Inquiry into the hyperinfectivity of *Vibrio cholerae*

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Abstract

Following host passage *Vibrio cholerae* enters a hyperinfectious phase. This transient phase of increased infectivity likely plays an important role in the rapid spread of cholera during outbreaks. As a complex phenotype, hyperinfectivity should be viewed as a collection of host-induced phenotypes that improve the fitness of *V. cholerae* during subsequent infection. To date, neither regulators of the hyperinfectivity nor important characteristics of the phase have been determined. We took a three-pronged approach to studying hyperinfectivity: genetic, proteomic and phenotypic. We used Tn-seq to examine the genes important for hyperinfectivity in the two main animal models; we determined rice water stool-associated proteome, and we determined stress resistance associated with host-passaged *V. cholerae*. Our data indicate that host-passaged *V. cholerae* reduces population diversity in the secondary infection, undergoes changes in susceptibility to stress and contains a unique set of membrane proteins not expressed during growth *in vitro*.

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BS – bile salt mixture
CDC – chenodeoxychoate
DC – deoxycholate
FASP – filter aided sample preparation
GC – glycocholate
GDC – glycodeoxychoate
ICDDR,B – International Center for Diarrheal Disease Research, Bangladesh
LC/MS/MS – 1-dimensional liquid chromatography tandem mass spectrometry
MudPIT – Multidimensional Protein Identification Technology
OMV – outer membrane vesicle
PBS – phosphate-buffered saline
RIVET – recombination-based *in vivo* expression technology
SC – Sodium cholate
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
TA – taurocholate
TCP – toxin-coregulated pilus
Tn-seq – transposon sequencing
WHO – world health organization

> "It travels along the great tracts of human intercourse, never going faster than people travel, and generally much more slowly."
> *John Snow on the Mode of Communication of Cholera p2*

Introduction

History of Pandemic Cholera

As global populations continued to urbanize and travel became easier, a particularly virulent strain of cholera spread from its genesis in the Ganges River across the world in pandemic waves (Pollitzer, 1959). The first recorded pandemic began in 1817 in the Ganges Delta and was carried throughout the subcontinent of India to Myanmar (then Burma), Thailand (Siam), Vietnam, Mauritius, Malaysia and Singapore. By 1821, it had spread to China, Russia, the Philippines, Iran, Iraq, Turkey and Georgia. However, after this impressive march, the disease disappeared from these locations and spread no further. It is thought that this was due to a particularly harsh winter (Burrows and Pollitzer, 1958; McNeill, 2010). In 1829, cholera again began racing around the world, reaching Moscow, the capital of Russia, in the fall of 1830, where failed efforts to contain the disease stoked riots (McNeill, 2010). Cholera finally reached Europe in 1831, affecting Austria, Hungary and Germany, and then traveling to England. From the British Isles, cholera traveled to North America for the first time (Kaper et al., 1995; Pollitzer, 1959; Snow, 1854). Cholera had circled the globe.

It was during the second pandemic, 1829-1849, that cholera fully captured the attention of the European medical community. Cholera was unknown to the vast majority of

Europeans. The medical community was unequipped to treat it. The populace was horrified as healthy people suddenly fell ill with large volumes of vomiting and diarrhea before becoming disoriented, listless and cold, with their faces taking on a sunken appearance, pulses weakening and eventually death. In the 1830's there were advocates for rehydration therapy as a treatment for cholera, with Dr. William Stevens testing an oral salt solution and Dr. Thomas Latta using intravenous saline infusions (Daly and DuPont, 2008). For various reasons, neither of these methods (now the prevailing treatment for cholera) were fully adapted for many years.

In 1849, Dr. John Snow, a 34-year-old London doctor, published the first edition of "On the Mode of the Transmission of Cholera". The data he collected for the 1849 publication was insufficient to prove that cholera was, in fact, transmitted by water, specifically water contaminated with human waste, as opposed to miasmas or 'bad air'. In the second edition, published in 1954, Snow expands on his work using data from the 1853-1854 outbreak tracing the water company, and ultimately the water source, for each death from cholera (Snow, 1854). This work, in combination with the isolation of Robert Koch's 'bacillus' as it was often called, was the final proof that disease could be caused by water contaminated by sewage and that one of Antonie van Leeuwenhoek's animalcules were the cause of cholera (Howard-Jones, 1984; Koch, 1884). This knowledge was the leverage necessary to change policies to focus on sewage treatment, the provision of clean water, personal hygiene and hygienic urban living conditions. Starting in 1858, London began to build a sewage system to divert untreated human waste away from the

Thames River and although it took another 50 years to completely eradicate cholera from Europe and North America, it began with the acceptance of germ theory.

The Seventh Pandemic

Over the years between the sixth and the seventh pandemics, 1923-1961, pandemic cholera retreated from most of the world while continuing to circulate and cause disease in Indonesia. Starting in 1961, cholera once again raced around the world in waves spread from the Indonesian island of Sulawesi. Cholera had become endemic in Indonesia and had continuously caused outbreaks for the past 52 years (Lacey, 1995; Safa et al., 2010). During the 1970's, cholera spread rapidly across Africa, which had been spared the pandemic in the late 1800's. In 1991, cholera entered Peru and an outbreak began that subsequently spread to 18 Latin American countries killing more than 10,000 people over the course of the next four years. Cholera continued to cause outbreaks and establish itself as endemic in countries around the world.

In 2012, 48 countries reported 245,393 cases and 3,034 deaths to the World Health Organization (WHO). Cholera is a politically, socially and economically inflammatory disease and has the ability to shut down tourism, trade and cause unrest. As a result, the reported numbers are a massive under representation of the number of cholera infections and deaths that occur. In addition, many countries lack the medical training and disease surveillance systems to accurately determine the cause of outbreaks of profuse watery diarrhea. The WHO has estimated that the true worldwide burden of cholera may be as

much as 10 – 20 times higher. In Bangladesh alone, there are an estimated 5,400 unreported deaths per year from cholera (Ali et al., 2012).

The most severe ongoing outbreak in the world is occurring in Haiti. In 2012, 245,393 cases of cholera were reported to the WHO, 112,076 were from Haiti a nation of 10 million people that occupies half the island of Hispaniola. In October of 2010, there was a spike in cases of profuse watery diarrhea at hospitals in towns along the Artibonite River. It quickly became clear that Haiti was facing the first cholera outbreak in history. Due to the lack of water and sanitation infrastructure, it was also clear that the outbreak had the possibility of spreading throughout the country and causing massive casualties. Within weeks of the start of the outbreak, a few cases quickly grew to hundreds and to thousands. In the past three years, there have been 679,637 cases and 8,297 deaths from cholera according to the Haitian ministry of health (MSPP, 2013). Cholera has spread from Haiti across the border to the Dominican Republic, across the Caribbean to Cuba and most recently to Mexico. Haiti has extremely low levels of clean water and sanitation infrastructure, a warm climate, frequent storms and flooding, making it particularly susceptible to endemic cholera.

At no time in history have people traveled around the world so fast and so far as they have in the jet age. The combination of increased ease of human mobility, increased population density around the world, rapid urbanization, a changing climate and a world where one third of the population still practices open defecation with some locations having as much as 90% of the population without access to appropriately isolated

sanitation, has created the perfect breeding ground for *V. cholerae*. This bacterium will continue to race around the world and exploit locations with little to no defenses. Until we are able to provide a reliable and inexpensive vaccine, treatments that stop the disease, or clean drinking water to the over 2.5 billion people who are at risk, this pandemic will not end.

Vibrio cholerae

Cholera is caused by the bacterium *Vibrio cholerae* and characterized by profuse secretory diarrhea often accompanied by vomiting. *V. cholerae* is a member of the *Vibrionaceae* family of Proteobacteria. It is a gram negative, highly motile, comma shaped rod that has the ability to persist in brackish water. *V. cholerae* is a facultative anaerobe and a halophile. It has two chromosomes that have coordinated replication and regulation; a large 2.96 megabase chromosome and a smaller 1.06 megabase chromosome and essential genes are found on both chromosomes (Heidelberg et al., 2000).

V. cholerae are classified serologically by a variable O-antigen of its lipopolysaccharide. While there are over 200 known serotypes, some of which can cause disease, there are two dominant classes of epidemic causing *V. cholerae*, O1 and O139. The *V. cholerae* O139 serogroup produces a polysaccharide capsule. The O1 serogroup is further split into Classical and El Tor biotypes. Classical O1 *V. cholerae* was responsible for every wave of pandemic cholera until 1961 when El Tor emerged (Kaper et al., 1995). In 1992, O139 emerged as the dominant disease causing *V. cholerae* in South Asia, however the strain

that caused the South American pandemic was the O1 El Tor biotype (Faruque et al., 2003; Kaper et al., 1995). Classical is now not found in the environment or in cholera patients while O139 still causes occasional disease. The dominant biotype of *V. cholerae* currently circling the globe is El Tor.

A cholera patient can shed up to 20 liters (over 5 gallons) of diarrhea per day for three to seven days. That level of fluid loss can rapidly cause death from dehydration, especially in the young, elderly, pregnant women or the malnourished. Cholera treatment focuses on preventing life-threatening dehydration by administering simple oral rehydration therapy (ORS, a salt and sugar solution), or intravenous rehydration with Lactated Ringer's solution or normal saline solution. Antibiotics can be administered to shorten the course of disease by up to half (Swerdlow and Ries, 1992).

V. cholerae hyperinfectivity

Cholera is contracted through ingestion of contaminated water or food and can spread very rapidly among susceptible populations. Experimental infections with healthy adult North American volunteers has led to the calculation of an infectious dose of 10^6-10^{11} CFU (Cash et al., 1974; Levine et al., 1979). Such a high infectious dose suggests that cholera should spread slowly through populations. In fact, several studies have employed mathematical modeling to try and reconcile this high infectious dose with the explosive spread of cholera outbreaks and were unable to do so. However, when they included the decreased infectious dose induced by host-passage, they were able to reconcile the

rapidly increasing number of cases that characterize a cholera outbreak with the high calculated infectious dose (Hartley et al., 2006).

Using *V. cholerae* isolated from the rice water stool of patients at the International Center for Diarrheal Disease Research (ICDDR,B) in Dhaka, Bangladesh, it was shown that host-passaged bacteria have a one-log lower ID50 (the dose at which 50% of mice are infected) relative to *in vitro* grown bacteria (Butler et al., 2006). This phenotype was maintained for at least five hours of incubation in a pond environment (Merrell et al, 2002). However, hyperinfectivity was lost after growth of rice water stool bacteria *in vitro*, suggesting this is a transient physiological state. *V. cholerae* isolated from mouse intestine were also found to have a one-log lower ID50 (Alam et al, 2005). Host passaged *V. cholerae* were also found to outcompete *in vitro* grown *V. cholerae* in a one-to-one competition in mice.

The existence of transient hyperinfectivity presents the possibility of two routes of cholera infection; one direct and one indirect. In the direct route, an individual is infected with *V. cholerae* that is still in its hyperinfectious phase. In the indirect route, infection is mediated by *V. cholerae* that has been in the environment for an extended period of time and is no longer hyper-infectious. While the vast majority of cholera research has focused on lab-passaged *V. cholerae*, which would represent the non-epidemic route of route of infection, little to no work has been done to interrogate the direct route of infection.

The molecular basis of hyperinfectivity

Several microarray studies have been undertaken to examine the genes that are expressed in rice water stool-derived *V. cholerae* (Merrell et al., 2002a; Nelson et al., 2008). In addition, RNA levels were also measured following experimental infection in infant rabbits using RNA-seq (Fang et al., 2012). Interestingly, it has been well documented that essential virulence factors are repressed at the end of infection. This stands in direct contrast to proteomic analysis that showed rice water stool *V. cholerae* contain all of its major virulence factors (Larocque et al., 2008).

Transcriptional analysis provides a picture of a transcriptional capacity at a particular point in time- it does not show the levels of the proteins that are necessary to mediate phenotypes. While proteomic analysis has also been used to try and answer this question, it is inherently difficult. Without the technology to amplify peptides in the same way that we can amplify DNA fragments, and with the variability of biochemical properties of proteins, issues of peptide abundance and bias are major hindrances to proteomic analysis. To date, one proteomic analysis has been published on host-passaged *V. cholerae* (Larocque et al., 2008). In this work, they determined the proteome of 32 patient samples using a non-quantitative gel-based method followed by tandem-mass spectrometry (LC-MS/MS). Of the 909 proteins they identified with one or more peptides, only one, OmpU, was found in all 32 patients. OmpU is known to be one of the most abundant proteins expressed in *V. cholerae* (Larocque et al., 2008). Other proteins that were found included major virulence factors such as the two cholera toxin subunits CtxAB, and toxin coregulated pilus TcpA. The genes of both these proteins have been shown to be repressed at the end of infection. While this data provides a partial list of proteins present

in rice water stool samples, it does not provide quantitative data. In addition, the question remains, as these bacteria enter the environment, which of these proteins persist?

In vitro induced hyperinfectivity

In addition to host passage, three *in vitro* conditions have been found to increase the infectivity of *V. cholerae*; the deletion of the gene *cheY-3* which results in a motile but non-chemotactic state, acid-pretreatment, biofilm formation (Angelichio et al., 2004; Butler and Camilli, 2004; Merrell et al., 2002b; Tamayo et al., 2010). These conditions all result in a lower infectious dose in infant mice. Interestingly, both biofilm formation and the deletion of *cheY-3* also result in the dispersal of infecting *V. cholerae* along the entire length of the small intestine of the rice water stool-derived *V. cholerae*, as opposed to *in vitro* grown *V. cholerae*, which at 5-10 hours post-infection, clusters in the distal portion of the small intestine (Alam et al., 2005; Tamayo et al., 2010). Although genes important for acid-adaptation induced hyperinfectivity have not been determined, the phosphate uptake transporter, *pst2*, was shown to be important for biofilm-induced-hyperinfectivity (Mudrak and Tamayo, 2012). Finally, the motile but non-chemotactic state has been speculated to cause *V. cholerae* to avoid signals drawing it towards the defensins in the crypts of the intestine, but this hypothesis has not been tested (Butler and Camilli, 2005).

Infection induced phenotypes in other bacterial pathogens

V. cholerae is not the only pathogen that shows a documented increase in infectivity following infection. *Legionella pneumophila*, *Citrobacter rodentium*, *Salmonella enterica*

and *Mycobacterium avium* have all been shown to increase infectivity following passage through a host (Cirillo et al., 1994; Wiles et al., 2005; Rasmussen et al., 2005; Cirillo et al., 1997). Varying amounts of data have been published on each of these organisms, but what is common among the information that is available is the fact that following a successful infection in a susceptible organism, they enter a transient phase of increased infectivity which is abrogated after growth in laboratory media. Existing knowledge about hyperinfectivity in these organisms may shed light on the phenotype in *V. cholerae* and provides a starting point for phenotypic characterization of this state.

L. pneumophila is the pathogen with the most thoroughly characterized hyper-infectious state. *L. pneumophila* is an intracellular pathogen that infects amoeba and can also infect human macrophages causing Legionnaires' disease, a deadly lung infection. Following infection of *Acanthamoeba castellanii*, a protozoan species that is a natural host, *L. pneumophila* enters a state referred to as Mature Intracellular Form (MIF), which is morphologically and phenotypically distinct from *in vitro* grown bacteria. These passaged bacteria are 10-fold more invasive in a secondary *A. castellanii* infection, and in macrophages. They are also 100-fold more invasive in epithelial cells (Cirillo et al., 1994). If bacteria from the primary infection are grown on solid media prior to infection, the increase in infectivity is eliminated. In addition to an increase in infectivity, passaged *L. pneumophila* also are highly resistant to detergent, antibiotic, pH and osmotic stress (Barker et al., 1995; Garduno et al., 2002). Interestingly, these MIFs accumulate high amounts of the carbon storage compound poly-β-hydroxybutyrate (PBHB) - a phenotype that mirrors human-passaged accumulation of glycogen in *V. cholerae*. The presence of

these compounds could contribute to resistance to stresses by serving as osmoprotectants (Bourassa and Camilli, 2009).

C. rodentium is a natural mouse pathogen that causes intestinal disease similar to enteropathogenic *E. coli* in humans. Following a primary mouse infection, *C. rodentium* exhibit a lower infectious dose in comparison to either *in vitro* grown bacteria or passaged bacteria grown in LB overnight (Wiles et al., 2005). They found that this decrease in infectious dose is coupled with, and likely mediated by, an increase in adhesion, micro-colony formation and actin polymerization on Hep-2 cells as well as increased persistence within the mouse intestine (Bishop et al., 2007).

Salmonella enterica serovar Typhimurium is an intracellular pathogen that infects a wide spectrum of hosts causing intestinal infection. It was found that infection of bovine calves with a particular strain resulted in increased infectivity (Rasmussen et al., 2005). Because the rumen of calves contains a number of protozoan species, they tested the effect of a mixture of rumen-isolated protozoa on the ability of the *S.* Typhimurium strain to invade Hep-2 cells *in vitro*. They found that bacterial infection of these protozoa greatly increased invasion of Hep-2 cells. In addition, successive passage through Hep-2 cells eliminated this advantage. Importantly, when this experiment was repeated with a non-invasive *S.* Typhimurium strain there was no increase in invasion. Furthermore, they showed that exposure to supernatant is not enough to stimulate an increase in invasiveness (Rasmussen et al., 2005). The increase is directly due to transient changes in the strain's gene regulation due to invasion of the protozoa.

M. avium is another intracellular pathogen in which an increase of infectivity has been shown following *A. castellanii* infection. When the *M. avium* are isolated from the *A. castellanii* and used to infect human peripheral blood monocyte-derived macrophages they are more infectious than *in vitro* grown bacteria (Cirillo et al., 1997). Furthermore, they show that mice co-infection with *A. castellanii* and *M. avium* increases the recovered CFU/ml in an *A. castellanii* dependent manner (Cirillo et al., 1997).

These examples illustrate several points: 1. Host-induced increase in infectivity is found in several bacterial species; 2. The increase in infectivity is transient, requiring that the infections take place in quick succession; 3. Simple exposure to the host is not sufficient to induce the change in the pathogen; 4. Work in *L. pneumophila* suggests that not only is infectivity affected, but also characteristics that may aid in resistance to host or environmental factors. These examples also raise the question of whether *V. cholerae* experiences similar infection induced increases in resistance to stresses, and if this plays a role in hyperinfectious spread.

What we know about host-passaged *V. cholerae* falls in line with these examples: *V. cholerae* exhibits a transient increase in infectivity that requires the establishment of a successful infection (Bishop, 2012; Butler et al., 2006; Merrell et al., 2002a). It has been shown that *V. cholerae* is prepared for environmental conditions as it nears the end of an infection, as illustrated by genes turned on late in mouse infection that are necessary for environmental survival but not infectivity (Schild et al., 2007). It is also known that

human-passaged *V. cholerae* contains glycogen, a carbon storage molecule, that helps it persist in the environment, possibly by helping it resist stresses (Bourassa and Camilli, 2009). However, unlike *L. pneumophila,* we have no clear idea as to what *V. cholerae* is prepared to face or the genes that are involved in mediating the hyper-infectious phenotype.

Cholera vaccination and treatment

Since the subcutaneous live attenuated *V. cholerae* vaccine of Dr. William Haffkine in 1892, several vaccines have been developed (Haffkine, 1895; Hankin, 1892). The current WHO approved vaccine, which is the vaccine likely to be deployed under emergency conditions, is BivWC. BivWC is marketed as "Shanchol" and "mORCVAX". Shanchol was licenced in 2009 and approved by the WHO in 2011 in the wake of the outbreak in Haiti. It is a bivalent inactivated vaccine composed of a combination of whole cell heat and formalin-killed El Tor and classical *V. cholerae* O1 and O139 plus LPS from each of the included strains (Fang et al., 2012).

In recent human trials of Shanchol conducted in India, it was found that it has an adjusted protective efficacy of 65% over five years. During the study period, of the 31,932 vaccine recipients, 69 sought treatment at a treatment center for cholera infection. Of the 34,968 placebo recipients, 219 sought treatment (Bhattacharya et al., 2013; Taylor et al., 2011). *V. cholerae* infection, both natural and experimental, results in 80-90% immunity for at least three years, the longest period that has been tested (Levine et al., 1981).

Given the hyperinfectious state which is likely to mediate most outbreak-associated cases of cholera, it is possible that the epitopes present on the heat and formalin inactivated *in vitro* grown *V. cholerae* that were used to create both Shanchol and other vaccines are insufficient to provide protective immunity. The work that has been done to understand epidemic cholera has mainly focused on microarray studies. While several microarray studies and follow-up experiments have been conducted, we still do not have a clear picture of how the phenotype is mediated and exactly what *V. cholerae* is prepared to face. Because microarray analysis provides a picture of a transcriptional profile and not the actual enzymes and proteins that are necessary to mediate phenotypes, an understanding of the proteins present during the hyperinfectious spread of cholera may provide better vaccine targets.

Animal models of infection

Humans are the only known natural vertebrate host of *V. cholerae*. In order to study pathogenesis and examine the genes required for infection, several experimental infections models have been established. The two most prevalent have been the rabbit ileal loop model, and colonization of infant mice. In 2010, Ritchie et al re-introduced the infant rabbit model adding cimetidine pre-treatment to increase the rate of successful infection. While infant mice neither respond to experimental cholera infection with diarrhea nor do they respond to cholera toxin, the required virulence genes and transcriptional changes in this model and in humans have significant overlap (Levine et al., 1988; Lombardo et al., 2007). Until the re-introduction of the infant rabbit model, the

ligated loop model was the only animal model that resulted in the accumulation of fluid during an experimental infection.

The infant-rabbit model proceeds by intra-gastric inoculation of three-day-old New Zealand white rabbits that have been treated with cimetidine three hours prior to infection. Following 14-17 hours of infection, these animals experience profuse watery diarrhea and, without careful monitoring, can die from the infection. Relatively pure *V. cholerae* can be isolated from cecum fluid upon dissection of an infected infant rabbit. This is in stark contrast to the infant mouse model, which requires homogenizing the small intestine tissue to extract the *V. cholerae* following infection.

Post-infection phenotypes in *Vibrio cholerae*

As introduced above, host-passaged *L. pneumophila* has been extensively characterized phenotypically and structurally. It is known that these bacteria are highly resistant to a myriad of stresses, store PHBA and have altered cell morphology. No such characterization has been carried out in *V. cholerae*. We hypothesize that *V. cholerae* leaves the host in a highly resilient state and has proteins that allow it to mediate a successful secondary infection and survive in the environment.

Previous work showed that towards the end of an experimental infection, *V. cholerae* turns on the transcription of genes necessary to survive in the aquatic environment. These genes were dubbed “late genes” (Schild et al., 2007). It was found that most of these genes are dispensable for animal infection, but without them, the bacteria were severely

attenuated for survival in pond water following infection. This work showed that during the late stages of infection, *V. cholerae* had the ability to prepare for the stresses of entering the environment.

In addition to the stresses it encounters entering the environment following infection, *V. cholerae* encounters multiple stresses upon entry and transit through a human host: drastic changes in temperature, pH, osmolarity, oxygen, exposure to gastric acid, sIgA, mucus, bile and other antimicrobial peptides. In transit to a host, through a host, to the environment, and back to a host, *V. cholerae* encounters many distinct environments. One of the stresses encountered while *V. cholerae* is establishing infection and replicating in the small intestine is bile. It is possible that *V. cholerae* adapts to those stresses during infection, therefore preparing it for the next infection.

Bile resistance

Like many other enteric bacteria that occupy bile-bathed niches such as the small intestine, *V. cholerae* has adapted methods both to resist the antimicrobial effects of bile and use bile as a signaling molecule to activate virulence gene expression (Hernandez et. al., 2012; Hung et. al., 2005; Yang et. al., 2012). *V. cholerae* infects and replicates within the small intestine where it encounters high levels of bile. An understanding of the ability of *V. cholerae* to resist and respond to the presence of bile may be important to the development of therapeutics.

Bile is a complex mixture of molecules produced in the liver, stored in the gallbladder and released into the proximal small intestine when food is ingested to promote lipid absorption. It is composed of inorganic salts, pigments, fatty acids, cholesterol, porphyrins, proteins and phospholipids (Das et al., 1996). In addition to playing an important role in digestion, bile also plays an important role in controlling antimicrobial populations in the small intestine due to its antimicrobial activity.

Approximately 60% of bile is made up of bile salts (Farina et al., 2009). Bile salts are a mixture of primary, secondary and conjugated salts. Primary bile salts are derived from cholesterol in the liver. These bile salts are converted to secondary bile salts by the microflora in the large intestine and then recycled via the enterohepatic system. Primary and secondary bile salts are conjugated to the amino acids glycine and taurine (Bilecen and Yildiz, 2009; Ripps and Shen, 2012).There are also active transport mechanisms for conjugated bile salts that controls their levels and distribution (St-Pierre et al., 2001).

Bile salts are secreted into bile which is stored in the gallbladder until secretion into the proximal small intestine. Concentrations in the small intestine range from 0.2-2% (Prouty and Gunn, 2000). The total concentration of bile salts varies depending on eating and diet. The ratio of conjugated to unconjugated bile in the small intestine changes drastically from the duodenum to the ileum. The proximal segment of the intestine has the highest concentration of bile acids with the vast majority of those biles salts present in conjugated form (Northfield and McColl, 1973).

In addition to its role in digestion, bile also acts to control bacterial populations. As they function to form mixed micelles with lipids, and assist in the uptake of lipid soluble nutrients, bile salts have the ability to chelate calcium and iron, disrupt membrane lipids and cause the dissociation of membrane proteins (Gunn, 2000). Bile salts can also damage DNA, cause the misfolding of proteins and induce plasmid curing.

Bacteria that occupy the small intestine are adapted to resist the antimicrobial effects of bile. Some bacteria such as *S. enterica* and *V. cholerae* are able to resist high concentrations. This property has been taken advantage of for the production of selective media such as MacConkey agar, *Salmonella-Shigella* agar and the *Vibrio* specific media, thiosulfate-citrate-bile salts-sucrose agar (TCBS) and taurocholate-tellurite-gelatin agar (TTGA). Several cellular functions have been identified as being important in bile resistance in enteric bacteria such as *Salmonella,* including changes in LPS, perturbations of the outer membrane that result in disruption of porins and Tol genes. While genes have been identified that are important for survival in bile, a comprehensive study of genes essential for *V. cholerae* resistance to bile has not been undertaken.

In addition to developing mechanisms to resist increased bile concentrations, some bacteria have also developed mechanisms for using bile as a signaling molecule. In *Salmonella, Shigella* and *Campylobacter* species and in *V. cholerae*, bile salts have been found to have an influence on virulence gene regulation (Yang et al., 2013). In *Salmonella,* bile exposure has also been shown to increase bile resistance. While many genes have been identified that are important for bile sensitivity a comprehensive screen

has not been conducted. Furthermore, while bile adaptation has been shown in *Salmonella* (Hernandez et. al., 2012), it has not been demonstrated in *V. cholerae*.

Materials and Methods

Bacterial strains, plasmids and growth conditions

V. cholerae O1 serogroup, El Tor biotype, streptomycin-resistant strain E7946 and O139 strain MO10 were used in this thesis. All gene deletion strains were constructed in the E7946 background using natural competence and the FRT/FLP recombinase system as previously described (De Souza Silva and Blokesch, 2010). All wild-type, mutant and complemented strains are listed in Table 1. Complementation plasmids were constructed using pMMB67EH, which contains a leaky promoter that can be induced with the addition of IPTG. *V. cholerae* and *E. coli* strains were all grown in LB broth or on LB agar unless otherwise noted. Streptomycin, Sm, was used at 100 mg/ml; Kanamycin, Kan, at 100 mg/ml; Ampicillin, Amp, at 100 mg/ml and at 50 mg/ml; Spectinomycin, Spec, at 100 mg/ml.

Mini-Tn*5* transposon library construction

To generate mini-Tn*5* transposon (mTn*5*) libraries, we transferred the pUT-mTn*5*Km2 plasmid from Sm10λpir to E7946 via conjugation. We selected for the transfer of the mTn*5*Km2 transposition into the *V. cholerae* genome as previously described (Merrell et al., 2002b). Resulting colonies were estimated and appropriate pool sizes were made by scraping up transconjugants and resuspending in LB. Multiple aliquots of each pool were diluted to OD600=1 and frozen at -80°C with 15% glycerol (v/v). The number of unique insertions in each pool was determined by transposon-genome junction sequencing

(transposon-sequencing (van Opijnen et al., 2009)). For mouse infections pools of 800-1200 were constructed. For rabbit infections pools of ~30,000 were constructed.

Rabbit Tn-seq: primary infections

In this infection model, we used transposon libraries composed of ~30,000 insertion mutants. Frozen library stocks were thawed and grown up in LB plus Kan selection to mid-exponential phase, OD_{600}=0.3-0.5. The mid-exponential cultures were then washed and resuspended in LB at pH 5.5. In preliminary experiments, we determined that this exposure to mildly acidic pH lowers the bottleneck by increasing infectivity of *V. cholerae* and allowing a larger number of mutants to participate in the infection (Merrell et al., 2002b). The library was incubated in acidified LB for one hour after which time it was washed in LB and resuspended in sodium bicarbonate, pH 8.5. Approximately $5x10^8$ CFU were immediately used as inoculum to infect three-day old New Zealand white rabbit kits (purchased from MillBrook Labs in Amherst, MA). Infected kits were administered 300 mg/kg cimetidine hydrochloride three hours prior to infection (Morton Grove Pharmaceuticals) by intragastric inoculation (Ritchie et al., 2010). After the onset of disease, approximately 14-16 hours post-inoculation, kits exhibit visible signs of lethargy, watery diarrhea and weight loss. They are then euthanized and dissected. The accumulated fluid in the cecum and surrounding small intestine segments of kits was removed by draining the dissected section. This fluid, which was on the order of 0.6 - 1.0 mL, was then spun at low-speeds ≤ 2,000 g to remove cecotropes and any host cells.

Rabbit Tn-seq: secondary infections

The resulting supernatant was then treated and used as an inoculum for another group of three-day-old kits in one of three ways. In the first iteration, the bacteria were directly removed from the cecum and small intestine and used to infect 3-day-old kits who were not cimetidine treated. In the second iteration, cecal fluid bacteria were pelleted by centrifugation at 20,000 x g for one minute. This cell pellet was then washed in warm saline (0.85% NaCl at pH 7.5) and re-suspended in warm sodium bi-carbonate equal to the starting volume. This was then used to infect each of two secondary kits which were not treated with cimetidine. The third iteration was identical to the first except the kits were treated with cimetidine three hours prior to infection. All secondary infections proceeded for 14-26 hours at which point the animals were euthanized. From each kit, fluid was collected from the cecum and small intestine. In addition, in some experiments the tissue of the small intestine and cecum were also collected, and separately, the remainder of the large intestine. The tissue samples were homogenized. All samples, both input and output, were outgrown in LB broth with Sm and Kan, to select for *V. cholerae* and the mTn*5*, at 30^{o}C for approximately 12 hours (Figure 1).

Mouse primary and secondary infection

Mini-Tn*5* libraries of ~1,000 mutant complexity were grown up to mid-exponential phase and approximately 1x10^{5} CFU were used to infect five- to six-day-old pups via intragastric inoculation as previously described (Angelichio et al., 1999). After 20 hours the mice were euthanized and small intestines were homogenized in 1 mL of saline and filtered first through 100 μm filters then through 40 μm filters, and diluted 1:5 in saline. Five- to six-day old pups were intragastrically inoculated with 50 μL of the filtered

primary infection homogenate. Twenty hours after inoculation the mice were euthanized, dissected and the small intestines were homogenized (Figure 2).

Input and output samples were diluted and plated for determination of viable counts. In addition, inputs and outputs were grown in LB plus Sm and Kan for 12 hours at 30^{o}C.

Library preparation and sequencing

Transposon junctions were amplified from total genomic DNA, barcoded, mixed and subjected to massively parallel sequencing as previously described (Klein et al., 2012) (Kamp, 2013).

Neutral Genes

To serve as a control for the analysis of our Tn-seq data, we generated a list of "neutral" loci in the *V. cholerae* genome that appear to be both functionally and transcriptionally dead. Authentic point mutations and/or mutants with transposon insertions were unchanged in abundance after 30 generations and therefore had no growth advantage or disadvantage (D. Lazinski, unpublished data). This list was checked against most available *V. cholerae* microarray data to ensure that there was no transcription during *in vitro* virulence gene expression microarrays, in human rice-water stool samples, in rabbit ligated loop fluid microarrays, and that the genes were found to be dispensable in signature tagged mutagenesis screens (Xu et al., 2003). Genes that were found to be expressed in the Human IVET screen or for which peptides were identified in the

published rice-water stool proteome data were also disqualified (Larocque et al., 2008; Lombardo et al., 2007). These criteria resulted in a list of nine genes listed in table 2.

Data Analysis

Sequencing results were uploaded to the Tufts University Galaxy server where the reads were mapped to the *V. cholerae* N16961 reference genome using Bowtie. The *V. cholerae* E7946 and N16961 genomes differ by only a small number of SNPs and INDELs (Lazinski and Camilli, 2013), and thus this reference genome was appropriate. The number of reads per insertion site were tabulated using a custom program called hopcount (Klein et al., 2012). Further analysis was performed using the statistical program STATA.

Rice water stool sample collection and incubation and preparation

Stool samples were collected at the Iccdr,B fresh from patients over the age of 15 with acute watery diarrhea and no pre-hospital treatment with antibiotics. These samples were then delivered to the laboratory at room temperature and examined by darkfield microscopy to confirm a *V. cholerae* positive sample. Dark field positive samples are characterized as having over 100 highly-motile (darting motility) bacteria per field when viewed under 40-times magnification. In addition, it is necessary that these bacteria be immobilized and comma shaped following the addition of serotype specific antibodies. Samples that meet these criteria were decanted into 50 ml conical tubes and clarified at 500 x g to remove host mucus. The supernatants were collected and CFU of *Vibrio* and non-*Vibrio* were determined by differential plating on TTGA and McConkey agar. The bacteria were then pelleted from the rice water stool supernatant. Each pellet was

resuspended in 8 ml of filter sterilized pond water (0.22 μm filtration immediately after collection then stored at room temperature). From each patient, one cell pellet was immediately frozen and the other was placed into a dialysis bag and incubated in 0.5 liters of unfiltered pond water for 24 hours. Following the 24 hour incubation the samples were plated for CFU. Samples were pelleted immediately after washing with pond water (T=0) and after 24 hours incubation in pond water (T=24). Pellets were frozen at -80^{o}C and sent to the laboratory of Dr. Aleksandra Sikora for membrane extraction and comparative proteome analysis.

Membrane protein extraction, ITRAQ & Data Analysis

Cell pellets were resuspended in PBS with protease inhibitors and lysed by passing three times through French Press (at 1100 psi). 40 ml of cold 0.1 M sodium carbonate was added to each 10 ml of cell lysate and the samples were sonicated. The sample were rotated for 1 h at 4^{o}C and sonicated after 30 min and 1 hour. Membrane proteins were then pelleted by ultracentrifugation for 1 hour at 100,000 x g. Membrane pellets were washed with PBS and reconstituted in 8 M urea, 0.04% SDS in 0.5 M Tetraethylammonium bromide (TEAB). Protein concentrations were then measured using 2D Quant Kit.

Isobaric tags for relative and absolute quantitation (ITRAQ) is a quantitative proteomics method in which each sample is labeled at free amines with Isobaric tags that allow for relative and absolute quantitation. The samples were differentially labeled, quantified and mixed. The mixed samples were fractionated by strong cation exchange chromatography

and then subjected to two-dimensional liquid chromatography and mass spectrometry. This data was analyzed as previously described (Sikora et al., 2011).

In vitro sample preparation

Wild type samples were grown to mid-exponential phase, OD_{600}= 0.3-0.5 or overnight to stationary phase. Samples were then washed, pelleted and frozen at -80^{o}C. These samples were then either prepared for ITRAQ or FASP analysis.

Filter-aided sample preparation

Total proteome was isolated from mid-exponential and stationary phase wild-type cultures by modifying the Filter-aided sample preparation (FASP) protocol for bacteria (Wiśniewski et al., 2009). Cell pellets were resuspended in freshly prepared lysis buffer that had been pre-heated to 95^{o}C. Lysis buffer was composed of 4% SDS, 100 mM Tris-HCl pH 7.6, 0.1 M DTT. This was then incubated at 75^{o}C for five minutes. Approximately 250 mg of total protein in 30 µl were mixed with 200 µl of fresh UA buffer (6 M Urea, 0.1 M Tris-HCl pH 8.5), and sonicated in a horn sonicator at 100% amplitude at 12^{o}C for three minutes to lyse DNA and further disrupt any unlysed cells. The sonicates were centrifuged at 5,000 x g at 20^{o}C for five minutes to remove any intact cells and large debris. The supernatants were removed and saved as aliquots. 230 µl of supernatant was applied to a Micron YM-30 30 kDa filter and centrifuged at 14,000 x g at 20°C for 15 minutes, and the eluent was discarded. The filter bound proteins were subjected to a series of washes: 200 µl of UA buffer was applied to the filter and centrifuged at 14,000 x g at 20°C for 15 minutes; 200 µl of freshly prepared IAA buffer

(0.05 M iodoacetamide, 6 M Urea, 0.1 M Tris-HCl pH 8.5) was applied to filter and agitated by shaking at 600 RPM at 37°C for 1 minute, then incubated static at 37°C for 20 minutes, and then centrifuged at 14,000 x g at 20°C for 10 minutes; the filter was then washed three times with 100 µl UA buffer, and then three times with 100 µl 0.05 M TEAB. 40 µl of TEAB with fresh mass spec grade trypsin at 1:100 enzyme to total protein ratio was added to the filer and agitated at 600 rpm at 37°C for 1 min, then incubated at 37°C for 4-18 hours in a damp chamber. To collect the tryptic peptides, the filter was transferred to a collection tube that had been pre-rinsed with acetonitrile and ultrapure water. The filter was centrifuged at 14,000 x g at 20°C for 10 minutes. 50 µl of ultra pure water was applied to the filter and centrifuged at 14,000 x g at 20°C for 10 minutes. The combined flow-through (eluent) was saved and analyzed for peptide content by running 10 µl on 10-20% Tricine Gel at 20 W for 3 hours. The gel was silver stained to check for a smear of peptides at ≤3 kDa molecular weight and absence of a protein band at the molecular weight of trypsin.

Identification of immunogenic proteins in outer membrane vesicles

Outer membrane vesicles, OMVs, were generated from *V. cholerae* strain E7646 as previously described (Schild et al., 2008). We separated 5ug or 0.5ug of OMVs on each of two 4-12% gradient gel using a standard sodium dodecyl sulfate-polyacrylamide (SDS-Page) procedure. One gel was transferred onto a nitrocellulose membrane (Life Technologies) and probed using anti-OMV antiserum provided by Anne Bishop(Bishop et al., 2012). The other gel was silver stained using a kit from Life Technologies. The gel and blot were checked for corresponding band sizes and protein bands of interest were

excised from the silver stained one-dimensional SDS page gels at the approximate location of a western blot detected protein. Protein identification was performed by the Tufts University Core Facility by Tandem mass spectra (LC/MS/MS) following in-gel digestion as previously described (Li et al., 2009).

Cloning and expression of potential antigens in *E. coli*

Genes were cloned into a modified pET15B vector containing a 6xHis tag linked to the protein by a TEV protease recognition site as previously described (Pratt et al., 2010). Proteins were then expressed in *E. coli* BL21 DE3 and isolated from inclusion bodies using a modified protocol (Sahu et al., 2008).

Immunization

Two 12 week-old BALB/c female mice were injected via intraperitoneal route with 100 µl of a 1:1 emulsion of the protein and Freund's complete adjuvant. Each pair was boosted with an i.p. injection after two weeks using an emulsion of 1:1 protein and Incomplete Freund's adjuvant. Two weeks later, mice were sacrificed by CO_2 asphyxiation. The chest cavity was opened, the left heart chamber was punctured with scissors and then the circulation was perfused by slow injection of 8 mL PBS into the right heart chamber. The blood and perfusate were collected in a sterile petri plate via spillage from the opened left ventricle, then pipetted into a 10 ml BD Vacutainer (367874) with sodium Heparin to prevent clotting, and mixed by inversion three times. Blood cells were pelleted at 4,000 x g for 30 minutes at 20^{o}C. The plasma supernatants

were saved in 15 mL conical tubes at 4°C after adding sodium azide to 0.05% as a preservative.

Testing post-infection phenotypes following experimental infant rabbit infection

3-day-old New Zealand White rabbits were treated with cimetidine three hours pre-infection then returned to their mothers. They were then infected with a *lacZ*⁻ derivative of *V. cholerae* E7946. Infection was allowed to proceed for 12-14 hours depending on how quickly cholera symptoms (diarrhea, sluggishness and weight-loss) manifested. Kits were euthanized and dissected and the fluid was drained from the cecum and adjacent small intestine. Cecal fluid was spun at 2,000 x g to remove cecotropes and any host cells. The supernatant was then spun at 10,000 x g to collect the *V. cholerae*. These bacteria were then washed with saline and mixed roughly 1:1 with wild type (*lacZ*⁺) *V. cholerae* that had grown to mid-exponential in LB and washed in saline. This mixture was then tested for survival under control and various stress conditions in 96 well plates. These tests were incubated at room temperature for various amounts of time before being diluted in saline and plated for CFU on LB agar supplemented with Sm and 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) at 60 μg/mL.

Bile sensitivity Tn-seq Screen

Aliquots of a mTn*10* library of 70,000 mutant complexity were thawed from the -80°C freezer, diluted and grown to an OD_{600}=0.3 (McDonough, unpublished). They were then washed in phosphate buffered saline with 1 mM IPTG to induce an outward reading promoter on the mTn*10*. IPTG was present in each subsequent step of the experiment.

The bacteria were resuspended in PBS or PBS plus 1% porcine bile (Sigma #B8631). After one-hour incubation, each pool was pelleted, washed, split in two equal pools and resuspended in either PBS or 1% bile salts (Sigma #B8756). After a one hour incubation, the resulting pools were plated for CFU and outgrown in LB for 12 hours at 30^{o}C to amplify surviving bacteria for genomic DNA preparation needed for performing Tn-seq.

Validation of bile sensitive mutants

Selected mutant strains and the *lacZ⁻ V. cholerae* were grown to mid-exponential phase in LB and washed with saline or PBS. They were then mixed 1:1 at an OD_{600}=1 and diluted 1:10 into saline or PBS with various concentrations of bile salts, porcine bile extract or specific bile salts at 100 μm. Following a 1 hour incubation at room temperature, each sample was serial diluted into PBS and plated on LB plates containing X-gal to titer CFU.

Results 1. Secondary infection Tn-seq screen

We infected two- or three-day-old infant rabbits with $5x10^8$ – $1x10^9$ CFU of high complexity mTn*5* transposon mutant libraries. We took advantage of the knowledge that acid tolerizing *V. cholerae* increases their infectivity (Angelichio et al., 2004), and we found that it alleviates an otherwise significant colonization bottleneck. With acid tolerized *V. cholerae* we were able infect a single animal with approximately 40,000 transposon mutants. When animals began to show symptoms of cholera following 12-17 hours of infection, they were euthanized and dissected. In infected animals, the cecum and adjacent small intestine were visibly distended with approximately 600 – 1000 µl of clear fluid. When this fluid was drained, and examined under the microscope, there were a large number of highly motile *V. cholerae* present, reminiscent of rice water stool samples.

Rabbit-passage induced hyperinfectivity

To determine if rabbit-passaged *V. cholerae* was more virulent than *in vitro* grown *V. cholerae*, as had been shown with human and mouse passaged bacteria, we mixed rabbit passed and *in vitro* grown 1:1 and infected infant mice. We found that the rabbit passaged *V. cholerae* was 2-100 times more virulent than *in vitro* grown (Figure 3).

Secondary infection in infant rabbits

In order to determine the genes that were necessary for the hyperinfectious phenotype, we used the cecal fluid derived *V. cholerae* to infect a new set of 3-day-old rabbits. In our first experiments, we directly inoculated the kits with unwashed cecal fluid, in the second we washed and buffered the cecal fluid derived bacteria and inoculated kits, in the third experimental protocol, we treated the secondary kits in the same way that the primary were treated: we washed and buffered the inoculum and we treated the secondary kits with cimetidine three hours before infection. Using all three of these methods, there was a severe bottleneck in the secondary infection that prevented Tn-seq analysis of the genes involved in this phenotype.

Secondary infection output complexity with unwashed inoculum

In order to determine the genes necessary for secondary infection in rabbits, we directly inoculated 200 µl of primary infection cecal fluid into naive three-day old kits that were otherwise untreated. This method resulted in a highly variable infection with some of the infections resulting in visible signs of cholera within 12 hours, but no with visible bacteria in the cecal fluid or recoverable CFU (Table 4). This suggested that perhaps cholera toxin, known to be found in rice water stool and known to be able to stimulate the symptoms of cholera, was inducing cholera symptoms too early in infection and therefore preventing the passaged bacteria from establishing an infection (Levine et al., 1983).

Secondary infection output complexity with washed inoculum

In an attempt to increase colonization upon secondary infection, steps were taken to remove extracellular cholera toxin from the cecal fluid-derived inoculum. The bacteria in the cecal fluid were pelleted and washed once in saline. The bacteria were then resuspended in saline and used to infect secondary kits that had not been pretreated with cimetidine. There were approximately 30,000 insertion sites in the initial input, *in vitro* grown and primary infection output samples, while there were only approximately 2,000 insertion sites in the secondary infection samples. Thus, a severe bottleneck during secondary infection was still present despite the washing of the inoculum.

Despite the bottleneck, an attempt was made to salvage the data. For the analysis, we required that an insertion site be present in each of the three samples of one kit: small intestine tissue, large intestine tissue and cecal fluid. Furthermore, they were required to be present in each of the four secondary kits. This resulted in 763 insertions to be analyzed. These 763 insertion sites are within 604 genes. Of these genes, only 43 have three or more insertion sites. The massive loss of complexity in the secondary infection resulted in a skewing of the number of reads per insertion site. In order to determine the relative importance of these genes in secondary infection, we had to compare the output to input ratio in both the primary and secondary infection. To do this, we standardized the data by ranking the output to input ratios using a percentile. This transformation creates datasets that have similar means and standard deviations. We used a paired t-test to compare the mean and variance of all the insertion sites in a particular gene in the *in vitro,* primary and secondary output samples. Using this test, we generated p-values to determine if there was a statistically significant difference between the abundance of the

analyzed genes in the input and the output samples. We found that many of the genes that were in statistically different percentile in the primary and the secondary infection were involved in flagella.

Interrogating the role of flagella in rabbit secondary infection

The data suggested a role for genes involved in flagellar synthesis and regulation in primary and secondary infection in rabbits. *In vitro* and in the primary infection, the ratio of these genes (*fliA*, *fliK*, *flrA*, *flaA*, *flgD*, *flgN*, *rpoN*) are at the top percentiles of their populations. In the secondary infection, however, these genes are in the lower and middle percentiles, 26^{th}-44^{th}. Their increased read numbers in the *in vitro* sample may be representative of a fitness advantage for not producing the energetically costly flagella during growth in rich media. In the primary infection, however, it is thought that flagella is necessary for *V. cholerae* to penetrate the mucus layer to act on the epithelium. In infant mice, amotile flagellar mutants are attenuated for infection (Richardson, 1991). In contrast, amotile mutants are still able to cause disease in humans, and they have been shown to have increased toxicity and up-regulation of toxin genes, including hemolysins, cholera toxin and *tcp* genes, under *in vitro* conditions (Gardel and Mekalanos, 1996). This change in toxin production, rather than or in addition to the growth advantage, could account for the increased representation of these mutants in the primary infection. In the secondary infection, the fact that these genes are not more abundant then in the primary infection, suggest that this toxin-induced phenomenon is not occurring.

To test the hypothesis that there are genes in the flagella biosynthesis pathway that are differentially important in the primary and secondary infections, we tested genes from various classes of the regulatory hierarchy *in vivo* competition in infant rabbits primary and secondary infection.

In order to determine the importance of these genes in secondary infection, we constructed a pool of deletion mutants with in-frame frt scars. This allowed testing the mutants *en masse* via massively parallel sequencing across the frt scar/chromosomal junctions, which serve as unique identifying sequences. The deletion mutants were tested for *in vitro* growth, primary and secondary infection in comparison with a frt-scar marked *lacZ* deletion strain (a neutral deletion) (Figure 7). With the exception of *rpoN*, which is known to have regulatory effects outside of flagella, no significant difference was found under any of these circumstances.

Secondary infection output complexity with washed inoculum and cimetidine treated kits

In an attempt to widen this bottleneck, we added the step of cimetidine pre-treating the secondary kits to block stomach acid production. This resulted in a widened bottleneck with secondary kits returning up to 12,000 of 30,000 insertion sites (Figure 4). However, when looking at the insertion sites that the kits have in common or when considering how the number of reads at each insertion site correlates, the variability is still quite severe, suggesting that the widened bottleneck is still too small to get useful data. Figure 5 shows that 582 insertion sites are common in the cecal fluid of all four secondary kits. Even

when discarding a kit that had a poor infection, there are still only 1,644 insertion sites (of a possible 11,415) that are common in all three secondary kits. When looking at correlations between matched primary and secondary kits, a similarly dire view emerges. There is little to no correlation between the number of reads at each insertion site in the two samples (Figure 5). This suggests that regardless of the efforts made to standardize the infection, the representation of insertions in these animals is mostly random.

Infections carried out in rabbits showed that, of the approximately 30,000 unique mTn*5* insertion mutants that were used to infect rabbits, 76-80% were able to participate in the primary infection while only 4-8% were able to participate in the secondary infection.

When the secondary kits were treated with the acid production blocker cimetidine, this participation rate increased to 12-65% in the secondary kits (Figure 5). However, although the same library was used in all infections, there is little overlap between insertions in the kits, and the correlation between the number of reads in the input (primary infection) and the output (secondary infection) is very low suggesting a massive stochastic loss of mutants, not specific selective pressure.

Mouse secondary infection Tn-seq

In order to examine which genes are important for primary and secondary infection in mice, we constructed 35 mTn*5* libraries, each ~1,000 insertion complexity, and carried out the experiment outlined in Figure 1. One mouse was infected with each library. The output of that individual was used as the input for one secondary mouse and also

outgrown *in vitro*. Analysis of the outgrown samples shows that 60 - 95% of mutants can participate in the primary infection while, with one exception, 2 - 50% of insertions can participate in the secondary infection (Figure 6). Because of the large bottleneck, we did not analyze secondary infection data further.

The low complexity of the secondary outputs suggests that very few *V. cholerae* are able to participate in the secondary infection. At the same time, the number of CFU in the output Table 3A shows that final CFU/ml for the secondary infection was very similar to the final CFU for the primary infection. This again suggests that the replication during the secondary infection is higher than in the primary infection.

Summary

In our initial rabbit infections, we found that some infections resulted in diarrhea and fluid accumulation in the cecum and small intestine but without any recoverable CFU. We hypothesized that this was due to cholera toxin in the cecal fluid and therefore decided to wash the bacteria isolated from the cecal fluid prior to their inoculation into secondary animals. We also added buffering to the inoculum as is done in the primary inoculation. This resulted in a wider, yet still narrow bottleneck. It is possible that cholera toxin present within the rabbit-passaged *V. cholerae* gets released upon inoculation into secondary animals and thus is still able to cause the bottleneck. It was also possible that not treating the secondary kits with cimetidine was resulting in the remaining bottleneck.

The final iteration of this protocol involved both washing and buffering the inoculum and treating the secondary kits with cimetidine three hours before infection. In this final

integration, the primary and secondary infection conditions are identical: inoculum is washed, resuspended in sodium bicarbonate buffer and administered to 3-day-old kits that were treated with cimetidine three-hours prior to infection. This also does not allow for a full representation of mutants in the secondary infection.

The presence of a bottleneck in secondary infection in two distinct animal models, and with different transposon libraries, suggests that there may be an upper limit to the number of *V. cholerae* that can participate in the secondary infection. There are two scenarios that might explain this bottleneck. In the first, only a percentage of the passaged bacteria are hyperinfectious, in the second, each bacterium has the ability to become hyperinfectious, but the first to come in contact with the mucosa begin the infection to the exclusion of others.

Results 2. Proteome of rice water stool *V. cholerae*

We undertook a study to determine the proteins that are present on the surface of rice water stool *V. cholerae*, but not present in *in vitro* grown samples. This data represents proteins that may be important for infection as well as proteins that might serve as protective antigens in a vaccine that are not currently present in the vaccines designed from *in vitro* grown bacteria. While this study is incomplete, it constitutes the first comparative proteomics study that not only represents the proteins present in rice water stool, but also presents the proteins that are stably maintained after entry into the environment. These proteins may be good targets around which to build a vaccine.

In order to determine proteins that are stably maintained on the surface of *V. cholerae* following entry into the environment, we measured the membrane proteome following human infection and after 24 hours of incubation in pond water (Table 5), and after 24 hours of incubation of the rice water stool bacteria in pond water. In addition, we measured the membrane and total proteomes of *in vitro* grown *V. cholerae*. Using this data, we have generated a list of predicted and known outer membrane proteins that are found in rice water stool but not in any of the *in vitro* samples (Table 7).

Rice water stool membrane proteome

We were able to measure comparative levels of 64 proteins from the inner and outer membranes of rice water stool-derived *V. cholerae* from two patients (Table 5). These were proteins that were identified with at least one peptide. Included were several

membrane associated virulence factors shown to be present in other studies of rice water stool proteins, including TcpA, TcpC and AcfA, as well as several proteins that had never previously been detected in rice water stool including OmpV, OmpA and OmpT. Among the most abundant proteins were HutA, IrgA and ViuA, all involved in iron acquisition, which is known to be important for human infection (Goldberg et al., 1992; Henderson and Payne, 1994). 19 of these proteins were found at similar abundance in both patient samples and were maintained for at 24 hours (Table 6).

In vitro proteome

We determined the proteome of two strains of *V. cholerae* using a method that solubilizes cells and digests proteins on the surface of a filter (Wiśniewski et al., 2009). Using tandem mass spectrometry and Mud-PIT, we identified between 150 and 215 proteins present in each of these samples. Because the data is only semi-quantitative, we can only determine the total percentage that each protein is of the sample. As expected, in strain E7946, OmpU is the most abundant protein. In strain MO10, the most highly abundant protein was maltoporin, OmpS. This is consistent with previous reports that the maltose operon is constitutively expressed in O139 strains (Calia et al., 1994).

Proteins recognized by anti-OMV antibodies

Outer membrane vesicles are made up of the outer membrane and proteins from the periplasm. Previous studies have shown that the dominant protective antigen in OMVs is the lipopolysaccharide O-antigen (Bishop et al., 2012; Leitner et al., 2013). To determine what protein antigens are present in OMVs, we performed a Western blot using serum

raised to outer membrane vesicles to detect proteins present in OMVs. We extracted bands from SDS page gel that corresponded to the size of recognized proteins. We determined a list of proteins present in OMVs and recognized by the anti-OMV antibodies (Table 8). Although it is not a complete list of proteins present in OMVs, it does represent possible antigens as well as a representation of proteins present on the outer membrane during *in vitro* growth.

Summary

In this study we determined a set of proteins that were present in host-passaged *V. cholerae* but not present in *in vitro* grown *V. cholerae*. Among these are proteins involved in iron acquisition (IrgA, HutA and ViuA) and known virulence factors (TcpC and AcfA), and proteins of uncharacterized function (VC0742 and VC2662). These proteins are not only present in rice water stool *V. cholerae,* but also maintained for 24 hours of pond incubation. Despite the fact that several of these genes have have been shown to be dispensable for survival in the pond environment (Hughes et al., 1995; Watnick et al., 1999), these proteins persist in *V. cholerae*. Further study is necessary to determine if the presence of these proteins contributes to the increased infectivity of host-passed rice water stool.

Additionaly, given the limitations of the current vaccine, these proteins may represent surface exposed epitopes that present in host-passaged *V. cholerae* and could be used to stimulate long lasting protective immunity. The ability of these proteins to produce

vibriocidal and protective antibodies should be tested. If they are protective, expression in OMVs could create a hyperinfections specific, heat stable cholerae vaccine.

Results 3: Post-infection Phenotypes

Infection induced stress resistance

We hypothesized that hyperinfectivity and dissemination fitness of stool shed *V. cholerae* may be due, at least in part, to the presence of a physiological state of stress resistance. To examine the ability of post-infection *V. cholerae* to survive stresses, we first compared rice water stool-derived with *in vitro* grown *V. cholerae* for their ability to survive in pond water compared to rich and minimal medium. A comparison of the physical properties of these media is shown in table 9. Rice water stool *V. cholerae* collected from patients at the ICDDR,B in Dhaka, where washed with filter sterilized pond water and incubated at room temperature. CFU were determined by plating directly from rice water stool, immediately after suspending in pond water and after five hours incubation (Figure 8). Following human infection, *V. cholera*e is able to survive entry into pond water over five hours. The *in vitro* grown lab strain was also tested for survival in pond water over five hours and we found that while *V. cholerae* grown in M9 minimal media plus glucose they survive to a similar level, when grown in LB there is a four-log decrease in plating efficiency after 5 hours (Figure 8). In both cases the bacteria were grown to mid-exponential phase.

To eliminate the possibility that this difference was due to salinity and osmolarity of the media used, we measured total dissolved solids, conductivity and salinity (Table 9). We found that M9 glucose, LB and rice water stool all had similar readings which suggests that these do not play a role in the survival of *V. cholerae*. These measurements show that

the substantial drop in salinity and in osmolarity that the bacteria experience is similar under each condition.

The pond water used in the experiment above represents some defined (e.g. low osmolarity, nutrient depleted) but mostly undefined stresses. Therefore, we next wanted to examine the ability of *V. cholerae* to survive specific defined stresses following infection. To do this, we took advantage of the fact that the *V. cholerae* that accumulates in the cecum of infected infant rabbits is a good proxy for human rice water stool (Ritchie et al., 2010), and can be cleanly isolated and then used in stress survival assays *in vitro*. Kits were infected with a *V. cholerae lacZ*$^-$ strain. Following infection, fluid was removed from the cecum, the bacteria were washed in saline and mixed 1:1 with wild type (*lacZ*$^+$) *V. cholerae* that had been grown in LB to mid-exponential phase (OD_{600}= 0.3 - 0.5). These mixtures were then placed into various stress or non-stress conditions: 0.85% saline (pH 7.5), saline pH 2.5, Organic Acids pH 4.5, 15% H_2O_2, distilled water, 1% (w/v) sodium deoxycholate and sodium cholate mixture (bile salts, Sigma), or LB broth. CFUs were determined at four time points: prior to placing into test condition, and at 10 minutes, 2 hours and 24 hours after placing into the test condition.

Following exposure to saline pH 2.5, organic acids or H_2O_2, there were no recoverable CFU from *in vitro* grown or rabbit-passaged *V. cholerae* (data not shown). Rabbit-passage did increase survival of *V. cholerae* in distilled H_2O and 1% bile salts. Rabbit-passaged substantially outcompeted *in vitro* grown in both conditions. Data from the two-hour time point is presented as a competitive index in figure 9.

V. cholerae survival is increased following exposure to porcine bile

Because the small intestine and cecum are bathed in bile, we hypothesized that *V. cholerae* may have evolved the ability to adapt to this stress, which could be demonstrated experimentally by showing that exposure to sub-lethal concentrations of bile salts would increase resistance to lethal doses of bile salts. To test this hypothesis, we exposed *in vitro* grown *V. cholerae* to various concentrations of resuspended porcine bile extract or PBS and measured the ability of these pretreated bacteria to survive exposure to 1% bile salts. We found that pretreatment with porcine bile extract resulted in a substantial survival advantage over control pretreatment with PBS (Figure 10).

The effect of various bile salts on the viability of *V. cholerae*

Bile is a complex set of molecules. The dominant component of bile is bile salts. We tested *V. cholerae* for its ability to survive in 100μM of specific bile salts compared to a 1% mixture of deoxycholate and sodium cholate (BS) (Figure 11). In addition, we examined the ability of individual bile salts to stimulate resistance to higher concentrations of bile salts (Figure 12). We found that not all individual bile salts decrease viability and not all bile salts increase resistance to lethal concentrations of BS. Of the individual salts, only 100μM deoxycholate has a significant effect on increasing resistance to lethal levels of BS (Figure 12).

Candidate genes involved in bile tolerance

To increase our understanding of how *V. cholerae* resists bile and becomes tolerant to bile, a Tn-seq selection was done to identify the genes that play demonstrable roles. A library of approximately 70,000 mTn*10* insertions was used for the selections (McDonough, unpublished). We selected for survival in BS, in 0.1% and 1% porcine bile extract. By comparing the abundance of mutants in the untreated control sample to the abundance in the bile treated samples, we were able to putatively identify new genes as well as confirm the importance of all genes that had been previously identified as being important in bile resistance (Bina and Mekalanos, 2001; Bina et al., 2006; Cerda-Maira et al., 2008; Nesper et al., 2001; Provenzano and Klose, 2000) with the exception of two, *galU* and *ompU*, that were not present in the input library (Table 10). An *ompU* null mutation is known to cause a moderate growth defect in rich media, and this is likely why this particular mutant was absent from our library. In all, 265 genes were identified that have three or more insertions and showed a statistically significant ($p \leq 0.01$) 2-fold difference between the bile and control treated samples, with most genes playing a positive role in resistance to bile (Table 11). Among the categories identified that had increased sensitivity were porins, transporters, quorum sensing genes and genes involved in biofilm formation. LPS biosynthetic genes were found to not be important for bile survival, although they have been found to be essential in *S.* Typhimurium (Hernandez et al., 2012).

OmpU is a porin that has been widely implicated in *V. cholerae* survival under various stresses including bile (Mathur and Waldor, 2004; Provenzano and Klose, 2000). As observed for the wild-type, an *ompU* deletion mutant is resistant to cholate, glycocholate,

glycodeoxycholate and taurocholate. It does have much higher susceptibility to chenodeoxycholate and deoxycholate than wild-type and this results in no viable CFU of the *ompU* mutant being recovered following exposure to these salts (Figure 13).

We examined three new genes identified in the Tn-seq screen, which represent different functional classes, for their resistance to bile salts; the porin gene *ompA*, the two-component system genes *carRS*, and the lipoprotein gene VC1755. OmpA is a major outer membrane protein that, in *E. coli*, has been shown to be important to resistance to several stresses including the bile salt sodium cholate, human serum, acid, sodium dodecyl sulfate (SDS) and high osmolarity (Wang, 2002). Although it has been suggested that in *V. cholerae* classical strain O395 *ompA* plays a role in bile resistance (Wibbenmeyer et al., 2002), it had not been demonstrated to our knowledge. CarRS encoding a cognate sensor kinase and response regulator, respectively, was found to be important for biofilm integrity in *V. cholerae* (Bilecen and Yildiz, 2009). The role of this system in bile salt resistance has not been determined in *V. cholerae,* but homologs in *E. coli,* RstAB and BaeRS, have been shown to be important in resistance to bile and other stresses (Guerrero et al., 2013; Zhou et al., 2003). VC1755 is an uncharacterized putative 21.9 kDa lipoprotein with no homology to any characterized proteins.

We found that disruption of these genes resulted in a decrease of viability of *V. cholerae* in 1% bile, 100 μM deoxycholate and 100 μM chenodeoxycholate (Figure 13). In the same fashion as the wild type, neither sodium cholate, nor any of the conjugated bile salts tested affected viability of the mutants. The viability of these deletion mutants were restored upon complementation *in trans* from a plasmid that has leaky expression in the

absence of inducer, or can be expressed at higher levels by addition of inducer (Figure 14). The *ompA* deletion was fully complemented even in the absence of inducer, while the *carRS* and VC1755 deletions required inducer to be fully complemented.

Summary

Following host passage, *V. cholerae* enters a transient state of hyperinfectivity. This largely uncharacterized phase of the *V. cholerae* life cycle should be thought of as a collection of phenotypes. In order to better mediate the next infection, host-passaged *V. cholerae* may be more resistant to the stresses encountered during the infectious process, including changes in pH, osmolarity, temperatures, oxygen concentrations and a myriad of antimicrobial defenses including bile. Bile poses a particular threat, not only because of its natural ability to disrupt lipid membranes, but also because it has the ability to chelate iron, which is important for the survival of *V. cholerae* (Sanyal et al., 1994). Because it occupies the small intestine as an infectious niche, *V. cholerae* has developed strategies to resist the high concentrations of bile in the small intestine and to use some of the bile components as signals stimulate virulence gene production.

We examined the effect of infant rabbit infection resistance to bile, and found increased resistance. We went on to find three previously unidentified proteins that are involved in the resistance of *V. cholerae* to bile. In addition, we show adaptation following experimental rabbit infection and following exposure to resuspended porcine bile extract *in vitro*. We also performed a high throughput genetic selection, resulting in the near comprehensive identification of the genes important for survival in bile. We validate the

importance of a porin, a lipoprotein and a two-component system that had not previously been shown to be important for survival in bile.

Discussion

In order to better understand the hyperinfectious phenotype of *V. cholerae*, we took a three-pronged approach to: 1) identify the genes involved, 2) determine associated proteins, and 3) characterize additional phenotypes related to stress survival. We found that in the two prevailing animal models of *V. cholerae* there is a severe bottleneck in secondary infection. We also identified proteins and phenotypes associated with the rice water stool *V. cholerae*. This suggests multiple new avenues for research into the hyperinfectious phenotypes and possible new vaccine antigens.

Secondary infection genetic selection

Prior to Tn-seq, transposon studies involved a population of transposon mutants being subjected to a selection condition after which the location of the transposon insertions in the surviving mutants were determined on a one-by-one basis using various methods (Merrell et al., 2002b; Tischler et al., 2002). In some cases, transposon mutant libraries were serially passaged through the condition to enrich for particularly fit mutants. This method whittled down the number of mutants necessary to sequence. The presence of particular mutants among the sequenced transposon insertions suggested that the mutants answered the selection. Massively parallel sequencing has allowed for a more detailed analysis of genes important for a particular phenotype. It has also provided the opportunity to assay all the genes in the genome simultaneously with multiple transposon insertions per gene (Van Opijnen, 2009). Current sequencing technology provides approximately 170 million reads that allows for the simultaneous measurement of many

different mutants. This allows the investigator to identify enriched (more fit), depleted (less fit) and neutral (dispensable) gene mutations.

We took advantage of this technology to test the viability of each of 30,000 mutants in secondary infection. Because the vast majority of genes are not necessary for either *in vitro* growth or primary infection and there is no significant bottleneck, we were able to determine the fitness of each gene under these conditions (Kamp, 2013). Sequencing the secondary infection output, however, revealed a significant bottleneck that severely decreased population complexity in a random manner. Even when taking the most simplistic view of the data and asking if there are any genes that are absolutely essential for secondary infection, and therefore never isolated from any of the 18 secondary infections kits sequenced, we find that every insertion in the primary infection appears in at least one animal in the rabbit secondary infection. This suggests that it is stochastic loss rather then selection that prevented us from identifying genes required for transmission of hyperinfectious *V. cholerae*.

We initially hypothesized that extracellular cholera toxin present in the cecal fluid from infected primary rabbits was responsible for causing the bottleneck in the secondary rabbits due to its action in eliciting a secretory response in the small intestine. In this scenario the immediate fluid buildup would prevent bacteria in the inoculum from establishing colonization. However, after removing extracellular cholera toxin from the inoculum by washing, the bottleneck was still apparent. However, our analysis and the analysis of other groups (Larocque et al., 2008) of the proteome of rice water stool *V.*

cholerae indicates that an intracellular pool of cholera toxin is present despite the downregulation of the *ctxAB* genes by this stage of the infection (Merrell et al., 2002a; Nelson et al., 2008). Thus, it is possible that a similar intracellular pool of cholera toxin in the cecal fluid *V. cholerae* gets released upon secondary infection and is sufficient to elicit fluid secretion and thus block colonization.

Experimental mouse *V. cholerae* infection is drastically different from infant rabbit infection. The pups do not produce watery diarrhea, nor do they respond appreciably to cholera toxin (Ritchie and Waldor, 2009). Therefore, we hypothesized that if the bottleneck in the secondary rabbit infection was due to immediate or early release of cholera toxin by *V. cholerae*, that such an effect would not manifest during secondary infection in infant mouse mice. As a result, we should be able to assay the entire transposon library for secondary infection. However, this also turned out not to be the case: While standard infection conditions in mice allowed for the establishment of 1,000 transposon mutants in the primary infection, most of the secondary infections allowed less than 200 mutants to establish colonization.

Achieving the same result in two very different animal models presents the possibility that *V. cholerae* has developed mechanisms to limit population diversity during hyperinfectious spread. This could function as a competitive mechanism to prevent other *V. cholerae* strains (e.g. cheaters that do not produce TCP or other colonization factors) from co-colonizing new hosts. Such a bottleneck may explain how cholera outbreaks gain their clonal nature despite the presence of several pathogenic strains in some patients and

in the environment (Chun et al., 2009; Kendall et al., 2010). In this scenario the most fit *V. cholerae* establishes infection and excludes others.

In this light, it is fair to ask the question: How can rice water stool *V. cholerae* be hyperinfectious, while at the same time, have the capacity to limit colonization of secondary infected hosts? The answer may lie in the different inoculum doses used in experimental infection versus natural transmission. In our Tn-seq experiments, we purposely used very high inoculum doses both in the primary and secondary infections so as not to lose complexity of the transposon library. However, natural infection with hyperinfectious *V. cholerae* may occur via doses that are many orders of magnitude lower that what we've used in our Tn-seq experiments. Indeed, the very nature of hyperinfectivity - having a lower infectious dose - supports the model that natural transmission occurs via low dose inocula.

Despite this severe bottleneck during secondary infection, which may be a function of high dose inocula, it may be possible to determine the genes that are required for secondary infection. In order to obtain robust and reliable data, secondary infections would have to be carried out using much lower doses of cecal fluid *V. cholerae*, and thus much lower complexity libraries. For example, one could use an ordered knockout library, and compete small-defined pools of 10 or 20 mutants at a time. The optimal complexity would have to be determined empirically. In addition, secondary infection of larger groups of animals per primary output inoculum would provide more robust data. Only by

showing that known neutral gene mutations reproducibly come out of the secondary infection outputs, would we be able to then ask whether essential transmission genes exist.

Rather than continuing this line of investigation, it would be interesting to explore whether *V. cholerae* is limiting population diversity via factors expressed or activated in primary infection. In order to identify proteins unique to this stage of infection, we have carried out a comparative proteomic screen on rice water stool derived *V. cholerae*.

Quantitative proteomic analysis of *in vitro* and rice water stool *Vibrio cholerae*

Outer membrane proteins such as the toxin co-regulated pilus (TCP) and the porin OmpU are essential for *V. cholerae* infection. While several microarray studies have shown that these genes are not being transcribed in rice water stool *V. cholerae*, LaRocque and others have shown that TcpA, the main pilin subunit, and other virulence factors are present in the proteome (Larocque et al., 2008; Merrell et al., 2002a; Schild et al., 2007). In order to explore the possibility that virulence-associated proteins are mediating the hyperinfectious phenotype, we wanted to establish that these proteins are stably maintained in rice water stool samples. We also wanted to determine if other outer membrane proteins would be only associated with rice water stool *V. cholerae* and not any *in vitro* grown samples as these proteins may represent appropriate targets to block pathogenesis of *V. cholerae* with a vaccine or treatment.

To determine the *in vitro* proteome, we used three different methods to identify proteins in the whole proteome as well as membrane-associated proteins. This data provided a

solid framework against which rice water stool *V. cholerae* membrane associated proteins could be compared. In addition, we used anti-OMV antiserum to determine the proteins in OMVs were recognized by serum. This analysis provided another look into the proteins of *in vitro* grown *V. cholerae*.

In order to establish the proteome of host passaged *V. cholerae*, we isolated bacteria from human rice water stool samples at the Icddr,B in Dhaka, Bangladesh. We were not only interested in knowing what the proteome of *V. cholerae* is immediately following infection, but also interested in having semi-quantitative data that showed which proteins were stably maintained after entry into the environment.

The role of rice water stool associated proteins in hyperinfectivity

We found that of the 64 proteins we identified, 19 were stably maintained, including proteins known to be important for virulence (Table 6). The levels of TcpC and AcfA were consistent over the two patient samples and did not change over 24 hours of pond incubation. This presents the possibility that these proteins are available at the end of this incubation period to participate in an infection. The maintenance of these protein levels may allow *V. cholerae* to circumvent the complex de-repression of its virulence genes that has been demonstrated using inocula grown in rich media, and perhaps begin using virulence factors earlier in infection. It would be fascinating to perform recombination-based *in vivo* expression technology (RIVET) screens on mouse or rabbit passaged *V. cholerae* to determine if the kinetics of virulence gene expression is different in secondary infection (Camilli and Mekalanos, 1995). However, by the end of primary

infection in mice, virulence genes resolve at the level of 90-100% making this experiment impossible (Lee et al., 2001).

There is one method that might help answer this question that would allow for dynamic measurement of expression rather than protein levels and takes advantage of *in vivo* imaging technology (Cara E Morin, 2009). In this experiment the lux operon is fused to a virulence gene promoter of interest and is present on a stable low copy plasmid. When this fusion is expressed, light is visible through the abdomen of a live infected infant mouse. By comparing the kinetics of light production in a primary and secondary mouse, it would be possible to determine gene expression kinetics. One potential caveat to this experiment is that the initial low numbers of *V. cholerae* may not produce sufficient light to detect even with a fully induced fusion. A way to circumvent this problem would be to employ short half-life fluorescent protein fusions (Nielsen et al., 2010) instead of a lux operon, then perform fluorescence microscopy on infected sections of secondary mouse small intestine.

In addition to the known virulence factors detected by our proteomic analysis, there are several proteins that have not previously been identified as important to primary infection including VC0742 and VC2662, neither of which have a characterized function. These proteins may be important for the hyperinfectious spread of *V. cholerae*, and their role in secondary infection should be determined.

The protective antigen potential of rice water stool associated proteins

In addition to being proteins that may play a role in secondary infection, these proteins may represent the long sought after protein antigen components of a long-lived and highly protective cholera vaccine. The current vaccines are all composed of different preparations of *in vitro* grown *V. cholerae*, and it is known that the LPS O-antigen is the dominant protective antigen elicited by these vaccines. However, the antibody response to this carbohydrate antigen wanes rapidly, much more so than to protein antigens (Harris et al., 2009). It is thought that the waning of anti-O-antigen antibodies is largely responsible for the waning of protective immunity afforded by these vaccines, which is on the order of 1-3 year (Sur et al., 2009). It is possible that the addition of these proteins to a vaccine would provide greater and/or more long lasting protection. Because of the focus on membrane bound proteins, it would be possible to integrate these proteins into an outer membrane based vaccine. It has been illustrated that expression of outer membrane and periplasmic proteins result in the presence of these proteins in OMVs (Schild et al., 2009). Outer membrane vesicles are ideal because they have been shown to provide protection in an infant mouse model and do not require cold-storage, a factor that drives up the price of vaccines and greatly complicates distribution (Bishop et al., 2010).

In order to determine if these proteins are appropriate for inclusion in a vaccine, there are several tests that should be carried out. The proteins should be expressed in *E. coli* to high levels and purified. This recombinant protein can then be used to vaccinate mice. Serum from these mice can be used in passive protection assays in both primary and secondary infection as well as vibriocidal antibody titers following primary infection.

V. cholerae post-infection phenotypes

Hyperinfectivity should be viewed as a collection of phenotypes. This increased ability to mediate a secondary infection might be due to one or a combination of phenotypes, which could include resistance to any of the barriers and stresses encountered during infection or a change in virulence gene regulation. Over the course of infection, *V. cholerae* encounter vastly different environments including drastic changes in temperature, pH, osmotic shocks, bile and antimicrobial peptides. It is possible that a single gene is not responsible for hyperinfectivity and that characterizing the various phenotypes of host-passaged *V. cholerae* may shed light on the mechanism of hyperinfectious spread.

In order to determine which stresses host-passaged *V. cholerae* was resistant to, we competed washed infant rabbit passaged *V. cholerae* against *in vitro* grown *V. cholerae.* We found that, among the conditions we tested, host-passaged *V. cholerae* are substantially more resistant to hypo-osmotic shock, incubation in distilled water, and to exposure to bile salts (a mixture of sodium cholate and deoxycholate). It turned out that the other stresses, including low pH, high pH and exposure to H_2O_2, were too strong to be survived and should be adjusted to increase survival.

Bile is a complex mixture that plays an important role in digestion and in minimizing the microbial populations in the small intestine. The cecum of an infant rabbit, which (along with the small intestine) is where *V. cholerae* accumulates during experimental infection, has a high concentration of bile (Das et al., 1996). Because bile salts are the major

component of bile, we decided to test which bile salts were most deleterious to the survival to *V. cholerae.* We found that at 100μM and under our test conditions (static incubation at room temperature in 0.85% saline pH 7.5 or PBS), deoxycholate and chenodeoxycholate where the only components that affected survival. This also held true when we tested an *ompU* deletion strain, which has been found to be highly sensitive to several stresses including exposure to bile.

In order to determine which genes and cellular functions are important for bile resistance, we tested a library of transposon mutants for their survival in bile salts and in porcine bile. We found that, in addition to every gene previously identified as playing a role in bile sensitivity, we also identified many new genes. In testing genes for their sensitivity to bile, we found that several candidate identified in the selection (*mshA*, *hapR*, *potD1*, *potD2*) did not reproduce when deleted and tested in 1:1 competition assays. When deleted individually each of these genes resulted in a rugose colony phenotype, which is known to increase resistance to bile salts. While we cannot explain why they are not attenuated in 1:1 competitions, it is possible that they were attenuated in the Tn-seq selection because they represented a much smaller proportion of the total population (where there were over 50,000 mutant present). This may suggest that, in order for the rugose phenotype to be protective, there needs to be a high enough local concentration of rugose bacteria for the phenotype to manifest.

The genes that did have a confirmed sensitivity to bile were *ompA*, encoding an uncharacterized porin, *carRS*, encoding a two-component system involved in biofilm

formation, and VC1755, encoding a putative lipoprotein. Deletion of any one of these genes resulted in a decreased (or eliminated) ability of *V. cholerae* to survive in 100μM deoxycholate. The level of sensitivity of these genes, in addition to the large number of genes identified in the selection was surprising. Given the bile-bathed environment that *V. cholerae* occupies during infection, we might have expected redundancy. To determine if these genes played a role in infection we used the infant mouse model to test the ability to compete 1:1 with a *lacZ* deletion mutant and found that they all behave the same as wild type in infection. This presents the possibility that during infection, multiple genes mediate bile resistance.

Investigating hyperinfectivity in *V. cholerae*

Following either human or experimental animal infection, *V. cholerae* enters a transient state of hyperinfectivity. During hyperinfectivity, the infectious dose, as measured in infant mice, is 10-fold lower than that of *in vitro* grown *V. cholerae*. This phenotype helps to explain how cholera outbreaks spread so rapidly through susceptible populations. While the complex virulence gene cascade is well characterized in *V. cholerae*, the period of hyperinfectivity represents a largely unstudied segment of the *V. cholerae* life cycle. In addition to the genes that potentially mediate and regulate this process, the characteristics of host-passaged *V. cholerae* that are responsible for hyperinfectivity have also gone unexamined. Knowledge about this distinct phase of the *V. cholerae* life cycle may allow for development of treatments and vaccines specifically tailored to the phenotypes of *V. cholerae* that are mediating an outbreak.

In this thesis, I undertook three distinct approaches towards understanding the hyperinfectious phase of *V. cholerae*: genetic, proteomic and phenotypic. Using a genetic approach, we combined transposon mutagenesis with highly parallel sequencing to determine genes important for the hyperinfectious phenotype. In the proteomic approach, we used comparative proteomic analysis to determine proteins stably maintained following human infection but not expressed *in vitro*. In the phenotypic approach, we examined stresses to which host-passaged *V. cholerae* are resistant and we determined genes involved in that resistance. Each of these approaches offered a view into the hyperinfectious phenotype and introduced many avenues for further investigation.

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