Supporting Information

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SI Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions. Mutants were generated in the *Salmonella* typhimurium 14028 background as described previously (1). Strains can express only *fliC* flagellin because of the deletion of *fljBA* (2). To construct the *fliA* reporter, the P*fliA*::*gfp* fusion was cloned from pDW3 (3) using primers 5′-CCCGAGTGGCTGATTTTATTCTGCTC-3′ and 5′-CGGGGCTTATTTGTATAGTTCATCCA-3′, ligated into pCR2.1, then digested out with BsoBI and EagI and ligated into pBR322. p*fliC* is pRP3, a pBR322 derivative in which *fliC* expression is driven from the native *fliC* promoter; this plasmid was a gift from Kelly Hughes (University of Utah, Salt Lake City, UT). To construct P_{araBAD}::*ydiV*, the *ydiV* gene was PCR-amplified from BC1662 using primers 5′-GAATTCATGGGACTGGCGTAATGATTG-3′ and 5′-GAGCTCTTATCGCTGAACGAGTTTAATG-3′. The resulting fragment was cloned into pCR2.1, sequence-confirmed, digested out with EcoRI and SalI, and cloned into pJN105 (4). The *flhD-lacZ* single-copy chromosomal translational fusion was generated as described previously (5). Plasmid pCE40 was integrated into an in-frame FRT site generated using PKD13. The *STM4226/7*::*tetRA* construct was a gift from Joyce Karlinsey (University of Washington, Seattle, WA); the *tetRA* tetracycline resistance cassette is inserted 83 bp downstream of the *STM4226* stop codon.

GFP Expression ex Vivo. Bacteria living in the spleens of infected mice were recovered, analyzed, and enumerated as described previously (3). By this point in the colonization process, residual fluorescence from the input culture is diluted below the level of detection (3). Statistical significance was determined using the Student nonpaired two-sample two-tailed *t* test.

Competitive Infections. Tissues were collected into 2 mL of sterile PBS on ice and homogenized. Then Triton X-100 was added to a final concentration of 0.5% to lyse cells. Homogenates were serially diluted and plated to selective media to determine colonization by each of the two strains.

Macrophages. Bone marrow macrophages derived from C57BL/6 mice were harvested and cultured as described previously (6). Macrophages were replated 24 h before infection in antibiotic-free DMEM with 5% FBS. At 1 h before infection, the medium was aspirated and replaced.

Immunoblot Analysis. For IL-1β detection, 2×10^5 bone marrow-derived macrophages were seeded per well of a 24-well plate and treated overnight with 100 ng/mL of LPS. At 1 h before the infection, macrophages were washed three times in PBS, and 5 mM glycine was added to the media (6). Bacteria were prepared as for cytotoxicity experiments. Macrophage culture supernatants were collected at indicated times, filtered for 2 min at 2,000 rpm using a 0.22-μm SPIN-X centrifuge filter (Costar), concentrated for 50 min at 20,800 × *g* and 4 °C using a Microcon 10,000-MWCO filter (Amicon), combined with SDS/PAGE sample buffer to 1×, and then stored at −20 °C. Macrophages were lysed in 25 μL of 2× SDS/PAGE sample buffer and then incubated for 10 min on ice; lysates were stored at −20 °C. Samples were thawed, boiled for 10 min, run on a 15% SDS/PAGE gel, and transferred to nitrocellulose overnight. Processed cytokine was detected using anti–IL-1β antibody (AF-401-NA; R&D Systems) and quantified using NIH ImageJ version 1.63.

Serum Cytokine Quantitation. Blood from infected C57BL/6 mice was collected by submandibular puncture into Microtainers (365967; BD Biosciences). Spleen homogenates were serially diluted and plated to determine colonization. The microtainers were stored for 1 h at 4 °C, then spun for 2 min at 20,800 × *g* to separate serum from other blood components. Serum was stored at −20 °C until analysis. Cytokine concentrations were measured on a FACSCanto flow cytometer (BD Biosciences) using the Mouse Inflammation Kit Cytometric Bead Array (552364) and the Mouse/Rat Soluble Protein Master Buffer Kit (558266) with the Mouse IL-1β Flex Set (560232).

Identification of GGDEF and EAL Domain-Containing Proteins and Construction of Fig. S1. *Salmonella* genes encoding GGDEF and EAL domains were identified using the Comprehensive Microbial Resource's TIGRFAM/Pfam search function. All hits were then used as starting points for individual homology searches using the Protein vs. All Alignment function of JCVI-CMR. Individual GGDEF and EAL domains were ranked for homology to the canonical sequence based on the bit score assigned by Pfam. Scores of <20 were classified as low, scores of 21–200 as medium, and scores of >200 as high. Protein domains were identified and mapped using the SMART database (http://smart.embl-heidelberg.de/).

1. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645.
2. Bonifield HR, Hughes KT (2003) Flagellar phase variation in *Salmonella enterica* is mediated by a posttranscriptional control mechanism. *J Bacteriol* 185:3567–3574.
3. Cummings LA, Wilkerson WD, Bergsbaken T, Cookson BT (2006) In vivo, fliC expression by *Salmonella enterica* serovar Typhimurium is heterogeneous, regulated by ClpX, and anatomically restricted. *Mol Microbiol* 61:795–809.
4. Newman JR, Fuqua C (1999) Broad-host-range expression vectors that carry the L-arabinose-inducible Escherichia coli araBAD promoter and the araC regulator. *Gene* 227:197–203.
5. Ellermeier CD, Janakiraman A, Slauch JM (2002) Construction of targeted single copy lac fusions using λ Red and FLP-mediated site-specific recombination in bacteria. *Gene* 290:153–161.
6. Fink SL, Cookson BT (2006) Caspase-1–dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cell Microbiol* 8:1812–1825.

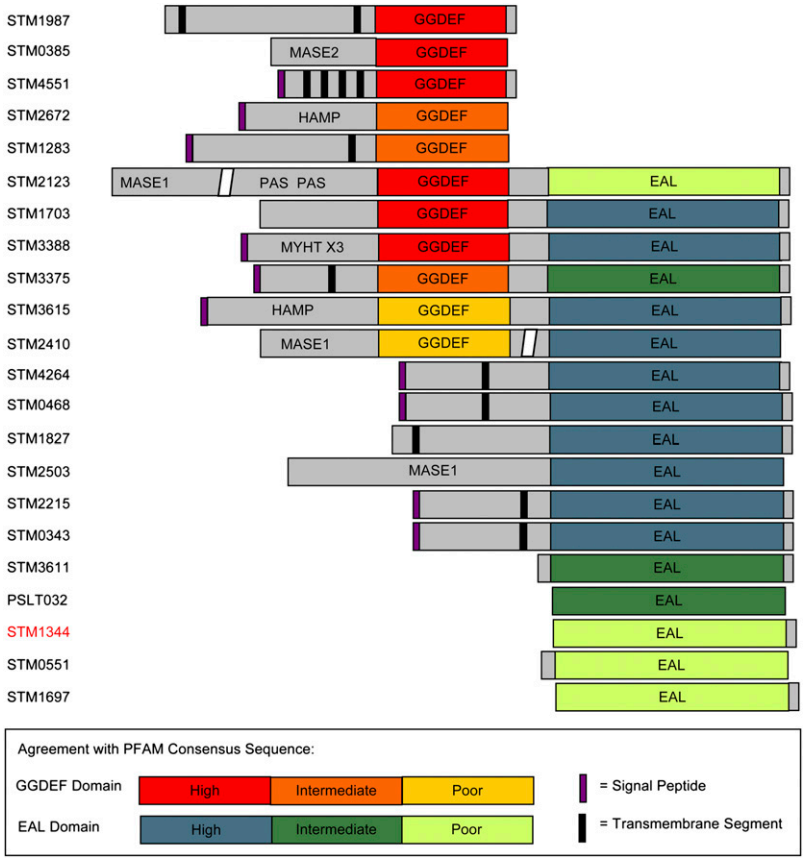

Fig. S1. Domain structures of GGDEF and EAL domain proteins encoded by *Salmonella* typhimurium. STM1344 is *ydiV*.

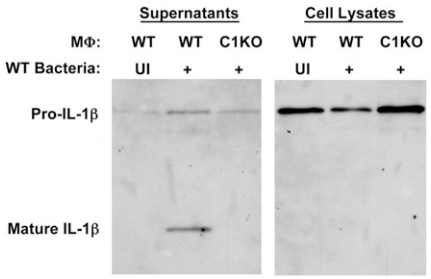

Fig. S2. Release of mature IL-1β is caspase-1–dependent. The cleaved form of IL-1β is found only in supernatants of infected WT macrophages, not caspase-1 KO macrophages; the proform is visible in cell lysates from both strains of macrophages. Macrophages were infected for 40 min. UI, uninfected.

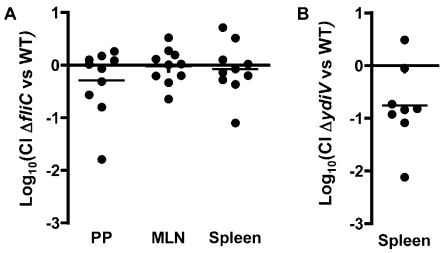

Fig. S3. (*A*) WT and Δ*fliC* are equally fit in vivo. Mice were orally gavaged with 10^6 cfu of Δ*fliC* (strain 1) and WT (strain 2), for a total of 2×10^6 cfu/mouse. On day 5 after infection, the animals were killed, tissue colonization was measured, and the competitive indices (CI) were calculated as described in *Materials and Methods*. Because *fliC* is normally repressed at systemic sites of infection, deletion of flagellins provides no competitive advantage in the WT mouse background, where *fliC* is subject to appropriate regulation. The WT and Δ*fliC* strains colonize equally well in all tissues analyzed ($P = 0.1826$, 0.8346, and 0.6820 for Peyer's patches, mesenteric lymph nodes, and spleens, respectively; one-sample *t* tests with hypothetical mean of 0). (*B*) Δ*ydiV* is attenuated for growth in the spleen. Mice were orally gavaged with 10^6 cfu of Δ*ydiV* (strain 1) and WT (strain 2), for a total of 2×10^6 cfu/mouse. On day 6 after infection, the animals were killed, tissue colonization was measured, and the CIs were calculated as described in *Materials and Methods*. The average $\log_{10}$(CI) is −0.7588 ($P = 0.0258$, one-sample *t* test with hypothetical mean of 0).

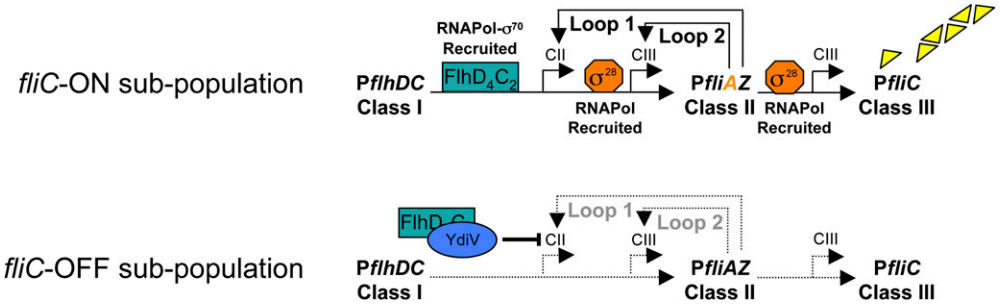

Fig. S4. YdiV controls bistable flagellar gene expression in *Salmonella*. (*Upper*) *fliA* is transcribed from both a class II promoter (CII) and a class III promoter (CIII), each of which is subject to a positive feedback loop. $FlhD_4C_2$ initiates transcription of *fliA* by recruiting the RNA polymerase-σ^{70} complex to the *fliA* CII promoter. The second gene in the *fliA* operon, *fliZ*, then posttranslationally increases the amount of $FlhD_4C_2$ to complete loop 1. In the second positive feedback loop, FliA (σ^{28}) drives its own expression by recruiting RNA polymerase to the *fliA* CIII promoter. (*Lower*) YdiV prevents $FlhD_4C_2$ from initiating transcription at class II promoters. Thus, YdiV acts upstream of the two *fliA* positive feedback loops to lock a subpopulation of cells in *fliA*/*fliC*-OFF mode.

Table S1. Strains and plasmids used in this study

Strain no. or plasmid name	Genotype or plasmid backbone vector	Source
Strain		
BC156	*Salmonella* typhimurium 14028	American Type Culture Collection
BC1662	BC156 *fljBA*::FRT	This study
BC1642	BC1662 *STM1703*::FRT	This study
BC1643	BC1662 *ydiV*::FRT	This study
BC1644	BC1662 *STM2123*::FRT	This study
BC1645	BC1662 *STM3611*::FRT	This study
BC1646	BC1662 *STM2410*::FRT	This study
BC1647	BC1662 *PSLT032*::FRT	This study
BC1648	BC1662 *STM1827*::FRT	This study
BC1649	BC1662 *STM0343*::FRT	This study
BC1650	BC1662 *STM3615*::FRT	This study
BC1651	BC1662 *STM3388*::FRT	This study
BC1652	BC1662 *STM0468*::FRT	This study
BC1653	BC1662 *STM4264*::FRT	This study
BC1654	BC1662 *STM2503*::FRT	This study
BC1655	BC1662 *STM3375*::FRT	This study
BC1656	BC1662 *STM2215*::FRT	This study
BC1657	BC1662 *STM1987*::FRT	This study
BC1658	BC1662 *STM2672*::FRT	This study
BC1659	BC1662 *STM4551*::FRT	This study
BC1660	BC1662 *STM1283*::FRT	This study
BC1661	BC1662 *STM0385*::FRT	This study
BC1693	BC1662 *STM1697*::FRT	This study
BC1694	BC1662 *STM0551*::FRT	This study
BC2062	BC1662 *sipB*::FRT	This study
BC725	BC1662 pBR322 CarbR	This study
BC1677	BC1643 pBR322 CarbR	This study
BC726	BC1662 *fliC*::FRT pBR322 CarbR	This study
BC1963	BC1662 *fliC*::FRT pRP3 CarbR	This study
BC1846	BC1643 *fliC*::FRT pBR322 CarbR	This study
BC1961	BC1643 *fliC*::FRT pRP3 CarbR	This study
BC1964	BC1643 pKAS32::P*fliC*::*gfp* CarbR	This study
BC1965	BC1662 pKAS32::P*fliC*::*gfp* CarbR	This study
BC2333	BC1643 pP*araBAD*::*ydiV* GentR	This study
BC1810	BC1662 pP*flhD*::*gfp* CarbR	This study
BC2847	BC1662 pP*fliA*::*gfp* CarbR	This study
BC1844	BC1643 pP*flhD*::*gfp* CarbR	This study
BC2848	BC1643 pP*fliA*::*gfp* CarbR	This study
BC2717	BC1662 *flhD*::*lacZ* translational fusion KanR	This study
BC2718	BC1643 *flhD*::*lacZ* translational fusion KanR	This study
BC2805	BC2717 *csrA*::FCF *flhD*::*lacZ* translational fusion CamR KanR	This study
BC2264	BC1662 FlhC::3× FLAG KanR	This study
BC2265	BC1643 FlhC::3× FLAG KanR	This study
BC2238	14028 *STM4226/7*::*tetRA* TetR	Joyce Karlinsey (University of Washington, Seattle, WA)
BC2647	BC1662 *fliC*::FRT *STM4226/7*::*tetRA* TetR	This study
BC1743	BC1643 *fliC*::FKF KanR	This study
BC1640	BC156 *fljBA*::FKF KanR	This study
BC2243	BC1662 *STM4226/7*::*tetRA* TetR	This study
BC1621	14028 y*diV*::FRT *fljBA*::FKF KanR	This study
Plasmids		
p*fliC*	pRP3, a pBR322 derivative, CarbR	Kelly Hughes (University of Utah, Salt Lake City, UT)
pP*araBAD*::*ydiV*	pJN105 derivative, CarbR	This study
pKAS32::P*fliC*::*gfp*	pKAS32 derivative, CarbR	Cummings et al. (6)
pP*fliA*::*gfp*	pBR322 derivative, CarbR	This study
pP*flhDC*::*gfp*	pBR322 derivative, CarbR	Cummings et al. (6)