



Environmental reservoirs and mechanisms of persistence of *Vibrio cholerae*

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It is now well accepted that *Vibrio cholerae*, the causative agent of the water-borne disease cholera, is acquired from environmental sources where it persists between outbreaks of the disease. Recent advances in molecular technology have demonstrated that this bacterium can be detected in areas where it has not previously been isolated, indicating a much broader, global distribution of this bacterium outside of endemic regions. The environmental persistence of *V. cholerae* in the aquatic environment can be attributed to multiple intra- and interspecific strategies such as responsive gene regulation and biofilm formation on biotic and abiotic surfaces, as well as interactions with a multitude of other organisms. This review will discuss some of the mechanisms that enable the persistence of this bacterium in the environment. In particular, we will discuss how *V. cholerae* can survive stressors such as starvation, temperature, and salinity fluctuations as well as how the organism persists under constant predation by heterotrophic protists.

Keywords: starvation adaptation, biofilms, chitin, zooplankton, protozoa, predation, stress, viable but non-culturable

INTRODUCTION

While it is likely to have been responsible for human infections and mortality throughout human history, cholera outbreaks have only been formally known to science since 1817 (Pollitzer, 1954). Sir John Snow was credited in 1849 as being the first person to connect contaminated water with cholera outbreaks and to use that information as an infection control strategy (Snow, 1855). In addition to being the genesis of modern epidemiology, his observation may also be the first study on the ecology of *Vibrio cholerae*. However, it took another 120 years for *V. cholerae* to be recognized as an autochthonous aquatic bacterium rather than a human pathogen that is a transient resident of the aquatic environment (Colwell et al., 1977). *V. cholerae* has over 200 serogroups, with O1 and O139 being the causative agents of cholera, due to their carriage of the genes encoding cholera toxin (CT) and the toxin co-regulated pilus (TCP; Chatterjee et al., 2007). Surveys performed in non-endemic areas have shown that the majority of *V. cholerae* strains isolated are non-toxigenic (Faruque et al., 2004; Haley et al., 2012; Islam et al., 2013), which suggests that associations with the human host is only one small aspect of the *V. cholerae* life cycle and is not necessary for environmental persistence.

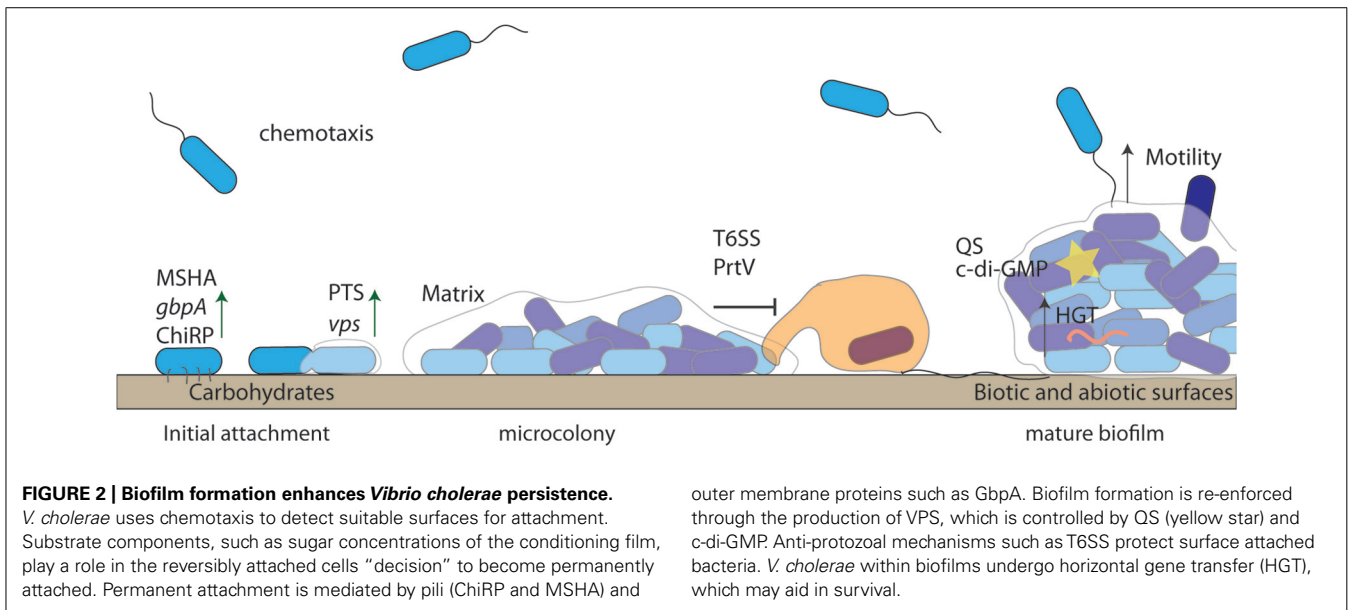
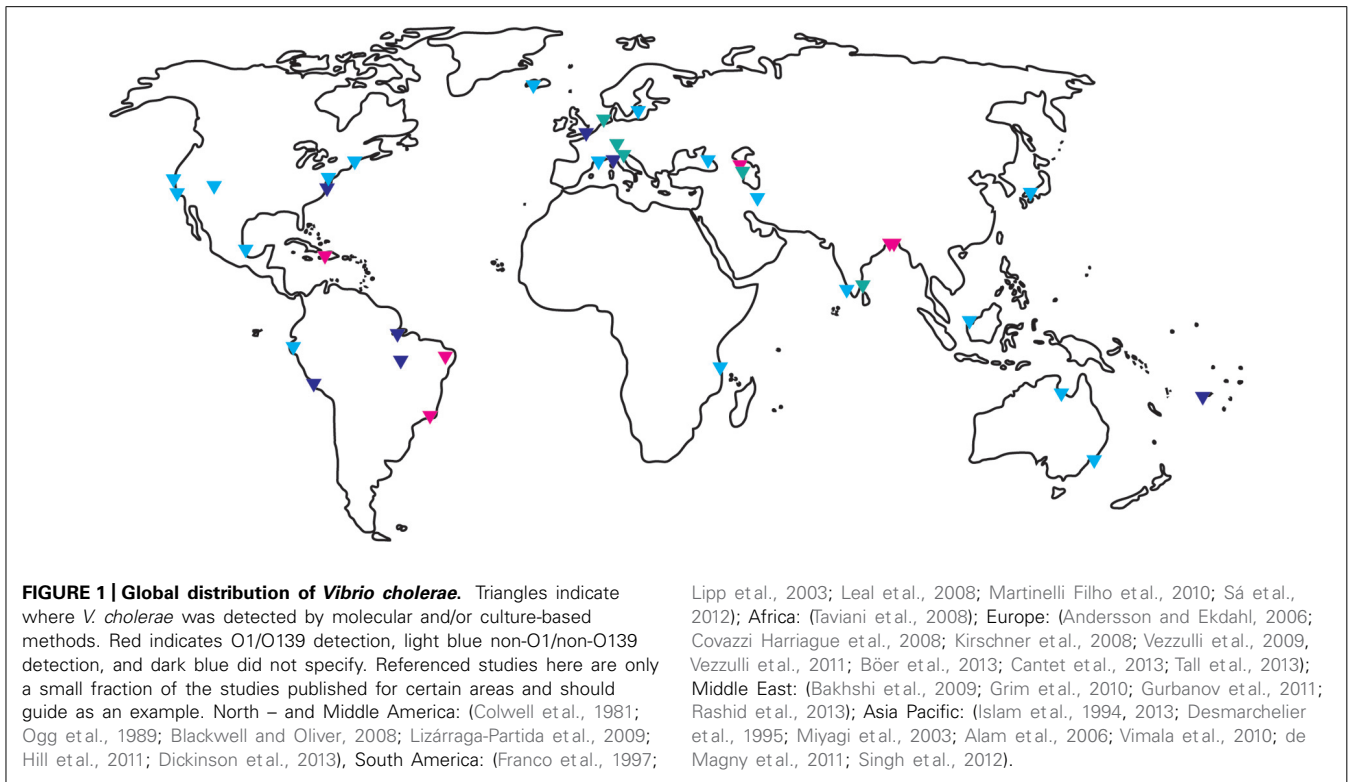
Vibrio cholerae inhabits a vast geographical range from the tropics (e.g., the Bay of Bengal where pandemics still occur, e.g., Albert et al., 1993; Huq et al., 2005; de Magny et al., 2011) to temperate waters world-wide (e.g., USA, South America, Australia, Sweden, and Italy, e.g., Vezzulli et al., 2009; Collin and Rehnstam-Holm, 2011; Schuster et al., 2011; Islam et al., 2013; Tall et al., 2013; **Figure 1**). An increasing understanding of the ecology of *V. cholerae*, along with advances in molecular detection

has demonstrated that this bacterium is a cosmopolitan aquatic species that is capable of causing illness in humans (Sack et al., 2004).

The capability to survive in many different environmental niches is largely due to the evolution of a range of adaptive responses that allow *V. cholerae* to survive stressors such as nutrient deprivation, fluctuations in salinity and temperature and to resist predation by heterotrophic protists and bacteriophage. One such strategy is the conversion into a viable but non-culturable (VBNC) state during unfavorable conditions (Colwell, 2000; Thomas et al., 2006). Additionally, *V. cholerae* attaches to abiotic and biotic surfaces (chitinous as well as gelatinous zoo- and phytoplankton) as biofilms (e.g., Huq et al., 1996; Akselman et al., 2010; Shikuma and Hadfield, 2010). Biofilm formation is associated with increased stress resistance, increased access to nutrients and as a means of dispersal when attached to living, mobile hosts (Costerton et al., 1995; Hall-Stoodley et al., 2004). Here, the current understanding of how *V. cholerae* is able to adapt to, and persist in the aquatic environment is summarized.

SURFACE COLONIZATION AND BIOFILM FORMATION ENHANCE *V. cholerae* PERSISTENCE

For aquatic bacteria, surface attachment provides a selective advantage through access to nutrients that accumulate at the liquid-surface interface (Dawson et al., 1981). Therefore, surface adhesion may be a survival strategy that allows bacteria to persist in nutrient-limited natural environments (Dawson et al., 1981; **Figure 2**). Additionally, some biotic surfaces may provide nutrients for attached bacteria (e.g., chitin; Nalin et al., 1979). Thus, it is not surprising that *V. cholerae* has been detected on many abiotic



and biotic surfaces, including ship hulls (Shikuma and Hadfield, 2010), zooplankton (Tamplin et al., 1990; Epstein, 1993; Huq et al., 2005; Turner et al., 2009), macroalgae (Hood and Winter, 1997), and as floating aggregates (Alam et al., 2006).

Vibrio cholerae attachment is mediated by pili, which are surface expressed proteins, comprised of pilin subunits that promote surface attachment and subsequent biofilm formation. The ability of *V. cholerae* to attach to a range of surfaces is reflected in the variation in pilin subunits, and hence variation in pili, expressed

by *V. cholerae* (Boyd and Waldor, 2002; Aagesen and Häse, 2012). One ecologically important substratum is chitin, and *V. cholerae*, as are most Vibrionaceae, is chitinolytic and possesses multiple conserved genes to attach to and degrade chitin (Meibom et al., 2004; Hunt et al., 2008). This organic polymer of *N*-acetylglucosamine (GlcNAc/NAG) is the second most abundant organic polymer in nature and is an excellent carbon source for bacteria (Rinaudo, 2006; Martínez et al., 2009). The binding of *V. cholerae* to chitin involves the GlcNAc binding protein, GbpA (Kirn et al., 2005;

Stauder et al., 2010), as well as the mannose sensitive hemagglutinin (MSHA), which is a type IV pilus (Chiavelli et al., 2001; **Figure 2**). Furthermore, the TCP, which is a colonization factor of human intestinal epithelia, has a dual role in association with chitin. TCP is required for differentiation of attached biofilms, and undifferentiated biofilms lacking TCP have reduced ecological fitness, as they are less efficient at degrading chitin (Reguera and Kolter, 2005).

After initial surface attachment, *V. cholerae* forms “matrix-enclosed, surface-associated communities” or biofilms (Yildiz and Visick, 2009). *V. cholerae* biofilm formation is enhanced through the actions of type IV pili, flagella and production of the biofilm matrix, Vibrio polysaccharide (VPS; Watnick and Kolter, 1999). VPS is involved in cell immobilization, microcolony formation, and biofilm maturation (Watnick and Kolter, 1999; Watnick et al., 2001). High and low VPS producing *V. cholerae* colony types are referred to as “rugose” and “smooth,” respectively, with the rugose having a higher protective effect toward a variety of stresses, including chlorine (Rice et al., 1992; Morris et al., 1996; Yildiz and Schoolnik, 1999), low pH (Zhu and Mekalanos, 2003), osmotic and oxidative stress (Wai et al., 1998), anti-bacterial serum (Morris et al., 1996), SDS (Fong et al., 2006), phage (Nesper et al., 2001), and heterotrophic protists (Sun et al., 2013). The importance of VPS for protection in the environment is still unknown as there are few published reports on the occurrence of rugose *V. cholerae* in the environment (Ali et al., 2002; Jubair et al., 2012).

The structural genes for VPS production are encoded on two carbohydrate biosynthesis operons located on the large chromosome, which encodes many essential housekeeping genes (Yildiz and Schoolnik, 1999; Fong et al., 2010). The *vpsI* operon contains the genes *vpsA* to *vpsK* and the *vpsII* operon contains the genes *vpsL* to *vpsQ*. The six genes located between the two *vps* operons (*rbmA–F*) are also involved in biofilm formation (Fong and Yildiz, 2007; Absalon et al., 2011; Berk et al., 2012). The requirement for sugars in the synthesis of VPS is an important determinant for the control of biofilm formation (discussed in Section “*V. cholerae* Responses to Environmental Stresses – Bottom-up Control of *V. cholerae*”). In addition to sugars, multiple regulators control the expression of VPS. For example VPS biosynthesis is positively regulated by VpsR (Yildiz et al., 2001) and VpsT (Casper-Lindley and Yildiz, 2004) in a c-di-GMP-dependent manner (Krsteva et al., 2010; Srivastava et al., 2011). C-di-GMP is an intracellular secondary messenger that controls the surface association of bacteria in response to environmental conditions (Yildiz, 2008).

Bacterial cell–cell communication, or quorum sensing (QS), is critical for biofilm maturation and subsequent dispersal (Liu et al., 2007; Muller et al., 2007). At high *V. cholerae* cell densities, the QS response regulator, HapR, positively regulates the transcription of *hapA* encoding the hemagglutinin protease (HAP; Jobling and Holmes, 1997; Zhu et al., 2002), *cytR*, a repressor of biofilm development, flagellum biosynthesis genes (Yildiz et al., 2004), and represses VPS production and *toxR*, the regulator of virulence (Jobling and Holmes, 1997; Zhu et al., 2002; Hammer and Bassler, 2003; Zhu and Mekalanos, 2003; Yildiz et al., 2004). It is proposed that the coordination of QS and c-di-GMP controlled traits allows for survival through

rapid adaptation to environmental conditions. For example, the switch from a free-swimming to an attached lifestyle (Yildiz and Visick, 2009; Srivastava and Waters, 2012) enables natural competency and horizontal gene transfer (HGT; Lo Scudato and Blokesch, 2012) as well as provides enhanced predation resistance (Matz et al., 2005). Mechanisms such as biofilm formation enable the persistence of *V. cholerae* and are not limited to interactions with nutritive biotic factors. Indeed, as described in the following sections, many abiotic factors including temperature, salinity, and pH influence the expression of adaptive traits.

“VIALE BUT NON-CULTURABLE” *V. cholerae* IN PLANKTON

In contrast to starved cells, VBNC cells fail to grow on culture media normally used to grow them, and are often reduced in size but remain metabolically active (Nilsson et al., 1991; McDougald et al., 1998; Oliver, 2010). Since the discovery that *V. cholerae* can enter the VBNC state (Xu et al., 1982), many bacteria, pathogens as well as non-pathogens, have been shown to enter the VBNC state under unfavorable conditions (McDougald et al., 1998, 1999; Oliver, 2005, 2010). Factors known to induce VBNC formation in *V. cholerae* include extremes in temperature and salinity as well as nutrient deprivation (Colwell et al., 1985; Ravel et al., 1995; Carroll et al., 2001; González-Escalona et al., 2006; Thomas et al., 2006; Mishra et al., 2012). VBNC cells of *V. cholerae* have been detected on the surface of higher organisms, such as crustaceans and algae in the plankton and benthos, attached to chironomid egg masses, as well as suspended in bacterioplankton (e.g., Louis et al., 2003; Binsztein et al., 2004; Alam et al., 2007; Halpern et al., 2007). Interestingly, *V. cholerae* appears predominantly as VBNC cells within the bacterioplankton and as culturable cells in biofilm consortia, either as aggregates or attached to biotic and abiotic surfaces (Alam et al., 2006). The importance of the VBNC state in cholera epidemiology was demonstrated by Mishra et al. (2012), where virulence and colonization traits were actively expressed in VBNC *V. cholerae* incubated in freshwater microcosms.

For the VBNC response to impart protection allowing for persistence during unfavorable conditions, the cells must be able to resuscitate and divide when conditions become favorable (McDougald et al., 1998). For example, *Vibrio vulnificus* enters the VBNC state and can be resuscitated when incubated in environmental diffusion chambers in the marine environment (Oliver et al., 1995). Just as there are numerous conditions that induce VBNC formation in different species, there are numerous factors that induce resuscitation, including temperature upshift (Nilsson et al., 1991; Mishra et al., 2012) or an increase in nutrients (Binsztein et al., 2004; Senoh et al., 2010).

VBNC *V. cholerae* cells have also been shown to regain culturability by passage through animal digestive tracts (Colwell et al., 1985; Alam et al., 2007; Asakura et al., 2007). Furthermore, the ingestion by human volunteers of *V. cholerae* cells that had been VBNC for as long as 7 weeks resulted in colonization of the intestine and excretion of culturable cells (Colwell et al., 1996). Thus, VBNC cells represent an important environmental reservoir of *V. cholerae* as an agent of disease. However, VBNC cells remain capable of resuscitation for a limited time, and eventually,

these cells lose the ability to resuscitate (Weichart et al., 1997). For example, VBNC cells can be resuscitated after co-incubation with eukaryotic cell lines, but resuscitation does not occur for cells that have been VBNC for a prolonged time (more than 91 days; Senoh et al., 2010).

Recently, QS has been implicated in the regulation of the VBNC state. For example, transition of culturable *V. cholerae* to the VBNC state involves biofilm formation and was shown to be dependent on QS (Kamruzzaman et al., 2010). In accordance with these results, VBNC cells from surface waters in Bangladesh have been resuscitated by natural or chemically synthesized QS autoinducers, as high colony forming unit (CFU) counts were detected after 4–5 h of exposure to two different autoinducers (Bari et al., 2013).

One hypothesis for the non-culturability of viable cells on common agar plates is that accumulation of reactive oxygen species (ROS) in the non-growing VBNC cells is detrimental when growth is initiated after nutrient addition. Thus, increased nutrient could lead to an imbalance in metabolism resulting in the production of ROS and cell death (Bloomfield et al., 1998). In fact, treatment of VBNC *Escherichia coli* with catalase or peroxide-degrading compounds can restore culturability (Mizunoe et al., 1999) and elimination of hydrogen peroxide from starved cultures of *E. coli* can prevent VBNC formation (Arana et al., 1992). Furthermore, loss of culturability of *V. vulnificus* under low temperature incubation has been correlated with loss of catalase activity, making the cells ROS sensitive (Kong et al., 2004).

It was recently hypothesized that VBNC cells resuscitate in a stochastic manner rather than in response to environmental parameters (Epstein, 2009). The authors argue that some cells of a dormant community will randomly revive from dormancy and if conditions are favorable, they will grow. Thus these revived cells can be likened to “scouts” inspecting environmental conditions (Buerger et al., 2012a,b). If conditions are not permissive for growth, the scouts will die, resulting in the loss of only a small fraction of the population. However, if conditions are favorable, then the genetic pool is amplified and maintained. The authors demonstrated that sampled marine and soil bacteria randomly became culturable during long term incubation in the wells of microtiter plates containing single cells. Furthermore, strains that were slow growing initially, with a cultivation time of 3–4 weeks could be sub-cultured within 48–72 h (Buerger et al., 2012b). In this way, the VBNC state represents a low cost population-based strategy that allows bacteria to remain dormant in the environment for extended periods, and to potentially either revive when an appropriate cue is present, e.g., an inducing signal, or to randomly test their environment to subsequently grow when conditions are favorable. Although stochastic VBNC resuscitation was not tested with *V. cholerae*, it has implications for identifying resuscitation cues and for understanding triggers of *V. cholerae* growth and cholera outbreaks.

Vibrio cholerae* RESPONSES TO ENVIRONMENTAL STRESSES – BOTTOM-UP CONTROL OF *V. cholerae

The occurrence of *Vibrio* spp. in the environment is correlated with temperature, salinity, and phyto- as well as zooplankton (Turner et al., 2009, 2013; Johnson et al., 2010; Asplund et al., 2011). High

water temperature is a strong predictor for the presence of *Vibrio* spp. (Blackwell and Oliver, 2008; Lama et al., 2011; Johnson et al., 2012), as they are mainly detected in warmer waters (above 15°C). Many studies have demonstrated that the abundance of *Vibrio* spp. follows a seasonal pattern that is dictated to a large extent by temperature (e.g., Louis et al., 2003; Binsztein et al., 2004). Increased temperature can influence the attachment of *V. cholerae* to chitinous zooplankton. At temperatures above 15°C, attachment to chitin increases significantly due to an increase in the expression of the MSHA pilus and the colonization factor, GbpA (Castro-Rosas and Escartín, 2005; Turner et al., 2009; Stauder et al., 2010). In contrast, despite the water temperatures in the Chesapeake Bay being above 19°C, *V. cholerae* was found more often in the water column, as planktonic cells, than attached to plankton (Louis et al., 2003). Thus, in addition to elevated temperature, other factors must influence biofilm formation or dispersal, demonstrating the importance of environmental surveying, collecting, and interpreting metadata to determine those factors that influence cholera epidemics.

Temperature fluctuations due to seasonal changes, as well as freshwater influx can strongly influence the salinity of marine water bodies. Open ocean waters have an average salinity of 35 ppt. However, near coastal and estuarine areas the salinity can drop due to freshwater input from rivers or rain run-off (Jutla et al., 2011), and can increase in areas with higher solar evaporation, especially in the tropics. *Vibrio* spp. grow preferably at salinities <25 ppt (e.g., Jiang, 2001; Thomas et al., 2006; Baker-Austin et al., 2010). In high salinity environments *V. cholerae* increases the production of the protective pigment, melanin (Coyne and al-Harthi, 1992), which provides UV resistance (Valeru et al., 2009). The relationship between *V. cholerae* occurrence and salinity appears to be variable, with some studies reporting a significant correlation (Singleton et al., 1982; Johnson et al., 2010), while others demonstrate a lack of correlation between the occurrence of the organism and salinity (Johnson et al., 2012). For example, Stauder et al. (2010) showed that different salinities had no effect on attachment to surfaces, which is important for environmental persistence (as discussed in Section “Association with Other Organisms”).

Seasonal fluctuations are often correlated with changing nutrient concentrations, as rain run-off is generally higher in spring/autumn and in coastal and estuarine areas. This can lead to higher phytoplankton abundance, followed by zooplankton blooms (e.g., Lobitz et al., 2000; Huq et al., 2005), which provide the chitinous surfaces that harbor bacteria such as *V. cholerae*. This may enable overall numbers of the organism to increase in the environment even though bacterivorous predators are also more abundant.

Nutrient sources in the environment are not uniformly distributed but occur as microscale patches, influenced by localized events such as cell lysis and waste excretion (Blackburn et al., 1998). Planktonic bacteria use motility and chemotaxis to take advantage of such nutrient patches (for a review of see, Stocker and Seymour, 2012). *V. cholerae* possesses a single sheathed polar flagella (Hranitzky et al., 1980) powered by sodium motive force (Kojima et al., 1999). The number of duplicated chemotaxis-related genes possessed by *V. cholerae* indicates the importance of this response for environmental survival (Heidelberg et al., 2000). *V. cholerae*

have multiple chemotaxis genes, however not all are required for chemotaxis under standard laboratory conditions, suggesting that the other genes act as accessory chemotactic genes or have as yet undiscovered functions in the environment (Gosink et al., 2002). *V. cholerae* has been shown to be chemotactic toward all amino acids (Freter and O'Brien, 1981), suggesting that proteins, peptides, or amino acids may be important nutrient sources in the aquatic environment. In addition, *V. cholerae* upregulates chemotaxis genes in response to chitin oligosaccharides, facilitating detection and attachment to chitinous organisms (Meibom et al., 2004).

The ability of *V. cholerae* to persist in the environment is intrinsically linked to biofilm formation and VPS synthesis, both of which allow for the exploitation of nutrients available at the surface. Concentrations of sugars, phosphorus, and nitrogen influence attachment and biofilm formation *V. cholerae* cells. The presence of glucose and mannose induce VPS synthesis during biofilm development (Kierek and Watnick, 2003; Moorthy and Watnick, 2004). The phosphoenolpyruvate phosphotransferase system (PTS) is one of the major sugar transport systems in *V. cholerae* and activation of PTS results in derepression of VPS gene transcription and thus increased biofilm formation (Houot and Watnick, 2008; Houot et al., 2010). In addition, a *V. cholerae* PTS that responds to intracellular nitrogen concentrations, is believed to repress VPS production, however the receptor molecule and signaling pathway are still unknown (Houot et al., 2010).

Phosphorous also affects surface colonization. In phosphorus depleted environments, *V. cholerae* adopts a free-swimming planktonic lifestyle that is mediated by a two-component system, PhoBR. The histidine kinase, PhoR, phosphorylates the response regulator, PhoB, resulting in further repression of VPS production (Pratt et al., 2009; Sultan et al., 2010).

Planktonic *V. cholerae* cells have been shown to settle in response to extracellular DNA (eDNA), which is a component of the pre-established biofilm matrix (Haugo and Watnick, 2002). This occurs by repression of CytR, which in turn represses VPS and biofilm formation (Haugo and Watnick, 2002). Since *V. cholerae* is rich in DNases (Focareta and Manning, 1991), the eDNA maybe utilized as a nutrient source (Seper et al., 2011).

Since nutrient availability fluctuates in the aquatic environment, the ability to store essential nutrients is an important trait for bacteria that live a "feast-to-famine lifestyle." In bacteria, glycogen is stored in granules and can serve as a carbon source during periods of starvation (Preiss and Romeo, 1994). Under nutrient rich conditions *V. cholerae* increases glycogen storage precursors (Kan et al., 2004). In addition, glycogen granules are present in nutrient poor rice water stools shed by patients with cholera (Bourassa and Camilli, 2009), indicating that glycogen storage may provide nutrients to *V. cholerae* as it passages from the human host into the aquatic environment. In addition to glycogen storage, the ability to store inorganic phosphorus (Pi) facilitates protection against environmental stresses such as acidity, salinity fluctuations, and the damaging effects of hydrogen peroxide, as it is required for activity of the general stress response regulator, RpoS (Jahid et al., 2006). *V. cholerae* is also able to store Pi within membrane bound granules at 100 times the concentrations achieved by *E. coli* (Ogawa et al., 2000). *V. cholerae* mutants deficient in Pi storage displayed

reduced attachment to abiotic surfaces, decreased motility and a delayed adaptation to high calcium media (200 mM) (Ogawa et al., 2000).

In addition to carbon and phosphorous, iron is also a growth limiting factor required for cellular metabolism as it is a component of many cofactors (Wackett et al., 1989) and has low solubility in aquatic environments (Martin, 1992). Iron concentrations in the aquatic environment are highly variable and are generally correlated with water depth (Martin and Michael Gordon, 1988). *V. cholerae* has evolved several iron transport systems and receptors that enable persistence in low iron environments (Heidelberg et al., 2000; Wyckoff et al., 2006, 2007). These iron acquisition systems include a catechol siderophore, vibriobactin (Griffiths et al., 1984), and a transport system, Feo, that can take up ferrous iron (Wyckoff et al., 2006). Most iron acquisition genes, such as *irgA* (Goldberg et al., 1991), are repressed by the ferric uptake regulator (Fur) in environments with sufficient iron, as Fe(II)-Fur binds to the promoter of iron-regulated genes, preventing their expression (Bagg and Neilands, 1987). *V. cholerae* can also make use of siderophores secreted by other microorganisms, such as fluvibactin synthesized by *Vibrio fluvialis*, as it possesses the required receptors and uptake systems (Yamamoto et al., 1993).

In nutrient limited environments, *V. cholerae* can enter a starvation state, in which the cells are non-growing but culturable. In a recent laboratory study, Jubair et al. (2012) described the long-term starvation survival of *V. cholerae* (700 days). The authors suggest the term "persister phenotype" to differentiate starved cells from the VBNC state. The growth of persister cells was enhanced in the presence of phosphate and chitin, both important nutrients, which further highlights their importance for *V. cholerae* survival. An earlier study on the behavior of *V. cholerae* starved for 40 days showed that chitin attachment ligands were maintained (Pruzzo et al., 2003). These findings demonstrate the importance of association with chitinous organisms with details of specific interactions discussed in the Section "Association with Other Organisms."

TOP-DOWN CONTROL BY PREDATORY MICROGRAZERS

While availability of nutrients exerts "bottom-up" control of *V. cholerae*, predation by heterotrophic protists is one of the major mortality factors faced by bacteria in the environment (Hahn and Höfle, 2001; Matz and Kjelleberg, 2005). As part of the bacterioplankton, *V. cholerae* is under constant predation pressure by phagotrophic protists and other bacterivorous members of the zooplankton community. The long-term persistence and seasonal accumulation of *V. cholerae* is dependent on how it responds to this stress. Microcosm studies of natural bacterioplankton communities from the Gulf of Mexico showed that ciliates and heterotrophic nanoflagellates (HNFs) efficiently eliminate *V. cholerae* from environmental water samples (Martínez Pérez et al., 2004). In addition, ciliates as well as flagellates can feed on *V. cholerae*, with grazing rates of up to 600–2,000 bacteria cell⁻¹ h⁻¹ (Macek et al., 1997). Control of *V. cholerae* numbers by heterotrophic protists was also demonstrated by Worden et al. (2006), where *V. cholerae* growth rates of up to 2.5 doublings per day were countered by heavy grazing mortality by HNFs. During intense phytoplankton blooms, these growth rates increased to more than four doublings per day, allowing

V. cholerae to overcome grazing pressure, potentially attaining sufficient numbers in the environment to reach an infectious dose.

Vibrio cholerae cells encased in a biofilm matrix are protected from predation by HNFs, while planktonic cells are rapidly eliminated (Matz et al., 2005). Predation induces biofilm formation and a smooth to rugose morphological shift, due to an increase in VPS production (Matz et al., 2005). VPS has subsequently been confirmed to be partly responsible for biofilm grazing resistance, where the *V. cholerae* cells encased in VPS were protected from predators (Sun et al., 2013). In addition to physical protection provided by biofilms, the high cell density in *V. cholerae* biofilms provides a sufficient quorum to promote expression of several QS-regulated anti-protozoal factors that cannot accumulate in the planktonic phase.

The importance of QS for protection from protozoal predation is supported by field tests demonstrating that QS-deficient *V. cholerae* was more susceptible to grazing than the wild type. However, the QS mutant strain did not lose all grazing resistance, suggesting that *V. cholerae* regulates anti-protozoal activities by a combination of QS and other regulatory systems (Erken et al., 2011). VPS mutants were less resistant than the wild type strain to surface grazing by the amoeba, *Acanthamoeba castellanii* and the HNF, *Rhynchomonas nasuta*, but were more resistant than the *hapR* mutant strain, indicating that QS is more protective than VPS against predators (Sun et al., 2013). QS has been shown to regulate secreted compounds that provide resistance from functionally different predators such as *Tetrahymena pyriformis*, *Cafeteria roenbergensis*, and *Caenorhabditis elegans*, e.g., an uncharacterized anti-protozoal factor (Matz et al., 2005) and the PrtV protease (Vaitkevicius et al., 2006).

The type VI secretion system (T6SS) also functions as an anti-predation mechanism that is inhibitory against *Dictyostelium discoideum*, mammalian macrophages, and *E. coli* (Pukatzki et al., 2006; MacIntyre et al., 2010). Three proteins (VgrG-1, -2, and -3) secreted by the T6SS form syringe-like structures, puncturing the cell membrane and delivering a virulence factor, VasX, into *D. discoideum* (Pukatzki et al., 2007; Miyata et al., 2011). The expression of another major component of T6SS, Hcp, is positively regulated by QS in *V. cholerae* (Ishikawa et al., 2009). Although all *V. cholerae* strains have this system, expression differs between them (Unterweger et al., 2012). For example, pandemic El Tor strains do not express T6SS under laboratory conditions while in some non-O1/non-O139 strains T6SS is constitutively expressed (Miyata et al., 2010).

Another mechanism for surviving protozoan predation is the ability of the bacterium to survive digestion. Both clinical and environmental strains of *V. cholerae* can survive intracellularly in a range of amoeba (Abd et al., 2004, 2005; Jain et al., 2006). Several studies have demonstrated that *V. cholerae* growth is enhanced when associated with free-living amoeba (Thom et al., 1992; Sandström et al., 2010; Valeru et al., 2012), further demonstrating the role amoeba play as environmental reservoirs of *V. cholerae*. In addition to surviving within amoebic trophozoites, *V. cholerae* cells have been found in the stress resistant cysts formed by amoeba, providing protection from environmental stresses (Thom et al., 1992; Abd et al., 2004), as well as a vehicle for dispersal throughout

the aquatic environment (Thom et al., 1992; Brown and Barker, 1999). Thus, amoeba cysts could potentially facilitate the spread of cholera (Winięcka-Krusnell and Linder, 2001). Although many reports have characterized the relationship between *V. cholerae* and amoeba (Thom et al., 1992; Abd et al., 2005, 2007; Sandström et al., 2010), very little is known about the mechanisms that facilitate intracellular survival, although survival of the acidic conditions encountered within the digestive vacuoles has been attributed to an inducible acid tolerance response (Merrell and Camilli, 1999). In addition, ToxR has been shown to be important for survival in amoeba (Valeru et al., 2012). The authors suggest that it may be the ToxR-regulated outer membrane proteins, OmpU and OmpT that are responsible for enhanced survival. Experimentally, attraction and attachment to a protozoan host cell has yet to be shown (Abd et al., 2009, 2011). However, adhesins such as MSHA or capsule/LPS O side chain are not involved (Lock et al., 1987; Abd et al., 2009).

There is a lack of knowledge regarding the type and function of other virulence factors in facilitating intracellular survival in protozoa, especially when compared with other bacteria such as *Legionella* spp. and *Salmonella* spp. (Rowbotham, 1980; Bozue and Johnson, 1996; Brandl et al., 2005). There are many important questions that need to be addressed regarding *V. cholerae*–protozoa interactions, including how prevalent these interactions are in the environment and whether they facilitate resuscitation from the VBNC state. In addition, it is important to explore the prevalence of survival and passage through predatory protists and whether the bacterium remains viable in fecal pellets. A further understanding of the roles higher organisms play in the enhancement of *V. cholerae* fitness traits is required to understand the persistence and spread of the pathogen in the environment.

In addition to aforementioned predation pressure by phagotrophic protists, phage, and predatory bacteria also affect the abundance and serogroup prevalence of *V. cholerae* in the environment. For example, the CTX ϕ phage carries the genes encoding CT and is required for conversion of non-toxigenic to toxigenic strains (Miller and Mekalanos, 1988; Pearson et al., 1993; Waldor and Mekalanos, 1996). Phage predation has shaped cholera epidemics in Bangladesh, where high concentrations of phage are detected after an initial peak in cholera cases and numbers of *V. cholerae* cells in the aquatic environment (Faruque et al., 2005). Following the increase in phage numbers, the number of cholera cases decreases. An increase in phage numbers in the environment was also correlated with an increase in *V. cholerae* lytic bacteriophage in patient stool samples, with one of the predominant bacteriophage species belonging to the *Myoviridae* family (Seed et al., 2011). Environmental surveys have detected *Myoviridae* in regions where cholera outbreaks have occurred, such as Peru (Talledo et al., 2003), Kolkata (Sen and Ghosh, 2005), and Kenya (Maina et al., 2013). Control of *V. cholerae* by phage is supported by a continuous culture experiment, which suggests that *V. cholerae* populations may be influenced by phage to a larger extent than by nutrient limitation (Wei et al., 2011). Predatory bacteria such as *Bdellovibrio* sp. also prey on *V. cholerae* (Chen et al., 2012) and might also shape *V. cholerae* occurrence in the environment. However, there is limited information on the interactions between predatory bacteria and *V. cholerae*.

ASSOCIATION WITH OTHER ORGANISMS

Vibrio cholerae is an integral part of the aquatic environment and in addition to heterotrophic protists, interacts with a wide range of organisms. It has been demonstrated to interact with water fowl (Halpern et al., 2008), fish (Kiiyukia et al., 1992; Senderovich et al., 2010), chironomids (Broza and Halpern, 2001; Halpern et al., 2006), mussels (Deriu et al., 2002; Collin et al., 2012), cyanobacteria (Islam et al., 1999), diatoms (Binsztein et al., 2004; Seeligmann et al., 2008), and dinoflagellates (Binsztein et al., 2004; Akselman et al., 2010; **Figure 3**). The association of *V. cholerae* with zooplankton has been a topic of study since the discovery of cells attached to the surface of copepods in the early 1980s (Huq et al., 1983; Tamplin et al., 1990). Zooplankton are an important part of the aquatic food web, grazing on autotrophic and heterotrophic bacterio-, nano-, and micro-plankton and are in turn preyed upon by larger plankton, such as insect and crustacean larvae and fish. One well-studied interaction is that between *V. cholerae* and chitinous zooplankton, e.g., copepods and cladocerans (Nalin et al., 1979; Huq et al., 1983; Rawlings et al., 2007). For example, pivotal experiments link the transmission of cholera with zooplankton (Huq et al., 1996, 2005; Colwell et al., 2003). In a now classic experiment, the filtration of water through readily available sari cloth reduced *V. cholerae* numbers by 99% (Huq et al., 1996). This method proved to be effective in field trials in reducing the incidence of cholera cases and was continued by villagers as a treatment for drinking water (Colwell et al., 2003; Huq et al., 2010). de Magny et al. (2011) suggested the use of different zooplankton to predict cholera epidemics as they demonstrated that the cladocerans, *Monia* spp. and

Diphanosoma spp. as well as the rotifer *Brachionus angularis*, were significantly correlated with the presence of *V. cholerae* and with cholera outbreaks. *V. cholerae* has repeatedly been found to be associated with the copepod *Acartia tonsa*, which appears to harbor higher numbers of *V. cholerae* than co-occurring copepods (e.g., Huq et al., 1983; Binsztein et al., 2004; Rawlings et al., 2007; Lizárraga-Partida et al., 2009, for further information, see Pruzzo et al., 2008).

The predominantly attached lifestyle of *V. cholerae* enables it to use many different biotic surfaces as nutrient sources. In addition to degrading chitin, *V. cholerae* has the ability to degrade the egg masses of chironomids (Broza and Halpern, 2001; Halpern et al., 2004). The production of the QS-regulated HAP is necessary for the degradation of the gelatinous matrix of the egg masses (Halpern et al., 2003). Although high numbers of *V. cholerae* were found attached to the egg masses (3.9×10^4 per egg mass; Halpern et al., 2007) 99.5% of the attached cells were other species, e.g., *Acinetobacter*, *Aeromonas*, *Klebsiella*, *Shewanella*, and *Pseudomonas*. These species may benefit from the nutrients that are released by *V. cholerae* as it degrades the egg mass. Alternatively, these species may have a negative impact on *V. cholerae* by expressing bacteriocins or competing for nutrients and space, which may in part explain why the majority of *V. cholerae* on the egg masses, 99.7%, were VBNC (Halpern, 2011). *V. cholerae* has been shown to be associated with chironomids in all four stages of development, from egg to adult (Broza and Halpern, 2001; Halpern et al., 2003; Broza et al., 2005), suggesting these insect eggs and larvae can serve as vectors for the transmission of cholera. Indeed, chironomids that were collected in air 3 km away from a water source

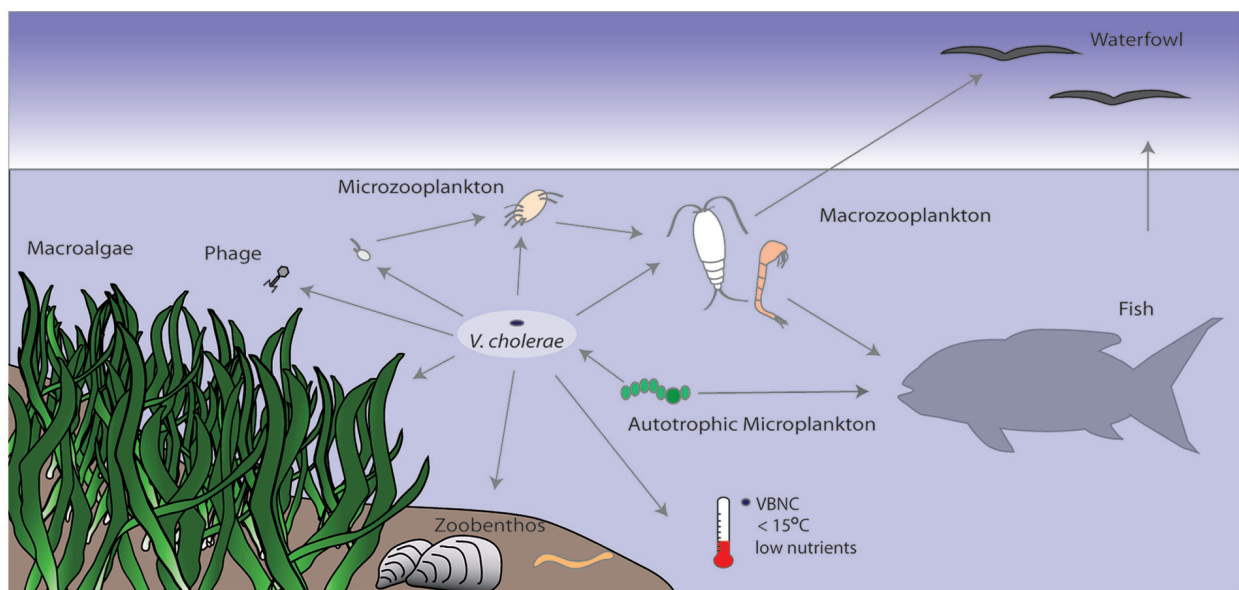


FIGURE 3 | *Vibrio cholerae* interactions with other organisms and the environment. *V. cholerae* is part of the bacterioplankton in aquatic environments. It is under predation pressure by protozoa and bacteriophage and is thus incorporated into the microbial loop. Low temperature and nutrient conditions can trigger the VBNC state, from which it resuscitates under more favorable conditions. *V. cholerae* can

also attach to autotrophic organisms such as phytoplankton or macroalgae, which can provide a carbon source. Attachment to chitinous zooplankton and gelatinous egg masses (e.g., chironomids) provide nutrients and also facilitate HGT. Fish and birds feed on plankton or mussels that might harbor *V. cholerae* and can potentially spread the bacterium across long distances.

were found to harbor *V. cholerae* and thus, these midges can carry the pathogen from one body of water to another (Broza et al., 2005). Although no toxigenic serogroups of *V. cholerae* have been detected in association with chironomids to date, it remains possible that these could also be associated with chironomids (Halpern, 2011).

The association between *V. cholerae* and phytoplankton has been well studied (e.g., Tamplin et al., 1990; Lobitz et al., 2000; Turner et al., 2009). Autotrophic protists, such as diatoms and dinoflagellates (Binsztein et al., 2004; Eiler et al., 2006), cyanobacteria (Islam et al., 1999; Eiler et al., 2007) as well as macroalgae (Vugia et al., 1997; Haley et al., 2012) support *V. cholerae* growth (e.g., Vezzulli et al., 2010). Various laboratory and environmental studies have shown that *V. cholerae* cells attach to microalgae cells. In a study off the coast of Argentina, Seeligmann et al. (2008) detected 1–10 VBNC *V. cholerae* cells attached to a single algal cell. It was suggested that attachment to phytoplankton might enable *V. cholerae* to survive prolonged exposure in freshwater environments due to the nutrients and salts excreted by the phytoplankton cells (Islam et al., 1989; Tamplin et al., 1990; Binsztein et al., 2004). Nutrients supplied by phytoplankton, e.g., due to a massive bloom, can also support explosive growth of *V. cholerae* (Mouriño-Pérez et al., 2003). In fact, remote sensing of chlorophyll *a* has been proposed as a method to predict cholera outbreaks (Lobitz et al., 2000).

Attachment of *V. cholerae* to macroalgae is induced by the plant-derived polyamine, norspermidine (Hamana and Matsuzaki, 1982). Norspermidine is bound by NspS, a periplasmic spermidine-binding protein that interacts with the periplasmic portion of the membrane protein, MbaA, resulting in induction of biofilm formation (Karatan et al., 2005). Mannitol, which is a product of photosynthesis by brown algae and secreted at high concentrations (Yamguchi et al., 1969; Ymele-Leki et al., 2013), induces *V. cholerae* settlement and VPS-dependent biofilm formation, resulting in transcription of *mtlA*, encoding a mannitol specific transport protein (Ymele-Leki et al., 2013). Once mannitol is transported into the *V. cholerae* cell it is possibly used as a carbon source or an osmoprotectant (Ymele-Leki et al., 2013).

Vibrio cholerae has also been detected in the gut of various species of fish that these feed on phyto- as well as zooplankton (Senderovich et al., 2010). *V. cholerae* numbers as high as 5×10^3 cells per gram of intestine content were detected in fish sampled from different marine and freshwater environments in Israel (Senderovich et al., 2010). Non-O1 *V. cholerae* has also been detected in the kidneys, livers, and spleens of diseased, or homogenates of healthy ayu fish sampled from different rivers in Japan (Kiiyukia et al., 1992). Although no isolate carried CT genes, the supernatant of the cultures produced fluid accumulation in suckling mice. In addition to fish, waterfowl have been connected to the spread of *V. cholerae* (Ogg et al., 1989). Sea birds feed on zooplankton as well as phytoplankton and come in contact with these organisms by swimming on the water. Here, these planktonic organisms can then attach to bird feathers and thus *V. cholerae* can potentially be spread by air (Halpern et al., 2008).

Most research on environmental *V. cholerae* has focused on the occurrence of the bacterium within the planktonic community or on the interactions of *V. cholerae* with planktonic organisms.

However, recent research has shown that benthic communities also harbor high numbers of Vibrios, including *V. cholerae* (e.g., Covazzi Harriague et al., 2008; Vezzulli et al., 2009; Collin and Rehnstam-Holm, 2011). For example, bivalves are benthic filter feeders connecting the plankton and the benthos by feeding on the plankton. Bivalves such as mussels and oysters can harbor high numbers of pathogenic *Vibrio* spp. in their tissue and are an important niche for these bacteria (e.g., Olafsen et al., 1993; Maugeri et al., 2001; Kirs et al., 2011). Food poisoning resulting from the ingestion of contaminated raw or undercooked seafood is a major threat to human health. While infection by *V. vulnificus* and *Vibrio parahaemolyticus* from ingestion of seafood are the most common (Wright and Harwood, 2013), mussels can also harbor high numbers of *V. cholerae* and thus are a potential health threat (e.g., Murphree and Tamplin, 1995; Bauer et al., 2006; Haley et al., 2012).

The bivalve immune system consists of hemocytes (phagocytic cells) and the hemolymph (i.e., lysosomal enzymes and antimicrobial peptides; Mitta et al., 2000; Pruzzo et al., 2005). In order to reside in bivalve tissues, bacteria need to survive the antimicrobial activity of the hemolymph and engulfment by the hemocytes. Vibrios are resistant to depuration treatments (Murphree and Tamplin, 1995) and show resistance toward the hemocytes of the blue mussel *Mytilus edulis* (Hernroth et al., 2010). Some Vibrios were able to inhibit filtration in adult *M. edulis*, which was not correlated with the binding of the bacteria to the gills of the mussels (Birkbeck et al., 1987), suggesting another mechanism is involved. Interestingly *V. cholerae* strains of different origin have different retention times in mussels (Collin et al., 2012). An environmental *V. cholerae* strain isolated from the blue mussel was both taken up and eliminated much faster than a clinical non-O1/O139 strain. The clinical strain had a much longer retention time, implying that pathogenic strains have better fitness in the mussel than environmental strains. This has implications for control measures such as depuration, as they will be less effective at removing clinical strains than environmental strains. In addition, Collin et al. (2012) identified a highly virulent El Tor strain that was not ingested at all, indicating that bivalves did not eliminate this pathogenic strain from the water column. These results highlight the importance of interaction of *V. cholerae* with other organisms in its environment and the evolution and selection for virulent strains.

In addition to being incorporated into the benthos by filter feeders, *V. cholerae* can be isolated from sediments in numbers that are much higher than in the planktonic phase (Covazzi Harriague et al., 2008; Vezzulli et al., 2009). Sediments may therefore also act as a reservoir for cholera, especially in colder months, seeding the water column when temperatures rise (Vezzulli et al., 2009). Interestingly, in this study nematodes accounted for the highest abundance of the meiofauna, and bacterivorous nematodes accounted for 50% of the total. This suggests that *Vibrio* spp. are under high grazing pressure and top-down control by these nematodes (Vezzulli et al., 2009). In a laboratory study with *C. elegans*, Vaitkevicius et al. (2006) showed that *V. cholerae* kills the nematode after ingestion by secreting the extracellular protease PrtV. Neither CT nor TCP were required for the killing. Interestingly, PrtV was also required to prevent grazing by the flagellate *C. roenbergensis* and the ciliate *T. pyriformis*. In a $\Delta hapR$ strain, the ability to

kill the nematode was strongly diminished. This is in accordance with the role of the QS response regulator, *hapR*, which is important for grazing resistance in the laboratory (Matz et al., 2005) as well as in the environment (Erken et al., 2011). Thus, *V. cholerae* has evolved or acquired a number of genetic systems that facilitate its ability to resist top-down control exerted by predatory eukaryotes.

CONCLUSIONS

Vibrio cholerae is a significant pathogen that has played an important role in human history. Its role in the spread of disease and in epidemics has been reported for more than 150 years and the organism has even played an important role in establishment of modern epidemiology. While the mechanisms leading to infection and epidemics have been well studied, the ecology and mechanisms that underpin environmental persistence have been less well documented. Interestingly, environmental *V. cholerae* strains are largely represented by non-toxigenic strains and indeed, environmental strains display a high degree of genetic variability which has been suggested to aid in *V. cholerae* environmental stress resistance and subsequent persistence. The bacterium has an array of genetic systems involved in stress resistance, when faced with nutrient starvation, iron limitation, or changes in salinity and temperature. One such adaptation is the ability to grow as a biofilm on a range of abiotic and biotic surfaces. This not only increases resistance to stress, but may also directly provide access to nutrients, such as when attached to chitinous surfaces. Biofilm formation has also been directly linked to avoidance of predation by microeukaryotes. Predation resistance can be provided either by physical protection offered by the biofilm, the production of anti-predator compounds or defensive molecules or both. Perhaps not surprisingly, some of the gene systems involved in anti-predator defenses are the same as those associated with virulence during human infection. This may support the co-incidental virulence hypothesis that suggests that virulence factors evolve, at least in part, from the competition between predator and prey rather than against a human host. *V. cholerae* is a common inhabitant of many marine and freshwater habitats and this is most likely because it has evolved a range of strategies to enable its persistence in the natural environment. The identification and elucidation of these mechanisms, from ecological, evolutionary and molecular perspectives are likely to deliver exciting discoveries for the next 150 years.

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