Regulation of phenotypic heterogeneity permits *Salmonella* evasion of the host caspase-1 inflammatory response

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Sensing and adapting to the environment is one strategy by which bacteria attempt to maximize fitness in an unpredictable world; another is the stochastic generation of phenotypically distinct subgroups within a genetically clonal population. In culture, *Salmonella* Typhimurium populations are bistable for the expression of flagellin. We report that YdiV controls this expression pattern by preventing transcription of the sigma factor that recruits RNA polymerase to the flagellin promoter. Bistability ensues when the sigma factor is repressed in a subpopulation of cells, resulting in two phenotypes: flagellin expressors and flagellin nonexpressors. Although the ability to swim is presumably a critical survival trait, flagellin activates eukaryotic defense pathways, and *Salmonella* restrict the production of flagellin during systemic infection. *Salmonella* mutants lacking YdiV are unable to fully repress flagellin at systemic sites, rendering them vulnerable to caspase-1 mediated colonization restriction. Thus, a regulatory mechanism producing bistability also impacts *Salmonella* virulence.

nongenetic variation | pyroptosis | bet-hedging | fliC | pathogenesis

Deterministic adaptation in response to environmental signals is a common and well-characterized strategy by which bacteria endure changing external conditions. Another strategy is bet-hedging, in the form of stochastically generated, phenotypically distinct subgroups within a genetically clonal population (1). Diversity may improve the chance that a clone will thrive in a fluctuating environment. In some cases, deterministic adaptation and stochastic survival strategies are integrated. For example, as a population of *Bacillus subtilis* enters the stationary phase, the probability that a subgroup of cells will become competent increases in response to environmental cues (2). The presence of two stable phenotypes within a genetically clonal population, under homogenous conditions, is termed bistability (1).

When the pathogen *Salmonella enterica* serovar Typhimurium is grown under homogenous conditions in rich media, transcription of the flagellin monomer *fliC* is bistable, with well-defined subpopulations of *fliC*-OFF and *fliC*-ON cells (3). In this organism, a three-class transcriptional cascade orders the production of flagellar proteins (4). Class I genes encode a transcriptional activation complex that is required for class II expression; class II proteins include the sigma factor (σ^{28}, produced from the *fliA* gene) that recruits RNA polymerase to class III promoters, including the *fliC* promoter. Flagellar gene expression is controlled by environmental signals, and WT *Salmonella* strains capable of causing systemic infection regulate the production of flagellin in a host compartment-specific manner (3). During oral infection of mice, the pathogen breaches the mucosal layer of the gut, colonizes the lymphoid tissue (Peyer's patches) (5), and finally establishes a niche within phagocytic cells that ferry the organism to the spleen and other systemic tissues (6, 7). *Salmonella* transcribe *fliC* within the Peyer's patches; however, *fliC* is not expressed at systemic sites (3).

Salmonella reside within macrophages in vivo (6) and can translocate flagellin into the cytosol of host cells via the *Salmonella* pathogenicity island 1 (SPI-1) type III secretion system (8). Macrophages interpret intracytosolic flagellin as a danger signal, and initiate the proinflammatory cell death program pyroptosis in response (9, 10). Pyroptosis depends on expression of caspase-1 and is characterized by the maturation and release of the proinflammatory cytokines IL-1β and IL-18 and lysis of the macrophage. Mice deficient in caspase-1, IL-1β, or IL-18 are more susceptible to systemic *Salmonella* infection than WT mice, underscoring the importance of this pathway to host resistance (11, 12). Therefore, the down-regulation of flagellin in systemic tissues may prevent host cell death and inflammation.

In many bacterial species, motility is regulated by GGDEF and EAL domain proteins (13). We hypothesized that members of this protein family would regulate *Salmonella* flagellar genes in vivo. Here we show that an EAL-like protein, YdiV, represses *fliC* transcription in a subpopulation of cells in culture, producing bistability. YdiV also represses flagellar genes in systemic tissues, thereby protecting *Salmonella* from caspase-1–mediated bacterial clearance. We demonstrate that *ydiV* controls phenotypic heterogeneity in vitro and in vivo and modulates *Salmonella* virulence.

Results

EAL-Like Protein YdiV Suppresses the Inflammatory Capacity of *Salmonella*. PFAM (14) and homology searches were used to identify the full complement of genes encoding GGDEF and EAL domain proteins in *Salmonella* (Fig. S1 and *SI Materials and Methods*). A targeted deletion was constructed in each gene, yielding a panel of 22 mutant strains. To identify GGDEF and EAL domain proteins that control *Salmonella* interactions with host phagocytes, murine bone marrow-derived macrophages were infected with individual mutants, and macrophage lysis was measured by lactate dehydrogenase release. We hypothesized that this screen would enable us to identify mutants with altered virulence.

Twenty-one of the mutations had no effect on the ability of the bacteria to kill macrophages, but deletion of *ydiV* significantly increased the cytotoxicity of *Salmonella* (Fig. 1*A*). Δ*ydiV* is on average 2.16 times more cytotoxic than WT, as noted by Hisert et al. (15). Enhanced killing by Δ*ydiV* was accompanied by accelerated release of mature IL-1β, an endogenous substrate of caspase-1, into the supernatant (Fig. 1*B*). After 20 min, Δ*ydiV*-infected macrophages released 6.2-fold more mature IL-1β into the supernatant than WT-infected macrophages as determined by densitometry (Fig. 1*B*). Release of mature IL-1β by macrophages was dependent on caspase-1 (Fig. S2).

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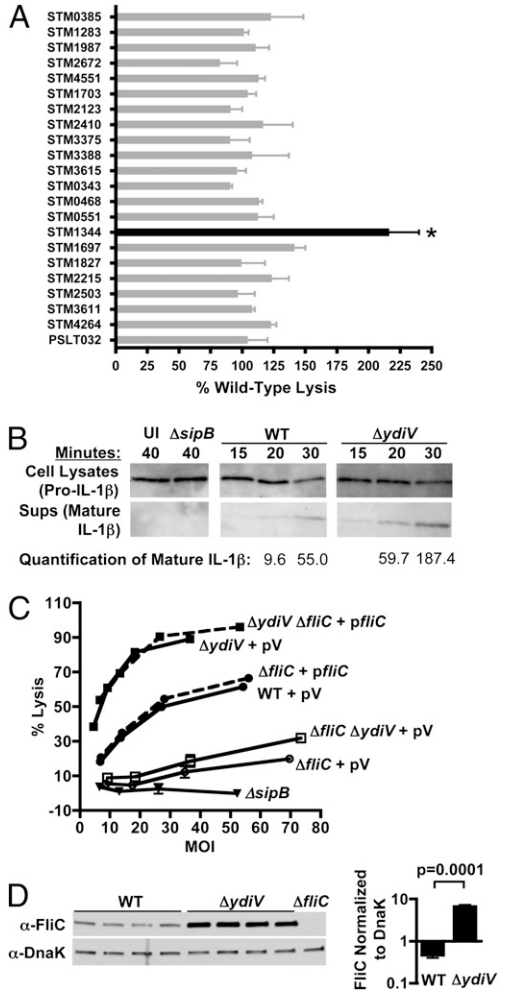

Fig. 1. An EAL domain protein modulates cytotoxicity by repressing *fliC*. (*A*) A panel of 22 GGDEF/EAL domain mutants was screened for the ability to trigger pyroptosis of bone marrow-derived macrophages. Macrophages were infected for 90 min at a multiplicity of infection (MOI) of 10. Only Δ*ydiV* (STM1344) was significantly more cytotoxic than WT *Salmonella* ($P < 0.05$). Error bars represent the SD of two independent experiments. (*B*) Macrophages infected with Δ*ydiV* (MOI = 12) release mature IL-1β into the supernatant more rapidly than macrophages infected with WT (MOI = 14). Values were determined using densitometry as described in *Materials and Methods*. Uninfected and Δ*sipB* (noncytotoxic control; MOI = 11) samples were collected at 40 min. (*C*) Hypercytotoxicity of Δ*ydiV* is dependent on *fliC*. Dashed lines represent strains in which the Δ*fliC* mutation was complemented. pV, vector control. (*D*) Δ*ydiV* overexpresses FliC. WT and Δ*ydiV* cultures were grown to exponential phase in LB and evaluated by Western blot analysis. Four independent samples of each strain are shown. FliC expression was normalized to the DnaK loading control.

Overexpression of FliC Accounts for the Hypercytotoxicity of the Δ*ydiV* Mutant. Because FliC triggers macrophage pyroptosis (9, 10), excess FliC expression could account for the enhanced cytotoxicity of Δ*ydiV*. Accordingly, *fliC* deletions were engineered into the Δ*ydiV* and WT backgrounds. Both the Δ*fliC* and Δ*ydiV* Δ*fliC* mutants killed poorly compared with the WT and Δ*ydiV* parent strains, demonstrating the contribution of flagellin to cytotoxicity in both backgrounds (Fig. 1*C*). Reintroduction of FliC completely restored the hypercytotoxic phenotype characteristic of the Δ*ydiV* parent strain. Western blot analysis confirmed that Δ*ydiV* cultures contained 14.15 times more FliC protein than WT (average WT FliC:DnaK ratio, 0.465; average Δ*ydiV* FliC:DnaK ratio, 6.578) (Fig. 1*D*). Taken together, these results demonstrate that YdiV represses *fliC*, and that the overproduction of flagellin by Δ*ydiV* stimulates macrophage pyroptosis and cytokine production.

YdiV Controls the Heterogeneity of *fliA* Transcription. Transcription of *fliC* in WT *Salmonella* is bistable (3). To evaluate whether YdiV regulates bistability, *fliC* transcription was examined in single cells using a chromosomally integrated reporter construct in which expression of GFP was driven from the *fliC* promoter (P*fliC*::*gfp*). This test revealed that the overexpression of FliC observed on Western blot analysis was due to a dramatic increase in the percentage of the bacterial population transcribing *fliC* in Δ*ydiV* cultures (Fig. 2 *A* and *B*). In contrast to WT, Δ*ydiV* cultures were unimodally *fliC*-ON; the average percentage of *fliC*-ON was 61.24% for WT and 91.44% for Δ*ydiV* ($P = 7.8 \times 10^{-10}$). Deletion of *ydiV* removed the persistently *fliC*-OFF subpopulation.

To test whether complementation could reconstitute *fliC* bistability in the Δ*ydiV* mutant, *ydiV* was cloned downstream of an arabinose-inducible promoter. Low concentrations of inducer restored a *fliC*-OFF population to the Δ*ydiV* P*fliC*::*gfp* strain; at the highest concentration of inducer, *fliC* transcription was reduced below the level of detection in most cells (Fig. 2*C*). We conclude that YdiV represses *fliC* expression, and that a YdiV-directed process occurring in a subpopulation of cells produces bistability of *fliC* transcription.

Products of the class I operon *flhDC* activate transcription of class II operons (Fig. 3*A*). The class II gene *fliA* encodes σ^{28}, which activates transcription of *fliC* and other class III genes. P*flhDC*::*gfp*, a transcriptional reporter construct in which GFP expression is driven from the class I promoter, was used to investigate whether YdiV controls flagellar gene expression from the top of the regulatory cascade (Fig. 3*A*). The mean fluorescent intensities (MFIs) of WT and Δ*ydiV* populations harboring this reporter were averaged over 10 trials per strain. These average MFIs did not differ significantly between the strains (Fig. 3*B*), indicating that YdiV does not regulate *flhDC* transcription. A *flhD-lacZ* translational reporter fusion revealed that the *flhDC* mRNA is translated equally by the WT and Δ*ydiV* strains (Fig. 3*C*).

The $FlhD_4C_2$ complex is posttranslationally regulated by proteolytic degradation. To examine whether YdiV regulates this process, a 3× FLAG epitope tag fused to the C terminus of FlhC was used to compare the amount of a master flagellar regulatory protein in the Δ*ydiV* and WT backgrounds (Fig. 3*D*). Western blot analysis revealed that Δ*ydiV* cultures contained similar amounts of FlhC::3×FLAG as WT cultures (Fig. 3*D*), demonstrating that YdiV does not reduce *fliC* expression by degrading $FlhD_4C_2$.

Wada et al. (16) showed that YdiV can bind to FlhD and prevent $FlhD_4C_2$ from binding the *fliA* promoter, thereby inhibiting *fliA* transcription. Thus, we hypothesized that YdiV might initiate phenotypic heterogeneity in flagellar gene expression by directly repressing *fliA*; this heterogeneity could then be transmitted down through the flagellar gene cascade to *fliC*. To test this hypothesis, we used a transcriptional reporter construct in which GFP expression was driven from the *fliA* promoter (Fig. 3*E*). The *fliA* transcriptional distributions of WT populations were bistable, with distinct peaks of *fliA*-OFF and *fliA*-ON cells. However, in the Δ*ydiV* strain, most cells were transcribing *fliA*; the average percentage of *fliA*-ON was 55.51% for WT and 87.03% for Δ*ydiV* ($P = 8.4 \times 10^{-17}$). Thus, the transcriptional profiles of *fliA* in WT and Δ*ydiV* closely mirrored those of *fliC* in the two backgrounds. We conclude that in WT *Salmonella*, YdiV prevents activation of *fliA* transcription in a subpopulation of cells. Because FliA is required for *fliC* expression, this repression causes bistability in the transcription of *fliC*.

YdiV Represses *fliC* Transcription in Vivo. *Salmonella* living within macrophages require ClpXP protease, which degrades $FlhD_4C_2$, to suppress *fliC* transcription (3, 17). YdiV also posttranslationally regulates the flagellar master regulatory proteins to repress *fliC*

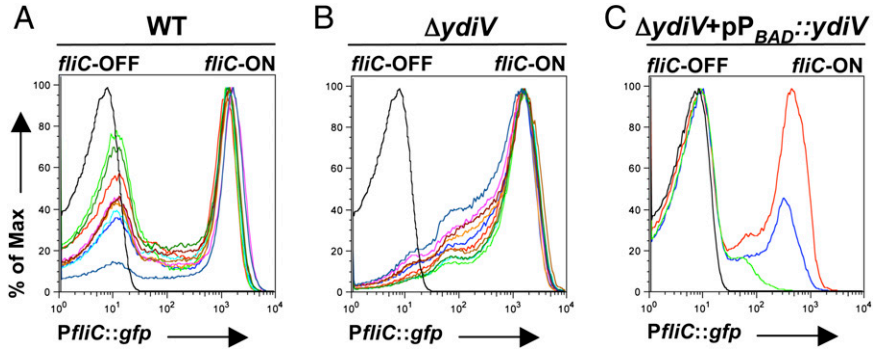

Fig. 2. Bistability of *fliC* depends on *ydiV*. (*A*) Transcriptional fusion of the *fliC* promoter to *gfp* reveals distinct populations of *fliC*-OFF and *fliC*-ON cells in WT when cultures are interrogated by flow cytometry. (*B*) In contrast, Δ*ydiV* demonstrates a unimodal distribution of predominantly *fliC*-ON cells. In *A* and *B*, 10 independent trials (colored traces) per strain are shown; the black trace represents GFP-negative control. MFIs of the *fliC*-ON populations are not significantly different between the two strains. *fliC*-ON is defined as all cells with fluorescence greater than that of the reporter-minus strain (average MFI, 1,084.7 for WT and 1,235.3 for Δ*ydiV*:; $P = 0.08$). (*C*) When expression of *ydiV* is driven from the *araBAD* promoter, increasing concentrations of inducer correspondingly reduce the *fliC*-ON population (red trace, 0.0002% L-arabinose; blue trace, 0.002%; green trace, 0.2%). All experiments were performed using exponential-phase cultures.

(16). Thus, we hypothesized that YdiV might repress *fliC* as *Salmonella* adapts to life within a host cell. To examine *fliC* transcription in vivo, WT and Δ*ydiV* strains harboring the P*fliC*::*gfp* reporter construct were orally inoculated into separate groups of C57BL/6 mice, and the percentage of bacteria transcribing *fliC* within splenocytes was determined. Indeed, 10-fold more Δ*ydiV* bacteria than WT bacteria activated the *fliC* transcriptional reporter (mean value, 0.90% vs. 0.09%), demonstrating that YdiV represses class III flagellar genes in vivo (Fig. 4). Thus, YdiV regulates phenotypic heterogeneity in vitro and in vivo and plays a role in the maintenance of the *fliC*-OFF state during growth in the spleen.

Δ*ydiV* Elicits an Enhanced Inflammatory Cytokine Response from the Host. Concentrations of IL-1β and TNF-α were higher in the sera of Δ*ydiV*-infected mice compared with WT-infected mice, indicating heightened activation of proinflammatory pathways and possibly pyroptotic cell death (Fig. 5 *A*–*C*). Activation of such mechanisms during colonization of systemic sites could promote bacterial clearance. If this were true, then we predicted that a Δ*ydiV* Δ*fliC* mutant would outcompete the Δ*ydiV* parent strain at systemic sites in the host.

YdiV Protects *Salmonella* from Pyroptosis. We used a murine competitive infection model to assess the benefit of YdiV-mediated *fliC* repression within systemic tissues. Orally introduced *Salmonella* initially colonize the gastrointestinal tract, then infect the Peyer's patches (5) and mesenteric lymph nodes (MLN), and finally travel through the blood and lymph to the spleen, liver, and other systemic sites (6, 7). When WT *Salmonella* was coinfected with its Δ*fliC* derivative, the two strains colonized the

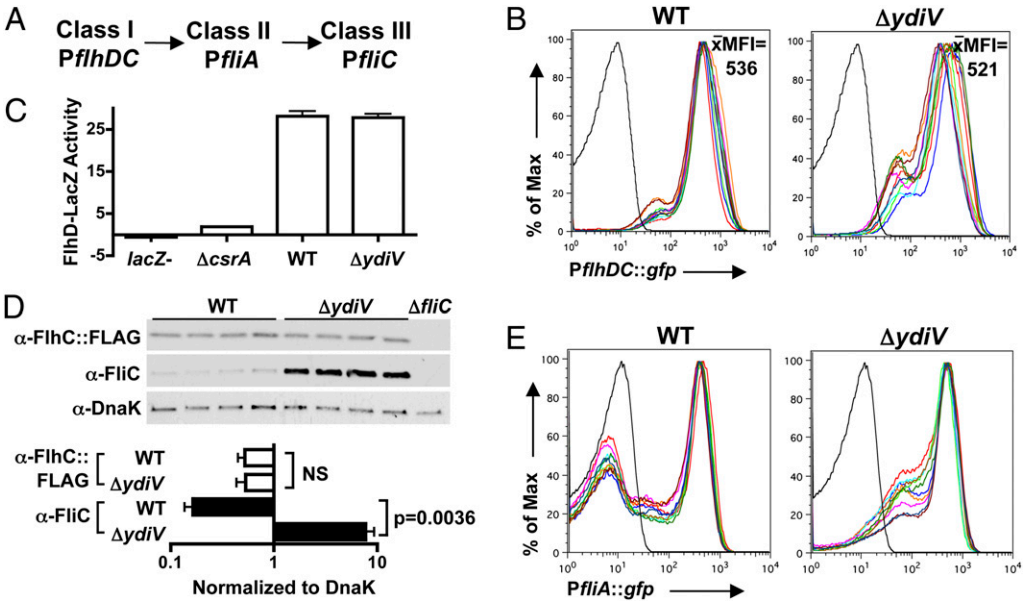

Fig. 3. YdiV controls the heterogeneity of *fliA* (σ^{28}) transcription. (*A*) The *Salmonella* flagellar gene regulatory cascade. (*B*) YdiV does not repress *flhDC* transcription. The average MFI of 10 independent trials per strain is reported. The black line represents GFP-negative control. (*C*) A *lacZ* translational reporter fusion to *flhD* reveals that YdiV does not repress the translation of class I proteins. *csrA* is required for efficient translation of the *flhDC* mRNA (32); thus, a Δ*csrA* mutant serves as a negative control. (*D*) Western blot analysis demonstrates that WT and Δ*ydiV Salmonella* contain similar amounts of FlhC protein. Four independent samples of each strain are shown. FlhC and FliC expression were normalized to the DnaK loading control for quantification. The Δ*fliC* mutant does not harbor the FlhC::3× FLAG construct. (*E*) YdiV represses *fliA* transcription. The black line represents GFP-negative control. All experiments were performed using exponential-phase cultures.

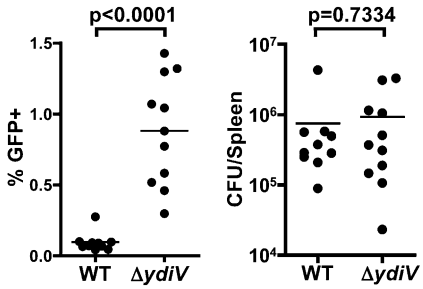

Fig. 4. Δ*ydiV Salmonella* overexpress *fliC* in systemic tissues. C57BL/6 mice were orally infected with 10^6 Δ*ydiV* or WT *Salmonella* harboring the P*fliC*::*gfp* reporter construct. Splenocytes were harvested from infected animals, and *fliC* expression by intracellular bacteria was quantified by flow cytometry as described in *Materials and Methods*. (*Left*) On average, 10 times more Δ*ydiV* than WT transcribed *fliC* in the spleen. (*Right*) To facilitate direct comparison of gene expression from Δ*ydiV* and WT bacteria, the two groups of mice were colonized to similar levels (*Materials and Methods*). Data from two independent experiments are combined.

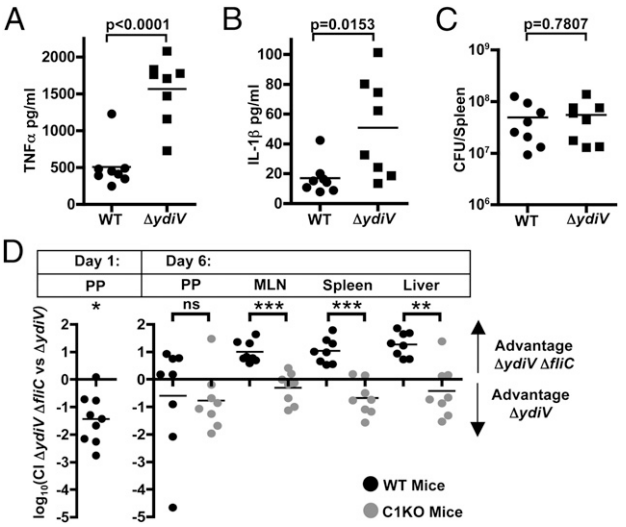

Fig. 5. YdiV is required to evade pyroptosis during infection. (*A*–*C*) Serum levels of TNFα and IL-1β were measured as described in *Materials and Methods* after oral infection with WT or Δ*ydiV*. Concentrations of TNFα (*A*) and IL-1β (*B*) were higher in the sera after infection with Δ*ydiV*. (*C*) To facilitate direct comparisons, the mice shown in *A* and *B* were colonized to similar levels with either WT or Δ*ydiV Salmonella* (*Materials and Methods*). (*D*) Mice were orally gavaged with 10^6 cfu of Δ*ydiV* (strain 2) and Δ*ydiV* Δ*fliC* (strain 1), for a total of 2×10^6 cfu/mouse. Tissue colonization was measured at the indicated times, and the CIs were calculated as described in *Materials and Methods*. Δ*ydiV* outcompetes Δ*ydiV* Δ*fliC* in colonizing the Peyer's patches after 1 d of infection (n = 9 mice; data combined from two independent experiments). However, on day 6, Δ*ydiV* Δ*fliC* outcompetes Δ*ydiV* in the systemic tissues. This advantage disappears when the same experiment is performed in caspase-1 KO animals. $*P < 0.01$; $**P < 0.001$;$***P < 0.0001$.

Peyer's patches, MLNs, and spleen of C57BL/6 mice equally well (Fig. S3*A*). Next, the Δ*ydiV* strain was competed against its Δ*fliC* derivative. Strikingly, on day 1 of the infection Δ*ydiV* was significantly more likely than Δ*ydiV* Δ*fliC* to be found in the Peyer's patches ($P = 0.0013$, one-sample t test with hypothetical mean of 0) (Fig. 5*D*). Despite this considerable advantage, on day 6, the Δ*ydiV* Δ*fliC* strain consistently outcompeted the Δ*ydiV* parent strain in the MLN, spleen, and liver (MLN, $P = 0.0002$; spleen, $P = 0.0003$; liver, $P < 0.0001$; one-sample t tests with hypothetical mean of 0) (Fig. 5*D*). No difference was observed in the Peyer's patches ($P = 0.4040$), where WT *Salmonella* normally transcribe flagellin late in infection (3). These data show that YdiV-dependent maintenance of the *fliC*-OFF state at systemic sites is critical for maximal colonization. When even a small percentage of *Salmonella* infecting systemic tissues are in the *fliC*-ON state (Fig. 4), a protective host response ensues (Fig. 5*D*).

The accelerated release of IL-1β by macrophages infected with Δ*ydiV* compared with WT (Fig. 1*B*), along with the enhanced serum levels of both IL-1β and TNF-α in Δ*ydiV*-infected mice (Fig. 5 *A*–*C*), suggest that YdiV-mediated *fliC* repression at systemic sites protects the pathogen against caspase-1–dependent proinflammatory pathways. To test this hypothesis, we competed Δ*ydiV* against Δ*ydiV* Δ*fliC* in caspase-1–deficient mice and measured bacterial colonization on day 6. In the MLN, spleen, and liver, the competitive indices (CIs) in caspase-1 deficient mice were significantly different from those observed during infection of WT animals (*P* values for comparisons between WT and caspase-1–deficient mice (Peyer's patches, $P = 0.8245$; MLN, $P < 0.0001$; spleen, $P < 0.0001$; liver, $P = 0.0004$; two-sample t tests) (Fig. 5*D*). The distributions of CIs were in fact skewed toward an advantage for Δ*ydiV* in the caspase-1 deficient mice, although this effect was only statistically significant in the spleen (Peyer's patches, $P = 0.0769$, MLN, $P = 0.1612$, spleen: $P = 0.0165$, liver: $P = 0.2405$, one-sample t tests with hypothetical mean of 0) (Fig. 5*D*). These data show that YdiV-mediated *fliC* repression allows *Salmonella* to circumvent a specific, protective host response.

Discussion

YdiV bears no close homology to any currently characterized protein domain. Its nearest relatives are the EAL domain proteins, many of which are phosphodiesterases that degrade the bacterial second messenger c-di-GMP. However, YdiV is a very poor match to the EAL domain consensus sequence, and neither degrades nor binds c-di-GMP in vitro (18). Wada et al. (16) demonstrated that YdiV binds to FlhD and inhibits the transcription of the class II gene *fliA*. Intriguingly, Wada et al. observed that YdiV deterministically repressed flagellar genes in response to nutritional cues. Based on those findings and our present observations, we conclude that YdiV controls both stochastic and deterministic gene expression, tuning flagellar gene transcription in reponse to environmental signals.

The Δ*ydiV* mutant is attenuated compared with WT in oral (average $\log_{10}$ CI, −0.7588; $P = 0.0258$) as well as i.p. and i.v. (15) infections (Fig. S3*B*). Intriguingly, the spread in our oral competition data demonstrates variation in the ability of Δ*ydiV* to compete with WT in the deep tissues, which is consistent with the heterogeneity in *fliC* expression that we observed in Δ*ydiV* populations ex vivo (Fig. 4) and the heterogeneity in the proinflammatory serum cytokine response that we found in mice infected with the Δ*ydiV* mutant (Fig. 5 *A*–*C*). In fact, deletion of *ydiV* increased heterogeneity in vivo, in contrast to our in vitro results. The majority of bacteria isolated from both WT- and Δ*ydiV*-infected mice were *fliC*-OFF (Fig. 4), most likely due to other regulatory mechanisms that repress *fliC* in systemic tissues. Indeed, we have previously shown that the protease ClpX represses *fliC* expression in macrophages (3). The presence of multiple mechanisms to ensure tight control of *fliC* reflects the importance of maintaining the *fliC*-OFF state in specific environments, including systemic tissues (Fig. 5*D*).

Genetic circuits that produce bistable patterns of gene expression often include positive feedback (1), and *fliA* is subject to two positive feedback loops (Fig. S4). The first loop increases *fliA* transcription by elevating the concentration of $FlhD_4C_2$ within the cell (19). In the second loop, FliA (σ^{28}) directly recruits RNA polymerase to the *fliA* promoter (20). Because nonlinear gene expression dynamics are essential components of other bistable circuits (1, 21), one or both of these loops might produce an acceleration of *fliA* transcription that could

potentially result in bistable expression of class III genes. In contrast, we discovered that bistability of *fliC* expression in *Salmonella* is regulated by YdiV, which controls a mechanism that operates upstream of both positive feedback loops (Fig. S4).

YdiV controls the proportion of cells in a population transcribing *fliA* and, consequently, the proportion transcribing *fliC*. This is likely accomplished by preventing the flagellar master regulatory complex from activating class II transcription, effectively locking a subpopulation of cells into *fliA*-OFF mode. Working with the laboratory *Salmonella* strain LT2, Saini et al. (22) demonstrated that flagellar class III transcription becomes unimodal when σ^{28} is unable to activate transcription at the *fliA* promoter. Therefore, we propose that *ydiV* controls bistability of *fliA* and *fliC* by ensuring that a subpopulation of cells is unable to activate *fliA* transcription (Fig. 3*E*), whereas the σ^{28} autoactivation loop likely controls the acceleration of *fliA* expression once *fliA* transcription begins (Fig. S4).

The Δ*ydiV* mutant is hypercytotoxic (Fig. 1 *A* and *C*) (15). We have demonstrated that this phenotype requires *fliC*, and that many more cells in Δ*ydiV* populations are transcribing *fliC* than in WT populations. An increase in the *fliC*-ON population could potentially have pleiotropic effects on cytotoxicity in this in vitro assay; expression of flagellar genes might increase both access to macrophages and the ability to deliver the toxic protein FliC. We used cytotoxicity as a screen to identify mutants that we hypothesized would have altered virulence, and thus focused subsequent experiments on the mechanism by which *ydiV* controls heterogeneity of *fliC* expression and the role of *ydiV* in maintaining the anatomical restriction of *fliC* expression in vivo.

Previous studies have demonstrated that *Salmonella* downregulate *fliC* during growth in the spleen (3), that FliC triggers caspase-1–dependent death of macrophages (10), and that caspase-1–deficient mice are more susceptible to *Salmonella* infection (11, 12). Miao et al. (23) recently found that an i.p.-introduced strain of *Salmonella*, engineered to express *fliC* within a macrophage, induces pyroptosis of host cells and is outcompeted by isogenic WT bacteria at systemic sites. Here we show that depletion of YdiV naturally relieves *fliC* repression in a small percentage of systemic *Salmonella*, which is sufficient to trigger the caspase-1–dependent protective host response. This response is highly localized, since coinfecting *fliC*-OFF bacteria are not cleared to the same extent as the *fliC*-ON strain. We can eliminate the possibility that the production of FliC causes a metabolic burden that constrains colonization. If FliC production posed a disadvantage, we would not expect the *fliC*-expressing Δ*ydiV* strain to able to colonize caspase-1 KO mice as as well or better than the Δ*fliC* Δ*ydiV* strain (Fig. 5*D*).

Many pathogenic bacteria either repress flagellar genes throughout infection [e.g., enteric *Yersinia* (24), *Listeria* (25)] or have completely lost the ability to express flagellar genes [e.g., *Shigella* (26), *Yersinia pestis* (24)], thus avoiding the potent host defense response to flagellin. In contrast, *Salmonella* have evolved to regulate flagellar genes in a host compartment-specific manner (3). This allows the pathogen to benefit from flagellar gene expression in favorable host environments. Flagellin is a proinflammatory molecule, and motile *Salmonella* grow more quickly than nonmotile strains in the inflamed intestine, because they are able to migrate to the nutrient-rich zone close to the epithelium (27). However, our data demonstrate that tight repression of flagellin is required for *Salmonella* to thrive in systemic tissues. YdiV modulates phenotypic heterogeneity in *Salmonella* populations; given the costs and rewards involved in flagellar gene expression, this specialization appears to provide a mechanism by which the bacteria maximize fitness in the host. Thus, a mechanism controlling bistable gene expression significantly impacts the ability of an organism to cause infection.

Materials and Methods

Bacterial Strains, Plasmids and Culture Conditions. The strains used in this study are listed in Table S1. As described previously, mutants were generated in the *Salmonella enterica* serovar Typhimurium 14028 background (28), and *fliC* and *flhDC* GFP reporters were constructed (3). The *flhD-lacZ* single-copy chromosomal translational fusion (29) has the reporter fused to the first 60 amino acids of FlhD. β-galactosidase activity was determined as described previously (30), and is reported as (activity units per A_{600} unit per mL of cell suspension) × 2,000, where activity units are μmol of ortho-nitrophenyl formed/min. The FlhC::3× FLAG tag fusion was a gift from the Rao laboratory (19). The *fliA* GFP reporter, p*fliC*, and pP_{BAD}::*ydiV* are described in *SI Materials and Methods*. Strains were grown in LB at 37 °C with aeration unless noted otherwise. Carbenicillin, kanamycin, and gentamicin were added at 100, 50, and 20 μg/mL, respectively.

Mice. C57BL/6 WT mice (Jackson Laboratories) and Casp1$^{-/-}$ mice (a gift from R. Flavell, Yale University, New Haven, CT) were housed under specific pathogen-free conditions in accordance with the University of Washington's Institutional Animal Care and Use Committee guidelines.

Competitive Infections. Bacteria were grown overnight in LB without aeration. Fasting mice received 10^6 of each strain orally in 200 μL of 5% sodium bicarbonate/PBS. Organs were harvested at indicated time points. CIs were calculated as the output ratio of strain 1 to strain 2 divided by the input ratio of strain 1 to strain 2 (31). All statistical tests were applied to the $\log_{10}$ of the CIs. A one-sample, two-tailed *t* test with a hypothetical mean of 0 was used to determine whether either strain demonstrated a significant colonization advantage within the individual tissues. A two-sample, two-tailed *t* test was used to compare the results obtained in WT mice with those obtained in caspase-1 KO mice.

GFP Expression ex Vivo. Fasting mice were orally gavaged with 10^6 bacteria in 200 μL of 5% sodium bicarbonate/PBS. The mice received 2 mg/mL of ampicillin in their drinking water during the fast and throughout the course of infection to maintain reporter constructs (3). Because the ability of Δ*ydiV* to compete with WT is variable (Fig. S3*B*), we harvested bacteria from mice at approximately the same stage of infection to evaluate gene expression by Δ*ydiV* and WT bacteria that had replicated to similar numbers within the host. Mice were killed on days 4 and 5, and bacteria from the spleens were analyzed as described previously (3).

Flow Cytometry. Exponential-phase bacteria were analyzed using a FACScan flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar). Cytokines in processed blood (see *SI Materials and Methods* for processing methods) were measured on a FACSCanto flow cytometer (BD Biosciences) using cytometric bead arrays (BD 552364 and 560232) in accordance with the manufacturer's instructions. Data were analyzed using FCAP Array software (BD Biosciences). Given the variable ability of Δ*ydiV* to compete with WT, we collected blood from infected mice on days 5 and 6 to evaluate host cytokine production in mice with similar levels of bacterial colonization.

Macrophages. Bone marrow macrophages derived from C57BL/6 mice were isolated and cultured as described previously (9). For macrophage infections, overnight bacterial cultures were diluted 1:15 and grown for 3 h, washed, and then resuspended in PBS. Bacteria were spun down onto macrophages at 200 × *g* for 10 min. Lactate dehydrogenase release was measured after a total of 90 min using the Promega CytoTox 96 Kit (G1780). Macrophages were seeded 2×10^4 per well in 96-well plates. Cytotoxicity was calculated as described previously (9).

Immunoblot Analysis. Western blot analyses were performed on exponential-phase bacterial cultures using standard techniques (3) and developed with Amersham ECL Western Blotting Detection Reagents. Anti-3× FLAG antibody (F3165; Sigma-Aldrich) and anti-mouse HRP-antibody (NA931; Amersham) were used to detect the FlhC::3× FLAG fusion. IL-1β detection was performed using 2×10^5 macrophages per well in 24-well plates (*SI Materials and Methods*). Macrophages were treated overnight with 100 ng/mL of LPS and infected in media containing 5 mM glycine (9). Bacteria were prepared as for the cytotoxicity experiments. Processed cytokine was detected using anti–IL-1β antibody (AF-401-NA; R&D Systems) and HRP-conjugated anti-goat antibody (SC2350; Santa Cruz Biotechnology). National Institutes of Health ImageJ version 1.63 was used to quantitate protein expression.

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