

MICROBIOLOGY

Stochastic transcriptional pulses orchestrate flagellar biosynthesis in *Escherichia coli*J. Mark Kim¹, Mayra Garcia-Alcala^{1,2}, Enrique Balleza^{1*}, Philippe Cluzel^{1†}

The classic picture of flagellum biosynthesis in *Escherichia coli*, inferred from population measurements, depicts a deterministic program where promoters are sequentially up-regulated and are maintained steadily active throughout exponential growth. However, complex regulatory dynamics at the single-cell level can be masked by bulk measurements. Here, we discover that in individual *E. coli* cells, flagellar promoters are stochastically activated in pulses. These pulses are coordinated within specific classes of promoters and comprise “on” and “off” states, each of which can span multiple generations. We demonstrate that in this pulsing program, the regulatory logic of flagellar assembly dictates which promoters skip pulses. Surprisingly, pulses do not require specific transcriptional or translational regulation of the flagellar master regulator, FlhDC, but instead appears to be essentially governed by an autonomous posttranslational circuit. Our results suggest that even topologically simple transcriptional networks can generate unexpectedly rich temporal dynamics and phenotypic heterogeneities.

INTRODUCTION

Transcriptional cascades form the basis for many complex genetic programs that underlie cell fate such as division and differentiation (1–3). The genetic network that governs flagellar synthesis in *Escherichia coli* (4) (Fig. 1A), a classic archetype of a transcriptional cascade, plays a crucial role in adapting to different lifestyles of the bacterium such as locomotion, biofilm formation, surface adhesion, and host invasion (5–8). In this network, transcription of the genes *flhDC* (class I genes) by a single promoter dictates the synthesis of the master regulator FlhD₄C₂ (henceforth abbreviated as FlhDC) that, in turn, activates the transcription of genes involved in the synthesis of the flagellar hook and basal body (class II genes) (4, 9). The alternate sigma factor, FliA, is expressed along with the other class II genes and mediates the expression of class III genes, which encode the flagellar filament and chemotactic signaling system (4, 9).

Time-course measurements of flagellar genes in bulk culture suggest that, when flagellar production is induced, the cascade switches on in a deterministic temporal sequence and that the promoters, once activated, remain continuously active over exponential growth (10, 11). However, such population measurements can mask biologically important hidden phenotypes. Even under homogeneous conditions, individual bacteria are capable of executing markedly distinct gene expression programs from the rest of the population (12–17). For example, flagellar gene expression in the closely related *Salmonella* appears to occur in only a subpopulation of cells (18, 19). On the other hand, there are notable differences in the proteins that regulate flagellar expression in *Salmonella* and *E. coli* (20, 21), including the master regulator FlhDC itself (22), which suggests that *E. coli* could have its own unique pattern of flagellar gene regulation. Tracking the temporal dynamics of the flagellar cascade in individual cells would not only uncover such patterns but also directly address whether

single cells activate flagellar promoters in a deterministic program as suggested by bulk measurements. In addition, it would distinguish whether any heterogeneity in flagellar gene expression within a population emerges due to a subset of cells that stably express flagella (akin to differentiation) or from each cell in the population dynamically sampling different phenotypes over time.

Prompted by these questions, we tracked flagellar promoter activity in individual *E. coli* cells across multiple cell divisions. Despite continuous transcription of the master regulator, downstream flagellar genes stochastically switched between periods of inactivity and vigorous transcription. We determined how the regulatory logic of the flagellar cascade operates in this dynamic context by analyzing how the pulses of activation are coordinated within and across the tiers of the cascade. Last, our experiments revealed that post-translational regulation plays an unexpected role in the origin of these pulses.

RESULTS

Monitoring flagellar gene expression in individual cells

We used a microfluidic device (23, 24) that allowed us to monitor promoter activity in individual cells via fluorescence microscopy while maintaining a constant growth environment over many divisions (Fig. 1B and fig. S1). In this device, cells are loaded into channels where one end is closed and the other end is open to microfluidic flow of fresh medium. In this way, cells are maintained indefinitely in exponential phase. Under this condition, we could dissociate the behavior of the flagellar system from its response to uncontrolled fluctuating environments and analyze with high precision temporal variations taking place in the flagellum regulatory network itself.

Operationally, we constructed *E. coli* strains in which we inserted in the chromosome a copy of a flagellar promoter of interest fused to the coding sequence of a yellow fluorescent protein (YFP) variant mVenus NB (see the “Construction of transcriptional reporter strains” section) (25). For *flhDC*, we also made a cis-insertion of YFP at the end of the *flhDC* transcript to monitor the production of the endogenous *flhDC* transcript. Using the microfluidic device, we could monitor YFP expression in the trapped “mother” cells that grow at the bottom of each channel for over 20 generations.

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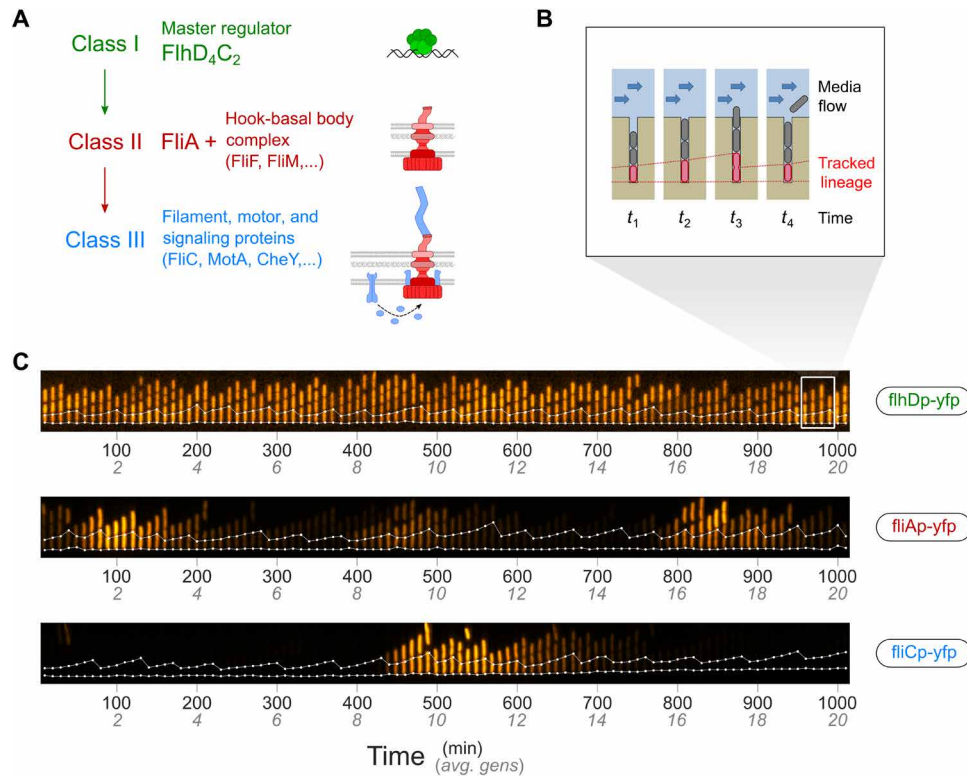


Fig. 1. Stochastic pulsing of flagellar promoters. (A) The flagellar operons are organized in a transcriptional three-tiered cascade and are accordingly labeled class I (green), class II (red), or class III (blue). For simplicity, our diagram omits various branching and feedback regulatory pathways (such as the anti- σ factor FlgM). (B) A microfluidic device (“mother machine”) constrains cells to grow in narrow linear tracks (fig. S1). As the cells grow and divide, they are flushed away at the open end of the tracks by a constant flow of medium, which ensures chemostatic growth. We monitor the cell confined at the bottom (the “mother cell,” red) to build a lineage over multiple cell divisions. (C) Kymographs illustrating the pulsating dynamics of flagellar promoters. Each kymograph shows fluorescence (false color, orange) from a strain harboring a YFP transcriptional fusion to a specific flagellar promoter from either class I (top), II (middle), or III (bottom). Cells were grown in Mops-rich defined medium at 34°C, and fluorescence images from each cell were acquired every 10 min. White dots indicate the location of the mother cell in each frame, which was identified via a constitutively expressed mCherry marker. Cells with the promoter encoding the class I master regulator (top) are continuously fluorescent. By contrast, class II (middle) and class III promoters (bottom) stochastically switch between active and inactive transcriptional states over multiple divisions.

Flagellar genes in *E. coli* are activated in stochastic pulses

First, we tracked the activity of promoters from the three classes separately (Fig. 1C). The promoter of the class I gene *flhDC* behaved like a standard constitutive promoter with a steady expression over time and small random fluctuations about the mean. However, class II promoters (exemplified by *fliA*) showed an unexpected pulsatile pattern of transcription: Cells exhibited long periods of inactivity lasting multiple generations, which were suddenly interrupted by several generations of high promoter activity, before switching back to inactivity. Class III promoters (exemplified by *fliC*) also pulsed, but with inactive periods that were much longer than that observed for class II promoters. We found that single cells grown in liquid culture also displayed highly heterogeneous flagellar gene expression, consistent with the pulsing behavior observed in our microfluidic device (figs. S2 and S3).

Pulses are coordinated within the same class of promoters

To characterize the promoter dynamics more precisely, for each mother cell lineage, we computed the promoter activity from the fluorescence signal time series (fig. S5; see the “Estimation of promoter activity from time-lapse data” section). Although the activity of the class I promoter fluctuated, the relative variability [as measured

by coefficient of variation (CV)] was similar to that of a constitutive promoter ($CV_{\text{class I}} = 1.1$ versus $CV_{\text{constitutive}} = 1.06$) (Fig. 2A and fig. S6). By contrast, class II and III promoters (Fig. 2, B and C) showed much larger variations in promoter activity relative to its mean ($CV_{\text{class II}} = 3.6$, $CV_{\text{class III}} = 4.4$). When we monitored two class II promoters within the same cell [via cyan fluorescent protein (CFP) and YFP], we found that the pair pulsed synchronously (Fig. 2B). Similarly, we found that class III promoters within the same cell also pulsed synchronously (Fig. 2C). This observation could be confirmed by the high correlation between the CFP and YFP reporters in cells corresponding to a pair of class II or a pair of class III promoters (Fig. 2, B and C, bottom, and fig. S7). On the basis of this observation, we subsequently focused on the promoters of *flhDC*, *fliF*, and *fliC* as representative promoters of classes I, II, and III, respectively.

The stochastic fluctuations in *flhDC* transcription were dynamically fast and distinct from the slower pulses in class II and III promoters as indicated by the autocorrelation functions of these three promoters. While the autocorrelation function of *flhDC* promoter activity decayed sharply and resembled that of uncorrelated transcriptional noise, the class II and III pulses showed longer correlation times, consistent with the longer time scale of the pulses (Fig. 2D). To quantify the duration of the pulses, we defined an operational

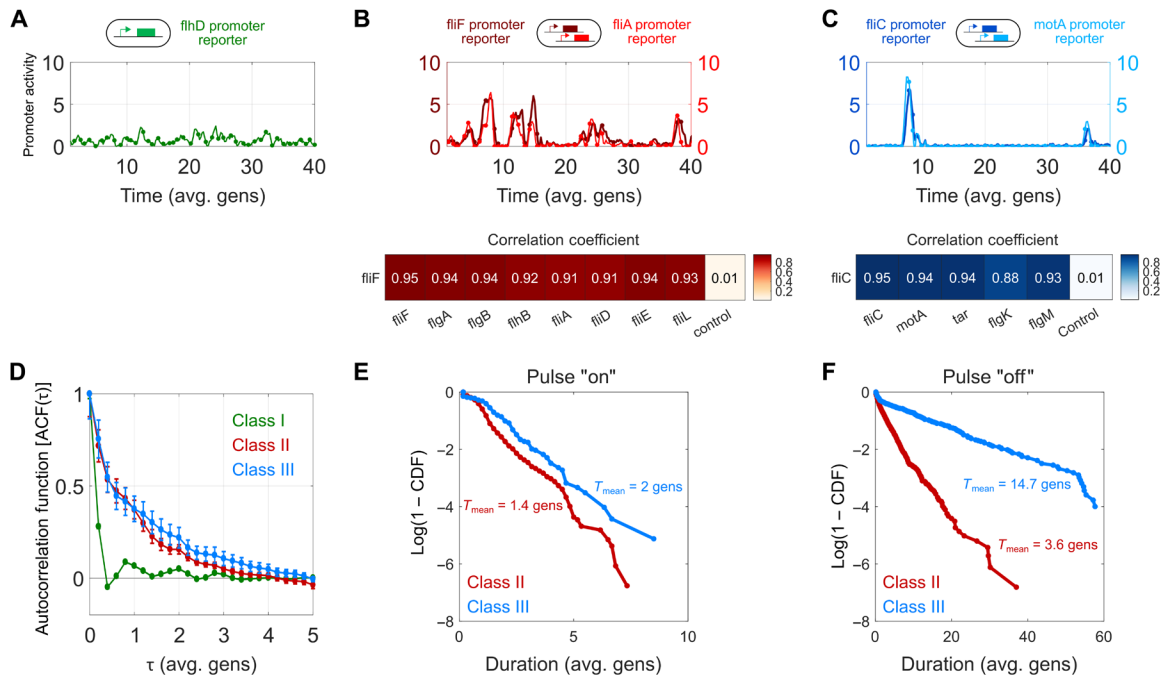


Fig. 2. Promoters across classes show distinct dynamics but pulse simultaneously within their own class (II or III). (A) Typical activity of the sole class I promoter, *flhD*, which controls the expression of the master regulator (green). Promoter activity is quantified by taking the cell growth-corrected time derivative of the associated fluorescence signal (see the “Estimation of promoter activity from time-lapse data” section). (B) (Top) Activity of *flhF* (dark red) and *flhA* (bright red) promoters within the same cell, representative of class II pulsing dynamics. (Bottom) Correlation between two class II gene reporters in the same cell as determined by flow cytometry. Each strain harbors a reference reporter consisting of the *flhF* promoter and CFP and a second class II promoter fused to YFP. The control promoter is a synthetic constitutive promoter. (C) Activity of *flhC* (dark blue) and *motA* (light blue) promoters within the same cell, representative of class III pulsing dynamics. (Bottom) Correlation between two class III gene reporters in the same cell as determined by flow cytometry. Similar to (B), each strain harbors a reference reporter consisting of the *flhC* promoter and CFP and a second class III promoter fused to YFP. The control promoter is again a synthetic constitutive promoter. (D) Normalized autocorrelation function of flagellar promoter activity of classes I (green), II (red), and III (blue), estimated from the activity of *flhD*, *flhA*, and *tar* promoters, respectively (see the “Correlation analysis” section). For each promoter, the autocorrelation was estimated from 50 lineages, each at least 40 generations long. (E) Cumulative distribution of the pulse on durations, plotted as $\log(1 - \text{CDF})$. The distributions for *flhF* (class II, red) and *flhC* (class III, blue) are shown. Durations were estimated from 50 lineages, each at least 60 generations long. (F) Cumulative distribution of the pulse off durations, plotted as $\log(1 - \text{CDF})$. The distributions for *flhF* (class II, red) and *flhC* (class III, blue) are shown. Durations were estimated from 50 lineages, each at least 60 generations long.

threshold based on the detection limit of our reporters and categorized the promoter as being “on” or “off” based on whether the activity was above or below that threshold. The on duration of the pulses was approximately exponentially distributed for both class II and class III pulses, with similar mean durations spanning one to two cell generations (Fig. 2E). The off durations were also approximately exponentially distributed. However, the average off duration of class II promoters was notably shorter (~4 generations) compared to that of class III promoters (~14 generations) (Fig. 2F). We note that these values may slightly underestimate the true on and off durations because we often observed long clusters of pulses interrupted by short periods of inactivity. Overall, our results suggest that despite being coupled in a linear cascade, each class of flagellar promoters has a distinct dynamical behavior.

Pulses do not require regulation of FlhDC transcription or translation by endogenous regulators

How might transcription of *flhDC* give rise to pulsatile behavior of class II promoters? Given that class II (and III) promoters appear to pulse despite relatively steady *flhDC* transcription, we first decided to examine whether any transcriptional or translational regulation of *flhDC* was necessary for class II pulses. Various studies have estab-

lished that transcription of *flhDC* is regulated by multiple pleiotropic transcription factors (26–29). In addition, small RNAs (sRNAs) have been shown to regulate the translation and/or lifetime of the *flhDC* mRNA by binding to the 5′ untranslated region (5′ UTR) (Fig. 3A, left) (30). To bypass this regulatory complexity, we replaced the ~2-kb region directly upstream of the endogenous *flhDC* coding sequence with synthetic sequences encoding constitutive promoters of various strength (“the Pro series”) (31). We also modified the DNA sequence corresponding to the 5′ UTR of the *flhDC* mRNA with a synthetic ribosomal binding site (RBS) sequence derived from the T7 phage. Consequently, in these strains, the regulation of FlhDC expression was isolated from any known transcriptional and translational regulators (Fig. 3A, right). Unexpectedly, when expression of *flhDC* was coupled to the synthetic promoter Pro4, downstream class II promoters exhibited pulses with similar dynamics and amplitudes than those observed in the presence of the native *flhDC* promoter (Fig. 3, B and C). Thus, class II pulses do not require flagellum-specific transcriptional or translational regulation of the master regulator.

We then asked whether posttranslational regulators of FlhDC activity could influence the dynamics of the observed class II pulses. To this end, we turned to the anti-FlhDC factor YdiV (32, 33). Although

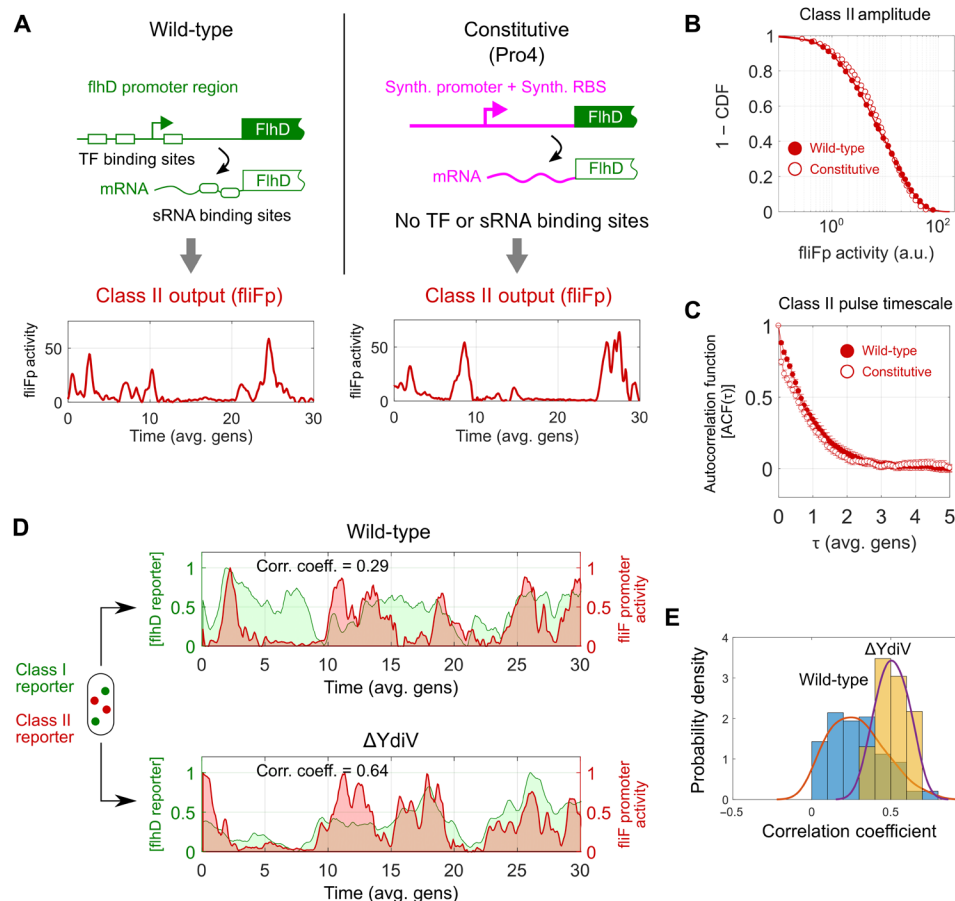


Fig. 3. Class II pulses do not require regulation of FlhDC transcription or translation by endogenous regulators. (A) (Top) Endogenous expression of FlhDC is driven by the class I promoter, which is regulated by multiple transcription factors (TF) (53). In addition, translation of FlhDC is regulated by several sRNAs interacting with the 5'UTR of the *FlhDC* transcript (top left). We replaced the native class I promoter with a synthetic constitutive promoter (Pro4)—in addition, we altered the 5'UTR so that the synthetic RBS, which drives FlhDC translation, is insensitive to sRNA regulation (top right). (Bottom) Typical class II promoter dynamics (*fliF* promoter, red) and wild-type (bottom left) and “constitutive” (bottom right) FlhDC promoters. (B) Cumulative distribution function (plotted as 1 – CDF) of class II promoter activity amplitudes from wild-type (solid red circle) and constitutive strains (open red circle). For each strain, we analyzed 50 lineages, each at least 30 generations long. (C) Normalized autocorrelation function of class II promoter activity, wild type (dark red), and synthetic constitutive (light red). For each strain, we analyzed 50 lineages, each at least 30 generations long. (D) Simultaneous measurements of fluorescence signal from the class I reporter (green) and activity of the class II promoter *fliFp* (red) within the same cell. The fluorescence signal from the class I reporter is a proxy for the concentration of proteins produced from the FlhDC promoter, while the class II promoter activity is the time derivative of the fluorescence signal from the class II reporter, which is a proxy for transcription from that promoter. Typical examples of wild-type (upper) and $\Delta YdiV$ cells (lower), along with the Pearson correlation coefficient for the class I and class II signals. For ease of visualization, each signal is normalized so that the minimum value of the signal is 0 and the maximum value of the signal is 1. Pearson correlation coefficient was computed on the raw data before normalization. (E) Normalized histogram showing distribution of correlation coefficients from 100 lineages for wild-type (blue) and $\Delta YdiV$ (yellow) strains. Solid lines, kernel density approximations of those distributions (red and purple, respectively). Each lineage is at least 30 generations long. a.u., arbitrary units.

the regulatory role of YdiV was well characterized in *Salmonella* (33), both the expression and regulatory significance of the *E. coli* homolog had previously been questioned (see the “Impact of class II and class III genes on class II pulses” section in the Supplementary Materials) (32, 34). To test whether YdiV plays a role in class II pulses, we first generated a strain harboring a cis-insertion of YFP at the 3'-end of the *flhDC* transcript and a trans-copy of the *fliF* promoter fused to CFP in another chromosomal locus. In wild-type cells, we observed that the fluctuations in YFP fluorescence levels, a proxy for the FlhDC concentration, occasionally coincided with class II activity. However, this correlation was very weak ($R = \sim 0.3$) (Fig. 3, D, top, and E). Upon deletion of YdiV, we found that class II promoter activity became considerably more correlated ($R \sim 0.6$) with YFP fluores-

cence (Fig. 3D, bottom, and E). Together, our results suggest that transcriptional “noise”—rather than deterministic transcriptional or translational regulation—of *flhDC*, coupled with posttranslational regulation of FlhDC by YdiV, generates the stochastic pulses of class II promoter activity. We found that flagellar genes such as *FliZ* and *FliT*, which have been implicated in posttranslational regulation of FlhDC in *Salmonella*, appeared to have a negligible effect on flagellar pulses in *E. coli* (see the “Impact of class II and class III genes on class II pulses” section in the Supplementary Materials). In addition, complementing $\Delta ydiV$ strains with a plasmid that steadily expressed YdiV from a synthetic constitutive promoter was sufficient to restore pulses (fig. S11). Thus, transcriptional feedback on YdiV is unnecessary for pulses in *E. coli*.

The propagation of pulses from class II to class III is gated by posttranslational regulation of FlhA

We also examined how pulses in class II promoters might be transmitted to class III promoters. Using a similar approach as for studying the transcriptional cascade from class I to class II, we generated a strain harboring both a copy of the class II (*fliF*) promoter driving CFP and a copy of the class III (*fliC*) promoter driving YFP. We then compared the class II fluorescence signal, a proxy for the concentration of proteins produced from class II promoters, to the activity of a class III promoter. We found that pulses in class II genes were not always accompanied by pulses in class III genes, which skipped in a stochastic manner some of the class II gene pulses (Fig. 4A). However, all class III pulses were always accompanied by class II pulses (Fig. 4A).

A well-characterized checkpoint regulates the transcriptional cascade from class II to class III promoters: Transcription of class III genes is regulated by the class II gene, the alternative sigma factor FlhA (35, 36). However, another class II gene, FlgM, binds to FlhA and acts as an inhibitor (37). Thus, FlhA remains initially inactive. When the basal body of the flagellar motor (encoded by the other class II genes) is assembled, it acts as a secretion system and exports FlgM from the cytoplasm (38, 39). This export results in the activation of FlhA. We hypothesized that this checkpoint pathway, and more directly FlgM, might be responsible for the “skipping” behavior of class III pulses.

To test this hypothesis, we simultaneously monitored the pulsating dynamics of the class II (*fliFp*) and class III (*fliCp*) promoters in an FlgM knockout strain (Δ flgM). In this mutant strain, class III promoters pulsed deterministically whenever class II genes pulsed (Fig. 4B). To better quantify this relationship, we plotted the mean activity of the class III promoter as a function of the class II reporter concentration. In wild-type cells, we observed that this relationship is sigmoidal, which is consistent with the hypothesis that a critical concentration of class II gene products is necessary for cooperative assembly of the basal body and export of FlgM. Such a sharp activation of FlgM export, in turn, frees the sigma factor FlhA for activation of class III genes. By contrast, in the Δ flgM mutant, the classes II and III relationship became linear, suggesting that class III pulses now simply mirror class II pulses (Fig. 4C).

Interactions between FlhDC and YdiV convert a deterministic program of flagellar gene expression into stochastic pulses

Last, we sought to examine how the dynamics of the flagellar network might change if we varied the mean levels of *flhDC* transcription. Using the previously described synthetic Pro series promoters, we measured the class II (*fliF*) promoter activity as a function of various levels of class I (*flhDC*) expression (31). Given our observation that the inherent noise of these synthetic promoters can lead to large changes in class II promoter activity (Fig. 3), rather than averaging all the single-cell data for a given strain, we divided class I reporter levels into five logarithmically spaced bins. Then, for each bin, we plotted the mean input (i.e., class I) against the mean of the corresponding output (i.e., class II) (see the “Input-output relationship between promoters across different classes” section). This procedure gave us greater resolution into how the class II promoter activity changes as a function of relatively small changes in class I. The resulting plot is analogous to a classic “dose-response” relationship.

We first examined the dose-response relationship in the Δ ydiV mutant to eliminate potentially confounding effects of the anti-FlhDC factor YdiV. For each strain, class II activity only fluctuated over a narrow range (Fig. 5, A and C). Notably, in the three strains with intermediate strength promoters that have extensive overlaps in class I expression level (P3, P4, and P5), class II expression across the strains varied nearly fivefold, although within each strain, class II activity mildly varied over class I fluctuations (Fig. 5A, inset). Such a behavior hints at a potential long-term “memory” that commits a strain to a level of class II expression over time.

In the presence of YdiV, there was a marked broadening of the dynamic range for class II activity for strains with intermediate class I expression (Fig. 5, B and D). Notably, a Δ ydiV mutant with P4 and a +YdiV strain with P5 had virtually identical mean class II expression levels, yet distribution of class II activity in the +YdiV strain was substantially wider (Fig. 5E). In effect, our observations suggest that in the absence of YdiV, small variations in FlhDC production are buffered from drastically altering the state of class II activation (Fig. 5, A and C): Cells are committed to maintaining a similar class II promoter activity over time. YdiV appears to break this stability by allowing noise in

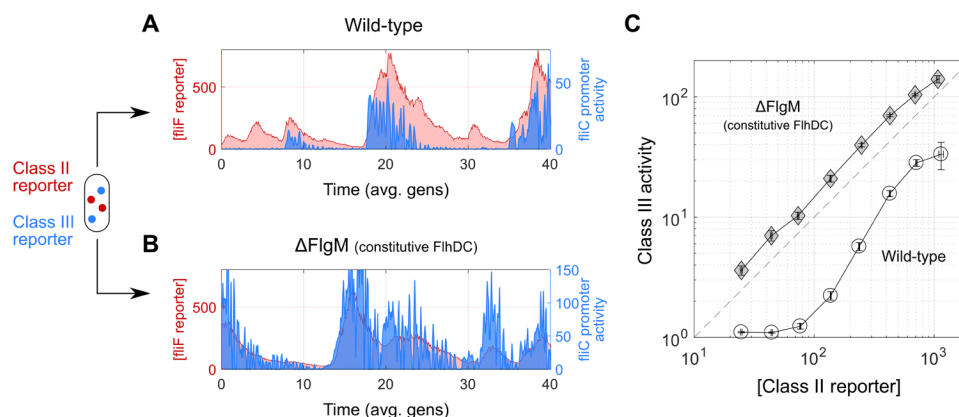


Fig. 4. Modulation of class III pulses by posttranslational regulation of the alternative sigma factor, FlhA. (A and B) Paired measurements of class II and class III within the same cells. We compared fluctuations in class II fluorescence signal (red, *fliFp*), a proxy for the concentration of proteins produced from class II promoters, to the activity of a class III promoter (*fliCp*, blue). Typical examples, (A) wild-type cells, and (B) a Δ flgM mutant that constitutively expresses the master regulator, FlhDC, to bypass any transcriptional feedback. (C) Mean class III activity as a function of the class II reporter concentration in cells harboring *fliF* and *fliC* promoter reporters. Binned average of single-cell measurements and wild-type (circles) and Δ flgM (diamonds) strains; Δ flgM mutant, same as in (B). For each strain, we analyzed 25 lineages, each at least 50 generations long.

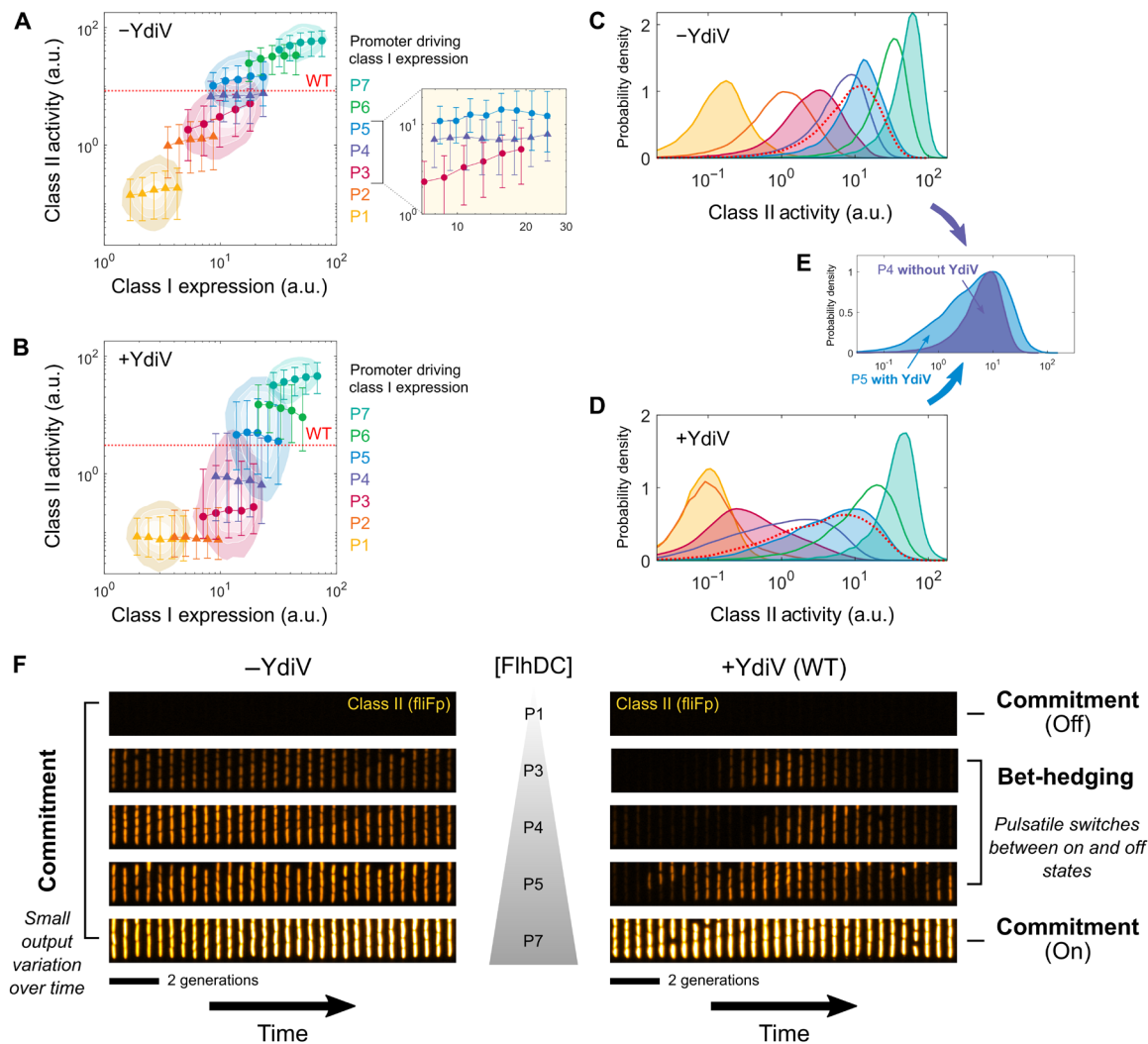


Fig. 5. YdiV underlies the dynamics of a pulsatile, bet-hedging circuit. (A and B) Input-output relationship between class I and class II. We generated strains where class I expression was driven by a synthetic promoter of different strength (P1 to P7, see the “Construction of strains with constitutively expressed FlhDC” section and table S3 from the Supplementary Materials) and plotted the class II (*fliFp*) promoter activity in those strains as a function of class I expression. For each strain, we divided class I reporter levels into five logarithmically spaced bins and plotted the mean input (i.e., class I) against the median of the corresponding output (i.e., class II) in each bin. Error bars indicate the 15 and 85% bounds of class II activity for each bin. Contour plots depicting the density of the paired class I and class II data for each strain are also shown. (A) shows Δ YdiV mutant strains, while (C) shows strains with wild-type YdiV. Dashed red line indicates mean class II promoter activity of strain with wild-type FlhDC promoter without [in (A)] and with [in (B)] YdiV. The inset in (A) highlights strains with promoters P3, P4, and P5 that have substantially overlapping class I expression yet show distinct class II outputs. For each strain in (A) and (B), we analyzed at least 50 lineages, each at least 20 generations long. (C and D) Distribution of class II promoter activities for each strain (C) without or (D) with YdiV. Shaded distributions correspond to P1, P3, P5, and P7. (E) Class II promoter activity distributions for P4 strain without YdiV (violet) and P5 strain with YdiV (blue). Both strains have similar mean class II activities (7.1 versus 7.4), but in the presence of YdiV, the class II activity distribution becomes considerably wider. (F) Commitment versus bet-hedging behavior in Δ YdiV and wild-type cells. Each strip is a typical kymograph of a strain harboring a class II promoter reporter expressing FlhDC from synthetic promoters.

FlhDC production to trigger a marked change in class II activity—i.e., cells periodically “escape” the state chosen by class I expression and sample active or inactive states (Fig. 5, B and D). Such a behavior would manifest itself as pulses of class II activation over time. Overall, our results suggest that in wild-type cells, YdiV plays a central role in generating pulses.

What might be the functional consequence of such a circuit? In the absence of YdiV, intermediate levels of FlhDC expression would cause *E. coli* cells to be committed to a certain level of flagellar synthesis—such a behavior would be beneficial in buffering small

changes in the environment but would also potentially force cells to a given genetic program for a long time (Fig. 5F, left). By contrast, with YdiV, a single cell can use a “compromise” solution where it can sample over time between active and inactive states of class II expression at intermediate levels of FlhDC (Fig. 5F, right).

DISCUSSION

Together, our results suggest that although the core architecture of the flagellar network appears to be a straightforward linear

transcriptional cascade, it exhibits a surprisingly rich and complex dynamical behavior—most notably a “pulsatile regime,” where continuous *flhDC* expression yields spontaneous switching between active and inactive states of flagellar transcription. Unlike transcriptional bursts, which are typically shorter in time scale (less than one generation), flagellar gene pulses span longer time periods to allow full activation of the cascade (as evidenced by class III activation), which suggests that pulses might be functional rather than simple noise.

The flagellum plays an essential role in chemotaxis and the initiation of biofilms (40, 41). However, its synthesis is associated with a sizeable growth cost (42). Because many conditions that trigger *E. coli* flagellar biosynthesis are also associated with decreased nutrient availability, pulsating dynamics may have evolved as a bet-hedging strategy to minimize cost. This strategy would yield a mixed population, with cells favoring either growth or flagellum synthesis. Similarly, pulses in the flagellar system might allow bacteria seeking to colonize host organisms to circumvent or minimize immune responses (8, 43, 44).

Our work also suggests that despite superficial similarities, *E. coli* regulates flagella in a distinct manner from *Salmonella*. In *E. coli*, we observed highly dynamic pulses of transcriptional activity across all class II and III promoters. Consequently, in flow cytometry measurements of flagellar genes, we observe a wide and smeared distribution, more notably for the frequently pulsing class II genes (fig. S12). By contrast, flow cytometry data in *Salmonella* show well-separated bimodal distributions for both class II and class III promoter activity (19). These differences suggest that flagellar genes in *E. coli* are considerably more dynamic and pulse at a much higher frequency than the previously reported bistable behavior in *Salmonella*, which is defined by more stable active or inactive states. Moreover, in *Salmonella*, the flagellar bistability requires a *FliZ*-mediated repression of *YdiV* (45–47). By contrast, we have found that in *E. coli*, *FliZ* knockout only affect mildly the pulses and that constitutive expression of *YdiV* is sufficient for pulses (see the “Impact of class II and class III genes on class II pulses” section in the Supplementary Materials). There is a growing body of evidence that while *E. coli* and *Salmonella* share many regulatory components for flagella, some are not conserved (21), and even conserved homologous proteins can show notable differences in behavior (20, 22). Our results suggest that those dissimilarities may give rise to drastically distinct dynamical behavior, which, in turn, may reflect differences in lifestyle and pathogenicity between these two organisms.

Broadly, our results highlight that even topologically simple transcriptional networks can generate unexpectedly rich temporal dynamics and phenotypic heterogeneities. We speculate that *E. coli* has evolved to operate the flagellar synthesis network in a “pulsatile” mode, which allows each cell to sample different flagellar phenotypes rather than allocating a subpopulation for fixed flagellar expression for extended periods of time.

MATERIALS AND METHODS

Chromosomal engineering

E. coli strains used in this study were engineered via Lambda red-mediated recombination (“red recombination”) (48). Parental strains were transformed with the helper plasmid pSIM5, and red recombination was performed using standard protocols (49). Strains were cured of pSIM5 (which harbors a temperature-sensitive origin of replication) by overnight growth at 37°C.

To generate point mutations or insert sequences without selective markers, we used a “scarless” chromosomal engineering technique—i.e.,

a genome-editing strategy that eliminates extraneous sequences such as antibiotic resistance cassettes (50). We modified a dual selection/counter-selection cassette in pKD45 (51) consisting of a kanamycin resistance marker and a toxin *ccdB* driven by a rhamnose-inducible promoter (*P_{rhaB}*). This original cassette required counter-selection to be done on minimal M9 plates containing rhamnose because the presence of other carbon sources allowed cells to avoid activation of the rhamnose promoter and escape counter-selection. Cell growth was extremely slow on these plates, requiring almost two full days of incubation before colonies became visible. To overcome this limitation, we replaced the rhamnose-inducible promoter with the arabinose-inducible *P_{araB}* using standard molecular biology techniques and isothermal assembly (52) using the NEB HiFi DNA Assembly Master Mix (New England Biolabs). The resulting kmR-araBp-ccdB (“KAC”) cassette allowed efficient counter-selection on LB plates containing 10% arabinose. We also constructed a variant of this cassette with the gentamicin resistance (*gmR*-araBp-ccdB or “GAC”).

Experimental background strains

The genetic background strain for this work was MG1655 (CGSC #6300), obtained from the Coli Genetic Stock Center (CGSC). A commonly used alternate stock of MG1655 (seq) (CGSC #7740) harbors an IS1 element insertion in the regulatory region of *FlhDC* (53). CGSC #6300 is the original isolate of MG1655 submitted to the CGSC by the Blattner laboratory, which does not have any IS elements in the regulatory region of *FlhDC*. See the “Impact of insertion element mutation on flagellar transcription” section in the Supplementary Materials.

A cassette constitutively expressing mCherry was inserted into the *IntS* locus to improve identification of cells in flow cytometry experiments and facilitate segmentation of cells in time-lapse experiments. An expression cassette containing (i) a Zeocin resistance marker, (ii) an RNA-I promoter from ColE1 fused to a coding sequence for mCherry, and (iii) two 100–base pair (bp)–long flanking sequences homologous to the *IntS* gene was ordered from IDT as a gBlock. This linear fragment was chromosomally inserted into the *IntS* locus via chromosomal engineering techniques described above.

For all strains used in time-lapse experiments, the scarless technique described above was used to introduce an E98K point mutation in *MotA* (54), which disables flagellar rotation and prevents cells from swimming out of the channels in the “mother machine.” Flow cytometry was used to confirm that this point mutation did not affect the pattern of flagellar gene expression. Lists of plasmids and strains are available in tables S1 and S2.

Construction of transcriptional reporter strains

Construction of cloning vectors

Cloning vectors pMK4 and pMK7 were constructed from pUA66 (55). pMK4 consists of a cloning site with two *Bsa* I sites: the RBS of gene 10 in T7 phage (“T7 RBS”) with the sequence TTTAAGAAG-GAGATATACAT and Venus NB (“VenNB”) (5). pMK7 consists of two *Bsa* I sites, a T7 RBS and SCFP3A (25), and the selective marker is replaced with ampicillin resistance. These vectors were assembled from linear fragments via isothermal assembly using the NEB HiFi DNA Assembly Master Mix (New England Biolabs) following manufacturer protocols. See the “Construction of cloning vectors for transcriptional reporters” section in the Supplementary Materials for details.

Construction of transcriptional reporter cassettes

A region of the MG1655 chromosome encompassing the flagellar promoter of interest and >30 bp of the protein coding sequences

flanking the promoter was amplified by polymerase chain reaction (PCR) to account for potentially unknown regulator binding sites. The PCR primers were designed to contain overhangs that contained Bsa I sites with compatible target sequences to pMK4 or pMK7. The PCR product was inserted into pMK4 or pMK7 via Golden Gate assembly using the previously described high-efficiency protocol of cycling between 3-min incubation at 37°C and 4-min incubation at 16°C for 25 cycles (56).

Chromosomal insertion of transcriptional reporters

The transcriptional reporters were inserted into one of two chromosomal loci: the lambda attB site or GalK. Both loci have previously been used for the insertion of chromosomal transcriptional reporters (57–59). The two sites are approximately 20 kb apart, which is not only sufficiently far to prevent read-through transcription (which is already low due to the strong *rrnB* T1 terminator at the end of each reporter cassette) but also sufficiently close to each other so that variations in copy number during replication can safely be ignored. To integrate transcriptional reporters into these sites, the reporter cassette was PCR-amplified from the plasmid template with primers containing at least 40-bp overhang homologous to the target locus. This PCR fragment was inserted chromosomally using the red recombination technique described above.

Construction of the class I transcriptional reporter

To monitor class I transcription, a cassette composed of the T7 RBS and VenNB was directly integrated into the 3'UTR of the endogenous FlhDC transcript using the scarless technique described above. See the “Construction of the class I transcriptional reporter” section in the Supplementary Materials for details.

Construction of strains with constitutively expressed FlhDC

A series of strains were constructed where the native class I promoter, *flhDp*, was replaced with a synthetic constitutive promoter of different transcriptional strength (the “Pro” promoters) (31). The native RBS for FlhD was also replaced with either the T7 RBS or a mutant RBS (“mut4”) with the sequence TTTAAGAATTGCATATACAT (mutated bases in bold). To construct these strains, plasmids consisting of a synthetic Pro promoter, a T7 or mut4 RBS, and FlhDC coding sequence were constructed. Subsequently, a linear fragment consisting of the Pro promoter, T7 or mut4 RBS, and FlhD, with overhangs homologous to the 5'-end of the *flhDp* was amplified from the plasmid template. This linear fragment was chromosomally inserted via red recombination. See the “Construction of strains with constitutively expressed FlhDC” section in the Supplementary Materials for more details.

Growth conditions

For all experiments, except where otherwise indicated, cells were grown in a modified version of the Neidhardt EZ-rich medium (Teknova), an optically clear rich defined medium based on a Mops buffer (60). The 0.2% glucose in the original formulation was replaced with 0.4% glycerol to prevent catabolite repression of flagellar synthesis.

For initial experiments, cells were grown at 34°C so that the width of the cell better matches the width of our initial microfluidic device. Subsequently, additional microfluidic devices with narrower channels permitted cell growth at 30°C, which, due to the slower generation time, also allowed more fields of view to be sampled in a single experiment. The pulsating dynamics were qualitatively similar at both temperatures. However, for any set of experiments where multiple strains were compared to each other (e.g., for pairwise

class II and III measurements, wild type versus mutant, and synthetic FlhDC expression), identical temperature and growth conditions were used to enable proper quantitative comparison.

Flow cytometry

For flow cytometry experiments, an overnight culture was diluted at least 1:3000 into 500 μ l of fresh growth medium. Cells were incubated at 30°C with shaking at 250 rpm for at least 6 hours before measurement. At this point, the cell density was typically around or below OD (optical density) = 0.1.

Cells were analyzed on a BD Fortessa flow cytometer (Becton Dickinson). The side-scatter (SSC-A) profile was used to first discriminate cells from background particles. Where possible, the constitutive red fluorescence from the mCherry marker was used as a second gate. The raw flow cytometry data were imported into MATLAB (MathWorks) and analyzed via custom software (figs. S3, S4, S7, S9, S10, and S12).

Microfluidic master and device fabrication

For initial experiments, microfluidic devices were fabricated from an epoxy replica of the mother machine described by Potvin-Trottier *et al.* (24) (fig. S1, design A). The replica mold was a gift from M. Cabeen (Harvard University). Subsequently, custom SU-8 molds were generated to build channels that better matched the typical cell width in our growth conditions (fig. S1, design B). See the “Microfluidic master fabrication” section in the Supplementary Materials for details.

To prepare a new microfluidic device out of the mold, dimethylsiloxane monomer (Sylgard 184, Dow Corning) was mixed with the curing agent at a 10:1 ratio, degassed, and poured over the mold. This mixture was degassed for an additional 1 hour and cured overnight at 65°C before being removed from the mold. Individual devices were first cut from the cured polydimethylsiloxane. Subsequently, inlets and outlets for each flow channel (“lane”) were created using a 0.75-mm biopsy punch. The device was treated with oxygen plasma in a plasma cleaner along with a 25 mm $\times$ 40 mm No. 1.5 coverglass (VWR) for 15 s at 30 W and an oxygen pressure of 200 mtorr. Following plasma treatment, the device and glass were bonded and incubated at least 1 hour at 65°C before use.

Microfluidic experimental setup

For time-lapse experiments, a passivating agent (Pluronic F-108, Sigma-Aldrich) was added to the growth medium at a final concentration of 0.85 g/liter. *E. coli* strains were first grown overnight in medium without the passivating agent. Cells were diluted the morning of the experiment 1:100 fold in fresh medium, containing the passivating agent, and allowed to grow to late exponential or early stationary phase. The reduced cell size at this growth phase improved the efficiency with which cells loaded in the device.

The cell culture was loaded into the inlet of the device by pipetting. The device was then centrifuged on a custom adaptor fit into a standard tabletop centrifuge at 6000g for 10 min. The inlets were connected to syringes filled with the growth medium (+passivating agent) via Tygon tubing (VWR; inside diameter 0.02 inch $\times$ outside diameter 0.06 inch). The flow-through was collected from the outlet via a second Tygon tubing into an empty beaker. The growth medium was first pumped at a rate of 35 μ l/min for at least 1 hour to allow the inlets and outlets to be cleared. Afterward, the flow rate was reduced to 4 to 5 μ l/min for the duration of the experiment.

Time-lapse fluorescence microscopy

The cells were allowed to adapt to growth in the device for at least two additional hours before imaging. Time-lapse images in experiments performed at 34°C were acquired on a Nikon Eclipse Ti-inverted microscope equipped with a 60× Plan Apo oil objective (numerical aperture, 1.4; Nikon), an Orca R2 charge-coupled device (CCD) camera (Hamamatsu), an automated xy stage (Ludl), and a SOLA light engine light-emitting diode (LED) excitation source (Lumencor). The microscope was surrounded by a temperature-controlled enclosure. The following filter sets were used for acquisition: YFP (Semrock YFP-2427A), CFP (Semrock CFP-2432A), and red fluorescent protein (RFP; Semrock mCherry-A). Automated time-lapse acquisition was controlled using custom MATLAB 2011a (MathWorks) scripts interfacing with μManager 1.4. Images were taken every 10 min, and focal drift was corrected via the Nikon PerfectFocus system and periodically recalibrated using z-stacks on a sacrificial position. Images in the RFP (the cell segmentation marker) were acquired at full camera resolution (1344 × 1024 pixels) to improve segmentation, while CFP and YFP images were acquired using 2 × 2 binning to reduce measurement noise. Short exposure times (typically 200 to 300 ms) and low illumination intensities (<30% of maximum illumination power) were used to minimize the effects of photobleaching.

Time-lapse images in subsequent experiments at 30°C were acquired on a Zeiss Axiovert 200M microscope equipped with a Plan-Apochromat 40×/1.3 Oil Ph3 Objective, a CCD camera (Hamamatsu C4742-98-24ERG), and a fluorescence excitation LED illumination source (SOLA SE II, Lumencor). Filters with the following specifications were used: for YFP [excitation (Ex), 500/24; dichroic (Di), 520; emission (Em), 542/27], CFP (Ex, 438/24; Di, 458; Em, 483/32), and RFP (Ex, 586/20; Di, 605; Em, 647/57). All filters were from Semrock. The variation in fluorescence intensity illumination across the field of view was less than 10% in all channels. The microscope setup was controlled using custom software on MATLAB 2013a (MathWorks) interfacing with μManager 1.4. Images were acquired every 5 min. Focal drift was corrected at each acquisition step via a custom autofocus routine, which acquires phase-contrast images at planes above and below the previously determined optimal autofocus plane and estimates the image plane with maximal contrast. Once the new focal plane was determined, images in the YFP, CFP, and RFP channels were acquired at full camera resolution (1344 × 1024 pixels). Again, short exposure times (typically 200 to 300 ms) and low illumination intensities (<15% of maximum illumination power) were used to minimize the effects of photobleaching.

Cell segmentation and tracking

Custom software in MATLAB implementing previously described algorithms (24, 61–63) was used to analyze time-lapse movies. RFP fluorescence was used as the “reference” image channel for segmentation. Parameters of the segmentation algorithm were optimized for image conditions. Following segmentation, the mother cell lineage was constructed by following the cell at the bottom of the growth channel (i.e., the mother cell) in each frame. “Cell divisions” events were identified by sudden decreases in cell area and corrected by manual review. Each lineage was tracked to the end of the experiment, until the mother cells were “lost” from the channels or until mother cells became filamentous or stopped growing.

For mother cells, in each frame, the mean fluorescence for all three (YFP, CFP, and RFP) fluorescence channels was determined by collecting and averaging the pixel values of the fluorescence image that

lie within the mask corresponding to the mother cell. The cell length was estimated by computing the distance between the top- and bottom-most pixels of the cell. See the “Cell segmentation and tracking” section in the Supplementary Materials for more details.

For the input-output relationship experiments where FlhDC expression was driven by promoters P1 to P7 (e.g., Fig. 5 from the main text), cell identification and tracking were supplemented with software designed for automated processing of Mother Machine experiments, named Molyso (64). No substantial differences were observed between fluorescence time-lapse traces generated by either software.

Estimation of promoter activity from time-lapse data

Promoter activity was estimated as previously described (24, 65). Briefly, if the total area of the cell is $A(t)$ and the average pixel intensity is $C(t)$, then $F_{\text{total}} = A(t)C(t)$. It follows that

$$\frac{1}{A} \frac{dF_{\text{total}}}{dt} = C \frac{1}{A} \frac{dA}{dt} + \frac{dC}{dt}$$

The left term $\frac{1}{A} \frac{dF_{\text{total}}}{dt}$, i.e., the “cell size normalized” production rate, was operationally defined as the promoter activity. To compute promoter activity, $\frac{1}{A} \frac{dA}{dt}$, i.e., the relative growth rate of the cell, was estimated by taking the log ratio of the initial and final area of the cell for each cell division. $\frac{dC}{dt}$ was estimated by smoothing our fluorescence traces with a Savitzky-Golay filter and taking the numerical derivative. See the “Estimation of promoter activity” section in the Supplementary Materials for more details.

Estimation of on and off states

To estimate the duration of on and off states, a heuristic threshold was defined. A “low detection threshold” based on cellular autofluorescence was used to detect all transcriptional events detectable above background noise. The statistics of short “bursty” transcriptional events were then used to define a second “high detection threshold.” Time periods when the flagellar promoter activity was continuously above this second threshold were defined as on periods. Similarly, off periods were defined as contiguous time periods, with promoter values below this threshold. See the “Estimation of “on” and “off” states” in the Supplementary Materials for more details.

Correlation analysis

To compute the average autocorrelation function for a given promoter, the autocorrelation function of each individual promoter activity trace (each corresponding to a single continuous mother cell lineage) was computed using the `xcov` function in MATLAB. Only traces with the same sample length (i.e., same number of time points) were used. The resulting autocorrelation functions for all the traces were then averaged.

The Pearson correlation coefficient between two promoter activity traces (or between YFP fluorescence and CFP promoter activity) was computed using the `corr` function in MATLAB. The mean correlation coefficient was then obtained by averaging the correlation coefficients from independent lineages.

Input-output relationship between promoters across different classes

Class II versus class III

Class II reporter fluorescence was divided into five logarithmically spaced bins that spanned minimum and maximum class II fluorescence

values. Paired class II/class III data points were sorted into each bin via class II fluorescence. Subsequently, for each bin, the mean input was determined by averaging class II fluorescence, while the mean output was determined by averaging the class III promoter activity of cells/observations within that bin.

Class I versus class II

The time-lapse data were binned into 2-hour windows (corresponding to about two cell divisions). Class I fluorescence and class II promoter activity were averaged for each time bin. The resulting time-averaged class I reporter values were then split into five logarithmically spaced amplitude bins spanning the minimum and maximum class I fluorescence values. For each amplitude bin, the mean input was determined by averaging the (time-averaged) class I fluorescence of all time bins with within that amplitude bin. The mean output was determined by averaging the (again, time-averaged) class II promoter activities of the same time-bin observations. In strains where FlhDC expression was driven by the *mut4* RBS, a correction factor (~0.221) was used to account for the difference in FlhDC production rate. See the “Input-output relationship between promoters across different classes” section in the Supplementary Materials for more details.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/6/eaax0947/DC1>

SUPPLEMENTAL STRAIN CONSTRUCTION METHODS

Fig. S1. Schematic of the microfluidic device.

Fig. S2. Flagellar gene expression is heterogeneous when cells are grown in liquid suspension.

Fig. S3. Flow cytometry analysis of flagellar gene expression for cells grown in liquid culture.

Fig. S4. The *cis*-YFP reporter in the FlhDC operon does not affect class II gene expression.

Fig. S5. Estimation of promoter activity from time-lapse data.

Fig. S6. Class I (flhDp) promoter activity resembles a constitutive promoter.

Fig. S7. Promoters within the same class show high correlation.

Fig. S8. Calibration of class I reporter for *mut4* and T7 RBS.

Fig. S9. An insertion element mutation in the FlhDC regulatory region results in high homogenous expression of flagellar genes.

Fig. S10. Effect of flagellar gene deletions on class II pulses.

Fig. S11. Constitutively expressed YdIV can restore class II promoter pulses in a *YdiIV* mutant of *E. coli*.

Fig. S12. Comparison of *Salmonella* and *E. coli* class II and III gene expression.

Table 1. List of plasmids.

Table 2. List of strains.

Table 3. List of the combination of promoter and RBS used to control class I expression and the notation used in this work to reference them.

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[View/request a protocol for this paper from Bio-protocol.](#)

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Acknowledgments: We thank M. Cabeen and J. Paulsson for sharing an early version of the microfluidic mother machine with our groups; C. Chang, M. Cabeen, N. Lord, T. Norman, and S. Canas Duarte for technical help with the microfluidic device; R. Losick and his laboratory for their microscope; and N. Kleckner, J. Paulsson, and K. Gibbs for their helpful discussions and feedback. **Funding:** This work was performed, in part, at the Center for Nanoscale Systems (CNS), a member of the National Nanotechnology Infrastructure Network (NNIN), which is supported by the NSF under award no. ECS-0335765. CNS is part of Harvard University. This work was supported by grant 1615487 from the NSF and, in part, by R01GM134275 from the NIH to P.C. M.G.-A. thanks M. Aldana for the scholarship received through grant PAPIIT-UNAM IN226917 and the Center for Complexity Sciences. **Author contributions:** J.M.K. and P.C. conceived the study. J.M.K. designed genetic constructs and experiments. J.M.K. and M.G.-A. constructed strains, performed experiments, and analyzed data, with help from E.B. M.G.-A. fabricated new microfluidic device masters. P.C. supervised the project. J.M.K. and P.C. prepared the manuscript with help from M.G.-A. J.M.K. and M.G.-A. wrote the Supplementary Materials. **Competing interests:** All authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the corresponding author.

Submitted 21 February 2019

Accepted 22 November 2019

Published 5 February 2020

10.1126/sciadv.aax0947

Citation: J. M. Kim, M. Garcia-Alcala, E. Balleza, P. Cluzel, Stochastic transcriptional pulses orchestrate flagellar biosynthesis in *Escherichia coli*. *Sci. Adv.* **6**, eaax0947 (2020).