

**A PilZ domain protein FlgZ mediates c-di-GMP-dependent
swarming motility control in *Pseudomonas aeruginosa***

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25 **ABSTRACT**

26 The second messenger cyclic diguanylate (c-di-GMP) is an important regulator of motility in
27 many bacterial species. In *Pseudomonas aeruginosa*, elevated levels of c-di-GMP promote
28 biofilm formation and repress flagellum-driven swarming motility. The rotation of *P.*
29 *aeruginosa*'s polar flagellum is controlled by two distinct stator complexes: MotAB, which
30 cannot support swarming motility, and MotCD, which promotes swarming motility. Here we
31 show that when c-di-GMP levels are elevated, swarming motility is repressed by the PilZ
32 domain-containing protein FlgZ and by Pel polysaccharide production. We demonstrate that
33 FlgZ interacts specifically with the motility-promoting stator protein MotC in a c-di-GMP-
34 dependent manner, and that a functional GFP-FlgZ fusion protein shows significantly reduced
35 polar localization in a strain lacking the MotCD stator. Our results establish FlgZ as a c-di-GMP
36 receptor affecting swarming motility by *P. aeruginosa* and support a model wherein c-di-GMP-
37 bound FlgZ impedes motility via its interaction with the MotCD stator.

38
39 **IMPORTANCE**

40 The regulation of surface-associated motility plays an important role in bacterial surface
41 colonization and biofilm formation. C-di-GMP signaling is a widespread means of controlling
42 bacterial motility, yet the mechanism whereby this signal controls surface-associated motility
43 remains poorly understood in *P. aeruginosa*. Here we identify a PilZ domain-containing, c-di-
44 GMP effector protein that contributes to c-di-GMP-mediated repression of swarming motility by
45 *P. aeruginosa*. We provide evidence that this effector FlgZ impacts swarming motility via its
46 interactions with flagellar stator protein MotC. Thus, we propose a new mechanism for c-di-
47 GMP-mediated regulation of motility for a bacterium with two flagellar stator sets, increasing

48 our understanding of surface-associated behaviors, a key prerequisite to identifying ways to
49 control the formation of biofilm communities.

50

51 INTRODUCTION

52 Cyclic diguanylate (c-di-GMP) is a ubiquitous bacterial second messenger responsible for
53 regulating a range of cellular processes including motility and biofilm formation (1). In general,
54 low intracellular c-di-GMP levels are associated with motile lifestyles, while elevated levels of c-
55 di-GMP promote surface attachment and sessile lifestyles (2, 3). C-di-GMP is synthesized from
56 two molecules of GTP by diguanylate cyclases (DGCs) and degraded by c-di-GMP-specific
57 phosphodiesterases (PDEs) (1, 4). Many DGCs and PDEs involved in motility regulation have
58 been characterized, but the mechanisms by which c-di-GMP regulates motility are poorly
59 understood in *Pseudomonas aeruginosa* and in other bacterial species.

60 To regulate numerous biological functions, c-di-GMP binds to specific effector proteins
61 or RNA [reviewed in (5)]. Recent studies have focused on identifying these c-di-GMP effectors
62 and their mechanisms for regulating c-di-GMP-dependent processes. One class of effectors is the
63 PilZ domain-containing protein family, which is characterized by a conserved c-di-GMP binding
64 motif, RxxxR - D/NxSxxG (6, 7). PilZ domain-containing proteins typically bind c-di-GMP, and
65 in the c-di-GMP-bound state influence cellular processes including polysaccharide production,
66 virulence, biofilm formation, and motility control (8-14). The PilZ domain-containing protein
67 YcgR of *Escherichia coli* and its homologs in *Salmonella enterica* and *Bacillus subtilis*, have
68 been shown to bind to c-di-GMP and to inhibit cellular motility in response to c-di-GMP (15-20).
69 Evidence suggests that these PilZ domain proteins impede flagellar function by directly
70 interacting with parts of the flagellar motor. In *E. coli*, interactions have been demonstrated

71 between YcgR and three different flagellar motor-associated proteins, MotA, FliG, and FliM (15,
72 17, 18). In *B. subtilis*, evidence suggests that the YcgR homolog YpfA (now called DgrA) (20)
73 interacts with MotA (16). The *P. aeruginosa* genome encodes seven PilZ domain-containing
74 proteins that have been shown to bind to c-di-GMP and an eighth PilZ domain protein that lacks
75 c-di-GMP binding, but no link between these proteins and flagellar motility has been established
76 in this organism (12, 21-23).

77 We have previously reported a connection between c-di-GMP-dependent repression of
78 swarming and the activity of flagellar stator proteins (24). Stator proteins form the ion-
79 translocating channels that are necessary for generating torque to power flagellar rotation (25,
80 26). *P. aeruginosa* and its relatives are distinguished from many other flagellated bacteria in that
81 they have two sets of proton-dependent stators, MotAB and MotCD (27, 28). Our previous
82 studies have shown that these stators play distinct roles in the control of surface-associated
83 swarming motility – one set of stators promotes swarming motility (MotCD) and a second set
84 (MotAB) prevents swarming motility. From this work, we suggested a model by which *P.*
85 *aeruginosa* controls swarming motility in response to c-di-GMP via a unique stator swapping
86 mechanism between these distinct MotAB and MotCD stator complexes (24). Specifically,
87 MotCD was more likely to be found co-localized with the motor as c-di-GMP levels decreased,
88 thereby presumably enabling surface motility (24).

89 Here we tested the importance of PilZ domain proteins in the control of *P. aeruginosa*
90 swarming motility, and demonstrate that the PilZ domain protein PA14_20700, named here FlgZ
91 after the *P. fluorescens* and *P. pudita* homologs (29), and the Pel polysaccharide contribute to c-
92 di-GMP-mediated swarming repression. We provide evidence that FlgZ interacts directly with
93 stator protein MotC, but does not interact with MotA. The function of FlgZ in swarming

94 repression and its ability to interact with MotC both depend on c-di-GMP binding. Furthermore,
95 we show that the localization of a GFP-FlgZ fusion to the pole of the cell is increased at high c-
96 di-GMP levels and depends on the presence of MotCD. Thus, we suggest that FlgZ functions to
97 repress swarming motility in response to c-di-GMP by specifically targeting the function of
98 MotCD, the swarming-promoting stator set, by preventing the engagement of MotCD with the
99 rotor.

100 **MATERIALS AND METHODS**

101 **Strains and media.** Bacterial strains used in this study are listed in Supplementary Table S1. *P.*
102 *aeruginosa* PA14, and *E. coli* S17-1 λ pir and BTH101 cells were routinely grown in lysogeny
103 broth (LB) or on LB solidified with 1.5% agar. When antibiotic selection was appropriate for *P.*
104 *aeruginosa*, gentamicin (Gm) was used at 30 μ g/ml. For *E. coli* selections, Gm was used at 10
105 μ g/ml, carbenicillin (Cb) at 50 μ g/ml, kanamycin (Kan) at 50 μ g/ml, and nalidixic acid (Nal) at
106 20 μ g/ml.

107 *Saccharomyces cerevisiae* strain InvSc1 (Invitrogen) was used for constructing plasmids
108 via *in vivo* homologous recombination (30). InvSc1 was grown in yeast extract-peptone-dextrose
109 (1% Bacto yeast extract, 2% Bacto peptone, and 2% dextrose). Synthetic defined medium
110 lacking uracil was used to select for plasmid-harboring yeast.

111
112 **Construction of mutant strains and plasmids.** Supplementary Table S2 lists all plasmids used in
113 this study. Primers used in plasmid and mutant construction are listed in Supplementary Table
114 S3. In-frame deletion mutants were constructed via allelic exchange as previously described (30).
115 Integrants were isolated on LB medium supplemented with Gm and Nal followed by sucrose
116 counter-selection. Resolved integrants were confirmed by PCR and sequencing.

117 Point mutations made to *flgZ* plasmids were generated using a modified protocol for *in*
118 *vitro* site-directed mutagenesis (31). Briefly, forward and reverse oligonucleotide primers were
119 designed to contain mismatches for generating the desired point mutation. These primers were
120 first used separately to amplify the parental vector using Phusion DNA polymerase (NEB) for
121 four cycles. Products from the forward and reverse reactions were then combined and amplified

122 for an additional 18 cycles. The parental, non-mutagenized plasmid was digested using DpnI

123 before transforming the products into *E. coli*.

124

125 **Swarming motility assays.** Swarming motility was tested by inoculating 2 µl of overnight
126 cultures onto M8 minimal salts medium supplemented with 0.53% agar and glucose (0.2%),
127 MgSO₄ (1mM), and casamino acids (0.5%), as reported (32). Arabinose was used at 0.2% where
128 indicated for expression plasmids with the P_{BAD} promoter. Swarm assays were incubated at 37°C
129 for 16-19 hours. Quantification of swarm zones was performed using ImageJ software (National
130 Institutes of Health) (33).

131

132 **Free-swimming capillary assay.** To perform a zero-flow, free-swimming capillary assay, 100 µl
133 of mid-log *P. aeruginosa* culture (OD_{600nm} between 0.6 and 1.0) were pelleted at 1000 x g for 1
134 min, and gently resuspended in 500 µl motility buffer (liquid medium of the same composition
135 as the swarm plates, but lacking agar). The resuspended cells were incubated at room
136 temperature for 20 min to allow recovery of motility, before 50 µl were drawn into a 0.2 x 2 mm
137 glass capillary tube by capillary action. The ends of the tube were sealed with silicon grease to
138 prevent evaporation and flow, the tube was attached to a glass slide, and bacteria were visualized
139 in phase contrast at 20x magnification at the center of the tube for 1-2 min with a video frame
140 rate of 20 ms.

141 The image segmentation software Tracker (34) was used to detect cells and form tracks,
142 using the “Threshold” algorithms with parameters, upper threshold = 0.52, lower threshold =
143 0.48, minimal cluster size = 12. To exclude wrong connections of tracks, a cut-off of 90 µm/s per
144 single frame was applied. More than 400 tracks from three videos per strain with a minimal track

length of 50 frames were analyzed, and swimming speeds of all detected bacteria, as well as bacteria classified as motile (swimming speed > 10 $\mu\text{m/s}$) were compared.

147

Western blot analysis of protein level. Determining the level of wild-type and mutant proteins in whole cell extracts was performed as reported (24) and is summarized as follows. Cells were cultured on 0.53% swarm medium for 16-19 hours. Cells were harvested from swarm medium using ethanol-washed plastic coverslips and then centrifuged for 1 min at room temperature (RT). Supernatants were removed and cell pellets were stored at -80°C prior to further processing. To generate cell lysates, cells were resuspended in buffer (200 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 mM MgCl_2 , cOmplete protease inhibitors (Roche Diagnostics Corp., Indianapolis, IN)), and benzonase nuclease (Novagen, San Diego, CA) was added to a final concentration of ~ 50 units/ml and lysed by French press or bead beating. For Western blotting, whole cell lysates were mixed with a final concentration of 1x SDS and 100 mM DTT. Samples were boiled for 5 min and resolved by SDS-PAGE using Any kD or 12% polyacrylamide gels (Bio-Rad). Proteins were transferred to a nitrocellulose membrane and probed with an anti-penta-His antibody (Qiagen, Valencia, CA). Nitrocellulose membranes were washed with TBS-Tween and then incubated with HRP-conjugated secondary antibodies for 30 min at RT. Proteins were detected using ECL Plus Western blotting substrate (Pierce).

163

C-di-GMP measurements. Cells were collected from swarm plates after incubation at 37°C for 18 hours. Nucleotide extraction was performed as previously described (35, 36). C-di-GMP measurement analysis was performed by liquid-chromatography mass spectrometry (LC-MS/MS) at the Mass Spectrometry Facility at Michigan State University.

168

169 **Bacterial two-hybrid analysis.** Protein-protein interactions were examined using the Bacterial
170 Adenylate Cyclase Two-Hybrid (BATCH) system obtained from Euromedex
171 (Souffelweyersheim, France) as previously described (37, 38). In this assay, full-length proteins
172 of interest are fused to either the T18 or T25 fragment of *Bordetella pertussis* adenylate cyclase
173 and then co-expressed in *E. coli* BTH101 cells. Interaction between the two hybrid proteins
174 functionally reconstitutes the catalytic domain of adenylate cyclase leading to cAMP synthesis
175 and transcriptional activation of the *lac* operon.

176 In this study, genes *motA*, *motC*, *fliG*, and *fliM* were each cloned into pKNT25, pKT25,
177 pUT18, and pUT18C vectors. All vectors containing the *motA* gene include a C-terminal 6xHis
178 tag. We tested pairwise interactions with T18 and T25 fusions in both orientations.
179 Transformants were 10-fold serially diluted and spotted (2 μ l) on LB agar containing Cb, Kan,
180 X-gal (40 μ g/ml), and IPTG (0.5 mM) and incubated for 40 h at 30 °C. Efficiency of interaction
181 was determined via β -galactosidase activity assays, as previously described (39).

182

183 **Immunoprecipitation assays.** Cells were collected from swarm plates as described above.
184 Swarm plates contained 0.2% arabinose to induce the expression of plasmid-based genes. Whole
185 cell lysates were prepared in lysis buffer containing 20 mM Tris pH 8, 10 mM MgCl₂, cOmplete
186 EDTA-free Protease Inhibitor Cocktail (Roche), benzonase nuclease, and 10mM imidazole. Each
187 immunoprecipitation contained 500 μ l lysate, 40 μ l ProBond nickel-chelating resin (Invitrogen),
188 and 0.8% Thesit (Sigma) to solubilize membranes. Immunoprecipitations were incubated at 4°C
189 for 75 min. When indicated, they contained 5 μ M c-di-GMP. The nickel resin was washed in
190 lysis buffer once for 15 min at 4 °C, then three times at RT with gentle shaking prior to SDS-

191 PAGE analysis. Immunoprecipitations containing 5 μ M c-di-GMP were washed with lysis buffer
192 containing 5 μ M c-di-GMP. For Western blotting, proteins were transferred to a nitrocellulose
193 membrane and probed with an anti-HA antibody (Covance, Princeton, NJ).

194

195 **Fluorescence microscopy and data processing.** *P. aeruginosa* for fluorescence images were
196 picked from the edges of swarm plate colonies, as previously described (24, 32), and
197 resuspended in motility buffer (liquid medium of the same composition as the swarm plates, but
198 lacking the agar). Two streaks of bacteria were resuspended in 800 μ l motility buffer and
199 centrifuged (3750 x g, 3 min). The bacterial pellet was gently resuspended in 25-50 μ l motility
200 buffer to ensure similar bacterial densities. 1.5 μ l of the resuspension was placed on a
201 microscope slide layered with a pad of 1.5% agarose in motility buffer, and covered with a cover
202 slip. Fluorescence microscopy was performed as previously described (40) with minor
203 modifications: a Deltavision Spectris optical sectioning microscope (Applied Precision)
204 equipped with a UPlanSApo 100 \times 1.40 oil objective (Olympus) combined with 1.6 \times auxiliary
205 magnification, resulting in a pixel size of 101.31 nm. An Evolve electron multiplying charge
206 coupled device (EMCCD) camera (Photometrics) was used to take differential interference
207 contrast (DIC) and fluorescence photomicrographs. For fluorophore visualization, the
208 GFP/hsGFP filter set (Ex 475/28 nm, Em 522/44 nm) and an EM gain of 50 was used. A stack of
209 20 DIC frames ($\Delta z = 100$ nm) were taken with 0.005 s. Fluorescence frames corresponding to the
210 center of the bacterium were acquired for 12 seconds (0.4 s exposure time, $\Delta t = 1$ sec). To
211 minimize the effect of cytosolic autofluorescence and to identify stable fluorescent foci, the first
212 three frames were discarded, and the remaining frames were averaged. The fluorescence
213 background of each averaged image was determined and max/min intensity was set to 9200 units

214 above and 800 below average, respectively using ImageJ (33). Polar fluorescent spots were then
215 identified by eye in a blind analysis. For each strain, more than 2000 bacteria in eleven fields of
216 view from three independent experiments were analyzed.

217

218 RESULTS

219 ***FlgZ and Pel mediate c-di-GMP-dependent repression of swarming motility in a mutant that***
220 ***makes high levels of c-di-GMP.*** In *P. aeruginosa*, deletion of the *bifA* gene, which encodes a c-
221 di-GMP-degrading phosphodiesterase, results in elevated intracellular c-di-GMP levels and
222 repression of swarming motility (41). We have previously demonstrated that the flagellar stator
223 protein MotA and pilin-associated protein PilY1 contribute to swarming repression in the $\Delta bifA$
224 mutant (24, 42). We expected that in addition to these factors, a c-di-GMP-binding effector
225 would likely be involved in swarming repression in response to high c-di-GMP levels. The *P.*
226 *aeruginosa* genome encodes eight PilZ domain-containing proteins, and seven of these PilZ
227 proteins have been shown to bind to c-di-GMP. One PilZ domain protein, PA14_20700, has a
228 YcgR domain also found in the YcgR protein, a c-di-GMP-responsive flagellar motility control
229 protein of *E. coli* and *S. enterica* (23). It was recently shown that an ortholog of PA14_20700 in
230 *P. fluorescens* participates in biofilm formation, and another ortholog in *P. putida* is able to
231 repress swimming motility when expressed from a plasmid (29). Based on these roles for similar
232 proteins, we focused on PA14_20700 as a potential candidate for c-di-GMP-mediated swarming
233 repression. Orthologs of PA14_20700 in *P. fluorescens* and *P. putida* are called *flgZ*, as the gene
234 is positioned downstream of the *flgMN* genes, so we will adopt that nomenclature for *P.*
235 *aeruginosa* and refer to PA14_20700 as the *flgZ* gene (29).

236 To investigate whether FlgZ participates in repression of swarming motility under
237 elevated c-di-GMP conditions in *P. aeruginosa*, we constructed a $\Delta bifA \Delta flgZ$ double mutant and
238 examined its swarming phenotype. As shown in Figure 1A, deletion of the *flgZ* gene did not
239 restore swarming to the $\Delta bifA$ mutant. In addition to its inability to swarm, another hallmark of
240 the $\Delta bifA$ mutant is increased production of the Pel polysaccharide component of extracellular
241 polymeric substances (EPS), which causes cells on swarm medium to appear wrinkly (41). We
242 noted that the $\Delta bifA \Delta flgZ$ mutant cells have a wrinkly appearance indicating that Pel production
243 remains high in this mutant. Pel plays important roles in cell aggregation, and we hypothesized
244 that elevated levels of Pel may be inhibiting cell mobility and masking any contributions of FlgZ
245 to regulating flagellar-mediated swarming motility in the $\Delta bifA$ mutant. Similarly, other
246 extracellular polysaccharides have been shown to impair cell motility in *S. enterica* (19), *B.*
247 *subtilis* (43), and *Listeria monocytogenes* (44).

248 To remove the potential influence of Pel on swarming motility, we introduced a mutation
249 in the *pelA* gene to eliminate a critical function required for Pel production (45). Deleting the
250 *pelA* gene in the *bifA* mutant did not restore swarming; however, when *flgZ* and *pelA* were both
251 deleted in a $\Delta bifA$ mutant background, swarming motility was largely restored compared to the
252 wild-type strain (Figure 1A). As reported previously, the $\Delta bifA \Delta pelA$ mutant was unable to
253 swarm (41) (Figure 1A). We complemented the $\Delta bifA \Delta flgZ \Delta pelA$ mutant by allelic replacement
254 of the *flgZ* deletion with a His-tagged wild-type *flgZ* allele (Figure 1A). The complemented
255 $\Delta bifA \Delta pelA \Delta flgZ::flgZ$ -His strain did not swarm, indicating that FlgZ participates in swarming
256 inhibition in the $\Delta bifA \Delta pelA$ mutant. Confirming the ability of the FlgZ-His protein to
257 complement the *flgZ* deletion, we could detect the FlgZ-His protein by Western blot performed
258 on cells harvested from a swarm agar plate (Figure 1A, bottom panel). To examine whether FlgZ

259 had an impact on swimming motility in addition to its effects on swarming, we performed a zero-
260 flow, free-swimming capillary assay to compare the single-cell motility of WT and $\Delta flgZ$ mutant
261 cells. We found that the $\Delta flgZ$ mutant swims slightly faster than the WT (Supplemental Figure
262 S1) indicating that FlgZ plays a role in inhibiting both swimming and swarming.

263 FlgZ is just one of eight PilZ domain-containing proteins in *P. aeruginosa* while *E. coli*
264 and *S. enterica* each have only two PilZ proteins. It is possible that in *P. aeruginosa* additional
265 PilZ domain proteins play roles in c-di-GMP-responsive swarming motility repression. To
266 examine whether other PilZ domain proteins participate in swarming motility repression, we
267 constructed deletions of each PilZ domain protein-encoding gene in $\Delta bifA$ and $\Delta bifA \Delta pelA$
268 mutant backgrounds. As shown in Supplemental Figure S2A, deletions of each of the seven
269 remaining PilZ domain proteins did not restore swarming to the $\Delta bifA$ mutant. When introduced
270 into the $\Delta bifA \Delta pelA$ mutant, only a mutation in *pilZ* showed a small increase in swarming, but
271 not to the extent observed in the $\Delta bifA \Delta pelA \Delta flgZ$ mutant (Supplemental Figure S2B). These
272 results indicate that FlgZ is the main c-di-GMP effector protein contributing to swarming
273 motility repression in the $\Delta bifA \Delta pelA$ mutant, although PilZ may also play a minor role in this
274 process. While PilZ does not appear to bind c-di-GMP (12, 23), this protein participates in Type
275 IV pili biogenesis (46), and we suspect that the defect in Type IV pili function is responsible for
276 the impact on swarming (35). Given the substantial role of FlgZ in regulating cell surface
277 motility, we focus on the contribution of this protein for the remainder of the work presented
278 here.

279 We next confirmed that the increased motility observed in $flgZ$ mutant strains was not the
280 result of a decrease in c-di-GMP levels. We used mass spectrometry to quantify intracellular c-
281 di-GMP in wild-type *P. aeruginosa* PA14 as well as the $\Delta flgZ$, $\Delta pelA$, $\Delta pelA \Delta flgZ$, $\Delta bifA$, $\Delta bifA$

282 $\Delta flgZ$, $\Delta bifA \Delta pelA$, and $\Delta bifA \Delta pelA \Delta flgZ$ mutants. In most cases, c-di-GMP levels did not
283 change significantly with the introduction of the $flgZ$ mutation (Supplemental Figure S3).
284 However, the $\Delta bifA \Delta flgZ \Delta pelA$ mutant had significantly higher c-di-GMP levels than the $\Delta bifA$
285 $\Delta pelA$ mutant (Supplemental Figure S3) despite that fact that the triple mutant shows increased
286 motility versus the double mutant; this result indicates that swarming in the $\Delta bifA \Delta flgZ \Delta pelA$
287 mutant is not due to decreased c-di-GMP levels.

288 To assess whether FlgZ and Pel contribute to swarming motility repression under
289 conditions of high c-di-GMP generally, and not specifically to the absence of $bifA$, we used
290 another strain with high c-di-GMP, which carries a mutation in the $hptB$ gene. The $hptB$ gene
291 encodes a histidine phosphotransfer protein involved in a complex regulatory cascade (47-49).
292 The $\Delta hptB$ mutant of *P. aeruginosa* PAK has been demonstrated to produce hyperbiofilms,
293 elevated Pel polysaccharide production, and elevated c-di-GMP (48, 50). By mass spectrometry
294 analysis, we found that in the *P. aeruginosa* PA14 strain, the $\Delta hptB$ mutant produces 4.3-fold
295 more c-di-GMP than wild type, while the $\Delta bifA$ mutant produces 12.8-fold more c-di-GMP than
296 wild type. Mirroring our observations in the $\Delta bifA$ mutant background, the $\Delta hptB$ single mutant
297 as well as the $\Delta hptB \Delta pelA$ and $\Delta hptB \Delta flgZ$ double mutants were unable to swarm; but
298 swarming was restored in a $\Delta hptB \Delta flgZ \Delta pelA$ triple mutant (Figure 1B). Notably, swarming
299 motility appeared to be fully restored to wild-type levels in the $\Delta hptB \Delta flgZ \Delta pelA$ mutant as
300 opposed to the partial restoration observed for the $\Delta bifA \Delta flgZ \Delta pelA$ mutant. We suspect that
301 this discrepancy is due to the difference in c-di-GMP levels measured for the $\Delta hptB$ and $\Delta bifA$
302 mutants.

303 In addition to the roles of FlgZ and Pel in motility repression in two distinct high c-di-
304 GMP mutant backgrounds, we found that a $\Delta pelA \Delta flgZ$ mutant hyper-swarms compared to the

305 wild-type strain (Figure 1C). The ability of *flgZ* and *pelA* mutations to alleviate swarming
306 repression in two high c-di-GMP backgrounds and in the wild-type background suggests that
307 FlgZ's impact on swarming is not due to the response to any particular mutation, but a general
308 function of FlgZ.

309

310 ***FlgZ's impact on swarming motility control depends on its conserved c-di-GMP binding motif.***

311 Amino acid residues of the conserved motifs RxxxR and D/NxSxxG are critical for c-di-GMP
312 binding in PilZ domain-containing proteins including *E. coli* YcgR (8), *P. aeruginosa* Alg44
313 (12), *C. crescentus* DgrA (10), *V. cholerae* PlzD (7), and *Borrelia burgdorferi* PlzA (13). The
314 PilZ domain of *P. aeruginosa* FlgZ contains these conserved residues, as demonstrated by
315 sequence alignment with PilZ domain-containing proteins of other species (Figure 2A). To test
316 whether FlgZ's role in swarming repression requires this c-di-GMP binding motif, we generated
317 strains in which we complemented the $\Delta bifA \Delta flgZ \Delta pelA$ mutant with a His-tagged variant of
318 FlgZ carrying single amino acid substitutions in the PilZ domain and tested the ability of these
319 mutant FlgZ proteins to restore swarming repression. A R140A substitution resulted in the
320 production of a detectable FlgZ protein that was no longer able to repress swarming (Figure 2B).
321 The R144A, R144D, D172A, and G177A mutant variants of FlgZ-His each resulted in a protein
322 that could not be detected by Western blot (Figure 2B). This result suggests that the conserved
323 R140 residue required for c-di-GMP binding is critical for FlgZ's role in repressing swarming in
324 response to c-di-GMP.

325

326 ***FlgZ interacts with the flagellar stator protein MotC.*** PilZ domain proteins in other bacteria
327 have been shown to influence motility via direct interactions with one or more components of the

328 flagellar motor. Thus, we hypothesized that FlgZ may impact swarming motility by a similar
329 mechanism. We employed a bacterial adenylate cyclase two-hybrid assay in *E. coli* to probe for
330 interactions between FlgZ and components of the *P. aeruginosa* flagellar motor: MotA, MotC,
331 FliG, and FliM. MotA and MotC are components of ion-translocating stators; and FliG and FliM
332 are the rotor component and switch protein of the motor, respectively. The motor requires MotC,
333 FliG, and FliM for functional swarming motility (51), whereas MotA prevents swarming motility
334 in *P. aeruginosa*.

335 Full-length proteins were fused to either the T18 or T25 subunit of adenylate cyclase and
336 these hybrid proteins were co-expressed in *E. coli* BTH101 to test each potential interaction. An
337 interaction between hybrid proteins can be detected as blue color on medium containing the
338 substrate X-gal. As shown in Figure 3A, FlgZ was found to interact with MotC, but not with
339 MotA, FliG, or FliM. To investigate whether the c-di-GMP binding region of FlgZ is important
340 for the interaction between FlgZ and MotC, we next tested the point mutant FlgZ (R140A) in the
341 bacterial two-hybrid assay. As shown in Figure 3B, FlgZ (R140A) was not able to interact with
342 MotC, indicating that residue R140 is critical for both FlgZ's function in swarming motility
343 repression and for the FlgZ-MotC interaction. As an additional negative control, we co-
344 transformed the pUT18 empty vector with T25-MotC, and saw no blue color as expected (Figure
345 3B). The strength of these interactions was quantified using β -galactosidase assays, and strains
346 co-expressing FlgZ and MotC fusion proteins showed significantly higher β -galactosidase than
347 the negative control (Figure 3C). Mutating R140 in the FlgZ protein resulted in a significant
348 reduction in interaction with MotC to a level observed for the FlgZ-MotA interaction (Figure
349 3C).

350 To further substantiate the evidence for physical interaction between FlgZ and MotC, we
351 assessed the ability of FlgZ and MotC to co-precipitate. To probe this interaction, FlgZ was C-
352 terminally HA-tagged in *P. aeruginosa* strains carrying overexpression plasmids pMotA-His or
353 pMotC-His. These His-tagged versions of MotA and MotC have been used previously by our
354 group and are functional in swarming assays (24). We used a nickel resin to enrich for MotA-His
355 or MotC-His and tested for FlgZ-HA co-precipitation with and without the addition of 5 μ M c-
356 di-GMP. Our results indicate that FlgZ interacts with MotC in the presence of 5 μ M c-di-GMP,
357 and does not interact with MotC in the absence of c-di-GMP (Figure 4). FlgZ does not interact
358 with MotA regardless of the presence of c-di-GMP. In strains lacking His-tagged MotA or MotC,
359 only background bands were detected (Figure 4, far right lanes).

360

361 ***FlgZ localization is consistent with an interaction between FlgZ and MotCD.*** Our data suggest
362 that there is a c-di-GMP-dependent interaction between FlgZ and MotC. To assess whether c-di-
363 GMP levels and the presence of MotCD stator sets influence subcellular localization of FlgZ, we
364 replaced the *flgZ* gene with *gfp-flgZ* at its native chromosomal locus. We complemented the
365 $\Delta bifA \Delta flgZ \Delta pelA$ mutant by allelic replacement of the *flgZ* deletion with *gfp-flgZ* to show that
366 this GFP fusion does not interfere with the function of FlgZ in swarming motility (Supplemental
367 Figure S4).

368 Previous work suggests that FlgZ-like proteins in *P. fluorescens*, *E. coli*, and *S. enterica*
369 localize to the flagellar basal body and that this localization is enhanced in mutants with high c-
370 di-GMP levels (15, 18, 29). In *P. aeruginosa* cells harvested from a swarm agar plate, we
371 observed that GFP-FlgZ could indeed localize to the pole (Figure 5A,B). Furthermore, the
372 percentage of cells with polarly localized GFP-FlgZ significantly increased in the $\Delta bifA$ mutant

373 (Figure 5A), which produces more c-di-GMP than the wild-type strain (Supplemental Figure S3).

374 This finding indicates that increased levels of c-di-GMP impact localization of FlgZ.

375 Given that we have demonstrated that FlgZ interacts with MotCD, we hypothesized that

376 the polar localization of FlgZ might be at least in part dependent upon MotCD. To test this

377 hypothesis, we introduced a $\Delta motCD$ mutation into our strain expressing the functional GFP-

378 FlgZ from its endogenous locus on the chromosome. In the $\Delta motCD$ mutant, we find

379 significantly fewer cells with polar localization of GFP-FlgZ compared to wild type (Figure 5B).

380 This finding is consistent with our hypothesis that FlgZ interacts with the flagellar machinery via

381 a direct interaction with MotC.

382

383 ***MotCD stator overexpression restores swarming motility in high c-di-GMP backgrounds***

384 ***lacking Pel.*** We previously demonstrated that the MotCD stator set is required for swarming

385 motility and that under conditions of high levels of c-di-GMP, there is a decrease in polar

386 localization of MotD (24). We hypothesized that overexpression of MotCD would increase the

387 presence of this stator at the flagellar motor and that this increase in MotCD could restore

388 swarming to high c-di-GMP backgrounds by over-coming the FlgZ-mediated swarming

389 repression mechanism.

390 We found that similar to the impact of deleting the *flgZ* gene, overexpression of MotCD

391 partially restores swarming motility to the $\Delta bifA \Delta pelA$ mutant (Figure 6A,B). However, MotCD

392 overexpression does not restore swarming motility in the $\Delta bifA$ mutant in which elevated Pel

393 polysaccharide is produced. This result is consistent with our hypothesis that the effect of c-di-

394 GMP on flagellar motor function can be masked by the contribution of Pel polysaccharide.

395

396 **DISCUSSION**

397 The second messenger c-di-GMP coordinately regulates motility and extracellular
398 polysaccharide synthesis to allow bacteria to efficiently transition between motile and sessile
399 lifestyles. Regulation via c-di-GMP can occur at the level of transcription as well as post-
400 transcriptionally and post-translationally (52). In *P. aeruginosa*, c-di-GMP regulates the
401 biosynthesis of both flagellar components and extracellular polysaccharide through the
402 transcription factor FleQ (53). Post-transcriptional regulation of extracellular polysaccharide
403 production is controlled by another c-di-GMP binding protein, PelD (54). Here we describe an
404 additional layer of motility regulation: the first PilZ domain protein in *P. aeruginosa* with a role
405 in flagellar-dependent swarming motility control.

406 Deletion mutants lacking the genes encoding each of the eight PilZ proteins of *P.*
407 *aeruginosa* revealed that c-di-GMP-mediated repression of swarming motility is largely restored
408 by the deletion of both *flgZ* and *pelA* genes. This finding indicates that inhibition of motility can
409 be controlled by FlgZ and highlights a role for Pel polysaccharide in this process. The finding
410 that Pel polysaccharide plays a role in motility repression in the absence of c-di-GMP effector
411 protein FlgZ, particularly in the $\Delta bifA \Delta flgZ$ and $\Delta hptB \Delta flgZ$ mutants, aligns with previous
412 findings that extracellular polysaccharides impair motility in other species *S. enterica* (19), *B.*
413 *subtilis* (43), and *L. monocytogenes* (44). However, *P. aeruginosa* is unique from these and other
414 organisms using c-di-GMP-dependent motility control, in that eliminating either the c-di-GMP
415 binding protein or the exopolysaccharide singly has no significant impact on motility in the high
416 c-di-GMP background.

417 It has been previously demonstrated that FlgZ binds c-di-GMP. We show that mutants
418 with an amino acid substitution in the conserved c-di-GMP binding motif of FlgZ behave like a

419 *flgZ* null mutant, suggesting that the PilZ domain of FlgZ is required for swarming motility
420 inhibition and consequently, that c-di-GMP binding is critical for FlgZ's function in swarming
421 motility control. We measured global levels of c-di-GMP in *flgZ* mutants to ensure that the
422 increased motility of the $\Delta bifA \Delta flgZ \Delta pelA$ mutant was not due to an overall decrease in c-di-
423 GMP. Unexpectedly, we found that the $\Delta bifA \Delta flgZ \Delta pelA$ mutant has elevated levels of c-di-
424 GMP compared to the $\Delta bifA \Delta pelA$ mutant despite the enhanced swarming motility of the $\Delta bifA$
425 $\Delta flgZ \Delta pelA$ triple mutant; we currently do not understand the basis for this observation.

426 *P. aeruginosa* has two stator sets to power a single flagellar motor, distinguishing it from
427 many organisms that use PilZ proteins to control motility. We hypothesize that c-di-GMP-bound
428 FlgZ inhibits swarming motility by specifically targeting the stator set that promotes swarming
429 motility – MotCD. This hypothesis is supported by bacterial two-hybrid assays, co-
430 immunoprecipitation experiments, and localization studies, all of which indicate a protein-
431 protein interaction between FlgZ and MotC. These experiments also demonstrate that the
432 presence of c-di-GMP and the conserved PilZ domain of FlgZ, which are necessary for motility
433 inhibition, are also required for FlgZ-MotC interaction.

434 By impacting flagellar motor function in response to c-di-GMP, FlgZ can allow *P.*
435 *aeruginosa* to regulate swarming motility after flagellar assembly is complete, thus enabling
436 cells to quickly adapt to changing environmental conditions. The mechanism by which FlgZ's
437 interaction with MotC may be altering MotCD engagement with the rotor will be the focus of
438 future studies. Interestingly, evidence for stator exchange in the context of a dual stator system
439 also comes from studies in *Shewanella oneidensis*, in which sodium-dependent PomAB and
440 proton-dependent MotAB drive rotation of a single flagellar rotor (55). In *S. oneidensis*, sodium
441 levels drive stator dynamics by influencing the incorporation efficiency of MotAB into the motor

442 (56), however the mechanism of stator switching in this organism is not yet clear. We propose
443 that due to the dynamic nature of the stator proteins with respect to the flagellar motor (57, 58),
444 in the case of *P. aeruginosa*, one possibility is that under conditions of high c-di-GMP, FlgZ
445 interacts with MotCD stators to prevent MotCD engagement with the flagellar rotor, leaving
446 room for increased incorporation of MotAB stators, which cannot drive swarming motility.
447 Perhaps analogous mechanisms function to promote stator exchange in other organisms.

448

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454 Facility for quantitative analysis of c-di-GMP.

455

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- 633

634 **FIGURES AND LEGENDS**

635

636 **Figure 1. FlgZ and Pel polysaccharide contribute to swarming motility repression.** (A) Top
637 panel: Representative swarm plates of strains indicated. Bottom panel: Western blot probed with
638 anti-His antibody to detect FlgZ-His expression in the $\Delta bifA \Delta pelA \Delta flgZ::flgZ$ -His strain in
639 which the *flgZ* gene deletion is complemented by allelic replacement resulting in expression of a
640 His-epitope tagged FlgZ protein. (B) Representative swarm plates of strains indicated. (C)
641 Representative swarm plates of the indicated strains. The values below the swarm plates indicate
642 the percentage (means \pm SEM, from three independent experiments of six plates each) of the
643 plate surface coverage of the mutant strains relative to that of the WT strain (set at 100%).
644 Significance was determined by analysis of variance and Dunnett's posttest comparison for
645 differences relative to the WT. *, $P < 0.05$ compared to WT.

646

647 **Figure 2. Conserved residues in the c-di-GMP binding domain are important for FlgZ**
648 **stability and function.** (A) Multiple sequence alignment of the predicted c-di-GMP binding
649 region of FlgZ in *P. aeruginosa* (*Pa*) with orthologs from *P. putida* (*Pp*), *P. fluorescens* (*Pf*), *S.*
650 *enterica* (*Se*), *E. coli* (*Ec*), and *B. subtilis* (*Bs*) along with other PilZ-domain containing proteins
651 from *C. crescentus* (*Cc*), and *V. cholerae* (*Vc*). The sequence alignment was generated by Clustal
652 Omega (59, 60) using the complete PilZ domain of each protein as predicted by SMART (61,
653 62). A portion of the alignment is shown here. Clustal Omega determined conservation of
654 residues. *, a fully conserved residue; :, a residue of strongly similar properties. Boxed conserved
655 residues were targeted for site-directed mutagenesis. Numbers correspond to the amino acid
656 residue in the *P. aeruginosa* FlgZ full-length protein. (B) Top panel: Representative swarm

assays of the strains indicated. Bottom panel: Protein levels by Western blotting and anti-His antibody to detect expression of the wild type and mutant FlgZ-His variants.

Figure 3. Detection of interaction between FlgZ and MotC by a bacterial two-hybrid analysis. (A-B) Full length *flgZ* and *flgZ*(R140A), as well as the flagellar motor genes *motA*, *motC*, *fliG*, and *fliM* were cloned into vectors pKNT25, pKT25, pUT18, or pUT18C, and co-transformed into *E. coli* BTH101 cells. The co-expressed fusion protein combinations for each transformation are indicated on the left. The transformants were 10-fold serial diluted, spotted (2 μ l) on LB agar containing Cb, Kan, X-gal, and IPTG, then incubated for 40 h at 30 °C. Cells co-transformed with empty vectors serve as negative control and cells co-transformed with leucine zipper vectors provided by the manufacturer (T18-zip and T25-zip) serve as a positive control. The degradation of X-gal (blue color) indicates positive protein-protein interaction. (C) Bacterial two-hybrid interactions were quantified by measuring β -galactosidase activity in transformants grown in LB broth supplemented with Cb and Kan overnight at 30 °C. The data represent three independent experiments with three or four biological replicates each, and values are reported as mean \pm SEM. Significance was determined by analysis of variance and Dunnett's posttest comparison for differences relative to the negative control (T18 + T25). n.s., not significant; ***, $P < 0.001$ (the positive control T18-zip + T25-zip was not included in the statistical analysis).

Figure 4. Immunoprecipitation analysis to assess FlgZ and MotC interaction.

Immunoprecipitations with a nickel-chelating resin were performed with cell lysates expressing FlgZ-HA, and MotA-His or MotC-His. Western blots of precipitate (top panel) and input

679 (bottom panel) are probed with anti-HA. Immunoprecipitations were performed with and without
680 5 μ M c-di-GMP, as indicated.

681

682 **Figure 5. Localization of GFP-FlgZ is impacted by c-di-GMP levels and MotCD. (A)**

683 Representative images of WT, $\Delta bifA$, and $\Delta motCD$ strains expressing GFP-FlgZ. (B) For each
684 strain, more than 2000 bacteria from three independent experiments were analyzed, and values
685 are reported as mean \pm SEM. Significance was determined by analysis of variance and Dunnett's
686 posttest comparison for differences relative to WT. ***, $P < 0.001$.

687

688 **Figure 6. MotCD overexpression restores c-di-GMP-inhibited swarming only in the**

689 **absence of Pel.** (A) Representative swarm plates of strains indicated carrying either an empty
690 vector or MotCD-His-expressing plasmid (pMotCD-His). Swarm plates contained 0.2%
691 arabinose. (B) Protein levels by Western blotting and anti-His antibody to detect expression of
692 pMotCD-His.

Fig. 1

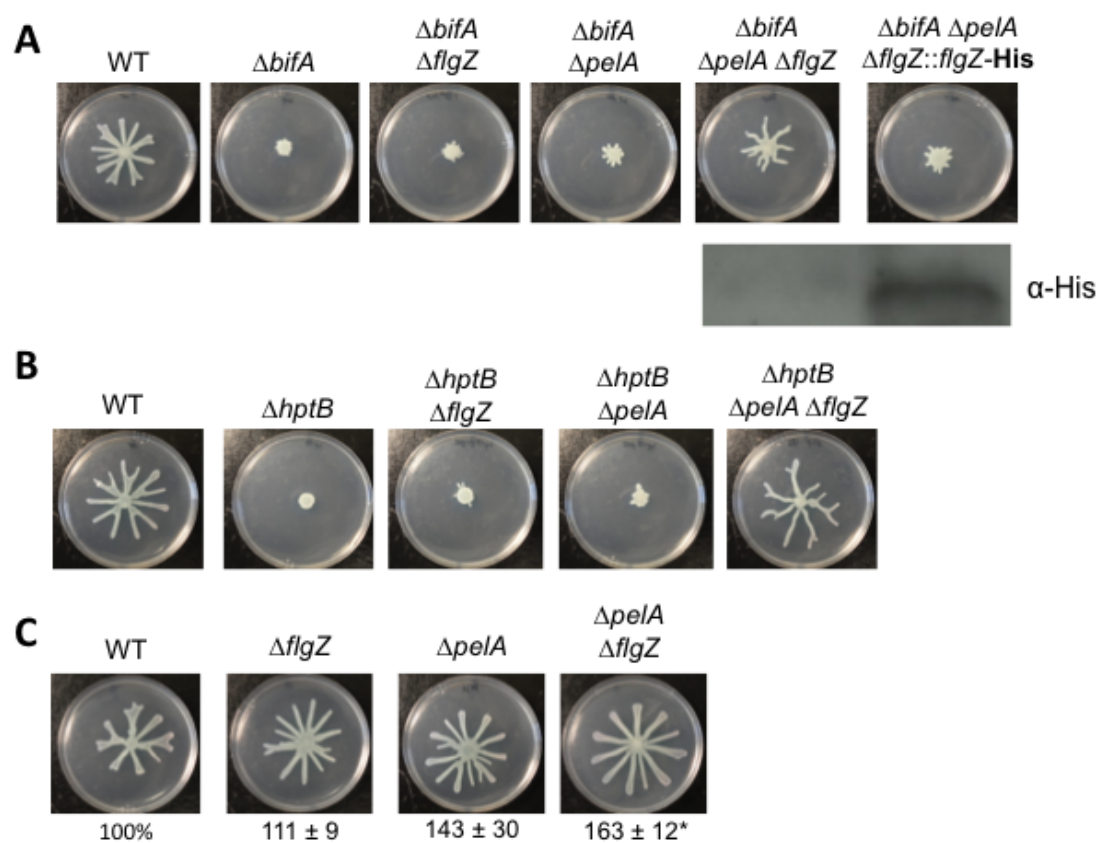


Fig. 2

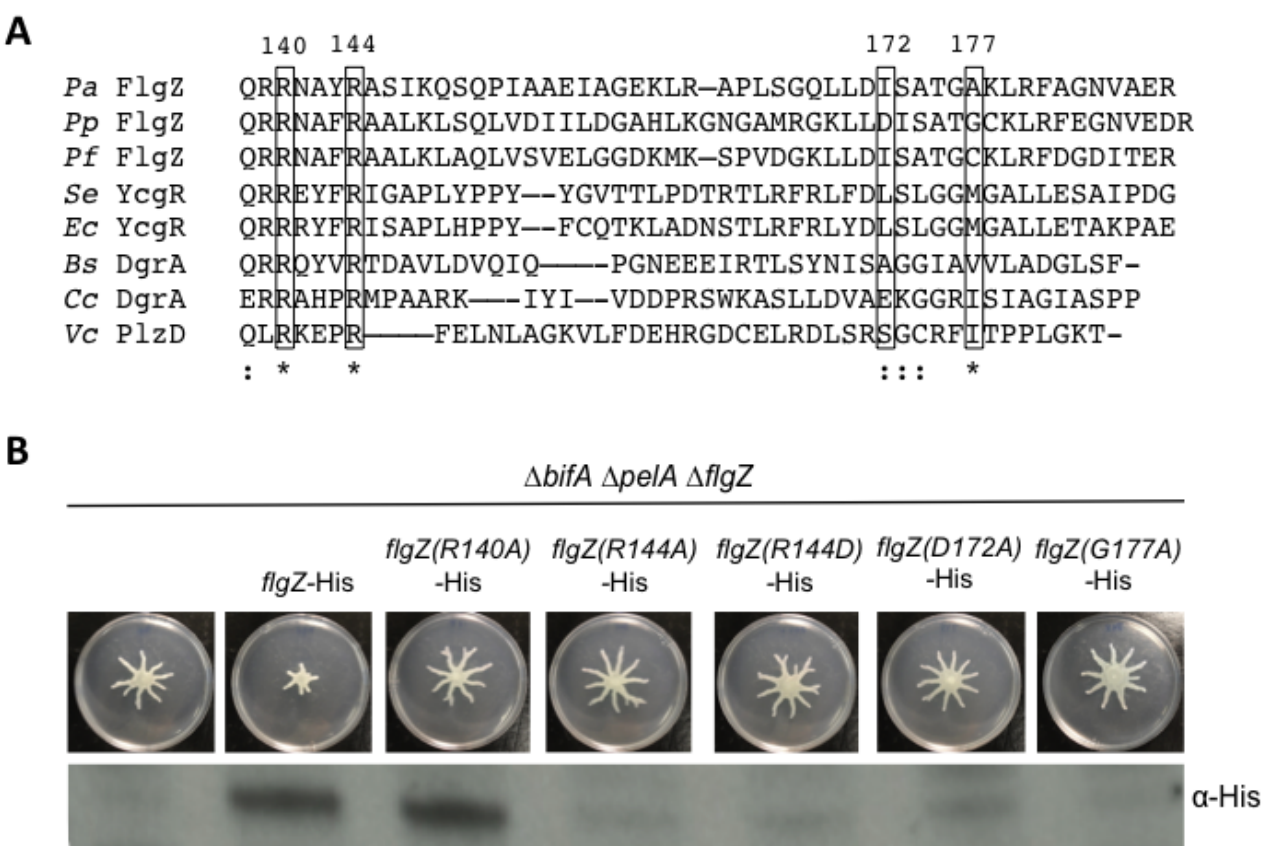


Fig. 3

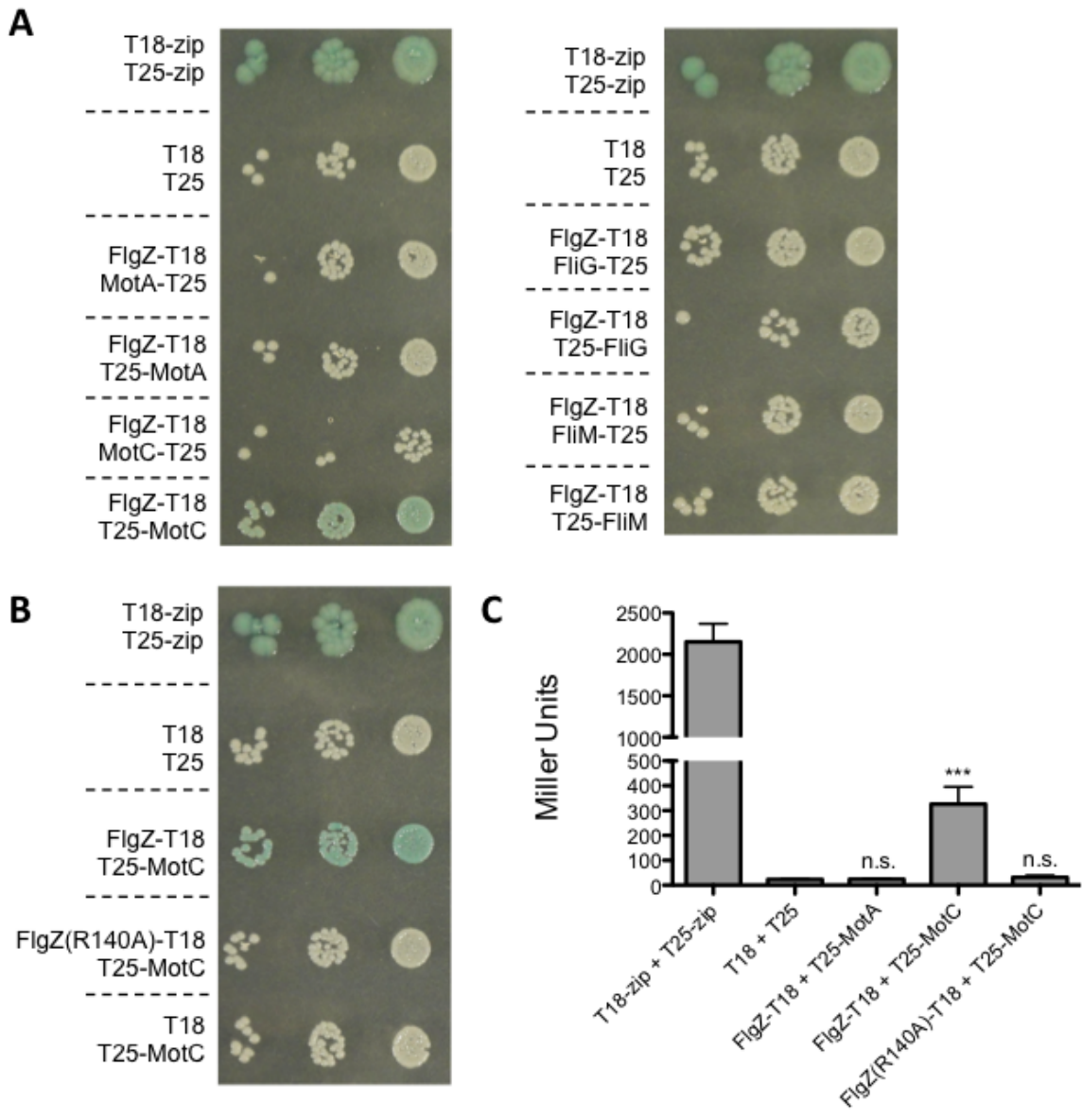


Fig. 4

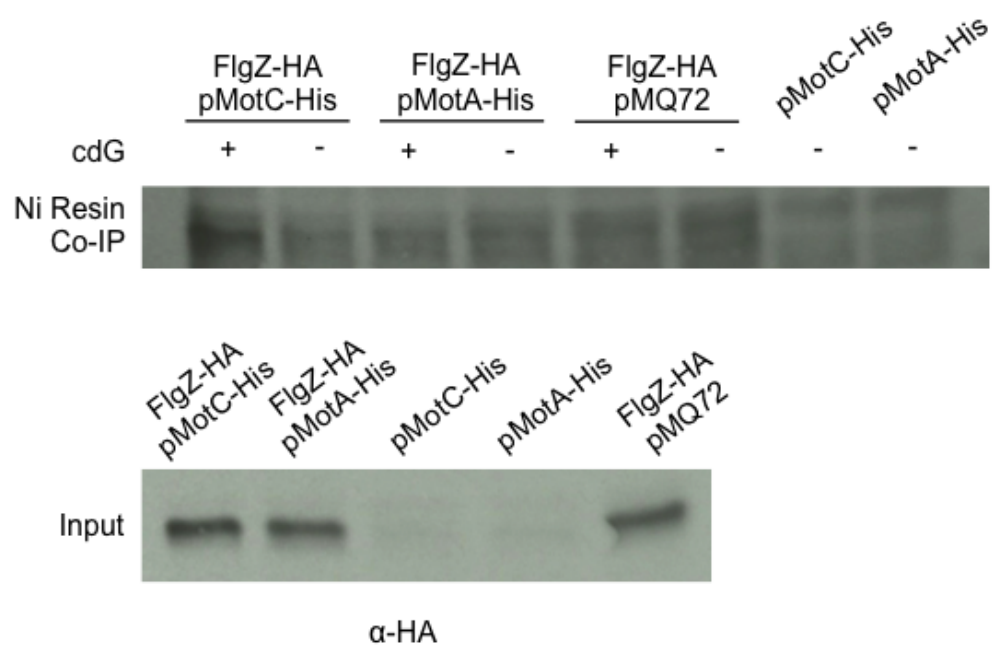


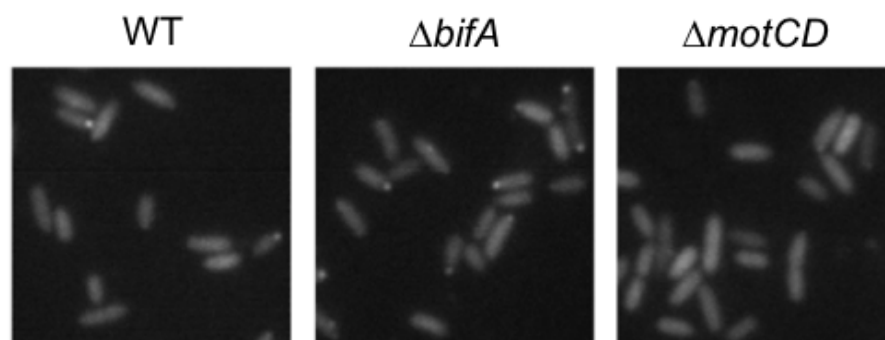
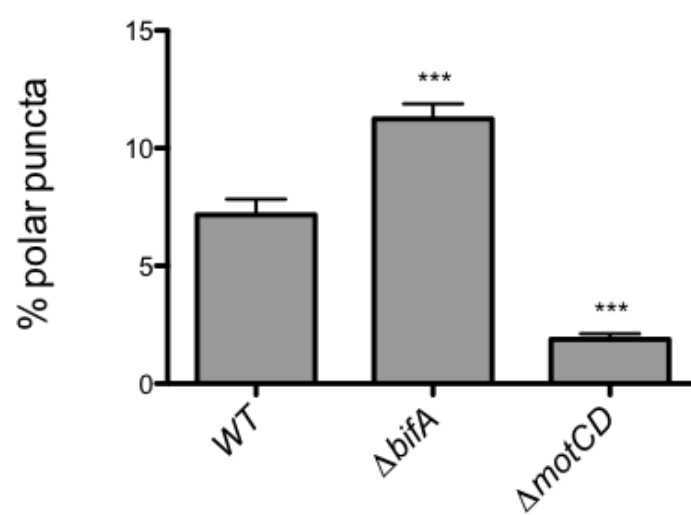
Fig. 5**A****B**

Fig. 6

