Hyperswarming adaptations in a bacterium improve collective motility without enhancing single cell motility†

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Pseudomonas aeruginosa is a monoflagellated bacterium that can use its single polar flagellum to swim through liquids and move collectively over semisolid surfaces, a behavior called swarming. Previous studies have shown that experimental evolution in swarming colonies leads to the selection of hyperswarming bacteria with multiple flagella. Here we show that the advantage of such hyperswarmer mutants cannot be explained simply by an increase in the raw swimming speed of individual bacteria in liquids. Cell tracking of time-lapse microscopy to quantify single-cell swimming patterns reveals that both wild-type and hyperswarvers alternate between forward and backward runs, rather than doing the run-and-tumble characteristic of enteric bacteria such as E. coli. High-throughput measurement of swimming speeds reveals that hyperswarvers do not swim faster than wild-type in liquid. Wild-type reverses swimming direction in sharp turns without a significant impact on its speed, whereas multilagellated hyperswarvers tend to alternate fast and slow runs and have wider turning angles. Nonetheless, macroscopic measurement of swimming and swarming speed in colonies shows that hyperswarvers expand faster than wild-type on surfaces and through soft agar matrices. A mathematical model explains how wider turning angles lead to faster spreading when swimming through agar. Our study describes for the first time the swimming patterns in multiflagellated P. aeruginosa mutants and reveals that collective and individual motility in bacteria are not necessarily correlated. Understanding bacterial adaptations to surface motility, such as hyperswarming, requires a collective behavior approach.

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen and an environmental microbe that is capable of living in a variety of environments. This versatile microbe has several types of motility including twitching, swimming and swarming motility. Swimming in P. aeruginosa is mediated by a single polar flagellum. Its flagellar motor can rotate in two directions, clockwise or counterclockwise, resulting in either forward or backward propulsion in aqueous environments. Swarming is a collective form of surface motility where a dense colony of bacteria migrates on a semi-solid surface, such as an agar gel. Like swimming, swarming motility requires flagellar motility but, in addition, it requires the production and secretion of rhamnolipid surfactants thought to reduce friction between bacteria and the surface. Swarming occurs in many species of bacteria. The fact that the same flagellar system that drives swimming also enables to swim in liquids raises the question of whether swimming and swarming are distinct phenomena. Here we present evidence that these are indeed distinct phenomena, at least in P. aeruginosa.

We analyze hyperswarmer mutants with dramatically enhanced swimming capabilities, obtained recently from experimental evolution. All independently evolved hyper-swammer mutants harbor single point mutations in the same protein, FleN, which is the anti-activator of the flagellar master regulator. The genetic causality of the hyperswarmer genotype was firmly established using microbial genetics techniques. Hyperswarvers have 2 or more polar flagella, in contrast with the single polar flagellum of wild-type (Fig. 1A and E). In a swimming assay, a single wild-type P. aeruginosa colony covers about \( \frac{1}{3} \) of the agar plate making characteristic branched patterns. In contrast, colonies of hyperswarvers can cover the whole agar plate and show no branching patterns (Fig. 1B). Hyperswarvers are also poor biofilm formers compared to wild-type. This supports the existence of an antagonism between biofilm formation and motility which may affect the pathogenicity of P. aeruginosa and may eventually lead to new therapies against biofilm formation.

Since the discovery of the multilagellated, hyperswarming FleN mutants is very recent, very little is still known about their swimming behavior. Completely knocking out FleN creates multilagellated mutants unable to swim, whereas...
our point mutation hyperswarvers show increased swimming in classical the soft agar assay (Fig. 1C and D). However, classical swimming assays still portray macroscopic motility behaviors that are not informative of single cell motility. Because flagella are required for both swimming and swarming, an intriguing question is if the increased number of flagella in hyperswarvers translates into enhanced swimming at the single-cell level. This study is, to the best of our knowledge, the first observation of swimming patterns in this kind of multi-flagellated mutant of *P. aeruginosa*.

We confirmed, with macroscopic measurements of swarming motility and swimming through soft agar, that hyperswarvers spread faster in these environments. Then we investigated whether hyperswarvers swim faster than wild-type *P. aeruginosa* in liquid by measuring swimming speeds using a single-cell tracking method and quantitative image analysis. Counter-intuitively, we observed that hyperswarvers do not swim faster than wild-type. Having multiple flagella does, however, increase the variability in swimming speed of single cells, which become much more likely to alter their speed after a turn and to make wider turns than wild-type. We also performed single-cell tracking through soft agar. We observed that wild-type *P. aeruginosa* swims slightly faster than hyperswarvers in this environment. However, hyperswarvers diffuse much faster than wild-type. A mathematical model with adjustable turning angle explains how a wider turning angle distribution can lead to a faster spreading. These contrasting observations support that in order to understand the evolutionary adaptations to swarming, such as hyperswarming, one must study bacterial motility from a collective behavior perspective.

### Results and discussion

#### Hyperswarvers move faster through and on agar gels

Swarming and swimming through agar in *P. aeruginosa* and its hyperswarmer mutants has only been compared through
end-point measurements. Here we measured the expansion speed by tracking the colony edge over time. We first quantified swimming in the classical microbiology swimming assay. This assay consists of observing the spreading of a colony as it swims through soft agar prepared at a concentration of 0.3% (w/v). In agreement with previous measurements, both hyperswarmer clones spread significantly faster than wild-type (Fig. 2). Next, we tracked the expansion of swarming colonies (Fig. 3). The total area of the colony (Fig. 3C) and the speed of the advancing edge (tracked by following the tip of the tendrils for wild-type, or the protrusions for the hyperswarmers, Fig. 3D) reveal that hyperswarmers are faster than wild-type (Fig. 2D, \( p < 0.001 \) by Kruskal–Wallis test). The typical swimming speed (1 mm h\(^{-1}\)) does not match the typical swarming speed (3 mm h\(^{-1}\)), reinforcing that these two motility modes differ mechanistically.

**Time lapse imaging of bacteria and flagellar filaments**

In order to observe *P. aeruginosa* swimming patterns in liquid we labeled bacteria using Alexa Fluor® 488 carboxylic acid, succinimidyl ester which binds to lysine residues. This dye will bind to any protein on the membrane of bacteria as well as their flagella. There are about 30 lysines per flagellin unit, and each flagellum is made of thousands of flagellins. We then imaged bacteria using fluorescence microscopy and constructed time-lapse videos at an average frame rate of 16.6 fps.

We first observed wild-type swimming patterns. The time-lapse videos confirmed that bacteria can swim both backwards, *i.e.* with their single flagellum ahead or behind according to the direction of movement. The movement is always along the long axis of the rod-shaped bacterium. In some instances the time-lapse captured a cell reversing the direction...
of movement (Fig. 4B), which it presumably does by changing the rotation of the flagellum.18

We selected hyperswarmer mutants from our previous study with two distinct FleN mutations (FleN(V18G) and FleN(W253C)), conferring on average 1.5 or 3 flagella per cell, respectively (Fig. 1E and 4C). Hyperswarmers apparently swim regularly, although the flagellar configuration varies from cell to cell. A fraction of the hyperswarmer cells show a tidy flagellar arrangement that is indistinguishable from wild-type. These cells could be part of the small fraction of the population that has a single flagellum, which is expected due to large variability in the number of flagella in these mutants.14 Alternatively, these cells could have multiple flagella that form a bundle, as happens in E. coli swimming runs. Nonetheless, the majority of the hyperswarmer cells display multiple flagella that are not in a bundle and therefore are clearly visible (Fig 4C). Even when the flagella do not bundle, the bacteria are still capable of swimming (Fig. 4A).

We tracked the cells in 2D and quantified the average running speed both in forward and backward runs (Fig. 4D). In all three strains there is no significant difference between forward and backward speeds. Even though the sample number is relatively small (427, 65 and 219 respectively, with around 80% running forward and 20% running backward) the comparison suggests that hyperswarms swim slower, rather than faster, than wild-type (Fig. 4D).

High throughput imaging of swimming trajectories in pseudo 3D

Our observations from fluorescent-labelled bacteria and their flagella suggest differences in the swimming patterns of wild-type and hyperswarms, but the statistics are not sufficient to inform on how bacteria change swimming direction. We therefore opted to track swimming unlabeled bacteria using phase contrast microscopy at lower magnification. Tracking unlabeled bacteria has the disadvantage that the flagella are no longer visible and, therefore, the swimming orientation may not be determined. However, this type of imaging presents several advantages. First, lower magnification allows a wider field of view and therefore much higher throughput to quantify their turning behaviors. Second, data can be obtained for longer periods as the illumination is not nearly as damaging to the cells as exposing the cells to an Hg lamp for fluorescence microscopy. Third, phase contrast microscopy allows estimating the position of bacteria in the third dimension: when the bacteria are exactly in the focal plane they are seen in black. Bacteria not directly in the focal plane, but close enough to remain visible, become blurry and have a characteristic gray value profile that can be used to estimate their vertical location (Fig. S1A–C†). We constructed a calibration curve of gray values as a function of the distance to the focal plane using immobilized bacteria. Then we applied this calibration to estimate the distance to the focal plane for swimming bacteria. With the reasonable assumption that bacteria do not reverse direction when crossing the focal plane, it is possible to transform 2D trajectories into pseudo 3D (Fig. S1D† inset). As expected, cell speed computed using pseudo 3D is measurably higher than the speed computed from 2D data alone (Fig. S1D†).

The swimming speeds of wild-type P. aeruginosa and the hyperswarming mutants quantified using this method show a large variance (Fig. 5). Wild-type has a median speed of 46.2 μm s⁻¹, but the fastest run recorded was faster than...
90 μm s⁻¹. The FleN(V178G) hyperswarmer had a median speed of 45.3 μm s⁻¹ whereas mutant FleN(W253C), which has more flagella, had a slower mean speed of 41.8 μm s⁻¹. It should be noted that the speeds measured by the pseudo 3D method were approximately twice as high as the speeds measured with a 2D method when the bacteria and their flagella were fluorescently labeled (Fig. 3C) and the mismatch cannot be explained from a simple conversion of 2D to 3D. The difference is most likely caused either by damage due to the excitation wavelength used in fluorescence imaging or by the labeling procedure itself, which involves binding of large quantities of Alexa molecules to the cell wall and flagella. Nonetheless, both the fluorescent data and the pseudo 3D data agree that hyperswarmer do not swim faster than wild-type in bulk liquid. The recorded mean speed for both hyperswarmer is actually slower than wild-type, although only the mutant with higher flagella number, FleN(W253C), is sufficiently different to pass a Kruskal–Wallis statistical significance test (p < 0.001). Altogether, the typical single-cell swimming speed is two orders of magnitude larger (50 μm s⁻¹, ~180 mm h⁻¹) than swimming speed (3 mm h⁻¹) or the speed of swimming through agar (1 mm h⁻¹).

Bacteria of other species are known to differentiate when swimming on agar.19–21 Evidence of cellular differentiation in P. aeruginosa swarming is less clear, although there are differences in gene expression.22 Therefore, it is relevant to confirm whether the previous results are still valid using cells harvested from swarms rather than cells taken from overnight shaken cultures. We collected cells from two locations: at the edge of the swarm, and at a distance of 3 mm from the edge. Cells were then diluted in 1× PBS and immediately used in tracking experiments. Wild-type cells were found to swim faster (39.7 μm s⁻¹ at the edge) than hyperswarmer (30.3 μm s⁻¹ at the edge for FleN(V178G), p < 0.001 by Kruskal–Wallis test, Fig. S2†). However, no difference of velocities could be detected between cells from the edge and cells further in the swarm, for both hyperswarmer and wild-type. During these observations, no significant difference of cell size or swimming pattern was noticed between cells collected at the edge and cells collected deeper in the swarm. These observations confirm that hyperswarmer cells do not swim faster than wild-type cells in liquid, even when cells are harvested directly from swarming colonies.

**Hyperswarmer have distinct swimming patterns**

The visual inspection of a large number of swimming trajectories suggested the following model of swimming behavior: wild-type makes long swim runs that end in a sharp turn. Each run is followed by another run in the opposite direction and is likely caused by a reversal of the rotation of the single polar flagellum. There seems to be no obvious qualitative difference between alternating runs. Hyperswarmer also swim in long runs and execute sharp turns between runs. However, the turning angles seem much wider than those in wild-type. Also, it is much more common to observe hyperswarmer changing running speed after a turn in swimming direction.

In order to quantify these visual observations, we computed the change in speed following a reversal in swimming direction (Fig. S3†). We detected “turns” by identifying consecutive movement vectors separated by a very sharp angle (<90°, Fig 6A). Using trajectories that have only a single “turn”, i.e. trajectories that include samples from two consecutive runs (two segments) we calculated the average speed of each segment, V₁ and V₂, and used those values to calculate the relative difference between consecutive runs, |V₁ – V₂|/(V₁ + V₂). The change in speed quantified using this method showed a significant difference between wild-type and hyperswarmer (Fig. 6B). All three strains had a high frequency of trajectories where the average speed before and after the turn was very similar ([V₁ – V₂]/(V₁ + V₂) ≈ 0). The frequency of turns with significant changes in speed drops sharply for the wild-type, meaning that bacteria continue with the same speed. In contrast, hyperswarmer mutants showed large differences in the speed of consecutive runs at a much higher frequency (p < 0.001 by Kruskal–Wallis test; for runs differing >20%).
Next, we quantified the distribution of turning angles. For this, we defined the direction of rotation as positive when bacteria turn counterclockwise in the images. This quantification revealed another significant difference in swimming patterns: while turns tended to be very sharp ($\theta = 0$), hyperswarmer show a wider distribution in turning angle than wild-type ($p < 0.001$ by Kruskal–Wallis test on the absolute values of turning angles). About 5% of the turns have an angle wider than 45° (Fig. 7).

**Hyperswarmer diffuse faster through agar**

To understand how the single-cell swimming patterns in liquid translate into bacterial spreading in a structured environment, we observed *P. aeruginosa* swimming through 0.3% (w/v) agar at the single-cell level. The trajectories of both wild type and hyperswarmer are similar to their trajectories in liquid, consisting of run and turns. Using automatic cell tracking, we could quantify the displacements and velocities. It should be noted that pseudo-3D tracking could not be performed in these conditions because of the poorer imaging quality through agar. Measured velocities are therefore only 2D velocities. Nonetheless, in this structured environment, hyperswarmer populations also spread faster, as shown by a higher diffusion coefficient extracted from mean-squared-displacement curves ($D = 29.8 \mu m^2 s^{-1}$ for wild-type, $D = 44.8 \mu m^2 s^{-1}$ for mutation FleN(W253C), $D = 41.6 \mu m^2 s^{-1}$ for mutation FleN(W253C), Fig. 8b).

**Modeling run-and-turn swimming explains the advantage of multiple flagella in spreading**

We showed previously that hyperswarmer tend to change directions with a wider angle than wild-type, when swimming in liquid. Here, a simple model is proposed to show the importance of the turning angle distribution. We started from a simplified wormlike-chain model, but with a change of direction after each run (Fig. S4a†). At each time step, a cell runs over a distance $L$, with a velocity $V$. After this run, the cell reverses its running direction, but with a turning angle theta $\theta$. The distribution of turning angle is peaked at $\pi$ and is assumed to be normal for simplicity with a standard deviation $\sigma$ (Fig. S4b†). With these hypotheses, the average squared distance from the origin after $n$ steps is

$$E[R^2] = L^2(n + \gamma)(1 - \gamma)$$  \hspace{1cm} (1)

(for large $n$), where $\gamma = \langle \cos(\theta) \rangle$. If $\sigma = 0$, then $\gamma = -1$ and $D = 0$. If $\sigma$ is high, then the distribution is flat, $\gamma = 0$, and $E[R^2] = Ln^{1/2}$. This model shows how the distribution of turning angle can change the spreading dynamics (Fig. S4c†). Using eqn (1), we are able to infer $\gamma$ from the experimental swimming velocities and diffusion coefficients. We estimate $L = 10 \mu m$ from...
trajectories (Fig. S5†), which we assume to be constant over strains because this is a property of the environment, and \( n = t/\tau \) (with \( \tau = L/V \)). Then \( \gamma = -0.40 \) (\( \sigma = 1.35 \)) for FleN(V178G), \( \gamma = -0.45 \) (\( \sigma = 1.26 \)) for FleN(W253C), and \( \gamma = -0.48 \) (\( \sigma = 1.21 \)) for wild-type. It should be noted that these values of \( \gamma \) do not allow comparing the model with the actual macroscopic spreading speed for three reasons. First, the spreading is linear in time, whereas the model predicts that the size of the colony goes with \( t^{1/2} \). Second, the actual distribution of distance exceeds the one predicted by the previous equation, as shown by 99% percentile distance obtained by simulation (Fig. S4d†). Third, several aspects of bacterial migration are not considered, such as cellular growth, chemotaxis, wide distribution of velocities, and wide distribution of run lengths. Nonetheless, the model supports that wider turning angle distributions can lead to a faster population spreading in such a structured environment.

**Conclusion**

How behaviors of dense groups can be extrapolated from the traits of individuals is a central problem in biology. A vast literature on collective behavior in animal groups such as flocks of bird (e.g. Cavagna\(^{24}\)) and schools of fish (e.g. Berdahl\(^{25}\)) supports that many group properties emerge from close interactions between individuals. Interactions between cells are also key in collective migratory patterns in cell biology\(^{26,27}\) and may help explain cancer invasiveness\(^{28}\) and tissue development.\(^{29}\) Interactions between cells can be mechanical but also chemical, through the release and detection of diffusible chemical substances that can travel relatively large distances.\(^{30,31}\) Swarming in *P. aeruginosa* is a model system that allows tackling this problem from a quantitative perspective. In spite of its simplicity, *P. aeruginosa* shares important features of multicellular organisms such as cell-to-cell communication\(^{32}\) and the sharing of secreted products\(^{13,14}\) which also act as long range morphogenic cell–cell modulators.\(^{7}\) These important features are combined with the simplicity of a bacterial model, such as short generation times and large populations numbers.

Here, we contrasted single-cell swimming behavior of *P. aeruginosa* to its motility at the collective level. We observed that wild-type *P. aeruginosa* cells swim in a back-and-forth pattern in liquid. These swimming patterns are consistent with recent rotational measurements from tethered *P. aeruginosa*\(^{16}\) and swimming patterns measured in *Pseudomonas fluorescens*,\(^{15}\) a non-pathogenic relative of *P. aeruginosa*. We also measured the single-cell swimming patterns of hyperswarmer mutants that we recently obtained by experimental evolution\(^{14}\) but saw no evidence of faster motility in liquid. Our observations, therefore, raise the question of how investigating swimming in liquid can help in understanding densely packed, surface-associated communities.

Measurements of change in speed after a turn and turning angles reveal that hyperswarmer do have differences in swimming behaviors in liquid compared to wild-type. The swimming behavior of hyperswarmer seems at odds with their advantage in swarming colonies since (1) their median swimming speed is not faster, (2) they tend to change speed more frequently after a sharp turn and (3) the turns tend to be wider than those of the wild-type. Extracted from swarms and diluted to liquid phase, hyperswarmer swim even slower than wild-type. Nonetheless, hyperswarmer do outperform wild-type *P. aeruginosa* in swarming competitions and they also do better in the traditional swimming assay. In a swarming colony, bacteria are closely packed and their displacement may involve long-range correlation and collective flow. Thus swimming is radically different from single-cell swimming motility.

The implications of these results for our understanding of hyperswarming evolution is that hyperswarmer evolve in the lab not due to faster swimming speed but because of a specific advantage in collective motility that occurs in the setting of a swarming colony. Our results also highlight the importance of the substrate as being key to the emergence of collective migration in *P. aeruginosa*. Both swimming patterns and swimming through soft agar are likely highly dependent on cell–cell interactions as well as modulated by the environment. Thus, to some extent, these migratory behaviors are not cell-autonomous. Swimming through agar, as wide as the mesh may be, is significantly different from swimming in liquid. Under those conditions, having multiple flagella allows *P. aeruginosa* to spread more, for example by introducing wider turning angles that accelerate population diffusion through the agar medium.

Our results therefore highlight that investigating swimming in liquid media alone may provide a limited view of the rich

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**Fig. 8** (a) Hyperswarmer swim faster than wild-type through 0.3% (w/v) agar. Dots denote median velocities. Lower and upper bound represent respectively 25% and 75% percentile of velocities distributions. (b) Mean-squared-displacement *P. aeruginosa* when swimming through soft agar.
motile behaviors of bacteria. Bacteria often do not live in low density-liquid cultures but rather in high-density communities associated with bio-gels or hard surfaces. Recent studies are going in this direction by investigating collective swimming patterns in high density populations,\textsuperscript{24} motility through mucin biogels\textsuperscript{17,18} and in partially saturated porous materials that mimic soil.\textsuperscript{39}

**Experimental methods**

**Bacterial strains**

All of the strains used in this study were based on the laboratory strain *Pseudomonas aeruginosa* PA14. The non-motile strain is isogenic to PA14, except for a clean deletion of *flgK* (encoding the flagellar hook protein FlgK; \(\Delta flgK^{\text{40}}\)). The two hyperswarmer strains used in this study have two different FleN mutations. Both came from the experimental evolution.\textsuperscript{14} The first FleN(V178G) was denominated as clone 4 in that study and the second FleN(W253C) was denominated as clone 10. Bacterial cells were routinely grown in LB Miller at 37 °C with aeration.

**Bacterial assays**

Swarming assays are performed in standard conditions using 0.5% (w/v) agar.\textsuperscript{33} Briefly: an overnight culture of bacteria is washed twice in 1 x PBS and 2 \(\mu\)L of the washed suspension are used to inoculate a swarming plate in the center. The plates are then flipped and imaged for 24 h at 37 °C. Swimming assays were performed with slight adaptations to standard protocol in 0.3% (w/v) agar plates.\textsuperscript{40} Cells from overnight cultures were washed twice in 1 x PBS before normalizing their density to 1 OD\textsubscript{600}. A pipet tip was then dipped into the washed suspension and used to point inoculate a swimming plate, then flipped and imaged for 16 h at 37 °C.

**Microscopy and cell tracking**

A PDMS layer is first made using Sylgard mix, laid between two Plexiglas sheets spaced by 400 \(\mu\)m PET spacer, and cured overnight at 65 °C. A 1 cm \(\times\) 1 cm square of PDMS is cut from this layer, and then punched using a 5 mm diameter punch tool. The PDMS square is then laid on a glass slide. A drop of 30 \(\mu\)L of a bacterial suspension is deposited in the round chamber, then covered by a coverslip. As glass is more hydrophobic than PDMS, the excess of water from drop is pushed out of the PDMS-coverslip slit, removing any remaining layer of water. The fluid is enclosed in this microchamber and the chamber shows only very limited internal flow. As bacterial swimming is disturbed when near a solid surface,\textsuperscript{41} observations were performed in the middle of the chamber (200 \(\mu\)m from any boundary).

Cells from the exponential growth phase in LB Miller are diluted to 0.005 OD\textsubscript{600} in 1 x PBS without centrifugation, as centrifugation can be harmful to flagella.\textsuperscript{42} Cells are then gently incubated at 37 °C on a rotary shaker for one hour. Finally, the cell solution is enclosed in the microchamber, and immediately imaged. Fluorescence imaging is carried out using an Axiovert 200M inverted microscope (Zeiss) equipped with a CoolSNAP ES CCD Camera (Photometrics) and Hg lamp, with a \(\times 40\) and a \(\times 63\) lens at 16.6 fps (acquisition time = 10 ms) during 12 seconds. The \(\times 63\) lens is used for high-resolution imaging (Fig. 2). Phase contrast imaging was carried out using a Zeiss video-microscope, with \(\times 10\) phase contrast lens, with Orca2-ER CCD camera (Hamamatsu), at 16 fps (acquisition time = 2 ms) during 20 seconds. A few hundred trajectories are recorded per video. A few tens of videos are shot for each strain.

**Flagellar staining**

Flagellar staining is adapted from Darnton:\textsuperscript{43} cells in exponential phase are gently washed three times in 1 x PBS (350 rcf for 10 min). The final pellet is adjusted to a volume of 0.5 mL of 1 x PBS (\(\sim\)1 OD\textsubscript{600}). 10 \(\mu\)L of a solution of Alexa Fluor 488 carboxylic acid, succinimidyl ester ( Molecular Probes) (10 mg mL\textsuperscript{-1} in dimethyl sulfoxide (DMSO)) are added, and the cells are labeled for 90 min at room temperature. Excess dye is removed by washing three times in 1 x PBS. The labeled cells are diluted to 0.1 OD\textsubscript{600} for observation.

**Conversion of 2D tracks into 3D**

Trajectories are extracted from images using Matlab and the Crocker algorithm.\textsuperscript{44} Bacteria are first tracked in two dimensions only. In phase contrast imaging, bacteria appear as black spots when in focus, and as white spots when out of focus. The background appears as a grey field. Using an “altitude versus gray value” calibration curve, the third dimension coordinate is added to bacterial locations. As gray value gives only absolute value of distance to focal plane, trajectories are considered to cross this focal plane only once: thus absolute altitude can be converted to relative altitude, then to a vertical component of speed.

**Determination of turning angles**

Turning events are first automatically detected using a \(\pi/2\) threshold for the angle between all consecutive velocity vectors of each trajectory. A second sorting was carried out manually to keep only trajectories where turning points split trajectories in two clear runs. Solely trajectories with one single turn and two runs were considered in the analysis. For the sake of precision, turning angles are not measured between consecutive frames but between three frames (\(\Delta t = 0.18\) seconds) on either side.

**Swarming speed measurement**

Swarming was quantified by tracking the edge of binary images obtained from direct thresholding. Tips or protrusions are first detected using distance transform (function \texttt{bwdist} in Matlab) of the binary image, then tracked using Crocker algorithm.\textsuperscript{44}

**Acknowledgements**

This work was supported by the Office of the Director, National Institutes of Health of the National Institutes of Health under Award Number DP2OD008440 to JBX. CCF is supported by Integrated Cancer Biology Program under grant U54...
CA14896704 grant as an independent fellow of the Computational Biology Center at Memorial Sloan-Kettering.

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