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Understanding the Genomic Influence and Virulence Capabilities of Environmentally Isolated Vibrios

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UNDERSTANDING THE GENOMIC INFLUENCE AND VIRULENCE CAPABILITIES OF ENVIRONMENTALLY ISOLATED VIBRIOS

by

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DEDICATION

I would like to dedicate this work to my parents, Terry and Dennis Pipes. Without the supportive and caring role that you both played throughout my graduate career, this work may have never come to fruition. I love you both so much and everything that you two have done for me during this time cannot be thanked enough.

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ABSTRACT

The genus *Vibrio* consists of Gram-negative bacteria that possess a curved rod shape and are routinely isolated from estuarine and coastal salt water. *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* are the major three species that contribute to human disease worldwide, and a variety of other *Vibrio* species contribute to persistent problems in the aquaculture and fishing industries. The CDC estimates that vibrios cause 80,000 cases of disease each year in the United States alone, however, this number is thought to be underestimated, since some disease is only self-limiting, meaning patients may not seek medical treatment and have an official diagnosis of vibriosis. Most cases in the United States are caused by *V. parahaemolyticus,* which infects humans after the consumption of contaminated raw or undercooked seafood, with raw oysters being the primary vector. *V. parahaemolyticus* typically causes mild gastroenteritis that usually resolves itself in a few days unless the patient is immunocompromised, where more serious infection can occur. *V. vulnificu*s has a much lower incidence of disease, with only about 50-100 cases occurring in the USA annually. However, this organism causes more severe infections, including necrotizing fasciitis and sepsis when introduced into an open wound and typically needs medical intervention. Warmer climates naturally support *Vibrio* growth, so with climate change on the rise, outbreaks of vibriosis are becoming more prevalent worldwide in all varying latitudes. This study focused on the genome distribution of various *Vibrio* species to identify the core genes that belong to all members of the genus, how pathogenicity and fitness islands play a role in the ecological

persistence of the organism, and how virulence factors shape the overall pathogenicity of *Vibrio vulnificus* by using an animal model of Zebrafish (*Danio rerio*).

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LIST OF ABBREVIATIONS

LPS.....Lipopolysaccharide

MLSA .....Multilocus Sequence Analysis

PAI .....Pathogenicity Island

recA..... Recombination Protein A (protein is RECA)

rtxA1..... Repeats in toxin gene (protein is RTXA1)

SLB .....Saline Luria-Bertani Broth

TA ..... Toxin-Antitoxin

TCBS..... Thiosulfate citrate bile salts sucrose agar

tdh .....thermostable direct hemolysin gene (protein is TDH)

T1SS.....Type One Secretion System

T3SS2.....Type Three Secretion System Two

vcgC .....virulence correlated gene clinical (protein is VCGC)

vcgE..... virulence correlated gene environmental(protein is VCGE)

vvhA..... *Vibrio vulnificus* hemolysin gene (protein is VVHA)

vvpe .....*Vibrio vulnificus* protease gene (protein is VVPE)

INTRODUCTION

The genus *Vibrio* is a diverse and ecologically important taxa of bacteria. These organisms are halophilic, Gram-negative, and belong to the largest class falling under the Proteobacteria, Gammaproteobacteria. This class of bacteria contains many prominent and well-studied genera of bacteria including *Escherichia*, *Pseudomonas*, *Salmonella*, *Shigella*, and many others. This class of bacteria are home to many opportunistic human pathogens, and specifically within the genus *Vibrio*, the three most well-known human pathogens include *Vibrio cholerae, Vibrio parahaemolyticus, and Vibrio vulnificus*.

In the United States alone, vibrios account for over 80,000 cases of illness annually, with 52,000 being a direct result from consuming contaminated seafood such as raw oysters or improperly handled cooked seafood (CDC, 2017). *Vibrio parahaemolyticus* accounts for most cases of vibriosis each year, with a little over half of the cases attributed to *V. parahaemolyticus* alone (CDC, 2017). Typical illness that results from a *V. parahaemolyticus* infection is acute gastroenteritis that is usually self-limiting and self-resolving with rest and hydration over 2-4 days (CDC, 2017). Common symptoms from a *V. parahaemolyticus* infection include vomiting, diarrhea, headache, nausea, and dehydration (Su and Liu, 2007). *Vibrio vulnificus*, a close relative of *V. parahaemolyticus*, however, can cause life-threatening wound infections. Many people with a *Vibrio vulnificus* infection require intensive care or limb amputations, and about 1 in 5 people with this infection die, sometimes within a day or two of becoming ill (CDC, 2017). Even though this organism causes severe disease, it typically only causes about

50 to 100 cases in the U.S. per year, but is especially dangerous in immunocompromised individuals, with liver disease being the most common underlying health condition of infected individuals (Heng et al., 2017). Its pathogenicity mechanism, like *V. parahaemolyticus* is very poorly understood despite decades worth of research, with only certain virulence factor genes of interest being implicated for both organism's pathogenicity.

Both *V. parahaemolyticus* and *V. vulnificus* thrive in warmer climates, and in regions where the water temperature does not dip below 15°C, these organisms can be routinely isolated from the environment year-round. Vibrios are typically associated with warmer, more tropical climates, but research is finding that these organisms are more commonly being reported in colder water regions like the Pacific Northwest (Abanto et al., 2020) and the Baltic Sea (Gyraite et al., 2019). With climate change on the rise, and the average sea surface temperature rate increasing at an alarming rate, vibrios will only continue to increase in abundance and expand globally in territory, even to higher latitudes that have never seen vibrios occur naturally before (EPA, 2021; Baker-Austin et al., 2013; Ford et al., 2020). Just between the years of 2006-2017, the average incidence of all *Vibrio* infections increased by 54% and with these organisms expanding on a global scale, there is serious threat of the emergence of isolates with epidemic potential (Abanto et al., 2020).

Not only is there a high impact cost in terms of human health with regards to vibrios, but there is also a high cost in terms of the aquaculture and fishery industry. *Vibrio parahaemolyticus* has been the cause of multiple shellfish bed closures, in many different states including colder water regions like Massachusetts (FSN, 2015). Oyster

beds and other shellfish harvesting was put on a 14-day halt back in 2015 after 6 people contracted an infection linked to *Vibrio parahaemolyticus* from the consumption of oysters from contaminated beds (FSN, 2015). These closures cause major problems for local economy and tourism. Other vibrios that continue to be problems for the aquaculture and shellfish industries include *Vibrio crassostreae*, *Vibrio alginolyticus*, *Vibrio coralliilyticus*, and *Vibrio splendidus*, all which greatly impact the Pacific oyster (*Crassostrea gigas*) and the Eastern oyster (*Crassostrea virginica*) (Bruto et al., 2017; Gonzalez-Escalona et al., 2006; Richards et al., 2014; Lacoste et al., 2001). Recent studies are now finding even more species of vibrios, such as *Vibrio jasicida* and *Vibrio rotiferianus*, routinely isolated from oysters and other shellfish that have not been seen before (Harrison et al., 2021). This continues to be a concern, with more species of vibrios being cultivated from the same location, as horizontal gene transfer of plasmids, virulence factor genes, and pathogenicity islands may begin to occur at higher rates.

Species of vibrios are found worldwide, in great numbers, and it is important to understand how these organisms persist in the environment, and what genes are shared across all species of vibrios. Because of the closely related nature of these organisms and how many species are routinely isolated from shared ecological niches, understanding how pathogenicity islands and fitness islands are distributed among environmentally isolated vibrios is an important piece to understanding how pathogenicity genes may be shared across the genus. Environmentally isolated vibrios that can cause disease are thought to be distributed in low abundance, however, when zebrafish are challenged with environmentally isolated strains of *Vibrio vulnificus*, we found that virulence did vary

greatly, but there was no indication that some of the pathogenicity “marker” genes played a role in predicting virulent versus non- virulent strains.

CHAPTER 1

DEFINING THE CORE GENOME OF THE GENUS, *VIBRIO*

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ABSTRACT

Vibrio is a large and highly diverse bacterial genus. Many species within this genus impact commercial fisheries and some can cause human diseases. There has been an increase in human *Vibrio*-related infections worldwide, which can be attributed in part to transfer of virulence genes among the *Vibrio* species. However, very little work has been done to evaluate the genetic plasticity of this genus. This study utilized high-quality genome sequences from strains of 100 individual *Vibrio* species of both clinical and environmental origin to examine the evolution and genome dynamics of the genus and tested a total of 153 genomes. We identified the *Vibrio* core genome, the genes that are required for a bacterial species to function as a *Vibrio*. The core genome is the portion of the genome that is shared across all species in the genus. It was found to be very small relative to those of comparable taxa, consisting of only 158 genes out of the 27,473 gene families that were represented throughout the entire genus.

INTRODUCTION

The vibrios comprise a highly diverse and well-studied bacterial genus. They are Gram negative, curved rod shaped, and belong to the class *Gammaproteobacteria*. Many *Vibrio* species are halophilic and thus associated with marine and estuarine ecosystems. The genus *Vibrio*, along with allied genera in the family *Vibironaceae*, constitutes one of the largest prokaryotic families, with more than 100 species identified and numerous genome sequences deposited in the NCBI GenBank. Vibrios are abundant and widespread among marine ecosystems, occurring as both free-living bacteria and symbionts of higher organisms.

Environmental strains of vibrios can severely impact aquaculture and wild shellfish harvests, such as those of the Eastern oyster (*Crassostrea virginica*) (Froelich et al., 2013) and the Asian tiger shrimp (*Penaeus monodon*) (Kaneko et al., 1998). Some of the vibrios can cause human infections, at times producing epidemic disease outbreaks (DePaola et al., 2000; Faruque et al., 2003; Karaolis et al., 1998; McLaughlin et al., 2005). It was thought that vibriosis infections only occur at latitudes having warm temperatures, but cases of vibriosis have been documented in cold water regions as well, including Europe and North America (Baker-Austin et al., 2013, 2016; Kumar et al., 2016; Vezzulli et al., 2016). Vibriosis outbreaks in cold water regions, such as those associated with the Pacific oyster (*Crassostrea gigas*), have increased in frequency (Baker-Austin et al., 2013; Vezzulli et al., 2013, 2016), perhaps due to rising sea surface temperatures (Baker-Austin et al., 2016; Goertz et al., 2013; Young et al., 2015).

Examples of vibrios that impact commercial species of invertebrates include *Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio splendidus,* and *Vibrio anguillarum*. In particular, *V. parahaemolyticus* and *V. vulnificus* may contaminate seafood harvested for human consumption and lead to cases of gastroenteritis and septicemia if consumed (Broberg et al., 2011; CDC, 2016; FDA, 2005). There are over 80,000 cases of vibriosis-related infections each year (CDC, 2016; Scallan et al., 2011). Hospitalization and mortality rates of *V. parahaemolyticus* gastroenteritis are not extremely high, 22% and 1%, respectively (Scallan et al., 2011). *V. vulnificus* cases are less common; only about 100 occur each year in the United States. However, the hospitalization and mortality rates caused by this bacterium are much higher, at 92% and 35%, respectively (Scallan et al., 2011). Similarly, *Vibrio cholerae,* the causative agent of cholera, is a freshwater-to-estuarine human pathogen that can produce fatal dehydration (Herrington et al., 1998; Orata et al., 2014; Vezzulli et al., 2010). It is estimated that worldwide there are about 2.0 million cases of cholera and roughly 95,000 cholera deaths annually (CDC, 2016).

Vibrios are able to occupy many different ecological niches very successfully. *V. cholerae* is one such example, as it is able to adhere to and hydrolyze chitinous exoskeletal materials of invertebrates which can serve as a source of carbon and nitrogen for the bacteria (Kaneko et al., 1975; Lovell, 2017). *V. cholerae* has a well-established association with copepods, which commonly serve as a vector of cholera infections in Bangladesh water systems (Bhowmick et al., 2006; Huq et al., 1983; Nalin, et al., 1979; Tamplin et al., 1990). Other vibrios persist in the environment by maintaining mutualistic relationships with host organisms. For example, *Vibrio fischeri* maintains a

mutualistic relationship with the Hawaiian bobtail squid (*Euprymna scolopes*) (Visick et al., 2000).

An important ecological application of this genomic comparison is that it can be applied as a reference for studying the community dynamics of vibrios. In particular, some species develop biofilms involving interactions among different populations (Hammer et al., 2003; Nyholm et al., 2000; Yildiz and Visick, 2009; Zhu et al., 2003). Biofilms offer a stable environment in which bacterial conjugation and recombination can occur, and recent research has shown that recombination may be commonplace among vibrios (Sawabe et al., 2007). The genus *Vibrio* includes numerous closely related species that cannot be differentiated by 16S rRNA gene sequences alone as this gene is too highly conserved (Thompson et al., 2004, 2005). There is a relatively high level of sharing of genes among the vibrios. This is due to co-occurrence of many vibrios together in microenvironments, such as biofilms and copepod exoskeletons, as well as shared community dynamics. High levels of sequence conservation among the vibrios are attributed to recombination, and genes that were once thought to be species specific have been identified in several *Vibrio* species (Klein et al., 2014).

Little work has been done comparing the genomes of many different vibrios and the genes that are shared among all *Vibrio* species are not well characterized. The goal of this *Vibrio* genome comparison was to examine and characterize genes that are shared among all *Vibrio* genomes to determine which genes are essential for a *Vibrio* species. Put another way, what genes make up the core genome of a *Vibrio*? We found that the core genome is extremely small. The accessory genome, or the genes that are common

to several species, but not found in all species, was much larger than the core genome, making up as much as 90% of the total genome.

MATERIALS AND METHODS

Eleven environmental *Vibrio* isolates were newly sequenced for this study. The isolates came from water and sediment samples from the relatively pristine North Inlet estuary near Georgetown, SC, USA (33°20'N, 79°12'W) in August and September in 2011 as described previously (Gutierrez West et al., 2013). Environmental *V. vulnificus* strains were isolated from low salinity environments from Winyah Bay and the Waccamaw River, also located near Georgetown, SC, USA. The water samples were plated on CHROMagar *Vibrio* (DRG International, NJ) for isolation of *V. vulnificus* strains following the US Food and Drug Administration protocol (DePaola et al., 2004). The newly sequenced vibrios included confirmed *V. parahaemolyticus* strains (JS-8-11-2 and TS-8-11-4), confirmed *Vibrio diabolicus* strains (CW-9-11-1 and JBS-8-11-1), confirmed *V. vulnificus* strains (WR1-BW, WR2-BW, WR2-BW2, and 05-25-BW5), a confirmed *Vibrio natriegens* species (T-C2-11), and two novel *Vibrio* species (JPW-9-11-11, 05-20-BW147). Accession numbers: GCA_013369315.1 (*V. parahaemolyticus* JS-8-11-2), GCA_003798505.1 (*V. parahaemolyticus* TS-8-11-4), GCA_013369375.1 (*V. vulnificus* WR1-BW), GCA_003798485.1 (*V. vulnificus* WR2-BW), GCA_013369285.1 (*V. vulnificus* WR2-BW2), GCA_013369015.1 (*V. vulnificus* 05-25-BW5), SZTN00000000 (*V. natriegens* T-C2-11), GCA_013369295.1 (*V. diabolicus* CW-9-11-1), and GCA_003798525.1 (*V. diabolicus* JBS-8-11-1), GCA_013369335.1 (*Vibrio* sp. JPW-9-11-11), GCA_013369385.1 (*Vibrio* sp. 05-20-BW147).

Pure bacterial cultures were routinely cultivated in liquid Saline Luria-Bertani Broth (SLB; per L 27 g NaCl, 10 g Tryptone, 5 g Yeast Extract). Genomic DNA was isolated using the Wizard Genomic DNA Purification kit (Promega, Madison, WI**)** following the protocol for Gram negative organisms. The only change to the protocol included increasing the incubation on ice from 5 minutes to 30 minutes to precipitate proteins. DNA quantity was measured via Quibit fluorescence analysis. Libraries were prepared and then sequenced using an Illumina MiSeq (V3 26300 base) at the Indiana University Center for Genomic Studies. Sequencing was done on a shared run as a part of The Genome Consortium for Active Teaching NextGenSequencing Group (GCAT-SEEK) (Buonaccorsi et al., 2011, 204). Sequencing reads were filtered (median phred score 0.20), trimmed (phred score 0.16), and assembled using the paired-end *de novo* assembly option in NextGENe V2.3.4.2 (SoftGenetics**,** State College, PA). The assembled genomes were uploaded to the Rapid Annotation with Subsystem Technology (RAST) web service (Aziz et al., 2008; Overbeek et al., 2005) for analysis, guided contig reordering, and assembly improvement. Genomes were aligned based on completed sequences using dotplot comparisons. This graphical method allows for alignment and reordering of the genome based on completed, closed chromosomes. The nucleotide sequences were translated to protein sequences which were used in the Bacterial Pan Genome Analysis (BPGA) pipeline.

A total of 153 (including the 11 newly sequenced for this study) *Vibrio* genomes were used to identify the core genome (Table 1.1). The data set included 100 different species, including 2 novel environmental *Vibrio* species. *Vibrio* species used in the study were chosen based on their varying impacts on human health, aquaculture and seafood

harvests, and interactions with other higher organisms. Sequences of *Vibrio* species were obtained from GenBank and at least one sequence every species that is currently represented in GenBank was obtained and used for this study. Type strains were included when possible. Representative genomes, which exemplify other genomes of a given species, were used when type strains were not available. These sequences were closed, ungapped chromosomes, or were in supercontig scaffold form.

All of the *Vibrio* genome sequences were translated into protein sequences and were uploaded into the BPGA pipeline (Chaudhari et al., 2015). The BPGA works by performing a USEARCH hit for clustering, using 50% sequence identity as its cutoff. MUSCLE was the alignment software used to align concatenated core genes. USEARCH was also used to assign predicted clustered orthologous gene (COG) families and KEGG IDs, through best hit identities based on respective reference databases.

Once the orthologous genes were concatenated through the BPGA, MEGA7 was used to build alignments and make phylogenetic trees (Kumar et al., 2016). Maximum likelihood trees were built using the fully concatenated core genomes, as well as the core genes of the genome of *Vibrio* that are found on chromosomes 1 and 2. Species level homology of *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* was also examined. Sequence based alignments were performed using RAST with different strains of each species and gene counts were acquired based on homologous genes. Core, accessory, and unique gene proportions per species were found.

RESULTS

This *Vibrio* sequence collection was assembled to facilitate the examination and comparison of both environmentally and clinically isolated strains. This study also

contributes to tracking the evolution of the *Vibrio* genus by identifying the genes that are shared among all species (core genome), along with identifying the mobile elements of the genus that are in high abundance, but not apparent in every species (accessory genome). The BPGA analysis showed the *Vibrio* core genome consists of 158 genes (Table 1.2). The core genome is defined as the genes that are shared across the genus in all the genomes that we examined. These genes may be involved in essential functions for the genus since they are seen in every species across the genome.

Most of the genes in the core genome are involved with genetic information processing and signaling, which includes genes involved in transcription, translation, replication, and recombination (Table 1.2). They accounted for 41% of the total core genome (Figure 1.4). Genes involved in metabolism (including carbohydrate, amino acid, vitamins, lipids, and nucleotides) also accounted for a substantial portion of the core, with these categories contributing to 36% of the core genome (Figure 1.4). Surprisingly, 10% of the core genome was made up of genes that have an unknown function, which means there is no current defined knowledge for what protein that gene encodes for, or what its purpose is (Figure 1.4).

It was also important to determine where in the genome the majority of the core genes were located since all *Vibrio* genomes are made up of two chromosomes, with the first chromosome being the larger of the two. We hypothesized that the core genome would be located on the first, more conserved chromosome, and the second chromosome would contain more of the accessory and unique genes. To test this hypothesis, we used a smaller subset of the 153 genomes where all the genes could be assigned to one of the two chromosomes. This subset included 26 genomes, which encompassed 8 *Vibrio*

species. This analysis showed that 93% of the core genes were found on chromosome 1, whereas only 7% of the core genes were on chromosome 2. The accessory genome predominates chromosome 2, with 84.6% of the genes that are found on chromosome 2 are included in the accessory genome.

The accessory genome is thought to carry the supplemental traits of the organism, including but not limited to pathogenicity markers, toxin-producing genes, and antibiotic resistance genes (Chaudhari et al., 2015). With *Vibrio*, we saw a much broader and more important role of the accessory genome. High variability within the accessory genome can be seen down to the species level. The total gene families that were found from the BPGA across the entire *Vibrio* genus totaled out to 27,473 unique gene families.

To better understand the core genome dynamics of individual species within the *Vibrio* genus, direct gene comparison counts obtained through RAST to study *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio cholerae*. These three species have the most impact on human health on a global scale, especially in immunocompromised individuals. Homologous gene comparisons revealed that *V. parahaemolyticus* and *V. vulnificus* share roughly 77-80% of their genes, with roughly 3-6% of the genes in each individual strain scored as unique or unshared with any other strain (Figures 1.5a-b). The rest, about 14-20%, constitutes the accessory genome, meaning the genes are shared with at least two other strains in this species-specific test. Only about 70% of the *V. cholerae* genome is shared among all strains, with a high accessory genome content of about 20-28%. Some individual strains, like *V. cholerae* RC385, had a high gene uniqueness percentage with 10% of its genes being unique to that strain alone (Figure 1.5c).

DISCUSSION

This study emphasized just how diverse the *Vibrio* genus truly is. The core genome was extremely small, and this gives insight into the genome dynamics among different species in the genus. The location of the core genes fell predominately on the first chromosome with 93% of the core genes found on chromosome one. Chromosome 1 is larger than chromosome 2 and is typically thought of as the more conserved and less genetically flexible of the two chromosomes that vibrios have (Okada et al., 2005). The majority of chromosome 2 was made up of accessory or unique genes. The fact that there were very few core genome genes on chromosome 2 may offer vibrios a selective advantage in the environment, as chromosome 2 can be used as a site of horizontal gene transfer and the addition of new DNA, without interfering with the core genes that are necessary for a species to be a *Vibrio*.

Based on the phylogeny results, the second chromosome is considered a site of recombination and lateral transfer of genes; many *Vibrio* species are known to bear integron and transposon genes on their second chromosome, which could allow for the inclusion of foreign DNA in the genome (Chen et al., 2011; Cohen et al., 2007; Dobrindt et al., 2004; Hacker et al., 1997, 2000; Klein et al., 2018). The second chromosome has also been shown to be the location of pathogenicity islands (PAIs) (Klein et al., 2018). The second chromosome is less conserved than the first, have fewer core genome genes, and act as a site for recombination and DNA integration. The phylogeny of the core genome that seen on chromosome 1 versus chromosome 2 depicts this. While looking at the core genome genes that are present on chromosome 1, there is still species-level resolution within the phylogenetic tree, whereas the core genome genes found on

chromosome 2 do not offer the same species resolution (Figure 1.2 and Figure 1.3). This supports the theory that the second chromosome is less stable and allows for more genome flexibility in recombination and uptake of foreign DNA (Hacker et al., 1997; Chen et al., 2011; Dohen et al., 2007).

Phylogenetic data also shows that the core genomes are conserved within most species, but the core genome phylogeny can show how genetic drift and evolution may have occurred based on species relationships (Figure 1.1). The phylogenetic analysis also showed some species may be heterotypic synonyms of other *Vibrio* strains, including *Vibrio antiquarius*. *V. antiquarius* EX25^{T} clustered in the same clade as *V. diabolicus*, with a bootstrap value of 100 (Figure 1.2). *V. antiquarius* was first isolated from a deep-sea hydrothermal vent along the East Pacific Rise (Hasan et al., 2015). *V. diabolicus* was also first isolated from a polychaete located at a deep-sea hydrothermal vent, however, this organism has also been found in less extreme locations such as estuarine sediment (Klein et al., 2014; Raguenes et al., 1997). Their genomic similarity and similar isolation location indicates that *V. antiquarius* was misidentified as a novel species; it is actually a strain of *V. diabolicus*. This has been notion has been supported by other studies that have demonstrated that there is high species similarity between these two organisms, and due to the ubiquitous nature of vibrios, may be an issue for other *Vibrio* species as well (Turner et al., 2018)

The second clade of organisms that did not have high species resolution based on the core genome included *V. lentus, V. crassostreae, V. cyclitrophicus, and V. splendidus.* Based on their core genome phylogeny, none of these strains grouped out with their respective species. For example, *V. lentus* 5F79 branched out with the *V. tasmaniensis*

clade and the other *V. lentus* strain 10N.22.54.E4 formed a clade with *V. crassostreae* 9CS106, *V. cyclitrohpicus* ECSMB14105, and *V. splendidus* 12B01 (Figure B.1). All four of these organisms have been isolated from bivalves such as oysters, mussles, and clams (Li et al., 2019; Gomez-Leon et al., 2005; Macian et al., 2001; Bruto et al., 2017). It is very likely that these organisms co-exist with one another and because of this, many of their genes may be shared and due to their close relatedness of their habitat and similar ecological properties, the genetic drift between species is extremely low, and researchers may not be able to determine species just on the core genes alone. Full genome sequencing may be required to aptly ID these organisms due to their similarity (Figure 1.1; Figure B.1).

Researchers have made distinctions between clinical, disease-causing strains versus purely environmental non-disease-causing strains of some *Vibrio* species, particularly *V. vulnificus* and *V. parahaemolyticus* (Kaysner and DePaola, 2004; Baker-Austin et al., 2008; Miyamoto et al., 1969; Shirai et al., 1990). It has been stated that many environmentally derived *Vibrio* species are nonpathogenic because they lack the proper virulence factors, with some studies indicating only 1-2% of environmentally isolated strains contain virulence factors like *tdh*, *trh*, and *tlh* for *Vibrio parahaemolyticus* (Kaysner and DePaola, 2004; Baker-Austin et al., 2008; Taniguchi et al., 1986). However, studies have shown that certain species-specific virulence factors ascribed to *V. vulnificus* and *V. parahaemolyticus* occur at higher frequencies than previously thought and also appear in other species (Gutierrez West 2013; Klein et al., 2014). Based on the phylogeny of the core genome, there is no difference between clinically derived strains and environmentally derived strains for *V. parahaemolyticus* and *V. vulnificus* (Figure

1.1). This indicates that these species would more than likely be able to undergo horizontal gene transfer and share potential virulence factors easily to different strains, which makes it very difficult to determine which environmental strains are pathogenic or not.

The vibrios have a very large total pangenome with over 27,000 gene families identified from the BPGA analysis, with the accessory genome making up typically two-thirds or more of a single strain's total genome. The pangenome indicates how adaptable an organism is; meaning the more diverse the genome is, the more ability the strain has to adapt and persist under a variety of ecological regimes. This large pangenome reflects the diverse fitness capabilities and the ecological persistence of the vibrios.

The accessory genome seems to play a more vital role in the way that vibrios persist in the environment. By having a small core genome, this allows for more genetic flexibility while in the environmental setting and may allow for more mobile genetic elements and horizontal gene transfer to take place since there are less "vital" genes present in the total genome (Oliveira et al., 2017). This dynamic has been seen in other similar genera compared to the *Vibrio* genus. For example, the genus *Photobacterium* is closely related to the genus *Vibrio* as both are in the family *Vibrionaceae*. Its core genome was found to be 1,232 genes, which makes up approximately 25% of the total genome (Machado and Gram, 2017). *Photobacterium* species are a highly diverse group of organisms that can persist in many different environmental niches; it is also the second largest known genus, with *Vibrio* being the only larger genus characterized to date (Machado and Gram, 2017). Another genus of bacteria that is similar to both *Photobacterium* and *Vibrio* with regard to size, diversity, and abundance in the

environment is the genus *Bacillus*. It is the largest and best characterized genus of the Gram-positive bacteria; there are over 300 species currently deposited into the NCBI GenBank. It also had a small core genome size (814 shared orthologous genes) and a very large pan-genome made up of over 19,000 gene families (Alcaraz et al., 2010) (comparatively *Vibrio* has 27,473 gene families and *Photobacterium* has 28,951 gene families in their pan-genomes). With this, it seems that bacterial genera that consist of opportunistic taxa and have the ability to grow under a wide variety of environmental conditions and exploit a wide variety of organic and inorganic substrates as nutrient sources, have smaller core genomes, and a much larger and variable accessory genome. In support of this idea, the genus *Listeria*, which contains organisms that have more specific nutritional and environmental needs, has a very large core genome making up about two-thirds of the entire genome, which is the exact opposite of *Vibrio* (den Bakker et al., 2010).

Vibrio species can undergo horizontal gene transfer with one another when they occupy the same environmental niche. With this, further studies examining the rates of lateral transfer among vibrios in the environment are needed. Vibrios are highly persistent in the environment and with climate change, are exhibiting longer periods of activity during the year (Lutz et al., 2013; Stauder et al., 2010; Vezzulli et al., 2010). Consequently, the pathogenicity loci and potential of these loci to be transferred laterally in naturally occurring environmental strains (Klein et al., 2018) are clearly important.

Table 1.1: Strain list and results from the Bacterial Pan Genome Analysis pipeline. Core genes represent the number of genes that all *Vibrio* genomes have. The number of accessory genes represents the number of genes that each individual strain has that is shared with at least one other *Vibrio* strain genome. The number of unique genes are the genes that are only present in one particular *Vibrio* genome. Type strain is represented with ^{T}. Strains in bold were newly sequenced for this study.

Organism name	**No. of accessory genes**	**No. of unique genes**	**No. of exclusively absent genes**
Vibrio aerogenes CECT 7868	3712	408	0
Vibrio aestuarianus 02041	2941	265	0
Vibrio alfacsensis 04Ya108	3673	533	0
Vibrio alginolyticus ATCC 17749^{T}	4136	64	1
Vibrio alginolyticus ZJ-T	4310	132	0
Vibrio algivorus SA2	2780	188	0
Vibrio anguillarum 775	3111	8	0
Vibrio anguillarum M3	3190	5	1
Vibrio anguillarum NB-10	3334	72	1
Vibrio antiquarius EX25	4188	81	0
Vibrio aphrogenes CA-1004	2515	169	0
Vibrio atlanticus CECT 7223^{T}	3839	138	0
Vibrio atypicus DSM 25292^{T}	3904	160	0
Vibrio azureus LC2-005	3744	18	0
Vibrio azureus NBRC 104587^{T}	3730	10	0
Vibrio barjaei 3062	4553	104	0
Vibrio bivalvicida 605	3951	187	0
Vibrio brasiliensis LMG 20546^{T}	3794	184	0
Vibrio breoganii FF50	3221	443	0

Vibrio breoganii ZF-29	3183	127	1
Vibrio campbellii ATCC BAA-1116	4209	149	0
Vibrio campbellii LMB29	4664	516	10
Vibrio caribbeanicus ATCC BAA-2122	2978	639	4
Vibrio casei DSM 22364^{T}	3086	171	0
Vibrio casei JB196	3042	160	1
Vibrio celticus CECT 7224^{T}	4106	7	0
Vibrio celticus RD-8-15	4117	2	0
Vibrio chagasii ECSMB14107	4054	107	4
Vibrio chagasii LC2-408	4001	91	0
Vibrio chemaguriensis Iso1	3998	63	3
Vibrio cholerae 2012Env-9	3295	90	0
*Vibrio cholera*e FORC_055	3148	158	0
Vibrio cholerae O1 biovar El Tor str. N16961	3240	91	0
Vibrio cidicii 2423-01 57	3576	126	1
Vibrio cidicii 2756-81^{T}	2535	233	36
Vibrio cincinnatiensis NCTC12012^{T}	2825	163	1
Vibrio comitans NBRC 102076	3394	201	0
Vibrio coralliilyticus OCN014	4529	188	3
Vibrio coralliilyticus RE98	4683	595	0
Vibrio coralliirubri corallo1^{T}	4205	134	0
Vibrio coralliirubri MARg	4362	111	0
Vibrio crassostreae 9CS106	3957	226	0
Vibrio crassostreae J2-9	4336	277	0
Vibrio cyclitrophicus ECSMB14105	3934	75	0
Vibrio cyclitrophicus FF274	4033	179	0
***Vibrio diabolicus* CW-9-11-1**	4246	104	0

***Vibrio diabolicus* JBS-8-11-1**	4192	126	0
Vibrio diabolicus CNCM I-1629^{T}	4235	201	0
Vibrio diazotrophicus NBRC 103148^{T}	3616	258	0
Vibrio europaeus PP-638^{T}	4405	228	0
Vibrio fluvialis AK 1296-A2-1	4006	80	0
Vibrio fluvialis ATCC 33809^{T}	3989	69	0
Vibrio fortis Dalian14	4133	222	0
Vibrio furnissii CIP 102972^{T}	4081	123	6
Vibrio furnissii NCTC 11218	4016	96	4
Vibrio galatheae S2757^{T}	3212	228	0
Vibrio gallaecicus DSM 23502^{T}	3727	324	0
Vibrio gazogenes ATCC 43942	3444	271	0
Vibrio gazogenes DSM 21264^{T}	3438	240	0
Vibrio gigantis LGP 13^{T}	4332	306	0
Vibrio halioticoli IAM 14596^{T}	2832	377	1
Vibrio hangzhouensis CGMCC 1-7062^{T}	3897	330	0
Vibrio harveyi ATCC 33843	4776	109	1
Vibrio harveyi ATCC 43516	4968	39	0
Vibrio harveyi CAIM 1792	4920	131	0
Vibrio hepatarius DSM 19134^{T}	3628	500	0
Vibrio hyugaensis 090810a^{T}	4448	85	0
Vibrio hyugaensis 100512A	4432	109	0
Vibrio ichthyoenteri ATCC 700023^{T}	3566	237	0
Vibrio injenensis M12-1144^{T}	2825	179	0
Vibrio inusitatus NBRC 102082^{T}	3284	211	0
Vibrio ishigakensis JCM19231^{T}	4022	620	2
Vibrio ishigakensis JCM19241	3990	595	4

Vibrio jasicida 090810c	4848	49	2
Vibrio jasicida BFLP-10	4892	45	0
Vibrio kanaloae 5S-149	3712	73	0
Vibrio lentus 5F79	4166	10	0
*Vibrio lentus*10N.222.54.E4	4375	101	0
Vibrio mangrovi CECT 7927^{T}	3428	543	0
Vibrio maritimus CAIM 1455^{T}	3263	584	25
Vibrio maritimus JCM 19235	2760	645	46
Vibrio mediterranei NBRC 15635^{T}	4567	140	0
Vibrio mediterranei QT6D1	4593	169	0
Vibrio metoecus 08-2459	3094	104	0
Vibrio metschnikovii CIP 69-14^{T}	2928	9	1
Vibrio metschnikovii NCTC8563	2842	6	3
Vibrio mexicanus CAIM 1540^{T}	2642	598	22
Vibrio mimicus ATCC 33654^{T}	3426	111	0
Vibrio mimicus SCCF01	3506	129	0
Vibrio mytili CAIM 528^{T}	3125	255	9
Vibrio natriegens ATCC 14048^{T}	4022	203	0
***Vibrio natriegens* T-C2-11**	3789	208	0
Vibrio navarrensis 0053-83	3315	89	0
Vibrio navarrensis ATCC 51183^{T}	3311	111	0
Vibrio neptunius S2394	4022	243	0
Vibrio nereis NBRC 15637^{T}	3303	151	0
Vibrio nigripulchritudo MADA3029	5041	295	0
Vibrio nigripulchritudo SFn1	5038	212	0
Vibrio ordalii ATCC 33509^{T}	2600	55	0
Vibrio orientalis ATCC 33934^{T}	3702	225	0

Vibrio ostreicida UCD-KL16	3329	344	0
Vibrio owensii 170302	4959	306	0
Vibrio owensii XSBZ03	4708	108	0
Vibrio pacinii DSM 19139^{T}	3378	282	0
Vibrio panuliri ASM193972	3661	267	0
Vibrio paracholerae 2016V-1111	3107	50	0
Vibrio parahaemolyticus 17802^{T}	4193	127	0
***Vibrio parahaemolyticus* JS-8-11-2**	4145	73	0
Vibrio parahaemolyticus O1K33 str. CDC_K4557	4258	67	0
Vibrio parahaemolyticus RIMD 2210633	4246	104	0
***Vibrio parahaemolyticus* TS-8-11-4**	4163	68	0
Vibrio pectenicida CAIM 594^{T}	3171	433	4
Vibrio penaeicida TUMSAT-NU1	4747	505	0
Vibrio ponticus CAIM 1731	3593	215	1
Vibrio ponticus JCM 19238^{T}	3583	723	0
Vibrio profundi TP187^{T}	3951	400	0
Vibrio proteolyticus NBRC 13287^{T}	3652	388	0
Vibrio qinghaiensis Q67^{T}	2939	114	0
Vibrio quintilis CECT 7734^{T}	3914	698	0
Vibrio renipiscarius DCR 1-4-2^{T}	3298	192	0
Vibrio rhizosphaerae DSM 18581^{T}	3239	256	0
Vibrio rotiferianus B64D1	4240	113	0
Vibrio rotiferianus HM-10	4284	154	0
Vibrio rumoiensis FERM P-14531^{T}	2974	296	2
Vibrio sagamiensis NBRC 104589^{T}	3267	304	0
Vibrio scophthalmi FP3289	3772	289	0
Vibrio scophthalmi VS-12	3686	154	0

Vibrio scophthalmi VS-05	3690	124	0
Vibrio sinaloensis DSM 21326	3961	187	0
Vibrio sonorensis CAIM 1076^T	2847	851	17
***Vibrio* sp 05-20 BW147**	3542	187	1
***Vibrio* sp JPW-9-11-1**	3340	245	0
Vibrio splendidus 12B01	4247	216	18
Vibrio splendidus ATCC 33789	4066	161	0
Vibrio superstes G3-29^T	3544	208	0
Vibrio taketomensis C4III291	3084	270	9
Vibrio tapetis CECT4600^T	3592	757	2
Vibrio tasmaniensis 5F-179	4170	30	0
Vibrio tasmaniensis LGP32	3832	100	1
Vibrio toranzoniae CECT 7225^T	3568	83	0
Vibrio toranzoniae R17	3616	87	0
Vibrio tritonius JCM 16456^T	3393	760	0
Vibrio tubiashii ATCC 19109^T	4322	404	0
***Vibrio vulnificus* 05-25-BW5**	3989	56	0
Vibrio vulnificus 93U204	4096	98	0
Vibrio vulnificus CMCP6	4169	160	0
***Vibrio vulnificus* WR1-BW**	3992	112	0
***Vibrio vulnificus* Aug WR2-BW**	4106	58	0
***Vibrio vulnificus* WR2-BW2**	4004	74	1
Vibrio vulnificus YJ016	4236	167	0
Vibrio xiamenensis CGMCC 1-10228	3624	862	0
Vbrio zhugei HBUAS61001^T	2673	436	1
Vibrio ziniensis ZWAL4003^T	3554	310	0

Table 1.2: KEGG orthology gene definitions of core genome.

Core Gene (Amino Acid Gene Length)	KEGG Orthology Gene Assignment	Definition
Gene_1 (513)	K02111	ATPF1A, atpA; F-type H+/Na+-transporting ATPase subunit alpha
Gene_2 (377)	K00526	E1.17.4.1B, nrdB, nrdF; ribonucleoside-diphosphate reductase beta chain
Gene_3 (469)	K01915	glnA, GLUL; glutamine synthetase
Gene_4 (419)	K03628	rho; transcription termination factor Rho
Gene_5 (429)	K03885	ndh; NADH:ubiquinone reductase (H+-translocating)
Gene_6 (416)	K00600	glyA, SHMT; glycine hydroxymethyltransferase
Gene_7 (434)	K00892	gsk; inosine kinase
Gene_8 (423)	K11754	folC; dihydrofolate synthase / folylpolyglutamate synthase
Gene_9 (398)	K00639	kbl, GCAT; glycine C-acetyltransferase
Gene_10 (362)	K01735	aroB; 3-dehydroquinate synthase
Gene_11 (340)	K19810	epmB; L-lysine 2,3-aminomutase
Gene_12 (346)	K01698	hemB, ALAD; porphobilinogen synthase
Gene_13 (354)	K01599	hemE, UROD; uroporphyrinogen decarboxylase
Gene_14 (346)		Gene of unknown function
Gene_15 (344)		methionine ABC transporter ATP-binding protein MetN
Gene_16 (325)	K04087	hflC; modulator of FtsH protease HflC
Gene_17 (337)	K01424	E3.5.1.1, ansA, ansB; L-asparaginase
Gene_18 (342)	K00674	dapD; 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase
Gene_19 (334)	K00145	argC; N-acetyl-gamma-glutamyl-phosphate reductase
Gene_20 (337)	K00133	asd; aspartate-semialdehyde dehydrogenase
Gene21 (321)	K03598	rseB; sigma-E factor negative regulatory protein RseB
Gene_22 (315)	K05541	dusC; tRNA-dihydrouridine synthase C
Gene_23 (314)	K01749	hemC, HMBS; hydroxymethylbilane synthase
Gene_24 (302)	K13283	fieF; ferrous-iron efflux pump FieF
Gene_25 (305)	K08974	K08974; putative membrane protein
Gene_26 (308)	K07106	murQ; N-acetylmuramic acid 6-phosphate etherase
Gene_27 (302)	K04763	xerD; integrase/recombinase XerD
Gene_28 (305)	K01963	accD; acetyl-CoA carboxylase carboxyl transferase subunit beta
Gene_29 (291)	K03442	mscS; small conductance mechanosensitive channel
Gene_30 (290)	K00963	UGP2, galU, galF; UTP--glucose-1-phosphate uridylyltransferase
Gene_31 (287)	K04083	hslO; molecular chaperone Hsp33
Gene_32 (294)	K00858	ppnK, NADK; NAD+ kinase

Gene_33 (280)	K01265	map; methionyl aminopeptidase
Gene_34 (291)	K07056	rsmI; 16S rRNA (cytidine1402-2'-O)-methyltransferase
Gene_35 (290)	K01902	sucD; succinyl-CoA synthetase alpha subunit
Gene_36 (269)	K00215	dapB; 4-hydroxy-tetrahydrodipicolinate reductase
Gene_37 (263)	K00930	argB; acetylglutamate kinase
Gene_38 (274)	K02886	RP-L2, MRPL2, rplB; large subunit ribosomal protein L2
Gene_39 (270)	K02564	nagB, GNPDA; glucosamine-6-phosphate deaminase
Gene_40 (269)	K02073	metQ; D-methionine transport system substrate-binding protein
Gene_41 (263)		Gene of unknown function
Gene_42 (267)	K01092	E3.1.3.25, IMPA, suhB; myo-inositol-1(or 4)-monophosphatase
Gene_43 (262)	K00677	lpxA; UDP-N-acetylglucosamine acyltransferase
Gene_44 (257)	K02500	hisF; imidazole glycerol-phosphate synthase subunit HisF
Gene_45 (250)	K00655	plsC; 1-acyl-sn-glycerol-3-phosphate acyltransferase
Gene_46 (250)	K00806	uppS; undecaprenyl diphosphate synthase
Gene_47 (223)		ABC transporter ATP-binding protein
Gene_48 (249)	K02342	dnaQ; DNA polymerase III subunit epsilon
Gene_49 (245)	K06177	rluA; tRNA pseudouridine32 synthase / 23S rRNA pseudouridine746 synthase
Gene_50 (249)	K01420	fnr; CRP/FNR family transcriptional regulator, anaerobic regulatory protein
Gene_51 (231)	K06183	rsuA; 16S rRNA pseudouridine516 synthase
Gene_52 (243)	K15396	trmJ; tRNA (cytidine32/uridine32-2'-O)-methyltransferase
Gene_53 (233)	K06997	yggS, PROSC; PLP dependent protein
Gene_54 (224)	K01783	rpe, RPE; ribulose-phosphate 3-epimerase
Gene_55 (234)	K03784	deoD; purine-nucleoside phosphorylase
Gene_56 (227)		Gene of unknown function
Gene_57 (239)	K03439	trmB, METTL1, TRM8; tRNA (guanine-N7-)-methyltransferase
Gene_58 (214)	K07323	mlaC; phospholipid transport system substrate-binding protein
Gene_59 (238)	K06920	queC; 7-cyano-7-deazaguanine synthase
Gene_60 (152)	K03635	MOCS2B, moaE; molybdopterin synthase catalytic subunit
Gene_61 (226)	K07220	K07220; uncharacterized protein
Gene_62 (197)	K09891	K09891; uncharacterized protein
Gene_63 (199)	K08316	rsmD; 16S rRNA (guanine966-N2)-methyltransferase
Gene_64 (213)	K00876	udk, UCK; uridine kinase
Gene_65 (223)	K00793	ribE, RIB5; riboflavin synthase
Gene_66 (210)	K11755	hisIE; phosphoribosyl-AMP cyclohydrolase / phosphoribosyl-ATP pyrophosphohydrolase

Gene_67 (215)	K00939	adk, AK; adenylate kinase
Gene_68 (193)	K25422	yceF; 7-methyl-GTP pyrophosphatase
Gene_69 (211)	K03186	ubiX, bsdB, PAD1; flavin prenyltransferase
Gene_70 (207)	K01625	eda; 2-dehydro-3-deoxyphosphogluconate aldolase / (4S)-4-hydroxy-2-oxoglutarate aldolase
Gene_71 (202)	K07184	ygiM; SH3 domain protein
Gene_72 (204)	K03687	GRPE; molecular chaperone GrpE
Gene_73 (208)	K03607	proQ; ProP effector
Gene_74 (210)	K10914	crp; CRP/FNR family transcriptional regulator, cyclic AMP receptor protein
Gene_75 (209)	K02906	RP-L3, MRPL3, rplC; large subunit ribosomal protein L3
Gene_76 (191)	K03271	gmhA, lpcA; D-sedoheptulose 7-phosphate isomerase
Gene_77 (196)	K07337	K07337; penicillin-binding protein activator
Gene_78 (196)	K05501	slmA, ttk; TetR/AcrR family transcriptional regulator
Gene_79 (176)	K02553	rraA, menG; regulator of ribonuclease activity A
Gene_80 (175)	K09889	yjgA; ribosome-associated protein
Gene_81 (188)	K02356	efp; elongation factor P
Gene_82 (156)	K03402	argR, ahrC; transcriptional regulator of arginine metabolism
Gene_83 (117)	K02884	RP-L19, MRPL19, rplS; large subunit ribosomal protein L19
Gene_84 (176)	K02860	rimM; 16S rRNA processing protein RimM
Gene_85 (181)	K03640	pal; peptidoglycan-associated lipoprotein
Gene_86 (149)	K03786	aroQ, qutE; 3-dehydroquinate dehydratase II
Gene_87 (172)	K07040	yceD, ylbN; DUF177 domain-containing protein
Gene_88 (177)	K02933	RP-L6, MRPL6, rplF; large subunit ribosomal protein L6
Gene_89 (160)	K03600	sspB; stringent starvation protein B
Gene_90 (172)	K00891	aroK, aroL; shikimate kinase
Gene_91 (164)	K03101	lspA; signal peptidase II
Gene_92 (169)	K00950	folK; 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase
Gene_93 (166)	K02988	RP-S5, MRPS5, rpsE; small subunit ribosomal protein S5
Gene_94 (143)		Gene of unknown function
Gene_95 (162)	K07740	rsd; regulator of sigma D
Gene_96 (159)	K03637	moaC, CNX3; cyclic pyranopterin monophosphate synthase
Gene_97 (164)	K01653	E2.2.1.6S, ilvH, ilvN; acetolactate synthase I/III small subunit
Gene_98 (164)	K02864	RP-L10, MRPL10, rplJ; large subunit ribosomal protein L10
Gene_99 (161)	K03664	smpB; SsrA-binding protein
Gene_100 (154)	K02160	accB, bccP; acetyl-CoA carboxylase biotin carboxyl carrier protein

Gene_101 (147)	K09160	K09160; uncharacterized protein
Gene_102 (155)	K03625	nusB; transcription antitermination protein NusB
Gene_103 (144)		Gene of unknown function
Gene_104 (156)	K00794	ribH, RIB4; 6,7-dimethyl-8-ribityllumazine synthase
Gene_105 (152)	K02372	fabZ; 3-hydroxyacyl-[acyl-carrier-protein] dehydratase
Gene_106 (150)	K09899	K09899; uncharacterized protein
Gene_107 (138)	K01759	GLO1, gloA; lactoylglutathione lyase
Gene_108 (141)	K00940	ndk, NME; nucleoside-diphosphate kinase
Gene_109 (117)		Gene of unknown function
Gene_110 (130)	K02834	rbfA; ribosome-binding factor A
Gene_111 (121)	K02458	gspI; general secretion pathway protein I
Gene_112 (131)	K02990	RP-S6, MRPS6, rpsF; small subunit ribosomal protein S6
Gene_113 (137)	K00997	acpS; holo-[acyl-carrier protein] synthase
Gene_114 (66)	K03563	csrA; carbon storage regulator
Gene_115 (128)	K04762	hslR; ribosome-associated heat shock protein Hsp15
Gene_116 (126)	K02437	gcvH, GCSH; glycine cleavage system H protein
Gene_117 (98)		Gene of unknown function
Gene_118 (130)	K02994	RP-S8, rpsH; small subunit ribosomal protein S8
Gene_119 (129)	K02948	RP-S11, MRPS11, rpsK; small subunit ribosomal protein S11
Gene_120 (70)	K03704	cspA; cold shock protein
Gene_121 (123)	K02935	RP-L7, MRPL12, rplL; large subunit ribosomal protein L7/L12
Gene_122 (121)	K03075	secG; preprotein translocase subunit SecG
Gene_123 (112)	K04751	glnB; nitrogen regulatory protein P-II 1
Gene_124 (108)	K05809	raiA; ribosome-associated inhibitor A
Gene_125 (117)	K02887	RP-L20, MRPL20, rplT; large subunit ribosomal protein L20
Gene_126 (117)	K02881	RP-L18, MRPL18, rplR; large subunit ribosomal protein L18
Gene_127 (95)		Gene of unknown function
Gene_128 (110)	K03210	yajC; preprotein translocase subunit YajC
Gene_129 (110)	K02890	RP-L22, MRPL22, rplV; large subunit ribosomal protein L22
Gene_130 (110)	K09747	ebfC; nucleoid-associated protein EbfC
Gene_131 (90)		Gene of unknown function
Gene_132 (105)	K09802	K09802; uncharacterized protein
Gene_133 (108)	K03671	trxA; thioredoxin 1
Gene_134 (106)	K06891	clpS; ATP-dependent Clp protease adaptor protein ClpS
Gene_135 (105)	K02895	RP-L24, MRPL24, rplX; large subunit ribosomal protein L24
Gene_136 (103)	K02946	RP-S10, MRPS10, rpsJ; small subunit ribosomal protein S10

Gene_137 (100)	K02892	RP-L23, MRPL23, rplW; large subunit ribosomal protein L23
Gene_138 (96)		Gene of unknown function
Gene_139 (96)	K04078	groES, HSPE1; chaperonin GroES
Gene_140 (95)	K05808	yhbH; putative sigma-54 modulation protein
Gene_141 (78)		Gene of unknown function
Gene_142 (87)		Gene of unknown function
Gene_143 (92)	K02897	RP-L25, rplY; large subunit ribosomal protein L25
Gene_144 (92)	K02965	RP-S19, rpsS; small subunit ribosomal protein S19
Gene_145 (90)		Gene of unknown function
Gene_146 (88)	K09806	ubiK; ubiquinone biosynthesis accessory factor UbiK
Gene_147 (86)	K09159	cptB; antitoxin CptB
Gene_148 (84)		Gene of unknown function
Gene_149 (84)	K02961	RP-S17, MRPS17, rpsQ; small subunit ribosomal protein S17
Gene_150 (78)		Gene of unknown function
Gene_151 (75)		Gene of unknown function
Gene_152 (70)	K09898	K09898; uncharacterized protein
Gene_153 (66)		Gene of unknown function
Gene_154 (64)	K02916	RP-L35, MRPL35, rpmI; large subunit ribosomal protein L35
Gene_155 (58)	K02907	RP-L30, MRPL30, rpmD; large subunit ribosomal protein L30
Gene_156 (59)	K09791	K09791; uncharacterized protein
Gene_157 (55)		Gene of unknown function
Gene_158 (37)		50S ribosomal protein L36

Figure 1.1: Maximum-likelihood phylogeny (Kimura 2-parameter model) of the concatenation of the core genome. The bootstrap values represent 1,000 replications, and all values from study are shown. The reference sequences were acquired from NCBI GenBank.

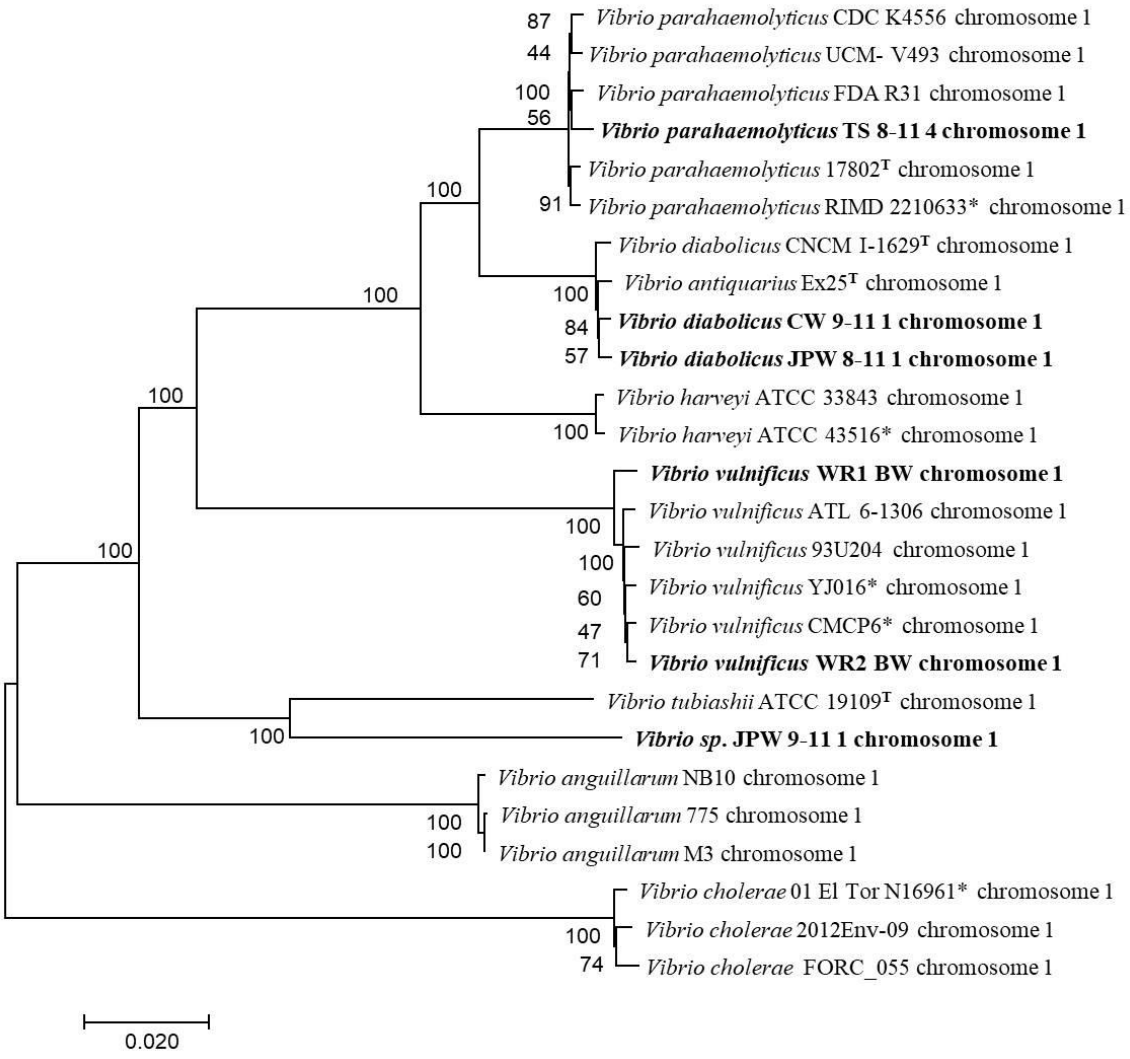


Figure 1.2: Maximum-likelihood phylogeny (Kimura 2-parameter model) of the concatenation of core genes found on chromosome 1. Bold indicates sequences obtained from this study. Superscript T (^{T}) represents type strains for the species. Asterisk (*) indicates representative organisms of that species. The bootstrap values represent 1,000 replications, and all values from study are shown. The reference sequences were acquired from NCBI GenBank.

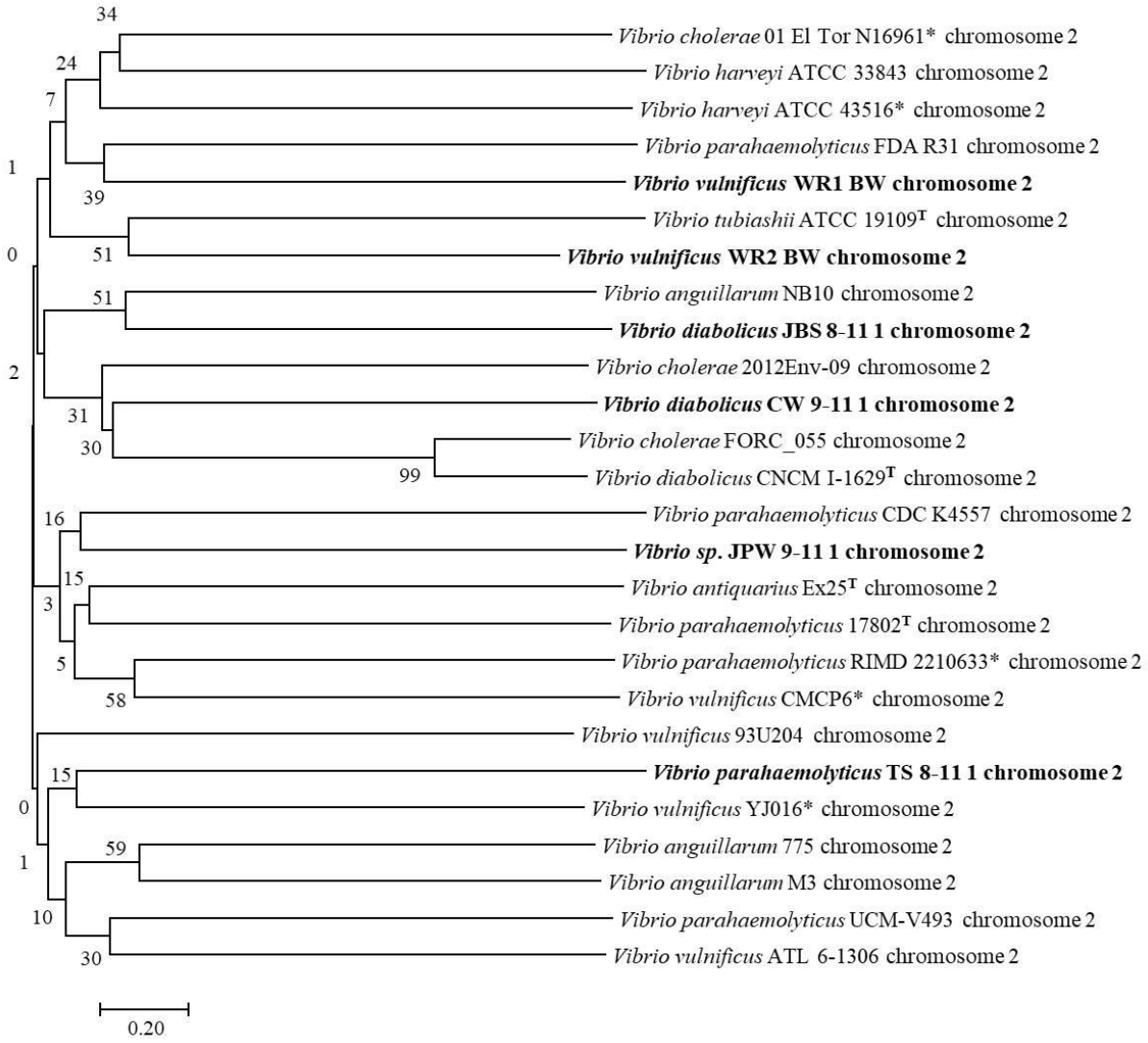
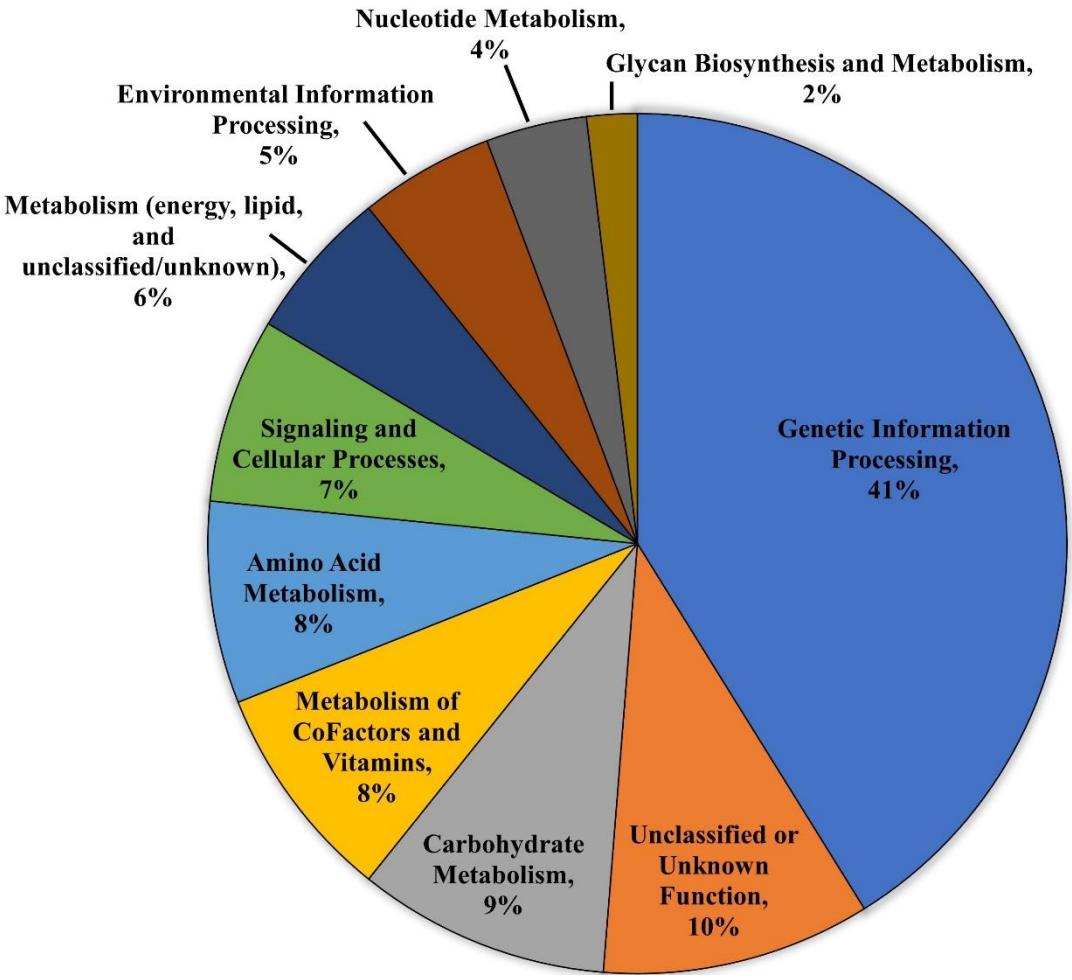
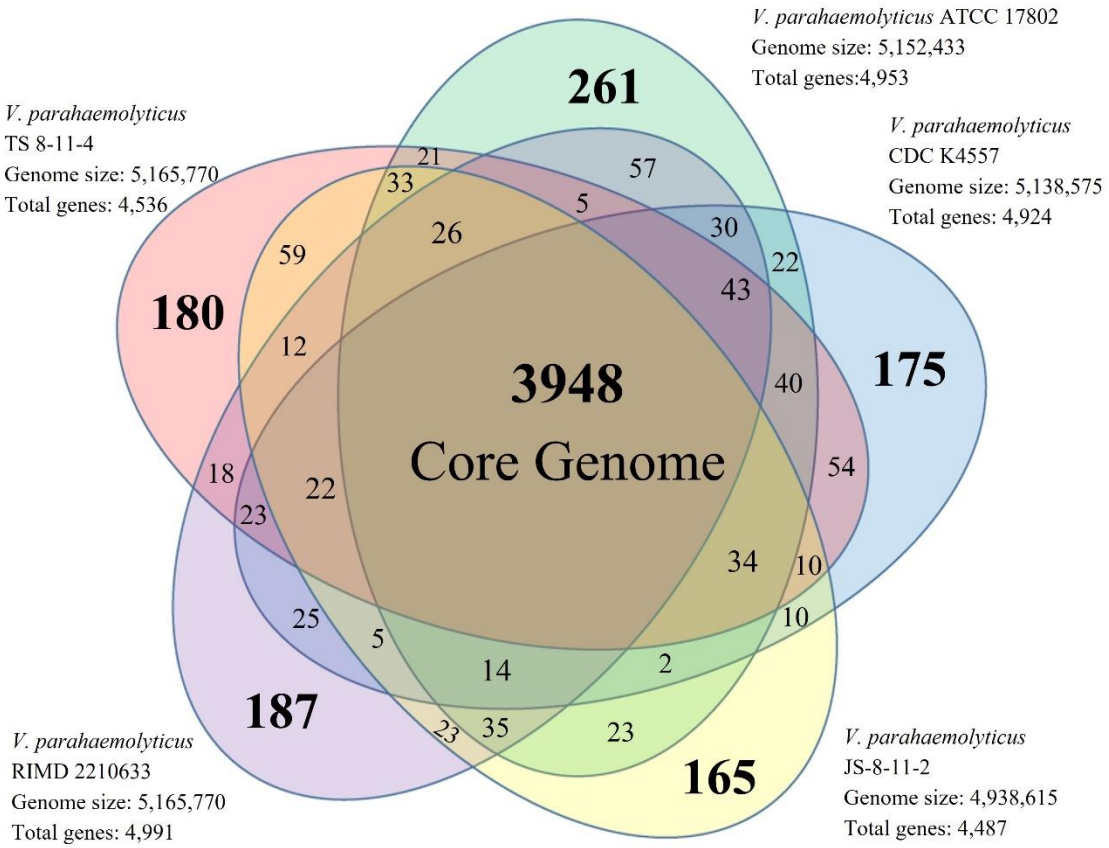


Figure 1.3: Maximum-likelihood phylogeny (Kimura 2-parameter model) of the concatenation of the core genes found on chromosome 2. Bold indicates sequences obtained from this study. Superscript T (^{T}) represents type strains for the species. Asterisk (*) indicates representative organisms of that species. The bootstrap values represent 1,000 replications, and all values from study are shown. The reference sequences were acquired from NCBI GenBank.



Figures 1.4: Percentage breakdown of core genome into KEGG Classification Categories.



Figures 1.5a: Homologous core genome and unique genes of the species *Vibrio parahaemolyticus* (a). Homology calculations were performed using sequence-based alignments in RAST.

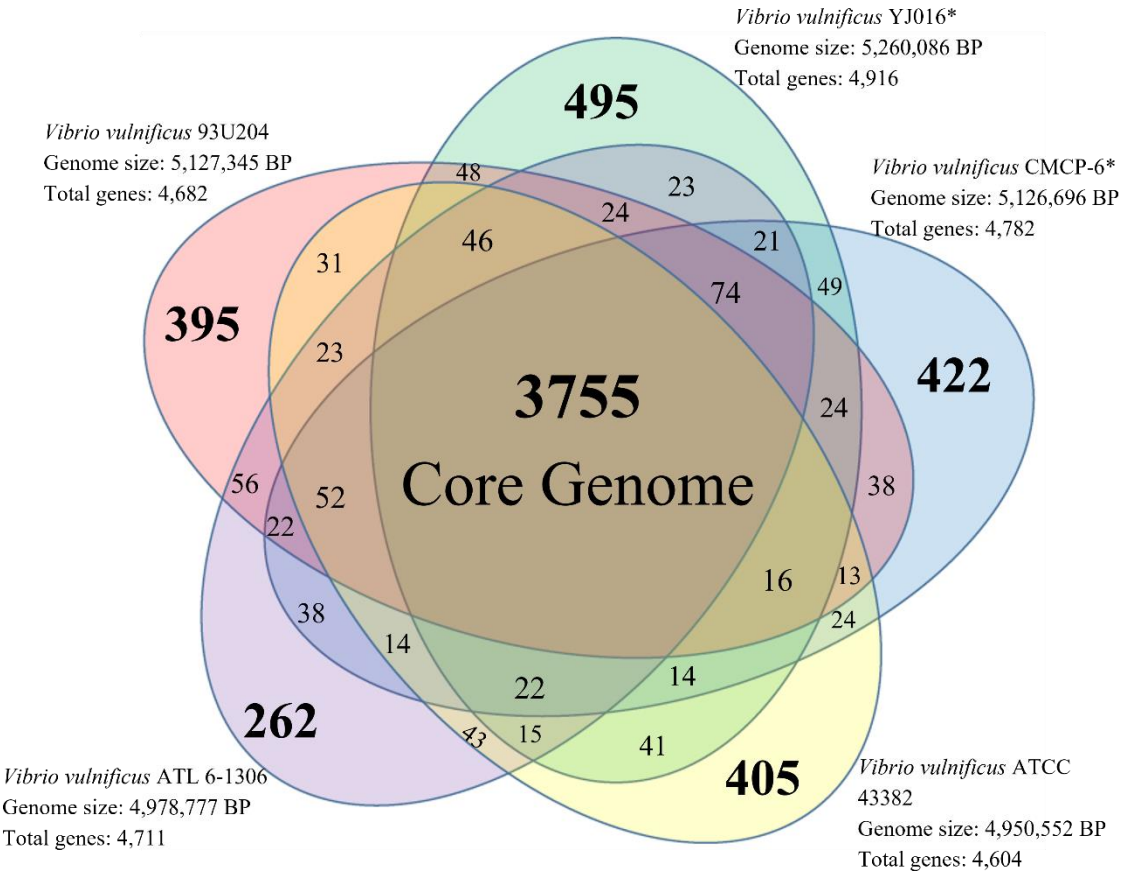


Figure 1.5b: Homologous core genome and unique genes of the species *Vibrio vulnificus*. Homology calculations were performed using sequence-based alignments in RAST. Asterisks denote representative genomes.

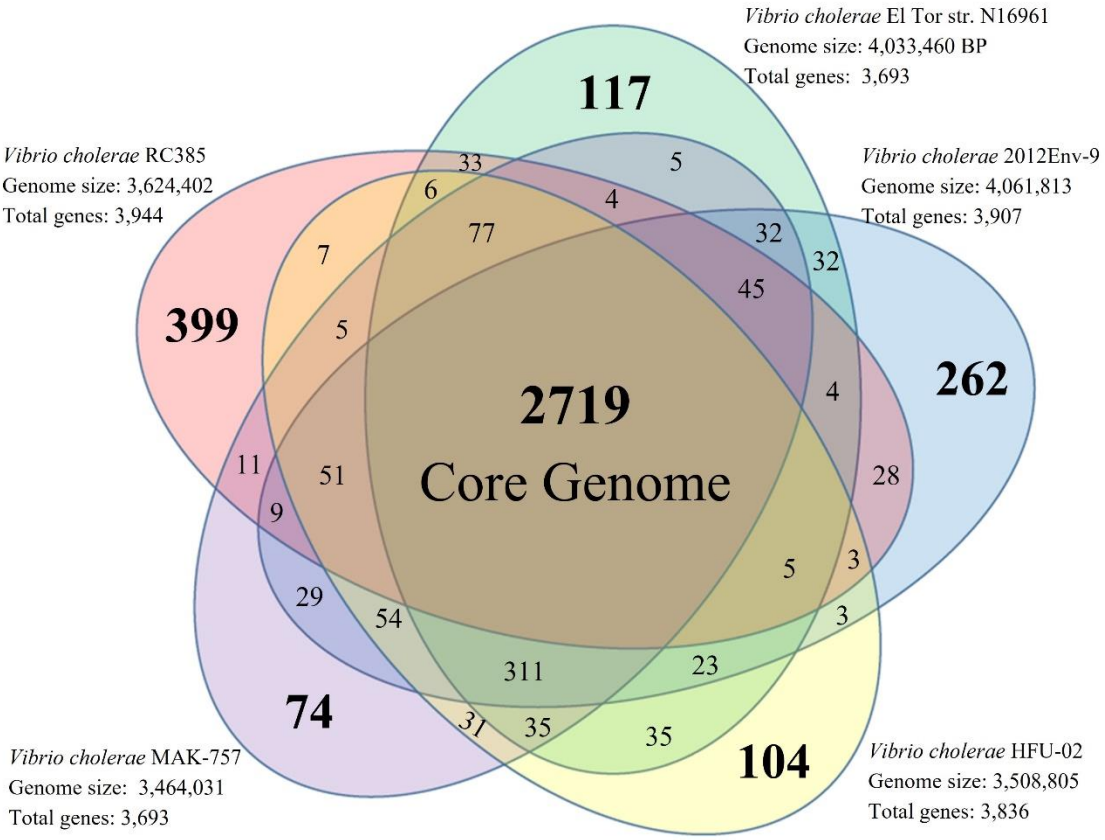


Figure 1.5c: Homologous core genome and unique genes of the species *Vibrio cholerae*. Homology calculations were performed using sequence-based alignments in RAST.

CHAPTER 2

OCCURRENCE AND SIGNIFICANCE OF PATHOGENICITY AND FITNESS ISLANDS IN ENVIRONMENTAL VIBRIOS

Klein SL[1], **Pipes SE[1]**, Lovell CR. 2018. Occurrence and significance of pathogenicity and fitness islands in environmental vibrios. AMB Express; 8(177)

ABSTRACT

Pathogenicity islands (PAIs) are large genomic regions that contain virulence genes, which aid pathogens in establishing infections. While PAIs in clinical strains (strains isolated from a human infection) are well-studied, less is known about the occurrence of PAIs in strains isolated from the environment. In this study we describe three PAIs found in environmental *Vibrio vulnificus* and *Vibrio parahaemolyticus* strains, as well as a genomic fitness island found in a *Vibrio diabolicus* strain. All four islands had markedly different GC profiles than the rest of the genome, indicating that all of these islands were acquired via lateral gene transfer. Genes on the PAIs and fitness island were characterized. The PAI found in *V. parahaemolyticus* contained the *tdh* gene, a collagenase gene, and genes involved in the Type 3 Secretion System II (T3SS2). A *V. vulnificus* environmental strain contained two PAIs, a small 25 kbp PAI and a larger 143 kbp PAI. Both PAIs contained virulence genes. Toxin-antitoxin (TA) genes were found in all three species: on the *V. diabolicus* fitness island, and on the *V. parahaemolyticus* and *V. vulnificus* PAIs.

INTRODUCTION

Vibrio parahaemolyticus and *Vibrio vulnificus* can cause illnesses in humans, with an estimated 80,000 cases occurring annually in the United States (Scallan et al., 2011; CDC, 2017). The hospitalization and mortality rates of *V. parahaemolyticus* gastroenteritis are 22% and 1%, respectively (Scallan et al., 2011). Although cases are usually mild and tend to resolve themselves after 1 to 3 days, *V. parahaemolyticus* is responsible for the majority of vibriosis cases (Scallan et al., 2011). *V. vulnificus* cases are less common; only about 100 occur each year in the United States. However, the hospitalization and mortality rates of this bacterium are much higher, at 92% and 35%, respectively (Scallan et al., 2011). *V. vulnificus* also causes sepsis and necrotizing fasciitis if it enters the body through an open wound. The majority of reported *V. vulnificus* cases are from wound infections (45%) and septicemia (43%); only 5% are gastroenteritis (Scallan et al., 2011). The mortality rate of *V. vulnificus* when it invades the bloodstream (sepsis) increases to 60%. Pathogenesis of both species is complex, and while some virulence factor genes have been implicated, the mechanisms underlying *V. vulnificus* and *V. parahaemolyticus* virulence are not well understood (Broberg et al., 2011; Lovell, 2017).

Pathogenicity Islands (PAIs), a subgroup of genomic islands that aid in and contribute to pathogenesis, have been found in clinical strains of both *V. vulnificus* and *V. parahaemolyticus*. PAIs are large chromosomal regions that are flanked by tRNA genes, and are usually associated with mobile genetic elements, such as phage, plasmid, integron, and transposon genes. A genomic island must contain at least one virulence gene, or gene that contributes to pathogenesis, to be considered a PAI. The size of PAIs

ranges from 10-200 kbp (Schmidt and Hensel, 2004; Hacker and Kaper, 2000; Hacker and Carniel, 2000) and the average *Vibrio* genome is 4.5 mb, meaning that a single PAI could make up as much as 4% of a *Vibrio* genome. PAIs are flanked by highly conserved tRNA genes that act as both integration and excision sites. The majority (approximately 75%) of PAIs discovered have tRNA flanking sequences (Schmidt and Hensel, 2000; Hacker and Kaper, 2000). Additionally, tRNA loci are often found on extrachromosomal elements, such as plasmids and bacteriophages. This indicates that the most likely mechanism for extrachromosomal element insertion is homologous recombination between the extrachromosomal element tRNA and PAI flanking tRNA loci (Hacker and Kaper, 2000).

There is considerable evidence that PAIs are acquired horizontally via one or more lateral transfer events. Within some PAIs there is evidence of one large transfer event, while other PAIs are more "mosaic-like." The "mosaic-like" composition of certain PAIs is caused by multiple, independent lateral transfer events (Hacker and Kaper, 2000; Schmidt and Hensel, 2004). PAIs usually differ in codon usage biases and have a markedly lower or higher GC content than the rest of the genome (Schmidt and Hensel, 2004; Hacker and Kaper, 2000; Hacker et al., 1997; Hacker and Carniel, 2000). This supports the idea that recognizable PAIs are incorporated into a genome via lateral gene transfer from a dissimilar or unrelated organism (donor) having differing GC content and codon usage than the recipient (Schmidt and Hensel, 2004). However, PAI GC content may not differ from that of the core genome if the donor and recipient microorganisms are closely related (Hacker and Kaper, 2000). Dissimilarities in base

composition confirm that detectable lateral transfer of PAIs must have been of recent origin, as insufficient time for genetic drift has passed (Schmidt and Hensel, 2004).

PAIs have been found in clinical strains of *V. vulnificus* and *V. parahaemolyticus* (e.g: Makino et al., 2003; Wang et al., 2006; Sugiyama et al., 2008; Quirke et al., 2006; Cohen et al., 2007). Nine PAIs have been identified in *V. parahaemolyticus*, with VPAI-1 and VPAI-7 (*V. parahaemolyticus* pathogenicity island one and *V. parahaemolyticus* pathogenicity island seven) being the most studied (Ceccarelli et al., 2013). VPAI-1 is a 22 kbp island that is found on chromosome 1 in some strains, and chromosome 2 in others (Wang et al., 2006; Chen et al., 2011). This observation provides evidence for the mobility of this genomic island. VPAI-7 is the largest *Vibrio* genomic island found to date. This island contains the virulence factors TDH (thermostable direct hemolysin) and Type III Secretion System 2 (T3SS2) (Makino et al., 2003; Sugiyama et al., 2008). Other names for VPAI-7 include VPaIα or *tdh*VPA (Xu et al., 2017) and parts of VPAI-7 have been found in other *Vibrio* species, such as *Vibrio mimicus* (Gennari et al., 2011).

Genomic islands have been found in *V. vulnificus* clinical strains YJ016 and CMCP6, with 14 regions ranging in size from 14-117 kpb. A superintegon (SI) and nine *V. vulnificus* genomic islands (VVI-I to VVI–IX) have been found in these clinical strains. PAIs have not been detected in environmentally derived *V. vulnificus* strains (Quirke et al., 2006). *V. vulnificus* VVI-I has been found in the *Vibrio cholerae* biotype El Tor and O139 serogroup. The functional role of this island has not been determined but its presence in *V. cholerae* supports the idea that these regions can be transferred to other closely related species (O'Shea et al., 2004).

Work on *Vibrio* PAIs is heavily skewed toward clinical strains, with the pathogenic potential of naturally-occurring (environmental) strains rarely considered. In this study, we characterized four genomic islands found in environmental *Vibrio* strains: a PAI within a *V. parahaemolyticus* strain, two novel PAIs within a *V. vulnificus* strain, and a novel fitness island found in a *Vibrio diabolicus* strain. Environmental *Vibrio* strains, and the PAIs within them, could serve as reservoirs for virulence genes.

MATERIALS AND METHODS

Strain Isolation

Environmental *V. parahaemolyticus* and *V. diabolicus* strains were isolated previously (Gutierrez West et al., 2013; Klein et al., 2014) from the pristine North Inlet salt marsh estuary near Georgetown, SC, USA (33°20'N, 79°12'W). Environmental *V. vulnificus* strains were also isolated near Georgetown, SC; however, they were isolated from lower salinity waters in Winyah Bay and the Waccamaw River. Water samples were plated on CHROMagar *Vibrio* (DRG International, NJ, USA) for isolation of *V. vulnificus* strains following the US Food and Drug Administration protocol (DePaola and Kaysner, 2004). *Vibrio* strains were routinely cultivated on saline Luria Agar (SLA; per L; 10 g tryptone, 5 g yeast extract, 27 g NaCl, 15 g Bacto Agar). *V. parahaemolyticus* TS-8-11-4 and *V. diabolicus* JBS-8-11-1 were deposited into the DSMZ Public Culture Collection and were assigned their respective accession numbers: DSM 107522 and DSM 107521.

Whole Genome Sequencing

Genomic DNA was isolated through the Wizard Genomic DNA Purification kit following the protocol for Gram negative organisms (Promega, Madison, WI, USA**)**.

After DNA was extracted, DNA quantity was measured via Quibit fluorimetry. Libraries were prepared and then sequenced using an Illumina MiSeq (V3 26300 base) at the Indiana University Center for Genomic Studies as a part of the Genome Consortium for Active Teaching NextGenSequencing Group (GCAT-SEEK) shared run (Buonaccorsi et al., 2011, 2014). Sequencing reads were filtered (median phred score 0.20), trimmed (phred score 0.16), and assembled using the paired-end *de novo* assembly option in NextGENe V2.3.4.2 (SoftGenetics**,** State College, PA, USA). The assembled genomes were uploaded to the Rapid Annotation with Subsystem Technology (RAST) web service (Aziz et al., 2008; Overbeek et al., 2014) for analysis, guided contig reordering and assembly improvement. Genomes were aligned based on completed sequences using dotplot comparisons. Whole genome sequence data obtained from this work were submitted to the NCBI GenBank and assigned the accession numbers: GCA_003798505.1, GCA_003798485.1, and GCA_003798525.1.

PAI detection and characterization

The fully sequenced genomes were uploaded to TUBIC (Tiajin University Bioinformatics Center) to determine their GC profiles (http://tubic.tju.edu.cn/). This tool displays GC content variation across a genome and can be useful for identifying genomic regions that differ from the rest of the genome in GC content (Gao and Zhang, 2006). Genomic islands that were detected via TUBIC were isolated and the island nucleotide sequence was uploaded to RAST to identify and characterize the specific genes found on the genomic islands (http://rast.nmpdr.org/). NCBI GenBank was also used to characterize genomic island genes (http://www.ncbi.nlm.nih.gov/genbank/). Gene sequences of interest were edited, and maximum-likelihood trees were constructed using

the Kimura 2-parameter model with MEGA version 7 ((Kumar et al., 2016; Tamura et al., 2015). DNAPlotter was used to visualize the circular chromosomes of the *Vibrio* strains (Carver et al., 2009).

RESULTS

V. parahaemolyticus island

V. parahaemolyticus strain TS-8-11-4 was isolated from salt marsh sediments (Gutierrez West et al., 2013; Klein et al., 2014) at the pristine North Inlet estuary in South Carolina, USA. This strain had a genome of 4.98 mbp; chromosome 1 was 3.19 mbp in length and chromosome 2 was 1.78 mbp in length. The majority of the genome contained a GC content of 45.57%, which is typical for *V. parahaemolyticus* (Farmer and Janda, 2005). However, this strain contained a 223 kbp island that had a markedly lower GC content (41.5%) not typical of *V. parahaemolyticus* (Figure 2.1A). The majority (69%) of genes on the TS-8-11-4 PAI could not be assigned specific identities and were thus designated hypothetical. The genomic island of *V. parahaemolyticus* TS 8-11-4 was on the second chromosome of and it harbored virulence genes (Figure 2.2A). The virulence factor genes that were found on this island included the thermostable direct hemolysin gene, genes involved in the Type Three Secretion System II (T3SS2), a collagenase gene, as well as capsule production genes.

V. vulnificus islands

V. vulnificus strain WR-2-BW was isolated near Georgetown, SC from Waccamaw River waters. Its genome (4.96 mpb) contained two chromosomes with the first chromosome larger (2.96 mbp) than the second (1.99 mbp). The average GC content of *V. vulnificus* ranges from 46-48% (Farmer and Janda, 2005), and the average GC

content of strain WR-2-BW was 46.83%. Two regions within the genome had GC contents that were markedly lower from the rest of the genome (Figure 2.1B). The first region had a GC content of 38.2% and the second region had a GC content of 42.5%; both of which are lower than the typical GC content of *V. vulnificus* strains. These regions were found on the second chromosome (Figure 2.2B). The first region was a 25 kbp island and the second region was a 143 kbp island. The 25 kbp island was 30 genes in length and had two genes that had virulence-related functions, which include a putative LPS biosynthesis protein gene and an O-antigen flippase *wzx* gene. The 143 kbp island contained the cytolysin gene *vvhB*, a chitinase gene, *tldD*/*tldE* proteolytic complex genes, and Type IV secretory pathway components. The 143 kbp genomic island was comprised of 160 genes in total, 63% of which were characterized as hypothetical or had unknown function.

***V. diabolicus* island**

V. diabolicus strain JBS-8-11-1 was isolated previously from North Inlet salt marsh sediments (Gutierrez West et al., 2013; Klein et al., 2014). Its genome (5.04 mbp) was comprised of two chromosomes, the first (3.23 mbp) being larger than the second (1.81 mbp). Its GC content was typical of other *V. diabolicus* genomes (44.91%) (Goudenege et al., 2014), except for a 182 kbp island, located on chromosome 2, which had a GC content of 40.8% (Figure 2.1C). Eighty-two % of the island consisted of hypothetical genes. This island harbored no known virulence genes; it is hereafter referred to as a fitness island (Figure 2.2C). Three genes, a phage DNA synthesis gene, a phage DNA replication gene, and a gene encoding a phage capsid protein, were located

very close to each other on the fitness island. Thirteen genes involved in toxin-antitoxin (TA) systems were located on the fitness island.

DISCUSSION

The genomic island of *V. parahaemolyticus* TS 8-11-4 was deemed a PAI due to the presence of virulence genes on this island, despite its environmental origin (Schmidt and Hensel, 2004; Hasan et al., 2010; Dobrindt et al., 2004). The thermostable direct hemolysin gene (*tdh*) was found on this island, as well as genes involved in the Type Three Secretion System II (T3SS2). Both the *tdh* gene and T3SS2 complex are the two major virulence factors implicated in *V. parahaemolyticus* pathogenesis (Makino et al., 2003; Park et al., 2004; Yanagihara et al., 2010). A collagenase gene was found on the island; collagenase is thought to be involved in *V. parahaemolyticus* virulence (Gode-Potratz et al., 2010). The genomic island of *V. parahaemolyticus* strain TS-8-11-4 is a PAI, and more specifically, because it contains *tdh* and T3SS2 genes, we designate this island as a VPAI-7 (VPaIα or *tdh*VPA) (Makino et al., 2003; Sugiyama et al., 2008; Xu et al., 2017).

Four genes involved in capsule production, as well as one integrase gene, and a Na+/H+ antiporter (*nhaA*) were also found on this PAI. Capsules aid pathogens in evasion of host immune defenses, establishing infections, and survival in harsh environments, such as the stomach. *V. parahaemolyticus* virulence is correlated with capsule production (Broberg et al., 2011; Letchumanan et al., 2014). One capsule gene had high homology with Gram positive capsule production genes. This is interesting because vibrios are Gram negative organisms, so this gene may have been acquired laterally. An integrase gene was found near the center of the island. Integrase genes are

associated with PAIs and function to integrate foreign DNA into the genome (Hacker and Kaper, 2000). Usually VPAI-7 islands do not contain an integrase gene, but they contain a few transposon genes instead (Ceccarelli et al., 2015). Finally, we determined that a *nhaA* gene is located on this genomic island. *nhaA* genes encode Na+/H+ antiporters, which transport ions to balance pH. Na+/H+ antiporters aid *V. cholerae* in environmental persistence (Vimont and Berche, 2000) and are essential for *Yersinia pestis* virulence (Minato et al., 2013).

Similar to *V. parahaemolyticus*, the two islands found for the *V. vulnificus* WR-2-BW strain are characterized as PAIs due to the presence of virulence genes and virulence-related genes. Two of these genes had virulence-related functions, a putative LPS biosynthesis protein gene and an O-antigen flippase *wzx* gene. These genes are virulence-associated factors, as they do not directly cause host cell damage, but they do contribute to pathogenesis, aiding in the establishment of infections. Lipopolysaccharide (LPS) is a main component of the outer membrane of Gram-negative bacteria and is a known pyrogen (fever-producing agent) (McPherson et al., 1991; Jones and Oliver, 2009). Phylogenies show that the LPS biosynthesis protein gene from *V. vulnificus* WR-2-BW was closely related to an LPS biosynthesis protein gene from a *Vibrio coralliilyticus* species. The O-antigen flippase *wzx* gene is part of the major class of O-antigen gene clusters, and it encodes a hydrophobic protein with 12 potential transmembrane segments (Liu et al., 1996).

A cytolysin secretion gene, *vvhB*, was found also found on the 143 kbp *V. vulnificus* island. Cytolysins lyse erythrocytes by forming small pores in the cytoplasmic membrane or binding to cholesterol to interrupt potassium and sodium ion channels (Choi

et al., 2002). In *V. vulnificus*, the expression and mechanism of cytolysins *vvhA* and *vvhB* are not fully understood, however, they are both believed to play a role in pathogenicity (Choi et al., 2002). They are homologous to a known *V. cholerae* El Tor hemolysin (Choi et al., 2002; Yamamoto et al., 1990). Phylogenies show that the *vvhB* gene in the *V. vulnificus* WR-2-BW strain was 99% identical to other *V. vulnificus vvhB* genes from other strains.

Other genes of interest on the 143 kbp PAI include a chitinase gene, *tldD*/*tldE* proteolytic complex genes, and Type IV secretory pathway components. In *Escherichia coli*, it was shown that the TldD and TldE proteins could be involved in regulating gyrase function as well as aiding in proteolytic activity (Allali et al., 2002). The chitinase gene had a 99% blast identity score to the chitinase gene found in the *V. vulnificus* YJ016 strain; however, the chitinase gene in YJ016 is located on the first chromosome and WR-2-BW's chitinase gene is located on the second chromosome. Chitinous exoskeletal materials of invertebrates can be a source of carbon and nitrogen for bacteria; vibrios in particular have a well-known association with marine copepods (Kaneko and Colwell, 1975; Lovell, 2017). *V. cholerae* has a well-studied association with copepods, which commonly serve as a vector of cholera infections in Bangladesh water systems (Tamplin et al., 1990). Chitinase has been identified as part of the mechanism for adsorption and attachment to copepods, which relates to its ability to colonize its host and degrade the host exoskeleton, increasing the overall ecological fitness of the vibrios (Huq et al., 1983; Nalin et al., 1979; Bhowmick et al., 2006).

V. diabolicus had a large genomic island that did not contain any virulence factors or virulence associated genes, which we defined as a fitness island, as it contained genes

that would aid the organism in persistence in the environment. Toxin-antitoxin (TA) systems are found either on plasmids, genomic islands, or within the chromosome and are made up of closely linked toxin and antitoxin genes. The encoded labile antitoxin protects the host from the stable toxin, while competitor cells that do not have the TA system (and respective antitoxin) are eliminated (Hayes, 2003; Van Melderen and Saavedra De Bast, 2009). Sometimes TA systems are referred to as "addiction modules" because the host cell is dependent on the antitoxin (Van Melderen and Saavedra De Bast, 2009). The toxin and respective antitoxin loci are usually found neighboring each other, often overlapping (Hayes, 2003). Seven Type II TA toxins were found on JBS-8-11-1's fitness island, along with their neighboring respective antitoxins. Type I TAs include RNA antitoxins, while Type II TAs have protein antitoxins (Hayes, 2003). The *relE*, *yafQ*, and *yoeB* toxin genes encode mRNA interferase endoribonucleases; all three of these toxin genes were detected on this fitness island. The *doc* toxin gene (death on curing) inhibits translation by blocking translation elongation at the 30S ribosomal subunit (Liu et al. 2008); three copies of the *doc* toxin gene and three copies of its antitoxin partner gene, *phd* (prevent host death) were found on JBS-8-11-1's fitness island. *doc* toxin genes and *phd* antitoxin genes are widespread in vibrios and were also found on *V. parahaemolyticus* strain TS-8-11-4's PAI as well as *V. vulnificus* strain WR-2-BW's PAI (Figure 2.3).

Lateral Gene Transfer in Environmental Strains

PAIs are present in environmental *Vibrio* strains and are most likely acquired via lateral gene transfer. All four of the islands described here have significant lower GC content than the rest of the genome, providing evidence that these islands originated from

a foreign source and were transferred into these genomes relatively recently. Additional evidence includes mobile genetic elements, such as phage and plasmid genes, integrases, and transposons. Virulence loci on VPAI-7 have been detected in environmental species that do not cause human infections: *Vibrio mimicus*, *Vibrio harveyi*, and *Vibrio natriegens* (Gennari et al., 2013; Klein et al., 2013). Clearly, lateral transfer of individual virulence loci and/or entire PAIs is occurring between and among environmental vibrios. It is well documented that *V. cholerae* enters a natural competency state in the presence of chitin or under low-nutrient conditions (Hazen et al., 2010; Metzger and Blokesch, 2016); however, less is known about uptake of exogenous DNA by other *Vibrio* species. Further studies examining the rates of lateral transfer among vibrios in the environment are needed. Vibrios survive, persist, and can undergo rapid population expansions (bloom) in coastal ecosystems. Consequently, the pathogenicity loci (and potential of said loci to be transferred laterally) of naturally occurring environmental strains are clearly important.

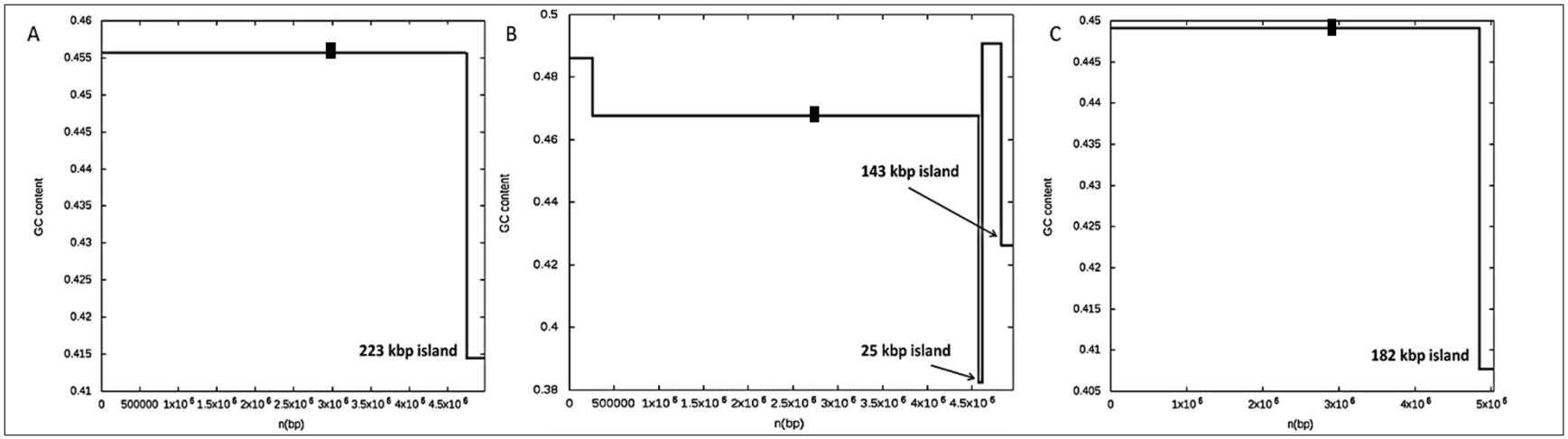


Figure 2.1 A-C: GC profile of (A) *Vibrio parahaemolyticus* environmental strain TS-8-11-4 (B) *Vibrio vulnificus* environmental strain WR-2-BW and (C) *Vibrio diabolicus* environmental strain JBS-8-11-1. All GC profiles were constructed using TUBIC software. Solid black line on graph chart indicates the end of the first chromosome and where the second chromosome begins.

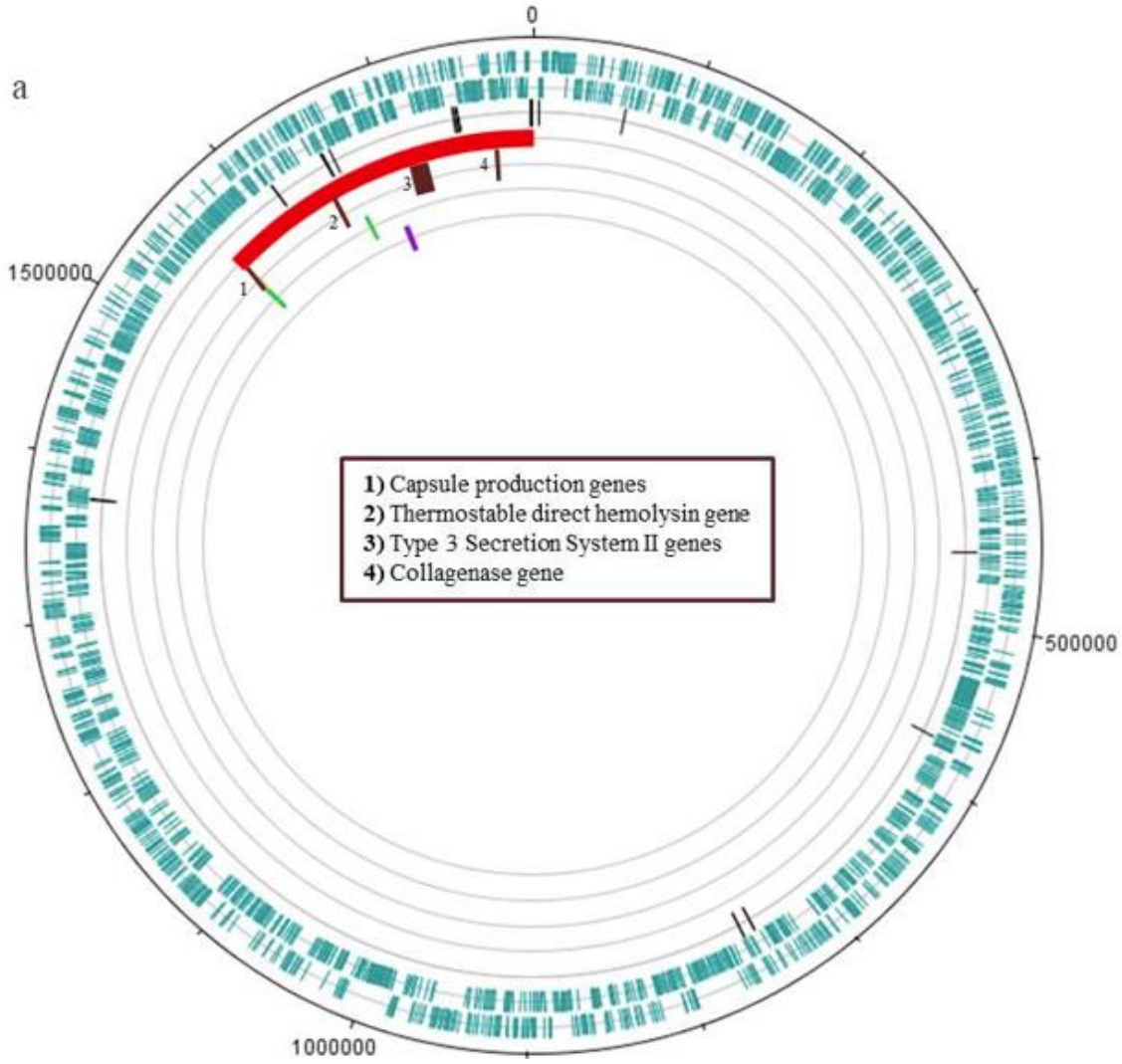


Figure 2.2a Circular presentation of the second chromosome of (a) *Vibrio parahaemolyticus* environmental strain TS-8-11-4. Track 1, forward coding sequences; track 2, reverse coding sequences; track 3, tRNA genes; track 4, red, pathogenicity islands, blue, genomic fitness islands; track 5, virulence and virulence-associated genes; track 6, genes involved in toxin-antitoxin systems; track 7, mobile genetic elements. Virulence and virulence-associated genes are numbered and are defined via the center text boxes.

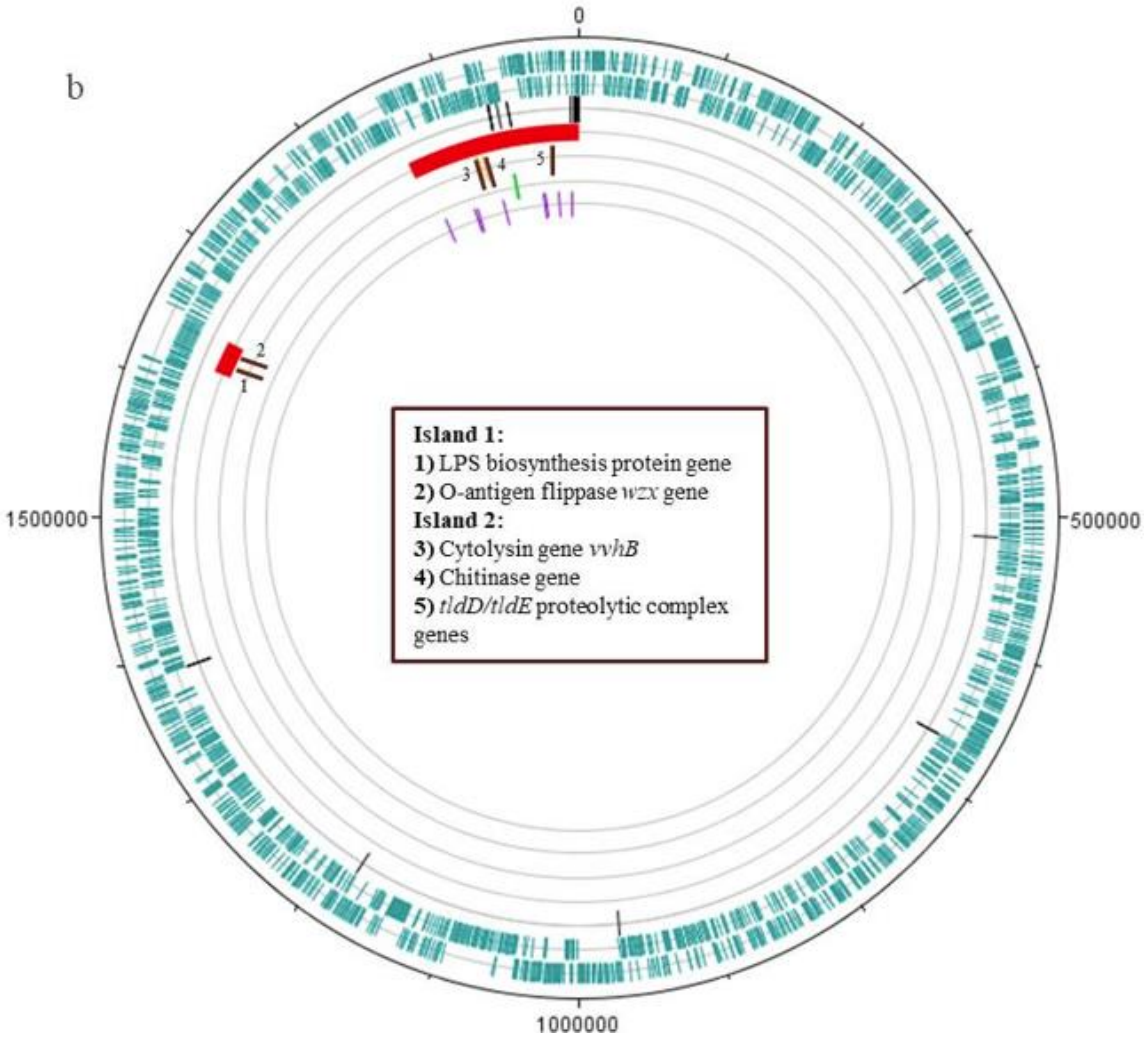


Figure 2.2b: Circular presentation of the second chromosome of (b) *Vibrio vulnificus* environmental strain WR2-BW. Track 1, forward coding sequences; track 2, reverse coding sequences; track 3, tRNA genes; track 4, red, pathogenicity islands, blue, genomic fitness islands; track 5, virulence and virulence-associated genes; track 6, genes involved in toxin-antitoxin systems; track 7, mobile genetic elements. Virulence and virulence-associated genes are numbered and are defined via the center text boxes.

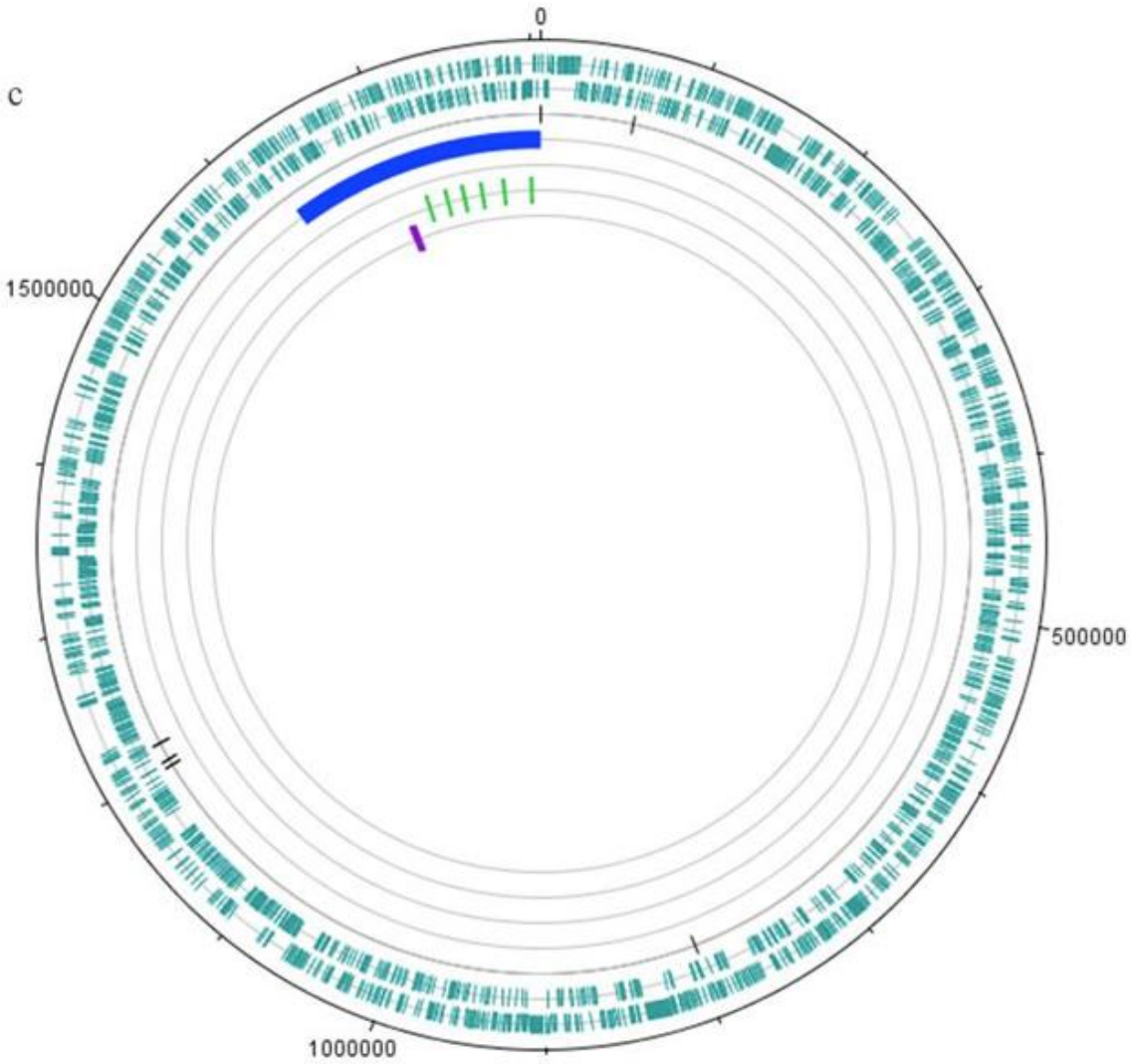


Figure 2.2c: Circular presentation of the second chromosome of (c) *Vibrio diabolicus* environmental strain JBS-8-11-1. Track 1, forward coding sequences; track 2, reverse coding sequences; track 3, tRNA genes; track 4, red, pathogenicity islands, blue, genomic fitness islands; track 5, virulence and virulence-associated genes; track 6, genes involved in toxin-antitoxin systems; track 7, mobile genetic elements.

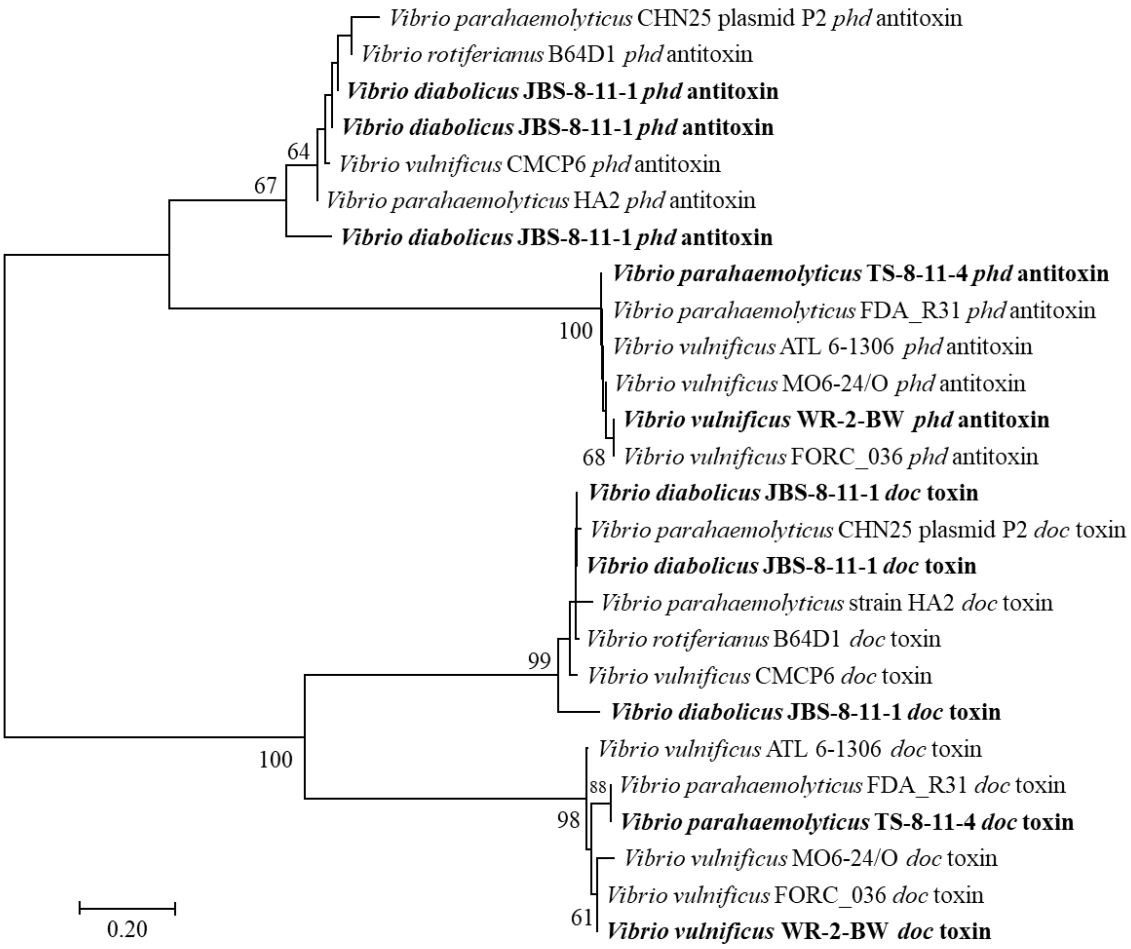


Figure 2.3: Maximum-likelihood phylogeny (Kimura 2-parameter model) of *doc* toxin genes and *phd* antitoxin genes. Bold indicates sequences obtained from this study. The bootstrap values represent 1,000 replications, and values of less than 50 are not shown. The reference sequences were acquired from NCBI GenBank.

CHAPTER 3

EXAMINATION OF THE VIRULENCE OF ENVIRONMENTALLY ISOLATED *VIBRIO VULNIFICUS* THROUGH A ZEBRAFISH MODEL

ABSTRACT

Vibrio vulnificus is Gram-negative, halophilic, environmental human pathogen whose pathogenicity and virulence mechanisms are poorly understood. There are roughly 100 cases of *Vibrio vulnificus* related infections yearly, and these infections result in hospitalizations 92% of the time, with a mortality rate of 35%. This infection is severe, with patients typically contracting the bacteria via an open wound when in the water and can result in necrotizing fasciitis (flesh-eating) and amputation of infected tissue. There have been several genes that have been implicated to contribute to the pathogenicity of this organism (*rtxA1*, *vvpE*, *vvhA*), but no defined mechanism for pathogenicity has been discovered. This study focused on environmentally isolated *Vibrio vulnificus* strains and used a Zebrafish (*Danio rerio*) assay to observe the virulence capabilities of these strains. The study found that virulence varied greatly between individual strains of the bacteria, and the commonly used marker gene of disease-causing strains of *vcgC*, did not accurately predict the more virulent strains. In fact, the least virulent strain from the study, *V. vulnificus* Sept WR1-BW6, which was positive for *vcgC*, *vvhA*, and *rtxA1*, did not produce severe disease in the fish, and was the only strain that did not produce one mortality throughout the study.

INTRODUCTION

Vibrio vulnificus is a Gram negative, halophilic bacterial species that is endemic to estuarine and coastal waters. This organism is a naturally occurring environmental human pathogen and is a great concern for public health due to its routine isolation from the water column, sediment, and shellfish. There are roughly 80,000 cases of vibriosis infections a year in the United States, and while the number of infections that stem from *V. vulnificus* infections is a relatively low percentage of the total number of cases, it can cause severe infection, and typically results in hospitalization (Scallan et al., 2011). *V. vulnificus* only accounts for about 100 occur each year in the United States, but the hospitalization and mortality rates of this bacterium are high, at 92% and 35%, respectively (Scallan et al. 2011). *V. vulnificus* also causes sepsis and necrotizing fasciitis if it enters the body through an open wound. The majority of reported *V. vulnificus* cases are from wound infections (45%) and septicemia (43%); only 5% are gastroenteritis (Scallan et al., 2011). The mortality rate of *V. vulnificus* when it invades the bloodstream (sepsis) increases to 60%. *V. vulnificus* alone is responsible for 95% of all seafood-related deaths in the United States, but it is still poorly understood as to why there aren't more cases of illness from this organism and what genes in the organism are directly involved in pathogenicity (Linkous and Oliver, 1999; Al-Assafi et al., 2014).

Even though it is known that this organism can cause severe disease in humans, little is known on the pathogenicity mechanism or what genes are involved in virulence for this species. There have been implicated virulence genes that are thought to be involved in the pathogenicity process of the organism, but unlike its relative *Vibrio cholerae*, there is still no defined pathway that fully explains how *V. vulnificus* causes

disease. *V. vulnificus* is commonly broken down into groups based on biotype due to their different biochemical and biological properties: biogroup 1, biogroup 2, and biogroup 3 (Linkous and Oliver, 1999; Oliver 1989). Biogroup 1 strains are most frequently isolated from clinical sources, meaning a patient who is actively suffering from a *V. vulnificus* infection. Biotype 2 was isolated from diseased eels and is thought to be rarely associated with human infections (Amaro and Biosca, 1996). Biotype 3 is a more recent addition to the clade of *V. vulnificus* and is currently only isolated in Israel (Efimov et al., 2013). Its genome is closer related to the Biotype 1 genomes at 90% similar, when compared to the Biotype 2 at 87%. Researchers have suggested that certain extracellular proteins released by the invading bacteria mediate the pathogenesis process by penetrating cellular barriers which causes tissue damage, especially to tissue of vascular nature (Al-Assafi et al., 2014; Jeong and Satchell, 2012).

Certain genes have been frequently implicated in *V. vulnificus*, and these genes include *vvhA* and *vvhB*, *rtxA1*, and *vvpE*. *V. vulnificus* is also commonly broken down into two genotypes, based on the presence of one of the two versions of virulence correlated gene (*vcg*) type E or type C, with biotype 1 strains containing the *vcgC* gene (Rosche et al., 2005). Previous studies have shown that 90% percent of clinically isolated strains of *V. vulnificus* possess the *vcgC* sequence variant of the gene, while 87% of environmental isolates possess the *vcgE* sequence variation (Rosche et al., 2005). The *vcg* gene has not been determined to code for any protein and does not seem to play a role in virulence. There have been many isolates of *Vibrio vulnificus* that have come from the environment that contain the *vcgC* variant of the gene, so this may not be an accurate way to predict virulent strains in the environmental setting (Klein, 2018).

Hemolysins (*vvhA* and *vvhB*), toxins (*rtxA1*), siderophores, v, outer membrane proteins and lipopolysaccharides (LPS), and flagella components all have been implicated in *V. vulnificus* (Kim et al., 2010; Yokochi et al., 2013; Jeong and Satchell 2013; Jones and Oliver, 2009; Goo et al., 2006; Kim et al., 2012). The gene *vvhA* encodes a hemolysin that induces cytolysis and death of erythrocytes by making small pores in the cell membrane (Kim et al., 2010). The protease *vvpE* has been shown to induce hemorrhagic damage and dermonecrosis, enhance vascular permeability and edema, and has also proven to be lethal to mice (Kothary and Kreger, 1987). *VvpE* may play important roles in the invasiveness of *V. vulnificus* by facilitating the proteolytic cleavage of IgA and lactoferrin (Kim et al., 2007). The *rtxA1* gene is a multifunctional cytotoxin toxin that can produce changes in cytoskeletal rearrangement, contact cytotoxicity, hemolysis, tissue invasion, and lethality in mice and is one of the most studied virulence factors of *Vibrio vulnificus* (Kim et al., 2016).

Understanding the virulence and pathogenicity of *Vibrio parahaemolyticus* and *Vibrio vulnificus* has been a focus for researchers for many years now. Studies have shown that strains of these organisms can produce cytotoxic effects in human epithelial cell lines, but research has heavily focused on the strains from clinically isolated sources (Hiyoshi et al., 2010; Raimondi et al., 2000). Little work has been done to test the virulence capabilities in environmentally derived strains, and many researchers believe that there are few strains that naturally persist in the environment can cause serious disease, or occur at low infrequent incidences (Kaysner and DePaola, 2004; Baker-Austin et al., 2008). However, when environmentally derived strains of *V. parahaemolyticus* and *V. vulnificus* were compared to clinical strains using a human gastrointestinal epithelial

cell line, both clinically and environmentally isolated strains caused similar degrees of damage to human cells in vitro (Klein, 2018).

Zebrafish (*Danio rerio*) have successfully been used to study the virulence and pathogenic capabilities of many different types of bacterial species, including *Vibrio parahaemolyticus* (Paranjpye et al., 2013; Neely et al., 2002; Bergeron et al., 2017). The use of zebrafish is a good model system to use due to the fact the fish have both an innate and adaptive immune system, similar to humans, so the symptomatic effects that the bacteria have on the fish could lead to conclusions how humans may react as well (Da'as et al., 2011; Stemple and Driever, 1996; Sullivan and Kim, 2008). This study's focus was to better understand how environmentally isolated *V. vulnificus* strains compare in terms of virulence to clinically isolated strains. It was found that the range in virulence differs greatly between strains, and the use of *vcgC* and *vcgE* presence as a potential predictor of genotype and virulence may not be a reliable tool.

MATERIALS AND METHODS

Strain Isolation

Environmental *V. vulnificus* strains were isolated near Georgetown, SC, USA (33°20'N, 79°12'W) from lower salinity waters in Winyah Bay and the Waccamaw River. Water samples were plated on CHROMagar *Vibrio* (DRG International, NJ, USA) for isolation of *V. vulnificus* strains following the US Food and Drug Administration protocol (DePaola and Kaysner 2004). *Vibrio* strains were routinely cultivated on saline Luria Agar (SLA; per L; 10 g tryptone, 5 g yeast extract, 15 g NaCl, 15 g Bacto Agar).

Whole Genome Sequencing

Genomic DNA was isolated through the Wizard Genomic DNA Purification kit following the protocol for Gram negative organisms (Promega, Madison, WI, USA**)**. After DNA was extracted, DNA quantity was measured via Quibit fluorimetry. Libraries were prepared and then the strain of *Vibrio vulnificus* Aug-WR2-BW were sequenced using an Illumina MiSeq (V3 26300 base) at the Indiana University Center for Genomic Studies as a part of the Genome Consortium for Active Teaching NextGenSequencing Group (GCAT-SEEK) shared run (Buonaccorsi et al. 2011, 2014). Sequencing reads were filtered (median phred score 0.20), trimmed (phred score 0.16), and assembled using the paired-end *de novo* assembly option in NextGENe V2.3.4.2 (SoftGenetics**,** State College, PA, USA). The assembled genome was uploaded to the Rapid Annotation with Subsystem Technology (RAST) web service (Aziz et al. 2008; Overbeek et al. 2014) for analysis, guided contig reordering and assembly improvement. The genome was aligned based on completed sequences using dotplot comparisons. Whole genome sequence data obtained from this work was submitted to the NCBI GenBank and assigned the accession number: GCA_003798485.1.

Zebrafish Husbandry and Care

Zebrafish (*Danio rerio*) Tübingen strain were bred and maintained in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institute of Health (NIH) Office of Laboratory Animal Welfare (OLAW) guidelines and under the protocols approved by Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina.

Bacterial Growth Conditions

Bacterial strains used in this study are listed in Table 3.1. *V. vulnificus* strains were grown overnight in 15 ppt NaCl SLA broth using a shaking incubator at 37°C. Cultures were washed twice with PBS before use as an inoculum. Serial 10-fold dilutions of cultures were plated on 15 ppt NaCl SLA to confirm the concentration of the inoculum. Controls for this experiment included PBS buffer as well as a non-virulent strain of *Vibrio, Vibrio pacinii* DSM 19139^{T}. *V. pacinii* was grown up overnight in 15 ppt NaCl SLA broth at 23°C.

PCR Virulence Gene Screening

Isolates were grown overnight at 37°C in SLB. The cells were then centrifuged and transferred to sterilized distilled water, where crude DNA was then extracted by a boiling method of the cells at 95-100°C for 20 minutes. For all PCR reactions, 1 µl of the sample was used in each reaction. The species of the bacteria was confirmed by amplification of the recombinase A gene (*recA*) (Thompson et al., 2005). A common housekeeping gene that is typically used for species identification is the 16s rRNA gene, however, in vibrios, this gene is too highly conserved and doesn't allow for species resolution. Amplicons were sent off for sequencing (Eurofins, Louisville KY) and a phylogenetic tree using comparison sequences from NCBI was created. The protocol and primers that were used followed the protocols outlined by Thomspon (2005). PCR products for recA (790 bp) were resolved on a 1.5% agarose gel and sequenced using an ABI Prism 3730 DNA analyzer. Once sequences were received, they were edited, and Maximum-likelihood phylogenetic trees were made using the Kimura 2 parameter model with Mega 7 (Kumar et al., 2016; Tamura et al., 2015).

All *V. vulnificus* strains used in the study were screened for the following virulence factors: *vvhA*, *vcgC*, *vcgE*, *vvpE*, and *rtxA1*. The virulence correlated genes (*vcgC* and *vcgE*) variants used as indicator genes to differentiate between strains thought as avirulent (*vcgE* positive) and pathogenic (*vcgC* positive). PCR primers of Warner and Oliver (2008) were used to amplify *vvhA* (410 bp), *vcgE* (199 bp), and *vcgC* (97 bp). The primers of Liu et al. (2007) were used to amplify the *rtxA1* gene and the primers of Jeong et al. (2001) were used to amplify a segment of the *vvpE* (697 bp) gene. Each reaction included the following: 10x PCR buffer (Qiagen), 1.25 units of Taq, 0.5 µM of each primer, 200 µM of each dNTP (Qiagen), and 150 µM MgCl (Qiagen); distilled water used in reactions was supplemented with 10% DMSO. The thermal cycling program used for detection of all *V. vulnificus* virulence genes was as follows: denaturation at 94°C for 3 min, followed by 29 cycles consisting of 94°C for 45s, 55°C for 45s, 72°C for 45s, and a final elongation of 72°C for 2 min. PCR products for all virulence genes were also resolved on a 1.5% agarose gel.

Intraperitoneal Challenge

For challenge experiments, zebrafish were anesthetized, and then were injected intraperitoneally (IP) midway between the pectoral fin and the anus with 10 ml of the inoculum using a Hamilton syringe and a 33-gauge needle (Hamilton, Franklin, Massachusetts, USA) following previously published protocols (Lefebvre et al., 2009; Paranjpe et al., 2013). Zebrafish were housed in individual glass aquariums, with water supplied from the main on-system water of the zebrafish breeding tanks. Water temperature was maintained at room temperature which was around 23°C. Tris-buffered tricaine was used to kill the fish on completion of the experiment after 3 days, if the fish

had not succumbed to the bacterial infection. All aquariums and water were disinfected with 10% bleach solution.

Virulence Evaluation and Statistical Analysis

Each strain was tested on three fish per trial, and each trial was repeated two times. Fish were monitored for 7 hours post initial IP injection. Fish were housed for 3 days and monitored for several hours each consecutive day. Fish were viewed grossly to determine any signs of external injury such as swelling, redness, and lesions, increase of fecal production, changes in swimming pattern, changes in breathing pattern, and death. To determine how the bacteria infiltrates the fish body, some fish were sectioned into three regions: head, abdomen, and tail regions. These sections were weighed and homogenized, and then resuspended into 900 microliters of PBS. These were then serially diluted 10-fold and were plated onto TCBS agar to re-isolate the *Vibrio* bacteria to get colony counts and determine the concentration of *Vibrio* cells per gram of fish tissue per region. A one-way ANOVA using SPSS was performed comparing the means of fish death for each clade of tested *Vibrio* strains: *vcgC*-positive, *vcgE*-positive, and both *vcgC* and *vcgE*- positive. The significance level for the test was set at a p-value of 0.05. A one-way ANOVA was also used to compare the means of fish death between the clinically isolated strains and the environmentally isolated strains. A significance level of 0.05 was also used.

Genome Gazing

Vibrio vulnificus WR2-BW, JY1701, and 27562^{T} genomes were used to genome gaze and compare the differences in gene makeup between the three strains. The RAST SEED Viewer application was used to compare the genomes of the three strains and were

compared using a sequence-based function (Aziz et al., 2008; Overbeek et al., 2005). The comparison tables were downloaded and using the most virulent strain from the three of them as the reference organism, gene profiles of missing genes from the two less virulent strains were categorized.

RESULTS

Gene sequences of the *recA* gene confirmed that the environmentally isolated bacterial species were in fact *Vibrio vulnificus*, based on the phylogeny and percent similarity identity scores compared to reference genes of the *recA* gene in confirmed and sequenced strains of *Vibrio vulnificus* from NCBI GenBank (Figure 3.1). In all the species that were confirmed to be *Vibrio vulnificus* that were used in the study, 100% tested positive for containing the *vvhA* gene. Of the 13 environmentally isolated strains used in this study, 7 strains (54%) were *vcgE* variant positive, 2 strains (15%) were *vcgC* variant positive, and 4 strains (30%) contained both variants of the *vcg* gene (Table 3.1). For the *vgcE* positive strains, all but 1 contained the *rtxA1* gene, and 5 of the 7 were positive for *vvpE*. Strain Sept WR1-BW4 was the only strain of the *vgcE* clade that was negative for *rtxA1*, and it was also negative for vvpE. The other strain that was negative for *vvpE* was Oct 05-25-BW. Looking at the *vcgC* clade, strain Oct SF 05-20-BW tested positive for both *rtxA1* and *vvpE*, whereas strain Aug WR1-BW6 tested positive for rtxA1 only. All strains that contained both *vcg* variants also contained rtxA1 and *vvpE* (Table 3.1).

Virulence varied greatly between individual strains. The clinically isolated strain ATCC BAA-86 was the most virulent, since in both trials with this organism, all 3 fish used in each trial were dead by 24 hrs. Comparatively, the least virulent strain was *V.*

vulnificus strain Aug WR1-BW6, as zero fish in both trials succumbed due to the bacterial infection, and the fish exhibited very little symptomatic response to the bacterial injection. The most common symptoms early in the infection included diarrhea and site injection redness and irritation. As the infection progressed, many fish displayed difficulty swimming and staying positively buoyant, signs of labored breathing, and many became very lethargic, choosing to lay prone on the bottom of the aquarium rather than swimming at all. In the more virulent strains, there was clear enterohemorrhagic activity (Figure B.2), and upon dissection, many fish that succumbed early in the infection had a much lower blood volume and clear tissue damage when compared to fish that survived the entire trial (Figure B.3). A total of 102 fish were used in this study (17 strains, two trials for each strain, each trial involving three inoculated fish). Out of the total study, 67 (65%) fish succumbed as a direct result of the *V. vulnificus* infection (Table 3.2). For the clinically isolated strains, 91% of the inoculated fish succumbed to the bacterial infection. For the environmentally isolated strains, 58% of the fish directly succumbed to the *Vibrio vulnificus* infection (Table 3.2).

A one-way ANOVA statistical test was completed to compare the means of fish killed between the three clades of tested strains: *vcgE*-positive, *vcgC*-positive, and both variant positive. The hypothesis was that the means between the different gene clades would produce a difference in mean fish killed during the trials. The study showed there was no statistically significant difference in the means between the three groups with a p-value of 0.687. The one-way ANOVA looking at the means of fish death between the clinically isolated strains and the environmentally isolated strains also showed no significant difference between the two, with a p-value of 0.051.

Fish that were injected with strains BAA-86 and 05-21-BW1 were used to determine cell recovery per gram of tissue in three sections of the fish: the head region, the abdomen region, and the tail region. These fish regions were homogenized and serially diluted onto TCBS agar. The results showed that the highest recovery of cells came from the abdomen region, with the average of 6 fish for BAA-86 being $1.58x10^{9}$ CFUs g^{-1}, the tail region with the next highest concentration at $1.07x10^{8}$ CFUs g^{-1}, and the head region with the lowest at $2.35x10^{7}$ CFUs g^{-1}. The results for environmental strain 05-21-BW1 held consistent when compared to BAA-86 with the abdomen region with the highest cells recovered per gram of tissue at $1.55x10^{9}$ CFUs g^{-1},but for the environmental strain, the head had the next highest cells recovered at $5.65x10^{7}$ CFUs g^{-1}, and the tail region with the lowest at $8.18x10^{6}$ CFUs g^{-1}.

Of the three strains that had full genome sequences, *V. vulnificus* JY1701 had the highest level of virulence, with the strain killing all 6. Both strain ATCC 27562^{T} and WR2-BW killed 4 fish. JY1701 and WR2-BW are *vcgE* positive strains, whereas ATCC 27562^{T} is a *vcgC* positive strain (Figure 3.2). Because JY1701 was the most virulent, this strain's genome was used as the reference genome when compared for genome gazing to see what different and unique genes the more virulent strain has when compared to less virulent strains. There was a total of 305 unique genes that were present in JY1701 that were not present in either WR2-BW and ATCC 27562^{T}. Only 47 of those 305 genes had a known function (Table 3.3). The rest of the unique genes (258 genes, or 84.5% of the total unique genes) were hypothetical proteins, genes of unknown function, or phage related genes. The genes that may pose some interest in terms of virulence that were present in *V. vulnificus* JY1701 that were not present in WR2-BW and ATCC 27562^{T}

include the following genes: T1SS secreted agglutinin RTX, virulence-associated E gene, a putative integrase, and a WzxE protein.

DISCUSSION

The pathogenicity of *V. vulnificus* seems to be very complex, undefined, and may not depend on just a handful of virulence factors. There seems to be a more complicated and less direct mechanism at work because virulence between strains of *V. vulnificus* isolated from the environment varied greatly, regardless of the presence or absence of the implicated virulence related genes. The clinically isolated strains that were *vcgC* positive did in fact show some of the highest virulence rates within the zebrafish, however, the presence of *vcgC* in our study did not necessarily reflect the highest virulence capabilities. Our study contained 7 strains that were positive for *vcgE* alone, and 6 of those 7 strains had at least the same level of virulence as the least virulent clinically isolated strain of *V. vulnificus* ATCC 27562^T; each of these strains killed at least 4 fish in total. The 3 least virulent strains from the study were *V. vulnificus* Sept WR1-BW6, June 05-25-SW1, and Sept 05-20-BW4, all of which had a copy of the *vcgC* gene.

Previous studies have shown that *V. vulnificus* isolates from oysters show an overwhelming proportion of *vcgE* positive strains, and this has been considered a reason why incidence of *V. vulnificus* infections is relatively low; if *vcgC* strains are not predominant, then it is less likely for a person to consume or encounter "virulent strains" (Warner and Oliver, 2008a). There may be an important ecological reason as to why the *vcgE* genotype does predominate in oysters. However, this genotyping protocol implies that the *vcgE* strains are incapable of causing disease, and our study clearly demonstrates that there are virulent *vcgE* strains. Zebrafish have well-developed innate and adaptive immune systems and could clearly survive some direct infections of *V. vulnificus* strains. It was also clearly not just the addition of live bacteria that killed the fish, as not a single fish that was inoculated with *Vibrio pacinii* DSM 19139^T, an avirulent bacterial species, died or showed any signs of illness or distress. Therefore, it seems

inappropriate to continue to separate *V. vulnificus* strains by genotype, or rely on *vcgE* status to identify pathogenic versus nonpathogenic status. In addition, cytotoxicity trials show that *vcgE* strains are capable of causing destruction of epithelial cells (Klein, 2018) and cause disease in organisms that have an immune system. Studies overwhelmingly focus on testing and researching strains that have been isolated in a clinical setting, and it seems researchers fail to remember that these organisms originated from the environment; there is no clinical case unless a patient contracts the bacteria from the environment.

Another factor that needs further study is looking at individual strain's iron acquisition while it is actively infecting a source. Strains that showed high virulence in the fish also seemed to deplete the fish of its blood, indicating that it may serve as a nutritional source of iron (Figure B.3). *V. vulnificus* strains are known to possess siderophores, or iron acquisition molecules, which are low-molecular-weight chelators that bind iron and are then returned and brought back into the cells (Simpson and Oliver, 1983). In many cases, blood serum iron is unavailable to microorganisms due to inhibitory effects, resulting in iron deprivation for microorganisms while in the blood stream (Weinberg et al., 1978). When iron-containing compounds that are more biologically available have been injected directly into the blood during animal infection models, there has been an increase in microbial numbers (Holbein et al., 1980; Kochan et al., 1978). Perhaps iron acquisition in *Vibrio vulnificus* plays a more important role in sustaining an infection and looking into the siderophore response between the virulent strains versus less virulent strains is important for future work.

Through genome gazing, it was determined that there were 305 genes that were unique to *V. vulnificus* JY1701 when compared to *V. vulnificus* ATCC 27562^{T} and Aug WR2-BW. Of those 305 genes, only 47 had a known or defined function and the rest was hypothetical, undefined, or phage related. There were only 4 genes that were of interest in terms of virulence-related function. Those genes included the T1SS secreted agglutinin RTX, virulence-associated E, putative integrase, and WzxE protein. Type 1 secretion systems (T1SS) are present in a lot of

different Gram-negative bacteria and are used to secrete and deliver different substrates such as proteases, lipases, and hemophores to target cells (Masi and Wandersman, 2010). The *rtxA1* toxin is known to be associated with Type 1 secretion models (Lee et al., 2008). Within all three genomes, there were multiple genes that related to the Type 1 Secretion System (T1SS) secreted agglutinin RTX, however, WR2-BW and ATCC 27562^{T} only contained two copies of the three genes that made of up the system and did not contain this gene in particular. The other two genes involved in the T1SS were 314 amino acids in length and 548 amino acids long. Both species, WR2-BW and ATCC 27562^{T}, contain the RTX protein, and contained some elements of the T1SS, however both strains are missing this gene that is 79 amino acids in length. Perhaps the RTX toxin wasn't being delivered into the host organism by strains lacking this gene at a rate equal to JY1701, which could explain its slightly less virulent effects.

The wzxE protein is interesting, because *Vibrio vulnificus* Aug WR2-BW does have a *wzx* gene that was found on a PAI (Klein et al., 2018), however, it is a slightly different gene. The gene that was located within the PAI of *V. vulnificus* Aug WR2-BW was a O-antigen flippase *wzx* gene. This gene is considered a virulence-associated factor, as they do not directly cause host cell damage, but they do contribute to pathogenesis, aiding in the establishment of infections. Lipopolysaccharide (LPS) is a main component of the outer membrane of Gram-negative bacteria and is a known pyrogen (fever-producing agent) (McPherson et al. 1991; Jones and Oliver 2009). The gene that was found in JY1701 belonged to the oligosaccharide flippase family based on the NCBI Protein Blast score (99%). Genes that fall under this category of translocation of lipid-linked oligosaccharides are used in activities such as cell wall construction, polysaccharide synthesis, and protein glycosylation, however the wzx/wzy pathway for

many aspects is undefined and research is still needed to fully understand this pathway and all its functions (Hong et al., 2017).

The virulence-associated E proteins belong to family of proteins that contain a p-loop motif, or phosphate-binding loops. Virulence-associated proteins have been identified in other microorganisms including *Streptococcus* and *Rhodococcus* species (Ji et al., 2016; Okoko et al., 2015). Mice that were exposed to a strain of *Streptococcus suis* serotype 2 that had a functional copy of the virulence-associated E protein (vapE) exhibited more severe symptoms, including depression, apathy, fever, anorexia, emaciation, swollen eyes, and neural disorders, and died within two days of infection. Mice that had a knocked-out version of vapE exhibited less severe clinical symptoms and all recovered within a week (Ji et al., 2016). The role that vapE plays in pathogenicity is poorly understood, but from these trials does show that it increased the virulence of this species of *Streptococcus*. Because of vapE's undefined overall function and role in pathogenicity mechanisms, it is unclear how its role in *Vibrio vulnificus* virulence is involved.

The putative integrase gene is not a direct virulence factor, per say, but may be involved in virulence in a different way, as a site of recombination and integration of foreign DNA (Hacker and Kaper 2000). *Vibrio cholerae*, a close relative of *Vibrio vulnificus*, also has a putative integrase gene that is found at the distal end of a large cluster of ToxR-regulated colonization genes of toxin-coregulated pilus (TCP) and accessory colonization factor (ACF) (Kovach et al., 1996). TCP and ACF are involved in colonization of host cells, and greatly aid in the ability to successfully colonize the host intestinal epithelial tissue (Kovach et al., 1996). What this means is that at one time, these

genes may have been obtained by *Vibrio cholerae* via horizontal gene transfer or other mobile genetic elements, which is why it is important to note that this putative integrase gene is present in strain JY1701, and not in the other two. *V. vulnificus* JY1701 may be able to obtain other PAIs, virulence factors, and other foreign DNA from other organisms at a much faster rate than the other two strains.

While more research needs to be done, it is important to know that virulence between different environmental strains varies greatly and cannot accurately be predicted based on genotype alone. Full genome sequencing of more environmental strains is needed to determine what additional genes may be involved in *V. vulnificus* virulence

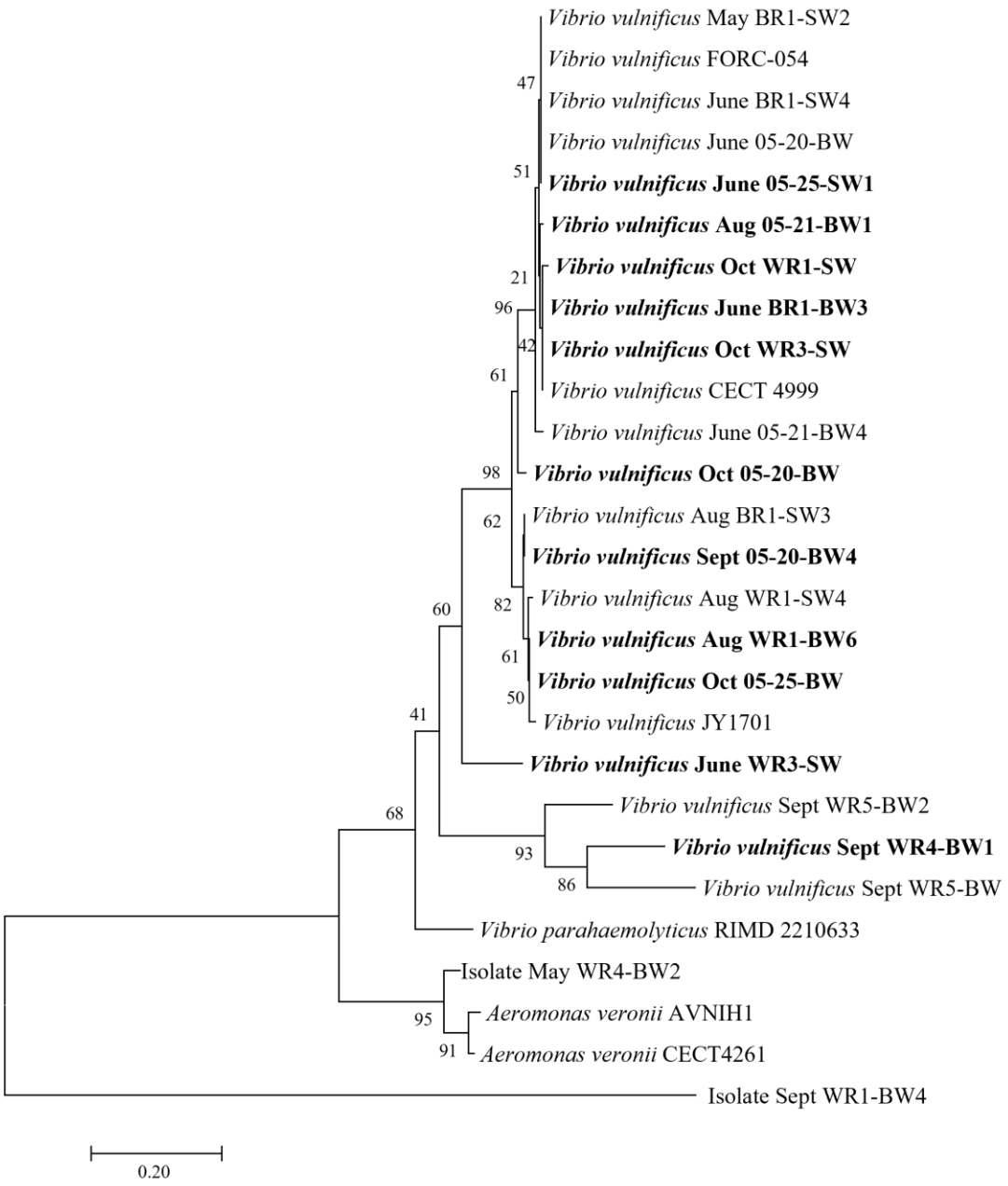


Figure 3.1: Maximum-likelihood phylogeny (Kimura 2-parameter model) of *recA* gene sequences. The bootstrap values represent 1,000 replications. The reference sequences were acquired from NCBI GenBank. Bolded sequences are the environmentally strains used in this study.

Month Isolated	Total # Strains	*vcgE* +	*vcgC* +	Both *vcgE* and *vcgC* +	*vvhA* +	*rtxA1* +	*vvpE* +
May	23	18	19	5	23	21	20
June	55	40	3	3	40	40	40
July	42	23	5	0	35	33	n/a
August	55	45	13	2	55	48	n/a
September	15	12	6	6	15	10	15
October	37	34	15	13	37	31	27
Totals:	227	172	61	29	205	183	102

Table 3.1: Gene distribution implicated virulence genes of *Vibrio vulnificus* strains isolated from the environment from Winyah Bay and the Waccamaw River.

	Genes Screened via PCR				
	vvhA	*vcgE*	*vcgC*	*rtxA1*	*vvpE*
Vibrio vulnificus Strain					
ATCC 27562^{T}	+	-	+	+	+
ATCC BAA-86	+	-	+	+	+
ATCC 33817	+	-	+	+	+
JY1701	+	+	-	+	-
June BR1-BW3	+	+	-	+	+
Aug WR2-BW	+	+	-	+	+
Aug 05-21 BW1	+	+	-	+	+
Sept WR4-BW1	+	+	-	-	-
Oct WR3-SW	+	+	-	+	+
Oct WR1-SW	+	+	-	+	+
Oct 05-25-BW	+	+	-	+	-
Aug WR1-BW6	+	-	+	+	-
Oct 05-20-BW	+	-	+	+	+
June WR3-SW	+	+	+	+	+
June 05-25-SW1	+	+	+	+	+
Aug 05-25-BW3	+	+	+	+	+
Sept 05-20-BW4	+	+	+	+	+

Table 3.2: Gene distribution of *Vibrio vulnificus* strains used in zebrafish inoculations. Bold indicates strains from a clinical source. Superscript ^{T} indicates type strain.

Fish Death Rate					
Vibrio vulnificus Strains Tested	12 hrs (dead / total tested)	24 hrs	48 hrs	72 hrs	Total fish that died during the trial
***V. vulnificus* ATCC-27562^T**	0 / 6	1 / 6	2 / 6	4 / 6	4
***V. vulnificus* ATCC BAA-86**	1 / 6	6 / 6	-----	-----	6
***V. vulnificus* ATCC 33817**	0 / 6	5 / 6	6 / 6	-----	6
***V. vulnificus* JY1701**	1 / 6	5 / 6	6 / 6	-----	6
V. vulnificus June BR1-BW3	0 / 6	0 / 6	3 / 6	4 / 6	4
V. vulnificus Aug WR2-BW	0 / 6	2 / 6	4 / 6	4 / 6	4
V. vulnificus Aug SF-05-21 BW1	0 / 6	4 / 6	4 / 6	4 / 6	4
V. vulnificus Sept WR4-BW1	0 / 6	0 / 6	1 / 6	2 / 6	2
V. vulnificus Oct WR3-SW	0 / 6	0 / 6	6 / 6	-----	6
V. vulnificus Oct WR1-SW	0 / 6	0 / 6	2 / 6	5 / 6	5
V. vulnificus Oct 05-25-BW	0 / 6	0 / 6	3 / 6	4 / 6	4
V. vulnificus Aug WR1-BW6	0 / 6	0 / 6	0 / 6	0 / 6	0
V. vulnificus Oct SF-05-20-BW	0 / 6	0 / 6	0 / 6	2 / 6	2
V. vulnificus June WR3-SW	0 / 6	4 / 6	5 / 6	6 / 6	6
V. vulnificus June 05-25-SW1	0 / 6	1 / 6	1 / 6	1 / 6	1
V. vulnificus Aug 05-25-BW3	0 / 6	0 / 6	0 / 6	3 / 6	3
V. vulnificus Sept 05-20-BW4	0 / 6	0 / 6	2 / 6	4 / 6	4

Table 3.3: Fish death rate of *Vibrio vulnificus* strains used in zebrafish inoculations. Totals come from two independent trials of each strain. Each trial was repeated twice. Bold indicates strains from a clinical source. Superscript ^{T} indicates type strain.

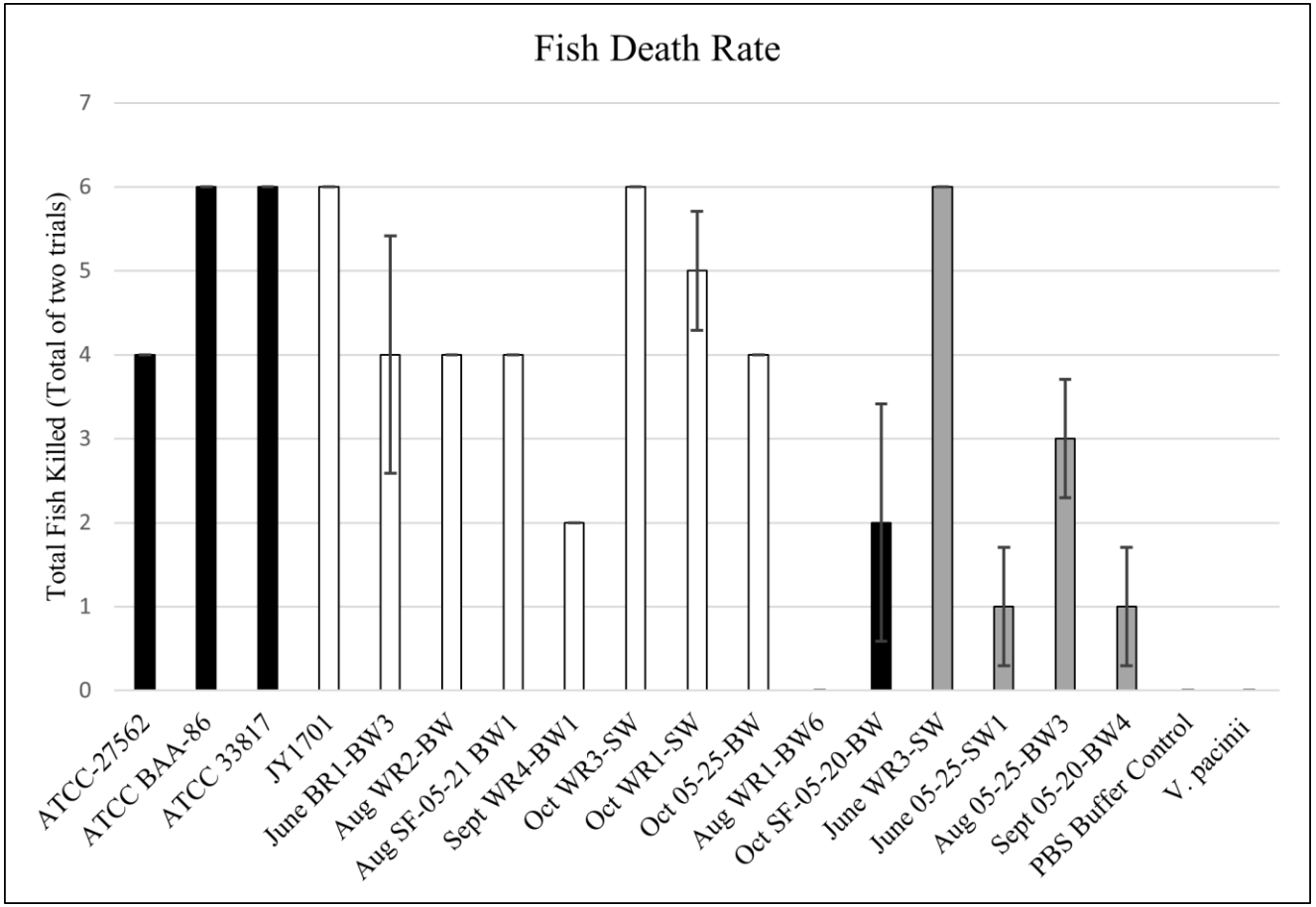


Figure 3.2: Death rate of fish totaled from two independent trials of *Vibrio vulnificus* strains. Clinically isolated strains include ATCC-27562^{T}, ATCC-BAA-86, ATCC 33817, and JY1701. All other strains are environmentally isolated. Controls for the experiment included fish injected with PBS buffer and an avirulent *Vibrio* species, *Vibrio pacinii*. Black bars indicate *vcgC* positive strains. White bars indicate *vgcE* positive strains. Grey bars indicate strains positive for both *vcgC* and *vcgE*. Error bars represent the standard deviation between the two trials.

Table 3.4: Results from genome gazing at the genes present and unique to strain *Vibrio vulnificus* JY1701 and not present in strains *V. vulnificus* 27562^T or WR2-BW. The genes in this table are genes with known functions. Hypothetical proteins, genes with unknown function, or phage related genes were not included. Bolded genes indicate genes that may have a possible virulence-related function.

Gene Number in *V. vulnificus* JY1701	Length of Gene (No. amino acids)	Function of Gene
424	53	Type cbb3 cytochrome oxidase biogenesis protein CcoI; Copper-translocating P-type ATPase (EC 3.6.3.4)
479	210	TonB-dependent receptor
480	483	TonB-dependent receptor
483	147	putative membrane protein
520	46	Maltoporin (maltose/maltodextrin high-affinity receptor, phage lambda receptor protein)
615	41	2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)
689	152	Rhs family protein
1138	239	Beta-1,4-galactosyltransferase
1141	393	UDP-Bac2Ac4Ac hydrolyzing 2-epimerase NeuC homolog
1143	213	4-amino-6-deoxy-N-Acetyl-D-hexosaminyl-(Lipid carrier) acetyltrasferase
1149	77	Acyl carrier protein, putative
1152	672	Acyl protein synthase/acyl-CoA reductase RfbN
1153	131	Acyl protein synthase/acyl-CoA reductase RfbN
1155	325	polysaccharide deacetylase
1171	141	S-adenosylhomocysteine hydrolase
1175	135	Structural protein P5
1178	157	Methyl-accepting chemotaxis protein
1179	124	Mg-dependent DNase
1183	278	Outer membrane receptor protein
1446	51	Neopullulanase (EC 3.2.1.135)
1791	266	Arginine/ornithine antiporter ArcD
2057	65	Lipid carrier : UDP-N-acetylgalactosaminyltransferase (EC 2.4.1.-) / Alpha-1,3-N-acetylgalactosamine transferase PglA (EC 2.4.1.-); Putative glycosyltransferase
2061	388	Glycosyl transferase, group 1
2063	**417**	**WzxE protein**
2215	931	Chromosome segregation ATPases

2267	587	DNA double-strand break repair Rad50 ATPase
2284	290	EF hand domain protein
2623	738	Translation-disabling ACNase RloC
2674	118	ORF2
2991	637	DNA helicase II related protein
3371	288	**putative integrase**
3374	**420**	**virulence-associated E**
3459	297	Type III restriction-modification system methylation subunit (EC 2.1.1.72)
3460	330	Type III restriction-modification system methylation subunit (EC 2.1.1.72)
3461	799	Type III restriction-modification system DNA endonuclease res (EC 3.1.21.5)
3636	38	Trehalose-6-phosphate hydrolase (EC 3.2.1.93)
3686	264	putative alpha-dextrin endo-1, 6-alpha-glucosidase
3890	161	GCN5-related N-acetyltransferase
3967	40	Alcohol dehydrogenase (EC 1.1.1.1)
4081	176	Predicted transcriptional regulator
4082	302	Predicted nucleotide-binding protein
4259	445	articulin, putative
4314	265	putative type II restriction endonuclease
4334	141	putative glyoxalase
4402	224	HAD superfamily hydrolase
4425	159	putative acetyltransferase
4461	**79**	**T1SS secreted agglutinin RTX**

CONCLUDING REMARKS

The vibrios make up a very important genus of bacteria in terms of both an ecological and human health impact. The genus *Vibrio* is a very diverse and ecologically persistent organism due to the genomic variability. These organisms are found in all kinds of estuarine and saltwater environments, such as sediment, water column, and associated with many times of marine fauna as well like oysters, copepods, spartina, clams, shrimp, and many others.

Due to their large, diverse nature, the core genome that encompasses all species within the genus is very small, with only 158 genes shared between all species. This small core genome may allow for high genetic plasticity, permitting a high level of adaptability to alter their genomes quickly, so they can survive changes in environmental conditions. Environmentally isolated vibrios may also play a much larger role in the ability to cause disease in humans, as a characterized and defined pathogenicity island of PAI-7 was found on *V. parahaemolyticus* JS-8-11-1's second chromosome, which had previously only ever been identified in clinically isolated strains of *V. parahaemolyticus*. Virulence levels greatly differ between strains of *Vibrio vulnificus* as demonstrated in zebrafish, and the current virulence marker genes that we have for *V. vulnificus* do not seem to accurately predict whether a strain may be virulent or not.

The pathogenicity mechanism of *V. parahaemolyticus* and *V. vulnificus* seems to be complicated and is clearly a pathway that is not cut and dry like these organism's relative of *Vibrio cholerae*. The current marker genes that we use to track potential pathogenic strains of *V. vulnificus* did not prove to be useful in this study. For example, many *vcgE* strains produced extreme disease and displayed high virulence in zebrafish, whereas a *vgcC* strain possessed very little virulent function at all. There is still a lot we do not understand about the genomes of these organisms, considering 10% of the core genome that's shared across the entire *Vibrio* genus has an undefined and unknown function. This is even seen within pathogenicity islands, where close to 65-70% of a pathogenicity island can be made of up genes of unknown function as well. The answer of virulence and pathogenicity of these organisms remains hidden within the genome, and as more research and understanding of how these organisms cause disease and persist in the environment, one day a mechanism can hopefully be defined.

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APPENDIX A

REPRINT PERMISSION

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APPENDIX B

SUPPLEMENTAL FIGURES

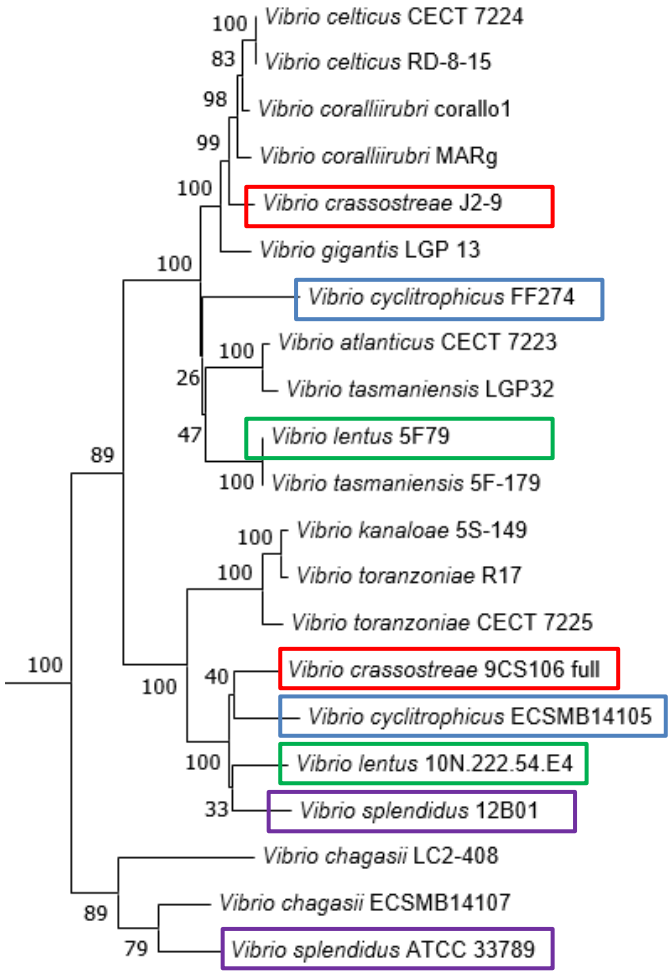

Figure B.1: A focused section of the phylogenetic tree from Chapter 1 Figure 1.1. There was lower species resolution using the core genome for the following *Vibrio* species: *Vibrio lentus*, *Vibrio crassostreae*, *Vibrio cyclotrophicus*, and *Vibrio splendidus*.

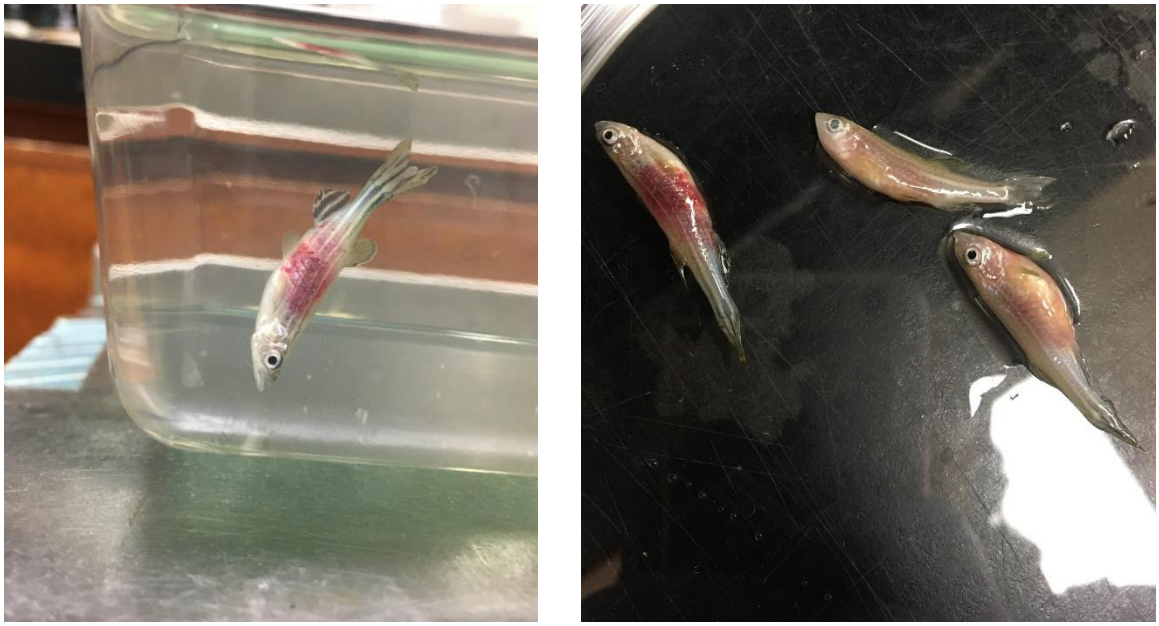

Figure B.2: Enterohemorrhagic damage in zebrafish due to the clinically isolated strain *Vibrio vulnificus* ATCC BAA-86.

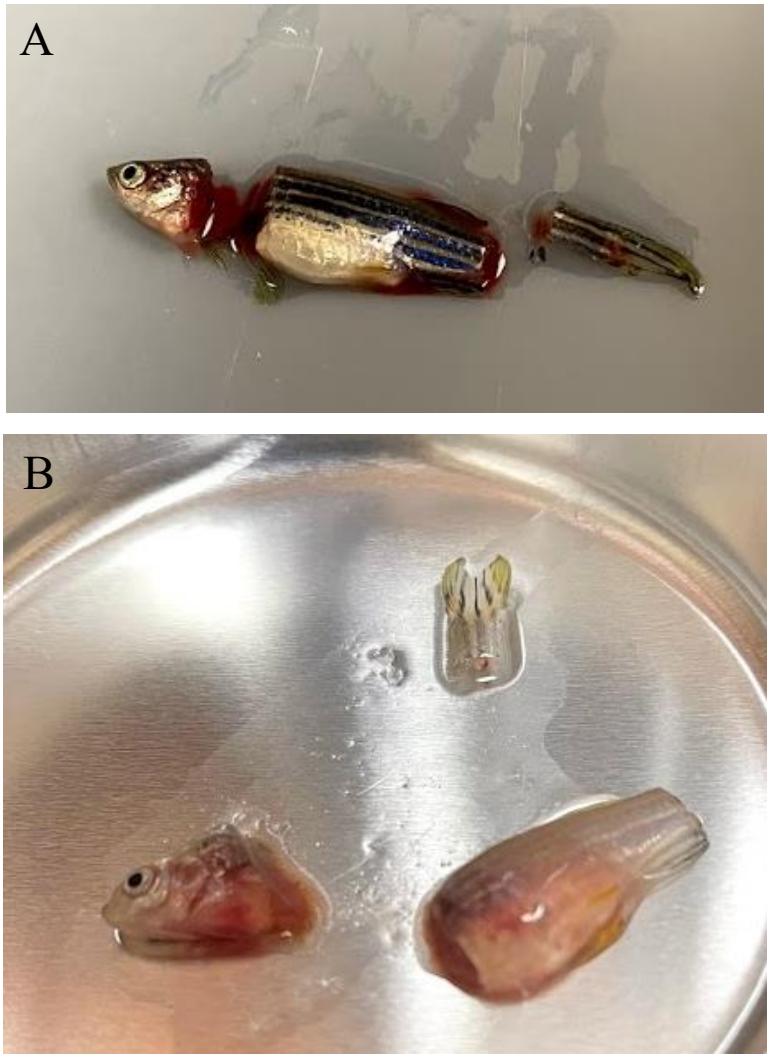

Figure B.3: The top picture (A) shows a dissected fish that survived all three days in a *Vibrio vulnificus* infection challenge from strain *Vibrio vulnificus* Aug WR1-BW6. There was very little blood loss and no apparent tissue damage. The bottom picture (B) shows a dissected fish that perished from an infection caused by *Vibrio vulnificus* ATCC BAA-86. There was blood utilization and metabolism by the bacteria and tissue damage as seen from the lack of blood and pale pallor of the tissue from the outside of the fish and the interior tissue had less structural integrity compared to fish A.