IN VITRO AND *IN VIVO* COLD SHOCK RESPONSE IN *VIBRIO VULNIFICUS*

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IN VITRO AND *IN VIVO* COLD SHOCK RESPONSE IN *VIBRIO VULNIFICUS*

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IN VITRO AND *IN VIVO* COLD SHOCK RESPONSE IN *VIBRIO VULNIFICUS*

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Suttinee Limthammahisorn, daughter of Pravit and Pranee Sukaphatana, was born on December 19, 1964, in Chonburi, Thailand. She graduated from Assumption College Sriracha High School, Chonburi, Thailand in 1983. She attended Kasetsart University, Thailand and graduated with a Bachelor of Science degree majoring in Fisheries in 1988. She obtained a Master of Science degree in Environmental Technology Management from Mahidol University, Thailand in 1997. She entered Graduate school in the Department of Fisheries and Allied Aquacultures at Auburn University in August 2003. She is a senior fishery biologist in Department of Fisheries, Thailand. She married Somkiat Limthammahisorn on December 31, 1990. They have one son, Meen (Max) Limthammahisorn.

DISSERTATION ABSTRACT

IN VITRO AND *IN VIVO* COLD SHOCK RESPONSE IN *VIBRIO VULNIFICUS*

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Vibrio vulnificus is a serious food-borne pathogen associated with the consumption of raw oysters. The Interstate Shellfish Sanitation Conference (ISSC) recommends refrigeration of Gulf Coast oysters after harvesting to minimize pathogen risks. Cooling temperature control of harvested oysters could potentially induce cold shock response in *V. vulnificus*. In order to determine the potential for cold shock mediated survival of *V. vulnificus*, my research investigated *in vitro* and *in vivo* cold shock response in *V. vulnificus* regarding its growth and the molecular mechanisms underlying this phenomenon.

The effect of cold shock on *V. vulnificus* cultures was investigated under ISSC guidelines by the drop plate method. *V. vulnificus* cultures were incubated at 35, 25, 20, and 15°C for 10 h, 12 h, 14 h, and 36 h, respectively, and then abruptly shifted to 7.2°C and 4°C for 168 h. The mean counts of *V. vulnificus* after 7 day refrigeration at 7.2°C

was significantly greater ($P<0.05$) than at 4°C in all treatments. Cold adaptation response enhanced cell culturability and was observed when cells were adapted to 15°C prior to cold shock at 7.2°C. When *V. vulnificus* was artificially inoculated into oysters, its growth during cold shock was investigated using colony hybridization. After oysters were kept at 35°C and 25°C for 10 h and 12 h, respectively, and abruptly shifted to 4°C, cells exhibited a moderate decline in culturability. Oysters kept at 15°C prior to a 4°C shift for 168h, showed a slight decline in *V. vulnificus* cell numbers. These findings suggested that incubation of *V. vulnificus* at 15°C could induce cold adaptation and should be avoided during post-harvest conditions.

Four cold shock genes (csp_s) encoding small cold shock proteins in *V. vulnificus* were cloned. Analysis of *in vitro* and *in vivo* cold shock gene expression was displayed by reverse transcriptase polymerase chain reaction (RT-PCR). All csp_s tested in this study can not be considered cold shock inducible genes under *in vitro* condition, while some csp_s showed weak induction after cold shock under *in vivo* experiments. Besides, the oxidative related genes (*oxyR* and *katG*) were induced during *in vitro* study, while these transcripts were not detected during *in vivo* experiment. The transcript levels of *16SrRNA* and *rpoS* were constant throughout the study. Overall, these results demonstrated that gene expression of *V. vulnificus* was different between *in vitro* and *in vivo* conditions during cold shock.

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I. INTRODUCTION

Vibrio vulnificus is a Gram-negative, ubiquitous, estuarine bacterium that is abundant in water, sediments, mollusks, crustaceans, and finfish along the U.S. Gulf Coast (38, 170). *Vibrio vulnificus* has been blamed for the reduced prices and consumer demand for oysters (*Crassostrea virginica*), especially Gulf Coast oysters, because of negative publicity associated with health risks. Oysters have served as vectors in the transfer of this indigenous estuarine pathogen to humans (38). Every year millions of Americans consume raw oysters. For some people, ingestion of raw or undercooked oysters containing this bacterium can cause serious illness and even death. This pathogen may cause severe wound infection and primary septicemia (99). Nearly all fatal primary septicemia cases are associated with the consumption of raw oysters. The United States Food and Drug Administration (USFDA), the Centers for Disease Control and Prevention (CDC), the Interstate Shellfish Sanitation Conference (ISSC), and the oyster industry faced with a major challenge in managing public health risk of *V. vulnificus*. In 2001, the ISSC proposed the "*Vibrio vulnificus* Risk Management Plan", and set a goal for reduction of illnesses caused by the consumption of commercially harvested raw or undercooked oysters by at least 60% by the year 2007 and 2008 (44). The ISSC recommended that post-harvest treated consumable oysters must contain less than 30 colony forming units of *V. vulnificus* per gram of oyster meat (45). Typically, the concentration of *V. vulnificus* at consumption is influenced by ambient air temperature during harvest, the time from harvest until the oysters are placed under refrigeration, the time it takes the oyster to cool down under refrigeration, and the length of refrigeration time until consumption (183). Potential infection by this bacterium could be reduced by limiting time from harvest to refrigeration in at-risk-harvesting areas and by using proper

refrigeration temperatures. In one effort to achieve the ISSC goal, restrictive shellstock temperature control measures have been recommended by FDA/ISSC and implemented by the industry. This control limits the time from harvesting of oysters to refrigeration. The length of time depends upon the average monthly maximum water temperature at the time of harvest. Once placed under temperature control, shellstock shall be maintained at 10°C or below throughout processing, distribution and storage (45).

However, many bacteria, especially food-borne pathogens have been reported to manifest their tolerance to cold temperatures by altering their cellular protein profiles and continuing to grow (6, 36, 55, 75, 90). This physiological response of living cells to temperature downshift is referred to as cold shock response (52). The cooling strategy recommended by ISSC may potentially cause a cold shock response in *V. vulnificus* inhabitating oysters. To date, cold shock response in *V. vulnificus* has not been fully elucidated although this pathogen greatly impacts oyster industry and public health. The impact of cold shock response on the physiology of this pathogenic microorganism in oysters under typical industry practices from harvest to consumption, particularly in regard to their survival, needs to be further investigated.

Although a wide array of experimental data addressed the role of cold shock response in many bacteria, no clear indication on the molecular mechanisms underlying this phenomenon has been proposed in *V. vulnificus* so far. Only two reports have investigated a *V. vulnificus* response mechanism to cold temperature. In 1995, McGovern and Oliver (110) showed that when *V. vulnificus* is subjected to a moderate temperature downshift from 23 to 13°C, protein synthesis declines but new cold-induced proteins are expressed. In 1999, Bryan et al. (17) reported that a brief cold adaptation

period for 3 h at 15°C enhanced survival of *V. vulnificus* at 6°C. They concluded that cold adaptive protective proteins might enhance survival and tolerance at cold temperature. Moreover, cold shock has been shown to alter gene expression in bacterial cells in order to compensate for this cold stress. Little is known about *V. vulnificus* gene expression in response to cold shock. While several reports have investigated *in vitro* and *in situ* gene expression of *V. vulnificus* during temperature downshift in the "viable but non-culturable" (VBNC) state (46, 160, 161), *in vivo* gene expression of this pathogen has not yet been reported in response to the cold or any other situation. Since oysters can act as reservoirs for *V. vulnificus,* it is crucial to study the cold shock gene expression while this pathogen is embedded in oysters. This scientific knowledge from *in vitro* and *in vivo* studies of cold shock response of *V. vulnificus* under USFDA/ISSC shellstock temperature control guidelines will assist in the development of an effective risk reduction strategy for *V. vulnificus* septicemia associated with the consumption of raw oysters.

II. LITERATURE REVIEW

Overview

Raw oysters have the potential to cause human disease because they serve as vectors for viral, bacterial and parasitic pathogens (156). The greatest numbers of seafood-associated illnesses are from the consumption of contaminated raw shellfish (such as mussels, oysters, clams) as a result of their filter feeding habit that concentrate particles present in the surrounding waters. Two general groups of pathogenic bacteria may be present in coastal sea water and may be entrapped by shellfish: the first group consists of nonindigenous bacterial pathogens that enter the water from animals and humans, the latter includes indigenous bacteria in the marine environment, mainly members of the family *Vibrionaceae* (148). Typically, bacterial pathogens associated with fecal contamination have represented only 4% of the shellfish associated outbreaks, while naturally-occurring bacteria accounted for 20% of shellfish-associated illnesses and 99% of the deaths. Most of these indigenous bacteria fall into the family *Vibrionaceae* which includes the genus *Vibrio* (101). The *Vibrio* genus comprises more than 30 species, some of them being pathogenic to human and had been associated with food-borne related diseases (22).

1. *Vibrio vulnificus*

Vibrio vulnificus is recognized as the most invasive and rapidly lethal *Vibrio* and is the leading cause of reported seafood-related deaths in the United States (11). It accounts for 95% of seafood related deaths in the U.S. It is an opportunistic human pathogen that can cause gastroenteritis, septicemia, and wound infections in humans. Typically, *V. vulnificus* infections are associated with the consumption of raw oysters and can produce rapidly fatal septicemia in individuals (151). In humans, *V. vulnificus* is associated with two disease patterns, primary septicemia and wound infection. The major pattern of infection is a rapidly progressing septicemia. Septicemia caused by this pathogen occur in individuals with chronic liver disease shortly after eating raw oyster to several days of infection, with a fatality rate of over 60%. In immunocompromised hosts, *V. vulnificus* infection can cause high fever, nausea, myalgia, and abdominal cramps 24 to 48 hours after eating contaminated food. Because the organism can cross the intestinal mucosa rapidly, sepsis and cutaneous bullae can occur within 36 hours of the initial onset of symptoms. Cases are most commonly reported during warm weather months (April-November) and often are associated with eating raw oysters. Progression of the illness can be very rapid from asymptomatic to death within 24 hours.

About 75% of patients with *Vibrio* septicemia have pre-existing hepatic diseases. Wound infections with *V. vulnificus* usually develop after trauma and exposure to the marine environment by handling seafood products and progress rapidly. Wound infections most commonly present cellulites, and the fatality rate can be up to 25%. About one-third of patients with wound infections have some underlying disease.

However, infections that occur through wounds are not limited to immunocompromised individuals. The etiological role of *V. vulnificus* in gastroenteritis has not been proven. An indole-negative biogroup of this Vibrio, which is pathogenic to eels, has also been associated with human infections (99).

Characteristics of *Vibrio vulnificus*

Vibrio vulnificus is a Gram-negative, oxidase-positive, obligately halophilic, motile, curved bacillus with a single polar flagellum. It belongs to the family *Vibrionaceae*, and is distinguished from other members of the *Vibrio* genus by its ability to ferment lactose. *Vibrio vulnificus* can grow in NaCl with concentration as high as 5% and does not require Mg^{+2} or K^{+} for its growth. Consequently, it can easily be cultured in a variety of media. *Vibrio vulnificus* was first isolated in 1964 by the U.S. Centers for Disease control (CDC), although at that time it was mis-identified as a virulent strain of *V. parahaemolyticus* (165). It was finally recognized as a distinct species in 1976 after researchers realized that many clinical cases of foodborne septicemias and wound infections were caused by a pathogen with unique characteristics from other *Vibrio* species. The name " vibrio" meaning "to quiver", and the word "vulnificus" is derived from Latin word "vulnus" meaning "wound" and was given its official taxonomic status in 1980 (116).

Vibrio vulnificus has been classified based on biotypes, lipopolysaccharide (LPS) antigens, capsule, and most recently genetic sequences. There are three biotypes of *V. vulnificus* according to their different biochemical and biological properties. Biotype 1

is ubiquitous in estuarine environments, is an opportunistic human pathogen, produces indole and ornithine decarboxylase, and exhibits several immunologically distinct lipopolysaccharide (LPS) types (66, 100). Biotype 2 is primarily related with eels but can be pathogenic to humans, is characterized by a homogeneous lipopolysaccharide (LPS), and is negative for indole production and ornithine decarboxylase (3, 10). A third group, biotype 3, was recently identified and causes wound infections and bacteremia in humans handling St. Peter's fish (*Tilapia* spp.) in Israel (12).

Geographical range distribution and environmental conditions

The presence of *V. vulnificus* is not associated with pollution. It is normally found in estuarine environments, flourishes in warm temperatures. It is part of the bacterial flora of the marine environment, and is distributed worldwide (175). *Vibrio vulnificus* has been isolated from waters of widely varying temperatures and salinities (187). Several studies along the Gulf of Mexico and the east and west coasts of the United States have shown strong relationships between salinity, water temperature, and the isolation of *V. vulnificus* (38, 125, 133, 134, 169). Prevalence of *V. vulnificus* in the marine ecosystem is correlated with seasonality since water temperature plays a significant role in controlling the densities of this bacterium. *Vibrio vulnificus* has been recovered from waters with a range of temperatures from 9°C to 31°C, but has seldom been recovered from cold waters and has frequently been found in high concentrations in shellfish and surface water during the summer, April to September, when water temperatures were above 25°C. In fact, *V. vulnificus* optimal temperature growth is

35°C, and like all human pathogens can also grow at 37°C. However, this bacterium has never been responsible for human infections in that area where summer temperatures are optimal for its growth (117). Under suboptimal temperatures the number of *V. vulnificus* increases during 6 days incubation at 13°C to 22°C, but *V .vulnificus* survives poorly at less than 8.5°C (85). Pfeffer et al. (145) reported that water temperature accounted for most of the variability in the concentration of *V .vulnificus*, and that low water temperatures (<10°C) have a negative effect for its isolation. There have been several theories to explain the seasonality observed in *V. vulnificus* isolation. One possible reason that might explain why *V. vulnificus* is difficult to culture during cold-water months is that this bacterium can enter a viable but nonculturable state known as VBNC. It has been proven that *V. vulnificus* enters a VBNC state at temperatures below 10°C under low nutrient conditions (126).

Typically, U.S. Gulf Coast oysters harbor about 10^3 to 10^4 *V. vulnificus* cells g^{-1} during the warmer months from April through October but typically less than 10 cells g^{-1} during colder months (32, 171). Illnesses caused by *V. vulnificus* have been reported every year, primarily along the Gulf of Mexico in the U.S. during the summer months. In general, the relationship between salinity and *V. vulnificus* presence is not as evident as between temperature and prevalence. It has been isolated from waters with a wide range of salinities from 1 to 34 parts per thousand (ppt) although estuarine waters with salinities between 6 and 16 ppt are considered optimal for *V. vulnificus* populations. Salinities greater than 25 ppt have been shown to adversely affect survival of this bacterium under laboratory conditions. However, *V. vulnificus* has been isolated from Mediterranean waters and shellfish with salinities around 35 ppt.

Isolation and identification methods for *Vibrio vulnificus*

Several isolation and identification techniques have been developed to distinguish *V. vulnificus* from other vibrios. Thiosulfate citrate bile salts sucrose (TCBS) agar was originally used for isolation of pathogenic vibrios and became widely used for the isolation of *Vibrio* spp. from environmental samples. This medium differentiates *V. cholerae* from *V. parahaemolyticus* and *V. vulnificus* on the basis of sucrose fermentation (85, 153). Sodium dodoecyl sulfate-polymyxin B-sucrose agar (SPS) was used to separate *V. vulnificus* from *V. parahaemolyticus*, which are both negative for sucrose fermentation and had similar morphology on TCBS (92). Many selective and differential media have been developed for *V. vulnificus* isolation including *V. vulnificus* (VV) agar (15), cellobiose-polymyxin B colistin (CPC) agar (107), modified CPC (mCPC) agar (171), *V. vulnificus* enumeration (VVE) agar (112), *V. vulnificus* agar (VVA) (188), cellobiose-colistin (CC) agar (65), *V. vulnificus* medium (VVM) (20), modified VVM (VVMc) agar (21). Confirmation assays for *V. vulnificus* include biochemical or physiological tests, immunological assays, DNA probes, and PCR analysis (60). For *V. vulnificus* enumeration in oysters, USFDA recommended two approaches. One uses a most probable number (MPN) enrichment step coupled with biochemical or molecular confirmation. The second performs a direct isolation on selective media followed by colony hybridization with a DNA probe (VVAP) (82). The VVAP probe method has been widely used for molecular confirmation of *V. vulnificus* (21, 65). A quantitative PCR or real-time PCR assay is now available for *V. vulnificus* detection (18, 138). However, this method does not discriminate live from dead cells.

Reverse transcription PCR is an alternative method that can be used to detect RNA from viable but nonculturable (VBNC) cells of *V. vulnificus* since only live cells yield a positive result (46).

Methods by reducing *Vibrio vulnificus* in oysters

In order to reduce the number of unwanted *V. vulnificus* cells to acceptable levels for human consumption, several studies have been investigating methods that will eliminate this pathogen. One of the most common methods in controlling pathogen proliferation in oysters is cold temperatures (30, 31, 129, 170). However these studies have shown that cold storage cannot eliminate this bacterium to safe levels for consumption. Thoroughly cooking the oysters then storing them under either hot (60°C or higher) or cold (4°C or lower) conditions completely eliminates the bacteria, but this methodology is unfit for the half-shell market (64). Ionized or gamma irradiation (69, 73) is effective at eliminating *Vibrio* but this process requires an expensive facility and is not yet federally approved. The use of additives such as organic acids (Generally Recognized as Safe, GRAS) (166, 167), butylated hydroxyanisole (BHA), or diacetyl (11), although effective in reducing *Vibrio* contamination, produce unacceptable flavor changes. The use of hot sauce (168) was not successful in reducing numbers of this bacterium. Depuration has been proved to be effective in removing indicator bacteria but it is ineffective in reducing the numbers of this pathogen. In addition, some bacteria may persist and even multiply during depuration (86, 170). Exposing oysters to high-salinity (30 to 34 ppt) environments seemed an effective method to eliminate *V. vulnificus* in

oysters; however, this method requires a period of time of more than one month (117). Rapid chilling by ice immersion at 7.2°C (149) has been shown to reduce *V. vulnificus*, but not to recommended limits. Recently, electrolyzed oxidizing water (150) was found to be effective in eliminating this bacteria in oysters, but this method directly increased the mortality of oysters.

To date, most efforts to reduce *Vibrio* contamination in live oysters have not been successful. Post-harvest treatments have been recommended in order to reduce *V. vulnificus* numbers in oysters. Currently, several effective post-harvest treatments have been used commercially and approved by the Food and Drug Administration. These methods include high hydrostatic pressure (9), low-temperature pasteurization (5), and individual quick freezing (63). These treatments; however, required the use of specialized equipment, which increases the cost of the post harvest process.

2. The United States Oyster industry

Oyster industry profile

Two major species of oysters are produced in the U.S.: *Crassostrea virginica* (Atlantic oyster or Eastern oyster) and the non-native *Crassostrea gigas* (Pacific oyster). The amount of eastern oysters accounts for roughly 75 percent of total U.S. harvests. The U.S. annual yields of eastern oysters were 10,112 metric tons in year 2005 (121). Oysters are typically produced in three different ways: natural, managed and cultivated. Natural oysters grow and reproduce without human intervention and are available for harvest by

anyone with the appropriate licenses and permits. In contrast, managed natural oysters are supervised by harvesters who scrape the oyster bed periodically to reduce clustering. In the case of cultivated oysters, immature oysters are transported to man-made beds where they are allowed to mature. In the Northwest and Northeast of the U.S., a significant amount of shellstock oysters are produced on cultivated beds, whereas in the Gulf waters historically, they have been primarily harvested from wild reefs (119).

All oyster dealers in the U.S. must be certified under the National Shellfish Sanitation Program (NSSP) in order to market oyster products in interstate commerce. Processing plants that ship oysters are certified as interstate or intrastate shippers. Interstate certified shippers may ship oysters across state lines, whereas intrastate certified shippers may ship oysters only within their state borders. Interstate shippers are inspected and certified by individual states, which later provide the list to the USFDA. The USFDA uses this information to publish the Interstate Certified Shellfish Shippers List. Intrastate shippers are also inspected and certified by individual states, but those states manage their own lists. State agencies involved in certification include the departments of health, marine resources, agriculture, national resources, and wildlife and fisheries (119). The FDA carefully monitors oysters from their growing waters through processing plants, and finally to retail outlets. The NSSP maintains a Manual of Operations, which is routinely revised by the Interstate Shellfish Sanitation Conference (ISSC), a voluntary group composed of members from FDA, state health regulatory agencies and industry (105).

Generally, there are four main sectors in the oyster industry; harvesters, wholesalers, processors, and retailers. Oyster harvesting includes wild and cultivated

oysters. Harvested oysters are generally delivered to wholesalers and processors, and in some cases, they are directly delivered to restaurants or other retail outlets. They are generally sold in dozens, by the bushel bag, or in bushel-fraction boxes (105). Processors normally sell their products as fresh, raw, shucked, processed halfshell, smoked, cooked, canned, and breaded oysters. Oysters generally reach consumers as live in the shell, fresh, frozen, or as canned products. Shucked oyster meats are graded and sold according to size in 8 ounce (226 g) and 12 ounce (342 g) cup, or in pint (0.55L), quart (1.1 L), or gallon (3.78 L) containers (144). Oyster harvesting is done by handpicking, tonging, and dredging from oyster boats. Oysters are harvested throughout the year; but the meat yield differs between seasons (104).

United States per capita oyster consumption was 0.11 kg in the year 2003. Consumers judge the quality of the raw half-shell oysters based on organoleptic characteristics including appearance (size, shape, color), odor, flavor (sweetness and saltiness), and texture (firmness). Consumers prefer cup-shaped oysters where the meat fills the shell. Color is less important to consumers, but unpleasant odors are regarded as an indication of spoilage. The public prefers fresh oysters that are mild and salty in flavor, have no off-flavor, and are tender (not mushy) in texture (26, 105). Individuals generally consume cooked oysters at home and both raw and cooked oysters in restaurants. Demand for in-shell oysters is assumed to be high during the summer travel and vacation season; whereas demand for shucked oysters peaks during the winter holiday season (4).

The U.S. imported approximately 10.48 million kilograms of farmed and wild oysters during 2004. The value of imports during that period was $ 47 million. South

Korea is the major exporter of oysters to the U.S., accounting for 53.6% of total U.S. oyster imports in 2006. The U.S. imports oysters in live, fresh, frozen, dried, salted, brine, canned smoked and canned forms. On the other hand, in 2004, the U.S. exported approximately 3.4 million kilograms of oysters overseas. France was the major importer of oysters accounting for 24 percent of total U.S. exports (105, 122).

Current regulation strategies for post-harvest oysters

In the U.S., the Interstate Shellfish Sanitation Conference (ISSC), a voluntary, cooperative association of states, U.S. Food and Drug Administration (FDA), National Marine Fisheries Service (NMFS), Environmental Protection Agency (EPA), and shellfish industry, adopted guidance to be incorporated in the National Shellfish Sanitation Program (NSSP) that requires states, which produce shellfish reported to harbor *V. vulnificus*, to develop "*Vibrio vulnificus* Risk Management Plans". The goal of this strategy is to reduce the rate of etiologically confirmed shellfish-borne *V. vulnificus* septicemia illnesses from the consumption of commercially harvested raw or undercooked oysters by at least 60 percent at the end of year 2008 (44). The core reporting states include Florida, Texas, Louisiana, and California (the biggest oyster importer). Additionally, in order to minimize the risk from this pathogen, ISSC adopted a " NSSP Guide for the Control of Molluscan Shellfish 2005". Under the section of "Control of shellfish harvesting" the time from harvesting to refrigeration is limited. The length of time depends upon the average monthly maximum water temperature (AMMWT) at the time of harvest. These controls set the maximum time interval from

harvest to refrigeration at 36 h when AMMWT in the harvest area is less than 18°C, 14 h when AMMWT is 18°C to 23°C, 12 h when AMMWT is 23°C to 28°C, and 10 h when AMMWT exceeds 28°C. Once placed under temperature control, shellstock shall be continuously maintained at 10°C or below until final sale to the consumer. This guideline suggests that consumable oysters should not contain more than 30 MPN of *V. vulnificus* per gram of oyster meat (45).

Currently, the USFDA and ISSC have approved the use of post-harvest treatments which was implemented by 2006 (Interstate Shellfish Sanitation Commission Model Ordinance 03-121) (60).

3. Cold shock response in bacteria

The cold shock response is a physiological response of living cells to temperature downshift. In terms of thermal stress, cold shock stress (temperature down shift) is poorly understood compared with heat shock. Most of the cold shock response studies have been performed with *Escherichia coli* and *Bacillus subtilis* as model system (51).

Microbial response to low temperature involves changes in cellular physiology that have been documented as survival strategy in other species. Several cold shock proteins have been identified not only in *E. coli* but also in many other bacteria, including mesophilic, psychrophilic, and psychrotrophic bacteria such as *Bacillus subtilis* (55), *Enterococcus faecalis* (139), and *Lactococcus lactis* (90, 140), *Lactococcus lactis subs. lactis*, *Lactobacillus helveticus*, *Streptococcus thermophilus*, *Pediococcus pentosaceus* (89), *Pseudomonas fragi* (61, 113, 114), *Arthrobacter globiformis* (7, 8), *Bacillus*

psychrophilus (184), *Listeria monocytogenes* (6, 147), *Pseudomonas putida* (59), *Salmonella typhimurium* (35), *Salmonella enteritidis* (75), *Aeromonas hydrophila* (71), *Vibrio vulnificus* (110), *Vibrio cholerae* (36), *Sinorhizobium meliloti* (123, 124), *Rhizobium* (28), and *Aquaspirillum articum* (152).

Because temperature has wide-ranging effects on growth and survival, bacteria have developed responses that allow them to adapt to change in temperature. The evolution of cold shock genes is one those adaptive mechanisms. Many species of bacteria have been shown to alter gene expression in response to a shift to low temperatures (6, 7, 55, 80, 109, 140). Since cold has a rate-depressing effect on almost all aspects of physiological performance, an increase in mRNA transcripts may not necessarily correspond to increased levels of the translated protein. Increases in mRNA may simply reflect changes in mRNA turnover parameters with cooling. These changes should not be associated with cold-shock gene induction.

In general, bacterial cells must cope with several recognized problems that arise following cold shock: a) membrane fluidity is too low, b) superhelical density of the DNA is too high for opening out the double helix, c) enzyme activities decrease profoundly, but probably to different extents, therefore protein levels must be adjusted, d) protein folding may be too low or inefficient, e) ribosomes must be adapted to function property at low temperatures, and f) secondary structures in RNA might affect initiation of translation (54, 78, 140). A decrease in membrane fluidity can be overcome by increasing an unsaturated fatty acid and di-unsaturated phospholipids in membrane. Cold shock proteins are transiently induced to overcome the translational block (72). Lowering the temperature, especially down to 1-8°C, brings up alterations in translational

processes, including the dissociation of polysomes and the accumulation of whole ribosomes and their subunits. It should be noted that cold shock suppresses protein synthesis just at the stage of initiation of RNA translation. Once the synthesis of a polypeptide chain has started, its elongation will continue irrespective of the cold shock conditions (54, 78).

Cold shock proteins have been classified based on level of increased expression after a temperature downshift. Class I Csps, which are at low level of expression at 37°C, increased synthesis in more than 10-fold in response to cold shock, whereas class II Csps are present at 37°C and increase modestly upon cold shock. However, the majority of class I and II Csps that have been described are chaperones involved in enabling ribosomes to translate mRNAs at low temperatures (173).

Cold shock synthesis of Csps seems to be regulated mainly at the post-transcriptional level. Individual mRNA for each Csp seems to play a main role in cold shock response. It is believed that the cold shock response serves as an adaptive mechanism, and it has been suggested that proteins of the CspA family, together with other Csps, play a role in protecting cells from damage due to the cold. Most of the free living bacteria possess at least one cold shock-inducible CspA homologue, which likely functions as an RNA chaperone (77).

In *E. coli*, when culture temperature of exponentially growing cells is shifted from 37 to 10°C, a lag phase is observed before growth is reinitiated (80). Similar to the heat shock response, to compensate for this stress, *E. coli* reacts to temperature downshifts by inducing a specific pattern of gene expression called “cold shock response”, which includes induction of a set of proteins defined as cold shock proteins (78, 80). These cold

shock proteins are typically classified into two groups based on their expression patterns (173). Class I cold shock proteins are expressed at an extremely low level at 37°C, but are dramatically induced upon cold shock. Class I includes CspA, CspB, CspG, CspI, CsdA, RbfA, NusA, and PNP. Class II cold shock proteins are expressed at a certain level at 37°C, and are induced moderately upon cold shock. Class II included IF-2, H-NS, RecA, α subunit of DNA gyrase, Hsc66, HscB, trigger factor, dihydrolipoamide acetyltransferase, and pyruvate dehydrogenase (lipoamide). Three of the cold shock proteins so far identified in *E. coli* appear to be associated with translation; RbfA, a ribosome binding factor, CsdA, an RNA unwinding protein, and IF2, an initiation factor (173). These proteins are thought to be involved in the preservation of ribosome function at colder temperature. In *E. coli*, where the induction of cold shock genes has been most extensively studied (173), nine genes encoding CspA-like proteins, *cspA* to *cspI* have been identified (190). Among them, *cspA* (50), *cspB* (97), *cspG* (120), and *cspI* (180) have shown to be cold shock inducible. Among cold shock inducible genes, *cspA* has been extensively investigated for its cold shock induction (190). Low temperature regulation of the *E. coli cspA* gene is involved in both transcriptional and post transcriptional mechanisms.

In *V. vulnificus,* when cells responded to a moderate temperature downshift, from 23°C to 13°C, a decline in protein synthesis was observed, but cells continued to grow at a lower rate (110). In contrast, when *V. vulnificus* was exposed to more dramatic temperature shifts, cell growth was halted and protein synthesis stopped (127). When *V. vulnificus* culture was shifted from 35°C to 6°C, it underwent a transition to the VBNC state, but when cells were adapted to 15°C prior to change to 6°C they remained viable

and culturable. This result suggested that the exposure to an intermediate cold temperature (15°C) caused a cold adaptive response helping *V. vulnificus* remain culturable at a much colder temperature. The survival and tolerance at cold temperatures could be due to the expression of cold adaptive proteins other than the major cold shock protein as found in *E. coli* and other microorganisms (17).

4. Genes involved in general stress responses

rpoS

The *rpoS* gene encodes a sigma factor, σ^S, RpoS, which is required for expression of a large number of genes in response to various stresses, including nutrient limitation, osmotic challenge, and during growth into stationary phase (103). The *rpoS* gene has been found in a variety of Gram-negative bacteria, and its function and regulation have been studied extensively in the enteric species *E. coli* and *Salmonella enterica* serovar typhimurium. RpoS can act as a virulence factor since it activates some of the genes necessary for pathogenesis in several bacteria, including *S. enterica*, *E. coli*, and *Shigella flexneri* (42, 135, 159). In fact, *rpoS* expression is induced when these bacteria enter mammalian cells (24). The *rpoS* levels tend to be very low in rapidly growing cells not exposed to any stress. However, the *rpoS* levels increase in response to a variety of environmental stresses, which included starvation for limiting nutrients, stationary phase, high osmolarity, and high or low temperature. RpoS is a highly unstable protein under non-stress conditions, but exposure to various stresses differentially affects *rpoS*

transcription and translation, as well as the rate of proteolysis. Research on the regulation of *rpoS* gene expression has mainly focused on the control of RpoS translation (96, 103) and RpoS proteolysis (96, 155)

oxyR

OxyR is a peroxide sensor and a transcriptional activator of the oxidase stress response. *OxyR* has been proved to be a positive regulator of the expression of nine hydrogen-peroxide-inducible genes, including those encoding catalase (*katG*), glutathione reductase (*gor*), glutaredoxin (*grxA*), alkyl hydroperoxide reductase (*ahpCF*), *oxyS* (a regulatory RNA), *fur* (ferric uptake regulation), and *dps* (a nonspecific DNA-binding gene) (27, 115). Catalase is part of a global system regulated by OxyR (27). OxyR-regulated enzymes are involved in the defense against reduced-oxygen compounds. *OxyR* is required for adaptation to H_2O_2 (74). An *oxyR* gene has been identified in 31 bacterial genomes (178). The position of *oxyR* is variable in the different bacterial genomes, but it is often next to a gene involved in oxidative stress protection and regulated by OxyR. In *E. coli* it is known that the expression of peroxide defense genes is activated by OxyR in the exponential phase. In the stationary phase, expression of these genes is controlled by the starvation-induced sigma factor RpoS. OxyR mediates a response to oxidative stress in *E. coli*. The change in *oxyR* expression during aerobic growth is completely abolished in the absence of oxygen (52).

katG

There are two types of structural catalases in bacteria: a bifuntionally catalase-peroxidase (HPI) and a monofunctional catalase (HPII). Most bacteria appear to express one or more catalases in response to peroxidase stress. *Vibrio vulnificus* has been shown to have only one catalase gene, *katG*, encoding the catalase-hydroperoxidase (94). *KatG* is induced during the bacterial exponential phase (174). The expression of the *katG* is controlled by the positive transcriptional activator OxyR, and it is induced in response to exogenous H_2O_2 (115), which directly sense oxidative stress (88, 193). Bacteria produce H_2O_2 under aerobic conditions to be toxic, and *katG* is primarily responsible for its detoxification (137).

5. Molecular diagnosis techniques

DNA probe hybridization

The ISSC established the methodology for enumerating *V. vulnificus* in shellfish under Chapter 9 of the FDA Bacteriological Analytical Manual, 9th edition, 2004 (176). Two analytical schemes for isolating and enumerating *V. vulnificus* are described. The first method utilizes the most probable number (MPN) analysis coupled with identification of suspect isolates using biochemical profiles, DNA probe colony hybridization, or Polymerase Chain Reaction (PCR). The second uses two direct plating methods followed by hybridization with DNA probes for colony identification. This

technique has been used to enumerate *V. vulnificus* in oysters in several studies (187, 188). This method provides a more rapid and accurate evaluation of post harvest treatment of oysters (60). The sensitivity of DNA probe hybridization assays for detection of vibrios in oyster homogenates approaches 10 CFU/g oyster meat.

The identification of bacteria by DNA probe hybridization is based on the presence and absence of particular genes. The physical basis for gene probe tests stem from the structure of DNA molecules themselves (84). DNA hybridization tests may be performed in many ways; one technique is the colony hybridization assay (58, 106). An aliquot of a homogenized food is spread on the appropriate agar plate medium. After incubation, the colony pattern is transferred to a solid support (usually membrane or paper filter). Then, the cells are lysed by a combination of high pH and temperature, which also denature and fix the DNA to the support. The solid supports with the attached target DNA are incubated with an enzyme-labeled specific probe. The labeled probe failing to attach to the target is removed by stringent washing (177).

The radioactive probe DNA that is bound to the target on the support is often detected by a colorimetric reaction. A chromogenic substrate then is added to the membrane. Where the probe-associated enzyme is present, a color spot will develop. Each spot represents a bacterial colony that has arisen from a single cell. The number of cells harboring the target gene in the original sample can be calculated by multiplying the number of spots by the dilution factor (177).

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is an approach for converting and amplifying a single-stranded RNA template to yield abundant double-stranded DNA products. This molecular approach was first described in 1987 (179) and its use in the analysis of steady-state messenger RNA levels has become increasingly widespread in recent years. Detection of low abundance mRNAs by RT-PCR has become a standard technique to determine gene expression in tissues and culture cells. RT-PCR is most useful to determine if a specific mRNA is present in sufficient numbers to be amplified to a detectable level and is a more sensitive alternative to Northern Blot Analysis (172). RT-PCR technique involves the amplification of target RNA molecules by sequentially combining reverse transcription (RT) and polymerase chain reaction (PCR). It is a technique similar to conventional PCR, except that the starting material is RNA rather than DNA. Because of this copy, a DNA must first be made from RNA, utilizing an enzyme known as reverse transcriptase. Once the copy of the DNA is made, the PCR proceeds as usual (136).

The greatest advantage of the RT-PCR in the analysis of mRNA includes its sensitivity, versatility, and rapid turn-around time (29). The major shortcoming of RT-PCR is reliability and accuracy because the results are based solely on the quality and quantity of starting RNA sample (189). Since RT-PCR was first described for the detection of low abundance mRNAs, the method has been extended to quantitative measurements of relative and absolute levels of single and multiple mRNAs. Methods for obtaining RNA for RT-PCR have also been extended to include analysis of mRNA

levels in clinical samples and even in single cells. Measurement of specific mRNA species is of major importance for studies on gene expression. RT-PCR is one of the most applied techniques in biomedical research for disease pathogenesis. The use of RT-PCR is also increasing in the clinical research and diagnostic fields where it is becoming a popular method for the identification of infectious agents, as well as for detecting markers indicatives of certain types of cancers and genetic disorders (16).

Several studies have investigated the potential of mRNA detection using reverse transcription-PCR (RT-PCR) as a viability marker (13, 46, 93, 158). Detection of mRNA is thought to be a good viability marker, due to its central role in cell metabolism and its very short half life. RT-PCR was used to study gene expression both i*n situ* and *in vitro* for VBNC state of *V. vulnificus* (160, 161).

Statement of research objectives:

In summary bacteria adapted to a period of low temperature growth could promote survival, which is referred as cold shock response. *Vibrio vulnificus*, a food-borne pathogen could potentially develop this cold shock phenomenon during exposure to low temperature. Oysters can serve as reservoirs for *V. vulnificus* and transmit this bacterium to humans through the ingestion of raw oysters. If the potential for cold shock-mediated survival of *V. vulnificus* exists, the cooling strategy recommended by USFDA/ISSC may potentially induce this response in *V. vulnificus* and result in its persistence in oyster. Not only could cold shock response affect microbial survival, but it may also alter gene expression. It was critical to examine the impact of

cold shock response of *V. vulnificus* in oysters regarding its survival and physiology. The expected outcome of this study is to provide scientific data for the development of control strategies of *V. vulnificus* risk associated with raw oyster consumption. The overall benefit would be to enhance quality and safety of shellfish to ensure consumer confidence in U.S. Gulf Coast oysters.

This research focuses on the cold shock response of *V. vulnificus* both in culture (*in vitro*) and in live oysters (*in vivo*). The objectives of this study are:

1. to investigate the effect of cold shock on growth of *V. vulnificus* under mimicked controlled shellstock temperature conditions as proposed by USFDA/ISSC *in vitro*.
2. to examine the effect of cold shock on *V. vulnificus* growth in shellstock oysters using colony blot hybridization as a tool to enumerate *V. vulnificus* persisting in oysters.
3. to determine which genes of *V. vulnificus* might be altered during cold shock
4. and to use RT-PCR technology to analyze *V. vulnificus* gene expression during cold shock both *in vitro* and *in vivo*.

III. COLD SHOCK EFFECT ON GROWTH RATE AND SURVIVAL OF *VIBRIO VULNIFICUS* IN CULTURE BROTH UNDER SHELLSTOCK TEMPERATURE CONTROL CONDITIONS

ABSTRACT

The growth rate of *V. vulnificus* was investigated at different temperatures. The drop plate method was used to determine the culturability of the cells. All the four strains tested displayed a similar growth rate when incubated at the same temperature ($P>0.05$), except at 20°C ($P<0.05$). At 35 and 25°C *V. vulnificus* cultures showed a mean growth rate of 1.23 and 1.169 $hour^{-1}$, respectively. At 20°C cells multiplied with a mean growth rate that ranged from 0.752 $hour^{-1}$ in clinical strains to 0.982 $hour^{-1}$ in environmental strains. In contrast, at 15°C *V. vulnificus* showed little growth with a mean growth rate of 0.004 $hour^{-1}$. At 4°C and 7.2°C cell numbers decreased over time until non-detectable levels were recorded at 72 h. The response of *V. vulnificus* to cold shock was investigated under ISSC guidelines. *Vibrio vulnificus* was grown at 35, 25, 20, and 15°C, and then subjected to subsequent low temperatures at 7.2 or 4°C for one week. *Vibrio vulnificus* cells rapidly multiplied at elevated temperatures (35, 25, and 20°C). After cold shock at 7.2°C cell numbers decreased by 1 to 2 log units. However, the highest reduction in culturability was observed after cold shock at 4°C. When cells were adapted to 15°C prior to cold shock at 7.2°C, a slight increase in cell counts were observed. Results suggested that a cold adaptive mediated survival mechanism in *V. vulnificus* existed when cells were adapted to 15°C prior to shock at 7.2°C. The effective refrigeration temperature in reducing the multiplication of *V. vulnificus* is at 4°C. However, optimal and sub-optimal growth temperatures (35, 25, 20°C) allowed *V. vulnificus* cell counts to reach high number that could not be reduced to safety levels by refrigeration conditions even at 4°C.

INTRODUCTION

Vibrio vulnificus is a Gram-negative bacterium naturally inhabitating marine and estuarine environments. *V. vulnificus* is a mesophilic bacterium with maximum growth occurring at 35°C, pH of 7.5 to 8.0, and with 2% NaCl present in the medium (68). In the U.S., *V. vulnificus* is a major concern for the public health authorities since 95% of all deaths associated to seafood consumption are caused by this bacterium (132). *V. vulnificus* infections can cause a primary septicemia with fatalities up to 60% (132), especially in patients with underlying diseases. The majority of those cases are associated to the consumption of raw oysters. High *V. vulnificus* counts in oysters are correlated with elevated water temperatures in summer months which explains the increase incidence of the disease during warmer months (157). These epidemiological findings suggest that infections caused by *V. vulnificus* are dose dependent. Although the infectious dose for *V. vulnificus* has not been established for humans, the invasiveness and high fatality rates that characterize *V. vulnificus* infections makes the reduction of *V. vulnificus* counts in oysters to non-detectable levels a primary goal for the Interstate Shellfish Sanitation Conference (ISSC), as well as for the Gulf Coast oyster industry.

Under optimal conditions, *V. vulnificus* exhibits one of the fastest growth rates of all bacteria but cell culturability rapidly declines when this bacterium is subjected to refrigeration temperatures (30, 33, 110, 128, 170). In order to minimize consumer risks, the ISSC has established specific guidelines for the control of molluscan shellfish at harvest, transportation, shucking, packing and shipping (44). Shellstock temperature control is a critical point within that set of guidelines. Shellstock temperature control is

defined as the management of the environmental temperature of shellstock by means of ice, mechanical refrigeration or other approved methods which are capable of lowering the temperature of the shellstock and which will maintain it at 10°C or less. A specific time-temperature matrix has been defined for molluscan shellfish collected from waters where problems associated to *V. vulnificus* have been reported. The warmer the water temperatures are at time of harvest, the shorter the time required from harvest to temperature control conditions (45).

Not only shellstock temperature control but also various actions have been adopted in order to minimize the risk of oyster containing *V. vulnificus*. ISSC has adopted industry-implemented post-harvest controls to reduce *V. vulnificus* loads in oyster shellstock, which may include time-temperature, post harvest treatment (i.e. hydrostatic pressure, cool pasteurization, IQF, and irradiation - pending approval), and rapid chilling. The post-harvest treatment technologies currently account for less than 10% of all domestic raw oyster sales in the U.S. (98). Moreover, these post-harvest treatments are effective for eliminating *V. vulnificus*, but each has some drawbacks. Cool pasteurization (5) may kill bacteria but can change the oyster flavor and texture. Hydrostatic pressure processing (9) can eliminate this bacterium but it cause the meat to detach from the shell. All of these processes kill the harvested oyster and increase product costs. The use of accurate shellstock time-temperature controls offers several advantages over post-harvest treatments since the procedure does not require specialized technology. The temperature control strategies for minimizing *V. vulnificus* risks by the ISSC were proposed following reports by Cook and Ruple (33). These authors stated that storage of summer harvested oyster shellstock from the U.S. Gulf Coast at 10°C prevented the multiplication of

V. vulnificus, however, recent studies have been shown that *V. vulnificus* can adapt to cold temperatures below 7.2°C, following exposure to intermediate temperatures (≈15°C) (17, 110). Previous literature has reported that 4°C seems to be more effective than 7°C in reducing *V. vulnificus* numbers to non-detectable levels (162).

Understanding the relationship between *V. vulnificus* numbers and temperature in culture broth may help predict the growth pattern of *V. vulnificus* in oysters. To better characterize the effects of refrigeration conditions in *V. vulnificus* growth, the objectives of this study were (a) to establish the growth rate of different *V. vulnificus* strains under different temperatures, and (b) to determine *V. vulnificus* growth under ISSC recommended shellstock temperature control conditions.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

Two clinical strains of *V. vulnificus*, ATCC 27562 (type strain), and C7184Tr (avirulent, translucent morphotype provided by J.D. Oliver, University of North Carolina, Charlotte, NC) were used in this study. Two environmental strains (VV3, VV4) isolated from eastern oyster (*Crassostrea virginica*) samples collected from Mobile Bay, Alabama were also included. Identification and classification of both environmental isolates were performed by API 20E (bioMerieux, Inc., Hazelwood, MO) and confirmed by PCR analysis with *V. vulnificus* specific primers (43). Cells were maintained and routinely grown on marine agar (MA) (Difco, Detroit, MI.) at 35°C.

Growth rate of *V. vulnificus*

Growth curves for *V. vulnificus* were determined by inoculating one single colony into 25 ml of marine broth (MB) (Difco) and grown in a shaker incubator at 35°C. At various time intervals, 5 ml aliquots of culture were taken and the optical density (OD) at 450 nm was determined. Simultaneously and in order to correlate OD with colony forming units (CFU), the drop plate method described by Herigstad et al. (62) was used. One hundred µl of culture broth was serially diluted and plated onto MA and incubated overnight at 35°C and colonies counted after 24 h incubation (Appendix. 1).

Growth curves at different temperatures (35, 25, 20, 15, 7.2, and 4°C), were determined following the above mentioned protocol with the following modifications. Four *V. vulnificus* strains were compared at the same time for each experiment. One hundred and fifty ml of MB was inoculated with cells at OD 0.025 which equals a cell count of approximately 10^4 CFU/ml. Flasks were then incubated at 35, 25, 20, 15, 7.2, and 4°C. Samples were taken at 0, 1, 2, 3, 4, 5, 6, 8, 10, and 12 h of incubation, viable plate counts were done in triplicate on MA plates by the drop plate method. For experiments carried out at 7.2°C and 4°C, cells were cultured and sampled for culturability until no CFU will be detected. For each temperature experiment, the cell growth rate was calculated by simple linear regression of ln (CFU/ml) over on time (hours) for five or six points in the linear portion of the growth curve.

Growth patterns under cold shock conditions

The effect of temperature downshift (cold shock) on *V. vulnificus* growth was studied by imitating the time-of-harvest to time-of-refrigeration conditions that are currently recommended by the ISSC (44). The culturability of the cells after cold shock was monitored and compared at two different refrigerated temperatures (7.2°C and 4°C). Four different growth temperatures and times prior to cold shock were compared: 35°C for 10 h, 25°C for 12 h, 20°C for 14 h, and 15°C for 36 h. After those incubation periods, cells were transferred to either 7.2°C or 4°C and incubated for 168 h. At various time intervals from initial growth to 168 h, the culturability of the cells was monitored by the drop plate method.

Statistical analysis

All bacterial samples were analyzed in triplicate. The standard error was calculated for all of the treatment replications. To determine if difference between data set existed, the Randomized Complete Block Design (RCBD), F-test was performed using one-way Analysis of Variance (ANOVA) analysis procedure in the Statistical Analysis System, SAS 9.1.3 (SAS Institute, Cary, NC).

RESULTS

Growth rate of *V. vulnificus*

All four strains exhibited a very similar growth rate (Fig. 1-6) when incubated at the same temperature ($P>0.05$), with the exception of temperature at 20°C ($P<0.05$). When cells were cultured at 35°C, *V. vulnificus* viable counts increased by 5 log over a period of 7 to 10 h, reaching 10^9 CFU/ml during the stationary phase. The growth rate at 35°C was between 1.218 to 1.250 $hour^{-1}$ (Fig.1). A similar result was obtained at 25°C where growth rates ranged from 1.149 to 1.174 $hour^{-1}$ (Fig.2). At 20°C only the two environmental strains were able to reach a viable count of 10^9 CFU/ml after a 12 h period. Both clinical strains showed a decline in cell growth by one to two log reductions with the growth rate ranged from 0.655 to 0.982 $hour^{-1}$ (Fig.3). Small positive growth was observed at 15°C with the growth rate ranged from 0.003 to 0.005 $hour^{-1}$ (Fig.4). When cells were incubated under refrigeration temperatures at 4°C, total viable counts started to decrease 12 h post-inoculation. Cells incubated at 7.2°C started to lose culturability 24 h post-inoculation. After 72 h of incubation, no viable cells were observed at either 7.2 or 4 °C. No difference between strains was observed in this experiment (Fig. 5-6).

Growth patterns under cold shock conditions

Figures 7 to 14 depict cold shock survival response of *V. vulnificus*, when cells were subjected to different temperatures and times growing conditions. Under ISSC guidelines conditions, *V. vulnificus* reached 10^9 CFU/ml when cells were incubated at 35 and 25°C for 10 and 12 h, respectively (Fig. 7-8). At 20°C, only environmental strains were able to reach those numbers after 14 h of incubation, while clinical strains plateaued at 10^8 CFU/ml (Fig. 9). At 15°C no growth was recorded prior to cold shock (Fig. 10). After cold shock at 7.2°C, a steady decrease in *V. vulnificus* cells numbers was observed at 35, 25, and 20°C. After 168 h of incubation at 7.2°C, an overall cell count reduction by one log was observed in all cases with the exception of VV3 and VV4 grown at 25°C (Fig. 7-9). After cold shock, these two strains experienced a reduction of two log units in cell count numbers. Nevertheless, when cells were allowed to grow up to 10^8 CFU/ml or higher levels, they maintained a cell culturability of 10^7 CFU/ml or higher during 7 days under refrigeration conditions at 7.2°C. Conversely, when cells were incubated at 15°C for 36 h and then shifted to 7.2°C, total cell counts slightly increased during the length of the study (Fig. 10). This increment in cell counts represented only a 0.2 log in magnitude but showed an adaptation response to 15°C.

A similar pattern was found when *V. vulnificus* cells were incubated at 35, 25, 20°C, and then shifted to 4°C (Figure 11-13). Cell counts started to decrease a few hours after cold shock. Cells grown at 35°C required up to 50 h of incubation at 4°C before starting to lose culturability. Cells grown at 25°C and 20°C showed a reduction in total cell counts in less than 12 h at 4°C. A similar trend was observed when cells were

subjected at 7.2°C (Fig. 7-9). When cells were incubated at 15°C for 36 h and then cold shocked at 4°C a decrease in total cell counts was observed starting 72 h after cold shock (Fig. 14). This result was different when cells were cold shocked at 7.2°C. After 168 h of incubation at 7.2°C, cell counts averaged 10^4 CFU/ml while after 168 h of incubation at 4°C remaining viable cells were down to 10^2 CFU/ml. Statistical analyses showed that the mean of *V. vulnificus* counts after 7 day refrigeration at 7.2°C were significantly greater ($P<0.05$) than at 4°C in all treatments.

DISCUSSION

The response of living cells to temperature downshift has been termed cold shock (72). Several factors have been reported to affect bacterial growth during cold shock including temperature (67), incubation time at low temperature (164), chill rate (111), phase of inoculums (111, 143), cell concentration (49, 111, 163), plating medium (49), and the opportunity for the cells to repair the damage caused by the cold shock (37). Several studies have reported cold shock resulted in the decline in cell culturability of *V. vulnificus* (30, 33, 81, 83, 118, 129). There has also been evidence that cold shock adaptation promoted survival of *V. vulnificus* (17).

Results of this study indicate there was no difference in the growth pattern between the clinical and environmental strains of *V. vulnificus* tested. The optimal temperature condition assayed was 35°C while incubation at 15°C or lower prevented *V. vulnificus* growth. The current data are in agreement with the maximum (37°C) and minimum (13°C) temperatures reported for *V. vulnificus* growth (81). This study

demonstrated that clinical strains ATCC 27562 and C7814Tr had a worse performance rate at 20°C than environmental strains, and suggests that environmental strains might be better adapted to suboptimal temperatures than clinical strains.

When *V. vulnificus* growing patterns were investigated under the time-of-harvest to time-of-refrigeration conditions recommended by the ISSC, the results revealed that all strains tested had similar growing patterns. *V. vulnificus* cells rapidly multiplied in culture broth at elevated temperature (35, 25, 20°C) while no positive growth was detected at 15°C and loss of culturability occurred under refrigeration conditions. Growth of *V. vulnificus in vitro* can be prevented when exposed to 7.2°C and 4°C, however, *V. vulnificus* cells, although unable to multiply at low temperatures, remain culturable for up to 7 days under refrigeration conditions. If *V. vulnificus* cell numbers are allowed to reach high numbers under favorable conditions, neither 7.2°C nor 4°C will reduce cell culturability to non-detectable levels (at least in a 7 day period).

Data presented here show that cold shock adaptation enhances survival of *V. vulnificus*, when cells are cultured *in vitro*. A cold adaptation response was observed after subjecting *V. vulnificus* cells to 15°C for a few hours before cold shock. In fact, cells adapted at 15°C were able to slightly increase their numbers at 7.2°C. This finding is in agreement with Bryan et al (17) who verified that a cold adaptation period at 15°C enhanced *V. vulnificus* survival at 6°C. This cold adaptive response in *V. vulnificus* induced at 15°C could prevent the cells from entering the VBNC state when exposed to a much colder temperature. McGovern and Oliver (110) reported the induction of cold response proteins in *V. vulnificus* after temperature downshift from 23°C to 12°C. This result suggested that cellular protein expression changed during or after temperature

shifting from high temperature to low temperature. The adaptation of *V. vulnificus* to cold temperature after priming at 15°C may be due to the induction of cold-adaptive protective proteins. Cold temperature adaptation effect was lost when cells were transferred to colder refrigeration conditions (4°C instead of 7.2°C). Not only were 15°C-acclimated *V. vulnificus* cells unable to grow at 4°C, but their numbers drastically declined over time. This striking difference between close temperature conditions has been pointed out in other studies. Stanford reported that 4°C and 7°C differently affected the rate of entry into dormancy (162). These stronger negative effects caused by 4°C versus 7°C might warrant the use of stringent refrigeration conditions by the shellfish industry.

The temperature and time of cold shock treatment clearly affects the cold shock response of *V. vulnificus*, which could enhance survival during cold stress conditions. The current findings show that the shellstock temperature control guidelines suggested by ISSC might not be effective in reducing *V. vulnificus* numbers. When *V. vulnificus* cells are exposed to optimal or suboptimal temperatures, cell numbers rapidly reached 10^8 to 10^9 CFU/ml, and which can be reduced by refrigeration only in a few orders of magnitude. Minimizing *V. vulnificus* growth by limiting the time from harvest to refrigeration should be strictly enforced. Further studies analyzing *V. vulnificus* response to cold shock *in vivo* should be carried out in order to provide the industry with safer practices.

In conclusion, this study showed that exposure of *V. vulnificus* to optimal or suboptimal temperatures for a brief period increases the number of cells to such high

levels that they can not be safely reduced by refrigeration afterwards. These data also suggest that 4°C is the appropriate refrigeration temperature.

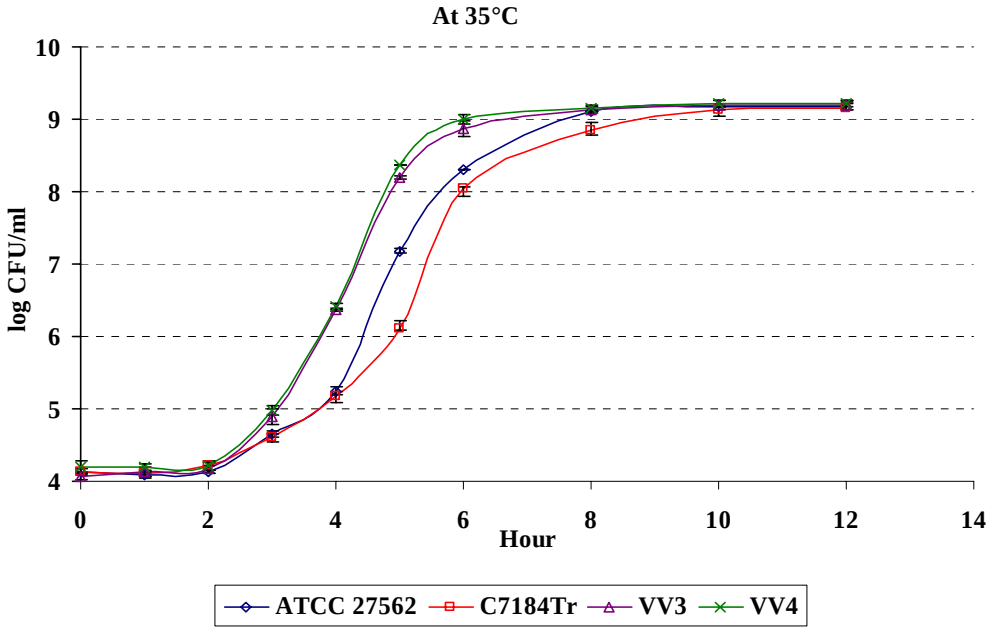

FIG. 1. Growth of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 at 35°C

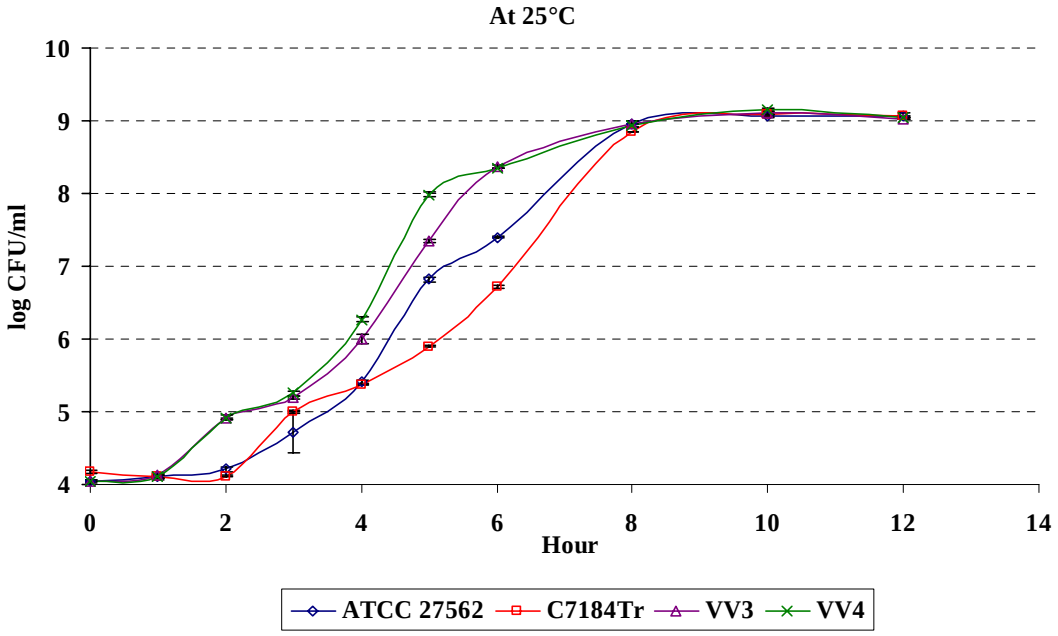

FIG. 2. Growth of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 at 25°C

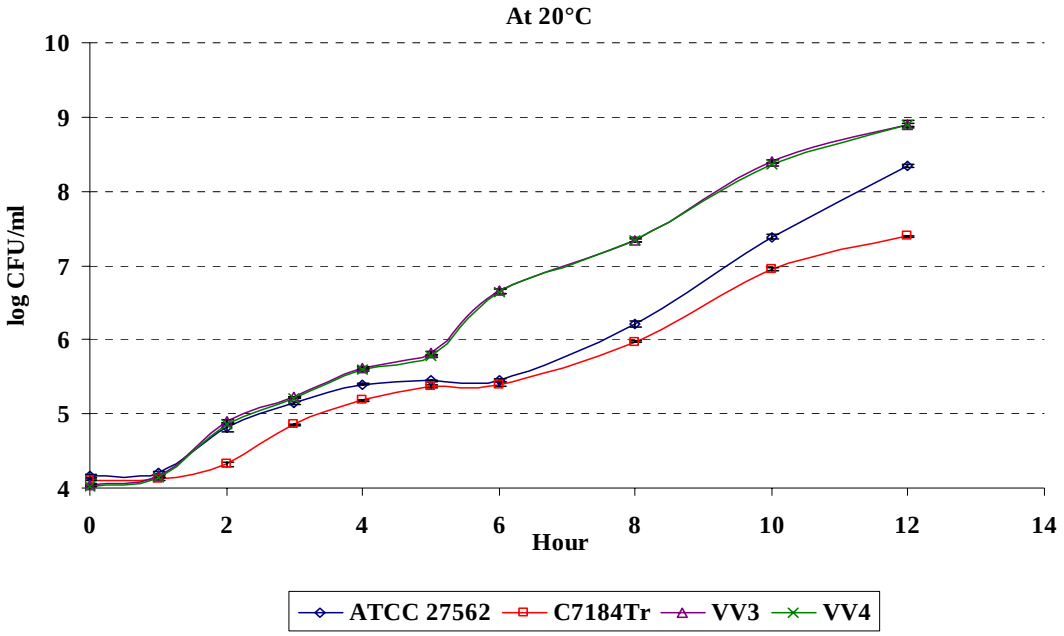

FIG. 3. Growth of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 at 20°C

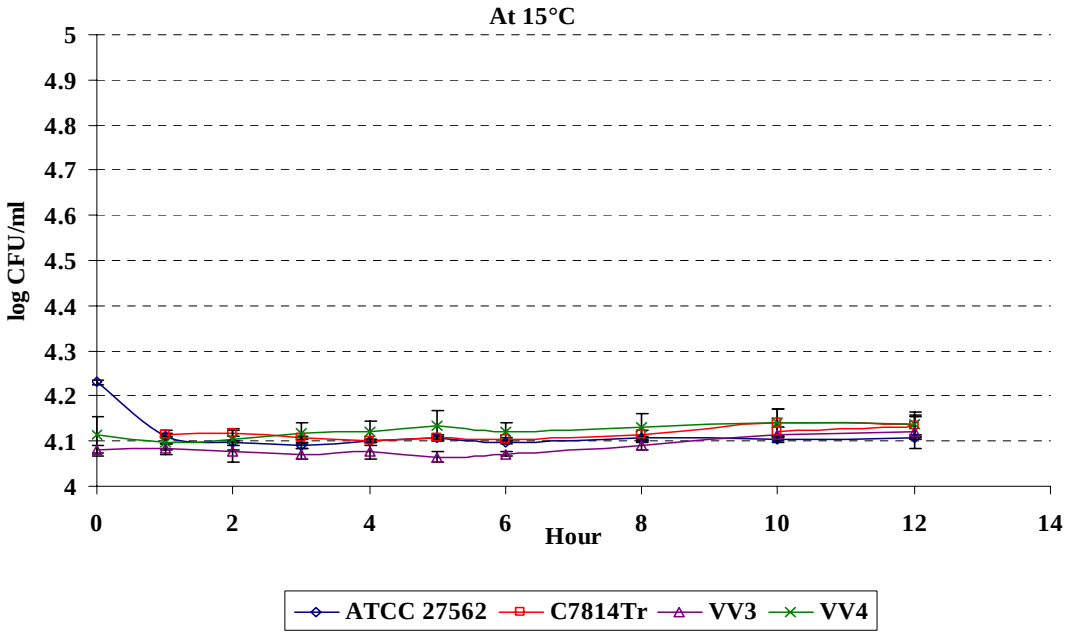

FIG. 4. Growth of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 at 15°C

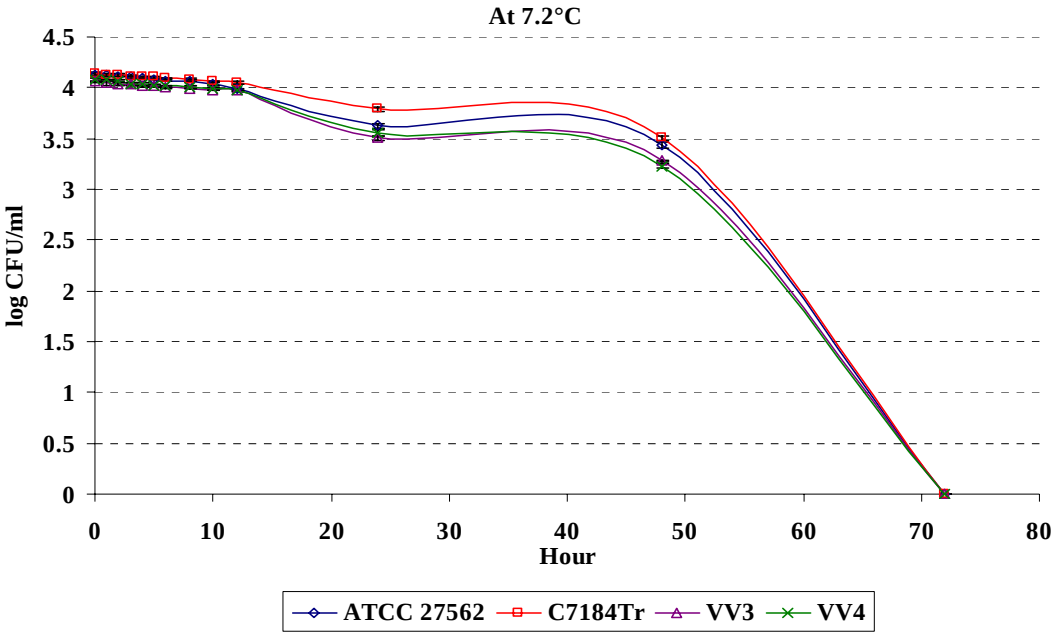

FIG. 5. Growth of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 at 7.2°C

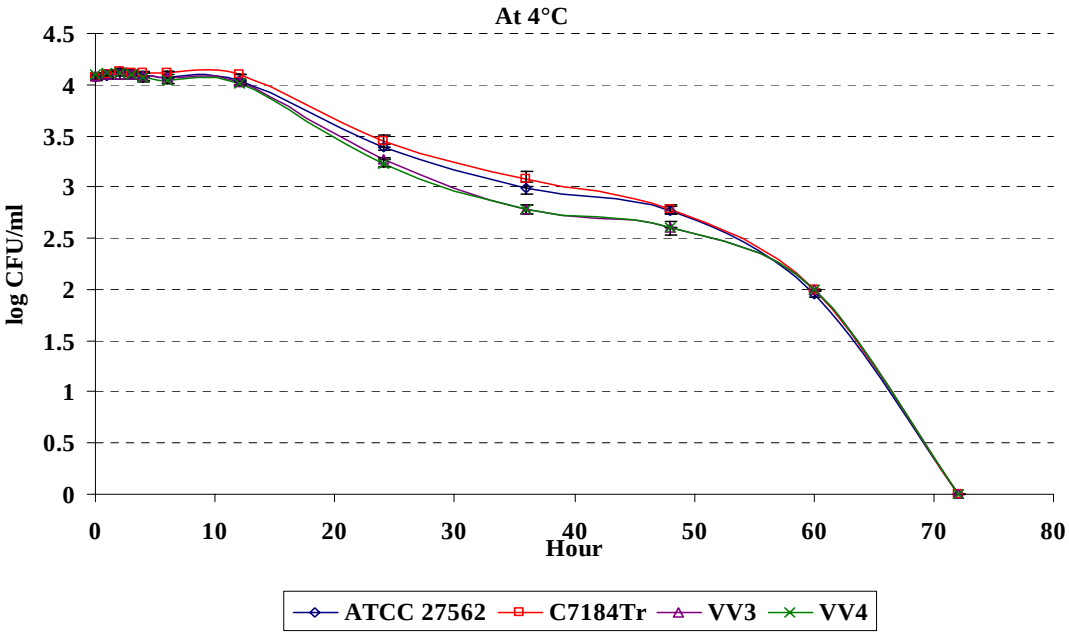

FIG. 6. Growth of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 at 4°C

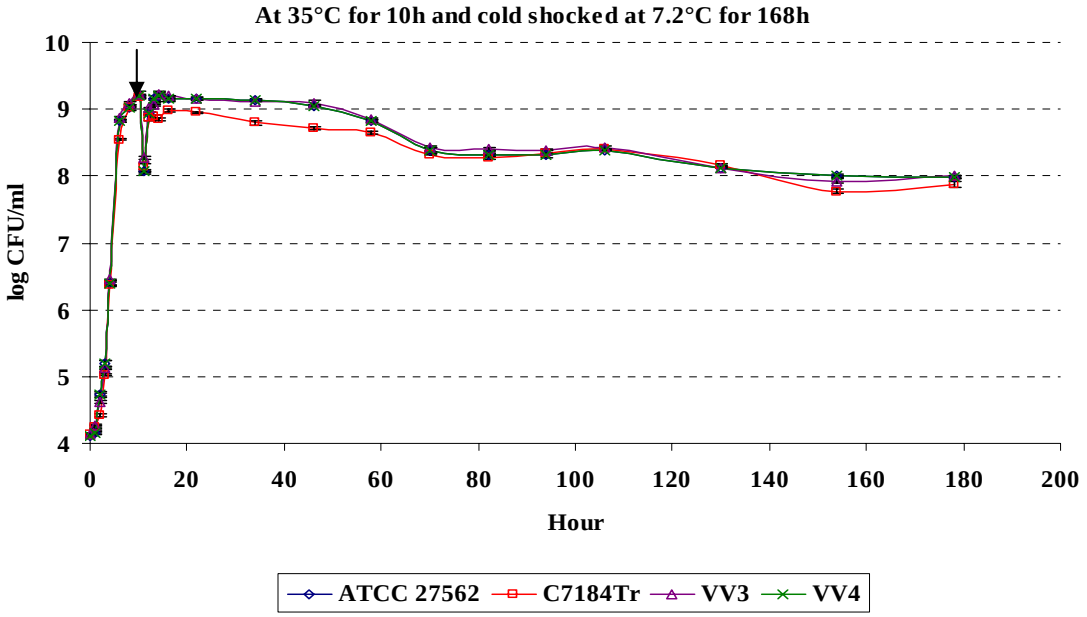

FIG. 7. Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 in MB when cells were incubated at 35°C for 10 h and cold shocked at 7.2°C for 168 h, vertical arrow indicated cold shock at 7.2°C.

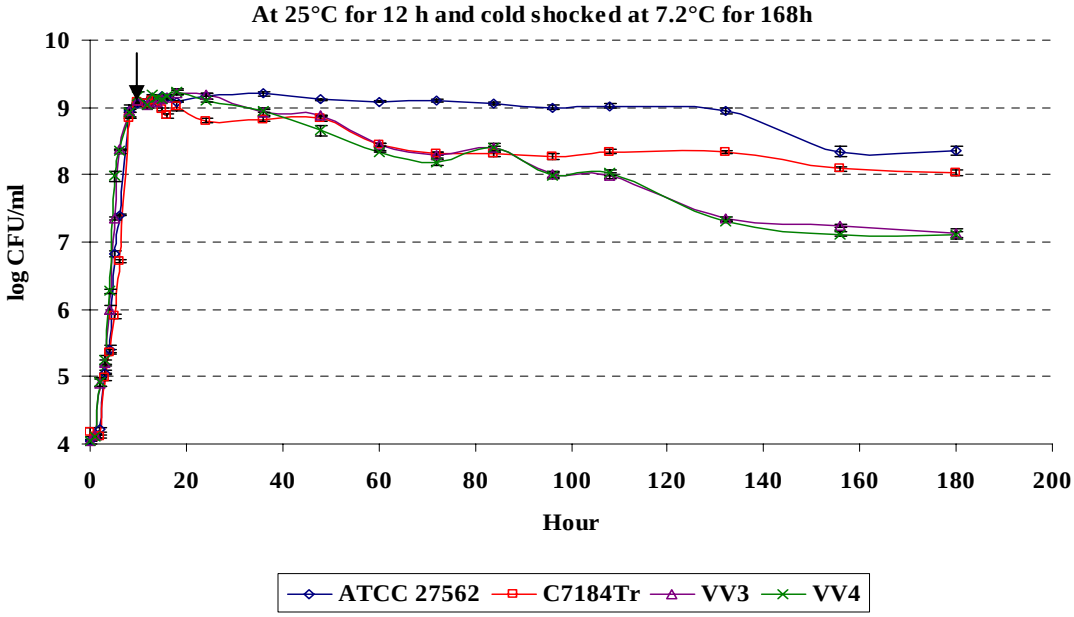

FIG. 8. Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 in MB when cells were incubated at 25°C for 12 h and cold shocked at 7.2°C for 168 h, vertical arrow indicated cold shock at 7.2°C.

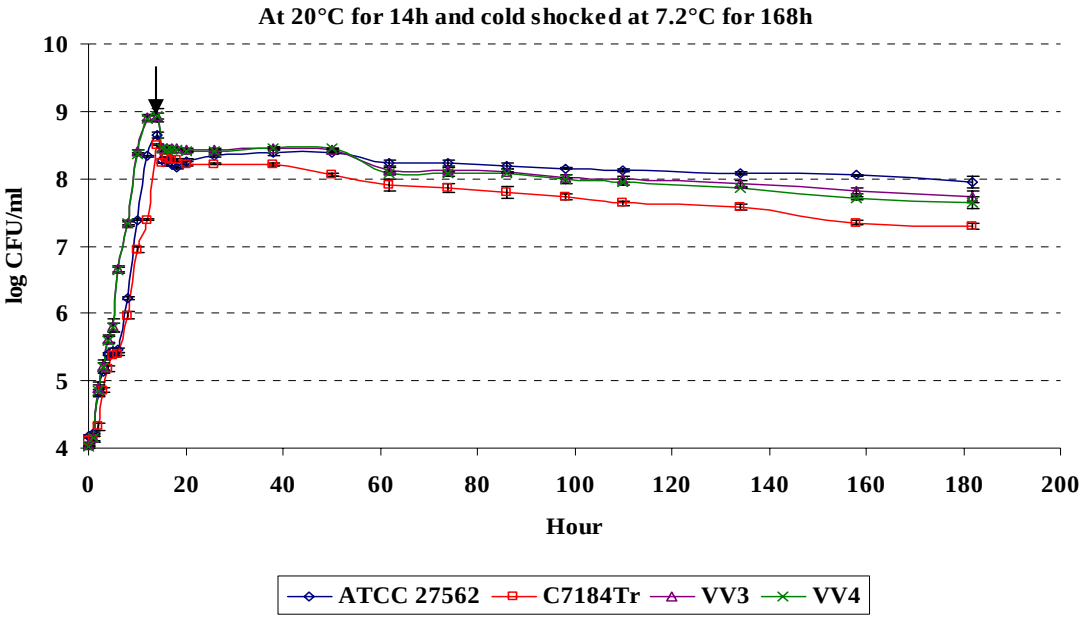

FIG. 9. Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 in MB when cells were incubated at 20°C for 14 h and cold shocked at 7.2°C for 168 h, vertical arrow indicated cold shock at 7.2°C.

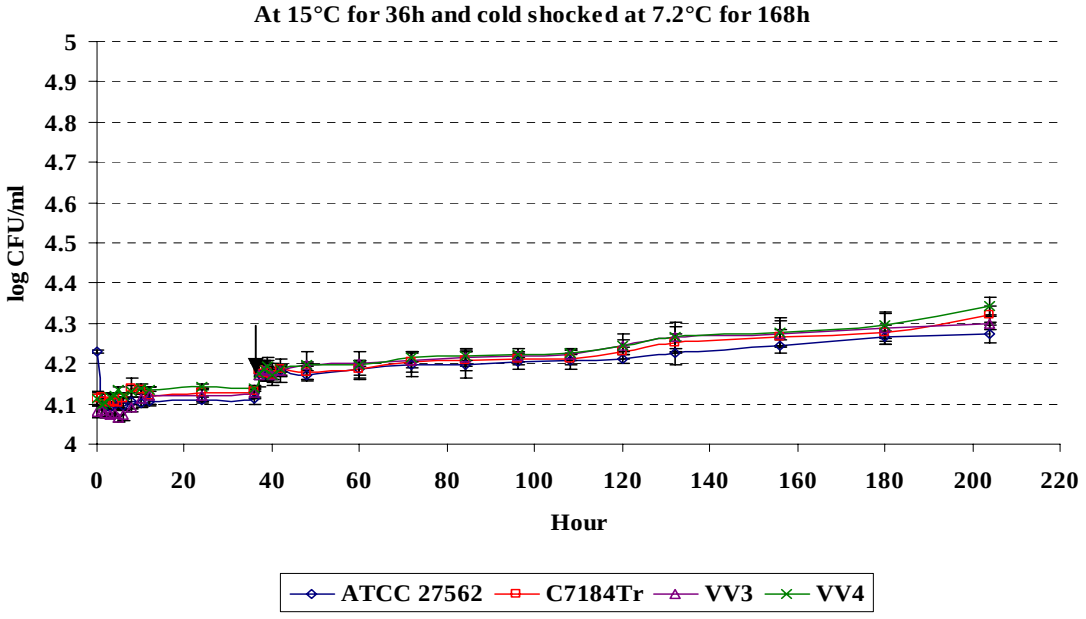

FIG. 10. Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 in MB when cells were incubated at 15°C for 36 h and cold shocked at 7.2°C for 168 h, vertical arrow indicated cold shock at 7.2°C.

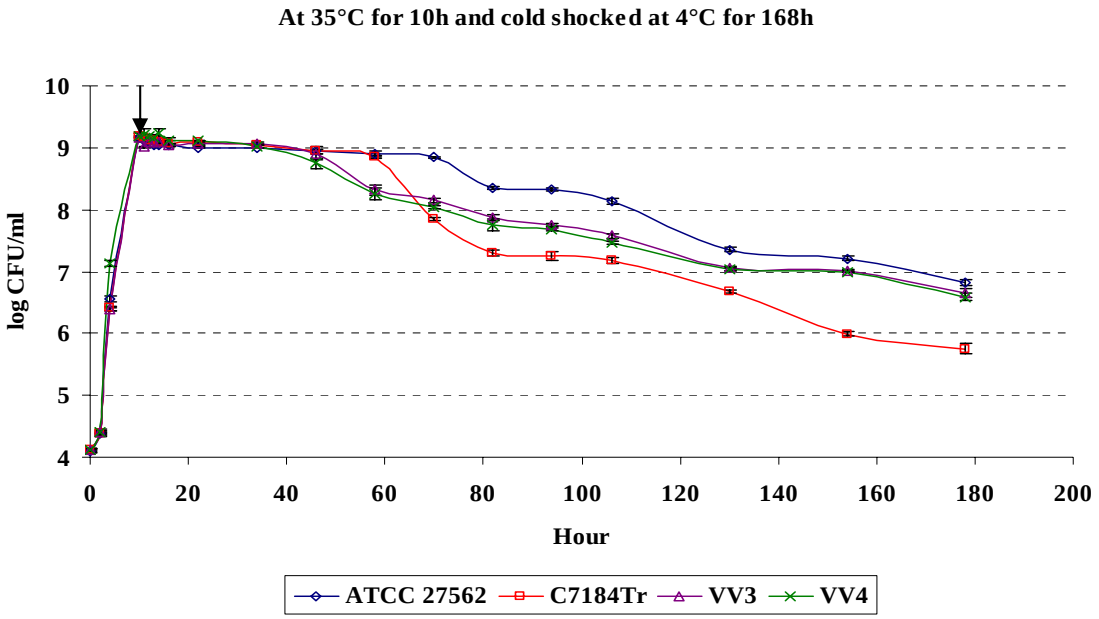

FIG. 11. Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 in MB when cells were incubated at 35°C for 10 h and cold shocked at 4°C for 168 h, vertical arrow indicated cold shock at 4°C.

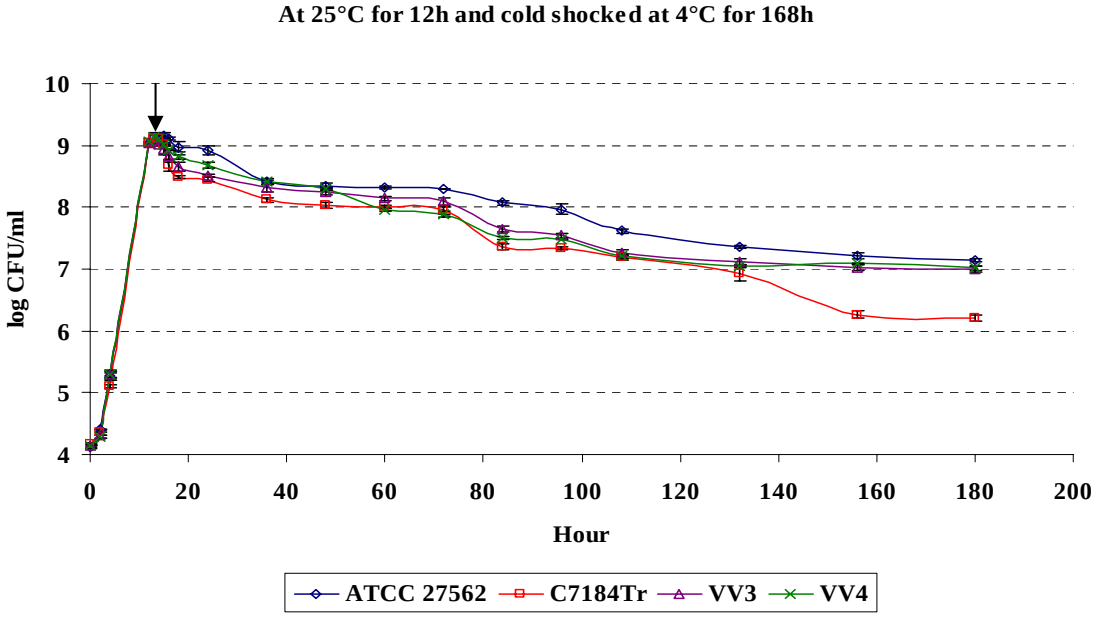

FIG. 12 Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 in MB when cells were incubated at 25°C for 12 h and cold shocked at 4°C for 168 h, vertical arrow indicated cold shock at 4°C.

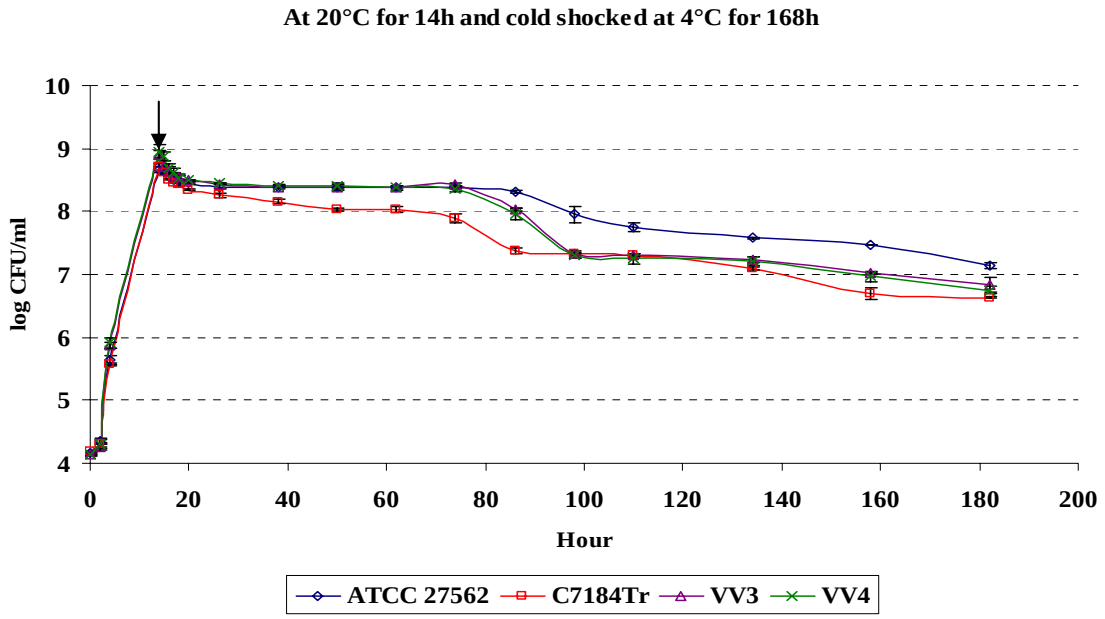

FIG. 13. Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 in MB when cells were incubated at 20°C for 14 h and cold shocked at 4°C for 168 h, vertical arrow indicated cold shock at 4°C.

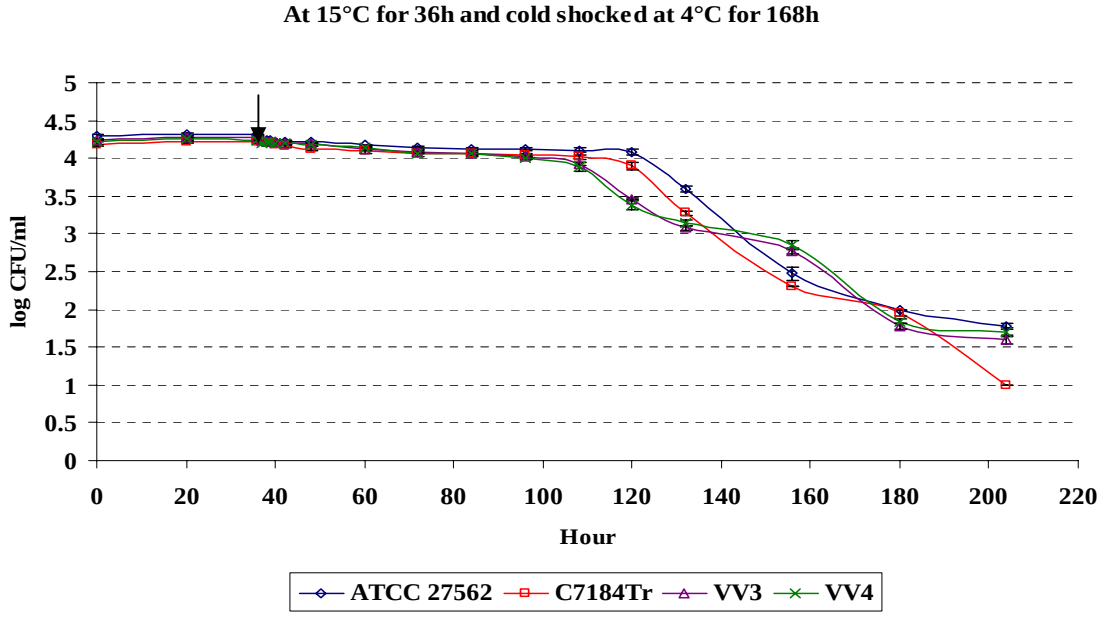

FIG. 14. Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4 in MB when cells were incubated at 15°C for 36 h and cold shocked at 4°C for 168 h, vertical arrow indicated cold shock at 4°C.

IV. EFFECT OF COLDSHOCKING ON SURVIVAL OF *VIBRIO VULNIFICUS* IN SHELLSTOCK OYSTERS

ABSTRACT

The response of *V. vulnificus* cells in oysters to cold shock was evaluated. Depurated oysters were inoculated with three strains of *V. vulnificus* at 10^6 CFU/g oyster meat. Both clinical and environmental strains of *V. vulnificus* presented similar growth numbers in oysters under the same treatment ($p>0.05$). Three cold shock conditions were examined. When oysters were transferred to 35 and 25°C, *V. vulnificus* numbers multiplied by a half log after 10 h and 12 h incubation, respectively. When oysters were shifted to 4°C for one week, *V. vulnificus* exhibited a moderate decline in cells viability. No growth of *V. vulnificus* was observed after oysters were adapted to 15°C for 36 h, but cell viability decreased slightly after they were cold shocked at 4°C for one week. This decline in cell culturability varied from experiment to experiment ($p<0.05$) but was generally on the order of a half to two log reduction after cold shock for one week. This study indicates that cells adapted to 15°C prior to change to 4°C enhanced cell culturability of *V. vulnificus* while it is embedded in oysters. Conditions that favor adaptation to cold temperatures should be avoided in *V. vulnificus* control strategies.

INTRODUCTION

Raw or under cooked oysters pose a human health risk, as these oysters tend to concentrate microorganisms from the surrounding waters via their filter feeding. In the U.S., *Vibrio vulnificus* is an autochthonous microorganisms in the Gulf of Mexico waters where its growth is favored by relatively high water temperatures (85). Gulf Coast oysters (*Crassostrea virginica*) have been reported to be the primary source for *V. vulnificus* related illnesses and deaths. From 1988-1996, Center for Disease Control and Prevention (CDC) received reports of 302 cases of *V. vulnificus* infections from the Gulf Coast States, of these, 141 cases were associated with seafood ingestion, and 96% of these cases were due to raw oyster consumption (19). *V. vulnificus* is the most serious food-borne pathogen with more deaths reported annually from this pathogen than any other seafood-related disease (11). This pathogen is known to cause gastroenteritis, severe wound infection and primary septicemia (99). Nearly all fatal primary septicemia cases were associated with the consumption of raw oysters. The case fatality rate for *V. vulnificus* septicemia exceeded 60% (19). High case-fatality rate by *V. vulnificus* was reported in persons with preexisting liver disease or immunocompromised conditions (165). This bacterium is one of the most costly in terms of economic loss. FDA estimated that *V. vulnificus*-related illnesses cost over $120 million annually, or about 60% of the total cost of shellfish-related illnesses each year (44).

Legal harvesting of oysters is limited to areas free of feacal contamination, however oysters harvested from approved sites may contain *V. vulnificus*. During summer, *V. vulnificus* levels in the Gulf Coast oysters can ranged from 10^3 to 10^5 CFU/g

oyster and appears to replicate within the oyster tissue (170). This bacterium may be presented in oysters in relative low numbers at harvest but may multiply to more hazardous levels if they are exposed to time or temperature abuse. Storage times and refrigerated temperature are known to effect the survival of *V. vulnificus*. Cook et al. (30) reported that *V. vulnificus* could multiply in the oyster shellstock if they are held at temperatures above 13°C. It has been proven that the shorter the time oyster shellstock remained outside refrigeration after harvest, the lower the number of *V. vulnificus* were detected in them (31). Conversely, refrigeration temperature is known to be detrimental to the survival of *V. vulnificus*. The majority of studies that focused on the effect of low temperatures to *V. vulnificus* survival pointed up the need to refrigerate oyster shellstock quickly after harvesting (30, 31, 81, 129). In order to limit the multiplication of *V. vulnificus* in post-harvest oysters, restrictive shellstock temperature control measures that requires refrigeration of Gulf coast oysters after harvesting have been recommended by the shellfish authorities, the Interstate Shellfish Sanitation Conference (ISSC) and implemented by the industry (44). However, fatalities associated to *V. vulnificus* infectious associated with the consumption of raw oysters have not decreased in recent years.

The cold shock phenomenon, a physiological response for survival strategy of living cells to temperature downshift has been reported in several bacteria (51). Bryan et al. (17) found that the effectiveness of rapid chilling or freezing of oysters to eliminate *V. vulnificus* levels in shellfish may be compromised by product handling procedures that permitted cold adaptation. When *V. vulnificus* culture was abruptly shifted from 35°C to 6°C conditions, a non-culturable state was induced, whereas cells

adapted to 15°C prior to change to 6°C condition, remained viable and culturable. Recently, Quevedo et al. (149) reported that chilling of oysters on ice at 7.2°C resulted in a small decline of endogenous *V. vulnificus* and concluded that this technique was not sufficient to eliminate this pathogen in postharvest oysters. Cooling regimes in control measures may potentially induce cold shock adaptation that could enhance *V. vulnificus* survival. It is critical to determine whether the potential for cold shock-mediated survival of *V. vulnificus* exists while it presents in oyster shellstock.

The effect of cold adaptation of this pathogenic microorganism in oysters under industry practices from harvest to consumption, particularly in regard to their survival need to be further investigated. The objective of this study was to examine the effect of cold shock to *V. vulnificus* growth in oyster shellstock.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Two clinical strains of *V. vulnificus*, ATCC 27562 (type strain), and C7184Tr (avirulent, translucent morphotype) provided by J.D. Oliver, University of North Carolina, Charlotte, NC and one environmental strain (VV3) isolated from oyster samples collected from Mobile Bay, Alabama were used in these studies. Cells were grown in marine broth with shaking and maintained in marine agar (MA) (Difco, Detroit, MI) at 35°C.

Depuration of oyster samples

Twelve hundred live shellstock eastern oysters, one year old, 50 to 80 mm in diameter, were provided by the Auburn University Shellfish Laboratory (AUSL), Department of Fisheries and Allied Aquacultures, Dauphin Island, AL. Oysters were acclimated in 19 mm mesh size Durethane oyster bags, (ADPI Enterprise Inc., Philadelphia, PA) for 2 weeks in filtered water flow-through system before depuration. Oysters were scrubbed with a sterile scrub-brush under running tap water to remove extraneous material. Cleaned oysters were treated under UV filtered seawater system (UV flow 3000 μw-sec/cm^2), using a water flow-through system (180 l/h) for 7 days at the AUSL. During depuration, oysters were fed twice a day with marine microalgae concentrate (Shellfish diet 1800, Reedmariculture, Campbell, CA). Salinity, temperature, and dissolved oxygen were determined everyday. The mortality of oysters was checked every morning before feeding.

Bacteriological analysis

Ten oysters were randomly selected before and during depuration once a day for bacteriological analysis as follows: total heterotrophic aerobic plate count, total *Vibrio* counts, and *V. vulnificus* counts. Oysters were washed and scrubbed in tap water with sterile brushes, shucked and weighed in sterile equipment to avoid cross-contamination. Oyster meat sample and the shell liquid from ten oysters were pooled together and homogenized using a MCI mini Waring® blender for 90s. The homogenate was then

diluted 1:1 (wt/wt) in phosphate-buffered saline (PBS) and homogenized for another 30s. Ten-fold dilutions from the homogenate were made with PBS. The sample (100µl) was spread with a sterile glass rod on MA in triplicate for total aerobic plate count. Another 100 µl was spread on Thiosulfate Citrate Bile-Salt and Sucrose (TCBS) agar (Difco) in triplicate for total *Vibrio* plate count. One hundred µl of homogenated oyster was spread on T1N1 agar (1%NaCl, 1% tryptone, 1.5% agar) in triplicate for *V. vulnificus* enumeration. All plates were dried at room temperature until homogenate was fully diffused in the agar. The plates were inverted and incubated 24 h at 35°C.

Vibrio vulnificus counts in oysters were enumerated using a species-specific oligonucleotide probe (*vvhA*) and colony blot hybridization procedure previously described by Quevedo et al (149). After 24 h incubation, colony lifts from T1N1 spread plates were overlaid with Whatman #541 filters papers. Filters with transferred colonies were heated to dryness in a microwave oven for 2 min on the highest setting in lysis solution (0.5 M NaOH, 1.5M NaCl), followed by neutralization in ammonium acetate buffer and washed in standard saline citrate buffer. Filters were treated with proteinase K (20µg/ml) and hybridized with the alkaline phosphatase-labeled *vvhA* probes (DNA Technologies, Aarhus, Denmark) under stringent conditions (56°C) for 1 h. Filters were washed in standard saline citrate buffer with 1% sodium dodoecyl sulfate at hybridization temperature, followed by additional washing with standard saline citrate buffer at room temperature to overcome nonspecific hybridizations signal. NBT/BCIP (nitrobluetetrazolium/5-bromo-4-chloro-3-indolylphosphate toluidinium) substrate (Roche Diagnosis, Indianapolis, Ind.) was used for detection of hybridization positive colony. Control filters were developed concurrently for all the time points.

Artificial inoculation

After one week, live depurated oysters were cooled on ice and immediately transported to the Bacterial Genomics Laboratory, Department of Fisheries and Allied Aquacultures, Auburn University, AL for cold shock experiments. Live depurated oysters were inoculated with *V. vulnificus* by self-inoculation filtration. Four 10 gallon (38 L) aquaria containing 27 L of autoclaved seawater was prepared. One hundred fifty oysters were placed into each aquarium at 25°C and allowed to acclimate up to 24 h. A fresh culture of each strain of *V. vulnificus* was grown in MB until exponential growth phase (10^8 CFU/ml, OD_{450}=0.15) was reached. Three liters of *V. vulnificus* culture were introduced into each aquarium at approximately 10^6 CFU per gram of oyster. Control aquaria contained oysters without introduced *V. vulnificus* and were acclimated as described for test aquaria.

Cold shock experiments

After *V. vulnificus* inoculations, oysters remained at 25°C overnight prior to temperature shift. To examine cold shock effect on *V. vulnificus* survival in oysters, three different cold shock conditions were compared. The first experiment, oysters were transferred to 35°C for 10 h, then chilled by ice-water immersion until the internal temperature in oysters reached 4°C. Oysters were then transferred to a 4°C incubator for 168 h. In the second experiment, oysters were transferred to 25°C for 12 h then chilled to 4°C and incubated for 168 h. For the last experiment, oysters were transferred to 15°C

for 36 h, and chilled to 4°C as described above and incubated at 4°C for 168 h. The internal temperature of the oysters was monitored using a digital thermometer inserted through a hole drilled in the shell to the meat of oysters throughout the study (5). The external ambient air temperature was also recorded and the pH of oyster meat measured periodically.

V. vulnificus enumeration in oysters

At various time intervals from t = 0 to t = 168 h, *V. vulnificus* counts in oysters were enumerated using a species-specific oligonucleotide probe (*vvhA*) and colony blot hybridization procedure previously described by Quevedo et al (149).

Statistical analysis

All bacterial samples were analyzed in triplicate. The standard error was calculated for all of the treatment replications. To determine if difference between data set existed, the Randomized Complete Block Design (RCBD) using F-test was performed using one-way ANOVA analysis procedure in the Statistical Analysis System, SAS 9.1.3 for Windows (SAS Institute, Cary, NC).

RESULTS

Total bacteria, total *Vibrio,* and *V. vulnificus* during depuration

Before oysters were depurated by UV filtered seawater system, the total bacteria count was 10^4 CFU/g oyster meat. After depuration for 7 days, CFU/g oyster declined about two log units. Similar decline in CFU/g oyster of total *Vibrio* count from TCBS plate was observed. Numbers of *V. vulnificus* in oysters was around 10^2 CFU/g oyster before depuration. After five days of depuration, no CFU/g oyster of *V. vulnificus* was detected (Fig. 15).

pH of oyster meat

The pH of oysters after artificially inoculation with *V. vulnificus* at 25°C was 6.6. After oysters were transferred to 35°C for 10 h the pH slightly increased, but then decreased to 6.2 after oysters were kept at 4°C for one week. When oysters were kept at 25°C for 12 h, pH remained constant. After oysters were shifted at 4ºC for one week, pH of oyster meat decreased from 6.6 to 6.2. When oysters were transferred to 15ºC for 36 h, the pH was constant. After oysters were shifted to 4ºC, the pH decreased from 6.6 to 6.3 (Fig. 16). The pH range across the study (pH 6.2 to 6.6) was lower than the optimal pH described for *V. vulnificus* (pH 7.5 to 8.0).

V. vulnificus growth in shellstock oysters during cold shock

Colony hybridization was used to enumerate *V. vulnificus* in oysters during cold shock. No *V. vulnificus* was detected in control oysters at anytime during any treatment. The average initial *V. vulnificus* numbers in artificially inoculated oysters for each experiment was 1×10^6 CFU/g oyster. When *V. vulnificus*-contaminated oysters were incubated at 35°C for 10 h, cells increased by one half log unit. After oysters were placed at 4°C cell numbers declined moderately by two log units, with the final counts of 3×10^4 CFU/g oyster meat (Fig 17). Similarly, when *V. vulnificus* was incubated at 25°C for 12 h, cell numbers slightly increased but after cold shock at 4°C numbers decreased by 1.5 log units, with the final counts of 6.5×10^4 CFU/g oyster meat (Fig 18). When *V. vulnificus* contaminated oysters were acclimated at 15°C for 36 h *V. vulnificus* numbers remained constant, and slightly decreased only by a half log unit, with final numbers around 5.3×10^5 CFU/g oyster meat after downshift to 4°C (Fig 19). Overall, *V. vulnificus* numbers were not significantly different among strains (ATCC 27562, C7184Tr, VV3) after cold shock for one week under the same temperature treatment ($p>0.05$). The decline in cell culturability varied from experiment to experiment ($p<0.05$) but was generally on the order of a half to two log reduction after cold shock for one week.

DISCUSSION

The present study successfully demonstrated a consistent method to artificially inoculate *V. vulnificus* into live oysters. Numbers of *V. vulnificus* found in oyster shellstock were very low (10^2 CFU/g oyster) before depuration treatment. After 5 days of depuration, the cells were not detected by colony hybridization. Previous literature reported that the sensitivity of DNA probe colony hybridization assays for detection of *V. vulnificus* approached 10 CFU/g oyster. The FDA currently recommends that postharvest-treatment should reduce *V. vulnificus* to non-detectable level (<30 CFU/g) (45). This result revealed that the depuration treatment used in this study was successful in eliminating *V. vulnificus* to non-detectable levels.

When *V. vulnificus*-contaminated oysters were kept in a warm environment (at 35 and 25°C), even for 10 to 14 h, the numbers of *V. vulnificus* in oysters increased by a half log. This finding supports previous reports of how elevated temperature abuse in oysters contributes to an increase in endogenous *V. vulnificus* levels in post-harvest shellstock oysters (30, 31, 81). Recently, Gooch (53) reported that *V. vulnificus* multiplied in live oysters held at 26°C with an increase of 1.2 log CFU/g 10 h post harvest. There was evidence that *V. vulnificus* growing *in vitro* had a faster growth rate than *in vivo* (14). The difference in *V. vulnificus* numbers in oysters may be attributed to oyster defense mechanisms, specifically in hemocytes which posses strong phagocytic properties and play a role in the survival of *V. vulnificus* in oyster tissues (47). Vibrio specific Bacteriophages are another factor that may effect populations of *V. vulnificus* within oysters (39).

The decrease in the persistence of viable *V. vulnificus* cells by one to two orders of magnitude was observed when oysters were transferred to cold temperature (4°C). This result is in agreement with Kasper and Tamplin (81), who reported one log reduction of natural *V. vulnificus* numbers during cold storage at 4°C. The same authors also concluded that this temperature is critical to control *V. vulnificus* growth in oyster shellstock. In this study, however, the reduction observed at this level would still leave large concentrations of *V. vulnificus* remaining in oysters. These data show that the endogenous population of *V. vulnificus* might behave similarly to the laboratory grown culture when it responded to the cold during embedded in oysters.

Vibrio vulnificus remained culturable but no growth was observed when oysters were transferred to 15°C. After a temperature down shift from 15°C to 4°C for one week, *V. vulnificus* numbers in oysters decreased by less than a half log unit. This result showed that *V. vulnificus* final counts at this temperature condition (15°C to 4°C) was one log higher in cell survival when compared with 35 and 25°C to 4°C. Data presented here documented cell adaptation to cold temperatures since 15°C enhanced cell culturability. This finding is in agreement with Datta and Bhadra (36) who reported that priming of cells at 15°C allow bacteria to adapt and grow at 10°C. Exposure to an intermediate temperature (15°C) may cause a cold adaptive response helping *V. vulnificus* remain in the culturable state when exposed to a much colder temperature (17). It is well documented that low temperatures can cause *V. vulnificus* cells to enter the viable but non-culturable (VBNC) state (182, 185). The decrease in cell culturability that was observed under refrigeration conditions during the study could be attributed to the VBNC state (131). The methodology used only accounted for culturable cells and the

effect, if any, of the VBNC was not measured. If the reduction in number of cells was by a reversion into the VBNC state, no net reduction may have occurred. Further investigation is necessary to evaluate the VBNC state during cold shock under shellstock temperature control recommended by ISSC. Moreover, the pH of the oysters slightly decreased throughout the cold shock study but probably did not effect *V. vulnificus* growth and survival in the current study. Optimum pH for *V. vulnificus* growth is around pH 7 and minimum growth pH is pH 5.0 (95). Goatcher (49) found pH not to be a factor in vibrio cell death in oysters following cold shock.

It was clear in this *in vivo* study that the capability for this pathogen to survive in oysters at cold temperature for a considerable period of time exists. Based on this result high temperatures and cold adaptation conditions should be avoided to reduce *V. vulnificus* numbers in oysters. This study suggests that cooling oysters at 4°C after harvesting could limit the multiplication of *V. vulnificus* while embedded in oyster shellstock, but might not be effective in order to reduce *V. vulnificus* numbers to the recommended limit by FDA (<30 CFU/g oyster). The guideline from ISSC should be evaluated due to the potential for cold adaptation-mediated survival of *V. vulnificus*.

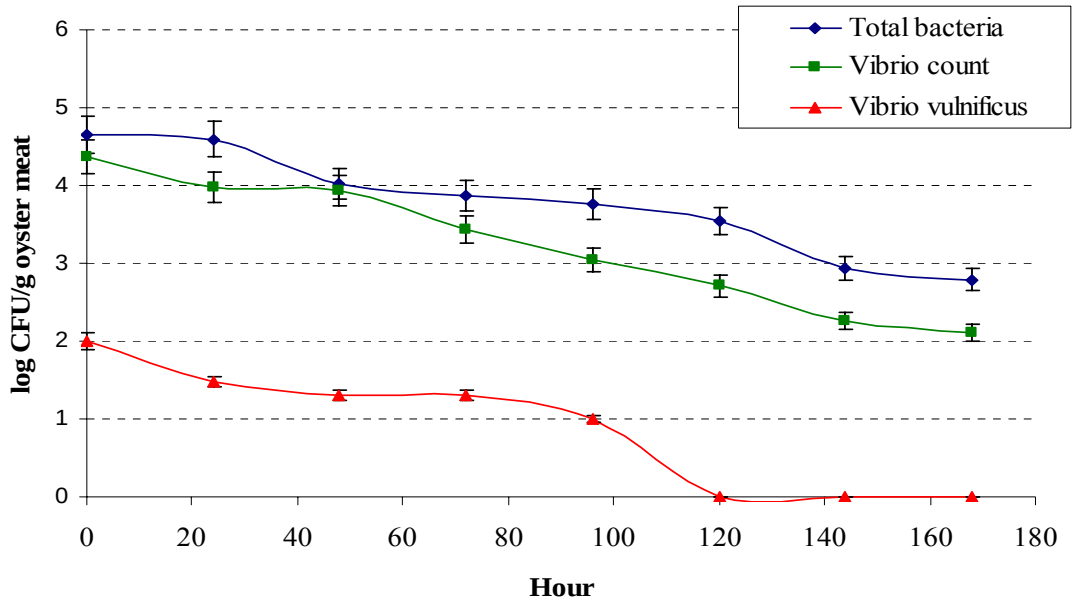

Fig. 15. Total heterotrophic bacteria, total *Vibrio*, and *V. vulnificus* (CFU/g oyster meat) during depuration of eastern oysters (*Crassostrea virginica*).

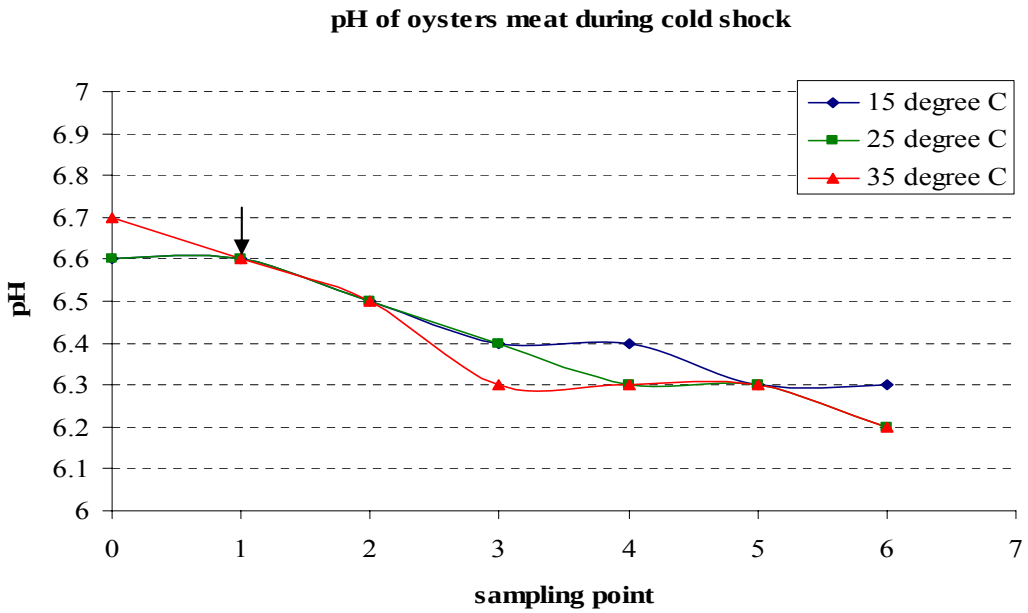

Fig. 16. pH of oyster meat during cold shock experiment, vertical arrow indicated cold shock point.

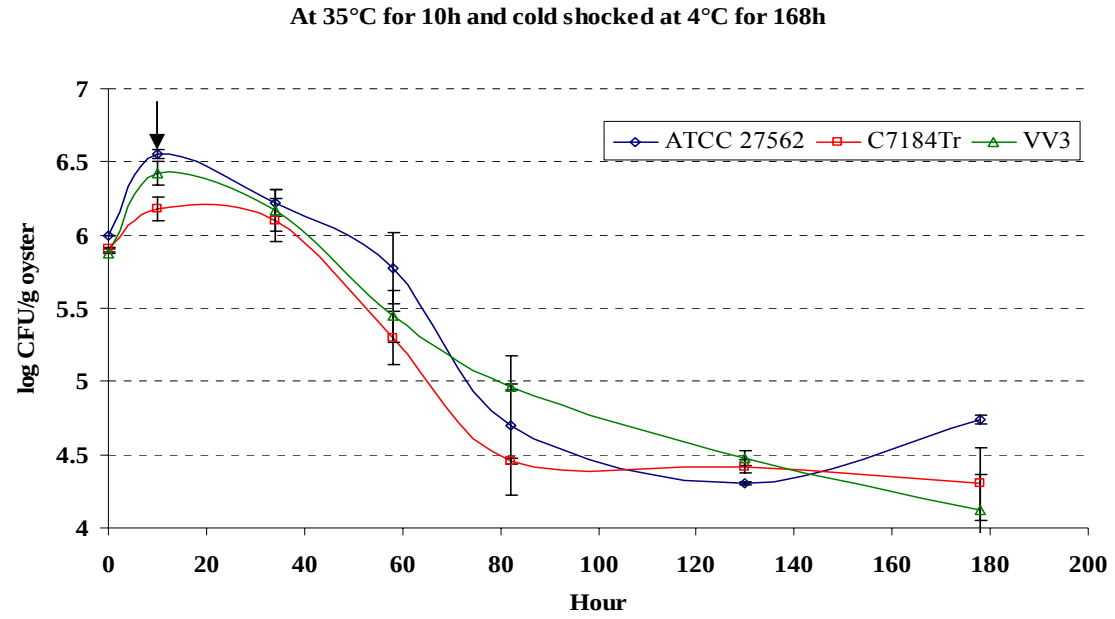

FIG. 17. Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, and VV3 in oysters when oysters were incubated at 35°C for 10 h and cold shocked at 4°C for 168 h, vertical arrow indicated cold shock at 4°C.

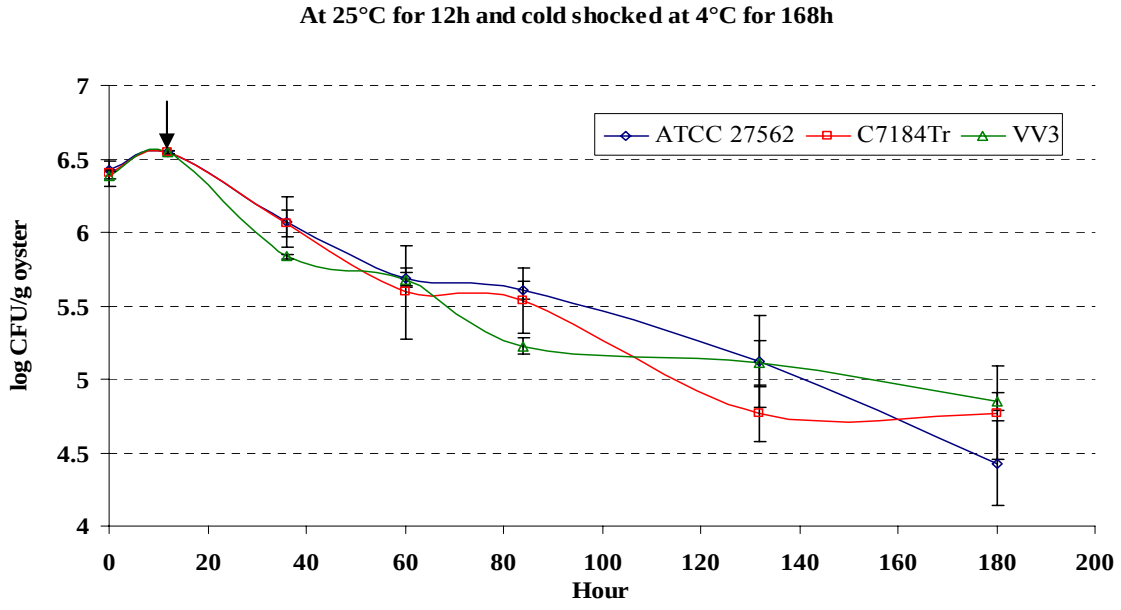

FIG. 18. Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, and VV3 in oysters when oysters were incubated at 25°C for 12 h and cold shocked at 4°C for 168 h, vertical arrow indicated cold shock at 4°C.

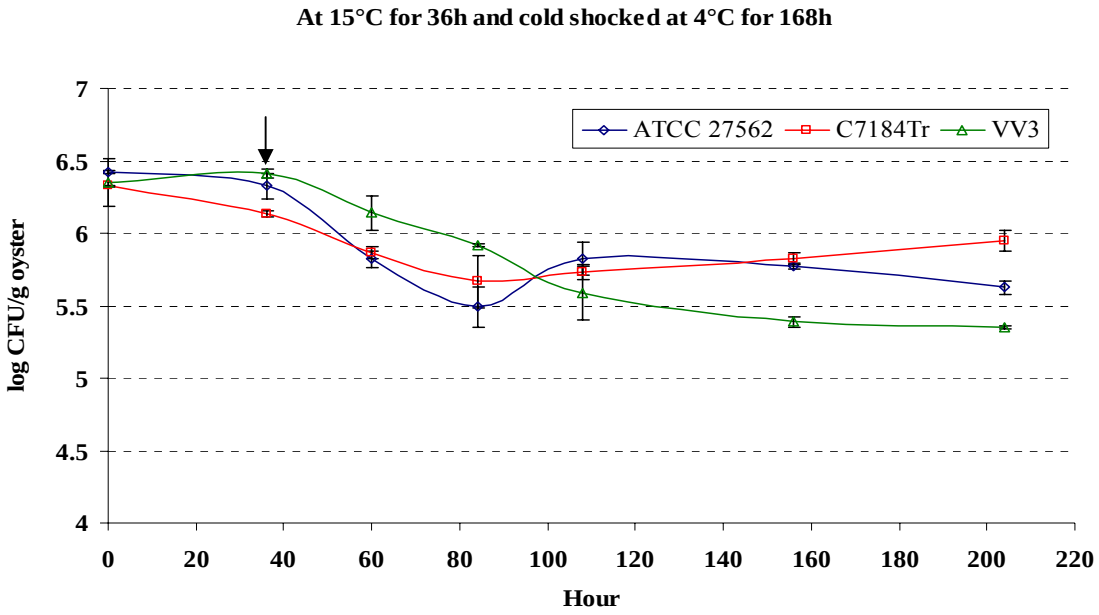

FIG. 19. Cold shock-survival response of *V. vulnificus* strain ATCC 27562, C7184Tr, and VV3 in oysters when oysters were incubated at 15°C for 36 h and cold shocked at 4°C for 168 h, vertical arrow indicated cold shock at 4°C.

V. *IN VITRO* COLD SHOCK GENE EXPRESSION OF *VIBRIO VULNIFICUS*

ABSTRACT

Four cold shock genes (*csp*$_s$) encoding small cold shock proteins of *V. vulnificus* were cloned. *In vitro* gene expression of *csp*$_s$ and stress-related genes in four different strains of *V. vulnificus* was analyzed by reverse transcriptase PCR. Two different temperature profiles were compared. First, *V. vulnificus* cultures were incubated at 35°C for 10 h and then cold shocked at 4°C for 168 h. The transcript levels of *csp5,* and a stress regulator gene, *rpoS*, were found to be constant in both clinical and environmental strains throughout the study. The synthesis of *katG*, a catalase gene, was detected at different time points after cold shock, while the transcript of *oxyR*, a transcriptional activator of oxidative stress response, was expressed at all time points. The induction of *csp1* and *csp3* varied among strains, while *csp4* transcript was down-regulated after cold shock in all strains. In the second experiment, after overnight incubation at 35°C, *V. vulnificus* cultures were transferred to 15°C for 36 h and then cold shocked at 4°C for 168 h. A constitutive expression of *csp1* and *rpoS* was observed, while transcription of *csp3* and *csp5* was discontinued after cold shock at 4°C. The synthesis of *oxyR* mRNA was induced during the acclimation step at 15°C. *KatG* gene expression was induced after shift to 4°C in some strains. The expression of *csp4* was repressed after cold shock at 4°C. The constitutive expression of *16SrRNA* indicated that *V. vulnificus* was viable during cold shock treatment. These results revealed that gene expression in *V. vulnificus* varied during cold shock *in vitro*. It can be concluded that the cold shock genes of *V. vulnificus* tested in this study are not cold inducible.

INTRODUCTION

Temperature has wide-ranging effects on microbial growth and survival, and therefore, bacteria have developed responses that allowed them to adapt to change in temperature. Bacteria can exhibit a cold shock response by altering gene expression in response to a shift to low temperature (6, 7, 55, 80, 109, 140). Cold shock response inhibits the synthesis of most cellular proteins resulting in a growth lag period, called acclimation phase (72). Cold shock proteins are synthesized to overcome the deleterious effects of cold shock (146). Cold shock proteins (Csps) are small proteins (around 7 kDa) and the Csp family has been found in nearly all bacteria (41). Several functions have been postulated for Csps. These proteins could act as a transcription activators, RNA chaperones and could help to minimize secondary folding (55, 56). Moreover, Csps has been reported to play a role in protection against freezing (186). Csps are not expressed only at low temperatures; they are also induced by other environmental stresses. Most of the cold shock response studies have been performed with *Escherichia coli* and *Bacillus subtilis* as model systems (51). Out of the nine Csps found in *E. coli*, five were not induced at low temperatures. CspA is considered essential for cold adaptation and has been found to be expressed during the stationary growth phase (50, 146). CspD was induced when under nutritional stress during the stationary growth phase (190). *Bacillus subtilis* possesses three Csps: CspB, CspC, and CspD that are induced upon cold shock (57). Recently, Datta and Bhadra (36) investigated the major cold shock proteins in *V. cholerae* and reported that when *V. cholerae* cells were shifted from 37°C to 15°C, CspV and CspA were produced at low temperature and concluded

that *cspV* was a cold shock inducible gene and was tightly regulated at the transcription level.

Vibrio vulnificus is a gram negative naturally occurring bacterium that causes septicemia, wound infections, and gastroenteritis. Transmission typically occurs through ingestion of contaminated raw or undercooked seafood, especially raw oysters harvested from the Gulf of Mexico during warm weather months (151). *Vibrio vulnificus* infections can develop into a fatal septicemia, especially in patients who suffer from immune disorders, liver disease, or hemochromatosis with death occurring within 48 h after onset of symptoms (48). The incidence of outbreak of *V. vulnificus* due to oysters is a significant concern of the oyster industry and public health agencies. Numerous studies have investigated the response of *V. vulnificus* to low temperatures (17, 110, 118, 128, 130, 149, 181, 185) although most of these studies focused on the viable but non-culturable (VBNC) state that can be induced in *V. vulnificus* when subjected to low temperatures. This VBNC state might explain the difficulty of culturing this bacterium from marine samples during winter months (for a recent review on the VBNC state see Oliver, 2005 (131)). However, the VBNC state is not the only response to cold temperatures observed in *V. vulnificus.* In 1995, McGovern and Oliver (110) showed that when *V. vulnificus* is subjected to a temperature downshift from 23°C to 13°C, protein synthesis is reduced but new cold-induced proteins are expressed. In addition, an adaptive response to cold temperatures in *V. vulnificus* has been described by Bryan et al.(17). Their study showed that *V. vulnificus* cells shifted from 35°C to 4°C entered the VBNC state in few days. However, when cells were acclimated from 35°C to 15°C and then shifted to 6°C, they remained viable for the duration of the study. Their

results also suggested that cold-induced proteins played a role in this cold adaptive response. However, no evidence of a cold shock inducible gene was reported and no gene involved in the cold shock response in *V. vulnificus* has yet been identified. To date, the molecular mechanisms by which *V. vulnificus* adapt to low temperature are not well understood. To better explain the physiological mechanisms response to cold shock at the molecular level, the small cold shock family genes (csp_s) obtained from two clinical and two environmental strains of *V. vulnificus* were cloned. Analysis of *V. vulnificus* cold shock genes encoding cold shock proteins (Csps) that exhibited significant increased or decreased in mRNA abundance caused by the temperature downshift were performed. Moreover, *rpoS*, the stress regulator which is used by bacteria in the adaptive response through various environmental stresses was examined. *KatG*, a periplasmic catalase gene (HPI) and *oxyR*, a positive regulator of catalase gene (both involved in oxidative stress) were also analyzed in response to exposure to cold temperature. Reverse transcription PCR was used to analyze gene expression of all these genes in all strains used in this study.

MATERIALS AND METHODS

1) Cloning of the small cold shock family genes (*csp*$_s$) of *V. vulnificus*

Bacterial strains and growth conditions

Two clinical strains of *V. vulnificus*, ATCC 27562 (type strain), and C7184Tr (avirulent, translucent morphotype, provided by J.D. Oliver, University of North Carolina, Charlotte, NC) were used in this study. Two environmental strains (VV3, VV4) isolated from oyster samples collected from Mobile Bay, AL were also included. Cells were grown in marine broth (Difco, Detroit, Mich.) at 35°C.

Primer design

Putative cold shock genes were identified in *V. vulnificus* based on amino acid-amino acid similarities with *V. cholerae* cold-shock proteins (Table 1). Open reading frame (ORF) sequences from five putative cold shock genes (*csp1-5*), (see Appendix 2 to 6), were obtained from the whole *V. vulnificus* genome sequence available at the National Center for Biotechnology Information (NCBI) (GenBank accession no. NC0044459, NC004460 for *V. vulnificus* CMCP6, and GenBank accession no. NC005139, NC005140 for *V. vulnificus* YJ016) (23, 91) (Table 2).

DNA extraction

DNA was extracted from the four strains of *V. vulnificus* (ATTC 27562, C7184Tr, VV3, and VV4) using DNeasy kit following the manufacturer's instructions (QIAGEN, Valencia, CA). Briefly, 1.5 ml of *V. vulnificus* cells were placed in a 2 ml microcentrifuge tube centrifuged for 10 min at 3,000×g. The supernate was extracted and resulting pellet was resuspended in 180 µl of buffer ATL, plus 20 µl of proteinase K (10 mg/ml). The sample was incubated at 55^{o}C for 3 h until cells were completely lysed or appeared viscous. Samples were vortexed for 15 s, with 200 µl of lysis buffer, followed by incubation at 70°C for 10 min. The resulting DNA precipitated with 200 µl ethanol 95% (vol/vol). Washing buffers (AW1 and AW2) were added sequentially to the DNeasy Mini spin column and centrifuged at 3,500×g. The supernatants were discarded, and the DNA was resuspended in distilled water. DNA was quantified by Gene Quant spectrophotometer (Amersham Pharmacia Biotech, Sweden) and stored at -20°C for amplification.

PCR condition and DNA amplification

Genes were PCR amplified in a total volume of 50 µl. Unless otherwise stated, all PCR reagents were purchased from Promega (Promega, Madison, WI). One hundred ng of DNA were used as template in a reaction containing; 0.2 U of Taq polymerase, 0.2 mM of dNTP mix, 1X PCR buffer, 2.5 mM $MgCl_2$ and 0.2 mM of each primer. Primers were designed in order to amplify the entire ORFs (Table 2). The reaction mix

was cycled through the following temperature profile: 95°C for 5 min; 95°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec for a total of 35 times. The PCR reaction was ended at 72°C for 10 min and thereafter cooled down to 4°C. PCR products were analyzed through a 1% agarose gel. Gels were run for about 60 min at 102 V in TAE 1X buffer (0.04 M Tris/acetate, EDTA 1mM) mixed with ethidium bromide (0.5µg/ml). A 100-bp ladder was used as a standard marker. Following electrophoresis, the PCR products were visualized under a UV (245 nm) transilluminator.

DNA purification

The selected DNA bands were purified from the gels using the GENECLEAN III kit (Q-BIO gene, Carlsbad, CA) followed the manufacturer's instruction. The DNA band from the agarose gel was excised and transferred to polypropylene tube. Six hundred µl of NaI solution was added to cover the gel and incubated at 50^{o}C in water bath for 5 min. After the agarose block was melted, the NaI/DNA solution was resuspended with 5 µl of EZ-GLASSMILK, mixed by vortexing 1 min, and the NaI/DNA/EZ-GLASSMILK solution was incubated at room temperature for 5 min (vortexed every two min). The solution was centrifuged at 11,000×g for 5 sec, the supernatant was discarded, and the pellet containing the EZ-GLASSMILK with bound DNA was saved. The washing step was done twice by adding 500 µl of provided washing solution, then the solution was resuspended and centrifuged at 10,000×g for 5 sec, and the supernatant was discarded. The resulting pellet was air dried and resuspended in 5 µl of ddH_2O. After centrifugation

at 11,000×g for 1 min the supernatant of DNA was removed and transferred to a new tube.

Cloning procedure

Ligation of purified DNA to linearized plasmid

The ligation mixture was prepared by using PGEM-T-easy vector system (Promega). The mixture of 5 µl of Genecleaned DNA from purification step included 1 µl of pGEM-T easy vector (50ng/µl), 5 µl of 2X rapid ligation buffer, and 1 µl of T4 DNA ligase (3U/µl) was incubated at room temperature for 3 h.

Transformation

The ligation mixture was mixed with 50 µl of JM 109 competent cells (Promega) and incubated on ice for 20 min. The bacterial-plasmid mixture was heat shocked by placing the mixture in a 42°C water bath for 45 sec without shaking. Tubes were removed and incubated on ice for another 5 min. The transformed bacterial mixture was pooled in a tube containing 900 µl Luria-Betani Medium (LB), incubated in a water bath at 37°C for 1 h, centrifuged for 1 min at 500×g, and resuspended in 75 µl of LB.

Plating of recombinant *E. coli* cells

Transformed *E. coli* cells were plated onto LB + ampicillin + IPTG/X-GAL agar and incubated at 37°C for 16-18 h for blue/white screening. White colonies were picked using a toothpick. Each colony was transferred to 5 ml LB broth/ 5µl ampicillin (50mg/ml), and incubated at 37°C for 16-18 h. Two ml of cell cultures were kept in 10% sterile glycerol at -80°C, while 3 ml of the cultures were used for plasmid isolation.

Plasmid isolation

Aurum™ plasmid mini kit (Bio-Rad laboratories, Hercules, CA) was used for plasmid isolation. Briefly, 1.5 ml of cell cultures was pelleted by centrifugation at 11,000×g for 5 min at room temperature, and the supernatant was removed by pipetting. Bacterial cell pellets were resuspended in 250 µl of resuspension solution by vortexing. The suspensions were mixed with 250 µl of lysis solution until the solution became viscous and slightly clear. The solutions were mixed with 350 µl of neutralization solution until a visible precipitate was formed. Then the neutralized lysate was centrifuged for 5 min. The supernatant (plasmid DNA) was washed twice with washing solution via a plasmid mini column then transferred to a new 1.5 ml eppendorf tube. Fifty µl of sterile deionized water was added to the column, incubated for 1 min, and centrifuged to elute the plasmid. The mini column was discarded and the eluted plasmid DNA stored at 4°C. Plasmid DNA samples were quantified using a GeneQuant Spectrophotometer (Amersham Pharmacia Biotech). Plasmid DNA was stored at -20°C.

Clone characterization/testing

In order to check the presence of insert, the *EcoRI* restriction digestion analysis was performed. The plasmid vector has two *EcoRI* restriction sites on its sequence. DNA sequences were inserted between these two *EcoRI* recognition sites. The *EcoRI* restriction digestion was used to digest the ligated DNA insert. Briefly, a mixture of 5µl of plasmid DNA, 1 µl of *EcoRI* enzyme (12U/µl), 2 µl of 10X buffer and 12 µl of deionized water was incubated at 37°C for 4 h. The restriction digestion products were resolved on 1 % agarose gels at 102 V in TAE 1X buffer (0.04 M Tris/acetate, EDTA 1mM) stained with ethidium bromide (0.5µl/ml) for 30 min. A 100-bp ladder was used as a standard marker. Following electrophoresis, the gel was visualized under a UV (245 nm) transilluminator.

Sequencing and alignment

Plasmid concentrations were adjusted to 200 ng/µl and sequenced at the Auburn University Genomics & Sequencing Lab (AU-GSL) using the T7 promoter and the ABI 3100 Genetic Analyzer. The sequences were assembled and aligned with the Vector NTI Advance 9.0 software (Informax, Frederick, MD). The nucleotide sequences of the *csp* genes of *V. vulnificus* were compared with that of other bacterial species using the BLAST program (Basic Local Alignment Search Tool) (1).

In vitro gene expression of cold shock genes and stress-related genes of *V. vulnificus* during cold shock

Bacterial collection during cold shock treatment

Expressions of four putative cold shock genes (csp_s) and additional genes involved in stress response; *rpoS*, *oxyR*, and *katG* of *V. vulnificus* were investigated under cold shock conditions. In order to mimic the conditions *V. vulnificus* might experience under the ISSC guidelines, the experiment used the following temperature profiles: a) cells (10^4 CFU/ml) were incubated at 35ºC for 10 h and b) at 15ºC for 36 h, and then transferred to an incubator shaker at 4ºC for up to 168 h. Samples were taken at different time points and kept in RNA*later* RNA stabilization solution (Ambion, Austin, TX.) and stored at 4ºC until RNA isolation analysis. All four strains were analyzed simultaneously.

Total RNA isolation and DNA elimination

RNA Plus mini kit (QIAGEN, Valencia, CA) was used to extract total RNA from all the bacterial samples (according to the manufacturer's protocol). Each bacterial sample was homogenized using QIAshredder homogenizers, and the lysate transferred to a gDNA Eliminator spin column. The column was centrifuged at 5,600×g, then discarded. The filtrate was mixed with 70% ethanol, and 750 µl of the sample was transferred to an RNeasy spin column, and centrifuged for 15 sec at 5,600×g. The filtrate

was discarded. Seven hundred µl of buffer RW1 was added to the column, and centrifuged at 5,600×g for another 15 sec to wash the spin column membrane. The filtrate was discarded. Then 500 µl of Buffer RPE was added to wash the column and centrifuged at 5,600×g. This wash step was repeated once. Then the RNA was eluted with 50 µl RNase-free water by centrifugation. RNA concentrations were quantified spectrophotometerically and stored at -80ºC. In order to eliminate carryover DNA in the RNA extract, 1 µg of RNA was treated for 30 min at 37°C with 150 U of DNase (Ambion). The DNase was then inactivated by heating at 65°C for 10 min.

Reverse transcriptase PCR

The presence and expression of *csp1*, *csp3*, *csp4*, *csp5*, *rpoS*, *oxyR*, and *katG* was tested using reverse transcriptase-PCR (RT-PCR) amplification. cDNA was synthesized using the cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ). According to the manufacturer's instructions, random hexamers were used to synthesize the first strand cDNA. Twenty µl of RNA solution (5µg total RNA plus RNase-free water) was heated to 65°C for 10 min, and then was chilled on ice. The cDNA synthesis reaction mixture consisted of 20 µl total RNA (3 µg/µl), 1 µl of primer, 1 µl of DDT solution, and 11 µl of Bulk first-strand reaction mix. The cDNA synthesis reaction was incubated at 37°C for 1h, then the completed first-strand reaction was heated to 90°C for 5 min to denature the RNA-cDNA duplex, and the reverse transcriptase was inactivated and chilled on ice.

PCR amplification was performed as followed: 2 µl of the cDNA was used as template. Primers targeting the eight genes were used for specific PCR (Table 3). The

absence of DNA contamination in RNA samples was verified by PCR using RNA as a template and internal control primers UFUL and URUL. A PCR negative control (no nucleic acid template) was also carried out. *V. vulnificus* DNA was used as a positive control for PCR. The expression level of the 16SrRNA gene was used as constitutive expression control (internal control) in the RT-PCR. All PCRs and RT-PCR were conducted using the appropriate annealing temperature according to the primer set used. PCR amplification followed this profile: initial heating step at 95°C for 5 min followed by 35 cycles of denaturation (95°C for 30 sec), annealing (temperature varying according to the primers used (Table 3) for 30 sec), and extension (72°C for 45 sec). The final extension ended at 72°C for 10 min and thereafter cooled to 4°C. The amplification product was separated by horizontal gel electrophoresis in a 1 % agarose gels at 102 V in TAE 1X buffer (0.04 M Tris/acetate, EDTA 1mM) stained with ethidium bromide (0.5µl/ml) for 30 min. A 100-bp ladder was used as a standard marker. Following electrophoresis, the gel was visualized under a UV light (245 nm) transilluminator.

RESULTS

Cloning of *csp$_s$* genes from *V. vulnificus*

By comparing the genome sequence of *V. vulnificus* strain YJ016 and CMCP6 (23, 91) with *V. cholerae*, several putative cold shock genes were identified. Using sequence data for the whole genome of *V. vulnificus* strain YJ016 and CMCP6 (23, 91), four putative cold shock genes in *V. vulnificus* YJ016 and five putative cold shock genes

in *V. vulnificus* CMCP6 were recognized (Table 2, nucleotide sequence see appendix 2-6). The four putative cold shock genes obtained from strain YJ016 shared 100% nucleotide similarity with those of strain CMCP6. These five predicted cold shock genes encoding for cold shock proteins were designated as *csp1*, *csp2*, *csp3*, *csp4*, and *csp5*. The entire sequences *csp1* to *5* of *V. vulnificus* were amplified from clinical (ATCC 27562, C7184Tr) and environmental strains (VV3, VV4). All *V. vulnificus* isolates produced PCR products of the expected size, except *csp2*. This gene identified in CMCP6 strains was not found in any of the four *V. vulnificus* strains analyzed, and therefore was not included in the study. PCR products of these four cold shock genes were amplified from the clinical and the environmental strains of *V. vulnificus* and were cloned. The isolates were sequenced and the amplified DNA sequences were compared for homologous amino acid sequences using BLAST. A BLAST-X analysis of predicted Csp sequences indicated that Csp1 obtained from *V. vulnificus* ATCC 27562, C7184Tr, VV3 and VV4 strains was 100% identical to cold shock protein of *V. vulnificus* CMCP6 and YJ016 (GenBank accession number NP761574 and NP 934297), and 81.4% identity to cold shock transcriptional regulator CspA from *V. cholerae* (TIGR accession number VCA0166). Csp3 showed 98% similarity with the sequence of cold shock-like protein, CspD of *V. vulnificus* CMCP6 and YJ016 (GenBank Accession number NP760979 and NP935117), and 81.6% identity to the cold shock-like protein from *V. cholerae* CspD (TIGR accession number VC1142). Csp4 protein sequence revealed 98% matching with the protein sequence of *V. vulnificus* cold shock protein from GenBank accession number NP762479 and 937123, and was 88.4% identical to a cold shock DNA-binding domain protein of *V. cholerae* (TIGR accession number VCA0184). The deduced sequence of

Csp5 was found to be 98% identical to cold shock protein of *V. vulnificus* CMCP6 and YJ016 (GenBank accession number NP762464 and NP937108), and was 85.7% identity to cold shock protein CspV of *V. cholerae* (TIGR accession number VCA0933) (Table 1). The deduced amino acid sequences for *csp1*, *csp3*, *csp4*, and *csp5* resulted in a 7 to 8 kDa proteins. The *csp1* and *csp3* genes are presented in the large chromosome (chromosome I), whereas the *csp4* and *csp5* are presented in the small chromosome (chromosome II). The M_w and isoelectric point (pI) were also calculated and found to be similar to the values described for *V. cholerae* CSPs (Table 1) (36).

In vitro expression of cold shock genes and stress-related genes of *V. vulnificus* during cold shock

Gene expression analysis of cold shock genes and stress-related genes were investigated in pure culture using RT-PCR technology at two different temperature profiles. In the first experiment, *V. vulnificus* was incubated at 35°C for 10 h (T_0) and cells transferred to 4°C for one week. The mRNA transcripts of the *16SrRNA* (house keeping gene), *rpoS*, a stress regulator gene, and *csp5*, a cold shock gene were detected and remained constant through the temperature downshift. Expression levels of the transcriptional activator of oxidative stress response, *oxyR,* were also detected at all time points although some intensity variations were observed. The expression of *csp1* and *csp3*, encoding cold shock proteins of *V. vulnificus* varied between strains. *Csp1* and *csp3* mRNA synthesis was induced 24 h after cold shock only in C7184Tr strain. These genes appeared to be constitutively expressed in ATCC 27562 and in both environmental

strains. In addition, induction of *katG* (a catalase gene) was observed in all strains of *V. vulnificus* but at different time points after cold shock from 35°C to 4°C. In contrast, cold shock appeared to repress *csp4* expression in all *V. vulnificus* strains used (Figure 20-21).

The second experiment investigated gene expression when aliquots of *V. vulnificus* cells grown overnight at 35°C (T_{0A}) were transferred to 15°C for 36 h (T_{0B}), and then cold shocked at 4°C for one week. Continuous expression of *16SrRNA*, *rpoS*, and *csp1* was observed at 35°C, 15°C, and 4°C in clinical and environmental strains across the duration of the study. Additionally, *csp3* and *csp5* transcripts were constantly detected from 35°C through the acclimation period at 15°C and then at 4°C although some variations in expression levels at 4°C were observed. *Csp4* expression was detected during the first cold shock (35°C to 15°C) and was down-regulated after transferred to refrigerate conditions. *OxyR* and *katG* showed different patterns of gene regulation. While *oxyR* mRNA was induced during the acclimation phase at 15°C, *katG* mRNA was induced after downshift to 4°C in some strains. Once expression of *oxyR* occurred, gene expression remained fairly constant in all strains. Interestingly, *katG* showed an induction pattern 48h after cells were transferred to 4°C only in the environmental strain VV3. On the contrary, VV4 expressed *katG* immediately after cold shock at 4°C but transcripts levels steady decreased overtime. Expression of *katG* in clinical strains was seldom detected (Fig.22-24).

DISCUSSION

Several food-borne pathogens have been reported to manifest their tolerance to cold temperatures by altering their cellular protein profiles and continuing to grow (6, 36, 55, 75, 90). The cold shock response is a physiological response of living cells to temperature downshift which involves de novo protein synthesis (52, 54, 123). From this current study, RT-PCR analysis revealed that *csp*$_s$ transcripts were constitutively expressed before and after cold shock with a few exceptions. Expression of *csp1*, *csp3*, and *csp5* was detected at all sampling times in strains ATCC 27562, VV3 and VV4 with or without the 15°C cold adaptation period. Interestingly, *csp1* and *csp3* were not expressed in the avirulent mutant C7184tr at 35°C but was induced 24 h post cold shock. However, *csp1* and *csp3* expression was detected in C7184Tr during the 15°C adaptation period. The induction of *csp1* transcript (homologous to *cspA*) in C7184Tr strain which has a translucent morphotype after cold shock may be essential for cell protection during cold adaptation. It is believed that the cold shock response serves as an adaptive function and it has been suggested that *cspA*, together with other *csp*$_s$, played a role in protecting cells from damage due to cold stress. The increased levels of *cspA* after cold shock have been reported to be necessary for compensating for higher stability of secondary structures in RNA at low temperature. The expression of *V. vulnificus csp1* and *csp5* (homologous genes to *cspA* and *cspV*, respectively) remained constant despite cold shock. This current result contrast the data reported from *V. cholerae* response to cold shock. Datta and Bhadra (36) reported a strong induction of *cspA* and *cspV* after cold shock at 15°C. The current result seems to indicate *V. vulnificus* cold shock response is

different from that trigger in *V. cholerae*. These present findings showed that all *V. vulnificus* cold shock genes tested in this study are not cold inducible. *Csp4* showed a different expression pattern in all strains. This gene was expressed at 35°C and 15°C but its expression faded away 24 to 48 h post cold shock at 4°C. Despite sequence similarity with other cold-inducible genes, *csp1*, *csp3*, *csp4*, and *csp5* cannot be considered class I *csp* genes (173), since no upregulation by cold shock was observed. Similarly, no differences in induction levels were detected (although some genes seemed to be repressed overtime at 4°C) and therefore did not follow the expression pattern of *csp* class II genes either. It can conclude that although *V. vulnificus* presented some of the *csp* genes described in other species, they are not upregulated during cold shock.

In addition to csp_s gene expression this study investigated other genes that have been implicated in response to environmental stressors in *V. vulnificus* (70, 94, 142, 154). As expected *rpoS,* a general stress regulator, was constitutively expressed thorough the study (160, 161). Generally, *rpoS* was expressed when response to various stress, including nutrient limitation, osmotic challenge, and during stationary growth phase (103). This finding suggests that the presence of *rpoS* transcript may be significant for cell survival during cold stress. *OxyR* regulated the expression of the periplasmic catalase (*katG*) in *V. vulnificus* and both genes have been shown to be repressed by low temperatures (94, 160). The current data show that levels of *oxyR* transcripts increased after 4°C cold shock while *katG* expression was slightly induced at 4°C between 60 min and 72 h post cold shock depending on strain. During the adaptation period at 15°C, *oxyR* was induced and reach high expression levels at 36 h. Expression levels decreased at 4°C overtime, except in strain VV3 which displayed high levels of *oxyR* throughout the

experiment. The expression of *oxyR*, a peroxide sensor in this research indicated *V. vulnificus* may experience a H_2O_2 toxicity under aerobic condition during growing in culture broth. Expression of *katG* during the 15°C period was weak but after cold shock at 4°C main differences were observed between strains. While the type strain ATCC 27562 did not expressed this gene, a weak expression was detected in C7184Tr after cold shock. Interestingly, VV3 and VV4 presented opposite expression patterns. *KatG* expression in VV3 was gradually induced after 6 h post cold shock. On the contrary, VV4 exhibited high levels of *katG* immediately after cold shock but expression faded out until non-detectable levels at 168 h post cold shock. Smith and Oliver (160) showed that *katG* expression could be detected in both clinical and environmental strains of *V. vulnificus* up to 240 h post cold shock at 4°C. However, these authors were inducing the VBNC state and cells were maintained in low nutrient medium. In this present study use of rich medium might explain the differences in *katG* and *oxyR* expression since reactive oxygen species (ROS) between high and low nutrient media are likely to be different.

Additionally, results show that the synthesis of the *16SrRNA* housekeeping gene was never repressed following an abrupt temperature downshift. This result is in agreement with previous studies where housekeeping genes were similarly expressed at optimal and low temperatures (72). Detection of ribosomal mRNA is thought to be a good viability marker in many bacteria (13, 46, 93, 158). The constitutive expression of *16SrRNA* mRNA indicated the viability of *V. vulnificus* occurred throughout the cold shock experiment.

In conclusion, no cold shock gene induction was observed in all four genes tested, although the differences in *oxyR* and *katG* expressions observed during cold shock suggest that *V. vulnificus* cells encountered not only cold stress but also oxidative stress. Further investigation of the role of these cold shock genes and stress-related genes and their regulation in *V. vulnificus* will help scientists to better understand the survival of this pathogen during cold shock.

TABLE 1. Computed theoretical pI and M_w values of cold shock proteins encoded by putative cold shock genes of *V. vulnificus*

Gene	Chromosome	pI	M_w (KDa)	GenBank Accession no.	Homologous gene in *V. cholerae*	TIGR Accession no. *V. cholerae* * (36)	% identity of amino acid with *V. cholerae*
csp1	chromosome I	8.09	7.599	NP761574	*cspA*	VCA0166	81.4
csp3	chromosome I	6.03	8.398	NP760979	*cspD*	VC1142	81.6
csp4	chromosome II	6.54	7.521	NP762479	DNA-binding domain	VCA0184	88.4
csp5	chromosome II	8.12	7.554	NP762464	*cspV*	VCA0933	85.7

* TIGR, The Institute for Genome Research, from Datta and Bhadra (36)

TABLE 2. Primers design for cold shock genes of *V. vulnificus*

Bacteria strain	GenBank Accession	Length bp	Tm (°C)	Primer sequences
V. vulnificus CMCP6 (Chromosome I)	VV12757	213	50	ATGTCTGGTAAAATGACTGG TTAGATAACTGTAACGTTAGAA
V. vulnificus YJ016 (Chromosome I) *(a)*	VV1504			
V. vulnificus CMCP6 (Chromosome I) *(b)*	VV12532	468	50	ATGAAAGGGAAGATTCTTCG CTACCAGCCGCCACATAA
V. vulnificus CMCP6 (Chromosome I)	VV12119	231	50	ATGTATAGCATGGCTACAG TTACTTCGTTGCTTGTACTT
V. vulnificus YJ016 (Chromosome I) *(c)*	VV2324			
V. vulnificus CMCP6 (Chromosome II)	VV20519	210	50	ATGTCTAACACAGTAACCG TTAAAGCAGTGTGATCTCTT
V. vulnificus YJ016 (Chromosome II) *(d)*	VVA1067			
V. vulnificus CMCP6 (Chromosome II)	VV20503	213	50	ATGTCTAATAAAGTAACTGGTT TTATAGCGCAGTTACGTTTG
V. vulnificus YJ016 (Chromosome II) (e)	VVA1052			

(a): designated *csp1*, *(b)*: designated *csp2*, *(c)*: designated *csp3*, *(d)*: designated *csp4*, and *(e)*: designated *csp5*

TABLE 3. Primers used for RT-PCR of cold shock genes and stress-related genes for the *in vitro* study

Gene	GenBank Accession Number	Characteristics	Size of amplified fragment (bp)	Tm (°C) used in RT-PCR	Primer sequence	References
csp1	VV12757 VV1504	Cold shock protein,	213	50	ATGTCTGGTAAAATGACTGG TTAGATAACTGTAACGTTAGAA	This study
csp3	VV12532	Cold shock-like protein (*CspD*)	231	50	ATGTATAGCATGGCTACAG TTACTTCGTTGCTTGTACTT	This study
csp4	VV12119 VV2324	Cold shock protein	210	50	ATGTCTAACACAGTAACCG TTAAAGCAGTGTGATCTCTT	This study
csp5	VV20519 VVA1067	Cold shock protein	213	50	ATGTCTAATAAAGTAACTGGTT TTATAGCGCAGTTACGTTTG	This study
oxyR	AY102627	Control *katG* expression, mediated catalase activity.	496	57	CGAATACCTAGTGGCTTTGGC GCATGGCACAGCAACACTTA	This study
rpoS	AY187681	Stress regulator	391	50	GTGCATTACGAGGCGATGAA ATTTTGCCCACATCGTCAAC	This study
katG	VV12755	Catalase gene	504	57	GTTGGTACCTCAACTACCGC GGATGTCTAGATTGAGGGCC	(142)
16SrRNA	X76333 X76334	Housekeeping gene	492	57	GCCTAACACATGCAAGTCGA CGTATTACCGCGGCTGCTGG	(25)

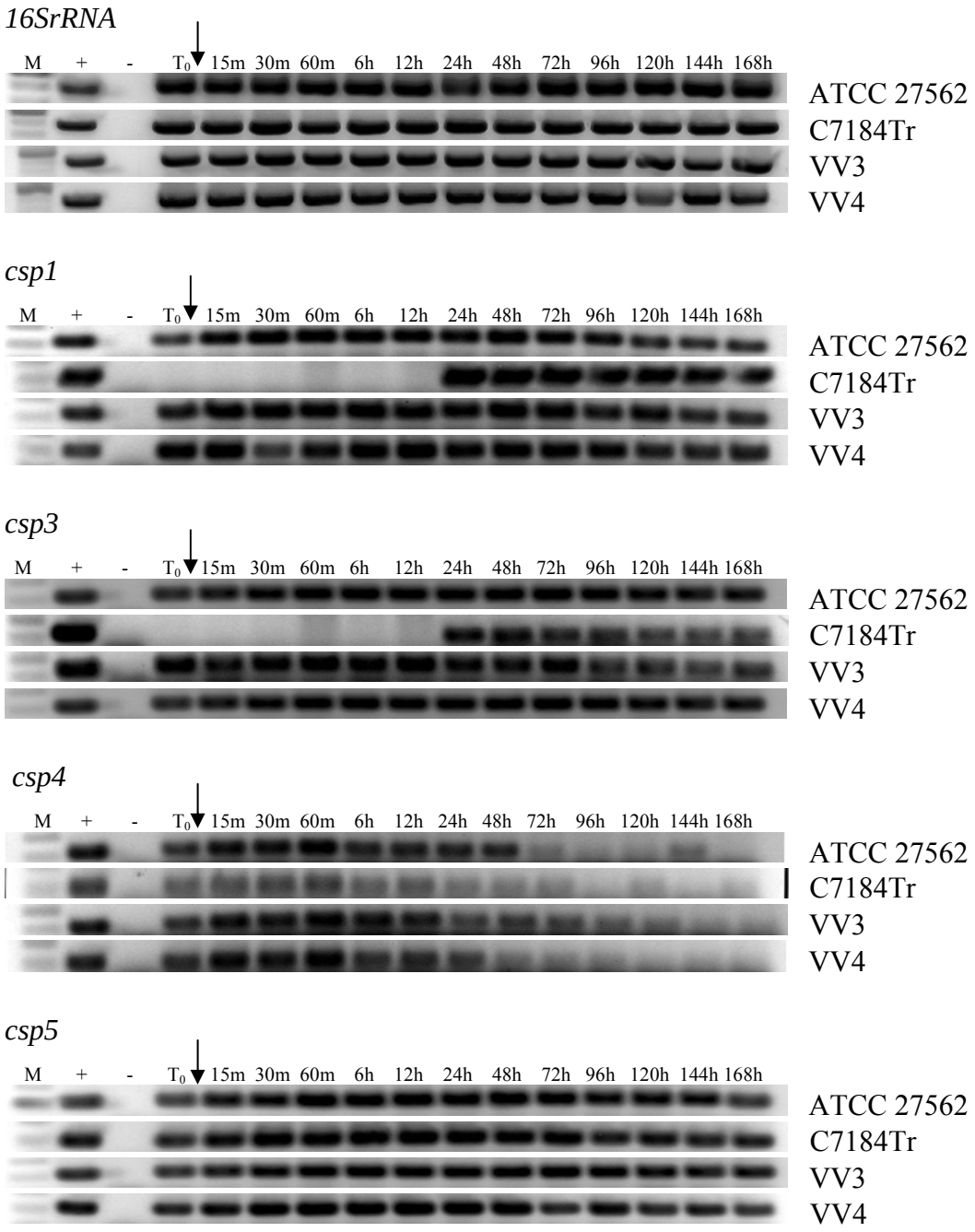

FIG. 20. *Vibrio vulnificus* gene expression profiles: internal control: *16SrRNA*, and cold shock genes: *csp1*, *csp3*, *csp4,* and *csp5* during cells were cold shocked from $35°C_{10\ h}$ to $4°C_{168\ h}$ (T_0: after 10 h incubation at 35°C, vertical arrow indicated cold shock at 4°C).

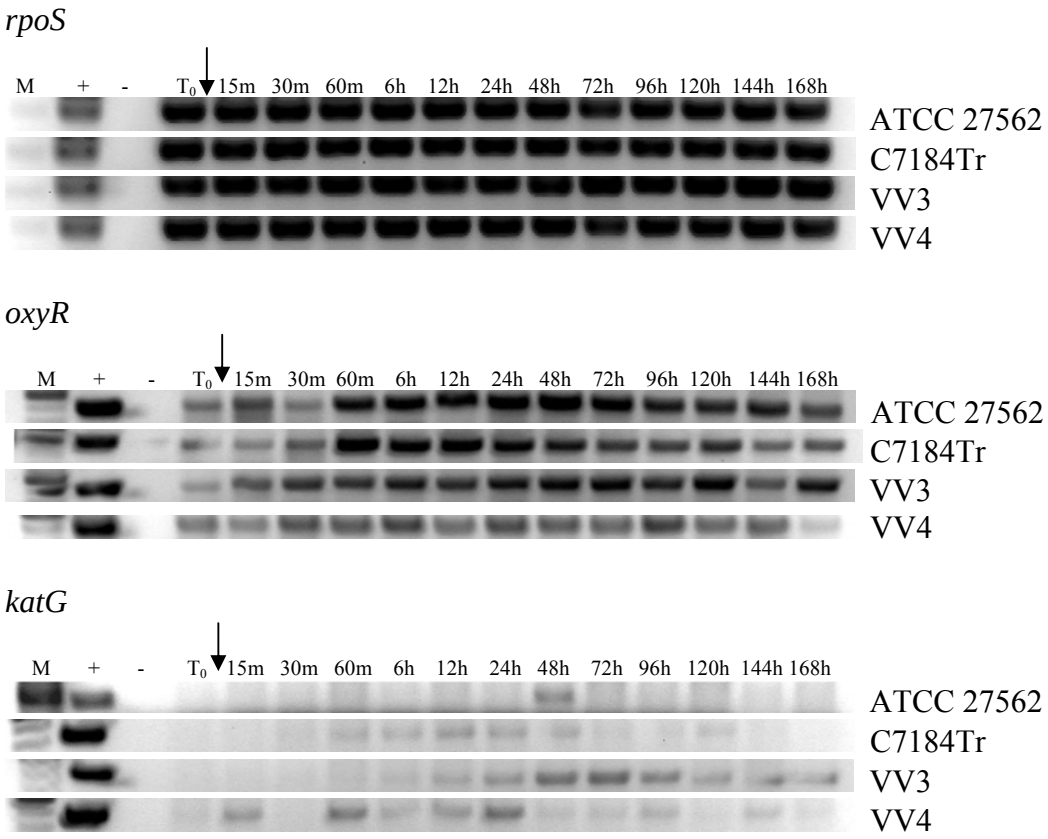

FIG. 21. *Vibrio vulnificus* gene expression profiles: stress-related genes: *rpoS*, *oxyR*, and *katG* during cells were cold shocked from 35°C$_{10\ h}$ to 4°C$_{168\ h}$ (T_0: after 10 h incubation at 35°C, vertical arrow indicated cold shock at 4°C).

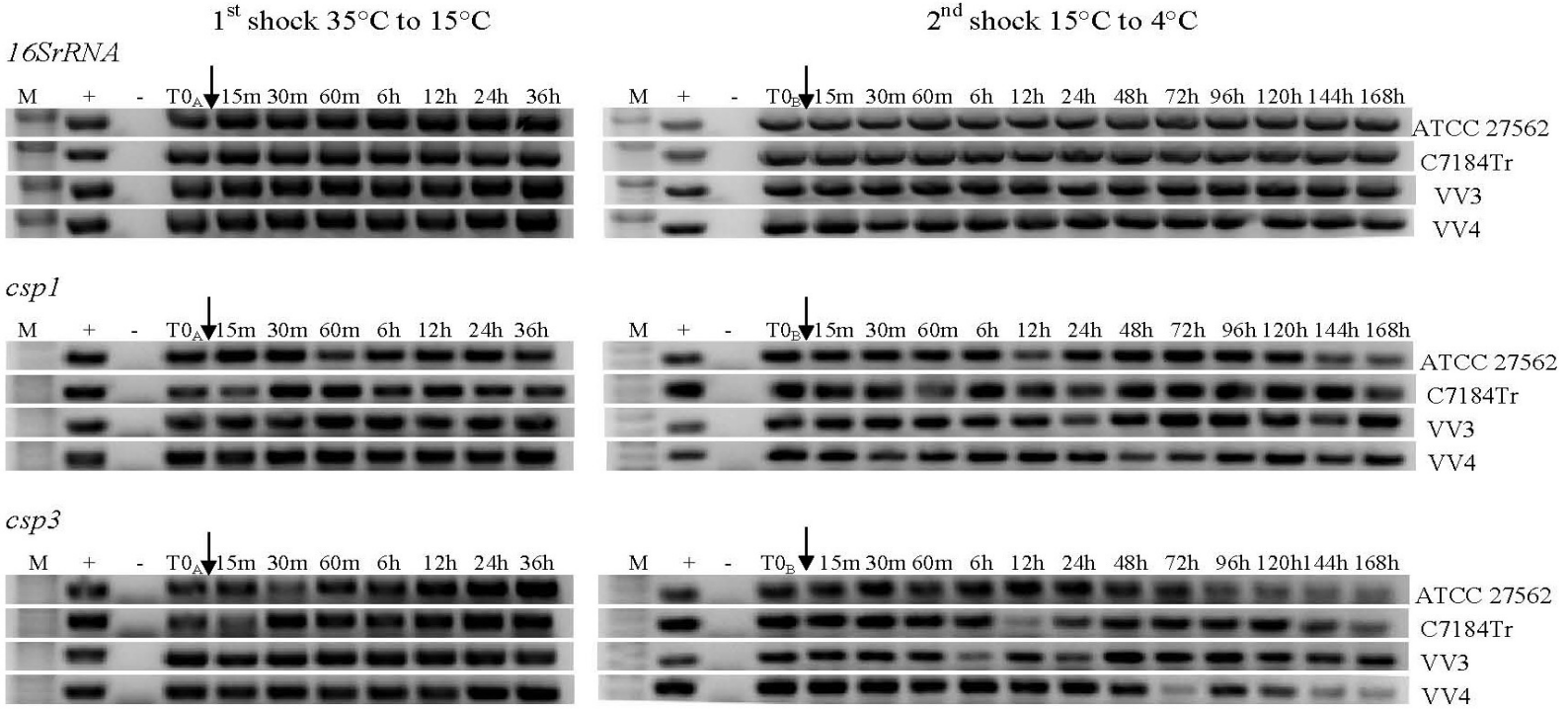

FIG. 22. *Vibrio vulnificus* gene expression profiles: internal control: *16RrRNA*, and cold shock genes: *csp1* and *csp3* during cells were cold shocked from $35°C_{10\,h}$ to $15°C_{36\,h}$, and from $15°C_{36\,h}$ to $4°C_{168\,h}$ ($T0_A$: after overnight incubation at 35°C, $T0_B$: after 36 h incubation at 15°C, the 1st vertical arrow indicated cold shock at 15°C, the 2nd vertical arrow indicated cold shock at 4°C).

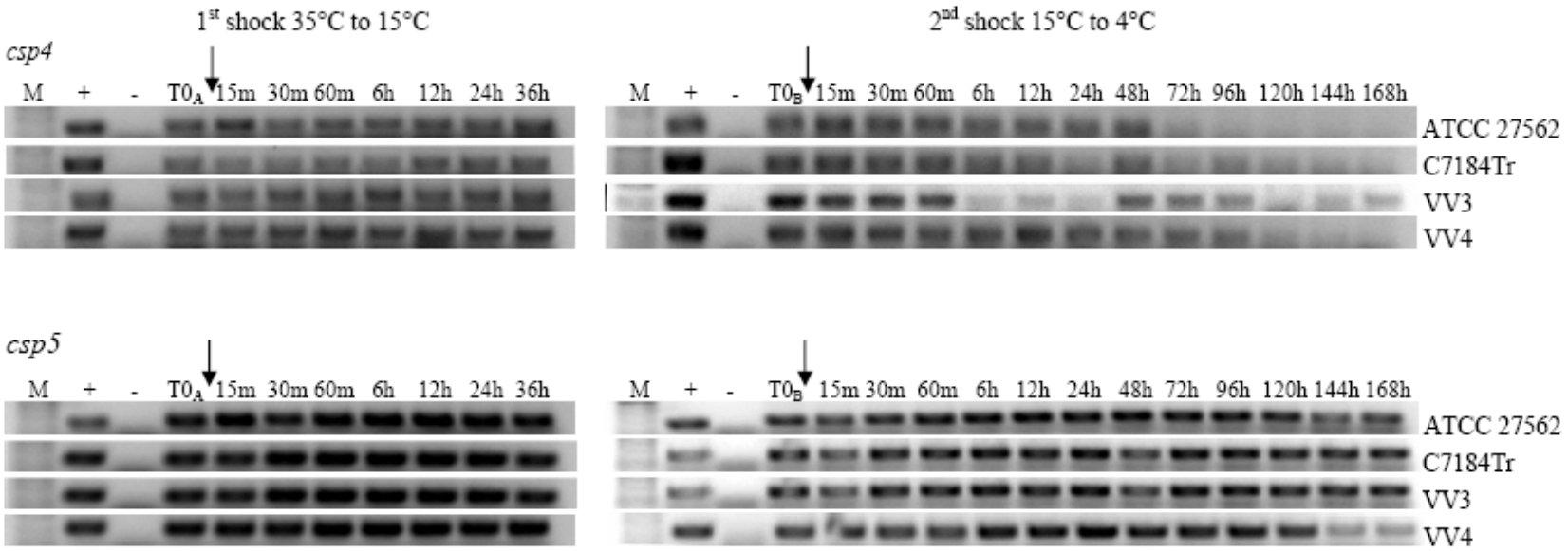

FIG. 23. *Vibrio vulnificus* gene expression profiles: cold shock genes: *csp4*, and *csp5* during cells were cold shocked from $35°C_{10\,h}$ to $15°C_{36\,h}$, and from $15°C_{36\,h}$ to $4°C_{168\,h}$ ($T0_A$: after overnight incubation at 35°C, $T0_B$: after 36 h incubation at 15°C, the 1st vertical arrow indicated cold shock at 15°C, the 2nd vertical arrow indicated cold shock at 4°C).

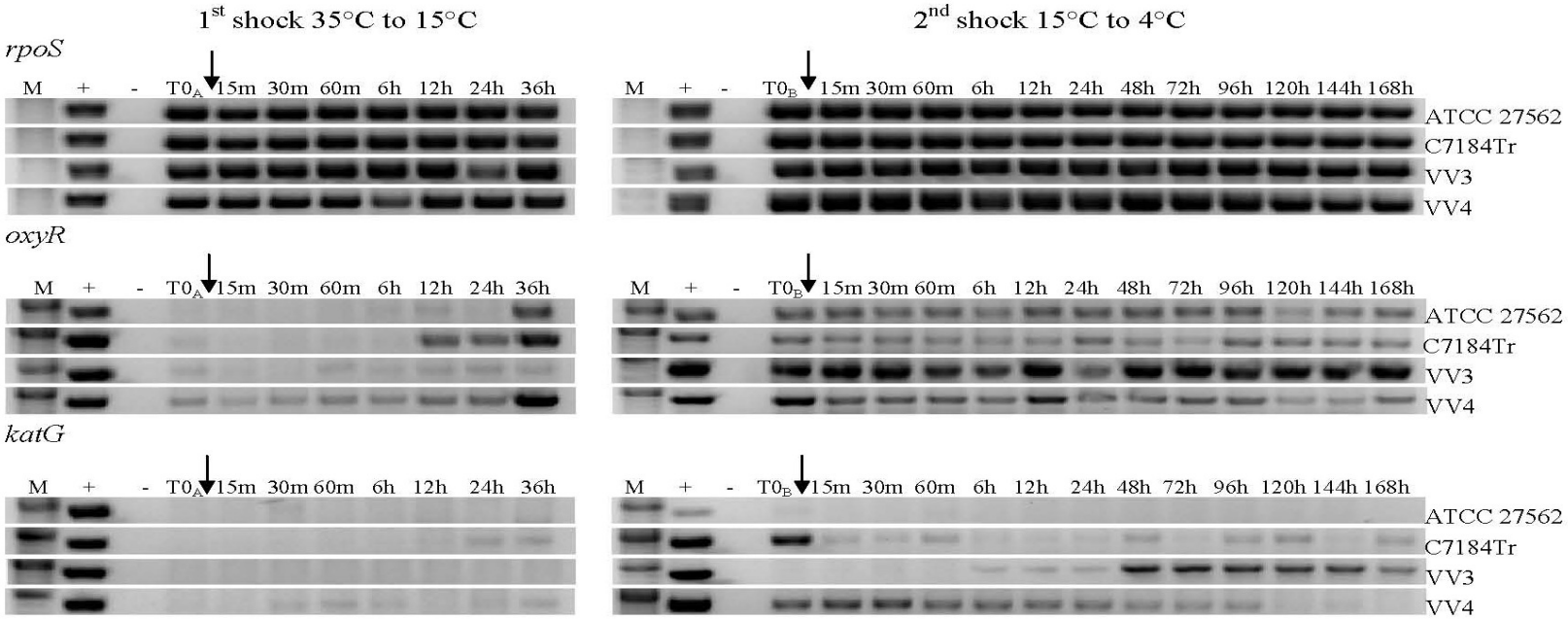

FIG. 24. *Vibrio vulnificus* gene expression profiles: stress-related genes: *rpoS*, *oxyR*, and *katG* during cells were cold shocked from $35°C_{10\ h}$ to $15°C_{36\ h}$, and from $15°C_{36\ h}$ to $4°C_{168\ h}$ ($T0_A$: after overnight incubation at 35°C, $T0_B$: after 36 h incubation at 15°C, the 1st vertical arrow indicated cold shock at 15°C, the 2nd vertical arrow indicated cold shock at 4°C).

VI. *IN VIVO* COLD SHOCK GENE EXPRESSION OF *VIBRIO VULNIFICUS*

ABSTRACT

Analysis of *V. vulnificus* gene expression in oysters (*in vivo*) during cold shock was performed at two different temperature conditions. RT-PCR was used to analyze the presence and expression of cold shock genes (csp_s) and stress-related genes of *V. vulnificus* in oysters. During the first experiment, oysters were contaminated with *V. vulnificus* overnight at 25°C, transferred to 35°C for 10 h, and then cold shocked at 4°C for 168 h. Expression of *16SrRNA,* a house keeping gene, and *rpoS*, a stress regulator was detected at all time points. The induction of *csp5*, a cold shock gene occurred after cold shock at 4°C, while the expression of *csp4* varied between strains. The expression of *csp1*, *csp3*, *oxyR* (which mediated catalase activity) and *katG*, a catalase gene were not detected throughout the study period in neither clinical nor environmental strains. In the second experiment, oysters were contaminated with *V. vulnificus* and after overnight at 25°C, were cold adapted at 15°C for 36 h prior change to 4°C for 168 h. Constitutive gene expression pattern of *16SrRNA* was observed whereas *rpoS* exhibited discontinuous gene expression throughout the study period. The transcript of *csp5* was not continuous expressed at all time points. The synthesis of *csp3* and *csp4* mRNA was weakly expressed and varied among strains. The expression of *oxyR* and *katG* mRNA was down-regulated during 35°C, 15°C, and in the cold. These result demonstrated that *V. vulnificus* displayed variations of *in vivo* cold shock genes expression. This is the first report on *V. vulnificus* gene expression under *in vivo* conditions.

INTRODUCTION

Vibrio vulnificus, a mesophilic shellfish-borne pathogen, is present as natural microbiota in warm coastal waters throughout most parts of the world (22, 85, 133, 169, 187). *V. vulnificus* thrives in warm seawater and is frequently isolated from shellfish from the Gulf of Mexico (85). Infection caused by this pathogen is a serious concern for the oyster industry and public health in the United States. Most cases of illnesses or fatality occurred from consumption of raw oysters harvested from the Gulf region (Alabama, Florida, Louisiana, Mississippi, and Texas) and a few cases from oysters harvested from the Northeast, and the Northwest in the U.S. (19). Primary septicemia, the most severe disease, was observed in individuals who had consumed raw oysters and was limited almost exclusive to persons with underlying liver disease (22). The occurrence and prevalence of *V. vulnificus* is seasonal with numbers peaking in summer month, with a range from May to October (32, 85). However, during the winter months, the decline in numbers of detectable *V. vulnificus* in coastal water and shellfish has been attributed to its entry into the viable but non-culturable (VBNC) state (30, 126).

Low temperature has been reported to be one of the most important factors in limiting *V. vulnificus* survival. *V. vulnificus* was not capable of growing when incubated at temperature below 10°C, however low temperatures are not lethal to *V. vulnificus* (33) since cells can entry to the VBNC state when temperature is decreased below 5°C (185). Bryan et al. (17) reported that a brief cold adaptation period for 3 h at 15°C enhanced survival of *V. vulnificus* at 6°C. They concluded that cold-adaptive protective proteins might enhance survival and tolerance at cold temperature. Moreover, a downshift to low

temperature has been previously considered to change protein expression in *V. vulnificus* (110). The physiological response for survival strategy of living cells to temperature downshift have been reported in several bacteria (51). Microbes exposed to abrupt temperature downshift underwent severe physiological disturbance, such as changes in supercoiled DNA, reduction in membrane fluidity, and the formation of stable secondary structures in DNA and RNA that impaired replication, transcription and protein synthesis. To overcome the injurious effects of cold shock and ensured that cellular activity will resume or be maintained at low temperature, bacteria have developed a transient adaptive response, the cold shock response (78, 141). During that period, the expression of a subset of specific proteins termed cold shock proteins (Csps) is induced (146, 173). Csps are widespread among bacteria (41) and are not only required during the cold shock adaptive response but also play a general role in cellular function adaptation to various growth conditions (40). Cold shock has been shown to alter genes expression in many species of bacteria (6, 7, 55, 80, 109, 140). However, little is known about *V. vulnificus* gene expression in response to cold shock. Cold shock response in *V. vulnificus* has not been fully elucidated although this pathogen greatly impacts the oyster industry and public health. The preventive measures suggested by USFDA/ISSC, required refrigeration of oysters after harvest, may potentially induce a cold shock phenomenon in *V. vulnificus*. The molecular mechanisms of *V. vulnificus* response to cold shock need to be unraveled.

While several reports have been investigated *in vitro* and *in situ* gene expression of *V. vulnificus* during the VBNC state (46, 160, 161), *in vivo* gene expression of this pathogen has not yet been attempted under any conditions. Since oysters can act as

reservoirs and transmission vectors for *V. vulnificus,* it is crucial to study the cold shock gene expression while this pathogen is embedded in oysters. Specifically, the investigation of cold shock response of *V. vulnificus* should be based on the result of the examination in oysters as results may differ from those obtained through artificial inoculation.

In an effort to elucidate the *in vivo* gene expression of *V. vulnificus* during cold shock, reverse transcriptase PCR (RT-PCR) technology was used to monitor the presence and the expression of cold shock genes (csp_s), encoding cold shock protein, and stress related genes in two clinical and one environmental strains. In this study, I examined the transcription of small putative cold shock family genes of *V. vulnificus* (*csp1*, *csp3*, *csp4*, and *csp5*), encoded for cold shock proteins. In addition, *rpoS*, the stress regulator which is used by cells in adaptive response in various environmental stresses was monitored. *KatG*, a periplasmic catalase gene (HPI) and *oxyR*, a positive regulator of catalase gene which both involved in oxidative stress were analyzed in response to exposure to cold temperature.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Two clinical strains of *V. vulnificus*, ATCC 27562 (type strain), and C7184Tr (avirulent, translucent morphotype) provided by J.D. Oliver, University of North Carolina, Charlotte, NC and one environmental strain (VV3) isolated from Mobile Bay,

AL were used in these study. Cells were grown in marine broth (MB) with shaking and maintained on marine agar (MA) (Difco, Detroit, MI) at 35°C

Oyster samples

One-year-old live shellstock eastern oysters (*Crassostrea virginica*) (50-75 mm diameter) were depurated using a UV filtered (UV flow 3000 μw-sec/cm^2), with a water flow-through system (180 l/h) for 7 days at Auburn University Shellfish Laboratory (AUSL), Department of Fisheries and Allied Aquacultures, Dauphin Island, AL and transported immediately to the Bacterial Genomics Laboratory, Department of Fisheries and Allied Aquacultures, Auburn University for processing. The oyster samples for each experiment were transported and stored at 4°C.

Artificial inoculation

Live depurated oysters were artificially-inoculated with *V. vulnificus* by self-inoculation filtration. Four aquaria (38 L) containing 27 L of autoclaved seawater were prepared. One hundred fifty oysters were placed into each aquarium at 25°C and were allowed to acclimate for 24 h. A fresh culture of each strain of *V. vulnificus* was grown in MB until exponential growth phase (10^8 CFU/ml, OD_{450}=0.15) was reached. In each aquarium, 3 L of *V. vulnificus* cultures were used to inoculate oysters at approximately 10^6 CFU per gram of oyster. Oysters with no *V. vulnificus* artificially inoculation (control) was prepared and acclimated at the same condition.

Cold shock experiment

After *V. vulnificus* inoculation, oysters remained at 25°C overnight prior to temperature downshift. Two different cold shock conditions were compared. During the first experiment oysters were transferred to 35°C for 10 h, and then chilled by ice-water immersion until the internal temperature in oysters reach to 4°C. Oysters were placed in a low temperature incubator at 4°C and incubated for 168 h. For the second experiment, inoculated oysters were transferred to 15°C for 36 h, chilled by ice-water immersion until the internal temperature reached 4°C. Oysters were incubated at 4°C for an additional 168 h. Internal oyster temperature and external ambient air temperature were monitored throughout the study.

Reverse transcription-PCR (RT-PCR) detection

(i) Sampling.

The gastro-intestinal tract of oysters containing with *V. vulnificus* was sampled from these two different cold shock experiments. Samples were collected at various time intervals from time 0 h to 168 h. Three oyster gastro-intestinal tracts were pooled from each time point and kept in RNalater RNA stabilization reagent (Ambion, Austin, TX) and stored at 4°C for further RNA extraction analysis.

(ii) RNA isolation and contaminated DNA elimination

Thirty milligrams of mixed tissue samples were used for RNA isolation. RNA Plus mini kit (QIAGEN, Inc., Valencia, CA) was used to extract total RNA from all oyster samples (according to the manufacturer's protocol, as described in Chapter IV). RNA concentrations were quantified in Gene Quant spectrophotometer and stored at -80°C until being processed. In order to eliminate carryover DNA in the RNA extract, 1 µg of RNA was treated for 30 min at 37°C with 150 U of DNase (Ambion, Austin, TX). Treated RNA was heated at 65°C for 10 min to inactivate DNase.

(iii) Reverse transcription or cDNA synthesis

cDNA synthesized using the cDNA synthesis kit from Amersham Biosciences. The presence and expression of seven genes in *V. vulnificus* was investigated using RT-PCR. Primers targeting four cold shock genes plus three stress-related genes were used (Table 4). The absence of DNA contamination in RNA samples was verified by PCR, using RNA as a template with primer target 16SrRNA universal primers (UFUL and URUL). Negative controls with no template nucleic acid were carried out. *V. vulnificus* DNA was used as a positive control for the reaction. Primers against *V. vulnificus* specific target sequences in the *16SrRNA* gene were used to detect *V. vulnificus* constitutive gene expression. All PCRs were conducted using the annealing temperature varying according to the primer set used. PCR amplification consisted of the following temperature profile: initial heating step at 95°C for 5 min followed by 35 cycles of

denaturation (95°C for 30 sec), annealing (temperature varying according to the primers used (Table 1) for 30 sec), and extension (72°C for 45 sec). The final extension was ended at 72°C for 10 min and thereafter cooled to 4°C. The amplification product was separated by horizontal gel electrophoresis in a 1% agarose gel and PCR products were visualized by ethidium bromide staining and UV translumination.

RESULTS

The initial mRNA analysis by RT-PCR was 18 h (overnight incubation) after oysters kept at 25°C were exposed *V. vulnificus*. After treatment oysters were transferred to either 35°C for 10 h and 15°C for 36 h and downshifted to 4°C for one week. *V. vulnificus* displayed variations of *in vivo* cold shock genes expression after a temperature downshift. During the first experiment, when oysters were transferred from 25°C to 35°C for 10 h, and abruptly shifted to 4°C (Fig 23, 24), the expression of *16SrRNA* and *rpoS* were detected or constitutively expressed at all time points in both clinical (ATCC 27562 and C7184Tr) and environmental strain (VV3). Both ATCC 27562 and C7184Tr strains exhibited weak induction of *csp4* transcript at 25°C whereas only C7184Tr transcript was induced at 35°C. After a change to cold temperature, this gene was early induced (after 60 min at 4°C) in both ATCC 27562 and C7184Tr, while this transcript exhibited late expression after 168 h incubation at 4°C in VV3. The *csp5* synthesis was early expressed after 30 min cold shock at 4°C in ATCC 27562, but detected later after 6 h cold incubation in both C7184Tr and VV3. The transcript of *csp1* and *csp3* were undetectable or down-regulated throughout the study period in both

clinical and environmental strains. The same expression pattern was shared by the two oxidative stress related genes, *oxyR* and *katG* which exclusively down-regulated during at 35 or 15°C and in the cold.

When oysters were transferred from 25°C to 15°C for 36 h and downshifted at 4°C (Fig 25, 26), the synthesis of *16SrRNA* was constantly expressed throughout the study period. *RpoS* was also detected but showed different intensity in mRNA expression at all time points in both clinical and environmental strains. The transcript of *csp3* was induced at 25°C in both C7184Tr and VV3, but not in ATCC 27562. After a change from 15°C to 4°C, this gene was weakly induced in both ATCC 27562 and C7184Tr, while it was not expressed in VV3. The induction of *csp4* mRNA was varied among strains. In ATCC 27562, this gene was induced at 25°C, after 36 h incubation at 15°C and the early incubation at 4°C. In C7184Tr, this gene was expressed at 25°C, the early period at 15°C, and during the beginning at 4°C, while this gene was discontinuously induced after the oysters were incubated at 15°C and 4°C in VV3. The transcription of *csp5* was not continuously expressed at all time points in clinical strains, whereas this transcript was detected and showed discontinuous intensity in mRNA abundance after 1h change to an acclimation temperature (15°C) in environmental strain (VV3). The synthesis of *csp1, oxyR,* and *katG* were down-regulated throughout the study period in both clinical and environmental strains.

DISCUSSION

Cold shock in microorganisms results in unstable metabolism and a longer lag phase. However, microbial growth after cold shock incubation for a lengthy period of time suggested cellular adaptive mechanisms with an altered or unaltered metabolic recovery (143). Therefore, the continued viability of *V. vulnificus* after incubation at 4°C demonstrated that *V. vulnificus* has the metabolic and genetic potential to survive at refrigerated temperatures. This research showed that upon cold shock some genes underwent a transient induction, some of which were found to decrease after cold shock. Jones et al. (79) hypothesized that the level of transcription is an important mechanism of regulation of cold shock protein synthesis. There was evidence that *V. vulnificus* survival and tolerance at cold temperature could be due to the expression of cold adaptive proteins (17). In the present study, the *csp* genes, encoding cold shock proteins were differentially expressed in response to cold shock *in vivo*. Some csp_s genes were repressed whereas others were induced after temperature downshift meaning that these small cold shock family genes include non-cold and cold shock inducible genes. The repression of *csp1*, which has high sequence identity with *cspA* of *V. cholerae* occurred throughout the study period. This was in contrast to the findings obtained in *V. cholerae* when cold shocked *in vitro* in which *cspA* was highly induced during exposure to a low temperature and weakly induced at 37°C (36). In *V. vulnificus* the expression of *csp3* and *csp4* varied among strains from treatment to treatment. The synthesis of *csp3* (encoded CspD), was repressed when oysters were transferred from 25°C to 35°C and abruptly shifted to 4°C. However, this gene was weakly induced at 25°C and at 4°C in C7184Tr but only induced

after a shift to 4°C in ATCC 27562. The expression of *csp4*, 86% identical to cold shock DNA-binding domain protein obtained from *V. cholerae,* occurred at both optimal temperature and at low temperature, while *csp5*, 84% identical to CspV encoded *cspV* of *V. cholerae* was induced during cold shock *in vivo*. This gene was detected at all time points but a discontinuous pattern was observed during cells adaptation to 15°C and to the cold. This result is in agreement with Datta and Bhadra (36). These authors reported that *cspV* was induced only after exposure to low temperature (15°C) *in vitro* and concluded that it was truly a product of cold shock inducible gene.

V. vulnificus possess one catalase gene: *katG* which is induced by H_2O_2. *KatG* is regulated by alternative sigma factor, a product of the *rpoS* gene under control of *oxyR* (94). It appeares that *rpoS* mRNA was expressed by all three *V. vulnificus* strains upon cold shock and growth at 35 or 15°C. *RpoS* is involved in bacterial survival under stress conditions (70, 154, 192). These results were similar as those previously reported during *in vitro* and *in situ* study (160, 161) suggesting that *rpoS* transcript plays a role in cell survival during both cold and optimal growth conditions. *KatG* and *oxyR* genes, which are involved in oxidative stress, were not detected at all time points under any condition. Jenkins et al. and Altuvia et al. (2, 76) stated that the loss of *oxyR* induction was accompanied by decreased in *katG* expression and increased cellular sensitivity to H_2O_2, consistent with inadequate antioxidant defenses in these cells. Also, low temperature has been reported in inhibiting *oxyR* mediated catalase activity in *V. vulnificus* (94). Smith and Oliver (160, 161) investigated gene expression *in vitro* and *in situ* and reported that the expression of *katG* was decreased during winter months in the VBNC state of *V. vulnificus*. These authors showed that when *V. vulnificus* was restored to higher

temperatures (>20°C), it resulted in the induction of this gene. The cold shock found in *V. vulnificus* has been described as a "peroxide sensitive"(14), however, the disappearance of *katG* and *oxyR* transcript of *V. vulnificus* may be due to cells experience the absence of oxygen during persistence in oyster tissue.

Last but not least, the constitutively expression of *16SrRNA* of *V. vulnificus* was observed in this study. The presence of this gene indicated viability of *V. vulnificus* cells. Oliver (131) indicated this is a key test to determine whether such cells were dead or alive. mRNA transcription has been reported to be a reliable marker of viable cells due to its short half life and its role in bacterial cell physiology (158). Several studies have demonstrated continued gene expression by cells in the VBNC state *in vitro* (34, 46, 102, 191). The current findings lead to the question if the decline in cell culturablility observed during cold shock was caused by dead cells or by cells entering into VBNC state. The expression of *16SrRNA* provided evidence that during cold shock, *V. vulnificus* cells were alive and suggested that cells might enter into the VBNC state resulting in the decline in CFU on media. The difference in CFU between these two temperature profiles may be due to the cold adaptive mechanism that enhanced cell culturability. In addition, variations of *V. vulnificus* gene regulation may enhance bacterial survival during cold shock *in vivo*. Expression of *16SrRNA* gene under cold shock should be quantified by using real-time RT-PCR or other quantification technique, in order to determine if a decrease in net gene expression occurred under cold temperatures.

The current study is the first report on *V. vulnificus* gene expression during cold shock *in vivo*. Cold adaptive response in *V. vulnificus* was observed at the molecular

level when cells were exposed to intermediate temperatures. However, further research is need to confirm this cold adaptation is truly compensated their survivals. The loss of culturable cells due to cells entering the VBNC state using cooling strategy under oyster industry practices is another aspect of great concern for their virulence. Investigation of the role of cold shock genes and other stress-related genes and their regulation in *V. vulnificus* may explain the survival of this pathogen during cold shock. Understanding the impact of cold stress response on the physiology of this pathogenic microorganism in shellfish, particularly in regard to their survival will improve strategies control of *V. vulnificus* risk.

TABLE 4. Primers used for RT-PCR of cold shock genes and stress-related genes for the *in vivo* study

Gene	GenBank Accession Number	Characteristics	Size of amplified fragment (bp)	Tm (°C) used in RT-PCR	Primer sequence	References
csp1	VV12757 VV1504	Cold shock protein,	213	50	ATGTCTGGTAAAATGACTGG TTAGATAACTGTAACGTTAGAA	This study
csp3	VV12532	Cold shock-like protein (*CspD*)	231	50	ATGTATAGCATGGCTACAG TTACTTCGTTGCTTGTACTT	This study
csp4	VV12119 VV2324	Cold shock protein	210	50	ATGTCTAACACAGTAACCG TTAAAGCAGTGTGATCTCTT	This study
csp5	VV20519 VVA1067	Cold shock protein	213	50	ATGTCTAATAAAGTAACTGGTT TTATAGCGCAGTTACGTTTG	This study
oxyR	AY102627	Control *katG* expression, mediated catalase activity.	496	57	CGAATACCTAGTGGCTTTGGC GCATGGCACAGCAACACTTA	This study
rpoS	AY187681	Stress regulator	391	50	GTGCATTACGAGGCGATGAA ATTTTGCCCACATCGTCAAC	This study
katG	VV12755	Catalase gene	504	57	GTTGGTACCTCAACTACCGC GGATGTCTAGATTGAGGGCC	(142)
16SrRNA	X74726	Housekeeping gene *Vv* Species-specific	273	55	TCTAGCGGAGACGCTGGA GCTCACTTTCGCAAGTTGGCC	(87, 108)
16SrRNA	X76333 X76334	Housekeeping gene	492	57	GCCTAACACATGCAAGTCGA CGTATTACCGCGGCTGCTGG	(25)

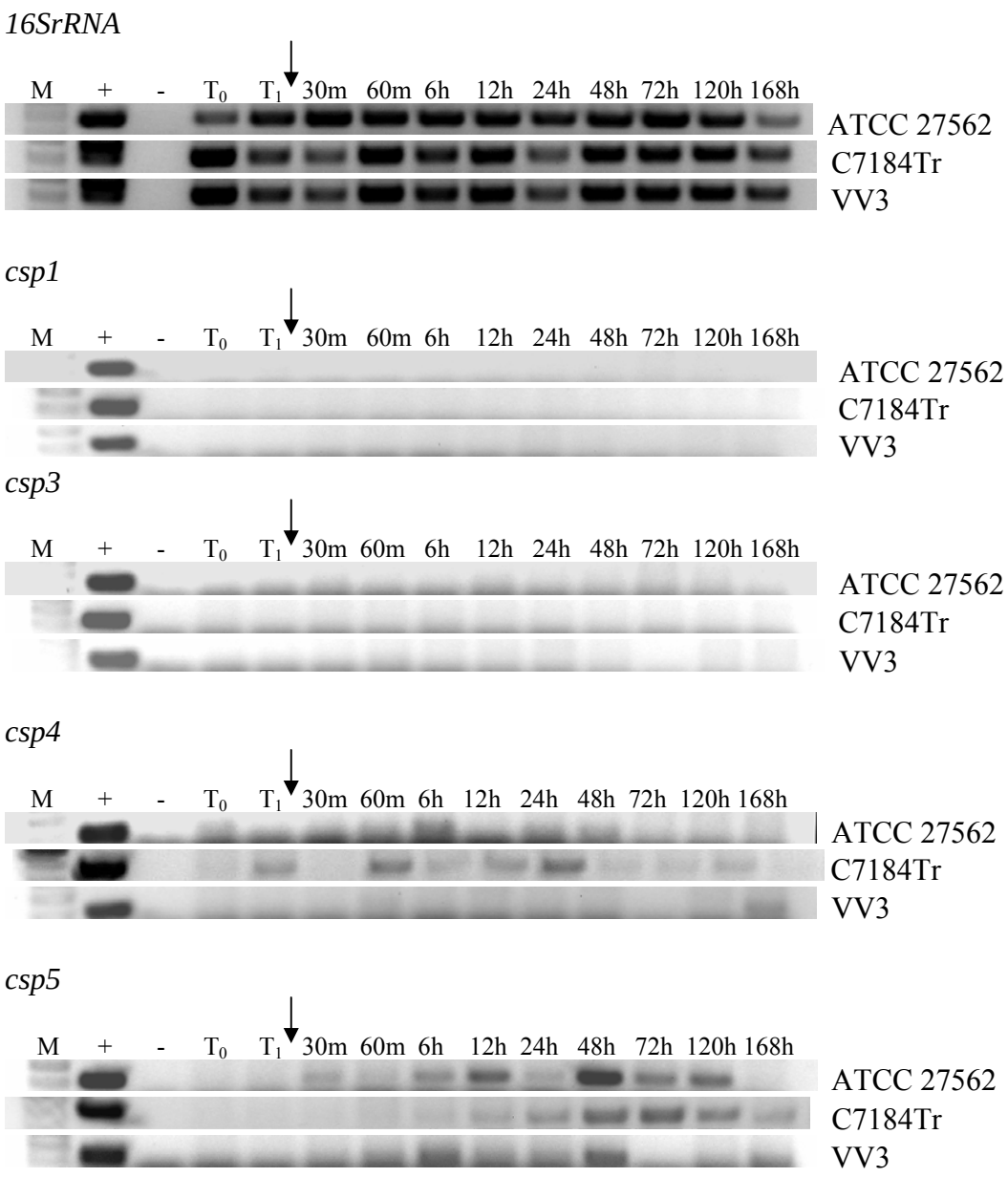

FIG. 25. *Vibrio vulnificus* gene expression profiles: internal control: *16SrRNA*, and cold shock genes: *csp1*, *csp3*, *csp4*, and *csp5* during oysters were cold shocked from $35°C_{10\ h}$ to $4°C_{168\ h}$ (T_0: after overnight incubation at 25°C, T_1: after 10 h incubation at 35°C, vertical arrow indicated cold shock at 4°C).

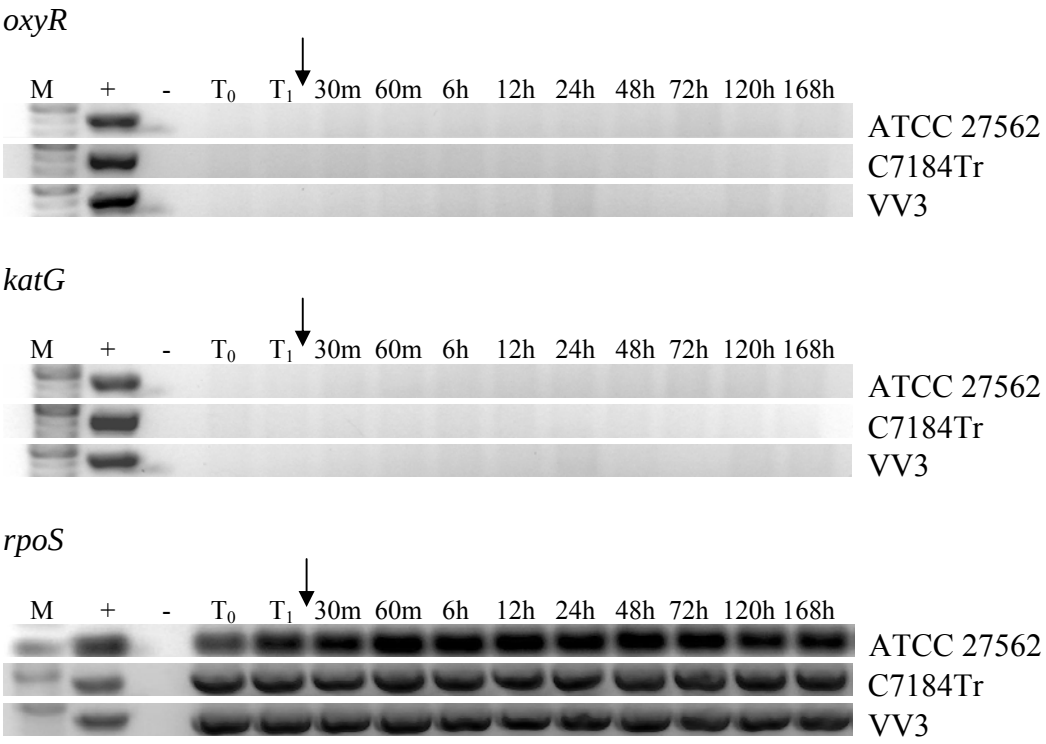

FIG. 26. *Vibrio vulnificus* gene expression profiles: stress-related genes: *oxyR*, *katG*, and *rpoS* during oysters were cold shocked from 35°C$_{10\ h}$ to 4°C$_{168\ h}$ (T_0: after overnight incubation at 25°C, T_1: after 10 h incubation at 35°C, vertical arrow indicated cold shock at 4°C).

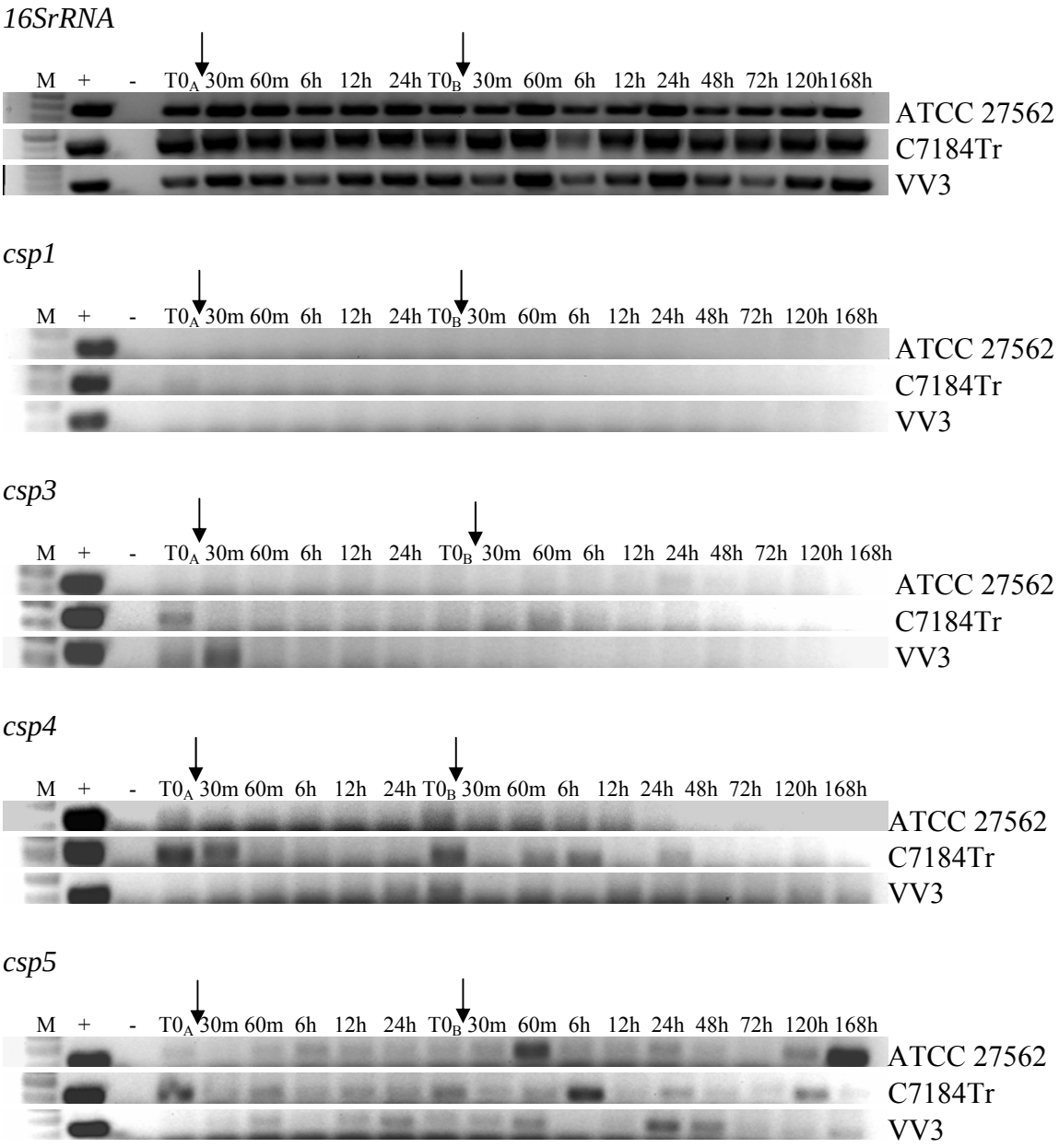

FIG. 27. *Vibrio vulnificus* gene expression profiles: internal control: *16SrRNA*, and cold shock genes: *csp1*, *csp3*, *csp4*, and *csp5* during oysters were cold shocked from $25°C_{overnight}$ to $15°C_{36\ h}$, and from $15°C_{36\ h}$ to $4°C_{168\ h}$ ($T0_A$: after overnight incubation at 25°C, $T0_B$: after 36 h incubation at 15°C, the 1st vertical arrow indicated cold shock at 15°C, the 2nd vertical arrow indicated cold shock at 4°C).

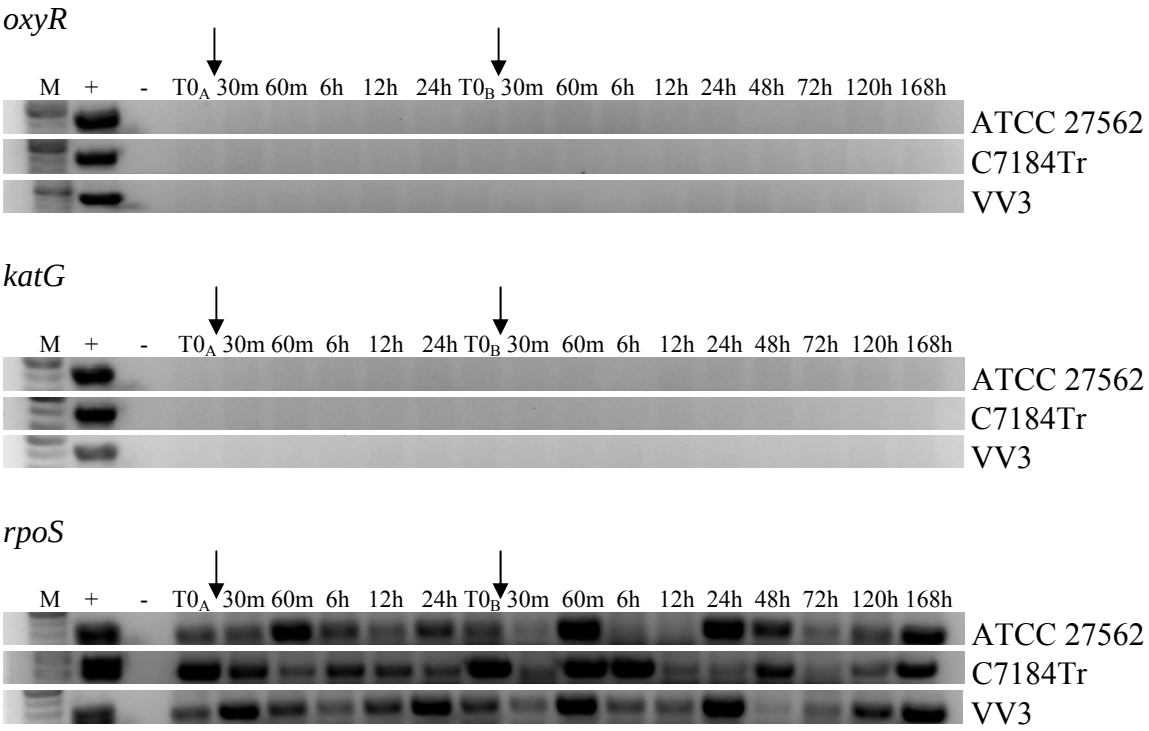

FIG. 28. *Vibrio vulnificus* gene expression profiles: stress-related genes: *oxyR*, *katG*, and *rpoS* during oysters were cold shocked from 25°C$_{overnight}$ to 15°C$_{36\ h}$, and from 15°C$_{36\ h}$ to 4°C$_{168\ h}$ (T0$_A$: after overnight incubation at 25°C, T0$_B$: after 36 h incubation at 15°C, the 1st vertical arrow indicated cold shock at 15°C, the 2nd vertical arrow indicated cold shock at 4°C).

VII. CONCLUSIONS

Cold shock response may significantly increase survival in bacteria exposed to low temperatures including shellfish-borne pathogens such as *V. vulnificus*. In this dissertation, the primary objective addressed issues relating to cold shock adaptive mechanism in *V. vulnificus* both under laboratory conditions (*in vitro*) and in shellstock oysters (*in vivo*) based on the use of current shellstock temperature guidelines recommended by ISSC. The molecular mechanism underlying this cold shock phenomenon was also investigated at the transcriptional level.

Overall results can be summarized as follows:

1) The negative effect on *V. vulnificus* cell culturabilty caused by 4°C is significantly greater than by 7.2°C. However, when *V. vulnificus* was exposed to optimal or suboptimal temperatures, neither 7.2°C nor 4°C reduced *V. vulnificus* cell culturability by less than factor of 100 during at a one-week period.

2) Cold shock adaptation mediated survival of *V. vulnificus* was found for both *in vitro* and *in vivo* experiments.

- Cells adaptation to 15°C prior to cold shock to 7.2°C enhanced *V. vulnificus* survival.

- Acclimation of infected oysters at 15°C resulted in *V. vulnificus* greater survival during refrigeration at 4°C.

3) The expression of cold shock genes (*csp*$_s$) of *V. vulnificus* varied among strains and differed between the *in vitro* and the *in vivo* conditions. Cold shock genes of *V. vulnificus* tested in this study (*csp1*, *csp3*, *csp4*, *csp5*) can not be considered cold shock inducible genes based on *in vitro* experiments. However, some weak inductions of *csp3*, *csp4*, and *csp5* were observed under *in vivo* conditions.

4) The continuous synthesis of *rpoS* in both *in vitro* and *in vivo* studies highlights the importance of this gene for cells viability.

5) The induction of *oxyR* and *katG* suggested that an oxidative stress response occurred during the *in vitro* study but not during the *in vivo* study.

6) The constant expression of *16SrRNA* in both *in vitro* and *in vivo* experiments indicated the viability of *V. vulnificus* cells through out the cold shock period.

From these cumulative findings I suggest that:

1) The stronger negative effects caused by 4°C versus 7.2°C might warrant the use of stringent refrigeration conditions by the shellfish industry.

2) *Vibrio vulnificus* should be kept below the growing temperature range (<15°C) at all times to prevent its multiplication.

3) Conditions that favor adaptation to cold temperatures should be avoided in *V. vulnificus* control strategies.

4) Minimizing *V. vulnificus* growth by limiting the time from harvest to refrigeration should be strictly enforced.

5) Whether net reduction of *V. vulnificus* cold shocked cells is due to cells entering the VBNC state need to be further evaluated. Analysis of *16SrRNA* gene

expression should be accurately quantified by real-time RT-PCR or other sensitive technique, in order to determine if a decrease in net gene expression occurred under cold temperatures. Additional studies on the VBNC state under ISSC guidelines conditions will probably unravel the risk of *V. vulnificus* infections due to VBNC cells.

6) *Vibrio vulnificus* response to cold shock is different from the elucidated in *V. cholerae*, since the homologous csp_s investigated were not cold inducible under *in vitro* conditions. *Vibrio vulnificus* has to harbor additional cold response mechanism that should be further identified and investigated.

Noteworthy, my dissertation is the first report examined the expression of cold shock genes (csp_s) in *V. vulnificus* under laboratory condition (*in vitro*), and while it was embedded in oysters (*in vivo*). The cumulative results obtained from this study highlight some very important aspects that need to be addressed from the perspective of seafood safety. My investigation "*In vitro* and *in vivo* cold shock response in *V. vulnificus*" may provide some accurate scientific data for control analysis of *V. vulnificus* septicemia associated with the consumption of raw oysters.

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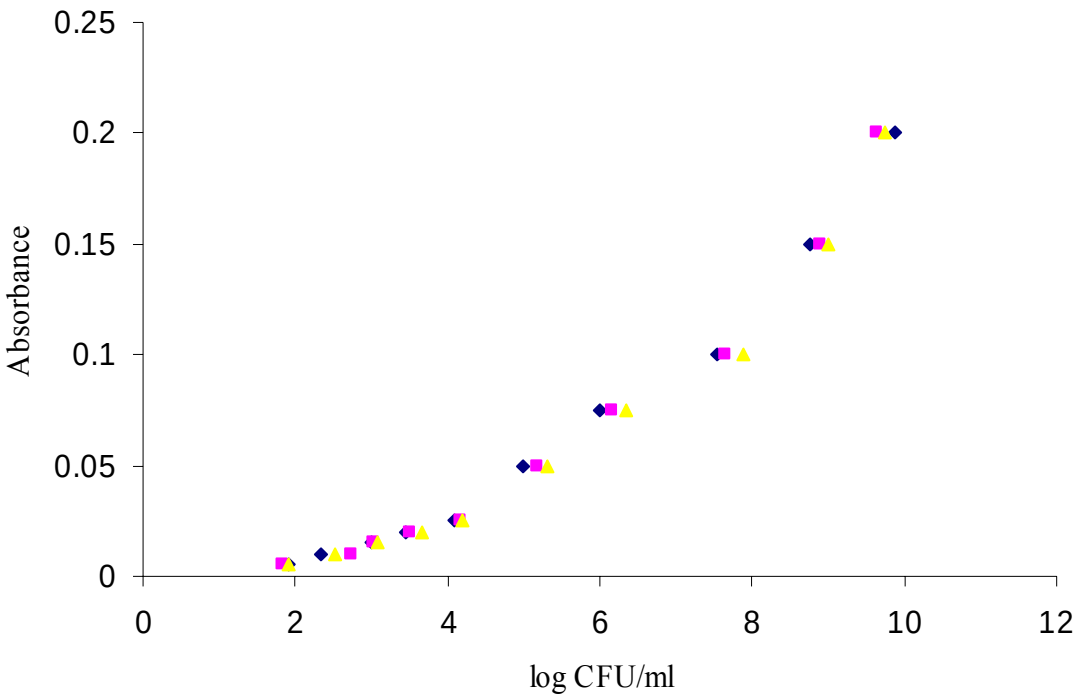
APPENDICES



Appendix 1. The correlation between the numbers of *V. vulnificus* (log CFU/ml) and the absorbance of *V. vulnificus* at 450 nm.

```
1-    M  S  G  K  M  T  G  T  V  K  W  F  N  E  T  K  G
      atg tct  ggt aaa atg act ggt aca gta aaa tgg  ttc aac gag act aaa ggt    -1

18-   F  G  F  I  S  Q  D  N  G  G  K  D  V  F  V  H  F
      ttt ggt  ttt  att  tct  caa gac aac ggc ggc aaa gat gta  ttc  gta cac ttc   -52

35-   R  A  I  V  S  E  G  F  K  T  L  A  E  G  Q  K  V
      cgt gct atc gtt  tct  gaa  ggc ttc  aaa act ctt gct gaa  ggt cag aaa gta   -103

52-   S  F  N  V  E  E  G  Q  K  G  P  Q  A  S  N  V  T
      agc ttc aac gtt gaa gaa  ggt caa aaa ggc cca caa  gct tct  aac gtt aca   -154

69-   V  I  *
      gtt atc taa                                                            -205
```

Appendix 2. Nucleotide and deduced amino acid sequence of cold shock protein *V. vulnificus* CMCP6 (GenBank accession number VV12757 and NP761574) and *V. vulnificus* YJ016 (GenBank accession number VV1504 and NP934297), designated *csp1* in this study. The nucleotides are numbered on the right and the amino acids are numbered on the left. An asterisk above the last three nucleotides indicates a stop codon.

```
1-    M  K  G  K  I  L  R  W  V  D  E  R  G  F  G  F  I
      atg aaa ggg aag att ctt cgc tgg gtt gat gaa cgt ggg ttt ggg ttc att    -1

18-   K  S  D  E  L  D  G  D  V  F  V  H  I  S  K  F  P
      aaa tct  gat  gag ctt gat ggc gac gta ttt gtt  cat ata tcc aag ttt ccc  -52

35-   Q  G  Y  R  R  P  Q  V  G  D  H  V  E  F  H  L  A
      caa ggc tat cgt cgc cca caa gta ggt gat cat gta gag ttt cat ctg gca    -103

52-   N  N  Q  P  K  L  S  A  A  S  A  R  L  I  G  V  E
      aat aat cag cct aaa  tta  agt gca gca agt gct cgt tta att ggc gtg gaa  -154

69-   P  Q  K  T  N  S  L  S  V  V  I  S  A  A  V  L  G
      cct caa aaa aca aac tct  ctt  tca  gtt  gta ata tca gca gct gtc cta ggt  -205

86-   L  L  G  A  G  L  Y  V  F  L  I  E  P  K  L  N  P
      tta tta  ggt gct  gga  tta  tac gtg  ttt  ctt atc gaa cct aaa ctg aac cca  -256

103-  A  Y  E  N  M  G  F  S  C  E  G  K  T  Y  C  S  E
      gcg tat gaa aat atg ggt  ttt  agt tgt  gaa ggt aaa acg tac tgt agc gaa  -307

120-  M  V  S  C  D  E  A  K  F  Y  L  S  N  C  P  N  V
      atg gtg tct tgt gac gaa gct  aag  ttt  tat  ttg  tca aac tgc ccg aac gta  -358

137-  K  I  D  G  D  R  D  G  I  P  C  E  S  Q  L  C  G
      aaa ata gac ggc gac cgt gat ggc atc cct tgt gag agc cag tta tgt ggc    -404

154-  G  W  *
      ggc tgg tag                                                          -460
```

Appendix 3. Nucleotide and deduced amino acid sequence of cold shock protein *V. vulnificus* CMCP6 (GenBank accession number VV12119 and NP761361), designated *csp2* in this study. The nucleotides are numbered on the right and the amino acids are numbered on the left. An asterisk above the last three nucleotides indicates a stop codon.

```
1-    M  Y  S  M  A  T  G  T  V  K  W  F  N  N  A  K  G
      atg tat agc atg gct aca ggt aca gta aag tgg ttc aat aac gcc aaa gga    -1

18-   F  G  F  I  C  S  D  Q  E  E  G  D  I  F  A  H  Y
      ttt ggt ttt atc tgt tca gat caa gag gaa ggc gac atc ttc gcc cac tac    -51

35-   S  T  I  Q  M  D  G  Y  R  T  L  K  A  G  Q  Q  V
      tca acg att cag atg gac ggt tat cgt aca ctg aaa gcg ggc caa caa gtc    -103

52-   T  Y  E  I  E  K  G  P  K  G  C  H  A  S  S  V  V
      acg tat gag att gag aaa ggt ccg aaa ggg tgt cac gct agc agc gtc gtt    -154

69-   P  L  E  V  Q  A  T  K  *
      cca ctc gaa gta caa gca acg aag taa                                   -205
```

Appendix 4. Nucleotide and deduced amino acid sequence of cold shock protein *V. vulnificus* CMCP6 (GenBank accession number VV12119 and NP760979) and *V. vulnificus* YJ016 (GenBank accession number VV2324 and NP935117), designated *csp3* in this study. The nucleotides are numbered on the right and the amino acids are numbered on the left. An asterisk above the last three nucleotides indicates a stop codon.

```
1-    M  S  N  T  V  T  G  T  V  K  W  F  N  E  T  K  G
      atg tct aac aca gta acc ggc acc gta aaa tgg ttt aac gaa act aaa ggc    -1

18-   F  G  F  I  K  Q  E  N  G  P  D  V  F  A  H  F  S
      ttt ggc ttc atc aag caa gaa aac ggc ccg gac  gtt ttt  gca cac ttc  tct   -51

35-   A  I  K  G  D  G  F  R  T  L  A  E  G  Q  K  V  S
      gct atc aaa ggt gat ggc ttc cgc act ctt  gct gaa ggt cag aaa gtt tct    -103

52-   F  V  I  S  Q  G  Q  K  G  P  Q  A  E  E  I  T  L
      ttc gtc atc tct caa ggc caa aaa ggt cct caa gca gaa gag atc aca ctg    -154

69-   L  *
      ctt taa                                                               -205
```

Appendix 5. Nucleotide and deduced amino acid sequence of cold shock protein *V. vulnificus* CMCP6 (GenBank accession number VV20519 and NP762479) and *V. vulnificus* YJ016 (GenBank accession number VVA1067 and NP937123), designated *csp4* in this study. The nucleotides are numbered on the right and the amino acids are numbered on the left. An asterisk above the last three nucleotides indicates a stop codon.

```
1-    M  S  N  K  V  T  G  S  V  K  W  F  N  E  T  K  G
      atg tct aat aaa gta act ggt tca gta aaa tgg ttt aac gaa acg aaa ggt    -1

18-   F  G  F  I  S  Q  D  N  G  G  N  D  V  F  V  H  F
      ttc ggt ttt att tct caa  gac aac  ggt ggt aac gat gtg ttt  gtt cac ttc   -51

35-   N  S  I  V  S  T  G  F  K  T  L  A  E  G  Q  R  V
      aac tct atc gtt tca aca ggt ttt aaa act ttg gca gaa ggc caa cgc gta    -103

52-   T  F  E  V  E  Q  G  K  K  G  P  Q  A  A  N  V  T
      aca ttt gaa gtt gag caa ggc aaa aaa ggc cca caa gct gca aac gta act    -154

69-   A  L  *
      gcg cta taa                                                          -205
```

Appendix 6. Nucleotide and deduced amino acid sequence of cold shock protein *V. vulnificus* CMCP6 (GenBank accession number VV20503 and NP762464) and *V. vulnificus* YJ016 (GenBank accession number VVA1052 and NP937108), designated *csp5* in this study. The nucleotides are numbered on the right and the amino acids are numbered on the left. An asterisk above the last three nucleotides indicates a stop codon.

Appendix 7. Similarities between *V. vulnificus* cold shock protein obtained from *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4, designated *csp1* in this study.

Protein	Species	% identity
Cold shock protein	*V. vulnificusCMCP6*	100
	V. vulnificusYJ016	
Cold shock transcriptional regulator CspA	*V. cholerae*	81
Cold shock transcriptional regulator CspA	*V. parahaemolyticus*	80
Cold shock transcriptional regulator CspA	*V. alginolyticus*	83
Putative cold shock-like protein	*Photobacterium profundum*	80
Cold shock protein, DNA binding	*Shewanella denitrifican*	80

Appendix 8. Similarities between *V. vulnificus* cold shock protein obtained from *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4, designated *csp3* in this study.

Protein	Species	% identity
Cold shock-like protein CspD	*V. vulnificusCMCP6* *V. vulnificusYJ016*	98
Cold shock-like protein CspD	*V. parahaemolyticus*	92
Cold shock-like protein CspD	*V. alginolyticus*	94
Cold shock-like protein CspD	*V. cholerae*	78
Cold shock-like protein CspD	*Vibrio sp.*	80
Cold shock-like protein CspD	*V. splendidus*	80

Appendix 9. Similarities between *V. vulnificus* cold shock protein obtained from *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4, designated *csp4* in this study.

Protein	Species	% identity
Cold shock protein	*V. vulnificusCMCP6* *V. vulnificusYJ016*	98
Cold shock DNA-binding domain protein	*V. parahaemolyticus*	86
Cold shock DNA-binding domain protein	*V. cholerae*	86
Cold shock protein	*Pseudoalteromonas tunicata*	81
Cold shock DNA-binding domain protein	*V. splendidus*	81
Cold shock protein	*Hahella chejuensis*	82

Appendix 10. Similarities between *V. vulnificus* cold shock protein obtained from *V. vulnificus* strain ATCC 27562, C7184Tr, VV3, and VV4, designated *csp5* in this study.

Protein	Species	% identity
Cold shock protein	*V. vulnificusCMCP6*	98
	V. vulnificusYJ016	
Cold shock transcriptional regulator CspD	*V. cholerae*	87
Cold shock domain family protein	*V. cholerae*	84
Cold shock protein CspV	*V. cholerae*	84
Cold shock transcriptional regulator CspD	*V. parahaemolyticus*	80
Cold shock transcriptional regulator CspD	*V. alginolyticus*	82