



A heritable iron memory enables decision-making in *Escherichia coli*

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The importance of memory in bacterial decision-making is relatively unexplored. We show here that a prior experience of swarming is remembered when *Escherichia coli* encounters a new surface, improving its future swarming efficiency. We conducted >10,000 single-cell swarm assays to discover that cells store memory in the form of cellular iron levels. This “iron” memory preexists in planktonic cells, but the act of swarming reinforces it. A cell with low iron initiates swarming early and is a better swimmer, while the opposite is true for a cell with high iron. The swarming potential of a mother cell, which tracks with its iron memory, is passed down to its fourth-generation daughter cells. This memory is naturally lost by the seventh generation, but artificially manipulating iron levels allows it to persist much longer. A mathematical model with a time-delay component faithfully recreates the observed dynamic interconversions between different swarming potentials. We demonstrate that cellular iron levels also track with biofilm formation and antibiotic tolerance, suggesting that iron memory may impact other physiologies.

swarming | iron | *E. coli* | antibiotics | memory

Constantly changing environments present a challenge to the survival of all organisms (1). They can adapt to change either by behavioral or phenotypic plasticity (2) but must decide among available phenotype choices (3). Response times during decision making can be critical for survival (3). Vertebrates use their nervous system for faster decision-making by storing information about their prior experiences (4). The process of storage and retrieval of information is called memory (4, 5), which can be stored for a few seconds to several years (6). A stronger memory can be brought to bear on the system by “conditioning” (7)—a process where repeated encounters with a stimulus can be stably linked to a specific response (8, 9), drastically reducing decision-making time (10).

Bacteria experience large environmental fluctuations to which they can readily adapt (11). They commonly do this by transducing environmental signals to elicit appropriate transcriptional responses (12) that occur on the time scale of minutes, dissipating in the absence of the signal. Keeping these systems always “on” is costly (12), so bacteria also employ less-costly bet-hedging strategies that stochastically switch among many alternative phenotypic states in similar time frames (13, 14). Alternatively, different cell types can preexist in an isogenic population and respond heterogeneously to antibiotic stress (15) or to starvation (16). All of these strategies come under the umbrella of bacterial memory (17).

Whether bacteria can store memory has been explored both theoretically (17, 18) and experimentally (19–22). Memory can influence the fitness of individuals (18) or of a community (19), as well as bacterial interaction with their host (20) and defense against phages (23). The mechanism of memory storage in bacteria involves genetic (5) or epigenetic factors (24, 25) such as type 1 fimbriae phase variation (5), motile-sessile transition (26), or chemotaxis (27–29). These varied molecular mechanisms of bacterial memory storage are more stimulus-specific in nature, in contrast to the nervous system where no matter the type of stimulus, the same molecular principle of storage is employed (4). The existence of the latter kind of memory mechanism in bacteria can only be conjectured from some recent reports (21, 22).

In this study, using *Escherichia coli* swarms as our experimental system, we asked a question not considered before: Is there a heritable memory in bacteria that is accessible to multiple stimuli? The choice of this system was based on observations that a prior experience of swarming hastens the onset of swarming (30). Swarming motility is a collective, flagella-driven, adaptation to colonizing the surface of semisolid media by population expansion (30–34). *E. coli* will typically swarm on semisolid media (~0.5% agar), but are nonmotile on solid or hard media (~1.5% agar). The “softer” agar is more conducive to swarming because among other properties, it also holds more water, allowing flagella to work on a surface terrain. Surface colonization presents varied challenges, both physical and nutritional (33). Nutritional challenges include acquisition of iron (35) leading to upregulation of efflux

Significance

In humans, repeated encounters with a wide variety of stimuli can reactivate specific neurons to produce long-term memories. We report here a multigenerational memory in *Escherichia coli* swarming motility, where bacteria “remember” their swarming experience for several generations. We show unambiguously that the molecular basis of this memory is the levels of available cellular iron. The act of swarming “conditions” the cells with this memory. Given the central role of iron in cellular metabolism, an iron-based memory might offer the advantage of providing a hub connecting various stress responses such as antibiotic survival and biofilms.

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pumps essential for the secretion of iron-binding siderophores into the extracellular matrix, as well as upregulation of iron import systems (36–40). When bacteria cultured in liquid media are transferred to swarm media, a long lag ensues before swarming begins (30). Widespread transcriptome changes accompany the onset of swarming (38, 41–44), revealing that the bacteria are adapting to a distinctly different environment during the lag phase. If, however, bacteria from a swarm are transferred to a fresh swarm media, the lag is considerably shortened (45), suggesting that the bacteria may be already “conditioned” to swarm.

To interrogate whether swarming bacteria “remember” the adaptation mechanisms employed while swarming, we designed an experimental setup that allowed monitoring of swarms initiated from >10,000 single cells. We report here that the bacteria indeed possess a heritable memory of the swarming state that is retained on the time scale of hours over at least four generations. We show that the mechanism of this type of memory is primarily related to how much iron is stored intracellularly. Since iron levels are important to several other physiological responses as our results confirm, we think that iron memory could be important there as well. Our findings suggest a more general physiological memory in *E. coli*.

Results

Swarming Proficiency Preexists in Planktonic Cells. The impetus for experiments performed in this study originated from a serendipitous observation that swarm cells plated for determining CFU counts (colony forming units) on freshly made (hence moist) hard agar, produced colonies whose phenotype was different from those made by planktonic cells (growing in liquid culture) plated on similar agar. Compared to planktonic (P) colonies, swarm-derived (S) colonies had more irregular margins, were larger, and flatter (when viewed from the side) (Fig. 1 *A* and *B*). We posited that these differences stemmed from prior conditioning of S cells, so they were attempting to swarm even on hard agar because the plates were still moist. We quantified these morphologies by measuring colony circularity (using both area and perimeter) and size (diameter) (Fig. 1 *C*). S colonies were significantly less circular and larger. On closer inspection, a fraction (~20%) of P-derived colonies also displayed S-colony characteristics, although less prominently (Fig. 1 *B*, *Top*; compare #9–10 with #1–8), suggesting that P-cells were also heterogeneous for swarming potential, with some inherently primed for swarming. To test the latter deduction, we performed single-cell inoculum (SCI) swarm assays, where P cell-derived swarms were initiated from single P cells isolated using the dilution-to-extinction method (46) targeted to yield an average of 0.5 cells per unit volume (Fig. 1 *D* and *SI Appendix*, Fig. S1 *A* and *B*). The sampling events would follow a Poisson distribution, so ~40% of seeded swarm plates would have received a cell. This was confirmed by parallel growth in microtiter plates (*SI Appendix*, Fig. S1 *C*, *Top*), which matched the expected positive sampling events with a high degree of precision (*SI Appendix*, Fig. S1 *D*). Therefore, in all SCI assays, at least 120 swarm plates were inoculated to gather data for 50 swarms (*SI Appendix*, Fig. S1 *C*, *Bottom*), again with high precision (*SI Appendix*, Fig. S1 *E*). The sample size was kept sufficiently large to increase confidence in the statistical analysis. Additionally, such Poisson distributions would sample multiple cells in ~5% of events. We could identify those events on a swarm plate by just counting the number of swarm “nucleation” centers and exclude them from our analysis (Fig. 1 *E*). In parallel, we compared data from SCIs vs. 100- or 10,000-cell inoculums, where any heterogeneity present in single cells is expected to be subsumed within the large cohort. The swarming proficiency (measured as the swarm diameter) of SCIs showed a

large variability, in contrast to the larger cell inoculums (see the endpoints of lines in each group) (Fig. 1 *F*). Comparison of this variability between the three datasets was complicated by the fact that each cohort had a different lag period as expected from the dependence of lag on cell-density (33, 47). A zone-adjustment was therefore performed by arbitrarily dividing the plate into 3 zones (C/M/O) (Fig. 1 *G*) and comparing swarm diameters of every cohort within each zone (Fig. 1 *H*; see *SI Appendix*, *SI Methods*). The zone-wise variability in frequency distributions of swarm diameters (5 mm bins) was then compared (Fig. 1 *I–K*), and the noise within each group was measured as the coefficient of variation, i.e., SD over mean (Fig. 1 *L–N*). In every zone, SCIs had significantly higher levels noise (a reflection of heterogeneity) compared to other cohorts, the maximal noise evident in the outermost zone O (Fig. 1 *K* and *N*). The large heterogeneity observed within the P cell-derived SCIs reveals that swarming proficiency preexists even in planktonic cells.

Swarming-Related Memory Is Present in Both P and S Cells. To explore whether the heterogeneity of swarming proficiency was random or inherited, we evaluated the swarming capability of offspring from single cells over several generations (Fig. 2 *A*). The starting cells represent our G0 generation “mothers” and were derived from both P and S conditions. We first standardized the established dilution-based protocol (46) to precisely collect daughter cells at different generations (*SI Appendix*, Fig. S2 *A–C*). Some G0 mothers ($n = 50$) were directly spotted on swarm plates (Fig. 2 *B* and *C* and *SI Appendix*, Fig. S2 *D*). Others were grown in 96-well plates for either 4 (Fig. 2 *D* and *E*) or 7 (Fig. 2 *F* and *G*) generations ($n = 25$, x axis) before plating single daughters of each mother (16 expected, y axis in Fig. 2 *D* and *F*) on swarm media (*SI Appendix*, Fig. S2 *D* and *F*). We initially probed other generations as well; but to reduce time and effort in these labor-intensive experiments, we confined our observations to these generations, which gave conclusive results during our initial screening. In the bubble plots (Fig. 2 *B–G*), the size of each bubble represents the swarm diameter and the color represents the noise within 16 daughters of the same mother. The behavioral patterns of P mothers (P→S data, *B–D–F*) was distinctly different from the S mothers (S→S data, *C–E–G*). P mothers (G0) showed the expected heterogeneity (see 1-cell data in Fig. 1 *F*) in swarming (Fig. 2 *B* and *SI Appendix*, Fig. S3). In contrast, all the G4 daughters of a single P mother were more homogeneous (Fig. 2 *D*, follow the data vertically or column-wise for the ~16 siblings). Progeny of some of these mothers (#11, #13, #14) showed variability, which could have come from experimental noise. A K-means clustering of the means derived from each column resulted in three distinct groupings labeled XS (poor), M (moderate), and L (efficient) swimmers. The majority patterns, i.e., consistent behavior of each sibling group, suggested that some information stored in the mother was passed down to the daughters. This information was lost by G7 as the variability returned (Fig. 2 *F* and *SI Appendix*, Fig. S3 *A*). In summary, P daughters inherit some form of memory that is intact up to four generations, but is lost by the seventh generation, restoring the original heterogeneity.

The same set of experiments performed on S mothers (S→S data, *C–E–G*) showed a very different outcome at G0 and G4. In contrast to P mothers, swarm diameters of S mothers at G0 were uniform (Fig. 2 *C* and *SI Appendix*, Fig. S3 *A*), suggesting that they retained “swarm” memory or were conditioned in some way while swarming. Similar to P cells, this memory was maintained in S daughters until G4, but with even lower noise (Fig. 2 *E*). In addition, the K-means clustering resulted in only in one group (L), in contrast to 3 groups in P daughters. By G7, the S daughters had lost this memory, similar

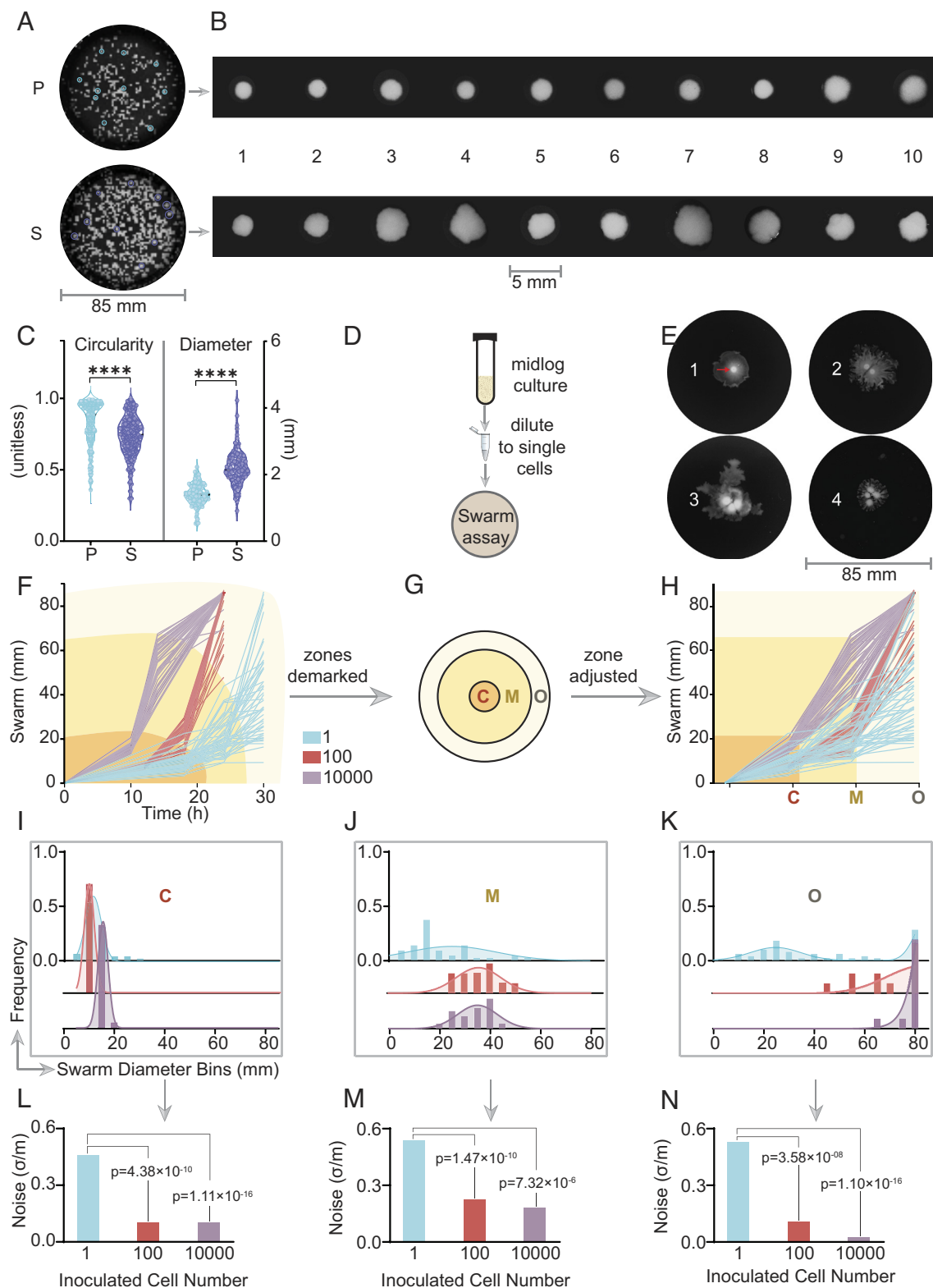


Fig. 1. Swarming proficiency preexists in planktonic cells. (A) Representative hard-agar plates showing CFUs of planktonic (P) cells from a mid-log phase liquid culture, and swarm (S) cells collected after growth for 16 h. In both cases, a 10^{-6} dilution of 0.5 OD cells was plated on hard agar plates and incubated for 16 h at 37 °C. (B) Blown-up images of 10 randomly selected colonies from A (indicated by colored circles). (C) Distribution of physical parameters of P and S colonies ($n = 218$). See *Methods* for circularity calculations. (**** $P < 0.0001$, Mann-Whitney test) (D) Flow chart of single P-cell swarm assays, where the dilution was estimated to yield a single cell in a unit volume of 4 μ L (see *SI Appendix, Fig. S1* and *Methods* for details). (E) Swarms with varying number (1 to 4) of swarm nucleation centers (red arrow) dictated by the initial number of cells spotted on the plate. (F) A spaghetti plot showing swarm diameters (y axis) measured over time (x axis). Each line represents a replicate. (G) Three arbitrary zones considered for adjustment: C, central; M, medial; O, outer. (H) zone-adjusted comparisons (see *Methods* and text). (I–K) Frequency distribution of swarm diameters (5 mm bins), color-coded as in (F). (L–N) Comparison of noise (SD/mean) within cohorts. *P* values were calculated from F-tests (*SI Appendix, SI Methods*).

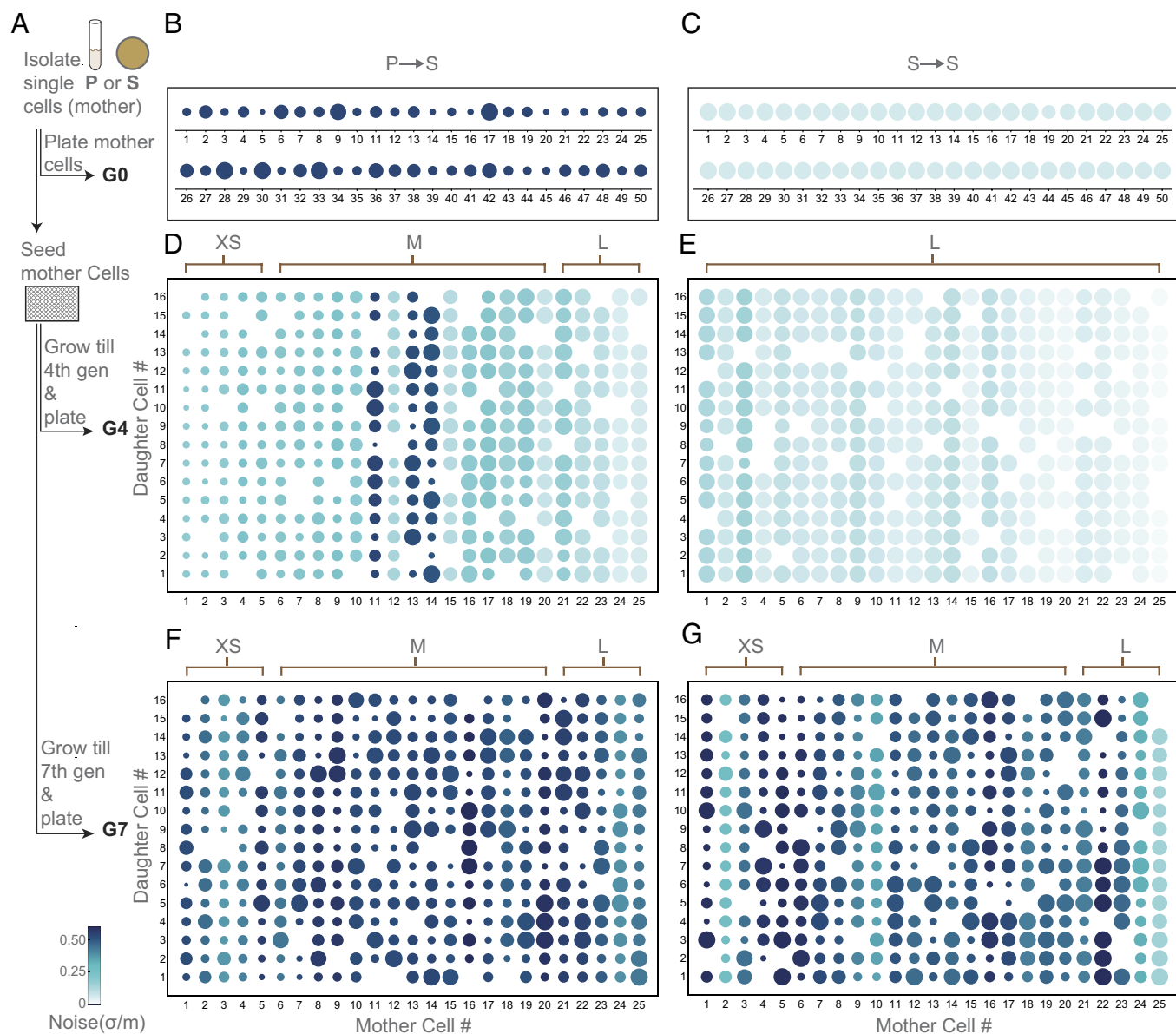


Fig. 2. Swarming-related memory is present in both *P* and *S* cells. (A) Flowchart summarizing the operations involved. *P* and *S* cells were collected and diluted to get single cells; these are the starter G0 cells, which were directly spotted on swarm plates ($P \rightarrow S$), as well as seeded in 96-well plates and grown for either 4 or 7 generations (see *SI Appendix*, Fig. S2 for the standardization process of the generations and statistics). All the resulting daughters (16 from each mother) were further similarly diluted and spotted separately on swarm plates. (B–G) Bubble-array plots where each bubble represents swarm diameters from SCIs (~1,700 total plates with swarms). The size of the bubble represents the swarm diameter and the color represents the noise within a group of daughters born from the same mother (B and C: G0; D and E: G4; F and G: G7; B, D, and F: *P* data; C, E, and G: *S* data). The noise data of each mother are shown in *SI Appendix*, Fig. S3A. Based on the daughters' ability to swarm in G4 and G7, a K-means clustering of mother cells resulted in three distinct groups; XS—poor, M—moderate, and L—efficient swimmers.

to G7 *P* cells (Fig. 2G and *SI Appendix*, Fig. S3A), but even here the noise was lower than in *P* cells. In summary, the uniform behavior of G0 *S* mothers, as well as the single cluster of their G4 daughters indicates that *S* cells are conditioned, and that this swarm memory is retained at least for 4 generations but lost by the 7th generation. That the different patterns of swarm memory are not due to *S*-cells experiencing an environmental change during dilution in bulk liquid, was ascertained by collecting G0 mothers from hard agar where the bacteria do not swarm, and monitoring their swarming proficiency after similar dilution. These showed similar variability as the *P* mothers, suggesting that the act of swarming is an important determinant of this memory (*SI Appendix*, Fig. S3B).

In conclusion, both *P* and *S* cells have a heritable multigenerational memory of swarming, henceforth referred to simply as

memory. While this memory is heterogeneously distributed in *P* cells, it is homogeneously present in *S* cells.

Cells Have an Iron Memory. What is the nature of this multigenerational memory? Given the significant variation in the swarming proficiency of SCIs, we reasoned that any factor(s) that alters this heterogeneity would provide insights into its nature. Since swarming involves both growth and motility, we first looked at the SCI variation in these two factors. We measured the total pixel intensity of each swarm as the growth variable (V_G), and the maximum swarm diameter as the motility variable (V_M) (*SI Appendix*, Fig. S4A and *SI Methods*). A K-means clustering (*SI Appendix*, Fig. S4B) of the V_G – V_M data from *P* mothers at G0 resulted in four distinct groups—poor

growth and motility (I), moderate growth and motility (II), moderate growth and good motility (III), and good growth and moderate motility (IV). The actual plate images from these clusters revealed that the clustering was real and could be visually correlated (SI Appendix, Fig. S4C). Cohorts that showed less variation (G4 P, G0 S, 100-cell, 10,000-cell) exhibited only two clusters (SI Appendix, Fig. S4B) but could not be visually correlated (SI Appendix, Fig. S4C), suggesting that only a single cluster was present in these samples. Interestingly, the four real clusters of G0 P cells that disappeared at G4, reappeared in G7 (SI Appendix, Fig. S4C, 1P-G7), consistent with memory retention for 4 generations but not for 7. Together, the clustering analysis confirmed that V_G and V_M are both important for swarming heterogeneity.

To explore environmental or genetic factors that might reduce the swarming heterogeneity of the P mother cells (measured as swarm diameters), we perturbed several factors prior to inoculation of these G0 cells on swarm plates. The environment was perturbed by changing several parameters such as media, temperature, pH, etc. (Fig. 3A, Environmental; see SI Appendix, Fig. S5A for noise data), but no apparent decrease in swarming heterogeneity was observed. These observations were further quantified by performing a principal component analysis (PCA, SI Appendix, SI Methods). Since the mean and the noise are major descriptors of sample data, these values were taken as variables for PCA (48). None of the environmental perturbations changed principal components significantly (Fig. 3B and SI Appendix, Fig. S5B). Genetic factors were assessed by introducing plasmids carrying representative genes that had been identified as contributing to *E. coli* swarm physiology (38, 44, 49, 50). These genes encoded for porins (*ompA*, *ompF*), iron regulation (*fepA*, *fur*), efflux (*evgA*, *marA*), redox (*katG*, *sodB*), and mechanosensing (*mscL*, *mscS*). Of these, perturbation of iron homeostasis significantly reduced noise and clustered the data very differently in PCA when compared to LB control (Fig. 3B, *fepA* and *fur*; Fig. 3B and SI Appendix, Fig. S5B). Iron starvation is known to be crucial signal for swarming (37, 44). *FepA* is an outer membrane transporter for ferric enterobactin, while *Fur* represses iron uptake genes (51). Compared to WT, $\Delta fepA$ and Δfur strains showed no significant differences in growth or swimming motility as assayed in soft agar (0.3% w/v) (SI Appendix, Fig. S6 A and B), yet had different swarming phenotypes: The $\Delta fepA$ strain was a poor swarmer, whereas Δfur did not swarm (SI Appendix, Fig. S6 C and D). The importance of iron in maintaining swarming heterogeneity was confirmed by growing P mothers in iron-rich ($FeCl_3$) or iron-starved conditions (deferrioxamine mesylate or DFO, an iron chelator) before conducting SCI assays (Fig. 3C). These treatments severely reduced the heterogeneity, creating defined peaks (Fig. 3C). A shift to the right on the graph indicates better swarming (+DFO), while that to the left indicates the opposite (+ $FeCl_3$), validating studies that iron starvation is a swarming signal (37, 50). Interestingly, neither DFO nor $FeCl_3$ had any effect on $\Delta fepA$ mothers (Fig. 3C), consistent with the fact that iron uptake is impaired in this mutant.

To investigate the effect of iron starvation on memory, we followed memory inheritance for G4, G7, and G12 daughters in WT P cells treated with DFO. Memory was now largely maintained till G7 (Fig. 3D) but was lost by G12, showing that experimentally induced iron starvation extends memory of high swarming potential beyond G4. On the other hand, increasing the intracellular iron through overexpression of *fepA* (expected to import iron) also extends memory of low swarming potential till G12, the last generation we tested (Fig. 3E).

In summary, intracellular levels of iron are an agent of memory that can be either environmentally or genetically controlled.

Swarming Iron Memory Tracks from Mother to Daughters. Most methods for measuring intracellular iron require complex endpoint chemistry (52) and those that measure iron in live cells can impact cellular physiologies (53). To avoid such experimentally induced perturbations while quantifying iron in our setup, we designed a fluorescent reporter-based iron biosensor (SI Appendix, Fig. S7). The reporter is sfGFP, transcribed from the Fur-regulated *E. coli fepA* promoter. *Fur* is the global repressor of iron uptake (35) (Fig. 3F). The *fepA* promoter was used to drive sfGFP because this gene has a strong *Fur* binding site (see Table I in ref. 54), is crucial for swarming (55), and is highly expressed during swarming (44). When intracellular iron levels are high, *Fur* stops further iron intake by complexing with iron and binding to a “Fur box” adjacent to promoters of hundreds of iron homeostasis genes (56) to repress transcription (Fig. 3F). When iron levels drop below a threshold, the system is depressed and genes involved in siderophore production, secretion (efflux pumps), and uptake (siderophore receptors) are expressed (Fig. 3F). Thus, high intracellular iron should cause strong suppression by *Fur*, yielding a low fluorescence in our setup and vice versa. The sfGFP fluorophore was expected to yield a signal strong enough (57) to be detected even under *Fur* repression. A control plasmid expressing sfGFP from a P_{trc} promoter in WT cells gave a homogeneous background signal (Fig. 4 A and B, Top), whereas sfGFP driven by the *fepA* promoter (P_{fepA}) produced a heterogeneous signal, reflecting heterogeneity of iron levels (Fig. 4 A and B, Middle). The ability of the biosensor to report on cellular iron levels was further tested by subjecting WT/ P_{fepA} sfGFP cells to $FeCl_3$ and DFO treatment. Not only did fluorescence decrease and increase, respectively, the heterogeneity in signals also reduced, as expected (SI Appendix, Fig. S6 E and F; heterogeneity due to different plasmid copy numbers is controlled for by the IPTG-induced, continuously “ON” sfGFP plasmid). When P_{fepA} sfGFP was introduced into the $\Delta fepA$ strain, which cannot import iron, the fluorescence signal became homogeneous, as expected if all cells had low iron (Fig. 4 A and B, Bottom; SI Appendix, Fig. S7B). Fluorescence-Activated Cell Sorting (FACS) analysis confirmed results from microscopy (Fig. 4C and SI Appendix, Fig. S8): Only WT/ P_{fepA} sfGFP showed heterogeneity (Fig. 4 C, Middle, green singlets). Having validated this construct as an iron biosensor, we used it next to validate iron memory in swarming, as well as other phenotypes expected to be impacted by iron levels (Fig. 4D).

FACS was used to first sort P cells harboring all the biosensor-harboring constructs through a predefined sort gate that included a range of signals covering low to high iron levels (SI Appendix, Figs. S8 and S9). Single cells covering this range were then dropped on swarm plates to measure swarming proficiency (Fig. 4D). Although cells with the control P_{trc} plasmid showed heterogeneity in swarming (Fig. 4 E, Left, follow x axis), no correlation between the sfGFP signal and swarming diameter was observed. As expected, however, a positive correlation between these two measurements was observed in WT/ P_{fepA} sfGFP G0 cells, i.e., cells with high sfGFP (low iron) were better swimmers (Fig. 4 E, Middle). In $\Delta fepA$ / P_{fepA} sfGFP strain, where iron import is impaired, we observed a clustering of swarm diameters (Fig. 4 E, Right, follow x axis), consistent with the reduction of heterogeneity (see Fig. 3B, $\Delta fepA$). Next, for each of the strains shown in Fig. 4E, we performed a multigeneration analysis with randomly sorted G0 cells (see SI Appendix, Fig. S9 for sort results). Five such random P mothers from each strain were propagated until G4 or G7 (Fig. 4 F and G). Unlike in Fig. 2A, where the physiological state of the seeded mothers was unknown, this time we had knowledge

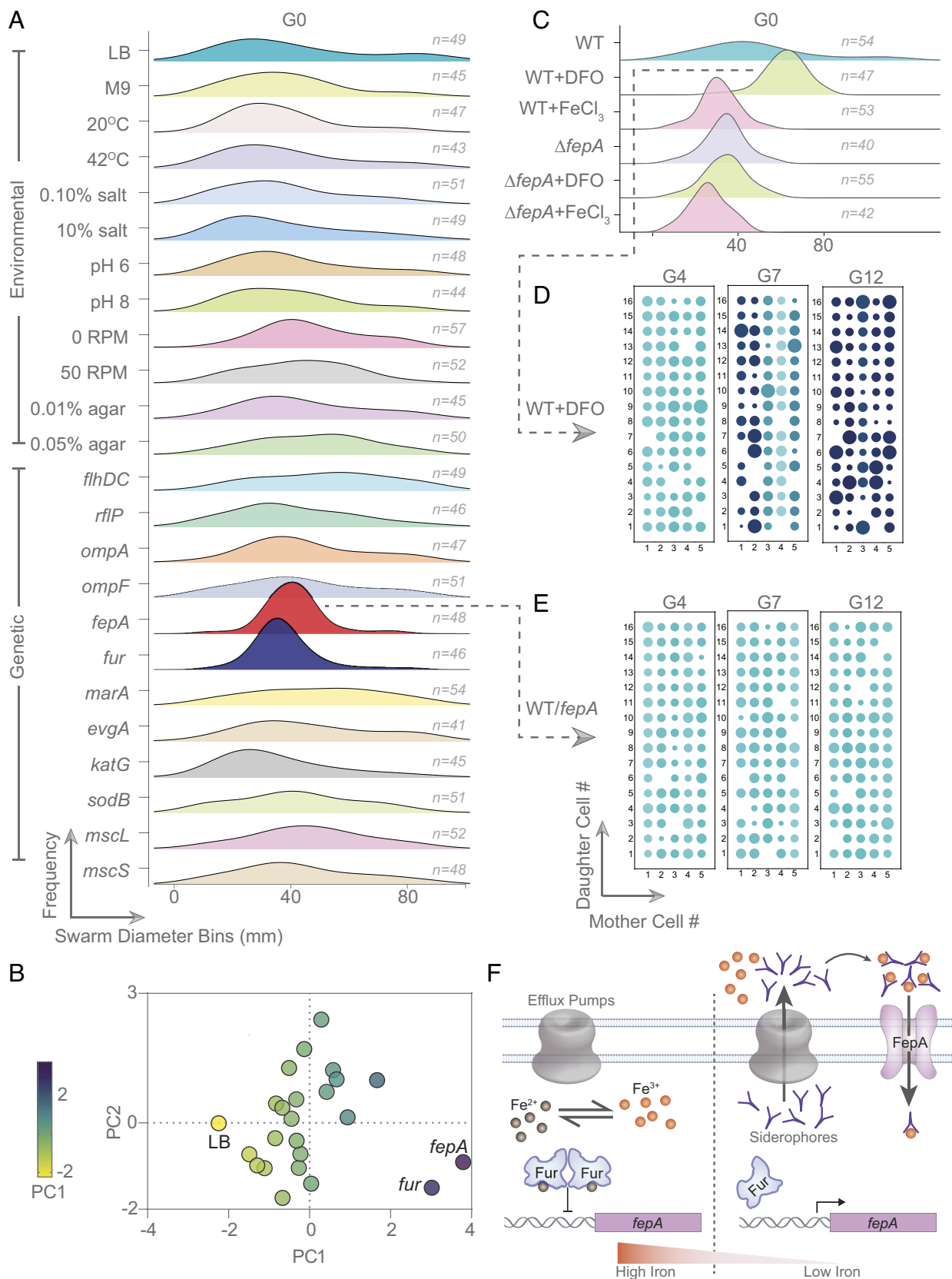


Fig. 3. Cells have an iron memory. (A) Ridgeline plots showing frequency distribution (0 to 1 in each plot) of swarm diameters of G0 P-cell SCIs (~2,400 total), where prior to plating, P mothers were either subjected to indicated growth environments (in liquid), or transformed with inducible plasmids expressing genes known to influence swarming. See *SI Appendix, Fig. S5A* for the noise data. (B) Principal Component Analysis of data from A. The mean, SD, and noise were considered as variables of each condition. Eigenvalues of PC1 (60.06%) and PC2 (39.87%) are expressed on the x- and y-axis, respectively. Each dot represents a condition, with relevant ones labeled. See *SI Appendix, Fig. S5B* for the biplot. (C) Ridgeline plots specific to WT or $\Delta fepA$ P cells experiencing iron-poor (DFO) or iron-rich (FeCl₃) conditions. (D and E) Bubble-array plot of G4, G7, and G12 daughter P cell swarm assays (as in Fig. 2) of WT strains that were either starved for iron (DFO) or had abundant iron. (F) Schematic of Iron homeostasis as controlled by Fur, the global negative regulator of intracellular iron. See text for details.

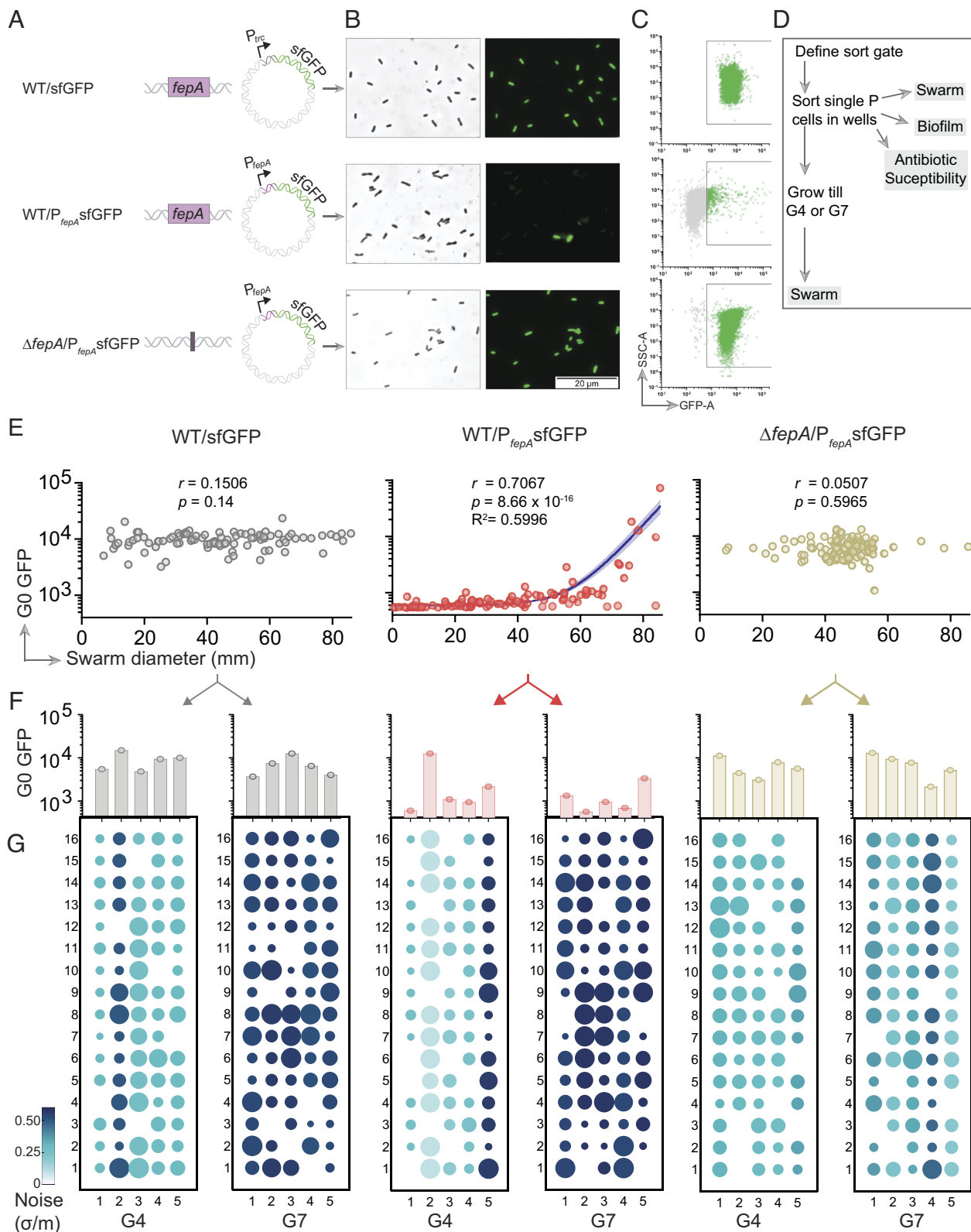


Fig. 4. Swarming iron memory tracks from mother to daughters. (A) WT or $\Delta fepA$ cells harboring P_{trc} -sfGFP or P_{fepA} -sfGFP plasmids. The P_{trc} promoter was induced by 1 mM IPTG. See *SI Appendix, Fig. S7A* for cloning details. (B) Brightfield (Left) and fluorescence (Right) microscopic images of strains depicted in A. (C) Results of FACS analysis of strains indicated in A. Singlets were gated based on SSC-A (side scatter channel—area) and GFP intensity. A sort gate was defined to collect singlets where the background fluorescence of WT cells without GFP was defined as the minimum threshold (See *SI Appendix, Fig. S8* and *Methods* for details). Gray, cells below threshold. Green, cells within the defined gate. (D) Flow chart of how sorted cells were used. (E) Correlation of single-cell GFP intensities (from FACS, $n = 96$) with swarming of G0 mother cells collected from the different strains indicated. r , Spearman correlation coefficient, P value as indicated. The blue line represents the exponential regression line with indicated R^2 values. The blue error band represents 95% CI. (F) Fluorescence intensities of sorted G0 mothers that were dropped into one well of a 96-well plate, indicated as bars. (G) The G4 or G7 daughters of the mothers with known GFP intensities (indicated as bars in F) were assayed for their swarming ability and the results are shown as bubble-matrix plots. See *SI Appendix, Fig. S9* for the FACS data of each experiment.

of their iron levels based on their fluorescence levels, shown as bars (Fig. 4*F*, *y* axis – log scales; high bars = low iron). The swarming proficiencies of each sibling set are plotted as before (follow the columns below each bar for daughter cell data). The swarm diameters of daughters correlated closely with iron levels of the mother (see *SI Appendix*, Fig. S7*C* for correlation data). For example, in the G4 cohort of WT/ P_{*fepA*}sfGFP mothers (Fig. 4*G*, third panel from the left), mother #1 (first column, short bar = high iron) birthed daughters that were all poor swimmers, while mother #2 (second column, higher bar = low iron) birthed daughters that were efficient swimmers. The G7 data from these mothers mirrored previous observations that iron memory is lost by G7 (Fig. 4*G*, fourth panel). In contrast, no such correlation between iron and swarm diameters was observed in the other two strains shown in Fig. 4*E*, *Left* and *Right*. In both strains, memory was maintained till G4 (Fig. 4*G*, first and fifth panels). As expected, the Δ *fepA* strain exhibited high sfGFP signals due to their low intracellular iron (Fig. 4*E*, right, follow *y* axis) when compared to WT (Fig. 4*E*, *Middle*). Interestingly, memory of low iron in this Δ *fepA* strain was maintained at least till G7, which is an improvement upon the G4 memory of WT cells (Fig. 4*G*, compare second with sixth panel). This observation, along with the data in Fig. 3*E*, revealed that both decreases or increases in iron levels can extend the span of memory beyond WT levels, although the phenotypes in both cases are at the opposite ends of the spectrum.

In summary, we constructed and validated an iron biosensor that can be used to quantify the iron memory and sort cells based on the quantification. Results from this biosensor showed a strong correlation of iron memory with swarming, which is propagated from mothers to daughters till G4 in WT cells.

A Mathematical Model that Explains the Propagation and Switching of Iron Memory States. To gain insight into the dynamic switching among three cell states—XS, M, and L—over multiple generations (Fig. 2), we used an ordinary differential equation model as our mathematical framework (*Methods*). The model assumes different rates of switching between the states (Fig. 5*A*). Starting from any state, cells could stochastically switch to other states over time, ultimately recreating the original phenotypic heterogeneity (Fig. 5*B–D*). In order to satisfy the curious observation that the S data had lower noise, i.e., S daughters took longer to switch compared to P daughters (compare Fig. 2*D* and *E*), a time delay component had to be incorporated. Model parameters such as the delay duration and kinetic rates were inferred by fitting the model to data at G4 (Fig. 2*D* and *E*), and these rates were further constrained so as to recreate the intercellular variation seen in single G0 mothers by G7 (*SI Appendix*, *SI Methods*). Our modeling results reveal that in the case of high to moderate iron states (XS and M), all three states started to appear during G3 to G4 transition (Fig. 5*B* and *C*). In contrast, the low iron state cells (L) initiated this process at G4 to G5 transition suggesting that conditioning (prior swarming experience) can enforce iron memory further than stochastic switching (Fig. 5*D*). When comparing fractions of different cell states, our model (solid lines, *B–D*) could capture the real data from Fig. 2 (dashed lines, *B–D*; see *SI Appendix*, Fig. S10), efficiently. In other words, no matter the starting cell state, the emerging population eventually converges to the original ratio of cell states. Using the model data, a cell lineage dendrogram was generated, which visually illustrates how these cell states can reappear (Fig. 5*E–G* and *SI Appendix*, *SI Methods*).

In summary, our mathematical model shows that specific rates of dissipation and acquisition of memory can be correlated with

the experimentally obtained fractions of cells in different iron states—thus linking the model with the experimental data.

Discussion

This study has found a new role for iron in bacterial behavior by demonstrating the existence of physiological “iron” memory in *E. coli* that persists over multiple generations. To our knowledge, this kind of memory has not been unearthed before, but there is no reason to think that it is limited to the iron pathway monitored in this study.

Iron Memory in Swarming. The original observation that led to the present study was a particular colony phenotype present uniformly in swarm (S) cells plated on hard agar, but present nonuniformly in planktonic (P) cells (Fig. 1*A–C*), suggesting that some attribute of S cells was prepresent in P cells. The latter deduction was confirmed by assaying the swarming potential of a large number of single P cells (Fig. 1*F–N*). To test whether the heterogeneity in swarming potential of these cells was random or inherited, the offspring of G0 P cells tracked over several generations, were found to inherit their starting swarming potential until G4 but not up to G7 (Fig. 2*B–D–F*). The swarming proficiency of S mothers was homogeneous, and also inherited until G4 and lost by G7 (Fig. 2*C–E–G*). Thus, both P and S cells have a heritable multigenerational memory of swarming.

Based on prior knowledge of gene regulation during swarming, we tested various candidates that might contribute to swarming memory (Fig. 3*A*, Genetic). Of these, only the over-expression of critical players in the iron acquisition pathway—*fepA* and *fur*—reduced the noise in G0 mothers significantly. Efflux pumps also play a role in this pathway because they are responsible for siderophore secretion (58). However, these pumps were not observed to contribute to memory (Fig. 3*A*, *marA* and *evgA*), ruling out the possibility that the heterogeneity of swarming is caused by the known heterogeneity in efflux pumps (59). The role of intracellular iron in encoding swarming memory was ascertained using an iron biosensor (Fig. 4). Swarming proficiency tracked with intracellular iron levels, i.e., cells with low iron were better swimmers and vice versa. Memory retention could be manipulated by decreasing or increasing iron levels (Fig. 3*D* and *E*), establishing a strong correlation of iron memory with swarming.

Mechanistic Insights into the Switching of Iron Memory States.

What explains the mixed nature of swarm phenotypes and their heritability (XS, M, and L in Fig. 2)? The short duration of this heritability excludes epigenetic mechanisms (60, 61) or bistable (62) and toggle switches (63). Although epigenetics might play a role in *fur* regulon (64), stochastic fluctuation (65, 66) could explain the observed fluctuations within the population (Fig. 5*A–D*). Stochasticity may arise due to intrinsic or extrinsic noise in gene expression (67, 68). The stochastic fluctuations in transcription and translation of a given gene constitute intrinsic noise (e.g., Fig. 3*A*, genetic), whereas the effect of other cellular components on the expression of that gene constitute extrinsic noise (e.g., iron manipulation in Fig. 3*C*). That iron memory could be prolonged beyond G4 by tweaking intrinsic or extrinsic components [possibly due to an increase in signal-to-noise ratio (69)] and can be interpreted as a form of conditioning (70–72). Additionally, a continuous loss of the Fe–S proteins due to noisy dilution of these proteins during cell division (73), and damage during aerobic respiration (74, 75), may further contribute to phenotype switching.

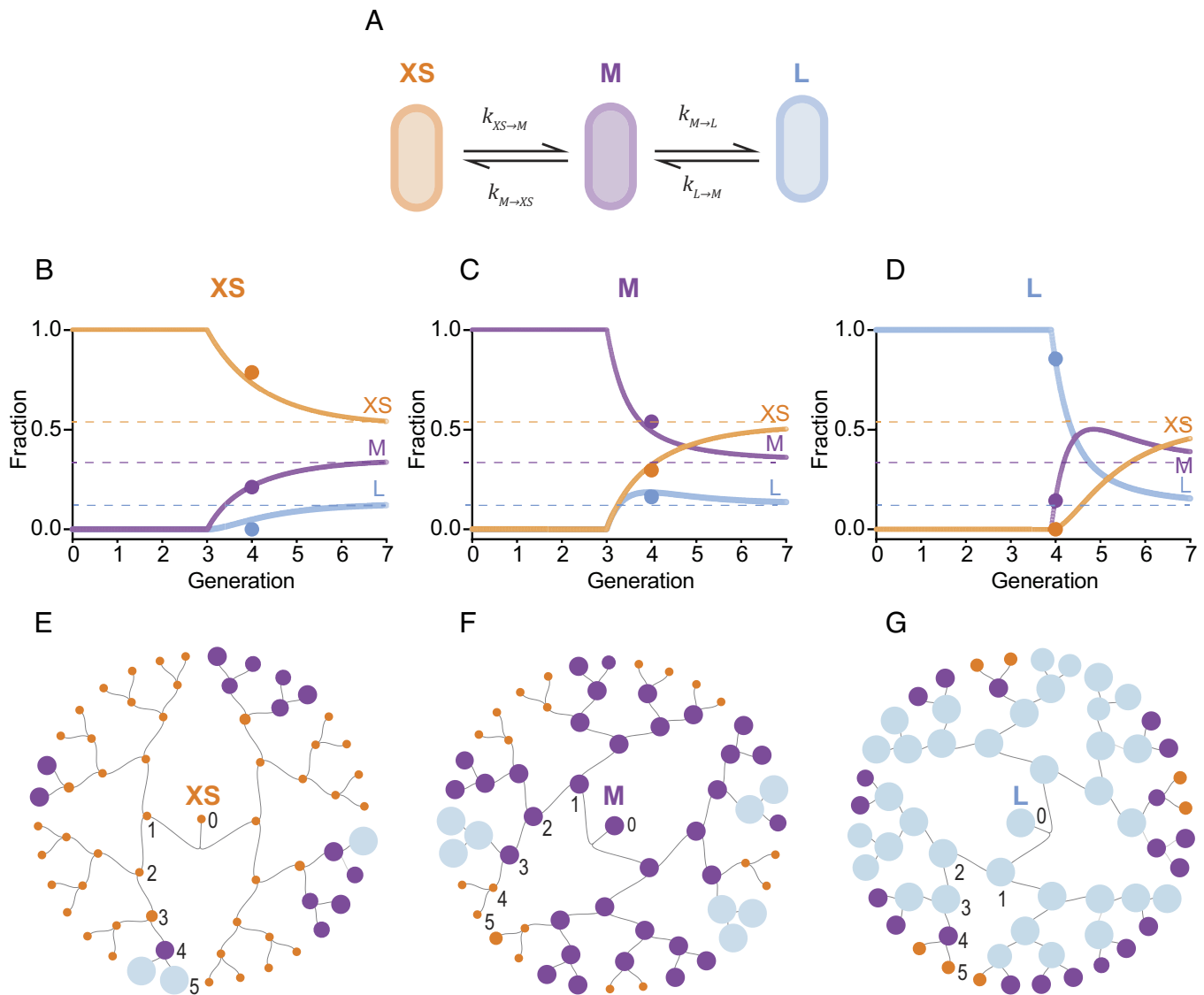


Fig. 5. A mathematical model that explains the propagation and switching of iron memory states. (A) Depiction of the mathematical framework used to model the data in Fig. 2. Briefly, XS, M, and L cells with poor, moderate, and efficient swarming capabilities, respectively, can switch to other states by the given rates with a time delay. See text and *Methods* for details. (B–D) Results from the time delay model showing how all three swarming states can appear starting from any cell type (as indicated on *Top*). The XS and M cells can maintain the memory till G3 and then start to switch to other cell types, whereas L cells take until G4 before switching. The dashed lines show the fractions of cell types obtained from G0 mother data in Fig. 2B (also see *SI Appendix, Fig. S10*). Irrespective of the initial cell type, the resulting population converges to the original ratio of the cell types. (E–G) A cell lineage illustrating the memory model. Starting from a specific cell type, the fractions of all three cell types in each of the G1 to G5 generations (1 to 5) were obtained from the model in (B–D) to get the approximate number of each cell type. That information was used to create the circular dendrograms.

A mathematical model was formulated to explain iron memory measured as swarming efficiency (not iron levels) of single cells over multiple generations (Fig. 2). The model consists of individual cells being in three states (XS, M, L) that dynamically switch amongst each other with a time-dependent rate (Fig. 5A). It is important to note that while we classify cells in three states, in reality there could be a continuum of swarming states that could be mapped into a continuum of iron level states. In such cell-state switching models, the underlying mechanism of switching may be a result of a gene product crossing a critical threshold level. Based on our data, we hypothesize that fluctuations in intracellular iron concentrations—that are by themselves driven by a myriad of factors such as stochastic expression of enzymes, transporters, and other regulators—could cause cells to switch between states based on iron levels crossing different thresholds. We believe that this can connect switching at a cell-state level to intracellular

molecular thresholds. More work is warranted to mechanistically probe these results using quantitative and dynamical intracellular iron measurements, combined with modeling of underlying networks.

Our model allowed us to discern a curious aspect of the molecular dynamics underlying this phenotypic memory. Transitions among cell states are often captured by constant rates that correspond to memoryless switching, i.e., the probability of a switch is independent of the time spent in a specific state. A key insight from our model fitting is that such memoryless switching cannot capture our experimental data, and incorporation of an explicit time delay (rather than a first-order delay) was necessary to reproduce the experimentally obtained fractions of different cell states (Fig. 5 B–D). Time delay often arises from operation of a feedback (76), or a slow incorporation of the input signal (77, 78), or a slow response time (77, 79). The differences in the delays specific

to different states (for example, it took 3 generations for XS/M cells to switch vs. 4 generations for L cells; Fig. 5 *B–D*) can be due to differences in feedback time or to the requirement of a series of rate-limiting steps involved in switching. The quantification of these delays and switching kinetics can be further validated in future and refined with additional experimental timepoints. This will also help in mapping the thresholds in mechanistic models of biochemical processes regulating iron levels.

Iron homeostasis is a complex, multistep, process. The flux of iron is controlled by the global regulator Fur, which controls the expression of dedicated iron transport membrane proteins, channels, and receptors (80). Ferrous iron can freely diffuse through the outer membrane porins to enter periplasm and enter the cytoplasm via various transport systems such as Feo. The transport of ferric iron is mediated by siderophores and their receptors such as FepA. Cytoplasmic iron can be stored in proteins such as bacterioferritins. When cells gather enough iron, the negative feedback loop of *fur* regulon is activated which shuts down the expression of iron uptake system (35). Although the *fur* regulon operates on a negative feedback loop (Fig. 3*F*) (81), the expression of *fur* itself is under positive regulation by the upstream regulators Crp (82), OxyR (83), and SoxS (84). Every step of this complex genetic circuit could have different rates of activity and feedback times depending on the cell states. For example, XS G0 mothers and their daughters would be in a Fur-repressed state till G3–4 when their iron pool goes below the threshold, so they switch to the Fur-derepressed M state. This would initiate the synthesis of iron import proteins. M cells could stay derepressed for another 3 to 4 generations, where they accumulate enough iron to switch to the L state. This timescale is consistent with previous calculations for *fur* regulon (81). In contrast, the switch of L back to M would require active degradation of the newly synthesized iron import proteins and mRNAs, which could take longer.

Iron Memory in Biofilm Formation and Antibiotic Survival.

Besides swarming, iron is also crucial for several other physiologies (85). We tested a few such phenotypes for their correlations with intracellular iron levels (*SI Appendix*, Figs. S11 *A–D* and S9). Recent studies have shown that iron is crucial for the decision to enter the biofilm state (86, 87), a contrasting sedentary lifestyle choice when compared to swarming (34). To test whether iron memory is a predictor of biofilm formation, we measured this ability in G0 P-cell SCIs using a standard crystal violet staining assay (88) (*SI Appendix*, Fig. S11*A*). As expected, we observed no correlation between iron and biofilm formation in the control WT/ P_{urc} -sfGFP strain (*SI Appendix*, Fig. S11 *A*, *Left*; see *SI Appendix*, Fig. S11*D* for correlation data) but a strong anticorrelation between low iron and biofilms in WT/ P_{fepA} sfGFP cells (*SI Appendix*, Fig. S11 *A*, *Middle*; Fig. 5*D*), i.e., more iron meant more biofilms. This anticorrelation was absent in strains lacking FepA (*SI Appendix*, Fig. S11 *A*, *Right*; *SI Appendix*, Fig. S11*D*). Iron is critical for redox homeostasis (35, 89), which is upended by antibiotics (90, 91). To test whether iron levels can be a predictor for antibiotic survival, sorted cells of the same three strains shown in *SI Appendix*, Fig. S11*A* were treated with $\sim 1/2$ MIC of the antibiotics Kanamycin (Kan) and Chloramphenicol (Cam) for 4 h. A positive correlation was observed between low iron and increased survival with both antibiotics (*SI Appendix*, Figs. S11 *B–D* and S9). This correlation was absent in $\Delta\text{fepA}/P_{\text{fepA}}$ -sfGFP or P_{urc} -sfGFP strains.

Although we have not followed these two physiologies for multiple generations as we did with swarming, the correlation between iron levels and the potential for establishing biofilms or for resisting antibiotics is not only highly significant but can be superimposed

on similar data for swarming (Fig. 4*E*), suggesting that iron memory is not restricted to swarming decisions alone.

Evolutionary Significance of Using Iron as Memory. Iron metabolism is critical for life (92) and for its evolution (93, 94). Changes in its metabolism affect mutation rates (89), which directly affect natural selection (95). Depending on the ecological niche, iron-limiting and iron-rich conditions can be both beneficial (35, 96) and harmful (97, 98). For example, cellular iron levels influence the rate of antibiotic resistance (89, 99), host–pathogen interactions (100–102), composition of the gut microbiome (19), and various other stresses in both clinical and natural settings (103). Frequent switching between different iron memory states would therefore be a bet-hedging strategy for maximizing survival in varied environments. Iron memory is therefore expected to be widespread in nature and to impact other physiologies. Two such other physiologies—biofilm formation and antibiotic survival—were also examined in this study. Swarming and biofilm formation are two opposite lifestyles. While low iron is a signal for swarming, high iron, a signal for biofilm formation (104), was positively correlated with biofilm development (*SI Appendix*, Fig. S11*A*). Low iron was also positively correlated with increased survival to antibiotics (*SI Appendix*, Fig. S11 *B* and *C*), likely because less cellular damage is expected when iron levels are low. Antibiotics trigger production of ROS (91), and high iron levels would increase ROS, increasing lethality (35). A multigenerational iron memory would improve the survival chances of at least some individuals within the population under antibiotic stress.

Why might iron pools be used for storing memory? Given the central role of iron in cellular metabolism, an iron-based memory offers the advantage of connecting various stress responses. For example, swarm cells are subject to surface stress (31) while at the same time exhibiting adaptation to antibiotic stress (44). We have shown in this study that iron memory is responsive to multiple stimuli: Swarming over a semisolid surface (Fig. 2, *S* data), extracellular iron (Fig. 3 *C–E*), and intracellular iron (Fig. 3*A*, *fepA* and *fur*). We expect this memory to also be jogged by multiple stimuli during biofilm formation (*SI Appendix*, Fig. S11*A*), where the stimuli could be any combination of surface contact, absence of movement, nutrient limitation, etc. (105). In the case of antibiotic challenge (*SI Appendix*, Fig. S11 *B* and *C*), the stimulus would be intracellular iron because increased ROS production is mitigated by ROS scavenger enzymes that use iron as a cofactor (106). It may be safe to say that iron memory impacts all iron-controlled behaviors.

Methods

The majority of the *Methods* are found in *SI Appendix*.

Dilution Method. The overall dilution-to-extinction method (46) used in this study is summarized in *SI Appendix*, Fig. S1*A*. Instead of FACS-based sorting, dilution was chosen as the primary method of single-cell isolation because of its low cost and the relative ease of inoculating a large number of swarm plates immediately after final dilution. First, the OD_{600} of the starting cell suspension—either P or S—was adjusted to 0.5. Then, 100 μL of the cell suspension was serially diluted by adding 900 μL of LB medium in each step. Any recalibration or change in pipettes (or change in personnel) was accompanied by redoing the standardization steps. The accuracy of dilution was always confirmed by CFU counts. Ascertaining that the fraction of wells with growth in a 96-well plate followed a Poisson distribution was an additional confirmation (*SI Appendix*, Fig. S1*B*).

SCI Swarm Assays. SCI swarm plates were prepared with 0.45% Eiken agar (Eiken Chem. Co. Japan) and 0.5% glucose. Swarm plates were dried for 8 h at room temperature before use. Then, 4 μL of the cell suspension was inoculated at

the center of each plate and incubated at 30 °C for 30 h. The maximum diameter of each swarm was physically measured with a ruler. For those swarms with low radial symmetry, i.e., with no apparent max diameter upon visual inspection, three measurements with a ruler were made at different degrees of rotation to the center of the plate, and the highest value chosen as the max diameter was recorded to be the diameter.

“Generation” Swarm Assay. The ideal time points for daughter-cell isolation from a specific generation were first standardized as summarized in *SI Appendix, Fig. S2 A–C*. A suspension of 0.5 cell/ 4 µL was prepared as described in the “Dilution method” section and used to inoculate 96-well plates prefilled with 196 µL of LB medium. The plates were covered with lids, taped on the sides with parafilm, and then incubated at 37 °C with shaking at 200 RPM. The number of cells per well was estimated by CFU counts at different times (*SI Appendix, Fig. S2A*). Expectedly, some wells did not receive any cells (*SI Appendix, Fig. S2B*). Once

the timepoints for specific generations were identified, the 96-well plates were inoculated and incubated as before, except, instead of CFU counts, the cells from G0, G4, and G7 were spotted on swarm plates (0.5 cell per plate) as summarized in *SI Appendix, Fig. S2 D–F*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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