Crash landing of *Vibrio cholerae* by MSHA pili-assisted braking and anchoring in a viscoelastic environment

Wenchao Zhang^{1,#}, Mei Luo^{2,#}, Chunying Feng¹, Huaqing Liu¹, Hong Zhang¹, Rachel R. Bennett^{3,*}, Andrew S. Utada^{4,5,*}, Zhi Liu^{2,*}, Kun Zhao^{1,*}

¹Frontier Science Center for Synthetic Biology and Key Laboratory of Systems Bioengineering (Ministry of Education), School of Chemical Engineering and Technology, Tianjin University, Tianjin, P.R. China

²Department of Biotechnology, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China

³School of Mathematics, University of Bristol, Bristol, UK

⁴Faculty of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan

⁵The Microbiology Research Center for Sustainability, University of Tsukuba, Ibaraki, Japan

*Address correspondence to: rachel.bennett@bristol.ac.uk, utada.andrew.gm@u.tsukuba.ac.jp, zhiliu@hust.edu.cn, or kunzhao@tju.edu.cn.

These authors contributed equally.

Abstract

Mannose-sensitive hemagglutinin (MSHA) pili and flagellum are critical for the surface attachment of *Vibrio cholerae*, the first step of *V. cholerae* colonization on host surfaces. However, the cell landing mechanism remains largely unknown, particularly in viscoelastic environments such as the mucus layers of intestines. Here, combining the cysteine-substitution-based labelling method with single-cell tracking techniques, we quantitatively characterized the landing of *V. cholerae* by directly observing both pili and flagellum of cells in a viscoelastic non-Newtonian solution consisting of 2% Luria-Bertani and 1% methylcellulose (LB+MC). The results show that MSHA pili are evenly distributed along the cell length and can stick to surfaces at any point along the filament. With such properties, MSHA pili are observed to act as a brake and anchor during cell landing which include three phases: running, lingering, and attaching. Importantly, loss of MSHA pili results in a more dramatic increase in mean path length in LB+MC than in 2% LB only or in 20% Ficoll solutions, indicating that the role of MSHA pili during cell landing is more apparent in viscoelastic non-Newtonian fluids than viscous Newtonian ones. Our work provides a detailed picture of the landing dynamics of *V. cholerae* under viscoelastic conditions, which can provide insights into ways to better control *V. cholerae* infections in real mucus-like environment.

Introduction

Vibrio cholerae, a human pathogen that causes the debilitating disease cholera, is a natural inhabitant of aquatic ecosystems (Almagromoreno *et al.*, 2015; Kaper *J B*, 1995). They can form biofilms on both biotic and abiotic surfaces, which increases their infectivity and environmental survival (Donlan *et al.*, 2002; Silva *et al.*, 2016; Teschler *et al.*, 2015; Yildiz *et al.*, 2009).

Bacterial appendages have been shown to play important roles in regulating bacterial activities especially biofilm formation during microbe-host interactions. The flagellum is required for biofilm formation in a variety of bacteria species, such as *E. coli* (Pratt *et al.*, 1998), *P. aeruginosa* (O'Toole *et al.*, 1998), and *V. cholerae* (Guttenplan *et al.*, 2013; Watnick *et al.*, 1999). Mutants lacking flagella in both *E. coli* and *Vibrio vulnificus* have been observed to be defective for attachment (Friedlander *et al.*, 2013; Lee *et al.*, 2004). Type IV pili (TFP) are another type of filamentous appendages commonly found on many bacteria and archaea, which have diverse functions such as cellular twitching motility, biofilm formation, horizontal gene transfer, and host colonization (Piepenbrink *et al.*, 2016). *P. aeruginosa* display two types of TFP-driven twitching motility (Gibiansky *et al.*, 2010). *Neisseria gonorrhoeae* have shown a TFP-dependent attachment, leading to the formation of microcolonies on host cell surfaces (Higashi *et al.*, 2007). In contrast, although *V. cholerae* biosynthesize three types of TFP that are expressed under different scenarios, they have not been observed to twitch on surfaces. These three pili are: chitin-regulated competence pili (ChiRP; formerly termed PilA), toxin co-regulated pili (TCP), and mannose-sensitive hemagglutinin type IV pili (MSHA) (Meibom *et al.*, 2004; Reguera *et al.*, 2005; Yildiz *et al.*, 2009). ChiRP pili were observed to be able to grasp extracellular DNA and transport it back to the cell surface via pili retraction (Ellison *et al.*, 2018). TCP pili are important for host colonization and pathogenesis (Kirn *et al.*, 2000; Thelin *et al.*, 1996). In contrast to these two types, MSHA pili are known to be important for surface attachment of *V. cholerae* (Utada *et al.*, 2014; Watnick *et al.*, 1999).

64 Motility has been shown to be a crucial element for *V. cholerae* colonization of the epithelium,
 65 leading to successful infection of the human host (Krukoniš et al., 2003; Tsou et al., 2008). Two types of
 66 near-surface motility, roaming and orbiting, were observed in *V. cholera* (Utada et al., 2014). It has
 67 been further suggested that *V. cholerae* synergistically employ the use of their flagella and MSHA pili to
 68 enable a hybrid surface motility that facilitates surface selection and attachment (Utada et al., 2014).
 69 However, there is a lack of direct observational evidence of the appendages in question. More
 70 importantly, the environmental niches *V. cholerae* encounter in their life cycle typically include
 71 viscoelastic mucus (Almagromoreno et al., 2015). The mucus layer of animal intestines is estimated to
 72 have a wide range of viscosities, varying anywhere from the viscosity of water (~1 cP) to 1000-fold
 73 higher (1000 cP) (Lai et al., 2009). How cells land on surfaces in such viscoelastic environments is still
 74 not clear. To answer these questions, direct live-cell visualization of the pili and flagellum in real-time in
 75 viscoelastic conditions is needed.

76 Recently, there have been significant advances in techniques for directly observing cell appendages
 77 (Blair et al., 2008; Ellison et al., 2019; Ellison et al., 2018; Ellison et al., 2017a; Nakane et al., 2017;
 78 Renault et al., 2017; Skerker et al., 2001; Talà et al., 2019). Among them, the cysteine substitution-
 79 based labelling method is specific and has been successfully applied to visualize tight adherence (TAD)
 80 pili of *C. crescentus* and type IV pili of *V. cholera* (Ellison et al., 2019).

81 In this paper, by combining a cysteine substitution-based labelling method with single-cell tracking,
 82 we directly observed the individual pili and flagellum of landing cells in viscoelastic media and revealed
 83 the dynamic landing sequence of *V. cholerae* as it makes initial surface attachment. The role of MSHA
 84 pili during cell landing in viscoelastic environment is demonstrated. Our work provides a detailed
 85 picture of the landing dynamics of *V. cholerae* under viscoelastic conditions, during which, the
 86 synergistic functions of MSHA pili and flagellum are elucidated.

Results

MSHA pili are evenly distributed along cell length with a constant length density

To visualize the MSHA pili, we constructed a mutant (MshAT70C) by cysteine substitution, which can subsequently be labeled with highly-specific maleimide dyes (*Figure 1a* and *Figure 1-Figure supplement 1*), following the protocol in Ellison *et al.* (Ellison *et al.*, 2019; Ellison *et al.*, 2017a). To observe the distribution of MSHA pili on the cell surface, we simultaneously stained the plasma membrane with FM4-64 in *Figure 1a*.

We visualized the positions of the different pili as the cell body rotates by recording high speed videos during surface landing (see one example of a three-dimensional model of a single cell reconstructed from the videos in *Figure 1-Figure supplement 2*). The results show evenly distributed MSHA pili along the cell length, indicating absence of preferred pili localization on the cell body. Quantitatively, we find that the majority of cells have approximately 3~7 MSHA pili, with 4 MSHA pili per cell being observed most frequently, as shown in *Figure 1b*. These results are in agreement with recent reports (Floyd *et al.*, 2020). Under our conditions, we observed MSHA pili growth (*Figure 1-Figure supplement 3a* and *3b*) but no retraction.

The number of MSHA pili appears to be positively correlated with cell length since it increases as the cell grows (*Figure 1-Figure supplement 3c*). Statistically, the number of MSHA pili shows a linear relationship with cell length (*Figure 1c*), indicating that the length density of MSHA pili is roughly constant for *V. cholerae*.

MSHA pili mediate *V. cholerae* landing by acting as a brake and anchor

The MSHA pili, which are uniformly distributed across the cell surface, play a crucial role in surface attachment of *V. cholerae* through pili-surface interactions (Utada *et al.*, 2014). To elucidate the role of MSHA pili in the landing dynamics under viscoelastic conditions, we directly visualize the fluorescently

110 labeled MSHA pili on *V. cholerae* swimming in a viscoelastic medium consisting of 2% LB and 1% MC
111 (LB+MC).

112 Consistent with previous reports in normal aqueous solutions (*Utada et al., 2014*), the WT strain in
113 LB+MC also exhibits orbiting behavior, characterized by multi-pass circular trajectories, and roaming
114 behavior, characterized by highly exploratory, diffusive, trajectories. Typical roaming and orbiting
115 trajectories in LB+MC are shown in Figure 2 (see more examples in *Figure 2-Figure supplement 1*).
116 The roaming cell traces out a path that is linear trajectory over short distances, with a radius of gyration
117 $R_g = 19.5 \mu\text{m}$, and an average speed of $1.7 \mu\text{m/s}$ (see *Figure 2a, b, Video 1*). In contrast, the orbiting cell
118 trajectory is much more circular with an average $R_g = 1.6 \mu\text{m}$ and an average speed of $1.1 \mu\text{m/s}$ (see
119 *Figure 2c, d, Video 2*). A 3D plot of speed plotted along the trajectory in both examples show that both
120 phenotypes make momentary pauses, where their speed slows down; this can be seen clearly in Figure
121 2b, where the cell motion near a surface displays a characteristic alternation between moving and
122 stopping (*Figure 2b and 2d*).

123 Such pauses are suggested to be caused by MSHA pili-surface interactions (*Utada et al., 2014*).
124 However, by recording fluorescence video sequences, we directly visualized the process, thereby
125 providing direct evidence that the pauses are due to transient contact between MSHA pili and surface.
126 We show a transient pili-surface contact during orbiting in Figure 2e. In a sequence of frames, we see a
127 transiently attached pilus become stretched due to cell motion away from the point of attachment.
128 Subsequently, this pilus detaches from the surface as the cell continues to move, as indicated with the
129 white arrowheads in Figure 2e (for more details, see Video 2). These results indicate that the MSHA pili
130 can work as a brake to abruptly slow-down cell motion by transiently attaching to the surface. This is
131 further confirmed by the observation that during the course of surface motion, different MSHA pili
132 attach and detach, switching dynamically as the cell uses these as transient attachment points (*Figure 2-*

133 *Figure supplement 2* and Video 3). Such a switching of the specific MSHA pili that are engaging the
 134 surface is caused by the rotation of cell body, which is required to balance the torque for flagellar
 135 rotation when cells swim. Thus, as the cell body rotates due to the rotation of the flagellar motor,
 136 different MSHA pili distributed on the cell body take turns approaching and receding from the surface.
 137 The switching of attached MSHA pili not only continues to slow-down cell motion but also changes the
 138 direction of motion. Taken together, this indicates that the pili distribution on the cell body may also
 139 affect cell-surface motion.

140 When the adhesion between MSHA pili and surface is sufficiently strong, the attachment point can act
 141 as an anchor point. We demonstrate this by showing the deflection of the trajectory of a swimming cell
 142 by the attachment of a, single, anchoring MSHA pilus; here, linear motion is bent into circular motion
 143 that is centered around the attachment point (see Video 4). We estimate the centripetal force for this
 144 motion to be on the order of 10^{-21} N, which is much smaller than the pN forces that pili can sustain
 145 (*Floyd et al., 2020; Maier et al., 2002*). The anchoring of MSHA pilus eventually leads to the
 146 irreversible attachment of the cell.

147 **The landing sequence of *V. cholerae* includes three phases**

148 To further clarify the landing process, we labelled both flagellum and pili simultaneously using
 149 MshAT70CFlaAA106CS107C mutant. Figure 3 shows an example of the complete landing process of
 150 an orbiting cell. Based on the pattern of motion displayed by the cell (*Figure 3a* and Video 5), we divide
 151 the landing process into three phases: running, lingering, and attaching. In the running phase (0-3.77 s),
 152 cells will swim and can perform roaming or orbiting. We note that misalignments between the flagellum
 153 and cell body axis tend to change the motion direction of the cell (*Figure 3a, b*). In the lingering phase
 154 (3.77-4.68 s), the cells demonstrate one of two states: a paused state or a tethered state, where the cell
 155 can move under the constraint of tethering pilus (see *Figure 3a* for the tethered state). At 3.77 s, one

156 pilus attaches to the surface and acts as an anchor point to prevent the cell from moving away. Finally,
 157 in the attaching phase (≥ 4.68 s), cells remain on the surface motionless during the observation period
 158 most likely since they have effected irreversible attachment. Upon irreversible cell attachment, some of
 159 the free MSHA pili become attached to the surface firmly while others demonstrate fluctuations
 160 punctuated with intermittent attachment to the surface (Video 6).

161 During cell landing, transitions between the running and lingering phase, as well as between the two
 162 states of lingering phase are observed. The measured conditional probabilities q_{ij} that a cell transitions
 163 from state i to j show that the running phase has a relatively lower q_{rt} to the tethered state ($\sim 22\%$) but a
 164 higher q_{rp} to the paused state ($\sim 78\%$). Similarly, the paused state has a higher q_{pr} than q_{pt} . In contrast, the
 165 tethered state shows similar q_{tr} and q_{tp} , which are 45% and 55%, respectively (Figure 3c).

166 The single-cell dynamics in each specific phase/state is also characterized quantitatively. In the
 167 running phase of *V. cholerae*, we found that the period for body rotation is generally distributed between
 168 0.25-2 s and is centered at ~ 0.7 s (the rotation rate was ~ 1.5 Hz) in LB+MC (Figure 4a). We measured
 169 the swimming speed, v , and the cell-body rotation rate, ω_c , for each cell, and plotted v as a function of ω_c
 170 (see Figure 4b). By fitting the data, we found that v linearly increases with ω_c with a slope of $|v/\omega_c| =$
 171 2.48 ± 0.04 $\mu\text{m}/\text{radian}$.

172 By contrast, a cell in the tethered state typically performs a circular motion around the attachment
 173 point (red dots in Figure 4c). The direction of the circular motion is also dynamic and can switch from
 174 counter-clockwise (CCW) to clockwise (CW) presumably due to a switch in the rotation direction of the
 175 flagellar motor (see 2.6 s, Figure 4c). Angular velocity is roughly constant during each circular-motion
 176 interval (i.e., in each CCW or CW period) and quickly changes sign after CCW-CW switching (Figure
 177 4d and Video 7). Due to the distribution of pili across the cell body, tethering can occur at a pole or
 178 under the body, which leads to cells standing vertically or lying down horizontally to the surface,

respectively. We find that standing tethered cells perform a faster circular motion (mean angular speed = 8.5 ± 1.9 rad/s) than lying ones (mean angular speed = 3.0 ± 2.1 rad/s) (*Figure 4e*). For the horizontal cells, different MSHA pili may be used to further anchor the cell to the surface. For example, two horizontally-tethered cells demonstrate different tethered-motion trajectories depending on the location of the anchoring MSHA pilus (*Figure 4-Figure supplement 1*). In addition to the fact that unattached pili may increase the likelihood that the cell will make irreversible attachment, we observe that MSHA pili appear to be able to attach to the surface along their entire length, and not just the tip (Video 8).

Interestingly, we find that the flagellum of attached cells frequently continues to rotate (Video 5, after 4.68 s), indicating that even after cell attachment, the flagellar motor is still active for some period. The flagellum will eventually stop rotating after a cell stay long enough on the surface (Video 9).

Role of MSHA pili in cell landing is more apparent in viscoelastic non-Newtonian fluids than viscous Newtonian ones

To further investigate the dependence of MSHA pili function and hence cell landing on viscoelasticity, we compared cell motion behavior obtained in 2% LB, which is a Newtonian-fluid and has a viscosity ~ 1 cP at 30 °C (*Utada et al., 2014*) and in 2% LB+1% MC, which is a non-Newtonian fluid and has a shear-dependent viscosity (*Figure 6*), for both WT and $\Delta mshA$ cells (*Figure 5* and *Figure 5-Figure supplement 1*).

We observed WT cells to demonstrate roaming and orbiting motilities in both solutions (*Figure 5a*). The histograms of deviation angle of each type of motility obtained in the two solutions are also similar (*Figure 5-Figure supplement 1a* and *b*). These results indicate that roaming and orbiting motilities of cells are robust against the tested viscoelastic environment. Although the general motility pattern is similar in both solutions, the motion of cells, as expected, is slowed significantly in LB+MC. The average speed of WT cells for near-surface motion is reduced by ~ 22 times from 86.7 ± 32.9 $\mu\text{m/s}$

(mean $\pm$ standard deviation) in 2% LB to 3.8 ± 2.6 $\mu\text{m/s}$ in LB+MC. Similarly, the average speed of $\Delta mshA$ cells is also decreased by ~ 12 times from 80.0 ± 15.0 $\mu\text{m/s}$ in 2% LB to 6.5 ± 1.4 $\mu\text{m/s}$ in LB+MC. The slow-down of motion can also be seen clearly from their mean square displacement curves (Figure 5-Figure supplement 1c, d), which show similar shape but very different time scales.

However, WT and $\Delta mshA$ cells also show differences in their motility behavior in these two solutions (Figure 5). In LB+MC, WT cells tend to land on the surface soon after approaching it (less than one round in orbiting motility) and more tethered motions are observed, which leads to more irregular and tortuous trajectories and smaller R_g for WT cells compared with the case of 2% LB (Figure 5c). By contrast, $\Delta mshA$ cells show very similar R_g distributions in the two types of solutions (Figure 5c). More interestingly, compared with WT, in LB+MC, a large proportion of $\Delta mshA$ cells show orbiting for a substantial large number of cycles, as shown in Figure 5b. Quantitatively, this can be seen by the calculated mean path length, $\bar{l}$, which is 39.7 ± 51.2 μm for WT and 72.5 ± 99.1 μm for $\Delta mshA$ in LB+MC, whereas the corresponding value in 2% LB is 58.7 ± 63.1 μm for WT and 47.2 ± 50.8 μm for $\Delta mshA$. To see how the role of MSHA pili varies with viscoelasticity, we can calculate the ratio of mean path length between $\Delta mshA$ and WT, $\bar{l}_{\Delta mshA}/\bar{l}_{WT}$, for each type of solution, which is ~ 1.8 in LB+MC and ~ 0.8 in 2% LB only, respectively (Figure 5d and e). So loss of MSHA pili results in a more dramatic increase in mean path length in LB+MC than in 2% LB. Moreover, such prolonged orbiting motions of $\Delta mshA$ were not observed in a 20% Ficoll solution (Figure 5), which shows a high viscosity but still belongs to a Newtonian fluid (Winet, 1976). As shown in Figure 5, the cell motility behaviors in 20% Ficoll are similar to those in 2% LB only except that the average cell speed for near-surface motion is dramatically slowed in 20% Ficoll, which is 9.3 ± 4.3 $\mu\text{m/s}$ for WT and 10.0 ± 3.5 $\mu\text{m/s}$ for $\Delta mshA$. The cell trajectories in 20% Ficoll are similar to those in 2% LB only and consequently, the ratio of $\bar{l}_{\Delta mshA}/\bar{l}_{WT}$ is ~ 0.75 in 20% Ficoll, very close to ~ 0.8 in 2% LB only.

Together, these results indicate that the elastic properties of viscoelastic solutions can also affect cell motility behavior, and the role of MSHA as a braking and anchoring machine in cell landing is more apparent in viscoelastic non-Newtonian fluids than viscous Newtonian fluids.

Discussion

The first step in *V. cholerae* biofilm formation is the transition from planktonic swimmers to stationary surface attached cells; this process is mediated by the landing process (Teschler *et al.*, 2015). In this study, the combination of cell appendage labelling with high-resolution spatio-temporal imaging allows us to quantitatively deconstruct the landing process into three stages: running, lingering, and attaching, as summarized in Figure 7. During the running phase, cell motion is powered by flagellar rotation, which simultaneously induces a counter-rotation of cell body. When swimming cells come to within a distance that is comparable to the length of a typical pilus from a surface, dangling pili may brush against the surface, thereby deflecting the trajectory. Typical MSHA pili are ~0.4-1.2 μm in length. During near surface swimming, cell body rotation actively brings MSHA pili into close proximity with the underlying surface where friction between pili and the surface can slow the cells, or, transient adhesions can be made, which may even arrest cell motion. Here, we make an analogy to the slow-down and stop effected by the brake system of a car. During near-surface swimming, it has been suggested that hydrodynamic forces cause the cell bodies of swimming rod-like bacteria take on a tilted, non-parallel, orientation to the surface (Vigeant *et al.*, 2002). In the case of *P. aeruginosa*, whose TFP are distributed with a strong bias toward a particular pole (Skerker *et al.*, 2001), pili-surface contact will depend on which pole is closer to the surface. In contrast, the homogeneous distribution of MSHA pili on *V. cholerae* (see Figure 1c) may be more efficient at slowing such tilted cell bodies by increasing the probability that pili encounter the surface.

247 If the contact-induced adhesion between MSHA pili and the surface is sufficiently strong to arrest
 248 forward motion, the cell will either pause or commence tethered motion centered about the point-of-
 249 adhesion. Cells rotating at an angle closer to the surface have a slower angular velocity (*Figure 4e*), to
 250 which hydrodynamic effect presumably has an important contribution (Bennett et al., 2016). This
 251 suggests that for cells demonstrating tethered motion, a progressive twisting of the surface-attached pilus
 252 fiber during the circular motion of cells may gradually cause the circular motion to stop by pulling the
 253 cell body ever closer to the surface. Although twitching has not been observed in *V. cholerae*, this is one
 254 mechanism by which retraction-like dynamics may be achieved (*Charles et al., 2019*), possibly in
 255 tandem with actual retraction of MSHA pili, which has been shown recently in a different strain of *V.*
 256 *cholera* (*Floyd et al., 2020*). Under our conditions, we have not observed MSHA pili retraction events
 257 nor have we seen bacterial cells that gradually acquire fluorescence when only maleimide dyes were
 258 used. These results are consistent since in bacteria where pilus retraction does occur, such as in the TAD
 259 pili of *Caulobacter crescentus* (*Ellison et al., 2017a*), ChiRP pili of *V. cholera* (*Ellison et al., 2018*), and
 260 TFP of *P. aeruginosa* (*Skerker et al., 2001*), the cell body gradually becomes fluorescent due to
 261 internalization of labeled pili by retraction. Such phenotypical differences may be due to the different
 262 experimental conditions used in each study and require more work to fully elucidate.

263 In addition to possible hydrodynamical effects, our observation that MSHA pili are able to adhere to
 264 surfaces along their entire length highlights their versatility and likely increases the chances of the
 265 formation of a cell-surface attachment. The ability to adhere not only at the distal tip, contrasts with the
 266 TFP of *P. aeruginosa* (*Skerker et al., 2001*) and ChiRP pili of *V. cholerae* (*Ellison et al., 2018*) who
 267 show the pilus-substrate interactions mainly mediated by the pilus tip. Thus, for *V. cholerae*, the strength
 268 of adhesion between a cell and a surface that is mediated by an individual MSHA pilus appears to be
 269 more complicated to model with a single point of attachment. Rather, cells can enhance the adhesion

270 strength by increasing both the length and the number of the MSHA pilus adhered to the surface. This
271 will facilitate cells to become irreversibly attached.

272 Similar running and lingering phases for cells near surface motion has also been reported in
273 enterohaemorrhagic *E. coli* (EHEC) cells (Perez Ipiña *et al.*, 2019), where results suggested that by
274 choosing the optimal transition rates, EHEC bacterial diffusivity is maximized and the surface
275 exploration efficiency is greatly improved. In a future work, it will be interesting to apply similar
276 analysis in *V. cholerae*.

277 In this study, the data collection of *V. cholerae* cells was performed mainly in the solution of 2%
278 LB+1% MC. Rheological measurements show that its viscosity is shear-dependent and has a higher
279 storage modulus than loss modulus in the tested oscillation frequency range, thus it is a non-Newtonian
280 fluid (Figure 6a-c). On the other hand, the rheological measurements of a mucin solution in 2% LB with
281 a mucin concentration of 18.7% (w/w), which was obtained by measuring the concentration of mixtures
282 scraped from the fresh mouse intestine surfaces, show similar viscoelastic behavior with a higher storage
283 modulus than loss modulus in the same tested oscillation frequency range (Figure 6d-f). So compared
284 with Newtonian Ficoll solutions, LB+MC is better to simulate the viscoelastic environment that *V.*
285 *cholerae* cells encounter in the mucus layer of animal intestines. In such viscoelastic environments,
286 Millet *et al.* (Millet *et al.*, 2014) observed considerable differences of bacterial localization in different
287 parts of small intestine and found that *V. cholerae* motility exhibits a regiospecific influence on
288 colonization, indicating viscoelastic intestinal mucin is a key factor limiting colonization. To directly
289 observe cell motion in mucus solutions made from lyophilized mucus powders or fresh mucus-
290 containing solutions scraped from the surfaces of mouse intestines is technically challenging due to the
291 complicated inhomogeneous environment with too many impurities (data not shown). In this work, by
292 direct visualization of pili and flagellum of cells during their landing process in LB+MC that mimic the

real mucus solutions, we find that *V. cholerae* cells can move well in this viscoelastic solution under our conditions. Moreover, we show that the effect of MSHA pili as a braking and anchoring machine on cell landing is more apparent in LB+MC than in 2% LB only or 20% Ficoll solutions, suggesting that MSHA pili might play an even more important role for cell surface attachment in viscoelastic non-Newtonian environments such as in the mucus layer of small intestines.

To summarize, in this work, using fluorescence imaging with labeled pili and flagellum, we show a comprehensive picture of the landing dynamics of *V. cholerae* cells in viscoelastic environments and provide a direct observational evidence exhibiting the role of MSHA pili during cell landing. We hope this can shed insights into the prevention and control of *V. cholerae* infections.

Materials and methods

Bacterial strains

Bacterial strains, plasmids, and primers used in this study are listed in Table 1. *V. cholerae* El Tor C6706 (Joelsson *et al.*, 2006) was used as a parental strain in this study. C6706 and mutants were grown at 30 °C or 37 °C in Luria-Bertani (LB) supplemented with 100 µg/mL streptomycin, 50 µg/mL kanamycin, 1 µg/mL chloromycetin where appropriate. *E. coli* strains harboring plasmids were grown at 37 °C in LB supplemented with 100 µg/mL ampicillin. The optical densities of bacterial cultures were measured at 600 nm (OD₆₀₀) using a UV-vis spectrophotometer.

Table 1. Strains, plasmids, and primers used in this study

Strain	Description	Source or reference
parent	C6706 Sm ^R	(Joelsson <i>et al.</i> , 2006)
$\Delta mshA$	C6706 Sm ^R , VC1807::Cm ^R , <i>mshA</i> knockout	This study
$\Delta flaA$	C6706 Sm ^R , VC1807::Cm ^R , <i>flaA</i> knockout	This study

MshA ^{T70C}	C6706 Sm ^R , VC1807::Km ^R , MshAT70C	(Ellison <i>et al.</i> , 2017b)
FlaA ^{A106C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAA106C	This study
FlaA ^{S107C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAS107C	This study
FlaA ^{A106CS107C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAA106CS107C	This study
FlaA ^{E332C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAE332C	This study
FlaA ^{G23C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAG23C	This study
FlaA ^{N26C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAN26C	This study
FlaA ^{N83C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAN83C	This study
FlaA ^{S325C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAS325C	This study
FlaA ^{S87C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAS87C	This study
FlaA ^{S376C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAS376C	This study
FlaA ^{V117C}	C6706 Sm ^R , VC1807::Cm ^R , FlaAV117C	This study
MshA ^{T70C} , Δ <i>flaA</i>	C6706 Sm ^R , VC1807::Km ^R , <i>flaA</i> knockout	This study
MshA ^{T70C} , FlaA ^{A106C}	C6706 Sm ^R , VC1807::Km ^R , FlaAA106C	This study
MshA ^{T70C} , FlaA ^{S107C}	C6706 Sm ^R , VC1807::Km ^R , FlaAS107C	This study
MshA ^{T70C} , FlaA ^{A106CS107}	C6706 Sm ^R , VC1807::Km ^R , FlaAA106CS107C	This study

312

313 **Flagellin and pilin mutagenesis**

314 Following the protocol in Ellison *et al.* (Ellison *et al.*, 2019; Ellison *et al.*, 2017a), we first predicted
315 10 amino acid residues in *V. cholerae* flagellin FlaA for cysteine replacement. Then the *flaA* knockout
316 and FlaA sequences containing the FlaAA106C, FlaAS107C, FlaAA106CS107C, FlaAE332C,
317 FlaAG23C, FlaAN26C, FlaAN83C, FlaAS325C, FlaAS87C, FlaAS376C, FlaAV117C knock-in were
318 constructed using the MuGENT method (Dalia *et al.*, 2014). The FlaAA106C, FlaAS107C, and
319 FlaAA106CS107C knock-in were constructed by cloning the fragment into the suicide vector pWM91
320 containing a *sacB* counter-selectable marker (Metcalfe *et al.*, 1996). The plasmids were introduced into *V.*

321 *cholerae* by conjugation and mutations were selected for double homologous recombination events. The
322 MshAT70C mutation can be successfully labeled with thiol-reactive maleimide dyes has been described
323 previously (Ellison *et al.*, 2017a), and MshAT70C was constructed using the MuGENT method to light
324 MSHA pilus. All mutants were confirmed by DNA sequencing.

325 **Hemagglutination assays**

326 Mannose-sensitive hemagglutination by *V. cholerae* was measured as described previously (Gardel *et*
327 *al.*, 1996). Briefly, bacteria were grown to the mid-logarithmic phase in LB medium. Initial
328 concentrations of approximately 10^{10} CFU/mL were two-fold diluted with KRT buffer in U-bottomed
329 wells of 96-sample microtiter dishes. Sheep erythrocytes were washed in PBS and resuspended in KRT
330 buffer for a final concentration of 10% vol/vol. Equivoluminal erythrocyte were added into serially
331 diluted bacterial suspensions and the plates were gently agitated at room temperature for 1 min. Samples
332 were checked for hemagglutination after 2 h at room temperature (RT).

333 The results of the Hemagglutination assay test show that MshAT70C displays similar behavior to WT,
334 which indicates that the point mutation in MSHA does not affect MSHA pilus function (Figure 1-Figure
335 supplement 4).

336 **Cell tracking and analysis**

337 **Preparation of viscous solutions**

338 To change the solution viscosity, methyl cellulose (MC) (M20, 4000 cp, Solarbio, China) solutions
339 were prepared by dissolving 0% and 1% (wt/vol) MC in 2% Luria-Bertani (LB) motility medium
340 (containing 171 mM NaCl). 20%(w/v) Ficoll 400 (MW=400 kDa, Yuanye Bio-Technology Co.,Ltd,
341 China) dissolved in 2% LB medium was also prepared for a control experiment.

342 **Preparation of the small intestinal mucus samples from ICR female mice**

343 Six-week-old female ICR mice were provided with drinking water with 10 g/L streptomycin and
 344 0.2g/L aspartame for two days. To clean the small intestine content, food will be removed before 24 h.
 345 Mice were sacrificed, the small intestine was cut open with sterile scissors and the mucus layer was
 346 gently extracted with a cell scraper. Samples were weighed to get wet weight first, then frozen overnight
 347 at -80 °C. After that, they were freeze-dried using a freeze-dryer (Labconco, the United States) in -40 °C
 348 low temperature vacuum. Next, these dried samples were weighted again to get dry weight. The
 349 obtained average value of dry/wet ratio from three repeats is 18.7%.

350 **Cell imaging**

351 For the *V. cholerae* motility observation in 2% LB without MC, overnight cultures in LB were
 352 resuspended and diluted with 2% LB to an OD₆₀₀ ranging from 0.01-0.03. Then the bacterial suspension
 353 was injected into a flow cell, which contained the same media. Imaging was performed using a Phantom
 354 V2512 high-speed camera (Vision Research, USA) collecting ~200 000 bright-field images at 5 ms
 355 resolution with a 100× oil objective on a Leica DMI8 inverted microscope (Leica, Germany) at a set
 356 temperature value of 30°C.

357 For the *V. cholerae* motility observation in 2% LB with 1% MC (henceforth, this medium is referred
 358 to LB+MC), overnight cultures in LB were resuspended and diluted with LB+MC to a final OD₆₀₀ of
 359 0.01-0.03. Then, the bacteria were incubated at 37°C for 20 min to allow them to adapt to the new
 360 environment and were then used immediately. Bacteria samples were pipetted onto standard microscope
 361 slides with a 8 mm diameter spot and then were sealed with a coverslip using a 1 mm thick secure spacer.
 362 Imaging was performed using EMCCD camera (Andor iXon Ultra 888) collecting ~10 000 bright-field
 363 images with a time resolution of 90ms at a set temperature value of 30°C. Similar protocols were carried
 364 out for observations in 20% Ficoll solutions and in the small intestinal mucus samples of mice.

365 **Cell-tracking algorithms and analysis**

The images were preprocessed using a combination of software and algorithms adapted from the methods described (Lee et al., 2016; Utada et al., 2014; Zhao et al., 2013) and written in MATLAB R2015a (Mathworks) by subtracting the background, scaling, smoothing and thresholding. After image processing in this way, the bacteria appear as bright regions. The bacteria shape was fit with a spherocylinder. Then the geometric information of the cell, such as location of the centroid and two poles, and the length and width of the bacterium were collected. Trajectory reconstruction was also achieved for further analysis.

The motility parameters (Utada et al., 2014), such as instantaneous speed, deviation angle, radius of gyration (R_g), MSD and mean path length $\bar{l}$ were calculated to further characterize the near-surface motility of *V. cholerae*. The instantaneous speed was calculated via $|r_{i+1}-r_i|/\Delta t$, where r_i is the cell position vector in frame i and Δt is the time interval between two consecutive frames. The deviation angle of cell motion is defined as the angle between its cell body axis and the direction of motion. The radius of gyration, R_g , is a statistical measure of the spatial extent of the domain of motion given by an ensemble of points that define a trajectory (Rubenstein, 2003). The square of this quantity is defined as $R_g^2 = \frac{1}{N} \sum_{i=1}^N (\vec{R}_i - \vec{R}_{cm})^2$, where N is the number of points in the tracked trajectory, $\vec{R}_i$ is the position vector corresponding to the i -th point on the trajectory, $\vec{R}_{cm}$ is the position vector of the center-of-mass. The MSD of cells was calculated via $\langle \Delta r^2(\tau) \rangle = \langle [r(t + \tau) - r(t)]^2 \rangle$, where $r(t)$ is the position vector of a cell at time t , and τ represents the time lag. The MSD provides information on the average displacement between points in the motility trajectory separated by a fixed time lag. Mean path length $\bar{l}$ were calculated as the average of the total travelling distance of each tracked cell in the field of view.

MSHA pilus labeling, imaging, and quantification

Pilin labeling was achieved using Alexa Fluor 488 C5 Maleimide (AF488-mal; ThermoFisher Scientific, cat. no. A10254) or Alexa Fluor 546 C5 Maleimide (AF546-mal; ThermoFisher Scientific,

cat. no. A10258), which were dissolved in DMSO, aliquoted, and stored at -20°C while being protected from light.

V. cholerae cultures were grown to mid-log phase (OD₆₀₀ 0.8-1.5) before labeling. ~100 µL of culture was mixed with dye at a final concentration of 25 µg/mL (Ellison *et al.*, 2017a) and incubated at RT for 5 min in the dark. Labeled cultures were harvested by centrifugation (900×g, 5 min) and washed twice with PBS, resuspended in 200 µL PBS and imaged immediately. Images were collected using an EMCCD camera on a Leica DMI8 inverted microscope equipped with an Adaptive Focus Control system. The fluorescence of cells labeled with AF488-mal and AF546-mal were detected with FITC and Rhod filter, respectively. The cell bodies were imaged using phase contrast microscopy.

To quantify the number of MSHA pili per cell and cell length, imaging was done under 0.2% PBS gellan gum pads. The cell lengths were measured using ImageJ.

We used AF546-mal and AF488-mal, in turn, for the two-color labeling to observe the growth of pili. We first, labeled log-phase cells with AF546-mal for the primary staining by incubating for 20 min, followed by two successive washes in PBS by centrifugation. The cells were then resuspended in LB and incubated for an additional 40 min at 30 °C. For the secondary staining, we incubated the cells in AF488-mal for 5 min, washed twice with PBS, and then imaged the cells immediately using phase contrast, FITC, and RhoD channels.

Fluorescence video acquisition of MSHA pilus-labelled cells motility in LB + MC

The labeled cells were centrifugated, resuspended in ~20 µL PBS, and then diluted in 500 µL of the viscous solution of LB + MC. The solution was then immediately pipetted onto a standard microscope slides. Fluorescence images were acquired at 130 ms intervals for a total of about 2-5 min. After a few minutes of fluorescence imaging, most cells in the field of view have attached to the surface, while the

fluorescence was bleached due to the continuous exposure. We recorded images from different locations to capture new instances of bacterial movement and adhesion events.

Rheological measurements

Rheological measurements of 1% MC+2% LB solution and 18.7% (w/w) mucin (Sigma, USA) in 2% LB solution were carried out on a rheometer (DHR-2, TA Instruments, Waters LLC) using a cone-plate geometry with a diameter of 40 mm and a cone angle of 5° at 26°C. Here, because when they were observed on a microscope, the real temperature of samples at the observation window site is lower than the set value of 30°C due to the relatively low thermal conductivity of glass, which is estimated to be 26 °C ~28 °C, the rheological measurements were performed at 26 °C. The viscosity curves were determined at shear rates of 0.01-1000/s. And the storage modulus and loss modulus as a function of the testing oscillation strain and oscillation frequency were also recorded. For each tested solution, it was left standing for 15 min prior to each measurement for equilibrium, and it was covered with a thin layer of silicon oil to prevent loss of moisture during measurement.

Statistical analysis

Statistical significance was determined with One way ANOVA followed by Tukey's multiple comparison test comparing the different groups. (*p < 0.05; **p < 0.01; ***p < 0.001). The data were analyzed using the Prism 5.0 software program (GraphPad Software, La Jolla, CA, USA).

429

430 **Acknowledgements**

431 We thank Zhanglin Hou and Thomas G. Mason for their help with scientific discussions.

432 **Additional information**

433 **Funding**

Funder	Grant reference number	Author
National Key R&D Program of China	2018YFA0902102	Kun Zhao, Wenchao Zhang, Chunying Feng
National Natural Science Foundation of China (NSFC)	31770132	Zhi Liu, Mei Luo
National Natural Science Foundation of China (NSFC)	81572050	Zhi Liu, Mei Luo
National Natural Science Foundation of China (NSFC)	21621004	Kun Zhao, Wenchao Zhang, Chunying Feng
University of Bristol	Vice-Chancellor's Fellowship	Rachel R. Bennett
Grant in aid for Young Scientists (B) from the Japanese Society for the Promotion of Science (JSPS)	17K15410	Andrew S. Utada

434

435 The funders had no role in study design, data collection and interpretation, or the decision to submit
436 the work for publication.

437

438 **Conflict of interest** The authors declare that they have no conflict of interest.

439 **Additional files**

440 Supplementary File 1. Including Supplementary table S1 and Supplementary references.

441 Source code 1. Source code for Figure 5a and b trajectory plots.

442 Transparent reporting form

443 **Data availability**

444 Source data files and/or MATLAB code have been provided for Figures 1-6.

Figures

Figure 1. MSHA pili are evenly distributed along cell length with a constant length density. (a) Examples of labeled MSHA pili observed on cell bodies. Green fluorescence showing the AF488-mal labeled MSHA pili, red fluorescence showing the FM4-64 labeled plasma membrane. (b) Distribution of pili number per cell cultivated in LB medium. $N_{\text{cell}} = 110$. (c) The MSHA pili number per cell is linearly correlated with the cell length. Cells with longer length were obtained by 30~50 min treatment using 10 μg/mL cephalixin. $N_{\text{cell}} = 368$.

Figure 1-source data 1. Figure 1b source data.

Figure 1-source data 2. Figure 1c source data.

Figure 1-Figure supplement 1. Labeling of *V. cholerae* MSHA pilus protein MshA with AF488-mal.

Figure 1-Figure supplement 2. A 3D view of a typical *V. cholerae* cell showing the whole body distribution of MSHA pili; This cell has 6 pili.

Figure 1-Figure supplement 3. MSHA pili labeling of the prepared samples. To evaluate changes to the MSHA pilus during cell growth, the MSHA pili were labelled with two different colored dyes, AF546-mal (red) and AF488-mal (green), at 0 min and 40 min, respectively. (a) Representative double-color labeling image of MshAT70C cell, showing the new separate pilus (top left in green) and the secondary segments (lower left, green) at the end of the primary segments (lower left, red). Scale bar, 2

464 μm . (b) Representative double-color labeling image for a newly dividing cell, which is only labelled
 465 with AF488-mal. (c) In situ observation of MSHA pili growth stained at 0 and 50 min with AF488-mal.
 466 The results show that during a period of 50 min, the length of the cell changes from 3.0 μm to 5.1 μm ,
 467 while the number of pili increases from 7 to 12.

468 **Figure 1-Figure supplement 4. Hemagglutination assays.** MshAT70C point mutation does not affect
 469 MSHA pilus function. *V. cholerae* strains were grown in LB medium and assayed for MSHA production
 470 by hemagglutination. Two-fold dilutions of mid-log cultures of bacteria (left to right) were assayed for
 471 their ability to agglutinate sheep erythrocytes. Assay was repeated three times, and representative results
 472 are showed.

473

Figure 2. Analysis of roaming and orbiting, using cells of MSHA labelled MshAT70C. The 3D plot and speed changes over time of representative (a-b) roaming and (c-d) orbiting cells, respectively. The magenta dashed lines in panel (a) and (c) are the trajectories of cells and the color maps indicate the deviation angle. The arrows in panel (b) represent temporary attachment between MSHA pili and surface, where the speeds are close to 0. (e) Time-sequence snapshots of the orbiting cell in panel (c-d) at 130 ms intervals (see Video 2 for more details). The arrowheads show the stretched pilus, which

corresponds to the red arrow in panel (d), indicating temporary attachment and stretching of pilus on the surface. Dashed lines indicate the estimated envelope of the cell body. Scale bar, 2 μm .

Figure 2-source data 1. Figure 2a source data.

Figure 2-source data 2. Figure 2b source data.

Figure 2-source data 3. Figure 2c source data.

Figure 2-source data 4. Figure 2d source data.

Figure 2-Figure supplement 1. Quantitative analysis of roaming and orbiting by MSHA labelled MshAT70C in 2% LB with 1% MC. (a) Trajectories and (b) speed of typical roaming cells; (c) Trajectories and (d) speed of typical orbiting cells.

Figure 2-Figure supplement 2. Switch of temporary attached pili. When transient pauses happened, the attached pilus could be switched from one to another or more. The arrows show the apparent pili attached with surface. Scale bar, 1 μm .

Figure 3. An example of a typical landing sequence of a *V. cholerae* cell with MSHA pili and flagella both labelled (MshAT70CFlaAA106CS107C). (a) Representative image sequences showing the behavior of MSHA pili and flagella. For easy identification, four pili of the example cell in Figure 3a were numbered from 1 to 4, which revolve around the major axis of the cell periodically as the cell swims. The white arrowheads indicated the orientation of cell body and flagellum. (b) A 3D plot of speed and deviation angle of the representative cell in panel (a) over its trajectory. The red arrow in panel (b) represents the position, where the pili touch surface, causing a deflection. (c) The conditional

504 probabilities q_{ij} that the bacterium transitions from state i to j . The number of transition events used for
 505 estimating these conditional probabilities is 666. r: running state, t: tethered state, p: paused state.

506 **Figure 3-source data 1. Figure 3b source data.**

507 **Figure 3-source data 2. Figure 3c source data.**

508

509

Figure 4. Characterization of running and tethered cells. (a) Distribution of the rotation period of cell body. The dashed line represents Gaussian fitting. A total of 416 rotation events from 54 cells were used for statistical analysis. (b) Measured relation between the rotation rate of cell body and the mean swimming speed of cell. The dotted line represents linear fitting result. $N_{\text{cell}} = 47$. (c) An example of a typical tethered motion, showing cell performing a circular motion around a center point (the red dot) with the direction of motion (noted by arrows) switched from CCW to CW. Scale bar, 2 μm . (d) The angular velocity of the tethered cell in panel (c) over a short duration showing a pair of CW (positive angular velocity) and CCW (negative angular velocity) intervals; (e) Distribution of angular speed of

519 circular motion for horizontal (241 intervals from 25 cells) and vertical (38 intervals from 5 cells)
520 tethered cells.

521 **Figure 4-source data 1. Figure 4a source data.**

522 **Figure 4-source data 2. Figure 4b source data.**

523 **Figure 4-source data 3. Figure 4d source data.**

524 **Figure 4-source data 4. Figure 4e source data.**

525 **Figure 4-Figure supplement 1. Examples show positions of two poles and centroid of tethered**
526 **motility.** The contributing MSHA pili were indicated by yellow arrows. (a) $\sim 1/2$ position; (b) $1/3$ or $2/3$
527 position.

528

529

Figure 5. Role of MSHA pili in cell landing is more apparent in viscoelastic non-Newtonian solutions than viscous Newtonian ones. (a) Examples of WT cell trajectories showing both roaming and orbiting motilities in 2% LB only, 2% LB with 1% MC and 2% LB with 20% Ficoll; (b) Examples of cell trajectories of $\Delta mshA$; (c) Histograms of R_g of WT and $\Delta mshA$ in different solutions; (d) A box

535 plot summary of path lengths of WT and $\Delta mshA$; (e) The ratio of mean path length between $\Delta mshA$ and
 536 WT, $\bar{l}_{\Delta mshA}/\bar{l}_{WT}$.

537 **Figure 5-source data 1. Figure 5c source data.**

538 **Figure 5-source data 2. Figure 5d source data.**

539 **Figure 5-source data 3. Figure 5e source data.**

540 **Figure 5-Figure supplement 1. Motility characterization of WT and $\Delta mshA$ cells in 2% LB only**
 541 **and in 2% LB with 1% MC.** (a) Histograms of deviation angle for WT in 2% LB only. (b) Histograms
 542 of deviation angle for WT in 2% LB+1% MC viscous solution. Black represents orbiting motility and
 543 red represents roaming motility. (c-d) Mean square displacements (MSDs) of WT (c), $\Delta mshA$ (d) in 2%
 544 LB only and 2% LB+1% MC viscous solution.

545

546

Figure 6. Viscoelasticity characterization of 1% MC solution in 2% LB (a-c) and a mucin solution of 18.7%(w/w) in 2% LB (d-f) at 26°C. (a) and (d) show viscosity as a function of shear rate. (b) and (e) display modulus as a function of the oscillation strain, using cone-plate geometry. (c) and (f) show modulus as a function of the oscillation frequency under an oscillation strain of 0.1%, using cone-plate geometry.

Figure 6-source data 1. Figure 6a source data.

Figure 6-source data 2. Figure 6b source data.

Figure 6-source data 3. Figure 6c source data.

Figure 6-source data 4. Figure 6d source data.

Figure 6-source data 5. Figure 6e source data.

Figure 6-source data 6. Figure 6f source data.

Figure 7. Diagrammatic sketch for the landing process of *V. cholera* cells on a substrate.

Video Legends

Video 1. Time-lapse fluorescence imaging showing a typical roaming cell (indicated by the arrowhead) with labeled MSHA pili in 2% LB+1% MC viscous medium. This video was shown every 390 ms for 98 s and displayed at 20 frames per second (fps).

Video 2. Time-lapse fluorescence imaging showing a typical orbiting cell with labeled MSHA pili in 2% LB+1% MC viscous medium. This video was recorded every 130 ms for 15 s and displayed at 10 fps.

570 **Video 3. Time-lapse fluorescence imaging showing switch of pili.** When transient pauses happened,
571 the attached pilus could be switched from one to another or more. See also Figure S4. This video was
572 recorded every 70 ms for 10 s and displayed at 5 fps.

573 **Video 4. Time-lapse fluorescence imaging showing linear motion bent into circular motion that is**
574 **centered around the attachment point between MSHA pili and the surface, which can act as an**
575 **anchor point.** This video was recorded every 130 ms for 8 s and displayed at 10 fps.

576 **Video 5. Time-lapse fluorescence imaging showing dynamic movements of a**
577 **MshAT70CFlaAA106CS107C cell with labeled flagellum and MSHA pili in 2% LB+1% MC**
578 **viscous medium.** This video was recorded every 130 ms for 13 s and displayed at 10 fps.

579 **Video 6. Time-lapse fluorescence imaging showing five MSHA pili of a WT cell stuck to the**
580 **surface and kept still or fluctuated frequently.** This video was recorded every 460 ms for 25 s and
581 displayed at 10 fps.

582 **Video 7. Time-lapse fluorescence imaging showing a typical tethered cell performing a circular**
583 **motion around a fixed point with the direction of motion switched from CCW to CW.** See also
584 Figure 4c. This video was recorded every 130 ms for 6 s and displayed at 5 fps.

585 **Video 8. Time-lapse fluorescence imaging showing different adhesion points of a pilus.** When the
586 tip of the pilus was free (~3.5 s), the upper part of the pilus was still capable of keeping the cell adhered.
587 This video was recorded every 130 ms for 13 s and displayed at 10 fps.

588 **Video 9. Time-lapse fluorescence imaging showing the motion evolution of flagellum from rotating**
589 **to stopping eventually.** This video was recorded every 130 ms for 10 s and displayed at 10 fps.

590

591

References

- Almagromoreno S, Pruss K, Taylor RK. 2015. Intestinal colonization dynamics of *Vibrio cholerae*. *PLoS Pathogens* **11**: e1004787. DOI:10.1371/journal.ppat.1004787, PMID: 25996593
- Bennett RR, Lee CK, De AJ, Nealson KH, Yildiz FH, O'Toole GA, Wong GC, Golestanian R. 2016. Species-dependent hydrodynamics of flagellum-tethered bacteria in early biofilm development. *Journal of the Royal Society Interface* **13**:20150966. DOI: 10.1098/rsif.2015.0966, PMID: 26864892
- Blair KM, Turner L, Winkelman JT, Berg HC, Kearns DB. 2008. A molecular clutch disables flagella in the *Bacillus subtilis* biofilm. *Science* **320**: 1636-1638. DOI:10.1126/science.1157877, PMID: 18566286
- Charles N, Gazzola M, Mahadevan L. 2019. Topology, geometry, and mechanics of strongly stretched and twisted filaments: solenoids, plectonemes, and artificial muscle fibers. *Physical Review Letters* **123**: 208003. DOI: 10.1103/PhysRevLett.123.208003, PMID: 31809094
- Chen X, Berg HC. 2000. Torque-speed relationship of the flagellar rotary motor of *Escherichia coli*. *Biophysical Journal* **78**: 1036-1041. DOI: 10.1016/S0006-3495(00)76662-8, PMID: 10653817
- Dalia AB, McDonough E, Camilli A. 2014. Multiplex genome editing by natural transformation. *PNAS* **111**: 8937-8942. DOI: 10.1073/pnas.1406478111, PMID: 24889608
- Darnton NC, Turner L, Rojevsky S, Berg HC. 2007. On torque and tumbling in swimming *Escherichia coli*. *Journal of Bacteriology* **189**: 1756-1764. DOI: 10.1128/JB.01501-06, PMID: 17189361
- Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews* **15**: 167-193. DOI: 10.1128/cmr.15.2.167-193.2002, PMID: 11932229
- Ellison CK, Dalia TN, Dalia AB, Brun YV. 2019. Real-time microscopy and physical perturbation of bacterial pili using maleimide-conjugated molecules. *Nat Protoc* **14**: 1803-1819. DOI: 10.1038/s41596-019-0162-6, PMID: 31028374

615 Ellison CK, Dalia TN, Vidal Ceballos A, Wang JC-Y, Biais N, Brun YV, Dalia AB. 2018. Retraction of
616 DNA-bound type IV competence pili initiates DNA uptake during natural transformation in *Vibrio*
617 *cholerae*. *Nature Microbiology* **3**: 773-780. DOI: 10.1038/s41564-018-0174-y, PMID: 29891864

618 Ellison CK, Kan J, Dillard RS, Kysela DT, Ducret A, Berne C, Hampton CM, Ke Z, Wright ER, Biais N.
619 2017. Obstruction of pilus retraction stimulates bacterial surface sensing. *Science* **358**: 535-538.
620 DOI: 10.1126/science.aan5706, PMID: 29074778

621 Floyd KA, Lee CK, Xian W, Nametalla M, Valentine A, Crair B, Zhu S, Hughes HQ, Chlebek JL, Wu DC,
622 Hwan Park J, Farhat AM, Lomba CJ, Ellison CK, Brun YV, Campos-Gomez J, Dalia AB, Liu J, Biais N,
623 Wong GCL et al. 2020. C-di-GMP modulates type IV MSHA pilus retraction and surface attachment in
624 *Vibrio cholerae*. *Nature Communication* **11**: 1549. DOI: 10.1038/s41467-020-15331-8,
625 PMID: 32214098

626 Friedlander RS, Vlamakis H, Kim P, Khan M, Kolter R, Aizenberg J. 2013. Bacterial flagella explore
627 microscale hummocks and hollows to increase adhesion. *PNAS* **110**: 5624-5629.
628 DOI: 10.1073/pnas.1219662110, PMID: 23509269

629 Gardel CL, Mekalanos JJ. 1996. Alterations in *Vibrio cholerae* motility phenotypes correlate with changes
630 in virulence factor expression. *Infect Immun* **64**: 2246-2255. PMID:8675334

631 Gibiansky ML, Conrad JC, Jin F, Gordon VD, Motto DA, Mathewson MA, Stopka WG, Zelasko DC,
632 Shrout JD, Wong GC. 2010. Bacteria use type IV pili to walk upright and detach from surfaces. *Science*
633 **330**: 197. DOI: 10.1126/science.1194238, PMID: 20929769

634 Guttenplan SB, Kearns DB. 2013. Regulation of flagellar motility during biofilm formation. *FEMS*
635 *Microbiology Reviews* **37**: 849-871. DOI: 10.1111/1574-6976.12018, PMID: 23480406

636 Higashi DL, Lee SW, Snyder A, Weyand NJ, Bakke A, So M. 2007. Dynamics of *Neisseria gonorrhoeae*
637 attachment: microcolony development, cortical plaque formation, and cytoprotection. *Infection and*
638 *Immunity* **75**: 4743-4753. DOI: 10.1128/IAI.00687-07, PMID: 17682045

639 Joelsson A, Liu Z, Zhu J. 2006. Genetic and phenotypic diversity of quorum-sensing systems in clinical
640 and environmental isolates of *Vibrio cholerae*. *Infect Immun* **74**: 1141-1147.
641 DOI: 10.1128/IAI.74.2.1141-1147.2006, PMID: 16428762

642 Kaper J B MJG, Levine M M. 1995. Cholera. *Clinical Microbiology Reviews* **8**: 48-86. PMID: 7704895

643 Kirn TJ, Lafferty MJ, Sandoe CMP, Taylor RK. 2000. Delineation of pilin domains required for bacterial
644 association into microcolonies and intestinal colonization by *Vibrio cholerae*. *Molecular Microbiology*
645 **35**: 896-910. DOI: 10.1046/j.1365-2958.2000.01764.x, PMID: 10692166

646 Krukons ES, Dirita VJ. 2003. From motility to virulence: Sensing and responding to environmental
647 signals in *Vibrio cholerae*. *Current Opinion in Microbiology* **6**: 186-190. DOI: 10.1016/s1369-
648 5274(03)00032-8, PMID: 12732310

649 Lai SK, Wang Y-Y, Wirtz D, Hanes J. 2009. Micro-and macrorheology of mucus. *Advanced Drug*
650 *Delivery Reviews* **61**: 86-100. DOI: 10.1016/j.addr.2008.09.012, PMID: 19166889

651 Lee CK, Kim AJ, Santos GS, Lai PY, Lee SY, Qiao DF, De AJ, Young TD, Chen Y, Rowe AR. 2016.
652 Evolution of cell size homeostasis and growth rate diversity during initial surface colonization of
653 *Shewanella oneidensis*. *Acs Nano* **10**:9183-9192. DOI: 10.1021/acsnano.6b05123, PMID: 27571459

654 Lee J-H, Rho JB, Park K-J, Kim CB, Han Y-S, Choi SH, Lee K-H, Park S-J. 2004. Role of flagellum and
655 motility in pathogenesis of *Vibrio vulnificus*. *Infection and Immunity* **72**: 4905-4910.
656 DOI: 10.1128/IAI.72.8.4905-4910.2004, PMID: 15271959

657 Magariyama Y, Kudo S. 2002. A mathematical explanation of an increase in bacterial swimming speed
658 with viscosity in linear-polymer solutions. *Biophysical Journal* **83**: 733-739. DOI: 10.1016/S0006-
659 3495(02)75204-1, PMID: 12124260

660 Maier B, Potter L, So M, Seifert HS, Sheetz MP. 2002. Single pilus motor forces exceed 100 pN. *PNAS* **99**:
661 16012-16017. DOI: 10.1073/pnas.242523299, PMID: 12446837

662 Meibom KL, Li XB, Nielsen AT, Wu CY, Roseman S, Schoolnik GK. 2004. The *Vibrio cholerae* chitin
663 utilization program. *PNAS* **101**: 2524-2529. DOI: 10.1073/pnas.0308707101, PMID: 14983042

664 Metcalf WW, Jiang W, Daniels LL, Kim SK, Haldimann A, Wanner BL. 1996. Conditionally replicative
665 and conjugative plasmids carrying lacZ alpha for cloning, mutagenesis, and allele replacement in
666 bacteria. *Plasmid* **35**: 1-13. DOI: 10.1006/plas.1996.0001, PMID: 8693022

667 Millet YA, Alvarez D, Ringgaard S, von Andrian UH, Davis BM, Waldor MK. 2014. Insights into *Vibrio*
668 *cholerae* intestinal colonization from monitoring fluorescently labeled bacteria. *PLoS Pathogens* **10**:
669 e1004405. DOI: 10.1371/journal.ppat.1004405, PMID: 25275396

670 Nakane D, Nishizaka T. 2017. Asymmetric distribution of type IV pili triggered by directional light in
671 unicellular cyanobacteria. *PNAS* **114**: 6593-6598. DOI: 10.1073/pnas.1702395114, PMID: 28584115

672 O'Toole GA, Kolter R. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa*
673 biofilm development. *Molecular Microbiology* **30**: 295–304. DOI: 10.1046/j.1365-
674 2958.1998.01062.x, PMID: 9791175

675 Perez Ipiña E, Otte S, Pontier-Bres R, Czerucka D, Peruani F. 2019. Bacteria display optimal transport
676 near surfaces. *Nature Physics* **15**: 610-615. DOI: 10.1038/s41567-019-0460-5

677 Piepenbrink KH, Sundberg EJ. 2016. Motility and adhesion through type IV pili in Gram-positive bacteria.
678 *Biochemical Society Transactions* **44**: 1659. DOI: 10.1042/BST20160221, PMID: 27913675.

679 Pratt LA, Kolter R. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility,
680 chemotaxis and type I pili. *Molecular Microbiology* **30**: 285-293. DOI: 10.1046/j.1365-
681 2958.1998.01061.x, PMID: 9791174

682 Reguera G, Kolter R. 2005. Virulence and the environment: a novel role for *Vibrio cholerae* toxin-
683 coregulated pili in biofilm formation on chitin. *Journal of Bacteriology* **187**: 3551-3555.
684 DOI: 10.1128/JB.187.10.3551-3555.2005, PMID: 15866944

685 Renault TT, Abraham AO, Bergmiller T, Paradis G, Rainville S, Charpentier E, Guet CC, Tu Y, Namba K,
686 Keener JP. 2017. Bacterial flagella grow through an injection-diffusion mechanism. *Elife* **6**: e23136.
687 DOI: 10.7554/eLife.23136, PMID: 28262091

688 Rubenstein MC, R. H. 2003. Polymer Physics. *Oxford University Press*: 60-66.

689 Samad T, Billings N, Birjiniuk A, Crouzier T, Doyle PS, Ribbeck K. 2017. Swimming bacteria promote
690 dispersal of non-motile staphylococcal species. *The ISME journal* **11**: 1933.DOI: 10.1038/ismej.2017.23,
691 PMID: 28398350

692 Silva AJ, Benitez JA. 2016. *Vibrio cholerae* biofilms and Cholera pathogenesis. *Plos Negl Trop Dis* **10**:
693 e0004330. DOI: 10.1371/journal.pntd.0004330, PMID: 26845681

694 Skerker JM, Berg HC. 2001. Direct observation of extension and retraction of type IV pili. *PNAS* **98**:
695 6901-6904. DOI: 10.1073/pnas.121171698, PMID: 11381130

696 Talà L, Fineberg A, Kukura P, Persat A. 2019. *Pseudomonas aeruginosa* orchestrates twitching motility
697 by sequential control of type IV pili movements. *Nature microbiology* **4**: 774-780.
698 DOI: 10.1038/s41564-019-0378-9, PMID: 30804544

699 Teschler JK, Zamoranosánchez D, Utada AS, Warner CJ, Wong GC, Linington RG, Yildiz FH. 2015.
700 Living in the matrix: assembly and control of *Vibrio cholerae* biofilms. *Nature Reviews Microbiology*
701 **13**: 255-268. DOI: 10.1038/nrmicro3433, PMID: 25895940.

Thelin KH, Taylor RK. 1996. Toxin-coregulated pilus, but not mannose-sensitive hemagglutinin, is required for colonization by *Vibrio cholerae* O1 El Tor biotype and O139 strains. *Infection & Immunity* **64**: 2853-2856.

Tsou A, Frey E, Hsiao A, Liu Z, Zhu J. 2008. Coordinated regulation of virulence by quorum sensing and motility pathways during the initial stages of *Vibrio cholerae* infection. *Communicative & integrative biology* **1**: 42-44. DOI: 10.4161/cib.1.1.6662, PMID: 19704787

Utada AS, Bennett RR, Fong JC, Gibiansky ML, Yildiz FH, Golestanian R, Wong GC. 2014. *Vibrio cholerae* use pili and flagella synergistically to effect motility switching and conditional surface attachment. *Nature Communication* **5**: 4913. DOI: 10.1038/ncomms5913, PMID: 25234699

Vigeant MA, Ford RM, Wagner M, Tamm LK. 2002. Reversible and irreversible adhesion of motile *Escherichia coli* cells analyzed by total internal reflection aqueous fluorescence microscopy. *Applied & Environmental Microbiology* **68**: 2794-2801. DOI: 10.1128/aem.68.6.2794-2801.2002, PMID: 12039734

Watnick PI, Kolter R. 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Molecular Microbiology* **34**: 586-595. DOI: 10.1046/j.1365-2958.1999.01624.x, PMID: 10564499

Winet H. 1976. Ciliary propulsion of objects in tubes: wall drag on swimming Tetrahymena (Ciliata) in the presence of mucin and other long-chain polymers. *Journal of Experimental Biology* **64**: 283.

Yildiz FH, Visick KL. 2009. *Vibrio* biofilms: so much the same yet so different. *Trends in Microbiology* **17**: 109-118. DOI: 10.1016/j.tim.2008.12.004, PMID: 19231189

Zhao K, Tseng BS, Beckerman B, Jin F, Gibiansky ML, Harrison JJ, Luijten E, Parsek MR, Wong GC. 2013. Psl trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms. *Nature* **497**: 388-391. DOI: 10.1038/nature12155, PMID: 23657259

724 Zöttl A, Yeomans JM. 2019. Enhanced bacterial swimming speeds in macromolecular polymer solutions.
 725 *Nature Physics*: 1.
 726