



## 29 Introduction

30 The bacterial flagellum is a macromolecular machine that transforms the movement of ions ( $H^+$  or  $Na^+$ ) across the  
31 cell membrane into a mechanical torque to move the bacterial cell through its environment[1]. In general, the  
32 flagellum consists of a cell-envelope-embedded motor, a hook which acts as a universal joint and a long propeller-  
33 like filament[2,3]. The motor can rotate the filament in either a counterclockwise or clockwise direction. For cells  
34 with a single flagellum this drives the cell forward or backward; for peritrichous cells this results in "run" or  
35 "tumble" movements. Flagella can also exhibit more complex behavior; it was recently reported that the *Shewanella*  
36 *putrefaciens* flagellum can wrap around the cell to mediate a screw-like motion that allows the cell to escape narrow  
37 traps[4]. Besides their role in motility, bacterial flagella participate in other vital activities of the cell such as biofilm  
38 formation[5]. Moreover, the virulence of many human pathogens depends directly on their flagella, with flagellated  
39 strains of *Pseudomonas aeruginosa* and *Legionella pneumophila* causing more serious infections with higher  
40 mortality rates[6,7]. *P. aeruginosa* lacking fully-assembled flagella cause no mortality and are 75% less likely to  
41 cause pneumonia in mice[6].

42  
43 The best-studied flagellar motor, in *Salmonella enterica*, consists of several sub-complexes, which we will describe  
44 in order from the inside out. On the cytoplasmic side are the inner-membrane-embedded MS ring (formed by the  
45 protein FliF) and the C-ring (aka the switch complex, formed by FliN, FliM and FliG). The C-ring encircles a type  
46 III secretion system (T3SS) export apparatus (FliH, FliI, FliJ, FlhA, FlhB, FliP, FliQ and FliR). Spanning the space  
47 from the inner membrane to the peptidoglycan cell wall is the ion channel (called the stator), a complex of two  
48 proteins (MotA and MotB) with 4:2 stoichiometry[8,9]. The interaction between the stator and the C-ring (FliG)  
49 generates the torque required to drive the flagellum. The MS ring is coupled to the extracellular hook (FlgE) through  
50 the rod (FlgB, FlgC, FlgF and FlgG). The rod is further surrounded by two other rings: the P- (peptidoglycan, FlgI)  
51 and the L- (lipopolysaccharide, FlgH) rings which act as bushings during rod rotation. Extending from the hook is  
52 the filament (FliC) which is many micrometers in length. In addition to these components, the assembly of the  
53 whole flagellar motor is a highly synchronized process that requires a plethora of additional chaperones and capping  
54 proteins[10–12].

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56 Recently, the development of electron cryo-tomography (ECT)[13,14] has allowed the determination of the  
57 complete structures of flagellar motors in their cellular milieu at macromolecular (~5 nm) resolution. ECT studies  
58 of many different bacterial species have revealed that while the core structure described above is conserved, the  
59 flagellar motor has evolved many species-specific adaptations to different environmental conditions[15–21]. For  
60 example, extra periplasmic rings were found to elaborate the canonical P- and L-rings in the motor of the  
61 Gammaproteobacteria *Vibrio* species. These rings are called the T-ring (MotX and Y) and H-ring (FlgO, P and  
62 T)[20]. Unlike the *S. enterica* motor described above, which is driven by H<sup>+</sup> ions, the motors of *Vibrio* and other  
63 marine bacteria employ different stators (PomA and PomB) which utilize Na<sup>+</sup>. These Na<sup>+</sup>-dependent stators generate  
64 higher torque (~2,200 pN) than H<sup>+</sup>-dependent stators (~1,200 pN), driving the motor at higher speeds (up to 1,700  
65 Hz compared to ~300 Hz in H<sup>+</sup>-driven motors)[22].

66

67 Most flagellated bacteria use a single stator system – either H<sup>+</sup>-driven or Na<sup>+</sup>-driven, depending on their  
68 environment. Some species, however, such as *Vibrio alginolyticus*, use two distinct types of motors to move in  
69 different environments: a polar Na<sup>+</sup>-driven flagellum and lateral H<sup>+</sup>-driven flagella. Still other species employ dual  
70 stator systems with a single flagellar motor, conferring an advantage for bacteria that experience a range of  
71 environments (see [23] and references therein). For example, *P. aeruginosa* employs a dual H<sup>+</sup>-driven stator system  
72 (MotAB and MotCD). While the MotAB system is sufficient to move the cell in a liquid environment[24], MotCD  
73 is necessary to allow the cell to move in more viscous conditions[25]. *Shewanella oneidensis* MR-1 combines both  
74 Na<sup>+</sup>- and H<sup>+</sup>-dependent stators in a single motor, enabling the bacterium to move efficiently under conditions of  
75 different pH and Na<sup>+</sup> concentration[26]. How these more elaborate motors may have evolved remains an open  
76 question.

77

78 Here, we used ECT to determine the first *in situ* structures of three Gammaproteobacteria flagellar motors with dual  
79 stator systems: in *L. pneumophila*, *P. aeruginosa* and *S. oneidensis* MR-1. *L. pneumophila* and *P. aeruginosa* have  
80 dual H<sup>+</sup>-dependent stator systems and *S. oneidensis* has a dual Na<sup>+</sup>-H<sup>+</sup>-dependent stator. This imaging, along with  
81 bioinformatics analysis, shows a correlation between the structural complexity of the motor and its stator system,  
82 suggesting a possible evolutionary pathway.

83

## 84 Results and Discussion

85 To determine the structures of the flagellar motors of *L. pneumophila*, *P. aeruginosa*, and *S. oneidensis* we imaged  
86 intact cells of each species in a hydrated frozen state using ECT. We identified clearly visible flagellar motors in the  
87 tomographic reconstructions and performed sub-tomogram averaging to enhance the signal-to-noise ratio,  
88 generating a 3D average of the motor of each species at macromolecular resolution (Fig. 1 and S1). While all three  
89 motors shared the conserved core structure of the flagellar motor, they exhibited different periplasmic decorations  
90 surrounding this conserved core. While the *S. oneidensis* and *P. aeruginosa* averages showed clear densities  
91 corresponding to the stators (Fig. 1 E, F, K and L, orange density), none were visible in the *L. pneumophila* average,  
92 suggesting that they were more variable, or dynamic. Interestingly, we observed a novel feature in the *S. oneidensis*  
93 motor: an extra ring outside the outer membrane (Fig. 1 A-F, purple density). This structure is reminiscent of the O-  
94 ring (outer membrane ring) described recently in the sheathed flagellum of *Vibrio alginolyticus*[17]. However, while  
95 the *V. alginolyticus* O-ring was associated with a 90° bend in the outer membrane, no such outer membrane bend  
96 was seen in the unsheathed *S. oneidensis* flagellum, so the function of this structure remains mysterious.

97

98 The most striking difference between the three motor structures was the L- and P-rings, which were highly  
99 elaborated in *S. oneidensis*. The *P. aeruginosa* and *L. pneumophila* motors lacked additional rings associated with  
100 the L-ring, but showed smaller elaborations of their P-rings. To determine whether flagellar motor structure  
101 correlates with motor type, we compared our three new ECT structures with those of the five previously-published  
102 Gammaproteobacteria motors (Fig. 2). Two motors (*Escherichia coli* and *S. enterica*) have a single H<sup>+</sup>-driven stator  
103 system, two motors have dual H<sup>+</sup>-dependent stator systems (*P. aeruginosa* and *L. pneumophila*), three motors have  
104 Na<sup>+</sup>-driven systems (the three *Vibrio* species) and one motor has a dual Na<sup>+</sup>-H<sup>+</sup>-driven system (*S. oneidensis*).  
105 Interestingly, we found that motors with similar stator type also shared similar structural characteristics. While the  
106 two motors with a single H<sup>+</sup>-dependent stator system did not show any periplasmic elaborations beyond the  
107 conserved flagellar core, the dual H<sup>+</sup>-dependent stator systems had an extra ring surrounding their P-ring, with no  
108 embellishment of the L-ring. The Na<sup>+</sup>-dependent motors of the *Vibrio spp.*, together with the Na<sup>+</sup>-H<sup>+</sup>-dependent  
109 motor of *S. oneidensis* have extra components surrounding both their P- and L- rings. In *Vibrio*, these extra  
110 periplasmic rings are known as the T-ring (surrounding the P- ring and formed by the MotX and MotY proteins) and  
111 the H-ring (surrounding the L-ring and consisting of the FlgO, FlgP and FlgT proteins). The presence of the T- and

112 H-rings was suggested to be specific to the Na<sup>+</sup>-driven *Vibrio* motors[20] with the FlgT protein required for the  
113 formation of both rings[27].

114

115 Previous studies showed that MotX and MotY are important for flagellar rotation in *S. oneidensis* but it was not  
116 known whether they form part of the motor or not[28]. Similarly, bioinformatics analysis and biochemical studies  
117 showed that MotY is involved in the function of the *P. aeruginosa* motor, but the structural basis of this role was not  
118 known[24]. We therefore performed a bioinformatics search for candidate homologs of MotX, MotY, FlgO, FlgP  
119 and FlgT in the genomes of *P. aeruginosa*, *L. pneumophila* and *S. oneidensis* to examine whether there is a  
120 correlation between the presence of homologous genes and the extra periplasmic rings observed in the ECT  
121 structures. While we found candidates for all five proteins constituting the T- and H-rings in *S. oneidensis* as  
122 previously suggested[29], only MotY candidates were found in *L. pneumophila* and *P. aeruginosa* (Table S1). This  
123 is in accordance with our ECT structures, which showed that *L. pneumophila* and *P. aeruginosa* motors have a ring  
124 surrounding only their P-rings while the *S. oneidensis* motor has rings surrounding both the P- and L-rings. These  
125 rings are likely T- and H-rings, respectively, as in *Vibrio*. The lack of candidate MotX homologs in the genomes of  
126 *L. pneumophila* and *P. aeruginosa* (Table S1) is consistent with their lack of PomB, the component of the Na<sup>+</sup>-  
127 dependent stator with which MotX interacts. Interestingly, the absence of candidates for FlgT in the *L. pneumophila*  
128 and *P. aeruginosa* genomes suggests that it may not be required for the recruitment of MotY as in *Vibrio* species.

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130 To see whether these correlations hold more broadly, we expanded our bioinformatics analysis to additional species  
131 of Gammaproteobacteria. We examined the genomes of species with single H<sup>+</sup>-driven stator systems (Table S2),  
132 dual H<sup>+</sup>-driven stator systems (Table S3) and Na<sup>+</sup>-driven stator systems (Table S4). Interestingly, we identified a  
133 second species, *Colwellia psychrerythraea* 34H, with a single motor and candidates for both PomAB (Na<sup>+</sup>-driven)  
134 and MotAB (H<sup>+</sup>-driven) stator systems, similar to *S. oneidensis* MR-1 (Table S5). In all species we examined, we  
135 observed the same pattern: (i) genomes of species with single H<sup>+</sup>-driven stator systems lacked homologs of H- or T-  
136 ring components; (ii) genomes of species with Na<sup>+</sup> stator systems contained homologs of all H- and T-ring  
137 components, and (iii) genomes of species with dual H<sup>+</sup>-driven stator systems contained candidate homologs only for  
138 the T-ring component MotY. The sole exception to this rule was *Chromohalobacter salexigens* DSM 3043, which

139 contained a homolog of FlgO in addition to MotY. None of the eight species with dual H<sup>+</sup>-driven stator systems we  
140 examined contained a homolog of FlgT, further suggesting that it is not essential for MotY stabilization in this group.  
141  
142 Together, our results from ECT imaging of flagellar motors *in situ* and bioinformatics analysis reveal a correlation  
143 between the structural complexity of the flagellar motor of Gammaproteobacteria and the type of its torque-  
144 generating unit, the stator (summarized in Fig. 3). Low-speed motors with single H<sup>+</sup>-stator systems have only the P-  
145 and L-ring, while high-speed motors using Na<sup>+</sup> have two extra periplasmic rings, the T- and H-rings. Unexpectedly,  
146 we find that motors with dual H<sup>+</sup>-driven stator systems represent a hybrid structure between the two, elaborating  
147 their P-rings with one of the five components of the T- and H-rings, MotY. This extra MotY ring might help to  
148 stabilize the motor under conditions of increased load, as in the viscous environment of the pulmonary system  
149 encountered by *L. pneumophila* and *P. aeruginosa*. These results therefore suggest an evolutionary pathway in  
150 which these pathogenic Gammaproteobacteria species could have borrowed a motor stabilization strategy from  
151 related Na<sup>+</sup>-driven motors to allow them to colonize animal hosts.

152

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244 **Figures**

245 **Figure 1**

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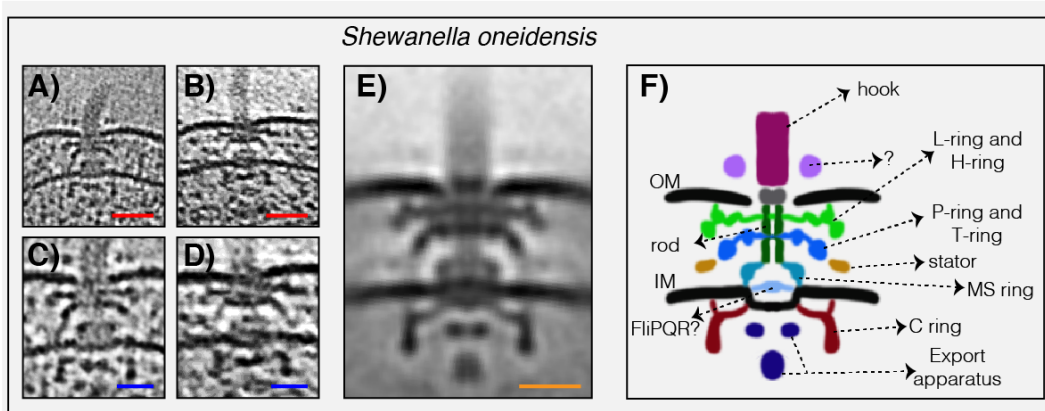
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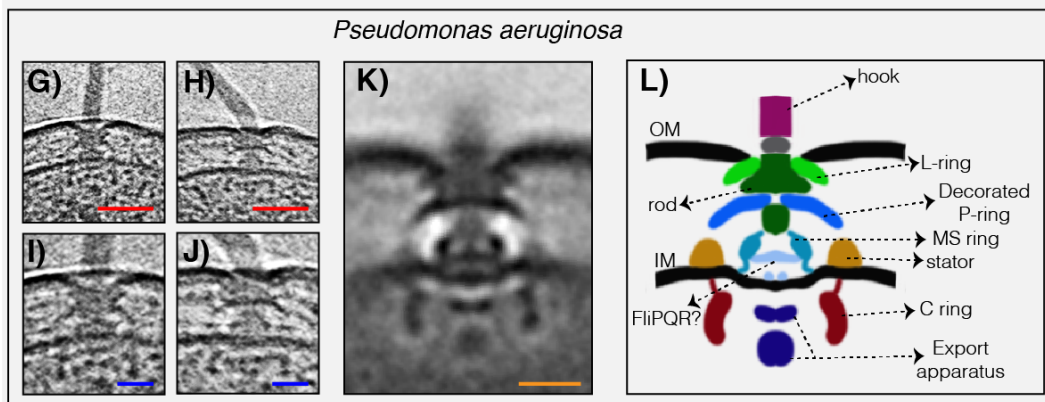
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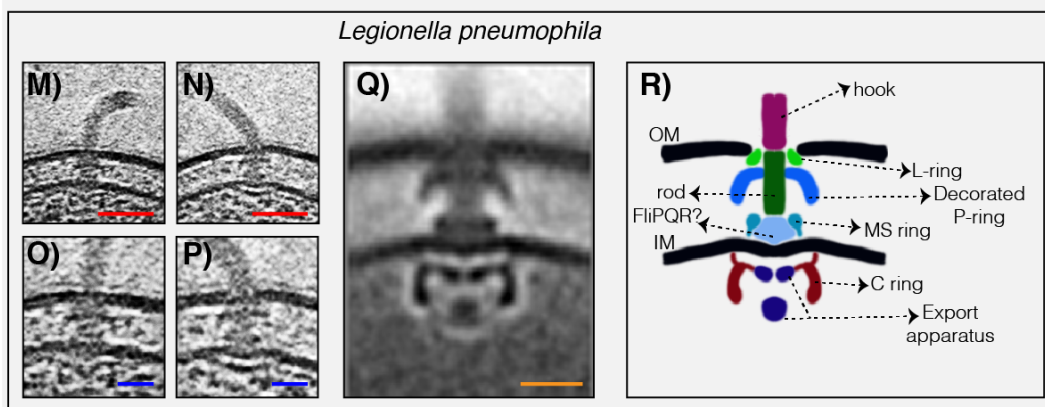
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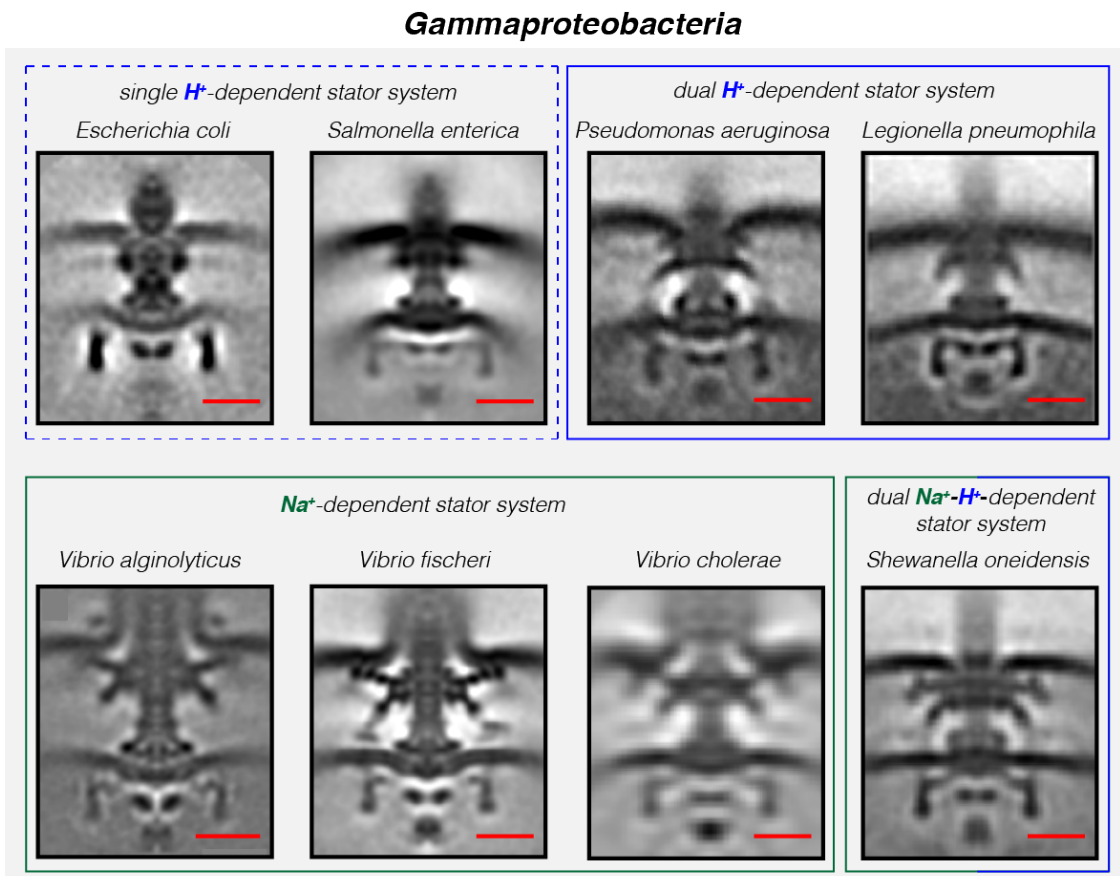
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272 **Figure 2**



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286 **Figure 3**

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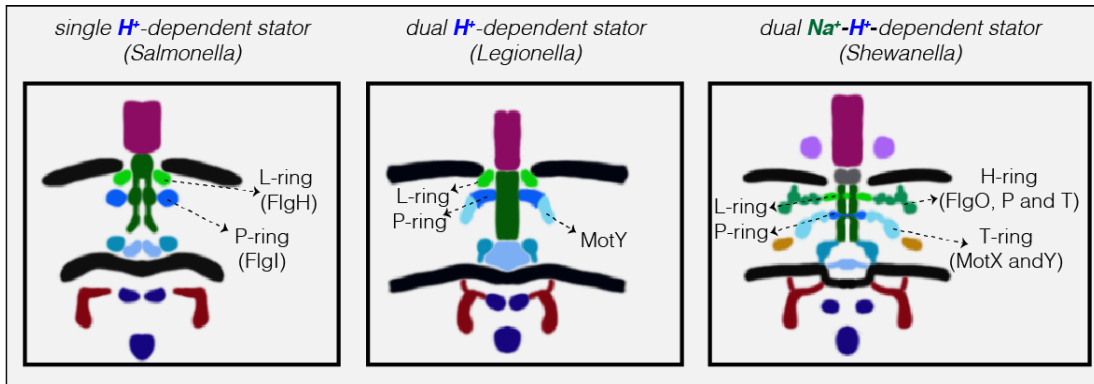
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## 298 **Figure legends**

### 299 **Figure 1: The structures of three dual-stator Gammaproteobacteria flagellar motors revealed by ECT. A & B)**

300 slices through *Shewanella oneidensis* MR-1 electron cryo-tomograms showing single polar flagella. **C & D)**  
301 zoomed-in views of the slices shown in **A** and **B** highlighting the flagellar motors. **E)** central slice through a sub-  
302 tomogram average of the *S. oneidensis* MR-1 flagellar motor. **F)** schematic representation of the sub-tomogram  
303 average shown in **E** with the major parts of the motor labeled. **G-L)** flagellar motor of *Pseudomonas aeruginosa*.  
304 Panels follow the same scheme as in **A-F** above. **M-R)** flagellar motor of *Legionella pneumophila*. Panels follow the  
305 same scheme as above. Scale bars 50 nm (red) and 20 nm (blue and orange).

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### 307 **Figure 2: Compilation of all Gammaproteobacteria flagellar motors imaged to date by ECT.** Central slices of

308 sub-tomogram averages are shown for the eight Gammaproteobacteria flagellar motors revealed by ECT, including  
309 the three structures solved in this study (*P. aeruginosa*, *L. pneumophila* and *S. oneidensis*). The motors are classified  
310 based on their stator system: single H<sup>+</sup>-driven (dashed blue box), dual H<sup>+</sup>-driven (blue box), Na<sup>+</sup>-driven (green box)  
311 or dual Na<sup>+</sup>-H<sup>+</sup>-driven (green-blue box). Scale bars are 20 nm.

312

### 313 **Figure 3: Models showing correlation between structural complexity of the flagellar motor and its stator type.**

314 Flagellar motors with single H<sup>+</sup>-driven stator systems (e.g. *Salmonella*) have P- and L-rings alone. Motors with dual  
315 H<sup>+</sup>-driven stator systems have an extra ring surrounding the P-ring formed by the MotY protein alone. Motors with  
316 Na<sup>+</sup>-driven motors have two periplasmic rings, the T-ring (MotX and MotY) and H-ring (FlgO, FlgP and FlgT),  
317 decorating the P- and L-rings respectively. Note that the boundaries between the P- and L-rings and their  
318 decorations are tentative in these schematics.

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325 **Supporting information:**

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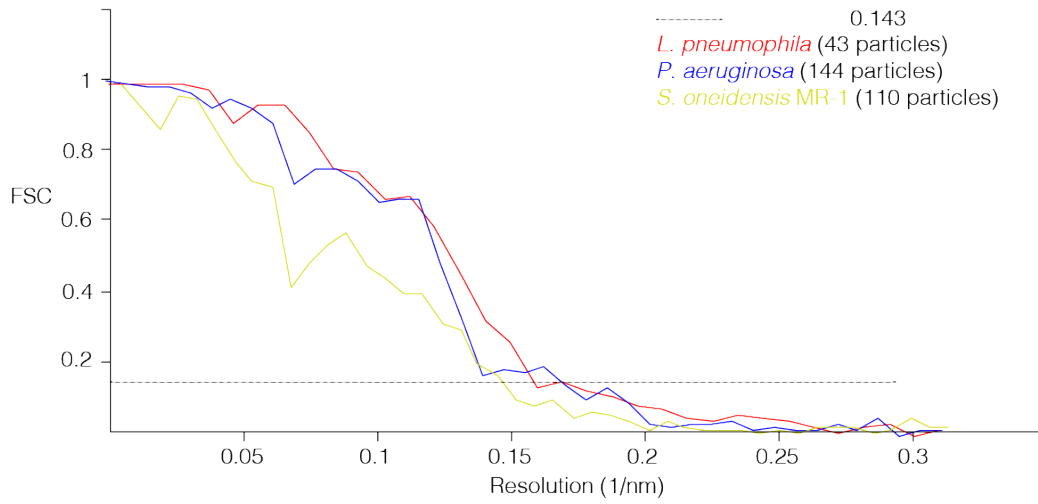
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**Figure S1:** Gold-standard FSC curves of sub-tomogram averages. Resolutions at a 0.143 cutoff (dashed line) are: *L.*

*pneumophila*, 6.4 nm; *P. aeruginosa*, 5.9 nm; *S. oneidensis* MR-1, 6.9 nm.

345 **Table S1. Candidate homologs of H- and T-ring components in species imaged in this study.**

Species	MotX candidate	MotY candidate	FlgO candidate	FlgP candidate	FlgT candidate
<i>Pseudomonas aeruginosa</i> (dual H <sup>+</sup> -driven stator)	-	+	-	-	-
<i>Legionella pneumophila</i> (dual H <sup>+</sup> -driven stator)	-	+	-	-	-
<i>Shewanella oneidensis</i> MR-1 (dual Na <sup>+</sup> -H <sup>+</sup> -driven stator)	+	+	+	+	+

346

347 **Table S2. Candidate homologs of H- and T-ring components in single H<sup>+</sup>-dependent stator systems.**

Species	MotX candidate	MotY candidate	FlgO candidate	FlgP candidate	FlgT candidate
<i>Escherichia coli</i>	-	-	-	-	-
<i>Salmonella enterica</i>	-	-	-	-	-
<i>Sodalis glossinidius</i>	-	-	-	-	-

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349 **Table S3. Candidate homologs of H- and T-ring components in dual H<sup>+</sup>-dependent stator systems.**

Species	MotX candidate	MotY candidate	FlgO candidate	FlgP candidate	FlgT candidate
<i>Azotobacter vinelandii</i> DJ	-	+	-	-	-
<i>Cellvibrio japonicus</i> Ueda107	-	+	-	-	-
<i>Chromohalobacter salexigens</i> DSM 3043	-	+	+	-	-
<i>Pseudomonas entomophila</i>	-	+	-	-	-
<i>Saccharophagus degradans</i> 2-40	-	+	-	-	-
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	-	+	-	-	-
<i>Teredinibacter turnerae</i> T7901	-	+	-	-	-
<i>Pseudomonas putida</i>	-	+	-	-	-

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351 **Table S4. Candidate homologs of H- and T-ring components in Na<sup>+</sup>-dependent stator systems.**

Species	MotX candidate	MotY candidate	FlgO candidate	FlgP candidate	FlgT candidate
<i>Colwellia psychrerythraea</i> 34H	+	+	+	+	+
<i>Vibrio alginolyticus</i>	+	+	+	+	+
<i>Vibrio fischeri</i>	+	+	+	+	+

352

353 **Table S5. Candidate homologs of stator system components in *Colwellia psychrerythraea* 34H.** *E*-values of

354 BLAST results are shown for each candidate locus (name in parentheses).

Species	MotA candidate	MotB candidate	PomA candidate	PomB candidate
<i>Colwellia psychrerythraea</i> 34H	8e-10 (CPS_1524)	4e-11 (CPS_1525)	2e-124 (CPS_1092)	4e-129 (CPS_1093)

355

356 **Table S6. Raw Blast results for all species in Tables S1-S5.** *E*-values are shown. For *E*-values exceeding the

357 cutoff, the top hit is listed in parentheses.

Species	MotX candidate	MotY candidate	FlgO candidate	FlgP candidate	FlgT candidate
<i>Azotobacter vinelandii</i> DJ	1e-06	8e-14 (Avin_48650)	0.032	0.32	4.2
<i>Cellvibrio japonicus</i> Ueda107	0.59	9e-28 (CJA_2588)	0.72	0.29	6e-09
<i>Chromohalobacter salexigens</i> DSM 3043	0.014	6e-13 (Csal_3309)	9e-16 (Csal_2511)	2.8	2.7
<i>Pseudomonas entomophila</i>	3e-05	2e-31 (PSEEN1209)	1.6	0.32	0.099
<i>Saccharophagus degradans</i> 2-40	6e-06	1e-37 (Sde_2427)	0.24	2.1	1.2
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	0.019	1e-13 (XCC1436)	3	4.5	0.021
<i>Teredinibacter turnerae</i> T7901	0.7	3e-35 (TERTU_3000)	1.4	0.061	1
<i>Pseudomonas putida</i>	0.005	7e-31 (PP_1087)	1	0.77	0.063
<i>Legionella pneumophila</i>	1e-08	3e-35 (lpg2962)	0.87	0.11	2.2



<i>Pseudomonas aeruginosa</i>	3e-05	2e-37 (PA3526)	0.047	0.63	0.19
<i>Escherichia coli</i>	9e-06	2e-09	0.19	0.84	0.19
<i>Salmonella enterica</i>	5e-07	5e-09	0.34	4.9	0.82
<i>Sodalis glossinidius</i>	4.4	1e-07	0.88	0.27	2.2
<i>Colwellia psychrerythraea</i> 34H	2e-63 (CPS_4618)	1e-73 (CPS_3471)	2e-59 (CPS_1469)	6e-28 (CPS_1470)	5e-38 (CPS_1468)
<i>Shewanella oneidensis</i> MR-1	2e-46 (SO_3936)	2e-80 (SO_2754)	2e-19 (SO_3257)	6e-31 (SO_3256)	3e-36 (SO_3258)

358

359

360 **Materials and Methods:**

361

362 **Strains and growth conditions:**

363 *Legionella pneumophila* (strain Lp02) cells were grown on plates of ACES [N-(2-acetamido)-2-aminoethanesulfonic  
364 acid]-buffered charcoal yeast extract agar (CYE) or in ACES-buffered yeast extract broth (AYE) with 100 µg/ml  
365 thymidine. Ferric nitrate and cysteine hydrochloride were added to the media. For ECT experiments, cells were  
366 harvested in early stationary phase.

367

368 *Shewanella oneidensis* MR-1 cells belonging to the strains listed in Table S7 were used in this study. They were  
369 grown using one of the following methods: Luria–Bertani (LB) broth culture, chemostat, the batch culture method or  
370 in a perfusion flow imaging platform. Detailed descriptions of these methods can be found in[30]. Briefly, in the  
371 chemostat method, 5 mL of a stationary-phase overnight LB culture was injected into a continuous flow bioreactor  
372 containing an operating liquid volume of 1 L of a defined medium[31], while dissolved oxygen tension (DOT) was  
373 maintained at 20%. After 20 h, and as the culture reached stationary phase, continuous flow of the defined  
374 medium[31] was started with a dilution rate of 0.05 h<sup>-1</sup> while DOT was still maintained at 20%. After 48 h of  
375 aerobic growth under continuous flow conditions, the DOT was manually reduced to 0%. O<sub>2</sub> served as the sole  
376 terminal electron acceptor throughout the experiment. pH was maintained at 7.0, temperature at 30 °C, and agitation  
377 at 200 rpm. Either 24 or 40 hours after DOT reached 0%, samples were taken from the chemostat for ECT imaging.

378

379 In the batch culture method, 200 µL of an overnight LB culture of *S. oneidensis* cells was added to each of two  
380 sealed and autoclaved serum bottles containing 60 mL of a defined medium[31]. One of the two bottles acted as a  
381 control and was not used for imaging. To this control bottle, 5 µM resazurin was added to indicate the O<sub>2</sub> levels in  
382 the medium. The bottles were then placed in an incubator at 30 °C, with shaking at 150 rpm until the color due to  
383 resazurin in the control bottle completely faded, indicating anaerobic conditions. At this point, samples were taken  
384 for ECT imaging from the bottle that did not contain resazurin.

385

386 For the perfusion flow imaging experiments, *S. oneidensis* cells were grown overnight in LB broth at 30 °C to an  
387 OD<sub>600</sub> of 2.4–2.8 and washed twice in a defined medium[31]. A glow-discharged, carbon-coated, R2/2, Au NH<sub>2</sub>

388 London finder Quantifoil EM was glued to a 43 mm × 50 mm no. 1 glass coverslip using waterproof silicone glue  
389 (General Electric Company) and let dry for ~30 min. Using a vacuum line, the perfusion chamber (model VC-LFR-  
390 25; C&L Instruments) was sealed against the grid-attached glass coverslip. A total of ~10 mL of the washed culture  
391 was injected into the chamber slowly to allow cells to settle on the grid surface, followed by a flow of sterile defined  
392 medium from an inverted serum bottle through a bubble trap (model 006BT-HF; Omnifit) into the perfusion  
393 chamber inlet. Subsequently, the flow of medium was stopped and the perfusion chamber was opened under sterile  
394 medium. The grid was then detached from the coverslip by scraping off the silicone glue at the grid edges using a  
395 22-gauge needle and rinsed by transferring three times in deionized water, before imaging by ECT.

396

397 Samples were also prepared from an aerobic *S. oneidensis* LB culture grown at 30 °C to an OD<sub>600</sub> of 2.4–2.8.

398

399 *Pseudomonas aeruginosa* PAO1 cells were first grown on LB plates at 37 °C overnight. Subsequently, cells were  
400 inoculated into 5 ml MOPS [(3-(*N*-morpholino) propanesulfonic acid)] Minimal Media Limited Nitrogen and grown  
401 for ~ 24 hours at 30 °C.

402

403 **Table S7. *S. oneidensis* strains used in this study**

404

Strain	Relevant genotype	Ref.
MR-1	Wild-type	[32]
<i>ΔpilMNOPO</i>	type IV pili biogenesis mutant	[33]
<i>ΔmshHIJKLMNEGBACDOPO</i>	Msh pili biogenesis mutant	[33]
<i>ΔpilM-Q, ΔmshH-Q</i>	mutant that lacks type IV and Msh pili biogenesis genes	[33]
<i>Δcrp</i>	Lacking the cAMP receptor protein (CRP)	[34]

405

406 **Sample preparation for electron cryo-tomography:**

407 Cells (*L. pneumophila*, *P. aeruginosa* and *S. oneidensis*) from batch cultures and chemostats were mixed with BSA  
408 (Bovine Serum Albumin)-treated 10-nm colloidal gold solution (Sigma-Aldrich, St. Louis, MO, USA) and 4 μL of  
409 this mixture was applied to a glow-discharged, carbon-coated, R2/2, 200 mesh copper Quantifoil grid (Quantifoil  
410 Micro Tools) in a Vitrobot Mark IV chamber (FEI). Excess liquid was blotted off and the grid was plunge frozen in  
411 a liquid ethane/propane mixture for ECT imaging.

412

413

414 **Electron cryo-tomography:**

415 Imaging of ECT samples (*S. oneidensis* and *P. aeruginosa*) was performed on an FEI Polara 300-keV field emission  
416 gun electron microscope (FEI company, Hillsboro, OR, USA) equipped with a Gatan image filter and K2 Summit  
417 counting electron-detector camera (Gatan, Pleasanton, CA, USA). Data were collected using the UCSF Tomography  
418 software[35], with each tilt series ranging from  $-60^\circ$  to  $60^\circ$  in  $1^\circ$  increments, an underfocus of  $\sim 5-10 \mu\text{m}$ , and a  
419 cumulative electron dose of  $\sim 130-160 \text{ e}^-/\text{A}^2$  for each individual tilt series. For *L. pneumophila* samples, imaging was  
420 done using an FEI Titan Krios 300 kV field emission gun transmission electron microscope equipped with a Gatan  
421 imaging filter and a K2 Summit direct electron detector in counting mode (Gatan). *L. pneumophila* data was also  
422 collected using UCSF Tomography software and a total dose of  $\sim 100 \text{ e}^-/\text{A}^2$  per tilt series with  $\sim 6 \mu\text{m}$  underfocus.

423

424 **Sub-tomogram averaging:**

425 The IMOD software package was used to calculate three-dimensional reconstructions of tilt series[36]. Alternatively,  
426 the images were aligned and contrast transfer function corrected using the IMOD software package before producing  
427 SIRT reconstructions using the TOMO3D program[37]. Sub-tomogram averages with 2-fold symmetrization along  
428 the particle Y-axis were produced using the PEET program[38].

429

430 **Bioinformatics analysis:**

431 Candidate H- and T-ring component genes were identified by sequence alignment of the following *Vibrio cholerae*  
432 proteins against the fully sequenced genomes of each bacterial species using BLASTP. The *Vibrio cholerae* proteins  
433 used were: MotX (Q9KNX9), MotY (Q9KT95), FlgO (Q9KQ00), FlgP (Q9KQ01) and FlgT (Q9KPZ9). Candidate  
434 stator homologs in *Colwellia psychrerythraea* 34H were identified by sequence alignment of PomAB proteins of *V.*  
435 *cholerae* (Q9KTL0 and Q9KTK9 respectively) and MotAB proteins of *E. coli* (P09348 and P0AF06 respectively)  
436 against its genome. The *C. psychrerythraea* 34H genome contains a single flagellar motor system[23]. Candidate  
437 MotX and MotY homologs identified were adjacent to the flagellar cluster in the genome, and for each stator system  
438 candidate homologs were characteristically located in tandem in the genome. The codes in parentheses represent  
439 Uniprot IDs. An *E*-value cutoff of  $< 1 \times 10^{-10}$  was used. The raw BLAST results for all species are shown in Table  
440 S6.

441