Ins and Outs of *Vibrio cholerae*

Vibrio cholerae transitions between the human gut and the aquatic environment are aided by specific shifts in gene expression

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The gram-negative, motile, curved-rod bacterium *Vibrio cholerae* can cause cholera—an acute, explosive diarrheal disease. Epidemic cholera was described in the 1500s, but reports of cholera-like symptoms date back much earlier, to the times of Hippocrates and the Buddha. In 1849 John Snow, a London physician, determined that cholera is transmitted by water, and in 1883 Robert Koch successfully isolated the cholera vibrio. Facilitated by the expansion of global trade routes, cholera spread worldwide in 1817 and remains a global health challenge.

Cholera patients typically lose large volumes of fluid. If left untreated, even previously healthy adult patients may become severely dehydrated and can die. Although rehydration therapy makes cholera survivable, it does not cure the diarrhea or prevent infections. Cholera remains a social and economic burden, especially in the developing world. In 2006 the World Health Organization reported a total of 236,896 cholera cases with 6,311 deaths worldwide. These figures likely greatly underestimate the toll of cholera. Many cases go unreported during seasonal outbreaks, and a substantial percentage of people die without diagnosis.

Summary

- The facultative pathogen *Vibrio cholerae* has adapted to survive and colonize two very different environments—the aquatic environment and the human gastrointestinal tract.
- The intracellular signal molecule c-di-GMP can inversely regulate genes specific for the aquatic environment or the host.
- Quorum sensing is one of the signals that helps to regulate c-di-GMP levels.
- While genes essential for colonization are induced early during infection, late in vivo induced genes can increase fitness for transition into the aquatic environment.

Two Serogroups Give Rise to Cholera Disease

Despite there being more than 200 known serogroups of *V. cholerae* in the aquatic environment, only strains of two serogroups, O1 and O139, are known to cause epidemic disease. O-antigen and core oligosaccharide of the O1 and O139 lipopolysaccharides, as well as the capsule of O139, contribute in colonization of the small intestine and adhesion to epithelial cells. Strains within serogroup O1 can be further divided into two biotypes, El Tor and classical. These two biotypes are based upon phenotypic traits, including phage and polymyxin B sensitivity or resistance. Classical and El Tor exhibit many differences, often at the level of gene regulation.

The genome sequence of the *V. cholerae* clinical isolate N16961 is of similar size to that of other γ-proteobacteria. In this case, N16961 contains 4.033 Mbp, which is similar in size to that of *Escherichia coli* K12 with 4.639 Mbp. However, the *V. cholerae* genome is split into two chromosomes, with one containing 2.961 Mbp and the other 1.072 Mbp, a feature that is seen in other *Vibrio* species. Having two smaller chromosomes, which replicate in a coordinated but independent fashion, might speed replication.

V. cholerae is closely related to other aquatic vibrios, some of which live closely

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with marine organisms either as symbionts, commensals, or pathogens. *V. cholerae* O1 likely arose from an aquatic ancestor that was nonpathogenic to humans. *V. cholerae* is not only transmitted via contaminated water, like other fecal-orally transmitted pathogens such as *Salmonella* or *E. coli*, but also spends considerable time as an aquatic organism in both fresh and salt water.

Since epidemiological record-keeping began in the early 19th century, eight cholera pandemics have been recorded. O1 classical caused the fifth and sixth pandemics and possibly the four earlier pandemics. In contrast, O1 El Tor is responsible for the seventh pandemic, which began in the 1960s. Although strains of the classical biotype are probably extinct in nature, both serotypes are still extensively studied in the laboratory, and differences that may explain the emergence of El Tor remain of great interest to researchers in the field.

Since its recent emergence in 1992, cholera infections caused by O139 have spread worldwide. Although O139 has not replaced O1 El Tor, it is responsible for an eighth cholera pandemic that is running in parallel with the seventh. Genetic analysis reveals O139 to be closely related to O1 El Tor, suggesting that O139 evolved from an El Tor O1 strain that acquired new LPS and capsule biosynthesis genes.

Life Cycle of *Vibrio cholerae*

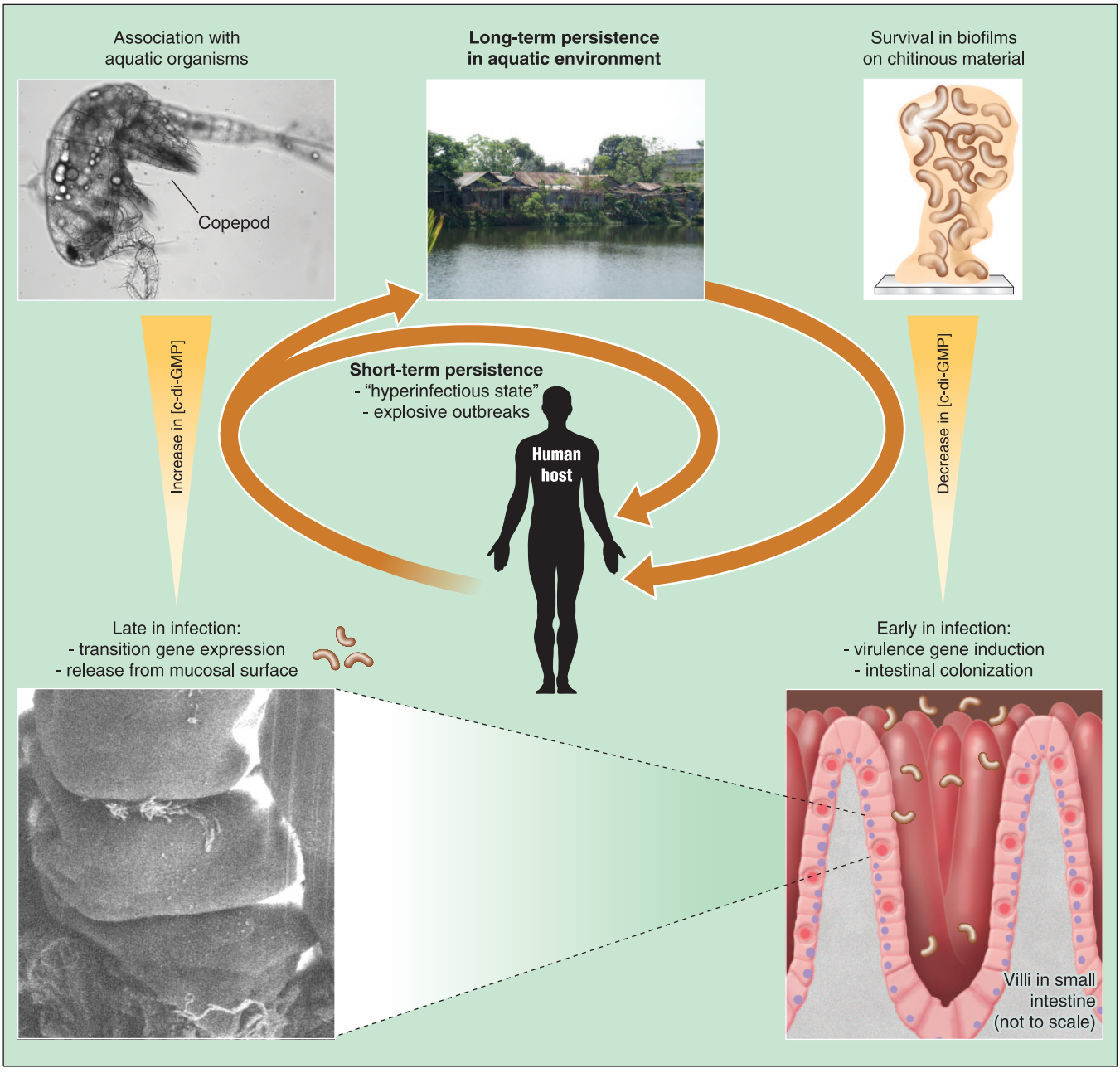
In our laboratory we are striving to gain a better understanding of the full life cycle (all of the "ins and outs") of the facultative pathogen *V. cholerae*. As a facultative pathogen, *V. cholerae* resides in two worlds (Fig. 1): as a natural inhabitant of aquatic ecosystems and as the causative agent of a severe diarrhea in the gastrointestinal tract of its human host. In the environment, *V. cholerae* is found in association with zooplankton, crustaceans, and egg masses of chironomids, commonly called "midges." A variety of factors that promote adherence to chitin surfaces of such organisms have been reported, including N-acetylglucosamine binding protein A (GbpA) as well as mannose-sensitive hemagglutinin (MSHA) and PilA pili. This association may provide an environmental advantage, since *V. cholerae* is capable of degrading chitin and using it as a carbon and nitrogen source. Furthermore, *V. cholerae* O1 and O139 form biofilms. In static cultures biofilm formation is dependent on biosynthesis of *Vibrio* exopolysaccharide (VPS). In contrast, in a flow cell system a *vps*-independent biofilm has recently been described by Alfred Spormann and colleagues at Stanford University, Calif. It is likely that survival in aquatic environments is aided by the formation of such multicellular structures.

When a human ingests *V. cholerae*, the bacteria face a sudden shift in temperature and osmolarity as well as the extreme acidity of the stomach. Later, in the human gut, the bacteria encounter alkaline pH, digestive enzymes, bile, and components of the innate immune system. In the small intestine, the polar flagellum propels individual *V. cholerae* cells into the mucus layer where they can attach to host epithelial cells, the major sites of bacterial proliferation.

In the mouse as well as in humans, *V. cholerae* induces several virulence factors, including cholera toxin (CTX) and the toxin-coregulated pilus (TCP), the major colonization factor required for microcolony formation. In mice this gene induction is rapid, occurring within the first five hours. The activity of CTX causes a massive secretory diarrhea that, if untreated, can result in hypotensive shock, organ failure, and death within a day. The ability of *V. cholerae* to replicate with a generation time of 20 minutes may be advantageous during the gastrointestinal (GI) tract colonization stage of its life cycle, when the potent effects of CTX provide only a short time in which to proliferate. To induce these virulence factors, *V. cholerae* relies on signal transduction and transcriptional regulatory cascades that include the two transmembrane regulators ToxR and TcpP, as well as the cytoplasmic regulator ToxT.

Cholera researchers are making great advances in understanding changes in gene expression at different stages in the *V. cholerae* life cycle. Our lab's research has focused on identifying in vivo-induced genes at early stages of infection, using recombination-based in vivo expression technology (RIVET) in combination with the infant mouse model. Briefly, the method relies on a library of strains carrying transcriptional fusions to *tnpR* (encoding a resolvase) that are not expressed in vitro. When a subset of these strains expresses the resolvase in vivo, they excise a cassette harboring selectable and counter-selectable markers that are used to identify the strains. Using microarray technol-

FIGURE 1



Model for the life cycle of *V. cholerae*. When humans ingest food or water containing *V. cholerae*, several bacterial genes are induced, including those encoding cholera toxin and toxin coregulated pilus (bottom right), while c-di-GMP levels drop (right). A scanning electron micrograph (bottom left) shows *V. cholerae* associated with a microvillus in the infant mouse small intestine (magnification, ×750; taken by Michael J. Angelichio). Inducing late genes (bottom left) may help to maintain an infection and increase fitness prior to the transition back into aquatic environments. Genes induced late during infection include DGCs, which are predicted to increase c-di-GMP level prior to release into the aquatic environment (left). After release into the environment hyperinfectious *V. cholerae* can infect a new human host (short-term persistence). Alternatively, *V. cholerae* may form associations with and biofilms upon chitinous material, facilitating long-term persistence in the aquatic environment. Pictures of the pond in Bangladesh and of a copepod (top) were taken by Lori Bourassa.

ogy, we and other research groups have analyzed the transcriptome of *V. cholerae* in stool from cholera patients to better understand what happens to the bacteria during human infections. A green fluorescent protein-based screen allowed Jun Zhu and co-workers at University of Pennsylvania in Philadelphia to identify genes downregulated in vivo. They demonstrated that the repression of the MSHA-operon is necessary to evade the host immune system. Gary K. Schoolnik and coworkers at Stanford University, Stanford, Calif., reported that once *V. cholerae* colonize the rabbit ileal loop, they produce a stationary-phase sigma factor (RpoS) that enables detachment from the epithelial surface. Whether this sigma factor is required for detachment during infection in an open intestinal tract is unclear. However, at some stage during the infection, bacteria detach from the epithelial layer and are shed in "rice-water" stool into the environment.

The "Ready" State of *V. cholerae*

V. cholerae shed by cholera patients are more infectious than laboratory-cultured bacteria when tested in mice. Mathematical modeling of human epidemiological data supports the existence of a hyperinfectivity phenomenon, as reported by David M. Hartley at the University of Maryland School of Medicine in Baltimore and his collaborators. Hyperinfectivity is maintained for at least 5 h in pond water, suggesting that expression of a subset of *V. cholerae* genes responsible for hyperinfectivity persists temporarily and could facilitate reinfection.

Siouxsie Wiles, Gordon Dougan, and Gad Frankel at Imperial College London in England have observed similar hyperinfectivity of naturally transmitted compared with laboratory-grown bacteria for *Citrobacter rodentium* after it passes through the mouse GI tract. Whether the mechanisms of hyperinfectivity for *V. cholerae* and *C. rodentium* differ or are conserved remains to be determined. However, Frankel and his collaborators also report that well-characterized virulence genes are strongly expressed after shedding of *C. rodentium* by mice, whereas in human stool the ToxR and ToxT regulons of *V. cholerae* are not.

Meanwhile, our analysis indicates that repression of chemotaxis in fresh stool-borne *V. cholerae* enhances virulence in a new host using the infant mouse model and can be observed phenotypically using chemotaxis assays. The nature of *V. cholerae* in stool samples and its effects on cholera transmission are just beginning to be investigated. For instance rice-water stool sometimes contains *V. cholerae* aggregates, some of which are associated with mucus, according to Stephen B. Calderwood and colleagues at Massachusetts General Hospital and Harvard Medical School in Boston, Mass., and Shah M. Faruque and colleagues at the International Centre for Diarrhoeal Disease Research in Bangladesh. Such aggregates could enhance environmental survival and affect rates of transmission.

Recently we identified *V. cholerae* genes specifically induced late in infection using a modified RIVET screen and the infant mouse model. Mutant analysis revealed that most of the late genes are necessary neither for survival in the host nor for maintenance of the infection. By establishing a novel transition assay, we could demonstrate that some of those late gene mutants, after in vivo passage, exhibit a fitness defect if incubated in pond water and/or rice-water stool. In vivo passage is essential to observe these phenotypes. When human volunteers ingested a RIVET library made in an attenuated vaccine-candidate strain of *V. cholerae*, we and James Kaper and colleagues at the University of Maryland in Baltimore identified a similar set of late-induced genes, indicating that this type of gene regulation occurs when this pathogen passes through human hosts.

The transition from host to environment is as harsh as from the environment to the host. In leaving the mammalian GI tract into fresh water, *V. cholerae* faces a 50-fold drop in osmolarity, a shift to lower temperature, and a massive shift in environmental salts and nutrients. Extensive changes in the transcriptome and proteome to adapt to the new conditions are energetically costly, but *V. cholerae* has the advantage that, through evolution, it can "prepare" for this transition. That is to say, a facultative pathogen like *V. cholerae* may induce genes that are beneficial (and repress genes that are detrimental) for environmental survival while still in the host, due to a selective advantage once in the aquatic environment. This idea is supported by the identification of operons involved in chitin utilization and the biosynthesis of MSHA as being induced late in infections.

A significant percentage of the late in vivo

induced genes is associated with uptake or transport systems. One explanation for this observation is that *V. cholerae* may need these systems ready to use and fully functional when it faces the aquatic environment. Expression of these uptake systems in vivo may allow *V. cholerae* to hoard factors that will be limited later in the environment, such as carbon sources.

V. cholerae Transitions Are Mediated by c-di-GMP Signaling

V. cholerae induces diguanylate cyclases (DGCs) at the late stage of infection, which would lead to an increase in cyclic-di-guanylate (c-di-GMP). c-di-GMP is an intracellular bacterial second messenger commonly found in gram-negative and some gram-positive organisms. DGCs synthesize c-di-GMP from GTP, while specific phosphodiesterases (PDEs) degrade c-di-GMP. *V. cholerae* encodes about 60 proteins containing a DGC, a PDE, or both domains, only a fraction of which have been demonstrated to be active.

In *V. cholerae* high levels of c-di-GMP enhance biofilm formation by increasing expression of *vps* genes, while inhibiting transcription of virulence and motility genes. In the classical biotype, mutation of *vieA*, which is a response regulator and PDE, elevates c-di-GMP resulting in enhanced biofilm and dramatically reduced motility and virulence. Furthermore, transcriptional changes in a *vieA* mutant are similar to those seen with ectopic DGC overexpression.

These data show that *vieA* is essential for c-di-GMP down-regulation in the classical biotype, yet in the El Tor biotype, *vieA* deletion mutants show no detectable phenotypes and relatively minor changes in gene transcription. This is one of many intriguing differences between O1 El Tor and classical. We suspect that one or more of the other more than 20 PDEs in *V. cholerae* are substituting for *vieA* in El Tor.

Based on such studies, we hypothesize that levels of c-di-GMP in *V. cholerae* are high in at least some aquatic environments, facilitating biofilm formation, but are reduced early during infection to allow proper expression of the virulence genes. Our finding that a mutant strain, which lacks three DGCs expressed late in infection, is less fit for transitioning from stool to pond water further suggests that elevated c-di-GMP plays an important role in this transition.

The molecules that link changes in c-di-GMP level with observed changes in gene transcription have proved difficult to identify. Using bioinformatic analysis, Dorit Amikam and Michael Galperin at the Tel-Hai Academic College in Jerusalem, Israel, and National Institutes of Health in Bethesda, Md., respectively, identified the PilZ domain as a putative c-di-GMP binding motif. The PilZ-domains of cellulose synthase in *Gluconacetobacter xylinus* and other PilZ-proteins in *E. coli* and *Caulobacter crescentus* mediate binding to c-di-GMP and control protein activity.

The *V. cholerae* genome encodes five PilZ-domain-containing proteins, designated PlzA-PlzE; among them, PlzB shows only weak homology to the PilZ-domain consensus. We find that both PlzD and PlzC bind c-di-GMP in vitro, while a *plzD* PilZ-domain point mutation abrogates binding. A double *plzCD* mutant is reduced in virulence in the murine model and this can be complemented by *plzD* on a plasmid, but not by the *plzD* PilZ-domain point mutant. We have yet to assign roles for the other predicted Plz-proteins in the life cycle of *V. cholerae*, and we do not understand the molecular mechanism by which Plz proteins transduce c-di-GMP signals. Further, because DGCs are found in organisms that lack any predicted PilZ-domain proteins, other classes of proteins likely bind and respond to c-di-GMP.

Recently, an exciting link has been identified between c-di-GMP signaling and bacterial cell density. Quorum sensing allows bacteria to sense their density and/or the density of other bacteria in their vicinity. In many other pathogenic bacteria at high density, quorum sensing activates virulence gene expression (enterohaemorrhagic *E. coli*) and/or biofilm formation (*Pseudomonas*). *V. cholerae* is surprisingly different in that at high density a key protein in its quorum sensing cascade (HapR) inhibits both virulence gene expression and biofilm formation. Intriguingly, *hapR* is often mutated in both environmental and clinical isolates such as the classical biotype strain O395. In addition, the binding site for HapR in the promoter of *aphA*, which is a key virulence gene regulator that is inhibited by HapR, is mutated in classical strain O395. These observations suggest that HapR is not essential to the *V. cholerae* life cycle. Low

cell density signaling through phospho-LuxO turns off *hapR*, enabling virulence and/or biofilm genes to be expressed. Recently, Bonnie L. Bassler at Princeton University, Princeton, N.J., discovered a new target for quorum sensing which is independent of *hapR*, the predicted DGC VCA0939. VCA0939 translation is activated by the same sRNAs that inhibit translation of *hapR* and destabilize the *hapR* mRNA. Whereas *hapR* mutations are common in clinical isolates, the quorum regulator RNA (*qrr*) sRNAs are highly conserved, suggesting that HapR-independent, sRNA-dependent branches of quorum sensing may be essential to *V. cholerae*. This opens up exciting new fields of investigation into HapR-independent quorum sensing and coordination between quorum sensing and c-di-GMP signaling in the *V. cholerae* life cycle.

Conclusions

In the last century improved sanitation and improvements in oral rehydration therapy have been the most important breakthroughs in cholera prevention and treatment. On the pathogenesis research front, the powerful combination of studies using transcriptional profiling or in vivo expression technologies, such as RIVET, is beginning to create a more comprehensive picture of the "ins and outs" of *V. cholerae* as it passes through very different environments between host and aquatic reservoirs.

Research into the regulation and characterization of MSHA is a good example of how information acquired by different approaches in different laboratories comes together to form a more coherent model. Initially thought to be an adherence factor for binding to epithelial cells, MSHA was later shown to be dispensable for colonization in vivo but important for biofilm formation and attachment to chitinous surfaces by Ronald K. Taylor and coworkers at Dartmouth Medical School, Hanover, N.H. Jun Zhu and colleagues at the University of Pennsylvania made it evident that the expression of MSHA has to be down-regulated in vivo to evade the host immune response. Now, we have identified MSHA biosynthesis genes as being induced in the late stage of infection, which leads to the idea that *V. cholerae* might, in this way, prepare itself for the transition into the aquatic environment at the end of the infection.

In summary, we hope that an improved understanding of the *V. cholerae* organism at all stages of its life cycle, in both host and aquatic environments, will help us to prevent disease with novel ways to block key steps in pathogenesis or transmission and to develop effective and inexpensive vaccines.

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