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Ecology And Virulence Capabilities Of Vibrios Isolated From The Pristine North Inlet Estuary

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ECOLOGY AND VIRULENCE CAPABILITIES OF VIBRIOS ISOLATED FROM THE
PRISTINE NORTH INLET ESTUARY

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ABSTRACT

Vibrio bacteria are Gram negative, motile organisms that occur naturally in most coastal and estuarine ecosystems. Some vibrios are important human pathogens, including *Vibrio parahaemolyticus* and *Vibrio vulnificus*. The CDC estimates that vibrios cause 80,000 cases of disease each year in the United States alone. Most cases are caused by *V. parahaemolyticus*, which infects humans after the consumption of contaminated raw or undercooked seafood, primarily oysters. *V. parahaemolyticus* causes mild gastroenteritis that is self-limiting unless the patient is immunocompromised. *V. vulnificus* has a much lower incidence of disease (100 cases in the USA yr^{-1}); however, this organism causes much more severe infections, including necrotizing fasciitis (flesh eating disease) and sepsis when introduced into an open wound. With global climate change, *Vibrio* outbreaks are expanding in size, frequency, and latitude. This investigation examined the reliability of using "species specific" marker genes to identify a *Vibrio* strain, the distribution of pathogenicity islands (PAIs) within *Vibrio* genomes, and the distributions of potential pathogenic *V. parahaemolyticus* within oysters and oyster tissues. We determined that some oysters, designated as "hot" oysters, can harbor significantly more vibrios than surrounding oysters. These "hot" oysters, which occur at low frequency, may explain the sporadic (and difficult to predict) nature of *V. parahaemolyticus* infections. The cytotoxic effects of environmental *Vibrio* strains and the interactions of vibrios with various marine microalgae were also studied.

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LIST OF ABBREVIATIONS

MLSA ....Multilocus sequence analysis

PAI ....Pathogenicity island

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

T3SS2..Type Three Secretion System Two

TCBS agar.. Thiosulfate citrate bile salts sucrose agar

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

tlh ....Thermostable labile hemolysin gene (protein is TLH)

trh..*tdh*-related gene (protein is TRH)

vscC2..*Vibrio* secretion gene (part of the T3SS2 complex)

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

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

INTRODUCTION

Vibrio parahaemolyticus is a human pathogen that occurs naturally in most estuarine and coastal ecosystems. When ingested in raw, undercooked, temperature abused, and/or mishandled oysters, this bacterium causes gastroenteritis. Symptoms usually manifest between 12-24 hours after ingestion of contaminated shellfish, but it can take as long as four days for symptoms to appear. Symptoms include diarrhea, nausea, vomiting, abdominal cramps, headache, fever, and chills. Most cases in healthy individuals are mild and self-limiting, meaning the infection runs its course after 2 to 3 days. Hospitalization is not common for *V. parahaemolyticus* infections unless the patient is immunocompromised. Tracking and monitoring outbreaks of *V. parahaemolyticus* is made difficult due to this problem of underreporting. However, the CDC estimates that there are 80,000 cases of vibriosis (*Vibrio* gastroenteritis) per year in the United States (2018).

V. parahaemolyticus is an environmental pathogen, meaning it occurs naturally in most coastal ecosystems. A contamination event is not required for *V. parahaemolyticus* to be introduced into oysters. With the right environmental parameters, such as elevated temperatures, densities of this organism in the environment will spike. But in recent years this organism has invaded to typically cold-water regions such as the Baltic Sea (Eiler et al., 2006) and Alaska (McLaughlin et al., 2005; Martinez-Urtaza et al., 2010). This organism has also recently been responsible for oyster bed closures in typically

cooler months, such as September and October. For example, oyster beds in Martha's

Vineyard, MA were closed in October 2013 and again in October 2017 due to confirmed

cases of *V. parahaemolyticus* disease (Interstate Shellfish Sanitation Conference (ISSC),

2018). Oyster beds in British Columbia were closed in September 2015 due to *V.

parahaemolyticus* infections (ISSC, 2018). Oyster recalls also followed these shellfish

bed closures. These oyster bed closures are of note because they happened in cold water

regions and during a time of year when it is thought that *V. parahaemolyticus* numbers

are low enough to not cause disease. Outbreaks of this organism are increasing in size,

frequency, and latitude. There is no way to accurately predict an outbreak of *V.

parahaemolyticus*. Shellfish monitoring programs do routine monitoring of *Vibrio* levels

in oysters; yet shellfish bed closures and recalls only occur after an outbreak (or a

confirmed case of vibriosis) has occurred (ISSC, 2018).

Not only do shellfish monitoring programs determine the number of *V.

parahaemolyticus* cells in shellfish, officials must also differentiate between avirulent

(non-pathogenic) and virulent (pathogenic) strains of this organism. The standard

method to determine if a strain of *V. parahaemolyticus* is pathogenic is via PCR

amplification of two genes, *tdh* (the thermostable direct hemolysin gene) and *trh* (the *tdh*-

related gene). *tdh* and *trh* have been implicated in *V. parahaemolyticus* virulence

(Miyamoto et al., 1969; Shirai et al., 1990; Honda and Iida, 1993; DePaola and Kaysner,

2004; Lovell, 2017). TDH and TRH are tetrameric proteins that act as porins and

facilitate efflux of divalent cations and other solutes from and influx of water molecules

into intestinal cells (Yanagihara et al., 2010; Broberg et al., 2011; Ohnishi et al., 2011).

In addition to *tdh* and *trh*, *V. parahaemolyticus* can utilize secretion systems to cause

damage to host cells (Makino et al., 2003). However, PCR amplification of *tdh* and/or *trh* is the standard method to determine the pathogenic potential of *V. parahaemolyticus* strains (Kaysner and DePaola, 2004).

Numerous studies have indicated that *tdh* and *trh* are found almost exclusively in clinical strains isolated from patients suffering from *V. parahaemolyticus* gastroenteritis (Miyamoto et al., 1969; Shirai et al., 1990). Only about 1 to 2% of screened *V. parahaemolyticus* strains not derived from infected humans ("environmental strains"), were reported to carry these genes (Kaysner and DePaola, 2004; Baker-Austin et al., 2008). Put another way, it was thought that only 1-2% of environmental *V. parahaemolyticus* strains were pathogenic and able to cause human infections. However, in 2013 Gutierrez-West redesigned the *tdh* and *trh* PCR primers used to amplify these genes. With the advent of new and improved methods for virulence factor gene amplification, detection of *tdh* and *trh* in environmental *V. parahaemolyticus* rose to approximately 50% (Gutierrez-West et al., 2013).

This investigation used the improved PCR primers of Gutierrez-West (2013) to determine the frequency of *tdh* and *trh* in oyster-derived environmental *V. parahaemolyticus* strains. We detected these genes, previously thought to be rare in environmental isolates, in approximately half of the oyster isolates. We also examined the reliability of using "species specific" marker genes to identify a *Vibrio* strain, the distribution of pathogenicity islands (PAIs) within *Vibrio* genomes, and the distributions of potential pathogenic *V. parahaemolyticus* within oysters and oyster tissues. We determined that some oysters, designated as "hot" oysters, can harbor significantly more vibrios than surrounding oysters. These "hot" oysters, which occur at low frequency,

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may explain the sporadic (and difficult to predict) nature of *V. parahaemolyticus* infections. The cytotoxic effects of environmental *Vibrio* strains and the interactions of vibrios with various marine microalgae were also studied.

CHAPTER 1

THE *VIBRIO PARAHAEMOLYTICUS* VIRULENCE RELATED GENES, *TDH*, *TLH*, AND *VSCC2* OCCUR IN OTHER *VIBRIONACEAE* SPECIES [1]

[1] Klein SL, West CK, Mejia DM, Lovell CR. 2014. Genes similar to the *Vibrio parahaemolyticus*-virulence related genes *tdh, tlh,* and *vscC2* occur in other *Vibrionaceae* species isolated from a pristine estuary. Appl. Environ. Microbiol. 80:595-602.

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ABSTRACT

Detection of the human pathogen *Vibrio parahaemolyticus* often relies on molecular biological analysis of species-specific virulence factor genes. These genes have been employed in determinations of *V. parahaemolyticus* population numbers and the prevalence of pathogenic *V. parahaemolyticus* strains. Strains of the *Vibrionaceae* species *Photobacterium damselae*, *Vibrio diabolicus*, *Vibrio harveyi*, *Vibrio natriegens*, and strains similar to *Vibrio tubiashii* were isolated from a pristine salt marsh estuary. These strains were examined for the *V. parahaemolyticus* hemolysin genes *tdh*, *trh*, and *tlh*, and for the *V. parahaemolyticus* Type III Secretion System 2α gene *vscC2* using established PCR primers and protocols. Virulence-related genes occurred at high frequencies in non-*V. parahaemolyticus Vibrionaceae* species. *V. diabolicus* was of particular interest as several strains were recovered and the large majority (>83%) contained virulence-related genes. Detection of these genes does not assure correct identification of virulent *V. parahaemolyticus*. Further, the occurrence of *V. parahaemolyticus*-like virulence factors in other vibrios potentially complicates tracking of outbreaks of *V. parahaemolyticus* infections

INTRODUCTION

Vibrio parahaemolyticus is the leading cause of seafood-associated gastroenteritis in the US and the world (FDA, 2005). Outbreaks of *V. parahaemolyticus* infections are increasing in frequency and expanding in geographic range (McLaughlin et al., 2005; Paranjype et al., 2012). This organism is ubiquitous in nearshore marine waters and cell numbers are typically highest in surficial sediments (West, 2012; Gartmon et al., unpublished) and in turbid waters bearing high loads of resuspended sediment (Parveen et

al., 2008, Johnson et al., 2010). Filter feeding bivalve mollusks, such as oysters and mussels, can concentrate *V. parahaemolyticus* and other pathogenic vibrios (e.g. Warner and Oliver, 2008; Froelich et al., 2013), resulting in levels in the mollusks capable of producing infection in a person that ingests them (DePaola et al., 1990). Virulent *V. parahaemolyticus* strains are clearly a concern for seafood safety and their detection is important anywhere that elevated levels of this organism are found.

Detection of *V. parahaemolyticus* in shellfish and environmental samples is typically based on molecular biological analysis of specific genes, particularly genes exclusive to this species and those strongly correlated with pathogenicity. The gene encoding the thermolabile hemolysin (TLH), designated *tlh*, encodes a phospholipase A2 (Zhang and Austin, 2005). While its contribution to *V. parahaemolyticus* pathogenicity is unknown, expression of this gene is upregulated under conditions mimicking the human intestine (Broberg et al., 2011; Gotoh et al., 2010). *tlh* is considered to be a species-specific marker for *V. parahaemolyticus* (Taniguchi et al., 1986; McCarthy et al., 1999) and is frequently employed to identify this species (Bej et al., 1999; DePaola et al., 2003; Nordstrom et al., 2007; Jones et al., 2012). Genes encoding the thermostable direct hemolysin (TDH) and the homologous thermostable direct hemolysin-related hemolysin (TRH), *tdh* and *trh*, respectively, have been implicated in *V. parahaemolyticus* virulence (Miyamoto et al., 1969; Shirai et al., 1990; Honda and Iida, 1993). TDH and TRH are tetrameric proteins that act as porins and facilitate efflux of divalent cations and other solutes from and influx of water molecules into intestinal cells (Yanagihara et al., 2010; Broberg et al., 2011; Ohnishi et al., 2011). Occurrence of *tdh* is correlated with the Kanagawa Phenomenon, a β-hemolytic reaction on saline blood agar (Wagatsuma Agar)

(Miyamoto et al., 1969). Numerous studies indicated that *tdh* and *trh* are found almost

exclusively in clinical strains isolated from patients suffering from *V. parahaemolyticus*

gastroenteritis (Miyamoto et al., 1969; Shirai et al., 1990). Only about 1 to 2% of

screened *V. parahaemolyticus* strains not derived from infected humans ("environmental

strains"), were reported to carry these genes (e.g. Baker-Austin et al., 2008). These

results were due, at least in part, to inadequate methodology and recent studies have

shown that high levels of environmental *V. parahaemolyticus*, 52% of strains from an

area supporting intensive shrimp aquaculture (Velazquez-Roman et al., 2012) and 48% of

strains from water and sediment in a pristine estuarine ecosystem (Gutierrez-West et al.,

2013), carry these genes. In addition, *V. parahaemolyticus* strains can encode two Type

Three Secretion Systems, T3SS1 on chromosome 1 and T3SS2 on chromosome 2

(Makino et al., 2003). T3SS1 has been found in all tested strains of *V. parahaemolyticus*

while T3SS2 has been reported in virulent strains (Makino et al., 2003; Park et al., 2004).

T3SSs are composed of 20-30 proteins and are responsible for translocating effector

proteins directly into host cell cytoplasm. There are over one hundred described effector

proteins having effects ranging from autophagy to cytotoxicity (Kodama et al., 2007;

Burdette et al., 2009). Additionally, there are two distinct types of T3SS2 (Okada et al.,

2009). T3SS2α has been found in strains that carry *tdh*, while T3SS2β is correlated with

trh (Okada et al., 2009; Noriea et al., 2010). Pathogenesis of *V. parahaemolyticus* does

not appear to rely solely on a given virulence function, rather virulence is complex and

different strains may employ somewhat different strategies.

     The utility of any of these genes as a molecular marker for *V. parahaemolyticus*

or for evaluation of the potential pathogenicity of *V. parahaemolyticus* strains relies upon

their specificity for *V. parahaemolyticus*. Sporadic reports of *V. parahaemolyticus*-like virulence genes in other species have appeared in the literature (Nishibuchi et al., 1996; Xie et al., 2005; Gonzalez-Escalona et al., 2006; Wang et al., 2007; Okada et al., 2010; Gennari et al., 2012), but little is known regarding the distributions of these genes among *Vibrionaceae*. In particular, occurrence of these genes in environmental strains has received very little attention. We screened a collection of *Vibrionaceae* strains isolated from a pristine estuary for *tlh*, *tdh* and *trh*, and for a gene encoding a T3SS2α outer membrane protein, *vscC2* (VPA1339), to evaluate the specificity of these genes for *V. parahaemolyticus*. Our results show that *tlh*, *tdh*, and *vscC2* are not found exclusively in *V. parahaemolyticus*.

MATERIALS AND METHODS

Sample site and strain isolation

Strains were isolated from the pristine North Inlet estuary near Georgetown, SC (33°20'N, 79°12'W). The North Inlet-Winyah Bay National Estuarine Research Reserve protects the third largest watershed on the east coast of the United States and 90% of the 18,916 acres is in its natural state. The keystone macrophyte *Spartina alterniflora* dominates the intertidal marsh except at lower salinity, higher elevations, where the subdominant *Juncus roemerianus* thrives. Fiddler crabs of the genus *Uca* are the biomass dominant fauna within the marsh. Their burrows are found throughout the intertidal and have been shown to contain high levels of *V. parahaemolyticus* (Gamble and Lovell, 2011).

Sampling trips were made in August 2011 and again in September 2011, to coincide with periods of elevated *Vibrio* numbers (Gamble and Lovell, 2011; West, 2012). Samples were collected at low tide from bulk sediment, *Uca* burrow lining sediment, *Uca* burrow water, sediment pore water, and creek water as described previously (Gamble and Lovell, 2011; West, 2012). All samples were diluted with phosphate buffered saline (400 mM NaCl, 1.75 mM NaPO$_4$, pH 7.4) and plated on Thiosulfate Citrate Bile salts Sucrose (TCBS) agar (BD, NJ) following the FDA protocol (DePaola and Kaysner, 2004) without the use of enrichment media. The TCBS plates were then incubated at 37ºC for 48 h. Well-isolated colonies were streaked onto fresh TCBS plates for further characterization. Green colonies (typical appearance of *V. parahaemolyticus*) were collected and routinely cultivated on saline Luria Agar (SLA; per L; 10 g tryptone, 5 g yeast extract, 27 g NaCl, 15 g Bacto Agar).

Strain designations indicate source (S, sediment; BS, burrow lining sediment; BW, burrow water; PW, porewater; CW, creek water), the month and year it was isolated (8-11 for August, 2011; 9-11 for September, 2011), and the isolate number. For example, JS-8-11-4 refers to isolate number 4 from *Juncus* zone sediment (JS) collected on August, 2011 (8-11).

Multilocus Sequence Analysis (MLSA)

The identities of the North Inlet *Vibrionaceae* isolates were determined by Multilocus Sequence Analysis of concatenated 16S ribosomal RNA gene, recombinase A (*recA*), RNA polymerase alpha subunit (*rpoA*), and gyrase B (*gyrB*) gene sequences. Bacterial genomic DNA was extracted using the Wizard Genomic DNA Purification kit

(Promega, Madison, WI) and diluted to 25 ng µl⁻¹. The PCR program used to amplify

recA, rpoA and *gyrB* consisted of an initial denaturation at 95°C for 5 min, three cycles of

95°C for 1 min, 55°C for 2 min 15 s, and 72°C for 1 min 15 s, then thirty cycles of 95°C

for 35 s, 55°C for 1 min 15 s and 72°C for 1 min 15 s and a final elongation at 72°C for 7

min. *recA* (~790 bp) and *rpoA* (~900 bp) amplification employed the primers of

Thompson et al. (2005) and *gyrB* (~800 bp) amplification employed the primers of Ast

and Dunlap (2004). 16S rRNA (~1500 bp) was amplified using the primers and

protocols of Lane (1991). PCR products were resolved on a 1.5% agarose gel and

sequenced using an ABI Prism 3730 DNA analyzer. Sequences were edited and

Maximum-likelihood phylogenies were constructed using the Kimura 2 parameter model

with Mega version 7 (Tamura et al., 2015). Sequence data of reference *Vibrionaceae*

species were obtained from the NCBI GenBank.

Recovery and Analysis of Hemolysin and T3SS2 Genes

All PCR reactions targeting *tdh, trh* and *tlh* were carried out in 25 µl volumes

using *Taq* DNA polymerase (Qiagen, Valencia, CA). The PCR thermocycling program,

conditions, and primers of Bej et al. (1999) were employed for amplification of *tlh*. PCR

amplifications of the *tdh* and *trh* genes used the primers of Gutierrez-West et al. (2013)

and were performed in separate reactions. Each reaction included the following: 1x PCR

buffer (Qiagen), 1.25 units of *Taq,* 0.5 µM of each primer, 200 µM of each dNTP

(Qiagen) and yielded single bands of 245 bp (*tdh*) or 410 bp (*trh*). The thermal cycling

program for the three hemolysin genes was: denaturation at 95°C for 5 min, followed by

40 cycles consisting of 95°C for 1 min, 62°C for 1 min, 72°C for 1 min, and a final

elongation of 72°C for 2 min.

The T3SS2α gene *vscC2* (VPA1339) encodes an outer membrane protein and has been used to determine the presence of T3SS2α, which is correlated with enterotoxicity due to *V. parahaemolyticus* (Park et al., 2004; Noriea et al., 2010). Primers designed by Noriea et al. (2010) were used to amplify an approximately 330 bp segment of *vscC2* in 25 µl volumes using *Taq* DNA polymerase (Qiagen, Valencia, CA). Each reaction included the following: 10x PCR buffer (Qiagen), 0.125 units of *Taq,* 1.25 µM of primer, 0.5 µM of each dNTP (Qiagen) and 2 µM MgCl. The thermal cycling program used for detection of *vscC2* was: denaturation at 94°C for 4 min, followed by 32 cycles consisting of 94°C for 45s, 60°C for 40s, 72°C for 45s, and a final elongation of 72°C for 7 min.

PCR products were sequenced and the resulting gene sequences were edited and Maximum-likelihood phylogenies were constructed using the Kimura 2 parameter model with Mega version 7 (Tamura et al., 2015). Sequence data obtained from this work were submitted to the NCBI GenBank and assigned the accession numbers: JX453017-JX453108, JX257004-JX257016, JX262950-JX262990, and KF197044-KF197068, KF569811-KF569825, and KF578086-KF578118.

RESULTS

Fifty-five strains of vibrios produced green (sucrose non-fermenting) colonies on TCBS agar. MLSA demonstrated that 18 of these strains were not *V. parahaemolyticus* (Figure 1.1). Twelve of the North Inlet strains were identified as *Vibrio diabolicus;* these strains grouped with the *V. diabolicus* type strain HE800[T] in the MLSA tree. Three strains grouped with the *Vibrio tubiashii* type strain LMG 10936[T], but were not similar enough to the type strain to be considered true *V. tubiashii* strains. These three strains (JPW-8-11-4, JPW-9-11-6, JPW-9-11-11) most likely represent a new species of *Vibrio*

that is related to *V. tubiashii* and are referred to in the present study as "*V. tubiashii*-like."

The other *Vibrionaceae* recovered from North Inlet were strains of *Vibrio harveyi* (TBS-9-11-8), *Vibrio natriegens* (TBW-8-11-5), and *Photobacterium damselae, subspecies damselae* (JPW-8-11-6).

All eighteen strains were screened for the three hemolysin genes (Table 1.1). Six *tlh* amplicons were detected. Two strains produced faint amplicons that after multiple attempts did not yield sequence data. This has been observed previously for some authentic *V. parahaemolyticus* strains (Gutierrez-West et al., 2013).   *tlh* gene sequences were recovered from the other four strains (Figure 1.2). The *V. diabolicus* strains JPW-9-11-8 and JPW-8-11-8 contained *tlh* sequences that were each 99.3% similar to *tlh* gene sequence from the *V. parahaemolyticus* type strain ATCC 17802^T. *tlh* was also detected in JPW-9-11-11 (*V. tubiashii*-like) and JPW-8-11-6 (*P. damselae*). *tlh* gene sequences from these species were 99.3% and 99.1% similar , respectively, to those from *V. parahaemolyticus* ATCC 17802^T *tlh*.

tdh amplicons were detected in 5 *V. diabolicus* strains and 1 *V. tubiashii*-like strain (Figure 1.3). Two of the strains produced faint amplicons that after multiple attempts did not yield sequence data, indicating that these strains might contain a variant of *tdh*. Four amplicons, all from *V. diabolicus*, yielded sequence data. Three of the *tdh* gene sequences had high similarities (97.6-99.2%) to the *tdh* sequence of the *V. parahaemolyticus* reference strain ATCC 33846, which was employed because the *V. parahaemolyticus* type strain ATCC 17802^T does not contain *tdh*. The *tdh* sequence from CW-9-11-1 appeared to be divergent, having only 83.2% similarity to the ATCC 33846 *tdh* sequence. The translated peptide encoded by the CW-9-11-1 *tdh* gene sequence was

75.9% similar to the peptide sequence from *V. parahaemolyticus* 33846. The hemolysin gene *trh* was not recovered from any of the non-*V. parahaemolyticus* strains examined in this study. This gene was also less common in North Inlet *V. parahaemolyticus* strains than *tlh* or *tdh* (Gutierrez-West et al., 2013).

The T3SS2α gene *vscC2* was detected at the highest frequency of the genes examined. Twelve of the 18 non-*V. parahaemolyticus* strains (67%) contained *vscC2*. *vscC2* was detected in 7 *V. diabolicus* strains, 2 *V. tubiashii*-like strains, and in a *P. damselae* strain, a *V. natriegens* strain, and a *V. harveyi* strain (Figure 1.4). All of the *vscC2* amplicons yielded sequences that were highly similar (92.9-98.8%) to the *vscC2* sequence from the *V. parahaemolyticus* clinical strain RIMD 2210633 in which *V. parahaemolyticus* T3SS2 was first described (Makino et al., 2003). In total, at least one *V. parahaemolyticus*-like virulence gene was detected in sixteen of the 18 non-*V. parahaemolyticus* strains (89%) from North Inlet. None of the genes of interest were detected in two of the *V. diabolicus* strains (strains JBS-8-11-1 and TBS-8-11-1).

DISCUSSION

All of the non-*V. parahaemolyticus* environmental *Vibrionaceae* strains examined in this study were identical in appearance to *V. parahaemolyticus* on TCBS agar. TCBS agar is a medium commonly used in the isolation of clinical and environmental vibrios; however, only 67% of the presumptive *V. parahaemolyticus* colonies were confirmed by *recA* phylogenetic analysis (Gutierrez-West et al., 2013) to be *V. parahaemolyticus*. Clearly, results of TCBS agar plating and confirming physiological testing of the kind used here must be supported by further analysis to determine the identities of environmental strains.

The thermolabile hemolysin gene *tlh* has provided a high throughput and convenient means to determine *V. parahaemolyticus* numbers in samples, but this analysis is clearly subject to false positives arising from other *Vibrio* species. Unlike previous studies (Xie et al., 2005), the *tlh* sequence data recovered from non-*V. parahaemolyticus* strains indicate that *tlh* gene sequences in *V. diabolicus*, *V. tubiashii*-like, and *P. damselae* are highly similar to those in *V. parahaemolyticus*. This makes unambiguous species discrimination, even employing gene sequences, much more difficult. The mol% G+C content of *tlh* (47.6%) (Taniguchi et al., 1986) corresponds to the mol% G+C contents of these four *Vibrionaceae* species (43-49%) (Farmer and Janda, 2005), which may indicate that any horizontal transfer of *tlh* within this family occurred far enough in the past for genetic drift to eliminate clear-cut evidence of this process. Some previous reports of *tlh* in non-*V. parahaemolyticus* vibrios attributed its detection to false positive results (e.g. Croci et al., 2007). False positive PCR amplification arising from sample contamination is certainly possible, but true *tlh* amplicons may have been recovered instead. Additionally, *tlh* is highly similar to homologous hemolysin genes found in other *Vibrio* species; including, *V. harveyi (vhh)*, *V. tubiashii (vth)*, and *V. vulnificus (vvh)*. Wang et al. (2007) determined that DNA probes for *tlh* and *vhh* could be used interchangeably, thus demonstrating that *tlh* probing for *V. parahaemolyticus* lacks the necessary specificity. Using *tlh* as the sole marker for *V. parahaemolyticus* densities may lead to overestimation of total *V. parahaemolyticus* counts by including other *Vibrio* species carrying *tlh* or similar hemolysins (*V. harveyi*, *V. tubiashii, V. vulnificus*). The extent of this overestimation may be variable among different systems

and sample types. It appears that no virulence factor gene sequence can be used to detect *V. parahaemolyticus* quantitatively without additional, supporting analyses.

The thermostable direct hemolysin gene (*tdh*) and a T3SS2α gene, both typical of virulent *V. parahaemolyticus* strains, are also not restricted to this species. In fact, virulence features generally attributed to *V. parahaemolyticus* were common in environmental *Vibrionaceae* strains that were isolated from an estuary having negligible human impacts. The apparent rarity of thermostable hemolysin genes in environmental *V. parahaemolyticus* strains was refuted by Gutierrez-West et al. (2013). Results of the present study extend those observations as frequencies of these genes in environmental non-*V. parahaemolyticus* are also quite high. The *Vibrionaceae* species in which these toxin genes were detected also expand the list of species that carry genes correlated with pathogenicity. *tdh* sequences have been recovered from clinical strains of *Vibrio cholerae* non-O1, *Vibrio mimicus*, and *Vibrio hollisae* (Nishibuchi et al., 1996), but environmental strains also carry this gene. Non-*V. parahaemolyticus tdh* sequences recovered in this study were similar to those of *V. parahaemolyticus tdh*, with the exception of *tdh* from *V. diabolicus* CW-9-11-1, which was clearly divergent from the other sequences and might represent a variant of *tdh*. It seems likely that these genes were transferred from an origin taxon to other *Vibrio* species, including, but perhaps not exclusively *V. parahaemolyticus*.

Recent studies have established a correlation between T3SS2 and hemolysin genes in *V. parahaemolyticus*, with T3SS2α distribution correlated with the presence of *tdh* and T3SS2β with *trh* (Okada et al., 2009; Noriea et al., 2010). We found no correlation between the T3SS2α marker gene *vscC2* and the presence of *tdh*. *vscC2* was

detected at high frequency in environmental *Vibrionaceae*. The *vscC2* gene sequences from *V. parahaemolyticus* strains are highly similar to those from non-*V. parahaemolyticus* strains. This gene has been reported in other vibrios, including *V. cholerae* non-O1 and *V. mimicus* strains (Okada et al., 2009; 2010). Evidence of *vscC2* in *V. diabolicus, P. damselae, V. natriegenes, V. harveyi*, and in *V. tubiashii*-like strains greatly expands the known distribution of the T3SS2α gene *vscC2* in *Vibrionaceae*.

V. parahaemolyticus* hemolysin genes and T3SS2 genes have been detected previously in the highly pathogenic *Vibrio cholerae* non-O1 but also in vibrios that rarely, if ever, cause illness in humans, including *V. alginolyticus*, *V. mimicus* and *V. hollisae* (Terai et al., 1991; Nishibuchi et al., 1996; Xie et al., 2005; Gonzalez-Escalona et al., 2006; Okada et al., 2010; Gennari et al., 2012). None of the non-*V. parahaemolyticus* strains we isolated belong to a species implicated in human infections, being either pathogenic to marine fauna (*V. tubiashii*, *V. harveyi* and *P. damselae*) or considered to be nonpathogenic (*V. diabolicus* and *V. natriegens*). The *V. diabolicus* strains are of particular interest because this species has seldom been reported since its description by Raguenes et al. (1997). The frequent recovery of *V. diabolicus* strains from the pristine North Inlet estuary suggests that this species is more broadly distributed than previously thought and the finding of *V. parahaemolyticus*-like virulence factor genes in this species implicates it as a reservoir for these genes. Additionally, the high similarity of the *V. diabolicus* and *V. parahaemolyticus tdh* and *vscC2* sequences may indicate that these genes are readily transferred among these and other *Vibrionaceae* species.
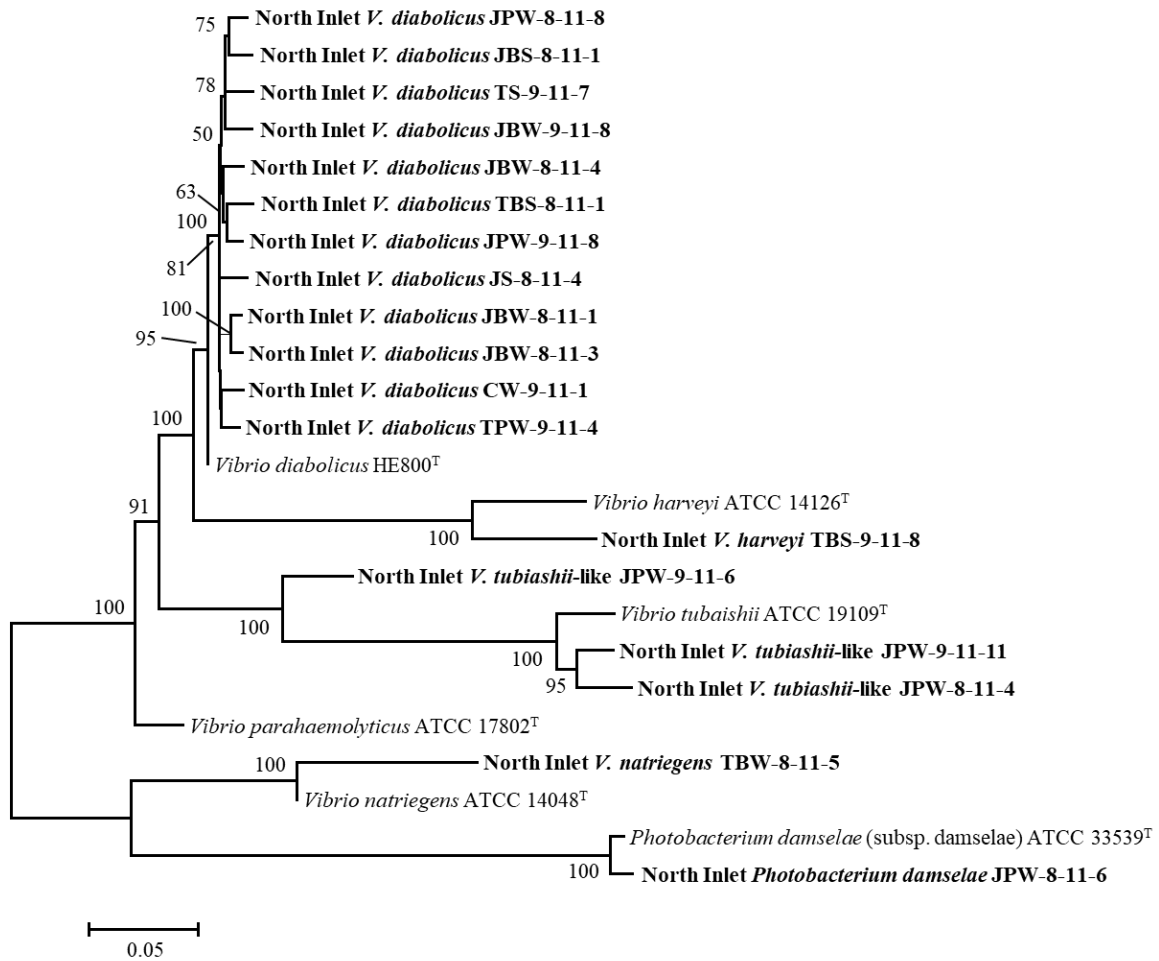
Figure 1.1. Maximum-likelihood phylogeny (Kimura 2 parameter model) of concatenated 16S rRNA gene, *recA, rpoA,* and *gyrB* sequences from environmental *Vibrionaceae* strains isolated from the North Inlet estuary. Bootstrap values represent 1000 replications and values less than 50 are not shown. Reference sequences were acquired from the NCBI GenBank

Table 1.1 Occurrence of genes and phenotypic characteristics considered diagnostic for virulence in *Vibrio parahaemolyticus* and also found in non-*V. parahaemolyticus* environmental *Vibrionaceae* strains

Strain designation	Species	*tlh*	*tdh*	*trh*	*vscC2*	colony appearance on TCBS agar
JBW 8:11 1	*V. diabolicus*	+	+	-	+	green
JPW 9:11 11	*V. tubiashii-* like	+	+	-	+	green
JPW 9:11 8	*V. diabolicus*	+	-	-	-	green
JPW 8:11 8	*V. diabolicus*	+	-	-	+	green
JPW 8:11 6	*P. damselae*	+	-	-	+	green
JPW 9:11 6	*V. tubiashii-* like	+	-	-	-	green
JBW 8:11 3	*V. diabolicus*	-	+	-	+	green
CW 9:11 1	*V. diabolicus*	-	+	-	+	green
JS 8:11 4	*V. diabolicus*	-	+	-	-	green
TPW 9:11 4	*V. diabolicus*	-	+	-	-	green
TBW 8:11 5	*V. natriegens*	-	-	-	+	green
JBW 8:11 4	*V. diabolicus*	-	-	-	+	green
JPW 8:11 4	*V. tubiashii-* like	-	-	-	+	green
JBW 9:11 8	*V. diabolicus*	-	-	-	+	green
TS 9:11 7	*V. diabolicus*	-	-	-	+	green
TBS 9:11 8	*V. harveyi*	-	-	-	+	green
JBS 8:11 1	*V. diabolicus*	-	-	-	-	green
TBS 8:11 1	*V. diabolicus*	-	-	-	-	green
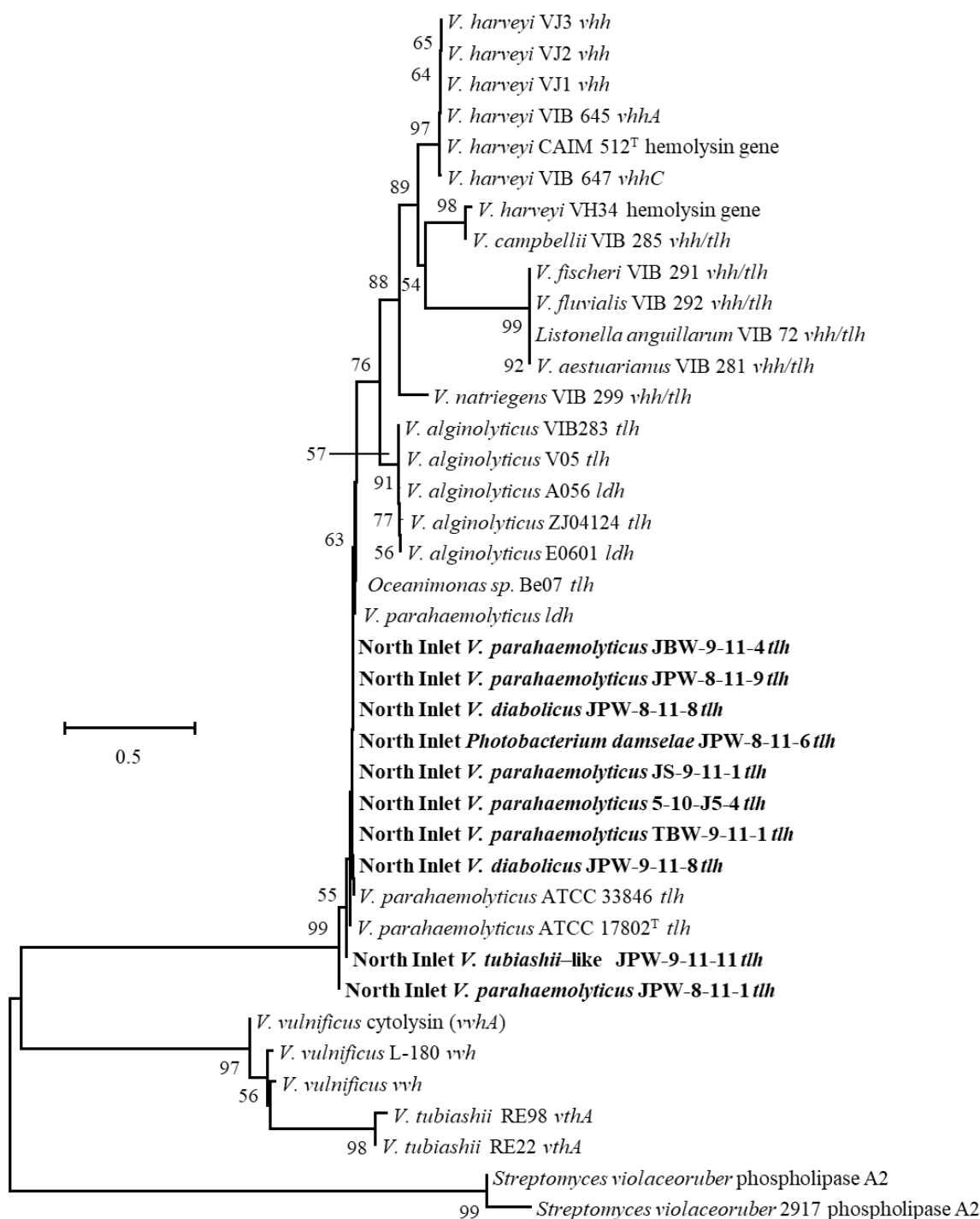
Figure 1.2. Maximum-likelihood phylogeny (Kimura 2 parameter model) of *tlh* gene sequences from environmental *Vibrionaceae* isolates. Gene sequences of hemolysins homologous to *tlh* are also shown. Bootstrap values represent 1000 replications and values less than 50 are not shown. Phospholipase A2 gene sequences from *Streptomyces violaceoruber* served as the outgroup. Reference sequences were acquired from the NCBI GenBank.
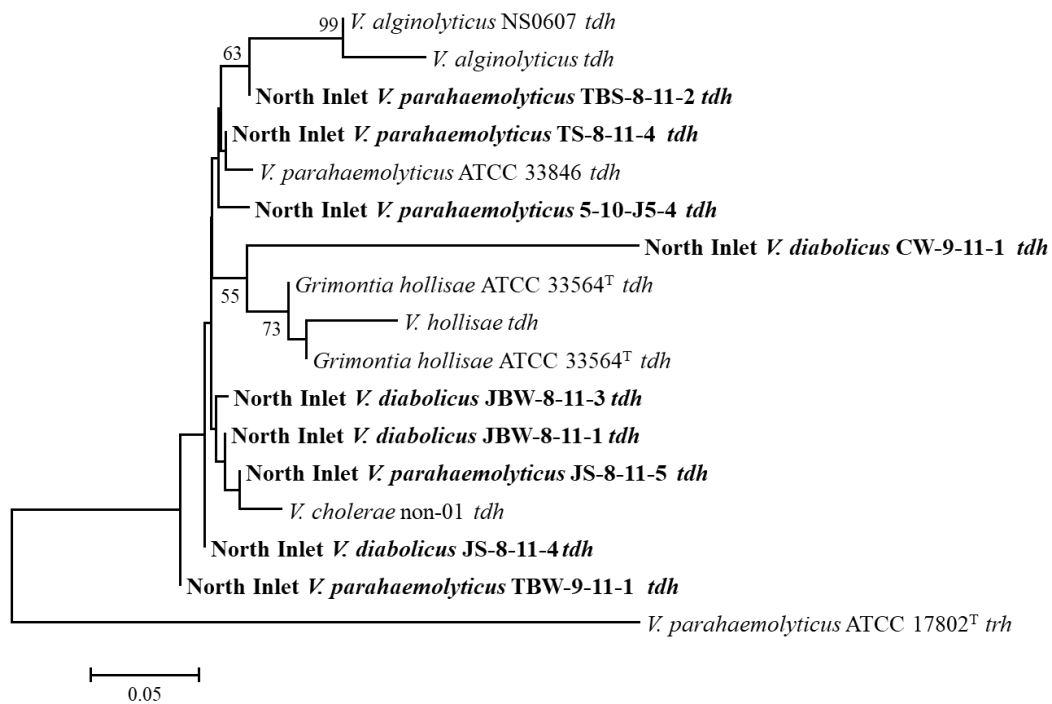
Figure 1.3. Maximum-likelihood phylogeny (Kimura 2 parameter model) of *tdh* gene sequences recovered from environmental *Vibrionaceae* isolates. Bootstrap values represent 1000 replications and values less than 50 are not shown. Reference sequences were obtained from the NCBI GenBank; the *trh* gene sequence of ATCC 17802[T] served as the outgroup.
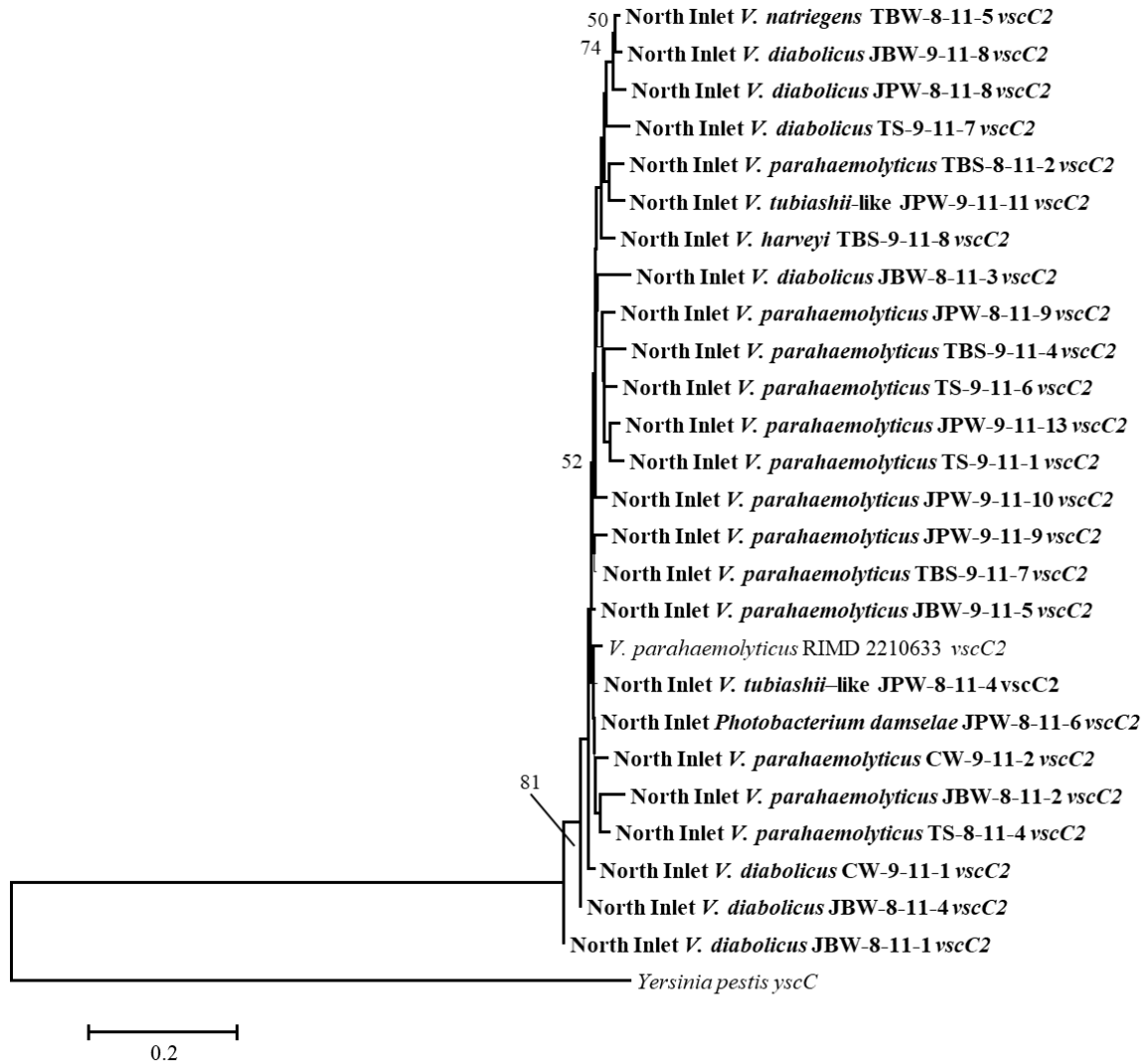
Figure 1.4. Maximum-likelihood phylogeny (Kimura 2 parameter model) of *vscC2* gene sequences recovered from environmental *Vibrionaceae* isolates. Bootstrap values represent 1000 replications; values less than 50 are not shown. The *yscC2* gene sequence of *Yersinia pestis* served as the outgroup and reference sequences were obtained from the NCBI GenBank

CHAPTER 2

THE HOT OYSTER: LEVELS OF VIRULENT *VIBRIO PARAHAEMOLYTICUS* STRAINS IN INDIVIDUAL OYSTERS [1]

ABSTRACT

Vibrio parahaemolyticus is the leading cause of seafood-associated gastroenteritis and is most commonly transmitted by raw oysters. Consequently, detection of virulent strains of this organism in oysters is a primary concern for seafood safety. *V. parahaemolyticus* levels were determined in 110 individual oysters harvested from two sampling sites in South Carolina, USA. The majority of oysters (98%) contained low levels of presumptive *V. parahaemolyticus*. However, two healthy oysters contained presumptive *V. parahaemolyticus* numbers that were unusually high. These two "hot" oysters contained levels of presumptive *V. parahaemolyticus* within the gills that were approximately 100-fold higher than the average for other oysters collected at the same date and location. Current *V. parahaemolyticus* detection practices require homogenizing a dozen oysters pooled together to determine *V. parahaemolyticus* numbers, a procedure that would dilute out *V. parahaemolyticus* in these "hot" oysters. This study demonstrates the variability of *V. parahaemolyticus* densities taken from healthy, neighboring individual oysters in the environment. Additionally, environmental *V. parahaemolyticus* isolates were screened for the virulence-related genes, *tdh* and *trh* using improved PCR primers and protocols. We detected these genes, previously thought to be rare in environmental isolates, in approximately half of the oyster isolates.

INTRODUCTION

Vibrio parahaemolyticus is a marine bacterium that can cause gastroenteritis in humans. Symptoms occur 24 to 72 h after ingestion of raw or undercooked seafood, usually oysters. More than 80,000 vibriosis cases occur in the United States each year (CDC, 2018); however, this may be an underestimate, considering that the majority of *V.*

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parahaemolyticus infections go unreported. *V. parahaemolyticus* occurs naturally in warm, brackish coastal environments and elevated cell densities are observed in surficial sediment (West, 2012), fiddler crab burrows (Gamble and Lovell, 2011), and bivalve mollusks (DePaola et al., 1990, Bisha et al., 2012). Detection of virulent *V. parahaemolyticus* in oysters is a primary concern for seafood safety.

Diagnostic monitoring of virulent *V. parahaemolyticus* relies on PCR detection of virulence-related hemolysin genes. The thermolabile hemolysin gene (*tlh*) is used as an indicator gene for *V. parahaemolyticus* (Taniguchi et al., 1986, Bej et al., 1999, DePaola et al., 2003, FDA, 2005). *tlh* encodes a phospholipase A2 (Zhang and Austin, 2005), but is not thought to contribute substantially to *V. parahaemolyticus* pathogenicity (Bisha et al., 2012). This gene was thought to be specific to *V. parahaemolyticus* but recent studies have found *tlh* in additional *Vibrio* species (Xie et al., 2005, Klein et al., 2014). Nonetheless, PCR amplification of *tlh* remains the most commonly used molecular method to identify *V. parahaemolyticus*. Two additional hemolysin genes, *tdh* and *trh*, have been correlated with virulent strains of *V. parahaemolyticus*. These genes encode the thermostable direct hemolysin (TDH) and the homologous *tdh*-related hemolysin (TRH). *tdh* and *trh* encode porins that insert into the membranes of host cells and cause a nonspecific efflux of divalent cations and water molecules (Raimondi et al., 2000, Yanagihara et al., 2010, Broberg et al., 2011, Ohnishi et al., 2011), which is consistent with involvement in *V. parahaemolyticus* pathogenesis.

tdh and *trh* were thought to occur only rarely in environmental *V. parahaemolyticus* strains (i.e. 1-2% of strains) (e.g. Pillot-Robert et al., 2004, Baker-Austin et al., 2008); however, recent studies employing improved detection methods have

found *tdh* and *trh* in roughly half of environmental *V. parahaemolyticus* strains isolated from sediment, water, and shrimp (Velazquez-Roman et al., 2012, Gutierrez-West et al., 2013, Klein et al., 2014). The distributions of *tdh* and *trh* in *V. parahaemolyticus* occurring in the Eastern oyster, *Crassostrea virginica*, has yet to be determined **using these improved methods**. The purpose of this study was to determine frequencies of the virulence-related genes *tdh* and *trh* within *Vibrio* isolates recovered from *C. virginica* oysters. Strong variability in *Vibrio* densities was observed between individual oysters, as well as among tissues of individual oysters.

MATERIALS AND METHODS

Sampling Sites, Oyster Harvesting and Oyster Processing

All environmental strains used in this study were isolated from two sampling sites: North Inlet Estuary at the Belle W. Baruch Institute near Georgetown, SC (33°20'N, 79°12'W) and Whale Branch near Beaufort, SC (32°30'N, 80°47'W). The pristine North Inlet-Winyah Bay Estuary is part of the National Estuarine Research Reserve System and consists of 18,916 acres of which more than 90% is in its natural state. No commercial harvesting of oysters is allowed in North Inlet, which served in this study as a comparison site. Whale Branch is a site of commercial oyster harvest.

C. virginica oysters were harvested May-October 2014 following practices commonly used by commercial oyster harvesters. Oysters were either collected immediately after low tide exposure or while still submerged; oysters in the high intertidal that were subjected to elevated temperatures during low tide exposure were not sampled. Discrete individuals were sampled, as well as oysters within clusters. All harvested oysters were larger than 6 cm from hinge to lip and were inspected for signs of

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boring sponge infection and weakness of the adductor muscle. Oysters showing any
overt sign of disease were not included in this study. Water samples were collected in
sterile 50 mL containers. After harvesting, the oysters and water samples were placed on
ice and immediately transported back to Columbia, SC for processing. All oysters and
water samples were processed within four hours of harvesting.

Oysters were scrubbed, shucked with a sterile knife, and weighed to determine
their fresh weight. Oysters were homogenized individually with sterile mortars and
pestles in order to observe differences in *Vibrio* populations between oysters. Oyster
homogenate was diluted with phosphate buffered saline (PBS) (400 mM NaCl, 1.75 mM
$NaPO_4$, pH 7.4), and plated on Thiosulfate Citrate Bile salts Sucrose (TCBS) agar (BD,
NJ) without the use of enrichment. TCBS plates were incubated at 37ºC for 48 h. To
avoid cross contamination, all implements were re-sterilized via ethanol flaming after
each oyster.

Vibrio distributions within the oyster were observed via dissection of selected
individuals. These oysters were scrubbed and shucked as described above. The oyster
gut and gills were weighed, homogenized separately, diluted with PBS and plated on
TCBS agar. The rest of the oyster (hereafter referred to as "oyster meat") was also
homogenized, diluted and plated separately. One mL of the oyster mantle fluid was
extracted using a sterile syringe, diluted with PBS, and plated on TCBS agar. Water
samples (50 mL) were filtered onto sterile 0.45 µm pore size polycarbonate membranes,
which were then placed in PBS, vortexed and aliquots plated on TCBS agar. These
TCBS plates were also incubated at 37ºC for 48 h.

Colony counts of both green (sucrose non-fermenters - typical appearance of *V. parahaemolyticus* and/or *Vibrio vulnificus*) and yellow colonies (sucrose fermenters - typical appearance of *Vibrio alginolyticus* and/or *Vibrio cholerae*) (DePaola and Kaysner, 2004) on TCBS agar were taken after the 48 h incubation. Colony forming units (CFU) g^{-1} oyster fresh weight was calculated. CFU g^{-1} fresh weight of oyster meat, gills, and guts were determined for the dissected oysters; as well as CFU mL^{-1} of oyster mantle fluid. In addition to *V. parahaemolyticus* counts from individual oysters, we also calculated the CFU g^{-1} if we had combined and homogenized all of the oysters from each sampling trip, following standard US Food and Drug Administration protocol (DePaola and Kaysner, 2004). Selected well-isolated colonies were streaked onto saline Luria Agar (SLA; per L; 10 g tryptone, 5 g yeast extract, 27 g NaCl, 15 g Bacto Agar) for purification and further characterization. Both green and yellow colonies were purified and routinely cultivated on SLA. To determine if there was one part of the oyster that contained significantly higher presumptive *V. parahaemolyticus*, a Ryan-Einot-Gabriel-Welsch (REGW) range test was performed employing the statistical software package SPSS version 20 (IBM, Armonk, NY).

PCR Protocols and Procedures

Colonies were screened for the *V. parahaemolyticus* hemolysin genes, *tlh*, *tdh*, and *trh*. Isolates not demonstrating the appearance of *V. parahaemolyticus* on TCBS agar (yellow colonies) were also selected for PCR screening due to reports that *V. parahaemolyticus* virulence genes can occur in non-*V. parahaemolyticus* species (Xie et al., 2005, Gonzalez-Escalona et al., 2006, Okada et al, 2010, Klein et al., 2014). Isolates were grown overnight at 37ºC in saline Luria broth (SLB) and crude DNA was extracted

by boiling the cells in distilled water for 15 min. All PCR reactions were completed

within three days of DNA extraction and used 1 µl of boiled DNA extract per reaction.

A PCR reaction targeting the housekeeping gene *recA* (recombinase A) was

performed on all isolates to confirm quality of the crude DNA extracts. All reactions

were carried out in 25 µl volumes using 1 µl of boiled DNA extract and *Taq* DNA

polymerase (Qiagen, Valencia, CA). *recA* (~790 bp) amplification employed the primers

and protocols of Thompson et al. (2005). PCR products were resolved on 1.5% agarose

gels and arbitrarily selected amplicons were sequenced. Sequencing was performed by

Eurofins Genomics (Louisville, KY) using an ABI Prism 3730 DNA analyzer.

Sequencing of the *recA* gene is sufficient to identify members of the *Vibrionaceae* family

at the level of species (Thompson et al., 2005) while *Vibrionaceae* 16S rRNA gene

sequences are too similar to support species-level resolution. PCR amplification of the

recA gene served as both a DNA quality control measure and a test for species, as a

selected few were sent out for sequencing**.** Sequences were edited and Maximum-

likelihood phylogeny were constructed using the Kimura 2 parameter model with Mega

version 7 (Tamura et al., 2015). Sequence data of reference *Vibrionaceae* species were

obtained from the NCBI GenBank database. *recA* sequence data determined in this study

were submitted to the NCBI GenBank and assigned the accession numbers: KU306238-

KU306262.

All PCR reactions targeting *tlh, tdh* and *trh* were also carried out in 25 µl volumes

using *Taq* DNA polymerase and 1 µL of boiled DNA extract. The PCR thermal cycling

program, conditions, and primers of Bej et al. (1999) were employed for amplification of

tlh (450 bp), the most commonly used molecular marker for *V. parahaemolyticus*. PCR

amplifications of the virulence-related *tdh* and *trh* genes used the primers and protocols

of Gutierrez-West et al. (2013) and were performed in separate reactions. PCR products

were resolved on 1.5% agarose gels. A one tailed two proportion z test was used to

determine if vibrios derived from independent sample types (water vs. oyster)

significantly differed in their virulence gene content. This test is appropriate for

comparisons of percentages or proportions of two independent groups, even when sample

sizes differ. A significance level of 0.05 was used.

RESULTS

Nine sampling trips were made between May and October 2014, five to North

Inlet estuary near Georgetown and four to Whale Branch in Beaufort. Six to 18 oysters

were collected during each sampling trip, totaling 110 oysters harvested throughout 2014.

These oysters were homogenized individually and plated on TCBS agar so that the

variability of green colonies (hereafter referred to as presumptive *V. parahaemolyticus*)

between individual oysters could be determined. Green colonies on TCBS are typically

confirmed to be *V. parahaemolyticus* via sequencing of specific housekeeping genes

(Gutierrez-West et al., 2013, Klein et al., 2014). Sixty-six oysters were harvested from

North Inlet; 44 were harvested from Whale Branch. The levels of presumptive *V.

parahaemolyticus* in 97% of individual oysters ranged from 0 CFU g^{-1} – 7,610 CFU g^{-1},

with high variability between individual oysters (average of 632 CFU g^{-1}, standard

deviation 1,054). High standard deviations reflect the high oyster-to-oyster variability in

V. parahaemolyticus densities. Ninety seven percent (107/110) of oysters contained

presumptive *V. parahaemolyticus* levels considered to be safe for consumption, that is,

less than 10^4 CFU g^{-1} (FDA, 2011). Two Whale Branch oysters (numbers 47 and 96)

contained levels higher than 10^4 CFU g^{-1} (Figure 2.1). Another Whale Branch oyster, number 104, contained 9,459 presumptive *V. parahaemolyticus* CFU g^{-1}; only 541 CFU g^{-1} below the maximum level considered safe for consumption.

Standard methods (DePaola and Kaysner, 2004) suggest homogenizing a dozen oysters to determine *V. parahaemolyticus* densities. This method would have diluted out *V. parahaemolyticus* densities from the "unsafe" oysters 47 and 96. If oyster 47 was pooled and homogenized with the other individuals sampled from the same date and oyster bed, the result would have been 1,343 CFU g^{-1}, which is well below the safe limit suggested by the FDA. The same dilution effect is seen with oyster 96; the pooled homogenate would have yielded 2,924 CFU g^{-1} (Figure 2.1). Combining many individuals for analysis does not reveal the high variability in *V. parahaemolyticus* densities between oysters and dilutes out potentially dangerous strains.

Oysters 47 and 96 were harvested when the water temperature was approximately 28ºC (June and August, respectively); oyster 104 was harvested in late September when the water temperature was approximately 24ºC. The highest levels of presumptive *V. parahaemolyticus* from oysters 47 and 96 were found in the gills; 32,500 CFU g^{-1} and 37,179 CFU g^{-1}, respectively. The presumptive *V. parahaemolyticus* densities in gills from these oysters are approximately 100-fold higher than the presumptive *V. parahaemolyticus* densities found in other dissected oyster gills (Figure 2.2A). Oyster 104 contained the highest numbers of presumptive *V. parahaemolyticus* within the mantle fluid (Figure 2.2B).

A total of 55 oysters were selected for dissections. With the exception of the anomalous "hot" oysters, presumptive *V. parahaemolyticus* densities within oyster gills (average of 293 CFU g^{-1}, standard deviation 982) and meat (average of 355 CFU g^{-1}, standard deviation 736) were lower than in the oyster mantle fluid (average of 1132 CFU g^{-1}, standard deviation 1,106) and gut (average of 964 CFU g^{-1}, standard deviation 1,427) (Figure 2.2A-D). However, even in the oyster gills and meat, standard deviations were high, meaning oyster-to-oyster variability in presumptive *V. parahaemolyticus* counts was also high (REGW Test- Table 2.1).

Only well isolated colonies recovered from oysters were streaked to purity and employed in PCR analysis. Colonies demonstrating the typical appearance of *V. parahaemolyticus* on TCBS agar (green) and colonies not appearing to be *V. parahaemolyticus* on TCBS agar (yellow) were selected for PCR screening; a total of 379 colonies were screened. Most of these (253) produced green colonies on TCBS agar; the other 126 produced yellow colonies. Most *Vibrio* strains (225) selected for PCR were isolated from North Inlet oysters; the other 154 *Vibrio* isolates were recovered from Whale Branch oysters.

Partial *recA* sequences were successfully amplified from all strains. This step ensured that the boiled extraction method produced DNA of adequate quality and concentration for use in further PCR reactions. Sequence data for 25 arbitrarily selected *recA* amplicons were obtained (Figure 2.3). Eight of these strains were identified as *Vibrio harveyi* based on their placement in the *recA* phylogenetic tree. *V. harveyi* varies in its utilization of sucrose (Farmer and Janda, 2005); five demonstrated sucrose fermentation (yellow colonies) on TCBS agar and three did not (green colonies). Three

Photobacterium damselae strains were recovered, as well as one *Vibrio brasiliensis* strain. *V. brasiliensis* was first isolated from scallop larvae and ferments sucrose; this strain produced yellow colonies on TCBS agar. *P. damselae* does not ferment sucrose (Farmer and Janda, 2005), which is consistent with its appearance on TCBS agar. Thirteen strains were confirmed to be *V. parahaemolyticus.* Eleven of these strains appeared green on TCBS agar, typical for *V. parahaemolyticus*; two strains appeared yellow on TCBS agar reflecting fermentation of sucrose to acidic end products. Out of the 17 presumptive *V. parahaemolyticus* strains (green colonies) chosen for *recA* sequencing, the majority (65%) were confirmed *V. parahaemolyticus*.

The 379 *Vibrio* isolates from oysters were then screened for the *V. parahaemolyticus* hemolysin genes, *tlh*, *tdh*, and *trh*. North Inlet oyster *Vibrio* isolates contained *tlh*, *tdh*, and *trh* at frequencies of 72%, 53%, and 58%, respectively (Table 2.2). Whale Branch oyster *Vibrio* isolates also contained relatively high frequencies of *tlh* (82%), *tdh* (38%), and *trh* (51%) (Table 2.3). The *V. parahaemolyticus* virulence-related hemolysin genes were frequently amplified from presumptive non-*V. parahaemolyticus* isolates, that is, isolates producing yellow colonies on TCBS agar. During October in North Inlet oysters, *tlh*, *tdh*, and *trh* were detected in 100%, 86%, and 100%, respectively, of yellow colonies. The high detection frequency of the "species specific" marker gene *tlh* in non-*V. parahaemolyticus* vibrios (as well as the absence of this marker from some authentic *V. parahaemolyticus* strains) (Gutierrez-West et al., 2013, Klein et al., 2014) indicate that it is insufficient for identification of *V. parahaemolyticus***.** *Vibrio* isolates recovered from the surrounding water in North Inlet were also screened for the three hemolysin genes. These 153 water isolates contained *tlh*, *tdh*, and *trh* at frequencies

of 58%, 22%, and 35%, respectively (Table 2.4). There was no significant difference in the frequency of *tlh* detected from oyster or water *Vibrio* isolates in North Inlet. However, the frequencies of *tdh* and *trh* were significantly higher (p values <0.001) in oyster isolates, compared to water isolates.

DISCUSSION

Standard methods to enumerate *Vibrio* populations within oysters usually require pooling a dozen individuals and homogenizing them together (DePaola and Kaysner, 2004). These protocols do not allow evaluation of oyster to oyster variability of *Vibrio* levels, which was a goal of this study. Kaufman et al. (2003) also examined variability between individuals and found that >90% of oysters sampled contained 200-2,000 CFU g^{-1} of *V. parahaemolyticus*. However, this group sampled one oyster with approximately 20,000 *V. parahaemolyticus* CFU g^{-1}; which was designated as a "hot" oyster. They hypothesized that these "hot" oysters, which occur at low frequency, may explain the sporadic nature of *V. parahaemolyticus* infections. Our results are consistent with this "hot oyster hypothesis." During 2014, we harvested two oysters we designate as "hot" due to their presumptive *V. parahaemolyticus* population densities being approximately 20 times higher than the average. These "hot" oysters were the only individuals out of 110 that would not be considered safe to consume with minimal cooking, according to US FDA standards (2011), with presumptive *V. parahaemolyticus* levels in these oysters exceeding 10^4 CFU g^{-1}. The specific mechanisms resulting in the "hot" oyster have yet to be determined, but may be related to oyster physiological stress due to temperature, salinity, disease, or other factors. It should be noted that the two "hot" oysters described here showed no signs of disease and were collected under conditions that would not be

considered overtly stressful. Recent studies (Jones et al., 2016) have suggested that low

tide exposure of oysters can result in an increase of *V. parahaemolyticus* and *V. vulnificus*

levels. All of the oysters we sampled were collected immediately after low tide exposure

or while still submerged; none were subjected to warm ambient air temperatures during

low tide exposure. The "hot" oysters' gills contained the highest levels of presumptive *V.*

parahaemolyticus. We determined that oysters harvested from the same oyster bed, at

the same date and time, contained extremely variable levels of *V. parahaemolyticus*.

While most of these densities remained well below the "safe limits" recommended by the

FDA, there was still high variability, as seen in the high standard deviations in the TCBS

counts data.

How a healthy oyster becomes "hot" by accumulating more *V. parahaemolyticus*

than neighboring oysters is not yet known. Further studies on the "hot" oyster are

needed. Perhaps mesocosm experiments would help to illuminate the mechanism behind

the "hot" oyster. Also, further studies examining how to lower *V. parahaemolyticus*

densities from "hot" oysters could be useful, especially to commercial harvesters. "Hot"

oysters are indistinguishable from other individuals, as they do not show any overt signs

of disease, so preventative measures used by commercial harvesters could stop these

"hot" oysters from reaching costumers. When Alaskan oysters produced cases of

vibriosis in consumers, oyster harvesters in Prince William Sound started lowering their

oyster cages below the thermocline ($<10^{\mathrm{o}}$ C) (Martinez-Urtaza et al., 2010). This

resulted in reduction of *V. parahaemolyticus* densities by 1 log (Martinez-Urtaza et al.,

2010). This wouldn't be possible in the SC systems we sampled, but other preventative

measures could be possible.

The infectious dose of *V. parahaemolyticus* is estimated to be 10^7 to 10^8 cells (Sanyal and Sen, 1974); however in a recent Alaskan *V. parahaemolyticus* outbreak, the dose was determined to be significantly lower (10^3 to 10^4 cells) (Martinez-Urtaza et al., 2010). This low infectious dose could have been due to an especially virulent *V. parahaemolyticus* strain, as 100% of Alaskan oysters tested positive for the *tdh* gene (Martinez-Urtaza et al., 2010). If the infectious dose of *V. parahaemolyticus* is as low as 10^3 cells, then the "hot" oysters found in SC would certainly be capable of causing disease, especially given the high detection of both *tdh* and *trh* genes within SC oysters. If the infectious dose is as high as 10^8 cells, then these oysters would not be expected to produce disease.

Most of the presumptive *V. parahaemolyticus* isolates we selected for *recA* analysis were later confirmed to be *V. parahaemolyticus*. Sequencing of housekeeping genes (excluding 16S rRNA genes for *Vibrionaceae*) remains the most reliable way to identify *Vibrio* species. Biochemical identifiers and molecular markers can be used as presumptive tests in strain identification, but these methods are not perfect. The content of *tlh*, *tdh*, or *trh* alone is also insufficient to confirm *V. parahaemolyticus* identity due to the occurrence of these genes in other *Vibrionaceae* (Xie et al., 2005, Gonzalez-Escalona et al., 2006, Okada et al, 2010; Klein et al., 2014). The majority of our green colonies (presumptive *V. parahaemolyticus*) were confirmed *V. parahaemolyticus*. We found no *V. vulnificus* during our sequencing efforts, even though *V. vulnificus* and *V. parahaemolyticus* can co-occur and look similar on TCBS agar (Jones et al. 2016); the systems we sampled have higher salinities than would favor growth of *V. vulnificus*. All

three *V. parahaemolyticus* virulence-related genes that we examined were routinely found in presumptive non-*V. parahaemolyticus* isolates (yellow colonies on TCBS agar).

Our findings support other recent studies in that *V. parahaemolyticus* virulence factor genes occur at relatively high frequencies in environmental isolates (Velazquez-Roman et al., 2012, Gutierrez-West et al., 2013, Klein et al., 2014). We detected more *tdh* and *trh* from oyster-derived vibrios than water-derived vibrios or sediment-derived vibrios from North Inlet estuary (Gutierrez-West et al., 2013). This could indicate that there is a positive selection for *tdh* and/or *trh*-containing *Vibrio* strains within the oyster niche. Perhaps these hemolysin genes (or other genetic loci linked to the hemolysin genes) aid in the persistence of vibrios within oyster tissues, acquisition of nutrients, or evasion of oyster hemocytes. Further study is required to determine if *tdh* and/or *trh* play a role in environmental persistence of *V. parahaemolyticus*.

Detection of hemolysin genes, especially *tdh* and *trh*, at such high frequencies in *C. virginica* oysters further confirms that these genes are not rare in environmental *Vibrio* isolates. *trh* and *tdh*, the most commonly cited virulence factors of *V. parahaemolyticus*, occurred in the majority of *Vibrio* isolates recovered from oysters. Perhaps *V. parahaemolyticus* gastroenteritis is induced after ingestion of a "hot" oyster, that is, an oyster enriched in vibrios to levels far exceeding the average. Since "hot" oysters occur so infrequently (1-3%), *V. parahaemolyticus* gastroenteritis remains a sporadic illness that is somewhat difficult to predict.
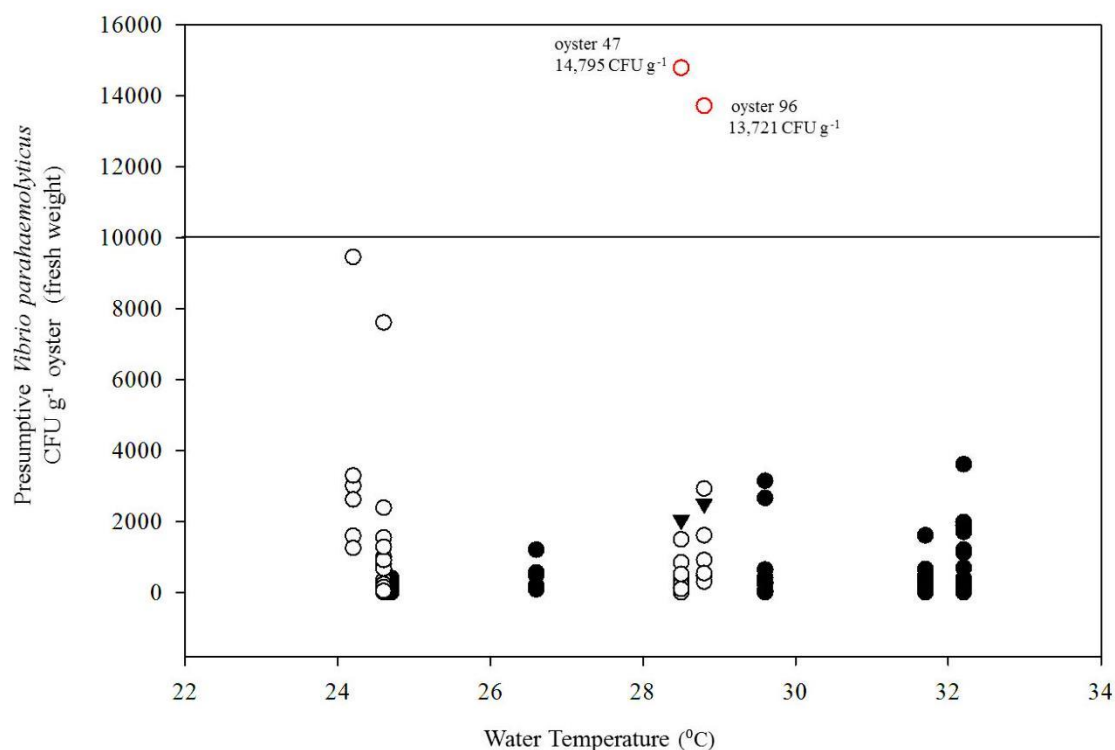
Figure 2.1. Presumptive *Vibrio parahaemolyticus* levels in individual *Crassostrea virginica* oysters. Closed circles are oysters from North Inlet Estuary and open circles are oysters from Whale Branch. Red open circles are the two "hot" oysters from Whale Branch. The horizontal line indicates the maximum level of *V. parahaemolyticus* (10,000 CFU g^{-1}) considered safe for consumption with minimal cooking by the FDA. Triangles indicate the *V. parahaemolyticus* levels (per g) if oysters were pooled and homogenized together, as standard methods suggest. This was only determined for the two sampling trips that reared "hot" oysters
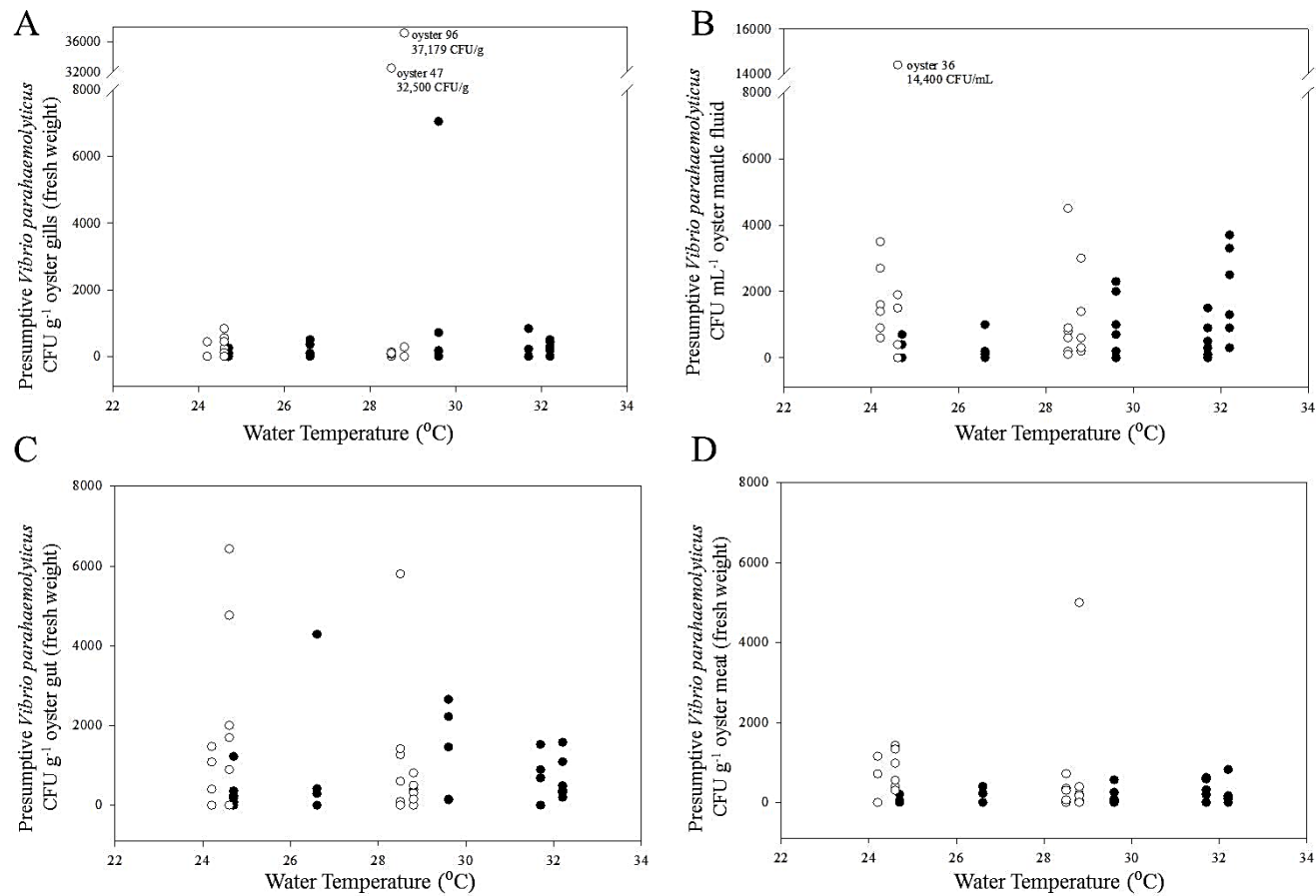
Figure 2.2. Presumptive *Vibrio parahaemolyticus* levels in dissected *Crassostrea virginica* oysters. Closed circles are oysters from North Inlet Estuary and open circles are oysters from Whale Branch. Dissected sections of the oysters include (A) the oyster gills, (B) the oyster mantle fluid, (C) the oyster gut, and (D) the oyster meat. The oyster's gut, gills, and mantle fluid were dissected out and all other tissues were considered oyster meat.

Table 2.1. Results of REGW range test for presumptive *Vibrio parahaemolyticus*. Homogeneous and statistically significant subsets are displayed. Values presented are based on observed means. Alpha = 0.05.

Homogeneous Subsets

Count

Ryan-Einot-Gabriel-Welsch F[a]

Location	N	Subset 1	Subset 2	Subset 3
Gills	52	292.80		
Meat	52	355.10	355.10	
Gut	52		964.20	964.20
Mantle Fluid	52			1132.69
Sig.		.969	.059	.794

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 2017252.306.

   a. Alpha = .05.
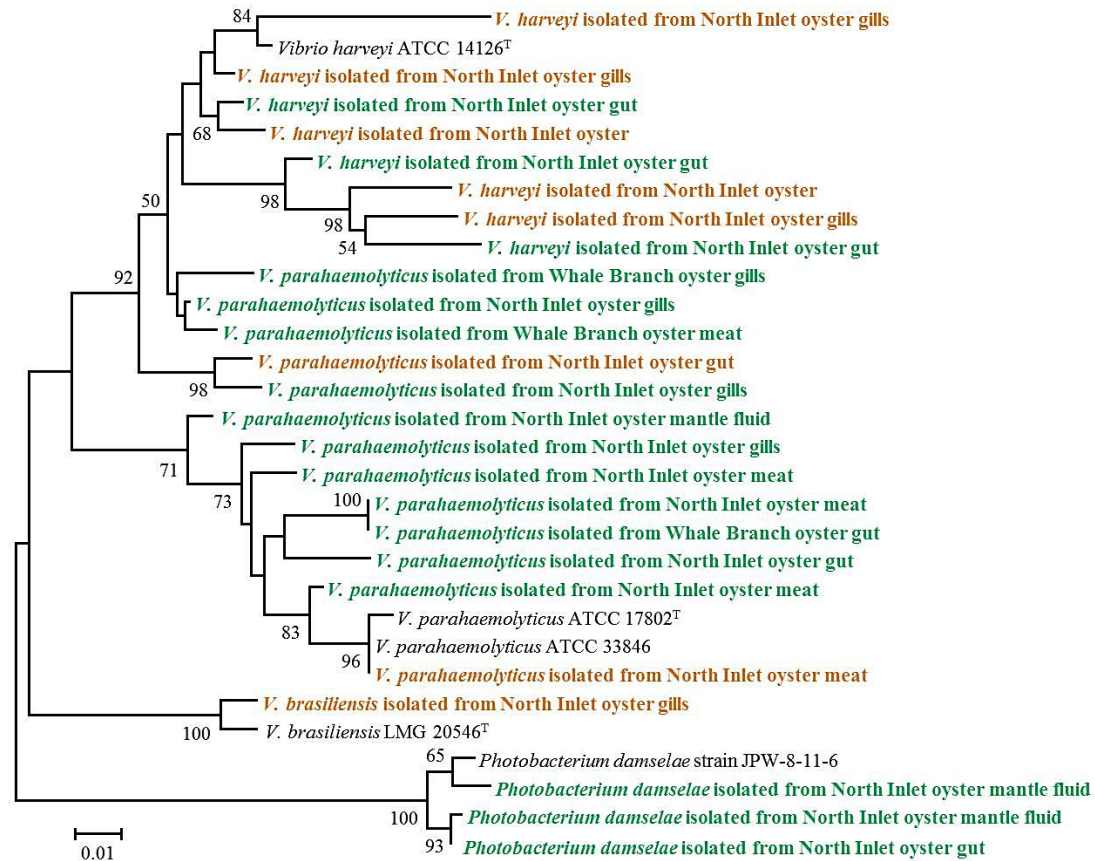
Figure 2.3. Maximum-likelihood phylogeny (Kimura 2-parameter model) of *recA* gene sequences recovered from oyster *Vibrio* strains. The bootstrap values represent 1,000 replications, and values of less than 50 are not shown. The reference sequences were acquired from NCBI GenBank. Green font indicates the isolate does not ferment sucrose (is green on TCBS agar); orange font indicates the isolate does ferment sucrose (is yellow on TCBS agar)

Table 2.2. Sampling date, physiological appearance on TCBS agar and *Vibrio parahaemolyticus* virulence-related gene distributions in *Vibrio* isolates recovered from North Inlet *Crassostrea virginica* oysters.

Date of sampling	Colony Apperance on TCBS agar	No. Isolates Chosen for PCR Screening	Percentage of…		
			tlh detected	*tdh* detected	*trh* detected
5/8/2014	green	27	78% (21/27)	60% (16/27)	26% (7/27)
5/8/2014	yellow	23	96% (22/23)	52% (12/23)	63% (17/27)
5/8/2014	green + yellow	50	86% (43/50)	56% (28/50)	48% (24/40)
6/27/2014	green	36	50% (18/36)	42% (15/36)	44% (16/36)
6/27/2014	yellow	12	67% (8/12)	58% (7/12)	50% (6/12)
6/27/2014	green + yellow	48	54% (26/48)	46% (22/48)	46% (22/48)
7/10/2014	green	28	86% (24/28)	50% (14/28)	21% (6/28)
7/10/2014	yellow	17	71% (12/17)	59% (10/17)	65% (11/17)
7/10/2014	green + yellow	45	80% (36/45)	53% (24/45)	38% (17/45)
7/22/2014	green	38	58% (22/38)	42% (16/38)	79% (30/38)
7/22/2014	yellow	19	58% (11/19)	47% (9/19)	84% (16/19)
7/22/2014	green + yellow	57	58% (33/57)	44% (25/57)	81% (46/57)
10/10/2014	green	11	82% (9/11)	82% (9/11)	73% (8/11)
10/10/2014	yellow	14	100% (14/14)	86% (12/14)	100% (14/14)
10/10/2014	green + yellow	25	92% (23/25)	84% (21/25)	88% (22/25)
total samplings	green	140	67%(94/140)	50%(70/140)	48%(67/140)
total samplings	yellow	85	79%(67/85)	59%(50/85)	75%(64/85)
total samplings	green + yellow	225	72%(161/225)	53%(120/225)	58%(131/225)

Table 2.3. Sampling date, physiological appearance on TCBS agar and *Vibrio parahaemolyticus* virulence-related gene distributions in *Vibrio* isolates recovered from Whale Branch *Crassostrea virginica* oysters.

Date of sampling	Apperance on TCBS agar	No. Isolates Chosen for PCR Screening	*tlh* detected	Percentage of... *tdh* detected	*trh* detected
5/23/2014	green	43	88% (38/43)	47% (20/43)	35% (15/43)
5/23/2014	yellow	24	75% (18/24)	50% (12/24)	38% (9/24)
5/23/2014	green + yellow	67	84% (56/67)	48% (32/67)	36% (24/67)
6/23/2014	green	47	72% (34/47)	63% (19/30)	90% (27/30)
6/23/2014	yellow	8	88% (7/8)	13% (1/8)	75% (6/8)
6/23/2014	green + yellow	55	75% (41/55)	36% (20/55)	60% (33/55)
9/28/2014	green	23	91% (21/23)	13% (3/23)	61% (14/23)
9/28/2014	yellow	9	89% (8/9)	33% (3/9)	89% (8/9)
9/28/2014	green + yellow	32	91% (29/32)	19% (6/32)	69% (22/32)
total samplings	green	113	82% (93/113)	37% (42/113)	50% (56/113)
total samplings	yellow	41	80% (33/41)	39% (16/41)	56% (23/41)
total samplings	green + yellow	154	82% (126/154)	38% (58/154)	51% (79/154)

Table 2.4. Physiological appearance on TCBS agar and *Vibrio parahaemolyticus* virulence-related gene distributions in *Vibrio* isolates recovered from North Inlet water.

Apperance on TCBS agar	No. Isolates Chosen for PCR Screening	*tlh* detected	Percentage of... *tdh* detected	*trh* detected
green	106	68% (72/106)	21% (22/106)	34% (36/106)
yellow	47	38% (18/47)	26% (12/47)	36% (17/47)
green + yellow	153	58% (90/153)	22% (34/153)	35% (53/153)

CHAPTER 3

PATHOGENICITY ISLAND OCCURRENCE IN ENVIRONMENTAL *VIBRIO* STRAINS[1]

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 a PAI found in an environmental *Vibrio parahaemolyticus* strain, as well as a genomic fitness island found in a *Vibrio diabolicus* strain. All islands had markedly different GC profiles than the rest of the genome, indicating that these islands were acquired via lateral gene transfer. Genes on the PAI 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). Toxin-antitoxin (TA) genes were found on the *V. diabolicus* fitness island and on the *V. parahaemolyticus* PAI.

INTRODUCTION

Some *Vibrio* species can cause illnesses in humans, with an estimated 80,000 cases occurring annually in the United States (Scallan et al., 2011; CDC, 2018). 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). Pathogenesis of this species is complex, and while some virulence factor genes have been implicated, the mechanisms underlying *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 *V. parahaemolyticus*

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(Makino et al., 2003). 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 mbp (Pipes et al., in preparation), 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; 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. parahaemolyticus* (e.g: Makino et al., 2003; Wang et al., 2006; Sugiyama et al., 2008). 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., 2012).

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 genomic islands found in environmental *Vibrio* strains: a PAI within a *V. parahaemolyticus* 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 and Whole Genome Sequencing

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). *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).

Genomic DNA was isolated through the Wizard Genomic DNA Purification kit following the protocol for Gram negative organisms (Promega, Madison, WI). 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). 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: PKQA00000000, PKPY00000000, and PKPZ00000000.

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. DNAPlotter was used to visualize the circular chromosomes of the *Vibrio* strains (Carver et al., 2009).

RESULTS AND DISCUSSION

The defining feature of a PAI is the presence of virulence genes, or genes employed in pathogenesis. Chromosomal regions that appear to be PAIs but lack virulence factor genes are considered to be genomic, metabolic, or fitness islands (Schmidt and Hensel, 2004). There is much controversy surrounding the occurrence of PAIs in non-pathogenic or non-clinical (environmental) strains. Some groups argue that PAIs only occur in pathogenic, disease-causing clinical strains and that PAIs found in non-clinical (environmental) isolates should be referred to as fitness islands (e.g. Hacker et al., 1997; Hacker and Carniel, 2000). Other groups state that as long as the genomic island contains at least one virulence factor gene, it is considered a PAI, regardless of the

origin of the strain (e.g. Schmidt and Hensel, 2004; Hasan et al., 2010; Dobrindt et al., 2004). We agree with the latter argument, especially considering previous work that demonstrated virulence genes can occur in a majority of non-clinical *Vibrio* strains (Gutierrez West et al., 2013; Klein et al., 2014; Klein and Lovell, 2017). Therefore, we define PAIs as genomic islands having at least one virulence factor gene, even if the PAI is found in an environmental strain.

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 has a genome of 4.98 mbp; chromosome 1 is 3.19 mbp in length and chromosome 2 is 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 not typical of *V. parahaemolyticus* (Figure 3.1A).

This genomic island lies on the second chromosome of TS-8-11-4 and it harbors virulence genes (Figure 3.2A). The thermostable direct hemolysin gene (*tdh*) was found on this island, as well as genes involved in the Type Three Secretion System II (T3SS2). 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 integron 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, meaning that this gene may have been acquired laterally. An integrase gene was found towards 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 does not contain an integrase gene, but a few transposon genes instead (Ceccarelli et al., 2013). 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). The majority (69%) of genes on the TS-8-11-4 PAI could not be assigned specific identities and were thus determined to be hypothetical.

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) is 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 (Figure 3.1B). 82% of the island consisted of hypothetical genes. This island harbored no known virulence genes; it is hereafter referred to as a fitness island (Figure 3.2B). 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. All three of these phage genes had high nucleotide sequence similarities to phage ϕX174 genes (100%, 99%, and 100%, respectively). At least part of this island was most likely inserted into JBS-8-11-1's genome from the same ϕX174 phage.

Thirteen genes involved in toxin-antitoxin (TA) systems were located on the fitness island. 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; without the TA system and encoded antitoxin, the host cell would die (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 3.3).

Lateral Gene Transfer in Environmental Strains

PAIs are present in environmental *Vibrio* strains and are most likely acquired via lateral gene transfer. All 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., 2012; Klein et al., 2014). 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 should be done on the rates of lateral transfer among vibrios in the environment. Because vibrios survive, persist and can undergo rapid population expansions (bloom) in coastal ecosystems, the pathogenic loci (and potential of said loci to be transferred laterally) of naturally occurring environmental strains should no longer be overlooked.
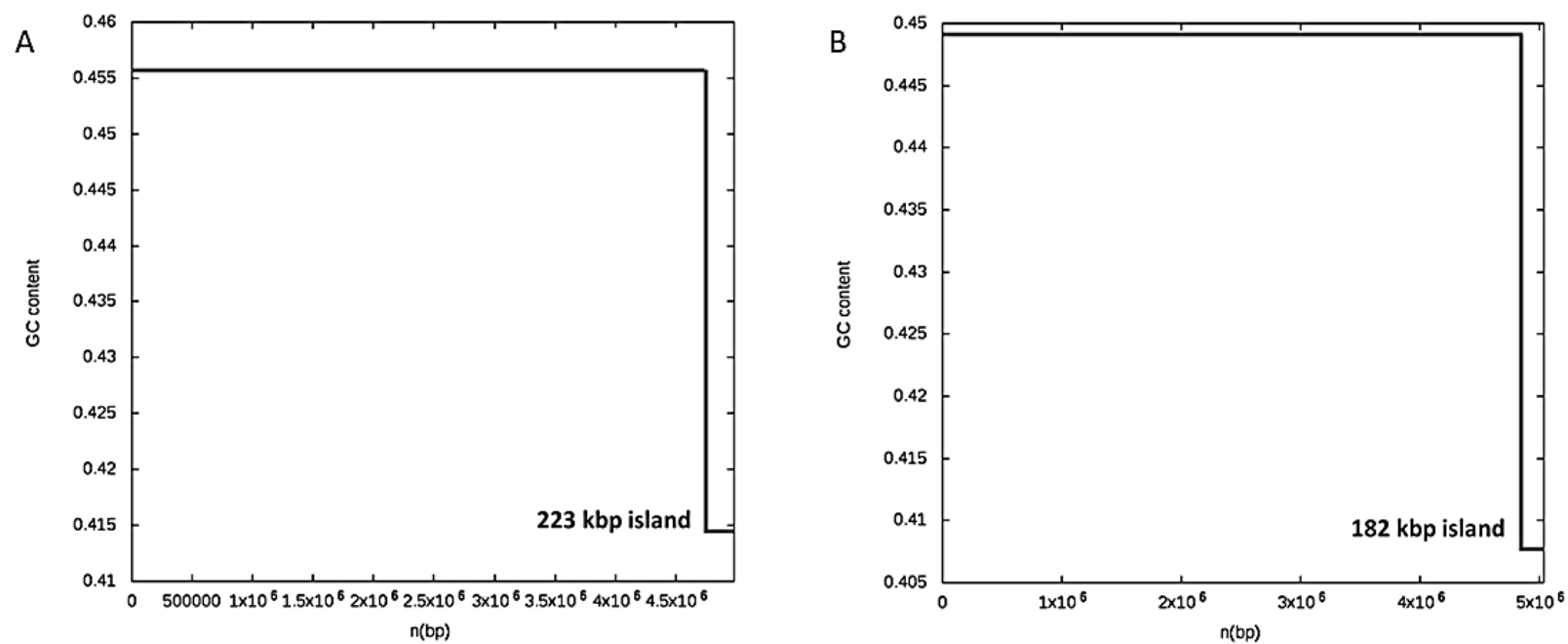
Figure 3.1. GC profile of (A) *Vibrio parahaemolyticus* environmental strain TS-8-11-4 and (B) *Vibrio diabolicus* environmental strain JBS-8-11-1. All GC profiles were made using TUBIC software.
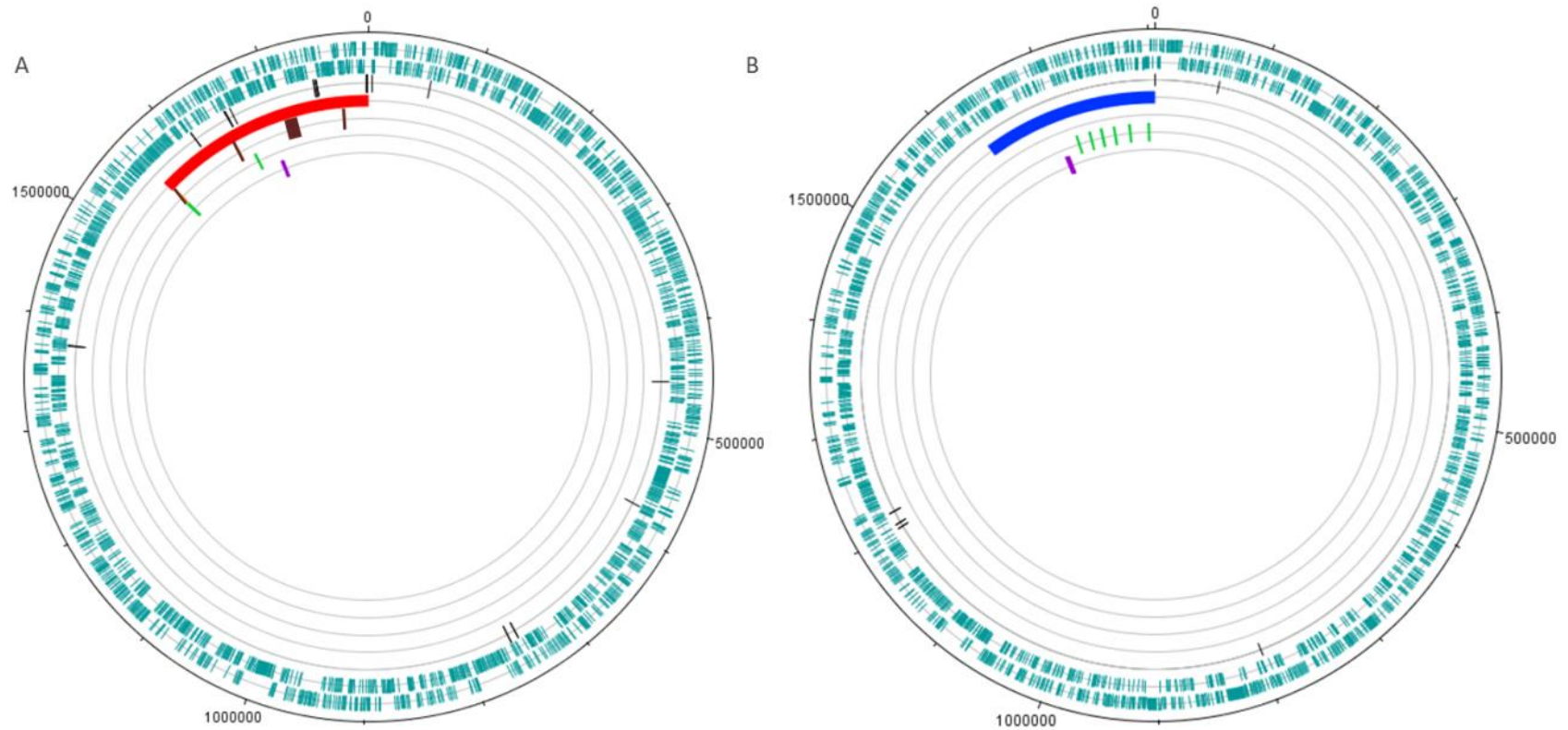
Figure 3.2. Circular presentation of the second chromosome of (A) *Vibrio parahaemolyticus* environmental strain TS-8-11-4, and (B) *Vibrio diabolicus* environmental strain JBS-8-11-1. Track one shows the forward coding sequences; track 2 is the reverse coding sequences. tRNA genes are shown on track 3 and islands are shown on track 4. Red indicates pathogenicity islands (PAIs); blue indicates genomic fitness islands. Virulence and virulence-associated genes are located on track 5, genes involved in toxin-antitoxin systems are on track 6, and mobile genetic elements are shown on track 7
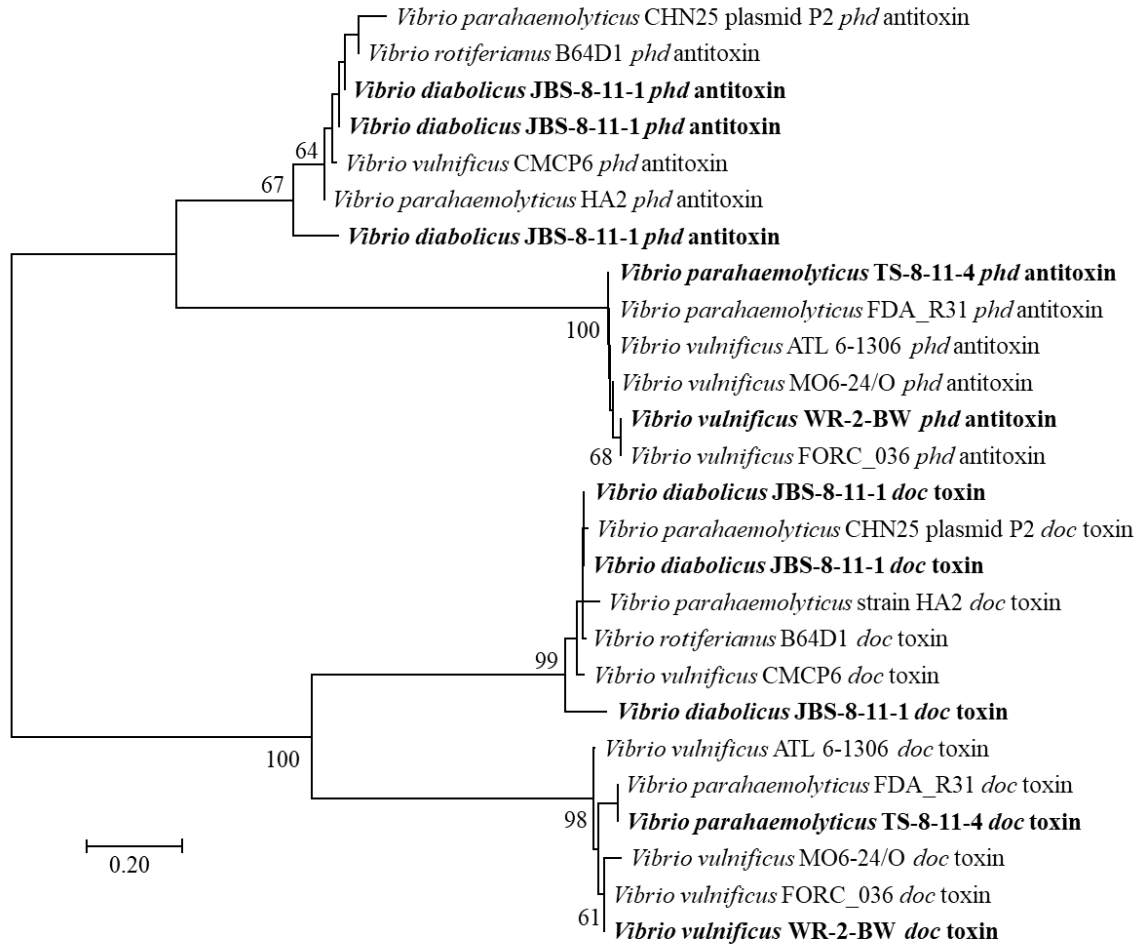
Figure 3.3. Maximum-likelihood phylogeny (Kimura 2-parameter model) of *doc* toxin genes and *phd* antitoxin genes. Bold indicates sequences obtained from this study. The bootstrap values represent 1,000 replications, and values of less than 50 are not shown. The reference sequences were acquired from NCBI GenBank

CHAPTER 4

CYTOTOXICITY OF ENVIRONMENTAL *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* STRAINS

ABSTRACT

Bacteria in the genus *Vibrio* occur naturally in coastal environments and some species pose significant threats to human health. Two pathogenic *Vibrio* species were used in this study, *Vibrio vulnificus* and *Vibrio parahaemolyticus*, both of which cause mild to severe gastroenteritis (vibriosis) after ingestion of raw or undercooked seafood. It is estimated there are 80,000 cases of vibriosis each year in the US. In addition to vibriosis, some *V. vulnificus* strains can infect open wounds to cause necrosis and invade the bloodstream to cause septicemia. The mortality rate of *V. vulnificus* septicemia is 50%. The release of lactate dehydrogenase (LDH) from cultured epithelial colorectal carcinoma cell line HCT 116 was measured to quantify the cytotoxic effect of both *Vibrio* species. LDH is released during tissue damage from eukaryotic cells that have lost membrane integrity. Tested strains of *V. vulnificus* had varying combinations of three virulence factor genes; the hemolysin gene, *vvhA*, the repeat toxin gene, *rtxA1*, and the *vvpE* gene, which encodes a metalloprotease. The *V. parahaemolyticus* virulence factors studied were the two hemolysin genes, *tdh* and *trh*, and the Type III Secretion System 2 marker gene *vscC2*. Both environmental and clinical strains of each species were used. We determined that even environmental *Vibrio* strains where no virulence factors were detected can cause significant damage to human colorectal epithelial cells. This suggests that the distinction between clinical and environmental *Vibrio* strains may not be as straightforward as previously thought.

INTRODUCTION

The CDC (2018) estimates that *Vibrio* bacteria cause 80,000 cases of gastroenteritis (vibriosis) each year in the United States alone. Most cases occur after the consumption of raw or undercooked seafood, primarily oysters. Vibriosis is a global issue wherever raw or undercooked seafood is ingested; it is estimated that in Japan, where raw seafood is prominent in many diets, 20-30% of food poisoning cases are caused by *Vibrio* (Toyofuku, 2013). Vibriosis is caused by two species, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Although cases are mild and tend to resolve themselves after 1 to 3 days, *V. parahaemolyticus* is responsible for the majority of vibriosis cases. The hospitalization and mortality rates of *V. parahaemolyticus* gastroenteritis are 22% and 1%, respectively (Scallan et al., 2011). *V. vulnificus* cases are less common; only about 100 occur each year in the United States (Scallan et al., 2011). However, the hospitalization and mortality rates of this bacterium are much higher, at 92% and 35%, respectively (Scallan et al., 2011). *V. vulnificus* can also cause sepsis and necrotizing fasciitis if introduced into open wounds. 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 et al., 2017). *V. parahaemolyticus* virulence was long thought to be caused by two hemolysin genes, *tdh* (thermostable direct hemolysin) and *trh* (*tdh*-related hemolysin) (Miyamoto et al., 1969;

Shirai et al., 1990; Honda and Iida, 1993; DePaola and Kaysner, 2004; Lovell, 2017). These genes encode toxic proteins that act as porins to disrupt host cell membranes (Yanagihara et al., 2010; Broberg et al., 2011; Ohnishi et al., 2011). However, recent whole genome sequencing of the RIMD strain has revealed secretion systems that may contribute to *V. parahaemolyticus* pathogenesis (Makino et al., 2003). Secretion systems inject toxic effector proteins into host cells and one of these secretion systems, the Type III Secretion System 2 (T3SS2) is thought to play a role in pathogenesis (Makino et al., 2003; Park et al, 2004).

The *V. vulnificus* mechanism of pathogenicity also remains unclear. Three genes are thought to contribute to *V. vulnificus* virulence, *vvhA*, *rtxA1*, and *vvpE*. (Kim et al., 2008; Warner and Oliver, 2008; Jones and Oliver, 2009). *vvhA* is another hemolysin gene that encodes a toxic porin; it is used as a marker for *V. vulnificus* because the majority of strains contain this gene (Warner and Oliver, 2008; Jones and Oliver, 2009). *rtxA1* encodes a repeat toxin and *vvpE* encodes a metalloprotease (Kim et al., 2008; Lee et al., 2016). It is still unclear if content of one, two, or all three of these virulence factor genes are required to cause infections in virulent *V. vulnificus* strains. An additional gene, the "virulence correlated gene" *vcg*, is used to differentiate pathogenic from non-pathogenic strains of *V. vulnificus*. *vcg* encodes a hypothetical protein of unknown function (Warner and Oliver, 2008; Jones and Oliver, 2009). While this gene is not thought to contribute directly to virulence, there is a correlation between content of the clinical variant of *vcg* and disease-causing strains. The *vcgE* variant has been reported as prevalent in environmental, avirulent *V. vulnificus*; the *vcgC* variant has mostly been

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found in clinical, disease causing strains (Warner and Oliver, 2008; Jones and Oliver, 2009).

Current thinking is that most environmental *Vibrio* strains are unlikely to cause infection. This was supported by reports that only 1-2% of environmental *V. parahaemolyticus* strains contained *tdh*, *trh*, and other virulence-related genes; and that most of the environmental (80-90%) *V. vulnificus* are of the avirulent *vcgE* variety. However, with recent improvements in detection of *V. parahaemolyticus* virulence-related genes, they have been detected at a higher frequency (50-70%) in environmental vibrios (Gutierrez-West et al., 2013; Klein et al., 2014; Klein and Lovell, 2017). Environmental strains are defined, in this study, as any strain isolated from a non-clinical, environmental source, such as water or sediment; clinical strains are any that were isolated from an infected patient. Establishing an infection in a human host requires more than just content of one or two virulence genes; but the pathogenic potential of environmental vibrios has been largely ignored and understudied. In this study we determine that environmental *Vibrio* strains are capable of causing cytotoxicity to human gastrointestinal epithelial cells *in vitro*. We compared the cytotoxic effect of environmental *Vibrio* strains to clinical strains and found that they both cause similar degrees of damage to human cells *in vitro*. We also evaluated correlations between cytotoxicity and detection of particular virulence genes.

MATERIALS AND METHODS

Environmental *Vibrio* strain isolation

V. parahaemolyticus strains were isolated from the pristine North Inlet salt marsh estuary near Georgetown, SC, USA (33°20'N, 79°12'W). Strains were isolated from water and sediment in August and September 2011. Strain designations indicate source (S, sediment; BS, burrow lining sediment; BW, burrow water; PW, porewater; CW, creek water), the month and year it was isolated (8-11 for August, 2011; 9-11 for September, 2011), and the isolate number. For example, JS-8-11-4 refers to isolate number 4 from *Juncus roemerianus* (black needle rush) zone sediment (JS) collected on August, 2011 (8-11). Presumptive vibrios were isolated on Thiosulfate Citrate Bile salts Sucrose (TCBS) agar (BD, NJ) without the use of an enrichment step.

V. vulnificus strains were isolated from lower salinity systems, Winyah Bay and the Waccamaw River near Georgetown, SC, USA. *V. vulnificus* isolates were not recovered from North Inlet estuary samples, most likely due to the high salinities (40 to 44 ppt) during sampling for strain isolation (West, 2012). Strains used in this study were isolated from bottom (BW) and surface water (SW) in August from oyster bed areas in Winyah Bay (shellfish isolates) and five sampling stations in the Waccamaw River (WR-1 through WR-5). For example, WR-4 SW 5 refers to isolate number 5 isolated from Waccamaw River sampling site 4 (WR-4) surface water (SW). Presumptive *V. vulnificus* were isolated on CHROMagar *Vibrio* (DRG International, Springfield, NJ) following the FDAs Bacteriological Analytical Manual protocol for isolation of vibrios (DePaola and Kaysner). Presumptive *Vibrio* isolates were collected and routinely cultivated on saline Luria Agar (SLA; per L; 10 g tryptone, 5 g yeast extract, 27 g NaCl, 15 g Bacto Agar). Along with environmental *Vibrio* isolates, we employed two clinical *V. parahaemolyticus* strains and three clinical *V. vulnificus* strains from the American Type Culture Collection

(ATCC). The two clinical *V. parahaemolyticus* strains were isolated from patients suffering from vibriosis (gastroenteritis) and the three clinical *V. vulnificus* strains were isolated from septic patients.

***Vibrio* DNA extractions and species identification**

Isolates were grown overnight at 37ºC in saline Luria broth (SLB) and crude DNA was extracted by boiling the cells (95-100ºC) in distilled water for 15 min. All PCR reactions were completed within three days of DNA extraction and used 1 µl of boiled DNA extract per reaction. Presumptive *V. parahaemolyticus* and *V. vulnificus* strains were confirmed via PCR amplification and sequence analysis of the housekeeping gene, recombinase A (*recA*) using the primers and protocols of Thompson (2005). The 16S rRNA gene is the standard for bacterial species identification but vibrio 16S rRNA genes are too similar for adequate resolution at the species level. Thompson et al. (2005) recommended using other housekeeping genes, such as *recA*, to identify *Vibrio*. *recA* PCR products (790 bp) were resolved on a 1.5% agarose gel and sequenced using an ABI Prism 3730 DNA analyzer. Sequences were edited and Maximum-likelihood phylogenies were constructed using the Kimura 2 parameter model with Mega version 7 (Tamura, 2015). Sequence data of reference *Vibrionaceae* species were obtained from the NCBI GenBank.

***Vibrio parahaemolyticus* virulence-correlated genes PCR protocols and procedures**

Confirmed *V. parahaemolyticus* strains used in cytotoxicity assays were screened for three virulence-correlated genes, *tdh*, *trh*, and *vscC2*. All PCR reactions targeting *tdh* and *trh* were carried out in 25 µl volumes using *Taq* DNA polymerase (Qiagen, Valencia,

CA). The PCR thermocycling program, conditions, and primers of Bej et al. (1999) were

employed for amplification of *tlh*. PCR amplifications of the *tdh* and *trh* genes used the

primers of Gutierrez-West et al. (2013) and were performed in separate reactions. 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 yielded single bands of 245 bp (*tdh*) or

410 bp (*trh*). The thermal cycling program for the three hemolysin genes was:

denaturation at 95°C for 5 min, followed by 40 cycles consisting of 95°C for 1 min, 62°C

for 1 min, 72°C for 1 min, and a final elongation of 72°C for 2 min.

The T3SS2α gene *vscC2* (VPA1339) encodes an outer membrane protein and has

been widely used as a marker for T3SS2α. Primers designed by Noreia et al. (2010) were

used to amplify a 330 bp segment of *vscC2* in 25 µl volumes using *Taq* DNA polymerase

(Qiagen, Valencia, CA). Each reaction included the following: 10x PCR buffer (Qiagen),

0.125 units of *Taq,* 1.25 µM of primer, 0.5 µM of each dNTP (Qiagen) and 2 µM MgCl.

The thermal cycling program used for detection of *vscC2* was: denaturation at 94°C for 4

min, followed by 32 cycles consisting of 94°C for 45s, 60°C for 40s, 72°C for 45s, and a

final elongation of 72°C for 7 min. PCR products were sequenced and the resulting gene

sequences were edited and Maximum-likelihood phylogenies were constructed using the

Kimura 2 parameter model with Mega version 7 (2015).

***Vibrio vulnificus* virulence-correlated genes PCR protocols and procedures**

Confirmed *V. vulnificus* strains used in cytotoxicity assays were screened for three

virulence-correlated genes, *vvhA*, *rtxA1*, and *vvpE. V. vulnificus* strains were also

screened for two variants of the *vcg* gene, *vcgE* and *vcgC*. Amplification of the *vcg*

variants is the standard method for differentiating pathogenic and non-pathogenic *V. vulnificus* (Warner and Oliver, 2008; Jones and Oliver, 2009).

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 a the *rtxA1* gene and the primers of Jeong et al. (2001) were used to amplify a 697 bp segment of the *vvpE* 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-correlated genes was: 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.

Colorectal Carcinoma Epithelial Cell Line

HCT 116 gastrointestinal epithelial cell line was used for cytotoxicity assays. This epithelial cell line was derived from an adult male suffering from colorectal carcinoma (ATCC, 2018). Since *V. parahaemolyticus* and *V. vulnificus* have been implicated in gastroenteritis infections, a gastrointestinal epithelial line was deemed most appropriate for this study. HCT 116 cells were grown up in RPMI 1640 medium (Corning, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 incubator at 37°C. Every three days, when the cells reached an approximate concentration of 10^5, the epithelial cells were trypsinized using 0.25% trypsin EDTA (Corning, Manassas, VA). This protocol breaks the adherent monolayer that the HCT

116 cells form while growing. After trypsinization, cells were counted using a hemocytometer and used in cytotoxicity assays.

Cytotoxicity Assays

Vibrio cells were grown in SLB at 37°C with shaking for 5 h. Vibrio culture media was discarded and cells were resuspended in phosphate buffered saline (PBS). Immediately after trypsinization, HCT 116 cells were diluted as needed, and co-incubated in a tissue-culture treated 96 well plate with Vibrio cells at a multiplicity of infection (MOI) of 100:1. A CytoTox-Fluor Cytotoxicity reagent (Promega, Madison, WI) was used, following the manufacturer's protocols. This assay uses a fluorogenic substrate to measure lactate dehydrogenase (LDH) activity, which is released from eukaryotic cells that have lost membrane integrity. The substrate gives no signal from live, intact cells. Each Vibrio strain was tested in 6 wells per 96 well plate. For true replication, each 96 well plate was repeated three times. Low controls included epithelial cells incubated with an avirulent Vibrio, Vibrio pacinii, and epithelial cells with no vibrios. The high control was epithelial cells co-incubated with 1% Triton X-100. Vibrio strains and epithelial cells were co-incubated for 2 h in a 5% CO_2 incubator at 37°C without shaking. Fluorescence was measured immediately after co-incubation and every hour using a SpectraMax Gemini EM microplate reader (Molecular Devices, Sunnyvale, CA). Percent cytotoxicity was calculated after 2 h co-incubation using the following equation:

$$Cytotoxicity\ (\%) = \left(\frac{experimental - low\ control}{high\ control - low\ control}\right) x\ 100$$

Percent cytotoxicity of vibrios were compared to the avirulent V. pacinii low control by using a One Way ANOVA. Multiple comparisons were made versus the control group

(*V. pacinii*) using the Holm-Sidak method (SigmaPlot, 2016). The significance level used was 0.05. Student's t-tests were used to compare the percent cytotoxicity of clinical and environmental strains.

Dose Response Curve

To determine the effect of *Vibrio* dosage on the *in vitro* experiments, vibrios were serially diluted in PBS. The diluted vibrios were co-incubated with the HCT 116 cells, the concentration of which was not altered. MOIs of 100:1, 75:1, 50:1, 25:1, and 1:1 were used in dose response curves. The CytoTox-Fluor Cytotoxicity reagent (Promega, Madison, WI) was added, fluorescence was measured hourly for 2 h, and percent cytotoxicity was calculated.

Adherent monolayer Coverage Assay and Trypan Blue Exclusion Assays

In order to visualize *Vibrio* damage to the HCT 116 epithelial cell adherent monolayer, *Vibrio* strains were used in a percent coverage assay. HCT 116 cells were grown for 3 days in a tissue-treated petri dish to reach an approximate concentration of 10^5. The *Vibrio* strain was added to the petri dish at a MOI of 100:1. Photographs of the HCT 116 cell monolayer were taken immediately after inoculation and every hour for 2 h at 20x magnification. Percent coverage of the HCT 116 epithelial cell monolayer was calculated using Vidana software (2017).

To determine if there was damage to the individual epithelial cells after adherent monolayer coverage assays, a trypan blue exclusion assay was used. Trypan blue only stains cells that have lost membrane integrity. If epithelial cells take up the trypan blue dye, they are no longer considered viable. A 0.4% solution of trypan blue in PBS was

used to stain cells and cell counts were performed using a hemocytometer. The percent
of viable cells in the population was calculated.

RESULTS

V. parahaemolyticus cytotoxicity

Thirteen *V. parahaemolyticus* strains were used in cytotoxicity assays (Table 4.1).
Two clinical isolates were used; these strains were isolated from patients suffering from
V. parahaemolyticus gastroenteritis. The *V. parahaemolyticus* type strain was used
(ATCC 17802^T) as well as a well-studied reference strain (ATCC 33846); both of these
strains contain at least one virulence factor gene. Eleven environmental *V.
parahaemolyticus* strains were used in cytotoxicity assays. Seven of these environmental
strains contained at least one virulence factor gene (*tdh, trh*, or *vscC2*), while no
virulence factors were detected in four strains.

All *V. parahaemolyticus* strains used in this study, regardless of isolation source
(clinical or environmental), were cytotoxic to HCT 116 epithelial cells (Figure 4.1).
There was no significant difference in cytotoxicity between clinical strains and
environmental strains (Student's t-test, p value=0.211). There was also no significant
difference in cytotoxicity between the environmental strains that contained virulence
factors and environmental strains where no virulence factors were detected (Student's t-
test, p value=0.362).

Dose response experiments were conducted at different MOIs (Figure 4.2). As
the concentration of *V. parahaemolyticus* cells decreased, so did the cytotoxic effect of
the vibrios. However, even at the extremely low MOI of 1:1, some cytotoxic effects were
observed. For example, environmental *V. parahaemolyticus* strain JS-9-11-6 caused

67.20% cytotoxicity at an MOI of 100:1; at an MOI of 1:1 the same strain caused 19.68% cytotoxicity. The cytotoxic effects were always detected, even at an MOI of 1:1.

V. vulnificus cytotoxicity

All 15 *V. vulnificus* strains used in cytotoxicity assays tested positive for the *vvhA* hemolysin gene, the *rtxA1* toxin gene, and the *vvpE* protease gene (Table 4.2). Three clinical strains were used; all were isolated from patients suffering from *V. vulnificus* sepsis and all clinical strains were determined to be *vcgC* variants. Twelve environmental strains were used; 6 *vcgC* variants and 6 *vcgE* variants. As with *V. parahaemolyticus*, all *V. vulnificus* strains were cytotoxic to gastrointestinal epithelial cells (Figure 4.3). We determined that there was no significant difference in cytotoxicity between clinical and environmental *V. vulnificus* strains (Student's t-test, p value=0.114). There was also no significant difference in cytotoxicity between *vcgC* variants and *vcgE* variants (Student's t-test, p value=0.552). As seen with *V. parahaemolyticus*, as the MOI decreased, so did the degree of cytotoxicity (Figure 4.4).

V. vulnificus-mediated adherent monolayer dysregulation

HCT 116 epithelial cells grow in an adherent monolayer connected by adherens and tight junctions (Niessen, 2007; ATCC, 2018). Cytotoxicity assays were performed while HCT 116 epithelial cells were in suspension. However, we wanted to test what effect vibrios had on the epithelial cell adherent monolayer, so co-incubations with vibrios were also performed while HCT 116 cells were growing in their monolayer adhered to the bottom of a tissue-treated petri dish. After a 2 h incubation, *V. vulnificus* caused disruption (dysregulation) of the epithelial cell monolayer, releasing it from the bottom of the petri dish (Figure 4.5). This was seen consistently after 2 h with all *V.*

vulnificus strains tested, regardless of isolation source or *vcg* variant. This disruptive effect on the epithelial cell monolayer was never observed with any *V. parahaemolyticus* strain tested. After the epithelial cell monolayer was dysregulated, a trypan blue exclusion assay was performed to determine how many individual cells were compromised during the monolayer disruption. We determined that >90% of the population of epithelial cells that had come into suspension due to *V. vulnificus*-mediated dysregulation of the monolayer had lost membrane integrity and were no longer viable (Table 4.3).

DISCUSSION

There have been many studies on the cytotoxic potential of *V. parahaemolyticus* and *V. vulnificus* (Raimondi et al., 2000; Park et al., 2004; Lee et al., 2007; Kim et al., 2008; Hiyoshi et al., 2010; Mahoney et al., 2010; Lee et al., 2016, etc). However, these studies either use inappropriate human cell lines as hosts (such as cervical cancer HeLa cells) or exclusively look at *Vibrio* strains isolated from a clinical source and therefore ignore the potential pathogenic effects of environmental strains. There is a fascination with *Vibrio* strains that have been isolated from sick patients, which causes a clinical strain bias. This also leads to the erroneous idea that environmental and clinical strains are inherently dissimilar. A study by Mahoney and company (2010) used 13 environmental *V. parahaemolyticus* strains in their cytotoxicity assays and they also found that environmental strains have cytotoxic capabilities like those of clinical strains. Putative virulence factor genes such as *tdh* and *trh* were not detected in any of the 13 strains Mahoney used (2010). To our knowledge, there have been no cytotoxicity assays done with environmental *V. vulnificus* strains.

One of most unexpected results of this study (and Mahoney's) is that *V. parahaemolyticus* strains having no detectable virulence factor genes can still cause significant (and dramatic) cytotoxicity to intestinal epithelial cells. We suggest three explanations for this observation. The first explanation is that *in vitro* cytotoxicity assays are unreliable indicators of actual virulence and pathogenic potential. This seems unlikely given that *in vitro* cytotoxicity assays like the kind employed in this study have been used abundantly for years to determine the pathogenic potential of microorganisms. The second explanation is that all *V. parahaemolyticus* strains (regardless of virulence gene content and isolation source) can cause disease. This would mean outbreaks solely rely on an increase in *V. parahaemolyticus* population densities, not virulence factor acquisition.

Another reason could be that the actual mechanism for *V. parahaemolyticus* virulence has nothing to do with *tdh*, *trh*, or *vscC2* and the correct *V. parahaemolyticus* virulence mechanism(s) have not yet been identified. More *V. parahaemolyticus* genomes need to be sequenced to determine if there are previously unrecognized virulence factors within the genome. It's important to note that before Makino et al. (2003) sequenced the first *V. parahaemolyticus* genome, it was not known that this organism could utilize secretion systems or that one *V. parahaemolyticus* strain could contain up to nine pathogenicity islands. With more high-quality genomic sequencing, our understanding of *V. parahaemolyticus* virulence will only grow.

All of the *V. vulnificus* strains tested in this study contained all virulence factors screened for (*vvhA*, *rtxA1*, and *vvpE*); these strains were divided based on whether they contained the E or C variant of the *vcg* gene. This is the standard method to determine

the pathogenic potential of *V. vulnificus*; however, we found no difference in cytotoxicity between *vcgE* and *vcgC* variants. The *vcg* gene, which encodes a hypothetical protein with unknown function, is an unreliable indicator of virulence in *V. vulnificus*. *vcgE* variants (thought to be only found in the environment) have also been isolated from a clinical source, a patient suffering from *V. vulnificus* sepsis (Warner and Oliver, 2008). However, there is no suitable replacement for the *vcg* gene as a *V. vulnificus* virulence-correlated marker at present.

We also found that *V. vulnificus* dysregulates the epithelial cell adherent monolayer. Many *V. vulnificus* infections start in the gastrointestinal tract and then turn septic when the bacterium then enters the bloodstream (Scallan et al., 2011). We propose *V. vulnificus* breaks down the epithelial cell layers, causing the intestinal epithelia to slough off and that is how *V. vulnificus* then invades the bloodstream to become septic. Lee et al. (2016) determined that the protease gene *vvpE* was responsible for disruption of tight junctions between epithelial cells. *V. vulnificus* mutants with a nonfunctional *vvpE* gene did not dysregulate the tight junctions, wild-type *V. vulnificus* with a fully functional *vvpE* gene caused dysregulation of the adherent monolayer. Our data support this study; all *V. vulnificus* strains tested contained the *vvpE* gene and all *V. vulnificus* strains tested caused dysregulation of the adherent monolayer. Our *V. parahaemolyticus* strains did not cause dysregulation to the epithelial cell monolayer. This was expected as *V. parahaemolyticus* infections are overwhelmingly gastrointestinal and no cases of *V. parahaemolyticus* gastroenteritis leading to sepsis have been reported.

The results of this study indicate that there is still much to be done in characterizing *Vibrio* virulence and pathogenesis. There are still no reliable molecular

markers of virulence for *V. parahaemolyticus* or *V. vulnificus*. There are still no reliable means to predict outbreaks of infections caused by either species as well. We suggest that genome sequencing of many more *Vibrio* strains and shifting away from the "clinical strain bias" in this field may remedy some of these problems

Table 4.1 *Vibrio parahaemolyticus* strains used in cytotoxicity assays. Bold indicates strains from a clinical source. [T] indicates type strain

Vibrio parahaemolyticus Strain designation	*tdh*	*trh*	*vscC2*
ATCC 17802[T]	-	+	+
ATCC 33846	+	-	+
TS 8-11-4	+	+	+
JBW 8-11-2	-	+	+
CW 9-11-2	+	-	+
JPW 9-11-10	+	-	+
JPW 8-11-9	-	-	+
JPW 9-11-13	-	-	+
JS 9-11-6	+	-	-
JS 8-11-6	-	-	-
JS 9-11-3	-	-	-
TS 9-11-5	-	-	-
JS 8-11-2	-	-	-
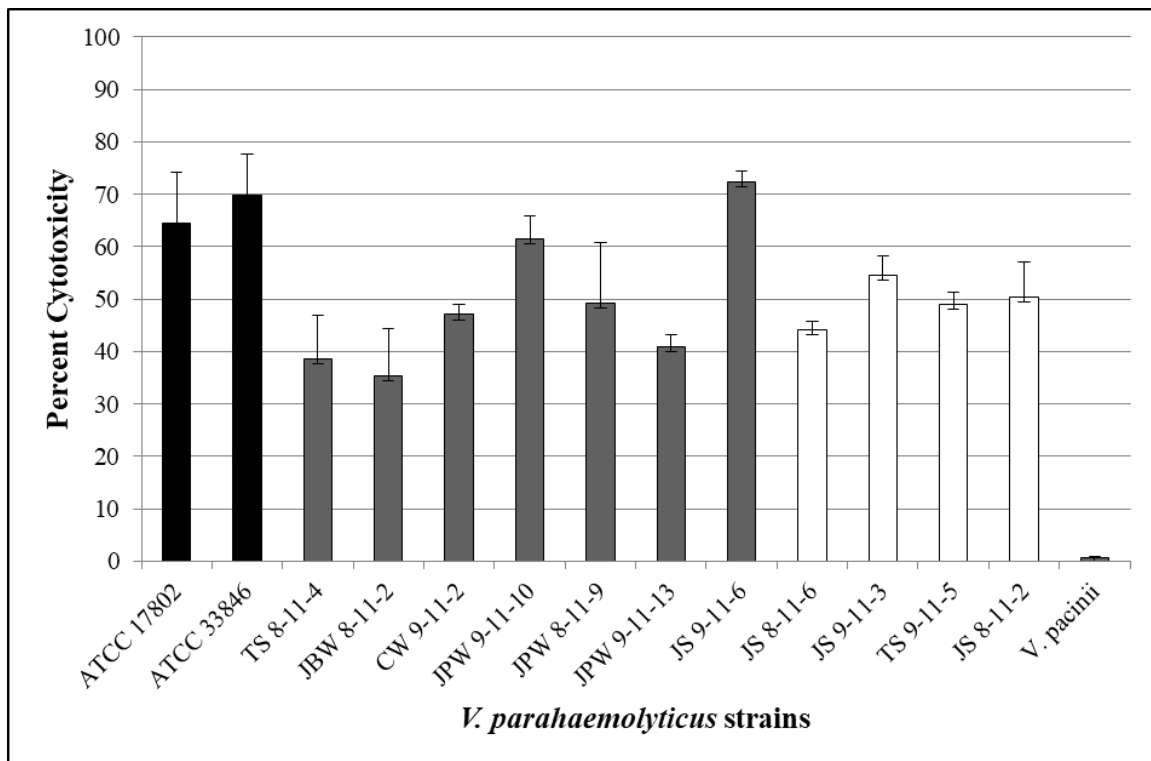
Figure 4.1. Release of lactate dehydrogenase (LDH) from intestinal epithelial cells when co-incubated with various *Vibrio* strains. Black bars represent *Vibrio parahaemolyticus* strains isolated from a clinical source. Dark gray bars represent environmental *V. parahaemolyticus* strains with various combinations of virulence factors. White bars represent environmental *V. parahaemolyticus* strains where no virulence factors were detected. The control was epithelial cells co-incubated with the avirulent *Vibrio*, *Vibrio pacinii.*

Environmental *V. parahaemolyticus* dose response curve
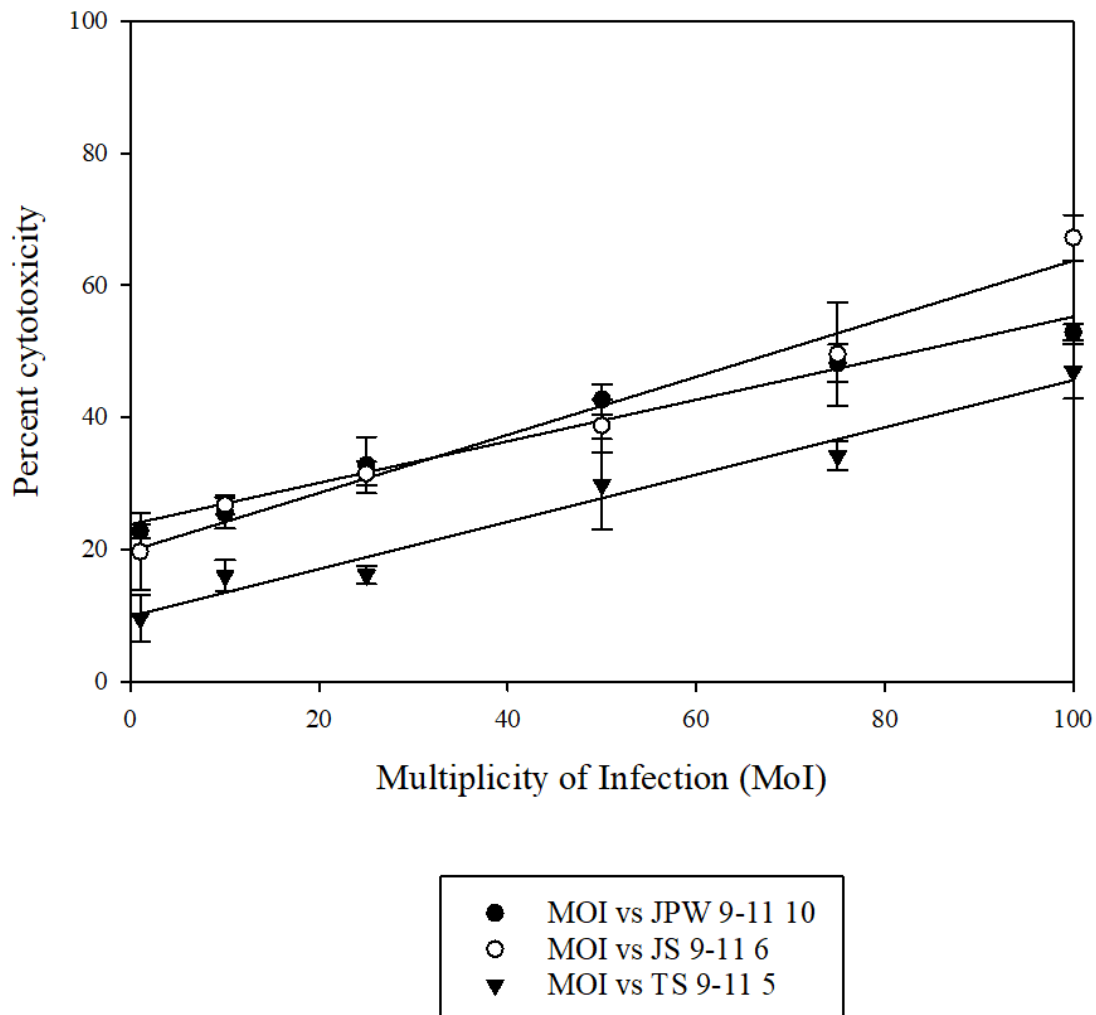
Figure 4.2. Dose response curve showing the cytotoxic effects of *V. parahaemolyticus* strains when co-incubated with HCT 116 epithelial cells at varying multiplicity of infections (MOIs). The concentration of HCT 116 epithelial cells did not change.

Table 4.2. *Vibrio vulnificus* strains used in cytotoxicity assays. Bold indicates strains from a clinical source. [T] indicates type strain

Vibrio vulnificus Strain designation	*vcg* variant	*vvhA*	*rtxA1*	*vvpE*
ATCC 27562[T]	*vcgC*	+	+	+
ATCC 33817	*vcgC*	+	+	+
ATCC BAA-86	*vcgC*	+	+	+
Aug WR-1 SW 4	*vcgC*	+	+	+
Aug WR-2 BW	*vcgC*	+	+	+
Aug WR-2 BW 2	*vcgC*	+	+	+
July shellfish 5-21 BW 3	*vcgC*	+	+	+
July WR-2 SW	*vcgC*	+	+	+
July BR-1 SW 2	*vcgC*	+	+	+
Aug WR-1 SW 6	*vcgE*	+	+	+
Aug shellfish 5-21 BW 1	*vcgE*	+	+	+
Aug shellfish 5-25 BW 3	*vcgE*	+	+	+
July BR-1 BW	*vcgE*	+	+	+
July shellfish 5-20 BW 5	*vcgE*	+	+	+
July shellfish 5-20 BW 6	*vcgE*	+	+	+
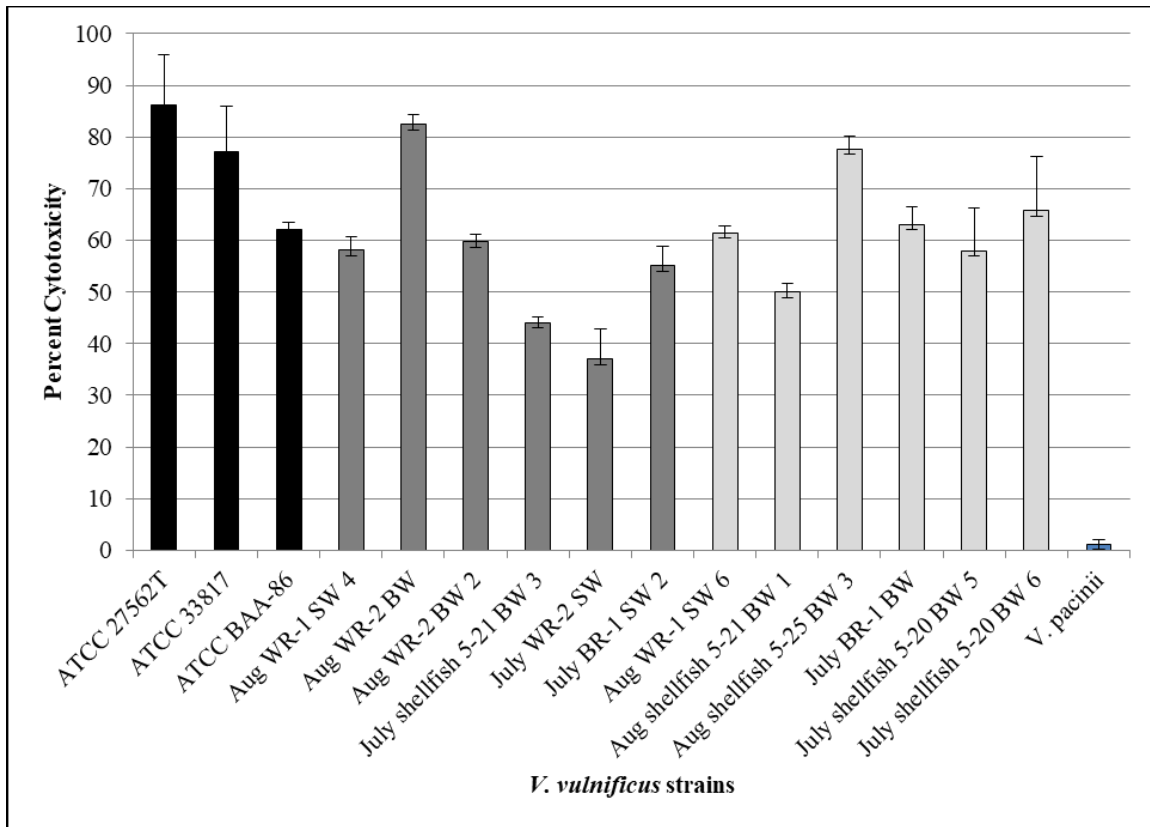
Figure 4.3. Release of lactate dehydrogenase (LDH) from intestinal epithelial cells when co-incubated with various *Vibrio* strains. Black bars represent *Vibrio vulnificus* strains isolated from a clinical source. Dark gray bars represent environmental *vcgC* variant *V. vulnificus* strains. Light gray bars represent environmental *vcgE* variant *V. vulnificus* strains. The control was epithelial cells co-incubated with the avirulent *Vibrio*, *Vibrio pacinii.*
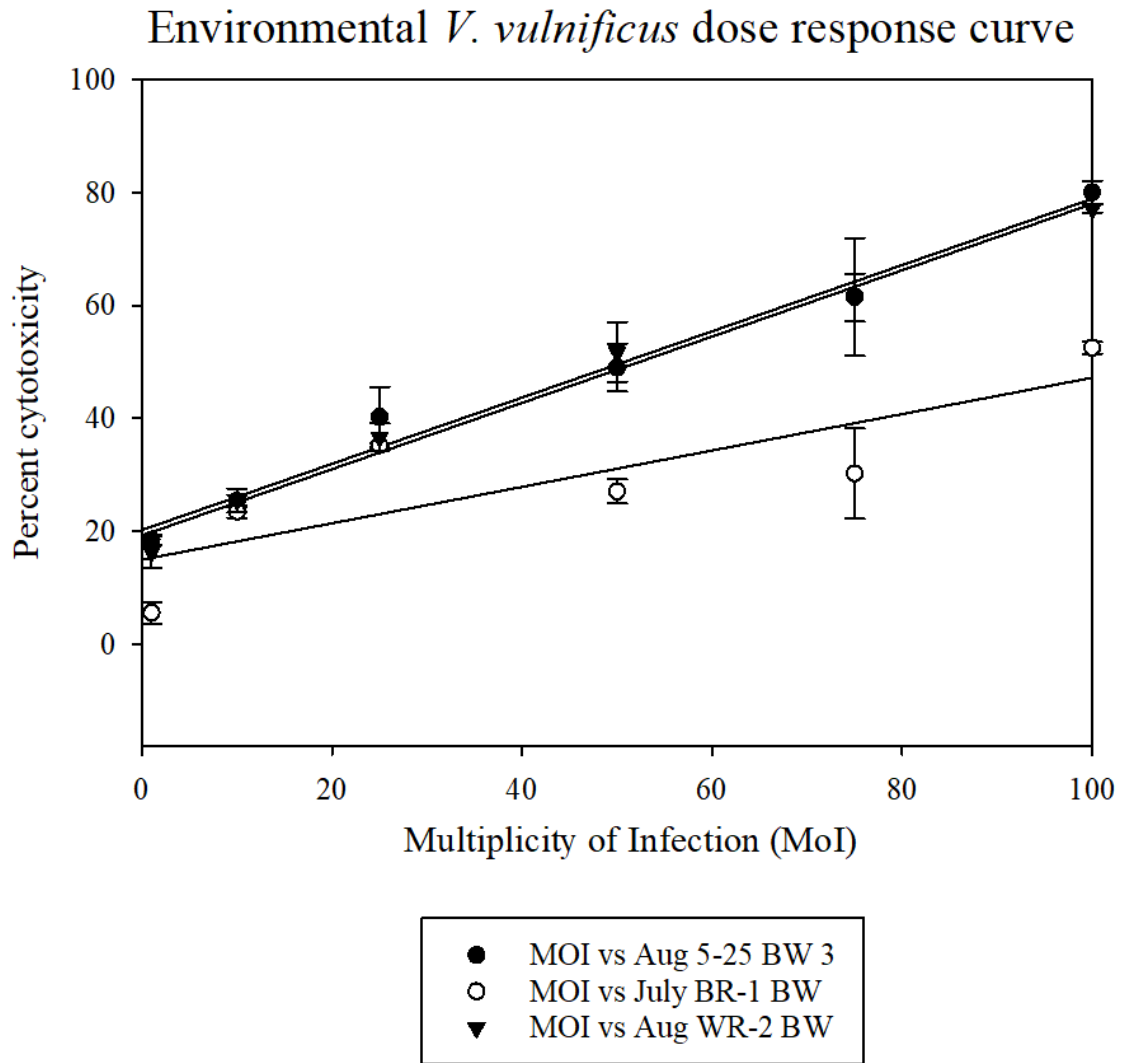
Figure 4.4. Dose response curve showing the cytotoxic effects of *V. vulnificus* strains when co-incubated with HCT 116 epithelial cells at varying multiplicity of infections (MOIs). The concentration of HCT 116 epithelial cells did not change.

T0- No *Vibrio* cells	T1- 1h with *Vibrio* cells	T0- 2h with *Vibrio* cells
55% coverage	**28% coverage**	**0% coverage**
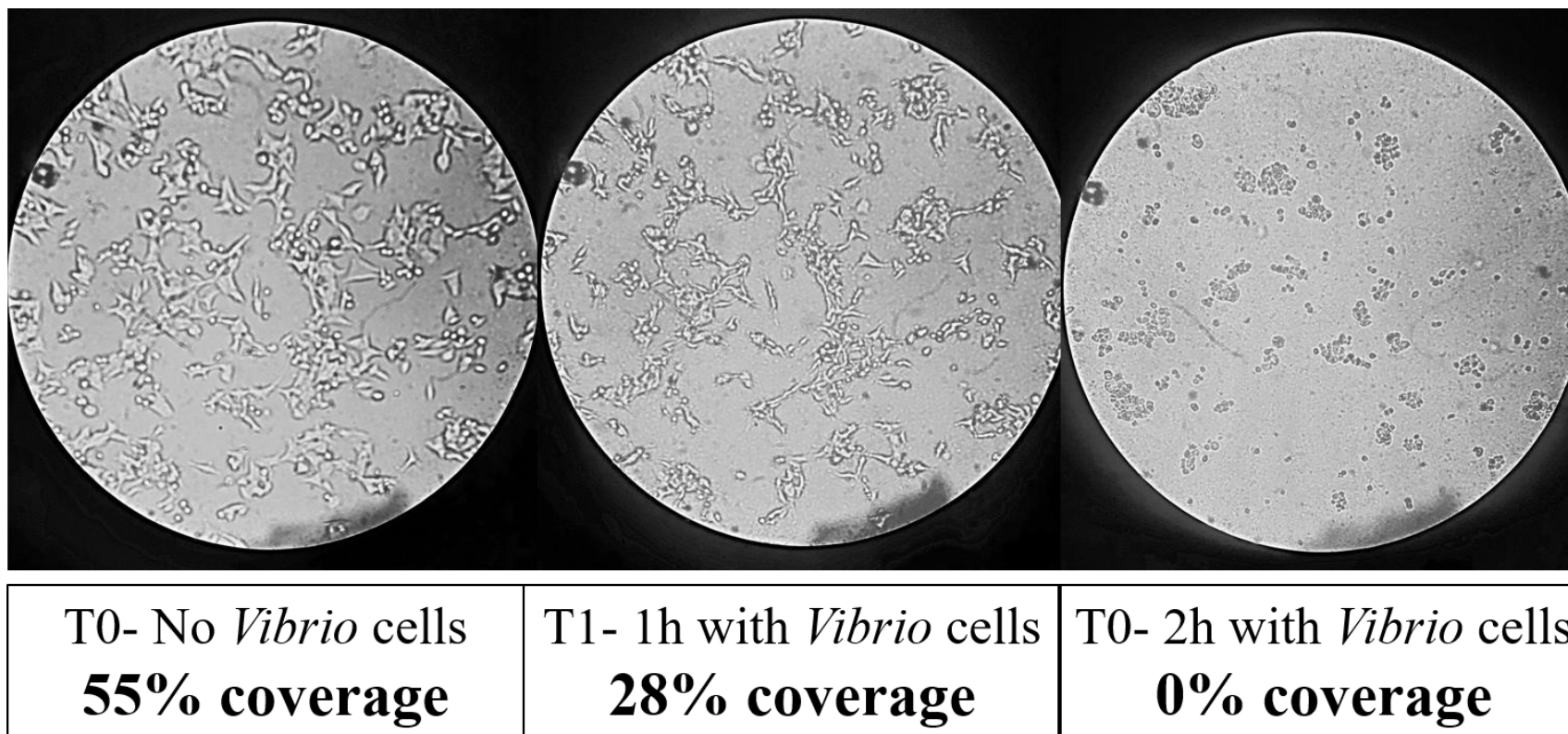
Figure 4.5. Percent coverage assay of HCT 116 epithelial cell adherent monolayer co-incubated with *V. vulnificus* environmental strain Aug WR-2 BW 2. Percent coverage was calculated using Vidana software. At 2 h the adherent layer was completely dysregulated and the epithelial cells were in suspension

Table 4.3. The percentage of dead cells after 2 h coverage assay (Figure 4.5) with *Vibrio vulnificus*. The negative controls were done using a trypsinization protocol to break up the adherent monolayer and get cells in suspension

	average % of dead cells	standard deviation
negative control	6.28	1.52
V. pacinii	15.45	5.76
27562T	95.22	4.82
WR-2-BW-2	92.17	3.40

CHAPTER 5

INTERACTIONS BETWEEN THE HUMAN PATHOGEN

VIBRIO PARAHAEMOLYTICUS AND COMMON MARINE

MICROALGAE [1]

ABSTRACT

Vibrio parahaemolyticus is a gastrointestinal pathogen that is abundant in coastal marine environments. Elevated numbers of *V. parahaemolyticus* have been correlated with marine microalgae blooms, particularly blooms of diatoms and dinoflagellates, but the nature of the relationship between *V. parahaemolyticus* and microalgae is unknown. We performed *in vitro* assays using 27 environmental *V. parahaemolyticus* strains and various phototrophs; a diatom, a dinoflagellate, unarmored and armored forms of a coccolithophore, and two species of cyanobacteria. The *V. parahaemolyticus* strains we employed contained different combinations of virulence-correlated genes, the hemolysin genes *tdh* and *trh*, the Type III Secretion System 2 (T3SS2) marker gene *vscC2*, and the Type VI Secretion System (T6SS) marker gene *vipA1*. We determined that all *V. parahaemolyticus* strains, even strains in which no virulence factor genes were detected, were able to cause decreases in diatom, dinoflagellate, and unarmored coccolithophore biomass *in vitro*. No correlation between content of any virulence gene and damage to microalgae was apparent. This may explain the recent correlations between *V. parahaemolyticus* and microalgae blooms.

INTRODUCTION

Vibrio parahaemolyticus, a common organism in coastal environments, is a significant and sometimes pandemic human pathogen. This organism and other vibrios are responsible for an estimated 80,000 cases of seafood-associated gastroenteritis per year in the United States (CDC, 2018). Most cases of *V. parahaemolyticus*-induced gastroenteritis are self-limiting and relatively mild, but infections can be deadly in immunocompromised individuals. The common mode of transmission of this bacterium

to the human host is ingestion of raw or undercooked shellfish, primarily oysters. Elevated densities of *V. parahaemolyticus* most often occur during the warm months and at warm locations (DePaola et al., 1990; Gamble and Lovell, 2011) but many recent large vibriosis outbreaks have occurred at locations not considered typical for this organism (McLaughlin et al., 2005; Paranjype et al., 2012).

V. parahaemolyticus not only persists, but can increase in population size very rapidly in coastal marine environments (Gamble and Lovell, 2011; West, 2012; DePaola et al., 2003). The abundance of *V. parahaemolyticus* as free-living cells in water is typically low (< 2,000 cells per liter) (West, 2012), but this organism can be very abundant in surficial sediment and in infaunal burrows (Gamble and Lovell, 2011; West, 2012; Gartmon et al., in preparation). *V. parahaemolyticus* also occurs at higher levels in shellfish (DePaola et al., 1990; 2003; Klein and Lovell, 2017), and in association with algal blooms (Turner et al., 2009; Rehnstam-Holm et al., 2010; Seong and Jeong, 2011; Turner et al., 2014; Main et al., 2015). Potential for a *V. parahaemolyticus* outbreak has often been predicted on the basis of local temperature, salinity, turbidity, and chlorophyll *a* concentrations (Carburlotto et al., 2010; Johnson et al., 2010). In addition, some correlations between *V. parahaemolyticus* densities and certain algal taxa, specifically diatoms and dinoflagellates (Turner et al., 2009; Eiler et al., 2006; Asplund et al., 2011) have been reported and elevated levels of *V. parahaemolyticus* can occur during dinoflagellate and diatom blooms (Rehnstam-Holm et al., 2010; Seong and Jeong, 2011). It is not yet known why *V. parahaemolyticus* co-occurs with marine microalgae in the water column or what kind of symbiosis is occuring between *V. parahaemolyticus* and microalgae.

We examined the ability of *V. parahaemolyticus* to decrease phototroph biomass. *In vivo* chlorophyll *a* served as a measure of phototroph biomass. Direct microscopic cell counts were also performed to confirm *in vivo* chlorophyll *a* data. Six phototrophs, including three species of microalgae and two species of cyanobacteria were incubated with several strains of *V. parahaemolyticus*. The phototrophs employed are abundant in marine environments and present a variety of cell wall surface structures and properties, providing insight into associations between susceptibility of the microalgae to *V. parahaemolyticus*.

MATERIALS AND METHODS

V. parahaemolyticus strain isolation and characterization

V. parahaemolyticus strains were isolated from the pristine North Inlet estuary near Georgetown, SC, USA (33°20'N, 79°12'W) in August and September 2011 as described previously (Gutierrez West et al., 2013). The North Inlet-Winyah Bay National Estuarine Research Reserve protects the third largest watershed on the east coast of the United States; and North Inlet is a bar built oligotrophic salt marsh where human impact is negligible (Dame et al., 2000; Buzzelli et al., 2004). Samples were diluted and plated directly onto Thiosulfate Citrate Bile Salts Sucrose agar (TCBS) (BD, NJ). The presumptive identification of all *V. parahaemolyticus* strains used in this study was confirmed by *recA* sequence analysis (Gutierrez West et al., 2013) using the PCR primers and protocols of Thompson et al. (2005).

Virulence gene PCR screening

Two virulence-related hemolysin genes, *tdh* and *trh*, have been correlated with pathogenesis in *V. parahaemolyticus*, and these hemolysin genes are frequently used as

molecular markers for strain virulence (Gutierrez West et al., 2013; DePaola and Kaysner, 2004). Additional virulence factors, specifically secretion systems, have been discovered with recent sequencing of *V. parahaemolyticus* genomes (Makino et al., 2003; Park et al., 2004; Okada et al., 2009). The Type III Secretion System (T3SS2) has also been implicated in *V. parahaemolyticus* virulence (Makino et al., 2003) and the outer membrane protein gene, *vscC2* is a useful marker for this structure (Noriea et al., 2010; Klein et al., 2014).

The Type VI Secretion System (T6SS) has also been detected in some *V. parahaemolyticus* isolates (Salomon et al., 2013). This secretion system has not been implicated in pathogenicity of *V. parahaemolyticus* to humans, but T6SS producing *V. parahaemolyticus* strains have been shown to cause damage to other prokaryotes *in vitro* when incubated on a surface (Salomon et al., 2013). Its impacts on eukaryotic microalgae are presently unknown.

V. parahaemolyticus strains were grown overnight at 37ºC in Saline Luria-Bertani Broth (SLB; per L 27 g NaCl, 10g Tryptone, 5 g Yeast Extract) and boiled extracts (15 min at 95-100°C) were prepared. All PCR reactions were completed within three days of DNA extraction and used 1 μl of boiled DNA extract per reaction. Strains were screened for the T6SS marker gene *vipA1* using the PCR primers and protocols of Salomon et al. (2013). PCR products were resolved on a 1.5% agarose gel and sequenced using an ABI Prism 3730 DNA analyzer to confirm gene identity. Sequences were analyzed using the Kimura 2 parameter model with Mega version 7 (Tamura et al., 2015). Sequence data obtained from this work were submitted to the NCBI GenBank and assigned the accession numbers KX171447- KX171449.

Cultivation of microalgae and cyanobacteria

Phototroph cultures were obtained from the Bigelow National Center for Marine Algae and Microbiota (Bigelow Center, East Boothbay, ME). Two species of cyanobacteria were used, *Prochlorococcus marinus* (CCMP 1986) and *Synechococcus bacillaris* (CCMP 1333). Three species of eukaryotic microalgae were used in this project, the diatom *Thallasiosira pseudonana* (CCMP 1335), the dinoflagellate *Prorocentrum minimum* (CCMP 695), and two strains of the coccolithophore, *Emiliania huxleyi* (CCMP 371 and CCMP 373). *T. pseudonana* and *P. minimum* are common in North Inlet and *E. huxleyi* CCMP 371 is a coccolith-producing (armored) form that causes extensive blooms. *E. huxleyi* CCMP 373 is an unarmored mutant phenotype. The phototrophs chosen had a variety of cell wall surface structures (Paasche, 2001; Armbrust et al., 2004; Heil et al., 2005; Javaheri et al., 2015) and each required its own growth medium (Appendix A). Microalgae and cyanobacteria were grown at 23°C with an 11 h light, 13 h dark cycle.

***In vitro* experiments**

V. parahaemolyticus strains were grown in SLB at 37°C with shaking. At 5 h, cultures were in exponential growth phase and yielded approximately 2×10^7 cells mL^{-1}. Cultures were centrifuged (600 x g), the supernatants discarded, and cell pellets resuspended in 33 ppt phosphate buffered saline (PBS) (400 mM NaCl, 1.75 mM NaPO$_4$, pH 7.4). Phototroph cultures were grown for 5-10 days, reaching approximately 2.0×10^5 cells mL^{-1}, then harvested by centrifugation (1075 x g) for 10 min, and the supernatants removed. Cells were resuspended in PBS. Bacterial strains and phototroph cultures were combined in 96 well microplates with a multiplicity of infection (MOI) of

approximately 100:1. *In vivo* chlorophyll *a* fluorescence was used as an indicator of phototroph condition, and was measured immediately after co-inoculation ($T_{initial}$) using a SpectraMax Gemini EM microplate reader (Molecular Devices, Sunnyvale, CA). Microplates were then incubated with light for 24 h at 25°C. After incubation, chlorophyll *a* fluorescence was measured (T_{final}). The percent difference between final and initial time points was determined by the formula: $((T_{final} - T_{initial}) / T_{initial})) \times 100$. After incubation, aliquots were observed under a Nikon Eclipse TS100 microscope to determine the effect of *V. parahaemolyticus* strains on microalgae. Microalgal cell counts were performed using a hemocytometer after the 24 h co-incubation.

Each *V. parahaemolyticus* strain was tested in 12 wells per 96 well plate. For true replication, each 96 well plate was repeated three times. Controls included replicates of phototrophs in appropriate media and replicates of phototrophs in PBS (with no *V. parahaemolyticus* added), against which experimental replicates were compared. *Vibrio pacinii*, an avirulent *Vibrio* (Gomez-Gil et al., 2003), was used as a non-*V. parahaemolyticus*, heterotrophic bacterial control. Changes in *in vivo* chlorophyll *a* fluorescence were compared to the control using One Way ANOVA. Multiple comparisons were made versus the control group using the Holm-Sidak method (SigmaPlot, 2016). The significance level used was 0.05.

All 29 *V. parahaemolyticus* strains were tested against the eukaryotic algae. A subset of these strains, some having the antibacterial mechanism T6SS (*vipA1*-positive) and some lacking it, was also tested against the cyanobacteria. We also used the T6SS-bearing POR1 strain, a derivative of the *V. parahaemolyticus* RIMD 2210633 reference

strain, and two POR1 derivatives. The POR1 derivatives were the T6SS-knockout strain, POR1Δhcp1, and the T6SS de-repressed strain, POR1Δhns.

Dose response

To determine the effect of *V. parahaemolyticus* dosage on the *in vitro* experiments, bacterial cultures were serially diluted (10^7 to 10^2) in PBS. These dilutions were incubated with unarmored *E. huxleyi*, the concentration of which was not altered.

RESULTS

Eukaryotic microalgae *in vitro* experiments

Twenty-nine *V. parahaemolyticus* strains, 27 of which were environmental strains isolated from North Inlet estuary, were incubated with microalgae. These environmental strains were previously confirmed to be *V. parahaemolyticus* via *recA* sequence analysis (Gutierrez West et al., 2013; Thompson et al., 2005). The two non-environmental strains were clinical isolates, the *trh*-bearing *V. parahaemolyticus* type strain ATCC 17802^T and the *tdh*-bearing reference strain ATCC 33846. Nineteen of the *V. parahaemolyticus* strains contained varying combinations of the virulence-related genes *tdh*, *trh*, *vscC2*, and *vipA1*; no virulence factor genes were detected in ten of our strains (Table 5.1).

Chlorophyll *a* is a strong indicator of phototroph health and biomass and was used to quantitatively measure the condition of each alga when exposed to *V. parahaemolyticus*. The unarmored coccolithophore *E. huxleyi* was most susceptible to *V. parahaemolyticus*, with chlorophyll *a* decreasing by 71.5-96.3% (Figure 5.1A) after incubation. Variability among replicates was extremely low. No correlations could be made between content of virulence gene(s) and unarmored *E. huxleyi* biomass loss. Instead, consistently strong decreases in chlorophyll *a* fluorescence were observed in the

presence of all *V. parahaemolyticus* strains (One Way ANOVA, all p values <0.001). The avirulent *Vibrio* control, *V. pacinii*, did not cause any decreases in chlorophyll *a* in unarmored *E. huxleyi* or any other microalga.

All *V. parahaemolyticus* strains tested caused a significant loss in the diatom *T. pseudonana* and the dinoflagellate *P. minimum* biomass (Figure 5.1 B and C). The decreases in chlorophyll *a* from these species were not as extreme as those of unarmored *E. huxleyi*, amounting to 15-50% (*T. pseudonana*: 15.3 to 48.0%; *P. minimum* 14.7 to 53.3%). Compared to controls, all *V. parahaemolyticus* strains tested caused significant chlorophyll *a* decreases in both algae (One Way ANOVA, all p values <0.001), regardless of virulence gene content.

The armored *E. huxleyi* showed highest variability when incubated with *V. parahaemolyticus* (Figure 5.2). Three different results were observed when the armored *E. huxleyi* was exposed to *V. parahaemolyticus* strains. (a) Some strains had no effect (13 strains, One Way ANOVA, p values 0.09-0.88, no growth stimulation). (b) Some produced significant inhibition of armored *E. huxleyi* growth (8 strains, One Way ANOVA, p values <0.001 to 0.009). When exposed to these strains, the armored *E. huxleyi* grew (i.e.: the chlorophyll *a* fluorescence (biomass) increased) but this growth was significantly less than the control with no *V. parahaemolyticus*. (c) Significant losses in *E. huxleyi* biomass, as seen by decreases in chlorophyll *a* fluorescence (8 strains, One Way ANOVA, all p values <0.001). Once again, we found no correlation between content of virulence factor gene(s) and decreases in chlorophyll *a* fluorescence. For example, strain TBS 8-11-3 caused significant armored *E. huxleyi* biomass loss, yet no virulence factor genes were detected in this strain.

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Direct microscopic cell counts on all microalgae were also performed to confirm *in vivo* chlorophyll *a* data (Figure 5.3). As expected, chlorophyll *a* decreases were accompanied by strong decreases in microscopically visible cells. *In vivo* chlorophyll *a* fluorescence provides a rapid, strong indirect measure of phototrophic biomass when working with relatively concentrated cultures (Butterwick et al., 1982; Kalaji et al., 2014).

Dose response

We conducted dose response experiments (Figure 5.4) challenging unarmored *E. huxleyi* with three of our environmental *V. parahaemolyticus* strains at levels of 10^7 to 10^2 cells mL^{-1}. We found that even at low *V. parahaemolyticus* concentrations, this pathogen was able to reduce the biomass of unarmored *E. huxleyi*. As expected, the degree chlorophyll *a* loss decreased with reduced *V. parahaemolyticus* concentrations. For example, *V. parahaemolyticus* strain TBW 9-11-1 caused an 83.1% decrease in unarmored *E. huxleyi* chlorophyll *a* fluorescence at 10^7 cells mL^{-1}, while at 10^2 cells mL^{-1} a 55.2% decrease in chlorophyll *a* fluorescence was observed.

***vipA1* in environmental *V. parahaemolyticus* strains**

Six of our 27 (22%) environmental *V. parahaemolyticus* strains carried the T6SS marker gene *vipA1*. *vipA1* was also detected in the clinical reference strains ATCC 17802^T and ATCC 33846. The T6SS is only expressed when *V. parahaemolyticus* is also expressing lateral flagella on a surface (Salomon et al., 2013); however we did not find the T6SS marker gene *vipA1* exclusively in strains isolated from surfaces. Three environmental strains that contained *vipA1* were isolated from sediment (JS 8-11-5, JS 8-

11-6, 5-10-J5-4); the other three were isolated from water (JPW 8-11-1, JPW 8-11-9, JBW 9-11-5). Sequence data recovered from these amplicons confirmed amplification of the *vipA1* gene.

Cyanobacteria *in vitro* experiments

Cyanobacteria were exposed to a subset of our environmental *V. parahaemolyticus* strains; some that contained the antibacterial mechanism T6SS and some that did not. We found that regardless of content of *vipA1*, all of the *V. parahaemolyticus* strains tested stimulated cyanobacterial growth (Figure 5.5). No decrease of biomass was observed in either species of cyanobacteria, rather there was a significant increase in chlorophyll *a* fluorescence during co-incubation with *V. parahaemolyticus* (One Way ANOVA, all p values <0.001). There was no significant difference in cyanobacterial stimulation between strains that contained T6SS and strains that did not (Student's t-test, p values 0.214 and 0.252). The de-repressed strain POR1Δhns, which constitutively expresses T6SS, also caused cyanobacterial stimulation. The cyanobacteria were the only phototrophs that were consistently stimulated by the presence of *V. parahaemolyticus*.

DISCUSSION

V. parahaemolyticus is able to cause varying degrees of marine microalgae biomass loss *in vitro*. The unarmored *E. huxleyi*, which was the most susceptible to *V. parahaemolyticus*, is rarely observed in the environment, yet grows well under laboratory conditions. It has been hypothesized that unarmored *E. huxleyi* rarely survives in the environment due high susceptibility to predation (Paasche, 2001). Our data support this,

as our environmental *V. parahaemolyticus* strains caused the greatest loss in unarmored *E. huxleyi* biomass. As microscopic observations demonstrate, intact unarmored *E. huxleyi* cells were not readily observed after incubations with *V. parahaemolyticus*. Conversely, the armored version of *E. huxleyi* was not very susceptible to *V. parahaemolyticus*; only eight of our *V. parahaemolyticus* strains reduced armored *E. huxleyi* biomass. We hypothesized that cell wall surface structures and properties may play a role in susceptibility of microalgae to *V. parahaemolyticus*. Our data confirm this; the unarmored *E. huxleyi* was consistently and severely susceptible to all *V. parahaemolyticus* strains, while only a few strains had a negative effect on the armored version. The CaCO$_3$ coccoliths must protect the armored *E. huxleyi* cell and make this phenotype of *E. huxleyi* less susceptible to *V. parahaemolyticus*.

The dinoflagellate and diatom were also susceptible to *V. parahaemolyticus*. The intensity of biomass loss caused by *V. parahaemolyticus* was similar for both of these algae, which have rigid cell wall structures. Cellulose thecal plates protect *P. minimum* and a silica frustule covers *T. pseudonana* (Heil et al., 2005; Javaheri et al., 2015). Although these microalgae are covered by cell walls composed of differing materials, they were similarly susceptible to *V. parahaemolyticus*. Correlations between elevated *V. parahaemolyticus* densities and dinoflagellate and diatom blooms have been reported (Turner et al., 2009; Eiler et al., 2006; Asplund et al., 2011). Release of DOC from microalgae due to excretion may contribute to the association between *V. parahaemolyticus* and algal blooms, but direct predation on algae by *V. parahaemolyticus* presents an interesting additional aspect to this association.

We found no correlation between content of *tdh*, *trh*, or *vscC2* and microalgae biomass loss. The hemolysin genes *tdh* and *trh* have long been used as molecular markers of *V. parahaemolyticus* virulence. However, our results are consistent with recent reports (Park et al., 2004; Lynch et al., 2005; Jones et al., 2012) that destruction of eukaryotic cells does not exclusively rely on these hemolysin genes. Content of the T3SS marker gene *vscC2* was also not correlated with algal loss. Ten of the *V. parahaemolyticus* strains used contained no virulence factors, yet were able to cause decreases in algae chlorophyll *a* fluorescence. Our data suggest that another mechanism(s) by which *V. parahaemolyticus* damages these eukaryotes must exist. Further studies on the mechanism *V. parahaemolyticus* uses to cause damage to marine microalgae are needed.

Cyanobacteria were tested against a subset of our *V. parahaemolyticus* strains, some that contained the antibacterial mechanism T6SS, and some that did not. In addition, we used a T6SS knockout mutant and a T6SS de-repressed strain. All strains of *V. parahaemolyticus* caused cyanobacterial growth stimulation. We found no evidence that *V. parahaemolyticus* can decrease cyanobacteria biomass. It has been observed that cyanobacteria grow better in non-axenic laboratory cultures and our finding of cyanobacterial stimulation when incubated with *V. parahaemolyticus* is consistent with this observation. Perhaps *V. parahaemolyticus* and other heterotrophic bacteria consume or neutralize some inhibitory byproducts of cyanobacterial growth. *In vitro*, the presence of *V. parahaemolyticus* results in cyanobacterial stimulation and microalgae biomass loss. In the environment, *V. parahaemolyticus* may affect phototrophic population dynamics. Further experimentation is needed to determine if *V. parahaemolyticus* can

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induce changes in marine phototroph populations, perhaps by selective predation upon phototrophs having more susceptible cell wall structures.

All of the *in vitro* experiments performed used a high dose of *V. parahaemolyticus* to assure observation of damage if such occurred. We also determined what would happen when the unarmored *E. huxleyi* was incubated with varying concentrations of *V. parahaemolyticus*, including concentrations that more accurately mimic *V. parahaemolyticus* densities observed in the environment. As shown by our dose response curves, the unarmored *E. huxleyi* was susceptible to *V. parahaemolyticus* at low concentrations. These low concentrations (10^3 or 10^2 cells mL^{-1}) are similar to *V. parahaemolyticus* concentrations found in surficial sediment and shellfish (DePaola et al., 2003; Gamble and Lovell, 2011; West, 2012; Klein and Lovell, 2017). *V. parahaemolyticus* at low, "environmental" doses can still damage unarmored *E. huxleyi*, meaning that this interaction is certainly possible in particular environments.

In salt marshes along the US east coast, there are high concentrations of marine microalgae and other phototrophs in surficial sediment. In North Inlet estuary, where our *V. parahaemolyticus* strains were isolated, chlorophyll *a* readings in the sediment can reach as high as 101.5 mg chlorophyll *a* m^{-2} (Pinckney and Zingmark, 1993; Pinckney et al., 1994). *V. parahaemolyticus* concentrations in surficial sediment, particularly around fiddler crab burrows, can reach levels as high as 10^5 cells mL^{-1} (Gamble and Lovell, 2011; West, 2012; Gartmon et al., in preparation). Clearly, phototrophs and *V. parahaemolyticus* occur, and even bloom, in the same environments. We propose that it is no coincidence *V. parahaemolyticus* populations co-occur with marine microalgae as

marine microalgae provide a nutrient-rich resource that *V. parahaemolyticus* can utilize in these environments.

Table 5.1 Distribution of virulence-related genes in *Vibrio parahaemolyticus* strains used in algal co-incubations

Strain designation	*tdh*	*trh*	*vscC2*	*vipA1*
TS 8-11-4	+	+	+	-
JBW 8-11-2	-	+	+	-
CW 9-11-2	+	-	+	-
JPW 9-11-9	+	-	+	-
JPW 9-11-10	+	-	+	-
JPW 8-11-1	+	+	-	+
5-10-J5-4	+	+	-	+
ATCC 17802[T]	-	+	-	+
JPW 8-11-9	-	-	+	+
JBW 9-11-5	-	-	+	+
JPW 9-11-13	-	-	+	-
TBS 9-11-7	-	-	+	-
TS 9-11-6	-	-	+	-
JS 8-11-1	+	-	-	-
TBW 9-11-1	+	-	-	-
JBS 8-11-4	+	-	-	-
ATCC 33846	+	-	-	+
JS 8-11-5	+	-	-	+
JS 8-11-6	-	-	-	+
JBS 9-11-1	-	-	-	-
JPW 9-11-4	-	-	-	-
JS 9-11-3	-	-	-	-
TBS 8-11-3	-	-	-	-
JBW 9-11-4	-	-	-	-
JS 8-11-7	-	-	-	-
JS 8-11-9	-	-	-	-
TS 9-11-5	-	-	-	-
TPW 9-11-2	-	-	-	-
JS 8-11-2	-	-	-	-
POR1	-	-	+	+
POR1 Δhcp1	-	-	+	-
POR1 Δhns	-	-	+	+

Figure 5.1. A-C. Changes in algal chlorophyll *a* fluorescence during 24 h incubation with *Vibrio parahaemolyticus* strains. Dark bars indicate *V. parahaemolyticus* strains containing at least one virulence factor gene, white bars indicate strains that had no virulence factor gene, and the light gray bars are the algal and *Vibrio pacinii* controls. Algae include (A) the unarmored coccolithophore *Emiliania huxleyi,* (B) the diatom *Thalassiosira pseudonana*, and (C) the dinoflagellate *Prorocentrum minimum*

Figure 5.2. Changes in the armored coccolithophore *E. huxleyi* chlorophyll *a* fluorescence during 24 h incubation with *Vibrio parahaemolyticus* strains. Dark bars indicate *V. parahaemolyticus* strains containing at least one virulence factor gene, white bars indicate strains that had no virulence factor gene, and the light gray bars are the algal and *Vibrio pacinii* controls. During incubation with the armored *E. huxleyi*, *V. parahaemolyticus* strains either (a) had no effect on *E. huxleyi*, (b) significantly inhibited *E. huxleyi* growth or (c) significantly damaged *E. huxleyi*.

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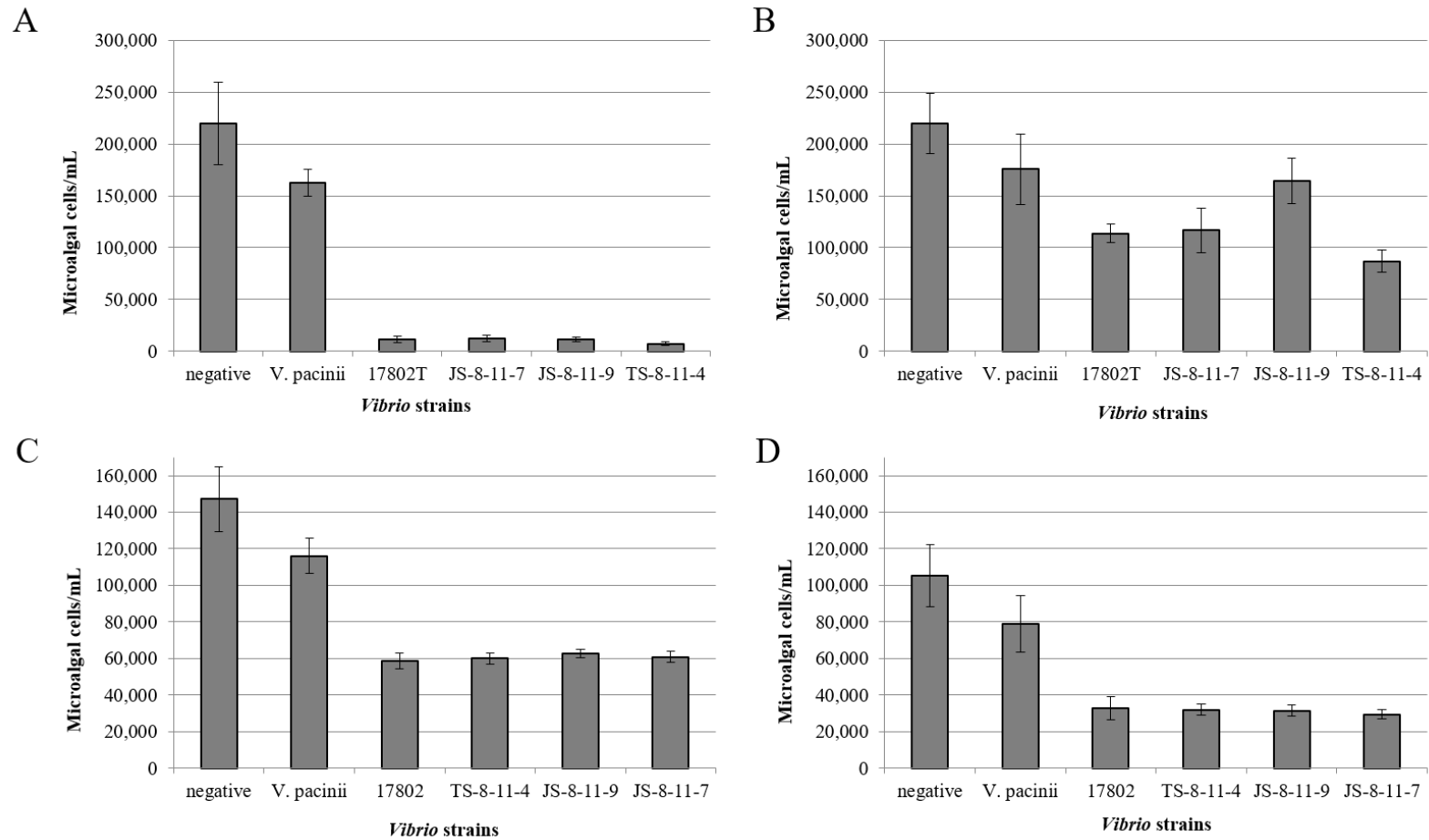
Figure 5.3. A-D. Microalgal cell densities after a 24 h incubation with *Vibrio parahaemolyticus* strains. Algae include (A) the unarmored coccolithophore *Emiliania huxleyi,* (B) the armored coccolithophore *Emiliania huxleyi* (C) the diatom *Thalassiosira pseudonana*, and (D) the dinoflagellate *Prorocentrum minimum*
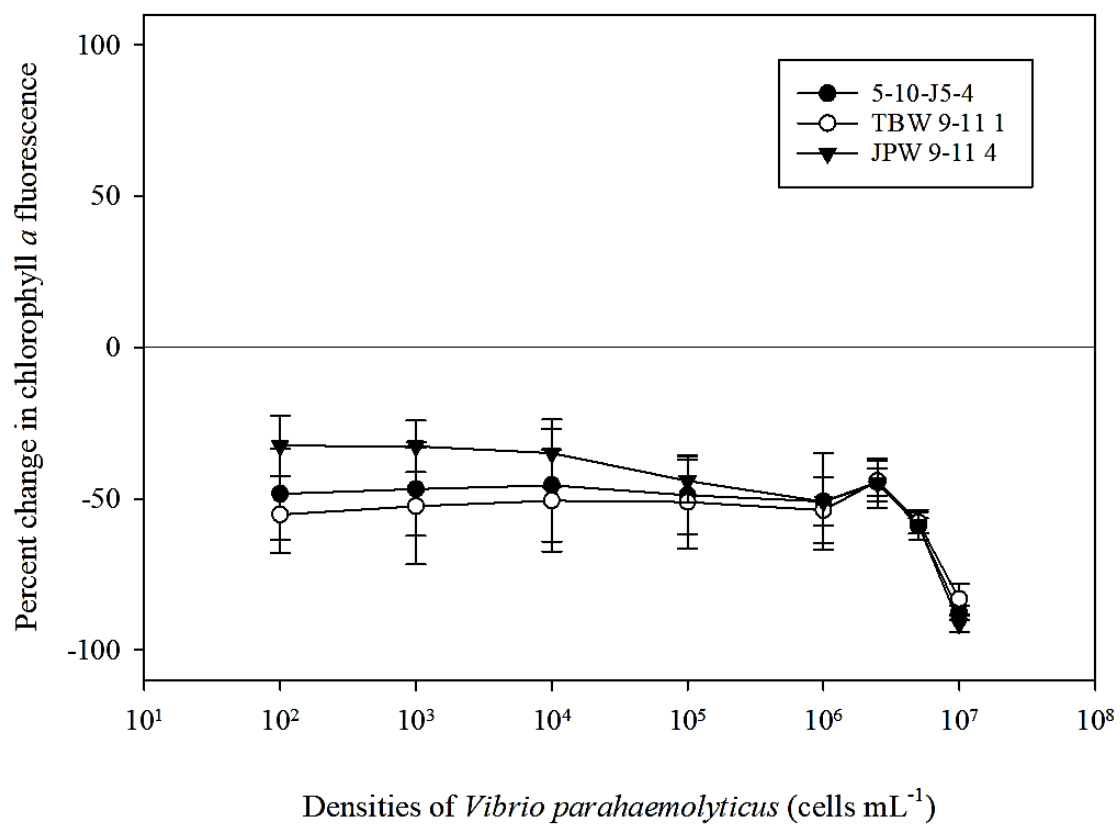
Figure 5.4. Dose response curve of unarmored coccolithophore *Emiliania huxleyi* chlorophyll *a* fluorescence during 24 h incubation with *Vibrio parahaemolyticus* strains at varying concentrations.
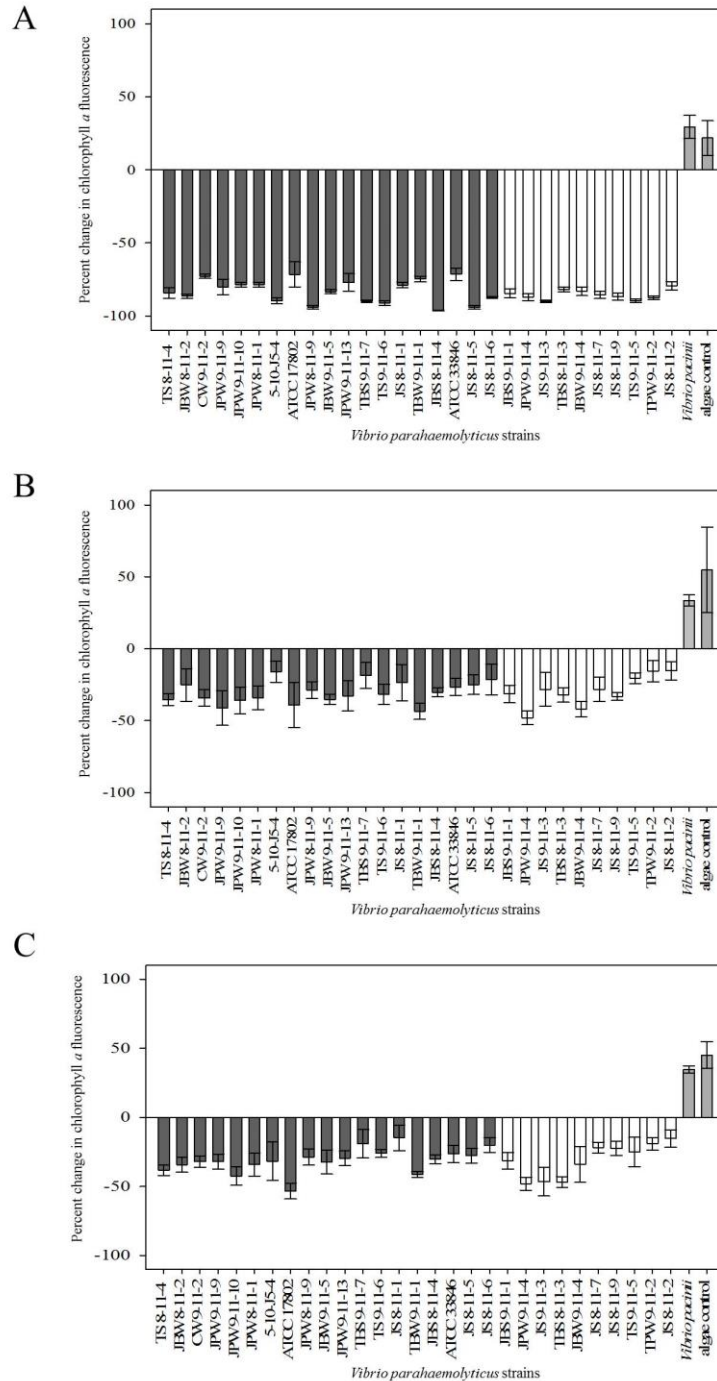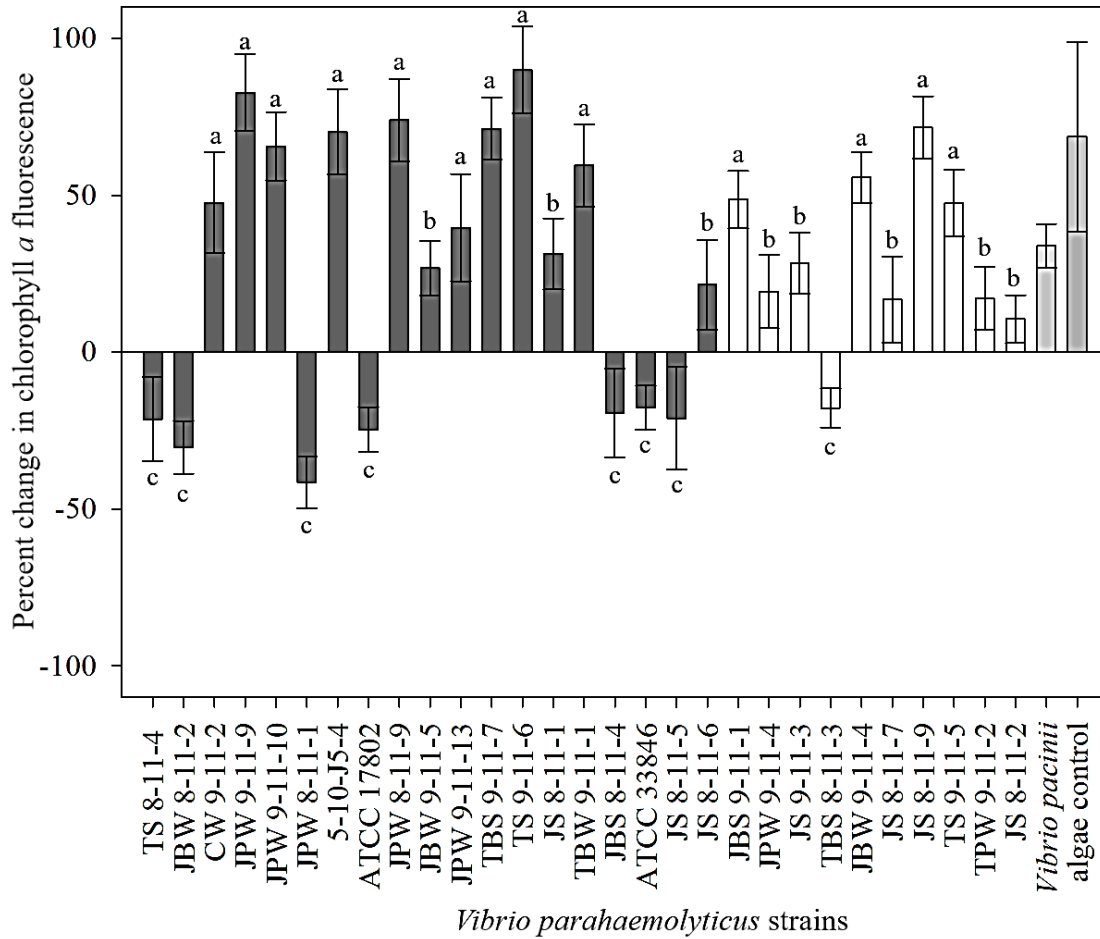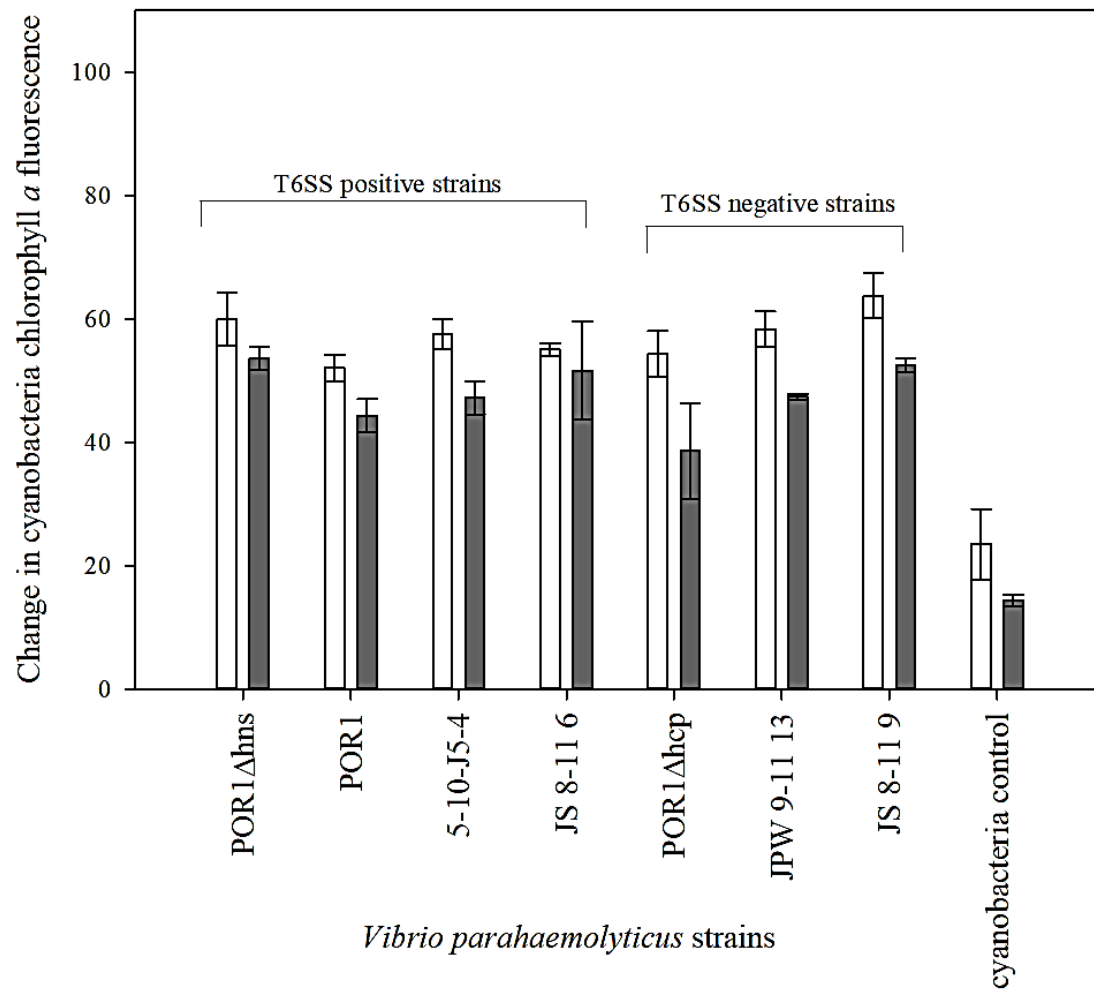
Figure 5.5. Changes in cyanobacterial chlorophyll *a* fluorescence during 24 h incubation with *Vibrio parahaemolyticus* strains. White bars indicate the cyanobacteria *Prochlorococcus marinus* was used and dark bars indicate the cyanobacteria *Synechococcus bacillaris* was used. A different subset of *V. parahaemolyticus* strains were used, some containing the antibacterial Type 6 Secretion System (T6SS) mechanism and some that did not. T6SS is de-repressed in POR1Δhns.

CONCLUDING REMARKS

Further studies are needed to accurately predict outbreaks of *V. parahaemolyticus* and to determine the virulence mechanism(s) of this organism. The interaction of *V. parahaemolyticus* with marine microalgae and detection of "hot" oysters provide some insight into where elevated numbers (blooms) of *V. parahaemolyticus* occur in estuarine environments. However, more work needs to be done into the mechanism of these interactions. It is still not known how a seemingly healthy oyster harbors significantly more *V. parahaemolyticus* than surrounding oysters exposed to the same environmental parameters. Determining what causes the anomalous "hot" oyster may offer a solution to avoid harvesting and serving these contaminated oysters to unsuspecting customers. However, since "hot" oysters occur so infrequently (1%), *V. parahaemolyticus* gastroenteritis remains a sporadic illness that is somewhat difficult to predict.

"A clear-cut, easily defined mechanism of *V. parahaemolyticus* pathogenesis has not emerged despite decades of intensive study (Lovell, 2017)."

Unlike *Vibrio cholerae*, which has two highly studied and well-defined virulence factors (CTX and TCP), the virulence mechanism of *V. parahaemolyticus* appears to be less obvious. The results of the cytotoxicity work indicate that *tdh*, *trh*, and T3SS2 are not the only virulence factors responsible for *V. parahaemolyticus* pathogenesis. Perhaps

the correct *V. parahaemolyticus* virulence mechanism(s) have not yet been identified. We suggest that more *V. parahaemolyticus* genomes need to be sequenced to determine if there are previously unrecognized virulence factors within the genome. One of the most interesting results of the PAI work is that >70% of a PAI can be compromised of undefined genes that encode hypothetical proteins. Perhaps one of these undefined genes encodes the true *V. parahaemolyticus* virulence mechanism. Or perhaps *V. parahaemolyticus* pathogenesis is more complicated that just having one or two virulence factor genes. Perhaps it is a complicated multistep infection model that utilizes numerous virulence factors working in synergy. With more high-quality genomic sequencing, our understanding of *V. parahaemolyticus* virulence will only grow.

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APPENDIX A

MARINE MICROALGAE MEDIA

Surface structure of microalgae and cyanobacteria

Two species of cyanobacteria were used in this study, *Prochlorococcus marinus* (CCMP 1986) and *Synechococcus bacillaris* (CCMP 1333). As is typical of gram negative prokaryotes, *P. marinus* and *S. bacillaris* have cell walls composed of peptidoglycan surrounded by an outer membrane and a glycocalyx of polysaccharides and polypeptides. Three species of eukaryotic microalgae were used, the diatom *Thallasiosira pseudonana* (CCMP 1335), the dinoflagellate *Prorocentrum minimum* (CCMP 695), and two strains of the coccolithophore, *Emiliania huxleyi* (CCMP 371 and CCMP 373). *E. huxleyi* CCMP 371 is a coccolith-producing (armored) form that often causes extensive blooms. *E. huxleyi* CCMP 373 is an unarmored mutant phenotype.

The bloom producing armored form can have as many as thirty $CaCO_3$ coccoliths forming multiple layers to protect the cell interior. The unarmored *E. huxleyi* lacks coccoliths and these strains are rarely isolated from the environment. Unarmored cells are thought to arise from armored cells via mutation; reversion back to the coccolith-forming morphology has not been reported (Paasche, 2001).

The centric diatom *T. pseudonana* is a model organism and was the first diatom chosen for genome sequencing (Armbrust et al., 2004). As is the case for all diatoms, the cell wall, or frustule of *T. pseudonana*, is composed of amorphous hydrated silica in a species-specific three-dimensional structure (Javaheri et al., 2005). The "petri dish" shape of centric diatoms is due to two unequal silicate halves (valves) that are connected by a series of girdles. Additionally, to prevent silica dissolution, the frustule is covered by an organic casing made up of glycoproteins (Javaheri et al., 2005). The dinoflagellate

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P. minimum is associated with harmful algal blooms (HABs) (Heil et al., 2005). *P. minimum* cells are protected by overlapping thecal plates composed of cellulose.

Cultivation of microalgae and cyanobacteria

Phototroph cultures were obtained from the Bigelow National Center for Marine Algae and Microbiota (Bigelow Center, East Boothbay, Maine). Each algal species required its own growth medium. Unarmored *E. huxleyi,* the dinoflagellate *P. minimum* and the cyanobacteria *S. bacillaris* was grown in F/2-Si (8.82×10^{-4} M NaNO$_3$, 3.62×10^{-5} M NaH$_2$PO$_4$·H$_2$O) supplemented F/2 trace metals (1.17×10^{-5} M Na$_2$EDTA·2H$_2$O, 1.17×10^{-5} M FeCl$_3$·6H$_2$O, 7.65×10^{-8} M ZnSO$_4$·7H$_2$O, 4.2×10^{-8} M CoCl$_2$·6H$_2$O, 9.1×10^{-7} M MnCl$_2$·4H$_2$O, 2.6×10^{-8} M Na$_2$MoO$_4$·2H$_2$O, 3.93×10^{-8} M CuSO$_4$·5H$_2$O) and with F/2 vitamins (2.96×10^{-7} M thiamine·HCl, 2.05×10^{-9} M biotin, 3.69×10^{-10} M cyanocobalamin). Armored *E. huxleyi* was grown in F/50, which is a 1/25 dilution of F/2-Si. *T. pseudonana* was grown in F/2+Si (add 1.06×10^{-4} M Na$_2$SiO$_3$·9H$_2$O to F/2-Si recipe). The cyanobacterium *P. marinus* was grown in Pro99 medium (5×10^{-5} M NaH$_2$PO$_4$·H$_2$O, 8×10^{-4} M NH$_4$Cl) supplemented with pro99 trace metals (1.17×10^{-6} M Na$_2$EDTA·2H$_2$O, 1.17×10^{-6} M FeCl$_3$·6H$_2$O, 8×10^{-9} M ZnSO$_4$·7H$_2$O, 5×10^{-9} M CoCl$_2$·6H$_2$O, 9×10^{-8} M MnCl$_2$·4H$_2$O, 3×10^{-9} M Na$_2$MoO$_4$·2H$_2$O, 1×10^{-8} M Na$_2$SeO$_3$, 1×10^{-8} M NiSO$_4$·6H$_2$O). All media recipes can be found on the Bigelow Center website (https://ncma.bigelow.org/algal-recipes). Microalgae and cyanobacteria were grown at 23°C with an 11 h light, 13 h dark cycle.

APPENDIX B

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