Regulation of Phenotypic Heterogeneity in *Salmonella*: Bifunctional Regulators, RcsB and YifA, Shape Flagellar Bistability

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Abstract

Regulation of Phenotypic Heterogeneity in *Salmonella:* Bifunctional Regulators, RcsB and YifA, Shape Flagellar Bistability

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Heterogeneous expression of flagellar and invasion genes is crucial for *Salmonella* virulence. Such a division of labor enables an invasive subpopulation to trigger inflammation in the infected host and alter the gut environment to favor growth of *Salmonella*. Meanwhile, non-flagellated cells are better able to evade host defenses systemically and persist. This bistable expression requires the master regulator of the flagellar cascade, $FlhD_4C_2$, to be kept in low concentration by post-translational regulators YdiV, ClpXP, and STM14_2047. In this study, we demonstrated novel roles for two previously identified regulators of flagellar expression. RcsB and YifA are independently able to repress the transcription of both *flhDC* and *STM14_2047* in several conditions, but their control of flagellar expression is environmentally responsive. We have identified conditions where RcsB and YifA act as either repressors or promoters of flagellar expression and hypothesize that this conditional regulation increases fitness in varied environments.

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Chapter 1. INTRODUCTION

1.1 Phenotypic Heterogeneity

Phenotypic heterogeneity occurs when genetically identical organisms exposed to homogenous growth conditions produce phenotypically distinct subpopulations (Avery et al., 2006). When there are two subpopulations, with one expressing a trait and the other not, expression is termed bistable (Veening et al., 2008). This pattern is common in the microbial world and can confer advantages to organisms through bet hedging and division of labor. Bet hedging refers to the resilience conferred to heterogeneous populations facing rapidly changing environments. Sensing and responding to any change in the environment, termed deterministic adaptation, requires time and pre-existing regulatory mechanisms to appropriately sense new conditions and express the correct suite of genes for a given situation. However, in certain cases, it may be that organisms encounter lethal or crippling changes in their environment too quickly to be able to effectively respond. By having multiple subpopulations, each with their own advantages in different conditions, phenotypic heterogeneity enables at least a subset of clonal cells to be able to survive and persist (de Jong et al., 2011). Division of labor refers to when a fraction of the population undertakes a given task for the benefit of the whole. As a result, multiple needs of a population can be met simultaneously by specialized subpopulations without the need for genetic change. It is of note that the ability of organisms to produce heterogeneity does not make deterministic adaptation obsolete (Lowery et al., 2017). Integration of environmental signals with the genetic circuitry that generates heterogeneity can alter the probability of each cell adopting a given phenotype. These responses to environmental signals

helps populations maximize fitness for the given environment while maintaining the ability to weather rapid changes or serve multiple functions.

Phenotypic heterogeneity originates from random variability in the concentration of an active and available master regulator. These master regulators are often kept at low concentrations within the cell by post-translational repressors that deactivate (Goldbeter et al., 1981), degrade (Buchler et al., 2005), or sequester them (Buchler et al., 2008). As a result, cells are left with very little functional master regulator and, following division, random probability will generate cells with subtle differences in the concentration of the master regulator. Ultrasensitive positive feedback loops are able to amplify even these tiny differences and create a system in which intermediate levels of gene expression are rendered unstable. The result is a bistable pattern of expression with two separate subpopulations that either express or do not express a given trait. Once bistable expression is established, hysteresis maintains cells in either the ON or OFF subpopulation, unless a strong enough signal prompts change (Ray et al., 2011).

The ability to generate heterogeneous populations without altering genetic content has been observed in several bacterial genera, including *Bacillus* (Veening et al., 2008), *Vibrio* (Nielsen et al., 2010), *Pseudomonas* (Rietsch et al., 2006), and *Salmonella enterica* serovar Typhimurium (Cummings et al., 2006). *Salmonella enterica* subspecies *enterica* serovar Typhimurium (from this point referred to as *S*. Typhimurium) thrives in a wide variety of environments and has been shown to heterogeneously express flagellar genes and virulence determinants. For this study, we use *S*. Typhimurium as a model of phenotypic heterogeneity and examine the regulatory circuitry that enables non-genetic diversity in a variety of conditions.

1.2 *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

S. Typhimurium is a Gram-negative bacillus that contributes significantly to the diarrheal

disease burden of both the developed and developing world. It, along with other additional non-typhoidal *Salmonella* serovars, is a causative agent of non-typhoidal Salmonellosis (NTS). Worldwide, it is estimated that there are 153 million cases of NTS annually (CDC). NTS often presents as acute diarrhea within three days of consuming contaminated food or water and typically resolves in 4-7 days without treatment in an otherwise healthy human host (WHO). However, infections can lead to severe systemic salmonellosis in hosts that are very young or immunocompromised, requiring supportive therapy and intravenous antibiotics while hospitalized (Okaro et al., 2012).

Following consumption of an infectious dose of organisms by the host, *S*. Typhimurium moves through the digestive tract, where it invades the intestines through the Peyer's patches of the ileum. A protein involved in flagellar regulation, FliZ, is also a positive regulator of *Salmonella* Pathogenicity Island 1 (SPI-1), which encodes the type III secretion system enabling invasion of the intestinal epithelium (Chubiz et al., 2010; Saini et al., 2010). FliZ is heterogeneously expressed, leading to a portion of flagellated cells expressing invasive capabilities as well. Heterogeneous expression of both flagella and the tightly linked SPI-1 genes has been shown to be crucial for S. Typhimurium virulence. In the gut of someone infected with *Salmonella*, there are distinct subpopulations that are motile and invasive and non-motile and non-invasive (Cummings et al., 2006; Ackerman et al., 2008). The invasive subpopulation is able to penetrate the intestinal epithelium and trigger an inflammatory response. Inflammation is lethal to the invading cells, but also results in the release of tetrathionate into the lumen, which *S*. Typhimurium has evolved to be able to use as an alternative electron acceptor. The availability of this additional resource enables further growth in the intestines (Stecher et al., 2007). The non-invasive cells in the lumen benefit from the actions of the altruistic invasive subpopulation,

outcompete the normal microbiota, and move out of the host to disseminate further (Ackermann, 2008).

In a healthy host, the infection and resulting diarrhea resolves in approximately a week without further spread beyond the intestinal epithelium, but in the case of systemic salmonellosis, the organisms are phagocytosed by macrophages and are able to establish a protective niche termed the *Salmonella*-containing vacuole (SCV) (Monack et al., 2004). Evasion of the host immune system in such cases is accomplished by the ability of *S.* Typhimurium to sense the conditions of the phagosome through the two-component system, PhoPQ. Once activated, PhoPQ represses inflammatory gene products, such as flagellin, and promotes SPI-2 effector proteins to modify the phagosome of the macrophage and evade killing (Tang et al., 2013). In mice infected with *S.* Typhimurium, the macrophages carrying the bacteria migrate to the mesenteric lymph nodes, then the spleen, where *S.* Typhimurium is able to persist (Monack et al., 2004). Failure to repress flagellin inside of the macrophage results in severe attenuation of strains (Stewart et al., 2011). The presence of flagellin within macrophages is sensed by the innate immune receptor NLRC4, and initiates pyroptosis, an inflammatory form of programmed cell death (Fink et al., 2007; Miao et al., 2011). During pyroptosis, caspase-1 is cleaved and activated, resulting in the maturation and secretion of IL-1β and IL-18. Ultimately, pores form in the plasma membrane of the macrophage, chromosomal DNA is cleaved, and the cell lyses (Bergsbaken et al., 2010). Mice lacking NLRC4, caspase-1, IL-1β, or IL-18 are more susceptible to systemic infection than wild-type mice (Miao et al., 2010; Lara-Tejero et al., 2006; Raupach et al., 2006), highlighting the importance of these genes in defense against infection. Beyond inflammasome activation, flagellin is sensed by TLR5 and is also a major antigen for T cells, thus there are multiple mechanisms of immune sensing of flagellin that could prevent

systemic spread if flagella are expressed in these conditions.

1.3 FLAGELLAR ASSEMBLY IN *SALMONELLA*

For both gastrointestinal and systemic infections, *S*. Typhimurium must maintain strict control of flagellar production and has evolved a complex regulatory network to meet that need. At the very backbone, flagellar production is the result of the successful expression of a three class genetic cascade. The class I operon encodes the subunits for the master regulatory complex $FlhD_4C_2$. This complex promotes transcription of class II genes by binding to the promoters and recruiting RNA polymerase. Class II encodes the base and motor of the flagellum, FliZ, and FliA, an alternative sigma factor that promotes expression of class III promoters. Class III includes genes for chemotaxis and movement and the flagellin protein that is polymerized to form the flagellar filament (Chevance & Hughes, 2008). There are two genes that express a flagellin protein monomer, *fljB* and *fliC*, which enable phase variation of flagellar antigens. The operon containing *fljB* also includes *fljA*, which represses transcription and translation of *fliC* to ensure that only one form of flagellin is expressed at a time (Bonfield & Hughes, 2003). We have chosen to study flagellar production in strains lacking *fljBA* in order to have a single clear reporter for class III expression.

Bistable flagellar production emerges following monomodal transcription of the class I operon, *flhDC*, and is dependent on the expression of post-translational regulators that keep $FlhD_4C_2$ concentrations low. There are several post-translational regulators of $FlhD_4C_2$. YdiV binds to the FlhD subunit and disrupts $FlhD_4C_2$ binding to class II promoters. Additionally, YdiV targets $FlhD_4C_2$ for degradation by the ClpXP protease (Wada et al., 2011; Li et al., 2012; Takaya et al., 2012). YdiV and a class II gene, FliZ, participate in a system of mutually repressing repressors that help shape flagellar bistability. The activity of YdiV results in lower

class II transcription, and FliZ, when expressed, transcriptionally represses YdiV. This system forms a net positive feedback loop to either reinforce the fate of the cell as flagellated or unflagellated (Stewart MK and Cookson BT, 2014). A more recently identified regulator, STM14_2047, functions similarly to YdiV and binds FlhD (Ahmad et al., 2013; Li et al., 2017). While this regulator has not been shown to prevent complex binding to class II promoters, it has been shown to prevent $FlhD_4C_2$ from recruiting RNA polymerase to class II genes (Li et al., 2017).

Much of the focus on bistable flagellar expression has been on the system of mutually repressing repressors, FliZ and YdiV, which destabilize intermediate states of gene expression. A high degree of disorganized heterogeneity for flagellar gene expression was observed in the absence of FliZ and YdiV (Stewart MK and Cookson BT, 2014). We hypothesized that this stemmed from unequal distribution of factors increasing and reducing the amount of activated $FlhD_4C_2$ complex between individual cells of a *Salmonella* population. Stochastic influences upon a system are predicted to be strongest when the number of molecules involved is low, thus strong repressors of early events in the flagellar gene expression cascade might be important regulators of heterogeneity. Accordingly, we tested whether a panel of *flhDC* transcriptional and translational activators and repressors impact the bistable expression of flagellar genes.

Chapter 2. METHODS

2.1 Growth Conditions

Salmonella were grown with shaking at 225rpm at 37 °C in the indicated conditions. Miller Luria Broth (Product #244620, Difco), LB with trace salt (5 g tryptone, 2.5 g yeast extract, 500 mL diH_2O), or SPI-2 media (recipe described in Miao et al., 2002) were used to assess flagellar regulation in relevant environmental conditions. Overnight cultures were backdiluted 1:100 and grown to indicated time points. Antibiotic concentrations were as follows: Carbenicillin, 100 µg/ml; kanamycin, 50 µg/ml; gentamicin, 20 µg/ml; tetracycline, 24 µg/mL. Chlortetracycline hydrochloride (Sigma #26430-5G, autoclaved with liquid media) and L-arabinose were added at the indicated concentrations.

2.2 Strain Creation

Experiments were conducted using *Salmonella enterica* subspecies *enterica* serovar Typhimurium strain 14028s. Tables of all strains, plasmids and primers used may be found in Appendix A.

2.2.1 *Gene Deletions*

Gene knockouts were generated using the method described in Datsenko and Wanner (2000). Briefly, PCR fragments were created that encoded a kanamycin resistance cassette flanked by recognition sites for the FLP recombinase and 40 bp regions of homology targeting the PCR fragment to the chromosome. The PCR fragments were then transformed into cells expressing the lambda-red recombinase from pKD46 and transformants were selected for by kanamycin resistance. After PCR confirmation of the deletion and loss of pKD46 by growth at

37 ^{o}C, the deletion constructs were transduced into strains lacking *fljBA*, the alternative flagellin gene, by P22 transduction. Finally, the cassette was excised by transformation with pCP20, which encodes the FLP recombinase. Finally, the strains were cured of pCP20 by incubation at 37 ^{o}C.

2.2.2 *Inducible and Complementation Constructs*

Tetracycline-inducible *flhDC* construct was a gift from the Hughes lab. Complementation of *rcsB* and *yifA* deletions could not be achieved with the insertion of a tetracycline-inducible promoter upstream of the start sites or by cloning the genes into plasmids without their native promoter. For the construction of complementation plasmids, PCR products containing each of the genes with their native promoters flanked by restriction sites for EcoRI and SacI were created. Inserts and the plasmid backbone, pJN105 (Newman & Fuqua, 1999), were digested with EcoRI and SacI and gel purified before being ligated with Quick T4 DNA ligase and transformed into TOP 10 competent *E. coli*. Gentamycin resistant transformants were selected. A diagnostic digest was performed to confirm the insert was present and the plasmids were sequenced to rule out any mutations.

2.2.3 *Epitope-Tagged Proteins*

3xFLAG-tagged versions of YdiV and STM14_2047 were created using the method described in Uzzau et. al (2001).

2.2.4 *lacZ Transcriptional Reporters*

Transcriptional reporters for genes of interest were created with a modified version of the protocol reported in Ellermeier et. al. (2002). The gene of interest was deleted as described in section 2.2.1 above. The conditionally-replicating plasmid pKG136 was the integrated into the

FRT scar via FLP recombinase, thus putting *lacZ* under control of the promoter of interest. Successful integrations were selected for by kanamycin resistance and PCR confirmed. The construct was transduced into the appropriate strains by phage P22.

2.2.5 *GFP Transcriptional Reporters for Single Cell Analysis*

For single cell transcriptional analysis, plasmids containing GFP driven by the promoters of *flhDC*, *fliAZ*, and *fliC* were transformed into cells and selectively maintained by growth in the presence of carbenicillin.

2.3 WESTERN BLOTTING

Overnight bacterial cultures were diluted 1:100 into fresh media and grown to the indicated time points before being spun down and resuspended in PBS. Cell concentrations were measured using the Beckman Coulter Multisizer 4 and normalized to 10^9 cells/mL. Samples were then boiled for 10 min, separated by 4-20% SDS-PAGE and transferred to nitrocellulose. Blots were probed with anti-FliC antibody (Hardy Diagnostics product number 293985, pre-absorbed with FliC–null *Salmonella* before use), anti-3xFLAG antibody (EMD Millipore, Chicago IL), or anti-DnaK antibody (StressGen, Biotechnologies, Victoria, BC Canada).

2.4 BETA-GALACTOSIDASE ASSAY

Overnight cultures were diluted 1:100 into fresh media and grown to the indicated time points. Cell concentrations were measured using the Beckman Coulter Multisizer 4 and normalized to 10^8 cells/mL in Z-buffer. Cells were permeabilized with 2.8 µL β-mercaptoethanol, 50 µL 0.1% SDS, and 100 µL $CHCl_3$ for fifteen minutes and plated in triplicate

in a 96-well clear bottom plate. 50 µL of 10mg/mL ONPG was added and absorbance was measured at 420 nm every five minutes for an hour.

2.5 Flow Cytometry to Assess GFP Fluorescence

Overnight cultures were backdiluted 1:100 5 and 2.5 hours before being spun down, fixed with 2% paraformaldehyde for 5 minutes on ice, and resuspended in filtered PBS. GFP fluorescence was measured using the Canto flow cytometer and analyzed using FlowJo software.

2.6 Statistical Analysis

T-tests were used to compare single knockout strains to WT levels with a p-value of <0.05 being termed significant (<0.05 = *, <0.01 = **, <0.005 = ***, <0.001 = ****). When multiple strains were compared to each other, a one-way ANOVA with correction for multiple comparisons was used. Notation directly over a column refers to the statistical differences between that strain and WT while comparisons between other strains are shown above brackets.

Chapter 3. RESULTS

3.1 A SCREEN OF *FLHDC* REGULATORS IDENTIFY *RCSB* AND *YIFA* AS STRONG REGULATORS OF FLAGELLAR EXPRESSION

A literature search was conducted to identify reported regulators of *flhDC* that may play a role in regulating bistable flagellar expression (Figure 3.1A). Eleven genes were identified: one transcriptional activator of *flhDC*, *hilD* (Kelly et al., 2004); eight transcriptional repressors, *lrhA* (Lehnen et al., 2002), *rcsB* (Wang et al., 2007), *yifA* (Ko et al., 2000), *ompR* (Shin and Park, 1995), *rtsB* (Ellermeier et al., 2003), *slyA* (Sporey and Stapleton et al., 2002), *fimZ* (Saini et al., 2010; Clegg and Hughes, 2001), and *ecnR* (Wozniak et al., 2009); and two post-transcriptional repressors, *barA* and *uvrY* (Teplitski et al., 2003). Single knockouts of each of these genes were created using the method described by Datesenko and Wanner (2001).

Knockouts of the genes of interest were examined for their effect on both the levels of transcription of the master regulator, *flhDC*, and the production of FliC after 2.5 hours of growth in LB media. For *flhDC* transcription, a chromosomal β-galactosidase fusion to the *flhDC* promoter was created and galactosidase activity was measured in a microtiter plate assay as a reporter for transcription levels (Figure 3.1B). FliC protein was measured in each strain using Western blotting and normalized to DnaK, then adjusted to percent of WT (Figure 3.1C). Of note, the knockout of *lrhA* showed increases in both *flhDC* transcription and FliC protein levels, consistent with its reported role as a repressor of flagellar expression. In contrast, two genes stood out that had counteracting effects at the different points of the flagellar cascade. While the loss of *rcsB* or *yifA* results in a significant increase in *flhDC* transcription, ultimately, these knockouts had lower FliC protein levels than were measured in WT. We then hypothesized that *rcsB* and *yifA* affected the flagellar cascade at multiple points.

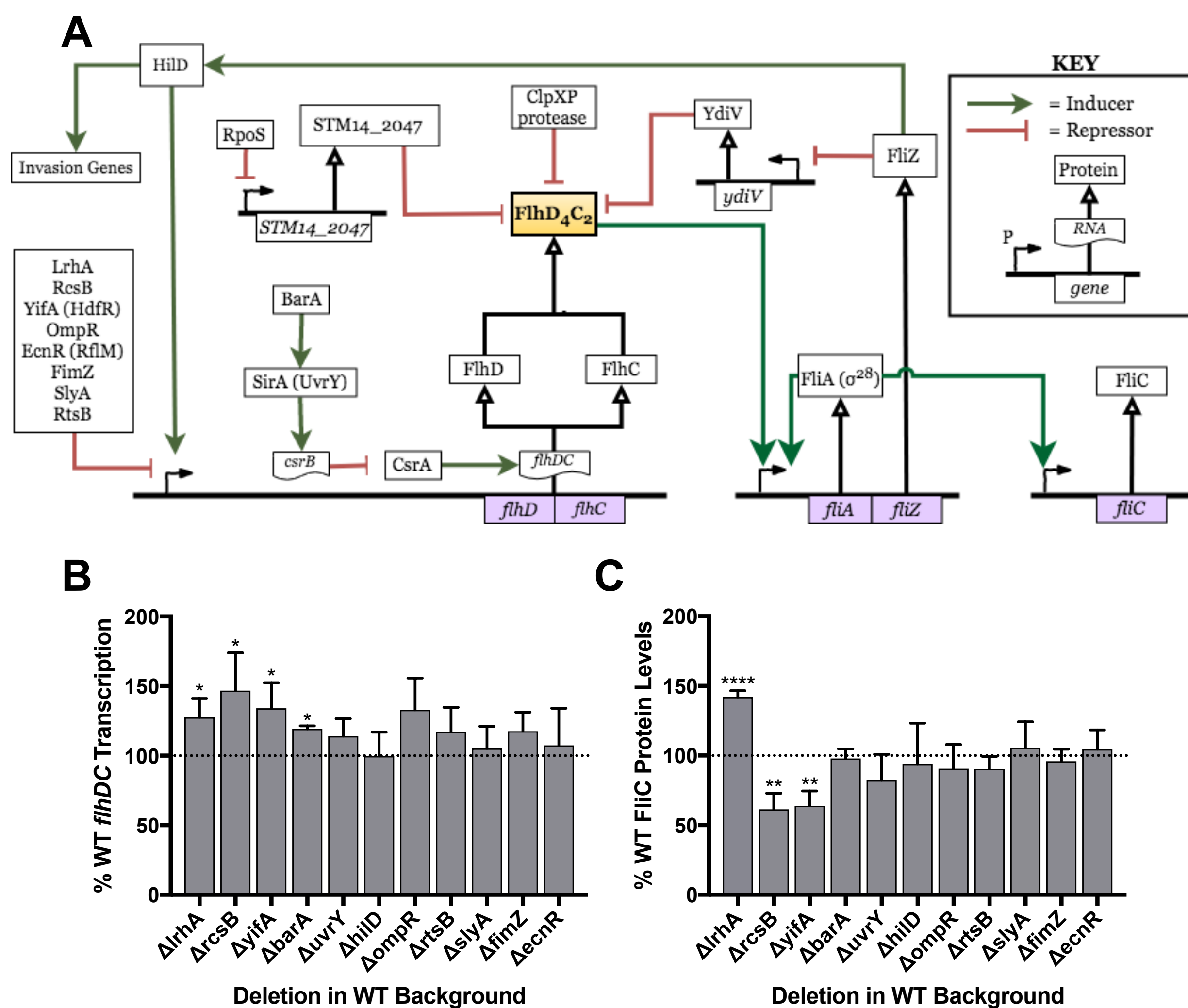

Figure 3.1. A screen of *flhDC* regulators reveals a class of regulators with counteracting effects on the flagellar cascade.

A. The compilation of a literature search focusing on regulation of the class I operon reveals a complex regulatory network to modulate expression within the flagellar cascade. Numerous transcriptional regulators impact the early levels of the master regulator, $FlhD_4C_2$. Post-translational repressors, YdiV, ClpXP, and STM14_2047, maintain low levels of active $FlhD_4C_2$ complex, which enables random variability in the concentration of the master regulator upon cell division.

B. A transcriptional, chromosomal β-galactosidase fusion to the *flhDC* promoter was used to investigate the impacts of single gene knockouts on *flhDC* transcription in LB media 2.5 hours after backdilution. Results were adjusted to %WT and one-sample t-tests were used to compare knockout strain *flhDC* transcription with that of WT.

C. Western blotting of FliC production 2.5 hours after backdilution in LB was normalized to DnaK and adjusted to %WT expression. One-sample t-tests were used to compare knockout strain FliC protein levels with that of WT.

RcsB and YifA were selected for closer examination of their effects on the flagellar cascade through single cell analysis. The promoters for *flhDC*, *fliA*, and *fliC* were fused to *gfp* and cloned into a plasmid to characterize transcription of these genes at the single cell level. These plasmids were then transformed into strains with knockout mutations for *rcsB* or *yifA*, along with a WT control. After 2.5 hours of growth in LB media, GFP fluorescence was measured using flow cytometry and analyzed in FloJo Software. Histograms of this data were generated to visualize the distribution of transcription levels within the population (Figure 3.2A). Strains containing knockout mutations of *rcsB* and *yifA* have unimodal transcription of *flhDC*, similar to the WT profile (left panels), but, beginning with *fliA* transcription (middle panels), the knockout strains depart dramatically from WT. Rather than the bistable histogram in WT *fliA* transcription (Top row, middle panels), there is a loss of bistability and a greater partitioning of cells outside of the ON gate (middle & bottom rows, middle panels). Ultimately, fewer cells expressing *fliC* are observed as well, consistent with the population average results obtained in the FliC western blots (Figure 3.1C). While roughly 65% of cells are expressing *fliC* at some level in the WT census (Figure 3.2A, top row, right panel), 30% and 45% of cells are transcribing *fliC* in the Δ*rcsB* and Δ*yifA* strains, respectively (middle and bottom rows, right panel).

Complementation plasmids containing *rcsB* or *yifA* with their native promoters were generated to confirm the role of these genes in flagellar expression. The region of *S.* Typhimurium 14028s chromosome containing *rcsB* or *yifA* and 200 bp preceding the start site was cloned into the pJN105 plasmid vector (Newman & Fuqua, 1999). Either complementation plasmid (pRcsB or pYifA) or an empty vector control was transformed into strains along with the P*fliC*::GFP reporter plasmids. Strains were grown for 2.5 hours post-backdilution into LB

and *fliC* transcription was examined using flow cytometry (Figure 3.2B). The empty vector controls for the complementation experiments (Figure 3.2B, left column) do not perfectly reflect the profile observed in the single knockouts (Figure 3.2A, right column), possibly due to the presence of both carbenicillin and gentamycin to maintain the complementation and reporter plasmids. However, comparisons between the empty vector controls and pRcsB or pYifA show significant differences. While the percent of cells in the ON gate is relatively unchanged between the empty vector control and the complementation plasmids, the profiles of the histograms are considerably different. Bistable expression is re-established with the addition of the pRcsB (Fig 3.2B, top row) or pYifA (Fig 3.2B, bottom row). Fewer cells expressing intermediate levels *fliC* were observed and the geometric mean fluorescent intensity of the *fliC*-ON subpopulation was significantly higher in the complementation plasmids compared to the empty vector control (Fig 3.2B, right column).

The drop in the number of cells expressing flagellar genes beginning at class II transcription in the knockout strains (Figure 3.2A) is consistent with repression of $FlhD_4C_2$ activity after *flhDC* has already been transcribed. Complementation with *rcsB* or *yifA* also restores bistable expression that was disrupted in the knockout strains. Following these observations, we hypothesized that RcsB and YifA may play a role in regulating one of the known post-translational repressors of $FlhD_4C_2$ that are required for bistable expression. We next examined strains containing knockout mutations of *rcsB* and *yifA* for expression of these repressor proteins in LB broth after 2.5 hours of growth. 3xFLAG-tagged YdiV constructs were examined by Western analysis in the WT background and strains containing knockout mutations in *rcsB* or *yifA*. Results were normalized to DnaK and adjusted to a percentage of WT. No effect was seen on YdiV protein levels (Figure 3.2C). Subsequently, protein levels of STM14_2047

were examined in the knockout strains using Westerns and a 3xFLAG-tagged STM14_2047 construct. The loss of *rcsB* or *yifA* resulted in significant increases in protein levels of STM14_2047 (Figure 3.2D). A chromosomal β-galactosidase fusion to the promoter of STM14_2047 was then transduced into WT, Δ*rcsB*, and Δ*yifA* strains for analysis of transcription of STM14_2047. Loss of either *rcsB* or *yifA* resulted in increases in transcription of *STM14_2047* as well (Figure 3.2E), indicating that these genes are transcriptional repressors of *STM14_2047*.

To determine if the decrease in *fliC* expression in the knockouts of *rcsB* or *yifA* was due to their repression of *STM14_2047* alone, transcription of *fliC* was examined by flow cytometry in double knockouts of *STM14_2047* and *rcsB* or *yifA*. In the absence of *STM14_2047*, the additional loss of *rcsB* or *yifA* showed no decrease in the proportion of cells expressing *fliC* (Figure 3.2F). Additionally, FliC production was measured by Western blotting in the double knockouts of *STM14_2047* and either *rcsB* or *yifA*. Not only did the double knockouts show no drop in FliC levels relative to the STM14_2047 knockout, but they produced greater amounts of FliC (Figure 3.2G). The observed increase is hypothesized to be due to derepression of *flhDC* transcription that results in increases in expression throughout the cascade. The increase in flagellar expression upon the loss of *rcsB* or *yifA* in the absence of STM14_2047 indicates that the decrease in flagellar expression seen in the *rcsB* or *yifA* single knockouts is due to their activity on *STM14_2047*.

In summary, RcsB and YifA are transcriptional repressors of both *flhDC* and *STM14_2047*, which are counteracting effects. Repression of *flhDC* transcription alone would result in lower levels of $FlhD_4C_2$ master complex and ultimately lower flagellar production, but the additional repression of *STM14_2047* serves to increase the levels of $FlhD_4C_2$ that is able to

promote class II transcription. These effects integrate to produce a net result on flagellar production. Thus, in rich media, RcsB and YifA promote flagellar expression by repressing transcription of STM14_2047, thus increasing the levels of active and available $FlhD_4C_2$ complex.

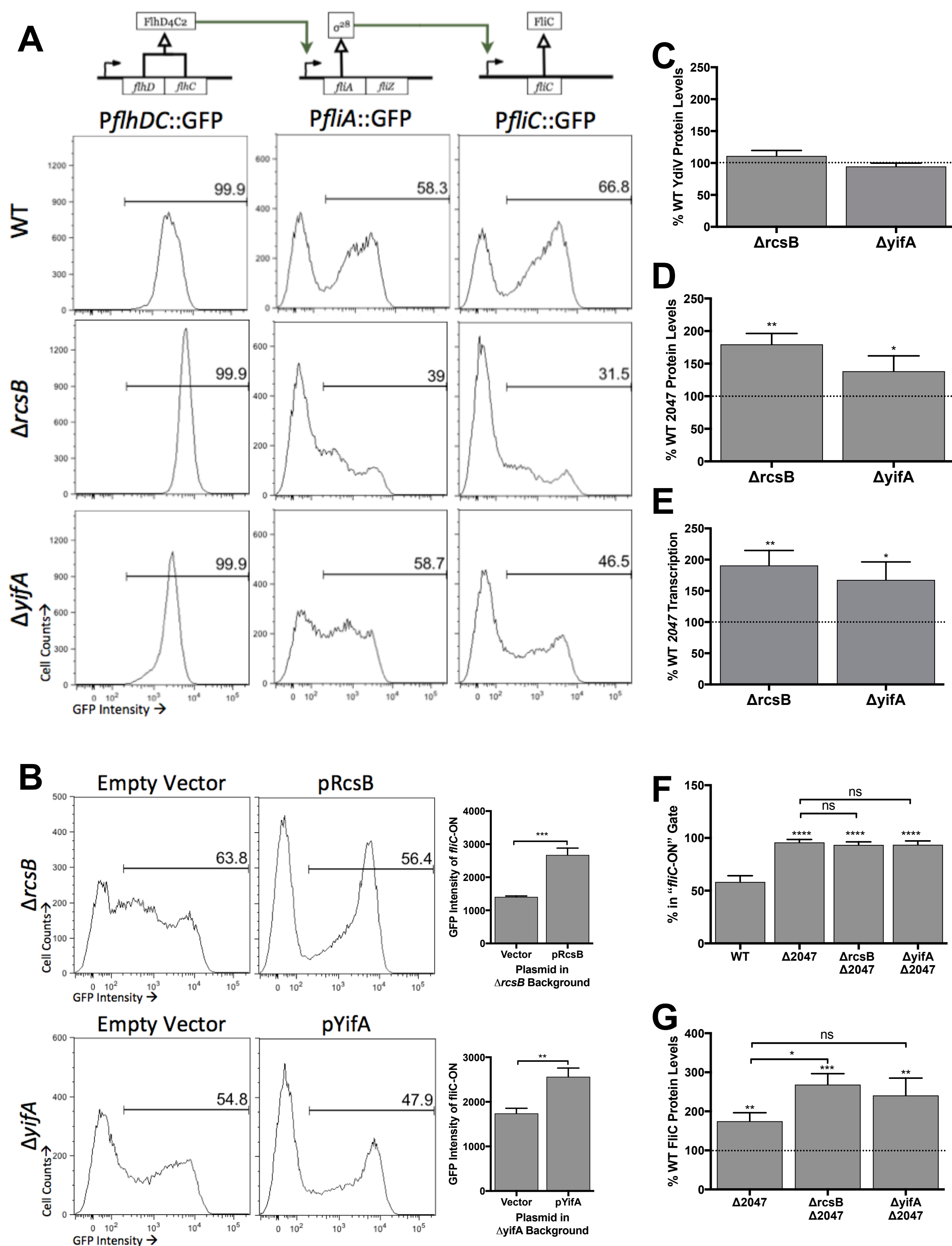

Figure 3.2. RcsB and YifA shape flagellar bistability in exponential phase growth.
A. The promoters for *flhDC*, *fliA*, and *fliC* were fused to *gfp* to characterize transcription at the single cell level using flow cytometry. Strains lacking *rcsB* and *yifA* have dramatically altered *fliA* and *fliC* transcription 2.5 hours after backdilution compared to WT.

B. The region of Stm 14028 chromosome containing *rcsB* or *yifA* and 200 bp preceding the start site was cloned into an arabinose-inducible plasmid. The complementation plasmid or an empty vector control was transformed into strains containing chromosomal knockouts of the gene being complemented and a GFP reporter fused to the promoter of *fliC*. Strains were grown for 2.5 hours post-backdilution and fliC transcription was examined using flow cytometry. One-sample t-tests were used to compare the geometric mean GFP intensity of the FliC-ON subpopulation in the complementation plasmid and the vector control.
C. A 3xFLAG-tagged version of YdiV was quantified 2.5 hours following backdilution into LB using Western analysis. Results were normalized to DnaK, and adjusted to %WT. One-sample t-tests were used to compare knockout strain YdiV protein levels with that of WT. No effect is seen upon YdiV protein levels by the loss of *rcsB* or *yifA*.
D. Chromosomal β-galactosidase transcriptional reporters show that loss of *rcsB* or *yifA* results in an increase in *STM14_2047* transcription 2.5 hours following backdilution into LB. One-sample t-tests were used to compare knockout strain transcription levels with that of WT.
E. A 3xFLAG-tagged version of STM14_2047 was quantified using Western analysis, normalized to DnaK, and adjusted to %WT. One-sample t-tests were used to compare knockout strain STM14_2047 protein levels with that of WT.
F. Double knockouts of STM14_2047 and either *rcsB* or *yifA* were generated and *fliC* transcription was measured by flow cytometry. The percent of cells expressing *fliC* in each strain were compared.
G. Double knockouts of *STM14_2047* and either *rcsB* or *yifA* have higher levels of FliC protein measured by Western.

3.2 Control of flagellar expression by RcsB and YifA is environmentally responsive.

We investigated the expression of STM14_2047 in diverse environmental conditions to better understand the magnitude of its role in flagellar regulation throughout *S*. Typhimurium's path from host to environment and back again. In each of these conditions, *S*. Typhimurium must appropriately regulate its flagella in order to effectively maximize its use of resources and disseminate to further hosts. Previous work by the Cookson lab has shown STM14_2047 is repressed by RpoS activity in both exponential and stationary phase (unpublished data). RpoS is a sigma factor involved with coordinating stationary phase gene expression and the general stress response (Hengge-Aronis, 2002). It is most strongly expressed during stationary phase growth, so gene expression in stationary phase growth was examined in two culture conditions, SPI-2 media (ST_{IN}) and LB broth (LB_{STAT}). SPI-2 media is an approximation of the conditions that *S*. Typhimurium would experience inside the *Salmonella*-containing vacuole (SCV) of a macrophage, where flagellin is strongly repressed to evade detection by the host immune system and pyroptotic cell death (Fink & Cookson, 2007). Key characteristics of these conditions include a pH of 5.0, minimal nutrients, and low concentration of Mg^{2+} (Miao et al., 2002). Additionally, low salt conditions reflect environments that *S*. Typhimurium might experience outside of a host, which present different requirements for survival of the bacteria. It has been reported that STM14_2047 is undetectable by chemiluminescent Western blots in LB media without added salt (Ahmad et al., 2013), but transcription of *STM14_2047* and flagellar expression were not examined in that condition. Consequently, LB media without added salt (LB_{EXP}-NaCl) was also selected for analysis.

STM14_2047 was measured at both the level of transcription and protein product. Transcription was measured using the β-galactosidase transcriptional reporter and reported in Miller units (Figure 3.3A). 3xFLAG-tagged STM14_2047 constructs were generated and protein levels were measured by Western analysis and reported as fluorescence per cell count (Figure 3.3B). When comparing transcription and protein levels, several trends were seen. First, relative transcription levels differ from the differences seen at the protein level. There is no significant difference between transcription levels of *STM14_2047* in LB_{STAT} and LB_{EXP} (Fig 3.3A, second and fourth column), but protein levels are roughly three fold higher in LB_{EXP} than LB_{STAT} (Fig 3.3B, second and fourth column). Similarly, no transcriptional differences are seen between ST_{IN} and LB_{EXP} (Fig 3.3A, first and fourth column) but protein levels are roughly six fold higher in LB_{EXP} (Fig 3.3B, first and fourth column). This would serve to indicate that the regulation of STM14_2047 during stationary phase growth involves post-transcriptional activity. Additionally, a decrease in STM14_2047 expression in media without added salt was observed, replicating results reported by Ahmad et al. (2013). It was further shown that the decrease originates with lower transcription of the gene that ultimately results in the diminished protein levels. Overall, the lowest expression of STM14_2047 was seen in ST_{IN}, intermediate expression was measured in LB_{STAT} and LB_{EXP}-NaCl, and the highest expression was observed in LB_{EXP}. The mechanisms for these regulations are as yet unknown and serve to show that there are additional regulatory inputs to be characterized for STM14_2047.

We hypothesized that RcsB and YifA could repress *flhDC* and *STM14_2047* in these alternative conditions and generate significant impacts on flagellar production as well. Transcription of both genes was measured using chromosomal β-galactosidase fusions to the promoters of interest and results were normalized to WT expression in each condition. Loss of

rcsB or *yifA* resulted in increased transcription of both *flhDC* and *STM14_2047* in all conditions tested, with the stronger effect being observed in *STM14_2047* (Figures 3.3C and 3.3D). The knockout strains displayed a roughly 100% increase in *STM14_2047* transcription while *flhDC* transcription increased 25-50%.

WT FliC levels in each condition were measured by Western and compared as fluorescence normalized to cell count (Figure 3.3E). FliC expression is very low in ST_{IN}, reflecting the need to repress flagellin and evade pyroptosis within macrophages. In LB_{STAT}, LB_{EXP}-NaCl, and LB_{EXP}, flagellar protein was high with no significant difference between the three. FliC expression was measured by Western analysis in Δ*rcsB* and Δ*yifA* strains in all conditions as well to gauge the effect of these genes on flagellar expression in various conditions. The results were normalized to DnaK and adjusted to percent WT in each condition. Notably, loss of *rcsB* or *yifA* produced both a large increase in flagellar expression relative to WT in ST_{IN} and a decrease in LB_{EXP} (Figure 3.3F).

When considering how RcsB and YifA could act as inducers or repressors of flagellar production, we hypothesized that the yet unidentified conditional regulators of STM14_2047 played a role. In all conditions, the loss of *rcsB* or *yifA* resulted in a roughly 100% increase in transcription of *STM14_2047* relative to WT (Figure 3.3D). However, WT levels vary in each condition and so a doubling of the low expression seen in ST_{IN} is considerably less than a doubling of the levels observed in LB_{EXP}, whose WT baseline is six-fold higher (Figure 3.3B). When FliC levels were measured in ST_{IN}, the loss of *rcsB* or *yifA* resulted in 5-10 fold increases in protein levels of FliC (Figure 3.3F). This would suggest that the derepression of *flhDC* transcription upon the loss of *rcsB* or *yifA* is relatively greater than the derepression of *STM14_2047*. These individual effects integrated to tip the balance towards a more flagellated

population. In LB_{EXP}, relatively greater levels of STM14_2047 protein lead to a significant repression of $FlhD_4C_2$ activity and resulted in an overall decrease in flagellar expression (Figure 3.3F). The intermediate levels of STM14_2047 found in LB_{STAT} and LB_{EXP}-NaCl appear to be near an inflection point between the two outcomes where the loss of *rcsB* or *yifA* generates similar relative changes in *flhDC* and *STM14_2047* transcription and results in no significant change from WT FliC levels or a slight increase (Figure 3.3F).

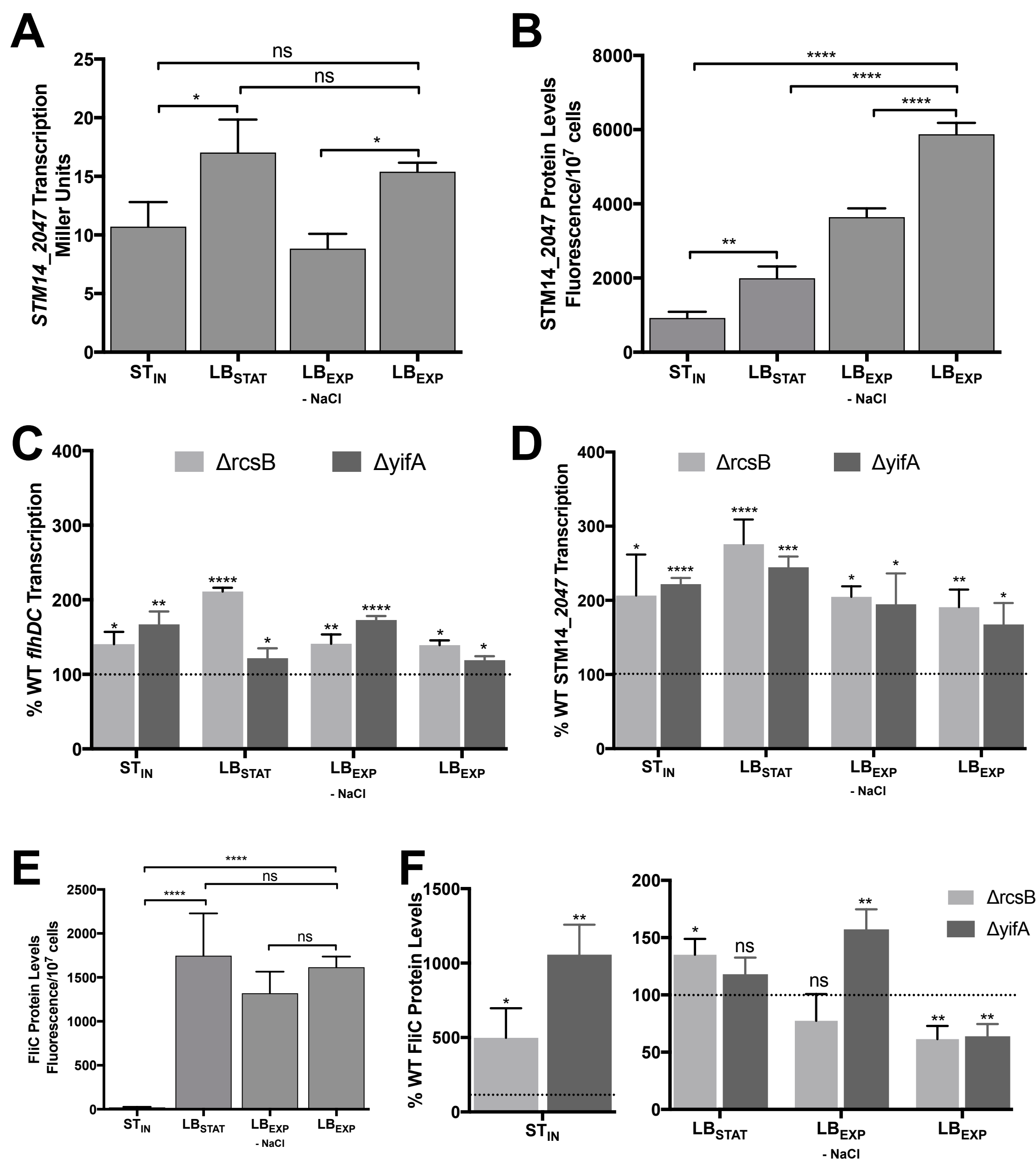

Figure 3.3. Control of flagellar expression by RcsB and YifA is environmentally responsive. A-B. The β-galactosidase transcriptional reporter and 3xFLAG-tagged constructs were used to examine WT STM14_2047 transcription and translation, respectively, in diverse environmental conditions. One way ANOVA with correction for multiple comparisons was used.
C-D. Chromosomal β-galactosidase reporters for *flhDC* and *STM14_2047* transcription show RcsB and YifA repress both genes in all conditions tested. Results were adjusted to %WT and one-sample t-tests were used to compare knockout strain transcription levels with WT.
E. WT FliC levels in diverse environmental conditions were measured by Western analysis and normalized to cell count.
F. FliC production was measured by Western analysis in all conditions tested. Results were normalized to DnaK, adjusted to %WT, and one-sample t-tests were used to compare knockout strain FliC protein levels with WT in each condition.

3.3 RcsB and YifA independently regulate *flhDC* and *STM14_2047*.

RcsB and YifA are two proteins that have remarkably similar effects at the same points in the flagellar cascade, which could be an indication that the two genes act as a sequential regulatory pathway. We next examined the independence of these genes in their repression of *flhDC* and *STM14_2047*.

For *flhDC* transcription after 2.5 hours of growth in LB, β-galactosidase transcriptional reporters show that knocking out both *rcsB* and *yifA* results in an additive effect on the depression of *flhDC* transcription, indicating that these genes do not work together under these conditions to repress *flhDC* (Figure 3.4A). Additionally, either complementation plasmid (pRcsB or pYifA) or an empty vector control was transformed into strains containing a chromosomal *flhDC* transcriptional reporter. β-galactosidase activity was measured with each gene complemented in the presence or absence of the other for epistatic analysis (Figure 3.4D). Regarding pRcsB, the addition of *rcsB* resulted in repression of *flhDC* transcription both in strains that had a functional *yifA* gene and that lacked *yifA*. This confirms RcsB's role as a repressor of *flhDC* and establishes its independence from YifA. Complementation with pYifA did not result in measurable decreases in *flhDC* transcription, which may indicate that YifA acts through RcsB, but further examination showed that the loss of YifA had no effect on RcsB activity (data not shown), which was examined through the transcription of a downstream gene, *rprA*, that depends on RcsB for expression (Hu et al., 2013).

Similar experiments were performed to confirm the independence of RcsB and YifA with respect to repression of *STM14_2047*. Using the β-galactosidase transcriptional reporter, we observe an increase in *STM14_2047* transcription relative to WT when both *rcsB* and *yifA* are

eliminated, with additive effects observed in some conditions. In LB_{EXP} or in ST_{IN} conditions, the increase in the double knockout is no greater than either of the single gene, but additive effects were seen in LB_{EXP} - NaCl and LB_{STAT}, supporting that the two genes can act independently (Figure 3.4B). It is possible that the additive effect on derepression of *STM14_2047* transcription in LB_{EXP} was too slight to be measured by the available methods, but by examining effects downstream in the flagellar cascade, we can gain further insight into the activity of STM14_2047 in the single and double knockouts. RcsB and YifA have been shown to act at two points in the cascade, *flhDC* and *STM14_2047*, and the decrease in *fliC* expression is a result of their action through STM14_2047. Transcription of *flhDC* was rendered independent of RcsB and YifA by replacing the native *flhDC* promoter with a tetracycline inducible promoter. Consequently, the only point through which RcsB or YifA could regulate *fliC* would be their repression of *STM14_2047* transcription. Transcription of *fliC* measured by flow cytometry may then be used as a downstream marker of STM14_2047 activity. In this scenario, the double knockout of *rcsB* and *yifA* results in a significantly lower percentage of cells that express *fliC* than either of the single knockouts, which are both lower than WT. These results are concordant with the derepression of *STM14_2047* by the loss of *rcsB* or *yifA* and an additive effect upon the loss of both (Figure 3.4C). Additionally, experiments were performed using pRcsB and pYifA as complementation plasmids and a 3xFLAG-tagged version of STM14_2047 to examine protein levels by Western. There appeared to be a trend for the complementation of *rcsB* in the presence of *yifA,* where STM14_2047 levels are lower than in the empty vector control, but that difference is not significant. None of the remaining complementation pairs showed a significant difference either.

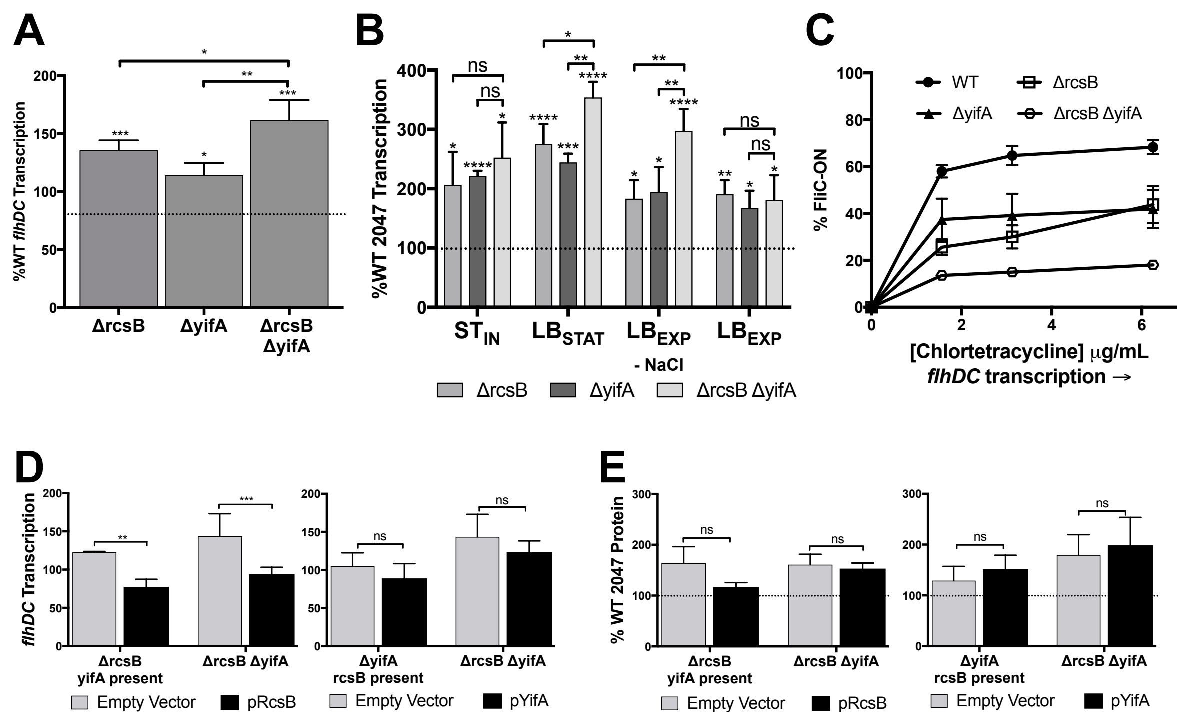

Figure 3.4. RcsB and YifA independently regulate *flhDC* and *STM14_2047*.
A. Transcription of *flhDC* is measured to asses the independence of RcsB and YifA. Chromosomal β-galactosidase reporters were used in strains lacking either *rcsB*, *yifA*, or both genes. A one-way ANOVA with correction for multiple comparisons was used to compare all strains.
B. Transcription of *STM14_2047* is measured to asses the independence of RcsB and YifA. *STM14_2047* transcription in strains lacking either *rcsB*, *yifA*, or both genes is measured using the chromosomal β-galactosidase reporter. A one-way ANOVA with correction for multiple comparisons was used to compare all strains.
C. Expression of *fliC* was used as an indicator of STM14_2047 activity to look for additive effects in the double knockout of *rcsB* and *yifA* in LB_{EXP}. The native promoter for *flhDC* was replaced with a tetracycline inducible construct by P22 transduction, rendering transcription of *flhDC* independent of RcsB and YifA. A plasmid containing the promoter for *fliC* fused to *gfp* was then used to obtain the results of *rcsB* and *yifA* single and double knockouts on *fliC* expression independent of their roles as regulators of *flhDC*.
D-E. Complementation experiments were performed for epistatic analysis to confirm independence of RcsB and YifA. The region of Stm 14028 chromosome containing *rcsB* or *yifA* and 200 bp preceding the start site was cloned into an arabinose inducible plasmid. The complementation plasmids (pRcsB or pYifA) or an empty vector control was transformed into strains containing chromosomal *flhDC* (D) or *STM14_2047* (E) transcriptional reporters. β-galactosidase activity was measured in strains lacking the chromosomal complemented gene only and strains without *rcsB* and *yifA* on the chromosome. A one-way ANOVA with correction for multiple comparisons was used to compare *flhDC* transcription in all strains.

3.4 THE DOUBLE KNOCKOUT OF RCSB AND YIFA RESULTS IN INCREASED FLAGELLAR EXPRESSION

Further investigation of the double knockout of *rcsB* and *yifA* showed drastic differences in flagellar gene expression between the single knockouts of each gene and the double knockout of both *rcsB* and *yifA*. GFP reporter plasmids for *flhDC* (Figure 3.5A), *fliA* (Figure 3.5B), and *fliC* (Figure 3.5C) transcription were transformed into strains lacking both *rcsB* and *yifA* and examined by flow cytometry after 2.5 hours of growth in LB media. The data was then analyzed in FloJo software to measure the percentage of cells expressing each gene (Figure 3.5D) and the GFP intensity of the ON subpopulations (Figure 3.5E). These results were compared to WT and each single knockout. Beginning with *flhDC*, there was monomodal high transcription of the master regulator in the double knockout (Figure 3.5A) and the GFP intensity is roughly 60% greater than WT (Figure 3.5E), indicating an increase in *flhDC* transcription with the loss of both genes. Moving through the cascade, the deletion of either *rcsB* or *yifA* resulted in a partitioning of cells into the OFF subpopulation (Figure 3.5D), but the double knockout has a majority of cells in the ON subpopulation (Figure 3.5B). An average of 96% of cells in the double knockout strain expressed *fliA* as compared to 65% in WT (Figure 3.5D). This promotion of the class II genes in the double knockout carried through into class III transcription (Figure 3.5C), with an average of 83% of cells expressing *fliC* as well (Figure 3.5D). The GFP intensity of the populations expressing *fliA* and *fliC* were not significantly higher than WT (Figure 3.5E), possibly due to the repression by larger amounts of STM14_2047 that also results from the loss of *rcsB* and *yifA* (Figure 3.4B).

We showed that when the native *flhDC* promoter is in place and susceptible to regulation by RcsB and YifA, there is an additive derepression of *flhDC* transcription after 2.5 hours of

growth in LB with the loss of both genes (Figure 3.4A). This additive derepression is not seen as readily in the transcription of *STM14_2047* in the same conditions (Figure 3.4B). This could suggest that the increase in *STM14_2047* transcription is smaller than the one observed in *flhDC* transcription. Additionally, a double knockout of *rcsB* and *yifA* resulted in a decrease in *fliC* transcription when the native *flhDC* promoter was replaced with a tetracycline-inducible promoter and held independent of action by RcsB and YifA (Figure 3.4C). The data suggest that RcsB and YifA must be able to act through *flhDC* in order to decrease flagellar expression relative to the double knockout. We have thus hypothesized that the stronger additive derepression of *flhDC* in the double knockout overwhelms a more modest derepression of *STM14_2047* transcription, resulting in more expression of class II genes, which is carried through to an increase in transcription of *fliC* as well.

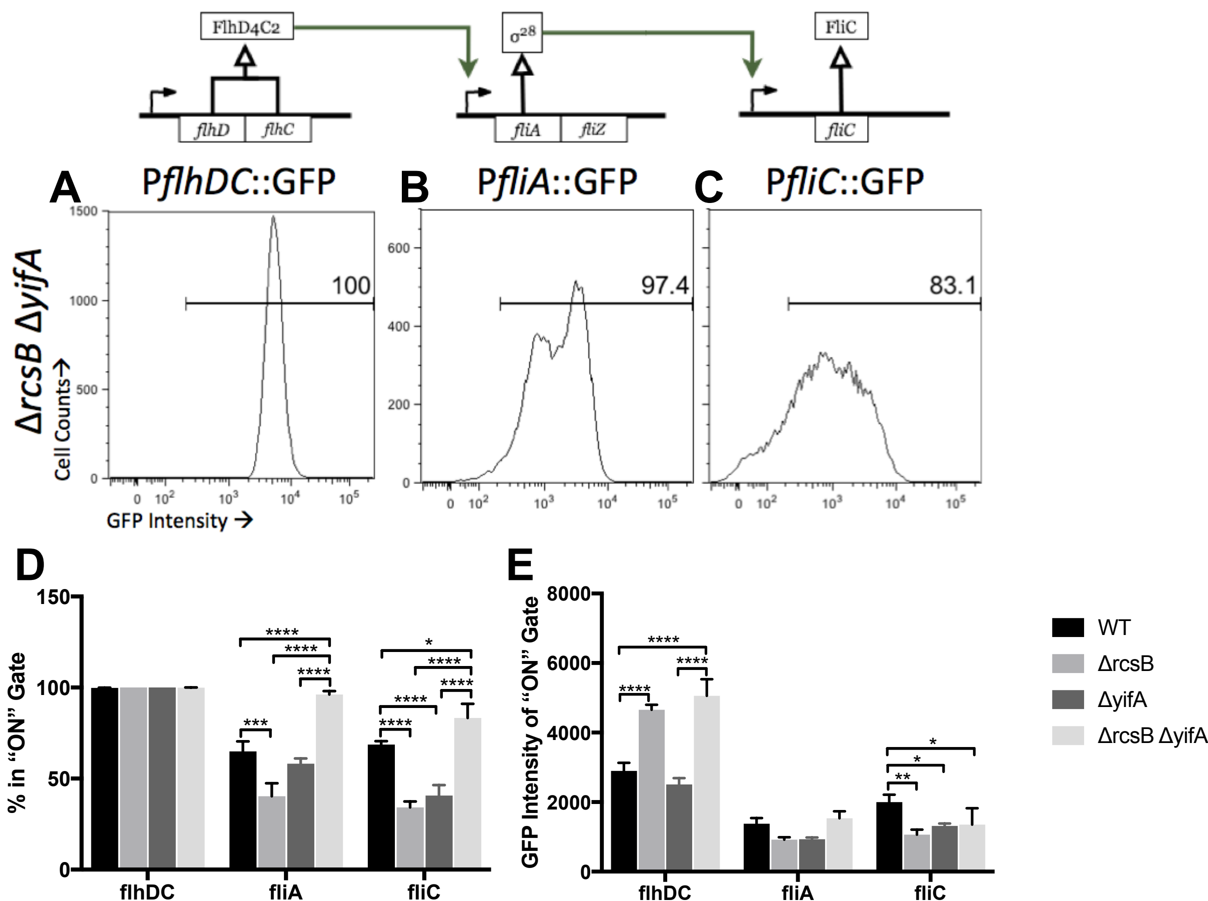

Figure 3.5. Transcription of the native promoter of *flhDC* increases in the double knockout of *rcsB* and *yifA* and results in increased expression throughout the cascade.
A-C. Single cell analysis of transcription of *flhDC* (A), *fliA* (B), and *fliC* (C) was done in the double knockout of *rcsB* and *yifA* using the *gfp* reporter plasmids and flow cytometry. Measurements were taken 2.5 hours after backdilution into LB broth.
D. The percent of the population expressing each class can be measured using gating in FlowJo software. A one-way ANOVA with correction for multiple comparisons was used to compare all strains. The loss of either *rcsB* or *yifA* has been shown to result in a significantly greater partitioning of cells into the OFF subpopulations in class II and III transcription, but the loss of both genes results in an increase in the percent of cells expressing class II and III genes.
E. Examination of the geometric mean fluorescent intensity of the ON populations shows significant increases in *flhDC* transcription in the double KO. A one-way ANOVA with correction for multiple comparisons was used to compare all strains.

Chapter 4. DISCUSSION

4.1 Flagellar bistability is a delicate balance between the master regulator and its post-translational repressors

In forming an understanding of the regulation of flagellar expression, we can think of the resulting bistable pattern as a delicate balance between the master regulator and its post-translational regulators. Any alteration in environmental condition or gene expression that increases *flhDC* or *STM14_2047* expression relative to its counterpart can lead to a greater probability for a cell to become flagellated or non-flagellated, respectively (Figure 4.1A). An increase in STM14_2047 relative to $FlhD_4C_2$ results in a strong repression of class II and III transcription because $FlhD_4C_2$ is bound by STM14_2047 and is unable to recruit RNA polymerase. This is what is observed in the case of the single knockouts of *rcsB* or *yifA* in LB_{EXP}, where STM14_2047 increases roughly 60-100% whereas *flhDC* transcription increases only about 20-30%. The result is a net repression of flagellar expression. Conversely, in the double knockout of both *rcsB* and *yifA*, the additive increase in $FlhD_4C_2$ surpasses the ability of STM14_2047 to repress it, resulting in successful promotion of the second and third classes in the flagellar cascade.

4.2 Environmental control of STM14_2047 occurs at multiple levels of expression and shapes bistability

The data that has been presented highlights the importance of examining regulation beyond transcription of a gene of interest. It has been reported previously that STM14_2047 is highly transcribed within macrophages, and it was hypothesized that it played a large role in repressing flagellar expression during systemic spread of *S.* Typhimurium (Li et al., 2017).

However, the research outlined in this paper shows that while STM14_2047 transcription may be high, post-transcriptional mechanisms limit STM14_2047 levels to the point that a slight increase in *flhDC* transcription resulted in a large increase in the number of cells expressing flagellin. Interestingly, the condition where the highest levels of STM14_2047 protein is observed is in rich media that reflects conditions found in host intestines. This is a condition where the expression of flagella and the related invasion genes are required for pathogenesis. I propose that rather than having a primary role of absolutely repressing $FlhD_4C_2$ activity, the role of STM14_2047 is to maintain the FliC-OFF subpopulation so that cells may benefit from the actions of the invasive subpopulation and disseminate to new hosts.

4.3 RcsB and YifA may maximize fitness in varied conditions

Salmonella is typically highly motile in nutrient-rich conditions, rather than low resource environments, which has been used to hypothesize that the role of flagellar expression in *Salmonella* has evolved to benefit host colonization rather than foraging (Koirala et al., 2014). In nutrient rich media, RcsB and YifA independently act as inducers of flagellar expression by repressing STM14_2047 to reinforce the motile phenotype where it has been hypothesized to be most useful (Figure 4.1B). Additionally, RcsB and YifA have been reported as inducers of curli and std fimbriae, respectively, which aid in attachment to the intestinal epithelium (Sharma et al., 2017; Clarke et al., 2010; Jakomin et al., 2008). Another possible benefit from these genes' regulation could be induction of SPI-1 genes through FliZ to promote invasion of the intestinal epithelium after attachment with the fimbriae. In intracellular conditions, however, where the presence of flagella results in pyroptosis, the roles of RcsB and YifA are reversed. This occurs because the repression of *flhDC* has a greater influence on flagellar expression than repression of *STM14_2047*, which is kept at very low levels by regulators yet to be identified. In each of these

situations, the actions by RcsB and YifA as either inducers or repressors of flagellar production could be to maximize the fitness of the cell in a given population.

Current literature focusing on the roles of RcsB and YifA in flagellar regulation has only identified their ability to repress *flhDC* transcription and thus labeled them as negative regulators of flagella. This has been the accepted narrative for a variety of reasons. RcsB and YifA have both been identified as inducers of fimbriae (Sharma et al., 2017; Clarke, 2010; Jakomin et al., 2008) and RcsB is a promoter of the colonic acid capsule (Clarke, 2010), both of which are characteristics that are not often coexpressed with flagella (Clegg and Hughes, 2002). Additionally, the strain of *Salmonella* that is often used in molecular studies of *Salmonella* motility is LT2. This common lab strain has attenuated virulence and there is a frameshift mutation in the *STM14_2047* gene, rendering it nonfunctional (Jarvik et al., 2010). We have demonstrated that in the absence of STM14_2047, loss of *rcsB* or *yifA* results in significant increases in flagellar expression. Consequently, motility or FliC expression in LT2 strains with knockout mutations in *rcsB* or *yifA* would be expected to show an increase relative to WT. However, even in studies that use the14028s strain, limiting to *flhDC* transcription as a proxy for expression in the entire flagellar cascade without looking at expression further downstream or phenotypic motility would result in a conclusion that *rcsB* and *yifA* repress motility in all conditions because the genes are repressors of *flhDC*. This further serves to highlight the importance of taking post-transcriptional regulation into account when assessing flagellar expression. Our data show that *flhDC* transcription should not be the only marker examined as a single reporter for flagellar expression because regulation further down the cascade can produce counteracting effects and lead to false conclusions.

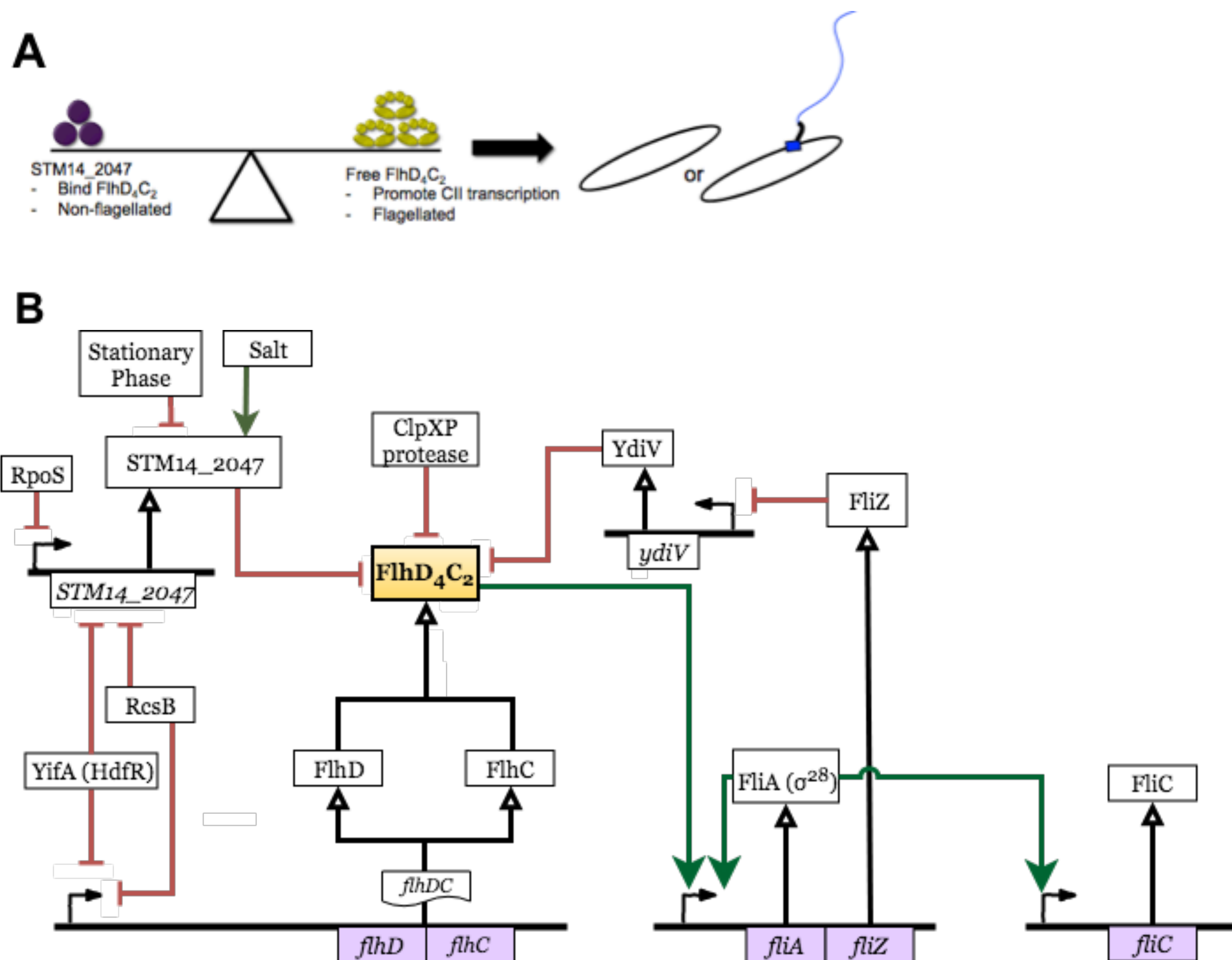

Figure 4.1. RcsB and YifA affect expression at multiple points in the flagellar cascade.
A. The flagellation state of each cell is the culmination of the balance between $FlhD_4C_2$ and its post-translational regulators. A relative increase in $FlhD_4C_2$ or STM14_2047 influences the probability that the cell will become flagellated or not, respectively.
B. Our experiments have shown that RcsB and YifA control flagellar expression at two points in the flagellar cascade, *flhDC* and *STM14_2047*. Due to their ability to act at these points, RcsB and YifA are able to either induce or repress flagellar production to maximize the fitness of the cell in a given environmental condition. When STM14_2047 is at higher levels of expression in exponential phase growth in rich media, the repression of STM14_2047 by RcsB and YifA serve to promote flagellar expression beginning at class II transcription despite a more modest repression of *flhDC*. However, there are additional regulators of *STM14_2047* that respond to environmental cues and limit STM14_2047's influence in stationary phase and low salt conditions. As a result, the primary roles of RcsB and YifA in these conditions are as repressors of *flhDC,* and ultimately *fliC*.

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APPENDIX A

Table 4.1. Strains used in this study

BC####	GENOTYPE	Source
BC1662	Stm 14028 *fljBA*::FRT	
BC3843	Stm 14028 *fljBA*::FRT *hilD*::FRT	This Study
BC3962	Stm 14028 *fljBA*::FRT *rcsB*::FRT	This Study
BC4033	Stm 14028 *fljBA*::FRT *lrhA*::FRT	This Study
BC4128	Stm 14028 *fljBA*::FRT *rtsB*::FRT	This Study
BC4129	Stm 14028 *fljBA*::FRT *slyA*::FRT	This Study
BC4130	Stm 14028 *fljBA*::FRT *fimZ*::FRT	This Study
BC4131	Stm 14028 *fljBA*::FRT *yifA*::FRT	This Study
BC4132	Stm 14028 *fljBA*::FRT *ompR*::FRT	This Study
BC4133	Stm 14028 *fljBA*::FRT *ecnR*::FRT	This Study
BC4134	Stm 14028 *fljBA*::FRT *barA*::FRT	This Study
BC4135	Stm 14028 *fljBA*::FRT *uvrY*::FRT	This Study
BC3956	Stm 14028 *fljBA*::FRT *STM14_2047*::FRT	Stewart et al., 2018
BC4520	Stm 14028 fljBA::FRT STM14_2047::FRT rcsB::FRT	This Study
BC4521	Stm 14028 fljBA::FRT STM14_2047::FRT yifA::FRT	This Study
BC4499	Stm 14028 fljBA::FRT rcsB::FRT yifA::FRT	This Study
BC3799	Stm 14028 *fljBA*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC3985	Stm 14028 *fljBA*::FRT *rcsB*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC4335	Stm 14028 *fljBA*::FRT *lrhA*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC4336	Stm 14028 *fljBA*::FRT *hilD*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC4337	Stm 14028 *fljBA*::FRT *rtsB*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC4338	Stm 14028 *fljBA*::FRT *slyA*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC4339	Stm 14028 *fljBA*::FRT *fimZ*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC4340	Stm 14028 *fljBA*::FRT *yifA*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC4341	Stm 14028 *fljBA*::FRT *ompR*::FRT *flhD*::FRT::pKG136 kanR	This Study

BC4342	Stm 14028 *fljBA*::FRT *ecnR*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC4343	Stm 14028 *fljBA*::FRT *barA*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC4344	Stm 14028 *fljBA*::FRT *uvrY*::FRT *flhD*::FRT::pKG136 kanR	This Study
BC4524	Stm 14028 fljBA::FRT rcsB::FRT yifA::FRT flhDC::FRT::pKG136 kanR	This Study
BC1810	Stm 14028 fljBA::FRT PflhD::GFP CarbR	Cummings et al, 2006
BC4410	Stm 14028 fljBA::FRT rcsB::FRT pPflhD:GFP CarbR	This Study
BC4415	Stm 14028 fljBA::FRT yifA::FRT pPflhD:GFP CarbR	This Study
BC4558	Stm 14028 fljBA::FRT rcsB::FRT yifA::FRT pPflhD::GFP CarbR	This Study
BC2847	Stm 14028 fljBA::FRT pPfliA::gfp CarbR	Cummings et al, 2006
BC4399	Stm 14028 fljBA::FRT rcsB::FRT pPfliA::gfp CarbR	This Study
BC4404	Stm 14028 fljBA::FRT yifA::FRT pPfliA::gfp CarbR	This Study
BC4559	Stm 14028 fljBA::FRT rcsB::FRT yifA::FRT pPfliA::gfp CarbR	This Study
BC2117	Stm 14028 fljBA::FRT pPfliC-GFP	Cummings et al, 2006
BC4388	Stm 14028 fljBA::FRT rcsB::FRT pPfliC::gfp CarbR	This Study
BC4393	Stm 14028 fljBA::FRT yifA::FRT pPfliC::gfp CarbR	This Study
BC4559	Stm 14028 fljBA::FRT rcsB::FRT yifA::FRT pPfliC::GFP CarbR	This Study
BC3998	Stm 14028 fljBA::FRT STM14_2047::FRT pPfliC::gfp	Stewart et al., 2018
BC4726	Stm 14028 fljBA::FRT STM14_2047::FRT rcsB::FRT pPfliC::gfp	This Study
BC4729	Stm 14028 fljBA::FRT STM14_2047::FRT yifA::FRT pPfliC::gfp	This Study
BC3418	Stm 14028 fljBA::FRT STM14_2047::FRT::pKG136 kanR	Stewart et al., 2018
BC3947	Stm 14028 fljBA::FRT rcsB::FRT STM14_2047::FRT::pKG136	This Study
BC4426	Stm 14028 fljBA::FRT yifA::FRT STM14_2047::FRT::pKG136 kanR	This Study
BC4500	Stm 14028 fljBA::FRT rcsB::FRT yifA::FRT STM14_2047::FRT::pKG136 kanR	This Study
BC4019	Stm 14028 fljBA::FRT STM14_2047::3XFLAG kanR	Stewart et al., 2018
BC4437	STM 14028 fljBA::FRT rcsB::FRT STM14_2047::3XFLAG kanR	This Study
BC4442	STM 14028 fljBA::FRT yifA::FRT STM14_2047::3XFLAG kanR	This Study
BC4501	Stm 14028 fljBA::FRT rcsB::FRT yifA::FRT STM14_2047::3xFLAG kanR	This Study
BC4004	Stm 14028 fljBA::FRT ydiV::3xFLAG kanR	This Study
BC4459	Stm 14028 fljBA::FRT rcsB::FRT ydiV::3xFLAG kanR	This Study

BC4464	Stm 14028 fljBA::FRT yifA::FRT ydiV::3xFLAG kanR	This Study

Table 4.2. Plasmids used in this study

Name	Description	Source
pKD4	Template plasmid for resistance cassette and FLP sites, kanR	Datsenko and Wanner, 2000
pKD46	Lambda red integration plasmid, carbR	Datsenko and Wanner, 2000
pCP20	FLP recombinase for closing cassette in gene deletions, carbR	Datsenko and Wanner, 2000
pKG136	LacZ and kanamycin resistance for transcriptional reporters, kanR	Ellermeier et al., 2001
pPflhDC::GFP	Reporter plasmid for flhDC transcription, carbR	Cummings et al., 2006
pPfliA::GFP	Reporter plasmid for fliA transcription, carbR	Cummings et al., 2006
pPfliC::GFP	Reporter plasmid for fliC transcription, carbR	Cummings et al., 2006
pJN105	Vector for complementation plasmids, gentR	Newman & Fuqua, 1999
pRcsB	Complementation plasmid with rcsB and native promoter, gentR	This Study
pYifA	Complementation plasmid with yifA and native promoter, gentR	This Study

Table 4.3. Primers used in this study

Name	Sequence
LR lrhA FWD	GGTCAGCCCGATATGACCCGCCAGTAAGTGAAAAATTATGGTGTAGGCTGGAGCTGCTTC
LR lrhA REV	TTTTTACATTTACGACTTAGCGCCTTTTGCCTGCGAGCTACATATGAATATCCTCCTTAG
LR rcsB FWD	GATGACCACCCGATTGTACTGTTCGGTATTCGCAAATCACGTGTAGGCTGGAGCTGCTTC
LR rcsB REV	GGTGACGGAAGAGAGATAGTTGAGCAGCGCGATATCATTCCATATGAATATCCTCCTTAG
LR yifA FWD	AAAACAAAACATTTAAATCATAACGACAAATAATTTTGTGGTGTAGGCTGGAGCTGCTTC
LR yifA REV	GTAATCGTAAGTTCCCTTCTTTTTCTTTTCATCATTTTCACATATGAATATCCTCCTTAG
LR barA FWD	TAACAGTGTGACCCTAATTGTCCCATAACGGAACTCCATGGTGTAGGCTGGAGCTGCTTC
LR barA REV	GGCCGGATAAGGCGGTCACGCCGCCATCCGGCGAATATCACATATGAATATCCTCCTTAG
LR uvrY FWD	CTATCAGTAGCGTTATCCCTATTCTGGAGATATTCCTTTGGTGTAGGCTGGAGCTGCTTC
LR uvrY REV	ACGGTTTTCAAAAACGCCTTTGCGTCAAATATTTCACTCACATATGAATATCCTCCTTAG
LR hilD FWD	GTAAGGAACATTAAAATAACATCAACAAAGGGATAATATGGTGTAGGCTGGAGCTGCTTC
LR hilD REV	ATAAAAATCTTTACTTAAGTGACAGATACAAAAAATGTTACATATGAATATCCTCCTTAG

LR ompR FWD	CACTTACATTTGTTGCGAACCTTTGGGAGTACAGACAATGGTGTAGGCTGGAGCTGCTTC
LR ompR REV	CGGGCAAATGAACTTCGCGGTGAGAAGCGCATTCGCCTCACATATGAATATCCTCCTTAG
LR rtsB FWD	CCTCTCGTCATCAATATGTTAATTGAGATATCTGACAATGGTGTAGGCTGGAGCTGCTTC
LR rtsB REV	TGCCTTGCCTACCACTCTACCAACATTTTAGGAAAAATTACATATGAATATCCTCCTTAG
LR slyA FWD	ATAATAACTTAGCAAGCTAATTATAAGGAGATGAAATTGGTGTAGGCTGGAGCTGCTTC
LR slyA REV	ACGTGTGGTCACATGGCCACACGTATGCCCCTGCACCTCACATATGAATATCCTCCTTAG
LR fimZ FWD	AAAACGAAGGACGCATAACAGTCTGAGGCATACAACAATGGTGTAGGCTGGAGCTGCTTC
LR fimZ REV	ATTGTGGCTCCCGAACGATAATTCGCCGGGAGTACATTTACATATGAATATCCTCCTTAG
LR ompR FWD	CACTTACATTTGTTGCGAACCTTTGGGAGTACAGACAATGGTGTAGGCTGGAGCTGCTTC
LR ompR REV	CGGGCAAATGAACTTCGCGGTGAGAAGCGCATTCGCCTCACATATGAATATCCTCCTTAG
LR ecnR FWD	AAGAAAGTGCTTTATCTACTTCCGGGTGCAAAAAACTATGGTGTAGGCTGGAGCTGCTTC
LR ecnR REV	GCAGTAATCATCAACGGTACGGCATGGCGTCGTACCGTTACATATGAATATCCTCCTTAG
RcsB comp FWD	TTTTTGAATTCCCAGCGGCTATTATGCGCTA
RcsB comp REV	TTTTTGAGCTCAACATAAAAGCGATTTATTC
YifA comp FWD	TTTTTGAATTCTCAGTCACAGGCTCACGCTT
YifA comp REV	TTTTTGAGCTCTCTTTTCATCATTTTCATTG

VITA

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EXPERIENCE

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Five week rotation covering standard practices in a clinical microbiology laboratory. Subjects included workup of blood, stool, wound, respiratory, fluid, and urine specimens for infectious bacteria and fungi, mass spectrometry, and some data entry into Sunquest.

MICROBIOLOGY TEACHING ASSISTANT Fall 2015 and Summer 2017

Duties: lesson planning, writing and grading exams, conducting office hours and review sessions, leading introductory lectures before lab, and helping students interpret lab protocols and results.

STUDENT RESEARCH ASSISTANT at Kavanagh Lab- University of Washington 2012 to 2016

Duties: DNA isolation, PCR, tissue culture of hepatocytes, protein quantification and analysis, making stock solutions, assisting in necropsies, and budget paperwork.

MICROPHILES PUBLIC OUTREACH LEAD 2015 - 2016

Instructor on basic microbiology to interested children in after school programs
Coordination of new and returning volunteers for each of the three programs
Outreach to professionals in microbiology for student field trips

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Duties: Process shellfish for both microbiological and chemical hazards and collect water samples throughout the Puget Sound.

PRESENTATIONS AND PUBLICATIONS

GRADUATE RESEARCH presented at Laboratory Medicine Research Conference

April 2017- Regulation of phenotypic heterogeneity in *Salmonella* flagellar expression

January 2018- A recombineering approach to unraveling clinically significant bacterial phenotypes

UNDERGRADUATE RESEARCH presented at university-wide and School of Public Health research symposiums

MAY 2015- An in vitro evaluation of silver ion toxicity with regards to glutathione production

MAY 2016- Adaptive regulation of glutathionylation in *gclm* null mice

Cartwright M, Schmuck S, Corredor C, Wang B, Scoville DK, Chisholm CR, et al. The pulmonary inflammatory response to multiwalled carbon nanotubes is influenced by gender and glutathione synthesis. *Redox Biol*. 2016; 9:264–275.

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