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# Expression of *Vibrio cholerae* Virulence Genes in Response to Environmental Signals

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## Abstract

*Vibrio cholerae*, the causative agent of Asiatic cholera, is a gram-negative motile bacterial species acquired via oral ingestion of contaminated food or water sources. The O1 serogroup of *V. cholerae* is responsible for pandemic cholera and is divided into two biotypes, classical and El Tor (Butterton and Calderwood, 1995; Mekalanos, 1985). The El Tor biotype is responsible for the current cholera pandemic. In the absence of disease, the vibrio life cycle consists of a free-swimming phase in marine and estuarine environments in association with zooplankton, crustaceans, insects, and water plants. Vibrios interact with various surfaces found in the environment to generate biofilms which may promote survival (Watnick *et al.*, 1999). Within the host the motile vibrios must evade the innate host defense mechanisms, penetrate the mucus layer covering the intestinal villi, adhere to and colonize the epithelial surface of the small intestine, assume a non-motile phase, replicate and cause disease by secreting numerous exoproteins at the site of infection (Oliver and Kaper, 1997). The voluminous diarrhea associated with cholera infection leads to the dissemination of the vibrios back into a watery environment and thus a continuation of the environmental phase of the life cycle. The host phase of the vibrio life cycle is only possible through the action of a group of virulence genes (ToxR-regulon) controlled by a complex and incompletely understood regulatory cascade. The ToxR regulon colonization and toxin genes are coordinately expressed in response to specific host signals that have yet to be completely defined (Skorupsky and Taylor 1997). Although little is known regarding the host signals that impact the ToxR regulatory cascade, it is clear that these intrainestinal signals play an important role in maximizing the ability of the vibrios to survive and multiply within the host. Key to understanding the complex events involved in the pathogenesis of *V. cholerae* will be elucidating the intrainestinal signaling molecules that trigger the expression of vibrio virulence genes. Understanding the molecular basis of this host-parasite interaction

will provide important information with respect to how pathogenic bacteria establish infection and provide insights leading to novel methods for treating and/or preventing bacterial infections. This review will summarize what is known regarding host signaling and the complex ToxR regulatory system employed by *V. cholerae* to coordinate virulence gene expression within the host.

## Introduction

At first glance the ToxR regulatory cascade (Figure 1) seems overly complex, however this may not be unreasonable given the evolution of this regulatory/virulence regulon. The two major virulence determinants of *V. cholerae* are encoded by two separate genetic elements; CTX $\phi$  which encodes the cholera toxin genes and VPI $\phi$  which encodes the genes required for toxin-coregulated pilus (TCP) production, accessory colonization factors (ACF) and the ToxT, TcpP, TcpH and TcpI regulatory proteins. ToxR which presumably evolved to control outer membrane synthesis in response to osmolarity somehow gained control of the genes encoded by the two "recently" acquired elements. Our ability to understand how this complex regulatory circuit functions both *in vitro* and *in vivo* is hampered by a general lack of information regarding the signal transduction pathways used by the individual components of ToxR/ToxT system. ToxR/ToxS homologues have been found in nontoxinogenic *V. cholerae* strains and in numerous vibrio species not associated with human disease. *Vibrio parahaemolyticus*, a halophilic organism associated with human gastroenteritis following ingestion of seafood, produces disease via the elaboration of a thermostable direct hemolysin. The *V. parahaemolyticus* *toxRS* operon is structurally and functionally similar to the *V. cholerae* *toxRS* operon and controls the synthesis of the thermostable direct hemolysin 2 (*tdh2*) gene and proteins other than TDH. Like *V. cholerae*, the *V. parahaemolyticus* *toxRS* operon is found in both clinical and environmental isolates suggesting that *toxR* is "ancestral" with respect to the *V. parahaemolyticus* genome when compared to the "recently" acquired *tdh* genes (Oliver and Kaper, 1997).

Since TCP is the receptor for the CTX $\phi$ , ancestral *V. cholerae* seemingly acquired the VPI $\phi$  element first and developed the ability to meld its ToxT-dependent regulatory circuit into the ToxR/ToxS system. At some later date *V. cholerae* acquired the CTX $\phi$  phage and again placed its regulation under control of the ToxR/ToxT system with additional input from the VPI $\phi$  encoded regulators such as ToxT. Recent genetic and genomic analyses aimed at characterizing the environmental regulation of the ToxR/ToxT virulence cascade have identified additional layers of regulation involving *V. cholerae* genes not associated with the CTX $\phi$  and VPI $\phi$  elements. The finding that classical

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# ToxR Regulon

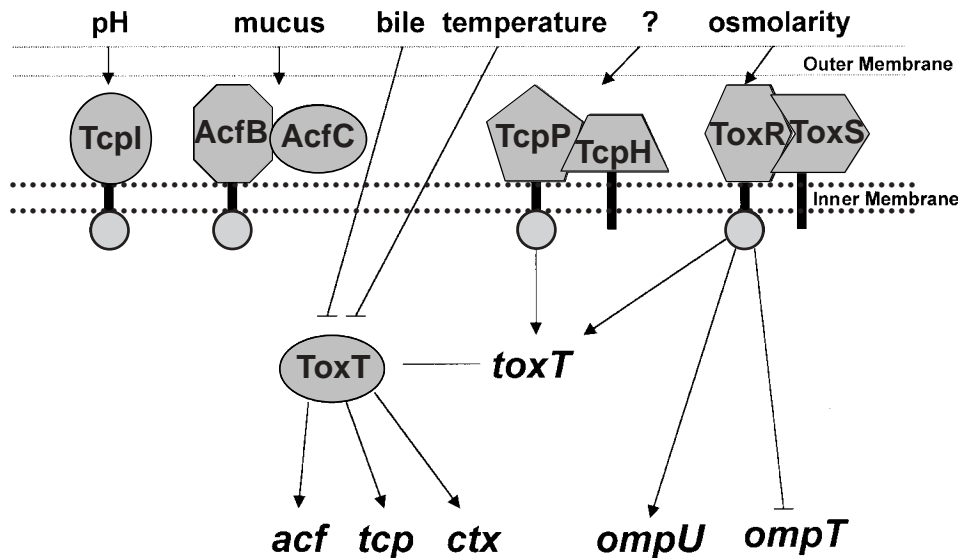


Figure 1. ToxR regulon proteins implicated in environmental sensing.

and El Tor strains of *V. cholerae* differentially regulate ToxR regulon genes may also be a result of selective evolution in that classical strains are more likely to cause symptomatic infections and are associated with waterborne transmission whereas El Tor strains are more frequently associated with non-waterborne sources such as contaminated food.

## Cholera Toxin Synthesis

*In vitro* studies aimed at maximizing cholera toxin synthesis in the laboratory have uncovered several variables that stimulate cholera toxin synthesis *in vitro* and led to the discovery of other virulence genes that are expressed under the same conditions (ToxR regulon). Surprisingly, cultures maintained at 30°C produce more toxin than cultures grown at 37°C, the temperature of the human small intestine. Growth of *V. cholerae* at low pH (6.5) with adequate aeration favors toxin production by Classical strains of *V. cholerae*. It has also been shown that the ion concentration of the growth medium, the presence of certain amino acids in glucose minimal medium (asparagine, serine, glutamate, and arginine), carbon dioxide levels and the addition of bile salts influence cholera toxin synthesis (Gupta and Chowdhury, 1997; Cotter and DiRita, 2000). The conditions that promote high-level synthesis of cholera toxin in classical strains fail to stimulate similar levels of cholera toxin in El Tor biotype strains. Maximum production of cholera toxin by El Tor strains requires AKI medium which contains tryptone and bicarbonate (Iwanaga *et al.*, 1986). Although the *in vitro* growth conditions that stimulate cholera toxin (CTX) and toxin coregulated pilus (TCP)

synthesis by the two biotypes differ significantly, the ToxR regulon must have similar function relevance *in vivo* since null mutations in *toxR* and *toxT* abolish CTX and TCP synthesis *in vitro* and attenuated virulence in intestinal colonization (Skorupski and Taylor, 1997). The reason for the differential ToxR regulon control exhibited by El Tor and classical strains of *V. cholerae* is based on the mechanism of *toxT* transcription initiation by TcpP which will be discussed in detail later in this article.

These early observations regarding environmental control of cholera toxin synthesis led to the identification of a coordinately regulated set of genes (ToxR regulon) (Peterson and Mekalanos, 1988) under the control of multiple overlapping regulatory systems (Higgins and DiRita, 1994; Hase and Mekalanos, 1998). The *in vivo* relevance of specific host intrainstestinal signals and the overlapping regulatory pathways affecting ToxR regulon gene expression that have been discovered in the laboratory are just now being elucidated. It seems clear at this point, however; that the ability of *V. cholerae* to modulate virulence gene expression in response to environmental conditions is essential for *in vivo* survival since strains lacking this ability due to a mutation in the *toxR* gene, the product of which is involved in signal-dependent virulence gene expression are deficient in intestinal colonization of human volunteers (Herrington *et al.*, 1988). A combination of *in vivo* genetic techniques using relevant animal models of cholera infection (see below) have begun shedding light on this fascinating area of pathogenesis. These studies which will be discussed below, have clearly demonstrated that virulence gene production is not a plus/minus phenomenon. *V. cholerae* appears to

posses the ability to modulate virulence gene expression in response to microenvironmental signals within the small intestine. These data demonstrate that the overlapping signal transduction pathways controlling virulence gene expression ensure the timely expression of vibrio genes required for intrainestinal survival.

### ToxR Regulon

As mentioned in the introduction, the expression of a large number of *V. cholerae* genes required for successful colonization of the human small intestine are induced in response to specific environmental conditions. These genes are members of the ToxR regulon and their expression is controlled by a cascade of regulatory proteins (ToxR, TcpP and ToxT) (Skorupski and Taylor, 1997). ToxR regulon genes fall into four general classes: cholera toxin (CTX) genes; toxin coregulated pilus biogenesis (TCP) genes; accessory colonization factor (ACF) genes; and ToxR-activated genes (TAG) of unknown function (Peterson and Mekalanos, 1988).

### Cholera Toxin

Cholera toxin is a multisubunit ADP-ribosylating toxin that binds to GM1 ganglioside found on intestinal epithelial cells. The A<sub>1</sub> subunit of the holotoxin activates the alpha subunit of Gs, a guanylnucleotide-binding protein involved in regulation of adenylate cyclase activity (Collier and Mekalanos, 1980; Gill and King, 1975). ADP-ribosylation of Gs results in high levels of cAMP and subsequent alterations in ion transport in villous and crypt cells of the intestinal mucosa. The overall effect is an increase in chloride secretion into the intestinal lumen and an inhibition of sodium absorption (Oliver and Kaper, 1997). The A and B subunits of cholera toxin are encoded by the *ctx* operon on a region of DNA termed the CTX genetic element originally thought to be a compound transposon associated with toxigenic strains of *V. cholerae* (Waldor and Mekalanos, 1996). It is now known that the *ctx* genes lie within the genome of a lysogenic filamentous phage (CTX $\phi$ ). The single-stranded CTX $\phi$  infects *V. cholerae* by absorbing to the toxin-coregulated pilus (TCP) which is the major colonization determinant of *V. cholerae*. Upon infection of *V. cholerae* the CTX $\phi$  genome integrates into the genome of *V. cholerae* to form a prophage. The 6.9-kb CTX $\phi$  genome has a modular structure composed of two functionally distinct domains, the core and the RS2 regions (Waldor and Mekalanos, 1996). The core region encodes cholera toxin and phage morphogenesis genes. RS2 encodes genes required for replication, integration, and regulation of CTX $\phi$  (Waldor *et al.*, 1997). Based on the distinct GC content of *ctxAB*, compared to the rest of the CTX $\phi$  genome it has been suggested that *ctxAB* genes were probably acquired by a precursor form of CTX $\phi$ . Comparative analysis of CTX $\phi$  from a number of *V. cholerae* strains indicate that acquisition of this phage by *Vibrio* sp. has occurred multiple times and has involved several CTX $\phi$  genotypes (Boyd *et al.*, 2000).

### Toxin Coregulated Pilus

The type IV pilus encoded by *V. cholerae* was named toxin coregulated pilus because its production parallels that of cholera toxin (Taylor *et al.*, 1987). TCP is composed of 7 nm filaments that form laterally associated bundles composed of the TcpA pilin subunit. The TCP structure is assembled as a polymer of repeating subunits of TcpA pilin that form long fibers, which laterally associate into bundles. Production of TCP on the vibrio surface leads to autoagglutination of the cells (Kirn *et al.*, 2000) and intestinal colonization since human volunteers given a *tcpA* derivative strain were not colonized and did not develop Asiatic cholera. The molecular mechanism by which TCP promotes intestinal colonization is not presently known. *In vitro* and *in vivo* analyses of *tcpA* mutants however, suggest that a major function of TCP is to mediate vibrio interaction through direct pilus-pilus contact which leads to microcolony formation, intestinal colonization and increased serum resistance (Kirn *et al.*, 2000). As is the case for the *ctxAB* genes, *V. cholerae* *tcpA* and TCP biogenesis genes are encoded by a genetic element (TCP-ACF pathogenicity island) with the characteristics of a phage (Kovach *et al.*, 1996; Karaolis *et al.*, 1999). Thus it appears that the two major virulence determinants of *V. cholerae*, which are coordinately expressed in response to a regulatory cascade that is influenced by *in vivo* signals are encoded by genetic elements that have been acquired by horizontal transmission.

### Accessory Colonization Factor

Intestinal mucus provides a chemotactic signal for *V. cholerae* whereby the vibrios direct their movement toward the intestinal surface. Directed motility coupled with the vibrios ability to secrete enzymes (mucinase, lipases, proteinases) capable of degrading mucus, maximize the ability of *V. cholerae* to burrow through the mucus to the surface of intestinal cells (Freter and O'Brien, 1981a; Freter and O'Brien, 1981b, Freter *et al.*, 1981a; Freter *et al.*, 1981b). Disruption of accessory colonization factor (ACF) genes results in altered swarm plate phenotypes which is characteristic of genes involved in chemotaxis (Huges *et al.*, 1994; Everiss *et al.*, 1994). These data coupled with amino acid alignment algorithms that show a close relatedness between AcfB and enteric methyl-accepting chemotaxis proteins suggest that ACF proteins play a role in a host-specific chemotaxis system that recognizes intestinal mucus (Everiss *et al.*, 1994).

The TCP and ACF gene clusters are physically linked on the *V. cholerae* chromosome and are flanked by bacteriophage attachment (*att*) half sites. Together these two loci constitute over 25 Kb of chromosomal DNA encoding an environmentally regulated "pathogenicity island". Genomic analysis of *V. cholerae* strains indicate that only vibrios capable of causing epidemic cholera possess this "pathogenicity island" (Kovach *et al.*, 1996). A recent study has proposed that the "pathogenicity island" carrying the TCP/ACF genes is a prophage (VPI $\phi$ ) (Karaolis *et al.*, 1999). As mentioned above, the genes encoding CTX are contained on CTX $\phi$  and the receptor for CTX $\phi$  is

TCP which is the coat protein for VPI $\phi$ . Remarkably, maximum expression of TCP presumably only occurs in the gastrointestinal tracts of humans. Indeed, CTX $\phi$  can efficiently convert TCP+ CTX $\phi$ - *V.cholerae* to CTX $\phi$ + most efficiently in experimentally infected mice (Waldor *et al.*, 1997). These data underline the importance of understanding the contribution of intestinal signals to the pathogenesis and spread of Asiatic cholera.

### Transcriptional Control of ToxR regulon Genes

Transcription of ToxR-activated genes is regulated by a complex regulatory cascade that responds to numerous yet to be identified host signal molecules (Figure 1). ToxR is a transcription factor located in the inner membrane that regulates the synthesis of cholera toxin, ToxT (itself a transcription factor), and the OmpU/OmpT porins with the help of ToxS. ToxT is a soluble transcription factor that amplifies its own expression and directly regulates the expression of cholera toxin, toxin coregulated pilus and accessory colonization factor genes. TcpP and TcpH are proteins that regulate CTX and TCP genes through ToxT. TcpI is a homologue of methyl-accepting chemotaxis proteins that negatively regulates TCP synthesis by an unknown mechanism. It is thought that ToxR and TcpP in response to particular environmental signals, act cooperatively to induce expression of *toxT*. ToxT, in turn, directly activates transcription of *ctx*, *tcp* and *acf* genes. Recent evidence suggest that ToxT may also respond to specific host signals in order to effect transcription activation (Schuhmacher and Klose, 1999). Null mutations in *toxR*, *toxT* and *tcpP* abolish *ctx*, *tcp* and *acf* expression and result in complete attenuation of colonization in animal models of cholera infection (Skorupski and Taylor, 1997; Cotter and DiRita, 2000).

### ToxR/ToxS

ToxR was identified based on its ability to activate *ctxAB* transcription in *E. coli* (Miller and Mekalanos, 1984). *toxR* encodes a 32-kDa integral membrane protein that contains a cytoplasmically located amino-terminal domain related to several prokaryotic transcriptional activators. The carboxy-terminal domain lies within the periplasmic space and is thought to be involved in sensing specific environmental conditions. ToxS is predominantly located within the periplasmic space and has been shown to interact with the periplasmic domain of ToxR (DiRita and Mekalanos, 1991). It has been proposed that the ToxR periplasmic domain senses osmolarity and is able to adopt a conformation that promotes transcriptional activation. A role of osmolarity in this process comes from a study in which the ToxR periplasmic domain was replaced with alkaline phosphatase. The ToxR-alkaline phosphatase fusion protein was able to activate *ctxAB* expression but was insensitive to high osmolarity, an *in vitro* growth condition that normally represses *ctxAB* expression (Miller *et al.*, 1987). This observation makes sense since ToxR is an "ancestral" gene that is present in other vibrio species which is involved in modulating OmpT and OmpU outer membrane protein levels in response to different salinity

levels found in the environment. Taken together these data suggest that ToxR/ToxS act as direct mediators of signal transduction via their ability to recognize environmental signals with their periplasmic domains and subsequently control transcription of ToxR regulon genes with the ToxR cytoplasmic domain. The exact nature of the osmolarity signal recognized by this system during intrainestinal infection remains to be determined. Optimal expression of cholera toxin and toxin coregulated pilus genes for example occurs *in vitro* in medium containing 0.1 molar NaCl. It is thought that the intestinal luminal osmolarity is equivalent to a NaCl concentration of at least 0.3 M (Cotter and DiRita, 2000).

*toxR* expression is negatively regulated by *htpG*, a gene that encodes a member of the HSP90 family of heat shock proteins. *toxR* and *htpG* are divergently transcribed from overlapping promoters. Thus *htpG* transcription negatively impacts *toxR* expression (Parsot and Mekalanos, 1990). These data suggest that conditions of stress, such as those encountered by *V. cholerae* within the stomach and small intestine (low pH, anoxia, bile salts) induce the expression of *htpG* which in turn inhibits the transcription of ToxR and subsequent ToxR-dependent virulence gene activation. Negative regulation of virulence gene expression during a maximum heat shock response may be biologically relevant since virulence gene activation during experimental infection of mice does not occur until 4-10 hours postinfection.

### ToxT

ToxT is a homologue of a large family of positive regulators referred to as the AraC/XlyS family. Like other members of this family *toxT* expression is controlled by environmental signals. Expression of *toxT* is controlled by regulators encoded by the ancestral chromosome (ToxRS) and by regulators encoded within the VPI $\phi$  element (TcpPH) (Cotter and DiRita, 2000). ToxT controls the transcription of genes contained on two separate mobile genetic elements *ctxAB* on CTX $\phi$  and *tcp/acf* genes on VPI $\phi$ . ToxT shares homology with the AraC family of proteins in the carboxy-terminus, which bind to specific DNA sequences and activate transcription (DiRita *et al.*, 1991). The amino termini of these proteins are quite divergent and are generally required for effector binding and dimerization. *toxT* expression is controlled by the same *in vitro* conditions that control cholera toxin and toxin coregulated pilus gene expression. *toxT* transcription is activated by ToxR and TcpP in response to various medium conditions *i.e.* temperature, osmolarity and pH. ToxT amplifies its own expression and regulates cholera toxin, toxin coregulated pilus and accessory colonization factor synthesis (Skorupski and Taylor, 1997). A recent study examining ToxT-dependent virulence factor expression revealed that several ToxR regulon genes are modulated by bile. ToxT-dependent expression of *ctxA* and *tcpA* was significantly decreased by the addition of 0.4% bile to the medium (Shuhmacher and Klose, 1999; Provenzano *et al.*, 2000). The authors suggest that presence of bile within the intestinal lumen where concentrations may be as high as 2% of individual bile salts would prevent ToxT-dependent

transcriptional activation of virulence genes. The authors also posit that since the concentration of bile presumably decreases at the surface of the epithelial cells lining the intestinal tract the repressive activity of bile would dissipate and thus allow for ToxT transcription of virulence genes.

### TcpP/TcpH

Recently, TcpP and TcpH which have been shown to regulate *ctx* and *tcp* expression via enhanced transcription of ToxT (Hase and Mekalanos, 1998; Krukoniš *et al.*, 2000). Although TcpP alone is sufficient for *toxT* expression, activation of *toxT* is significantly increased when *tcpH* is present. TcpP shares a similar periplasmic and cytoplasmic topology within the inner membrane to that of ToxR. Interestingly, TcpH also shares a similar membrane topology *i.e.* a large periplasmic carboxy-terminal domain and a small periplasmic amino-terminal tail to that of ToxS. ToxR/ToxS and TcpP/TcpH seem to function synergistically to activate expression of the *toxT* promoter. The expression of *tcpPH* is influenced by temperature and pH in similar fashion to that of *ctx* and *tcp*, two genes further down the regulatory cascade (Hase and Mekalanos, 1998).

These data indicate that like ToxR/ToxS, TcpP and TcpH represent a membrane bound transcriptional regulation complex that activates gene expression in response to particular environmental signals. The membrane location of TcpP/TcpH invokes a mechanism for sensing environmental signals yet it appears that expression of *tcpPH* rather than the activity of TcpP/TcpH controls *toxT* expression. *tcpPH* transcription is regulated by culture conditions that control *toxT*, and subsequently *ctx* and *tcp* expression. As mentioned earlier in this article, strains of classical and El Tor biotype *V. cholerae* synthesize CTX and TCP under different *in vitro* culture conditions. Recent studies examining *tcpPH* expression suggest that it is the differential control of *tcpPH* transcription in classical and El Tor biotype vibrios that is responsible for the differences in the *in vitro* *ctx* and *tcp* expression patterns observed between the two biotypes (Carroll *et al.*, 1997; Murley *et al.*, 1999). A single base-pair difference at positions -65 and -66 of the classical and El Tor *tcpPH* promoters was found to be responsible for this differential regulation. The presence of either an A or a G at position -65 or -66 conferred the classical or El Tor biospecific pattern of toxin and TCP expression in response to environmental signals (Kovacikova and Skorupski, 2000).

### AphA/AphB

A recent study identified two genes *aphA* and *aphB* (activator of *tcpP* and *tcpH* expression) that are required for maximum expression of *tcpP* (Skorupski and Taylor, 1999; Kovacikova and Skorupski, 2000). Although these two genes are located on the ancestral *V. cholerae* chromosome they may lie at or near the top of the virulence cascade that controls *toxT* expression. Neither *aphA* nor *aphB* is regulated by environmental signals known to activate *toxT* transcription. It seems likely therefore that AphA and AphB may function in recognition and or transduction of environmental signals required for proper

ToxR regulon activation. AphB is a member of the LysR family of transcriptional regulators whereas AphA has no known homologues. The overproduction of AphB in El Tor biotype strains results in increased expression of *tcpPH* and *tcpA* (Kovacikova and Skorupski, 2000). AphA and AphB appear to co-operate in order to activate the expression of the TcpP and TcpH transcription factors. Recent studies have identified distinct AphA and AphB binding sites within the *tcpPH* promoter. Interestingly, the AphB site encompasses the -65, -66 site identified above as being critical in the biotype specific expression of virulence genes (Kovacikova and Skorupski, 2000).

### cAMP-CRP

Mutations that derepress toxin expression under the normally repressive temperature of 37°C identified the cAMP-CRP system as a negative regulator of cholera toxin synthesis (Skorupski and Taylor, 1997). Interestingly a putative cAMP-CRP binding site overlaps the -35 site of the *tcpA* promoter and thus this site may prevent activation of *tcpA* in a ToxT dependent fashion. However, purified CRP does not appear to bind in a cAMP-dependent manner to the *tcpA*, *toxT* or *ctx* promoters. More recent studies examining *tcpPH* expression have identified a cAMP-CRP binding site within the AphA and AphB binding regions of the *tcpPH* promoter (Kovacikova and Skorupski, 2001). Thus the negative influence of cAMP-CRP on virulence gene expression is likely a result of its ability to influence AphA/AphB-dependent transcriptional activation of *tcpPH* under various growth conditions. Of note is the fact that ToxR regulon expression in response to pH and temperature is altered in the absence of a functional cAMP-CRP system suggesting that intestinal carbon and energy sources impact the way that the ToxR/ToxT regulatory cascade responds in the intestine. High intracellular levels of cAMP are typically seen in growth on poor carbon sources. Low levels of cAMP are detected in bacteria cultured in nutrient rich media. It is not clear at this point why cAMP-CRP would repress the ToxR regulon *in vitro* when grown in LB medium. The authors of this article suggest that under these conditions the vibrios metabolize so quickly that they exhaust their carbon sources (Skorupski and Taylor, 1997). What is the possible relevance of this regulation to intrainstestinal production of virulence factors? The down regulation of the ToxR regulon when cAMP levels are high (low nutrient environment encountered by free living vibrios) may serve to limit toxin and pilus synthesis in non-productive environments. On the other hand, the high nutrient environment of the intestine would favor toxin and pilus gene expression.

### H-NS

H-NS is a nucleoid-associated protein involved in the maintenance of chromosomal architecture and is also involved in silencing environmentally regulated gene expression during growth under nonpermissive conditions. H-NS is thought to influence gene regulation by organizing promoter/regulatory regions into nucleoprotein complexes. These complexes are formed in response to environmental

signals such as osmolarity, temperature, anaerobiosis, pH and growth phase. In most cases, H-NS negatively influences gene expression. In *V. cholerae*, deletion of *hns* results in high, nearly constitutive levels of cholera toxin, toxin-coregulated pilus and ToxT-encoding genes. H-NS influences ToxR regulon gene expression by exerting a negative effect on at least three promoters, *toxT*, *tcpA* and *ctx* with its largest repressive effect on the *toxT* promoter (Nye *et al.*, 2000). Thus H-NS negatively influences multiple levels of gene expression within the ToxR regulon and suggest that transcriptional activator proteins within the regulatory cascade function to counteract the repressive effects of H-NS at the *toxT*, *tcpA* and *ctx* promoters.

### Quorum Sensing

As can be seen above, production of virulence factors by *V. cholerae* is strongly influenced by environmental conditions. The ToxR regulon signal transduction cascade is responsible for sensing and integrating a seemingly complex amount of environmental information in order to spatially and temporally coordinate virulence gene expression. Recently it was discovered that in addition to the known components of the ToxR signaling circuit, quorum sensing regulation is involved in virulence gene expression (Zhu *et al.*, 2002). Quorum sensing regulates bacterial processes that are more effective when a population of bacterial cells acts in a coordinated fashion. A link between quorum sensing and bacterial virulence has previously been shown for *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Marine vibrios use quorum sensing to regulate bioluminescence. A *luxO* mutant of *V. cholerae* was found to be defective in intestinal colonization. LuxO is a response regulator that negatively regulates bioluminescence. Interestingly, the ToxR regulon is repressed in the *luxO* mutant and this repression is mediated by HapR a homologue of LuxR a protein required for transcription of genes encoding luciferase. HapR was shown to repress the expression of TcpP and in turn ToxR regulon expression (Zhu *et al.*, 2002). This is in contrast to other pathogens in which quorum sensing activates virulence gene expression at high cell densities. LuxO and HapR were also shown to regulate motility, protease production, and biofilm formation. The authors of this work suggest that quorum sensing modulates the expression of blocks of virulence genes in a reciprocal fashion during intestinal colonization. They further suggest that quorum sensing mediated repression of vibrio virulence genes may promote detachment of *V. cholerae* from the epithelium. This action could then serve to promote the establishment of new foci of infection within the small intestine and/or promote the exit of the vibrios from the host.

### Motility and Virulence Factor Production

*V. cholerae* adherence to the epithelial cells of the small intestine represents the final step in the colonization process. A key step that precedes enterocyte colonization is the ability of the vibrios to penetrate the mucus layer covering these cells. Intestinal mucus provides a chemotactic signal for *V. cholerae* whereby the vibrios direct

their movement toward the intestinal surface. *V. cholerae* are motile by the action of a single, polar, sheathed flagellum. Numerous studies have shown that motility and chemotaxis are important *V. cholerae* virulence properties (Guenzel and Berry, 1975; Freter *et al.*, 1981; Richardson, 1991; Gardel and Mekalanos, 1996). The flagellum has also been implicated in vibrio adherence to host tissue via the activity of a fucose-sensitive flagellum associated hemagglutinin (Attridge and Rowley, 1983). The precise contribution of the flagellar structure to intestinal colonization remains unknown. It has been hypothesized that the flagellum functions solely to promote motility and chemotaxis in order to enhance *V. cholerae* host-cell interactions. The resulting close association of the bacterial cell to host tissue would promote interactions between host-cell receptors and vibrio cell-surface adhesions. Alternatively the flagellar structure may actually bear an adhesion and be directly involved in vibrio binding to host cells.

A recent study examining vibrio motility and virulence gene expression uncovered a remarkable relationship between the two. Hyperswarming vibrio cells were found to be defective in TCP synthesis, toxin production and production of a cell-associated hemolysin while non-motile vibrios demonstrated increased expression of these virulence genes (Gardel and Mekalanos, 1996). Thus there is an inverse relationship between motility and virulence factor expression. These data suggest that early in the colonization process when the vibrios are actively swimming through the intestinal mucus layer virulence factors such as TCP and CTX are not produced. Upon reaching the underlying enterocytes motility would no longer be beneficial and maximal expression of toxin and pilus genes would occur. This mechanism would provide for temporal and spatial regulation of virulence factor expression at the appropriate time and place within the intestine.

### Methyl-Accepting Chemotaxis Proteins

In the previous section the role for motility in virulence gene expression was outlined. There also appears to be a role for vibrio chemotaxis proteins in the expression of ToxR regulon genes. The TCP gene cluster encodes a protein, TcpI which has been shown to negatively regulate the synthesis of the major pilin subunit of TCP (*tcpA*) in response to pH. *tcpI* mutants synthesize TCP at elevated pH levels that are normally repressive for pilus production (Harkey *et al.*, 1994). The ability of *tcpI* mutants to relieve pH repression of pilus synthesis suggests that TcpI may negatively regulate *tcpA* and subsequent *toxT* expression in response to pH. *V. cholerae tcpI* mutants display altered swarming in semisolid media compared to wild type vibrios. This observation indicates that the TcpI protein in addition to regulating pilus synthesis, is capable of interacting with the chemotaxis machinery of *V. cholerae*. Interestingly, TcpI is a homologue of enteric methyl-accepting chemotaxis proteins (Harkey *et al.*, 1994), a family of membrane spanning proteins involved in a signal transduction cascade which allows cytoplasmic response regulators to respond to external environmental signals. The influence of pH on

TCP synthesis *in vitro* may be a biologically significant finding since a pH gradient exists in the intestine. The pH in the lumen is relatively neutral to slightly alkaline whereas at the surface of the microvilli the pH is somewhat acidic. As mentioned earlier, optimal toxin/pilus synthesis occurs *in vitro* when the vibrios are cultured in acidic conditions with little to no synthesis at neutral to basic pH. The fact that vibrio *tcpI* mutants express *tcpA* at elevated pH, is consistent with the idea that *tcpI* somehow recognizes pH via its large periplasmic domain in order to activate *tcpA* transcription via its cytoplasmic domain. This may provide the vibrios a mechanism to activate *tcpA* expression when the vibrios encounter the appropriate microenvironment (*i.e.* the brush border of the small intestine).

**Accessory Colonization Factor (ACF)**

A subset of genes found within the VPI $\phi$  pathogenicity element encodes a set of proteins required for efficient intestinal colonization known as accessory colonization factor. The ACF and TCP genes clusters are physically and transcriptionally linked and thus may encode functions that are related. Mutations within 3 of the 4 *acf* genes produce altered motility/chemotaxis phenotypes. Interestingly, one of the genes (*acfB*) encodes a protein that is highly homologous to the TcpI protein described above and to enteric methyl-accepting chemotaxis proteins involved in bacterial chemotaxis (Everiss *et al.*, 1994). AcfB is predicted to share a number of structural features with the MCPs, including the number and organization of the transmembrane regions, the size of the cytoplasmic domains, and spatial conservation of the highly conserved signaling domain. Increased expression of *acfB* within vibrio

cells results in decreased swarm plate activity reinforcing the idea that AcfB is structurally and functionally related to enteric methyl-accepting chemotaxis proteins (Everiss *et al.*, 1994). The *acfC* gene lies immediately downstream of *acfB* which encodes a protein with significant homology to enteric sulfate binding proteins. Sulfate binding proteins from *E. coli* and *Salmonella* contain two signature sequences that are highly conserved among all sulfate binding proteins. The greatest similarity shared between AcfC and this family of proteins is located in the region containing the two signature sequences. Because mucus is rich in sulfated compounds, we tested the hypothesis that AcfB and AcfC represent a signal transduction pathway for vibrio recognition of, and chemotaxis toward sulfated compounds. *In vitro* capillary tube chemotaxis assays revealed that *V. cholerae acfC* mutants lack the ability to swim toward a gradient of galactose-6-sulfate as well as intestinal mucus (unpublished observation). We hypothesize that AcfC recognizes sulfated moieties present in mucus, and via an interaction with AcfB, provide the motile vibrios with a mechanism to guide them toward the intestinal mucosa. It had been known for some time that strains carrying *acf* mutations demonstrated reduced pilus and toxin synthesis. Interestingly, vibrios overexpressing AcfB from a multicopy plasmid agglutinate better than wild type strains. Agglutination is a property that is associated with the toxin-coregulated pilus and thus it appears as if AcfB is also required for efficient pilus synthesis (unpublished observation). The observation that AcfB is involved in regulating pilus gene expression may be significant given the role of TcpI (an MCP-like protein related to AcfB) in regulating pilus synthesis in response to environmental signals. These data suggest that AcfB

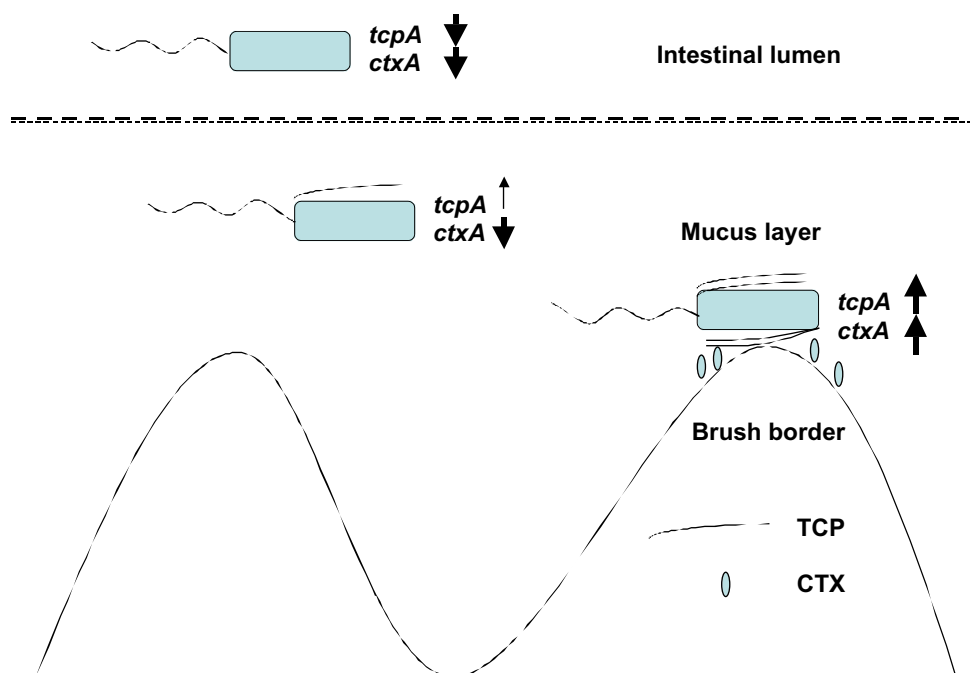


Figure 2. Synthesis of cholera toxin and toxin-coregulated pilus *in vivo*.

promotes the synthesis of CTX and TCP in response to intestinal mucus. The link between CTX and TCP synthesis suggest that ToxR/ToxT/TcpP regulatory cascade is involved.

### ***In vivo* Signaling**

Nearly all the information we have regarding the *V. cholerae* ToxR regulatory cascade is based on *in vitro* data. In some cases, such as the repressive effect that 37°C has on toxin and pilus synthesis, it is not readily apparent how this environmental signal could have the same effect inside the human gastrointestinal tract. An ingenious genetic approach using recombinase directed fusions to *tcpA* and *ctxA* promoters was recently employed during experimental infection of mice in an attempt to shed light on the function of the ToxR regulatory network *in vivo* (Lee *et al.*, 1999). Recombination-based *in vivo* expression technology (RIVET) utilizes a site-specific DNA recombinase that catalyzes excision of a selectable substrate gene (tetracycline resistance) from the bacterial genome as a transcriptional reporter. The site specific DNA recombinase gene (*tnpR*) is transcriptionally fused to a virulence gene promoter (*ctxA*, *tcpA*). Production of TnpR results in the conversion of the cell to tetracycline sensitivity due to the excision and loss of the *tet* gene. Thus the loss of the selectable marker serves as an *ex post facto* indicator of increased transcription of the gene fusion. The beauty of this system is that virulence gene activity can be monitored not only as a function of time but also as a function of the microenvironment the organisms finds itself in during infection. RIVET analysis revealed that intraintestinal activation of cholera toxin and toxin coregulated pilus genes are temporally distinct with induction of *ctxA* transcription following transcriptional induction of *tcpA* (Figure 2). These experiments confirmed that ToxT is required for expression of *tcpA* and *ctxA* *in vivo*. Perhaps not surprisingly, the activation patterns mediated by regulatory proteins previously shown to influence *in vitro* expression of *tcpA* was significantly different than during *in vitro* growth. ToxR/ToxS and TcpP/TcpH are both required for activation of *tcpA* *in vitro*, however either ToxR/ToxS or TcpP/TcpH is sufficient *in vivo*. *ctxA* activation requires both ToxR/ToxS and TcpP/TcpH *in vitro* whereas *in vivo* only ToxR/ToxS is required (not TcpP/TcpH). Remarkably, expression of *tcpA* occurs in two distinct phases with the 2-4 hr post infection phase dependent on TcpP/TcpH whereas during the second later 6-10 hr postinfection phase, ToxR/ToxS seems to be more important (Lee *et al.*, 1999). Based on the intragastric location of the vibrios at these time points, the authors speculate that the initial induction of *tcpA* is due to signals present in the lumen of the small intestine whereas the second more pronounced induction of *tcpA* transcription is mediated by signals present in the brush border. RIVET monitoring of the *ctxA* promoter revealed that its expression does not exhibit early phase induction and does not require TcpP/TcpH. Induction of *ctxA* occurred about 4 hr postinfection and was dependent on ToxR. These data suggest that maximum expression of *tcpA* requires high levels of ToxT brought about by the cooperative action of ToxR/ToxS and TcpP/TcpH whereas

transcription of *ctxAB* *in vivo* is less sensitive to ToxT levels, but also requires ToxR/ToxS for activation.

The identification of luminal and brush border signals by RIVET analysis is interesting given the phenotype of TcpI and AcfB mutants. AcfB mutants are partially defective in producing the toxin coregulated pilus and are also reduced for toxin production. Studies implicating AcfB in mucus recognition make this intestinal component a possible candidate for the luminal signal discovered by RIVET analysis. The ability of *tcpI* mutants to synthesize TCP at elevated pH levels that are normally repressive for TCP production suggests that *tcpI* may negatively regulate *tcpA* expression in response to local pH. Once the vibrios leave the lumen of the intestine which is relatively neutral and reach the surface of the microvilli where the pH is more acidic, TcpI would no longer repress TCP synthesis and thus full blown expression of *tcpA* could occur. Another possible brush border signal may be bile. Bile also causes TCP production to be shut down. Since the bile concentration in the lumen of the intestine is higher than that found at the surface of the microvilli, the ability of bile to repress TCP production would disappear as the vibrios reach the surface of the enterocytes (Shuhmacher and Klose, 1999).

### **Summary**

*Vibrio cholerae*, a member of the normal free-living microflora of estuarine waters, has evolved a sophisticated regulatory circuit to control the production of two key virulence factors at different times and places within the human small intestine to produce the devastating pandemic disease, Asiatic cholera. These events are all the more remarkable given the manner in which *V. cholerae* has acquired the pilus genes required for intestinal colonization, the toxin genes responsible for the pathological changes to the intestines of infected individuals and the sensory/regulatory protein genes required for the coordinated expression of the toxin/pilus genes in response to specific intestinal signaling molecules. Much of the information we have gleaned regarding the structure and function of the ToxR/ToxT/TcpP regulatory cascade is the result of *in vitro* studies examining transcriptional activation patterns of ToxR regulon genes in response to various culture conditions that alter toxin/pilus production. The development of *in vivo* methods of monitoring ToxR regulon gene expression patterns have begun to elucidate a spatial/temporal pattern of virulence gene regulation involving each of the components of the ToxR/ToxT/TcpP regulatory cascade. A component of the *V. cholerae* virulence cascade that remains difficult to address is the specific nature of the intestinal signals recognized by the various periplasmic/inner membrane signaling components of the ToxR regulon. A thorough understanding of ToxR regulon signal transduction pathways is important in that it may lead to the development of novel strategies for disrupting the *V. cholerae* virulence cascade.

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