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Integration of Global Regulatory Mechanisms Controlling *Vibrio Cholerae* Behavior

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1. Introduction

Cholera is an acute water-borne diarrheal disease caused by the facultative Gram-negative bacterium *Vibrio cholerae* of serogroup O1 of the classical and El Tor biotypes and serogroup O139. Characteristics of this bacterium are its comma-shaped morphology, expression of a fast-rotating polar flagellum, and production of cholera toxin (CT). The O1 *V. cholerae* serogroup contains a common A antigen and can be subdivided in Ogawa and Inaba serotypes on the basis of serotype-specific antigens B and C, respectively (Kaper et al., 1995). Mankind has experienced seven recorded cholera pandemics. The seventh and current pandemic is characterized by the predominance of the O1 serogroup El Tor biotype, with periodic emergence of O139 strains, which exhibit a new lipopolysaccharide (LPS) and a capsule (Albert, 1994). Cholera, which continues to be a major public health concern in endemic areas of South Asia and Africa, is estimated to cause 5.5 million cases of disease and 130,000 deaths per year. The disease, which commonly occurs as rapidly spreading and difficult to contain outbreaks in low-income countries, is a common sequel of natural and human disasters. The typical cholera symptoms include a profuse rice-watery diarrhea and vomiting. If untreated, this condition can lead to severe dehydration, electrolyte imbalances, and death. Cholera infections can be effectively treated with oral rehydration and, in cases of severe illness, with antibiotics. Antibiotic treatment lessens the duration of illness and reduces the excretion of highly infective *Vibrios* (Nelson et al., 2011). The downside however, is the emergence of multiple-antibiotic resistant O1 and O139 strains (Das & Kaur, 2008; Roychowdhury et al., 2008; Okeke et al., 2007; Mwansa et al., 2007; Faruque et al., 2007).

As illustrated in Fig. 1, the cholera bacterium is fundamentally an organism adapted to the aquatic environment, which has evolved to maximize the benefit of being casually ingested by humans. The goal of this chapter is to examine the global regulatory mechanisms that assist the cholera bacterium in colonizing the small bowel of humans and persisting in the aquatic environment.

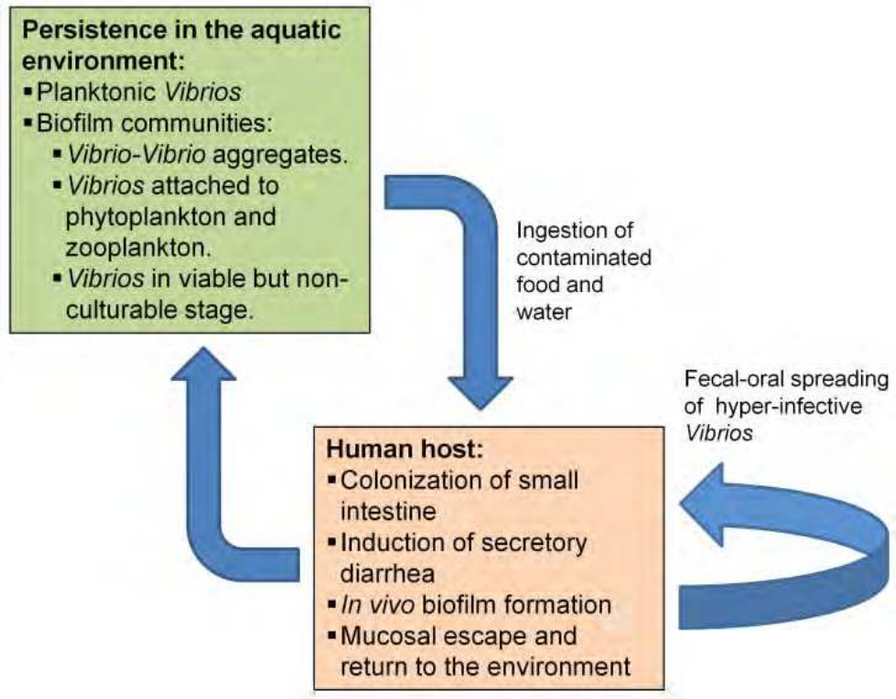

Fig. 1. The *V. cholerae* life cycle

2. The Vibrio cholerae dual life cycle

2.1 *Vibrio cholerae* persistence in the environment

V. cholerae occur globally in most estuaries and coastal ecosystems, where their concentrations range from 10^1 to 10^4 cells per mL and can reach 10^6 cells per g of sediment (Urakawa & Rivera, 2006). In nature, *Vibrios* are subject to various physical and chemical environmental stresses, which include nutrient limitation, extreme temperatures, and oxidative stress. The persistence of *Vibrios* in the aquatic environment is additionally challenged by protozoan grazing and bacteriophage infection (Matz et al., 2005; Jensen et al., 2006; Faruque et al. 2005, 2005a). The bacterium can be found in the form of planktonic free-swimming cells or as sessile biofilm communities associated with phytoplankton and zooplankton (Watnick & Kolter, 1999; Kierek & Watnick, 2003; Huq et al., 1983; Islam et al., 1990; Kaper et al., 1979). The capacity of *V. cholerae* to form biofilm communities has been proposed to be involved in bacterial survival in the aquatic environment (Faruque et al., 2006: Joelsson et al., 2006: Matz et al., 2005: Schoolnik & Yildiz, 2000). Biofilm formation and adoption of a rugose colonial morphology correlate with the production of *V. cholerae* exopolysaccharide (*vps*) (Yildiz & Schoolnik, 1999). The *V. cholerae* rugose colonial variant described by White (1938) is more resistant to chlorinated water (Morris et al., 1996: Rice et al., 1992) and to osmotic and oxidative stresses (Wai et al., 1998: Yildiz & Schoolnik, 1999). In aquatic ecosystems, *V. cholerae* can also be found in the form of large biofilm aggregates of partially dormant cells that resist cultivation in conventional media but can be recovered as virulent *Vibrios* by animal passage (Faruque et al., 2006). These biofilm aggregates, named conditionally viable environmental cells (CVEC), appear to be similar to previously described viable but not culturable cells (Xu et al. 1982). The role of these biofilm aggregates in infection is considered below.

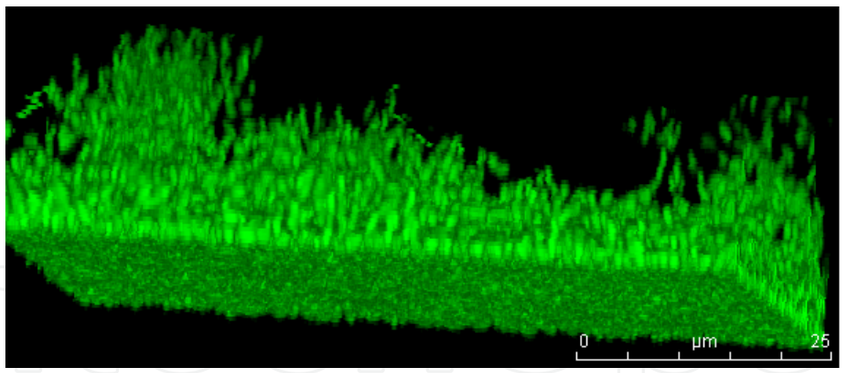

Fig. 2. Confocal microscopy three-dimensional image of a *V. cholerae* biofilm stained with the fluorescent dye, SYRO-9, and imaged using 485- and 498-nm excitation and emission wavelength, respectively. Biofilm developmental is initiated by a reversible surface attachment, followed by changes in gene expression patterns conducive to more permanent adherence, synthesis of exopolysaccharide matrix material, and building of three-dimensional columnar aggregates and channels.

2.2 Transition of Vibrio cholerae between the aquatic environment and the human intestine

A cholera infection starts with human ingestion of *V. cholerae* present in contaminated water or food (Fig. 1). Infecting *Vibrios* that survive passage through the acidic stomach compartment progress to the nutrient-rich environment of the human small intestine. It has been suggested that *V. cholerae* biofilm aggregates are more resistant to the initial low pH stress (Zhu & Mekalanos, 2003). Mutations in *vps* genes that block biofilm matrix exopolysaccharide biosynthesis impair colonization in the suckling mouse model (Fong et al., 2010). In addition, deletion of genes encoding the alternative stress-related sigma factors σ^S and σ^E inhibit bacterial colonization (Merrel et al., 2000; Kovacikova & Skorupski, 2002). *Vibrios* use their fast-rotating polar flagellum to swim toward and bind to the mucus layer through their LPS (Benitez et al., 1997), the GbpA adhesin (Bhowmick et al., 2008; Jude et al., 2009), and other factors. Subsequent colonization requires expression of the toxin co-regulated pilus (TCP) (Herrington et al., 1988). Intestinal fluid secretion results from production by colonizing *Vibrios* of CT, which acts by increasing the cAMP content of host cells. Dissemination of the infection throughout the small bowel most likely involves detachment of *Vibrios* in a motile stage that could swim toward and adhere to other sites along the small intestine. *Vibrio* detachment and adherence could create new infective foci and enhance the severity of the disease. In the course of this process, however, *Vibrios* that detach but fail to adhere and establish new infection foci can be cleared from the small intestine by peristalsis (Walker & Owen, 1990) and excreted in the rice-watery diarrhea. As the overall population of *Vibrios* increases, and nutrients become in short supply, detachment predominates over re-colonization, a process also known as mucosal escape (Nielsen et al., 2006). Late in infection and, in preparation for their extra-intestinal life, *Vibrios* associate into biofilm aggregates prior to exiting the host (Faruque et al., 2006). Such biofilms, formed *in vivo*, are in a stage of transient hyperinfectivity (Tamayo et al., 2010) that enhances their dissemination through the fecal-oral route (Merrel et al., 2002) (Fig. 1). This view of the time course of a cholera infection is consistent with the presence of highly motile planktonic *Vibrios* and biofilm aggregates in freshly shed cholera stools.

The timing of events that occur during infection is difficult to ascertain, since current models likely yield average data from *Vibrio* subpopulations at different stages of the infective process. A promising approach in this direction has been the development of a recombination-based *in vivo* expression technology (RIVET) (Camilli & Mekalanos, 1995; Lee et al., 1999). With this approach, it has been reported that *tcpA* and *ctxA* are expressed within the first 6 h of infection in the infant mouse intestine (Lee et al., 1999). Nevertheless, much remains to be learned about the events, occurring later in infection, that are involved in bacterial dissemination within the host and their exit to the environment.

3. Major virulence factors

V. cholerae O1 and O139 strains, which cause epidemic cholera, exhibit three major characteristics: (a) production of CT, (b) expression of TCP, and (c) expression of a sheathed polar flagellum. *V. cholerae* produces additional potentially toxic factors, such hemagglutinin (HA)/protease (Hase & Finkelstein, 1991), hemolysin (Nagamune et al., 1996), the repeat toxin (RTX) (Lin et al., 1999), the zonula occludens toxin (Fasano et al., 1991), and the accessory cholera enterotoxin (Trucksis et al., 1993). The potential contributions of these secondary factors to the infective process has been reviewed by Fullner (2003). The regulatory pathways that control virulence, motility, and biofilm formation have been extensively studied and reviewed elsewhere (Childers & Klose, 2007; Matson et al., 2007). In this chapter, we discuss how these regulatory pathways are interconnected.

3.1 Cholera toxin and the toxin co-regulated pilus

CT is an ADP-ribosyl transferase responsible for the profuse rice-watery diarrhea typical of this disease (Finkelstein, 1992; Kaper et al., 1995). It is composed of one A subunit (CTA) which catalyzes NAD-dependent ADP-ribosylation of host adenylate cyclase and five B subunits (CTB) that carry the ganglioside GM_1 receptor binding site (Finkelstein, 1992). The genes encoding CTA (*ctxA*) and CTB (*ctxB*) are located in the genome of the filamentous phage CTXΦ (Waldor & Mekalanos, 1996). The CTXΦ receptor is the type IV pilus and colonization factor TCP (Waldor & Mekalanos, 1996). The expression of CT and TCP is co-regulated by a complex regulatory network. At the top of the regulatory cascade, the regulator AphA enhances transcription of the transmembrane regulators TcpP and TcpH (Hase & Mekalanos, 1998; Kovacikova & Skorupski, 2001). AphA alone cannot activate transcription of *tcpPH*, but requires interaction with the LysR-type regulator, AphB which binds downstream of the AphA binding site to the *tcpPH* promoter (Kovacikova & Skorupski, 2001). TcpPH, in concert with the transmembrane regulators ToxR and ToxS (Miller & Mekalanos, 1985: Miller et al., 1989) (Fig. 3), activates expression of the soluble regulator, ToxT (DiRita et al., 1991). Finally, ToxT interacts with the *ctxA* and *tcpA* promoters to activate production of CT and TCP (DiRita et al., 1991).

3.2 Motility

Motility is necessary for *V. cholerae* to establish infections, for colonization of the small intestine, to detach and spread along the small intestine, and/or to exit the host and return to the environment (Butler & Camilli, 2004; Lee et al., 2001; Nielsen et al., 2006; Silva et al., 2006). In addition, shedding of *V. cholerae* flagellins induce an inflammatory response in the

host by interacting with Toll-like receptor V to induce the production of pro-inflammatory interleukin-8 (Harrison et al., 2008; Rui et al., 2010; Xicohtencalt-Cortes, et al. 2006). Flagellar motility also influences the expression of CT and TCP (Gardel & Mekalanos, 1996; Hase, 2001; Hase & Mekalanos, 1999: Hase et al., 2001; Silva et al., 2006; Syed et al., 2009).

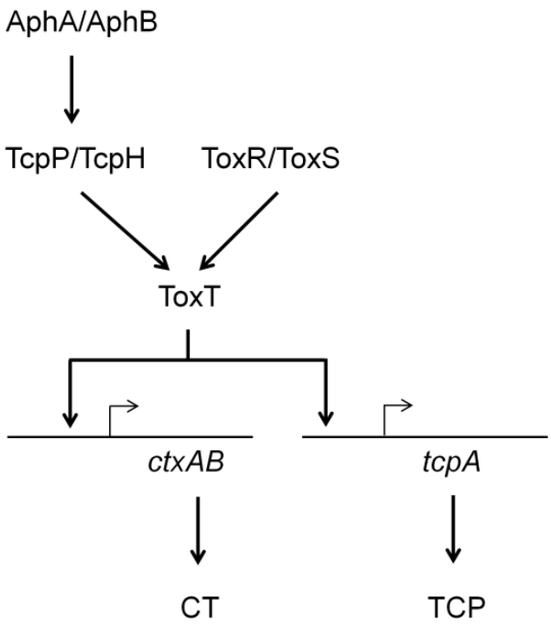

Fig. 3. Regulation of CT and TCP expression

Motility is a complex phenotype that requires (a) the synthesis and export of the flagellum and its motor, (b) coupling of the flagellum motor to an energy source and (c) coupling of flagellum rotation to numerous chemosensory pathways. The *V. cholerae* genome encodes multiple flagellin genes (*fllaABCDE*), but only flagellin mutants lacking FlaA are non-flagellated (Klose & Mekalanos, 1998; Klose et al., 1998; Klose & Mekalanos, 1998a). The expression of motility requires a hierarchical regulatory cascade involving the alternative RNA polymerase (RNAP) subunits, σ^{54} and σ^{28} and the σ^{54}-dependent transcriptional activators, FlrA and FlrC (Correa et al., 2004; Correa & Klose, 2005; Correa et al., 2000; Correa et al., 2005; Prouty et al., 2001; Syed et al., 2009).

The *V. cholerae* polar flagellum is powered by sodium motive force (Kojima et al., 1999). *V. cholerae* expresses Na^+ pumps such as the Na^+-translocating NADH: quinone oxidoreductase and multiple Na^+/H^+ antiporters responsible for maintaining the inward Na^+ gradient that drives flagellum rotation (Hase et al., 2001). Genes required for flagellum rotation include *pomA* (*motA*), *pomB* (*motB*), *motX, motY, fliG, fliM and fliN*. Inactivation of *motA motB*, *motY* or *motX* by mutation abolish motility but does not prevent flagellum assembly (Boles & McCarter, 2000; Kim & McCarter, 2000; McCarter, 2001). MotA and MotB translocate Na^+ by forming the Na^+ conducting channel; MotX and MotY are required for torque generation (Asai et al., 1997). FliG, FliM and FliN, also required for torque generation, form the switch complex at the base of the flagellum basal body (Boles & McCarter, 2000; McCarter, 2001). The direction of flagellum rotation (clockwise or counterclockwise) is dictated by the interaction between the response regulator CheY3 and the FliM component of the motor

(Berg, 2003; Boin et al., 2008; Hyakutake et al., 2005). The *V. cholerae* genome contains numerous chemotaxis-related genes, including multiple methyl-accepting chemotaxis proteins (MCP), methyltransferases (CheR), methylesterases (CheB), linker proteins (CheW), histidine kinases (CheA), and response regulators (CheY), mostly located in three clusters (Boin et al., 2008). Only a limited number of genes, however, have been demonstrated to be essential for chemotaxis. These genes include *cheA*-2 (Gosink et al., 2002), *cheR*-2 (Boin et al., 2004) and *cheY*-3 (Hyakutake et al., 2005). The function of other chemotaxis genes and the reason for their redundancy are not understood.

Transcription hierarchy class	**Class I**	**Class II**	**Class III**	**Class IV**
Upstream regulator	-	RpoN (σ^{54}) and FlrA	RpoN (σ^{54}) and FlrC	FliA (σ^{28})
Genes	*flrA*	*flrBC* *fliEFGHIJ* *flhA operon* *fliA*	*flgBCDEFGHIJ* *fliKLMNOPQ* *motY* *flaA* *flhB* *flgKLOPT*	*motAB* *motX* *flaBCDE*
Function	σ^{54}-dependent activator	Regulatory factors, MS ring-switch and export components	Basal body-hook, major flagellin, motor component	Alternative flagellins, anti-sigma factor FlgM, motor components

Table 1. Transcriptional organization of motility genes (adapted from Syed et al., 2009; Prouty et al., 2001).

We recently developed and validated a high-throughput screening assay for inhibitors of *V. cholerae* motility (Rasmussen et al., 2010). A new inhibitor consisting of a quinazoline 2,4-diamino analog (Q24DA) induced a flagellated, non-motile phenotype and was specific for the Na^+-dependent polar flagellum motor of pathogenic *Vibrios* (Rasmussen et al., 2010). While some motility mutants express more CT and TCP (Silva et al., 2006, Syed et al. 2009), blocking motility with Q24DA diminished CT and TCP expression. Thus, the relationship between motility and CT expression could be more complex than anticipated by genetic studies. Identification of the molecular target of Q24DA and other inhibitors is required to clarify the disconnection between the genetic and chemical approaches.

3.3 Hemagglutinin/protease

Numerous *V. cholerae* strains of the El Tor biotype express a Zn-dependent metalloprotease (mucinase) known as hemagglutinin (HA)/protease (Finkelstein et al., 1983; Hase & Finkelstein, 1991). HA/protease enhances enterotoxicity in the rabbit ileal loop model of cholera (Ichinose et al., 1994; Silva et al., 2006) and contributes to live vaccine candidates' reactogenicity in humans (Benitez et al., 1999; Garcia et al., 2005). In cell culture, HA/protease perturbs the paracellular barrier of intestinal epithelial cells (Mel et al., 2000: Wu et al., 1996) by acting on tight junction-associated proteins (Wu et al., 2000). A second

proposed role for HA/protease is to facilitate *V. cholerae* detachment from the intestinal mucosa when infecting *Vibrios* reach a high cell density (Finkelstein et al., 1992; Benitez et al., 1997; Silva et al., 2003; Silva et al., 2006; Robert et al., 1996). Consistently, inactivation of *hapA* encoding HA/protease enhances adherence to mucin-coated polystyrene plates (Silva et al., 2006), adherence to mucin-secreting differentiated HT29-18N2 cultured cells (Benitez et al., 1997), and colonization of the suckling mouse intestine (Robert et al., 1996; Silva et al., 2006). The mucinase activity of HA/protease (Finkelstein et al., 1983), together with its capacity to cleave the mucin-binding adhesin GbpA (Jude et al., 2009) at high cell density, has provided a mechanism supporting the "detachase" function attributed to this protein. The high viscosity of the mucus layer promotes breakage and loss of the polar flagellum (Liu et al., 2008). We have proposed that production of extracellular proteases facilitates preservation of the flagellum of *V. cholerae* during detachment by decreasing the viscosity of the medium (Silva et al., 2003). This could result from HA/protease degradation of preexisting mucin (Finkelstein et al., 1983) and cleavage of the GbpA adhesin, which enhances the production of intestinal mucins (Bhowmick et al., 2008).

4. Global regulatory networks controlling *Vibrio cholerae* behavior

In a dynamic environment, the capacity of *V. cholerae* to switch between planktonic and sessile life styles or from virulence to detachment mode in response to environmental changes is essential. In the following sections, we discuss our current understanding of how *V. cholerae* integrates overlapping extracellular stimuli to adopt one or the other lifestyle.

4.1 Adenylate cyclase and cAMP signaling

Cyclic AMP (cAMP) is synthesized from ATP by the activity of adenylate cyclase. *V. cholerae* possesses only one adenylate cyclase, which belongs to the type-I (enterobacterial) class (Danchin, 1993: Baker et al., 2004). This enzyme is monomeric and consists of an N-terminal catalytic domain and a C-terminal regulatory domain. The C-terminal regulatory domain contains the His residue suggested to be phosphorylated by the phospho-EIIA^{glc} component of the phosphoenolpyruvate phosphotransferase system (PTS), leading to its activation (Baker et al., 2004). The PTS is a phosphoryl cascade that allows the transport and phosphorylation of sugars (Deutscher et al., 2006; Deutscher, 2008). It acts as sensory system, feeding information to adenylate cyclase to regulate bacterial behavior in response to the availability of sugars in the medium and the energy state of the cell (Lengeler et al., 2009). In the PTS, phosphate is transferred from phosphoenolpyruvate to a sugar by a pathway that sequentially involves enzyme I (EI), the protein HPr, and a sugar-specific enzyme II (EII) complex. The different EII complexes are characterized by their domains (A, B, C) present either on a single or distinct polypeptide chains. In the presence of a rapidly metabolizable sugar (i.e., D-glucose) phospho-EIIA^{glc} donates its phosphate to the sugar, leading to lower adenylate cyclase activity and lower intracellular concentrations of cAMP.

4.2 Cyclic diguanylate

In a broad spectrum of bacterial species, the second messenger, cyclic diguanylic acid (c-di-GMP), regulates the transition between sessile and motile lifestyle by activating biofilm formation and inhibiting motility (D'Argenio & Miller, 2004; Hengge 2009; Simm et al., 2004;

Tamayo et al., 2007). Cyclic di-GMP is synthesized from GTP by GGDEF domain family proteins that exhibit diguanylate cyclase (DGC) activity. On the other hand, proteins of the EAL and HD-GYP families exhibit a phosphodiesterase (PDE) activity degrading c-di-GMP to GMP (Galperin, 2004). The *V. cholerae* genome contains 31 genes encoding GGDEF domain family proteins; 10 genes encoding proteins with GGDEF and EAL domains; 12 genes encoding proteins with only EAL domains; and 9 genes encoding proteins with HD-GYP domains (Galperin, 2004). In *V. cholerae*, over-expression of the DGC, VCA0956, abolishes swimming, whereas expression of the PDE, VieA, enhances it (Tischler & Camilli, 2004). Transcriptional profiling has revealed that genes involved in flagellum biosynthesis, motility, and chemotaxis are repressed in response to an increase in intracellular c-di-GMP (Beyhan et al., 2006). The signaling pathways responsible for the phenotypic consequences of increasing the c-di-GMP pool are not fully understood. Potential c-di-GMP binding proteins include those containing the PilZ domain (Pratt et al., 2007). The *V. cholerae* genome contains five PilZ domain proteins (Pratt et al., 2007). Of these, PlzA and PlzE appear to be essential; PlzB, PlzC, and PlzD affect *V. cholerae* motility; and PlzC and PlzD bind to c-di-GMP *in vitro*. The positive regulator of biofilm formation, VpsT, can directly senses c-di-GMP to modulate motility and biofilm formation (Krasteva et al., 2010). Finally, there are two riboswitches responsive to c-di-GMP changes in the *V. cholerae* genome (Sudarsan et al., 2008). The function of these riboswitches in cholera infections is currently unknown.

4.3 Quorum sensing

Quorum sensing is a process by which bacteria communicate with one another by secreting extracellular signaling molecules termed autoinducers. In *V. cholerae*, two autoinducer/sensor systems have been identified. System 1 consists of cholera autoinducer 1 (CAI-1, 3-hydroxytridecane-4-one), synthesized by the activity of CqsA, and its cognate receptor, CqsS (Higgins et al., 2006: Miller et al., 2002). System 2 consists of an AI-2 molecule (a furanosyl borate diester), synthesized by the activity of LuxS, and its cognate receptor, LuxPQ (Chen et al., 2002; Miller et al., 2002). Sensory information is fed through a phosphorelay system to LuxO. At low cell density, the autokinase domains of CqsS and LuxPQ become phosphorylated, and phosphate is transferred to LuxU and then LuxO (Miller et al., 2002). Phospho-LuxO then activates expression of multiple, redundant small regulatory RNAs (sRNAs or *qrr*), which promotes translation of the mRNA encoding AphA and destabilize the *hapR* mRNA (Lenz et al., 2004; Rutherford et al., 2011). In addition, the global regulator, CsrA, and the small nucleoid protein factor for inversion stimulation (Fis) enhance phospho-LuxO activity to promote degradation of *hapR* mRNA at low cell density (Lenz et al., 2005; Lenz et al., 2007). When the amount of CAI-1 and AI-2 produced by growing bacteria reaches a threshold value, CqsS and LuxPQ switch from kinase activity to phosphatase. The flow of phosphate is reversed, and phospho-LuxO becomes de-phosphorylated and inactive (Miller et al., 2002). At this stage (high cell density), HapR is expressed (Zhu et al., 2002). The consequences of HapR expression include (a) diminished expression of CT and TCP due to transcriptional repression of *aphA* (Kovacikova and Skorupski, 2002a; Lin et al., 2007), (b) inhibition of *vps* expression (Waters et al., 2008), and (c) activation of *hapA* encoding HA/protease (Jobling & Holmes, 1997). The transition into or out from the quorum-sensing mode appears to be finely regulated by additional mechanisms. For instance, upon transiting into quorum-sensing mode (high cell density),

HapR binds to and represses its own promoter (Lin et al., 2005). Conversely, upon dilution of a high density culture, HapR activates the transcription of *qrr* sRNAs to promote rapid degradation of its own mRNA (Svenningsen et al., 2008). Further, at low cell density, the LuxR-type regulator, VqmA, enhances *hapR* transcription (Liu et al., 2006). In conclusion, at low cell density, *V. cholerae* expresses CT, TCP, and synthesizes matrix exopolysaccharide (*vps*); at high cell density, these functions are repressed, and production of HA/protease is activated (Fig. 4).

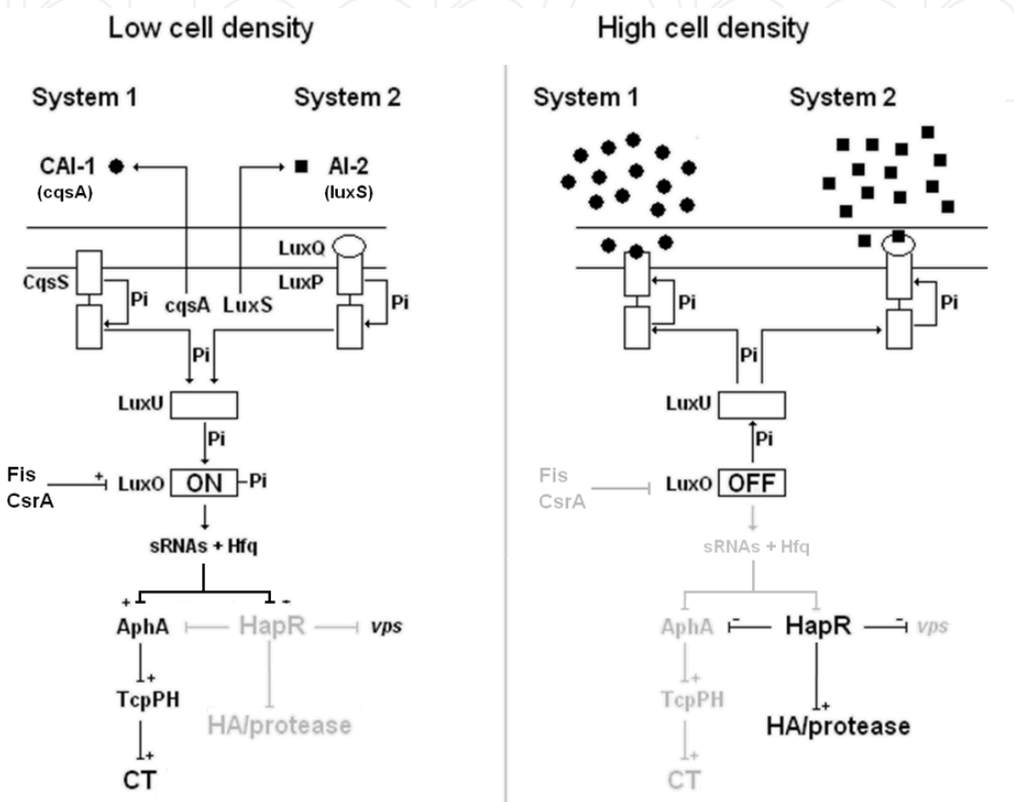

Fig. 4. Quorum sensing in *Vibrio cholerae*

4.4 Quorum sensing regulation of biofilm formation

The formation of a three-dimensional, mature biofilms involves a complex genetic program that includes the expression of motility and mannose-sensitive hemagglutinin for surface attachment and monolayer formation, as well as the biosynthesis of an exopolysaccharide matrix (Watnick & Kolter, 1999). Biofilm formation precedes adoption of the conditionally viable environmental cell stage described in section 1.3 (Kamruzzaman et al., 2010). In *V. cholerae*, biofilm formation is repressed by the master quorum sensing-regulator, HapR (Zhu & Mekalanos, 2003; Hammer & Bassler, 2003; Yildiz et al., 2004). The genes responsible for *vps* biosynthesis are clustered in two operons in which *vpsA* and *vpsL* are the first genes of operon I and II, respectively (Yildiz & Schoolnik, 1999). The expression of *vps* genes is regulated by a complex network involving a growing number of factors. For instance, the second messenger, c-di-GMP, enhances *vps* expression (Fong & Yildiz, 2008; Beyhan et al., 2006; Beyhan et al., 2007; Lim et al., 2006; Lim et al., 2007; Tischler & Camilli, 2004). Biofilm

formation is also modulated by interplay between the positive transcription regulators, VpsT (Casper-Lindley & Yildiz, 2004) and VpsR (Yildiz et al., 2001), and the negative regulator, CytR (Haugo & Watnick, 2002). In addition, *vps* expression is modulated by the PhoBR two-component regulatory system (Pratts et al., 2009, 2010; Sultan et al., 2010) and by components of the PTS phosphoryl cascade (Houot et al., 2008; 2010, 2010a).

4.5 The cAMP receptor protein (CRP)

CRP is a member of the CRP/FNR family of transcriptional regulators known for its role in carbon catabolite repression, a process in which the presence of a favorable carbon source in the medium inhibits expression of enzymes involved in the catabolism of other carbon sources (Brückner & Titgemeyer, 2002; Stülke and Hillen, 1999). Activation of adenylate cyclase leads to high intracellular levels of cAMP. Then, cAMP binds to CRP to form a complex that acts at responsive promoters to activate or repress transcription (Brückner & Titgemeyer, 2002; Stülke & Hillen, 1999) The cAMP-CRP complex binds as a dimer to the consensus sequence TGTGA-(N_6)-TCACAA which can be found within, adjacent to or upstream from responsive promoters. The complex is believed to assist in binding of RNAP to the promoter by bending the DNA molecule. *V. cholerae crp* mutants form small colonies, are less motile, do not express *hapA* (Benitez et al., 2001) and are defective in colonization of the suckling mouse intestine (Skorupski and Taylor, 1997). The cAMP-CRP complex negatively affects CT and TCP expression by directly repressing the *tcpPH* promoter (Skorupski & Taylor, 1997; Kovacikova & Skorupski, 2001). The fact that *crp* mutants show reduced colonization in the suckling mouse, although expressing elevated TCP, suggests that CRP is required for the expression of additional colonization factors.

As a global regulator, CRP indirectly affects the expression of many genes by controlling the expression of a broad range of transcriptional factors. As an example, an isogenic Δ*crp* mutant of *V. cholerae* strain C7258 expressed elevated *fis* mRNA and lower levels of mRNAs encoding the general stress response regulator, RpoS, and the histone-like nucleoid structuring protein (H-NS) (Fig. 5) (Silva and Benitez, 2004; Liang et al., 2007). Gene expression profiling of a *crp* deletion mutant revealed 174 differentially expressed genes. With the exception of conserved hypothetical proteins, most differentially expressed genes fell into the functional categories of energy metabolism, transport and binding protein, and cellular processes (Fig. 6) (Liang et al., 2007). Furthermore, 77 % of the differentially expressed genes were down-regulated, suggesting that CRP most frequently acts as a positive regulator in *V. cholerae*. The *crp* mutant exhibited diminished expression of genes involved in motility and chemotaxis, outer membrane protein expression, genes specifically induced in rabbit ileal loops, and *rpoE* encoding σ^E (Liang et al., 2007). These data explain the colonization defect exhibited by *crp* mutants. Among the differentially expressed genes, *cqsA* (VCA0523) encoding CAI-1 synthase and *hapR*, encoding the master quorum sensing regulator, HapR, were diminished. Another gene (VC0291), annotated as coding for a NifR3/Smm1 family protein was up-regulated in the *crp* mutant. Tn5 insertions in this locus reduced the expression of the small nucleoid protein, Fis (Lenz & Bassler, 2007), a regulator that enhances degradation of *hapR* mRNA at low cell density (Lenz & Bassler, 2007). Since VC0291 and *fis* are predicted to be part of an operon (Osuna et al., 1995), this finding is consistent with CRP being a repressor of Fis (Fig. 5).

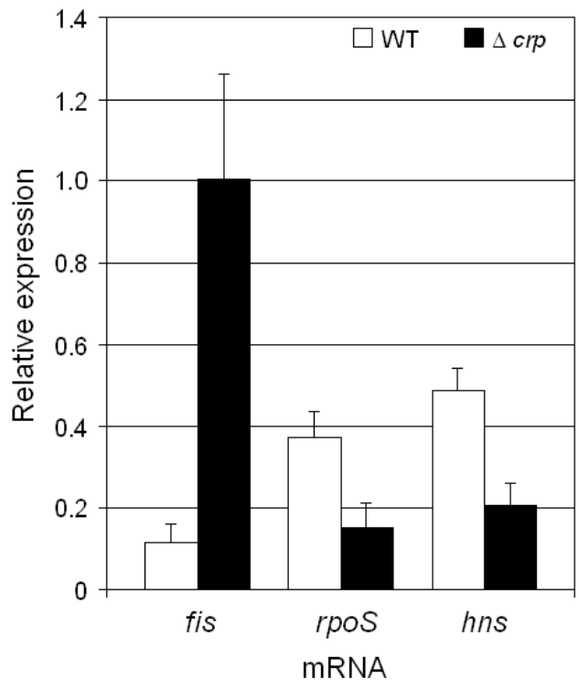

Fig. 5. Relative expression measured by quantitative, real-time reverse transcription PCR of global regulators *fis*, *rpoS,* and *hns* in a *V. cholerae crp* deletion mutant standardized by *recA* mRNA levels.

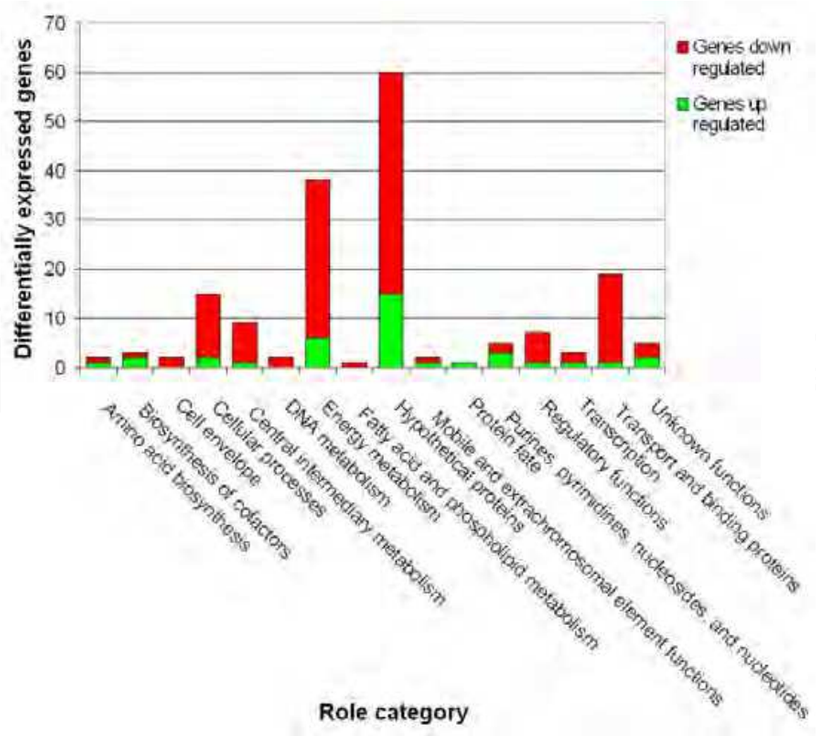

Fig. 6. Global regulation by the cAMP receptor protein.

4.6 Quorum modulation: Integration of cell density and carbon source sensory information

Since the *crp* mutant expresses reduced *cqsA* and *hapR* mRNA, we used a bioassay to compare the production of CAI-1 in wild-type and mutant backgrounds using a *V. cholerae* Δ*cqsA*Δ*luxP* reporter containing the *V. harveyi lux* operon on a cosmid (Miller et al., 2002). This reporter strain does not make its own CAI-1 nor does it respond to AI-2. Exogenous CAI-1 from a cell-free culture supernatant activates expression of HapR, which in turn induces the *lux* operon to make light. As shown in Fig. 7, no CAI-1 can be detected in culture supernatants of Δ*crp* and Δ*cya* (adenylate cyclase). The *crp*^{c} allele containing the amino acid substitutions T127L/S128A encodes a CRP protein that activates transcription in the absence of cAMP (Krueger et al., 1998; Shi et al., 1999; Wang et al., 2000). As shown in Fig. 7, introduction of this constitutive allele into a Δ*cya* mutant restored expression of HapR and light production. Furthermore, quorum sensing was restored in Δ*crp* and Δ*cya* mutants by introducing the corresponding genes on a plasmid vector and, in the case of a Δ*cya* mutant, by adding cAMP or the cAMP analog, 7-deaza-cAMP, to the culture medium (Liang et al., 2008). The mechanism by which the cAMP-CRP complex regulates *cqsA* expression is not known, although there is evidence suggesting a posttranscriptional regulation (Liang et al., 2008).

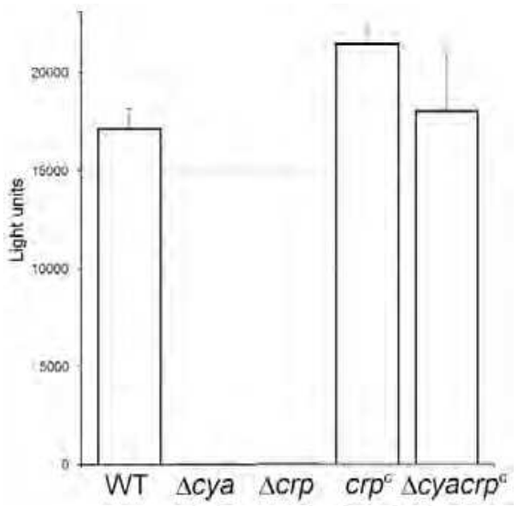

Fig. 7. The cAMP receptor protein is required for the biosynthesis of the *Vibrio cholerae* major autoinducer.

In section 4.5., we showed that cAMP-CRP controls quorum sensing by activating *cqsA* (Fig. 7) and repressing *fis* (Fig. 5). These findings suggest that the intensity of bacterial cell-to-cell communication is modulated by environmental signals other than population density, such as the type and availability of carbon sources. Thus, we propose a new level of regulation, termed quorum modulation, mediated in this case by cAMP. Quorum modulation functions in the following way. Under environmental conditions conducive to low intracellular cAMP levels (i.e., high glucose), the amount of CAI-1 produced per cell is diminished. The *V. cholerae* population would require a higher quorum (i.e., cells/ml) to activate HapR. Conversely, under conditions conducive to high cAMP levels (i.e., low glucose) the production of CAI-1 per cell is enhanced, and the bacterial population requires a lower quorum to activate HapR. Thus, quorum modulation controls the cell density at which *V.*

cholerae switches its metabolism to the quorum-sensing mode. As a consequence, commitment of the bacterial population to enter the quorum sensing-mode and turn on the HapR transcriptional program is placed in context with other features of the environment. This principle is illustrated in Fig. 8. Consistent with this scheme, the cell density at which *V. cholerae* enters the quorum-sensing mode is increased by addition of glucose to the medium (to lower the cAMP pool) and diminished by addition of cAMP (Liang et al., 2008).

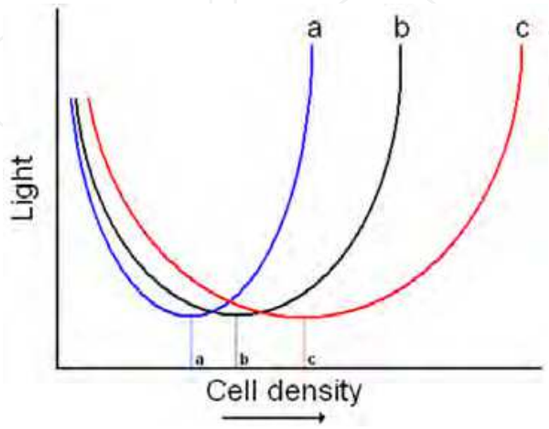

Fig. 8. Quorum modulation. When a culture at high cell density is diluted in broth, quorum sensing (i.e., light) is turned off. As the population increases, a threshold cell density is attained (quorum), at which quorum sensing is activated to generate a U-shape curve (b). The rate of autoinducer biosynthesis determines the cell density at which the bacterial population enters the quorum-sensing mode. A condition that enhances autoinducer biosynthesis lowers the threshold (a). A condition that inhibits autoinducer biosynthesis increases the threshold (c).

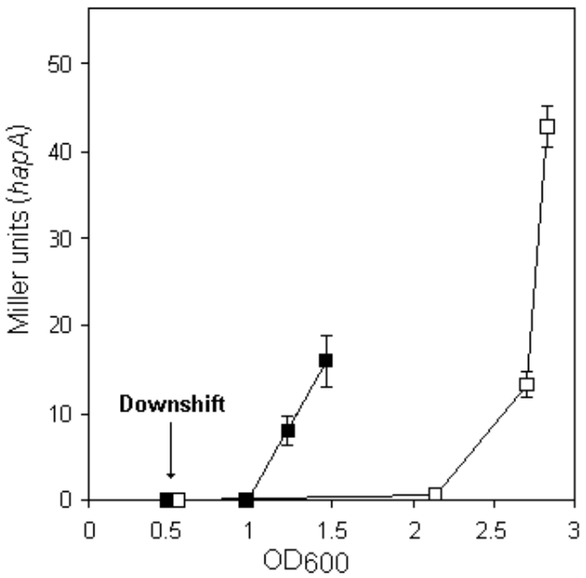

Fig. 9. Effect of a nutritional downshift on the cell density required for expression of a quorum sensing-regulated *hapA-lacZ* promoter fusion measured as β-galactosidase activity (Miller units) (Silva and Benitez, 2004).

A practical example of how quorum modulation works is provided in Fig. 9. In this case, a *V. cholerae* strain containing a chromosomally integrated *hapA-lacZ* promoter fusion is grown in rich medium, and, at an optical density of 0.5, half of the culture is nutritionally downshifted by centrifugation and reconstitution in a medium of diminished strength. This experiment shows that *hapA* expression can be detected at a lower cell density in nutritionally downshifted cells (Silva and Benitez, 2004). Quorum modulation is not restricted to carbon regulation of cellular cAMP levels, as other environmental conditions might influence autoinducer biosynthesis or even autoinducer stability in the medium.

4.7 Interplay between quorum sensing and cAMP in the fine regulation of matrix exopolysaccharide expression

Over-expression of *vps* in *hapR* mutants gives rise to the rugose colonial morphology (Yildiz et al., 2004). Although HapR is not detected in *crp* and *cya* mutants, these strains still produce the smooth colonial variant (Liang et al., 2007, 2007a). As shown in Fig. 10, the rugose colonial morphology of a *hapR* mutant is turned to smooth by deletion of *crp* or *cya,* and the resulting smooth strains can be converted back to rugose by introduction of the *crp* and *cya* genes on a plasmid vector. These results suggest that formation of the cAMP-CRP complex has a dual effect on *vps* expression by activating quorum sensing (a negative effect) and by enhancing the expression of a positive factor. In our strain, the positive factor was the regulator, VpsR (Liang et al., 2007a).

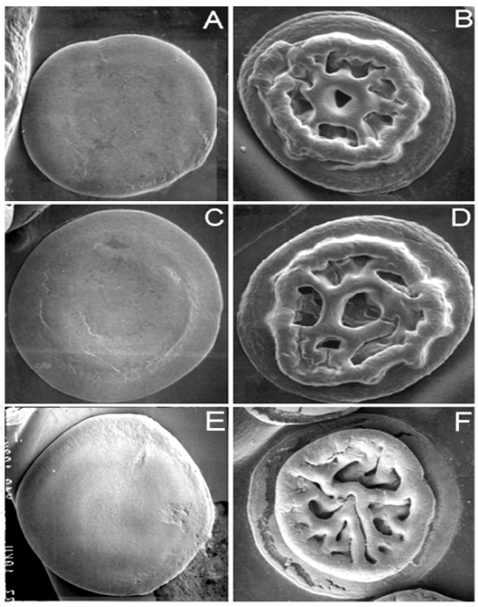

Fig. 10. Scanning electron microscopy of *V. cholerae* Δ*crp*, Δ*cya*, and Δ*hapR* colonies. A, Δ*crp*; B, Δ*hapR*; C, Δ*hapR*Δ*crp*; D, Δ*hapR*Δ*crp* transformed with *crp* plasmid; E, Δ*hapR*Δ*cya*; F, Δ*hapR*Δ*cya* transformed with *cya* plasmid

Fig. 11 schematically illustrates the dual input of adenylate cyclase and CRP on *V. cholerae* expression of *vps.* In this model, deletion of *crp* results in diminished expression of the positive regulator, VpsR, which is required for expression of rugose colonial morphology. This event, however, is partially compensated for by reduced expression of the negative

regulator HapR. As a result, Δ*crp* mutants express elevated *vpsA* and *vpsL* compared to the wild-type strain, but this increase is not large enough to induce rugose colonial morphology. Maximal exopolysaccharide expression requires inactivation of HapR (repressor) but an active *crp* allele for enhancing VpsR (activator). In a different strain, however, CRP repressed VpsR, suggesting that there is strain variability in the regulation of this protein (Fong et al., 2008).

In addition to the abovementioned regulatory effects of *crp* and *cya* on *vps* expression, several components of the PTS that function upstream of adenylate cyclase have their own regulatory input on *vps* gene expression. For instance, in minimal medium, phosphoryl transfer from EI to HPr and FPr represses *vps* expression (Houot et al., 2008); in LB medium, glucose-specific $EIIA^{glc}$ and nitrogen-specific $EIIA^{Ntr1}$ and $EIIA^{Ntr2}$ activate and repress *vps* expression, respectively (Houot et al., 2010).

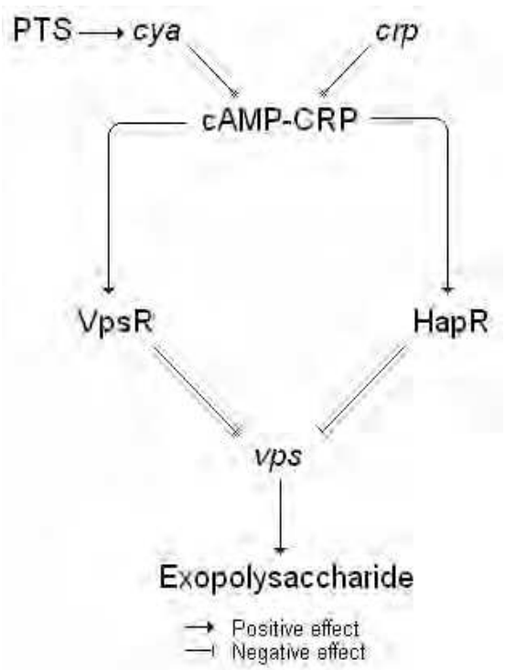

Fig. 11. Model for the dual regulatory input of the cAMP-CRP complex on biosynthesis of *V. cholerae* matrix exopolysaccharide.

4.8 Effect of extracellular phosphate on vps expression and biofilm formation

Since freshwater and estuarine ecosystems where *Vibrios* survive and persist outside the human host are limited in phosphate content (Benitez-Nelson, 2000; Correl, 1999), *V. cholerae* build stores of intracellular polyphosphate (poly-P) (Ogawa et al., 2000). The enzyme polyphosphate kinase (PPK) is responsible for the synthesis of poly-P from ATP (Ahn and Kornberg, 1990). A *V. cholerae ppk* mutant exhibits a reduced capacity to withstand conditions of low pH, high salinity, and oxidative stress in low- phosphate medium (Jahid et al., 2006). These findings underline the importance of phosphate homeostasis as well as sensing and responding to changes in extracellular phosphate in the *V. cholerae* life cycle. In *E. coli*, deprivation of phosphate induces the expression of the PhoB regulon (Lamarche et

al., 2008). PhoB is part of the PhoR/PhoB two-component regulatory system. PhoR is an inner membrane histidine kinase that responds to periplasmic orthophosphate through its interaction with the phosphate transport system. Under conditions of phosphate limitation, phosphorus is transferred from phospho-PhoR to the response regulator PhoB. Phospho-PhoB then binds to DNA pho boxes to activate or repress the transcription of target genes (Lamarche et al., 2008). A proteomic comparison of wild-type and *phoB V. cholerae* strains revealed 140 differentially expressed proteins (von Kruger et al., 2006). A *V. cholerae phoB* mutant colonized rabbit ileal loops to a lesser extent suggesting a role for this regulator in intestinal colonization and pathogenesis (von Kruger et al., 1999). More recently, it was shown that PhoB negatively affects CT and TCP expression by repressing the *tcpPH* promoter (Pratt et al., 2010). In addition, PhoB negatively regulates biofilm formation in *V. cholerae* of classical and El Tor biotypes (Pratt et al., 2009; Sultan et al., 2010). A comparison of the levels of expression of known regulators of *vps* and biofilm between wild-type and Δ*phoB V. cholerae* of the El Tor biotype revealed that VpsR, is negatively regulated by PhoB (Sultan et al., 2010).

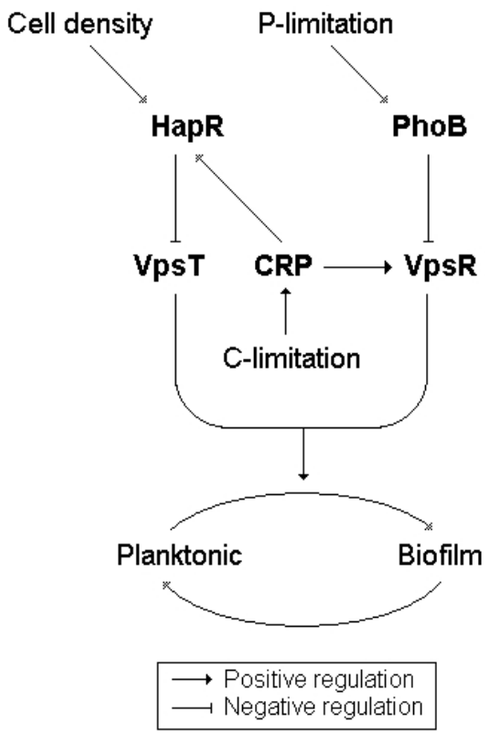

Fig. 12. Model for the integration of cell density, carbon and phosphorus sensory information in the regulation of biofilm formation.

Since VpsR is positively modulated by CRP (Fig. 11), we propose that VpsR has an essential function in biofilm formation by acting as a receiver of external carbon and phosphorus sensory information to modulate biosynthesis of the exopolysaccharide matrix. PhoB and CRP exhibit antagonistic effects on VpsR (Fig. 12). The parallel function of multiple signaling pathways with opposing and/or re-enforcing effects appears to be a common theme in metabolic regulation. We suggest that such a regulatory architecture allows

bacteria to adjust their response quantitatively to chemical and physical changes in the extracellular milieu. For instance, in defined chemical media, maximal VpsR expression would be expected to occur under conditions of low glucose concentration (high cellular cAMP) and high phosphate (PhoB inactive). Furthermore, according to the scheme presented in Fig. 12, the regulator VpsT acts as a receiver of population density and carbon sensory information; VpsR acts as a receiver of carbon and phosphate sensory information to regulate the transition between the planktonic and biofilm life styles.

4.9 Enhancement of motility and detachment by the general stress response regulator, RpoS

As shown in Fig. 5, CRP enhances expression of the general response regulator, RpoS. The *rpoS* gene encodes the RNAP σ^S subunit, which regulates expression of more than 100 genes in response to starvation and other stresses such as osmotic shock, acid shock, and temperature changes (Hengge-Aronis, 2002). It is not clear what makes a given promoter selective for transcription by RpoS. Several promoter elements have been described including an upstream (UP) element, a more degenerate -35 region, a cytosine at -13, and a AT-rich region downstream from -10 (Hengge-Aronis, 2002a; Typas et al., 2007). It also has been suggested that histone-like proteins such as H-NS, the Leucine-responsive protein (Lrp), or the integration host factor (IHF) can contribute to σ^S promoter selectivity (Hengge-Aronis, 2002a; Hengge-Aronis, 1999; Typas et al., 2007). For instance, many σ^S-dependent genes are repressed by H-NS, and association of RNAP with σ^S on these promoters may overcome H-NS transcriptional repression (Barth et al., 1995; Bouvier et al., 1998; Hengge-Aronis, 1999). In *E. coli*, the intracellular level of σ^S is controlled at the levels of transcription, translation, and protein stability (Hengge-Aronis, 2002; Nogueira & Springer, 2000; Vicente et al., 1999). The regulation of RpoS expression in *V. cholerae* is less understood but, relative to *E. coli*, there are differences that likely reflect adaptation to distinct environments. In contrast to *E. coli*, *V. cholerae* mutants that produce diminished guanosine tetraphosphate (ppGpp) and poly-P are not affected in *rpoS* expression (Jahid et al., 2006, Silva and Benitez, 2006). Moreover, deletion of Hfq, a factor that enhances *rpoS* translation in *E. coli*, has no effect on expression of *V. cholerae* RpoS (Ding et al., 2005). Additionally, we have shown that H-NS, which negatively influences *rpoS* translation in *E. coli*, has the opposite effect in *V. cholerae* (Silva et al., 2008). We have constructed an RpoS reporter strain expressing an RpoS-FLAG protein from native *rpoS* transcription and translation signals (Wang et al., 2010). In rich tryptone soy broth, RpoS was detected in the late logarithmic phase and after the population entered quorum-sensing mode (Wang et al., 2010). The quorum-sensing regulator, HapR, enhanced RpoS expression.

Transcription hierarchy	Increase in c-di-GMP	Deletion of *rpoS*
Class II	*flhF, fliFGHIN, fliA (σ28)*	*flhAF, fliFGJ*
Class III	*flaAGI, flgBCDEFGI*	*flgBCDEFGHIJL, fliMNOPQ, flaA*
Class IV	*flaCD, flgM*	*flaBCDE*

Table 2. Comparison of motility genes differentially expressed in response to an increase in c-di-GMP and to deletion of *rpoS* (adapted from Beyhan et al., 2006; Nielsen et al., 2007).

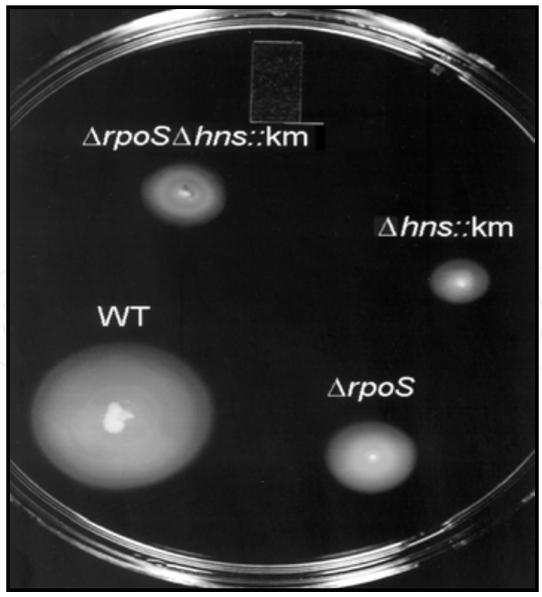

Fig. 13. Motility of *V. cholerae rpoS* and *hns* (Δ*hns*::km) mutants in swarm agar plates

V. cholerae rpoS mutants are more sensitive to starvation, high osmolarity, and oxidative stresses, are less motile than their wild-type precursors (Fig. 13), and do not express *hapA* (Nielsen et al., 2006; Silva et al., 2008; Yildiz & Schoolnik, 1998; Silva & Benitez, 2004). We have recently shown that, similar to HapR, expression of RpoS acts to diminish the c-di-GMP pool (Wang et al., 2011). Gene profiling experiments have shown that RpoS positively controls the expression of several proteins putatively identified as PDEs (Nielsen et al., 2006). As shown in Table 2, both deletion of *rpoS* and artificial enhancement of the c-di-GMP pool modulate the expression of class II through IV hierarchy motility genes. The data suggest that RpoS enhances motility by diminishing the c-di-GMP pool and by acting at an early step of the motility transcription hierarchy, such as RpoN and/or FlrA.

A common approach to investigate the phenotypic consequences of changes in intracellular c-di-GMP content is to increase or diminish the c-di-GMP pool by over-expressing a DGC or PDE, respectively. Using this method, we have investigated the effect of artificially altering the c-di-GMP pool on HapR and HA/protease expression. These studies revealed a complex interplay between c-di-GMP, HapR, VpsT, and RpoS that favors detachment of *V. cholerae* at high cell density (Fig. 13). Increasing the c-di-GMP pool enhances the expression of the c-di-GMP sensing protein, VpsT (Beyhan et al., 2006), which acts as a repressor of HapR (Yildiz et al., 2004). In the model shown in Fig. 14, expression of HapR at high cell density results in lower c-di-GMP content (Waters et al., 2008); lowering of c-di-GMP further enhances HapR, generating a double-negative regulatory loop that requires VpsT; HapR positively enhances RpoS expression (Joelsson et al., 2007); and the elevated expression of RpoS feeds into the regulatory loop by diminishing the intracellular concentration of c-di-GMP. The concurrent activation of HapR and RpoS results in elevated expression of HA/protease and motility, which promotes detachment. By promoting multiple cycles of detachment and re-colonization, the coordinate expression of HA/protease and motility could contribute to the dissemination of colonizing *Vibrios* along the small intestine.

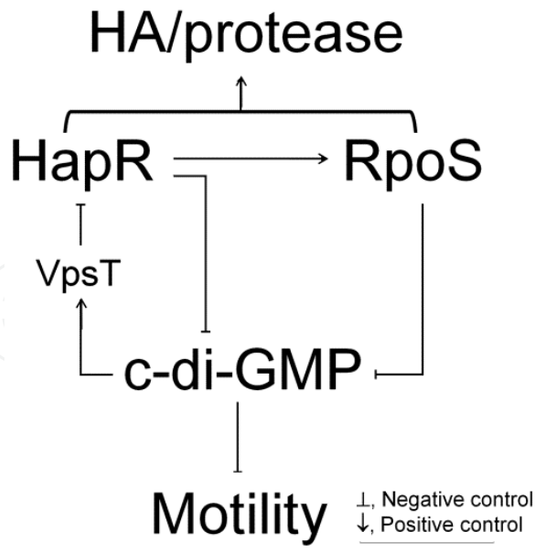

Fig. 14. Coordinate regulation of HA/protease and motility by c-di-GMP, HapR, VpsT and RpoS.

4.10 The histone-like nucleoid structuring protein (H-NS) represses the expression of virulence genes

H-NS belongs to a family of small nucleoid-associated proteins that include its paralog StpA; Fis, the heat-unstable protein (HU); and IHF (Dorman, 2004; Dorman & Deighan, 2003). In *E. coli,* H-NS is a 15-kDa, highly abundant protein present at about 20,000 copies per cell; it was initially characterized for its capacity to mediate DNA condensation (Dorman, 2004; Dorman & Deighan, 2003). Mutations that inactivate *hns* are pleiotropic, suggesting that H-NS influences a broad spectrum of physiological processes (Atlung & Hansen, 2002; Atlung & Ingmer, 1997; Hommais et al., 2001). The H-NS proteins of *E. coli* and *V. cholerae* contain an N-terminal oligomerization domain connected by a flexible linker to a nucleic acid binding domain (Atlung & Ingmer, 1997; Cerdan et al., 2003; Dorman, 2004; Nye & Taylor, 2003). Both oligomerization and DNA binding are essential for the biological activities of H-NS, which include DNA condensation and regulation of transcription (Dame et al., 2001; Spurio et al., 1997). In regulation of transcription, H-NS most commonly negatively affects gene expression by binding to promoters exhibiting AT-rich, highly curved DNA regions that contain clusters of the more conserved 10 bp motif, TCGATAAATT (Lang et al., 2007; Owen-Hughes et al., 1992; Uegushi & Mizuno, 1993). In *V. cholerae*, *hns* mutants form small colonies, are incapable of using β-glucosides as a carbon source, exhibit diminished motility (Fig. 12) and intestinal colonization capacity, and show altered responses to environmental stresses (Ghosh et al., 2006; Krishnan et al., 2004; Silva et al., 2008; Tending et al., 2000; Silva et al. 2008). An emerging function of H-NS is the transcriptional silencing of horizontally acquired genes (Lucchini et al., 2006; Navarre et al., 2006; Oshima et al., 2006). Consistent with this role, H-NS silences expression of virulence genes in *V. cholerae* by acting at different levels of the ToxR regulatory cascade (Fig. 3), which include the *toxT*, *tcpA* and *ctxA* promoters (Nye et al., 2000). Binding of H-NS to a promoter apparently inhibits transcription by a bridging mechanism consisting of cross-linking DNA segments in a manner that traps RNAP (Dorman & Kane, 2009). There is

considerable evidence indicating that H-NS-mediated repression can be antagonized in response to environmental stimuli that activate the expression of other regulators whose binding sites overlap that of H-NS (anti-bridging) (Dorman & Kane, 2009). For instance, the small nucleoid protein, Fis opposes the repression activity of H-NS at a various promoters (Dorman & Kane, 2009). The IHF alleviates H-NS silencing of *S. enterica hilA* (Queiroz et al., 2011), *E. coli csgD* (Ogasawara et al., 2010), *Shigella flexneri vir* genes (Porter & Dorman, 1997), and bacteriophage Mu early promoter (Van Ulsen et al., 1996). In the case of *V. cholerae*, transcriptional silencing of the *tcpA* and *ctxA* promoters by H-NS is antagonized by the AraC-like transcriptional regulator, ToxT, and by IHF (Stonehouse et al., 2008, 2010; Yu & DiRita, 2002). In theory, other small nucleoid proteins, such as Lrp and HU, can exhibit the anti-bridging activity required to attenuate H-NS repression (Dorman & Kane, 2009).

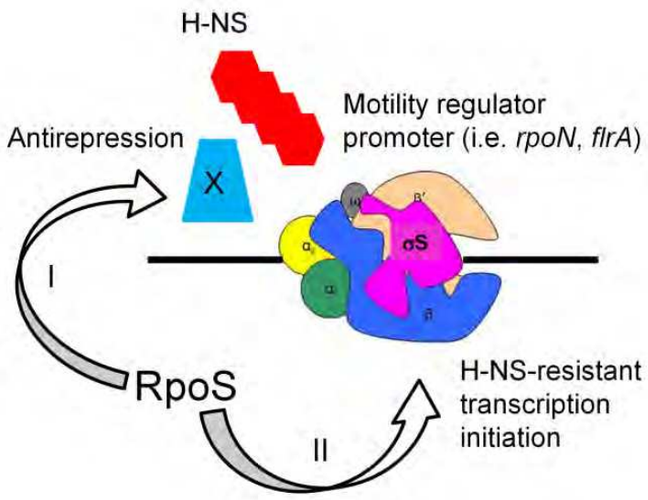

Fig. 15. Hypothetical model for the interaction of RpoS and H-NS in the regulation of *V. cholerae* motility. RpoS could enhance the transcription of motility by inducing the expression of an antirepressor (I), and by binding to core RNAP to promote transcription initiation that is H-NS-resistant.

The effect of H-NS on motility of *V. cholerae* is not fully understood. On one hand, H-NS could enhance motility by positively affecting the expression of RpoS (Silva et al., 2008). This effect, however, is modest compared to the reduction in motility observed for *hns* mutants (Fig. 13). Bright-field microscopy has revealed that *hns* mutants are flagellated nut non-motile. In *E. coli*, H-NS binds to the switch protein, FliG, to enhance motility, and *hns* mutants express a paralyzed flagellum (Donato & Kawula, 1998). A similar mechanism may function in *V. cholerae*. Moreover, in the absence of RpoS, H-NS appears to function as a repressor of the motility genes, *flaA*, *flaC* and *motY* (Silva et al., 2008). This suggests that, in addition to lowering the c-di-GMP pool, RpoS enhances motility by attenuating H-NS repression. A speculative model for the anti-repressor function of RpoS is shown in Fig. 15. Based on data derived by transcriptional profiling, we suggest that RpoS is most likely to act at the *rpoN* and/or *flrA* promoters to enhance expression of downstream class II-IV motility genes (Table 2). In the stationary phase, transcription initiation at the *rpoN* and *flrA* promoters by RNAP containing σ^S could be more resistant to H-NS repression (Barth et al., 1995; Bouvier et al., 1998; Hengge-Aronis, 1999). In addition, RpoS could enhance the

expression of other nucleoid-associated proteins, such as IHF to attenuate H-NS repression by an anti-repressor (anti-bridging) mechanism (Dorman & Kane, 2009).

5. CRP as a master regulator of *V. cholerae* behavior

As described in the preceding sections, CRP acts as an upstream master regulator modulating quorum sensing, virulence, stress response, motility, and biofilm development. Thus, in Fig. 16, we provide an integrative model for CRP regulation of *V. cholerae* behavior. The CRP protein is activated when the intracellular concentration of cAMP is increased due to PTS activation of adenylate cyclase. Thus, the PTS system acts as the primary carbon source sensing mechanism. The state of activity of CRP controls the execution of secondary genetic programs mediated by HapR, RpoS and H-NS that modulate *V. cholerae* switching from planktonic to sessile life styles or from virulence to detachment. For instance, in its active state, CRP enhances the expression of (a) HapR by activating CAI-1 biosynthesis and repressing Fis, (b) RpoS, and (c) H-NS. The expression of HapR and RpoS enhances motility and activates HA/protease, favoring detachment. Simultaneous activation of HapR and H-NS leads to quorum-sensing repression of *aphA* and to H-NS-mediated transcriptional silencing of *toxT, ctxA,* and *tcpA* to diminish expression of virulence genes. By enhancing HapR, RpoS and H-NS, formation of the cAMP-CRP complex favors the motile planktonic stage; the opposite is true for biofilm formation.

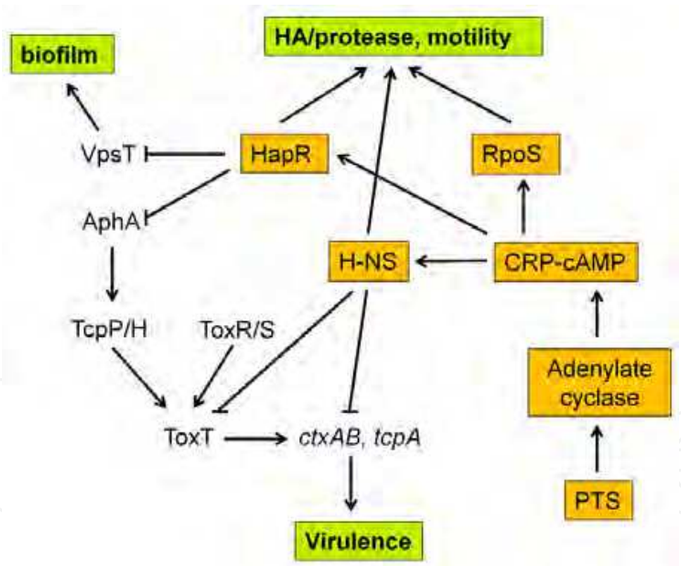

Fig. 16. Multilevel regulation of *V. cholerae* behavior by CRP.

6. Significance to anti-virulence drug discovery

The increase in our understanding of the regulatory pathways that control *V. cholerae* behavior creates the possibility of identifying new drugs to block infection and to prevent biofilm development. This is particularly relevant due to the emergence of antibiotic-resistant *V. cholerae* (Das & Kaur, 2008; Roychowdhury et al., 2008; Okeke et al., 2007; Mwansa et al., 2000). Consequently, development of anti-virulence drugs is proceeding

(Waldor, 2006). An example is the small molecule inhibitor of *V. cholerae* intestinal colonization, virstatin [N-(1, 8-(naphthalimide)-n-butyric acid], which was identified in a high-throughput phenotypic screen (Hung et al., 2005; Shakhnovich et al., 2007, 2007a). This molecule inhibits *ctxAB* and *tcpA* transcription by preventing dimerization of their positive regulator, ToxT (Shakhnovich et al., 2007a). Another example is the newly identified inhibitor of the Na^+-dependent flagellar motor, Q24DA, which could prevent infection dissemination by blocking motility and indirectly diminishing CT and TCP secretion (Rasmussen et al., 2010. An attractive target to block infection is the quorum- sensing phosphoryl cascade leading to the expression of HapR (Higgins et al., 2007). Pretreatment of mice with commensal bacteria engineered to express CAI-1 affords protection against a cholera challenge (Duan and March, 2010). A second set of attractive targets are the PTS components that modulate biofilm formation (Houot et al., 2008, 2010, 2010a). A comparative genomic analysis of 202 fully sequenced genomes (174 bacterial, 19 archaeal, and 9 eukaryotic) did not reveal components of the PTS system in eukaryotic cells (Barabote et al., 2005). A third potential target is CRP, whose cyclic nucleotide binding (CNB) domain is substantially different from eukaryotic cAMP binding proteins, with substitutions in highly conserved positions within the phosphate-binding cassette that determine ligand specificity (Kannan et al., 2007). These differences can be exploited to identify and/or synthesize CRP ligands selective for the bacterial CNB domain. Based on the results described in this chapter, CRP agonists would be expected to inhibit expression of virulence genes and biofilm formation while favoring motility and detachment. An advantage of targeting the PTS and CRP is their broad range. For instance, CRP modulates the expression of virulence factors in bacterial pathogens such as *V. vulnificus*, *Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica*, *Y. pestis,* and *Pseudomonas aeruginosa*. The genes encoding global regulators, such as CRP, and the virulence factors under its control are generally not essential. Thus, contrary to anti-bacterial drugs, inhibitors of expression of virulence genes are not expected to exert a high selective pressure for the dissemination of resistance or to impact the commensal flora. Finally, targeting global regulators such as CRP, RpoS and H-NS could significantly impair bacterial *in vivo* stress response allowing the host to clear the infection and diminish the use of antibiotics. The use of anti-virulence versus anti-bacterial therapies is still a matter of debate, particularly in regard to pathogens capable of long-term persistence in the host, which is not the case in cholera.

7. Conclusions

The bacterial pathogen *V. cholerae* has evolved to colonize the human small bowel efficiently and to persist in aquatic environments. A sophisticated regulatory network allows the cholera bacterium to modify its behavior in response to the environmental changes dictated by its dual life cycle. Studies conducted in the last few decades have revealed that:

a. *V. cholerae* can switch from virulence to detachment modes and from motile to sessile (biofilm) life styles to maximize fitness.
b. The cAMP receptor protein acts as an upstream master regulator of *V. cholerae* behavior by controlling the execution of secondary regulatory pathways, such as quorum sensing (HapR), RpoS, and H-NS.
c. Efficient regulation requires substantial molecular cross-talking between regulatory modules, as exemplified by the interplays between quorum sensing (HapR) and RpoS

expression as well as the interactions between RpoS and H-NS repression in the regulation of expression of motility genes.

d. A salient feature of this complex global regulatory network is the occurrence of parallel or overlapping regulatory outputs with opposing or re-enforcing effects to fine-tune bacterial responses to environmental stresses. In this chapter, this principle is illustrated by the dual effect of CRP on *vps* expression involving HapR and VpsR, and the opposing effects of CRP and PhoB on the expression of VpsR.

e. As we continue to develop a better understanding of the *V. cholerae* regulatory landscape, it should become possible to identify small molecules capable of shifting bacterial behavior from pathogenic to commensal.

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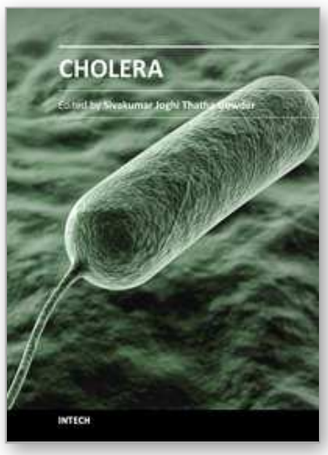

Cholera

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Cholera, a problem in Third World countries, is a complicated diarrheal disease caused by the bacterium Vibrio cholerae. The latest outbreak in Haiti and surrounding areas in 2010 illustrated that cholera remains a serious threat to public health and safety. With advancements in research, cholera can be prevented and effectively treated. Irrespective of "Military" or "Monetary" power, with one's "Own Power", we can defeat this disease. The book "Cholera" is a valuable resource of power (knowledge) not only for cholera researchers but for anyone interested in promoting the health of people. Experts from different parts of the world have contributed to this important work thereby generating this power. Key features include the history of cholera, geographical distribution of the disease, mode of transmission, Vibrio cholerae activities, characterization of cholera toxin, cholera antagonists and preventive measures.

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