

PNAS PLUS

# Importance of positioning for microbial evolution

Wook Kim<sup>a,b,c,1</sup>, Fernando Racimo<sup>c,2</sup>, Jonas Schluter<sup>a,b</sup>, Stuart B. Levy<sup>d</sup>, and Kevin R. Foster<sup>a,b,c,1</sup>

<sup>a</sup>Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom; <sup>b</sup>Oxford Centre for Integrative Systems Biology, University of Oxford, Oxford OX1 3QU, United Kingdom; <sup>c</sup>Faculty of Arts & Sciences Center for Systems Biology, Harvard University, Cambridge, MA 02138; and <sup>d</sup>Department of Molecular Biology and Microbiology, Center for Adaptation Genetics and Drug Resistance, Tufts University School of Medicine, Boston, MA 02111

Edited by Joan E. Strassmann, Washington University in St. Louis, St. Louis, MO, and approved March 17, 2014 (received for review December 19, 2013)

Microbes commonly live in dense surface-attached communities where cells layer on top of one another such that only those at the edges have unimpeded access to limiting nutrients and space. Theory predicts that this simple spatial effect, akin to plants competing for light in a forest, generates strong natural selection on microbial phenotypes. However, we require direct empirical tests of the importance of this spatial structuring. Here we show that spontaneous mutants repeatedly arise, push their way to the surface, and dominate colonies of the bacterium Pseudomonas fluorescens Pf0-1. Microscopy and modeling suggests that these mutants use secretions to expand and push themselves up to the growth surface to gain the best access to oxygen. Physically mixing the cells in the colony, or introducing space limitations, largely removes the mutant's advantage, showing a key link between fitness and the ability of the cells to position themselves in the colony. We next follow over 500 independent adaptation events and show that all occur through mutation of a single repressor of secretions, RsmE, but that the mutants differ in competitiveness. This process allows us to map the genetic basis of their adaptation at high molecular resolution and we show how evolutionary competitiveness is explained by the specific effects of each mutation. By combining population level and molecular analyses, we demonstrate how living in dense microbial communities can generate strong natural selection to reach the growing edge.

bacteria | biofilm | experimental evolution | social interaction

**M** icrobes commonly live in dense surface-attached communities where cell division results in layer upon layer of cells lying on top of one another (1–4). Key examples include biofilms and the closely related colony mode of growth (5). These types of structured communities are not only common but also central to many of the ways that bacteria affect us, which includes an important role in health and disease via commensal gut communities and chronic infections (6, 7). In addition, these microbial communities are central to bioremediation, such as in wastewater treatment, but also biofouling in industry and shipping (8).

Why do microbes form these structured surface-associated communities? A key explanation is that the process allows cells to establish themselves in the best environment for growth and survival. This theory is well illustrated by the adaptive radiation of *Pseudomonas fluorescens* SBW25 in undisturbed liquid cultures (9). Here, specific mutants arise that colonize the glass surface of test tubes and generate a mat-like biofilm across the air–liquid interface, which subsequently collapses via the re-emergence of non–mat-forming strains. From such examples, we can understand the processes driving the formation and breakdown of biofilm communities. However, many such communities persist for long periods, over many generations (8, 10, 11). Understanding microbial phenotypes, therefore, also requires us to study the processes that affect cells within these dense communities.

A key feature of growth within a biofilm or colony is an extremely high cell density relative to liquid growth. This high cell density readily generates localized nutrient limitation and, particularly, the existence of microgradients, whereby the availability of nutrients decreases the further one goes into the cell mass and away from the nutrient source (12). There is good evidence that these gradients commonly occur in a range of systems, both when nutrients come from the surface to which cells attach, and when nutrients diffuse in from the environment (13–17). Moreover, there is good reason to believe that these microgradients will be central to understanding microbial phenotypes.

A growing body of theory emphasizes how strong nutrient gradients mean that cells near the nutrient source are able to divide but cells in the center of a colony or biofilm often cannot (18–20). In evolutionary terms, the difference between the two positions can be stark: division versus dormancy or death. A key corollary is that cells will benefit from investing in phenotypes that solely act to allow them to reach the growing edge ahead of a competitor. An analogy from macroscopic biology is the evolution of woody tissue in plants to grow tall and gain better access to light than competitors. In microbes, candidate mechanisms from the theory that may enable competition for the growing edge include secreted polymers, which allow a genotype to expand to reach growth surfaces, and cell motility (18, 20). In sum, given that microbes commonly live with both spatial structure and nutrient limitation, natural selection to gain the best position within biofilm-type communities should be central to understanding microbial phenotypes. However, we need direct tests of this hypothesis.

Colonies have long been considered a model laboratory system to study spatially structured microbial communities (12, 21–23), and are one of the main experimental approaches in studying biofilms (5). Here we use experimental evolution in laboratory colonies to study competition in microbial groups. Although artificial, our goal is to use a defined and tractable system to dissect key principles responsible for evolutionary success within dense microbial communities. We show that

## Significance

Microbes commonly form dense communities that are central to many diseases and bioremediation. Here we demonstrate a simple and general principle of living in dense communities: microbes will commonly compete to reach nutrients at the community edge, akin to plants competing for light. We follow the evolution of a highly competitive genotype in colonies of a soil bacterium. We show that the genotype gains its advantage not from its intrinsic growth rate but by positioning itself at the surface of the community, where it gains preferential access to oxygen. A large-scale genetic analysis reveals striking parallel evolution and evidence of strong natural selection. Our work suggests that positioning is a major basis for evolutionary competition in dense microbial communities.

Author contributions: W.K., S.B.L., and K.R.F. designed research; W.K., F.R., and J.S. performed research; W.K. and K.R.F. analyzed data; and W.K. and K.R.F. wrote the paper. The authors declare no conflict of interest

This article is a PNAS Direct Submission.

<sup>1</sup>To whom correspondence may be addressed. E-mail: wook.kim@zoo.ox.ac.uk or kevin. foster@zoo.ox.ac.uk.

<sup>&</sup>lt;sup>2</sup>Present address: Department of Integrative Biology, University of California, Berkeley, CA 94720.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10. 1073/pnas.1323632111/-/DCSupplemental.

mutants arise repeatedly de novo within colonies of the bacterium *P. fluorescens* Pf0-1 and outcompete the parent. Importantly, we find their evolution occurs not because of intrinsically faster growth but rather the ability to expand as a group and eventually dominate the surface of the colony. We also follow the process at the genetic level, which reveals striking parallel evolution that is consistent with very strong natural selection to reach the surface of the colonies. Accordingly, the study has two main components: (*i*) the characterization of a bacterial phenotype that results from strong natural selection on cells to position themselves at the growth surfaces, and (*ii*) the genetic analysis of how this strong natural selection is manifest at the molecular scale.

# **Results and Discussion**

**Mucoid Variants Repeatedly Emerge as a Dominant Genotype.** Mucoid variants reliably arise in colonies of the soil bacterium *P. fluorescens* Pf0-1. These variants emerge initially as distinct spots in colonies before spreading into patches and finally emerging and spreading across the surface of the colony (Fig. 1 *A* and *B* and Fig. S1*A*). Isolating a subset of the variants and replating them multiple times as pure colonies revealed a stable phenotype



**Fig. 1.** Emergence and reproductive dominance of the mucoid variants. (*A*) Propagation of a WT colony consistently leads to the emergence of mucoid variants that appear to overtake the WT population over time. Indicated above each panel is the number of days postinoculation. (Scale bars, 5 mm.) (*B*) Discrete patches of tagged MV (GFP) emerge to the surface when introduced concurrently with WT (DsRed-Express) (*Left*). The mixed population was initially seeded at MV:WT ratio of  $10^{-5}$ :1, and visualized by fluorescence microscopy. (Scale bar, 2 mm.) (*Right*) Magnification of the section of the colony bound by the rectangle. (Scale bar, 0.2 mm.) (*C*–*E*) Results of competitions between MV and WT commenced at different starting ratios (MV:WT): (C) 1:1, (*D*)  $10^{-3}$ :1, and (*E*)  $10^{-5}$ :1. MV and WT were tagged with kanamycin and streptomycin resistance cassettes, respectively. The graphs show the mean population size of MV (red circle) and WT (blue circle) in CFU obtained from destructively sampling three independent populations at each interval over a period of 7 d. (*F*) Results of competition between MV tagged with kanamycin resistance (red) and MV tagged with streptomycin resistance (blue). Error bars represent the 95% confidence interval.

consistent with a genetic mutation. We therefore labeled a representative strain of the variant, herein mucoid variant (MV), and the wild-type (WT), with neutral antibiotic-resistance markers to track their frequency in mixed colonies. MV cells were seeded at various initial frequencies in mixed colonies with the WT and the frequency of the two genotypes was tracked over time by destructively sampling colonies each day (Fig. 1 *C-F*). This process revealed that MV cells are under strong positive selection in mixed colonies and rapidly increase in frequency as the colony grows. This effect is strongest when MV cells are seeded at very low frequencies but, even when MV cells initially vastly outnumber the WT cells (Fig. S1 *B–D*), they retain their fitness advantage.

MV's dominance over the WT could simply be a result of acquiring the ability to grow faster than its counterpart within the given environment. We thus compared the growth rate of the two strains when alone as single genotypes. In strong contrast to the fitness difference seen in mixed colonies, we could detect no differences in the growth rate of the two strains when alone, either over timescales of hours or days, in liquid or on plates (Fig. S1 *E* and *F*). The fitness advantage of the MV, therefore, rests upon the interactions between cells of the two genotypes.

The fact that mucoid variants readily emerge from any given WT colony indicates that they could already be present and perhaps even selected for in the WT population before plate inoculation. However, we found that mucoid variants also emerge within colonies commenced from a single WT cell (Fig. S14). We provide additional evidence that the causal mutation occurs de novo in each experiment after the WT populations are

seeded on the plates in SI Text, Estimation of the Timeline of rsmE Mutations.

A Model for the Evolutionary Advantage of the MV. The fact that the MV does not grow faster than WT when alone suggests that the MV does not gain its advantage from a simple growth-promoting secretion, like an enzyme that provides nutrients. This aspect and its mucoid appearance suggest a model where the MV uses secretions to reduce cell density and expand outwards and upwards. Moving to the surface will allow cells to gain the best access to oxygen, which is strongly growth-limiting within colonies (13–16, 21). We used confocal imaging to assess the spatial patterning of the MV within WT colonies. Importantly, imaging was done noninvasively without a coverslip added to the colony, but we still achieve single-cell resolution. Consistent with our model, the MV does indeed display a much lower cell density than the WT and, moreover, it forms expanding patches at the surface of the colony that push up and out (Figs. 2 and 3*E* and Fig. S2).

The model is also consistent with the theoretical work discussed in the introduction that suggests that volume expansion by polymers can allow cells to push themselves to the edge of a submerged biofilm (18–20). We therefore adapted these models for colonies rather than submerged biofilms to show the same processes play out (*SI Text, Individual-Based Simulation of WT-MV Competitions*, and Fig. 3 and Fig. S3). Finally, general support for the idea that polymer secretion can provide a competitive advantage to a secreting strain comes from Nadell and Bassler, who showed a polymer-secreting genotype of *Vibrio cholerae* makes more robust biofilms than a nonproducer genotype, even when the two genotypes are mixed in direct competition (24).



Fig. 2. Confocal imaging of colony competitions reveals that MV's density is initially much lower than WT. Mixed populations of fluorescently tagged  $\Delta rsmE$  (DsRed-Express) and WT (GFP) were seeded at the initial ratio of  $10^{-5}$ :1 ( $\Delta rsmE$ :WT). Independent colonies were visualized over time by fluorescence microscopy (*Left*) and confocal microscopy (*Center* and *Right*, rendered in 3D). The images also show how MV comes to dominate the surface of the colony.



**Fig. 3.** Individual-based simulations. (A) Snapshot from a 2D simulation of an 870- $\mu$ m-wide cross-section of a colony growing on agar; MV in red, WT in green. (*B*) Fraction of the total mass belonging to the MV over 50 h in six independent simulations (black line: simulation shown in *A*, *C*, and *D*); initial fraction 0.05. *Inset* is a boxplot showing the relative fitness (W) of the mucoid variants at t = 50 h; the asterisk (\*) means results are significantly different from equal fitness (W = 1), Wilcoxon signed-rank test (*P* = 0.0313). (*C*) Close-up of a region from the simulated colony. Because of the secretion of polymers, mucoid variant cells are less densely packed than WT cells. (*D*) Oxygen concentration profile in the simulation of the region shown in *C*). More oxygen is available in the region of mucoid variant cells because of the lower local cell density. (*E*) Confocal microscopy image of a colony of MV cells expressing DSRed-Express and WT cells expressing GFP. (Scale bars, 50  $\mu$ m.)

However, in this case the primary benefit came from better attachment to the substratum in a flow environment rather than competition for the growing edge.

**MV Cells' Fitness Rests upon Positioning Themselves in the Colony.** Our model for the evolutionary advantage of MV cells is that they use secretion to push themselves up and out and dominate growth surfaces (Figs. 1*B* and 2 and Fig. S2*A*). Specifically, it suggests that the ability of MV cells to gain a preferential position in the colony is what leads to their evolutionary advantage (Fig. 2) (18, 19). If correct, this model predicts that disrupting spatial structure will prevent MV cells from preferentially colonizing growth surfaces in mixed genotype populations. We tested this theory by having the two genotypes compete in shaking and standing liquid culture, where spatial structure is absent or modest. As predicted, we observe little or no fitness difference between the two genotypes under these conditions (Fig. 4*A*).

Although the liquid experiments support our predictions, liquid culture and colonies differ in many ways and it is not clear that the MV's ability to gain a better position in a colony is the causal factor explaining the fitness of the MV in colonies. We, therefore, performed a second assay to more directly test the importance of cell position for fitness. In addition to our normal experimental design, we added new treatments where each day we physically mixed up the colony using a pipette tip or a microbiological loop (Fig. 4). Although such a coarse manipulation will not fully disturb all patches of MV cells, this had a strong effect on the fitness of MV cells. MV fitness was greatly reduced close to the level observed in liquid competitions. Disrupting

E1642 | www.pnas.org/cgi/doi/10.1073/pnas.1323632111

spatial structure in this way likely has two effects. First, it prevents MV cells from preferentially localizing themselves near the growing edge at the top of the colony. Second, mixing cells means that the secretions provided by MV cells could also help WT cells. Both effects may be important in reducing the performance of MV cells in mixed colonies. We, therefore, designed a complementary experiment to test the idea that the ability of MV cells to position themselves at the top of the colony is specifically necessary for their evolutionary advantage. Here, we had the two genotypes compete in colonies that were grown under an agar pad, which prevents upwards spreading and is intended to allow all cells equal access to nutrients and oxygen (Fig. S4); this again removed the fitness advantage of the MV. Together, these experiments suggest that the ability to position themselves in the colony is necessary for the strong natural selection on MV cells.

**MV Selection Results from a Mutation Within the** *rsmE* Locus. To further understand the MV phenotype, we next sought to identify the underlying genetic changes. We used whole-genome pyrosequencing to identify potential causal mutations in one evolved isolate. This process revealed a deletion of a single nucleotide at the 126th position within the coding sequence of *rsmE*, which causes a frameshift, leading to a premature termination codon and the probable loss of protein function. RsmE functions as a specific repressing clamp in the GacA/S regulon by binding to regulatory small-RNAs and mRNA. The Gac system and its homologs have been a focal point of research for many years, as they regulate the production of various factors impli-



Fig. 4. Spatiogenetic structure is essential for the fitness of MVs. (A) Effect of physical mixing of colonies and liquid cultures on the relative fitness of  $\Delta rsmE$  compared with WT after 4 d of incubation. WT and  $\Delta rsmE$  were tagged with streptomycin or kanamycin resistance cassettes, respectively. All competitions were seeded at the starting ratio of  $10^{-5}$ :1 ( $\Delta rsmE:WT$ ). Colonies were either left undisturbed or mixed daily with a pipette tip or sterile loop, and liquid cultures were incubated either standing or constantly shaking. The datapoints represent the mean ( $\Delta rsmE$  over WT) from three independent populations, and the error bars represent the SD. The undisturbed colony treatment was found to be significantly different against all other treatments (two-tailed t test; P < 0.0001). (B) Visual assessment of physical mixing on competitions between the  $\Delta rsmE$  mutant and WT by fluorescence microscopy ( $\Delta rsmE$  tagged with GFP, WT untagged) after 4 d of incubation. Competitions were seeded at the starting ratio of  $10^{-5}$ :1 ( $\Delta rsmE$ : WT) and either left undisturbed or mixed daily with a pipette tip or sterile loop. (Scale bars, 2 mm.)

cated in metabolism, host-colonization, and pathogenesis in a wide range of organisms (25). Finding mutations in the Gac regulon is consistent with other data from *Pseudomonas* spp., which suggests that the Gac pathway is a common mutational target, which includes evidence that *gac* genes undergo reversible mutations (26) and *gac* mutants frequently evolve during rhizosphere colonization (27).

RsmE is one of three Rsm proteins in the Pf0-1 genome. Among the pseudomonads, Rsm proteins are known to promote motility (28) and suppress various secreted products, such as protease, extracellular polysaccharides, and a quorum-sensing system that regulates phenazine biosynthesis (25, 29-31). Given the nature of such components, the Gac/Rsm regulatory cascade has previously been proposed as a key modulator of bacterial social behavior (25). Pf0-1 also appears to have a suppressed Gac regulon (32), suggesting that our WT strain has a low activity of the Gac pathway relative to other strains of *P. fluorescens*. Accordingly, mutation in *rsmE* is a way to activate the corresponding component of the Gac regulon and generate a more social phenotype. To confirm that the identified mutation was directly responsible for the MV phenotype, we constructed a strain from the WT harboring the same single-nucleotide deletion, and a second strain by deleting the entire *rsmE* locus. These strains appear phenotypically identical to the MV (Fig. 5A) and displayed the same strong evolutionary advantage in competitions with the WT (Fig. 5B). This finding supports the notion that the original mutation in the MV results in loss of RsmE function and is sufficient to cause the MV phenotype. Note that RsmE is a global repressor, so although the mutations cause a loss of function at the level of RsmE, the dominant phenotypic outcome is predicted to be an increase in multiple types of secretion under the control of the Gac/Rsm regulatory cascade (25, 29-31).

Strong Natural Selection Drives Parallel Evolution at the rsmE Locus. Our data are consistent with strong natural selection on cells to gain the best position within a bacterial community. If correct, we should also see evidence of this selection at the genetic level. Strong natural selection for a particular phenotype is often associated with parallel evolution whereby the same phenotype, and potentially genotype, arises reliably whenever an organism experiences the environmental conditions of interest. A familiar example from bacteria is the evolution of antibiotic resistance (33, 34). Consistent with parallel evolution, preliminary work suggested that *rsmE* mutants were a common occurrence in our experimental set-up. We therefore isolated and sequenced the rsmE locus in 565 independently derived mucoid variants to determine how many of the variants contained a mutation to a new *rsmE* allele (Fig. 6A and Table S1). Every MV strain harbored a single instance of mutated rsmE allele with changes either in the coding (534 mutants) or the upstream regulatory sequence (31 mutants), an example of perfectly repeatable evolution at the level of the gene. Cataloguing the mutations by type reveals a diverse array throughout the length of the *rsmE* locus: 322 base pair substitutions (282 missense and 40 nonsense), 190 deletions, and 22 insertions within the coding sequence, and 24 base pair substitutions, 6 deletions, and 1 insertion within the upstream sequence. Given their nature and the physical organization of the *rsmE* gene, these mutations are unlikely to exert any polar effects. Few exceptions may be the insertion sequence element insertions and the large deletions that extend beyond the coding sequence (Fig. 6A).

In addition to strong natural selection, parallel evolution can occur as a result of mutational bias if certain genes—in our case *rsmE*—mutate more often than is typical. Inspection of the *rsmE* sequence data, however, does not suggest a raised mutation rate. Specifically, we do not see any synonymous substitutions in *rsmE* (Table S2) and we find only one loss-of-function mutation per



**Fig. 5.** Mutation in *rsmE* triggers the secretion of products leading to the MV phenotype. (A) Phenotypic comparison of WT, MV, and engineered *rsmE* mutant colonies following 2 d of growth: "N" shows the relative mucoidy of colonies without polycarbonate membrane, "S" shows colony spreading on the shiny side of the membrane, and "D" shows biosurfactant production on the dull side of the membrane. (Scale bars, 5 mm.) (*B*) Results of competitions between WT and mutants in colony. Competitions were initiated at the mutant: WT ratio of  $10^{-5}$ :1. WT was tagged with streptomycin resistance and all mutants were tagged with kanamycin resistance. Error bars represent the SD of the mean relative fitness (mutant over WT) calculated from destructively sampling three independent populations after 4 and 7 d of incubation. There were no significant differences among the mutants on either day (Kruskal–Wallis, P = 0.4296 and 0.0665, respectively).

clone (additional such mutations would be neutral). To further evaluate the possible role of mutational bias in our system, we estimated the mutation rate at the *rsmE* locus using a modified form of the methodology of Lang and Murray (35). The analysis revealed that the mutation rate at *rsmE* is well within the estimates of the genome average and effective target size of *rsmE* (SI Text, Comparison of Rates Between the Emergence of Mucoid Variants and Mutations in *rsmE*). In addition, the great majority of mutations in *rsmE*, if not all, occur de novo in each experiment after the cells have been plated (SI Text, Estimation of the Timeline of *rsmE* Mutations, and Fig. S1H). We, therefore, conclude that our identifications of mutations in *rsmE* is the product of strong natural selection, not an elevated mutation rate.

We can then always link the emergence of any one mucoid variant to a single mutation in the same gene, *rsmE*. This approach raises a rare opportunity to explore in fine detail how the idiosyncrasies of different mutations impact upon evolutionary competitiveness. We therefore explored this by comparing a representative subset of the mutants in competition with the WT in two ways. In the first assay, we mixed each mutant at the ratio of  $1:10^5$  in pairwise competition with the WT to see how well







D



**Fig. 6.** Molecular map and competitive phenotypes of individually derived *rsmE* mutations. (A) A schematic of mutations identified in each MV emerging from independent WT populations. Each mutant is classified into two categories based on the assay shown in *B*: strong competitor phenotype similar to  $\Delta rsmE$  (red) and weak competitor phenotype closer to WT (blue). Mapped to the amino acid sequence of RsmE are missense mutations (denoted by corresponding substitutions), nonsense mutations (#), insertions (triangles denoting insertion site and size; IS denotes insertion sequence element), and deletions (horizontal bars showing size). Substitution mutations in the 5' UTR are denoted by the actual nucleotide substitutions, and the Shine-Dalgarno sequence is shown in gray. Arrowhead denotes deletions that extend beyond the range. (*B*) Comparison of the colony spreading phenotype of representative mutant strains on the shiny side of the polycarbonate membrane.  $\Delta 57$ -61 denotes the amino acid residues that are deleted, which is listed under the  $\Delta 171$ -181 genotype in Table S1. (Scale bars, 2 mm.) (C) All mutants outcompete the WT, but not to the same extent. Comparison of competition outcome after 4 d between WT and select MVs representing different classes of mutations. MVs were seeded at 10<sup>-5</sup> frequency against WT. Boxplots illustrate the distribution (medians, upper and lower quartiles, and outliers) of the relative frequency of mutant over WT among six competition replicates. Multiple occurrences of the knock-out control case ( $\Delta rsmE$  vs. WT) and six strains were significantly weaker (blue). (*D*) Structural representation of the RsmE dimer (gray) bound to two cognate mRNA molecules (yellow) as determined previously by NMR (39). Missense mutations have either a large (red) or small (blue) effect on RsmE activity as shown in the other panels of the figure. Image was rendered by PyMOL (PDB accession ID 2JPP).

they proliferate during colony growth (Fig. 6C). The second assay mimicked our mutant isolation process by seeding a small number of mutant cells with a large number of WT and then counting how many of the mutants successfully emerged to the top of the colony (Fig. S5; see *SI Text, Comparison of Emergence Rates Between Mucoid Variants* for detailed analysis). Both assays showed that all mutants outcompete the WT, but that the mutants differed in their competitive abilities against the WT.

The competition assays are difficult to do on a large scale. We therefore sought a proxy phenotype that would allow us to assess the competitive ability in all of our mutants. One major difference was revealed when the strains were propagated on top of polycarbonate membranes (16). The two sides of these membranes have different properties. One side is visibly dull and the other side is shiny (Materials and Methods). Here, we found that the MV and the constructed *rsmE* mutants could spread across the shiny surface, whereas WT could not, leading to a clear diagnostic phenotype (Fig. 5A). Moreover, none of the strains could spread across the dull side; however, a zone of transparent biosurfactant-like substance was visible around the MV genotypes but not the WT. We posit that this RsmE-regulated secreted product reduces surface tension and allows colonies to spread (36) across the shiny polycarbonate membrane. The dull side physically hinders expansion of the colony but not that of the secreted product.

The spreading phenotype of the mutants mapped predictably onto the two measures of competitive ability. In particular, mutants that appeared more like the WT performed relatively poorly in competition, whereas mutants that appeared like the  $\Delta rsmE$  strain performed relatively well against the WT (Figs. 5A and 6A and B). There exists a possibility that secondary mutations could account for the observed variations in competitiveness. However, this is unlikely given that independent strains that share the same mutation fall into the same measure of competitive ability and most fit mutants are predicted to be knockouts (Fig. 6C). In addition, as we show in the next section, the competitive ability of the mutants also makes sense in terms of precisely where the *rsmE* mutations are found.

The difference in the ability to expand on membranes might indicate a role for flagella-driven motility rather than secretion in the competitive ability of the MV. However, the introduction of a flagellin mutation in the MV had no effect in competitions against WT (Fig. S6). Although active motility appears unimportant, we do not exclude a role for passive motility in the fitness advantage of the MV over WT. In our simulations (Fig. 3) secretions act by carrying cells along with them, which means that secreting cells move further than nonsecretors. A related observation has been made in *Pseudomonas aeruginosa*, whereby cells slide on a surface independent of flagella, specifically when the type-iv pili are absent (37). Our particular WT strain does not exhibit twitching motility (38), thus a similar mechanism may aid MV cells to reach the growing edge of the colony.

Competitiveness Can Be Explained in Terms of Molecular Structure

and Function. We find then that all mutants outcompete the WT, but they differ in their relative ability to compete. The variability among mutants is consistent with differences in the production of the secretions that help groups of MV cells to gain preferential access to the growing edge in the colony in competition with the WT. We next sought to understand the molecular basis for this variability. Using the spreading phenotype as a proxy for competitive ability against WT, we divided the different mutants into two classes: strong competitors (more secretion) and weak competitors (less secretion). Fig. 6A summarizes the position of the different mutations found among the 565 mutants (full details in Tables S1 and S2). For many positions, we found the same mutation multiple times, up to a maximum of 69 cases of a particular single nucleotide substitution (Arg44). Moreover, we found 7 of 7 possible loss of start codon mutations and 11 of 14 possible non-

sense mutations in *rsmE*, where two of the three missing nonsense mutations are expected to be silent as they occur at the nonfunctional tail end of the protein (39, 40). Because we know which of these classes of mutations lead to a loss of function, we can then estimate that we have found more than 95% (21 of 22) of the other loss-of-function mutations (35). Although approximate only, this calculation suggests that we have a detailed molecular map of the possible routes to the origin of the MV phenotype.

We used this map to evaluate how well one can translate the degree of adaptation at the population (colony) level into processes at the molecular level. We first considered insertion and deletion mutations (indels), which are expected to lead to a nonfunctional protein because of frameshift. Consistent with this result, we found that most indels cause a strong competitor phenotype, including multiple cases of insertion sequence element insertion (Fig. 6A). The tail end of the protein, however, has indels with both the strong and the weak competitor phenotype. Here, the observed deletion within the Gly54 codon leads to an immediate truncation of the protein at the next position and the strong competitor phenotype. This finding fits with the prediction that the tail end of the protein has little functional importance but from position 55 onwards (39, 40). However, if the tail end of the protein is not functionally important, why do we also see weak competitor phenotypes in this region? All of the weak competitor phenotypes in this region result from frameshift that add 59-66 amino acids to the carboxyl-terminus of RsmE, which is a significant bulk to a protein that is normally only 64 amino acids in length. We also found clear patterns for the single-nucleotide substitutions. At the carboxyl terminus, there were again a number of weak competitors that resulted from the loss of the stop codon and the addition of 18 amino acids to the protein. At the other end of the locus, the 5' UTR is associated with weak competitor mutations with a single exception in the Shine-Dalgarno sequence.

All nonsense mutations that we found led to the strong competitor phenotype. In contrast, the missense mutation spectrum showed that only specific amino acids in specific positions lead to the MV adaptation (Table S2). This finding is of course expected but it does appear that RsmE is a robust protein (41), in the sense that very few missense mutations lead to a complete loss of function (see Table S2 for robustness calculation). Moreover, in contrast to nonsense mutations, the missense mutations that we found caused both weak and strong competitors. At first glance, the distribution of the two phenotypes caused by a missense mutation appeared to be arbitrary across the linear sequence (Fig. 6A). However, there are detailed structure-function predictions for the binding of RsmE, and its closely related homolog CsrA, to its cognate mRNA (39, 40). These studies immediately provide insight on our data as we found many cases of mutation at the two residues, Leu4 and Arg44 (Fig. 6A), that were subjected to additional in vivo and in vitro functional studies on the basis of their predicted importance (39). However, we sought to further test the explanatory power of the structure-function data in our evolutionary experiment. We, therefore, plotted the positions of the missense mutations on the RsmE-mRNA structure by competitive class. It is striking that the strong and weak competitor mutations each fall out into discrete regions within the structure. Strong competitor residues cluster at the interface of RsmE-mRNA, and weak competitor residues cluster more at the interface between the two dimers of RsmE (Fig. 6D). The distinction between the two classes of competitive adaptation by missense mutation, therefore, appears to arise from the modulation of different classes of molecular interaction.

What Maintains *rsmE* Expression in Nature? We observed the reliable mutation of *rsmE* in our experiments. What then maintains *rsmE* expression under natural conditions? We speculate that two key factors are at play. The first factor is that there may be

hidden fitness costs to the loss of rsmE that are not seen in the laboratory. We observed no fitness cost associated with mutation in *rsmE* under laboratory conditions, even though it is associated with the expression of multiple secretions. The lack of costs may partly result from *rsmE* being expressed primarily at stationary phase rather than during exponential growth (29). Loss of rsmE, therefore, will tend to derepress secretions when cells are not growing at their fastest, which can limit the fitness costs of the secretions (36). Nevertheless, loss of rsmE may have fitness costs under some natural condition that we are unable to recapitulate in the laboratory. The second factor is that our WT strain (Pf0-1) may be more likely than some natural isolates to lose rsmE function. The production of numerous extracellular secretions appears to correspond to the relative activity of the Gac system. Activity in the Gac pathway is variable in the pseudomonads (26, 27) and Pf-01 is known to have a suppressed Gac system (32). This finding suggests that Pf-01 has a relatively low level of secretions to begin with and, under conditions that favor secretion, it may be more likely to lose *rsmE* function than a natural isolate that is already a strong secretor. In the end, experimental evolution studies are limited by the fact that what happens in the laboratory may not reflect precisely what happens in natural systems. However, there is natural variation in Rsm homolog copy number in the pseudomonads (25, 28, 42), so the gain and loss of Rsm proteins is part of the evolutionary trajectory of natural systems.

# Conclusion

Microbes growing on a solid surface, such as submerged biofilms and colonies, commonly form dense communities that rapidly deplete incoming nutrients. This general effect is predicted to exert strong natural selection on cells to gain the best access to nutrients, in a similar way to plants competing to gain the best access to light. Here we have described a series of simple evolutionary experiments that suggest that pushing to the surface can provide large fitness benefits. MV cells spontaneously arise within colonies and use secretions to collectively expand and push themselves to the surface of WT colonies. Notably, the MV cells show no growth rate advantage when in single genotype colonies; the evolutionary process demonstrably rests upon social interaction between MV and WT cells within a colony. In addition, we find that moving cells around or limiting their ability to form thick-layered colonies removes the evolutionary advantage of MV cells. We also find evidence of the importance of this competition at the genetic level: it leads to strong natural selection and parallel evolution comparable to the clearest known examples, such as the evolution of antibiotic resistance (33, 34). We used this evidence to generate a fine-scale map of the mutations that cause the MV phenotype and explain their competitiveness in terms of molecular structure and function. Our work suggests that strategies that allow microbes to reach the edge of dense communities will often be under strong natural selection.

### **Materials and Methods**

An extended version of the materials and methods used can be found in *SI Materials and Methods*.

**Bacterial Strains.** *P. fluorescens* Pf0-1 is a natural strain (43) that was directly isolated from soil by S.B.L. All mucoid variants described in this study were derived from individually isolated or spotted colonies of Pf0-1 on Difco *Pseudomonas* agar F (PAF), which is a commercial formulation of King's Medium B (KMB) (44). The evolution of mucoid variants is observed in minimal and complex media supplemented with glycerol or glucose as carbon source. One mucoid variant, MV, was designated as the prototype for detailed analyses.

Estimation of Frequency in Single and Mixed Genotype Populations. Competition experiments in colonies were carried out by mixing strains and spotting in 20- $\mu$ L volumes on PAF plates. Individual strains were tagged with either

a kanamycin or streptomycin resistance cassette, which are neutral in *P. fluorescens* Pf0-1 (45) (Fig. 1*F*). Colonies were harvested and the frequency of individual strains was estimated by serial dilutions and plating on selective media. Individual strains were also tagged with cassettes encoding GFP, YFP, or DsRedExpress proteins (46), and competition was visualized by fluorescence and confocal laser scanning microscopy. For competition experiments in liquid, 20  $\mu$ L of the mixture was inoculated into test tubes containing 2 mL of KMB or PAF without agar. The tubes were incubated either shaking or left standing undisturbed, and frequency was estimated as above. The outcome of each competition was analyzed by comparing both the raw CFU data and calculating the relative fitness (W) (47), or as noted otherwise.

Genome Sequencing, Identification, and Confirmation of the Causal Mutation. Whole-genome sequencing (454 FLX) was carried out by the Washington Genome Sequencing Center (St. Louis, MO), to compare MV to its parent strain. A single nucleotide (A) deletion at the 126th position of the coding DNA sequence of the *rsmE* gene was confirmed to be the causal mutation, resulting in the mucoid phenotype by introducing the same single-nucleotide deletion (*rsmE*pm) or deleting the entire *rsmE* locus ( $\Delta rsmE$ ) in the parent strain.

**Mutant Construction and Tagging.** Mutants were constructed using the gene splicing by overlap extension method (48) and homologous recombination as previously outlined (49). Primers complimentary to regions flanking the targeted gene were used to monitor the proper replacement with the mutant constructs by PCR, and confirmed by sequencing both template strands. The mini*Tn*7 system was used to tag the chromosomes of the strains used in this study using established procedures (46).

**Colony and Biosurfactant Spreading Assay on Polycarbonate Membranes.** Nuclepore polycarbonate membrane (Whatman) was laid on top of PAF plates using sterile forceps and spotted with overnight cultures. The smooth and shiny side of the membrane was spotted to compare the colony spreading phenotype and the matted and dull side was spotted for visualizing biosurfactant production.

Individual-Based Simulations. An extension of an established framework was used for the computer simulation of bacterial growth (18, 19, 50–52) to model a cross-section of a mixed colony of WT and MV cells. In the simulations, cells are considered to be spheres that metabolize diffusing nutrients that grow and eventually divide. Through this activity, local concentration gradients of nutrients arise. Multigrid solvers were used to calculate the steady-state solution of the 2D diffusion reaction equations that return these gradients in each iteration. Both cell types have the same growth rate but only the MV secretes polymers. Differences in the relative fitness (W) were calculated as described for experimental competitions. See Table S3 for a summary of the parameters used in the simulations.

**Parallel Evolution Experiments.** Overnight WT cultures (20  $\mu$ L) were spotted on PAF plates and incubated for 4 d at room temperature until mucoid variants became clearly visible. A single variant was randomly isolated from each single WT colony, with one exception being that three spatially separated patches of variants were isolated from a common WT colony. Each variant was purified and phenotype confirmed on fresh PAF plates. The mutation in each variant was identified by sequencing PCR products amplified with primers specific to regions flanking the *rsmE* locus.

Statistical Analyses. Given that the sample sizes were too small (n = 3) for the Mann–Whitney test, a two-tailed t test was used to compare the relative fitness differences between any two given strains. A Kruskal–Wallis test was applied to compare the relative fitness of the constructed *rsmE* mutants to the MV. The Kruskal–Wallis test, corrected for multiple comparisons (Tukey's honestly significant difference criterion), was used to compare CFU ratios of different mucoid variants to the WT. A two-tailed Mann–Whitney test was applied to compare the emergence ratios of different mucoid variants. The Wilcoxon signed-rank test was applied to compare relative fitness in the simulations. Bonferoni correction was applied when making multiple pairwise comparisons, and the relevant values for the n and  $\alpha$  parameters are indicated for each test where appropriate. All statistical tests were conducted using Matlab.

Imaging. Still pictures of colonies were generated using the CanoScanLiDE 200 flatbed scanner (Canon) or the EOS 30D DSLR camera (Canon), and images

were scaled to calibrated dimensions using the ImageJ software (53). Fluorescently tagged strains were imaged using the Typhoon 9400 scanner (GE Healthcare) and the associated ImageQuant TL software as described elsewhere (36), the SteREO Lumar.V12 microscope (Zeiss) under the NeoLumar S 0.8× objective lens and the associated AxioVision software, or the Axio Zoom.V16 microscope (Zeiss) under the PlanApo Z 0.5× objective lens and the associated Zen software. Confocal imaging was carried out on the LSM 700 laser scanning microscope (Zeiss) using the 20× and 50× objectives and the associated Zen software. A square piece of agar containing the entire colony was cut out and placed on slides without a coverslip for confocal

- 1. Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: From the natural environment to infectious diseases. *Nat Rev Microbiol* 2(2):95–108.
- Kolter R, Greenberg EP (2006) Microbial sciences: The superficial life of microbes. Nature 441(7091):300–302.
- 3. Monds RD, O'Toole GA (2009) The developmental model of microbial biofilms: Ten years of a paradigm up for review. *Trends Microbiol* 17(2):73–87.
- Nadell CD, Xavier JB, Foster KR (2009) The sociobiology of biofilms. FEMS Microbiol Rev 33(1):206–224.
- Branda SS, Vik S, Friedman L, Kolter R (2005) Biofilms: The matrix revisited. Trends Microbiol 13(1):20–26.
- Stewart PS (2002) Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol 292(2):107–113.
- Costerton JW, Montanaro L, Arciola CR (2005) Biofilm in implant infections: Its production and regulation. Int J Artif Organs 28(11):1062–1068.
- Jass J, Walker J (2000) Industrial Biofouling: Detection, Prevention and Control, eds Walker J, Surman S, Jass J (John Wiley & Sons, New York).
- Rainey PB, Rainey K (2003) Evolution of cooperation and conflict in experimental bacterial populations. *Nature* 425(6953):72–74.
- 10. Gellatly SL, Hancock REW (2013) *Pseudomonas aeruginosa*: New insights into pathogenesis and host defenses. *Pathog Dis* 67(3):159–173.
- 11. Des Marais DJ (1990) Microbial mats and the early evolution of life. *Trends Ecol Evol* 5(5):140–144.
- Stewart PS, Franklin MJ (2008) Physiological heterogeneity in biofilms. Nat Rev Microbiol 6(3):199–210.
- 13. Pirt SJ (1967) A kinetic study of the mode of growth of surface colonies of bacteria and fungi. J Gen Microbiol 47(2):181–197.
- 14. Wimpenny JW, Lewis MW (1977) The growth and respiration of bacterial colonies. *J Gen Microbiol* 103(1):9–18.
- Peters AC, Wimpenny JW, Coombs JP (1987) Oxygen profiles in, and in the agar beneath, colonies of *Bacillus cereus*, *Staphylococcus albus* and *Escherichia coli*. J Gen Microbiol 133(5):1257–1263.
- Walters MC, 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS (2003) Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* 47(1):317–323.
- Xu KD, Stewart PS, Xia F, Huang CT, McFeters GA (1998) Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl Environ Microbiol* 64(10):4035–4039.
- Xavier JB, Foster KR (2007) Cooperation and conflict in microbial biofilms. Proc Natl Acad Sci USA 104(3):876–881.
- Nadell CD, Xavier JB, Levin SA, Foster KR (2008) The evolution of quorum sensing in bacterial biofilms. *PLoS Biol* 6(1):e14.
- Xavier JB, Martinez-Garcia E, Foster KR (2009) Social evolution of spatial patterns in bacterial biofilms: When conflict drives disorder. Am Nat 174(1):1–12.
- Rani SA, et al. (2007) Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J Bacteriol* 189(11):4223–4233.
- 22. Shapiro JA (1992) Pattern and control in bacterial colony development. *Sci Prog* 76(301–302 Pt 3–4):399–424.
- López D, Vlamakis H, Losick R, Kolter R (2009) Cannibalism enhances biofilm development in Bacillus subtilis. Mol Microbiol 74(3):609–618.
- Nadell CD, Bassler BL (2011) A fitness trade-off between local competition and dispersal in Vibrio cholerae biofilms. Proc Natl Acad Sci USA 108(34):14181–14185.
- Lapouge K, Schubert M, Allain FH-T, Haas D (2008) Gac/Rsm signal transduction pathway of gamma-proteobacteria: From RNA recognition to regulation of social behaviour. *Mol Microbiol* 67(2):241–253.
- van den Broek D, Chin-A-Woeng TFC, Bloemberg GV, Lugtenberg BJJ (2005) Molecular nature of spontaneous modifications in gacS which cause colony phase variation in *Pseudomonas* sp. strain PCL1171. J Bacteriol 187(2):593–600.
- Martínez-Granero F, Rivilla R, Martín M (2006) Rhizosphere selection of highly motile phenotypic variants of *Pseudomonas fluorescens* with enhanced competitive colonization ability. *Appl Environ Microbiol* 72(5):3429–3434.

imaging. For all other imaging procedures, entire plates were imaged without disturbing the agar surface.

ACKNOWLEDGMENTS. We thank M. Cant, L. Keller, G. Lang, M. Laub, A. Murray, C. Nadell, B. Stern, K. Verstrepen, S. West, and J. Xavier for providing comments, and S. Mitri for assistance on statistical analyses. These studies were funded by Natural Sciences and Engineering Research Council of Canada Postdoctoral Fellowship (to W.K.), US Department of Agriculture Grants 2006-35604-16673 and 2010-04952 (to S.B.L.), National Institute of General Medical Sciences Center of Excellence Grant 5P50 GM 068763 (to K.R.F.), and European Research Council Grant 242670 (to K.R.F.).

- Martínez-Granero F, et al. (2012) The Gac-Rsm and SadB signal transduction pathways converge on AlgU to downregulate motility in *Pseudomonas fluorescens*. *PLoS ONE* 7 (2):e31765.
- Reimmann C, Valverde C, Kay E, Haas D (2005) Posttranscriptional repression of GacS/ GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain *Pseudomonas fluorescens* CHA0. J Bacteriol 187(1):276–285.
- Irie Y, et al. (2010) Pseudomonas aeruginosa biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. Mol Microbiol 78(1):158–172.
- Wang D, et al. (2013) Roles of the Gac-Rsm pathway in the regulation of phenazine biosynthesis in *Pseudomonas chlororaphis* 30-84. *Microbiologyopen* 2(3):505–524.
- Loper JE, et al. (2012) Comparative genomics of plant-associated Pseudomonas spp.: Insights into diversity and inheritance of traits involved in multitrophic interactions. PLoS Genet 8(7):e1002784.
- 33. Sandgren A, et al. (2009) Tuberculosis drug resistance mutation database. *PLoS Med* 6(2):e2.
- MacLean RC, Buckling A (2009) The distribution of fitness effects of beneficial mutations in *Pseudomonas aeruginosa*. *PLoS Genet* 5(3):e1000406.
- Lang GI, Murray AW (2008) Estimating the per-base-pair mutation rate in the yeast Saccharomyces cerevisiae. Genetics 178(1):67–82.
- Xavier JB, Kim W, Foster KR (2011) A molecular mechanism that stabilizes cooperative secretions in *Pseudomonas aeruginosa*. Mol Microbiol 79(1):166–179.
- Murray TS, Kazmierczak BI (2008) Pseudomonas aeruginosa exhibits sliding motility in the absence of type IV pili and flagella. J Bacteriol 190(8):2700–2708.
- Barton MD, Petronio M, Giarrizzo JG, Bowling BV, Barton HA (2013) The genome of *Pseudomonas fluorescens* strain R124 demonstrates phenotypic adaptation to the mineral environment. J Bacteriol 195(21):4793–4803.
- Schubert M, et al. (2007) Molecular basis of messenger RNA recognition by the specific bacterial repressing clamp RsmA/CsrA. Nat Struct Mol Biol 14(9):807–813.
- Mercante J, Suzuki K, Cheng X, Babitzke P, Romeo T (2006) Comprehensive alaninescanning mutagenesis of *Escherichia coli* CsrA defines two subdomains of critical functional importance. *J Biol Chem* 281(42):31832–31842.
- Guo HH, Choe J, Loeb LA (2004) Protein tolerance to random amino acid change. Proc Natl Acad Sci USA 101(25):9205–9210.
- Morris ER, et al. (2013) Structural rearrangement in an RsmA/CsrA ortholog of Pseudomonas aeruginosa creates a dimeric RNA-binding protein, RsmN. Structure 21 (9):1659–1671.
- Compeau G, Al-Achi BJ, Platsouka E, Levy SB (1988) Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. *Appl Environ Microbiol* 54(10):2432–2438.
- King EO, Ward MK, Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescin. J Lab Clin Med 44(2):301–307.
- Silby MW, Nicoll JS, Levy SB (2009) Requirement of polyphosphate by *Pseudomonas* fluorescens Pf0-1 for competitive fitness and heat tolerance in laboratory media and sterile soil. *Appl Environ Microbiol* 75(12):3872–3881.
- Lambertsen L, Sternberg C, Molin S (2004) Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environ Microbiol* 6(7):726–732.
- Lenski RE, Rose MR, Simpson SC, Tadler SC (1991) Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am Nat* 138(6):1315–1341.
- Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. *Gene* 77 (1):61–68.
- Silby MW, Levy SB (2004) Use of in vivo expression technology to identify genes important in growth and survival of *Pseudomonas fluorescens* Pf0-1 in soil: Discovery of expressed sequences with novel genetic organization. *J Bacteriol* 186(21):7411–7419.
- Nadell CD, Foster KR, Xavier JB (2010) Emergence of spatial structure in cell groups and the evolution of cooperation. *PLOS Comput Biol* 6(3):e1000716.
- Schluter J, Foster KR (2012) The evolution of mutualism in gut microbiota via host epithelial selection. *PLoS Biol* 10(11):e1001424.
- Mitri S, Xavier JB, Foster KR (2011) Social evolution in multispecies biofilms. Proc Natl Acad Sci USA 108(Suppl 2):10839–10846.
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9(7):671–675.

# **Supporting Information**

# Kim et al. 10.1073/pnas.1323632111

# **SI Materials and Methods**

Bacterial Strains and Growth Conditions. Pseudomonas fluorescens Pf0-1 is a soil isolate (1), the WT parent strain of all mucoid variants isolated and described in this study (Table S1). One of the mucoid variants was randomly chosen as the prototype and designated as MV. All routine cloning was done in Escherichia coli 10B (Invitrogen), and E. coli S17.1 $\lambda$ pir (2) was used as the donor strain in conjugations. P. fluorescens strains were routinely grown in Pseudomonas minimal medium (PMM) (3) at 30 °C and E. coli strains in Luria Broth (LB) at 37 °C, or as stated otherwise. Liquid cultures were shaken in test tubes at 250 rpm. The evolution of mucoid variants is observed in minimal and complex media supplemented with glycerol or glucose as carbon source. King's Medium B (KMB) (4) or Pseudomonas agar F (PAF, a commercial formulation of KMB) was used as the base for detailed evolution and competition analyses. When necessary, a given medium was solidified with agar [1.5% (wt/vol)] and supplemented with the following antibiotics: ampicillin (100  $\mu$ g/mL), kanamycin (50  $\mu$ g/mL), streptomycin (50  $\mu$ g/mL), or gentamicin (30 µg/mL). Complex media components were Difco-branded and obtained from BD and all other chemicals were obtained from Sigma.

**Measurement of Growth.** For measurement of growth on agar, 20-µL overnight cultures were spotted and absorbed onto the surface of PAF medium (25 mL per plate), which was left to dry for 1 d at room temperature before inoculation. Plates were incubated at room temperature and colonies were harvested over time into test tubes containing 5 mL PBS using bent-glass Pasteur pipettes. Cell suspensions were vortexed until clumps were no longer visible and then serially diluted (10-fold) in fresh PMM and enumerated on PMM-agar. For measurement of growth in liquid, overnight cultures were diluted into KMB and optical density at 600 nm was measured over 24–48 h (30 °C, constant shaking) in the Bioscreen C MBR (Oy Growth Curves Ab) or the Infinite M200 PRO (Tecan).

Competition Experiments. Overnight cultures (1.5 mL) were washed in fresh PMM and resuspended in 1.0 mL PMM. Suspensions were serially diluted accordingly in PMM and mixed with equal volumes of the competing strain suspension. Each mixture was serially diluted and plated out on antibiotic plates to estimate the initial population size of the competing strains. For competition experiments on agar, colonies were spotted using 20 µL of the mixture on PAF plates, incubated, and harvested as described above. Resulting cell suspensions were serially diluted in PMM and enumerated on PMM-agar plates supplemented with streptomycin or kanamycin. For competition experiments in liquid, 20 µL of the mixture was inoculated into test tubes containing 2 mL of KMB or PAF with the agar subtracted. The tubes were either left standing undisturbed in tube racks or shaken at 250 rpm. The liquid cultures were enumerated over time by serially diluting 50-µL samples and plating on PMM-agar plates containing streptomycin or kanamycin. Competition experiments were also set up using fluorescently tagged strains and visualized over time by various imaging procedures described below. The outcome of each competition was analyzed by comparing both the raw CFU data and calculating the relative fitness (W) (5), or as noted otherwise.

**Spatial Disruption Experiments.** Competition experiments were set up on PAF plates as described above, but the colonies were

disrupted by mixing or padding. For the mixing experiment, the colonies were either left alone or disturbed daily: either using a pipette tip (repeated horizontal and vertical motions) or a sterile plastic loop (repeated orbital motion) to physically mix the colony. For the padding experiment, the colonies were either left alone or covered by a thin layer of agarose [3% (wt/vol), prepared between two layers of sterile plastic under pressure]. Fluorescently tagged strains were used in both mixing and padding experiments and each colony was visualized under a fluorescent microscope after 4 d of incubation. Strains tagged with antibiotic resistance were also used in mixing experiments, and the colonies were enumerated on PMM-agar plates supplemented with kanamycin or streptomycin, as described above.

Genome Sequencing and Identification and Confirmation of the Causal Mutation. Genomic DNA from one variant was sequenced to identify the causal mutation that leads to the MV phenotype. Genomic DNA was isolated and purified using the Genomic-tip 100/G, Genomic DNA Buffer Set, lysozyme, proteinase K, and RNase A, as instructed by the manufacturer in the Genomic DNA Handbook (Qiagen). Genomic DNA samples were submitted to the Washington University Genome Sequencing Center (St. Louis, MO) for 454 FLX random fragment library construction and sequencing. Greater than 87 Mbp of sequence data were retrieved with an average read length of 224 bp, representing ~14× genome coverage. Contigs were aligned against the published P. fluorescens Pf0-1 genome sequence (6) using the EagleView software (7) and sequences compared using BLAST (8). More than 50 individual cases of sequence mismatches were observed in the dataset; however, the majority were associated with homopolymeric sequences and were thus filtered out, leaving five candidates. A single nucleotide (A) deletion at the 126th position of the coding DNA sequence of the rsmE gene was chosen as the primary candidate because its homologs had already been implicated in modulating social behavior in bacteria (9). To confirm the presence of the deletion mutation, rsmE and its flanking regions in both the WT and MV were PCRamplified using primers csrA1 (5'-TTGCGCATCCACACTC-TTGC) and csrA2 (5'-GGTGGGGGGAATGGCAATACG), and both strands sequenced using internal primers csrA1B (5'-TTCGCCACATCCTGCCAATG) and csrA2B (5'-TCATTGG-CGCGCAGGCAAAC). The single-nucleotide deletion was confirmed to be present in the MV but not in WT. The Phusion High-Fidelity DNA Polymerase (Finnzymes) was used in all PCR reactions described in this study unless stated otherwise.

Mutant Construction and Tagging. The gene splicing by overlap extension (SOE) method (10) was used as previously outlined (11) to create mutations in *rsmE* by homologous recombination. The 126th nucleotide (A) of the *rsmE* gene was deleted in the WT by introducing the appropriate mutation in the SOE primers: rsmEpm5f (5'-CAGCAGGCGCCGTTACTACC) and rsmEpm5r (5'-GCTGGTAGATCTCTTCCCGGTGACTGCAACGTTCT-TCGGAGC) for the 5' fragment and rsmEpm3f (5'-GCTCCG-AAGAACGTTGCAGTCACCGGGAAGAGATCTACCAGC) and rsmEpm3r (5'-GAAGATGGCGTTGTTCGTGC) for the 3' fragment. The entire *rsmE* gene was deleted in WT using primers rsmEd5f (5'-ACAAAGCCGTGCTCGATCAG) and rsmEd5r (5'-GGCTACTGACTGCGATAGGGCGGTCTTCTCCTTGAT-TGCTTTGTAGG) for the 5' fragment and rsmEd3f (5'-CCT-ACAAAGCAATCAAGGAGAAGACCGCCCTATCGCAGTC-AGTAGCC) and rsmEd3r (5'-GGTGTTGCTCATCACTGGCG)

for the 3' fragment. Platinum Taq DNA Polymerase High Fidelity (Invitrogen) was used in the PCR reactions to facilitate the downstream T-A cloning process. The two fragments in each set were joined and cloned into the pGEM-T Easy vector system (Promega) then subcloned into the EcoRI site of the suicide plasmid pMQ30 (12). Primers rsmE1 (5'-CGCTGGCATCCT-TGATGACG) and rsmE2 (5'-TCTGGATCCGGTGAGGTCGC) were used to monitor the replacement of the WT rsmE gene with the mutant constructs by PCR and subsequently confirmed by sequencing both strands as described above. The single-nucleotide deletion strain was named rsmEpm and the complete deletion strain was named  $\Delta rsmE$ . The entire *fliC* gene was deleted in MV in the same manner as above using primers fliCd5f (5'-GCTGCAAGGCTGGATAGACG) and fliCd5r (5'-CGCCAA-AACTCATTCCGAAACCATGACGAATTCCTCGTTGG) for the 5' fragment and fliCd3f (5'-CCAACGAGGAATTCGTCA-TGGTTTCGGAATGAGTTTTGGCG) and fliCd3r (5'-ACT-TGCCATGTGCATCTCCC) for the 3' fragment. Primers fliC1 (5'-ACCCATGCCGGCTGGAGTGATG) and fliC2 (5'-TTG-CACCGATGTCCAGGCCG) were used to confirm the deletion of the *fliC* gene. The miniTn7 system was used to tag the chromosomes of the strains used in this study using established procedures. Strains used in competition experiments were tagged with neutral kanamycin or streptomycin resistance cassettes (13). These specific markers allow the identification of the different genotypes of P. fluorescens Pf0-1 within mixed populations while not affecting the relative fitness of cells (14). Strains used in microscopy were tagged with GFP, YFP, or DsRed-Express proteins (15). All primers used in this study were obtained from Integrated DNA Technologies and Sanger-based sequencing was carried out by GENEWIZ or Source BioScience. DNA fragments were purified using the QIAquick Kit (Qiagen) and plasmids were extracted using the QIAprep Kit (Qiagen). All enzymes were purchased from New England Biolabs.

### Colony and Biosurfactant Spreading Assay on Polycarbonate Membranes.

Nuclepore polycarbonate membrane (90- to 142-mm diameter, 0.4-µm pore size; Whatman) was laid on top of PAF plates using sterile forceps, and overnight cultures (2 µL for the mutant library from the parallel evolution experiments, or 20 µL for individual analyses) were spotted on top of the membrane. One side of the membrane appears smooth and shiny whereas the other side appears matted and dull. According to Whatman, the apparent differences stem from the manufacturing process where the duller side faces the open air and the shinier side makes constant contact against the preparatory surface. These physical properties persist across the scale of both phase-contrast and atomic force microscopy, where the surface of the dull side is significantly less uniform to that of the shiny side. Plates were inverted after the spots had been adsorbed and incubated at room temperature.

Measurement of Colony Density. The height (Z) of fluorescently tagged colonies was measured across the diameter (X) in reference to the agar surface by confocal microscopy. The Z dimension was calibrated to one edge of the colony (i.e., single layer of cells) and measurements were made in 0.5-mm increments of X across the center of the colony to the other edge. Given that there were small differences in Z between the two edges, the slope of the base was calculated and each measured Zvalue across the colony was normalized accordingly. This generated a cross-sectional map across the center of the colony (i.e., Z dimension for the y axis and X dimension for the x axis), which was relatively symmetric. The cross-section was sliced into individual trapezoids (triangles for the two edges) for each X increment from the edges to the center. Each slice was converted into rectangles by keeping the X constant, and cylindrical volume was calculated as a function of the radius (X). Final volume was calculated by averaging the measurements obtained from each half of the cross-section. Following the confocal analysis, each colony was harvested and the population size estimated by serial dilutions and plating as described above. The mean density from three independent colonies was calculated as CFU/mm<sup>3</sup> for each day.

Individual-Based Simulations. An individual-based simulation framework was used that captures bacterial growth and the concentration gradients of oxygen originating from diffusion and bacterial consumption. The parameters used in the simulations are summarized in Table S3. Fifty hours of growth was simulated for a cross-section of a bacterial colony initially seeded with 413 WT and 22 MV cells (i.e., initial relative frequency of MV is 0.05). As observed in our experiments, both cell types were assumed to grow equally fast. We extended an established framework that had been developed and tested over the last 15 y to understand and predict the behavior of bacterial communities. Recently, such simulations have been applied to understand the evolution and ecology of microbial groups (16-20), which have subsequently been validated experimentally (21, 22). The model assumptions, justifications, and implementation are extensively discussed elsewhere (23-26). Briefly, bacterial cells are modeled as growing and dividing spheres that metabolize oxygen in a continuous concentration field that is updated for each iteration by solving the 2D reaction-diffusion equations to steady state using a multigrid solver. Cell growth is calculated by solving the Monod equation based on the local oxygen concentration. We have focused on oxygen as the only nutrient for cells because both in simulations with explicit calculation of glycerol diffusion from the agar and consumption in the colony, as well as in empirical studies (27, 28), oxygen has been found to be the key limiting factor for growth in a colony. In the simulation, oxygen originates from the air above the colony and the agar below and diffuses through a thin diffusion layer (10 µm) above the colony and the agar (simulated thickness is 250 µm, and a constant boundary condition with fixed, low oxygen concentration further below) (Table S3). Cells grow, divide and, in case of MV cells, secrete polymers modeled as inactive spheres. Growth, division and polymer secretion leads to pushing away neighboring cells and expansion of the colony. We considered two patterns of cell division. The first was to assume that cells that divide produce new cells in a random direction. However, observations of the colonies revealed frequent vertical alignment of rod-shaped P. fluorescens cells at the interface between the genotypes (Fig. S2A). Therefore, we also tested the effects of simulated vertical cell division by enforcing a rule that newborn cells are placed either below or above the mother cell. This assumption of cell division direction does not affect conclusions as both reveal conditions where secretor cells have an evolutionary advantage over nonsecretor cells (Fig. 3 and Fig. S3). However, stochastic loss of genotypes was more likely in the random model, which did not reflect our observations that rare lineages often persist in the colonies. We, therefore, focused on the vertical alignment model (Fig. 3), which also better reflects the microscopy.

**Parallel Evolution Experiments.** Overnight WT cultures (20  $\mu$ L) were spotted on PAF plates and incubated for 4 d at room temperature until mucoid variants became clearly visible. A single variant was randomly isolated from each single WT colony, with one exception being that three spatially separated patches of variants were isolated from a common WT colony. Each variant was purified and phenotype confirmed on fresh PAF plates. The *rsmE* locus and its flanking regions were sequenced in each variant by using primers csrA1/2 and csrA1B/2B, as described above.

Statistical Analyses. Given that the sample sizes were too small (n = 3) for the Mann–Whitney test, a two-tailed *t* test was used to compare the relative fitness differences between any two given strains. A Kruskal–Wallis test was applied to compare the relative fitness of the constructed *rsmE* mutants to MV. A Kruskal–Wallis test, corrected for multiple comparisons (Tukey's honestly significant difference criterion), was used to compare CFU ratios of different mucoid variants to the WT. A two-tailed Mann–Whitney test was applied to compare the emergence ratios of different mucoid variants. The Wilcoxon signed-rank test was applied to compare relative fitness in the simulations. Bonferoni correction was applied when making multiple pairwise comparisons, and the relevant values for the *n* and  $\alpha$  parameters are indicated for each test where appropriate. All statistical tests were conducted using Matlab.

Imaging. Still pictures of colonies were generated using the CanoScanLiDE 200 flatbed scanner (Canon) or the EOS 30D DSLR camera (Canon), and images were scaled to calibrated dimensions using the ImageJ software (29). Fluorescently tagged strains were imaged using the Typhoon 9400 scanner (GE Healthcare) and the associated ImageQuant TL software as described elsewhere (30), the SteREO Lumar.V12 microscope (Zeiss) under the NeoLumar S 0.8× objective lens and the associated AxioVision software, or the Axio Zoom.V16 microscope (Zeiss) under the PlanApo Z 0.5x objective lens and the associated Zen software. Confocal imaging was carried out on the LSM 700 laser scanning microscope (Zeiss) using the 20× and 50x objectives and the associated Zen software. A square piece of agar containing the entire colony was cut out and placed on slides without a coverslip for confocal imaging. For all other imaging procedures, entire plates were imaged without disturbing the agar surface.

# SI Text

Estimation of the Timeline of *rsmE* Mutations. To estimate the timeline of mutations in *rsmE*, we compared the onset of MV emergence in pure WT colonies (i.e., natural) relative to colonies spiked with a small number of GFP-tagged MV (MVG) cells. Competition experiments were set up on PAF plates between WT and MVG using mixtures set at the following ratios (WT:MVG):  $1:1 \times 10^{-6}$ ,  $1:2 \times 10^{-6}$ , and  $1:3 \times 10^{-6}$ . The control group consisted of an equal volume of dH<sub>2</sub>O rather than the MVG suspension in the mixtures. The GFP-tagging procedure used in this study renders MVG resistant to gentamicin (15), so the population size of WT and MVG in each inoculate was estimated by serially diluting and plating out the mixture on PMM-agar plates with or without gentamicin supplementation.

Colonies were monitored daily under the fluorescent microscope over a period of 6 d and the results are summarized in Fig. S1*H*. As expected, all mucoid patches that emerged from the initially pure WT populations were nonfluorescent. The spiked fluorescent mucoid cells (i.e., MVG) emerge as visible patches after 2 d, reach their peak on the third day, and plateau thereafter. In contrast, very few nonfluorescent mucoid patches are visible after 3 d in both pure and spiked WT populations, and these then continue to increase in frequency gradually throughout the duration of the experiment. This finding implies that new MV cells emerge from WT in the MV-WT competitions and likely reduce the fitness differences measured at later time points (Fig. 1).

Spiking in a known number of fluorescent mucoid cells also allows us to estimate the probability that a particular mucoid variant cell will successfully form a patch and emerge from the surface of the colony. Specifically, we can compare the relative proportions of the introduced MVG cells that emerge from the WT population as discrete mucoid patches. Measurements made at three different initial frequencies revealed that ~50% of

Kim et al. www.pnas.org/cgi/content/short/1323632111

introduced MVG consistently emerge as independent patches (Fig. S11). These data will include some cases where a single mucoid patch emerges from a mixture of multiple MVG cells that happened to start near one another. However, this effect does not seem to be important because we observe strikingly similar proportions of emergence across competitions commenced at different relative frequencies. A cell that harbors the causal mutations thus appears to have a very good chance of emerging as an independent mucoid patch. Collectively, these results suggest that the causal mutations most likely occur de novo in each experiment after the WT populations are seeded on the plates, and are subsequently selected for independently.

Individual-Based Simulation of WT-MV Competitions. Previous theory on the use of polymers in competition for the growing edge was based on liquid submerged biofilms rather than colonies. We therefore modified our individual-based simulation system to capture the competition of a mucoid strain, which makes a bulky secretion that spaces out cells, versus a nonproducer in a colony setting. Parameters for the simulation came from measured values in our experiment and the literature (Table S3). Consistent with studies that measure oxygen levels and growth patterns within colonies (27, 28, 31–33), the key limiting nutrient in the model was found to be oxygen rather than the carbon source, so we focused our analysis on access to oxygen.

The simulation shows that, as for submerged biofilms, secreting strains can have a competitive advantage (Fig. 3 and Fig. S3). However, we found that lateral expansion is more important in colonies relative to upwards expansion, which is the dominant benefit in submerged biofilms. This lateral expansion allows a strain to conquer the region near the surface of the colony where there is best access to oxygen diffusing from above. The strength of this evolutionary advantage was not as great as that seen in the experiments, so we may not capture all of the processes at play. Nevertheless, the simulation demonstrates that our model for secretion-driven expansion can indeed provide an evolutionary advantage in colonies.

Comparison of Rates Between the Emergence of Mucoid Variants and Mutations in *rsmE*. The data presented in Fig. S1*H* was further probed to assess whether the rate of emergence was in accordance with the predicted mutation rates. Mucoid colonies emerging from WT control populations were cumulatively scored over the duration of the experiments and the mean estimates gathered from three independent experiments were used in the calculations. We observed 37 mucoid colonies emerging from each of the WT populations following 6 d of incubation. Experiments that seeded a known number of GFP-labeled *rsmE* mutant cells into colonies indicate that approximately half emerge from the surface in any experiment (Fig. S1*I*). Therefore, the number of loss-of-function *rsmE* mutants arising in the average experiment is  $\sim$ 80.

The size of the initial WT population was  $3.05 \times 10^6$  CFU, which expanded to  $1.89 \times 10^{10}$  CFU after 6 d, representing ~ $1.89 \times 10^{10}$  individual replication events. The genome size of the WT strain *P. fluorescens* Pf0-1 is  $6.43 \times 10^6$  bp (6), so the effective genome size is  $1.28 \times 10^7$  bp because both strands are copied during each replication event. The mutation rate during a genome replication event ( $5.40 \times 10^{-10}$  mutations per base pair per generation) is widely perceived to be similar among bacteria (34). Incorporating this as a proxy for the mutation rate in our experimental system, we estimate the total number of mutations accumulated over the duration to be:  $(1.89 \times 10^{10}) \times$  $(1.28 \times 10^7) \times (5.40 \times 10^{-10}) = 1.31 \times 10^8$ . The fact that this number is larger than the size of the genome generates an expectation that every nucleotide in the genome will be mutated in at least one cell during the course of the experiment. Although this is only a crude estimate, it gives an idea of the extreme levels of genetic variability present in the colony. For *rsmE* specifically, given its size of 195 bp (a  $3.02 \times 10^{-5}$  proportion of the genome) the expected number of mutations in *rsmE* based upon the genome average mutation rate is ~4,000 per experiment. Comparison of this expectation with the observation of around 80 *rsmE* mutants per experiments would suggest that mutation rate at *rsmE* is if anything below the genome average. However, this comparison rests upon the incorrect assumption that every mutation in *rsmE* leads to a loss of function. Thus, it is necessary to correct the genome-based estimate of *rsmE* mutation rate for the proportion of mutations that will cause a loss of function.

The goal then is to determine the effective target size for rsmE, which can be estimated by multiplying the size of the gene by the probability that any given mutation will result in the mucoid phenotype. Our estimations are based on the methods described by Lang and Murray (35) using the collection of mutations identified in this study (Table S1). The collection comprises 212 insertions and deletions (collectively referred to as "indels") and 322 base pair substitutions (BPS). The 31 mutations (7 indel and 24 BPS) identified solely in the 5' untranslated region are excluded from calculations because it is not possible to meaning-fully predict the probability of loss of function mutations outside the coding sequence.

We have identified 11 unique (40 in total) nonsense substitutions, which represent 79% of the 14 possible nonsense substitutions in *rsmE*. There are 26 unique substitutions within the collection of 282 missense mutations. Under the assumption that the same proportion of missense substitutions was isolated as in the nonsense collection, we predict that  $14/11 \times 26 = 33$ possible missense mutations will result in loss of function. This result gives a total of 14 + 33 = 47 BPS mutations that will lead to loss of function. In comparing these mutations with all possible BPS in *rsmE*, we must also consider the fact that each base can mutate to three different base pairs so each specific mutation event is only one of three options for its particular position. Therefore, we divide the number of nonsense and missense substitutions by three to generate an effective number of base pairs that will result in a loss of function; this leads to an estimate of 16 base pairs ( $\tau_{BPS}$ ), which is the *rsmE*-specific target size for all BPS. Moving to indels, under the assumption that any insertion or deletion is deleterious to RsmE and thus produces the mucoid phenotype, the rsmE-specific target size for indels is 195 base pairs ( $\tau_{indels}$ ).

To combine  $\tau_{BPS}$  and  $\tau_{indels}$  into a single effective target size, we must next estimate the relative probability that a mutation results in a BPS versus an indel. We can do this using the distribution of BPS (322 of 534 = 60%) and indels (212 of 534 = 40%) in our mutant collection. Notably, the overrepresentation of BPS relative to indels is in stark contrast to the target size predictions of 16 bases for BPS and 195 bases for indels. This result implies that indel mutations are much less probable than BPS [as also found by Lang and Murray (35)]. We can use this discrepancy to estimate the relative probability of the two major classes of mutation. This result reveals that the relative

- Compeau G, Al-Achi BJ, Platsouka E, Levy SB (1988) Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. *Appl Environ Microbiol* 54(10):2432–2438.
- Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in Gram negative bacteria. Nat Biotechnol 1:784–791.
- Kirner S, et al. (1996) The non-haem chloroperoxidase from Pseudomonas fluorescens and its relationship to pyrrolnitrin biosynthesis. Microbiology 142(Pt 8):2129–2135.
- King EO, Ward MK, Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescin. J Lab Clin Med 44(2):301–307.
- Lenski RE, Rose MR, Simpson SC, Tadler SC (1991) Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am Nat* 138(6):1315–1341.
- Silby MW, et al. (2009) Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. Genome Biol 10(5):R51.

probability of substitution mutations is 0.95  $[f_{BPS} = (0.6/0.08)/((0.6/0.08) + 0.4)]$ , and that for the indels is 0.05  $(f_{indels})$ , which is again in agreement with the data of Lang and Murray. Combining the target sizes with these relative probabilities of mutation gives us a final effective target size of  $(\tau_{BPS} \times f_{BPS}) + (\tau_{indels} \times f_{indels}) = 25$  base pairs.

We can now combine the final effective target size with our earlier estimate of the number of mutations per experiment, which predicts that there should be ~510 loss-of-function mutations in rsmE per experiment. Although lower than the naive estimate of 4,000 mutations, this value is still well above the observed mutation rate of ~80 mutations per experiment. Therefore, we conclude that there is no clear evidence of a raised mutation rate in rsmE and that the emergence of rsmEmutants is better explained by the observed strong natural selection (Fig. 1).

Comparison of Emergence Rates Between Mucoid Variants. The frequencies with which we find the different mutants are extremely variable (Tables S1 and S2). For example, nonsense mutants tend to be underrepresented relative to missense mutants. As such, we were interested in whether some of the strong competitors exhibited differences in phenotype during the isolation process that we do not see in our other phenotypic assays. We, therefore, compared the relative proportions of emergence among the individual MVs using the same technique as described for Fig. S1H. We compared the proportion of emergence in WT colonies spiked with known number of MV cells. Competition experiments were set up on PAF plates between the selected MV and WT tagged with either GFP (WTG) or YFP (WTY) using mixtures set at the ratio (WT:MV) of  $1:3 \times$  $10^{-6}$ . The initial population size of each MV (69 ± 5; mean ± 95% confidence interval) was estimated by plating out the serially diluted culture before mixing with WTG or WTY. Previous experiments revealed that the optimal time point for assessing emergence was 3 d following inoculation (Fig. S1H).

Six independent competitions were set up for each MV against WTG or WTY. Colonies were visualized by fluorescent microscopy and the discrete nonfluorescent MV subcolonies were counted. There were no significant differences between the WTG and WTY competitions (nonparametric Mann-Whitney test, with Bonferroni correction for multiple comparisons, all P >0.0023), so the results were averaged across all 12 competitions for each MV strain. The relative proportions of each introduced MV cells that emerged from the WT population are summarized in Fig. S5. Given the small sample size of 12 for each competition, a nonparametric Mann-Whitney test was applied. The data show a high variance and so are not definitive. Nevertheless, they are consistent with the view that all strong competitor mutants behave similarly in the emergence assay because we did not find large or significant differences among the different mutants tested. The one exception is the L23P mutant, a weak competitor phenotype, which had a significantly weaker emergence rate than some of the other mutants in the pairwise comparisons (P < 0.05).

- Huang W, Marth G (2008) EagleView: A genome assembly viewer for next-generation sequencing technologies. *Genome Res* 18(9):1538–1543.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410.
- Lapouge K, Schubert M, Allain FH-T, Haas D (2008) Gac/Rsm signal transduction pathway of gamma-proteobacteria: From RNA recognition to regulation of social behaviour. *Mol Microbiol* 67(2):241–253.
- Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. *Gene* 77(1):61–68.
- Silby MW, Levy SB (2004) Use of in vivo expression technology to identify genes important in growth and survival of *Pseudomonas fluorescens* Pf0-1 in soil: Discovery of expressed sequences with novel genetic organization. *J Bacteriol* 186(21):7411–7419.
- Shanks RMQ, Caiazza NC, Hinsa SM, Toutain CM, O'Toole GA (2006) Saccharomyces cerevisiae-based molecular tool kit for manipulation of genes from Gram-negative bacteria. Appl Environ Microbiol 72(7):5027–5036.

- Kim W, Levy SB (2008) Increased fitness of Pseudomonas fluorescens Pf0-1 leucine auxotrophs in soil. Appl Environ Microbiol 74(12):3644–3651.
- Silby MW, Nicoll JS, Levy SB (2009) Requirement of polyphosphate by *Pseudomonas fluorescens* Pf0-1 for competitive fitness and heat tolerance in laboratory media and sterile soil. *Appl Environ Microbiol* 75(12):3872–3881.
- Lambertsen L, Sternberg C, Molin S (2004) Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environ Microbiol* 6(7):726–732.
- Nadell CD, Xavier JB, Levin SA, Foster KR (2008) The evolution of quorum sensing in bacterial biofilms. *PLoS Biol* 6(1):e14.
- 17. Xavier JB, Foster KR (2007) Cooperation and conflict in microbial biofilms. Proc Natl Acad Sci USA 104(3):876–881.
- Nadell CD, Foster KR, Xavier JB (2010) Emergence of spatial structure in cell groups and the evolution of cooperation. *PLOS Comput Biol* 6(3):e1000716.
- Schluter J, Foster KR (2012) The evolution of mutualism in gut microbiota via host epithelial selection. *PLoS Biol* 10(11):e1001424.
- Mitri S, Xavier JB, Foster KR (2011) Social evolution in multispecies biofilms. Proc Natl Acad Sci USA 108(Suppl 2):10839–10846.
- Korolev KS, Xavier JB, Nelson DR, Foster KR (2011) A quantitative test of population genetics using spatiogenetic patterns in bacterial colonies. Am Nat 178(4):538–552.
- Nadell CD, Bassler BL (2011) A fitness trade-off between local competition and dispersal in Vibrio cholerae biofilms. Proc Natl Acad Sci USA 108(34):14181–14185.
- Xavier JB, Picioreanu C, van Loosdrecht MCM (2005) A framework for multidimensional modelling of activity and structure of multispecies biofilms. *Environ Microbiol* 7(8):1085–1103.
- Xavier JB, De Kreuk MK, Picioreanu C, Van Loosdrecht MCM (2007) Multi-scale individual-based model of microbial and bioconversion dynamics in aerobic granular sludge. *Environ Sci Technol* 41(18):6410–6417.

- Kreft JU, Picioreanu C, Wimpenny JW, van Loosdrecht MC (2001) Individual-based modelling of biofilms. *Microbiology* 147(Pt 11):2897–2912.
- Lardon LA, et al. (2011) iDynoMiCS: Next-generation individual-based modelling of biofilms. Environ Microbiol 13(9):2416–2434.
- Rani SA, et al. (2007) Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. J Bacteriol 189(11):4223–4233.
- Walters MC, 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS (2003) Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* 47(1):317–323.
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9(7):671–675.
- Xavier JB, Kim W, Foster KR (2011) A molecular mechanism that stabilizes cooperative secretions in *Pseudomonas aeruginosa*. Mol Microbiol 79(1):166–179.
- Peters AC, Wimpenny JW, Coombs JP (1987) Oxygen profiles in, and in the agar beneath, colonies of Bacillus cereus, Staphylococcus albus and Escherichia coli. J Gen Microbiol 133(5):1257–1263.
- Wimpenny JW, Lewis MW (1977) The growth and respiration of bacterial colonies. J Gen Microbiol 103(1):9–18.
- Pirt SJ (1967) A kinetic study of the mode of growth of surface colonies of bacteria and fungi. J Gen Microbiol 47(2):181–197.
- Drake JW, Charlesworth B, Charlesworth D, Crow JF (1998) Rates of spontaneous mutation. *Genetics* 148(4):1667–1686.
- Lang GI, Murray AW (2008) Estimating the per-base-pair mutation rate in the yeast Saccharomyces cerevisiae. Genetics 178(1):67–82.



Fig. S1. Emergence of MV and comparison of MV and WT growth in single genotype and mixed cultures. (A) Emergence of mucoid variants in a colony initiated from a single WT cell. An isolated colony on PAF derived from serially diluting and plating out WT was observed over time (shown above each panel is the number of days postinoculation). (Scale bars, 5 mm.) (B and C) MV dominates WT in mixed colonies independent of the initial frequency. Results of competitions between MV and WT, where MV was initially underrepresented (B) or overrepresented (C) by 1,000-fold. The graphs show the mean population size of MV (red circle) and WT (blue circle) in CFU obtained from destructively sampling three independent populations at each interval. (D) Visual presentation of competition between fluorescently tagged MV (green) and WT (red) each starting at a low frequency. Both strains were diluted 10<sup>-5</sup> from the stock preparations before mixing and imaged over time (number of days postinoculation). Shown are individual and overlay of images obtained from scanning with red and green lasers. (E) Growth profiles of single genotypes in in liquid KMB as measured by optical density at 600 nm. Genotypes are WT, streptomycin resistance tagged WT (WTS), MV, and kanamycin resistance tagged MV (MVK). Results from six replicates are shown in each graph along with the mean doubling time ± 95% confidence interval, and the regression coefficient of the data points used to calculate the doubling time. (F and G) Growth profiles of single genotype colonies on PAF plates in short (F) and long (G) timescales. Genotypes are WT (blue), MV (red), and  $\Delta rsmE$  (green). The datapoints represent the mean CFU estimated from destructively sampling three populations. (H) Rate of emergence of MV from WT colonies where differing numbers of MV are spiked in at the start. Zero or ~30, 60, or 90 GFP-tagged MV (MVG) cells were mixed with WT. The zero case shows the rate of emergence of de novo mutants, whereas the GFP-spiking treatments show the rate of emergence of MV cells that are there from the beginning. Each datapoint represents the mean from three independent populations. (/) Graph depicting the mean proportion of patches observed after 6 d of incubation from the estimated number of introduced MVG cells. The initial frequency of introduced MVG cells for each competition is indicated below the x axis. The mean is from nine independent competitions and the mean from all 27 experiments combined is shown at the far right. Error bars represent the 95% confidence interval (B, C, F, and G) or the SD (H and I) of the mean.



**Fig. S2.** Confocal laser scanning microscopy images of mixed colonies and estimation of population density in single genotype colonies. (A) Cells tend to align vertically in regions of competition between the genotypes. Mixed populations of fluorescently tagged  $\Delta rsmE$  (DsRed-Express) and WT (GFP) were seeded at the initial ratio of  $10^{-5}$ :1 ( $\Delta rsmE$ :WT) and are imaged here on day 4. (Scale bar,  $10 \ \mu$ m.) (B) Comparison of density differences between unmixed WT and MV colonies over time. Density was estimated by combining confocal microscopy estimates of colony volume with destructive sampling to obtain cell number at each time point. Error bars represent the SD of the mean of three colonies. The *P* values obtained from a two-tailed *t* test were 0.0554, 0.001, and 0.0025 over the 3 d, respectively.



**Fig. S3.** Individual-based simulations with cell division at a random angle. (*A*) Snapshot from a 2D simulation of an 870- $\mu$ m-wide cross-section of a colony growing on agar; MV in red, WT in green. (*B*) Fraction of the mucoid variant of total biomass over 50 h in six independent simulations (black line: simulation shown in *A*, *C*, and *D*); initial fraction 0.05. (*Inset*) The MV is fitter than the WT. The boxplot shows the relative fitness (W) of the MV at *t* = 50 h; the asterisk (\*) means results significantly different from equal fitness (W = 1), Wilcoxon signed-rank test (*P* = 0.0313). (*C*) Close-up of a region from the simulated colony. Because of the secretion of polymers, mucoid variant cells are less densely packed than WT cells. (*D*) Oxygen concentration profile in the simulation of the region shown in *C*. More oxygen is available in the region of mucoid variant cells because of the lower local cell density. (*E*) Confocal microscopy image of a colony of MV cells expressing DsRed-Express and WT cells expressing GFP. (Scale bars, 50  $\mu$ m.)



**Fig. S4.** Effect of limiting upward expansion on competitive ability of  $\Delta rsmE$ . GFP-tagged  $\Delta rsmE$  was mixed with unlabeled WT at a starting ratio of  $10^{-2}$ :1. After spotting the mixture on the agar surface, colonies were incubated either undisturbed (*Left*) or covered with a thin layer of agarose (*Right*). Images were captured after four days of incubation by fluorescent microscopy. (Scale bars, 2 mm.)



**Fig. S5.** Comparison of emergence of various MVs from spiked WT populations. A boxplot illustrating the distribution of proportions of MVs that emerged 3 d after mixing ~70 CFU with dense population of WT (~10<sup>7</sup> CFU). WT was tagged with YFP or GFP and the MVs were untagged, thus nonfluorescent patches that emerged were counted. Each box plot shows the median (inner circle), upper and lower quartiles (edges of box), and outliers (outer circle) among 12 replicates. Multiple occurrences of the same genotype indicate independently isolated mutants. According to a nonparametric Mann–Whitney test (n = 12, P < 0.05), only the L23P mutant showed any differences among the group.



**Fig. S6.** Loss of flagella has no bearing on MV's fitness. Results of competitions between MV( $\Delta fliC$ ) and WT commenced at 10<sup>-5</sup>:1 ratio. WT was tagged with streptomycin resistance and MV( $\Delta fliC$ ) was tagged with kanamycin resistance. The graph shows the mean population size of MV( $\Delta fliC$ ) (red circle) and WT (blue circle) in CFU obtained from destructively sampling three independent populations at each interval. Error bars represent the 95% confidence interval.

### 9 of 14

Table S1.	Summar	/ of	<sup>-</sup> mutations	identified	in	the	parallel	evolution	study
		-							

Location <sup>†</sup>	- Mutation <sup>‡</sup>	Consequence <sup>§</sup>	Genotype <sup>¶</sup>	Frequency	
U->65	Δ268	Entire gene deletion	∆-17-(ORF+56)	1	
U-65	∆299	Partial gene deletion	∆-106-193	1	
U-59	∆360	Partial gene deletion	∆-184-176	1	
U-45	Δ260	Partial gene deletion	∆-125-135	1	
U-42	Δ207	Partial gene deletion	∆-81-126	1	
U-40	Δ133	Partial gene deletion	∆-13-120	1	
U-14	Δ51	Partial gene deletion	Δ-11-40	1	
U-10	$\Delta 40$	Partial gene deletion	Δ-10-30	1	
U-6	Δ324	Partial gene deletion	∆-307-17	1	
U	Δ149	Upstream deletion	∆-161-(-13)	2	
U	Δ149	Upstream deletion	∆-155-(-7)	1	
U	Δ119	Upstream deletion	∆-132-(-14)	1	
U	Δ77	Upstream deletion	∆-80-(-4)	1	
U	G→A	Substitution (5' UTR)	G→A (-30)	1	
U	T→A	Substitution (5' UTR)	T→A (-29)	6	
U	A→C	Substitution (5' UTR)	A→C (-23)	5	
U	CTACA→CTA([/S]CTA)CA <sup>II</sup>	Disrupted 5' UTR (IS)	/S+3 (-22)	1	
U	A→G	Substitution (5' UTR)	A→G (-19)	3	
U	A→C	Substitution (5' UTR)	A→C (-15)	1	
Ū	AAGGAGA→A****A	Disrupted SD	∆-11-(-7)	1	
Ŭ	AAGGAGA→AAGAAGA	Disrupted SD	G→A (-9)	8	
1		Missense (START lost)	M11	3	
1	ATG→AAG	Missense (START lost)	M1K	1	
1	ATG→AGG	Missense (START lost)	M1R	3	
1		Missonso (START lost)	MIT	2	
1		Missonso (START lost)	N/11	51	
1		Missonso (START lost)	N/11	2	
1		Missonso (START lost)	N/11	1	
1		Framashift (IC)		1	
1 2	AIG→A([//]CCA)IG	Frameshift (deletion)	1011101+13+4	1	
1-5		Frameshitt (deletion)	Δ5-7 A 1 1F	1	
1–5 2				I C	
2	CTG→CCG	Missense	LZP	6	
2	CTG→CAG	Missense	L2Q	2	
2		Nilssense	LZR	3	
2		Frameshitt (Insertion)	L25+1	1	
2		Framesnitt (insertion)		1	
3	AIA→A(C)IA	Frameshitt (Insertion)	131+1	1	
3	AIA→AI*	Frameshift (deletion)	Δ9	4	
4		Missense	L4P	62	
4	CIC→(A)CIC	Frameshift (insertion)	L4I+1	1	
5	ACC→CCC	Missense	T5P	6	
6	CGC→CAC	Missense	R6H	2	
6	CGC→*GC	Frameshift (deletion)	Δ16	1	
7	AAA→TAA	Nonsense	K7#	6	
7	AAA→(T)AAA	Frameshift (insertion)	K7#+1	1	
7	AAA→AA*	Frameshift (deletion)	Δ21	2	
7–11	Δ13	Frameshift (deletion)	∆20–32	1	
7–11	Δ11	Frameshift (deletion)	∆21–31	2	
8	GTC→(C)GTC	Frameshift (insertion)	V8R+1	1	
8	GTC→(A)GTC	Frameshift (insertion)	V8S+1	1	
8	GTC→*TC	Frameshift (deletion)	Δ22	1	
8–25	Δ52	In-frame deletion	∆22–73	2	
8–38	Δ91	Frameshift (deletion)	∆22–112	1	
9	GGT→(G)GGT	Frameshift (insertion)	G9G+1	1	
9–11	$\Delta 7$	Frameshift (deletion)	∆27–33	1	
10	GAA→TAA	Nonsense	E10#	2	
10	GAA→GA*	Frameshift (deletion)	Δ30	2	
10–14	Δ11	Frameshift (deletion)	∆30–40	2	
11	AGC→AG([ <i>IS</i> ]AAG)C <sup>d</sup>	Frameshift (IS)	S11S+/S+3	2	
11–14	Δ10	Frameshift (deletion)	∆32–41	4	
11–14	Δ11	Frameshift (deletion)	∆32–42	4	
12	ATA→AT*	Frameshift (deletion)	Δ36	2	
13	AAC→AA(A)C	Frameshift (insertion)	N13K+1	1	

PNAS PNAS

Table S1.	Cont.			
Location <sup>†</sup>	Mutation <sup>‡</sup>	Consequence <sup>§</sup>	Genotype <sup>¶</sup>	Frequency
13	AAC→A*C	Frameshift (deletion)	Δ38	1
13	AAC→AA*	Frameshift (deletion)	Δ39	5
13–14	$\Delta 4$	Frameshift (deletion)	∆39–42	1
14	ATT→AAT	Missense	114N	1
14–15	$\Delta 4$	Frameshift (deletion)	∆40–43	1
20	ATC→AGC	Missense	1205	1
21	ACC→CCC	Missense	T21P	2
22	ATT→AT(C)T	Frameshift (insertion)	I22I+1	1
22–30	Δ25	Frameshift (deletion)	∆64–88	1
23	CTC→C(GACATCACGATCACCATTC)TC	Frameshift (insertion)	L23R+19	1
23	CTC→CCC	Missense	L23P	6
24-33	Δ27	Frameshift (deletion)	∆/1-9/	1
25-31	Δ16	Frameshift (deletion)	∆74–89	1
25-31		Frameshift (deletion)	Δ/4–92	1
27		Frameshift (deletion)	Δ81 028#	2
20		Frameshift (deletion)	Q20#	3
20			029#	11
29		Frameshift (deletion)	\Q25# ∧87	1
29_50	×63	In-frame deletion	A86_1/18	1
31	CGA→TGA	Nonsense	R31#	4
32		Missense	132N	
34	ATC→*TC	Frameshift (deletion)	∆100	1
34	ATC→AT*	Frameshift (deletion)	∆102	1
34–37	Δ10	Frameshift (deletion)	$\Delta 100 - 109$	1
34–39	Δ15	In-frame deletion	$\Delta 102 - 116$	1
36	GCT→(T)GCT	Frameshift (insertion)	A36C+1	1
36	GCT→*CT	Frameshift (deletion)	∆106	5
36	GCT→G*T	Frameshift (deletion)	∆107	3
36	Δ2	Frameshift (deletion)	∆106–107	4
36	Δ3	In-frame deletion	∆106–108	1
36–37	Δ5	Frameshift (deletion)	∆106–110	1
36–38	Δ9	In-frame deletion	∆106–114	1
36–39	Δ11	Frameshift (deletion)	∆106–116	2
36–39	Δ12	In-frame deletion	∆106–117	1
36–39	Δ9	In-frame deletion	∆107–115	1
36–39	Δ10	Frameshift (deletion)	∆107–116	2
36–39	Δ11	Frameshift (deletion)	∆107–117	3
36–40	Δ12	In-frame deletion	∆107–118	5
36–41		Frameshift (deletion)	∆106–122	1
37	$CCG \rightarrow C([/S]CTC)CG''$	Frameshift (IS)	P37L+/S+3	3
37	CCG→C(CGAAGAA)CG	Frameshift (insertion)	P3/P+/	1
37	CCG→C^G	Frameshift (deletion)		1
37-39		Frameshift (deletion)		1
0C		Nonsense Framashift (dalation)	N30#	1
28 12	AAG→A*G ∧ 15	In-frame deletion	A112_126	2
30-42	A5	Frameshift (deletion)	∆112=120 ∧117_121	1
39-47	A10	Frameshift (deletion)	∆116–121 ∆116–125	1
40	GTT→GT*	Frameshift (deletion)	∆120	15
40-41	Δ <b>4</b>	Frameshift (deletion)	∆119–122	15
40-41		Frameshift (deletion)	∆120–121	2
40-43		Frameshift (deletion)	∆119–128	- 1
40-44	Δ13	Frameshift (deletion)	Δ119–131	3
40–45	Δ15	In-frame deletion	∆119–133	-
41	GCA→*CA	Frameshift (deletion)	Δ121	24
41	GCA→G*A	Frameshift (deletion)	∆122	3
41	Δ2	Frameshift (deletion)	∆121–122	2
42	GTA→GAA	Missense	V42E	10
42	GTA→GT*	Frameshift (deletion)	∆126	6
42–43	Δ3	In-frame deletion	∆125–127	1
42–46	Δ11	Frameshift (deletion)	∆126–136	2

PNAS PNAS

Table S1.	Cont.			
$Location^{\dagger}$	Mutation <sup>‡</sup>	Consequence <sup>§</sup>	Genotype <sup>¶</sup>	Frequency
43	CAC→CCC	Missense	H43P	1
43	CAC→*AC	Frameshift (deletion)	Δ127	5
43–46	Δ10	Frameshift (deletion)	∆127–136	1
43–47	Δ14	Frameshift (deletion)	∆128–141	1
43–48	Δ16	Frameshift (deletion)	∆127–142	1
44	CGG→TGG	Missense	R44W	12
44	CGG→CCG	Missense	R44P	1
44	CGG→CAG	Missense	R44Q	69
45	GAA→TAA	Nonsense	E45#	2
45	GAA→GA(GT)A	Frameshift (insertion)	E45E+2	1
46	GAG→TAG	Nonsense	E46#	3
47	ATC→*TC	Frameshift (deletion)	Δ139	9
48	TAC→TAG	Nonsense	Y48#	5
48	TAC→*AC	Frameshift (deletion)	Δ142	1
48–49	$\Delta 4$	Frameshift (deletion)	∆142–145	1
49	CAG→TAG	Nonsense	Q49#	1
49	CAG→(T)CAG	Frameshift (insertion)	Q49S+1	1
49	CAG→*AG	Frameshift (deletion)	Δ145	1
49–53	Δ14	Frameshift (deletion)	∆145–158	1
49–53	Δ13	Frameshift (deletion)	∆146–158	1
49–54	Δ16	Frameshift (deletion)	∆145–160	1
50–51	Δ2	Frameshift (deletion)	∆150–151	1
52	Δ2	Frameshift (deletion)	∆155–156	1
52	CAG→TAG	Nonsense	Q52#	2
53–56	Δ11	Frameshift (deletion)	∆157–167	1
54	GGC→G*C	Frameshift (deletion)	Δ161	2
57–61	Δ11	In-frame deletion	∆171–181	1
59–61	Δ5	Frameshift (deletion)	∆177–181	1
61	CCA→(G)CCA	Frameshift (insertion)	P61A+1	1
65	TGA→CGA	Missense (STOP lost)	#65R	24
65	TGA→TTA	Missense (STOP lost)	#65L	1
65	TGA→TGG	Missense (STOP lost)	#65W	1

Each variant was isolated from a unique WT population, with the exception of three belonging to the #65L, L4P, and  $\Delta$ -11-(-7) genotypes that were isolated from discrete patches emerging from a common WT population. <sup>†</sup>Location of mutation with respect to the relative positions within the amino acid sequence; U denotes the region upstream from the START site; U->65 denotes the entire ORF and flanking regions.

<sup>\*</sup>Changes in the nucleotide sequence; \* denotes a single nucleotide deletion;  $\Delta$  denotes the number of nucleotides deleted; inserted nucleotides are indicated within parentheses; underlined nucleotides represent the Shine-Dalgarno sequence.

<sup>§</sup>Partial gene deletion includes coding and upstream regions; 5' UTR denotes the five prime untranslated region; STOP lost mutation results in read through of 18 additional amino acids; SD denotes the Shine-Dalgarno sequence.

<sup>6</sup>The # denotes a STOP codon;  $\Delta N$  denotes the relative nucleotide positions of deletion within the coding sequence; position of mutations in the region upstream from the coding sequence is indicated within the parentheses relative to the START codon, and the nucleotide substitution is shown where applicable; + denotes the number of nucleotides inserted or an insertion sequence element.

<sup>II</sup>IS denotes the insertion of a 1,313-bp insertion sequence element. All but one share exact nucleotide sequence identity as those annotated in the WT genome as Pfl01\_0068-0069 or Pfl01\_1346-13477. There are two additional IS elements in the genome (Pfl01\_2031-2032 and Pfl01\_2130-2131) that differ by a single nucleotide. One of the three in the P37L+/S+3 genotype shares exact nucleotide sequence identity with those in the latter group.

SANG SAN

Table S2.	Summary of possible and	identified substitutions	within the coding sequence	of rsmE
	· · · · · · · · · · · · · · · · · · ·			

WT	sequence		First position				Second position				Third position									
No.	Codon	AA	Т	s	Т	v	T	/	Г	s	Т	v	Т	v	Ts	5	T۱	/	T	v
1	ATG	М	GTG	V	CTG	L (3)	TTG	L	ACG	T (9)	AAG	K (1)	AGG	R (1)	ATA	I (51)	ATC	I (1)	ATT	I (3)
2	CTG	L	TTG	L	ATG	М	GTG	V	CCG	P (6)	CAG	Q (2)	CGG	R (3)	CTA	L	СТС	L	CTT	L
3	ATA	I	GTA	V	СТА	L	TTA	F	ACA	Т	AAA	К	AGA	R	ATG	М	ATC	Ι	ATT	Т
4	СТС	L	TTC	F	ATC	I	GTC	V	ссс	P (62)	CAC	Н	CGC	R	CTT	L	СТА	L	CTG	L
5	ACC	т	GCC	А	ССС	P (6)	тсс	S	ATC	I	AAC	Ν	AGC	S	ACT	т	ACA	т	ACG	т
6	CGC	R	TGC	С	AGC	S	GGC	G	CAC	H (2)	ссс	Р	стс	L	CGT	R	CGA	R	CGG	R
7	(AAA)	К	GAA	Е	CAA	Q	TAA	# (6)	AGA	R	ACA	т	ATA	I	AAG	К	AAC	Ν	AAT	Ν
8	GTC	v	ATC	Ι	стс	L	TTC	F	GCC	А	GAC	D	GGC	G	GTT	V	GTA	V	GTG	V
9	GGT	G	AGT	S	CGT	R	TGT	С	GAT	D	GCT	А	GTT	V	GGC	G	GGA	G	GGG	G
10	(GAA)	Е	AAA	К	CAA	Q	TAA	# (2)	GGA	G	GCA	А	GTA	V	GAG	Е	GAC	D	GAT	D
11	AGC	s	GGC	G	CGC	R	TGC	C	AAC	Ν	ACC	т	ATC	I	AGT	s	AGA	R	AGG	R
12	ATA	I	GTA	V	СТА	L	TTA	F	ACA	т	AAA	к	AGA	R	ATG	М	ATC	Ι	ATT	Т
13	AAC	Ν	GAC	D	CAC	н	TAC	Y	AGC	S	ACC	т	ATC	T	AAT	Ν	AAA	к	AAG	к
14	ATT	I	GTT	V	CTT	L	TTT	F	ACT	т	AAT	N (1)	AGT	S	ATC	I	ATA	Т	ATG	м
15	GGT	G	AGT	s	CGT	R	TGT	с	GAT	D	GCT	A	GTT	V	GGC	G	GGA	G	GGG	G
16	GAT	D	AAT	N	CAT	н	TAT	Y	GGT	G	GCT	A	GTT	V	GAC	D	GAA	E	GAG	E
17	GAC	D	AAC	N	CAC	н	TAC	Y	GGC	G	GCC	A	GTC	V	GAT	D	GAA	E	GAG	E
18	ATC	-	GTC	v	СТС	L	TTC	F	ACC	т	AAC	N	AGC	s	ATT	1	ATA	-	ATG	M
19	ACG	т	GCG	Δ	CCG	P	TCG	s	ATG	M	AAG	к	AGG	R	ACA	т	ACC	т	ACT	т
20	ATC	I.	GTC	V	СТС	L	TTC	F	ACC	т	AAC	N	AGC	S (1)	ATT	I	ATA	I	ATG	M
21	ACC	т	GCC	Δ	ССС	P (2)	тсс	s	ATC	1	AAC	N	AGC	S	ACT	т	ACA	т	ACG	т
22	ATT	·	GTT	V	CTT	L	TTT	F	ACT	т	AAT	N	AGT	s	ATC	·	ATA	· I	ATG	M
23	СТС	, i	TTC	F	ATC	-	GTC	V	ССС	P (6)	CAC	н	CGC	R	CTT	, i	СТА	I	CTG	1
24	GGC	G	AGC	s	CGC	R	TGC	c	GAC	D	GCC	Α	GTC	v	GGT	G	GGA	G	GGG	G
25	GTC	v	ATC	J	СТС	1	TTC	F	GCC	A	GAC	D	GGC	Ğ	GTT	v	GTA	v	GTG	v
26	AGC	5	GGC	G	CGC	R	тас	C		N	ACC	т	ATC	1	AGT	5	AGA	R	AGG	R
27	GGC	G	AGC	s	CGC	R	тас	c	GAC	D	GCC	Δ	GTC	v	GGT	G	GGA	G	GGG	G
28	(CAG)	0	TAG	# (3)	AAG	ĸ	GAG	F	CGG	R	CCG	P	СТБ		CAA	0	CAC	н	CAT	н
29	(CAA)	õ	тдд	# (11)		ĸ	GAA	F	CGA	R	CCA	P	СТА	-	CAG	õ	CAC	н	CAT	н
30	GTT	v	ΔΤΤ	/	СТТ	1	ттт	F	GCT	Δ	GAT	D	GGT	G	GTC	v	GTA	v	GTG	v
31	(CGA)	R	TGA	# (4)	AGA	R	GGA	G	CAA	0	CCA	P	СТА	I	CGG	R	CGC	R	CGT	R
32	ATC	1	GTC	( .,	СТС	1	TTC	F	ACC	т		N (1)	AGC	- s	ΔΤΤ	1	ΔΤΔ		ATG	м
33	GGC	G	AGC	s	CGC	R	тас	C	GAC	D	GCC	Α	GTC	v	GGT	G	GGA	G	GGG	G
34	ATC	I	GTC	v	СТС	1	TTC	F		т		N	AGC	s	ΔΤΤ	I	ΔΤΔ	I	ATG	м
35	AAT	N	GAT	D	CAT	н	тат	Y	AGT	s	ACT	т	ATT	J	AAC	N		ĸ	AAG	к
36	GCT	Δ	ACT	т	ССТ	P	тст	s	GTT	V	GAT	D.	GGT	G	GCC	Δ	GCA	Δ	GCG	Δ
37		P	TCG	s	ACG	т	GCG	Δ	СТБ	i	CAG	0	CGG	R		P		P	ССТ	P
38		ĸ	GAG	F	CAG	0	TAG	# (1)	466	R	ACG	т	ATG	м		ĸ	ΔΔΟ	N	ΔΔΤ	N
39		N	GAC	D	CAC	ч	TAC	Y	AGC	s		т	ΔΤΟ		ΔΔΤ	N		ĸ	AAG	ĸ
40	GTT	v	ΔΤΤ	I	СТТ		TTT	F	GCT	Δ	GAT	, D	GGT	G	GTC	V	GTA	V	GTG	V
/11	GCA	Å		т		Þ	тса	ç	GTA	v	GAA	F	GGA	G	GCG	Å	GCC	Å	GCT	^
41	GTA	v	ΑΤΑ	1	СТА	r I	тта	J	GCA	Δ	GAA	E (10)	GGA	G	GTG	v	GTC	v	GTT	v
72 /12	CAC	ч	TAC	v		L N	GAC	L L		R		P (1)	CTC	ı	CAT	ч	CA ^	°	CAG	°
45 AA	CAC	P	TCC	I \\\ (12)		P	GEE	G		(60)	CCC	P (1)	СТС	ь 1		P		Q P	CAG	γ P
 // E	(C ^ ^)	Г.		v (12)	CV V	0		# (2)		C (05)	GCA	^	GTA	L \/	GVC	Г.	CUC CAC		CAT	
45 16		F		ĸ		Q O		# (2) # (2)	GGG	G	GCA	A 	GTG	v V	GAG	F	GAC	D	GAT	D
-0 /17		-	GTC	N V	СЛО	ې ۱	TTC	F	ACC	т	ΔΛΟ	N		¢		ь 1	ΔΤΛ	ı		м
-77 48	(TAC)	v	CAC	ч		N	GAC	י ח	TGC	ć		ç	TTC	F	тат	v	TAA	" #	TAG	# (5)
49		0	TAG	# (1)		ĸ	GAG	F	CGG	R	000	P	СТС		CAA	0	CAC	"	САТ	н <del>н</del>
	(0,10)	~					0/10	-					0.0	-	0.4	~	0.70		0.11	

PNAS PNAS

Table S2. Cont.

LAS PNAS

WT sequence First position						Second position					Third position									
No.	Codon	AA	1	l s	T۱	/	T۱	/	T	5	T۱	v	Т	v	1	<b>Ts</b>	T۱	/	T۱	v
50	CGC	R	TGC	С	AGC	S	GGC	G	CAC	Н	ссс	Р	СТС	L	CGT	R	CGA	R	CGG	R
51	ATC	Т	GTC	V	СТС	L	TTC	F	ACC	Т	AAC	Ν	AGC	S	ATT	Т	ATA	Ι	ATG	М
52	(CAG)	Q	TAG	# (2)	AAG	К	GAG	Е	CGG	R	CCG	Р	CTG	L	CAA	Q	CAC	Н	CAT	н
53	GCG	А	ACG	А	CCG	Р	TCG	S	GTG	V	GAG	Е	GGG	G	GCA	А	GCC	А	GCT	А
54	GGC	G	AGC	S	CGC	R	TGC	С	GAC	D	GCC	А	GTC	V	GGT	G	GGA	G	GGG	G
55	CTG	L	TTG	L	ATG	М	GTG	V	CCG	Р	CAG	Q	CGG	R	CTA	L	CTC	L	CTT	L
56	ACC	Т	GCC	А	CCC	Р	тсс	S	ATC	Ι	AAC	Ν	AGC	S	ACT	Т	ACA	Т	ACG	т
57	GCT	А	ACT	Т	CCT	Р	тст	S	GTT	V	GAT	D	GGT	G	GCC	А	GCA	А	GCG	А
58	CCG	Р	TCG	S	ACG	Т	GCG	А	CTG	L	CAG	Q	CGG	R	CCA	Р	CCC	Ρ	CCT	Р
59	GAC	D	AAC	Ν	CAC	Н	TAC	Υ	GGC	G	GCC	А	GTC	V	GAT	D	GAA	Е	GAG	Е
60	(AAG)	К	GAG	Е	CAG	Q	TAG	#	AGG	R	ACG	Т	ATG	Μ	AAA	К	AAC	Ν	AAT	Ν
61	CCA	Р	TCA	S	ACA	Т	GCA	А	СТА	L	CAA	Q	CGA	R	CCG	Р	CCC	Ρ	CCT	Р
62	(CAA)	Q	TAA	#	AAA	К	GAA	Е	CGA	R	CCA	Р	CTA	L	CAG	Q	CAC	Н	CAT	Н
63	ACG	т	GCG	А	CCG	Р	TCG	S	ATG	М	AAG	К	AGG	R	ACA	Т	ACC	Т	ACT	т
64	ССТ	Ρ	тст	S	ACT	Т	GCT	А	CTT	L	CAT	н	CGT	R	CCC	Р	CCA	Ρ	CCG	Ρ
65	TGA	#	CGA	R (24)	AGA	R	GGA	G	TAA	#	TCA	S	TTA	L (1)	TGG	W (1)	TGC	С	TGT	С

Possible substitutions and corresponding amino acid changes are grouped based on the three positions of the codon; Ts denotes transition and Tv denotes transversion; codons within parentheses are pretermination codons and # denotes the STOP codon; identified substitutions are highlighted and frequency noted within parentheses; substitutions highlighted in red or blue resulted in large or reduced colony spreading, respectively, as shown in Fig. 6. Robustness was calculated as follows: [8 (number of observed missense substitutions that resulted in a knock-out phenotype (i.e., highlighted in red), excluding the START/STOP codons) / 0.95 (estimation of missense coverage based on nonsense and START codon mutation identification)]/409 (total number of possible missense substitutions available, excluding the nonsense and START/STOP codons) = 2%.

Table S3.	Parameters	used in	the	individual-based	simulations

Parameter	Value	Source
Diffusion coefficient O <sub>2</sub>	7.2e6 (μm²/h)	(1)
Monod constant O <sub>2</sub>	3.5e-5 (g/L)	(2)
Maximum growth rate cells	0.5 (h <sup>-1</sup> )	Approximated
Specific masses		(2)
Cells	220 [g(carbon)/L]	
Polymer	44 [g(carbon)/L]	
Maximum cell diameter	1 (μm)	
Boundary conditions		
Periodic boundaries at the sides		
O <sub>2</sub> concentrations bulk air/colony boundary layer interface	8.90e-3 (g/L)	(3)
$O_2$ concentration bulk agar below 250 $\mu m$	8.90e-4 (g/L)	(1, 3–6)

1. Picioreanu C, Vanloosdrecht M, Heijnen J (1997) Modelling the effect of oxygen concentration on nitrite accumulation in a biofilm airlift suspension reactor. Water Sci Technol 36(1):147–156.

2. Nadell CD, Xavier JB, Levin SA, Foster KR (2008) The evolution of quorum sensing in bacterial biofilms. PLoS Biol 6(1):e14.

3. Xavier JB, Foster KR (2007) Cooperation and conflict in microbial biofilms. Proc Natl Acad Sci USA 104(3):876-881.

4. Rani SA, et al. (2007) Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. J Bacteriol 189(11): 4223–4233.

5. Walters MC, 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS (2003) Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas* aeruginosa biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother 47(1):317–323.

6. Peters AC, Wimpenny JW, Coombs JP (1987) Oxygen profiles in, and in the agar beneath, colonies of Bacillus cereus, Staphylococcus albus and Escherichia coli. J Gen Microbiol 133(5): 1257–1263.