Alkaline pH increases swimming speed and facilitates mucus penetration for *Vibrio cholerae*.

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Abstract

Intestinal mucus is the first line of defense against intestinal pathogens. It acts as a physical barrier between epithelial tissues and the lumen that enteropathogens must overcome to establish a successful infection. We investigated the motile behavior of two *V. cholerae* strains (El Tor C6706 and Classical O395) in mucus using single cell tracking in unprocessed porcine intestinal mucus. We determined that *V. cholerae* is able to penetrate mucus using flagellar motility and that alkaline pH increases swimming speed, and consequently, improves mucus penetration. Microrheological measurements indicate that changes in pH between 6 and 8 (the physiological range for the human small intestine) had little effect on the viscoelastic properties of mucus. Finally, we determined that acidic pH promotes surface attachment by activating the mannose-sensitive haemagglutinin (MshA) pilus in *V. cholerae* El Tor C6706 without a measurable change in the total cellular concentration of the secondary messenger cyclic dimeric guanosine monophosphate (c-di-GMP). Overall, our results support that pH is an important factor affecting the motile behavior of *V. cholerae* and its ability to penetrate mucus. Therefore, changes in pH along the human small intestine may play a role in determining the preferred site for *V. cholerae* during infection.
The diarrheal disease cholera is still a burden for populations in developing countries with poor sanitation. To develop effective vaccines and prevention strategies against *Vibrio cholerae*, we must understand the initial steps of infection leading to the colonization of the small intestine. To infect the host and deliver the cholera toxin, *V. cholerae* has to penetrate the mucus layer protecting the intestinal tissues. However, the interaction of *V. cholerae* with intestinal mucus has not been extensively investigated. In this report, we demonstrated using single cell tracking that *V. cholerae* is able to penetrate intestinal mucus using flagellar motility. In addition, we observed that alkaline pH improves the ability of *V. cholerae* to penetrate mucus. This finding has important implications for understanding the dynamics of infection because pH varies significantly along the small intestine, between individuals, and between species. Blocking mucus penetration by interfering with flagellar motility in *V. cholerae*, reinforcing the mucosa, controlling intestinal pH, or manipulating the intestinal microbiome, will offer new strategies to fight cholera.
**Introduction**

*Vibrio cholerae* is the cause of an ongoing cholera pandemic with up to 4 million cases per year from regions of the world that do not have access to potable water (1). Without proper rehydration and antibiotic treatments, severe diarrhea triggered by the cholera toxin can be fatal (2). Preventative measures and vaccines against *V. cholerae* have had partial success (3, 4), but cholera outbreaks are still a significant burden for populations living in developing regions or after natural disaster, such as Bangladesh and Haiti (1).

*V. cholerae* is represented by more than 200 serogroups that are endemic to sea and brackish waters and often found associated with copepods (5, 6). However, only the O1 and 0139 serogroups have been associated with cholera, the diarrheal disease in humans (7). Within the O1 serogroup, the Classical biotype dominated the first 6 recorded cholera pandemics. The ongoing 7th pandemic is dominated by the El Tor biotype, which has rapidly displaced the Classical biotype in the environment (8, 9). Although similar, the two biotypes have differences in their genetic makeups, signaling dynamics, and behaviors (10–12). The relative importance of these unique traits has not been fully elucidated yet.

*V. cholerae* colonize the mucus of the small intestine without invading epithelial cells. When reaching the intestinal crypts, *V. cholerae* secretes the cholera toxin, which targets epithelial cells to activate the chlorine channel proteins and consequently trigger a massive efflux of chlorine ions and water into the intestinal lumen. Many aspects of *V. cholerae* physiology and the regulation virulence factor expression have been investigated to recapitulate the dynamics...
of infection after ingestion (13–15), such as pili production (16), type 6 secretion system (17),
quorum sensing (18), biofilm formation (19), and flagellar motility (20). While these different
behaviors have been shown to contribute to *V. cholerae* success during infection, the specific
sequence of events and site-specific activities in the intestine are still under investigation.

Flagellar motility is essential for *V. cholerae* infection. Studies of transcription profiles and
screens of mutant libraries during the infection of animal models and humans identified genes
involved in chemotaxis and motility functions (21). Non-motile *V. cholerae* mutants have
reduced virulence and intestinal colonization (22–24). In addition, previous work supports that
protective immunity is mostly provided by mucosal antibodies that inhibit *V. cholerae* motility
through bivalent binding of the O-antigen (25). Motility may not be required for survival and
growth in the intestine since non-motile mutants do not appear to suffer a large competitive
disadvantage when inoculated with motile *V. cholerae* (26). However, flagellar motility is likely
necessary to penetrate the mucus layer protecting the intestinal tissue and reach epithelial cells
to deliver the cholera toxin.

Mucus is a complex hydrogel made of mucins (2-10% w/v), lipids, and DNA (27) and is difficult
for motile bacteria to penetrate. Mucins are large and highly glycosylated proteins cross-linked
by disulfide bonds reinforced by hydrophobic interactions to form a tight mesh. The intestinal
mucus layer is continuously renewed by secretion of highly O-glycosylated MUC2 mucin by
goblet cells (240 ± 60 µm per hour) (28). Consequently, mucus forms a selective diffusion
barrier undergoing continuous regeneration, the rate of which can increase in response to
threats such as the cholera toxin (29). Histological analyses revealed that the inner part of the
mucus layer is mostly free of bacteria (30). In the small intestine, the mucus layer is thinner in
the proximal part (~200 μm) than the distal part (~500 μm) (31). These observations raise the
questions of how *V. cholerae* can penetrate mucus and why it preferably infects the distal small
intestine where the mucosa is thicker.

Few studies have directly observed the motile behavior of individual bacteria in mucus to
characterize the strategy used to compromise the protective layer. *Helicobacter pylori*, which
colonizes the thick mucus layer of the stomach, facilitates flagellar motility through mucus by
enzymatically increasing the local pH to liquify the mucus gel structure (32, 33). It is also
believed that the helical cell shape of both *H. pylori* and *Campylobacter jejuni*, which colonizes
the thick mucus layer of the cecum, facilitates mucus penetration by allowing the body to push
against the mucin matrix like a corkscrew (34, 35). Recent work demonstrated that the
peritrichous rod-shaped bacteria, *Escherichia coli* and *Bacillus subtilis*, are able to penetrate
cervical mucus by taking advantage of water channels created by shear forces during secretion
(36). The behavior of *V. cholerae* in mucus has not been described.

In this study, we characterized the behavior of individual cells from two *V. cholerae* strains in
unprocessed porcine intestinal mucus and tested if *V. cholerae* alters the rheological properties
of mucus over time. We demonstrated that *V. cholerae* is able to swim through porcine
intestinal mucus even without measurable changes in mucus rheology and measured that
porcine intestinal mucus is not sensitive to change in pH between 6 and 8. However, alkaline
conditions dramatically increase swimming speed and mucus penetration for *V. cholerae*. These
results shed light on how *V. cholerae* is able to overcome the defensive mucus layer and the
role of intestinal pH during the initial stage of infection.
Results

V. cholerae can penetrate intestinal mucus using flagellar motility

We tracked fluorescently labeled V. cholerae Classical O395 in unprocessed mucus that was scraped from the medial part of the small intestine of an adult pig (Figure 1AB). Porcine mucus has been shown to be the most comparable to human mucus regarding structure and thickness when compared to several animal models and also act as a physical barrier between intestinal tissues and bacteria in the lumen (37, 38). As expected, the movement of V. cholerae, as quantified by the trajectory effective diffusion coefficient (Figure 1C), is severely impaired in mucus when compared to swimming in a liquid environment (Figure 1D). To determine the proportion of cells using flagellar motility to swim through mucus, we also measured the effective diffusion coefficient of non-flagellated cells (flrA mutant) and determined that a diffusion coefficient above $10^{-0.5} \, \mu m^2/s$ was evidence of flagellar motility (Figure 1D). Most wild-type cells (~75%) were trapped and unable to swim through the mucus mesh. The rest of the population (~25%) was able to swim through the mucus while being caught in the mucus mesh only intermittently. Because cells were not moving freely and did not have a constant diffusion coefficient, the reported diffusion coefficient represents an average over the entire length of each trajectory.

Alkaline pH improves the motility of V. cholerae in intestinal mucus

V. cholerae appears to colonize preferentially the lower part of the small intestine (ileum) where the mucus layer is thicker (31). The ileum is also the most alkaline region of the small intestine (pH 7-8), whereas the jejunum (upper part) is slightly acidic (pH 6-7) (39). Therefore,
we tested if pH influenced the motile behavior of *V. cholerae* in intestinal mucus. We equilibrated unprocessed porcine intestinal mucus with phosphate saline buffer at pH 6, 7, and 8. We then tracked the swimming behavior of both *V. cholerae* Classical O395 and El Tor C6706 in mucus at each pH. The proportions of swimming cells and the swimming speeds increased as pH increased for both strains (p-values < 10^-4) (Figure 2AB). At pH 8, 51% of Classical O395 and 76% of El Tor C6706 were able to swim through the mucus. Directional persistence (the time scale at which cells change direction) did not show a response indicating that the reversal frequency of the flagellar motor was not affect by the change in pH (Figure 2C). Overall, alkaline pH improves the motility of *V. cholerae* in mucus, but pH could be affecting either the rheological properties of mucus or the physiology of *V. cholerae*.

**Change in pH between 6 and 8 had little effects on the mucus rheological properties**

To test if pH affects the structure of mucus, we tracked the motion of 1 µm fluorescent polystyrene beads coated with polyethylene glycol that were mixed in the same mucus samples used to track *V. cholerae*. The thermally driven diffusive behavior of beads is affected by the viscoelastic properties of mucus. The 1 µm beads had a sub-diffusive behavior (slope of the mean-squared displacement < 1) indicating that the motion of the beads was constrained by the mucin matrix (Figure 3A) (27). The mucin matrix pore sizes were previously estimated to be ~240 nm using electron microscopy (28, 40). Consequently, the diffusive motion of 1 µm beads and similarly sized bacteria such as *V. cholerae* is severely diminished in mucus.

The loss (viscous) and storage (elastic) moduli of the mucus can be calculated from mean-squared displacement of the beads with respect to time using the generalized Stokes-Einstein relation (41). This analysis indicated that the viscosity and elasticity of the porcine intestinal...
mucus did not change substantially when pH was equilibrated at 6, 7, or 8 (Figure 3BC). We also determined that a prolonged incubation (1 hour) of mucus with *V. cholerae* El Tor C6706 had no measurable effect on the mucus rheology at pH 8. The average diffusion coefficient of non-motile *V. cholerae* (*flrA*) decreased slightly after 1 hour in mucus when compared to 15 minutes (Figure 3D). Incubation of mucus with *V. cholerae* El Tor C6706 at pH 6 and 7 produced identical results (Figure S1). Therefore, we concluded that the improved motility of *V. cholerae* in mucus at pH 8 is likely not attributed to changes in the mucus structure.

Previous studies have characterized the behavior of *V. cholerae* in mucus reconstituted from purified mucin commercially available (42, 43). We characterized the rheological properties of solutions of mucins from bovine sub-maxillary glands and porcine stomach purchased commercially. We used a 3% w/v concentration, which is comparable to native mucus (27, 44), in phosphate saline buffer at pH 8. The beads had purely diffusive trajectories indicating that the solutions were viscous but not elastic (Figure S2A). The storage and loss moduli of the purified mucin solutions were lower than our porcine mucus sample (Figure S2BC). Therefore, the purified mucins failed to reconstitute the gel structure of native mucus when dissolved in solution likely because they do not spontaneously crosslink. This result indicates that the physical structure of mucus reconstituted from purified mucins is not comparable to unprocessed mucus.

**Alkaline pH promotes the spread of *V. cholerae* colonies in soft agar**

To test the effect of pH on *V. cholerae* motility in the traditional soft agar assay, we measured the spread of colonies in M9 salts supplemented with pyruvate, tryptone, and 0.3% w/v agar (Figure 4A). Both Classical 0395 and El Tor C6706 formed significantly larger colonies at alkaline
pH (p-values < 10^{-4}) (Figure 4B). The colony morphology of the El Tor C6706 was denser and rugged at the edge when compared to the Classical 0395. One of the differences between the two strains is that Classical does not elaborate the MshA (mannose-sensitive hemagglutinin) pilus that mediates cell attachment (45–47). We inactivated mshA in the El Tor background to test if MshA affects colony morphology (Figure 4A). The colonies of the mshA mutant had smoother edges, spread further (p-values < 10^{-4}) (Figure 4B), but remained dense like the wild type. Overall, *V. cholerae* spreads further in soft agar at alkaline pH.

Colony spreading is a function of cell motility and chemotaxis to self-generated chemical gradients, but also a function of growth rate (48–50). *V. cholerae* growth is known to be sensitive to acidic pH (51). Therefore, we also measured growth rates in batch cultures at pH 6, 7, and 8 in M9 salts supplemented with pyruvate at 37°C (Figure 4C). At neutral pH, El Tor C6706 grew ~60% faster (63 minutes generation time) than Classical O395 (98 minutes generation time). pH had only a small effect on the generation time of Classical O395. El Tor C6706 grew fastest at pH 7 and 8 (63 minutes and 59 minutes generation times) but significantly slower at pH 6 (103 minutes) (p-values < 10^{-4}). The expression of MshA had a very small but measurable effect on the generation time of El Tor C6706. The effect of pH on growth rate may explain why colony spreading was reduced for El Tor C6706. However, these results do not explain why Classical O395 was similarly affected by pH and spread faster than El Tor C6706 in soft agar. Therefore, we hypothesized that pH affects *V. cholerae* flagellar motility directly.
**V. cholerae swims faster at alkaline pH**

To characterize how the swimming behavior of *V. cholerae* is affected by pH more directly, we tracked single cells swimming in a liquid environment between 2 glass coverslips (~10 µm in height). The diffusion coefficient of 1 µm beads and non-motile cells (*flrA* mutant) is distributed between 0.1 and 10 µm²/s in liquid. Therefore, trajectories with an effective diffusion coefficient below 10 µm²/s were categorized as non-motile in the different conditions tested and excluded from the calculations of swimming parameters.

For Classical O395, most cells were highly motile near the end of the exponential growth phase. The diffusion coefficient and swimming speed of the motile population increased upon transfer from the spent growth medium to fresh medium at all pH (p-value < 10⁻⁴) likely because of the replenishment of the energy source (addition of pyruvate to spent medium had an identical effect, data not shown). In fresh medium, Classical O395 was most diffusive at alkaline pH (p-value < 10⁻⁴) (Figure 5A). Both swimming speed and the frequency at which cells change direction by reversing the flagellar motor rotation affects diffusion coefficient. However, analysis of the trajectories revealed that only swimming speed was affected by pH (p-value < 10⁻⁴) (Figure 5B). On the other hand, the directional persistence of the cell trajectories did not change substantially, indicating that the reversal frequency of the flagellar motor was not affected by pH in Classical O395 (Figure 5C).

Tracking of El Tor C6706 revealed a more complex behavioral response to change in pH. Upon transfer from the growth medium to pH 6, two third of the population became non-motile (Figure 5D) while at pH 7 and 8 the response was like that of Classical O395. We hypothesized that MshA-mediated surface attachment was activated in El Tor C6706 at acidic pH, so we also
tracked the swimming behavior a mshA mutant at pH 6 and 8. The mshA mutant was fully motile at pH 6 (Figure 5D), thus, we concluded that El Tor C6706 activates MshA-mediated attachment at acidic pH but not at neutral or alkaline pH in our growth conditions. These results are consistent with the observation that the presence of MshA reduces the spread of colonies on soft agar (Figure 4). In the absence of MshA, El Tor C6706 swimming speed more than doubled between pH 6 and 8 (p-value < 10^{-4}) (Figure 5E), while the directional persistence was unaffected (Figure 5F).

The second messenger c-di-GMP regulates many behavioral responses in V. cholerae, including flagellar motility and surface attachment (52–54). To test if the cytoplasmic c-di-GMP concentration changes after a shift in pH, we quantified the bulk c-di-GMP concentrations after transfer to buffer solution at different pH using mass spectrometry with El Tor C6706 sampled during the early stationary phase. No measurable change in the total c-di-GMP concentration could be attributed to change in pH (Figure S3). Our results cannot exclude that pH activates c-di-GMP signaling through localized pathways as previously demonstrated in V. cholerae (55) or Escherichia coli (56) or that c-di-GMP changed and returned to the pre-stimulus concentrations during the incubation period (15 minutes). Overall, the increase in swimming speed in both V. cholerae strains is likely the main factor underlying improved motility in intestinal mucus and soft agar at alkaline pH.

Inhibiting the Na⁺-translocating NADH:quinone oxidoreductase (Na⁺-NQR) in V. cholerae reduces swimming speed and hydrogel penetration.

V. cholerae uses a sodium motive force to power its flagellar motor (57). Therefore, change in pH is unlikely to have a direct effect on the flagellar motor torque and rotation speed in V.
cholerae. However, maintaining a strong sodium gradient across the cell membrane when the motor is rotating at high speed is energetically costly (58). V. cholerae uses several sodium transporter but most of the sodium export is done by the NADH:quinone oxidoreductase (Na\(^+\)-NQR) as part of the respiratory chain (59). Activity of the Na\(^+\)-NQR pump is strongest at alkaline pH while cells are respiring (60). Previous studies showed that Vibrio alginolyticus is unable to maintain a strong sodium potential across the cell membrane when the cell environment becomes acidic (61). Therefore, the reduction of swimming speed we observed at acidic pH is likely the result of the reduction of the Na\(^+\)-NQR pump activity.

To test if Na\(^+\)-NQR activity plays a role in the ability of V. cholerae to penetrate mucus, we added 2-n-Heptyl-4-hydroxyquinoline N-oxide (HQNO), a strong inhibitor of Na\(^+\)-NQR activity (57). Unfortunately, mucus has a strong binding affinity to HQNO, which becomes unavailable to inhibit the Na\(^+\)-NQR pump. Mucus has been previously shown to bind similar small molecules with high affinity (62). Buffer containing 100 µM HQNO recovered after incubation with porcine intestinal mucus had no effect on V. cholerae swimming speed or behavior.

Instead, we tested the effect of HQNO on V. cholerae motility in liquid and agarose gel at pH 8. Low melting temperature agarose at 0.3% w/v forms a hydrogel similar to our porcine intestinal mucus samples but with larger mesh pores and less viscosity and elasticity (Figure S2). As observed with mucus, agarose gel impaired the motility of V. cholerae but did not completely abolished it (as expected from the soft agar plate assays). HQNO did not appear to interact with agarose as it dramatically reduced the effective diffusion coefficients of both V. cholerae strains (p-values < 10\(^{-4}\)) (Figure 6A). Most cells were unable to swim through the agarose gel in the presence of HQNO (diffusion coefficient < 10\(^{-0.5}\) µm\(^2\)/s) supporting that the ability to maintain a
strong sodium gradient is required for *V. cholerae* to escape the gel matrix using flagellar motility.

To test that HQNO did not block rotation of flagellar motor, we characterized the dose response of *V. cholerae* swimming speed at low viscosity (in liquid). The swimming speed of the motile cell population decreased in a dose-dependent manner with increasing concentration of HQNO (Figure 6B). The effect was more pronounced at acidic pH, indicating a possible synergistic interaction between the effect of low pH and HQNO binding in the pump channel. Swimming speed was very low at 100 µM HQNO but both strains were still motile. Overall, our results are consistent with a model that link the reduced activity of the Na⁺-NQR pump at acidic pH to the observed reduction in swimming speed and motility in porcine intestinal mucus.

**Discussion**

In this work, we demonstrated that *V. cholerae* can penetrate intestinal mucus using flagellar motility. We extracted mucosa from a pig small intestine and characterized its viscoelastic properties to examine the physical challenge motile bacterial pathogens have to overcome to reach the epithelial tissues from the intestinal lumen. Unprocessed intestinal mucus is a viscoelastic hydrogel with a pore size estimated to be between 200 nm and 1 µm from our microrheological analyses and previous imaging (28, 40). *V. cholerae* is small enough to swim through mucus using flagellar motility. However, many cells were trapped in the mucin matrix.
and the effective diffusion coefficient of free-swimming cells was severely reduced when compared to swimming in liquid.

Previous studies suggested that secreted proteases help *V. cholerae* colonize the intestinal mucus layer by degrading mucins (63, 64). In the conditions we tested, incubation of *V. cholerae* in unprocessed porcine intestinal mucus did not produce measurable changes in the mucus rheological properties suggesting that secreted proteases may not be required during the initial stages of infection when the number of *V. cholerae* is low. Another study proposed that *V. cholerae* shears or loses its flagellum in the presence of bovine mucin and initiates the expression of virulence factors (42). In this study, we found that *V. cholerae* rapidly dies in bovine mucin solutions unless dissolved in rich media (likely quenching an unidentified toxic compound). Dead cells showed the expected Brownian motion consistent with previous observations (42). We found that *V. cholerae* can grow in unprocessed porcine intestinal mucus and that the motile behavior stays steady suggesting that the integrity of the flagellum is not compromised. These results indicate that, beside the physical interactions with the mucus matrix, there were no measurable biological interactions between *V. cholerae* and mucus in our experimental conditions.

The diffusion coefficient we observed for motile *V. cholerae* in mucus is sufficient for cells to reach epithelial tissues during infection of the human small intestine. Previous studies have indicated that directional motion controlled by chemotaxis is not required for *V. cholerae* to infect the host (26, 65, 66). Therefore, *V. cholerae* is likely performing a diffusive random walk through the mucosa. The typical thickness of mucus in the human small intestine is in the order of a few hundred micrometers and grows about 240 μm per hour (28). The typical first-passage
time of a diffusive trajectory can be calculated as the square of the distance to cross divided by twice the diffusion coefficient (67). From our results, we estimate that the typical time \( V.\) cholerae would take to penetrate 400 \( \mu \)m of the small intestine mucosa at pH 8 is about 2 hours, which is comparable to the time it takes to grow mucosa of that thickness. Therefore, in the absence of factors that interfere with flagellar motility, \( V.\) cholerae is intrinsically capable of overcoming the physical barrier formed by intestinal mucus using flagellar motility even without a chemotactic response.

The dynamics of infection of the human small intestine by \( V.\) cholerae has not been firmly established, partially because of the limitations of existing animal models (68). The early infection steps may differ significantly between animal models and humans. Studies done on infant rabbits and mice indicate that in the early stage of infection planktonic \( V.\) cholerae cells are distributed throughout the small intestine. Then, the bacterial load drops in proximal and medial small intestine while the surviving cells preferentially colonize the distal small intestine (26, 69). Only, a small fraction of cells is able to penetrate the mucus layer protecting epithelial tissues. In the later stage of the infection, \( V.\) cholerae repopulates all parts of the small intestine (69, 70). Previous studies provided conflicting evidence supporting the role of flagellar motility during infection (20). Some studies found that non-motile cells are less infectious (71, 72), while others reported that there is no difference and that non-motile cells can reach the epithelial crypts in infant mice (26). Therefore, the route to the epithelium may vary between experimental models.

The pH gradient along the length of the small intestine may contribute to the preferred site of infection for \( V.\) cholerae. In humans, the proximal small intestine is slightly acidic (pH 6.3-6.5)
while the distal part is slightly alkaline (pH 7.5-7.8) (39, 73). *V. cholerae* is able to grow between pH 6.5 and 9, but its preferred pH is that of sea water at ~8 (74). Acidic pH regulates expression of virulence factors in *V. cholerae*. The production of cholera toxin and toxin-coregulated pili is maximal at pH 6.6 (75, 76). Our results showed that MshA affected the motility of El Tor C6706 at acidic pH when grown on soft agar but did not have measurable effect in porcine intestinal mucus, consistent with previous observation that MshA is likely not involved during host infection (77). On the other hand, high gastrointestinal pH increases the susceptibility of *V. cholerae* infection (78) and lactic acid producing bacteria, such as *Lactococcus lactis*, provide some protection against *V. cholerae* infections (79).

Our results showed that alkaline pH increases swimming speed and improves the ability of *V. cholerae* to penetrate intestinal mucus. Because *V. cholerae*’s flagellar rotation is powered by the transmembrane sodium gradient, the effect of environmental pH on flagellar motility is likely indirect. The main sodium pump of *V. cholerae*, Na\(^+\)-NQR, has increased activity at alkaline pH and no activity at acidic pH, thereby, affecting the sodium potential across the membrane (60, 61). In this study, inhibiting Na\(^+\)-NQR with HQNO had the same effect as reducing pH on motility, presumably because the sodium motive force is weakened. In addition, a previous study reported that a mutant strain lacking NqrA (a subunit of the Na\(^+\)-NQR complex) is defective at colonizing infant mice (80) and inhibiting Na\(^+\)-NQR activity decreased the production of cholera toxin (81). Our model is that *V. cholerae* has difficulty maintaining a strong sodium motive force at acidic pH, reducing the cells capacity to penetrate mucus and reach the epithelium. In addition, acidic pH reduces the production of cholera toxin, which is essential to disrupt the normal function of the small intestine to provide a competitive...
advantage to *V. cholerae*. Therefore, the preferred site of infection of *V. cholerae* in the human small intestine is likely in the ileum where the pH is alkaline.

**Methods**

**Bacterial strains**

*V. cholerae* strains used in this study were El Tor C6706str2 (82) and Classical O395 (83) biotypes. Our wild type El Tor strain has a functional *luxO* gene. Strains were fluorescently labelled with the expression of the green fluorescent protein expressed from a constitutive cytochrome c *V. cholerae* promoter on a p15a plasmid derivative (gift from Dr. Christopher Waters). The inactivation of *mshA* in the El Tor C6706 background was generated by recombining genomic DNA of mutant EC4926 from the defined transposon mutant library (84) using natural transformation (85). The El Tor *flrA* mutant was generated from previous work (54).

**Growth conditions**

M9 minimal salts (52 mM Na₂HPO₄, 18 mM K₂HPO₄, 18.69 mM NH₄Cl, 2 mM MgSO₄) were supplemented with 10 μM FeSO₄, 20 μM C₆H₉Na₃O₉ and 36.4 mM Sodium pyruvate. The pH of the growth medium was adjusted with HCl to the desired value. *V. cholerae* was grown shaking (200 r.p.m.) in liquid cultures at 37°C. Kanamycin was added to 50 μg/ml when needed. For all experiments, *V. cholerae* cultures were sampled at early stationary phase (1.9 x 10⁹ c.f.u./mL).

Soft agar plates were prepared with the same medium with the addition of 0.1% w/v Tryptone and 0.3% w/v Bacto agar (BD). Plates were inoculated with 5 μl of saturated liquid cultures (5.8
x 10^6 cells) on the agar surface and incubated at 37°C for 12 hours before measuring colony

**Mucus preparation**

Small intestines were obtained from a freshly slaughtered adult pig at the Meat Lab at Michigan State University (USDA permit #137 from establishment #10053). The animal was slaughtered as part of the normal work of the abattoir according to the rules set by the Michigan State University Institutional Animal Care and Use Committee (IACUC). The small intestines were acquired from the abattoir with prior consent. The mucosa was gently scraped from the medial part of small intestine and frozen in liquid nitrogen before storage at -80°C. For each experiment, mucus samples were warmed to 37°C temperature and equilibrated for 1 hour in 10 volumes excess of M9 salts buffered to the desired pH. Bovine submaxillary gland mucin (M3895, Sigma-Aldrich) solution was prepared at 3% w/v in LB medium adjusted to pH 8.0 with sodium hydroxide. Non soluble particles were separated from the preparation by centrifugation at 21,130 r.c.f for 10 minutes. Porcine stomach (M2378, Sigma-Aldrich) mucin solution was prepared at 3% w/v in M9 salts at pH 8.0. The survival rate of *V. cholerae* in BSG was calculated by enumerating colonies on LB agar plate supplemented with 50 µg/ml kanamycin. Fluorescent beads were added to the samples at 0.15% w/v and gently mixed.

**Single cell tracking**

*V. cholerae* cells were tracked in liquid medium following the protocol previously described (86). Briefly, *V. cholerae* cells in the early stationary growth phase were diluted to 1.9 x 10^7 cells/mL in fresh medium adjusted to pH 6, 7, or 8. Cells were incubated shaking at 37°C for 15 minutes before tracking to allow for adaptation of the chemotaxis response.
Polyvinylpyrrolidone (PVP) was added at 0.05% w/v to the samples to prevent attachment on the glass slide. 6 µl of each sample dropped on a glass slide and trapped under a 22 x 22 mm, #1.5 coverslip sealed with wax and paraffin to create a thin water film (10±2 µm) for video microscopy. For tracking in mucus or low-melting temperature agarose, a 130 µm spacer was added between the slide and the coverslip and fluorescently labelled cells were used. The samples were kept at 37°C during tracking. Images of swimming cells were recorded using a sCMOS camera (Andor Zyla 4.2, Oxford Instruments) at 20 frames per second using a 40X objective (Plan Fluor 40x, Nikon Instruments, Inc.) mounted on an inverted microscope (Eclipse Ti-E, Nikon Instruments, Inc.). Cell were illuminated using phase contrast in liquid or epifluorescence in mucus and agarose. Images were analyzed to detect and localize cells using custom scripts (86) and cell trajectories were reconstructed using the µ-track package (87). The analysis and plots of the cell trajectory statistics were done in MATLAB (The Mathworks, Inc.) as previously described (86).

Passive micro rheology of mucus and agarose gel

The viscoelasticity of mucus and agarose were measured by tracking the passive diffusion of 1 µm fluorescent polystyrene beads (F8814, ThermoFisher Scientific). To prevent electrostatic or hydrophobic interactions between the beads and the gels, beads were coated with polyethylene glycol (PEG MW 2,000Da). Coating was done by crosslinking carboxyl groups on the surface of the beads with diamine-PEG following the previously described protocol (88). Beads 0.5% w/v and Triton (X-100, Sigma-Aldrich) 0.01% w/v were added to samples and mixed gently. Epifluorescence signal from the beads were recorded using a sCMOS camera (Andor Zyla 4.2, Oxford Instruments) at 100 frames per second using a 100X objective (Plan Fluor 100x,
Nikon Instruments, Inc.) and a 1.5X multiplier mounted on an inverted microscope (Eclipse Ti-E, Nikon Instruments, Inc.). Images were analyzed to detect and localize beads using custom scripts and trajectories were reconstructed using the μ-track package (87). The beads trajectories were manually inspected to remove artifact and erroneous linking. Systematic drift of the trajectories was corrected prior to calculating the bead average mean-squared displacement (MSD) and velocity autocorrelation (VAC) as a function of time. The VAC was fitted to a degree six polynomial multiplied by an exponential decay function. The VAC function was integrated according the Green-Kubo relation (89, 90) to obtain a function that can also be fitted to the MSD with the same parameters. The VAC and MSD were fitted simultaneously using nonlinear least-square regression to separate the dynamic properties of the beads from the tracking noise. The fitted parameters were then used to calculate the storage and loss moduli of the sample according to the generalized Stokes–Einstein equation (91). The analysis and plots of the bead diffusive behavior were done in MATLAB (The Mathworks, Inc.).

**Growth rate analysis**

The growth rates of bacterial cultures were calculated by recording the change in optical density at 590 nm of 200 µL cultures in 96-well plates (Corning, CLS3595) using a Sunrise plate reader (Tecan Trading AG, Switzerland). Cultures were inoculated with 1.6 x 10⁶ c.f.u./mL cells in the exponential growth phase and incubated at 37°C with intermittently shaking every 10 mins for 24 hours. Precautions were taken to limit evaporation.

**c-di-GMP quantification**

The concentration of c-di-GMP was measured as previously described (92). Briefly, 2x10⁸ cells sampled during the exponential growth phase were collected on a PTFE membrane filter (0.2
µm) from each condition. Membranes were submerged and mixed in extraction buffer (40% v/v acetonitrile, 40% v/v methanol, 0.1 N formic acid) for 30 minutes. The extraction solution was spiked with a known amount of N\textsuperscript{15}-labeled c-di-GMP to normalize sample loss across samples during extraction. Non-soluble cell debris were separated by centrifugation (21,130 r.c.f. for 2 minutes). The soluble fractions were dried in vacuum overnight and resuspended in 100µl distilled water prior to identification and quantification using mass spectrometry (Quattro Premier XE mass spectrometer, Waters Corp.). c-di-GMP and N\textsuperscript{15}-labeled c-di-GMP were detected simultaneously at m/z 699.16 and at m/z 689.16, respectively.

**Statistical analyses**

Statistical significance of the different effects was calculated using Bayesian sampling of linear mixed-effect models taking into account experimental treatments and random effects from replication. The effect of pH on motility in mucus and in liquid was modeled as Response ~ Strain*pH + (1|Replicate) using a log normal link function. The addition of HQNO was modeled as an additional interaction with concentration modeled as a monotonic relationship. The effect of pH on motility in soft agar and growth rate was modeled as Response ~ Strain*pH + (1|Replicate) using a normal link function. Models were compiled and sampled using the RSTAN (93) and BRMS packages (94, 95) in R (96). The plots were generated using the ggplot2 (97) and tidybayes (98) packages.
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Author contributions

Y. S. D and N. T. Q. N. conceived and designed the study. N. T. Q. N., H. J. W., and J. S. L. performed the experiments. Y. S. D. and N. T. Q. N. analyzed the data and wrote the manuscript with input from all authors.

Declaration of interests

The authors declare no competing interests.
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Figure 1. *V. cholerae* Classical O395 flagellar motility through unprocessed porcine intestinal mucus (PIM). (A) Mucus scraped from the medial part of the small intestine of an adult pig. (B) Representative epi-fluorescence image at 40X magnification of *V. cholerae* Classical O395 (expressing the green fluorescent protein) swimming in unprocessed pig intestinal mucus between two glass coverslips. (C) Motile cells can be distinguished from non-motile by comparing the trajectories effective diffusion coefficients. (D) Distributions of diffusion coefficients from individual trajectories in liquid and PIM. Motile wild-type *V. cholerae* O395 (WT) was compared to a non-motile mutant (*flrA*) in PIM. Each distribution represents 3 to 12 replicates combining between 500 and 6,000 individual trajectories (between 250 and 1,700 minutes of cumulative time).

Figure 2. Effects of pH on the motility of *V. cholerae* through porcine intestinal mucus. (A) Distributions of diffusion coefficient from individual trajectories in mucus buffered at different pH. Cells with a diffusion coefficient < $10^{-0.5}$ µm$^2$/s were categorized as non-motile or trapped and were excluded from the following analyses. (B) Distributions of swimming speed from the motile cell populations. (C) Distributions of directional persistence time scales from the motile cell populations. Each distribution represents 8 to 12 replicates combining between 6,000 and 19,000 individual trajectories (between 1,000 and 2,600 minutes of cumulative time). Circles: means for the motile populations.
**Figure 3.** Passive microrheology of porcine intestinal mucus. (A) Mean-squared displacement (Mean sq. disp.) of PEG-coated 1 µm polystyrene beads with respect to time at different pH (represented by different colors) and after incubation with *V. cholerae*. The data points (circles) are the average of trajectories from 4 to 6 replicates (10 to 25 individual trajectories). A polynomial fit to the data was used to calculate the storage and loss moduli using the generalized Strokes-Einstein relation. (B) Storage moduli (elasticity) of porcine intestinal mucus at different pH. (C) Loss moduli (viscosity) of porcine intestinal mucus at different pH. (D) Distributions of the diffusion coefficient of non-motile *V. cholerae* (*flrA*) after incubation in mucus at pH 8. Each distribution represents 6 replicates combining between 1,000 and 2,000 individual trajectories (~150 minutes of cumulative time). Circles: means.

**Figure 4.** Effects of pH on the spreading of *V. cholerae* colonies in soft agar. (A) Representative colonies from the Classical O395 and El Tor C6706 (wild type and *mshA*) at different pH (white bar is 10 mm). (B) Measured colony diameters at different pH for all experimental replicates. (C) Measured growth rates in batch cultures at different pH for all experimental replicates.

**Figure 5.** Effects of pH on *V. cholerae* flagellar motility. (A) Distributions of diffusion coefficient of Classical O395 from single-cell trajectories in spent medium (Spent) or in fresh medium at different pH. Trajectory below 10 µm²/s were categorized as non-motile and excluded from the remaining analyses. (B) Distributions of swimming speed from the motile cell populations. (C) Distributions of trajectory directional persistence from the motile cell populations. (D)
Distributions of diffusion coefficient of El Tor C6706 from single-cell trajectories in spent medium (Spent) or in fresh medium at different pH. A mshA mutant was also tracked (mshA).

(E) Distributions of swimming speed from the motile cell populations. (F) Distributions of trajectory directional persistence from the motile cell populations. Each distribution represents 3 replicates combining between 2,000 and 10,000 individual trajectories (between 100 and 500 minutes of cumulative time). Circles: means for the motile populations.

Figure 6. Effects of inhibiting the Na⁺-NQR pump on flagellar motility in V. cholerae. (A) Distributions of diffusion coefficient in liquid and 0.3% w/v agarose buffered at pH 8 and with the addition of 100 μM HQNO. Each distribution represents 6 replicates combining between 1,000 and 3,000 individual trajectories (~1,000 minutes of cumulative time). (B) Distributions of swimming speed at different pH as function of HQNO concentration. Each distribution represents at least 6 replicates combining between 2,000 and 6,000 individual trajectories (between 500 and 1,000 minutes of cumulative time). Circles: means for the motile populations.
