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Joseph W. Francis
The Master's College

Todd C. Wood
Core Academy of Science

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A CREATIONIST PERSPECTIVE ON THE ORIGIN OF PATHOGENIC *VIBRIO CHOLERAE* AND *VIBRIO CHOLERAE* TOXIN (CT)

Joseph W. Francis, The Master's College, 21726 Placerita Canyon Rd, Santa Clarita Ca, 91321
Todd Charles Wood, Core Academy of Science, Dayton, TN 37321

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ABSTRACT

Many microbial pathogens and toxins exhibit elaborate mechanisms of engagement with mammalian cell structure and cell biochemistry. For instance, some toxins gain entrance into cells using exquisite secretory devices and, once inside cells, interact in very specific ways with intracellular membrane trafficking factors and cell signaling components. Because many microbial pathogens and their associated toxins appear to be created to interact with human cells, they are difficult to explain within the context of a "good creation." In this paper, we examine the pathogenic role of *V. cholerae*, the cholera toxin (CT) and other associated virulence factors, and their origin in the context of the creation model. We examine the literature and use methods of genomic comparison to investigate the origin of CT and the corresponding origin of cholera. Our results are consistent with a model of nonpathogenic function for CT prior and even after the Fall. We suggest that the originally beneficial function of CT has been subsequently modified by the presence of phages and mobile genetic elements.

INTRODUCTION

Vibrio cholerae is a pathogen which interacts very specifically with human tissue. Several pathogenic strains were responsible for seven devastating cholera pandemics in the last few centuries (Colwell, 2006). Cholera spreads rapidly through human populations primarily by unsanitary living conditions in densely populated areas (Griffeth et al., 2006), and kills human hosts by causing a rapid and devastating dehydration, which is essentially a physiological response to the toxin (Sack *et al.*, 2006). *V. cholerae* possess myriad virulence factors, including cholera toxin (CT), the primary toxin responsible for massive water and electrolyte loss from the human body (Francis and Wood, 2008, 2009; Bharati, 2011).

The virulence factors of cholera have been shown to participate in both pathogenesis and infection. However, it has been revealed upon closer inspection that many of the virulence factors participate in an environmental role of *V. cholerae* in the aquatic environment. The fact that *V. cholerae* may play an environmental role in nature is predicted by the creation model (Francis, 2008). In fact, many vibrios specialize in binding to aquatic organisms, specifically to exoskeletal chitin (one of the most abundant complex carbohydrates on earth) (Table 1). Perhaps they participate in the breakdown and recycling of elements from this rich source of nitrogen and

carbon, suggesting that the original purpose of *V. cholerae* was to promote nutrient cycling. However, a role for CT in this process or any other non-pathogenic environmental processes has yet to be determined (Francis and Wood, 2009). Furthermore, the specific function of CT appears to be a "better fit" with regard to the human intestinal environment than the chitinous surface of aquatic organisms. Because the toxins produced by *V. cholerae* appear to be created to interact with human cells, they are difficult to explain within the context of a "good creation." [Gen 1] (Purdom, 2009; Francis, 2003).

Table 1: Vibrio bacteria associate with many aquatic creatures

Flatfish	Bleached coral
Jackmackerel	Oysters
Salmonids	Polycheates
Sea bream	Sea urchina
Shrimps	Squid
Oysters	Scallopa
Sharks	Prawns
Abalones	Turban shella
Rotifers	Isopods
Blue crabs	

CT is a binary toxin, where one part of the toxin possesses ADP-ribosylation activity (Bharati, 2011; Patton *et al.*, 2000; Masignani *et al.*, 2000), and the other is involved in transport of the toxin to cells. Curiously, other pathogenic bacteria use ADP-ribosylation to cause toxicity and pathogenesis in the human body. For instance, diphtheria toxin poisons human cells by causing ADP-ribosylation of a uniquely modified amino acid, dipthamide, primarily found on a crucial translation factor (Liu *et al*., 2004). This supports the concept that some microbial toxins appear to be designed to interact with the human body. In light of these host-microbe interactions, how do we incorporate these observations of specific toxicity into the creation model? Were microbial toxins designed to interact with the human body? If so, what was their original function? Theoretical creation microbiology suggests that CT has two possible origins; as a factor that was modified from its original good function or one that has been displaced from its beneficial context of its original operation (Francis and Wood, 2009; Francis, 2008, 2009).

An overview of *Vibrio* genera

V. cholerae is a flagellated, gram negative, curved rod and a member of the Vibrionaceae family. Of the 81 species of vibrios (Thompson *et al.*, 2007), only 12 are known to cause disease and only a few *V. cholerae* strains cause cholera (Thompson and Swings, 2006; Nishibuchi, 2006).

All vibrio species are known to be aquatic and require moderate to low salt concentrations for growth in culture. Vibrios are found in deep sea, surface, brackish, fresh and estuarine waterways. Vibrio can dominate other bacterial species in some waterways, especially in the warm summer months (Thompson and Polz, 2006). Up to 35% and 40% of the bacteria in the ocean waters off of the southeastern U.S and Hong Kong, respectively, are vibrios during warm months (Thompson and Polz, 2006; Colwell, 2006). In warm waters, vibrio associate strongly with zooplankton and are known to completely cover the outer and inner chitinous surfaces of

small planktonic creatures such as copepods (Faruque and Nair, 2006). This fact has led to several methods for preventing cholera transmission including the simple method of filtering drinking water with cloth which traps the larger vibrio-rich planktonic organisms.

Several vibrios possess specific surface receptors for binding to chitin and many vibrio species possess specific chitinase enzymes (Meibom *et al.*, 2004). It appears that a primary role of vibrios may be to promote the recycling of biological carbon and nitrogen from chitin in aquatic systems. Consistent with this role is the observation that vibrios can cause the release of carbon from polycyclic aromatic hydrocarbons (PAH) as part of a mechanism to metabolize petroleum (Urakawa and Rivera, 2006). Vibrios occur frequently in the digestive tract and surfaces of marine animals (Table 1). In some cases, vibrios are symbiotic partners in mutualistic relationships, which include relationships with squid, sea urchins and abalones (Fabiano *et al*., 2004).

Mutualistic and parasitic associations of vibrio

Pathogenic vibrios may have originated from vibrios that participate in symbiosis and, thus, understanding their mutualistic lifestyles may help inform how pathogenesis arose. Several vibrio species are involved in providing bioluminescence for fish or squid hosts to assist in obtaining prey or for defensive purposes (Thompson *et al*., 2004). Perhaps the most well studied mutualism is the association of *Vibrio fischeri* with the Hawaiian bobtail squid. In this relationship, *V. fischeri* populates a specific, ventrally-located light organ in the squid. The downward directed light from the ventral surface of the squid provides counter-illumination; a well-known defense mechanism used by several aquatic species, whereby the provision of light prevents the creature from projecting a shadow which can be detected by predators who swim below the bobtail squid (Francis, 2012). *V. fischeri* utilize a host of virulence-like factors to promote the relationship between bacteria and squid, and also appear to possess some genes associated with the pathogenesis-causing mobile genetic elements found in pathogenic *V. cholerae* (Francis, 2006; Nyholm and McFall-Ngai, 2004).

Symbiotic vibrios have also been detected living with sea urchins and have been shown to fix nitrogen and to supply this nitrogen directly to their invertebrate hosts (Urakawa and Rivera, 2006). Vibrios have also been detected in symbiosis with abalones and help to break down alginate, a seaweed polysaccharide, in the abalone gut. The partnership also appears to be important in the early lifecycle of the juvenile abalones (Sawabe *et al.*, 2007). Vibrios also participate in light-producing symbiotic relationships with several species of fish, including the ponyfish and pinecone fish (Fitzgerald, 1977; Dunlap, 1985).

Virulence factors of *V. cholerae* and the molecular mechanisms of cholera disease

Before we examine possible theories for the origin of pathogenic *V. cholerae* and its virulence factors within a creation context, we first describe its pathogenic lifestyle and virulence factors with a focus on CT, the main causative agent of cholera.

The primary toxin of the cholera syndrome is CT, which promotes the life threatening water loss through poisoning of the cells of the intestinal lumen and subsequent induction of a severe

pseudomembranous colitis. *V. cholerae* possess many virulence factors, all of which may or may not participate in the promotion of diarrhea (Table 2). The large amount of fluid extruded from the human body becomes dilute in particulates and is, therefore, characterized as a watery grey "rice stool." This results in severe dehydration and causes death within hours after onset of water loss. Rehydration is the main cure for cholera along with, but not necessarily including, the use of antibiotics.

Table 2: Virulence factors of *V. cholerae*.

Zot (zona occludens toxin)
ACE (accessory cholera toxin)
TCP (toxin coregulated pilus)
Polar flagellum
LPS (Iipopolysaccharide)
PG (peptidoglycan) Capsule
RTX (actin cross-linking toxin)
Hly (hemolysin)
Colonization binding protein
Mannose sensitive hemagglutinin
ToxR
AphA and AphB (transcription regulators)
GbpA (GlcNAc-binding protein A)

Curiously, CT is only found in a few strains of *V. cholerae* and it resides on a mobile genetic element (Waldor and Mekalanos, 1996; Davis *et al*., 2000). At first glance, it might appear that CT is an acquired toxin and was perhaps not originally intended to be part of *V. cholerae* nor its life cycle. However, a closer examination of the molecular mechanisms involved in CT function gives one the impression that CT is part of an elaborate system that *V. cholerae* uses to invade, infect and poison the human intestine (de Haan and Hirst, 2004; Matson *et al.*, 2007). The system of CT acquisition, CT gene activation, CT secretion, and CT adherence to and alteration of host cells is extremely intricate (Figure 1).

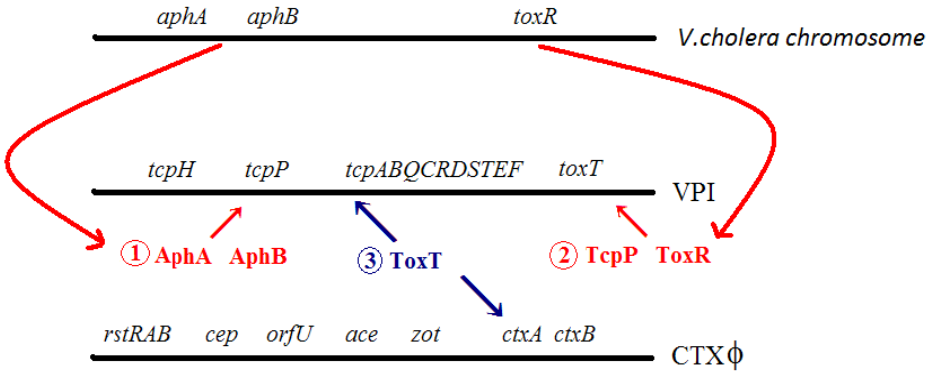

Figure 1. Three genetic elements are involved in *V. cholerae* infection of the human intestine; the *V. cholera*e chromosome, VPI and CTXΦ phage. Transcription factors and genes from the three separate genetic elements interact in a complex cascade to activate the production of cholera toxin (*ctxAB* genes) in *V. cholerae*.

CT Acquisition

Two chromosomes and two genetic elements are involved in *V. cholerae* infection of the human intestine and in the production and dissemination of CT: the *V. cholerae* chromosomes, a pathogenecity island (VPI), and CTX phage (CTXΦ) (Figure 1). Both VPI and CTXΦ are mobile genetic elements (Reichow *et al.*, 2010) but can also be found incorporated into the chromosomes. Many virulence factor genes in pathogenic bacteria are found clustered on such islands, which often bear nucleotide sequences indicative of mobile genetic elements (Purdom, 2009). VPI does not appear to be an active mobile genetic element and, thus, more is known about CTXΦ, a mobile genetic element that carries the CT gene. Infection of *V. cholerae* by CTXΦ promotes induction of pathogenic *V. cholerae*. All pathogenic isolates of *V. cholerae* carry another pathogenecity island designated as VPI-2, but it is not currently known how it contributes to CT biology (Reichow *et al.*, 2010).

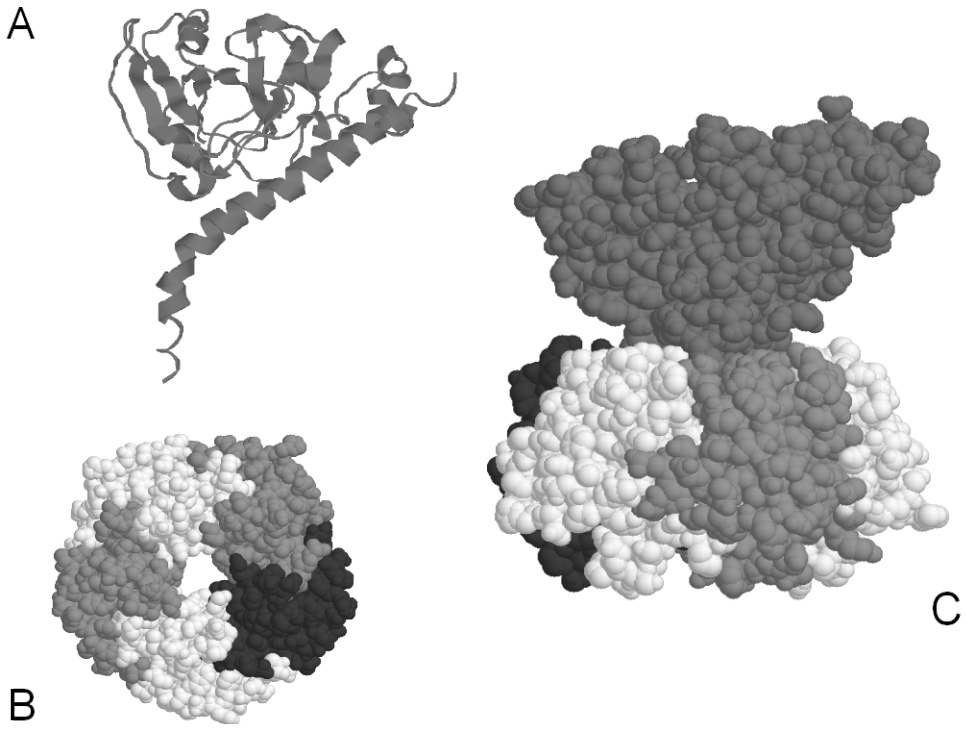

Figure 2. X-ray crystallographic structures of cholera toxin from Zhang *et al*. (1995). (A) The A chain is shown in a structural ribbon diagram to highlight the extended alpha helix that binds it to the B chain pentamer (B). The full complex is shown as a spacefill structure (C). Coordinates for Zhang *et al*.'s structure were obtained from the PDB (www.rcsb.org, ID 1XTC).

CTXΦ is a filamentous phage which infects only *V. cholerae* that express a pilus called the toxin coregulated pilus (TCP) (Waldor and Mekalanos, 1996; Davis *et al.*, 2000). TCP is an adherence factor, which also is thought to be involved in helping *V. cholerae* colonize the intestinal lumen. The TCP gene is located on VPI (Figure 1). Once the CTXΦ genome gains entry into *V. cholerae*, the CT genes are integrated into the bacterial genome at specific locations. CT

integration cannot occur without an antirepressor protein supplied by yet another phage RS1. RS1 is a satellite phage associated with CTXΦ (Faruque *et al.*, 2003).

CT gene activation

The activation of CT can be broken down into a three step process. Step 1: The activation of the CT gene involves all three genetic elements starting with the transcription of *aphA*, *aphB* and the *toxR* genes on the *V. cholerae* chromosome (Figure 1). The transcription of *aphA* and *aphB* is one of the earliest events in activation of CT and these genes are influenced by temperature, salt concentration, pH, and possibly cell concentration. The *aphA* and *aphB* gene products, AphA and AphB, activate the production of TcpP. TcpP is part of an operon that makes up the genes and proteins of the toxin coregulated pilus (TCP).

Step 2: TcpP works in conjunction with ToxR to activate the transcription of *toxT*. The *tcpP* and *toxT* genes reside on the VPI (Figure 1).

Step 3: ToxT activates the transcription of both major virulence factors; TCP on the VPI, and CT (*ctxA* and *ctxB* genes) on the phage genome. Curiously, the global regulator *toxR* gene is thought to reside in most all vibrio genomes. Another regulator of this system, HapR (not shown) is involved in controlling AphA and AphB. It decreases production of these early factors and makes a protease which promotes detachment of bacteria from tissues.

CT secretion

CT is secreted by a large complex secretory apparatus called the type II secretion system (T2SS). This secretion system spans the inner and outer membranes of *V. cholerae* and is made of more than 14 proteins; the entire apparatus is preferentially located at the flagellum-containing pole of the cell (Reichow *et. al*., 2010; Davis *et al*., 2000; Sikora, 2013). We speculate that this might help direct the toxin toward the host cells, because attachment of the bacteria is mediated by the flagellum. The control of phage secretion levels and the directionality of CT secretion would suggest that *V. cholerae* is designed to be localized near a certain host cell population.

During the secretion process, the CT protein begins folding in the periplasmic space and it is secreted in its folded but non-activated state. CT is made of two major subunits called the A and B subunits. Once out, the A subunit is then subjected to proteolytic cleavage, producing A1 and A2 fragments, which are essential for toxin action (Hirst, 1999). The proteolysis can take place in the extracellular environment or by proteases within the host intestinal cell. Thus, it appears there are several designed “fail safe” mechanisms to prevent the toxin from premature activation and possible destruction of the *V. cholerae* host cell.

CT adherence and intoxication of host cells

Due to the intricacies of CT activation and secretion, it would be logical to hypothesize that the binding of the CT is very specific for the cell it infects; however, this is not the case. CT binding is fairly promiscuous, with the primary receptor being GM1-ganglioside. The B subunit of CT is the primary binding receptor and has been shown to bind to several other glyco-proteins (Blank

et al., 2007; Kuziemko *et al*., 1996). Perhaps this promiscuity is part of a system designed to ensure that CT binds to its targeted tissue location.

After binding to the intestinal cell surface, the B subunit chaperones the A subunit across the cell membrane by inducing several possible endocytic mechanisms and engages the retrograde membrane traffic of the cell. CT holoenzyme (intact enzyme with all A and B subunits) enters the trans-Golgi network via a coated vesicle and is most likely guided in this retrograde movement by several host cell factors (vanden Broeck *et al*., 2007; de Haan and Hirst, 2004). Once in the ER, it is thought that the A1 subunit disassociates from the holoenzyme, unfolds, and enters a degradation pathway for misfolded proteins. Remarkably, the A1-subunit is not digested (most misfolded proteins are digested), but is instead translocated to the cytosol. Once in the cytosol, the A1-subunit refolds and activates the adenylate cyclase enzyme by ribosylation of the G protein associated with the adenylate cyclase. CT does not display specificity for substrates, yet it appears to be specifically directed toward the G protein of the adenylate cyclase where it has an effect. The adenylate cyclase is permanently activated, causing high sustained levels of cAMP, which activate the chloride ion channels causing massive loss of chloride ions and corresponding loss of salt and water.

Creationist theories about the origin of *V. cholerae* and its virulence factors

The specificity with which *V. cholerae* interacts with human cells and the human intestine gives the appearance that *V. cholerae* is designed to interact with the human intestine, or perhaps the mammalian intestinal environment. Since there was no death or disease in the Garden, this leads us to postulate at least three hypotheses about the origin of *V. cholerae* and its virulence factors:

1. *V. cholerae* utilized its virulence factors for a beneficial function in the open ocean environment and the Fall presented new environmental conditions that allowed its spread into human environments.
2. *V. cholerae* utilized its virulence factors for a beneficial function in the intestinal environment or other internal organs and modification or displacement caused it to be detrimental.
3. *V. cholerae* obtained its virulence factors from outside sources and was initially created without virulence factors.

Since hypothesis 3 assumes that the virulence factors were pre-existing and does not directly address the question of their origin as possible beneficial factors, we will focus on hypothesis 1 and 2, examine the factors which tend to support these hypotheses, and attempt to determine if one hypothesis is more viable than the other. In addition, since both hypotheses 1 and 2 assume that the virulence factors once had beneficial functions, and CT is the most crucial factor in the promotion of cholera, and a natural beneficial function of CT has not been determined, we will focus on the biology and natural history of CT.

Cholera toxin (CT)

CT is classified as an AB toxin. AB toxins are proteins constructed of a B subunit that binds to the surface of the host cell and promotes the uptake of the enzymatic A subunit, which then alters

the normal biochemistry of the cell (Patton *et al*., 2000; Masignani *et al*., 2000). The A subunit is comprised of two protein chains proteolytically derived from a single precursor (Zhang *et al*., 1995). The smaller A2 chain is helical and binds the globular A1 chain to a pentameric ring of B subunits (Figure 2). Most AB toxins are ADP-ribosyltransferases that operate by modifying host factors by transferring an ADP-ribosyl group to cell factors. ADP ribosyltransferases are fairly ubiquitous in organisms except for a few invertebrates and have been noted to be involved in regulating enzyme activity, cell signaling, protein synthesis, cell proliferation and nucleic acid metabolism (Fieldhouse and Merrill, 2008). Also the nitrogenase function of some symbiotic bacteria is controlled by ADP-ribosylation (Liu and Kahn, 1995). Thus it is possible to postulate, based on the evidence of ADP-ribosylation factors in general, that CT could have once had a beneficial function or currently does have a beneficial function as an ADP-ribosylation factor in an as yet undiscovered biological system.

RESULTS

To search for the origin and possible beneficial function of CT we compared its sequence with currently available bacterial genomes. Both CT subunits (A and B) share significant sequence similarity with the protein subunits of *E. coli* heat labile enterotoxin (Dallas and Falkow, 1980).

We searched for potential homologues of each CT subunit using NCBI's web-based BLAST (Johnson *et al*., 2008). For the B subunit, we found only the previously-reported *E. coli* enterotoxin proteins as well as a protein from *Citrobacter freundii*. Karasawa *et al*. (2002) report that the B subunit gene from *Citrobacter* does not have a corresponding A subunit homologue from the same species. Our BLAST search with the A subunit sequence yielded significant results from the beta proteobacterium *Burkholderia*, the gamma proteobacteria *Xanthomonas*, *Serratia*, and *Proteus*, the spirochaete *Leptospira*, *Flavobacterium*, and the rice blast fungus *Magnaporthe oryzae*. The *Magnaporthe* homologues were previously noted by Dean *et al*. (2005), who conjectured that they might be involved in ADP-ribosylating rice G proteins.

We used MEGA (Tamura *et al*., 2007) to align 24 protein sequences homologous to the CT A subunit and to create a neighbor-joining tree from percent differences of the alignment of 103 amino acid positions. All seven *Burkholderia* homologues formed a branch, as did the five proteins from *Leptospira*, and the two *Magnaporthe* homologues (Figure 3). All of the sequences were substantially different from the original CT or enterotoxin proteins, implying that the proteins from species other than *Vibrio* or *E. coli* may not have an actual genetic ancestor in common.

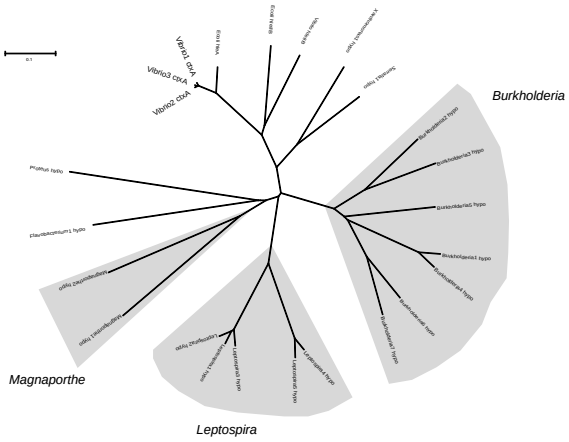

Figure 3. Neighbor-joining tree for 24 protein sequences homologous to cholera toxin A chain. Putative hypothetical proteins are designated as "hypo." Sequence accession codes are YP_004937111 (Vibrio1 ctxA), NP_231100 (Vibrio2 ctxA), ZP_01682692 (Vibrio3 ctxA),YP_001451390 (Ecoli hleA), ZP_06662161 (Ecoli hleIIB) ZP_01956924 (Vibrio hleIIB), ZP_06729314 (Xanthomonas1), ZP_08038623 (Serratia1), YP_004843256 (Flavobacterium1), ZP_09266663 (Leptospira1), YP_798503 (Leptospira2), ZP_09255309 (Leptospira3), ZP_09265263 (Leptospira4), YP_797867 (Leptospira5), YP_560254 (Burkholderia1), YP_553467 (Burkholderia2), ZP_09816229 (Burkholderia3), ZP_09820935 (Burkholderia4), YP_553552 (Burkholderia5), ZP_09822299 (Burkholderia6), YP_558853 (Burkholderia7), XP_369014 (Magnaporthe1), ZP_03802707 (Proteus1), and XP_363025 (Magnaporthe2). For details on tree construction, see text.

To further investigate potential origins of cholera toxin genes, we calculated codon frequencies for 1,840 bacterial genomes obtained from GenBank and compared them to the codon frequencies of the *ctxA* and *ctxB* genes using the frequency of preferred codon statistic. We found that the best matches to both genes among 20 complete *Vibrio* genomes came from *V. fischeri* rather than *V. cholerae*. Overall, the best codon usage matches to *ctxA* came from *Wolbachia* endosymbionts, and the best matches to *ctxB* came from *Cellulophaga algicola* and three *Rickettsia typhi* genomes (Table 3). Adding to the mystery is the fact that there is also evidence to support that CT was derived from a yet unknown ancient genetic mobile element, because its GC content is different from the GC content of CTXΦ, its current mobile genetic element (Faruque and Mekalanos, 2012).

Table 3. Frequency of preferred codons based on 1,840 microbial genomes.

	ctxA			ctxB		
Top four matches	*Wolbachia*	UID61645	0.583	*Cellulophaga*	UID62159	0.624
	Wolbachia	UID57851	0.583	*Rickettsia*	UID158357	0.624
	Wolbachia	UID58107	0.583	*Rickettsia*	UID158161	0.624
	Wolbachia	UID59371	0.583	*Rickettsia*	UID58063	0.624
Top Four *Vibrio* matches	*fischeri*	UID58163	0.521	*fischeri*	UID58163	0.600
	fischeri	UID58907	0.521	*fischeri*	UID58907	0.600
	EJY3	UID83161	0.459	*anguillarum*	UID68057	0.504
	splendidus	UID59353	0.459	EJY3	UID83161	0.504

DISCUSSION

Considering the sequence and codon usage data (Figure 3 and Table 3), one conclusion is that CT appears to have been derived from a more ancient genomic element than CTXΦ. Thus if we consider that the strongest matches for codon usage, sequence, GC content, and structure and function include certain strains of *E. coli* and *Vibrio fischeri*, perhaps we can postulate that a symbiont like *V. fischeri* once possessed CT (the CT genes are not detectable in the genome of *V. fischeri* but, intriguingly, the genes of the CTXΦ are present and *V. fischeri* possesses a functional analogue of CT). Supporting this hypothesis is the fact that the ecological environment of *V. cholerae* overlaps with both *E. coli* and *V. fischeri*, such that horizontal gene transfer would be possible.

Evidence in support of the hypothesis that *V. cholerae* utilized its virulence factors for a beneficial function in the aquatic environment (hypothesis 1)

We find the following supporting evidence in favor of the hypothesis that the virulence factors of *V. cholerae* originally induced chitin breakdown:

1. *V. cholerae* possesses a complex chitin utilization program including 16 genes involved in chitin metabolism (Meibom *et al.*, 2004).
2. Several *V. cholerae* functions which promote pathogenesis also are involved in chitin metabolism, including chemotaxis, adherence, secretion, and biofilm formation (Francis, 2006; Kirn *et al*., 2005).
3. *V. cholerae* becomes more competent in the presence of chitin. Competency of bacteria is often associated with the insertion of genetic mobile elements which promote disease. The role of chitin-induced competency is not known, but it may allow vibrios to obtain genetic elements which promote the breakdown of specific forms of chitin. It is also possible that this heightened level of competency promoted or promotes the uptake of CTXΦ (Meibom *et al.*, 2005).

Some virulence factors which participate in causing cholera are also known to participate in chitin metabolism. For instance, the toxin coregulated pilus (TCP), the receptor for CTXΦ, is also involved in promoting biofilm formation in environmental strains of *V. cholerae;* this is predicted to aid in the concentration and localization of chitinases secreted by *V. cholerae.* Without a mechanism to contain the chitinases, they would dilute rapidly in the open ocean (Reguera and Kolter, 2005). In addition, the GlcNAc-binding protein (GbpA), which binds intestinal mucus carbohydrates also binds to chitin (Kirn *et al*., 2005).

Evidence in support of the hypothesis that *V. cholerae* utilized its virulence factors for a beneficial function in the intestinal or other internal organ environment (hypothesis 2)

1. Vibrio species related to *V. cholerae* live in symbiotic relationship with many organisms and are found in the gastrointestinal tract or other internal organs of some organisms (Fabiano, 2004).

2. CT function and acquisition is complex and appears to be designed for enclosed spaces of internal organs. CT toxin is carried by an unusual non-lytic phage (Waldor and Mekalanos, 1996). Phage infection of *V. cholerae* is thought to occur in the intestinal environment. This is supported by the observation that potentially pathogenic or environmental *V. cholerae* acquired from the aquatic environment do not carry an active CT gene (Faruque *et al*., 1998). Also, the enclosed space of internal organs would allow for more efficient phage infection compared to the waters of the open ocean.
3. Some *V. cholerae* genes which code for virulence factors, including those associated with the *V. cholerae* CTXΦ, are also found in the symbiotic vibrio, *V. fischeri*, which resides in the internal light organ of the Hawaiian bobtail squid. Curiously, the *V. fischeri* CTXΦ-like mobile genetic element does not possess the CT gene. However, *V. fischeri* possess ADP-ribosylation factors and also expel excess *V. fischeri* daily, consistent with the fact that an agent which causes expulsion is present in *V. fischeri* (Francis, 2006).
4. Some virulence factors found in *V. cholerae* are used by symbiotic *V. fischeri* to promote symbiosis. In this case, the virulence factors cause an inflammation shortly after the preliminary capture of the bacterial symbionts by the squid. The inflammation prevents other bacteria from infecting the squid and is an essential part of establishing the symbiotic partnership (Francis, 2006; Reich and Schoolnik, 2006).
5. Expulsion of bacteria appears to be an essential process of some vibrio-host symbiotic relationships. In the case of the vibrio-squid, or vibrio-ponyfish symbiosis, daily or frequent expulsion of bacteria from the host is part of the symbiotic life cycle (Nyholm and McFall-Ngai, 2004; Wada *et al*., 2004). Expulsion allows the bacteria to continue in a growth cycle which is essential for quorum sensing molecules to be released. Quorum sensing molecules promote light production among the bacterial symbionts. Also, expulsion seeds the surrounding area with free-living bacteria that can repopulate hosts or initiate the first population of a young sterile host. CT is involved in bacterial expulsion from the human intestine, but has not yet been shown to be involved in expulsion in symbiont hosts.
6. *V. cholerae* is abundant in estuarine waterways and is found attached to the chitin in the intestine of some aquatic animals in this environment where it is postulated to aid in salt metabolism as creatures move from a freshwater to saltwater environment. This is consistent with the action of CT which promotes salt excretion from the intestinal environment (Francis and Wood, 2009; Colwell *et al.*, 1984).

A proposed model of CT origin and function

It appears from this analysis that *V. cholerae* could play a role in both chitin metabolism and promotion of symbiosis. However, there is currently more circumstantial data to suggest that CT is expressed and functions better in the internal spaces of living hosts. There is some intriguing evidence to suggest that *V. cholerae* may use the chitinous surface of the internal organs of certain creatures (*e.g.*, blue crab and Hawaiian bobtail squid) to gain access to the intestinal lumen or other internal organs. This is supported by recent data which shows that one of the virulence factors, GbpA, possesses domains that specifically bind to chitin and intestinal mucus, but do not bind well to individual GlcNAc residues found on individual intestinal cells (Wong *et al.*, 2011). This is also supported by a recent study which shows that *V. fischeri* gains access to the light organ by a chitin chemotaxis signal (Mandel *et al*., 2012).

We propose the following model for the origin and function of CT:

1. CT was designed to be a modular transmissable factor which could be "plugged in" to vibrio bacterial symbionts. This would primarily occur in vibrios that achieve colonization within an enclosed space conducive to phage infection and quorum sensing, such as the intestinal environment or light organ of the host symbiont.
2. Vibrios gain access to the intestinal lumen or other receptive internal organ in invertebrate marine organisms by chemotaxis and binding to chitin or other exposed polysaccharides. The promiscuous carbohydrate-binding activity of vibrios ensures that they will attach to the intestinal surface or other type of symbiotic organ of many organisms. CT is secreted when certain cell concentrations and environmental factors are present.
3. Depending on the symbiont in which it resides, CT promotes salt metabolism or maintenance of optimal bacterial numbers by causing secretion of salt or the cyclic expulsion of excess bacteria.
4. The toxicity of CT is controlled by phage induction, repressors at the genetic level and the requirement for certain environmental triggering factors such as temperature and salt concentration. In addition, quorum sensing induction at high cell density shuts off TCP and CT production and colonization preventing uncontrolled expulsion of the bacteria.

Is this bad design?

It could be postulated that, because of the abundance of vibrios in the aquatic environment and the mobility of the CT genes, human infection is inevitable, given the fact that humans live near aquatic environments. However, a closer look at the infections caused by *V. cholerae* shows that even though pandemics have occurred, the pandemics tend to occur only in certain regions. This is an intriguing paradox, because *V. cholerae* is found in most waterways across the globe. In addition, a high concentration of vibrio must occur in waterways and a high number must be ingested for cholera illness to occur. It has also been shown that *V. cholerae* concentrations are modulated in waterways by natural predation and lytic phages (Nelson *et al.*, 2008). This also corresponds with the fact that cholera illness tends to be seasonal and occurs after planktonic blooms in the warm spring and summer months (Sack and Mekalanos, 2005). Consistent with this is the fascinating story about the recent cholera outbreak in Haiti, which occurred after the destructive earthquake in 2010. In this case, cholera was introduced to this area by a non-native aid worker. Apparently, there was not a high enough dose of local *V. cholerae* in the local waterways to cause cholera, even though unsanitary conditions were ripe for a cholera epidemic. The last reported cases of cholera in Haiti were over a century ago (Anonymous, 2010). Thus, these historical accounts and data suggest that cholera is not easily contracted by humans. Certain environmental conditions, including sea surface temperatures and monsoons all correlate with incidences of cholera outbreaks, suggesting a possible explanation for how *V. cholerae* and its associated toxins would exist in the benign environment of the Garden without causing the cholera illness.

CONCLUSIONS

There is an abundance of data showing that *V. cholerae* and its pathogenic strains live as autochthonous residents of marine waterways and perform beneficial functions involving nutrient cycling in those locations. This may represent a remnant of the original niche of *V.*

cholerae. In support of the postulated environmental roles, many of *V. cholerae*'s virulence factors have been discovered to participate in ecological functions, suggesting that *V. cholerae* is more fit for the aquatic environment than the human intestine where it causes a severe life-threatening diarrhea. In contrast, *V. cholerae*'s primary toxin, CT, has no known role in the aquatic environment and appears to be released and work optimally only in the human intestine, where it moves in an intricate retrograde fashion through the infected cells to gain access to the cytosol while avoiding degradation.

In this paper we propose several speculative models for how CT may participate in the aquatic ecological role of *V. cholerae*, including as a factor which performs vital functions and promotes a sustained relationship between *V. cholerae* and invertebrate hosts in the estuarine environment. Several indirect indicators show that CT may be involved in salt regulation in invertebrates which traverse estuarine waterways and may serve a role in maintaining bacterial cell concentration in symbiont hosts. Our research suggest that CT may indeed have an environmental role as part of the *V. cholerae* life cycle and that *V. cholerae* obtained mechanisms to invade and colonize the human gut. Two recent studies show that a single change in a regulatory protein or acquisition of sialic acid metabolism genes can promote the association of vibrios with biological organisms that were not able to be colonized with vibrio bacteria before they obtained these genetic sequences (Mandel *et al.*, 2009; Almagro-Moreno, 2009).

Future areas of study include examining vibrios for regulatory factors or structural genes which they obtained by HGT which allowed them to colonize and parasitize biological organisms.

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