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Mechanisms of the evolutionary arms race between *Vibrio cholerae* and Vibriophage clinical isolates

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Summary. This review highlights recent findings on the evolutionary arms race between the causative agent of cholera *Vibrio cholerae* and virulent bacteriophages (phages) ICP1, ICP2, and ICP3 isolated from cholera patient stool samples. We discuss mechanisms of phage resistance such as a unique phage-inhibitory chromosomal island and mutations that affect phage receptor expression. We also discuss the molecular characterization of ICP1 and its unique CRISPR-Cas system, which it uses to combat the phage-inhibitory chromosomal island. The role of phages in the life cycle of *V. cholerae* has been increasingly recognized and investigated in the past decade. This article will review hypotheses as to how the predator-prey relationship may have an impact on infections within individuals and on the self-limiting nature of cholera epidemics. In addition, we put forth a strategy of using phages as an intervention to reduce household transmission of cholera within a community.

Keywords: *Vibrio cholerae* · Vibriophage · evolution · cholera

Introduction

Bacteriophages, or phages for short, are found throughout the biosphere. There are an estimated 10^{31} phages in the world [18]. As viruses of bacteria, phages play an important role in the regulation of bacterial populations. Indeed, when phages were first discovered by Felix d'Herelle in 1917, he proposed that the predator-prey relationship may contribute to controlling the natural population of pathogens such as *Vibrio cholerae*, the causative agent of cholera [6]. Cholera is an acute gastrointestinal disease that is characterized by the rapid onset of vomiting and profuse, secretory diarrhea. It is caused by ingestion of water or food that has been contaminated with *V. cholerae*, a Gram-negative bacterium that resides in brackish coastal waters and estuaries [14]. Cholera is a significant burden on global health, particularly in developing countries where water sanitation services are not readily accessible. The World Health Organization estimates that there are approximately 2.8 million cases of cholera each year in endemic countries, which are predominantly in Africa and Asia [1].

Cholera is an ancient disease; descriptions in Sanskrit of cholera-like symptoms have been found and dated back to the 5th century BC. Sometimes referred to as "Asiatic cholera", it has been endemic in South Asia, especially the Ganges delta region, since recorded history [32]. Naturally, populations of phages capable of infecting *V. cholerae* also are present in cholera-endemic regions. Before phages were identified, historical reports note that there were certain elements in the Ganges and Yamuna Rivers in India that can protect against cholera. In 1896, Ernest Hankin passed the water through fine porcelain filters and suggested that there was an unidentified substance in the filtrate that is responsible for killing *V. cholerae*. He further hypothesized that it perhaps plays a role in limiting the spread of cholera epidemics [13,33].

D'Herelle also identified phages from cholera patient stool samples during his work in the 1920s and used them to launch a phage therapy trial in India known as "The Bacteriophage Inquiry" [34]. Initial reports showed consistent observations

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that oral phage therapy resulted in reduced severity and duration of cholera symptoms as well as a decrease in mortality rates [8]. When phages were added to water wells, there were no further cases in villages that previously had cholera [7]. The sum of these results suggested that the introduced phage populations were capable of controlling the *V. cholerae* population in both clinical and environmental contexts.

Since then, there have been a number of cholera phage collections maintained globally. Phage typing has been used to identify *V. cholerae* strains and has contributed greatly to understanding cholera epidemiology. In 1968, Basu and Mukerjee developed a typing scheme using five groups of phages, allowing them to successfully identify 3,464 strains from different epidemics between 1937 and 1966 [2]. Additional updated phage collection schemes [3,4]. There are also a large number of cholera phages stored at the Eliava Institute in Tbilisi, Georgia, the majority of which were isolated from aquatic environments. A recent publication mentions that there are 71 phages collected from 2006 to 2009 alone [9]. Similar collections are maintained at institutions in Russia and China as well.

In recent years, there has been a renewed interest in cholera phages and their study using modern molecular methods [10,23,28]. At the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Faruque et al. have isolated at least 18 cholera phages, known as the JSF series [12]. Our lab has isolated and sequenced the genomes of three distinct phages, referred to as the ICP phages, from 31 Bangladeshi clinical stool samples that span a 10-year period from 2001 to 2010 [28]. This review will discuss the co-evolutionary dynamics between *V. cholerae* and the ICP phages as well as implications for the role of phage in cholera epidemics.

The discovery of the ICP phages

Through a collaboration with the ICDDR,B and Massachusetts General Hospital (MGH), a postdoctoral scholar in our lab at the time, Dr. Kimberley Seed, tested clinical samples for phage presence by plaque assay. Three novel, virulent phages were identified and designated as ICP1, ICP2, and ICP3 (Table 1). ICP1 is specific for O1 serogroup *V. cholerae*; however, the host ranges for ICP2 and ICP3 are broader and include some non-O1 serogroup *V. cholerae* strains. Using primers specific for the DNA polymerase gene of each phage, Dr. Seed screened total DNA from the cholera patient stool samples by PCR to determine prevalence. While the presence of ICP2 and ICP3 were more sporadic, ICP1 was present in all stool samples tested [28].

Co-evolutionary dynamics between the ICP phages and *V. cholerae*

V. cholerae has evolved multiple strategies to evade phage infection. For instance, Zahid et al. have demonstrated how down-regulation of cyclic AMP and the cyclic AMP receptor protein through mutations in the *cyaA* or *crp* genes, respectively, can confer resistance to multiple environmental cholera phages from the JSF series [36]. The work in our lab and that of Dr. Seed's in her lab at the University of California, Berkeley has focused on understanding the co-evolutionary arms race between the ICP phages and *V. cholerae* through the use of comparative genomics and molecular biology approaches. A major theme that has emerged from these studies, described below, is that the ICP phages have evolved to use cell surface receptors that are critical virulence factors of *V. cholerae*, thus limiting the ability of *V. cholerae* to escape phage predation during infection of humans.

ICP1 and the O1 antigen receptor. To identify a mechanism for ICP1 resistance, *V. cholerae* spontaneous mutants that formed small colonies within otherwise clear zones of plaques were genome sequenced and compared to the sequence of the parent strain [29]. Single nucleotide deletions were identified in the poly-A tracts of two lipopolysaccharide (LPS) O-antigen genes *wbeL* and *manA*, which are responsible for tetronate and perosamine biosynthesis, respectively. These mutations, which shift the reading frame of the genes they reside in and cause premature termination, were sufficient to confer ICP1 resistance. In addition, purified LPS preparations from O1 *V. cholerae* strains were able to reduce ICP1 plaque formation, while those from non-O1 strains were not [28]. Taken together, these lines of evidence suggest that the receptor for ICP1 infection is the O1 antigen of *V. cholerae* LPS.

As in the example above, one mechanism to prevent phage infection is eliminating, altering, or reducing the amount of

Table 1. Genome characteristics of the sequenced ICP phages isolated from cholera patients at the ICDDR,B

Phage	Taxonomic family	Genome size (bp)	G+C content (%)	No. of predicted CDSs	% CDSs similar to known proteins
ICP1	*Myoviridae*	125,956	37.1	230	12
ICP2	*Podoviridae*	49,675	42.7	73	19
ICP3	*Podoviridae*	39,162	42.9	54	48

phage receptor on the bacterial surface. Bacteria can modify the availability of the receptor through phase variation, allowing for a heterogeneous population to ensure survival. The example above illustrates this phase variation mechanism, whereby *V. cholerae* undergoes slipped-strand mispairing in the *wbeL* and *manA* poly-A tracts to modify the availability of its O1 antigen. The LPS O1 antigen is normally expressed constitutively and, for *V. cholerae*, is required for intestinal colonization and is therefore considered a major virulence factor [5]. Competition assays in the infant mouse model of *V. cholerae* colonization were performed to assess the ability of *wbeL* and *manA* mutants to colonize the small intestine. Both showed decreased fitness, with the *wbeL* mutant being the more severely compromised [29]. Therefore, under ICP1 predation, the *V. cholerae* population is forced to survive by shifting towards an attenuated virulence phenotype.

ICP1 and its CRISPR/Cas system. The consistent presence of ICP1 in the face of ongoing cholera epidemics in Bangladesh, however, implies that *V. cholerae* has evolved a mechanism to resist ICP1 infection while remaining virulent. A large fraction of *V. cholerae* clinical isolates from Bangladesh were found to encode an 18-kb phage-inhibitory chromosomal island, which is referred to as a phage-inducible chromosomal island-like element (PLE) [30]. The PLE is specifically activated by ICP1 infection and inhibits ICP1 replication. Activation includes excision from the chromosome and subsequent replication as an episome. PLEs can be horizontally transferred by natural transformation or by ICP1 transduction [24], the latter implying that PLE DNA is packaged into virions. This mechanism of phage resistance is akin to abortive infection, where the infected cell sacrifices itself to block phage reproduction, thus protecting the neighboring cells.

ICP1 has evolved a mechanism to overcome the PLE defense mechanism by encoding a CRISPR/Cas system. CRISPR/Cas systems are sequence-specific, adaptive immunity mechanisms typically used by bacteria and archaea to protect themselves from invading nucleic acids such as phage DNA. However, five ICP1 phages, which were isolated from cholera stool samples spanning multiple years at the ICDDR,B, encoded a CRISPR/Cas system with spacers that were 100% identical to sequences found within the *V. cholerae* PLE. The ICP1 CRISPR/Cas is also capable of acquiring new spacers during the phage infection process, which confers specific targeting of new PLE sequences and the restoration of ICP1 replication. Therefore, ICP1 has successfully co-opted the use of a classically microbial adaptive immune system to allow for its own propagation within its host, a mechanism that has not previously been demonstrated in phages [30].

ICP2 and the OmpU receptor. We previously described ICP2 as a virulent phage found sporadically in cholera patient stool samples from Bangladesh. While testing for the presence of phages within Haitian cholera patient samples, our lab, in collaboration with others, identified one sample from 2013 with a high titer of phage [31]. Whole-genome sequencing revealed this phage to have 84% identity over 93% of its genome to an ICP2 isolate from Bangladesh in 2011. The Haitian ICP2 isolate is the first lytic phage reported to be associated with epidemic cholera in Haiti [31].

Most of the *V. cholerae* single colony isolates that were recovered from the same clinical sample as the Haitian ICP2 were resistant to its infection. By comparative genomics, we determined that the ICP2-resistant bacteria had mutations in the *ompU* gene, which encodes the major outer membrane porin OmpU. Using Western blotting, we determined that wild-type amounts of OmpU were present in the outer membranes of these mutants, but the mutations were sufficient to confer ICP2 resistance. The mutations were mapped onto a predicted membrane topology of OmpU and shown to lie within two outer loops, implying that they may disrupt the interaction between OmpU and ICP2 tail fibers [31]. OmpU expression is induced during infection where it plays a major role in resistance to organic acids [20], anionic detergents [27], bile [35], and antimicrobial peptides [19]. A number of assays were performed to determine whether the OmpU mutants were attenuated. No detectable reduction in fitness was observed in the presence of bile or when the mutants were competed with the wild type strain in pond water. There was a mild competitive defect for two of the mutants when passaged multiple times in LB medium, implying a mild defect in the context of rapid growth and replication [31]. This may explain why these ICP2-resistant OmpU variants have not become fixed in the *V. cholerae* population in Bangladesh or Haiti.

ICP2 and the ToxR major virulence gene regulator. Whole-genome sequencing also revealed several ICP2-resistant isolates from Bangladeshi cholera patient stool samples with null mutations in the *toxR* gene. ToxR is the direct transcriptional activator of a number of virulence factors, including OmpU. ICP2 sensitivity in the ToxR mutants was restored by expressing *ompU in trans*, indicating that ICP2 resistance is mediated through the reduced expression of OmpU. Competitions between each clinical ToxR mutant and its isogenic wild-type ToxR revertant strain were performed in the infant mouse model of cholera, and the null mutants were 100- to 1000-fold attenuated [31]. These results are consistent with the inability of these mutant ToxR proteins to activate downstream virulence genes reported by other labs [21,25], thereby the ICP2-resistant ToxR mutants would be attenuated for infection [15].

Role of virulent phages in cholera epidemics

Seasonal variations of phage levels in the environment were discovered in Kolkata, India as early as 1930 [26]. In endem-

ic settings, cholera epidemics are self-limiting in nature; it has been suggested that virulent phages play a role in modulating the course of epidemics [10,11,16,22,23]. Based on epidemiological data collected from Dhaka, Bangladesh, Faruque et al. developed a model suggesting that virulent phages can attenuate cholera epidemics [16]. This model, which is based on observations that the rise of *V. cholerae* in the environment at the peak of epidemics is usually followed by a rise of cholera phages, suggests that the bloom of cholera phages reduces the *V. cholerae* population to the point that the epidemic peters out. A critical assumption of this model is that the initial drop in cholera cases should coincide with high levels of ambient phage. This assumption has been challenged by King et al., as the data show that the number of cholera cases began to decline while the phage numbers were still low [17]. Doubtless, phage predation plays a critical role within patients during the course of cholera infection as well as in its transmission to others or to the environment. More detailed clinical data are needed, however, before a causal role for phages in limiting cholera epidemics can be drawn.

Conclusions

In this review, we have discussed recent literature regarding the arms race between *V. cholerae* and its phages in the context of infection. Adaptations to phage predation are shown to involve trade-offs in fitness, which can impact virulence, transmission, and seeding of environmental reservoirs. By further understanding and characterizing the molecular mechanisms of these predator-prey relationships, we envision using phages as a rapid-acting intervention for at-risk populations, such as household contacts of cholera patients, to immediately protect against contracting the disease themselves. In this manner, phage prophylaxis can represent a fast and specific tool to reduce the burden of bacterial infections on global health.

Competing interests. Authors declare that no competing interests exist.

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