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MEETING REVIEW

Environmental Role of Pathogenic Traits in *Vibrio cholerae*

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ABSTRACT *Vibrio cholerae* is a natural inhabitant of aquatic ecosystems. Some strains of *V. cholerae* can colonize human hosts and cause cholera, a profuse watery diarrhea. The major pathogenicity factors and virulence regulators of *V. cholerae* are encoded either in mobile genetic elements acquired in the environment (e.g., pathogenicity islands or lysogenic phages) or in the core genome. Several lines of evidence indicate that the emergence of numerous virulence traits of *V. cholerae* occurred in its natural environment, due to biotic and abiotic pressures. Here, we discuss the connections between the human host and the potential ecological roles of these virulence traits. Elucidating these connections will help us understand the emergence of this organism and other facultative bacterial pathogens.

KEYWORDS *Vibrio cholerae*, virulence, environment, emergence, pathogen ecology, evolution, virulence factors

Facultative pathogens do not rely on their human hosts for survival and long-term persistence. Some members of the family of aquatic bacteria *Vibrionaceae* represent several distinct paradigms of facultative and emergent pathogens. Although some species, such as *Vibrio vulnificus*, *Vibrio parahaemolyticus*, or *Vibrio cholerae*, can cause disease in humans, they are natural inhabitants of estuarine and brackish environments and most strains are nonpathogenic (1–4). *V. cholerae*, the etiological agent of the severe diarrheal disease cholera, is the most widely studied pathogenic species of the *Vibrionaceae*. Cholera remains a major scourge in places with limited access to clean drinking water and with poor sanitation (5, 6). There have been cholera outbreaks in places as diverse as South America, the Caribbean, South Asia, Africa, and the Middle East. Although cholera cases are often unreported, there are an estimated 3 to 5 million cases per year globally (5, 6). The largest epidemic in the world is currently taking place in Yemen, where there have been over 1,000,000 suspected cholera cases (7–10).

Among the >200 known serogroups of *V. cholerae*, only the O1 and O139 serogroups have been associated with cholera symptoms (5, 6). Both serogroups belong to a clade of phylogenetically confined strains of *V. cholerae*, the pandemic genome (PG) group (11–13). To date, only strains from this group have been found to cause cholera in humans; however, other strains of *V. cholerae* (non-O1/non-O139) can cause gastrointestinal infections (14, 15). Numerous virulence factors of *V. cholerae* are encoded within mobile genetic elements and were horizontally acquired by pathogenic strains (16). For instance, cholera toxin (CT), the source of profuse watery diarrhea, is encoded within the CTXϕ lysogenic phage (17) and toxin-coregulated pilus (TCP), an essential colonization factor (18), is encoded within *Vibrio* pathogenicity island 1 (VPI-1) (19). However, other factors, such as *N*-acetylglucosamine-binding protein A (GbpA), an adhesin involved in attachment to intestinal epithelial cells, and the inner membrane-localized virulence regulator ToxR, are encoded in the core genome of both clinical and environmental strains (20, 21).

In its natural environment, *V. cholerae* is frequently found in association with other

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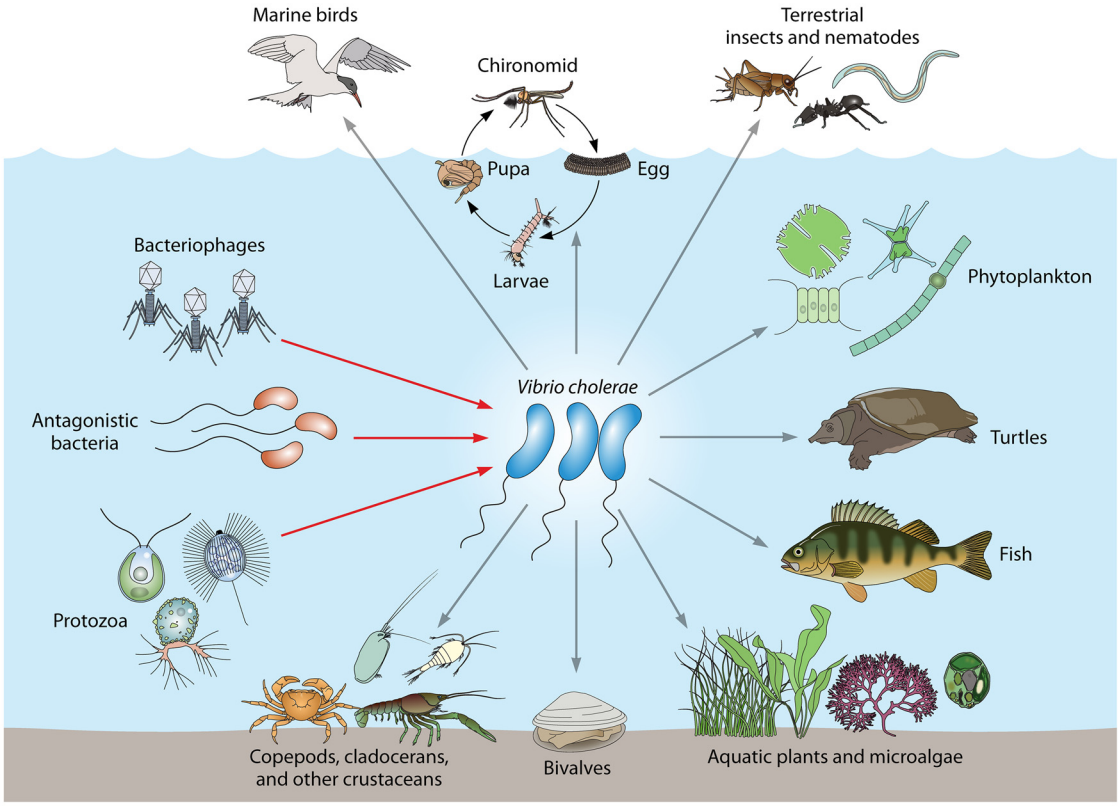

FIG 1 *Vibrio cholerae* interactions in its natural environment. The associations of *V. cholerae* with reservoirs and antagonistic organisms that shape its virulence potential are shown. Gray arrows indicate reservoirs, such as crustaceans, copepods, chironomid egg masses, phytoplankton, fish, turtles, aquatic birds, shellfish, and protozoa. Red arrows indicate antagonistic relationships with protists, bacteriophages, and predatory bacteria.

aquatic organisms, such as copepods and crustaceans (22–25), arthropods and chironomid egg masses (26–28), cyanobacteria (29, 30), shellfish (31, 32), waterfowl (33), and fish (34–36) (Fig. 1). In addition, *V. cholerae* generally faces a wide range of abiotic and biotic stressors that pose threats to its survival, such as nutrient limitations, pH changes, temperature and salinity fluctuations, grazing by protozoa, and phage predation (Fig. 1) (37–44). It appears that some of the mechanisms that allow the bacteria to colonize and to persist in their natural environment provide preadaptations for virulence in human hosts (Fig. 2).

Humans play an unquestionable role in the emergence and evolution of pathogenic *V. cholerae*, by selecting and amplifying virulent clones and their traits (44–46). In recent years, however, several virulence and colonization factors of *V. cholerae* have been found to play roles in the survival and persistence of the bacteria in their natural environment (Fig. 2). In this review, we discuss the environmental roles of several *V. cholerae* virulence factors that are involved in a wide variety of functions, such as colonization, motility, adhesion, biofilm formation, quorum sensing (QS), and toxin secretion. Overall, we highlight some of the factors that, together with host selective pressures, could have led to the emergence of pathogenic traits in *V. cholerae*.

TYPE VI SECRETION SYSTEM

Some non-O1/non-O139 strains of *V. cholerae* can cause gastrointestinal infections (14, 15). *V. cholerae* V52, a strain that belongs to the O37 serogroup, encodes a nanosyringe-like system termed the type VI secretion system (T6SS), which induces inflammatory diarrhea, facilitates replication of *V. cholerae* within the rabbit intestine, and plays a role in competing against the gut microbiota (Fig. 2A) (15, 47, 48). Since the seminal discovery by Pukatzki et al. (15), T6SSs have been described in *V. cholerae* O1

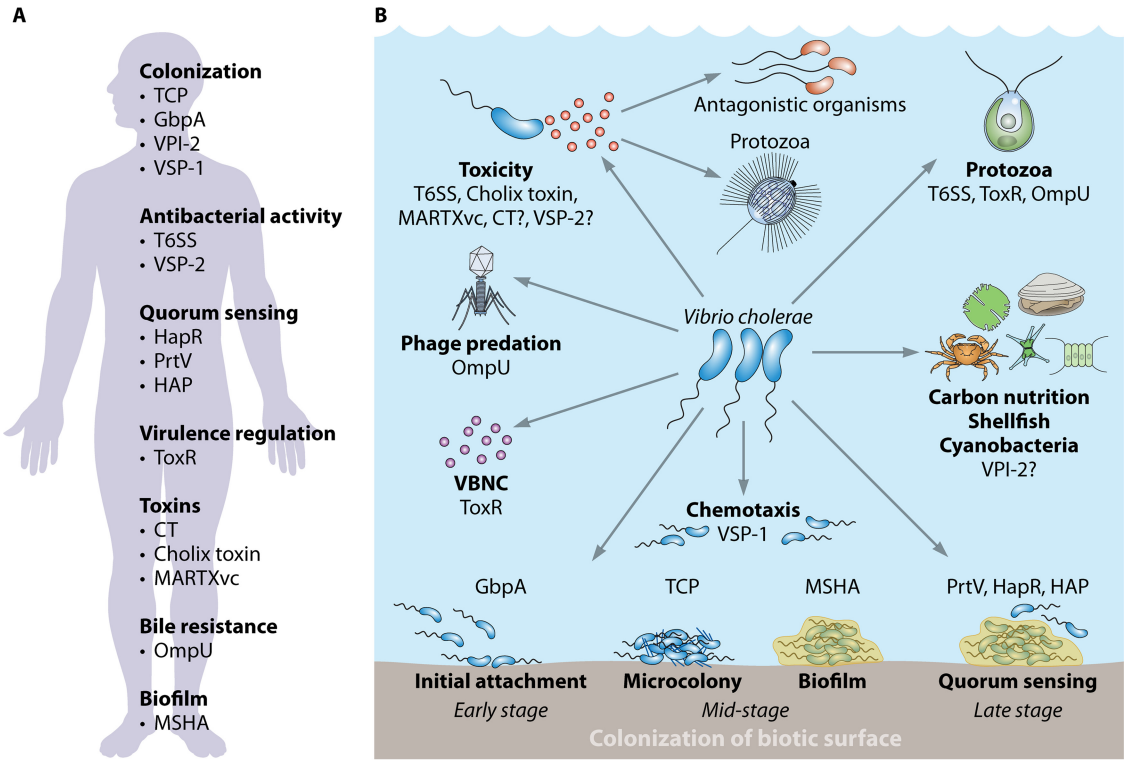

FIG 2 Convergence of the aquatic environment and the human host. Factors involved in *Vibrio cholerae* colonization, survival, and toxicity in the human host (A) and the aquatic environment (B) are shown. MSHA, mannose-sensitive hemagglutinin; TCP, toxin-coregulated pilus; GbpA, *N*-acetylglucosamine-binding protein A; VPI-2, *Vibrio* pathogenicity island 2; VSP-1, *Vibrio* seventh pandemic island I; HAP, hemagglutinin protease; PrtV, *Vibrio* metalloprotease; CT, cholera toxin; MARTXvc, multifunctional autoprocessing repeats-in-toxin; T6SS, type VI secretion system; VSP-2, *Vibrio* seventh pandemic island II; VBNC, viable but nonculturable. Question marks indicate hypothetical roles or connections.

strains and other bacterial species (48–52). It was recently shown that T6SS inactivation attenuates *V. cholerae* pathogenesis in *Drosophila melanogaster* (53). Interestingly, the T6SS can be reactivated in the presence of commensal gut bacteria such as *Acetobacter pasteurianus* (53). The roles of the T6SS in intestinal colonization, virulence, and antagonistic interactions with gut microbes are governed by diverse regulatory mechanisms such as QS or carbon utilization and chitin-induced natural competency pathways (50, 52, 54, 55). Recent findings show a direct regulatory relationship between the T6SS and QS; however, the possible contribution of the T6SS to the virulence regulatory cascade needs further elucidation (see below) (48). Besides its critical role in the host, the T6SS plays a major role in the environmental survival of *V. cholerae* (15, 49–52). In the environment, the T6SS confers protection against predators, aids in competition against antagonistic microorganisms, and facilitates gene acquisition and horizontal gene transfer (48). The T6SS secretes self-protecting proteins (TsiV1, TsiV2, and TsiV3) and toxic effector proteins (VasX, TseL, and VgrG-3), which provide a competitive advantage over other bacterial species in the natural environment and mediate cytotoxicity to both mammalian cell lines and the soil-living amoeba *Dictyostelium discoideum* (Fig. 2B) (15, 49–51). Secretion of toxins and effectors by the T6SS provides a selective advantage during interspecies competition against numerous species, such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (47). Interestingly, besides serving as a predatory killing device, the T6SS is part of the competence regulon in *V. cholerae* (56, 57). Borgeoud and colleagues showed that the T6SS-encoding gene cluster is under the positive control of the competence regulators TfoX and QstR and fosters horizontal gene transfer by making exogenous DNA accessible to *V. cholerae* cells (56, 57). All of these findings highlight the critical roles of the T6SS both in the host and in the natural environment, allowing *V. cholerae* to prey on other microorganisms and also acquire novel genetic traits (Fig. 2).

QUORUM SENSING

QS is a phenomenon by which bacteria monitor their cell population density through the extracellular accumulation of signaling molecules called autoinducers (58–62). Expression of *hapR*, a negative regulator of virulence, is repressed at low cell densities; however, during the late stages of colonization, when cell numbers are high, *hapR* becomes derepressed, thus negatively affecting virulence gene expression (Fig. 2A) (59, 62). The signaling molecules produced from QS at high cell densities also facilitate cellular processes that cause increased motility, repression of *Vibrio* polysaccharide (VPS) production, downregulation of TCP and CT, upregulation of the T6SS, and protease secretion (58–64). At high cell densities, quorum regulatory small RNAs become activated by HapR to activate T6SS genes, a phenomenon that is conserved across *V. cholerae* strains (65). Zheng et al. reported that the activity of the T6SS in *V. cholerae* is controlled by the combined actions of LuxO, a QS response regulator, and TsrA, a global regulator of *V. cholerae* (54). The authors found that TsrA represses the production of the T6SS substrate Hcp (54). Disruption of LuxO and TsrA activates the T6SS, thus increasing intestinal colonization in the mouse model and inflammatory diarrhea in infant rabbits (54). The influence of QS on the survivability and persistence of *V. cholerae* in aquatic habitats has been discussed previously (66–68). The production of HapR in the natural environment plays a role in preventing the bacteria from protozoal grazing through secretion of PrtV and, at high cell densities, regulates the transcription of *hapA*, which encodes a hemagglutinin protease (HAP) that cleaves biofilm proteins (58–62). PrtV plays a role in bacterial survivability against predators such as phages, protozoa, and bacteriovorous organisms such as *Cafeteria roenbergensis* and *Tetrahymena pyriformis* (69, 70). In the human host, PrtV mediates degradation of the epithelial extracellular matrix and blood components and induces an inflammatory response (Fig. 2A) (69, 71). HAP is a HapR-regulated metalloprotease that cleaves proteins in the biofilm matrix when the cell density increases, thus possibly facilitating bacterial cell dispersal in the late stages of colonization (58, 59, 72, 73). In the aquatic environment, HAP digests the gelatinous matrix of chironomid egg masses, mediates associations with cyanobacteria, and aids in dissolving organic matter, thereby releasing nutrients for *V. cholerae* cells (Fig. 2B) (74, 75). Recently, Kamareddine et al. reported a direct relationship between QS and the intestinal colonization of an arthropod host by *V. cholerae* (76). They showed that QS-mediated intestinal colonization promotes *Drosophila melanogaster* survival and reduction of succinate uptake by the bacteria (76).

N-ACETYLGLUCOSAMINE-BINDING PROTEIN A

In its natural environment, *V. cholerae* can be typically found in association with the chitinaceous exoskeleton of crustaceans (22, 37, 38). GbpA is a chitin-binding protein that is highly conserved on the core genome of members of the family *Vibrionaceae* (20, 77, 78). GbpA promotes adherence, colonization, and interactions with various environmental biotic surfaces, such as crustacean shells, mussel hemocytes, and bivalves and their hepatopancreatic cells (Fig. 2B) (20, 77–79). Chitin is one of the most abundant carbon sources in the aquatic environment; therefore, binding to and degrading chitin provide a competitive advantage for *V. cholerae* outside the human host (80, 81). Recently, Wang et al. showed active interactions of GbpA during the intestinal colonization of soft-shelled turtles (Fig. 1) (82). These findings prompted the authors to propose the turtle gut as an alternative model system for *V. cholerae* colonization (82). In addition, GbpA has been shown to mediate attachment to human intestinal epithelial cells and is required for successful gut colonization, which provides a direct link between environmental and host colonization of *V. cholerae* (20).

TOXIN-COREGULATED PILUS

TCP, a type IV pilus, is an essential colonization factor that mediates microcolony formation in the intestine (18). Microcolonies are clusters of *V. cholerae* cells that confer numerous properties to the bacteria (83). For instance, TCP enhances attachment to intestinal epithelial cells, facilitates bacterium-bacterium interactions by tethering cells

together, mediates secretion of the colonization factor TcpF, and provides protection against antimicrobial agents (84–86). The ability to form microcolonies correlates with the ability to colonize infant mice and humans (Fig. 2A) (18, 84–86). In addition, TCP also acts as the receptor of the CTXϕ phage (17). In aquatic environments, together with other pili such as mannose-sensitive hemagglutinin (MSHA) and chitin-regulated pilus (ChiRP), TCP mediates attachment to and colonization of the chitinaceous surface of copepods (Fig. 2B) (80, 87). Furthermore, it has been shown that mutant strains that do not secrete TCP are unable to form differentiated biofilms on those surfaces, which leads to increased sensitivity to stressors (87). Overall, it appears that the ability of *V. cholerae* to colonize crustaceans provides the bacteria with the ability to form microcolonies in the human gut.

CHOLERA TOXIN

The production of CT in the intestine is directly responsible for the severity of the profuse diarrhea associated with cholera (5, 6). CT constitutively activates adenylate cyclase by ADP-ribosylating a coupled G-protein, which leads to increased intracellular cAMP levels (5, 6). This prompts the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel to be constitutively opened, Cl^- to be effluxed with sodium, and water to follow passively (5, 6). Although a direct environmental role of CT has yet to be reported, it has been shown that, due to the lysogenic nature of the CTXϕ phage, the insertion and deletion of this phage can enable gene recombination, which leads to diversity within the pandemic strains (17, 88–90). This serves as an opportunity to increase the pathogenic potential of pandemic strains (17, 88–90). Intriguingly, *V. cholerae* secretes CT while associated with the cyanobacterium *Rhizoclonium fontanum*; the biological reason behind this remains unknown (91). Furthermore, studies have shown that CT causes protein trafficking and death of *D. melanogaster* (28). CT also causes disruption of exocyst trafficking, which induces the breakdown of intestinal adherens junctions in both *D. melanogaster* and mammalian intestines in a manner dependent on Rab11, a conserved G protein (92). These unresolved associations indicate that CT plays a role in the environment; however, more research needs to be conducted in order to establish an evolutionary origin of the toxin. It was previously hypothesized that, given its inherent function, CT might act as an osmoregulator when produced in the gills of crustaceans, providing an advantage to the crustaceans as they move into environments of increasing salinity (22, 23, 25, 93, 94). It is tempting to speculate that *V. cholerae* might establish a symbiotic relationship with those crustaceans, obtaining a suitable place to attach and to feed while providing the host with a powerful osmoregulator.

ToxR AND OUTER MEMBRANE PORIN U

The transmembrane transcriptional activator ToxR is encoded in the core genome of every sequenced member of the family *Vibrionaceae* (21, 95). It influences the expression of numerous genes (~150 genes) involved in diverse cellular functions (96–99). In association with TcpP, ToxR is required for transcription of the gene encoding ToxT, which regulates the expression of the major pathogenicity factors of *V. cholerae* (e.g., TCP and CT) (21, 100–105). When *V. cholerae* cells are exposed to nutrient limitations at alkaline pH, ToxR is proteolyzed via a process that involves the site 2 protease RseP and is dependent on the sigma E-dependent envelope stress response (106–109). The proteolysis of ToxR is associated with the entry of *V. cholerae* into a dormant state called viable but nonculturable (VBNC) (106, 107). When conditions are not suitable for growth, *V. cholerae* enters a dormant state (VBNC) in which it loses culturability and adopts a viable coccoid form, which appears to facilitate its survival and persistence in the environment (106, 107). It seems possible that ToxR evolved as a nutrient sensor in the *Vibrionaceae* and was adopted by the virulence cascade as a means to detect the presence of the host, as it is intrinsically associated with nutrient abundance.

In response to the nutritional status of the cell, ToxR also reciprocally regulates the expression of the outer membrane porin genes *ompU* and *ompT* (96, 109–112). It has

been shown that OmpU provides resistance to bile and organic acids and confers an advantage in intestinal colonization (113–115). OmpU also confers resistance against phage predation, facilitates survival inside the amoebal lysosome, and is involved in biofilm formation (44, 116, 117). These traits provide an evolutionary advantage in the natural environment of *V. cholerae* that likely led to the emergence of virulence traits (Fig. 2B) (44, 116, 117).

VIBRIO SEVENTH PANDEMIC ISLANDS

El Tor strains are responsible for the seventh and current pandemic of cholera. There are numerous traits that distinguish El Tor from classic as strains, among them the presence of two gene clusters, i.e., *Vibrio* seventh pandemic (VSP) islands I and II (118). Although the phenotypic functions provided by these clusters are not completely understood, recent work has revealed some roles of the VSP islands (119). Davies et al. showed that VspR, a transcriptional factor encoded in VSP-I, is regulated by the master regulator of virulence in *V. cholerae*, ToxT, through the small RNA TarB (119). Repression of VspR by TarB is associated with lower levels of intestinal colonization as well as decreased chemotaxis (119). Interestingly, VSP-I was also found in nonpandemic strains of *V. cholerae*, and it has been suggested to have an environmental role related to chemotaxis (120). It has also been reported that the presence of VSP-II in clinical and environmental strains might be associated with environmental survival and fitness of the bacteria (121–123). Comparative genomic analysis of *V. cholerae* El Tor N16961 and a group of *V. cholerae* strains that caused an outbreak in Florida associated with oyster consumption revealed the presence of a novel bacteriocin and a pyocin protein in the VSP-II elements of the *V. cholerae* Florida group (123). Numerous microorganisms secrete bacteriocin and other antimicrobial peptides in order to protect themselves from other microorganisms (124). Furthermore, pyocin mediates cytotoxicity toward other inhabitants of its natural environment, such as the fish pathogen *Vibrio anguillarum* (123, 125). Overall, these findings indicate that VSP-II might provide a competitive advantage to *V. cholerae* El Tor versus other microbial marine dwellers.

VIBRIO PATHOGENICITY ISLAND 2

VPI-2 is a 57.3-kb horizontally acquired region present in pandemic strains of *V. cholerae* (16, 126). VPI-2 includes genes for sialic acid (*N*-acetylneuraminic acid) utilization (16, 126). Sialic acids or nonulosonic acids constitute a family of 9-carbon amino sugars that are prevalent in mucus-rich environments (127). VPI-2 includes the genes necessary for the scavenging, transport, and catabolism of sialic acid (127, 128). NanH, a neuraminidase that allows for the scavenging of sialic acid, converts higher-order gangliosides found in the intestinal mucus into GM1 gangliosides, thus unmasking the CT receptors (129, 130). The capacity to utilize sialic acid as a carbon and energy source provides *V. cholerae* with a competitive advantage in the mucus-rich environment of the gut, where sialic acid availability is extensive (131). The ability to use sialic acid likely confers a competitive advantage in the natural ecosystem of *V. cholerae*, as the molecule is present in the mucilaginous sheath of cyanobacteria, the guts of fish, and the mucus-rich gills of oysters (31, 35, 132, 133). Furthermore, the catabolic pathways of sialic acid and *N*-acetylglucosamine (the monomer of chitin) converge, suggesting a synergistic relationship between the two pathways and the different hosts of *V. cholerae*.

MANNOSE-SENSITIVE HEMAGGLUTININ AND BIOFILM FORMATION

V. cholerae O1 El Tor and O139 strains produce a second type IV pilus, MSHA (134–137). MSHA promotes attachment of *V. cholerae* to abiotic surfaces and the exoskeleton of crustaceans and mediates biofilm formation (134–137). Strains with functional MSHA are able to adhere to and colonize both abiotic and biotic surfaces, independent of the surface chemistry (77, 78, 137). MSHA provides a major advantage for persistence of *V. cholerae* in its natural environment, due to its role in attachment to various substrates (Fig. 2B) (37, 38, 77, 78, 137). Furthermore, biofilm acts as a

reservoir of VBNC *Vibrio cholerae* O1 cells between epidemics and promotes long-term survivability of the bacterium in the ecological niches it colonizes (138, 139). Interestingly, the role of MSHA and biofilm formation in human pathogenesis remains puzzling (140). *V. cholerae* cells that are ingested as part of a biofilm can successfully survive the low pH of the stomach (141). Furthermore, while forming biofilm, *V. cholerae* can be found in a hyperinfectious physiological state that reduces its infectious dose (142, 143). However, the inability of *V. cholerae* cells to repress MSHA biosynthesis prevents colonization of the mouse intestine in the presence of secretory IgA (144). Furthermore, TcpJ, a prepilin peptidase encoded within the TCP operon, cleaves the primary structural pilin of MSHA, indicating that TCP and MSHA play antagonistic roles *in vivo* (145). Thus, it appears that biofilm formation and MSHA biosynthesis have a precise spatiotemporal pattern that provides advantages at some specific stages during host and environmental colonization (Fig. 2) (140).

OTHER TOXINS

Cholix toxin. Cholix toxin has been found to be cytotoxic toward eukaryotic cell lines (146, 147). The cytotoxic effect is caused by protein synthesis inhibition in the cytoplasm of the host cells (146). The inhibition can potentially damage cellular functions due to a modification of translational elongation factor 2 in the eukaryotic ribosome (146). The diversity of cholix toxin genes is high among different strains that have been isolated from both the environment and patients (148). Cholix toxin also plays a role in the environmental survivability and fitness of *V. cholerae* strains, as it is cytotoxic toward yeast cells, *Artemia nauplii*, and other crustaceans (147, 149).

Multifunctional autoprocessing repeats-in-toxin. Multifunctional autoprocessing repeats-in-toxin (MARTXvc) has been found to enhance the colonization ability of *V. cholerae in vivo* (150–152). MARTXvc inhibits phagocytosis and intestinal clearance of the bacterial cells (150–152). MARTXvc has also been hypothesized to play a part in niche adaptation and to be involved in the pathogenesis of various marine organisms (150). Some members of the repeats-in-toxin (RTX) family play a defensive role in the environment as bacteriocins, indicating that these effectors evolved as a natural defense mechanism for bacteria (150, 153, 154).

CONCLUSIONS

Humans play an undisputable role in the emergence and selective amplification of virulence traits in *V. cholerae* (44–46). As discussed above, however, the environmental roles of some virulence factors of *V. cholerae* appear to confer prolonged survivability of the bacterium in the aquatic environment and also increase its the ability to colonize and infect the human host (e.g., GbpA) or express virulence factors (e.g., ToxR) (Fig. 2). It remains to be determined which other abiotic and biotic factors have driven the emergence of virulence traits of *V. cholerae* in its natural environment. We recently discovered that pandemic *V. cholerae* strains encode allelic variations in core genes in the form of virulence adaptive polymorphisms (VAPs) that enhance their pathogenic potential (117). VAPs confer preadaptations to virulence prior to the acquisition of virulence genes such as CT or TCP and are also encoded by environmental strains (117). Since some of the virulence traits of *V. cholerae* appear to have evolved prior to host colonization, we speculate that VAPs circulate in nonpathogenic environmental populations of *V. cholerae* and are selected for and enriched in the environment. Combined with the presence of selective pressures such as grazing, phage predation, and environmental fluctuations, it is possible that the bacteria are prompted to regularly adapt and to develop novel defensive strategies, which might drive the emergence of virulence properties (155–157). Multidisciplinary approaches that integrate fields such as genomics, evolutionary ecology, and pathogenesis might provide us with the knowledge and tools to understand the sets of conditions and the environmental drivers that lead to the emergence and acquisition of virulent traits in bacterial populations.

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