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Strain to strain differences in the growth, survival and adaptation of Vibrio vulnificus and Vibrio parahaemolyticus in broth

Veronica Elaine Burnham
Louisiana State University and Agricultural and Mechanical College, vburnh1@lsu.edu

STRAIN TO STRAIN DIFFERENCES IN THE GROWTH, SURVIVAL AND ADAPTATION OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* IN BROTH

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Dedicated to
My Mother,
the late Sylvia Burnham

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ABSTRACT

Vibrio vulnificus and *Vibrio parahaemolyticus* are natural inhabitants of estuarine environments of the Gulf of Mexico. *V. vulnificus* is the leading cause of death, while *V. parahaemolyticus* is a leading cause of foodborne gastroenteritis from the consumption of seafood in the United States. Refrigeration is commonly used as a preservation method to control the growth of microorganisms in food. The ability of some microorganisms to adapt, as a survival response when exposed to a downshift in temperature, could compromised efforts to use low temperature storage to reduce the risk of foodborne illnesses. Limited research is available on the growth characteristics of different strains of *V. vulnificus* and *V. parahaemolyticus*, or on the adaptation response to cold shock on their survival. This study was conducted to determine if strain-to-strain differences exist in the growth and survival of *V. vulnificus* and *V. parahaemolyticus* at refrigeration temperatures, and to determine if these strains exhibit a cold temperature adaptation response. Results obtained from this study show that various *V. vulnificus* and *V. parahaemolyticus* strains grown in tryptic soy broth have significant differences in growth and survival when stored at 5, 8 or 10^{o}C over 10 days. *V. vulnificus* strains were able to survive but not grow when shifted from 37^{o}C to storage at 5 or 8^{o}C, while most of these strains were able to grow at 10^{o}C. *V. parahaemolyticus* strains survived but did not grow when shifted from 37^{o}C to 5^{o}C. During storage at 8 or 10^{o}C however, *V. parahaemolyticus* strains were able to grow. When these strains were adapted at an intermediate temperature of 15^{o}C for 4 hours, this resulted in an enhanced survival of *V. vulnificus* strains. This adaptation response however varied between strains. Not all of the *V. parahaemolyticus* strains had an enhanced survival when exposed to an

intermediate temperature of 15^{o}C. The cold adaptation response was more sustained for the *V. vulnificus* strains at some temperatures tested, while for the *V. parahaemolyticus* strains that had an adaptation response, this response was generally short lived.

CHAPTER 1

INTRODUCTION

Vibrio vulnificus and *Vibrio parahaemolyticus* are important foodborne pathogens found naturally in estuarine environments of the Gulf of Mexico, and the Atlantic and Western coasts of the United States. Oysters and other molluscan shellfish concentrate *Vibrio* bacteria from the surrounding water during filter feeding (Panicker et al. 2004). *V. vulnificus* is the leading cause of death from the consumption of seafood in the United States (Hlady and Klontz 1996), with the majority of cases resulting from the consumption of raw oysters harvested from the Gulf of Mexico during the summer months (Rippey 1994). *V. vulnificus* can cause gastrointestinal disease as well as wound infections, both of which can progress to a fatal septicemia in individuals who are immunocompromised or have elevated serum iron resulting from cirrhosis due to alcoholism (Oliver and Kaper 2001).

Vibrio parahaemolyticus is a leading cause of foodborne gastroenteritis (Joseph et al. 1983) that is often associated with consuming raw or undercooked seafood (Blake et al. 1980). The bacteria was first isolated and implicated in an outbreak of food poisoning in Japan in 1950 (Fujino et al. 1953), and has been associated with outbreaks of illness in the United States since 1969. Prior to 1997, foodborne illnesses caused by *V. parahaemolyticus* were sporadic and commonly associated with crabs, oysters, shrimp and lobsters. In 1997 and 1998 there were more than 700 cases of illness due to *V. parahaemolyticus* in the United States, many of which resulted from consuming raw oysters. Warmer than usual water temperatures was suggested to be the cause of these outbreaks (FAO/WHO 2001).

The time of year oysters are harvested, as well as the methods used for harvesting and handling can have an impact on the concentration of *V. vulnificus* and *V.*

parahaemolyticus found in these shellfish. Studies by Gooch et al. (2002) demonstrated that elevated water temperatures can increase the numbers of *V. parahaemolyticus* in oysters at harvest, and that after harvest numbers can multiply rapidly in live oysters held at 26^{o} C, with the potential for creating food safety hazards for at-risk individuals consuming raw oysters. Food safety guidelines provided by the National Shellfish Sanitation Program indicate that shellfish-borne *Vibrio* infections can be prevented by cooking seafood thoroughly, keeping them from being cross contamination after cooking, and eating them promptly or storing them at hot (60°C or higher) or cold (4°C or lower) temperatures (FDA 2003). The problem of foodborne infections associated with *V. vulnificus* and *V. parahaemolyticus* continues to persist since oysters are not always stored at appropriate temperatures during harvesting and processing, coupled with the fact that many consumers still prefer to consume oysters raw. Effective measures to control the levels of these pathogens in raw oysters, therefore, remain a challenge facing regulatory agencies.

The foodborne outbreaks caused by *V. parahaemolyticus* in 1997 and 1998 resulted in the Food and Drug Administration (2001) conducting a draft risk assessment to determine the health impact on individuals consuming raw oysters containing pathogenic *V. parahaemolyticus* and to review programs related to the regulation of *V. parahaemolyticus* in raw shellfish to ensure that these programs were effective. Due to limited data on the post-harvest growth of *V. parahaemolyticus* in oysters, the risk assessment assumed the growth rate of pathogenic *V. parahaemolyticus* in oysters to be one fourth that in broth culture at all temperatures, based on the model of Miles et al. (1997), and studies on the growth rate of *V. parahaemolyticus* at 26^{o}C in oysters,

conducted by Gooch et al. (1999). The possible impact of cold temperature adaptation of the organism was not taken into account during the development of the risk assessment.

The objectives of this study were to determine if differences exist in the growth and survival rates of various strains of *V. vulnificus* and *V. parahaemolyticus* during cold storage, and to determine if these strains exhibit a cold temperature adaptation response.

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CHAPTER 2

LITERATURE REVIEW

VIBRIO PARAHAEMOLYTICUS

Vibrionaceae are curved gram-negative rods that commonly live in water and includes the genus *Vibrio* (Lederberg 1992) which consists of at least twenty-eight species (Jay 1986). *V. parahaemolyticus* is a facultatively anaerobic, halophilic, motile bacterium. It is often associated with *V. vulnificus* in estuarine and coastal waters and in seafood. *V. parahaemolyticus* can grow in media containing 1 to 8% NaCl, but optimum growth occurs at a salt concentration of 2 to 4%. Optimum pH for growth is 7.6 - 8.6. The organism grows at temperatures between 10 and 44^{o}C, but the optimum temperature for growth is 30 to 35 ^{o}C. No growth occurs at temperatures below 4^{o}C. The bacteria grows well on thiosulphate citrate bile salt sucrose (TCBS) and vibrio agar, but may also grow on MacConkey and deoxycholate-lactose containing 2% NaCl (Rose 1983). Optimum water activity for growth is 0.992 (Jay 1986). Johnston and Brown (2002) determined the thermal death time (D-value) for one strain of *V. parahaemolyticus* to be 1.75 min at 55^{o}C in Artificial Seawater (ASW).

All strains of *V. parahaemolyticus* produce a thermolabile hemolysin (TLH) which is species specific (Taniguchi et al. 1986), while most clinical strains possess a *tdh* gene and produce a thermostable direct hemolysin (TDH) and are designated as Kanagawa phenomenon (KP) positive (Wong et al. 2000). The Kanagawa reaction is the production of β-hemolysis on Wagatsuma agar, and is considered a good means of differentiating between human pathogenic and non-pathogenic strains of *V. parahaemolyticus* (Park et al. 2004). In the 1980s there were reports of cases of gastroenteritis caused by KP negative strains of *V. parahaemolyticus* that produced a TDH-related hemolysin (TRH) (Okuda et al. 1997). TDH and TRH are considered the

main virulence factors for *V. parahaemolyticus* (Nishibuchi and Kaper 1990). Environmental isolates of *V. parahaemolyticus* rarely produce TDH or TRH, however isolates from patients with diarrhea produce TDH or TRH or both (Park et al. 2004).

Vibrio parahaemolyticus produces three surface antigens, H antigens (flagellar antigens) which are common to all strains, heat-stable somatic O antigens (lipopolysaccharides), and heat-labile capsular K antigens (capsular polysaccharide) (FDA 2001). With conventional serotyping based on O and K antigens, isolates of *V. parahaemolyticus* can be differentiated into 13 O groups and 71 K groups (Wong et al. 2000). K antigens have been shown to play a role in the adherence of the bacteria to its target cells (Hsieh et al. 2003).

VIBRIO VULNIFICUS

Vibrio vulnificus can be differentiated taxonomically from *V. parahaemolyticus* primarily by differences of the ONPG (o-nitrophenyl-β-d-galactopyranoside), salt tolerance, cellobiose and lactose reactions (FDA 2001). The bacteria produces green colonies similar to *V. parahaemolyticus* on TCBS agar, and yellow colonies on modified cellobiose-polymyxinB-colistin (mCPC) and cellobiose-colistin (CC) agar (FDA 2001). The bacteria require 0.5 to 2% NaCl for optimal growth in culture media (Wright et al. 1995). Optimum temperature for growth is 37^{o}C (Kelly 1982). Johnston and Brown (2002) determined the D-value for one strain of *V. vulnificus* to be 12 s at 55^{o}C in ASW.

Vibrio vulnificus strains are grouped into three biotypes. Biotype 1 is mainly pathogenic to humans, biotype 2 is pathogenic to eels and biotype 3 has been identified in humans handling fish in Israel (Bisharat et al. 1999). Clinical infections of humans with

biotype 1 usually arise from a single strain (Jackson et al. 1997) even though one shellfish can contain numerous strains.

Vibrio vulnificus secretes a number of toxins including iron, lipopolysaccharide (LPS), capsular polysaccharide (CPS), and cytolysin which have all been implicated in the pathogenesis of the disease (Chiang and Chuang 2003). Iron plays an important role in the pathogenesis of *V. vulnificus*, with the bacteria relying on iron imbalances in the host and on acquisition of iron. The organism grows more rapidly in the blood of persons with hemochromatosis (Bullen et al. 1991).

Capsular polysaccharide is an important virulence factor for *V. vulnificus,* preventing phagocytosis of the bacteria by the host cells (Strom and Paranjpye 2000). Strains with capsules are opaque, while strains with little or no capsular material are translucent (Wright et al. 1999). Opaque strains are associated with the utilization of iron for growth and high levels of virulence (Harris-Young et al. 1993). According to Smith and Siebeling (2002) clinical strains have capsules, but in the laboratory, CPS is often lost, resulting in a shift from opaque to translucent colonies, which are no longer virulent.

Cytolysin produced by *V. vulnificus* is haemolytic in nature and can cause lysis of erythrocytes by the formation of pores in the membranes of host cells (Kim et al. 1993) which can lead to dilation of vessels and edema (Gulig et al. 2004). The bacteria can also construct LPS which has been shown to cause mortality in mice (McPherson et al. 1991).

EFFECTS OF TEMPERATURE ON GROWTH

The Gulf Coast is associated with the majority of *Vibrio* infections in the United States (Cook et al. 2001); however, the presence of *V. vulnificus* has also been reported

along the East Coast (Pfeffer et al. 2003) and West Coast of the United States (Kaysner et al. (1987). Investigations into factors that may affect levels of *Vibrio* in coastal waters were conducted by Pfeffer et al. (2003). The study which was carried out along the Eastern U.S. Coast found that while levels of V*ibrio* were controlled by several factors including temperature, turbidity, levels of dissolved oxygen, estuarine bacteria and coliforms, most of the variability in the concentrations of *V. vulnificus* and other *Vibrio* species was controlled by water temperature.

The highest concentration of *V. vulnificus* can be found in the water column and in shellfish when water temperatures are typically above 20^{o}C (Randa et al. 2004). A study carried out by Motes et al. (1998) demonstrated that a similar seasonal distribution of *V. vulnificus* occurs in oysters from different Gulf Coast sites, with numbers of the organism increasing as water temperature increased up to 26^{o} C, then levelling off. The counts ranged from 10^3 to 10^4 organisms per g of oyster meat from May to October and fell to < 10 per g from late December through mid-March. In March and April it then rose sharply again to summer levels. Another study carried out by Pfeffer et al. (2003) on water samples taken from the East Coast found that *V. vulnificus* was isolated only when water temperatures were between 15 and 27^{o}C.

A study carried out by Gooch et al. (2002) showed that the influence of water temperature on the growth of *V. parahaemolyticus* was similar to that seen with *V. vulnificus.* The study showed that from April to December, when water temperatures at harvest from Mobile Bay, Alabama, were >20^{o} C, the harvest density of *V. parahaemolyticus* in oysters was 130 CFU/g, but when water temperatures were < 20^{o} C, the harvest density was 15 CFU/g. Another study by DePaola et al. (2002) found that

densities of *V. parahaemolyticus* in oysters ranged from 100 to 1,000 CFU g^{-1} from April through November at Cedar Point, Alabama, and were < 100 CFU g^{-1} from December through March. Cook et al. (2001) in a national survey carried out between 1998 and 1999 also showed that densities of both *V. vulnificus* and *V. parahaemolyticus* in harvested oysters followed a seasonal distribution with highest densities occurring from the Gulf Coast during the summer and with densities of *V. vulnificus* being higher than *V. parahaemolyticus* in Gulf Coast oysters.

The length of time as well as the temperature at which shellstock oysters are stored after harvest, can also influence the growth of *Vibrio*. Cook (1994) showed that when oysters are stored at 18°C and under ambient conditions, the numbers of *V. vulnificus* are greater than they were at harvest, indicating that *V. vulnificus* can multiply in unchilled shellstock oysters. The study further found that the numbers of *V. vulnificus* increased in excess of one log unit during the first 12 h of storage with only minimum growth thereafter. These findings support reports of multiplication of *V. vulnificus* in postharvest shellstock oysters by Kaspar and Tamplin (1993) who showed that the pathogen increased in shellstock oysters by more than 100-fold when stored at 30° C.

Similar findings were demonstrated for *V. parahaemolyticus* by Gooch et al. (2002), who showed that after harvest, *V. parahaemolyticus* multiplied rapidly in live oysters held at 26°C, showing a 50-fold increase at 10 h and 790 fold-increase at 24 h (April through December). A mathematical model developed by Miles et al. (1997) to predict the effects of temperature and water activity on the growth rate of *V. parahaemolyticus*, found the growth rate at 26°C to be four times higher in tryptic soy broth than was reported in shellstock oysters stored at the same temperature.

EFFECTS OF SALINITY ON GROWTH

Vibrio vulnificus and *V. parahaemolyticus* undergo seasonal fluctuations in estuarine and coastal waters, with the levels of the bacteria strongly correlated to water temperature. The effects of salinity on abundance of these bacteria are however less clear. Kaspar and Tamplin (1993), found that at salinities between 5 and 25 ppt (at 14^{o}C), the numbers of *V. vulnificus* in seawater increased or remained constant after 6 days of incubation, while at salinities between 30 to 38 ppt numbers decreased by 58 to 83%. Motes et al. (1998) also found that higher levels of *V. vulnificus* were found in oysters when salinities ranged from 5 to 25 ppt. and that numbers were lower at salinities higher than 28 ppt. Another study by Randa et al. (2004) however found that regardless of water temperature, high levels of *V. vulnificus* were seen at salinities between 5 and 10 ppt. suggesting that this was the optimal range for survival of the organism.

EFFECTS OF COLD STORAGE ON GROWTH AND SURVIVAL

Refrigeration is currently used as means of controlling the growth of *Vibrio* in postharvest oysters. A study by Cook and Ruple (1989) showed that *V. vulnificus* does not multiply in shellfish held at temperatures below 10^{o}C. The National Shellfish Sanitation Program now requires interstate shipment of live shellstock to be maintained at temperatures of 10^{o}C or less in instances where transport exceeds 2 h (FDA 2003).

Other researchers have shown however, that while refrigeration controls the growth of *Vibrio*, this preservation method may not destroy the bacteria. A study carried out by Cook and Ruple (1992) showed that processed oyster meat stored on crushed ice for 3 and 7days, resulted in a one-log and two-log reduction of *V. vulnificus* respectively, but did not destroy the bacteria. In other studies, Kasper and Tamplin (1993) found that

numbers of *V. vulnificus* in shellstock oysters were reduced by 10 fold after 14 days when stored at 2°C to 4°C, and by 100 fold when stored for 14 days at 0°C, while Kaysner et al. (1989) showed that *V. vulnificus* cultures stored in BHI broth at 0.5°C, survived for 5 days.

The effects of cold storage on the survival of *V. parahaemolyticus* are similar to those seen with *V. vulnificus*. Johnson et al. (1973) showed that *V. parahaemolyticus* in shellstock oysters refrigerated at 4°C, survived for at least 3 weeks.

VIABLE BUT NON-CULTURABLE STATE

There is an abundance of evidence linking water temperature to the seasonal distribution of *V. vulnificus*, however, it still remains unclear if the decline in numbers of the bacteria during the winter results from mortality due to an unfavourable decrease in water temperature or is due to the bacteria entering the viable but nonculturable (VBNC) state (Randa et al. 2004).

In the VBNC state, bacterial cells remain viable but are unable to grow on routine microbiological media. For the state to be recognized as a survival response, the cells must be able to resuscitate from this state and be detected again on microbiological media (Bates and Oliver 2004). Wolf and Oliver (1992) reported that *V. vulnificus* can be prompted to enter the VBNC state by incubation at 5°C. Other studies by Johnston and Brown (2002) showed that when cells of *V. vulnificus* and *V. parahaemolyticus* lost their culturability, they underwent a shape change from rods to coccoid cells. By the use of light microscopy it was observed that the coccoid-shaped cells reverted to the normal rod shape as the cells became culturable again after a 24 hour upshift in temperature to 37°C. This suggested that the increase in numbers of the organism seen after the temperature

upshift was not due to the growth of a few survivors, but due to previously non culturable cells reverting to the culturable state (Johnston and Brown 2002). Resuscitation of VBNC cells was also demonstrated by Nilsson et al. (1991) who showed that *V. vulnificus* can be brought from the nonculturable to the culturable state by holding at 23^{o}C for 2 days. Another study by Bates and Oliver (2004) found that they were no longer able to resuscitate strains of *V. parahaemolyticus* by a temperature upshift after cells had been in a VBNC state for one week.

Randa et al. (2004) has suggested that culture based methods used in the past to identify and enumerate *V. vulnificus,* allowed cells in the VBNC state to escape detection. The researchers concluded that newer techniques using Polymerase chain reaction (PCR) were more effective in detecting cells of *V. vulnificus* that are in a VBNC state. In their study, they were unable to detect VBNC cells of *V. vulnificus* from water during the winter using an assay adapted to detect VBNC cells, and suggested that the lack of detection was due to the cells being absent from the water column and not just due to the cells entering a VBNC state. They further proposed that the absence of the organism from the water column during the colder periods was as a result of the sediment serving as a refuge for a subpopulation of the organism over the winter, and that elevated levels of the bacteria in the water column during the summer resulted subsequent to turbulence of the water following adverse weather conditions in the spring.

Other investigators (Barer and Harwood 1999) have concluded that the appearance of resuscitation following the addition of nutrients to nonculturable cells could be due to the presence of low numbers of culturable cells that respond and multiply following the addition of nutrient, giving the appearance of resuscitation. Bates and

Oliver (2004) disagreed, however, that cells recovered after a temperature upshift were due to the regrowth of a few surviving cells. They conducted a resuscitation study which involved <0.025 to <0.1 culturable cells, leading them to conclude that the culturability they saw could not have been as a result of residual culturable cells but due to the resuscitation of nonculturable cells.

The type of media used can also have an impact on numbers of injured cells that are resuscitated. Recovery of *Vibrio* on selective media is reduced (Kaysner et al. 1989), since injured cells are sensitive to selective agents present in the media. According to Alam et al. (2001), non-selective media may be advantageous to the recovery of injured cells that are sensitive to these selective agents.

STRAIN-TO-STRAIN DIFFERENCES

In a study which compared strains, Bates and Oliver (2004) observed considerable variation in the time it took for different *V. parahaemolyticus* strains incubated at 5^{o}C in ASW to enter the VBNC state. Strain SAK11 took an average of 5.4 days, while strain SPRC851, both clinical strains, took an average of 11.9 days. Another study by Jiang and Chai (1996) found that a Kanagawa phenomenon (KP) positive and KP negative strain of *V. parahaemolyticus* maintained in Modified Morita mineral salts solution (MMS) at 3.5^{o}C took 1 to 2 weeks and 3 weeks respectively to enter a VBNC state. The study further found that the KP positive strain reached the VBNC state at around 50 days when starved, while it took the KP negative strain under similar conditions, approximately 80 days. Another study by Wong et al. (2004) determined that the resuscitation of *V. parahaemolyticus* from the VBNC state was dependant on strain.

METHODS OF IDENTIFICATION AND ENUMERTION

The most probable number (MPN) technique is commonly used to enumerate *V. parahaemolyticus* and *V. vulnificus*. This technique is however lengthy, requiring several days. New techniques such as PCR and gene probes have been developed for identification of these organisms, and have the advantage of being more rapid. Fatty acid profiles and enzyme assays have also been developed to detect *V. vulnificus* (FDA 2001).

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CHAPTER 3

GROWTH AND SURVIVAL DIFFERENCES OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* STRAINS DURING COLD STORAGE

INTRODUCTION

Vibrio vulnificus and *V. parahaemolyticus* are the most common *Vibrios* associated with seafood illness in the United States (Hlady 1997). A report by the CDC (2006) indicated that infections caused by pathogens transmitted commonly through food have declined, or are approaching targeted national levels, with the exception of *Vibrio* infections, which are on the increase, and therefore require further action to prevent foodborne illnesses. Foodborne infections caused by *V. vulnificus* is one of the most severe, with mortality rates as high as 60% in individuals with liver disease or who are immunocompromised (Kumamoto et al. 1998).

There are three syndromes associated with disease caused by *V. vulnificus*, primary septicaemia, gastrointestinal disease and wound infection. Primary septicaemia and gastrointestinal infections are associated with the consumption of raw oysters, with gastrointestinal infections causing vomiting, diarrhea and abdominal pain. Wound infections occur when broken skin is exposed to water containing *V. vulnificus* (Chiang and Chuang 2003). Both septicaemia and wound infections can cause rapid multiplication of the pathogen in the tissues of the host, resulting in severe damage to the tissues of the skin.

Infections due to *V. parahaemolyticus* have increased worldwide in recent years. The largest outbreak of *V. parahaemolyticus* in the United States in 1998 was linked to the consumption of oysters in Texas, in which all clinical isolates were from a single clone of the O3:K6 serotype (DePaola et al. 2000). This strain appeared in India in 1996, but had not previously been detected in coastal waters of the U.S. (Wong et al. 2000).

Infections caused by *V. parahaemolyticus* result in diarrhea, abdominal cramps, nausea and vomiting.

The highest concentration of *Vibrio* can be found in the water column and in shellfish when water temperatures are typically above 20^{o}C (Randa et al. 2004). Motes et al. (1998) found that higher levels of *V. vulnificus* were present in oysters when salinities ranged from 5 to 25 ppt. and that numbers were lower at salinities higher than 28 ppt.

Vibrio parahaemolyticus is suggested to be sensitive to cool temperatures, but refrigeration and freezing cannot be relied upon to destroy the organism (Beuchat 1975). Temperature abuse of oyster meat following refrigeration or freezing could therefore still pose a food safety hazard. Cook and Ruple (1992) showed that the number of *V. vulnificus* in shucked and shellstock oysters held below 5^{o}C, decreased in numbers but could still be cultured after 14 and 21 days, respectively. In the same study, freezing also significantly reduced the numbers of the organism, but it was still possible to culture the bacteria from oysters that were stored for 12 weeks at -20^{o}C. Another study by Gooche et al. (2002) showed a six-fold decrease (0.8 log CFU/g) of naturally occurring *V. parahaemolyticus* in shellstock oysters after 14 to 17 days of refrigeration at 3^{o}C, while Cook and Ruple (1989) showed that levels of *Vibrio* in shellstock oysters did not increase during storage below 10^{o}C.

Commercial oysters are typically stored at 5 to10^{o}C, for approximately 7.7 days between first refrigeration and retail (FDA 2005). The National Shellfish Sanitation Program (2003) requires that during transport, shellstock oysters be cooled to an internal body temperature of 10^{o}C or less, when transportation exceeds 2 hours, and that during processing and shipment, shucked oysters be maintained at 7.2^{o}C or less.

Efforts to minimize foodborne illnesses through proper refrigeration of post harvest oysters may be compromised, if *Vibrio* strains exhibit significant differences in survival rates at refrigeration temperatures. There is limited research currently available on whether growth and survival differences occur between various strains of *V. vulnificus* and *V. parahaemolyticus.* This study was therefore conducted to determine if strain-to-strain differences exist in the growth and survival of *V. vulnificus* and *V. parahaemolyticus* at low temperatures.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PREPARATION OF INOCULUM

Eight *V. vulnificus* and eight *V. parahaemolyticus* strains were used during this study. Four of the *V. vulnificus* strains used were clinical isolates. Of these, strains 33816, 33815 and 29306 were obtained from the American Type Culture Collection (ATCC), Manassas, Va., and strain 1007 was obtained from Dr. Simonson, Louisiana State University Agricultural Center. The remaining four *V. vulnificus* strains used were environmental isolates. Of these, strains WR1 and 515-4C2 were obtained from Dr. Oliver, University of North Carolina at Charlotte, and strains 541(O) 49C and 0106-14 were obtained from the Food Safety / Food Microbiology laboratory at Louisiana State University Agricultural Center. Four of the *V. parahaemolyticus* strains used were clinical isolates, and the remaining four were environmental isolates. Two of the clinical isolates (33847 and 49529) were obtained from ATCC and the remaining two (TX2103 and CT6636), from the FDA (Yeung et al. 2002). Three of the environmental *V. parahaemolyticus* isolates (8332924, NY477 and M350A) were obtained from the FDA

(Yeung et al. 2002), and the remaining isolate, 541(O)57C from the Food Safety / Food Microbiology laboratory at Louisiana State University Agricultural Center.

Stock strains were maintained on 2% NaCl supplemented tryptic soy agar ($TSAN_2$) slants, at room temperature. Prior to use, each strain was transferred two consecutive times in 2% NaCl supplemented tryptic soy broth ($TSBN_2$) and incubated at 37^{o}C for 12 h. After incubation, the cultures were centrifuged at 10,000 rpm for 10 minutes, the supernatant discarded, and the bacterial pellet re-suspended into 10 ml of Phosphate Buffered Saline (PBS) two consecutive times. Serial 10-fold dilutions of the re-suspended cultures were carried out to achieve a final cell count of 10^6 CFU/ml.

BACTERIOLOGICAL MEDIA

All of the media used in this experiment have been previously described in the U.S. Food and Drug Administration Bacteriological Analytical Manual *Online* (2001). Non-selective Tryptic Soy Broth and Tryptic Soy Agar were supplemented with 2% NaCl for culturing and enumerating *V. vulnificus* and *V. parahaemolyticus*.

COLD STORGE STUDY

Ninety-nine ml of tryptic soy broth supplemented with 2% NaCl was added to specimen cups then the cups were prechilled to 5^{o}C. The cups were inoculated with one ml of washed overnight cultures of eight strains of *V. vulnificus* and eight strains of *V. parahaemolyticus* that had been grown at 37^{o}C in tryptic soy broth supplemented with 2% NaCl. This gave an initial count of about 10^6 log CFU/ml. The cups were then stored at 5, 8 or 10^{o}C for 10 days. Bacterial counts were determined every other day by plating on tryptic soy agar supplemented with 2% NaCl.

STATISTICAL ANALYSIS

Differences in growth and survival rates between the various strains were analyzed for significance using Student's t test following one-way analysis of variance (ANOVA) JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA). The statistical difference was set at $p<0.05$. All experiments were done in duplicate.

RESULTS

VIBRIO VULNIFICUS

Vibrio vulnificus strains stored in tryptic soy broth at 5°C for 10 days showed significant differences in survival rates throughout the duration of the experiment (Table 1). At day 2, *V. vulnificus* strain 1007 had significantly lower viable counts than the other strains, with 3.56 log CFU/ml. *V. vulnificus* strain 33816 had the highest counts for the same day, with 4.27 log CFU/ml. At day 4, *V. vulnificus* strain 33815 counts had decreased to 2.49 log CFU/ml, which were the lowest viable counts of the eight strains tested, while *V. vulnificus* strain 33816 had the highest counts, with 3.64 log CFU/ml. *V. vulnificus* strain 515-4C2 with counts of 1.97 log CFU/ml, had the best survival rate compared to the other strains by the end of the testing period. The stain with the next best survival rate at day 10 was *V. vulnificus* strain 0106-14, with counts of 1.48 log CFU/ml, followed by *V. vulnificus* strain 33816 with counts of 1.45 log CFU/ml. *V. vulnificus* strains 1007 and 29306 had declined to non-detectable levels at day 10, while *V. vulnificus* strain 33815 was unable to survive as well as the others, and had declined to non-detectable levels at day 8. All strains had a gradual reduction in viable plate counts as the experiment progressed.

Table 1 . Viable counts of *V. vulnificus* strains after cold storage at 5°C in tryptic soy broth supplemented with 2% NaCl over 10 days.

	log CFU/ml[a]					
Strain	**0 Day**	**2 Day**	**4 Day**	**6 Day**	**8 Day**	**10 Day**
1007	6.34 ± 0.07 ab	3.56 ± 0.10 c	2.70 ± 0.16 d	1.88 ± 0.39 b	1.67 ± 0.26 b	ND[b] c
33816	5.13 ± 0.05 c	4.27 ± 0.08 a	3.64 ± 0.21 a	2.36 ± 0.06 ab	1.84 ± 0.34 ab	1.45 ± 0.21 ab
33815	6.34 ± 0.03 ab	3.84 ± 0.15 b	2.49 ± 0.12 d	0.65 ± 0.92 c	ND[b] c	ND[b] c
29306	6.08 ± 0.10 b	4.09 ± 0.13 a	3.24 ± 0.04 bc	2.85 ± 0.03 a	0.65 ± 0.92 c	ND[b] c
WR1	6.60 ± 0.23 b	3.84 ± 0.07 b	3.40 ± 0.10 ab	2.66 ± 0.08 ab	1.72 ± 0.33 ab	1.00 ± 0.00 bc
515-4C2	6.41 ± 0.12 ab	4.10 ± 0.06 a	3.38 ± 0.03 ab	3.06 ± 0.16 a	2.45 ± 0.21 ab	1.97 ± 0.37 a
541(0)49C	6.59 ± 0.20 a	4.22 ± 0.06 a	3.50 ± 0.12 ab	2.55 ± 0.08 ab	1.80 ± 0.28 ab	0.65 ± 0.92 bc
0106-14	6.61 ± 0.30 a	4.19 ± 0.08 a	3.05 ± 0.06 c	2.84 ± 0.11 a	2.63 ± 0.28 a	1.48 ± 0.67 ab

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).
[b] ND = non detectable.

Table 2. Viable counts of *V. vulnificus* strains after cold storage at 8°C in tryptic soy broth supplemented with 2% NaCl over 10 days.

	log CFU/ml[a]					
Strain	**0 Day**	**2 Day**	**4 Day**	**6 Day**	**8 Day**	**10 Day**
1007	6.36 ± 0.04 bc	3.57 ± 0.60 d	2.86 ± 0.09 e	2.27 ± 0.13 d	1.98 ± 0.18 c	ND[b] d
33816	5.19 ± 0.09 d	4.88 ± 0.09 a	4.08 ± 0.06 a	2.90 ± 0.10 c	2.38 ± 0.05 bc	1.95 ± 0.13 ab
33815	6.36 ± 0.03 bc	3.66 ± 0.20 cd	2.75 ± 0.10 e	1.95 ± 0.13 e	0.50 ± 0.71 d	ND[b] d
29306	6.11 ± 0.08 c	4.17 ± 0.05 bc	3.31 ± 0.03 d	3.24 ± 0.05 b	1.95 ± 0.07 c	1.15 ± 0.21 c
WR1	6.70 ± 0.11 a	4.11 ± 0.04 bc	3.52 ± 0.20 cd	2.96 ± 0.06 bc	2.72 ± 0.12 ab	1.24 ± 0.34 c
515-4C2	6.44 ± 0.11 ab	4.25 ± 0.02 b	3.80 ± 0.12 b	3.20 ± 0.08 bc	2.67 ± 0.08 ab	2.23 ± 0.16 a
541(0)49C	6.72 ± 0.06 a	4.23 ± 0.02 b	3.76 ± 0.06 bc	2.97 ± 0.08 bc	2.52 ± 0.15 bc	ND[b] d
0106-14	6.71 ± 0.13 a	4.26 ± 0.08 b	3.86 ± 0.07 ab	3.71 ± 0.28 a	3.20 ± 0.08 a	1.83 ± 0.18 b

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).
[b] ND = non detectable.

Vibrio vulnificus strains stored in tryptic soy broth at 8°C, all had a gradual reduction in viable cell counts over the experimental period (Table 2). Significant differences in survival rates occurred between the various strains over the 10 days of the experiment. At day 2, *V. vulnificus* strain 1007 had significantly lower viable counts than all the other strains tested, with 3.57 log CFU/ml, while *V. vulnificus* strain 33816 had the highest viable counts with 4.88 log CFU/ml. At day 6, *V. vulnificus* strain 33815 had significantly lower viable counts than all the other strains tested, with 1.95 log CFU/ml, while *V. vulnificus* strain 29306 had the highest viable counts for the same period, with 3.24 log CFU/ml. Five of the eight *V. vulnificus* strains (33816, 29306, WR1, 515-4C2 and 0106-14) survived for the entire duration of the experiment at 8°C. Strain 515-4C2 had a gradual reduction in viable counts from day 0 with 6.44 log CFU/ml, to day 10 with 2.23 log CFU/ml. This strain had the highest survival rate at day 10. Strains 1007, 33815 and 541(0)49C survived for only 8 days. Survival rates for *V. vulnificus* strain 33815 ranged from 6.36 log CFU/ml on day 0, to 0.50 log CFU/ml on day 8, and had reached non-detectable levels by day 10.

V. vulnificus strains stored in tryptic soy broth at 10°C for 10 days had significant differences in growth and survival between the various strains, throughout the duration of the experiment (Table 3). At day 2, *V. vulnificus* strain 33815 with 4.07 log CFU/ml had the lowest viable counts of the eight strains tested, while strain 515-4C2 had the highest counts with 5.76 log CFU/ml. At day 6, *V. vulnificus* strain 33815 continued to have the lowest viable counts, with 2.80 log CFU/ml, and strain 515-4C2 the highest counts with 7.82 log CFU/ml. This trend continued at day 8, with viable counts for *V. vulnificus* strain 33815 being 0.65 log CFU/ml, while strain 515-4C2 had viable counts of 7.85 log

CFU/ml. At day 10, the strain with the best survival rates was 541(O)49C with 8.02 log CFU/ml. *V. vulnificus* strain 33815 was unable to survive as well as the others. This strain was the only one that showed a steady decline in viable counts over the course of the experiment, reaching non-detectable levels at day 10.

VIBRIO PARAHAEMOLYTICUS

Vibrio parahaemolyticus strains stored in tryptic soy broth at 5°C for 10 days survived for the duration of the experiment, with all strains having a steady decline in viable counts over the 10 days (Table 4). Differences in survival rates were evident between the various strains at this temperature. At day 2, *V. parahaemolyticus* strain 33847 with counts of 5.20 log CFU/ml, had significantly lower viable counts than the other *V. parahaemolyticus* strains tested, while *V. parahaemolyticus* strain NY477 had the highest viable counts of that day, with 6.67 log CFU/ml. At day 6, *V. parahaemolyticus* strain 33847 still had the lowest viable counts with 4.91 log CFU/ml, while *V. parahaemolyticus* strain CT6636 had the highest counts, with 5.95 log CFU/ml. *V. parahaemolyticus* strain 33847 had a decline in viable counts from 6.34 log CFU/ml on day 0 to 3.46 log CFU/ml on day 10, and was unable to survive as well as the other strains at 5°C. *V. parahaemolyticus* strain 541(0)57C had a decline in viable counts from 6.68 log CFU/ml on day 0 to 5.28 log CFU/ml on day 10, and was able to survive better than all the other strains tested.

Vibrio parahaemolyticus strains stored in tryptic soy broth at 8°C for 10 days, had significant differences in growth and survival rates between days 2 and 10 (Table 5). At day 2, *V. parahaemolyticus* strain 33847, with 5.34 log CFU/ml had significantly lower counts than the other strains tested, while *V. parahaemolyticus* strain 8332924 had the

highest counts for the same period, with 7.2 log CFU/ml. At day 8 the trend remained the same for these two strains, with viable counts for *V. parahaemolyticus* strain 33847 remaining the lowest, at 4.86 log CFU/ml, and for *V. parahaemolyticus* strain 8332924 the highest, with 8.10 log CFU/ml. *V. parahaemolyticus* strains 33847 and 541(0)57C, had a steady decline in viable counts over the ten days of storage, with viable counts for *V. parahaemolyticus* strain 33847 declining from 6.35 log CFU/ml at day 0, to 4.86 log CFU/ml at day 10. Viable counts for *V. parahaemolyticus* strain 541(O)57C declined from 6.96 log CFU/ml at day 0, to 4.80 log CFU/ml at day 10. This strain was unable to survive as well as the others at 8^{o}C. *V. parahaemolyticus* Strain M350A was able to survive better than the other *V. parahaemolyticus* strains at day 10, with counts of 7.97 log CFU/ml. This strain had a steady increase in viable counts from 6.62 log CFU/ml on day 0, to 8.23 log CFU/ml on day 6, but had a slight reduction in counts at day 10.

Vibrio parahaemolyticus strains stored in tryptic soy broth at 10^{o}C for 10 days all had an overall increase in viable counts over the 10 days (Table 6). Significant differences in growth occurred between the various strains throughout the duration of the experiment. At day 2, *V. parahaemolyticus* strain 33847 had significantly lower viable counts than all the other strains, with 5.90 log CFU/ml, while *V. parahaemolyticus* strain CT6636 had the highest viable counts for that day with 8.37 log CFU/ml. At day 4, *V. parahaemolyticus* strain 33847 continued to have the lowest viable counts, with 6.20 log CFU/ml, and strain 49529 the highest counts with 8.85 log CFU/ml. At day 6, the trend continued, with *V. parahaemolyticus* strain 33847 still having the lowest counts, of

Table 3. Viable counts of *V. vulnificus* strains after cold storage at 10°C in tryptic soy broth supplemented with 2% NaCl, over 10 days.

	log CFU/ml[a]					
Strain	**0 Day**	**2 Day**	**4 Day**	**6 Day**	**8 Day**	**10 Day**
1007	6.37 ± 0.06 ab	4.14 ± 0.06 d	3.86 ± 0.09 f	5.24 ± 0.13 e	6.08 ± 0.06 b	7.08 ± 0.21 cd
33816	5.22 ± 0.09 c	5.60 ± 0.06 a	6.51 ± 0.15 b	6.93 ± 0.38 b	7.05 ± 0.28 a	7.40 ± 0.08 bc
33815	6.39 ± 0.05 ab	4.07 ± 0.05 d	3.14 ± 0.05 g	2.80 ± 0.08 f	0.65 ± 0.92 c	ND[b] f
29306	6.14 ± 0.05 b	4.88 ± 0.09 c	5.23 ± 0.07 e	5.35 ± 0.16 e	6.13 ± 0.18 b	6.31 ± 0.04 e
WR1	6.79 ± 0.11 ab	5.72 ± 0.05 a	6.09 ± 0.06 c	6.58 ± 0.16 bc	7.10 ± 0.04 a	7.71 ± 0.13 ab
515-4C2	6.67 ± 0.14 a	5.76 ± 0.14 a	7.25 ± 0.05 a	7.82 ± 0.11 a	7.85 ± 0.12 a	6.50 ± 0.13 e
541(0)49C	6.85 ± 0.13 a	5.11 ± 0.07 b	5.46 ± 0.08 d	6.07 ± 0.10 d	7.70 ± 0.13 a	8.02 ± 0.15 a
0106-14	6.76 ± 0.13 a	4.70 ± 0.11 c	5.04 ± 0.07 e	6.25 ± 0.07 cd	7.43 ± 0.04 a	7.00 ± 0.31 d

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).
[b] ND = non detectable.

Table 4. Viable counts of *V. parahaemolyticus* strains after cold storage at 5°C in tryptic soy broth supplemented with 2% NaCl over 10 days.

	log CFU/ml[a]					
Strain	**0 Day**	**2 Day**	**4 Day**	**6 Day**	**8 Day**	**10 Day**
33847	6.34 ± 0.18 b	5.20 ± 0.15 c	5.04 ± 0.08 d	4.91 ± 0.04 c	4.05 ± 0.00 c	3.46 ± 0.28 c
49529	6.60 ± 0.26 ab	5.86 ± 0.08 b	5.61 ± 0.17 cd	5.58 ± 0.08 abc	5.00 ± 0.61 ab	4.85 ± 0.47 ab
TX2103	6.63 ± 0.20 ab	5.73 ± 0.35 ab	5.72 ± 0.01 bc	5.34 ± 0.16 bc	4.82 ± 0.16 b	4.23 ± 0.22 bc
CT6636	6.59 ± 0.18 ab	6.37 ± 0.36 a	6.41 ± 0.66 a	5.95 ± 0.45 a	5.55 ± 0.23 a	5.17 ± 0.66 ab
8332924	6.63 ± 0.08 ab	6.39 ± 0.21 a	6.29 ± 0.21 ab	5.85 ± 0.64 ab	4.91 ± 0.35 ab	4.84 ± 0.74 ab
NY477	6.89 ± 0.37 a	6.67 ± 0.21 a	6.35 ± 0.28 ab	5.73 ± 0.30 ab	5.34 ± 0.20 ab	4.51 ± 0.08 ab
M350A	6.68 ± 0.20 ab	6.16 ± 0.04 ab	5.94 ± 0.23 abc	5.65 ± 0.30 ab	4.79 ± 0.13 b	4.41 ± 0.31 abc
541(O)57C	6.68 ± 0.16 ab	5.77 ± 0.13 b	5.50 ± 0.02 cd	5.39 ± 0.08 abc	5.37 ± 0.20 ab	5.28 ± 0.11 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

Table 5. Viable counts of *V. parahaemolyticus* strains after cold storage at 8°C in tryptic soy broth supplemented with 2% NaCl over 10 days.

	log CFU/ml[a]					
Strain	**0 Day**	**2 Day**	**4 Day**	**6 Day**	**8 Day**	**10 Day**
33847	6.35 ± 0.06 a	5.34 ± 0.33 c	5.05 ± 0.08 d	5.08 ± 0.21 d	4.86 ± 0.33 b	4.86 ± 0.61 c
49529	6.72 ± 0.21 a	6.05 ± 0.06 b	6.25 ± 0.01 c	7.16 ± 0.50 bc	7.62 ± 0.81 a	7.65 ± 0.23 a
TX2103	6.65 ± 0.28 a	6.13 ± 0.07 b	6.90 ± 0.01 b	6.91 ± 0.25 c	7.08 ± 0.25 a	7.41 ± 0.14 a
CT6636	6.67 ± 0.14 a	6.94 ± 0.06 a	8.15 ± 0.04 a	8.07 ± 0.52 a	5.69 ± 0.60 b	6.12 ± 0.16 b
8332924	6.62 ± 0.34 a	7.20 ± 0.25 a	8.17 ± 0.44 a	8.09 ± 0.49 abc	8.10 ± 0.54 a	7.89 ± 0.94 a
NY477	6.77 ± 0.28 a	7.06 ± 0.21 a	7.57 ± 0.49 a	7.86 ± 0.12 ab	8.05 ± 0.33 a	7.81 ± 0.28 a
M350A	6.62 ± 0.33 a	7.19 ± 0.31 a	7.97 ± 0.54 a	8.23 ± 0.29 a	7.90 ± 0.03 a	7.97 ± 0.06 a
541(O)57C	6.96 ± 0.38 a	6.40 ± 0.17 b	6.11 ± 0.03 c	5.71 ± 0.42 d	5.21 ± 0.04 b	4.80 ± 0.14 c

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

Table 6. Viable counts of *V. parahaemolyticus* strains after cold storage at 10°C in tryptic soy broth supplemented with 2% NaCl over 10 days.

	log CFU/ml[a]					
Strain	**0 Day**	**2 Day**	**4 Day**	**6 Day**	**8 Day**	**10 Day**
33847	6.39 ± 0.13 b	5.90 ± 0.54 c	6.20 ± 0.7 d	5.22 ± 0.37 d	5.92 ± 0.57 e	6.77 ± 0.44 c
49529	6.77 ± 0.20 ab	7.81 ± 0.32 ab	8.85 ± 0.44 a	7.99 ± 0.28 bc	7.71 ± 0.21 cd	7.70 ± 0.13 b
TX2103	6.80 ± 0.05 ab	7.13 ± 0.18 b	7.92 ± 0.02 bc	8.46 ± 0.42 ab	8.57 ± 0.08 a	7.75 ± 0.15 b
CT 6636	6.75 ± 0.23 ab	8.37 ± 0.30 a	8.56 ± 0.18 abc	8.77 ± 0.16 a	7.88 ± 0.16 bc	7.75 ± 0.16 ab
8332924	6.49 ± 0.08 b	7.94 ± 0.47 ab	8.61 ± 0.12 ab	8.59 ± 0.09 a	8.22 ± 0.27 abc	7.96 ± 0.21 ab
NY477	6.94 ± 0.25 a	7.78 ± 0.11 ab	8.54 ± 0.16 abc	8.38 ± 0.03 ab	8.40 ± 0.12 ab	8.31 ± 0.10 a
M350A	6.69 ± 0.32 ab	7.96 ± 0.35 ab	8.67 ± 0.02 a	8.26 ± 0.03 ab	8.01 ± 0.14 abc	8.02 ± 0.40 ab
541(O)57C	6.58 ± 0.16 ab	7.72 ± 0.40 ab	7.88 ± 0.02 c	7.53 ± 0.07 c	7.05 ± 0.38 d	7.63 ± 0.06 b

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

5.22 log CFU/ml, and strain CT6636 the highest, with 8.77 log CFU/ml. The best growing *V. parahaemolyticus* strain at day 10 was NY477. This strain had an increase in viable counts from 6.94 log CFU/ml at day 0, to 8.31 log CFU/ml at day 10. *V. parahaemolyticus* strain 33847, was unable to survive as well as the other strains at day 10, with viable counts of 6.77 log CFU/ml.

DISCUSSION

Vibrio vulnificus and *V. parahaemolyticus* strains grown at 5, 8 or10^{o}C exhibited significant differences in growth and survival rates at all the temperatures tested. These differences were evident throughout the 10 days of the experiment. We found that *V. vulnificus* strains were able to survive but not grow at 5^{o}C. Survival rates differed among the strains, with five of the strains surviving for the duration of the experiment at this temperature, while the remaining three strains reached non-detectable levels between days 8 and 10. *V. vulnificus* strains stored at 8^{o}C for 10 days had no increase in viable counts over the duration of the experiment. Differences in survival rates were evident between the various strains at this temperature. Five of the eight *V. vulnificus* strains were able to survive to day 10, while the remaining three strains reached non-detectable levels at day 10. The survival rate for strain 33815 at day 10 was 1.95 log CFU/ml, while *V. vulnificus* strain 33815 reached non-detectable levels at day 8.

The temperature range allowing the growth of *V. vulnificus* in Defined Medium (DM) was demonstrated by McGovern and Oliver (1995) to be between 10 to 42^{o}C, while another study carried out in BHI broth by Kaysner et al. (1989) showed that at 10^{o}C, *V. vulnificus* survived for 8 days. We showed that some *V. vulnificus* strains grew

at 10°C, while others survived. At 10°C, seven of the eight *V. vulnificus* strains had increases in viable counts over the 10 days of storage, with significant differences in growth being evident between the various strains. *V. vulnificus* strain 541(O)49C had the highest viable counts of 8.02 log CFU/ml at the end of the experiment. *V. vulnificus* strain 33815 did not grow at this temperature and had dropped to non-detectable levels by day 10.

We found that at 5°C, all *V. parahaemolyticus* strains were able to survive for the duration of the experiment, but were not able to grow. Survival rates at this temperature varied significantly between the different strains. At the end of the experiment, *V. parahaemolyticus* strain 33847 had viable counts of 3.46 log CFU/ml compared to counts of 5.28 log CFU/ml for *V. parahaemolyticus* strain 541(O)57C. At 10°C all of the *V. parahaemolyticus* strains had an overall increase in viable counts, with significant differences in growth occurring between the various strains. The best growing strain at day 10 at this temperature was *V. parahaemolyticus* strain NY477, with viable counts of 8.31 log CFU/ml, compared to *V. parahaemolyticus* strain 33847, with counts of 6.77 log CFU/ml. In a 24 hour study carried out by Miles et al. (1997) using tryptic soy broth, *V. parahaemolyticus* strains grew between 8.3 and 45.3°C. The study also suggested that there might be minor strain-to-strain variations between the four strains tested in the experiment. We found significant differences in the growth and survival rates of the various *V. parahaemolyticus* strains at all temperatures tested. At 8°C, five of the eight *V. parahaemolyticus* strains had an increase in viable counts over the course of the experiment. *V. parahaemolyticus* strain M350A had growth over the 10 days of storage, and had the highest viable counts of 7.97 log CFU/ml at day 10. *V. parahaemolyticus*

strain 541(O)57C had a steady decline in viable counts over the course of the experiment, and was unable to survive as well as the other strains, with counts of 4.80 log CFU/ml at day 10.

Oliver et al. (1991) showed that when *V. vulnificus* cells were shifted from 37^{o}C to 5^{o}C, plate counts declined gradually, with cells exhibited an immediate decrease in culturability. The study found that *V.vulnificus* cells enter a viable but non culturable state when exposed to temperatures below 10^{o}C. The VBNC state is a physiological state in which bacterial cells remain viable but are unable to grow on routine microbiological media. In this state, cell size, DNA, RNA and protein synthesis are decreased (Oliver 1993). The VBNC state is thought to be a survival response to adverse environmental conditions (Warner and Oliver 1998).

Another study by Bates and Oliver (2004) determined that the time required for Kanagawa Phenomenon positive and Kanagawa Phenomenon negative cells of *V. parahaemolyticus* to enter the VBNC state (at 5^{o}C) in ASW was 6.9 days and 7.2 days respectively. Other studies by Johnston and Brown (2002) showed that cells of *V. vulnificus* and *V. parahaemolyticus* lost their culturability after storage in ASW at low temperatures (4^{o}C), whilst undergoing a shape change from rods to coccoid cells. These cells could not be grown on selective or non-selective agar, but appeared to be metabolically active. The cells became culturable again following an upshift in temperature. Another study by Bogosian et al. (2000) found however that additional culturable cells seen following an upshift in temperature, resulted from growth of residual culturable cells, and determined that non culturable cells were in fact dead. In our study, the decline in viable plate counts seen with *V. vulnificus* cultures stored at 5 and 8^{o}C and

V. parahaemolyticus cultures stored at 5°C may be attributed either to the cells entering a VBNC state, or to actual death of the cells. Variation in survival rates seen between the different strains could be either due to the various strains entering a VBNC state at different times, or due to strains that are more susceptible to cold temperatures dying at a faster rate.

A study carried out by Herendeen et al. (1979) using *E. coli* cultures found that when the cultures are held within the normal temperature range of growth, the levels of most proteins did not change significantly. When these cultures were transferred to extreme temperatures (high or low) outside of the normal temperature range for growth, some proteins that were previously barely detectable, either increased or decreased dramatically. Changes in the level of production of certain proteins, following up or down shifts in temperature, impact on the ability of some bacteria to survive under adverse conditions. Datta and Bhadra (2003) found that when *Vibrio cholera* cells were shifted from 37°C to 15°C, there was significant production of two major cold shock proteins which degraded when the cells were shifted back to 37°C. The study suggested that these proteins were necessary for the bacteria to adapt at a lower temperature. In our study, the various *V. vulnificus* and *V. parahaemolyticus* strains had significant differences in growth and survival rates at all the temperatures tested. These differences may be attributed to variations in the levels and or types of proteins produced by the different strains in response to the downshift in temperature. These differences in growth and survival rates could have an impact on the safety of seafood stored at refrigeration temperatures.

In 2005, the FDA published a risk assessment on the public health impact of pathogenic *V. parahaemolyticus* in raw oysters (FDA 2005). In the risk assessment, the growth rate of *V. parahaemolyticus* in oysters was estimated based on studies by Miles et al. (1997) and Gooch et al. (2002). The study by Gooch et al. was limited to growth in oysters at only one temperature (26^{o}C). Due to limited data on the post-harvest growth of *V. parahaemolyticus* in oysters, a model for growth of *V. parahaemolyticus* carried out in broth (Miles et al. 1997) was used to predict the growth of *V. parahaemolyticus* in oysters at all temperatures, based on the results of the study in oysters at 26^{o}C by Gooch et al. (2002). Our research has shown that various *V. vulnificus* and *V. parahaemolyticus* strains vary significantly in their ability to survive and grow at refrigeration temperatures. This data may be useful in updating risk assessment models for these pathogens.

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CHAPTER 4

ADAPTATION DIFFERENCES OF *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* STRAINS DURING COLD STORAGE

INTRODUCTION

Storage at low temperatures is an established preservation method used to control the growth of microorganisms in food. The National Shellfish Sanitation Program recommends the storage of shucked and shellstock oysters to be between 7.2 to 10^{o}C during transport, processing and shipment. The ability of some microorganisms to adapt, as a survival response, when exposed to a downshift in temperature, could compromised efforts to use temperature control to reduce the risk of *Vbrio* infections associated with the consumption of raw oysters.

An adaptation response of microorganisms to cold temperatures has been documented in several species, including *Escherichia coli* (Jones et al. 1987), *Bacillus subtilis* (Willimsky et al. 1992), and *Listeria monocytogenes* (Bayles et al. 1996). A study by Jones et al. (1987) showed that when cultures of *E. coli* were shifted from 37 to 10^{o}C, a 4-h lag phase occurred, during which time protein synthesis was reduced. Due to the downshift in temperature, the production of several cold shock proteins (Csps) were induced, with the protein CspA of molecular mass 7.4 kDa being dramatically induced (Phadtare et al. 1999). The cold shock response is required for cellular adaptation to low temperatures (Weining et al. 1996). Another study by Datta and Bhadra (2003) found that a cold shock protein, (CspV), of molecular mass 7.5 kDa, was induced upon exposure of *Vibrio cholera* to a downshift in temperature.

Limited research is available on the adaptive response to cold shock on the survival of *V. vulnificus* and *V. parahaemolyticus*. This study was conducted to determine if different *V. vulnificus* and *V. parahaemolyticus* strains exhibit a cold temperature adaptation response.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PREPARATION OF INOCULUM

Eight *V. vulnificus* and eight *V. parahaemolyticus* strains were used during this study. Four of the *V. vulnificus* strains used were clinical isolates. Of these, strains 33816, 33815 and 29306 were obtained from the American Type Culture Collection (ATCC), Manassas, Va., and strain 1007 was obtained from Dr. Simonson, Louisiana State University Agricultural Center. The remaining four *V. vulnificus* strains used were environmental isolates. Of these, strains WR1 and 515-4C2 were obtained from Dr. Oliver, University of North Carolina at Charlotte, and strains 541(O) 49C and 0106-14 were obtained from the Food Safety / Food Microbiology laboratory at Louisiana State University Agricultural Center. Four of the *V. parahaemolyticus* strains used were clinical isolates, and the remaining four were environmental isolates. Two of the clinical isolates (33847 and 49529) were obtained from ATCC and the remaining two (TX2103 and CT6636), from the FDA (Yeung et al. 2002). Three of the environmental *V. parahaemolyticus* isolates (8332924, NY477 and M350A) were obtained from the FDA (Yeung et al. 2002), and the remaining isolate, 541(O)57C from the Food Safety / Food Microbiology laboratory at Louisiana State University Agricultural Center.

Stock strains were maintained on 2% NaCl supplemented tryptic soy agar ($TSAN_2$) slants, at room temperature. Prior to use, each strain was transferred two consecutive times in 2% NaCl supplemented tryptic soy broth ($TSBN_2$) and incubated at 37^oC for 12 h. After incubation, the cultures were centrifuged at 10,000 rpm for 10 minutes, the supernatant discarded, and the bacterial pellet re-suspended into 10 ml of

Phosphate Buffered Saline (PBS) two consecutive times. Serial 10-fold dilutions of the re-suspended cultures were carried out to achieve a final cell count of 10^6 CFU/ml.

COLD TEMPERATURE ADAPTATION

Ninety-nine ml of tryptic soy broth supplemented with 2% NaCl was added to specimen cups then the cups were prechilled to 15°C. The cups were inoculated with one ml of washed overnight cultures of eight strains of *V. vulnificus* and eight strains of *V. parahaemolyticus* that had been grown at 37°C in tryptic soy broth supplemented with 2% NaCl. This gave an initial count of about 10^6 log CFU/ml. The cups were stored at 15°C for 4 hours (Bryan et al. 1999). At the end of 4 hours, the cups were stored at 5, 8 or 10°C for 9 days. Bacterial counts were determined by plating on tryptic soy agar supplemented with 2% NaCl.

STATISTICAL ANALYSIS

The ability of the various strains to undergo cold temperature adaptation was analyzed for significance using Student's t test following one-way analysis of variance (ANOVA) JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA). The statistical difference was set at $p<0.05$. All experiments were done in duplicate.

RESULTS

VIBRIO VULNIFICUS

Cold adapted *V. vulnificus* strains stored at 5 and 8°C for 9 days were able to survive but not grow (Tables 7 to 14). At 5°C all of the cold adapted *V. vulnificus* strains survived to the end of the experiment, with the exception of V. *vulnificus* strain 1007, which had reached non-detectable levels at day 9 (Table 7). At 8°C, all cold adapted *V. vulnificus* strains survived the entire duration of the experiment, but did not grow, while

at 10°C all cold adapted *V. vulnificus* strains with the exception of *V. vulnificus* strain 33815, were able to grow (Table 9).

Vibrio vulnificus strain 1007 cold adapted then stored at 5°C (Table 7), had significantly higher viable counts than the non-cold adapted counts, at days 3 and 5. This increase was higher at day 5, with a 0.76 log CFU/ml difference from the non-cold adapted counts. At 8°C, the cold adapted *V. vulnificus* strain 1007, had a significant increase in viable counts at day 5, compared to the non-cold adapted counts, while at 10°C, the increase occurred at days 3, 5 and 7.

Vibrio vulnificus strain 33816 cold adapted then stored at 5°C (Table 8) had significantly higher viable counts than the non-cold adapted counts throughout the 9 days of the experiment. This increase was highest at day 9, with a 1.31 log CFU/ml difference between the cold adapted *V. vulnificus* strain 33816 counts and the non-cold adapted counts. A similar trend was seen with cold adapted *V. vulnificus* strain 33816 stored at 8°C. The increase at this temperature was highest at day 7, with a 1.58 log CFU/ml difference between the cold adapted and the non-cold adapted counts. At 10°C, a significant increase was evident only up to day 1.

Cold adapted *V. vulnificus* strain 33815 stored at 5 and 8°C, had significantly higher viable counts than the non-cold adapted counts at days 3, 5, 7, and 9 (Table 9). At 5°C, the increase was highest at day 5, with a 3.21 log CFU/ml difference between the cold adapted *V. vulnificus* strain 33815 counts and the non-cold adapted counts. At 8°C, the increase was highest at day 9, with a 2.49 log CFU/ml difference in bacterial counts. At 10°C, the cold adapted *V. vulnificus* strain 33815 had higher viable counts at days 3, 5, 7 and 9, compared to the non-cold adapted counts. This increase was highest at day 9

Table 7 . Viable counts of *V. vulnificus* strain 1007 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a] 0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	**Cold Adapted**	6.37 ± 0.06 a	4.52 ± 0.13 a	3.60 ± 0.09 a	2.79 ± 0.09 a	1.95 ± 0.13 a	ND[c] a
	Cold Storage	6.34 ± 0.07 a	4.39 ± 0.13 a	3.09 ± 0.08 b	2.03 ± 0.11 b	1.76 ± 0.40 a	ND[c] a
8°C	**Cold Adapted**	6.38 ± 0.06 a	4.62 ± 0.26 a	3.83 ± 0.09 a	2.95 ± 0.12 a	2.30 ± 0.09 a	1.69 ± 0.13 a
	Cold Storage	6.36 ± 0.04 a	4.60 ± 0.11 a	3.42 ± 0.14 a	2.45 ± 0.06 b	2.14 ± 0.13 a	1.67 ± 0.26 a
10°C	**Cold Adapted**	6.40 ± 0.05 a	4.85 ± 0.12 a	5.03 ± 0.13 a	5.32 ± 0.16 a	7.33 ± 0.05 a	7.55 ± 0.20 a
	Cold Storage	6.37 ± 0.06 a	4.77 ± 0.14 a	3.93 ± 0.21 b	3.71 ± 0.10 b	5.47 ± 0.06 b	6.91 ± 0.30 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).
[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days.
[c] ND = non detectable.

Table 8. Viable counts of *V. vulnificus* strain 33816 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a] 0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	**Cold Adapted**	6.34 ± 0.16 a	5.74 ± 0.07 a	4.76 ± 0.06 a	4.30 ± 0.01 a	3.32 ± 0.04 a	2.85 ± 0.08 a
	Cold Storage	5.13 ± 0.05 b	5.06 ± 0.15 b	3.74 ± 0.10 b	3.05 ± 0.07 b	2.35 ± 0.16 b	1.54 ± 0.34 b
8°C	**Cold Adapted**	6.34 ± 0.18 a	5.86 ± 0.08 a	5.43 ± 0.06 a	4.91 ± 0.04 a	4.08 ± 0.11 a	3.20 ± 0.06 a
	Cold Storage	5.19 ± 0.09 b	5.12 ± 0.05 b	4.57 ± 0.24 b	3.56 ± 0.13 b	2.50 ± 0.12 b	2.30 ± 0.09 b
10°C	**Cold Adapted**	6.35 ± 0.16 a	6.03 ± 0.14 a	5.97 ± 0.26 a	6.96 ± 0.35 a	7.22 ± 0.08 a	7.37 ± 0.06 a
	Cold Storage	5.22 ± 0.09 b	5.23 ± 0.11 b	5.92 ± 0.12 a	6.86 ± 0.11 a	7.04 ± 0.11 a	7.11 ± 0.07 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).
[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days.

Table 9. Viable counts of *V. vulnificus* strain 33815 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a] 0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	**Cold Adapted**	6.37 ± 0.04 a	5.10 ± 0.23 a	4.20 ± 0.05 a	3.86 ± 0.37 a	2.83 ± 0.18 a	1.24 ± 0.34 a
	Cold Storage	6.34 ± 0.03 a	4.56 ± 0.11 a	2.71 ± 0.07 b	0.65 ± 0.92 b	0.50 ± 0.71 b	ND[c] b
8°C	**Cold Adapted**	6.39 ± 0.05 a	5.30 ± 0.22 a	4.75 ± 0.15 a	3.98 ± 0.11 a	2.79 ± 0.35 a	2.49 ± 0.12 a
	Cold Storage	6.36 ± 0.03 a	4.85 ± 0.11 a	2.82 ± 0.13 b	2.20 ± 0.12 b	0.65 ± 0.92 b	ND[c] b
10°C	**Cold Adapted**	6.40 ± 0.04 a	5.23 ± 0.20 a	4.94 ± 0.08 a	4.12 ± 0.08 a	3.08 ± 0.06 a	2.78 ± 0.23 a
	Cold Storage	6.39 ± 0.05 a	4.91 ± 0.30 a	3.56 ± 0.11 b	3.04 ± 0.08 b	2.50 ± 0.13 b	0.50 ± 0.71 b

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days.

[c] ND = non detectable.

with a 2.28 log CFU/ml difference between the cold adapted and non-cold adapted counts.

Vibrio vulnificus strain 29306 cold adapted then stored at 5, 8 and 10°C, had significantly higher viable counts than the non-cold adapted counts at 5, 8 and 10°C at days 5 and 7, 7 and 9, and 3, 5, 7 and 9, respectively (Table 10). At 5°C, the increase was higher at day 7, with a 1.20 log CFU/ml difference between the cold adapted *V. vulnificus* strain 29306 counts and the non-cold adapted counts. At 8°C, the increase was higher at day 9, with a 1.12 log CFU/ml difference between the cold adapted and the non-cold adapted counts, and at 10°C the highest increase occurred at days 3 and 5 with a 0.58 log CFU/ml difference between the cold adapted *V. vulnificus* strain 29306 counts and the non-cold adapted counts.

Vibrio vulnificus strain WR1 cold adapted then stored at 5, 8 and 10°C, had significantly higher viable counts than the non-cold adapted counts (Table 11). At 5°C, the increase was evident at days 3, 5 and 7, and at 8 and 10°C, at days 3, 5, 7 and 9. At 5°C, the greatest difference occurred at day 7, with a 1.41 log CFU/ml increase in the cold adapted *V. vulnificus* strain WRI counts compared to the non-cold adapted counts. At 8°C, the increase was highest at day 9, with a 1.54 log CFU/ml difference between the cold adapted and non-cold adapted counts, and 10°C, the greatest difference was at day 3, with a 1.56 log CFU/ml increase in cold adapted strain WR1 counts compared to the non-cold adapted counts.

Vibrio vulnificus strain 515-42C cold adapted then stored at 8°C had no significant differences in viable counts compared to the non-cold adapted counts (Table 12). However at 5 and 10°C, significant differences were seen at days 3 and 5, and

Table 10. Viable counts of *V. vulnificus* strain 29306 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a]					
		0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	Cold Adapted	6.15 ± 0.04 a	4.68 ± 0.24 a	3.69 ± 0.25 a	3.40 ± 0.05 a	2.65 ± 0.16 a	1.83 ± 0.18 a
	Cold Storage	6.08 ± 0.10 a	4.41 ± 0.15 a	3.38 ± 0.08 a	3.12 ± 0.05 b	1.45 ± 0.71 b	0.65 ± 0.92 a
8°C	Cold Adapted	6.18 ± 0.09 a	4.73 ± 0.23 a	3.86 ± 0.25 a	3.59 ± 0.26 a	3.15 ± 0.08 a	2.36 ± 0.11 a
	Cold Storage	6.11 ± 0.08 a	4.65 ± 0.20 a	3.55 ± 0.08 a	3.41 ± 0.05 a	2.69 ± 0.02 b	1.24 ± 0.34 b
10°C	Cold Adapted	6.18 ± 0.21 a	4.76 ± 0.06 a	5.79 ± 0.09 a	5.85 ± 0.15 a	6.76 ± 0.08 a	6.63 ± 0.13 a
	Cold Storage	6.14 ± 0.05 a	4.80 ± 0.13 a	5.21 ± 0.06 b	5.27 ± 0.05 b	6.21 ± 0.59 b	6.20 ± 0.04 b

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days.

Table 11. Viable counts of *V. vulnificus* strain WR1 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a]					
		0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	Cold Adapted	6.77 ± 0.16 a	4.96 ± 0.13 a	4.37 ± 0.06 a	3.74 ± 0.10 a	3.35 ± 0.21 a	2.81 ± 0.12 a
	Cold Storage	6.60 ± 0.23 a	4.90 ± 0.12 a	3.58 ± 0.03 b	2.89 ± 0.06 b	1.94 ± 0.34 b	0.65 ± 0.92 a
8°C	Cold Adapted	6.85 ± 0.10 a	5.05 ± 0.12 a	4.80 ± 0.03 a	3.92 ± 0.10 a	3.46 ± 0.10 a	2.89 ± 0.08 a
	Cold Storage	6.70 ± 0.11 a	4.96 ± 0.13 a	3.61 ± 0.25 b	3.17 ± 0.06 b	2.89 ± 0.05 b	1.35 ± 0.50 b
10°C	Cold Adapted	6.89 ± 0.08 a	5.91 ± 0.11 a	7.23 ± 0.20 a	7.37 ± 0.06 a	7.58 ± 0.14 a	7.79 ± 0.14 a
	Cold Storage	6.79 ± 0.11 a	5.84 ± 0.12 a	5.67 ± 0.10 b	6.49 ± 0.12 b	6.65 ± 0.18 b	7.58 ± 0.18 b

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days.

Table 12. Viable counts of *V. vulnificus* strain 515-4C2 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a] 0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	**Cold Adapted**	6.49 ± 0.12 a	4.86 ± 0.21 a	4.40 ± 0.16 a	3.81 ± 0.15 a	2.77 ± 0.22 a	2.23 ± 0.11 a
	Cold Storage	6.41 ± 0.12 a	4.73 ± 0.23 a	3.65 ± 0.12 b	3.23 ± 0.04 b	2.78 ± 0.10 a	2.16 ± 0.17 a
8°C	**Cold Adapted**	6.66 ± 0.14 a	4.93 ± 0.15 a	4.55 ± 0.20 a	3.94 ± 0.08 a	2.92 ± 0.25 a	2.43 ± 0.04 a
	Cold Storage	6.44 ± 0.11 a	4.93 ± 0.04 a	3.94 ± 0.10 a	3.76 ± 0.06 a	2.86 ± 0.06 a	2.29 ± 0.13 a
10°C	**Cold Adapted**	6.70 ± 0.13 a	5.90 ± 0.15 a	7.40 ± 0.03 a	7.73 ± 0.17 a	7.98 ± 0.14 a	6.74 ± 0.16 a
	Cold Storage	6.67 ± 0.14 a	5.05 ± 0.11 b	6.32 ± 0.04 b	7.67 ± 0.08 a	7.87 ± 0.44 a	6.65 ± 0.07 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C,then stored at 5, 8 or 10°C for 9 days.

Table 13. Viable counts of *V. vulnificus* strain 541(0)49C after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a]					
		0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	Cold Adapted	6.76 ± 0.33 a	4.79 ± 0.11 a	4.11 ± 0.04 a	3.27 ± 0.10 a	2.50 ± 0.10 a	2.00 ± 0.13 a
	Cold Storage	6.59 ± 0.20 a	4.54 ± 0.20 a	3.82 ± 0.10 a	2.80 ± 0.06 a	2.02 ± 0.23 a	0.65 ± 0.92 a
8°C	Cold Adapted	6.76 ± 0.50 a	4.89 ± 0.08 a	4.45 ± 0.05 a	3.52 ± 0.15 a	2.80 ± 0.11 a	2.08 ± 0.05 a
	Cold Storage	6.72 ± 0.06 a	4.65 ± 0.16 a	3.73 ± 0.45 a	3.09 ± 0.04 a	2.73 ± 0.15 a	0.50 ± 0.71 a
10°C	Cold Adapted	6.91 ± 0.13 a	4.96 ± 0.08 a	5.89 ± 0.05 a	5.96 ± 0.11 a	6.56 ± 0.21 a	7.98 ± 0.26 a
	Cold Storage	6.85 ± 0.13 a	4.68 ± 0.16 a	5.36 ± 0.06 b	5.58 ± 0.18 a	6.47 ± 0.15 a	7.97 ± 0.10 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C,then stored at 5, 8 or 10°C for 9 days.

Table 14. Viable counts of *V. vulnificus* strain 0106-14 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a]					
		0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	Cold Adapted	6.72 ± 0.13 a	5.36 ± 0.08 a	5.06 ± 0.08 a	4.15 ± 0.08 a	4.03 ± 0.22 a	2.56 ± 0.08 a
	Cold Storage	6.61 ± 0.30 a	4.81 ± 0.18 a	3.49 ± 0.12 b	2.92 ± 0.10 b	2.81 ± 0.08 b	1.90 ± 0.07 b
8°C	Cold Adapted	6.74 ± 0.28 a	5.59 ± 0.22 a	5.04 ± 0.29 a	4.52 ± 0.15 a	4.27 ± 0.04 a	2.84 ± 0.17 a
	Cold Storage	6.71 ± 0.13 a	4.83 ± 0.11 b	3.91 ± 0.04 b	3.82 ± 0.17 b	3.47 ± 0.08 b	2.60 ± 0.06 a
10°C	Cold Adapted	6.76 ± 0.23 a	5.93 ± 0.10 a	7.13 ± 0.03 a	7.31 ± 0.16 a	7.60 ± 0.28 a	7.21 ± 0.04 a
	Cold Storage	6.76 ± 0.13 a	4.96 ± 0.11 b	4.80 ± 0.16 b	5.87 ± 0.11 b	7.54 ± 0.08 a	7.13 ± 0.18 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C,then stored at 5, 8 or 10°C for 9 days.

Table 15. Viable counts of *V. parahaemolyticus* strain 33847 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a] 0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	**Cold Adapted**	6.78 ± 0.06 a	6.67 ± 0.06 a	5.52 ± 0.13 a	5.58 ± 0.24 a	4.99 ± 0.16 a	3.92 + 0.23 a
	Cold Storage	6.34 ± 0.18 a	5.94 ± 0.18 b	5.03 ± 0.03 b	5.09 ± 0.10 a	4.57 ± 0.08 a	3.53 + 0.29 a
8°C	**Cold Adapted**	6.69 ± 0.25 a	6.46 ± 0.06 a	6.31 ± 0.09 a	5.26 ± 0.13 a	5.18 ± 0.08 a	4.98 + 0.12 a
	Cold Storage	6.35 ± 0.16 a	5.82 ± 0.16 b	5.29 ± 0.04 b	4.86 ± 0.24 a	4.95 ± 0.27 a	4.80 + 0.30 a
10°C	**Cold Adapted**	6.87 ± 0.11 a	7.04 ± 0.12 a	7.79 ± 0.05 a	7.80 ± 0.21 a	7.83 ± 0.21 a	6.34 + 0.11 a
	Cold Storage	6.39 ± 0.13 a	5.95 ± 0.25 b	6.09 ± 0.55 b	5.61 ± 0.44 b	5.10 ± 0.31 b	5.78 + 0.13 b

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days.

days 1 and 3, respectively. At both 5 and 10°C, the increase was highest at day 3, with 0.75 and 1.08 log CFU/ml differences respectively, between the cold adapted *V. vulnificus* strain 515-42C counts and the non-cold adapted counts.

Vibrio vulnificus strain 541(O) 49C cold adapted then stored at 5 and 8 °C (Table 13), had no significant differences in viable counts compared to the non-cold adapted counts. At 10°C, the only significant difference occurred at day 3, with the cold adapted *V. vulnificus* strain 541(O)49C having higher counts than the non-cold adapted counts.

Vibrio vulnificus strain 0106-14 cold adapted then stored at 5, 8 and 10°C, had significantly higher viable counts compared to the non-cold adapted counts (Table 14). Significant differences occurred at 5°C for days 3, 5, 7 and 9, at 8°C, for days 1, 3, 5 and 7, and at 10°C, for days 1, 3 and 5. At 5°C, the increase was highest at day 3, with a 1.57 log CFU/ml difference between the cold adapted *V. vulnificus* strain 0106-14 counts and the non-cold adapted counts. At 8°C, the increase was highest at day 3, with a 1.13 log CFU/ml difference, and at 10°C, the increase was also highest at day 3, with a 2.33 log CFU/ml difference between the cold adapted and the non-cold adapted counts.

VIBRIO PARAHAEMOLYTICUS

Some of the *V. parahaemolyticus* strains that were cold adapted prior to storage at 5, 8 or 10°C, showed significant increases in viable plate counts compared to non-cold adapted counts. Other strains however, showed no significant differences between the cold adapted counts and non-cold adapted counts. *V. parahaemolyticus* strain 33847 cold adapted then stored at 5, 8 and 10°C (Table 15), had significantly higher viable counts at days 1 and 3, at both 5 and 8°C as compared to the non-cold adapted counts. At 10°C, the

counts were higher at days 1, 3, 5, 7 and 9. At 5°C, the increase was highest at day 1, with a 0.73 log CFU/ml difference between the cold adapted *V. parahaemolyticus* strain 33847 counts and the non-cold adapted counts, while at 8°C, the highest increase was seen at day 3 with a 1.02 log CFU/ml difference between the cold adapted and the non-cold adapted counts. At 10°C, the increase was highest at day 7, with a 2.73 log CFU/ml difference between the cold adapted and the non-cold adapted *V. parahaemolyticus* strain 33847 counts.

V. parahaemolyticus strain 49529 cold adapted then stored at 10°C, had no significant increases in viable counts compared to non-cold adapted counts (Table 16). However, at 5 and 8°C significant differences were seen at day 1 and days 1, 3 and 5, respectively. At 8°C, the highest increase was at day 5, with a 1.28 log CFU/ml difference between the cold adapted *V. parahaemolyticus* strain 49529 counts as compared to non-cold adapted counts.

V. parahaemolyticus strain TX2103 cold adapted then stored at 5 and 8°C, had significant increases in viable counts compared to non-cold adapted counts (Table 17). At 5°C, significant differences occurred at days 3 and 5, and at 8°C at days 1, 3, 5 and 7. *V. parahaemolyticus* strain TX2103 cold adapted then stored at 10°C had no significant increases in viable counts compared to non-cold adapted counts. At 5°C, the greatest difference occurred at day 5, with a 1.05 log CFU/ml increase in the cold adapted counts compared to the non-cold adapted counts. At 8°C, the highest increase was at day 7 with a 0.46 log CFU/ml difference between the cold adapted and the non-cold adapted counts.

Table 16. Viable counts of *V. parahaemolyticus* strain 49529 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a] 0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	**Cold Adapted**	7.52 ± 0.14 a	7.17 ± 0.06 a	6.33 ± 0.13 a	6.21 ± 0.13 a	6.14 ± 0.11 a	5.53 ± 0.08 a
	Cold Storage	6.60 ± 0.26 a	6.32 ± 0.23 b	5.57 ± 0.06 a	5.48 ± 0.08 a	5.51 ± 0.14 a	4.94 ± 0.41 a
8°C	**Cold Adapted**	7.36 ± 0.08 a	7.19 ± 0.07 a	7.08 ± 0.21 a	7.77 ± 0.12 a	7.90 ± 0.18 a	8.30 ± 0.15 a
	Cold Storage	6.72 ± 0.21 a	6.39 ± 0.06 b	6.15 ± 0.02 b	6.49 ± 0.04 b	7.35 ± 0.74 a	7.59 ± 0.27 a
10°C	**Cold Adapted**	7.37 ± 0.11 a	7.44 ± 0.08 a	8.52 ± 0.08 a	8.48 ± 0.08 a	8.28 ± 0.04 a	8.17 ± 0.11 a
	Cold Storage	6.77 ± 0.20 a	6.81 ± 0.20 a	8.47 ± 0.51 a	8.17 ± 0.09 a	8.05 ± 0.42 a	7.72 ± 0.51 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, and then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, and then stored at 5, 8 or 10°C for 9 days.

Table 17. Viable counts of *V. parahaemolyticus* strain TX 2103 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a] 0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	**Cold Adapted**	6.71 ± 0.19 a	6.54 ± 0.07 a	6.45 ± 0.08 a	6.38 ± 0.07 a	5.52 ± 0.08 a	4.64 ± 0.32 a
	Cold Storage	6.63 ± 0.20 a	6.20 ± 0.48 a	5.88 ± 0.06 b	5.33 ± 0.20 b	5.28 ± 0.21 a	4.48 ± 0.05 a
8°C	**Cold Adapted**	6.78 ± 0.09 a	6.52 ± 0.06 a	7.01 ± 0.35 a	7.22 ± 0.03 a	7.33 ± 0.07 a	7.43 ± 0.08 a
	Cold Storage	6.65 ± 0.28 a	6.19 ± 0.06 b	6.58 ± 0.17 b	7.02 ± 0.06 b	6.87 ± 0.78 b	7.36 ± 0.23 a
10°C	**Cold Adapted**	6.92 ± 0.12 a	7.39 ± 0.15 a	8.06 ± 0.23 a	8.74 ± 0.21 a	8.41 ± 0.15 a	8.02 ± 0.10 a
	Cold Storage	6.80 ± 0.05 a	6.69 ± 0.39 a	7.52 ± 0.04 a	8.26 ± 0.33 a	8.57 ± 0.06 a	7.78 ± 0.45 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37 C, and then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37 C, and then stored at 5, 8 or 10°C for 9 days.

Table 18. Viable counts of *V. parahaemolyticus* strain CT6636 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a]					
		0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	Cold Adapted	6.91 ± 0.21 a	6.79 ± 0.21 a	6.70 ± 0.16 a	6.21 ± 0.21 a	6.18 ± 0.13 a	5.46 ± 0.07 a
	Cold Storage	6.59 ± 0.18 a	6.40 ± 0.33 a	6.48 ± 0.76 a	5.82 ± 0.27 a	5.71 ± 0.14 a	5.21 ± 0.29 a
8°C	Cold Adapted	6.90 ± 0.13 a	6.95 ± 0.08 a	7.94 ± 0.16 a	8.41 ± 0.12 a	7.62 ± 0.31 a	7.09 ± 0.19 a
	Cold Storage	6.67 ± 0.14 a	6.60 ± 0.01 b	7.71 ± 0.01 a	7.94 ± 0.81 a	6.23 ± 0.35 a	5.90 ± 0.40 a
10°C	Cold Adapted	7.41 ± 0.03 a	8.11 ± 0.08 a	8.49 ± 0.08 a	8.56 ± 0.10 a	8.65 ± 0.23 a	8.16 ± 0.13 a
	Cold Storage	6.75 ± 0.23 a	7.05 ± 0.31 b	8.68 ± 0.28 a	8.56 ± 0.04 a	8.53 ± 0.00 a	7.76 ± 0.18 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, and then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days.

Table 19. Viable counts of *V. parahaemolyticus* strain 8332924 after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a]					
		0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	Cold Adapted	7.20 ± 0.15 a	7.19 ± 0.04 a	6.54 ± 0.08 a	6.38 ± 0.09 a	5.85 ± 0.16 a	5.36 ± 0.06 a
	Cold Storage	6.63 ± 0.08 b	6.60 ± 0.17 b	6.22 ± 0.03 b	5.95 ± 0.20 a	5.42 ± 0.17 a	4.91 ± 0.35 a
8°C	Cold Adapted	7.23 ± 0.23 a	7.23 ± 0.13 a	7.65 ± 0.12 a	8.02 ± 0.13 a	8.23 ± 0.18 a	7.64 ± 0.04 a
	Cold Storage	6.62 ± 0.34 a	6.61 ± 0.10 b	7.96 ± 0.67 a	8.33 ± 0.46 a	8.09 ± 0.50 a	8.00 ± 0.69 a
10°C	Cold Adapted	7.24 ± 0.16 a	7.86 ± 0.10 a	8.65 ± 0.12 a	8.47 ± 0.07 a	8.37 ± 0.07 a	8.26 ± 0.10 a
	Cold Storage	6.49 ± 0.08 a	7.14 ± 0.21 b	8.79 ± 0.19a	8.63 ± 0.29 a	8.31 ± 0.00 a	8.13 ± 0.02 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, and then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days.

Table 20 . Viable counts of *V. parahaemolyticus* strain NY477 after cold adaptation at 15°C compared to cold Storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a] 0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	**Cold Adapted**	6.96 ± 0.28 a	6.96 ± 0.26 a	7.06 ± 0.25 a	6.13 ± 0.11 a	5.93 ± 0.18 a	5.86 ± 0.19 a
	Cold Storage	6.89 ± 0.37 a	6.77 ± 0.01 a	6.48 ± 0.02 a	5.90 ± 0.26 a	5.39 ± 0.05 a	5.10 ± 0.40 a
8°C	**Cold Adapted**	7.00 ± 0.16 a	7.10 ± 0.09 a	7.58 ± 0.13 a	7.94 ± 0.11 a	8.15 ± 0.23 a	8.26 ± 0.18 a
	Cold Storage	6.77 ± 0.28 a	6.80 ± 0.01 b	7.32 ± 0.30 a	7.93 ± 0.84 a	7.82 ± 0.39 a	8.01 ± 0.33 a
10°C	**Cold Adapted**	7.14 ± 0.13 a	7.64 ± 0.11 a	8.54 ± 0.07 a	8.45 ± 0.05 a	8.42 ± 0.04 a	8.27 ± 0.03 a
	Cold Storage	6.94 ± 0.25 a	7.03 ± 0.08 b	8.40 ± 0.28 a	8.55 ± 0.04 a	8.30 ± 0.04 a	8.36 ± 0.13 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, then stored at 5, 8 or 10 C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days.

Table 21. Viable counts of *V. parahaemolyticus* strain M350A after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a] 0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	**Cold Adapted**	6.66 ± 0.18 a	6.21 ± 0.11 a	6.22 ± 0.04 a	5.81 ± 0.28 a	5.40 ± 0.08 a	4.87 ± 0.10 a
	Cold Storage	6.68 ± 0.20 a	6.31 ± 0.22 a	6.17 ± 0.44 a	5.67 ± 0.43 a	5.22 ± 0.52 a	4.46 ± 0.19 a
8°C	**Cold Adapted**	6.85 ± 0.09 a	6.30 ± 0.06 a	7.30 ± 0.13 a	7.95 ± 0.10 a	8.25 ± 0.17 a	8.10 ± 0.14 a
	Cold Storage	6.62 ± 0.33 a	6.27 ± 0.02 a	7.70 ± 0.23 a	8.14 ± 0.49 a	8.02 ± 0.09 a	7.87 ± 0.08 a
10°C	**Cold Adapted**	6.92 ± 0.12 a	7.25 ± 0.08 a	8.30 ± 0.13 a	8.35 ± 0.11 a	8.29 ± 0.09 a	8.18 ± 0.16 a
	Cold Storage	6.69 ± 0.32 a	6.93 ± 0.20 a	8.67 ± 0.10 a	8.54 ± 0.18 a	8.15 ± 0.09 a	8.04 ± 0.33 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, and then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37°C, then stored at 5, 8 or 10°C for 9 days.

Table 22. Viable counts of *V. parahaemolyticus* strain 541(0)57C after cold adaptation at 15°C compared to cold storage at different temperatures over 9 days.

Temperature	[b]Treatment	log CFU/ml[a] 0 Day	1 Day	3 Day	5 Day	7 Day	9 Day
5°C	**Cold Adapted**	6.82 ± 0.17 a	6.71 ± 0.14 a	6.20 ± 0.16 a	5.80 ± 0.18 a	5.59 ± 0.03 a	5.20 ± 0.10 a
	Cold Storage	6.68 ± 0.16 a	6.36 ± 0.15 a	5.81 ± 0.02 a	5.51 ± 0.02 a	5.36 ± 0.28 a	5.02 ± 0.13 a
8°C	**Cold Adapted**	7.25 ± 0.10 a	6.88 ± 0.23 a	6.72 ± 0.16 a	5.94 ± 0.16 a	5.78 ± 0.06 a	5.35 ± 0.04 a
	Cold Storage	6.96 ± 0.38 a	6.45 ± 0.06 a	6.68 ± 0.11 a	5.61 ± 0.00 a	5.40 ± 0.34 a	4.96 ± 0.14 a
10°C	**Cold Adapted**	6.99 ± 0.15 a	7.35 ± 0.13 a	8.17 ± 0.11 a	8.19 ± 0.11 a	7.73 ± 0.16 a	7.43 ± 0.12 a
	Cold Storage	6.58 ± 0.16 a	6.96 ± 0.06 a	8.00 ± 0.01 a	7.72 ± 0.41 a	7.68 ± 0.10 a	7.33 ± 0.28 a

[a] All analyses were based on two separate experiments with each mean ± standard deviation being the average of two determinations. Means for each sampling day (vertical columns) followed by different letters, were significantly different from each other ($P \leq 0.05$). Analysis for significance was carried out using Student's t test following one-way analysis of variance (ANOVA) of JMP-IN (version 4.0.03, SAS Inst. Inc., Cary, North Carolina, USA).

[b] Cold Adapted cultures were exposed to 15°C for 4 hours following incubation at 37°C, and then stored at 5, 8 or 10°C for 9 days. Cold Storage cultures were inoculated into 5°C broth following incubation at 37 C, then stored at 5, 8 or 10°C for 9 days.

V. parahaemolyticus strain CT6636 (Table 18) and strain NY477 (Table 20) cold adapted then stored at 8 and 10°C, had significant increases in viable counts compared to non-cold adapted counts, only at day 1. At 5°C, cold adapted *V. parahaemolyticus* strains CT6636 and NY477 had no increases in viable counts compared to the non-cold adapted counts.

Vibrio parahaemolyticus strain 8332924 cold adapted prior to storage at 5, 8 and 10°C (Table 19) had significant increases in viable counts compared to non-cold adapted cultures. At 5°C, significant differences occurred at days 1 and 3, while at 8 and 10°C, significant differences occurred at day 1.

Vibrio parahaemolyticus strain M350A (Table 21) and strain 541(0)57C (Table 22) cold adapted and stored at 5, 8 and 10°C, had no significant increases in viable counts compared to non-cold adapted counts at any of the temperatures tested.

DISCUSSION

Vibrio vulnificus strains shifted from 37 to 15°C prior to storage at 5 or 8°C (cold adapted) had better survival rates than cultures shifted directly to 5 or 8°C without prior exposure to an intermediate temperature. Strains stored at 10°C following exposure to 15°C had better growth rates than strains shifted directly to 10°C. The duration of the response however differed between the various strains, with some strains having increased survival rates throughout the duration of the experiment at some of the temperatures tested, while others had significant increases in growth or survival rates for shorter periods of time.

Bryan et al.(1999) showed that when a *V. vulnificus* culture incubated at 35°C was exposed to 15°C for 3 hours prior to storage at 6°C, this culture had enhanced

survival over the 6 days of the experiment, compared to the non-cold adapted culture that was shifted from 35°C directly to 6°C. Bang and Drake (2002) also showed that *V. vulnificus* cultures that were cold adapted by holding them at 15° C for 4 h had more tolerance to cold temperatures (5°C). The study also found that the response differed among strains, with the response being more enhanced in one of three strains tested. In our study we found that cold adapted *V. vulnificus* strains had an enhanced ability to survive at a lower temperature. The duration of the response however varied at different temperatures for the various strains. Cold adapted *V. vulnificus* strain 33816 had enhanced survival throughout the duration of the experiment at both 5 and 8°C as compared to the non-cold adapted strain. At 10°C however, cold adapted *V. vulnificus* strain 33816 had enhanced survival only at day 1 compared to the non-cold adapted strain. Cold adapted *V. vulnificus* strain 33815 had an enhanced survival at days 3, 5, 7 and 9 compared to the non-cold adapted strain, at all temperatures tested, while cold adapted strain 541(O)49C did not show significant enhanced survival at 5 or 8°C compared to the non-cold adapted strain.

Lin et al. (2003) showed that when a *V. parahaemolyticus* strain incubated at 37°C was subjected to cold shock at 20 or 15°C prior to storage at 5°C for 6 days, the cold adapted strain had a better survival percentage than the control which was downshifted from 37 to 5°C, without exposure to an intermediate temperature. In our study we found that, six of the eight *V. parahaemolyticus* cultures that were shifted from 37°C to 15°C prior to storage at 5, 8 or 10°C exhibited better survival rates than cultures shifted directly from 37°C to 5, 8 or 10°C. The response varied between strains, but in many cases was short-lived, often only lasting between 1 to 3 days. Overall, the cold adaptation response

appeared to be more enhanced in *V. vulnificus* strains than they were in *V. parahaemolyticus* strains. It is possible that *V. parahaemolyticus* strains downshifted from 37°C directly to 5, 8 or 10°C, were able to adapt, hence the minimal differences in survival rates seen between these strains and the cold adapted strains.

Microorganisms have been shown to adapt when exposed to cold temperatures, as a survival response. Cold shock reduces the efficiency of translation, transcription and DNA replication, which are overcome by the induction of cold-shock proteins (Phadtare et al. 1999). McGovern and Oliver (1995) showed that when *V. vulnificus* cultures were downshifted from 42 to 10°C, this resulted in the production of forty proteins being synthesized at higher levels during the cold stress response, with one designated 1005 of molecular weigh 16.4 kDa being increased by a factor of 35 within an hour of the downshift in temperature. This protein was absent when the culture was held at room temperature. In our study, differences in the cold adaptation responses seen between the various *V. vulnificus* and some *V. parahaemolyticus* strains, suggest that the ability to produce cold shock proteins following a downshift in temperature may vary between strains, ultimately influencing the ability of the organism to survive under adverse conditions.

More research is needed to obtain a clearer understanding as to whether the variations in the cold adaptation response seen between different *V. vulnificus* and *V. parahaemolyticus* strains are due to the production of different cold shock proteins following a downshift in temperature, or due to the production of the same proteins but at varying levels.

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CHAPTER 5

CONCLUSION

Results obtained from this study have shown *V. vulnificus* and *V. parahaemolyticus* strains grown in tryptic soy broth exhibit significant differences in growth and survival when stored at 5, 8 and 10°C. The *V. vulnificus* strains were able to survive but not grow when shifted from 37°C to storage at 5 and 8°C, while most *V. vulnificus* strains were able to growth at 10°C. The *V. parahaemolyticus* strains survived but did not grow when shifted from 37°C to storage at 5°C. At 8 and 10°C however, *V. parahaemolyticus* strains were able to grow. When these strains were adapted to an intermediate temperature of 15°C for 4 hours, this resulted in an enhanced survival of *V. vulnificus* strains. This adaptation response however varied between strains. Not all of the *V. parahaemolyticus* strains showed an enhanced survival when exposed to an intermediate temperature of 15°C. The cold adaptation response was more sustained for the *V. vulnificus* strains at some temperatures tested, while for the *V. parahaemolyticus* strains exhibiting a response, this response was generally short lived, usually lasting only 1 to 3 days.

VITA

The author was born in Silver Spring, Maryland. She resided in Georgetown, Guyana, where she received her high school education. She later attended Tuskegee University where she received a Doctor of Veterinary Medicine degree in May 1989. She practiced as a veterinarian for fourteen years in Georgetown, Guyana. In August 2004 she started pursuing a Master of Science degree in food science at Louisiana State University.