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T6SS intraspecific competition orchestrates *Vibrio cholerae* genotypic diversity

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Summary. *Vibrio cholerae* is a diverse species that inhabits a wide range of environments from copepods in brackish water to the intestines of humans. In order to remain competitive, *V. cholerae* uses the versatile type-VI secretion system (T6SS) to secrete anti-prokaryotic and anti-eukaryotic effectors. In addition to competing with other bacterial species, *V. cholerae* strains also compete with one another. Some strains are able to coexist, and are referred to as belonging to the same compatibility group. Challenged by diverse competitors in various environments, different *V. choleare* strains secrete different combination of effectors – presumably to best suit their niche. Interestingly, all pandemic *V. cholerae* strains encode the same three effectors. In addition to the diversity displayed in the encoded effectors, the regulation of *V. cholerae* also differs between strains. Two main layers of regulation appear to exist. One strategy connects T6SS activity with behavior that is suited to fighting eukaryotic cells, while the other is linked with natural competence – the ability of the bacterium to acquire and incorporate extracellular DNA. This relationship between bacterial killing and natural competence is potentially a source of diversification for *V. cholerae* as it has been shown to incorporate the DNA of cells recently killed through T6SS activity. It is through this process that we hypothesize the transfer of virulence factors, including T6SS effector modules, to happen. Switching of T6SS effectors has the potential to change the range of competitors *V. cholerae* can kill and to newly define which strains *V. cholerae* can co-exist with, two important parameters for survival in diverse environments.

Keywords: *Vibrio cholerae* · T6SS · competition · evolution

Introduction

Over millions of years, bacteria have evolved mechanisms to compete against each other for limited resources, inhabiting nearly every environmental niche on the planet [19, 47]. Evolution of virulence strategies have permitted bacteria to infect higher vertebrates and expand their niche repertoire. Competitive tactics to fight for resources include secreted colicins, antibiotics, siderophores and contact-dependent secretion systems to engage in cell-cell mediated killing while avoiding detection of the immune system. These mechanisms have enabled bacteria to adapt to unique niches by acquiring genetic elements and developing strategies for protection from predation [19, 29, 35, 47, 50].

Mechanisms of predation protection have been studied extensively in *Vibrio cholerae*, the Gram-negative, marine bacterium that causes a dramatic form of diarrheal disease in humans known as cholera [41, 45, 46, 56]. Over 200 serogroups comprise the species *V. cholerae*, some of which are primary environmental and are present in the marine environment year round [58]. Other strains bloom during epidemics, and are optimized for causing disease in the human host [15, 16]. One consequence of this diversity is increasing the variety of organisms that *V. cholerae* must compete with.

In environmental reservoirs, *V. cholerae's* ability to recycle *N*-acetylglucosamine, a carbon source sequestered in the chitin polymer, challenging these bacteria to inhabit multiple diverse

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microbial environments, including the surface of copepods [22, 37, 44]. *V. cholerae* contends for resources with competing strains of the same species, other *Vibrios*, and a range of additional bacteria and grazing eukaryotic amoeba. *V. cholerae* bacteria employ various techniques, including adherence molecules and biofilm production, iron scavenging molecules, such as vibriobactin, and toxins, including those produced by the type 6 secretion system (T6SS) to negotiate this social complexity [18, 24, 31, 34]. Numerous factors, including *V. cholerae* proliferation on copepods and changes in phage populations cause unpredictable *V. cholerae* blooms to reach titres sufficient to become infectious to humans [16, 22, 58]. Most notable are *V. cholerae* strains that belong to the O1/O139 serogroup, as these strains have been implicated in all *V. cholerae* pandemics [14]. Non-O1/O139 strains have been implicated in local outbreaks and also represent a significant health burden [43].

When susceptible human hosts consume contaminated water, *V. cholerae* confronts a considerably distinct host environment and activates acid-response pathways to survive passage through the stomach acid [38, 39]. In the small intestine, *V. cholerae* navigates a number of chemical and physical barriers such as mucin, bile detergents, and the host microbiome [5, 21, 45, 49]. Upon establishing intimate contact with the epithelial layer, *V. cholerae* cells co-agglutinate as a result of expression of a Type-IV pilus known as Toxin Co-regulated Pilus (TCP), and then secrete cholera toxin (CT) resulting in massive watery efflux characteristic of cholera diarrhea which functions to disperse *V. cholerae* back into the environment [20, 28]. How diverse *Vibrio cholerae* compete in such a wide array of environments with diverse competitors is currently unknown. We propose that among many factors, the T6SS secretes highly specific effectors contributes to the needs of individual strains.

Genes coding the T6SS have thus far been identified in all *V. cholerae* strains examined to date [26, 55]. Structurally, the T6SS is a molecular contractile toxin delivery device that bacteria engage to inject a protein spear decorated with effectors across the cell boundary of adjacent eukaryotic and prokaryotic cell, resulting in lysis of the targeted cell [3, 34, 46]. The T6SS of *V. cholerae* secretes effectors that degrade lipids, peptidoglycan, purportedly DNA, as well as form pores that act on the outer membrane of the prey bacteria [8, 32, 42]. The T6SS also secretes effectors that lead to pore formation and actin crosslinking in eukaryotic cells; however, how significant these observations are to *V. cholerae* pathogenesis remains to be clarified. Actin-crosslinking takes place *in-vivo* when *V. cholerae* bacteria traverse the small intestine [33]. Although the host cells subjected to actin crosslinking have not been identified, this mechanism likely serves to immobilize approaching immune cells, allowing *V. cholerae* to establish an infection [33].

Within bacterial communities, T6SS-mediated attacks can be protected. *V. cholerae* synthesizes immunity proteins that sequester cognate T6SS effectors expressed by sister, or kin cells [12, 55]. Immunity proteins that protect cells from effector alleles of kin cells are ineffective against effectors encoded by alleles of non-kin bacteria belonging to the same species. Taken together, these observations suggest that "compatibility rules" allow distinct strains of *V. cholerae* with identical effector modules to coexist, thereby giving rise to a unique self-recognition system [55]. Conversely, *V. cholerae* strains expressing dissimilar effector/immunity pairs are unable to share a niche as one of the two s trains will be excluded [51, 52, 55]. Each *V. cholerae* strain examined to date encodes three distinct effector/immunity alleles within the three T6SS gene clusters. So far, we identified a total of nineteen effectors across the three clusters, but expect that number to increase as additional strain sequences become available [26]. We assigned each module a letter as an identifier to distinguish strains able to coexist from those that compete against each other. In addition to these three clusters, some strains, including pandemic O1 strains encode a fourth T6SS effector, *tseH,* in an additional cluster; strains that do not have this effector have no replacement [1]. All sequenced pathogenic *V. cholerae* strains harbored the same effector/immunity module set, TseL/TsiV1, VasX/TsiV2 and VgrG3/TsiV3, we called the AAA compatibility group regardless of serogroup; these included all pandemic O1/O139 strains [55]. Virtually all strains belonging to the AAA compatibility group we examined shared the presence of the horizontally-acquired genetic elements *Virulence Pathogenicity Island I* (VPI-1) and *Cholera Toxin prophage* (CTX-Φ) essential for pandemic spread [55]. In contrast, environmental strains available for examination displayed highly diverse effector/immunity allele pairs, belonged to a wide range of serogroups and did not harbor VPI-1 and CTX-Φ. Furthermore, laboratory experiments demonstrated the AAA effector/immunity allele pair to be by far the most effective at killing non-kin *V. cholerae*. Collectively, these results suggest that non-toxigenic strains are unable to coexist with each other or with toxigenic *V. cholerae* strains, but that toxigenic strains even belonging to different serogroups are compatible and can coexist.

The three T6SS effector/immunity allele pairs reside within three highly conserved gene clusters that have considerably lower GC contents compared to the surrounding sequences, supporting the hypothesis that they are horizontally mobile among *V. cholerae* strains [55]. Further evidence supporting this hypothesis is the observation that *V. cholerae* T6SS regulation and natural competence pathways are linked because *V. cholerae* growth on chitin activates both DNA acquisition and T6SS expression [7, 59].

Observations that T6SS compatibility groups define the competitive behaviour of *V. cholerae* strains and that effector/immunity allele pairs may be freely exchanged in nature and in the host collectively suggest a critical role for the T6SS in evolution of not only the competitive behaviour of the pathogen, but also in the acquisition of virulence factors [51]. This review will outline the diversity of the T6SS in *V. cholerae*, both on a genetic and regulatory level and discuss the consequences of T6SS competition driven exchange of genetic information.

The Conserved Structure of the T6SS

In contrast to the remarkable diversity of T6SS effectors, the core structural components of the T6SS are highly conserved amongst distantly related *V. cholerae* strains and other *Vibrio* species [55]. T6SS genes are distributed over both *V. cholerae* chromosomes and consist of three clusters: a large cluster and two auxiliary clusters. The large cluster encodes the majority of the structural T6SS components, including the outer sheath proteins, VipA/B; key proteins for the tip of the T6SS, VgrG3 and a PAAR protein; and proteins that assemble at the inner and outer membranes [10]. Additionally, the large cluster encodes a gene necessary for disassembly of the T6SS, *clpV*, and an essential transcriptional regulator, *vasH* [6, 27]. The two auxiliary clusters also encode structural components; Hcp-1 and Hcp-2 as well as the tip proteins VgrG1 and VgrG3 respectively and the inner tube proteins. Also encoded in each cluster is an effector module, consisting of an adaptor, effector and immunity gene [53]. The fourth and last cluster appears in several *V. cholerae* strains, including pandemics, where it either encodes an amidase, or no effector at all. While structural T6SS components have >95% identity over 37 sequenced strains, effector module DNA sequences have <30% identity among the same strain set. Further genetic differences are highlighted by GC-content divergence between effector modules and core regions. Effector modules harbor a 6-13% lower GC-content than the core structural components indicating that these DNA sequences were acquired independently [55]. This initial observation provoked the hypothesis that effector modules mobilize and are freely exchanged among *V. cholerae* strains. Together, this describes a T6SS in *V. cholerae* that is highly conserved in regions coding for the core structural components and assembly yet highly diverse in effector module sequences.

Regulation of the T6SS

The T6SS of *V. cholerae* is tightly regulated and subject to distinct layers of regulation in different strains. Briefly, among pandemic *V. cholerae* strains, T6SS regulation is controlled by three principal transcriptional regulators: VasH, TfoY and TfoX [27, 40]. The large cluster becomes transcriptionally activated first, by either TfoY or TfoX. While both of these activators act on the large T6SS gene cluster and thus effector modules, each also drives independent processes that depend on *V. cholerae*'s lifestyle. TfoX is activated in the presence of chitin and co-regulates chitin catabolism and DNA uptake, whereas TfoY's response to decreased cyclic-di-GMP levels in the cell encourages anti-eukaryotic behavior such as upregulation of motility and hemolysin production, while inhibiting cell attachment [40]. These distinct pathways infer multiple roles for the T6SS based on the environment *V. cholerae* confronts; regardless, both pathways result in the transcription of the large T6SS gene cluster, including the regulator, *vasH*. VasH is a sigma-54 dependent transcription factor encoded in the large T6SS cluster that positively regulates the two auxiliary clusters essential for T6SS activity [40]. In addition to VasH, the quorum sensing-regulated transcription factor HapR - induced at high cell density - binds to *hcp-1* and *hcp-2* promoters and positively regulates the T6SS auxiliary clusters [48].

In *V. cholerae,* the T6SS is negatively regulated by sRNAs through two distinct mechanisms related to quorum sensing. In response to low cell densities, LuxO is phosphorylated thereby producing quorum regulatory sRNAs. These small RNAs bind to and negatively regulate the 5' untranslated regions of the mRNA for *hapR* and the large T6SS cluster. This is a two-pronged regulator silencing network that shuts down expression of genes residing in auxiliary clusters through the downregulation of *hapR* and also of large cluster genes directly. Interestingly, this layer of regulation also exists in non-pandemic strains suggesting a conserved relationship between quorum sensing and the T6SS in this species [48].

V. cholerae bacteria modulate T6SS activity in response to a wide variety of environmental cues; some of these function as "on/off" switches, while others modulate the intensity of the response. For example, mucin, chitin and high-osmolality have been shown to induce T6SS in a variety of toxigenic and non-toxigenic strains, while bile salts and thiourea influence the magnitude of an already active T6SS [2, 7, 23].

As a general rule, pandemic O1 strains appear to regulate T6SS expression differently than non-patient derived strains. One comprehensive study showed that a constitutively active T6SS under laboratory conditions is rare amongst clinical El Tor O1 strains (<15%), but common among environmentally derived strains (<90%)[4]. This correlates with *V. cholerae*'s natural competence on chitin as more environmental than clinical strains incorporated exogenous DNA (33.3% vs 13.8%). This profound regulatory difference might provide insights into how different sets of strains use the T6SS as it pertains to their individual lifestyles. Furthermore, the different regulatory cues that the strains respond to gives insight into where they use their T6SS.

Effector Diversity and Compatibility groups

The discovery that *V. cholerae*'s T6SS has antibacterial activity led to experiments showing that O1 *V. choleare* strains were immune to the fate of the bacterial killing by V52, a toxigenic non-O1 strain that expresses T6SS constitutively [34]. Curiously, V52 was able to kill environmental *V. cholerae* isolates endemic to the lower Rio Grande delta that also express T6SS constitutively, yet these same environmental strains were able to kill O1 [52]. We hypothesized that O1 strains express immunity proteins cognate to the effectors expressed by V52 (O37 serogroup), but not to the effector proteins of *V. cholerae* endemic to the Rio Grande delta. This was confirmed when two groups independently concurrently demonstrated that open reading

frames downstream of T6SS effectors – VCA1419, VCA022 and VC0124 – encoded immunity proteins [12, 55]. Interestingly, O1 strains do not express T6SS constitutively under laboratory conditions, yet still retained immunity against V52 suggesting that immunity is regulated independently from the rest of the T6SS [42]. Later, a promoter region was identified in the 5' region of the effector genes that constitutively provide T6SS immunity. Immunity genes appear to be expressed constitutively under laboratory conditions in O1 strains even in the absence of T6SS activation [42]. However, immunity gene regulation becomes less clear in the host. Using the infant rabbit model investigators found that both *tsiV1* and *tsiV3* are upregulated three-fold higher than their respective effector. Interestingly, *tsiV2* and its cognate effector *vasX* were not significantly upregulated in the infant rabbit [17].

Next, we performed a comprehensive bioinformatics analysis to categorize *V. cholerae* strains based on immunity genes sequences to lay the roadmap of the competitive relationship between *V. cholerae* strains. Pairwise competition assays were performed to test the hypothesis that strains encoding the same immunity genes would be able to coexist while strains encoding a different complement of immunity genes will compete [55]. Strains that coexisted based on their T6SS immunity genes were said to belong to the same compatibility group. Sharing a compatibility group is hypothesized to allow strains to share DNA, a niche and interact with one another. Our study and others identified 19 distinct effectors across the three clusters, with 2 possible effectors in auxiliary cluster one, 5 possible effectors in auxiliary cluster two, and 12 possible effectors in the larger cluster – a total of 120 potential combinations [26]. Perhaps most important is the observation that all toxigenic *V. cholerae* strains that have caused epidemics, including pandemic strains, all encode the same effector/immunity pairs, given the designation AAA.

Delivery of distinct effectors through a highly conserved structure requires adaptor proteins to mediate the biochemical/physical interaction. Modular T6SS adaptor proteins (Tap or Tec) having a domain that bind effectors and another that interacts with VgrG trimers at the tip of the T6SS were reported by several investigators to be ubiquitous in all *V. cholerae* strains and other Gram-negative species [30, 54].

The notion of compatibility groups was recently expanded upon by an in-depth study examining over 400 *V. cholerae* strains isolated from five different locations within Oyster Pond, MA, USA. Kirchberger *et al.* found that *V. cholerae* isolated from the same collection site all shared the same compatibility group, yet compatibility groups were distinct across different sites [25]. The authors hypothesized that homogeneity of *V. cholerae* at any given site is driven by the T6SS, resulting in incompatible strains being excluded.

Another important aspect of compatibility are so-called orphan immunity genes consisting of open reading frames that bear considerable homology to immunity genes but are not positioned directly downstream of a cognate effector but still exist within a given T6SS gene cluster [26]. All AAA-module strains harbor a single orphan immunity gene downstream of the *tsiV1* immunity gene outside of the T6SS auxiliary cluster 1, yet other *V. cholerae* strains have several orphan immunity genes in long arrays following all three T6SS gene clusters. While it is not yet known if these purported genes are active and provide protection to other effector genes, RNAseq data from *V. cholerae* demonstrate that the orphan immunity gene downstream of *tsiV1* is activated along with the rest of the cluster when T6SS is induced through *tfoX* overexpression [7]. Additional immunity genes could offer a resistance mechanism for *V. cholerae* to effectors other than the ones they encode, providing a mechanism by which incompatible strains could coexist in a heterogeneous environmental niche.

Membership to a compatibility group dictates the outcome of competition, occupation of a niche, ability to participate in co-infections and the ability to share DNA. Understanding how compatibility groups are acquired and maintained is critical to understanding *V. cholerae* biology. While the consequences of compatibility grouping are coming to light in environmental and laboratory conditions, the impact they have during colonization and pathogenesis remain unclear. However, the universality of the AAA compatibly group in pandemic strains suggests they are critical for *in-vivo* fitness.

Compatibility group switching

Co-regulation of T6SS and natural competence invites an intriguing hypothesis whereby T6SS mediated killing causes release of extracellular prey DNA (eDNA), which could then be acquired by the predator strains [7]. This could result in the acquisition of potentially any gene sequence including new virulence factors and/or T6SS effector modules. This notion is supported by the observations that both T6SS and natural competence are activated when *V. cholerae* is grown on chitin under nutrient-limiting conditions. Natural competence following T6SS killing has been observed under laboratory conditions; effector modules marked with antibiotic resistance cassettes have been shown to be horizontally mobilized and integrated into the genome resulting in a change in competitive behavior [51].

When *V. cholerae* binds chitin, chitinases are upregulated and secreted. Oligomeric chitin is sensed by ChiS which activates TfoX – acting as a transcription factor for natural competence genes such as pilin, *comEA,* and *dprA,* as well as the T6SS [59]. The regulatory connection between the T6SS and natural competence is also functionally linked as chitin-mediated transformation following T6SS-mediated killing has been reported by multiple groups [7, 51, 59]. In addition to chitin, mucin appears to be sensed and responded to analogously in this manner [9]. We propose a model whereby *V. cholerae* in the environment competes and exchanges genetic information (including T6SS-effector modules) with members of the same

species giving rise to a pool of diverse genotypes; akin to *genetic card reshuffling*. A surviving heterogeneous pool of *V. cholerae* ingested by the human host are then subjected to selective pressure that favors acquisition of virulence factors which provide an advantage in this host followed by amplification where those *V. cholerae* selected for multiply to high titers before being release back into the environment thereby giving rise to a clonal lineage sharing T6SS modules optimized for human infection. In addition to acquiring T6SS modules, we hypothesize that host-directed virulence factors, or any genetic elements increasing fitness in the host are also being exchanged and subsequently selected for (Figure 1). Evidence for the exchange of multiple genetic components in a single bacterium emerged from a technique called multiplex genome editing by natural transformation (MuGENT) which was developed to manipulate multiple loci on the same genome simultaneously [11]. *V. cholerae* bacteria are grown on chitin and antibiotic-marked and unmarked DNA is added to the culture. Over half of the bacterial population that integrates antibiotic-marked DNA also integrates unmarked DNA, providing evidence to support a model whereby *V. cholerae* recombines additional genetic elements into their genome while reconfiguring their T6SS effector clusters [11]. Experiments performed with *V. cholerae* grown on chitin suggest that non-toxigenic strains are more likely to become competent than toxigenic strains suggesting that the flow of genetic material may move preferentially from toxigenic genes to non-toxigenic strains [4].

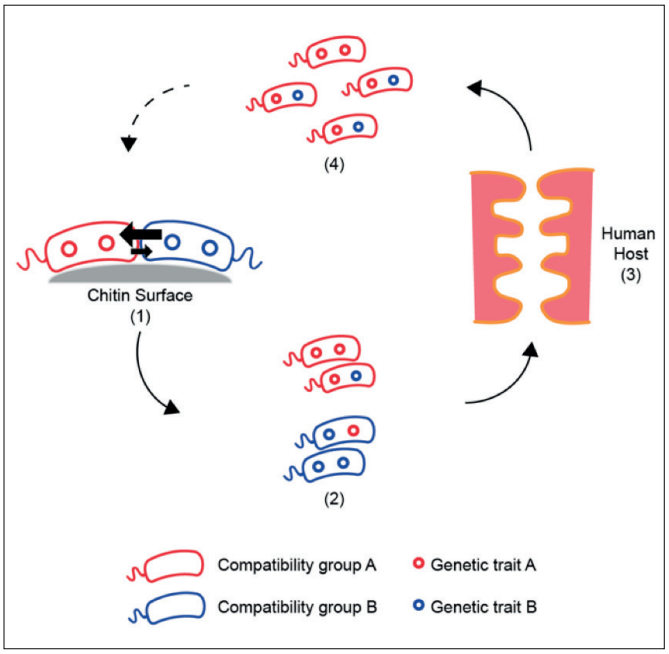

Fig. 1. Model for the diversification of *V. cholerae* in the environment and selection in the host. 1. *V. cholerae* strains of different compatibility groups (indicated by differently coloured bacteria) encounter each other on chitin surfaces in environmental reservoirs. Because they have incompatible effector modules, they kill each other in a T6SS-dependent manner and incorporate new DNA when co-existing on chitin (black arrows). The net flow of genetic information is towards bacteria with a dominant T6SS effector set (AAA). **2.** Uptake of genetic traits results in heterologous bacteria from various compatibly groups with different combinations of genetic traits (coloured circles within bacteria). Compatible bacteria (despite differences in genomic content) can coexist in environmental reservoirs. **3.** Infection of the human host by a mixed inoculum of strains. **4.** Human host selects for toxigenic bacteria of one compatibility group. Previous acquisition of genetic traits beneficial for persistence in the host (blue circle within red bacterium) allows *V. cholera* to exit the host in increased numbers.

Discussion

Despite the diversity of T6SS effectors and regulation, many conserved elements of the T6SS both genetically and functionally conserved. All tested strains display antimicrobial activity against *E. coli* [4, 34, 52]. This ubiquitous feature implies a universal function for the T6SS to be used in competition with other Gram-negative species. Within the species, the T6SS is used for either self-recognition, phase separation or exclusion. Self-recognition, through shared immunity genes, allows the strains to coexist and share a niche [51, 55]. Phase separation occurs when two bacteria compete with one another but no strain is able to overtake the other, instead they form their own clusters [34, 36]. Finally, if the T6SS of one strain overpowers the other, one *V. cholerae* strain can kill the other strain – completely excluding them from the niche [52].

Beyond conserved functional roles, on the genetic level, the T6SS is highly conserved throughout all structural and assembly/disassembly genes. Additionally, this conservation could be exploited through the development of therapeutic drugs targeted towards the T6SS. In stark contrast, effector module genes display variability both on a genetic and functional level. The interaction between highly conserved and variable protein domains requires the presence of adaptor proteins to link these two components. Indeed, a bipartite adaptor protein links the conserved core T6SS structural tip with the diverse effector proteins [54]. This variability in T6SS effectors amongst *V. cholerae* species leads to competition between the strains. Interestingly, despite this considerable divergence, all O1 pandemic strains encode the same effector set and can therefore coexist [55].

Regulation of the T6SS represents another source of diversity. Patient-derived strains tend to encode a tightly regulated T6SS that is activated by mucin and non-functional under laboratory conditions whereas environmental strains engage in T6SS-mediated killing under the same conditions; i.e. express the system constitutively [2, 4]. Additionally, environmental strains appear to activate *tfoX* expression when grown on chitin, which further induces the T6SS [7]. This observation is consistent with the life-style differences between pathogenic and environmental *V. cholerae* as the T6SS is likely utilized differently by the two groups of strains. Although the lines are blurred, TfoY-based regulation may benefit epidemic strains and TfoX-dependent regulation may favor environmental strains

enhancing genetic exchange [40]. This would also explain the increased T6SS effector module diversity among non-pandemic strains compared to pandemic strains as their T6SS appears to be more likely regulated with natural competence [4, 25].

Horizontal gene transfer following T6SS-mediated attack may lead to the acquisition of eDNA that can recombine anywhere in the genome [7, 11]. This DNA may mediate a fitness advantage as it is acquired from living cells that were actively killed by the T6SS and not from dead cells that potentially died as a result of their low fitness [57]. This process might contribute to the diversity of the species in two ways: by mediating the uptake of new (T6SS-independent) traits and acquisition of novel T6SS effector modules.

The second scenario presents a conceptual paradox: a predator strain acquiring an effector/immunity allele pair from a defeated, lysed prey makes it vulnerable to its own kin and would be selected against. This problem is seemingly solved by the acquisition of orphan immunity genes – an array of genes that are found immediately downstream of T6SS effector modules [26]. These open reading frames are co-regulated with the T6SS and share high sequence identity to T6SS immunity genes found in other strains [7]. Such mechanisms would allow strains to develop immunity to several effectors, expanding their niche and microbial community by now coexisting with other compatibility groups. Nevertheless, through sequential rounds of exchange, cassettes of orphan immunity genes could be acquired downstream of the effector module, suggesting that successive rounds of competition and competence diversify *V. cholerae*'s T6SS effector/immunity modules [26].

The diversity of T6SS effectors could help different *V. cholerae* acquire competitive mechanisms beneficial in different environments. For example, for a strain that encounters eukaryotic phagocytes, the acquisition of the anti-eukaryotic effectors VasX and the actin-crosslinking domain of VgrG1 would provide a competitive advantage [41, 46]. VasX presents an interesting example of an effector that displays cross-kingdom toxicity targeting both eukaryotic and prokaryotic cells. Such an effector would presumably help *V. cholerae* in a wide range of environments. This presents an interesting example whereby a set of T6SS might expand the niche of *V. cholerae.*

Bioinformatics analysis of the distribution of T6SS effectors reveals a clear difference between clinical and environmental strains [26, 55]. This is an indication that the T6SS effectors, at minimum, correlate with the lifestyle of a *V. cholerae* strain. The increased diversity amongst the T6SS repertoire of environmental strains could reflect the diversity of environmental reservoirs *V. cholerae* inhabits. *V. cholerae* could potentially constantly modify their compatibility group to best fit their environment as a result of the highly modular nature of the effector/immunity alleles. On the other end of the spectrum, the observation that all toxigenic strains encode the same T6SS effectors raises many interesting questions. For example, the AAA combination may provide the best competitive advantage amongst other *V. cholerae* isolates within a single host. In a host, competition between a heterogeneous inoculum of *V. cholerae* would lead to selection of a clonal lineage and subsequent expansion of toxigenic AAA strains, which may explain why cholera diarrhea has been described as a virtually pure culture of clonal bacteria [13]. Alternatively, this combination could be best suited for outcompeting commensals and phagocytic immune cells in the gut. Indeed, several groups have shown that the T6SS of pandemic strains is activated *in-vivo* supporting a potential role in pathogenesis.

Intraspecies competition has long been studied as a contributor to diversity [50]. That a diverse bacterial species like *V. cholerae* employs numerous mechanisms of intraspecific competition should therefore not be surprising. How the T6SS contributes to such diversity has yet to be clarified; however, if acquisition of a new effector set facilitates *V. cholerae* niche expansion, a given strain will likely accumulate secondary mutations that differ from those found in strains inhabiting other niches. In support of this theory, evolutionary trees built from bioinformatics analysis of conserved genes have shown separating branches of the tree correlates with changes in T6SS compatibility groups [26, 55].

The ability to exclude other strains from a bacterial niche is one important role of T6SS activity; however, the link with natural competence is a separate facet. This dual role places the T6SS at a key position in the natural history of a diverse species. This molecular mechanism facilitating diversity could continue to allow *V. cholerae* to adapt to the human host, selecting for novel strains that could pose new challenges to human health.

Competing interests. Authors declare that no competing interests exist.

References

1. Altindis E, Dong T, Catalano C, Mekalanos J (2015) Secretome Analysis of *Vibrio cholerae* Type VI Secretion System Reveals a New Effector-Immunity Pair. mBio 6:e00075-15
2. Bachmann V, Kostiuk B, Unterweger D, Diaz-Satizabal L, Ogg S, Pukatzki S (2015) Bile salts modulate the mucin-activated type VI secretion system of pandemic *Vibrio cholerae*. PLoS Negl Trop Dis 9:e0004031
3. Basler M, Pilhofer M, Henderson PG, Jensen JG, Mekalanos J (2012) Type VI secretion requires a dynamic contractile phage tail-like structure. Nature 483:182–186
4. Bernardy EE, Turnsek MA, Wilson SK, Tarr CL, Hammer BK (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
5. Bhowmick R, Ghosal A, Das B, Koley H, Saha DR, Ganguly S, Nandy RK, Bhadra RK, Chatterjee NS (2008) Intestinal adherence of *Vibrio cholerae* involves a coordinated interaction between colonization factor GbpA and mucin. Infect Immun 76:4968–4977
6. Bönemann G, Pietrosiuk A, Diemand A, Zentgraf H, Mogk A (2009) Remodeling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. EMBO J 28:315–325

7. Borgeaud S, Metzger LC, Scrignari T, Blokesch M (2015) The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. Science 347:63–67
8. Brooks TM, Unterweger D, Bachmann V, Kostiuk B, Pukatzki S (2013) Lytic activity of the *Vibrio cholerae* type VI secretion toxin VgrG-3 is inhibited by the antitoxin TsaB. J Biol Chem 288:7618–7625
9. Chourashi R, Mondal M, Sinha R, Debnath A, Das S, Koley H, Chatterjee NS (2016) Role of a sensor histidine kinase ChiS of *Vibrio cholerae* in pathogenesis. Int J Med Microbiol IJMM 306:657–665
10. Cianfanelli FR, Monlezun L, Coulthurst SJ (2016) Aim, load, fire: the type VI secretion system, a bacterial nanoweapon. Trends Microbiol 24:51–62
11. Dalia AB, McDonough E, Camilli A (2014) Multiplex genome editing by natural transformation. Proc Natl Acad Sci U S A 111:8937–8942
12. Dong TG, Ho BT, Yoder-Himes DR, Mekalanos JJ (2013) Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. Proc Natl Acad Sci USA 110:2623–2628
13. Dutta D, Chowdhury G, Pazhani GP, Guin S, Dutta S, Ghosh S, Rajendran K, Nandy RK, Mukhopadhyay AK, Bhattacharya MK, Mitra U, Takeda Y, Nair GB, Ramamurthy T (2013) *Vibrio cholerae* Non-O1, Non-O139 Serogroups and Cholera-like Diarrhea, Kolkata, India. Emerg Infect Dis 19:464–467
14. 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 USA 99:1556–1561
15. Epstein PR (1993) Algal blooms in the spread and persistence of cholera. Biosystems 31:209–221
16. Faruque SM, Naser IB, Islam MJ, Faruque ASG, Ghosh AN, Nair GB, Sack DA, Mekalanos JJ (2005) Seasonal epidemics of cholera inversely correlate with the prevalence of environmental cholera phages. Proc Natl Acad Sci U S A 102:1702–1707
17. Fu Y, Waldor MK, Mekalanos JJ (2013) Tn-Seq analysis of *Vibrio cholerae* intestinal colonization reveals a role for T6SS-mediated antibacterial activity in the host. Cell Host Microbe 14:652–663
18. Griffiths GL, Sigel SP, Payne SM, Neilands JB (1984) Vibriobactin, a siderophore from *Vibrio cholerae*. J Biol Chem 259:383–385
19. Hibbing ME, Fuqua C, Parsek MR, Peterson SB (2010) Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Microbiol 8:15
20. Holmgren J (1981) Actions of cholera toxin and the prevention and treatment of cholera. Nature 292:413–417
21. Hsiao A, Shamsir Ahmed AM, Subramanian S, Griffin NW, Drewry LL, Petri WA, Haque R, Ahmed T, Gordon JI (2014) Members of the human gut microbiota involved in recovery from *Vibrio cholerae* infection. Nature 515:423–426
22. Huq A, Small EB, West PA, Huq MI, Rahman R, Colwell RR (1983) Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Appl Environ Microbiol 45:275–283
23. Ishikawa T, Sabharwal D, Bröms J, Milton DL, Sjöstedt A, Uhlin BE, Wai SN (2012) Pathoadaptive Conditional Regulation of the Type VI Secretion System in *Vibrio cholerae* O1 Strains. Infect Immun 80:575–584
24. J Kirn T, Jude B, K Taylor R (2006) A colonization factor links *Vibrio cholerae* environmental survival and human infection. Nature 438:863-866
25. Kirchberger PC, Orata FD, Barlow EJ, Kauffman KM, Case RJ, Polz MF, Boucher Y (2016) A small number of phylogenetically distinct clonal complexes dominate a coastal *Vibrio cholerae* population. Appl Environ Microbiol 82:5576–5586
26. 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
27. Kitaoka M, Miyata ST, Brooks TM, Unterweger D, Pukatzki S (2011) VasH Is a Transcriptional Regulator of the Type VI Secretion System Functional in Endemic and Pandemic *Vibrio cholerae*. J Bacteriol 193:6471–6482
28. Krebs SJ, Taylor RK (2011) Protection and Attachment of *Vibrio cholerae* Mediated by the Toxin-Coregulated Pilus in the Infant Mouse Model. J Bacteriol 193:5260–5270
29. Ley RE, Peterson DA, Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837–848
30. Liang X, Moore R, Wilton M, Wong MJQ, Lam L, Dong TG (2015) Identification of divergent type VI secretion effectors using a conserved chaperone domain. Proc Natl Acad Sci U S A 112:9106–9111
31. Long RA, Rowley DC, Zamora E, Liu J, Bartlett DH, Azam F (2005) Antagonistic Interactions among Marine Bacteria Impede the Proliferation of *Vibrio cholerae*. Appl Environ Microbiol 71:8531–8536
32. Ma L-S, Hachani A, Lin J-S, Filloux A, Lai E-M (2014) Agrobacterium tumefaciens Deploys a Superfamily of Type VI Secretion DNase Effectors as Weapons for Interbacterial Competition In Planta. Cell Host Microbe 16:94–104
33. Ma AT, Mekalanos JJ (2010) In vivo actin cross-linking induced by *Vibrio cholerae* type VI secretion system is associated with intestinal inflammation. Proc Natl Acad Sci USA 107:4365–4370
34. MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S (2010) The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. Proc Natl Acad Sci USA 107:19520–19524
35. Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH, Kjelleberg S (2005) Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. Proc Natl Acad Sci U S A 102:16819–16824
36. McNally L, Bernardy E, Thomas J, Kalziqi A, Pentz J, Brown SP, Hammer BK, Yunker PJ, Ratcliff WC (2017) Killing by Type VI secretion drives genetic phase separation and correlates with increased cooperation. Nat Commun 8:ncomms14371
37. Meibom KL, Li XB, Nielsen AT, Wu C-Y, Roseman S, Schoolnik GK (2004) The *Vibrio cholerae* chitin utilization program. Proc Natl Acad Sci U S A 101:2524–2529
38. Merrell DS, Bailey C, Kaper JB, Camilli A (2001) The ToxR-mediated organic acid tolerance response of *Vibrio cholerae* requires OmpU. J Bacteriol 183:2746–2754
39. Merrell DS, Hava DL, Camilli A (2002) Identification of novel factors involved in colonization and acid tolerance of *Vibrio cholerae*. Mol Microbiol 43:1471–1491
40. 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
41. Miyata ST, Kitaoka M, Brooks TM, McAuley SB, Pukatzki S (2011) *Vibrio cholerae* requires the type VI secretion system virulence factor VasX to kill Dictyostelium discoideum. Infect Immun 79:2941–2949
42. Miyata ST, Unterweger D, Rudko SP, Pukatzki S (2013) Dual expression profile of type VI secretion system immunity genes protects pandemic *Vibrio cholerae*. PLoS Pathog 9:e1003752
43. Morris JJ (1990) Non-O group 1 *Vibrio cholerae*: a look at the epidemiology of an occasional pathogen. Epidemiol Rev 12:179–191
44. Nalin DR, Daya V, Reid A, Levine MM, Cisneros L (1979) Adsorption and growth of *Vibrio cholerae* on chitin. Infect Immun 25:768–770
45. Provenzano D, Klose KE (2000) Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes *Vibrio cholerae* bile resistance, virulence factor expression, and intestinal colonization. Proc Natl Acad Sci USA 97:10220–10224
46. Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, Heidelberg JF, Mekalanos JJ (2006) Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. Proc Natl Acad Sci USA 103:1528–1533
47. Riley MA, Gordon DM (1999) The ecological role of bacteriocins in bacterial competition. Trends Microbiol 7:129–133
48. Shao Y, Bassler BL (2014) Quorum regulatory small RNAs repress type VI secretion in *Vibrio cholerae*. Mol Microbiol 92:921–930
49. Silva AJ, Pham K, Benitez JA (2003) Haemagglutinin/protease expression and mucin gel penetration in El Tor biotype *Vibrio cholerae*. Microbiology 149:1883–1891
50. Svanbäck R, Bolnick DI (2007) Intraspecific competition drives increased resource use diversity within a natural population. Proc R Soc Lond B Biol Sci 274:839–844

51. Thomas J, Watve SS, Ratcliff WC, Hammer BK (2017) Horizontal Gene Transfer of Functional Type VI Killing Genes by Natural Transformation. mBio 8:e00654-17
52. 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
53. Unterweger D, Kostiuk B, Ötjengerdes R, Wilton A, Diaz-Satizabal L, Pukatzki S (2015) Chimeric adaptor proteins translocate diverse type VI secretion system effectors in *Vibrio cholerae*. EMBO J 34: 2198-2210
54. Unterweger D, Kostiuk B, Pukatzki S (2017) Adaptor proteins of type VI secretion system effectors. Trends Microbiol 25:8–10
55. 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. DOI: 10.1038/ncomms4549
56. Vaitkevicius K, Lindmark B, Ou G, Song T, Toma C, Iwanaga M, Zhu J, Andersson A, Hammarström M-L, Tuck S, Wai SN (2006) A *Vibrio cholerae* protease needed for killing of Caenorhabditis elegans has a role in protection from natural predator grazing. Proc Natl Acad Sci USA 103:9280–9285
57. Veening J-W, Blokesch M (2017) Interbacterial predation as a strategy for DNA acquisition in naturally competent bacteria. Nat Rev Microbiol 15:621–629
58. Vezzulli L, Pruzzo C, Huq A, Colwell RR (2010) Environmental reservoirs of *Vibrio cholerae* and their role in cholera. Environ Microbiol Rep 2:27–33
59. 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