THE EFFECT OF POLYAMINES AND BIOFILM FORMATION ON ZEBRAFISH COLONIZATION BY *VIBRIO CHOLERAE*

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by
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Abstract

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Vibrio cholerae, the intestinal pathogen responsible for the diarrheal disease cholera, is an aquatic bacterium that utilizes biofilms as an integral part of its life cycle. Biofilms are clusters of cells, which are surrounded by and contained within a matrix that is self-produced by the bacteria. These biofilms have been shown to protect bacteria from environmental stresses as well as facilitate adhesion to biotic and abiotic surfaces and are thought to aid in the transmission of *V. cholerae* into its hosts. It has previously been shown that polyamines play an important role in regulating biofilm formation and several genes encoding components of polyamine synthesis and transport pathways are upregulated in animal models of infection. Zebrafish have been used in previous colonization studies of *V. cholerae* and are established as an effective model organism. In this work, the zebrafish model was used to determine the colonization efficiency of wild-type bacteria compared to mutant strains that were deficient in regulating biofilm formation as well as in polyamine signaling, transport, and synthesis pathways. Results to date indicate that Δ*vpsA* mutants, which lack the ability to form mature biofilms, appear to have a competitive advantage in early stages of colonization of zebrafish. The

NspS/MbaA biofilm regulation pathway, however, has no impact on early colonization efficacy. Disruption of putrescine transport appears to confer a competitive advantage in zebrafish colonization. In contrast, disrupting norspermidine and spermidine import appears to confer a disadvantage in zebrafish colonization. Furthermore, disruption of the synthesis of the polyamines norspermidine and putrescine negatively impacts *V. cholerae's* ability to outcompete microbiota local to the zebrafish intestine. Our results suggest that several polyamines have a definite impact on the ability of *V. cholerae* to colonize the zebrafish intestine.

Acknowledgements

I would like to thank Dr. Mary Kinkel and Dr. Ted Zerucha for all of their assistance in serving on my committee and training me in zebrafish husbandry as well as showing me effective techniques for the surgical portions of this work. I would like to convey my most sincere gratitude and respect for my advisor, Dr. Ece Karatan. She has helped me improve the quality and depth of my thinking and helped cultivate in me a higher capacity in and appreciation of good research. I am grateful for the time, effort, and experience which she so freely shared with me and know that it will have great impact on my future endeavors in the sciences.

There are many others among both faculty and students that have contributed to making this work better, always with positive and constructive attitudes that reflect on the culture that the Appalachian State Biology Department has cultivated.

I would like to thank the Office of Student Research for its support of this project, both through direct funding of certain aspects of my research as well as in assistance with travelling to conferences where I was able to interact with other scientists and gain new perspectives. I would also like to thank the Appalachian State Biology Department for its assistance in funding this work.

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Introduction

V. cholerae is a motile, gram negative, curved rod-shaped bacterium that is found in aquatic environments. While *V. cholerae* is a natural inhabitant of aquatic ecosystems, there are two serogroups, O1 and O139, which are able to colonize humans and cause the severe diarrheal disease cholera. The severity of the diarrhea is due to the actions of cholera toxin (CT) that infectious *V. cholerae* produces. Cholera is spread through the consumption of contaminated water and is known to cause epidemics, typically in developing countries [1].

In order for successful infection of a human host, *V. cholerae* must first survive passage through the gastric barrier of the stomach. One process that aids in overcoming this barrier is the formation of biofilms. Biofilms are thought to play a role in assisting in passage through the acidic environment of the stomach and are known to generate a hyper-infectious state within *V. cholerae* [2]. Biofilms consist of bacterial cells embedded in a self-produced matrix consisting of exopolysaccharides, proteins, and genetic material [2]. When *V. cholerae* enters the intestine various signals cause an upregulation of the genes associated with virulence and it begins to produce and secrete CT. CT is an AB type toxin that is composed of one A subunit and five identical B subunits that are able to bind to the ganglioside GM_1 on cell membranes [3]. CT binds to the epithelial cell apical membrane through its B subunit which tethers the toxin to the membrane [4]. Through retrograde transport, the A subunit travels in a vesical to the endoplasmic reticulum (ER) of the target cell [4]. CT enters the ER while still fully folded, but once in the ER, the host chaperone protein disulfide isomerase (PDI) recognizes the A1 chain and dissociates it from the B subunits. The A subunit is comprised of two peptides that are linked by noncovalent interactions and one disulfide bond. During the translocation process this

disulfide bond is reduced which leads to the disassociation of the enzymatically active A1 peptide [5]. After separation from the B subunits, retro translocation is thought to occur by passage of the A subunit through the ER's sec61 channel [4] (Figure 1). This active A1 subunit is able to activate adenylate cyclase within the cytoplasm of the target cell. This activation occurs through the manipulation of the G protein $G_s\alpha$, a GTPase present in epithelial cells. GTPases regulate biochemical processes within the cell utilizing a common mechanism which allows them to activate or inactivate a signal transduction cascade [6]. Upon binding of GTP, the GTPase is activated and stimulates adenylate cyclase and cyclic adenosine monophosphate (cAMP) production. When GTP is hydrolyzed to GDP the GTPase reverts to its inactive state.

When CT is introduced to the cytoplasm of the cell, it binds to adenylate cyclase and inhibits the hydrolysis of GTP into GDP leading to constitutive activation of adenylate cyclase and increased production of cAMP [3]. High cAMP levels activate the cystic fibrosis transmembrane conductance regulator (CFTR) and lead to increased trans-epithelial secretion of chloride ions [7]. This provides the driving force for the secretion of Na+ and Cl- ions as well as water from the cytoplasm of intestinal epithelial cells into the lumen of the intestine [8].

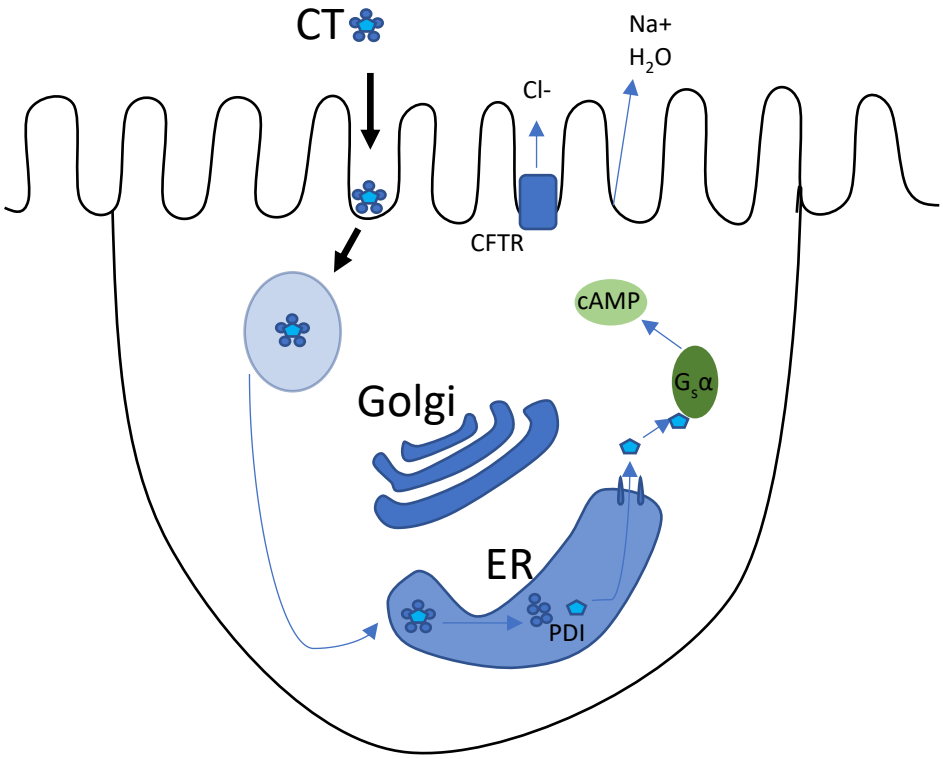

Figure 1. Simplified process of retrograde transport of cholera toxin into the ER of an intestinal epithelial cell. The whole CT is sent to the ER, where the A and B subunits are separated. The activated A subunit then is released into the cytoplasm, where it binds to $G_s\alpha$. This constitutively activates $G_s\alpha$ and stimulates excess cAMP production, leading to secretion of water and ions, accounting for the severe diarrhea the disease is known for. (Modified from Lencer, 2004)

V. cholerae makes use of both a motile planktonic state and a stationary biofilm state in order to effectively survive in its environment as well as within the host in the event of an infection. In its planktonic state *V. cholerae* is motile and utilizes its polar flagellum to swim through the water, while in a biofilm community, *V. cholerae* does not produce its flagellum and is stationary. In order to form a biofilm, *V. cholerae* generally must find a suitable surface to attach to (Figure 2).

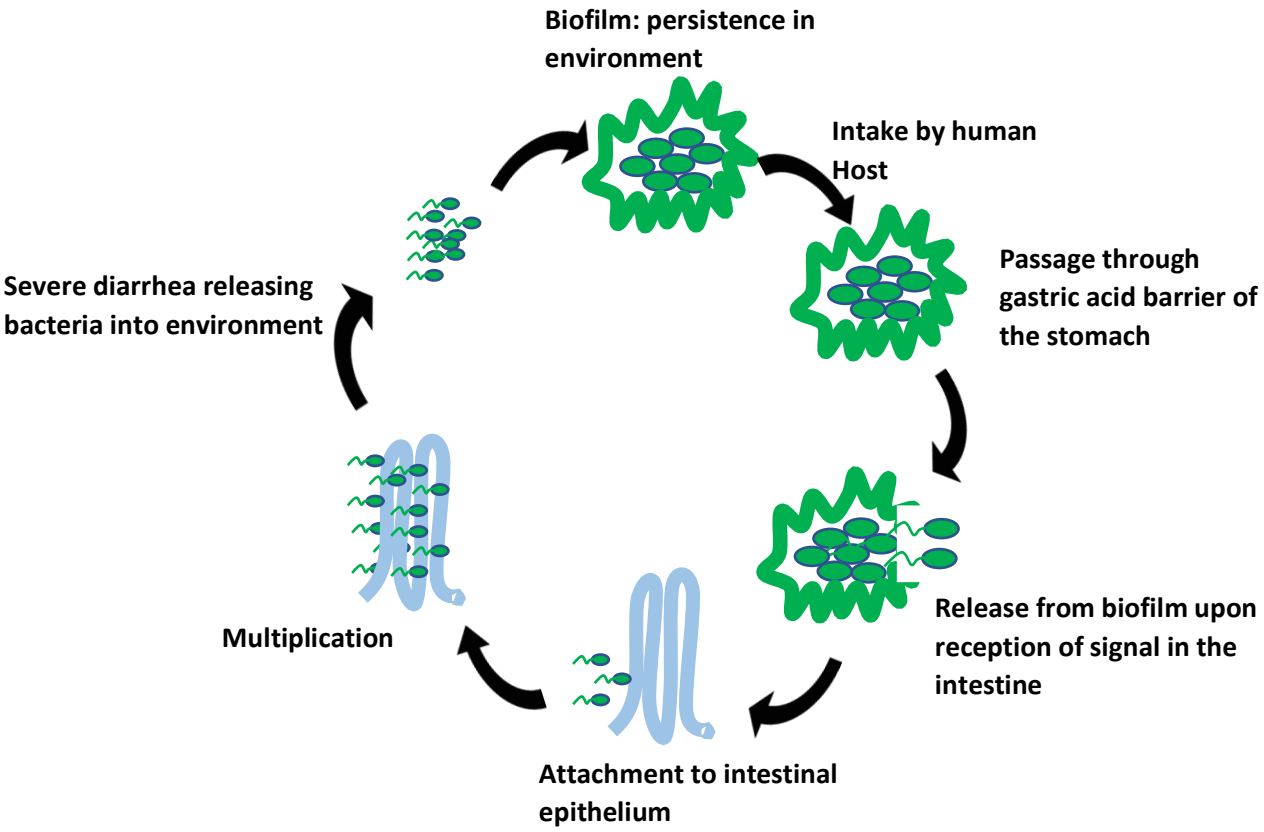

Figure 2. *V. cholerae's* lifecycle is modulated in part by external signals, some of which control biofilm formation. Biofilms play a role in persistence in the aquatic environment, shown at the top of the figure. As *V. cholerae* comes into contact with a human host, its lifestyle undergoes changes that eventually lead to disease. Biofilms are thought to aid in passage through the gastric barrier, after which signaling that occurs in the intestine prompts a release from the biofilm state. After releasing from the biofilm state, attachment to intestinal epithelia occurs and as the bacteria multiply, they produce their toxins leading to the diarrheal disease cholera. The resultant diarrhea enables reintroduction to the environment where biofilms may once again form to aid in persistence.

To find a suitable surface for attachment, *V. cholerae* use both their polar flagella as well as their mannose-sensitive hemagglutinin (MSHA) type IV pili [8]. The flagella and pili are responsible for two complementary motility behaviors known as roaming and orbiting respectively. The roaming motility is characterized by persistent trajectories which allow *V. cholerae* to travel long distances, while the orbiting motility involves smaller circular

trajectories, which allow for prolonged and repeated contact with encountered surfaces. The function of flagella and roaming motility in regard to biofilm formation is in aiding the *V. cholerae* in reaching a surface to which it may attach. While swimming in its planktonic state, nearby surfaces will cause the cell to experience hydrodynamic forces that both attract them towards the surface, and cause them to swim in circular trajectories. Once this broad swimming action brings *V. cholerae* into contact with a surface, the MSHA pili are able to facilitate a change in motility from the roaming behavior to the circular orbiting behavior. Orbiting allows the MSHA pili ample opportunity to interact with an encountered surface, and should the surface prove to have a high enough affinity for the MSHA pili, will lead to permanent attachment to the surface with which they have come in contact with [9]. Following the initial stages of cell attachment, the presence of certain sugars such as the monosaccharide mannose [10] will induce the production of the extracellular matrix [11]. The primary component of the biofilm matrix is Vibrio Polysaccharide (VPS) which makes up approximately 50% of the mass of the biofilm matrix and is secreted from *V. cholerae* cells shortly after the initial attachment. The *vps* genes that encode proteins required for the formation of this polysaccharide are found on *V. cholerae's* large chromosome in two operons; *vpsA-K* and *vpsL*-Q [12,13]. The deletion of the *vpsA-K* operon is sufficient to prevent the formation of biofilms.

Outside of a host organism *V. cholerae* lives within the aquatic environment. It has been shown that *V. cholerae* is able to attach to surfaces that are provided by plants, algae, zooplankton, crustaceans, as well as insects [14]. One factor that plays a role in this attachment is the MSHA type IV pili. This MSHA pili is characterized as a chitin binding protein that binds zooplankton within both O139 and O1 El Tor strains of *V. cholerae* [15]. It is believed that the ability of *V. cholerae* to associate with the previously mentioned surface types may prove

protection to the bacteria living within the aquatic environment and that this attachment and subsequent biofilm formation plays an important role facilitating persistence of *V. cholerae* as a part of its life cycle [16]. Furthermore, *V. cholerae* secrete chitinase enzymes, which are thought to be required for chitin utilization as a carbon source. The combination of these binding proteins and chitinase enzymes suggest that there is an important association between *V. cholerae,* and chitinous surfaces found within its native environment [16].This is not surprising considering *V. cholerae* is known to proliferate while attached to or associated with copepods, which utilize chitin as a primary component of their exoskeleton. Additionally, chironomid egg masses also serve as a reservoir for *V. cholerae* [16]. It has been hypothesized that both copepods and chironomids are consumed and dispersed by migratory birds, which may allow *V. cholerae* to more easily spread between bodies of water both on and between continents [17].

It is also suggested that fish act as important reservoirs of *V. cholerae* which is evidenced by the association of the disease cholera with the consumption of fish based seafood in various parts of the world [18,19]. An analysis of various fish in different habitats, it was found that several species of fish contain *V. cholerae* in their digestive tracks [17]. This study found that 71% of freshwater fish sampled were positive for *V. cholerae*, while 60% of fish taken from the Sea of Galilee and 50% of fish sampled from rivers were positive for *V. cholerae.* Due to the estimation that 70% of human fish consumption is of farm-raised fish, the potential impact that this relationship might have on fish farming was considered. Out of eight fish species that were sampled from farm ponds, seven tested positive for *V. cholerae* [17]. It has been hypothesized that fish may actively benefit from *V. cholerae* strains within their intestine due to the capacity of *V. cholerae* to digest chitin. In support of this hypothesis, all *V. cholerae* isolated from fish in the study mentioned above showed the ability to degrade chitin. This relationship is thought to be

commensal as the fish would benefit from having digestive enzymes, while the *V. cholerae* would gain a host which may disperse them farther than otherwise possible. This dispersion could be accomplished through migration of the fish themselves or by means such as predation of the host fish by migratory birds. It is important to note that this study only succeeded in isolating *V. cholerae* of non-O1/O139 serogroups and these *V. cholerae* species inhabiting the fish were likely non-pathogenic. However, it is believed that the non-O1/O139 serogroups are likely to inhabit the same environments as the pathogenic serotypes, making this information relevant in how we perceive the life cycle of pathogenic *V. cholerae*.

Understanding the lifestyle of *V. cholerae* is critical to understanding how to avoid and combat the infectious outbreaks that this pathogen can cause. Its survival in the environment appears to be strongly tied to its ability to form and disperse from biofilms depending upon the environmental stimulation that it receives. In most cases, biofilm formation and virulence are inversely regulated, but further research is required to elucidate how the regulation of these pathways influence each other. Moving forward, the ability to understand or manipulate one of these processes should allow understanding or manipulation of the other and may provide useful knowledge of other bacterial species as well.

One of the objectives of my project was to determine whether any polyamine pathways that the Karatan lab had studied previously, played a role in colonization of the intestine. Polyamines are small organic molecules containing two or more amine groups and are positively charged at physiological pH [20]. They are ubiquitous and are utilized by nearly all living cells [21]. I sought to determine the impact of several polyamine pathways through direct competition assays using the *Danio rerio* (zebrafish) animal model. In addition to the benefit of their relatively low cost and the ease of their care, zebrafish were chosen as a model organism for this

project for several reasons. Zebrafish serve as a natural host to *V. cholerae* [22] and are colonized without any artificial manipulation required. *V. cholerae* colonizes the small intestine in humans, with preferential colonization occurring in the distal region of the small intestine [23]. When zebrafish are exposed to *V. cholerae*, they are colonized through uptake of contaminated water and this colonization occurs in their intestine. These similarities with human infection by *V. cholerae* make zebrafish an ideal animal model to study *V. cholerae* [22].

Based on current understanding, the human stomach is an obstacle that impedes colonization by *V. cholerae* at least in part by virtue of its high acidity. In human volunteer trials, doses of 10^{11} CFU (Colony Forming Units) of *V. cholerae* were required for consistent symptoms of infection in adults. This requirement is lowered to 10^{6} CFU through the addition of sodium bicarbonate, which aids in neutralizing the acidity in the stomach [1]. While this experiment specifically addressed stomach acidity, another study showed that sodium bicarbonate stimulates CT production [24]. Subsequent research confirmed this observation, showing that bicarbonate also induces the production of the toxin co-regulated pilus (TCP), which is required for virulence, and introduced a model which might more thoroughly explain this interaction [25]. Under static growth conditions, bicarbonate was found to induce expression of both CT and TCP.

It was proposed that bicarbonate is utilized during infection as an effector molecule to induce virulence [25]. The model proposes that as *V. cholerae* enters the lumen of the small intestine, *V. cholerae* encounters an unknown primary signal which induces transcription of *toxT*. ToxT is a regulatory protein that directly activates transcription of the genes that encode CT and TCP, as well as other virulence genes. In the lumen, there are lower levels of pancreatic bicarbonate compared to the mucus layer due to diffusion throughout the intestine. However, at

later stages of infection, *V. cholerae* enters the mucus layer, which contains a higher concentration of bicarbonate being secreted by the intestinal epithelial cells [26]. This increased bicarbonate concentration leads to enhanced ToxT activity and high expression of TCP and CT.

It is established that *V. cholerae* does not function well in low pH environments and that the acidic phase of digestion in human stomachs creates a barrier that *V. cholerae* must overcome in order to proceed down the gastrointestinal tract [1]. One mechanism by which *V. cholerae* overcomes this obstacle is believed to be the adoption of the biofilm state. One relevant benefit of biofilms in *V. cholerae* is that biofilms increase resistance to low pH levels, which assists the bacteria in surviving passage through the stomach and into the intestine. In considering obstacles to *V. cholerae* colonization, one key distinction between zebrafish and human digestive systems is that zebrafish are part of a family known as cyprinids that have no stomach [27,28]. Instead, cyprinids have an expanded intestinal bulb and lack a gastric acid barrier.

Based on the current model of *V. cholerae* infection, it is not the structure of the stomach that serves as a barrier for the bacteria, but rather the high levels of acidity that planktonic *V. cholerae* does not tolerate well. Data suggests that part of the reason such a high inoculum size is required for *V. cholerae* is that the majority of bacteria are killed by the acidic pH of the stomach at the time of ingestion [29]. A previous study performed in adult zebrafish surveyed pH levels at different locations along the full length of the intestine. This showed that the pH levels never fell below a value of 7.5 under homeostatic conditions [30]. This strongly suggests that zebrafish intestines do not have any regions which are high in acid content and thus, that *V. cholerae* will not be exposed to a low pH environment when colonizing the zebrafish intestine. Although the absence of a stomach is expected to lower the required infectious load to infect zebrafish, the

lack of stomach or low pH region in cyprinids is not expected to alter the mechanics of colonization beyond that initial infectious load requirement.

In this study, the impact of signaling, transport, and/or synthesis pathways for four polyamines on colonization of zebrafish by *V. cholerae* were analyzed. The four polyamines utilized in these experiments were; putrescine, spermine, spermidine, and norspermidine (Figure 3).

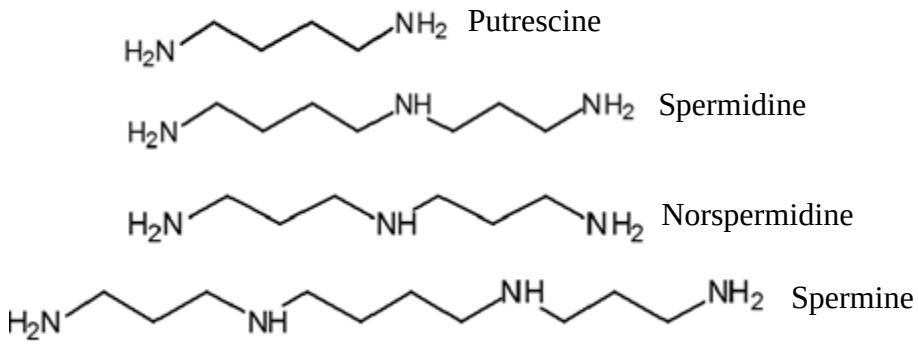

Figure 3. Chemical structures of the polyamines putrescine, spermidine, norspermidine, and spermine.

Of these polyamines, spermine, putrescine and spermidine are present within the human intestine, while norspermidine is often present within the native environment of *V. cholerae* [31]. Norspermidine functions as an environmental signal that positively regulates *V. cholerae* biofilm formation. *V. cholerae* senses, imports, and synthesizes this polyamine, indicating that it serves an important capacity within the lifestyle of this bacterium [31]. Spermidine and spermine both reduce the formation of biofilm and are thought to play similar roles in *V. cholerae's* lifecycle. Spermidine is not thought to be present in high enough concentrations in the human intestines, but spermine is present at nearly 50 μM, which is sufficient for biofilm inhibition [32]. It is

known that spermine and spermidine are produced by zebrafish and it remains a possibility that these polyamines may play a role in zebrafish colonization which may aid in environmental persistence through this animal vector [33]. High concentrations of putrescine can reduce levels of the protein TcpA, which makes up the majority of the TCP, by up to 50% [20]. This in turn was shown to decrease agglutination of *V. cholerae* cells, which is an important stage in the infection process in humans.

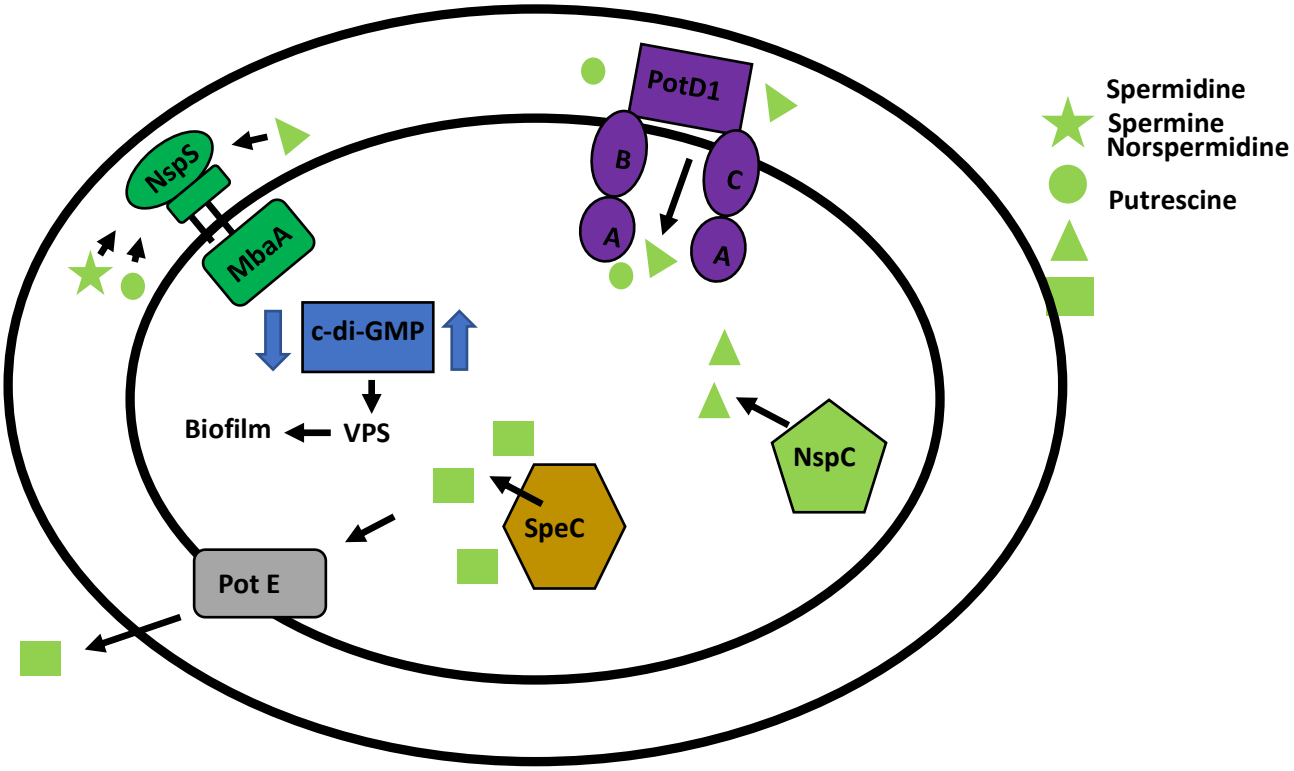

Figure 4. The four polyamines utilized in this study are all involved with multiple proteins within a *V. cholerae* cell. Spermine, spermidine, and norspermidine are involved with the biofilm regulation NspS/MbaA pathway. Norspermidine and spermidine are both imported by the PotABCD1 transporter. NspC is responsible for synthesis of norspermidine. SpeC synthesizes putrescine, and the PotE transporter protein exports putrescine from the cytoplasm of the cell.

The NspS/MbaA pathway is a proposed pathway that enables *V. cholerae* to regulate the biofilm state based on the presence of environmental polyamines. This pathway has been shown

to increase biofilm formation in the presence of norspermidine and decrease biofilm formation in the presence of spermine or spermidine [32,34,35]. NspS is proposed to bind to environmental polyamines and to the periplasmic region of the MbaA protein (Figure 4). Depending on the polyamine NspS binds, it is proposed that it alters the conformation of MbaA and regulates whether the phosphodiesterase region of MbaA is functional or not. Phosphodiesterases degrade the secondary messenger c-di-GMP, and increased c-di-GMP concentration increases biofilm formation, while decreased levels of c-di-GMP decrease biofilm formation. The NspS/MbaA pathway allows for altered biofilm formation in the presence of the three polyamines spermine, spermidine, and norspermidine. Because there are many pathways to regulate biofilm formation, a Δ*vpsA-K* mutant (referred to as Δ*vpsA)* was utilized to examine the colonization impact of biofilm formation outside of any potential impact that polyamines might have. The PotABCD1 transport complex is able to import the polyamines spermidine and norspermidine and may also have an undetermined role in biofilm repression [34,36]. The NspC protein synthesizes norspermidine and overexpression of NspC has been shown to enhance biofilm formation in an unknown manner [37,38]. SpeC is responsible for the synthesis of putrescine, with previous experiments showing an upregulation of the genes coding for this protein in infant mice and rabbit models [39–42]. The PotE transport protein is responsible for transporting putrescine out of the cytoplasm and the gene for this protein is also found to be upregulated in infant mice and rabbit models [39–42]. Clearly, polyamines play an important role in the life of *V. cholerae* and this study was aimed at determining how that role impacted colonization of zebrafish by this pathogen. The objective of this study was to one, determine the impact of biofilm formation on *V. cholerae* colonization in zebrafish, two, determine the impact of polyamine sensing pathways affecting biofilm formation on *V. cholerae* colonization of zebrafish, and three, to determine if

polyamine transport and synthesis pathways in *V. cholerae* have an impact on colonization of zebrafish.

Materials and Methods

Ethics Statement

All experiments involving zebrafish were carried out in accordance with protocols approved by the Appalachian State University Institutional Animal Care and Use Committee.

Zebrafish Husbandry

All zebrafish used in this study were obtained from the Zerucha Lab zebrafish colony in the animal facility of Appalachian State University, which is covered by IACUC protocol 17-13 and 18-09. Prior to treatment, fish were kept in one-liter to four-liter aquaria housed in an Aquatic Habitats Z-Mod system, or in a ten-gallon stand-alone aquarium, located in the animal facility. Water quality indicators such as pH, temperature, and conductivity were monitored daily and fish were fed a combination of dry food with or without live brine shrimp at least once a day. Up to four adult fish infected with *V. cholerae* were placed into covered one liter autoclavable plastic aquaria (capable of holding 7 adult fish) containing conditioned water obtained from the Aquatic Habitats Z- Mod system and transported to the laboratory.

Bacterial Strains

The *V. cholerae* strain utilized in this study was O139 MO10. All gene deletions were obtained in this O139 background and in an O139 *lacZ* mutant background that does not carry a functional *lacZ* gene.

The *V. cholerae* strains used for these experiments had a streptomycin resistance gene; therefore, streptomycin (SM) was used at 100 µg/mL both in Luria Bertani (LB) liquid media

(per liter: 10g tryptone, 5g yeast extract, 10g NaCl, and 15g agar) and agar (per liter: 10g tryptone, 5g yeast extract, 10g NaCl, and 15g agar). *V. cholerae* was grown at 27°C with shaking at 200rpm in a New Brunswick Scientific I2400 incubator.

Strains of interest were *V. cholerae* strains which have deletions in genes coding for proteins that play a role in either biofilm development or polyamine detection, synthesis, or transport. Mutant strains utilized in this study consisted of: Δ*vpsA*, Δ*nspS*, Δ*mbaA*, Δ*potE*, Δ*potA*, Δ*nspC*, and Δ*speC* (Table 1).

Table 1. Bacterial strains.

Strain	Genotype	Reference/Source
PW 249	MO10, clinical isolate of *V. cholerae* O139 from India, Sm^R	[43]
PW 357	MO10 *lacZ::vpsLp* → *lacZ*, Sm^R	[44]
PW 514	MO10*lacZ*::*vpsLp*→*lacZ*, Δ*nspS*, Sm^R	[35]
PW396	MO10, Δ*vpsA*-*vpsK*, Sm^R	[45]
PW444	MO10 *lacZ*::*vpsLp*→*lacZ*, Δ*mbaA*, Sm^R	[44]
AK314	MO10 *nspC*::*kan*, Kan^R, Sm^R	[31]
AK871	MO10 *lacZ*::*vpsLp*→*lacZ* →*lacZ*, Δ*speC* Sm^R	[46],(Robinson and Karatan unpublished)
AK429	MO10 *lacZ*::*vpsLp*→*lacZ*, Δ*potA* Sm^R	[39]
AK535	MO10 *lacZ*::*vpsLp*→*lacZ*, Δ*potE* Sm^R	[46]

Bacterial culturing and inoculation

All experiments involving live *V. cholerae* were carried out exclusively in the Karatan laboratory, which has Biosafety Level 2 designation. Strains utilized in experiments were first streaked for isolation on LB plates with 100µg/ml streptomycin and incubated overnight at 27°C. After isolation, *V. cholerae* colonies were selected and transferred using a toothpick into a culture tube containing 2ml LB broth with 100µg/ml streptomycin. This culture tube was then incubated overnight in a shaking incubator and 27ºC and 200rpm. Following overnight incubation, a 1:100 dilution of the overnight culture was generated for day culturing through the transfer of 20µL of overnight culture to new culture tubes for day culturing. The day cultures were performed under the same conditions as the overnight cultures and within the same solution with antibiotics. Shaking incubation continued until the mid-log phase of growth was achieved. The mid log phase in these experiments was defined by an optical density (OD) reading of 0.3-0.4 at 655nm using 100µL of bacterial culture read in a BIO-RAD Microplate Reader model 680. One milliliter of this day culture was spun down at 4000 x g for five minutes and then washed three times in sterile Phosphate Buffered Saline (PBS) solution. Following the wash cycles, the pellet was transferred and resuspended in one ml of sterile PBS and added to the tank containing the fish. Approximately 10^8 wild-type and mutant *V. cholerae* was added to each tank for co-infection experiments, where both strains competed for colonization of the zebrafish intestine.

After allowing circulation for 10 minutes, a water sample was taken, serially diluted, and plated in order to calculate the initial number of bacteria and the ratio of the mutant and wild-type *V. cholerae* strains. Inoculation of the water in this manner was sufficient for intestinal colonization of the zebrafish as described previously [22].

Euthanasia and dissection of zebrafish

Twenty-four hours after inoculation of tank water with *V. cholerae*, the zebrafish were sacrificed by euthanization with 300 mg/L Tricaine. After the addition of Tricaine to the tank, 20 minutes was allowed for the drug to take effect and death was confirmed via absence of gill movement for 20 seconds. Prior to dissection, fish were rinsed in ethanol to remove surface bacterial contamination. After lightly blotting dry, zebrafish were placed in a dissection apparatus. The apparatus consisted of a small open container with dental wax folded into the bottom to serve as a surface for pinning. Sterile PBS solution was added to the apparatus to a depth of approximately three centimeters and the dissection was performed within this solution (Figure 5).

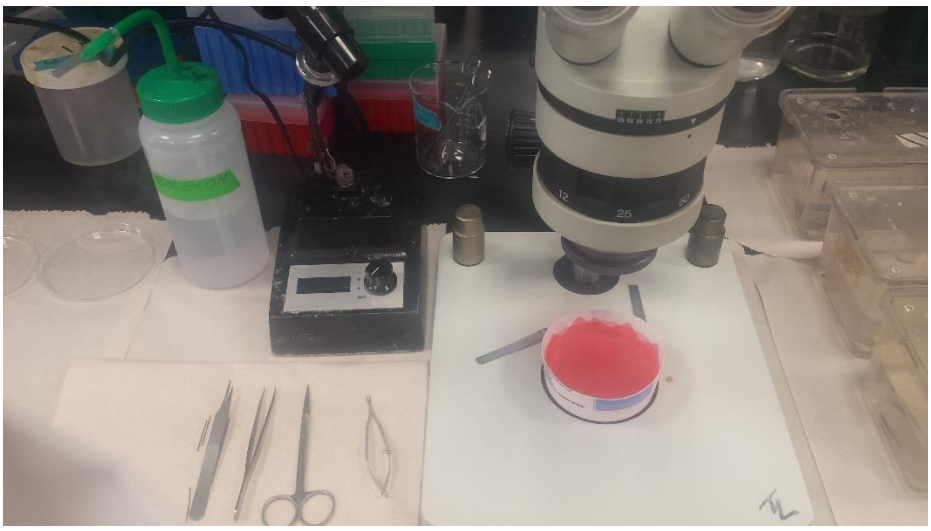

Figure 5. A picture of the instruments and setup for the dissection workstation. The dissection apparatus is placed under the lens of the dissection microscope with the light source, forceps and scissors located to the left of the microscope. 70% ethanol is present in the green squeeze bottle and paper towels are present for the blotting of zebrafish.

The zebrafish were initially pinned down through the fleshy portion of the tail and the head was removed at the gills using Mayo scissors (Fine Science Tools #14110-15). The

abdominal cavity was then opened by cutting along the length of the fish skin using Vannas spring scissors (Fine Science Tools #15007-08). Intestines were surgically removed using forceps in conjunction with the Vannas spring scissors and placed into a microcentrifuge tube containing 500µL sterile PBS and glass beads. The microcentrifuge tubes containing the beads and intestine were vortexed at maximum speed for 10-20 minutes to assure homogenization of the intestines. Following homogenization 100µL of the homogenate was pipetted from the microcentrifuge tube and serially diluted.

Enumeration of *Vibrio*

Dilutions were plated on LB media with added streptomycin (LB+SM) and allowed to grow overnight. From these plated dilutions a plate was chosen which contained 30-300 colony forming units and these colonies were patched onto corresponding pairs of plates chosen to facilitate selection and differentiation and grown overnight at 27ºC.

The first plates used in the patching process were Thiosulfate Citrate Bile Salts Sucrose (TCBS) plates, which are highly selective for *Vibrio* growth. They also differentiate between *V. cholerae* and other species of *Vibrio*. In TCBS Agar, yeast extract and peptone provide nitrogen and vitamins, while sodium citrate, sodium thiosulfate, oxgall, and cholate are selective agents. TCBS medium has an increased pH to enhance *V. cholerae* growth because this bacterium is sensitive to acidic environments and a high sodium concentration favors *V. cholerae* which is halotolerant over other bacteria that may be present in the zebrafish intestine. *V. cholerae* breaks down the sucrose which is in the TCBS agar and lowers the pH. This allows the bromothymol blue/thymol blue pH indicator within the TCBS agar to a yield a yellow color change in the agar

and allows identification of *V. cholerae.* Colonies that either did not grow or did not produce yellow color on this agar were ignored.

The second set of patch plates were used for differentiation and were LB+SM plates with added 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). X-Gal is broken down by beta-galactosidase, which is produced by bacteria with a functional *lacZ* gene encoding this enzyme. The breakdown of X-Gal yields a blue precipitate at the site of enzymatic activity, enabling differentiation between mutant and wild type strains through simple visual analysis. It is important to note that the Δ*vpsA* mutant used in this study was generated within a *lacZ+* background and thus was compared to an otherwise wild-type *lacZ-* strain for this specific competition assay. Furthermore, due to statistically different competitive index values between the *lacZ-* and *lacZ+* background strains, all mutant strains are compared against their respective backgrounds to establish significance of results.

Using these colony counts, a competitive index was generated by dividing the colony forming units (CFU) of the mutant strain *V. cholerae* by the CFU of the wild-type *V. cholerae.* A number greater than one was interpreted as the mutant strain having a competitive disadvantage while a number lower than one meant that the introduced mutation was advantageous to intestinal colonization.

Each group of experimental fish was comprised of four zebrafish that had been inoculated with both wild-type and mutant strains of *V. cholerae*. Three to five replicates of each competition experiment were performed. Results were graphed using GraphPad Prism software. The competitive index was graphed with each individual fish represented by a single data point and p-values were calculated within this program using Welch's *t*-test, or unequal variances *t*-test. This test is an adaptation of a Student's *t*-test that is considered more reliable when two

samples have unequal sample sizes [47]. In analyzing the data sets involving the Δ*potA* and Δ*vpsA* mutant strains, a GraphPad Prism function to detect outliers was utilized and outliers were removed from the analysis. The competitive index values were normalized using the competitive index generated from the CFU counts of tank water at the time of inoculation to account for any initial disparities between strain populations. Specifically, a competitive index score was generated for the inoculated water at the time of infection and the competitive index for each colonized zebrafish was divided by this number.

Results

Comparison of colonization rates between *lacZ*- and *lacZ*+ strains

To determine the impact that mutations in various genes had on the colonization of zebrafish intestines by *V. cholerae*, I performed competition experiments. In these experiments, I co-infected adult zebrafish with wild-type *V. cholerae* and one of several mutant *V. cholerae* strains with gene deletions. After 24 hours, I euthanized the zebrafish and dissected out their intestines and homogenized them. Following homogenization, I serially diluted the homogenate in PBS and plated the dilutions on selective and differential plates to determine the CFU counts of wild-type and mutant strain *V. cholerae.* I then generated a competitive index for each infected fish by dividing the number of mutant strain CFU by the number of wild-type strain CFU.

Mutant *V. cholerae* strains were differentiated from wild-type strains by a disruption of the *lacZ* gene except in the case of the Δ*vpsA* mutant which had a functional *lacZ* gene. Initially, a functional *lacZ* gene was not thought to have any impact on colonization by *V. cholerae* and thus would serve as an effective means to differentiate strains without impacting their viability in colonization. However, our results revealed that the wild-type strains with a mutation in the *lacZ* gene displayed a disadvantage in colonization when co-infected with *lacZ*+ *V. cholerae.* Due to this inherent disadvantage, the competitive indices of all *V. cholerae* mutations are plotted alongside the competitive index of their respective background strains and the statistical significance of any difference is calculated using a Welch's t-test. Values above one indicates that the mutant strain held a competitive advantage over the wild-type strain, while a value below one is indicative of a competitive disadvantage. The difference between the *lacZ*- and *lacZ*+ backgrounds is shown below in Figure 6.

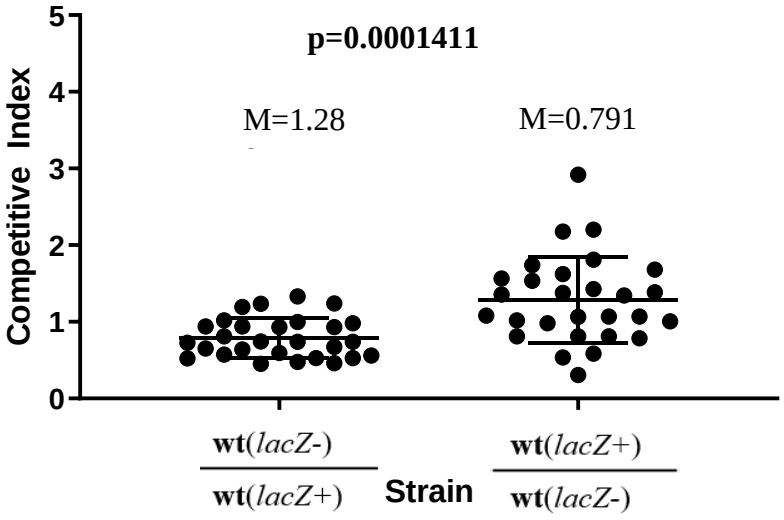

Figure 6. The competitive index of the wild-type (*lacZ*$^{-}$) *V. cholerae* shown alongside the competitive index of the wild-type (*lac*$^{+}$) *V. cholerae* when competing with each other. These values give the expected competitive index scores of the background strains housing the tested mutations. Each data point represents an individual fish which was co-infected with both wild-type (*lacZ*$^{-}$) and wild-type (*lacZ*$^{+}$). On the left, wild-type (*lacZ*$^{-}$) CFU are divided by wild-type (*lacZ*$^{+}$) CFU to form the index values for the wild-type (*lacZ*$^{-}$) strain. On the right, wild-type (*lacZ*$^{+}$) CFU are divided by wild-type (*lacZ*$^{-}$) CFU to form the index values for the wild-type (*lacZ*$^{+}$) strain. Twenty-eight zebrafish from seven different infection experiments make up the individual data points used in these graphs. The M values indicate the means of the wild-type (*lacZ*$^{-}$) and wild-type (*lacZ*$^{+}$) competitive index values from zebrafish co-infected with both strains.

Impact of biofilm formation on *V. cholerae* colonization of zebrafish intestine

To determine the impact of biofilm formation on colonization of zebrafish by *V. cholerae*, a Δ*vpsA* strain of *V. cholerae* incapable of biofilm formation was used. Formation of biofilms generally requires the presence of the exopolysaccharide Vibrio Polysaccharide (VPS), which is required for the generation of three dimensional biofilm structures [48]. The Δ*vpsA* mutant lacks *vpsA-vpsK* genes which are required for the synthesis of VPS; therefore, this mutant is unable to produce biofilms. The data gathered in the competitive colonization assays show that

Δ*vpsA V. cholerae* mutants gain a competitive advantage over the wild-type *V. cholerae* (Figure 7).

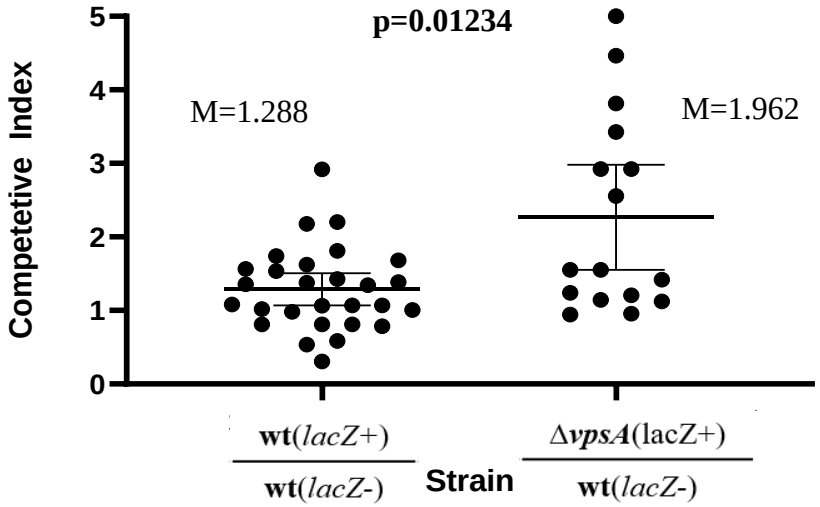

Figure 7. The competitive index of the Δ*vpsA* mutant (right) plotted alongside the competitive index of the wild-type (*lacZ*+) *V. cholerae* (left). Wild-type experiments were performed separately and are utilized to differentiate background strain impact from mutant strain impact. Sixteen zebrafish from five different infection experiments make up the individual data points for the Δ*vpsA* competitive index values with outliers removed. The p value indicates the significance of the difference between the wild-type (*lacZ*+) and Δ*vpsA* strains.

The Δ*vpsA* mutant displays a competitive advantage when compared to the wild-type *V. cholerae*, suggesting that the inability to produce biofilms is beneficial to 24-hour colonization levels of *V. cholerae* within zebrafish.

Impact of the NspS/MbaA signaling pathway on *V. cholerae* colonization of zebrafish intestine

A Δ*nspS* mutant was utilized to determine if the ability to detect spermine by the MbaA/NspS biofilm regulatory pathway would have an impact on colonization efficiency. The

nspS gene encodes a protein that detects the polyamine spermine which is thought to be present in the intestine of almost all vertebrates including zebrafish. Additionally, the Δ*nspS* mutant displays decreased levels of biofilm formation, presumedly because it is unable to inhibit the phosphodiesterase activity of MbaA [32]. Results from co-infection competitive assays show a lack of any significant difference in colonization efficiency as seen in figure 8 below.

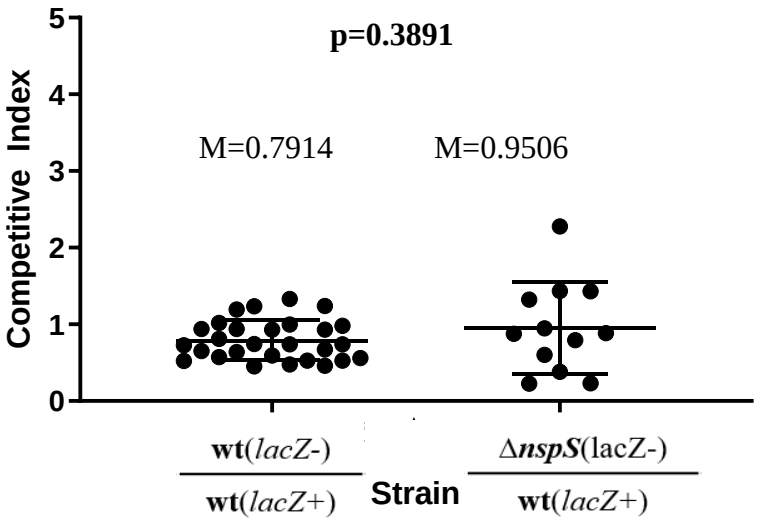

Figure 8. The competitive index of the wild-type (*lacZ-*) *V. cholerae* (left) plotted alongside the competitive index of the Δ*nspS* mutant (right). Twelve zebrafish from three different infection experiments make up the individual data points for the Δ*nspS* competitive index values. Wild-type experiments were performed separately and are utilized to differentiate background strain impact from mutant strain impact. The p value indicates the significance of the difference between the wild-type (*lacZ-*) and Δ*nspS V. cholerae* strains.

Additional co-infections examining the impact of the NspS/MbaA on colonization were performed using the Δ*mbaA* mutant. This mutant displays enhanced biofilm formation compared to wild-type strains which is thought to be due to activity of the EAL domain of the MbaA

protein. EAL domains exhibit phosphodiesterase activity and this is thought to lower local levels of the secondary messenger cyclic-di-GMP [32]. Elevated levels of this secondary messenger has been associated with increased biofilm formation and diminished levels correspond to decreased biofilm formation [49]. In figure 9 it can be seen that the Δ*mbaA* mutant does not seem to have either an advantage or disadvantage in terms of colonization efficiency relative to the wild-type strain.

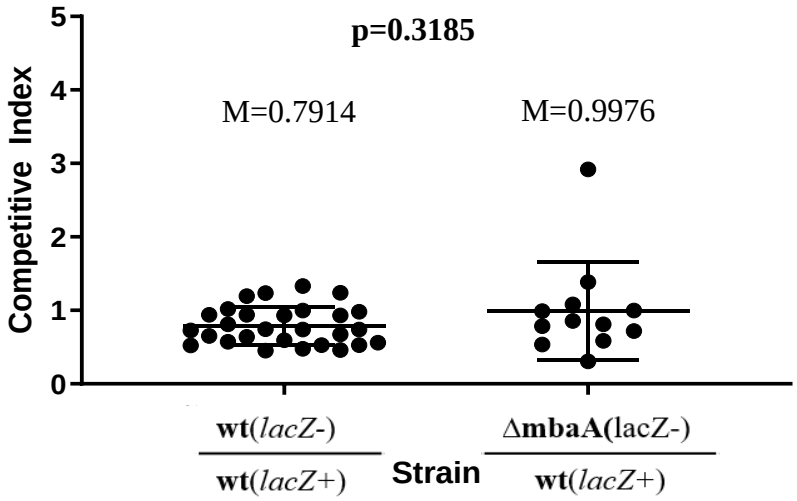

Figure 9. The competitive index of the wild-type (*lacZ*-) *V. cholerae* (left) plotted alongside the competitive index of the Δ*mbaA* mutant (right). Twelve zebrafish from three different infection experiments make up the individual data points for the Δ*mbaA* competitive index values. Wild-type experiments were performed separately and are utilized to differentiate background strain impact from mutant strain impact. The p value indicates the significance of the difference between the wild-type (*lacZ*-) and Δ*mbaA V. cholerae* strains.

Impact of polyamine transport on *V. cholerae* colonization of zebrafish intestine

The polyamine spermidine is known to be present in animal intestines [50] and is thought to be present within the zebrafish intestine with a potentially important role in the *V. cholerae* lifecycle. To analyze the impact of spermidine import on *V. cholerae* colonization, a Δ*potA*

mutant was included in the competitive assays. The PotABCD1 protein transport system imports the polyamine spermidine from the environment into the cytoplasm and it was hypothesized that if spermidine import played a key role in colonization, the Δ*potA* mutant would suffer a deficiency in colonization compared to wild-type *V. cholerae*. As seen in figure 10, co-infection experiments with wild type *V. cholerae* and the Δ*potA* mutant reveal a comparative disadvantage for the Δ*potA* strain in colonization efficiency.

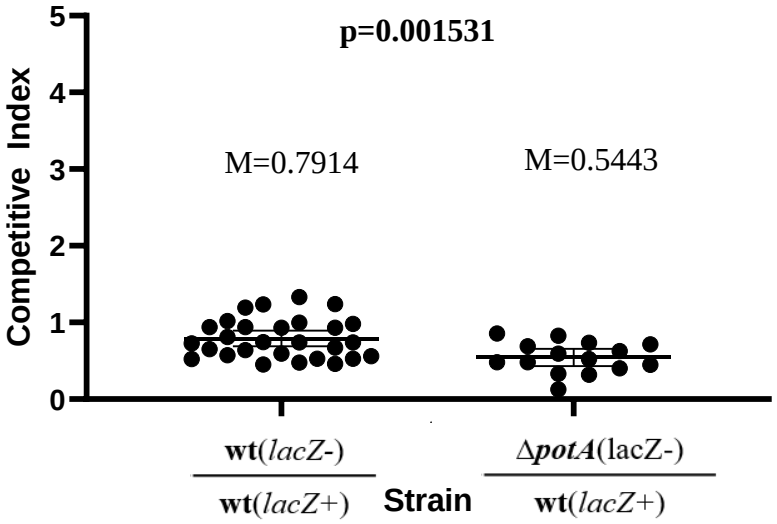

Figure 10. The competitive index of the wild-type (*lacZ*-) *V. cholerae* (left) plotted alongside the competitive index of the Δ*potA* mutant (right). After removal of one outlier value, fifteen zebrafish from four different infection experiments make up the individual data points for the Δ*potA* competitive index values. Wild-type experiments were performed separately and are utilized to differentiate background strain impact from mutant strain impact. The p value indicates the significance of the difference between the wild-type (*lacZ*-) and Δ*potA V. cholerae* strains.

The PotE protein in *V. cholerae* is responsible for the export of the polyamine putrescine from the cytoplasm to the environment. Expression of the *potE* gene was found to be upregulated during colonization in infant mice and rabbits [51]. I hypothesized that due to this upregulation in the infection of mammalian animal models, disruption of the *potE* gene would negatively

impact fish colonization. However, co-infection experiments showed that the Δ*potE* mutant displayed a colonization advantage on average compared to wild-type *V. cholerae*, although the distribution of competitive index values is somewhat bimodal (Figure 11).

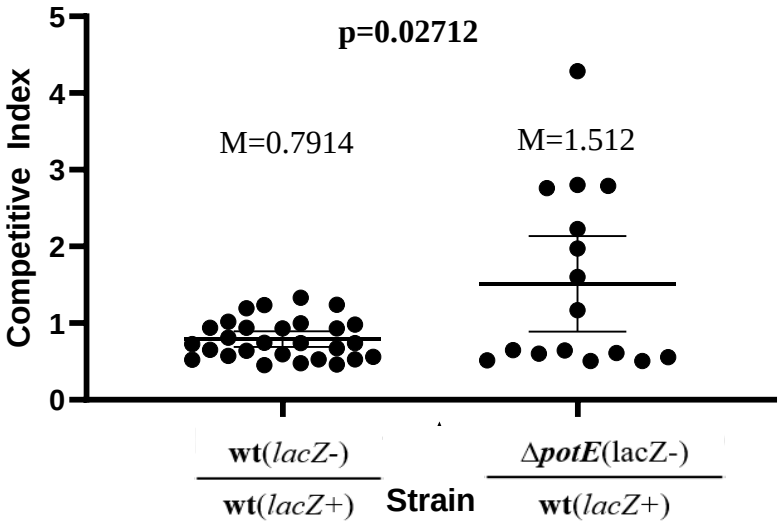

Figure 11. The competitive index of the wild-type (*lacZ*-) *V. cholerae* (left) plotted alongside the competitive index of the Δ*potE* mutant (right). Sixteen zebrafish from four different infection experiments make up the individual data points for the Δ*potE* competitive index values. Wild-type experiments were performed separately and are utilized to differentiate background strain impact from mutant strain impact. The p value indicates the significance of the difference between the wild-type (*lacZ*-) and Δ*potE V. cholerae* strains.

Impact of polyamine synthesis on *V. cholerae* colonization of zebrafish intestine

To ascertain the effect of putrescine synthesis on *V. cholerae* colonization of zebrafish, a Δ*speC* mutant was utilized (Isenhower, J., Robinson, M., and Karatan, E., unpublished). SpeC is responsible for the synthesis of the polyamine putrescine and the expression of *speC* is also upregulated during colonization in infant mice and rabbits. Furthermore, disruption of this gene leads to fitness defects in the aquatic environment, which is suggestive of an important function

in *V. cholerae* physiology [51]. For these reasons, I hypothesized that disruption of this gene would negatively impact zebrafish colonization. Results of infection experiments indicate that disruption of the *speC* gene causes an increase in the efficacy of *V. cholerae* in colonizing zebrafish when compared to the wild-type *V. cholerae* it is co-infected with (Figure 12). However, the Δ*speC* mutant seemed to also confer a lessened ability to clear or outcompete the normal zebrafish microbiota. On average in these experiments, fish co-infected with the WT(*lacZ*$^{+}$) and WT(*lacZ*$^{-}$) *V. cholerae* yielded approximately 98% *V. cholerae* CFU with only 2% of the CFU belonging to non-*Vibrio* bacteria. Fish co-infected with wild-type *V. cholerae* and Δ*speC V. cholerae* strain yielded approximately 73% of *V. cholerae* CFU with 27% of CFU comprised of non-*Vibrio* bacteria.

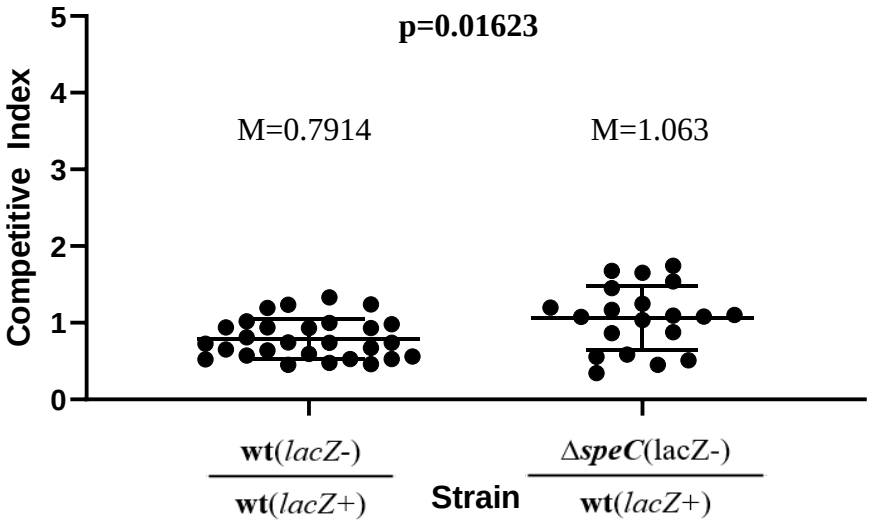

Figure 12. The competitive index of the wild-type (*lacZ*-) *V. cholerae* (left) plotted alongside the competitive index of the Δ*speC* mutant (right). Twenty zebrafish from five different infection experiments make up the individual data points for the Δ*speC* competitive index values. Wild-type experiments were performed separately and are utilized to differentiate background strain impact from mutant strain impact. The p value indicates the significance of the difference between the wild-type (*lacZ*-) and Δ*speC* strains.

The effect of norspermidine synthesis on *V. cholerae* colonization of zebrafish was analyzed by utilizing a Δ*nspC* mutant which is unable to synthesize the polyamine norspermidine [36]. Norspermidine is present in the environment that *V. cholerae* normally inhabits and plays a role in the life cycle of *V. cholerae* as a positive regulator of biofilm [31]. Deletion of the *nspC* gene was shown to lead to increased colonization when compared to wild-type *V. cholerae* colonizing the same zebrafish (Figure 13). However, the Δ*nspC* mutant also showed a decreased ability to clear or outcompete the normal zebrafish microbiota. Zebrafish co-infected with wild-type *V. cholerae* and Δ*nspC V. cholerae* strain yielded approximately 71% of *V. cholerae* CFU with 29% of CFU comprising non-*Vibrio* bacteria.

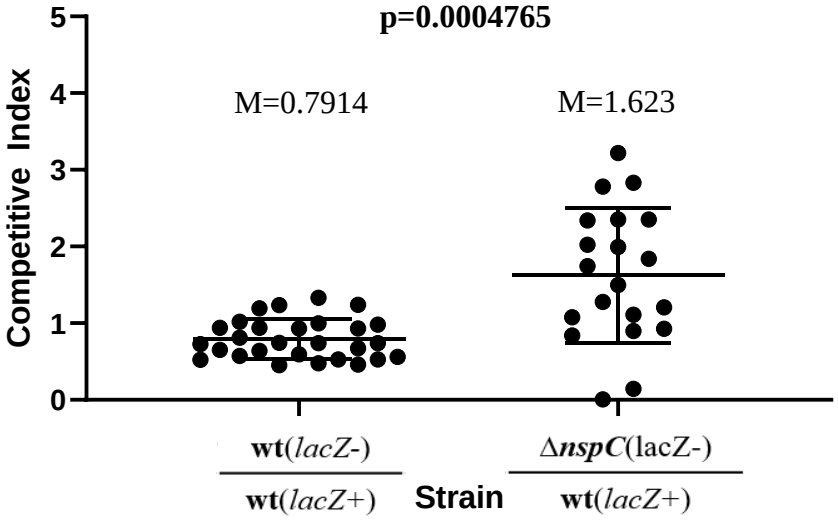

Figure 13. The competitive index of the wild-type (*lacZ*-) *V. cholerae* (left) plotted alongside the competitive index of the Δ*nspC* mutant (right). Twenty zebrafish from five different infection experiments make up the individual data points for the Δ*nspC* competitive index values. Wild-type experiments were performed separately and are utilized to differentiate background strain impact from mutant strain impact. The p value indicates the significance of the difference between the wild-type (*lacZ*-) and Δ*nspC* strains.

Discussion

Impact of Biofilm formation on colonization

In *V. cholerae,* the biofilm state is thought to be critical to the survival of this bacterium, both within a host and within the environment [35]. Furthermore, due to the importance of the biofilm state at producing a hyper infectious state in humans [52,53], researchers hypothesized that disruption of VPS production would have a negative effect on colonization of the zebrafish intestine. Contrary to this hypothesis, I observed an increase in colonization competitiveness of the Δ*vpsA* mutant, which is unable to produce VPS and form mature biofilms. The requirement for biofilm formation in *Drosophila melanogaster* colonization contrasts with the results seen in my experiments. In *D. melanogaster,* VPS-dependent biofilms are required for successful colonization by *V. cholerae* [54]. These contrasting effects are likely the result of the innate differences in the vertebrate *D. rerio* and invertebrate *D. melanogaster* animal models.

In general biofilm and virulence genes are inversely regulated in human hosts and it has been shown that there are genes upregulated late in the infection cycle that increase the formation of biofilms [55]. While biofilms are thought to play a protective role in initiating human infections by assisting in passage through the gastric barrier, zebrafish are cyprinids, and as such, they have no stomach and are not expected to have any highly acidic regions within their digestive tract. Cyprinids from various regions and on various diets were tested and found to not have highly acidic regions in their digestive tract, a difference from the human intestinal tract [27]. Thus, the biofilm state would not be expected to confer an advantage in bypassing innate host defenses as it would in humans and *Drosophilla*. It is possible that the zebrafish colonization advantage seen in the Δ*vpsA* mutant is due to energy conserved from being completely unable to synthesize VPS. There also remains the potential that the biofilm state does

play some role in colonization, but the biofilm deficient Δ*vpsA* strain is able to become enmeshed in, or benefit from, the biofilm generated by the wild-type strain it is co-inoculated with.

It is intriguing that the VPS biofilm mutant displayed a phenotype that was different from the NspS/MbaA biofilm regulation mutants. This lab has previously shown that the deletion of *nspS* decreases biofilm development while the deletion of *mbaA* increases biofilm development. It was hypothesized that disruption of the *nspS* gene and the resulting inability to detect norspermidine or spermine within the zebrafish intestine would negatively impact *V. cholerae's* ability to colonize. My experiments revealed a lack of significant effect for the Δ*nspS* mutant on colonization efficacy of *V. cholerae*. Given that the decreased biofilm phenotype from the Δ*vpsA* mutant was shown to have a competitive advantage relative to the wild-type *V. choelrae*, it is unclear why the Δ*nspS* mutant has no effect on colonization.

While the Δ*nspS* and Δ*mbaA* mutant strains had no discernable colonization impact, despite their altered biofilm phenotypes, the inability to generate the primary biofilm component VPS seems to be beneficial for colonization of the zebrafish intestine. An additional explanation for the lack of impact by these two mutants may be that the *nspS* and *mbaA* genes are more important for late infection and transition from the host environment to the aquatic environment, than initial colonization of the intestine. Additionally, in the Δ*nspS* mutant strain, VPS synthesis may still be sufficiently activated through a different pathway within *V. cholerae*. In this circumstance, the advantage gained by not producing biofilms in the Δ*vpsA* mutant would not occur in the Δ*nspS* mutant, offering a possible explanation for the difference in colonization between Δ*nspS* and Δ*vpsA* strains.

Additionally, based on the competitive advantage displayed by the Δ*vpsA* strain, the Δ*mbaA* mutant with its increased biofilm levels would be expected to have some level of disadvantage when compared to the wild-type. This was not the case, with no significant difference being seen between the Δ*mbaA* mutant and the wild-type *V. cholerae*. Given that no colonization difference is displayed by either the Δ*nspS* or Δ*mbaA* mutant, it is likely that the NspS/MbaA pathway does not play a role in early colonization in zebrafish. I propose that the biofilm state may primarily benefits *V. cholerae* in transitioning from the zebrafish host to the aquatic environment, while providing no benefit to early colonization.

Impact of polyamine synthesis on zebrafish colonization

As the polyamine norspermidine is a major polyamine synthesized by *V. cholerae,* the potential impact of norspermidine synthesis on colonization was analyzed using a *nspC* mutant. The Δ*nspC* mutant strain displays a competitive advantage when compared to the wild-type *V. cholerae* it is co-infected with, however, co-infections involving this Δ*nspC* mutant are unable to displace or outcompete the normal microbiota effectively. This is unlikely to be a result of not secreting norspermidine as *V. cholerae* does not secrete this polyamine into the extracellular environment. The synthesis of norspermidine through NspC is part of a complex regulatory pathway and disruption of this pathway has been linked to reductions in growth and biofilm formation [37]. These results combined indicate the possibility that the Δ*nspC* mutant alters the immediate environment in some way that is detrimental to *V. cholerae's* ability to clear the local microbiota, as well as reducing the ability of wild-type *V. cholerae* to effectively colonize. The deletion of *nspC* leads to a build-up of the norspermidine precursor carboxynorspermidine [37], which may be involved in an unknown signaling pathway or otherwise impede normal cell

behavior. Additional experiments are required to elucidate the mechanistic causes of these observations.

Putrescine synthesis was analyzed utilizing the Δ*speC* mutant which lacks the ability to synthesize this polyamine through the ornithine decarboxylase pathway [46]. This mutant also displays a competitive advantage, but this competitive advantage is accompanied by the inability to clear local microbiota effectively in fish that are co-infected with Δ*speC* and wild-type *V. cholerae*. It is unclear why the inability to synthesize putrescine would lead to less effective clearing of local microbiota. One possibility relates to the fact that when ornithine to putrescine synthesis is inhibited, there is a build-up of the diamine cadaverine within *V. cholerae* [46]. In *V. cholerae* cadaverine plays an important role in the acid tolerance response that aids in its passage through the stomach [56]. When the ornithine decarboxylase SpeC is impaired, this may falsely signal that the cell is in an acidic environment and that there is a need to modify behavior and growth patterns to adjust for this perceived threat.

The known mechanism by which *V. cholerae* clears the microbiota in zebrafish is through the action of its Type Six Secretion System (T6SS). The T6SS in *V. cholerae* has been shown to modulate the intestinal microenvironment within zebrafish by stimulating gut motility and leading to displacement of the host microbiota [57]. Entry of *V. cholerae* to the intestinal lumen is an important regulator of the T6SS, and the T6SS is known to respond to host mucin, bile, and indole, all of which are found within the intestine [58]. As T6SS targets cells within the intestine [59], this suggests that the T6SS would not be active while in a highly acidic environment such as the stomach. It is possible that if *V. cholerae* cells are growing in a more acid tolerant or survival-oriented phenotype, such as may occur in the Δ*speC* mutant, normal T6SS system activity may be impacted in favor of these more defensive growth strategies

associated with low pH and passage through the gastric barrier. If this occurred, the altered T6SS activity of the Δ*speC* mutant could potentially lead to incomplete clearing of local microbiota. While the exact mechanism by which the Δ*speC* mutant impacts colonization is unclear, the mutation is possibly having an effect on the wild-type *V. cholerae* as well. The Δ*speC* mutant appears unable to effectively clear the microbiota, but this would not be expected to confer the competitive advantage relative to the wild-type *V. cholerae* that is observed. Thus, it is possible that the Δ*speC* mutant is detrimentally impacting the co-inoculated wild-type *V. cholerae* while also inhibiting the mutant strain from effectively clearing the local microbiota.

Impact of the polyamine transport on zebrafish colonization

The polyamine norspermidine is a major polyamine which is present in the native environment of *V. cholerae* and it has been shown that exogenous norspermidine is an important signal that supports biofilm formation in *V. cholerae* [31]. The Δ*potA* mutant is unable to import the polyamines norspermidine and spermidine. Use of this strain tests the hypothesis that acquisition of external norspermidine and spermidine plays a role in colonization in the zebrafish model. While the Δ*potA* mutant strain shows a competitive disadvantage compared to the wild-type *V. cholerae*, it must be determined if this is due to the uptake of norspermidine, spermidine, or both. Although the Δ*potA* mutant is unable to import norspermidine, it still has the capacity to synthesize this polyamine. However, *V. cholerae* does not have the ability to synthesize spermidine, therefore, it is likely that the colonization effect seen is due primarily to the uptake of spermidine rather than norspermidine. It must be noted however, that while it is known that zebrafish produce spermidine, the presence of spermidine within the intestine has not been analyzed. Disruption of the PotABCD1 transport system in *V. cholerae* has previously been

shown to result in increased levels of biofilm production [34] and there is a small possibility that the disadvantage in colonization is due to this increased biofilm formation. However, Δ*mbaA* strains also form increased biofilms and have no change to colonization efficiency. This suggests that the alteration in colonization efficacy is due to disrupted polyamine transport rather than increased biofilm formation. Further, spermidine uptake may represent a signal that modulates bacterial behavior in a manner favorable to host colonization.

In addition to spermidine uptake, the impact of the putrescine transporter PotE on colonization was analyzed. The Δ*potE* mutant [46], experienced a competitive advantage when compared to the wild-type *V. cholerae*. Previous research within the Karatan lab indicates that disruption of the PotE transporter protein does not alter levels of putrescine within the cell; therefore, the advantage seen is unlikely to be related directly to levels of the polyamine putrescine. In *V. cholerae*, there are two pathways by which putrescine can be synthesized. One is the ornithine to putrescine pathway, which is facilitated by the SpeC protein. The other method of putrescine synthesis is the arginine to agmatine to putrescine pathway facilitated by SpeB [46]. PotE functions by exchanging ornithine for putrescine and inhibiting PotE function has been shown to cause an increase in cadaverine levels by an unknown mechanism [46]. Interestingly, this increase was observed with and without supplemented ornithine. As putrescine levels have not been shown to change in the absence of PotE, one possibility is that PotE in some way impacts colonization through a build-up of the stress-associated cadaverine. It is also possible that PotE has an as yet unknown function relating to regulating cadaverine levels within *V. cholerae*. All results are summarized in Table 2.

Table 2. Summary of all strains utilized in these experiments with regard to the pathway being interrupted, the impact that each mutation has on biofilm formation, and the competitiveness of each mutant strain against co-inoculated wild-type *V. cholerae*.

Strain	Pathway Interrupted	Biofilm Phenotype	Competition against wild-type
Δ*vpsA*	Biofilm Formation	Inhibited	Advantage
Δ*nspS*	Polyamine Signaling	Decreased	No Impact
Δ*mbaA*	Polyamine Signaling	Increased	No Impact
Δ*potA*	Polyamine Transport	Increased	Disadvantage
Δ*potE*	Polyamine Transport	Decreased	Advantage
Δ*nspC*	Polyamine Synthesis	Decreased	Advantage
Δ*speC*	Polyamine Synthesis	Decreased	Advantage

Conclusions

The findings of this study strongly suggest that polyamine pathways within *V. cholerae* play a role in its colonization of potential animal vectors. The import of environmental polyamines appears to play a beneficial role in colonization, while the synthesis of the two polyamines spermidine and putrescine may play a role in cell to cell signaling or in the competition of *V. cholerae* with native intestinal microbiomes. The NspS/MbaA biofilm regulation system does not appear to play any role in the colonization of zebrafish, despite biofilm inhibition through *vps* gene deletions proving to be beneficial to early stages of colonization. It is unclear why the inhibition of a putrescine exporter would benefit *V. cholerae* and further details must be elucidated. Results to date suggest that the polyamine pathways are having an impact on competitiveness of *V. cholerae* outside of their roles in biofilms.

While the competitive advantages between the mutant and wild-type strains were relatively minor, small competitive advantages can generate large population differences over many generations. Indeed, small advantages due to differences in cellular processes such as polyamine utilization may account for why one strain of *V. cholerae* is able to become predominant over another. Further study is required to fully understand how the regulation of various polyamines may impact colonization, virulence, and persistence of *V. cholerae*.

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Vita

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