Constitutive Type VI Secretion System Expression Gives Vibrio cholerae Intra- and Interspecific Competitive Advantages

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Constitutive Type VI Secretion System Expression Gives *Vibrio cholerae* Intra- and Interspecific Competitive Advantages

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**Abstract**

The type VI secretion system (T6SS) mediates protein translocation across the cell membrane of Gram-negative bacteria, including *Vibrio cholerae* – the causative agent of cholera. All *V. cholerae* strains examined to date harbor gene clusters encoding a T6SS. Structural similarity and sequence homology between components of the T6SS and the T4 bacteriophage cell-puncturing device suggest that the T6SS functions as a contractile molecular syringe to inject effector molecules into prokaryotic and eukaryotic target cells. Regulation of the T6SS is critical. A subset of *V. cholerae* strains, including the clinical O37 serogroup strain V52, express T6SS constitutively. In contrast, pandemic strains impose tight control that can be genetically disrupted: mutations in the quorum sensing gene luxO and the newly described regulator gene tsa lead to constitutive T6SS expression in the El Tor strain C6706. In this report, we examined environmental *V. cholerae* isolates from the Rio Grande with regard to T6SS regulation. Rough *V. cholerae* lacking O-antigen carried a nonsense mutation in the gene encoding the global T6SS regulator VasH and did not display virulent behavior towards *Escherichia coli* and other environmental bacteria. In contrast, smooth *V. cholerae* strains engaged constitutively in type VI-mediated secretion and displayed virulence towards prokaryotes (*E. coli* and other environmental bacteria) and a eukaryote (the social amoeba *Dictyostelium discoideum*). Furthermore, smooth *V. cholerae* strains were able to outcompete each other in a T6SS-dependent manner. The work presented here suggests that constitutive T6SS expression provides *V. cholerae* with an advantage in intraspecific and interspecific competition.

**Introduction**

The Gram-negative bacterium *Vibrio cholerae* is the causative agent of the acute diarrheal disease cholera and remains a serious health risk to humans. In addition to the two main virulence factors needed to cause massive watery diarrhea–cholera toxin [1] and the toxin coregulated pilus [2] – the bacterium utilizes accessory virulence factors also capable of causing diarrheal disease. Accessory toxins such as hemolysin (HlyA) and actin-cross-linking repeats-in-toxin (RtxA) have been reported to be virulence mechanisms exploited by some strains [3].

Another such accessory virulence factor is the type VI secretion system (T6SS), which confers cytotoxic effects against both prokaryotic and eukaryotic cells [4–6]. Bacteria have developed numerous mechanisms to export proteins, including toxins, across their cell walls into the surrounding environment or into host cells. To date, six distinctive pathways, collectively called secretion systems and classified into type I to type VI (T1SS – T6SS), have been identified in Gram-negative bacteria [7]. The T6SS of *V. cholerae* mediates cytotoxicity towards eukaryotic hosts, including murine macrophages [5,8,9] and the amoeba *Dictyostelium discoideum* [4]. The *V. cholerae* T6SS is encoded by three gene clusters on two separate chromosomes: one large cluster (VCA0107 – VCA0124) [10] and two small auxiliary clusters (VCA0017 – VCA0021 and VCA1145 – VCA1141). Bioinformatic analyses and a series of experimental approaches have elucidated the functions of several genes belonging to the *V. cholerae* T6SS clusters. For example, the Hcp protein [11], secreted by bacteria with a functional T6SS, forms a nanotube structure with an internal
diameter of 4 nm [12]. Three VgrG proteins were shown to interact with each other to form a trimeric complex that structurally resembles a T4-bacteriophage gp5-gp27 tail spike complex [9], but unlike their phage counterparts lack an internal channel [13]. The current working model of the T6SS is based on these observations and the finding that Hcp and VgrG are codependent for secretion. The model proposes that the Hcp nanotube, decorated with a VgrG trimer at its top, is pushed through the bacterial envelope of the predator cell and into the prokaryotic or eukaryotic target cell. It is suggested that cytoplasmic VipA and VipB (VCA0107 and VCA0108) form a contractile sheath around the Hcp tube similar to the T4 phage outer sheath; contraction of the VipAB sheath erects the Hcp tube from the predator cell [14]. The VgrG cap might mediate toxicity via the C-terminal extensions of evolved VgrGs upon delivery into the target cell [5]. Alternatively, the cap might dissociate from the Hcp nanotube to allow delivery of soluble toxin(s) or effector molecule(s) through the Hcp conduit [13]. VasH (VCA0117) acts as a sigma-54 activator protein and controls transcription of T6SS genes including hcp and vgrG. We recently reported that the V. cholerae T6SS also exerts contact-dependent killing properties against other Gram-negative bacteria such as Escherichia coli [6]. This finding suggests that V. cholerae may employ the T6SS to compete with commensal bacteria in the human intestine and/or environmental reservoirs.

The environmental reservoirs of V. cholerae (river deltas with brackish waters, oceans, and deep seas [15]) are as diverse as the environmental reservoirs. We focused on the Rio Grande, a river that empties into the Gulf of Mexico and is considered to be a major reservoir of unique, nonpandemic O1 El Tor strains responsible for sporadic food-born cholera in the summer [21]. Environmental V. cholerae isolates (RGVCs) collected at two locations along the Rio Grande were examined to test whether constitutive T6SS expression is prevalent in V. cholerae exposed to microbial competitors and predators.

### Materials and Methods

#### Strains and Culture Conditions

A streptomycin-resistant V. cholerae strain V52 (O37 serogroup) lacking hlyA, rtxA, and hlyD genes [4] was used as a T6SS-positive strain in all experiments presented in this study. DH5αpir and SM102pir were used for cloning, and mating of pWM91-based plasmids, respectively. The strains and plasmids used in this study are listed in Table 1. Unless stated otherwise, bacteria were grown in a Luria-Bertani (LB) broth at 37°C with shaking (200 rpm). Rifampicin-resistant (50 µg·mL⁻¹) Vibrio communis, Vibrio harveyi, Vibrio cholerae, and Salmonella typhimurium were grown on LB agar plates supplemented with 100 µg·mL⁻¹ ampicillin.

### Table 1. Bacterial strains and plasmids.

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibrio cholerae V52</td>
<td>O37 serogroup strain, ΔhapA, ΔrtsA, ΔNlyA, smR</td>
<td>[25]</td>
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<tr>
<td>Vibrio cholerae V52 ΔvasK</td>
<td>V52 mutant lacking vasK (VCA0120)</td>
<td>[25]</td>
</tr>
<tr>
<td>DL2111, DL2112, DL4211, DL4215</td>
<td>Environmental isolates collected in this study (see Table 3).</td>
<td>This study</td>
</tr>
<tr>
<td>DL4211 ΔvasK</td>
<td>DL4211 mutant lacking vasK (VCA0120)</td>
<td>This study</td>
</tr>
<tr>
<td>DL4215 ΔvasK</td>
<td>DL4215 mutant lacking vasK (VCA0120)</td>
<td>This study</td>
</tr>
<tr>
<td>Escherichia coli DH5α λpir</td>
<td>hfaA2 Δ(orf-F-lacZ)U169 phoA glnIV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 thi-1 hsdR17</td>
<td>Provenzano Laboratory (University of Texas at Brownsville)</td>
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<td>Escherichia coli SM100 λpir</td>
<td>KmR, thi-1, thr, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, pir</td>
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<td>Escherichia coli MG1655</td>
<td>F- lambda- ilvG- rfb-50 rph-1, RifR</td>
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<tr>
<td>Klebsiella pneumoniae</td>
<td>Wild-type, T6SS-negative control</td>
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<td><strong>Plasmids</strong></td>
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<tr>
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<td>pBAD vector, pBR322 ori, araC, KanR</td>
<td>[39]</td>
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<tr>
<td>pBAD18-vasH/myc</td>
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<td>[16]</td>
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<tr>
<td>pBAD24</td>
<td>pBAD vector, pBR322 araC, AmpR</td>
<td>[39]</td>
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<tr>
<td>pBAD24-vasK</td>
<td>pBAD24 vector, pBR322 araC, AmpR of the Vibrio cholerae strain V52</td>
<td>[6]</td>
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<td>pWM91</td>
<td>oriR6K mobrB4 lacI pTAC trp mini-Tn10Km Clm1 AmpR</td>
<td>[23]</td>
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<tr>
<td>pGEM-T-easy</td>
<td>Vector for cloning PCR products, AmpR</td>
<td>Promega</td>
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doi:10.1371/journal.pone.0048320.t001
DNA Sequence Analysis and Protein Structure Prediction Analysis

Nucleotide sequence analyses and alignments were performed with MacVector software (version 11.0.2).

16S Ribosomal Sequencing

Primers binding to conserved 16S ribosomal gene sequences were used to PCR-amplify the 16S ribosomal sequences from environmental bacterial isolates. Primer sequences are summarized in Table 2. DNA sequencing was performed at the University of Alberta Applied Genomics Centre and species were identified using BLASTn.

Table 2. Primers.

<table>
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<tr>
<td>S-vasH-myc</td>
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<td>S-vasK-pBAD24</td>
<td>TTGAAATCCATGAGGAAATTCCTT</td>
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</tr>
<tr>
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<td>S'-16S Universal (U1115R)</td>
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<table>
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<th>Serogroup</th>
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<td>frameshift, H116D, Q278L, T449A, T456I</td>
</tr>
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<td>O123</td>
<td>H116D, T449A</td>
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<td>4215</td>
<td>O113</td>
<td>H116D, T441S, P447S, T449V</td>
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<tr>
<td>N16961</td>
<td>O1</td>
<td>H116D, T449A</td>
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doi:10.1371/journal.pone.0048320.t002
doi:10.1371/journal.pone.0048320.g001

Figure 1. Ability of RGVC isolates to kill E. coli. Rough RGVC isolates DL2111 and DL2112, and smooth RGVC isolates DL4211 and DL4215 were tested for their ability to confer T6SS-mediated prokaryotic killing. V52 and V52ΔvasK were used as virulent and avirulent controls, respectively. V. cholerae and E. coli were mixed in a 10:1 ratio and incubated for 4 hours at 37°C. Bacterial spots were resuspended, serially diluted, and plated on E. coli-selective media to determine the number of surviving E. coli. The averages and standard deviations of two independent experiments, each performed in duplicates are shown.

doi:10.1371/journal.pone.0048320.g001
an equal volume of lysis buffer and boiled for 10 minutes. Samples were subjected to SDS-PAGE (10% acrylamide) and analyzed by western blotting using a rabbit polyclonal antibody against DnaK (Stressgen, diluted 1:15,000), mouse anti-RNAP (Neoclone, diluted 1:1000), mouse anti-beta-lactamase (Sigma, diluted 1:200), and polyclonal rabbit anti-Hcp [5] antiserum (diluted 1:500). Secondary antibodies used were goat anti-mouse horseradish peroxidase (HRP) and goat anti-rabbit HRP (both Santa Cruz, diluted 1:3000).

D. discoideum Plaque Assays

100 μL of overnight bacterial culture and 10^5 D. discoideum AX3 cells were spread on SM/5 plates [22]. Arabinose (0.1%) was added to SM/5 plates when indicated. Plates were incubated at 22°C for 3 days to assess the number of plaques.

Bacterial Killing Assay

Bacterial strains were grown as lawns on LB-agar plates with appropriate antibiotics. Environmental non-V. cholerae strains were grown on 1/2 YTSS agar plates with appropriate antibiotics. Streptomycin-resistant (rifampicin-sensitive) predator and rifampicin-resistant (streptomycin-sensitive) prey were harvested and mixed at a 10:1 ratio with volumes normalized by OD600 readings. 25 μL of the mixed bacterial culture was spotted onto prewarmed LB-agar (or 1/2 YTSS agar plates for mixtures containing non-V. cholerae strains) and incubated at 37°C (or 30°C for non-V. cholerae strains) for 4 h. Bacterial spots were harvested and the CFU-mL⁻¹ of surviving prey and predator were measured by serial dilution and selective growth on agar containing 50 μg·mL⁻¹ rifampicin and 100 μg·mL⁻¹ streptomycin, respectively. Where applicable, arabinose was added to LB plates at a final concentration of 0.1% to induce expression from the P_BAD promoter during the 4 hour incubation.

![Figure 2. RGVC isolates with a constitutive T6SS kill D. discoideum.](image)

10^5 D. discoideum cells were plated with indicated bacteria on SM/5 agar plates that support bacterial but not amoeboid growth. Plaques formed by D. discoideum were counted on the third day of incubation. The graph summarizes the results of two independent experiments. Standard deviations are shown. KP: Klebsiella pneumoniae.
doi:10.1371/journal.pone.0048320.g002

![Figure 3. RGVC isolates differ in T6SS regulation.](image)

Indicated RGVC isolates and V52 (positive control) were cultured to mid-logarithmic phase of growth followed by centrifugal separation of pellets and culture supernatants. Supernatant portions were concentrated by TCA precipitation and both fractions were subjected to SDS-PAGE followed by western blotting using the antibodies indicated. Experiments were repeated at least three times with equivalent results.
doi:10.1371/journal.pone.0048320.g003
DNA manipulations

3’-Myc-tagged vasH was PCR-amplified from V. cholerae V52 chromosomal DNA with primers 5’vasH and 3’vasH:myc (Table 1). The resulting PCR product was restricted with 5’-EcoRI and 3’-XbaI, cloned into pGEM T-easy (Promega), and subcloned into pBAD18.

In-frame deletion of vasK was performed as described by Metcalf et al. [23] using the pWM91-based vasK knockout construct [9]. During sucrose selection, sucrose concentration was increased from 6% to 20% for all RGVC gene deletions because these isolates exhibited increased tolerance to sucrose compared to V52.

For complementation, vasK was amplified from V52 chromosomal DNA using primers 5’-vasK-pBAD24 and 3’-vasK-pBAD24 (Table 1). The resulting PCR product was purified using the Qiagen PCR cleanup kit, digested with EcoRI and XbaI, and cloned into pBAD24.

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**Results**

RGVC Isolates Exhibit T6SS-Mediated Antimicrobial Properties

We previously demonstrated that clinical V. cholerae O37 serogroup strain V52 uses its T6SS to kill E. coli and Salmonella Typhimurium [6]. To determine the role of the T6SS in environmental strains, we employed two different types of V. cholerae isolated from the Rio Grande: smooth isolates with distinct O-antigens as part of their lipopolysaccharides (LPS), and rough isolates that lack O-antigen (Table 3). Due to concerns that rough bacteria are genetically unstable because the lack of O-antigen allows the uptake of chromosomal DNA [24], we assessed the virulence potential of two separately isolated but genetically identical rough isolates DL2111 and DL2112 (as determined by deep sequencing [Illumina platform] of a polymorphic 22-kb fragment [Genbank accession numbers JX669612 and JX669613]) to minimize the chance of phenotypic variation due to genetic exchange.

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**Figure 4.** Complementation of a vasK null-mutation restores T6SS-dependent secretion and virulence. (A) VasK-mutants of smooth RGVC isolates carrying a plasmid for arabinose-induced vasK expression were cultured to midlogarithmic phase of growth in the presence or absence of 0.1% arabinose. V52 and the isogenic vasK mutant were used as positive and negative controls, respectively. Pellets and culture supernatants were separated by centrifugation. The supernatant portions were concentrated by TCA precipitation and both fractions were subjected to SDS-PAGE followed by western blotting using the antibodies indicated. (B) Survival of E. coli MG1655 after mixing with V. cholerae. V. cholerae and E. coli were mixed in a 10:1 ratio and incubated for 4 hours at 37°C before the resulting spots were resuspended, serially diluted, and plated on E. coli-selective media. Data represent the averages of three independent experiments. Standard deviations are included. (C) Survival of D. discoideum after mixing with V. cholerae. D. discoideum was plated with V. cholerae and the number of plaques formed by surviving D. discoideum were counted after a 3-day incubation at 22°C. Data are representative of three independent experiments. Standard deviations are shown.

doi:10.1371/journal.pone.0048320.g004
To determine whether environmental RGVC *V. cholerae* are capable of killing bacteria, we performed an *E. coli* killing assay (Figure 1). RGVC isolates and *E. coli* strain MG1655 were spotted on LB nutrient agar plates, and the number of surviving MG1655 cells was determined after a 4-hour incubation at 37°C. V52 and V52ΔvasK were used as virulent and avirulent controls, respectively. The presence of V52 resulted in an average 5-log reduction of viable *E. coli*. Smooth isolates DL4211 and DL4215 killed *E. coli* at levels comparable to V52 (Figure 1). In contrast, both rough isolates, DL2111 and DL2112, were unable to kill *E. coli* prey. In summary, smooth RGVC isolates readily killed *E. coli* while rough RGVC isolates appeared to be attenuated.

RGVC Isolates Display T6SS-Mediated Virulence Towards *D. discoideum*

The clinical *V. cholerae* O37 serogroup strain V52 displays T6SS-dependent cytotoxicity towards the social amoeba *D. discoideum* [4]. We tested whether RGVC isolates were also capable of evading amoeboid grazing by killing the eukaryotic predator. RGVC isolates were plated together with amoebae on nutrient agar plates that exclusively support bacterial growth. For amoebae to survive on agar plates, they must obtain nutrients from phagocytosed bacteria. This amoeboid grazing behavior on bacteria results in the formation of plaques—clear zones in the bacterial lawn that are devoid of bacteria [25]. The T6SS mediates bacterial virulence towards *D. discoideum* and abrogates plaque formation. Wild-type V52 and *Klebsiella pneumoniae* were used as virulent (no plaques) and avirulent (plaque formation) controls, respectively. Smooth isolates DL4211 and DL4215 killed *D. discoideum* at levels comparable to V52. In contrast, rough DL2111 and DL2112 did not kill *D. discoideum* similar to the T6SS-null mutant V52ΔvasK and the avirulent *Klebsiella pneumoniae* negative control (Figure 2).

Expression of Hcp in RGVC Isolates

Next, we set out to test whether RGVC isolates were able to produce and secrete the T6SS hallmark protein Hcp because experimental results presented thus far suggested that *V. cholerae*’s ability to kill bacterial competitors or eukaryotic predators [6] could be mediated by the T6SS. As shown in Figure 3, smooth isolates DL4211 and DL4215 produced Hcp at sufficient levels to be detected by western blots probed with Hcp antiserum. In contrast, rough isolates did not produce or secrete Hcp. The

Figure 5. Alignment of VasH polypeptide sequences of RGVC isolates. VasH of V52, N16961, and four RGVC isolates were aligned. In the rough isolates, a guanine was inserted at position 157 of vasH to restore the open reading frame. Colored bars indicate substitutions compared to VasH from V52.
doi:10.1371/journal.pone.0048320.g005

Figure 6. VasH complementation restores Hcp synthesis but not secretion in rough RGVC isolates. *V. cholerae* isolates were transformed with pBAD18-vasH-myc. The isolates were cultured to midlogarithmic phase of growth in the presence or absence of 0.1% arabinose. Pellets and culture supernatants were separated by centrifugation. The supernatant portions were concentrated by TCA precipitation and both fractions were subjected to SDS-PAGE followed by western blotting using the antibodies indicated. Data are representative of three independent experiments.
doi:10.1371/journal.pone.0048320.g006
presence of Hcp correlated with virulence as the smooth isolates secreted Hcp (Figure 3) and killed *E. coli* (Figure 1) as well as *D. discoideum* (Figure 2), while rough isolates did not produce Hcp and appeared to be attenuated.

**RGVC Isolates Engage in T6SS-Mediated Secretion and Virulence**

To determine whether killing of *E. coli* (Figure 1) and *D. discoideum* (Figure 2) depends on a functional T6SS, we performed killing assays and plaque assays with DL4211ΔvasK and DL4215ΔvasK as a predator. VasK is an inner membrane protein believed to provide the energy for T6SS-mediated secretion [26,27]. VasK is, therefore, crucial for a functional T6SS. As shown in figure 4A, parental V52, DL4211, and DL4215 constitutively produced and secreted Hcp, while deletion of *vasK* blocked secretion but not synthesis of Hcp. To complement the *vasK* chromosomal deletion, *vasK* from V52 was cloned downstream of an arabinose-inducible promoter in the plasmid pBAD24 and introduced into DL4211ΔvasK (DL4211ΔvasK/pvasK) and DL4215ΔvasK (DL4215ΔvasK/pvasK). Trans complementation of *vasK* restored Hcp secretion in V52 and the two smooth isolates (Figure 4A). To assess the role of T6SS in killing *E. coli*, we incubated *E. coli* with various *V. cholerae* isolates and determined the number of surviving *E. coli* after a 4-hour

Figure 7. RGVC isolates kill bacterial neighbors. *V. cholerae* and prey bacteria were mixed in a 10:1 ratio and incubated on ½ YTSS agar for 4 hours at 30°C. Bacterial spots were resuspended, serially diluted, and plated on selective YTSS agar to determine the number of surviving prey. The average and standard deviations of three independent experiments, each performed in duplicates, are shown.

doi:10.1371/journal.pone.0048320.g007
incubation at 37°C (Figure 4B). VasK mutants of V52, DL4211, and DL4215 lost their ability to kill *E. coli*, but providing vasK in *trans* restored virulence. Furthermore, amoebae were unable to form plaques in lawns of V52, DL4211, and DL4215, but did so in lawns of V52ΔvasK, DL4211ΔvasK and DL4215ΔvasK (Figure 4C). Complemented isolates, V52ΔvasK/pvasK, DL4211ΔvasK/pvasK

**Figure 8. T6SS-dependent competition among *V. cholerae* isolates.** (A–C) Smooth *V. cholerae* isolates successfully competed with each other and outcompeted the rough isolates in a T6SS-dependent manner. All combinations among the isolates and their isogenic vasK mutants were tested in a killing assay. Predator- and prey-*V. cholerae* were mixed in a 10:1 ratio and incubated for 4 hours at 37°C. Bacterial spots were resuspended, serially diluted, and plated on selective media to determine the number of surviving prey. The number of surviving prey in the presence of T6SS⁺ or T6SS⁻ predator are shown. (D) Arrows indicate the competitive relationship between isolates such that the arrow points from the predator towards the prey. Arrow thickness indicates relative killing efficiency. T6SS-dependence of the killing phenotype was confirmed by employing the vasK-deficient predator of each *V. cholerae* isolate examined. To avoid killing of the predator, vasK-deficient prey of smooth T6SS⁺ isolates were used. The average and standard deviations of two independent experiments, each performed in duplicates, are shown.

doi:10.1371/journal.pone.0048320.g008
and DL4215ΔvasK/pvasK, regained virulence towards D. discoideum (Figure 4C). Although the wild-type phenotype of DL4211 could not be fully complemented by episomal expression ofvasK, the complemented phenotype is statistically significant (unpaired t-test, p=0.0116). We conclude that smooth RGVC isolates conferred T6SS-mediated virulence towards E. coli and D. discoideum, demonstrating that the virulence phenotype described in Figures 1 and 2 is T6SS-dependent.

**Rough RGVC Isolates Carry Unique vasH Sequences**

We previously showed that the global transcriptional activator VasH is essential for expression of hcp and other T6SS genes. As the rough isolates failed to synthesize Hcp (Figure 3), we tested whether these isolates carried a nonfunctional vasH allele. The 1594 nucleotide-long vasH sequences of V52 and RGVC isolates were PCR-amplified and their polypeptide sequences aligned. The rough RGVC isolates were missing a guanine in codon 157 (ΔG157) which resulted in a frameshift. To include vasH of the rough isolates in our comparative analysis, we restored the vasH reading frame by in-silico insertion of G157. We found that all RGVC VasH sequences aligned with V52 and N16961 as well as with each other (Figure 5). Therefore, vasH is conserved in environmental (RGVC), pandemic (N16961), and endemic (V52) V. cholerae strains. The repaired vasH open reading frame closely resembled vasH from N16961 with only two unique substitutions (Q278L and T456I). Smooth RGVC isolate DL2111 carried an intact VasH gene identical to N16961; DL4215 differed from N16961 and V52 by three and four residues, respectively (Table 3). Substitutions of histidine to aspartic acid at position 116 (H116D) and threonine to alanine at position 449 (T449A) appear to be conserved in vasH sequences of V52 and RGVC isolates (Figure 5). Therefore, V. cholerae isolates carry a nonsense mutation and are likely to produce a truncated 63 amino acid-long VasH mutant protein.

### Table 4. Secretion and virulence phenotypes of RGVC isolates.

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<th>Prokaryotic Killing</th>
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<tbody>
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<td>+++</td>
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<td>DL2111</td>
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<td>DL2112</td>
<td>–</td>
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**Smooth RGVC Isolates Use Their T6SS to Compete with Natural Neighbors**

Because RGVC isolates with active T6SSs kill E. coli, we hypothesized that RGVC isolates use their T6SS to compete with other bacteria in their environmental niche. To test this hypothesis, we isolated three environmental bacterial non-V. cholerae strains from estuaries where the Rio Grande meets the Gulf of Mexico. Sequencing of 16S-rRNA identified these bacterial species as Vibrio communis, Vibrio harveyi, and Pseudoalteromonas phenolica (data not shown). We then tested whether DL4211 and DL4215 were able to kill these environmental bacteria in a T6SS-dependent fashion. As shown in Figure 7, both DL4211 and DL4215 killed all three environmental isolates. The observed killing required a functional T6SS, as isogenic vasK mutants lost their ability to kill. Killing of the environmental bacteria was restored by complementing the vasK mutant backgrounds with episomal vasK in trans. Therefore, we propose that constitutive expression of T6SS genes provides smooth RGVC isolates with the means to kill both their bacterial neighbors and potential eukaryotic predators.

**Smooth RGVC Isolates Use Their T6SS for Intraspecific Competition**

*V. cholerae* O37 strain V52 kills *E. coli* and *S. Typhimurium*, but is unable to kill other *V. cholerae*, including the O1 serogroup N16961 (El Tor) and O395 (classical biotype) strains [6]. Accordingly, the T6SS− isolates V52, DL4211 and DL4215 also exhibited immunity, because we did not observe a decline in viable CFUs when we recovered these isolates from single-isolate spots on LB agar plates after a 4-hour incubation (data not shown). We hypothesized that *V. cholerae* employs an immunity system that provides protection against T6SS-mediated toxicity. A functional link between T6SS and toxin/antitoxin systems has been established in *Pseudomonas aeruginosa* and *Burkholderia* species [28,29], which employ antitoxin proteins to counteract T6SS effectors [28]. VCA0124, an open reading frame downstream of the T6SS effector gene vgrG3 (VCA0123), has been implicated as an antitoxin gene in *V. cholerae* [30]. As RGVCs killed close relatives such as *V. harveyi* (Figure 7), we wondered if the RGVC isolates have the ability to kill each other. We hypothesized that if RGVC isolates use different toxins (and antitoxins), the T6SS might be used for intraspecific competition. We predicted that immunity of an RGVC isolate would be lost when approached by a *V. cholerae* bacterium with a different set of T6SS toxins to which the former lacks the corresponding antitoxin gene. To test this hypothesis, we mixed V52, DL4211, and DL4215 (predators) with smooth and rough RGVC isolates as prey bacteria. To eliminate the killing activity of smooth T6SS+ prey, we used vasK-deficient mutants with a disabled T6SS as prey. Rough wild-type RGVC isolates were used as prey since they do not express Hcp (Figure 3).
Discussion

We examined environmental smooth and rough V. cholerae isolates (RGVCs) collected at two locations along the Rio Grande to study T6SS regulation in V. cholerae exposed to microbial competitors and predators.

Our study showed that smooth RGVC isolates use their T6SS to kill other Gram-negative bacteria isolated from the Rio Grande delta. Deletion of the T6SS gene vasK resulted in a loss of bacterial killing. Importantly, the killing phenotype was restored by vasK complementation in trans. The requirement of VasK for killing implies that a constitutively active T6SS provides smooth RGVC isolates with a competitive advantage compared to their bacterial neighbors. By killing other bacteria, RGVC isolates might enhance their own survival in their environmental niche. In addition, we found that V. cholerae isolates use their T6SS to compete against each other.

In our experiments, Hcp synthesis and secretion correlated with eukaryotic and prokaryotic host cell killing (Table 4). For example, smooth Hcp-secreting RGVC isolates DL211 and DL215 (Figure 3) displayed full virulence towards E. coli (Figure 1) and D. discoideum (Figure 2). Rough RGVC isolates with their framseshift mutations in the T6SS transcriptional activator gene vasH did not produce or secrete Hcp, and their virulence was attenuated. Sequencing and gene alignments of the T6SS transcriptional activator vasH in rough strains indicated a missing guanine at position 157 in rough isolates, resulting in a frameshift mutation. Because VasH was recently implicated in regulating expression of T6SS during their complex life cycle remains to be determined.

It is becoming increasingly clear from our investigation and other reports that T6SS expression is prevalent in V. cholerae exposed to microbial competitors and predators until virulence factors such as cholera toxin and toxin-coregulated pilus genes are acquired. However, how V. cholerae regulate expression of T6SS during their complex life cycle remains to be determined.

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Author Contributions

Conceived and designed the experiments: DU MK STM DP SP. Performed the experiments: DU MK STM VB TB JM OS DS JDG. Analyzed the data: DU MK STM VB TB JM OS DS JDG DP SP. Contributed reagents/materials/analysis tools: DP. Wrote the paper: DU MK DP SP.
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