biophysica Acta-General Subjects, 2000. **1475**(2): p. 169-174.
 111. Schafer, M., C. Schmitz, and G. Horneck, *High sensitivity of Deinococcus radiodurans to photodynamically-produced singlet oxygen*. International Journal of Radiation Biology, 1998. **74**(2): p. 249-253.
 112. Walther, J., et al., *Protochlorophyllide: a new photosensitizer for the photodynamic inactivation of Gram-positive and Gram-negative bacteria*. Fems Microbiology Letters, 2009. **290**(2): p. 156-163.
 113. Friedrich, C.L., et al., *Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria*. Antimicrob Agents Chemother, 2000. **44**(8): p. 2086-92.
 114. Malik, Z., H. Ladan, and Y. Nitzan, *Photodynamic Inactivation of Gram-Negative Bacteria - Problems and Possible Solutions*. Journal of Photochemistry and Photobiology B-Biology, 1992. **14**(3): p. 262-266.

Chapter 2

Loss of Collective Motion in Swarming Bacteria Undergoing Stress

This chapter is adapted from: "*Loss of collective motion in swarming bacteria undergoing stress*", Shengtao Lu, Wuguo Bi, Fang Liu, Xiangyang Wu, Bengang Xing and Edwing K. L. Yeow, *Physical Review Letters*, **111**, 208101 (2013)."

2.1 Abstract

The collective motion of *Bacillus subtilis* in the presence of a photosensitizer is disrupted by reactive oxygen species when exposed to light of sufficient dosages and is partially recovered when light irradiation is suspended. The transition from a highly collective to a more random motion is modeled using an improved self-propelled model with alignment rule. The increment in noise level describes the enhanced uncertainty in the motion of swarming bacteria under stress as observed experimentally.

2.2 Introduction

Collective motion of living organisms is ubiquitous in nature and can be found in schools of fish, flocks of birds and herds of cattle. In biology, certain bacteria such as the Gram-positive *Bacillus subtilis* undergo cell differentiation into swarmer cells that display a high number of flagella and move rapidly across the surface of a semisolid media in multicellular rafts or clusters [1, 2]. Experimental studies on the collective motion of bacteria have so far been limited to understanding the swarming dynamics of *healthy* cells [3-5]. Several theoretical models have also been developed to investigate coherent motion and the onset of collectivity starting from a non-equilibrium state [6-20]. Self-propelled particle models such as the Vicsek model [6, 7] have been extensively used to analyze the nature of phase transition in collective motion. Unfortunately, laboratory observations of the transition from a highly collective to a more random motion are scarce [10]. Therefore, the lack of experimental studies renders the characterization of most theoretical models incomplete [10].

The photoactivation of a photosensitizer by light produces reactive oxygen species that kill bacteria (photodynamic therapy PDT) [21, 22]. In this work, PDT is utilized to alter the motion of swarming bacteria under stress without direct mechanical intervention on the cells. Bacteria within clusters undergo a transition from a highly collective to a more random motion *via* a simple and convenient method. Swarming bacteria, often found in biofilms, migrate rapidly across host tissues and aid in the spread of microbial diseases. In order to use PDT as an

antimicrobial treatment against swarming bacteria, it is important to understand how PDT alters the collective motion of such cells. In this study, wild type *B. subtilis* (strain 3610), photosensitizer protoporphyrin IX (PpIX) and 0.5% LB agar are used. The latter ensures the presence of a thin layer of water that is needed for cell motility [1]. A simple self-propelled model that takes into account the alignment of bacteria to the direction of motion is developed and used to simulate the effects of PDT on the collective behavior of cells.

2.3 Experimental

2.3.1 Culture Conditions and Preparation of Bacteria

Wild type *B. subtilis* (strain 3610) was purchased from ATCC. The culture media LB (tryptone 10 g L⁻¹, yeast extract 5 g L⁻¹ and NaCl 5 g L⁻¹ in water) was first sterilized in an autoclave machine (Hirayama) at 120°C for 20 min. *B. subtilis* culture was grown in LB at 37°C overnight before being diluted 40× and then allowed to grow for a further 4 h at 37°C to reach an optical density at 600 nm (OD₆₀₀) of ~ 0.9 (*i.e.*, at the end of the log phase). An LB agar (0.5% agarose in LB media) film of ~ 1 mm thickness was prepared in a homemade sample chamber and pre-dried for 1 h before use. The agar gel was inoculated by dropping 1 µL of the bacteria culture onto the surface and dried at room temperature for 0.5 h before being incubated at 37°C and 80% humidity for 2 h. After the initial swarming lag of 2 h, dendritic colony patterns of *B. subtilis*, both in the absence and presence of PpIX, are observed on 0.5%

agar (see Fig. 2.1 for the colony pattern taken for stained bacteria).

FIG. 2.1 Dendritic colony pattern of stained *B. subtilis* on agar after incubation at 37°C for 2 h.

For the photodynamic inactivation experiment, protoporphyrin IX (PpIX, Sigma-Aldrich) was used as the photosensitizer. 10 μL of DMSO solution of PpIX (14 μM) was added into 2 mL of *B. subtilis* culture (when $\text{OD}_{600} \sim 0.9$, see above) and 2 mL of 0.5% LB agar to achieve a photosensitizer concentration of 0.07 μM . Similar method was used to prepare samples with 0.007 μM and 0.35 μM of PpIX. The procedure to prepare swarming bacteria was similar to that for the unstained *B. subtilis* as described above.

2.3.2 Microscopy Setup and Data Analysis

The microscopy setup consists of an inverted microscope (IX 71, Olympus) coupled to a highly sensitive CCD camera (CascadeII 512B, Photometrics). A halogen

lamp source (Photonik optical illuminator), with tunable power from 8 mW cm^{-2} to 110 mW cm^{-2} , was fixed above the bacteria sample and used to observe bacteria motion and activate photodynamic effects. The transmitted light from the sample was passed through an air objective lens ($20\times$, N.A. = 0.40, Olympus), attenuated by a neutral density filter (OD = 2.0, Edmund Optics), 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 chamber was maintained at $30\text{-}35^\circ\text{C}$ and 80% humidity *via* a heating coil wrapped around the chamber.

Bacteria motion was analyzed using particle image velocimetry (PIV) (PIVlab 1.31 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). From the recorded consecutive microscopy images, 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.

2.3.3 Survival Assay for Swarming *B. subtilis* under Photodynamic Action

Two sets of swarming *B. subtilis* bacteria in the presence of $0.07 \mu\text{M}$ PpIX were grown on the surface of agar as described above. One set was irradiated with light of

power intensity $I = 100 \text{ mW cm}^{-2}$ for 8 min while the other set was not exposed to light. The bacteria on the agars were subsequently added into separate PBS solutions (2 mL) and shaken for 1 h at 37°C. 10 μL of each solution was spread onto agar plates and allowed to be cultivated overnight. The cell viability of each sample was then obtained by counting colony forming unit per mL (CFU mL^{-1}).

2.3.4 Photodynamic inhibition mechanism study

B. subtilis that did not undergo a swarming lag period were cultured overnight at 37°C and separated from the LB media by centrifugation. The residue was re-suspended in PBS ($\text{OD} \sim 0.9$), incubated for 3 h at 37°C, and then divided into 3 equal portions. Each portion was centrifuged and the residue was suspended in PBS buffer prepared in H_2O (culture A), PBS buffer prepared in 90% D_2O and 10% H_2O (culture B) and PBS buffer containing catalase (20 mg L^{-1}) (culture C). PpIX was added into each of the culture A, B and C so that the final concentration of the photosensitizer is $0.1 \mu\text{M}$ and the samples were allowed to be incubated at 37°C for 1 hour. For each of the culture A, B and C, 1 mL of the sample in an eppendorf tube was exposed to white light of $I_h = 100 \text{ mW cm}^{-2}$ for 5 min while another 1 mL of the sample was kept in the dark. The cell viability of each of the sample was subsequently determined by counting the number of CFU per mL, and the results subjected to statistical analysis.

2.3.5 Modeling

In the self-propelled particle model simulation, N bacteria (or particles) are initially homogeneously distributed a two-dimensional square of size $D \times D$. 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 $\Delta t (= 1)$, bacterium i is displaced by a magnitude of $v = 0.1$ (velocity) in the direction of $\theta_i(t + \Delta t)$. In the simulation, the direction of motion at each time step Δt is computed using the expression

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

where the extent of interaction between bacteria is accounted for by considering the angle of motion of the i th bacterium and its n_i neighboring j bacteria located within an interaction active area of radius x (*i.e.*, from the index of summation $\langle i,j \rangle$). The interaction parameter $\beta_{i,j} = \{1 + [(\vec{v}_i \cdot \vec{v}_j)/(|\vec{v}_i| |\vec{v}_j|)]\}/2$, ranging from 0 to 1, acts as a weighting factor that determines the amount of contribution of each interaction between two bacteria, based on their mutual alignment, to the direction of motion such that $\beta_{i,j}$ is high for bacteria that are well aligned (*i.e.*, found within the same cluster) and low for bacteria that are poorly aligned (*i.e.*, bacteria found in different clusters). An intrinsic noise defined by $\eta \xi_i(t)$ where η is the amplitude and $\xi_i(t)$ is a random number uniformly distributed between $-\pi$ and π is considered in the simulation [7]. The noise is ascribed to the bacterium's uncertainty in its motion after receiving an average 'signal' from the neighboring j bacteria [23]. Throughout the simulation, the speed of the bacteria is assumed to be constant. The primary aim of

our numerical study is to understand how photodynamic action in the form of an increment in noise intensity affects the collectivity, and thus speed variation was not considered.

Periodic boundary condition is applied during the simulation. Both volume exclusion and geometric constraints are implemented as described in Ref. [6, 24]. Basically, a short-range hard-core repulsion is introduced such that when the separation between particles is closer than 1 (*i.e.*, the repulsion radius), the particles are repelled from each other and move in a direction given by

$$\theta_i^{t+1} = \text{Arg} \left[\sum_{\langle i,j \rangle} e^{i \text{Arg} [\bar{R}_i^t - \bar{R}_j^t]} \right]$$

where $\bar{R}_j^t$ is the position of particle j within the repulsion radius of particle i at time t .

The simulation is performed by taking $\Delta t = 1$ as the unit of time. A reasonable experimental depiction is obtained when $N = 1000$ and $D = 120$.

For swarming cells, Zhang *et al.* have experimentally shown that changing the average speed of the swarming bacteria have negligible effects on the spatial correlation of the cells [25]. Furthermore, when the flagella are damaged by reactive oxygen species, the speed is decreased due to a weakened propelling action whereas the collectivity is reduced because of the disruption in inter-cell flagella interaction. Therefore, the change in the average speed due to photodynamic effects should not have a significant impact on the collectivity of the bacteria. It is thus reasonable to consider the evolution of the collective behavior as an independent process in our computational simulation. The primary aim of our theoretical study is to understand how photodynamic action in the form of an enhancement in noise intensity affects the

collectivity, and speed variation, which will complicate the modeling and phase transition analysis, was therefore not included.

2.4 Results and discussion

2.4.1 Bacterial uptake of PpIX

The amount of photosensitizer taken up by *B. subtilis* ($OD_{600} \sim 0.9$) in the culture media was determined using a fluorescence spectrometer (Eclipse, Varian). Various samples of bacteria were cultured for 2 h in the absence and presence of different concentrations ($0.07 - 0.7 \mu\text{M}$) of PpIX. The samples were subsequently centrifuged and the emission spectra of the supernatant solutions containing PpIX not taken up by the bacteria were measured using the method described in ref. R4. A calibration curve of the fluorescence intensity at 604 nm vs. concentration of PpIX used was constructed (Fig. 2.2). The supernatant solution without photosensitizer (*i.e.*, initial PpIX concentration of 0) was then spiked with PpIX to form a final concentration of $0.07 \mu\text{M}$ and the emission intensity at 604 nm was recorded. From the calibration curve, the concentration of PpIX in a culture media containing *B. subtilis* ($OD_{600} \sim 0.9$) needed to achieve a concentration of $0.07 \mu\text{M}$ of PpIX not taken up by the bacteria is determined. It was found that $\sim 60\%$ of the photosensitizer is taken up by the bacteria. The fluorescent image of the stained cells is presented in Fig. 2.3. Similar procedure was also used to show that $\sim 60\%$ of the initial photosensitizer concentration is taken up by the bacteria to achieve $0.35 \mu\text{M}$ of photosensitizer not taken up by the bacteria.

FIG. 2.2 The calibration curve of the fluorescence intensity of free PpIX in the supernatant solutions ($\lambda_{\text{ex}} = 408 \text{ nm}$, $\lambda_{\text{em}} = 604 \text{ nm}$) vs. initial concentration of photosensitizer.

FIG. 2.3 Fluorescence microscopy image of *B. subtilis* stained with PpIX (recorded using a confocal fluorescence microscope, MicroTime 200, PicoQuant, $\lambda_{\text{ex}} = 405 \text{ nm}$).

The bacteria were prepared in the presence of 0.07 μM PpIX as above.

2.4.2 Photodynamic inhibition activity disrupts bacterial swarming

Movie 2.1 shows the evolution of motion of *B. subtilis* in the presence of 0.07 μM PpIX, where the bacteria are initially irradiated with white light of low power ($I_l = 8 \text{ mW cm}^{-2}$) for the first 6.3 s, followed by light of a relatively higher power ($I_h = 100 \text{ mW cm}^{-2}$) for the rest of the microscopy measurement (94 s). During exposure to low light intensity, it is observed that close ($< 100 \mu\text{m}$) to the colony edge, where a monolayer of bacteria is formed, *B. subtilis* are transported rapidly across the surface in multicellular clusters (Movie 2.1). Within each cluster, the bacteria move side-by-side each other, and the overall collective motion is similar to that previously reported for swarming phenotype of *B. subtilis* [4, 25].

Fig. 2.4 shows the instantaneous velocity field image (from particle image velocimetry) of *B. subtilis* in the upper right quarter of the CCD image from Movie 2.1 at time $t = 6.3 \text{ s}$ after the start of the measurement. The arrow corresponds to the velocity vector and indicates the average direction and magnitude of velocity for a particular interrogation window. Several meandering jets of collectively high velocities are seen (*e.g.*, jets centered at $(x = 75 \mu\text{m}, y = 105 \mu\text{m})$ and $(110 \mu\text{m}, 95 \mu\text{m})$); confirming that the bacteria undergo collective motion. The average speed (V) for a particular area of interest is calculated from $V = \sum_i |\vec{v}_i| / n$ [25, 26], where $\vec{v}_i$ and n are the velocity vector of the i -th interrogation window and the total number of interrogation windows, respectively. Fig. 2.5(a) shows that V remains relatively constant ($48 \pm 4 \mu\text{m s}^{-1}$) throughout the time duration that the bacteria are exposed to light of I_l intensity. The light dosage is too low to activate a photodynamic effect on

the speed of the swarming bacteria as discussed below.

FIG. 2.4 Instantaneous velocity field image of *B. subtilis* when the cells are exposed to (a) light of $I_l = 8 \text{ mW cm}^{-2}$ at $t = 6.3 \text{ s}$ and (b) light of $I_h = 100 \text{ mW cm}^{-2}$ at $t = 13.3 \text{ s}$. The arrow corresponds to the velocity vector and both the arrow length and color indicate the magnitude of velocity (see color bar).

When the irradiating light is switched from $I_l = 8 \text{ mW cm}^{-2}$ to $I_h = 100 \text{ mW cm}^{-2}$ at $t = 6.3 \text{ s}$, the motion of the stained bacteria is substantially affected (Movie 2.1). For an example, the velocity field image at $t = 13.3 \text{ s}$ after the start of the experiment (or 7 s after the switch to I_h) displays a drastic drop in the number of high magnitude velocity vectors when compared to $t = 6.3 \text{ s}$ (Fig. 2.4(b)). Fig. 2.5(a) clearly shows that V is quickly reduced to approximately half of the initial value (*i.e.*, $V \sim 20 \text{ } \mu\text{m s}^{-1}$) upon exposure to light of I_h intensity before decreasing gradually until all the bacteria become immotile at *ca.* 60 s (Movie 2.1). The time-dependent change of V in Fig. 2.5(a) is described using a double-exponential decay function with a short lifetime component of 0.54 s and a long lifetime component of 10.0 s ; implying that the effects of photodynamic action on the motion of swarming *B. subtilis* is important and occurs, at least, *via* a two-step process (*i.e.*, a rapid process followed by a relatively slower one).

To further investigate the influence of photodynamic action on the extent in which the bacteria move in a coordinated manner, the spatial correlation function of instantaneous velocity (C_v) is computed [25, 26]: $C_v = \langle \vec{v}_i \cdot \vec{v}_j \rangle_{ij} / \langle \vec{v}_i \cdot \vec{v}_i \rangle_i$, where $\vec{v}_i$ and $\vec{v}_j$ are the velocity vectors of two interrogation windows with a center-to-center distance r , and $\langle \dots \rangle_{ij}$ is the ensemble average over all possible ij pairs. The C_v vs. r plots for $t = 6.3, 7.7$ and 13.3 s after the start of the experiment are given in Fig. 2.5(b). By defining the average spatial correlation length (L) to be the r value when C_v falls to one standard deviation above the noise level, we obtain $L = 19.6, 12.9$ and $11.6 \text{ } \mu\text{m}$ for $t = 6.3, 7.7$ and 13.3 s , respectively. L is the highest for low light intensity I_l since

bacteria are found in relatively big packs and move collectively in the same direction within individual clusters.

FIG. 2.5 (a) The average speed of bacteria (V) vs. t for *B. subtilis* irradiated with light of $I_l = 8 \text{ mW cm}^{-2}$ for the first 6.3 s before the light is switched to $I_h = 100 \text{ mW cm}^{-2}$. (b) The spatial correlation function of instantaneous velocity vs. distance corresponding to (a) for $t = 6.3$ ($\square$, red), 7.7 ($\circ$, green, i in (a)) and 13.3 (Δ , blue, ii in (a)) s.

Two important features are noted when light of intensity I_h is used: the spatial correlation length L decreases with increasing light exposure time (Fig. 2.5(b)) and a positive correlation exists between L and V . Zhang *et al.* have demonstrated that the spatial correlation length of healthy swarming bacteria is only weakly dependent on the average velocity [25]. Thus the positive correlation between V and L observed in this study further supports the conclusion that photodynamic effects are responsible for the light dosage-dependent decay of the average speed of bacteria. As the motion of *B. subtilis* becomes sluggish, the bacteria display reduced tendencies to form large packs capable of surging forward at high velocities (Movie 2.1). Instead, packs of significantly smaller sizes, compared to healthy clusters, are seen. As light illumination progresses, more cells break away from their clusters and attempt to swim randomly while being pushed and jostled by oncoming bacteria. Individual cells are mobile because of the presence of sufficient water on the agar surface and a lack of a capillary force to clamp the bacteria onto the substrate [27]. The overall reduction in cluster size and a less ordered motion within individual clusters give rise to the observed decrease in L (Fig. 2.5(b)). Swarming cells in clusters undergo a transition from a highly collective to a more random motion in the presence of photodynamic effects.

Irradiating *B. subtilis* in the *absence* of PpIX is used to confirm that the presence of both photosensitizer and light of sufficient dosages are required to alter the dynamics of swarming motion. In this case, the motion of unstained bacteria that are exposed first to light of I_l intensity for 6.7 s followed by I_h intensity for 28.3 s does not

display significant variation in either the average speed (*i.e.*, $V = 51 \pm 5 \mu\text{m s}^{-1}$, Fig. 2.6) or collective behavior (Movie 2.2); implying that the motion of unstained *B. subtilis* is not affected by the light dosages used in this study. For the stained bacteria of Movie 2.1 and Fig. 2.5(a), the mean speed of motile bacteria when low power light (*e.g.*, I_l) and short exposure times are utilized displays similar behavior to that of the unstained bacteria; implying that photodynamic effects are negligible at low light dosages. On the other hand, the collective motion is disrupted when a higher light intensity (*e.g.*, I_h) is used which is unambiguously attributed to the presence of PpIX and the influence of photodynamic action. Upon long exposure to I_h light (*e.g.*, 8 min), close to 100% of cells are killed by photodynamic action (Fig. 2.7). Furthermore, the time required to completely immobilize the bacteria is dependent on the concentration of the photosensitizer used. When the concentration of PpIX is 0.007 μM (diluted 10 $\times$), the bacteria are still mobile even after 100 s exposure to light of I_h intensity. On the other hand, when the amount of PpIX is increased to 0.35 μM (concentrated 5 $\times$), the motion of the bacteria is arrested within 20 s of I_h light illumination. In this case, the average speed decays with lifetime components of 0.26 s and 3.8 s (Fig. 2.8, Movie 2.3).

FIG. 2.6 Average speed vs. time for unstained bacteria exposed to light of intensity $I_1 = 8 \text{ mW cm}^{-2}$ for 6.7 s followed by light of $I_h = 100 \text{ mW cm}^{-2}$ for the rest of the measurement.

FIG. 2.7 Viability results for the same concentrations of swarming phenotype of stained *B. subtilis* that are (A) exposed to light of intensity $I_h = 100 \text{ mW cm}^{-2}$ for 8 min and (B) kept in the dark. No living bacteria are observed in (A) since the bacteria are killed by photodynamic action, whereas thousands of living bacteria are found in (B).

FIG. 2.8 The average speed of bacteria (V) vs. time (t) for *B. subtilis* irradiated with light of intensity $I_l = 8 \text{ mW cm}^{-2}$ for the first 7.4 s before the light is switched to intensity $I_h = 100 \text{ mW cm}^{-2}$. The decay of the average speed is described using a double exponential function with lifetime components of 0.26 and 3.8 s. Concentration of PpIX used is 0.35 μM .

2.4.3 Recovery of collective motion after photodynamic inhibition

To examine if the collective motion and average speed are recovered after applying photodynamic action, swarming *B. subtilis* in the presence of 0.07 μM PpIX are first exposed to light of I_l intensity for 7.3 s, followed by light of I_h intensity for 14.7 s before the intensity is reverted back to I_l . The real-time evolution of motion and time-resolved average speed are provided in Movie 2.4 and Fig. 2.9(a), respectively. When light of I_h intensity is turned on, the decrease in V follows a double exponential decay function with a short lifetime of 0.49 s and a long lifetime of 9.8 s (Fig. 2.9(a));

in line with the behavior reported in Fig. 2.5(a). After the light is switched from I_h back to I_l at $t = 22$ s, the bacteria regain their speed such that V increases from $5.1 \mu\text{m s}^{-1}$ at $t = 22$ s to $31 \mu\text{m s}^{-1}$ at $t = 100$ s. Within the time window of the experiment, V does not return to its original value of $50.0 \mu\text{m s}^{-1}$ obtained before photodynamic action. The partial recovery of V is described using a double exponential rise function with lifetime components of 0.24 s and 15.7 s.

Apart from the partial recovery of V , the bacteria also regain their collective motion as soon as light of I_h intensity is turned off (see Movie 2.4). The spatial correlation functions calculated for $t = 7.1$, 14.0 and 90.0 s are presented in Fig. 2.9(b). The correlation length L for $t = 14.0$ s is $9.0 \mu\text{m}$ which is shorter than $L = 18.5 \mu\text{m}$ for $t = 7.1$ s due to the partial lose of collectivity when light of I_h intensity is in operation. The coherent behavior improves when the I_h intensity light is suspended and the bacteria reform clusters of bigger sizes (Movie 2.4). For an example, at $t = 14.0$ s, the bacteria move in clusters containing a few bacteria (*e.g.*, 3-5), whereas at $t = 90.0$ s, the bacteria regroup themselves and move in packs as large as tens of bacteria (*e.g.*, 30–40) in size. This is supported by the larger L value ($= 14.3 \mu\text{m}$) obtained for $t = 90.0$ s.

FIG. 2.9 (a) V vs. t for *B. subtilis* irradiated with light of I_1 for the first 7.3 s followed by light of I_h for 14.7 s before reverting back to I_1 at $t = 22$ s. (b) The spatial correlation function of instantaneous velocity vs. distance corresponding to (a) for $t = 7.1$ ($\square$, red), 14.0 ($\circ$, green, i in (a)) and 90.0 (Δ , blue, ii in (a)) s.

2.4.4 Simulation

To gain a better understanding of the transition from collective motion within clusters (*i.e.*, before light illumination) to a more random-type (*i.e.*, after light illumination) motion, the collective dynamics of moving bacteria is simulated using a simple self-propelled particle model that accounts for volume exclusion and geometric constraints (see Experimental). The rod-like geometry of the cells may play a role in the clustering [28], however, this effect is not included since it is presently unclear whether elongation, which increases the aspect ratio of the rod, is a requirement for swarming motility [2]. Another possible mechanism behind the coherent motion is hydrodynamic interactions [14]. Copeland *et al.* have observed that at high cell density, inter-cellular flagella bundling occurs and they have proposed that communication between swarming cells is responsible for orchestrating the coordinated motion [1, 29].

The Vicsek model does not distinguish between intra-cluster interaction amongst bacteria found in similar clusters and inter-cluster interaction between bacteria in different clusters; resulting in a long-range effect whereby the motion of one cluster influences the motion of neighboring clusters. This gives rise to packs moving in the same direction and an overall high order parameter. In our experiment, bacteria within clusters move coherently whereas the clusters move in random direction. The alignment factor ensures that bacteria within individual clusters move collectively. It is worth mention that Ginelli *et al.* [12] have also introduced an alignment parameter in their self-propelled particle model that takes on either of two values; 1 for well

aligned particles and -1 for poorly aligned particles. This description is too coarse when describing bacteria motion. In particular, it is not observed from our experimental data that poorly aligned cells are able to provide a strong influence on bacterial motion

The absence of photodynamic action is modeled by choosing $x = 5$ and $\eta = 0.4$ for the first 15000 time steps where large clusters of particles (size of *ca.* 20 – 30 particles each) are seen at dimensionless time $t^* = 15000$ (Fig. 2.10(a)). Photodynamic action is simulated by increasing η to 0.5 in the next 15000 time steps where a more homogeneous spread of the particles is noted at $t^* = 30000$ (Fig. 2.10(b)). We observed that the dimensionless correlation length decreases as η increases and this trend is consistently seen for several runs performed with different time steps (Fig. 2.11). Therefore, the conclusion that a smaller correlation length is obtained when the noise η is enhanced is valid. This is clearly due to an increase in the randomness in motion. A reverse process (*i.e.*, less correlated and random-type to a more correlated and collective motion) is achieved by reducing the noise η as depicted in Fig. 2.12.

FIG. 2.10 Location of particles for last 20 time steps before and including t^* are given in (a) and (b) for $t^* = 15000$ and 30000, respectively.

FIG. 2.11 The correlation functions obtained from our model for the last time step when $\eta = 0.4$ and total time steps is (a) 15000, (b) 30000, (c) 45000 and (d) 60000. The correlation lengths are 20.1, 19.6, 20.7 and 22.8 for (a), (b), (c) and (d), respectively. The correlation functions for the last time step when the simulation of (a)-(d) is allowed to run for another (e) 15000, (f) 30000, (g) 45000 and (h) 60000 steps with an increase in η to 0.5. The correlation lengths are 10.4, 6.9, 7.0 and 10.8 for (e), (f), (g) and (h), respectively. The correlation is higher for $\eta = 0.4$ compared to $\eta = 0.5$.

FIG. 2.12 Simulation run with $\eta = 0.4$ for 15000 time steps followed by $\eta = 0.5$ for the next 15000 time steps and finally $\eta = 0.42$ for the last 15000 time steps. The correlation functions obtained for the last time step when $\eta = 0.4$ (a), 0.5 (b), and 0.42 (c). The correlation lengths are 20.1, 10.4, and 17.0 for (a), (b) and (c), respectively.

The dimensionless correlation length obtained from the spatial correlation function (Fig. 2.13) decreases from 20.1 (~ 4 times of x) to 10.4 (~ 2 times of x) when η is increased by 1.25 times. At sufficiently high η , the noise overrides the averaged signal received by a bacterium resulting in the cell moving in a direction not dictated by its neighbors. This behavior is in qualitative agreement with the experimental observation reported in Fig. 2.5(b); demonstrating that photodynamic action can be reasonably represented by a slight increment in intrinsic noise. In other words, when incident light on the bacteria is increased, PpIX forms toxic reactive oxygen species that gradually attack and destroy the cells such that bacteria undergo stress and move in a disorderly manner with little influence from their neighbors.

FIG. 2.13 The spatial correlation function from modeling for (a) $\eta = 0.4$ and (b) $\eta = 0.5$.

2.4.5 Mechanism study of photodynamic inhibition

Type II mechanism (by singlet oxygen $^1\text{O}_2$) is often favored outside the cell [30] due to the relatively short life-time of $^1\text{O}_2$ in an aqueous environment and the presence of $^1\text{O}_2$ quenchers inside the cell. In general, porphyrin-based photodynamic action has been proposed to occur *via* the adverse effects of singlet oxygen [31].

The role of $^1\text{O}_2$ (type II mechanism) in the photodynamic action-induced damage of *B. subtilis* is assessed by conducting viability tests on stained cells in their planktonic state in PBS buffers prepared in H_2O (culture A) and 90% D_2O /10% H_2O mixture (culture B). For bacteria that are not exposed to light (*i.e.*, the control experiment), cells from both cultures A and B display similar degree of survival ($\sim 1.1 \times 10^9$ CFU mL^{-1} , Fig. 2.14). This suggests that different solvents have no obvious effects on cell viability when the bacteria are kept in the dark. On the other hand, when the bacteria are illuminated with light of I_2 intensity for 5 min, the viability assay shows a CFU mL^{-1} reduction of 0.61- $\log_{10}$ and 1.8- $\log_{10}$ for cultures A and B, respectively (Fig. 2.14). The greater enhancement of bacteria death in D_2O -rich solvent clearly demonstrates that a type II mechanism is involved in the destruction of *B. subtilis* since $^1\text{O}_2$ has a longer lifetime in D_2O (65 μs) than in H_2O (3.7 μs) [32]. Attempts to understand the dynamics of swarming phenotype in a D_2O -rich medium were not successful since the unstained bacteria displayed significantly reduced motility. This is due, in part, to the solvent-isotope effects of D_2O on the flagellar rotary motor [33].

FIG. 2.14 The viability assay results for *B. subtilis* in the planktonic state from culture A (PBS buffer prepared in water), culture B (PBS prepared in 90% D₂O/10% water mixture) and culture C (presence of catalase in aqueous PBS). In the absence of light illumination (blue columns), the viability of the cells from all 3 cultures show close to similar viability (*i.e.*, $1.06(\pm 0.12) \times 10^9$, $1.11(\pm 0.04) \times 10^9$ and $1.12(\pm 0.10) \times 10^9$ CFU mL⁻¹ for cultures A, B and C, respectively). The light treated samples (red columns) display reduced viability (*i.e.*, $2.64(\pm 0.37) \times 10^8$, $1.66(\pm 1.74) \times 10^7$ and $2.46(\pm 0.48) \times 10^8$ CFU mL⁻¹ for cultures A, B and C, respectively).

In order to determine if H₂O₂, a common reactive oxygen species generated *via* type I mechanism, is responsible for the death of *B. subtilis* during photodynamic action, the viability assay is performed on stained cells in their planktonic state in PBS buffer containing catalase (20 mg L⁻¹) (culture C). The population of culture C bacteria kept in the dark is $\sim 1.1 \times 10^9$ CFU mL⁻¹ and is reduced by 0.65-log₁₀ CFU

mL^{-1} when irradiated with light of I_2 intensity for 5 min (Fig. 2.14). The survival behavior of *B. subtilis* from culture *C* is similar to that observed for culture *A*; indicating that H_2O_2 may not play a significant role in photodynamic action.

The general ‘kill’ mechanism that reactive oxygen species adopt is to damage both the cell wall and membrane of the bacteria, leading to extensive membrane peptide cross-linking, deactivation of membrane enzyme activity, or leakage of intracellular content after prolonged exposure to light of sufficient intensity [21, 34]. The rapid decrease of V in Fig. 2.5(a) suggests that the initial photo-oxidative shock on the cells is significant and arises from a reactive oxygen species-induced perturbation (most likely singlet oxygen $^1\text{O}_2$, Fig. 2.14) of the cell membrane and flagella. In particular, the flagellar filament which is important for swarming motion [1], hook and proton channels in the stator are found at the cell exterior/wall and are readily exposed to the photosensitizer that can form reactive oxygen species that quickly impair the normal functions of the flagella; *e.g.*, causing intracellular flagella to bundle and rotate at a slower rate, impeding cell-to-cell interaction and reducing the overall speed and collectivity of the bacteria.

The subsequent slower decay of V in Fig. 2.5(a) is likely due to a combination of the on-going photo-oxidative stress on the flagella and reactive oxygen species damage on the cell membrane. The latter is a relatively slow process (*ca.* a few minutes to completely kill) based on the light dosage used here and as reported elsewhere [35]. In this way, the internal energy of a bacterium is reduced when its membrane undergoes $^1\text{O}_2$ -induced damages such that the speed of motion and ability

to interact with neighboring bacteria are gradually affected with irradiation time. The bacteria stop moving when the driving force arising from flagella, cell-to-cell contact and internal energy is no longer able to overcome the resistive force of environmental viscosity. With continued exposure to light, the stationary cells will eventually be killed by photodynamic action.

The post-photodynamic recovery of V and L in Figs. 9(a) and 9(b) indicates that the bacteria are able to undergo a repair mechanism to recover parts of their collective behavior and speed. It would seem most likely that swarming *B. subtilis* possess a core set of singlet oxygen response genes that allow the bacteria to protect and repair themselves against $^1\text{O}_2$ photo-oxidative stress [36]. It has been found that the bacteria have multiple ways to protect and recover themselves from photooxidative damage. For example, the carotenoids in cells are prominent quenchers of ROS. When cells are exposed to photo-induced ROS the carotenoids readily quench ROS. Carotenoids are always found in cells and can react very quickly to surrounding ROS[37]. Other mechanisms are induced by photooxidative stress, such as the expression of glutathione peroxidase, which is found to rapidly degrade ROS. This process needs time to upregulate relevant genes (usually takes several to tens of minutes). The gene expression can be upregulated up to 16 folds compared to non-stressed conditions, which effectively degrades ROS. This process is also accompanied by the synthesis of new biomolecules and substitution of damaged parts. It is necessary to note that several issues are still unclear at the present moment such as the reason behind the two-stage motion recovery process seen in Fig. 2.9(a). A likely explanation, which

mirrors the two-stage disruption process, is the combination of these two distinct mechanisms, which displays an efficient repair of motion-related parts (*e.g.* flagella), giving rise to a fast initial recovery, followed by a slower repair of the other parts of the cell damaged by $^1\text{O}_2$.

2.5 Conclusion

We have visualized, in real-time, the evolution of motion of swarming bacteria undergoing stress due to photodynamic action. Bacteria within clusters lose their collective behavior, and undergo a transition from a highly collective motion to a more random motion with a reduction in the average speed. The collectivity is partially recovered upon the suspension of light irradiation. The photodynamic effect is treated as an increment in intrinsic noise in our improved self-propelled model. Our experimental study provides a platform (*i.e.*, by varying the amount of photosensitizer and light intensity) to test out hypothesis proposed in previous self-propelled models (*e.g.*, dynamics of transition). In addition, PDT techniques may be used to drive or control the motion of synthetic active particles [38, 39].

2.6 Movie captions

Movie 2.1 The real time motion of swarming *B. subtilis* in the presence of 0.07 μM PpIX on agar (0.5% agarose in LB media). The bacteria are irradiated with light of intensity $I_l = 8 \text{ mW cm}^{-2}$ for the first 6.3 s before it is switched to light of intensity $I_h = 100 \text{ mW cm}^{-2}$. The dimension of an image is $120 \times 120 \mu\text{m}^2$. The density of cells is $\sim 1300\text{-}1400$.

Movie 2.2 The real time motion of unstained swarming *B. subtilis* (*i.e.*, in the absence of a photosensitizer) on agar (0.5% agarose in LB media). The bacteria are irradiated with light of intensity $I_l = 8 \text{ mW cm}^{-2}$ for the first 6.7 s before it is switched to light of intensity $I_h = 100 \text{ mW cm}^{-2}$. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 2.3 The real time motion of swarming *B. subtilis* in the presence of 0.35 μM PpIX on agar (0.5% agarose in LB media). The bacteria are irradiated with light of intensity $I_l = 8 \text{ mW cm}^{-2}$ for the first 7 s before it is switched to light of intensity $I_h = 100 \text{ mW cm}^{-2}$. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 2.4 The real time motion of swarming *B. subtilis* in the presence of 0.07 μM PpIX on agar (0.5% agarose in LB media). The bacteria are irradiated with light of intensity $I_l = 8 \text{ mW cm}^{-2}$ for the first 7.3 s, followed by light of intensity $I_h = 100 \text{ mW cm}^{-2}$ for the next 14.7 s before the intensity is switched back to light of intensity I_l . The dimension of an image is $120 \times 120 \mu\text{m}^2$.

2.7 Bibliography

1. Copeland, M.F. and D.B. Weibel, *Bacterial swarming: a model system for studying dynamic self-assembly*. Soft Matter, 2009. **5**(6): p. 1174-1187.
2. Kearns, D.B., *A field guide to bacterial swarming motility*. Nature Reviews Microbiology, 2010. **8**(9): p. 634-644.
3. Chen, X., *et al.*, *Scale-Invariant Correlations in Dynamic Bacterial Clusters*. Physical Review Letters, 2012. **108**(14): p. 148101.
4. Zhang, H.P., *et al.*, *Collective motion and density fluctuations in bacterial colonies*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(31): p. 13626-13630.
5. Darnton, N.C., *et al.*, *Dynamics of Bacterial Swarming*. Biophysical Journal, 2010. **98**(10): p. 2082-2090.
6. Vicsek, T. and A. Zafeiris, *Collective motion*. Physics Reports-Review Section of Physics Letters, 2012. **517**(3-4): p. 71-140.
7. Vicsek, T., *et al.*, *Novel Type of Phase-Transition in a System of Self-Driven Particles*. Physical Review Letters, 1995. **75**(6): p. 1226-1229.
8. Gregoire, G. and H. Chate, *Onset of collective and cohesive motion*. Physical Review Letters, 2004. **92**(2): p. 025702.
9. Baglietto, G. and E.V. Albano, *Nature of the order-disorder transition in the Vicsek model for the collective motion of self-propelled particles*. Physical Review E, 2009. **80**(5): p. 050103(R).
10. Szabo, B., *et al.*, *Phase transition in the collective migration of tissue cells:*

- Experiment and model*. Physical Review E, 2006. **74**(6): p. 061908.
11. Pedley, T.J. and J.O. Kessler, *Hydrodynamic Phenomena in Suspensions of Swimming Microorganisms*. Annual Review of Fluid Mechanics, 1992. **24**: p. 313-358.
 12. Ginelli, F., *et al.*, *Large-Scale Collective Properties of Self-Propelled Rods*. Physical Review Letters, 2010. **104**(18): p. 184502.
 13. Sokolov, A., *et al.*, *Concentration dependence of the collective dynamics of swimming bacteria*. Physical Review Letters, 2007. **98**(15): p. 158102.
 14. Leoni, M. and T.B. Liverpool, *Swimmers in Thin Films: From Swarming to Hydrodynamic Instabilities*. Physical Review Letters, 2010. **105**(23): p. 238102.
 15. Aranson, I.S., *et al.*, *Model for dynamical coherence in thin films of self-propelled microorganisms*. Physical Review E, 2007. **75**(4): p. 040901(R).
 16. Baskaran, A. and M.C. Marchetti, *Enhanced Diffusion and Ordering of Self-Propelled Rods*. Physical Review Letters, 2008. **101**(26): p. 268101.
 17. Toner, J., *Birth, Death, and Flight: A Theory of Malthusian Flocks*. Physical Review Letters, 2012. **108**(8): p. 088102.
 18. Simha, R.A. and S. Ramaswamy, *Hydrodynamic fluctuations and instabilities in ordered suspensions of self-propelled particles*. Physical Review Letters, 2002. **89**(5): p. 058101.
 19. Saintillan, D. and M.J. Shelley, *Instabilities, pattern formation, and mixing in active suspensions*. Physics of Fluids, 2008. **20**(12): p. 123304.
 20. Baskaran, A. and M.C. Marchetti, *Statistical mechanics and hydrodynamics of*

bacterial suspensions. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(37): p. 15567-15572.

21. Hamblin, M.R. and T. Hasan, *Photodynamic therapy: a new antimicrobial approach to infectious disease?* Photochemical & Photobiological Sciences, 2004. **3**(5): p. 436-450.

22. Xing, B.G., *et al.*, *Multifunctional divalent vancomycin: the fluorescent imaging and photodynamic antimicrobial properties for drug resistant bacteria*. Chemical Communications, 2011. **47**(5): p. 1601-1603.

23. Pimentel, J.A., *et al.*, *Intrinsic and extrinsic noise effects on phase transitions of network models with applications to swarming systems*. Physical Review E, 2008. **77**(6): p. 061138.

24. Czirok, A., *et al.*, *Formation of complex bacterial colonies via self-generated vortices*. Physical Review E, 1996. **54**(2): p. 1791-1801.

25. Zhang, H.P., *et al.*, *Swarming dynamics in bacterial colonies*. Epl, 2009. **87**(4): p. 48011.

26. Steager, E.B., C.B. Kim, and M.J. Kim, *Dynamics of pattern formation in bacterial swarms*. Physics of Fluids, 2008. **20**(7): p. 073601.

27. Hamze, K., *et al.*, *Single-cell analysis in situ in a Bacillus subtilis swarming community identifies distinct spatially separated subpopulations differentially expressing hag (flagellin), including specialized swarmers*. Microbiology-Sgm, 2011. **157**: p. 2456-2469.

28. Peruani, F., A. Deutsch, and M. Bar, *Nonequilibrium clustering of self-propelled*

- rods. Physical Review E, 2006. **74**(3): p. 030904 (R).
29. Copeland, M.F., *et al.*, *Studying the Dynamics of Flagella in Multicellular Communities of Escherichia coli by Using Biarsenical Dyes*. Applied and Environmental Microbiology, 2010. **76**(4): p. 1241-1250.
30. O'Rourke, J.F. and B.C. Dowds, *Dye-mediated photodynamic inactivation of Bacillus subtilis*. Biochemical Society Transactions, 1992. **20**: p. 76S.
31. Jori, G. and M.R. Hamblin, *et al.*, *Photodynamic Inactivation of Microbial Pathogens: Medical and Environmental Applications*. Royal Society of Chemistry, London, 2011: p. 1-18.
32. Ogilby, P.R. and C.S. Foote, *Chemistry of Singlet Oxygen* .36. *Singlet Molecular-Oxygen (1-Delta-G) Luminescence in Solution Following Pulsed Laser Excitation - Solvent Deuterium-Isotope Effects on the Lifetime of Singlet Oxygen*. Journal of the American Chemical Society, 1982. **104**(7): p. 2069-2070.
33. Yuan, J.H. and H.C. Berg, *Thermal and Solvent-Isotope Effects on the Flagellar Rotary Motor near Zero Load*. Biophysical Journal, 2010. **98**(10): p. 2121-2126.
34. Wainwright, M., *Photodynamic antimicrobial chemotherapy (PACT)*. Journal of Antimicrobial Chemotherapy, 1998. **42**(1): p. 13-28.
35. Ergaieg, K., *et al.*, *Involvement of both Type I and Type II mechanisms in Gram-positive and Gram-negative bacteria photosensitization by a meso-substituted cationic porphyrin*. Solar Energy, 2008. **82**(12): p. 1107-1117.
36. Ziegelhoffer, E.C. and T.J. Donohue, *Bacterial responses to photo-oxidative stress*. Nature Reviews Microbiology, 2009. **7**(12): p. 856-863.

37. Glaeser, J. and G. Klug, *Photo-oxidative stress in Rhodobacter sphaeroides: protective role of carotenoids and expression of selected genes*. Microbiology-Sgm, 2005. **151**: p. 1927-1938.
38. Ebbens, S.J. and J.R. Howse, *In pursuit of propulsion at the nanoscale*. Soft Matter, 2010. **6**(4): p. 726-738.
39. Paxton, W.F., et al., *Chemical locomotion*. Angewandte Chemie-International Edition, 2006. **45**(33): p. 5420-5429.

Chapter 3

Non-toxic Colloidal Particles Impede Antibiotics Resistance of Swarming Bacteria by Disrupting Collectivity and Speed

This chapter is adapted from: "*Nontoxic Colloidal Particles Impede Antibiotic Resistance of Swarming Bacteria by Disrupting Collective Motion and Speed*, Shengtao Lu, Fang Liu, Bengang Xing and Edwing K. L. Yeow, Physical Review E, **92**,062706 (2015)."

3.1 Abstract

Monolayer of swarming *Bacillus subtilis* on semi-solid agar display elevated resistance against antibiotics due to their collective behavior and motility. The drug resistance is impeded when the collective motion of bacteria is judiciously disrupted using non-toxic polystyrene (PS) colloidal particles immobilized on the agar surface. The colloidal particles block and hinder the motion of the cells, causing cohesive rafts of bacteria to lose their collectivity and speed. In this case, the lack of strong group dynamics results in the bacteria becoming vulnerable to antibiotics. The negative correlation between the degree of collectivity and PS particle density is examined using an improved self-propelled model that takes in to account inter-particle alignment and hard-core repulsion.

3.2 Introduction

Collective motion is widely observed in nature (*e.g.*, in bird flocks, insect and single cell organism swarms, etc.), and is known to affect population-wide social behavior (*e.g.*, escape from predators) [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 and is an integral part of pathogenesis [19-32]. 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 seen in swarming *S. enterica* 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 enable swarming *S. enterica* to protect themselves against antibiotics [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]. An advantage of studying *B. subtilis* is the formation of a monolayer of swarming bacteria that stretches hundreds to thousands of μm from the edge into the colony interior. This allows the quantitative investigation of the two-dimensional collective motion of cells to be conveniently conducted using microscopy techniques [29, 40]. In this Letter, 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 lethal concentrations of antibiotics. We designed a simple method to impede drug resistance by judiciously disrupting swarming motion. Non-toxic 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. 3.1). In turn, the bacteria drastically lose their defense 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 non-motile particles and an alignment rule are systematically implemented [12, 29].

FIG. 3.1 Scheme showing bacteria swarming in large rafts in the non-PS particle zone. The collectivity is disrupted in the presence of colloidal particles which results in more bacteria becoming vulnerable to the antibiotic.

3.3 Experimental

3.3.1 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. 10% (w/w) polystyrene (PS) beads (1.1 μm in diameter, Sigma-Aldrich) suspended in water was diluted to the desired concentration using filter-sterile water.

3.3.2 Growth Conditions

B. subtilis was cultured in LB broth (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl in water) at 37 °C until an OD₆₀₀ 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 petridish and dried for 1 h. To obtain swarming cells, 2 μL of the bacterial culture was inoculated at the centre 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 and the surface density of PS particles were 0.06, 0.015 and 0.0035 particles μm^{-2} respectively. The plate was subsequently dried until

the PS colloidal particles remain immobilized on the agar surface.

3.3.3 Antimicrobial Assays

Disc diffusion method was used to assess bacterial sensitivity to antibiotics (*i.e.*, Tob or Van). The antibiotic discs were made from circular filter paper (6 mm-diameter) and sterilized under a UV-ozone lamp for 30 min. 5 μL of freshly prepared 150 mg mL^{-1} tobramycin or 6 mg mL^{-1} vancomycin solution was then added onto the paper discs and dried. The final drug concentrations were 750 μg tobramycin and 30 μg vancomycin per disc ($\mu\text{g}/\text{pc}$). 1 to 1.5 h after inoculating the center of the agar with bacteria, an antibiotic disc was placed 25 mm from the inoculum. The inhibition zone size (r) was defined as the shortest distance from the edge of the antibiotic disc 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 disc 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, colloidal particles (0.06 particles μm^{-2})

were first immobilized on the agar surface before the bacteria was inoculated.

Live/dead staining was performed by slicing out the agar carrying the secondary front and placed upside-down on a glass coverslip containing 10 μL of the live/dead stain (Baclight, Invitrogen) and incubated for 15 min. The fluorescent image was collected using a fluorescent microscopy (Nikon Eclipse TE2000-E).

Viability assay for planktonic cells with and without PS particles was conducted. Overnight 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 was collected from bacterial culture at 0, 1, 2, 3 and 4h after dilution, and evenly applied on the surface of a LB-agar plate with an L-shape spreader. The plate was incubated overnight, and the colony number was counted the next day.

3.3.4 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 $\times$, N.A. = 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 - 35 $^{\circ}\text{C}$ *via* a heating plate. The

diffraction of light from colloidal particles was subtracted.

Bacterial motion was analyzed using particle image velocimetry (PIV) (PIVlab 1.31 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, 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 $\langle \dots \rangle_{ij}$ 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 3 times. For the inhibition zone size experiment, we repeated the experiment at least 5 times. All experiments were highly reproducible (e.g., the error for the observed correlation lengths was $< \pm 0.5$).

3.3.5 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 corresponding 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. 3.2. We defined the nearest immobilized neighbors for each stationary particle to be the 4 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 $\theta_i(t + \Delta t)$.

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, 44]:

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

where $\eta \xi_i(t)$ is an intrinsic noise with intensity η and ξ is a random number between $-\pi$ and π . 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 non-motile). In the simulation, the density of moving particles is 0.07, $v = 0.1$, $\eta = 0.4$ and $x = 5$ [29].

The intrinsic noise $\eta \xi_i(t)$ is ascribed to the bacterium's intrinsic uncertainty in its motion after receiving an average 'signal' from the neighboring j bacteria [12, 17, 44-46]. In this case, η is the intensity of the noise and $\xi_i(t)$ is a δ -correlated white noise that is uniformly distributed between $-\pi$ and π [12, 17, 44-46]. This means that after receiving an average signal, the particle can move with an equal probability in any direction between $-\eta\pi$ to $\eta\pi$ with respect to the average signal. 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 non-toxic 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. The values of $v = 0.1$, $\eta = 0.4$ and $x = 5$ follow the study reported in ref. [29].

Periodical boundary condition was applied, and the total number of time-steps for each run is 15000.

FIG. 3.2 The distribution of stationary particles for $C_p = 0$ (a), 0.0034 (b), 0.014 (c) and 0.054 (d).

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 centres 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. 3.3). 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 centres 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 Fig. 3.3. 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 3 simulation runs were repeated for each condition and the results were reproducible (*i.e.*, error for the calculated correlation length L was $< \pm 1.0$).

FIG. 3.3 Schematic illustration of the incoming and outgoing velocity for the hard-core repulsion model that takes into account the ‘incoming’ angle. x and y are the vertical and horizontal axis, respectively. V_v is the vertical component of the incoming and outgoing velocity along the x -axis, and V_p and V_p' are the horizontal components of the incoming velocity and outgoing velocity, respectively. The outgoing velocity is constructed from V_v and V_p' .

3.4 Results and discussion

3.4.1 Swarming motility protect single-layered bacteria in lethal concentrations of drugs

For swarming *B. subtilis*, the size of the inhibition zone, determined from disc diffusion experiments (Fig. 3.4(c) and Fig 3.5(c)), are $r = 1.3 \pm 0.6$ mm and 2.5 ± 0.3 mm for Tob (750 $\mu\text{g/pc}$) and Van (30 $\mu\text{g/pc}$), respectively (Fig. 3.6). A single layer of bacteria advances beyond the primary front (multilayered colony) and continues to expand toward the disc, forming a secondary front (monolayered colony) before coming to a halt [36]. In comparison, lethal amounts of drugs are determined to be within 8.2 ± 0.2 mm and 4.6 ± 0.2 mm from the Tob and Van discs, respectively (Fig. 3.6 and Fig. 3.7). All inhibition zone sizes are measured between 4.5 to 5 h after incubation to ensure that drug diffusion is standardized in all experiments.

FIG. 3.4 Swarming colony morphology recorded at various times after inoculation t_i for tobramycin (Tob, (a) $t_i = 150$ min, (b) $t_i = 210$ min and (c) $t_i = 240$ min). The agar is inoculated at the center (y) and the drug disc x is placed at the top of the agar plate. The shortest distances between colony edge and drug disc are 9.4, 3.5, 1.0 for (a), (b) and (c) respectively.

FIG. 3.5 Swarming colony morphology recorded at various times after inoculation t_i for vancomycin (Van, (a) $t_i = 150$ min, (b) $t_i = 210$ min and (c) $t_i = 240$ min). The agar is inoculated at the center (y) and the drug disc x is placed at the top of the agar plate. The shortest distances between colony edge and drug disc are 9.3, 3.1 and 2.5 mm for (a), (b) and (c), respectively.

FIG. 3.6 Inhibition zone sizes of planktonic and swarming cells in the absence and presence of colloidal particles for Tob and Van.

FIG. 3.7 Inhibition zones of planktonic *B. subtilis* for Tob in the (a) absence and (c) presence of PS particles, and Van in the (b) absence and (d) presence of PS particles. The density of particle is $0.06 \text{ particles } \mu\text{m}^{-2}$. The inhibition zone sizes are $8.2 \pm 0.2 \text{ mm}$, $4.6 \pm 0.2 \text{ mm}$, $8.2 \pm 0.3 \text{ mm}$ and $4.3 \pm 0.2 \text{ mm}$ for (a), (b), (c) and (d), respectively. Disc diffusion experiment shows that for planktonic cells on hard agar (*i.e.*, 3%), non-motile bacteria within an inhibition zone of $r = 8.2 \pm 0.2 \text{ mm}$ and $4.6 \pm 0.2 \text{ mm}$ for Tob and Van, respectively, are either killed or unable to proliferate. Since the size of the inhibition zone is not dependent on agar concentrations [36], lethal amounts of drugs are found within $8.2 \pm 0.2 \text{ mm}$ and $4.6 \pm 0.2 \text{ mm}$ from the Tob and Van discs, respectively.

In order to unravel the dynamics of collective motion of swarming bacteria as they move toward the antibiotic disc, real-time imaging of cells close to the secondary front (*i.e.*, $< 200 \mu\text{m}$ 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. 3.4(a)). Movie 3.1 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 $L = 13.1 \mu\text{m}$ (Fig. 3.8). When $t_i = 210$ min, the swarm monolayer is exposed to Tob (Fig. 3.4(b)). The bacteria continue to move cohesively in rafts, albeit with reduced collectivity (*i.e.*, $L = 10.0 \mu\text{m}$, Fig. 3.8) and significantly slower speed (*i.e.*, $V = 15.8 \pm 0.4 \mu\text{m s}^{-1}$) (Movie 3.2). 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 $D = 0.048 \text{ cells } \mu\text{m}^{-2}$ to $D = 0.090 \text{ cells } \mu\text{m}^{-2}$ 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.

FIG. 3.8 The spatial correlation function of instantaneous velocity (C_v) vs. distance for Tob at $t_i = 150$ min (red), 210 min (green) and 240 min (blue). The inset shows the corresponding L and V .

As the colony continues to expand closer to the antibiotic disc, a larger fraction of the cells experiences high dosages of Tob. For example, at $t_i = 240$ min (Movie 3.3), the swarm stops advancing (Fig. 3.4(c)). Apart from a lack of sufficient surfactin secreted to induce proper swarm expansion, the slow moving bacteria behind the thick layer (3 to 4 cell length) 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. 3.8). 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 3.4). However, a large portion of cells located further away from the front remain mobile (*e.g.*, $\sim 500 \mu\text{m}$, see Movie 3.5). About 90% of frontal cells are not killed by Tob as demonstrated by live/dead

staining experiments (Fig. 3.9). When the drug used is Van, the correlation length and average speed of *B. subtilis* display similar qualitative behavior as observed for Tob (Fig. 3.10). Reductions in both spatial correlation and speed were also observed for swarming cells experiencing external stress due to toxic oxygen species [29].

FIG. 3.9 Live-dead staining of bacteria close to the secondary front of colony at $t_i = 270$ min. Live bacteria are stained green (*ca.* 90%) and dead bacteria are stained red. Experimental details are described in section 3.3.3.

FIG. 3.10 The spatial correlation function of instantaneous velocity vs. distance for Van at $t_i = 150$ min (red), 210 min (green), and 240 min (blue). The insets show the corresponding correlation lengths of C_v and average speed. When the drug used is Van, the correlation length and average speed of *B. subtilis* display similar qualitative behavior as observed for Tob. $L = 14.4 \mu\text{m}$ and $V = 25.3 \pm 0.9 \mu\text{m s}^{-1}$ at $t_i = 150$ min, $L = 9.6 \mu\text{m}$ and $V = 12.8 \pm 0.3 \mu\text{m s}^{-1}$ at $t_i = 210$ min, and $L = 6.5 \mu\text{m}$ and $V = 8.7 \pm 0.3 \mu\text{m s}^{-1}$ at $t_i = 240$ min.

3.4.2 Polystyrene particles on the surface disrupt swarming motion

Polystyrene (PS) particles with 1.1 μm diameter, approximately the width of the bacteria, are used in this study to disrupt the collective behavior of *B. subtilis*.

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 culture with the same cell concentrations were prepared; one without PS colloidal particles and the other with particles μm^{-2} PS particles. Colony forming unit (CFU) assay was conducted for both cell cultures after incubation for 0, 1, 2, 3 and 4 h. Fig. 3.11 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 do not affect the viability and proliferation of the bacteria.

FIG. 3.11 The growth curves of bacterial culture with (black) and without (red) PS particles ($0.06 \text{ particles } \mu\text{m}^{-2}$) for different incubation times.

FIG. 3.12 Swarming colony of *B. subtilis* on a semi-solid agar (0.5%). The cell culture used to inoculate the agar was first incubated with a PS colloidal suspension (1% (w/w)).

Fig. 3.12 shows the bacterial colony formed from an inoculum of *B. subtilis* and PS colloidal particles (1% (w/w)). Both bacteria and PS particles were co-incubated 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.*, see Fig. 3.4(a)). Furthermore, swarming bacteria is observed in the monolayer leading to the colony front (see Movie 3.6). The corresponding correlation length is $13.7 \mu\text{m}$ and average speed is 25.4 ± 0.9 (close to the values for healthy bacteria); indicating that the PS particles do not affect cell differentiation from planktonic to swarming phenotypes.

Swarming bacteria are also observed to spread on agar surfaces containing immobilized PS particles. Fig. 3.13(a) shows the colony patterns in the PS particle (top half of agar plate, particle density $C_p = 0.06$ particles μm^{-2}) and non-PS particle (bottom half) zones. 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. Furthermore, *B. subtilis* do not adhere to the surfaces of the inert colloidal particles (Movie 3.7).

FIG. 3.13 Colony morphology in PS particle zone (top half) and non-PS particle zone (bottom half): (a) in the absence of antibiotics, and in the presence of (b) Tob and (c) Van. The arrow in (c) indicates the secondary front. The particle density $C_p = 0.06$ particles μm^{-2} .

To make sure that the particles do not hinder front propagation and colony expansion, colony expansion on both PS particle zone and non-PS particle zone is measured. Fig. 3.14 shows the distance of the colony front from the point of inoculum (*i.e.*, centre of agar) at various times for both the colony in the PS particle (0.06 particles μm^{-2}) and non-PS particle zones. We observed that the distances of the colony front from the centre of agar do 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 $\mu\text{m s}^{-1}$ 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 min).

FIG. 3.14 The distance from the colony front to the point of inoculum (*i.e.*, centre of the agar) X for the colony in the non-PS particle zone ($\circ$) and PS particle zone ($\square$) at different times.

For a swarming colony *without* PS particles, a layer of stalled bacteria is found at the edge of the colony as seen in Movie 3.1 and in ref. [47]. As discussed by Darnton *et al.* [47], 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 *with* PS particles, a layer of stalled bacteria is not formed because the colloidal particles break up the aggregation of the frontal cells (see Movie 3.8). Smaller packs and individual motile bacteria are seen at the colony front. We note from Movie 3.8 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 and the bacterial speed is lower than normally swarming bacteria.

The collective motion of the monolayered 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, particle density on the agar surface $C_p = 0.06$), as illustrated in Movie 3.7. The PIV image in Fig. 3.15 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 $(x = 83$

μm , $y = 39 \mu\text{m}$)). 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 therefore forced to squeeze through the gaps between PS particles which leads to the observed diminished collectivity and speed.

When C_p increases, both the collectivity (L) and speed of swarming bacteria (V) decrease. Since the speed of healthy swarming bacteria increases with an increase in bacterial density D [47], a similar D ($\sim 0.07 \text{ cells } \mu\text{m}^{-2}$) is maintained throughout the experiment at various C_p . The L (V) values for $C_p = 0.06, 0.015, 0.0035$ (Fig. 3.16) and $0 \text{ particles } \mu\text{m}^{-2}$ are $7.8 \mu\text{m}$ ($11.7 \pm 0.4 \mu\text{m s}^{-1}$), $12.9 \mu\text{m}$ ($23.8 \pm 2.4 \mu\text{m s}^{-1}$), $14.9 \mu\text{m}$ ($34.1 \pm 2.9 \mu\text{m s}^{-1}$) and $18.4 \mu\text{m}$ ($42.7 \pm 6.9 \mu\text{m s}^{-1}$), respectively (Fig. 3.17). 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.

FIG. 3.15 Instantaneous velocity field image of *B. subtilis* (Movie 3.7). The green line divides the PS-particle zone (left of the dividing line) from the non-PS particle zone (right of the dividing line).

FIG. 3.16 Particle density $C_p = 0.0035$ particles μm^{-2} (a) and 0.015 particles μm^{-2} (b).

FIG. 3.17 Spatial correlation function of instantaneous velocity for different PS particle densities: $\diamond$ 0.06 (cyan), Δ 0.015 (blue), $\circ$ 0.0035 (green) and $\square$ 0 (red) particles μm^{-2} . The inset shows the corresponding L and V .

3.4.3 Simulation

Computational simulation *via* a simple self-propelled particle model is used to model the motion of swarming cells in the presence of colloidal particles [12, 29]. 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 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 (Fig. 3.18). when the surface density of immobile particles $C_p' = 0.014, 0.0034$ and 0.054 , respectively, are used in the simulation. 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 particle size) for $C_p' = 0.0034$. This distance is reduced to $l = 4.7$ (*ca.* 1 - 2 particle size) for $C_p' = 0.054$ (Fig. 3.2). 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. The relationship between collectivity and particle density seen in the simulation study is in line with the experimental observation.

FIG. 3.18 The spatial correlation functions computed at the 15000th time-step in the (a) absence of stationary particles and (b), (c), (d) presence of stationary particles (surface density = 0.0034 (b), 0.014 (c), and 0.054 (d)).

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. 3.7), which are not significantly different from the values determined for planktonic cells in the absence of PS particles (Fig. 3.6). 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.

3.4.4 Disruption of swarming motion leads to reduced antibiotic resistance for swarming colony

The effect of PS particles on the drug-resistance properties of swarming bacteria is studied by placing an antibiotic disc at both ends of the agar plate equidistant from the point of inoculation (*i.e.*, center of culture plate) (Fig. 3.19). 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. Fig. 3.19(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 $r = 5.5 \pm 0.9$ mm which is significantly larger than $r = 1.3 \pm 0.6$ mm observed in the non-PS particle zone (Fig. 3.6). 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. 3.13(c)). Similar qualitative behavior is observed when different drug concentrations are used (see Fig. 3.20 for Tob disc concentration of 150 $\mu\text{g}/\text{pc}$). 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.

To investigate if PS particle density influences the Tob-resistance properties of swarming bacteria, drug diffusion experiments were further conducted using different colloidal particle densities. The inhibition zone sizes are determined to be 5.5 ± 0.9 mm, 2.0 ± 0.1 mm, 1.4 ± 0.4 mm and 1.3 ± 0.6 mm for PS particle densities 0.06, 0.015 and 0.0035 and 0 particles μm^{-2} , respectively (Fig. 3.21). The enhanced

drug-resistance property is not significantly affected at low colloidal particle densities. For example, when the PS particle density utilized is 0.0035, the inhibition zone size is similar to that noted in the absence of colloidal particles. In this case, the swarming bacteria persist to display strong collectivity and fast mobility, and the relatively intact group dynamics allow the cells to remain resilient against drugs. When a high particle density is used, the inhibition zone is large (*e.g.*, 5.5 ± 0.9 mm) suggesting that drug-resistance due to strong multicellular group dynamics is lost. The disrupted swarm is easily damaged when compared to cells in either non-PS or low PS particle zones. This means that the disrupted swarm colony is not able to resist the antibiotic and move close to the drug disc. 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.

FIG. 3.19 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 shortest distances between the colony edge and drug disc in the PS particle zone are 13.3, 6.2, 5.2 and 5.1 mm for (a), (b), (c) and (d), respectively.

FIG. 3.20 When Tob concentration is 150 $\mu\text{g}/\text{pc}$, the swarming bacteria overwhelm the whole non-PS particle area, leaving no inhibition zone (lower half). On the other hand, in the PS particle zone ($C_p = 0.06$), an inhibition zone of 1.7 ± 0.3 mm is still clearly observed (upper half).

FIG. 3.21 (A) Colony morphology of swarming *B. subtilis* with different PS particle densities C_p : (a) 0.0035, (b) 0.015 and (c) 0.06 μm^{-2} . x is the Tob disc and y is the inoculum. (B) The inhibition zone sizes in the absence and presence of different C_p .

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. 3.19(a)) where a distinct edge of jammed bacteria is not observed (Movie 3.8 and Fig. 3.22(a)). 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. The cell density is $0.05 \text{ cells } \mu\text{m}^{-2}$ which is close to the density without particles. When exposed to Tob between $t_i = 210$ min (Fig. 3.19(b)) and $t_i = 270$ min (Fig. 3.19(d)), the shortest distance from the colony edge to the drug disc 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 disc, the swarm spreads at a slower rate due to more efficient cell damage by Tob.

FIG. 3.22 Microscopy images of bacteria in the presence of immobilized particles at (a) $t_i = 150$ min and (b) $t_i = 240$ min.

3.4.5 Effective cell translocation and exchange by collective raft motion increases antibiotic resistance in single-layered bacterial colony

Resistance observed in the absence of PS particles can be rationalized as follows. Movie 3.2 shows that there are bacteria close to the swarm edge that 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*. We performed particle tracking to demonstrate the extent of transportation and exchange of cells in the monolayer. Fig. 3.23 shows typical trajectories of motion of single bacteria *close* to the colony front and initially away from the edge of the observation window without colloidal particles and drugs (a), with colloidal particles (b), and without colloidal particles but exposed to drug (c). We note that in the absence of colloidal particles and drug, there exist bacteria that are capable of moving long distances away from the colony edge and toward the colony interior (Fig. 3.23(a)). In particular, bacteria 1, 2 and 3 in Fig. 3.23(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. 3.23(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. 3.23(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. 3.23(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.

FIG. 3.23 (a) Trajectories of motion of 5 bacteria close to the colony front on agar without both PS particles and drug. S1, S2, S3, S4, S5 are the starting points of the 5 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 5 bacteria close to the colony front on agar with PS particles. S1, S2, S3, S4, S5 are the starting points of the 5 bacteria. (c) Trajectories of motion of 5 bacteria close to the colony front on agar without PS particles but exposed to drug. S1, S2, S3, S4, S5 are the starting points of the 5 bacteria. The trajectories were calculated using Movie 3.1 (150 min without PS particles) for (a), Movie 3.8 (150 min with PS particles) for (b) and Movie 3.2 (210 min without PS particles) for (c) and the observation time is from 13.3 to 15 s.

We selected 10 bacteria each from Movie 3.1 and S2 that move efficiently away from the front to the colony interior and calculated their mean square displacement (MSD) to illustrate the effects of stationary hurdles. The MSD of bacterium i is calculated from:

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

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 RMSD for 10 typical bacteria that move long distances in the absence of colloidal particles in Movie 3.1 and S2 are 39 μm and 29 μm , respectively. On the other hand, the average RMSD for 10 typical bacteria in the presence of colloidal particles (Movie 3.8) is 10 μm . Clearly, the RMSD without colloidal particles is larger than the RMSD with colloidal particles.

Furthermore, close to the border between the primary and secondary fronts (*ca.* 8 – 10 mm away from edge), a constant exchange between cells in the drug-exposed monolayer with cells in the more protected multilayered colony takes place (Movie 3.9) [35]. It is also observed that motile bacteria moving into the advancing front squeeze out stalled bacteria at the edge (Movie 3.10 and Fig. 3.24). 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 damages and to continue spreading.

FIG. 3.24 A cartoon depicting the exchange between motile swarming cells (blue dots) with cells found at the colony edge (red dots) (Movie 3.10, 210 min). A pack of swarming cells move rapidly from behind the edge (dark yellow) towards the colony front (light yellow) so that temporarily stalled cells are squeezed out.

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 cripple their ability to effectively return to the inner colony (Movie 3.8 and Fig. 3.23). When exposed to drug, such behavior will prevent an efficient relocation of frontal cells away from areas of lethal drug concentrations, 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.* 2-3 cell length) but densely packed monolayer of cells ($\sim 0.17 \text{ cells } \mu\text{m}^{-2}$) with negligible motion followed by a multilayer of bacteria are observed (*e.g.*, Movie 3.11 and Fig. 3.22(b) for $t_i = 240 \text{ min}$). In single-layered colonies, swarming bacteria remain relatively more resilient when compared to their PS particle-disrupted counterparts. When swarming behavior is disrupted, the bacteria become highly susceptible to the drugs regardless of the mechanism of action

imposed by the antibiotics (Vancomycin acts by inhibiting the synthesis of cell wall in gram-positive bacteria. It is able to bind to the D-Ala-D-Ala residue to prevent the cross-linking of monomers that forms the peptidoglycan backbone of the cell wall. Tobramycin acts by inhibition bacterial protein synthesis. It binds to the bacterial 30S and 50S ribosome to prevent them from forming the 70S complex, which is essential in the translation from mRNA to protein). This clearly underscores the importance of collective dynamics in elevating drug resistance.

3.5 Conclusion

Rapid coordinated motion within multicellular packs is visualized in real-time and found to be instrumental in ensuring that single-layered bacteria located at the expanding colony front suffer minimal damages when in contact with antibiotics. In this manner, we have designed a simple method to impede drug resistance by judiciously disrupting swarming motion. Non-toxic 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.

The disruption of collective motion is simulated using an improved self-propelled Vicsek model where hard-core repulsion between particles and an alignment rule are systematically implemented. 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 the result agree with experimental observations.

The bacteria lose their defense against tobramycin and vancomycin drastically when their motion speed and collectivity is disrupted, indicating that swarming motion dynamics may also play an important role in antibiotic resistance in single-layered colonies as it does in multilayered colonies.

3.6 Movie captions

Movie 3.1 The real time motion of swarming *B. subtilis* close to secondary front at t_i = 150 min. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 3.2 The real time motion of swarming *B. subtilis* close to secondary front at t_i = 210 min. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 3.3 The real time motion of swarming *B. subtilis* close to secondary front at t_i = 240 min. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 3.4 The real time motion of swarming *B. subtilis* close to secondary front at t_i = 270 min. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 3.5 The real time motion of swarming *B. subtilis* in a region *ca.* 500 μm from the colony edge at t_i = 270 min. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 3.6 The real time motion of swarming *B. subtilis* close to secondary front at t_i = 150 min. The inoculum consists of *B. subtilis* and PS colloidal particles (1% (w/w)). The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 3.7 The real time motion of swarming *B. subtilis* moving on agar where approximately half the surface contain immobilized PS particles. The colloidal

particles are the black circular dots and the particle density is $0.06 \text{ particles } \mu\text{m}^{-2}$. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 3.8 The real time motion of swarming *B. subtilis* close to secondary front at $t_i = 150 \text{ min}$ and in the presence of PS particles. The colloidal particles are the black circular dots and the particle density is $0.06 \text{ particles } \mu\text{m}^{-2}$. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 3.9 The continuous exchange of bacteria in the monolayer (right of screen) with bacteria in the multilayered colony (left of screen) at $t_i = 270 \text{ min}$. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

Movie 3.10 A movie depicting the exchange between motile swarming cells (blue) with cells temporarily stalled at the colony edge (red) at $t_i = 210 \text{ min}$. A pack of swarming cells move rapidly from behind the edge towards the colony front so that temporarily stalled cells are squeezed out. The speed of the movie is reduced by $3\times$. The dimension of an image is $47 \times 47 \mu\text{m}^2$.

Movie 3.11 The real time motion of swarming *B. subtilis* close to secondary front at $t_i = 240 \text{ min}$ and in the presence of PS particles. The colloidal particles are the black circular dots and the particle density is $0.06 \text{ particles } \mu\text{m}^{-2}$. The dimension of an image is $120 \times 120 \mu\text{m}^2$.

3.7 Bibliography

1. Vicsek, T., *A question of scale*. Nature, 2001. **411**(6836): p. 421-421.
2. Becco, C., et al., *Experimental evidences of a structural and dynamical transition in fish school*. Physica a-Statistical Mechanics and Its Applications, 2006. **367**: p. 487-493.
3. Buhl, J., et al., *From disorder to order in marching locusts*. Science, 2006. **312**(5778): p. 1402-1406.
4. Ward, A.J.W., et al., *Quorum decision-making facilitates information transfer in fish shoals*. Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(19): p. 6948-6953.
5. Ballerini, M., et al., *Empirical investigation of starling flocks: a benchmark study in collective animal behaviour*. Animal Behaviour, 2008. **76**: p. 201-215.
6. Moussaid, M., D. Helbing, and G. Theraulaz, *How simple rules determine pedestrian behavior and crowd disasters*. Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(17): p. 6884-6888.
7. Friedl, P. and D. Gilmour, *Collective cell migration in morphogenesis, regeneration and cancer*. Nature Reviews Molecular Cell Biology, 2009. **10**(7): p. 445-457.
8. Szabo, B., et al., *Phase transition in the collective migration of tissue cells: Experiment and model*. Physical Review E, 2006. **74**(6): p. 061908.
9. Szabo, A., et al., *Collective cell motion in endothelial monolayers*. Physical Biology, 2010. **7**(4): p. 046007.

10. Helbing, D., I. Farkas, and T. Vicsek, *Simulating dynamical features of escape panic*. Nature, 2000. **407**(6803): p. 487-490.
11. Helbing, D., et al., *Active walker model for the formation of human and animal trail systems*. Physical Review E, 1997. **56**(3): p. 2527-2539.
12. Vicsek, T., et al., *Novel Type of Phase-Transition in a System of Self-Driven Particles*. Physical Review Letters, 1995. **75**(6): p. 1226-1229.
13. Vicsek, T. and A. Zafeiris, *Collective motion*. Physics Reports-Review Section of Physics Letters, 2012. **517**(3-4): p. 71-140.
14. Bertin, E., M. Droz, and G. Gregoire, *Hydrodynamic equations for self-propelled particles: microscopic derivation and stability analysis*. Journal of Physics a-Mathematical and Theoretical, 2009. **42**(44): p. 445001.
15. Huepe, C. and M. Aldana, *Intermittency and clustering in a system of self-driven particles*. Physical Review Letters, 2004. **92**(16): p. 168701.
16. Ginelli, F. and H. Chate, *Relevance of Metric-Free Interactions in Flocking Phenomena*. Physical Review Letters, 2010. **105**(16): p. 168103.
17. Baglietto, G. and E.V. Albano, *Nature of the order-disorder transition in the Vicsek model for the collective motion of self-propelled particles*. Physical Review E, 2009. **80**(5): p. 050103.
18. Chate, H., et al., *Collective motion of self-propelled particles interacting without cohesion*. Physical Review E, 2008. **77**(4): p. 046113.
19. Cisneros, L.H., et al., *Fluid dynamics of self-propelled microorganisms, from individuals to concentrated populations*. Experiments in Fluids, 2007. **43**(5): p.

737-753.

20. Sokolov, A., et al., *Concentration dependence of the collective dynamics of swimming bacteria*. Physical Review Letters, 2007. **98**(15): p. 158102.

21. Huepe, C. and M. Aldana, *New tools for characterizing swarming systems: A comparison of minimal models*. Physica a-Statistical Mechanics and Its Applications, 2008. **387**(12): p. 2809-2822.

22. Romensky, M., V. Lobaskin, and T. Ihle, *Tricritical points in a Vicsek model of self-propelled particles with bounded confidence*. Physical Review E, 2014. **90**(6): p. 063315.

23. Chou, Y.L. and T. Ihle, *Active matter beyond mean-field: Ring-kinetic theory for self-propelled particles*. Physical Review E, 2015. **91**(2): p. 022103.

24. Peruani, F., et al., *Traffic Jams, Gliders, and Bands in the Quest for Collective Motion of Self-Propelled Particles*. Physical Review Letters, 2011. **106**(12): p. 128101.

25. Peruani, F., A. Deutsch, and M. Bar, *Nonequilibrium clustering of self-propelled rods*. Physical Review E, 2006. **74**(3): p. 030904 (R).

26. Ginelli, F., et al., *Large-Scale Collective Properties of Self-Propelled Rods*. Physical Review Letters, 2010. **104**(18): p. 184502.

27. Wu, Y.L., et al., *Periodic reversal of direction allows Myxobacteria to swarm*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(4): p. 1222-1227.

28. Czirok, A., M. Matsushita, and T. Vicsek, *Theory of periodic swarming of*

- bacteria: Application to Proteus mirabilis*. Physical Review E, 2001. **63**(3): p. 031915.
29. Lu, S.T., et al., *Loss of Collective Motion in Swarming Bacteria Undergoing Stress*. Physical Review Letters, 2013. **111**(20): p. 208101
30. Kearns, D.B., *A field guide to bacterial swarming motility*. Nature Reviews Microbiology, 2010. **8**(9): p. 634-644.
31. Copeland, M.F. and D.B. Weibel, *Bacterial swarming: a model system for studying dynamic self-assembly*. Soft Matter, 2009. **5**(6): p. 1174-1187.
32. Partridge, J.D. and R.M. Harshey, *Swarming: Flexible Roaming Plans*. Journal of Bacteriology, 2013. **195**(5): p. 909-918.
33. Kim, W., et al., *Swarm-cell differentiation in Salmonella enterica serovar typhimurium results in elevated resistance to multiple antibiotics*. Journal of Bacteriology, 2003. **185**(10): p. 3111-3117.
34. Kim, W. and M.G. Surette, *Swarming populations of Salmonella represent a unique physiological state coupled to multiple mechanisms of antibiotic resistance*. Biol Proced Online, 2003. **5**: p. 189-196.
35. Butler, M.T., Q.F. Wang, and R.M. Harshey, *Cell density and mobility protect swarming bacteria against antibiotics*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(8): p. 3776-3781.
36. Lai, S., J. Tremblay, and E. Deziel, *Swarming motility: a multicellular behaviour conferring antimicrobial resistance*. Environmental Microbiology, 2009. **11**(1): p. 126-136.

37. Fernandez, L., E.B.M. Breidenstein, and R.E.W. Hancock, *Creeping baselines and adaptive resistance to antibiotics*. Drug Resistance Updates, 2011. **14**(1): p. 1-21.
38. Lee, H.H., et al., *Bacterial charity work leads to population-wide resistance*. Nature, 2010. **467**(7311): p. 82-U113.
39. Benisty, S., et al., *Antibiotic-Induced Anomalous Statistics of Collective Bacterial Swarming*. Physical Review Letters, 2015. **114**(1): p. 018105.
40. Zhang, H.P., et al., *Collective motion and density fluctuations in bacterial colonies*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(31): p. 13626-13630.
41. Kearns, D.B. and R. Losick, *Swarming motility in undomesticated Bacillus subtilis*. Molecular Microbiology, 2003. **49**(3): p. 581-590.
42. Kearns, D.B., et al., *Genes governing swarming in Bacillus subtilis and evidence for a phase variation mechanism controlling surface motility*. Molecular Microbiology, 2004. **52**(2): p. 357-369.
43. Calvio, C., et al., *Autoregulation of swrAA and motility in Bacillus subtilis*. Journal of Bacteriology, 2008. **190**(16): p. 5720-5728.
44. Czirok, A., et al., *Formation of complex bacterial colonies via self-generated vortices*. Physical Review E, 1996. **54**(2): p. 1791-1801.
45. Pimentel, J.A., et al., *Intrinsic and extrinsic noise effects on phase transitions of network models with applications to swarming systems*. Physical Review E, 2008. **77**(6): p. 061138.
46. Aldana, M., H. Larralde, and B. Vazquez, *On the Emergence of Collective Order*

in Swarming Systems: A Recent Debate. International Journal of Modern Physics B, 2009. **23**(18): p. 3459-3483.

47. Darnton, N.C., et al., *Dynamics of Bacterial Swarming*. Biophysical Journal, 2010. **98**(10): p. 2082-2090.

Chapter 4

Position-Correlated Hierarchy in a Self-Propelled Particle Model Describes Collective Turns in Natural Flocks

4.1 Abstract

Position-correlated hierarchy is introduced by adding a weightage parameter to Vicsek model (VM) to simulate collective motion systems. The new position-correlated hierarchical self-propelled particle (PCH-SPP) model displays a rapid, linear-styled transfer of motion information during group turning: a phenomenon not observed in simulations by the VM. With the PCH-SPP model, the collective turning of bird flocks is reproduced by simulation. The model overcomes the inability of VM in simulating collective turning phenomenon by enhancing the propagation of information without having significant difference in the steady-state phase behavior of the VM simulations.

4.2 Introduction

Collective motion is a common phenomenon occurring in bird flocks, fish schools, and swarming bacterial colonies [1-11]. The modeling of such systems reveals important knowledge on biological behavior as well as active matter properties [2, 6, 8-15]. It is widely believed that in the biological world, collective motion is orchestrated by each individual receiving sensory signals such as vision, sound and chemical information [16]. Therefore, the rule for signal transferring is important for the determination of alignment [17].

A minimal self-propelled particle (SPP) model was proposed by Vicsek (*i.e.* Vicsek model, VM) in 1995 [18]. In this model, a group of self-propelled particles achieve system-wide coherency by localized aligning interaction. The VM has been successful in modeling bacterial swarming and steady state animal collective motion [19].

However, despite its simplicity and ability to describe collective motion, VM has its limitations. As all particles in the VM have the same strength in influencing their neighbors, the perturbation information (*e.g.* orientation change of a small portion of the particles) will be dampened in the averaging process [17]. Therefore, in the VM simulation, after reaching a highly coherent motion, the group will tend to "ignore" the perturbation and maintain its original motion orientation. This leads to unwanted behavior in real life (*e.g.* inability to effectively avoid predators) [16]. Many observations on the stress response of animal groups go against the VM's prediction. In 1973 Radakov first reported that by perturbing only a small part of a fish school,

the change in direction propagates rapidly across the whole school [20]. A mathematical analysis based on the observation of flocks of starling was reported in 2014 by Attansi *et. al.* In their experiment they found that changes in direction propagates robustly throughout the whole flock with a linear dispersion law [17]. The results contrast sharply with the diffusive information transfer predicted by the current VM [18].

By adopting a simple modification, we introduce the position-correlated hierarchy into the local interaction in VM. And By enhancing signal information propagation, the renovated position-correlated hierarchical self-propelled particle model (PCH-SPP model) has successfully overcome the VM's inability to simulate rapid group response to direction change without inducing significant differences in steady-state behavior. The PCH-SPP model expands the SPP model to many more applications. Further exploitation of the new model will lead to new explorations in biology, ecology and social behavior.

4.3 Experimental

4.3.1 Model description

In a self-propelled particle (SPP) model, the position of particle i at time $(t + 1)$ is given by $r_i(t + 1) = r_i(t) + v_i(t + 1)$, where $r_i(t)$ and $v_i(t + 1)$ are the position and velocity of the particle at time t and $(t + 1)$, respectively. The angle of motion of the i th particle at time $(t + 1)$ is determined by the average angle of motion of its neighboring particles at time t :

$$\theta_i(t + 1) = \text{Arg} \left[\sum_{\langle i, j \rangle} \alpha_j e^{i\theta_j(t)} \right] + \eta \xi_i(t) \quad (1)$$

where $\theta_j(t)$ is particle j 's angle of motion. The speed of each particle is assumed to be the same. A circle, centered at particle i , of radius x is the interaction area that contains neighboring particles j that directly influence the direction of motion of particle i . The intrinsic noise $\eta \xi_i(t)$ describes particle i 's uncertainty in motion after receiving an average velocity information from its neighboring particles. ξ is a δ -correlated white noise uniformly distributed between $-\pi$ and π , and η controls the noise intensity. The larger the value of η , the more chaotic and disorder the system becomes. When the noise is maximum (*i.e.*, $\eta = 1$), all the particles in the system perform random walks, whereas in the absence of noise (*i.e.*, $\eta = 0$) and at steady-state, the particles move collectively with the same velocity [18, 21].

The weightage parameter α_j in eq. (1) defines the contribution of the angle of motion of particle j to the direction of motion of particle i . To describe a turning event, all the particles in the system are initially made to move cohesively in a straight

direction with similar velocity (*i.e.*, flocking). The ultimate aim is to allow a particle, chosen randomly within the flock, initiate turning and induce other particles to undergo step-wise collective turning. The initiator is analogous to the fish (in the school) or bird (in the flock) that first encounters an agitation or prey, and rapidly changes its direction pathway while sending a signal to the rest of the team members to follow suit [22]. In the SPP model, the initiator transfers turning information to the rest of the particles in the flock *via* the interaction rule provided in eq. (1). We now examine the effects of α_j on the mode and efficiency of information transfer and its overall effect on collective flock turning.

In the Vicsek model (VM), all the neighboring particles j within the interaction area contribute equally to the velocity of particle i (*i.e.*, $\alpha_j = 1$), resulting in a diffusive and inefficient propagation of information (see discussion below) [18]. To facilitate and enhance information transfer from the initiator to distal particles, a position-correlated hierarchy SPP model (PCH-SPP) is implemented. The PCH-SPP model is based on the intuitive reasoning that particles that lie closer to the initiator (s), receive the correct turning information more effectively than those located further away from s . The former group of particles, in turn, has a larger influence on the velocity of particle i . This creates a hierarchy amongst neighboring particles within an interaction area based on their relative positions with respect to s . The α_j parameter in eq. (1) is thus expressed as:

$$\alpha_j = \begin{cases} \varepsilon_j > 1 \text{ for } (R_{js} < R_{is}) \\ 1 \text{ for } (R_{js} \geq R_{is}) \end{cases} \quad (2)$$

where R_{js} and R_{is} are the distances of particles j and i from s , respectively. Within a

localized interaction area, if particle j is closer to s as compared to particle j' (i.e., $R_{js} < R_{j's} < R_{is}$), the contribution to the velocity of particle i from particle j is higher than particle j' (i.e., $\varepsilon_j > \varepsilon_{j'} > 1$). Since the interaction radius x is much smaller than the size of the flock, it is assumed, without loss of generality, that the influence coefficient ε_j value is the same for all relevant particles in an interaction area.

4.3.2 Characterization of the PCH-SPP model

The relationship between phase behavior change with noise density and particle density are investigated. The simulation is carried out in a 20×20 square lattice with periodical boundary condition. The interaction radius $x = 2.0$ and particle speed $v_0 = 0.1$. Both the orientation of the direction vector and position of particles are randomly distributed throughout the lattice. In the VM, the initiator s is not included and $\alpha_j = 1$. In the PCH-SPP model, s is placed at the center of the square with a constant velocity $\mathbf{v}_s^T = (0.1, 0)$ throughout the simulation, and $\varepsilon_j = 2.0$.

The effects of noise intensity is performed by varying η from 0 to 1 at an increment interval of 0.05 while maintaining a constant particle number $N = 200$. The dependence of phase behavior on N is conducted by using N values ranging from 25 to 400 at an incremental interval of 25. In this case, η is kept constant at a value of 0.2. The initiator particle is not included in the calculation of the order parameter value. 2000 time-steps are used in each simulation run, and the system equilibrates within the first 1000 time-steps. 50 simulation runs are performed for each condition.

The phase behavior is characterized using the order parameter [18, 21]. The average value of the order parameter from $t = 1001$ to 2000 in 50 simulation runs is calculated for each condition:

$$\varphi(\eta, N) = \frac{1}{5 \times 10^4} \sum_{m=1}^{50} \sum_{t=1001}^{2000} \varphi(m, t)_{\eta, N} \quad (3)$$

where $\varphi(m, t)_{\eta, N}$ is the order parameter at time-step t in simulation m with noise intensity η and particle number N .

4.3.3 2-D information propagation experiment

We illustrate the dynamics of information propagation for a 2-D square lattice consisting of 625 (or 25×25) uniformly distributed particles. The positions of the particles are fixed throughout the simulation and only the orientation of their direction vectors vary with time. The distance between adjacent particles is 1 (unit length), and the particle at the center of the square lattice (*i.e.*, at position $(x = 13, y = 13)$) is chosen to be the initiator s . The direction vector of s is set as $\mathbf{v}_s^T = (0, 1)$ (*i.e.*, parallel to the y -axis) throughout the simulation, whereas the initial direction vectors of non-initiator particles are $\mathbf{v}^T = (1, 0)$ (*i.e.*, parallel to the x -axis). The direction vectors at $t = 0$ is illustrated in Fig. 4.1. The direction angle (φ) of a particle is defined as the angle between the corresponding direction vector and x -axis. Therefore, $\theta = 0^\circ$ for non-initiator particles at time $t = 0$ and $\theta_s = 90^\circ$ for s at all times. The dynamics of information transfer from s to non-initiator particles is obtained from the time-resolved variation of φ .

In the simulation, an interaction radius $x = 1.5$ is chosen such that each particle i is influenced by 8 neighboring particles (except for particles at the edges of the lattice which are influenced by either 3 or 5 neighboring particles). In both the VM and PCH-SPP model, $\alpha_j = 2$ for interactions that directly involve the initiator s . On the other hand, $\alpha_j = 1$ for non-initiator particles j in the Vicsek model. To demonstrate the effects of position-correlated hierarchy on the dynamics of information transfer, $\varepsilon_j = 2$ is implemented in the PCH-SPP model.

FIG. 4.1 Initial orientation of the 25×25 particles used in the information propagation simulation. Initiator s is placed at the center with θ_s fixed at 90° , and $\theta_i = 0^\circ$ for all other particles at $t = 0$.

4.3.4 2-D collective turning simulation

VM and PCH-SPP are used to simulate a 16×16 particle array with an initiator s . The distance between closest particles is 1. Particle speed v_0 is 0.1 step^{-1} . Interaction radius x is 1.5. $\varepsilon = 8$. Noise is set as 0 for convenience of analyze. s is placed 0.5 away from the rightmost column of particles. All particles and s has the same initial velocity $(0.1, 0)$. At the 100th step we assume the emergence of a predator from the right side. Then the initiator will lead the flock to evade predator by changing route. from step 101 to step 200, θ_s increases 1.8° per step. Thus s will take a 180° turn in a semicircle trajectory. At 200th step the velocity of s is $(-0.1, 0)$. From then on s will keep this velocity until the end of simulation. The simulation lasts 300 steps.

4.4 Results and discussion

4.4.1 Characterization of PCH-SPP

The average order parameter ϕ plotted against noise intensity η and particle number N are shown in Fig. 4.2(a) and 4.2(b), respectively. From the plots, it is clear that the phase behaviors of the VM and PCH-SPP model are similar. This suggests that introducing an initiator and hierarchy amongst particles do not significantly interfere with the system's steady-state behavior. The position-correlated hierarchy only serves to improve information transfer during the turning (as discussed below), and does not affect phase transition.

FIG. 4.2 (a) The steady-state order parameter ϕ of the VM (red) and PCH-SPP model (black) at different noise intensity η . The particle number $N = 200$. (b) The steady-state order parameter of the VM (red) and PCH-SPP model (black) with different particle number N and $\eta = 0.2$.

4.4.2 Information propagation

Movie 4.1 and 4.2 show the orientation change in the direction vector of non-initiator particles after receiving direction information from s during the first 200 time steps of the VM and PCH-SPP model runs, respectively. Clearly, at the end of 200 time steps, all the non-initiator vectors have perfectly aligned themselves with the initiator's direction vector in the PCH-SPP model (Movie 4.2). However, in the Vicsek model, direction vectors of non-initiator particles located far from s display only small changes in their direction, implying that information from s is not efficiently transported to these particles (Movie 4.1).

FIG. 4.3 (a) Velocity of particle array at step 50 in VM simulation. (b) Velocity of particle array at step 50 in PCH-SPP simulation. Signal source s is placed at the center with θ_s fixed at 90° , and every normal particle's θ_i is 0° . The vectors indicate the velocity direction of corresponding particle. Each vector is color mapped so that when θ_i increases from 0° to 90° the vector changes from blue to red. (c) and (d) 3-D surface plotting of θ against the corresponding x - and y -coordinates in (a) and (b), respectively. The z -axis value is the θ_i of each particle.

The orientation of the direction vector taken at the 50th time-step ($t = 50$) is shown in Fig. 4.3(a) and Fig. 4.3(b) for the VM and PCH-SPP model, respectively. This time is chosen for analysis because all the non-initiator particles are still in the process of alignment (*i.e.*, all particles have not reached the steady-state angular angle of 90°). It is observed that in the PCH-SPP model, all the non-initiator particles have attained $\theta > 10^\circ$, whereas in the VM, only 16 % of the particles around s have $\theta > 10^\circ$. The plot of θ against the corresponding x - and y -coordinates at $t = 50$ is given in Fig. 4.3(c) and Fig. 4.3(d) for the VM and PCH-SPP model, respectively. Clearly, the change in θ with respect to particle location is symmetrical about s . Furthermore, a faster decrease in θ across particles and away from s is seen in the VM as compared to the PCH-SPP model. In the VM, θ decays mono-exponentially for particles lying along the x -axis with a decay rate of 2.7 (Fig. 4.4). In contrast, θ decreases linearly with increasing spatial separation between the particle and s (*i.e.*, gradient of the liner fit = -4.87 in Fig. 4.4). In the VM, damping of information propagation restricts information from being relayed to particles that are separated far from s . Such detrimental behavior is not present in the PCH-SPP model where information is observed to be efficiently transported across several particles in a linear mode.

FIG. 4.4 θ of particles at row 13 (*i.e.* particles with coordination $(x,13)$) in Fig. 4.3 (c) (black) and Fig. 4.3 (d) (red) respectively. As the system is symmetrical, only half of the row (from $(14,13)$ to $(25,13)$) is plotted. It is worth mention that particle $(13,13)$ is the initiator s , thus the x value is positively correlated to the distance between particle and s .

The information propagation speed C_s is obtained by plotting the distance of non-initiator particle from s (l) vs. the corresponding time taken for the particle to attain $\theta = 45^\circ$ (t_{turn}). In the case of the VM, a non-linear plot is observed (Fig. 4.5). On the other hand, in the PCH-SPP model, a linear relationship, with a gradient of $C_s = 0.183$, exists between l and t_{turn} . Attanasi *et al.* have demonstrated that in starling flocks, l increases linearly with t_{turn} . Therefore, the PCH-SPP model introduced here clearly describes the dynamics of information propagation occurring in natural flocks [17].

FIG. 4.5 (a) The distance l travelled by information plotted against time (t_{turn}) for the VM (black) and PCH-SPP model (red). (b) A zoomed-in graph of the information propagation plot for the PCH-SPP model. The blue line indicates the linear fit.

4.4.3 Influence of weightage parameter ε on C_s

The dynamics of information propagation in the PCH-SPP model is influenced by ε . In Fig. 4.6(a), the linear plots of l vs. t_{turn} for different ε values are presented. The gradient for each ε values (*i.e.*, C_s) are shown in Fig. 4.6(b), where it is clearly observed that for higher ε values, information is propagated across particles at a higher speed. When ε is small C_s could change greatly with a little change in ε . And as ε gets larger C_s grows more insensitive to ε . The convergence of the relationship between C_s and ε is intuitive.

FIG. 4.6 (a) Information propagation plot of PCH-SPP simulations under different influence coefficient ε . The ε values used are: 1.5 (wine), 2 (dark yellow), 3 (magenta), 4 (cyan), 8 (blue), 16 (green), 32 (red) and 64 (black). (b) The information propagation speed C_s calculated from (a), each point is plotted in the same color as the plots in (a) with the corresponding ε .

4.4.4 Simulating collective turning in 2-D

With the renovated PCH-SPP the observation on real animal flock collective turning can be simulated. In this simulation we focus on reproducing the fast decision making in group orientation change due to enhanced information propagation instead of reproducing every single detail in reality, thus we simulate in 2-D to make data analysis convenient. Movie 4.3 and 4.4 shows the simulation of a 16×16 particle array with an initiator s (black square in the movie). Trajectories of s (black dash line) and representative particles are plotted in Fig. 4.7(a) and Fig. 4.7(b) for VM and

PCH-SPP simulation respectively. Representative particles are taken from VM and PCH-SPP at the same initial positions.

In PCH-SPP simulation (Movie 4.4), all particles show uniform trajectories (P1-P4 in Fig. 4.7(b)) regardless of the particle's position. This indicates the information from s is well preserved all the way to the most distant particles from s during transfer. The whole group is perfectly aligned with s before and after the turn. It is worth mention that from the movie it is shown that the turning of the group is not simultaneous: particle closer to s turn first, and then adjacent particles follows. This sequential manner in turning indicates the time needed for information propagation through particles. A turning delay exists between the particles. Due to such delay slight group deformation after turning is observed (Fig. 4.7(d)). A larger ε can be used to lower the deformation as the information transferring is faster.

In the VM simulation (Movie 4.3), from the trajectories it is shown that there are several types of particles based on their interaction with s (Fig. 4.7(a)). Though the influencing power of s is very strong, due to poor information propagation, when s starts to turn, only the particles closest to it will be strongly affected (e.g. P1 in Fig. 4.7(a)). And when some of such affected particles move out of influence area of s they soon lost alignment with s (e.g. P2 and P3 in Fig. 4.7(a)). Most particles which do not have close contact with s will keep their initial velocity with minimal change (e.g. P4 in Fig. 4.7(a)). The different types of particles form discrete clusters moving towards different directions. The group is scattered and group coherency is lost irreversibly (Fig. 4.7(c)).

Fig. 4.7 (a), (b) representative trajectories of particles from simulation in VM (a) and PCH-SPP (b). Trajectory of initiator s is plotted with black dash line. P1 - P4 marks the initial position of the four tracked particles. (c),(d) Particle motion at step 300 in VM (c) and PCH-SPP (d) simulations. Arrows are used to indicate the motion directions of discrete particle clusters. The particles are color mapped so that when θ_i increases from 0-180° the particle changes gradually from blue to red.

4.5 Conclusion

The information transfer is increased greatly by introducing a hierarchy determined by the particle's position. By enhancing signal information propagation, the renovated PCH-SPP has successfully reproduced the collective turning phenomenon observed in nature such as bird flocks by computational simulation. Furthermore, the steady state collective behavior remains unchanged. Thus this model can be used as an alternative for VM in many situations. By overcoming the fault that VM faces in collective turning. PCH-SPP expands the SPP models to more applications.

4.6 Movie captions

Movie 4.1 The information propagation in 2-D in VM simulation. The center is the initiator. The color of an vector is determined by its angle. Each vector is color mapped so that when θ_i increases from 0° to 90° the vector changes from blue to red. The frame rate of the movie is 50 fps.

Movie 4.2 The information propagation in 2-D in PCH-SPP simulation. The center is the initiator. The color of an vector is determined by its angle. Each vector is color mapped so that when θ_i increases from 0° to 90° the vector changes from blue to red. The frame rate of the movie is 50 fps.

Movie 4.3 2-D simulation of collective turning of a 16×16 particle array with an initiator s using VM. s take a 180° turn from the 100^{th} step to the 200^{th} step. Each particle is color mapped so that when θ_i increases from 0° to 180° the vector changes from blue to red. The frame rate of the movie is 30 fps.

Movie 4.4 2-D simulation of collective turning of a 16×16 particle array with an initiator s using PCH-SPP. s take a 180° turn from the 100^{th} step to the 200^{th} step. Each particle is color mapped so that when θ_i increases from 0° to 180° the vector changes from blue to red. The frame rate of the movie is 30 fps.

4.7 Bibliography

1. Katz, Y., et al., *Inferring the structure and dynamics of interactions in schooling fish*. Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(46): p. 18720-18725.
2. Makris, N.C., et al., *Critical Population Density Triggers Rapid Formation of Vast Oceanic Fish Shoals*. Science, 2009. **323**(5922): p. 1734-1737.
3. Ward, A.J.W., et al., *Quorum decision-making facilitates information transfer in fish shoals*. Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(19): p. 6948-6953.
4. Bajec, I.L. and F.H. Heppner, *Organized flight in birds*. Animal Behaviour, 2009. **78**(4): p. 777-789.
5. Ballerini, M., et al., *Empirical investigation of starling flocks: a benchmark study in collective animal behaviour*. Animal Behaviour, 2008. **76**: p. 201-215.
6. Bode, N.W.F., D.W. Franks, and A.J. Wood, *Limited interactions in flocks: relating model simulations to empirical data*. Journal of the Royal Society Interface, 2011. **8**(55): p. 301-304.
7. Cavagna, A., et al., *Scale-free correlations in starling flocks*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(26): p. 11865-11870.
8. Nagy, M., et al., *Hierarchical group dynamics in pigeon flocks*. Nature, 2010. **464**(7290): p. 890-U99.
9. Czirok, A., et al., *Formation of complex bacterial colonies via self-generated*

- vortices*. Physical Review E, 1996. **54**(2): p. 1791-1801.
10. Czirok, A., M. Matsushita, and T. Vicsek, *Theory of periodic swarming of bacteria: Application to Proteus mirabilis*. Physical Review E, 2001. **63**(3): p. 031915.
 11. Lu, S.T., et al., *Loss of Collective Motion in Swarming Bacteria Undergoing Stress*. Physical Review Letters, 2013. **111**(20): p. 208101
 12. Aldana, M., H. Larralde, and B. Vazquez, *On the Emergence of Collective Order in Swarming Systems: A Recent Debate*. International Journal of Modern Physics B, 2009. **23**(18): p. 3459-3483.
 13. Cavagna, A., et al., *Flocking and Turning: a New Model for Self-organized Collective Motion*. Journal of Statistical Physics, 2015. **158**(3): p. 601-627.
 14. Huepe, C. and M. Aldana, *New tools for characterizing swarming systems: A comparison of minimal models*. Physica a-Statistical Mechanics and Its Applications, 2008. **387**(12): p. 2809-2822.
 15. Morin, A., et al., *Collective motion with anticipation: Flocking, spinning, and swarming*. Physical Review E, 2015. **91**(1).
 16. Couzin, I., *Collective minds*. Nature, 2007. **445**(7129): p. 715-715.
 17. Attanasi, A., et al., *Information transfer and behavioural inertia in starling flocks*. Nature Physics, 2014. **10**(9): p. 692-697.
 18. Vicsek, T., et al., *Novel Type of Phase-Transition in a System of Self-Driven Particles*. Physical Review Letters, 1995. **75**(6): p. 1226-1229.
 19. Vicsek, T. and A. Zafeiris, *Collective motion*. Physics Reports-Review Section of

Physics Letters, 2012. **517**(3-4): p. 71-140.

20. Radakov, D.V., *Schooling in The Ecology of Fish*. Wiley, New York, 1973.

21. Pimentel, J.A., et al., *Intrinsic and extrinsic noise effects on phase transitions of network models with applications to swarming systems*. Physical Review E, 2008. **77**(6): p. 061138.

22. Couzin, I.D., et al., *Effective leadership and decision-making in animal groups on the move*. Nature, 2005. **433**(7025): p. 513-516.

4.8 Perspective

In this thesis, we have studied the responses of bacterial swarming motion to photodynamic inhibition. The loss of swarming motility is drastic as soon as a sub-lethal amount of photodynamic oxidative stress is applied. As it is reported that swarming motion protects the colony against antibiotics, our result indicates the possibility that a therapy combining traditional antibiotic treatment with photodynamic therapy may yield higher killing efficacy of swarming bacteria. We are curious about how the bacterial resistance to multiple drugs will change after photodynamic inhibition is applied. This is one direction of our future research.

We also successfully reduced swarming bacterial drug resistance by using polystyrene particles to disrupt the motion collectivity and speed. This finding have given direct evidence that the swarming motion dynamics can affect the drug resistance of a colony. The future work continues to obtain a deeper understanding of how collectivity and speed influence drug resistance of swarming bacterial colony, and developing new anti-surface infection methods by exploiting this property of swarming bacteria. More different drugs and swarming bacterial strains should be tested in the future to further expand the result of our study to applications.

We have constructed a new model (PCH-SPP model) based on the Vicsek model. In this thesis, we focus on reproducing the collective turning of bird flocks. Different parameters can be used to adjust the information transfer manner, thus this model can be utilized to simulate more collective motion systems observed in nature. The new model can also be applied with other modifications to the Vicsek model in

the future (*e.g.* the adding of attraction/repulsion forces).