From motility to virulence: sensing and responding to environmental signals in *Vibrio cholerae*
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Sensing its changing environment is key for *Vibrio cholerae* when making the transition from an aquatic lifestyle to one more suited to a human host. An inverse correlation between motility and virulence gene expression has been reported, with the NADH : ubiquinone oxidoreductase system which powers motility by generating a sodium-motive force, playing a pivotal role. Recent studies have demonstrated that bile inhibits activity of the transcription factor ToxT, a protein responsible for direct activation of numerous virulence gene promoters. In addition, recent technological advances have allowed for the analysis of promoters. In-vivo expression technology has revealed that the toxin-coregulated pilus (a colonization factor) is expressed before cholera toxin. Components of an acid-tolerance response system have also been found using this method as well as signature-tagged mutagenesis. Finally, a role for quorum sensing in regulation of virulence gene expression has recently been established.

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**Abbreviations**

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<tr>
<th>ATR</th>
<th>acid-tolerance response</th>
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<tr>
<td>CT</td>
<td>cholera toxin</td>
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<tr>
<td>NQR</td>
<td>NADH ubiquinone oxidoreductase</td>
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<td>Omp</td>
<td>outer membrane porin</td>
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<td>RIVET</td>
<td>recombinase-based in vivo expression technology</td>
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<td>STM</td>
<td>signature-tagged mutagenesis</td>
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<td>TCP</td>
<td>toxin-coregulated pilus</td>
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**Introduction**
Many bacterial pathogens use classical two-component systems for sensing and responding to signals encountered during infection of a host. *Vibrio cholerae*, the agent of human cholera, uses a related membrane-localized transcription system in which a periplasmic domain of indeterminate function is joined to a response-regulator-like DNA binding and activation domain by a transmembrane helix. Therefore, membrane-localized transcription factors, such as those in *V. cholerae*, share features of both parts of the two-component sensory transduction system in a single protein [1]. In *V. cholerae* two membrane-localized transcription factors, ToxR and TcpP, activate the promoter for the cytoplasmic transcription factor, ToxT. In turn, ToxT, activates numerous promoters required for colonization and disease in the host, including those for cholera toxin (CT) and the toxin coregulated pilus (TCP) genes.

Much of what we know about regulation in this system has been elucidated *in vitro*; however, recent advances have allowed investigators to begin to explore *in vivo* requirements for the successful colonization of an animal host. In particular, although *in vitro* experiments are invaluable for the identification of numerous regulators and *de facto* virulence factors, some curiosities have emphasized the artificial nature of *in vitro* experiments. These include the necessity of growing classical *V. cholerae* strains at 37°C for maximal virulence gene expression (a condition obviously not found in the small intestine of the host) and the two-step incubation process known as AKI for virulence expression in El Tor (the current pandemic) isolates of *V. cholerae*.

In this review, we focus on recent studies aimed at determining the nature of environmental regulation of virulence in *V. cholerae*, paying special attention to *in vivo* studies.

**Virulence gene regulation in response to environmental cues**
Before the discovery of ToxR as a regulator of virulence in *V. cholerae*, it was understood that in classical strains, expression of virulence genes required culturing the strains at pH 6.5 and 30°C (so-called ToxR-inducing conditions; [1]). A decade after the discovery of ToxR, a second membrane-localized factor, TcpP, was shown to be necessary for virulence gene expression [2]. Given the membrane topology of ToxR and TcpP, an attractive hypothesis has long been that their periplasmic domains play a role in signal recognition as potential signals may have ready access to the periplasmic space. However, at least for ToxR, the periplasmic domain is dispensable for the regulation of virulence gene expression [3,4] and it is now evident that transcription of tcpP is the event most directly controlled by pH and temperature [5,6].
This latter observation has moved the question of environmental regulation to the control of tcpP expression. Two activators, AphA and AphB, regulate tcpP in both classical and El Tor strains of V. cholerae, however, their expression is pH- and temperature-independent. The current hypothesis is that one or both of these regulators requires specific environmental conditions to become active. The product of another gene, pepA, plays a role in pH regulation in classical V. cholerae strains, although the mechanism of this regulation is unclear [7].

Other in vitro studies have attempted to address the role of potential in vivo signals such as bile on virulence gene regulation. Numerous studies have demonstrated that growth of V. cholerae in the presence of bile results in decreased virulence gene expression and increased motility [8,9]. Klose and Schumacher [9] showed that bile specifically affects the activity of the ToxT protein and suggest that this may be a way of coordinating bacterial swimming and colonization activities [9]. According to this hypothesis, once a bacterium swims to a deeper location in the mucus gel lining the intestine, the bile concentration will decrease and inhibition of ToxT activity will be relieved, resulting in expression of the TCP required for effective colonization. This hypothesis supports the implied model of ‘motility first, virulence gene expression second’, and further suggests why these two phenotypes are linked [10]. The phenotypic link between these opposing activities has been studied in detail by Häse et al. [11,12], who showed that inhibition of motility by increasing the medium viscosity, or addition of ionophores that disrupts ion gradients used to energize motility, results in increased toxT expression. Later studies however, demonstrated this induction to be flagellum-independent [12].

A potential drawback to the hypothesis of motility-directed escape from the inhibitory effects of bile is that by following its chemotactic senses the bacterium might be delivered to a hostile host environment at the surface of intestinal cells. Two groups, working nearly 20 years apart with vastly different experimental approaches, have reported that motile but non-chemotactic mutants of V. cholerae out compete wild-type V. cholerae [13,14**]. Using recombinase-based in vivo expression technology (RIVET), Camilli and co-workers [14**] found that mutations affecting chemotaxis prevent in vivo expression of both toxT and the ToxT-dependent gene ctxA. Closer analysis demonstrated that these strains were not completely defective for toxT or ctxA induction, but rather in the timing of their expression. Once identified, various chemotactic mutants were analyzed for their ability to compete with a wild-type isogenic derivative of V. cholerae and were found to outcompete the wild-type strain. A similar finding was first reported by Freter and O’Brien [13], who showed that undefined chemotactic mutants outcompete wild-type V. cholerae in the infant mouse.

This was not owing to increased growth or colonization of the non-chemotactic mutant but rather increased death of the wild-type strain [13]. Freter and O’Brien also suggested that by swimming through the mucus layer to the close proximity of the intestinal epithelial surface, wild-type V. cholerae exposes itself to bactericidal compounds that compromise its viability. Thus, induction of CT, although surely playing a role in dissemination of V. cholerae from host to host, might also cause dilution of potentially bactericidal compounds present at the surface of the host intestinal epithelium (Figure 1).

Bile also inhibits the growth of V. cholerae in vitro; this sensitivity is controlled by two outer membrane porins, OmpU and OmpT, both regulated by ToxR. ompU expression is activated by ToxR whereas ompT expression is repressed [15,16]. OmpU protects against bile sensitivity and addition of bile can increase the levels of...
OmpU in the outer membrane [17]. In addition, the presence of OmpT exacerbates bile sensitivity [18]. In an elegant series of experiments, ToxR-dependent expression profiles of ompU and ompT were inverted by switching their promoters. The resulting ectopic expression of OmpT, in place of OmpU, resulted in increased bile sensitivity and, unexpectedly, a 100-fold decrease in both CT expression and transduction of the CTX phage, which indicated decreased levels of the phage receptor TCP [19]. These defects were paralleled by a twofold decrease in toxT expression [19], suggesting a previously unknown link between expression of porins and toxT, and also demonstrating how a modest decrease in toxT expression in vitro can lead to large effects in downstream targets. The inappropriate expression of OmpT also led to a 100-fold reduction in infant mouse colonization in a competition experiment [19], supporting the in vitro observations. Subsequent work suggests it is the presence of increased levels of OmpT, not decreased levels of OmpU, which leads to this colonization defect [18].

**Studying V. cholerae gene expression during infection**

Camilli and colleagues have made great use of RIVET to monitor the expression of specific genes during infection of a host with V. cholerae. This technique is based on the use of a resolvase reporter that, when expressed from a promoter under investigation, catalyzes the resolution of res sites that flank a tetracycline-resistance marker. Upon resolution, the marker gene is deleted and the progeny become sensitive to tetracycline. Thus, the dynamics of gene expression, with particular respect to spatial and temporal considerations, can be monitored during animal passage by assessing the fraction of a population that becomes sensitive to tetracycline after infection with an inoculum that contains 100% of resistant organisms [20].

RIVET experiments demonstrated that the TCP is expressed before CT during infection, and that a second signal for toxin production might be encountered by the bacterium following colonization [21]. It was also suggested that the expression of TCP might be required for CTX induction, although analysis of a tcpA mutant (pilus subunit) in this system leads to greatly reduced numbers of recovered bacteria (~100-fold decrease), thereby complicating the interpretation of the experiment. In vitro experiments have demonstrated that the CT promoter (ctxA) is more strongly repressed by the global repressor H-NS than tcpA [22]. This might lead to the delay in ctxA expression during infection.

The RIVET system has also been used to identify and characterize several genes that are induced in vivo, which was the original application of this powerful tool [20,23]. One such gene, cadA, encodes a lysine decarboxylase and plays a key role in the acid-tolerance response (ATR) in V. cholerae, which is important for surviving passage through the stomach [23]. Cells that had been pre-adapted to a moderately low pH (pH 5.7) outcompeted unadapted, wild-type V. cholerae in a mixed infection of a mouse model. In addition, ToxR was demonstrated to play a role in ATR mediated by organic acids, but not by hydrochloric acid [23]. It should be emphasized that the choice of animal model was critical for such experiments, as the initial screen was performed in an adult rabbit ligated-loop model and fewer than half of the genes identified as induced in this model were induced in the infant mouse model [23]. Which model more closely reflects the human host is an open question.

Studies using stool from human cholera patients in Bangladesh suggest that human-passaged V. cholerae is also in an enhanced virulent state, as measured by competition with broth-grown strains in the infant mouse model [24*]. Stool bacteria outcompete broth-grown strains 10–100-fold [24*]. Although this competitive advantage of stool bacteria was maintained following a 5-hour incubation in pond water, it was lost after a single laboratory passage [24*]. These studies are tempered slightly by an inherent, potentially confounding feature of the experiment, that the stool bacteria are in an unknown growth phase (directly from a human host) and are competing against an overnight broth culture of V. cholerae, which must first outgrow lag phase to begin competition for growth and colonization in the mouse [24*].

Microarray analysis of the freshly isolated human-passaged V. cholerae suggested that nutrient acquisition and motility was upregulated, whereas chemotaxis was downregulated, but again the caveat of which normalizing reference conditions are appropriate for calibrating expression levels must be considered [24*]. Another interesting feature of the microarray data was that the previously identified ATR genes from the rabbit studies were not induced in freshly isolated human-passaged V. cholerae, thus calling into question the role of ATR in human transmission.

Another technique that has been used to investigate the environments encountered during infection is signature-tagged mutagenesis (STM, [25]). Using STM, after passage through an animal, genes containing insertion mutations whose products are required for colonization or survival are eliminated from the inoculum, and can be identified by comparison with the input pool. Two screens using this technique with V. cholerae have been reported [26,27]. The first, emphasized the role of the TCP biosynthetic genes for colonization as well as the importance of an intact O-antigen [26]. Other metabolic genes (for purine and biotin synthesis, as well as magnesium and phosphate transport) were also found to be required, perhaps hinting at either limiting nutrients in the gut or potential signals for virulence induction in vivo [26]. All of these mutants were severely defective for
colonization in the infant mouse model. A second more extensive screen was performed more recently, identifying 95 different mutants, including those with insertions in genes for TCP and O-antigen synthesis, various metabolic functions, new members of the ATR system, and numerous regulators and transporters [27]. The severity of the attenuation of most of these mutants was not assessed, as only 9 of 95 mutants were characterized in a competition assay [27]. Two disrupted genes are involved in the NQR complex, an energy generating system required for the sodium-motive force that powers flagellar rotation in V. cholerae and other marine microbes. Components of this system had been identified previously as playing a role in toxT expression in vitro [2]. This result again emphasized the already appreciated critical tie between motility and virulence gene expression [10,11].

It has also been revealed recently that quorum sensing plays a role in the regulation of virulence in V. cholerae [28**]. This is achieved through the action of LuxO, whose activity depends on no fewer than three autoinducer signaling circuits [29*] leading to the repression of HapR, a repressor of tcpP expression [28**,30*]. During early to mid-log growth, hapR expression is repressed by LuxO. On entering late-log phase, HapR is de-repressed and the elevated levels lead to repression of virulence gene expression, possibly explaining the well-described phenomenon of mid-log induction of virulence gene expression in vitro, occurring in the narrow window of a few hours [31,32]. Although these studies indicate a clear role for quorum sensing in V. cholerae pathogenesis, the fact that a hapR mutant strain maintains colonization suggests that other mechanisms allow proper spatiotemporal coordination of motility and virulence factor activation in the absence of tcpP repression.

Conclusions
Of the more than 100 known serogroups of V. cholerae, only two have been associated with epidemic cholera, O1 and O139. The latter is likely to have emerged recently as a competition assay [27]. Two disrupted genes are of special interest

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In these studies, stools were harvested directly from cholera patients and tested for their ability to colonize the infant mouse. Freshly harvested strains were able to outcompete broth-grown cultures. In addition, the authors incubated freshly harvested human stool isolates in Vibrio-free pond water at room temperature for 5 hours and the strains maintained their hyper infectivity. The hyperinfectious character was lost, however, upon colony purification and in vitro culturing.


In this study, a luxO mutant was found to be severely defective for mouse colonization, but if a luxO hapR double mutant was constructed, colonization was restored. Experiments using a hapR-lacZ reporter demonstrated that LuxO represses hapR either directly or indirectly during early-mid-log phase. This repression allows virulence gene expression to be initiated during this window of opportunity.


In this study, it was revealed that Vibrio cholerae possesses at least three quorum-sensing circuits, based on the fact that a csqS luxQ double mutant (autoinducer sensors I and II) still responds to cell density for induction of the V. harveyi luciferase operon. Furthermore, the various signaling systems were shown to play a role in regulating tcpP expression and virulence factor production.


In this study, HapR was shown to repress the aphA promoter, and DNaseI footprinting confirmed a HapR-binding site in the aphA promoter. AphA is required for activation of tcpP and thus virulence gene expression.
