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Convergent Evolution of Hyperswarming Leads to Impaired Biofilm Formation in Pathogenic Bacteria

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SUMMARY

Most bacteria in nature live in surface-associated communities rather than planktonic populations. Nonetheless, how surface-associated environments shape bacterial evolutionary adaptation remains poorly understood. Here, we show that subjecting *Pseudomonas aeruginosa* to repeated rounds of swarming, a collective form of surface migration, drives remarkable parallel evolution toward a hyperswarmer phenotype. In all independently evolved hyperswarmers, the reproducible hyperswarming phenotype is caused by parallel point mutations in a flagellar synthesis regulator, FleN, which locks the naturally monoflagellated bacteria in a multiflagellated state and confers a growth rate-independent advantage in swarming. Although hyperswarmers outcompete the ancestral strain in swarming competitions, they are strongly outcompeted in biofilm formation, which is an essential trait for *P. aeruginosa* in environmental and clinical settings. The finding that evolution in swarming colonies reliably produces evolution of poor biofilm formers supports the existence of an evolutionary trade-off between motility and biofilm formation.

INTRODUCTION

In nature, bacteria are generally not found in free-swimming, planktonic states but rather living in dense, surface-associated communities called biofilms (Kolter and Greenberg, 2006). Still, much of our knowledge of bacteriology comes from studying bacterial populations in liquid culture, neglecting this very spatial structure (Mitri et al., 2011). When studying bacterial evolution, in particular, most experiments are done in well-mixed liquid cultures (e.g., Blount et al., 2008; Chou et al., 2011; Lenski et al., 1991; Perfeito et al., 2007; Tenaillon et al., 2012; Woods et al., 2006). Bacterial evolution is central to many natural and human activities, from the improvement of bioremediation (Smidt and de Vos, 2004) and the treatment of infectious disease (Ensminger et al., 2012; Lieberman et al., 2011; Taubes, 2008) to the evolution of life on Earth (David and Alm, 2011; Dietrich et al., 2006; Kasting and Siefert, 2002). The structure of natural surface-associated communities likely plays a major role in bacterial evolutionary adaptation (Hibbing et al., 2010; Nadell et al., 2010; Xavier et al., 2009).

Here, we investigate evolution in swarming colonies of *Pseudomonas aeruginosa*. Swarming is a collective form of motility over soft surfaces (Kearns, 2010; Köhler et al., 2000; Rashid and Kornberg, 2000). A versatile environmental microbe, *P. aeruginosa* is an opportunistic pathogen notorious for causing diverse infections at multiple sites, including wounds, the circulatory system, the urinary tract, and the lungs of patients with cystic fibrosis. As for all disease-causing organisms, evolutionary adaptation is central to *P. aeruginosa* pathogenesis (Cattoir et al., 2013; Oliver et al., 2000; Smith et al., 2006; Weigand and Sundin, 2012; Yang et al., 2011); however, we have little understanding of selection pressures governing its evolution in surface-associated communities. *P. aeruginosa* is a well-known biofilm former, and its biofilms are notoriously difficult to eradicate by conventional antibiotic treatment (Costerton et al., 1999). Biofilm formation is, therefore, being studied extensively in search of novel therapeutic approaches (reviewed in Boyle et al., 2013). Swarming motility raises equally interesting implications. Previous studies have shown that *P. aeruginosa* cells in swarming colonies can have distinct phenotypes from planktonic cultures, including gene expression (Tremblay and Déziel, 2010) and increased antibiotic resistance (Lai et al., 2009). In addition, the self-produced biosurfactants required for swarming motility (Caiazza et al., 2005; Déziel et al., 2003) are important for biofilm maintenance and dispersion (Boles et al., 2005; Lequette and Greenberg, 2005) as well as to kill immune cells (Jensen et al., 2007). Biofilm formation and swarming

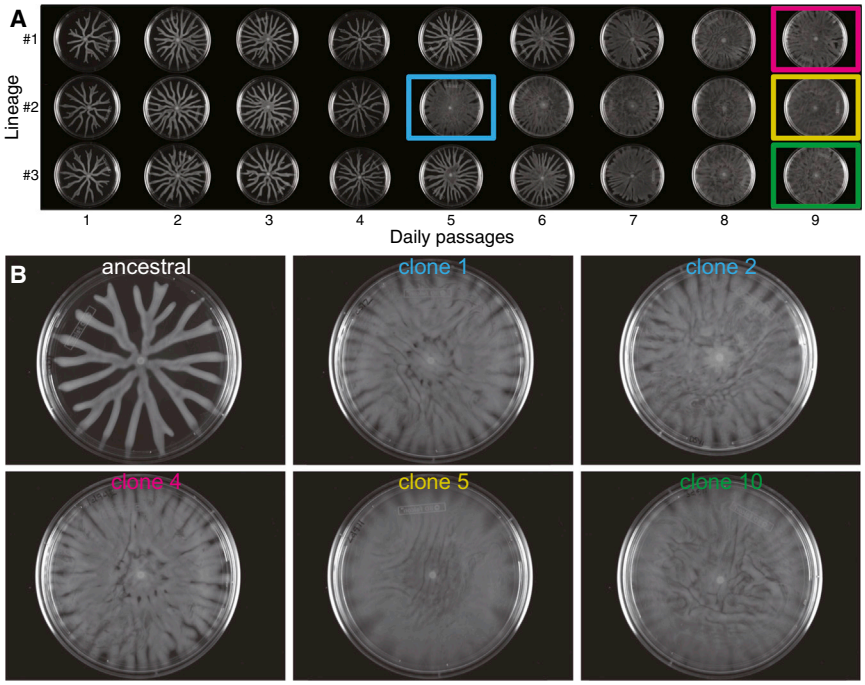


Figure 1. Experimental Evolution of Swarming Motility Produces a Stable and Heritable Hyperswarmer Phenotype

(A) Three independent lineages (1–3) were subjected to experimental evolution by sequential passages of growth in swarming media. After each 24 hr swarming interval, the entire colony was flushed off the plate, and a 1/1,500 fraction of the recovered population was point inoculated onto a fresh swarming plate. Lineage #2 acquired a hyperswarming phenotype at day 5, whereas lineages #1 and #3 only did so at day 7. The colonies outlined in color were selected for clonal isolation procedures. The color-coding scheme (cyan for lineage #2 at day 5, magenta for lineage #1 at day 9, yellow for lineage #2 at day 9, and green for lineage #3 at day 9) is maintained throughout the paper.

(B) Hyperswarming is stable and heritable. Swarming colonies of the ancestral strain and clones isolated from each of the colonies outlined in (A) are shown.

See also Movie S1.

motility are inversely regulated (Baraquet et al., 2012; Caiazza et al., 2007; Kuchma et al., 2007; Lee et al., 2007), and the regulation involves the second messenger cyclic diguanylate (c-di-GMP): high levels of c-di-GMP induce biofilm formation and suppress swarming motility (Baraquet et al., 2012). This inverse regulation could be of interest for novel therapeutics against biofilm formation.

Swarming colonies of *P. aeruginosa* make characteristic branching patterns of striking regularity (see Movie S1). This phenotype requires coordination of several pathways, including flagellar motility (Köhler et al., 2000), cell-cell signaling/quorum sensing (Köhler et al., 2000; Xavier et al., 2011), and biosurfactant secretion (Caiazza et al., 2005; Déziel et al., 2003). Screening for genes affecting swarming motility yielded over 200 hits in diverse functional categories, including motility, transport secretion, metabolism, and transcriptional regulation (Yeung et al., 2009). Given the multifactorial nature of swarming motility, one would naively expect that repeated rounds of swarming in independent lineages could lead to divergent evolution. Such evolutionary diversification has been observed before in bacterial experimental evolution, even with bacteria facing relatively well-defined evolutionary pressures such as new nutrients (Chou et al., 2011; Herring et al., 2006; Woods et al., 2006), a change in temperature (Bennett and Lenski, 2007; Lenski and Bennett, 1993; Tenaillon et al., 2012), or the overexpression of costly genes (Chou and Marx, 2012).

Here, we show that repeated passaging of *P. aeruginosa* on swarming plates leads to striking parallel molecular evolution. After only a few daily passages, we see emergence of a hyperswarming phenotype where the colony covers the entire plate. Hyperswarming is caused by single-point mutations in the flagellar synthesis regulator FleN, which cause the bacteria to assemble multiple polar flagella and gain a strong, growth rate-independent advantage in swarming competitions. Importantly, hyperswarmers become poor biofilm formers and are outcompeted by the ancestral strain in biofilm competitions. Experimental evolution in swarming thus provides a unique example of parallel evolution and suggests an evolutionary trade-off between motility and biofilm formation.

RESULTS

Experimental Swarming Evolution Yields Hyperswarmers

Three independent lineages initiated from a common ancestor strain, *P. aeruginosa* PA14, were submitted to experimental evolution through consecutive rounds of swarming. After every 24 hr period of swarming, each colony was harvested in its entirety from the swarming plate and resuspended in a test tube. A 1/1,500 fraction of each of the harvested populations was point inoculated onto the center of a fresh swarming plate. This procedure was repeated daily for 9 days.

Over time, all three lineages lost the distinctive branching pattern of wild-type swarming in favor of a plate-covering morphology (Figure 1A; Movie S1). We call this phenotype hyperswarming. Lineage #2 started showing hyperswarming at day 5, whereas the other two lineages acquired the phenotype later. All 12 isolated clones showed round hyperswarming colonies (Figure 1B; Movie S1), confirming heritability and stability of hyperswarming.

We observed subtle, yet reproducible, differences in colony morphology between different hyperswarmer clones. This suggested that we had obtained an unknown number of distinct clones. We therefore undertook five quantitative phenotypic

assays in order to determine the number of distinct clones. The measured phenotypes were swimming motility, twitching motility, biosurfactant production, and the amount of attached and suspended cells in biofilm assays (Figure 2A). The wild-type strain provided a reference for all assays. In addition, two nonmotile clones (*flgK*$^-$ and *pilB*$^-$) served as negative controls for the motility and biofilm assays (O'Toole and Kolter, 1998).

In each of the phenotypic assays, hyperswarmers showed differences compared to wild-type but to varying degrees (Figure 2A). To ascertain the number of distinct clones, the strains were grouped according to their performances in the phenotypic assays using hierarchical clustering (Figure 2B) (Maynard et al., 2010; Xie et al., 2011). Wild-type clustered separately from all other strains, and the two nonmotile mutants clustered together. We determined that there were likely three clusters of hyperswarmers: one cluster consisting of clones 1, 3, and 4; one consisting of clones 5–8; and the last consisting of clones 2 and 9–12. Cell size clearly set apart clone 5 from clones 2 and 10 (Figure S1). The fact that the two clones isolated from the same plate (clones 1 and 2 isolated from lineage #2 at day 5) were placed in different groups suggested that population #2 was polyclonal at this early stage. All other clones (clones 3–12), which were isolated from day 9, grouped according to their plate of origin. Based on the clustering, we chose the two early clones (1 and 2) and one clone from each of the late populations (clones 4, 5, and 10, respectively) for further study.

Hyperswarmers Outcompete Ancestral Strain in Swarming Competitions

The consistent emergence of hyperswarmers from independent lineages suggested a competitive advantage for these clones over the ancestral strain. An obvious way to gain a competitive advantage is an increased growth rate. However, the growth rates measured in liquid cultures with the same nutrient composition as swarming plates through growth curve synchronization (van Ditmarsch and Xavier, 2011) (Figure S2A) revealed that hyperswarmers grow slightly slower than the ancestral strain (Figure 3A). We then tested whether hyperswarmers would lose a direct competition against the ancestral strain in liquid cultures. These competition experiments indeed showed that hyperswarmers have a disadvantage in liquid competition (Figure 3B). Although there are important differences between growth in liquid and the spatially structured environment of a swarming plate, the fact that hyperswarmers (1) have a lower growth rate in liquid and (2) are outcompeted in liquid coculture with the ancestral suggests that the process driving the evolution of hyperswarmers is growth independent.

In contrast, similar competitions in swarming plates confirmed that the hyperswarmers have a significant selective advantage in swarming against the ancestral strain (Figure 3C). To evaluate how hyperswarmers manage to win swarming competitions, we imaged competition plates and observed that hyperswarmers localized preferentially at the leading edge of swarming colonies (Figure 3D). These competitions further support that growth rate is not the cause of the emergence of hyperswarmers. Rather, they suggest that the hyperswarmers gain an ecological advantage by getting prime access to nutrients as the colony expands, a phenomenon studied in depth with theoretical models (e.g., Xavier and Foster, 2007; Xavier et al., 2009). The loss of branching in hyperswarmer colonies is not caused by a loss of the repulsion effect (Caiazza et al., 2005; Tremblay et al., 2007) because hyperswarmers are still affected in proximity of a surfactant-producing but immotile *flgK*$^-$ strain (Figures S2B and S2C; third segment of Movie S1).

Hyperswarming Is Caused by Parallel Point Mutations in *fleN*

After determining stability and heritability of hyperswarming, we sequenced the whole genome of the five selected hyperswarmer clones and the ancestral strain in search of mutations causing hyperswarming. Using the deposited reference genome of *P. aeruginosa*, UCBPP-PA14, from http://www.pseudomonas.com (Winsor et al., 2011) as the scaffold for read mapping, we compared each of the hyperswarmers to the ancestral strain, so as to specifically call mutations between hyperswarmers and our lab strain. The analysis identified only SNPs in one gene. The mutated gene, *fleN* (PA14_45640), encodes the flagellar synthesis regulator FleN (Dasgupta et al., 2000), suggesting that the mutations affected flagellar motility. We found two distinct *fleN* mutations: clones 1 and 4 harbored mutation FleN(V178G), and clones 2, 5, and 10 harbored FleN(W253C) (Figure 4A). Targeted resequencing of *fleN* in the remaining seven hyperswarmers revealed that they also harbored single-point mutations in *fleN* (Figure 4A). Interestingly, the *fleN* mutations agreed with the hierarchical clustering carried out earlier (Figure 2B), confirming that lineage #2 was indeed polyclonal at day 5.

Because none of the SNPs found in the hyperswarmers is present in any of the *Pseudomonas* spp. genomes publicly available (Winsor et al., 2011) (Figure 4A), we proceeded to confirm causality of *fleN* mutations for hyperswarming. The expression level of *fleN* is vital for its proper functioning, and *fleN* overexpression yields nonflagellated cells, whereas a knockout yields multiflagellated but nonmotile cells (Dasgupta et al., 2000). We thus opted for in *cis* complementation to ensure appropriate expression. Allelic replacement of wild-type *fleN* with mutated *fleN* produced the hyperswarmer morphology; conversely, the replacement of mutant *fleN* with the wild-type sequence reverted the swarming morphology to the wild-type, branched colony morphology in all clones (Figure 4B). Moreover, all the other phenotypes assessed previously were also complemented by the allelic replacements (Figure S3A). Together, the complementation experiments provide definitive proof that the SNPs identified are necessary and sufficient to cause hyperswarming. In addition, we confirmed that the *fleN* mutations do not constitute complete loss of function in FleN because a Δ*fleN* strain is impaired in swarming motility and behaves different from any hyperswarmer clone in the other phenotypes as well (Figures 4B and S3A). No explanatory SNPs were found for the different cell size in clone 5, but *fleN* complementation proved any additional unknown mutation would not be sufficient for the hyperswarming phenotype (Figure 4B).

Parallel Evolution of Multiflagellated Hyperswarmers

The finding that three independent lineages of experimental evolution led to hyperswarmers through distinct point mutations in

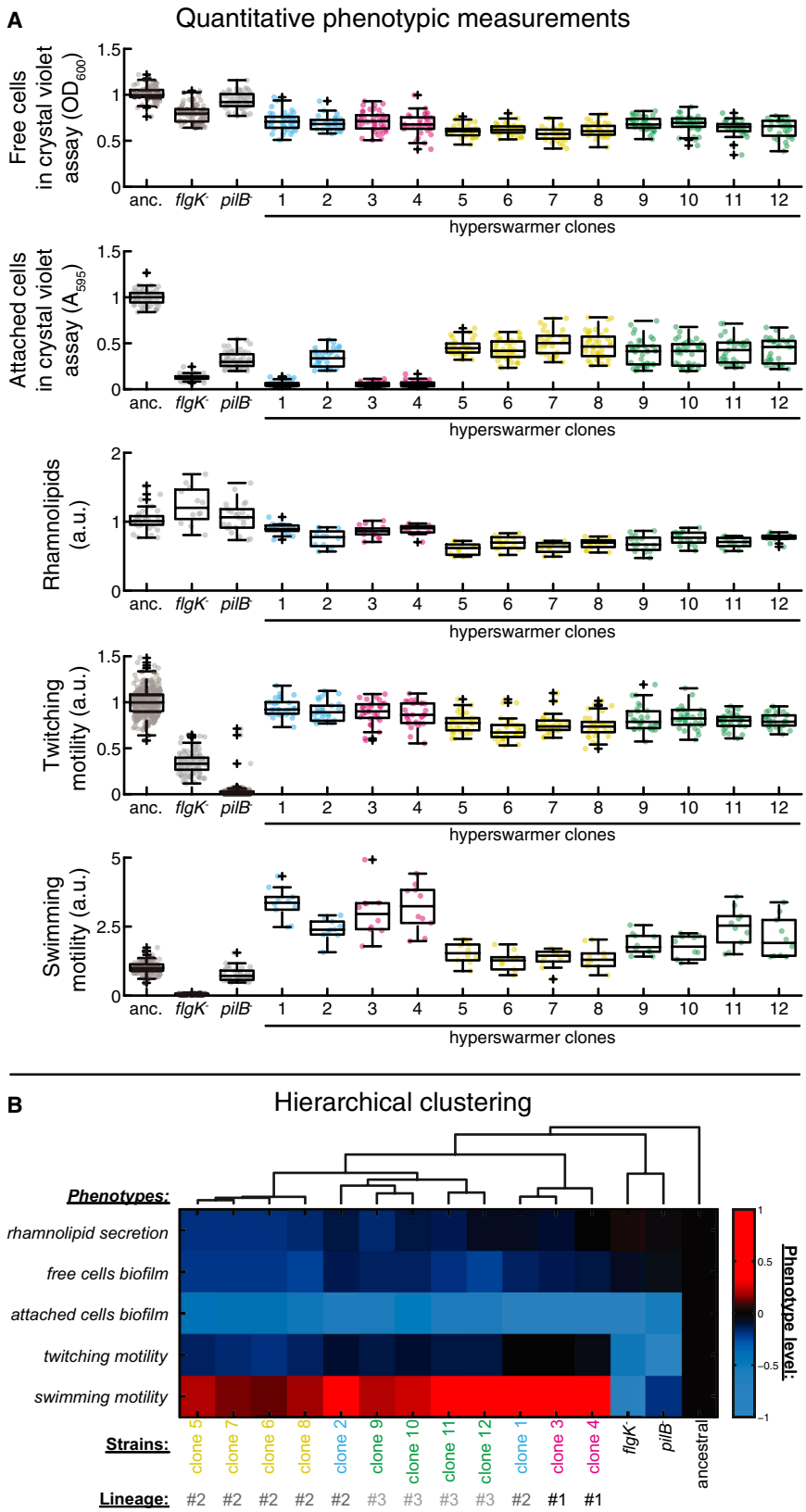


Figure 2. Hierarchical Clustering of Quantitative Phenotypic Assays Suggested the Existence of Three Distinct Hyperswarmer Clones

(A) Quantitative phenotypic assays were performed on the ancestral strain (denoted as "anc."), 2 nonmotile clones (*flgK*⁻ and *pilB*⁻), and 12 hyperswarmer clones. Free cells and attached cells in a crystal violet biofilm assay were quantified, together with rhamnolipid secretion (using the sulfuric acid anthrone assay), twitching motility, and swimming motility. All measurements were normalized to the ancestral strain. On each box, the central mark is the median, and the edges of the box are the 25th and 75th percentiles among experimental replicates.

(B) Phenotypic strain grouping using hierarchical clustering is illustrated. Blue indicates a decrease compared to the ancestral strain, and red indicates an increase compared to the ancestral strain (the ancestral strain on the right-hand side is black because all phenotypes are normalized to it). The nonmotile mutants cluster separately from hyperswarmers and from the ancestral strain. Within the hyperswarmer clones, there are three apparent clusters: clones 1, 3, and 4; clones 2 and 9–12; and clones 5–8. After clustering, clones 1, 2, 4, 5, and 10 were selected for use in the following studies. Clone 5 was indeed different from clones 2 and 10 when looking at cell size (see Figure S1).

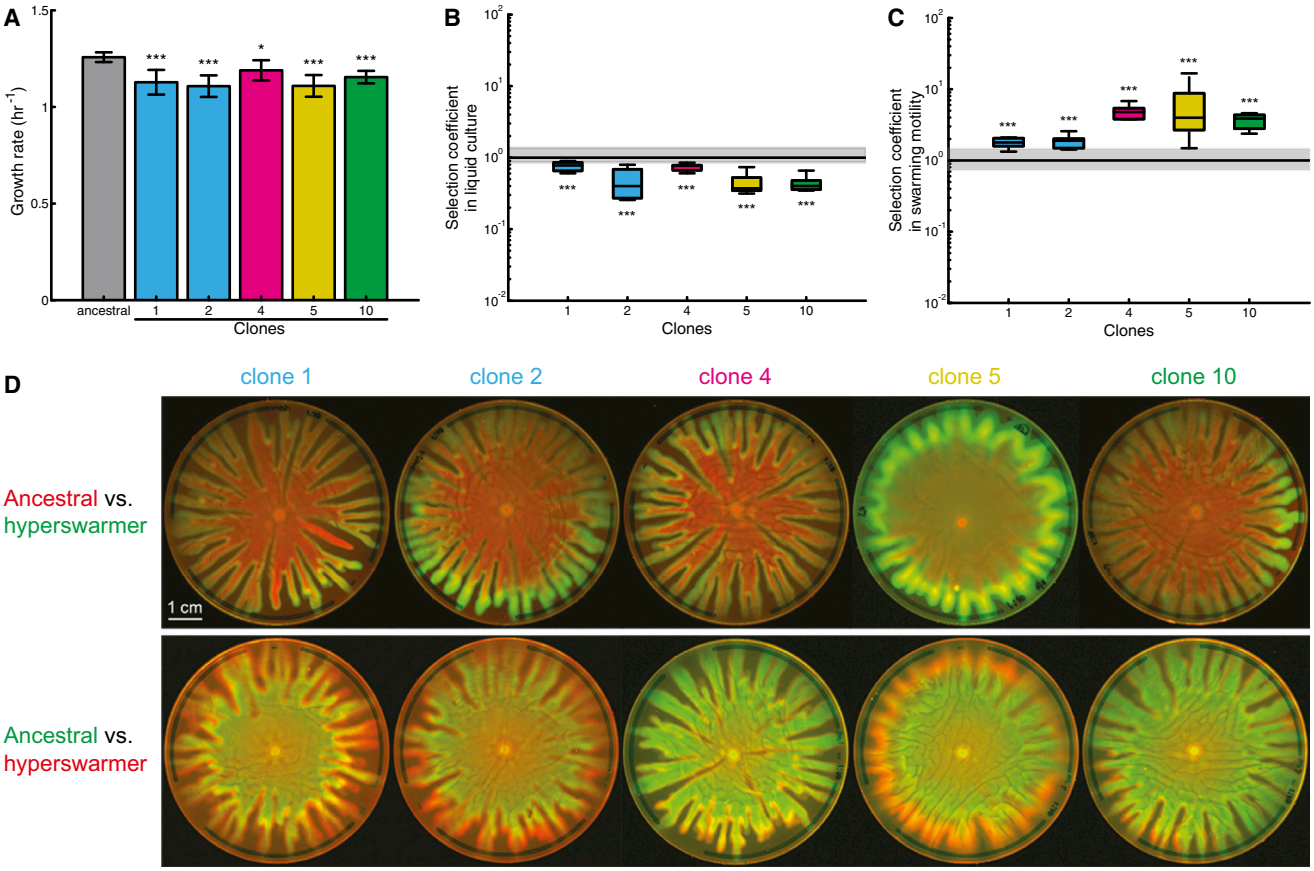


Figure 3. Hyperswarmers Lose Competitions in Liquid Culture against the Ancestral Strain but Prevail in Swarming Competitions by Segregating at the Leading Edges of Expanding Swarming Colonies

(A) Growth rates of the ancestral strain and five hyperswarmer clones reveal that hyperswarmers grow slower in liquid culture (see Figure S2A for actual determinations). Error bars represent 95% confidence level from the linear regression.

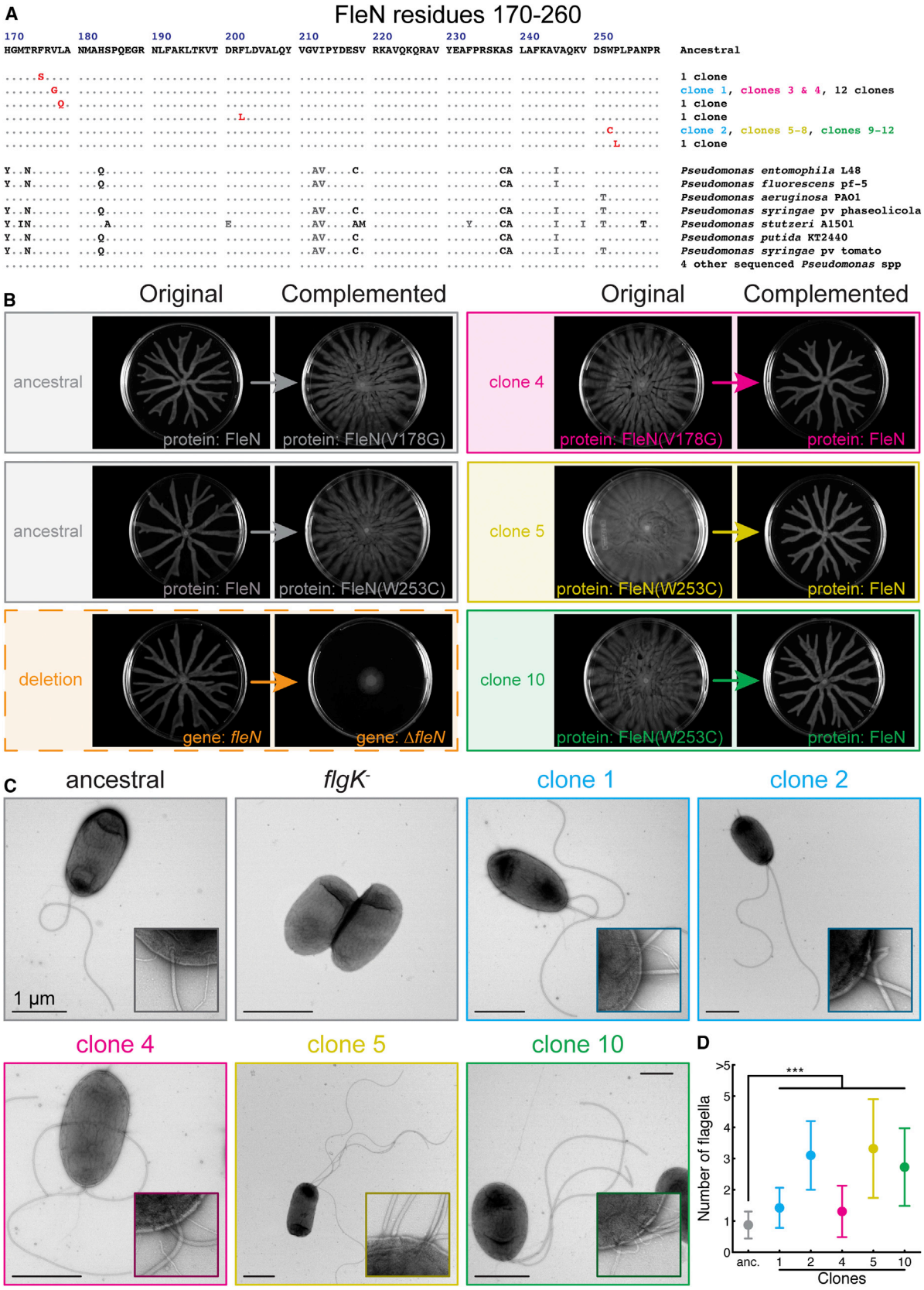
(B and C) Competitions between hyperswarmers and the ancestral strain in liquid media (B) and swarming plates (C) are shown. The neutral selection coefficient of 1 is marked with the black line. Each competition was started at a 1:1 ratio of hyperswarmer to ancestral. The error margins of the neutral selection coefficients (gray area) were experimentally determined by competing wild-type against itself in the appropriate settings. The selection coefficients represent the ratios of hyperswarmers to ancestral before and after the competition divided over each other. A selection coefficient of >1 means the hyperswarmer wins, whereas a selection coefficient of <1 means the hyperswarmer loses. On each box, the central mark is the median, and the edges of the box are the 25th and 75th percentiles among experimental replicates.

(D) Hyperswarmers are enriched at the leading edge of swarms. Fluorescence scans of swarms initiated with a 10:1 ratio of ancestral in green to hyperswarmer in red (top row) or the inverse (bottom row) are presented. See Figure S2B for repulsion assays.

*$p < 0.05$, ***$p < 0.005$.

fleN suggested that swarming selection was driving parallel molecular evolution. Out of 24 additional experimental evolution lineages, 16 resulted in hyperswarming (the remaining 9 lineages collapsed due to a phage outburst, and those lineages were excluded from further analysis; data not shown). All new hyperswarmer clones (1 from each lineage) harbored *fleN* mutations, and 12 harbored FleN(V178G); the remaining 4 revealed four additional *fleN* mutations: FleN(F176S), FleN(L179Q), FleN(F203L), and FleN(P254L) (Figure 4A).

At the molecular level, FleN has a dual role: influencing flagellar synthesis and chemotaxis as the antiactivator of the flagellar master regulator FleQ, and maintaining flagellar number and polarity due to its interaction with FlhF (Balaban and Hendrixson, 2011; Kazmierczak and Hendrixson, 2013; Kusumoto et al., 2006; Murray and Kazmierczak, 2006). As stated previously, in the complete absence of FleN, *P. aeruginosa* becomes multiflagellated but suffers from a chemotactic defect, impairing motility (Dasgupta et al., 2000) (Figures 4B and S3A). Furthermore, overexpression of FleN leads to nonflagellated cells (Dasgupta et al., 2000, 2003; Dasgupta and Ramphal, 2001). Due to increased swimming and swarming motility of hyperswarmers (Figures 2A, 3C, and 3D), we decided to investigate whether the *fleN* mutations affected flagellar synthesis. We did so in two ways. First, we constructed a flagellin (FliC) promoter-based fluorescence reporter (*attB*::P_{fliC}-*gfp*). FliC is the major extracellular component of the flagellar filament. In liquid culture, hyperswarmers expressed *fliC* earlier and at higher levels than the ancestral strain (Figure S3C), suggesting upregulation of flagellar



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synthesis. Second, the ancestral strain, as expected, was monoflagellated as observed by electron microscopy (EM) and flagella counts (Figures 4C, 4D, and S3B), although there are conditions where *P. aeruginosa* may have one to two flagella (Köhler et al., 2000; Rashid and Kornberg, 2000). The hyperswarmers were confirmed to be polar multiflagellates (Figures 4C, 4D, and S3B). It should be noted that the cells visualized by EM originated from stationary-phase liquid cultures, supporting that *fleN* mutations lock hyperswarmers in a multiflagellated state.

Previous studies have shown that both *P. aeruginosa* PAK and *P. putida* became polar multiflagellates when FlhF is overexpressed (Pandza et al., 2000; Schniederberend et al., 2013). Because FlhF and FleN are binding partners (Balaban and Hendrixson, 2011; Kazmierczak and Hendrixson, 2013; Kusumoto et al., 2006; Murray and Kazmierczak, 2006), it is possible that if their interaction is hampered by the *fleN* mutations, the cells may be experiencing a relatively higher activity of FlhF. However, swarming motility in a constitutive FlhF expression strain was indistinguishable from the ancestral (Figure S3D), suggesting that the hyperswarmer phenotype is not due to a relatively higher activity of FlhF.

Hyperswarming Comes at the Expense of Biofilm Formation

Because single-nucleotide changes can transform *P. aeruginosa* into a hyperswarmer and thus produce a pronounced competitive advantage in swarming, we wondered whether hyperswarmers exist in nature. So, we investigated swarming motility in 47 isolates of *P. aeruginosa*: 18 environmental isolates from contaminated soils in Canada (Deziel et al., 1996), and 29 clinical isolates from patients at Memorial Sloan-Kettering Cancer Center (Figure 5). All of these strains came from direct isolations and have undergone few generations in a laboratory setting. They should, consequently, represent the natural diversity of *P. aeruginosa* more appropriately. None of these strains showed hyperswarming (Figure 5). In fact, hyperswarmers clearly occupy a distinct region within the space of colony morphologies when compared to wild-type strains. None of the five best swarmers from the clinical strains had coding mutations in *fleN* compared to our wild-type.

Although all the clinical strains originated from the same hospital, we observed a large variance in their swarming phenotype (Figure 5), suggesting that the strain library is significantly diverse. We selected one of the best swarmers among these isolates and subjected it to experimental evolution. The selected clinical strain harbored four noncoding mutations in its *fleN* sequence, showing that it is genotypically distinct from PA14. Nonetheless, as with PA14, this isolate evolved a hyperswarmer phenotype, in this case, via the mutation FleN(V178G) (Figure S4). The observation that another strain can also evolve hyperswarming through FleN point mutations shows that convergent evolution can occur beyond PA14.

The fact that none of our environmental and clinical isolates showed a hyperswarming phenotype (Figure 5) raises the question whether there is an opposing selective pressure in the wild. Such an effect could be caused by antagonistic pleiotropy

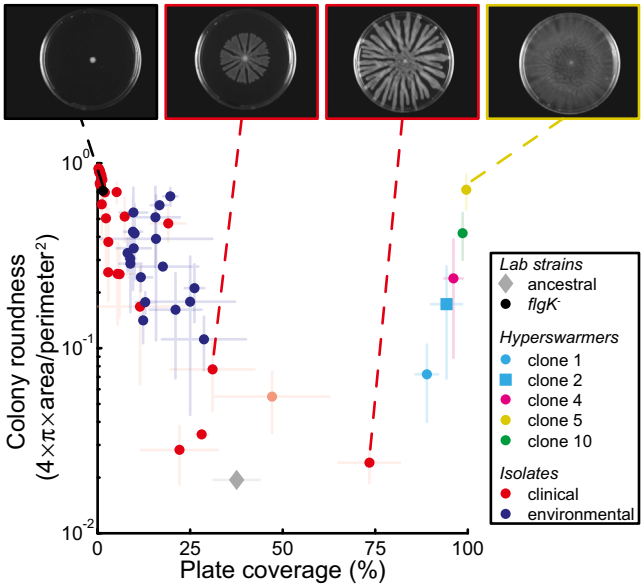

Figure 5. Hyperswarming Is Not Found in Clinical or Environmental Isolates

The shape of swarming colonies was quantified and plotted in 2D using plate coverage and colony roundness: calculated by (4 × π × area)/perimeter^2. The ancestral strain and a nonflagellated clone (*flgK*$^-$) were included as controls. A total of 47 nonlaboratory strains (18 environmental isolates from hydrocarbon-contaminated soil in Canada, shown in blue; and 29 clinical isolates from patients at Memorial Sloan-Kettering Cancer Center, shown in red) were compared to five hyperswarmer clones (color coded according to their plate of origin) in standard swarming assays. Representative photos of swarming colonies are shown above for illustration. In orange is the clinical isolate that was subjected to experimental evolution (see ancestral and hyperswarmer for this strain in Figure S4). Error bars represent the SD among experimental replicates.

Figure 4. Hyperswarming Is Caused by Point Mutations in *fleN*, which Produce Polar Multiflagellated Cells

(A) Six distinct *fleN* point mutations were identified from experimental evolution in swarming motility. FleN(V178G) was found in clones 1, 3, and 4 of the initial experiment, and FleN(W253C) was found in clones 2 and 5–12. FleN(V178G) emerged another 11 times, and FleN(F176S), FleN(L179Q), FleN(F203L), and FleN(P254L) were all encountered once in independent runs. These mutations are not found in the deposited *Pseudomonas* spp. genomes.

(B) *fleN* mutations are necessary and sufficient to cause hyperswarming. In *cis* genetic complementation was performed through allelic replacement. When converting wild-type *fleN* to either FleN(V178G) (top left) or FleN(W253C) (middle left), colony morphology changed from wild-type into hyperswarming. Conversely, the reversion of clones back to wild-type (right-hand column) yielded wild-type morphologies. Hyperswarming was not recapitulated by Δ*fleN* (lower left). (See Figure S3A for phenotypic assays of all complemented clones.)

(C) Transmission EM of the ancestral strain, a nonflagellated clone (*flgK*$^-$), and the five hyperswarmer clones shows that hyperswarmers have become multiflagellated. Insets show 88,000× magnification of the cell pole.

(D) The distribution of flagella in the ancestral strain and all five hyperswarmer clones is shown (see Figure S3B for the histograms and Figure S3C for *fliC* expression in liquid culture).

Error bars represent the SD of flagella number.

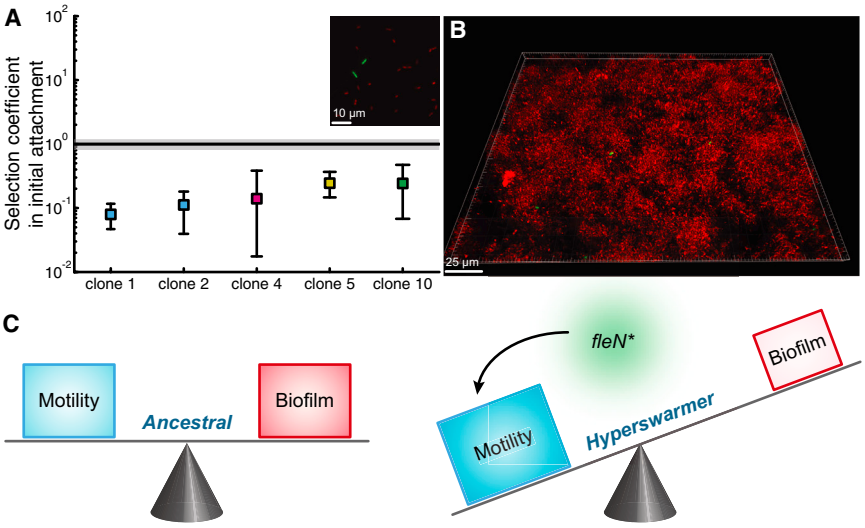

Figure 6. Hyperswarmers Lose Biofilm Competitions against the Ancestral Strain, Indicating an Evolutionary Trade-Off between Motility and Biofilm Formation

(A) Hyperswarmer clones lose against the ancestral strain already at the phase of initial attachment to a biofilm substratum. Slide biofilms were inoculated at a 1:1 ratio and incubated for 6 hr before imaging the attached cells (see inset where ancestral is red, and clone 4 is green). The black line indicates the neutral selection coefficient of 1. These experiments were performed in duplicate (also for the neutral selection coefficient). The 95% confidence intervals (both for neutral and actual selection coefficients) were determined through the probability density functions of the counts.

(B) Full-grown, flow cell biofilm of clone 4 (green) and ancestral (red) is presented. The biofilm was inoculated at a 1:1 ratio and then grown for 24 hr before imaging. The image is a deconvolved 3D projection of the biofilm.

(C) In the ancestral strain, motility and biofilm formation are balanced. The *fleN* mutations in hyperswarmers, however, cause an imbalance by which the highly motile mutant lacks in biofilm formation. (See Figure S5 for all biofilm competition data and Spearman correlation between swarming and biofilm formation in the clinical and environmental isolates.)

(Taylor and Buckling, 2011) or an evolutionary trade-off (Shoval et al., 2012), where the improvement in one trait comes at the expense of another.

Biofilm formation is an essential trait for *P. aeruginosa*, both in environmental and clinical settings (Costerton et al., 1999; Kolter and Greenberg, 2006), and is known to be inversely regulated to swarming motility (Baraquet et al., 2012; Boyle et al., 2013; Caiazza et al., 2007; Kuchma et al., 2007; Lee et al., 2007). An inverse relationship has previously been shown between swarming motility and biofilm formation in a cohort of 237 clinical isolates (Murray et al., 2010). Our compendium of isolates shows a similar trend where good biofilm formers tend to be poor swarmers, whereas poor biofilm formers can be good swarmers (Figure S5E).

We had already observed that hyperswarmer clones are poor biofilm formers in monocultures (Figure 2A), so we proceeded to check their competitive ability against the ancestral strain in mixed biofilms. These competitions revealed that hyperswarmers start losing at the initial attachment phase (Figure 6A) and become severely outnumbered in mature biofilms, both in flow cell (Figure 6B) and slide biofilm assays (Figure S5A–S5C). Hyperswarmers were strongly outcompeted even when hyperswarmers were inoculated up to a 100-fold excess compared to the ancestral strain (Figure S5D). The experiments suggest that biofilm formation impairment of hyperswarmers lies in an attachment problem (Figure 6A), whereas wild-type cells in the same culture do manage to attach and grow a mature biofilm. Therefore, the *fleN* mutations seem to tip the balance between biofilm and motility (Caiazza et al., 2007; Shrout et al., 2006) in favor of swarming motility in hyperswarmers but at the expense of biofilm formation (Figure 6C).

DISCUSSION

Here, we show that evolution in surface-associated swarming colonies produces parallel molecular evolution of multiflagellated hyperswarmers. Swarming motility in *P. aeruginosa* is a social trait involving many molecular pathways (Caiazza et al., 2005; Déziel et al., 2003; Köhler et al., 2000; Rashid and Kornberg, 2000; Xavier et al., 2011), and a priori prediction of evolutionary trajectories is complicated due to its multifactorial nature. So, the consistent emergence of hyperswarmers in independent lineages is surprising all the more because all hyperswarmers have single-point mutations in the same region of the flagellar synthesis regulator FleN. This is a striking example of parallel molecular evolution. In addition, the emergence of the most predominant mutation—FleN(V178G)—in experimental evolution started from a clinical isolate shows the convergent molecular evolution in strains other than PA14.

These results raise an intriguing question: if *P. aeruginosa* can be transformed from a monoflagellated (monotrichous) into a polar multiflagellated (lophotrichous) bacterium by single-point mutations, why is *P. aeruginosa* monoflagellated in nature? The answer to this question may lie in the fact that hyperswarmers are poor biofilm formers that lose against wild-type in biofilm competitions. Because biofilms are an essential part of the *P. aeruginosa* lifestyle, hyperswarmers likely face a strong counterselection in the wild, preventing their fixation there. Interestingly, nonflagellated mutants are also poor biofilm formers (O'Toole and Kolter, 1998), suggesting that fine-tuning of flagellar motility is essential for biofilm formation.

Swarming is common in *P. aeruginosa* strains and has been reported by many labs (e.g., Breidenstein et al., 2012; Kuchma et al., 2010; Lai et al., 2009; Morris et al., 2011; Murray et al., 2010; Shrout et al., 2006; Takahashi et al., 2008; Tremblay and Déziel, 2008; Yeung et al., 2009, 2012). Nonetheless, swarming is a conditional phenotype that varies significantly with media composition and agar hardness. For example, round *P. aeruginosa* swarms without branches have been reported before in specific media conditions (e.g., Breidenstein et al., 2012; Morris et al., 2011; Takahashi et al., 2008; Yeung et al.,

2012), even in wild-type strains. In contrast, the present study focused on the transition from branched swarms to round swarms while keeping the media composition and hardness constant, in order to determine the underlying genetic mechanism.

Importantly, these hyperswarmer clones could not have been found in traditional liquid culture-based experimental evolution. Hyperswarmers invariably grow slower than the ancestral strain in liquid culture and thus would not get fixed in evolving populations. In contrast, in swarming plates, hyperswarmers emerge by virtue of their superior motility, which allows them to segregate at the leading edge of colonies, thereby gaining prime access to free space and unused nutrients. Therefore, hyperswarming, like certain other bacterial traits such as biofilm-building polymer secretion (Nadell and Bassler, 2011; Xavier and Foster, 2007), requires spatial structure to be selected.

We also would not expect to find these hyperswarmers in genetic knockout screens. As shown in the present study, a *fleN* knockout actually swims and swarms worse than the wild-type. In contrast to a *fleN* knockout, the point mutations in hyperswarmers are restricted to the 3′-terminal region of *fleN*, and these mutants swim far better than the wild-type. A transposon screen generated superior swarmers (Yeung et al., 2009), but these mutants were not related to flagella. One example is a *pqsH* knockout, which is a synthase for the extracellular signaling molecule PQS. The *pqsH*$^-$ hyperswarmers made larger swarming colonies that still retained the branching pattern of the wild-type. Therefore, our plate-covering *fleN*-mutated hyperswarmers would probably outcompete these transposon mutants.

Interestingly, in that same transposon screen, mutants with swarming defects generally showed biofilm overproduction, whereas mutants with enhanced swarming showed normal or decreased biofilm ability (Yeung et al., 2009). This adds to the mounting evidence for an inverse regulation between biofilm formation and motility (Caiazza et al., 2007; Yeung et al., 2011), which, as shown here, leads to an evolutionary trade-off.

The molecular mechanism underlying the hyperswarming phenotype is currently unknown. Our experiments suggest, however, that the observed changes are subtle because neither a *fleN* deletion nor constitutive *flhF* expression (both of which should create multiple polar flagella) recapitulates the hyperswarming morphology.

Placed in a broader perspective, our experiments in spatially structured swarming populations support that adaptive evolutionary paths can be, at least to some extent, predictable. Beyond *P. aeruginosa*, can we expect parallel evolution to be a general feature? Recent experiments on the adaptation of *Escherichia coli* to growth in high temperatures provide a counterexample and reinforce the notion that adaptive convergence can occur through diverse molecular changes (Tenaillon et al., 2012). Furthermore, studies in other bacterial pathogens, both in laboratory populations (Ensminger et al., 2012) and epidemics (Lieberman et al., 2011; Snitkin et al., 2012), suggest that adaptations to a host immune system can be divergent. Our evolutionary experiments seem to be uncommon among bacteria and thus constitute a unique experimental model of highly reproducible molecular adaptation. Nevertheless, parallel evolution is important in nature (Christin et al., 2010), as illustrated by a recent finding that herbivorous insects facing the same selective pressure exhibit parallel molecular changes across species (Zhen et al., 2012). Which conditions lead to convergent rather than divergent evolutionary adaptations is therefore a major open question in evolutionary biology. The reproducible evolution of hyperswarmers in *P. aeruginosa* provides an experimental system to address this question.

EXPERIMENTAL PROCEDURES

Swarming Motility

Swarming assays and competitions were performed as previously described (Xavier et al., 2011). The selection coefficient is defined as the final ratio of hyperswarmers to ancestral over the initial ratio of hyperswarmers to ancestral. Competitions were performed in both color combinations to exclude a possible selective advantage of either fluorescent marker (GFP or DsRedExpress). The neutral selection coefficient was determined by using wild-type in both colors. Repulsion assays were performed in standard swarming conditions.

Experimental Evolution

Experimental evolution was carried out in standard swarming conditions. After 24 hr, the entire swarming colony was collected from the plate and used to inoculate a fresh swarming plate with a population bottleneck of 1/1,500. Every cell suspension was glycerol stocked at −80°C.

Phenotypic Assays

Swimming motility was performed according to Murray and Kazmierczak (2006) with minor adaptations. Twitching motility was also described elsewhere by O'Toole and Kolter (1998), with minor adaptations. Rhamnolipid production was quantified using the sulfuric acid anthrone assay as described previously (Xavier et al., 2011). Monoculture biofilms were performed using a standard 96-well crystal violet biofilm assay (O'Toole and Kolter, 1998) with some adaptations. Biofilm competitions were performed using the tilted glass slip biofilm method (Xavier et al., 2009), with minor adaptations. Cell sizes were measured from overnight cultures diluted 1:2,000 and left to grow for 5 hr at 37°C before gently diluting them out in order to visualize the cells by microscope. GFP-labeled strains were used to facilitate imaging (see Extended Experimental Procedures).

Growth Curves, Calculation of Growth Rates, and *fliC* Expression

Growth rates were measured using the growth curve synchronization method (van Ditmarsch and Xavier, 2011). The liquid media were of the same composition as swarming media. The *fliC* promoter was PCR amplified directly from the *P. aeruginosa* PA14 genome using primers from a previous study by Wyckoff et al. (2002). The promoter (P_{fliC}) was used to replace P_{rhlAB} in pYL122 (Lequette and Greenberg, 2005). The construct was mobilized into *P. aeruginosa* following four-parental mating and then integrated into the *attB* site. *fliC* expression was monitored through GFP.

Whole-Genome Sequencing and SNP Calling

P. aeruginosa PA14 whole-genome sequencing was done using Applied Biosystems SOLiD sequencing (Bedford) with average 100× coverage. Duplicate reads were removed. Only areas of the genome with at least 10× coverage in high-quality score reads were included in the analysis to allow SNP calling (≥94.35% of each sequenced genome). The ancestral and five hyperswarmers were genotyped with the GATK Unified Genotyper (McKenna et al., 2010). Sequence differences between the deposited genome and our lab strain genome were not included in this direct comparison; thus, the mutations defining the hyperswarmers were called specifically.

Targeted Resequencing of *fleN*

fleN was PCR amplified from the *P. aeruginosa* genome using primers fleN_fwd and fleN_rev (see Extended Experimental Procedures). Fragments were sequenced in both directions with the Sanger method.

Strain Creation through Allelic Exchange

Site-directed mutagenesis of *fleN* was performed through homologous recombination (Shanks et al., 2006), with minor adaptations (see Extended Experimental Procedures). To create a constitutive FlhF-expressing strain, the $P_{A1/04/03}$ promoter (Lambertsen et al., 2004; Lanzer and Bujard, 1988) was introduced in front of *flhF*.

Transmission EM

Transmission EM was performed directly from overnight cultures. All acquired photos were used to assess the distribution of the number of flagella by manual counts.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.07.026.

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