Published in final edited form as:
Mol Microbiol. 2014 December ; 94(6): 1272–1284. doi:10.1111/mmi.12828.

Mutually repressing repressor functions and multi-layered cellular heterogeneity regulate the bistable *Salmonella fliC* census

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Summary

Bistable flagellar and virulence gene expression generates specialized *Salmonella* subpopulations with distinct functions. Repressing flagellar genes allows *Salmonella* to evade caspase-1 mediated host defenses and enhances systemic colonization. By definition, bistability arises when intermediate states of gene expression are rendered unstable by the underlying genetic circuitry. We demonstrate sustained bistable *fliC* expression in virulent *Salmonella* 14028 and document dynamic control of the distribution, or single-cell census, of flagellar gene expression by the mutually repressing repressors YdiV and FliZ. YdiV partitions cells into the *fliC*-OFF subpopulation, while FliZ partitions cells into the *fliC*-HIGH subpopulation at late timepoints during growth. Bistability of Δ*fliZ* populations and *ydiV*-independent FliZ control of flagellar gene expression provide evidence that the YdiV-FliZ mutually repressing repressor circuit is not required for bistability. Repression and activation by YdiV and FliZ (respectively) can shape the census of *fliC* expression independently, and bistability collapses into a predominantly intermediate population in the absence of both regulators. Metered expression of YdiV and FliZ reveals variable sensitivity to these regulators and defines conditions where expression of FliZ enhances *fliC* expression and where FliZ does not alter the *fliC* census. Thus, this evolved genetic circuitry coordinates multiple layers of regulatory heterogeneity into a binary response.

Keywords

bistability; *Salmonella*; hysteresis; heterogeneity; flagella; stochastic

Introduction

Transcriptional responses at a promoter can be characterized as graded or binary (Biggar & Crabtree, 2001, Temme *et al.*, 2008). In a graded response, all levels of expression from fully off to fully on are stable and the response to an activation signal is continuous, while a binary or switch-like response implies only two levels of activity, on and off. A switch-like and heterogeneous response within a population of single cells generates a bistable

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The authors declare no conflict of interest.

distribution, or census, of gene expression (Veening *et al.*, 2008). Many cellular programs that direct construction of complex structures (flagella and other surface organelles), entail irreversible differentiation (sporulation), or risk a significant fitness cost in some other way (growth arrest leading to persistence, competence for DNA uptake) are controlled by genetic circuitry imparting bistable expression of key phenotypic determinants (Cummings *et al.*, 2006, Stewart *et al.*, 2011, Temme et al., 2008, Sturm *et al.*, 2011, Chung *et al.*, 1994, Bigger, 1944, Moyed & Bertrand, 1983, Smits *et al.*, 2005).

Bistability requires a promoter signal-response function characterized by non-linear kinetics, positive feedback, and hysteresis (Veening *et al.*, 2008, Ray *et al.*, 2011). Non-linear kinetics coupled with positive feedback imparts high promoter sensitivity to supra-threshold signal concentrations, generating a strong, rapid transcriptional response. In hysteretic systems, distinct signal threshold levels initiate switching from OFF to ON versus ON to OFF. Hysteresis stabilizes cells in the OFF- or ON-state, pending a decisive change in signal concentration (Ray *et al.*, 2011). Specific biochemical modules by which non-linearity, positive feedback and hysteresis are integrated into a genetic program can be transcriptional or non-transcriptional, including post-translational mechanisms such as phosphorylation, proteolytic degradation or physical sequestration (Chen & Arkin, 2012, Ray et al., 2011).

Our model system for evolved genetic circuitry conferring bistability is the *Salmonella enterica* serovar Typhimurium flagellar regulon. Host compartment-specific flagellar regulation is critical to *Salmonella* virulence. Flagellar gene expression promotes intestinal colonization by conferring motility (Stecher *et al.*, 2008) and activating expression of invasion genes (Chubiz *et al.*, 2010). Bistable flagellar and invasion gene expression generates distinct subpopulations that 1) invade the intestinal epithelium and activate inflammation, and 2) thrive on host products released into the gut lumen during inflammation, thereby outcompeting the intestinal microbiota and promoting transmission (Ackermann *et al.*, 2008). In contrast, failure to repress flagellar genes in systemic tissues (where *Salmonella* live within phagocytes) is attenuating (Stewart et al., 2011, Miao *et al.*, 2010). *Salmonella* signaling networks have evolved such that the flagellar regulon receives input from numerous environmental, physiological and metabolic pathways, many of which help *Salmonella* orient to host micro-environments (Bader *et al.*, 2003, Adams *et al.*, 2001, Spory *et al.*, 2002, Lin *et al.*, 2008, Wozniak *et al.*, 2009, Clegg & Hughes, 2002, Lawhon *et al.*, 2003, Mouslim & Hughes, 2014).

Salmonella flagella are constructed via the sequential expression of three classes of genes (figure 1). Class I comprises one operon, *flhDC*, encoding master regulatory proteins required to initiate class II expression. Cellular signaling pathways converge here to control the amount and activity of the $FlhD_4C_2$ complex, modulating its transcription (Mouslim & Hughes, 2014), translation (Wei *et al.*, 2001, Jonas *et al.*, 2010, Lawhon *et al.*, 2003), activation (Takaya et al., 2006) and activity/stability (Tomoyasu *et al.*, 2002, Saini *et al.*, 2008, Wada *et al.*, 2011a, Takaya *et al.*, 2012, Li *et al.*, 2012, Ahmad *et al.*, 2013). Class II genes encode proteins of the hook/basal body (HBB), while class III genes encode the flagellar filament monomers (FliC and FljB) and proteins required for motor function and chemotaxis (Chevance & Hughes, 2008). Class II and III promoters also drive expression of multiple positive and negative regulators of the cascade (Wozniak *et al.*, 2010), including

the sigma factor (FliA) required for class III expression (Chevance & Hughes, 2008), the anti-sigma factor (FlgM) that sequesters FliA until HBB assembly is complete (Karlinsey *et al.*, 2000, Hughes *et al.*, 1993), and FliZ, an activator of class II promoters (Kutsukake *et al.*, 1999).

The three-class flagellar gene expression cascade includes biochemical features that generate positive feedback and ultrasensitivity (Ray et al., 2011) in other bistable systems, both natural (evolved) and synthetic. Complex genetic architecture comprising multiple positive and negative feedback loops (Kutsukake et al., 1999, Yamamoto & Kutsukake, 2006, Kalir *et al.*, 2005), sequestration of the sigma factor FliA by the anti-sigma factor FlgM, and a system of mutually repressing repressors formed by FliZ and a protein encoded outside the regulon, YdiV (Wada *et al.*, 2011b, Wozniak et al., 2009), are candidate mechanisms that might contribute to bistable expression of class II or III genes (Ray et al., 2011, Chen & Arkin, 2012, Dubnau & Losick, 2006). WT *Salmonella* populations comprise a subpopulation transcribing *fliA* and a subpopulation in which *fliA* transcription is post-translationally blocked by YdiV (Stewart et al., 2011), resulting in bistable *fliA* and *fliC* expression. YdiV binds FlhD, inhibits $FlhD_4C_2$ complex binding to DNA, and targets the complex to the ClpXP protease for degradation (Wada et al., 2011a, Takaya et al., 2012, Li et al., 2012). FliZ increases the level of class I proteins by an unknown mechanism, and represses *ydiV* transcriptionally. Thus YdiV and FliZ are mutually repressing repressors with opposing effects at the same important control point of flagellar gene expression, the $FlhD_4C_2$ complex. Repression of *fliC* by YdiV is required for full virulence (Stewart et al., 2011, Hisert *et al.*, 2005). FliZ is also necessary for virulence, as a positive regulator of the *Salmonella* Pathogenicity Island-1 locus (Iyoda *et al.*, 2001, Lucas *et al.*, 2000, Chubiz *et al.*, 2010). Whether FliZ's role in flagellar regulation contributes to infection is unknown.

We investigated the contribution of this mutually repressing repressor genetic circuit to *fliC* bistability, using flow cytometry to plot the single-cell census, or distribution, of *fliC* expression for WT and mutant populations. Bistable populations were maintained from 2–5 hours after backdilution, and the repressor YdiV maintained the *fliC*-OFF subpopulation throughout 5 hours of growth. FliZ was required for WT levels of *fliC* expression, however, bistable populations continued to form in a Δ*fliZ* strain, indicating that reciprocal repression by YdiV and FliZ is not crucial for *fliC* bistability. Instead, the individual roles of YdiV and FliZ are important, such that bistability collapses into a predominantly intermediate population in the absence of both regulators. Dose-dependent induction or metering of YdiV and FliZ production from separate exogenous promoters revealed underlying cell-to-cell heterogeneity in factors required for these regulators to modulate *fliC* expression. We propose that input from multiple pathways tuning transcription, translation, activation and stability of the $FlhD_4C_2$ complex generates population diversity in $FlhD_4C_2$ activity, determining which cells are locked into *fliC*-OFF mode and which activate *fliC* expression.

Results

The kinetic role of FliZ in *fliC* bistability

The census of *fliC* expression in populations of the virulent *Salmonella enterica* serovar Typhimurium strain 14028 was analyzed by flow cytometry, using a *fliC*-GFP

transcriptional reporter (Cummings et al., 2006). (See figure 1 for an overview of flagellar regulation in *Salmonella*.) In order to quantitatively describe our observations, the range of fluorescence observed was divided into three gates: *fliC*-OFF, *fliC*-INT and *fliC*-HIGH (see figure S1 for a detailed description of gating). Percent gate occupation in these experiments depends upon fluorescent intensity of individual bacteria in the populations. When fluorescent proteins are used as reporters of bacterial gene transcription, fluorescent intensity is determined by the relative kinetics of promoter activity, dilution due to cell division, and proteolytic degradation. Proteolytic degradation is negligible in these experiments, due to the 9.5-day fluorescence half-life (Cummings et al., 2006) of the GFP (GFPmut3, (Cormack *et al.*, 1996)) in this construct. Additionally, fluorescence of GFPmut3 is detectible within 8 minutes of transcriptional induction (Cormack et al., 1996). Thus, fluorescent intensity of *Salmonella* in these populations is a function of promoter activity and dilution due to growth. Similar growth curves and generation times for the four strain backgrounds used in this study (figure S2) indicate that comparisons of percent gate occupation can be made across strains at individual timepoints. Trends in gate occupation observed across timepoints for a single strain reflect changes in both promoter activity and growth rate during those intervals. Strains are in exponential growth phase between 1.5–3 hours after backdilution and post-exponential phase during the remainder of the timecourse (figure S2). Expression of a constitutive promoter is unimodal for all strain backgrounds used in this study (figures S3 and S4B).

Wild-type populations demonstrated well-defined bimodal *fliC* expression distributions at all timepoints tested (figure 2A, top panels, histograms from a representative experiment and average subpopulation percentages from three independent experiments). (See supplemental material for subpopulation percentages and mean fluorescent intensities from individual experiments, and tables 1 and S1 for strain specifics.) This kinetic analysis confirms that two stable states of *fliC* expression are maintained throughout the growth curve in *Salmonella* strain 14028, in contrast to strain LT2 (figure S4A) where flagellar class III expression becomes activated in all cells of the population during this timeframe (Saini *et al.*, 2010). *fliC* expression increased over time, as cells were partitioned from the *fliC*-INT gate to the *fliC*-HIGH gate. The *fliC*-HIGH subpopulation grew significantly between 2–3 hours ($p<0.001$) and 3–4 hours ($p<0.01$), with corresponding decreases in the size of the *fliC*-INT subpopulation over both intervals ($p<0.001$).

In order to define the role of FliZ in shaping the census of *fliC* expression in a bistable population, *fliC* expression in a Δ*fliZ* mutant was next examined. Δ*fliZ* populations also proved bistable throughout the timecourse (figure 2A, bottom panels, histograms from a representative experiment and average subpopulation percentages from three independent experiments). When kinetic plots of subpopulation sizes were compared, the population distributions of *fliC* expression for WT and the Δ*fliZ* mutant were indistinguishable at 2 and 3 hours (figures 2B,C). At 4 hours, however, the WT *fliC*-HIGH subpopulation was larger (figure 2B) than that of the Δ*fliZ* mutant, and this difference increased between 4 and 5 hours. The opposite trend was observed when the sizes of the *fliC*-INT subpopulations were compared (figure 2C), whereby at 5 hours the WT *fliC*-INT subpopulation was smaller than that of the Δ*fliZ* mutant. The average mean fluorescent intensity (MFI, measured in arbitrary

fluorescence units, AFU) of the WT *fliC*-HIGH subpopulations was significantly higher than the average MFI of the Δ*fliZ* mutant *fliC*-HIGH subpopulations (at 5 hours: WT *fliC*-HIGH MFI=1588 AFU and Δ*fliZ* mutant *fliC*-HIGH MFI=1415 AFU, $p<0.001$), consistent with the observation that the peak of *fliC*-expressing cells for Δ*fliZ* mutant is shifted toward lower levels of expression, spanning the junction between the *fliC*-INT and *fliC*-HIGH gates. Individual plots of the population distributions for WT (figure 2D) and the Δ*fliZ* mutant (figure 2E) highlight this *fliZ*- and time-dependent partitioning of cells from the *fliC*-INT subpopulation to the *fliC*-HIGH subpopulation.

Thus, in a bistable strain, FliZ increases the probability that an individual cell will maximize *fliC* expression, in a growth-phase dependent manner. Intriguingly, many cells are able to maximize *fliC* expression in the absence of FliZ, revealing biochemical heterogeneity in these well-mixed, homogeneously grown cultures.

FliZ controls flagellar gene expression via *ydiV*-dependent and -independent mechanisms

Two mechanisms have been proposed to account for FliZ-mediated upregulation of flagellar genes. Whether these activities constitute a single pathway or distinct pathways was unknown. FliZ and YdiV participate in a genetic circuit of mutually repressing repressors (figure 1), wherein FliZ repression of *ydiV* is predicted to increase flagellar class II gene expression because YdiV inhibits binding of the $FlhD_4C_2$ complex to DNA and targets the complex for ClpXP-mediated proteolysis (Takaya et al., 2012, Wada et al., 2011a). Additionally, FliZ increases the level of the class I protein FlhC concurrently with *fliC* transcription (Saini et al., 2008); specifics of this pathway have not been elucidated, and *ydiV*-dependence of the finding was not tested. We investigated whether the impact of either mechanism varied with time in our system.

A β-galactosidase reporter fusion was used to measure *ydiV* transcription in the presence and absence of FliZ. In WT cells, *ydiV* was transcribed at similar levels at both 2.5 and 5 hours (figure 3A, compare black bars across timepoints, difference is not significant), and there were no significant differences between the WT, Δ*fliZ*, and Δ*fliZ* p*fliZ* strains at 2.5 hours. In contrast, the temporal difference in *ydiV* transcription for the Δ*fliZ* mutant was highly significant (figure 3A, compare grey bars across timepoints), and at 5 hours *ydiV* was more highly transcribed in *fliZ* than in the WT strain (compare black bar to grey bar). Complementation with exogenous FliZ was significant at 5 hours (compare grey bar to white bar). These data demonstrate that FliZ represses *ydiV* to a greater extent as cells approach stationary phase (figure S2).

FlhC levels were measured by western blot and are reported for the Δ*ydiV*, Δ*fliZ*, and Δ*ydiV* Δ*fliZ* mutants as the percentage of WT FlhC at 2.5 or 5 hours (figure 3B). FliZ did not regulate FlhC at 2.5 hours (figure 3B, none of the mutants differed significantly from WT which is set at 100%). However, at 5 hours, FliZ was required to stabilize WT FlhC levels (figure 3B, FlhC in Δ*fliZ* mutant is 34.8% of WT, $p=0.0014$, and FlhC in Δ*ydiV* Δ*fliZ* mutant is 37% of WT, $p=0.0041$). Thus, both the kinetic pattern of *ydiV* repression by FliZ (figure 3A) and FlhC regulation by FliZ (figure 3B) support our *fliC* transcription timecourse, in which FliZ increases *fliC* expression at later timepoints during growth (figure 2A-E).

To determine whether FliZ requires *ydiV* in order to augment the amount of FlhC protein, FlhC levels between the Δ*ydiV* single mutant and the Δ*ydiV* Δ*fliZ* double mutant were compared (figure 3B, compare black bars to white bars within timepoints). FliZ represses *ydiV* transcription (Wada et al., 2011b, Wozniak et al., 2009). If this were the sole mechanism by which FliZ increased FlhC, the amount of FlhC should be identical between Δ*ydiV* and Δ*ydiV* Δ*fliZ*. FliZ significantly increases the amount of FlhC in a Δ*ydiV* background at 5 hours (FlhC in Δ*ydiV*=102.5% WT, FlhC in Δ*ydiV* Δ*fliZ*=37% WT, $p<0.001$). Therefore, FliZ also plays a *ydiV*-independent role in flagellar gene regulation.

We hypothesized that if FliZ can boost *fliC* expression by a *ydiV*-independent mechanism, Δ*ydiV* single and Δ*ydiV* Δ*fliZ* double mutant populations would have distinct patterns of *fliC* expression (figure 4A, second and fourth rows, histograms from a representative experiment and average subpopulation percentages from three independent experiments). Indeed, the percentage of cells in the *fliC*-HIGH gate was significantly larger for Δ*ydiV* than for Δ*ydiV* Δ*fliZ* when distributions of *fliC* expression were compared at 4 and 5 hours (figure 4B, black circles). In contrast, the *fliC*-INT subpopulations were significantly larger for Δ*ydiV* Δ*fliZ* than for Δ*ydiV* (figure 4B, dark grey triangles). Thus, *fliZ* significantly shapes these distributions even in the absence of *ydiV*.

The changing role of YdiV during growth

At 2 hours, there were significantly more *fliC*-HIGH cells and significantly fewer *fliC*-OFF cells for the Δ*ydiV* mutant compared to WT (figures 4A,C,E,F), while the *fliC*-INT subpopulations did not significantly differ in size. Thus YdiV partitions cells from the *fliC*-HIGH gate to the *fliC*-OFF gate early during growth.

This kinetic analysis of *fliC* expression in the Δ*ydiV* mutant revealed significant *ydiV*-independent *fliC* repression at 4 and 5 hours. This manifested as 1) a drop in the percentage of *fliC*-HIGH cells across time for Δ*ydiV* (figure 4C, black circles, compare 2 hours to 5 hours, $p<0.001$) and 2) an increase in the percentage of *fliC*-OFF cells across time (figure 4C, light grey squares, compare 2 hours to 5 hours, $p<0.05$). Furthermore, the average MFI for cells in the *fliC*-INT gate fell significantly from 2 hours (246.7 AFU) to 5 hours (144.7 AFU, $p<0.01$) for the Δ*ydiV* mutant, indicating a shift to a lower level of *fliC* expression for this subpopulation. From 3 hours on, significant differences in the *fliC* expression distributions between WT and the Δ*ydiV* mutant occurred in the *fliC*-OFF and *fliC*-INT subpopulations (figures 4A,C,E,F), while the sizes of the *fliC*-HIGH subpopulations did not significantly differ. These data demonstrate a changing role for YdiV during growth whereby YdiV partitions cells into *fliC*-OFF from *fliC*-HIGH at 2 hours, and from *fliC*-INT at later timepoints (3, 4 and 5 hours).

The census of *fliC* expression is inverted in the absence of YdiV and FliZ

We predicted that deleting both *ydiV* and *fliZ* would invert the WT census of *fliC* expression as cells approach stationary phase, since YdiV is critical to maintain the *fliC*-OFF subpopulation, and FliZ is required to maximize *fliC* expression under these conditions. In WT distributions, intermediate states of *fliC* expression are unstable compared to the *fliC*-OFF and *fliC*-HIGH states (figures 2A,D and 4A,E). This observation suggested the

possibility that intermediate states of *fliC* expression might be strongly favored in the absence of YdiV-mediated repression and FliZ-mediated enhancement. This proved to be true at late timepoints (4 and 5 hours, figures 4A (bottom panels),D), when the *fliC*-INT subpopulation of Δ*ydiV* Δ*fliZ* distributions contained a significantly higher percentage of cells than either the *fliC*-OFF or *fliC*-HIGH gates, which did not differ from one another (at 4 hours, *fliC*-INT compared to *fliC*-OFF, $p<0.001$, and to *fliC*-HIGH, $p<0.001$; at 5 hours, *fliC*-INT compared to *fliC*-OFF, $p<0.001$, and to *fliC*-HIGH, $p<0.001$).

Multiple layers of heterogeneity shape the *fliC* expression census

We hypothesized that modulating production of YdiV and FliZ would reveal the extent to which *fliC* expression can be controlled by these two regulators. Accordingly, a system with which to study the sensitivity of *fliC* transcription to perturbation by YdiV and FliZ was constructed. Regulator production is metered from separate exogenous promoters (figure 5A), YdiV according to the concentration of chlortetracycline, and FliZ according to the concentration of L-arabinose. Neither promoter inherently produces bistable populations in the presence or absence of inducer (figure S5). Bimodal distributions of cells in many panels of figure 5B evince switch-like, heterogeneous control of *fliC* expression in the metered strain.

Metered induction of YdiV reduced *fliC* expression, partitioning cells into the *fliC*-OFF subpopulation. Without FliZ (figure 5B, panels 1–3 and 4–6, histograms and subpopulation percentages from a single experiment), *fliC* transcriptional responses to YdiV induction were similar at the two timepoints tested. At 2.5 hours, titration of YdiV into the system increases the percentage of the population in the *fliC*-OFF gate from 20.7% (panel 1) to 58.6% (2) to 76.3% (3). At 5 hours, the *fliC*-OFF population rose from 24% (panel 4), to 63.7% (5) and 79.3% (6).

At 2.5 hours, no YdiV-independent role of FliZ was observed (figure 5B, compare panels 1,7 and 13). FliZ boosted *fliC* expression only when *ydiV* was concurrently induced (figure 5B, columns 2 and 3). In the context of high YdiV expression, strong induction of *fliZ* increased the *fliC*-HIGH subpopulation from 6.9% (panel 3) to 26% (15) and reduced the *fliC*-OFF subpopulation from 76.3% (panel 3) to 55.5% (15). The *fliC*-INT subpopulation remained nearly constant at 16.8–18.4% (figure 5B, panels 3,9 and 15).

At 5 hours, in the absence of YdiV and FliZ, the largest subpopulation is *fliC*-INT at 41.5% (figure 5B, panel 4). This mirrors the inverted census of *fliC* expression observed for the Δ*ydiV* Δ*fliZ* strain in figure 4A (fourth row, 4 and 5 hours). At 5 hours, as at 2.5, metered YdiV expression partitioned cells into the *fliC*-OFF subpopulation at all strengths of FliZ induction (figure 5B, compare panels 4 to 6, 10 to 12 and 16 to 18). However, in contrast to observations at 2.5 hours, metered induction of FliZ now partitioned cells into *fliC*-HIGH in the presence and absence of YdiV (figure 5B, compare *fliC*-HIGH subpopulations in panels 4–6, where FliZ is not induced, to *fliC*-HIGH subpopulations in panels 10–12 and 16–18). This demonstrates that FliZ is able to counter both YdiV-dependent and –independent *fliC* repression at 5 hours. Thus, the impact of FliZ upon the *fliC* census is enhanced at 5 hours compared to 2.5 hours under conditions of metered expression. These data are consistent

with the requirement for FliZ to maintain both WT *fliC* expression at late timepoints (figure 2A-E) and WT levels of FlhC in a *ydiV*-independent manner at 5 hours (figure 3B).

Partitioning of 92.6% of cells into the *fliC*-HIGH gate with strong *fliZ* induction at 5 hours (figure 5B, panel 16) shows that most cells in the population contained all cellular factors required for *fliC* expression except for FliZ at this time. Intriguingly, though *fliZ* was induced throughout the population (figure S5) in our metered strain, the ability of FliZ to counter YdiV-dependent (figure 5B, panels 11,12,17,18) and –independent (figure 5B, panels 10,16) *fliC* repression varies from cell to cell. Similarly, at both timepoints, the addition of increasing amounts of YdiV partitions some cells from *fliC*-ON (*fliC*-INT + *fliC*-HIGH) to the *fliC*-OFF subpopulation, but cell-to-cell sensitivity to the repressor varies (compare column 1 data to columns 2 and 3 for both timepoints). By titrating expression levels of *ydiV* and *fliZ*, we reveal heterogeneity in cellular factors that potentiate the impact of these two important regulators upon *fliC* expression.

Discussion

YdiV and FliZ destabilize intermediate states of *fliC* expression

Previously, we showed that the virulent *Salmonella* strain 14028 demonstrates bistable *fliC* expression at a single timepoint (Stewart *et al.*, 2011), and the present data show that bistable distributions of *fliC* expression in 14028 persist through 5 hours of growth. Using these kinetic analyses, we define the roles of two flagellar regulators in subpopulation flux and the maintenance of *fliC* bistability. The activator FliZ partitions cells from intermediate to high levels of *fliC* expression at late timepoints. This finding is supported by a study of flagellar gene expression dynamics in the transiently bimodal strain LT2, showing that a class III gene was expressed at a lower intensity at late timepoints in a Δ*fliZ* mutant (Saini et al., 2010). YdiV partitions cells from *fliC*-HIGH (at early timepoints) or *fliC*-INT (at later timepoints) into the *fliC*-OFF subpopulation. Thus, both regulators contribute to the instability of intermediate states of expression, organizing the population into tightly defined *fliC*-OFF and *fliC*-HIGH subpopulations.

Individual roles of YdiV and FliZ shape *fliC* bistability, while reciprocal repression is dispensible

FliZ and YdiV are mututally repressing repressors, a genetic feature demonstrated to contribute to phenotypic heterogeneity in other systems. The positive feedback loops formed when FliZ increases class I protein levels or decreases *ydiV* transcription were candidate hysteretic mechanisms for *fliC* regulation. Positive feedback loops could lock cells into the *fliC*-ON state by increasing the level of a positive regulator or decreasing the level of a negative regulator of flagellar class II gene expression. Here we demonstrate bistability in the absence of FliZ (figures 2A and 4A). Therefore, neither coordinated downregulation of *ydiV* by FliZ as flagellar gene expression increases nor the positive feedback loop formed by FliZ's impact upon class I proteins is required for cells to activate *fliC* expression or persist in *fliC*-ON mode across time. Additionally, the present work shows that these two activities of FliZ constitute distinct pathways. FliZ represses *ydiV*, and increases both class I protein levels (figure 3B) and *fliC* expression in the absence of *ydiV* (figure 4A,B). These regulators

therefore play important individual roles in *fliC* expression, and bistable population distributions continue to form in the absence of the mututally repressing repressor circuit.

FliZ activity is temporally regulated

The impact of FliZ upon *fliC* expression was most evident at 5 hours in both the WT (figure 2A,B,C) and engineered backgrounds (figure 5B). At the population level, this suggests temporal fluctuations in average concentrations of cellular factors required for FliZ to activate *fliC* expression, most likely regulators of class I. Transcription of class I genes is tightly controlled by growth-phase specific expression of multiple positive and negative regulators (Mouslim & Hughes, 2014). One positive transcriptional regulator of *flhDC*, HilD, is itself more highly expressed as growth progresses (Mouslim & Hughes, 2014, Kröger *et al.*, 2013) and is a master activator of *Salmonella* Pathogenicity Island-1 transcription (Schechter *et al.*, 1999). HilD is post-translationally activated by FliZ (Chubiz *et al.*, 2010), and therefore represents a potential growth-phase specific mechanism by which FliZ could upregulate *flhDC* transcription coordinately with SPI-1 genes. However, control of class I proteins by FliZ is reported to be predominantly post-translational (Saini *et al.*, 2008), and availability of cellular factors impacting translation, activation, and stability of $FlhD_4C_2$ may also fluctuate with growth phase. These hypotheses will be tested in future studies.

Model of YdiV-dependent hysteresis in *fliC* heterogeneity

Metered expression (figure 5B) revealed that sensitivity to YdiV and FliZ varies from cell to cell within populations. The presence of a YdiV-dependent *fliC*-OFF subpopulation in the WT census (figures 2A,4A, top panels) suggests variable sensitivity, but could also be explained by a variable distribution of YdiV across the population. In the metered strain, however, variable responses occurred with uniform induction of these regulators (figure S5). We observed that addition of FliZ organized a population with broadly heterogeneous *fliC* expression into a single *fliC*-HIGH peak (figure 5B, compare panel 4 to panel 16), demonstrating that these cells contained all factors required for *fliC* expression, and suggesting that post-translationally increasing class I protein levels (figure 3B) was critical.

We propose a model in which variable sensitivity to YdiV and FliZ is predicated upon apparently stochastic cell-to-cell differences (Elowitz *et al.*, 2002) in the amount and activity of the $FlhD_4C_2$ complex. Production, activity and stability of $FlhD_4C_2$ are tuned by many regulators, in accordance with information flow through diverse signaling pathways (RcsB (Wang *et al.*, 2007), RflM (Singer *et al.*, 2013), SlyA (Mouslim & Hughes, 2014), LrhA (Mouslim & Hughes, 2014), RtsB (Ellermeier & Slauch, 2003), FimZ (Clegg & Hughes, 2002), HilD (Singer *et al.*, 2014), CsrA (Lawhon et al., 2003), DnaK (Takaya et al., 2006), ClpX (Tomoyasu et al., 2002), HNS (Kutsukake, 1997), cAMP-CRP (Komeda *et al.*, 1976), and others). Inputs from multiple cellular systems include simultaneous positive and negative regulation (Mouslim & Hughes, 2014). Cumulatively, small cell-to-cell variations in regulatory input from multiple pathways could generate population heterogeneity in the net level of activated $FlhD_4C_2$ complex. Cells low in activated $FlhD_4C_2$ would require more FliZ to activate *fliC* expression, and cells with higher concentrations of activated $FlhD_4C_2$ would require more YdiV to switch off *fliC* expression.

YdiV is required for a stable *fliC*-OFF peak (figure 4A), and for *fliC* repression and *Salmonella* virulence in host systemic tissues (Stewart et al., 2011). Li et al. recently proposed a model based upon work in *E. coli* whereby up to four YdiV molecules can bind to FlhD in the $FlhD_4C_2$ ring (Li *et al.*, 2012). The $FlhD_4C_2$ conformation was unaffected by the addition of one or two molecules of YdiV, but the third and fourth distorted the ring, abrogating DNA binding and flagellar class II transcription. Depending upon the strength of the association, $YdiV_4$-$FlhD_4C_2$ could act as a dead-end complex (Igoshin *et al.*, 2008, Ray et al., 2011). High concentrations of YdiV are also reported to mediate degradation of $FlhD_4C_2$ by ClpXP (Takaya et al., 2012). Dead-end complex formation and/or YdiV-driven $FlhD_4C_2$ degradation could impart hysteresis. Such cells without sufficient $FlhD_4C_2$ would be locked into *fliC*-OFF mode until the relative concentrations of factors preventing and promoting flagellar gene expression shift decisively, perhaps due to unequal partitioning to daughter cells during division. Thus, we provide evidence that coordinated input from a landscape of heterogeneously distributed cellular factors shapes the binary output of this evolved genetic circuit.

Experimental Procedures

Bacterial Strains and Growth Conditions

Strains and plasmids used in this study are listed in tables 1 and S1. Mutants were generated in the *Salmonella enterica* serovar Typhimurium 14028 background as previously described (Datsenko & Wanner, 2000). Strains express only *fliC* flagellin due to deletion of *fljBA* (Bonifield & Hughes, 2003). The FlhC::3XFLAG construct was a gift from the Rao lab (Saini et al., 2008), and the *ydiV*::FKF::pKG136 and T-POP::*ydiV* (*ydiV*240::Tn10dTc(del-25)) constructs were gifts from the Hughes lab (Wozniak et al., 2009). Constructs were transferred between *Salmonella* strains by P22 transduction (Maloy *et al.*, 1996). *Salmonella* were grown at 37°C with shaking at 225 rpm in Miller Luria Broth (Product #244620, Difco) with appropriate antibiotics, backdiluted 1:100 after overnight incubation, and grown to indicated timepoints. Carbenicillin, kanamycin and gentamicin were added at 100μg/ml, 50μg/ml and 20μg/ml, respectively. Chlortetracycline hydrochloride (Sigma #26430-5G, autoclaved with liquid media) and L-arabinose were added at the indicated concentrations. Growth curves were generated (figure S2) by using a Beckman Coulter Multisizer 4 to measure the density of bacterial cultures every 30 minutes from backdilution through five hours of growth. Generation times were calculated as follows (Fuchs & Kroger, 1999): $t_g=(t-t_0)/[(\log(N_t/N_0))*3.3]$, where t_g=generation time, N=number of cells at time t, and N_0=number of cells at time t_0.

Construction of p*fliZ* and p*gfp*

The *fliZ* coding sequence from *Salmonella* 14028 and the *gfp* coding sequence from pDW5 (Cummings et al., 2006) were amplified using primers GAATTCGTATAGACTACCAGGAGTTC and GAGCTCGACTCTGCTACATCTTATGC, or GAATTCGATTTAAGAAGGAGATATAC and GAGCTCCAGGTCTGGACATTTATTTG, respectively. PCR products were cloned into pCR2.1, sequenced, digested out with EcoRI and SacI, and ligated into EcoRI/SacI

digested pJN105 (Newman & Fuqua, 1999). For both plasmids, L-arabinose induces transcription of the insert from P_{araBAD}.

Flow Cytometry

Samples were diluted into 0.5ml 0.2% sodium azide in filtered PBS. Unless otherwise noted (Figures S3-S5), 100,000 events were collected on a Becton Dickinson FACScan using the following parameters: FSC-H E01 log, SSC-H 400 log, SSC threshold of 129, FL1 750 log, FL2 and FL3 150 log. Data were analyzed using FlowJo (Tree Star).

Western Blotting

At indicated timepoints cells were pelleted and resuspended in PBS. Concentrations were determined using a Beckman Coulter Multisizer 4 and adjusted to 5×10^8 cells/ml. Proteins from 5×10^6 cells per sample were separated by SDS-PAGE electrophoresis on a 4–20% gel and transferred to nitrocellulose overnight at 30V. FlhC detected was normalized to DnaK detected for each lane and to FlhC/DnaK detected for the WT strain at the specified timepoint within that experiment. Primary antibodies used were α-3XFLAG (F3165, Sigma-Aldrich) and α-DnaK (SPA-880, Assay Designs). Goat α-Mouse secondary antibody (926-32280, 800CW) was purchased from Li-Cor. Western blots were visualized and proteins were quantified on the Li-Cor Odyssey CLx Imaging System.

β-galactosidase Activity

β-galactosidase activities were determined as described previously (Slauch & Silhavy, 1991) with the following modifications. At the indicated timepoints, bacterial cultures were centrifuged and resuspended in Z-buffer. Concentrations were determined using a Beckman Coulter Multisizer 4 and adjusted to 10^8 cells/ml. β-galactosidase activity of 100μl of cell suspension was measured in triplicate in 96-well plate format by the kinetic method of (Slauch & Silhavy, 1991) on a Molecular Devices SpectraMax M3 and is reported as [μmoles of ortho-nitrophenyl formed/minute/ml of cell suspension]·2×10^8.

Statistics

All statistical comparisons were performed using GraphPad Prism version 6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant U19 AI 090882A and a University of Washington Cellular and Molecular Biology Training Grant.

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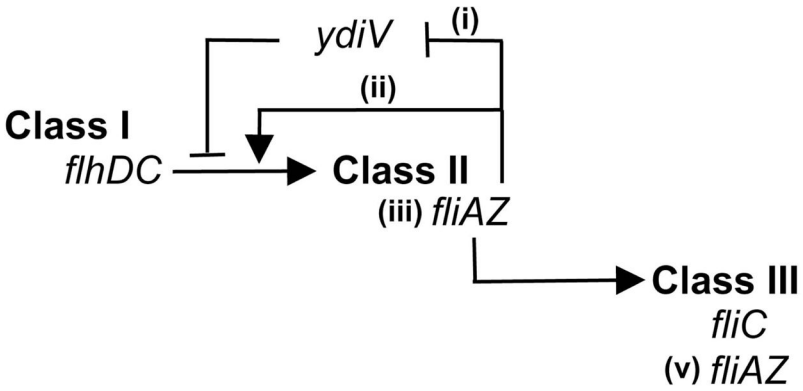


Figure 1.

The flagellar three class regulatory cascade. Proteins encoded by *flhDC*, the sole class I operon, form a complex required to activate class II gene transcription. Class II genes encode structural proteins of the platform that anchors the flagellum within the bacterial cell envelope (the basal body) and of the flexible linker that connects the platform with the filament (the hook), as well as the regulators *fliZ* and *fliA*. FliZ enhances class II gene expression, forming a positive feedback loop. Two mechanisms have been proposed to account for this loop. FliZ represses the class II repressor *ydiV* (i); thus *fliZ* and *ydiV* are mutually repressing repressors with opposing effects upon class II transcription. FliZ has also been shown to augment the level of the class I protein FlhC (ii). FliA is the sigma factor required for transcription of class III genes, including the flagellin filament protein FliC. The *fliAZ* operon is transcribed from both a class II (iii) and a class III (v) promoter, thus forming another positive feedback loop (the sigma factor activates transcription of its own operon).

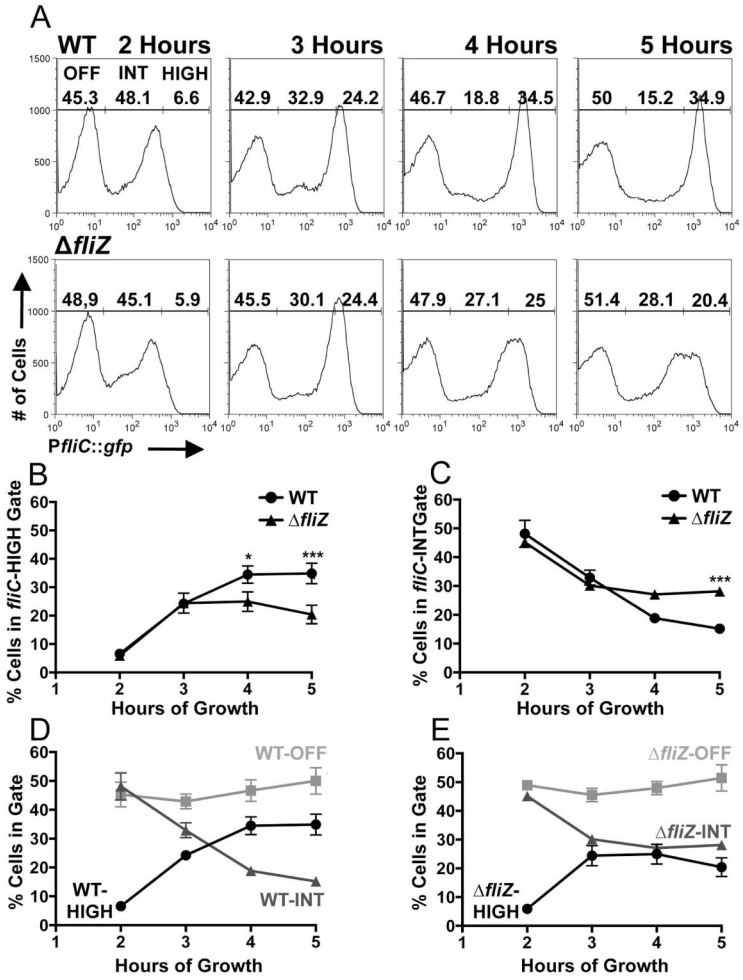


Figure 2.

fliZ is required to maximize the *fliC*-HIGH subpopulation at late timepoints. A) A transcriptional fusion of the *fliC* promoter to *gfp* (p*PfliC::gfp*) was used with flow cytometry to take a census of *fliC*-expression in WT (BC2117) and Δ*fliZ* (BC2119) *Salmonella* populations as the cultures progressed through five hours of growth after backdilution (figure S2). B and C) Percentages of cells falling within the *fliC*-HIGH (B) and *fliC*-INT (C) gates for WT and the Δ*fliZ* mutant. D and E) Kinetic plots of subpopulation flux for WT (D) and Δ*fliZ* (E). Histograms in (A) depict the results of one representative experiment; associated subpopulation percentages in (A) are the means of three independent experiments. Line graphs in (B–D) represent the means with standard deviation of the three independent experiments. One-way ANOVA with Tukey's multiple comparisons test was used to compare all strain/timepoint values. *p<0.05, **p<0.01, ***p<0.001.

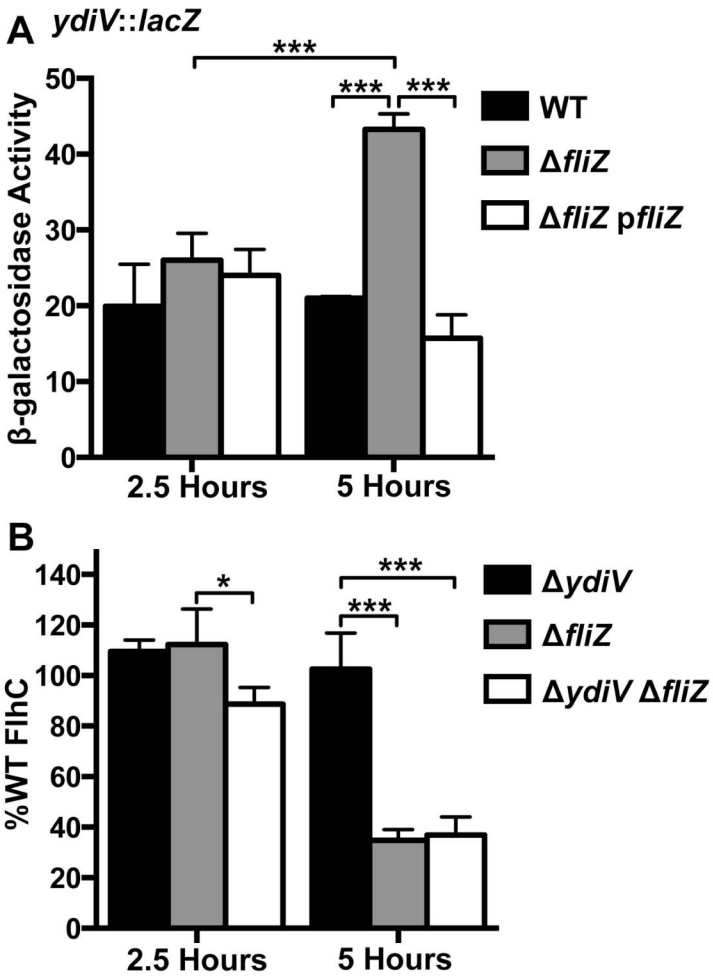
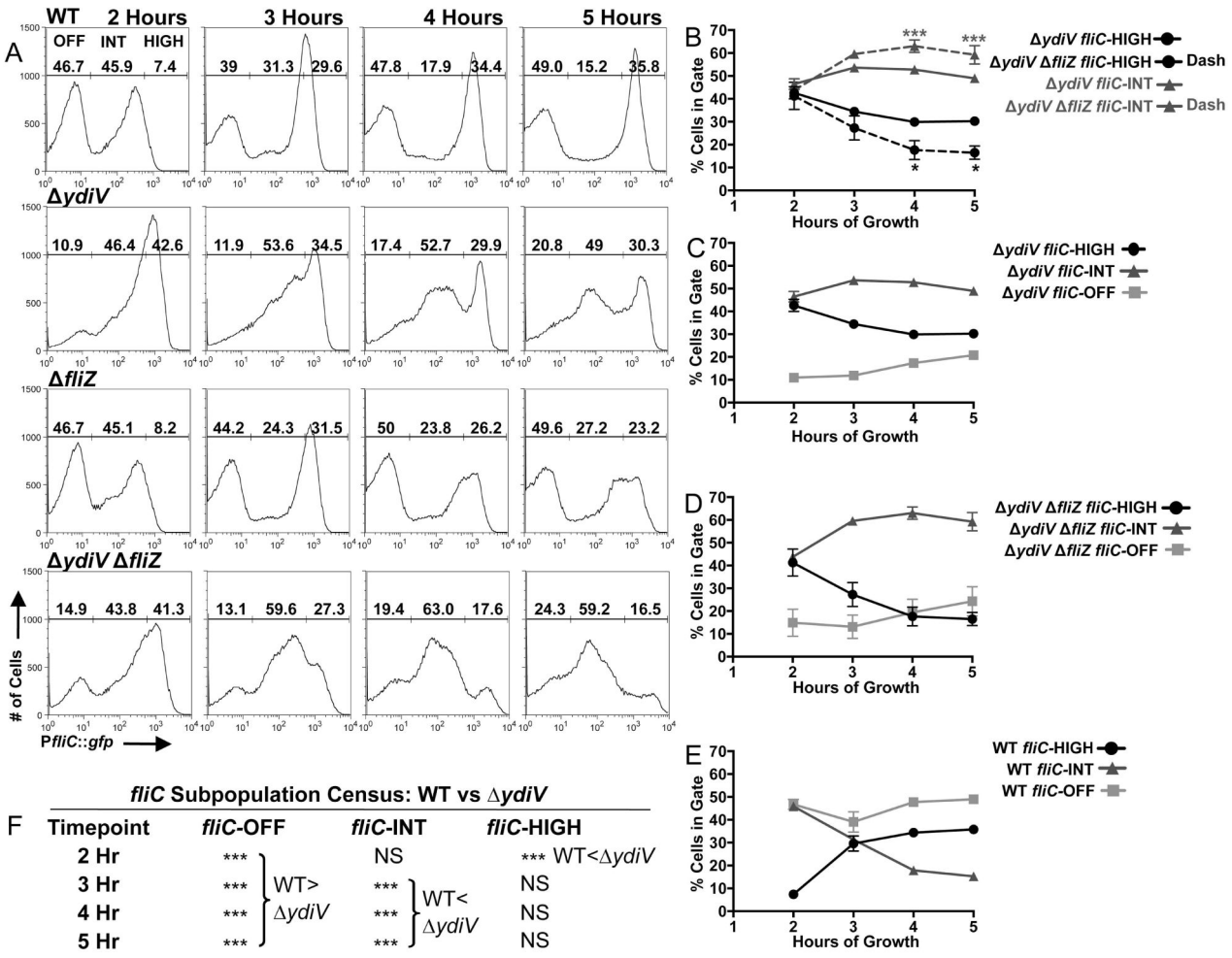


Figure 3.

fliZ impacts flagellar regulators via a mechanism that is independent of the mututally repressing repressors genetic circuit. A) A transcriptional, chromosomal β-galactosidase fusion to the *ydiV* promoter was used to characterize the kinetics of FliZ-mediated *ydiV* repression. 0.01% L-arabinose (to induce Δ*fliZ* transcription from P_{araBAD} in the complementation strain, Δ*fliZ* p*fliZ*) was added to all samples 2.5 hours before cells were lysed and β-galactosidase activity was measured. One-way ANOVA with Tukey's multiple comparisons test was used to compare all strain/timepoint values. (WT: BC2307, Δ*fliZ*: BC3276, Δ*fliZ* p*fliZ*: BC3277) B) FliZ increases the amount of FlhC by a *ydiV*-independent mechanism. A 3XFLAG tagged version of the class I protein FlhC was detected by western blot, normalized to DnaK expression, and adjusted to %WT expression by timepoint. One-sample t-tests with hypothetical value of 100 were used to compare mutant FlhC levels to WT. One-way ANOVA with Tukey's multiple comparisons test was used to compare FlhC values within timepoints. (WT (not shown, used to compute %WT values): BC2264, Δ*ydiV*: BC2265, Δ*fliZ*: BC2266, Δ*ydiV* Δ*fliZ*: BC2638) Bars in (A) and (B) represent the mean plus standard deviation of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.



fliC Subpopulation Census: WT vs ΔydiV

F

Timepoint	fliC-OFF		fliC-INT		fliC-HIGH
2 Hr	***	WT> ΔydiV	NS		*** WT<ΔydiV
3 Hr	***		***	WT< ΔydiV	NS
4 Hr	***		***		NS
5 Hr	***		***		NS

Figure 4.

Inversion of bistability in the absence of *fliZ* and *ydiV*. A) Flow cytometry census of *fliC* expression for WT (BC2117) and the Δ*ydiV* (BC2118), Δ*fliZ* (BC2119), and Δ*ydiV* Δ*fliZ* (BC2675) mutants. B) Comparisons of *fliC*-HIGH and *fliC*-INT subpopulations for the Δ*ydiV* and Δ*ydiV* Δ*fliZ* mutants. Black asterisks (bottom of figure) reference comparisons between population percentages in the *fliC*-HIGH gate, dark grey asterisks (top of figure) reference comparisons between population percentages in the *fliC*-INT gate. There were no significant differences between the Δ*ydiV* and Δ*ydiV* Δ*fliZ* strains in the sizes of the *fliC*-OFF subpopulations. C, D and E) Kinetics of subpopulation flux for strains Δ*ydiV* (C), Δ*ydiV* Δ*fliZ* (D), and WT (E, from this set of experiments, distinct from WT data represented in figure 2). F) Significance of comparisons between WT and the Δ*ydiV* mutant. Histograms in (A) depict the results of one representative experiment; associated subpopulation percentages in (A) are the means of three independent experiments. Line graphs in (B–E) represent the means with standard deviation of the three independent experiments. One-way ANOVA with Tukey's multiple comparisons test was used to compare all strain/timepoint values. *p<0.05, **p<0.01, ***p<0.001.

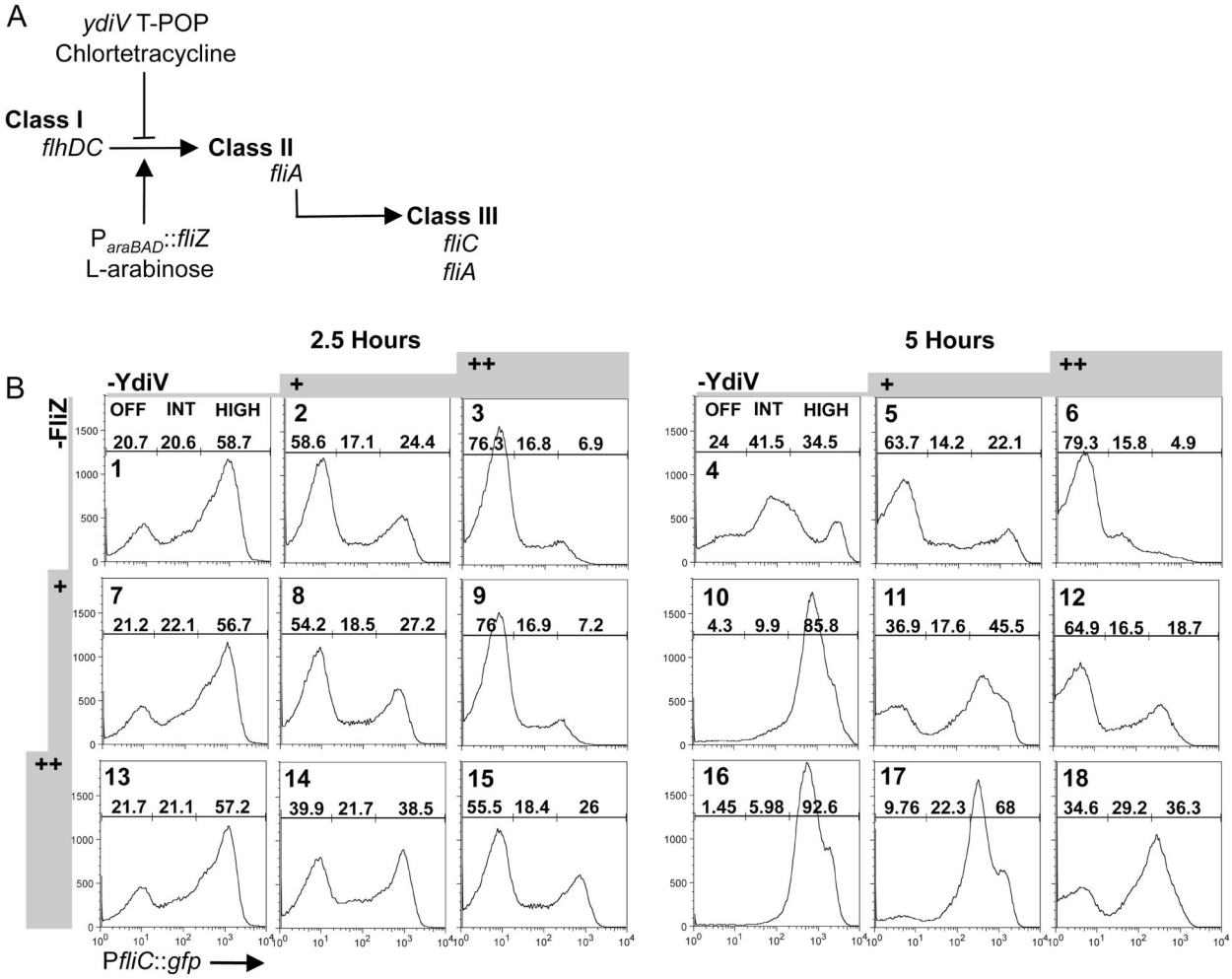


Figure 5.

Multi-layered heterogeneity regulates *fliC* expression. A) Schematic of our metered system (BC3314). YdiV and/or FliZ are expressed with the addition of chlortetracycline (+1.6 or ++3.1μg ml^{-1}) (T-POP) and/or L-arabinose (+0.01 or ++0.1%) (P_{araBAD}), respectively, and *fliC* transcription is measured using the GFP reporter fusion. B) FliZ's impact upon *fliC* transcription remains time-dependent, and heterogeneity in *fliC* expression is maintained in the metered system. Populations were grown in media +/− chlortetracycline for 2.5 or 5 hours, and L-arabinose was added 2.5 hours before harvesting the cells at both timepoints.

Table 1

Strains and plasmids used in this study

Strains		
BC2117	*Salmonella* Typhimurium 14028 *fljBA*::FRT p*fliC::gfp*, carbR	This Study
BC2118	*Salmonella* Typhimurium 14028 *fljBA*::FRT *ydiV*::FRT p*fliC::gfp*, carbR	This Study
BC2119	*Salmonella* Typhimurium 14028 *fljBA*::FRT *fliZ*::FRT p*fliC::gfp*, carbR	This Study
BC2675	*Salmonella* Typhimurium 14028 *fljBA*::FRT *ydiV*::FRT *fliZ*::FRT p*fliC::gfp*, carbR	This Study
BC2264	*Salmonella* Typhimurium 14028 *fljBA*::FRT FlhC::3XFLAG, kanR	Stewart et al., 2011
BC2265	*Salmonella* Typhimurium 14028 *fljBA*::FRT *ydiV*::FRT FlhC::3XFLAG, kanR	Stewart et al., 2011
BC2266	*Salmonella* Typhimurium 14028 *fljBA*::FRT *fliZ*::FRT FlhC::3XFLAG, kanR	This Study
BC2638	*Salmonella* Typhimurium 14028 *fljBA*::FRT *ydiV*::FRT *fliZ*::FRT FlhC::3XFLAG, kanR	This Study
BC2307	*Salmonella* Typhimurium 14028 *fljBA*::FRT *ydiV*::FKF::pKG136, kanR	This Study
BC3276	*Salmonella* Typhimurium 14028 *fljBA*::FRT *fliZ*::FRT *ydiV*::FKF::pKG136 pJN105, kanR gentR	This Study
BC3277	*Salmonella* Typhimurium 14028 *fljBA*::FRT *fliZ*::FRT *ydiV*::FKF::pKG136 p*fliZ*, kanR gentR	This Study
BC3314	*Salmonella* Typhimurium 14028 *fljBA*::FRT *fliZ*::FRT *ydiV* T-POP, p*fliZ* p*fliC::gfp*, tetR gentR carbR	This Study
Plasmids		
p*fliC::gfp*	pSRB1, P*fliC::gfp* construct in pBR322 vector	Cummings et al., 2006
pJN105	Vector, arabinose-inducible expression of inserts (ParaBAD)	Newman et al., 1999
p*fliZ*	*Salmonella* Typhimurium 14028 *fliZ* cloned into pJN105	This Study