AMERICAN SOCIETY FOR MICROBIOLOGY | mBio®

RESEARCH ARTICLE

Evolution of a *cis*-Acting SNP That Controls Type VI Secretion in *Vibrio cholerae*

Siu Lung Ng,[a,c,d] Sophia Kammann,[a,c,d] Gabi Steinbach,[b,d] Tobias Hoffmann,[a,c] Peter J. Yunker,[b,d] Brian K. Hammer[a,c,d]

[a]School of Biological Sciences, Georgia Institute of Technology, Atlanta, Georgia, USA
[b]School of Physics, Georgia Institute of Technology, Atlanta, Georgia, USA
[c]Parker H. Petit Institute for Bioengineering & Bioscience, Georgia Institute of Technology, Atlanta, Georgia, USA
[d]Center for Microbial Diseases and Infection, Georgia Institute of Technology, Atlanta, Georgia, USA

ABSTRACT Mutations in regulatory mechanisms that control gene expression contribute to phenotypic diversity and thus facilitate the adaptation of microbes and other organisms to new niches. Comparative genomics can be used to infer rewiring of regulatory architecture based on large effect mutations like loss or acquisition of transcription factors but may be insufficient to identify small changes in noncoding, intergenic DNA sequence of regulatory elements that drive phenotypic divergence. In human-derived *Vibrio cholerae*, the response to distinct chemical cues triggers production of multiple transcription factors that can regulate the type VI secretion system (T6), a broadly distributed weapon for interbacterial competition. However, to date, the signaling network remains poorly understood because no regulatory element has been identified for the major T6 locus. Here we identify a conserved *cis*-acting single nucleotide polymorphism (SNP) controlling T6 transcription and activity. Sequence alignment of the T6 regulatory region from diverse *V. cholerae* strains revealed conservation of the SNP that we rewired to interconvert *V. cholerae* T6 activity between chitin-inducible and constitutive states. This study supports a model of pathogen evolution through a noncoding *cis*-regulatory mutation and preexisting, active transcription factors that confers a different fitness advantage to tightly regulated strains inside a human host and unfettered strains adapted to environmental niches.

IMPORTANCE Organisms sense external cues with regulatory circuits that trigger the production of transcription factors, which bind specific DNA sequences at promoters ("*cis*" regulatory elements) to activate target genes. Mutations of transcription factors or their regulatory elements create phenotypic diversity, allowing exploitation of new niches. Waterborne pathogen *Vibrio cholerae* encodes the type VI secretion system "nanoweapon" to kill competitor cells when activated. Despite identification of several transcription factors, no regulatory element has been identified in the promoter of the major type VI locus, to date. Combining phenotypic, genetic, and genomic analysis of diverse *V. cholerae* strains, we discovered a single nucleotide polymorphism in the type VI promoter that switches its killing activity between a constitutive state beneficial outside hosts and an inducible state for constraint in a host. Our results support a role for noncoding DNA in adaptation of this pathogen.

KEYWORDS vibrio cholerae, evolution, gene regulation, secretion systems, signal transduction, transcription factors

A central role in the dynamic, temporal control of gene expression is played by transcription factors (TFs), diffusible "*trans*" products that bind to molecular switches within DNA sequences termed "*cis*"-regulatory elements (CREs). In eukaryotes, where horizontal gene transfer (HGT) is rare, mutations in CREs that alter TF binding sites are

Editor Gisela Storz, National Institute of Child Health and Human Development (NICHD)

Address correspondence to Brian K. Hammer, brian.hammer@biology.gatech.edu.

The authors declare no conflict of interest.

Received 13 February 2022
Accepted 29 April 2022
Published 23 May 2022

major contributors to phenotypic diversity (1–3). In bacteria, pervasive HGT can alter entire regulatory circuits that allow adaptation to new niches, as prominently demonstrated in *Vibrio fischeri*, where host range is altered by the presence or absence of a histidine kinase RcsS, which regulates biofilm and colonization genes via indirect mechanisms (4, 5). By contrast, specific mutations at CREs in noncoding DNA are more difficult to identify and receive less attention as drivers of phenotypic divergence and evolutionary adaptation (6). Thus, elucidation of how microbes adapt to new niches, a process of fundamental importance in bacterial pathogenesis, requires coupling of genome-wide computational methods with experimental approaches to map the *cis*- and *trans*-regulatory interactions across and within species.

To understand how mutations play a role in microbial adaptation, pathogenic viruses and bacteria with lifestyles that exploit niches within and outside a human host are of great interest. Following ingestion, pandemic strains of the bacterium *Vibrio cholerae* can colonize the human gastrointestinal tract and secrete the cholera toxin that leads to the often fatal diarrhea responsible for seven pandemics to date (7–9). Conversely, *V. cholerae* isolated from nonhuman niches lack the horizontally acquired prophage that carries the cholera toxin, and cause mild illness (10). By contrast, all sequenced *V. cholerae* encode a type VI secretion system (T6), a broadly distributed "nano-harpoon" weapon that injects toxic effector proteins into neighboring bacterial cells, leading to cell envelope damage and cell lysis (11, 12). Due to its broad distribution among bacteria including those of the human gut, there is intense interest in understanding the T6 interactions between our microbiota and foreign pathogens, and whether they can be manipulated to influence health (13).

V. cholerae obtained from humans carry a limited arsenal of effectors and a T6 believed to be tailored for *in vivo* success (11, 14–19), while strains from nonhuman niches encode a more diverse effector repertoire (11, 14, 20, 21). To date, however, adaptative evolution mechanisms of T6 regulation in *V. cholerae* derived from nonhuman sources have largely been overlooked. Since the discovery of T6, studies of human-derived strains identify two primary TFs for T6 activation (22–26). T6 control in pandemic strains (e.g., C6706 and A1552) requires QstR, which is positively regulated by multiple external cues, including chitin that triggers TfoX production, and quorum-sensing autoinducers that control the well-studied LuxO/HapR regulatory circuit (27–30). QstR also contains a C-terminal DNA binding domain postulated to interact with a presumptive CRE of the major T6 gene cluster, yet how QstR-DNA interaction affects T6 transcription remains unclear (23, 27). On the other hand, T6 regulation in nonpandemic strain V52, which causes mild disease, requires TfoY. Expression of *tfoY* is modulatable by the intracellular second messenger 3′,5′-cyclic diguanylic acid (c-di-GMP) (25, 26). At low c-di-GMP levels, *tfoY* expression is posttranscriptionally regulated by a *cis*-acting riboswitch located upstream of the gene. At high c-di-GMP levels *tfoY* is regulated by transcription factor VpsR, which binds the second messenger (31). Despite significant progress over the past decade in uncovering the signaling systems that modulate QstR and TfoY, the mechanisms by which these two regulatory proteins control gene expression remain unclear. Similarly, direct regulators of T6 transcription, still remain elusive, with only one putative T6 CRE described (23). Elucidation of the differences in intraspecies T6 regulatory mechanisms between diverse *V. cholerae* isolates will provide insights into how pathogens emerge from nonpathogenic progenitors.

To understand the regulatory differences in *V. cholerae* strains, we examine several environmental isolates that exhibit T6-mediated killing (32). Despite encoding functional signaling circuity and TFs, we find that QstR is dispensable for killing and that TfoY plays only a minor role for killing in the strains tested. Thus, existing regulatory models fail to explain the T6 control in *V. cholerae* from human and nonhuman sources. Genomic analysis identifies one conserved noncoding single-nucleotide polymorphism (SNP) that we show interconverts *V. cholerae* T6 activity between chitin-inducible and constitutive states, which are QstR-dependent and TfoY-independent, respectively. We demonstrate

that noncoding SNPs can rewire *cis*-regulatory elements, which may aid in adaptation of bacteria to different niches, including the human host.

RESULTS AND DISCUSSION

Constitutive, *in vitro* T6 activity requires neither QstR nor TfoY in many environmental *V. cholerae* isolates. In pandemic C6706, high cell density conditions (HCD) and chitin are required for induction of *qstR* which leads to activation of T6 genes. In the absence of chitin, C6706 with *qstR* expressed from a heterologous promoter (defined here as *qstR**) reduces survival of *Escherichia coli* "target" cells in coculture by over 4-orders of magnitude (~10,000), compared with wildtype (WT) C6706, a T6 strain with a mutation in an essential structural gene (Δ*vasK*), and a strain with a Δ*qstR* mutation (Fig. 1A) (29). Deletion of *tfoY* does not reduce the killing activity of the T6$^+$ *qstR** strain, but eliminates the robust killing in the nonpandemic strain V52 (serogroup O37), which requires TfoY but not QstR (Fig. 1B) (26).

To determine whether QstR or TfoY participates in control of the T6 in nonhuman derived strains, we examined 3223-74, a genetically amenable, T6-proficient environmental strain (32). Like V52, 3223-74 does not require QstR to efficiently kill *E. coli* in conditions without chitin, but surprisingly, also does not require TfoY. Isogenic strains carrying the Δ*tfoY* and Δ*qstR* Δ*tfoY* mutations retain >99.99% killing activity, with only modest *E. coli* survival (Fig. 1C). Gene fusions of the 5′ intergenic region (IGR) of the major T6 cluster of each strain fused to green fluorescent protein (*gfp*) confirm that transcriptional differences account for the killing observed, with maximal *gfp* expression mirroring activity (i.e., low *E. coli* survival with high *gfp* expression, and *vice versa*) (Fig. 1D to F). To confirm that expression of the major T6 loci is not influenced by transcriptional read-through from a regulatory element upstream of the IGR, a T7 terminator (33) was inserted directly after the stop codon of *vca0106* in *V. cholerae* with activated T6 (Fig. S1). We observed no differences in T6 killing, demonstrating that the IGR is sufficient for control of the major T6 locus. Confocal microscopy reinforces the negligible role of TfoY on killing by 3223-74, with a Δ*tfoY* mutation having little effect on killing WT (Fig. 1G). Transcription of plasmid-borne reporters is significantly higher in *V. cholerae* than in *E. coli* (Fig. S2), supporting a hypothesis that an additional *V. cholerae*-specific regulator of the T6 may remain to be identified.

To probe each strain's T6-related regulatory circuitry, we measured canonical behaviors under the control of HapR, QstR, and TfoY; quorum sensing (QS) controlled bioluminescence, natural transformation, and motility, respectively (31, 34, 35). As expected, each TF is intact in C6706; but like several *V. cholerae* strains, V52 lacks a functional *hapR* gene that prevents QS and natural transformation (36, 37). Nonetheless, V52 encodes a functional *tfoY* that controls motility (Fig. 2A and B) (26). Interestingly, the regulatory circuity of *V. cholerae* 3223-74 is intact, like C6706, confirming that it encodes functional TFs (Fig. 2C), which are nonetheless expendable for T6-mediated killing. Nucleoid associated proteins (NAPs) that bind DNA both specifically and nonspecifically (39) may contribute to T6 transcription. NAPs participate in regulation of many promoters in numerous bacteria including *Vibrios* (40), yet NAP regulation and expression levels may differ in C6706 and 3223-74 (41). It is also possible that T6 regulation is complex and involves more than one TF specific to *V. cholerae*.

A SNP in the T6 intergenic region confers QstR-dependency. Human and environmental isolates of *V. cholerae* we have characterized prior (32) share ≥97% average nucleotide identity with many chromosomal differences (11). Yet, inspection of the T6 IGRs of C6706, V52, and 3223-74 revealed only 17 SNPs and three multinucleotide polymorphisms (Fig. 3A), which we hypothesized could contribute to the differences in T6 transcription and killing activity observed. To address this, we replaced the T6 IGR of C6706 on the chromosome with that from V52 and 3223-74 and measured killing activity. While C6706 carrying the *qstR** allele, but not WT, adeptly kills *E. coli*, both IGR replacements increase the killing efficiency of WT C6706 by 5- to 6-orders of magnitude (Fig. 3B), mimicking the robust killing observed by WT V52 and 3223-74 (Fig. 1B and C). Deletion of *tfoY* but not *qstR* in C6706 with V52's IGR increases *E. coli* survival

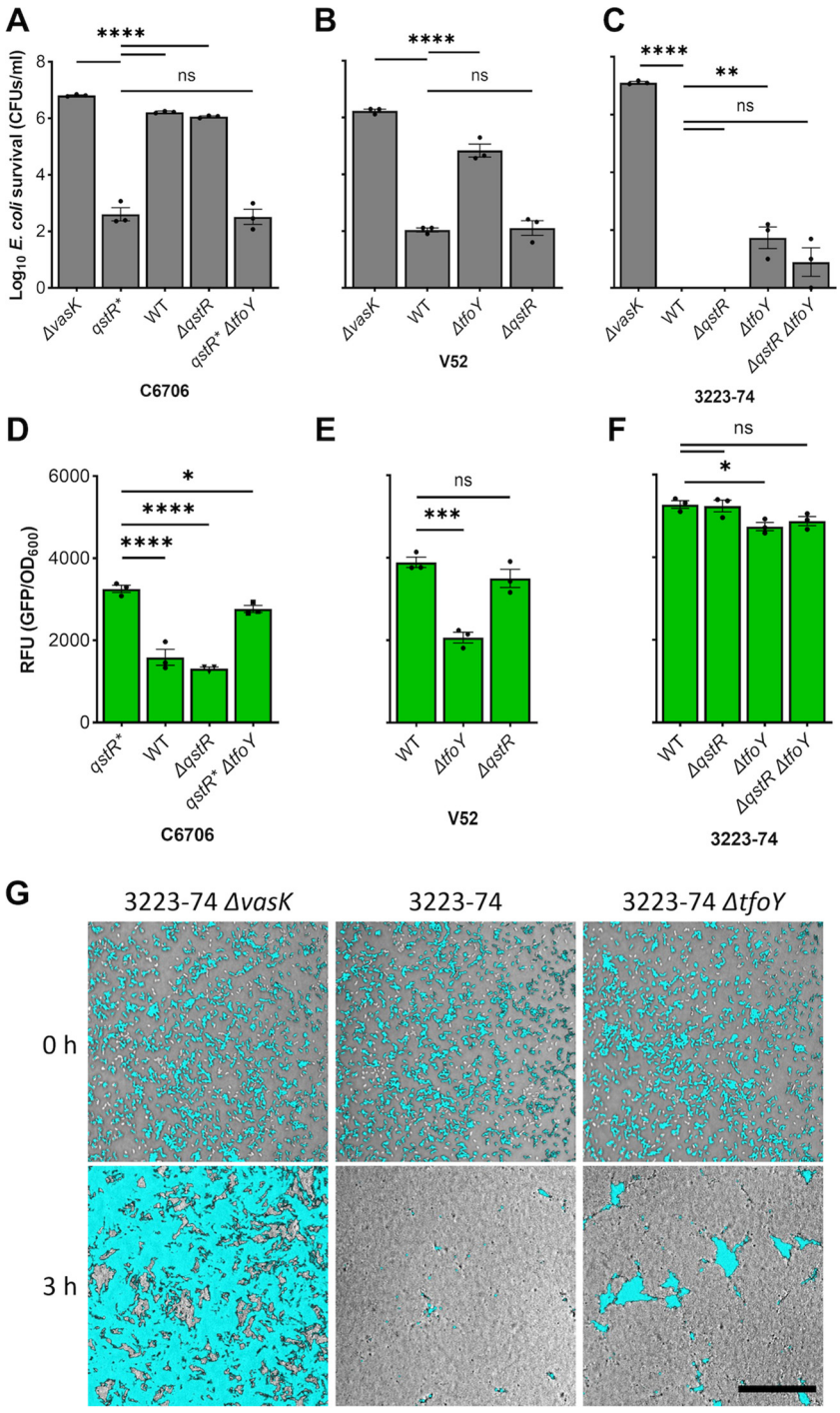

FIG 1 *Vibrio cholerae* 3223-74 T6 activity is QstR- and TfoY-independent. (A to C) *V. cholerae* strains with the indicated genotypes were cocultured with chloramphenicol resistant (Cm^r) *E. coli* followed by determination of *E. coli* survival by counting of CFU on LB agar with Cm. A *V. cholerae* Δ*vasK* mutant defective in T6 assembly served as a T6- negative control. (D to F) Relative Fluorescence Units are from reporters with *gfp* fused to the intergenic region 5′ of *vipA* derived from the strains shown. The mean value ± S.E. from cocultures (A to C) and monocultures (D to F) are derived from three independent biological replicates. A one-way ANOVA with Dunnett *post hoc* test was conducted to determine the significance: ns denotes not significant, ****, $P \leq 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$. (G) *E. coli* cells expressing constitutive *gfp* were competed against 3223-74, with the same frame imaged at 0 h and 3 h by confocal microscopy. In the images, *gfp* signal from the *E. coli* is overlaid on top of bright-light images of the coculture. Scale bar = 50 μm.

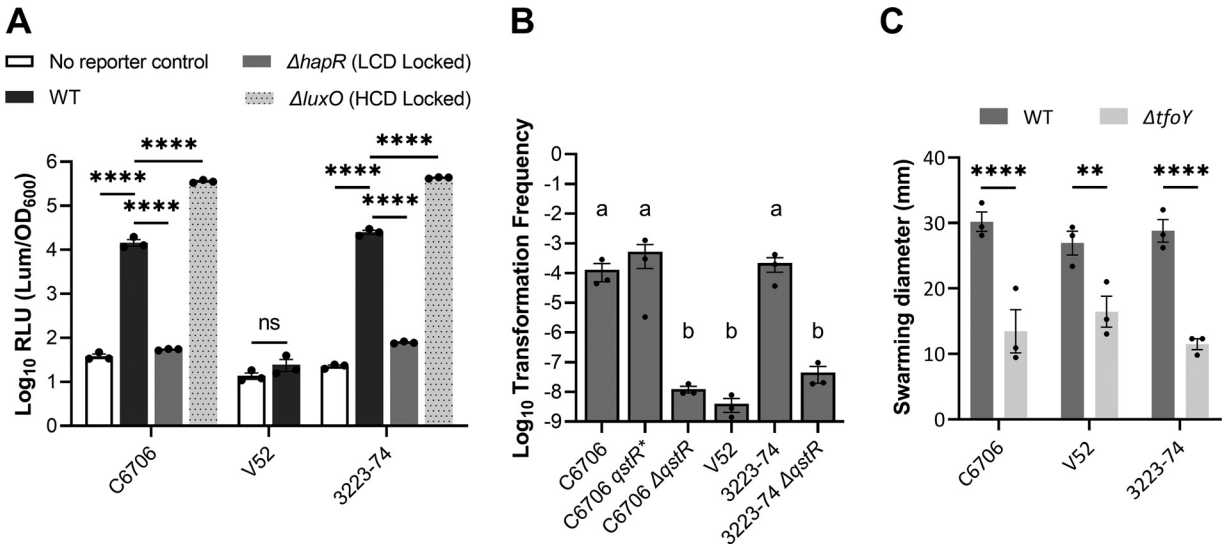

FIG 2 *Vibrio cholerae* 3223-74 encodes functional HapR, QstR, and TfoY. (A) *V. cholerae* strains with and without a QS-dependent *lux* reporter cosmid (pBB1) were grown in liquid LB with relative luminescence units per OD_{600} measured at HCD (OD_{600} = 0.6-0.8). Statistical analyses were conducted with one-way ANOVA with Tukey *post hoc* test (C6706 and 3223-74) and one-tailed Student's *t* test (V52). The Δ*hapR* mutant is defective at QS and effectively "locked" at low cell density, while the Δ*luxO* mutant that constitutively produces HapR is effectively "locked" at high cell density. (B) *V. cholerae* strains with the indicated genotypes were grown in ASW with crab shell and exogenous Spec-marked genomic DNA. Transformation frequency = $Spec^r$ CFU mL^{-1}/total CFU mL^{-1}. Statistical analyses were conducted with one-way ANOVA with Tukey *post hoc* test. Letters "a" and "b" identify statistically significance ($P \leq 0.05$) of transformation frequency between *V. cholerae* strains. (C) *V. cholerae* strains were inoculated on 0.3% LB agar and grew overnight. Statistical analyses were conducted with one-tailed Student's *t* test. Colony diameters were physically measured from the furthest edges. All data shown are the mean ± S.E. from 3 independent biological replicates. ns: not significant, ****, $P \leq 0.0001$; **, $P \leq 0.01$.

(~ 2-logs), as observed with V52, but does not alter *E. coli* survival with 3223-74's IGR (Fig. 3B; Fig. S3). Chromosomal transcriptional *gfp* reporters with identical mutations were elevated relative to WT C6706 in each IGR replacement strain (Fig. 3C), consistent with the enhanced killing detected. How TfoY controls gene expression is currently unknown and beyond the scope here. However, we speculate that slight differences detected in survival but not T6 transcription when *tfoY* is deleted from C6706 carrying the IGR of V52 may result from indirect effects of TfoY, or a factor(s) specific to V52 and absence in C6706. These results support a hypothesis that a novel CRE lies within the IGR 5′ of the T6 locus, despite a lack of any known direct TF-DNA interactions at this locus identified to date.

To begin mapping the T6 IGR region and SNP locations, we experimentally determined the transcriptional start site (+1) by 5′ rapid amplification of cDNA ends (Materials and Methods). The +1 of transcription resides 320 nucleotides (nt) 5′ of the ATG of the first T6 gene (*vipA*, *vca0107*), and adjacent to a putative promoter with 8/12 identical nucleotides compared with the consensus sigma70-dependent promoter (Fig. 3A). The +1 is consistent with paired-end RNA-seq results we have reported prior (29). Because the majority of 5′ untranslated regions (UTRs) in *V. cholerae* are 20 to 40 nt, with few exceeding 300 nt (42), we speculate that the 320 nt 5′ UTR of the major T6 gene cluster may be posttranscriptionally regulated, beyond the sRNA interactions already described near the ribosome binding site (RBS) (43). Alignment of the IGRs of C6706 and V52 reveals a single SNP at −68, with a guanine (G) in C6706 at that position and a thymine (T) in V52 (Fig. 3A).

The replacement of the C6706 IGR with V52 was effectively a G-68T mutation (Fig. 3B and C), thus we further tested whether G was necessary for QstR activation by replacing the T with a G at position −68 (T-68G) in the 3223-74 WT, *qstR**, and Δ*qstR* backgrounds. The T-68G mutation significantly increases *E. coli* survival and decreases T6 transcription in WT 3223-74 and the Δ*qstR* derivative, with killing restored in the strain with the *qstR** allele (Fig. 3D and E). Thus, a G at position −68 confers inducible,

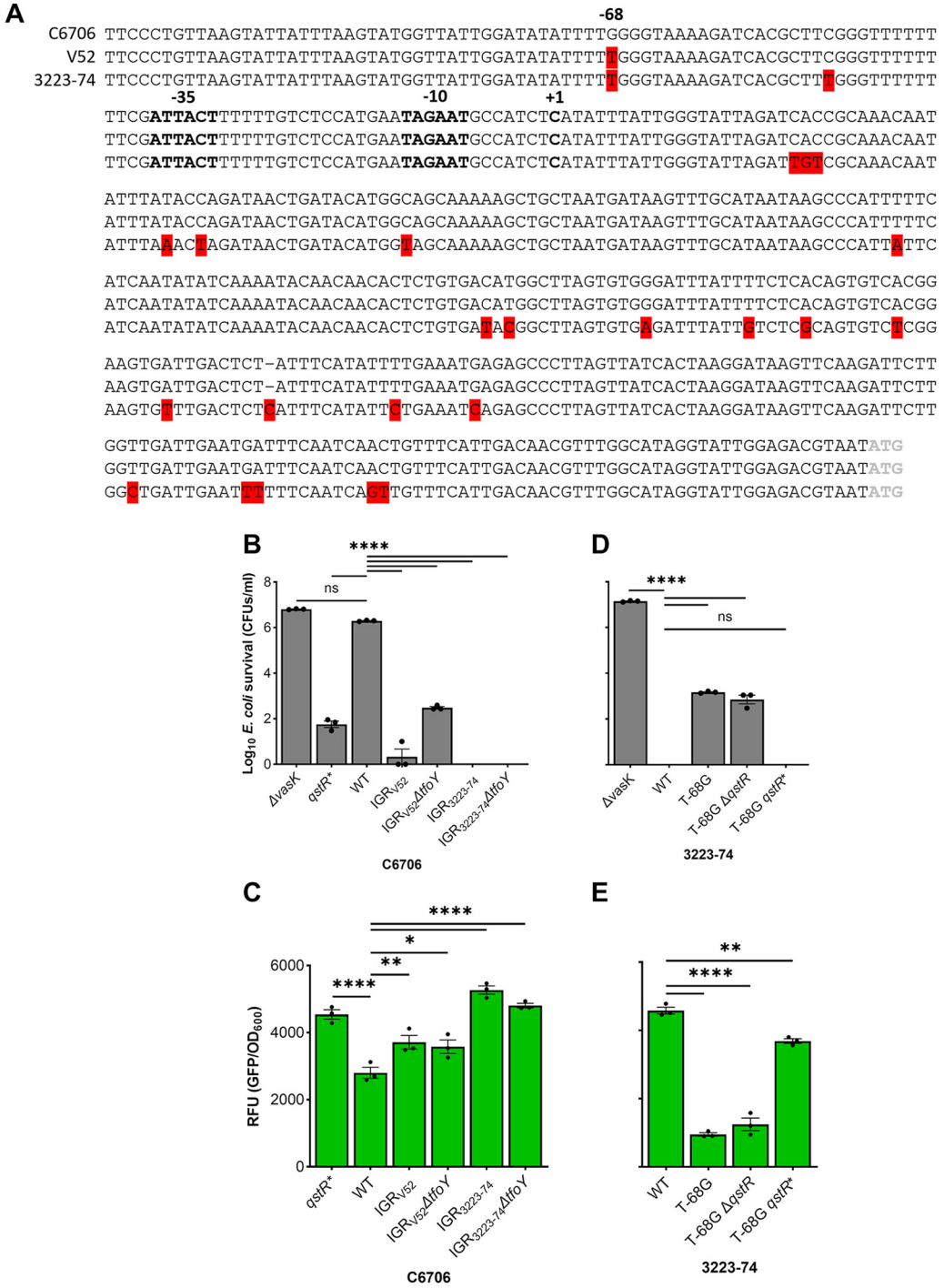

FIG 3 G-68T mutation abolishes QstR dependence in C6706 and T-68G confers QstR dependence to 3223-74. (A) Alignment of the IGR upstream of *vipA* was conducted using MUSCLE. SNPs and MNPs are highlighted in red, one gap indicated with a "–," the putative promoter and the transcriptional start site (TSS; +1) in bold, and the start codon of *vipA* in gray. (B) the C6706 5′ IGR of *vipA* was replaced with the IGR from either V52 or 3223-74. (D) A T-68G mutation in the 5′ IGR of *vipA* was introduced into 3223-74 with different *qstR* alleles. Competition assays were conducted by coculturing *V. cholerae* killers and Cm^r *E. coli* target followed by determination of *E. coli* survival by counting of CFU (CFU) on LB agar with Cm. The *V. cholerae* Δ*vasK* mutant unable to assemble a functional T6 served as a T6- negative control. (C, E) Shown are fluorescence levels of transcriptional reporters with *gfp* fused to corresponding IGRs of *vipA* expressed in either C6706 (C) or 3223-74 (E). Shown are mean values ± S.E. from three independent biological replicates of cocultures (B and D) and monocultures (C and E). A one-way ANOVA with Dunnett *post hoc* test was conducted to determine the significance. ns, not significant; ****, $P \leq 0.0001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

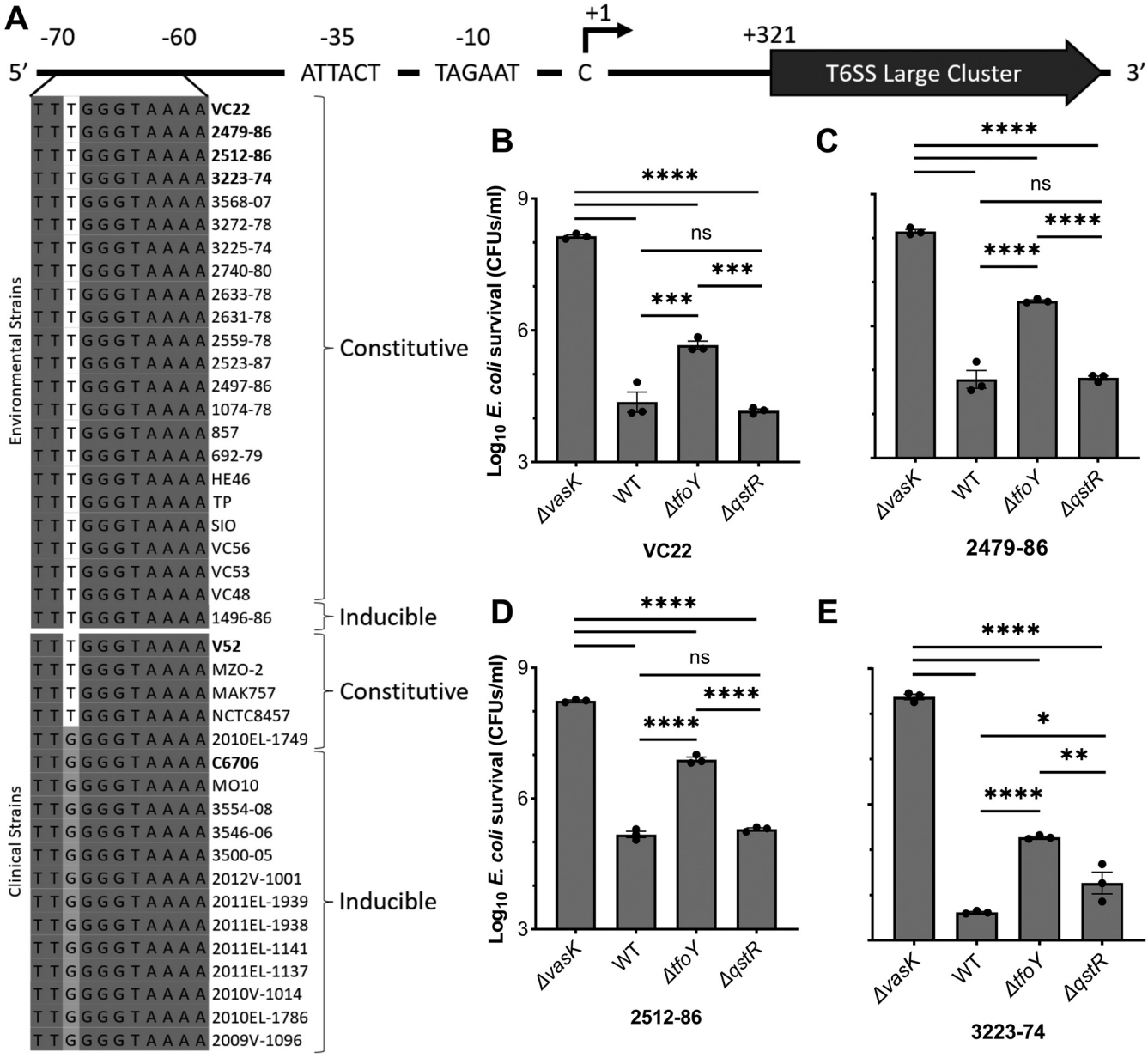

FIG 4 Environmental *V. cholerae* isolates encode a T at position −68 while human, chitin-induced isolates encode a G. (A) A SNP at position −68 in the IGR of the major T6 cluster controls killing activity. Conserved nucleotides are in dark gray and the SNP of interest is highlighted in white/gray. T6 control was categorized as described (32). (B to E) Survival of *E. coli* following competition assays with WT *V. cholerae* strains and mutants was determined by CFU counts. The *V. cholerae* Δ*vasK* mutant served as a T6- negative control. Data shown are mean values ± S.E. of three independent biological replicates. A one-way ANOVA with Tukey *post hoc* test was conducted to determine the significance. ns, not significant; ****, $P \leq 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

QstR-control, while a T results in constitutive killing *in vitro*, consistent with results recently reported during manuscript revision (44). Based on these results we predicted this SNP is a result of adaptive evolution to control T6 activity in different environments.

The SNP at −68 is evolutionarily conserved. To determine whether the SNP at −68 is prevalent in *V. cholerae*, we aligned the T6 IGR sequences of diverse strains that we have characterized prior for T6 killing activity (Fig. 4A) (32). Consistent with prior studies (11, 14, 16, 18), our phylogenetic analysis (Materials and Methods) of the T6 IGRs places human strains in a distinct clade, with the exception of two O1 strains isolated nearly a century ago (NCTC8457 and MAK757), and two non-O1 strains (MZO-2 O14 and V52 O37; Fig. S4). All 23 environmental isolates carry the T-68 SNP and displays constitutive T6 activity, with one exception that is chitin-inducible (1496-86) (Fig. 4A; Fig. S4). By contrast,

the 18 human-derived isolates tested carry either G or T at the −68 position (Fig. 4A; Fig. S4). The 13 chitin-inducible human isolates carry a G; five show constitutive activity and carry a T, like environmental strains, with one exception that is constitutive yet carries the G (2010EL-1749) (Fig. 4A; Fig. S4). Neither C nor A are observed at −68 in any stains tested, although both pyrimidine nucleotides (T and C) confer constitutive killing at −68, and both purines (G and A) behave similarly (Fig. S5). The focal SNP location is distal from the promoter, but inconsistent with AT-rich "UP-elements" that reside immediately upstream of the promoter at −38 to −59 and interact directly with the alpha subunit of RNAP (45). We propose the SNP is more likely a component of a CRE for a TF to be determined. Indeed, transversion mutations have greater effects of TF binding than transitions, as noted here (Fig. S5) likely due to changes in shape of the DNA backbone or DNA-amino acid contacts (46, 47).

We examined regulation of three additional genetically manipulatable environmental strains (VC22, 2479-89, and 2512-86) that exhibit T6 killing (32). Like 3223-74, QstR is expendable in each strain (Fig. 4B to E) while TfoY contributes to some extent in activating T6, with varying *E. coli* recovery observed in each derivative carrying the Δ*tfoY* mutation (Fig. 4B to E). Taken together, our findings reveal that the constitutive T6 killing activity of environmental *V. cholerae* is driven by a T at position −68, which obviates the QstR requirement, and permits modest TfoY regulation.

Bacterial adaptation to unexploited niches can be the result of horizontal gene transfer events (5) as well as mutations in protein coding and promoter regions (48, 49). Here we describe an intergenic non coding SNP that coordinates adaptation by altering T6 control between two states—one that is inducible and the other that displays constitutive activity. While the type VI secretion system was first described in *V. cholerae* in 2006, the knowledge of its regulation remains largely restricted to human isolates, and the identity of a TF that directly controls the major T6 cluster remains elusive to this date (22, 24). We speculate that the focal SNP we identified at position −68 is a component of a CRE that contributes to pathoadaptation (Fig. 3A), a result of adaptive evolution, which allows *V. cholerae* to carefully control the T6 expression in specific environments. Our results are consistent with the hypothesis that constitutive T6 is beneficial in aquatic environments outside a human host (50), with varying degrees of TfoY contribution, which may act directly or indirectly at the transcriptional or posttranscriptional level (Fig. 3A; Fig. 4B to E; Fig. S6). During human infection where selection promotes dampened T6, *V. cholerae* with a T-to-G mutation (inducible T6) are favored. In fact, T6-deficient human isolates (e.g., O395) have been reported to have less competitive fitness in human intestinal colonization and infection (19, 51). Although low level, basal expression of T6 contributes to pathogenesis of C6706 (52), overexpression of T6 may be deleterious *in vivo*. Indeed, we have reported prior that *V. cholerae* with constitutive T6 induces violent peristaltic contractions in a fish host (53), which may disrupt the interaction between *V. cholerae* and the gut microflora.

There remains a pressing public health need to understand the emergence of pathogens from environmental reservoirs (54). Efforts such as microbial genome wide association studies (55) to identify genetic variants in genomes that are associated with phenotypes like virulence and antibiotic sensitivity will be bolstered by knowledge of the ecological and evolutionary processes that promote pathogen-host association. Defining the plasticity of the regulatory circuity controlling the T6 weapon will provide insights into the role of polymorphisms in the evolution of this and other pathogens.

MATERIALS AND METHODS

Bacterial growth conditions and plasmid constructions. All *V. cholerae* and *E. coli* (Table S1) strains were grown aerobically at 37°C overnight in lysogeny broth (LB) with constant shaking or statically on LB agar. Ampicillin (100 μg/mL), kanamycin (50 μg/mL), chloramphenicol (10 μg/mL), spectinomycin (100 μg/mL), streptomycin (5 mg/mL), sucrose (20% wt/vol), and diaminopimelic acid (50 μg/mL) were supplemented where appropriate.

Plasmids (Table S2) used were constructed with DNA restriction nucleases (Promega, WI, USA), Gibson Assembly mix (New England Biolabs, MA, USA), and PCR amplification (Qiagen, Hilden, Germany) by PCR with Q5 polymerase (New England Biolabs, MA, USA), and primers (Table S3) generated by Eton

Bioscience Inc. (NC, USA) or Eurofins Genomics (KY, USA). All reagents were used according to the manufacturer's instructions. Plasmids were confirmed by PCR and Sanger sequencing by Eton Bioscience Inc. (NC, USA).

***V. cholerae* mutant construction.** All genetically engineered strains of *V. cholerae* were constructed with established allelic exchange methods using vector pKAS32 (56) and pRE118 (Addgene - Plasmid #43830). All insertions, deletions, and mutations were confirmed by PCR and Sanger sequencing conducted by Eton Bioscience Inc. (NC, USA). Primers used are in Table S3.

Fluorescence microscopy. Variants of *V. cholerae* strain 3223-74 and an *E. coli* MG1655 strain with *gfp* introduced into the chromosome were separately back-diluted 1:100 and incubated at 37°C for 3 h. *V. cholerae* and *E. coli* were normalized to OD_{600} = 1 and mixed in a 1:5 ratio. A 2 μL aliquot of a mixed culture was inoculated on LB agar and allowed to dry. Cells were imaged before and after a 3 h of incubation at 37°C and 96% to 100% humidity using an Eclipse Ti-E Nikon (NY, USA) inverted microscope with a Perfect Focus System and camera previously described (11). The images were processed with ImageJ (35).

Motility assay. Overnight cultures of *V. cholerae* were diluted to OD_{600} = 0.1, and 1 μL inoculated onto predried LB plates with 0.3% agar. Cells were incubated at 37°C statically overnight, with motility determined by measuring the swarming diameter.

Transformation assay. Chitin-induced transformation frequency was measured as described with defined artificial seawater (450 mM NaCl, 10 mM KCl, 9 mM $CaCl_2$, 30 mM $MgCl_2 \cdot 6H_2O$, and 16 mM $MgSO_4 \cdot 7H_2O$; pH 7.8) (57). Bacteria were incubated with extracellular DNA in triplicate wells containing crab shell tabs, and transformation frequency calculated as Spectinomycin resistant (Spec^r) CFU mL^{-1}/ total CFU mL^{-1}.

QS-dependent luciferase assay. A previously described, pBB1 cosmid was used as a QS-dependent *lux* reporter in *V. cholerae* (58). Overnight cultures of the *V. cholerae* strains were diluted to OD_{600} = 0.001 in liquid LB in microtiter plates and incubated at 37°C with shaking. The OD_{600} and luminescence were measured each hour with a BioTek (VT, USA) Synergy H1 microplate reader to calculate relative luminescence units (RLU) as luminescence/OD_{600}. *V. cholerae* without the cosmid served as a negative control (no reporter control). Data were collected when OD_{600} = 0.6 to 0.8. LB medium was used to blank the microplate reader for OD_{600} and luminescence readings.

Green fluorescent protein gene transcriptional reporter quantification. Overnight cultures of *V. cholerae* or *E. coli* were diluted 1:100 and incubated at 37°C for 3 h. To enhance the translation of *gfp*, the sequence of the native RBS (12 nt sequence) was replaced with the T7 RBS (12 nt sequence) in the primers used to make the fusions. Cm was added to maintain the plasmid-borne versions of reporters that were cloned into plasmid pSLS3. Then, 300 μL aliquots were transferred to black microtiter plates to read the OD_{600} and GFP fluorescence (Excitation: 485, Emission: 528) with a BioTek Synergy H1 microplate reader (VT, USA) to calculate relative fluorescence units (RFU) as fluorescence/OD_{600}. LB medium was used as the blank for the OD_{600}. Strain lacking reporters were used to blank the spectrophotometer for GFP fluorescence measurements.

T6-mediated killing assay. Overnight cultures of *V. cholerae* or *E. coli* were back-diluted 1:100 and incubated at 37°C for 3 h. *V. cholerae* strains and the Cm^r *E. coli* target were normalized to OD_{600} = 1 and then mixed at a ratio of either 10:1 or 1:5. A 50 μL mixed culture was spotted onto LB agar and dried. After a 3 h of incubation at 37°C, cells were resuspended in 5 mL of LB, and serial dilutions were conducted. Finally, the resuspension was inoculated on a LB agar containing Cm to select for the surviving *E. coli*, which was incubated overnight at 37°C and the *E. coli* colonies were counted and shown as CFU mL^{-1}.

RNA extraction and determination of the +1 of transcription by 5′-RACE. Overnight cultures of *V. cholerae* were back-diluted 1:100 and incubated at 37°C for 3 h before lysing. Three independent cultures of T6-active *V. cholerae* C6706 *qstR** and 3223-74 WT were harvested by centrifugation at room temperature. RNA isolation, genomic DNA removal, and RNA cleanup were performed as previously described (59). Genomic DNA contamination was confirmed by conducting PCR with primer pair specific for 16S rRNA loci (*rrsA*) as previously described (Table S3) (60). RNA purity was confirmed by NanoDrop (260/280 $\approx$ 2.0).

5′-RACE (Invitrogen, MA, USA) was conducted according to the manufacturer's protocol with slight modifications. Specifically, SuperScript IV reverse transcriptase (Invitrogen, MA, USA) was used to complete the first strand cDNA synthesis. Two *vipA*-specific primers (GT3056 and GT3060) were used to identify the +1 of transcription for the major T6 gene cluster (Table S3). PCR products were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany) or Zymoclean gel DNA recovery kit (Zymo Research, CA, USA). Sanger sequencing was conducted by Eton Bioscience Inc. (NC, USA) with the corresponding nesting primer (Table S3).

Genomic and phylogenetic analysis. Genome sequences of *V. cholerae* strains were collected from NCBI Genome database (Table S4) (61). The IGR upstream of major T6 cluster was extracted, aligned, and presented using BLAST+ v2.2.18 (62), MUSCLE v3.8 (https://www.ebi.ac.uk/Tools/msa/muscle/) (63, 64), and ESPript 3.0 (https://espript.ibcp.fr/) (38). The DNA sequence of the IGR was used for phylogenetic analysis, and the phylogenetic tree was constructed by the Maximum likelihood method using MEGA11 (65, 66).

For 2012V-1001, 2011EL-1939, 2011EL-1938, and 2011EL-1141 that do not have genome sequence available, colony PCR was conducted to amplify the 5′ IGR of the major T6 cluster using OneTaq DNA polymerase (New England Biolabs, MA, USA). PCR products were confirmed with gel electrophoresis and Sanger sequencing by Eton Bioscience Inc. (NC, USA) with the identical primer pair (Table S3).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 1.3 MB.
FIG S2, TIF file, 1.2 MB.
FIG S3, TIF file, 1.1 MB.
FIG S4, TIF file, 0.4 MB.
FIG S5, TIF file, 0.3 MB.
FIG S6, TIF file, 2.8 MB.
TABLE S1, XLSX file, 0.02 MB.
TABLE S2, XLSX file, 0.02 MB.
TABLE S3, XLSX file, 0.02 MB.
TABLE S4, XLSX file, 0.02 MB.

ACKNOWLEDGMENTS

We thank Dr. Jyl S. Matson for assistance with RNA isolation and Dr. Marvin Whiteley and current and past members of the Hammer Lab for critiques and discussion, specifically, Dr. Samit Watve and Rakin Choudhury for bioinformatic advice and assistance.

The authors have no competing interests.

REFERENCES

1. Wray GA. 2007. The evolutionary significance of cis-regulatory mutations. Nat Rev Genet 8:206–216. https://doi.org/10.1038/nrg2063.
2. Stern DL, Orgogozo V. 2008. The loci of evolution: how predictable is genetic evolution? Evolution 62:2155–2177. https://doi.org/10.1111/j.1558-5646.2008.00450.x.
3. Wittkopp PJ, Kalay G. 2011. Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. Nat Rev Genet 13:59–69. https://doi.org/10.1038/nrg3095.
4. Norsworthy AN, Visick KL. 2015. Signaling between two interacting sensor kinases promotes biofilms and colonization by a bacterial symbiont. Mol Microbiol 96:233–248. https://doi.org/10.1111/mmi.12932.
5. Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, Ruby EG. 2009. A single regulatory gene is sufficient to alter bacterial host range. Nature 458:215–218. https://doi.org/10.1038/nature07660.
6. Perez JC, Groisman EA. 2009. Evolution of transcriptional regulatory circuits in bacteria. Cell 138:233–244. https://doi.org/10.1016/j.cell.2009.07.002.
7. Dziejman M, Balon E, Boyd D, Fraser CM, Heidelberg JF, Mekalanos JJ. 2002. Comparative genomic analysis of Vibrio cholerae: genes that correlate with cholera endemic and pandemic disease. Proc Natl Acad Sci U S A 99:1556–1561. https://doi.org/10.1073/pnas.042667999.
8. Faruque SM, Albert MJ, Mekalanos JJ. 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol Mol Biol Rev 62:1301–1314. https://doi.org/10.1128/MMBR.62.4.1301-1314.1998.
9. Karaolis DKR, Johnson JA, Bailey CC, Boedeker EC, Kaper JB, Reeves PR. 1998. A Vibrio cholerae pathogenicity island associated with epidemic and pandemic strains. Proc Natl Acad Sci U S A 95:3134–3139. https://doi.org/10.1073/pnas.95.6.3134.
10. Pruzzo C, Vezzulli L, Colwell RR. 2008. Global impact of Vibrio cholerae interactions with chitin. Environ Microbiol 10:1400–1410. https://doi.org/10.1111/j.1462-2920.2007.01559.x.
11. Crisan CV, Chande AT, Williams K, Raghuram V, Rishishwar L, Steinbach G, Watve SS, Yunker P, Jordan IK, Hammer BK. 2019. Analysis of Vibrio cholerae genomes identifies new type VI secretion system gene clusters. Genome Biol 20:163. https://doi.org/10.1186/s13059-019-1765-5.
12. MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S. 2010. The Vibrio cholerae type VI secretion system displays antimicrobial properties. Proc Natl Acad Sci U S A 107:19520–19524. https://doi.org/10.1073/pnas.1012931107.
13. Gallegos-Monterrosa R, Coulthurst SJ. 2021. The ecological impact of a bacterial weapon: microbial interactions and the Type VI secretion system. FEMS Microbiol Rev 45. https://doi.org/10.1093/femsre/fuab033.
14. Hussain NAS, Kirchberger PC, Case RJ, Boucher YF. 2021. Modular molecular weaponry plays a key role in competition within an environmental Vibrio cholerae population. Front Microbiol 12. https://doi.org/10.3389/fmicb.2021.671092.
15. Ishikawa T, Sabharwal D, Bröms J, Milton DL, Sjöstedt A, Uhlin BE, Wai SN, Camilli A. 2012. Pathoadaptive conditional regulation of the type VI secretion system in Vibrio cholerae O1 strains. Infect Immun 80:575–584. https://doi.org/10.1128/IAI.05510-11.
16. Kirchberger PC, Unterweger D, Provenzano D, Pukatzki S, Boucher Y. 2017. Sequential displacement of type VI secretion system effector genes leads to evolution of diverse immunity gene arrays in Vibrio cholerae. Sci Rep 7:45133. https://doi.org/10.1038/srep45133.
17. Santoriello FJ, Pukatzki S. 2021. When the pandemic opts for the lockdown: secretion system evolution in the cholera bacterium. Microb Cell 8:69–72. https://doi.org/10.15698/mic2021.03.744.
18. Unterweger D, Miyata ST, Bachmann V, Brooks TM, Mullins T, Kostiuk B, Provenzano D, Pukatzki S. 2014. The Vibrio cholerae type VI secretion system employs diverse effector modules for intraspecific competition. Nat Commun 5:3549. https://doi.org/10.1038/ncomms4549.
19. Kostiuk B, Santoriello FJ, Diaz-Satizabal L, Bisaro F, Lee K-J, Dhody AN, Provenzano D, Unterweger D, Pukatzki S. 2021. Type VI secretion system mutations reduced competitive fitness of classical Vibrio cholerae biotype. Nat Commun 12:6457. https://doi.org/10.1038/s41467-021-26847-y.
20. Crisan CV, Chandrashekar H, Everly C, Steinbach G, Hill SE, Yunker PJ, Lieberman RR, Hammer BK, Ellermeier CD. 2021. A new contact killing toxin permeabilizes cells and belongs to a broadly distributed protein family. mSphere 6:e00318-21. https://doi.org/10.1128/mSphere.00318-21.
21. Drebes Dörr NC, Blokesch M. 2020. Interbacterial competition and antipredatory behaviour of environmental Vibrio cholerae strains. Environ Microbiol 22:4485–4504. https://doi.org/10.1111/1462-2920.15224.
22. Crisan CV, Hammer BK. 2020. The Vibrio cholerae type VI secretion system: toxins, regulators and consequences. Environ Microbiol 22:4112–4122. https://doi.org/10.1111/1462-2920.14976.
23. Jaskolska M, Stutzmann S, Stoudmann C, Blokesch M. 2018. QstR-dependent regulation of natural competence and type VI secretion in Vibrio cholerae. Nucleic Acids Res 46:10619–10634. https://doi.org/10.1093/nar/gky717.
24. Joshi A, Kostiuk B, Rogers A, Teschler J, Pukatzki S, Yildiz FH. 2017. Rules of engagement: the type VI secretion system in Vibrio cholerae. Trends Microbiol 25:267–279. https://doi.org/10.1016/j.tim.2016.12.003.
25. Joshi A, Mahmoud SA, Kim SK, Ogdahl JL, Lee VT, Chien P, Yildiz FH. 2020. c-di-GMP inhibits LonA-dependent proteolysis of TfoY in Vibrio cholerae. PLoS Genet 16:e1008897. https://doi.org/10.1371/journal.pgen.1008897.
26. Metzger LC, Stutzmann S, Scrignari T, Van der Henst C, Matthey N, Blokesch M. 2016. Independent regulation of type VI secretion in Vibrio cholerae by TfoX and TfoY. Cell Rep 15:951–958. https://doi.org/10.1016/j.celrep.2016.03.092.
27. Lo Scrudato M, Blokesch M. 2013. A transcriptional regulator linking quorum sensing and chitin induction to render Vibrio cholerae naturally transformable. Nucleic Acids Res 41:3644–3658. https://doi.org/10.1093/nar/gkt041.
28. Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK. 2005. Chitin induces natural competence in Vibrio cholerae. Science 310:1824–1827. https://doi.org/10.1126/science.1120096.
29. Watve SS, Thomas J, Hammer BK. 2015. CytR is a global positive regulator of competence, type VI secretion, and chitinases in Vibrio cholerae. PLoS One 10:e0138834. https://doi.org/10.1371/journal.pone.0138834.

30. Tsou AM, Cai T, Liu Z, Zhu J, Kulkarni RV. 2009. Regulatory targets of quorum sensing in Vibrio cholerae: evidence for two distinct HapR-binding motifs. Nucleic Acids Res 37:2747–2756. https://doi.org/10.1093/nar/gkp121.
31. Pursley BR, Maiden MM, Hsieh ML, Fernandez NL, Severin GB, Waters CM. 2018. Cyclic di-GMP Regulates TfoY in Vibrio cholerae to control motility by both transcriptional and posttranscriptional mechanisms. J Bacteriol 200. https://doi.org/10.1128/JB.00578-17.
32. Bernardy EE, Turnsek MA, Wilson SK, Tarr CL, Hammer BK, Liu S-J. 2016. Diversity of clinical and environmental isolates of Vibrio cholerae in natural transformation and contact-dependent bacterial killing indicative of type VI secretion system activity. Appl Environ Microbiol 82:2833–2842. https://doi.org/10.1128/AEM.00351-16.
33. Dunn JJ, Studier FW. 1983. Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. J Mol Biol 166: 477–535. https://doi.org/10.1016/S0022-2836(83)80282-4.
34. Jobling MG, Holmes RK. 1997. Characterization of hapR, a positive regulator of the Vibrio cholerae HA/protease gene hap, and its identification as a functional homologue of the Vibrio harveyi luxR gene. Mol Microbiol 26:1023–1034. https://doi.org/10.1046/j.1365-2958.1997.6402011.x.
35. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675. https://doi.org/10.1038/nmeth.2089.
36. Zheng J, Shin OS, Cameron DE, Mekalanos JJ. 2010. Quorum sensing and a global regulator TsrA control expression of type VI secretion and virulence in Vibrio cholerae. Proc Natl Acad Sci U S A 107:21128–21133. https://doi.org/10.1073/pnas.1014998107.
37. Joelsson A, Liu Z, Zhu J. 2006. Genetic and phenotypic diversity of quorum-sensing systems in clinical and environmental isolates of Vibrio cholerae. Infect Immun 74:1141–1147. https://doi.org/10.1128/IAI.74.2.1141-1147.2006.
38. Robert X, Gouet P. 2014. Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res 42:W320–W324. https://doi.org/10.1093/nar/gku316.
39. Holowka J, Zakrzewska-Czerwinska J. 2020. Nucleoid associated proteins: the small organizers that help to cope with stress. Front Microbiol 11. https://doi.org/10.3389/fmicb.2020.00590.
40. Ayala JC, Silva AJ, Benitez JA. 2017. H-NS: an overarching regulator of the Vibrio cholerae life cycle. Res Microbiol 168:16–25. https://doi.org/10.1016/j.resmic.2016.07.007.
41. Chaparian RR, Olney SG, Hustmyer CM, Rowe-Magnus DA, van Kessel JC. 2016. Integration host factor and LuxR synergistically bind DNA to coactivate quorum-sensing genes in Vibrio harveyi. Mol Microbiol 101:823–840. https://doi.org/10.1111/mmi.13425.
42. Papenfort K, Forstner KU, Cong JP, Sharma CM, Bassler BL. 2015. Differential RNA-seq of Vibrio cholerae identifies the VqmR small RNA as a regulator of biofilm formation. Proc Natl Acad Sci U S A 112:E766–E775. https://doi.org/10.1073/pnas.1500203112.
43. Shao Y, Bassler BL. 2014. Quorum regulatory small RNAs repress type VI secretion in Vibrio cholerae. Mol Microbiol 92:921–930. https://doi.org/10.1111/mmi.12599.
44. Drebes Dorr NC, Proutiere A, Jaskolska M, Stutzmann S, Bader L, Blokesch M. 2022. Single nucleotide polymorphism determines constitutive versus inducible type VI secretion in Vibrio cholerae. ISME J Epub 2022/04/13. https://doi.org/10.1038/s41396-022-01234-7.
45. Gourse RL, Ross W, Gaal T. 2000. UPs and downs in bacterial transcription initiation: the role of the alpha subunit of RNA polymerase in promoter recognition. Mol Microbiol 37:687–695. https://doi.org/10.1046/j.1365-2958.2000.01972.x.
46. Guo C, McDowell IC, Nodzenski M, Scholtens DM, Allen AS, Lowe WL, Reddy TE. 2017. Transversions have larger regulatory effects than transitions. BMC Genomics 18:394. https://doi.org/10.1186/s12864-017-3785-4.
47. Slattery M, Zhou T, Yang L, Dantas Machado AC, Gordân R, Rohs R. 2014. Absence of a simple code: how transcription factors read the genome. Trends Biochem Sci 39:381–399. https://doi.org/10.1016/j.tibs.2014.07.002.
48. MacKenzie KD, Wang Y, Musicha P, Hansen EG, Palmer MB, Herman DJ, Feasey NA, White AP. 2019. Parallel evolution leading to impaired biofilm formation in invasive Salmonella strains. PLoS Genet 15:e1008233. https://doi.org/10.1371/journal.pgen.1008233.
49. Osborne SE, Walthers D, Tomljenovic AM, Mulder DT, Silphaduang U, Duong N, Lowden MJ, Wickham ME, Waller RF, Kenney LJ, Coombes BK. 2009. Pathogenic adaptation of intracellular bacteria by rewiring a cis-regulatory input function. Proc Natl Acad Sci U S A 106:3982–3987. https://doi.org/10.1073/pnas.0811669106.
50. Unterweger D, Kitaoka M, Miyata ST, Bachmann V, Brooks TM, Moloney J, Sosa O, Silva D, Duran-Gonzalez J, Provenzano D, Pukatzki S. 2012. Constitutive type VI secretion system expression gives Vibrio cholerae intra- and interspecific competitive advantages. PLoS One 7:e48320. https://doi.org/10.1371/journal.pone.0048320.
51. Fu Y, Waldor MK, Mekalanos JJ. 2013. Tn-Seq analysis of Vibrio cholerae intestinal colonization reveals a role for T6-mediated antibacterial activity in the host. Cell Host Microbe 14:652–663. https://doi.org/10.1016/j.chom.2013.11.001.
52. Zhao W, Caro F, Robins W, Mekalanos JJ. 2018. Antagonism toward the intestinal microbiota and its effect on Vibrio cholerae virulence. Science 359:210–213. https://doi.org/10.1126/science.aap8775.
53. Logan SL, Thomas J, Yan J, Baker RP, Shields DS, Xavier JB, Hammer BK, Parthasarathy R. 2018. The Vibrio cholerae type VI secretion system can modulate host intestinal mechanics to displace gut bacterial symbionts. Proc Natl Acad Sci U S A 115:E3779–E3787. https://doi.org/10.1073/pnas.1720133115.
54. Seal S, Dharmarajan G, Khan I. 2021. Evolution of pathogen tolerance and emerging infections: A missing experimental paradigm. Elife 10. https://doi.org/10.7554/eLife.68874.
55. San JE, Baichoo S, Kanzi A, Moosa Y, Lessells R, Fonseca V, Mogaka J, Power R, de Oliveira T. 2019. Current affairs of microbial genome-wide association studies: approaches, bottlenecks and analytical pitfalls. Front Microbiol 10: 3119. https://doi.org/10.3389/fmicb.2019.03119.
56. Skorupski K, Taylor RK. 1996. Positive selection vectors for allelic exchange. Gene 169:47–52. https://doi.org/10.1016/0378-1119(95)00793-8.
57. Watve SS, Bernardy EE, Hammer BK. 2014. Vibrio cholerae: measuring natural transformation frequency. Curr Protoc Microbiol 35:4 1–12. https://doi.org/10.1002/9780471729259.mc06a04s35.
58. Miller MB, Skorupski K, Lenz DH, Taylor RK, Bassler BL. 2002. Parallel quorum sensing systems converge to regulate virulence in Vibrio cholerae. Cell 110:303–314. https://doi.org/10.1016/S0092-8674(02)00829-2.
59. Matson JS. 2018. Preparation of Vibrio cholerae Samples for RNA-seq Analysis. Methods Mol Biol 1839:29–38. https://doi.org/10.1007/978-1-4939-8685-9_3.
60. Manera K, Caro F, Li H, Pei TT, Hersch SJ, Mekalanos JJ, Dong TG. 2021. Sensing of intracellular Hcp levels controls T6 expression in Vibrio cholerae. Proc Natl Acad Sci U S A 118. https://doi.org/10.1073/pnas.2104813118.
61. Sayers EW, Agarwala R, Bolton EE, Brister JR, Canese K, Clark K, Connor R, Fiorini N, Funk K, Hefferon T, Holmes JB, Kim S, Kimchi A, Kitts PA, Lathrop S, Lu Z, Madden TL, Marchler-Bauer A, Phan L, Schneider VA, Schoch CL, Pruitt KD, Ostell J. 2019. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 47:D23–D28. https://doi.org/10.1093/nar/gky1069.
62. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. 2009. BLAST+: architecture and applications. BMC Bioinformatics 10:421. https://doi.org/10.1186/1471-2105-10-421.
63. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797. https://doi.org/10.1093/nar/gkh340.
64. Madeira F, Park YM, Lee J, Buso N, Gur T, Madhusoodanan N, Basutkar P, Tivey ARN, Potter SC, Finn RD, Lopez R. 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res 47:W636–W641. https://doi.org/10.1093/nar/gkz268.
65. Felsenstein J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376. https://doi.org/10.1007/BF01734359.
66. Tamura K, Stecher G, Kumar S. 2021. MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol 38:3022–3027. https://doi.org/10.1093/molbev/msab120.