Cell density and mobility protect swarming bacteria against antibiotics

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Edited by Raghavendra Gadagkar, Indian Institute of Science, Bangalore, India, and approved December 9, 2009 (received for review September 23, 2009)

Swarming bacteria move in multicellular groups and exhibit adaptive resistance to multiple antibiotics. Analysis of this phenomenon has revealed the protective power of high cell densities to withstand exposure to otherwise lethal antibiotic concentrations. We find that high densities promote bacterial survival, even in a nonswarming state, but that the ability to move, as well as the speed of movement, confers an added advantage, making swarming an effective strategy for prevailing against antimicrobials. We find no evidence of induced resistance pathways or quorum-sensing mechanisms controlling this group resistance, which occurs at a cost to cells directly exposed to the antibiotic. This work has relevance to the adaptive antibiotic resistance of bacterial biofilms.

swarming motility | antibiotic resistance | group trait | surfactants | biofilms

Swarming is defined as flagella-driven bacterial group motility over a surface, which is observed in the laboratory on media solidified with agar (1–4). The percentage of agar is critical for enabling swarming. Some bacteria like *Vibrio parahaemolyticus* and *Proteus mirabilis* can swarm readily on higher percentage agar (1.5–3%; referred to here as hard agar), whereas others like *Salmonella*, *Escherichia coli*, *Serratia*, *Pseudomonas*, and *Bacillus* swarm only on lower percentage agar (0.5–0.8%; referred to here as medium agar to distinguish it from even lower percentage soft agar in which the bacteria swim individually within water-filled channels inside the agar). Hard-agar swarmers differentiate into specialized swarm cells that are elongated and have increased flagella. Medium-agar swarmers generally do not display a similar differentiated morphology (5, 6). In many of the latter class of swarmers (e.g., *Serratia, Pseudomonas*, *Bacillus*), movement is enabled by powerful extracellular surfactants whose synthesis is under quorum-sensing control (7, 8). Surfactants lower surface tension and allow rapid colony expansion (9–11). *Salmonella* and *E. coli* do not appear to make such surfactants (12).

An elevated resistance to multiple antibiotics has been reported for swarming populations of *Salmonella enterica* (13, 14), *Pseudomonas aeruginosa* (15), and a variety of other medium-agar swarmers, including *Serratia marcescens* and *Bacillus subtilis* (16). This resistance was reported to be linked specifically to swarming and was not observed in the same bacteria growing on hard agar, where they cannot move, or in soft agar, where they swim inside the agar. Therefore, the resistance was attributed to a physiology specific to swarmer cells. The resistance was not attributable to selection for antibiotic-resistant mutants, because the swarmer cells were killed with lethal doses of antibiotic when inoculated in fresh liquid media (13, 15), reminiscent of the nongenetic or "adaptive resistance" seen in bacterial biofilms (17).

The present study was initiated to reexamine the data showing that the adaptive resistance of *Salmonella* swarmers is attributable to their special physiology, a conclusion at odds with a microarray study that found essentially similar genome-wide expression profiles (a proxy for physiology) for *Salmonella* growing on medium vs. hard agar (i.e., swarming vs. nonswarming conditions, respectively) (6). We show here that adaptive resistance is a property of high cell densities within the swarming colony and not of swarming-specific physiology as concluded earlier. We test predictions of the *Salmonella* results in two other swarming bacteria—*Bacillus* and *Serratia*—and show that cell density and mobility are common protective features for survival against antimicrobials.

Results and Discussion

Antibiotic Resistance Is a Property of High Cell Density and Is Favored by Mobility. In soft agar (0.3%), cells swim individually inside the agar and are referred to as swimmers. In medium agar (0.6%), cells move as a group on the surface and are referred to as swarmers. The original E-test strip assay showing differential antibiotic resistance of swimmer and swarmer cells of *Salmonella* (13, 14) is shown in Fig. 1*A*. The strips have a predefined gradient of antibiotic concentrations (highest at the top end), and the antibiotic diffuses into the surrounding medium when the strip is placed on the surface of the agar. The three antibiotics tested target different processes in the cell, namely, DNA replication (ciprofloxacin), protein synthesis (kanamycin), and membrane integrity (polymyxin). Bacteria are inoculated at points indicated by the asterisks, from which they migrate outward (the antibiotics do not elicit a chemotactic response). When they encounter the antibiotic, both swimmers and swarmers are expected to stop as a result of cell killing. A pear-shaped clear zone was visible for the swimmers, delineating the area into which the antibiotic had diffused, and arrested their migration. The lower end of this zone marks the minimum inhibitory concentration (MIC) for swimmers. Swarmers displayed no inhibition zones on these plates, consistent with earlier results showing that swarmers are resistant to higher antibiotic concentrations than swimmers. However, this test might potentially overestimate MIC values for swarmers because they achieve higher cell densities than swimmers. Sampling of local cell density showed that the advancing edge of a swarm colony has ~30-fold higher cell density compared with that of a swimming edge and ~75-fold higher density than an exponentially growing broth culture (Fig. 1*B* and Fig. S1). The high density is a property of surface growth, and similar densities are achieved on both medium and hard agar (see below).

To test if the higher resistance exhibited by swarmer cells is attributable to their higher cell density rather than their swarmer cell status, the surface of medium- and hard-agar plates was uniformly inoculated with a low density of cells (Fig. 2*A*; 0-h time point). E-test strips containing ciprofloxacin were applied on the surface of these plates at various times from 0 to 3 h of growth (i.e., at increasing cell density), and the plates were photographed 3 h after the application (Fig. 2*B*). On hard agar, where cells cannot move, the inhibition zone around the E-test strips was maximal when cell density was low (0–1.5 h), diminished significantly after 2 h of growth, and was erased by 3 h, showing a

Author contributions: M.T.B, Q.W. and R.M.H. designed research; M.T.B. and Q.W. performed research; R.M.H. contributed new reagents/analytic tools; R.M.H. analyzed data; and R.M.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0910934107/DCSupplemental.

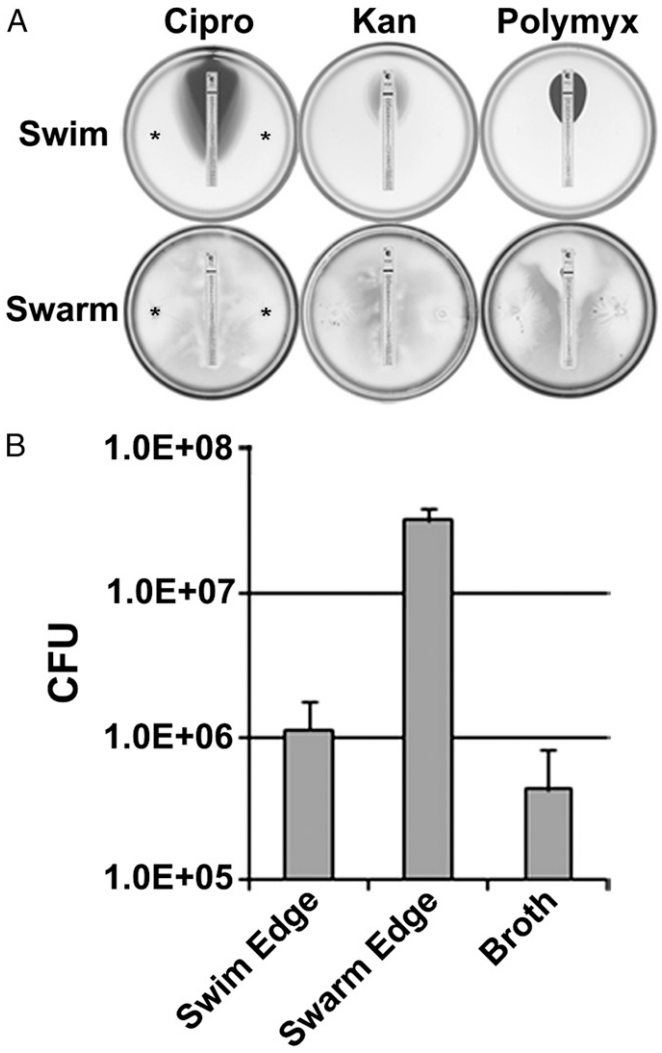

Fig. 1. Antibiotic response and cell densities of bacteria moving within soft agar (swim) or over the surface of medium agar (swarm). (*A*) E-test strips containing a gradient of indicated antibiotics (decreasing from top to bottom) were placed in the center of swim (0.3%) or swarm (0.6%) agar plates. Bacteria (*Salmonella*) were inoculated at a point indicated by the asterisk and allowed to migrate outward. Plates were incubated at 37°C overnight and photographed against a black background so that zones of bacterial colonization appear white and uncolonized agar appears black. Cipro, ciprofloxacin; Kan, kanamycin; Polymyx, polymyxin. (*B*) Relative local cell densities of indicated *Salmonella* cultures determined by controlled sampling using the flat end of a cylindrical culture stick (see *Methods*). The cell numbers represent cells per stick sampled (cfus). Measurements of swim and swarm edges were taken ~6 h after the initial inoculation (swarming motility initiates at ~3 h). The accuracy range of this method was tested using broth-grown cells concentrated to various degrees, as shown in Fig. S1.

clear dependence on cell density. [Cells are still in the exponential phase of growth at these time points (6)]. On medium agar, where cells swarm, the antibiotic zone was colonized even earlier, at 1.5 h. If observed at 4 h after E-test strip application, no inhibition zones remained on swarm agar even on the 0-h plate (also see swarm plates in Fig. 1*A*). This experiment demonstrates the importance of high cell density in promoting growth in the antibiotic zones, irrespective of swarmer cell status. However, the swarmer population apparently has the additional advantage of mobility, promoting earlier migration into these zones after a buildup of cell density. We note that the previous conclusion that nonswarmers do not exhibit resistance was arrived at by placing E-strips on hard agar seeded with low cell densities of bacteria, similar to the 0-h sample in Fig. 2*A* (13, 14).

Cell density dependence of antibiotic resistance could also be observed in concentrated broth-grown cells (Fig. S2).

Border-Crossing Assay to Measure Adaptive Resistance of Swimming vs. Swarming Bacteria. The antibiotic zone around the E-test strips is narrow and readily infiltrated by swarmers. To test how far swarmers would travel on a wider antibiotic surface, we set up a test we call the "border-crossing test," where the border is a plastic barrier dividing a Petri plate into two chambers. Media was poured into both chambers, but antibiotic was added only to the right chamber (Fig. 3*A*). A thin (~1-mm tall) agar bridge was constructed over the barrier (*Methods*), which allowed the bacteria inoculated in the left chamber to cross over and migrate to the right. The narrow bridge minimized antibiotic diffusion, as seen by lack of significant growth inhibition left of the border. The cross-border plates gave swarmers more time to declare their MICs, because it takes several generations for *Salmonella* at the border to colonize the entire right chamber on control plates (5–6 h at 37 °C). Both swimmers and swarmers had to navigate the border crossing in a similar space. Swarmers were clearly able to move on higher antibiotic media than swimmers, although the rate of advancement of the swarm front decreased with increasing antibiotic until the swarmers were eventually arrested at the border. It took 10-fold higher kanamycin and 200-fold higher ciprofloxacin to arrest swarmers compared with that required to arrest swimmers at the border (Fig. 3*A*).

If cells surviving on the antibiotic surface had turned on pathways to enable resistance, they should exhibit a growth advantage when transferred to fresh swarm media containing similar antibiotic concentrations. Transferred cells were picked from the moving edge to ascertain that they were still in the exponential phase of growth. Such a transfer maintains the physiological status of the cells but does not deliver the original high cell densities. These cells were killed on transfer, ascertaining that their antibiotic resistance was not induced (Fig. 3B). When plated clonally, similar results were obtained (i.e., the resistant population was unable to grow from single cells on transfer to either medium- or hard-agar antibiotic plates) (Fig. S3). We conclude that some feature of the swarming colony other than long-lived induced resistance must contribute to its survival; the ability to swarm acts as a preadaptation for survival in an antibiotic-containing solid environment.

Swarming Bacteria Sustain Cell Death While Navigating the Antibiotic Surface. If antibiotic resistance is not induced but is somehow afforded by high cell density and an ability to move, a subpopulation of cells that is in direct or prolonged contact with the antibiotic likely gets killed. The advancing edge of a swarming *Salmonella* colony has an ~2–3 cell-wide zone consisting of a monolayer of cells but is generally multilayered behind this edge, with cells moving continuously through these layers (Movie S1). Survivors are likely those that are in the upper layers or those that minimize their exposure to the antibiotic by circulation through the multilayers. To test if cells from the antibiotic region are killed, they were treated with live/dead stain that stains live cells green and dead cells red. Cell death was clearly apparent in cells taken from the antibiotic regions (Fig. 3*C*), consistent with the visibly lower growth resulting from cells transferred from the antibiotic region compared with those from the control region (Fig. 3*B*, *Left*). Thus, the swarming colony endures death of a subpopulation while continuing to move.

Quorum-Sensing Regulators Are Not Involved in Tolerance to Antibiotics. In a process referred to as quorum-sensing, bacteria can produce and detect signaling molecules to control their behavior in response to variation in cell density (18). To determine if

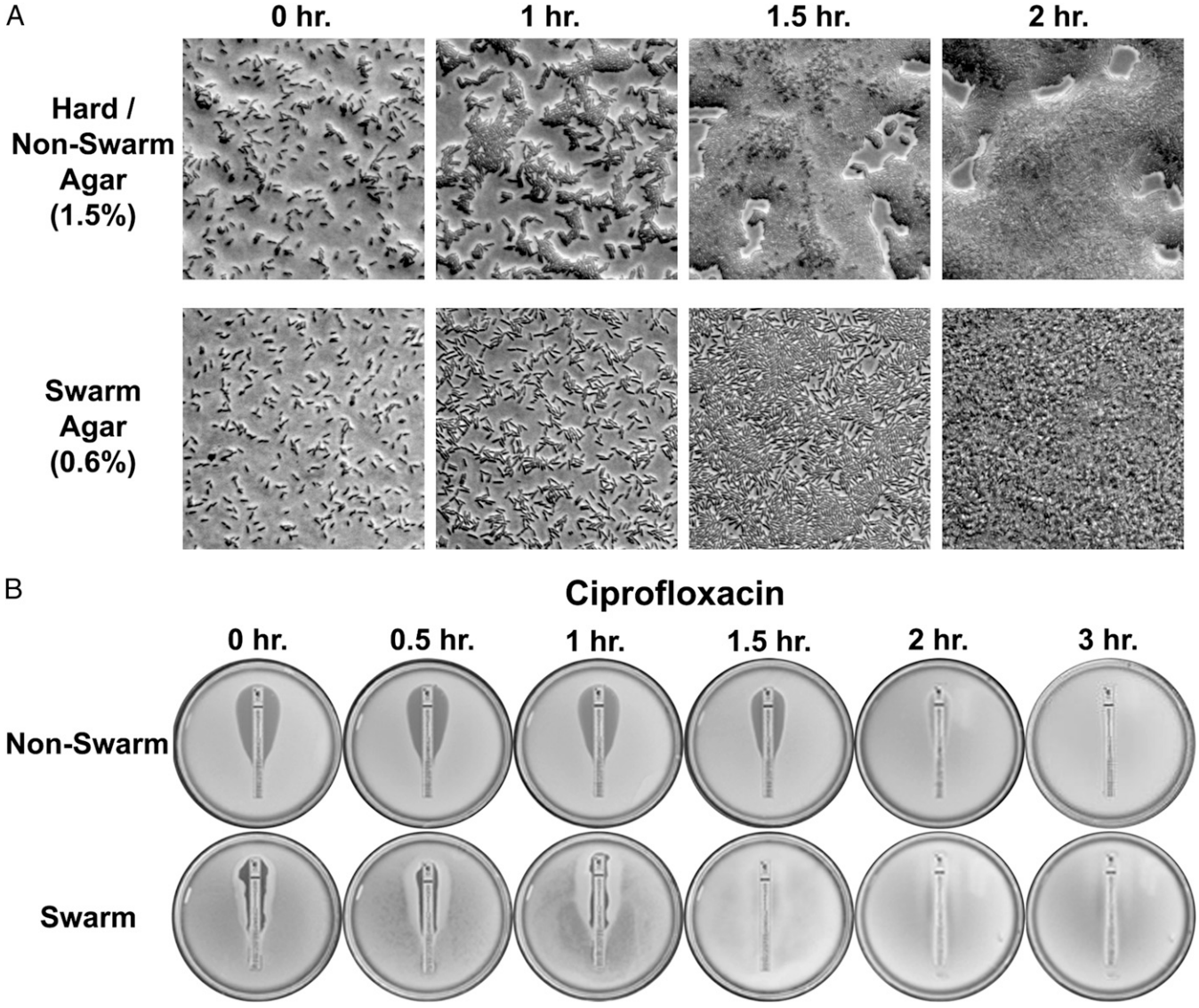

Fig. 2. Nonswarmers show cell density-dependent antibiotic resistance. (*A*) Phase-contrast images (100× magnification) showing the density distribution of cells during 0–2 h of growth on the surface of swarm and nonswarm hard-agar plates. The pour-and-drain method of inoculation from a broth culture at an OD_{600} of ~0.7 was used to get an initially uniform distribution of cells on the agar surface (6). At 0 h, the cell density is low and cells do not touch each other. Cell density increases continuously with time, and growth rates on both sets of plates are similar (see figure 4 of ref. 6). Cells tend to grow in aggregates on the hard agar, likely because the surface of hard agar appears not to be as smooth as that of the swarm agar. Cell density becomes confluent by 2 h; some clear pockets remain on hard agar, likely attributable to the initial uneven distribution of cells. Motility initiates on swarm plates between 2 and 2.5 h. *(B)* Ciprofloxacin E-test strips were applied to the surface of plates shown in *A* at indicated times after the plates had been evenly inoculated. Plates were photographed 3 h after E-test strip application. Similar results were obtained with kanamycin and polymyxin. The opaque halo around the clear zones surrounding the E-strips on 0–1-h swarm plates likely results from accumulating dead cells that migrate into this region (Fig. 3*C*).

the high cell densities of swarming bacteria turn on quorum-sensing pathways that aid migration on the antibiotic surface, we tested a *luxS* mutant defective in synthesis of the only known quorum-sensing signaling molecule in *Salmonella*, an *N*-acylhomoserine lactone derivative called AI-2 (19). AI-2 synthesis has been reported to be up-regulated in *Salmonella* swarmers (20), and there is a report that AI-2 affects antibiotic susceptibility of *Streptococcus anginosus* (21). A *luxS* mutant showed antibiotic resistance similar to the WT control (Fig. S4). Thus, AI-2 is not necessary for the ability of *Salmonella* swarmers to migrate into antibiotic zones.

We conclude from the experiments in Figs. 1–3 and Figs. S2–S4 that adaptive resistance or tolerance to antibiotics is not a property of a gene expression program specific to *Salmonella* swarmers, as concluded earlier (13, 14), but is rather afforded by high cell densities. The known quorum-sensing pathway in *Salmonella* does not play a role in adaptive resistance. Although all cells—swarmers, nonswarmers, and broth-grown swimmers—can tolerate higher antibiotic concentrations at high cell densities, the ability to move gives swarmers an added advantage in overriding the antibiotic. Individuals within the dense-moving group, likely those directly exposed to the antibiotic, undergo cell death, protecting cells that are likely not directly exposed.

Faster Migration Enables Higher Adaptive Resistance. Because cell density and mobility are apparently the only protective features of the swarm and a subset of cells is being killed by the antibiotic, slower swarmers would be expected to suffer higher casualties because of longer exposure to the antibiotic, and would therefore invade less

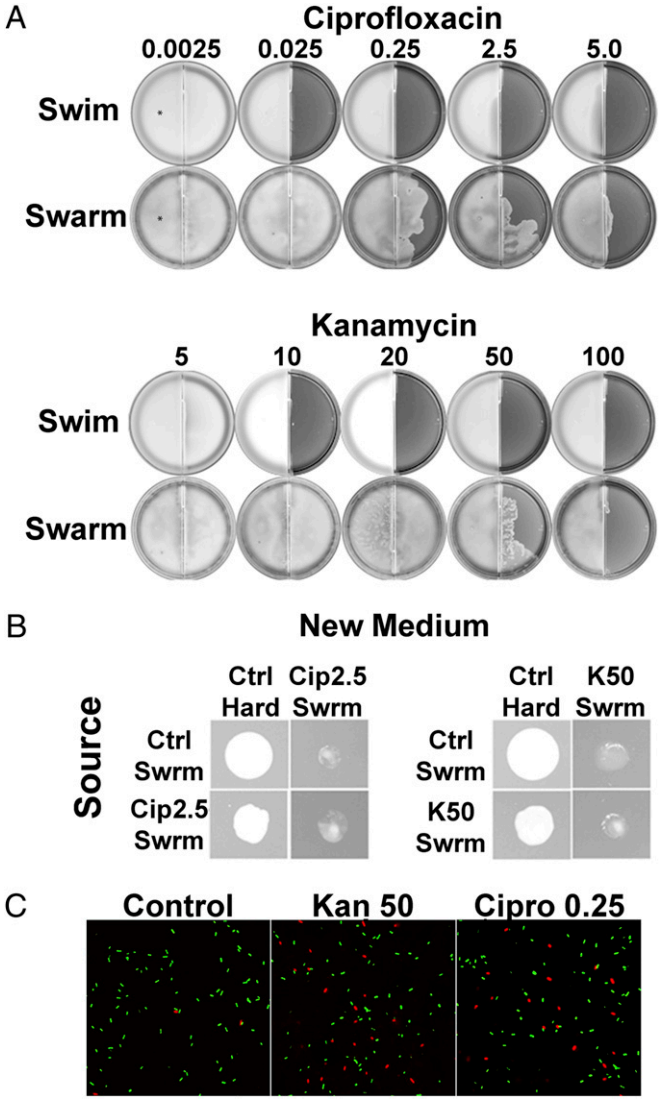

Fig. 3. Border-crossing assay, adaptive resistance, and cell death in *Salmonella*. (*A*) Cells were inoculated in the left no-antibiotic chamber and allowed to migrate to the right antibiotic-containing chamber (*Methods*). Numbers refer to μg/mL of indicated antibiotic. Plates were incubated at 37 °C for 16 h, which is the time it took for bacteria in the no-antibiotic control plates to colonize the entire right chamber. (*B*) Antibiotic sensitivity of swarmer cells that crossed the border on ciprofloxacin (Cip) and kanamycin (K) plates. Cells just behind the edge of the moving front were transferred by the flat end of a cylindrical toothpick to fresh swarm plates containing the same antibiotic concentration from which cells were picked (Fig. S3). Controls (Ctrl) included cells from the no-antibiotic side. The control no-antibiotic plates were solidified with 1.5% (w/v) agar to prevent swarming. (*C*) Swarmer cells from indicated plates stained with the live/dead stain. The red cell fraction was 6% on the control plates, 38% on kanamycin (Kan) 20, and 30% on ciprofloxacin (Cipro) 0.25 (*Methods*).

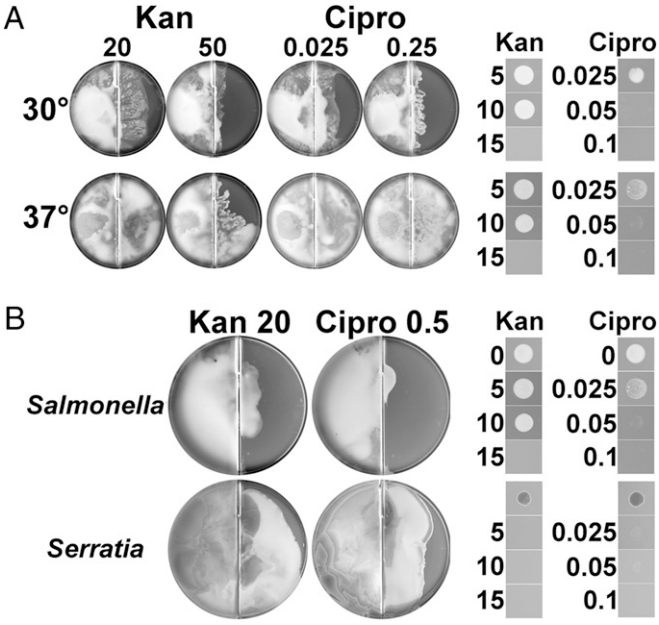

Fig. 4. Faster migration enables higher adaptive resistance. (*A*) Cross-border swarm plates were inoculated with *Salmonella* as described in Fig. 3*A*. The 37 °C and 30 °C plates were incubated for 16 and 26 h, respectively, the time at which control plates were fully colonized. Further incubation did not promote additional migration. Sensitivity of corresponding broth cultures (OD_{600} ~0.7) spotted on indicated (μg/mL) antibiotic plates is shown on the right. (*B*) Plates were inoculated with either *Salmonella* or *Serratia* and incubated at 30 °C. The experiment was stopped at 12 h when *Serratia* colonized the entire right side on control plates. *Salmonella* had just arrived at the border in 12 h and did not cross the border significantly on the antibiotic plates, even when incubated for longer times. We note that experiments in *A* and *B* were performed on different days; thus, the 30 °C *Salmonella* plates cannot strictly be compared between the two panels. The strips on the right show antibiotic sensitivity of corresponding broth-grown cultures as described in *A*. The dark color of *Serratia* is attributable to a red pigment that accumulates during overnight growth. Cipro, ciprofloxacin; Kan, kanamycin.

territory compared with faster swarmers. To test this prediction, we compared resistance to kanamycin and ciprofloxacin at two different temperatures, which promote different rates of movement. At 30 °C and 37 °C, the *Salmonella* swarming fronts move at the average rate of 1.5 mm/h and 5 mm/h, respectively. A temperature of 37 °C enabled migration over higher antibiotic concentrations than one of 30°C (Fig. 4*A*, *Left*), even though sensitivities of broth-grown cells to these antibiotics are similar at both temperatures (Fig. 4*A*, *Right*). Similarly, a *Salmonella* mutant that swarms at a slower rate was unable to move over antibiotic concentrations easily colonized by the WT at the same temperature (Fig. S5). These data show a direct relation between adaptive resistance and swarming speed, and they support the notion that the exposure time of the group to the antibiotic is a critical factor affecting resistance.

Testing Predictions of *Salmonella* Results in Other Swarming Bacteria. The swarming behavior of many medium-agar swarmers, particularly those with peritrichous flagella, is very similar. Swarming initiates only after a buildup of cell density; cells at the edge of the colony are not as motile as those immediately behind; multilayered bacterial rafts swirl in different directions in highly motile regions; and cells are motile only in groups, becoming immotile if accidentally isolated. This shared behavior likely comes from a common cell shape, common flagellar mechanics, and common challenge of moving against surface friction. Because adaptive resistance to antimicrobials is exhibited by many medium-agar swarmers, it is reasonable to assume that the survival strategy used by *Salmonella* will be shared by these other swarmers. We therefore tested two predictions from the *Salmonella* results in other swarming bacteria that show adaptive resistance—*S. marcescens* and *B. subtilis*.

The prediction that adaptive resistance should be governed by swarming speed was satisfied in *Salmonella* in two experimental setups: at two different temperatures with WT (Fig. 4*A*) and at the same temperature with WT vs. a slow-swarming mutant (Fig. S5). To extend these results further, we compared two different bacteria—*S. enterica* and *S. marcescens*—that swarm at different speeds at the same temperature. The swarming dynamics and group morphology of these bacteria are otherwise indistinguish-

able (Movie S2, compare with Movie S1). The faster speed of *Serratia* is attributable to a secreted lipopeptide surfactant (9, 22). Under conditions optimal for *Serratia* motility (30°C), the swarming front moves at the average rate of 7 mm/h in *Serratia* and 1.5 mm/h in *Salmonella*. Cross-border experiments comparing migration of the bacteria on kanamycin and ciprofloxacin plates are shown in Fig. 4*B*. Broth-grown cells of both bacteria are sensitive to low levels of these antibiotics (Fig. 4*B*, *Right*). *Serratia* is more sensitive to kanamycin than *Salmonella*, yet it efficiently colonized kanamycin 20 as well as ciprofloxacin 0.5 plates, whereas *Salmonella* was stopped near the border. Thus, the relation between adaptive resistance and swarming speed observed in *Salmonella* could be extended to *Serratia*.

Another prediction of the *Salmonella* experiments is that the cells that get killed are those directly exposed to the antibiotic, whereas those that are protected are in the interior of the multilayered swarming colony. To test this, we turned to *B. subtilis*, which initially sends forth a monolayer of cells, exposing them directly to the antibiotic (10). The monolayer migrates rapidly, aided by a lipopeptide surfactant (23) (Movies S3 and S4). The colony later becomes multilayered, attributable both to subsequent waves of bacteria that travel over the monolayer and growth within the monolayer. The behavior of *Bacillus* with increasing polymyxin concentrations is shown in Fig. 5*A*. The initial monolayer was seen traversing the right chamber at all antibiotic concentrations (likely aided by rapid spreading of the surfactant), although its rate of advancement decreased with increasing polymyxin. Live/dead staining revealed increasing cell death in this monolayer with increasing antibiotic (Fig. 5*B*). Cells from the left continued to move in and swarm over the monolayer, but they were stalled at the border at higher antibiotic concentrations. To test if a multilayered swarm would offer more protection, we altered the experimental setup, allowing swarmer cells to build up density in the left chamber before pouring antibiotic media into the right chamber. As a dense colony, cells were able to cross over and survive on higher antibiotic zones compared with a single-layered colony (Fig. 5*C*; compare polymyxin 20 and polymyxin 50 in Fig. 5C with similar plates in Fig. 5A). These experiments demonstrate both that cells directly in contact with the antibiotic get killed and that a multilayered colony is at an advantage compared with a monolayered colony while navigating antimicrobial territory, satisfying the expectations from the *Salmonella* results.

That observations with swarming *Salmonella* can be extended to different bacterial genera speaks to a commonly conserved behavior of their swarms.

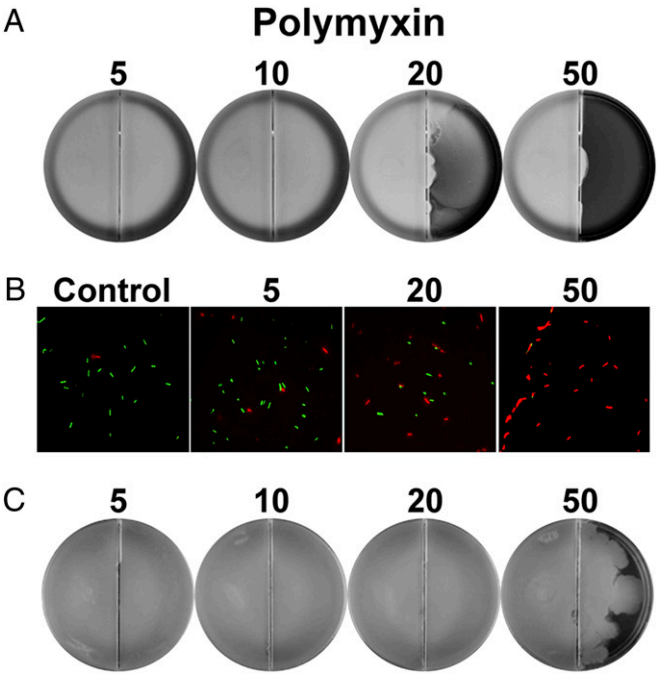

Fig. 5. Behavior of *Bacillus* in the cross-border assay. (*A*) Cross-border migration of *B. subtilis* over increasing polymyxin concentrations. Numbers refer to μg/mL. Plates were incubated at 37 °C for 16 h. (*B*) Increasing cell death in the monolayer swarms on polymyxin plates stained with live/dead stain. The fraction of red cells was 8% on the control plates, 15% on polymyxin 5, 20% on polymyxin 20, and 80% on polymyxin 50 in monolayer samples from plates shown in *A*. (*C*) As in *A*, except that cells were allowed to build up cell density in the left chamber (16 h at 37 °C) before pouring antibiotic media into the right chamber. The plates were incubated for another 16 h.

Adaptive Resistance: Self-Sacrifice or Selfish Behavior? Our study has shown that high bacterial densities promote survival of swarming bacteria in certain types of harsh environments. The survival occurs without apparently altering gene expression but at a cost to some individuals. Swarms typically move after reaching a density threshold. Movement to a different location involves risk, and in some cases, part of or all the moving swarm could get wiped out. This cost of movement might appear to represent a form of self-sacrifice or "altruism," a trait particularly observed in species with complex social structures (24). However, migration can be favored as a "selfish" trait, even when the death rate during movement is high (25), such that the death that occurs in a swarm does not, by itself, point to altruism. From another perspective, there is a clear group benefit of high density in a swarm. Group benefits are sometimes associated with (or identified with) altruistic behavior. At face value, however, our observations could sit well with more than one model in which group benefit follows from phenotypic heterogeneity and intercellular interactions with selection at the level of the individual (26), combined with a Poisson-type distribution of group sizes in each generation (27), or "safety in numbers" leading to "byproduct benefit" (28). None of these models require altruism. To all appearances, our observations would favor the selfish model, in which all cells are actively trying to stay alive but some get caught in the swarm in a way that leads to their death. For example, survival may be highest on top (furthest from the antibiotic), but some bacteria just get "piled on" and die because they cannot get off the bottom in time. In general, there may be positions within a swarm that are better for survival than others, and if bacteria are capable of sensing where those best locations lie, there could be considerable selfishness to individual bacterial attempts to reach those positions, leading to classic "selfish herd" dynamics (29). We point to a parallel behavior of red fire ants, which, when floods arrive, survive by binding together the entire colony into a dense ball that floats on the flood waters until the ants drift to higher ground (Fig. S6). The ants constantly reposition themselves to minimize their exposure to water (30).

Whatever the underlying mechanism and evolutionary basis, a group-level trait, namely, swarming behavior, confers a fitness advantage to individual members of the group when the environment contains something harmful. The mobility of the swarm allows it to "outrun" harsh conditions to reach safer ground. The dead bacteria in immediate contact with the antibiotic might provide a physical barrier that protects those on top. They might also feed the group with nutrients released on their death, as seen during the cannibalistic behavior of *B. subtilis* bacteria, which feed on their siblings to delay committing to spore formation (31).

In summary, three different swarming bacteria exhibit a common survival strategy against antibiotics. This strategy involves maintaining high cell density, circulating within the multilayered colony to minimize exposure to the antibiotic, and the death of individuals that are directly exposed.

Methods

Strains and Growth Conditions. WT *Salmonella enterica* serovar Typhimurium (strain 14028) and its mutant *luxS* strain have been described (12, 32), as has WT *Serratia marcescens* (strain 274) (22). The *flhE* mutant of *S. enterica* is a complete gene deletion, constructed by Jaemin Lee (University of Texas, Austin). WT *Bacillus subtilis* strain 3610 was obtained from Daniel Kearns (Indiana University, IN). All strains were grown in LB (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) broth. LB plates (25 mL) were solidified with 0.3%, 0.6%, or 1.5% Bacto agar (Difco). For *S. enterica*, 0.5% glucose was added to swarm plates. Plates were allowed to dry on the bench top overnight and were used the next day. For *S. marcescens,* optimal motility is observed at 30°C and is inhibited at 37°C (22). The optimal temperature for *S. enterica* and *B. subtilis* motility is 37°C. Border-crossing plates were prepared by pouring 30 mL of LB swarm media into each chamber in a two-step process: The antibiotic media were poured first and allowed to harden before the nonantibiotic side was filled. Before the latter media hardened, a sterile culture stick was introduced into the molten media and the meniscus was dragged over the plastic border, connecting the two sides with an ~1-mm tall agar bridge. Motility plates were inoculated with 5 μL of an exponentially growing broth culture at an OD_{600} of ~0.7. The drop was allowed to dry for ~15 min with the lid off and was then transferred to the incubator. Plates were photographed using either a BioRad Geldoc system or a "bucket of light" device (33) and a Canon Rebel XSI digital camera.

Cell Density Measurements. Cell density measurements were determined by controlled sampling using the flat end of a round culture stick (2-mm diameter). The stick was held upright and gently touched to the culture to be sampled (swimming, swarming, or broth culture). The stick was then rinsed in 1 mL of LB. The sampling procedure was repeated two more times, using a different stick each time, and the bacteria from all three samplings were collected in the same 1 mL of LB. Serial dilutions of this tube were plated on LB agar plates to determine the cfus. The cell numbers were normalized to cells per sampling stick. Three independent experimental repeats of this method yielded similar cell numbers. Broth-grown cells were concentrated as follows: 15 mL of an exponentially growing LB culture at an OD_{600} of ~0.7 was pelleted by centrifugation at 5,000 × *g*, diluted to various extents with LB, and sampled with the end of a culture stick as described previously.

E-Test Assay. The E-test strip comprises a predefined gradient of antibiotic concentrations on a plastic strip. The strips were purchased from AB Biodisk and applied to the surface of swim or swarm plates before spot inoculation of cells on either side. On hard-agar plates, broth-grown cells were uniformly inoculated by the pour-and-drain method described by Wang et al. (6) before application of the strips. All assays were repeated at least three times.

Live/Dead Staining. The stain was purchased from Invitrogen, and cells were stained according to the manufacturer's specifications. The kit includes two nucleic acid stains—green-fluorescent STYO 9 and red-fluorescent propidium iodide (PI). STYO 9 labels both live and dead bacteria alike, whereas PI reduces STYO 9 stain intensity only after crossing damaged cellular membranes. To determine red/green cell numbers, at least 500 cells were counted in each sample analyzed.

Microscopy. Phase-contrast images were obtained with a DP-12 digital camera (Olympus) attached to an Olympus BH2 microscope. Red and green fluorescence of cells stained with live/dead stain was monitored using an Olympus BX60 microscope equipped with a Photometrics Quantix camera system. Optimal excitation wavelengths for STYO and PI are 480 nm and 490 nm, respectively. Red and green images were captured and overlaid using MetaMorph software (Molecular Devices Corporation) or MDC. Movies of swarming bacteria were recorded using an Olympus IX50 microscope, maintained in a temperature- and humidity-controlled environment, and equipped with LD ×20 and ×60 phase-contrast objective lenses. Motion was captured with a digital camera at 30 frames per second and a spatial resolution of 640 × 480 pixels. To improve clarity and minimize vibration attributable to bacterial motion, 11 mL of LB swarm medium was used per plate.

ACKNOWLEDGMENTS. We thank Avraham Be'er for use of his microscope facility and help with recording the movies. We are grateful to James Bull, Raghavendra Gadagkar, Richard Meyer, and Vidyanand Nanjundiah for their comments and ideas on evolutionary mechanisms and to David Moynahan for sharing his fire ant image. This work was supported by National Institutes of Health Grant GM 57400.

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