LATERAL GENE TRANSFER OF PANDEMIC *VIBRIO PARAHAEMOLYTICUS* GENOMIC ISLAND GENES.

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LATERAL GENE TRANSFER OF PANDEMIC *VIBRIO PARAHAEMOLYTICUS* GENOMIC ISLAND GENES.

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This thesis is dedicated to Mom, Dad, Nancy, Sandy, Ernie, Al, and Abigail. I cannot thank you all enough for your love and support.

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LIST OF SYMBOLS AND ABBREVIATIONS

LGT	Lateral Gene Transfer
MGE	Mobile Genetic Element
TDH	Thermostabile direct hemolysin
TL	Thermolabile hemolysin
TRH	Thermostabile direct related hemolysin
T3SS	Type III Secretion System
T3SS1	Type III Secretion System gene cluster 1
T3SS2	Type III Secretion System gene cluster 2
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
OpaR	Opacity Regulator
KP	Kanagawa Phenomenon
VpaI	*Vibrio parahaemolyticus* Island
TCBS	Thiosulfate Citrate Bile Salts Sucrose
MLST	Major Locus Sequence Typing

SUMMARY

Vibrio parahaemolyticus is an emerging enteric pathogen. The O3:K6 serotype has become pandemic, and the genome has been sequenced for one strain of this serotype (20). The thermostabile direct hemolysin (*tdh*), thermostabile related hemolysin (*trh*), and two distinct Type III Secretion Systems (T3SS) have been implicated in virulence. T3SSs secrete their own virulence proteins called effectors, and the hemolysins are secreted independently of the T3SS. Deletion studies have determined that the T3SS alone are sufficient to induce eukaryotic cell apoptosis. The T3SS loci are found within two separate genomic islands. Because genomic islands are known to be disseminated by Lateral Gene Transfer (LGT), we examined the distribution of the pandemic genomic island genes among clinical and environmental *V. parahaemolyticus* isolates and also among closely-related environmental *Vibrio* spp. using primers designed to amplify hemolysins and T3SS effectors previously characterized for *V. parahaemolyticus* strain RIMD2210633 (23). We also examined the distribution of a hypothetical bacteriocin, encoded by *vpa1263*, within another genomic island that is similar to the bacteriocins produced by *Escherichia coli*. Preliminary screens for the bacteriocin gene suggest that many clinical strains and some environmental strains contain *vpa1263*. PCR screens for the T3SS effector genes have shown that 22% of closely-related environmental *Vibrio* spp. contain at least one T3SS1 or one T3SS2 effector gene. Strains selected as closely-related environmental *Vibrio* spp. for this analysis were identified as yellow sucrose-fermenting colonies on the selective Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar, while environmental isolates of *V. parahaemolyticus* were identified as green colonies on TCBS. Because PCR screens have detected the presence of the *V. parahaemolyticus*

thermolabile hemolysin (*tl*) within strains that grew yellow on TCBS, we may have overestimated the distribution of T3SS effector genes. Alternately, the *Vibrio parahaemolyticus tl* gene could be transferred to closely-related species, or *V. parahaemolyticus* may have aquired the ability to ferment sucrose. While sequencing of the effector genes and the housekeeping gene *recA* are ongoing, preliminary results suggest that the *tl* positive isolates that grow yellow on TCBS, may actually be *V. parahaemolyticus*. Since *V. parahaemolyticus* may have acquired the ability to ferment sucrose, the TCBS plating method may not be conclusive enough for the separation of *Vibrio* spp.

CHAPTER 1

INTRODUCTION

1.1 *Vibrio parahaemolyticus*: an emerging pathogen

Vibrio parahaemolyticus is an emerging enteric pathogen that was first discovered in Japan in 1950 as the cause of a food poisoning outbreak (14). This Gram-negative marine bacterium is now the major cause of seafood borne gastroenteritis within the United States and the major cause of all foodborne infections in Asian countries (14). Although humans mainly become infected with the bacterium by consuming raw or undercooked shellfish, septicemia can also result from wound infections (14, 26). Nevertheless, infections are thought to be underreported (6). Prior to 1996, pathogenic strains exhibited diverse serotypes; however, since this time, the O3:K6 serotype and related strains have become pandemic (14). As a result of the pathogen's emergence, the genome has been sequenced for the RIMD2210633 strain of the O3:K6 serotype. Two virulence genes have been previously identified from *V. parahaemolyticus* pathogenic strains. These genes include the thermostable direct hemolysin (*tdh*) and the *tdh*-related hemolysin (*trh*) (26). More recently, the Type III Secretion System (T3SS) effector genes have been shown to contribute to the virulence mechanism of *V. parahaemolyticus* (3, 14, 25, 26) . Although the presence of hemolysins has been used as an indicator of the pathogenicity of clinical isolates, hemolysin-deficient strains have recently been shown to be pathogenic (26). The indication that screening for hemolysins alone may underestimate pathogens has spurred further research of the T3SSs. While researching

the function of potential virulence genes will benefit the medical community in diagnosing and treating infections, few studies have examined the role these genes play in the emergence of the pathogen. Epidemics of *V. parahaemolyticus* infection have become more frequent and more globally distributed within the last 10 years (14); however, the pathogen's emergence remains to be elucidated.

To address the pathogen's emergence, Hurley et al. 2006 examined the distribution of T3SS effector genes between pathogenic clinical and non-pathogenic environmental *V. parahaemolyticus* samples collected over several years (14), but these researchers have not sequenced the effector genes to determine their diversity within the species. This study examined the genomic island distribution primarily among clinical *V. parahaemolyticus* strains. To date, there have been no studies that have analyzed the sequence diversity of the T3SS effector genes between the pathogenic and the non-pathogenic environmental isolates. Furthermore, studying only *V. parahaemolyticus* isolates without examining other species of *Vibrio* from the same niche (14) may underestimate the reservoir of virulence genes. This is because the Lateral Gene Transfer (LGT) of pandemic *V. parahaemolyticus* virulence genes to non-*V. parahaemolyticus* strains or vice versa will affect the distribution of the genes. The distribution of *V. parahaemolyticus* virulence genes by LGT was briefly assessed (26) in the study by Park et al, but the researchers used a hybridization technique that was too stringent to detect nucleotide substitutions. They showed that T3SS genes were present in *V. alginolyticus, V. harveyi,* and *V. tubiashii* strains (26); however, no studies to date have examined whether co-occurring non-*V. parahaemolyticus* strains in the environment may serve as a reservoir for T3SS genes. Therefore, we propose that a polymerase chain reaction (PCR)

screen for the pandemic virulence genes and subsequent gene sequencing may be more appropriate for examining virulence gene distribution by LGT.

LGT can also disseminate large gene clusters called genomic islands. Seven genomic islands have been identified within the RIMD2210633 genome and are denoted as VPaI-1 through VPaI-7 (14). Because the hemolysins and two T3SS gene clusters are located within the genomic islands VPa-1 and VPaI-7, we hypothesized that the virulence genes undergo LGT and that this may explain the pathogen's emergence. We have previous evidence to support this hypothesis based on the identification of a genomic island gene on the plasmid of an environmental *V. parahaemolyticus* isolate. This gene is a putative bacteriocin and will be discussed in Chapter 2. Briefly, the gene was isolated from the environmental *V. parahaemolyticus* 22702 strain, and it was 96% identical to the *vpa1263* gene found within VPaI-6 of the pandemic O3:K6 serotype. Thus more broadly, we hypothesize that pandemic *V. parahaemolyticus* genomic island genes, which include the hemolysins, the T3SSs, and the putative bacteriocin will be present in environmental *V. parahaemolyticus* and other co-occurring *Vibrio* species (Figure 1.1).

To more accurately determine the size of the potential *V. parahaemolyticus* virulence gene reservoir, we will:

1. Examine the distribution of T3SS effector protein-encoding genes and hemolysin genes in *V. parahaemolyticus* environmental isolates and other co-occurring *Vibrio* species.
2. Determine the sequence diversity of T3SS effector protein-encoding genes to examine the evolution of these genes and the emergence of the pathogen.

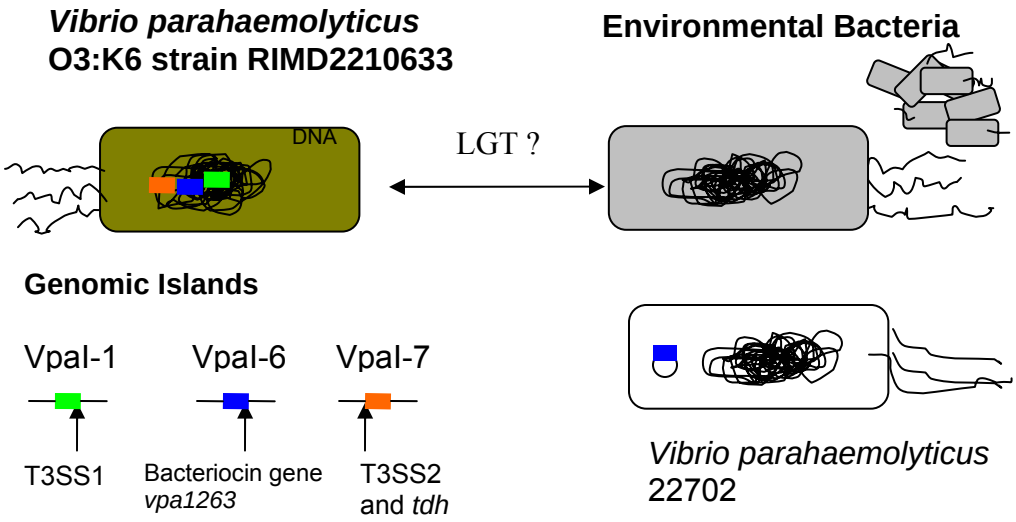

Figure 1.1 Lateral Gene Transfer of *V. parahaemolyticus* genomic island genes. *V. parahaemolyticus* strain RIMD2210633 contains 7 genomic islands. VpaI-1, VpaI-6, and VpaI-7 are shown. VpaI-1 encodes a T3SS1 injectisome and its associated effector proteins VP1680 and VP1686. VpaI-7 encodes the T3SS2 injectisome, the T3SS2 translocator protein VPA1354, the T3SS2 effector proteins VPA1346 and VPA1362, and two copies of Tdh. The environmental strain of *V. parahaemolyticus* 22702 contains a plasmid encoding *vpa1263*. Our study will examine whether the bacteriocin gene and the T3SS effector genes, and hemolysin genes undergo lateral gene transfer.

1.2 *Vibrio parahaemolyticus* virulence genes

The most well-studied *V. parahaemolyticus* virulence genes are the hemolysins (23). These include the thermostable direct hemolysin (*tdh*) and the thermostable direct-related hemolysin (*trh*) (23). The proteins encoded by these genes were named hemolysins due to their ability to lyse erythrocytes. Hemolysins contribute to lysis by forming pores in bacterial membranes, which modifies the ion balance (5). Typically, only 1-6% of *V. parahaemolyticus* strains in the environment have been shown to have either *tdh* or *trh* (18). Expression of either gene results in the β- type hemolysis observed Wagatsuma agar, which is known as the Kanagawa Phenomenon (KP). The KP detection

assay has served as the clinical confirmation of pathogenicity (26). Because the observation of KP is time consuming and some clinical strains have been isolated that lack *tdh* and are KP negative (2), the following alternative in vitro diagnostic measures have emerged: immunological techniques employing monoclonal antibodies specific for hemolysins (2), multiplex PCR reactions to amplify hemolysin genes (2), and pulsed-field gel electrophoresis (PFGE) of restriction endonuclease sites (21, 26). Nevertheless, observation of KP and serotyping remains standard in identifying *V. parahaemolyticus* pathogenic strains. The thermostabile direct hemolysin related gene, *trh*, was examined in multiple *Vibrio* spp., and was observed in *Vibrio alginolyticus* (12). One recent study showed that *tdh* can be spontaneously deleted due to its association with insertion sequences, which suggests that rearrangement and LGT of *tdh* may be occurring (16).

After the RIMD2210633 strain had its genome sequenced, two additional virulence mechanisms were discovered. These include two distinct Type III Secretion Systems (T3SS) known as T3SS1 and T3SS2. To date there have been no studies that have examined the reservoir of T3SS genes in environmental *Vibrio* populations.

1.3 Bacterial Secretion Systems

Currently, six bacterial secretion systems have been identified (30).The secretion system basal components are structurally similar to flagellum basal body (8). Gram-positive bacteria tend to secrete extracellular proteins via a general secretion pathway, but because Gram negative bacteria contain a periplasmic space and an outer membrane, they often possess specialized secretion systems (28). Both the T3SS and the type 4 secretion system (T4SS) have been identified in *V. parahaemolyticus* and are involved with

virulence and injection of effector proteins into the cytosol of a host cell (4, 8). The T4SS is found in *Helicobacter pylori*, *Legionella pneumophila* and *Bordetella* pertussis (4, 28). Secretion systems have been found on pathogenicity islands (28). Recently, a Type 6 Secretion System was reported in *Vibrio parahaemolyticus*, and it was predicted to have homology to a strain of *V. harveyi* (5), that we used as an outgroup in our gene trees in this study.

1.3.1 Type III Secretion Systems

Several Gram-negative bacteria including *Shigella* spp., and *Salmonella* spp. (20), enteropathogenic and enterohemorrhagic *Escherichia coli*, *Yersinia* spp., and *Pseudomonas* spp. contain complex T3SS (4). The T3SS apparatus, or injectisome (7), is essentially a small needle comprised of about 20 proteins (3, 25) that pierces the cell membrane of a eukaryotic cell and injects effector molecules into the host cell cytosol. T3SS structures are highly conserved between bacteria while the effectors are more variable (25). Once the effectors are injected into eukaryotes they often target the cytoskeleton (4, 6) and disrupt signaling pathways (3). One pathway that is often disrupted is the NFκB (4). For example, the *Vibrio parahaemolyticus* T3SS1 effector VP1686 has been shown to result in apoptosis through the inhibiton of NFκB (3).

1.4 Bacteriocins

Bacteriocins are protein antimicrobials secreted by bacteria that typically inhibit closely related bacterial species (1, 31) by either forming pores in the cytoplasmic membranes, non-specifically degrading nucleic acids, cleaving 16S rRNA or tRNA, or inhibiting peptidoglycan synthesis (1). Often, bacteria that produce bacteriocins contain immunity proteins to protect themselves from their own bacteriocin (1, 31). The protein domains of a bacteriocin can be separated based on local function (1). It is believed that bacteriocins result in enhanced fitness for a cell through the competitive inhibition of bacteria that share the same niche (1, 31). Bacteriocins have been identified from both Gram-negative and Gram-positive bacteria. The bacteriocins produced by *Escherichia coli* are referred to as colicins (27). *Vibrio cholerae* (27), *Vibrio Vulnificus* (27, 29), and *Vibrio harveyi* (27) each have their own bacteriocins. Research with the mucosal pathogen *Streptococcus pneumoniae* shows a competitive advantage for bacteriocin production in the nasopharynx (31), while studies show colicin producing *E. coli* can gain a competitive advantage in the intestines (17)

Currently, the Food and Drug Administration agency of the USDA has approved the bacteriocin nisin for use in agricultural products. Nisin is produced by *Lactobacilus lactis* (1, 10), but because bacteriocins must be over-expressed and purified in bulk to be practical for the agricultural industry as topical antimicrobials, several companies have begun using live cultures of commensal bacteria in their products (24). This practice is termed probiotics and occurs naturally in dairy products (24), particularly yogurt.

1.5 Lateral Gene Transfer

The transfer of genetic material between an organism or cell and its offspring is considered vertical gene transfer. In contrast, Lateral Gene Transfer (LGT) is the transfer of genetic material between different species (32).Genetic material that is disseminated by LGT is often transferred via Mobile Genetic Elements (MGEs) (11). MGEs include bacteriophages, plasmids, transposons, and insertion sequences (Figure 1.2). Abnormal GC nucleotide content of a gene or genomic island relative to a genome is often used to determine whether LGT has occurred (15).

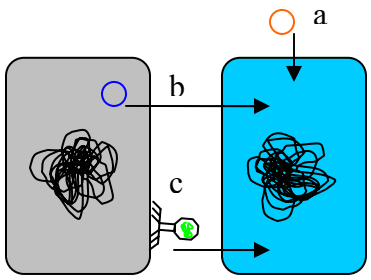

Figure 1.2 Lateral Gene Transfer. Bacteria can acquire novel genetic material from the environment by transformation (a), directly from another bacterium by conjugation (b), or through viral nucleic acid integration and subsequent transduction (c).

1.6 Genomic Islands

Genomic islands are large regions of the genome characteristically flanked by direct repeats and either integrase or transposase genes, which suggests the distribution of genomic islands is mediated by MGEs (14). MGEs can contribute to the evolution of bacterial pathogenicity by transferring virulence genes. For example, cholera toxin deficient strains of *V. cholerae* that express the toxin co-regulated pilus (TCP) can acquire the *ctxAB* cholera toxin genes from the CTXΦ phage and become pathogenic (34). Likewise, pathogenicity can be imparted following the acquisition of a

pathogenicity island, which is simply a genomic island carrying one or more virulence genes. The RIMD2210633 strain is proposed to have increased virulence due to the presence of predicted pathogenicity islands (14).

1.7 Genomic Islands of *V. parahaemolyticus*

Seven genomic islands were identified in *V. parahaemolyticus* and are denoted VPaI-1 through VPaI-7 (14). Virulence genes were identified on the predicted 10-kb pathogenicity island VPaI-1 and the 81-kb region VPaI-7. The T3SS1 gene cluster is located within VPaI-1 on chromosome I, and the T3SS2 gene cluster is located within VPaI-7 on chromosome 2. T3SS2 was present in all pandemic strains analyzed (23), and T3SS1 was found in all *V. parahaemolyticus* isolates examined. In addition to the T3SS2 gene cluster, VpaI-7 (23) also carries two copies of the thermostable direct hemolysin (*tdh*) gene (23). Since LGT can disseminate genes carried within genomic islands, we studied the distribution and diversity of the T3SS effector genes and the hemolysin genes,

A separate genomic island identified among *V. parahaemolyticus* pandemic strains, VPaI-6, contains the gene *vpa1263*, which is a putative bacteriocin gene (14). This gene is homologous to the colicins of *Escherchia* coli. Since LGT can disseminate genes carried within genomic islands, we also studied the distribution of the hypothetical bacteriocin gene.

CHAPTER 2

VIBRIO PARAHAEMOLYTICUS PUTATIVE BACTERIOCIN

2.1 The putative *V. parahaemolyticus* bacteriocin gene *vpa1263*

The *V. parahaemolyticus* O3:K6 serotype strain RIMD2210633 contains the gene *vpa1263* within the VpaI-6 genomic island. This gene shares a conserved domain with the colicins of *Escherichia coli* and s-type pyocins of *Streptococcus* spp. We hypothesized that the hypothetical protein encoded by this gene is a bacteriocin that inhibits growth of *V. parahaemolyticus* and related *Vibrio* spp. To determine whether the bacteriocin is functional, we used the flip-agar technique previously developed to screen for bacteriocinogenic activity of *Vibrio. harveyi* (22). *vpa1263* is the first bacteriocin-like gene to be reported for *V. parahaemolyticus*.

2.2 Experimental design for bacteriocin function

We used the flip-agar technique to determine whether any strains were bacteriocinogenic. In this method, a 10ul sample of 1x 10^9 cells/ml culture of RIMD2210633 was spotted on the agar and incubated at 30˚C for 24 hours. After incubation, the agar was inverted into the lid of the Petri dish and an indicator strain was plated on the newly exposed agar at approximately 10^7 cells/ml. In this assay bacteriocins that are produced would have diffused through the media during the overnight incubation and inhibit the growth of indicator strains.

To ensure that bacteriocin production is due to *vpa1263*, we are currently conducting a gene deletion to assess whether bacteriocin production is abolished in its

absence. The deletion is based on homologous recombination of the flanking regions of a fusion product of the *vpa1263* gene. We designed primers to amplify flanking regions of the *vpa1263* gene then fused them together. Using restriction digests and ligations we constructed a suicide vector containing the flanking DNA regions (Figure 2.1). This vector was then used to force the *vpa1263* gene from the bacterial chromosome by homologous recombination with the flanking regions (Figure 2.1). Due to the size of the gene and the occurrence of spontaneous mutations in the *sacB* gene of the suicide vector, we have had difficulty obtaining colonies that have undergone homologous recombination and loss of the suicide vector. Spontaneous mutations in *sacB* eliminate the sucrose sensitivity counter-selection required for loss of the suicide vector. We are investigating whether we can use an alternate vector that does not rely on sucrose sensitivity for loss of the vector and completion of the gene deletion.

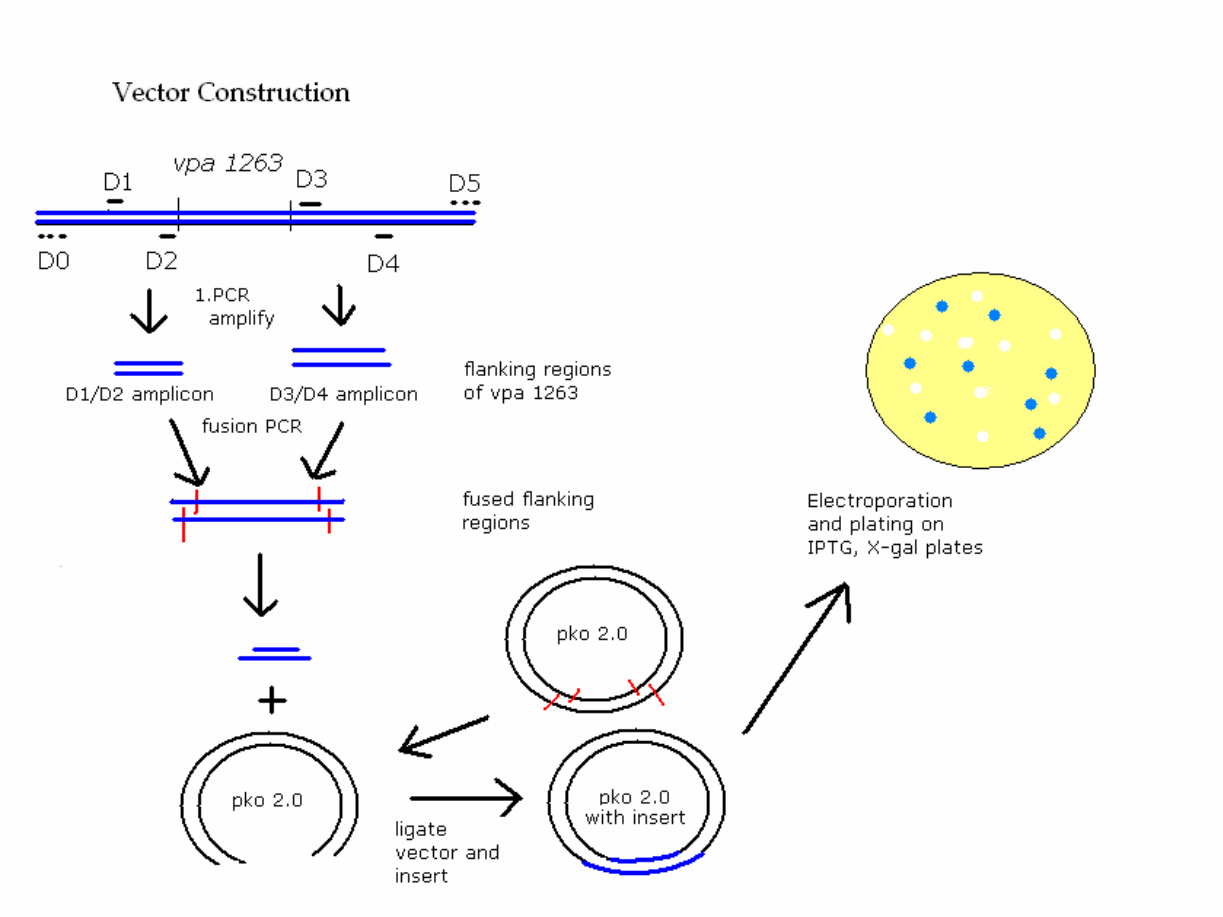

Figure 2.1. Vector construction for *vpa1263* deletion. DNA flanking the bacteriocin was amplified separately using primers D1, D2, and D3, D4 respectively. The D1/D2 amplicon and the D3/D4 amplicon were then fused together using the external D1 and D4 primers. Fusion was possible because the D2 and D3 primers were designed with homologous ends. The resulting amplicon and the suicide vector pko2.0 were separately double digested with the same restriction enzymes ApaI and SacI / XhoI. The digested amplicon was then inserted into the vector. The resulting vector was then electroporated into electrocompetent *E. coli* cells and white colonies were selected as successful transformations.

2.3 Distribution of *vpa1263*

Additionally, we discovered that the plasmid p22702B, isolated from the *V. parahaemolyticus* strain 22702, contains a gene with 96% homology to the *vpa1263* gene found in RIMD2210633. Because the RIMD2210633 *vpa1263* gene is located within the genomic island VPaI-6, we further hypothesized that the gene may be undergoing LGT

mediated by MGEs. To determine whether the gene occurs more frequently in environmental populations independent of the pandemic VPaI-6 genomic island, we decided to collect environmental samples and use polymerase chain reaction (PCR) (Figure 2.2) to detect the presence of the gene.

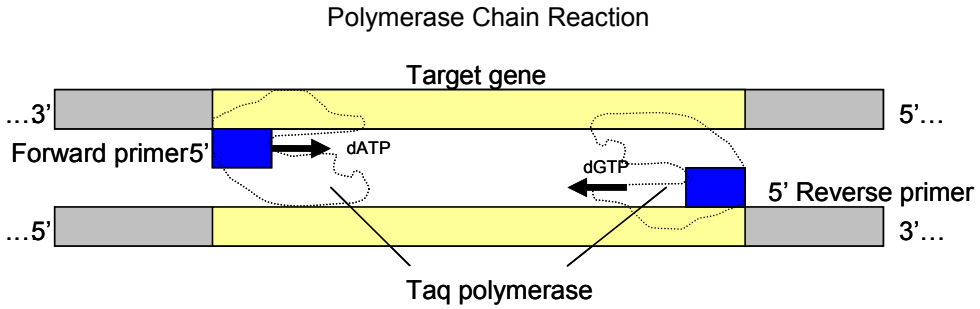

Figure 2.2. PCR annealing step.

2.4 Experimental design to determine *vpa1263* distribution

We collected sediment and water samples from marshes located at Skidaway Island GA, North Inlet, NC, and Apalachicola, FL in September 2006 and 2007. Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (Difco), was prepared to culture the bacteria and identify *Vibrio* spp. The sucrose in the green TCBS media is not fermented by *V. parahaemolyticus* and the colony remains green. Other *Vibrio* species, such as *Vibrio cholerae* and *Vibrio alginolyticus*, appear yellow following the fermentation of sucrose(13). It should be noted that *Vibrio vulnificus* also appears green on TCBS (13), and further identification through Major Locus Sequencing Typing (MLST) of conserved housekeeping genes is often necessary to determine whether green colonies on TCBS are indeed *V. parahemolyticus*. We isolated presumptive *V. parahaemolyticus* green colonies and the yellow co-occurring *Vibrio* species and extracted their DNA by boiling a colony

with nuclease free water. To screen for the presence of *vpa1263* in our isolates we designed the following forward and reverse primers.

Forward 5'- GCTCTGCTTAGTTACAACGACCCA -3'

Reverse 5'- ATTTGGGAAGGTATTGGAGCACCC -3'

These primers yield an amplicon of 2126-bp, and were designed using the Primer3 website and purchased from Integrated DNA Technologies (IDT). Additionally, we used the IDT OligoAnalyzer internet feature to design primers that are unlikely to form homodimers, heterodimers, or hairpins, which would decrease the efficiency of the amplification. We used Polymerase Chain Reaction (PCR) to amplify presumptive *vpa1263* genes to detectable concentrations from our isolates in a thermocycler. PCR reagents were purchased from New England Biolabs. The final volume of the PCR reaction was 20ul with the final concentrations: 0.5uM for *vpa1263* forward and reverse primers, 1Xbuffer, 0.02 U/ul taq polymerase, 2.5mM $MgCl_2$, and 0.5mM dNTPs. The following PCR cycle conditions were used: an initial denaturation step at 98°C for 2 minutes, 35 cycles of (denaturation at 98°C for 15 seconds, annealing at 58°C for 30 seconds, and elongation at 72°C for 1 minute), and a final elongation at 72°C for 5 minutes. The PCR amplicons were separated by size using agarose gel electrophoresis and were visualized via the incorporation of Ethidium Bromide and viewed under UV with a gel doc. Bands were compared to a 2-log ladder.

2.5 Results for bacteriocin function

Bacteriocin production by the RIMD2210633 strain was observed with fine-texture Acros agar (Figure 2.3), as shown by the zones of inhibition within the indicator growth, but was not observed with the coarse Difco agar. This suggests that the bacteriocin's activity is limited by diffusion. Table 2.1 shows that RIMD2210633 inhibited 44% of *V. parahaemolyticus* clinical isolates and 39% of *V. parahaemolyticus* environmental isolates. Bacteriocin production from RIMD2210633 did not inhibit the more distantly related species of *V. harveyi* and *V. fischeri*. RIMD2210633 was able to inhibit itself when grown as both a producer and an indicator.

Preliminary data suggests that deletion of the quorum-sensing regulator *opaR* resulted in a loss of the inhibitory activity (data not shown). Further experiments are necessary to confirm the role of quorum-sensing for regulation of bacteriocin production.

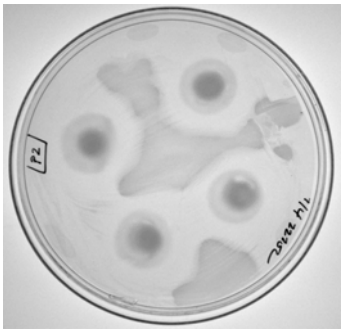

Figure 2.3. Flip agar bacteriocin screening assay. Zones of clearing surrounding RIMD221063 show the inhibition of an environmental indicator strain.

Table 2.1. Summary of Bacteriocin Activity

Strains	Activity (mm)	No. inhibited (%)
Environmental *V. parahaemolyticus*	++++	7/18 (39)
Clinical *V. parahaemolyticus*	++++	7/16 (44)
V. harveyi	-	0/1
V. fischeri	-	0/1

* Vp clinical strains isolated from patients include RIM2210633 and 25 *V. parahaemolyticus* strains from the CDC. Environmental isolates were taken from marshes located at Skidaway Island Georgia, North Carolina, and Florida in 2006 and 2007.

2.6 Results for the distribution of *vpa1263*

To determine whether the putative bacteriocin gene *vpa1263* may be undergoing LGT, we examined the distribution of *vpa1263* within clinical and environmental isolates (Table 2.2). We also examined the distribution of an adjacent gene on VpaI-6, *vpa1260*, to assess whether *vpa1263* is likely to be present on the genomic island or if it may be independent of VpaI-6. In 60 environmental isolates examined, we found that 17% contained vpa*1260* alone, 30% contained *vpa1263* alone, and 17% contained both *vpa1263* and *vpa1260*. All nine O3:K6 clinical isolates examined were double positive for *vpa1260* and *vpa1263*, while only 27% of non-O3:K6 clinical isolates were positive for both genes. Environmental isolates have a higher frequency of *vpa1260* alone and *vpa1263* alone than either the O3:K6 clinical strains or non-O3:K6 clinical strains, while the clinical isolates have a higher incidence for both genes. A greater percentage of environmental isolates contain *vpa1263* (47%) relative to non-O3:K6 clinical strains (34%).

Table 2.2. Frequency of *vpa1263* and the VPaI-6 gene *vpa1260*

		Frequency (%)		
Strain Source	No.	*vpa1260* only	*vpa1263* only	*vpa1260* and *vpa1263*
Environmentals	60	4 (17)	7 (30)	4 (17)
Clinicals O3:K6	9	-	-	9 (100)
Clinicals non-O3:K6	30	3 (10)	2 (7)	8 (27)

* Vp clinical strains isolated from patients include RIM2210633 and 25 *V. parahaemolyticus* strains from the CDC. Environmental isolates were taken from marshes located at Skidaway Island Georgia, North Carolina, and Florida in 2006 and 2007.

2.7 Discussion

The flip agar method shows that *V. parahaemolyticus* strain RIMD2210633 produces a bacteriocin-like inhibitory substance (Figure 2.3) that can inhibit the growth of both clinical and environmental strains of *V. parahaemolyticus*, but is unable to inhibit *V. haveryi* or *V. fischeri*. This may indicate that the *V. parahaemolyticus* bacteriocin has a narrow range of inhibition; however, because we only examined two other species (Table 2.1), bacteriocin screening with a larger sample of other *Vibrio* species is necessary to determine an inhibitory spectrum. To confirm that *vpa1263* is responsible for bacteriocin production, the generation of a deletion mutant is ongoing; nevertheless, a deletion in *opaR* showed loss of inhibitory activity. **Because the *opaR* deletion strain showed diminished bacteriocin production, we suspect that bacteriocin production may be regulated by quorum sensing; however, further experiments are required to confirm the role of quorum-sensing**.

Expression analysis with RT-PCR through varying stages of the growth cycle may provide insight into circumstances that lead to bacteriocin production. If these studies suggest that *vpa1263* is expressed in greater concentrations at higher cell densities, then there may be a selective advantage for producing the bacteriocin at the population level even without immunity proteins.

The gene distribution for *vpa1260* and *vpa1263* within environmental isolates shows that *vpa1260* is present in environmental isolates just as frequently by itself as it is to be found with *vpa1263* (Table 2.2). This finding may indicate that *vpa1260* is just as likely to be independent of VpaI-6 as it is to be found within the genomic island. Because the percentage of environmental strains that contain *vpa1263* alone (30%) is greater than the percentage with both *vpa1263* and *vpa1260* (17%), this may suggest that *vpa1263* is more likely to be found independent of VpaI-6 than within the genomic island. Alternately, these findings may indicate that either the *vpa1263* primers anneal with greater efficiency, or *vpa1260* may have greater sequence diversity than *vpa1263*. Our result that fewer non-O3:K6 isolates (27%) are positive for both *vpa1263* and *vpa1260* than the O3:K6 strains (100%) is in concordance with prior studies that suggest the O3:K6 serotype and other pandemic isolates have a higher incidence of genomic island genes, such as the T3SS2 gene cluster. Interestingly, environmental isolates seem to be more likely to contain *vpa1263* than non-O3:K6 clinical strains. This finding may relate to the fact that bacteriocins are generally active only against closely related strains. Since *V. parahaemolyticus* is a marine bacterium it is more likely to encounter similar strains in the ocean than in the gastrointestinal tract. The O3:K6 serotype may be more likely to contain the bacteriocin gene than environmental isolates simply because it is

more likely to contain the genomic island, or because a pandemic strain has more opportunity to associate with other environmental isolates and acquire genes. Regardless of where the gene originated, the bacteriocin may be indirectly involved with pathogenicity by increasing the fitness of the O3:K6 serotype in the environment or within the intestines where the bacteriocin may increase fitness for the bacterium relative to non-pathogenic commensal bacteria.

CHAPTER 3

VIBRIO PARAHAEMOLYTICUS TYPE III SECRETION SYSTEM

3.1 *Vibrio parahaemolyticus* Type III Secretion System

Following the genome sequencing of RIMD2210633, the type III secretion system (T3SS) was proposed as an additional virulence mechanism to explain the occurrence of KP negative pathogens (23, 26). The T3SS apparatus, or injectisome (7), is essentially a small needle comprised of about 20 proteins (3) that pierces the cell membrane of a eukaryotic cell and injects effector molecules into the host cell cytosol where they disrupt signaling pathways (3, 7). T3SS structures are highly conserved between bacteria while the effectors are more variable (3, 7, 23, 26)

Unlike many other bacterial species, *V. parahaemolyticus* has been shown to possess two distinct T3SSs (3, 7, 23, 26).

3.1.1 *Vibrio parahaemolyticus* T3SS1

The T3SS1 gene cluster is located on chromosome I, (14, 23). The T3SS1 apparatus resembles that of *Yersinia* spp. (3, 26), but there is no similarity between the *Yersinia* effectors and those of *V. parahaemolyticus*. All *V. parahaemolyticus* strains examined have the T3SS1 (26) and the GC content of the loci is within range of the 45.4% GC content of the genome (20). One of the T3SS1 apparatus genes examined in this study is *vp1670* (23).

3.1.2 *Vibrio parahaemolyticus* T332

The T3SS2 gene cluster is located on chromosome II, within the 80-kb genomic island VpaI-7. The GC content of the T3SS2 loci is 39.8% (5, 20), which is suggestive of LGT. Although the T3SS2 cluster appeared to be unique to *V. parahaemolyticus* KP^+ strains (26), it has since been shown that non-O1, non-O139 *V. cholerae* strains also have the T3SS2 cluster (9, 23), which is also suggests LGT of VpaI-7. One of the T3SS2 translocator genes examined in this study is *vpa1354*.

3.2 *Vibrio parahaemolyticus* T3SS effector molecules

V. parahaemolyticus effector molecules show little similarity to other known proteins and the extent of the diversity of the *V. parahaemolyticus* T3SS effectors is not known (3, 26). One study showed the secretion of four T3SS1 dependent effector proteins by 2D gel electrophoresis (25) Deletion of *vp1680* was demonstrated to reduce cytotoxicity to HeLa cells (25, 26) Furthermore, a mechanism describing another T3SS1 effector protein, VP1686, was shown to induce apoptosis through inhibition of the NFκB transcription factor in macrophage populations (3). In a separate study, cell death was due to autophagy (6). Although, the T3SS2 effector knock-out strains were not cytotoxic to HeLa cells, they appeared to be enterotoxic in a rabbit ileal loop test (25, 26). Another study confirms that the T3SS2 non-O1 *V. cholerae* strain colonized infant mouse intestines and caused diarrhea and death in rabbits (9). More functional studies must be undertaken to assign phenotypes to the twelve hypothetical proteins found within the T3SS1 (26) and the hypothetical proteins of T3SS2. Additionally, related species should be examined for the same genes to determine if LGT is occurring between species.

3.3 Experimental Design

Environmental samples were collected from marshes at Skidaway, GA and Apalachicola, FL and were spread on TCBS plates. We isolated *Vibrio parahaemolyticus* green colonies and the yellow co-occurring Vibrio species by patching to M10 media. Colonies were grown overnight in M10 broth and stored at -80˚C. The DNA templates for PCR were made by extracting their DNA by boiling a colony resuspended in nuclease free water. Primers located in table 3.1 were developed in a previous study (23). These primers were used for the initial screen to determine the distribution of T3SS effector genes in our samples. PCR reagents were obtained from New England Biolabs. The concentrations and cycle conditions were the same as in section 2.4. Amplicons were applied to a 0.7% agarose gel and were separated by electrophoresis. Strains were compared to a 2-log ladder and to the positive control from RIMD221063 to verify approximate gene length.

Table 3.1. PCR primers used to screen hemolysin and T3SS associated genes

Gene	Primer	Amplicon size (bp)	5' - 3' primer sequence	Length	T_m	%GC	Reference
tl	L-tl	450	AAAGCGGATTATGCAGAAGCACTG	24	64	46	(2)
	R-tl		GCTACTTTCTAGCATTTTCTCTGC	24	62	42	
tdh	L-tdh	269	GTAAAGGTCTCTGACTTTTGGAC	23	61	43	(2)
	R-tdh		TGGAATAGAACCTTCATCTTCACC	24	62	42	
trh	trh-F4	359	CTGAATCMCCAGTTAASGC	19	59	50	(2)
	trh-R4		ATGYCCATTKCCGCTCTC	18	60	55	
vp1680	VP1680-F	386	GTCAAGCTGAGCGCCAC	17	62	64	(33)
	VP1680-R		CACTGATGGCGTATCTTGGTC	21	63	52	
vp1686	VP1686-F	450	TGG CGG CTG CTC AGA AAA ATC CAG A	25	62	52	This study
	VP1686-R		AAG CCT TCG ATG TGT TGC TTC AAC A	25	59	44	
vpa1346	YopP-F	393	CAATGTTGGCTATTCGGTTGCAGTCTGTATG	31	62	45	(33)
	YopP-R		CGTCCAACTCTATTGTTGTGATATGGCGTTG	31	62	45	
vpa1362	VPA1362-F	397	GAGCAACCGAACGCATCGC	19	64	63	(23)
	VPA1362-R		GCAGGTATCGCATCTTCAGC	20	62	55	
vp1670	*VP1670-F*	338	GCGTCACTGCATTCGACAGG	20	64	60	This study
	VP1670-R		GACTCGTGTGACTCTGCTG	19	62	57	

Following the initial PCR screen to determine the distribution of the T3SS effector genes in our clinical and environmental samples, we designed primers to amplify a larger region of the genes from the positive strains for nucleotide sequencing. Additionally, we added M13 tags to the end of these primers (Table 3.2) so we could sequence directly with M13 forward and M13 reverse primers. Primers for the T3SS effector genes were ordered from IDT and a final concentration of 0.5uM of forward and reverse primers were combined with 25 ul of 2X iProof High Fidelity GC Master Mix (BioRad) for a total reaction volume of 50ul. The PCR was run in a thermocycler according the cycle conditions: initial denaturation at 98°C for 30 seconds, 35 cycles of (98°C for 10 seconds, 58°C for 30 seconds, 72°C for 1 minute), and a final elongation at 72°C for 10 minutes. The amplicons were applied to gel electrophoresis and the DNA was purified from the gel using a Sigma gel extraction kit. The DNA was quantified with a Nanodrop. We sequenced the genes using an ABI 3134 in the Georgia Institute of Technology Genomic Facility. Cycle sequencing conditions were 95°C for 15 seconds, 30 cycles of (95°C for 15 seconds, 55°C for 10 seconds, and 60°C for 4 minutes). Forward and reverse sequencing reactions were carried out separately in 10ul reaction volumes with 2.5uM forward M13 or 2.5uM reverse M13 primers, 1.75ul of 5X Dilution Buffer, 6.75ul DNA template and dH_20 mixture with 70ng template, and 0.5ul BigDye reaction mix added last to protect from light. We aligned nucleotide sequences for the T3SS1 effectors *vp1680*, *vp1686*, and the housekeeping gene *recA*., determined pairwise distances, and constructed phylogenies using MEGA (19). The *V. harveyi* ATCC BAA-1116 strain was used as an outgroup for all gene trees. The *V. cholerae* 0395 strain, and the *E. coli* K12 M1655 strain were also used as outgroups in the *recA* tree.

Table 3.2. PCR primers used to sequence T3SS effectors.

Primers	Amplicon size (bp)	5' - 3' primer sequence
VP1680-FM13		tgtaaaacgacggccagt CAG GCA TCA GCC AAT CCC AAT CTT C
VP1680-RM13	949	CAGGAAACAGCTATGACCG TCA GTC CGT CAG GCT ACC GAG
VP1686-FM13		tgtaaaacgacggccagtGCCATCGGTAAAGCCGTGATAACC
VP1686-RM13	990	CAGGAAACAGCTATGACCGTGAAGGCAAACTCAGCATTGGTGG
VPA1362-FM13		tgtaaaacgacggccagtGAG CAA CCG AAC GCA TCG CT
VPA1362-RM13	~1000	CAGGAAACAGCTATGACCGCGCAGAGAACCAAGAATCTCTGTGG
VPA1346-FM13		tgtaaaacgacggccagt CGT CCA ACT CTA TTG TTG TGA TAT GGC G
VPA1346-RM13	750	CAGGAAACAGCTATGACCG GGG CTC TGA TCT TCG TGA ACA G

3.4 Results

We collected environmental samples from Skidaway, Georgia and Apalachicola, FL in September 2007 and plated the samples on TCBS agar (Figure 3.1A) to separate *Vibrio parahaemolyticus* green colonies and yellow co-occurring *Vibrio* species. Figure 3.1B shows the characteristic blue-green appearance of a pure culture of *V. parahaemolyticus* strain RIMD2210633 on TCBS agar, while Figure 3.1B shows the yellow appearance of sucrose fermenting non-*V. parahaemolyticus* strain Y265. All of the other 39 yellow isolates in this experiment appeared similar to Y265.

Figure 3.1. TCBS plates. (A) mixed environmental culture. (B) pure culture of RIMD2210633. (C) pure culture of *non-V. parahaemolyticus* environmental strain Y265.

PCR templates for all the clinical and environmental strains were obtained as described in section 3.3. PCR results from the hemolysin and T3SS effector screen are shown in Figure 3.2. No PCR amplicons were obtained for *tdh* or *trh* within environmental isolates that appeared yellow on TCBS (data not shown), but the *V. parahaemolyticus* thermolabile hemolysin gene, *tl,* was amplified from 19 of these isolates. Figure 3.2A shows 10 of these strains. Additionally, strains SG189 and SG219 were green on TCBS but were *tl* negative. The *tl* results are summarized in Table 3.3

PCR results for the T3SS1 apparatus gene *vp1670* and effector proteins *vp1680* and *vp1686* are shown in Figure 3.2B-E and are summarized in Table 3.3. PCR analyses indicated that 22% of environmental isolates that grew yellow on TCBS contain at least one T3SS1 or T3SS2 effector gene, while 11% of these presumptive non-*V. parahaemolyticus* isolates contain at least two effector genes from either T3SS1, T3SS2, or both (Table 3.5). Clinical isolates showed a greater frequency for the *vp1680* and *vpa1362* effectors but a lower frequency for *vp1686* and *vpa1362* than *Vibrio parahaemolyticus* environmental isolates (Table 3.4). Environmental isolates that grew yellow on TCBS had fewer effector genes than all *V. parahaemolyticus* clinical and environmental isolates examined (Table 3.4).

New primers were designed to amplify approximately 1kb of the effector genes for sequencing, as described in section 3.3, from *V. parahaemolyticus* clinical isolates, environmental isolates, and the yellow TCBS strains that were determined to contain the genes shown in Table 3.3. Effector genes were amplified and gel extracted from clinical isolates and environmental *V. parahaemolyticus* isolates, but few 1kb effector genes from the yellow TCBS strains with could be amplified. Most of the amplicons from the yellow TCBS isolates were in such low yield that they could not be sequenced. For example, *vp1680* and *vp1686* were both amplified from Y169 (Figure 3.3) in duplicate to increase the DNA concentration following gel extraction, but we did not obtain sequences for these genes. Furthermore, while the initial PCR confirmed that Y128 was positive for both *vp1680* and *vp1686*, the *vp1686* effector gene from Y128 (Figure 3.6) was the only effector that we managed to obtain a sequence for from all the strains listed in Table 3.3.

Nucleotide sequences for *vp1680*, *vp1686*, and *recA* were aligned, as described in section 3.3, from *V. parahaemolyticus* clinical and environmental strains. In addition, *vp1686* and *recA* were sequenced from Y128. A segment of the *vp1680* alignment is shown in Figure 3.4 and a segment of the *vp1686* alignment is shown in Figure 3.7. Pairwise gene differences showed that all *vp1680* and *vp1686* environmental sequences were at least 97% identical to the published nucleotide sequences from RIMD2210633. The *vp1680* effector gene from the *V. parahaemolyticus* AF91 was 99% identical to *vp1680* from RIMD2210633. Gene trees are shown for *vp1680* in Figure 3.5, for *vp1686* in Figure 3.8, and for *recA* in Figure 3.9. All of the *recA* sequences from the environmental isolates are more closely related to the *recA* sequence from the ATCC BAA-1116 strain of *V. harveyi* than to *V. cholerae* strain O395. The *recA* tree also shows

that the *V. parahaemolyticus* strain AF91 groups with RIMD2210633 in the O3:K6 clade. Additionally, *vp1680* from AF91 appears to be closely related to the *vp1680* from RIMD2210633.

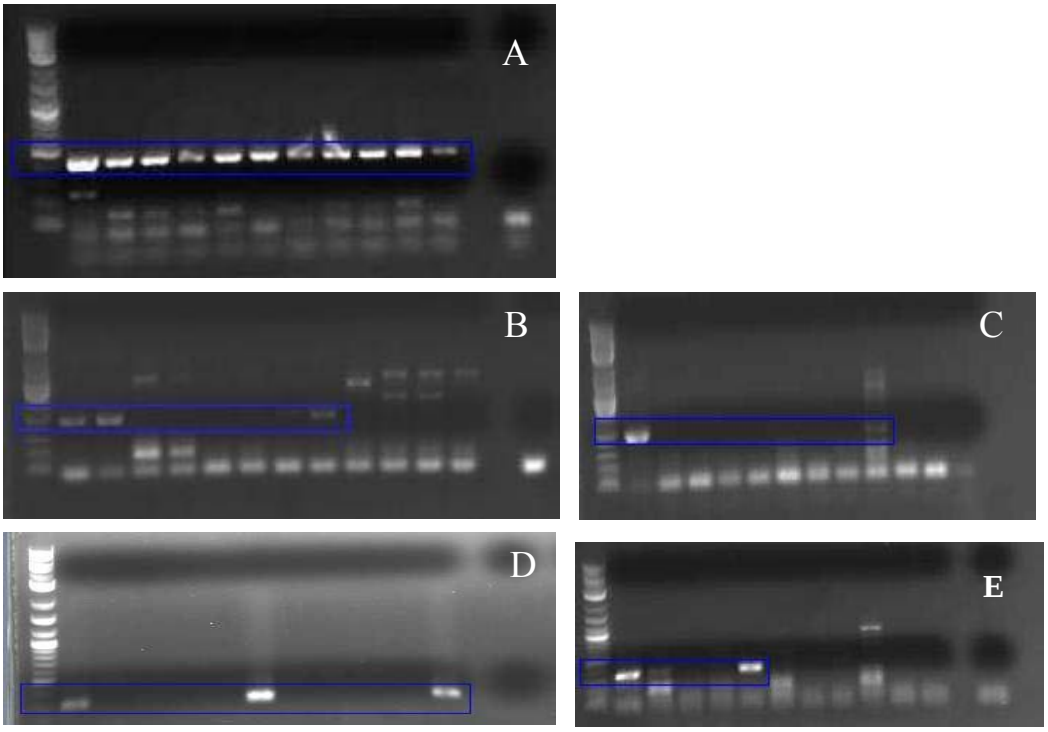

Figure 3.2. *tl* and T3SS1 genes from yellow environmental strains. *tl* is 450bp and *vp1680* is 386 bp. *vp1670* is 338bp (A) tl^+ strains. Lane 1 is the 2-log ladder and lane 2 is RIMD2210633. Lanes 3- 12 are strains Y97, Y106, Y110, Y117, Y128, Y136, Y137, Y150, Y157, Y169, Y172, Y174, Y186, Y193, and Y203. (B) and (C) are *vp1680* amplicons. In (B), Lane 1 is the 2-log ladder and lanes 1 and 2 are duplicates of the *vp1680* from RIMD2210633. The following lanes are representative samples of the environmental strains that appeared yellow on TCBS. The strain Y169 is positive. (C) is another representative sample of *vp1680*. RIMD2210633 is shown in lane 2. Y217 is positive. (D) and (E) are representative samples of *vp1670* from various environmental isolates that grew yellow on TCBS. RIMD2210633 is shown in lane 2 for both gels. In (D), Y128 and Y169 are positive for *vp1670*, and Y172 and Y193 are shown to be positive for vp1670 in (E).

Table 3.3. T3SS1 and T3SS2 apparatus and effector gene distribution for presumptive non - *Vibrio parahaemolyticus* strains.

Non-Vp environmental	*tl*	*vp1670*	*vp1680*	*vp1686*	*vpa1354*	*vpa1346*	*vpa1362*
Y1	-	-	-	-	-	-	-
Y3	-	-	-	-	-	-	-
Y12	-	-	-	-	-	-	-
Y17	+	-	-	-	-	-	-
Y26	+	-	-	-	-	-	-
Y36	-	-	-	-	-	-	-
Y44	-	-	-	-	-	-	-
Y54	-	-	-	-	-	-	-
Y63	-	-	-	-	-	-	-
Y89	-	-	-	-	-	-	-
Y97	+	-	-	-	?	-	+
Y106	+	-	-	-	-	-	-
Y110	+	-	-	-	-	-	-
Y117	+	-	-	-	-	-	-
Y128	+	+	+	+	-	-	+
Y136	+	-	-	-	-	-	-
Y137	+	-	-	-	-	-	-
Y150	+	-	-	-	-	-	-
Y157	+	-	-	-	-	-	-
Y169	+	+	+	+	-	-	-
Y172	+	+	-	+	-	-	-
Y174	+	-	-	-	-	-	-
Y186	+	-	-	-	-	-	-
Y193	+	+	-	-	-	-	-
Y203	+	-	-	-	-	-	-
Y207	-	-	-	-	-	-	-
Y216	-	-	-	-	-	-	-
Y217	-	?	+	-	-	-	-
Y220	-	-	-	-	-	-	-
Y224	-	-	-	-	-	-	-
Y231	-	-	-	-	-	-	-
Y234	-	-	-	-	-	-	-
Y239	-	-	-	-	-	-	-
Y249	+	-	-	-	-	-	+
Y256	+	-	-	-	-	-	-
Y265	-	-	-	-	-	-	-
Y275	-	-	-	-	-	-	-
Y285	-	-	-	-	-	-	-
Y290	-	-	-	-	-	-	-
Y297	-	-	-	-	-	-	-
SG189	-		-	+		-	+
SG219	-		+	+		-	-
SG395	-		-	-		+	+
SG291	-		-	+		-	-

* All strains shown are presumptive non-*Vibrio parahaemolyticus* strains shown due to either yellow growth on TCBS or absence of *tl*. The prefix Y indicates yellow growth on TCBS medium. Highlighted strains contain at least one T3SS effector. Strains with borders on the left are either yellow strains with the *V. parahaemolyticus* hemolysin *tl* gene or *tl*$^-$ strains that appear green on TCBS.

Table 3.4. T3SS effector distribution among environmental *Vibrio* spp. and *V. parahaemolyticus* clinical isolates.

	(frequency)			
	T3SS1 effectors		T3SS2 effectors	
Source	VP1680	VP1686	VPA1346	VPA1362
Vp clinical (n = 26)	(96.2) 25	(65.4) 17	(15.4) 4	(88.5) 23
Vp environmental (n = 37)	(91.9) 34	(83.8) 31	(27.0) 10	(64.9) 24
Yellow colonies (non-Vp, n = 46)	(6.5) 3	(13.0) 6	(2.2) 1	(10.9) 5

* Vp clinical strains isolated from patients include RIM2210633 and 25 *V. parahaemolyticus* strains from the CDC. T3SS1 effector genes *vp1680*, *vp1686*, and T3SS2 effector genes *vpa1346* and *vpa1362*, were identified using a PCR screen using primers previously developed (11).

Table 3.5. Percent of non- *V. parahaemolyticus* strains with two effector genes.

Virulence genes	Strains	Percent
Non-Vp T3SS1 effectors *vp1680*$^+$ and *vp1686*$^+$	Y169, SG219	4.3%
Non-Vp T3SS2 effector *vpa1346*$^+$ and *vp1362*$^+$ strains	SG395	2.2%
Non-Vp mixed T3SS effectors	Y128, SG189	4.3%

*Mixed T3SS effectors are strains positive for either *vp1680* or *vp1686* and also positive for either *vpa1346* or *vpa1362*.

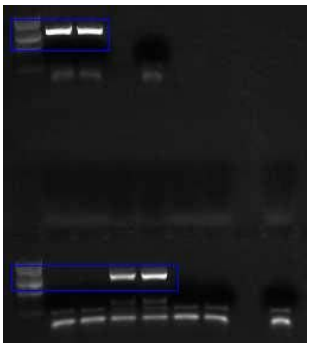

Figure 3.3. PCR amplification of *vp1680* and *vp1686*. Top 949-bp *vp1680* from Y169 and bottom 990-bp *vp1686* genes from Y169. Reactions were duplicated to increase the amplicons' DNA concentrations.

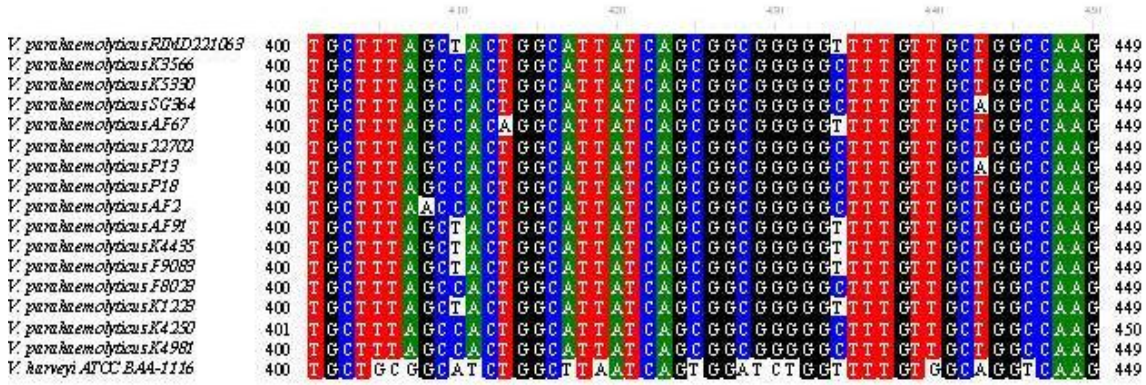

Figure 3.4. Partial alignment of the *vp1680*. Nucleotide sequences are from *Vibrio parahaemolyticus* clinical and environmental strains. The alignment was generated in MEGA (19) using ClustalW.

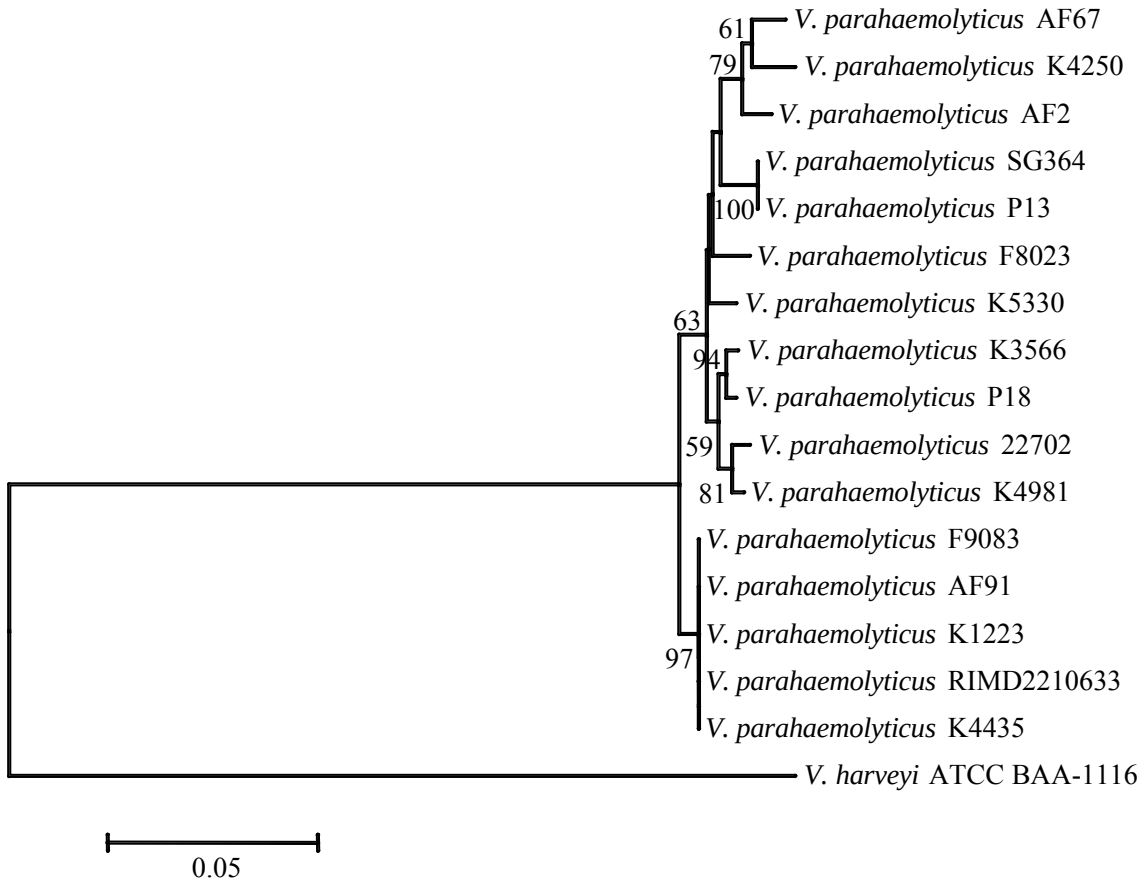

Figure 3.5. *vp1680* phylogeny. A neighbor-joining tree of *vp1680* gene sequence constructed with the kimura 2-parameter model in MEGA 3 with 1,000 bootstrap replications. Values shown are ≥50. The outgroup is a hypothetical protein-encoding gene from *V. harveyi*.

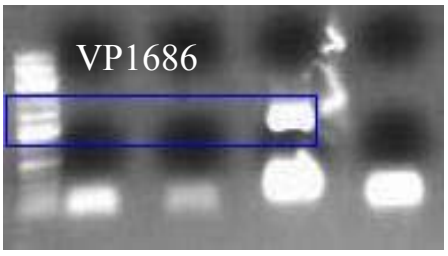

Figure 3.6. *vp1686* amplicon (990-bp) from Y128.

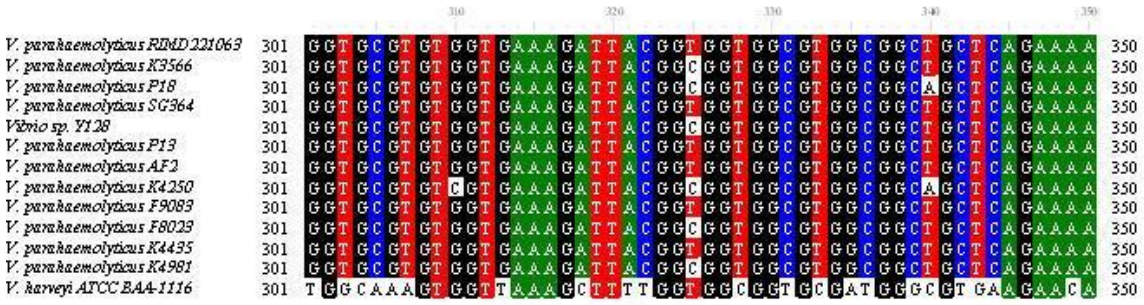

Figure 3.7. Partial alignment of *vp1686*. Nucleotide sequences are from *V. parahaemolyticus* clinical and environmental strains that were green on TCBS with the exception of Y128, which was yellow on TCBS. The alignment was generated in MEGA (19) using ClustalW.

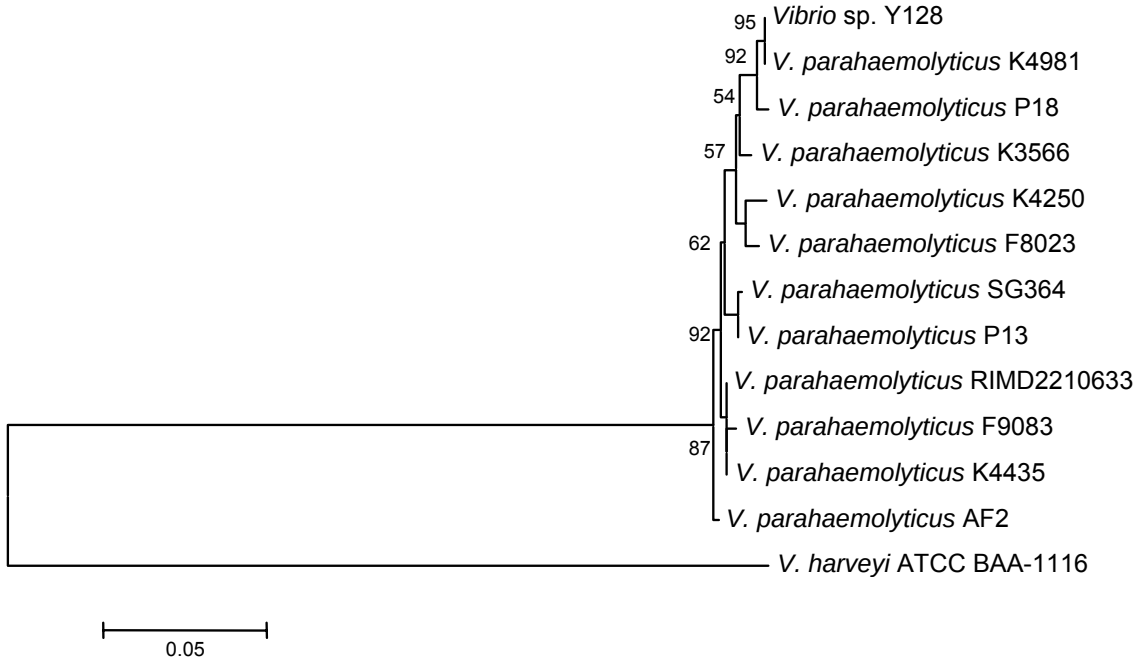

Figure 3.8. *vp1686* phylogeny. A neighbor-joining tree of *vp1686* gene sequence constructed with the kimura 2-parameter model in MEGA 3 with 1,000 bootstrap replications. Values shown are ≥50. The outgroup is a *V. harveyi* hypothetical protein-encoding gene.

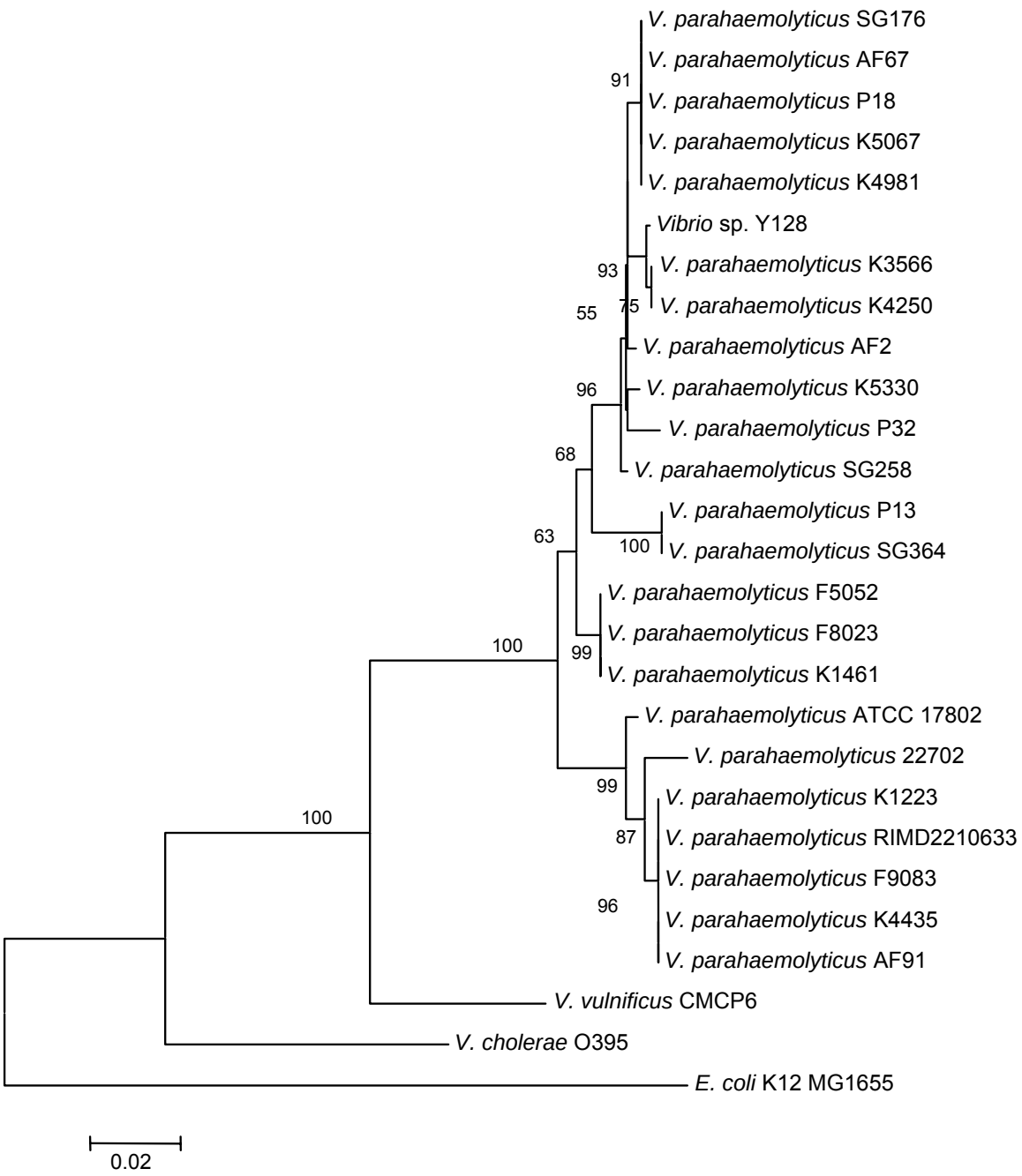

Figure 3.9. *recA* phylogeny. Neighbor-joining tree of *recA* housekeeping gene sequences constructed with the kimura 2-parameter model in MEGA (19) with 1000 bootstrap replications. Values ≥50 are shown. Outgroups include *V. harveyi*, *V. cholerae*, and *E. coli*.

3.5 Discussion

Our data shows that 22% of non- *Vibrio parahaemolyticus* environmental isolates contain at least one T3SS1 or one T3SS2 effector gene (Table 3.4), while 11% contain two T3SS1 effector genes, two T3SS2 effector genes, or one effector gene from each gene cluster (Table 3.5). These percentages may be overestimates considering several of the yellow isolates were actually tl^+ (Table 3.3). Since the presence of *tl* is often used to identify *V. parahaemolyticus*, this indicates that either the commonly used TCBS screen is not discriminatory enough to identify *Vibrio* species or that some *V. parahaemolyticus* strains may have acquired the ability to ferment sucrose. Alternately, the *tl* gene itself may have undergone LGT from a *V. parahaemolyticus* strain to another *Vibrio* spp. Since housekeeping genes are conserved among species, analyzing their nucleotide sequences for mutations is useful for identifying species. Species are more closely related if their housekeeping genes are more similar. Sequencing of *recA* is ongoing to determine whether these strains are *V. parahaemolyticus* or another *Vibrio* spp. Because the phylogeny for *recA* shows that the *recA* sequence from Y128 groups with the *V. parahaemolyticus recA* sequences instead of one of the outgroups (Figure 3.9), we expect that Y128 is a strain of *V. parahaemolyticus* that can ferment sucrose. Sequencing of *tl* amplicons is ongoing to determine whether the gene is undergoing LGT, or if *V. parahaemolyticus* isolates have actually acquired the ability to ferment sucrose. If this strain has acquired the ability to ferment sucrose, then more microbiological plating or biochemical reactions may be necessary to identify *V. parahaemolyticus* from other species of bacteria. Due to difficulties with TCBS, one study shows that a more complex and time consuming two step enrichment technique with the non-selective Tripticase Soy

Broth, followed by a selective salt polymixin broth, and chromogenic agar plating will display violet colonies as *V. parahaemolyticus* due to a beta galactisidase substrate (13). In this method, violet colonies are more easily detected as compared to green colonies on TCBS.

Interestingly, Figure 3.9 shows that *V. parahaemolyticus* environmental isolates appear to be more closely related to the ATCC BAA-1116 strain of *V. harveyi* than to the *V. cholerae* strain O395 based on *recA* sequences. Although this may be expected for a housekeeping gene, prior research has shown that non-01 and non-0139 strains of *V. cholerae* contain the *V. parahaemolyticus* T3SS2 gene cluster (9). Sequencing is ongoing for the T3SS2 translocator gene *vpa1354* and T3SS2 effectors *vpa1346* and *vpa1362* from environmental isolates to examine their similarity to the RIMD2210633 genes. If the *recA* sequences of the environmental strains group with *V. harveyi* but the T3SS2 translocator and effector genes group with *V. cholerae*, then LGT of the T3SS2 loci may be occurring. However, select housekeeping genes should be analyzed prior to examining the diversity of the T3SS2 effector sequences in case the strains that grew yellow on TCBS are identified as sucrose fermenting *Vibrio parahaemolyticus* isolates.

The gene trees for both *vp1680* (Figure 3.5) and *recA* (Figure 3.9) show that the environmental *V. parahaemolyticus* strain AF91 grouped within the O3:K6 pandemic serotype, which indicates that we may have isolated a pandemic strain from sediment collected in FL. More housekeeping genes must be sequenced from this strain to be certain that it is related to the clonal pandemic strain. Also, the environmental *V. parahaemolyticus* isolate 22702 is closely related to the O3:K6 serogroup based on *recA*

analysis alone (Figure 3.9); however, sequence analysis of other housekeeping genes indicates 22702 is not related to the O3:K6 strains.

CHAPTER 4
CONCLUSION

In conclusion, we have evidence that the pandemic O3:K6 bacteriocin gene *vpa1263* is distributed among environmental *V. parahaemolyticus isolates*. Furthermore, we have shown that the RIMD2210633 strain produces the bacteriocin on fine-texture agar. Bacteriocin screens are ongoing to determine whether the environmental isolates that contain the gene can produce a functional bacteriocin. Expression analysis with RT-PCR during the varying stages of growth could provide insight into the circumstances surrounding bacteriocin production.

While we have evidence for the distribution of T3SS effector genes within strains that grow yellow on TCBS, we cannot definitively state that LGT is occurring for these genes because the TCBS screening technique may not be discriminatory enough to identify *V. parahaemolyticus*. This is because both *vp1686* and the *recA* sequences for one of the strains that grew yellow on TCBS suggest that the bacterium is actually a *V. parahaemolyticus* that can ferment sucrose. Because the result may still be due to LGT of the *tl,* we plan to continue sequencing housekeeping genes and start sequencing *tl*.

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