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The Effects Of Motility And Chemotaxis On Vibrio Cholerae Colonization In Zebrafish

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THE EFFECTS OF MOTILITY AND CHEMOTAXIS ON VIBRIO CHOLERAE COLONIZATION IN ZEBRAFISH

by

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THESIS

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GENERAL INTRODUCTION

Cholera Disease

Cholera is an acute intestinal infection, accompanied by voluminous, watery diarrhea, and is characterized by severe dehydration. The watery diarrhea is referred to as “rice-water stool” as it is similar in appearance to the leftover water after washing rice. Diarrhea may progress to fluid losses of up to one liter per hour (1). Other symptoms include vomiting, abdominal discomfort or cramping, lethargy, dry mouth, cold clammy skin, decreased skin turgor, and wrinkled hands and feet (2). Muscle cramping and weakness are common due to electrolyte loss. Fever is considered rare and may be associated with a secondary infection. In extreme cases, excessive diarrheal illness can lead to severe dehydration and even death.

Rehydration is the primary treatment for patients with a cholera infection. Oral rehydration solution (ORS), introduced in the late 1960s, is currently the preferred treatment for cholera. ORS is designed to replace previous and continuing fluid loss by maximizing sodium uptake in the small intestine (3, 4). The World Health Organization (WHO) sets strict guidelines on the various salts and glucose to be included in the ORS to maximize its effectiveness (5). Antibiotics may also be added to the treatment regimen to decrease severity and duration of symptoms. Various findings indicate that antibiotics can reduce stool volume by 8-92%, shorten diarrhea duration by 50-56%, and decrease bacterial shedding by 26-83% (6-10).

While cholera treatment can be quite effective, preventing an outbreak would be ideal. Although cholera vaccines exist, there is not yet a vaccine with long-term or complete protection. Currently, two oral cholera vaccines (OCV), termed Dukoral and Shanchol, are considered safe and effective, and are licensed and available. Both
vaccines contain killed whole cell *V. cholerae* O1. Additionally, the Dukoral vaccine contains recombinant cholera toxin B subunit, and the Shanchol vaccine contains *V. cholerae* O139 (11). These vaccines are estimated to be 50-85% protective for two to three years (12-15). The WHO highly recommends the addition OCV into the current cholera control program in endemic areas, however, issues including cost and delivery still remain (11).

The World Health Organization estimates there to be 1.4-4.3 million cases of cholera per year (16). Approximately 75% of the infected individuals will show no symptoms, but will typically still shed the bacteria 24 hours after infection. These asymptomatic carriers are still able to infect others (17, 18). Of the symptomatic individuals, approximately 20% will experience the intestinal infection with severe, watery diarrhea and dehydration. These individuals can begin to shed *V. cholerae* in their stools before the onset of symptoms (19, 20), and will continue to shed bacteria 7-14 days after infection (21, 22). If untreated, the fatality rate for severe cholera cases is approximately 50% (23). However, if treated, the fatality rate drops to around 1% (24). Currently, there are approximately 100,000 to 120,000 deaths per year caused by cholera.

**History**

Cholera has been around for centuries, especially in Southern Asia, but the causative agent was not originally known. The first written accounts of a cholera-like disease date back as far as 500 BC in Sanskrit (2). Initially, cholera occurred in epidemics around the Ganges delta region with high mortality rates. It wasn’t until 1817 that cholera spread out of the Indian subcontinent, spanning as far as southern
Russian, causing its first pandemic. Filth and poor infrastructure, and the development of trade and transportation, allowed cholera the ability to spread and flourish (25). The second pandemic started in 1826, reaching the United Kingdom by 1831 and the United States in 1832 (25). Since the first pandemic in 1817, there have been a total of seven pandemics. The current (seventh), ongoing pandemic began in 1961 in Indonesia (26).

Cholera was originally thought to spread by an unhealthy smell or vapor, known as miasma. However, in 1854, London physician, John Snow, suggested that the disease was caused by contaminated drinking water, based on his pioneering epidemiological study in the Soho neighborhood of London (27). Separately, in 1854, Filippo Pacini observed comma-shaped objects under the microscope in stool samples from deceased cholera patients. He described these objects as infectious and the causative agent of the disease (28). However, Pacini’s publication was not widely recognized. It wasn’t until 1884, one year after Pacini’s death, that Robert Koch isolated and rediscovered the etiological agent of cholera (29), therefore receiving credit for many years as initially discovering *Vibrio cholerae* (28). In 1965, Pacini was finally recognized for his initial discovery of *V. cholerae* when the judicial commission of the international committee on bacteriological nomenclature officially changed the name of the bacteria to "*Vibrio cholerae* Pacini 1854," (30).

*Vibrio cholerae*

In 1884, the bacterium *Vibrio cholerae* was definitively determined to be the causative agent of the disease cholera (29). *V. cholerae* is a Gram-negative, curved rod-shaped bacterium belonging to the Vibrionaceae family. *V. cholerae* is classified into various serogroups by the O antigen lipopolysaccharide (31). There are over 200
serogroups (32, 33), but only the O1 and O139 serogroups cause epidemic and pandemic disease. The *V. cholerae* O1 serogroup can be further divided into two biotypes, classical and El Tor (23). Classical *V. cholerae* O1 was presumably the cause of the first six pandemics, while the seventh pandemic has been caused by the El Tor biotype. The El Tor biotype seems to have displaced the classical biotype, which appears to have become extinct in the environment (34). Previously, the O1 serogroup was thought to be the only cause of cholera, but the discovery of *V. cholerae* O139 in 1992 proved to be a new cause of the disease (35-37). The *V. cholerae* O1 and O139 serogroups currently coexist, but O139 was thought in the 1990s to be the cause of an upcoming (eighth) pandemic of cholera (23). In 2002 in Bangladesh, the number of cholera O139 associated cases exceeded the number of cholera El Tor associated cases (38). Similarly, in 2013, China reported 49 cholera cases, of which 37 were caused by O139, and 12 were caused by O1 (16). In recent years, the primary cause of cholera in most of the world has been “El Tor variant” strains, which have genomes that are largely similar to older El Tor strains but carry some classical genes as well. El Tor variants cause a more severe form of cholera than typical El Tor strains (39-41).

**Global Impact of Cholera**

In 2013, 129,064 cases of cholera and 2,102 related deaths were reported to the WHO by 47 countries. However, it is estimated that there are 1.4-4.3 million cases of cholera per year with 28,000-142,000 deaths around the world (16). The significant under-reporting is likely caused by the presumed negative impact it would have on travel and trade within infected populations. Other factors potentially influencing case report discrepancies include surveillance system limitations, case definition
inconsistencies, and lack of laboratory diagnostic tools. For example, *Vibrio cholerae* is environmentally prevalent in the Ganges delta region, however, in 2013, India only reported 6,008 cases, about 5% of the worldwide cases. They report over 2 million cases per year of acute watery diarrhea, 22% of which are estimated to be caused by *V. cholerae* and not reported (42). This is an example of inconsistencies in case definition and diagnosis.

In addition to the ancestral home of cholera in southern Asia, cholera is also currently endemic in central Africa. During a ten-year interval, from 1995-2005, there was a total of 632 cholera outbreaks reported worldwide; 66% of the total cases, and 87.6% of the fatal cases were reported from sub-Saharan Africa (43). Additionally, besides the cholera outbreak in Haiti in 2010 (to be discussed), Africa is experiencing some of the worst cholera epidemics. In 2013, 22 countries in Africa reported a total of 56,329 cases and 1,367 cholera deaths, the highest worldwide case fatality rate at 2.43% (16).

Cholera has also struck the western hemisphere, in some cases causing severe epidemics and many deaths. Most recently, on January 10, 2010, a massive earthquake struck Haiti. United Nations soldiers from Nepal were part of a group sent to Haiti to aid in recovery from this earthquake. However, apparently one or more of these soldiers was actively infected with cholerae, and due to inadequate sanitation, the infected stool leaked into the Artibonite River, resulting in a significant cholera outbreak (44-47). Haiti has not been previously exposed to cholera in at least 100 years and therefore had a naïve and susceptible population (48). The first case of *Vibrio cholerae* O1 El Tor in Haiti was reported on October 21, 2010, near the Nepali U.N. base (47). Within one week, a total of 4,722 cases and 303 deaths had been reported (49). Twenty-nine days
after the first reported case, *V. cholerae* had reached all 10 administrative departments (states) of Haiti. Various international public health organizations came together to help in the efforts to minimize damage, including the Ministry of Public Health and Population, the Pan American Health Organization and the Centers for Disease Control and Prevention (50). The Pan American Health Organization stated emphasis of the response would be on “1) minimizing mortality by using oral rehydration for most cases and intravenous rehydration for severely ill patients and 2) preventing infection by promoting water treatment, adequate sanitation and hygiene, and safe food preparation,” (51). Despite these efforts, in October 2012, two years after the first case, Haiti reported a total of 604,634 cases of cholera and 7,436 deaths since the origin of the epidemic (52). In 2013, Haiti continued to report a significant number of cholera cases, 60,763, 43.7% of the worldwide cases (16). Cholera is currently endemic in Haiti and significant numbers of cholera patients continue to be admitted to clinics each month.

**Vibrio cholerae Life Cycle**

*Vibrio cholerae* is naturally found in aquatic environments, such as freshwater ponds and rivers, and estuaries and brackish waters (53-55), where it can form commensal relationships with shellfish, copepods (crustaceans), algae, chironomid eggs masses, and can associate with various surfaces, forming biofilms (56-62). Associating with copepods and egg masses, as well as being able to form biofilms, is assumed to be a protective mechanism *V. cholerae* uses in potentially harsh environments. Being able to form biofilms allows the bacterial cells to persist between epidemic periods. *V. cholerae* also has the ability to switch to a viable, but non-
culturable state, during times of nutrient deprivation (63, 64). This is another survival strategy in which *V. cholerae* can persist in poor environmental conditions and can still infect a host.

*V. cholerae* has also been shown to be associated with various aquatic birds, such as pelicans, herons, gulls, and geese (65-67), within their fecal matter as well as externally attached to their feet and feathers (66, 68). Vertebrate fish have also recently been described as environmental reservoirs for *V. cholerae* (69-71). It has been suggested that *V. cholerae*, uses fish as a vector, both for increasing bacteria population and for transportation over longer distances. It is therefore hypothesized that fish and aquatic birds could be possible disseminators of *V. cholerae* between different bodies of water (72).

These organisms, from shellfish to copepods, are considered to be environmental reservoirs for *V. cholerae*. Environmental reservoirs are defined as “location out of the human body within the niche favouring bacterial persistence and replication in the environment and pathogen transmission susceptible host,” (73). Organisms, other than humans, such as adult chironomids (flying insects), fish, and aquatic birds, are considered to be hosts, in which a host is defined as “a living organism that temporarily harbours the pathogen, generally providing nourishment and shelter,” (73).

*V. cholerae* can dissociate from these various reservoirs and be ingested by humans in the form of contaminated water. It can also remain associated with environmental hosts and reservoirs, such as shellfish, which can then be ingested by humans as contaminated food. Humans are typically the only host of *V. cholerae* to get the disease cholera. However, a very high infectious dose, around $10^6$ to $10^{11}$ bacteria,
is required (22). In the environment and during early stages of infection, motility and chemotaxis genes are highly expressed. Once ingested by humans, _V. cholerae_ uses the expression of these genes to pass through the acidity of the stomach and bile from the duodenum, both of which are toxic to the bacteria (74, 75). _V. cholerae_ is able to pass through the stomach and into the small intestine where it colonizes the surface of the epithelium in the intestinal crypts and villi surfaces (76-78). To colonize successfully, _V. cholerae_ must downregulate motility, and upregulate virulence gene expression, which results in production of colonization factors (74, 75). Symptoms, as previously described, typically result 12-72 hours after initial colonization (17). Through either vomiting or diarrhea, an infected individual can shed hyperinfectious _V. cholerae_ back into the environment (79). The recently shed _V. cholerae_ remain in this hyperinfectious state for at least 5 hours after reentering the aquatic environment. This appears to be an effective way to enhance transmission in heavily crowded areas where it is likely another person can come in contact with the bacterium and substantially fewer bacterial cells are required to cause an infection (79, 80).

Once back in the aquatic environment, _V. cholerae_ exists in two states: free-swimming planktonic cells or fixed cells attached to various surfaces (57, 58, 60, 81). As a planktonic cell, _V. cholerae_ can persist in the previously mentioned viable, but non-culturable state (64). These cells cannot be cultured on standard media, but still perform basic metabolic processes, such as protein synthesis (82), and are still able to infect a host, and therefore are able to regain the ability to multiply (83). Other _V. cholerae_ cells, associated with various organisms, such as crustaceans, and attached to surfaces forming biofilms, can use chitin as a carbon and nitrogen source for survival (84-87).
**V. cholerae Pathogenesis Genes**

In order for *V. cholerae* to initiate disease, it must produce two main virulence factors: cholera toxin (CT) and toxin co-regulated pilus (TCP). These genes are located on the mobile genetic elements cholera toxin bacteriophage (CTXφ) and the *Vibrio* pathogenicity island (VPI), respectively (88-90). The CTXφ is found in all pandemic environmental isolates of *V. cholerae*, but is rarely found in non-O1/O139 strains (91). Besides CT, the CTXφ also encodes other accessory toxins, but the role of these toxins in pathogenesis is not well defined (92-94). Along with TCP, the VPI encodes various accessory virulence factors; however, much like the accessory toxins, the role these factors play in pathogenesis remains unclear (95, 96).

CT, essential for cholera symptoms, disrupts ion transport by the intestinal epithelial cells, leading to water and electrolyte loss, and consequently, severe diarrhea. CT consists of five smaller B subunits, responsible for binding the toxin to the target, the GM₁ ganglioside receptor, and one larger A subunit, which enzymatically acts to change the small intestine to a secreting organ instead of an absorptive one (97, 98). Ultimately, once inside the host cell, the A subunit causes an increase in cAMP, leading to secretion of chloride ions and decreased sodium uptake (99, 100). This ion imbalance causes massive secretion of water from the cells lining the lumen of the intestine (101, 102). This fluid accumulation in the intestine is the source of the characteristic watery diarrhea associated with the disease cholera.

TCP, a type IV bundle-forming pilus, is required for colonization in both animal models and human volunteers (89, 96, 103, 104). TCP is coordinately regulated with CT, therefore leading to the term “toxin co-regulated” (88, 89). The exact role TCP plays
Motility and Chemotaxis

The roles of motility and chemotaxis in *Vibrio cholerae* colonization of the human gut remain unclear; different strains, mutations, and animal models can show contradictory results (105-109). As previously stated, *V. cholerae* is highly motile, with a single, polar, sheathed flagellum. Flagella are cell surface organelles designed for locomotion. Bacterial flagella consist of the basal body, the flagellar hook, and the flagellar filament (110). The flagellar hook attaches the actual filament to the basal body. The basal body, positioned within the cell envelope, functions as the rotary motor driven by ions, specifically Na+, moving across the membrane (111-115). However, flagellar assembly is dependent upon external Na+ levels (116). If Na+ concentrations surrounding the cell decrease, the basal body will disassemble. The reason behind this physiological occurrence is not yet known.

When there are adequate levels of Na+ surrounding a cell, the assembly of the flagellum, including the basal body, flagellar hook, and flagellar filament, is successful. The basal body consists of two parts: the stator and the rotor. The basic structure of the *V. cholerae* flagellar base is shown schematically in **Figure 1**. The stator, or stationary portion, includes MotA and MotB and functions as the sodium driven motor. As ions pass through the MotA/MotB complex, the stator coupled to the rotor creates torque. The rotor, or rotary portion, contains various rings that extend through multiple
membranes and are connected by the proximal rod, which attaches to the hook. These rings have diverse functions. The C ring, composed of FliG, FliM and FliN, also considered the switch complex, is the main trigger for directional switch of flagellar rotation. The MS ring, P ring, and L ring, located in the cytoplasmic membrane, peptidoglycan layer, and outer membrane respectively (115, 117), do not rotate but function as bushing around the proximal rod of the flagellum. Lastly, the T ring, which surrounds the periplasmic side of the P ring (118), consists of MotX and MotY, which are thought to stabilize the stator surrounding the rotor, and are considered to be essential for motor function (118-121).

*V. cholerae* uses its flagellum for motility and chemotaxis to move through a host toward a prime site of colonization. In general, chemotaxis is the ability of bacteria to move with or against a chemical gradient. This process in *V. cholerae* is very complex with three chemotaxis operons, 68 open reading frames (ORF), and multiple gene duplications. Only one of the three operons is said to be essential for chemotaxis (122, 123). Of the 68 ORFs, 46 are believed to encode possible methyl-accepting chemotaxis proteins (MCP), and 22 are homologues for *che* genes, including five *cheY*, three *cheA*, three *cheB*, and two *cheW* gene duplications (124). MCPs are the proteins that sense the chemical signals from outside of the cell and trigger downstream chemotactic and motility effects. With so many MCPs, *V. cholerae* has the ability to travel toward and away from many signals. The complexity of this system is presumably because *V. cholerae* needs to be able to survive in many different aquatic environments.

Chemotaxis in *V. cholerae* (diagramed in **Figure 2**) is initiated when a MCP senses a chemical attractant or repellent outside of the cell. The MCP signals the
sensor kinase, CheA, to autophosphorylate. CheA, associated with cytoplasmic linker protein CheW, can then transfer the phosphate to three different proteins. Two mechanisms of negative feedback include the phosphate being transferred to either CheB or CheV. Phosphorylated CheB causes demethylation of the MCP, therefore ceasing signal output, and phosphorylated CheV prevents the signal from the MCP from reaching CheA. CheA can also transfer its phosphate to CheY, the response regulator. CheY then triggers the C ring, specifically FliM, to switch flagellar rotation. The C ring then signals the motor, composed of MotA and MotB, to switch direction. Very little is known about how CheY signals the C ring, and how the C ring signals the motor.

Many studies on the roles of motility and chemotaxis in host colonization have been performed with contradictory results. In one study, various chemotaxis genes from the El Tor biotype were deleted and colonization in the infant mouse model was measured. Multiple *V. cholerae* non-chemotactic mutants actually hypercolonized the infant mouse intestine (125). This same study reported that a non-chemotactic mutant colonized the entire length of the intestine, whereas the wild-type strain was localized to the distal portion of the infant mouse intestine (125), suggesting *V. cholerae* uses chemotaxis to find a preferred site of colonization. It has also been suggested by Freter *et al.* (107, 108) that non-chemotactic mutants likely lack the ability to enter the intestinal crypts, and therefore are not killed by the innate immune system. Consequently, it is assumed non-chemotactic *V. cholerae* outcompeting wild-type in intestinal colonization is due to the fact that it is avoiding killing by the immune system.

In another study, various chemotaxis and motility mutants from the classical and El Tor biotypes were tested for colonization in three separate animal models (109). There were varying results between the different strains and models. Richardson (109)
also proposed that motility likely increases the number of interactions between the bacterium and intestinal epithelium. In addition to motility, chemotactic responses can also enhance the number of interactions. Ultimately, this study concluded that motility is indeed a major factor contributing to pathogenicity and colonization.

**Zebrasfish and Other Models**

Various model organisms have been used to demonstrate *Vibrio cholerae* colonization, infection and pathogenesis. Common mammalian animal models include the suckling mouse model (126, 127), and the rabbit ligated ileal loop and the removable intestinal tie-adult rabbit diarrhea (RITARD) models (128-130). However, these animal models are not natural hosts for *V. cholerae* infection. The suckling mouse does not demonstrate signs of pathogenesis and the adult rabbits develop a disease unlike that of the human cholera infection. Adult rabbits also required various manipulations including a clearing of the intestinal microbiota and survival surgery. However, a new model for *V. cholerae* colonization has been recently described.

The adult zebrafish has been described as a natural host model for *V. cholerae* colonization (70). Zebrafish become colonized naturally, with no manipulation. According to a previous study, *V. cholerae* was found colonizing the intestines of 10 different wild-caught fish species in Israel (69). Another study found that between 10% and 17% of tilapia were colonized by *V. cholerae* during the warm season in Burkina Faso (71). This suggests that *V. cholerae* may use vertebrate fish as both a vector for transport to various locations, as well as a reservoir to increase bacterial population.

The zebrafish intestine consists of one long tube that folds over twice in the abdomen connecting the esophagus to the anus. Zebrafish do not have an acidified
stomach and there is no distinction between the small and large intestine. However, variances in morphologies throughout the intestine suggest a functional differentiation. The morphology of the mucosal columnar epithelial cells and increasing number of goblet cells support this idea (131). Also, progressing from rostral-to-caudal direction, the tube narrows and mini folds within the lumen become progressively shorter. These mini folds are significantly larger than the microvilli within a mammal intestine (132). However, a study by Runft et al. (70) presented data where wild-type *V. cholerae* was able to reach and presumably colonize within these intestinal folds in the zebrafish intestine.

The goal of this work is to better understand how motility and chemotaxis effect *Vibrio cholerae* colonization in a rather new model, the zebrafish. Zebrafish are suggested to be a natural host to *V. cholerae* as they become colonized with no manipulation. Although extensively studied in other animal models regarding their role in pathogenesis, not much is certain about how chemotaxis and motility effect colonization and disease. A list of strains used in this work is included in Table 1. This thesis characterizes the roles various chemotaxis and motility genes play in colonization in the zebrafish model.
FIGURE 1. Flagellar structure. The flagellar basal body consists of two main parts, the stator and the rotor. The stator, or stationary portion, included MotA and MotB and function as the sodium driven motor. The rotor, or rotary portion, includes the L ring, P ring, and MS ring, located in the outer membrane, peptidoglycan layer, and cytoplasmic membrane respectively. These rings do not rotate, but act as bushing around the proximal rod. The rotor also includes the C ring, which is responsible for directional switch of the flagellum. Lastly, the T ring, surrounding the periplasmic side of the P ring, is thought to stabilize the stator around the rotor.
FIGURE 2. Chemotaxis in Vibrio cholerae. Chemotaxis is initiated when a methyl-accepting chemotaxis protein (MCP), senses a chemical single, a chemoattractant or chemorepellent, from outside the cell. The MCP signals the sensor kinase, CheA, associated with cytoplasmic linker protein CheW, to autophosphorylate. CheA can negatively regulate chemotaxis by transferring its phosphate to either CheB, which demethylates the MCP, or to CheV, which inhibits the MCP signal from reaching CheA. CheA can also transfer its phosphate to CheY, the response regulator. CheY then signals the C ring to switch flagellar rotation. The C ring then signals the motor, composed of MotA and MotB, to switch direction.
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*Table 1.* List of strains used in this work.
CHAPTER ONE

The effects of motility and chemotaxis on *Vibrio cholerae* colonization in zebrafish

ABSTRACT

*Vibrio cholerae*, the causative agent of the diarrheal disease cholera, is a gram-negative, curved rod-shaped bacterium, with a single polar flagellum. *V. cholerae* is naturally found in aquatic environments and is highly motile. When it enters a human host, *V. cholerae* uses flagellar motility and chemotaxis to pass through the stomach and into the small intestine. Once in the small intestine, motility genes are downregulated and virulence gene expression is upregulated. *V. cholerae* motility and chemotaxis effects have not yet been studied in a zebrafish model, a natural host of this bacterium. We have predicted that *V. cholerae* in frame deletions of vital motility and chemotaxis genes, such as *flaA*, *cheY*-3, and *motY*, would decrease the ability of *V. cholerae* to colonize the zebrafish intestine. However, we find instead that the deletion of chemotaxis gene *cheY*-3, actually significantly increases the ability of *V. cholerae* to colonize the zebrafish intestine, and only the deletion of motility gene *motY* decreases the ability of *V. cholerae* to colonize.

INTRODUCTION

The disease cholera is characterized as an intestinal infection, accompanied by voluminous, watery diarrhea, leading to severe dehydration and even death. The bacterium responsible for this disease, *Vibrio cholerae*, is a gram-negative curved rod-shaped bacterium with a single polar flagellum. *V. cholerae* is highly motile and uses this ability, along with chemotaxis, to navigate through a host, and toward a prime site of
colonization. Although extensively studied in their roles in regard to pathogenesis, little is known about the effects motility and chemotaxis have on colonization. To further characterized their importance, we selected various chemotaxis and motility genes to delete from the *V. cholerae* El Tor biotype N16961. These deletion mutants include Δ*cheY*-3, Δ*cheY*-4, Δ*cheA*-2, Δ*flaA*, Δ*motA*, Δ*motB*, Δ*motAB*, and Δ*motY*. The reason for selection of these specific genes is described below.

Chemotaxis in *Vibrio* cholerae is a very complex process, so we wanted to select some genes that are thought to be key players in this system. Based on previous studies on chemotaxis, we chose to focus on *cheY*-3, *cheY*-4, and *cheA*-2. According to sequence analysis, of the five *cheY* homologues in *V. cholerae*, *cheY*-3 is most similar to the *E. coli* CheY protein (124). Also, a *cheY*-3 transposon insertion mutant has been reported as being non-chemotactic as seen on swarm plate analysis (125). It has also been stated that only *V. cholerae cheY*-3, and not *cheY*-1, *cheY*-2, or *cheY*-4, can affect flagellar rotation, and only a *cheY*-3 deletion shows this chemotaxis defect (124, 133, 134). That being said, we also chose to delete *cheY*-4 because it is suggested to be involved in attachment to host cells and migration towards the intestine (134). CheY-4 is also thought to modulate chemotaxis indirectly by regulating the expression of other unknown factors (134). Our third chemotaxis target was *cheA*-2, the presumed sensor kinase responsible for receiving the signal from the MCP and in turn initiating chemotaxis. Of the three *cheA* homologues, only *cheA*-2 is thought to be essential for chemotaxis (123). A *cheA*-2 transposon insertion mutant is show to be non-chemotactic (124), and the *V. cholerae cheA*-2 gene actually encodes a functional homologue of the *E. coli* CheA protein (123). We hypothesized that *V. cholerae* mutants deficient in chemotaxis would have decreased colonization in the zebrafish compared to wild-type.
We also created four *Vibrio cholerae* motility mutants, ΔmotA, ΔmotB, ΔmotAB, and ΔmotY, and a flagellin mutant, ΔflaA. The flaA deletion is non-motile and non-chemotactic (125). *V. cholerae* has five flagellin genes, flaA-E, however, flaB-E are not essential for flagellar synthesis and only a mutation in flaA results in the motility defect (135). Based on an electron micrograph from a previous study, we can assume our flaA deletion mutant does not have a flagellum (125). We also generated four mutants, ΔmotA, ΔmotB, ΔmotAB, and ΔmotY, with flagellar motor defects. MotA and MotB function as the sodium-driven rotary motor, therefore, deletions in these genes result in a non-functioning or non-existent flagellar motor. MotY is considered to be essential for motor function (136), and is thought to stabilize the motor around the rotor (118-121). We hypothesize that non-motile *V. cholerae* mutants would colonize the zebrafish less than wild-type.

In this study we characterized the effects various *Vibrio cholerae* chemotaxis and motility genes have on colonization in the zebrafish. We report that deletion of cheY-3, rendering *V. cholerae* motile but non-chemotactic, significantly increases colonization levels compared to wild-type. However, deletion of other chemotaxis genes, such as cheY-4 and cheA-2, does not significantly alter colonization levels. We also show that deletion of motY, rendering *V. cholerae* non-motile, significantly decreases colonization levels in the zebrafish intestine. However, deletion of other motility genes, such as motA and motB, does not significantly alter colonization levels. Deletion of the flagellum, flaA, from *V. cholerae* also does not significantly change colonization levels compared to wild-type.
MATERIALS AND METHODS

**V. cholerae strains and growth conditions.** All *V. cholerae* strains used in this study are derived from the El Tor biotype N16961 and are listed in Table 1. Strains were maintained in LB (Luria Broth) containing 20% glycerol and stored at -70°C. Overnight cultures were grown in LB with an antibiotic concentration of 100 µl/ml streptomycin for approximately 16 hours shaking at 37°C.

**Strain construction.** All *V. cholerae* mutants in this work were created with in-frame deletions of the various genes. Site-directed deletion mutants were completed using splicing by overlap extension PCR (137). PCR products, with specific restriction enzymes, were inserted into suicide vector, pKAS32, carrying an ampicillin resistance marker. The plasmid was then transformed into *E. coli* SM10 λpir and subsequently mated with *V. cholerae* N16961. Mated cultures were plated on *V. cholerae* selective TCBS plates and then hi-strep (1 mg/ml streptomycin) LB plates. Deletions were confirmed using PCR.

**V. cholerae motility analysis and chemotactic swarm assay.** Bacterial cell motility was determined by visualizing 5 µl of overnight or sub-culture under a light microscope and determining motile or non-motile based on observed swimming. To measure chemotactic ability, swarm agar plates (1% tryptone, 0.5% NaCl, 0.3% agar) were spot inoculated with 1 µl of normalized overnight cultures of wild-type or mutant strains of *V. cholerae*. The plates were incubated at room temperature for 24 hours and measured for swarm diameter.

**Zebrafish.** Experiments were performed using six to nine month old ZDR wild-type zebrafish. The zebrafish were euthanized in 100 ml of a double dose (336 µl/ml) of Tricaine (ethyl-3 aminobenzoate methanesulfonate salt; catalog no. A50040; Sigma)
solution. The fish remained in the solution for 25-30 minutes. Zebrafish were infected and dissected as described below. All animal protocols were approved by the Wayne State University IACUC committee.

**Inoculation of Zebrafish via water.** Bacterial cultures were washed twice in phosphate-buffered saline (PBS) and re-suspended to the correct concentration using PBS. Cultures were washed by centrifugation and re-suspension unless otherwise noted. Prior to adding the bacterial cultures to the fish water, four to five zebrafish were placed in a 400 ml beaker with a perforated lid containing 200 ml of tank water (sterilized ddH2O with 60 mg/liter of Instant Ocean aquarium salts [138]). One milliliter of bacterial inoculum with concentrations ranging from 10^8 to 10^9 CFU per beaker (5x10^5-6 CFU/ml) was added to the fish infection beakers. Each beaker was placed in a glass front incubator set at 27°C for the duration of the experiment.

Several experiments included the addition of a wash step of the fish between four and six hours post infection. Fish infection beakers were drained of water, and fresh 200 ml of tank water was added. Fish were allowed to swim for five minutes before this wash was repeated. Lastly, fish infection beakers were again drained of their water, and fish were transferred to fresh beakers with fresh 200 ml of tank water. Beakers were then returned to the incubator for the reminder of the experiment.

**Determination of V. cholerae colonization in zebrafish intestine.** At 24 hours post infection, fish were removed from the beakers and euthanized as described above. Intestines were aseptically removed, placed in 900 µl of LB containing 1 mm glass beads, and homogenized using a mini-beadbeater, in three pulses for 1 minute, with a 2 minute-on ice-intermittent period. Serial dilutions of the homogenate were performed and plated onto selective media for enumeration.
RESULTS

All mutant strains have decreased chemotaxis compared to wild type. To ultimately test the effects chemotaxis and motility have on colonization in the zebrafish model, we created mutants that exhibited various defects in these areas. Mutants were constructed from parental strain V. cholerae El Tor N16961. In frame deletions were executed for the following genes: cheY-3, cheY-4, cheA-2, motA, motB, motAB, motY, and flaA. Overnight and sub-cultures of the mutants were visualized using light microscopy and determined to be either motile or non-motile compared to wild-type by observed swimming. Non-motile mutants included ΔmotA, ΔmotB, ΔmotAB, ΔmotY, and ΔflaA. Mutants with motility similar to wild-type levels include ΔcheY-3, ΔcheY-4, and ΔcheA-2.

All mutant strains were also tested on swarm agar to quantify chemotactic properties. Bacterial cultures were inoculated into swarm plates as described above, and incubated overnight at room temperature. Plates were photographed (Figure 3a) and swarm diameter was measured (Figure 3b). All V. cholerae chemotaxis and motility mutants (average diameters of 1.5 mm to 12.5 mm) showed significantly less swarm diameter than wild-type (average 15.5 mm). Our results support those from a previous swarm plate analysis study (125) where cheY-3 and cheA-2 transposon insertion mutants rendered the bacteria non-chemotactic and in-frame deletions of flagellar motor genes, motAB and motY, and flagellin gene, flaA, generated mutants that are non-motile.

Deletion of cheY-3 leads to significantly increased colonization in zebrafish. V. cholerae presumably uses chemotaxis to move through a host to the prime site of colonization, and therefore, chemotaxis is assumed to be essential for
colonization and disease. In this study, a non-chemotactic *V. cholerae* mutant, ΔcheY-3, was able to colonize the zebrafish intestine to levels significantly higher than wild-type (Figure 4). Our study corresponds to a previous study where a cheY-3 transposon insertion mutant, and a cheY-3 deletion mutant were able to outcompete wild-type *V. cholerae* in the infant mouse model (140).

Other *V. cholerae* chemotaxis mutants do not show the same increased colonization in the zebrafish as our ΔcheY-3 mutant. Our results of the cheY-4 deletion show no significant colonization differences in zebrafish compared to wild-type (Figure 5A-C). This result supports previous findings suggesting cheY-4 does not affect chemotaxis (134). Our result of the cheA-2 deletion also shows no significant colonization differences in the zebrafish compared to wild-type *V. cholerae* (Figure 5A-C). These mutants, ΔcheY-4 and ΔcheA-2, were tested in the zebrafish using three different procedures. The results shown in Figure 5A and Figure 5B were performed using procedures described previously. The third set of results (Figure 5C) was performed by washing the bacteria by vacuum filtration instead of centrifugation prior to infection. Previous studies have shown that high-speed centrifugation can actually alter the bacterial cell surface and the viability of the organism (141-142). Through preliminary testing, and visualization using light microscopy, we determined that centrifuged bacterial cells exhibited decreased motility compared to vacuum filtered cultures. Vacuum filtration was then tested to see if there was a difference in colonization by possible bacterial damage during high-speed centrifugation. Ultimately, vacuum filtration compared to centrifugation appeared to not alter colonization levels in the zebrafish model, and therefore, we concluded that there were not significant differences between procedures (Figure 5A-C).
Deletion of *motY* leads to significantly decreased colonization in zebrafish.

Motility of *V. cholerae* is considered to be a major factor contributing to pathogenesis and colonization. In human infection, *V. cholerae* uses motility to pass through the stomach and into the small intestine, where it colonizes. Although the anatomy of zebrafish and humans is quite different, we assumed *V. cholerae* would use motility to navigate through the zebrafish intestinal tract. Our results show, that a *V. cholerae motY* deletion mutant colonizes significantly less than wild-type (Figure 5A). The other results for ΔmotY infections (Figure 5B, C), using the alternate infection procedures, do not show this difference. It is possible more replications using these procedures are needed for more definitive results.

While the *motY* deletion mutant colonizes significantly less than wild-type in the zebrafish model, other deletions affecting motility do not alter colonization. We show that deletions disabling the flagellar motor, ΔmotA, ΔmotB, and ΔmotAB, do not colonize significantly differently from wild-type *V. cholerae* (Figure 6). While there do appear to be differences in colonization, these variances are not significant. Our study also indicates that deletion of the *V. cholerae* flagellar filament, flaA, does not result in colonization levels different than wild-type (Figure 4).
Our findings indicate that while all of the mutants show significantly decreased swarm diameters compared to wild-type, only a few mutants show significantly altered colonization differences in the zebrafish. Non-motile *Vibrio cholerae*, including ΔflaA, ΔmotA, ΔmotB, and ΔmotAB mutants, have no significant defect in colonization. Even with deletion of the flagellum and deletion of the flagellar motor, *V. cholerae* still has the ability to enter and colonize the zebrafish intestine. This could be due to the fact that *V. cholerae* and the zebrafish are placed within a confined space. While, the mutant *V. cholerae* cannot swim, the zebrafish are continually moving and stirring up the water. We can assume that while swimming, the fish come in contact with and ingest many *V. cholerae* bacterial cells. The *V. cholerae* cells do not appear to need to swim to reach the zebrafish intestine.

We have also shown that various chemotaxis mutants, including the cheY-4 and cheA-2 deletions, do not colonize significantly differently from wild-type *V. cholerae* in the zebrafish model. It is possible that the other cheA homologues can compensate for the cheA-2 deletion. Furthermore, cheY-4 has previously been stated as appearing to not have an effect on chemotaxis (139). Unlike CheY-3, CheY-4 cannot bind the C ring to switch flagellar rotation (139). CheY-4 could however pull the phosphate from CheY-3. A study by Hyakutake *et. al.* (134) suggests that CheY-4 could regulate the expression of certain factors that in turn modulate chemotaxis indirectly. Our results can neither confirm nor deny these suggestions.

Some *V. cholerae* genes however, do appear to affect colonization within the zebrafish model. The deletion of cheY-3 for example, surprisingly shows a significant increase in colonization in the zebrafish compared to wild-type *V. cholerae*, while the
deletion of motY shows a significant decrease in colonization compared to wild-type. The motY deletion was the only non-motile mutant to appear to significantly impact colonization in the zebrafish. It is unclear why one non-motile mutant colonized significantly less than wild-type when the others, ΔflaA, ΔmotA, ΔmotB, and ΔmotAB, demonstrated no significant difference.

As stated, one of our chemotaxis mutants, the V. cholerae cheY-3 deletion, colonized to significantly higher levels than wild-type in the zebrafish model. As chemotaxis is defined as the ability of an organism to move toward or away from a chemical signal, in our model, chemotaxis is presumably the ability of V. cholerae to move toward a desired site of colonization. Without the ability to do this, we assumed that a non-chemotactic mutant would show decreased colonization levels. This is, however, not the case in the zebrafish model. A non-chemotactic V. cholerae cheY-3 deletion mutant colonizes significantly higher than wild-type in the zebrafish model. There are a couple of explanations to describe why this is the case. It is possible that the non-chemotactic V. cholerae cannot get within the intestinal folds of the zebrafish intestine, cannot reach the intestinal epithelial cells and therefore, cannot be fought off by the immune system. This has been previously postulated to be the case in mice (107, 108). Another possibility for this colonization increase could be that the mutant V. cholerae are unable to chemotax to the prime site of colonization, and therefore, the bacterial cells colonize the entire length of the fish intestine. In the human gut, V. cholerae is known to colonize the upper small intestine. While zebrafish do not have distinct large and small intestines, there do appear to be differences in morphologies, suggesting a functional differentiation throughout the intestine. Experiments comparing rostral and distal colonization in the zebrafish have not yet been performed. However,
Lee et al. (125), did find that a *V. cholerae cheY-3* transposon insertion mutant was able to colonize the entire length of the small intestine of infant mice, where wild-type *V. cholerae* primarily colonized the lower small intestine.

This study is meant to characterize the effects of chemotaxis and motility on colonization in the zebrafish model. Since vertebrate fish have been proposed as a natural host for *Vibrio cholerae* we hope these findings can shed some light on the importance of motility in the *V. cholerae* life cycle. Within a confined space, *V. cholerae* does not necessarily need all genes associated with chemotaxis and motility to colonize a natural host, like the zebrafish, successfully. However, this may not be true when *V. cholerae* is in its natural aquatic environment, such as a freshwater pond or river. Although *V. cholerae* chemotaxis and motility are stated as important in pathogenesis, the direct effects they have on colonization in the human intestine remain unknown.
FIGURE 3. Motility and chemotaxis in semisolid agar. (A). Chemotactic ability of each mutant was determined by stabbing overnight cultures of cells into swarm plates. Strain genetic backgrounds are indicated by each swarm location. Assay was performed in triplicate, two plates are not shown. (B). Swarm diameters were measured in triplicate and compared to wild-type. Statistical significance was determined by one-way ANOVA, *** (p<0.001).
FIGURE 4. Zebrafish colonization assay: wild-type *Vibrio cholerae*, and Δ*cheY*-3 and Δ*flaA* mutants. *V. cholerae* Δ*cheY*-3 mutant colonizes significantly higher than wild-type in the zebrafish model. Each symbol represents the data from one zebrafish. Statistical significance was determined by one-way ANOVA, * (p<0.05). Values are normalized to WT as follows: 

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\frac{\text{cfu/ml}_{\text{WT}}}{\text{cfu/ml}_{\text{mut}}} \times \text{cfu/intestine}_{\text{mut}}
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FIGURE 5. Zebrafish colonization assays: wild-type *Vibrio cholerae*, and ΔcheY-4, ΔcheA-2, and ΔmotY mutants. (A) *V. cholerae* ΔmotY mutant colonizes significantly lower than wild-type in the zebrafish model. (B) Infections were performed with the wash step of the fish. Mutants do not colonize significantly differently than wild-type. (C) Prior to fish infections, bacterial cultures were washed by vacuum filtration, not centrifugation. Mutants do not colonize significantly differently from wild-type. Each symbol represents the data from one zebrafish. Values are normalized to WT as previously stated. Statistical significance was determined by one-way ANOVA, * (p<0.05).
FIGURE 6. Zebrafish colonization assay: wild-type *Vibrio cholerae*, and Δ*motA*, Δ*motB* and Δ*motAB* mutants. Infections were performed with the wash of the fish. Mutants do not colonize significantly different than wild-type. Each symbol represents the data from one zebrafish. Values are normalized to WT as previously stated.
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ABSTRACT

THE EFFECTS OF MOTILITY AND CHEMOTAXIS ON VIBRIO CHOLERAE COLONIZATION IN ZEBRAFISH

by

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Major: Immunology & Microbiology

Degree: Master of Science

*Vibrio cholerae*, the cause of the diarrheal disease cholera, is a gram-negative, curved rod-shaped bacterium, with a single polar flagellum. *V. cholerae* is naturally found in aquatic environments and is highly motile. When it enters a human host, *V. cholerae* uses flagellar motility to pass through the stomach and into the small intestine. Once in the small intestine, motility genes are downregulated and virulence gene expression is upregulated. *V. cholerae* motility and chemotaxis effects have not yet been studied in a zebrafish model, a natural host of this bacterium. We hypothesize that *V. cholerae* in frame deletions of vital motility and chemotaxis proteins, such as *flaA*, *cheY*-3, and *motY*, would decrease the ability of *V. cholerae* to colonize the zebrafish intestine. However, the deletion of chemotaxis gene *cheY*-3 actually significantly increases the ability of *V. cholerae* to colonize the zebrafish intestine, and only the deletion of motility gene *motY* significantly decreases colonization compared to wild-type.
AUTOBIOGRAPHICAL STATEMENT

Born and raised in Michigan, I graduated from Novi High School in 2008, and began my undergraduate education at Grand Valley State University. I initially majored in Health Professions with a plan to attend graduate school to become a Doctor of Physical Therapy. Realizing this was not the profession for me, I switched to a Biomedical Science major where I was introduced to Microbiology and Virology courses. After graduating from Grand Valley State University in 2012, I took a year off to coach varsity and youth volleyball, and temporarily work in a restaurant. Realizing I wanted to return to graduate school with a focus on Microbiology, I applied to the Wayne State University Immunology and Microbiology program. I was accepted to start the Fall semester of 2013. I initially planned to join a Virology lab, but after completing three rotations, two bacteriology and one virology rotation, I decided to join Jeff Withey’s lab to work with *Vibrio cholerae*. Being new to the lab experience, I learned many useful techniques from various lab members. This lab has prepared me to be a successful scientist and to continue working at the bench. After completion of my M.S., I plan to continue bench work in academia, or industry research labs.