Report

Current Biology

Colonization, Competition, and Dispersal of Pathogens in Fluid Flow Networks

Highlights

- *P. aeruginosa* disperses upstream in branched, vasculature-like fluid networks
- Upstream dispersal gives *P. aeruginosa* a selective advantage
- This selective advantage enables competition against faster-growing pathogens
- Interplay between fluids and bacterial motility shapes multi-species communities

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In Brief

Siryaporn et al. find that the major human pathogen *P. aeruginosa* disperses *upstream* in vasculature-like fluid networks. This movement enables the bacterium to compete (and win) against other bacteria for nutrients, an effect that the authors can inhibit synthetically. These findings highlight a new strategy that bacteria may use to colonize hosts.

Siryaporn et al., 2015, Current Biology *25*, 1201–1207
May 4, 2015
http://dx.doi.org/10.1016/j.cub.2015.02.074

CellPress

Current Biology
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Colonization, Competition, and Dispersal of Pathogens in Fluid Flow Networks

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http://dx.doi.org/10.1016/j.cub.2015.02.074

SUMMARY

The colonization of bacteria in complex fluid flow networks, such as those found in host vasculature, remains poorly understood. Recently, it was reported that many bacteria, including *Bacillus subtilis* [1], *Escherichia coli* [2], and *Pseudomonas aeruginosa* [3, 4], can move in the opposite direction of fluid flow. Upstream movement results from the interplay between fluid shear stress and bacterial motility structures, and such rheotactic-like behavior is predicted to occur for a wide range of conditions [1]. Given the potential ubiquity of upstream movement, its impact on population-level behaviors within hosts could be significant. Here, we find that *P. aeruginosa* communities use a diverse set of motility strategies, including a novel surface-motility mechanism characterized by counter-advection and transverse diffusion, to rapidly disperse throughout vasculature-like flow networks. These motility modalities give *P. aeruginosa* a selective growth advantage, enabling it to self-segregate from other human pathogens such as *Proteus mirabilis* and *Staphylococcus aureus* that outcompete *P. aeruginosa* in well-mixed non-flow environments. We develop a quantitative model of bacterial colonization in flow networks, confirm our model in vivo in plant vasculature, and validate a key prediction that colonization and dispersal can be inhibited by modifying surface chemistry. Our results show that the interaction between flow mechanics and motility structures shapes the formation of mixed-species communities and suggest a general mechanism by which bacteria could colonize hosts. Furthermore, our results suggest novel strategies for tuning the composition of multi-species bacterial communities in hosts, preventing inappropriate colonization in medical devices, and combatting bacterial infections.

RESULTS

Pseudomonas aeruginosa is an opportunistic pathogen that infects a broad range of hosts including plants and animals. In humans, it is a major cause of vascular-related illnesses including lung infections, urinary tract infections, bacteremia, and sepsis [5–7]. In fluid flow environments, *P. aeruginosa* cells attach to surfaces using type IV pili (TFPs). These TFPs are localized to the bacterial cell poles [8] such that upon attaching to the surface, flow causes the bacteria to orient with the TFP pole pointed in the opposite direction of the flow (Figure 1A) [3, 4]. The repeated extension and retraction of TFPs in this position drives *P. aeruginosa* to move upstream along the surface (Figure 1A). The upstream movement is a direct response to surface shear stress and is not due to chemotaxis [4]. *P. aeruginosa* cells also swim through fluid environments using flagella, but upstream movement occurs without flagella [4].

While individual bacteria move upstream over short distances and small timescales [1, 2, 4], it is unknown whether populations can migrate physiological distances relevant for infection or colonize surfaces for extended periods in fluid flow. In particular, *P. aeruginosa* cells are dislodged from the surface by the fluid flow force and are subsequently pushed downstream [4]. This finding raises a paradox: if cells are ejected from the surface and move backward along a flow streamline, the same streamline would carry cells back downstream upon detachment from the surface, nullifying any effect of the upstream movement. Furthermore, although individual cells move upstream, it is unknown whether this mechanism could drive the expansion of a multi-cellular population.

Upstream Dispersal Involves Multiple Phenotypically Diverse Single-Cell Motility Modes

To investigate *P. aeruginosa* colonization dynamics in flow, we first imaged the leading edge of a population in a linear microfluidic channel. Cell-free medium was flowed steadily through the device (200 × 50 μm, width × height) at wall shear stresses between 0.2 to 2 Pa (1–10 μl/min, v_{fluid} = 2–20 mm/s), which correspond to the dimensions, shear stresses, and flow speeds typically observed in the vasculature of plants and animals [9–12]. Note that these flow rates are significantly higher than typical bacterial swimming rates. Cells were initially seeded at one side of the channel behind a "start line" (Figure 1B). In the presence of flow, cells exhibited three distinct motility behaviors: (1) movement in the opposite direction of the flow toward the "finish line" (700 μm upstream), (2) detachment from the surface (and subsequent downstream movement with the flow), or (3) no motility. After 2 hr, the fastest cells at the leading edge reached the upstream finish line (Figure 1B), whereas slower cells

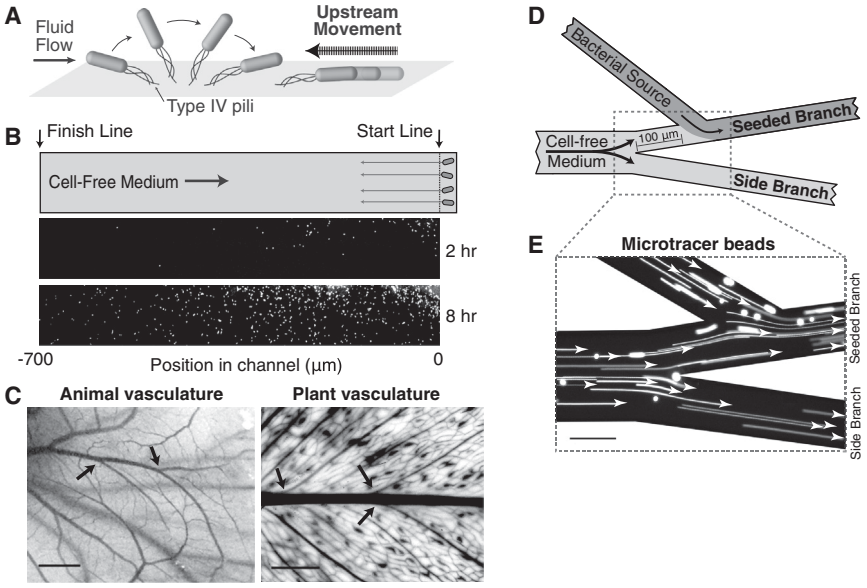

Figure 1. Upstream Dispersal of *P. aeruginosa* Populations in Flow Networks

(A) Schematic summarizing upstream movement by *P. aeruginosa*. Fluid flow orients polar-attached cells such that the attachment pole is positioned upstream and pilus retraction moves cells upstream.

(B) *P. aeruginosa* surface colonization in a linear microfluidic channel. Cells are loaded behind a "start line" in which cell-free medium flows from the left to right. Within 2 hr, cells at the leading edge of the population reach the "finish line."

(C) Vascular flow networks in chicken embryos and *E. aureum* plant leaves contain many branching intersections (arrows).

(D) Schematic of a branched flow network microfluidic device used to track colonization, competition, and dispersal. Cell-free medium flowing through the main branch diverges into two branches. Cells are inoculated into the "seeded branch." Arrows indicate the direction of flow.

(E) Tracking of fluorescent micro-tracer beads indicates a flow pattern that is laminar, stable, and uni-directional.

Scale bars represent 2 mm in (C) and 100 μm in (E). See also Figure S1 and Movie S1.

continued upstream migration. Cells were also found at the start line and downstream. The dispersal of the population to upstream and downstream regions in the device, which we refer to collectively as "upstream dispersal," is due to the phenotypic diversity of motility modes within a single population. As the behavioral heterogeneity at the single-cell level benefits the community as a whole, this could represent a form of bet hedging by *P. aeruginosa* that enables the species to explore upstream and downstream environments while maintaining colonization of the initial environment.

P. aeruginosa Gains a Selective Growth Advantage by Dispersing Upstream

We next sought to determine whether upstream dispersal provides bacteria with a selective growth advantage over bacteria that lack this motility mode. For these studies, we modified the microfluidic device to incorporate a branched network geometry, which enabled us to quantify colonization, competition, and dispersal in the same device. Branched networks are a defining feature of animal and plant vasculatures (Figure 1C), with bifurcation of vasculature promoting the uniform distribution of nutrients [13, 14]. To establish the flow pattern in this complex network (Figure 1D), we imaged micro-tracer beads that verified that the flow was laminar, stable, and uni-directional (Figure 1E, Movie S1).

We inoculated one branch, the "seeded branch" (Figure 1D), with an equal number of wild-type *P. aeruginosa* (expressing GFP) and Δ*pilTU* mutants (expressing mCherry) that are defective in surface motility because they lack the TFP PilT and PilU retraction motors [8] but retain swimming motility (Figure S1A). After 15 hr, wild-type cells colonized both the seeded and side branches, whereas the Δ*pilTU* cells colonized only the seeded branch (Figure 2A; Movie S2A). We collected the fluid effluent from each branch, which contains both planktonic (swimming) cells and cells that detached from the surface, and cultured the effluent for 3.5 hr at 37°C in order to characterize cell physiology and determined the relative proportion of wild-type and Δ*pilTU* cells using single-cell fluorescence microscopy. The effluent from the seeded branch contained ~2.4×10^9 cells, of which 60% were wild-type and 40% were Δ*pilTU* cells (Figures 2B–2D). In contrast, the side branch contained only wild-type cells (Figures 2A–2D). The total number of wild-type cells in both branches outnumbered Δ*pilTU* cells by more than 2-fold (~1.3×10^9 more cells), indicating that upstream migration results in a selective growth advantage. The greater number of wild-type cells is not due to a difference in growth rates as both strains are represented comparably in planktonic co-culture competitions (Figure S1B). Furthermore, wild-type cells from the side branch were 40% larger than those from the seeded branch (Figures 2C and S1C), indicating the increased availability of nutrients in the side branch [15]. Thus, by entering the side branch, the upstream-migrating population escapes nutrient limitation imposed by the competing Δ*pilTU* cells.

Dispersal through Zigzag Paths on Surfaces

Given that cells that move upstream should simply return downstream along the same streamline when they are released from the surface, our findings raised the question of how *P. aeruginosa* dispersed to the side branches. By tracking the movements of individual cells (Figure 2E), we found that upstream motility has a component whose direction is perpendicular to the flow. *P. aeruginosa* cells always migrated in a zigzag path, with some trajectories crossing over into streamlines that flow into the side branch. Cells that entered these streamlines were frequently detached from the surface, carried into the side branch, and subsequently re-attached to the surface of the side branch (Movie S2A). This zigzag motion was observed in upstream movement in linear channels [4] and in a network geometry in which branches converge at the intersection instead of diverging (Figure S2A; Movie S2B). The motion can be attributed

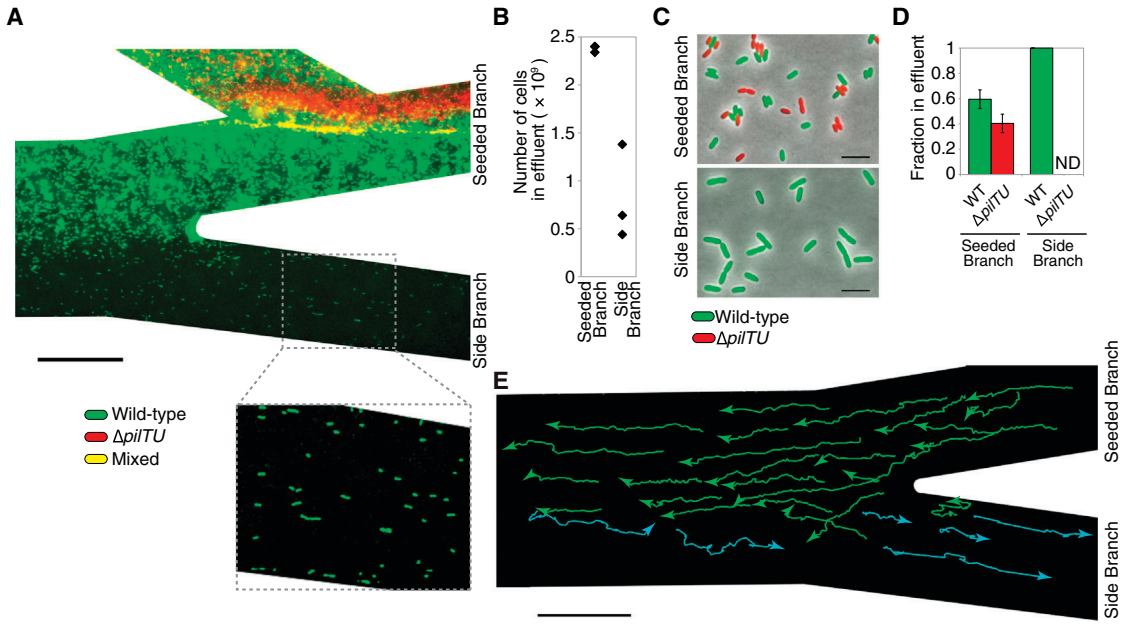

Figure 2. Upstream Dispersal Provides *P. aeruginosa* with a Selective Growth Advantage in Flow

(A) Surface colonization of wild-type (green) and pilus-defective Δ*pilTU* (red) *P. aeruginosa* cells in a branched network. An equal number of each strain was initially co-inoculated into the seeded branch (not shown). After 15 hr, wild-type cells colonized all areas of the device, whereas Δ*pilTU* cells remained in the seeded branch. The effluent (containing planktonic and surface-detached cells) from each branch was collected and cultured for an additional 3.5 hr.

(B–D) The total number of cells in each effluent was measured through optical density (B) and the effluent population composition was determined using fluorescence microscopy (C and D) (see the Supplemental Experimental Procedures). Δ*pilTU* cells were not detected (ND) in the side branch. Error bars in (D) indicate the SD.

(E) Trajectories of individual wild-type cells on surfaces, which move upstream (green) or downstream (cyan) in "zigzag" paths that cross laterally into different streamlines. Each trajectory was acquired at 30 s intervals for 30 min.

Scale bars indicate 100 μm in (A) and (E) and 5 μm in (C). All experiments were performed in triplicate. See also Figure S2 and Movie S2.

to the radial organization of TFPs at the cell pole (Figure S2B) and their non-synchronous activity [16]. Zigzag trajectories are also observed through flagellar-mediated swimming in sperm rheotaxis [17], indicating that similar upstream motions can be achieved using distinct motility mechanisms.

An alternative explanation for the presence of bacteria in the side channels is through flagella-mediated upstream movement. However, Δ*pilTU* mutants retain swimming motility (Figure S1A) but do not reach the side channel (Figures 2A–2D), supporting the conclusion that migration to the side channel is driven by surface motility. We also tested whether *B. subtilis* or *E. coli* migrates toward the side channel, as both have previously been shown to move upstream through flagella-mediated rheotactic motion [1, 2]. After 15 hr of continuous flow in which either species was inoculated into the seeded channel, we found no cells on the surface or in effluent from the side channel (Figures S2C and S2D), suggesting that the flagella-mediated rheotactic behavior does not result in dispersal. Together, our data show that colonization and dispersal is due to surface-mediated upstream migration.

Selective Advantage Enables Competition against Faster-Growing Pathogens

In natural settings, bacteria compete against other species for shared resources [18]. To determine whether upstream dispersal confers *P. aeruginosa* with a competitive advantage against other species, we co-inoculated the seeded branch with equal numbers of *P. aeruginosa* and *Proteus mirabilis*, a Gram-negative bacterium that grows at a faster rate than *P. aeruginosa* (Figures S3A and S3B) and moves on surfaces at significantly higher velocities (Figure S3C; Movie S3). Both pathogens cause nosocomial infections, colonize the urinary tract and gut, and may be considered natural competitors [5, 19–23]. In addition, *P. mirabilis* swims using a flagella-mediated mechanism similar to that of *E. coli* [24, 25], which suggests that *P. mirabilis* can move upstream.

We flowed media continuously for 15 hr (Figure 3A), collected effluent containing planktonic and surface-detached cells from each branch, and performed the analysis described above (Figures 3B–3D). As predicted from its higher growth rate, *P. mirabilis* outgrew *P. aeruginosa* in the seeded branch and accounted for 85% of the bacteria in the branch effluent (Figure 3D). In contrast, only *P. aeruginosa* cells colonized the side branch (Figures 3A and 3C–3D; Movie S4A). Examination of individual cell trajectories showed that *P. mirabilis* cells did not disperse upstream (Movie S4A), thereby explaining their restriction to the seeded branch. Furthermore, *P. aeruginosa* cells in the seeded branch were 29% smaller than those in the side branch (Figures 3C and S3D), indicating that *P. aeruginosa* cells were nutrient limited in the co-culture with *P. mirabilis*. These results demonstrate that upstream migration by a surface-motility mechanism enables *P. aeruginosa* to self-segregate from the

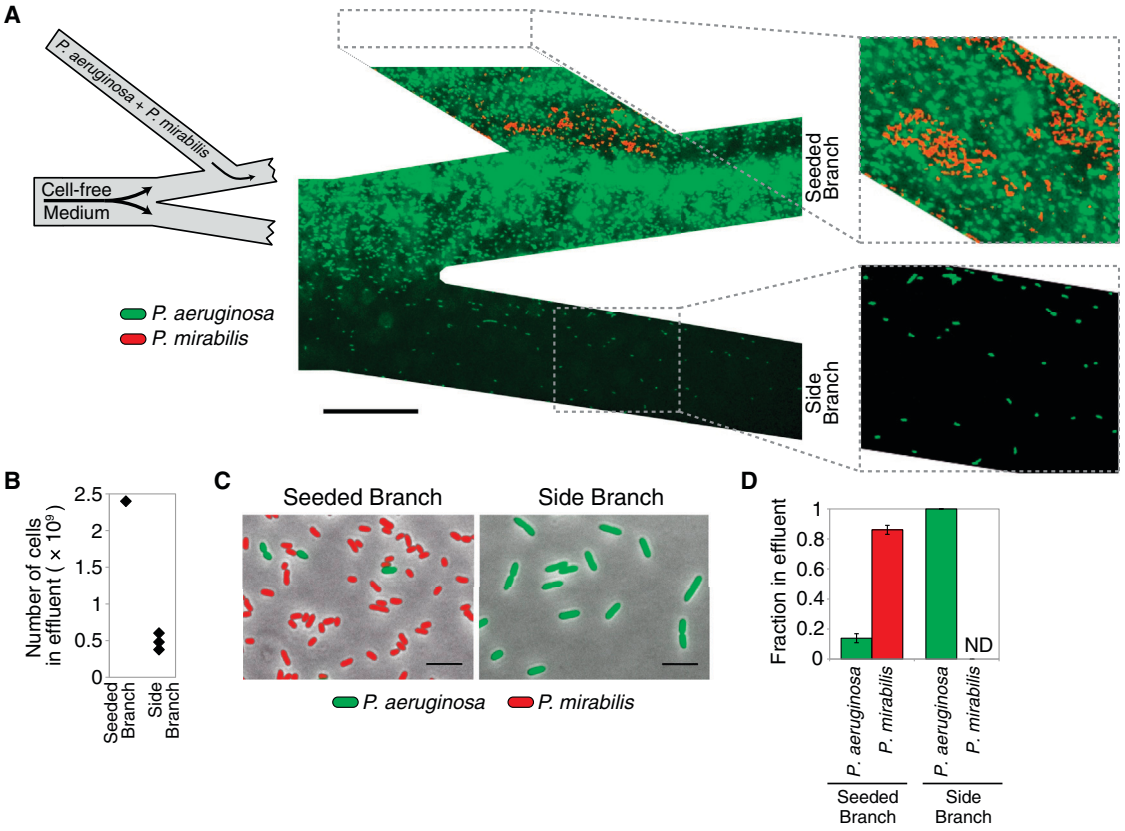

Figure 3. Pathogens Self-Segregate to Co-exist in a Branched Flow Network

(A) Surface colonization of *P. aeruginosa* (green) and *P. mirabilis* (red) cells in a branched network. Both strains were initially co-inoculated in equal numbers into the seeded branch. After 15 hr of continuous flow, *P. aeruginosa* colonized the upper, seeded, and side branches, whereas *P. mirabilis* colonized only the seeded branch. The dashed box (top) shows a region that is 100 μm upstream from the indicated section.

(B–D) The effluent (containing planktonic and surface-detached cells) from each branch was analyzed for (B) total cell number using optical density and (C and D) population composition using fluorescence microscopy. *P. mirabilis* was the dominant species in the seeded branch, whereas *P. aeruginosa* was the only species that colonized the side branch. ND indicates cells were not detected. Error bars in (D) indicate the SD.

Scale bars in (A) and (C) indicate 100 μm and 5 μm, respectively. Experiments were performed in triplicate. See also Figure S3 and Movies S3 and S4.

co-culture to colonize a separate niche in flow. *P. aeruginosa* thus gains a competitive advantage over a bacterium that would otherwise outgrow *P. aeruginosa* in the absence of flow. We observed similar findings when *P. aeruginosa* was co-cultured with *Staphylococcus aureus* (Figure S3E; Movie S4B), which co-colonizes the lungs of cystic fibrosis patients alongside *P. aeruginosa* [26], and with the pathogen *Salmonella enterica* serovar Typhimurium (Figure S3F). These results demonstrate that in a flow environment, bacterial species can self-organize to co-exist in separate micro-environments, despite one species having an apparent growth advantage over the other.

Upstream Dispersal Is Characterized by Counter-advection and Lateral Diffusion

To improve our ability to understand and ultimately disrupt *P. aeruginosa* dispersal and colonization, we developed a quantitative model of upstream dispersal (for a detailed derivation, see the Supplemental Results). Upstream migration within a population is a phenotypically diverse cycle consisting of upstream movement on surfaces, detachment from the surface, and downstream re-attachment to the surface (Figure 4A). As cells move upstream along the flow axis (x axis), cells also move and repeatedly switch direction along the transverse (y) axis, resulting in a zigzag path (Figure 2E). This behavior in single cells is described by the differential equation

$$\frac{\partial\sigma(x,y,t)}{\partial t} = (\alpha-\beta)\sigma(x,y,t) - v\frac{\partial\sigma(x,y,t)}{\partial x} + \mathrm{D}\frac{\partial^2\sigma(x,y,t)}{\partial y^2},$$

where $\sigma(x,y,t)$ is the population density of cells at positions x and y and at time t that travel upstream at a velocity v, duplicate at a rate α, and detach from the surface at a rate β; D is the effective diffusion coefficient. Upstream dispersal is thus a novel type of movement that is *counter-advective* along the flow axis and *diffusive* along the lateral axis. We solved this equation (see the Supplemental Results) for a population of cells with distinct upstream velocities [4] in a branched flow network. Our model (see the Supplemental Results for parameters) accurately predicts the population density distribution data in linear channels (Figures 4B and 4C) and in branched flow networks and shows that the number of cells that enter the side branch (Figure 4D) increases exponentially with time (Figure 4E). As bacterial growth is also exponential with time, the combined effects of

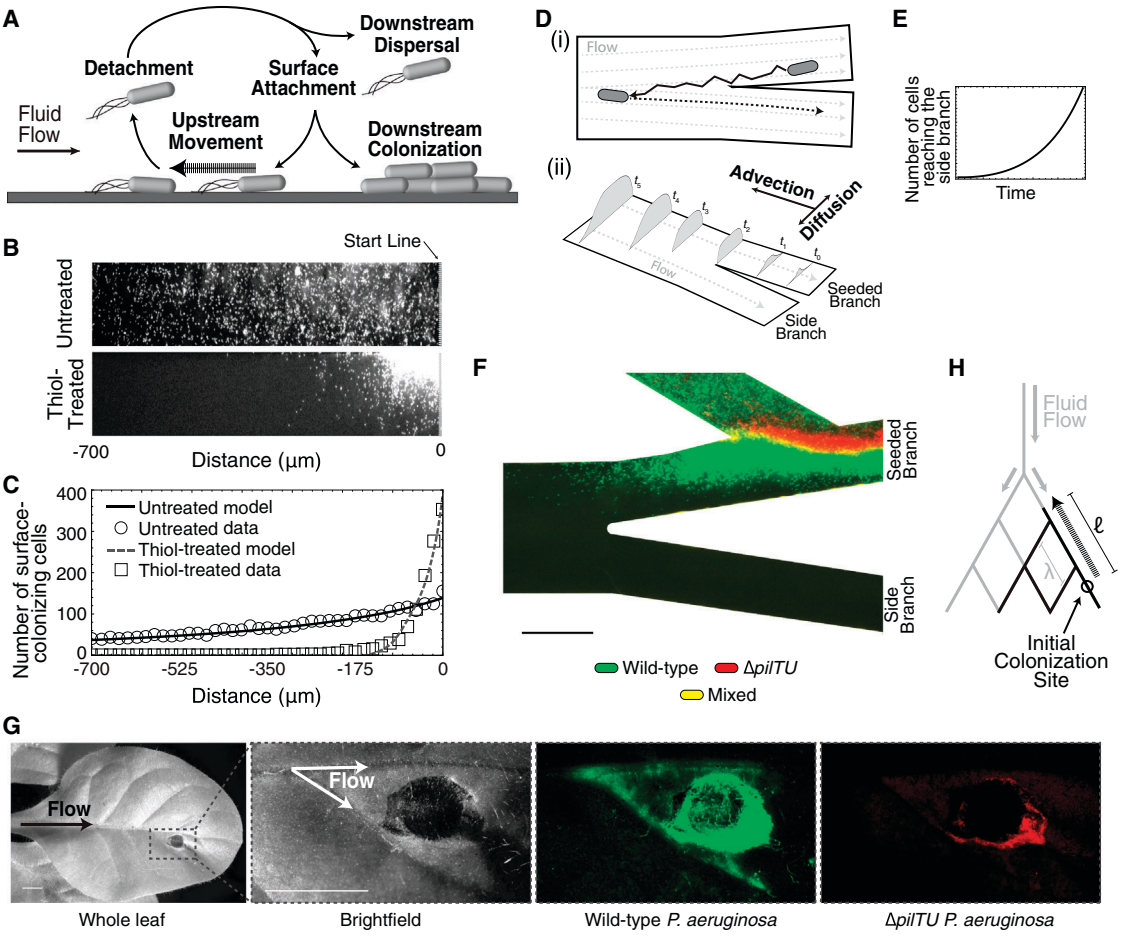

Figure 4. Theoretical and Natural Host Models of Upstream Dispersal

(A) Schematic illustrating the diverse motility modes observed during upstream dispersal.

(B) Surface colonization of wild-type *P. aeruginosa* after 15 hr of flow in untreated and thiol-treated linear channels.

(C) Corresponding population density data for untreated (circles) or thiol-treated (boxes) devices and population densities predicted by our upstream dispersal model (lines).

(D) Schematic depicting the mechanism of upstream dispersal. (i) Cells move upstream in a zigzag trajectory on surfaces, enter side branch streamlines, detach from the surface, and are carried downstream by the flow. (ii) Time evolution of a surface population that advances toward a branching intersection through counter-advection and lateral diffusion.

(E) Prediction from our model that the number of cells entering side-branch streamlines increases exponentially with time.

(F) Surface colonization of wild-type (green) and surface-motility-defective Δ*pilTU* (red) *P. aeruginosa* cells in a thiol-treated device after 15 hr of continuous flow. No cells were observed in the side channel (see Figure S4A).

(G) Plant colonization assay in which a tobacco plant leaf was inoculated with equal numbers of wild-type and Δ*pilTU P. aeruginosa* cells. After 7 days, wild-type cells (green) were observed in the upstream vasculature, whereas Δ*pilTU* cells (red) were found at the inoculation site.

(H) Schematic showing a generalized branched flow network (gray) with characteristic pore spacing λ and colonization of the network by bacterial communities (black) that migrate upstream a distance ℓ.

Scale bars represent 100 μm in (F) and 5 mm in (G). See also Figure S4.

exponential influx and growth result in rapid colonization of the side branch.

We tested a prediction of our model that decreasing the surface detachment rate (β) and migration velocity (v) restricts population expansion and prevents dispersal to the side branch. We coated channel surfaces with the thiol compound (3-mercaptopropyl)trimethoxysilane to promote sulfide bond formation with proteins on the bacterial cell surface, thereby increasing cell adhesion to the surface (i.e., making the surface more "sticky"). Thiol-treated linear channels inhibited the expansion of the *P. aeruginosa* population in linear channels (Figures 4B and 4C) and prevented cells from entering the side branch (Figures 4F and S4A), in good agreement with the predictions of our model of upstream dispersal.

Upstream Dispersal Promotes Colonization in Plant Vasculature

To establish the role of upstream dispersal in the colonization of the vasculature of a natural host of *P. aeruginosa*, we followed colonization of the tobacco plant *Nicotiana tabacum.* In these plants, fluid and nutrients flow from the main stem toward the periphery of the leaf vasculature in a branched network (Figure 4G).

We inoculated plant leaves with an equal mixture of wild-type *P. aeruginosa* (expressing GFP) and Δ*pilTU* cells (expressing mCherry) at the periphery using syringe infiltration. After 7 days, wild-type cells were found in the vasculature upstream from the inoculation site (toward the main stem) (Figures 4G and S4B–S4D). In contrast, Δ*pilTU* cells were found only at the inoculation site, resulting in a much more localized infection. Our results are thus consistent with upstream motility representing an important factor driving branched vasculature colonization in vivo.

DISCUSSION

Understanding how single-cell behaviors give rise to the population-level dynamics of *P. aeruginosa* in flow provides a quantitative framework for how *P. aeruginosa* colonization is established and how infection spreads within a host. Our results suggest that *P. aeruginosa* responds to fluid flow as a host cue by moving in the opposite direction of the flow. By employing diversified strategies of migrating upstream, downstream, and laterally through counter-advection and diffusion, *P. aeruginosa* communities gain a selective growth advantage in fluid flow. This combination of behaviors enables *P. aeruginosa* populations to disperse rapidly throughout a fluid-filled vascular network since the population expands into a new branch each time it reaches an intersection (Figure 4H). Since the number of cells that reach a branch junction is exponential with respect to time (Figure 4E), our model suggests that the upstream advancement of *P. aeruginosa* to a central branch node is catastrophic for the host. Indeed, our experiments demonstrate that only a small number of cells need to reach the branch junction for the establishment of large populations downstream (Figures 2A, 2B, 3A, and 3B). To more fully understand the dispersal of bacteria through generalized flow networks, it will be necessary to integrate additional factors into the model, including the network topology, pore spacing between branch intersections (λ), upstream migration distance (ℓ), and migration velocity (Figure 4H). It will also prove important to study the effects of time-periodic flow patterns such as those observed in the urinary tract.

Importantly, our model highlights strategies for minimizing colonization of flow networks. Specifically, the advantage gained by upstream dispersal can be eliminated by increasing surface adhesion. These findings have immediate implications for the design of medical devices in order to contain *P. aeruginosa* colonization and suggest alternative approaches to disrupting *P. aeruginosa* colonization and infection within hosts.

The ability of bacteria to self-segregate into stably co-existing niches has significant implications for pathogens and beneficial commensals. In particular, as fluid flow is prevalent in the digestive tract, understanding how different bacterial species disperse and establish colonization in flow advances our understanding of the forces that shape the composition and structure of microbial communities, as well as our ability to manipulate these populations to improve health. We find that unexpected population-level dynamics emerge from the complex interactions between shear forces, the polar organization of bacterial motility structures, and the architecture of fluidic networks. For example, the polar localization of TFPs may have evolved as an adaptation to promote competition in branched flow networks. As many bacteria possess polar-localized TFPs [8], upstream dispersal may be a generalized mechanism by which surface-motile bacteria colonize hosts. Thus, while flow has thus far been largely considered in the context of its effects on the motility of individual bacteria, our study shows that flow is a critical factor that drives the colonization, competition, and dispersal of bacterial populations in host environments. Finally, taken together with recent findings [27], the upstream dispersal of *P. aeruginosa* provides further evidence that the bacterial response to mechanical forces plays an important role in pathogenic processes.

EXPERIMENTAL PROCEDURES

Methods Summary

Strains were grown in lysogeny broth to mid-exponential phase. For competition experiments, strains were grown separately and mixed in equal number immediately before loading into devices. Microfluidic devices were constructed using standard soft photolithography techniques. Effluent from each branch of the device was collected into culture tubes and incubated for 3.5 hr at 37°C in order to amplify the total number of cells for single-cell microscopy analysis. The total cell number in the effluent was measured by optical density. *P. aeruginosa* colonization was tracked in *Nicotiana tabacum* tobacco plants that were exposed continuously to a fluorescent growth light for 7 days. Details on our model of bacterial dispersal by upstream migration, bacterial strains and culture conditions, microfluidic device construction and experimental conditions, fluorescence microscopy, leaf vasculature imaging, swimming assays, planktonic growth rate measurements and competitions, plant colonization, transmission electron microscopy and surface velocity measurements are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, four figures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.02.074.

AUTHOR CONTRIBUTIONS

A.S. and M.K.K. designed and carried out experiments, performed data analysis, and wrote and revised the manuscript. A.S. constructed the quantitative model, and H.A.S. revised it. Y.S. carried out experiments. H.A.S. and Z.G. designed experiments and provided feedback on results. All authors discussed the results and revised the manuscript.

ACKNOWLEDGMENTS

We thank A. Binns and N. Ouzounov for tobacco plants and J. Gleghorn for an image of chicken embryo vasculature. This work was supported by the NIH Director's New Innovator Award (1DP2OD004389) to Z.G., a National Science Foundation grant (1330288) to Z.G. and H.A.S., an NIH NRSA postdoctoral fellowship (F32AI095002) to A.S., and a graduate fellowship from the STX Scholarship Foundation to M.K.K.

Received: September 23, 2014
Revised: January 2, 2015
Accepted: February 26, 2015
Published: April 2, 2015

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