Characterizing clinically isolated *Vibrio cholerae* bacteriophages for the development of a phage prophylaxis cocktail

A thesis

submitted by

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In partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

In

Molecular Microbiology

TUFTS UNIVERSITY

Sackler School of Graduate Biomedical Sciences

October, 2016

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ACKNOWLEDGEMENTS

I am honored to have had the opportunity to complete my PhD research in the laboratory of Andrew Camilli. The Camilli lab has been an amazing place of scholarship and friendship, and my time here has allowed me to push myself in science beyond what I could have ever imagined when I first walked into this lab four years ago. With each passing year, Andy continues to impress me with his wisdom and innovation at the bench, but he has also inspired me with his compassion and his generosity. He is thoughtful, both in his science and in his mentorship, and his down-to-earth and open-minded leadership style is one that I hope to cultivate myself one day. Andy has my utmost respect, even though it may not be obvious through my many layers of sarcasm, and I have been very privileged in my graduate school career to have him as a mentor. Thank you, Dr. Camilli!

I have been lucky to have worked with two generations of Camillian scientists during my time here and have formed friendships with remarkable people. Thank you for making every day in lab enjoyable, for lunchroom educational chats, for being fellow riders on the struggle bus, and for just simply listening when life gets rough.

Dave Lazinski has been a major driving force throughout my PhD. He challenges me in every way and forces me to think past my assumptions. He is one of the main reasons I am the scientist I am today, which he may or may not take as a compliment!

My Lion Bay baymates, Rebecca Walsh and Revati Masilamani, were there to encourage me in my fledging microbiologist days. They never failed to make me laugh, although admittedly that does not require much encouragement. Thank you for the late-night musical reenactments, the midday Chipotle craving runs, and the Disney memories.

EmilyKate McDonough has to be one of the warmest people I know. She was the first one who talked to me when I rotated, and she instantly made me feel welcome. Thank you for always brightening up the lab with your smile. Kimberley Ellis trained me in phage biology, and I would have been hopeless without her advice and encouragement. Thank you, Kim, for introducing me to the life of a phageologist.

Team Phage, where do I even start? I heard rumors that the last year of a PhD is harrowing, but I never felt that way because you all made the work fun. Lynne Cairns and Andrea Wong have seen me at my best and my worst this past year, and somehow they still let me be a part of the team. Thanks for the love, both! Lynne, thank you for being my phage partner-in-crime. With all the late-night animal experiments, wine-sponsored business plan writing, and Phage Bay coffee runs, we have spent so much time together. You would think that we had our share of each other during the week, but then we meet up on the weekends to go to Target, to watch a movie, or to eat at every new delicious restaurant we discover. Thank you for being such an amazing friend both in and out of the lab!

Thank you to my clinical co-mentor, Dr. Stephen Calderwood, for keeping my focus on the application of my work. Every meeting inspired me to learn more about phage therapy and do more for public health. Thank you to my committee members, Profs. Ralph Isberg, Carol Kumamoto, and Wai-Leung Ng, for demanding my best and pushing me to think about my science in different ways. I thoroughly enjoyed our meetings because we always had such lively discussions with different perspectives. Thank you for your wise guidance and your creative ideas!

Thank you to our amazing office – Verna, Ann, Mike, Rima, and Molly. I am quite sure this department would fall apart without you. Thank you for always being the optimistic cheerleaders and for supporting me throughout my PhD. You all always make me smile and make me feel better about science!

I have been lucky to have amazing people all throughout my life who have been my most enthusiastic supporters. Emily and Riley, thank you for always answering the phone and the ensuing laughter and encouragement. Jamal, thank you for being the best friend that I do not know what I do without. And Eric, thank you for your endless support and faith in me over the past two years. Without it, graduate school would have been much more difficult.

Lastly, I would like to thank my family. My sweetheart of a nephew, Jack, reminds me that there is more to life than test tubes and pipettes. My brother-in-law, Jim, is a walking encyclopedia who is always teaching me something new. My amazing sister, Weiwei, is my role model for everything. Her kind and generous spirit, her endless capacity for knowledge, her insatiable curiosity – she inspires me in so many ways to always be a better person. Finally, the people to whom I owe everything, my incredible parents. They have sacrificed so much so that my sister and I could have everything. I have not always been the easiest daughter, but through it all, they were unwavering in their love and support. They always taught us to be the best we can be, and it is their faith and belief in me that has pushed me to go for my dreams. Mom and Dad, without you, I would not be the person I am today. 如果沒有你們的愛和稀牲，不可能會有今天的我。謝謝，爸爸媽媽！

ABSTRACT

Vibrio cholerae is the causative agent of cholera, a severe diarrheal disease that is a substantial health burden on the developing world. It is a Gram-negative bacterium that lives in the aquatic ecosystem and can colonize the human small intestine upon ingestion. Virulent bacteriophages are hypothesized to play a crucial role in modulating the dynamics of cholera epidemics and the progression of disease. Studies to date, however, have not demonstrated this in the context of human cholera infection. Therefore, the goals of my thesis are to understand the impact of intra-patient phage predation on *V. cholerae* population structure and how phages can be used to prevent disease.

In the first study, we investigated the heterogeneity of phage resistance in Bangladeshi and Haitian stool samples containing *V. cholerae* and high titers of the virulent phage ICP2. Using whole-genome sequencing, we determined that ICP2 uses the major outer membrane protein OmpU as its receptor to initiate infection. The potential fitness consequences of OmpU mutants were addressed using assays measuring *in vitro* growth, bile sensitivity, and colonization in an infant mouse model. We were also able to recapitulate the infection dynamics thought to occur in human cholera patients that lead to the selection of OmpU mutants. Our results indicate phage pressure during infection results in an altered *V. cholerae* population structure that exits the host.

In the second study, we determined that ICP2 is widespread in Haiti. We purified 34 ICP2 isolates from clinical samples collected from 2012 to 2014 and performed phylogenetic analyses to determine evolutionary relationships. Although there was a high degree of similarity, the relatedness does not seem to be associated with geographic location or time of isolation.

In the third study, we developed a prophylactic three-phage cocktail for the prevention of cholera. Oral administration of the phages up to 24 hours before *V. cholerae* challenge reduced colonization of the intestinal tract and prevented the onset of disease symptoms in two animal models of cholera pathogenesis. For acute infections, such as cholera, phage prophylaxis could provide a strategy to limit the impact of bacterial disease on human health.

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ABBREVIATIONS

Abi	Abortive infection
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
Amp50	50 μg/ml ampicllin
ATP	Adenosine triphosphate
BRED	Bacteriophage recombineering of electroporated DNA
CDS	Coding sequence
CFU	Colony-forming units
CI	Competitive index
CT	Cholera toxin
DAP	diaminopimelic acid
EDTA	Ethylenediaminetetraacetic acid
EOP	Efficiency of plating
FDA	Food and Drug Administration
GMP	Good manufacturing practice
GTR	Generalized time-reversible
HIIET	Hirszfeld Institute of Immunology and Experimental Therapy
ICDDR,B	International Centre for Diarrhoeal Disease Research, Bangladesh
IP1	Internal protein 1
IPTG	Iso-propyl-β-D-thiogalactopyranoside
IVET	*In vivo* expression technology
LADS	Lethal agent delivery systems

LB	Luria-Bertani
LPS	Lipopolysaccharide
MINUSTAH	United Nations Stabilization Mission in Haiti
MOI	Multiplicity of infection
OCV	Oral cholera vaccine
OMV	Outer membrane vesicle
ORS	Oral rehydration solution
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFU	Plaque-forming units
PICI	Phage-inducible chromosomal island
PLE	PICI-like element
pMMB_OmpU	pMMB76EH vector containing *ompU*
Prn	Pertactin
RF	Replicative form
R-M	Restriction-modification
RS2	Repeat sequence region of CTXϕ
SaPI	*Staphylococcus aureus* pathogenicity island
SDS	Sodium dodecyl sulfate
Sie	Superinfection exclusion systems
Sm	Streptomycin
Sm100	100 μg/ml streptomycin
STE	Salty Tris-HCl buffer with EDTA

TA	Toxin-antitoxin
TCP	Toxin-coregulated pilus
TEM	Transmission electron microscopy
UF	University of Florida
VNC	Viable, but non-culturable
VPI-1	*Vibrio* pathogenicity island 1
VPI-2	*Vibrio* pathogenicity island 2
VPS	*Vibrio* polysaccharide
WHO	World Health Organization
X-gal	5-bromo-4chloro-3indolyl-β-D-galactopyranoside

Chapter 1: Introduction

1.1. The disease cholera

Cholera is an acute gastrointestinal disease characterized by the rapid onset of profuse, secretory diarrhea. If left untreated, the infection leads to severe dehydration and death from circulatory collapse. It is caused by the Gram-negative bacterium *Vibrio cholerae*, which resides in brackish coastal waters and estuaries (Harris, LaRocque, Qadri, Ryan, & Calderwood, 2012; Morris, 2003). Cholera is a significant burden on global health, particularly in developing countries where water sanitation services are not readily accessible. The cause of cholera outbreaks, fecally contaminated drinking water, is always the same, the only difference being the conditions that lead to this dangerous situation. Poor sanitary conditions and overcrowding, especially in the aftermath of a natural disaster or in war-torn countries, are ripe conditions for the start of an outbreak.

Diarrheal diseases, including cholera, are the second leading cause of mortality worldwide in children under five years old (L. Liu et al., 2015). The World Health Organization (WHO) estimates that there are 2.8 million cases of cholera, with an uncertainty range of 1.2 to 4.3 million, each year in endemic countries, which are predominantly in Africa and Asia. About 91,000 of these, with an uncertainty range of 28,000 to 142,000, are fatal (Ali et al., 2012). These estimates are significantly higher than the reported number of cases; many cases are not recorded by countries because there are limitations in their surveillance systems. Countries also fear there will be trade and travel sanctions as a result (WHO, 2015).

1.1.1. Brief history of cholera

Cholera is an ancient disease; descriptions in Sanskrit of cholera-like symptoms have been found and dated back to the 5th century BC (Siddique & Cash, 2014).

Sometimes referred to as "Asiatic cholera", it has been endemic in South Asia, especially the Ganges delta region, since recorded history. Fear of the disease is evident in the culture; in Kolkata, India, a cholera temple for the goddess Ola Beebe was built for protection (Sack, Sack, Nair, & Siddique, 2004) in the 18th century, most likely around 1720 (Sen, 2012). The modern history of cholera began in 1817 when an epidemic broke out in Bengal, the first of seven pandemics on record. It spread beyond India in three directions (west, east, and south) and continued to cause disease for seven years (Siddique & Cash, 2014). The second pandemic (1829-1851) can also be traced back to Bengal, spreading to western countries including the United States for the first time (Duffy, 1971).

Physicians at the time believed that the source of cholera was "miasma" or foul air; however, in 1854, during the third pandemic (Siddique & Cash, 2014), John Snow conducted the first epidemiological study in London and showed that the disease was associated with contaminated well water (Johnson, 2006). Comma-shaped forms from cholera stool samples were also first recognized under a microscope that same year by Italian scientist Filippo Pacini (Harris et al., 2012). It was independently isolated by Robert Koch during his research in Egypt (Koch, 1884), which continued in Kolkata, India (Harris et al., 2012). Three more pandemics struck, spreading to almost every continent (Siddique & Cash, 2014). Cholera did not persist in any of the new geographical areas though, only continuing to be endemic in the Ganges delta (Sack et al., 2004). The damage was done, however, and it came to be viewed as a major public health disaster that would require government intervention. The devastation it caused in

the United States, especially the epidemic in New York City, led to the establishment of the first Board of Health, and cholera became the first reportable disease (Duffy, 1971).

It has been suggested that the seventh, current pandemic began in the Bay of Bengal and has now invaded nearly every continent by way of at least three independent waves (Mutreja et al., 2011). As of 2012, 51 out of 148 countries were classified by the WHO as cholera-endemic. Another 18 countries reported cholera cases but did not meet the criterion for endemic cholera. The WHO estimates that 1.4 billion of the world's population is at risk for cholera, with the most vulnerable populations in southern Asia and Africa (Ali et al., 2012).

1.1.2. Epidemiology

V. cholerae is classified into more than 200 serogroups based on the O-antigen of its lipopolysaccharide (Morris, 2003). Only the O1 and O139 serogroups cause epidemic cholera, with O1 being further classified into the classical and El Tor biotypes (Sack et al., 2004). Each biotype is then broken down into two major serotypes, Ogawa and Inaba, which individually take responsibility for outbreaks, but over time can replace one another in endemic areas (Longini et al., 2002). The epidemic O139 strain is believed to have originated from an O1 El Tor strain that replaced its O-antigen biosynthetic locus through horizontal gene exchange (Waldor, Colwell, & Mekalanos, 1994). Although the classical biotype is considered responsible for the fifth and sixth pandemics, El Tor is considered responsible for the current pandemic and has largely replaced the classical biotype (Harris et al., 2012).

After an epidemic passes through a region, cholera may settle into an endemic pattern of seasonal outbreaks. In Asia, cholera occurs after the monsoon season when

there is an increase in flooding (de Magny et al., 2008; Schwartz et al., 2006; Sebastian et al., 2015). When outbreaks occurred in South America in the early 1990s during the summer months, there were *V. cholerae* blooms in the water two months prior, presumably due to increases in water temperature (Morris, 2003). This may relate to algal blooms, which often precede cholera outbreaks in temperate parts of the world (Epstein, 1993). The correlation of cholera outbreaks with algal blooms is thought to be due to subsequent increases in chitinous planktonic forms coupled with *V. cholerae'*s ability to utilize chitin as a carbon and nitrogen source (Meibom et al., 2004). In addition to climate variability, the population levels of immunity from previous outbreaks may also play a role (Koelle, Rodó, Pascual, Yunus, & Mostafa, 2005). In immunologically naïve populations, such as Haiti before the outbreak began in 2010, cholera affects all age groups and results in high fatality rates (Harris et al., 2010). The spread of disease is particularly rapid in areas with high population density and poor sanitation and health infrastructure (Harris et al., 2012).

1.1.2.1. Outbreaks in Bangladesh

Bangladesh is a South Asian country with a population of 161 million people and considered to be among the most densely populated countries in the world. This historically poor country is starting to undergo rapid economic development (World Bank, 2015). Although significant improvements have been made to the health status of its people, diarrheal disease was still the number one cause of hospitalization in 2008 (Bharati & Bhattacharya, 2014).

Cholera is both a rural and urban disease in Bangladesh and occurs in endemic and epidemic patterns throughout the country due to frequent flooding and cyclones.

Tube wells were once considered a safe source for drinking water but are now contaminated with arsenic, forcing people to drink from contaminated surface water supplies (Smith, Lingas, & Rahman, 2000). There is currently no surveillance system in place, and most of the data for the country is generated by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Estimates from the ICDDR,B put the number of severe cholera cases at 300,000 per year; of note, for each hospitalized case, there are three more in the community, which puts the total number of infections at 1.2 million. Cholera is not officially reported from Bangladesh due to the aforementioned reasons (Bharati & Bhattacharya, 2014).

1.1.2.2. Outbreaks in Haiti

Haiti is the poorest country in the Western Hemisphere. In 2015, approximately 60% of the population had access to improved drinking-water sources, and only approximately 25% had access to improved sanitation (WHO, 2015). The development of sustainable infrastructure in Haiti is blocked by political instability and lack of effective investment. Cholera appeared in Haiti in October 2010 (Centers for Disease & Prevention, 2010), and the epidemic accounted for 57% of all cholera cases and 53% of all cholera deaths reported to the WHO in 2010 (Barzilay et al., 2013).

Epidemiological investigations revealed the source of the outbreak as the upstream region of the Artibonite River. Insufficient sanitation conditions at the United Nations Stabilization Mission in Haiti (MINUSTAH) camp allowed human fecal waste to enter the Meye Tributary System and distribute fully into the Artibonite River Delta (Lantagne, Nair, Lanata, & Cravioto, 2014). Molecular analysis investigations also showed that Haitian strains are clonal, indicating a single-source introduction. In

addition, they were highly similar though not identical to South Asian strains of *V. cholerae* O1 (Chin et al., 2011). As of August 2016, Secretary General Ban Ki-Moon has acknowledged the accidental role the UN played in the initial outbreak and promised to deliver a new response plan within the next two months (Katz, 2016).

1.1.3. Transmission

Asymptomatic cholera patients generally shed bacteria for only a few days, but symptomatic patients can shed bacteria up to two weeks (Harris et al., 2012). Household transmission of cholera contributes significantly to outbreaks; at least 20% of the household contacts will develop symptoms within two days of the index case presentation (Sugimoto et al., 2014; Weil et al., 2009). *V. cholerae* in human stool are present both as individual planktonic cells and as aggregates (Faruque, Biswas, et al., 2006; Nelson et al., 2007). Once in the aquatic environment, the bacteria persist in a free-living state or associate with phytoplankton, zooplankton, or other detritus (Nelson, Harris, Morris, Calderwood, & Camilli, 2009).

As mentioned previously, there is an increase in the prevalence of pathogenic strains in the environment prior to the peak of a cholera epidemic. Bacteriophages (phages) of O1 and O139 *V. cholerae* are consistently found in the same environmental reservoirs (Faruque, Naser, et al., 2005; Seed et al., 2011). Interestingly, there is an inverse association between phage density and cholera cases, suggesting that phages may temper the severity of outbreaks and even shorten their duration (Faruque, Islam, et al., 2005).

The infectious dose of *V. cholerae* in humans varies greatly depending on the host and the bacterial strain. In healthy North American volunteers, doses of 10^8 to 10^{11}

bacteria are required for consistent colonization; this number, however, drops to 10^4 to 10^8 when a bicarbonate buffer is used to neutralize stomach acid, suggesting that food can act as a vehicle for bacteria inoculation (Cash et al., 1970). In addition, O1 *V. cholerae* leaving a host have a hyperinfectious phenotype, which means the infectious dose is 10-100 times lower than for bacteria from the environment. This condition persists in water from 5 to 24 hours (Merrell et al., 2002), suggesting an even larger role for person-to-person transmission than previously anticipated.

1.1.4. Host susceptibility

There are a number of host factors that contribute to cholera susceptibility. Patients with blood type O have been associated with severe symptoms in a number of different populations (Harris et al., 2005). Interestingly, there is a low prevalence of this blood group in cholera-endemic areas, suggesting there is evolutionary pressure against this phenotype (Glass et al., 1985). Genetic factors may also play a role, as results from the study of Bangladeshi families showed that first-degree relatives of cholera patients were more susceptible than less closely related family members (Harris et al., 2008).

Although malnutrition is not a risk factor, it appears that vitamin A deficiency is associated with an increased risk of infection (Harris et al., 2008). Zinc is a nutrient that is rapidly depleted during diarrheal disease, and zinc supplementation to children with cholera has been shown to decrease both duration and stool output (Roy et al., 2008). Both vitamin A and zinc are known to contribute to mucosal immunity (Calder, 2002).

1.1.5. Clinical presentation and diagnosis

After an incubation period between 18 hours and 5 days, the patient will have experience diarrhea and vomiting. The most distinctive characteristic is rice-water stool, which can lead to up to 500-1000 mL/hour of fluid loss (Sack et al., 2004). *V. cholerae* is an extracellular pathogen that colonizes epithelial surfaces in the small intestine and secretes cholera toxin, which will be described in more detail in next section. The secretory diarrheal response is a direct result of cholera toxin acting on adsorptive epithelial cells. Although purging secretory diarrhea is generally painless, patients may experience cramping or abdominal discomfort. If left untreated, death rates are upwards of 70% (Harris et al., 2012). Complications from cholera include electrolyte imbalance and low blood glucose concentrations, usually as a result of insufficient or inappropriate fluids being given. Worst-case scenarios for patients surviving acute cholera include acute renal failure, miscarriages, and premature deliveries (Sack et al., 2004).

Depending on the setting, cholera presentation can vary in severity. In endemic regions, asymptomatic or mildly symptomatic *V. cholerae* infection occurs in 40 to 80% of cases. Such mild diarrhea has a clinical presentation similar to other gastrointestinal diseases, making it difficult to have an accurate diagnosis. Young children are generally most affected by cholera. During epidemics, however, adults contract severe infections just as frequently as children (Harris et al., 2012).

In areas where microbiology laboratories are available, cholera diagnoses are confirmed by isolation of *V. cholerae* from stool on selective media. It can also be rapidly diagnosed from fresh stool samples using darkfield microscopy (Benenson, Islam, & Greenough III, 1964), with a positive-identification rate of 50% (Nelson et al., 2007).

Other assays such as immunoassays, dipstick detection, PCR, and phage typing have a sensitivity of 97% and are used in the field where there is limited laboratory capacity (Alam et al., 2010).

1.1.6. Treatments

The current standard treatment for cholera is rehydration, in particular using oral rehydration solution approved by the WHO in the mid-1970s (Greenough, 2004) that has equimolar concentrations of sodium and glucose for enhanced sodium uptake in the small intestine (Harris et al., 2012). Because of rehydration treatment, the mortality of severe cholera is now reduced to less than 0.2%, even in resource-limited settings. In many cases, however, treatment is not readily accessible. In severe cases, the initial fluid loss needs to be replaced within 3-4 hours of presentation (Harris et al., 2012). It is not always feasible for patients to reach a clinic in time. Cholera is a rapid disease; in Haiti, the median time between the onset of symptoms and death was 12 hours (Centers for Disease & Prevention, 2010).

In some cases of moderate to severe dehydration, antibiotics are used to shorten the duration and volume of fluid loss and can be administered once the patient is rehydrated and vomiting has stopped (Harris et al., 2012). It also reduces the bacterial shedding time to one to two days (Lindenbaum, Greenough, & Islam, 1967). Many epidemic strains are resistant, however, to recommended antibiotics (Morris, 2003), a trend we will explore in a later section. Therefore, its use should be prudent and based on antimicrobial resistance patterns in the area (Harris et al., 2012; WHO, 2015).

1.1.7. Immune response to cholera

Cholera is considered a non-inflammatory infection. In terms of the innate response, there is an upregulation of pro-inflammatory cytokines and migration of neutrophils to the lamina propria; however, there is no gross change to the mucosa or the integrity of the small intestine (Leung et al., 2011; Nelson et al., 2009).

It is a well-recognized that natural cholera infections in endemic settings confer protection against recurrent infections (J. D. Clemens et al., 1991), a phenomenon that was also observed in North American volunteers challenged and re-challenged with cholera (Cash et al., 1970). The best measure for an immune response is serum vibriocidal antibody titer, though they may not contribute directly to protection since *V. cholerae* is non-invasive (Nelson et al., 2009). Most likely, the basis for protection is mucosal immunity, primarily the IgA secretory antibodies specific to the O-antigen of lipopolysaccharide (J. Clemens & Holmgren, 2014) and to the B unit of cholera toxin (Harris et al., 2008). Both serum titers and mucosal secretory IgA levels return to baseline levels in months and may be undetectable, despite continued protection against future infections (Cash et al., 1970). It is possible that the memory response is responsible, as a memory B cell response can be induced by exposure to *V. cholerae* for at least a year after the initial infection (Harris et al., 2009).

1.1.8. Prevention

The best prevention is improved water sanitation infrastructure, but this is not accessible to 663 million people in the world (WHO, 2015). Although the provision of safe water and adequate sanitation is a Millennium Development Goal, it will take decades to achieve (Ryan, 2011). Increased surveillance systems are also needed, and

local microbiological laboratory capacities need to be further strengthened for improving diagnosis. Health education campaigns that are adapted to local cultures should promote hygiene practices (George et al., 2016; WHO, 2015). These efforts have been in place for decades, however, and have not made a significant impact on decreasing the burden of cholera in these regions, which suggests that further action, such as vaccination campaigns, are needed (Ivers, Farmer, Almazor, & Léandre, 2010).

Oral cholera vaccines (OCV) are safe and effectively target induction of mucosal immunity (J. Clemens & Holmgren, 2014; Harris et al., 2012). There are currently two that are WHO pre-qualified, Dukoral® and Shanchol® (WHO, 2015). Dukoral® is a killed whole-cell vaccine that contains several biotypes and serotypes of *V. cholerae* O1 and is supplemented with the recombinant B subunit of cholera toxin (Harris et al., 2012). It is primarily used for travelers to cholera-endemic areas (Harris, 2016). Shanchol® is a bivalent vaccine that includes inactivated *V. cholerae* O1 and O139 but does not contain the recombinant B subunit (Harris et al., 2012). Compared to Dukoral, it confers longer-lasting protection and is much less expensive (Harris, 2016).

Dukoral® is administered in either two or three doses, depending on the age of the recipient. Protection can be expected 1 week after the last dose (WHO, 2015). Clinical trials in Bangladesh and Peru have shown it confers 85% protection for 4-6 months in all age groups (Hill, Ford, & Lalloo, 2006; WHO, 2015). It cannot, however, be administered to children under two years. Shanchol® is administered in two doses given two weeks apart to all older than one-year-old. It provides 67% protection for at least 2 years post-vaccination in areas endemic with *V. cholerae* O1 (Qadri et al., 2015; WHO, 2015). A field trial in Kolkata, India obtained 65% protection for up to 5 years

(Ali et al., 2013). In rural Haiti, vaccine efficacy ranged from 58% to 63% from 4 months to 24 months after vaccination; however, the number of vaccination cases was small with only 47 individuals (Ivers et al., 2015; Luquero & Sack, 2015). To counteract the problem of low supply, single-dose regimens of Shanchol® were tested in Bangladesh. The efficacy was 63% among all cholera episodes, and a single dose is suggested to be an efficient strategy for children older than 5 years and in adults (Qadri et al., 2016). As a response to these studies, the WHO has established a global OCV stockpile of 2 million doses for outbreak control and emergencies (WHO, 2015).

The feasibility of mass OCV campaigns, however, still leaves much to be desired. As mentioned previously, cholera spreads rapidly through a community and can kill within hours of symptom onset. When immediate protection is needed, it is not realistic to assume that OCV campaigns are sufficient. There is currently no standard-of-care for household contacts of cholera patients (personal communication with J.B. Harris). As household transmission contributes greatly to the spread of disease in a community, there is a window of opportunity for a targeted intervention that will immediately protect those at greatest risk of infection.

1.2. *Vibrio cholerae*

Vibrio cholerae is the causative agent of the disease cholera. It is a member of the Vibrionaceae family and is a motile, Gram-negative curved rod (Morris, 2003). It is a natural inhabitant of aquatic ecosystems, such as rivers and estuaries (Colwell, 1996; Harris et al., 2012), and the human gastrointestinal tract, thus making it a facultative human pathogen. In its marine environment, it can also be found attached to free-living

cells or biotic and abiotic surfaces, as previously mentioned (Colwell, 1996). In this section, we will describe the life cycle of *V. cholerae* in water as well as its pathogenesis inside the human host.

1.2.1. *V. cholerae* survival in the environment

V. cholerae is a natural inhabitant of freshwater, brackish, and coastal water habitats (Colwell & Spira, 1992), where it can associate with several environmental hosts. As mentioned previously, it is able to colonize zooplankton and phytoplankton (Butler & Camilli, 2005) due to its interaction with chitin, the most abundant organic polymer in the aquatic environment (Pruzzo, Vezzulli, & Colwell, 2008). One critical factor involved in this adherence is the mannose-sensitive hemagglutinin type IV pili, which are expressed by O1 El Tor and O139 strains (Chiavelli, Marsh, & Taylor, 2001). GbpA (VCA0811), a secreted protein, has also been shown to mediate attachment to chitin as well as epithelial cell surfaces, playing a critical colonization role in both contexts (Kirn, Jude, & Taylor, 2005). *V. cholerae* also encodes chitinases to use chitin as a carbon and nitrogen source (Meibom et al., 2004). Chitin has also been demonstrated to induce natural competence in *V. cholerae*, suggesting that lateral gene transfer occurs primarily in the aquatic environment (Meibom, Blokesch, Dolganov, Wu, & Schoolnik, 2005).

It is generally assumed that the association of *V. cholerae* with marine organisms provides protection against the harsh conditions of the aquatic habitat. To persist in the environment in between epidemics, O1 El Tor and O139 *V. cholerae* form biofilms (Watnick & Kolter, 1999). Biofilm formation involves the production of *Vibrio* polysaccharide (VPS) (Watnick & Kolter, 1999) and three matrix proteins, which allow for dynamic and flexible structures to encase the bacteria (Berk et al., 2012).

V. cholerae are also capable of switching into a viable, but non-culturable (VNC) state as a response to nutrient deprivation (Roszak & Colwell, 1987). The bacteria maintain their basic metabolism processes such as protein synthesis and respiration but are considered dormant. They can be resuscitated upon receiving the appropriate signal, such as the transition from the aquatic environment into the human intestinal tract (Reidl & Klose, 2002).

1.2.2. Pathogenesis in the human intestinal tract

Infection generally starts with oral ingestion of water or food contaminated with *V. cholerae*. The bacteria then pass through the stomach where most are killed by gastric acid. Survivors proceed into the small intestine where they penetrate into the mucus lining that coats the epithelial cells. Upon colonization, the bacteria secrete cholera toxin, one of *V. cholerae*'s major virulence factors that is responsible for the symptoms of cholera (Holmgren, 1981).

The two major virulence factors of *V. cholerae* are cholera toxin (CT) and the toxin-coregulated pilus (TCP) (Nelson et al., 2009). CT is a secreted protein that consists of one A subunit associated with five B subunits (Gill & Meren, 1978). The B subunit pentamer binds to the ganglioside GM_1 on the surface of epithelial cells and allows the A subunit to be translocated intracellularly. Once inside, the A subunit indirectly activates adenylate cyclase through ADP-ribosylation of a regulatory G-protein subunit, thus raising the intracellular level of cyclic AMP. This results in chloride secretion into the intestinal lumen and ultimately secretory diarrhea (Cassel & Pfeuffer, 1978; Gill & Meren, 1978). The genome of the filamentous and lysogenic phage CTXϕ contains the genes for CT. There are different versions of the CTXϕ in the classical and El Tor strains

that are dependent on biotype; it can insert itself into one or two attachment sites in the *V. cholerae* genome or replicate on its own as a episome (Waldor & Mekalanos, 1996). The receptor for CTXϕ is the other major virulence factor TCP (Waldor & Mekalanos, 1996), which is required for colonization (R. K. Taylor, Miller, Furlong, & Mekalanos, 1987). The major function of TCP is to mediate pilus-pilus contact in order to stabilize microcolony formation on the surface of the epithelium (Kirn, Lafferty, Sandoe, & Taylor, 2000). It is encoded on the *Vibrio* pathogenicity island (VPI-1) (Karaolis et al., 1998; Karaolis, Somara, Maneval, Johnson, & Kaper, 1999), which is carried by all seventh pandemic strains of *V. cholerae* O1 El Tor.

Using various transposon library-based methods in animal models of *V. cholerae* infection, a number of genes have been newly identified or confirmed as important for survival in the host (Fu, Waldor, & Mekalanos, 2013; Kamp, Patimalla-Dipali, Lazinski, Wallace-Gadsden, & Camilli, 2013; Pritchard et al., 2014). Of them, a large number are required for purine and pyrimidine biosynthesis, amino acid metabolism, and O-antigen biosynthesis. Included as well is the well-known virulence factor ToxR (Kamp et al., 2013), an inner membrane transcription factor that stimulates expression of virulence genes such as *ompU* (Krukonis & DiRita, 2003; Miller & Mekalanos, 1984).

1.2.2.1. The O-antigen of lipopolysaccharide

In Gram-negative bacteria, the outer membrane layer is dominated by the presence of lipopolysaccharide (LPS). There are three components: lipid A, core oligosaccharide, and the O-antigen. As previously mentioned, *V. cholerae* serogroups are categorized by the varying structures of the O-antigen. Since the experiments conducted for this thesis

use the E7946 strain of *V. cholerae*, which has the O1 Ogawa antigen, the introduction here will only discuss this O-antigen.

The O1 antigen comprises 12-18 repeating units of α(1,2)-linked D-perosamine residues. The amino acids are then further modified by acylation with tetronate. In the literature, the genes currently described as being required for O1 antigen synthesis are located on chromosome 1 of the *V. cholerae* O1 N16961 genome between open reading frames VC0240 (*gmhD*) and VC0264 (*rjg*) (Chatterjee & Chaudhuri, 2004). The genes in this region have been categorized into five groups according to putative function: perosamine biosynthesis (VC0241-0244), O-antigen transport (VC0246-0247), tetronate biosynthesis (VC0248-0252), O-antigen modification (VC0258), and additional genes essential for O-antigen biosynthesis (VC0259-VC0260, VC0263) (Seed et al., 2012).

The O-antigen serves a protective role for the bacterium against various environmental stresses such as the host immune response and antibiotics (Nikaido, 1994; Raetz & Whitfield, 2002). Consequently, because it is so prominent on the surface of the outer membrane, it is the target of the immune system, as described previously, and often used as a receptor for phage infection. Due to these selection pressures, the O-antigen is highly variable. The two serotypes for the O1 antigen are Ogawa and Inaba, which are characterized by the presence or absence, respectively, of a terminal methyl group (Hisatsune, Kondo, Isshiki, Iguchi, & Haishima, 1993). During outbreaks, the epidemic strain can undergo serotype conversion (Koelle, Pascual, & Yunus, 2006) through spontaneous mutations in *wbeT* (VC0258) (Stroeher, Karageorgos, Morona, & Manning, 1992), a predicted methylase.

Seed et al. demonstrated O-antigen phase variance due to slipped strand mispairing in genes *wbeL* and *manA*, which are responsible for tetronate biosynthesis and perosamine biosynthesis, respectively. In addition, the authors performed competitions in the infant mouse model of *V. cholerae* to assess the ability of *wbeL* and *manA* mutants to colonize the small intestine. Both showed varying degrees of decreased colonization (Seed et al., 2012). These results confirm the transposon-based analysis performed by Chiang & Mekalanos (Chiang & Mekalanos, 1998) that O-antigen biosynthesis is critical for colonization in the infant mouse model.

1.2.2.2. Outer membrane porin OmpU

Outer membrane porin OmpU is a 37-kDa protein (Sperandio et al., 1996) whose expression is positively regulated by the critical regulator ToxR (Crawford, Kaper, & DiRita, 1998). Sperandio et al. were the first to characterize it, and they demonstrated that it bound to immobilized fibronectin, a matrix glycoprotein of eukaryotic cells, and also played a role in adherence to HEp-2 cells, an epithelial cell line that is now considered a HeLa contaminant (Sperandio, Giron, Silveira, & Kaper, 1995). The role of OmpU in adherence was later questioned by a separate group who demonstrated that treating bacteria in advance with anti-OmpU Fab did not inhibit its adhesion to rabbit intestine (Nakasone & Iwanaga, 1998). These contradicting results may be due to a difference in *V. cholerae* strains and the source of OmpU preparations. Sperandio et al. extracted OmpU from an SDS-polyacrylamide gel (Sperandio et al., 1995), whereas Nakasone and Iwanaga purified OmpU from the outer membrane fraction of bacteria culture (Nakasone & Iwanaga, 1998).

Sperandio et al. also demonstrated that OmpU was highly immunogenic as post-*V. cholerae*-challenge sera from volunteers strongly recognized OmpU, as assayed by Western blotting (Sperandio et al., 1995). At the time, OmpU was not yet regarded as a virulence factor, although it was considered as possibly important since its expression is co-regulated with known virulence factors such as CT and TCP (Miller & Mekalanos, 1988).

Chakrabarti et al. demonstrated the ability of OmpU to form a functional porin using liposomes. OmpU can constitute as much as 60% of the total outer membrane protein depending on the osmolarity of the growth conditions. It is a homotrimer that requires the association of calcium ions; removal of them results in irreversible disruption of its structure as well as its porin function (S. R. Chakrabarti, Chaudhuri, Sen, & Das, 1996). Based on these data, OmpU research then focused on its functionality and the possible protection its expression can confer.

OmpU expression was shown to be crucial for resistance to organic acids (Merrell, Bailey, Kaper, & Camilli, 2001), anionic detergents (Provenzano, Lauriano, & Klose, 2001), bile (Wibbenmeyer, Provenzano, Landry, Klose, & Delcour, 2002), and antimicrobial peptides (Mathur & Waldor, 2004); all of which *V. cholerae* would encounter during its time in the gastrointestinal tract. The role of OmpU in virulence, however, is debatable. Provenzano et al. demonstrated that Δ*ompU* strains were not attenuated for colonization using a competition assay in the infant mouse model (Provenzano et al., 2001), but others have previously reported difficulties in generating strains with disrupted *ompU*, leading to the belief that it is an essential gene (Li, Crawford, DiRita, & Kaper, 2000; Sperandio et al., 1996). Recent transposon mutant

library analyses also have contradicting results (Fu et al., 2013; Kamp et al., 2013; Pritchard et al., 2014). OmpU and another outer membrane porin OmpT are differentially regulated by ToxR. In the absence of OmpU expression, there is increased expression of OmpT (Provenzano et al., 2001), suggesting that the porins may compensate each other in function. Further experiments are needed to investigate the nuances of varying OmpU mutations and their effects on the protein's structure to determine if there is ultimately any effect on *V. cholerae* virulence in the host.

1.2.3. Late-stage infection and dissemination

As *V. cholerae* prepares to exit from the host, processes involved in coping with nutritional limitations are initiated. Recycling of amino acids, proteins, and cell wall components is upregulated (Kamp et al., 2013) to take advantage of the components present and conserve energy. In addition, a large fraction of the bacteria become motile and thus may be prepared to chemotax to nutrative surfaces in the environment. Many of the genes induced during late infection impact bacterial survival in the aquatic environment or transmission to a new host (Kamp et al., 2013; Schild et al., 2007).

1.2.4. Antibiotic resistance

Although earlier epidemic strains of *V. cholerae* were sensitive to antibiotics, nearly all epidemic strains isolated in the past decade are resistant to co-trimoxazole and streptomycin (Faruque, Islam, et al., 2006). This resistance is mediated by an acquired mobile element SXT (Waldor, Tschäpe, & Mekalanos, 1996). In recent years, O1 strains have emerged from Asia that are resistant to tetracycline, erthryomycin, and/or ciproflaxin (H. B. Kim et al., 2010; Mandal, Dinoop, & Parija, 2012). In some of these

strains, the mechanism is due to acquired resistance genes in the SXT element. The increasing antibiotic resistance identified in recent clinical isolates of *V. cholerae* highlights the importance of prudent antibiotic use when treating patients. Antibiotics are being used as prophylaxis in some countries (Reveiz et al., 2011), although it is not recommended by the WHO (WHO, 2015). These findings in *V. cholerae* only add to the long list of pathogens that are now resistant to multiple antimicrobials and underscore the need for a comprehensive and interdisciplinary set of solutions to combat this increasingly dire problem.

1.2.5. Animal models of *V. cholerae* infection

The most commonly used animal model of cholera is the infant mouse (Klose, 2000). It was validated as a model for *V. cholerae* colonization when TCP and ToxR were shown to be essential factors (Peterson & Mekalanos, 1988; R. K. Taylor et al., 1987). It has been useful in determining a number of *V. cholerae* genes that are explicitly expressed in the murine small intestine and not during laboratory growth through the use of *in vivo* expression technology (IVET) (Camilli & Mekalanos, 1995). The infant mouse model, however, fails to mimic some aspects of human cholera. One major class of genes that is important for pathogenesis is motility, as non-motile mutants cause reduced disease symptoms in humans (Kenner et al., 1995). Non-motile *V. cholerae*, however, show no defects in colonization of infant mice, as measured by competition assays (Gardel & Mekalanos, 1996; Richardson, 1991). In addition, infant mice do not exhibit symptoms such as secretory diarrhea during *V. cholerae* infection.

On the other hand, the Waldor lab has developed an infant rabbit model for *V. cholerae* pathogenesis. This model was original developed in the late 19th century

(Pollitzer, 1959), but was seldom used because of irreproducibility of infection. Ritchie et al. showed that pre-treatment of infant rabbits with a histamine blocker (e.g. cimetidine), which decreases acid production in the stomach, increased the reproducibility of *V. cholerae* infection. Infected infant rabbits have symptoms similar to severe cholera in humans in that rabbits are colonized in a TCP-dependent mechanism and develop CT-dependent secretory diarrhea and bowel distension (Ritchie, Rui, Bronson, & Waldor, 2010). Disease progression is heterogeneous, but symptoms usually develop within 12 to 14 hours of inoculation (Abel & Waldor, 2014). Overall, it is the better animal model for evaluating *V. cholerae* pathogenesis and cholera disease progression.

1.3. Phage biology

Bacteriophages, or phages for short, seem to be everywhere in the biosphere. The total number of phages in the world is estimated to be 10^{31} to 10^{32} total (Kutter & Sulakvelidze, 2005). They are viruses of bacteria and thus play an important role in regulating microbial populations. Phages were independently discovered by Frederick Twort and Felix d'Hérelle in 1915 and 1917, respectively, and since then, several model types such as λ, T4, and T7 have been well-studied. In addition, phages are increasingly being used in a variety of applications. Phage research has provided much of the foundational knowledge in molecular biology today as well as the development of many standard techniques used in labs worldwide. In this section, we will discuss the discovery of phages, the nature of phages and their life cycles, mechanisms of phage resistance, and the co-evolutionary dynamics between bacteria and phages.

1.3.1. The discovery of phages

In 1915, British pathologist Frederick Twort described the "glassy transformation" of micrococci colonies (Twort, 1915). When he inoculated a fresh colony with a bit of material from the watery colony, the mucoid appearance could be induced and propagated indefinitely (Summers, 2005). He wrote that the agent for the transformation "might be considered as an acute infectious disease of micrococci" (Twort, 1915). In 1917, French-Canadian microbiologist Felix d'Hérelle independently isolated a microbe that was "antagonistic" to bacteria, resulting in their lysis in liquid culture and death in patches on the surface of agar plates. He believed this was the work of "ultraviruses" that invaded the bacteria and multiplied, so he called the microbes "bacteriophage", or bacteria-eater (d'Hérelle, 1917).

At the time, d'Hérelle was working at the Pasteur institute, and he was called upon to investigate an outbreak of bacillary dysentery amongst French soldiers. He filtered the clinical samples and looked for these invisible viruses, demonstrating that they were capable of multiplying in waves indefinitely if given a bacterial population. D'Hérelle later continued his phage work by advocating for its use as a therapeutic agent, which will be elaborated upon in a later section about phage therapy. Not everyone agreed, however, that phages were viruses of bacteria. In fact, leading authorities at the time believed it was an induced endogenous bacterial enzyme that was autocatalytic, an argument that Twort supported. For ten years, the controversy surrounding the biological nature of phages went on and became known as the "Twort-d'Hérelle Controversy". The advent of electron microscopy in 1940 allowed phages, which range in size from 25 to

200 nm in length, to be directly visualized, leading to the widespread adoption of d'Hérelle's view that phages are viruses of bacteria (Summers, 2005).

1.3.2. The nature of phages

Phages are at once simple and complex. There are many varieties and still more to be discovered as we increase in our abilities and capacities to culture and sequence them. At their most basic, each phage particle contains a nucleic acid genome (DNA or RNA) that is enclosed in a protein or lipoprotein coat known as the capsid. Like all viruses, they are absolute parasites; thus, they are found in large quantities wherever their hosts reside (Kutter & Sulakvelidze, 2005). Most often, phage isolation is from sewage and feces, soil, and natural bodies of water. Without their hosts, phages can maintain their ability to infect for long periods of time, as long as there is no damage from external agents. As phages are diverse, they also vary greatly in their sensitivity to chemical and physical agents. In general, however, all phages tend to be susceptible to UV light and protein-denaturing agents, although the latter depends on concentration and temperature (Guttman, Raya, & Kutter, 2005).

Over 95% of the phages sequenced to date are tailed phages with dsDNA genomes that are classified as members of the order Caudovirales. There are three families within Caudovirales defined by their tail morphologies: *Siphoviridae* have long, flexible tails; *Myoviridae* have double-layered, contractile tails; and *Podoviridae* have short, stubby tails. The remaining phages are tailless and are categorized into other orders as ten families based on shape, presence of a lipid coat, genome type, and method of escape from the bacterial cell (Guttman et al., 2005). As the focus of the research presented in this dissertation is on tailed phages, we will not go into the details of tailless

phages in this chapter. A comprehensive review of phage classification by Hans Ackermann (Ackermann, 2005) is available, if there is further interest.

1.3.3. Phage life cycles

Phage-host interactions can be studied using the single-step growth curve, which was standardized by Ellis and Delbrück (Ellis & Delbrück, 1939). Phages and the appropriate host bacteria are mixed together at a low multiplicity of infection (MOI), which is the ratio of number of phage particles to number of bacteria. After adsorption, samples are plated at various time points, depending on the phage being characterized, to determine infective centers, which produce a single plaque in a lawn of host bacteria growing within a low-percentage agar or agarose nutrient layer. For a characteristic amount of time known as the latent period, the number of plaques remains constant. It then rises sharply as each bacterium lyses and releases completed phage particles. The burst size refers to the ratio between the number of plaques obtained before and after this lysis event (Hyman & Abedon, 2009). Although the latent period and the burst size are characteristic for each phage, they can be affected by the host bacterial strain used, growth medium, and the temperature (Abedon, Herschler, & Stopar, 2001).

There is also a phenomenon where it seems like all phages disappear for a time, which is known as the eclipse period and was first observed by Doermann in 1953 (Doermann, 1953). During this time, only naked phage DNA is present in the cell. Typical single-step growth curve methods will use chloroform to cause bacterial lysis and plate the mixture to measure the eclipse period and the rate of phage particle synthesis (Hyman & Abedon, 2009). This process involves a number of tightly regulated steps,

which make up the lytic life cycle of what are called virulent phages. The efficiency and timing of each step are very much affected by the metabolic state of the host.

Infection begins with adsorption of tail fibers to specific receptors on the surface of the bacterium. Many phages require clusters of a specific, abundant receptor to position the tail fibers in the proper arrangement. This is needed to trigger the structural rearrangement of the baseplate, which is a multiprotein complex that controls the contraction of the tail sheath and, consequently, the injection of DNA into the bacterial cell (Guttman et al., 2005). The minimal components of this triggering mechanism are universally conserved in all contractile injection systems, such as the type VI secretion system, and has recently been crystallized by Taylor et al. for the T4 baseplate (N. M. I. Taylor et al., 2016).

After irreversible attachment of the phage to the surface of the bacterium, the phage genome passes through the tail sheath into the cell. The tail tip generally has an enzymatic mechanism that penetrates the peptidoglycan layer and inner membrane to allow the release of DNA directly into the bacterial cytoplasm. Once inside, the DNA is subject to host exonuclease enzymatic activities (endonucleases are discussed below); thus, many phages circularize their DNA immediately upon entry or have terminal redundancies to protect their linear ends. The bacterial RNA polymerase then recognizes the strong phage promoters, leading to transcription of immediate early genes whose products begin the process of taking over the bacterial metabolism for phage assembly (Guttman et al., 2005).

The final step in the virulent phage life cycle is bacterial lysis, which includes two proteins: a lysin and a holin. The lysin is an enzyme the degrades the cell wall

peptidoglycan while the holin is a protein that forms a hole in the bacterial cell membrane and allows for the lysin to reach its target (Fischetti, 2001). Holins control the length of the infective cycle; it accumulates in the membrane until, at a specific programmed time, the membrane is suddenly permeabilized (I.-N. Wang, Smith, & Young, 2000). As such, holins are under evolutionary pressure to achieve lysis at the optimal time (N. Wang, 2006).

Virulent phages can only replicate through the lytic cycle, resulting in immediate lysis and production of new progeny. Temperate phages, on the other hand, can enter lysogeny, which is characterized by the integration of the virus into the bacterial genome as a prophage or its existence as a stable episome. Persistence is indefinite, and regulation of lysogeny is mainly under the control of the repressor gene *cI* (Gandon, 2016). Under bacterial stress conditions, prophages can exit lysogeny and enter the lytic cycle (Salmond & Fineran, 2015).

1.3.4. Mechanisms for phage resistance

When there are large populations of phages and bacteria co-habiting the same environment, there are continuous cycles of co-evolution. Phage-resistant bacteria emerge and expand while phages counter these mechanisms to further produce on new hosts. In this section, we will discuss the known mechanisms for phage resistance and the corresponding strategies phages use to bypass them.

1.3.4.1. Preventing phage adsorption

There are three known mechanisms to prevent the first step of phage infection: blocking the phage receptor(s), producing extracellular matrix to prevent access, and producing competitive inhibitors.

Bacteria can modify the availability of the phage receptor through phase variation, allowing for a heterogeneous population of bacteria to ensure survival. The most well-characterized example of this is the two-component regulatory system BvgAS used by *Bordetella* species to express adhesions and toxins. When bacteria are Bvg^+, they express colonization and virulence factors, including the phage receptor pertactin (Prn) (Bock & Gross, 2001). Unsurprisingly, the phage BPP-1 is associated with clinical isolates of *Bordetella bronchiseptica* and is able to infect Bvg^+ bacteria 1 million-fold better than Bvg^- bacteria (M. Liu et al., 2002). Interestingly, despite the fact that Prn is not present on the surface of Bvg^- bacteria, BPP-1 is still able to infect them. Using a reverse-transcriptase-dependent process, nucleotide substitutions are introduced into a variable region of the phage gene *mtd*, which encodes a protein responsible for host recognition (Doulatov et al., 2004; M. Liu et al., 2002). This mechanism is not exclusive to *Bordetella* phages alone; comparative genome analyses revealed that the genes involved can be found in other phage species as well, such as the *V. harveyi* phage VHML (Medhekar & Miller, 2007).

A phase variation mechanism has been identified in *V. cholerae* as well, as previously mentioned. The LPS O1 antigen is expressed in the intestinal tract during infection for efficient colonization and undergoes phase variation through slipped-strand mispairing. Specifically, O-antigen mutants were found to have single nucleotide

deletions in the poly-A tracts of two O-antigen biosynthesis genes *wbeL* and *manA*. Virulent *V. cholerae* phages that use the O1 antigen as a receptor have been found in cholera samples. Bacteria phase variants can then protect the population from phage predation by altering their O-antigen levels (Seed et al., 2012).

Bacteria commonly develop adsorption resistance through mutational loss or alteration of phage receptors. In most cases, phages can acquire a compensating adaptation through host range mutations that alter the tail fibers to recognize the altered receptor. Such mutations have been found in a variety of phages, but the best-characterized interactions have been in *Escherichia coli* phages by the Lenski Lab. Meyer et al. demonstrated the λ phage's ability to infect its host through a new receptor by changing its tropism from the original receptor LamB to OmpF. This arose through mutations in λ's host recognition protein, J, after *E. coli* evolved reduced expression of LamB (Meyer et al., 2012).

Phage receptors can be hidden behind a physical barrier such as a capsule or other extracellular polymers. The capsular polysaccharide K antigen of *E. coli* can interfere with T7 infection (Scholl, Adhya, & Merril, 2005). The phages, to no surprise, have evolved abilities to break through the polysaccharide barrier with tail proteins that possess depolymerization activity (Nimmich, 1997). As another strategy, bacteria can produce molecules to competitively compete with the phage. For example, *E. coli* phages T1 and T5 use the iron transporter FhuA to initiate infection. *E. coli* secretes an anti-microbial molecule microcin J25 during conditions of nutrition depletion; not only does it inhibit the growth of related bacterial strains, it also binds to FhuA and allows the bacterium to avoid phage infection (Destoumieux-Garzón et al., 2005).

1.3.4.2. Preventing DNA entry

To block the entry of DNA, superinfection exclusion systems (Sie) confer immunity against specific phages. The genes involved tend to be found in prophages, suggesting that they act to protect a lysogenized host from further infection by closely related phages (Seed, 2015). For the purposes of this introduction, only Sie systems in Gram-negative bacteria will be discussed.

The *E. coli* phage T4 has two Sie systems encoded by genes *imm* and *sp*. They act separately and have different mechanisms. Imm prevents transfer by changing the conformation of the injection site. It is predicted to be localized to the membrane, but it needs to be associated with another membrane protein in order to achieve complete exclusion of phage DNA (M. J. Lu, Stierhof, & Henning, 1993). Sp inhibits the activity of the T4 lysozyme by preventing the degradation of peptidoglycan and thereby entry of phage DNA (M.-J. Lu & Henning, 1994). The Sim and SieA systems have been associated with prophages of other species as well such as those of Enterobacteriaceae (Kliem & Dreiseikelmann, 1989) and *Salmonella* spp. (Hofer, Ruge, & Dreiseikelmann, 1995).

Kappa phages are temperate phages widely distributed in *V. cholerae* O1 El Tor (Takeya, Shimodori, & Gomez, 1967). It encodes an exported product called Glo that is expressed during the lysogenic phase. Nesper et al. present data that Glo participates in a superinfection exclusion mechanism. Through cell fractionation and Western blot analysis, the authors determined that the Glo protein was located in the periplasm. By expressing Glo from a plasmid, they saw a reduction in plaque formation as well as a change in plaque morphology towards turbid and small plaques. When *glo* was mutated,

there was an increase in the number of plaques formed as well as an increase in lysogeny formation of superinfecting challenge phages (Nesper, Blaß, Fountoulakis, & Reidl, 1999). There have not been further investigations into the mechanism of exclusion, but presumably there is a specific protein-protein interaction that inhibits DNA entry.

There are many details regarding the superinfection exclusion mechanisms that have not yet been elucidated. These mechanisms protect not only the bacterium but the surrounding bacterial population as well from phage infection as the superinfecting phage is rendered unviable. It would be interesting to investigate how these systems evolved and whether there are conserved parts of the system across different species of lysogenic phages.

1.3.4.3. Restriction-modification systems

Many bacteria possess restriction-modification (R-M) systems, whose main function is to protect the cell against invading DNA by rapidly degrading it. They are widely distributed and varied; there are at least four types known (Roberts et al., 2003). When phage DNA enters the cell, it is either recognized by a endonuclease restriction enzyme and degraded, or it is methylated by the cognate modification enzyme and protected from restriction, thus allowing the lytic cycle to begin. The restriction enzyme is generally more active than the methylase, meaning the incoming phage DNA is usually cleaved. However, the rare phages that escape restriction by becoming methylated will yield progeny that are methylated. Many bacteria encode multiple R-M systems to provide some redundancy of protection (Labrie, Samson, & Moineau, 2010).

A search for R-M systems in the *V. cholerae* E7946 genome reveals Type I restriction enzyme HsdR (VC1765) and methylase HsdM (VC1769). They are encoded

by genes in the pathogenicity island VPI-2 (Jermyn & Boyd, 2002), a 57-kb genetic element that was most likely acquired just before or after the emergence of the toxigenic seventh pandemic El Tor strain (Faruque & Mekalanos, 2003). Classical Type I restriction enzymes are usually composed of three subunits encoded by *hsdR*, *hsdM*, and *hsdS*. The S subunit has two target recognition domains that allow for sequence specificity for the other subunits (Murray, 2000; Tock & Dryden, 2005). The E7946 adjacent gene VC1768 encodes a putative HsdS subunit.

Phages have evolved several strategies to counter R-M systems. One such way is introducing point mutations into recognition sites, so that there are none in the phage genome. For example, the T7 phage genome is devoid of EcoR*II* sites (5'-CCA/TGG-3') (Tock & Dryden, 2005). Some phages also enhance the methylase activity to avoid restriction. The protein Ral is responsible for this for phage λ (Zabeau, Friedman, Van Montagu, & Schell, 1980). Phage genomes can also incorporate modified bases, as is the case for T4 where it contains hydroxymethylcytosine (HMC) instead of cytosine. *E. coli*, however, acquired the ability to attack the modified phage DNA by using a modification-dependent system that targets methylated or hydroxymethylated bases (Raleigh & Wilson, 1986). T4 is still resistant to this system because its HMC residues are glucosylated. Continuing this co-evolutionary arms race, *E. coli* strain CT596 possesses a two-component system encoded by a prophage, and it is able to restrict glucose-modified bases using GmrS and GmrD proteins. Some T4 phages have yet another strategy and encode for internal protein I (IPI) to disable the GmrS-D system through direct interaction (Bair, Rifat, & Black, 2007). Yet again, some bacterial strains can get past IPI

by using a single GmrS/GmrD-like fusion enzyme (Rifat, Wright, Varney, Weber, & Black, 2008).

1.3.4.4. Abortive infections

Abortive infection (Abi) systems lead to the death of the infected bacterium, as opposed to the phage resistance mechanisms described above. They target steps in the phage multiplication system, but even now their mechanisms are not completely understood. The most well-characterized Abi system is the two-component system, RexA and RexB, that provides protection against T4 infection. When the phage DNA-protein complex is assembled, RexA is activated, which acts as an intracellular sensor to activate RexB. RexB is embedded in the membrane as an ion channel, and it reduces the membrane potential. This leads to a drop in the bacterium's intracellular ATP levels, which stops the phage infection (Snyder, 1995). In response, T4 encodes two proteins, RIIA and RIIB; however, it only provides partial protection from the Abi system, since overproduction of the Rex proteins confers phage resistance. If T4 also has a mutation in *motA*, a transcription factor that activates middle gene promoters, then it is completely insensitive to the Rex system (Labrie et al., 2010). *E. coli* has more diverse Abi systems though they will not be further described here.

Toxin-antitoxin (TA) systems have recently been shown to mediate Abi as well (Samson, Bélanger, & Moineau, 2013). For example, the ToxIN TA system of *Pectobacterium atrosepticum* protects against phage infections (Fineran et al., 2009). ToxI is the RNA antitoxin molecule, and ToxN is the endoribonuclease toxin. ToxI neutralizes the activity of ToxN, which would otherwise cleave bacterial RNA to initiate cell death (Blower, Salmond, & Luisi, 2011) to cause abortive infection in the event of

phage infection. A phage has been identified to encode a ToxI-like molecule, which is capable of neutralizing ToxN during phage infection (Blower et al., 2012), thereby hijacking the TA system to protect itself.

A new mechanism that is dependent on the eukaryotic-like serine/threonine kinase Stk2 has recently been identified in Staphylococci. Stk2 becomes activated only if the temperate phage phiNM1 enters the lytic cycle, in which case it triggers cell death. To identify what phage protein activates Stk2, Depardieu et al. isolated phage mutants that were capable of undergoing lytic infection without triggering Abi. The authors identified phage protein PacK and demonstrated that it is sufficient to trigger Stk2-mediated cell death. Of note, however, Stk2 can provide resistance to phages that do not have *pacK*, indicating that Stk2 can be activated by other phage proteins (Depardieu et al., 2016).

Abortive infection has been observed in *V. cholerae* El Tor. Chowdhury et al. observed that most of the early phage proteins were made following adsorption of phage phi149, but only some of the late proteins were synthesized. Pulse-labeling revealed that the late proteins were only transiently stable, and thus the authors did not observe plaques (Chowdhury, Biswas, & Das, 1989). It is suggested that two bacterial proteins (14 kDa and 22 kDa) may be responsible. It is possible that the 14-kDa protein is embedded in the inner membrane and protrudes into the cytoplasm to hinder the packaging of DNA into assembled phage capsids. Although these two proteins are found in classical strains of *V. cholerae* as well, they do not seem to have the same Abi effect (Biswas, Chowdhury, & Das, 1992).

1.3.4.5. Assembly Interference

Phage-inducible chromosomal islands (PICIs) are well-characterized in Gram-positive bacteria (Novick, Christie, & Penadés, 2010). Acting as phage parasites, PICIs are capable of interfering with helper phage reproduction by packaging their own DNA into capsids produced by the helper phage. The most characterized PICIs are the *Staphylococcus aureus* pathogenicity islands (SaPIs), which reside stably in the bacterial chromosome until they are induced to excise and replicate upon helper phage infection (Ram et al., 2012). A PICI-like element (PLE) in *V. cholerae* has been shown to interfere with virulent phage production (Seed, Lazinski, Calderwood, & Camilli, 2013), although the mechanism is currently unknown. This interaction will be explored in further detail in the next chapter.

1.3.4.6. Trade-offs

Biological adaptations can often have negative effects on fitness. In the case of phage resistance, trade-offs in *E. coli* are well-documented. For example, acquiring resistance to λ phage also means a loss in the ability to use maltodextrins since the glycoporin channels are either lost or altered. Resistance to T4 is acquired by mutations in *fhuA*, which results in a decrease in iron uptake (Ferenci, 2016). In the case of *V. cholerae*, resistance to virulent phage infection often results in decreased virulence in the host (Seed et al., 2012), a relationship that we will explore in the next chapter.

1.3.5. Roles of phages in bacterial pathogenicity and evolution

It is estimated that approximately 2×10^{16} phage-mediated gene transfer events occur every second (Brüssow, Canchaya, & Hardt, 2004). The process of lysogenic

conversion, a mechanism by which a prophage provides genes that benefit the bacterium, was first observed in the 1950s (Freeman, 1951). Its importance in pathogenicity was brought to light in 1996 when it was discovered that cholera toxin was encoded by a filamentous lysogenic phage CTXϕ (Waldor & Mekalanos, 1996). The mechanism by which this happened will be further explored in the next chapter.

During the 1990s, genome sequencing revealed the abundance of prophages in bacterial genomes and their importance as genetic variation between strains. In the case of *Streptococcus pyogenes*, prophages make up approximately 10% of its genome and encode virulence factors. Prophages can also drive evolutionary changes through inversions or deletions, if they contain homologous regions (Brüssow et al., 2004).

Phages also allow for horizontal gene transfer between bacteria, of which much is due to generalized transduction. Generalized transduction is when bacterial DNA is accidentally packaged during phage replication and delivered to another bacterium upon phage infection (Zinder & Lederberg, 1952). Specialized transduction is when bacterial DNA adjacent to the integrated prophage is transferred due to an imprecise excision. These mechanisms allow for the transfer of antibiotic resistance, virulence, and other genes, thus facilitating the evolution of bacteria (Modi, Lee, Spina, & Collins, 2013; Penadés, Chen, Quiles-Puchalt, Carpena, & Novick, 2015).

1.4. *Vibrio cholerae* phages

Phages have played a remarkable role in the evolution of *Vibrio cholerae* through the mediation of lysogenic conversion, horizontal gene transfer, and bactericidal selection (Seed et al., 2014; Waldor & Mekalanos, 1996). Indeed, with respect to the latter, it has been suggested that the self-limiting nature of cholera epidemics may in part be due to

virulent phage predation (Faruque, Islam, et al., 2005; Faruque, Naser, et al., 2005). In this chapter, we will discuss the diversity of *V. cholerae* phages (Vibriophages), in particular the virulent ICP phages isolated from Bangladeshi clinical samples, and how co-evolutionary phage-bacteria dynamics play a role in cholera epidemics.

1.4.1. A survey of Vibriophages

The use of phage typing to identify *V. cholerae* O1 strains has contributed greatly to understanding cholera epidemiology. In 1968, Basu and Mukerjee developed a new typing scheme that involved five groups of phages, allowing for the successful identification of 3464 strains of *V. cholerae* El Tor from different epidemics between 1937 and 1966 (Basu & Mukerjee, 1968). It allowed epidemiologists to trace the spread of *V. cholerae* from one region to another, a more robust method than following chronic carriers and the spread of infection through individuals. As the host evolved, new phage typing schemes emerged for O1 (Chattopadhyay et al., 1993) as well as O139 (A. K. Chakrabarti et al., 2000).

In addition to this collection, there are a great number of Vibriophages stored at the Eliava Institute in Tbilisi, Georgia, the majority of which were isolated from aquatic environments. There is discrepancy as to the exact number of Vibriophages stored, however, since the online catalog mentions 68 and a recent publication from the institute mentions 71 from 2006 to 2009 alone (Elbakidze et al., 2015). Nonetheless, the diversity of this collection is vast and could provide a resource for research and applications in the future.

Collections of Vibriophages also exist at institutes in Russia and China. Due to the language barrier in the published literature, however, it is not possible to know more

information about the species diversity and their biological characteristics. In recent years, a number of international phage conferences have taken place in these two countries, suggesting open communication within the phage research community and possibilities for further collaborations.

1.4.1.1. Temperate phages involved in virulence

As alluded to previously, phages have played a critical role in the evolution of *V. cholerae*, most famously in the acquisition of the cholera toxin genes by lysogenic conversion. To date, at least three temperate phages have been implicated in evolution of virulence in *V. cholerae*.

CTXϕ. CTXϕ is the most well-known, as it encodes three toxins, one of which is cholera toxin (CT). As previously mentioned, CT is a AB_5 toxin that affects ion transport of epithelial cells in the small intestine. It is encoded by *ctxAB* on the CTXϕ genome. CTXϕ is able to integrate into the *V. cholerae* genome as a stable lysogen or be maintained as an episome (Waldor & Mekalanos, 1996). The genome can be divided into two functional domains: the 4.7-kb core region, which carries the *ctxAB* operon, and the 2.4-kb repeat sequence region (RS2). The genes in the RS2 region (*rstR*, *rstA*, and *rstB*) encode proteins involved in regulation, replication, and integration of CTXϕ (Waldor, Rubin, Pearson, Kimsey, & Mekalanos, 1997). The core region also contains genes *ace* and *zot* (Waldor & Mekalanos, 1996). The Ace and Zot proteins are also enterotoxins (Fasano et al., 1991; Trucksis, Galen, Michalski, Fasano, & Kaper, 1993); however, they are primarily structural and assembly proteins for CTXϕ, implying that entertoxicity is most likely a secondary trait that is the consequence of bacterial infection in the human

host. Ace is a minor coat protein, and Zot is part of the secretion protein complex (Davis & Waldor, 2003; Waldor & Mekalanos, 1996).

The lysogeny of CTXϕ is maintained by the actions of the phage repressor protein RstR. Davis et al. analyzed the *rstR* sequences of closely related *V. cholerae* strains and demonstrated there was considerable variation (Davis, Kimsey, Chang, & Waldor, 1999). This suggests a possible mechanism for polylysogeny, a phenomenon that was also observed with another known temperate phage ϕP15 (Espeland, Lipp, Huq, & Colwell, 2004). Unlike other integrated prophages, CTXϕ does not induce its own replication. Instead, it relies on the anti-repressor protein RstC, which is encoded by another closely located phage genome RS1, for its replication and transmission (Davis, Kimsey, Kane, & Waldor, 2002).

RS1ϕ. RS1ϕ is a satellite phage that contains *rstR*, *rstA*, and *rstB* genes that are homologous to the RS2 region in the CTXϕ genome. As mentioned above, it also encodes the anti-repressor RstC protein for CTXϕ replication and transmission, as it causes the RstR repressor to aggregate (Davis et al., 2002). The production of CTXϕ requires a tandem array of CTX prophages (CTX-CTX) or a single prophage followed by RS1 (CTX-RS1) (Davis & Waldor, 2000). As a satellite phage, RS1ϕ is dependent on CTXϕ for its own packaging and transmission (Davis et al., 2002; Faruque et al., 2002). Using a kanamycin-marked copy of a replicative-form (RF) plasmid of RS1ϕ, Faruque et al. transduced *V. cholerae* isolates to become kanamycin-resistant. These isolates already harbor the CTXϕ genome, and it was observed that only these transductants were capable of producing RS1ϕ (Faruque et al., 2002). Production of RS1ϕ particles can also occur

using functions encoded by another filamentous phage KSF-1ϕ (Faruque, Kamruzzaman, Sack, Mekalanos, & Nair, 2003), resulting in TCP-independent RS1ϕ infection.

There are two lineages of CTXϕ within isolates of classical and El Tor biotypes , which indicate that these isolates may have acquired CTXϕ independently (Boyd, Heilpern, & Waldor, 2000). As tandem repeats of the CTX prophage or RS1 prophage do not exist in classical strains of *V. cholerae*, these isolates do not produce CTXϕ, although they produce CT and the prophage is integrated into two separate sites in the genome. Classical strain CTX prophages are present as solitary prophages or as truncated, fused prophages. On the other hand, the RS1 prophage is present in the *V. cholerae* El Tor isolates from the seventh pandemic (Davis, Moyer, Boyd, & Waldor, 2000). The production of infectious CTXϕ has contributed greatly to the dissemination of *ctxAB* genes among *V. cholerae* El Tor isolates, and may have contributed to the eventual displacement of classical strains as causes of pandemic cholera.

CP-T1. The only known generalized transducing phage of *V. cholerae* is CP-T1, a member of the *Myoviridiae* family, which was isolated by Ogg et al. through UV induction (Ogg, Timme, & Alemohammad, 1981). Transducing frequencies vary between 10^{-5} to 10^{-8} (Hava & Camilli, 2001), and it has been demonstrated that it is capable of transducing markers between classical and El Tor strains (Hava & Camilli, 2001; Ogg et al., 1981). O'Shea and Boyd demonstrated that CP-T1 is capable of mediating generalized transduction to transfer the pathogenicity island VP1 between *V. cholerae* O1 strains using a kanamycin-marked copy of VP1 (O'Shea & Boyd, 2002). It has also been suggested that CP-T1 mediates generalized transduction of the entire CTXϕ genome to classical and El Tor isolates (Boyd & Waldor, 1999), especially for *V. cholerae* isolates

that contain the CTX prophage but lack the *tcp* genes encoding the TCP receptor for CTXϕ (Ghosh et al., 1997).

VPI. There is significant controversy as to whether or not the pathogenicity island VPI, is encoded by a phage. The VPI is a large chromosomal region, approximately 39.5 kb in size, that encodes several virulence genes and a phage-like integrase. It has a GC content that differs from the host chromosome (Karaolis et al., 1998). The *tcp* gene cluster encoding the TCP was initially defined as part of this pathogenicity island. Then, in 1999, Karaolis et al. proposed that TCP is encoded by a filamentous phage VPIϕ, where the protein monomers that make up the pilus structure TcpA are also the VPIϕ coat proteins. The authors presented data to show that recipients were transduced with cell-free phage preparations and identified a double-stranded replicative form of the VPIϕ DNA (Karaolis et al., 1999). Skeptics, however, argue there is a lack of similarity between the VPI sequence and that of canonical filamentous phages (Davis & Waldor, 2003).

Further skepticism arose when the authors of the initial study were unable to show that there was transfer of VPIϕ among *V. cholerae* O1 isolates. In addition, the authors claimed that they were able to induce VPIϕ production using mitomycin or UV light from a collection of TCP-positive clinical and environmental *V. cholerae* isolates (Karaolis et al., 1999). Faruque et al., however, were unable to confirm these results, despite using the same induction conditions (Faruque, Zhu, Kamruzzaman, & Mekalanos, 2003). In addition, Faruque et al. used a PCR assay with a sensitivity of one phage per 10^8 bacteria to detect VPIϕ and CTXϕ DNA from phage preparations, using the *tcpA* and *ctxA* genes as templates. Although there were positive results for *ctxA*, results were negative for *tcpA*

(Faruque, Zhu, et al., 2003). The growth conditions and strains between the two studies were different, which may account for the discrepancy in results. Additional studies are needed, however, to confirm or reject the existence of VPIϕ. Nonetheless, there is a strong argument for the horizontal transfer of these genes; in particular, sequence homology analyses show that this region is highly similar to one in *V. mimicus*, suggesting a recent transfer (Boyd, Moyer, Shi, & Waldor, 2000).

1.4.1.2. Virulent phages of V. cholerae

Historically, it has been recognized that there are certain elements in the Ganges and Yamuna rivers in India that can protect against cholera. In 1896, the British bacteriologist Ernest Hankin reported the presence of antibacterial activity against *V. cholerae* in these waters. He passed the water through fine porcelain filters and suggested that an unidentified substance in the filtrate was responsible for killing the bacteria and perhaps in limiting the spread of cholera epidemics (Sulakvelidze, Alavidze, & Morris, 2001). D'Hérelle also identified Vibriophages in the 1920s and used them to treat cholera patients in India (Summers, 2001) during a phage therapy trial that we will discuss in further detail in the next section.

There has been a renewed interest in virulent Vibriophages in the past decade due to their isolation from environmental and clinical samples in cholera-endemic areas (Faruque, Naser, et al., 2005; Nelson et al., 2008; Seed et al., 2011). From Bangladesh, Faruque et al. have isolated at least 27 distinct Vibrophages (known as the JSF series), of which 18 produce clear plaque morphology and do not produce lysogens (Faruque, 2014; Faruque, Islam, et al., 2005). The genome sequences, however, have not been published, and thus it is unclear if these truly represent 27 distinct phages.

Seed et al. isolated and sequenced the genomes of phages from 31 Bangladeshi clinical stool samples that spanned a 10-year-period from 2001 to 2010. The samples were tested for phage presence by plaque assay using *V. cholerae* El Tor as the host strain. Three distinct, novel phages designated ICP1, ICP2, and ICP3 were discovered. The host range of ICP1 is specific for O1 strains; however, the host ranges of ICP2 and ICP3 are not limited to the O1 serotype. Using primers specific for the DNA polymerase of each ICP phage, the authors screened total genomic DNA from the stool samples by PCR to determine phage prevalence. ICP1 was present in all stool samples while the presences of ICP2 and ICP3 were more sporadic (Seed et al., 2011).

ICP1. ICP1 is a member of the *Myoviridiae* family with a 125,956 bp genome. The GC content is 37%, 10% lower than that of its host *V. cholerae* O1. Transmission electron microscopy (TEM) yields morphological characteristics of an 86-nm long icosahedral head and 106-nm long, 17-nm wide tail. There are a total of 230 predicted coding sequences (CDSs), the majority of which (75%) have no identifiable homologs (Seed et al., 2011). Through plaque inhibition assays and the sequences of ICP1-resistant *V. cholerae*, *V. cholerae* O1 antigen was determined as the receptor (Seed et al., 2011; Seed et al., 2012).

ICP2. ICP2 is a member of the *Podoviridae* family with a 49,675 bp genome that encodes 73 predicted proteins. TEM images show a 60-nm long capsid with 30-nm long, 8-nm wide tails. Uniquely, ORFs 41 and 42 appear to be involved in cobalamin (vitamin B_{12}) synthesis as they encode putative CobT and CobS cobalt chelatase subunits, respectively. This system has not been previously identified in phage genomes and may enhance the host bacterium's metabolism during phage infection, thereby increasing

ICP2 reproduction (Seed et al., 2011). In Chapter 4, we will present results to demonstrate that the major outer membrane porin OmpU is the receptor for ICP2 infection.

ICP3. ICP3 is a member of the *Podoviridae* family with a 39,162 bp genome that encodes 54 predicted proteins. It is a member of the T7-like viruses, since 43% of its proteins are in common with coliphage T7. By TEM, it is also morphologically indistinguishable from T7, with a 60-nm long capsid and 17-nm long, 8-nm wide tails (Seed et al., 2011). With T7, initial interactions of its tail fibers (gp17) begin with the bacterium's LPS; however, successful infection only occurs when one or both of the tail fiber proteins (gp11 and gp12) interact with unknown bacterial surface proteins to further orient the phage for a successful interaction (Kemp, Garcia, & Molineux, 2005). This may be true for ICP3 infection as well. Seed et al. established that ICP3 is not O1-specific (Seed et al., 2011); however, we have not been able to identify the co-receptors on *V. cholerae*'s surfaces that are additionally needed for successful infection (unpublished data).

1.4.2. Vibriophages and cholera epidemics

Season variations of phage levels in the environment were discovered in Kolkata, India as early as 1930 (Pasricha, de Monte, & Gupta, 1930). In endemic settings, cholera epidemics are self-limiting in nature; it has been suggested the lytic phages can play a role in modulating their course (Faruque, Islam, et al., 2005; Faruque, Naser, et al., 2005; Jensen, Faruque, Mekalanos, & Levin, 2006; Nelson et al., 2008; Nelson et al., 2009). Based on epidemiologic data collected, Faruque et al. developed a model that predicted a rise of *V. cholerae* in the environment is quickly followed by a rise of Vibriophages

(Jensen et al., 2006). This model explains the changing prevalence of phages in the waters of Dhaka, Bangladesh seemingly in response to the number of cholera patients.

Faruque et al. collected data on the levels of phage JSF4 and the epidemic *V. cholerae* O1 strain every 48 to 72 hours for 17 weeks. The phage levels in the stool samples of 387 cholera patients were also measured (Faruque, Naser, et al., 2005). The phage concentrations, however, were measured within one order of magnitude, calling into question the significance of the observed rise and fall in levels. In addition, the number of cholera cases were extrapolated from a 2% surveillance sample of all patients who presented at the ICDDR,B for treatment by using data from every 50th patient (Faruque, Naser, et al., 2005). There was no separation of patients into cohorts based on characteristics, nor was there a control group where samples were taken from individuals with no cholera but lived in the same communities. Furthermore, patients come to the ICDDR,B from varying regions of the country. Collecting environmental samples from only the waters in the city of Dhaka will not provide an accurate picture as patients most likely shed *V. cholerae* and Vibriophages in other regions. 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 stringent and detailed clinical data are needed, however, before a causal role for phages in limiting cholera epidemics can be drawn.

1.4.3. Co-evolutionary dynamics between *V. cholerae* and virulent phages

V. cholerae has evolved multiple strategies to evade phage infection. The co-culture of *V. cholerae* O1 with ICP1 causes the emergence of rough mutants, which are

V. cholerae that lack the O1 antigen (Seed et al., 2012; Zahid et al., 2008). We have described previously the slip-strand mispairing mechanism by which *V. cholerae* alters its O1 antigen level to avoid ICP1 infection. These rough mutants, in turn, lose their virulence in the context of the host, as shown by competitions in the infant mouse model with isolates that have their O1 antigen intact (Seed et al., 2012). We have also discussed how several *V. cholerae* strains harbor PICI-like elements (PLE) that interfere with ICP1 assembly during infection (Seed et al., 2013). Zahid et al. have also demonstrated how downregulation of cyclic AMP and the cyclic AMP receptor protein through mutations in the *cyaA* or *crp* genes, respectively, can confer resistance to multiple environmental phages from the JSF series (Zahid et al., 2010). Lastly, in Chapter 4, we demonstrate how *V. cholerae* mutates its outer membrane porin OmpU to evade ICP2 infection and yet keep its functionality.

Vibriophages, in turn, have also evolved strategies to overcome *V. cholerae*'s phage resistance. Most interesting is the discovery of a CRISPR/Cas system encoded by the ICP1 genome. The CRISPR/Cas system is a sequence-specific mechanism by which bacteria protect themselves from invading nucleic acids, such as phage DNA. Yet, Seed et al. isolated five ICP1-related phages from cholera stool samples at the ICDDR,B that encode a CRISPR/Cas system (Seed et al., 2013) between ORF 87 and ORF 88 of the ancestral ICP1 genome sequenced in 2011 (Seed et al., 2011). The majority of the spacers are 100% identical to sequences found within the 18-kb PLE. Therefore, ICP1 has successfully co-opted the use of a classically bacterial adaptive immune system to allow its own propagation within its host bacterium (Seed et al., 2013).

1.5. Phage therapy

Prior to the widespread use of antibiotics, phages were used to treat bacterial infections. Although clinical studies were not pursued in the United States and most of Western Europe after the discovery of penicillin in the 1940s, phages continued to be used by medical personnel in the former Soviet Union, eastern Europe, and France. As antibiotic use is declining in efficacy and its problematic consequences are becoming rapidly apparent, it is clear that new antimicrobial strategies need to be pursued. Phage therapy is now being revisited as a possible method, and lessons from the past could aid this endeavor. In this section, we will introduce the history of phage therapy and the numerous ways phages can be used to control bacterial infections. We will go into detail about the progress of current phage therapy trials as well as the issues that have slowed or halted their progress.

1.5.1. The beginning of phage therapy

Soon after d'Hérelle discovered phages, he decided to test his belief in their therapeutic capabilities (Summers, 2001). He began first with animals, treating avian typhosis caused by *Salmonella gallinarum* in the field and *Shigella dysenteriae* rabbit infections in the lab (d'Hérelle & Smith, 1926). In the field, chickens in certain pens were treated with phage orally, while some were left untreated. Then, both groups were exposed to chickens infected with *S. gallinarum*. The phage prophylaxis offered a high degree of protection. Encouraged by these results, d'Hérelle scaled up the experiment to the numerous flocks on farms in rural France where the epidemic was particularly severe (Summers, 2001). Again, the phage-treated animals had fewer deaths with shorter disease durations and were prevented from second infections. These results were confirmed by a

similar trial for the same disease in Holland by Kramer (d'Hérelle & Smith, 1926). Although these trials were not conducted in a double-blind fashion, such rigorous standards were not yet employed during d'Hérelle's day.

Since the animal trials were successful, d'Hérelle sought to perform human trials. At the time, it was not uncommon to experiment on one's self. D'Hérelle determined the safety of his *Shigella* phage preparations by ingesting increasing quantities, "aged from six days to a month" (d'Hérelle & Smith, 1926). He also injected himself subcutaneously with a 40-day-old preparation. His family members and co-workers were also subjected to both procedures. None of the subjects had any symptoms (d'Hérelle & Smith, 1926). This was considered sufficient to evaluate the safety of the *Shigella* phage preparations, and d'Hérelle proceeded to evaluate his treatment on dysentery patients (d'Hérelle & Smith, 1926).

The work that attracted the most attention to phage therapy, however, was treating bubonic plague victims. When d'Hérelle was stationed in Alexandria, Egypt at the League of Nations Quarantine Station, four patients on a ship passing through the Suez Canal had contracted the plague. D'Hérelle treated the patients with phage cocktails by directly injecting them into the buboes (Summers, 2001). The recoveries were remarkable and reported in the French medical periodical *La Presse médicale* (d'Hérelle, Malone, & Lahiri, 1930), resulting in the widespread knowledge of phage therapy.

Shortly after, d'Hérelle established "The Bacteriophage Inquiry" in India to study the application of phage therapy in India, especially for cholera epidemics (Summers, 2001) as they were regularly associated with the occurrence of religious festivals and pilgrimages. Initial reports from India in the 1920s and 1930s showed consistent

observations that the severity and duration of cholera symptoms as well as its overall mortality were reduced in patients given phages orally (d'Hérelle et al., 1930; Summers, 1993). D'Hérelle also added cholera phages to wells and took note of how many cholera cases occurred in the villages that received phages and those that did not. There were no cases of cholera in villages that were previously contaminated but had phage-treated their wells (d'Hérelle & Malone, 1927).

The purported success of phage therapy resulted in pharmaceutical companies churning out phage cocktail products. Eli Lilly, E.R. Squibb & Sons, and Swan-Myers all began producing therapies that were unlicensed. At the time, the United States Food and Drug Administration was still a fledging agency; therefore, the commercial phage products were not approved. More labs began using phages to treat a variety of bacterial diseases ranging from wound infections to gastrointestinal maladies. Driven by his strong belief in the power of phage therapy, d'Hérelle went on to establish the Eliava Institute in Georgia, an organization that to this day continues to produce phages for therapeutic cocktails (Kuchment, 2011).

1.5.2. Abandonment of phage therapy in the West

The literature on phage therapy was very conflicting. The field could not agree on how a phage worked inside a person or if it worked at all. There was no knowledge on phage biology in terms of its nature and its mechanisms. No attempt was made to standardize phage stocks or titers, making comparisons between therapeutic trials difficult. Many of the commercialized products were inactive due to the preservatives. Patient trials in the United States were conducted without controls or on too small populations with self-limiting conditions (Summers, 2001). The phenomenon of phage

resistance was also observed in 1941 and became the basis for Luria & Delbrück in their work on the nature of bacterial mutation (Luria & Delbrück, 1943). Even though resistance to antibiotic therapy was also noted as a clinical problem at the time, phage resistance became part of the established canon as it was constantly being exploited in the molecular biology community (Summers, 2001). Finally, the medical community came out with reviews in the Journal of the American Medical Association (Eaton & Bayne-Jones, 1934), firmly saying no to phage therapy. Combined with the widespread use of antibiotics, the enthusiasm in the West towards phage research as a treatment waned (Kuchment, 2011).

In the Soviet Union, however, phages continued to be used, and phage therapy became an ideological rebellion against the West. The State Serum and Vaccine Institute in what is now Tbilisi, Georgia, was a major source of phages and was held up as the success of Soviet Science against the capitalist ideas of the West. In the United States especially, it was important to maintain a distance from anything Soviet, even if it was medicine; thus, the idea of phage therapy became tainted as well (Summers, 2001).

1.5.3. Phage therapy in the Eastern Europe

Much of the detailed knowledge the research and medical communities have on phage therapy comes from Eastern Europe and the former Soviet Union. The two main institutions who are actively involved in research and production are the Eliava Institute of Bacteriophage, Microbiology, and Virology, or Eliava Institute for short, in Tbilisi, Georgia and the Hirszfeld Institute of Immunology and Experimental Therapy (HIIET) in Wroclaw, Poland (Sulakvelidze et al., 2001). The Republic of Georgia is the one country in the world where phage therapy is a part of the standard medical practice, and hospitals

routinely use it both for prophylaxis and treatment (Kutter et al., 2010). As the vast majority of literature from Eastern Europe are written in Russian, the discussion below is based on English-language reviews summarizing Georgian and Polish studies.

1.5.3.1. Georgia

The Eliava Institute became a key branch of the Soviet Ministry of Health. Bacterial samples from all over would be sent to the institute to develop phage cocktails. They performed extensive testing for safety and efficacy, and research conferences were held regularly; however, little was published in journals because research findings were treated as military secrets. D'Hérelle's two major cocktail formulations are still the primary cocktails in use, although they are upgraded every 6 months following testing against current epidemic strains to ensure a relevant and broad host range. *Pyophage* targets pus-causing infections, including those caused by *Staphylococcus aureus*, *E. coli*, and *Pseudomonas aeruginosa*. The other cocktail *Intestiphage* targets 23 different enteric bacteria and is an over-the-counter product used primarily to treat traveler's diarrhea (Kutter et al., 2010).

Combating enteric disease has long been a research mission of the institute. A prophylactic trial for dysentery in the early 1960s involved 30,769 children under the age of seven. Children living on one side of the street were given a phage cocktail in the form of a dry tablet targeted against *Shigella* spp., while children on the other side of the street were given a placebo. Subjects were followed for 109 days by weekly nurse visits, and there was a 3.8-fold decrease in dysentery incidence. Unfortunately, the publication for this data was 68 lines long and written in Russian. The data is available, however, and it has been used to further develop the tablet method. In addition to enteric diseases, the

Eliava Institute also focused on surgical and wound infections, although those studies will not be discussed in this chapter. Please read the 2010 review from Kutter et al. for more details, if interested (Kutter et al., 2010).

1.5.3.2. Poland

Phage therapy of humans in Poland has dated back to at least 1925 (Górski, Borysowski, Miedzybrodzki, & Weber-Dabrowska, 2007), when anti-staphylococcal treatments were used. At the Hirszfeld Institute, phages are selectively selected for each patient. Since the 1970s, over 2,000 patients have been treated at the institute, although these were not controlled clinical trials. These patients were treated with phage only after all other approaches had failed (Kutter et al., 2010). The most detailed English language reports were published by Slopek et al. A series of seven papers describes the administration of phage through various methods to 550 bacterial septicemia patients in total (Slopek, Weber-Dabrowska, Dabrowski, & Kucharewicz-Krukowska, 1986). Phages were administered orally, directly onto wounds using phage-soaked dressings, or directly onto the eye, middle ear, or nasal mucosa. For oral administration, the phages were mixed with baking soda or bicarbonate mineral water a few minutes prior to neutralize the gastric acid (Sulakvelidze et al., 2001). The rate of success, which was marked as having negative bacteria cultures, was 92%.

1.5.4. Renewed Interest in the West

Phage therapy has been practiced in France since 1919, since d'Hérelle gave phage preparations to dysentery patients (Summers, 1993). His laboratory at the Pasteur Institute in Paris was the first to produce commercial phage cocktails, which were still

manufactured until 1978. Even through the mid-1990s, the Pasteur Institute would continue to supply small amounts of phage preparations if requested. After production stopped, physicians who were interested in using phage therapy had trouble obtaining suitable phage preparations. The approach in Georgia and Russia is to use as many phages as possible in the cocktail, which is not a strategy that is well-accepted by regulators. In addition, there was a lack of interest in funding phage therapy clinical trials.

In the early 2000s, two U.S. companies, Intralytix and Exponential Biotherapies, made major progress in funding clinical trials with phages targeting the hospital-acquired infection vancomycin-resistant enterococcus. Exponential Biotherapies even carried out a small Phase I trial in England. With the Dotcom crash, however, the major investors pulled their funding, and Intralytix was forced to focus their products on less risky food-safety targets (Kutter et al., 2010), a mission which they still uphold today (Intralytix). In recent years, however, momentum in the United States and Western Europe seems to be building again.

1.5.5. Phage therapy strategies

There are many applications for phages, many of which are standardized tools used in modern molecular biology. These include using phages as cloning vectors for delivery of DNA into bacteria (Westwater & Schofield, 2005), vectors for targeted gene delivery to mammalian cells (Larocca, Witte, Johnson, Pierce, & Baird, 1998), and pathogen diagnostics (Rees & Loessner, 2005). For our introduction, however, we will focus on recent uses of phages in treating bacterial infections. We will discuss phages in

the context of lethal agent delivery vehicles, phage-component therapies, intact-phage cocktails, and genetically engineered phages.

1.5.5.1. Lethal agent delivery vehicles

As opposed to traditional phage therapy approaches, lethal agent delivery systems (LADS) do not kill their hosts via the lytic cycle. LADS use a phage-based *in vivo* packaging system to create delivery vehicles that inject genetic information encoding antimicrobial agents into the targeted pathogens (Westwater & Schofield, 2005). Westwater et al. demonstrated the concept using the ability of a non-lytic filamentous phage to package and deliver phagemids encoding the toxins Gef and ChpBK to *E. coli*. There was an approximately 1000-fold reduction in colony-forming units (CFU) when expression was induced, implying that Gef and ChpBK were able to exert a bactericidal effect. To demonstrate an effect *in vivo*, mice were infected with a single dose of *E. coli* injected intraperitoneally. Mice treated with LADS showed a significant reduction in the number of bacteria circulating in the blood (Westwater et al., 2003). The LADS were injected within 5 minutes of the *E. coli* injection, however, which does not establish efficacy in the case of an established infection where the *E. coli* have had time to express proteins to adapt to the new environment and multiply to a higher load. Nonetheless, this proof-of-concept study provides an alternative approach in using phages to treat bacterial infections.

1.5.5.2. Phage-component therapies

One of the most promising phage components that has been developed for commercial therapeutic use is the lysin. Late in the lytic cycle, lysins translocate to the

bacterial cell wall and damage its integrity by hydrolyzing the bonds in peptidoglycan. There are three types: N-acetylmuramidases (lysozymes) that act on sugar moieties, endopeptidases that act on the peptide cross bridge, and N-acetylmuramyl-L-alanine amidases (amidases) that act on the amide bond connecting the sugar and peptide moieties (Young, 1992).

It has been possible to purify recombinant forms of these lysins, which are capable of lysing sensitive bacteria upon contact. Binding is strong, with affinity constants of 3-6 x 10^{-8} M (Loessner, Kramer, Ebel, & Scherer, 2002). Research has focused on lysins that can kill a whole variety of Gram-positive bacteria, including *Streptococcus pneumoniae* (Loeffler, Djurkovic, & Fischetti, 2003; Loeffler, Nelson, & Fischetti, 2001) and *Bacillus anthracis* (Schuch, Nelson, & Fischetti, 2002). Their use has also been described in treating systemic bacterial infections for anthrax (Schuch et al., 2002; Yoong, Schuch, Nelson, & Fischetti, 2004), pneumococcal septicemia (Loeffler et al., 2003), and enterococcal septicemia (Yoong et al., 2004).

The biotechnology company Contrafect has recently completed a Phase 1 trial in the U.S. for its product CF-301, which is a lysin that is effective against *Staphylococcus aureus* bloodstream infections, including septicemia and endocarditis. In animal models, combination treatment using CF-301 and vancomycin or daptomycin increased survival significantly in comparison to using antibiotic treatment alone (Schuch et al., 2014). It is the first and only lysin to enter human clinical trials in the U.S.

1.5.5.3. Intact-phage cocktail therapies

Intact-phage cocktail therapies have several advantages over antibiotics. They have high specificity, usually only recognizing a limited number of strains. This reduces the negative effects of disrupting the microflora of the host. On the other hand, it also requires the selection of an effective phage for a selected target. Intact phages are also capable of "auto-dosing"; as phages propagate on their host, they are capable of increasing their numbers several orders of magnitude to continually reduce the numbers of their target bacteria. From a development point of view, phages are more rapidly isolated than antibiotics, allowing for tailored treatments for the bacterial infection as well as a quicker turnaround in the case of phage resistance.

There are several limitations as well, however. From a regulatory point of view, it may be difficult to receive approval for a formulation that may constantly be updated as new epidemic bacterial strains emerge. There is one FDA-approved precedent that could be applied to phage therapy; cocktails can be regulated in the same manner as the FluMist® influenza vaccine, which is reformulated every year to counter predicted circulating flu strains (Marwick, 2000). Instead of running through clinical trials for each year's formulation, the FDA approved the process used in developing the vaccine (Keen, 2012). From a pharmacokinetics angle, wild-type phage particles may be rapidly eliminated by the body's immune system, depending on the administration route. The circulatory time can be improved, however, by selecting for long-circulating mutants (Merril et al., 1996) or by shielding the phages in a non-immunogenic polymer such as polyethylene glycol (K. P. Kim et al., 2008). As the most promising phage therapies in

clinical trials are intact-phage cocktails, we will discuss this strategy further in the following section.

1.5.5.4. Genetically engineered phages

Until recently, the efficient and targeted modification of virulent phage genomes was difficult. New techniques have recently emerged though and can be useful in improving the antimicrobial effect of therapeutic phages. BRED is bacteriophage recombineering of electroporated DNA and has been developed successfully for the efficient manipulation of *Mycobacterium* phages (Marinelli et al., 2008), although it has been used for others such as *E. coli* phages as well (Fehér, Karcagi, Blattner, & Pósfai, 2012; Marinelli, Hatfull, & Piuri, 2012). Lu et al. have proprosed inserting the whole phage genome inside a yeast artificial chromosome vector by homologous recombination for propagation in the yeast host. Yeast recombineering techniques can then be applied for manipulating the phage genome (T. K. T. Lu et al., 2016). Additionally, the CRISPR-Cas system has recently been developed as a tool for manipulating the genome of phages (Box, McGuffie, O'Hara, & Seed, 2016; Martel & Moineau, 2014).

The specificity of phages has been described as a benefit, but it can also be a limitation as well from a regulatory standpoint. Therefore, many groups have worked to expand host ranges. Using homologous recombination, Yoichi et al. were able to change the host specificity of the coliphage T2 by swapping out genes encoding its putative host binding proteins for homologous genes from the genome of phage PP01. The host range of T2 was subsequently changed to that of PP01 (Yoichi, Abe, Miyanaga, Unno, & Tanji, 2005). Studies have also explored the genetic modification of temperate phages to become permanently virulent (lytic). The loss of lysogeny can be accomplished by

mutating the *vir* gene, which prevents the binding of the repressor protein to the operator and allows the phage to proceed with the lytic cycle (Rapson, Burden, Glancey, Hodgson, & Mann, 2002).

Engineered phages have also been shown to work in conjunction with antibiotic therapy. Lu and Collins overexpressed the LexA3 repressor and the SoxR regulator using a modified version of M13 phage to suppress the SOS response and DNA repair in *E. coli*. As it has been previously shown that bactericidal antibiotics are more effective when the SOS response is disabled, Lu and Collins were able to rescue infected mice using antibiotics in conjunction with the modified phage (T. K. Lu & Collins, 2009).

1.5.6. Phage therapy clinical trials

In recent years, there have been an increase in the number of phage therapy products that have entered clinical trials, both from the academic and private sectors. For example, a British phage therapy company, Biocontrol Limited (recently acquired by AmpliPhi Biosciences) obtained regulatory approval for a phase I/II clinical trial for a six-phage cocktail to treat chronic otitis (Wright, Hawkins, Änggård, & Harper, 2009). It was a successful trial, and Biocontrol Limited at the time released plans for progressing to large-scale phase III trials. Since their acquisition by AmpliPhi Biosciences, however, there has not been further news. AmpliPhi itself though has two cocktails against *Staphylococcus aureus* in wound infections and chronic rhinosinusitis that are in phase I trials (AmpliPhi).

In the U.S., the Wound Care Center in Lubbock, Texas initiated a phase I prospective, double-blind trial to evaluate the safety of the phage preparation WPP-201 developed by Intralytix (Kutter et al., 2010). The cocktail contained eight environmental

phages that targeted *P. aeruginosa*, *S. aureus*, and *E. coli*; the formulation was applied directly to primary dressing to target infections in venous leg ulcers (ClinicalTrials.gov, 2011). As expected, there were no clinical safety concerns with WPP-201, and the center will most likely proceed with phase II trials (Kutter et al., 2010). The two recent major clinical trials in phage therapy, however, are PhagoBurn and the Nestlé-sponsored *E. coli* phage cocktail.

1.5.6.1. PhagoBurn

A group of Belgian surgeons and scientists were interested in the possibilities of treating *S. aureus* and *P. aeruginosa* burn wound infections, which are a major cause of morbidity and mortality (Altoparlak, Erol, Akcay, Celebi, & Kadanali, 2004; Church, Elsayed, Reid, Winston, & Lindsay, 2006). They established a collaboration with Georgian and Russian scientists and started a phase I clinical trial with nine burn patients infected with *P. aeruginosa* and/or *S. aureus*. The BFC-1 cocktail consisted of three phages, two against *P. aeruginosa* and one against *S. aureus*, and it was applied on burn wounds ten times per patient. Subjects were monitored over the course of three weeks, and biopsy samples were taken to monitor bacteria counts before and after each application (Merabishvili et al., 2009). The results were unclear as to whether or not the phage application had an effect.

There were a number of issues during the trial. The gold standard for measuring bacterial load was biopsy samples (Kutter et al., 2010), but this proved to be cumbersome in practice as it necessitates the administration of local anesthesia and also required complex sample processing. Also, there were delays in receiving antimicrobial susceptibility reports for patients, further delaying trial enrollment. During the wait,

patients were still treated with topical antimicrobials in dressings as well as systemic antibiotics. Therefore, the bacterial numbers were already quite low at entry into the study. Despite this, the researchers continued to pursue the idea and eventually built it into an international consortium (Kutter et al., 2010), which is now known as PhagoBurn.

PhagoBurn is a project funded by the European Union that launched on June 1st, 2013 (PhagoBurn, 2016). It began as a response to the 2012 European Commission call for grant proposals for new antibacterial products, when the French Ministry of Defense partnered with several pharmaceutical companies. Pherecydes Pharam developed two phage cocktails, 12 and 13 phages each, for *E. coli* and *P. aeruginosa* infections. Phages were isolated from sewage running underground Parisian hospitals (Servick, 2016). Five partners are involved from France, Belgium, and Switzerland: two French pharmaceutical companies (Pherecydes Pharam and Clean Cells) and three hospital burn units (Percy military hospital in France, Queen Astrid military hospital in Belgium, and Centre Hospitalier Universitaire Vaudois in Switzerland) (PhagoBurn, 2016). This is the first time such an extensive collaboration for phage therapy has been sponsored in Western Europe. PhagoBurn began enrolling patients in July 2015 for its phase I/II clinical study to assess the safety, efficacy, and pharmacodynamics of the two phage cocktails to treat either *E. coli* or *P. aeruginosa* burn infections (PhagoBurn, 2016). Patients would either receive the cocktail or silver sulfadizine, an antibacterial cream that is the standard-of-care for burn infections (ClinicalTrials.gov, 2015).

As of now, the trial is still enrolling patients (ClinicalTrials.gov, 2015). The goal was to have the first round of results by the summer of 2016; however, there have been a number of barriers and delays, forcing the project to decrease in its size and scope. With

each new phage, the production steps had to be validated and documented; the process went to 20 months, exceeding well past the planned 12 months to establish good manufacturing practice (GMP). In addition, the goal of 220 patients, considered to be realistic at the time, was far too ambitious. In the first six months of recruitment, there were only 15 eligible *P. aeruginosa* patients and one *E. coli* patient. As the cocktails are highly targeted to only one bacterial species, the patient eligibility requirements were restricted to single-species infections. Most burn patients, however, are colonized with more than one species. Lastly, the subject recruitment was put on hold in January 2016 due to missed regulatory deadlines. As there are so many phages, demonstrating stability of each cocktail became a major hurdle. As of May 2016, the *E. coli* study has been dropped entirely, and only the 110-person *P. aeruginosa* trial remains. Now the focus is on reducing the number of phage strains in the formula, which Pherecydes Pharm worries will render it less effective and allow bacteria to evolve resistance quickly. Despite the disappointments, PhagoBurn continues and hopes to finish enrollment by the end of the year to present data in February or March of 2017 (Servick, 2016).

1.5.6.2. The Nestlé-ICDDR,B phage therapy experience

For over 20 years, the Nestlé Research Center in Switzerland and the ICDDR,B have been collaborating in efforts to use phage therapy to treat *E. coli*. Stool samples from Bangladeshi children who were hospitalized for diarrhea were screened for coliphage, and 29% were positive (Chibani-Chennoufi et al., 2004). Many T4-like phages were isolated, and cocktails were needed to achieve a coverage of about 50%, based on the pathogen collections from Mexico and Bangladesh that were being tested. The inclusion of more phage strains, however, introduces interference. Unsurprisingly, the

coverage rate of the cocktails also depended on the origin of the *E. coli* collection and if they were isolated from patients in the hospital or field studies (Bourdin, Navarro, et al., 2014). Therefore, it was decided that the cocktails should be tailored to specific epidemiological contexts. On the other hand, the lack of broad host-range phages combined with the genetic variability of epidemic *E. coli* strains complicated the situation.

As the cocktail would be orally administered, the absence of LPS was not requested in manufacturing since there is evidence that the gut is tolerant to LPS (Harper et al., 2011). The cocktail consisted of nine distinct T4-like phages that were stable for at least two years at 4°C. Upon addition of magnesium ions, the phages were stabilized and could be kept at 30°C for at least a month without any loss in titer (Bourdin, Schmitt, et al., 2014). Animal studies were performed in mice, where 10^3 to 10^6 PFU/ml of drinking water was administered. Phage retention in the absence of the host was measured by assaying the feces, and phages were recovered in a dose-dependent manner. *In vivo* lysis of *E. coli* using this model was also observed (Chibani-Chennoufi et al., 2004).

For two days, adult human volunteers from Switzerland drank the cocktail diluted in mineral water with no buffer to protect against stomach acidity. Phage titers in the stool reached 3 x 10^4 PFU/g of stool and were eliminated over the next two days. The commensal *E. coli* levels in the gut did not decrease, suggesting that they were not targeted by the cocktail. There were no adverse clinical effects observed in the volunteers, nor was there any phage or phage-specific antibodies detected in the serum (Bruttin & Brüssow, 2005). The cocktail was then given to healthy adult volunteers from Bangladesh. Fecal recovery of the phage was low in comparison to the Swiss study; 1%

of the phage inoculum was recovered, in comparison to 10% of the phage inoculum was recovered from the feces of Swiss volunteers. Again, there were no observable adverse effects, and the gut microbiota were unaffected (Sarker et al., 2012). A similar clinical study with adult Bangladeshi volunteers was performed using a commercial phage product from the Russian phage company Microgen for the treatment of *E. coli* and/or *Proteus* infections. Results were similar for this product as well (McCallin et al., 2013).

The next step was a phase II clinical trial, where 400 children with acute diarrhea were screened. 280 of these children were excluded, as they were infected with another pathogen other than *E. coli*. The remaining children were randomized and then received either the Nestlé cocktail, the Microgen product, or a placebo consisting of oral rehydration solution (ORS) supplemented with zinc. There were significant fecal phage titers in comparison to the placebo recipients; however, both products did not perform better than the placebo when WHO-recommended diarrhea parameters were measured. Stool loss, frequency, and consistency were evaluated to determine if there was any effect on disease progression (Sarker et al., 2016).

In-depth microbiological analysis was performed on the stool samples to understand what led to the failure of the trial. Only 60% of the enrolled children were diagnosed as having an *E. coli* infection; however, there was no observed beneficial effect of phage therapy even when the analysis was limited to this subset when compared to the placebo. There were several issues that came to light. The total count of *E. coli*, including pathogenic and commensal strains, represented less than 5% of the total fecal bacterial population (Sarker et al., 2016), meaning the pathogen did not grow to dominate the gut as expected. In the complex gut environment, it is possible that the phages could

not reach the *E. coli* if it was only present at such low levels. In addition, upon testing *E. coli* isolates from the stool sample against the two phage cocktails, coverage was only 50% (Sarker et al., 2016). Lastly, despite screening patients for single-species infections, co-infections were frequent. Mixed infections account for 25% of the acute diarrhea cases in Bangladeshi children (Kotloff et al., 2013; J. Liu et al., 2014; Taniuchi et al., 2013). It would be difficult in this context to pinpoint which pathogen is the driver for the symptoms of the disease if using a specifically targeted phage cocktail.

1.5.7. Previous attempts at cholera phage therapy

As previously mentioned, d'Hérelle demonstrated the efficacy of oral phage therapy and environmental phage prophylaxis for cholera. In the late 1960s, the WHO set up a phage therapy trial for cholera in Dacca, East Pakistan. At first, the study used high doses of phage, using multiplicity of infections (MOIs) ranging from 100 to 200, to test the idea of killing the bacteria but allowing for minimal rounds of phage replication. Phage therapy was compared with tetracycline treatment, and the control was fluid replacement alone. Patients were monitored for stool output, duration of diarrhea, fecal *V. cholerae* counts, and fecal phage titers. The high dose of phage resulted in a reduction in *V. cholerae* excreted but not in clinical improvement (Monsur et al., 1970). A larger study was then carried out, which allowed for two different methods of phage administration to compare with tetracycline: oral alone and oral combined with intramuscular injection. The MOI was 0.05 to 0.1, and unsurprisingly there were no significant effects (Marčuk et al., 1971). At the time, the WHO did not test the susceptibility of the *V. cholerae* to the phage stocks. They also did not characterize the phages that were employed.

Other attempts to use phages to prevent or treat cholera have produced mixed results. Dutta et al. showed that a single phage type given one hour before *V. cholerae* challenge in an infant rabbit model prevented onset of cholera symptoms (Dutta & Panse, 1963); however, Jaiswal et al. showed that a cocktail of five lytic bacteriophage types given 6 or 12 hours prior to *V. cholerae* challenge in an adult rabbit model did not affect the course of infection (Jaiswal, Koley, Ghosh, Palit, & Sarkar, 2013). The conflicting results in these studies could again be attributed to poor understanding of the basic biology of the phages used, as well as differences between the animal models used.

Chapter 2: Materials and methods

2.1. Bacterial strains and growth conditions

Strains utilized in this thesis dissertation are listed in tables located within the chapters in which they were used. Stool specimens and *V. cholerae* isolates from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) were previously collected and stored in cryoprotected form at -80°C in the Camilli Lab (Seed et al., 2011). *V. cholerae* isolates from Haitian patient samples utilized in Chapter 3 were isolated by postdoctoral fellow Dr. Kimberely Seed on Luria-Bertani (LB) agar with sulfamethoxazole (24 μg/ml), trimethoprim (32 μg/ml), nalidixic acid (20 μg/ml), and streptomycin (Sm) (100 μg/ml). In Chapter 3, mutations were introduced into *V. cholerae* strains by Dr. Seed by allelic exchange using pCVD442-lac (Seed et al., 2014). *V. cholerae* strains were grown in LB broth supplemented with 100 μg/ml Sm (Sm100) at 37°C with aeration for liquid culture, unless otherwise indicated.

2.2. Complementing *ompU*

In Chapter 3, the *ompU* gene was complemented using the low copy number, stable pMMB67EH vector, which has an iso-propyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter and encodes ampicillin resistance. Primers (IDT) used in *ompU* cloning are in Table 2.1.

Name	Sequence (5' to 3')	Description
pMMB_OmpU_F	GCGCGAATTCATGGACAATAAA TTAGGACTTAATAAG	Forward primer for cloning *ompU* into pMMB67EH vector using the EcoR*I* restriction site
pMMB_OmpU_R	GCGCGCATGCTTAGAAGTCGTA ACGTAGACCG	Reverse primer for cloning *ompU* into pMMB67EH using the Sph*I* restriction site

Table 2.1. Primers used for cloning *ompU* into the pMMB67EH vector

Wild-type *V. cholerae* E7946 *ompU* was amplified using primers pMMB_OmpU_F and pMMB_OmpU_R. The 50 µl PCR reaction was performed using the Phusion® High-Fidelity DNA Polymerase (New England BioLabs) to yield a 1050 bp product. Briefly, the reaction proceeded as follows: 98°C for 3 minutes; 30 cycles of 98°C for 10 seconds, 62°C for 30 seconds, and 72°C for 40 seconds; 72°C for 4 minutes.

Restriction digests using enzymes EcoR*I* and Sph*I* (New England BioLabs) for the *ompU* fragment and pMMB67EH vector were incubated at 37°C overnight. Restriction enzymes were inactivated by incubating reactions at 70°C for 20 minutes. The vector was then treated with Antarctic Phosphatase (New England BioLabs) at 24°C for 20 minutes followed by inactivation at 70°C for 20 minutes, followed by a QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. The ligation reaction used the T4 DNA ligase (New England BioLabs) and was incubated overnight at 16°C followed by inactivation at 65°C for 20 minutes. It was then transformed into *E. coli* DH5α electrocompetent cells and plated onto LB agar plates supplemented with 50 µg/ml ampicillin (Amp50) for incubation at 37°C overnight.

E. coli isolates that grew on the ampicillin selection plates were picked for colony PCR using primers specific for the pMMB67EH vector backbone. The 25 µl PCR

reaction was performed using the REDTaq® DNA Polymerase (Sigma-Aldrich). Briefly, the reaction proceeded as follows: 95°C for 10 minutes; 30 cycles of 98°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds; 72°C for 2 minutes. Successfully transformed isolates were colony-purified and grown in LB Amp50 broth. The plasmids containing *ompU* (pMMB_OmpU) were extracted using the QIAprep Spin Miniprep Kit (Qiagen), according to manufacturer's instructions. Electrocompetent *E. coli* MFDpir was transformed with pMMB_OmpU and plated onto LB Amp50 plates supplemented with 0.3 mM diaminopimelic acid (DAP). Successful transformants were colony-purified and stored at -80°C in 20% glycerol for future use.

Matings were set up between desired *V. cholerae* strains and *E. coli* MFDpir containing pMMB_OmpU on filters and LB plates supplemented with DAP, and they were incubated at 37°C for two hours. The mating mix was re-suspended in LB broth plus DAP and plated onto LB Sm100 Amp50 plates for overnight incubation at 30°C. Transformants were tested for the presence of pMMB_OmpU using the REDTaq colony PCR, as previously described. Strains containing the pMMB_OmpU plasmid were colony-purified and stored at -80°C in 20% glycerol.

Expression of *ompU* from the pMMB_OmpU plasmid was induced by addition of 1 mM IPTG to the culture during exponential growth (OD_{600} = 0.6). Cultures were incubated for 20 minutes static at room temperature. Induction of *ompU* expression was inspected by running the outer membrane fractions of induced cultures through a protein gel and staining with Lumitein, as described in a later section.

2.3. Phage strains and growth conditions

Phages utilized in this thesis dissertation are in Tables located according to the chapter in which they were used. *V. cholerae* phages ICP1, ICP2, and ICP3 were previously isolated from Bangladeshi cholera rice-water stool samples (Seed et al., 2011). Phage ICP2_2013_A_Haiti from Chapter 4 was isolated from Haitian cholera rice-water stool samples by Dr. Seed. All other ICP2 Haitian isolates were isolated from stool samples collected from the National Lab of Haiti in Port-Au-Prince in collaboration with Dr. Jacques Boncy and from various clinics in collaboration with the University of Florida (Dr. Glen Morris, Dr. Afsar Ali, and Mr. Meer Alam). The isolation protocol is detailed in a later section.

High-titer phage stocks were prepared by growth on LB agar plates followed by polyethylene glycol (PEG) precipitation. Each phage was grown on soft agarose (LB broth supplemented with 0.3% agarose) overlays with the appropriate host *V. cholerae* strain grown to mid-exponential phase. Plates were incubated at 37°C for 3 (for ICP3) or 4 (for all other phages) hours until the plaques became confluent. The overlays were then incubated with STE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA) overnight at 4°C while rocking. The STE-phage solution was centrifuged at 5000 x *g* for 10 minutes at 4°C, sterile-filtered using 0.22 μm Steriflip® units (EMD Millipore), and incubated with 1X PEG (4% PEG 8000, 0.5 M NaCl) at 4°C for 1 to 3 days to allow for phage precipitation. Phages were then harvested by ultracentrifugation at 10,000 x *g* for 15 minutes at 4°C, and the phage pellet was re-suspended in STE buffer for storage at 4°C.

2.4. Plaque assays and calculating efficiency of plating

Plaque assays using Costar® 6-well plates (Corning) were performed to determine titer of stocks on a susceptible host strain of *V. cholerae* as well as effiency of plating (EOP), which is the relative number of plaques that a phage stock produces on a different host strain or under altered conditions. *V. cholerae* strains were grown in LB Sm100 broth to mid-exponential phase at 37°C with aeration. The phage stock was serially diluted 1:10 in LB broth until the desired concentration was reached. For each well of a 6-well plate, 90 µl of phage were mixed with 90 µl of mid-exponential *V. cholerae*. To synchronize the start of the infection, the bacteria-phage mix was incubated for 10 minutes statically at room temperature to allow the phage to adsorb. After adsorption, each bacteria-phage mixture was added to 3 ml of molten 0.3% agarose (~50°C) per well. The 6-well plate was gently swirled to mix and allowed to solidify. Plates were incubated at 37°C for 3 to 4 hours, or until plaques formed. For EOP assays, each phage was titered on a selected *V. cholerae* isolate and on wild type E7946. The EOP was calculated by dividing the titer on the isolate by the titer on E7946.

2.5. Phage identification with plaque PCR

To identify which of the ICP phages formed a plaque of interest, plaque PCR was performed. Two primer sets were used to identify each ICP phage (Table 2.2). Plaques were inoculated into 10 µl of distilled water for use in plaque PCR reactions. For ICP1 and ICP3, 25 µl PCR reactions were performed using the Phusion® High-Fidelity DNA Polymerase (New England BioLabs). Briefly, the PCR reaction proceeded as follows: 98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 60°C for ICP1 or 55°C for ICP3

for 30 seconds, 72°C for 30 seconds; 72°C for 10 minutes. For ICP2, 25 µl PCR reactions were performed using the Q5® High-Fidelity DNA Polymerase (New England BioLabs). Briefly, the reaction proceeded as follows: 98°C for 1 minute; 30 cycles of 98°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds; 72°C for 2 minutes.

Name	Sequence (5' to 3')	Description	Product (bp)
ICP1gp58F	AACGCTGCTTTTCCTTTTGA	Forward primer for ancestral ICP1 (Seed et al., 2011) in ORF58	182
ICP1gp58R	CCCAGCATTGAGGACACTT	Reverse primer for ancestral ICP1 (Seed et al., 2011) in ORF58	
ICP1gp182F	GGTGGTTCAGCCAATATCCT	Forward primer for ancestral ICP1 (Seed et al., 2011) in ORF181	287
ICP1gp182R	CAACACTCTTCGTGGTGTGAA	Reverse primer for ancestral ICP1 (Seed et al., 2011) in ORF181	
ICP2gp6F	GACCGTGAGATGGATGCTGC	Forward primer for ancestral ICP2 and ICP2_2013_A_Haiti in OR6	227
ICP2gp6R	GCTTGGTTCATCCAACGGTC	Reverse primer for ancestral ICP2 and ICP2_2013_A_Haiti in OR6	
ICP2gp41F	CCAGAAGCTCCAAAGAAACC	Forward primer for ancestral ICP2 and ICP2_2013_A_Haiti in ORF41	215
ICP2gp41R	GCAAGAGCAAGGTCATCATC	Reverse primer for ancestral ICP2 and ICP2_2013_A_Haiti in ORF41	
ICP3gp5F	ATTGTCGAGTGGGACAAAGG	Forward primer for ancestral ICP3 (Seed et al., 2011) in ORF5	168
ICP3gp5R	ACCAACTCGACGCATAGCTT	Reverse primer for ancestral ICP3 (Seed et al., 2011) in ORF5	
ICP3gp1.3F	AGTTTGAGCAACAACTTCACGA	Forward primer for ancestral ICP3 (Seed et al., 2011) in ORF1	348
ICP3gp1.3R	TGAATGAAACTGCTTTGAATGG	Reverse primer for ancestral ICP3 (Seed et al., 2011) in ORF1	

Table 2.2. Primers used to identify phages using plaque PCR

2.6. Preparation of outer membrane fractions and Western blotting

V. cholerae cultures were grown to mid-exponential growth phase in LB broth at 37°C with aeration. Bacteria were then re-suspended in 200 mM Tris-HCl, pH 8.0 and 2 mM EDTA. Sucrose was added to a final concentration of 20% followed by lysozyme treatment at 37°C for 10 minutes. Samples were freeze-thawed twice using a dry ice/ethanol bath followed by DNAse I treatment at room temperature for 20 minutes. To pellet the membrane fraction, samples were spun for 5 minutes at 16,100 x *g*. Pellets were re-suspended in 1% Triton X-100, 10 mM $MgCl_2$, and 50 mM Tris-HCl, pH 8.0 and incubated at 37°C for 20 minutes. To separate the inner membrane from the outer membrane, samples were spun again for 5 minutes at 16,100 x *g*. The pellets, which now contain the outer membrane proteins, were re-suspended in 200 mM Tris-HCl, pH 8.0.

Outer membrane fractions were boiled for 10 minutes in sample buffer containing sodium dodecyl sulfate and β-mercaptoethanol. Samples were separated on a NuPAGE 4-12% Bis-Tris polyacrylamide gel (Life Technologies). To visualize the outer membrane protein profile, the gels were stained with Lumitein™ protein gel stain (Biotium) for 90 minutes shaking followed by a de-staining in water for 20 minutes. Fluorescence bands were visualized at an excitation wavelength of 488 nm.

For Western blotting, protein gels containing outer membrane fractions were transferred to a nitrocellulose membrane. Membranes were probed with rabbit polyclonal antisera against *V. cholerae* OmpU (gift of James Kaper). A Cy5 goat anti-rabbit antibody was used to develop the blot, and fluorescence was visualized at an excitation wavelength of 647 nm.

2.7. Bile survival assay

V. cholerae strains were grown in LB broth to OD_{600} = 0.5 and assessed for their ability to survive bile. Bacteria were spun down at 21,130 x *g* for 1 minute. Pellets were split in two and re-suspended in 0.85% NaCl alone or 0.85% NaCl with 0.2% porcine bile (Sigma-Aldrich). Samples were incubated for one hour static at room temperature. Percent survival was determined by plating serial dilutions on LB Sm100 agar plates.

2.8. Pond water fitness assay

V. cholerae were grown at 37°C overnight with aeration to stationary phase. The E7946 Δ*lacZ* strain was mixed with the (*lacZ*$^+$) strain of interest in a 1:1 competition. The mixture was pelleted by centrifugation at 21,130 x *g* for 1 minute, and pellets were re-suspended in filter-sterilized pond water. A sample was taken and plated for the input ratio. Competitions were incubated at 30°C static for 48 hours then serially diluted for CFU/ml enumeration on LB Sm100 agar plates supplemented with 40 μg/ml 5-bromo-4-chloro-3indolyl-β-D-galactopyranoside (X-gal). The competitive index (CI) was calculated as the output ratio of the mutant compared to the control strain normalized to the input ratio.

2.9. *In vitro* competition assay

Overnight plated *V. cholerae* cultures were scraped up and re-suspended in LB broth to OD_{600} = 1.0. The E7946 Δ*lacZ* strain was mixed with the strain of interest in a 1:1 competition. An aliquot of the mixture was plated to calculate the input ratio, and another aliquot of 10^4 CFU was used to inoculate 2 ml of LB Sm100 broth and incubated

overnight at 37°C with aeration. The next day, overnight competitions were back-diluted, and 10^4 CFU was taken to inoculate 2 ml of LB Sm100 broth. Again, the competition was incubated overnight at 37°C with aeration. This process was repeated one more time for a total of 3 passages in LB Sm100 broth overnight, resulting in approximately 58 generations. Competitions were serially diluted and plated onto LB Sm100 agar plates to enumerate CFU/ml. The CI was calculated as the output ratio of the mutant compared to the control strain normalized to the input ratio.

2.10. Animal competition experiments

All animal experiments were in accordance with the rules of the Department of Laboratory Animal Medicine at Tufts Medical Center and the Institutional Animal Care and Use Committee. Litters were housed with their dam or doe with food and water *ad libitum* prior to infection. Post-infection, litters were kept separate from their dam or doe and were monitored for health status up to a maximum of 20 or 24 hours, at which time all animals were euthanized and the experiment terminated.

2.11. *In vivo* competition in infant mouse model

In vivo competition experiments were done using 4-5 day old CD-1 mice. The inoculum was prepared as a 1:1 mixture of the strain of interest (*lacZ*$^+$) and the appropriate control strain (Δ*lacZ*). Mice were infected orogastrically with approximately 5 x 10^5 CFU and euthanized 24 hours post-infection. Small intestines were homogenized in 1 ml LB broth with 20% glycerol, diluted in LB broth, and plated onto LB Sm100 agar plates supplemented with 40 μg/ml X-gal. The CI was calculated as the output ratio of the mutant compared to the control strain normalized to the input ratio. *In vitro* controls

were included for each of these experiments in which the same inoculum was diluted 1:100 into five independent LB Sm100 broth cultures. The output CI ratios were determined in the same manner on LB Sm100 X-gal agar plates as previously described.

2.12. *In vivo* competition in infant rabbit model

Three-day-old New Zealand White infant rabbits (MillBrook Labs) were pre-treated intraperitoneally with 300 mg/kg of the histamine inhibitor Cimetidine-HCl (Morton Grove Pharmaceuticals) 3 hours prior to infection to reduce acid production in the stomach. Rabbits were orogastrically inoculated with approximately 5 x 10^8 CFU in 2.5% sodium bicarbonate buffer (pH 9.0). The inoculum was a 1:1 (for experiments without phage) or 1:10 (for experiments with phage) mixture of mutant (*lacZ*$^+$) to E7946 (Δ*lacZ*). For experiments with phage, phage were added immediately before inoculation to each *V. cholerae* inoculum to limit phage adsorption *ex vivo*. Rabbits were sacrificed approximately 12 hours post-infection, and the cecal fluid was collected. As previously described, competing strains were enumerated on LB Sm100 plates supplemented with 40 μg/ml X-gal, and the CI indices were calculated.

2.13. Isolation of *V. cholerae* and phages from clinical samples

Clinical samples from the National Lab of Haiti were originally stored at -80°C without cryoprotectant; therefore, approximately 100 μl of the sample was thawed and plated on LB agar plates with no selection. For a subset of samples, we were able to recover viable *V. cholerae* presumably due to the fortuitous presence of natural cryoprotectants in the rice-water stool. Following single-colony purification, isolates were verified to be *V. cholerae* O1 by microscope and by agglutination with O1 Ogawa

serum. *V. cholerae* were isolated from clinical samples from the collaboration with University of Florida in a similar manner, except the thawed sample was plated onto LB Sm100 plates.

For ICP2 isolates isolated from the National Lab clinical samples, enrichment was used to detect live phage. Ten ml cultures of the E7946 Δ*wbeL* mutant were grown to mid-exponential growth phase. This strain is a rough mutant, meaning it lacks its O-antigen. It was previously determined that ICP2 infects rough mutants better than wild-type strains by two-fold. Approximately 100 μl of each clinical sample was thawed and used to inoculate the cultures, which were left to incubate at 37°C overnight with aeration. The cultures were then spun down at 21,130 x *g* for 5 minutes, and the supernatants were filtered using 0.22 μm Millex-GP syringe filter units (EMD Millipore). The filtered samples were serially diluted and titered for presence of phage.

For ICP2 isolates from Haiti stored at the University of Florida, phages were not enriched from the clinical sample. Isolates were detected through direct titer of approximately 100 μl of rice-water stool using a *V. cholerae* isolate from the same patient sample.

2.14. Preparing libraries of *V. cholerae* and phage genomic DNA for whole-genome sequencing

V. cholerae genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen), according to manufacturer's instructions. For phage genomic DNA, 85 μl of high-titer phage stock was incubated with DNase I (New England BioLabs) and 625 μg of RNase A (Sigma-Aldrich) at 37°C for 30 minutes to degrade contaminating host cell

DNA and RNA. The DNaseI was inactivated at 75°C for 10 minutes. The mixture was then used in the DNeasy Blood & Tissue Kit (Qiagen), according to manufacturer's instructions.

V. cholerae and phage genomic libraries were generated using homopolymer tail-mediated ligation PCR (Lazinski & Camilli, 2013) for Chapter 3 or the Nextera XT DNA Library Preparation Kit (Illumina) for Chapters 4 and 5, according to manufacturer's instructions. Sequencing was conducted at the Tufts University Core Facility using an Illumina Hi-Seq. *V. cholerae* whole-genome libraries were prepared for single-end 150 nucleotide sequencing, while phage whole-genome libraries were prepared for single-end 50 nucleotide sequencing.

2.15. Phage genome analysis

Phage genomes were assembled using CLC Workbench 9.0.1 software (Qiagen). Reads were trimmed to Q30, and *de novo* assemblies were performed using a minimum contig length of 300 bp and similarity fraction of 0.9. Genomes were then compared at the nucleotide level using the progressiveMauve algorithm (Darling, Mau, & Perna, 2010).

2.16. Phylogenetic analyses of phage genomes

De novo assemblies were exported as FASTA files and imported for alignment into MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Variants were extracted as FASTA files and re-aligned in CLC Workbench to perform tree model testing. The tree was assembled using the neighbor-joining model, generalized time-reversible (GTR) (Tavaré, 1986), using 4-category rate variation and topology variation.

2.17. *In vitro* phage killing assay

Overnight cultures of *V. cholerae* E7946 were diluted back to OD_{600} = 0.05 in 10 ml of LB Sm100 broth and grown at 37°C with aeration. After 15 minutes, phages were added to each culture at an MOI of 1. The assay went for 24 hours, with samples collected every hour from 0 to 8, then at 12 and 24 hours. At each timepoint, OD_{600} readings were measured, and samples were taken to enumerate CFU/ml and PFU/ml by plating onto LB Sm100 agar plates and plaque assays, respectively.

2.18. *In vivo* models for phage retention and phage prophylaxis

Infections of infant mice with *V. cholerae* were performed as previously described, with the exception of the "high" infectious dose experiment in Chapter 5 where approximately 5 x 10^7 CFU were used to inoculate mice. Phages were re-suspended in filter-sterilized PBS for inocula. Small intestines were dissected and homogenized in LB broth with 20% glycerol. *V. cholerae* were enumerated as previously described on LB Sm100 agar plates. A limited number of *V. cholerae* colonies were colony-purified and assessed for resistance to ICP phage by EOP assays. To extract phage from small intestine homogenates, an aliquot of the homogenate was treated with chloroform and centrifuged at 10,000 x *g* for 5 minutes. The supernatant was collected and used in plaque assays to calculate PFU/intestine.

Infections of infant rabbits with *V. cholerae* were performed as previously described, with the exception of pre-treatment with 2 μg/g of body weight of the alternative histamine inhibitor Ranitidine-HCl (Caraco Pharmaceutical Laboratories). Rabbits were also from a different vendor for these experiments (Charles River

Laboratories). Phages were re-suspended in filter-sterilized PBS for inocula. Intestines were dissected and homogenized in LB broth with 20% glycerol. *V. cholerae* and phage were enumerated as previously described for infant mouse experiments. Surviving *V. cholerae* colonies were colony-purified and assessed for resistance to ICP phage by EOP assays.

2.19. Variant analysis of phage-resistant isolates

Illumina DNA sequence reads from each *V. cholerae* phage-resistant isolate were mapped to *V. cholerae* wild-type strain N16961 (accession numbers NC_002505.1 and NC_002506.1) using CLC Genomics Workbench 8 (CLC Bio). To determine the mutations that may confer phage resistance, variant analysis was performed on mapped reads with a frequency threshold of 20%. Results were compared to E7946 variants (Lazinski & Camilli, 2013) to remove those found in the wild-type inoculum. Surviving *V. cholerae* isolates that were determined to be sensitive to all three phages were also sequenced, and resulting variants were also removed from resistant isolates variant analyses. The frequency threshold in the final analysis is 51%, with the exception of variants known to be the consequence of slipped-strand mispairing in poly-A tracts of the genome (Seed et al., 2012).

Chapter 3: Intra-patient ICP2 predation affects *V. cholerae* population structure

The majority of the figures and tables presented within this chapter have been adapted from the following publication, as indicated in the legends, for which Dr. Seed and I performed all of the experiments:

Seed, K.D., **Yen, M**., Shapiro, B.D., Hilaire, I.J., Charles, R.C., Teng, J.E., Ivers, L.C., Boncy, J., Harris, J.B., and Camilli, A. (2014). Evolutionary consequences of intra-patient phage predation on microbial populations. *eLife*. **3**:e03497.

In Bangladesh, lytic phages are hypothesized to impact the levels of *V. cholerae* in the environment and, thus, the cholera disease burden. The assumption central to this hypothesis is the ability of phages to prey on *V. cholerae* in the small intestine following their co-ingestion. The molecular evidence to support this, however, is lacking. In this chapter, we will demonstrate the ability of a virulent phage ICP2 to modulate the levels of its host *V. cholerae* within the small intestines of an animal model. We will also provide evidence that phages impose significant bactericidal pressure during the course of infection, resulting in an altered *V. cholerae* population structure exiting the host.

3.1. Background

3.1.1. Isolated phage from Haiti is closely related to ICP2 isolates from Bangladesh

Seed et al. described a species of *V. cholerae*-specific virulent phage named ICP2. It is a podovirus found sporadically in cholera patient stool samples collected from 2001 to 2010 in Bangladesh (2011). As a study to assess the geographic diversity of virulent cholera phages found within clinical samples, Dr. Seed used plaque assays to test for the presence of phages in nine Haitian patient stool samples collected in 2013. One sample had a high phage titer of 10^8 PFU/ml and a *V. cholerae* titer of 10^5 CFU/ml. Plaques were isolated, and DNA was purified and whole-genome sequenced, thereby revealing 84% identity over 93% of its genome to ICP2_2011_A, a clinical ICP2 isolate collected from Bangladesh in 2011. The Haitian phage was therefore named ICP2_2013_A_Haiti, and its genome was aligned with the three available ICP2 isolates from Bangladesh using the progressiveMauve algorithm (Darling et al., 2010) (Figure 3.1). ICP2_2013_A_Haiti is similar but clearly distinct from the Bangladeshi ICP2 isolates. It is also the first reported lytic phage associated with the cholera epidemic in Haiti.

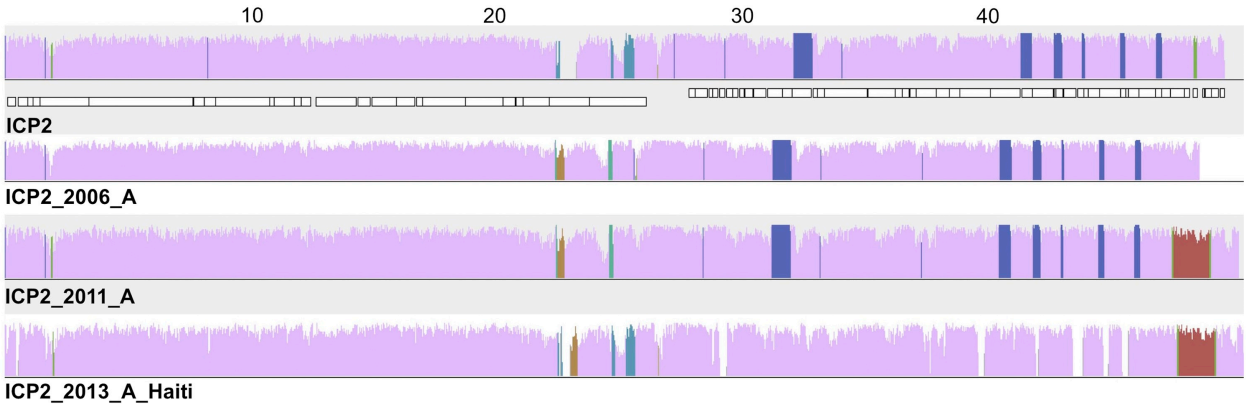


Figure 3.1. ICP2_2013_A_Haiti is similar but distinct to Bangladeshi ICP2 phages

Genomes from ICP2 isolates collected from cholera patients in Dhaka, Bangladesh in 2004, 2006, and 2011 were compared with the genome of an ICP2 isolate from Haiti collected in 2013 using progressiveMauve software. Above the ICP2 genome, there are numbers showing the distance in kilobases. The height of the similarity profile represents the relative degree of nucleotide similarity between aligned regions. Blocks of the genome are colored according to their presence within the isolates, with light purple representing the highly conserved backbone genome. The white boxes represent the annotated genes in the ICP2 genome, where the genes that are transcribed from the negative strand are on the lower row. Figure is adapted from Seed, K.D., Yen, M. et al. (2014).

3.1.2. Mutations in ompU *and* toxR *genes identified in ICP2-resistant clinical isolates*

Upon further investigation, it was revealed that 267 out of the 269 of the *V. cholerae* isolates recovered from the same clinical sample as ICP2_2013_A_Haiti were resistant to infection by the phage. This is a remarkable observation, considering the majority of phage-containing clinical samples usually have a high frequency of phage-sensitive *V. cholerae* isolates. To identify the cause of phage resistance, eight phage-resistant isolates and two phage-sensitive isolates were subjected to whole-genome sequencing. All ten isolates were isogenic, as expected due to the clonal expansion of *V. cholerae* in the patient (Seed et al., 2014). In the phage-resistant isolates, however, heterogeneous mutations were found within the *ompU* gene that encodes the major outer membrane porin OmpU. Upon further sequencing of the *ompU* genes of 11 phage-resistant *V. cholerae* isolates from the same clinical sample, a total of six alleles were identified.

	Presence of OmpU mutants among isolates collected in the following year[a]										
	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Total isolates:	••••	•••••	•••••	•••••••	••••	•••••••	••••	••••	••••	••••	•••••••
OmpU A195T[b]						•					
OmpU V324F	••					••					
OmpU G325D							••	•			

[a]Closed circles represent the number of single *V. cholerae* O1 El Tor isolates from the different stool samples collected in the given year. [b]The number of isolates with the noted mutation is indicated in the appropriate year. If OmpU was wild-type in all the isolates, the space is left blank.

Figure 3.2. Presence of OmpU mutants in Bangladeshi clinical samples

Three OmpU mutants were identified from clinical samples collected from 2001 to 2011 at the International Centre for Diarrheal Disease Research, Bangladesh (Seed et al., 2011). Figure is adapted from Seed, K.D., Yen, M. et al. (2014).

OmpU mutants were also observed in clinical isolates collected from Bangladesh between 2001 and 2011 (Figure 3.2). The mutant G325D was identified in clinical isolates from Haiti and from Bangladesh. In total, eight mutant *ompU* alleles were identified from the Haitian and Bangladeshi clinical isolates (Figure 3.3A). Seven out of the eight mutant proteins were altered in two predicted extracellular loops (L4 and L8) of OmpU (Figure 3.3B). The 8th mutant had an altered transmembrane segment next to L3. These data suggest ICP2 initiates infection of *V. cholerae* through interaction with these extracellular loops of OmpU.

An ICP2-positive stool sample collected in 2011 in Bangladesh was also tested for *V. cholerae* population heterogeneity. A total of 46 single-colony isolates were tested for ICP2_2011_A sensitivity, and 22% were phage-resistant. Again, whole-genome sequencing analysis of four phage-resistant and two phage-sensitive isolates showed that they were all isogenic except for nonsense mutations in *toxR*, which is the transcriptional activator of *ompU*. An additional eight phage-resistant isolates from the same stool sample were whole-genome sequenced, resulting in the identification of multiple unique *toxR* alleles (Figure 3.3C).

Each of the *ompU* and *toxR* alleles from the clinical isolates were used to replace their wild-type counterpart in the virulent *V. cholerae* lab strain E7946 (Table 3.1). In this chapter, we will present the results from testing these strains for ICP2 resistance, the presence of OmpU in the outer membrane, and the potential consequences of the mutations on *in vitro* and *in vivo* fitness. We propose that these mutant alleles arose due to ICP2 predation during cholera infection in an individual.

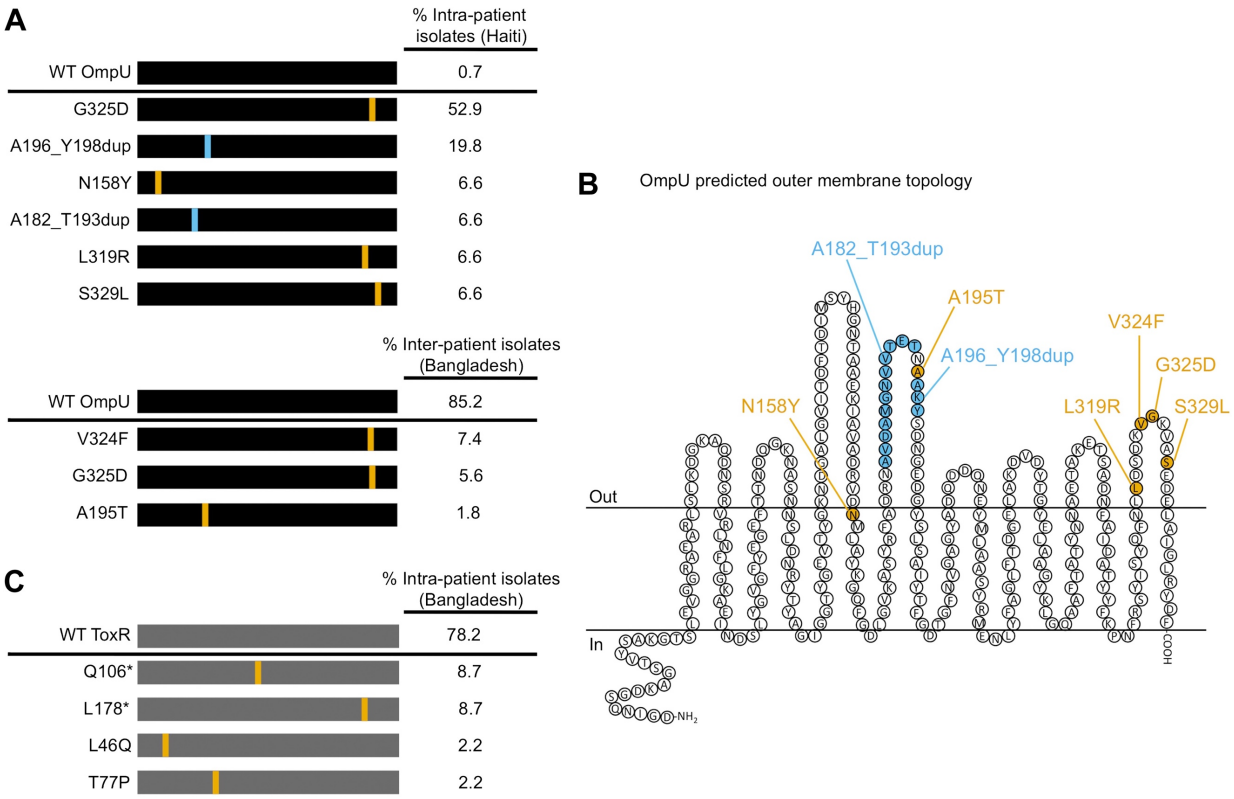


Figure 3.3. Frequency of OmpU and ToxR mutants within and between cholera patients from Haiti and Bangladesh

The black and gray bars represent the whole *ompU* and *toxR* genes, respectively. Amino acid substitutions or nonsense mutations (indicated by the asterisks) are represented in orange, and duplications are in blue. (a) The frequency of OmpU mutants found within a single Haitian stool sample containing 10^8 PFU/ml of ICP2_2013_A_Haiti (top) and from 54 different patient samples from Bangladesh (bottom). (b) Pred-TMBB (Bagos, Liakopoulos, Spyropoulos, & Hamodrakas, 2004) was used to predict the membrane topology of mature OmpU. Locations of the amino acid substitutions or duplications are indicated and color-coded in the same manner. (c) The frequency of ToxR mutants found within a single stool sample from Bangladesh containing 10^9 PFU/ml of ICP2_2011_A. Figure is adapted from Seed, K.D., Yen, M. et al. (2014).

Table 3.1. Strains used in Chapter 3

Strain	Genotype and description
WT	Spontaneous Sm^R derivative of E7946, El Tor Ogawa
OmpU strains	
KS611	*Vibrio cholerae* O1 Ogawa; single isolate from Haitian patient number 35; Sm^R, OmpU A196_Y198dup
KS708	*Vibrio cholerae* O1 Ogawa; single isolate from Haitian patient number 35, Sm^R
KS744	*Vibrio cholerae* O1 Ogawa; single isolate from Haitian patient number 35, Sm^R
KS761	*Vibrio cholerae* O1 Ogawa; single isolate from Haitian patient number 35, Sm^R, OmpU N158Y
KS760	*Vibrio cholerae* O1 Ogawa; single isolate from Haitian patient number 35, Sm^R, OmpU S329L
KS758	*Vibrio cholerae* O1 Ogawa; single isolate from Haitian patient number 35, Sm^R, OmpU A182_T193dup
KS759	*Vibrio cholerae* O1 Ogawa; single isolate from Haitian patient number 35, Sm^R, OmpU L319R
KS746	*Vibrio cholerae* O1 Ogawa; single isolate from Haitian patient number 35, Sm^R, OmpU G325D
KS747	*Vibrio cholerae* O1 Ogawa; single isolate from Haitian patient number 35, Sm^R, OmpU G325D
KS748	*Vibrio cholerae* O1 Ogawa; single isolate from Haitian patient number 35, Sm^R, OmpU G325D
AC2846	E7946 Δ*ompU*
AC3745	E7946 Δ*lacZ*
AC4608	E7946 Δ*ompU* pMMB67EH::*ompU,* Amp^R, Sm^R
AC4609	E7946 Δ*ompU* pMMB67EH*,* Amp^R, Sm^R
KS745	E7946 OmpU G325D
KS667	E7946 OmpU A196_Y198dup
KS658	E7946 OmpU A195T
KS672	E7946 OmpU V324F
KS822	E7946 OmpU A182_T193dup
KS823	E7946 OmpU L319R
KS824	E7946 OmpU S329L
KS825	E7946 OmpU N158Y

Sm^R, streptomycin-resistant; Amp^R, ampicillin-resistant.

Table 3.1. continued

Strain	Genotype and description
	ToxR strains
AC598	E7946 Δ *toxR*
KS720	*Vibrio cholerae* O1 Ogawa; single isolate from ICDDR,B patient number 2, Sm^R
KS721	*Vibrio cholerae* O1 Ogawa; single isolate from ICDDR,B patient number 2, Sm^R
KS722	*Vibrio cholerae* O1 Ogawa; single isolate from ICDDR,B patient number 2, Sm^R, ToxR Q106*
KS732	*Vibrio cholerae* O1 Ogawa; single isolate from ICDDR,B patient number 2, Sm^R, ToxR L178*
KS772	*Vibrio cholerae* O1 Ogawa; single isolate from ICDDR,B patient number 2, Sm^R, ToxR L46Q
KS773	*Vibrio cholerae* O1 Ogawa; single isolate from ICDDR,B patient number 2, Sm^R, ToxR T77P
KS778	E7946 ToxR Q106*
KS779	E7946 ToxR L178*
KS788	E7946 ToxR T77P
KS789	E7946 ToxR L46Q
KS786	KS732 ToxR WT, Δ *lacZ*
KS787	KS722 ToxR WT, Δ *lacZ*
KS793	KS772 ToxR WT, Δ *lacZ*
KS794	KS773 ToxR WT, Δ *lacZ*
KS756	KS722 pMMB67EH::*ompU,* Amp^R, Sm^R
KS757	KS732 pMMB67EH::*ompU,* Amp^R, Sm^R

Sm^R, streptomycin-resistant; Amp^R, ampicillin-resistant.

3.2. Results and Discussion

3.2.1. ompU *mutations do not affect OmpU expression but confer ICP2 resistance*

To determine whether the *ompU* alleles confer ICP2 resistance, four were selected for efficiency of plating (EOP) assays using ICP2_2013_A_Haiti. A histogram is used to represent the ratio of plaques of a mutant *V. cholerae* strain (OmpU*) to that of the wild-type (Figure 3.4A). In addition, a Δ*ompU* strain was used as a negative control. The

Δ*ompU* mutant was complemented *in trans* by induction of wild-type *ompU* carried on the plasmid pMMB67EH, which shows that wild-type sensitivity to ICP2_2013_A_Haiti is restored upon OmpU expression. Three of the OmpU* (A196_Y198dup, V324F, and G325D) were resistant to ICP2_2013_A_Haiti infection by this assay. One OmpU* (A195T) had partial sensitivity as there were a hundred-fold fewer plaques in comparison to a wild-type infection. Plaques on this strain were also turbid, unlike the clear plaques with crisp boundaries on the wild-type strain, which is indicative of a less efficient infection.

To determine whether OmpU was present on the cell surface of OmpU*, they were tested by Western blotting of outer membrane fractions (Figure 3.4A). All four OmpU* produced normal amounts of OmpU on their surface yet are resistant to some degree to ICP2_2013_A_Haiti, indicating that ICP2 uses OmpU as its receptor to initiate infection. To determine whether or not the remaining four OmpU* (A182_193dup, L319R, S329L, and N158Y) also produce normal amounts of OmpU on their surfaces, we assayed the outer membrane fractions by Western blot as well (Figure 3.4B). In line with our prior results, these strains also had OmpU present on their surfaces. As the predicted outer membrane topology of OmpU suggests (Figure 3.3), all identified mutations are located in or near two predicted extracellular loops and therefore may disrupt the interaction between OmpU and the ICP2 tail fibers.

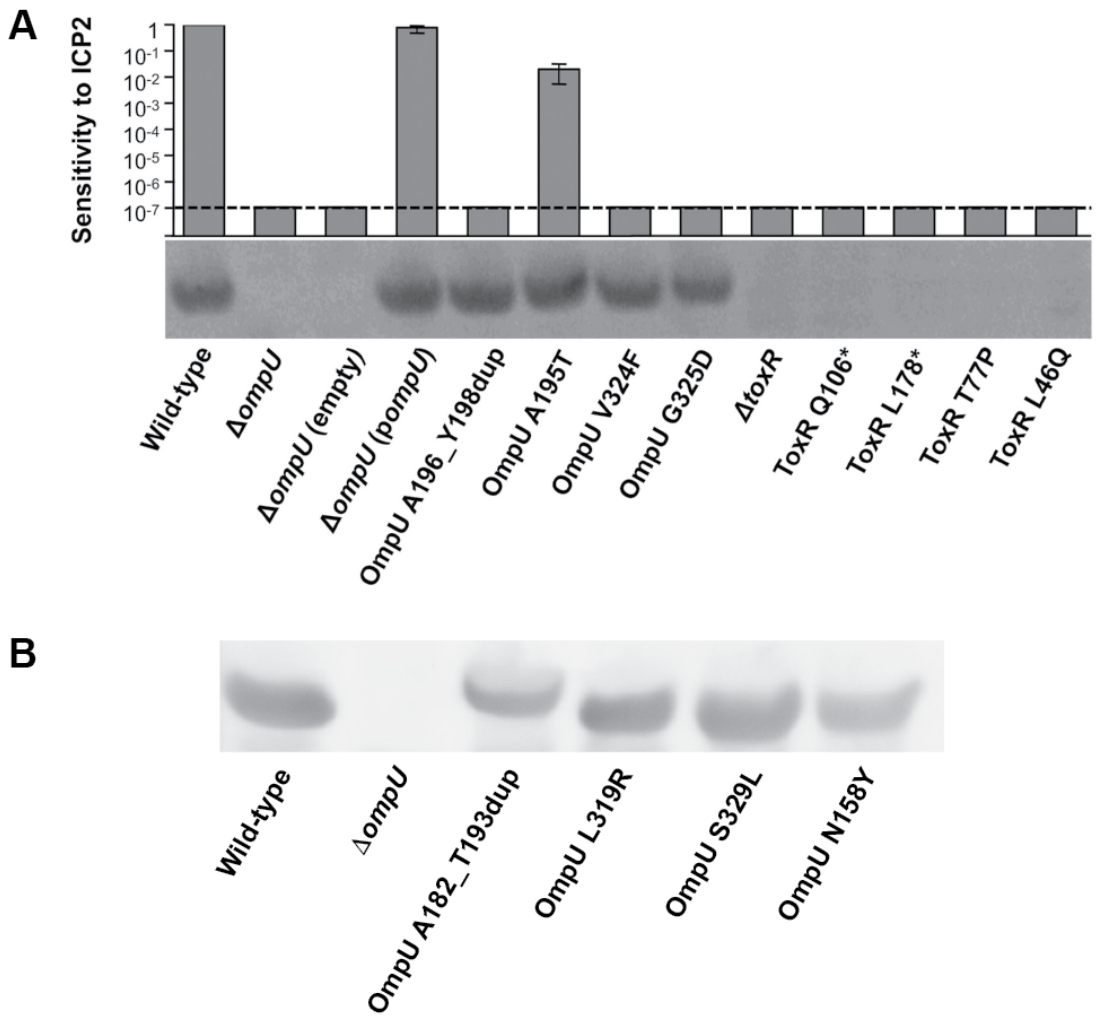


Figure 3.4. ICP2 sensitivity of OmpU and ToxR mutants and their OmpU expression

(a) Efficiency of plating is the ratio of ICP2_2013_A_Haiti plaques on the mutant *V. cholerae* strain to those on the wild-type strain and depicted as a histogram. The limit of detection for this assay is 10^{-7}. Outer membrane protein fractions were prepared from mid-exponential growth phase cultures matched by equivalent OD_{600} units. The samples were then separated by SDS-PAGE, and Western blots were performed using rabbit polyclonal antisera against OmpU for the four most common OmpU mutants and all the ToxR mutants, and for (b) the remaining OmpU mutants identified.

3.2.2. toxR *mutations affect OmpU expression and confer ICP2 resistance*

Since ToxR is the direct transcriptional activator of *ompU*, we hypothesized that the *toxR* mutant alleles would also confer ICP2 resistance. As a negative control, we also tested a Δ*toxR* mutant. We used EOPs to determine the resistance of the ToxR mutants (ToxR*) to ICP2_2013_A_Haiti (Figure 3.4A). All four ToxR* (Q106*, L178*, T77P, and L46Q) were resistant to infection. We hypothesized that ToxR* would also have low levels of OmpU on their surfaces as its transcription is likely disrupted. Using Western Blots, OmpU was undetectable in the outer membrane fractions of ToxR* (Figure 3.4A).

To test if ICP2 resistance is mediated through loss of OmpU expression, we expressed *ompU in trans* using pMMB67EH in ToxR* as well as reverted the *toxR* mutant alleles back to wild-type in the clinical strains. All strains had restored sensitivity to ICP2_2013_A_Haiti as determined by EOP as well as restored OmpU presence in their outer membrane fractions (Figure 3.5). These results suggest that the *toxR* alleles are necessary and sufficient for ICP2 resistance, which is due to loss of OmpU expression.

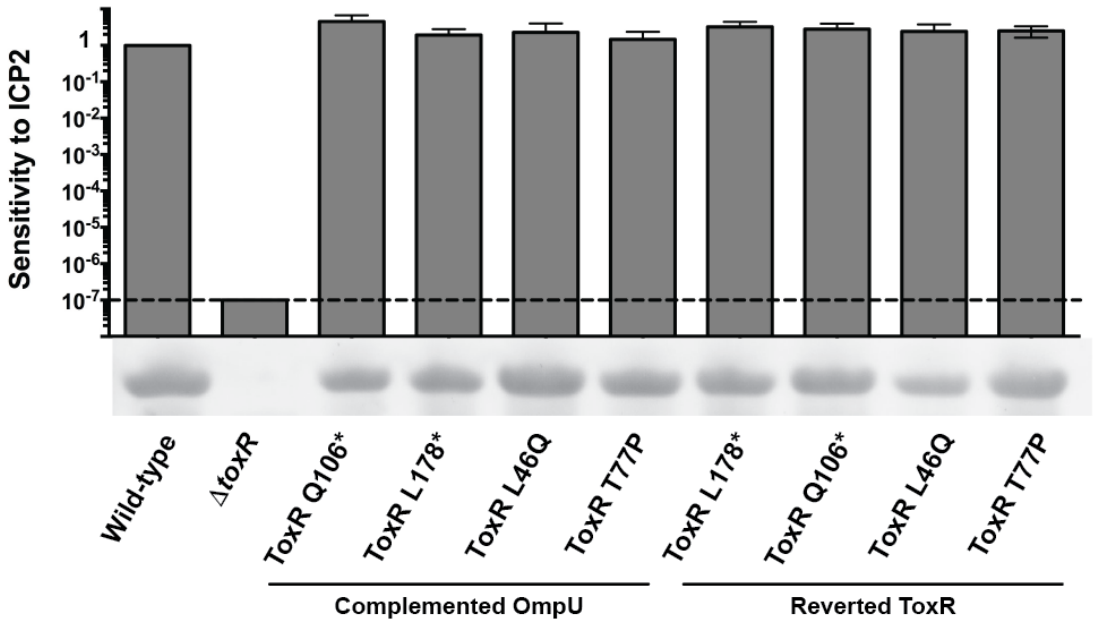


Figure 3.5. ICP2 sensitivity and OmpU expression of complemented and reverted ToxR mutants

ToxR mutants were complemented with OmpU by inducing *ompU* expression *in trans* from the pMMB76EH vector. Clinical isolates where the *toxR* mutant alleles were identified were also reverted back to wild-type *toxR*. EOP is the ratio of ICP2_2013_A_Haiti plaques on the mutant *V. cholerae* strain to those on the wild-type strain and depicted as a histogram. The limit of detection for this assay is 10^{-7}. Outer membrane fractions were prepared from mid-exponential cultures matched by equivalent OD_{600} units. Samples were then separated by SDS-PAGE, and Western blots were performed using rabbit polyclonal antisera against OmpU.

3.2.3. Fitness characterization of OmpU mutants

V. cholerae Tn-Seq analyses have shown that OmpU is critical for fitness in an infant rabbit infection model (Fu et al., 2013; Kamp et al., 2013) and for dissemination from the host into pond water (Kamp et al., 2013). In addition, OmpU plays an important role in protecting *V. cholerae* against the bactericidal effect of intestinal organic acids (Merrell et al., 2001), cationic peptides (Mathur & Waldor, 2004), and bile salts (Provenzano et al., 2001). To address the potential consequences of the identified ICP2-resistance mutations on *V. cholerae* fitness, the four most frequently isolated *ompU* alleles were tested in various survival and growth assays *in vitro*.

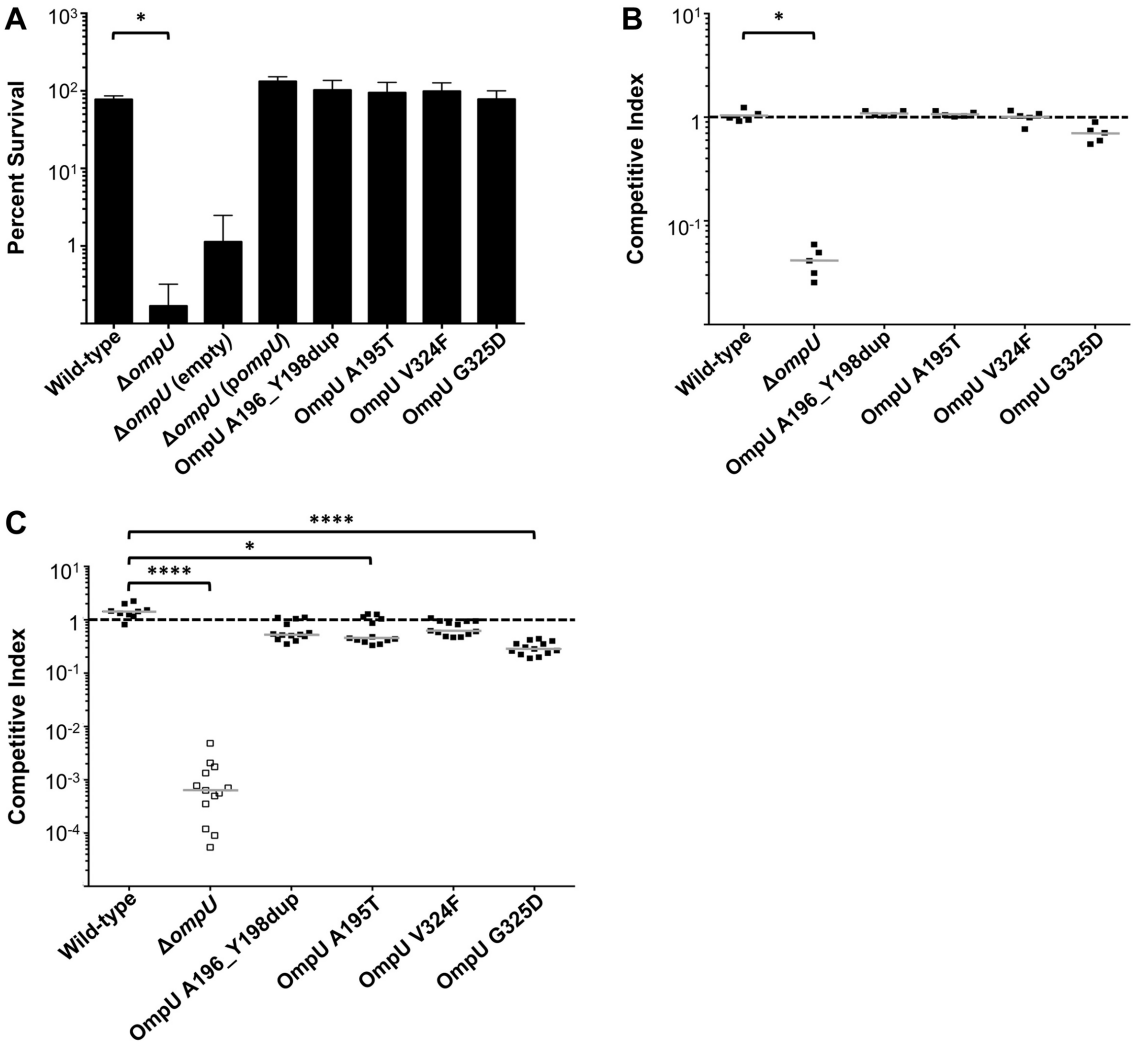


Figure 3.6. The fitness of clinically relevant OmpU mutations

(a) Clinically relevant OmpU mutants retain fitness in the presence of 0.2% porcine bile at room temperature for one hour. *$p < 0.05$ for the compared data sets using the Mann-Whitney U Test. (b) OmpU mutants retain competitive fitness in pond water at 30°C for 48 hours. *$p < 0.05$ for the compared data sets using the Mann-Whitney U Test. (c) OmpU mutants have slight competitive fitness defects when serially passed in LB broth for approximately 58 generations. *$p < 0.05$ or ****$p < 0.0001$ significantly different from wild-type using Kruskal-Wallis and *post hoc* Dunn's multiple comparison tests. Figure is adapted from Seed, K.D., Yen, M. et al. (2014).

OmpU* were assessed for their ability to survive in 0.2% porcine bile for one hour at room temperature (Figure 3.6A). All four OmpU* survive as well as wild-type in the presence of bile. We then tested their competitive fitness in pond water for 48 hours at 30°C (Figure 3.6B) and found that all four OmpU* survive as well as wild-type in pond water also. When three of the OmpU mutants were tested in a 1:1 competition with wild-type in the infant mouse model, they were competitively fit as well (Figure 3.7). These results are consistent with their wild-type levels of OmpU expression (Figure 3.4A). There is a mild competitive defect, however, when OmpU*, particularly mutants A195T and G325D, are passaged multiple times in LB medium (Figure 3.6C). The low prevalence of ICP2 in Bangladeshi clinical samples (Seed et al., 2011) coupled with the mild growth defect of OmpU* may explain why the ICP2-resistant OmpU variants are not fixed in the *V. cholerae* population (Figure 3.3).

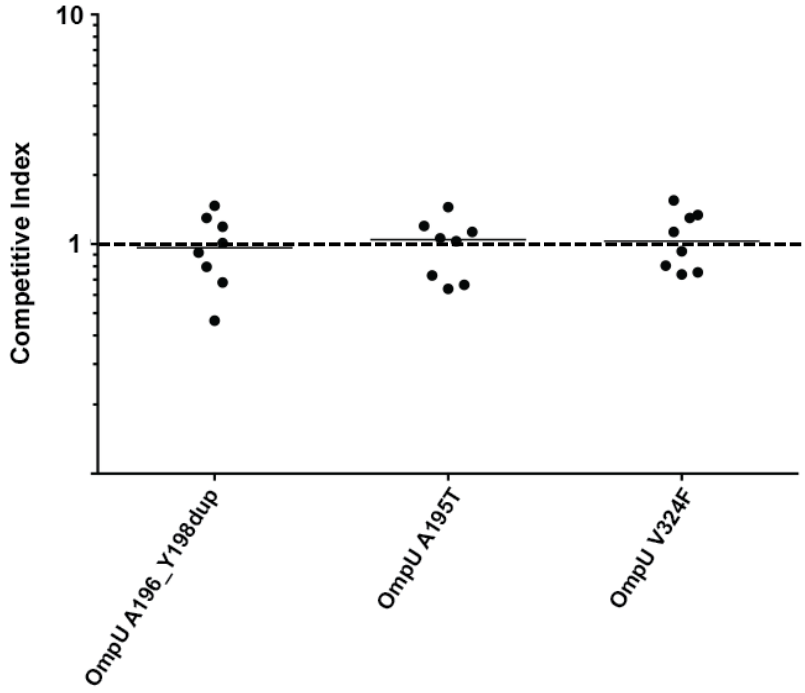


Figure 3.7. OmpU mutants are competitively fit in the infant mouse model

Three OmpU mutants (A196_Y198dup, A195T, and V324F) were competed 1:1 with wild-type *V. cholerae* E7946 in the infant mouse model of colonization. Horizontal bars indicate the median of each data set, and each circle represents the competition index from one animal.

3.2.4. In vivo *fitness characterization of ToxR mutants*

We assessed four ToxR* for virulence in the infant mouse model of intestinal colonization. All four strains were indistinguishable from a Δ*toxR* mutant and determined to be avirulent (Figure 3.8A). If these mutations are sufficient for avirulence, it is curious that they were identified in clinical isolates of *V. cholerae*. It is possible that the clinical strains containing these mutations harbor compensatory mutations. To address this hypothesis, competitions between each clinical ToxR point mutant and its isogenic wild-type ToxR revertant strain were performed in infant mice (Figure 3.8B). The clinical strains were 100- to 1000-fold attenuated, indicating that there are no compensatory mutations. The two non-synonymous mutations map to the cytoplasmic amino terminus (D17-E112) of ToxR, which has homology to the winged helix-turn-helix family of transcription activators. This region is involved in DNA binding and transcriptional activation (Miller, Taylor, & Mekalanos, 1987; Ottemann, DiRita, & Mekalanos, 1992), and it has been shown that point mutations decrease activation of *ompU* and virulence genes (Morgan et al., 2011; Ottemann et al., 1992).

Our results are consistent with the inability of mutant ToxR proteins to activate downstream genes. Therefore, these ToxR mutant strains would not be able to colonize the human small intestine and be recovered in the secretory diarrhea if they were ingested (Herrington et al., 1988). These data support our model that these mutations arose during cholera infection due to ICP2 predation in the small intestine. In this individual, selection of ICP2-resistant mutants most likely occurred late in infection when the expression of colonization and virulence genes were no longer required (Merrell et al., 2002).

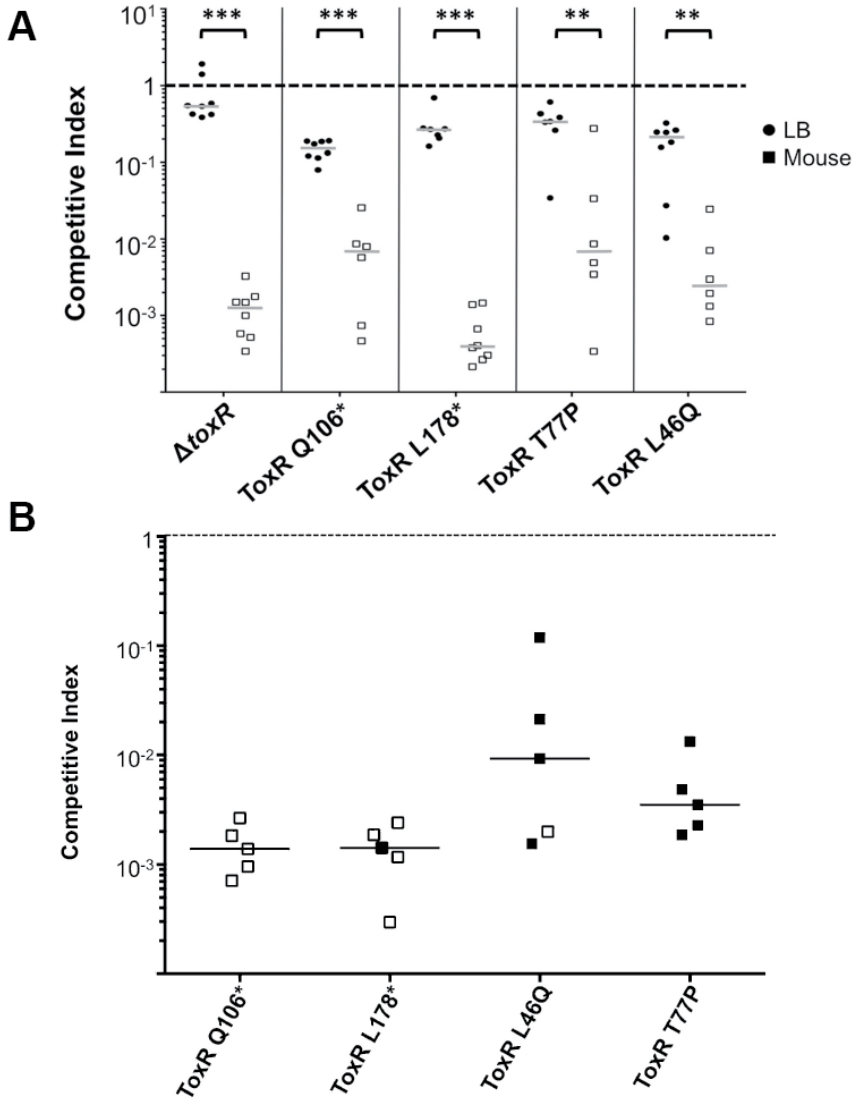


Figure 3.8. The fitness cost of clinically relevant ToxR mutations

The infant mouse colonization model for *V. cholerae* was used to determine the fitness of clinically relevant ToxR mutations. (a) ToxR mutants are attenuated *in vivo* when the allele is placed in a clean genetic background. $^{**}p < 0.01$ or $^{***}p < 0.001$ as compared to the *in vitro* median using Mann-Whitney U tests. (b) Each clinical ToxR mutant strain was then competed against its isogenic ToxR wild-type revertant strain. The horizontal bars indicate the median of each data set, and open symbols represent data below the limit of detection. Figure is adapted from Seed, K.D., Yen, M. et al. (2014).

3.2.5. ICP2 predation selects for OmpU mutant in infant rabbit model

We propose that phage predation during the course of infection leads to the selection of resistant bacterial mutants within the human patient, thereby altering the population structure of *V. cholerae* in the small intestine. To test this hypothesis, we administered a 1:10 mix of the OmpU(G325D) mutant to wild-type *V. cholerae* in the absence or presence of ICP2_2013_A_Haiti to infant rabbits (Figure 3.9). Of note, in the absence of ICP2, the OmpU(G325D) mutant was fully virulent. In the presence of ICP2 at a multiplicity of infection (MOI) of either 0.1 or 0.01, there was a 10,000-fold enrichment of OmpU(G325D) mutant over wild-type during infection. These data suggest that phage predation imposes a strong selective pressure during *V. cholerae* infection. This experiment emulates our model of what happens during human infections when *V. cholerae* and phages are ingested together. It is also the first reported evidence supporting the concept of phage predation altering the population structure of *V. cholerae* in the context of a diarrheal disease model.

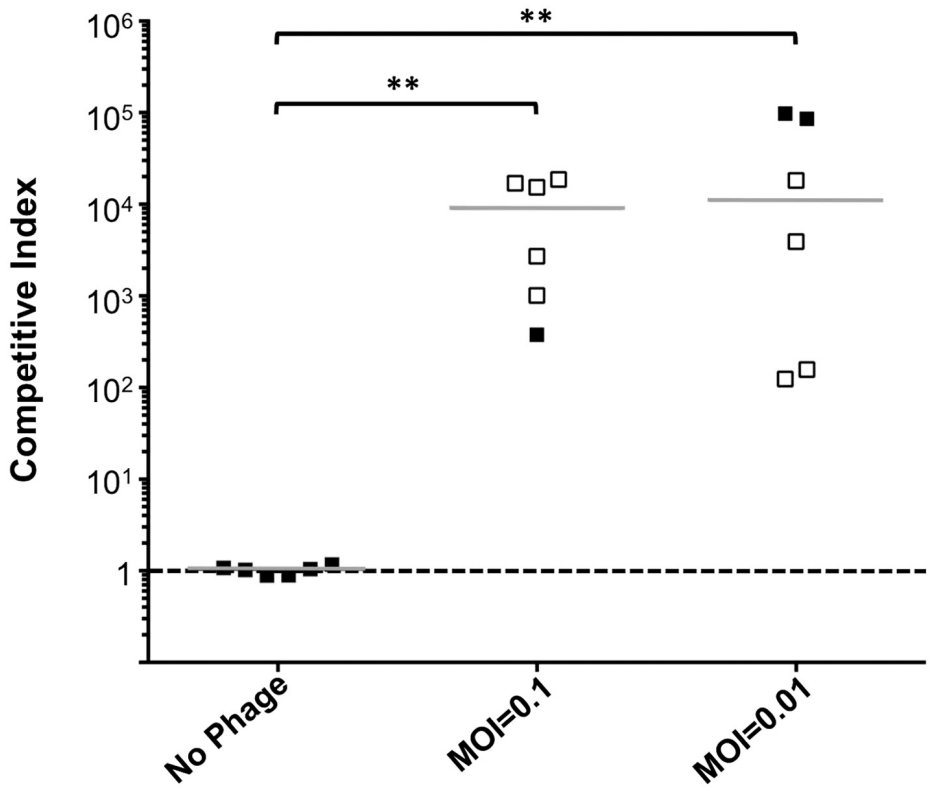


Figure 3.9. ICP2 predation within the small intestine leads to enrichment of the OmpU mutant over wild-type

Using the infant rabbit model of pathogenesis, the OmpU(G325D) mutant competed with wild-type in a 1:10 ratio in the absence or presence of ICP2_2013_A_Haiti at the multiplicity of infection of 0.1 or 0.01. Competitive indices were determined at 12 hours post-infection. $^{**}p < 0.01$, significantly different from the no-phage control by the Kruskal-Wallis and *post hoc* Dunn's multiple comparison tests. Horizontal lines indicate the median for each data set, and open symbols represent below the limit of detection. Figure is adapted from Seed, K.D., Yen, M. et al. (2014).

3.3. Conclusions

In this chapter, we have shown that lytic phages impose significant bactericidal pressure during the natural course of infection in humans. The adaptations to resist phage infection involve tradeoffs in evolutionary fitness, thereby affecting *V. cholerae* transmission and the seeding of environmental reservoirs. Our results provide the first molecular evidence for the impact of phage predation on shaping the bacterial population structure during the course of human infection.

Chapter 4: Investigation of ICP2 diversity in Haiti

ICP2 is the first virulent phage observed to be associated with the Haitian cholera epidemic (Seed et al., 2014). Through collaborations with the University of Florida and the National Lab of Haiti, we demonstrate in this chapter that ICP2 presence is widespread in Haiti. Furthermore, ICP2 isolates are highly similar to one another, although the relatedness is not associated with geography or time of isolation.

4.1. Results

4.1.1. Surveillance of clinical ICP2 in Haiti from 2012-2014

To determine if ICP2 is widespread in Haiti, we collaborated with Dr. Jacques Boncy at the National Lab in Port-au-Prince and Drs. Glen Morris and Afsar Ali (University of Florida) at their Haiti Research Lab in Christianville to collect patient stool samples. From the National Lab, a total of 85 samples were collected and processed for phage detection (Table 4.1). Samples were collected from patients that entered clinics from December 2012 to March 2013. Due to lack of resources, the lab had not been able to process the backlog of additional stored clinical samples to determine if the etiological agent for the patients' acute secretory diarrhea was *V. cholerae*.

Nonetheless, we proceeded to test the confirmed cholera patient samples for the presence of ICP2. We extracted total DNA from the stool samples and used two primer sets to probe for the presence of ICP2 DNA (Table 2.2, Chapter 2). ICP2 was detected by both primer sets in 35 out of the 85 samples tested. To further confirm ICP2 presence, we enriched the ICP2-positive samples for live phage. We attempted to isolate *V. cholerae* from each sample by plating onto LB Sm100 plates for use as phage enrichment host strains. More selective media was not used since samples were frozen at -80°C without

cryoprotectant. 33 out of 85 samples yielded *V. cholerae* colonies (Table 4.1, Column 4), indicating that rice-water stool may have cryoprotectant properties on its own.

To enrich for live phage, approximately 100 μl of the frozen stool sample was incubated overnight with 10 ml of *V. cholerae* culture at 37°C with aeration. The host strain used was a rough mutant of the wild-type E7946 strain, as we have previously determined that ICP2 infects rough mutants twice as well compared to wild-type (data not shown). If available, we also used a *V. cholerae* isolate that was isolated from the same clinical sample. Following overnight incubation, the supernatants were collected and tested for phage presence by plaque assay. Thirty-one supernatants tested positive for phage (Table 4.1, Column 5), and plaques were purified and prepped for whole-genome sequencing.

Table 4.1. Clinical samples from National Lab of Haiti

Clinical Sample	Region of Isolation	Date of Isolation	*V. cholerae* isolation?	ICP2 isolation?
PAP 1	Artibonite	27-Dec-12	-	-
PAP 2	Artibonite	27-Dec-12	-	Yes
PAP 3	Sud-Est	28-Dec-12	Ogawa	-
PAP 4	Ouest	4-Jan-13	Ogawa	Yes
PAP 5	Ouest	4-Jan-13	-	-
PAP 6	Ouest	4-Jan-13	-	-
PAP 7	Ouest	4-Jan-13	-	-
PAP 8	Sud-Est	7-Jan-13	Ogawa	-
PAP 9	Sud-Est	7-Jan-13	-	-
PAP 10	Sud-Est	7-Jan-13	Ogawa	-
PAP 11	Artibonite	8-Jan-13	-	-
PAP 12	Artibonite	8-Jan-13	-	Yes
PAP 13	Artibonite	8-Jan-13	-	-
PAP 14	Artibonite	8-Jan-13	-	-
PAP 15	Ouest	8-Jan-13	-	-
PAP 16	Ouest	8-Jan-13	-	-
PAP 17	Ouest	8-Jan-13	Ogawa	-
PAP 18	Ouest	8-Jan-13	-	Yes
PAP 19	Sud-Est	9-Jan-13	Ogawa	-
PAP 20	Sud-Est	9-Jan-13	-	-
PAP 21	Sud-Est	9-Jan-13	-	-
PAP 22	Sud-Est	9-Jan-13	-	-
PAP 23	Artibonite	10-Jan-13	-	-
PAP 24	Artibonite	10-Jan-13	-	-
PAP 25	Ouest	10-Jan-13	Ogawa	-
PAP 26	Ouest	10-Jan-13	Ogawa	-
PAP 27	Sud-Est	11-Jan-13	-	-
PAP 28	Sud-Est	11-Jan-13	-	-
PAP 29	Ouest	11-Jan-13	-	Yes
PAP 30	Ouest	11-Jan-13	Ogawa	-
PAP 31	Ouest	11-Jan-13	Ogawa	Yes
PAP 32	Artibonite	15-Jan-13	-	Yes
PAP 33	Artibonite	15-Jan-13	-	Yes
PAP 34	Artibonite	15-Jan-13	-	Yes
PAP 35	Ouest	15-Jan-13	Ogawa	-

Table 4.1. continued

Clinical Sample	Region of Isolation	Date of Isolation	*V. cholerae* isolation?	ICP2 isolation?
PAP 36	Ouest	16-Jan-13	Ogawa	-
PAP 37	Ouest	16-Jan-13	-	Yes
PAP 38	Ouest	16-Jan-13	-	-
PAP 39	Artibonite	17-Jan-13	-	-
PAP 40	Artibonite	17-Jan-13	-	Yes
PAP 41	Ouest	18-Jan-13	Ogawa	-
PAP 42	Ouest	18-Jan-13	Ogawa	-
PAP 43	Artibonite	22-Jan-13	-	Yes
PAP 44	Artibonite	22-Jan-13	-	Yes
PAP 45	Artibonite	22-Jan-13	-	-
PAP 46	Ouest	22-Jan-13	-	-
PAP 47	Ouest	22-Jan-13	-	-
PAP 48	Ouest	23-Jan-13	-	Yes
PAP 49	Ouest	23-Jan-13	-	Yes
PAP 50	Sud-Est	29-Jan-13	-	-
PAP 51	Sud-Est	29-Jan-13	-	Yes
PAP 52	Ouest	29-Jan-13	-	Yes
PAP 53	Ouest	29-Jan-13	-	-
PAP 54	Sud-Est	30-Jan-13	-	-
PAP 55	Ouest	30-Jan-13	-	-
PAP 56	Ouest	30-Jan-13	-	Yes
PAP 57	Artibonite	31-Jan-13	Ogawa	-
PAP 58	Artibonite	31-Jan-13	-	Yes
PAP 59	Sud-Est	1-Feb-13	-	-
PAP 60	Sud-Est	1-Feb-13	-	-
PAP 61	Artibonite	5-Feb-13	-	Yes
PAP 62	Artibonite	5-Feb-13	-	Yes
PAP 63	Ouest	6-Feb-13	-	Yes
PAP 64	Ouest	6-Feb-13	-	-
PAP 65	Ouest	6-Feb-13	Ogawa	Yes
PAP 66	Artibonite	7-Feb-13	-	Yes
PAP 67	Ouest	8-Feb-13	-	-
PAP 68	Ouest	8-Feb-13	Ogawa	Yes
PAP 69	Artibonite	14-Feb-13	Ogawa	Yes
PAP 70	Artibonite	14-Feb-13	Ogawa	Yes

Clinical Sample	Region of Isolation	Date of Isolation	*V. cholerae* isolation?	ICP2 isolation?
PAP 74	Ouest	14-Feb-13	Ogawa	Yes
PAP 75	Artibonite	19-Feb-13	Ogawa	Yes
PAP 76	Artibonite	19-Feb-13	-	Yes
PAP 77	Ouest	19-Feb-13	Ogawa	-
PAP 78	Ouest	19-Feb-13	Ogawa	-
PAP 79	Ouest	20-Feb-13	Ogawa	Yes
PAP 80	Ouest	20-Feb-13	Ogawa	-
PAP 81	Ouest	22-Feb-13	Ogawa	Yes
PAP 82	Sud-Est	22-Feb-13	-	-
PAP 83	Sud-Est	25-Feb-13	Ogawa	-
PAP 84	Artibonite	26-Feb-13	Ogawa	Yes
PAP 85	Artibonite	26-Feb-13	Ogawa	-
PAP 86	Ouest	26-Feb-13	Ogawa	Yes
PAP 87	Sud-Est	27-Feb-13	Ogawa	-
PAP 88	Ouest	27-Feb-13	Ogawa	-
PAP 89	Ouest	27-Feb-13	Ogawa	-
PAP 90	Artibonite	28-Feb-13	Ogawa	-
PAP 91	Ouest	1-Mar-13	-	-

Table 4.1. Clinical samples from National Lab of Haiti

Clinical samples were collected from December 2012 to March 2013 from three regions of Haiti. Although saved without cryoprotectant, samples were screened for the presence of culturable *V. cholerae* and tested for serotype by serum agglutination. Samples were then enriched for ICP2 phage by overnight culture with an O1 antigen mutant of *V. cholerae* E7946.

From the University of Florida (UF) collaboration, a total of 20 stool samples were collected and processed for phage detection (Table 4.2). I collected one of these samples in August 2014 during my clinical practicum in Haiti; otherwise, the stool samples were collected and stored with 20% glycerol by Meer Alam, a graduate student in the Ali Lab, and sent to us for processing. Patient samples were collected from August 2014 to April 2015. As these samples were stored with cryprotectant, we performed plaque assays on them directly without enrichment to isolate live phages. Out of the 20, 12 samples yielded high-titers of phage. Ten plaques per sample were stored and assayed for ICP2 presence by plaque PCR. Interestingly, only three of these samples yielded ICP2 plaques, implying there is another phage that is associated with Haitian cholera samples. This phage was sequenced, determined to be novel, and subsequently named MAA01, but this story is outside the scope of this dissertation.

Clinical sample	Region of isolation	Date of isolation	ICP2 isolated?
UF-CTC	Artibonite	19-Aug-14	Yes
UF-1638	Artibonite	2-Sept-14	-
UF-1651	Sud-Est	11-Sept-14	Yes
UF-1655	Sud-Est	11-Sept-14	-
UF-1708	Ouest	24-Oct-14	-
UF-1738	Ouest	19-Nov-14	Yes
UF-1757	Ouest	27-Nov-14	-
UF-1761	Ouest	28-Nov-2014	-
UF-1766	Ouest	11-Dec-14	-
UF-1865	Sud-Est	11-Mar-15	-
UF-1866	Sud-Est	11-Mar-15	-
UF-1880	Sud-Est	28-Mar-15	-
UF-1884	Sud-Est	8-Apr-15	-
UF-1891	Sud-Est	10-Apr-2015	-
UF-1892	Sud-Est	14-Apr-2015	-
UF-1893	Sud-Est	14-Apr-2015	-
UF-1894	Sud-Est	14-Apr-2015	-
UF-6024	Ouest	26-Jan-2015	-
UF-6051	Ouest	28-Jan-2015	-
UF-6073	Ouest	30-Jan-2015	-

Table 4.2. Clinical samples from University of Florida collaboration

Frozen clinical samples with cryoprotectant were tested for the presence of phage by direct titer. The host strain was a Haitian clinical isolate (UF456).

Collectively, we have 34 ICP2 isolates spanning December 2012 to November 2014. The samples were collected from hospitals in three different departments, or regions, of Haiti: Artibonite, Ouest, and Sud-Est. ICP2 isolates from the National Lab samples were plaque-purified and expanded for high-titer stocks on the rough mutant of wild-type E7946, as not all samples that yielded ICP2 plaques also yielded *V. cholerae* isolates. It is possible that the clinical ICP2 isolates would infect clinical *V. cholerae* isolates significantly better than the rough mutant, opening up the possibility that passaging ICP2 on the rough mutant would introduce variation in the ICP2 genomes due to selection. However, this was determined not to be the case by EOP assay (data not shown). Therefore, we proceeded with using National Lab ICP2 isolates prepared using the rough mutant as the host *V. cholerae* strain for whole-genome sequencing. For the UF ICP2 isolates, a clinical *V. cholerae* isolate UF456 was used as the host strain for plaque purification and high-titer stock preparation. Similarly, ICP2 isolates do not infect UF456 better than clinical *V. cholerae* isolates from the same sample. UF ICP2 isolates were subsequently prepared using UF456 as the *V. cholerae* host strain and submitted for whole-genome sequencing.

4.1.2. Mauve alignments of ICP2 genomes

Genomes were assembled using CLC Workbench 9 software and aligned with the progressiveMauve algorithm (Darling et al., 2010) (Figure 4.1). The alignment revealed that the 34 isolates have a high degree of similarity. There were only six regions that showed some degree of variability. The first is around 18 kb (light green) and is annotated as part of the portal protein, which plays a critical role in assembling the head as well as in DNA entry and release (Aksyuk & Rossmann, 2011). The second region is near 23 kb (light yellow) and is annotated as part of the tail fiber protein. Interestingly, only the UF-CTC isolate is different in this region, implying that it may have an altered host range. The third region is near 34 kb (light brown) and is annotated as part of the CobT protein. As previously mentioned, the CobT/CobS homologs were identified in the ICP2 genome and had not previously been observed in other phage genomes. It has been hypothesized that ICP2 can synthesize these enzymes to enhance the metabolism of its bacterial host to increase its own proliferation (Seed et al., 2011).

Lastly, several ICP2 isolates are missing a large region from 48 to 49 kb that is predicted to encode a class III ribonucleotide reductase, which has been shown to be expressed under anaerobic conditions during T4 infection of *E. coli* (Jordan & Reichard, 1998) in order to enzymatically convert ribonucleotides to deoxyribonucleotides. The other two regions around 43 kb (blue) and 46 kb (dark yellow) are annotated as part of hypothetical proteins. An NCBI Blast search did not report similarities to other proteins outside of those found in other cataloged ICP2 isolates.

Figure 4.1. ProgressiveMauve alignment of ICP2 isolates

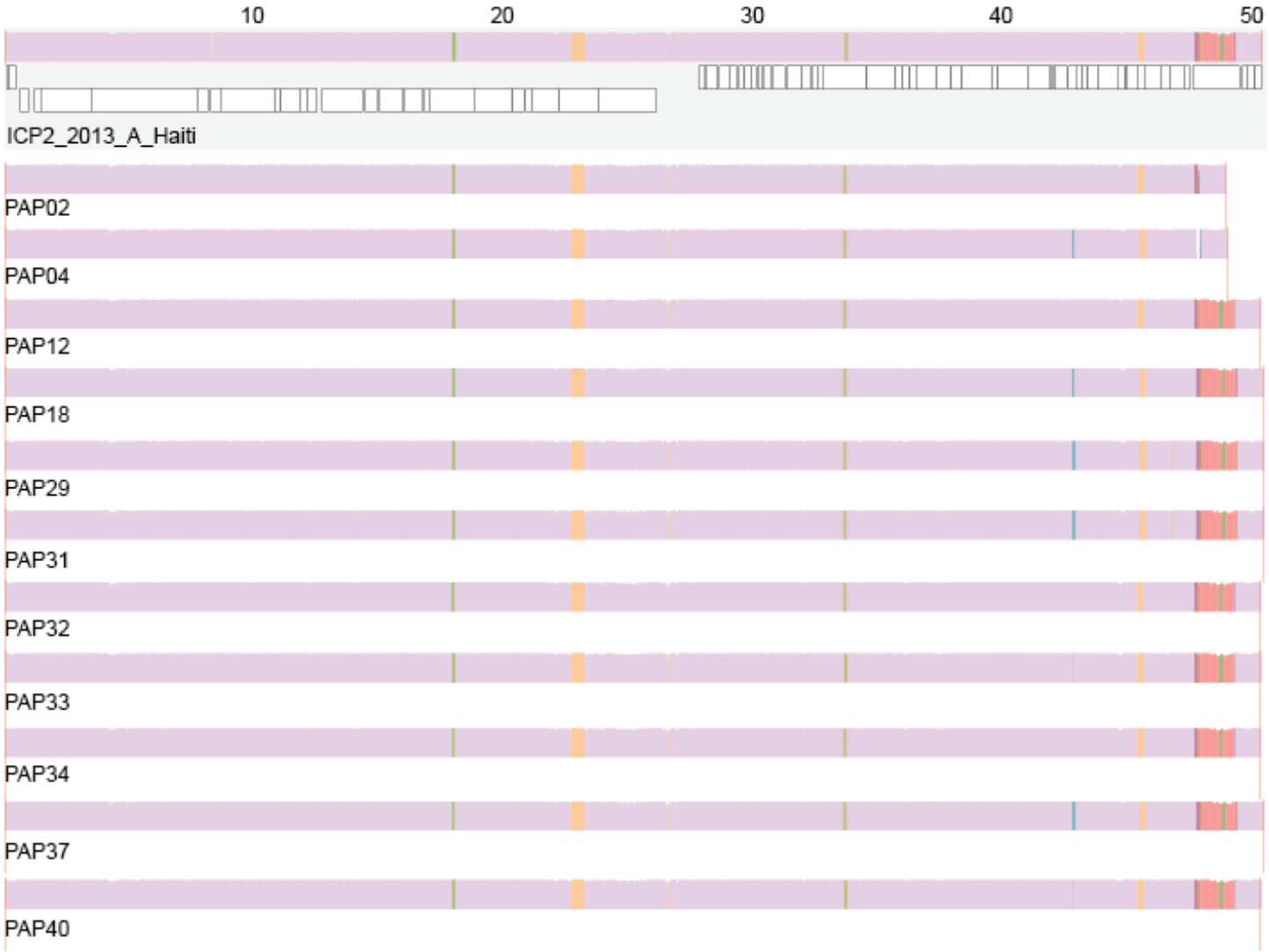


Figure 4.1. continued


10
20
30
40
50
ICP2_2013_A_Haiti
PAP68
PAP69
PAP70
PAP73
PAP74
PAP76
PAP79
PAP81
PAP84
UF-CTC
UF-1651
UF-1738

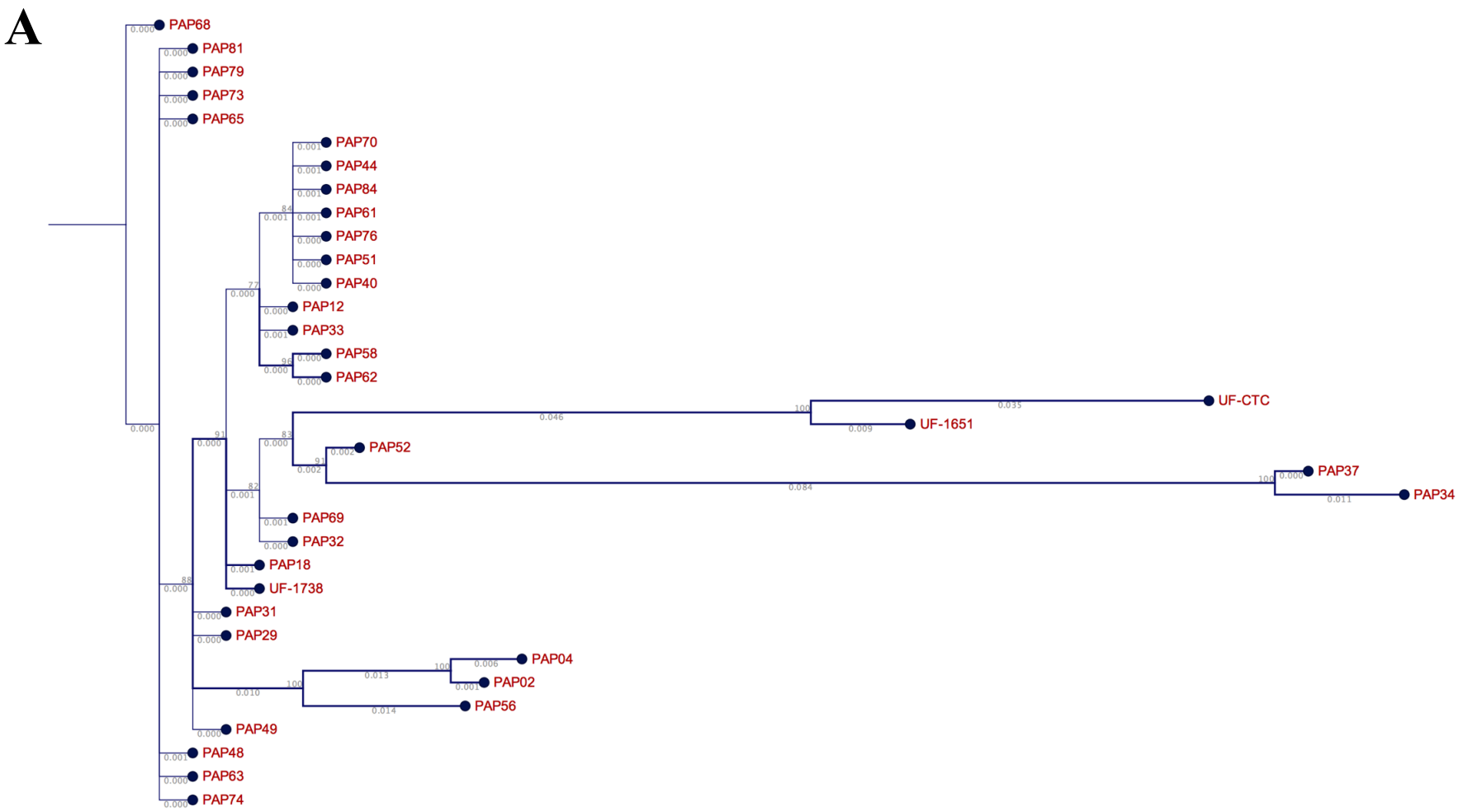
Figure 4.1. ProgressiveMauve alignment of ICP2 isolates

Comparison of Haitian ICP2 isolates from 2012 to 2014 using the progressiveMauve algorithm. The height of the similarity profile (y axis) indicates the degree of nucleotide similarity between the aligned regions. Regions that are common to multiple isolates are color-coded, where light purple represents the highly conserved backbone genome. Annotated genes of reference genome ICP2_2013_A_Haiti (Seed et al., 2014) are depicted in white boxes at the top, where genes that are transcribed from the negative strand are displaced downward. The numbers above the reference genome indicates the distance in kilobases.

4.2.3. Phylogenetic tree of Haitian ICP2 isolates

To determine the phylogenetic relationship among the Haitian ICP2 isolates, we used the neighbor-joining model, generalized time-reversible (GTR) (Tavaré, 1986), to build a tree from aligned variants (Figure 4.2). Bootstrap analysis was performed with 100 replicates, and bootstrap values above a threshold value of 70% are reported (Figure 4.2A). It appears that the relatedness is independent of time of isolation (Figure 4.2B). For example, UF-1738 was isolated in 2014, but it is more closely related to phages isolated in 2013 than the other two ICP2 phages isolated in 2014. Relatedness also seems to be independent of geographic location (Figure 4.2B). For example, PAP51 was isolated from the Sud-Est region, but it is more closely related to phages isolated from the Artibonite region.

Figure 4.2. Phylogenetic tree of Haitian ICP2 isolates



B

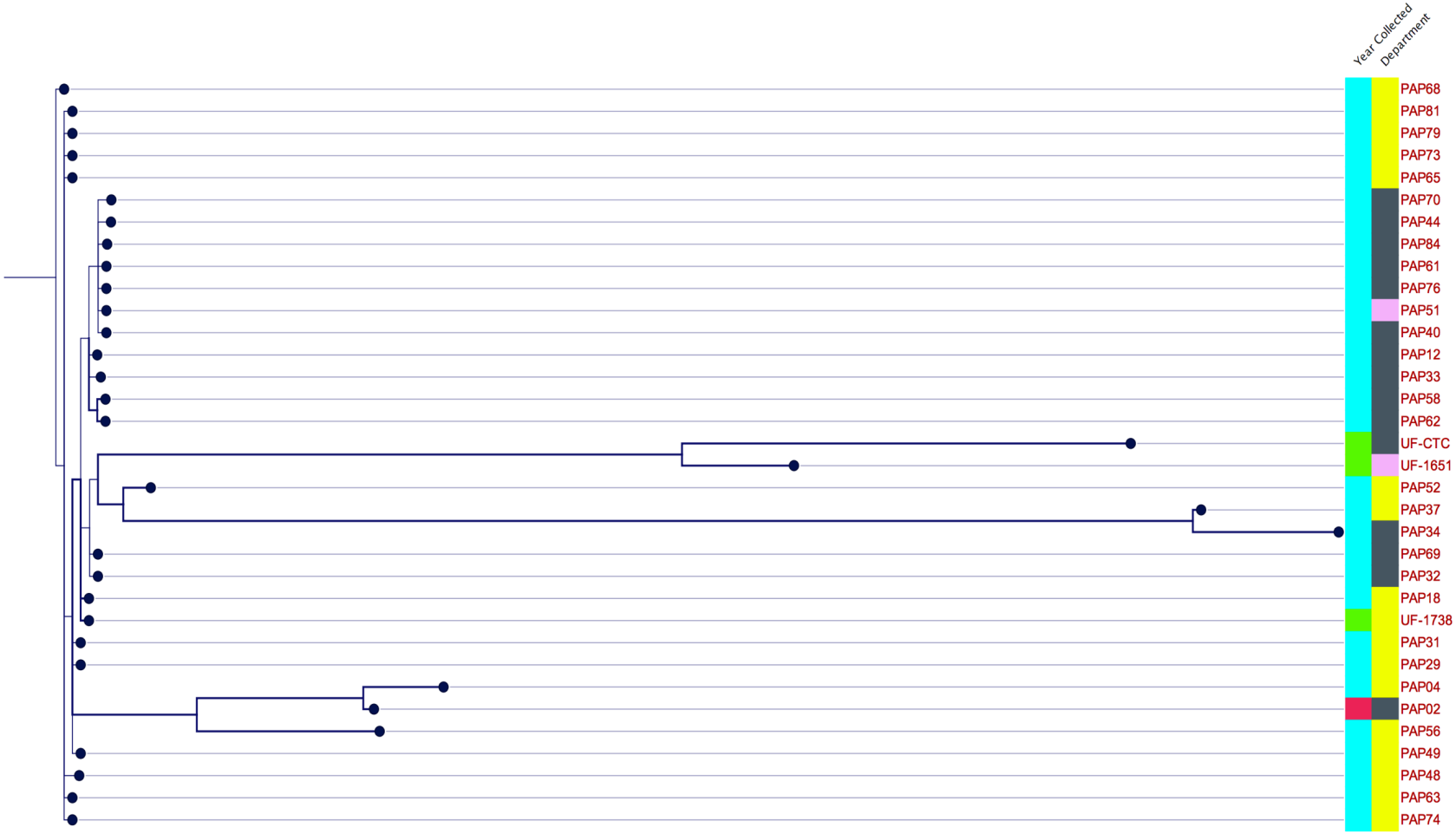

Year Collected
Department
PAP68
PAP81
PAP79
PAP73
PAP65
PAP70
PAP44
PAP84
PAP61
PAP76
PAP51
PAP40
PAP12
PAP33
PAP58
PAP62
UF-CTC
UF-1651
PAP52
PAP37
PAP34
PAP69
PAP32
PAP18
UF-1738
PAP31
PAP29
PAP04
PAP02
PAP56
PAP49
PAP48
PAP63
PAP74

Figure 4.2. Phylogenetic tree of Haitian ICP2 isolates

A phylogenetic tree was constructed using the neighbor-joining method, general time-reversible modeling, with 100 bootstrap replicates. (A) The bootstrap threshold value was set at 70%, and nodes with bootstrap values ≥ 90% are highlighted using bolded lines. Branch lengths are reported, with a minimum of 0.00029 (displayed as 0.000). (B) Metadata includes the year of isolation and the geographic location. For the year collected, red is 2012; blue is 2013; and green is 2014. For the geographic location, dark grey is the Artibonite department; yellow is the Ouest; and pink is the Sud-Est.

4.2. Conclusions

ICP2 is the first phage that was discovered to be associated with clinical samples from the Haitian cholera epidemic (Seed et al., 2014). We have demonstrated in this chapter that ICP2 is widespread, as it was isolated from patients in three geographic regions of Haiti. Alignments of *de novo* assembled genomes revealed a high degree of similarity among the isolates; however, phylogenetic analysis showed no association between relatedness and geographical or temporal isolation.

Chapter 5: Using a phage prophylaxis approach to prevent cholera

The experiments presented in this chapter were performed in collaboration with Dr. Lynne Cairns.

Effective prevention strategies are critical in reducing the disease burden due to bacterial infections. For cholera, however, there is currently an unmet need for the immediate prevention of household transmission, which is a major contributor to its rapid spread within a community. In this chapter, we use a cocktail of three virulent phages to show that oral phage prophylaxis is a successful approach for preventing cholera. We will demonstrate that oral administration of our phage cocktail up to 24 hours before *V. cholerae* challenge reduced colonization of the small intestine and prevented the onset of symptoms. Our study provides an important proof-of-principle that phage prophylaxis could be utilized as a strategy to limit the impact of bacterial disease on human health.

5.1. Background

Recent widespread cholera epidemics in disaster-stricken or war-torn countries such as Haiti (Luquero et al., 2016) and Iraq (Bagcchi, 2016) highlight the vulnerabilities of these populations to sudden outbreaks. The current recommended preventatives include mass vaccination campaigns with the WHO-prequalified oral cholera vaccine (OCV) (Qadri et al., 2016) and increased hygiene and sanitation practices (D. L. Taylor, Kahawita, Cairncross, & Ensink, 2015). There are currently two OCVs that are stockpiled for cholera outbreak control. Dukoral® requires two doses given at least one week apart. Earliest onset of protection against cholera develops seven days after the second dose. The preferred OCV Shanchol™ was prequalified more recently in 2011. Similarly, it also requires two doses given at an interval of two weeks, with the earliest onset of protection developing seven to ten days after the second dose (WHO, 2015).

Although the efficacies of Dukoral® and Shanchol™ in both endemic and non-endemic settings are greater than fifty percent, there is still a wide range of coverage,

depending on the country, as previously mentioned. In addition, effective deployment of OCVs during an ongoing outbreak is logistically challenging as multiple steps of administration are needed for protection. This requires patient adherence for full efficacy and at least two to three weeks post-vaccination before at-risk populations will begin to see protective effects. Although OCV campaigns may be effective in the long-term, they are not logistically feasible for immediate protection in the event of an outbreak.

Recently, it has been shown that household transmission is a major contributor to the rapid spread of *V. cholerae* within communities. Household members have an increased risk of contracting cholera, and they present with cholera symptoms two to three days after the initial patient becomes sick (Harris et al., 2008; Weil et al., 2009). Currently, there is no clinical intervention to stem the household spread of cholera by use of a rapid prophylactic treatment. To this end, we have harnessed the specificity and quick-acting properties of phages as a prophylaxis therapy for cholera.

In this chapter, we will show that a cocktail of three well-studied lytic phages ICP1, ICP2, and ICP3 is successful in two animal models of cholera pathogenesis as an approach to prevent disease. The phages target different receptors, thereby reducing the likelihood of multi-phage-resistant *V. cholerae* isolates in the surviving population. We will show the phage resistance profiles of the surviving *V. cholerae* isolates from both animal models and provide genomic analyses to determine the mechanisms of resistance. This important proof-of-principle study demonstrates the successful use of phage prophylaxis to prevent disease caused by an environmentally transmitted mucosal pathogen. Experiments in this chapter were designed and performed in collaboration with postdoctoral fellow Dr. Lynne Cairns in the Camilli lab.

Strain	Genotype and description
WT	Spontaneous Sm^R derivative of E7946, O1 El Tor Ogawa
AC2846	E7946 Δ*ompU*
AC4653	E7946 Δ*wbeL*
AC4741	*Vibrio cholerae* O1 El Tor Ogawa Sm^R, PLE-negative

Sm^R, streptomycin-resistant; PLE refers to PICI-like element.

Table 5.1. Strains used in Chapter 5

Bacteriophage	Description
ICP1	ICP1_2011_A; *Myoviridae* (Seed et al., 2011)
ICP2	ICP2_2004_A; *Podoviridae* (Seed et al., 2011)
ICP3	ICP3_2006_A; *Podoviridae* (Seed et al., 2011)

Table 5.2. Bacteriophages used in Chapter 5

5.2. Results

5.2.1. The ICP cocktail kills V. cholerae in vitro

The ICP cocktail comprises lytic *V. cholerae* phages ICP1, ICP2, and ICP3 in equal parts. ICP1 uses the O1 antigen of LPS as its receptor (Seed et al., 2011; Seed et al., 2012), and we have determined previously (Chapter 4) that the receptor for ICP2 is the major outer membrane porin OmpU. As of yet, we have not determined definitively the receptor for ICP3, but we believe that LPS is involved (data not shown). Nonetheless, there is diversity in the receptors used to initiate infection. Therefore, we hypothesize the ICP cocktail is more effective at killing *V. cholerae* than each phage in isolation.

To test this hypothesis *in vitro*, we grew up cultures of *V. cholerae* E7946 in the absence and presence of phage at an MOI of 1. Samples were collected every hour up to hour 8 and then again at 12 and 24 hours. Each sample was enumerated for CFU per ml (Figure 5.1A), and the phage fraction was isolated and enumerated for PFU per ml (Figure 5.1B). Although all cultures grown in the presence of phage suffered a large decrease in bacterial titer in the initial hour, cultures with either ICP1 or ICP3 rebounded quickly within 4 and 6 hours, respectively. Cultures with ICP2 alone had suppressed growth initially but were equal in bacteria number to the no-phage control by 24 hours. In contrast, cultures grown in the presence of the ICP cocktail do not fully recover within 24 hours. These results show that the ICP cocktail is more effective than each phage alone at killing *V. cholerae in vitro*.

It is not surprising that the *V. cholerae* population rebounds in the presence of the phage cocktail. In comparison to other phage killing experiments published in phage therapy literature, the MOI used is relatively low. In addition, the starting number of

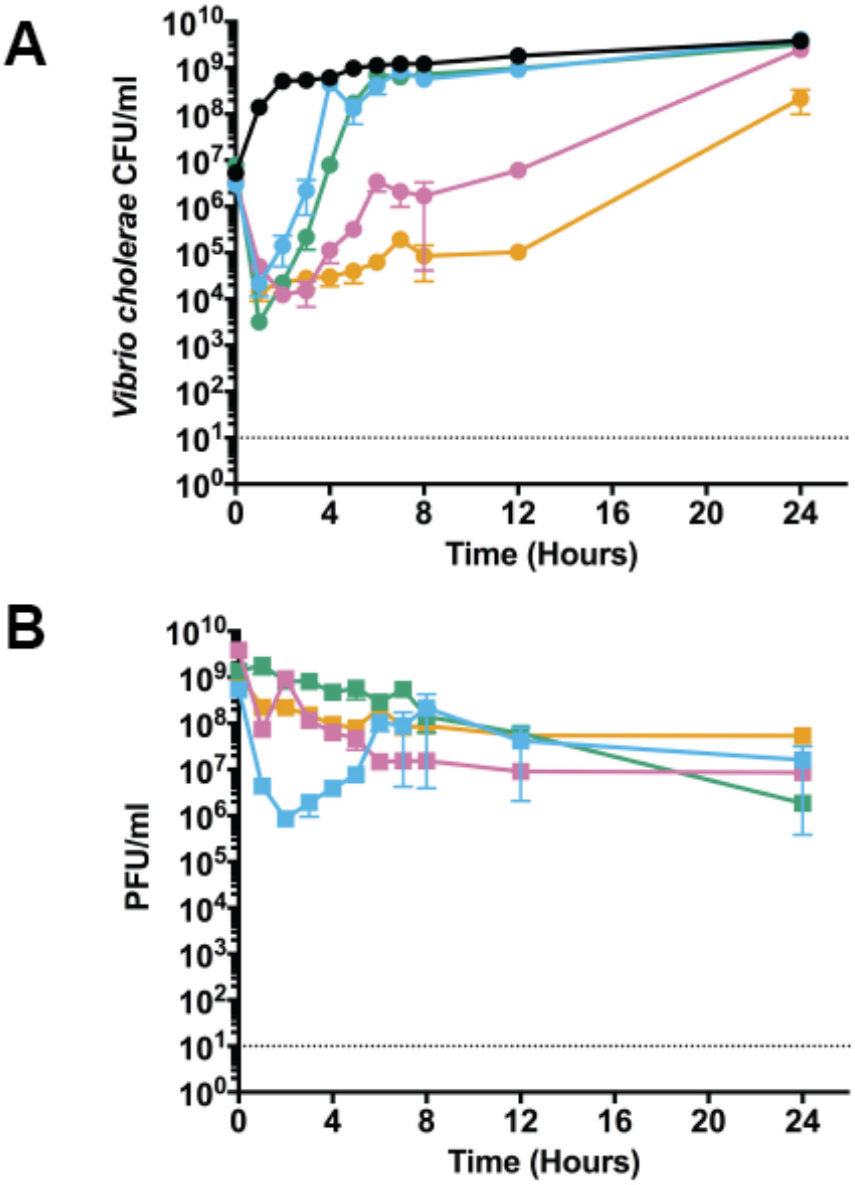

A
Vibrio cholerae CFU/ml
Time (Hours)
B
PFU/ml
Time (Hours)

Figure 5.1. The ICP cocktail is superior at killing *V. cholerae in vitro*

Growth curves of *V. cholerae* E7946 in the absence and presence of phage predation. Cultures were grown in 10 ml of LB supplemented with 100 μg/ml streptomycin in the absence of phage (black) or with ICP1 (blue), ICP2 (pink), ICP3 (green), or the ICP phage cocktail (orange). Phages were added to mid-exponential growth phase cultures at an MOI of 1. Samples of each culture were taken every hour until hour 8, then again at 12 and 24 hours. (a) *V. cholerae* were enumerated for CFU/ml by plating onto LB plates supplemented with 100 μg/ml streptomycin. (b) The phage fraction was isolated from samples and enumerated for PFU/ml by plaque assay. The data plotted are the averages of five independent replicates for each condition, and error bars represent the standard error of the mean. Dotted lines indicate the limit of detection.

bacteria was approximately 5 x 10^7 CFU. Most likely, there are a number of existing phage-resistant mutants in the starting *V. cholerae* population that are positively selected when the culture is grown in the presence of phage. The phage enumeration data support this hypothesis as the titers are 100- to 1000-fold lower at 24 hours than the initial phage population (Figure 5.1B). This is presumably due to the lack of sensitive hosts in the culture to continue phage replication.

5.2.2. The ICP cocktail prevents V. cholerae *colonization better than single phages in mice*

As the ICP cocktail was more effective in killing *V. cholerae* than each phage alone, we hypothesized this would also be the case *in vivo*. We performed prophylaxis experiments using the infant mouse model of *V. cholerae* colonization and hypothesized that the ICP cocktail would be effective in preventing *V. cholerae* infection. There were five groups of mice, and each received either (i) no phage, (ii) ICP1, (iii) ICP2, (iv) ICP3, or (v) the ICP cocktail with approximate MOIs of 2 to 80. Phages were administered orogastrically 3 hours prior to infection with 5 x 10^5 CFU of *V. cholerae*. The 3-hour time point was chosen as it takes under 3 hours for the bolus of intragastrically injected material to pass through the small intestine of an infant mouse. Thus, only phages retained in the small intestine will be present at the time of *V. cholerae* challenge, thus modeling prophylaxis in humans. Of note, the MOI for all prophylaxis experiments is calculated based on the number of PFU in the phage inoculum at the time of administration. The MOI, however, is not accurate in *in vivo* settings due to the architecture of the small intestine. The entire phage inoculum is also not likely to be

retained, resulting in a smaller number of phages that are able to infect the *V. cholerae* upon challenge.

Mice were sacrificed 24 hours post-*V. cholerae* challenge, and *V. cholerae* and phages in the small intestine were enumerated with plating and plaque assays, respectively, following tissue homogenization. In all conditions where phage was administered, the number of surviving *V. cholerae* in the small intestine was reduced by at least two orders of magnitude in comparison to the no-phage control (Figure 5.2A). Out of the single phage-conditions, ICP3 performed the best; however, as expected, the ICP cocktail was the most effective, with no *V. cholerae* detectable in the intestinal homogenates of 4 out of 5 mice ($p < 0.01$). These results demonstrate that the ICP cocktail is more effective than each phage in isolation at preventing *V. cholerae* colonization in the infant mouse model. In addition, phages were detected, often at high titer, in the homogenates at the end of the experiment (Figure 5.2B), suggesting that ICP phages can survive and persist in the small intestine.

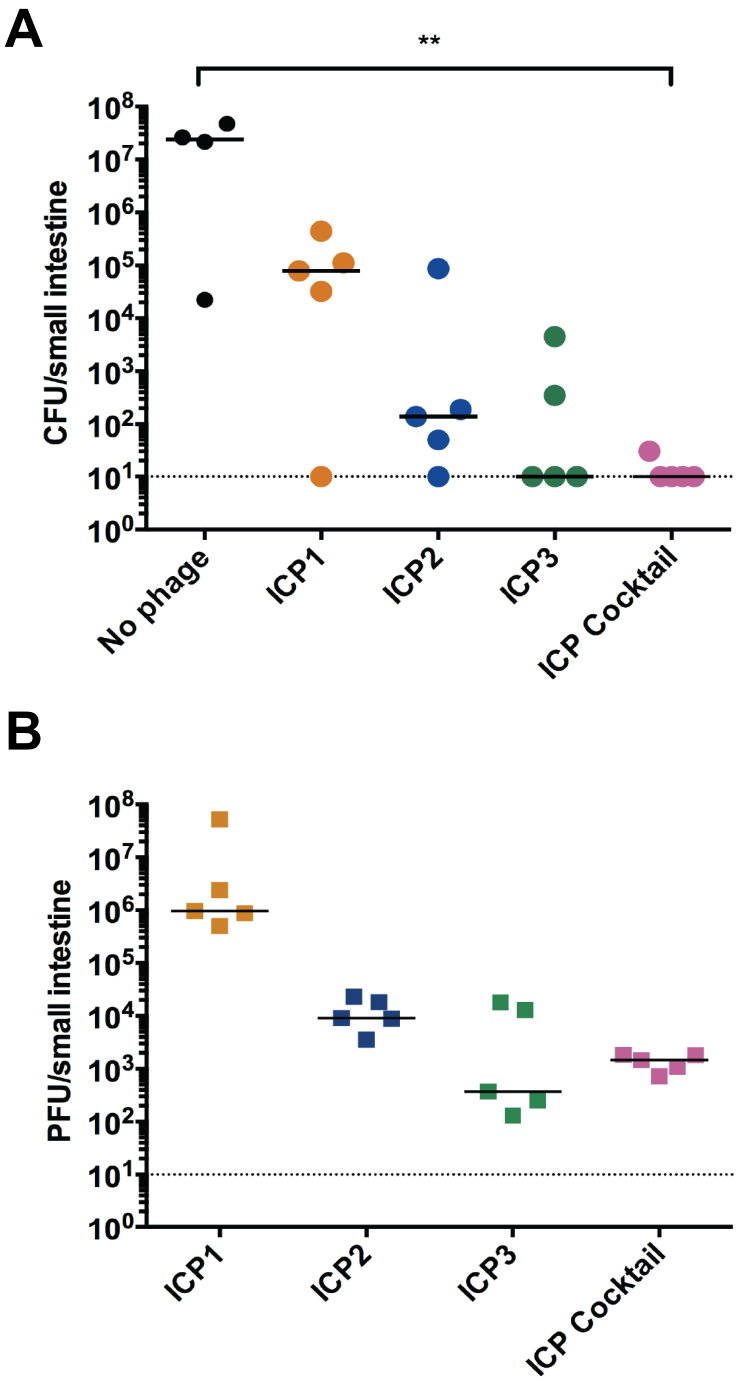

A
**
CFU/small intestine
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
No phage
ICP1
ICP2
ICP3
ICP Cocktail
B
PFU/small intestine
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
ICP1
ICP2
ICP3
ICP Cocktail

Figure 5.2. The ICP cocktail is more effective than single phages at reducing *V. cholerae* colonization in the infant mouse small intestine

Mice were dosed orogastrically with ICP1 (orange), ICP2 (blue), ICP3 (green), or the ICP cocktail (pink) at an MOI of 2-80. One group received no phage at all (black). Three hours later, mice were challenged with 5 x 10^5 CFU *V. cholerae* E7946. At 24 hours post-challenge, animals were sacrificed and the small intestines were homogenized. (a) *V. cholerae* were enumerated as CFU/small intestine by plating onto LB plates supplemented with 100 μg/ml streptomycin. (b) Phages were enumerated as PFU/small intestine by plaque assay. Dotted lines indicate the limit of detection, and the horizontal solid bars represent the medians. Each shape represents one animal. Significance was calculated using the Kruskal-Wallis and Dunn's post-hoc multiple comparisons tests. **, $p < 0.01$.

5.2.3. Phages are retained in the infant mouse small intestine up to 24 hours

It was necessary to establish if phages could survive in the small intestine for long periods of time in the absence of their bacterial host. Understanding the retention kinetics for each phage will determine whether phage prophylaxis will be effective if administered more than 3 hours before *V. cholerae* challenge. Each ICP phage was orogastrically administered in isolation (approximately 10^8 PFU per mouse), and mice were sacrificed after 3, 6, 12, or 24 hours. Phages were then enumerated from small intestine homogenates using plaque assays (Figure 5.3). At 3 hours, phages were largely retained, supporting the model that retained ICP phages were most likely responsible for decreasing the *V. cholerae* burdens in the previous experiment (Figure 5.3). After 6 to 12 hours, the phage titers had only dropped 10- to 100-fold. ICP1 and ICP2 were still retained at approximately 10^5 PFU per small intestine at 24 hours, similar to their titers at 12 hours. This may indicate there is a threshold amount of ICP1 or ICP2 that is retained despite peristalsis in the intestinal tract. ICP3 titers, on the other hand, fell to 10^2 to 10^4 PFU per small intestine. These results further show that ICP phages can survive in the infant mouse intestinal tract for at least 24 hours in the absence of their *V. cholerae* host.

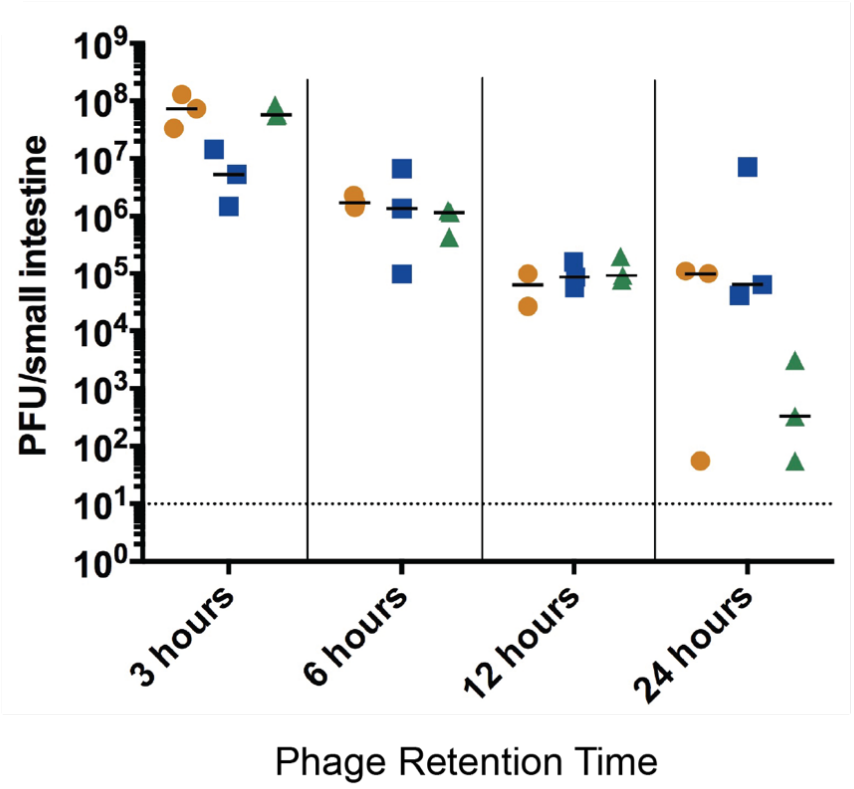
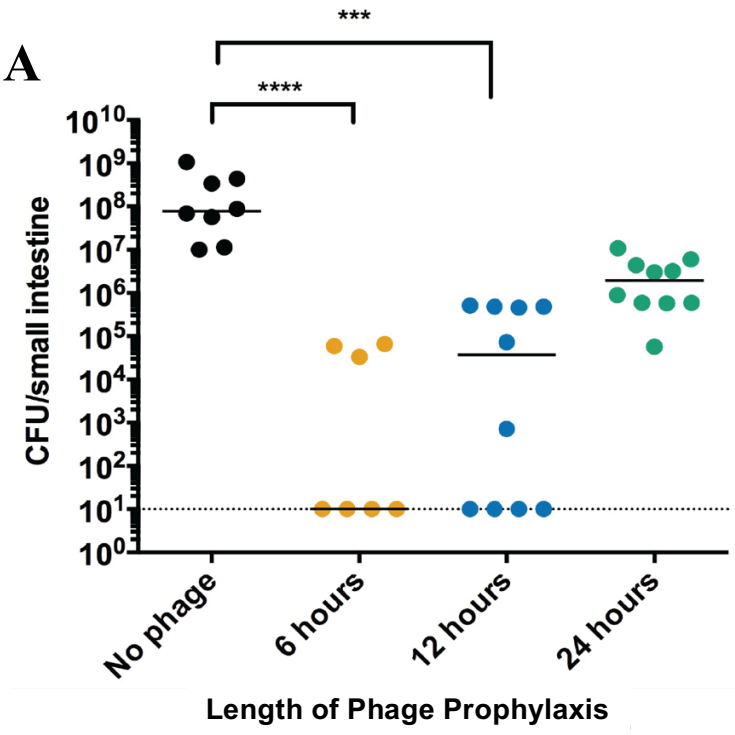


Figure 5.3. ICP phages are retained in the infant mouse small intestine

Mice were orogastrically dosed with approximately 10^8 PFU of ICP1 (orange), ICP2 (blue), and ICP3 (green) without their host *V. cholerae* E7946. After 3, 6, 12, or 24 hours, the animals were sacrificed, and the small intestines were homogenized. Phages were enumerated as PFU/small intestine by plaque assay. The dotted line indicates the limit of detection, and the horizontal solid bars represent the medians. Each shape represents one animal.

5.2.4. The ICP cocktail prevents V. cholerae *colonization in mice when administered up to 24 hours prior to challenge*

As phages were retained up to 24 hours in the absence of their host, we hypothesized the ICP cocktail remains protective up to 24 hours prior to *V. cholerae* challenge in the infant mouse model. The ICP cocktail was administered to infant mice 6, 12, or 24 hours at MOIs of 20 to 30 before challenge with 5×10^5 CFU *V. cholerae*. A control group did not receive phage. The 6-hour prophylaxis was most successful (Figure 5.4A). The number of surviving *V. cholerae* decreased by at least 1000-fold in comparison to the no-phage group, and there were no detectable bacteria for 4 out of the 7 mice ($p < 0.0001$). The 12-hour prophylaxis was also successful as the number of surviving *V. cholerae* was significantly lower ($p < 0.001$), where 4 mice did not have detectable bacteria. The number of *V. cholerae* was also reduced in the 24-hour prophylaxis group, albeit by only approximately 100-fold. Phages were also detectable for all mice that received prophylaxis treatment (Figure 5.4B).


A
***
****
CFU/small intestine
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
No phage
6 hours
12 hours
24 hours
Length of Phage Prophylaxis

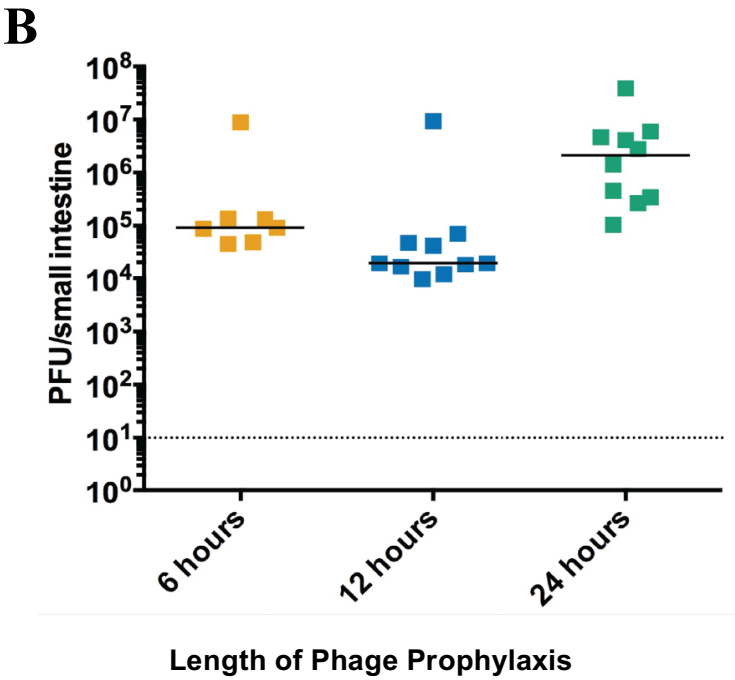
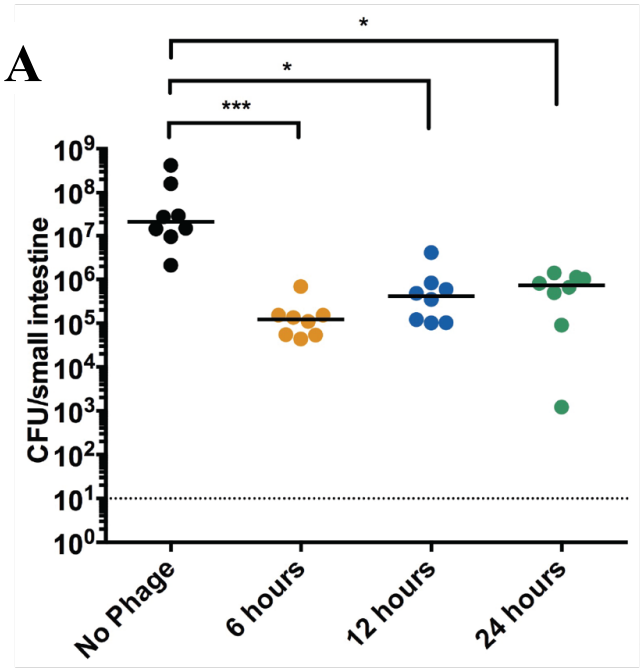

B
PFU/small intestine
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
6 hours
12 hours
24 hours
Length of Phage Prophylaxis

Figure 5.4. ICP cocktail reduced *V. cholerae* burden when administered up to 24 hours prior to challenge

Infant mice were orogastrically dosed with the ICP cocktail 6 (orange), 12 (blue), or 24 (green) hours prior to challenge with 5 x 10^5 CFU *V. cholerae* E7946 at MOIs of 20 to 30. A control group did not receive phage (black). Twenty-four hours post-challenge, the animals were sacrificed, and the small intestines were homogenized. (a) *V. cholerae* was enumerated for CFU/ml by plating onto LB plates supplemented with 100 μg/ml streptomycin. (b) Phages were enumerated for PFU/ml by plaque assay. Dotted lines indicate the limit of detection, and the horizontal solid bars represent the medians. Each shape represents one animal. Significance was calculated using the Kruskal-Wallis and Dunn's post-hoc multiple comparisons tests. ***, $p < 0.001$; ****, $p < 0.0001$.

To determine if the ICP cocktail can prevent colonization when mice are given a higher challenge dose, the experiment was repeated with an inoculum of 5 x10^7 CFU of *V. cholerae* per mouse, a dose approximately 100-fold higher than used in the experiment shown in Figure 5.4. The ICP cocktail is effective in significantly reducing *V. cholerae* colonization by at least two orders of magnitude when administered at either 6, 12, or 24 hours prior to a high-dose challenge (Figure 5.5A). Phages were again detected at similar levels in all three phage-treated groups (Figure 5.5B). These results support the hypothesis that a phage cocktail can be used to prevent *V. cholerae* colonization.


A
*
*
***
CFU/small intestine
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
No Phage
6 hours
12 hours
24 hours
Length of Phage Prophylaxis

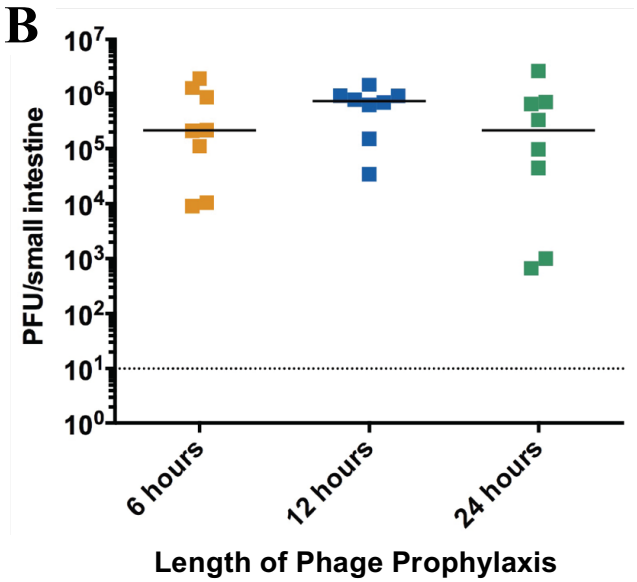

B
PFU/small intestine
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
6 hours
12 hours
24 hours
Length of Phage Prophylaxis

Figure 5.5. The ICP cocktail reduces *V. cholerae* burden when mice are challenged with a high dose

Infant mice were orogastrically dosed with the ICP cocktail 6 (orange), 12 (blue), or 24 (green) hours prior to challenge with 5 x 10^7 CFU *V. cholerae* E7946. A control group did not receive phage (black). Twenty-four hours post-challenge, the animals were sacrificed, and the small intestines were homogenized. (a) *V. cholerae* was enumerated for CFU/ml by plating onto LB plates supplemented with 100 μg/ml streptomycin. (b) Phages were enumerated for PFU/ml by plaque assay. Dotted lines indicate the limit of detection, and the horizontal solid bars represent the medians. Each shape represents one animal. Significance was calculated using the Kruskal-Wallis and Dunn's post-hoc multiple comparisons tests. *, $p < 0.1$; ***, $p < 0.001$.

5.2.5. *Phage resistance phenotypes and corresponding mutations of surviving* V. cholerae *isolates from mice*

V. cholerae can be isolated from the small intestines of several mice that were dosed with the ICP cocktail. Due to the complexity of the gut environment, it is possible that some fraction of these bacteria survived because they failed to encounter the ICP phages. Another fraction, however, may contain genetic mutations that allowed them to escape phage predation. To determine the phage resistance profile of the surviving bacteria population, *V. cholerae* isolates from the experiment described in Figure 5.4 were colony-purified and used in efficiency of plating (EOP) assays with each of the ICP phages (Table 5.3). All isolates from mice in the 6-hour and 12-hour prophylaxis groups were sensitive to all three ICP phages. The majority of isolates from mice in the 24-hour prophylaxis group were also sensitive to all three ICP phages; however, a small number showed differing resistance phenotypes (Table 5.4).

Table 5.3. Sensitivity of surviving mouse isolates to ICP phages

S = EOP > 10^{-1}; PS = 10^{-5} < EOP < 10^{-1}; R = EOP < 10^{-6} (limit of detection).
TURBID* = plaques were turbid instead of clear.

Mouse	Isolate	Efficiency of Plating			Resistance		
		ICP1	ICP2	ICP3	ICP1	ICP2	ICP3
6h Prophylaxis							
EM2	1	1.26E+00	6.25E-01	9.09E-01	S	S	S
	2	3.13E+00	6.88E-01	1.09E+00	S	S	S
	3	4.48E+00	1.08E+00	7.27E-01	S	S	S
	4	2.60E+00	1.10E+00	1.09E+00	S	S	S
	5	3.44E+00	1.33E+00	1.15E+00	S	S	S
	6	1.10E+01	1.58E+00	1.00E+00	S	S	S
	7	1.23E+00	9.38E-01	1.00E+00	S	S	S
	8	1.13E+00	1.04E+00	2.91E+00	S	S	S
	9	1.00E+00	8.75E-01	2.91E-01	S	S	S
	10	8.23E-01	1.13E+00	2.70E-01	S	S	S
EM3	1	1.45E+00	7.62E-01	1.09E+00	S	S	S
	2	1.25E+00	1.79E+00	1.12E+00	S	S	S
	3	9.25E-01	1.77E+00	1.45E+00	S	S	S
	4	1.93E+00	1.51E+00	1.30E+00	S	S	S
	5	6.50E-01	2.56E+00	1.21E+00	S	S	S
	6	2.65E-01	9.70E-01	1.58E+00	S	S	S
	7	3.90E-01	2.74E+00	3.39E-01	S	S	S
	8	1.00E+00	9.20E-01	1.18E+00	S	S	S
	9	1.55E+00	2.02E+00	7.88E-01	S	S	S
	10	3.83E+00	2.26E+00	1.42E+00	S	S	S
EM7	1	8.66E-01	7.97E+00	2.39E-01	S	S	S
	2	5.91E-01	1.11E+01	1.48E+00	S	S	S
	3	1.39E+00	1.18E+01	9.39E-01	S	S	S
	4	7.72E-01	7.47E+00	1.39E+00	S	S	S
	5	8.90E-01	6.84E+00	1.00E+00	S	S	S
	6	6.93E-01	5.57E+00	1.48E+00	S	S	S
	7	6.30E-01	5.06E+00	9.70E-01	S	S	S
	8	2.36E-01	4.56E+00	1.33E+00	S	S	S
	9	6.22E-01	4.94E+00	1.27E+00	S	S	S
	10	7.72E-01	6.58E+00	9.70E-01	S	S	S

Table 5.3. continued

Mouse	Isolate	Efficiency of Plating			Resistance		
		ICP1	ICP2	ICP3	ICP1	ICP2	ICP3
12h Prophylaxis							
CM3	1	1.25E+00	6.86E-01	8.64E-01	S	S	S
	2	7.95E-01	1.10E+00	1.37E+00	S	S	S
	3	1.64E+00	1.18E+00	1.14E+00	S	S	S
	4	1.75E+00	1.10E+00	1.14E+00	S	S	S
	5	1.82E+00	1.03E+00	1.14E+00	S	S	S
	6	8.86E-01	7.64E-01	1.14E+00	S	S	S
	7	1.75E+00	7.55E-01	1.17E+00	S	S	S
	8	3.48E+00	7.09E-01	1.02E+00	S	S	S
	9	3.98E+00	6.91E-01	1.05E+00	S	S	S
	10	1.00E+00	9.18E-01	7.63E-01	S	S	S
CM4	1	8.39E-01	9.79E-01	1.63E+00	S	S	S
	2	5.36E-01	7.02E-01	1.45E+00	S	S	S
	3	1.16E+00	7.11E-01	1.15E+00	S	S	S
	4	1.23E+00	7.19E-01	1.03E+00	S	S	S
	5	8.21E-01	5.54E-01	1.40E+00	S	S	S
	6	1.25E+00	7.36E-01	1.80E+00	S	S	S
	7	1.86E-01	7.27E-01	1.40E+00	S	S	S
	8	9.03E-01	5.87E-01	1.78E+00	S	S	S
	9	2.04E-01	6.36E-01	1.20E+00	S	S	S
	10	5.18E-01	6.86E-01	1.05E+00	S	S	S
CM7	1	1.22E+00	1.09E+01	1.02E+00	S	S	S
	2	2.44E+00	1.99E+01	1.42E+00	S	S	S
	3	1.19E+00	5.87E-01	9.32E-01	S	S	S
	4	1.66E+00	1.19E+01	1.31E+00	S	S	S
	5	8.89E-01	5.78E+00	6.78E-01	S	S	S
	6	1.31E+00	7.59E+00	6.10E-01	S	S	S
	7	4.63E+00	7.24E+00	8.31E-01	S	S	S
	8	4.03E+00	3.97E+00	1.19E+00	S	S	S
	9	2.88E+00	6.69E-01	1.10E+00	S	S	S
	10	1.38E+00	8.19E+00	1.25E+00	S	S	S

Table 5.3. continued

Mouse	Isolate	Efficiency of Plating			Resistance		
		ICP1	ICP2	ICP3	ICP1	ICP2	ICP3
12h prophylaxis							
CM9	1	1.08E+00	2.02E+00	5.83E-01	S	S	S
	2	8.60E-01	4.88E-01	3.11E+00	S	S	S
	3	7.53E-01	9.60E-01	1.12E+00	S	S	S
	4	8.60E-01	7.57E-01	6.80E-01	S	S	S
	5	1.29E+00	1.23E+00	5.15E-01	S	S	S
	6	1.12E+00	2.02E+00	5.53E-01	S	S	S
	7	6.02E-01	6.78E-01	7.67E-01	S	S	S
	8	7.96E-01	7.02E-01	8.81E-01	S	S	S
	9	7.63E-01	7.27E-01	1.10E+00	S	S	S
	10	8.92E-01	8.18E-01	8.98E-01	S	S	S
CM10	1	1.44E+00	4.07E-01	3.67E-01	S	S	S
	2	1.44E+00	3.23E-01	1.18E+00	S	S	S
	3	1.50E+00	3.43E-01	8.48E-01	S	S	S
	4	1.69E+00	3.80E-01	1.45E+00	S	S	S
	5	1.72E+00	4.87E-01	1.18E+00	S	S	S
	6	2.66E+00	3.20E-01	1.06E+00	S	S	S
	7	1.56E+00	3.67E-01	8.79E-01	S	S	S
	8	1.41E+00	3.30E-01	1.09E+00	S	S	S
	9	1.53E+00	2.73E+00	1.27E+00	S	S	S
	10	1.53E+00	1.30E+00	9.39E-01	S	S	S
24h Prophylaxis							
AM1	1	8.67E+00	7.19E+00	1.10E+00	S	S	S
	2	1.16E+00	1.61E+00	1.49E+00	S	S	S
	3	1.71E+00	1.89E+00	1.10E+00	S	S	S
	4	1.20E+00	5.26E+00	1.04E+00	S	S	S
	5	1.20E+00	2.18E+00	8.81E-01	S	S	S
	6	1.53E+00	1.84E+00	7.16E-01	S	S	S
	7	1.89E+00	2.67E+00	5.97E-01	S	S	S
	8	1.40E+00	6.32E+00	8.96E-01	S	S	S
	9	1.22E+00	7.02E+00	8.06E-01	S	S	S
	10	1.44E+00	2.32E+01	8.81E-01	S	S	S

Table 5.3. continued

Mouse	Isolate	Efficiency of Plating			Resistance		
		ICP1	ICP2	ICP3	ICP1	ICP2	ICP3
24h Prophylaxis							
AM2	1	9.53E-01	1.05E+00	1.27E+00	S	S	S
	2	5.19E+00	1.05E+00	1.12E+00	S	S	S
	3	3.77E+00	1.25E+00	1.18E+00	S	S	S
	4	1.48E+00	1.10E+00	9.40E-01	S	S	S
	5	6.89E-01	1.10E+00	7.76E-01	S	S	S
	6	7.45E-01	7.91E-01	9.85E-01	S	S	S
	7	1.23E+00	1.03E+00	6.27E-01	S	S	S
	8	9.53E-01	1.42E+00	7.76E-01	S	S	S
	9	1.26E+00	6.18E-01	9.40E-01	S	S	S
	10	7.92E-01	5.64E-01	8.51E-01	S	S	S
DM1	1	3.45E-07	1.67E+00	1.00E-06	PS	S	R
	2	1.79E+00	1.67E+00	7.16E-01	S	S	S
	3	1.82E+00	3.71E+00	9.50E-01	S	S	S
	4	1.17E+00	3.33E+00	1.00E-06	S	S	R
	5	1.65E+00	1.29E+00	7.89E-01	S	S	S
	6	8.16E-01	6.50E-01	7.44E-01	S	S	S
	7	1.10E+00	TURBID*	7.37E-01	S	PS	S
	8	1.72E-05	2.08E+00	1.00E-06	PS	S	R
	9	1.82E+00	2.54E-01	1.00E-06	S	S	R
	10	1.06E+00	1.96E+00	1.03E+00	S	S	S
DM2	1	1.04E+00	5.29E-01	1.03E+00	S	S	S
	2	1.20E+00	8.97E-01	9.88E-01	S	S	S
	3	1.59E+00	TURBID*	8.26E-01	S	PS	S
	4	2.45E+00	3.21E+00	6.63E-01	S	S	S
	5	8.78E-01	1.17E+00	7.09E-01	S	S	S
	6	9.39E-01	8.75E-01	9.07E-01	S	S	S
	7	1.14E+00	2.17E+00	8.02E-01	S	S	S
	8	8.98E-01	1.96E+00	8.14E-01	S	S	S
	9	9.39E-01	3.71E-01	7.79E-01	S	S	S
	10	9.80E-01	1.04E+00	1.01E+00	S	S	S

Table 5.3. continued

Mouse	Isolate	Efficiency of Plating			Resistance		
		ICP1	ICP2	ICP3	ICP1	ICP2	ICP3
24h Prophylaxis							
DM3	1	1.41E+00	1.22E+00	9.50E-01	S	S	S
	2	6.94E-01	8.15E-01	1.63E+00	S	S	S
	3	1.96E+00	1.15E+00	7.25E-01	S	S	S
	4	1.16E+00	5.89E-01	1.65E+00	S	S	S
	5	6.12E-01	6.22E-01	9.75E-01	S	S	S
	6	1.20E+00	4.78E-01	8.75E-01	S	S	S
	7	5.92E-01	4.67E-01	7.75E-01	S	S	S
	8	4.41E-01	1.07E+00	3.65E-01	S	S	S
	9	1.04E+00	4.11E-01	3.33E-01	S	S	S
	10	6.12E-01	3.11E-01	3.18E-01	S	S	S
DM4	1	1.22E+00	1.00E-06	3.78E-01	S	R	S
	2	9.59E-01	9.63E-01	8.65E-01	S	S	S
	3	1.27E+00	1.04E+00	8.92E-01	S	S	S
	4	1.02E+00	4.30E-01	9.73E-01	S	S	S
	5	1.14E+00	1.00E-06	8.92E-01	S	R	S
	6	5.92E-01	2.44E-01	8.92E-01	S	S	S
	7	1.00E+00	4.26E-01	1.92E-01	S	S	S
	8	7.19E-01	8.52E-01	1.00E+00	S	S	S
	9	7.02E-01	1.00E-06	1.57E-01	S	R	S
	10	8.57E-01	1.00E-06	3.41E-01	S	R	S
DM5	1	1.83E+00	7.19E-01	7.40E-01	S	S	S
	2	4.83E-05	2.98E+00	TURBID*	PS	S	PS
	3	1.00E+00	5.21E-01	8.08E-01	S	S	S
	4	1.08E+00	1.69E-01	7.26E-01	S	S	S
	5	1.05E+00	1.00E-06	6.71E-01	S	R	S
	6	2.41E+00	1.00E-06	4.25E-01	S	R	S
	7	3.76E+00	1.00E-06	4.11E-01	S	S	S
	8	5.03E+00	9.57E-01	5.48E-01	S	S	S
	9	2.48E-05	2.91E+00	1.00E-06	PS	S	R
	10	5.03E+00	1.10E+00	6.58E-01	S	S	S

Table 5.3. continued

Mouse	Isolate	Efficiency of Plating			Resistance		
		ICP1	ICP2	ICP3	ICP1	ICP2	ICP3
24h Prophylaxis							
DM6	1	1.22E+00	1.00E-06	4.37E-01	S	R	S
	2	9.74E-01	1.00E-06	6.34E-01	S	R	S
	3	1.04E+00	9.77E-01	7.04E-01	S	S	S
	4	1.54E+00	1.00E-06	4.08E-01	S	R	S
	5	1.00E+00	1.00E-06	4.37E-01	S	R	S
	6	3.03E-01	1.00E-06	4.69E-01	S	R	S
	7	8.25E-01	1.41E-02	6.06E-01	S	PS	S
	9	6.67E-01	1.53E-02	7.04E-01	S	PS	S
	10	1.83E-01	1.00E-06	6.48E-01	S	R	S
DM7	1	7.85E+00	1.34E+00	1.61E+00	S	S	S
	2	1.35E+00	1.30E+00	1.68E+00	S	S	S
	3	5.08E+00	1.05E+00	1.13E+00	S	S	S
	4	6.46E+00	1.19E+00	1.09E+00	S	S	S
	5	5.47E+00	1.27E+00	7.79E-01	S	S	S
	6	3.70E+00	1.00E-06	6.95E-01	S	R	S
	7	7.57E-01	1.13E+00	7.58E-01	S	S	S
	8	1.81E+00	1.94E+00	4.74E-01	S	S	S
	9	1.78E+00	9.81E-01	3.33E+00	S	S	S
	10	7.84E-01	1.00E-06	3.94E+00	S	R	S
DM8	1	1.76E+00	6.79E-01	1.15E+00	S	S	S
	2	1.43E+00	1.11E+00	1.00E+00	S	S	S
	3	1.65E+00	1.17E+00	8.84E-01	S	S	S
	4	1.16E+00	1.19E+00	7.58E-01	S	S	S
	5	1.97E+00	1.34E+00	7.16E-01	S	S	S
	6	1.68E+00	1.25E+00	8.21E-01	S	S	S
	7	1.76E+00	7.55E-01	5.79E-01	S	S	S
	8	8.92E-01	1.15E+00	8.32E-01	S	S	S
	9	1.62E+00	7.55E-01	4.74E-01	S	S	S
	10	1.76E+00	9.06E-01	6.00E-01	S	S	S

Resistance Class	Length of Prophylaxis		
	6h (30)	12h (50)	24h (98)
S to all ICP	30 (100%)	50 (100%)	79 (81%)
R to ICP1 only	-	-	-
R to ICP2 only	-	-	11 (11%)
R to ICP3 only	-	-	1 (1%)
PS to ICP1 only	-	-	2 (2%)
ICP1 (PS) and ICP3 (PS)	-	-	1 (1%)
ICP1 (PS) and ICP3 (R)	-	-	4 (4%)
ICP1 (R) and ICP3 (PS)	-	-	-
R to all ICP	-	-	-

Table 5.4. Phage resistance of surviving *V. cholerae* isolates from phage prophylaxis in the infant mouse

Summary of the phage resistance phenotypes of isolates that survived the phage prophylaxis treatment shown in Fig. 5.4. Resistance was measured by efficiency of plating assays. Isolates were described as resistant (R) when EOP = 1 x 10^{-6}, sensitive (S) when EOP = 1, and partially sensitive (PS) when plaques appeared turbid or when the EOP was between 1 x 10^{-1} and 1 x 10^{-5}. The data presented refer to the number of isolates displaying a phenotype out of the total number of isolates tested for each time-point.

To uncover the genetic basis for phage resistance, 24 isolates of varying phage resistance phenotypes were analyzed by whole-genome sequencing followed by variant analysis. Our lab has shown previously that slipped-strand mispairing within three poly-A tracts of O1 antigen biosynthesis genes may result in abnormal O1 antigen production and confer ICP1 resistance (Seed et al., 2012). In agreement, mutations in ICP1- and ICP3-resistant isolates were found in O1 antigen biosynthesis genes located on chromosome 1 of the *V. cholerae* N16961 genome between open reading frames VC0240 (*gmhD*) and VC0264 (*rjg*) (Chatterjee & Chaudhuri, 2004) (Table 5.5). Our results show that ICP1 and ICP3 escape mutants occur at low frequency in prophylactically treated and challenged infant mice. When they do occur, however, O1 antigen mutations dominate. This is not of major concern given that strains carrying such mutations have previously been shown to be avirulent (Seed et al., 2012). ICP2 escape mutants were more common, occuring in 11% of isolates from the 24-hour time point. In Chapter 4, we have shown that mutations in *ompU* and *toxR* confer ICP2 resistance. Consistent with these findings, mutations in ICP2-resistant isolates were identified in open reading frames VC0633 (*ompU*) or VC0984 (*toxR*). Eight of the sequenced strains revealed no mutations to explain their phage-resistance phenotypes. This is most likely due to incomplete genome coverage during sequencing.

5.2.6. Phages are retained in the infant rabbit small intestine up to 24 hours

To further assess the ability of the ICP cocktail to prevent cholera, phage prophylaxis experiments were performed with the infant rabbit model. In contrast to the infant mouse model, infant rabbits develop the profuse secretory diarrhea associated with cholera. We first determined whether the ICP phages could be retained in the rabbit intestinal tract in the absence of their *V. cholerae* host. Phages were administered orogastrically, and infant rabbits were dosed with approximately 3 x 10^8 PFU of the ICP cocktail, with each ICP phage present in equal numbers. After 3 or 24 hours, animals were sacrificed and the intestines homogenized to enumerate the phages retained (Figure 5.6). At 3 hours, the phage titer decreased to approximately 6 x 10^7 PFU, with a retention rate of 20%. After 24 hours, approximately 8 x 10^5 PFU could still be detected in the intestine. We hypothesized this amount of phage would be sufficient in preventing cholera symptoms when infant rabbits are challenged with *V. cholerae*.

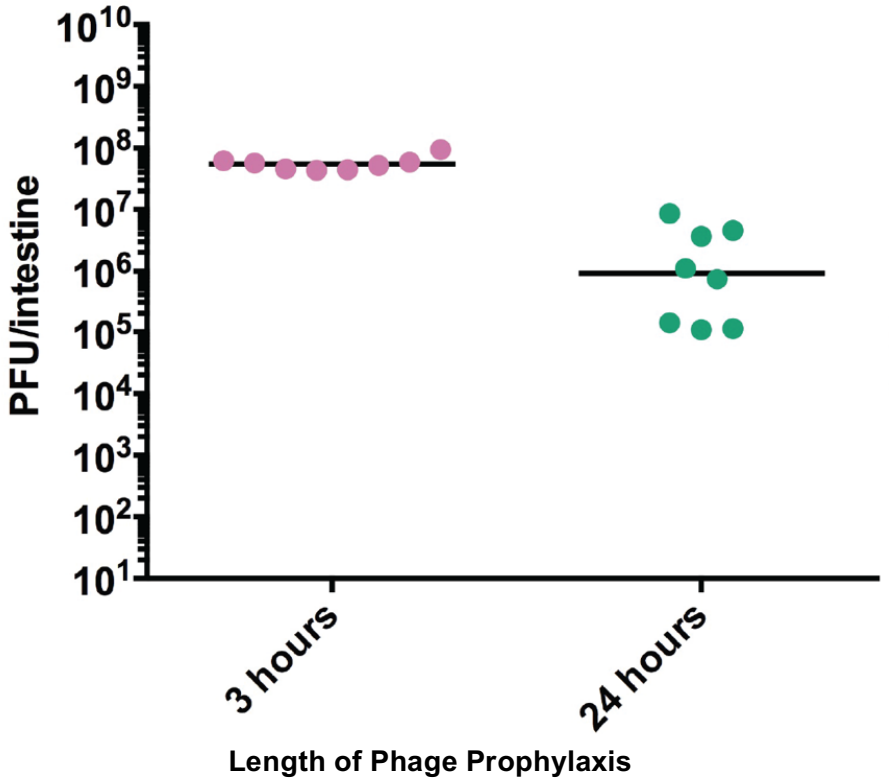
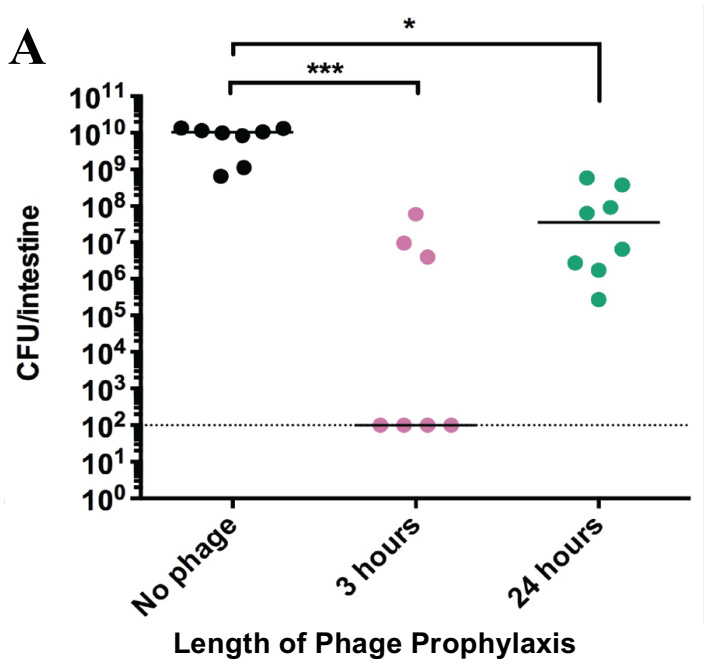


Figure 5.6. ICP phages are retained in the rabbit intestinal tract

Infant rabbits were orogastrically dosed with ca. 3 x 10^8 PFU of the ICP cocktail, with each ICP phage in equal numbers. Animals were sacrificed either 3 (pink) or 24 (green) hours after dosing, and the intestinal tracts were homogenized. Phage retention was measured in the absence of the *V. cholerae* E7946 host by enumerating phage as PFU/intestine using plaque assays. The limit of detection is 10 phages per intestine, and the horizontal solid bars represent the medians. Each shape represents one animal.

5.2.7. The ICP cocktail prevents cholera symptoms in rabbits when administered up to 24 hours prior to challenge

To determine if the ICP cocktail could protect rabbits from developing cholera symptoms, phages were orogastrically administered to two groups of infant rabbits either 3 or 24 hours prior to challenge with 5 x 10^8 CFU *V. cholerae*, with MOIs ranging from 2 to 10. A control group did not receive phage. Animals were monitored for symptoms of cholera, specifically weight loss and the presence of secretory diarrhea. In line with previously published observations (Abel & Waldor, 2014), the no-phage control group showed cholera-like symptoms 12 to 14 hours post-infection, had substantial weight loss, and were subsequently euthanized. Each rabbit had a distended cecum, which accumulated approximately 0.5 to 1 ml of fluid, a symptom of *V. cholerae* infection. Approximately 10^9 to 10^{10} CFU were detectable from cecal fluid (data not shown) and intestinal homogenates (Figure 5.7A) from this group.


A
*
***
CFU/intestine
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
No phage
3 hours
24 hours
Length of Phage Prophylaxis

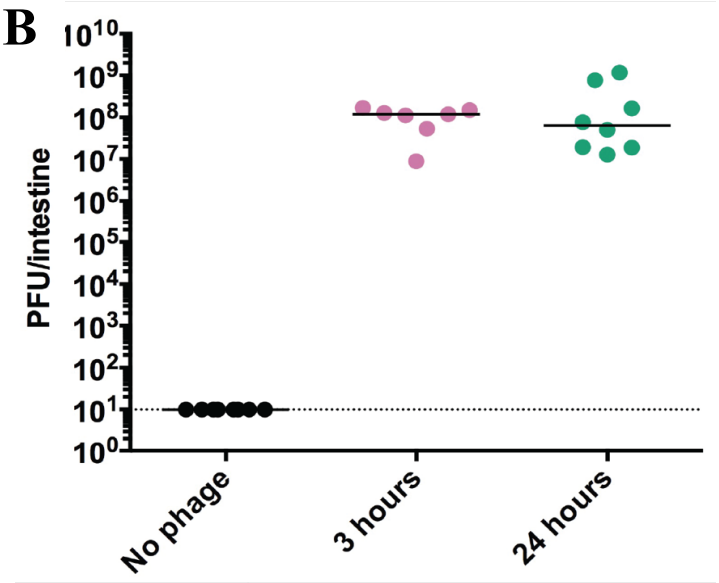

B
PFU/intestine
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
No phage
3 hours
24 hours
Length of Phage Prophylaxis

Figure 5.7. The ICP cocktail reduces *V. cholerae* burden when administered up to 24 hours prior to challenge in the infant rabbit model.

Infant rabbits were orogastrically dosed with the ICP cocktail either 3 (pink) or 24 (green) hours prior to challenge with 5 x 10^8 CFU *V. cholerae* E7946 for MOIs from 2 to 10. A control group did not receive phage (black). At the end of the infection period (either 14 or 20 hours after *V. cholerae* challenge), rabbits were sacrificed, and the intestinal tracts were homogenized. (a) *V. cholerae* was enumerated for CFU/intestine by plating onto LB plates supplemented with 100 μg/ml streptomycin. (b) Phages were enumerated for PFU/intestine by plaque assay. Dotted lines indicate the limit of detection, and the horizontal solid bars represent the medians. Each shape represents one animal. Significance was calculated using the Kruskal-Wallis and Dunn's post-hoc multiple comparisons tests. *, $p < 0.1$; ***, $p < 0.001$.

In the 3-hour prophylaxis group, bacteria could not be detected for 4 out of the 7 rabbits, indicating these animals were cleared of the *V. cholerae* infection (Figure 5.7A). In the remaining 3 rabbits, there was a 10- to 100,000-fold decrease in bacterial load in comparison to the non-treated group ($p < 0.001$). In the 24-hour group, there was a similar decrease in magnitude of 10- to 100,000-fold in all treated rabbits when compared to those that did not receive phage ($p < 0.05$). Despite the observed bacterial load, there was no evidence of any significant weight loss or secretory diarrhea from rabbits in either of the phage-dosed groups up to 20 hours after *V. cholerae* challenge (Figure 5.8). Upon dissection of the phage-treated animals, only one rabbit in the 24-hour group showed cecal fluid accumulation of approximately 0.1 ml; the other animals did not have cecal fluid. In addition, approximately 10^6 to 10^9 PFU was enumerated from the intestinal homogenates of the phage-treated animals (Figure 5.7B), suggesting the ICP phages were retained persisted in the intestinal tract. These results suggest that the ICP cocktail successfully protected the infant rabbits from cholera symptoms over the course of the experiment.

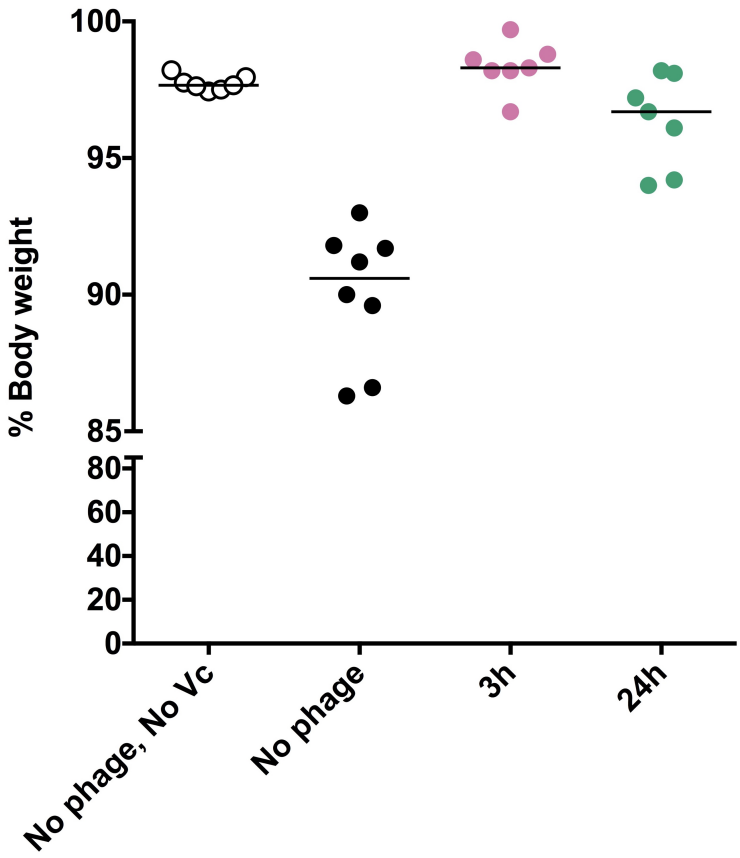


Figure 5.8. ICP cocktail prophylaxis prevents secretory diarrhea in the infant rabbit

Weight loss was calculated when animals from Figure 7 were euthanized. Animals that received only *V. cholerae* were euthanized when 10-15% of the body weight was lost, according to IACUC protocols. Phage-treated animals were euthanized at 20 hours post-*V. cholerae* challenge. The horizontal solid bars represent the medians; Each shape represents one animal. No significance was calculated due to the difference in endpoints for each group.

5.2.8. Phage resistance phenotypes and corresponding mutations of surviving V. cholerae isolates from rabbits

Similar to the mouse experiments, it was possible to isolate surviving *V. cholerae* from the intestines of rabbits treated with ICP cocktail prophylactically. To determine the phage resistance phenotype of these bacteria, *V. cholerae* isolates were colony-purified and used in EOP assays with each of the ICP phages (Table 5.6). No isolates were resistant to all three phages, but 39% were resistant to both ICP1 and ICP3 (Table 5.7).

To determine the genetic basis for phage resistance, 36 *V. cholerae* isolates with varying phage resistance phenotypes were submitted for whole-genome sequencing followed by variant analysis. Results were consistent with mutations found in ICP1- and ICP3-resistant isolates from the mouse experiment in Figure 5.4, as there were mutations identified in O1 antigen biosynthesis genes for 33 of the sequenced strains. Similarly, these are not of major concern as we predict these strains would be avirulent (Seed et al., 2012). For the remaining three strains, no mutations related to known phage-resistance strategies were detected in two of them, and no mutations at all were detected in one. This is most likely due to incomplete genome sequence coverage. From the phage resistance data in the two animal models tested, we conclude that the leading source of phage resistance is the mutation of genes required for assembly of the phage receptors.

Table 5.5. Sensitivity of surviving rabbit isolates to ICP phages

S = EOP > 10^{-1}; PS = 10^{-5} < EOP < 10^{-1}; R = EOP < 10^{-6} (limit of detection).
TURBID* = plaques were turbid instead of clear.

Rabbit	Isolate	Efficiency of Plating			Resistance		
		ICP1	ICP2	ICP3	ICP1	ICP2	ICP3
3h Prophylaxis							
N	1	1.00E-06	2.60E-01	1.00E-06	R	S	R
	2	TURBID*	2.97E-01	1.00E-06	PS	S	R
	3	1.46E+00	7.36E-01	1.49E+00	S	S	S
	7	3.81E-05	5.83E-01	3.03E-05	PS	S	PS
	8	1.00E-06	8.38E-01	TURBID*	R	S	PS
	9	1.87E+00	7.00E-01	1.40E+00	S	S	S
P	3	4.30E-06	2.18E-01	1.00E-06	PS	S	R
	4	1.00E-06	6.17E-01	1.00E-06	R	S	R
	5	1.00E-06	1.72E-01	1.00E-06	R	S	R
	7	1.00E-06	1.70E-01	3.45E-05	R	S	PS
	8	1.00E-06	1.72E-01	TURBID*	R	S	PS
	9	5.43E-07	1.45E-01	TURBID*	PS	S	PS
	10	1.00E-06	1.90E-01	1.00E-06	R	S	R
R	1	1.00E-06	1.26E+00	1.00E-06	R	S	R
	2	1.09E+00	1.41E+00	1.27E+00	S	S	S
	4	1.00E-06	8.84E-01	1.00E-06	R	S	R
	5	1.00E-06	7.68E-01	1.80E-01	R	S	S
	8	1.00E-06	9.42E-01	1.00E-06	R	S	R
	9	1.17E+00	2.21E+00	8.00E-01	S	S	S
	10	1.00E-06	TURBID*	1.00E-06	R	PS	R

Table 5.5 continued

Rabbit	Isolate	Efficiency of Plating			Resistance		
		ICP1	ICP2	ICP3	ICP1	ICP2	ICP3
24h Prophylaxis							
A	1	1.00E-06	1.83E+00	1.06E-01	R	S	S
	2	1.00E-06	9.76E-01	1.00E-06	R	S	R
	3	1.00E-06	1.31E+00	1.00E-06	R	S	R
	4	1.00E-06	1.38E+00	1.00E-06	R	S	R
	5	1.00E-06	2.07E+00	1.00E-06	R	S	R
	6	9.21E-01	9.21E-01	2.36E-01	S	S	S
	7	1.00E-06	2.21E+00	1.00E-06	R	S	R
	8	1.00E-06	1.74E+00	1.00E-06	R	S	R
	9	1.00E-06	2.52E+00	TURBID*	R	S	PS
	10	1.00E-06	2.14E+00	1.00E-06	R	S	R
B	1	9.74E-01	1.33E+00	3.45E-01	S	S	S
	2	1.24E+00	1.57E+00	2.91E-01	S	S	S
	4	1.00E-06	1.71E+00	TURBID*	R	S	PS
	6	1.00E-06	2.57E+00	1.00E-06	R	S	R
	8	1.00E-06	2.07E+00	TURBID*	R	S	PS
	9	1.00E-06	2.02E+00	1.00E-06	R	S	R
	10	4.78E+00	2.02E+00	6.44E-01	S	S	S
C	1	2.78E+00	9.15E-01	7.31E-01	S	S	S
	2	4.21E-01	2.26E+00	2.21E-01	S	S	S
	3	1.00E-06	1.53E+00	1.00E-06	R	S	R
	4	1.00E-06	1.45E+00	1.00E-06	R	S	R
	5	1.14E+00	1.09E+00	3.64E-01	S	S	S
	6	1.00E-06	1.47E+00	1.00E-06	R	S	R
	7	8.16E-01	7.79E-01	6.85E-01	S	S	S
	8	1.00E-06	1.68E+00	TURBID*	R	S	PS
	9	2.22E+00	1.49E+00	3.11E-01	S	S	S
	10	5.68E-01	1.00E+00	3.70E-01	S	S	S

Table 5.5 continued

Rabbit	Isolate	Efficiency of Plating			Resistance		
		ICP1	ICP2	ICP3	ICP1	ICP2	ICP3
24h Prophylaxis							
D	1	1.00E-06	1.43E+00	TURBID*	R	S	PS
	2	1.00E-06	3.93E+00	1.00E-06	R	S	R
	3	1.00E-06	1.30E+00	1.00E-06	R	S	R
	4	1.00E-06	1.17E+00	1.00E-06	R	S	R
	5	1.00E-06	6.98E-01	1.00E-06	R	S	R
	6	1.00E-06	1.89E+00	1.00E-06	R	S	R
	7	1.00E+00	1.04E+00	1.00E+00	S	S	S
	8	1.00E-06	1.74E+00	1.00E-06	R	S	R
	9	TURBID*	2.30E+00	TURBID*	PS	S	PS
	10	1.00E-06	5.28E-01	1.00E-06	R	S	R
E	1	1.78E+00	8.57E-01	1.65E+00	S	S	S
	2	1.49E+00	9.11E-01	1.44E+00	S	S	S
	3	2.35E+00	3.61E-01	1.30E+00	S	S	S
	4	1.00E-06	8.93E-01	TURBID*	R	S	PS
	5	1.00E-06	1.00E+00	1.00E-06	R	S	R
	6	1.00E-06	3.07E-01	TURBID*	R	S	PS
	7	2.10E+00	1.43E+00	1.71E+00	S	S	S
	8	1.18E-04	1.28E+00	6.21E-01	PS	S	S
	9	1.00E-06	1.16E-01	1.00E-06	R	S	R
	10	3.54E-01	6.07E-01	2.15E-01	S	S	PS
F	1	TURBID*	8.21E-01	TURBID*	PS	S	PS
	2	1.16E+00	3.16E-01	3.80E-01	S	S	S
	3	1.00E-06	7.14E-01	TURBID*	R	S	PS
	4	8.95E-01	5.36E-01	3.00E-01	S	S	S
	5	1.00E-06	1.76E-01	1.00E-06	R	S	R
	6	1.00E-06	3.59E-01	2.00E-01	R	S	S
	8	1.00E-06	3.07E-01	1.00E-06	R	S	R
	9	1.00E-06	5.36E-01	1.00E-06	R	S	R
	10	7.21E-01	3.02E-01	2.33E-01	S	S	S

Table 5.5 continued

Rabbit	Isolate	Efficiency of Plating			Resistance		
		ICP1	ICP2	ICP3	ICP1	ICP2	ICP3
24h Prophylaxis							
H	1	1.00E-06	3.28E-01	1.06E-01	R	S	S
	2	1.11E+00	9.79E-01	1.25E+00	S	S	S
	3	6.67E-01	1.16E+00	1.25E+00	S	S	S
	4	1.00E-06	3.63E-01	1.00E-06	R	S	R
	5	1.00E-06	4.56E-01	1.00E-06	R	S	R
	6	7.00E+00	5.15E-01	1.28E+00	S	S	S
	7	9.70E-01	2.11E-01	1.45E+00	S	S	S
	8	TURBID*	3.73E-02	TURBID*	PS	PS	PS
	9	6.11E+00	9.57E-01	1.49E+00	S	S	S
	10	1.10E+00	2.65E-01	1.19E+00	S	S	S
J	1	7.28E-01	5.00E-01	9.73E-01	S	S	S
	2	8.45E-01	3.58E-01	8.78E-01	S	S	S
	3	2.91E+00	3.43E-01	7.57E-01	S	S	S
	4	1.00E-06	3.09E-01	1.00E-05	R	S	R
	5	4.66E-01	3.19E-01	8.51E-01	S	S	S
	6	5.44E+00	2.40E-01	1.56E+00	S	S	S
	7	1.00E-06	2.75E-01	TURBID*	R	S	PS
	8	1.00E-06	4.46E-01	1.00E-06	R	S	R
	9	1.00E-06	2.25E-01	1.00E-06	R	S	R
	10	1.00E-06	3.87E-01	1.00E-06	R	S	R

Resistance Class	Length of Prophylaxis	
	3h (20)	24h (76)
S to all ICP	4 (20%)	30 (39%)
R to ICP1 only	1 (5%)	2 (3%)
R to ICP2 only	-	-
R to ICP3 only	-	-
ICP1 (PS) and ICP3 (PS)	2 (10%)	2 (3%)
ICP1 (PS) and ICP3 (R)	2 (10%)	-
ICP1 (R) and ICP3 (PS)	3 (15%)	8 (11%)
ICP1 (R) and ICP3 (R)	7 (35%)	30 (39%)
R to all ICP	-	-

Table 5.6. Phage resistance of surviving *V. cholerae* isolates from phage prophylaxis in the infant rabbit

Summary of the phage resistance phenotypes of isolates that survived the phage prophylaxis treatment shown in Fig. 4. Resistance was measured by efficiency of plating assays. Isolates were described as resistant (R) when EOP = 1 x 10^{-6}, sensitive (S) when EOP = 1, and partially sensitive (PS) when plaques appeared turbid or when the EOP was between 1 x 10^{-1} and 1 x 10^{-5}. The data presented refer to the number of isolates displaying a phenotype out of the total number of isolates tested for each time-point.

5.3. Conclusions

In this chapter, we have demonstrated that the ICP phage cocktail is efficient at killing *V. cholerae* both *in vitro* and *in vivo* in two animal models. It reduces or prevents *V. cholerae* colonization of the small intestine and provides protection against the onset of cholera symptoms in the infant rabbit model. In addition, *V. cholerae* isolates shed by phage-treated animals that are resistant to the ICP phages as a result of mutations in the receptor genes are expected to be avirulent. To our knowledge, this study is the first to show that prophylactic administration of a phage cocktail is protective against cholera. Current phage therapy research is focused on treating ongoing infections. Our study highlights the potential of phage therapy in prophylaxis, specifically for at-risk individuals. Phage prophylaxis for mucosal pathogens represents a fast and specific strategy for restricting the impact of bacterial infections on human health.

Chapter 6: Discussion and future directions

In recent years, there has been a great deal of interest in the interaction between *V. cholerae* and its virulent phages. Numerous studies have focused on isolating phages from the environment to understand their basic biology for potential applications in phage therapy. Faruque and colleagues have hypothesized that phages may play a role in tempering the severity of cholera epidemics (Faruque, Islam, et al., 2005; Jensen et al., 2006). A previous paper from the Camilli Lab reported the association of virulent Vibriophages ICP1, ICP2, and ICP3 with Bangladeshi cholera patient rice-water stool samples (Seed et al., 2011), and detailed investigations into ICP1 have explored its evolutionary relationship with *V. cholerae* (Seed et al., 2012; Seed et al., 2013). In this thesis dissertation, we further characterize the basic biology of ICP2 and its impact on *V. cholerae* population structure in the context of human infection. In addition, we collaborated with two institutions to study its widespread presence in Haitian clinical samples. Lastly, we report the successful use of a phage cocktail comprising ICP1, ICP2, and ICP3 in preventing *V. cholerae* colonization and the onset of disease symptoms in two animal models. Through investigation of the interaction between *V. cholerae* and its virulent phages, we have demonstrated that phage predation is prevalent during cholera epidemics and may play a critical role in shaping the course of infection within the human host.

Through whole-genome sequencing analysis, we previously identified eight duplication and point mutants of the major outer membrane porin OmpU in clinical *V. cholerae* isolates from Bangladesh and Haiti that were ICP2-resistant. Using plaque assay and Western blotting, we demonstrated that these *ompU* mutations conferred ICP2 resistance. As these mutations map to two predicted extracellular loops, we hypothesized

that ICP2 uses OmpU as its receptor, and the initial interaction between the phage tail fibers and OmpU may be disrupted by these mutations.

We had previously attempted to verify this model using plaque inhibition assays. Using synthesized peptides of the two loops, we immunized mice in order to obtain polyclonal antibodies for use in inhibiting ICP2 binding to OmpU during plaque assays. This approach was unsuccessful, most likely due to differences in the conformation of the extracellular loops by themselves as peptides and the folded structure of OmpU embedded in the outer membrane of *V. cholerae.* We then attempted using outer membrane vesicles (OMVs) extracted from wild-type *V. cholerae* and various OmpU mutants to block ICP2 plaque formation. Although there appeared to be some degree of inhibition, results were inconsistent.

Previous reports have shown direct interactions between phages and outer membrane protein receptors. For example, the *Shigella flexneri* phage Sf6 uses OmpA and OmpC to initiate infection. Casjens et al. (2004) and Zhao et al. (2011) show that OmpA and OmpC proteins co-purify with Sf6 phages, even after cesium chloride purification. Parent et al. (2012) then demonstrated that OMVs that had OmpA and OmpC were attached to Sf6 tails. To show a direct interaction, Parent et al. (2014) presented cryo-transmission electron microscopy images that show a decreased association of $OmpA^-/OmpC^-$ OMVs with Sf6 in comparison to wild-type OMVs. Similar methods could be employed to show the direct association of OmpU with ICP2 tail fibers. Polyclonal antibodies against OmpU can also be used in plaque inhibition assays.

Using a number of *in vitro* assays, we demonstrated that the OmpU point mutants were functional in bile and pond water survival. They also retained competitive fitness in

comparison with wild-type when tested in the infant mouse model. A search of the 39 genome sequences of clinical *V. cholerae* El Tor strains publicly available revealed that the wild-type extracellular L4 and L8 loop sequences were conserved in 37 genomes. The remaining two sequences are the genomes of the OmpU mutants we entered into the database from patient rice-water stools that had ICP2 present. These results imply that the *ompU* mutant alleles do not become fixed in the *V. cholerae* population. Our *in vitro* competitions in LB broth demonstrated a slight fitness defect for two OmpU mutants after 58 generations, which may be due to the general role OmpU has in growth.

As mentioned in the introduction, there is conflicting literature as to whether or not OmpU is truly a virulence factor. Provenzano et al. showed that Δ*ompU* strains retained competitive fitness in the infant mouse model (Provenzano et al., 2001), and transposon mutant library analyses of *V. cholerae* also present contradicting results (Fu et al., 2013; Kamp et al., 2013; Pritchard et al., 2014). Competitions can be performed between wild-type, Δ*ompU*, and OmpU point and duplication mutants in the infant rabbit model to determine if there is truly a virulence defect. The infant rabbit model is better than the infant mouse model for *V. cholerae* pathogenesis and may reveal a fitness defect in OmpU mutants that was not detected in previous assays.

Whole-genome sequencing analyses of ICP2-resistant clinical isolates also identified a number of null mutations in *toxR*. Through plaque assay and Western blotting, we demonstrated that ICP2 resistance was conferred by these mutations and mediated by the loss of OmpU expression on the outer membrane surface. We also showed that these ToxR mutants are attenuated for virulence in the infant mouse model, implying that the selection of these ICP2-resistant mutants most likely occurred late in

infection. This hypothesis can be tested *in vivo* using a competition assay between the ToxR mutants and wild-type in the presence of ICP2. Instead of measuring the competitive indicies and phage levels at the end of 24 hours, *V. cholerae* and phage population dynamics can be monitored throughout the infection and assayed at various time points. A similar experiment could be conducted using only wild-type or wild-type in competition with OmpU mutants. This would reveal more details about the kinetics of phage infection within the small intestine throughout infection.

We have demonstrated that ICP2 predation selects for the OmpU mutant in the infant rabbit model, the first reported evidence supporting the concept that phage predation can alter *V. cholerae* population structure during the course of infection. Interestingly, we also isolated ICP2 mutants from the *in vivo* experiment that were able to infect the OmpU mutant. Preliminary whole-genome analyses of these phage mutants suggest that point mutations in the predicted tail fiber genes may allow for increased promiscuity in host range. Interestingly, these ICP2 mutants were as capable as wild-type phage at infecting wild-type *V. cholerae*. To identify receptor binding proteins for ICP2, we can use a recently developed assay that uses phage genome expression libraries and protein screens (Simpson, Sacher, & Szymanski, 2016). Recombinant ICP2 tail fiber mutants can then be generated using the aforementioned methods in Chapter 1 and then tested for their ability to infect a variety of OmpU mutants. By identifying ICP2 mutant and OmpU mutant pairings, we can biochemically characterize the initial stages of ICP2 infection. This work will be continued in collaboration with Andrea Wong, a graduate student in the Camilli Lab.

It may be possible to isolate ICP2 mutants in the original rice-water stool samples from which the OmpU mutants were identified. Plaque assays using the OmpU mutants as host strains can be performed, and plaque PCR can be used to screen plaques for the prevalence of ICP2 mutants. If there is a relatively high level of ICP2 mutants in the stool samples in comparison to wild-type ICP2, it is possible that multiple rounds of phage replication in parallel with *V. cholerae* proliferation allows for the selection of ICP2 mutants. This would suggest that the arms race between *V. cholerae* and ICP2 can occur rapidly within an individual during the progression of disease.

We have shown that ICP2, a phage originally isolated from Bangladeshi clinical samples, is widespread in Haitian clinical samples from 2012 to 2014. Attempts have been made to isolate ICP2 from clinical samples that were collected earlier in the epidemic. Four rice-water stools from March 2011 were assayed for phage by directly titering the thawed clinical sample and by overnight enrichment with E7946 or its rough mutant. Both methods did not reveal the presence of phage. Total genomic DNA can be extracted from the clinical sample and probed with diagnostic PCR primers for the presence of phage. If there are positive results, it would not be difficult to extract the phage genome. Such a result would show that ICP2 was present early on in the cholera epidemic in Haiti. Attempts to acquire other clinical samples collected before 2012 have been difficult as many institutions save the *V. cholerae* isolates rather than an aliquot of the rice-water stool.

Based on the high degree of similarity among the Haitian ICP2 isolates and the similarity to Bangladeshi ICP2 isolates (Seed et al., 2014), we hypothesize that there was a single-source introduction of ICP2 in Haiti. We have attempted to calculate a molecular

clock to date the ancestral Haitian ICP2. The molecular clock hypothesis states that DNA and protein sequences evolve at a relatively constant rate over time. This hypothesis is controversial, but evolutionary biologists have relaxed the model to avoid an overly simplistic definition. There are two types of relaxed molecular clock models. The first allows for the rate to vary over time, but the variation centers around an average value. The second allows the rate to evolve over time, assuming the rate of evolution is tied to other biological characteristics known to undergo evolution (Ho, 2008).

For molecular clock analysis, we must first determine if there is a phylogenetic signal and if there is recombination among the ICP2 genomes in question. Using the TreePuzzle software (Schmidt, Strimmer, Vingron, & von Haeseler, 2002), we have determined the presence of a phylogenetic signal. Future steps will include testing the molecular clock assumption by inputting the maximum likelihood phylogenetic tree into the TempEst software (Rambaut, Lam, Carvalho, & Pybus, 2016). If the assumption holds, we will calculate the molecular clock using the BEAST software (Drummond, Suchard, Xie, & Rambaut, 2012). It is possible that the temporal scope of our Haitian ICP2 isolates is not wide enough, especially considering a majority of the isolates were collected within three months. Ongoing collaboration with the University of Florida will continue to investigate the prevalence of ICP2 from 2015 onwards. *De novo* assemblies of new ICP2 genomes will be added to increase the likelihood of calculating a molecular clock. Although molecular clock analysis has not been performed on phages of the same species isolated from clinical samples, experimental phylogenetic analyses have been performed using *in vitro* propagation of T7. Sousa et al. (2008) reported that only methods that encompassed a molecular clock recovered the true phylogeny.

It has been strongly suggested that the origin of the Haiti outbreak strain came from Nepal (Chin et al., 2011; Frerichs, Keim, Barrais, & Piarroux, 2012; Katz, 2016). It would be interesting to look for Vibriophages in Nepalese clinical samples from 2010 onward to compare genomes if ICP2 is present. As previously mentioned, however, it may be difficult to obtain rice water stool samples as they are generally not stored.

Another possibility is that ICP2 was not introduced into Haiti; rather, it was in the environment and was able to increase its numbers when epidemic *V. cholerae* was introduced. This model, however, requires that ICP2 is detected in environmental samples, which has not been observed thus far. If ICP2 was isolated from the environment, it would also be difficult to determine the directionality of events. ICP2 could have been present in the environment and preyed upon local, environmental, non-epidemic *V. cholerae*, or ICP2 could have been shed into the environment by cholera patients.

We have demonstrated that there is a high degree of similarity among Haitian ICP2 isolates, although this relatedness does not seem to be associated with geographic location or time of isolation. This may be due to the closing of cholera clinics during 2014, which forced patients to go to clinics that are far from where they ingested *V. cholerae* and ICP2. To truly associate an ICP2 isolate with a geographic location, records would have to indicate the location of the patient's home rather than the clinic to which the patient was admitted.

We have shown that there are six variable regions amongst the Haitian ICP2 isolates, four of which have annotated functions that may impact fitness. The variable region at 18 kb is annotated as the portal protein, which is the central component of the

molecular motor used to package DNA during the assembly process or to eject DNA out of the capsid during bacterial cell infection (Hendrix, 1978). Homologs of the portal protein have been used as a marker to study phage diversity, analogous to the 16S rRNA gene used for microbes although not as universal. The portal protein gene 20 (g20) has been used to study the diversity of *Myoviridae* (Breitbart et al., 2004; Breitbart et al., 2002; DeLong et al., 2006) as it is ubiquitous among T4-like myoviruses. The evolution of g20 most likely is constrained as the mechanism is geometrically precise (Coombs & Eiserling, 1977; Hsiao & Black, 1978). PAP70 and PAP79 are the only two isolates that differ in this region, implying that their variation may impact fitness negatively.

The 34 kb region is annotated as the CobT protein, which has not been observed in a phage genome prior to ICP2 (Seed et al., 2011). CobT is a subunit of the cobalt chelatases, and it plays a role in synthesizing the cofactor cobalamin (vitamin B_{12}) in prokaryotes (Rodionov, Vitreschak, Mironov, & Gelfand, 2003), although it has not been well-characterized in *V. cholerae*. It is possible, however, that ICP2 encodes CobT and CobS to enhance *V. cholerae* metabolism, which would increase the efficacy of its own proliferation. PAP44 is the only ICP2 isolate that varies in this region.

Approximately 2 kilobases are missing near 48 kb in ICP2 isolates PAP02, PAP04, and PAP56. This genomic region is annotated as a class III ribonucleotide reductase (RNRs) that is expressed under anaerobic conditions during T4 infection of *E. coli* (Jordan & Reichard, 1998). It is responsible for enzymatically converting ribonucleotides to deoxyribonucleotides. RNRs are prevalent in environmental viral metagenomes, with class III RNRs present in 28% of the 128 double-stranded phage genomes analyzed by Dwivedi et al. (2013). Class III RNRs are anaerobic enzymes that

are inactivated by oxygen (Nordlund & Reichard, 2006). Liu et al. demonstrated that under oxygen-limiting conditions, *V. cholerae* virulence genes are highly expressed. This may be a thiol-based switch mechanism to sense the intestinal environment (2011). Perhaps ICP2 uses the expression of its Class III RNR to sense that it is in an anaerobic environment and takes advantage of *V. cholerae* when it is increasing its metabolism.

The three regions described thus far can affect the kinetics of infection. To start, single-step growth curves can be measured for ICP2 isolates that vary in these regions to determine if there is any change in the eclipse time or the burst size. Additionally, to test for differences in the kinetics of phage DNA injection, time-lapse fluorescence microscopy can be used to monitor genome ejection at the single virion level (Parent et al., 2014). It would also be interesting to perform RNA-Seq during ICP2 infection to determine the timing of CobT/CobS and class III RNR expression.

The 23 kb region is annotated as the tail fiber protein, and only the UF-CTC isolate is different in this genome segment. It may be that the *V. cholerae* from this clinical sample have a different OmpU to resist ICP2 infection, and UF-CTC has a paired mutated tail fiber protein to counteract it, similar to what we have observed *in vitro* as mentioned previously. The population structure of *V. cholerae* and UF-CTC with regards to OmpU and the tail fiber, respectively, can be assessed by PCR to further elucidate the interaction.

We demonstrated the *in vitro* efficacy of the ICP cocktail in killing *V. cholerae* as well as the *in vivo* efficacy of using the ICP cocktail as phage prophylaxis for up to 24 hours prior to *V. cholerae* challenge in two animal models. The ICP phages were retained in the small intestine of both the infant mouse and the infant rabbit in the absence of *V.*

cholerae up to 24 hours. This is not surprising given that the ICP phages were isolated from clinical samples, where they presumably have evolved to infect *V. cholerae* where its metabolism is the highest. Barr et al. previously proposed a bacteriophage adherence to mucus model that provides a non-host-derived immunity at the mucosal surface. Through metagenomics analysis, the authors identified phage Ig-like domains that can bind to variable glycan residues that coat the mucin glocyprotein component of mucus (Barr et al., 2013). Analysis of the ICP phages, however, did not reveal these Ig-like domains, suggesting a different mechanism of adherence.

Although these proof-of-concept experiments suggest that phages can be used as an effective prophylaxis measure to prevent bacterial infections, there is still much to be done. The ICP cocktail used in these experiments is composed of equal parts of each phage. Based on the single-phage retention experiments in the infant mouse, ICP1 and ICP2 are retained the best while ICP3 drops approximately six orders of magnitude in titer over 24 hours. On the other hand, ICP3 is the most virulent of the three, i.e., it forms larger plaques and was able to reduce the load of *V. cholerae* in infant mice more effectively than ICP1 or ICP2 alone. Therefore, the cocktail formulation should be adjusted to reflect the observed kinetics. For example, there should be an increase in the number of ICP3 phages are in the cocktail to counter the lack of adherence in the small intestine.

In the infant rabbit prophylaxis experiments, there were a few surviving *V. cholerae* isolates. It would be prudent to increase the length of time the animals are monitored to determine whether or not symptoms arise due to the surviving *V. cholerae* population. The phage population should be monitored for changes in titer as well. To

obtain a clearer picture of the kinetics of killing in a cocktail context, the titers for the individual ICP phages should be determined during the course of the prophylaxis experiments. This could be determined by screening plaques by PCR or by performing plaque assays with single-phage resistant *V. cholerae* strains. Total genomic DNA could also be extracted from the small intestine homogenates and subjected to qPCR with diagnostic ICP phage primers. With a longer time course, we will also test the *V. cholerae* population at various time points for phage resistance. We have demonstrated that none of the surviving *V. cholerae* isolates from either animal model developed resistance to all three ICP phages. By using the same methods to monitor the *V. cholerae* population during a longer prophylaxis experiment, we can determine if this result continues to hold true. In addition, the phage resistance we have observed thus far was due to changes in the receptors. It would be interesting to see if there are any other mechanisms of resistance that become prevalent with more rounds of *V. cholerae* and phage proliferation.

The phage resistance pattern of the surviving *V. cholerae* isolates from the rabbits differs from the pattern exhibited by surviving *V. cholerae* isolates from the mouse. None of the *V. cholerae* isolates from the rabbit prophylaxis experiment had resistance to ICP2, whereas ICP2 resistance was frequent in the surviving *V. cholerae* isolates from mice. We had previously determined that clinically relevant point mutations in *ompU*, i.e. those that were found amongst ICP2 escape mutants isolated from human rice-water stool samples, did not result in avirulence in the infant mouse model (Chapter 4), but there are conflicting results as to whether or not OmpU is essential in the infant rabbit model (Fu et al., 2013; Kamp et al., 2013; Pritchard et al., 2014). It is possible that *ompU* mutations

that confer ICP2 resistance do arise during the course of the rabbit prophylaxis experiment; however, these *V. cholerae* strains may be selected against during the infection. Therefore, it is possible that we would not see them in the surviving *V. cholerae* population from phage-treated rabbits.

Another possibility is that the difference is due to the stochastic nature of pre-existing mutants in the inoculum. As these resistance patterns are from one prophylaxis experiment per model, it is possible that repeated experiments will reveal similar phage resistance distributions between the two animals. The results as they stand do not allow for the inference of concrete differences as to how ICP2 resistance affects *V. cholerae* virulence in the animal. As for ICP1 and ICP3 resistance, there are a number of genes that can be mutated that result in an abnormal or truncated O1 antigen. Therefore, the probability of resistance arising to these two phages is quite high, especially in the infant rabbit prophylaxis experiments as the inoculum dose is 1000-fold higher. As mentioned previously, however, O1 antigen mutants are avirulent and likely will be counter-selected during human infection (Seed et al., 2012), and we hypothesize that they are not capable of producing an infection if transmitted to another human host. This is consistent with observations in our lab, as O1 antigen mutants are not found in rice-water stools isolated at the ICDDR,B despite the high prevalence of ICP1.

We tested the ICP cocktail's ability to kill the *V. cholerae* El Tor O1 Ogawa E7946 strain. A panel of more recent circulating epidemic isolates should be tested, especially those from outside Bangladesh. This will determine whether or not phage cocktails should be tailored to the cholera epidemic and given to patients in the geographic region from which the phages are isolated. The O1 El Tor strains of the

ongoing 7th pandemic have evolved over time in three separate waves. The Wave 3 strains isolated from Haiti have recently been reported to be more virulent, have increased motility, and are reduced in the ability to form biofilms (Satchell et al., 2016). The ICP cocktail should be tested on Wave 3 strains to determine efficacy.

It should be determined whether or not the ICP phages are immunogenic. As the phages were isolated from clinical samples, it is reasonable to assume that Bangladeshi people are frequently exposed to them. The route of exposure, however, is through the gastrointestinal tract and thus subject to immune tolerance. Therefore, it is unlikely the ICP phages would travel to the rest of the body to invoke the immune response. A collaboration is currently ongoing with Drs. Jason Harris and Ana Weil at MGH to determine whether there are antibodies in the serum of cholera patients who have any of the ICP phages in their stool. A number of patient samples also have corresponding antibody-secreting cells, where we can investigate whether anti-phage IgA antibodies are secreted. This is perhaps more relevant in the context of infection as IgA antibodies act in the gastrointestinal tract. In addition, if there is an antibody response, it is important to determine whether or not the antibodies can neutralize the phages. Antibodies against the ICP phages should be generated and used in plaque inhibition assays.

The ultimate goal is to use the ICP cocktail as a prophylactic intervention for household contacts of cholera patients. Once the cocktail formulation is finalized, we would ideally proceed to Phase I clinical trials, in which we would collect information on safety, lack of disruption to the gut microbiota, survival of the phages during passage through the gastrointestinal tract, and memory B-cell response to the phages. Those enrolled in the Phase I study would most likely be North American volunteers.

The Nestlé *E. coli* phage cocktail story, however, has demonstrated that volunteers from Bangladesh, where pathogenic *E. coli* is endemic, have differing results. Therefore, we would most likely also conduct a Phase I study with Bangladeshi volunteers as well. Because is it very unlikely we would be able to challenge human volunteers with *V. cholerae*, we would have to rely upon experiments done in the infant mouse and rabbit models to optimize dosage and frequency of ingestion of the phage cocktail for maximal protection from cholera, and we would then scale these to the larger human body size.

Upon getting positive results from Phase I studies, we would proceed to a small-scale Phase II clinical trial, in which the ability of the phage cocktail to limit household transmission is tested in Dhaka, Bangladesh. This is the ideal location for such a study for a number of reasons. Firstly, ICP1, ICP2 and ICP3 are naturally present in this area, having been found in cholera patient rice-water stools, and thus we would not be introducing new phages into the study location. Secondly, there are two reliable cholera epidemics per year in Bangladesh, during which thousands of cholera patients are treated at the ICDDR,B in Dhaka. Thirdly, the ICDDR,B has a rich history of conducting clinical and field trials on enrolled patients and their household members. Because household transmission of cholera is extraordinarily high (15-20%) and with a peak incidence two days after the index case presents, we should be able to show efficacy with good statistical power with perhaps only a hundred households enrolled. In such a field trial, the families of confirmed cholera patients would be enrolled the same day the patient enters the hospital. All members of the household would begin ingesting the phage cocktail daily or perhaps twice daily starting the day

or day after of enrollment. Household members would continue ingesting the phage cocktail for seven days. Data on symptoms and shedding of *V. cholerae,* isolated from rectal swabs, would be collected daily over a period of two weeks, and the incidence of household transmission recorded. Ideally, the results would be compared to a control arm, in which household members not given phage prophylaxis or given heat-killed phage cocktail were monitored in parallel as currently there is no standard-of-care for them.

Concluding Remarks

In this dissertation, we have provided evidence that phage predation can play a critical role within the context of human disease. This dynamic will be further explored with the goal of using phage prophylaxis to decrease the burden of bacterial infections on global health.

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