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MINIREVIEW

Regulatory Networks Controlling *Vibrio cholerae* Virulence Gene Expression▽

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Cholera, a severe disease caused by *Vibrio cholerae* bacteria, has had a central role in the history of infectious disease research. The cholera studies of John Snow and Robert Koch, among many others, largely gave birth to modern epidemiology and microbiology. Despite its long history as a research target, cholera continues to afflict approximately 5 million people each year and remains an important public health problem in many areas of the globe. Here we review the current knowledge of the complex regulatory network used by *V. cholerae* to control expression of its virulence determinants.

FEATURES OF *VIBRIO CHOLERAE* PATHOGENICITY

Cholera, which is characterized by voluminous watery diarrhea, is produced when the gram-negative curved bacillus *V. cholerae* colonizes the upper small intestine of its human host. *V. cholerae* is found throughout the world in coastal areas, most often associated with aquatic fauna such as copepods and shellfish, and is transmitted to humans by contaminated food or water. Since 1817, seven cholera pandemics have been recorded, with the most recent, ongoing pandemic having begun in 1961. Although more than 200 different serogroups have been isolated from the environment, the O1 serogroup of *V. cholerae* was responsible for all seven pandemics. However, beginning in 1992, O139 serogroup strains were found to cause outbreaks of cholera as well; O139 *V. cholerae* is sometimes referred to as the eighth cholera pandemic strain.

V. cholerae O1 exist in two biotypes: classical and El Tor. Classical *V. cholerae* was responsible for the first six cholera pandemics, whereas the seventh pandemic has been caused by El Tor *V. cholerae*. The two *V. cholerae* biotypes differ considerably. El Tor strains generally cause a milder form of cholera than that caused by classical strains and apparently evolved as better survivors in the aquatic environment; currently, El Tor strains are predominant everywhere in the world that *V. cholerae* O1 can be found. *V. cholerae* O139 likely arose by seroconversion of O1 El Tor strains (8, 72). In addition, there are subtle differences in the way that El Tor strains and classical strains regulate expression of key virulence factors, as will be discussed below.

The clinical aspects of cholera are primarily induced by the activity of cholera toxin (CT), a bipartite toxin that consists of a single active A subunit and five B subunits that bind the toxin to the GM_1 ganglioside on the surface of the intestinal epithelium. Once inside epithelial cells, a proteolytically derived fragment of the CT-A subunit, CT-A_1, ADP-ribosylates $G_\alpha s$ protein, resulting in constitutive cyclic AMP production. This leads to massive secretion of chloride and water into the lumen of the intestine. Cholera patients can lose up to 20 liters of fluid within a 24-h period, resulting in rapid dehydration, and >50% of cholera patients die without treatment. However, if patients are rehydrated orally and/or intravenously, mortality rates decrease to ~1%.

Aside from CT, the other major *V. cholerae* virulence factor is the toxin-coregulated pilus (TCP), a type IV pilus that is required for intestinal colonization. The TCP causes aggregation of *V. cholerae* and induces microcolony formation within the intestine. However, the TCP is most likely not directly responsible for adhesion of *V. cholerae* to the intestinal epithelium. In addition to its role in colonization, the TCP acts as the receptor for the bacteriophage, CTXΦ, which harbors the *ctxAB* genes encoding the CT. The genes for TCP biogenesis are located in the *Vibrio* pathogenicity island in a large operon beginning with *tcpA*, which encodes the pilin subunit. The expression of other genes located within the *Vibrio* pathogenicity island is coordinately regulated with TCP expression by the same set of transcription regulators (see below). These other genes include the *acfA-D* genes, which encode accessory colonization factors. Insertional disruption of any of the *acf* genes results in a reduction in intestinal colonization by *V. cholerae*, as assessed in the infant mouse cholera model, but their function in colonization and/or pathogenesis is indeterminate.

Our purpose here is to review recent literature regarding the complex regulatory network that leads to expression of CT, TCP, and other virulence-associated genes. We explore *V. cholerae* virulence regulation by beginning with ToxT, the regulator most directly responsible for virulence factor expression, and then move on to describe the multiple regulatory inputs and pathways that contribute to whether the cell expresses ToxT or not.

TRANSCRIPTION ACTIVATION BY ToxT

The primary direct transcriptional activator of the *V. cholerae* virulence genes is ToxT protein (Fig. 1). ToxT is a member of the large AraC/XylS protein family (40); this family shares

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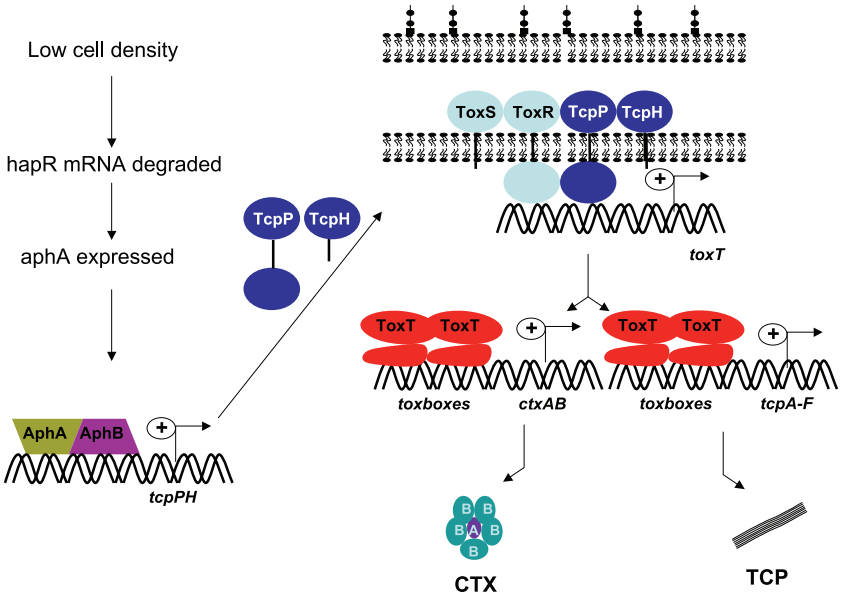

FIG. 1. Pathway by which cell density regulates expression of the major *V. cholerae* virulence factors CT and TCP. See the text for details.

an ~100-amino-acid domain that conveys DNA binding, via two helix-turn-helix motifs, and transcription activation activities (31, 63). ToxT has the AraC family domain at its C terminus; the remaining N-terminal 176 amino acids presumably form a secondary domain within ToxT (NTD), but the function of this domain is unclear. BLAST searches with just the ToxT NTD sequence return no homology to any other protein, and significant variation is found within the NTD in environmental *V. cholerae* strains (24), whereas the AraC family domain is nearly invariant among all known ToxT sequences. AraC family proteins consisting of only the conserved family domain exist, as do other AraC family proteins with a secondary domain either N or C terminal to the family domain activities (31, 63). Commonly, these secondary domains are involved in effector binding and/or multimerization. The natural effector of ToxT is proposed to be bile, which causes a decrease in the expression of CT and TCP (34, 84). Mutational analysis indicates that bile likely interacts with the ToxT NTD (79). A synthetic compound, virstatin, identified as an inhibitor of *ctx* gene expression in a high-throughput screen also inhibits ToxT activity by interacting with the ToxT NTD in the same region where bile is hypothesized to bind, suggesting that there is an effector-binding patch in this portion of the NTD (43).

Results of in vitro experiments suggest that ToxT binds to DNA as individual monomers (93, 94). Nevertheless, bacterial two-hybrid experiments suggest that ToxT NTDs can interact with each other (18), although full-length ToxT did not behave as a dimer in one of these studies (85). If dimerization of the NTD does occur, perhaps it occurs subsequent to DNA binding. Further evidence of NTD dimerization is that a truncated *toxT* allele expressing residues 6 to 167 acts in a dominant-negative fashion (85). Virstatin inhibits dimerization of ToxT in a bacterial two-hybrid assay and causes monomer accumulation in gel filtration studies but does not strongly inhibit ToxT-dependent activation of genes other than *ctx*, suggesting that dimerization is not an absolute requirement for ToxT to activate gene expression (85). As for the mechanism of dimerization, alanine scanning mutagenesis identified only one NTD mutation, F151A, that decreased apparent dimerization by as much as 50% (18). This could reflect a complex interaction surface between the two monomers that is not easily disrupted by any single mutation.

ToxT activates transcription by binding to a degenerate 13-bp DNA sequence, the toxbox (94) (Fig. 1). Toxboxes are located upstream of all known ToxT-activated genes. However, the configurations of toxboxes differ at different promoters. For example, at *tcpA*, ToxT binds to two toxboxes organized as a direct repeat between positions −44 and −67 relative to the start of transcription. In contrast, between the divergent *acfA* and *acfD* genes, ToxT binds to two toxboxes organized as an inverted repeat (93). From this pair of toxboxes both *acfA* and *acfD* transcription is activated by ToxT. ToxT activates transcription of *aldA*, which encodes an aldehyde dehydrogenase of unknown function in pathogenesis, by binding to a single toxbox (95). Toxboxes are always located upstream of, and not overlapping, the core −35 promoter element, suggesting that ToxT-activated promoters are all of the class I variety, and thus ToxT directly interacts with the α subunit C-terminal domain (α-CTD) of RNA polymerase to activate transcription (11). Overexpression of α subunit deleted for the CTD causes a large decrease in activation of *tcpA* by ToxT in accordance with this model (41).

Activation of *ctxAB* and *tcpA* transcription by ToxT is counteracted by the histone-like protein H-NS, which binds to the same DNA region (33, 76, 101). At the *ctxAB* promoter, H-NS strongly represses transcription, and much of the function of ToxT at *ctxAB* is to antirepress by competing with H-NS for DNA binding. However, once bound to the DNA, ToxT also directly activates *ctxAB* expression, presumably by interacting with RNA polymerase (101). At the *tcpA* promoter, the effect of H-NS is much less dramatic, and ToxT is primarily a direct activator of transcription. H-NS further reduces virulence gene

expression by binding to the *toxT* promoter region and repressing *toxT* transcription (76).

CONTROL OVER *toxT* EXPRESSION

TcpPH/ToxRS. The complex pathway of *V. cholerae* regulating *toxT* expression and, consequently, downstream virulence genes including *ctxAB* and *tcp* is often referred to as the "ToxR regulon," after the first identified positive regulator (77, 87) (Fig. 1). ToxR is a bitopic membrane protein containing a cytoplasmically localized DNA-binding/transcription activation domain, a transmembrane domain, and a periplasmic domain of unknown function. The amino-terminal DNA-binding domain is called a winged helix-turn-helix domain and is similar to that of the OmpR/PhoB family of transcriptional activators (64, 65). Wild-type ToxR activity requires the presence of another protein called ToxS. ToxS is also localized to the inner membrane but is thought to reside predominantly in the periplasm, where ToxR and ToxS are hypothesized to interact. The exact role of ToxS is unclear, but it appears to serve as an effector of ToxR function, perhaps by influencing stability and/or enhancing dimerization of ToxR (21, 23, 78).

To regulate expression of ToxT, ToxR acts in conjunction with a second transcription activator, TcpP, which, like ToxR, is membrane localized and has a cytoplasmic DNA-binding/activation and periplasmic domains. TcpP also requires the presence of a membrane-bound effector protein, TcpH, with which TcpP is thought to interact through its periplasmic domain (5, 15). Transcription of the operon encoding these two genes is responsive to environmental signals such as temperature and pH (6) and the production of specific autoinducers (discussed below).

Along with control over its gene expression, levels of TcpP are regulated by interaction with TcpH. In cells lacking TcpH TcpP is rapidly degraded (5). TcpP is also degraded in wild-type ($TcpH^+$) cells under conditions unfavorable for virulence gene expression in vitro, such as growth at pH 8.5 and 37°C (66). TcpP degradation is a regulated proteolytic event that requires at least two proteases working in sequence and is thus similar to the process by which a transmembrane protein called RseA—and its homologues—are degraded in *Escherichia coli* and other bacteria (2, 66) In fact, two-site proteolytic liberation of transcription factors from the membrane—termed regulated intramembrane proteolysis—is broadly conserved, being found in both prokaryotes and eukaryotes (10, 62).

In *E. coli*, envelope damage (or indicators of it, such as the presence of unfolded outer membrane proteins) induces proteolysis of an anti-sigma factor called RseA. RseA works by sequestering an alternative sigma factor, sigma E, to the membrane, thereby inactivating it. Sigma E is required for the cell to respond to the consequences of a damaged envelope; thus, under envelope stress, RseA is degraded in two distinct and successive steps by DegS and YaeL (also called RseP) (2). Such proteases, working in sequence, are generally termed site-1 and site-2 proteases. After RseA is eliminated, sigma E is released from the membrane and activates genes whose products are needed for surviving the envelope stress (this pathway is reviewed in reference 1).

For TcpP degradation, the consequence of the regulated intramembrane proteolysis is to destroy a transcription factor rather than to release one from inhibition, but the degradation process is partially conserved nonetheless. In TcpP degradation, the *V. cholerae* YaeL homologue serves as the site-2 protease, as in the degradation of *E. coli* RseA. However, the site-1 protease for TcpP has yet to be identified; it is not DegS (66). That DegS is not the site-1 protease suggests that the initiating signal for TcpP degradation may be distinct from signals that trigger sigma-E-dependent gene expression. In addition to the identity of the site −1 protease, a key unanswered question is the mechanism by which TcpH is released, allowing access of the proteases to TcpP. Given that regulation of TcpPH occurs both transcriptionally and posttranslationally, it appears that the activity of these regulators is a critical checkpoint in the cell.

Binding sites for ToxR and TcpP on the *toxT* promoter are located close together, with TcpP binding from positions −54 to −32 and ToxR binding upstream, from positions −104 to −68 (53). ToxR and TcpP appear to bind with different affinities to the *toxT* promoter, with ToxR binding more avidly (53). Overexpression of TcpP obviates the requirement for ToxR in *toxT* activation, but the converse is not true (37, 39, 53, 74). This suggests that TcpP is more directly responsible for transcription activation (i.e., stimulation of RNA polymerase) and that ToxR plays an indirect role. A working hypothesis is that ToxR provides *toxT* promoter recognition, and interaction of ToxR with TcpP fixes the latter into position for stimulating RNA polymerase, thus activating transcription. ToxR may also directly activate the *ctxAB* promoter within the host; how that may occur is not clear, although bile acids can stimulate partial activation of *ctxAB* by ToxR in vitro, and this could perhaps also occur in vivo (42, 55, 71).

Independently of TcpP, ToxR reciprocally regulates the production of two outer membrane proteins, OmpU and OmpT (19, 58, 70). OmpU requires ToxR for expression and confers resistance to some antimicrobial peptides and may also function as an adhesin (88). OmpT is maximally expressed in cells lacking ToxR, and its role in virulence has not been determined. Appropriate expression of these porins by ToxR is important for *V. cholerae* bile resistance, for intestinal colonization in mice, and for resistance to organic acids (58, 80). ToxR activates expression of *ompU* by binding DNA within the *ompU* promoter without the need for additional cofactors other than ToxS and RNA polymerase (19). *ompT* repression requires ToxR binding to a region of the *ompT* promoter where CRP is predicted to bind, thereby interfering with CRP activation of *ompT* transcription (58, 59). Another feature of ToxR regulation at the *ompU* and *ompT* promoters is that the ToxR winged-helix domain does not have to be localized to the membrane to bind those promoters, unlike the case for activation at the *toxT* promoter (20).

AphAB. The *toxRS* operon appears to be constitutively active, but *tcpPH* transcription is regulated by two activators, AphA and AphB, which are encoded by unlinked genes on the *V. cholerae* large chromosome (Fig. 1). AphA is in a family of regulatory proteins with homology to the PadR repressor, which controls expression of genes involved in the detoxification of phenolic acids (4). AphA binds the *tcpPH* promoter at a region of partial dyad symmetry between positions −101 and −71 relative to the transcriptional start site (51). AphA cannot activate transcription of *tcpPH* alone, requiring interaction

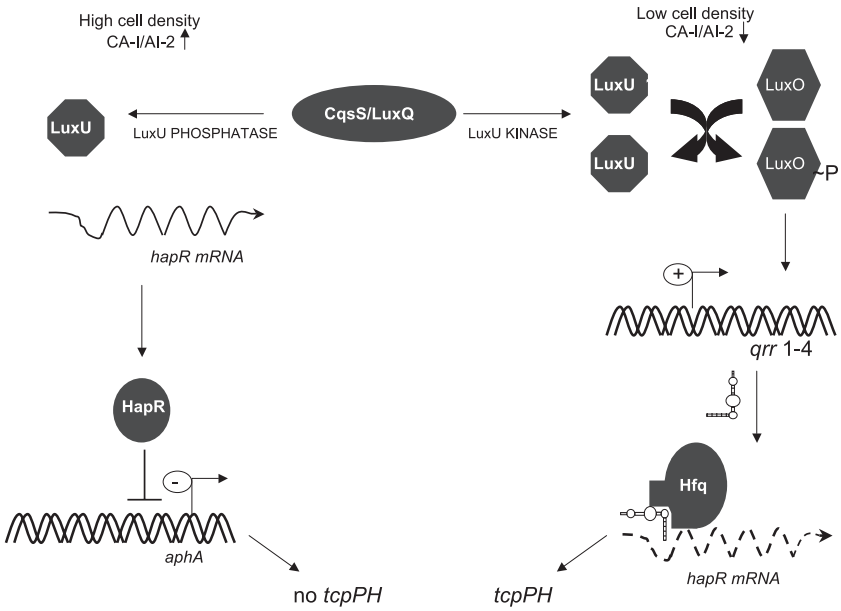

FIG. 2. Mechanism of quorum sensing using small RNAs that controls HapR levels in *V. cholerae*. See the text for details.

with the LysR-type regulator AphB that binds downstream of AphA, between positions −78 and −43 relative to the transcriptional start site (48, 51). This interaction is thought to stabilize AphB binding to its recognition site and result in activation of the *tcpPH* promoter under appropriate environmental conditions. Thus, activation of *tcpPH* by AphA and AphB is conceptually similar in mechanism to activation of *toxT* by ToxR and TcpP. The observation that El Tor and classical biotype *V. cholerae* regulate *toxT* differently (22) was explained by the discovery of a single base pair difference between the classical and El Tor *tcpPH* promoters that alters the ability of AphB to bind (49, 50). As discussed in greater detail below, AphA serves as a link between quorum sensing and virulence gene expression in some strains of *V. cholerae*, since its expression is subject to control by a mechanism involving bacterial cell density.

AphA regulates the expression of several other genes not associated with the *Vibrio* pathogenicity island (47). A set of genes involved in the biosynthesis of acetoin under some conditions is strongly repressed by AphA. Acetoin is a metabolic end product synthesized by a variety of bacteria with a neutral pH, as opposed to the organic acids that can be produced when bacteria are grown in the presence of excess glucose or other carbohydrates. Because *V. cholerae* is very acid sensitive, accumulation of acidic fermentation products in the growth medium results in a loss of viability. This metabolic regulation pathway may contribute to an important difference between the classical and El Tor biotypes of *V. cholerae*. Classical strains grow poorly in media containing high levels of glucose and lose viability due to production of organic acids and the resulting acidification of the growth medium (100). However, strains of the El Tor biotype grow much better in the presence of excess carbohydrates due to production of the neutral end products acetoin and 2,3-butanediol. AphA may repress acetoin biosynthesis more actively in classical strains than in El Tor, leading to increased fitness of the El Tor biotype in the environment and in the host.

QUORUM SENSING, SMALL RNA MOLECULES, AND HapR

Multiple quorum-sensing systems in *V. cholerae* act in parallel to regulate virulence gene expression, biofilm formation, and protease production (69). The central regulator ultimately under the control of these multiple systems is HapR, which links quorum sensing and virulence regulation through its control over *aphA* expression (52) (Fig. 2).

Quorum sensing system 1 is composed of the CAI-1 autoinducer, the structure of which is unknown, and a two-component sensor-kinase CqsS. System 2 is composed of AI-2 (a furanosyl borate diester), the periplasmic binding protein LuxP, and the two-component sensor LuxQ (17) (Fig. 2). The sensory information from both of these systems is conveyed through a phosphorelay mechanism that leads to a transcription factor called LuxO. A third system involves the VarS/VarA sensor kinase/response regulator pair, which activates expression of three small RNAs (CsrB, CsrC, and CsrD) (56). These sRNAs serve to inhibit the activity of the global regulatory protein, CsrA, which activates LuxO function independently of LuxU (69). The action of CsrA on LuxO is not direct, and the molecular mechanism of this event is unknown.

At low cell density, corresponding to a low autoinducer concentration, LuxU is phosphorylated by the sensor kinases and transfers its phosphate to LuxO (26, 27). LuxO~P activates the expression of four regulatory RNAs termed Qrr1 to -4 (for quorum-regulatory RNA) (57, 60). These sRNAs, together with the chaperone Hfq, bind the *hapR* mRNA and destabilize it. The result is that very little HapR protein is produced at low cell density. Alternatively, at high cell density the autoinducer concentrations increase, and the sensors CqsS

and LuxQ act as phosphatases rather than kinases. This leads to dephosphorylation of LuxO, rendering it inactive and consequently the *qrr* genes are not expressed. The result is stable production of *hapR* mRNA and its translation into HapR protein (Fig. 2).

HapR, a LuxR homolog, directly activates or represses a variety of genes. Virulence activation is downregulated by HapR because it represses *aphA* transcription (52). Biofilm formation is downregulated by HapR because it represses the *vps* operon encoding the polysaccharide component of the *V. cholerae* biofilm (see below). HapR upregulates expression of *hapA*, encoding the secreted hemagglutinin/protease that is responsible for detachment of bacteria from the intestinal epithelium in a process, also controlled by the stationary-phase sigma factor RpoS, called the "mucosal escape response" (25, 45, 75). *hapR* expression itself is repressed at high cell densities by HapR protein; however, the significance of this autorepression is currently unknown (61).

While quorum sensing is important in a variety of *Vibrio* species, some *V. cholerae* strains (including the sequenced El Tor N16961 strain) are not able to respond to the sensory input due to a frameshift mutation in *hapR* (102). Therefore, a functional quorum-sensing system does not appear to be an absolute requirement for virulence and biofilm formation in *V. cholerae*.

BIOFILM FORMATION AND THE VIRULENCE CONNECTION

Because of the HapR-dependent link between virulence expression and biofilm formation, it is worth reviewing what is understood of the process of biofilm formation. *V. cholerae* can survive both in environmental reservoirs and in human hosts. The ability to switch between two different phase variants, termed smooth and rugose, is thought to play a role in this survival. Phenotypic traits associated with the rugose phenotype include increased ability to form biofilms. Rugose variants produce VPS (for *Vibrio* polysaccharide), an exopolysaccharide that enables them to form well-developed biofilms and resist a variety of environmental stresses (73, 82, 92). The *vps* genes are located on the large chromosome of *V. cholerae* in two clusters: *vps*I (*vpsA-K*) and *vps*II (*vpsL-Q*) (92, 99); mutation in any of these genes results in a smooth-colony phenotype and reduced ability to form biofilms.

Transcription of the *vps* genes is controlled by VpsR, a σ^{54}-dependent two-component response regulator (98). The signals that lead to VpsR phosphorylation, and thus activation, are not known, and its cognate histidine kinase has not been identified. Transcription of the *vps* genes is also activated by another two-component response regulator, VpsT (16). VpsR and VpsT activate *vpsA* and *vpsL* expression and positively regulate their own and each other's transcription. Disruption of *vpsR* or *vpsT* in the rugose variant yields smooth colonies and prevents the formation of mature biofilms.

V. cholerae significantly enhances biofilm formation in response to bile acids (44). This is dependent on the *vps* genes and requires posttranslational activation of VpsR, and VpsT is not required. As noted above, HapR, the previously described quorum-sensing transcriptional regulator, represses the *vps* genes, as does CytR, a repressor of nucleoside uptake and catabolism (38).

VieSAB AND SECOND MESSENGER SIGNALING

Through HapR, quorum-sensing regulation controls both expression of virulence factors and formation of biofilms. The VieSAB signal transduction system also couples virulence gene expression and biofilm formation. The VieSAB proteins share similarities to sensor-kinase/response regulator two-component regulatory systems but deviate from the norm by having two putative response regulators, VieA and VieB. VieA appears to be a typical response regulator, with both a helix-turn-helix DNA-binding domain and a phosphoreceiver domain, while VieB contains only the phosphoreceiver domain and lacks a DNA-binding motif. VieS, the sensor kinase, was first identified in an in vivo promoter-trap experiment screening for positive regulators of *ctxA* expression (54). The screen identified a transposon insertion mutation in *vieS* that failed to induce expression of a *ctxA:tnpR* fusion during infection of infant mice. Although the *vieSAB* genes are located together on the large chromosome, it appears that the genes are differentially expressed. *vieB* expression only occurs in vivo during infection and requires TCP-mediated colonization (55). *vieS* is constitutively expressed during in vitro growth, whereas *vieA* expression is dependent on the presence of VieA during in vitro growth (54). A microarray study showed that VieA regulates 401 genes in the classical strain O395 but only 5 genes in the El Tor strain A1552. In the classical strain, *toxT* and ToxT-regulated genes were underexpressed in the *vieA* mutant, relative to the wild type. In addition, *vieA* mutant O395 expressed lower levels of genes for motility regulation, flagellum production, and the sigma-E regulon (7).

VieA works through a second messenger whose importance in bacterial cell signaling is becoming increasingly apparent. This molecule is 3′,5′-cyclic diguanylic acid (c-di-GMP), which controls a range of physiological and behavioral properties in both gram-negative and gram-positive bacteria (for a review, see reference 14). Biosynthesis and degradation of c-di-GMP is carried out through the activities of diguanylate cyclases and phosphodiesterases A, respectively. Two specific protein motifs have been associated with these activities: diguanylate cyclases typically have the conserved amino acid motif GGDEF, while phosphodiesterases A have the motif EAL (3). VieA contains an EAL domain, which acts as a c-di-GMP phosphodiesterase in vitro and in vivo (89). Through this activity, VieA maintains low intracellular c-di-GMP levels, negatively regulating biofilm formation and positively regulating virulence gene expression, including that of *toxT* (91).

CHITIN BINDING, REGULATION, AND COMPETENCE

V. cholerae is often found in the aquatic environment attached to the chitinous exoskeletons of zooplankton. This attachment likely enhances the survival of *Vibrio* species in the environment by providing a source of carbon and nitrogen, as well as a surface for the formation of biofilms. *V. cholerae* expresses a protein called GbpA, which binds to GlcNAc, a widespread compound that is both the constituent of chitin and a common modification of glycoproteins and lipids located on

the intestinal epithelium (46). GbpA is required for efficient intestinal colonization by *V. cholerae* and therefore links an environmental survival strategy—binding to chitinous structures—and human disease. Also, TCP production contributes to the colonization of chitinaceous surfaces due to the fact that *V. cholerae* TCP mutants are not able to form differentiated biofilms on chitin and do not form microcolonies (81). This suggests that *V. cholerae* producing TCP may have a fitness advantage in the environment over those that do not.

Various *V. cholerae* strains can become competent for natural transformation when grown on chitin (67). HapR is required for transformation competence, and high cell density positively controls this phenotype by relieving LuxO-dependent repression of HapR synthesis. Chitin-induced serogroup conversion mediated by natural transformation has been demonstrated in an experimental setting designed to mimic aquatic reservoirs, suggesting the importance of this process in the natural history of *V. cholerae* (9).

MOTILITY, CHEMOTAXIS, VIRULENCE, AND HOST-HOST TRANSMISSION

V. cholerae have a single, polar flagellum and are highly motile. There is significant dispute in the literature over the importance of motility for *V. cholerae* colonization of the intestine. Conflicting results have been obtained using different animal models and with El Tor versus classical biotype *V. cholerae* (28, 30, 32, 83, 90, 96, 97). Evidence from experiments using defined nonmotile mutant *V. cholerae* strains suggests that motility is indeed required for pathogenesis (54, 86). Work from many labs using both the infant mouse and rabbit ligated ileal loop models has indicated than nonmotile *V. cholerae* are defective at intestinal colonization. Furthermore, motility and expression of virulence genes are inversely correlated (32, 34–36). The currently favored model for *V. cholerae* infection is that motile vibrios localize to the crypts of the small intestine, after which their motility is reduced and virulence genes are expressed.

The role of chemotaxis in *V. cholerae* colonization has also been disputed in the literature and remains a subject of debate. Nonchemotactic *V. cholerae* colonize the intestine well but do so aberrantly (12, 29, 54). In the infant mouse, chemotactic *V. cholerae* colonize the lower small intestine, whereas nonchemotactic *V. cholerae* colonize throughout the small intestine, which results in higher numbers of colonizing bacteria compared to the wild type.

V. cholerae appears to become more transmissible by growing within the host during natural infection, a phenotype associated with changes in chemotaxis gene expression. This hypothesis derives from experiments showing that *V. cholerae* in human cholera stool outcompeted laboratory-grown, stationary-phase *V. cholerae* by 10- to 100-fold in an infant mouse model (68). This phenotype was maintained when the stool *V. cholerae* was incubated in pond water for up to 5 h but not after growth in LB medium. Competitive advantage of the stool *V. cholerae* was associated with repression of the *cheW* and *cheR* loci, whose products are required for chemotaxis (12, 13). Based on these studies, it was been proposed that *V. cholerae* repress chemotaxis when leaving the host. In contrast, work with rabbit ileal loops studying the mucosal escape response mentioned above showed that bacteria detach from the epithelial surface in a process that requires the stationary-phase sigma factor RpoS (75). One function of RpoS is to activate motility and chemotaxis functions. Further work is required to determine the precise role of chemotaxis during different phases of *V. cholerae* infection and escape from the host.

CONCLUSIONS

As befitting a pathogen that also is very successful living outside of human hosts, virulence gene regulation in *V. cholerae* is complex and linked very closely to other regulatory pathways in the cell. While expression of ToxT is the final committed step in virulence gene expression, there is not a direct, linear pathway from any one particular signal leading to transcription of *toxT*. Unlike other pathogens in which a principal difference in lifestyle is simply whether they are in the host or outside of the host, modulation of virulence gene expression in *V. cholerae* is intimately tied to whether or not the microbe is growing at high cell density or low cell density, with HapR levels serving as a measure of the two states. Each state might conceivably be achieved within a single infection, since a study of temporal and spatial patterns of virulence gene expression in vivo demonstrated that only a small fraction of the infecting inoculum may ultimately reach a site required to stimulate virulence gene expression to high levels (55). However, *V. cholerae* is also capable of growing to high levels during the course of an infection, after which it exits very efficiently and in large numbers. That virulence expression and the proposed mucosal escape response is so closely tied to HapR, which is controlled by cell density, makes sense in this "colonization/growth/escape" view of the infection. Even though we are coming to some broad understanding of this regulatory network, numerous questions remain. Are there yet-uncharacterized physiological states controlled by any of the major regulators in this system? The answer to that is almost certainly yes, as transcription profiling experiments to study the global effects of many of these regulators make clear. Further, the connection between motility and virulence is conceptually very appealing, but a precise mechanism linking these two complex traits has yet to be definitively established. Finally, the genetic program that controls the transmission state of *V. cholerae* is just now becoming appreciated, and future work will surely uncover interesting new knowledge in this important stage of *V. cholerae* regulation.

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