The Vibrio cholerae type VI secretion system can modulate host intestinal mechanics to displace gut bacterial symbionts

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Host-associated microbiota help defend against bacterial pathogens; however, the mechanisms by which pathogens overcome this defense remain largely unknown. We developed a zebrafish model and used live imaging to directly study how the human pathogen Vibrio cholerae invades the intestine. The gut microbiota of fish monocolonized by symbiotic strain Aeromonas veronii was displaced by V. cholerae expressing its type VI secretion system (T6SS), a syringe-like apparatus that deploys effector proteins into target cells. Surprisingly, displacement was independent of T6SS-mediated killing of A. veronii, driven instead by T6SS-induced enhancement of zebrafish intestinal movements that led to expulsion of the resident microbiota by the host. Deleting an actin cross-linking domain from the T6SS apparatus returned intestinal motility to normal and thwarted expulsion, without weakening V. cholerae’s ability to kill A. veronii in vitro. Our finding that bacteria can manipulate host physiology to influence intermicrobial competition has implications for both pathogenesis and microbiome engineering.

Vibrio cholerae | zebrafish | type VI secretion system | microbiota | peristalsis

The consortium of microbes that make up the human microbiome plays important roles in health and disease (1, 2). In the gastrointestinal tract, where most animal-associated microbiota reside and where the potential interface of interspecies contact is large, symbiotic microbes prevent colonization by pathogens, a function termed “colonization resistance” (3–6). Colonization resistance can be thwarted by pathogens as the first stage of infectious disease, but the mechanisms of this interspecies competition remain unclear. By understanding how pathogens interact with symbiotic communities, we may more rationally design future therapies focused on targeting the pathogens themselves, or on engineering the host microbiome to better resist disruption. However, uncovering these mechanisms has proven challenging due to the difficulties associated with in situ monitoring of intestinal microbial populations and precise control of pathogenic phenotype expression.

We consider the transient human pathogen Vibrio cholerae, which can successfully colonize the human gut following ingestion of contaminated food or water. There, it causes diarrhea that may return the microbe to aquatic reservoirs in even larger numbers, leading to outbreaks. Cholera diarrhea causes severe dehydration and can be fatal if untreated. Recent epidemics in Haiti and Africa highlight that V. cholerae remains a major global problem and underscore that a better mechanistic understanding of the lifestyle of this microbe can help control future cholera outbreaks and infection (7).

V. cholerae can form biofilms on chitinous substrates, such as the exoskeleton of crustaceans (8), and can colonize the gut of birds and fish, which may promote transmission in aquatic environments and perhaps contribute to human disease outbreaks (9, 10). Within a human host, a complex set of signaling systems and external cues regulate colonization and disease factors, such as biofilm formation, chemotaxis-guided flagella, toxin-coregulated pilis, several adhesins, and cell shape features, to ensure access of the microbe to the intestinal surface (11, 12). Toxigenic isolates that carry the CTXphi prophage secrete the potent cholera toxin, which triggers rapid fluid loss and massive diarrhea. While the cholera toxin itself serves as a competition factor by promoting dispersal of the gut microbes, less well understood are the additional factors that enable V. cholerae cells entering the gut to compete with the daunting assemblage of microbiota they encounter. Recent human studies have shown that cholera diarrhea disturbs the composition of the symbiotic intestinal microbiota (13), and studies in humans combined with mammalian animal models have suggested that the microbiome composition affects how the host recovers from the disease (14).

Here we sought to discover how V. cholerae may overcome resident microbial symbionts to invade a host intestine. We focused on the role of the type VI secretion system (T6SS), a syringe-like protein apparatus present in nearly 25% of all Gram-negative bacteria that inflicts damage on target cells and external cues regulate colonization and disease factors, such as biofilm formation, chemotaxis-guided flagella, toxin-coregulated pilis, several adhesins, and cell shape features, to ensure access of the microbe to the intestinal surface (11, 12). Toxigenic isolates that carry the CTXphi prophage secrete the potent cholera toxin, which triggers rapid fluid loss and massive diarrhea. While the cholera toxin itself serves as a competition factor by promoting dispersal of the gut microbes, less well understood are the additional factors that enable V. cholerae cells entering the gut to compete with the daunting assemblage of microbiota they encounter. Recent human studies have shown that cholera diarrhea disturbs the composition of the symbiotic intestinal microbiota (13), and studies in humans combined with mammalian animal models have suggested that the microbiome composition affects how the host recovers from the disease (14).

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Significance

Vibrio cholerae, the causative agent of the diarrheal disease cholera, uses the syringe-like type VI secretion system (T6SS) to pierce adjacent cells. To investigate the role of the T6SS in invasion of intestines already occupied by symbiotic microbes, we genetically engineered V. cholerae strains and performed live 3D imaging in zebrafish to find that V. cholerae can expel a resident bacterial species in a T6SS-dependent manner. Surprisingly, the T6SS acts primarily to increase the strength of gut contractions, rather than directly killing the bacterial competitor. Deletion of an actin cross-linking domain from the T6SS returned gut activity to normal and eliminated V. cholerae’s competitive advantage. These findings reveal a strategy by which pathogens can manipulate host biomechanics to redefine gut communities.


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to eukaryotic cells, as well as bacteria that lack immunity proteins (15–17). T6 activity in nontoxigenic environmental isolates and in toxigenic CTXphi isolates derived from clinical sources is controlled by diverse regulatory systems and external cues (18, 19). Recently, a role for T6-mediated microbial intercellular interactions within the mouse gut has been demonstrated for Shigella and Salmonella infections (20, 21). Bacteroides can use the T6SS to compete with other bacteria to maintain their presence in the mouse gut (22, 23). T6SS genes also have been detected in the human gut microbiome (24, 25). Together, the foregoing evidence suggests that T6SSs merit increased attention for their role in the initiation and development of cholera, and also in mediating microbial–microbe and microbe–host interactions in the gut microbiome.

Investigating the potential role of the V. cholerae T6SS in intestinal invasion is challenging in humans, and even in mammalian model organisms, due to the complexity of colonization and infection processes and the difficulty of in vivo imaging. In contrast, zebrafish are a powerful laboratory model for the direct observation and experimental control of microbiome interactions. Germ-free and gnotobiotic protocols allow precise control of intestinal microbiota composition (26). Zebrafish are relatively transparent at larval stages. Thus, light sheet fluorescence microscopy (27–29) can be used to capture detailed 3D images of fluorescent-labeled bacteria spanning the entire gut, over many hours, to monitor both sudden and longer-term transitions in bacterial populations (30), and differential interference contrast microscopy (DICM) can capture the dynamics of unlabeled intestinal tissue in the same animal (31).

Mammalian models for V. cholerae infection have revealed modest contributions of the T6SS to fitness in the infant rabbit (32), and fluid accumulation and immune modulation in the infant mouse (33, 34). However, these organisms, unlike fish (35) or humans, are not natural V. cholerae hosts (36). Recent studies have demonstrated the utility of the zebrafish as a model for cholera intestinal colonization, pathogenesis, and transmission (37), revealing that, for example, fish colonization is independent of cholera toxin (38). Together, these features make the zebrafish an ideal model for studying the dynamics of vertebrate gut colonization by V. cholerae, and specifically the role of its T6SS.

In this study, we used a combination of microbial genetics, in vitro experiments, and quantitative in vivo imaging in zebrafish to determine the role of the T6SS of V. cholerae in gut colonization. We exploited the known regulation pathways of T6SS (39–41) to genetically manipulate the human pathogenic V. cholerae wild-type El Tor strain C6706 to constitutively express functional, defective, or altered T6SS machinery, and to generate strains lacking T6SS immunity. We then imaged at high resolution the invasion by V. cholerae of zebrafish intestines that were previously colonized by a zebrafish-symbiont Aeromonas species. Our experiments revealed a strongly T6SS-dependent displacement of the resident bacteria. This displacement took the form of sudden collapses in Aeromonas populations via ejection of aggregated bacteria from the gut, similar to the collapses previously reported for Aeromonas when challenged by symbiotic Vibrio sp. ZWU0020, a species commonly isolated from zebrafish intestines (30). We found that the expression of a functional T6SS by V. cholerae induced a large increase in the amplitude of the peristaltic movements in the host intestine. Deletion of the actin cross-linking domain (ACD) of one of the T6SS spike proteins returned zebrafish gut activity to normal and eliminated V. cholerae’s ability to expel the symbiotic Aeromonas from the gut, without affecting its ability to kill Aeromonas cells in vitro.

Our finding that the bacterial T6SS can induce organ-level physiological changes in an animal host that displaces resident microbiota expands the array of known molecular mechanisms by which pathogens can leverage host–microbe interactions to redetermine microbial community composition, and also suggests that the T6SS can be rationally manipulated to deliberately engineer the human microbiome.

Results

Human-Derived V. cholerae Colonizes the Larval Zebrafish Intestine but Exhibits Weak Intraspess T6SS-Mediated Killing in Vivo. A streptomycin-resistant mutant of patient-derived El Tor biotype C6706 served as a wild-type strain (denoted T6SSWT1) because of its proficiency at T6-mediated bacterial killing (40, 42). T6SS and immunity genes are well characterized in this strain, allowing construction of variants that differed in T6SS expression, immunity, and functionality (Fig. 1A). A strain constitutive for T6SS expression, termed T6SSα, was previously constructed by replacement of the native gstR promoter, and a T6SS- derivative of this strain was constructed by deletion of the vask gene (Δvask) (43). Further deletion of three T6 immunity genes (istIV–3) generated a T6SS– Imm– strain. Each strain was labeled fluoroscently with a chromosomally introduced teal or orange fluorescent protein to enable microscopy (43) (Table S1).

To determine whether the human-derived V. cholerae and its variants could colonize the larval zebrafish gut, we inoculated flasks housing germ-free larvae with a single bacterial strain at 5 days post-fertilization (dfp). We then dissected the gut at 6 dfp and determined intestinal bacterial abundance by serial plating and counting of colony-forming units (CFUs). For comparison, we also considered a previously examined zebrafish symbiont, ZWU0020, assigned to the genus Vibrio (30, 44). All V. cholerae strains examined could colonize the larval zebrafish intestine robustly to an abundance of approximately 106 CFU per gut, which is roughly one order of magnitude lower than that of the symbiotic Vibrio (Fig. 1 B–D). Direct observation by light sheet fluorescence microscopy at 5 dfp showed that each strain of V. cholerae was abundant and highly motile in the intestinal lumen (Fig. 1C and Movie S1).

We then asked whether we could detect signatures of T6SS-mediated intraspecies competition in vitro and in vivo. For the in vitro assays, we mixed two V. cholerae strains in liquid culture at an approximate 1:1 ratio (measured as 1.2 ± 0.5 across all pairs of strains). One of these was always the T6SS– Imm– strain, which, lacking immunity to T6SS, served as a “target” for interbacterial killing (40). We spotted the mix onto nylon membranes on agar plates and allowed the microbes to interact in close proximity for 3 h. We then quantified killing by measuring the ratio of CFU counts for each pair of strains, which we distinguished by their fluorescent markers. T6SS+ and T6SS+ WT strains were only slightly enhanced compared with the target; however, the T6SS+ strain dominated the mixture, indicating T6SS-mediated killing (Fig. 1E), consistent with previous in vitro work (40). In vivo, we coinoculated zebrafish flasks at 5 dfp with the orange-labeled T6SS– Imm+ strain and one of either the teal-labeled wild-type, T6SS– defective, or T6SS– constitutive strains at an approximate 1:1 initial ratio. (The initial ratios were 0.94 ± 0.06, 1.2 ± 0.1, and 1.05 ± 0.10, respectively, for these three strain combinations.) We determined their ratios in the fish at 6 dpf using gut dissection and plating, again differentiating the strains by their fluorescent markers. T6SS– and T6SS+ WT strains were only slightly enhanced compared with the target; however, the T6SS+ strain dominated the mixture, indicating T6SS-mediated killing (Fig. 1E), consistent with previous in vitro work (40). In vivo, we coinoculated zebrafish flasks at 5 dfp with the orange-labeled T6SS– Imm+ strain and one of either the teal-labeled wild-type, T6SS– defective, or T6SS– constitutive strains at an approximate 1:1 initial ratio. (The initial ratios were 0.94 ± 0.06, 1.2 ± 0.1, and 1.05 ± 0.10, respectively, for these three strain combinations.) We determined their ratios in the fish at 6 dfp using gut dissection and plating, again differentiating the strains by their fluorescent markers. T6SS– and T6SS+ WT strains were only slightly enhanced compared with the target; however, the T6SS+ strain dominated the mixture, indicating T6SS-mediated killing (Fig. 1E), consistent with previous in vitro work (40).

Constitutive Expression of T6SS Potentiates V. cholerae Invasion of Zebrafish Intestines Occupied by a Symbiotic Species. We next addressed the key question of whether the T6SS can affect the ability to invade an established intestinal microbial community.
Fig. 1. (A) Genes of the V. cholerae C6706 type VI secretion system. T6SS genes are primarily organized in three operons that are transcriptionally activated through the regulator QstR. The main cluster (M) encodes most of the T6SS structural proteins, including the assembly ATPase VasK (black), while the major Hcp subunit is encoded on the auxiliary clusters Aux1 and Aux2. Each cluster terminates in genes encoding antibacterial effectors (TseL, VasX, and VgrG-3) and their respective immunity proteins (TsiV1–3). Each cluster also encodes proteins that form a spike at the apex of the apparatus, VgrG-1–3. Two of the three VgrG proteins are multifunctional; VgrG1 contains a C-terminal ACD (black), and VgrG3 has a muramidase domain that serves as an antibacterial effector. (B) A larval zebrafish at 5 dpf with the intestine colored for illustration by orally gavaged phenol red dye. (Scale bar: 1 mm.) (C) Light sheet fluorescence image of wild-type V. cholerae expressing orange fluorescent protein in the larval zebrafish intestine. The region shown roughly corresponds to the box in B, with the luminary boundary roughly indicated by the yellow dotted line. Individual motile bacteria are evident, as is the background autofluorescence of the gut lumen. See also Movie S1. (Scale bar: 10 µm.) (D) Abundance of Vibrio strains in the larval zebrafish intestine at 24 h postinoculation. All V. cholerae strains robustly colonize to approximately 10^8 bacteria per fish, roughly an order of magnitude lower than a symbiotic Vibrio species, ZWU0020 (rightmost data points). Measurements from individual fish at 6 dpf are shown in gray, averages are indicated by solid colored circles, and black error bars represent quartiles. For the distribution of abundances of each V. cholerae strain compared with the symbiotic Vibrio, \( P < 0.001 \) for a two-sample Kolmogorov–Smirnov (KS) test. (E) Ratios of V. cholerae strains in an in vitro competition assay. Each indicated strain was mixed 1:1 with T6SS^-Imm^- as a target, and then spotted onto a nylon membrane on agar. Ratios were determined from CFU counts following 3 h of incubation. The T6SS^+ strain exhibits a large competitive advantage over the T6SS^-Imm^- strain. For each pair of strains, \( P = 0.01 \), two-sample KS test. (F) Ratios of V. cholerae strains in the larval zebrafish intestine at 24 h after coinoculation. At 5 dpf, fish were coinoculated with T6SS^-Imm^- as a target and the wild-type, T6SS^-WT, or T6SS^+ strain. At 6 dpf, the ratio of the abundance of these strains to the target T6SS^-Imm^- was measured. The T6SS^+ and wild-type strains exhibit a slightly greater competitive advantage over the T6SS^-Imm^- strain compared with the T6SS^-strain. Two-sample KS tests give \( P = 0.08 \) for comparison of the T6SS^- and T6SS^+WT ratios and \( P = 0.9 \) for comparison the T6SS^- and T6SS^+ ratios. In E and F, measurements from individual fish are shown in gray, averages are indicated by solid circles, and quartiles are represented by black lines.
We used as our target species *Aeromonas veronii* strain ZOR0001 (hereinafter referred to as *Aeromonas*), a Gram-negative bacterium native to and commonly found in the zebrafish intestine (45). Previous work has shown that this strain can robustly monoclonize germ-free larval zebrafish at $10^3$–$10^5$ bacteria per gut (29, 44). *Aeromonas* forms dense bacterial aggregates in vivo (29) and can be invaded by symbiotic *Vibrio* sp. ZWU0020 (30).

We first determined whether *Aeromonas* was susceptible to T6S-mediated killing by *V. cholerae* in vitro. We mixed *Aeromonas* and *V. cholerae* strains in liquid culture and spotted them onto nylon membranes as in the previously described in vitro experiments. We then quantified killing by measuring *Aeromonas* CFUs counts before and after the membrane incubation. *Aeromonas* CFUs counts when mixed with T6SS$^+$ *V. cholerae* were indistinguishable from those of a control mix of *Aeromonas* with *Aeromonas* (Fig. 2A). Wild-type *V. cholerae*, and particularly the T6SS$^+$ strain, decreased *Aeromonas* CFUs counts significantly, indicating high interspecies killing rates (Fig. 2A and Movies S2–S4), consistent with previously reported in vitro results with an *Escherichia coli* target (19).

To determine the role of the T6SS in vivo, we monoclonized zebrafish by inoculating flasks containing germ-free larvae with *Aeromonas* at 4 dpf and then inoculated with one of the *V. cholerae* strains at 5 dpf (Fig. 2B; approximately 30 animals per *V. cholerae* strain). Gut dissection and serial plating at 6 dpf revealed dramatic differences in *Aeromonas* abundance, depending on the T6SS of the invading strain. *Aeromonas* challenged by T6SS$^−$ or T6SS$^{\text{Imm}^−}$ *V. cholerae* persisted in the gut at approximately 1,000 CFU per fish on average (Fig. 2C, top two panels); however, *Aeromonas* challenged by the T6SS$^+$ *V. cholerae* fell to single-digit numbers, with zero detectable *Aeromonas* in $>$50% of the fish (Fig. 2C, bottom panel). *Aeromonas* challenged by the wild-type *V. cholerae* showed intermediate numbers between the T6SS$^+$-challenged and the T6SS$^+$-challenged strains (Fig. 2C, third panel). Live imaging at 24 h after the *V. cholerae* inoculation demonstrated the differential impacts on the resident *Aeromonas*, with large populations consisting of dense clusters and discrete individuals in the gut of larvae challenged by T6SS$^+$ *V. cholerae* (Fig. 2D) and few *Aeromonas* remaining in the gut of larvae challenged by T6SS$^+$ *V. cholerae* (Fig. 2E). Each of the invading *V. cholerae* strains was present at 6 dpf at approximately $10^4$ CFU per gut (Fig. S1B).

**Aeromonas Are Expelled in Frequent Sudden Collapses from Fish Guts Invaded by T6SS-Expressing V. cholerae.** To better characterize the strong T6SS-mediated effect of *V. cholerae* on gut-resident *Aeromonas*, we monitored bacterial population dynamics over 12–17 h using light sheet fluorescence microscopy, capturing a 3D image spanning the entire larval intestine every 20 min. We used the same inoculation protocol and began live imaging at 8 h after *V. cholerae* inoculation.

In previous work, we found that *Aeromonas* populations residing in the zebrafish intestine can be punctuated by occasional large collapses corresponding to ejection from the gut. In fish monoclonized with *Aeromonas*, these collapses occurred at a mean rate of $p_c = 0.04 \pm 0.02$ h$^{-1}$, but in fish invaded by the symbiont *Vibrio* ZWU0020, the rate of collapse increased to $p_c = 0.07 \pm 0.02$ h$^{-1}$ (30). Here, as in that previous work, we defined a collapse as a population drop of at least a factor of 10 in one 20-min time interval, together with at a drop of at least a factor of 2 relative to the original population at the subsequent time step. The *Aeromonas* population was strikingly stable when invaded by the T6SS$^+$ strains; we observed no collapses during the entire 58.0-h and 70.3-h total imaging times for T6SS$^−$ (n = 5 fish) and T6SS$^{\text{Imm}^−}$ (n = 6 fish) *V. cholerae* challenges, respectively (Fig. 3A, first two panels). Challenge by the wild-type *V. cholerae*

![Fig. 2.](image-url)
resulted in two population collapses in 72.7 h, corresponding to a collapse rate of $p_c = 0.03 \pm 0.02$ h$^{-1}$ (Fig. 3A, third panel; $n = 6$ fish). Challenge by T6SS$^+$ V. cholerae gave rise to large and frequent collapses, a total of eight in 64.3 h (Fig. 3A, fourth panel; $n = 5$ fish), corresponding to a collapse rate of $p_c = 0.12 \pm 0.04$ h$^{-1}$, nearly twice as large as that induced by the fish-symbiont Vibrio ZWU0020 (30) (Fig. 3B–D and Movies S5 and S6).

Constitutively Expressed T6SS Alters the Intestinal Movements of Larval Zebrafish in an ACD-Dependent Manner. The larval zebrafish intestine, like the intestines of other animals, has periodic propagative contractions that drive the motion of dense aggregates of Aeromonas and can ultimately cause their ejection (30). We tested whether the collapses in the Aeromonas populations observed in the T6SS$^+$ competition (Fig. 3B–D) could be due to greater gut motility. We compared intestinal movements of germ-free fish and fish monoassociated with the various V. cholerae strains using DICM, which allowed direct visualization of the intestinal epithelial tissue and luminal space (Fig. 4A) (31). We then used image velocimetry techniques to quantify the frequency and amplitude of intestinal contractile waves (Fig. 4B–D) (30, 46). None of the strains altered the frequency of peristaltic contractions compared with germ-free fish (Fig. 4E); however, the amplitude of the contractions was greatly enhanced.

Fig. 3. (A) Time series of Aeromonas populations in larval zebrafish intestines when challenged by different strains of V. cholerae, derived from light sheet fluorescence imaging. Beginning at 8 h after Vibrio inoculation, fish were imaged every 20 min for 12–17 h. Each curve is from a different zebrafish; $n = 6, 5, 6$, and 5 for challenge by T6SS$^{-}$ Imm$^{-}$, T6SS$^{-}$, T6SS$^{WT}$, and T6SS$^+$ V. cholerae, respectively. When invaded by T6SS$^+$ V. cholerae, overall Aeromonas abundance is low, and collapses in populations of $>1$ order of magnitude are evident. (B–D) Maximum intensity projections of a 3D light sheet image stack of Aeromonas in a larval zebrafish intestine invaded by T6SS$^+$ V. cholerae at 9.3, 10.7, and 16.3 h after the start of imaging. A collapse of the Aeromonas population is evident as time progresses. See also Movie S5 (T6SS$^+$) and Movie S6 (T6SS$^{-}$ Imm$^{-}$). Yellow dotted lines indicate the rough luminary boundary. (Scale bar: 200 μm.)
in the fish colonized with the T6SS+ strain, but not with other strains (Fig. 4F and Movies S7 and S8). The magnitude of the effect—a roughly 200% increase in the amplitude of contractions compared with germ-free fish—was remarkable and unexpected. For comparison, treatment with the neurotransmitter acetylcholine or deletion of all enteric neurons induces at most a change in peristaltic amplitude of approximately 40% (46).

Although this T6SS-dependent alteration of host gut motility was unexpected, there are well-established precedents for T6SS-mediated V. cholerae interactions with eukaryotic cells driven by an ACD present in the C terminus of the VgrG-1 spike protein of the T6 secretion apparatus (33). We hypothesized that the V. cholerae-mediated change in peristaltic amplitude of approximately 40% (46).

For comparison, treatment with the neurotransmitter acetylcholine or deletion of all enteric neurons induces at most a change in peristaltic amplitude of approximately 40% (46).

We then tested the ability of T6SS+ ACD− V. cholerae to invade an intestinal Aeromonas population using the same zebrafish invasion assay described above. While the Aeromonas population dropped precipitously following T6SS+ V. cholerae introduction, Aeromonas after T6SS+ ACD− V. cholerae introduction remained abundant, averaging approximately 1,000 CFU per fish (n = 31 fish), similar to the numbers seen when challenged by T6SS− strains (Fig. 4H). T6SS+ ACD− V. cholerae was nonetheless present in the gut at high abundance, approximately 10^6 CFU per gut (Fig. S1B). This experiment demonstrates that removing the T6SS ACD eliminates V. cholerae’s ability to displace a competitor, despite an otherwise functional T6SS capable of killing in vitro. Taken together, these results show that the ability of V. cholerae to dominate a gut colonized by Aeromonas works specifically by increasing the amplitude of host peristalsis in a manner dependent on the VgrG-1 ACD.

Discussion

We have shown that V. cholerae can use its type VI secretion system to amplify the intestinal contractions in zebrafish and induce the host to expel from its gut a resident microbiota of the genus Aeromonas. The coupling of T6SS activity to host contractions depended on an ACD of the T6SS apparatus; when the ACD was deleted, V. cholerae could no longer induce enhanced host contractions, and dense Aeromonas communities remained in the gut. The effect on the host peristalsis was specific; deleting the ACD did not affect the ability of V. cholerae to kill Aeromonas on contact, and did not affect the ability of V. cholerae to enter and occupy the host intestine. V. cholerae itself seemed unaffected by the enhanced intestinal motility, likely due to its ability to remain planktonic and motile inside the zebrafish gut (Fig. 1C and Movie S1), which is similar to what was seen previously for the highly motile symbiont Vibrio ZWU0020 (30, 47).

Taken together, our results show that an enteric colonizer (V. cholerae) can use a previously undiscovered host-microbe interaction (T6SS-dependent enhancement of gut contractions) to influence the population dynamics of a competitor (Aeromonas).

The existence of this interspecies interaction raises intriguing questions and opens avenues for future research. Perhaps at the forefront is the question of what leads T6SS-affected host cells, likely lining the gut, to unleash large contractions of the entire organ. The host cellular mechanism is likely complex and could involve one or more cell types: immune cells, which at larval stages in zebrafish include neutrophils and macrophages that can
respond to microbial presence (44) and may take up T6SS-active intestinal bacteria; epithelial cells, varieties of which line the gut and lie in close proximity to luminal bacteria; cells of the enteric nervous system, which include enteric neurons that coordinate muscle activity; and the smooth muscle cells that are the proximate cause of contractions. Identifying the cells and signaling mechanisms that link the bacterial T6SS to intestinal mechanics is a challenging but attainable goal for research groups in the years to come. The tools of transgenic and gnotobiotic zebrafish together with 3D live imaging and image analysis empower future studies to discover new links between cellular processes and macroscopic organ behavior.

This newly discovered T6SS function is important in its own right, standing out from previously identified mechanisms for orchestrating the ecology of the microbiome (48) because it highlights the role of the host fluid-mechanical environment in shaping gut population dynamics, an emergent theme in contemporary microbiome research (30, 47, 49, 50). The T6SS can mediate interbacterial toxicity (51) and may be an important weapon that bacteria use to fight each other within the animal intestinal microbiome (22, 24, 25, 34). A recent report suggests that the host immune system also may play a role in this T6SS warfare (34). Given the prevalence of T6SS among bacteria, the possibility that gut-colonizing bacteria can use the T6SS to manipulate host peristalsis suggests that this could be a common tactic for bacteria to indirectly influence interbacterial competition. Moreover, exogenous delivery of T6SS proteins, or their engineering into otherwise beneficial microbes, could offer a new path to therapeutic modulation of human gastrointestinal activity.

Our observations may also inform our understanding of T6SS regulation. Previous studies have shown that chitinase material triggers T6SS activity in V. cholerae (19, 36, 40); chitin can be found in crab shells, zooplankton exoskeletons, and marine snow commonly colonized by Vibrios in aquatic environments (52, 53). We found that wild-type C6706 V. cholerae, but not T6SS− derivatives, was capable of modest killing of Aeromonas in vitro (Fig. 24), consistent with previously reported findings for C6706 T6 killing of an E. coli target (19). We also observed small reductions in Aeromonas counts and rare extinction events in vivo (Figs. 2C and 34). Since the germ-free zebrafish used here were not provided with a chitin source, it is interesting to speculate that we recorded chitin production recently documented within the juvenile zebrafish gut (54) induced the wild-type V. cholerae T6-mediated activity observed here. Furthermore, the presence of chitin in fish diets, either naturally or in aquaculture, may alter the abundance of V. cholerae in the fish intestinal microbiome, with potential implications for the transmission of disease to humans from aquatic reservoirs. Further studies will determine the contribution of chitin signaling in T6 expression by V. cholerae in fish intestinal environments.

Most directly, our work sheds light on the role of the T6SS in the ecological dynamics of V. cholerae colonization of a vertebrate host. The ability of the T6SS to amplify host intestinal mechanics was previously undetected, likely for three reasons. First, the development of cholera in humans is a complex, multifactorial process in which the role of T6SS may be confounded by other factors, most importantly the strong effects of the cholera toxin. Second, the animal models typically used in cholera research are not native cholera hosts, and the mechanisms of their colonization may be different; however, fish naturally host V. cholerae, and because zebrafish colonization depends less on other factors, we could detect the effects of T6SS on intestinal movements, and we could then use genetically modified V. cholerae strains to confirm the molecular mechanism. Third, the zebrafish model allows direct, quantitative, in vivo imaging using modern microscopy methods, in contrast to indirect, static, DNA- or RNA-sequencing–based assays typically used to study mouse or human microbiomes. In vivo imaging greatly facilitates observations of intestinal activity and enables the discovery of sudden spatiotemporal changes in bacterial distributions. How our findings may map onto V. cholerae coloni- zation in humans is unknown, but a role for T6SS-mediated activity is certainly plausible. Establishing this will require further investigation, especially for the potential aim of designing therapies that target the T6SS to prevent colonization in humans or in environmental reservoirs, such as fish. Nonetheless, our results enhance our understanding of the strategies and abilities of V. cholerae, a pathogen that continues to impact millions of people worldwide.

Materials and Methods

Ethics Statement. All experiments involving zebrafish were carried out in accordance with protocols approved by the University of Oregon’s Institutional Animal Care and Use Committee and followed standard methods (55).

Gnotobiotic Techniques. Wild-type larval zebrafish devoid of microbes were derived as described previously (26). In brief, fertile eggs were collected and placed in a sterile antibiotic embryo media solution consisting of 100 μg/mL ampicillin, 250 ng/mL amphotericin B, 10 μg/mL gentamicyn, 1 μg/mL tetracycline, and 1 μg/mL chloramphenicol for approximately 6 h. The eggs were then washed in a sodium hypochlorite solution and a polyvinylpyrrolidone-iodine solution. Washed embryos were distributed in sets of 15 into tissue culture flasks containing 15 μL of sterile embryo media. Flasks of larval zebrafish were inspected for sterility before being used in experiments.

Bacterial Strains and Culture Conditions. A. veronii (ZOR0001; PRJNA205571) and Vibrio (ZWU0002, PRJNA205585) were isolated from the zebrafish intestinal tract as described previously (45). These strains were fluorescently labeled with EGF or dTomato for imaging experiments using methods similar to those described previously (30, 56).

70 μL V. cholerae strains were derivatives of El Tor C6706 str−2 (Table S1). Bacterial cultures were routinely grown at 30 °C or 37 °C in Lysogeny broth (LB) with shaking, or statically on LB agar. In-frame deletion mutants and promoter replacements in V. cholerae were constructed using a previously described allelic-exchange method (57). Standard molecular biology-based methods were used for DNA manipulations. DNA-modifying enzymes and restriction nucleases (Promega and New England BioLabs), Gibson assembly mix (New England BioLabs), and Q5, Phusion, and OneTaq DNA polymerases (New England BioLabs) were used following the manufacturer’s instructions. All recombinant DNA constructs were verified by Sanger sequencing (Eurofins).

Culture-Based Quantification of Bacterial Populations. Germ-free larval zebrafish were inoculated with select bacterial strains as described previously (30, 44). Bacteria were grown on a shaker in Luria broth for 10–14 h at 30 °C, prepared for inoculation by pelleting for 2 min at 7,000 × g, and washed once in sterile embryo media before inoculation. An inoculum of 106 CFU/mL was used for Aeromonas ZOR0001 (PRJNA205571) and Vibrio ZWU0002 (PRJNA205585) strains, and an inoculum of 107 CFU/mL was used for V. cholerae strains. Bacterial inoculums were added directly to tissue culture flasks containing germ-free larval zebrafish.

In Vitro Measurements of Bacterial Competition. For in vitro killing assays, bacterial strains were inoculated from glycerol stock and shaken in LB at 30 °C or 37 °C overnight. The cells were then harvested, washed twice in sterile PBS, and normalized to OD600 = 1 in PBS. Pairs of strains were mixed at a 1:1 ratio, and 25 μL of the liquid was spotted onto a 0.20-mm diameter porous nylon membrane filter (EMD Millipore) placed on an LB agar plate. After being allowed to dry, the plate was incubated at 37 °C for 3 h. Each membrane was then carefully removed from the agar plate and vortexed in sterile PBS for 1 min. The killing rate was assessed by comparing the target cell numbers before and after incubation by plating and counting CFUs. An antibiotic-resistant marker (streptomycin or gentamicin) inserted into the target strain chromosome enabled discrimination of target cells for CFU counting.

For in vitro time-lapse fluorescence microscopy, bacterial strains were inoculated from glycerol stocks and shaken in LB at 30 °C or 37 °C overnight. The overnight culture was brought back to exponential phase by diluting 70 μL culture into 4 mL of fresh LB and shaking for 3 h at 37 °C. Frames and coverslips (Thermo Fisher Scientific) were used to form an agar pad using 1% low-melting-point agarose in PBS. Exponential-phase cells were centrifuged at 6,000 × g for 1 min and resuspended in fresh LB. Then 1 μL of
mixed cells (vol:vol ratio = 1:1) was spotted onto the agar pad, allowed to dry, and then covered with a coverslip. The fluorescent-labeled cells were imaged in a single fluorescence channel (647 nm excitation and 659 nm emission) using a 63× oil immersion objective lens on an inverted wide-field fluorescence microscope (Zeiss AxioObserver.Z1). Acquired images were processed with customized MATLAB scripts.

In vitro time-lapse images were processed by aligning to correct for field drift and cropping to keep areas that overlap in all images, and then identifying cells by thresholding based on fluorescence intensity, identifying connected above-threshold pixels as bacterial objects. The MATLAB script used to perform this analysis is available at https://github.com/Jinyuan1998/TeSSinivtro_imageAnalysis.

Light Sheet Fluorescence Microscopy. Light sheet microscopy was performed on a home-built light sheet microscope based on the design of Keller et al. (27) as described previously (29, 58). In brief, the beams from either of two continuous-wave lasers (Coherent Saphire, 448 nm and 561 nm) were rapidly scanned using a galvanometer mirror and then demagnified to create a thin sheet of excitation light perpendicular to and at the focus of an imaging objective lens. The specimen was moved through this sheet in 1-μm steps, and fluorescence emission was captured to create a 3D image. To image the entire larval zebrafish gut, four subregions were imaged and later manually registered and stitched. All exposure times were 30 ms, and the excitation laser power was set to 5 mW measured at the laser output. A 5.5-megapixel scientific-grade complementary metal oxide semiconductor camera ( Cooke Corporation) with a 40× 1.0 NA objective lens (Zeiss) was used for all light sheet imaging. For time series imaging, scans were done at 20 min intervals for 12–17 h.

Sample Handling and Mounting for Imaging. Specimens were prepared for imaging as described previously (29). Larval zebrafish were anesthetized using tricaine methanesulfonate at 120 μg/mL, placed in melted 0.5% agarose gel at no more than 42 °C, and pulled individually into glass capillaries. Each capillary was then mounted on a holder on a computer-controlled translation stage, and each fish was extruded in a plug of gel into a speciﬁc specimen chamber ﬁlled with sterile embryo media and tricaine methanesulfonate. The plug was extruded by the head. The specimen chamber was maintained at 28 °C. All time-series experiments were performed overnight beginning in the evening.

Imaging-Based Quantification of Bacterial Populations. In vivo gut bacterial populations (Fig. 3) were quantiﬁed from light sheet images using an analysis pipeline as described previously (29). In brief, bacterial aggregates and individual bacteria were identiﬁed separately. The number of bacteria was detected using a wavelet-based spot detection algorithm, with autocorrelation of the motility, and the amplitudes and frequencies of gut motions along the anteroposterior (AP) axis were obtained using a previously described analysis pipeline (46). In brief, the AP component of the vector field was averaged along the dorsoventral direction, resulting in a scalar motility measure at each position along the gut axis and at each time point. The frequency of gut contractions was calculated as the location of the ﬁrst peak in the temporal autocorrelation of the motility. The amplitude was calculated as the square root of the spatially averaged power spectrum at the previously determined frequency, providing the magnitude of the periodic motion.

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