Driving the expression of the *Salmonella enterica* sv Typhimurium flagellum using *flhDC* from *Escherichia coli* results in key regulatory and cellular differences.

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Running Title: *E. coli flhDC* function in *S. enterica*

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ABSTRACT

The flagellar systems of *Escherichia coli* and *Salmonella enterica* exhibit a significant level of genetic and functional synteny. Both systems are controlled by the flagellar specific master regulator $FlhD_4C_2$. Since the early days of genetic analyses of flagellar systems it has been known that *E. coli flhDC* can complement a Δ*flhDC* mutant in *S. enterica*. The genomic revolution has identified how genetic changes to transcription factors and / or DNA binding sites can impact the phenotypic outcome across related species. We were therefore interested in asking: using modern tools to interrogate flagellar gene expression and assembly, what would the impact be of replacing the *flhDC* coding sequences in *S. enterica* for the *E. coli* genes at the *flhDC S. entercia* chromosomal locus? We show that even though all strains created are motile, flagellar gene expression is measurably lower when $flhDC_{EC}$ are present. These changes can be attributed to the impact of $FlhD_4C_2$ DNA recognition and the protein-protein interactions required to generate a stable $FlhD_4C_2$ complex. Furthermore, our data suggests that in *E. coli* the internal flagellar FliT regulatory feedback loop has a marked difference with respect to output of the flagellar systems. We argue due diligence is required in making assumptions based on heterologous expression of regulators and that even systems showing significant synteny may not behave in exactly the same manner.

IMPORTANCE

The bacterial motility organelle known as the flagellum is shared across many bacterial species. *Escherichia coli* and *Salmonella enterica* have underpinned our appreciation of how bacteria express and assemble the bacterial flagellum for over half a century. We show that even though the *E. coli* and *S. enterica* flagellar systems look genetically identical, they input regulatory signals into the flagellar system differently. Our conclusions

54 are based on experiments where we carefully transfer the master flagellar regulator from
55 *E. coli* into the *S. enterica* chromosome and measure a range of outputs relating to
56 flagellar gene expression, assembly and functional output.
57
58

INTRODUCTION

The flagellum in the enteric bacteria, *Escherichia coli* and *Salmonella enterica*, has been studied extensively for over fifty years and provides the canonical example for bacterial motility. These studies have revealed not only the complex structure of the enteric flagellum but also its role in host colonization, pathogenesis, and cellular physiology (1-4). In addition, these studies have identified many of the complex regulatory processes that coordinate the assembly and control of this exquisitely complex biological machine (3-5).

The flagellum in *E. coli* and *S. enterica* are structurally very similar and are often tacitly assumed to be effectively identical aside from differences in the filament structure. However, in the case of regulation, these assumptions are based more on sequence similarity rather than on actual experimental data (5) (6). Indeed, a number of studies have shown that these two systems are regulated in entirely different manners in response to environmental signals despite strong gene synteny. For example, many common *E. coli* strains are motile only during growth in nutrient-poor conditions whereas many common *S. enterica* strains are motile only during growth in nutrient-rich conditions (7). In addition, *E. coli* is more motile at 30°C than at 37°C whereas motility *S. enterica* is generally insensitive to these temperature differences (8). *E. coli flhDC* are transcribed from a single transcriptional start site that is responsive to OmpR, RcsB and CRP regulation, to name only a few regulatory inputs (8). In contrast *S. enterica flhDC* transcription is significantly more complex with up to 5 transcriptional start sites, albeit with only a subset being responsible for the majority of *flhDC* transcription (9).

Part of the problem is that different questions have been asked when studying the regulation of motility in these two bacterial species. Most studies in *E. coli* have focused on the

85 environmental signals and associate regulatory process that induce bacterial motility. In
86 particular, they have focused on the processes that regulate the expression of the master
87 flagellar regulator, $FlhD_4C_2$ (8). Most studies in *S. enterica*, on the other hand, have focused
88 on the regulatory processes that coordinate the assembly process following induction (4). In
89 particular, they have focused on the downstream regulatory processes induced by $FlhD_4C_2$
90 (3).
91
92 Despite differences in regulation, the protein subunits of master flagellar regulators, FlhC
93 and FlhD, exhibit high sequence similarity sharing 94 and 92% identity, respectively,
94 between *E. coli* and *S. enterica*. Given that modifications to transcription factors and/or
95 promoter structure can lead to divergence in regulatory circuits (10), we were interested in
96 how $FlhD_4C_2$ functions in different genetic backgrounds? Previously, it was shown that *E.*
97 *coli flhDC* can complement a Δ*flhDC* mutant in *S. enterica*, suggesting that these proteins
98 are functions identical in the two bacterial species (11). However, it is not clear whether
99 they are regulated in the same manner. We, therefore, investigated the impact of replacing
100 the native master regulator in *S. enterica* with the one from *E. coli*. Defining the impact of
101 known $FlhD_4C_2$ regulators such as ClpP, YdiV, FliT and FliZ on the two complexes
102 suggest that these two species have adapted in how they perceive $FlhD_4C_2$. We argue that
103 these phenotypic differences arise from adaptations *E. coli* and *S. enterica* have made
104 during evolution to expand or modify cellular function with respect to movement within
105 specific environmental niches.
106

RESULTS

Orthologous flhDC *from* E. coli *can functionally complement* flhDC *in* S. enterica

Given the similarities between the flagellar systems in *S. enterica* and *E. coli*, we sought to determine whether the $FlhD_4C_2$ master regulator is functionally equivalent in these two species of bacteria. To test this hypothesis, we replaced the *flhDC* genes in *S. enterica* *flhDC*$_{SE}$) with the *flhDC* genes from *E. coli* (*flhDC*$_{EC}$). The reason that we performed these experiments in *S. enterica* rather than *E. coli* was that the flagellar system is better characterized in the former, particularly with regards to transcriptional regulation. To avoid plasmid associated artefacts associated with the ectopic expression of *flhDC,* we replaced the entire *S. enterica flhDC* operon with the *flhDC* operon from *E. coli* at the native chromosomal locus (Figure S1).

We first tested whether *flhDC*$_{EC}$ was motile as determined using soft-agar motility plates. As shown in Figures 1A and B, these strains formed rings similar to the wild type. These results demonstrate that *flhDC*$_{EC}$ is functional in *S. enterica.* However, motility plates measure both motility and chemotaxis and do not provide any insights regarding possibly changes in the number of flagella per cell. To determine the impact *flhDC*$_{EC}$ had upon flagellar numbers we used a FliM-GFP fusion as a proxy for flagellar numbers (Figure 1C). When this fluorescent protein fusion is expressed in cells, it forms spots associated with nascent C-rings that loosely correlate with the number of flagella (12-14). By counting the number of spots per cell, we can determine the number of flagella made per cell. As shown Figure 1C, *flhDC*$_{EC}$ did not change flagellar numbers as compared to the wild type. These results demonstrate *flhDC*$_{EC}$ induces flagellar gene expression at similar levels as the wild type.

133

134 *flhDC requires a specific transcription rate to maintain optimal flagellar numbers*

135

136 The flagellar network in *S. enterica* contains a number of feedback loops to ensure that the
137 cells regulate the number of flagella produced (4). One possibility is that these feedback
138 loops mask any differences in $FlhD_4C_{2EC}$ activity. To test this hypothesis, we replaced the
139 native P_{flhD} promoter with the tetracycline-inducble $P_{tetA}/_{tetR}$ promoters. We then measured
140 flagellar gene expression using a luciferase reporter system (15). In this case, a consistent
141 and significant change in flagellar gene expression was observed when comparing
142 $FlhD_4C_{2EC}$ to $FlhD_4C_{2SE}$ activity (Figure 2). Maximal expression of P_{flgA} and P_{fliC} , chosen to
143 reflect flagellar gene expression at different stages of flagellar assembly (5), for both
144 complexes was observed between 10 and 25 ng/ml of anhydrotetracycline, when *flhDC*
145 transcription was from P_{tetA} (Figure 2A and B). In contrast, P_{tetR}, the weaker of the two
146 tetracycline inducible promoters, reached a maximal output between 50 to 100 ng/ml
147 anhydrotetracycline. In both scenarios the output for $FlhD_4C_{2EC}$ control was lower than for
148 the native $FlhD_4C_{2SE}$ complex (Figure 2A and 2B).

149

150 We also measured the number of FliM-GFP foci at different anhydrotetracycline
151 concentrations. P_{tetR}::*flhDC* expression generated on average of approximately two FliM-
152 foci per cell at 25 ng/ml of anhydrotetracycline for both $FlhD_4C_2$ complexes (Figure 2C). In
153 contrast, 5 ng/ml induction of the P_{tetA}::$flhDC_{EC}$ strain was sufficient to generate typical
154 FliM-foci numbers (approx. 8 flagellar foci per cell). Even with the strong decrease in
155 average foci per cell at these levels of induction, the number of basal bodies observed is
156 sufficient to allow motility at comparable levels in the motility agar assay (Figure S2).

157

158 *Replacement of* flhC *but not* flhD *in* S. enterica *with the* E. coli *orthologs affects motility*

159

160 The hetero-oligomeric regulator $FlhD_4C_2$ is unusual in bacteria as the majority of
161 transcriptional regulators are believed to be homo-oligomeric complexes. To determine the
162 relative contributions of the two subunits, we individually replaced the *flhC* or *flhD* genes
163 from *S. enterica* with their ortholog from *E. coli* (Figure S1). When we tested the two
164 strains using motility plates, we found that motility was inhibited in the strain where $flhC_{EC}$
165 replaced the native *S. enterica flhC* (Figure 3A; blue bars), with an 88% reduction in
166 swarm diameter when compared to WT *S. enterica*. The introduction of $flhD_{EC}$ compared
167 to $flhDC_{EC}$ or $flhDC_{SE}$ produced swarms of a comparable size (Figure 3A; blue bars).
168
169 Using the dose-dependent inducible P_{tetA} promoter(16) we observed that P_{tetA} expression
170 of $flhC_{EC}$ led to reduced P_{flgA} transcription and strongly reduced P_{fliC} transcription (Figure
171 4). Strains expressing $flhD_{EC}$ in *S. enterica* showed a mild increase in P_{flgA} gene
172 expression and a similar response for P_{fliC}, although these changes were not significant (P
173 = 0.32) (Figure 4). These data suggest that the combination of $FlhD_{SE}$ and $FlhC_{EC}$
174 generates an inefficient $FlhD_4C_2$ complex, resulting in reduced motility.
175
176 *Orthologous FlhC and FlhD interaction is species specific and a key determinant of*
177 *promoter recognition by the $FlhD_4C_2$ complex*
178
179 The results above demonstrate that $flhC_{EC}$ is not functionally identical to $flhC_{ST}$. One
180 possibility is that that $FlhC_{EC}$ is impaired in $FlhD_4C_2$ for DNA-binding. Alternatively, the
181 stability of the $FlhD_4C_2$ complex is reduced in the $flhC_{EC}$ strain, leading to reduced $FlhD_4C_2$
182 activity. To test these hypotheses, we purified all combinations of the $FlhD_4C_2$ complex
183 using affinity (Ni+ and heparin) chromatography (Figure 5A). In each complex, FlhD was
184 tagged with a carboxy-terminal hexa-histidine to facilitate affinity purification. Such

185 expression constructs have previously been used successfully to purify the $FlhD_4C_2$
186 complex (17, 18). Using either Ni+ affinity or heparin purification, we observed complete
187 complex retrieval for three combinations (Figure 5A). FlhC recovery was less efficient in
188 the $FlhD_{SE}/FlhC_{EC}$ complex. In contrast, no $FlhD_{SE}/FlhC_{EC}$ complex was recovered via
189 Heparin purification, used to mimic DNA during protein purification of DNA-binding proteins
190 (Figure 5A). This suggests that the $FlhD_{SE}/FlhC_{EC}$ complex is less stable, resulting on a
191 lower yield of complex retrieval.
192
193 We next used the EMSA assays to test all four protein complexes for their ability to bind
194 the *S. enterica* P_{flgAB} promoter region. Quantification of the DNA shifts showed that
195 complexes containing the orthologous $FlhC_{EC}$ reduced the P_{flgAB} promoter binding profile,
196 compared to $FlhC_{SE}$ complexes (Figure 5B). This is consistent with FlhC being the DNA
197 binding subunit of the complex and the variation in $FlhD_4C_2$ activated promoter-binding
198 sites between *S. enterica* and *E. coli* (19). Therefore, these results suggest that FlhC is a
199 key determinant of DNA binding ability. Furthermore, the reduction in $FlhC_{EC}$ motility and
200 flagellar gene expression in *S. enterica* is a result of the $FlhD_{SE}/FlhC_{EC}$ complex being
201 unstable, ultimately reducing the cellular concentration of the $FlhD_4C_2$ complex.
202
203 *$FlhD_4C_{2EC}$ responds to proteolytic regulation*
204
205 *S. enterica* and *E. coli* both regulate the $FlhD_4C_2$ complex through ClpXP-mediated
206 proteolytic degradation. Proteolytic degradation of $FlhD_4C_2$ plays a fundamental role in
207 facilitating rapid responses to environmental changes that require motility (20, 21). The
208 $FlhD_4C_2$ complex has a very short half-live of approximately 2-3 minutes (22). Proteolytic
209 degradation of FlhD and FlhC is regulated in *E. coli* and *S. enterica* by YdiV (23).

However, *ydiV* is not expressed under standard laboratory conditions in model *E. coli* strains, suggesting that ClpXP activity is modulated in a species-specific manner (7).

Previous work has shown that YdiV delivers $FlhD_4C_2$ complexes to ClpXP for degradation (24). We have assessed the impact on motility for Δ*clpP* and Δ*ydiV* mutations (Figure 3). The Δ*clpP* and Δ*ydiV* mutants exhibited improved motility and flagellar gene expression, including the $FlhD_{SE}$/$FlhC_{EC}$ strain (Figure 3A and B). These results suggest that proteolytic degradation mechanism of FlhD and FlhC, and its regulation, is common to *E. coli* and *S. enterica*.

To complement the motility assays, we investigated how Δ*clpP* and Δ*ydiV* mutations impact the number of FliM-foci in cell. Both Δ*clpP* and Δ*ydiV* mutants showed an increased number of FliM-foci compared to the wild type (Figure 6 A-C). For $flhC_{EC}$ strain, FliM-foci were observed in 13% of the population where individual cells exhibited just one or two foci. However, the Δ*clpP* or Δ*ydiV* mutants increased the flagellated population of the $flhC_{EC}$ strains to 51 and 46 % respectively, albeit with the majority still possessing only a single FliM focus (Figure 6 B and C).

FliT and FliZ regulation of $FlhD_4C_2$ complexes

$FlhD_4C_2$ activity has an additional level of regulation in *S. enterica* via the flagellar-specific regulators FliT and FliZ. FliT functions as an export chaperone for the filament cap protein, FliD, and is a regulator of $FlhD_4C_2$ activity (17, 25). FliT disrupts the $FlhD_4C_2$ complex but is unable to disrupt a $FlhD_4C_2$:DNA complex. Therefore, FliT modulates availability of $FlhD_4C_2$ complexes for promoter binding (17). In contrast, FliZ is a negative regulator of

235 *ydiV* expression and thus increases the number of $FlhD_4C_2$ complexes in *S. enterica* (26,
236 27).
237
238 In motility assays of Δ*fliT* mutants, we observed a difference between our different *flhDC*
239 strains. Motility is increased in a Δ*fliT* mutant background in *S. enterica* ((28) and Figure
240 3A). However, when $flhDC_{EC}$ and $flhD_{EC}$ replaced the native genes, a reduced swarm size
241 was observed (Figure 3A). Furthermore, quantification of P_{fliC} activity agreed with the
242 motility profile for Δ*fliT* mutants, where $flhDC_{EC}$ and $flhD_{EC}$ containing strains had reduced
243 promoter activity compared to wild type (Figure 3B). This suggests that the $FlhD_4C_2$
244 complexes are being perceived differently by FliT in *S. enterica*. The results for Δ*clpP* and
245 Δ*ydiV* mutants suggests that this is not due to protein stability, as all complex
246 combinations reacted in a comparable fashion (Figure 3).
247
248 In contrast, the loss of *fliZ* resulted in a consistent reduction in motility, except for the
249 $flhC_{EC}$ strain. However, as the $flhC_{EC}$ strain was already impaired in motility, it is possible
250 that the resolution of the motility assay was unable to identify differences in Δ*fliZ* mutant.
251 Flagellar gene expression activity did, however, suggest a 2-fold drop in P_{fliC} expression in
252 the $flhC_{EC}$ Δ*fliZ* strain as compared to the otherwise wild-type (Figure 3B).
253
254 Analysis of FliM-foci distribution in Δ*fliT* mutant reinforced the observed discrimination of
255 $flhDC_{EC}$ and $flhD_{EC}$ gene replacements. Calculating the average foci per cell, *S. enterica*
256 Δ*fliT* mutants showed an increased average number of foci per cell from 2.9 to 6.3, while
257 the $flhD_{EC}$ ($fliT^+$: 3.4 versus Δ*fliT*: 4.2) and $flhDC_{EC}$ replacements ($fliT^+$: 3.6 versus Δ*fliT*:
258 2.7) exhibited no significant changes (Figure 7A). Interestingly, in a Δ*fliZ* mutant
259 background, the FliM-foci analysis was able to differentiate $flhDC_{EC}$ and $flhD_{EC}$ from the

native *S. enterica flhDC* strain. Both replacements exhibited an increase in the average foci compared to *S. enterica* Δ*fliZ* (Figure 7A).

These data suggest that there is a fundamental difference in how the $FlhD_4C_2$ complexes in *E. coli* and *S. enterica* respond to, at least, FliT regulation. There are two explanations for this: a) the *E. coli* combinations are being regulated via an unidentified mechanism in *S. enterica* or b) that they are insensitive to FliT regulation. Both arguments predict that in *E. coli* $FlhD_4C_2$ may respond differently to FliT regulation. Comparing *S. enterica* and *E. coli* does indeed identify a difference in the response to a Δ*fliT* mutant. While a Δ*fliT* mutant in *S. enterica* leads to a consistent increase in FliM-foci, no significant difference is noted for an *E. coli* Δ*fliT* mutant compared to *E. coli* wild type (Figure 7B). This suggests that the regulatory impact of FliT is very different in these two flagellar systems and the role FliT plays in *S. enterica* is potentially adaptive and species specific.

DISCUSSION

Two model flagellar systems that form the foundation of the flagellar field are those from the enteric species *E. coli* and *S. enterica*. These two systems have led to key discoveries in relation to many aspects of flagellar structure, type 3 secretion, flagellar cell biology and the regulation of flagellar assembly. Textbook explanations suggest that most flagellar systems are being activated, regulated and built according to the models for *E. coli* and *S. enterica*. Modifications of transcriptional regulatory circuits contribute to the phenotypic diversity we see in closely related gene sets and we are only now able to investigate this in depth due to the tools available. Here we have taken a simple step and asked how do orthologous $FlhD_4C_2$ complexes function in the closely related species *E. coli* and *S. enterica?*

At the onset of our work it was known that $FlhD_4C_2$ from *E. coli* could sustain motility in *S. enterica*(11). Our work was focussed on understanding and defining the species-specific differences in the regulon of two orthologous genes. Here we took advantage of the well-defined flagellar assembly tools to measure outputs such as, motility, flagellar assembly per cell and flagellar gene expression. Bioinformatic analysis identifies only an 8 and 6% identity difference between FlhD and FlhC in *E. coli* and *S. enterica* respectively, suggesting that these proteins function in an analogous fashion. It is well established that related taxa usually rely on orthologous regulators to coordinate response to a given signal (10).

The fine detail of the differences in the $FlhD_4C_2$ complexes only became apparent when we began to focus on their effect on flagellar gene expression and flagellar assembly. In all of our assays $FlhD_4C_{2EC}$ exhibited a reduction in flagellar gene expression compared to

301 $FlhD_4C_{2SE}$. Biochemical analysis of isolated complexes showed that $FlhC_{EC}$ had weaker
302 DNA binding ability to the P_{flgAB} promoter region from *S. enterica,* consistent with previous
303 investigations into $FlhD_4C_2$ DNA binding activity (19). The isolation of $FlhD_4C_2$ complexes
304 from our strains suggested that a key aspect of the phenotypes we observed, was the
305 stability of the complexes formed.
306
307 With respect to *flhDC* transcription we show a discrepancy in flagellar numbers defined by
308 FliM-foci when using P_{tetA}/P_{tetR}::*flhDC* expression. This was somewhat surprising as all
309 constructs exhibited good swarming ability on motility agar plates (Figure S2). Original
310 studies on the regulation of P_{tetA}/P_{tetR} from Tn10 have shown that these two promoters
311 have differing activities but both respond to TetR regulation. We show that even though
312 maximal activity of P_{flgA} and P_{fliC} can reach 40-50% of P_{tetA}::*flhDC* expression for P_{tetR}
313 strains, this results in an average of 2 flagella per cell. This suggests that even though the
314 majority of the literature states that *E. coli* and *S. enterica* produce between 4 and 8
315 flagella per cell, only 1 or 2 per cell is needed for an optimal output of the system with
316 respect to motility agar assays.
317
318 It has been shown that FliT interacts with FlhC and that in *S. enterica* the output of this
319 circuit is to destabilize $FlhD_4C_2$ complexes that are not bound to DNA. Our data suggests
320 that this level of regulation does not impact *E. coli* FlhC. The nature of the adaptability
321 needed by the favourable conditions to drive motility in *E. coli* may have led to the FliT
322 regulatory input becoming less critical. Similarly, the impact of FliZ regulation becomes
323 apparent for $FlhD_{EC}$ containing complexes when we assess flagellar numbers. FliZ
324 regulates the transcription of *ydiV* in *S. enterica* (27). It is plausible that the impact in
325 changing *ydiV* regulation is the source of this differentiation, especially as YdiV is
326 proposed to interact with $FlhD_{SE}$. Furthermore, we know that *ydiV* is not expressed in

model *E. coli* strains, strengthening the argument that $FlhD_{EC}$ has adapted to the absence of YdiV or vice versa $FlhD_{SE}$ to YdiV.

Importantly our analysis shows that even though these two systems are genetically similar, investigation of $FlhD_4C_2$ activity identifies subtle but key differences into how the $FlhD_4C_2$ complex is modulated in two closely related species. We argue that this is a valid example of the caution needed in the age of synthetic biology to exploit heterologous systems in alternative species or chassis'. Our data shows that even systems showing significant synteny may not behave in exactly the same manner and due diligence is required in making assumptions based on heterologous expression.

MATERIALS AND METHODS

Bacterial Strains and Growth conditions

S. enterica and *E. coli* strains used in this study have been previously described elsewhere (12, 15, 17, 28). This study used *S. enterica* serovar Typhimurium strain LT2 as the chassis for all experiments. *E. coli* genetic material was derived from MG1655. All strains were grown at either 30°C or 37°C in Luria Bertani Broth (LB) either on 1.5% agar plates or shaken in liquid cultures at 160 rpm (17). Antibiotics used in this study have been described elsewhere (29). Motility assays used motility agar (17) incubated at 37°C for 6 to 8 hours. Motility swarms were quantified using images captured on a standard gel doc system with a ruler in the field of view and quantified using ImageJ to measure the vertical and horizontal diameter using the average as the swarm size. All motility assays were performed in triplicate using single batches of motility agar.

Genetic Manipulations

For the replacement of *flhDC* coding sequences the modified lambda red recombination system described by Blank et al (2011) was used (30). Deletion of *clpP, ydiV, fliT* and *fliZ* was performed using the pKD system described by Datsenko and Wanner (2000) (31). P_{tetA} / P_{tetR} replacements of the P_{flhDC} region was also performed using Datsenko and Wanner (2000) with the template being Tn10*d*Tc (32). For Blank et al (2011) replacement experiments we used autoclaved chlortetracycline instead of anhydrotetracycline as described for the preparation of Tetracycline sensitive plates (33). All other gene replacements were performed as previously described (17). All primers used for these genetic manipulations are available on request.

364

365 *Quantification of flagellar gene expression*

366

367 Flagellar gene expression assays were performed using the plasmids pRG39::cat (P_{fliC})
368 and pRG52::cat (P_{flgA}) (15). Both plasmids were transformed into strains using
369 electroporation. Gene expression was quantified as described previously and analysis was
370 based on a minimum of n = 3 repeats for each strain tested (15).

371

372 *Quantification of FliM-GFP foci*

373

374 FliM-GFP foci were quantified using Microbetracker on images captured using a Nikon Ti
375 inverted microscope using filters and exposure times described previously (14). Strains
376 were grown to an OD600 of 0.5 to 0.6 and cells immobilised using a 1 % agarose pad
377 containing 10 % LB (14, 17). For each strain a minimum of 5 fields of view were captured
378 from 3 independent repeats. This allowed analysis of approximately 1000 - 1500 cells per
379 strain. For the comparison of FliM foci in *E. coli* Δ*fliT* to *S. enterica* Δ*fliT* shown in Figure
380 7B the chemostat growth system described by Sim et al (2017) was used. For this
381 experiment the growth rate of both strains was similar to batch culture in LB at 37°C where
382 the media used was a MinE base with 0.1% Yeast extract and 0.2% glucose (14, 17).

383

384 *Purification of $FlhD_4C_2$ complexes*

385

386 Purification of proteins complexes was based on previously described methods (17). Wild
387 type $FlhD_4C_{2SE}$ was purified using pPA158. The other 3 complexes were purified from
388 plasmids generated using the New England Biolabs NEBuilder DNA Assembly kit on the
389 backbone of pPA158. The *E. coli* strain BL21 was used for all protein induction

experiments prior to protein purification using either a pre-equilibrated 5ml His-trap column or a 5ml heparin column (GE Healthcare). Proteins were visualised using Tricine-based SDS polyacrylamide gel electrophoresis and standard commassie blue staining (17).

Electrophoretic mobility shift assay (EMSA).

All EMSA assays were performed using Ni++ (his-trap) purified proteins as this allowed analysis of all four complexes (Figure 5A). Buffer exchange from elution buffer to a 100mM Tris-HCl, 300 mM NaCl 1mM DTT (pH 7.9) buffer was performed through 10 cycles of protein concentration in VivaSpin columns with 20 ml buffer reduced to 5 ml per round of centrifugation at 4500 rpm. A protein concentration range of 100 to 700 nM was used with 80 ng / ml of a PCR product containing P_{flgAB} from *S. enterica*. After incubation bound and unbound DNA were resolved using 5% acrylamide gels made with 1x TBE buffer. Quantification of gel images was performed using ImageJ.

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FIGURES and LEGENDS

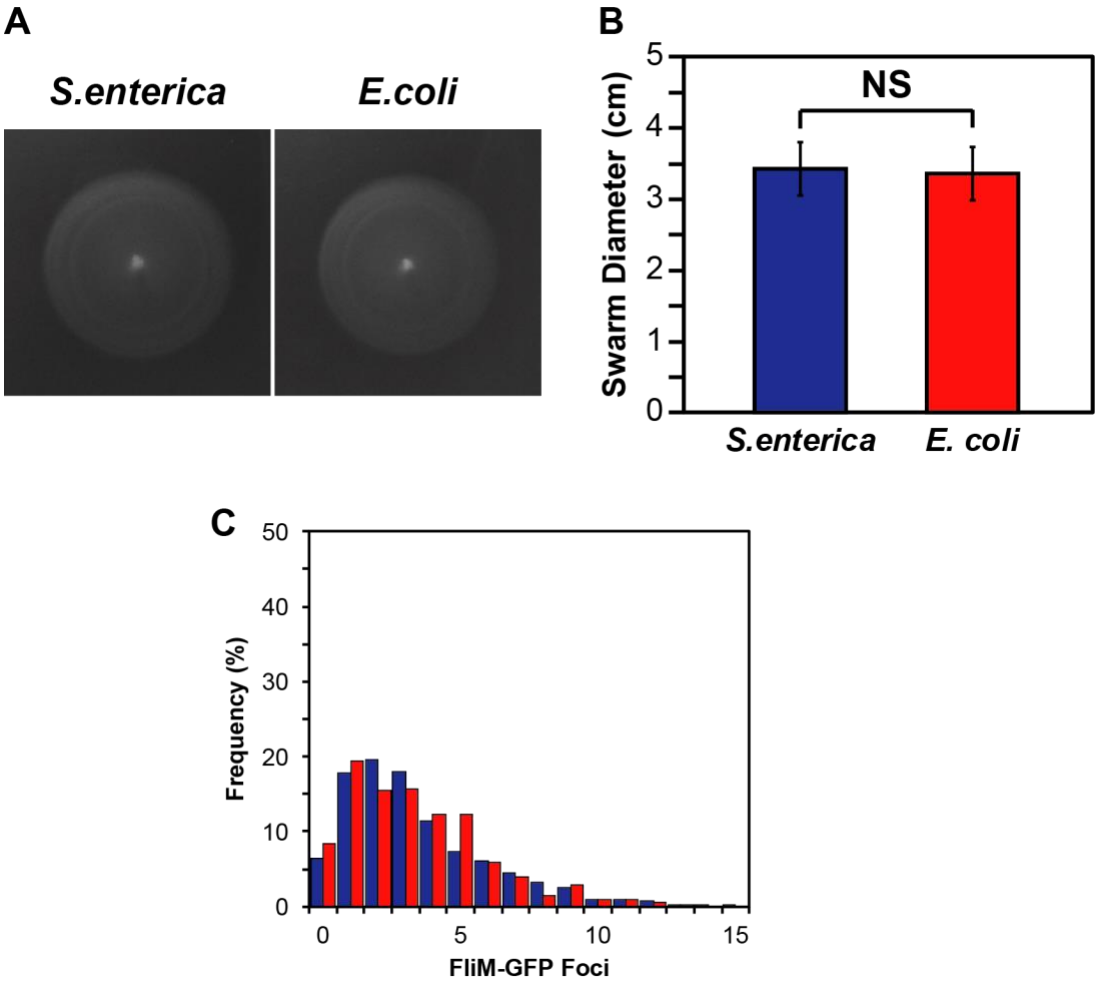

Figure 1 A. Motility of *flhDC*$_{ST}$ and *flhDC*$_{EC}$ driven by P$_{flhDC}$. **B.** Quantification of swarms produced in motility agar after 6 to 8 hours incubation. Error bars indicate calculated standard deviations. **C.** Percentage frequency of FliM-GFP foci for *flhDC*$_{EC}$ compared to *S. enterica* with *flhDC* under the control of P$_{flhDC}$. Colors of bars in the graph correspond to the source of *flhDC* as shown in (**B**).

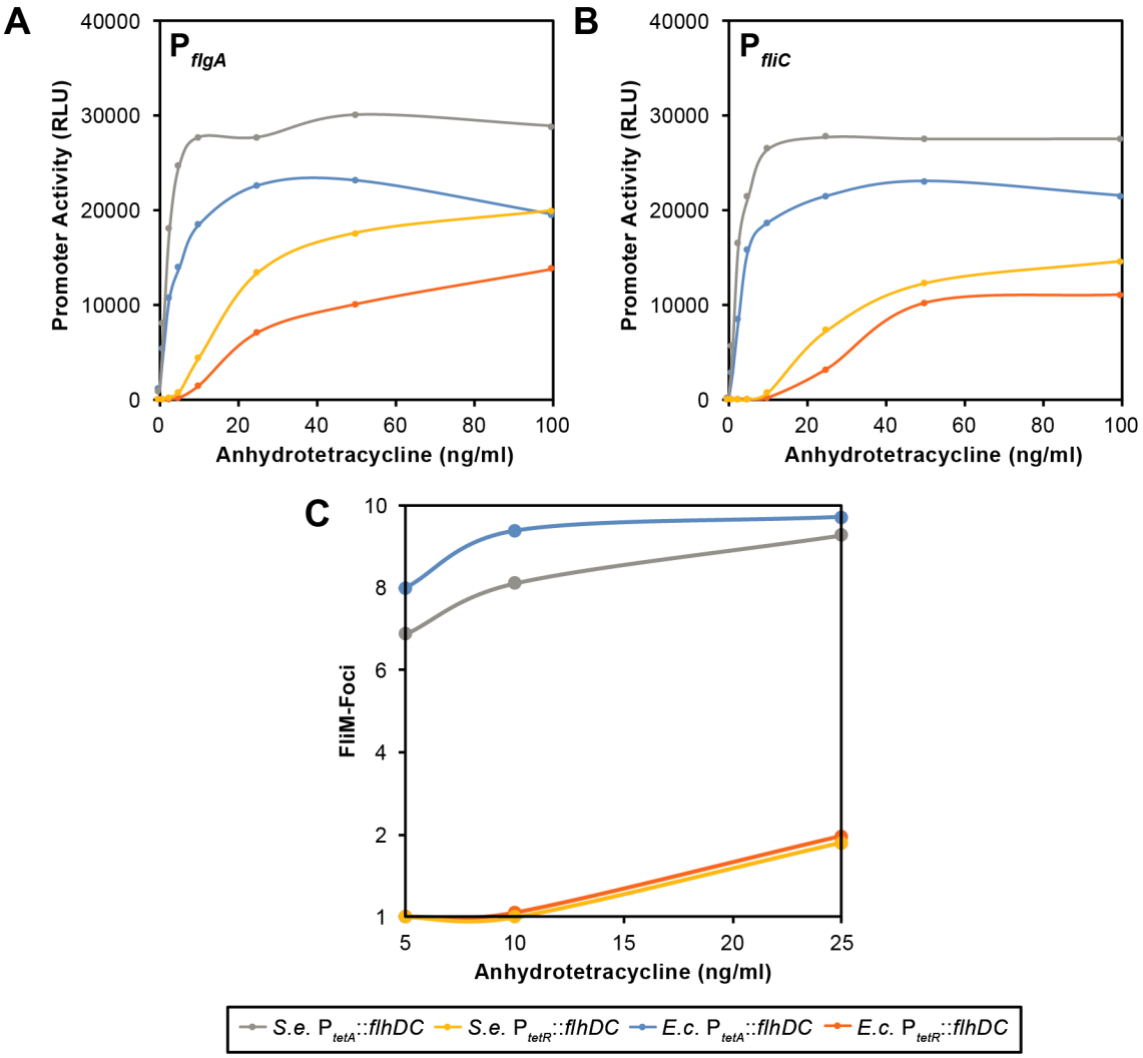

Figure 2 Titration of P_{tetA}*::flhDC*$_{ST/EC}$ and P_{tetR}*::flhDC*$_{ST/EC}$ activity suggests a given rate of transcription drives optimal flagellar assembly. **A.** Activity of P_{flgA} in response to P_{tetA} or P_{tetR} transcription of *flhDC* from *S. enterica* (S.e.) or *E. coli* (E.c.). **B.** Activity of P_{fliC} in response to P_{tetA} or P_{tetR} transcription of *flhDC*. **C.** flagellar numbers as defined by FliM-foci in response to P_{tetA} or P_{tetR} transcription of *flhDC.*

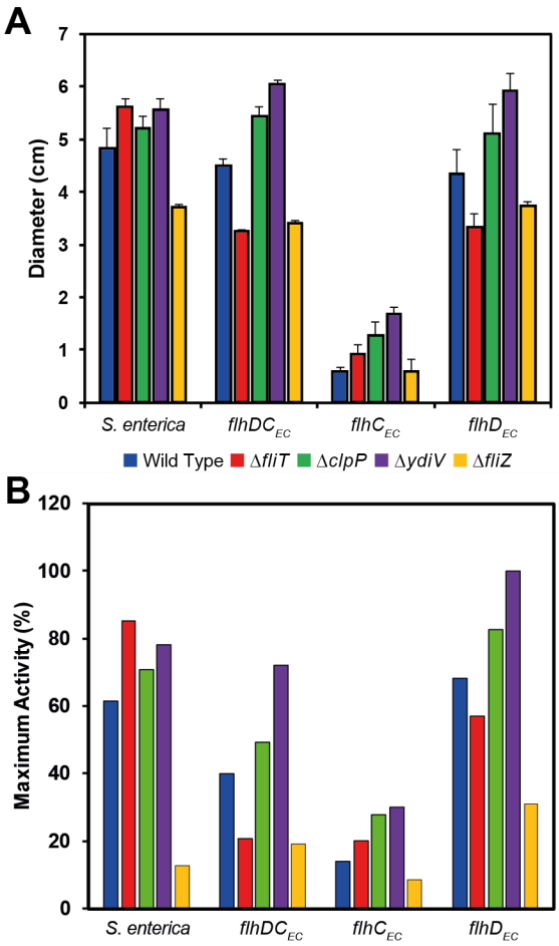

Figure 3 Motility phenotypes and gene expression of *flhDC*$_{ST}$, *flhDC*$_{EC}$, *flhD*$_{EC}$ and *flhC*$_{EC}$ strains in the absence of known $FlhD_4C_2$ regulators. **A.** Quantification of n = 3 swarms per strain produced in motility agar after 6 to 8 hours incubation at 37°C. Error bars indicate calculated standard deviations. **B.** Relative activity of P_{fliC} in all strains as a percent of the maximal activity observed in *flhD*$_{EC}$ Δ*ydiV*.

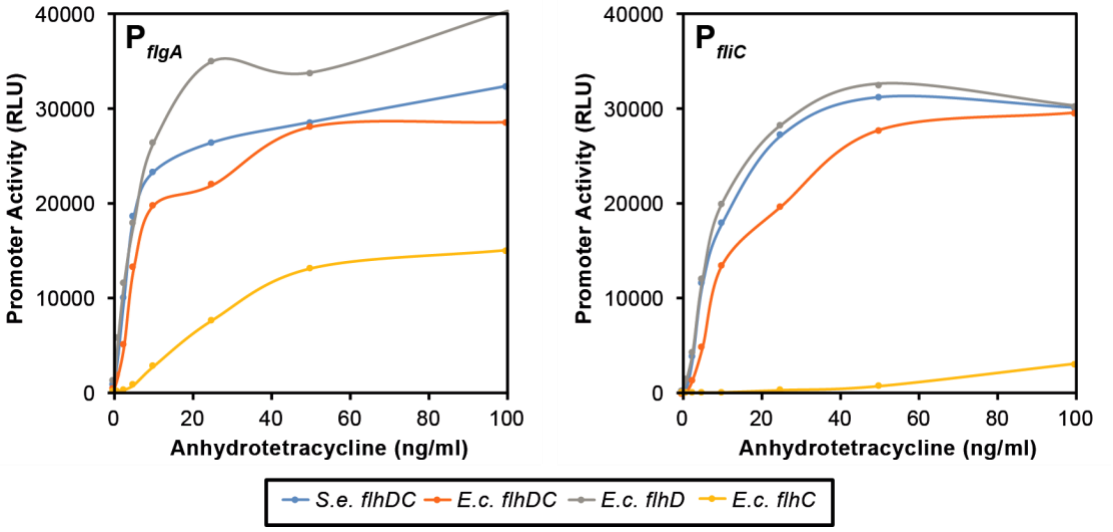

Figure 4 Titration of P_{tetA}::*flhDC* for *S. enterica*, *flhDC*$_{EC}$, *flhD*$_{EC}$ and *flhC*$_{EC}$ suggests that *flhC*$_{EC}$ exhibits low motility due reduced P_{flgA} activity and a strong reduction in P_{fliC} activity. Inducible expression was driven from the P_{tetA} promoter within the TetRA cassette of Tn10. The data shown in both panels is significant using ANOVA statistical analysis $P < 0.05$.

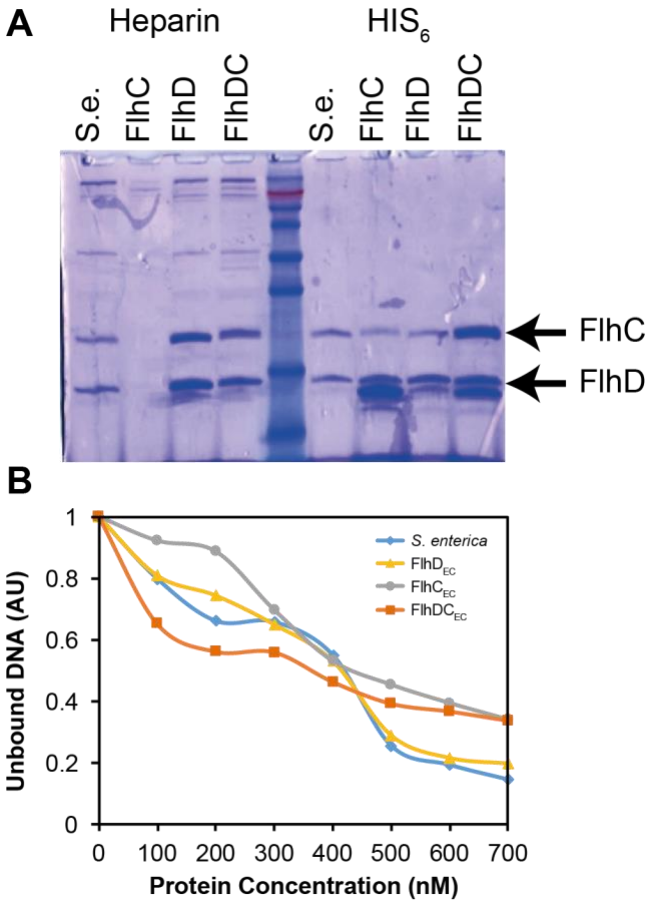

Figure 5 The $FlhD_{ST}FlhC_{EC}$ complex is an active but unstable complex. **A.** Protein gel showing purified complexes with either HIS_6 or Heparin based purification protocols. The nature of the FlhDC complex allows isolation of both proteins in these assays. Arrows indicate the FlhC and FlhD bands. **B.** Quantification of EMSA to define the binding ability of the complex combinations compared to *S. enterica* $FlhD_4C_2$. Data shows that whenever $FlhC_{EC}$ is present a reduced level of binding to P_{flgAB} was observed.

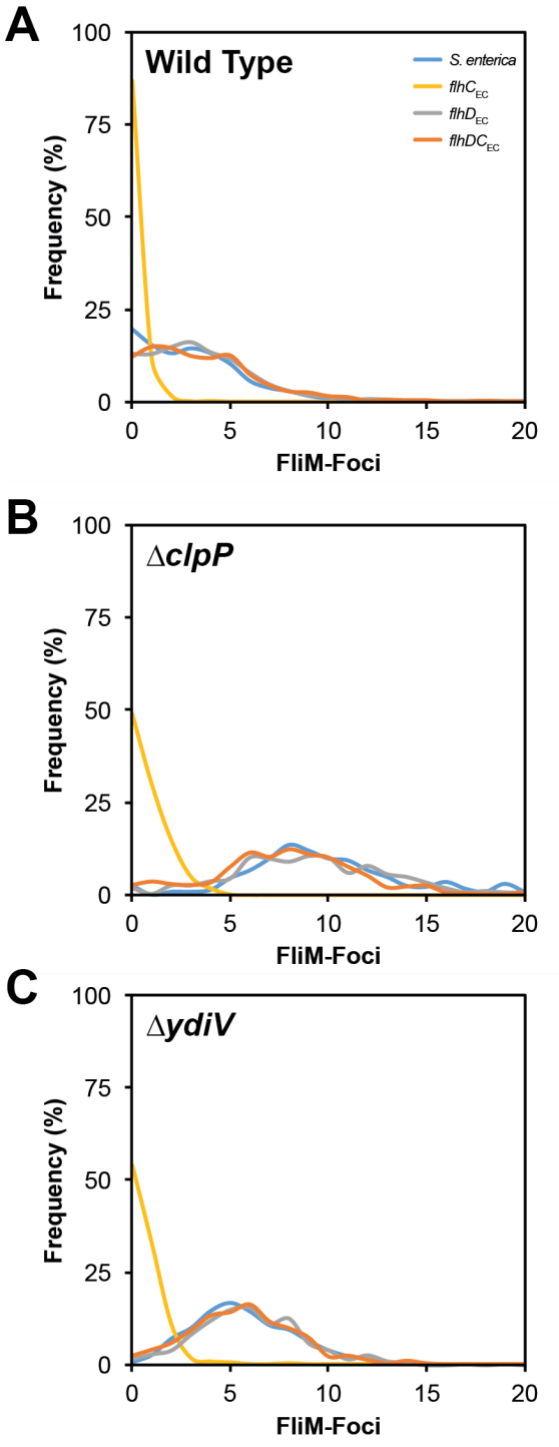

Figure 6 Impact of protein stability regulators of $FlhD_4C_2$ on flagellar numbers as defined by FliM-foci. Quantification of FliM-foci was performed using the semi-automatic protocols defined with in Microbetracker. **A.** Wild Type foci distribution; **B.** Δ*clpP*; **C.** Δ*ydiV*.

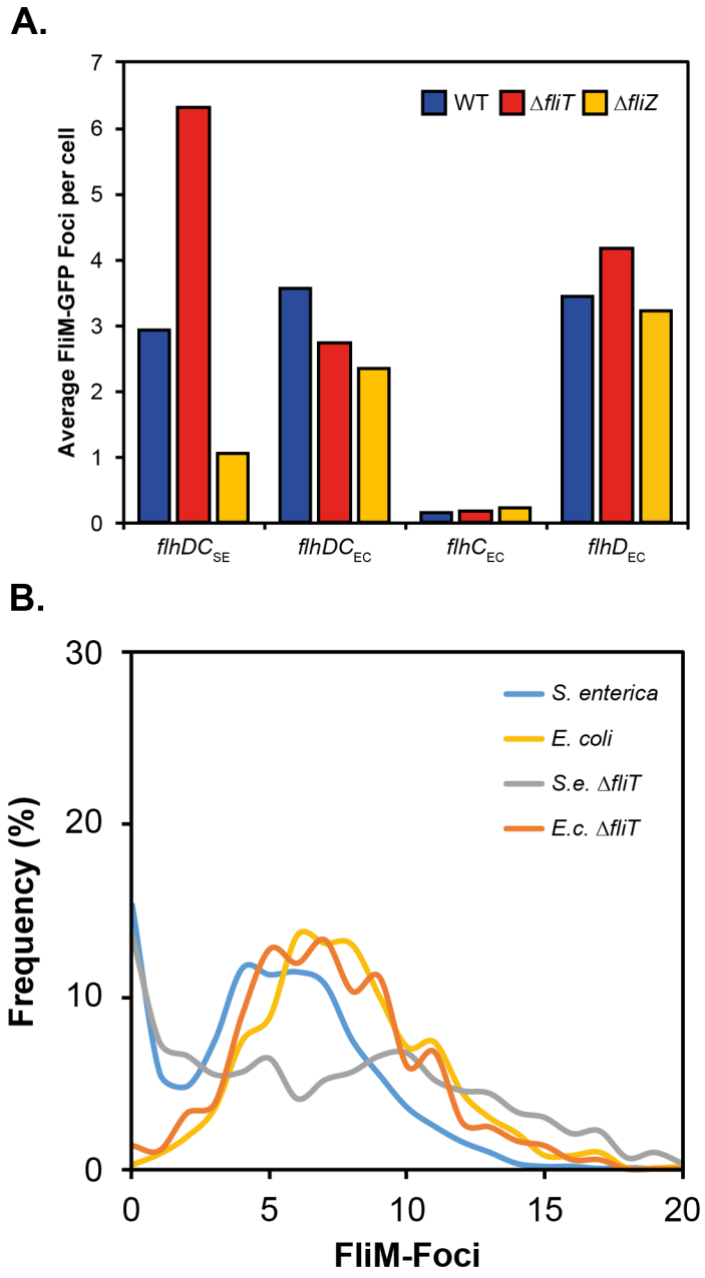

Figure 7 FliT and FliZ regulation reflects when $FlhC_{EC}$ or $FlhD_{EC}$ are present. **A.** FliM-Foci quantification is consistent with the observed motility phenotype of Δ*fliT* mutants. For Δ*fliZ* FliM-foci numbers discriminate between the source of FlhD, $FlhD_{SE}$ exhibits a consistnet drop in foci while $FlhD_{EC}$ containing strains show comparable foci averages. **B.** Testing the hypothesis that Δ*fliT* mutants respond differently in *E. coli* compared to *S. enterica*. Note: this experiment in **(B)** uses the species *E. coli* and *S. enterica* not engineered replacements.