

1 **Motile ghosts of the halophilic archaeon,**
2 ***Haloferax volcanii***

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10 **Author Contributions:**

11 Y.K. and R.M.B designed the research. Y.K. performed all experiments and
12 obtained all data; N.M. helped genetics, biochemistry, and preparation of figures;
13 Z.L, F.B., T.EF.Q., C.v.d.D and S.-V. A. helped genetics; R.I helped the ghost
14 experiments; N.M. and R.M.B helped microscope measurements; Y.K., and
15 R.M.B. wrote the paper.

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18

19 **Summary**

20 Motility is seen across all domains of life ¹. Prokaryotes exhibit various types of motilities,
21 such as gliding, swimming, and twitching, driven by supramolecular motility machinery
22 composed of multiple different proteins ². In archaea only swimming motility is reported,
23 driven by the archaellum (archaeal flagellum), a reversible rotary motor consisting of a
24 torque-generating motor and a helical filament which acts as a propeller ^{3,4}. Unlike the
25 bacterial flagellar motor (BFM), adenosine triphosphate (ATP) hydrolysis probably drives
26 both motor rotation and filamentous assembly in the archaellum ^{5,6}. However, direct
27 evidence is still lacking due to the lack of a versatile model system. Here we present a
28 membrane-permeabilized ghost system that enables the manipulation of intracellular
29 contents, analogous to the triton model in eukaryotic flagella ⁷ and gliding *Mycoplasma*
30 ^{8,9}. We observed high nucleotide selectivity for ATP driving motor rotation, negative
31 cooperativity in ATP hydrolysis and the energetic requirement for at least 12 ATP
32 molecules to be hydrolyzed per revolution of the motor. The response regulator CheY
33 increased motor switching from counterclockwise (CCW) to clockwise (CW) rotation,
34 which is the opposite of a previous report ¹⁰. Finally, we constructed the torque-speed
35 curve at various [ATP]s and discuss rotary models in which the archaellum has
36 characteristics of both the BFM and F₁-ATPase. Because archaea share similar cell
37 division and chemotaxis machinery with other domains of life ^{11,12}, our ghost model will
38 be an important tool for the exploration of the universality, diversity, and evolution of
39 biomolecular machinery.

40 The archaeellar motor has no homology with the BFM, but is evolutionarily and
41 structurally related to bacterial type IV pili (T4P) for surface motility³. In Euryarchaeota,
42 the filament is encoded by two genes, *flgA* (*flaA* in *Methanococcus*) and *flgB* (*flaB* in
43 *Methanococcus*), and the motor eight *flaC-J* (see Ref. 3 for details in Crenarchaeota).
44 Euryarchaeota encode the full set of a chemotaxis system, *cheA*, *B*, *C*, *D*, *R*, *W*, and *Y*,
45 like flagellated bacteria, which might have been acquired by horizontal gene transfer from
46 *Bacillus/Thermotoga* groups¹¹.

47 Figure 1a (top) shows the current association of functions with the motor genes, based
48 on analysis of mutants and biochemical data: FlaC/D/E as switching proteins for the
49 directional switch of archaeellum rotation coupled with the signals from the chemotaxis
50 system¹³; FlaG and FlaF complex interacting with the surface layer (S-layer), with FlaF
51 regulating FlaG filament assembly^{14,15}; FlaH as a regulator of the switch between
52 assembly of the archaeella and rotation¹⁶; FlaI as the ATP-driven motor for both assembly
53 and rotation⁵; FlaJ as the membrane-spanning component. An inhibitor of proton
54 translocating ATP synthases reduced both intracellular [ATP] and swimming speed in
55 *Halobacterium salinarum*⁶, suggesting that archaeellar rotation is driven by ATP
56 hydrolysis at FlaI. However, direct evidence is lacking due to the lack of a reconstituted
57 system. There is also no direct evidence as to which components are anchored to the cell
58 and which rotate with the filament: Figure 1b illustrates possibilities which we discuss
59 below.

60 Here we present an *in vitro* experimental system for the archaeellum, similar to the
61 Triton model for the eukaryotic flagellum⁷ and the permeabilized ghost model for gliding
62 *Mycoplasma mobile*^{8,9}. We use the halophilic archaeon *Haloferax volcanii*. *Hfx. volcanii*
63 possesses multiple polar archaeella and swims at 2-4 $\mu\text{m s}^{-1}$ at room temperature, with CW

64 rotation more efficient for propulsion than CCW (Fig. 1c *top*, Supplementary Result 1
65 and Supplementary Video 1)¹⁷. We increased the fraction of swimming *Hfx. volcanii* cells
66 from 20-30 % to 80 % by adding 20 mM CaCl₂ (Supplementary Figure 1a).

67 To prepare our experimental model system, we suspended motile cells in buffers
68 containing detergent (0.015 % sodium cholate) and 2.5 mM ATP (Fig. 1d). Fluorescent
69 imaging revealed that ghosts still possessed archaellar filaments, the cell membrane, and
70 S-layer (Fig. 1c *bottom* and Supplementary Figure 2). The detergent reduced the refractive
71 index of cells, indicating permeabilization of the cell membrane and corresponding loss
72 of cytoplasm. Remarkably, the permeabilized cells still swam (Supplementary Video 2
73 and Fig. 1d *lower right*). We named them “ghosts,” as in similar experiments on
74 *Mycoplasma mobile*⁹. Fig. 1e shows a typical example of a live swimming cell changing
75 to a ghost, marked by a sudden change of image density at 8.75 sec. The solution
76 contained 2.5 mM ATP and the swimming speed did not change dramatically when this
77 cell became a ghost (Fig. 1f, see Supplementary Figure 3a for another example). Fig. 1g
78 shows histograms of swimming speeds of cells, before and after adding detergent,
79 indicating that ghosts swim at the same speed as live cells in this, saturating, ATP
80 concentration ($P = 0.421834 > 0.05$ by *t*-test, ratio 0.93 ± 0.24 , $n = 24$, Supplementary
81 Figure 3b). Wild-type ghosts showed a single speed peak around $1.5 \mu\text{m s}^{-1}$ in detergent
82 (Fig. 1g, bottom), in contrast to peaks at ~ 1.7 and $3 \mu\text{m s}^{-1}$ for the same cells without
83 detergent (Supplementary Figure 1b). If CW rotation is associated with the faster peak¹⁷,
84 and is suppressed by detergent, this is consistent with our lack of observation of CW
85 rotation of beads in the presence of detergent (see below). The lack of the $3 \mu\text{m s}^{-1}$ peak
86 in cells lacking CheY (Fig. 1g, *top* and Supplementary Figure 4) would then indicate that
87 CheY is required for CW rotation, as in the bacterial flagellar motor¹⁸. However, we

88 found it difficult to track swimming ghosts due to their low contrast, and were not able to
89 determine the direction of archaellar filament rotation.

90 To overcome these difficulties, we established a ghost-bead assay for measuring ATP-
91 coupled motor rotation (Fig. 2a). We attached cells with sheared, biotinylated archaellar
92 filaments nonspecifically to the cover glass surface, and then introduced 500 nm
93 streptavidin beads which attached to the filaments (Material and Methods and
94 Supplementary Result 2). Addition of 0.1 mg ml⁻¹ streptavidin (which would crosslink
95 adjacent filaments in a rotating bundle) did not stop bead rotation, indicating that shearing
96 removed most filaments and rotating beads are attached to a single archaellum¹⁹
97 (Supplementary Video 3). For the preparation of ghosts, live cells labelled with rotating
98 beads were treated in a flow chamber with detergent (0.03 % sodium cholate, as for
99 swimming cells) for less than 30 sec to permeabilize their cell membrane, and
100 subsequently the detergent was replaced with buffer containing ATP. Motor rotation was
101 stopped by permeabilization and reactivated by the addition of ATP (Supplementary
102 Video 4, Fig. 2b). Although beads on ghost cells rotated only CCW in the presence of
103 detergent (n = 11, see Supplementary Result 3 and Supplementary Figure 7), we observed
104 both directions of rotation after detergent removal (Fig. 2c). We did not see any
105 differences between CW and CCW rotation rates (Supplementary Figure 8) and therefore
106 analyzed speeds collectively.

107 We next investigated the effect of different nucleotide triphosphates (NTPs). Previous
108 *in vitro* experiments showed that purified FlaI hydrolyzes different NTPs at similar rates
109²⁰. However, the archaellar rotational rates in ghosts in 10 mM GTP, CTP, and UTP were
110 5-10 times slower than in ATP (Fig. 2d). This suggests that the motor complex might
111 increase the selectivity of FlaI for nucleotides and/or prevent extra energy consumption

112 *in vivo* like the endopeptidase Clp (see Fig. 1B in Ref. 21). We also tested the inhibitory
113 effects on rotation of ADP, ADP+Pi, and the non-hydrolysable ATP analog ATP- γ -S
114 (adenosine 5'-[γ -thio]triphosphate). We saw no rotation with ATP- γ -S alone. We
115 measured the rotation rates of 500 nm beads attached to archaella in ghosts over a range
116 of [ATP] between 63 μ M and 10 mM, with and without each of ADP (2 mM) , ADP+Pi
117 (each 2mM) and ATP- γ -S (0.5 mM). Figure 2e shows the results as a Lineweaver-Burk
118 plot. All 3 caused large reduction of rotation rates at lower [ATP], but much smaller
119 reductions of f_{\max} , indicating competitive inhibition. The inhibitor constants, K_i , were
120 estimated to be 1.94 mM for ADP (Ocher), 1.22 mM for ADP.Pi (Green), and 0.11 mM
121 for ATP- γ -S (Blue). We also observed modest effects of pH, and ion concentration on
122 rotation (Supplementary Result 4).

123 Although we expected bi-directional rotational to be mediated by the response regulator
124 CheY¹⁰, live cells without CheY were observed to rotate in either direction, without
125 switching during our typical recording time of 30 s ($n = 5$ for CW rotation, $n = 76$ for
126 CCW rotation). To observe the role of archaeal CheY in motor switching, we extended
127 our recording time to 300 s. Switching from CCW to CW rotation was frequent in wild
128 type live cells, but rare in Δ CheY live cells even during 5 min recordings (Fig. 3 and
129 Supplementary Figure 10). Wild type ghosts still switched, but the bias and fraction of
130 switching cells were changed, suggesting the chemotaxis system was still active, but
131 altered (Supplementary Table 3).

132 Figure 4a shows the dependence of rotation speed (f , revs per second) of 200, 500, and
133 970 nm beads upon [ATP] in the range 8 μ M to 10 mM. Michaelis-Menten fits to the data
134 (solid lines) are poor below 30 μ M ATP. Figure 4b shows the relationship between
135 $\log([ATP])$ and $\log(f / (f_{\max} - f))$, where f_{\max} is estimated by Michaelis-Menten plot (Fig.

136 4a), and the slope represents Hill coefficients of 0.63, 0.82 and 0.89 for 200 nm, 500 nm,
137 970 nm beads respectively. This result indicates negative cooperativity in ATP-driven
138 archaellar rotation, (see below for discussion). Figure 4c shows the relationships between
139 torque, speed and [ATP] for archaellar rotation. The maximum motor torque was
140 estimated to be ~200 pN nm for live cells and ~170 pN nm for ghosts, comparable to *Hbt.*
141 *salinarum* live-cell experiments (160 pN nm)²².

142 Our estimated maximum torque of ~170 pN nm corresponds to the motor doing work
143 for a single rotation ($2\pi T$, ~1000 pN nm) equivalent to the free energy of hydrolysis of
144 ~12 ATP molecules per revolution, assuming the free energy of 80-90 pN nm per ATP
145 molecule. Conservation of energy therefore sets a lower limit of 12 /rev/motor on the ATP
146 hydrolysis rate, ~15 times higher than that measured *in vitro* for FlaI²³. This indicates
147 that motor assembly enhances ATPase activity in the archaellum, as observed in other
148 systems; for example the PilC-PilT interaction in T4P²⁴ and β - and γ -subunit interaction
149 in F₁-ATPase²⁵. Hydrolysis of 12 ATP molecules per revolution is consistent with
150 previous reports²² and with models of a 2-fold FlaJ rotor rotating within a 6-fold FlaI
151 ATPase^{22,26}.

152 Negative cooperativity in archaellar rotation at low [ATP] (Fig. 4a-b) might be explained
153 by a mechanism similar to that proposed for F₁-ATPase, where bi-site and tri-site
154 hydrolysis correspond to nucleotide occupancy of the three catalytic sites alternating
155 between 1 and 2 or between 2 and 3 or three, respectively²⁷, and the hydrolysis rate is
156 slower when only 1 site is occupied. In this scenario, negative cooperativity would be the
157 result of the same interactions within the FlaI hexamer that power rotation^{22,26}. Negative
158 cooperativity could also arise from communication between FlaI and FlaH rings, similar
159 to inter-ring effects in chaperonins²⁸. Although FlaH has only the Walker A motif, ATP

160 binding is known to modulate the interaction between hexameric rings of FlaI and FlaH
161 ¹⁶.

162 Our finding that the time-averaged motor torque decreases with increasing speed at low
163 loads (Fig. 4c) differs from a previous report²², which assumed constant torque
164 irrespective of viscous load and speed, and explained the observed speed variations by
165 assuming an extra contribution to the viscous drag from an unseen remnant of the filament.
166 The required length of these remnants (ξ , 0.8 pN nm s) would be about 4 μm ²², which
167 seems unlikely given our observation that most filaments are removed by shearing. The
168 curves in figure 4c are qualitatively similar to those reported for the BFM with varying
169 ion-motive force ²⁹. By contrast, the equivalent data for F₁-ATPase correspond to
170 Michaelis-Menten kinetics and torque that decreases linearly with increasing speed (see
171 Fig. 2 in Ref. ³⁰). Simple models for the torque-speed relationships, similar to those
172 applied to the BFM ^{29,31}, ^{29,31} and high-resolution detection of steps in rotation ³² using
173 gold nanoparticles ^{30,33} may reveal the details of the rotation mechanism of the archaellum
174 in future.

175 So far, there is no direct evidence as to which components of the archaellum are fixed
176 relative to the cell (“stator”) and which rotate with the filament (“rotor”). FlaF and G are
177 most likely part of the stator, anchored to the S-layer. Previous reports indicate interaction
178 between FlaF and the S-layer and a deficiency in swimming motility of S-layer deleted
179 cells ^{14,15}. Our observations of increased motility with [CaCl₂] and the speed fluctuations
180 at low [CaCl₂] (Supplementary Figures 1 and 9), given that calcium stabilizes S-layer ³⁴,
181 support this hypothesis. Homology to F₁-ATPase and T4P are generally taken to favour a
182 model where rotation of a FlaJ dimer within the central core of the FlaI hexamer is driven
183 by cyclic changes in the conformation of the FlaI hexameric ATPase, coupled to ATP

184 hydrolysis^{22,26}. In this model, FlaJ is the rotor and all other motor components are the
185 stator (Figure 1b, left). For switching, changes caused by CheY binding, presumably
186 somewhere on FlaC/D/E, would have to propagate all the way to the core of FlaI, which
187 would need separate mechanochemical cycles for CW and CCW rotation. Figure 1b, right,
188 illustrates the other extreme possibility, most similar to the BFM. In this model,
189 conformational changes in FlaI would push on FlaF/G, either directly or via FlaC/D/E. In
190 the latter case, the switch mechanism could reside within FlaC/D/E and FlaI need not
191 have separate modes for CW and CCW rotation. Intermediate models are also possible
192 (Figure 1b, middle). Our ghost model may allow labelling of archaeellar components to
193 observe directly which are part of the rotor^{35,36}, analysis of rotational steps as in isolated
194 F₁ and other molecular motors^{30,37,38}, and direct investigations of the role of CheY.

195 Our finding that archaeal CheY increases CW bias (Fig. 3) is inconsistent with previous
196 reports¹⁰ that the role of archaeal CheY in *Hbt. salinarum* enhances switching from CW
197 to CCW rotation, as revealed by a dark-field microscopy of swimming cells^{10,39}. This
198 study measured static filaments to be right-handed in *Hbt. salinarum* M175, and inferred
199 from this that CW rotation propels a cell forward, CCW backwards. We speculate that the
200 contradictory result might be due to misinterpretation of filament helicity caused by errors
201 in accounting for reflections in the microscope optics - cryo-EM data show left-handed
202 helicity in *Hbt. salinarum* M175, supporting our conclusion⁴⁰. With due care to account
203 for reflections in our microscope (Supplementary figure 6), our bead assay is a direct
204 observation of the rotational direction of the motor.

205 Our ghost assay represents the first experimental system that allows manipulation of the
206 thermodynamic driving force for an archaeal molecular motor, following previous
207 examples including eukaryotic linear motors⁴¹, the PomAB-type BFM³⁸, and

208 *Mycoplasma* gliding motor⁹. We anticipate that this assay will be helpful for other
209 biological systems. Archaea display chemotactic and cell division machinery acquired by
210 horizontal gene transfer from bacteria^{11,12}. Although the archaeellum and bacterial
211 flagellum are completely different motility systems, they share common chemotactic
212 proteins. Theoretically, only our ghost technique allows monitoring of the effect of
213 purified CheY isolated from different hosts on motor switching. Similarly, our ghost cells
214 offer the potential to manipulate and study the archaeal cell division machinery as with
215 *in vitro* ghost models of *Schizosaccharomyces pombe*⁴². Ghost archaea offer the
216 advantages of both *in vivo* and *in vitro* experimental methods and will allow the exploration
217 of the universality, diversity, and evolution of biomolecules in microorganisms.
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219

220 **Material and Methods**

221 **Strain and Cultivation**

222 Strains, plasmids, and primers are summarized in Supplementary Table 1, 2. *Haloferax*.
223 *volcanii* (*Hfx. volcanii*) cells were grown at 42°C on a modified 1.5 % Ca agar plate (2.0
224 M NaCl, 0.17 M Na₂SO₄, 0.18 M MgCl₂, 0.06 M KCl, 0.5% (wt/vol) casamino acid,
225 0.002% (wt/vol) biotin, 0.005% (wt/vol) thiamine hydrochloride, 0.01% (wt/vol) L-
226 tryptophane, 0.01% (wt/vol) uracil, 10 mM HEPES-NaOH (pH 6.8) and 1.5% (wt/vol)
227 Agar). Note that 20 mM CaCl₂ should be added (Supplementary Result 1). Colonies were
228 scratched by the tip of a micropipette and subsequently suspended in 5ml of Ca liquid
229 medium. After 3h incubation at 37°C, the culture was centrifuged at 5,000 r.p.m and
230 concentrated to 100 times volume. The 20 µl culture was poured into 25-ml fresh Ca
231 medium and again grown for 21 h with shaking of 200 r.p.m at 40°C. The final of an
232 optical density would be around 0.07.

233 Gene manipulation based on selection with uracil in $\Delta pyrE2$ strains was carried out with
234 PEG 600, as described previously⁴³. For the creation of KO strains, plasmids based on
235 pTA131 were used carrying a *pyrE2* cassette in addition to ~1000-bp flanking regions of
236 the targeted gene. *flgA1(A124C)* was expressed by tryptophane promotor (Supplementary
237 Result 2).

238 **Preparation of biotinylated cells**

239 The culture of *Hfx. volcanii* Cys mutant was centrifuged and suspended into buffer A (1.5
240 M KCl, 1 M MgCl₂, 10 mM HEPES-NaOH pH 7.0). Cells were chemically modified with
241 1 mg ml⁻¹ biotin-PEG2-maleimide (Thermo Fischer) for 1 h at room temperature, and
242 excess biotin was removed with 5,000 g centrifugation at R.T for 4 min.

243 **Motility assay on soft-agar plate**

244 A single colony was inoculated on a 0.25% (wt/vol) Ca-agar plate and incubated at 37°C
245 for 3-5 days. Images were taken with a digital camera (EOS kiss X7; Canon).

246 **Microscopy**

247 All experiments were carried under an upright microscope (Eclipse Ci; Nikon) equipped
248 with a 40× objective (EC Plan-Neofluar 40 with Ph and 0.75 N.A.; Nikon) or 100×
249 objective, a CMOS camera (LRH1540; Digimo). Images were recorded at 100 fps for 10-
250 30 sec. For a motility experiment at 45°C, a phase-contrast microscope (Axio Observer;
251 Zeiss) equipped with a 40× objective (EC Plan-Neofluar 40 with Ph and 0.75 N.A.; Zeiss),
252 a CMOS camera (H1540; Digimo), and an optical table (Vision Isolation; Newport) were
253 used.

254 For fluorescent experiment, a fluorescent microscope (Nikon Eclipse Ti; Nikon)
255 equipped with a 100× objective (CFI Plan Apo 100 with Ph and 1.45 N.A.; Nikon), a laser
256 (Nikon D=eclipse C1), an EMCCD camera (ixon⁺ DU897; Andor), and an optical table
257 (Newport) were used. The dichroic mirror and emitter were Z532RDC (C104891,
258 Chroma) and 89006-ET-ECFP/EYFP/mcherry (Chroma) for an FM4-64 experiment,
259 Z442RDC (C104887, Chroma) and 89006-ET-ECFP/EYFP/mcherry for an S-layer
260 experiment, and Z442RDC and ET525/50m (Chroma) for a Dylight488 experiment.

261 **Construction of swimming ghosts**

262 The flow chamber was composed of a 22×22 coverslip and slide glass. Two pieces of
263 double-sided tape, cut to a length of ~30 mm, were used as spacers between coverslips
264 ¹⁷. Two tapes were fixed with a ~5 mm interval, and the final volume was ~15 µl. The
265 glass surface was modified with a Ca medium containing 5 mg ml⁻¹ bovine serum

266 albumin (BSA) to avoid cells attaching to a glass surface.

267 To construct swimming ghosts, 10 μ l of cell culture in Ca medium and buffer B (2.4 M
268 KCl, 0.5 M NaCl, 0.2 M MgCl₂, 0.1 M CaCl₂, 10 mM HEPES-NaOH pH 7.2) containing
269 1 mg ml⁻¹ DNase, 5 mM ATP (A2383, Sigma Aldrich), and 0.03 % sodium cholate
270 (Sigma Aldrich) was mixed in an Eppendorf tube. Subsequently, the 20 μ l mixture was
271 infused into the flow chamber.

272 Phase-contrast images were captured at 20 frames s⁻¹ for 15 sec. Swimming trajectories
273 were determined by the centroid positions of cells and subjected to analysis using Igor
274 pro. Given the trajectory of cells, $\mathbf{r}(t) = [x(t), y(t)]$, the swimming velocity $\mathbf{v}(t)$ was
275 defined as $\mathbf{v}(t) = \frac{\mathbf{r}(t + \Delta t) - \mathbf{r}(t)}{\Delta t}$.

276 **Bead assay**

277 For the observation of a rotational bead attached to an archaeellar filament, archaeellar
278 filaments were sheared by 30 times pipetting with 200 μ l pipette (F123601, Gilson),
279 infused into a flow chamber and kept for 10 min. Streptavidin-conjugated fluorescent
280 beads (200 nm (F6774, Molecular probes), 500 nm (18720, Polysciences) or 970 nm
281 (PMC 1N, Bangs lab)) in buffer B (2.4 M KCl, 0.5 M NaCl, 0.2 M MgCl₂, 0.1 M CaCl₂
282 10 mM HEPES-NaOH pH 7.2, 0.5 mg ml⁻¹ BSA (Sigma Aldrich)) were added into the
283 flow chamber, incubated for 15 min, and then rinsed with buffer to remove unbound beads.
284 The solution was replaced to buffer B containing 0.03 % sodium cholate hydrate (C1254,
285 Sigma Aldrich) and 1 mg ml⁻¹ DNase. When the optical density of cells was decreased,
286 buffer B was replaced with buffer A containing ATP. Rotary ghosts were prepared within
287 1 min (Fig. 2b). For pH measurements, the following buffer was used: Bis-Tris HCl for
288 pH5.7 and 6.1 experiments; HEPES-NaOH for a pH8.0 experiment; and Tris-HCl for
289 pH8.6 and pH9.3 experiments (Supplementary Figure 9). For nucleotides experiments,

290 ADP (A2754, Sigma Aldrich), ATP- γ -S (A1388, Sigma Aldrich), GTP (ab146528,
291 Abcam), CTP (R0451, Thermo Fischer Scientific), and UTP (R0471, Thermo Fischer
292 Scientific) were used. Most data were collected at 100 frames s⁻¹ for 10 sec.

293 Bead position was determined by centroid fitting, giving cell trajectories, $\mathbf{r}(t) = [x(t),$
294 $y(t)]$. The rotation rate was determined from either Fourier transform analysis (Fig. 2b) or
295 a fit with a linear function to time course of bead rotation. (Fig. 2c). The rotational torque
296 against viscous drag was estimated as $T = 2\pi f \zeta$, where f is rotational speed and $\zeta =$
297 $8\pi\eta a^3 + 6\pi\eta ar^2$ the viscous drag coefficient, with r the radius of rotation (major axis of
298 ellipse), a the bead radius, and η the viscosity. We neglected the viscous drag of filaments
299 ²², which is expected to be negligible compared to these beads ⁴⁴.

300 To measure the viscosity of the medium, we tracked diffusing fluorescent beads for 30
301 sec at 100 fps and performed an analysis of their mean-squared displacement versus time.
302 From this analysis, the viscosities are estimated to be 0.0025 Pa·s in buffer, 0.0039 Pa·s
303 in buffer + ficoll 5 %, and 0.0072 Pa·s in buffer + ficoll 10 % at 25 °C, which are slightly
304 higher than a previous estimate ²². We inferred that this discrepancy might be due to the
305 proximity of the glass surface ⁴⁵.

306 **Fluorescent experiments**

307 For visualization of archaeellar filaments, biotinylated cells were subsequently incubated
308 with 0.1 mg ml⁻¹ Dylight488-streptavidin (21832, Invitrogen) for 3 min, washed by
309 centrifugation, and resuspended.

310 FM4-64 (F34653, Life Sciences) was used to stain the archaeal cell membrane. The
311 powder was dissolved by buffer B (1.5 M KCl, 1 M MgCl₂, 10 mM HEPES-NaOH pH
312 7.0), and the cells were incubated for 30 min. The extra dye was removed by
313 centrifugation. For microscopic measurements, the glass surface was cleaned using a

314 plasma cleaner (PDC-002; Harrick plasm).

315 Quantum dots 605 (Q10101MP, Invitrogen) was used to stain the archaeal cell surface,

316 S-layer¹⁷. Cells were biotinylated with biotin-NHS-ester (21330, ThermoFisher) and

317 incubated for 15 min at R.T. Extra biotin was washed by centrifugation. Biotinylated cells

318 were subsequently incubated with the buffer containing QD605 at a molar ratio of 400:1

319 for 3 min, washed by centrifugation, and resuspended.

320

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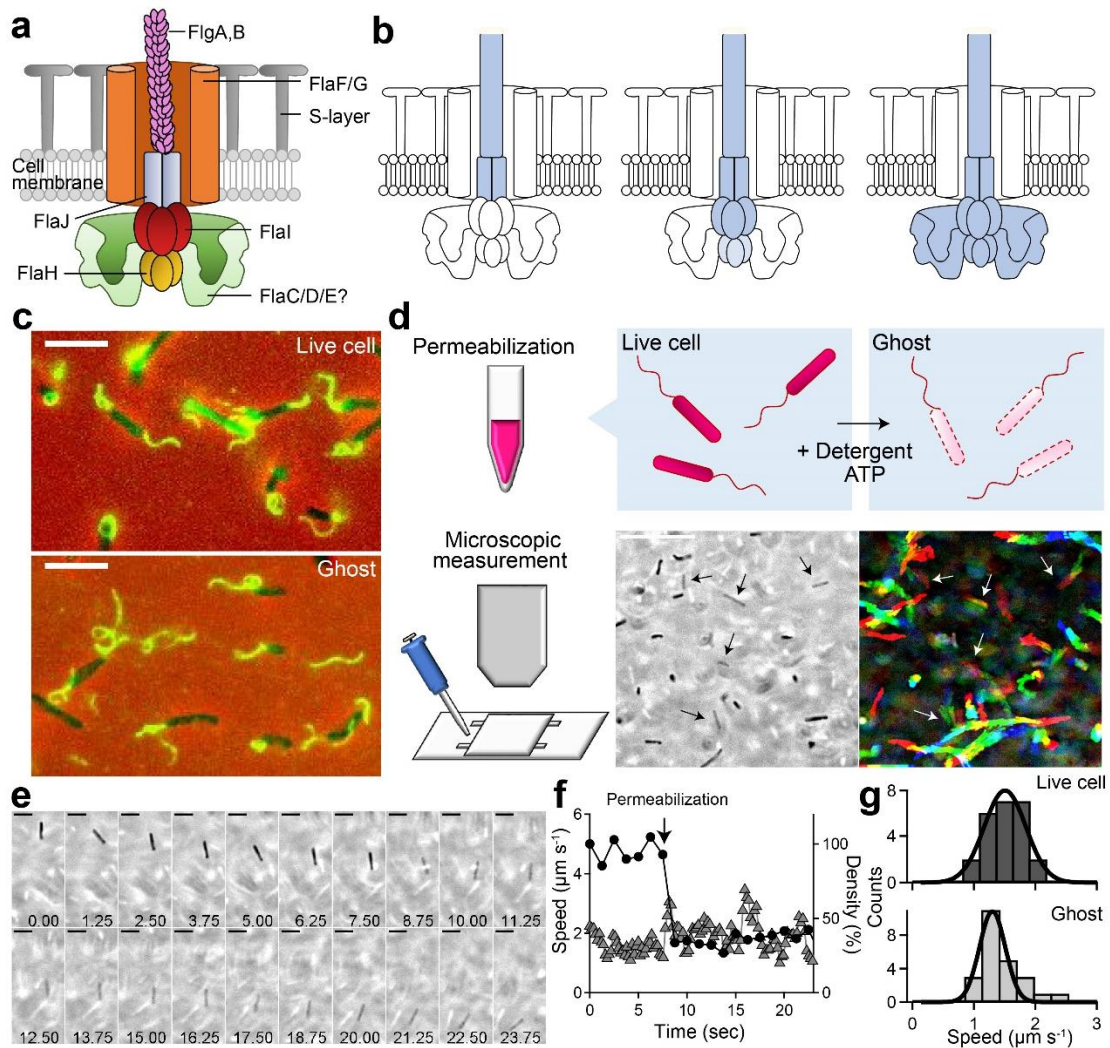
332 **Conflict of interest**

333 The authors declare no competing interests.

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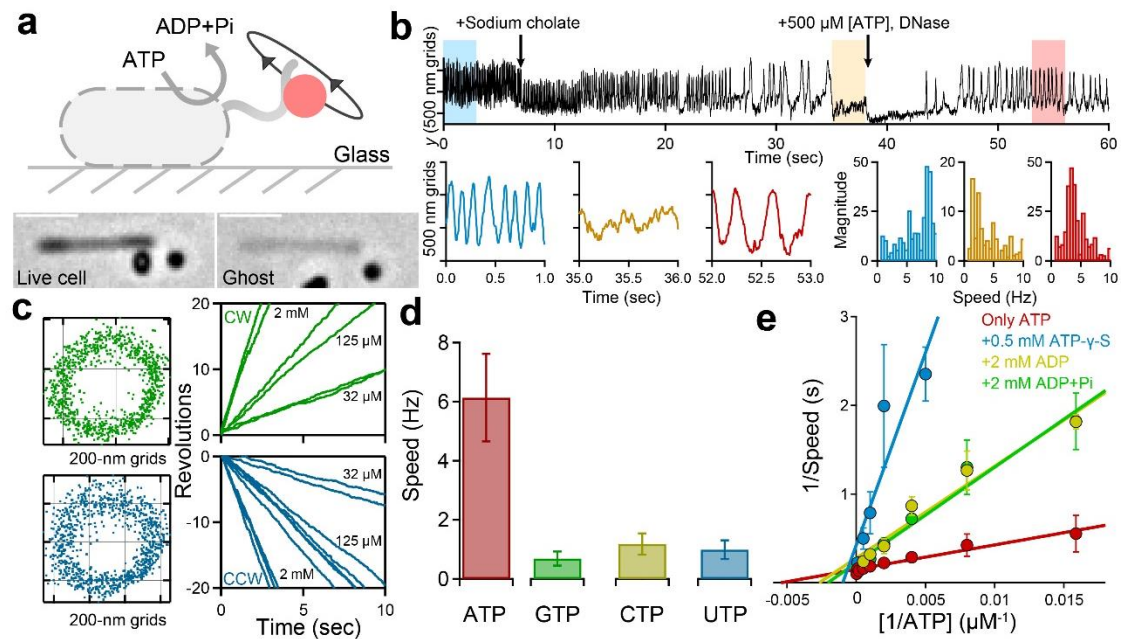


337

338 Figure 1 Swimming ghosts

339 (a) The current model of the archaeellar motor in Euryarchaeota (details in main
 340 text). (b) Different possibilities for which components of the archaellum are fixed
 341 relative to the cell (stator, white) and which rotate with the filament (rotor, blue).
 342 Details are described in the main text. (c) Merged phase-contrast and fluorescent
 343 images of live cells (*top*) and ghosts (*bottom*) labeled with streptavidin-dylight488
 344 which binds to biotinylated filaments. (d) Procedures to observe swimming ghosts.
 345 Live cells were permeabilized in a tube, then induced into a flow chamber to
 346 observe swimming motility. *Lower middle*: Phase-contrast image. Black arrows

347 indicate ghosts. Scale bar, 10 μm . Lower right: Sequential images with 0.5-s
348 intervals, integrated for 30 sec with the intermittent color code “red \rightarrow yellow \rightarrow
349 green \rightarrow cyan \rightarrow blue.” White arrows indicate trajectories of ghosts. (e)
350 Sequential images of a change from a live cell to ghost. Scale bar, 5 μm . (f) Time
351 course of swimming speed (triangles) and cell optical density (circles) from (d).
352 Arrow indicates the time of permeabilization. (g) Histograms of swimming speed,
353 $1.51 \pm 0.34 \mu\text{m s}^{-1}$ before permeabilization (live cells) and $1.30 \pm 0.21 \mu\text{m s}^{-1}$ after
354 permeabilization (ghosts), (mean \pm SD, n = 24).
355



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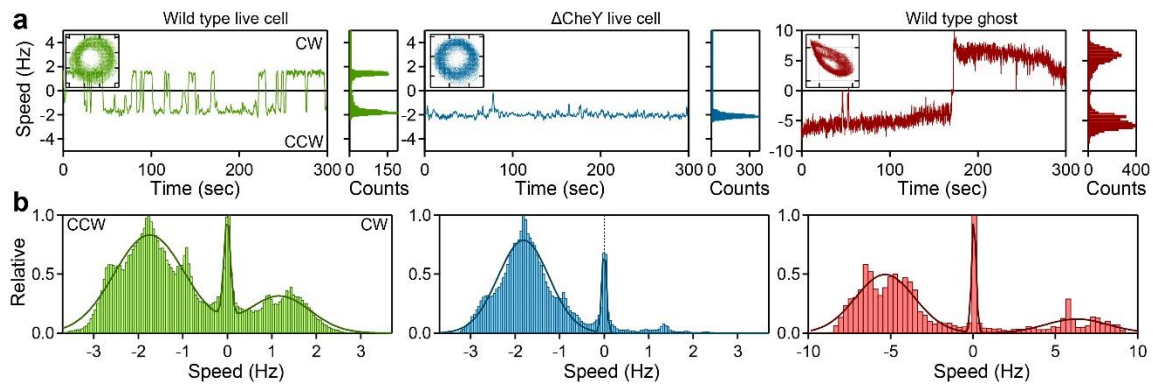
357 **Figure 2 Visualization of motor rotation in ghosts, via beads**
 358 **attached to archaeallar filaments**

359 (a) Schematic of experimental setup (top) and phase-contrast images of a live
 360 cell (lower left) becoming a ghost (lower right). Scale bar, 5 μm . (b) Top: Time
 361 course of bead location (y -coordinate) during ghost preparation. Bottom: Shaded
 362 sections (top) are expanded (left); with corresponding speed distributions by
 363 Fourier transform analysis (right), using the same colours as top. Blue shows the
 364 live cell, orange the motor stopped after treatment with detergent, red the motor
 365 re-activated after addition of ATP. Rotation cannot be measured during media
 366 exchange time, ~ 10 s (Data from Supplementary Video 4). (c) Left: x - y plots of
 367 locations of two different beads attached to archaeallar filaments. Green and blue
 368 represent CW and CCW rotation, respectively. Right: Angle vs time for [the same
 369 two/similar (delete whichever is not true)] beads. The slopes decrease in
 370 proportion to $[\text{ATP}]$ in both directions, indicating ATP-coupled rotation. (d)

371 Rotation rate for different nucleotide triphosphates at 10 mM. The mean \pm SD
372 were 6.14 ± 1.48 Hz for ATP (n = 43), 0.69 ± 0.24 Hz for GTP (n = 30), $1.18 \pm$
373 0.36 Hz for CTP (n = 32), and 0.99 ± 0.32 Hz for UTP (n = 29). (e) Lineweaver-
374 Burk plot of rotation rate and inhibitors. Blue, green, ocher, and red represent
375 data with 2 mM ADP (n = 140), 2 mM ADP+Pi (n = 114), 0.5 mM ATP- γ -S (n =
376 118), and without inhibitors (n = 345), respectively. Data are representative of
377 three independent experiments.

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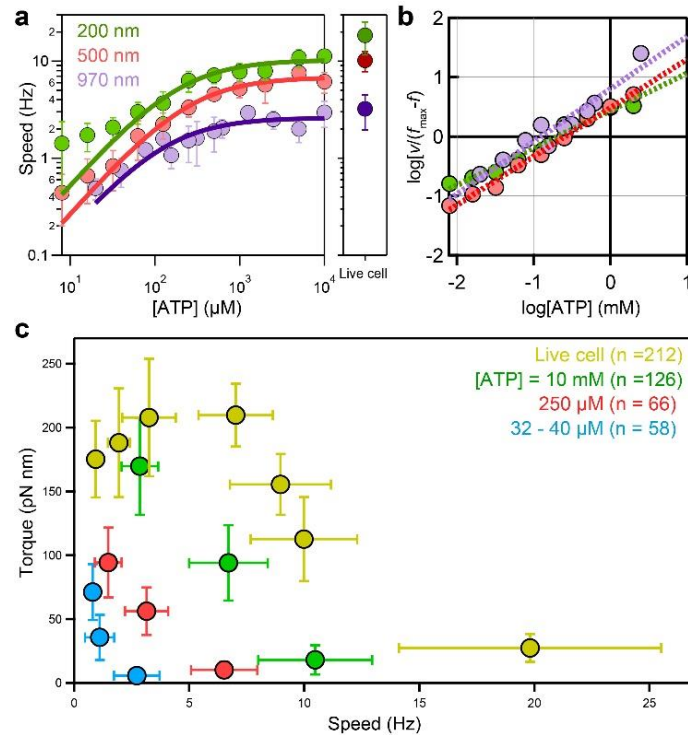


380

381 **Figure 3 Archaeal CheY-mediated motor switching**

382 (a) *Left*: Representative time course of rotation rate for 5 min, using 970 nm bead
383 in wild type- and Δ CheY live cells, and 500 nm bead in wild type ghosts. Positive
384 and negative speeds represent CW and CCW rotation, respectively. *Inset*: y-x
385 plots of bead rotation. Grids represent 500 nm. *Right*: Histogram of rotation rate
386 of each cell. (b) Population histograms of rotation rate. The solid line represents
387 a Gaussian distribution, where the peak and SD were 1.17 ± 0.31 Hz for CW
388 rotation and 1.75 ± 0.79 Hz for CCW rotation in wild type cells (46 cells); $1.81 \pm$
389 0.57 Hz for CCW rotation in the Δ CheY live cells (54 cells); and 6.31 ± 1.90 Hz
390 for CW rotation and 5.34 ± 1.79 Hz for CCW rotation in wild type ghosts (28 cells).
391 Data are representative of two independent experiments.

392



393 **Figure 4 ATP- and load-dependent archaeal motor rotation**

394 (a) Rotation rates of 200 (green), 500 (red) and 970 nm beads (blue) attached to
 395 archaeellar filaments, vs [ATP]. The solid lines show a fit to the Michaelis-Menten
 396 equation $\frac{f_{max} \times [ATP]}{K_m + [ATP]}$; where f_{max} and K_m are 10.3 Hz, 188 μM for 200 nm beads (n
 397 = 287), 6.8 Hz and 249 μM for 500 nm beads (n = 438), and 2.6 Hz and 132 μM
 398 for 1000 nm beads (n = 303). *Right*: corresponding rotation rates of live cells;
 399 18.47 ± 6.86 Hz for 200 nm beads (n = 19), 10.17 ± 2.39 Hz for 500 nm beads (n
 400 = 32) and 3.22 ± 1.27 Hz for 1000 nm beads (n = 72). (b) A Hill plot of the same
 401 data. The Hill coefficient, determined from the slope of the plots, was 0.63 for 200
 402 nm beads, 0.82 for 500 nm beads, and 0.89 for 970 nm beads. (c) Torque vs
 403 speed (mean \pm SD), of live cells and ghosts at various [ATP]. Torque was
 404 estimated as $T = 2\pi f \xi$, where f is rotation speed and $\xi = 8\pi\eta a^3 + 6\pi\eta a r^2$ the viscous
 405 drag coefficient of the bead. ξ was varied by using bead size ($d = 2a = 200, 500,$

406 970 nm) and viscosity ($\eta = 2.5, 3.9, 7.2$ mPa·s in buffer, 5% and 10% Ficoll
407 respectively). r is the major axis of the ellipse describing the orbit of the bead
408 center. [ATP] and number of cells are indicated.

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