Vibrios associated with marine samples from the Northern Gulf of Mexico: implications for human health

by

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Abstract

In this dissertation, I investigated the distribution and prevalence of two human-pathogenic *Vibrio* species (*V. vulnificus* and *V. parahaemolyticus*) in non-shellfish samples including fish, bait shrimp, water, sand and crude oil material released by the *Deepwater Horizon* oil spill along the Northern Gulf of Mexico (GoM) coast.

In my study, the *Vibrio* counts were enumerated in samples by using the most probable number procedure or by direct plate counting. In general, *V. vulnificus* isolates recovered from different samples were genotyped based on the polymorphism present in 16S rRNA or the *vcg* (virulence correlated gene) locus. Amplified fragment length polymorphism (AFLP) was used to resolve the genetic diversity within *V. vulnificus* population isolated from fish. PCR analysis was used to screen for virulence factor genes (*trh* and *tdh*) in *V. parahaemolyticus* isolates yielded from bait shrimp. A series of laboratory microcosm experiments and an allele-specific quantitative PCR (ASqPCR) technique were designed and utilized to reveal the relationship between two *V. vulnificus* 16S rRNA types and environmental factors (temperature and salinity).

In summary, research data showed that the human pathogen *V. vulnificus* is commonly found in non-shellfish samples (fish, bait shrimp and tar ball) of the Northern GoM coast. Moreover, I discovered a higher percentage of strains of great virulence potential in fish and shrimp than those previously reported in oysters. I proved that 16S type B strains outcompete type A strains at warmer temperatures explaining why more

cases of vibriosis due to this pathogen occur at the end of summer. Finally, the effects of the *Deepwater Horizon* oil spill significantly increased the presence of *V. vulnificus* in beach samples. Overall, my research shows that recreational activities conducted in the Northern GoM coast have an intrinsic risk of exposure to *V. vulnificus*.

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List of Abbreviations

AFLP	Amplified fragment length polymorphism
ASqPCR	Allele specific quantitative PCR
GoM	Gulf of Mexico
mCPC	modified Colistin Polymixin-B Cellobiose agar
MDS	Multidimensional scaling
RFLP	Restriction fragment length polymorphism
rrn	16S rRNA operon
TCBS	thiosulfate-citrate-bile salts-sucrose agar
TDH	thermostable direct hemolysin
TRH	TDH-related hemolysin

CHAPTER 1. LITERATURE REVIEW

Gulf of Mexico Ecosystem

The GoM is a semi-enclosed sea and is the ninth largest body of water in the world. It is bordered by the United States to the north (Florida, Alabama, Mississippi, Louisiana, Texas), five Mexican states to the west (Tamaulipas, Veracruz, Tabasco, Campeche, Yucatan), and the island of Cuba to the southeast. The GoM region covers more than 1,942,500 km^2 including open water areas and coastal wetlands with input from 33 major river systems (1). In total, there are 207 significant estuarine systems, and extensive barrier-islands with coastal lagoons, both in the United States and Mexico. Every year ca. 60% rainfall in continental American and about 40% in Mexico discharge into the Gulf (1). GoM habitats include coastal wetlands, submerged vegetation, upland areas, and marine and offshore areas. Due to its location, the GoM is transitional between temperate and tropical climate and comprises both elements. The diverse marine habitats and climate conditions teems a high biodiversity in the GoM. A recent inventory compiled by Felder and Camp (2) describes about 15,419 eukaryotic species which are distributed among over 40 phyla. This number is estimated to cover roughly 80% to 85% of the GoM eukaryotic taxa. However, the number of microbial species is not determined in the inventory. Whitman et al. (3) estimated that the number of prokaryotic cells in ocean and oceanic subsurface are 1.2×10^{29} and 3.5×10^{30}, respectively. These

microorganisms play a wide range of ecological and biogeochemical roles (i.e. carbon recycling) in the GoM ecosystems (4). In addition to these roles, several groups of microbes can be pathogenic to marine animals and humans. Among those, ,members of the family of Vibrionaceae have a long history of being associated with human diseases (2). However, the distribution of pathogenic species of vibrios in the GoM environment is poorly study, in particular, their associations with fish is largely unknown.

The genus *Vibrio*

The genus *Vibrio* is a major taxa of culturable heterotrophic bacteria commonly found in marine and estuarine environments (5, 6). This genus was first described by the Italian physician Filippo Pacini in 1854 while he was studying cholera as a medical student. This genus is classified into family Vibrionaceae, which, as of 2012, is the only family in the order Vibrionales. The family includes the genus *Vibrio* (111 species) and other 10 genera: *Aliivibrio* (6 species), *Allomonas* (1 species), *Beneckea* (11 species), *Catenococcus* (1species), *Enterovibrio* (4 species), *Grimontia* (1species) *Listonella* (3 species), *Lucibacterium* (1species), *Photobacterium* (24 species) and *Salinivibrio* (4 species) (7). According to the *Bergey's Manual of Systematic Bacteriology* (8), *Vibrio* spp. are characterized by being small (0.5-0.8 × 1.4-2.6 μm), comma-shaped rods with polar flagella enclosed in a sheath. They are motile, facultative anaerobes, and oxidase positive (except for 2 species). All *Vibrio* species can ferment D-glucose without producing gas and are able to reduce nitrate to nitrite.

Pathogenic *Vibrio spp.* Within the genus *Vibrio*, there are several pathogenic species that can cause disease in humans and marine animals (8, 9). Historically, *Vibrio*

cholera, V. parahaemolyticus and *V. vulnificus* has been recognized as three most serious human pathogens in the genus (10-12) but other species including *V. alginolyticus, V. fluvialis, V. furnissii, V. mimicus, V. metschnikovii,* and *V. cincinnatiensis* have also been reported as the cause of illness in humans (13-19) . Regarding aquatic animals, vibriosis is one of the most serious disease affecting marine invertebrate and vertebrates. Some of the *Vibrio* species that cause significant economic losses in finfish, shellfish, and shrimp marine culture are *Listonella* (formerly *Vibrio*) *anguillarum, V. ordalii, V. salmonicida, V. tapetis, V. harveyi,* and *V. vulnificus* biotype 2 (20-23). Other less common *Vibrio* species that cause disease in marine animals are *V. splendidus, V. neptunius, V. alginolyticus, V. parahaemolyticus,* and *V. penaeicida.* In addition, *V. coralliilyticus* has been associated with coral (*Pocillopora damicornis*) bleaching, (24-29). As more studies on aquatic animal health are published, the list of *Vibrio* species recognized as putative or opportunistic pathogens continues to increase.

Host-*Vibrio* interactions. Many species of *Vibrio* form symbiotic and commensal relationships with aquatic animals. As a part of the natural microbiota of estuarine and marine ecosystems, *Vibrio* species frequently encounter a wide-range of hosts such as zooplankton, shellfish, crustacean and fishes. These interactions can range from mutualistic symbiosis to pathogenic relationships.

Vibrio cholerae, the bacterium that causes cholera, can attach to chitinaceous zooplankton, particularly copepods (30). This kind of association with zooplankton is proven to be a key factor in deciphering the global nature of cholera epidemics, because *V. cholerae* can use zooplankton as a vector and colonize to new environments (31, 32).

In addition, zooplankton (live or dead) can support the growth of some *Vibrio* species such as *V. parahaemolyticus*, suggesting that zooplankton can act as possible reservoir for this pathogen in the environment (33). Furthermore, a more recent study conducted by Turner et al. (34) demonstrated that plankton composition modulates the presence of culturable *Vibrio* spp. thus, indicating the potential complex interactions between zooplankton and *Vibrio* species. Finally, Senderovich et al. (35) reported that *V. cholerae* can be found in the gastrointestinal tract of fish to where arrives through the food chain thus, suggesting that fish can serve as a reservoir for the bacterium.

Vibrio species are frequently found in shellfish as well. Marine bivalves such as oysters and mussels that are filter feeders accumulate microbiota including vibrios from surrounding waters (36). For example, high concentrations (10^5 CFU g^{-1}) of *V. vulnificus* are commonly found in GoM oysters (37). *Vibrio vulnificus* is recognized as the most invasive and rapidly lethal *Vibrio* species for humans (38) but it is a mere commensal to the oysters. Oysters harvested from waters in where the bacterium is present, become passive carriers for this opportunistic human pathogen. Interestingly, an earlier study showed *V. vulnificus* is more persistent in oyster tissues than fecal coliforms (e.g. *Escherichia coli*) and therefore more difficult to remove during post- harvest processes of shellfish, including depuration (39). A study conducted by Paranjpye et al. showed that *V. vulnificus* mutants that lack pili had a significantly reduced ability to persist in oyster (*Crassostrea virginica*) tissues (40). This finding suggested that type IV pili expressed by *V. vulnificus* are involved in attachment and colonization of oysters.

In crustaceans, *Vibrio* spp. accumulate in the digestive tract and the hepatopancreas where they can reach high numbers (5). Oxley et al. analyzed the gut microbiota of banana prawn (*Penaeus merguiensis*), showing that *Vibrio* species (including *V. parahaemolyticus*) were the dominant group in the gut of healthy cultured prawns (41). Another study also found high concentration (from 10^4 to 10^5 CFU g^{-1} tissue) of *Vibrio* spp. in the hepatopancreas of healthy shrimp (*Penaeus vannamei*) juveniles (42).

Vibrio communities are often part of the normal microbiota associated with fishes. In an early survey, Grimes et al. recovered *Vibrio* spp. from blood, liver and other internal organs of healthy sharks (43, 44). This finding was further confirmed by routine surveys of captive elasmobranches in which bacteria were isolated from the blood of healthy animals (45). It has been hypothesized that the bacteria found in the blood of elasmobranches could play a role in osmoregulation as most of the recovered isolates can hydrolyze urea (46, 47). In addition to sharks, *Vibrio* spp. have been reported from internal organs of healthy farmed turbot (*Scophthalmus maximus*) (48). However, the role that these bacteria play in host health and homeostasis is unclear and needs further investigation.

Vibrio vulnificus

Vibrio vulnificus was first isolated by the US Centers for Disease Control (CDC) in 1964 but misidentified as a virulent strain of *V. parahaemolyticus*. In 1976, it was referred to as "lactose fermenting vibrio", which was one of main characteristics that distinguished this organism from two other closely related species: *V. parahaemolyticus*

and *V. alginolyticus* (49). The bacterium was formally described as *Vibrio vulnificus* by Farmer et al. in 1979 (50). The epithet 'vulnificus' derives from the Latin 'wound', as the first cases on infections caused by *V. vulnificus* involved wound infections.

Ecology. *Vibrio vulnificus* is an autochthonous estuarine and marine bacterium that can be found in temperate and tropical climates worldwide (51). In the United States, it has been isolated from the Atlantic, Pacific, and GoM coasts including estuaries in those areas (52-56). In the natural environment, *V. vulnificus* is generally a free-living bacterium that is commonly found in water or sediment (57). In addition, it has been associated with shellfish (ie. oyster and clams) (52, 58), intestine of fishes (59), plankton (54), and seaweeds (60). Notably, the prevalence and abundance of *V. vulnificus* is governed by environmental factors, particularly by temperature and salinity (55, 61-63). Several studies have showed that the occurrence of *V. vulnificus* usually undergoes a seasonal pattern that is dependent on temperature. When water temperature is above 20˚C, *V. vulnificus* can be easily recovered from seawater and shellfish as long as the salinity remains within moderate values (15 ppt ~ 25 ppt). Consequently, in our latitude, *V. vulnificus* is abundant during the summer months while remains in low to non-detectable numbers in winter. During the colder months, the low concentration of *V. vulnificus* numbers in the water column led to hypothesize that the bacterium entered the 'viable but not culturable' state in response to cold conditions (64, 65). The term 'viable but not culturable' (VBNC) for bacterial cells was first coined by Roszak and Colwell (66) which refers to a life stage characterized by cells that still retain metabolic function but are unable to grow on culturable media under laboratory conditions. Although unable

to grow on media, *V. vulnificus* cells in this state retain their viability and, most important, their virulence (67). Under *in vitro* conditions, it was possible to resuscitated *V. vulnificus* cells that had entered the VBNC state by temperature upshift (68). However, the 'resuscitation' of the VBNC cells in the environment has not been demonstrated and the true relevance of VBNC forms remains a subject of controversy (69).

An alternative explanation for the decrease in *V. vulnificus* numbers in the water column during the winter months is that the vectors/hosts carrying this bacterium also decrease in winter. A recent study using culture independent methods provided evidence that supports the second hypothesis. The authors showed that the significant reduction of population size in winter is the primary factor for the observed decline in *V. vulnificus* colony counts and not the bacteria entering the VBNC state (53). According to this theory, the sediment will act as main reservoir for *V. vulnificus* during the winter months.

In addition to temperature, the dynamics of *V. vulnificus* is strongly correlated to the salinity of the marine environment. Although this bacterium has been isolated from waters with salinities ranging from 1 ppt to 34 ppt, most isolates are recovered from salinities between 15 ppt to 25 ppt (63, 70). The growth of *V. vulnificus* is negatively influenced by salinities greater than 25 ppt (63, 71). For instance, the low incidence of *V. vulnificus* among *Vibrio* isolates from seawater of the Mediterranean coast has been correlated to the constant high salinity (above 30.7 ppt) typical from the area (72). The northern GoM coast, where low to medium salinities (5 ppt ~ 25 ppt facilitates the abundance of *V. vulnificus*, whereas estuaries in similar latitudes on the Atlantic coast are

generally too saline (>28 ppt) to accommodate for this organism (63). It was found that the concentration of *V. vulnificus* was influenced primarily by water temperature in coastal waters of the northern GoM, although *V. vulnificus* numbers are significantly reduced in salinities higher than 26 ppt. Reversely, salinity is the main factor when moderate temperatures occur year round (55). In general, the interrelationship of temperature and salinity plays a complex role that controls the distribution and fluctuation of this organism in the aquatic environments. Other factors such as dissolved oxygen (73), turbidity (74), chlorophyll (75), plankton (34) and phages (76) have also been found related with the ecology of *V. vulnificus*.

Biotypes. The species *V. vulnificus* is divided into three biotypes which are characterized by specific biochemical properties (19, 77). Table 1 summarizes the discriminative biochemical tests between all three biotypes. Biotype 1 strains are positive for indole production and ornithine decarboxylation. Biotype 1 the predominant biotype in the USA and responsible for all human infections reported in this country (10, 78). Biotype 2 strains are negative for indole and ornithine decarboxylation, and have been associated with severe outbreaks of vibriosis in eels (77, 79). However, at least one biotype 2 strain has been isolated from a wound infection in the USA (80). Biotype 2 strains are immunologically identical, sharing a common lipopolysaccharide profile that differs from that of biotype 1 strains (81). In some studies, biotype 2 is referred to as serovar E (from eel) although a few non-serovar E biotype 2 strains have also been reported (82). Biotype 3 was first reported by Bisharat et al. in 1999 (83). Isolates in this subgroup were associated with wound infection in fishermen that have been in contact

with tilapia infected with *V. vulnificus*. Biotype 3 strains differ phenotypically from biotype 1 and 2 strains in five phenotypic characteristics: they are negative for citrate, and o-nitrophenyl-β-D-galactopyranoside tests, and also negative for salicin, cellobiose and lactose fermentation. A genetic study using multilocus sequence typing in where 10 housekeeping genes were analyzed, indicated that biotype 3 is a hybrid of biotypes 1 and 2 (84).

Genotypes. *V. vulnificus* is a heterogenetically diverse species. Environmental isolates of *V. vulnificus*, even those isolated from a single oyster, display a remarkable degree of genetic variation (85). Conversely, clinical isolates are more similar to each other. Early studies indicated that only a small fraction of the strains isolated from shellfish caused infections in humans but the search for a specific virulence-marker continues these days (86). The main difficulty for finding a virulence-associated marker relies on the fact that the both clinical and environmental isolates presented all the known putative virulence factors described for biotype 1 (70). For the past 15 years, several research groups have been trying to find molecular markers that could separate the putative more virulent isolates from the rest. The two most common single locus genotyping methods used for the genotyping of *V. vulnificus* are herein discussed.

In one genetic analysis on *V. vulnificus*, Warner et al. employed randomly amplified polymorphic DNA (RAPD) PCR to characterize the strains at the subspecies level (87). This study showed that all of the clinical strains produced an additional band that was rarely amplified in environmental strains. In a follow-up study, this fragment of genomic DNA (named as *virulence correlated gene* or *vcg*) was sequenced and analyzed,

and the consistent sequence variations were highly correlated with the isolation source, where clinical and environmental isolates were significantly associated with *vcg*C and *vcg*E type, respectively (88). The other widely-used subtyping scheme also separated the *V. vulnificus* strains into two subspecies groups, designated as type A and type B according to the 16S rRNA sequence polymorphisms (89). In total, there are 17 different nucleotides in the 16S rRNA gene that can differentiate type A and B. Originally, this polymorphism was identified in the type strain of the species *V. vulnificus* strain ATCC27562 and in a clinical isolate C7184 (89) (GenBank accession number X76333 and X 76334, respectively). In a later study by Nilsson et al. (90), a good correlation was found between 16S genotypes and source of isolation in a large collection of *V. vulnificus* isolates. In that study, 94% of nonclinical isolates were type A (31 of 33), while type B comprised the majority (76%) of human clinical strains (26 of 34). Using real-time PCR for the rapid determination of 16S rRNA types, Vickery et al. also proved the existence of a hybrid type AB that contains both A-type and B-type 16S rRNA gene alleles (91). The AB-type strains were associated with nonclinical sources, and shared higher genetic similarity with A-type strains than with B-type (92, 93). Both typing methods concur in defining two major genotypes within *V. vulnificus* that exhibit a strong correlation with source of isolation. However, a recent study demonstrated that strains of 16S rRNA type A, AB and *vcg*E could be highly virulent in a mouse model, indicating that genotypes are correlated with but do not equate to virulence potential in the species *V. vulnificus* (94). Hence, a specific virulence marker is still lacking for this species.

Virulence and Pathogenesis. The virulence of *V. vulnificus* has been inferred to be multifactorial (95-97). An arsenal of virulence factors have been identified in *V. vulnificus* including capsular polysaccharide, the ability to acquire iron, lipopolysaccharides, pili, and the RtxA1 toxin (encoded by the *rtxA1* gene). In addition, other putative virulence factors like extracellular enzymes metalloprotease (encoded by *vvp*E) and hemolysin-cytosin (encoded by *vvhA* gene) have been proposed (98). Nonetheless, these enzymes showed no definitive role in the pathogenesis of *V. vulnificus* in mammalian models (99). To date, all the identified virulence factors can only partially explained the extreme tissue damage and high mortality observed in both primary septicemia and wound infections caused by *V. vulnificus*. The full pathogenesis of this bacterium is still not fully understood.

One of the best studied virulence factors in *V. vulnificus* is the capsular polysaccharide (CPS) (100, 101). Early morphological observations identified encapsulated strains that formed opaque colonies and translucent colonies that had little or no capsule (100). A positive correlation has been found between the presence and amount of CPS in *V. vulnificus* isolates with virulence in mice (101). The expression of capsular polysaccharides contributes to the virulence in *V. vulnificus* with two roles. First, the presence of CPS on the cell surface helps the bacterium eluding the host defenses since capsule products confer *V. vulnificus* resistance against the bactericidal effects of serum (102). The presence of capsule also provides resistance against opsoization by complement and thus avoidance of phagocytosis by macrophages (103).

Second, CPS partially contributes to septic shock formation through the production of inflammation-associated cytokines (108).

The ability to acquire iron from the host's transferrin is another important virulence factor present in *V. vulnificus*. In human serum, most of the iron is bound to transferring making it unavailable to infectious organisms. In order to establish a successful infection, *V. vulnificus* developed systems to overcome this iron limitation. Indeed, multiple systems for iron acquisition (or siderophores) have been observed in *V. vulnificus* (104). The bacteria produce two types of siderophores: a catechol and hydroxymate siderophores, which are primarily involved in iron acquisition (105).

Lipopolysaccharide (LPS) is the putative virulence factor that likely causes the septic shock observed in *V. vulnificus* septicemia cases through the induction of host pyrogenic responses (106). Others also suggested that the some symptoms such as fever, tissue edema, and hypotension in response to LPS (107).

In bacteria, the adherence to hosts is often mediated by pili. Gander et al. reported that clinical *V. vulnificus* isolates from blood or wounds of infected individuals averaged higher numbers of individual pilus fibers per cell than environmental isolates (108). While another study indicates that type IV pili are conserved in both clinical and environmental strains, and has a role in adherence to human epithelial cells as well in biofilm forming (109). It was demonstrated that the decreased virulence is also detected in a mutant strain that is lacking of a type IV prepilin peptidase (109).

The ability of *V. vulnificus* to cause disease has also been associated with a cytotoxin called RtxA1 toxin (110-112). RtxA1 toxin causes aggregation of actin fibers

and plasma membrane blebs which culminated in a necrotic cell death in host epithelial cells. As a result, it allows *V. vulnificus* cells to invade the bloodstream by crossing the intestine epithelium cells. Furthermore, RtxA1-mediated cytotoxicity would be contact-dependent and toxin production was introduced by host cell contact (113). Overall, RtxA1 toxin is an important virulence factor by the intragastric route of infection in animal models, and likely plays a significant role in the food-borne infection related to *V. vulnificus*.

In addition to virulence factors described above, *V. vulnificus* cells produce multiple enzymatic factors like such as metalloprotease, hemolycin-cytolysin, chitinase, chondroitinase, collagenase, deoxyribonucleic, elastase, gelatinase, lecithinase, mucinase and phospholipase. These extracellular proteins may play an important role in taking advantage of the host throughout the course of infection.

***Vibrio vulnificus* infections and Public Health**

In the United States, surveillance for Vibriosis has been conducted for more than two decades. There are two main surveillance data bases maintained by Center for Disease Control and Prevention (CDC) that record *V. vulnificus* infections. COVIS was initiated by CDC, FDA, and the GoM Coast states (Alabama, Florida, Louisiana, Mississippi, and Texas) in 1988. By 1997, nearly all states were voluntarily reporting into COVIS. In 2007, vibriosis became nationally notifiable and all 50 states are mandated to report any vibriosis case to the CDC. The second data base is the 10-state Foodborne Diseases Active Surveillance Network (FoodNet). COVIS is a passive surveillance, while FoodNet is an active surveillance in sentinel populations for

laboratory confirmed *Vibrio* infections. Through COVIS and FoodNet, Newton et al. examined all cases of vibriosis reported to the CDC from 1996 to 2010 and found that the incidence of vibriosis increased from 1996 to 2010. In this study they also found that *V. vulnificus* was the most commonly reported species (114). Unfortunately, this finding indicates that the current prevention efforts have not been effective to decrease vibriosis.

Epidemiology. Geographically, cases of human infections caused by *V. vulnificus* have been reported from most locations where the bacterium was found in the environment (51, 115-118). In the United States, vibriosis associated with *V. vulnificus* occur most commonly in the GoM Coast region due to the warmer water temperature and consumption of raw shellfish (17, 119). The incidence of reported infections according community-based data in coastal regions is ca. 0.5 cases/100,000 population/year (120). Using data from two CDC surveillance systems, an analysis showed an increased in the incidence of infections from 1996 to 2010 (the peaked was observed in 2010) (114). This report reviewed all 1446 cases of *V. vulnificus* infections reported to the Cholera and Other Vibrio Illness and Surveillance (COVIS) and all 193 cases reported to the Foodborne Diseases Active Surveillance Network (FoodNet). The hospitalization rate of infections with *V. vulnificus* was high (86%, COVIS) and the overall mortality in the US was higher (ca. 30%) than that occurred in vibriosis caused by other *Vibrio* spp. The demographic analysis shows that the illnesses typically occurred in males (68% of total cases) of 40-49 years of age (19% COVIS, 21% in FoodNet). Most infections occurred primarily during the warmer months of the year and peaked in July and August. This results is not surprising since *V. vulnificus* proliferates in warmer water with temperature

above 20˚C. *Vibrio vulnificus* infections can be acquired through two routes: i) infection following ingestion of raw or undercooked seafood, particularly oysters, containing this pathogen (121); ii) infection of preexisting wounds or those incurred during seawater-associated activities, for instance, a penetrating injury by fish fin or cut obtained in shucking oyster (51).

The most common clinical manifestations associated with *V. vulnificus* illness are gastroenteritis, primary septicemia and wound infection. Gastroenteritis can be characterized by enteric symptoms in which a stool culture yielded *V. vulnificus,* and clinical presentations of wound infection and septicemia are excluded. Retrospective studies showed that symptoms in patients with this type gastroenteritis encompass predominantly diarrhea, followed by abdominal cramps, nausea, vomiting, fever, and bloody stools (17, 122). Gastroenteritis induced by *V. vulnificus* is usually self-limiting and not life-threatening and is therefore thought to be largely underreported.

Primary septicemia is defined as a systemic illness characterized by fever and shock and in which *V. vulnificus* was isolated from blood or other sites typically sterile, but no wound infection preceding illness was reported (70). Nearly all the cases of primary septicemia were followed by consumption of raw or undercooked oyster or other raw seafood (119, 121). Main symptoms include fever, chills, nausea and hypertension (systolic blood pressure < 85 mm Hg). Notably, secondary lesions develop on either extremities or the trunk as a direct result of sepsis in approximately two thirds of the cases (123). The time for developing symptoms after ingesting oyster is typically less than 36 hours, ranging from 7 hours to 10 days (78). The reported mortality rate with this

illness is between 40% and 60% (123). However, it can reach up to 90% for those who become hypotensive within 12 hours of initial presentation (120).

Wound infections are characterized by the inflammation at the wound site where *V. vulnificus* is directly isolated. They differ from primary septicemia only in the presence of a cutaneous portal of entry. Symptoms of wound infections include pain, erythema and edema at wound site where the pathogen was isolated, which can rapidly progress to cellulitis, bullous lesions and necrosis, and it could even develop into secondary bacteremia in some patients (78). Symptoms began within 4 h to 4 days in general although the onset of the disease typically occurs around 24 hours post-exposure (51, 124). The fatality rate for *V. vulnificus* wound infection is approximately 20% (120, 125).

Epidemiological studies showed that patient with infections caused by *V. vulnificus* were probably predisposed by certain underlying medical conditions (13, 17, 119, 126). Typical conditions found in patients suffering from *V. vulnificus* infections include liver diseases, immune disorders (i.e. AIDS) and some chronic conditions (i.e. diabetes mellitus). Preexisting conditions are more prevalent among primary septicemia patients than in those with either gastroenteritis or wound infections. Liver disease was the most common preexisting condition in all primary septicemia patients (> 80%). Why patients with underlying diseases are more susceptible to *V. vulnificus* infections? It is speculated that the elevated iron level in serum associated with those diseases favors pathogen growth while suppressing the host immune system (127, 128). People with chronic a liver disease or compromised immune system have been described to be up 80

times more likely to develop primary sepsis than healthy individuals. By contrast, *V. vulnificus*-induced gastroenteritis and wound infections do not seem to be favored by preexisting conditions and can occur in healthy individuals (119). However, severe outcomes like necrotizing fasciitis or secondary septicemia are complications of wound infections that are more common in those who belong to the previously mentioned high-risk group.

Interestingly, males markedly outnumbered females in terms of *V. vulnificus*-related wound infections and primary septicemia (51, 129, 130), and one single study found it was due to the protection that estrogen provides to host against *V. vulnificus* (131). Other risk factors may also contribute to *Vibrio vulnificus* infections, i.e. alcohol abuse (132) and advance age (129, 133). Given all those potential predictors for host susceptibility, a report shows that the susceptible individuals represent between 7% and 16% of the adult population in the USA (134). However, the *V. vulnificus* infections are rare and it is estimated that less than one case of *V. vulnificus*-induced illness occurs per 10,000 meals of raw oysters served (134). There are some explanations as to why only a few cases of primary septicemia are reported annually. One possible explanation is that not all *V. vulnificus* strains are equally pathogenic and only a subset is able to cause disease in humans. Another explanation could be that only a subset of the susceptible risk population is indeed a true-high risk group (135). Currently, it is necessary to characterize all risk factors in susceptible hosts and the virulence factors of *V. vulnificus* strains before we can assess the true infection risks.

Foodborne infections. As an etiologic agent for vibriosis, *V. vulnificus* is both a foodborne and a nonfoodborne pathogen. As foodborne pathogen, it is largely associated with the consumption of raw oysters and primary septicemia. Oysters are filter feeders that concentrate *V. vulnificus* from water column into their digestive tracts. Since *V. vulnificus* is a natural component of the GoM coast aquatic inhabits, elimination of this bacterium from oyster tissues is difficult unless they are processed. In fact, high concentrations of *V. vulnificus* ($>10^3$ per g) are typically found in GoM oysters during the summer months (63). On the other hand, oysters are popular seafood in US and it is estimated that about 20 million Americans consume raw oysters annually (136). From a public health point of view, raw oysters harvested from the GoM should undergo postharvest processing, especially during summer months, to ensure the safety of the product. For instance, the state of California has banned the sale of untreated raw oysters from the GoM states during summer months starting spring 2003. In 2009, the U.S. Food and Drug Administration (FDA) was planning to impose a ban on live oyster sales in the Gulf region during the warmer months to eliminate deaths related to the consumption raw oyster containing *V. vulnificus* (137). The proposed regulation would require all oysters from the GoM region harvested between May and October to be processed post-harvest. However, the plan was not implemented due to unclear impact to the oyster industries in the GoM region which supports local community economy. Indeed, the GoM is the major harvesting region for eastern oyster (*Crassostrea virginica*) producing about 90% of all the eastern oysters in the US and accounting for $ 64,244,826 annually (138). Eventually, it is believed that federal regulatory mandates and market constraints would be set up

according to different states to mandate the post-harvest processing for raw oyster production in the GoM.

Nonfoodborne infections. In addition to foodborne infections, *V. vulnificus* is also an important nonfoodborne pathogen in US. Using data from COVIS, Dechet et al. reviewed all the nonfoodborne *Vibrio* infections (NFVI) from 1997 to 2006 in the US, showing that *V. vulnificus* infections constituted 35% of those cases, and 72% of which was reported from residents of GoM Coast states (129). Herein, the NFVI mostly refer to wound infections with documented direct contact with salt water, marine wildlife, raw seafood, or seafood drippings (not including consumption of raw seafood). Among those with a pre-existing wound or a cut acquired on site, up to 70% of exposures were related with marine recreational water activities, including boating, surfing, swimming, and shore walking. Reversely, wound infections caused by *V. vulnificus* were more frequently followed by handling or cleaning seafood (58% of all cases) than by recreational activities (129). Notably, nonfoodborne *V. vulnificus* infections has also been linked to fishing and handling fishing equipment. Indeed, recreational fishing is a favorite outdoor activity in US very popular along the GoM coast. The northern GoM is one of top destinations for recreational anglers where an estimated >2.8 million anglers participate in more than 7 million fishing trips annually (139). Although many people engage in recreational activities in marine and estuarine waters and handle fish or bait shrimp there, little information is available regarding the prevalence and distribution of *V. vulnificus* in GoM fishes and bait shrimp. This highlights the need to identify the risk factors

associated with wound infections caused by *V. vulnificus,* particularly those related to fishing.

***Vibrio* sp. infections after environmental disasters.** The risk for illness caused by vibrios after natural disasters along the GoM Coast is also a public health concern. For instance, during the aftermath of hurricane Katrina, August and September 2005, 22 cases of Vibrio-wound infection resulting in 5 deaths were reported. People become in contact with warm and low salinity water while suffering injuries caused by debris (140). More recently, the man-made disaster -the 2010 *BP Deepwater Horizon* oil spill (DHOS) potentially changed the natural environment of *V. vulnificus* in some areas along the GoM coast. The spill released approximately 4.9 million barrels (779 million L) of Louisiana light sweet crude oil (from the MC-252 Macondo well) into the north-central GoM over a period from 20 April 2010 through 15 July 2010 (141). It is not clear the exact effect that this amount of allochthonous carbon had on vibrios living in the affected areas. Specifically, the effect that that carbon had on human pathogens is unknown. Natural or man-made disasters often offer fertile grounds to address basic and applied research questions. Understanding the ecosystem resilience during and after catastrophic events will help developing better response efforts including preparation for infectious diseases and possible outbreaks.

In summary, *V. vulnificus* is an autochthonous inhabitant of the GoM coast and a potential threat to public health. Humans can be easily exposed to this bacterium by eating raw oysters during the summer months and by performing recreational activities in, on, or around seawater. Thus, a better understanding of the ecology of *V. vulnificus* is

critical to fully delineate a comprehensive risk assessment plan that can be used by public health authorities to educate the public and reduce the number of vibrio cases in the population.

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CHAPTER 2. OBJECTIVES

The overarching goal of my research was to help public health specialists to prevention and control human vibriosis in the United States by studying the zoonotic bacterial agents *V. vulnificus* and *V. parahaemolyticus*. I focused on understanding the distribution and prevalence of these two pathogenic bacteria in non-shellfish samples including fish, bait shrimp, water, sand and crude oil material released by the *Deepwater Horizon* oil spill. The null hypothesis (H_0) for my dissertation research was that recreational activities performed at the GoM coast are not associated with a higher risk of contracting vibriosis. To test the above hypothesis, I proposed the following objectives:

1. Estimate the prevalence of *V. vulnificus* on skin and mucus of fishes.
    a. If samples yielded *V. vulnificus* isolates, those will be typified at the subspecies level to determine any correlation between *V. vulnificus* types and environmental or biological factors.
2. Enumerate *V. vulnificus* and *V. parahaemolyticus* in bait shrimp.
    a. Genotype recovered *V. vulnificus* isolates and screen for virulence-factor genes in *V. parahaemolyticus* isolates.
3. Enumerate the *V. vulnificus* in water, sand and tarball collected from GoM Coastal region and statistically analyze the difference of concentration between different type of samples.

4. Assess the adaptation of *V. vulnificus* strains of 16S type A and type B to various environmental conditions by using laboratory microcosms combined with allele specific quantitative PCR technique.

CHAPTER 3. PREVALENCE AND POPULATION STRUCTURE OF *VIBRIO VULNIFICUS* ON FISHES FROM THE NORTHERN GULF OF MEXICO

Abstract

The prevalence of *Vibrio vulnificus* on the external surfaces of fish from the northern GoM was determined in this study. A collection of 244 fish comprising 20 species was analyzed during the course of 12 sampling trips over a 16-month period. The prevalence of *V. vulnificus* was 37% but increased up to 69% in summer. A positive correlation was found between the percentages of *V. vulnificus*-positive fish (Vv+) and water temperatures, while salinity and Vv+ prevalence were inversely correlated. A general lineal model (Vv+% = 0.5930 - 0.02818 × salinity+ 0.01406 × water temperature) was applied to best fit the data. Analysis of population structure was carried out using 244 isolates recovered from fish. Ascription to 16S rRNA gene types indicated that 157 isolates were type A (62%), 72 (29%) were type B and 22 (9%) were type AB. The percentage of type B isolates, considered to have greater virulence potential, was higher than previously reported in oyster samples from the northern GoM. Amplified fragment length polymorphism (AFLP) was used to resolve the genetic diversity within the species. One hundred and twenty one unique AFLP profiles were found among all analyzed isolates resulting in a calculated Simpson's index of diversity of 0.991. AFLP profiles were not grouped based on collection date, fish species, temperature or salinity, but isolates were clustered into 2 main groups that correlated precisely with 16S type.

The population of *V. vulnificus* associated with fishes from the northern GoM is heterogeneous and includes strains of great virulence potential.

Introduction

Vibrio vulnificus is a Gram-negative bacterium commonly found in estuarine and coastal habitats throughout the northern GoM (1, 2). This species is an opportunistic human pathogen that can cause primary septicemia, wound infection, and gastroenteritis in susceptible individuals (3). Gastroenteritis is the more benign but less common clinical syndrome associated with *V. vulnificus* infections that typically courses as a self-limited illness. Conversely, primary septicemia is the most common and severe manifestation of *V. vulnificus*-associated illnesses, having a mortality rate of more than 50% (3-5). Both gastroenteritis and primary septicemia are associated with the consumption of raw shellfish harboring the pathogen, particularly the Eastern oyster (*Crassostrea virginica*) (3). In addition, *V. vulnificus* can produce severe skin and soft-tissue infections in patients with preexisting wounds who come in contact with the bacterium via seawater or by handling seafood or who sustain an injury while exposed to those sources (6).

Ecological studies have shown a seasonal pattern wherein the number of *V. vulnificus* in oysters, seawater and sediments increased with warmer temperatures (7-10). Predictably, the incidence of wound infections has been found to be positively correlated with warm temperatures (11, 12). According to the Cholera and Other *Vibrio* Illnesses Surveillance (COVIS), a *Vibrio* spp. wound infection is recorded as such when the pathogen is cultured from wound and the patient is reported to have sustained a wound or

having a pre-existing one while exposed to marine or estuarine water or by physical contact with marine wildlife in the seven days prior illness onset (13). In most clinical cases, patients reported that they had handled seafood prior to the onset of the disease but the data do not specify the kind of handled seafood (e.g., shellfish, crustaceans, or fish) (4, 11, 14). However, the only two documented outbreaks of *V. vulnificus* involving wound infections were attributed to handling farm raised fish in Israel (15) or from injuries sustained prior to or during a fishing contest in Texas (12), resulting in 62 and 5 cases, respectively.

Recreational fishing is a main service industry for the US, generating large revenues for local coastal communities (16). The northern GoM is a top destination for recreational anglers where an estimated >2.8 million anglers participate in more than 7 million fishing trips annually (17). Although many people recreate in marine and estuarine waters and handle fish there, little information is available regarding the prevalence and distribution of *V. vulnificus* in GoM fishes. DePaola et al. (18) enumerated the density of *V. vulnificus* in the intestine of estuarine fishes of Mississippi and Alabama, reporting higher levels (10^5- 10^8 CFU/g) in fish intestine than in the surrounding seawater and sediments; suggesting that fish may be reservoirs for *V. vulnificus*. However, it is noteworthy that those authors did not analyze the external surfaces of those fish. Anglers may sustain puncture wounds, lacerations, or bites from live fish or they may be incidentally punctured or cut by dead fish during routine recreational angling activities (e.g., de-hooking fish, filleting) (19). During these handling events, anglers may be exposed to bacteria present in the skin and mucus of their catch.

The purpose of this study was (i) to document the prevalence of *V. vulnificus* on the body surface of a group of estuarine fishes commonly caught by recreational fishermen in the GoM and (ii) to characterize the population structure of *V. vulnificus* in those fishes.

Material and methods

Sample collection. Sampling began during November 2009 and continued through March 2011 at regular intervals except during December 2009 and January and February 2010. Sampling sites were selected based on accessibility and considered to be representative of public fishing piers in Alabama and Mississippi. Locations included Dauphin Island and Gulf Shores in Alabama and Ocean Springs in Mississippi (Figure 3-1). Table 3-1 summarizes collection dates, locations and numbers of fish analyzed per collection event. Seawater surface temperature (at 1 m depth) was measured in situ using a mercury-in-glass thermometer (SargentWelch, USA). Salinities were measured with a handheld refractometer (Vital Sine™ Model SR-6). Fishing efforts lasted between 4 and 8 h. Fish were captured using standard baited hooks and standard 20 pound-test monofilament fishing line on standard spinning reels. Hooked fish were deliberately exhausted in ambient water before being raised from the water, secured and suspended in air by the angler grasping the leader base or hook shaft, and then touched only by a second worker donning sterile surgical gloves and equipped with flamed and ethanol-rinsed, heavy-gauge scissors. In coordination with raising the exhausted, immobilized fish from the water, the second worker approached and immediately excised a portion (about 1 cm^2) of the dorsal fin and placed the excised tissue in the tube containing 10 ml

of APW. Hence, no sampled fish was placed on any surface or touched by a second person before each sample was collected by the surgical-gloved worker. Each sample was enriched overnight in APW at room temperature (approximately 25 ℃). All fish were identified according to Carpenter (20), ordinal classification of fishes follows Nelson (21) and common names for fishes follows Eschmeyer (22).

Bacteriological analysis. Upon arrival to the laboratory, 100 µl of APW cultures were plated onto modified cellobiose-polymyxin B-colistin (mCPC) (23) and thiosulfate citrate bile salts sucrose (TCBS) (BD, Becton, Dickinson & Co., Franklin Lakes, NJ) agar plates and incubated overnight at 30 ℃. Three colonies displaying the typical *V. vulnificus* morphology (24) were randomly selected from each selective media and re-isolated on Marine Agar (MA) (BD). Putative isolates recovered from TCBS and mCPC agar were subjected to colony dot-blot hybridization according to the protocol described by Wright et al. (25). Briefly, putative isolates were cultured in Marine Broth (MB) (BD) overnight in a 96-well microtiter plate and approximately 5 µl of each culture was transferred to mCPC using a multiple channel replicator and allowed to grow overnight at 35 ℃. Colonies were lifted onto Whatman® 541 filter papers, followed by hybridization using an alkaline phosphate-conjugated oligonucleotide (5'-GAGCTGTCACGGCAGT-TGGAACCA-3') (DNATechnology A/S, Risskov, Denmark) that recognizes a specific sequence in the *V. vulnificus* hemolysin gene. Positive isolates were stored at -80 ℃ as glycerol stocks (MB supplemented with 20% glycerol) for further testing.

Ascription of *V. vulnificus* isolates to biotypes. A total of 251 *V. vulnificus* isolates recovered from fish and 8 reference strains (Table 3-2) were included in the

genetic analysis. DNA was extracted from all isolates using standard protocols (26). All *V. vulnificus* isolates were subjected to a multiplex PCR assay for biotype ascription according to Sanjuán et al. (2007) (27). In short, PCR was performed in a 25 µl reaction volume containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside, 0.1 µM of primer vvhA-F (5'-CGCCACCCACTTTCGGGCC-3') and vvhA-R (5'-CCGC-GGTACAGGTTGGCGC-3'), 0.2 µM of primer Bt2-F (5'-AGAGATGGAAGAAACA-GGCG-3') and Bt2-R (5'-GGACAGATATAAGGGCAAATGG-3'), 1.5 U Go*Taq* DNA polymerase and 1 µl DNA template (20 ng), and dH_2O up to 25 µl. Unless stated otherwise, all molecular reagents were purchased from Promega (Madison, WI, USA). PCR reaction was carried out on a Bio-Rad PTC-0200 DNA Engine Cycler (Bio-Rad, Valencia, CA) with cycling profile as following: an initial denaturation step 94 ℃ for 10 min, 35 cycles of 94 ℃ for 30 sec, 60 ℃ for 45 sec and 72 ℃ for 1 min, and a final extension step of 72 ℃ for 10 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized with UV light by staining with ethidium bromide. Ascription to biotype 1-3 or biotype 2 was based on amplicon(s) size (the method does not discriminate between biotypes 1 and 3).

16S-Restriction Fragment Length Polymorphism (RFLP) typing. 16S *rrn* genotype was determined by RFLP according to Nilsson et al. (28). A 492 bp region of 16S rRNA gene of *V. vulnificus* was amplified using primers UFUL (5'-GCCTAACAC-ATGCAAGTCGA-3') and URUL (5'-CGTATTACCGCGGCTGCTGG-3'). PCR reaction was performed as described above with the only difference being the annealing temperature of 57 ℃. PCR products were verified by 2% agarose gel electrophoresis.

Restriction endonuclease digestion of amplified product was performed in 20 µl reaction including 10 µl of amplicon, 2 µl of 10× buffer B, 0.2 µl of acetylated BSA (10 µg/ml), 0.5 µl of *Alu*I (10U/ µl) and sterile dH_2O up to 20 µl. Digestion was carried at 37 ℃ for 2 hr after which DNA fragments were separated by electrophoresis on 4% agarose gel. 16S *rrn* types were ascribed based on profiles described by Nilsson et al. (29)

Amplified Fragment Length Polymorphism (AFLP). AFLP reactions were carried out as described by Arias et al. (30). Briefly, 100 ng of RNase-treated genomic DNA was double digested with *Taq*I and *Hin*dIII. Following digestion, specific *Taq*I and *Hin*dIII adaptors were ligated to the restriction fragments and subsequently amplified by PCR using primers T000 (5'CGATGAGTCCTGACCGAA-3') and H00A (5'-GAACTGCGTACCAGCTTA-3'), selective bases at the3'end are underlined. *Hin*dIII primer (H00A) was labeled with an IR700 fluorochrome from LI-COR (LI-COR, Lincoln, NE, USA). PCR amplifications were performed with the following cycle profile: cycle 1, 60 s at 94 ℃, 30 s 65 ℃, and 60 s at 72 ℃; cycles 2 to 12, 30 s at 94 ℃, 30 s at annealing temperatures 0.7 ℃ lower than that used for each previous cycle, starting at 64.3 ℃, and 60 s at 72 ℃; cycles 13 to 24, 30 s at 94 ℃, 30 s at 56 ℃, and 60 s at 72 ℃. After completion of the cycling program, 5 µl of AFLP Blue Stop Solution (LI-COR) was added to the reaction mixtures. Prior to gel loading, the samples were heated for 5 m at 94 ℃ then rapidly cooled on ice to prevent reannealing. The PCR products were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (LI-COR) following manufacturer's instructions.

Data analysis. The BioNumerics 6.6 software suite (Applied Maths, Saint Martens-Latem, Belgium) was used for AFLP data analysis. Pairwise similarities were calculated using Pearson correlation coefficient with a 0.5% optimization. Using the similarity matrix as an input, a dendrogram was constructed with arithmetic averages algorithm (UPGMA). The Jackknife group separation method (based on maximal similarities between isolates) was used to assess the fidelity of the clustering analysis. Only bands within the range of 100-530 bp and with at least 8% of minimum profiling were considered in the analysis. Transversal clustering was performed based on the swapped data matrix of profile similarities (fingerprint patterns, horizontal cluster) and characters (band classes, vertical cluster). In transversal clustering, the isolates are grouped by fingerprint patterns (similarities based on band position and intensity, Pearson correlation coefficient), while the character are sorted by means of value of band classes (0 absent and 1 present, Jaccard coefficient). The diversity of the *V. vulnificus* isolates from each fish species was compared by generating rarefaction curves using the software Diversity version 1.4 (Hunt Mountain Software, Athens, GA).

The SAS Software 9.2 version (SAS Institute, Cary, N.C.) was applied to analyze the relationship between environmental factors and the percentages of fish harboring *Vibrio vulnificus* (Vv+%). The percentage, namely, is the frequency of fish fin clips that yielded confirmed *V. vulnificus* isolates and was calculated for each sampling event. Pearson correlation coefficient was used to estimate the relationship between percentages of Vv+ fish and environmental factors (water temperature and salinity). A general linear

regression analysis was used to quantify the trends observed between Vv+ fish and variation in salinity and water temperature.

Results

Prevalence of *V. vulnificus* in fish. A collection of 244 fish were randomly sampled during 12 sampling trips. Overall, 90 (37%) individual fish (each represented by an excised dorsal fin sample) yielded *V. vulnificus* isolates, and therefore were considered positive (Vv+) for harboring the bacterium (Table 3-1). The prevalence of *V. vulnificus* in fish varied between sampling events from 0-78%. *Vibrio vulnificus* was recovered from a large diversity of fish species (Table 3-3) with prevalence ranging from 0% to 58%. However, the numbers of analyzed fish were not equal across all species due to variable capture success. Because of this limitation, comparing the prevalence of *V. vulnificus* among fish species was not statistically feasible. *Vibrio vulnificus* was documented from any fish species wherein more than 3 individuals of that species were sampled. The order Perciformes was best represented in our sampling, with 63 of 171 (37%) individual fish harboring *V. vulnificus.* Among fishes wherein >10 individuals were analyzed, Atlantic croaker and southern flounder had the highest prevalence at 58% and 53%, respectively, while striped mullet had the lowest at 25%.

Environmental parameters fluctuated as expected during the study, with water temperatures ranging from 16 ℃ to 31 ℃ and salinity from 8 ppt to 33 ppt (Figure 3-2). Percentage of Vv+ fish and salinity were inversely correlated (Pearson's correlation coefficient $r = -0.91077$, $n=12$, $p<0.0001$). By contrast, a positive correlation was found between Vv+ fish and water temperature (Pearson's correlation coefficient $r = 0.62481$,

n=12, p=0.0298). General linear models were applied to describe the changes in percentages of Vv+ fish and the environmental factors (salinity and water temperature) analyzed in the study. The linear regression model identified these two environmental factors as having significant effect on the percentage of Vv+ fish (Vv+%). The model with two significant factors that best fits the data is as follows: Vv+% = 0.5930 - 0.02818 × salinity+ 0.01406 × water temperature. Parameter estimate statistics are included in Table 3-4 and the surface plot for the model equation is shown in Figure 3-3. The coefficient of determination (r^2) for the model was 0.9041, which denotes that 90% of the observed variation is explained by the independent variables. When an interaction term between salinity and water temperature was introduced to the model, the *p*-value indicated the interaction term was not significant (*p*-value= 0.9571). Essentially, this indicates that the number of Vv+ fish was independently affected by salinity and temperature but that both parameters were not linked to each other. In addition, and to test if the collections sites influenced the percentages of Vv+ fish, two dummy variables (S1 and S2) accounting for two of the collection areas (Dauphin Island and Ocean Springs) were brought into the model. The result of t-test on parameters S1 (*p*-value= 0.6413) and S2 (*p*-value= 0.9199) indicated that site was not significant.

Population structure of *V. vulnificus* on fish. Out of more than 500 putative *V. vulnificus* colonies recovered from selective media, 270 isolates were positive by colony dot-blot hybridization and, out of those, 251 were positive by *vvh*A-specific PCR. None of 251 isolates were Biotype 2 based on multiplex PCR (27). 16S-RFLP typing classified the isolates in 3 types (16S type A, B and AB) as previously reported (29). According to

this classification method, 157 isolates (62%) were 16S type A, 72 (29%) were type B and 22 (9%) were type AB. Representatives of 16S type A and B were recovered in every sampling, except for March-2010 where only 16S type A isolates were obtained. Figure 3-4 shows the temporal distribution of 16S types. The percentage of 16S type B isolates varied from 0% to 50% but no clear correlation between salinity, water temperature or fish species and 16S type could be inferred from our data.

Eight additional strains, proved to be virulent in a mouse model (31), were included as references in the AFLP analysis. All but 12 fish isolates were typeable by AFLP (untypeable isolates consistently produced profiles of weak intensity). AFLP profiles were highly informative with an average 95 bands per profile ranging from 50 to 700 bp. In order to test reproducibility of the AFLP method, we performed 3 independent AFLP experiments using 10 fish isolates (data not shown). Based on the variability observed, we selected 90% as our threshold for considering an AFLP type unique as previously described (32). Profiles displaying more than 90% similarity were ascribed to the same AFLP type while similarities lower than 90% indicate different AFLP profiles. A total of 121 unique AFLP types were defined within the 239 isolates of *V. vulnificus* (Table 3-3). Rarefaction curves were generated for each fish species in where 10 or more isolates from the same fish species had been typed (data not shown). In all cases, the slope of the curves was similar and the number of AFLP types observed linearly increased with the total number of *V. vulnificus* analyzed. These results suggest that the genetic diversity of the isolates was similar in all fishes.

Figure 3-5 displays a multiscaling dimensional (MSD) analysis of the similarity among all isolates derived from the AFLP cluster analysis. Two non-overlapping clusters could be clearly delineated that correlated with 16S type ascription. Cluster I grouped all 16S type B isolates at 70% similarity (see Figure 3-6), while cluster II consisted of 148 isolates including all 16S type A and type AB at 71% similarity. All *V. vulnificus* isolates shared a similarity of 65% or higher. Cluster I and cluster II were comprised of 36 and 85 unique AFLP types, respectively. Group separation statistics based on Jackknife analysis confirmed the statistically significant correlation between AFLP clusters I and II and 16 types B and A-AB. Resampling of the data showed a 100% agreement between assigned 16S type B and randomly selected subgroups (for types A and AB the agreement was 96% and 83%, respectively).

Overall, isolates did not cluster based on collection date, geographic location or fish species (data not shown). Moreover, *V. vulnificus* isolates recovered from the same individual fish were not more related to each other than to those isolates from other fish. As an example of how *V. vulnificus* types did not show any specificity for fish species, Atlantic croaker isolates (the fish species that yielded the most *V. vulnificus* isolates) were distributed across the entire AFLP-based dendrogram (Figure 3-6).

The band matching analysis revealed a total of 119 polymorphic bands responsible for AFLP cluster ascription out of 123 total observed bands. Transversal clustering identified the AFLP bands, or markers, characteristic of 16S type B isolates. In Figure 3-6, the isolates were clustered based on band profiles, while the character was grouped according to values in the band Table 3-which reflected band classes of

individual band profiles (1 present, 0 absent). In the two-dimensional clustering (data not shown), the isolates and band classes were arranged according to their relatedness. For example, band classes (labeled by band length) 116 bp, 156 bp, 200 bp, 253 bp, 343 bp, 386 bp, and 491 bp were more abundant in Cluster I (16S type B isolates). These band classes are AFLP markers for 16S type B isolates (highlighted area in Figure 3-6).

Discussion

Few studies have documented prevalence and distribution of *V. vulnificus* in wild fish (18), despite the fact that wound infections caused by this bacterium have been reported in fishermen worldwide (11, 15, 33, 34). In the US, wound infections attributed to *V. vulnificus* have been reported from US GoM Coast states since the species was first described in 1976 (5, 14, 35, 36). Approximately 30 confirmed cases occurred annually in the GoM coast region (13), and most of them purportedly linked to exposure to seawater or seafood (11). However, it is not possible to account for how many cases of wound infections were acquired by direct contact with fish based on epidemiological studies. Our data showed that *V. vulnificus* occurs on the fins of fishes from the northern GoM, with an overall prevalence of 37% although prevalence increased to 69% during the warmer months (June-September). It is noteworthy that during these months, *V. vulnificus* was recovered at all sampling events. This pathogen was isolated from a wide taxonomic and phylogenetic spectrum of fishes, including 16 species representing 15 genera, 8 families, and 6 orders (Table 3-3). Interestingly, the highest prevalence of *V. vulnificus* was found in two bottom feeder fish (Atlantic croaker and southern flounder), which have been shown to have high densities of this bacterium in their gut (18).

However, our findings suggest that *V. vulnificus* is a transient member of the microbiota (37) associated with fish fins surfaces because it was not found on each fish even when several individuals from the same species were collected at the same time.

The occurrence of *V. vulnificus* on fish fins may be higher than that reported here since we used a culture-based method to estimate prevalence. The success rate of recovering environmental bacteria with culture-based methods varies depending on several factors, but it tends to underestimate original densities. For instance, both APW and mCPC have been reported to favor the growth of *V. vulnificus* biotype 1 over biotype 2 (38). Similarly, Chase and Harwood (39) showed than biotype 1 grows better than biotypes 2 and 3 under identical conditions. These factors may have negatively influenced the recovery of biotype 2 in our study. In addition, culture methods disallow for recovery of viable but not culturable (VBNC) forms of *V. vulnificus*, a well-described life stage of this bacterium (40) although the abundance of VBNC forms in the environment is not well known (9). The protocol used herein (enrichment in APW followed by plating onto mCPC) could have preferentially enriched for *V. vulnificus* biotype 1 but, overall, it provided a simple and inexpensive methodology for successfully recovering *V. vulnificus* from fish.

The frequency of detection of *V. vulnificus* in fish was a factor of salinity and temperature. These environmental factors are known to influence *V. vulnificus* abundance and distribution in the marine environment (2, 7, 41). Temperature is positively correlated with *V. vulnificus* presence while salinity is negatively correlated. However, it has been suggested that salinity becomes the main factor influencing the abundance of *V.*

vulnificus in oysters when salinities exceed 30 ppt, irrespectively of water temperature (42). Our field data support this hypothesis since we failed to recover *V. vulnificus* from fish when salinities were higher than 29 ppt even when water temperatures were above 18 ℃. Based on our data, prevalence of *V. vulnificus* in fish fins was more affected by salinity than by water temperature, and this was particularly noticeable from June through September in where water temperatures remained over 25 ℃. Our model predicts that salinity accounted for 83% of the variability of Vv+ fish; whereas, temperature explained only 7% of the variability. Nevertheless, this model may not be accurate when water temperatures and salinities are outside the ranges observed during our study.

Epidemiological data suggest that most *V. vulnificus* strains must present little risk to the susceptible population because of its abundance in marine samples and the small number of clinical cases observed per year (3, 43, 44). The first marker to be used as an indicator for virulence was a polymorphism present in the 16S rRNA gene that classifies the isolates as 16S type A and 16S type B; in addition, some strains can present both 16S alleles and are classified as 16S type AB. Based on epidemiological data, up to 75% of clinical *V. vulnificus* isolates are 16S type B (this percentage increased up to 94% when clinical fatalities were considered) (29). However, Thiaville et al. (31) showed that *V. vulnificus* genotypes were correlated with but did not predict virulence in mice using a skin-infection model. Isolates of *V. vulnificus* are typically referred to as of "clinical origin" (including primary septicemia and wound infection) or "environmental origin" making it difficult to determine what percentage of wound infections are caused by 16S type B (29, 31, 45). The majority (62%) of the fish isolates recovered in this study were

classified as 16S type A (up to 71% if we include type AB) but at 29%, the number of type B isolates was higher than expected based on previous studies from the same area (29, 46, 47). Although significant correlations were not noted with any environmental parameter, the highest proportion of type B relative to A and AB was observed during the warmest months, which is consistent with similar observations of *V. vulnificus* populations in Galveston Bay, TX (48).

AFLP revealed two main genetic groups within the species that correlated well with 16S type ascription. The division of the species into two main genetic groups has been shown previously using up to 14 different loci including markers for virulence (44, 49, 50) and prompted Rosche et al. (2010) to hypothesize that this pattern is indicative of speciation. Our results, derived from a whole-genome fingerprinting method, are in agreement with this hypothesis. In terms of strain characterization, AFLP provided a high level of resolution and confirmed the high genetic diversity present in the species with a calculated Simpson's index of diversity of 0.991 (51). This value is in range with that previously reported by Arias et al. (52) when isolates from a broader geographic area were characterized by AFLP. In some cases, isolates recovered from different fish species (i.e., red drum and Atlantic croaker) and collected from different locations at different times, shared highly similar AFPL profiles (at 95% similarity), while in other examples isolates recovered from the same fish (i.e., Atlantic croaker) clustered in distinct AFLP types (Table 3-3). *Vibrio vulnificus* isolates associated with fish were highly heterogeneous, and we could not determine spatiotemporal- or fish-specific patterns that linked *V. vulnificus* types with environmental parameters or fish species. The inclusion of

reference strains in the AFLP analysis further confirmed the lack of uniqueness among fish isolates since some of them shared high similarities with reference strains including those of clinical origin. This indicates that fish from the nearshore waters of the northern GoM harbor *V. vulnificus* genotypes of great virulent potential for humans.

In summary, this study presents new data on the prevalence of *V. vulnificus* on fish and presents a novel statistical model for predicting its occurrence. Our data indicate that this bacterium has a broad, seasonally-dependent distribution among fishes ranging in coastal areas of the north-central GoM. The high genetic heterogeneity found among *V. vulnificus* isolates from fish resembles those found in other samples from the same area (including Eastern oysters), but the population structure differs as the percentage of 16S type B from fish was higher than expected. *Vibrio vulnificus* appears to be a transient member of the fish surface microbiota as it was affected by changes in environmental parameters, mainly salinity. Potentially pathogenic strains of *V. vulnificus* were recovered from fish, based on 16S ascription and AFLP similarity with clinical strains. Although reported cases of wound infections caused by this pathogen are clearly low proportional to the numbers of anglers, fish captured, and fishing trips taken in the GoM, our distributional data show that fishermen could likely be inoculated by *V. vulnificus* during fish handling and processing if punctured, cut, or abraded, particularly if the fish was captured in an estuary.

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Table 3-1. Temporal and spatial distribution of fishing efforts summarizing number of fish analyzed and number of fish positive for *Vibrio vulnificus* (Vv+).

Date (mm/dd/yy)	Sites	No. of fish	No. of Vv+ fish
11-17-09	OS[a]	19	9
03-26-10	OS	30	10
06-02-10	DI[b]	20	12
06-16-10	DI	25	18
07-18-10	OS	23	18
08-18-10	DI	23	6
09/18/10	OS	18	11
09/18/10	DI	10	4
10-16-10	GS[c]	25	0
11-12-10	OS	18	0
11-28-2010	OS	25	2
3-17-2011	GS	8	0
Total		244	90

[a] Ocean Springs, Mississippi

[b] Dauphin Island, Alabama

[c] Gulf Shores, Alabama

Table 3-2. Reference strains.

In this study	Strain[a]	Source	Origin	Date	16S[d]	*vcg*[e]	Virulence[f]
R-1	CDC 9060-96	Clinical[b]	TX	1996	B	C	Virulent
R-2	CDC 9070-96	Clinical[c]	TX	1996	B	C	Virulent
R-3	ATL-9824	Clinical[c]	TX	1994	B	C	Virulent
R-4	98-640-DP-E9	Oyster	LA	1998	A	E	Virulent
R-5	99-625 DP-D8	Oyster	TX	1999	AB	E	Virulent
R-6	246-0058	Clinical	FL	-	A	E	Virulent
R-7	99-609 DP-A4	Oyster	OR	1999	A	E	Virulent
R-8	99-537 DP-G7	Oyster	MD	1999	A	E	Virulent

[a] For a full description of these strains see Thiaville et al. (2011)

[b] Fatal outcome.

[c] patient recovered from infection.

[d] Typed according to Nilsson et al. (2003)

[e] Typed according to Rosche et al. (2005)

[f] Based on Thiaville et al. (2011)

Table 3-3. Occurrence of *Vibrio vulnificus* in fish collected during the study

Fish species (order: family), common name	No. Vv+ fish/ No. total fish	No. of recovered isolates	AFLP Type[a]
Dasyatis sabina (Myliobatiformes: Dasyatidae), Atlantic stingray	1/3	3	55
Elops saurus (Elopiformes: Elopidae), ladyfish	3/9	11	6, 36, 62, 64, 79, 95, 100
Brevoortia patronus (Clupeiformes: Clupeidae), Gulf menhaden	0/1	0	-
Dorosoma petenense (Clupeiformes: Clupeidae), threadfin shad	0/1	0	-
Bagre marinus (Siluriformes: Ariidae), gafftopsail sea catfish	2/2	2	2
Ariopsis felis (Siluriformes: Ariidae), hardhead sea catfish	6/9	16	7, 11, 12, 14, 26, 37, 43, 59, 94, 98
Opsanus beta (Batrachoidiformes: Batrachoididae), Gulf toadfish	0/1	0	-
Mugil cephalus (Mugiliformes: Mugilidae), flathead grey mullet	6/24	16	5, 82, 84, 86, 111, 117, 118
Strongylura marina (Beloniformes: Belonidae), Atlantic needlefish	0/2	0	-
Prionotus tribulus (Scorpaeniformes: Triglidae), bighead searobin	0/2	0	-
Echeneis naucrates (Perciformes: Echeneidae), live sharksucker	0/2	0	-
Caranx sp. (Perciformes: Carangidae)	0/1	0	-
Selene vomer (Perciformes: Carangidae), lookdown	0/1	0	-
Orthopristis chrysoptera (Perciformes: Haemulidae), pigfish	2/7	9	21, 80
Archosargus probatocephalus (Perciformes: Sparidae), sheepshead	1/6	2	116
Lagodon rhomboides (Perciformes: Sparidae), pinfish	7/23	18	1, 4, 25, 28, 39, 47, 69, 73, 82, 97, 102, 103

[a] only 239 out of 251 isolates were typeable by AFLP.

Table 3-3. Continued

Fish species (order: family), common name	No. Vv+ fish/ No. total fish	No. of recovered isolates	AFLP Type[a]
Bairdiella chrysoura (Perciformes: Sciaenidae), silver perch	5/24	13	60, 81, 85, 94, 99, 100, 106
Cynoscion arenarius (Perciformes: Sciaenidae), sand weakfish	11/28	32	13, 17, 22, 26, 29, 43, 44, 50, 52, 57, 63, 75, 79, 89, 94, 96, 117, 120
Cynoscion nebulosus (Perciformes: Sciaenidae), spotted weakfish	7/16	23	10, 15, 17, 33, 34, 35, 45, 66, 90, 91, 121
Leiostomus xanthurus (Perciformes: Sciaenidae), spot croaker	0/3	0	-
Menticirrhus sp. (Perciformes: Sciaenidae)	2/9	8	36, 51, 109
Micropogonias undulatus (Perciformes: Sciaenidae), Atlantic croaker	22/38	67	3, 8, 9, 10, 11, 15, 16, 19, 20, 23, 24, 38, 40, 41, 46, 48, 50, 54, 58, 61, 65, 67, 68, 70, 72, 77, 78, 82, 87, 92, 101, 104, 105, 108, 113
Pogonias cromis (Perciformes: Sciaenidae), black drum	0/1	0	-
Sciaenops ocellatus (Perciformes: Sciaenidae), red drum	3/5	7	15, 18, 64, 85, 112
Chaetodipterus faber (Perciformes: Ephippidae), Atlantic spadefish	3/3	8	27, 28, 49, 71, 114
Scomberomorus cavalla (Perciformes: Scombridae), king mackerel	0/1	0	-
Scomberomorus maculatus (Perciformes: Scombridae), Atlantic Spanish mackerel	0/3	0	-
Paralichthys lethostigma (Pleuronectiformes: Paralichthyidae), southern flounder	9/17	16	12, 31, 74, 76, 81, 83, 88, 93
Total	90/242	251	

[a] only 239 out of 251 isolates were typeable by AFLP.

Table 3-4. Statistical values for the multiple linear regression model.

Parameter	Estimate	Standard Error	t -value	Pr>|t|
Intercept	0.5930	0.1786	3.3200	0.0089
Salinity	-0.0282	0.0041	-6.9500	<.0001
Water Temperature	0.0141	0.0053	2.6500	0.0266

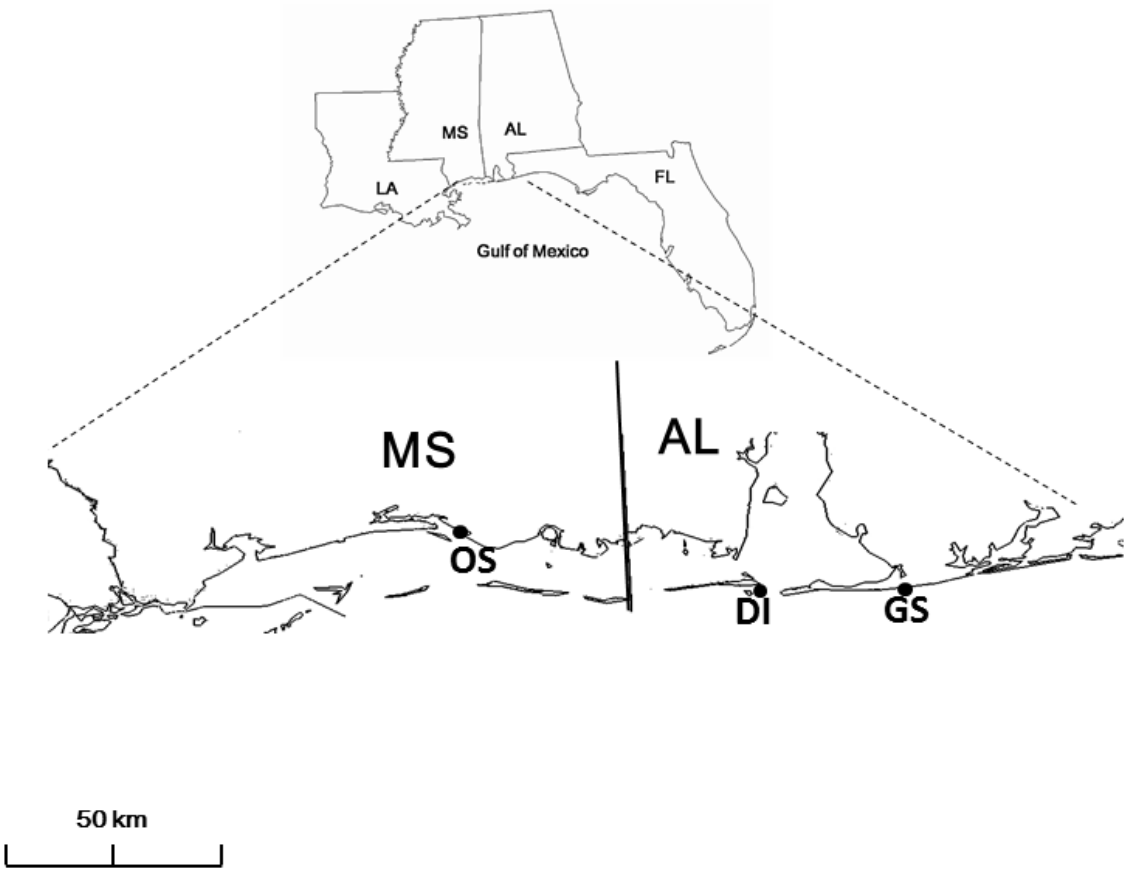

Figure 3-1. North-central GoM showing collecting sites: DI, Dauphin Island, GS, Gulf Shores, and OS, Ocean Springs. MS, Mississippi; AL, Alabama.

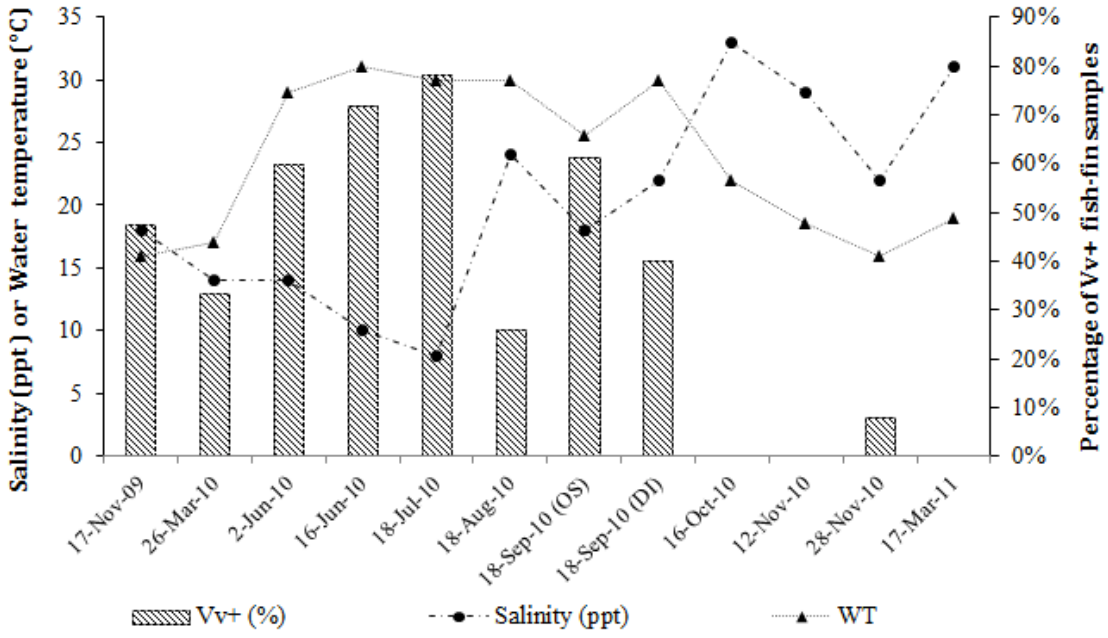

Figure 3-2. Distribution of *Vibrio vulnificus*-positive fish throughout the study (bars) in relationship to salinity and water temperature.

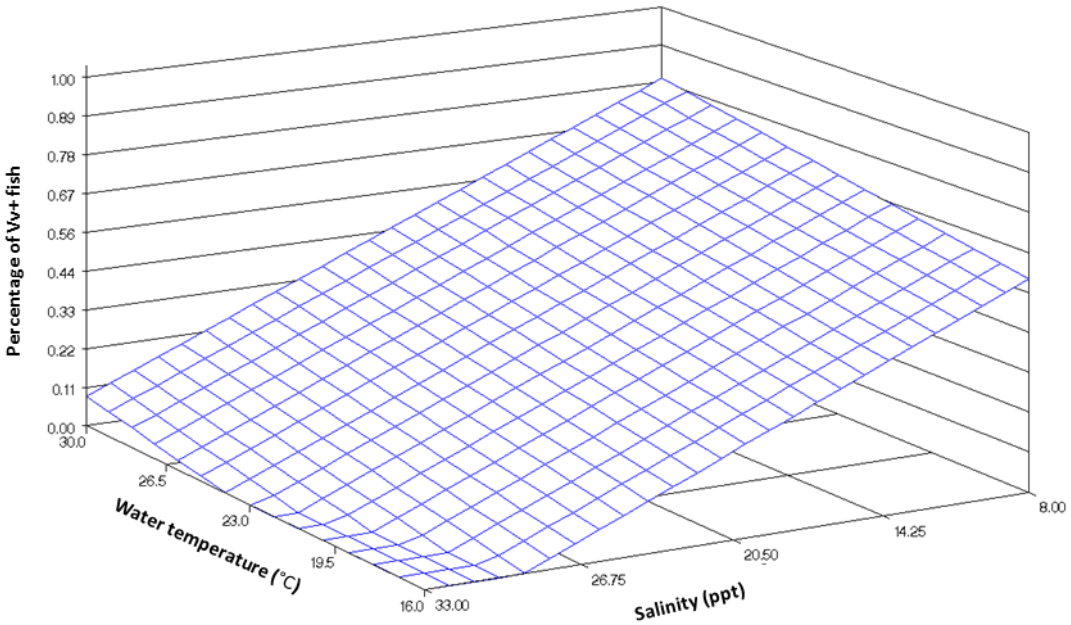

Figure 3-3. Surface plot for model equation: (Vv+)% = 0.5930 - 0.02818 × salinity + 0.01406 × water temperature

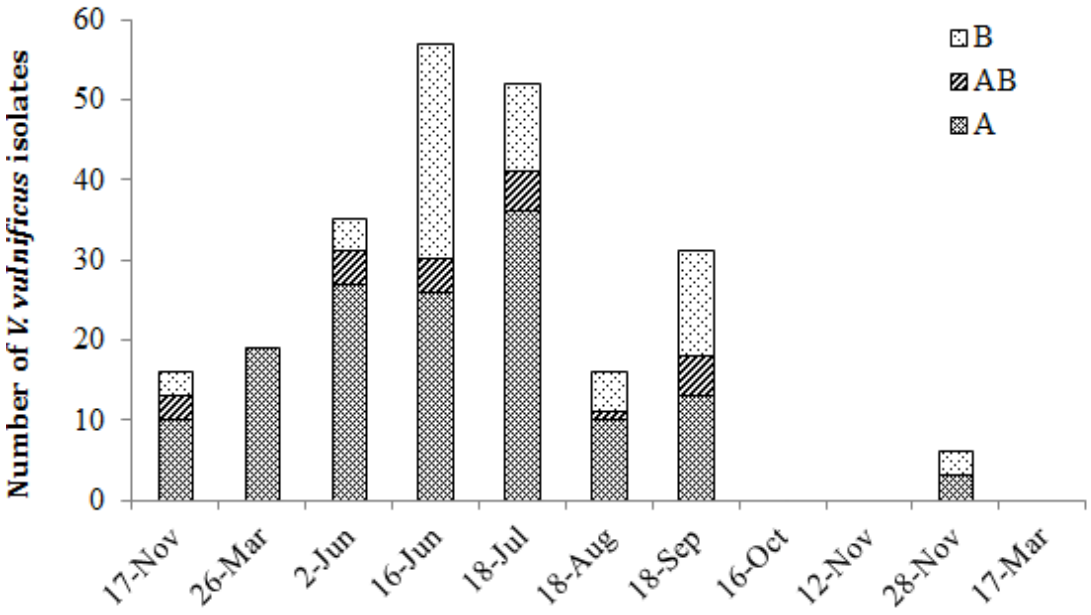

Figure 3-4. Distribution of *Vibrio vulnificus* 16S type A, AB, and B across the study.

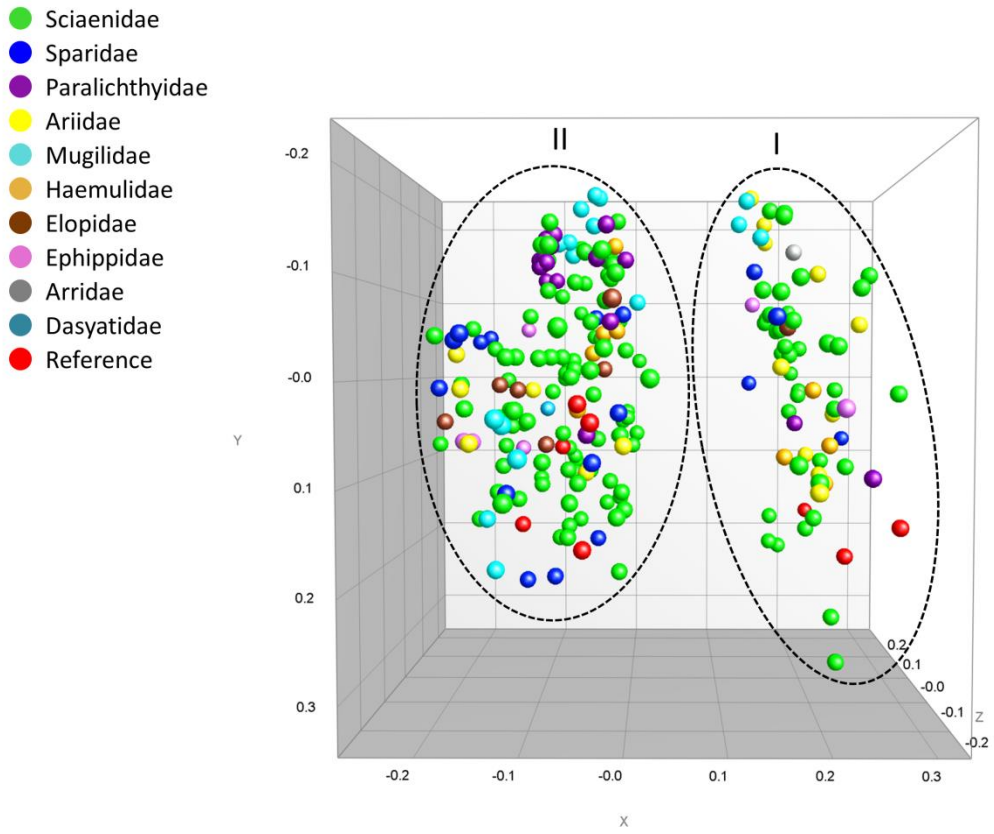

Figure 3-5. Multidimensional Scaling (MDS) representation of the similarity matrix generated by AFLP cluster analysis. Each of 251 *V. vulnificus* isolates is represented by a dot and the distance between dots represents relatedness obtained from the similarity matrix. Isolates are colored based on origin (fish family or reference strains). Dotted lines highlight the two main clusters observed in the analysis. Cluster II groups all *V. vulnificus* 16S type A and AB and cluster I groups 16S type B.

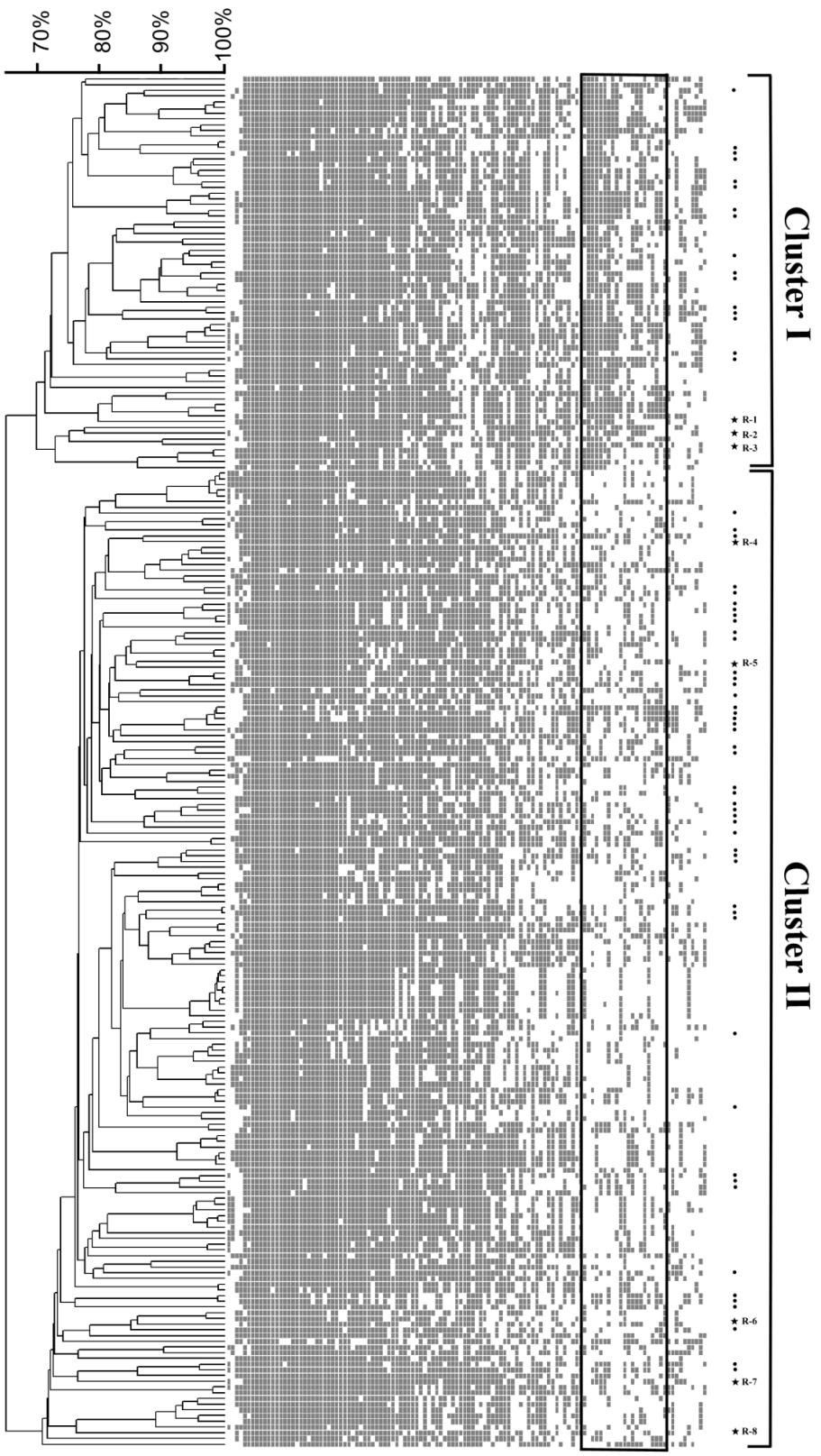
70%
80%
90%
100%
Cluster I
Cluster II
R-1
R-2
R-3
R-4
R-5
R-6
R-7
R-8

Figure 3-6. Clustering analysis based on AFLP fingerprint analysis. Composite matrix of band classes based on transversal clustering of *V. vulnificus* AFLP fingerprints. Scale represent percent of similarity based on Person's product moment correlation coefficient. The two main observed clusters (I and II) are delineated at 70% similarity. AFLP markers identified by transversal clustering and used for cluster delineation are framed. Reference strains are indicated with an asterisk. *Vibrio vulnificus* strains isolated from Atlantic croaker are marked with a dot.

CHAPTER 4. OCCURRENCE OF *VIBRIO VULNIFICUS* AND *V. PARAHAEMOLYTICUS* IN BAIT SHRIMP FROM ALABAMA AND MISSISSIPPI

Abstract

The prevalence of the human pathogens *Vibrio parahaemolyticus* and *V. vulnificus* in bait shrimp species was analyzed in this study. Shrimps were obtained from commercial bait shops along the coast of Alabama and Mississippi from March 2010 to January 2011. The levels of both *Vibrio* species were under the detection limit in November and March, and reached the highest level during the summer months ($>1.1 \times 10^5$ MPN per gram shrimp tissue for both Vibrio species). Randomly selected isolates of both species were type using virulence-related typing schemes. Out of 26 *V. vulnificus* isolates analyzed, 69% were 16S type B and *vcg*C type indicating the virulence potential of the isolates. By contrast, none of the 33 *V. parahaemolyticus* isolates was positive for *tdh* and *trh* hemolysin genes. This study confirms bait shrimp as potential vector for *V. vulnificus* infections.

Introduction

Bacteria of the genus *Vibrio* are key constituents of the microbiota of estuarine and coastal environments of the GoM in where they (1). Although most *Vibrio* species have never been associated with clinical cases, approximately a dozen *Vibrio* species have been demonstrated to be pathogenic to animals or humans (2). In the United States, according to CDC data from 1996 to 2010, *Vibrio parahaemolyticus* and *V. vulnificus* are the leading cause of *Vibrio* infections in humans (3). There are clinical manifestations of illness caused

by *V. parahaemolyticus* and *V. vulnificus*: gastroenteritis, wound infections, and septicemia (4). Septicemia, the most severe illness, and gastroenteritis are typically contracted by ingestion of raw or poorly cooked seafood while wound infections occur when compromised skin is exposed to seafood or seawater (5).

Many studies have focused on the importance of *V. parahaemolyticus* and *V. vulnificus* as foodborne pathogens in the US (6, 7) and risk assessments plans have been developed for both species (8-12). In addition, they also play a substantial role in nonfoodborne infections associated with activities such as seafood handling, swimming, wading and recreational fishing (13). However, less information is known about the risk factors of nonfoodborne *Vibrio* infections involved in recreational activities, including fishing. In present study, we investigated the distribution of *V. vulnificus* and *V. parahaemolyticus* in bait shrimp (*Penaeus* spp.) sold at coastal bait shops in Alabama and Mississippi. To enumerate these bacteria in shrimp, we used the most probable number (MPN) procedure according to *Bacterial Analytical Manual* (14). Furthermore, we examined the virulence potential of *V. vulnificus* and *V. parahaemolyticus* isolates recovered from bait shrimp using current typing schemes (9, 15, 16).

Material and methods

Sample collection. From March 2010 to January 2011 (except April, May and December in 2010), bait shrimps were purchased monthly from local baits shops, one located in Dauphin Island , Alabama (DI, AL) and one in Ocean Spring , Mississippi (OS, MS). Both facilities were on the water and shrimp were kept in holding tanks under flow-through conditions (pumps were used to circulate water continuously from Mobile bay (DI, AL) or

the intracoastal water way OS, MS. The shop owners decline to disclose the amount of time they kept the shrimp in those systems but they confirmed they were caught offshore using traditional trawling methods (17). One additional sample, July 2010, was collected from a marsh area in OS by using a castnet. Shrimp samples were transported to the laboratory with aeration within 5 hours of collection.

Sample analysis. Samples were analyzed following the 3-tube Most Probable Number (MPN) method described in the U.S. Food and Drug Administration's Bacteriological Analytical Manual (BAM) (14). Briefly, 12 shrimps were patted-dried, weighted, diluted 1:1 in phosphate-buffered saline (PBS), and homogenized for 90 sec in a blender (Osterizer®, USA) at maximum speed. The first 1:10 dilution corresponded to 20 g of the homogenate into 80 ml sterile PBS. Samples were subsequently diluted in PBS until 10^{-5} g. Samples were incubated at 35 ℃ for 18 h. For *V. vulnificus* enumeration, an aliquot of each tube was streaked onto modified Colistin Polymixin-B Cellobiose (mCPC) agar plates (18) and incubated at 35˚C for 18 h. Three putative colonies from each plate were transferred to a *V. vulnificus* Agar (VVA), and incubated at 35˚C for 18 h. Colonies on VVA were lifted onto Whatman 541 filter papers, and confirmed by alkaline phosphate-conjugated oligonucleotide probe (5'-GAGC TGTCACGGCAGTTGGAACCA-3') (DNATechnology A/S, Risskov, Denmark) that recognizes a specific sequence in the *V. vulnificus* hemolysin gene (*vvh*A) (19). MPN of *V. vulnificus* MPN g^{-1} was subsequently determined using the chart located in the BAM to interpret the results (20). The enumeration of *V. parahaemolyticus* was performed following similar protocols (14) with the exception of selective media and specific probe. For isolation of *V. parahaemolyticus*, the selective medium used was thiosulfate-

citrate-bile salts-sucrose agar (TCBS) (BD, Becton, Dickinson & Co., Franklin Lakes, NJ). Putative *V. parahaemolyticus* colonies were subsequently incubated on T1N3 agar (1% tryptone, 3% NaCl, and 1.5% agar). A hybridization probe (5'-AAAGCGGAT-TATGCAGAAGCACT-3') targeting the thermolabile hemolysin (*tlh*) gene was used to confirm the *V. parahaemolyticus* isolates (21).

Isolate selection. A subset of confirmed *V. vulnificus* and *V. parahaemolyticus* isolates were arbitrarily selected for genetic typing. A total of *26 V. vulnificus* and 36 *V. parahaemolyticus* isolates were stored in semisolid marine agar (marine broth [BD] with 0.3% agar) at room temperature until further use. Template DNA was prepared using a rapid boiling method as follows. Five colonies from each isolate were selected from a 24-hour culture on Marine Agar (BD) plates, and resuspended in a centrifuge tube with 100 µl sterile distilled H_2O. Proteinase K was added to the cell suspension to a final concentration of 30 unit/µl. After 20 min digestion at 55 ℃, the lysate was heated to 100 ℃ for 15 min, and spun down at 15,000 g for 5 min. The supernatant was transferred to a new tube and used as template DNA. Species-specific PCR reactions using primers listed in Table 4- 1 were applied to selected isolates to further confirmed their ascription to species.

***Vibrio vulnificus* typing.** All *V. vulnificus* isolates were ascribed to biotype as per by Sanju án et al. (22). Assignment to the virulence correlated gene types *vcgE* (environmental type) and *vcgC* (clinical type) were performed as described by Rosche et al. (16). 16S rRNA genotyping was performed by amplifying a conserved fragment of the 16S rRNA gene followed by restriction fragment length polymorphisms (RFLP) analysis according to Nilsson et al (15).

***Vibrio parahaemolyticus* PCR analysis.** All *V. parahaemolyticus* isolates were screened for *tdh* and *trh* genes in a multiplex PCR assay. PCR was performed in a 25-µl reaction volume containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM each deoxynucleoside, 0.2 µM primers Bt2-F and Bt2-R , 1.5 U Go*Taq* DNA polymerase, 1 µl DNA template, and distilled H_2O (dH_2O) up to 25 µl. Primer concentrations varied depending on the reaction (Table 4- 1). Unless stated otherwise, all molecular reagents were purchased from Promega (Madison, WI). PCR was carried out on a Bio-Rad PTC-0200 DNA engine cycler (Bio-Rad, Valencia, CA) with the following cycling profile: initial denaturation at 94 ℃ for 3 min, followed by 35 cycles as shown in Table 4- 1, and ended with a final extension at 72 ° for 10 min. PCR products was confirmed by determining the presence and sizes of products using agarose gel electrophoresis. DNA in gels was stained with ethidium bromide and visualized with UV light in a UVP BioSpectrum 300 Imaging system (city, state). An additional step was involved in RFLP for the *V. vulnificus* 16S rRNA genotyping. Restriction endonuclease digestion of amplified product was performed in a 20µl reaction volume including 10 µl of amplicon, 2 µl of 10× restriction buffer B (Promega), 0.2 µl of acetylated bovine serum albumin (10 µg·ml^{-1}), 0.5 µl of *Alu*I (10 U·$µl^{-1}$), and sterile dH_2O up to 20 µl. Digestion was carried at 37 ℃ for 2 h, after which DNA fragments were separated by electrophoresis on a 4% agarose gel.

Results and discussion

Recreational fishing is one major industry in GoM States and generate large revenues for local communities (23). For this region, 2.7 million fishermen participated in the recreational fishing and took nearly 22 million fishing trips in 2010 (24). Live shrimps are

popular bait along the US GoM coast where recreational fishermen typically purchased them at their local bait shops. For instance, recreational anglers aiming at spotted seatrout (*Cynoscion nebulosus*), red drum (*Sciaenops ocellata*) and flounder (*Paralichthys spp.*) prefer to use live bait shrimp. Therefore, handling live bait shrimp is common for a large number of saltwater fishermen. Epidemiologically, anglers may be incidentally punctured or cut by bait shrimp while handling or hooking the shrimp (25). During these handling events, anglers may be exposed to bacteria present in the external or internal organs of the shrimp. Thus, the objective of this study was to assess if bait shrimp could act as vector for pathogenic vibrios.

Our results showed that both species of pathogenic vibrios occur at high levels in bait shrimp. Eight out of 13 and 9 out of 13 samples tested contained $>10^4$ MPN/g of *V. vulnificus* and *V. parahaemolyticus*, respectively. Only 4 samples had non-detectable (<3 MPN/g) of either pathogen. Not surprisingly, negative samples occurred during cold temperature months (January and March) when levels of *V. vulnificus* and *V. parahaemolyticus* are low in water, sediments, and shellfish (8, 26).Conversely, high levels ($>10^5$ MPN/g) were obtained in all samples tested from June through August. However, levels of both species remained high ($>10^4$ MPN/g) during transition months (October and November).

All randomly selected positive *V. vulnificus* isolates (n=26) were confirmed by specific PCR assay. All these isolates were assigned to biotype1or 3 strains (Table 4- 3). Thirty one percent of *V. vulnificus* isolates were ascribed to 16S type A, and 69 % to 16S type B. Results of *vcg* genotyping were in accordance with 16S typing as all *vcg*C isolates were 16S type B and all *vcg*E isolates were identified as 16S type A. In this study, a high

percent (69%) of *V. vulnificus* isolates recovered from shrimp were classified as 16S type B/*vcg*C. The species *V. vulnificus* can be divided into distinct genetic groups according to two typing schemes. The first method uses a polymorphism present in the 16S rRNA gene that divides the species into 16S A, B and the hybrid AB types (15, 27, 28). The second methods, uses a marker referred to as 'virulence correlated gene' that classifies the isolates into two types: *vcg*C type and *vcg*E type (16, 29). In general, 16S type A and AB isolates are *vcg*E type while 16S type B strain are *vcg*C type (30). The justification for these typing schemes derives from epidemiological data that showed a strong correlation between clinical isolates and 16S and *vcg* types. While most (>80%) of clinical isolates (>95% when only fatality cases are considered) are 16S type B (*vcg*C) the majority of the environmental isolates are 16S type A and AB (*vcg*E) (15, 29, 31). Our data contradicts previous study in that most of our isolates were 16S type B (*vcg*C), which denotes a risk of contracting *V. vulnificus* wound infection by handling bait shrimp particularly due to the elevated numbers of this bacterium present in the shrimp (except during the winter months). However, due to the arbitrarily selection and the low number of isolates tested, it is possible that our isolates may not be accurately represent the *V. vulnificus* population in bait shrimp.

All 33 *V. parahaemolyticus* isolates that tested positive with the *tlh* probe were verified by specific. All isolates were negative for the *tdh* and *trh* genes (Table 4- 4). In *V. parahaemolyticus*, thermostable direct hemolysin (TDH, encoded by *tdh*) and TDH-related hemolysin (TRH, encoded by *trh*) are considered virulence markers because they cause the hemolytic activity known as the Kanagawa phenomenon (32-34). The pathogenicity of the bacterium has been strongly related with these two hemolysins and the genes codifying for

them are commonly found in clinical strains rather than in environmental isolates (35). Epidemiological data (reviewed by Nishibuchi et al.) have shown that that only 1% or 2% of nonclinical strains carry the *tdh* gene (36). Therefore, the absence of these two hemolysin genes among the *V. parahaemolyticus* isolates obtained from shrimp isolates indicated that these isolates were not potentially pathogenic for humans.

In summary, I found a high prevalence of *V. vulnificus* and *V. parahaemolyticus* in bait shrimp. Bacterial density was high throughout the year with the exception of the winter months. Interestingly, the percentage of potentially virulent *V. vulnificus* was higher than those reported from shellfish and other marine samples. Conversely, all of the *V. parahaemolyticus* isolates were negative for virulence markers. Our data suggest that handling bait shrimp has an intrinsic risk of exposure to pathogenic *V. vulnificus*. GoM fishermen should be aware of this risk and seek immediate medical attention if wounds inflicted while handling bait shrimp become infected.

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Table 4-1. DNA regions targeted for PCR amplification in this study; primer names, sequences, and values for the amplification reaction conditions that varied among primer sets; amplicon size; and literature sources of the oligonucleotide primers used.

PCR assay*	Primer name and sequence (5'→3')	PCR condition (temp., time)	Primer con. (μM)	Amplicon size (bp)	References
VV					
*vvh*A	Vvh-785F (CCGCGGTACAGGTTGGCGC)	94 ℃, 30s→57 ℃, 45s→72 ℃, 60s	0.1	519	a
	Vvh-1303R (CGCCACCCACTTTCGGGCC)		0.1		
Bt-2	Bt2-F (GGACAGATATAAGGGCAAATGG)	94 ℃, 60s→60 ℃, 45s→72 ℃, 60s	0.2	344	b
	Bt2-R (AGAGATGGAAGAAACAGGCG)		0.2		
*vcg*C	P1 (ACCTGCCGATAGCGATCT)	94 ℃, 20s→50 ℃, 20s→72 ℃, 20s	0.2	277	c
	P3 (CGCTTAGGATGATCGGTG)		0.2		
*vcg*E	P2 (CTCAATTGACAATGATCT)	94 ℃, 20s→50 ℃, 20s→72 ℃, 20s	0.2	277	c
	P3 (CGCTTAGGATGATCGGTG)		0.2		
rrn typing	UFUL (GCCTAACACATGCAAGTCGA)	94 ℃, 30s→57 ℃, 30s→72 ℃, 30s	0.2	492	d
	URUL (CGTATTACCGCGGCTGCTGG)		0.2		
VP					
tlh	L-TL (AAAGCGGATTATGCAGAAGCACTG)	94 ℃, 60s→58 ℃, 60s→72 ℃, 120s	0.5	450	e
	R-TL (GCTACTTTCTAGCATTTTCTCTGC)		0.5		
tdh	VPTDH-L (GTAAAGGTCTCTGACTTTTGGAC)	94 ℃, 60s→55 ℃, 60s→72 ℃, 60s	0.5	424	e
	VPTDH-R (TGGAATAGAACCTTCATCTTCACC)		0.5		
trh	VPTRH-L (TTGGCTTCGATATTTTCAGTATCT)	94 ℃, 60s→55 ℃, 60s→72 ℃, 120s	0.5	500	e
	VPTRH-R (CATAACAAACATATGCCCATTTCCG)				

[*] VV refers to *V. vulnificus* and VP to *V. parahaemolyticus*.

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Table 4-2. Density of *Vibrio vulnificus* and *V. parahaemolyticus* in bait shrimp.

Date	*V. vulnificus* density (MPN·g^{-1})		*V. parahaemolyticus* density (MPN·g^{-1})	
(mm/dd/yy)	DI, AL[a]	OS, MS[b]	DI, AL	OS, MS
03/26/10	<3	<3	<3	<3
06/02/10	$>1.1\times10^5$	$>1.1\times10^5$	1.1×10^5	$>1.1\times10^5$
07/17/10	ND[c]	$>1.1\times10^5$	ND	$>1.1\times10^5$
08/19/10	$>1.1\times10^5$	ND	$>1.1\times10^5$	ND
09/18/10	2.8×10^3	$>1.1\times10^5$	$>1.1\times10^5$	$>1.1\times10^5$
10/18/10	1.1×10^5	ND	$>1.1\times10^5$	ND
11/12/10	1.1×10^5	2.4×10^4	4.6×10^4	$>1.1\times10^5$
01/29/11	<3	<3	<3	<3

[a] Dauphin Island, Alabama

[b]Ocean Springs, Mississippi

[c] Not determined.

Table 4-3. Result of analyzed *V. vulnificus* isolates for PCR analysis and for *rrn* and *vcg* types

Isolate code	Site	Date	VvhA	Bt-2	16S-type	Vcg C	Vcg E
DC-2	DI, AL	06/03/10	+	-	B	+	-
SC-2	OS, MS	06/03/10	+	-	A	-	+
SC-1	OS, MS	06/03/10	+	-	B	+	-
SC-4	OS, MS	07/18/10	+	-	A	-	+
SC-7	OS, MS	07/18/10	+	-	A	-	+
SC-8	OS, MS	07/18/10	+	-	A	-	+
SC-9	OS, MS	07/18/10	+	-	A	-	+
SC-11	OS, MS	07/18/10	+	-	A	-	+
SC-21	OS, MS	07/18/10	+	-	A	-	+
SC-19	OS, MS	07/18/10	+	-	A	-	+
SC-13	OS, MS	07/18/10	+	-	B	+	-
SC-3	OS, MS	07/18/10	+	-	B	+	-
SC-5	OS, MS	07/18/10	+	-	B	+	-
SC-10	OS, MS	07/18/10	+	-	B	+	-
SC-15	OS, MS	07/18/10	+	-	B	+	-
SC-16	OS, MS	07/18/10	+	-	B	+	-
SC-18	OS, MS	07/18/10	+	-	B	+	-
SC-23	OS, MS	07/18/10	+	-	B	+	-
DC-12	DI, AL	08/19/10	+	-	B	+	-
DC-3	DI, AL	08/19/10	+	-	B	+	-
DC-4	DI, AL	08/19/10	+	-	B	+	-
DC-7	DI, AL	08/19/10	+	-	B	+	-
DC-9	DI, AL	08/19/10	+	-	B	+	-
DC-16	DI, AL	08/19/10	+	-	B	+	-
DC-21	DI, AL	08/19/10	+	-	B	+	-
DC-22	DI, AL	08/19/10	+	-	B	+	-

Table 4-4. Result of *V. parahaemolyticus* isolates for PCR analysis on virulence factor genes

Isolate code	Site	Date	*tlh*	*tdh*	*trh*
ST-1	DI, AL	06/02/10	+	-	-
ST-2	DI, AL	06/02/10	+	-	-
ST-1	OS, MS	06/02/10	+	-	-
ST-2	OS, MS	06/02/10	+	-	-
ST-4	OS, MS	07/18/10	+	-	-
ST-5	OS, MS	07/18/10	+	-	-
ST-6	OS, MS	07/18/10	+	-	-
ST-7	OS, MS	07/18/10	+	-	-
ST-8	OS, MS	07/18/10	+	-	-
ST-9	OS, MS	07/18/10	+	-	-
ST-10	OS, MS	07/18/10	+	-	-
ST-12	OS, MS	07/18/10	+	-	-
ST-13	OS, MS	07/18/10	+	-	-
ST-14	OS, MS	07/18/10	+	-	-
ST-15	OS, MS	07/18/10	+	-	-
ST-3	DI, AL	08/19/10	+	-	-
ST-7	DI, AL	08/19/10	+	-	-
ST-9	DI, AL	08/19/10	+	-	-
ST-10	DI, AL	08/19/10	+	-	-
ST-11	DI, AL	08/19/10	+	-	-
ST-13	DI, AL	08/19/10	+	-	-
ST-14	DI, AL	08/19/10	+	-	-
ST-15	DI, AL	08/19/10	+	-	-
ST-16	DI, AL	08/19/10	+	-	-
ST-17	DI, AL	08/19/10	+	-	-
ST-18	DI, AL	08/19/10	+	-	-
ST-19	DI, AL	08/19/10	+	-	-
ST-20	DI, AL	08/19/10	+	-	-
ST-24	DI, AL	09/18/10	+	-	-
ST-26	DI, AL	09/18/10	+	-	-
ST-16	OS, MS	09/18/10	+	-	-
ST-18	OS, MS	09/18/10	+	-	-

CHAPTER 5. HIGH NUMBERS OF *VIBRIO VULNIFICUS* IN TAR BALLS COLLECTED FROM OILED AREAS OF THE NORTH-CENTRAL GULF OF MEXICO FOLLOWING THE 2010 BP *DEEPWATER HORIZON* OIL SPILL

Abstract

The *Deepwater Horizon* Oil Spill was the largest oil spill in USA history releasing approximately 4.9 million barrels of crude oil into the Gulf of Mexico. Soon after the spill started, tar balls and other forms of weathered oil appeared in large numbers on beaches in Mississippi and Alabama. In this study, we analyzed tar balls for total aerobic bacterial counts and also for the presence of *Vibrio vulnificus*, a human pathogen known to be abundant in the GoM Coast environment and capable of causing severe wound infections by contact with contaminated surfaces. Our results showed that total aerobic bacterial counts were significantly higher in tar balls than in sand and seawater collected at the same location. In addition, *V. vulnificus* numbers were 10× higher in tar balls than in sand and up to 100× higher than in seawater. Densities of *V. vulnificus* were higher than 10^5 colony forming units/g of tar ball in all samples analyzed. Our data suggest that tar balls can act as reservoirs for bacteria including human pathogens.

Introduction

The 2010 BP *Deepwater Horizon* Oil Spill (DHOS) released approximately 4.9 million barrels (779 million L) of Louisiana light sweet crude oil (from the MC-252

Macondo well) into the north-central Gulf of Mexico over a period from 20 April 2010 through 15 July 2010 (FISG, 2010). An estimated 1.1 million barrels (22%) of DHOS oil may still exist in the Gulf of Mexico basin as (i) surface oil (light sheen), (ii) shoreline tar balls, and (iii) sediment oil or submerged "oil mats" associated with the benthos (1). Because microbes utilize myriad organic compounds as sources of carbon and energy, soon after the DHOS event began it was widely publicized that endemic gamma-proteobacteria could significantly affect the environmental fate and ecological impacts of the spilled oil (2). Subsequently, and as expected, the large-scale influx of DHOS allochthonous carbon markedly increased the number of heterotrophic microbes in the water column and a high rate of hydrocarbon biodegradation was documented from within the DHOS oil plume (3). The few existing DHOS-related microbial studies to date have focused on the interactions between the oil plume and pelagic microbes (3), but no published study has analyzed bacteria on the large amounts of shore-cast weathered oil (e.g., tar balls) from within DHOS-effected coastal zone habitats (i.e., marshes and beaches).

Among marine gamma-proteobacteria, *Vibrio* spp. are well-studied microorganisms because they (i) include human pathogens, (ii) are abundant in coastal ecosystems, (iii) are critical to the carbon cycle, and (iv) are highly effective at degrading varied carbon sources including some polycyclic aromatic hydrocarbons (PAH) (4). The human pathogen *Vibrio vulnificus* is of special concern for public health authorities in coastal areas of the southeastern United States because it is the leading cause of seafood-borne fatalities nationwide (5). In addition to primary septicemia after ingestion of

contaminated seafood, cutaneous exposure to seawater, fish, shellfish, or fishing gear contaminated with this bacterium can cause severe wound infections. Signs of infection include inflammation and necrotizing fasciitis plus cellulitis that can lead to secondary septicemia. The fatality rate for patients contracting wound infections caused by *V. vulnificus* is 20–30% (6).

Since the DHOS, many citizens have encountered tar balls by stepping on them or inspecting them while recreating on beaches; therefore, quantifying bacteria in tar ball is epidemiologically relevant since very little information is available regarding weathered oil as source of bacterial contamination. The objective of this study was to quantify total bacterial numbers in tar balls that appeared in great numbers on Mississippi and Alabama beaches after the DHOS. In addition, and because of the epidemiological relevance of *V. vulnificus* in the Gulf Coast, the presence of this human pathogen was also evaluated.

Material and methods

Sample collection. During July through October 2010, sand (200 g/collection), tar balls (200 g/collection), and seawater (1 L/collection) were collected aseptically from the intertidal (swash zone) of three beaches in Alabama and two in Mississippi (Table 5-1; FigureV-1). Air temperature was measured *in situ.* Samples were placed in insulated coolers with ice packs immediately after collection and during transport to the Aquatic Microbiology Laboratory, Auburn University (internal temperature of the coolers remained between 15-20 ℃).

Enumeration of *V. vulnificus*. Upon arrival to the laboratory, seawater (500 ml) was centrifuged at 5,000 *g* at 20 ℃ for 30 min. Pellets were re-suspended in 10 ml (50:1)

phosphate buffered saline (PBS), and shaken vigorously to dislodge bacteria from suspended aggregates. Cell suspensions were 10-fold diluted in PBS to the 10^{-7} dilution. Sand (15 g of wet sand) was mixed with 15 ml of PBS and vortexed for 2 min to detach bacterial cells from sand grains. Large particles were allowed to be settled by gravity and 2 ml of the supernatant was diluted into 8 ml of PBS. Ten-fold serial dilutions in PBS were carried out to the 10^{-7} dilution. Tar balls were prepared similar to sand samples, except that tar ball material (15 g) was broken down using a sterile inoculation loop prior to emulsification in PBS and a longer shaking time was employed (15 min). One hundred µl of each dilution were plated onto T1N1 agar (1% tryptone, 1% NaCl, 1.5% agar) in triplicate and incubated for 48 h at 30 ℃. Total aerobic bacterial (TAB) counts were calculated as colony forming units (CFU) per ml (seawater) or gram (sand and tar ball) of undiluted sample. To quantify *V. vulnificus*, colonies were transferred to a 90-mm Whatman no. 541 filter paper (Whatman, USA), followed by colony-dot-blot DNA hybridization using a specific *V. vulnificus* probe (7) as described in Food and Drug Administration Bacteriological Analytical Manual (FDA-BAM) (8). Positive dots were recorded from each filter paper as *V. vulnificus* counts from that dilution.

Statistical analysis. Using Statistical Analysis System v. 9.2 (SAS Institute Inc., Cary, NC), both TAB and *V. vulnificus* counts were converted to base 10 logarithms to fit the model assumption of normal distribution. One-way analysis of variance (ANOVA) was used to determine the differences in *V. vulnificus* counts and Welch's ANOVA (allowing for unequal variance) in TAB in all samples. If either ANOVA or Welch's ANOVA was statistically significant, Tukey's method and Scheffe's method were applied

to perform post hoc, pair-wise comparisons at $\alpha=0.05$ for the means of log *V. vulnificus* counts or the Dunnett's T3 test (allowing unequal variance) as post hoc, pair-wise comparisons for TAB at $\alpha=0.05$.

Results and discussion

Shoreline tar ball density and abundance is used to estimate the amount of spilled oil within an area (9) as part of the shoreline cleanup assessment team process (10). In our collection sites, tar ball density was in the range of 20-40 tar balls per m^2, with an average size of 3 cm. Tar balls are considered more of a nuisance than a public health concern with cutaneous allergic reactions due to hydrocarbons as primary hazard (11). Tar balls have not reportedly been identified as a source of infectious disease, but in this study we found TAB counts significantly higher in tar ball samples ($5.1x10^6$ to $8.3x10^6$ CFU/g) than in seawater ($3.5x10^3$ to $3.1x10^4$ CFU/ml) and sand ($1.9x10^5$ to $4.2x10^5$ CFU/g) (Welch's ANOVA p <0.0001; Dunnett's T3 p<0.05) (Table 5-2). The leves of TAB we described are on the upper range of those previously reported ($3x10^4$ to $3x10^6$ CFU/g) from tar ball samples collected in Nigeria (12), one of the few studies in where bacterial counts in tar balls have been measured. Interestingly, counts of the human pathogen *V. vulnificus* were significantly higher in tar ball samples than in any other sample analyzed (ANOVA p <0.0001, Tukey's HSD p<0.05). Numbers of *V. vulnificus* in tar balls were $>10^1$ higher than in sand and $>10^2$ higher than in seawater. *Vibrio vulnificus* numbers in tar balls were $>10^5$ CFU/g in all cases, reaching $>10^6$ CFU/g in 4 out of 5 samples analyzed (Table 5-2).

To test if *V. vulnificus* biodegraded tar balls, we attempted to culture several

strains of this bacterium on tar ball-enriched seawater agar (12) (data not shown). No visible growth was observed on culture plates after 7 days of incubation. It is plausible that *V. vulnificus*, although probably not actively consuming organic compounds directly from the tar balls could benefit from byproducts of the microbes that do degrade weathered oil.

Our values for *V. vulnificus* in seawater and sand were comparable to those previously reported in Gulf of Mexico seawater, sediment, and oysters (13). However, the high number of *V. vulnificus* recovered from tar balls is noteworthy because environmental samples rarely contain more than 10^5 CFU of *V. vulnificus* per gram (14). Therefore, the high number of *V. vulnificus* ($>10^6$ CFU/g) we document in tar balls from Alabama and Mississippi beaches has clear public health implications. Tar balls are sticky, especially during the warmer months, difficult to remove (11), and upon contact with skin abrasions may vector *V. vulnificus* and lead to severe wound infections. Persons who have immunocompromising conditions (such as liver disease) are particularly at risk for *V. vulnificus* infections (6) and should avoid contact with contaminated sources.

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Table 5-1. Sample collection data.

Collection date (dd/mm/yyyy)	Location	Geographic coordinates	Air temperature (°C)
27/07/2010	Dauphin Island, AL	30°14'54"N; 88°07'40"W	28.5
30/07/2010	Ship Island, AL	30°12'46"N; 88°58'08"W	30.7
06/09/2010	Gulfport, MS	30°22'07"N; 89°04'50"W	27.6
26/09/2010	Fort Morgan, AL	30°13'30"N; 88°00'33"W	26.5
17/10/2010	Gulf Shores, AL	30°14'55"N; 87°40'05"W	23.5

Table 5-2. Total aerobic bacterial counts (TAB) and *Vibrio vulnificus* counts (VVC). Values represent average ± standard deviation of repeat plate counts of each sample. Significantly different means ($P<0.05$) within each sampling date are noted with superscripts a, b, and c.

Date	Location	Sample*	Total bacterial count (CFU/ml or CFU/g)	*V. vulnificus* count (CFU/ml or CFU/g)
27-07-2010	Dauphin Island, AL	SW	$3.1 \pm 0.4 \times 10^{4a}$	$1.0 \pm 0.0 \times 10^{3a}$
		S	$4.2 \pm 0.9 \times 10^{5b}$	$3.0 \pm 1.4 \times 10^{4b}$
		TB	$5.1 \pm 1.2 \times 10^{6c}$	$2.8 \pm 1.0 \times 10^{6c}$
30-07-2010	Ship Island, MS	SW	$4.3 \pm 0.4 \times 10^{3a}$	$5.0 \pm 2.8 \times 10^{2a}$
		S	ND†	ND
		TB	$8.3 \pm 0.2 \times 10^{6b}$	$3.5 \pm 0.7 \times 10^{5b}$
06-09-2010	Gulfport, MS	SW	$5.4 \pm 1.9 \times 10^{3a}$	$5.5 \pm 2.1 \times 10^{2a}$
		S	$3.7 \pm 0.5 \times 10^{5b}$	$1.6 \pm 0.4 \times 10^{5b}$
		TB	$7.1 \pm 1.2 \times 10^{6c}$	$2.8 \pm 0.4 \times 10^{6c}$
26-09-2010	Fort Morgan, AL	SW	$3.5 \pm 0.3 \times 10^{3a}$	$6.7 \pm 5.8 \times 10^{a}$
		S	$3.8 \pm 0.2 \times 10^{5b}$	$1.0 \pm 1.0 \times 10^{4b}$
		TB	$5.4 \pm 0.2 \times 10^{6c}$	$2.7 \pm 0.6 \times 10^{5c}$
17-10-2010	Gulf Shores, AL	SW	$1.9 \pm 0.7 \times 10^{4a}$	$8.3 \pm 0.6 \times 10^{3a}$
		S	$1.9 \pm 0.7 \times 10^{4b}$	$1.3 \pm 0.6 \times 10^{3b}$
		TB	$8.0 \pm 1.5 \times 10^{6c}$	$3.3 \pm 0.5 \times 10^{6c}$

* SW stands for sea water; S, sand and TB, tar ball.

† ND, not determined.

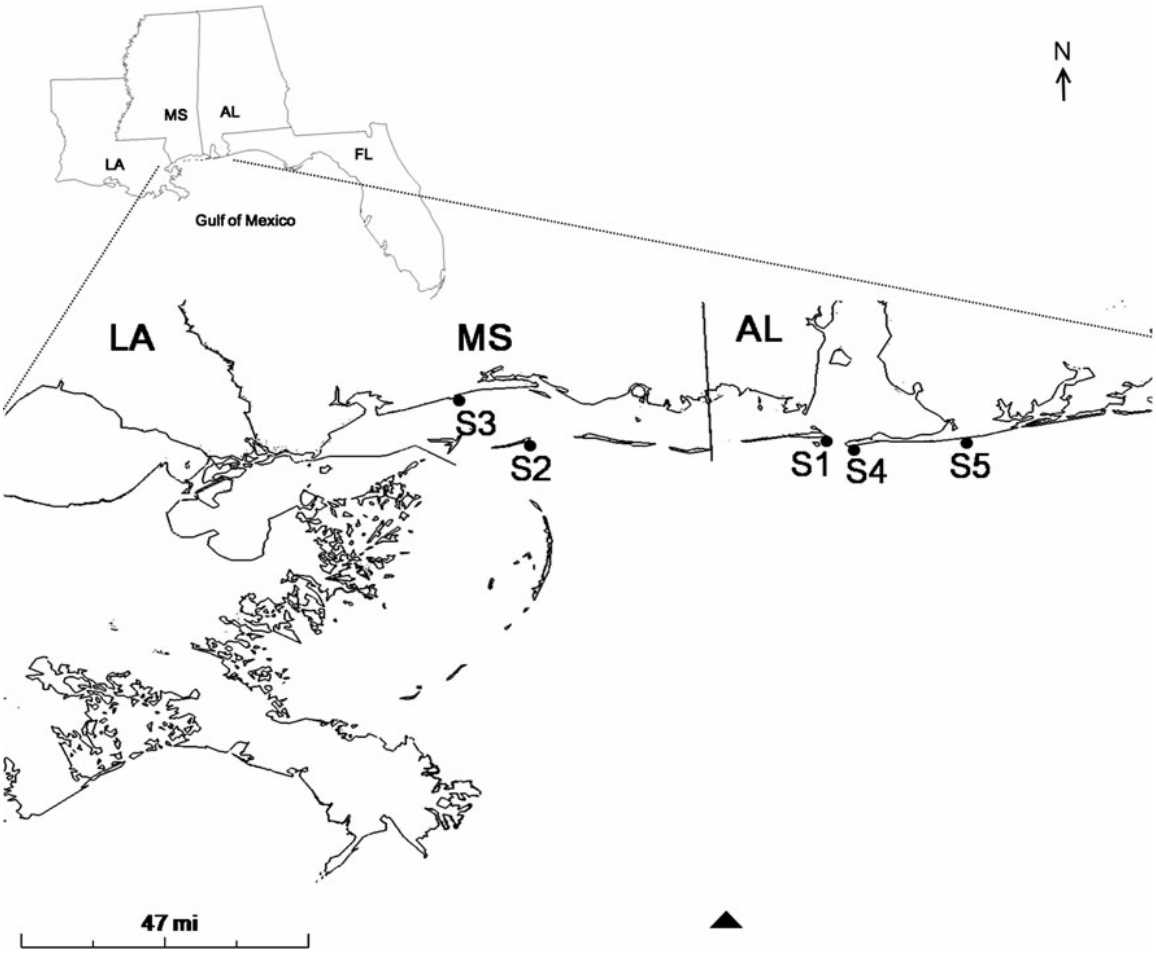

Figure 5-1. North-central Gulf of Mexico showing collecting sites: S1, Dauphin Island; S2, Ship Island; S3, Gulfport; S4, Fort Morgan; S5, Gulf Shores. Triangle indicates position of *BP Deepwater Horizon* wellsite, Mississippi Canyon Block 252 of the Gulf of Mexico.

CHAPTER 6. EFFECT OF TEMPERATURE AND SALINITY ON THE DYNAMICS OF VIBRIO VULNIFICUS 16S rRNA GENOTYPES REVEALED BY ALLELE-SPECIFIC QUANTIFICATION PCR

Abstract

Previous studies suggested the existence of two ecotypes within the species *Vibrio vulnificus* as indicated by molecular markers using the dimorphism on the *virulence correlated gene (vcg)* and the16S rDNA loci. To test whether 16S rRNA types are induced under different environmental conditions and thus could explain the ecotypes observed in the environment a series of experiments were carried out in this study. First, I investigated 16S type A and type B strains exhibited the same growth patterns when growing individually than when they were co-cultured (A-type: B-type strain, 50:50) under microcosms conditions. Different temperatures and salinities were assayed to determine the growth patterns of 16S type A and B strains. An allele-specific quantitative PCR (ASqPCR) was developed to quantify the proportion shift of the two genotypes (A and B) in the mixed culture. Results showed that the B-type strain was better fit than the A-type strain at high temperature (37˚C and 42˚C), while the opposite was true at low temperatures (15˚C, 10.5˚C) and at a low salinity (5 ppt). ASqPCR was also used to quantify the expression levels of the two 16S rRNA transcripts (A and B) in a hybrid (type AB) strain in response to temperature shift and different osmotic conditions. Results from this experiment showed no significant difference in gene expression between 16S rRNA type A and B within the same cells, indicating that 16S type B gene was not differentially regulated than 16S type A gene.

Introduction

Vibrio vulnificus is a Gram negative, motile, halophilic bacterium commonly found in estuarine and marine environments around the world (1). Although it can be isolated through a broad range of temperatures and salinities, this bacterium prefers warm waters (≥20˚C) of moderate salinities (5‰ to 25‰) (2, 3). *Vibrio vulnificus* is an opportunistic human pathogen that can cause severe illnesses such as primary septicemia and wound infections (4-7). Consumption of raw oysters is primarily linked to septicemia cases while wound infections can occur by direct exposure to contaminated seafood or seawater (for a review see Shapiro et al., 2002). Overall, the incidence of *V. vulnificus* infections in the USA is low, with an average of about 100 cases per year (8), but their severity and associated high mortality have significant implications for public health specialists (5, 9-11). Epidemiological studies showed that not everyone presented the same risk of contracting a septicemia caused by *V. vulnificus* (7) since more than 95% of septicemia patients suffered from underlying diseases, primarily liver disorders (4, 11) . Nevertheless, and based on the number of raw oysters consumed in the USA annually, the estimated number of *V. vulnificus* cases affecting the 'at risk population' is significantly higher than the observed number of cases**.** This paradox could be explained by an unequal distribution of virulence within *V. vulnificus* isolates (4, 12).

To investigate if clinical isolates represented a subset of the total *V. vulnificus* population, molecular epidemiology studies were carried out to identify virulence-associated loci. A study by Nilsson et al. (2003) was the first one to establish a correlation between clinical origin and genotyping (13). The authors used a dimorphism present in 16S rRNA gene sequence of *V. vulnificus* that have been previously described by Azanr et al. (14). Using this typing scheme, *V. vulnificus* isolates can be classified as 16S rRNA gene type A, and B. In addition, some strains

present both alleles and are classified as 16S type AB (15). While most (76.4%) clinical isolates are type B, the majority of environmental isolates are type A or AB (16, 17). Similar and additional marker was found in a region referred to as 'virulence correlate gene' (18, 19). Following this classification scheme, most clinical isolates have the allele *vcgC* while environmental strains are primarily classified as *vcgE* (19). Subsequent studies showed that both genotyping methods are generally in agreement (16, 17). Clinical strains classified as 16S type B present the *vcgC* allele while 16S type A/AB isolates are *vcgE*.

Both *rrn* and *vcg* typing systems have been used in to follow the instraspecies dynamics of *V. vulnificus* in the natural environment. Lin et al. showed that the distribution of 16S type A/AB and type B in the water column of an estuarine in the GoM was mediated by temperature (20). The 16S type A strains dominated the *V. vulnificus* populations in the colder months, while type B strains peaked in September after a period of high water temperatures. A similar dynamic of *V. vulnificus* subpopulations was described in estuarine waters in North Carolina. The proportion of *vcgC* strains increased as water warmed up and reached its highest point in August (21). These findings suggested that the distribution of 16S types A and B in the environment was not uniform. It is plausible that environmental factors such as temperature and salinity shape the distribution of *V. vulnificus* subpopulations in the marine environment.

The objective of this study was to assess the influence of temperature and salinity in a co-culture of *V. vulnificus* 16S type A and B under controlled conditions. I hypothesized that under co-culture conditions the growth patterns of 16S type A and B differ, with one type being favor the other. To test this hypothesis, we designed a series of microcosm experiments and designed an allele-specific quantitative PCR (ASqPCR) technique to measure the frequency of two different 16S rRNA gene alleles in a given sample. In addition, we applied ASqPCR to measure

the gene expression of alleles 16S type A and B in a *V. vulnificus* type AB strain to investigate if environmental factors directly influenced gene expression of 16S rDNA alleles.

Material and methods

Bacterial strains. A total of six *V. vulnificus* strains were used in the study (Table 6-1). The original description of the 16S rRNA gene polymorphism used two clinical strains, the type strain ATCC 27562 and stain C7481 (14). In this study, the 16S rRNA gene was amplified, cloned and sequenced from strains ATCC 27562 and C7481-T. These sequences were considered as the reference sequences for 16S type A (ATTCC 27562) and B (C7481-T). My strain selection for 16S reference types was based on the study by Aznar et al. (year) who used the same strains to first describe the polymorphism present in the 16S rRNA gene of *V. vulnificus.* C7481-T is a translucent, avirulent morphotype of C7481, kindly provided by Dr. J. Oliver while ATCC 27562 is the type strain of the species. Environmental strain VV3 (16S type A) and clinical strain CMCP6 (type B) were used for mixed-culture microcosm experiments. Strains VV3 and CMCP6 were preferred over ATCC 27562 and C7481-T since VV3 was originally isolated from GoM coast oysters and CMCP6 was a clinical isolate proved to be virulent in a mouse model (22) . ATCC 27562 has shown to have both variants of the 16S rRNA gene (23) and, although of clinical origin, is more related to the *V. vulnificus* 16S type A group (24). Strain C7184-T was not chosen for the microcosm experiment due to its lack of capsule that might have interfered with its overall fitness. Strain CDC 9030-95 and CN 7501 were used in cold and heat-shock experiments; both strains were isolated from GoM coast oysters and are Type AB. Prior to use, all strains were confirmed as *V. vulnificus* using species specific PCR (25) and *vvh*A probe (26). Their genotypes were verified by using RFLP as previously described (13).

Growth curves. *Vibrio vulnificus* strain VV3 (16S type A) and CMCP6 (16S type B) were individually cultured in 30-ml CAYEG (3% Casamino Acids [Amresco, Solon, OH], 0.3% yeast extract [Becton, Dickinson and Co. Spark, MD], 0.2% glucose, 0.05% KH_2PO4, pH 8.4) broth (27) at 22˚C overnight. Bacterial cells were span down for 5 min at 5,000 *g* and washed once in 1× phosphate buffered saline (PBS, pH 7.4). Then each pellet was resuspended individually in PBS and calibrated to an equal OD_{600} value of 0.5 (3.0×10^8 CFUs·ml^{-1}) as the inoculum for strain VV3 and CMCP6. Modified M9 (mM9) medium was used for the inoculation as per Chase et al. (28). mM9 contained 23.2mM Na_2HPO_4, 11.02 mM KH_2PO_4, 9.34mM NH_4Cl, 1.0mM $MgSO_4$ and was supplemented with 0.06% casamino acids, 0.006% yeast extract and 4.5mM glucose (28). The base salinity of mM9 was 5 ppt. To obtain the different salinities required for the study, mM9 was supplemented with sodium chloride. Microcosms were prepared by inoculating 100 µl of the inoculum into 100 ml of mM9 medium at the assayed salinity with an initial bacterial concentration of 3×10^5 CFUs·ml^{-1}. Microcosms were incubated with shaking (100 rpm) at the corresponding temperature. Growth curves were determined at each of the 5 temperatures assayed (10.5˚C±0.5˚C, 15±0.5˚C, 22±1˚C, 37±0.5˚C˚C and 42±0.5˚C˚C) with a salinity of 20ppt and 3 salinity levels (5 ppt, 20 ppt and 33 ppt) with a set temperature of 22˚C. Each microcosm experiment was performed in duplicate. Cell growth was quantified by measuring OD_{600} of the cultures. Growth curves for each strain under each condition were generated by plotting mean OD_{600} values against culture time.

Construction of plasmid standards and sequencing analysis. Reference 16S type A and type B sequences were generated by inserting the corresponding 16S rRNA gene fragments into pCR®4-TOPO® vector (Invitrogen, San Diego, CA), cloned, and verified by sequencing. In short, near-complete 16S rRNA genes were amplified using universal primers 63V and 1387R

(Table 6-2). The PCR was performed under a thermocycling profile: initiated with a 5-min denaturation at 95 ℃ followed by 30 cycles of 94 ℃ (45 s), 55 ℃ (45 s), and 72 ℃ (60 s), with a final extension at 72 ℃ for 10 min. PCR products were purified by 1.5% agarose gels, and extracted using GEANCLEAN® Turbo Kit (MP Biomedicals, Solon, OH). The purified PCR products were cloned using a TOPO TA cloning kit (Invitrogen, San Diego, CA). Cloned 16S rRNA genes were sequenced with an ABI 3730xl DNA sequencer and with vector primers T3 and T7 or primers 537F and CD-R that targeted conserved internal 16S rRNA sequences. The sequencing was conducted by Lucigen Corporation (Middleton, WI).

Plasmids extraction and copy number determination. Sequence-verified plasmids containing standard 16S type A (PL-*rrn*A) and type B (PL-*rrn*B) were purified from *E. coli* cells using an Aurum Plasmid mini kit (Bio-Rad Laboratories, Richmond, CA). The concentration of plasmid was quantitated on a NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE). Copy number of plasmid per microliter (CMP $\cdot\mu l^{-1}$) was calculated according to the equation: $\mathrm{CMP}\cdot\mu l^{-1} = \frac{\mathrm{COP}\times 10^{-9}}{600\ \mathrm{g\cdot mole^{-1}}\times \mathrm{Plasmid\ size}} \times \mathrm{Avogadro's\ number}$, where COP is concentration of plasmid with unit ng $\cdot\mu l^{-1}$, plasmid size is the number of nucleotide base pair of dsDNA, and Avogadro's number is equal to 6.022×10^{23} molecules $\cdot mole^{-1}$.

Allele-specific primer design. 16 rRNA gene sequences of *V. vulnificus* strain ATCC27562, C7184 and CMCP6 were retrieved from GenBank. Two allele-specific forward primers and a shared reverse primer were manually designed and tested on primer Express software 3.0 (Applied Biosystems, Foster City, CA) and IDT SciTools Oligo Analyzer 3.0 software (Integrated DNA Technologies, Coralville, IA). A single nucleotide polymorphism was placed at the 3 end (position -1 or -2) on two forward primers (Table 6-2). One additional mismatch was incorporated in the allele specific primer near the 3 end between position -2 and -

4. Primers were designed with melting point between 58˚C and 60˚C. The specificity of primer sequences were assessed by using NCBI online tool Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All the primers were screened based on criteria as described by Liu et al. (29) In short, Ct values of qPCR reaction meet the criteria that $Ct_{B-A} \geq 8$ against pure PL-*rrn*B – pure PL-*rrn*A and $Ct_{B-A} \leq 1$ against 50:50 PL-*rrn*B: PL-*rrn*A mixture (Figure 6-1). The standard *rrn* plasmid template was diluted to 10^8 copies $\cdot\mu l^{-1}$. The selected primes for this study amplify a fragment of 125 bp from 16S type A strain and of 123 bp from 16S type B strain (Figure 6-2).

Quantitative Real-Time PCR assay. Quantitative PCR (qPCR) were performed in 25 µl reaction volumes containing 1 µl of template, 200 nM of forward and reverse primer, respectively, and 1 × Power SYBR® Green PCR Master Mix (Applied Biosystems [AB], Warrington, UK) and molecular-grade water. For each sample, reactions for both primer ASVvA and ASVvB were performed on ABI PRISM™ 96-well optical reaction plate (AB, Forster City, CA). Each plate included non-template control (NTC) and pure A-type and B-type plasmid-construct positive control. All reactions were executed in triplicate. Amplification and florescence were performed on a 7500 Real Time PCR System (Applied Biosystems) using the following profile: 10 min at 95˚C for polymerase activation, 40 cycles of 15sec at 95˚C and 1 min at 60˚C. A panel of annealing temperatures (60˚C, 62˚C and 64˚C) and extension time (35s, 45s and 60s) were compared during the optimization. Dissociation curve analyses were performed using the instrument's default setting immediately after each PCR run. qPCR products from 12 well of each plate were randomly selected and analyzed on a 2% agarose gel to confirm a single amplified band of a correct size. Linearity of amplification of each primer pair was assessed according to the determination coefficient (R^2). The R^2 of the regression curve was

estimated through the plotting of $\log_{10}$ initial template copies (10^6, 10^7, 10^8 and 10^9 CMP $\cdot\mu l^{-1}$ of the standard plasmids) against Ct values. The PCR efficiency were calculated using the equation of 'E= $10^{(-1/S)} - 1$', where *S* is the slope of regression curve. The proportion of 16S type B allele in a mixed (c)DNA sample was obtained using equation adapted from Greer et al. (30): Percent of type B 16S rDNA $= \frac{1}{(2^{\Delta\Delta Ct}+1)} \times 100\%$, where ΔCt = (mean Ct of type B specific qPCR – mean Ct of type A allele specific qPCR) and ΔΔCt = ΔCt – normalization value. The mean Ct values were produced from three repeats of the template on a plate, and normalization value was derived from ΔCt 50:50 ratio in order to correct for unequal amplification efficiency or plasmid concentrations (Figure 6-1).

Assay verification. A panel of artificial mixtures of PL-*rrn*A and PL-*rrn*B was analyzed with ASqPCR to assess amplification and quantification accuracy (Table 6-3). To test the reproducibility and repeatability of the assays, three repeated runs of qPCR were carried out. The intra-run and inter-run standard deviations of Ct values were then calculated. In addition to using plasmid as template DNA, calibrated mixtures of strain VV3 (16S type A) and CMCP6 (16S type B) cells were tested. For this assay, we assumed that that both strains had same number of repeated 16S rRNA operon copies in their genome based on the genome sequence information available for the species *V. vulnificus* (31). To generate the mixtures, we quantitate the bacterial cells by plate count and OD_{600} value and made a series of 16S type B:16S type A (5:95, 10:90, 90:10, 95:5) mixtures with a final concentration of 3.0×10^8 CFUs $\cdot ml^{-1}$. Genomic DNA was extracted by using the rapid boiling protocol (see below).

16S type A and type B strain mixed-culture microcosm. The individual inoculum for strain VV3 or CMCP6 in PBS (OD = 0.5) was prepared as described above. Equal volume of these two inoculums were mixed to produce the mixed inoculum (VV3:CMCP6=50:50). Mixed-

culture microcosms were then generated by diluting 100 µl of the mixed inoculum into 100 ml of mM9 medium at the assayed salinity (see below). The initial bacterial concentration of microcosms was ca. 3×10^5 CFUs $\cdot ml^{-1}$. Microcosms were incubated with shaking (100 rpm) at the corresponding temperature. Each microcosm experiment was carried out in triplicate.

Environmental variables tested. The influence of temperature and salinity on the growth dynamics of 16S type A and B mixed cultures was assayed as follows. When temperature was the tested variable, salinity was fixed at 20 ppt and four temperatures were compared: 15˚C, 22˚C, 37˚C and 42˚C. mM9 broth was equilibrate to each tested temperature prior inoculation. When salinity was the tested variable, three salinities at 5 ppt, 20 ppt and 33 ppt were tested at 22˚C. When cultures reached the middle of the exponential phase (OD_{600} value 0.6), samples (1 ml of culture medium) were collected in duplicate from each replicate (microcosm). For each temperature and salinity assayed, a total of 6 samples (3 microcosm replicates $\times 2$ samples from each microcosm) were analyzed.

Cell viability at low temperature. To determine if there was a difference in cell viability between 16S type A and type B strains under low temperature, the following study was carried out. A mixed inoculum of strain VV3 and CMCP6 50:50 was made as described above. Because cells were not expected to grow at the assayed low temperature, instead of inoculating 100 µl of the inoculum into 100 ml modified M9 medium as before, I diluted 10 ml of the mixed inoculum into 90 ml of mM9 medium (salinity 20 ppt) to obtain sufficient cells for DNA extraction. Thus, the initial cell density was 3×10^7 CFUs $\cdot ml^{-1}$. Microcosms were incubated at 10.5 ℃ with shaking (100 rpm). Samples for DNA extraction were taken at 0, 24, and 48 h after inoculation as processed as before. In addition, single-strain cell suspensions (3×10^7 CFUs $\cdot ml^{-1}$) of each individual strain were inoculated in parallel. The viability of each individual strain as

well as the mixed culture was at 10.5 ℃ was assessed by using L7012 LIVE/DEAD® BacLight Viability kit (Invitrogen) at 24 and 48 h post-inoculation.

DNA extraction. Bacterial cells were immediately pelleted from 1 ml samples by centrifugation at 10,000 *g* for 1min. Pellets were washed in 1×PBS, resuspended into 350 µl of 1× TE buffer (10mM Tris, 1mM EDTA, pH8.0) and subsequently transferred into a sterile 2 ml screw-cap tube containing 200 mg 150-212 µm acid-wash glass beads (Sigma-Aldrich Co. St. Louis, MD). Cell disruption was performed by vortexing the tube horizontally at maximum scale for 5 min on Vortex-Genie® 2 mixer (Mo Bio Laboratory Inc.). The lysates in tubes were heated at 100˚C for 15 min and cooled on ice followed by centrifugation of 16,000 *g* for 5 min. The supernatant was carefully transferred to a new tube and stored at -80˚C until used as DNA templates.

Microcosms with 16S type AB strains. Two 16S type AB strains, CN 7501and CDC 9030-95, were used to determine if the expression of 16S rRNA gene alleles was differentially influenced by temperature or salinity. Cold and heat shock (C/HS) as well as changes in salinity were assayed as follows. For each 16S type AB strain, the inoculum was prepared as described above. A 100 µl of inoculum was inoculated into 100 ml sterile mM9 medium (for C/HS treatments salinity was maintained at 20 ppt while for the salinity assays temperature was maintained at 22 ℃). In C/HS experiments, the culture was initially grown at 22˚C to an OD_{600} of ca. 0.5 before being transferred to 10.5˚C (CS) or 42˚C (HS) for 30 min. Samples (1 ml of the culture) for RNA extraction were taken from each microcosm in duplicate immediately after C/HS. As control, one set of microcosm was incubated at 22˚C throughout the experiment. For the salinity test, cultures were sampled when they reached an OD_{600} of 0.6 at 22˚C under different salinities (5 ppt, 20 ppt and 33 ppt). Samples were centrifuged at 10,000 *g* for 1min and

immediately preserved in RNA*later* solution (Ambion, Austin, TX) at 4˚C according to the manufacturer's instructions. Samples were taken in duplicate from each microcosm, and RNA extractions of two repeats were pooled before reverse transcription. RNA was isolated and reversely transcribed to cDNA within 24 hours (see below).

Isolation of RNA and preparation of cDNA for 16S type AB strains. Total bacterial RNA was isolated using RNeasy® Mini Kit (Qiagen, Inc., Valencia, CA). In brief, 16S type AB cells were pelleted from RNA*later* suspensions by centrifugation at 10,000 *g* for 30 sec, and resuspended in 700 µl of lysis buffer RLT of the RNeasy Kit (added with 1% of β-mercaptoethanol) after the removal of the supernatant. The resuspension was transferred to a 2 ml screw cap tube filled with 100 mg 150-212 µm acid-washed glass beads. Cells homogenization was performed in a mini-bead beater (Biospec Products, Bartlesville, OK) for 30 sec twice with a 15 sec on-ice off in between and immediately cooled down on ice. Additional steps followed manufacturer's instructions (32). DNA was removed from RNA samples by using a TURBO DNase-free kit (Ambion, Cambridgeshire, UK) following the protocol for rigorous DNase treatment. Treated RNA was confirmed to contain an insignificant amount of gDNA as indicated by a Ct value >32 when RNA was directly used as template in ASqPCR. RNA concentration was determined with a NanoDrop® ND-1000 spectrophotometer. Each of RNA sample was adjusted to a concentration of 100 ng $\cdot\mu l^{-1}$ with RNase-free sterile water. One µg of RNA was reversely transcribed to cDNA by using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). cDNA samples were stored at -80˚C until further analysis.

Statistical analysis. The one-way or two-way ANOVA with Tukey's post hoc analysis was used to determine the significance of the frequency of 16S type B strain in mixed-culture

microcosms or B-type 16S rRNA in type AB strain cells under different conditions. The ratio of A-type and B-type 16S rDNA allele copies in 16S type AB strain was tested by pairwise t-test. The statistical analysis was performed on the SAS Software 9.2 version (SAS Institute, Cary, N.C.).

Results

Growth curves of strain VV3 and CMCP6. Growth curves are shown in Fig 3 (temperature gradient) and Fig 4 (salinity gradient). Both strains displayed similar growth patterns at 15 ℃, 22 ℃ and 37 ℃ while no growth was observed at 10.5 ℃. Interestingly, only 16S type B CMCP6 strain grew at 42˚C while no growth was observed in the 16S type strain VV3. Salinity had little effect on the growth curves and both strain produced similar growing patterns under the three salinities tested. However, 16S type B strain CMCP6 exhibited a shorter lag phase than 16S type A VV3 strain at 20 ppt salinity (Figure 6-4).

ASqPCR assay validation. PL-*rrn*A construct was amplified with specific primer ASVvA and PL-*rrn*B construct with primer ASVvB. Dissociation curves generated only a single peak for each type amplicon (Tm with a slight difference: ca. 82˚C for amplicon A and ca. 83˚C for amplicon B), and no peaks that indicates the presence of primer-dimers. The ASqPCR parameters for A type (ASVvA) and B type (ASVvB) are shown in Table 6-3. The relatively low PCR efficiencies were expected due to the intentional mismatches introduced into the primers to balance the specificity. The short length region containing the A/B polymorphism in the 16S gene limited option for specific primer design resulting in the two ASqPCR reactions having slight different amplification efficiencies. However, both reactions have a good linearity indicated by the coefficient of determination R^2 >0.99 for the regression curve (Ct value against $\log_{10}{}^{[\text{plasmid copy number}]}$). The average intra-run and inter-run standard deviations of Ct value for A

type and B type reaction were lower than 0.18, indicating a good reproducibility between PCR runs. Testing the artificial mixtures of PL-*rrn*B from 1% to 95% at 10^8 copies $\cdot\mu l^{-1}$ displayed a good correlation between the expected percentage and the calculated percentage (Table 6-4). To test the ASqPCR efficacy when cell mixtures were used we compared the calculated value of 16S type B percentage with the expected value determined by plate count. The percentage of 16S type B cells calculated in the four tested ratios was as follows: 1) for a A:B ratio of 5:95 the calculated 16S type percent was 3.5%; 2) for 10:90 was 7.6%; 3) for 90:10 was 90.2%; and 4) for 95:5 was 95.4%. In general, the deviation between the expected value and calculated value was small, although it was slightly higher when the percent of 16S B type cells was low.

Dynamics of 16S type A and type B strains under co-culture conditions. Figure 6-5 summarizes the results of the co-culture experiments. At low growth temperature (15˚C), my results showed that 16S type A VV3 strain constituted 97.8% of the population in the mixed culture when the culture reached the late exponential phase. Conversely, 16S type B CMCP6 strain outcompeted VV3 at 37˚C with 70.4% of cells estimated to be 16S type B. At 42˚C, practically the whole population was composed of 16S type B cells (99.9%) (Figure 6-5A). When 16S type A and B co-cultures were incubated at room temperature the percentage of 16S type B cells was close to 50% (49.6%) indicating that this temperature does not play a selective role for either 16S type. Regarding salinity, the 16S type A outcompeted 16S type B cells under low-salinity conditions (5ppt) with 16S type B cells remaining below 0.4% at the late exponential phase. Similar percentages (~50%) of both strains were detected under salinities of 20 ppt and 33 ppt (Figure 6-5B), suggesting these salinities do not favor any 16S type.

Survivability at 10.5˚C. The effect of cold temperature (lower than the minimum temperature require for growth) on both 16S types was assessed during a 48-hour incubation

period at 10.5˚C. The results of dual fluoresce staining on each single strain showed that the density of viable cells decreased between 24 h and 48 h for both strains (Figure 6-6). However, based on qualitative microscopy data, more 16S type A cells remained viable at 24 h and 48 h than 16S type B. ASqPCR analysis on the co-cultured microcosm confirmed the results observed by microscopy since the percentage of 16S type B strain (both live and dead cells with intact genomic DNA) declined from the initial 62.7% to 23.4% at 24 h and to a final 9.9 % after 48 hours at 10.5˚C (Figure 6-7). Here we have an assumption that dead cells of each genotype strain were lysed at same rate.

Determination of 16S rRNA alleles copy number in 16S type AB strains. The frequency of the 16S type B allele in the genomic DNA extracted from the 16S type AB strain was determined by ASqPCR. Results showed that in strain CN 7501 the copy number of 16S type B allele was 10.87% ± 0.66% (mean ± SD, measured in 3 independent DNA extractions) in strain CN 7501 while in strain CDC 9030-95 was 12.17% ± 1.55%. Statistical analysis using pairwise t-test comparison indicated that the ratio of A: B alleles are significantly equal to 9:1 ($p=0.58$ for strain CN 7501 and $p=0.15$ for CDC 9030-95, $n=3$, $\alpha=0.01$). These results were taken into account when calculating the gene expression levels in the cold/heat shock experiments.

Expression of 16S alleles in the 16S type AB strains in response to cold/heat shock and salinity. Changes in temperature did not significantly ($\alpha=0.01$) influence the expression level of 16S type B gene in either strain tested. Levels of 16S type B gene transcript remained the same in cold and heat-shocked treated cultures as they were in controls and represented approximately 15% of the total detected 16S rRNA gene transcripts. Similarly, no significant

differences were found in 16S allele expression when strains were incubated at different salinities (Figure 6-8).

Discussion

The goal of this study was to determine if there was a difference in the growth pattern of 16S type A and type B of *V. vulnificus* could explain why 16S type B is more abundant at the end of the summer period. The ASqPCR method I designed for this study allowed me to determine the proportion of cells belonging to each 16S genotype in a mixed culture. My data showed that when both types were cultured individually, their growth curves were similar at 15 ℃, 22 ℃, and 35 ℃ while only 16S type B strain grew at 42 ℃. However, when strains were co-cultured, the increase of 16S type B cells at 35 ℃ was significant, indicating this type outcompeted 16S type A at this temperature. As expected, since 16S type A did not grow at 42 ℃, when both strains were co-cultured at this temperature, only 16S type B was detected. Conversely, 16S type A outperformed 16S type B cells at 15 ℃ although both strains displayed similar individual growth patterns at 15 ℃.

The structural variation of *V. vulnificus* population in the environment is influenced by water temperature. The seasonal dynamic of *V. vulnificus* 16S type A and type B subpopulations in the water column has been observed in the Galveston Bay, TX and in Alligator Bay, NC (20, 33). In both studies, the percentage of 16S type B (*vcg*C) strains increased with water temperature, and peaked at the end of summer (when seawater temperatures was above 30˚C). These observations obtained from field surveys are supported by the results of my *in vitro* study. *In vitro*, the proportion of 16S type B cells increased from ca. 50% to 77% at 37˚C and up to > 99 % at 42˚C when the mixed culture reached to an OD of 0.6. Thus, it is likely that warmer water temperature acts as a selecting force that increases the proportion of 16S type B strains

during the summer. Moreover, this adaptation to warmer temperatures displayed by 16S type B might explain why 16S type B are responsible for the majority of *V. vulnificus* infections in humans (13, 19). It is possible that the human body temperature (37˚C) acts as a filter when *V. vulnificus* enters the human host allowing 16S type B cells to outgrow 16S type A cells. Thus, it is plausible that the main difference between 16S type A and B is not based on virulence factors but in the adaptation to warmer temperatures displayed by 16S type B. This notion is supported by the fact that when tested in mice, a correlation between 16S type B and virulence was not found and strains of both types (A and B) were found to cause disease in this mammal model (22).

My results also suggest that 16S type A strains are better adapted to low temperatures than 16S type B. The 16S type A strain increased its percentage in relationship to type B under low temperature (15˚C) conditions. In addition, more 16S type A type cells remained viable after 48-hour incubation at below-growth temperature (10.5˚C). These findings agree with previous results from field studies. In Lin and Schwarz's study, both genotypes of *V. vulnificus* were undetectable during the winter months but 16S type A strains were recovered earlier in spring than 16S type B. Similarly, data from the Warner and Oliver's study demonstrated that a higher proportion of 16S type A was present in the water column when the bacterium was first recovered in spring before the summer bloom (33).

Additionally, 16S type A *V. vulnificus* strains seemed better adapted to low salinities than 16S type B strains. My *in vitro* results showed a competitive advantage of the 16S type A strain over the 16S type B strain in the microcosm with a salinity of 5 ppt. Herein, my study supports the idea (34) that two ecotypes are present within the species *V. vulnificus* and that they are better adapted to different environmental conditions that play a significant role in the life cycle of an

estuarine bacteria such are temperature and salinity. It needs to be noted that although the 16S marker used targets a very small fragment of the *V. vulnificus* genome, a perfect correlation exists between the two genotypes found within the species using AFLP analysis (whole genome typing method) and 16S type A/AB and B (24, 35). Therefore, is expected that many other loci besides the 16S rRNA gene are implied in the observed adaptations to temperature and salinity.

The presence of both 16S haplotypes in 16S type AB strains posits an interesting evolutionary question as duplicate genes tend to share the same sequence through homogenization processes analogous to eukaryotic gene conversion. Members of the genus *Vibrio* to carry more than one *rrn* operon and intragenomic heterogeneity is not uncommon. It has been postulated that bacteria with more *rrn* operons can adapt quicker to changes in the environment than those with fewer copies. *Vibrio vulnificus* contains 9 *rrn* copies and a study by Arias et al. (36) revealed a high degree of interoperonic heterogeneity. Are these different operons the result of a recent lateral transfer event and thus have not had time to converge through homogenization? Or, do they offer an evolutionary advantage to the cell and thus they have been maintained over time? Recently, is has been demonstrated that different 16S haplotypes can be differentially expressed within the same cell depending on environmental factors (37). To determine if this was the case in *V. vulnificus*, I measured the expression level of each 16S rDNA allele within the same cell under different environmental conditions. If the 16S rRNA variants in the *V. vulnificus* hybrid strain were functionally specialized, I expected to observe changes in the frequency of 16S rRNA type A/B expressed in the cells. My results showed that both 16S rDNA variants were expressed in the 16S type AB strain cells. However, the proportion of A/B transcripts indicated no selective expression of either variant as levels of both types remained statistically identical regardless of the treatment applied.

In summary, under microcosm conditions, I showed that 16S type A and type B of *V. vulnificus* significantly different in their growth response to different temperature and salinity regimes. Interestingly, some of those differences were only observed when both types were co-cultured indicating that *V. vulnificus* subpopulation dynamics in nature are influenced by temperature and salinity. In hybrid 16S type AB strains, the two 16S alleles are not regulated differently from each other when exposed to temperature changes and different osmolality, which suggest that the better fitness observed in the co-culture experiments was not due to the expression of a specific 16S alleles but likely to other loci. This study provided additional evidence for the delineation of two *V. vulnificus* ecotypes.

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35. **Wood RR, Arias CR.** 2012. Distribution and survival of *Vibrio vulnificus* genotypes in postharvest Gulf Coast (USA) oysters under refrigeration. Journal of Applied Microbiology **113:**172-180.

36. **Tao Z, Bullard S, Arias C.** 2011. High numbers of *Vibrio vulnificus* in tar balls collected from oiled areas of the North-Central Gulf of Mexico following the 2010 BP deepwater horizon oil spill. EcoHealth **8:**507-511.

37. **López-López A, Benlloch S, Bonfá M, Rodríguez-Valera F, Mira A.** 2007. Intragenomic 16S rDNA Divergence in *Haloarcula marismortui* is an adaptation to different temperatures. Journal of molecular evolution **65:**687-696.

Table 6-1. *Vibrio vulnificus* strains

Strain ID	Source	16S type
CN 7501	Fish, Alabama, USA	AB
CDC 90 1506	Clinical, Florida, USA	AB
CMCP6	Clinical, South Korean	A
VV3	Oyster, Alabama, USA	B
ATCC 27562	Human blood, Florida, USA	A
C7184	Human blood, USA	B

Table 6-2. Primers for sequencing and ASqPCR.

Assay	Sequence (5'→3')	Reference
Sequencing primers		
63V	CAGGCCTAACACA TGCA AGTC	a
1387R	GGGCGGWGTGTACAAGGC	a
533 F	GTGCCAGCAGCCGCGGTAA	b
CD-R	CTTGTGCGGGCCCCCGTCAATTC	c
ASqPCR primers*		
Fw.(ASVvA)	CGATGGCTAATACCGCATCATA**A**	This study
Fw.(ASVvB)	ATGGCTAATACCGCATCAT**G**C	This study
Rev.(Common)	AGGGATCGTCGCCTTGGT	This study

*For allele specific qPCR primes, the incorporated mismatch is shown as underlined, and the target single-nucleotide mutation locus is shown as bolded.

Table 6-2 literature references:

a. **Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC,** Hiom SJ, Wade WG. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied and environmental microbiology 64:795-799.

b. **Weisburg WG, Barns SM, Pelletier DA, Lane DJ.** 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of bacteriology 173:697-703.

c. **Rudi K, Skulberg OM, Jakobsen KS.** 1998. Evolution of Cyanobacteria by Exchange of Genetic Material among Phyletically Related Strains. Journal of bacteriology 180:3453-3461.

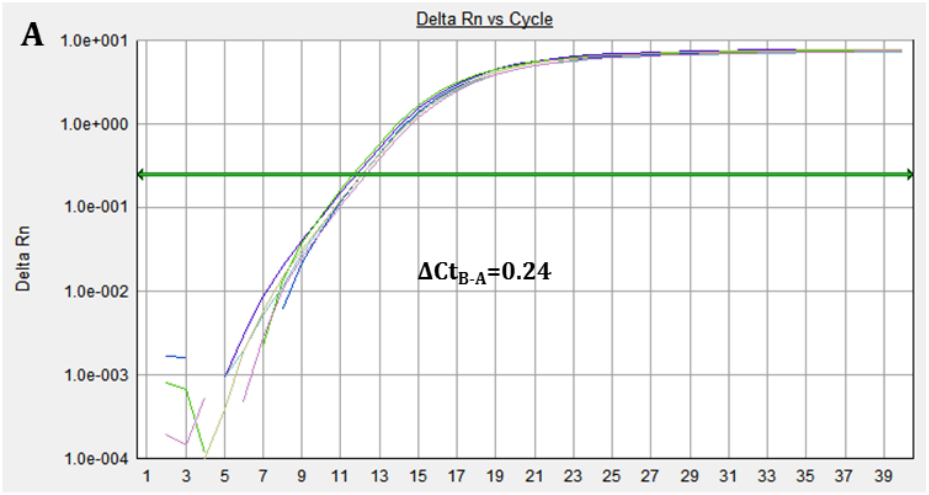
Table 6-3. Slopes, Intercepts, R^2, efficiencies, intra/inter -run standard deviations of two ASqPCR amplifications.

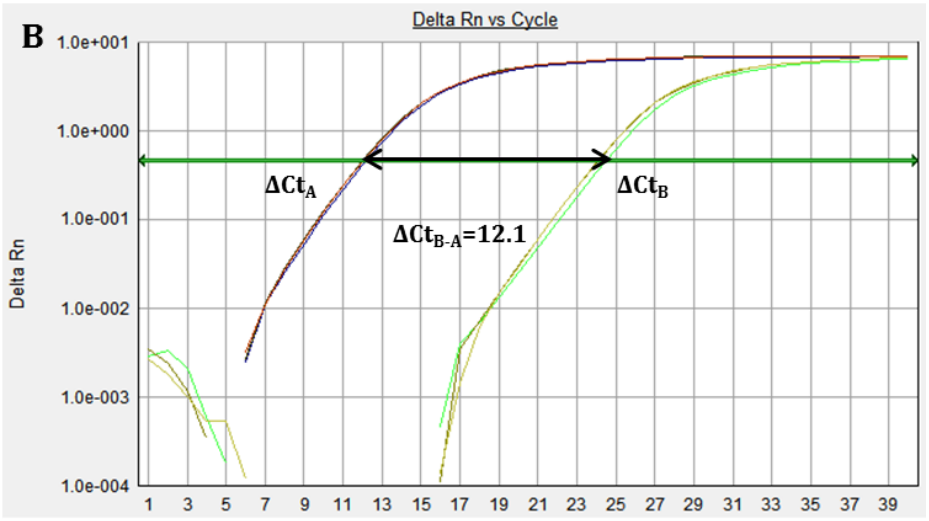
qPCR reaction	Slope	Intercept	R^2	Efficiency	Ct value intra-run SD. average (range)	Ct value inter-run SD. average (range)
A type (ASVvA)	3.814	42.217	>0.99	83%	0.17 (0.03-0.49)	0.14 (0.03-0.25)
B type (ASVvB)	3.574	40.429	>0.99	90%	0.18 (0.02-0.35)	0.14 (0.03-0.25)

Table 6-4. Relative 16S rRNA allele frequencies using standards of mixed plasmid constructs

Ratio (B:A)	ΔCt_{B-A}	ΔCt_{B-A} (Nor.)	Expected % B	Calculated % B
100:0	11.57	11.33	0	0.04
1:99	7.40	7.16	1	0.69
10:90	9.48	9.24	10	9.50
90:10	-2.78	-3.02	90	89.00
95:5	-3.72	-3.96	95	94.00
100:0	-9.10	-9.34	100	99.85
50:50	0.24[a]	0.00	-	-

These assays were evaluated at 10^8 total copies per reaction. [a] The ΔCt_{B-A} for ratio 50:50 should be equal to zero, where the deviation reflected the differences in amplification efficiencies and plasmids copy number in the reaction. ΔCt_{B-A} (Nor.) are the results of ΔCt_{B-A} subtracted with the normalization value for the assay dynamic range (see the text). These normalized values were used to calculate the percentage of 16S genotype B plasmid in the mixture.


A
Delta Rn vs Cycle
Delta Rn
1.0e+001
1.0e+000
1.0e-001
1.0e-002
1.0e-003
1.0e-004
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
ΔCt$_{B-A}$=0.24


B
Delta Rn vs Cycle
Delta Rn
1.0e+001
1.0e+000
1.0e-001
1.0e-002
1.0e-003
1.0e-004
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
ΔCt$_A$
ΔCt$_B$
ΔCt$_{B-A}$=12.1

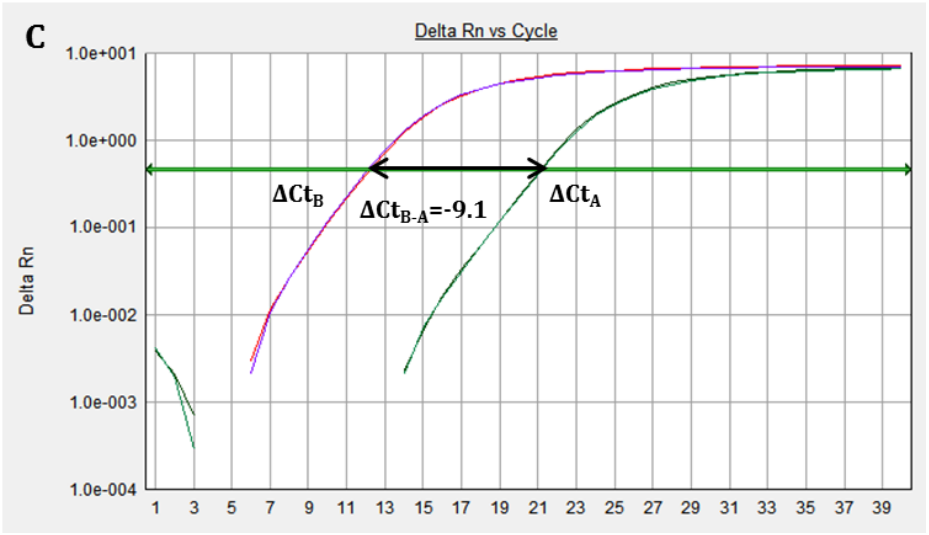

C
Delta Rn vs Cycle
Delta Rn
1.0e+001
1.0e+000
1.0e-001
1.0e-002
1.0e-003
1.0e-004
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
ΔCt$_B$
ΔCt$_{B-A}$=-9.1
ΔCt$_A$

Figure 6-1. ASqPCR amplification plots of 10^8 copies of (A) 50:50 standard *rrn* plasmid construct of A-type : B-type, (B) pure A-type plasmid, and (C) pure B-type plasmid. The ΔCt_{B-A} with A : B (50:50) was the normalization value. The graph shows that this value calculated at 10^8 copies was 0.24. In this study, the average normalization value across the entire dynamic range from 10^6 to 10^9 copies was generated to use for the ASqPCR assay. The average ΔCt was also equals to 0.24.

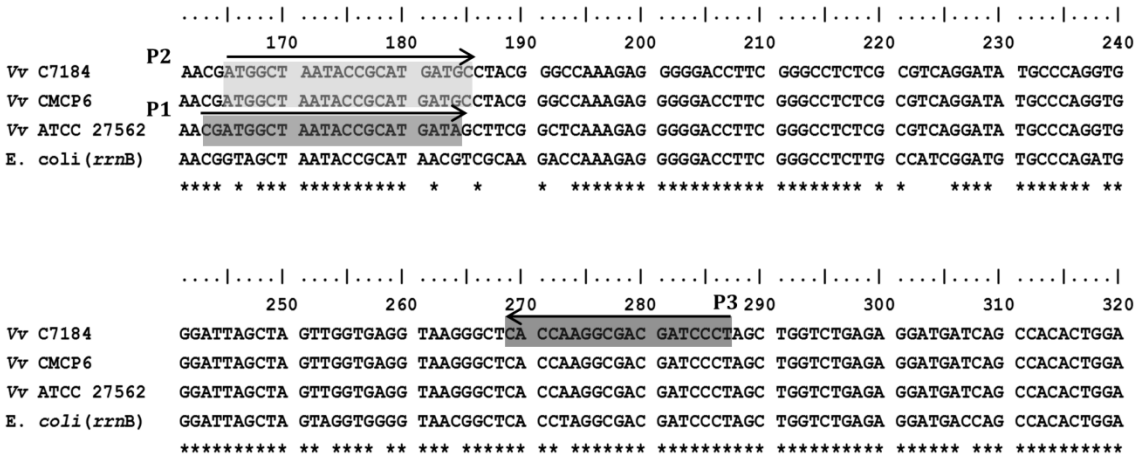


Figure 6-2. Partial 16S rDNA sequence alignment of *V. vulnificus* strain C7184 (GenBank accession no.: X76334), CMCP6 (NR 074889), ATCC 27562 (X76333) and *E. coli* (J01695). Shading indicates the ASqPCR primer positions: P1, P2 and P3 refer to primer ASVvA, ASVvB and common reverse primer, respectively. Numbers on top ruler denote position in the *Escherichia coli* reference sequence.

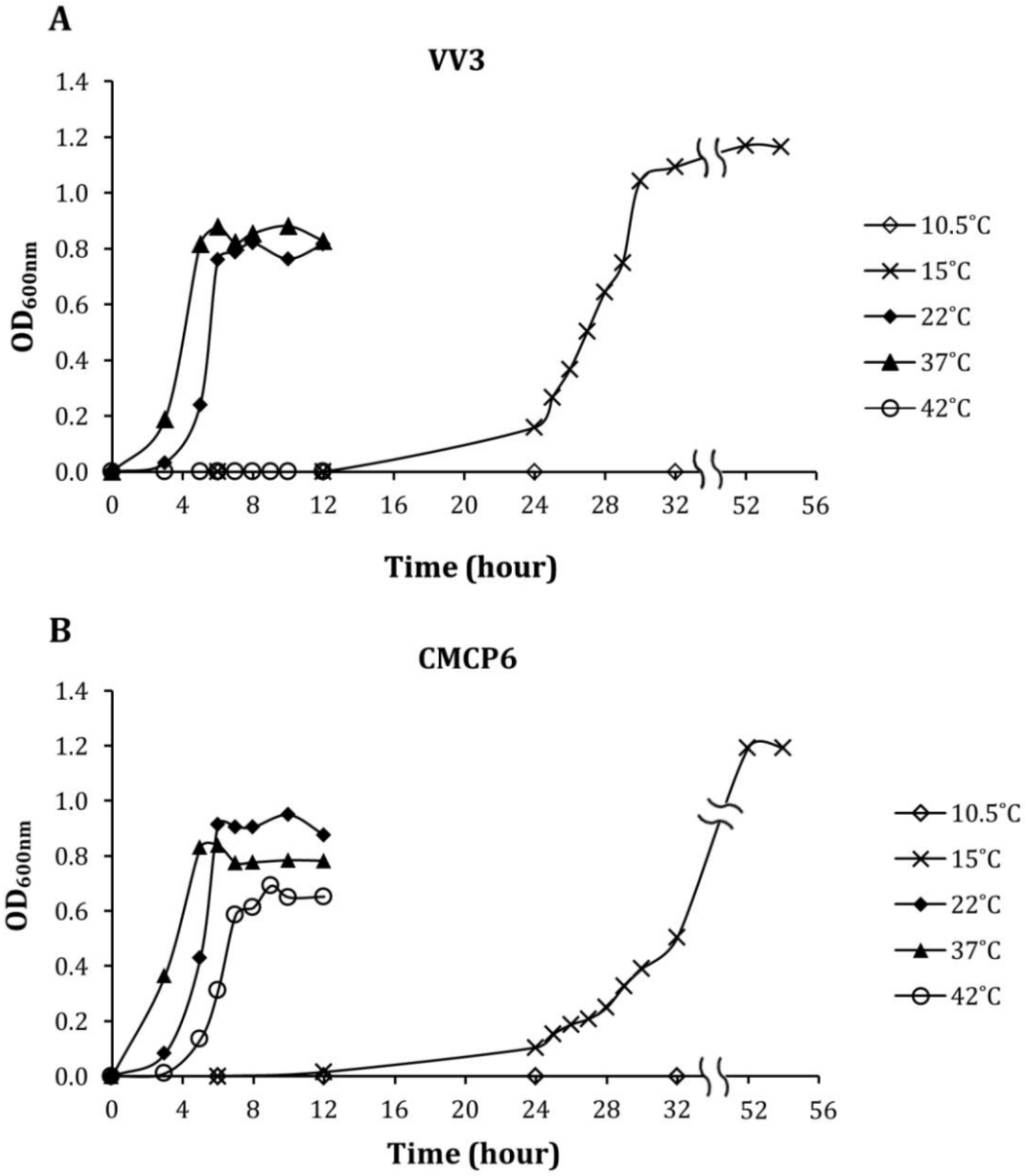


Figure 6-3. Growth curves of *V. vulnificus* across 5 temperature gradients (with a salinity of 20ppt): (A) strain VV3; (B) strain CMCP6. Data are the average values of two replicates.

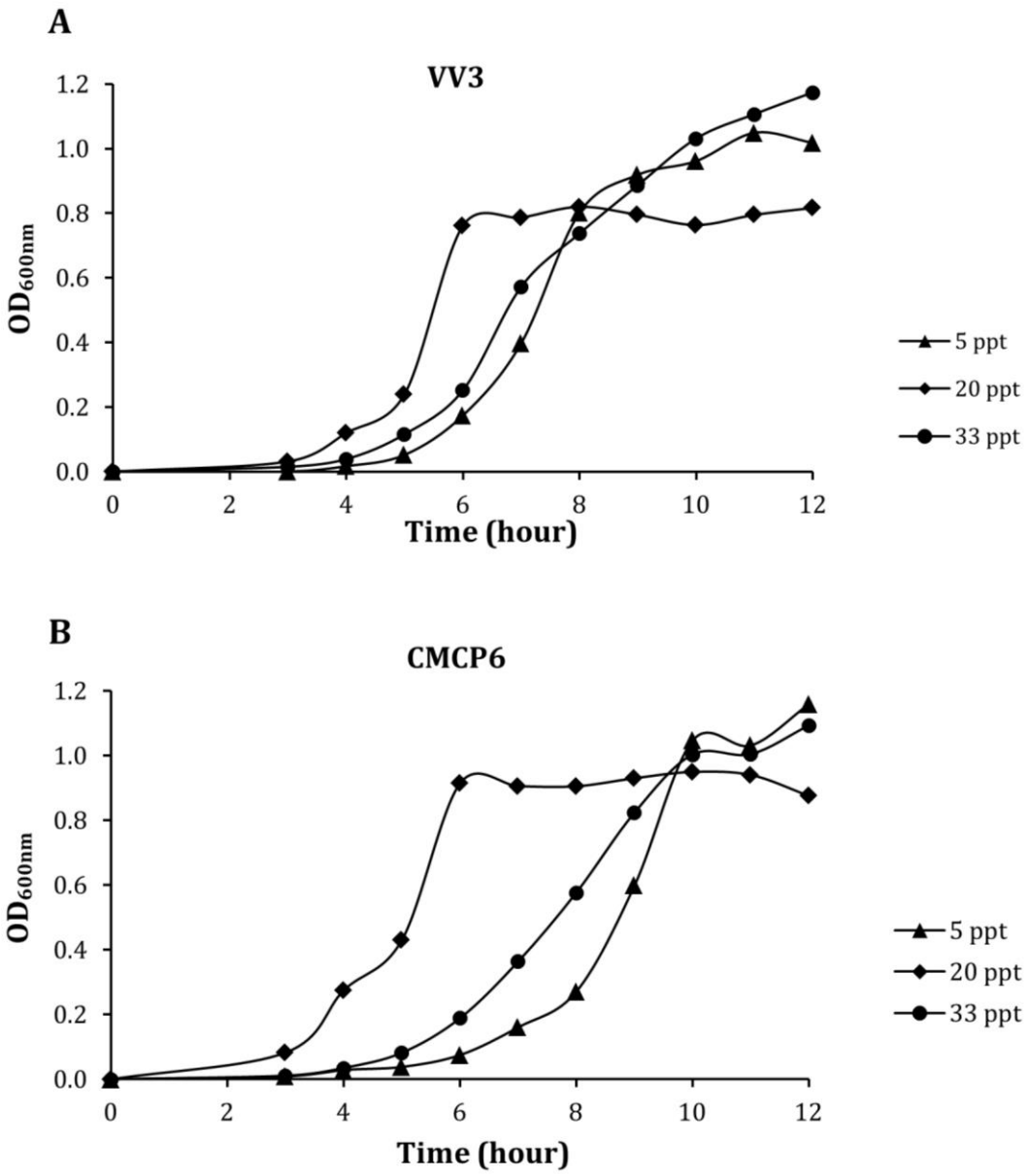


Figure 6-4. Growth curves of *V. vulnificus* across 3 salinity gradient (at 22˚C): (A) strain CMCP6; (B) strain VV3. Data are the average values of two replicates.

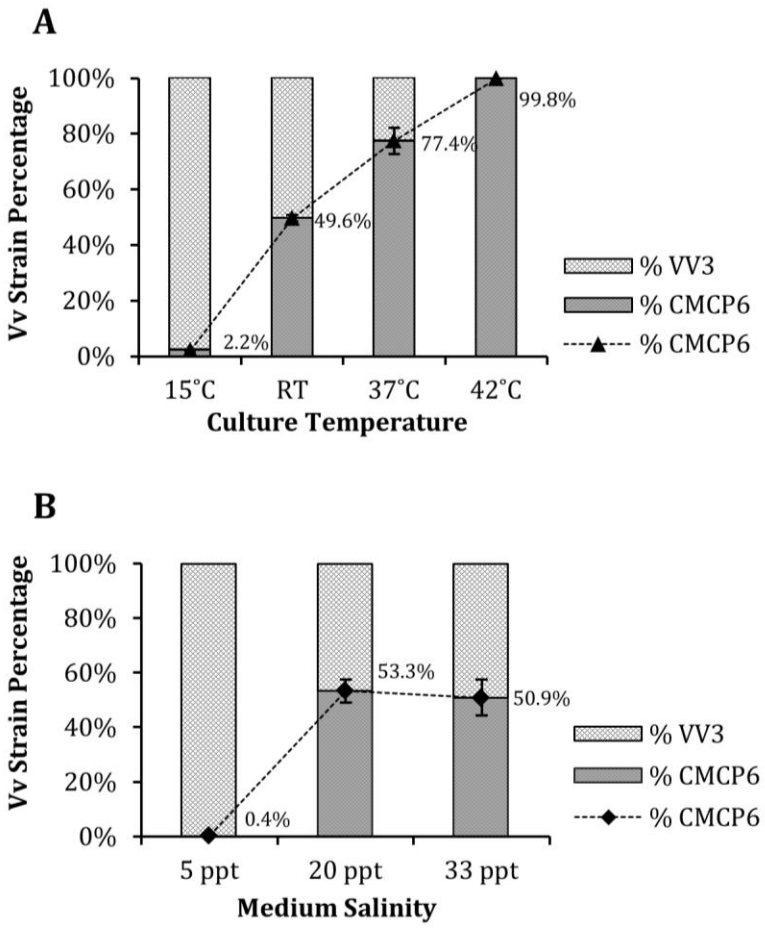


Figure 6-5. Growth dynamic of the co-culture of two genotypes in the mixed-culture microcosm at different conditions when the OD reaches to 0.6: (A) across a temperature gradient (medium salinity 20ppt); (B) across different salinities (at 22˚C).

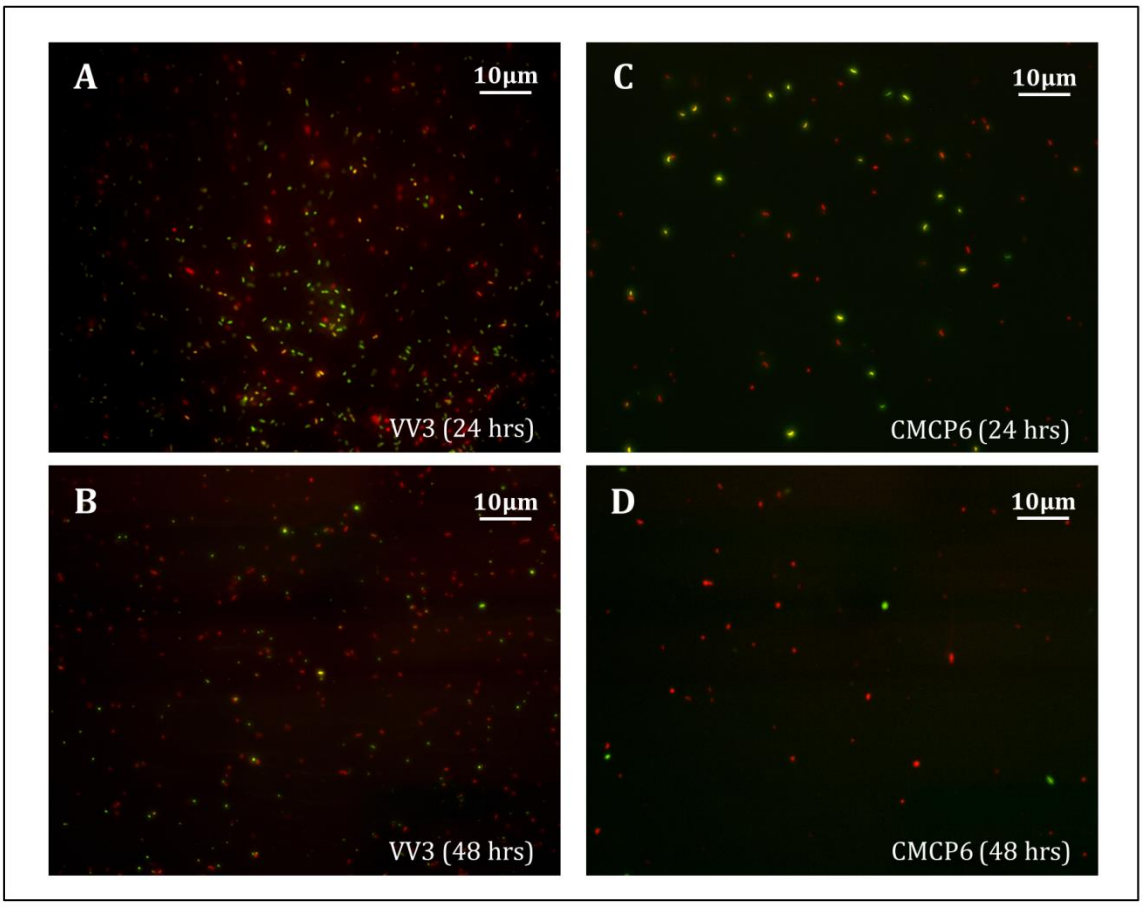


Figure 6-6. Photographs of *V. vulnificus* strain CMCP6 and VV3 epiflourescence microscopic observations after 24-h and 48-h incaution at 10.5˚C. Bacteria were stained with L7012 LIVE/DEAD® BacLight Viability kit before the microscopic observation at 320× magnification. Cells fluorescing green are considered alive while red fluorescing cells are considered dead.

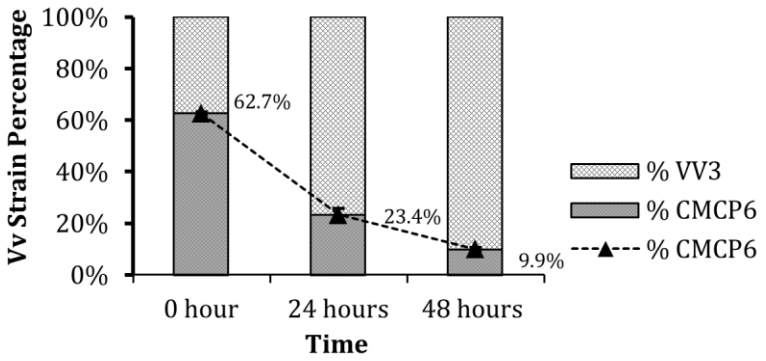


Figure 6-7. The genotype dynamic incubating at 10.5˚C at 0h, 24h and 48h (below minimum growth temperature; OD value declined over time, see text).

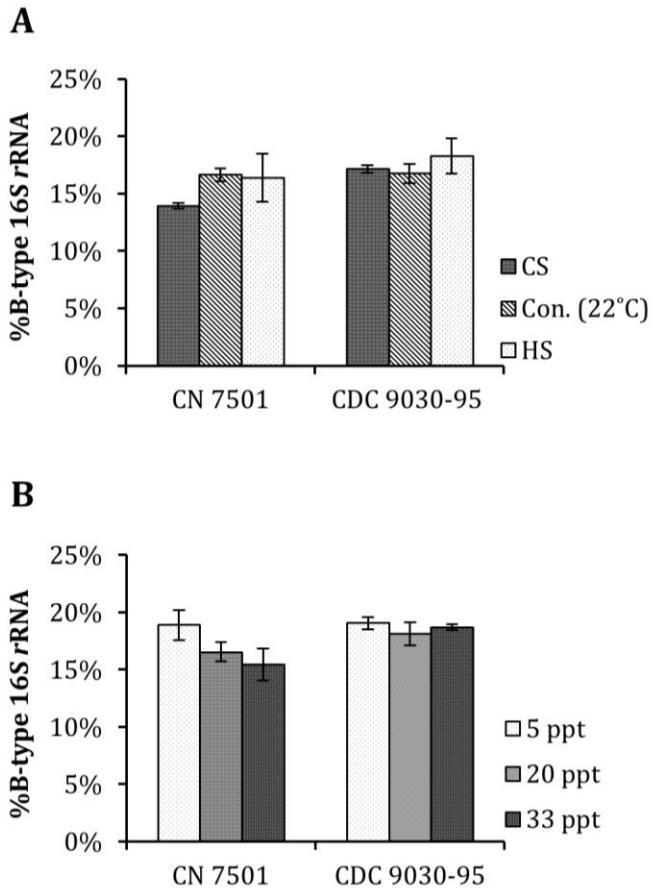


Figure 6-8. Expression level of B-type 16S rRNA (rrn) gene relative to the expressed 16S rRNA genes (A and B) through cold/heat shock (medium salinity 20ppt); (C) across a salinity gradient (at 22˚C). Means ± SD are shown. The frequencies of B-type 16S rRNA do not vary significantly with cold/heat shock treatments or under different salinities. The significance level of α=0.01was used.

CHAPTER 7. SUMMARY AND CONCLUSION

In this dissertation, I investigated the distribution and prevalence of two human-pathogenic *Vibrio* species (*V. vulnificus* and *V. parahaemolyticus*) in non-shellfish samples including fish, bait shrimp, water, sand and crude oil material released by the *Deepwater Horizon* oil spill along the Northern GoM coast.

Results from the fish investigation showed that 37% of all sampled fish (n=242) were positive for *V. vulnificus* presence on skin and mucus. A total of 244 *V. vulnificus* isolates were recovered from fish and further analyzed to determine the population structure of this species in fish. Ascription to 16S rRNA gene type indicated that 157 isolates were type A (62%), 72 (29%) were type B (significantly correlated with clinical cases) and 22 (9%) were type AB. Amplified fragment length polymorphism (AFLP) was used to resolve the genetic diversity within the species. One hundred and twenty one unique AFLP profiles were found among all analyzed isolates resulting in a calculated Simpson's index of diversity of 0.991. AFLP profiles were not grouped based on collection date, fish species, temperature or salinity, but isolates were clustered into 2 main groups that correlated precisely with 16S type.

A second investigation focused on the prevalence of two human pathogens, *Vibrio parahaemolyticus* and *V. vulnificus*, in bait shrimp obtained from commercial bait shops along the coast of Alabama and Mississippi. Both *Vibrio* species were below the

detection limit in November and March, and reached the highest level during the summer months (>1.1×10^5 Most Probable Number (MPN) per gram shrimp tissue) in both *Vibrio* species. Randomly selected isolates of both species were typed using virulence-related typing schemes. Out of 26 *V. vulnificus* isolates analyzed, 69% were 16S type B indicating the virulence potential of the isolates. By contrast, none of the 33 *V. parahaemolyticus* isolates was positive for *tdh* and *trh* hemolysin (virulence factors) genes.

Soon after *Deepwater Horizon* Oil Spill in the Gulf in 2010, I investigated if the large number of weathered oil (tar ball) present on beaches in Alabama and Mississippi acted as a sink for bacteria. My results showed that total aerobic bacterial counts were significantly higher in tar balls than in sand and seawater collected at the same location. Densities of *V. vulnificus* were higher than 10^5 colony forming units (CFU)/g of tar ball in all samples analyzed. In addition, *V. vulnificus* numbers were $10\times$ higher in tar balls than in sand and up to $100\times$ higher than in seawater.

Lastly, I used microcosm experiments in combination with allelic quantitative PCR technique, to demonstrate that *V. vulnificus*16S type B strain is better fit than type A strains under high environmental temperatures (37˚C and 42˚C). Conversely, type A grows or survives better than B-type at low temperature (15˚C, 10.5˚C) and low salinity (5 ppt). These findings further support the notion that posits two different ecotypes (16S type A and B) are present within the *V. vulnificus* species.

In summary, research data showed that the human pathogen *V. vulnificus* is commonly found in non-shellfish samples of the Northern GoM coast. Moreover, I

discovered a higher percentage of strains of great virulence potential in fish and shrimp than those previously reported in oysters. I proved that 16S type B strains outcompete type A strains at warmer temperatures explaining why more cases of vibriosis due to this pathogen occur at the end of summer. Finally, the effects of the *Deepwater Horizon* oil spill significantly increased the presence of *V. vulnificus* in beach samples. Overall, my research shows that recreational activities conducted in the Northern GoM coast have an intrinsic risk of exposure to *V. vulnificus*.

APPENDIX 1. DIVERSITY OF CULTURABLE BACTERIA FROM BLOOD VASCUAR SYSTEM OF LESSER ELECTRIC RAYS (*NARCINE BANCROFTII*) CAPTURED IN THE NORTHERN GULF OF MEXICO OFF ALABAMA

Abstract

The prevalence and taxonomic diversity of culturable bacteria recovered from the blood vascular system of healthy, grossly normal and showing no clinical sign of disease, lesser electric rays (*Narcine bancroftii*) captured from open beach habitat in the north-central GoM are reported herein. The blood of 9 of 10 rays was positive for bacteria, and bacterial isolates (n=83) were identified by partial 16S rRNA gene sequences. *Vibrio* spp. comprised 53% of all isolates and was recovered from all blood culture-positive rays. Among them, *V. harveyi* (n=14) and *V. campbellii* (n=11) were most common, followed by a group of unidentified *Vibrio* sp. (n=10) related to *V. nigripulchritudo.* Isolates representing species of *Pseudoaltermonas* (n=13), *Shewanella* (n=5), *Anphritea* (n=3), *Nautella* (n=3), and *Arenibacter* (n=1) were also recovered from ray blood. Higher bacterial diversity was observed in blood cultured on marine agar relative to blood agar but Gram positive bacteria were isolated from the latter only. Partial 16S rRNA gene sequences of the ray bacterial isolates were compared phylogenetically to those from related type strains. Most isolates were identified to the level of species but some clustered independently from reference strains, likely representing new species of *Vibrio, Amphritea, Shewanella*, and *Tenacibaculum.* The present study is the first record of any

bacterium from this ray species and reveals a taxonomically and phylogenetically diverse microbiota associated with its blood. Moreover, these data document that the presence of bacteria in elasmobranch blood is not coincident with clinical signs of disease; thereby rejecting the paradigm of septicemia indicating a disease condition in aquatic vertebrates.

Introduction

Little published information exists on the biodiversity, prevalence, and physiological effects of bacteria that infect the blood and other tissues of cartilaginous fishes (Chondrichthyes: sharks, skates, rays, and chimaeras) (1, 2) (Table 1). Based on a perusal of the published literature, *Vibri*o spp. comprise a large component of the normal microbiota in shark blood (3, 4). Based on this foundational taxonomic work with blood-borne bacteria in sharks, seemingly, the classical assumption that bacterial presence in blood indicates disease is no longer robust: (i) healthy captive elasmobranchs have urea-hydrolyzing bacteria in their blood vascular system (1) that may aid in osmoregulation, (ii) bacteria have been isolated from liver, spleen, kidney, and pancreas of healthy sharks and rays (1, 3) (Table 1), and (iii) Mylniczenko et al. (1) reported that 27% (50% when only rays were considered) of healthy captive and free-ranging elasmobranchs (n=80) yielded positive blood cultures. Yet, other bacteria, including *Vibrio* spp., recovered from sharks are indeed considered opportunistic pathogens (4). Hence, whether the taxonomic spectrum of these elasmobranch-associated bacteria comprises opportunistic/obligate pathogens, benign commensals, or bona fide tissue-dwelling symbionts that serve a critical role in elasmobranch physiology is indeterminate. Nevertheless, documenting microbial taxonomic diversity in other elasmobranch lineages is a good first step towards

testing such hypotheses. No such detailed study has been published based on materials sampled from non-shark elasmobranchs, i.e., skates, rays, or chimaeras.

The lesser electric ray, *Narcine bancroftii* (Griffith and Smith, 1834), (Torpediniformes: Narcinidae; syn. *Narcine brasiliensis*) ranges in shallow waters of tropical and sub-tropical continental shelves to 37 m depth, including the GoM, the Caribbean Sea, and the islands of the West Indies (5). Three other species of Narcinidae have geographic ranges that overlap with *N. bancroftii*, but *N. bancroftii* is the only narcinid that reportedly ranges in the north-central GoM (= the focus area for the present study)(6). This ray is a slow swimming fish that can be seasonally aggregated on sandbars and surf zones along open beaches and barrier islands. It can be regionally abundant in summer months, during which time pregnant females birth viviparous offspring, but then moves to offshore deep waters in winter (7). During Fall (Aug-Oct) in the northern GoM, lesser electric rays can be observed commonly by snorkeling in waters of 0.2−3.0 m; with the spiracles of the nearly completely buried rays appearing as characteristic holes in the sand (SAB, personal observations). Perhaps because this ray species is seldom landed by commercial fishermen, has no recreational or commercial value, and is typically hidden, nearly completely buried in sand, it is rarely included in faunal surveys of beach habitat in the GoM. They seldom are landed by recreational fishermen, and few local citizens are aware that they range in the shallow waters (<1 m) of open beaches. Perhaps as a result, there is little substantive quantitative information on the abundance and population structure of this species throughout its range or in the

northern GoM. Concomitantly, we know little of its general biology, symbionts (e.g., parasites and microbiota), and diseases.

The 2010 BP *Deepwater Horizon* oil spill has focused a torrent of media attention on the littoral zone of the GoM, including estuarine, seagrass bed, and beach communities, with the implication being that the health of aquatic vertebrates and invertebrates in those areas are a relative metric from which oil impacts can be measured. As such, the interplay between bacterial communities, fish health in the coastal zone, and basic science on the ecology and evolution of symbionts in sharks and rays is a fruitful area for study. Herein, we examined and characterized the bacterial community present in the blood vascular system of wild-caught lesser electric rays captured from beach habitat in the northern GoM off Alabama. The principal aim of this study was to document the taxonomic and phylogenetic diversity of bacteria that infected their blood, thereby contributing to a broader understanding of bacterial symbionts and/or potential pathogens of elasmobranchs.

Material and methods

Sample collection. The studied rays were hand-netted off Fort Morgan, Alabama (30˚13′45″N, 87˚54′7″W), maintained alive in enclosed plastic transport containers filled with water from the collection site and fitted with water pumps and aerators powered by a car battery, and transported alive to Auburn University (within 5 h after collection). Ten lesser electric rays (24-47 cm in total length; 7 females and 3 males) were analyzed in the study. Immediately before necropsy, each ray was euthanized with an overdoes of tricaine methanesulfonate (MS-222), and immediately thereafter the area of skin

circumscribed by the gill slits, mouth, and pectoral girdle was dried with a clean paper towel, disinfected with 70% ethanol, and cut away to expose the pericardial chamber. The exposed surfaces of the heart, including ventricle and conus arteriosus, were disinfected with 70% ethanol before a blood sample was taken by inserting a sterile syringe into the lumen of the heart. Each blood sample from each ray was immediately spread onto blood agar (BA) (MOLTOX, Boone, NC) and Marine Agar (MA) (Difco, Sparks, MD) using aseptic methods. Agar plates were incubated at 28˚C for 48 h under aerobic conditions. A representative of each colony type on the primary isolation plate was re-streaked on MA to obtain pure cultures for identification. A total of 86 single isolates were preserved as glycerol stocks (Marine broth supplemented with 20% glycerol) at -80˚C until subsequent analysis. Individual blood samples were labeled NB-01 through NB-09. The isolates were designated as FMR (Fort Morgan Ray) followed by the colony number.

Bacterial identification. Bacterial isolates were identified by partially sequencing the 16S rRNA gene. DNA template was prepared using a rapid boiling method as follows. Five colonies from a pure isolate were selected from a 24-h culture on MA, and re-suspended in a centrifuge tube with 100 µl sterile distilled H_2O. Proteinase K was added to the cell suspension to a final concentration of 30 unit/µl. After 20 min digestion at 55 ℃, the lysate was heated to 100 ℃ for 15 min, and spun down at 15,000 *g* for 5 min. The supernatant was transferred to a new tube and used as template DNA. The nearly-complete16S rRNA gene of each isolate was amplified using the following primers: 63V (forward) 5'-CAGGCCTAACACATGCAAGTC-3', and 1387R (reverse)

5'- GGGCGGWGTGTACAAGGC-3'. PCR conditions and reagents have been described elsewhere (8).

Sequence analysis. Sequence trace files were edited with BioEdit version 7.1.9 (9) to remove noise and untrusted ends. Sequences (n=3) having < 500 bp or > 3 ambiguous positions were excluded from the analysis. The resulting 83 sequences were assigned to taxonomic units by i) the Ribosomal Database Project (RDP) Naïve Bayesian Classifier (10) with a confidence threshold of 80%, ii) GreenGenes web classification tool (11), iii) BLAST (12) with a cut-off point for species ascription at 97% sequence similarity or higher (13).

Phylogenetic analysis. Partial 16Sr RNA gene sequences were aligned using Clustal X2 (14). Multiple sequence alignment (MSA) was conducted by trimming the sequences to cover the entire alignment and subsequent realignment. The trimmed MSA spanned the hypervariable V2, V3 and V4 regions corresponding to the *Escherichia coli* 16S rRNA gene base pair positions (15). Sequences of the type strains identified as nearest to the ray isolates by RDP and BLAST were incorporated into the phylogenetic trees as reference. Phylogenetic analysis was conducted in MEGA 5.0 software (16). Trees were constructed using the neighbor-joining method (17) with the Jukes-Cantor correction (18). The partial 16S rRNA gene sequences of bacterial isolates recovered from rays were submitted to the GenBank nucleotide sequence database (accession numbers KC439161 to KC439252).

Results

Isolate identification. All blood samples but one (NB-09) were culture positive, although the number of colony types (from approximately 3 to 16) varied among specimens (Figure 1). A total of 83 pure isolates were recovered from blood samples. Isolates were recovered on both marine agar (45 colonies) and blood agar (38 colonies) culture media. Three isolates yielded poor 16S rRNA gene sequence quality and were removed from the study. The remaining 83 sequences were ascribed to specific taxa using three databases. Overall, results from RDP, GreenGene, and NCBI were in agreement and isolates were identified unambiguously to genus. Isolates were classified into 14 genera, 11 families, 6 orders, 4 classes, and 3 phyla. The majority of the isolates (91.5%) were ascribed to the phylum Proteobacteria, followed by the Bacteroidetes (6.0%) and the Actinobacteria (2.4%). In a few cases, there was a disagreement between the results obtained from different databases. For example, GreenGenes could not place 4 Flavobacteriaceae isolates below family; whereas, RDP ascribed them to *Arenibacter* sp. Five isolates were ascribed to *Vibrio* by GreenGenes but RDP ascribed them to Vibrionaceae. We resolved the divergence by assigning the sequence to the lowest taxonomic level.

Among the Proteobacteria, 73 isolates were Gammaproteobacteria while only 3 isolates were identified as Alphaproteobacteria. Within the Gammaproteobacteria, *Vibrio* was the predominant genus with 45 isolates. Other isolates representing genera of Gammaproteobacteria comprised *Pseudoalteromonas* (n=13), *Shewanella* (n=5), *Ferrimonas* (n=2), *Amphritea* (n=3), *Photobacterium* (n=2), *Thalassomonas* (n=2) and *Pseudomonas* (n=1). All Alphaproteobacteria were assigned to *Nautella* (n=3). Figure 1

shows the distribution of the predominant genera in each individual fish. *Vibrio* was the only genus recovered from all blood culture-positive fish. In fact, it was the most common genus in all fish except in NB-07 from which only 4 isolates were recovered and all of them belonged to different genera. *Pseudoalteromonas* and *Shewanella* were recovered from 5 and 4 rays, respectively. MA not only yielded more isolates but also provided a higher diversity of genera than BA (Figure 2), but a few genera (Gram positive bacteria) were recovered on BA only.

Phylogenetic analysis. The majority of *Vibrio* isolates (35 out of 45) comprised 3 clades (Figure 3). Clade I included *V. harveyi* (n=14) plus the types species; Clade II = 11 ray isolates of *V. campbelli/V. sagamiensis* (the partial 16S rRNA gene sequence used did not allow for differentiation between these two species); Clade III = 10 isolates not ascribed to any reference or type strain sequence but with *V. nigripulchritudo* as the closest relative.

Analysis of the non-*Vibrio* Gammaproteobacteria isolates resulted in 9 principal groups (Figure 4). A *Pseudoalteromonas* clade had 13 ray isolates ascribed to *P. phenolica* (n=7), *P. prydzensis* (n=1), and *P. spongiae* (n=4). Two of the ray isolates could not be ascribed to named species. In two instances, a few ray isolates shared identical sequences but did not cluster with any reference strain. Five isolates within the *Shewanella* clade had a nonculterable bacterium as its closest neighbor and could not be assigned to any named species of *Shewanella*. Similarly, within the *Amphritea* clade, 3 isolates clustered separately from all known species of *Amphritea*.

The only two Actinobacteria recovered clustered along with *Micrococcus luteus/M. yunnanensis* and *Microbacterium hominis* (Figure 5). Two isolates of Bacteroidetes clustered with *Arenibacter nanhaiticus*, and another was the sister taxon to *Zhouia amylolytica*. The remaining two isolates were most similar to *Tenibacillum* sp. but could not be ascribed to a named species.

Discussion

We document that the blood of wild-caught lesser electric rays has bacteria. A perusal of the primary literature on the physiology, diseases, and health of elasmobranchs confirms that the blood of seemingly healthy wild-caught and captive elasmobranchs is neither sterile (i.e., free of living organisms) nor aseptic (i.e., free of microorganisms) (Table 1). While our results certainly contradict the paradigm that fish blood is sterile (19) and that isolation of bacteria from blood or other tissues results from contamination during necropsy (20), they agree with other published data suggesting presence of bacteria in elasmobranch tissues is not uncommon (21).

Similar to the tissues of other vertebrates, elasmobranch blood harbors, in addition to bacteria, a diverse assemblage of parasites, including flagellates, amoebas, apicomplexans, microsporidians, and ciliates (see Goertz (22) and references therein). Elasmobranch blood comprises food for an array of metazoan parasites; including at least some platyhelminths (23, 24), isopods, copepods, and leeches (25). In addition, blood is the principal site of infection for a diversity of metazoan parasites that invade/colonize, mature, copulate, release progeny, and die in the blood vascular system of their shark and ray hosts (26-28). Still other metazoan parasites that infect internal tissues but occur as

adults extra-intestinally may even foray into or transit within/through the blood vascular system in search of somatic muscle and epithelia where adult parasites deposit their eggs, e.g., species of *Huffmanela* (29, 30). In each aforementioned example, the blood is either invaded or exploited by a parasite, thereby plausibly exposing the blood vascular system to the milieu of bacteria ubiquitous in the aquatic environment.

Bacteria, while markedly less studied in elasmobranchs than the aforementioned symbionts, have been detected in various elasmobranch tissues, including blood, liver, muscle, and epithelium (1, 31, 32) (Table 1). Similar to previous researchers who have documented bacteria in elasmobranchs (3), that healthy sharks can harbor bacteria in blood is common knowledge also among the aquatic animal health professionals who monitor the well being of captive elasmobranchs in the aquarium industry (32). In fact, a significant contribution to our understanding of blood physiology and disease has emerged from the aquarium industry's monitoring of the health of sharks and rays as exhibit animals. From those observations numerous antimicrobial chemical therapies for captive elasmobranchs have been developed (33). What proportion of the bacteria known to associate with elasmobranchs are obligate pathogens is indeterminate; however, at least several are alleged primary pathogens, e.g., *Vibrio harveyi* (as *V. carchariae*) (4, 34-36), *Aeromonas salmonicida* (37), and "*Flavobacterium* sp." (32). Taken together, however, one should not assume an elasmobranch is diseased if its blood is infected with parasites or bacteria nor should one assume a link between presence/absence of parasites and that of bacteria in the blood of elasmobranchs. We speculate that the blood vascular system is vulnerable to bacterial infections (i) *indirectly* (= via bacteria adhered to or

residing within invading parasites) and (ii) *directly* (= via colonizing blood at the site of a hemorrhagic skin or gill lesion associated with intense infection by an ectoparasite population). Besides symbioses, the blood of elasmobranchs can be similarly exposed if the host is bitten by another predator or even the male mating partner (38). Doubtless, ample plausible scenarios exist for how the blood of elasmobranchs can be exposed to bacteria; however, we still lack a firm understanding of the taxonomic and phylogenetic diversity of bacteria that live in the blood of sharks and rays.

Most isolates recovered from lesser electric rays belonged to the phylum Proteobacteria (91.5%), which is a result that is in agreement with previous reports (3, 21). As expected, several species of *Vibrio* were isolated, including *V. harveyi* (17% of all isolates) and *V. campbellii* (13%). These *Vibrio* spp. have been previously reported as part of the normal flora in sharks (3). Conversely, we failed to recover any isolate ascribed to *V. alginolyticus*, a species common in sharks (4). Similarly, the common marine bacterium *Photobacterium damselae*, which has been isolated from internal organs of healthy fish (1, 39), was not isolated during our study. These discrepancies could be a factor of host-specificity, culture medium used for isolation, culture conditions, and habitat characteristics comprising the geographic locality where the rays were captured (19). Species of *Pseudoalteromonas* and *Shewanella* were common in the blood of the rays studied herein, and isolates representing these genera frequently have been reported from fish; however, only in a few instances have they been isolated from viscera. Some of the lesser-known bacteria isolated in the present study include *Amphritea atlantica*, *Arenibacter nanhaiticus*, and *Zhouia amylolytica.* These species or

their closest phylogenetic species were first discovered in marine sediments (40-43). Species of *Vibrio*, *Pseudoalteromonas*, and *Shewanella* have also been recovered from marine sediments (44-46). Dean and Motta (47) theorized that the suction feeding behavior of the lesser electric ray facilitates the ingestion of sediment, and we think it is plausible that many bacteria would also be ingested during this feeding activity; however, we lack adequate behavior observations and microbial data to accept or reject this notion. The interstitial/benthic habitat of the lesser electric ray could drive the taxonomic composition of the microbiota. Regardless, how bacteria enter the blood is unknown and also seemingly exceedingly difficult to test in an open, natural system. A comparison of the present results with those from a pelagic ray that is phylogenetically-related to *Narcine* may be informative along these lines.

The culture techniques used in this study likely underestimated the bacteria diversity of the tested samples since it is likely that only 1-10% of all bacteria can be cultured under laboratory conditions (48). We chose a culture-based strategy because culture methods are still the "gold standard" in fish disease diagnostics laboratories (49) and because the low cost associated with this approach makes it seemingly more accessible to a broader spectrum of researchers. The type of culture medium, even general media such as MA and BA, inadvertently can select for specific bacterial groups, and we observed this in the present study: species of *Pseudoalteromonas*, *Amphritea*, *Nautella*, *Arenibacter*, *Tenacibaculum*, and *Zhouia* were recovered on MA only; whereas, species of *Ferrimonas*, *Microbacterium*, *Micrococcus*, *Photobacterium*, and *Pseudomonas* were recovered on BA only.

In summary, the present study reported a high prevalence of bacteria in the blood of wild, apparently healthy lesser electric rays. *Vibrio* spp. were found in all but one individual and included opportunistic fish pathogens (*V. harveyi*) and potentially unnamed species (Figure 1, clade 3). Non-*Vibrio* Proteobacteria were also common and contained putative unnamed species within the *Shewanella* and *Amphritea* clades. These putative new species require further corroboration by full-length sequence of their 16S rRNA gene and additional taxonomic markers. Taken together, these insights on the elasmobranch microbiota are relevant to the fundamental ecology and evolutionary biology of aquatic symbioses. They are also vital to husbandry and veterinary staffers who are employed by the aquarium industry and tasked with keeping exhibited sharks and rays healthy; oftentimes following protocols that use blood picture as a means of assessing overall health status of the exhibited elasmobranch.

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Table AP-1. Taxonomic diversity of bacterial isolates from reportedly asymptomatic elasmobranchs.*

Host species	Bacterial species	Tissue
Carcharhinus acronotus (blacknose shark)	*Staphylococcus epidermidis*	blood[a]
Carcharhinus limbatus (blacktip shark)	*Photobacterium damsela*	blood[a, c]
	Vibrio alginolyticus	blood[a, c]
	Vibrio parahaemolyticus	blood[a, b]
	Photobacterium sp.	kidney[c]
	Vibrio furnissii	kidney[b]
	Vibrio harveyi	kidney[c]
	Vibrio sp.	kidney[c]
	Vibrio sp.	liver[b]
	Vibrio harveyi	liver[c]
	Vibrio alginolyticus	pancreas[c]
	Photobacterium damsela	spleen[c]
Carcharhinus melanopterus (blackfin reef shark)	*Aeromonas hydrophila*	blood[a]
	Aeromonas caviae	blood[a]
	Alcaligenes spp.	blood[a]
	Chryseomonas luteola	blood[a]
	Citrobacter freundii	blood[a]
	Citrobacter youngae	blood[a]
	Morganella morganii	blood[a]
	Pasteurella pneumotropica	blood[a]
	Photobacterium damsela	blood[a]
	Pseudomonas aeruginosa	blood[a]
	Pseudomonas alcaligenes	blood[a]
	Pseudomonas fluorescens	blood[a]
	Pseudomonas stutzeri	blood[a]
	Shewanella putrefaciens	blood[a]
	Sphingomonas paucimobilis	blood[a]
	Staphylococcus epidermidis	blood[a]
	Vibrio alginolyticus	blood[a]
Carcharhinus plumbes (sandbar shark)	*Vibrio vulnificus*	blood[a]
	Staphylococcus epidermidis	blood[a]
Cephaloscyllium ventriosum (swell shark)	*Staphylococcus epidermidis*	blood[a]
	Stenotrophomonas maltophilia	blood[a]
Chiloscyllium plagiosum (whitespotted bamboo shark)	*Vibrio alginolyticus*	blood[a]
	Vibrio vulnificus	blood[a]
Galeocerdo cuvier (tiger shark)	*Vibrio alginolyticus*	blood[c]
	Vibrio sp.	blood[c]
	Vibrio alginolyticus	liver[c]
Ginglymostoma cirratum (nurse shark)	*Photobacterium damsela*	blood[c]
	Vibrio harveyi	liver[b]
	Vibrio harveyi	pancreas[b]
	Vibrio harveyi	spleen[b]
Himantura granulata (Mangrove whiptail ray)	*Proteus vulgaris*	blood[a]
	Vibrio vulgaris	blood[a]
	Morganella morganii	blood[a]
	Photobacterium damsela	blood[a]
	Vibrio alginolyticus	blood[a]

Table AP-1(Continued)

Host species	Bacterial species	Tissue
Mustelus canis	*Vibrio metschnikovii*	trunk kidney[d]
(spiny dogfish)	*Photobacterium damsela*	trunk kidney[d]
Mustelus canis	*Pseudomonas* sp.	trunk kidney[d]
(spiny dogfish)		
Negaprion brevirostris	*Vibrio alginolyticus*	blood[b, c]
(lemon shark)	*Vibrio* sp.	blood[b, c]
	Photobacterium damsela	blood[b]
	Vibrio alginolyticus	eye[b]
	Vibrio sp.	eye[b]
	Photobacterium damsela	gall bladder[b]
	Vibrio harveyi	gill slit[b]
	Vibrio furnissii	gill slit[b]
	Vibrio sp.	gill slit[b]
	Vibrio alginolyticus	muscle[c]
	Vibrio harveyi	muscle[c]
	Aeromonas salmonicida	muscle[c]
	Photobacterium damsela	kidney[b]
	Photobacterium damsela	spleen[c]
Orectolobus japonicus	*Photobacterium damsela*	blood[a]
(bearded shark)		
Potamotrygon sp.	*Plesiomonas shigelloides*	blood[a]
(freshwater stingrays)	*Pseudomonas fluorescens*	blood[a]
	Pseudomonas putida	blood[a]
	Vibrio fluvialis	blood[a]
	Staphylococcus epidermidis	blood[a]
Pristis zijsron	*Pasteurella pneumotropica*	blood[a]
(Narrowsnout sawfish)		
Rhizoprionodon terraenovae	*Vibrio* sp.	liver[b]
(sharpnose shark)		
Squalus acanthias	*Alteromonas* sp.	trunk kidney[d]
(smooth dogfish)	*Vibrio alginolyticus*	trunk idney[d]
	Shewanella putrefaciens	trunk kidney[d]
	Photobacterium damsela	trunk kidney[d]
	Vibrio fluvialis	trunk kidney[d]
	Pseudomonas putida	trunk kidney[d]
Triaenodon obesus	*Moraxella* spp.	blood[a]
(whitetip reef shark)	*Streptococcus* group D	blood[a]
	Pasteurella pneumotropica	blood[a]
	Photobacterium damsela	blood[a]
	Shewanella putrefaciens	blood[a]
	Staphylococcus epidermidis	blood[a]
	Proteus vulgaris	blood[a]
Triakis semifasciata	*Vibrio alginolyticus*	blood[a]
(leopard shark)		

*Based on the published literature and from search results of keywords bacteria + elasmobranch' retrieved from bibliographic databases provided by ISI Web of

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Table AP-1 literature references:

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c. **Grimes DJ, Jacobs D, Swartz DG, Brayton PR, Colwell RR.** 1993. Numerical taxonomy of Gram-negative, oxidase-positive rods from Carcharhinid sharks. International Journal of Systematic Bacteriology 1993:88-98.

d. **Borucinska JD, Frascas S.** 2002. Naturally occurring lesions and micro-organisms in two species of free living sharks: the spiny dog fish, *Squalus acanthias* L., and the smooth dogifhs, *Mustelus canis* (Mitchill), from the north-western Atlantic. Journal of Fish Diseases 25:287-298.

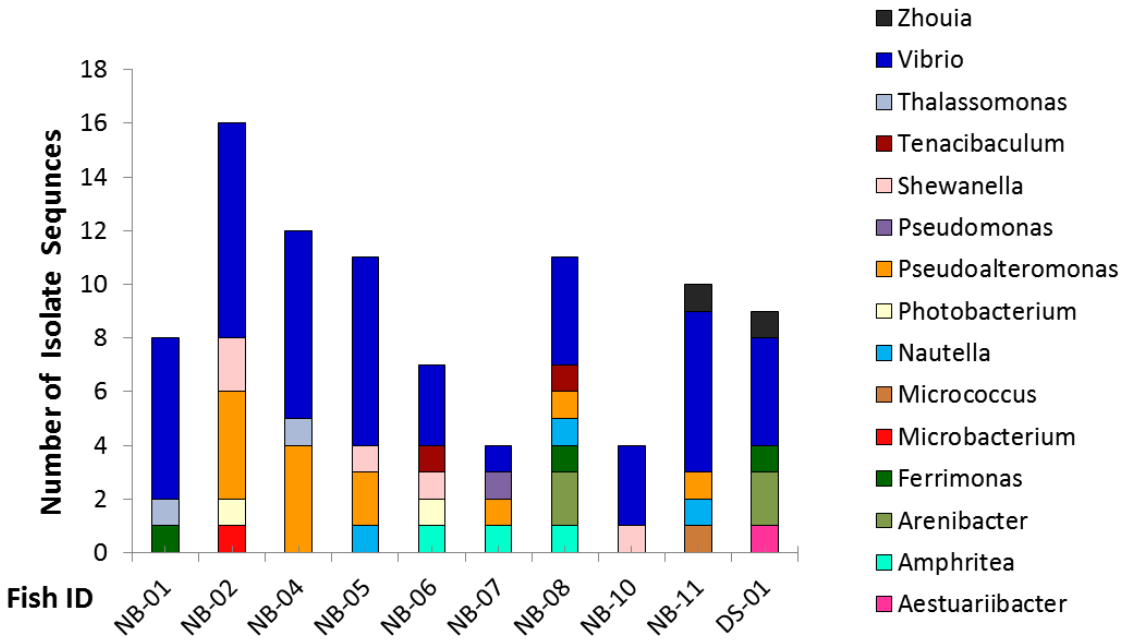

Figure AP-1. Bacteria recovered from blood of lesser electric rays (*Narcine bancroftii*, NB).

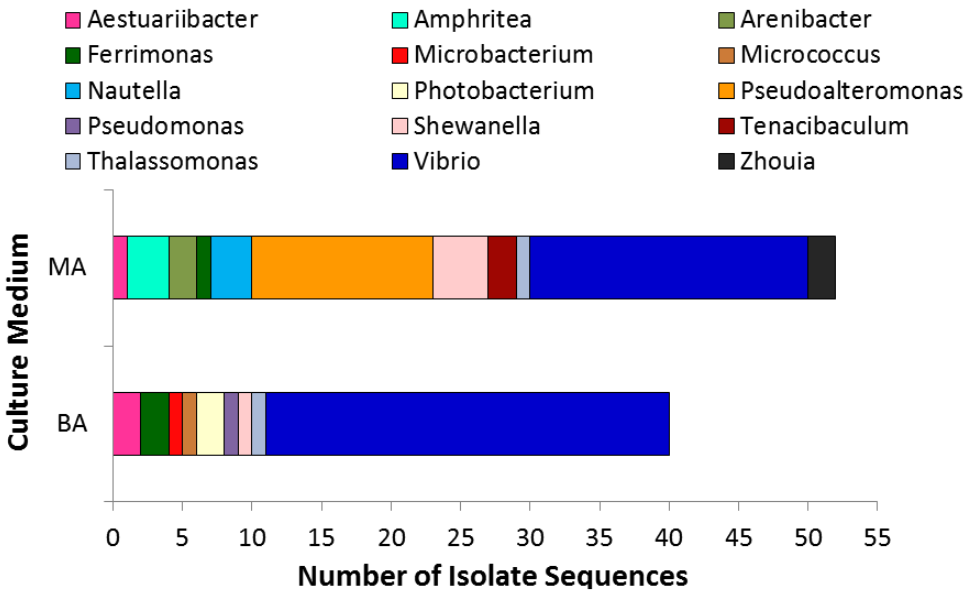

Figure AP-2. Distribution of isolates from blood of lesser electric rays (*Narcine bancroftii*) cultured in marine agar (MA) and blood agar (BA).

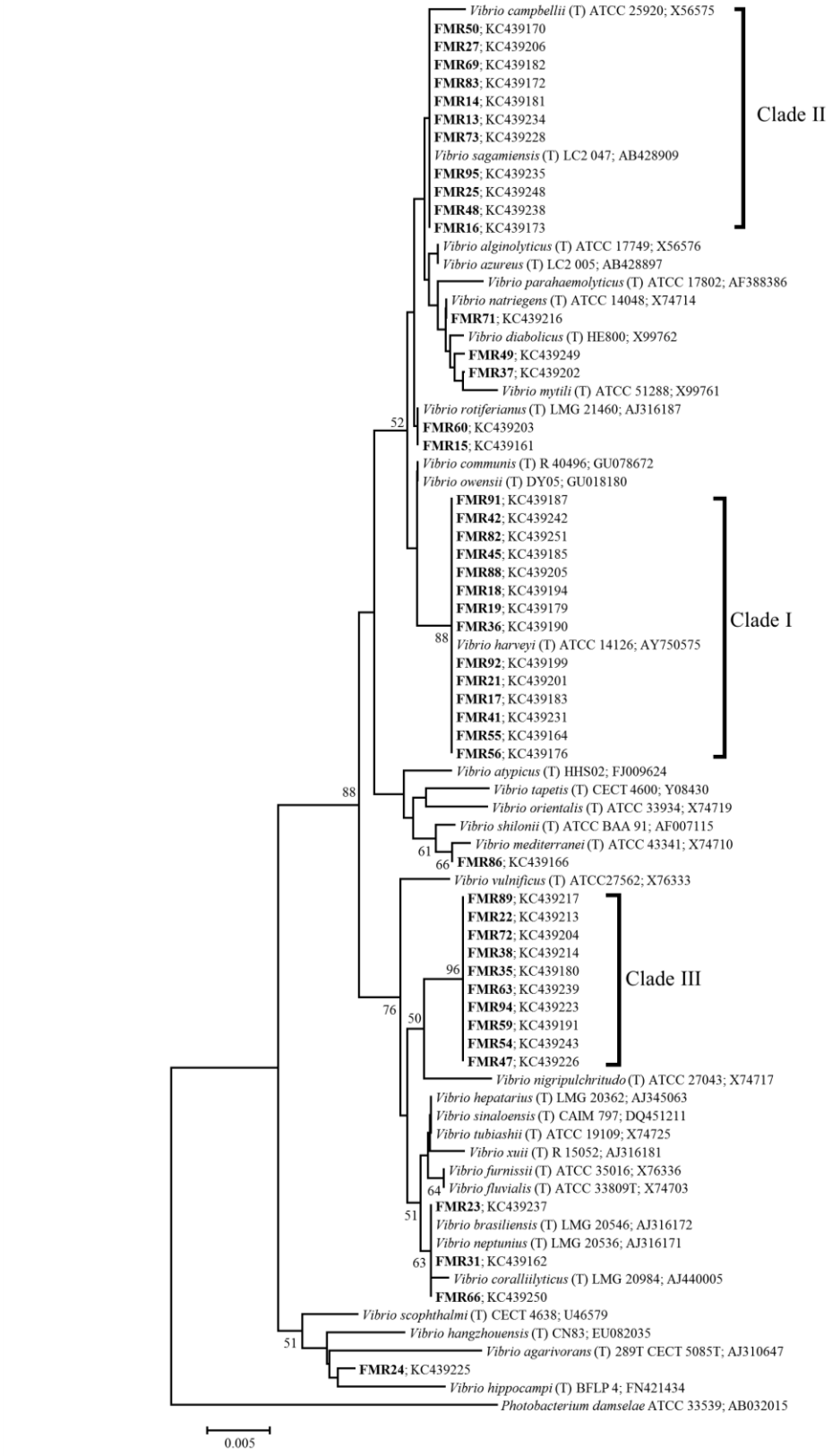
Vibrio campbellii (T) ATCC 25920; X56575
FMR50; KC439170
FMR27; KC439206
FMR69; KC439182
FMR83; KC439172
FMR14; KC439181
FMR13; KC439234
FMR73; KC439228
Vibrio sagamiensis (T) LC2 047; AB428909
FMR95; KC439235
FMR25; KC439248
FMR48; KC439238
FMR16; KC439173
Clade II
Vibrio alginolyticus (T) ATCC 17749; X56576
Vibrio azureus (T) LC2 005; AB428897
Vibrio parahaemolyticus (T) ATCC 17802; AF388386
Vibrio natriegens (T) ATCC 14048; X74714
FMR71; KC439216
Vibrio diabolicus (T) HE800; X99762
FMR49; KC439249
FMR37; KC439202
Vibrio mytili (T) ATCC 51288; X99761
Vibrio rotiferianus (T) LMG 21460; AJ316187
52
FMR60; KC439203
FMR15; KC439161
Vibrio communis (T) R 40496; GU078672
Vibrio owensii (T) DY05; GU018180
FMR91; KC439187
FMR42; KC439242
FMR82; KC439251
FMR45; KC439185
FMR88; KC439205
FMR18; KC439194
FMR19; KC439179
FMR36; KC439190
88
Vibrio harveyi (T) ATCC 14126; AY750575
FMR92; KC439199
FMR21; KC439201
FMR17; KC439183
FMR41; KC439231
FMR55; KC439164
FMR56; KC439176
Clade I
Vibrio atypicus (T) HHS02; FJ009624
88
Vibrio tapetis (T) CECT 4600; Y08430
Vibrio orientalis (T) ATCC 33934; X74719
Vibrio shilonii (T) ATCC BAA 91; AF007115
61
Vibrio mediterranei (T) ATCC 43341; X74710
66
FMR86; KC439166
Vibrio vulnificus (T) ATCC27562; X76333
FMR89; KC439217
FMR22; KC439213
FMR72; KC439204
FMR38; KC439214
96
FMR35; KC439180
FMR63; KC439239
FMR94; KC439223
FMR59; KC439191
FMR54; KC439243
FMR47; KC439226
Clade III
76
50
Vibrio nigripulchritudo (T) ATCC 27043; X74717
Vibrio hepatarius (T) LMG 20362; AJ345063
Vibrio sinaloensis (T) CAIM 797; DQ451211
Vibrio tubiashii (T) ATCC 19109; X74725
Vibrio xuii (T) R 15052; AJ316181
Vibrio furnissii (T) ATCC 35016; X76336
64
Vibrio fluvialis (T) ATCC 33809T; X74703
51
FMR23; KC439237
Vibrio brasiliensis (T) LMG 20546; AJ316172
Vibrio neptunius (T) LMG 20536; AJ316171
63
FMR31; KC439162
Vibrio coralliilyticus (T) LMG 20984; AJ440005
FMR66; KC439250
Vibrio scophthalmi (T) CECT 4638; U46579
Vibrio hangzhouensis (T) CN83; EU082035
51
Vibrio agarivorans (T) 289T CECT 5085T; AJ310647
FMR24; KC439225
Vibrio hippocampi (T) BFLP 4; FN421434
Photobacterium damselae ATCC 33539; AB032015
0.005

Figure AP-3. Phylogeny (partial 16S rRNA gene sequences) of bacterial isolates from blood of *Narcine bancroftii* and ascribed to species of *Vibrio*. Isolate number is followed by GenBank accession number. Sequences from type strains or the closest match were used for comparison. The tree topology was obtained by the neighbor-joining methods (Jukes-Cantor correction). The three main clades are highlighted. Numbers at nodes indicate boostrap values (1000 replicates). Bar = 0.5% sequence divergence.

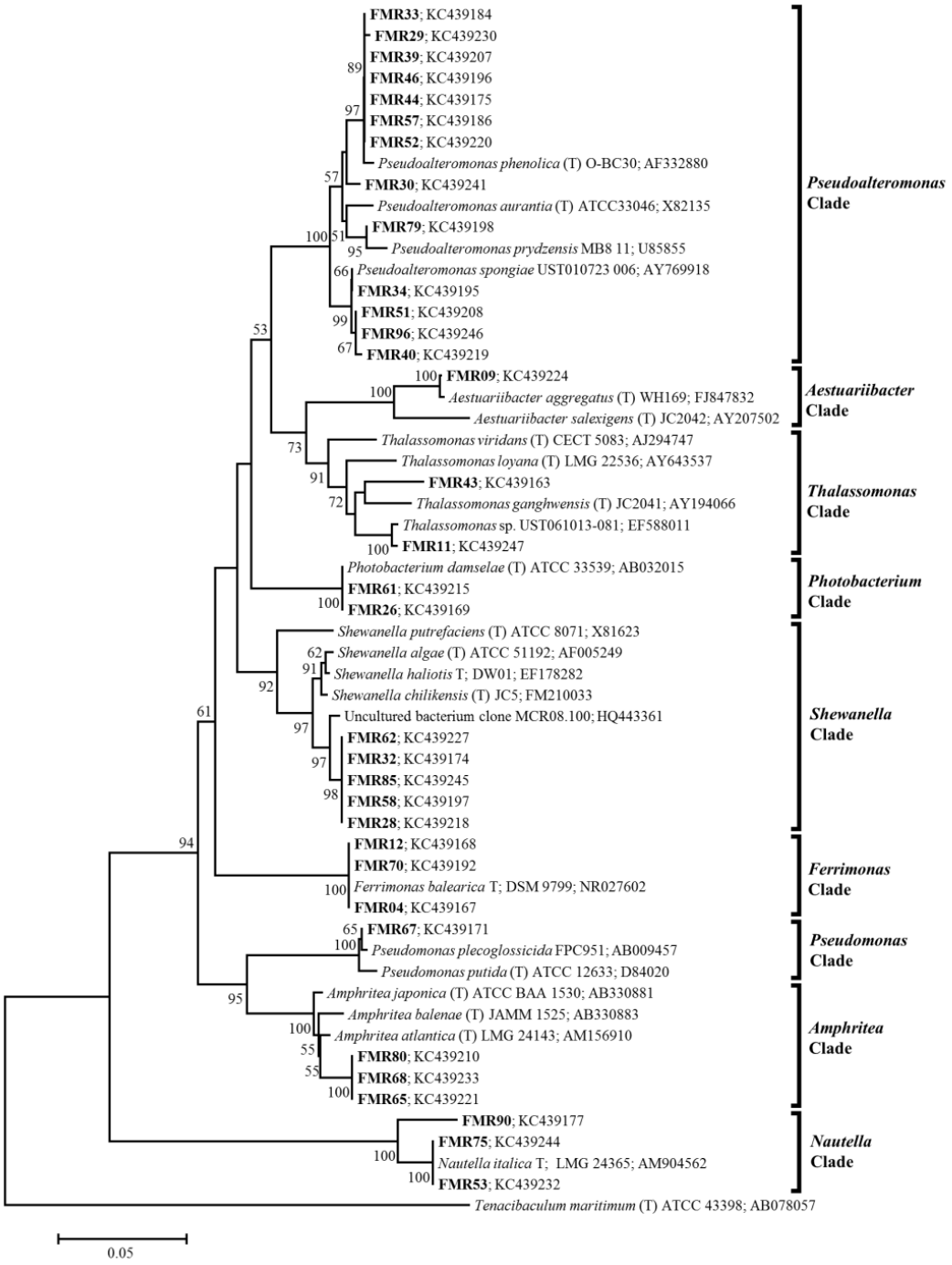
FMR33; KC439184
FMR29; KC439230
FMR39; KC439207
FMR46; KC439196
FMR44; KC439175
FMR57; KC439186
FMR52; KC439220
Pseudoalteromonas phenolica (T) O-BC30; AF332880
FMR30; KC439241
Pseudoalteromonas aurantia (T) ATCC33046; X82135
FMR79; KC439198
Pseudoalteromonas prydzensis MB8 11; U85855
Pseudoalteromonas spongiae UST010723 006; AY769918
FMR34; KC439195
FMR51; KC439208
FMR96; KC439246
FMR40; KC439219
FMR09; KC439224
Aestuariibacter aggregatus (T) WH169; FJ847832
Aestuariibacter salexigens (T) JC2042; AY207502
Thalassomonas viridans (T) CECT 5083; AJ294747
Thalassomonas loyana (T) LMG 22536; AY643537
FMR43; KC439163
Thalassomonas ganghwensis (T) JC2041; AY194066
Thalassomonas sp. UST061013-081; EF588011
FMR11; KC439247
Photobacterium damselae (T) ATCC 33539; AB032015
FMR61; KC439215
FMR26; KC439169
Shewanella putrefaciens (T) ATCC 8071; X81623
Shewanella algae (T) ATCC 51192; AF005249
Shewanella haliotis T; DW01; EF178282
Shewanella chilikensis (T) JC5; FM210033
Uncultured bacterium clone MCR08.100; HQ443361
FMR62; KC439227
FMR32; KC439174
FMR85; KC439245
FMR58; KC439197
FMR28; KC439218
FMR12; KC439168
FMR70; KC439192
Ferrimonas balearica T; DSM 9799; NR027602
FMR04; KC439167
FMR67; KC439171
Pseudomonas plecoglossicida FPC951; AB009457
Pseudomonas putida (T) ATCC 12633; D84020
Amphritea japonica (T) ATCC BAA 1530; AB330881
Amphritea balenae (T) JAMM 1525; AB330883
Amphritea atlantica (T) LMG 24143; AM156910
FMR80; KC439210
FMR68; KC439233
FMR65; KC439221
FMR90; KC439177
FMR75; KC439244
Nautella italica T; LMG 24365; AM904562
FMR53; KC439232
Tenacibaculum maritimum (T) ATCC 43398; AB078057
Pseudoalteromonas Clade
Aestuariibacter Clade
Thalassomonas Clade
Photobacterium Clade
Shewanella Clade
Ferrimonas Clade
Pseudomonas Clade
Amphritea Clade
Nautella Clade
0.05

Figure AP-4. Phylogeny (partial 16S rRNA gene sequences) of bacterial isolates from blood of *Narcine bancroftii* and assigned as non-*Vibrio* Proteobacteria species. Isolate number is followed by GenBank accession number. Sequences from type strains, or the closest match, were used for comparison. The tree topology was obtained by the neighbor-joining methods (Jukes-Cantor correction). Each genus-clade is highlighted. Numbers at nodes indicate bootstrap values (1000 replicates). Bar = 5% sequence divergence.

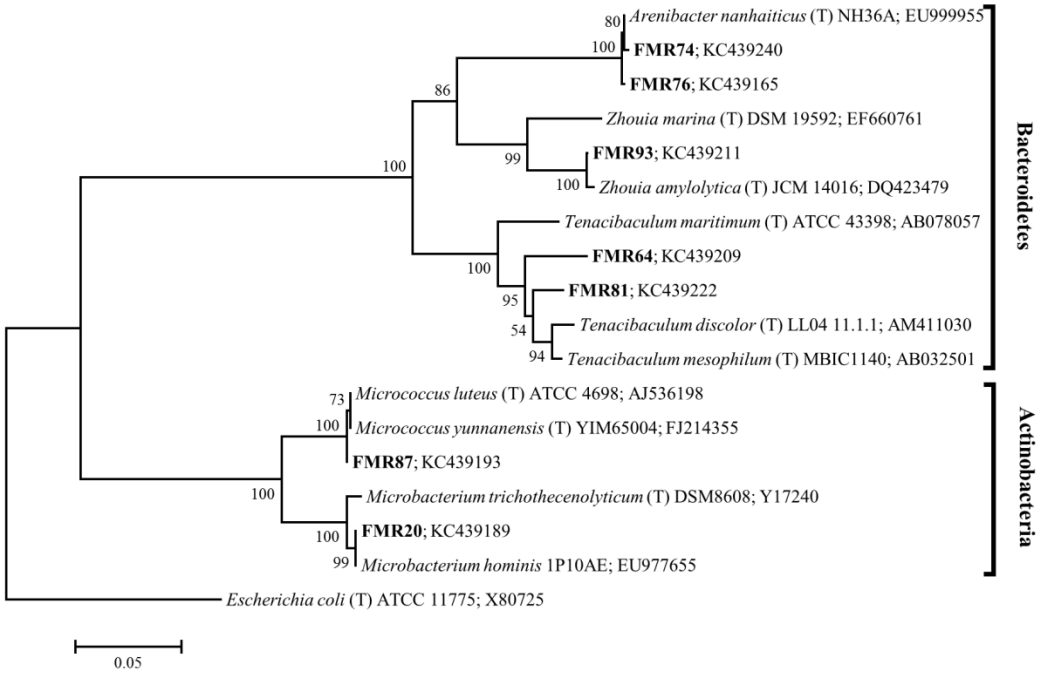

Figure AP-5. Phylogeny (partial 16S rRNA gene sequences) of bacterial isolates from blood of *Narcine bancroftii* and assigned as non-Proteobacteria. Isolate number is followed by GenBank accession number. Sequences from type strains or the closest match were used for comparison. The tree topology was obtained by the neighbor-joining methods (Jukes-Cantor correction). Each genus-clade is highlighted. Numbers at nodes indicate bootstrap values (1000 replicates). Scale bar represents 5% sequence divergence.