bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1 Driving the expression of the *Salmonella enterica* sv Typhimurium flagellum using *flhDC*
2 from *Escherichia coli* results in key regulatory and cellular differences.
3
4 Ayman Albanna[1,2,6], Martin Sim[1,2§], Paul A Hoskisson[3], Colin Gillespie[4], Christopher V.
5 Rao[5] and Phillip Aldridge[1,2]
6
7 1: Centre for Bacterial Cell Biology, Baddiley Clark Building, Newcastle University,
8 Richardson Road, Newcastle upon Tyne, United Kingdom, NE2 4AX. UK.
9 2: Institute for Cell and Molecular Biosciences, Newcastle University, Framlington Place,
10 Newcastle upon Tyne, United Kingdom, NE2 4HH. UK.
11 3: Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde,
12 Glasgow, United Kingdom G4 0RE. UK.
13 4: School of Mathematics & Statistics, Herschel Building, Newcastle University, Newcastle
14 upon Tyne, United Kingdom, NE1 7RU. UK.
15 5: Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-
16 Champaign, Urbana, Illinois, United States, 61801.
17 6: College of environmental science & technology, Mosul University, Iraq 41002
18
19 Running Title: *E. coli flhDC* function in *S. enterica*
20
21 *Corresponding author. Centre for Bacterial Cell Biology, Baddiley Clark Building,
22 Newcastle University, Richardson Road, Newcastle upon Tyne, United Kingdom, NE2
23 4AX. UK. Phone: +44-191-2083218. Email: phillip.aldridge@ncl.ac.uk.
24
25 § Present address: Isomerase Therapeutics Ltd., Chesterford Research Park, Cambridge,
26 CB10 1XL. UK.
27

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

28 **ABSTRACT**

29

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

47

48 **IMPORTANCE**

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

59 **INTRODUCTION**

60

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

67

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

82

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

107 **RESULTS**

108

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

110

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

120

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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*

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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).

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

210 However, *ydiV* is not expressed under standard laboratory conditions in model *E. coli*
211 strains, suggesting that ClpXP activity is modulated in a species-specific manner (7).
212
213 Previous work has shown that YdiV delivers $FlhD_4C_2$ complexes to ClpXP for degradation
214 (24). We have assessed the impact on motility for Δ*clpP* and Δ*ydiV* mutations (Figure 3).
215 The Δ*clpP* and Δ*ydiV* mutants exhibited improved motility and flagellar gene expression,
216 including the $FlhD_{SE}$/$FlhC_{EC}$ strain (Figure 3A and B). These results suggest that
217 proteolytic degradation mechanism of FlhD and FlhC, and its regulation, is common to *E.*
218 *coli* and *S. enterica*.
219
220 To complement the motility assays, we investigated how Δ*clpP* and Δ*ydiV* mutations
221 impact the number of FliM-foci in cell. Both Δ*clpP* and Δ*ydiV* mutants showed an increased
222 number of FliM-foci compared to the wild type (Figure 6 A-C). For *flhC*$_{EC}$ strain, FliM-foci
223 were observed in 13% of the population where individual cells exhibited just one or two
224 foci. However, the Δ*clpP* or Δ*ydiV* mutants increased the flagellated population of the
225 *flhC*$_{EC}$ strains to 51 and 46 % respectively, albeit with the majority still possessing only a
226 single FliM focus (Figure 6 B and C).
227
228 *FliT and FliZ regulation of $FlhD_4C_2$ complexes*
229
230 $FlhD_4C_2$ activity has an additional level of regulation in *S. enterica* via the flagellar-specific
231 regulators FliT and FliZ. FliT functions as an export chaperone for the filament cap protein,
232 FliD, and is a regulator of $FlhD_4C_2$ activity (17, 25). FliT disrupts the $FlhD_4C_2$ complex but
233 is unable to disrupt a $FlhD_4C_2$:DNA complex. Therefore, FliT modulates availability of
234 $FlhD_4C_2$ complexes for promoter binding (17). In contrast, FliZ is a negative regulator of

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

260 native *S. enterica flhDC* strain. Both replacements exhibited an increase in the average
261 foci compared to *S. enterica* Δ*fliZ* (Figure 7A).
262
263 These data suggest that there is a fundamental difference in how the $FlhD_4C_2$ complexes
264 in *E. coli* and *S. enterica* respond to, at least, FliT regulation. There are two explanations
265 for this: a) the *E. coli* combinations are being regulated via an unidentified mechanism in
266 *S. enterica* or b) that they are insensitive to FliT regulation. Both arguments predict that in
267 *E. coli* $FlhD_4C_2$ may respond differently to FliT regulation. Comparing *S. enterica* and *E.*
268 *coli* does indeed identify a difference in the response to a Δ*fliT* mutant. While a Δ*fliT*
269 mutant in *S. enterica* leads to a consistent increase in FliM-foci, no significant difference is
270 noted for an *E. coli* Δ*fliT* mutant compared to *E. coli* wild type (Figure 7B). This suggests
271 that the regulatory impact of FliT is very different in these two flagellar systems and the
272 role FliT plays in *S. enterica* is potentially adaptive and species specific.
273
274

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

275 **DISCUSSION**

276

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

287

288 At the onset of our work it was known that $FlhD_4C_2$ from *E. coli* could sustain motility in *S.*
289 *enterica*(11). Our work was focussed on understanding and defining the species-specific
290 differences in the regulon of two orthologous genes. Here we took advantage of the well-
291 defined flagellar assembly tools to measure outputs such as, motility, flagellar assembly
292 per cell and flagellar gene expression. Bioinformatic analysis identifies only an 8 and 6%
293 identity difference between FlhD and FlhC in *E. coli* and *S. enterica* respectively,
294 suggesting that these proteins function in an analogous fashion. It is well established that
295 related taxa usually rely on orthologous regulators to coordinate response to a given signal
296 (10).

297

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

327 model *E. coli* strains, strengthening the argument that $FlhD_{EC}$ has adapted to the absence
328 of YdiV or vice versa $FlhD_{SE}$ to YdiV.
329
330 Importantly our analysis shows that even though these two systems are genetically similar,
331 investigation of $FlhD_4C_2$ activity identifies subtle but key differences into how the $FlhD_4C_2$
332 complex is modulated in two closely related species. We argue that this is a valid example
333 of the caution needed in the age of synthetic biology to exploit heterologous systems in
334 alternative species or chassis'. Our data shows that even systems showing significant
335 synteny may not behave in exactly the same manner and due diligence is required in
336 making assumptions based on heterologous expression.
337

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

338 **MATERIALS AND METHODS**

339

340 *Bacterial Strains and Growth conditions*

341

342 *S. enterica* and *E. coli* strains used in this study have been previously described elsewhere
343 (12, 15, 17, 28). This study used *S. enterica* serovar Typhimurium strain LT2 as the
344 chassis for all experiments. *E. coli* genetic material was derived from MG1655. All strains
345 were grown at either 30°C or 37°C in Luria Bertani Broth (LB) either on 1.5% agar plates
346 or shaken in liquid cultures at 160 rpm (17). Antibiotics used in this study have been
347 described elsewhere (29). Motility assays used motility agar (17) incubated at 37°C for 6 to
348 8 hours. Motility swarms were quantified using images captured on a standard gel doc
349 system with a ruler in the field of view and quantified using ImageJ to measure the vertical
350 and horizontal diameter using the average as the swarm size. All motility assays were
351 performed in triplicate using single batches of motility agar.

352

353 *Genetic Manipulations*

354

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

390 experiments prior to protein purification using either a pre-equilibrated 5ml His-trap column
391 or a 5ml heparin column (GE Healthcare). Proteins were visualised using Tricine-based
392 SDS polyacrylamide gel electrophoresis and standard commassie blue staining (17).
393
394 *Electrophoretic mobility shift assay (EMSA).*
395
396 All EMSA assays were performed using Ni++ (his-trap) purified proteins as this allowed
397 analysis of all four complexes (Figure 5A). Buffer exchange from elution buffer to a 100mM
398 Tris-HCl, 300 mM NaCl 1mM DTT (pH 7.9) buffer was performed through 10 cycles of
399 protein concentration in VivaSpin columns with 20 ml buffer reduced to 5 ml per round of
400 centrifugation at 4500 rpm. A protein concentration range of 100 to 700 nM was used with
401 80 ng / ml of a PCR product containing P_{flgAB} from *S. enterica*. After incubation bound and
402 unbound DNA were resolved using 5% acrylamide gels made with 1x TBE buffer.
403 Quantification of gel images was performed using ImageJ.
404

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

405 **ACKNOWLEDGEMENTS**
406
407 PDA would like to recognize the internal financial support of ICAMB during this study. The
408 stipend and research costs for the PhD of AA was provided by The Ministry of Higher
409 Education and Scientific Research (Iraq). We would like to thank the financial support of
410 the Newcastle University Faculty of Medicine for providing the John William Luccok and
411 Ernest Jeffcock Research PhD Studentship to MS for this study. PAH would like to
412 acknowledge the support of iUK/BBSRC (grant: BB/N023544/1), NERC (grant:
413 NE/M001415/1), the University of Strathclyde and the Microbiology Society for funding. We
414 would also like to thank all lab members for feedback on the project during the
415 experimental and writing phases.
416

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

417 **REFERENCES**

418

419 1. **Duan Q**, **Zhou M**, **Zhu L**, **Zhu G**. 2013. Flagella and bacterial pathogenicity. J Basic
420 Microbiol **53**:1–8.

421 2. **Minamino T**, **Imada K**, **Namba K**. 2008. Mechanisms of type III protein export for
422 bacterial flagellar assembly. Mol Biosyst **4**:1105–1115.

423 3. **Chevance FFV**, **Hughes KT**. 2008. Coordinating assembly of a bacterial
424 macromolecular machine. Nat Rev Micro **6**:455–465.

425 4. **Aldridge P**, **Hughes KT**. 2002. Regulation of flagellar assembly. Curr Opin
426 Microbiol **5**:160–165.

427 5. **Chilcott GS**, **Hughes KT**. 2000. Coupling of flagellar gene expression to flagellar
428 assembly in Salmonella enterica serovar typhimurium and Escherichia coli. Microbiol
429 Mol Biol Rev **64**:694–708.

430 6. **Minamino T**, **Namba K**. 2004. Self-assembly and type III protein export of the
431 bacterial flagellum. J Mol Microbiol Biotechnol **7**:5–17.

432 7. **Wada T**, **Hatamoto Y**, **Kutsukake K**. 2012. Functional and expressional analyses of
433 the anti-FlhD4C2 factor gene ydiV in Escherichia coli. Microbiology **158**:1533–1542.

434 8. **Soutourina OA**, **Bertin PN**. 2003. Regulation cascade of flagellar expression in
435 Gram-negative bacteria. FEMS Microbiol Rev **27**:505–523.

436 9. **Mouslim C**, **Hughes KT**. 2014. The effect of cell growth phase on the regulatory
437 cross-talk between flagellar and Spi1 virulence gene expression. PLoS Pathog
438 **10**:e1003987.

439 10. **Perez JC**, **Groisman EA**. 2009. Evolution of transcriptional regulatory circuits in
440 bacteria. Cell **138**:233–244.

441 11. **Kutsukake K**, **Iino T**, **Komeda Y**, **Yamaguchi S**. 1980. Functional homology of fla
442 genes between Salmonella typhimurium and Escherichia coli. Mol Gen Genet
443 **178**:59–67.

444 12. **Aldridge P**, **Karlinsey JE**, **Becker E**, **Chevance FFV**, **Hughes KT**. 2006. Flk
445 prevents premature secretion of the anti-sigma factor FlgM into the periplasm. Mol
446 Microbiol **60**:630–643.

447 13. **Delalez NJ**, **Wadhams GH**, **Rosser G**, **Xue Q**, **Brown MT**, **Dobbie IM**, **Berry RM**,
448 **Leake MC**, **Armitage JP**. 2010. Signal-dependent turnover of the bacterial flagellar
449 switch protein FliM. Proceedings of the National Academy of Sciences **107**:11347–
450 11351.

451 14. **Sim M**, **Koirala S**, **Picton D**, **Strahl H**, **Hoskisson PA**, **Rao CV**, **Gillespie CS**,
452 **Aldridge PD**. 2017. Growth rate control of flagellar assembly in Escherichia coli
453 strain RP437. Sci Rep **7**:41189.

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

454 15. **Brown JD**, **Saini S**, **Aldridge C**, **Herbert J**, **Rao CV**, **Aldridge PD**. 2008. The rate
455 of protein secretion dictates the temporal dynamics of flagellar gene expression. Mol
456 Microbiol **70**:924–937.

457 16. **Bertrand KP**, **Postle K**, **Wray LV**, **Reznikoff WS**. 1984. Construction of a single-
458 copy promoter vector and its use in analysis of regulation of the transposon Tn10
459 tetracycline resistance determinant. J Bacteriol **158**:910–919.

460 17. **Aldridge C**, **Poonchareon K**, **Saini S**, **Ewen T**, **Soloyva A**, **Rao CV**, **Imada K**,
461 **Minamino T**, **Aldridge PD**. 2010. The interaction dynamics of a negative feedback
462 loop regulates flagellar number in Salmonella enterica serovar Typhimurium. Mol
463 Microbiol **78**:1416–1430.

464 18. **Wang S**, **Fleming RT**, **Westbrook EM**, **Matsumura P**, **McKay DB**. 2006. Structure
465 of the Escherichia coli FlhDC complex, a prokaryotic heteromeric regulator of
466 transcription. Journal of Molecular Biology **355**:798–808.

467 19. **Stafford GP**, **Ogi T**, **Hughes C**. 2005. Binding and transcriptional activation of non-
468 flagellar genes by the Escherichia coli flagellar master regulator FlhD2C2.
469 Microbiology (Reading, Engl) **151**:1779–1788.

470 20. **Kitagawa R**, **Takaya A**, **Yamamoto T**. 2011. Dual regulatory pathways of flagellar
471 gene expression by ClpXP protease in enterohaemorrhagic Escherichia coli.
472 Microbiology **157**:3094–3103.

473 21. **Tomoyasu T**, **Ohkishi T**, **Ukyo Y**, **Tokumitsu A**, **Takaya A**, **Suzuki M**, **Sekiya K**,
474 **Matsui H**, **Kutsukake K**, **Yamamoto T**. 2002. The ClpXP ATP-dependent protease
475 regulates flagellum synthesis in Salmonella enterica serovar typhimurium. J
476 Bacteriol **184**:645–653.

477 22. **Claret L**, **Hughes C**. 2000. Rapid Turnover of FlhD and FlhC, the Flagellar Regulon
478 Transcriptional Activator Proteins, during Proteus Swarming. J Bacteriol **182**:833–
479 836.

480 23. **Wada T**, **Morizane T**, **Abo T**, **Tominaga A**, **Inoue-Tanaka K**, **Kutsukake K**. 2011.
481 EAL domain protein YdiV acts as an anti-FlhD4C2 factor responsible for nutritional
482 control of the flagellar regulon in Salmonella enterica Serovar Typhimurium. J
483 Bacteriol **193**:1600–1611.

484 24. **Takaya A**, **Erhardt M**, **Karata K**, **Winterberg K**, **Yamamoto T**, **Hughes KT**. 2012.
485 YdiV: a dual function protein that targets FlhDC for ClpXP-dependent degradation by
486 promoting release of DNA-bound FlhDC complex. Mol Microbiol **83**:1268–1284.

487 25. **Bennett JC**, **Thomas J**, **Fraser GM**, **Hughes C**. 2001. Substrate complexes and
488 domain organization of the Salmonella flagellar export chaperones FlgN and FliT.
489 Mol Microbiol **39**:781–791.

490 26. **Saini S**, **Brown JD**, **Aldridge PD**, **Rao CV**. 2008. FliZ Is a posttranslational activator
491 of FlhD4C2-dependent flagellar gene expression. J Bacteriol **190**:4979–4988.

492 27. **Wada T**, **Tanabe Y**, **Kutsukake K**. 2011. FliZ Acts as a Repressor of the ydiV Gene,
493 Which Encodes an Anti-FlhD4C2 Factor of the Flagellar Regulon in Salmonella
494 enterica Serovar Typhimurium. J Bacteriol **193**:5191–5198.

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

495 28. **Aldridge P**, **Karlinsey J**, **Hughes KT**. 2003. The type III secretion chaperone FlgN
496 regulates flagellar assembly via a negative feedback loop containing its chaperone
497 substrates FlgK and FlgL. Mol Microbiol **49**:1333–1345.

498 29. **Bonifield HR**, **Hughes KT**. 2003. Flagellar phase variation in Salmonella enterica is
499 mediated by a posttranscriptional control mechanism. J Bacteriol **185**:3567–3574.

500 30. **Blank K**, **Hensel M**, **Gerlach RG**. 2011. Rapid and highly efficient method for
501 scarless mutagenesis within the Salmonella enterica chromosome. PLoS ONE
502 **6**:e15763.

503 31. **Datsenko KA**, **Wanner BL**. 2000. One-step inactivation of chromosomal genes in
504 Escherichia coli K-12 using PCR products. Proc Natl Acad Sci USA **97**:6640–6645.

505 32. **Rappleye CA**, **Roth JR**. 1997. A Tn10 derivative (T-POP) for isolation of insertions
506 with conditional (tetracycline-dependent) phenotypes. J Bacteriol **179**:5827–5834.

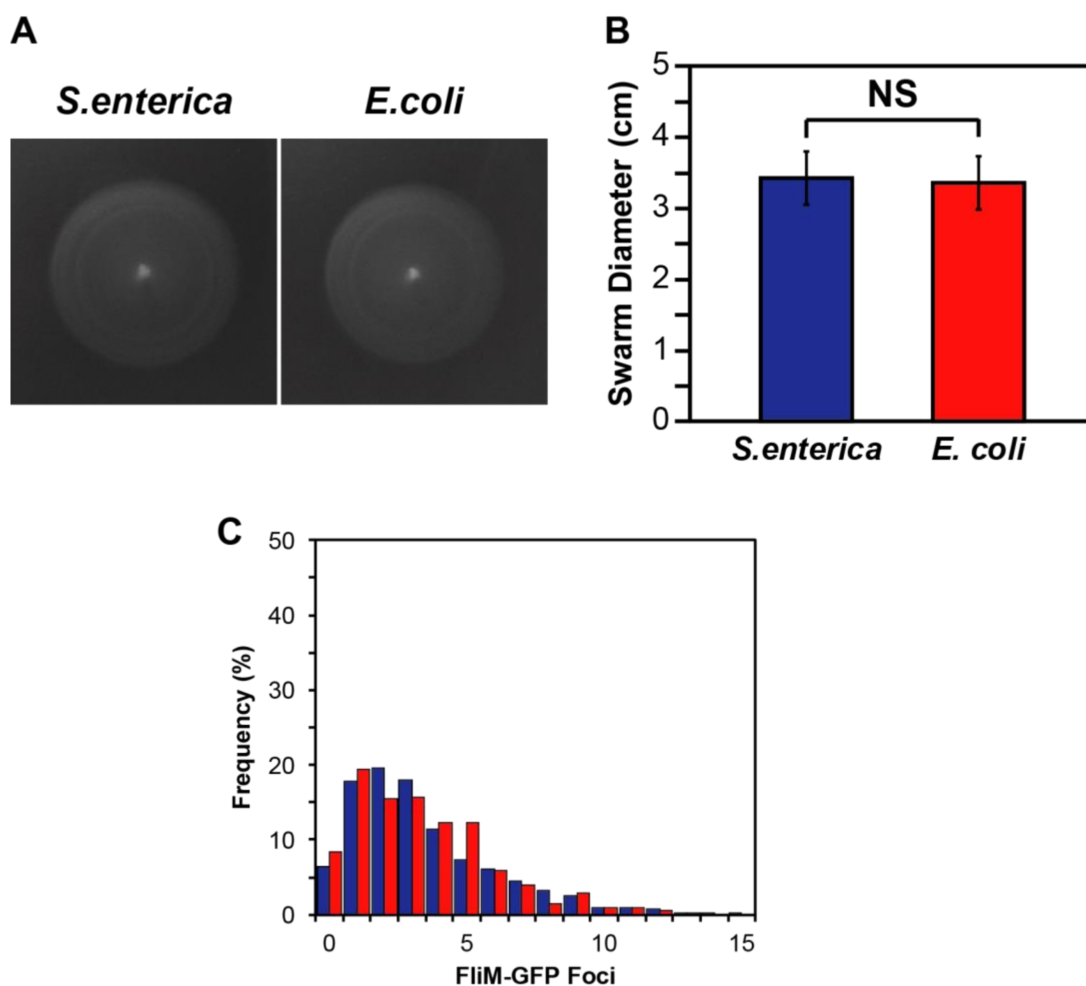
507 33. **Maloy SR**, **Nunn WD**. 1981. Selection for loss of tetracycline resistance by
508 Escherichia coli. J Bacteriol **145**:1110–1111.

509

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

510 **FIGURES and LEGENDS**

511



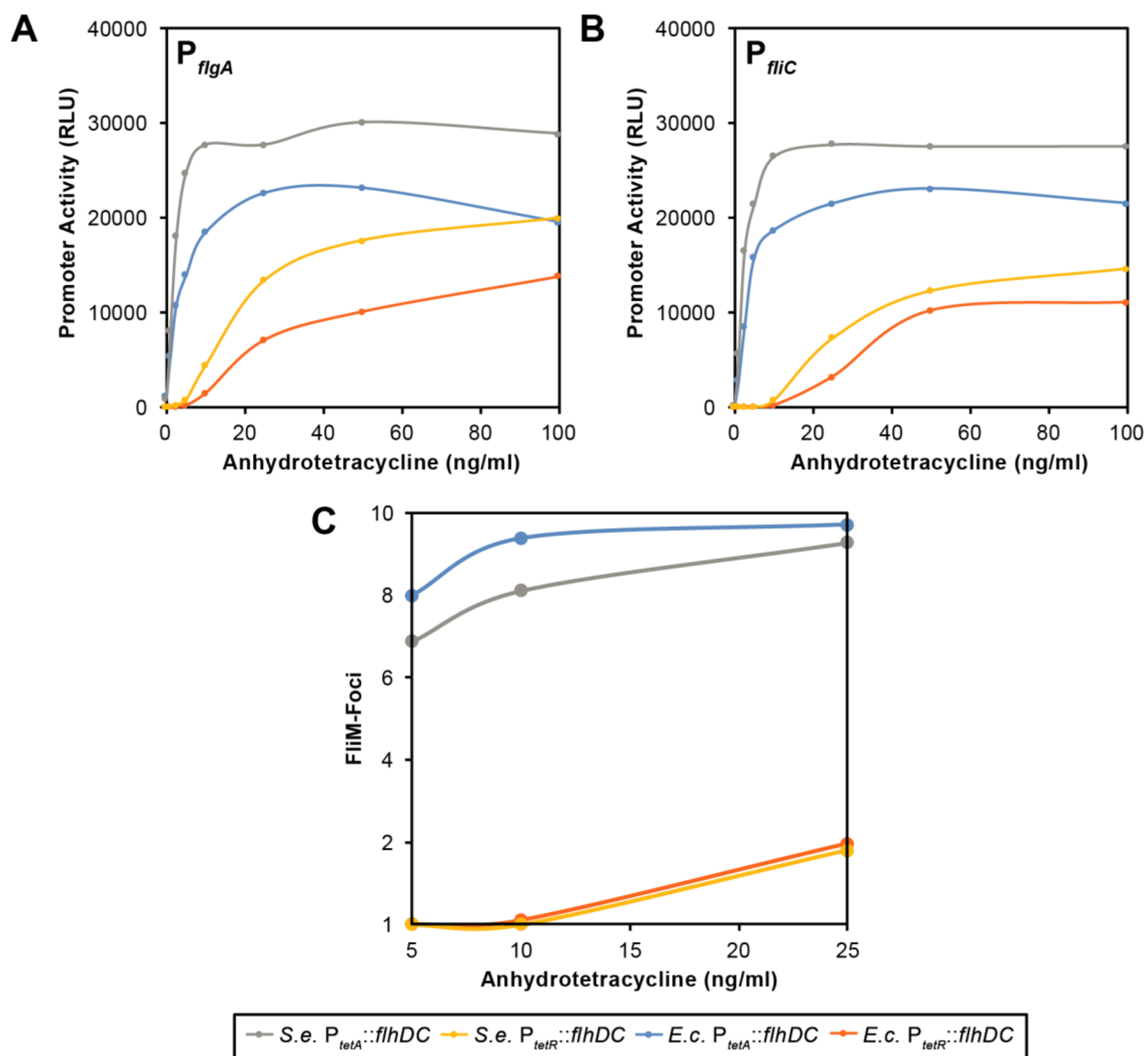
512

513

514 **Figure 1 A.** Motility of *flhDC*$_{ST}$ and *flhDC*$_{EC}$ driven by P$_{flhDC}$. **B.** Quantification of swarms
515 produced in motility agar after 6 to 8 hours incubation. Error bars indicate calculated
516 standard deviations. **C.** Percentage frequency of FliM-GFP foci for *flhDC*$_{EC}$ compared to *S.*
517 *enterica* with *flhDC* under the control of P$_{flhDC}$. Colors of bars in the graph correspond to
518 the source of *flhDC* as shown in (**B**).
519

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

520
521

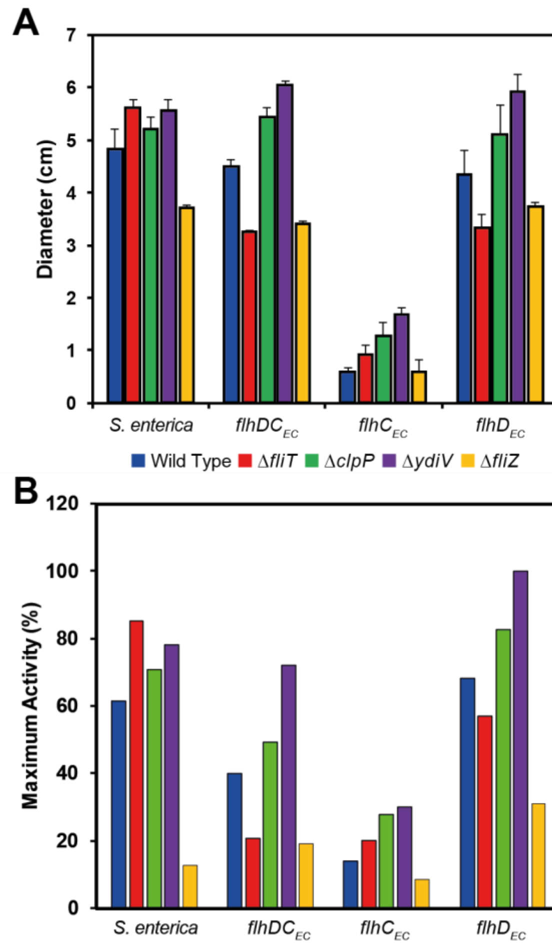


522
523

524 **Figure 2** Titration of P_{tetA}*::flhDC*$_{ST/EC}$ and P_{tetR}*::flhDC*$_{ST/EC}$ activity suggests a given rate of
525 transcription drives optimal flagellar assembly. **A.** Activity of P_{flgA} in response to P_{tetA} or
526 P_{tetR} transcription of *flhDC* from *S. enterica* (S.e.) or *E. coli* (E.c.). **B.** Activity of P_{fliC} in
527 response to P_{tetA} or P_{tetR} transcription of *flhDC.* **C.** flagellar numbers as defined by FliM-foci
528 in response to P_{tetA} or P_{tetR} transcription of *flhDC.*
529

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

530



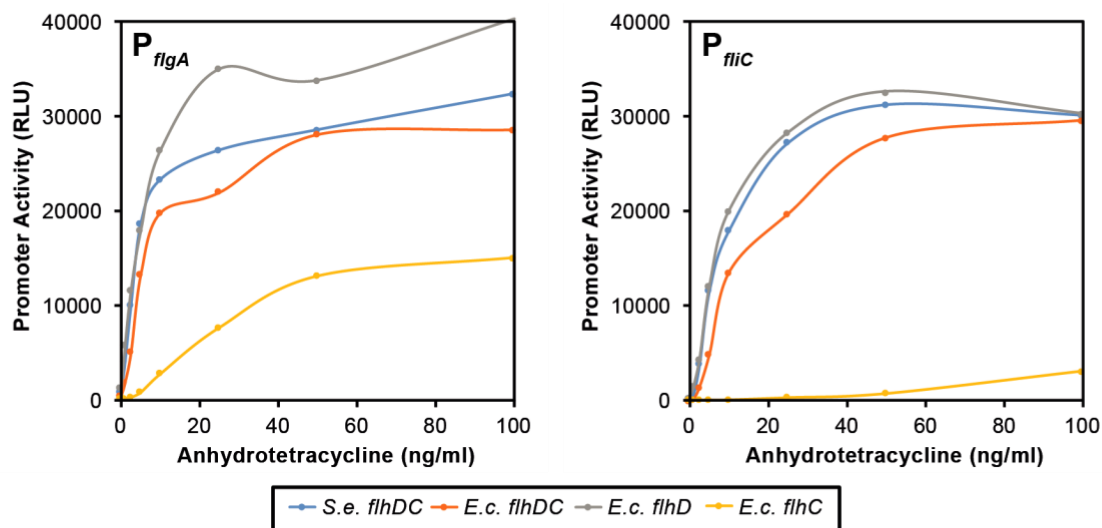
531

532

533 **Figure 3** Motility phenotypes and gene expression of *flhDC*$_{ST}$, *flhDC*$_{EC}$, *flhD*$_{EC}$ and *flhC*$_{EC}$
534 strains in the absence of known $FlhD_4C_2$ regulators. **A.** Quantification of n = 3 swarms per
535 strain produced in motility agar after 6 to 8 hours incubation at 37°C. Error bars indicate
536 calculated standard deviations. **B.** Relative activity of P$_{fliC}$ in all strains as a percent of the
537 maximal activity observed in *flhD*$_{EC}$ Δ*ydiV*.
538

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

539



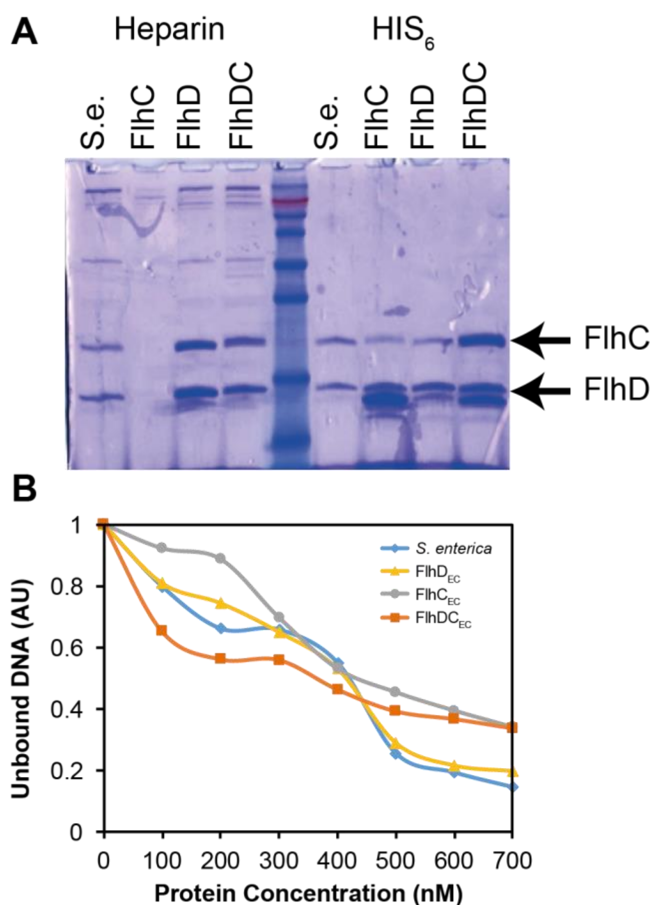
540

541

542 **Figure 4** Titration of P_{tetA}::*flhDC* for *S. enterica*, *flhDC*$_{EC}$, *flhD*$_{EC}$ and *flhC*$_{EC}$ suggests that
543 *flhC*$_{EC}$ exhibits low motility due reduced P_{flgA} activity and a strong reduction in P_{fliC} activity.
544 Inducible expression was driven from the P_{tetA} promoter within the TetRA cassette of Tn10.
545 The data shown in both panels is significant using ANOVA statistical analysis $P < 0.05$.
546

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

547



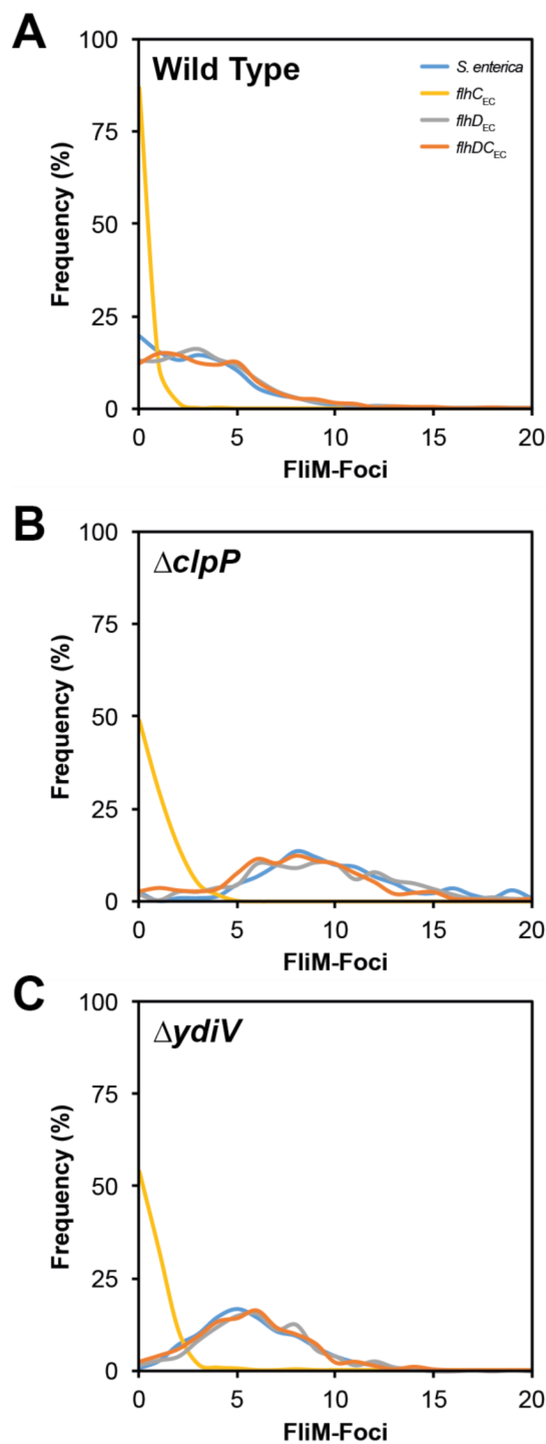
548

549

550 **Figure 5** The $FlhD_{ST}FlhC_{EC}$ complex is an active but unstable complex. **A.** Protein gel
551 showing purified complexes with either HIS_6 or Heparin based purification protocols. The
552 nature of the FlhDC complex allows isolation of both proteins in these assays. Arrows
553 indicate the FlhC and FlhD bands. **B.** Quantification of EMSA to define the binding ability
554 of the complex combinations compared to *S. enterica* $FlhD_4C_2$. Data shows that whenever
555 $FlhC_{EC}$ is present a reduced level of binding to P_{flgAB} was observed.
556

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

557



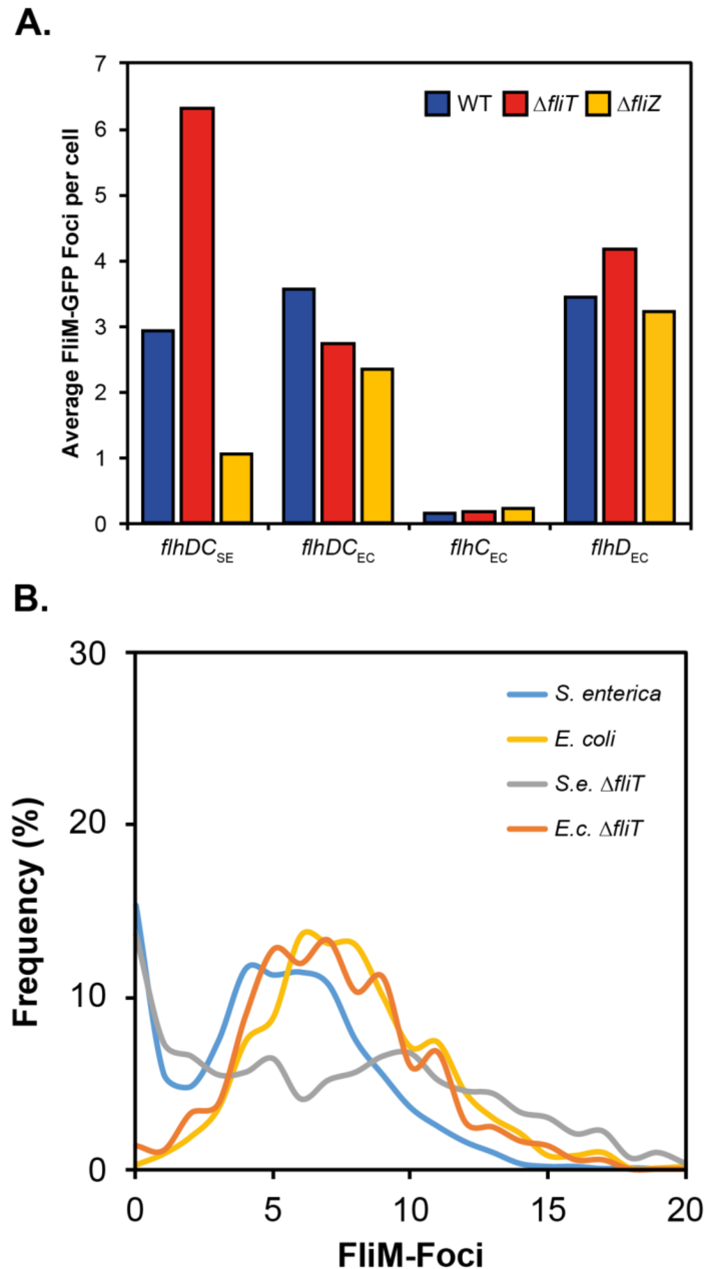
558

559

560 **Figure 6** Impact of protein stability regulators of $FlhD_4C_2$ on flagellar numbers as defined
561 by FliM-foci. Quantification of FliM-foci was performed using the semi-automatic protocols
562 defined with in Microbetracker. **A.** Wild Type foci distribution; **B.** Δ*clpP*; **C.** Δ*ydiV*.
563

bioRxiv preprint doi: https://doi.org/10.1101/333146; this version posted May 29, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

564



565
566
567 **Figure 7** FliT and FliZ regulation reflects when $FlhC_{EC}$ or $FlhD_{EC}$ are present. **A.** FliM-Foci
568 quantification is consistent with the observed motility phenotype of Δ*fliT* mutants. For Δ*fliZ*
569 FliM-foci numbers discriminate between the source of FlhD, $FlhD_{SE}$ exhibits a consistnet
570 drop in foci while $FlhD_{EC}$ containing strains show comparable foci averages. **B.** Testing the
571 hypothesis that Δ*fliT* mutants respond differently in *E. coli* compared to *S. enterica*. Note:
572 this experiment in **(B)** uses the species *E. coli* and *S. enterica* not engineered
573 replacements.
574
575
576