Hidden Dimensions of *Vibrio cholerae* Pathogenesis

Newer techniques help in analyzing growth of this microorganism in the intestinal tract and in identifying candidate vaccine antigens

Gonzalo Osorio and Andrew Camilli

Studying microbial virulence factors during infections requires care. Because the microbes recovered from infected tissues are in small number, the factors being sought can easily disappear, be lost, or be overlooked, even if those cells are immediately grown in vitro to try to produce more of those factors for study. Indeed, detecting in vivo-induced virulence factors is subject to a kind of uncertainty principle, whereby analytic procedures might perturb or even destroy the system. What really happens during an infection is mostly unknown and remains in a black box.

However, we believe that many microbial and host factors are involved in this complex, dynamic process—one with specific spatio-temporal coordinates and many factors and other variables that are being discovered for at least some microbial pathogens. Nineteenth-century Scottish physicist James Clerk Maxwell imagined a tiny “demon” to be used to challenge the Second Law of Thermodynamics. The demon’s small size permitted it to observe events that are happening on a microscopic scale. Without Maxwell’s demon to tell them what happens in vivo, microbiologists developed several ingenious methods over the past decade to analyze complex host-pathogen systems, including among others in vivo expression technology (IVET) and signature-tagged mutagenesis (STM). Recently, we have been applying these and other methods to learn more about *Vibrio cholerae* pathogenesis and its environmental persistence.

**Much about *Vibrio cholerae* Infections Remains in a Black Box**

*Vibrio cholerae* causes the acute diarrheal disease, called cholera, that has been known at least since the times of Hippocrates. Since 1817, there have been seven cholera pandemics, the most recent beginning in 1961 and reaching South America by the early 1990s. Nowadays cholera continues to threaten large portions of the world’s population, mainly in underdeveloped and poor countries, where it is spread via the fecal-oral route.

In areas where cholera is endemic, the organism persists in an aquatic niche between periodic outbreaks, thus making it a facultative human pathogen. Within aquatic environments, it attaches to surfaces of plants, green algae, copepods, crustaceans, and insects (Fig. 1). Although some cholera outbreaks coincide with seasonal algal blooms, the factors that enable this pathogen to persist are poorly understood or characterized.

One of the big challenges is to develop an effective vaccine to protect against *V. cholerae* in endemic areas. Because *V. cholerae* resides on the small intestinal mucosa in its human host, a
vaccine presumably would need to trigger a protective response at this site. In fact, several live-attenuated, candidate vaccine strains with the capacity to colonize the human intestine are immunogenic but also, unfortunately, also reactogenic. Thus, these candidate vaccines tend to produce mild diarrhea, malaise, nausea, vomiting, abdominal cramps, low grade fever, and headache—even though genes encoding the main toxins were deleted.

The residual bacterial reactogenicity factors remain unknown. An important challenge, therefore, is to uncouple these phenomena to obtain colonizing strains that are immunogenic without being reactogenic. We suspect that the expression of some of these immunogenic and reactogenic factors is being induced in vivo.

Analytic Methods Enable Us To Peer into the V. cholerae Black Box

Using IVET and STM helps us to reduce our time and labor when doing V. cholerae mutant hunts, enabling us to test large numbers of strains in parallel. IVET methods are based on constructing transcriptional fusion libraries in pathogens, in which only strains that have an induced transcriptional fusion in vivo survive and can be recovered from the host. In a more recent version of IVET, the in vivo-induced fusions generate heritable changes in the microorganism that subsequently can be detected.

Meanwhile, STM uses pools of random insertion mutation strains, wherein each strain harbors an insertional element uniquely marked with a short DNA sequence tag. The tags are monitored at each step of the procedure by PCR amplification and hybridization to master dot blots containing an ordered array of the tags. After infection, surviving bacteria are recovered and the tags checked, allowing the detection of avirulent strains that were lost in vivo. Such strains have mutations in genes necessary for infection that can be studied further. Though not the focus of this article, we and another group have recently used STM to identify more than 100 V. cholerae genes that are necessary for infecting infant mice, but are not required for growth in rich media in vitro. We are studying these genes, some of which enable V. cholerae to withstand chemical stresses.

In addition to IVET and STM, other recently designed methods are helping us to identify and monitor bacterial gene expression in vivo, including differential fluorescence induction (DFI), in which green fluorescent protein (GFP) fusions are analyzed using fluorescence-activated-cell sorting (FACS) technology; in vivo-induced antigen technology (IVIAT), in which expression libraries are used to identify antigenic proteins in serum from convalescent cholera patients; and real-time PCR, in which mRNA levels from in vivo samples can be measured quantitatively.

RIVET Is Like Having a Maxwell’s Demon

We are particularly pleased with—and are extensively using—the version of IVET called re-
The Explorer Phenotype

Although in an earlier era, Andrew Camilli might have been a explorer of some sort, he became a molecular biologist and is happy with his choice. “What keeps me going every day is my interest in discovery,” says this cholera expert from Tufts University Medical School in Boston. “I look at science as a way of fulfilling my need to be an explorer. If this were 1,000 years ago, I think I’d be on a sailing ship. I think I have an explorer phenotype.”

This nautical fantasy seems curious for someone born and reared in a land-locked automotive town—Flint, Mich.—the fifth of 7 children of a banker and a homemaker. He became the only scientist in the family. “I was always interested in science,” Camilli recalls. “On weekends my father would take us to the Flint public library, and I think I was the only one among our family who enjoyed talking to him about whatever science books he was reading. He helped me pick out books on various science things, and, in a way, was my teacher.”

Camilli began his college career at the University of Michigan studying computer science but eventually switched to wetter subject matter. “At that point, I didn’t know anything about molecular biology,” he says. “I thought I’d be a good programmer. Instead, I ended up finding it quite dry and boring. Then I took a human genetics course, and saw that biology was much more fascinating than computer science.”

He started graduate school at Washington University in St. Louis, but transferred after his first year to the University of Pennsylvania, where his mentor relocated. He received his Ph.D. in microbiology in 1992. As a graduate student, he studied the behavior of Listeria monocytogenes, a bacterial pathogen that is often food borne. In planning for postdoctoral work, he became fascinated with Vibrio cholerae, the bacterial agent responsible for cholera, particularly after learning the details of an unsuccessful vaccine clinical trial.

“I was reading about cholera and the results of testing a vaccine strain that had been attenuated by deleting the genes for the enzymatic subunit of cholera toxin,” he says. “With a live but attenuated vaccine strain the patients shouldn’t have gotten sick—but they did. While it was not as severe as the real disease, they still got diarrhea, which told us that something additional was going on. That’s when I got interested in cholera.” Camilli made up his mind to study cholera in the lab of John Mekalanos, chair of the microbiology and molecular genetics department at Harvard Medical School in Boston, who had been studying Vibrio cholerae for years.

This microorganism lives mostly in water, such as ponds and estuaries, and does not need humans to survive, Camilli says. Nonetheless, once humans accidentally consume this microorganism, it thrives in the small intestine. “Life in the water is very tough for this bacterium,” he says. “There are not many nutrients, and there are other organisms trying to eat it. That’s why it has evolved to take advantage of the occasional human host.”

Camilli and other scientists believe that V. cholerae changes its gene expression pattern upon entering the small intestine. Hence, he and his colleagues are using gene reporter technology—they call it recombination-based in vivo expression technology, or RIVET—to understand details of the infectious process in hopes of learning better ways to prevent or treat it. This is a variation on a theme termed IVET that was originally invented by his mentor John Mekalanos and two former postdocs, Michael Mahan and James Slauch.

“We want to know what does the surface of the bacterium look like during infection,” Camilli continues. “We want to know what are the antigens relevant to infection. The infection is almost certainly a dynamic process. When the bacteria first come in, they will have certain antigens on the surface—but these may be different ten minutes later, or hours later. Also, when they first come in, they have to attach to the small intestine. If they don’t attach, they get washed out,” he adds. “One possibility of attack is to prevent attachment. Another idea is to attack parts of the bacteria needed for its initial growth period. People don’t get sick for 18 to 48 hours after they ingest the bacteria—the bug has to grow up in pretty big numbers first.”

Camilli, who is married to a marketing researcher and the father of three children, hopes that his research will contribute to the development of a more effective vaccine against a devastating disease that remains all too common in developing parts of the world, including India and sub-Saharan Africa. “I’d be very happy if our data could help in the development of a vaccine,” he says. “Yeah, I would love that.”

Marlene Cimons
Marlene Cimons is a freelance writer who lives in Bethesda, Md.
combination-based in vivo expression technology (RIVET). RIVET was designed as a promoter trap. Hence, it uses as a transcriptional reporter the *tnpR* gene, which codes for the site-specific DNA resolvase enzyme from Tn10.

This system is suitable for detecting in vivo-induced (*ivi*) genes, including ones transiently induced or induced only to low levels. TnpR mediates recombination between two specific target DNA sequences, called *res* sites. Two *res* sites are naturally found in the cointegrate intermediate formed during transposition of Tn3 family elements, including Tn10 from a donor to a recipient DNA circle. The recombination reaction resolves the cointegrate intermediate back into the donor and modified recipient DNA molecules.

For our purposes, two *res* sites are inserted into a neutral site in the genome surrounding a reporter gene, such as an antibiotic resistance gene. Next, restriction enzyme-digested genomic fragments of the microorganism are cloned upstream of the promoterless *tnpR* on a conditional plasmid. After the plasmid library is introduced into *V. cholerae*, the nonreplicating plasmids integrate by homologous recombination to generate merodiploid strains. If a particular chromosomal-*tnpR* fusion is expressed during infection, TnpR will catalyze excision of the reporter gene from the genome.

An important preliminary step in any RIVET screen for *ivi* genes is the careful in vitro selection of unresolved or poorly resolved strains from the library for passaging through animals. This step is critical, and we liken it to the risk ships faced when passing between the threatening monsters Scylla and Charybdis from Greek mythology (Fig. 2). If only strains with undetectable levels of resolution in vitro are chosen, most of these also may resolve poorly in vivo (Charybdis). However, if strains that resolve a little in vitro are used, they can behave as random noise and dominate the collection of resolved strains detected postinfection (Scylla).

We believe that it is better to err on the side of Charybdis (Fig. 2). The final chosen strains are then pooled and inoculated into animals. After the infection has proceeded for a period, the resolved strains are screened from among the bacteria recovered from infected tissues. This screening originally entailed replica plating of colonies on agar supplemented with a specific antibiotic because, after resolution, the strains convert from antibiotic resistance to sensitivity. Therefore, the RIVET can detect *ivi* genes ex vivo, acting as if a microbial version of Maxwell’s demon were introduced to observe and report which genes are turned on in the animal.

We are starting to use RIVET to identify *V. cholerae* genes that are induced when it colonizes the cyanobacterium *Anabaena* sp. In using cyanobacteria as hosts, we hope to identify genes that enable *V. cholerae* to survive in aquatic environments and, thereby, to better understand the ecology of this pathogen.

We have also been using RIVET to study *V. cholerae* *ivi* gene expression in intestinal space and time of infection. For instance, *vieB* encodes a putative response regulator of a three-component signal transduction system. We find that *vieB* is transcriptionally induced three hours after infection starts, primarily within the duodenum of the host animal. Recently, we also studied expression of the *tcpA* and *ctxA* virulence genes, which encode a pilin subunit and catalytic subunit of cholera toxin, respectively. Like *vieB*, *ctxA* is induced in a delayed manner, whereas *tcpA* is induced earlier. Pilus production apparently is required for later induction of *vieB* and *ctxA*, indicating that expression of virulence genes, analogous to a developmental pathway, is hierarchical and interdependent. Thus, RIVET can be used both to identify *ivi*
genes and to study their spatio-temporal dimensions.

Second-Generation RIVET

RIVET-based screens suffer three primary limitations. First, because the site-specific DNA recombinase need act at only one or a few substrates per cell, a low level of expression of tnpR is sufficient to catalyze resolution. Although this exquisite sensitivity allows us to detect transient and low-level gene inductions, it fails to identify virulence genes with high levels of transcription during in vitro growth because such strains cannot be constructed in the unresolved state. Second, because ex vivo screening of strains containing active ivi-tnpR fusions requires replica plating, the procedure is laborious. Third, RIVET does not quantitate gene expression as reliably as do traditional reporter genes such as lacZ or pboA.

To overcome the replica-plating limitation, we designed a res cassette that incorporates the counter-selectable sacB gene in addition to an antibiotic-resistance gene. SacB, a levansucrase enzyme from Bacillus subtilis, confers sucrose sensitivity. Because strains that resolve can be selected directly by plating on agar containing 10% sucrose, this positive and negative selectable res cassette eliminates the need for replica plating.

To detect ivi genes with higher levels of basal transcription in vitro, we constructed three different tnpR fusion libraries, each using a different tnpR allele with down-mutations in the ribosome binding site. These tnpR alleles show a range of translational efficiencies and therefore produce less resolvase for any given level of transcription. This strategy is termed the tunable RIVET because the recombination reaction can be tuned to detect a great variety of induced ivi genes.

Soon after we put these improvements together (Fig. 3), we began to screen new libraries in mice. In the process, we uncovered an additional confounding variable of the RIVET methodology: Some strains with undetectable levels of resolution in vitro (<0.1%) have a low but reproducible level of resolution in vivo (1–5%), whereas others show undetectable resolution both in vitro and in vivo (Charybdis).

Is this observed increase in resolution of some strains during infections in mice a biological phenomenon? Perhaps the low levels of resolution observed in vitro represent a very small proportion of the infecting bacteria that, in the presence of unknown signals, partially or fully induces transcription of that particular gene. On the other hand, transcriptional “noise” may be higher for some genes during infection. If this final explanation is correct, then such noise could limit all highly sensitive in vivo expression detection technologies.

Approximately 20% of the putative V. cholerae ivi genes that we discovered are located in
open reading frames (ORFs), but in the antisense DNA strand. Are these antisense transcripts biologically meaningful? Many other bacterial RNA molecules appear to regulate a variety of cellular phenomena, making it seem likely that these *V. cholerae* transcripts are part of this group of regulatory RNA molecules, which we think of as “the dark matter of genomics.” Analyzing this dark matter will probably provide important clues about how microorganisms regulate themselves.

Remarkably, many sense-stranded *ivi* genes seem not to be essential for virulence when tested as single null mutant strains. This finding appears to fall within the context of an emerging paradigm, termed biological redundancy, which posits that at least some critical functions of bacteria, such as surviving nutritional or chemical stresses during infection, are mediated by multiple functionally redundant (or partially redundant) pathways. Only disruption of all pathways within a functionally redundant group will yield a strong mutant phenotype.

**Future Challenges**

One challenge to face in controlling *V. cholerae* outbreaks is to better understand the survival and dynamics of this microorganism in aquatic habitats. It is important to learn more about how *V. cholerae* interacts with its planktonic hosts and about its poorly understood viable but nonculturable state. Identifying and characterizing *V. cholerae* factors that determine its survival is a related part of this endeavor.

Another challenge entails developing a safe and effective vaccine to protect against cholera. It will depend in part on identifying and characterizing immunogenic factors and reactogenic factors, and understanding what role they play during infections. If some of these factors could be uncoupled from the infectious process, we may learn how to produce better live-attenuated vaccine strains. Finally, we have just begun to uncover some the hidden dimensions of infection. We hope that the improved version of RIVET and related in vivo technologies will help us in probing the complex nature of pathogenesis.

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**SUGGESTED READING**


