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4
5 *Title:* “**Diverse high-torque bacterial flagellar motors assemble wider stator rings using a**
6 **conserved protein scaffold**”

7 *Short title:* “**Evolved higher torque in bacterial flagella**”

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29 **Abstract**

30 Although it is known that diverse bacterial flagellar motors produce different torques, the
31 mechanism underlying this is unknown. To better understand this, we combined genetic
32 analyses with electron cryo-tomography subtomogram averaging to determine *in situ* structures
33 of flagellar motors that produce different torques. Our results unambiguously locate the torque-
34 generating stator complexes for the first time, and show that diverse high-torque motors employ
35 variants of an ancestrally-related family of structures to scaffold incorporation of additional stator
36 complexes at wider radii from the axial driveshaft than in the model enteric motor. We identify
37 the protein components of these additional scaffold structures and elucidate their sequential
38 assembly, demonstrating that they are required for stator complex incorporation. These novel
39 proteins are widespread, suggesting that different bacteria have tailored torques to specific
40 environments by scaffolding alternative stator placement and number. Our results quantitatively
41 account for different motor torques, complete assignment of locations of the major flagellar
42 components, and provide crucial constraints for understanding mechanisms of torque
43 generation and multiprotein complex evolution.

44 **Significance statement**

45 Many bacteria swim using helical propellers called flagella. Intriguingly, different bacteria show
46 different swimming abilities, strikingly illustrated by the abilities of some to bore through viscous
47 fluids (e.g., gastrointestinal mucous), while others are completely immobilized. Here we use 3D
48 electron microscopy to show that differences can be rationalized by the structures of their
49 torque-generating motors. We show that two diverse high-torque motors position additional
50 torque-generating complexes at wider radii from the axial driveshaft than in the model enteric

bacteria, consistent with exertion of greater leverage to rotate the flagellum – and thus greater torque generation. Intriguingly, these torque-generating complexes are scaffolded at wider radii by a conserved yet divergent family of structures, suggesting an ancient origin of reconfiguring torque output.

\body

Introduction

Flagellated bacteria have tailored their motility to diverse habitats. For example, the enteric model organisms *Salmonella enterica* serovar Typhimurium and *Escherichia coli* colonize animal digestive tracts and can reside outside a host, assembling flagella over their cell body to swim. There is, however, a diverse spectrum of flagellar swimming ability seen across the bacterial kingdom. *Caulobacter crescentus* inhabits low-nutrient freshwater environments where it swims using a high-efficiency flagellar motor (1, 2), while *Vibrio* species produce high-speed, sodium-driven polar flagella to capitalize upon the high sodium gradient of their marine habitat (3). On the other hand, the ϵ -proteobacteria and spirochaetes, many of which thrive exclusively in association with a host, have evolved characteristically rapid and powerful swimming capabilities that enable them to bore through mucous layers coating epithelial cells, or between tissues. Indeed, the ϵ -proteobacteria *Campylobacter jejuni* and *Helicobacter pylori* are capable of continued swimming in high-viscosity media that immobilize *E. coli* or *Vibrio* cells (4–6), with similar behaviour observed for spirochaetes (7, 8).

Despite differences in swimming ability, however, the flagellar motor is composed of a conserved core of approximately 20 structural proteins (9). The mechanism of flagellar motility is

72 conserved (10), with torque generated by rotor and stator components (9). Stator complexes,
73 heterooligomers of four MotA and two MotB proteins, are thought to form a ring that surround
74 the axial driveshaft. Transmembrane helices of MotA and MotB form an ion channel, and MotB
75 features a large periplasmic domain that binds peptidoglycan (11, 12) and the flagellar structural
76 component the P-ring (13). The stator complex couples ion flux to exertion of force on the
77 cytoplasmic rotor ring (the C-ring), which transmits torque to the axial driveshaft (the rod),
78 universal joint (the hook), and helical propeller (the filament), culminating in propulsion of the
79 bacterium. Biophysical (14) and freeze-fracture (15) studies together with modeling (16) have
80 proposed that a tight ring of approximately 11 stator complexes dynamically assemble around
81 the rod above the outer lobe of the C-ring in closely-related *Salmonella* and *E. coli* motors (to
82 which we collectively refer to as the 'enteric motor'). Yet despite these conclusions, and while
83 structures observed in subtomogram averages have been proposed to be the stator complexes
84 (17–19), the locations and stoichiometries of the stator complexes remain to be confirmed.

85 How can we explain the wide diversity in flagellar swimming abilities in the context of a
86 conserved core flagellar motor? Biophysical studies suggest that the source of the difference
87 lies, at least in part, in variations in the mechanical output of the motors themselves. Torques of
88 motors from different bacteria have been shown to range over an order of magnitude, and
89 torque correlates with swimming speed and the ability of bacteria to propel themselves through
90 different viscosities, indicating that adaptations are likely to be at the level of the motor itself
91 (torque also varies within a single species, up to a maximum value, as a function of the number
92 of stator complexes incorporated into the motor (14)). For example, *C. crescentus* motors have
93 been measured to produce torques of 350 pN nm (2). Estimates for the torque of the enteric

94 motor ranges from ~1300 to ~2000 pN nm (20, 21). The ϵ -proteobacterium *H. pylori* has been
95 estimated to swim with torque of 3600 pN nm (22), and spirochaetes are capable of swimming
96 with 4000 pN nm torque (21, 23). Sodium-driven *Vibrio* spp. motor torques have been measured
97 between approximately 2000 – 4000 pN nM (24), dependent upon the magnitude of the sodium
98 gradient. It is noteworthy, however, that an estimated sodium motive force in *Vibrio* spp. that is
99 lower than the standard *E. coli* proton motive force nevertheless drives the *Vibrio* motor with
100 higher torque than the *E. coli* motor (24, 25), further suggesting that torque differences likely
101 exist at the level of the motor. The molecular mechanism underlying how different motors might
102 produce different torques, however, has not been investigated.

103 The simplest scenario for tuning motor torque would be evolved adaptation of motor
104 architecture. Supportive of this, we recently showed that many motors have evolved novel
105 structures not found in the well-studied enteric motors (18), and observed that C-ring radius
106 varies between species (17, 18). One of the most widespread novel structures is a periplasmic
107 'basal disk' directly beneath the outer membrane, often co-occurring with varied uncharacterized
108 additional structures, which we collectively term 'disk complexes'. Consistently, disk complexes
109 have only been seen in motors that produce higher torque than in *E. coli* or *Salmonella*. For
110 example, the sodium-driven ~2000+ pN nm torque motors of *Vibrio* species assemble a disk
111 complex featuring a basal disk beneath the outer membrane (18) in addition to smaller H- and T-
112 rings composed of FlgOT and MotXY, respectively (26, 27). It has been shown that the T-ring
113 interacts with *Vibrio* spp. stator complexes (28), although the exact location and number of
114 *Vibrio* spp. stator complexes remains unclear. ϵ -proteobacteria such as *Helicobacter* species,
115 *Campylobacter jejuni*, and *Wolinella succinogenes* also assemble disk complexes composed of

large basal disks beneath the outer membrane together with additional, smaller, disks (18, 29). Although these and other cases of additional disks have been reported (18, 30), their relation to flagellar function remain enigmatic, and it is unclear if these widespread disk complexes are homologous or analogous.

In this study, we hypothesized that bacteria have tuned their swimming abilities by evolving structural adaptations to their flagellar motors that would result in altered torque generation. Using electron cryo-tomography and subtomogram averaging, we visualized that *Vibrio* polar γ -proteobacterial and *Campylobacter* ϵ -proteobacterial flagellar motors incorporate 13 and 17 stator complexes, respectively, compared to the ~11 in enteric bacteria. In both cases, these stator complexes are scaffolded into wider stator rings relative to the enteric motor by novel components of their respective disk complexes. The wider *C. jejuni* stator ring is further reflected in a considerably wider rotor C-ring. Further analysis of the components of the *Vibrio* and *C. jejuni* disk complexes reveals that they share a core protein, FlgP, but each have acquired diverse additional components to form divergent disk complex architectures. We conclude by showing that our structural data of wider stator rings featuring additional stator complexes can quantitatively account for the differences in torque between different flagellar motors.

Results

Structures of three flagellar motors that produce different torques are structurally diverse

We hypothesized that disk complexes might offer a structural rationale for differences in motor torque. To explore this hypothesis, we performed a comparative electron cryo-tomography study

of high-torque motor structures *in situ*, using *Vibrio fischeri* and *C. jejuni* as models for *Vibrio* spp. and ϵ -proteobacteria respectively, and compared them to the *Salmonella* motor as a well-studied model enteric motor. For data collection we developed a high-throughput electron cryo-tomography pipeline on a 200kV FEG electron microscope with side-entry cryo-holder and direct electron detector camera, and imaged motors *in situ* in *Salmonella*, *V. fischeri*, and *C. jejuni*. For *Salmonella*, we used a mutant that produced minicells (Fig. 1a) to circumvent problems caused by excessive inelastic and multiple electron scattering (31). In contrast, we were able to image motors in WT *V. fischeri* and *C. jejuni* (Fig. 1b,c).

Subtomogram averaging hundreds of motors enabled us to determine structures (Fig. 1a-i) to resolutions sufficient to discern individual protein domains (Fig. S1). Parts of the *V. fischeri* and *C. jejuni* motors showed clear 13-fold and 17-fold symmetry around their axes of rotation, respectively (Fig. 2, Fig. S2); this symmetry was imposed to improve contrast. The *Salmonella* motor exhibited minor flexion in the rod between the MS- and P-rings, necessitating separate averaging of the cytoplasmic and periplasmic sections, and merging the two structures at this flexion point. Since no symmetry was evident in the *Salmonella* motor, we applied 11-fold symmetry based on the consensus of stator complex symmetry in *Salmonella* and *E. coli* (14, 32). Neither this 11-fold symmetry nor other symmetries clarified any symmetric features in our reconstructions. Our *Salmonella* structure reproduced expected features seen in previous studies (31, 33) (Fig. 1d,g).

The *V. fischeri* and *C. jejuni* motors featured disk complexes with prominent basal disks beneath the outer membrane (Figs 1h,i). We could also visualize the previously described H- and T-rings in the *V. fischeri* motor, although positioned 12 nm from the inner membrane, further than the 5

nm recently proposed in closely related *Vibrio alginolyticus* (34). In *C. jejuni*, two additional disks were present between the basal disk and the inner membrane, which we annotated as a periplasmic 'medial disk' and inner membrane-associated 'proximal disk' (Fig. 1 f,i). Although the *C. jejuni* basal disk did not exhibit any clear symmetry, the periplasmic medial disk and inner membrane-associated proximal disk formed rings with clear 17-fold symmetry (Fig. 2 i-k, Fig. S2). Curiously, the cup-shaped *C. jejuni* basal disk appeared to push the outer membrane away from the cell body at increased radius from the motor axis, producing a concave indentation outer membrane distortion around the motor; the significance of this distortion is unclear, particularly in light of the observation that the closely-related *H. pylori* motor has a flat basal disk and does not feature a corresponding indentation (18).

High-torque motors incorporate additional stator complexes

We investigated our speculation that disk complexes might contribute to the ability of these motors to generate high torque by examining the torque-generating stator complexes. The enteric flagellar motor has been suggested to incorporate approximately 11 stator complexes that interact with the outer lobe of the C-ring in the cytoplasm, and the peptidoglycan and the P-ring (13). The number of stator complexes incorporated into the enteric motor is proportional to the load on the motor (35, 36). Because blotting during the sample vitrification process for cryo-tomographic imaging necessitated that we imaged in low viscosity growth medium, we anticipated that low motor load would result in low stator complex occupancy, and our subtomogram averaging procedure would not resolve stator complexes. Consistent with this, no candidate stator complex densities were observed in the periplasm in the *Salmonella* motor structure (Fig. 1d,g). Although no stator complexes were observed, the outer lobe of the C-ring,

181 which is believed to be the C-terminal domain of FliG upon which stator complexes exert force,
182 was positioned at a 20 nm radius from the flagellar rotational axis.

183 In contrast, in both *V. fischeri* and *C. jejuni* we observed periplasmic densities connecting
184 through the inner membrane to the outer lobe of the C-ring where the stator complexes are
185 expected to reside (Fig. 2a,b,i,j) with 13-fold and 17-fold symmetry as noted above. This odd-
186 numbered symmetry produced asymmetric cross-sections of both *V. fischeri* and *C. jejuni*
187 motors (Figs. 1, 2). For *V. fischeri*, these proposed stator complexes were consistent with the
188 size of a recent single-particle reconstruction of the *Vibrio* stator complex (16). The densities
189 spanned from the C-ring to approximately 10 nm above the inner membrane, as previously
190 predicted (34), arranged in a 17 nm-radius ring with 13-fold rotational symmetry, and contacted
191 the 16 nm-radius T-ring (Fig. 2a-d, red arrows; continuous density best visualized in Fig. 1h) as
192 previously demonstrated (28). The lever contact point of this putative stator complex with the C-
193 ring – i.e., the FliG_C:MotA interface and the radius at which torque would be applied – was at a
194 radius of 21.5 nm from the flagellar axis of rotation (Fig. 2a-d, blue arrows). Corresponding
195 putative stator complex densities in *C. jejuni* formed a wider 30 nm-radius ring with 17-fold
196 symmetry extending from the outer lobe of the C-ring to merge with the proximal disk
197 approximately 10 nm above the inner membrane (Fig. 2i-l, red arrows). The putative FliG_C:MotA
198 interface lever contact point was at a radius of 26.5 nm (Fig. 2i-l, blue arrows). The 26 nm-
199 radius *C. jejuni* C-ring was correspondingly wider than the 22-nm radius *Salmonella* or 23-nm
200 radius *V. fischeri* C-rings, spaced by an additional component on its interior surface (Fig. 2i,j,
201 yellow arrow). This observation suggests that the *C. jejuni* C-ring incorporates additional

subunits of FliG, FliM, and FliN (and possibly FliN paralog FliY (37)) to construct a wider C-ring relative to the *Salmonella* motor.

To test our hypothesis that these densities were the stator complexes, we deleted *motB* homologs in *C. jejuni* and *V. fischeri* (in *V. fischeri*, gene *motB1*, encoding a PomB-type protein from the sodium-driven stator variant (38)). In both species, the proposed MotB densities were lost, supporting our hypothesis (Fig. 2e-n). In the *V. fischeri* WT motor, MotB was less dense than the T-ring densities, which may reflect partial occupancy of the stator complexes in the *Vibrio* motor (39). In the *C. jejuni* motor, deletion of *motB* resulted in loss of part of the proximal disk, indicating that MotB is one component of a multi-protein proximal disk assembly (Fig. 2m-p). Although we cannot rule out the possibility that this density loss is a result of deletion of *motB* indirectly impacting assembly of the proximal disk, the fact that this is the expected location of MotB together with an unambiguous similar result in *V. fischeri* overwhelmingly supports assignment of this density as MotB. Loss of MotB did not appear to have as large of an impact on the structure of the motor due to the remainder of the proximal disk continuing to occupy this location. We also noted that the outer lobes and lever contact points of the *Vibrio* and *C. jejuni* C-rings became less well-resolved in Δ *motB* mutants, which likely indicates C-ring flexibility when not engaged by stator complexes (Fig. 2f,n). We conclude that *V. fischeri* and *C. jejuni* incorporate wider rings of 13 and 17 stator complexes into their motors, respectively (Fig. 2c,k). The observation that stator complexes in both organisms associate with their respective disk complexes supports the role of disk complexes as scaffolds for wider stator rings relative to the model enteric motors of *Salmonella* and *E. coli*.

Basal disks are homologous

224 Since the fully-assembled *Campylobacter* and *Vibrio* disk complexes play common roles in
225 binding stator complexes for incorporation and orientation into the flagellar motor, we asked
226 whether these structures convergently or divergently evolved by probing whether some disk
227 complex components are homologous. Both *V. fischeri* and *C. jejuni* disk complexes featured
228 outer membrane-associated basal disks. Consistent with our previous work on *Vibrio cholerae*
229 (18), the *V. fischeri* basal disk was ~28nm in radius and attached to the H-ring as previously
230 described in work on partial motors from *Vibrio alginolyticus* (26) (Fig. 2a and 3a). In addition,
231 the *Vibrio* basal disk is formed of concentric circles and appeared to contact the outer
232 membrane at the base of the flagellar sheath, an unusual extension of the outer membrane
233 present in *Vibrio* spp. and some other bacteria (40). In comparison, the *C. jejuni* basal disk
234 attached to the motor via the P-ring and varied in radius between cells, with a mean of 42nm.
235 We did not resolve discrete concentric circles suggesting that the *C. jejuni* disk is an unbounded
236 spiral assembly as noted in close relative *W. succinogenes* (29) (Fig. 2i).

237 A candidate component of the basal disk in both organisms was FlgP, an abundant outer
238 membrane-associated protein required for motility (but not flagellation) that we and others
239 previously identified in *C. jejuni* (41) and *Vibrios* (38, 42) but absent from *Salmonella*. To test
240 whether FlgP forms the basal disks in both organisms, we determined structures of $\Delta flgP$
241 flagellar motors. In *V. fischeri* $\Delta flgP$, motors were very seldom seen, and did not assemble the
242 extracellular hook/filament, making it technically challenging to derive an averaged structure.
243 Nevertheless, we were able to collect sufficient data to determine a subtomogram average
244 structure that unambiguously revealed absence of the basal disk and stator complexes (Fig.
245 3a). Similarly the *C. jejuni* $\Delta flgP$ motor revealed loss of the basal disk (Fig. 4a,b), and became

flexible between the MS- and P-rings, requiring separate averaging of cytoplasmic and periplasmic parts as with *Salmonella*. In *C. jejuni*, the medial and proximal disks were also lost, likely due to requirement of the basal disk for assembly of these lower disks (Fig. 4a,b). Deletion of *flgQ*, a gene adjacent to *flgP* encoding a protein required for FlgP stability and localization to the outer membrane, resulted in a structure indiscernible from the Δ *flgP* motor (41) (Fig. 4a,b). The non-functional *C. jejuni* Δ *flgP* and Δ *flgQ* motors closely resembled the functional *Salmonella* motor with the exception of the wider C-ring. These findings suggest that the disk complex is essential to maintain stator complex interactions with FliG relocated in a wider C-ring. We conclude that basal disks in *V. fischeri* and *C. jejuni* are homologous based on the facts that FlgP is abundant in the outer membrane in both *C. jejuni* and *V. cholera* (41, 42) (Fig. 5a), and exclusively the basal disk was lost in *V. fischeri* Δ *flgP*. Nevertheless, the proteins share just 25% sequence identity, which together with the large-scale differences in the two FlgP basal disks and respective other disk complex structures, suggests an ancient divergence.

The V. fischeri disk complex has evolved to become essential

Previous work identified other components of the *Vibrio* disk complex besides FlgP (26). In *Vibrio* species, additional structures composed of FlgO and FlgT (the H-ring), and MotX and MotY (the T-ring) are present in the flagellar motor (Fig. 1e,h), with the T-ring required for binding stator complexes for assembly into the motor (Fig. 1e,h; Fig. 2a,b; Fig. 3a). In comparing the tomograms of flagellar motors of different *Vibrio* disk and stator mutants, an order of assembly of disk complex biogenesis is suggested (Fig. 3b). We propose that in *Vibrio* spp., a core flagellar motor structurally similar to the *Salmonella* motor assembles first, followed by incorporation of the H- and T-rings on the L- and P-rings, respectively (Fig. 3b). Formation of

268 the H-ring likely provides a platform for assembly of FlgP to form the basal disk, which attaches
269 to the periplasmic face of the outer membrane (Fig. 3b). Because the T-ring contacts the stator
270 complex, but no stators are evident in the motor of the $\Delta flgP$ mutant, we suspect formation of
271 the basal disk may alter H- and T-ring architecture that ultimately influences the ability of the T-
272 ring to interact with stator complexes for incorporation into the motor.

273 *The C. jejuni disk complex is required for incorporation of the stator complexes*

274 To better understand the mechanism of stator scaffolding by the previously uncharacterized *C.*
275 *jejuni* disk complex, we sought to identify its remaining components. We employed transposon
276 (Tn) mutagenesis to identify proteins not present in *Salmonella* that are required for flagellar
277 motility in *C. jejuni* but not for biogenesis of an extracellular flagellar filament (indicating that
278 flagellar biogenesis is disrupted) (Table 1). For this approach, we conducted a three-step
279 screening process with a *C. jejuni* Tn mutant library carrying *astA*-transcriptional reporter to a
280 non-essential flagellar gene (see *Materials and Methods*). We first identified Tn mutants that
281 expressed the transcriptional reporter as an indicator of normal flagellar gene expression. Next,
282 we identified mutants that were non-motile in motility agar. Finally, we collected the non-motile
283 mutants that aggregated in standing broth cultures, a property of flagellated *C. jejuni* strains, to
284 identify non-motile mutants that retained flagellation. This screen identified 117 genes, with the
285 greatest number of Tn insertions in two genes, *pflA* and *pflB* (Table 1). PflA and PflB were
286 previously identified as interacting proteins essential for *C. jejuni* motility, but the reason for their
287 requirement for motility was unknown (43). PflA contains a predicted secretion signal for
288 periplasmic localization, while PflB is a predicted bitopic inner membrane protein with a small N-
289 terminal cytoplasmic domain and a 700-amino acid periplasmic domain. Intriguingly, PflA and

290 PflB contain multiple TPR motifs, which are associated with scaffolding roles in multiprotein
291 complexes (44).

292 We next compared motor structures of *C. jejuni* mutants lacking a specific disk protein with
293 immunoblot analysis, tracking subcellular location of disk proteins to provide insights into the co-
294 dependencies of disk and core flagellar motor proteins. Consistent with our visualization of *C.*
295 *jejuni* FlgP forming a basal disk attached to the P-ring and outer membrane, FlgP stability and
296 outer membrane-association was dependent on most core motor components. In type III
297 secretion system (e.g., *fliP*, *flhA*) and proximal rod (*fliE*, *flgB*, *flgC*) mutants, FlgP was generally
298 absent or present at only low levels in lysates (Fig. 5a). In the cytoplasm of some of these
299 mutants, we detected a low level of FlgP in the cytoplasm; however, FlgP was not detected in
300 the cytoplasm of WT *C. jejuni*, presumably due to efficient targeting of the protein to the outer
301 membrane (Fig. 5a). In P-ring (*flgI*), L-ring (*flgH*), or distal rod (*flgF*, *flgG*) mutants that do not
302 assemble L- or P-rings, FlgP was stable and accumulated in the cytoplasm and inner
303 membrane, but did not fully localize to the outer membrane (Fig. 5a). FlgP localization was
304 unaffected by deletion of *pflA* or *pflB* (Fig. 5b), although these mutants were non-motile.

305 To determine whether PflA and PflB formed part of the *C. jejuni* disk complex, we determined
306 motor structures for each mutant using electron cryo-tomography and subtomogram averaging
307 (Fig. 4a). Each motor exhibited flexibility, necessitating merging separate periplasmic and
308 cytoplasmic structures. As reported above, the *C. jejuni* Δ *motB* motor assembled a partial
309 proximal disk due to loss of MotB (Figs. 2m,n; 4a). In the *C. jejuni* Δ *pflB* mutant, the entire
310 proximal disk was lost, including MotB (Fig. 4a). Further supporting evidence that PflB could
311 form the proximal disk that appears to contact the inner membrane towards its center was our

312 fractionation of PflB with inner membrane proteins in *C. jejuni* (Fig. 5b). These results suggest
313 that PflB forms a majority of the proximal disk and functions to incorporate stator complexes into
314 the peripheral region of the proximal disk in the *C. jejuni* motor, which accounts for the
315 requirement of PflB for motility.

316 We next compared the structures of the *C. jejuni* $\Delta pflB$ and $\Delta pflA$ motors. The only difference
317 between these motors was the absence of the inner lobe of the medial disk associated with the
318 periplasm in $\Delta pflA$. Consistent with this finding, we were able to localize PflA to the periplasm of
319 *C. jejuni* (Fig.5b). A previous study detected interactions between PflA and PflB in *C. jejuni*
320 (43). We were also able to identify a co-dependence in stability between PflA and PflB. Although
321 PflB was not required for WT levels of PflA stability or localization to the periplasm, PflA was
322 required for full stability and inner membrane localization of PflB (Fig. 5b). These data suggest
323 that PflA polymerization into a part (or all) of the medial disk is required to form a platform for
324 PflB to form the proximal disk to subsequently incorporate stator complexes. This is reflected by
325 loss of both disks in the $\Delta pflA$ motor. Of note, we observed a disordered vestige of the medial
326 disk beneath the basal disk in both $\Delta pflA$ and $\Delta pflB$ motors (Fig. 4a). Currently, the identification
327 of this density is unknown. This density could be FlgQ that assists FlgP assembly into the basal
328 disk, an as-yet unidentified protein that forms part of the medial disk with PflA, or peptidoglycan
329 that becomes ordered upon formation of the FlgP basal disk. If this component is peptidoglycan,
330 it could be bound by MotB upon incorporation of stator complexes into the proximal disk and the
331 flagellar motor. Relevant areas of the *V. fischeri* and *C. jejuni* MotB proteins conform to the
332 peptidoglycan-binding consensus sequence.

Intriguingly, the stability of the MotA and MotB proteins of the stator complex did not appear to be affected by mutation of any disk component (Fig. 5c) and localized to the inner membrane in each mutant. These findings further support the function of the disk complex is to incorporate stator complexes into the flagellar motor, and are not simply required for stability or localization of stator complexes into the inner membrane.

Discussion

In this work, we sought to determine whether the generation of high torque by flagellar motors of specific bacterial species could be functionally attributed to the unique disk complexes associated with their respective motors. For this approach, we analyzed the flagellar motors of two bacterial species, *V. fischeri* and *C. jejuni*, with a combination of genetic and biochemical analysis of bacterial mutants and electron cryo-tomographic imaging, and compared them to the enteric motor assembled in *Salmonella*. The results of our studies indicate that the disk complexes of these two organisms, while displaying structural diversity and composed of both homologous and unrelated proteins, share a common role in forming a platform for the integration of different numbers of stator complexes into the flagellar motor. Furthermore, these disk complexes not only integrate more stator complexes into a motor, but also place them at wider radii from the center of the motor axis of rotation relative to the enteric motor. As discussed in detail below, we propose that this creation of a wider stator ring with an increased number of stator complexes will inevitably result in increased torque in these motors, which directly affects the swimming ability of the respective bacteria with higher swimming velocities and migration through environments with increased viscosity.

A proposed biogenesis order for assembly of the C. jejuni disk complex

355 Although parts of the purified *Vibrio* motor have previously been studied, no components of the
356 *C. jejuni* disk complex had previously been identified. Identifying components of the *C. jejuni*
357 motor and examining the structures of mutants of these components enable us to propose an
358 assembly pathway for biogenesis of a functional *C. jejuni* flagellar motor (Fig. 4b). This
359 biogenesis pathway is likely divided into two stages. The first stage is formation of a non-
360 functional *C. jejuni* core flagellar motor that resembles the (functional) *Salmonella* motor, as
361 evidenced by the non-functional $\Delta flgP$ or $\Delta flgQ$ *C. jejuni* motors that fail to incorporate any disk
362 complex components (Fig. 4a). The second stage involves the sequential assembly of each disk
363 in the disk complex, ultimately enabling correct incorporation of the stator complexes to produce
364 a functional motor. Consistent with our findings that FlgP requires core flagellar components for
365 outer membrane association, we suspect that FlgP polymerizes into the basal disk (with
366 assistance from FlgQ) only after the core flagellar motor forms to provide an attachment site for
367 the basal disk (possibly at the P- and/or L-ring). Besides FlgQ, no other disk complex proteins
368 are required for FlgP to localize to the outer membrane or form the basal disk (Fig. 4a, 4b, 5a,
369 and 5b). Formation of the basal disk provides a platform and site of interaction with PflA to form
370 the inner node of the medial disk (Fig. 4a,b). PflA assembly as the medial disk subsequently
371 forms an assembly platform for PflB to assemble into the proximal disk, together with the stator
372 complexes (Fig. 4a,b). The disk complex therefore forms a separate and previously
373 unappreciated additional biogenesis step in ϵ -proteobacteria that occurs after the conserved
374 flagellar assembly pathway of the flagellar type III secretory system, rotor, switch, rod, hook,
375 and filament.

376 *FlgP-based disk complexes are widespread*

377 Because *C. jejuni* and *V. fischeri* FlgP proteins share low sequence identity, FlgP may represent
378 an ancient recruitment to the ancestral motor, followed by later independent evolutionary
379 recruitment of the remaining *Vibrio* or ϵ -proteobacterial disk proteins to create motors with disk
380 complexes which function to incorporate additional stator units into the motor. The speculation
381 that FlgP recruitment was the first step in the evolution of disk complexes is further supported by
382 the morphological divergence and mutually exclusive sets of additional accessory proteins
383 between the two structural variants. Widespread presence of FlgP and other *C. jejuni* disk
384 complex proteins FlgQ, PflA, and PflB across the ϵ -proteobacteria, together with previous
385 observations of flagellar motors incorporating disk complexes (18, 29), confirm that other ϵ -
386 proteobacteria such as *Helicobacter* and *Wolinella* genera also assemble *C. jejuni*-type
387 homologous disk complexes. FlgP is also widespread amongst many γ -proteobacterial genera
388 including *Vibrio*, *Shewanella* and *Rhodobacter*, but in these genera, components of the *Vibrio*
389 disk complex MotX, MotY, FlgO and FlgT are encoded, consistent with assembly of *Vibrio*-type
390 disk complex homologs (45, 46).

391 Intriguingly, however, the widespread FlgP basal disks do not directly interact with the stator
392 complexes in either of the *Vibrio* and ϵ -proteobacterial disk complex variants. In *V. fischeri*, FlgP
393 connects the T-ring to the outer membrane while components of the H-ring interact with the
394 stator complexes. In the absence of FlgP and the basal disk, the T- and H-rings are produced,
395 but we suspect that their architecture may be altered so that these rings cannot interact with a
396 stator complex. In *C. jejuni*, the FlgP basal disk is required to assemble the remainder of the
397 disk complex, with the proximal disk responsible for incorporation of stator complexes.
398 Nevertheless, the common roles in ultimately creating scaffolds for stator complex incorporation

suggests that their common ancestor had the same stator complex scaffolding function, a function that was retained during divergence of the two forms of disk complexes by incorporation of the additional proteins. Alternatively the two disk complex lineages may have independently evolved roles to scaffold additional stator complexes. We are now pursuing a follow-up study to understand the phylogeny and probe the origins of these disk complexes.

Interactions of stator complexes with flagellar motors vary in motile bacterial species

The interactions of stator complexes with the disk complexes of the *Vibrio* and *Campylobacter* motors are different to those of stator complex interactions with the enteric motor. In the *Salmonella* enteric motor, the periplasmic region of MotB in a stator complex contacts the P-ring to anchor to the flagellar structure, in addition to contacting the peptidoglycan to tether the motor to the cell wall (13). The *Vibrio* stator complex also interacts with peptidoglycan, but additional interactions with the T-ring allows for incorporating the stators at a wider position in the flagellar motor compared to *Salmonella*. Currently, it is unknown if the *C. jejuni* stator complexes also interact with peptidoglycan, although the core OmpA peptidoglycan-binding domain motif is intact (47) and MotB from closely-related *H. pylori* has been co-crystallized with a bound glycan strand (48). Our work suggests that MotB must also interact with PflB, since these two proteins co-localize in subtomogram averages, and deletion of *pflB* results in failure of MotB to incorporate into the motor, further supporting our conclusion that the disk complex functions to scaffold stator complexes.

We did not observe stator complex densities in the *Salmonella* motor despite evidence that *Salmonella* minicells swim (31), consistent with previous studies failing to visualize *Salmonella* stator complexes (18, 31). Because *Salmonella* stator complexes are dynamic (49) we suspect

that under our low load conditions few are engaged and will thus be difficult to resolve without increasing stator complex occupancy, larger datasets, and classification approaches. Comparing the *Salmonella* motor to *V. fischeri* and *C. jejuni* further suggests that *Salmonella* incorporates no more than 11 stator complexes, as a stator ring immediately around the rod would be too narrow to incorporate more. This has been previously noted in a model of stator complex incorporation into the *V. alginolyticus* motor – when not taking into account the additional stator radius provided by the scaffolding role of the T-ring (16).

A structural rationale that quantitatively accounts for higher torque generation

Piecing together this work, and interpreting previous work (18, 19), we see a compelling correlation between the torque of a flagellar motor and its architecture. In addition to the results reported here, the ~4000 pN nm-torque spirochaete flagellar motor has 16 proposed stator complex densities (17, 19) in a 30-nm radius ring (although it is curious that the spirochaetes have convergently evolved an alternative stator scaffold, the P-collar, which shares no homologous components with the *Vibrio* or ϵ -proteobacterial disk complexes).

Can this data quantitatively predict observed motor torques? Given the torque of a single stator complex in enteric bacteria is 146 pN nm (14), our data, consistent with others, show that the lever contact point at which MotA contacts the outer lobe of FliG to apply force is approximately 20 nm from the motor axis of rotation (31, 33), leading to the estimation of the force exerted by a single stator complex as approximately 7.3 pN. There is good evidence to make the assumption that stator complexes exert a constant force in all bacteria, due to that fact that proton motive forces across bacteria are consistently reported between -100 and -200 mV (50), although it should be noted that sodium-driven motors may be powered by a higher sodium motive force

443 than proton motive force. While sodium motive force increases with increasing external sodium
444 ion concentration, it plateaus at around 250 mM external sodium concentration with a maximum
445 sodium motive force not exceeding -200 mV (25, 51). From this, we can predict the torque of a
446 motor, given the radius of stator complexes around the axis of rotation, and the number of stator
447 complexes, by making the approximation that torque from multiple stator complexes is roughly
448 additive (14). We have sufficient structural data to make this prediction for four groups of
449 organisms (Fig. 6a): enteric bacteria such as *Salmonella* (11 stator complexes exerting force at
450 a radius of 20 nm); *Vibrio* spp. (13 stator complexes exerting force at a lever contact point of
451 21.5 nm); ϵ -proteobacteria (17 stator complexes exerting force at a lever contact point of 26.5
452 nm); and spirochaetes (16 stator complexes exerting force at a lever contact point of 30.5 nm).
453 Multiplying stator complex count, lever contact point radius, and estimated 7.3 pN force exerted
454 per stator complex, we observe excellent correlation between our predicted and the observed
455 torque magnitudes for all (Fig. 6b): predicted torque of 1606 pN nm for enteric bacteria
456 (observed of 1260 pN nm torque (14), with some estimates of 2000 pN nm (20)); 2040 pN nm
457 for *Vibrio* (observed of 2200 pN nm at low sodium concentrations, increasing with higher
458 concentrations), 3288 pN nm for ϵ -proteobacteria (observed of 3600 pN nm); and 3562 pN nm
459 for spirochaetes (observed of 4000 pN nm). Although this is clearly a simplification of the
460 process, and biophysical studies reveal that additional stator complexes provide incrementally
461 smaller torque contributions (14), we believe the salient features of our model's predictions are
462 compelling. Another possibility is that ϵ -proteobacteria assemble more stator complexes at
463 wider radii to accommodate a lower proton motive force. The proton motive force of *H. pylori*,
464 however, has been reported as a relatively high value of -220 mV (52). Alternatively, additional
465 stator complexes may act as more sensitive mechanosensors.

466 In all organisms, C-ring radius tends to scale with both stator ring radius (as would be expected
467 to maintain MotA:FliG_C interaction), and motor torque. Curiously, however, while this correlation
468 is clear, it is not tightly constrained. For example, while ϵ -proteobacteria and spirochaetes have
469 wide stator- and C-rings, the spirochaete C-ring is wider than in ϵ -proteobacteria, while
470 conversely the ϵ -proteobacterial stator ring is wider than in spirochaetes. The observation that
471 these variables scale together, but without strict correlation, provides a mechanistic insight: no
472 strict stoichiometric or symmetric correspondence is necessary for motor function. This also
473 suggests a clear 'reducibly complex' evolutionary pathway to evolve higher-torque motors, as
474 the increase in radius of C-ring and stator rings can be progressively and asynchronously
475 staggered over evolutionary time through addition of spacers while maintaining a functional
476 motor.

477 Together these results indicate that different bacteria have modified their motors to produce
478 torques suited to their environments. *C. crescentus* has evolved a low-torque, efficient motor
479 suited to its low nutrient, low-viscosity freshwater habitat, while the ϵ -proteobacteria and
480 spirochaetes have evolved high-torque motors that require high energy expenditure to bore
481 through viscous mucous and tissue. Such modification of the mechanical output of a molecular
482 machine is not without precedent, as similar observations have been shown in the unrelated F-
483 ATPase rotor ring (53).

484 Our observations propose a mechanistic model for how different bacterial flagellar motors
485 produce different torques. By assembling wider stator rings using a proteinaceous scaffold,
486 individual stator complexes will exert greater leverage on the axial driveshaft of the motor,
487 producing greater torque. Additionally, more stator complexes can be accommodated around

the motor, further increasing torque. Besides describing the evolution of higher torque, this study also illustrates a mechanism for adapting mechanical output that might be capitalized upon in future synthetic repurposing of molecular machinery.

Materials and Methods

Construction of *C. jejuni* mutants. All strains and plasmids used are listed in Supporting Tables 1 and 2, respectively. *C. jejuni* mutants were constructed by electroporation as previously described (54). For removal from the *C. jejuni* 81-176 Sm^R (DRH212) chromosome, genes were first amplified by PCR using primers containing 5' BamHI restriction sites. Each amplified fragment contained the gene of interest with approximately 750 flanking regions. Cloning of fragments into pUC19 resulted in plasmids pLKB652 (pUC19::*pflB*), pDRH2526 (pUC19::*pflA*), pDRH2312 (pUC19::*motAB*). A *Sma*I-digested *cat-rpsL* cassette was ligated into the *Sma*I site of *pflB* in pLKB652 to create pLKB658, the *EcoRV* site of *pflA* in pDRH2526 to create pDRH2352, and the *Spe*I site of *motA* in pDRH2312 to create pDRH3330. To insert the *cat-rpsL* cassette in *motB*, site-directed mutagenesis was used to create an *EcoRV* site within *motB* to form pDRH2324 followed by insertion of *cat-rpsL* to generate pDRH3331.

Plasmids were electroporated into *C. jejuni* 81-176 Sm^R (DRH212) to inactivate each respective gene on the chromosome by insertion of the *cat-rpsL* cassette. In addition, the previously constructed plasmids pDRH2534, pDRH2536, and pALU101 were used to interrupt *flgF*, *flgI*, and *flgH* with a *cat-rpsL* cassette on the *C. jejuni* 81-176 Sm^R (DRH212) chromosome. Transformants were recovered on MH agar containing chloramphenicol and mutants were verified by colony PCR to recover the following isogenic mutants ABT337 (*pflB*::*cat-rpsL*),

509 ALU103 (*flgH::cat-rpsL*), DAR866 (*pflA::cat-rpsL*), DRH2550 (*flgF::cat-rpsL*), DRH2553 (*flgI::cat-*
510 *rpsL*), MB1225 (*motA::cat-rpsL*), and MB1226 (*motB::cat-rpsL*).

511 In-frame deletions of genes were created with specific primers using PCR-mediated
512 mutagenesis or SOEing mutagenesis (55, 56). Resulting plasmids were verified by sequencing
513 to form pABT324 (pUC19:: $\Delta pflB$), pDRH2745 (pUC19:: $\Delta pflA$), pDAR1012 (pUC19:: $\Delta motA$), and
514 pDAR1013 (pUC19:: $\Delta motB$). Deletions of genes were achieved by electroporating
515 corresponding plasmids into strains containing genes interrupted by insertion of the *cat-rpsL*
516 cassette. Transformants were recovered on MH agar containing 0.5, 1, or 2 mg/ml streptomycin
517 and then screened for chloramphenicol sensitivity. Deletions were verified by PCR, which
518 resulted in the creation of isogenic 81-176 Sm^R mutants containing deletions: $\Delta pflB$ (DAR981),
519 $\Delta pflA$ (DAR1124), $\Delta motA$ (DAR1131), $\Delta motB$ (DAR1066), $\Delta flgH$ (DRH2449), and $\Delta flgF$
520 (SNJ922).

521 **Bacterial growth.** *Salmonella* strain TH16943 (*Para-ftsZ*) was grown overnight in LB broth from
522 freezer stocks at 37°C shaking at 225rpm. Overnight culture and 5% L-arabinose were added
523 (50µl each) to 5ml fresh LB broth and incubated for an additional 5 h. To enrich for minicells 3ml
524 of this culture were spun at 6000rpm for five minutes and the supernatant recovered. The
525 supernatant was spin-concentrated by centrifugation at 18000rpm for 5 minutes and the
526 resultant pellet resuspended in approximately 50 µl LB broth and immediately plunge-frozen. *V.*
527 *fischeri* was grown overnight at 28°C shaking at 225rpm in LBS with 35mM MgSO₄. Cultures
528 were reinoculated into fresh LBS and harvested in early log phase and spin-concentrated to
529 OD₆₀₀ ~ 20 and immediately plunge-frozen. *C. jejuni* were grown for 48 to 60 hours on MH
530 agar under microaerobic conditions using CampyPak sachets (Oxoid) at 37°C. Cultures were

531 generously restreaked and returned to incubate for a further 16 hours. Bacteria were harvested
532 by resuspension into approximately 1ml MH broth to OD600 ~ 10.0 and immediately plunge-
533 frozen.

534 **EM sample preparation, data collection and tomogram reconstruction.** 200 mesh Quantifoil
535 R2/2 grids were glow-discharged for 60s at 10mA. A solution of 10-nm colloidal gold was added
536 to cells immediately before plunge freezing. A 3 μ L droplet of the sample solution was applied to
537 the glow-discharged EM grid, then blotted and plunge-frozen into liquid ethane-propane mixture
538 using a Vitrobot plunge-freezing robot (FEI Company) using 60 to 180s wait time, 5 to 10s blot
539 time, and blot offsets between -2 and -5. Grids were stored under liquid nitrogen until data
540 collection.

541 Tilt series were collected on a 200kV FEI Tecnai TF20 FEG transmission electron microscope
542 (FEI Company) equipped with a Falcon II direct electron detector camera (FEI Company) using
543 Gatan 914 or 626 cryo-holders. Tilt series were recorded from -61° to +61° with an increment of
544 3° collected defocus between -4 μ m and -10 μ m using Leginon automated data collection
545 software (57) at nominal magnification of 50000x, and binned 4x to final pixel size of 0.81nm.
546 Cumulative doses of approximately to 120 e⁻/Å² were used. Overnight data collection was
547 facilitated by addition of a 3L cold trap dewar and automated refilling of the cryo-holder dewar
548 triggered by a custom-written Leginon node interfaced with a computer-controlled liquid nitrogen
549 pump (Norhof LN2 systems, Maarssen, Netherlands).

550 Tomograms were reconstructed automatically using RAPTOR (58) and the IMOD package (59).
551 Low-defocus images were low-pass filtered to remove data beyond 3.5 nm⁻¹. High-defocus
552 datasets were CTF-corrected using TomoCTF (60).

Sub-tomogram extraction, alignment and averaging. Positions of flagellar motors in tomograms were initially aligned manually along their rotational axes. Datasets were halved and the PEET package (61) was used for iterative sub-tomogram extraction, fine alignment and averaging of the two halves independently. Resolution was estimated by 'Gold Standard FSC' by correlating the two halves of the dataset using Fourier shell correlation (FSC) (Supporting Fig. 1). *Salmonella* motors and *C. jejuni* $\Delta pflA$, $\Delta pflB$, $\Delta flgP$ and $\Delta flgQ$ exhibited flexibility between top and bottom parts of the motor. In these cases individual averages were constructed for top and bottom segments. These were ultimately merged into a composite structure in analogy to recent single particle studies (62).

Transposon mutagenesis and screening of *C. jejuni* 81-176. To identify potential disk mutants of *C. jejuni* 81-176, we conducted a three-step transposon mutagenesis screening procedure to identify flagellated but non-motile mutants. *C. jejuni* 81-176 $Sm^R \Delta astA flaB::astA$ chromosomal DNA was used in *in vitro* transposition reactions with the *darkhelmet* Tn as described previously (54, 63, 64). Tn mutants were recovered on MH agar containing chloramphenicol, kanamycin, and 35 μ g/ml 5-bromo-4-chloro-3-indolyl sulfate. Approximately 8000 colonies displaying a blue phenotype indicating an intact transcriptional pathway for expression of flagellar genes were stabbed in 0.4% MH motility agar and incubated for 24 h in microaerobic conditions. Mutants with impaired motility were recovered and grown for an additional 48 h. Mutants were then restreaked and grown for another 16 h and resuspended to an OD_{600} of 0.8 in MH broth in 15 ml conical tubes. After at least 1 h incubation at room temperature, mutants that aggregated as determined by visual inspection of tubes (indicating

574 production of flagella) were saved to identify the location of the transposon insertion by direct
575 DNA sequencing with a primer annealing to the end of the Tn.

576 **Antisera generation.** Primers were designed to amplify the coding sequence of *motA* from
577 codon 2 through the stop codon with in-frame BamHI and XhoI restriction sites added to the 5'
578 ends of the primers. PCR-amplified *motA* was ligated into pET21a digested with BamHI and
579 XhoI, which allowed for the expression of MotA with a C-terminal 6XHis-tag in pDAR1180.

580 Primers were designed to amplify the coding sequence of *motB* from codon 2 through the
581 penultimate codon with in-frame NdeI and PstI restriction sites added to the 5' end of the
582 primers. In addition, one primer contained codons for a C-terminal 6XHis-tag to be added to the
583 penultimate codon on *motB*. PCR-amplified *motB* was ligated into pT7-7 digested with NdeI and
584 PstI, which allowed for the expression of MotB-6XHis and created pDAR906.

585 Primers were designed to amplify the coding sequence of *pflB* from codons for V103 to L304
586 with in frame BamHI and PstI sites added to the 5' end of the primers. Additionally, one primer
587 contained codons for a C-terminal 6XHis-tag to be added after codon 304. After digestion, the
588 PCR-amplified *pflB* fragment was ligated into BamHI- and PstI-digested pMalC2X allowing for
589 expression of a fragment of PflB_{V103-L304} fused to MBP with a C-terminal 6XHis-tag to create
590 pDAR2226.

591 Primers were designed to amplify the coding sequence of *pflA* from codon 2 to the stop codon
592 with in frame BamHI and PstI sites added to the 5' end of the primers. In addition one primer
593 also contained codons for a C-terminal 6XHis tag to be added to the last codon. After digestion,

594 PCR-amplified *pflA* was ligated into BamHI- and PstI-digested pMalP2X, which allowed for
595 expression of PflA fused to MBP with a C-terminal 6XHis tag to create pDAR2111.

596 For induction of MotA-6XHis, MotB-6XHis, PflB_{V103-L304}-MBP-6XHis, and PflA-MBP-6XHis, the
597 respective plasmids were transformed into BL21 (DE3). Bacteria were then grown in 500 ml LB
598 to an OD₆₀₀ of 0.4. For induction, IPTG was added to a final concentration of 1 mM and the
599 cultures were incubated for an additional 3h.

600 Bacteria expressing recombinant proteins were harvested by centrifugation and lysed with an
601 Emulsiflex C5 cell disrupter (Avesin). Lysis buffer for purification of MotB-6XHis contained 0.5
602 M sucrose and 0.2% Zwittergen. Lysis buffer for MotA-6XHis purification contained 0.5 M
603 sucrose, 0.2% Zwittergen and 8 M urea. PflA-MBP-6XHis and PflB_{V103-L304}-MBP-6XHis proteins
604 were purified in denaturing conditions with 8 M urea. All proteins were purified by Ni-NTA
605 chromatography. Purified proteins were used to immunize mice or guinea pigs for generation of
606 polyclonal antisera.

607 **Fractionation of *C. jejuni* strains and analysis of proteins.** Recovery of whole-cell lysates
608 and fraction of *C. jejuni* strains into subcellular compartments for analysis of localization of
609 proteins from the inner membrane, outer membrane, periplasm, and cytoplasm were performed
610 as previously described (65). Proteins from each fraction representing equivalent cell numbers
611 (approximately 200 µl of a *C. jejuni* culture at an OD₆₀₀ 0.8) were loaded onto 10% SDS-PAGE
612 gels and then transferred to membranes for immunoblot analysis.

613 For immunoblotting analyses, the following primary antisera were used at the following dilutions:
614 FlgP M4 or M5 (1:2000 (41)), MotA M219 (1:500), MotB M195 (1:500), PflA M231 (1:500), and
615 PflB GP143 (1:500). Secondary antisera were used at a dilution of 1:15,000.

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625 **Supplementary information**

626 There are two Supporting figures and two Supporting tables associated with this manuscript.

627 **Author contributions**

628 MB designed and performed electron cryo-tomography and data analysis. DAR designed and
629 created *C. jejuni* mutants and conducted immunoblotting studies. CAB designed and created *V.*
630 *fischeri* mutants with input from EGR. MB, GJJ and DRH conceived the project. MB and DRH
631 interpreted results and wrote the paper with input from other authors.

632 **Author information**

633 The authors declare no competing financial interests. All structures have been deposited to the
634 Electron Microscopy Data Bank with accession codes: *Salmonella* WT: EMD-3154, *V. fischeri*
635 WT: EMD-3155, *C. jejuni* WT: EMD-3150, *V. fischeri* Δ *motB*: EMD-3156, *C. jejuni* Δ *motB*: EMD-
636 3157, *C. jejuni* Δ *flgQ*: EMD-3158, *C. jejuni* Δ *flgP*: EMD-3159, *C. jejuni* Δ *pflA*: EMD-3160, *C.*
637 *jejuni* Δ *pflB*: EMD-3161, *V. fischeri* Δ *flgP*: EMD-3162. Correspondence should be addressed to
638 MB (mbeeby@imperial.ac.uk) or DRH (David.Hendrixson@UTSouthwestern.edu).

639

Figure legends

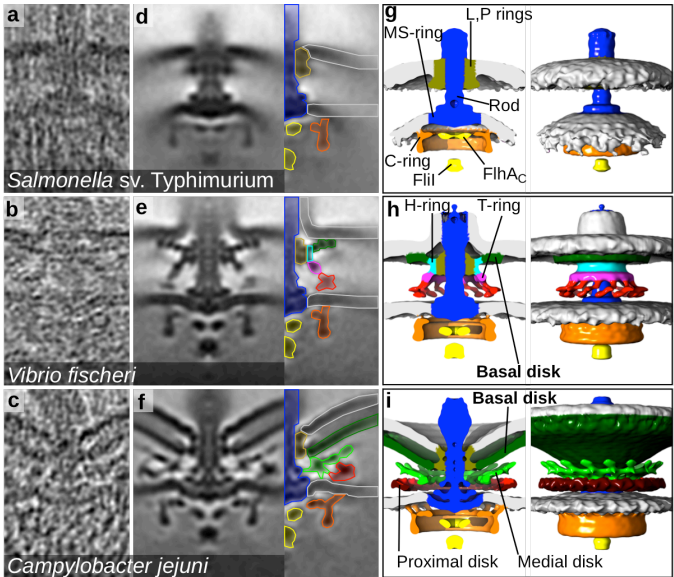


Figure 1: High-torque bacterial flagellar motors assemble large periplasmic disk complexes. Tomographic slices through intact cells of (a) *Salmonella*, (b) *V. fischeri* and (c) *C. jejuni* showing individual flagellar motors. Height of all image panels is 100nm. (d-f): 100nm x 100nm 0.81 nm-thick slices through subtomogram averages of hundreds of motors, with colour key to named regions of the motor, of (d) *Salmonella* (average of 286 motors), (e) *V. fischeri* (average of 302 motors) and (f) *C. jejuni* (average of 156 motors) (g-i): isosurface renderings of motors shown in (d-f). Colours correspond to colours used in colour key of (d-f). Flil and FlhA_C are components of the flagellar type III secretion system.

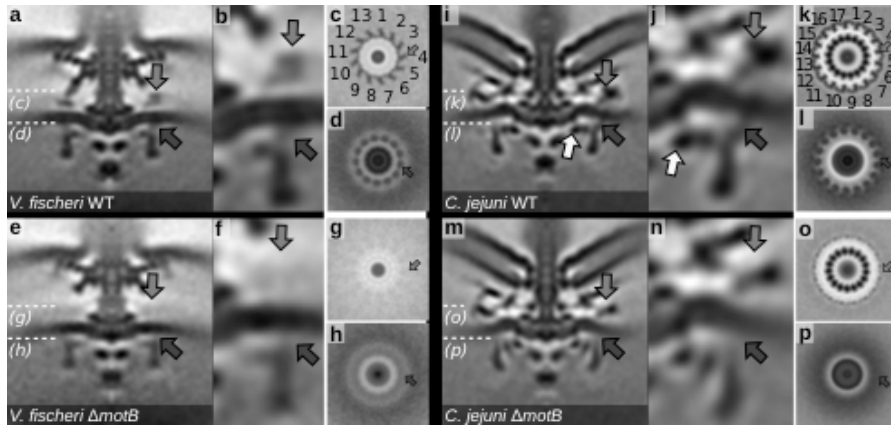


Figure 2: Disk complexes scaffold alternative stator complex location and number. (a)

WT *Vibrio fischeri* motor highlighting putative MotB (red arrow) and putative FliG_C:MotA interface (blue arrow). (b) close-up of putative MotB density and FliG_C:MotA interface (c,d) 13-fold symmetry through stator and C-ring planes as labelled with white dotted lines in (a). (e-h): equivalent images as (a-d) except for *V. fischeri* Δ motB, confirming identity of proposed stator complexes. (i-l): equivalent images for WT *C. jejuni* and (m-p) for *C. jejuni* Δ motB. Note 17 stator complex densities around observed in *C. jejuni*. Yellow arrow in (i,j) highlights an additional density on inner lobe of *C. jejuni* C-ring compared to *Salmonella* and *V. fischeri*. Note that the odd-numbered stator counts results in asymmetric appearance of motor cross-sections.

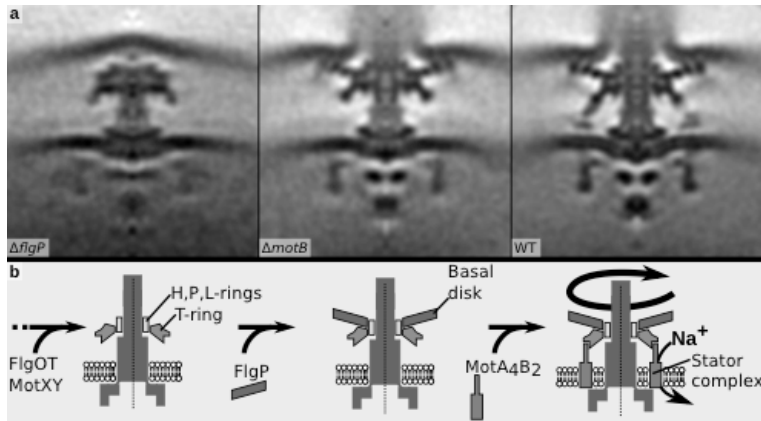


Figure 3: Assembly of the stator complexes in *V. fischeri* requires FlgP. (a) *V. fischeri* flagellar motors from $\Delta flgP$, $\Delta motB$ and WT strains (left to right). (b) Model of assembly of the *V. fischeri* disk complex. Early stage disk complex components MotXY and FlgOT assemble prior to FlgP incorporation, followed by assembly of the stator complex ring. Images are 100nm x 100nm 0.81 nm-thick slices through subtomogram averages.

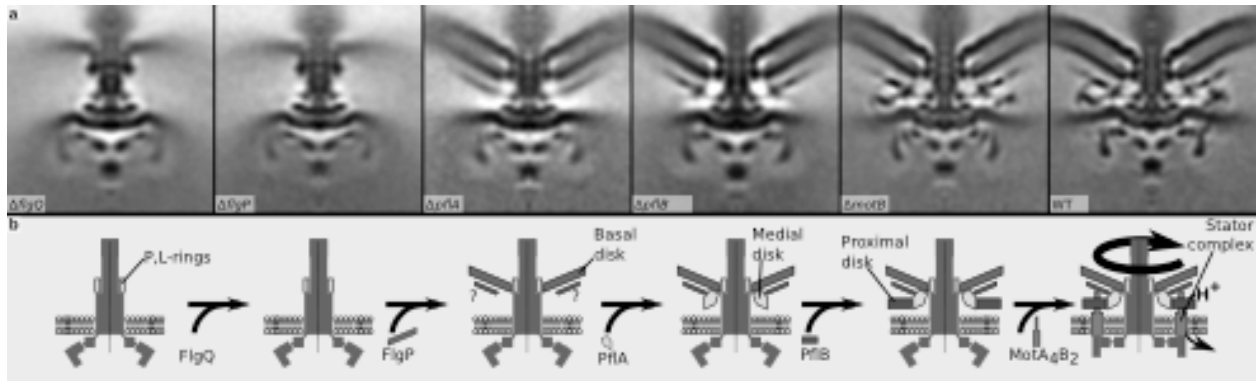


Figure 4: Protein location within the *C. jejuni* disk complex, and hierarchical assembly pathway revealed by subtomogram averaging of WT and mutant motors. (a) Subtomogram averages arranged to illustrate the hierarchical assembly dependencies of the *C. jejuni* disk complex. Note that the outer membrane is poorly resolved in $\Delta flgQ$ and $\Delta flgP$ mutants because without the basal disk as an anchor the membrane is in a different position in each component subtomogram (b) Model of the hierarchical assembly of the *C. jejuni* disk complex. Images are 100nm x 100nm 0.81 nm-thick slices through subtomogram averages.

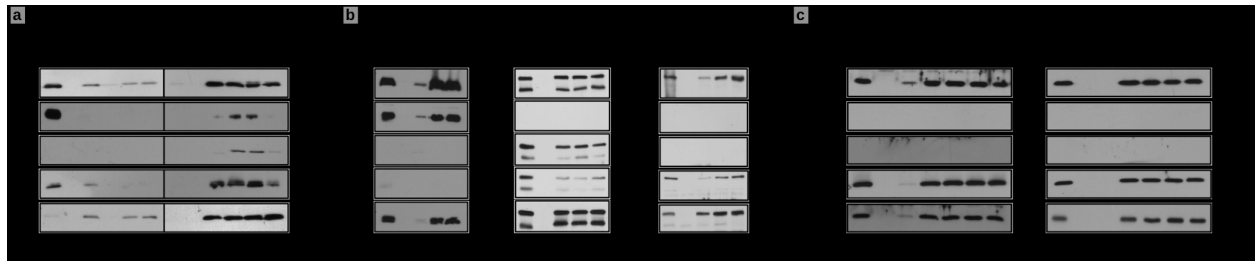


Figure 5: Localization interdependencies of four components of the *C. jejuni* disk complex (a-c) Immunoblot analysis of localization of FlgP, PflA, PflB, MotA, and MotB in various WT *C. jejuni* and mutant strains. (a) FlgP subcellular localization in flagellar T3SS, rod, and ring mutants. (b) Subcellular localization of FlgP, PflA, and PflB in specific flagellar disk mutants. (c) Localization of MotA and MotB stator proteins in flagellar disk complex mutants. For (a-c), all proteins were detected with specific antiserum. WCL: whole-cell lysate, OM: outer membrane, Peri: periplasm, IM: inner membrane, Cyt: cytoplasm. For all immunoblots, equivalent amounts of cell cultures were lysed and subcellular compartments fractionated.

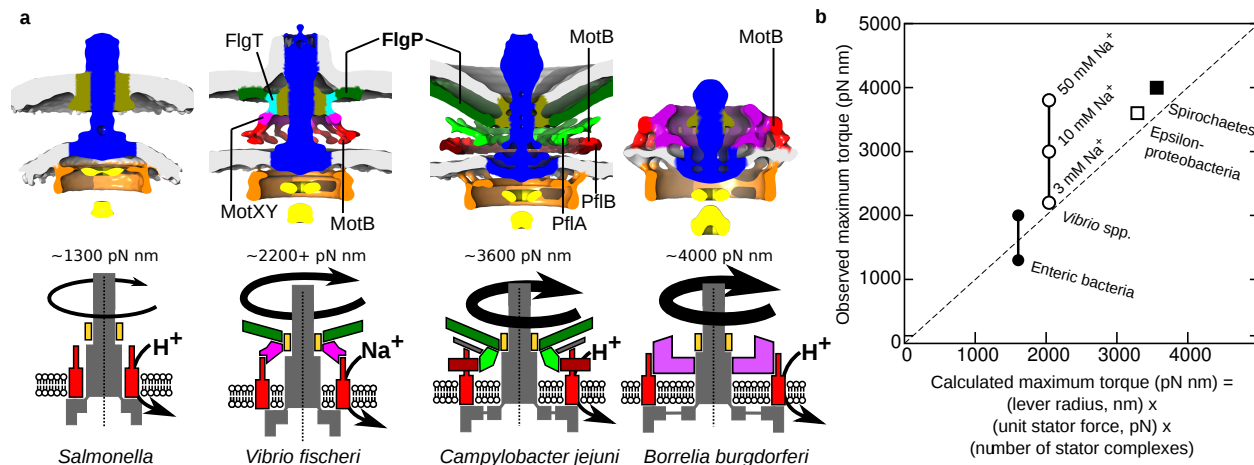


Figure 6: Wider stators featuring more stator complexes quantitatively accounts for torque diversity. (a) Disk complexes scaffold stators to increase number of stators, and radius of the stator complex ring. Stator number and increasing wider C-ring in each flagellar motor directly correlate with torque produced by each motor. (b) Comparison of predicted and observed torques of flagellar motors from various species. Filled circles: enteric bacteria; open circles: *Vibrio* spp.; open squares: ϵ -proteobacteria; filled squares: spirochaetes. Where no direct torque measurement *C. jejuni* and *B. burgdorferi* torques are inferred from closely-related species with similar motors (*H. pylori* and *Leptospira*, respectively). Relative torque strengths are validated by different speeds and abilities of different bacteria to swim through viscous fluids (1, 4, 7). Dotted line represents perfect prediction.

	Gene	Number of independent transposon mutants isolated in this gene.	Verified or proposed function
Flagellar motor protein	<i>motA</i>	9	Motor stator protein
	<i>motB</i>	2	Motor stator protein
	<i>fliM</i>	6	Motor switch protein
	<i>fliY</i>	1	Motor switch protein

Chemotaxis system	<i>cheA</i>	1	Chemosensory histidine kinase
	<i>cheY</i>	2	Chemosensory response regulator
Flagellar structural components	<i>flaA</i>	1	Major flagellin
	<i>flab</i>	1	Minor flagellin
	<i>flag</i>	1	Flagellin-like protein
	<i>flgH</i>	2	L-ring protein
	<i>fliW</i>	1	Putative flagellar chaperone
	<i>flgN</i>	1	Flagellar protein chaperone
	<i>pseE</i>	3	Pseudoamino acid biosynthetic protein; flagellin glycosylation
Flagellar gene or	<i>fliA</i>	6	σ^{28}

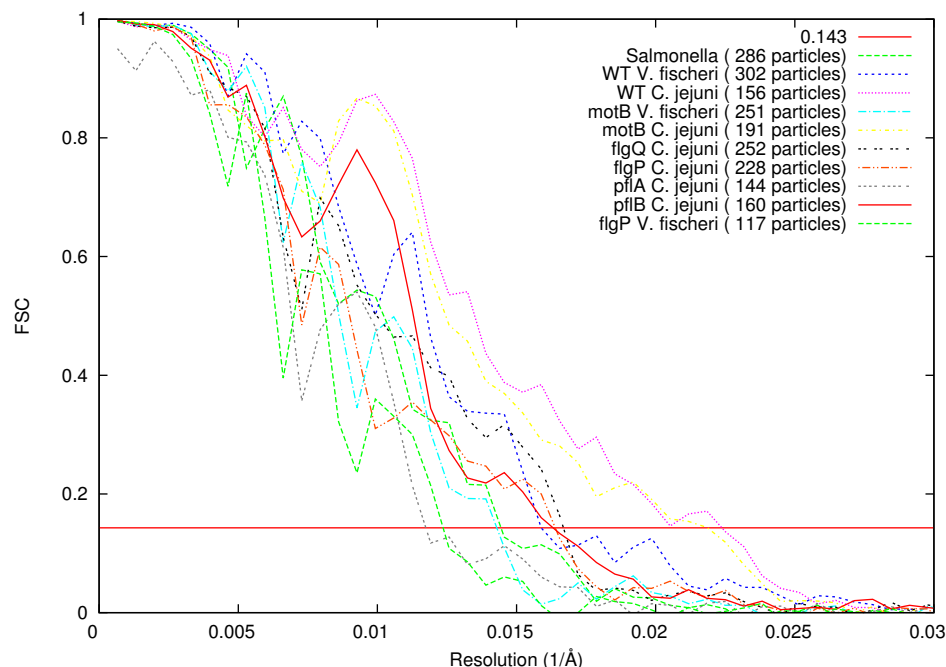
biosynthesis regulators			
	<i>flgM</i>	1	Anti- σ factor for σ^{28}
	<i>flhF</i>	1	Flagellar biosynthesis GTPase
Motility determinants (unknown functions)	<i>pflA</i>	23	Paralyzed flagellar protein
	<i>pflB</i>	19	TPR-containing inner membrane protein
	<i>Cjj81176_0276</i>	3	Hypothetical protein
Other factors	<i>clpA</i>	1	ATP-binding component of ClgAP protease
	<i>comEA</i>	1	Competence protein
	<i>fedA</i>	1	Putative hemerythrin

	<i>fedC</i>	1	DnaJ-domain containing protein
	<i>polA</i>	1	DNA polymerase (This Tn is at 3' of <i>polA</i> and immediately upstream of <i>motAB</i> , which encode the stator proteins required for motility)
	<i>pglE</i>	1	Capsule biosynthesis protein
	<i>Cjj81176_0246</i>	1	Hypothetical protein
	<i>Cjj81176_0294</i>	1	Hypothetical protein
	<i>Cjj81176_0481</i>	4	Hypothetical protein
	<i>miaB</i>	1	Adenosine tRNA methyltransferase
	<i>selA</i>	1	Selenocysteine synthase
	Unknown	19	

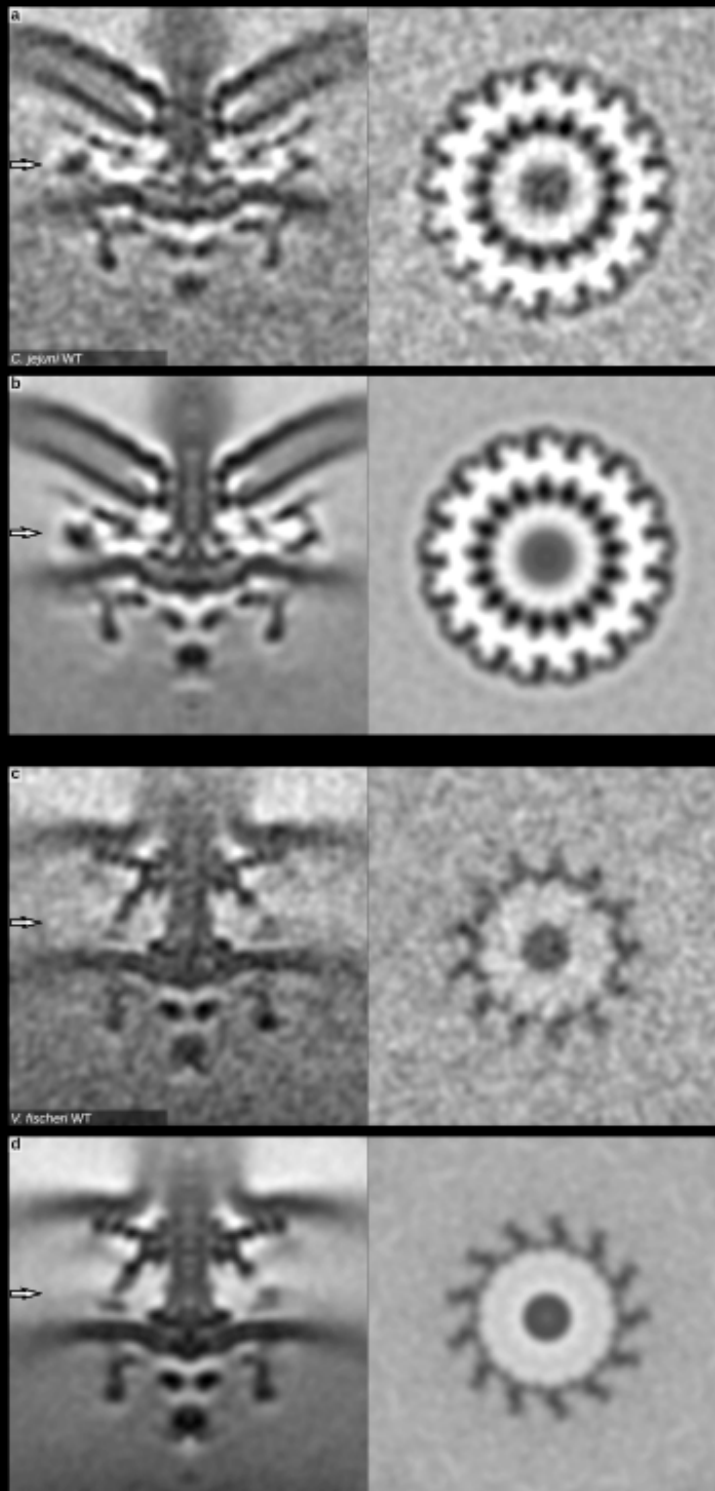
701

702 **Table 1: *C. jejuni* Tn mutants that produce flagella that do not support motility.** Numbers
703 beside each gene represent the number of independent Tn insertions identified in the mutant
704 pool.

705



Supporting Figure 1: Gold standard FSC curves of subtomogram averages. Datasets were fully separated into two half datasets and two independent reconstructions calculated using different template subtomograms from each dataset (59). FSC curves were calculated between the two aligned averages. Subsequently these two averages were averaged together and symmetrized to form the final averaged structure. Resolutions in Angstroms at 0.143 threshold are: *Salmonella* WT: 69.4, *V. fischeri* WT: 63.0, *C. jejuni* WT: 44.7, *V. fischeri* Δ *motB*: 70.3, *C. jejuni* Δ *motB*: 45.8, *C. jejuni* Δ *flgQ*: 60.1, *C. jejuni* Δ *flgP*: 61.2, *C. jejuni* Δ *pflA*: 85.3, *C. jejuni* Δ *pflB*: 61.9, *V. fischeri* Δ *flgP*: 81.0.



717 **Supporting Figure 2: Unsymmetrized motors showing clear symmetries.** (a) *C. jejuni*
718 motors (left) exhibited clear 17-fold symmetry (right) which was (b) applied to improve signal to
719 noise while (c) *V. fischeri* exhibited 13-fold symmetry which was (d) applied to improve signal-to-
720 noise.

721

Strain	Genotype	Source/Reference
DH5α	<i>E. coli supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Invitrogen
TH16943	<i>Salmonella enterica</i> Para ftsZ	Prof. Kelly Hughes, U. Utah.
81-176	Wild-type <i>C. jejuni</i> clinical isolate	(66)
DRH212	81-176 <i>rpsL</i> Sm	(54)
DRH979	81-176 <i>rpsL</i> Sm Δ <i>astA</i> Δ <i>flhA</i>	(63)
DRH1016	81-176 <i>rpsL</i> Sm Δ <i>astA</i> Δ <i>fliP</i>	(63)
DRH2070	81-176 <i>rpsL</i> Sm Δ <i>flgP</i>	(41)
DRH2071	81-176 <i>rpsL</i> Sm Δ <i>flgQ</i>	(41)
DRH2449	81-176 <i>rpsL</i> Sm Δ <i>flgH</i>	This study
DRH2550	81-176 <i>rpsL</i> Sm <i>flgF::cat-rpsL</i>	This study
DRH2553	81-176 <i>rpsL</i> Sm <i>flgI::cat-rpsL</i>	This study
DAR866	81-176 <i>rpsL</i> Sm <i>pflA::cat-rpsL</i>	This study
DAR981	81-176 <i>rpsL</i> Sm Δ <i>pflB</i>	This study
DAR1066	81-176 <i>rpsL</i> Sm Δ <i>motB</i>	This study

DAR1124	81-176 <i>rpsL</i> Sm Δ <i>pflA</i>	This study
DAR1131	81-176 <i>rpsL</i> Sm Δ <i>motA</i>	This study
ABT337	81-176 <i>rpsL</i> Sm <i>pflB</i> :: <i>cat-rpsL</i>	This study
MB1225	81-176 <i>rpsL</i> Sm <i>motA</i> :: <i>cat-rpsL</i>	This study
MB1226	81-176 <i>rpsL</i> Sm <i>motB</i> :: <i>cat-rpsL</i>	This study
SNJ915	81-176 <i>rpsL</i> Sm Δ <i>astA</i> Δ <i>fliE</i>	(67)
SNJ922	81-176 <i>rpsL</i> Sm Δ <i>flgF</i>	This study
SNJ925	81-176 <i>rpsL</i> Sm Δ <i>astA</i> Δ <i>flgG</i>	(68)
SNJ1046	81-176 <i>rpsL</i> Sm Δ <i>astA</i> Δ <i>flgB</i>	(69)
SNJ1048	81-176 <i>rpsL</i> Sm Δ <i>astA</i> Δ <i>flgC</i>	(69)
ALU103	81-176 <i>rpsL</i> Sm <i>flgH</i> :: <i>cat-rpsL</i>	This study
MJM1100	<i>V. fischeri</i> ES114, sequenced wild-type light organ isolate	(70)
MB06265	MJM1100 <i>flgP</i> :: <i>Tnrm</i>	(38)
MB06357	MJM1100 <i>motBI</i> :: <i>Tnrm</i>	(38)

722

723 **Supporting Table 1: Strains used in the study.**

Plasmid	Genotype/Description	Source/Reference
pET21a	Amp ^R	Novagen
pT7-7	Amp ^R	(71)
pUC19	Amp ^R	New England Biolabs
pMalP2X	Amp ^R	New England Biolabs
pMalC2C	Amp ^R	New England Biolabs
pDRH265	pUC19:: <i>cat-rpsL</i>	(54)
pDRH1955	pUC19:: <i>flgP</i> containing <i>cat-rpsL</i> in the NheI site of <i>flgP</i>	(41)
pDRH1957	pUC19:: Δ <i>flgP</i>	(41)
pDRH1958	pUC19:: Δ <i>flgQ</i>	(41)
pDRH2029	pUC19:: <i>flgQ</i> containing <i>cat-rpsL</i> in the MscI site of <i>flgQ</i>	(41)
pDRH2312	pUC19 containing <i>motAB</i> with 750 bases of upstream and downstream sequence cloned into the BamHI site	This study
pDRH2324	pUC19:: <i>motB</i> with the creation of an EcoRV site within the coding sequence of <i>motB</i>	This study
pDRH2422	pUC19:: Δ <i>flgH</i>	(69)
pDRH2504	pUC19:: Δ <i>flgF</i>	(69)

pDRH2526	pUC19 containing <i>pflA</i> with 750 bases of upstream and downstream sequence cloned into the BamHI site	This study
pDRH2532	pUC19:: <i>pflA</i> with a <i>cat-rpsL</i> cassette cloned into the EcoRV site of <i>pflA</i>	This study
pDRH2533	Same as pDRH2532 with <i>cat-rpsL</i> cassette cloned into the EcoRV site of <i>pflA</i> in the opposite orientation	This study
pDRH2534	pUC19:: <i>flgF</i> containing <i>cat-rpsL</i> in the EcoRV site of <i>flgF</i>	(69)
pDRH2536	pUC19 :: <i>flgI</i> containing <i>cat-rpsL</i> in the EcoRV site of <i>flgI</i>	(69)
pDRH2745	pUC19 with fusion of DNA upstream and downstream of <i>pflA</i> to delete the entire coding sequence.	This study
pDRH3330	pUC19:: <i>motA</i> with a <i>cat-rpsL</i> cassette cloned into the SpeI site of <i>motA</i>	This study
pDRH3331	pDRH2324 with a <i>cat-rpsL</i> cassette cloned into the EcoRV site within <i>motB</i>	This study
pDAR906	pT7-7 with <i>motB</i> coding sequence containing 3' in-frame fusion of codons for 6XHis tag cloned into the NdeI and PstI sites	This study
pDAR1012	pUC19 with an in-frame deletion of <i>motA</i> fusing the start codon to codon 252	This study
pDAR1013	pUC19 with an in-frame deletion of <i>motB</i> fusing codon 58 to codon 212	This study

pDAR1180	pET21a with <i>motA</i> coding sequence cloned into the BamHI and XhoI sites	This study
pDAR2107	pMalP2X with <i>pflA</i> coding sequence and 3' sequence encoding for 6XHis tag cloned into the BamHI and PstI sites	This study
pDAR2226	pMalC2X with <i>pflB</i> coding sequence from codons 103 to 304 cloned into the BamHI and PstI sites	This study
pLKB652	pUC19 containing <i>pflB</i> with 750 bases of upstream and downstream sequence cloned into the BamHI site	This study
pLKB658	pUC19:: <i>pflB</i> with a <i>cat-rpsL</i> cassette cloned into the SmaI site of <i>pflB</i>	This study
pABT324	pUC19 with an in-frame deletion of <i>pflB</i> fusing codons 5 to 808	This study
pALU101	pUC19:: <i>flgH</i> containing <i>cat-rspL</i> in the PmeI site of <i>flgH</i>	(69)

725

726 **Supporting Table 2: Plasmids used in this study**