Analysis of bacterial communities in the eastern oyster (*Crassostrea virginica*) with emphasis on *Vibrio vulnificus* dynamics under refrigeration

by

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Abstract

In this study we determined the effect of refrigeration on the total bacterial, total *Vibrio* spp., and *V. vulnificus* populations present within the eastern oyster (*Crassostrea virginica*), and the effects of cold shock (35°C to 4°C) on the complete *V. vulnificus* transcriptome. Oysters from two different locations, the Auburn University Shellfish Laboratory, Dauphin Island, AL, and a commercial processor, were compared during two weeks under refrigeration conditions. During the course of the experiment, total aerobic bacteria counts increased by two logs. Ribosomal Intergenic Sequence Analysis (RISA) and Denaturing Gradient Gel Electrophoresis (DGGE) were used to determine changes within the total population, while DGGE was used to evaluate changes of the *Vibrio* spp. population over the two week period. RISA-derived data showed that the microbial communities at Day 1 were clearly different from both Day 7 and 14 samples. Within the Day 1 cluster, samples were subdivided based on location. On Day 7 and 14, samples could also be subdivided by date and origin. *Vibrio*-specific DGGE also allowed for the clustering of samples by location on Day 1, which also clustered away from Days 7 and 14. Bands corresponding to both *V. parahaemolyticus* and *V. vulnificus* decreased in prevalence, while those corresponding to *V. mimicus* increased during the two weeks of refrigeration, particularly on Day 7. Interestingly, *V. vulnificus* counts determined by colony dot-blot hybridization remained unchanged throughout the experiment. *Vibrio vulnificus* isolates were recovered from oysters and genotyped as 16S type A, B or AB by

restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (ALFP) analyses. 16S type B isolates (typically associated with clinical cases) comprised 53% of the isolates. An important note was the identification of 16S type B-specific AFLP bands that differentiated type B from type A and AB. A DNA microarray of *V. vulnificus* showed that 165 genes out of 4,488 altered their expression profiles by more than twofold. The highest induction observed occurred in two of the five categorized cold shock genes, *cspA* and *cspB*, which showed a complementary and persistent expression pattern during cold shock suggesting a homologous role. Other genes showing a significant fold increase included ribosomal genes, protein folding regulators, and membrane genes.

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Table of Contents

List of Tables

List of Figures

I. Literature Review

Easter Oyster (*Crassostrea virginica*) Biology

Morphology. The eastern oyster (*Crassostrea virginica*) (Gmelin, 1791) belongs to the phylum Mollusca, class Bivalvia, order Ostreoida and family Ostreidae. Other common names are American oyster, Virginia oyster, and the Atlantic oyster *(7, 152)*. Eastern oysters are monomyarian lamellibranchs, or single muscled bivalves, exhibiting bilateral asymmetry and a restricted coelom. The valves are asymmetrical with the left being thicker and more deeply cupped than the right *(90, 117)*. There is no gap between the two halves when the oyster is closed. When settling on substrate, the oyster always does so on the left valve *(7)*. The shape and thickness of the shell is variable. These parameters differ depending on the environmental conditions in which the oyster grows. Umboes, or the pointed dorsal area located along with the hinge, are curved and point toward the posterior end of the oyster. Shells are thicker when growing on hard substrates. In silty environments or on reefs, umboes generally grow straight, but shells are more fragile than those growing on hard substrates. Solitary oysters found on hard substrates are usually rounded with radial ridges and foliated processes while those growing on soft substrates and reefs are more slender with few ridges The unitless "index of shape" ((height + width)/length) varies from 0.5 to 1.3 in southern populations and from 0.6 to 1.2 in northern populations *(7, 158)*.

The interior of the shell has a purple pigmented adductor muscle scar, slightly to the posterior and ventral sides of the oyster, which is used to differentiate the eastern oyster from related species. *Crassostrea* spp. have a well developed promyal chamber in which the dorsal end of the right exhalant chamber is expanded to allow for trapping

saltwater. This system to trap water is believed to aid in allowing a greater tolerance of salinity fluctuations which occur frequently in estuarine environments *(158)*. Overall, oysters exhibit great morphological plasticity as adults, but quite static morphology as larvae. The former is due to the great amount of environmental variability in individual locations and lack of selection on adult forms and the latter is due to evolutionary forces constraining the single motile life stage upon which the species is dependent for long-term persistence in a highly variable and ever changing estuarine environment *(7, 28)*.

The oyster body consists mainly of a large visceral mass, two mantle skirts, a mantle cavity, a large adductor muscle, a pair of gills, and a pair of labial palps. There is no foot, no anterior adductor muscle, and no siphons. The oysters contains a digestive system, rudimentary nervous system, circulatory system, excretory system and a reproductive system *(90)*.

Reproduction and Growth. Eastern oysters are protandric, meaning that they exhibit male reproductive system initially in life. Later, they switch to female and possibly revert back to male. This change can occur as frequently as yearly depending on the response of the oyster to environmental, nutritional and/or physiological stresses *(166)*. Sex determination may also depend on proximity and sex of the nearest neighbors, with oysters changing to the sex of the minority when grown in densely populated clusters. Sex reversal usually occurs between spawning seasons when the gonad is undifferentiated *(7, 166)*. Oyster fecundity is difficult to establish and is attributed to the long, intermittent spawning periods and redevelopment throughout the year. In addition, assessment of the sex state of oyster is further hindered by gonadal tissue that is diffuse and integrated into surrounding tissue. Despite the variability in the sex state within the

eastern oyster, spawning itself is initiated by defined parameters, which can include any combination of factors, such as water temperature, salinity and physiochemical interactions. Temperature is the most common associated parameter for spawning. Oysters in the northern regions typically spawn when the temperature reaches between 60 and 68°F, while oysters in southern waters spawn when the temperature reaches about 68°F. This spawning can occur throughout the warm months in each respective region, particularly when the salinity is higher than 10 practical salinity units (psu) *(7, 173)*.

The eastern oyster is nonincubatory and releases its eggs and sperm directly into the water. Fertilization takes place during this free floating stage. After fertilization, oysters develop through several free-swimming larval stages including trocophore, veliger, pediveliger, and spat before attaching to a hard substrate and becoming sessile *(7, 90, 173)*. Trochophore is the first larval stage and is formed four to six hours following fertilization and lasts approximately one to two days. Oysters in this larval stage do not feed. In the subsequent larval stages the oysters are planktotrophic. These oysters feed on small plants and animals. Veliger stages, which last up to two months, include several morphological changes to the larvae resulting in a fully developed larva possessing a well-developed foot. The foot is used for locomotion when seeking a place to attach after settling on appropriate substrate. Upon setting, the foot is reabsorbed and a final metamorphosis occurs leading to an attached oyster *(90)*.

The growth rate of the eastern oysters is largely dependent on temperature and the availability of food. Oysters undergo rapid growth during the first six months of life, growing as much as 10 mm/month, but slow throughout the rest of their life *(7, 145)*. Oysters typically reach 15 cm in length in five to six years. The left valve grows faster

than the right, therefore older oysters have a larger attached valve *(28)*. Because of temperature and nutrient differences, harvest size (76-90 mm) is reached in the Gulf of Mexico 18-24 months after setting whereas northern oysters, such as those from Long Island Sound, can take 4-5 years to reach a similar size *(7)*. Oyster can live 25-30 years and reach a terminal size of 20-35 cm in length. These large individuals are usually associated with undisturbed bottoms where commercial fishing is prohibited *(28)*. Oysters are filter feeders, feeding primarily on phytoplankton and suspended detritus. Food items range in size from 1-30 μm. Filtration rates have been reported to range from 1.5- 10.0 L/h/g dry tissue weight. The eastern oyster is capable of adjusting feeding rates depending on the size, type and composition of the available food source *(90)*.

The Gulf of Mexico Oyster Industry

The two major species of oysters produced in the United States are the eastern oyster (*Crassostrea virginica*) and the pacific oyster (*Crassostrea gigas*). Between 2004 and 2009, the eastern oyster landings accounted for 65,451.2 metric tons totaling $505,761,664.00, representing 66% of the total U.S. oyster production, while the pacific oyster landings accounted for 33,156.3 metric tons totaling $246,983,634.00 or roughly half the tonnage and value of the eastern oyster *(124)*. Most eastern oysters are now harvested from the Gulf of Mexico, which is the major harvesting site for this species in the U.S. In 2009, the amount of eastern oysters landed in the Gulf of Mexico accounted for roughly 91% of all the eastern oysters harvested from all around the U.S. The value of

landings in the Gulf has increased yearly from $20,138,817 in 1980 to $72,779,342 by 2009 *(124)*.

Oyster harvesting in the U.S. can be done throughout the year. Eastern oysters are harvested using equipment such as dredges or tongs, or are harvested by hand. In the Louisiana and Texas fisheries, dredging is the most common harvesting method. In 2002, dredging accounted for 99% of the harvest in Louisiana and 85% of the harvest in Mississippi. Historically, dredges been the primary equipment used in Texas *(108)*. Other harvesting methods generally account for less than one percent of the total oyster catch *(17)*. In Alabama and Florida, harvesting oysters with tongs or by hand are the only harvesting methods allowed on public reefs *(108)*.

When oysters are harvested, they are transported to wholesalers and/or processors for treatment and packing before being distributed to retailers and consumers. Some may be directly delivered from the harvester to restaurants or retailers without processing. Oysters can be sold as whole oysters, shucked, and half-shell processed. Additionally, they can be kept fresh and alive, frozen, pasteurized, smoked or canned. Oysters can be consumed raw or cooked based on consumer's taste and the quality of freshness, flavor, odor, and texture. In-shell oyster demand appears to be in higher in summer than in other seasons, whereas shucked oyster demand tends to increase during winter season *(4)*.

Microbial Contents of Oysters and Food Safety

Bivalves such as oysters are filter feeders that concentrate microorganisms in their digestive tracts. Because many people eat raw or under cook oysters, there is a concern

over the safety of such products. In fact, the number of heterotrophic bacteria is greater in bivalve shellfish than in its surrounding water *(92)*. Human pathogens may easily become concentrated within these oysters that are eaten raw or undercooked *(34)*. Most raw oysters are consumed in the warmer months. During these months when the water is at a higher temperature, the oysters are likely to have a higher number of bacteria from the genus *Vibrio,* for instance, than the ones collected from cold water *(29, 89, 137)*. It is during colder months that the oysters are better able to clear the bacterial load during filter feeding than when the water temperatures are warmer *(70)*. There are however, some genera of bacteria that seem to be better suited for survival within the oyster during periods of cooler water temperatures. These include *Pseudomonas, Moraxella,* and *Acinetobacter.* Each of these genera of bacteria have been found to be thriving within fish and shellfish stored under refrigeration *(12)*.

Several genera of bacteria have been isolated from oysters such as *Aeromonas, Acinetobacter, Alcaligenes, Achomobacter, Alteromonas, Campylobacter, Clostridium, Marinomonas, Flavobacterium*/*Cytophaga, Proteus, Pseudomonas, Pseudoalteromonas, Nocardia, Serratia, Salmonella, Escherichia, Enterococci, Enterobacter, Shewanella, Micrococcus, Bacillus, Lactobacillus, Corynebacterium, Staphylococcus, Vibrio,* and *Corynebacterium (11, 33, 57, 60, 80, 94, 119, 143, 147, 179).* These include bacterial species that are ubiquitous to the oyster's waters and those found due to contamination of water bodies by humans.

Concerning food safety, threats to humans can come from both pathogens that have been concentrated in the oyster from its surrounding water and those introduced through improper handling of the product after harvest. Specific pathogens typically

associated with shellfish and aquatic environments in general include *Vibrio cholerae, V. vulnificus, V. parahaemolyticus, Listeria monocytogenes, Clostridium botulinum, Plesiomonas* spp. and *Aeromonas* spp. Naturally occurring bacteria accounted for 20% of shellfish-related illnesses and 99% of the deaths in the United States *(104).* Species of bacteria that are known to be unnatural contaminants include *Salmonella* spp., pathogenic *Escherichia coli, Shigella* spp., *Campylobacter* spp., and *Yersinia enterocolitica*. These species are usually in the environment as a result of fecal contamination. Bacterial pathogens associated with fecal contamination represent only 4% of the shellfish-associated outbreaks. Non-indigenous species may also be introduced after harvest, typically during transport and processing. These species my include *Bacillus cereus, L. monocytogenes, Staphylococcus aureus* and *C. perfringens (114, 142, 143).*

Of particular concern are the members of the Vibrionaceae family, which comprise the largest bacterial community in oysters, representing up to 20% of the total flora *(26). Vibrio* spp. are not associated or correlated with fecal bacteria *(104).* The genus *Vibrio* consists of more than 40 species, some of them pathogenic to humans *(30). Vibrio vulnificus, V. parahaemolyticus, V. alginolyticus, V. splendidus, V. harveyi, V. phosphoreum, V. cholerae, V. crassostreae, V. aestuarianus, V. natriegens, V. campbellii, V. fluvialis, V. hollisae, V. mimicus,* and *V. pelagius* are usually isolated from oysters *(32, 38, 41, 44, 45, 49, 51, 59, 144, 154, 168). Vibrio* spp., in particular *V. vulnificus* and *V. parahaemolyticus*, have been shown to pose a significant health risk to consumers of raw or undercooked seafood. *Vibrio vulnificus* has been linked to primary septicemia resulting from the ingestion of raw shellfish, with a death rate as high as 50% in patients with preexisting conditions such as hemochromatosis, cirrhosis, diabetes, immuno-

compromising diseases, and kidney failure *(66)*. *Vibrio parahaemolyticus* is the leading cause of *Vibrio*-associated gastroenteritis in the United States. More infrequently, two other *Vibrio* species, *V. mimicus* and *V. hollisae*, have been associated with gastroenteritis resulting from the consumption of raw or undercooked seafoods *(1, 39, 142)*. In 2011, an outbreak of *V. cholerae* affected eight people in Florida and was linked to the consumption of raw oysters (Dr. Wright, University of Florida, personal communication)

Post Harvest Treatments of oysters

There are several post harvest treatment methods that can be used to reduce the number of potentially pathogenic bacteria in oysters *(24, 42, 120)*. These post harvest methods include depuration, high salinity relaying, high hydrostatic pressure treatment, gamma irradiation, heat/cool pasteurization, and individually quick freezing *(5, 98, 118)*.

Depuration and Relaying. Depuration is the process of controlled purification whereby shellfish are placed in disinfected, recirculating seawater and allowed to actively filter feed for 3 to 4 days *(65)*. This allows the oysters to filter in an optimum water environment in which the clearing of bacterial contaminants is favorable. The use of this practice is quite limited in the U.S. but extensive in Europe. Depuration has been proven to be useful in reducing the number of indicator bacteria but not the number of pathogens. Some bacteria have been shown to continue to persist within the oyster *(88, 161)*. The results appear to be inconclusive on the actual effectiveness of this treatment. It has been shown that oysters artificially inoculated with pathogenic *Vibrio vulnificus* strains were able to clear the pathogen to non-detectable numbers within 48 hours. However,

naturally occurring strains were not able to be cleared during the same study *(65)*. Another study showed that *V. vulnificus* appears to be slowly depurated from oysters with complete elimination after 16 days *(88)*. UV light has shown to be effective at helping to clear bacteria in recirculating water systems when the water temperature was 15°C but not when the water temperature was above 21°C *(161)*.

Relaying is the process of moving shellfish from a restricted harvesting area to an open area where natural cleansing can occur. It has been shown that relaying oysters into waters of higher salinity than those of harvest for 7 days can reduce the number of *V. vulnificus*. However, some batches required 1 month or longer to reduce to <10 cells per gram *(85)*. For example, Motes and Depaola *(118)* demonstrated that relaying decreased *V. vulnificus* levels from 10^3 colony forming units (CFU) per ml to <10 most probable number (MPN) per g within 17 to 49 days.

High hydrostatic pressure (HHP). This is a non-thermal process that subjects foods to hydrostatic pressures up to 1035 megapascals (MPa) *(24)*. The oysters are placed into large tanks that are filled with water and electronically controlled to pressurize the tank to a desired level. After treatment, the oysters may be sold in-shell wrapped with a plastic band to hold the shell firmly shut and are shipped to wholesalers/retailers for distribution to consumers. They can also be shucked into half-shell or frozen using liquid nitrogen in order to lengthen shelf-life. High hydrostatic pressure causes changes in essential enzymes of microorganisms and their membrane permeability *(71)*. The method is also referred to as High-Pressure Processing (HPP) or ultra high-pressure processing (UHP). High hydrostatic pressure processing was first applied commercially to oysters in the summer of 1999 in Louisiana *(120)*. When using

pressure to inactivate microorganisms, the treatment depends on the intensity of the pressure and the length of exposure *(78)*. In general, *Vibrio* species are extremely sensitive to pressure. It has been demonstrated that *V. parahaemolyticus* is rapidly reduced to nondetectable levels at pressures higher than 172 MPa when suspended in clam juice *(160)*. It was also reported that treatment with hydrostatic pressure of 250 MPa for 10 min at 25 °C reduced *V. vulnificus* in pure culture to nondetectable levels *(16)*. This method is considered effective and is one of the most commonly used technologies for food preservation since it has the ability to reduce and/or to destroy the microbial community present in the food, lengthening shelf-life, while providing a safer and better quality food, and increasing market value. Another advantage of this technology is that it does not use heat, therefore, sensory and nutritional attributes of the product remain virtually unaffected, yielding products with better quality than those processed by traditional methods *(143, 147, 150)*.

Gamma irradiation. Gamma or ionizing irradiation can be used to eliminate bacteria from oysters. The process was discovered in the 1920's. It was then used to preserve different types of food, such as fruits, vegetables, dairy products, and meat, during World War II *(170)*. Irradiation is considered one of the most efficient technological processes for the reduction of microorganisms in food. It can cause damages to cells by altering their genetic materials. It has been successfully used as a tool to reduce pathogenic bacteria, eliminate parasites, and decrease post-harvest sprouting in many products *(163)*. One study found that a 2 kilogray (kGy) dose of gamma radiation applied to oysters was sufficient for pasteurization without causing changes in organoleptic quality. After this treatment, total bacterial counts decreased by

99% *(127)*. Andrews et al *(5)* showed that ionizing irradiation doses of 1.0 kGy reduced *V. vulnificus* at initial inoculum of 10^7 CFU/g to non-detectable levels when applied to whole shell oysters. Oysters inoculated with *V. parahaemolyticus* 03:K6 (10^4 CFU/g) reached nondetectable levels after treatment with 1.5 kGy. The majority of the oysters survived the treatment. Further, sensory data showed that consumers could not tell a difference between irradiated and nonirradiated oysters. Recently, the FDA approved irradiation as a food additive for seafood, including oysters. However, consumer perception and lack of knowledge on the long terms effects on consumers of gamma irradiated foods have stalled the widespread acceptance of this method.

Heat/cool pasteurization. Pasteurization is a long used method for preserving foods. Pasteurization is the process of treating a food by heating it to a certain temperature for a specified time to reduce the numbers of harmful organisms such as bacteria, viruses, and molds to safe levels for human consumption without affecting the flavor or quality of the food. Pasteurization has been used to process milk, beer, wine, fruit juices, cheese, and egg products. There are various types of pasteurization available for food processing. High temperature-short time pasteurization uses temperatures from 71.5°C to 74°C for about 15 to 30 seconds. Ultra-high temperature treatment, also called ultra-heat treatment (UHT) uses a much shorter time, around 1-2 seconds, at temperatures exceeding 135°C. UHT reduces the processing time which minimizes the spoiling of nutrients within the foods *(6)*. Low temperature pasteurization has been used on oysters. The process was first implemented commercially in 1997 at a plant in Louisiana. The process consists of submerging oysters in a tank of water at 50ºC for 24 minutes. The oysters are then submerged into a tank of cold water at 4ºC for 15 min *(120)*. It has been

shown that low temperature pasteurization at 50°C for up to 15 minutes can reduce *V. vulnificus* and *V. parahaemolyticus* to non-detectable levels. In addition, spoilage bacteria can also be reduced by 10^2 to 10^3 CFU/g, which has been shown to increase the shelf life of oysters up to 7 days beyond that of unprocessed oysters *(6)*.

A similar process has already been implemented in the oyster industry, primarily in North and South Carolina. This method has been used to aid in shucking oysters. The process involves submerging about 70 chilled oysters in wire baskets into a tank containing approximately 850 L of potable water at a temperature of 67°C for about 5 minutes, depending on oyster size and relative oyster condition. After heat-shocking, the oysters are cooled by spraying for 1 min with potable water prior to shucking and washing. It was shown that this commercial heat-shock process reduces *V. vulnificus* levels by 2 to 4 $\log_{10}$ CFU, while no reduction in *V. vulnificus* levels were observed in oysters that were merely washed *(74)*.

Individually quick freezing (IQF). Freezing has been shown to be effective for preserving foods for a long period of time. It can stop the biological activity of some microbes while at the same time preserving others. Despite this, freezing foods for preservation has a well established safety record, with few cases of illness linked directly to frozen foods. Freezing affects microorganisms via physical and chemical effects and even possibly through the induction of genetic changes. In fact studies have proposed that many pathogenic microorganisms may be sub-lethally injured by freezing *(8)*. The technique has been applied to oysters since 1988. With this method, oysters are shucked on the half shell and sent through a freezer tunnel that rapidly cools the oysters using liquid carbon dioxide *(120)*. Oysters treated in this manner have a shelf life of up to one

year *(151)*. It has been shown that freezing reduces the levels of *Vibrio* species in shellfish although it does not eliminate the organism, even after frozen storage for up to 12 weeks. A temperature of -20°C was more effective for inactivating *V. vulnificus* than was 0°C. At -80 °C, *V. vulnificus* and *V. parahaemolyticus* cell numbers in brain heart infusion broth supplemented with 3% NaCl dropped by one $\log_{10}$ CFU/g during the freezing process but remained stable thereafter for 35 days *(13)*. Johnson and Brown also demonstrated that *Vibrio* organisms, whether in the culturable or the non-culturable form, were not inactivated by freezing at -20°C *(81)*. The Interstate Shellfish Sanitation Commission (ISSC) has adopted freezing combined with frozen storage as an acceptable means for post-harvest treatment to control *V. vulnificus* and *V. parahaemolyticus*. A number of firms now use this process.

Refrigeration. Refrigeration alone is also considered and used as a storage and preservation method for oysters, particularly for raw oysters. One study investigated the effects of various storage temperatures (10, 22, 30°C) on oysters and found that members of the Vibrionaceae family increased in concentration in shellstock oysters stored at 22 and 30°C, while 10°C storage prevented growth *(37)*. Later, the same group observed that *V. vulnificus* did not multiply in oysters stored below 13°C and growth at 18°C was significantly slower than at ambient air temperature (23 to 34°C) *(34)*. After 14 to 21 days of refrigerated storage, *V. vulnificus* was shown to decline to non-detectable levels *(36)*. However, it was later shown by Prapaiwong et al *(143)* that the concentration of *V. vulnificus* isolated in one sampling of raw oysters (10^3/g of oyster meat) remained constant throughout 21 days of storage under refrigeration.

Because some bacteria, *V. vulnificus* in particular, can grow at tremendous rates in oysters whose temperature is not properly controlled, guidelines were put into place to manage the time from oyster harvest to refrigeration. In 1993, the National Shellfish Sanitation Program (NSSP) set forth the first refrigeration guidelines for raw molluscan shellfish. These were updated in 1995, stating that shellstock must be placed under temperature control within 12-14 h of harvest, depending on the average monthly maximum water temperature *(35)*. As of the 2007 revision of the Guide for the Control of Molluscan Shellfish even more stringent requirements are mandatory for the harvest of oysters. In harvest waters where there have been no identified cases of infection of *V. vulnificus*, the harvest requirements are broken up into three levels. In waters where the current average monthly air temperature (CAMAT) is <18°C, up to 36 hours may be taken to get the oysters down to a temperature control point of 10°C or less. Oysters harvested from waters where the CAMAT is 19 - 27°C must be placed in the proper storage temperature within 24 hours, and oysters harvested from waters where the CAMAT is ≥27 °C must be placed in the proper storage temperature within 20 hours. Waters where two or more *V. vulnificus* reports have been confirmed require a different set of standards. Oysters coming from those waters, with a current water temperature of ≤18°C, 18- 23°C, >23 - 28°C, and >28°C, have 36, 14, 12 and 10 hours respectively to be placed under temperature control *(54)*.

Vibrio vulnificus

The genus *Vibrio* belongs to the family Vibrionaceae. This family consists of seven genera as of 2011: *Allivibrio* (6 species), *Enterovibrio* (4 species), *Salinivibrio* (6 species), *Catenococcus* (1 species), *Grimontia* (1 species), *Vibrio* (89 species), and *Photobacterium* (21 species) *(107).* In addition, it has been suggested that the genus *Listonella* be added to the Vibrionaceae family and that two of its species be renamed as *Vibrio* sp. *(165).* Vibrios are ubiquitous in the marine environment and all species, except *Vibrio cholera* and *Vibrio mimicus*, require sodium chloride supplementation of media for growth. Of the 13 pathogenic vibrios, *V. cholerae* O1, *V. parahaemolyticus,* and *V. vulnificus* are considered the most significant. Members of the *Vibrio* genus are straight or curved Gram-negative, nonspore-forming rods, 0.5 to 0.8 μm in width and 1.4 to 2.6 μm in length *(46, 113).* They are motile by a single polar flagellum and are either aerobic or facultatively anaerobic. Most species produce oxidase and catalase and ferment glucose without producing gas *(113).* Two of the most distinctive reactions of *V. vulnificus* are fermentation of lactose and production of β -D-galactosidase and these tests can be used to distinguish it from the related *Vibrio parahaemolyticus (77).* *Vibrio vulnificus* was first isolated in 1964 by the U.S. Centers for Disease control (CDC), although at that time it was misidentified as a virulent strain of *V. parahaemolyticus (159).* In 1976 it was recognized as a unique species when researchers realized that wound infections were caused by a species with unique characteristics compared to other *Vibrio* spp. In 1980 it was officially given its taxonomical name: " vibrio", meaning "to quiver", and "vulnificus", derived from Latin meaning "wound" *(116).*

Ecology. *Vibrio vulnificus* is ubiquitously found throughout the estuarine environment. It can frequently be isolated in high numbers from bivalves, crustaceans,

finfish, sediment, and plankton *(40, 46)*. In the United States, the bacterium can be found primarily in coastal waters from Maine down the eastern coast and all along the Gulf of Mexico. It has been isolated from waters of widely varying temperatures and salinities *(180)*. It has been demonstrated that at salinities between 5 and 25 ppt, *V. vulnificus* numbers can increase *(86)*. However, when salinities are 30, 35, and 38 ppt, *V. vulnificus* numbers decrease by 58, 88, and 83%, respectively. The same trend was reported by Motes et al. *(118)*, who observed lower numbers of *V. vulnificus* at salinities above 28 ppt.

The prevalence of the *V. vulnificus* has been shown to be associated with individual seasons of the year because of the change in water temperatures. It is frequently isolated from warmer waters, particularly when the temperature is $\geq$ 25°C, as is common from April to September. Although not as frequent as with warmer waters, it has been isolated from the colder waters. In fact *V. vulnificus* has been isolated from waters ranging from 9 to 31°C. Its optimal growth occurs at 35°C, however it has never been responsible for human infections in areas where summer water temperatures reach 35°C *(118)*. When grown under sub optimum temperatures (<25°C), *V. vulnificus* was able to proliferate from 13°C to 22°C, but was not able to survive as well when grown under 8.5°C *(87)*. This apparent seasonality has been attempted to be explained by many researchers. 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 (VBNC). It has been proven that *V. vulnificus* enters a VBNC state at temperatures below 10°C under low nutrient conditions *(129)*. Typically, U.S. Gulf Coast oysters harbor between 10^3 to 10^4 *V. vulnificus* cells/g during the warmer months

from April through October but typically less than 10 cells/g during colder months *(36)*. Some believe that the bacterium alternates between a planktonic state, in which it colonizes both plankton and animals, and dormant state where it settles into the sediment. The settling would occur in the cooler seasons when the water temperatures are not optimum for growth. Then in the warmer seasons, they would begin to proliferate and move into the water column to again colonize its summer hosts *(46, 180)*.

Pathogenesis. *Vibrio vulnificus* exists as both virulent and avirulent strains. Virulence factors have been reported for *V. vulnificus* and include the presence of a polysaccharide capsule, various extracellular enzymes, and the ability to obtain iron from transferrin *(103)*. A capsule causes opacity of colonies which makes the presence of the capsule easy to determine. Encapsulated isolates can undergo a reversible phase variation to a translucent colony phenotype that is correlated with reduced capsular polysaccharide (CPS). These translucent strains are less virulent than opaque strains. Wright et al. *(182)* reported that translucent strains had a lethal dose up to four times higher than that of opaque strains. Another study found that translucent colonies of *V. vulnificus* were avirulent in mice *(115)*. It has been shown that infection with *V. vulnificus* elicits an antibody response specific to the capsule and *V. vulnificus* relies on the capsule to resist host defenses during systemic disease *(46)*.

Several extracellular enzymes also play a role in *V. vulnificus* pathogenicity. The enzymes lecithinase, lipase, caseinolytic protease and DNase were present in >90% of the *V. vulnificus* strains screened by Moreno and Landgraf *(115)*. Systemic infections may be made possible by one of these proteases as it was discovered that 91% of the clinical and environmental strains of *V. vulnificus* produced a protease that was capable of

breaking down native albumin *(131)*. A separate metalloprotease containing a zinc atom was also discovered that is able to degrade a number of biologically important host-associated proteins, including elastin, fibrinogen, and plasma protease inhibitors, with the degradation of the latter aiding in a vascular permeability-enhancing action of the bacteria *(155)*. The extracellular enzyme commonly associated with *V. vulnificus* is the exotoxin cytolysin/hemolysin (vvhA). It is a heat-labile enzyme that lyses mammalian erythrocytes and is cytotoxic to a variety of mammalian cell lines *(63, 159)*. Antibodies to the enzyme have been reported in infected mice, which leads to the belief that the enzyme has a role in pathogenicity *(64)*. The same group also demonstrated that mice injected with the hemolysin developed skin damage similar to that of infected humans. In another study mice were inoculated with 10^7 CFU of a clinical *V. vulnificus* isolate. Bacterial cells recovered from the host liver were shown to be expressing the *vvh*A gene product which leads to the belief that the protein itself is produced *in vivo* and in association with particular tissues *(96)*.

It was previously shown that *V. vulnificus* cannot grow on iron limited media. Morris et al. showed that the bacteria was not able to grow in the presence of 30% saturated transferrin while some isolates were able to grown in the presence of 100% saturated transferrin indicating the importance of iron *(116)*. Transferrin is an iron transport protein and, because free iron is virtually absent in the body, pathogenic bacteria like *V. vulnificus* may have evolved mechanisms to scavenge iron from the iron transport proteins *(159)*. *Vibrio vulnificus* produces two siderophores, hydroxymate and phenolate, that when mutated have been shown to lead to a reduction in virulence *(105)*.

Epidemiology. *Vibrio vulnificus* infections present in one of three ways, a primary septicemia, gastroenteritis, or a wound infection, at the proportion of 17, 24, and 54% respectively *(75)*. Fatality rates from that study were 1% for gastroenteritis, 5% for wound infection, and 44% for septicemia. Sixty-eight percent of gastroenteritis and 83% of primary septicemia cases were associated with raw oyster consumption while 91% of the primary septicemia cases and 86% of the wound infections occurred in the months of April through October, with 48% of those with primary septicemia reporting pre-existing liver disease.

Wound infections caused by *V. vulnificus* are usually started as redness and swelling with the pain at the site of the wound. The illness will progressively affect the whole body and it is fatal about 20% of the time. To prevent death, destructive surgical treatments are needed such as amputations. The symptoms begin within 24 hours of the initial infection (CDC 2006). The most common ways of contracting this type of infection are wound exposure to seawater, seafood drippings, or punctures by fins or bones with 69% of the reported wound infections being among oyster shuckers and commercial fisherman *(159)*.

Gastroenteritis is identified when *V. vulnificus* is isolated from stool alone. Gastroenteritis caused by *V. vulnificus* may go unreported since it is not life threatening and symptoms are rarely severe enough to warrant medical attention. Symptoms of gastroenteritis are fever, diarrhea, abdominal cramps, nausea, and vomiting *(159)*.

Primary septicemia is the worst manifestation of an infection of *V. vulnificus.* This is usually associated with the consumption of raw shellfish. It is characterized by isolation of the bacteria from blood or any other site which is normally sterile *(46)*.

Typically occurring in immune-compromised individuals or those with underlying liver disease, the disease presents itself within 36 hours after ingesting the etiological agent. The most common symptoms are fever, chills and pain to the extremities. Within as little as 24 hours post infection, secondary lesions such as cellulitis, bullae or ecchymosis are observed on the cutaneous surface of the extremities. These lesions almost always become necrotic and require surgical amputation. After several days, the mental status of patients begins to decline sharply. Tetracycline is the most effective antibiotic used to treat these infections in combination with fluid replacement *(46, 159)*.

Isolation and Detection. There are several selective culture media that can be used to isolate *V. vulnificus*. The Cellobiose-Collistin-Polymyxin B (CPC) agar *(109)* and its modified version known as mCPC *(162)* were developed to specifically isolate *V. vulnificus* from environmental samples. These media are specific for *V. vulnificus* due to its resistance to polymyxin B and colistin, as well as its ability to ferment cellobiose and its ability to survive the high incubation temperature of 40ºC, which limits the growth of other marine bacteria. Another medium designed to select for *V. vulnificus* is thiosulphate citrate bile salts sucrose (TCBS) agar. This is a more general medium since it was designed to isolate pathogenic vibrios. Because of the alkaline pH, bile, and 1% NaCl, non desirable bacteria are inhibited while *V. vulnificus*, *V. parahaemolyticus*, and *V. cholera* grow and be differentiated. However, this medium is not as selective as mCPC for *V. vulnificus* isolation from environmental samples *(76)*. *Vibrio vulnificus* agar (VVA) was also created to help select specifically for *V. vulnificus*. It is a non-antibiotic agar containing cellobiose which is used more as a preliminary selective agar prior to other identification methods such as DNA hybridizations *(76)*. The DNA

hybridizations use a specific DNA probe to detect the DNA of the target bacteria. The most commonly used *V. vulnificus*-specific probe targets the hemolysin (*vvhA*) gene. Probes are linked to an alkaline phosphatase reporter to visualize which specific colonies were positive *(181)*. A combination of these methods is used in the United States Food and Drug Administrations' Bacteriological Analytical Manual (BAM) to both detect *V. vulnificus* by plating on the selective media, and enumerating using the DNA hybridization probe and MPN *(169)*.

PCR has been used to identify both actively growing *V. vulnificus* and those that are thought to be in the viable but non culturable (VBNC) state. Brauns et al. *(22)* detected both states of *V. vulnificus* by PCR amplification using primers targeting a 340-bp fragment of the hemolysin gene (*vvhA*). As little as 31 ng of DNA can be used for detection in unculturable cells and only 72 pg of DNA for culturable cells. Identification of virulent strains can be done by identifying virulence genes. The virulence correlate genes (*vcg*) have been identified and designated *vcg*E and *vcg*C. These genes show a strong correlation with environmental and clinical origin, respectively *(47, 149)*. Recently, it has been proposed that these two genes reflect two different ecotypes within the species, with the *vcg*E type better adapted for conditions present in oysters while *vcg*C would be favored during the stressful transition from seawater/oyster to human *(148)*. This hypothesis is based on the higher survival rate of *vcg*C strains under osmotic shock and heat stress in comparison with *vcg*E strains.

The other genetic marker for identifying specific *V. vulnificus* strains is the 16S rRNA gene. Nilsson et al. *(126)* found a good correlation between 16S rRNA gene type and isolates of clinical origin. Analyzing polymorphisms present in a 492 bp region of the

16S rRNA gene, they observed that 94% of all environmental *V. vulnificus* isolates could be classified as16S rRNA type A while 76% of clinical isolates were 16S rRNA gene type B.

A quantitative PCR method has also been created to detect *V. vulnificus.* A TaqMan real-time PCR assay targeting the hemolysin gene (*vvhA*) of *V. vulnificus* has a detection limit of 72 fg/μL of genomic DNA *(25).* Later, a SYBR Green-based real-time PCR (qPCR) method was used to identify *V. vulnificus* in shellfish and Gulf waters *(135).* The detection limit of the assay was 10^2 CFU *V. vulnificus*/g of oyster tissue homogenate, or 10^2 CFU/10 mL water, as applied to samples without prior cultural enrichment. An improved probe based on qPCR was developed in 2005 that had detection limits of 1 pg/μL of purified DNA, 10^3 CFU/mL of pure culture, and 1 CFU/g of oyster (after a 5-h enrichment) *(134).* In order to differentiate live from dead cells, another method was created that used the DNA intercalating agent ethidium monoazide (EMA) *(175).*

The species *V. vulnificus* has also been classified into different biotypes based on lipopolysaccharide (LPS) antigens, and capsules. Three biotypes of *V. vulnificus* exist 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 *(82, 103).* Biotype 2 is primarily related with diseased eels but can be pathogenic to humans; it is characterized by a homogeneous lipopolysaccharide (LPS); and is negative for indole production and ornithine decarboxylase *(3, 18).* Biotype 3 was more recently identified and causes wound infections and bacteremia in humans handling tilapia in Israel *(19).*

Cold Shock Response

Vibrio vulnificus is able to elicit what is known as a cold shock response. Many species of bacteria have been shown to alter gene expression in response to a shift to low temperatures *(14, 15, 62, 84, 110, 136)*. This is a physiological response of living cells to temperature downshift. Most of the information available on cold shock has been collected from *Escherichia coli* and *Bacillus subtilis*. In relation to heat shock, the cold shock response is poorly understood. The reduction in temperature causes several problems for the bacteria including decreased membrane fluidity , high superhelical density of the DNA that impairs DNA replication, decreased enzyme activities leading to inefficient completion of chemical processes, inefficient protein folding , function adaptation by ribosomes , and the inhibition of initiation of translation in RNA due to secondary structures *(62, 83, 138)*.

Cold shock proteins (Csps) have been classified based on expression after a temperature downshift. Class I Csps, which are expressed at low levels at 37°C, increased synthesis by more than 10-fold in response to cold shock, whereas class II Csps 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 *(164)*. Class I includes CspA, CspB, CspG, CspI, CsdA, RbfA, NusA, and PNP, while Class II cold shock proteins include IF-2, H-NS, RecA, α subunit of DNA gyrase, Hsc66, HscB, trigger factor, dihydrolipoamide acetyltransferase, and pyruvate dehydrogenase (lipoamide). Most of the free living bacteria possess at least one cold

shock-inducible CspA, the most characterized cold shock protein which likely functions as an RNA chaperone. In *E. coli*, three of the cold shock proteins, RbfA, a ribosome binding factor, CsdA, an RNA unwinding protein, and IF2, an initiation factor, appear to be associated with translation *(164)*. The purpose of these proteins is thought to aid in ribosomal functioning at low temperatures. Of the nine (*cspA-cspI*) genes encoding CspA-like proteins in *E. coli*, only four (*cspA*, *cspB*, *cspG*, and *cspI*) have been shown to be inducible by cold *(61, 97, 123, 174)*.

Vibrio vulnificus also produces a cold shock response when exposed to a temperature downshift. Bryan et al. *(23)* showed that when *V. vulnificus* was shifted from 35^{o}C to 6°C abruptly, it entered the VBNC state. But, when the culture was allowed to acclimate for a short period of time at 15°C, it remained culturable. This clearly showed that the bacteria had time to adjust to the cold temperature.

II. Objectives

I hypothesize that *V. vulnificus* is capable of adapting and surviving in oysters under refrigeration conditions and therefore, there may be a greater risk than perceived when using refrigeration to store oysters that are to be consumed raw. To prove my hypothesis I proposed the following objectives:

1. Enumerate the total bacterial population and the *Vibrio* spp. population present in raw oysters initially and throughout a two week storage in refrigeration and identify the change in those populations over time.
2. Enumerate the number of *Vibrio vulnificus* present in oysters during a two week refrigerated storage and determine the change, if any, in the ratio of the potentially more virulent 16S type B to 16S type A. Examine the isolates recovered genetically to determine a link between 16S type and location sampled or date removed from refrigeration.
3. Examine the global transcriptome change that occurs in *V. vulnificus* during a cold shock event and identify which gene and gene classes are induced or repressed at various temperatures during the temperature change.

III. Microbial community dynamics in the eastern oyster (*Crassostrea virginica*) under refrigeration

Abstract

In this study we determined the effect of refrigeration on the microbial communities present within the eastern oyster (*Crassostrea virginica*). Oysters from two different locations, the Auburn University Shellfish Laboratory, Dauphin Island, AL, and a commercial processor, were compared during two weeks under refrigeration conditions. During the course of the experiment, total aerobic bacteria counts increased by two logs. Ribosomal intergenic sequence analysis (RISA) and denaturing gradient gel electrophoresis (DGGE) were used to determine changes within the total bacterial population over the two-week period while a *Vibrio*-specific DGGE examined only that subset of the population. The resolution of the RISA proved to be more sensitive than that of the DGGE. Day 1 samples from both locations were clustered separately from both Day 7 and 14 samples and subdivided based on location. Day 7 and 14 samples also subdivided into individual date and origin groups. *Vibrio*-specific DGGE provided similar results with Day 1 samples from both locations clustering away from Days 7 and 14. Bands corresponding to both *V. parahaemolyticus* and *V. vulnificus* decreased in prevalence while those corresponding to *V. mimicus* increased during the two week of refrigeration, particularly on Day 7. Multidimentional scaling plots allowed visualization of the shifts in both total bacteria and *Vibrio* sp. over the course of experiment. Sequencing data mimicked that from the DGGE clusterings. Sequences from clones using *Vibrio*-specific primers followed the same patterns. *Vibrio parahaemolyticus* and *V. vulnificus* consisted of 44% and 13% respectively of the sequenced clones from the laboratory grown oysters on Day 1 and then decreased to 2% and 0% respectively by Day

14. Processor *V. parahaemolyticus* constituted 21% of the Day 1 clones and fell to 0% by Day 14. *Vibrio vulnificus* was not identified by sequencing in processor samples.

Introduction

The Eastern oyster (*Crassostrea virginica*) is a key economical and environmental species to many states in the U.S., particularly in the Gulf Coast region. Landings in the United States have totaled more than $85 million in the last ten years with the Gulf Coast region comprising $64 million *(125)*. A main concern with the consumption of oysters is the health risk involved. This is because of the fact that most oysters are eaten alive, raw, or undercooked, thereby increasing the risk associated with their consumption. The reason for increased risk is that oysters act as natural reservoirs for a variety of bacteria since they are filter feeders and tend to concentrate bacteria present in the surrounding waters *(40, 43)*. Consuming raw foods potentially exposes humans to a variety of microbes. Bacteria, including pathogens and spoilage organisms, may be introduced into the oyster via natural means, i.e. the feeding mechanism of the oyster, or through improper handling after harvest. Pathogens typically associated with shellfish and aquatic environments in general include *Vibrio cholerae, V. vulnificus, V. parahaemolyticus, Listeria monocytogenes*, *Clostridium botulinum, Plesiomonas* spp. and *Aeromonas* spp. Non-indigenous bacterial contaminants include *Salmonella* spp., pathogenic *Escherichia coli, Shigella* spp., *Campylobacter* spp., and *Yersinia enterocolitica*. Typically these species are in the environment as a result of fecal contamination. Non-indigenous species may also be introduced after harvest, typically during transport and processing. These species my include *Bacillus cereus, L. monocytogenes, Staphylococcus aureus* and *C. perfringens (114, 142, 143).* Of particular concern are the members of the *Vibrio* community. Members of the Vibrionaceae family

comprise the majority of the total bacterial community in oysters, representing up to 20% of the total flora *(26)*. *Vibrio* spp., in particular *V. vulnificus* and *V. parahaemolyticus*, have been shown to pose a significant health risk to consumers of raw or undercooked seafood. *Vibrio vulnificus* has been linked to primary septicemia resulting from the ingestion of raw shellfish with a death rate as high as 50% in patients with preexisting conditions such as hemochromatosis, cirrhosis, diabetes, immuno-compromising diseases, and kidney failure *(66)*. *Vibrio parahaemolyticus* is the leading cause of *Vibrio*-associated gastroenteritis in the United States. More infrequently, two other *Vibrio* species, *V. mimicus* and *V. hollisae*, have been associated with gastroenteritis resulting from the consumption of raw or undercooked seafoods *(1, 39, 142)*.

There are several post harvest treatment methods that can be used to reduce the number of potentially pathogenic bacteria in oysters *(24, 42, 120)*. These post harvest methods include depuration, high hydrostatic pressure treatment, high salinity relaying, gamma irradiation, heat/cool pasteurization, and individually quick freezing *(5, 98, 118)*. However, the majority of oysters that are consumed raw are preserved only by refrigeration. One reason consumers show preference to raw oysters over post harvest treated oysters is the change in the organoleptic properties of the oysters such as texture, odor and flavor which make post harvest treated oysters less appealing to eat *(106)*. Because of this demand for what is perceived as a higher quality, fresher product, many consumers are at risk for exposure to the bacteria that are present within the oyster.

This study focused on the bacterial communities present within the oysters at the time they are put into refrigeration and how they changed during the two week time period that is considered to be the shelf life of raw oysters. Total bacterial communities

were observed in two separate samplings of oysters using Automated Ribosomal Intergenic Sequence Analysis (RISA) and Denaturing Gradient Gel Electrophoresis (DGGE) as well as sequencing. In addition, because of the relevance of pathogenic *Vibrio* sp. in public health, we specifically monitored the changes of the *Vibrio* community in oysters during refrigeration.

Materials and Methods

Oyster Collection and Storage. Live oysters were collected from two locations, the Auburn University Shellfish Laboratory, Dauphin Island, AL, and a commercial processor in Bayou LA Batre, AL. Laboratory oysters were grown on long lines in baskets suspended off bottom in Mobile Bay. Processor oysters were grown on commercial oysters reefs located in Apalachicola, FL. Oysters were transported on ice to the Department of Fisheries and Allied Aquacultures, Aquatic Microbiology Laboratory in Auburn, AL and placed in refrigeration at 6°C.

Total Bacterial enumeration and DNA extraction. Oysters were processed for bacterial enumeration upon arrival (Day 1). Two more samples were taken on days 7 and 14. Twelve oysters were taken on each date for sampling. The oysters were homogenized individually with a tissue tearer. Following homogenization the samples were serially diluted to obtain a final dilution of 1:100,000. One hundred microliters of each dilution was plated in triplicate on Marine Agar (MA) and incubated over night at 35°C to obtain total aerobic bacterial counts. Twenty five milligrams of tissue

homogenate was taken for DNA extraction. The DNA was extracted using the Qiagen DNeasy Blood and Tissue kit (Valencia, CA) per the manufacturer's instructions.

RISA. Ribosomal intergenic sequence analysis was accomplished by amplification of the sequence present between the 16S and the 23S rRNA genes using the universal primers: ITS-F (5'-GTCGTAACAAGGTAGCCGTA-3') and ITS-R (5'-GCCAAGGCATCCACC-3') *(27)*. Approximately 100 ng of DNA extracted from oyster homogenate was amplified in a reaction containing 1X PCR buffer, 0.5 U of Taq polymerase, 0.4 mM of each deoxynucleoside triphosphate and 0.4 µM of each primer in a final volume of 25 µl. PCR conditions were as follows: 95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 55°C for 45 s and 72°C for 1 min. The PCR products were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (LI-COR, Lincoln, NE, USA) following manufacturer's instructions. RISA images were processed with BioNumerics v. 6.6 (Applied Maths, Austin, TX, USA).

Total bacteria and *Vibrio*-specific 16S PCR. PCR was performed on purified DNA samples using the universal primers from Muyzer et al. *(121)* to amplifiy the 16S rDNA from the total bacterial population present. The reaction consisted of the primers GC341F (5'-CCTACGGGAGGCAGCAG -3'), which contained a 40 bp GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') and 517R (5'-ATTACCGCGGCTGCTGG-3'). Approximately 10 ng of DNA extracted from oyster homogenate was amplified in a reaction containing 1X PCR buffer, 0.3 U of Taq polymerase (Promega, Madison, WI), 0.2 mM of each deoxynucleoside triphosphate and 0.4 µM of each primer in a final volume of 25 µl. PCR conditions were as follows: 95°C for 5 min, followed by 21 cycles of 95°C for 1 m, 65-55°C @ -0.5°C/cycle for 1 m and

72°C for 3 min followed by another 15 cycles of 95°C for 1 m, 55°C for 1 m and 72°C for 3 min and a final extension cycle of 10 m. *Vibrio* specific PCR was performed using the primers GC567F (5′-GGCGTAAAGCGCATGCAGGT-3′), which contained a 40 bp clamp (5'- CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3'), and 680R (5′-GAATTCTACCCCCCTCTACAG-3′) from Eiler and Bertilsson *(48)*. Approximately 10 ng of DNA was amplified in a reaction containing 1X PCR buffer, 1.2 U of Taq polymerase (Promega), 0.4 mM of each deoxynucleoside triphosphate and 1.2 µM of each primer in a final volume of 20 µl. PCR conditions were as follows: 95°C for 8 min, followed by 35 cycles of 95°C for 1 m then 64°C for 3 min.

DGGE. DGGE was then performed on the 16S rDNA PCR reactions in the Bio-Rad DCode Universal Mutation Detection System (Hercules, CA, USA). The process was carried out using a 1 mm thick 8% (wt/vol) polyacrylamide gel with a 35 to 60% denaturant gradient of formamide and urea in 1X TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM Na_2 EDTA; pH 7.4). PCR products were electrophoresed though the gel by applying 80 V for 18 h at 60 °C. *Vibrio* specific PCR products were processed using a similar procedure except for the gradient used that was 40 to 55%. Following electrophoresis, all gels were stained with 1X TAE running buffer containing 0.5 µg/ml ethidium bromide for 15 m followed by destaining for 10 m in H_2O. The gels were visualized using the UVP Biospectrum 310 imager (Upland, CA, USA).

***Vibrio* sp. Cloning and Sequencing.** A GC567R–680R clone library was constructed from PCR products amplified with the *Vibrio*-specific primers. The products were cloned into the pCR-4-TOPO vector and transformed into competent *E. coli* One Shot TOP10 using the TOPO-TA cloning kit for sequencing (Invitrogen, San Diego, CA,

USA). Forty eight clones were selected from each of the laboratory and processor samples taken on each of the 3 sampling days giving a total of 288 clones. Clones were automatically sequenced using an ABI 3730xl sequencer at Lucigen Corp. (Madison, WI, USA).

Data Analysis. Both RISA and DGGE gel sets were analyzed using the same procedure. Following conversion, normalization and background subtraction, levels of similarity between fingerprints were calculated with the Pearson product moment correlation coefficient. Cluster analysis was performed using the unweighted pair-group method with arithmetic mean (UPGMA) with branch quality assessed using the cophenetic correlation. Transversal clustering was then performed in order to obtain relevant band classes associated with specific clusters of sample fingerprints. To aid in the interpretation of the large data sets of both the RISA and DGGE samples, the similarity matrix generated above was then used to produce a multidimentional scaling analysis (MDS). The DGGE data were then subjected to principal component analysis to determine sample relatedness and further quantitate variability. Sequences obtained were aligned, annotated and analyzed for similarity using the same methods as fingerprint data. Analysis was performed using BioNumerics v 6.6 (Applied Maths Inc., Austin, TX, USA).

Results

Total bacteria enumeration. During the two week refrigerated period total bacterial numbers increased at a rate of one log per week as shown in Table 1. An

analysis of variance showed this to be a significant increase ($p<0.05$). The increase in bacterial concentration was observed in both oyster samples.

RISA. RISA profiles were generated from all oyster samples. After creating a similarity matrix using the Pearson product moment correlation a dendrogram was derived by UPGMA cluster analysis. The dendrogram is shown if Figure 1. The RISA profiles from the 72 oysters sampled over the 14 day period. Of these, 67 were usable profiles. Each individual profile was comprised by an average of 130 bands ranging from 100 to 700 bp. All samples clustered at 22% or greater similarity. Day 1 samples clustered separately from day 7 and day 14 samples at 37% similarity. The laboratory and processor day 1 oyster samples themselves were divided into two distinct clusters at 43 and 45% similarity respectively. Approximately, 20 bands were specific to day 1 laboratory and processor samples which contributed to those samples grouping away from the remaining samples. The absence of approximately 11 bands from the laboratory samples allowed for the separation of day 1 laboratory and processor groups. A second major cluster was formed that diverged at 30% similarity into two groups containing the 88% (15 of 17) of the remaining processor samples in the first and the remaining laboratory samples in the second. The laboratory cluster then again diverged at 35% into two groups representing day 7 and day 14 samples, which were grouped based on 7 and 11 bands respectively. A multidimensional scaling (MDS) plot was created to better visualize the groupings and the strong correlations observed between both location and sample dates. This is shown in Figure 2.

DGGE. DGGE was performed on all oyster samples to look at both the total bacterial populations and specifically, the *Vibrio* sp. population. Total bacteria DGGE

gels contained between 17 and 37 distinct bands. A dendrogram was created using the same methods as with RISA. The lower resolution DGGE method was not able to clearly distinguish samples based on sampling date but was able to form groups based on oyster locations. Principal component analysis confirmed the high degree of variability within these samples. The first five components were able to account for 59.7% of the total variability. The dendrogram is shown in Figure 3. Analysis of the dendrogram revealed three main clusters. Cluster 1 grouped at 50.7% similarity and consisted of 91% processor samples and 9% laboratory samples. Transversal clustering and discriminative band class analysis with BioNumerics showed approximately 5 band classes to be responsible for this grouping. Cluster 2 grouped at 53.8% similarity and consisted of 50% processor and 50% laboratory samples. Further analysis revealed that although cluster 2 contained half of processor samples, 70% of those samples grouped out together at 84% similarity. These were distinguished by the presence of an intense three band class cluster containing bands located at 35.9, 39.6, and 72.2% along the length of the gel when read from the low denaturant top to the higher denaturant bottom. These positions correspond to denaturant concentrations of 44, 45, and 53% respectively based on a linear curve of denaturant along the course of the gel. Twelve of the remaining cluster 2 samples formed another minor group at 84.2% similarity. This group was distinguished by the presence of an intense band at 37.8% along the gel, which corresponded to a denaturant concentration of 44.5%. It was also characterized by the absence of a group of band classes ranging from 15 to 31%, which corresponded to a denaturant range of 38.75 to 42.75%. Cluster 3 grouped at a similarity of 47.4% and was also further divided into 2 more closely related groups. The first of these grouped at 53.8% similarity and

contained 11 laboratory samples out of 14. Discriminative character analysis revealed three main band classes responsible for this group. Those included the band classes located at 45.7, 49.3, and 53.4% along the gel representing denaturant concentrations of 46 to 48%. The second subgroup of cluster 3 contained 17 samples of which 8 were laboratory and 9 were processor oysters. This group was determined via a large cluster of band classes that ranged across the denaturant gradient. In particular was a cluster of 8 intense bands ranging from 32-50% along the gel, or 43 to 47.5% denaturant concentrations.

Vibrio spp. specific DGGE was performed in all oyster samples using as similar methodology as previously described. A total of 33 band classes were found across all samples with each sample having an average of 10 bands. Unlike the DGGE which focused on the total bacterial composition of the samples, *Vibrio* sp. specific DGGE was able to produce results which grouped the samples by both sampling date and location of harvest. Figure 4 shows the dendrogram of the sample fingerprints transversed with that of the band classes. At 20.1% similarity all samples were split into two main groups. Group 1 consisted of all Day 0 samples from both laboratory and processor origins. A group of three Day 7 laboratory samples were also included and were further grouped out at 50.7% followed by a two Day 7 and one Day 14 sample, which grouped at 78.7%. The remaining samples in that group were all Day 0 and, with the exception of two processor samples, separated into laboratory and processor sample groups at 76.3%. Transversal clustering and discriminatory analysis revealed a cluster of 8 band classes responsible for the Day 0 clustering shown in group 1. These bands occurred at 21.5, 33.4, 44.6, 52.7, 55.0, 56.8, 62.4, and 66.9% along the length of the gel corresponding to denaturant

concentrations of 43.2 to 50.0% denaturant. Group 2 clustered together at 43.5% similarity. At 58.6% similarity a cluster formed consisting of 9 laboratory and 2 processor Day 7 samples. Most Day 14 laboratory samples grouped at 80.9% while Day 7 processor samples grouped into two small clusters at 84.7 and 85.6%. The remaining samples clustered at 71.2% similarity and consisted of 1 Day 7 laboratory, 3 Day 7 processor, 10 Day 14 processor, and 3 Day 14 laboratory samples. The sampling day/origin groupings for this experiment are more easily seen in the multidimemtional scaling plot. The MDS plot is shown in Figure 5. Band classes responsible for the clustering in group 2 included those at positions 5.2, 7.0, 16.9, 28.9, 41.5, 59.9, and 80.0% along the length of the gel.

Common *Vibrio* species were used as a ladder for the DGGE. Three band classes present in the oyster profiles correlated directly with three of the standards, *V. mimicus, V. parahaemolyticus, and V. vulnificus*, which were located at positions 27.6, 56.6, and 66.5% respectively along the length of the gel. A significant decrease in the presence of the band class that was associated with the *V. vulnificus* standard was observed. The same was true for the band class associated with the *V. parahaemolyticus* standard. The band class associated with *V. mimicus*, on the other hand, showed a significant increase from Day 1 to Day 7 and then a significant decrease in its presence from Day 7 to Day 14.

Sequencing of *Vibrio* sp. specific DGGE PCR products. Sequencing was conducted to identify the *Vibrio* sp. present during the refrigeration process over the two-week experiment. Of the 288 clones sequenced, 265 provided useable sequences. One hundred twenty three sequences (46%) could not be identified beyond the genus level

using these primers. Those that were identified at the species level included *V. parahaemolyticus* and *V. vulnificus*. The presence of these two species followed the same general trend of decreased presence after Day 1 that was noted in the DGGE procedure. All of the *Vibiro* species identified using the primers from the *Vibrio* sp. specific DGGE are shown in Table 2.

Discussion

This experiment examined the dynamics of the bacterial population within the eastern oyster during two weeks of post harvest refrigeration. During this time, the total bacterial load began at 10^7 CFU/g and rose at a rate of one log per week. Previous studies have shown that during the summer months, total bacterial counts were in the range of 10^7 to 10^8 CFU/g and were maintained up to 21 days during refrigeration *(143)*. Although starting at the same load, our data indicated an increase in bacterial numbers during the experiment. Quevedo et al. *(146)* noted a similar 1 log increase during 14 days of refrigeration. Discrepancies in the maintenance of the bacterial load between studies could be attributed to various factors including the handling of the oysters prior to the experiment, how quickly the oysters were brought down to refrigeration temperature, or the quality of refrigeration system used during the experiment.

RISA was used as a culture independent method to further examine the total bacterial population present in each of the samples taken over the two-week period. RISA has been shown to be a useful technique to survey a population because of its rapid and high-resolution nature *(10, 56)*. The bacterial profiles of refrigerated oysters sampled

at Day 1 seemed to consist of a markedly different community than that of both Day 7 and 14 samples. Nevertheless, Day 7 and Day 14 profiles were not identical and showed a high degree of dissimilarity from each other. Bacterial community profiles obtained from oysters from a commercial processor and the Auburn University Shellfish laboratory showed sufficient dissimilarity and clustered separately. Unfortunately, in general, because of the highly complex fingerprints along with the 0.2 mm sequencing gel format used in this experiment, obtaining gel slabs for sequencing was impossible. With approximately 130 bands per samples, the profiles generated here were no exception. What could be inferred is that, at each of the time points the oysters were sampled, a highly variable but significantly different community was present. This also holds true when looking at the oyster from each location. As seen in the MDS plots, there is a high degree of dissimilarity within each specific group as shown by the spread of the samples. But there is also enough diversity between each group, either date or location, that separate clusters are apparent in the plot. The diversity seen may also be misinterpreted. An underrepresentation may occur because of alignments of intergenic sequence fragments of the same length from multiple species. Overestimations of variability in the population diversity could be attributed to intergenic sequence fragments of multiple lengths from the same species *(67, 122)*. In addition, errors in molecular methods of DNA extraction and PCR including DNA shearing, incomplete PCR, PCR bias toward more prevalent sequences, and amplification errors, including chimeras, could also contribute to error in variability estimations *(56, 141, 171)*. But whether the quantity of diverse bacterial species is represented accurately or not, it is clear that population shifted during refrigeration over the two week time course.

Further, DGGE was incorporated in the experimental design to provide a more common approach to bacterial population diversity estimation within the oyster during refrigeration. The set of primers used to investigate the total bacterial population within the oyster tissue was chosen because it provided the clearest bands with the lowest background. As with all DGGE, there is a degree of background to deal with. Usually these occur for the same reasons described above for RISA except in this case the errors can occur because of heterologous rRNA operons creating multiple bands from one species or poor detection due to rare community members *(93)*. Proper band optimization and position tolerance along with increased stringency on band presence within the analysis software limited anomalies during the analysis. In this instance, the variability of total bacteria present within a single oyster in addition to the intrinsic variability of the DGGE method created difficulty in discovering commonalities within sample groups. With that being said, small clusters were derived which contained a significant amount of individual groups based more on location than on sampling date. This indicates that the procedure was more effective in discriminating larger population differences that occur because of the distance between the oyster harvest areas, which could account for distinct differences in the individual bacterial communities.

Vibrio-specific DGGE gave a clearer picture of the populations present in the oyster samples. Less background yielded clearer images that were easier to analyze. These samples followed the same clustering pattern as those seen by RISA. Day 1 samples clustered together as being significantly dissimilar to the banding patterns present in both Day 7 and Day 14 samples. In addition, the profiles based on location were also quite diverse but could be separated as shown in the MDS plot. Interestingly,

the diversity shown by the MDS plot, which grouped samples according to sample date, clearly reflected the changes in the *Vibrio* community over time. Day 1 samples have a tight cluster of sample points indicating a low diversity population. Day 7 samples indicate that the bacterial community moved to a more diverse community with several species making up a large proportion of the population. Finally, Day 14 samples again formed a tighter group indicating a divergence from the Day 1 and 7 samples and also a lack of total population diversity. In summary, the bacterial community shifted from a low diverse population at Day 1 to a different but also low diverse community by Day 14 with a transition in between in where the population composition was richer. Our data suggest that the predominant bacteria at Day 1 were replaced over time by bacterial species able to proliferate under low temperatures.

Sequencing of the *Vibiro* specific PCR products further supports the profiles generated with DGGE. Although many species were unable to be identified due to the relatively short length of the PCR product, enough data were available to see the dynamic proportion of bacterial community members. This was easiest to see when looking at laboratory derived oyster samples. At Day 1 *V. parahaemolyticus* made up a large portion of the total community with only a few identified remaining members present. As the samples refrigerated for another 7 days the community became more diverse with the number of identified members doubling. By day 14 the number of identified species again reduced, with different species present that could not be identified in the first sampling. In conclusion, each of the methods showed that there is a growing and dynamic bacterial population present within raw refrigerated oysters. The refrigeration practice itself does not limit the growth of all species present. Further evaluations need to

be made to find a more specific community profile at each time point during the refrigeration process to help identify critical control points where certain potential pathogens may proliferate. Therefore, changes in refrigeration temperature standards may be necessary to control these potentially virulent species from becoming a problem.

Table 1. Total aerobic bacterial counts recovered from raw oysters during this study

Origin	Day	*n*	Total Bacteria Counts (CFU/g)[a] ± SD
Lab oysters	1	12	$1.1 \times 10^{6} \pm 9.1 \times 10^{4}$
	7	12	$3.9 \times 10^{7} \pm 5.9 \times 10^{6}$
	14	12	$3.6 \times 10^{8} \pm 3.8 \times 10^{7}$
Proc. oysters	1	12	$2.1 \times 10^{6} \pm 3.4 \times 10^{5}$
	7	12	$7.0 \times 10^{7} \pm 2.1 \times 10^{7}$
	14	12	$2.7 \times 10^{8} \pm 5.3 \times 10^{7}$

[a]Both Laboratory and Processor samples held at 6°C showed a significant change in total bacteria counts between each successive sampling date ($p < 0.05$).

Table 2. *Vibiro* species identified using *Vibiro* spp. specific primers.

Location	**Day 0**		**Day 7**		**Day 14**	
Laboratory	*V. parahaemolyticus*	44%	*V.* spp.	60%	*V.* spp.	53%
	V. spp.	22%	Uncultured bacteria	13%	*Listonella anguillarum*	27%
	V. shilonii	21%	*V. parahaemolyticus*	9%	*V. diazotrophicus*	11%
	V. vulnificus	13%	*V. diazotrophicus*	6%	Uncultured bacteria	5%
			V. shilonii	6%	*V. parahaemolyticus*	2%
			V. ponticus	2%	*V. shilonii*	2%
			Listonella anguillarum	4%		
Processor	*V.* spp.	57%	*V. diazotrophicus*	27%	*V.* spp.	59%
	V. parahaemolyticus	21%	Uncultured bacteria	25%	*Listonella anguillarum*	18%
	Uncultured bacteria	10%	*V.* spp.	25%	Uncultured bacteria	12%
	V. ponticus	7%	*Listonella anguillarum*	21%	*V. shilonii*	9%
	V. shilonii	5%	*V. parahaemolyticus*	2%	*V. vulnificus*	2%

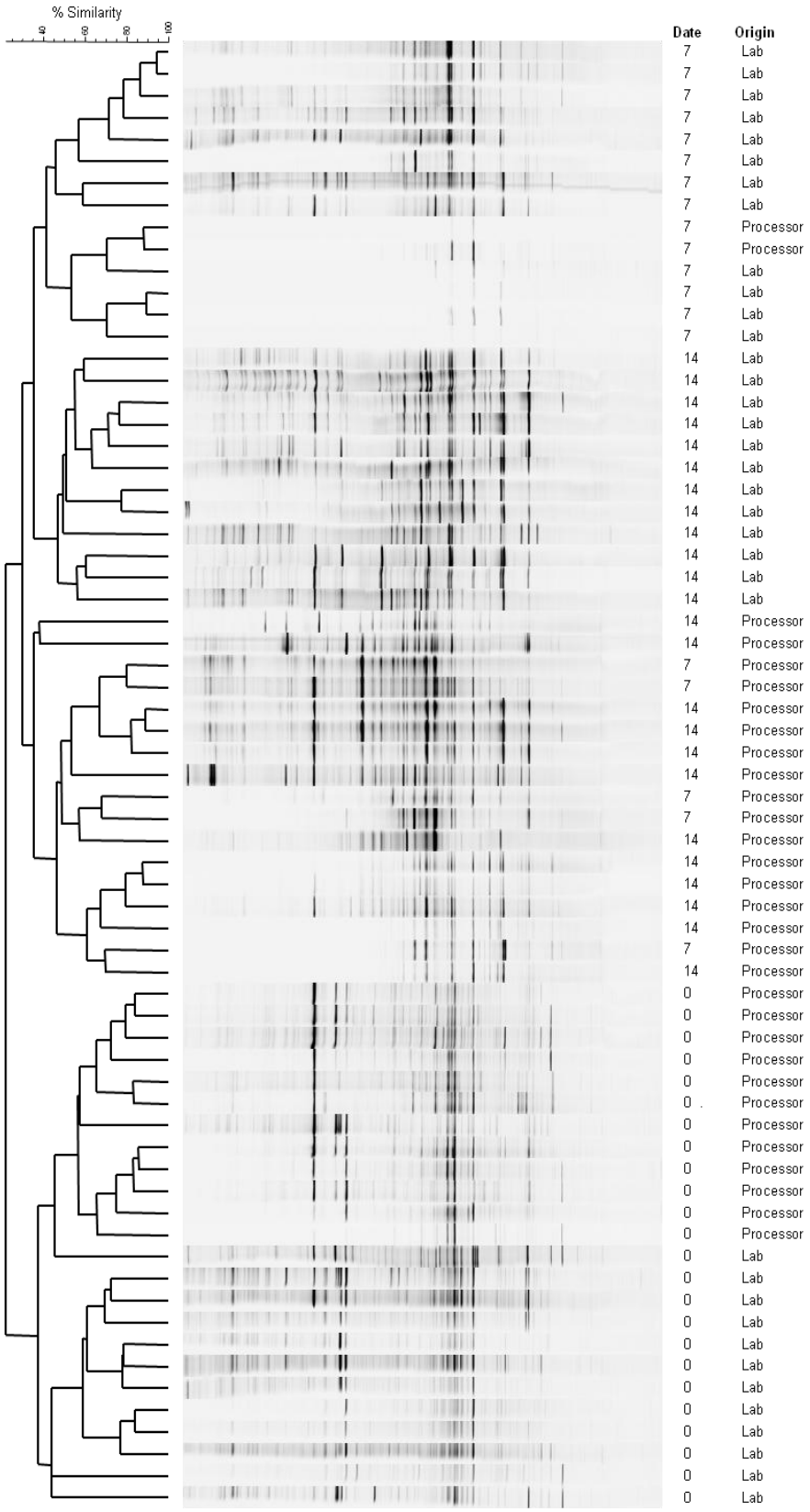
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Figure 1. RISA patterns of bacteria present in oyster samples. The dendrogram was derived by UPGMA cluster analysis of the RISA profiles from 67 individual oysters. The tracks show the processed band patterns after conversion, normalization, and background subtraction. To the right are the listing for the sample dates (Day 1, 7, or 14) and the oyster origin location (Lab or Processor).

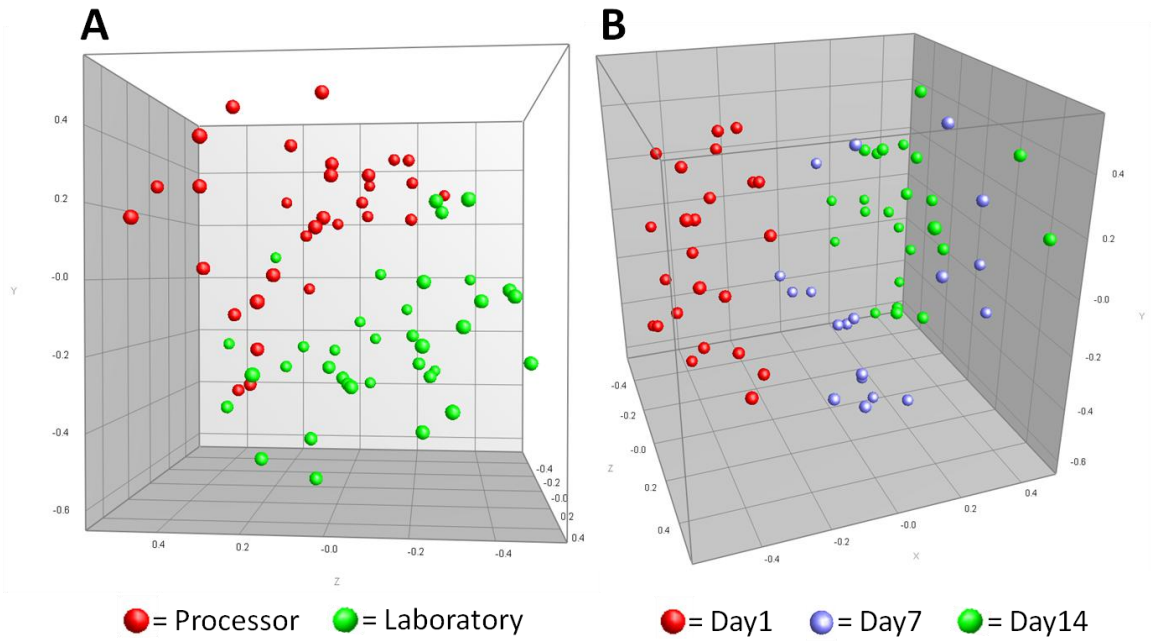

Figure 2. Multidimensional scaling (MDS) plot showing the relatedness of individual bacterial 16-23S intergenic sequences based on RISA data. Grouped by A) Location and B) Date sampled.

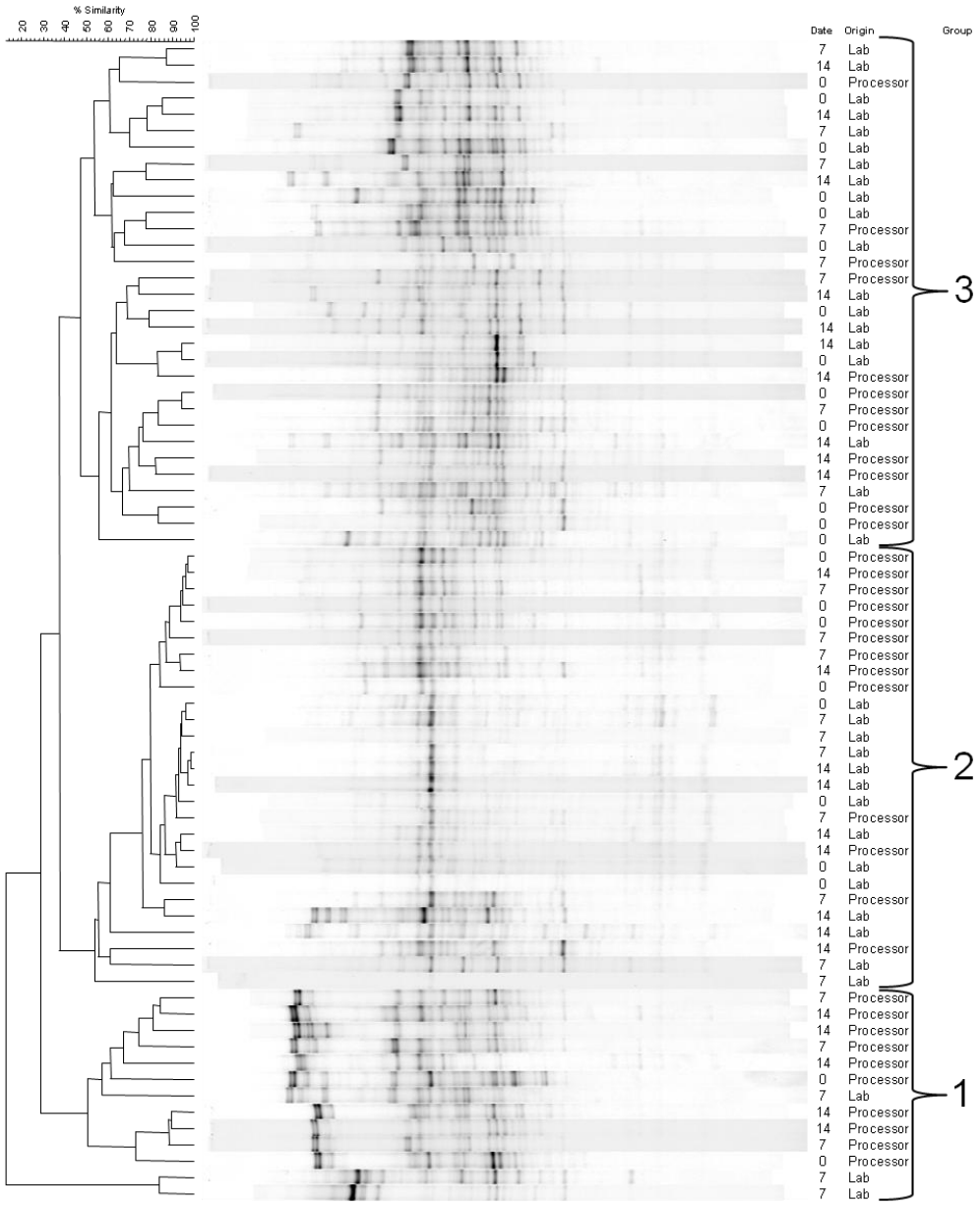

Figure 3. Dendrogram of DGGE 16S rDNA fingerprints for total bacteria present based. Group numbers indicate major clusters

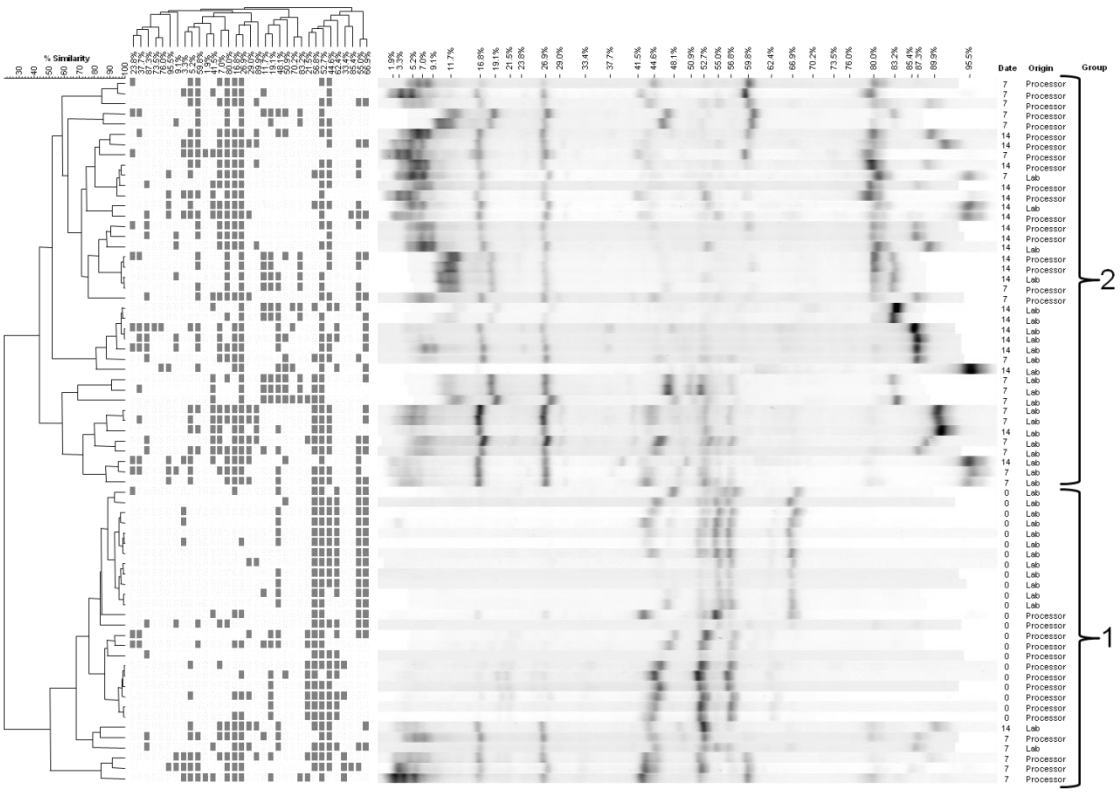

Figure 4. Dendrogram of *Vibrio*-specific DGGE fingerprints. Band class presence indicated by black blocks in the band class table preceding gel image. Group numbers indicate major clusters derived by UPGMA cluster analysis

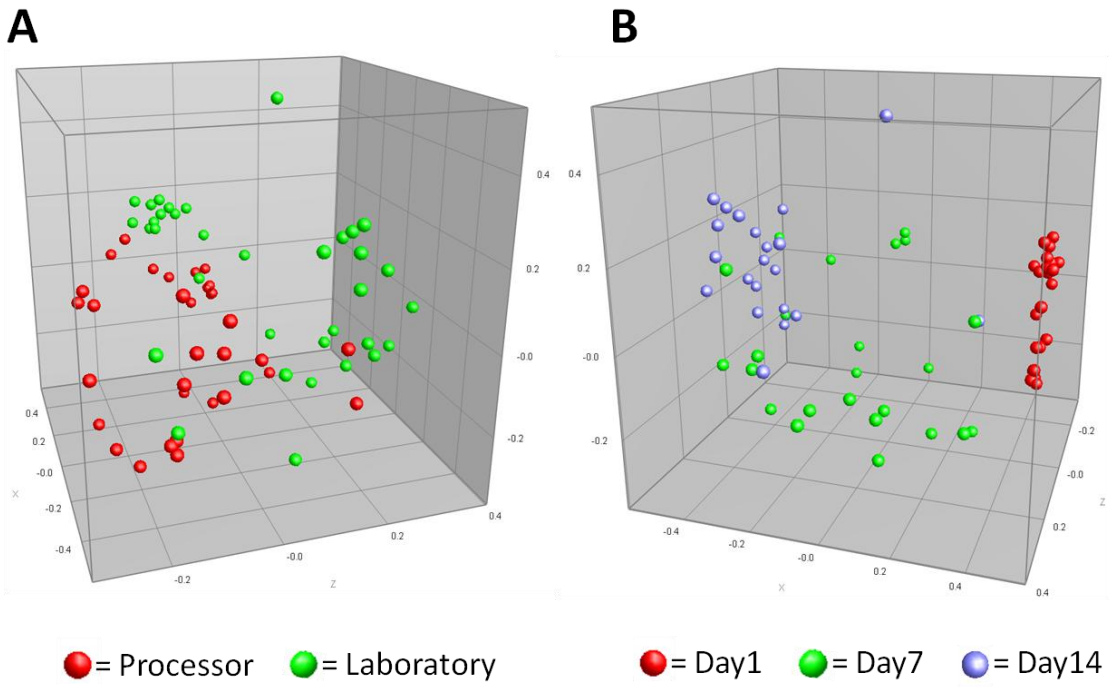

Figure 5. Multidimensional scaling (MDS) plot showing the relatedness of individual *Vibrio* sp. specific DGGE fingerprints. Grouped by A) Location and B) Date sampled.

IV. Dynamics of *Vibrio vulnificus* genotypes in oysters (*Crassostrea virginica*) under refrigeration

Abstract

In this study we determined the effect of refrigeration on the seafood-borne human pathogen *Vibrio vulnificus* in terms of strain selection and persistence. Naturally occurring numbers of *V. vulnificus* in oysters from two different locations were compared during two weeks under refrigeration conditions. During the course of the experiment total aerobic bacteria counts increased by two logs while numbers of *V. vulnificus* remained unchanged. At different time points, *V. vulnificus* isolates were recovered from oysters and genotyped as 16S type A, B or AB by using a restriction fragment length polymorphism (RFLP) analysis. 16S type B isolates (typically associated with clinical cases) comprised 53% of the isolates recovered, which accounted for more than both 16S type A or type AB combined. Further analysis using AFLP revealed a high level of heterogeneity throughout all isolates collected. This held true for both comparisons between 16S types and among individual isolates of the same type. Of important note was the identification of 16S type B-specific AFLP bands that can discriminate type B from type A and AB.

Introduction

Eastern oyster (*Crassostrea virginica)* landings in the United States over the last ten years have had an average annual value of over $85 million. States along the Gulf of Mexico including Alabama, Florida, Louisiana, Mississippi, and Texas account for 75% of that revenue totaling more than $64 million *(124)*. With this large industry along the coast, there is also a large consumer market. A main concern with the consumption of oysters is the intrinsic health risks associated with eating a raw or undercooked product. Oysters are filter feeders that have been shown as natural reservoirs of microbes *(40, 43)*. Among those bacteria commonly found in oysters, *Vibrio vulnificus* is of special concern because it has been reported as the leading cause of seafood related deaths in the United States *(29)*. Individuals with predisposing conditions such as hemochromatosis, cirrhosis, diabetes, immuno compromising diseases, and kidney failure requiring dialysis are particularly at risk *(66)*. *Vibrio vulnificus* infections which lead to a primary septicemia have been shown to have a fatality rate of up to 60% which is almost exclusively associated with the consumption of raw or undercooked oysters *(101, 130)*.

Warmer temperatures are correlated with high *V. vulnificus* numbers in oysters as well as with high disease incidence, suggesting that infection risk is dose dependent *(101, 153)*. Therefore, controlling the dose ingested by consumers is important for disease prevention. The concentration of *V. vulnificus* at consumption is influenced not only by the initial number of the bacteria in the oyster at the time of harvest but by the 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, any post

harvest treatment applied, and the length of refrigeration time until consumption *(135)*. Under refrigeration conditions, i.e. ≤ 7°C, oysters have been shown to have a shelf life of two weeks until spoilage was detected *(55, 132, 156)*.

Numbers of *V. vulnificus* in refrigerated oysters at market can reach 10^5 MPN/g during the summer *(42)*. According to the Food and Drug Administration, and based on consumers practices, at least 12 to 60 million people are at risk of contracting *V. vulnificus* through consumption of raw oysters. However, infections caused by this bacterium are rare (30-40 cases annually), which prompted the question are all *V. vulnificus* strains equally pathogenic for humans? *(148)*. Nilsson et al. *(126)* found a good correlation between 16S rRNA gene type and isolates of clinical origin. Analyzing polymorphisms present in a 492 bp region of the 16S rRNA gene, they observed that 94% of all environmental *V. vulnificus* isolates could be classified as16S rRNA type A while 76% of clinical isolates were 16S rRNA gene type B (this percent increased to 94% when considering clinical fatalities). Another genetic marker used to identify strains of clinical origin is the virulence-correlated gene (*vcg*). Two alleles have been identified for this gene, *vcg*E and *vcg*C showing a strong correlation with environmental and clinical origin, respectively *(47, 149)*. Recently, it has been proposed that these two genetic groups reflect two different ecotypes within the species, with the *vcg*E type (which has a strong correlation to 16S type A) is better adapted for conditions present in oysters while *vcg*C (which has a strong correlation to 16S type B) would be favored during the stressful transition from seawater/oyster to human *(148)*. This hypothesis is formulated based on the higher survival rate of *vcg*C strains under osmotic shock and heat stress in comparison with *vcg*E strains. The objective of this study was to monitor subspecies

variability of *V. vulnificus* in refrigerated oysters using molecular markers during the accepted two-week shelf life of this product.

Materials and Methods

Oyster collection. Live oysters were provided by the Auburn University Shellfish Laboratory, referred to in this study as "laboratory oysters," Dauphin Island, AL, and a commercial oyster processor, referred to as "processor oysters", in Bayou La Batre, AL. Laboratory oysters (approximately 2 years old) were grown on long lines in baskets suspended off bottom in Mobile Bay. Processor oysters were harvested from commercial oysters reefs located in Apalachicola, FL (during Summer 2010, the Deepwater Horizon Oil Spill closed oysters beds in Alabama). The locations are shown in Figure 6. Oysters were transported on ice to the Department of Fisheries and Allied Aquacultures, Aquatic Microbiology Laboratory in Auburn, AL. During transport, ambient temperature within the transport vessel ranged from 6-10°C as monitored via remote thermometer. Upon arrival, the oysters were placed under refrigeration at 6°C.

Total aerobic bacteria (TAB) and *Vibrio vulnificus* enumeration. Oysters were analyzed upon arrival to the laboratory (day 0) and at days 7 and 14. Twelve laboratory and 12 processor oysters were sampled at each of the three dates and processed according to the U.S. Food and Drug Administration Bacterial Analytical Manual (USFDA BAM) *(169)* Most Probable Number (MPN) method for enumerating *V. vulnificus* in raw oysters with the following modification for calculating TAB counts. Each set of 12 oysters was homogenized with a tissue tearer (VWR. USA) and admixed with and an equal volume of

phosphate buffered saline (PBS) creating a 1:2 dilution. Twenty grams of this mixture was added to 80 ml of PBS to obtain a 1:10 dilution. Serial dilutions were then made to obtain a final dilution of 1:100000. One hundred microliters of each dilution was plated in triplicate on Marine Agar (MA) and incubated over night at 35°C to obtain TAB counts. The remainder of the method followed the published protocol with enumeration of *V. vulnificus* performed following the MPN method located in the USFDA BAM *(169)*.

***Vibrio vulnificus* genotyping.** Fifty *V. vulnificus*-positive colonies were randomly selected from both type of samples and from all sampling times totaling 300 isolates. Cultures were purified and cultured on Marine Agar (BD Difco, Franklin Lakes, NJ) before DNA was extracted using the Qiagen DNeasy Blood and Tissue kit (Valencia, CA). Isolates were confirmed as *V. vulnificus* using the specific hemolysin (*vvh*) PCR protocol according the USFDA *(169)*. Strains were ascribed to 16S type A or B according to Nilsson et al. *(126)*. Briefly, amplification of 16S rDNA was carried out with primers UFUL (5'- GCCTAACACATGCAAGTCGA-3') and UFUR (5'-CGTATTACCGCGGCTGCTGG-3'). Following digestion with *Alu*I, restriction fragments were electrophoresed in a 3% TAE Agarose-1000 (Invitrogen, Carlsbad, CA, USA) gel for 45 min at 80V, stained with ethidium bromide, and photographed under UV light. Amplified Fragment Length Polymorphism (AFLP) fingerprintings were determined as previously described by Arias et al. *(9)* with the following modifications. PCR amplifications were performed using the MJ Research PTC-200 Thermocycler with the following cycle profile: cycle 1, 60 s at 94ºC, 30 s 65ºC, and 60 s at 72ºC; cycles 2 to 12, 30 s at 94ºC, 30 s at annealing temperatures 0.7ºC lower than that used for each

previous cycle, starting at 64.3ºC, and 60 s at 72ºC; cycles 13 to 24, 30 s at 94ºC, 30 s at 56ºC, and 60 s at 72ºC. After completion of the cycling program, 5 µL of AFLPBlue Stop Solution (LI-COR) was added to the reaction mixtures. Prior to gel loading, the samples were heated for 5 m at 94ºC then rapidly cooled on ice to prevent reannealing. The PCR products were electrophoresed on the NEN Global Edition IR2 DNA Analyzer (LI-COR) following manufacturer's instructions.

Statistical Analysis. Bacterial enumeration at each sampling time was performed in triplicate. Differences in the total aerobic bacterial counts for both laboratory and processor obtained oysters were analyzed using the one-way ANOVA procedure in PSI-Plot 9.01 (Poly Software International, INC, Pearl River, NY). Ascription of *V. vulnificus* strains to ribosomal types 16S-A and 16S-B was done according to the restriction fragments size *(126).* AFLP images were processed with BioNumerics v 6.5 (Applied Maths Inc., Austin, TX, USA). After normalization, AFLP similarities were calculated using the Pearson Product Moment Correlation and cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with branch quality assessed using the cophenetic correlation.

Results

TAB counts and *V. vulnificus* enumeration. Over a two-week period, TAB counts increased at a rate of one log per week (Table 1). The increase in bacterial counts taken at each sampling time was shown to be statistically significant ($p<0.05$). A similar

growth pattern was observed in both laboratory oysters and those obtained from the commercial processor as shown in Table 1 ($p<0.05$).

Vibrio vulnificus counts were determined using the FDA MPN method and the results are shown in Table 1. Initial loads of *V. vulnificus* in laboratory oysters were 1.1 x 10^5 MPN/g of tissue while commercial processor oysters had a 1 log lower load (1.0 x 10^4 MPN/g). At 7 days under refrigeration, *V. vulnificus* loads in laboratory oysters remained high at 1.1 x 10^5 MPN/g but processor oysters had an increase of at least one log to numbers comparable to the laboratory oysters. Final laboratory samples taken on day 14 had a slight decrease in *V. vulnificus* (8.0 x 10^4 MPN/g of oyster tissue). On day 14 processor samples remained at 1.1 x 10^5 MPN/g. No statistically significant change was observed in *V. vulnificus* numbers during the refrigeration period in either sample.

Characterization of *Vibrio vulnificus* isolates recovered from refrigerated oysters. Duplicate VVA plates were made during the MPN procedure to allow for MPN positive isolates to be recovered. Three hundred *V. vulnificus* isolates confirmed as positive by the MPN procedure were taken for further evaluation. These consisted of 50 isolates from both lab and processor oysters at day 1, 7 and 14. These isolates were first subjected to specific PCR to confirm their identity as *V. vulnificus.* Of the 300 isolates taken, 294 were positive for *V. vulnificus*-specific *vvh* gene. These isolates were ascribed to 16S type A or B by amplification of a fragment of the 16S rRNA gene followed by RFLP analysis. In total, 87 isolates were 16S type A, while 154 were type B, and 53 were type AB. Based on sample origin, laboratory oysters yielded 46 isolates identified as16S type A, 92 as type B, and 12 as type AB. Within laboratory oysters, the number of 16S type B isolates was significantly higher than types A and AB. *Vibrio vulnificus* isolates

from processor oysters were classified as 41 type A, 62 type B, and 41 type AB. Although more 16S type B isolates were identified from processor oysters than types A and AB, the actual differences were not significant. Figure 7 shows the percentage of each type isolated on each of the three sampling dates. Over the two-week course there was no statistically significant change in the ratios of 16S types A, B, or AB isolated during each sampling. Overall, numbers of type B isolates were significantly higher than types A and AB. All significant values were determined at the $p<0.05$ level. These results are summarized in Figure 8.

AFLP fingerprinting was then used to provide a higher resolution analysis of the confirmed *V. vulnificus* isolates. After conversion, normalization, and background subtraction, the AFLP patterns contained between 95 and 130 bands for each isolate. The 294 isolates formed two major clusters at a similarity of 62%. The first clustered at 63% and consisted only of those isolates that were ascribed to be 16S type B. The second group clustered at 66% and consisted of isolates ascribed to the 16S A and AB types. Figure 9 shows the AFLP data visualized multidimensional scaling (MDS) plot. Composite analysis which consisted of creating a binary matrix of band classes was then performed on the two groups to determine which bands were responsible for the dissimilarity. Using this data, discriminatory band classes were identified. The major cluster which contained types A and AB was distinguished based on approximately 33 out of the possible 171 identified band classes across all profiles while the type B cluster was differentiated based on approximately 41 of the possible 171 band classes. Excluding 16 outliers, all 16S type A, AB, and B isolates were included within one of the 2 clusters. Further, using the Pearson Product Moment Correlation to create a similarity

index of the band classes themselves followed by cluster analysis using UPGMA, a transversal clustering was performed to more narrowly identify discriminatory bands that were unique to the 16S types. With this, two distinct clusters of band classes were formed. One group was specific to type B and the other group was specific to both types A and AB. The transversal clustering showing the two groups is shown in Figure 10. From the clustering approximately 20 band classes were shown responsible for grouping 16S types A and AB while approximately 25 band classes grouped 16S type B.

Discussion

Previous studies have shown that total bacterial communities within the Easter oyster (*Crassostrea virginica*) during the summer months were in the range of 10^7 to 10^8 CFU/g and that those levels were maintained during refrigeration for up to 21 days *(143)*. In that study, the culturable bacterial community load fluctuated within the 2 log range. Sequencing of community members showed that during the summer months, oysters contained 5% *Actinobacteria*, 5% *Flavobacteria*, and 89% *γ-Proteobacteria*. Within the *γ-Proteobacteria*, the major genera were composed of *Vibrio* and *Shewanella*, which represented 55.7 and 11.4% of the whole bacterial community, respectively. Pathogens found included *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, and *Aeromonas hydrophila*. *Vibrio vulnificus* were found to be present at a rate of 10^3 CFU/g at the start of refrigeration and maintained that level for the course of the 21 day experiment *(143)*. Hernández-Zárate and Olmos-Soto found similar communities including *γ-Proteobacteria* and low G + C Gram positive bacteria *(73)*. In our study, we observed a

similar total bacterial load of 10^7 CFU/g at the onset of the experiment. However, we observed a one log increase in total bacteria as the refrigeration time progressed. This mirrors the study of Quevedo, et al *(146)* who also saw a 1 log increase in total bacterial counts after 14 days of refrigeration. This difference in the maintenance of the bacterial loads between studies could be attributed to several factors including the handling of the oysters prior to the experiment, how quickly the oysters were brought down to refrigeration temperature, or the quality of refrigeration system used during the experiment. Our study showed that the concentration of *V. vulnificus* remained steady throughout the experiment. Because of the MPN method used, this also indicates the viability of the potentially pathogenic *V. vulnificus* found in refrigerated oysters. The MPN procedure is carried out by sampling the oysters at each time point and enriching for *V. vulnificus*, consequently only viable cells are counted. Our results showed that the refrigeration had no detrimental effects on *V. vulnificus*. This could point to a serious flaw in the refrigeration practices that are currently implemented under the National Shellfish Sanitation Program (NSSP) *(53)*, indicating that the time from harvest to refrigeration is slow enough to allow adaptation to cold shock. This has been shown in many organisms *(100, 140, 183)* including *V. vulnificus (23)*. This could also be an indication that the temperature of refrigeration as outlined by the NSSP is not sufficient enough to prevent proliferation.

Most *V. vulnificus* strains routinely isolated from environmental samples are type A while most strains isolated from clinical samples are type B *(68, 126, 172)*. In this study, 52% of the isolates taken from the MPN procedure were of the B type. This is in stark contrast to the study from which the RFLP method we employed was derived *(126)*.

In that study only 6% of the oyster isolates observed were type B. One reason for the difference may be sampling techniques. The isolates used by Nilsson et al. *(126)* were isolated on LB agar, a general medium while the isolates from this study were acquired through the selective MPN procedure which uses an alkaline enrichment followed by plating on two different selective media, VVA and mCPC. It is plausible that type B *V. vulnificus* may have a selective advantage for growth on these media although this hypothesis needs further testing. A more likely explanation is seasonal variation of 16S types. A study by Lin and Schwarz *(102)* showed that during the warmer months, particularly in the months of August through October when the water was warmer and the salinity was higher, 16S type B isolates were more prevalent. In another study, Kim and Jeong *(91)* also found a high prevalence of type B than had been previously reported from the US. In their study, 65% *V. vulnificus* isolates were type B while only 35% were found to be type A. This study took place in Korea in August when the water temperature was 22°C. The current study used samples from Alabama and Florida, along the northern Gulf of Mexico. The water temperature was 28°C during the month of July when these oysters were harvested. In contrast, the previously mentioned study by Nilsson et al. *(126)* used isolates that were isolated mainly during the cooler months of spring and fall, which may have a direct effect on the different types of *V. vulnificus* observed.

AFLP data showed that all 294 *V. vulnificus* isolates used in the experiment were genetically heterogeneous. Even with each isolate producing between 95 and 130 bands out of a pool of 171 total band classes across all isolates, the method was able to distinguish 16S types with almost 100% correlation to RFLP typing. Using transversal

clustering of band classes against complete isolate fingerprints, a narrower range of bands were able to be identified which were able to correlate to either the 16S type A or AB classes or to the type B class. Outliers from either group were most likely the result of amplification or restriction errors as they all clustered with less similarity to two major groups and were all genetically dissimilar to each other. Within each major 16S group there was also a great degree of genetic diversity. This in itself makes it difficult to obtain a suitable method for identifying potentially virulent strains when looking beyond a single locus. In spite of this fact, the AFLP data obtained here may provide multiple and valuable markers for the various 16S types. There was no selection during the refrigeration process for any of the 16S types, nor was there a difference in the results obtained by using isolates derived from oysters which came from different waters. But with the potentially virulent 16S rDNA type strains found at a higher rate than previously reported in the United States *(126, 172)*, having useful markers could help to identify potentially hazardous water bodies or oyster beds. A larger group of markers could also help with tracking of clinical infection cases to particular sites. Although our experiment did not find a direct correlation to 16S type and refrigeration time or harvest location, we were able to analyze a large sample size of *V. vulnificus* isolates providing a greater view of the diversity present throughout the genome.

In conclusion, this study demonstrated that over the course of the two week shelf life of the eastern oyster during refrigeration bacterial activity does not cease. In fact, total bacterial numbers increased showing that many of the species within the oyster not only survive but thrive during refrigeration. Specifically, *V. vulnificus* was also able to remain at a relatively consistent concentration throughout the experiment indicating the

ineffectiveness of refrigeration on reducing their numbers. The majority of *V. vulnificus* isolated were 16S type B. There was no reduction in the 16S type ratio over the course of the experiment indicating that refrigeration places no selective pressure on any 16S type *V. vulnificus*. This also shows that refrigeration alone may not help reduce the chance of exposure of humans consuming the oysters to the potentially virulent bacteria.

Table 3. Total bacteria counts and *V. vulnificus* Most Probable Number per gram oyster meat

Origin	Day	*n*	Total Bacteria Counts (CFU/g)[a] ± SD	V.v. MPN/g
Lab oysters	1	12	$1.1 \times 10^6 \pm 9.1 \times 10^4$	1.1×10^5
	7	12	$3.9 \times 10^7 \pm 5.9 \times 10^6$	1.1×10^5
	14	12	$3.6 \times 10^8 \pm 3.8 \times 10^7$	8.0×10^4
Proc. oysters	1	12	$2.1 \times 10^6 \pm 3.4 \times 10^5$	1.1×10^4
	7	12	$7.0 \times 10^7 \pm 2.1 \times 10^7$	1.1×10^5
	14	12	$2.7 \times 10^8 \pm 5.3 \times 10^7$	1.1×10^5

[a]Both Laboratory and Processor samples showed a significant change in total bacteria counts between each successive sampling date ($p > 0.05$).

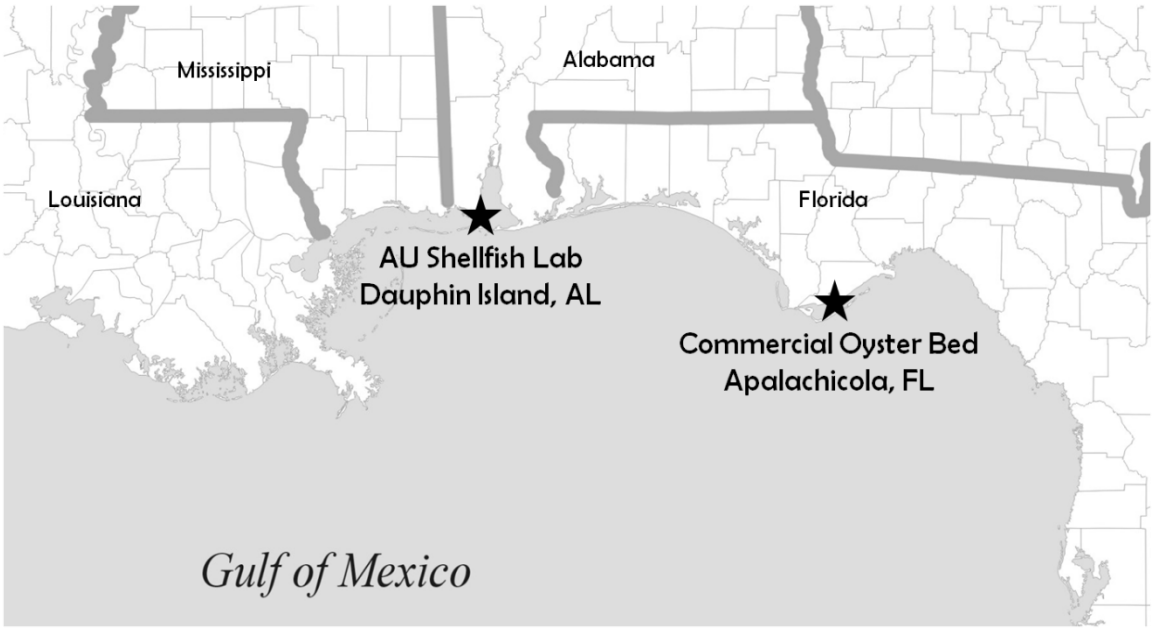

Figure 6. Map showing the locations at where the oysters were harvested used in this study. A) Auburn University Shellfish Laboratory, Dauphin Island, AL. B) Commercial oyster bed for processor, Apalachicola, FL

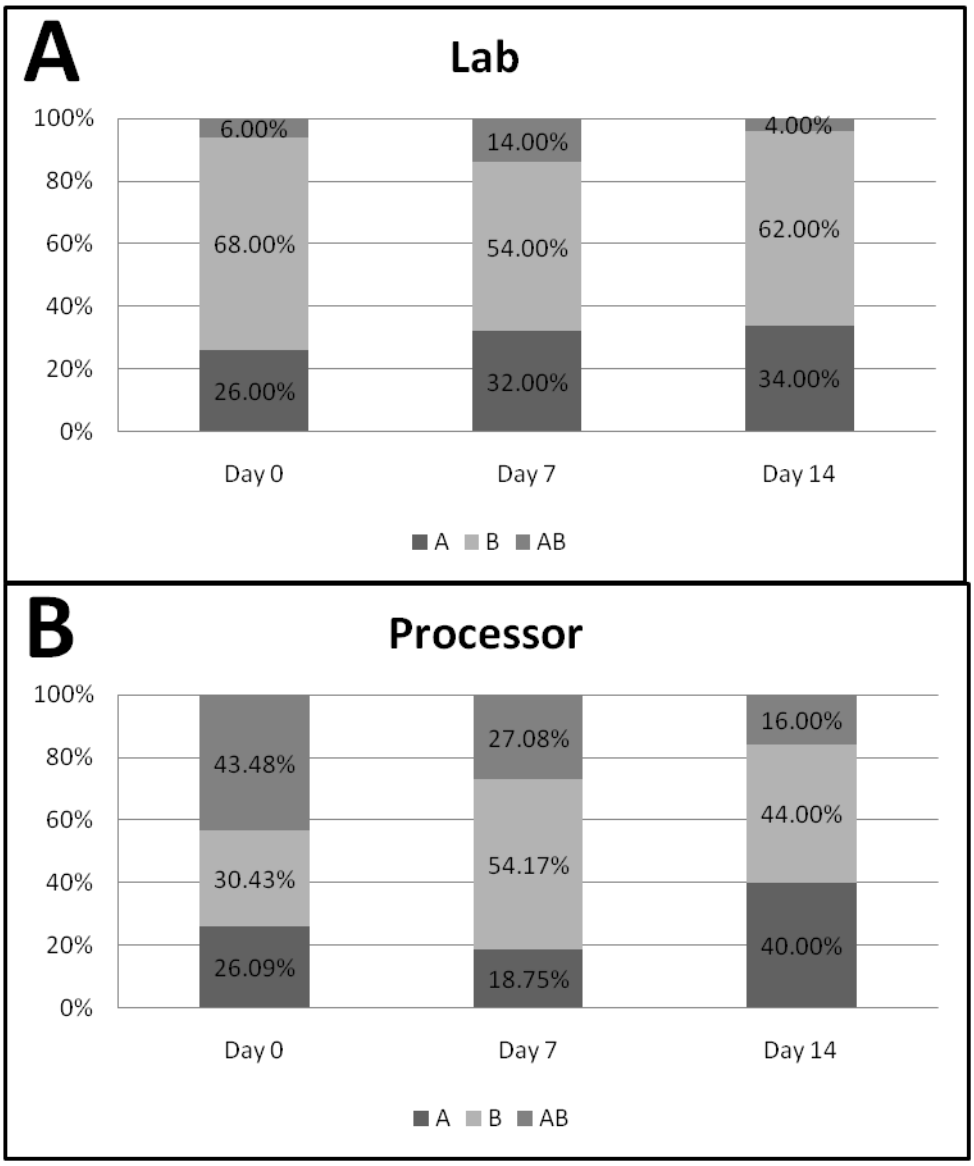

Figure 7. Percentage of total *V. vulnificus* 16S types isolated from each sampling date.

A) Laboratory oyster isolates. B) Processor oyster isolates

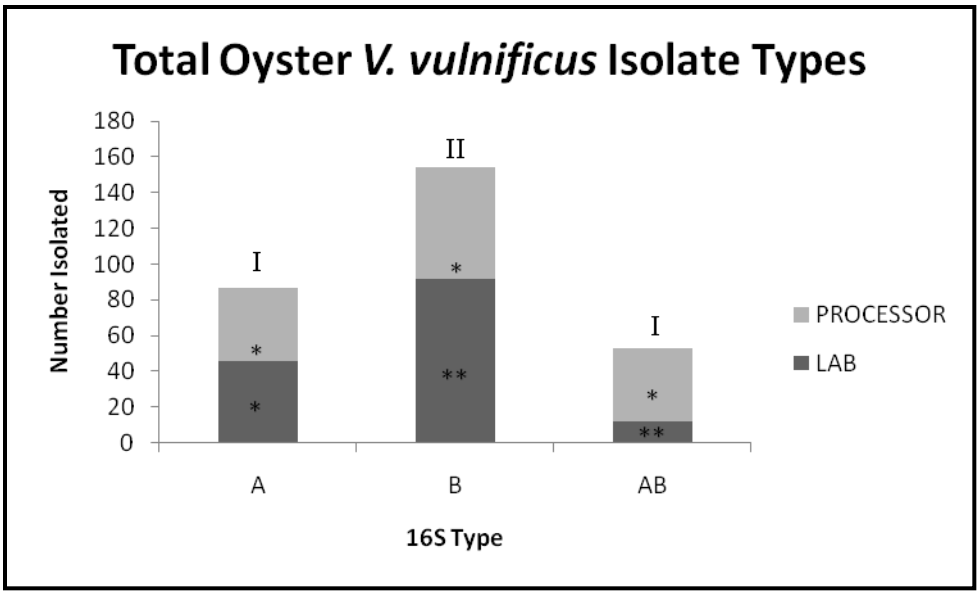

Figure 8. Graph showing the total number of *V. vulnificus* 16S types based on RFLP analysis. Each column shows the contribution of location to the total number isolated. Asterisks indicate if a significant difference between samples in that column is present while roman numerals indicate significance between columns ($p<0.05$)

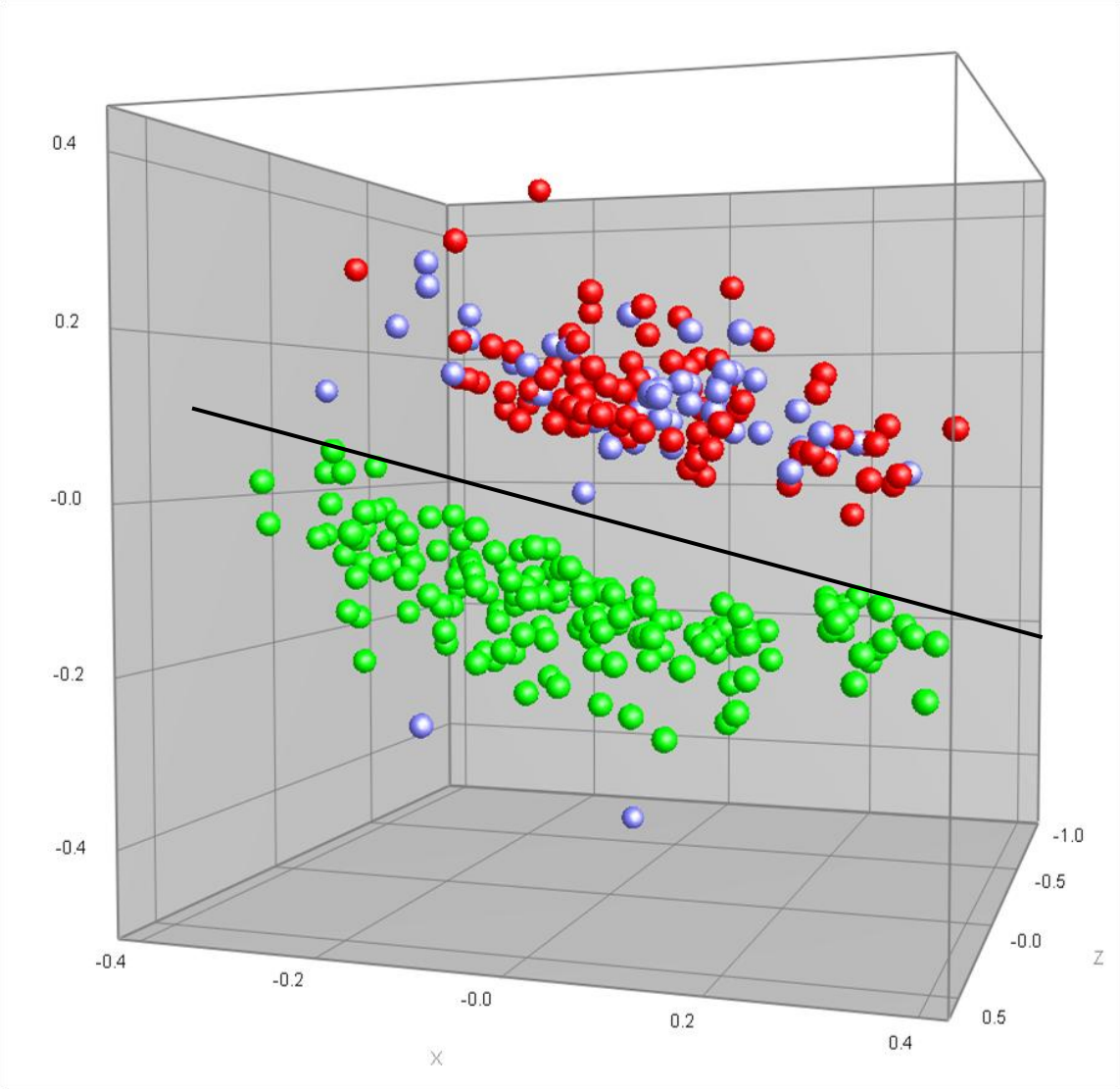

Figure 9. Multidimentional Scaling (MDS) performed on the AFLP patterns of 294 *V. vulnificus* isolates. Distance between entries represents dissimilarities obtained from the similarity matrix. Type B isolates (green) dominate one group, while Type A (red) and Type AB (blue) together represent the second group

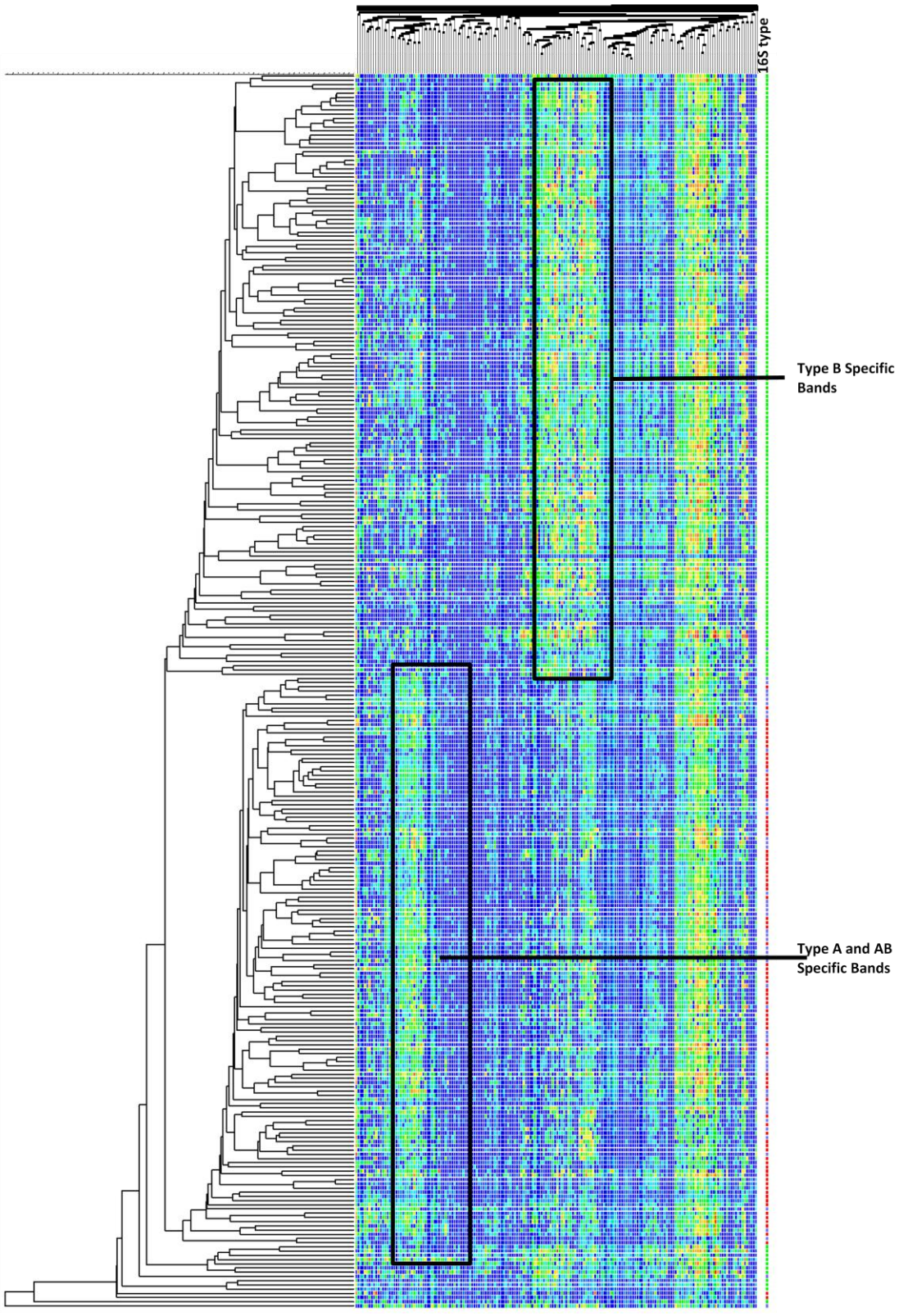
16S type
Type B Specific Bands
Type A and AB Specific Bands

Figure 10. Composite matrix of band classes based on transversal clustering of *V. vulnificus* AFLP fingerprints (vertical dendrogram) and band classes (horizontal dendrogram). 16S rDNA type is indicated by color in the right hand column: Type A-Red, Type B-Green, Type AB-Blue.

V. Evaluation of global gene expression during cold shock in the human pathogen *Vibrio vulnificus*

Abstract

Vibrio vulnificus can adapt to cold temperatures by changing the expression profiles of certain genes and their resulting proteins. In this study, the complete *V. vulnificus* transcriptome was analyzed under cold shock by looking at gene expression changes occurring during the shift from 35°C to 4°C. A DNA microarray-based global transcript profiling of *V. vulnificus* showed that 165 genes out of 4,488 altered their expression profiles by more than twofold. From 35°C to 10°C, an overall gene repression was observed while changes occurring below 10°C mainly resulted in upregulation. The highest induction observed occurred in two of the five categorized cold shock genes, *cspA* and *cspB*, which showed a complementary expression pattern during cold shock suggesting a homologous role. Other genes showing a significant fold increase included ribosomal genes, protein folding regulators, and membrane genes. Repressions were observed in all orthologous groups. Genes with top fold changes in repression include those coding for catalytic enzymes responsible for non temperature-related stress regulation. These included antioxidants, sugar uptake, and amino acid scavengers. *Vibrio vulnificus* maintained a high level of *cspA* and *cspB* transcripts during the entire experiment suggesting that these class I cold shock genes are require beyond the initial phase of the acclimation period.

Introduction

Vibrio vulnificus is a Gram negative food borne pathogen that has been identified as the leading cause of reported seafood related deaths in the United States *(29)*. This pathogen naturally occurs in estuarine and marine environments where it is a common member of the microbiota in warm coastal waters around the world *(180)*. It is known to cause sepsis in individuals with various predisposing conditions such as hemochromatosis, cirrhosis, diabetes, immuno compromising diseases, and kidney failure requiring dialysis *(66)*. Infections caused by *V. vulnificus* typically course as a primary septicemia with a fatality rate as high as 60% *(101, 130)*, which is almost exclusively associated to the consumption of raw oysters. Millions of oysters are eaten raw every year by Americans. Because oysters act as natural reservoirs for the bacterium *(40)*, there is potential for unintentional proliferation of *V. vulnificus* if the temperature of the oysters is not carefully controlled after harvest. *Vibrio vulnificus* is a mesophilic bacterium with an optimal growth temperature of 35°C and a generation time of 18 to 20 min *(135)*. The concentration of *V. vulnificus* at consumption is influenced not only by the initial dose of the bacterium in the oyster but 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 *(135)*. Therefore, restrictive shellstock control measures that require appropriate post-harvest refrigeration of oysters from the Gulf of Mexico have been implemented by the United States Food and Drug Administration (USFDA) under the National Shellfish Sanitation Program (NSSP) and have been accepted by the industry *(52, 101)*. However, even after

the implementation of refrigeration guidelines, infections caused by *V. vulnificus* have not declined overtime.

Vibrio vulnificus, like many other bacteria, is capable of developing a cold shock response, which allows the bacteria to adapt to lower temperatures while maintaining its active metabolism. Several studies have shown the adaptation of *V. vulnificus* to cold temperatures although most of them focused on the viable but nonculturable (VBNC) state *(23, 112)*. McGovern and Oliver *(112)* noted that during the moderate temperature downshift from 23°C to 13°C, up to 40 proteins were constitutively expressed. During this time a reduction in protein synthesis occurred, but growth continued. It was reported that after a 3 h acclimation period at 15°C the survival of *V. vulnificus* increased during exposure to 6°C *(23)*. Using standard and RT-PCR, Smith and Oliver *(157)* investigated the expression of putative virulence factors (*wza*, *wzb*, and *vvhA*), a protein synthesis factor (*tufA*), and stress response factors (*rpoS* and *katG*) while the pathogen naturally entered the VBNC state in an estuarine environment during the winter months. These authors suggested that the natural role of hemolysin (*vvhA*) in *V. vulnificus* could be related to osmoprotection and/or the cold shock response. Previous studies by our group examined the response of four putative cold shock genes (*csp1*, *csp2*, *csp4*, and *csp5)* and three stress-related genes (*oxyR*, *catG*, and *rpoS*) in *V. vulnificus* under shellstock temperature control conditions *(100, 101)*. Induction of some cold shock genes was observed during refrigeration but discrepancies in cold shock genes expression were found between *in vitro (100)* and *in vivo* studies *(101)*. All these studies used techniques that, although accurate, offered a limited the view of the overall expression status of the transcriptome during the downshift in temperature.

To further expand our knowledge of gene expression and regulation during cold shock in *V. vulnificus,* the present study analyzed the complete transcriptome displayed by this bacterium when subjected to cold shock by commercial refrigeration practices by using a microarray design capable of probing the entire genome. In order to identify key temperature points linked to the cascade of genetic modifications that takes place during cold, the downshift in temperature from 35°C to 4°C was broken into five steps. To provide a corroboration and validation to the microarray experiment, Real-Time Reverse Transcriptase PCR was performed using selected genes.

Materials and Methods

Bacterial strains and growth conditions. The type strain of *V. vulnificus* ATCC 27562 was used in this study. Prior to use, cells were maintained in 20% glycerol stocks and kept frozen at -80°C. Cells were then allowed to grow initially in marine broth (MB) (Difco, Detroit, MI) at 35°C for 24 h under shaking (100 rpm) and then transferred to 50 ml T1N1 (1% tryptone and 1% sodium chloride) broth and incubated for eight h to mid log phase.

Cold Shock and RNA Extraction. Cells were cold shocked by removing the culture from the 35°C incubator and immediately placing in a 4°C refrigerator with shaking. Five temperature ranges were selected based on the thermal profile generated during refrigeration. The time to bring the temperature down from 35-4°C was about one hour. Fifteen degrees was chosen as the end to the first temperature range because of information from previous studies that showed this as a major thermal point in the cold

shock process *(100, 101)*. The remaining ranges were chosen by selecting points resulting in even distribution over the remaining time frame. As the culture cooled to the predetermined temperatures of 35, 14, 10.5, 7.5, 6.0 and 4.5°C over a time span of 57 min (temperature was monitored in the culture broth), 5 ml of culture was transferred to 15 ml centrifuge tubes containing 10 ml of RNAProtect (QIAGEN, Valencia, CA) and RNA was extracted using the RNeasy kit (QIAGEN, Valencia, CA) following manufactures' instructions. RNA was eluted into 150 µl water and was quantified using the NanoDrop spectrophotometer and its integrity was checked by running 10 µl of each sample on a 1% agarose gel containing 6.7% formaldehyde. RNA samples were stored at -20°C until needed.

Microarray and data analysis. RNA was sent to Roche Nimblegen Inc. (Madison, WI) (30 µg per sample) for microarray assays. Using Genebank Accession numbers NC_004456 and NC_004460 representing chromosome 1 and chromosome 2 respectively of CMCP6 *V. vulnificus* strain, 6 microarray slides were produced. The Maskless Array Synthesizer was used to create the microarray consisting of 60-mer probes representing 4,488 genes that were synthesized on the slide. Seventeen 60-mer probes were constructed per gene target with five replicates of each set. Including those used for alignment and intensity validation, each array consisted of 385,000 probes. Each probe covered an area of 16 x 16µm, which created an array area of 17.4 x 13mm on the 25 x 76mm slide. The RNA was then converted to cDNA, labeled with Cy3 and hybridized to the array following standard protocols.

Arrays were scanned by Roche Nimblegen Inc. and data were normalized using quantile normalization *(21)*. Gene calls were made using the Robust Multichip Average

(RMA) *(79)*. Microarray data were analyzed using ArrayStar v2.1 (DNAStar, Inc.). Student's t-tests using Benjamin Hochberg correction was performed to compare the differences between individual samples.

Primer/Probe Design and Real-Time RT-PCR. To validate the results obtained during the microarray analysis RT- PCR was performed. Twenty four genes were selected for the validation process. These genes were selected by taking the top two expressed and top two repressed genes at each time point. In addition, the two remaining cold shock genes that were included in the microarray but did not have an up or down regulation of twofold or greater were selected. The gene VV1_0485 which codes for a DNA uptake lipoprotein was selected as an endogenous control because it had the least variation in expression throughout the cold shock experiment. The 16S rRNA gene was also included in the PCR. This was not used as an endogenous control as is normally done because it was not included on the microarray available from Roche Nimblegen Inc. Primers and probes were designed for Real-Time PCR using Vector NTI v. 10 (Invitrogen Inc. Carlsbad, CA) (Table 1).

A second cold shock experiment was carried out in order to extract RNA for Real-Time PCR. Similarly as described above, cells were exposed to cold shock by removing a culture from the 35°C incubator and immediately placing in a 4°C refrigerator with shaking. In this second experiment, more temperature points were tested. As the culture cooled to the predetermined temperatures of 35.0, 24.0, 14.0, 12.0, 10.5, 9.0, 7.5, 6.0, 5.0, 4.5, and 4.0°C, 5 ml of culture was transferred to 15 ml centrifuge tubes containing 10 ml of RNAProtect (QIAGEN, Valencia, CA) and the RNA was extracted using the RNeasy kit (QIAGEN, Valencia, CA) as described above.

The RNA samples were standardized to 40 ng/µl and reverse transcription was performed using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA). Briefly, reverse transcriptase master mix was made using 2 µl 10X Buffer, 0.8 µl dNTPs, 2.0 µl 10X Random Primers, 1.0 µl Reverse Transcriptase and 4.2 µl water per sample. This 10 µl master mix was combined with 10µl RNA. The complete mix was incubated at the following temperatures: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. The cDNA, at a concentration of 20 ng/µl, was stored at -20°C until needed.

Real-Time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The complete PCR mix contained 25µl SYBR Green PCR Master Mix, 4.5 µl of each primer (10 µM), 11 µl water, and 5 µl template cDNA. The reactions were carried out in an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Analysis was performed with the Relative Quantitation program of the ABI 7500 using the endogenous control listed above as a baseline for expression levels.

Results

Microarray analysis of cold shock on *V. vulnificus*. Samples of *V. vulnificus* cells were taken at various times during the induced cold shock to determine their transcriptome pattern by microarray. Following microarray analysis with the ArrayStar v2.1 (DNAStar, Inc.) software, genes with a fold change of two or higher were identified

for each temperature range. In total, only 3.7% (165/4,488) of the genes showed a difference in expression of twofold or higher. Using the ArrayStar software, the expression profiles of these genes were generated throughout the complete cold shock event and were grouped into eight distinct clusters based on their similarity by Standard Pearson Correlation Coefficient. The clusters are shown in Figure 11. Cluster A had a pattern that initially increased from 35-14°C then leveled off until 7.5°C when it began to decline. One hypothetical gene was included in this set. Cluster B maintained a steady expression with only a spike at 10.5°C. This cluster contained two genes VV1_0784 and VV2_0399, which code for adenylosuccinate lyase-like protein and acetlytransferase respectively. In cluster C, there were 6 genes consisting of mainly hypothetical genes and a transcriptional regulator VV2_0113. These genes maintained a steady expression with a slight repression at 10.5°C followed by a moderate induction at 7.5°C and another repression at 6°C. Cluster D contained 4 genes with a strong induction upon initial cold shock then continued to steadily increase in expression. Only four genes (VV2_0503, VV1_2575, VV1_0956, and VV2_0147) were grouped in this set that include two main cold shock genes (*cspA* and *cspB*). Cluster E grouped 9 genes that maintained steady expression until a moderate repression at 10.5°C followed by an induction at 7.5°C and continued steady expression until 4°C. Cluster F consisted of 7 genes which maintain a stable expression profile until 7.5°C when an induction was observed followed by a moderate repression at 6°C and another induction at 4°C. Within cluster G a fairly consistent expression pattern was detected in 6 genes with only an induction at 7.5°C observed that was maintained until the end of the experiment. Finally, cluster H was comprised of 130 genes. This large group had the generalized expression pattern of a

moderate repression from 35°C to 7.5°C followed by variable patterns until 4°C was reached.

The 165 genes with expressions that were either up regulated or down regulated two fold or higher were then placed into temperature ranges where these peak expressions were observed. When the temperature downshifted from 35°C to 14°C, a total of 4 genes had expressions and 64 had repressions of ≥ 2 fold. The next temperature ranges of 14-10.5°C, 10.5-7.5°C, 7.5-6°C, and 6-4°C yielded 2 and 36, 11 and 0, 56 and 9, and 7 and 0 genes being significantly up and down regulated, respectively. Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), the genes were assigned to known classifications or predicted classifications according to ontologically similar genes in the database. These gene classifications include general cell processes, environmental information processing, genetic information processing, amino acid metabolism, carbohydrate metabolism, energy metabolism, and other metabolism groups. The remaining genes are classified as having miscellaneous functions. These may either have no similarity to other genes or are poorly classified at the time the information was collected. Genes identified by microarray analysis and placed into these groups are shown in Table 2.

In the first temperature range, that included the start of the cold shock event, all but four of the 68 genes were down regulated. Two of the four up regulated genes were cold shock genes. These two genes, VV2_0503 and VV1_2757, code for proteins in the cold shock protein family (CspA and CspB, respectively). Upon cold shock, both genes were up regulated by 8.05 and 2.78 fold, correspondingly. A third cold shock protein gene was also present in this set, VV1_2119 (cold shock-like protein CspD). This gene

was down regulated by 2.14 fold. The following temperature range of 14-10.5°C showed a continued down regulation across the board including cold shock gene VV1_2757. With the exception of the genes coding for adenylosuccinate lyase-like protein and acetyltransferase, all significant regulations were negative. At the 10.5°C mark the gene regulation pattern changed dramatically. During the 10.5-7.5°C temperature range only 11 genes were significantly expressed. Each of these was up regulated as opposed to the previous temperature shift. Out of these 11 genes only 5 were non-hypothetical genes and included one codifying for the universal stress protein B (*uspB*) and a predicted transcriptional regulator. From 7.5 to 6°C there were 65 genes being significantly regulated including *cspA*. At this temperature range only 8 were being repressed. In the 6-4°C range only 7 genes showed significant regulation with each of these being induced.

Real-Time RT-PCR Analysis. Twenty-four genes were selected to be used in Real-Time RT-PCR as a validation of the microarray results. Comparison of the microarray and the Real-Time RT-PCR fluorescence profiles at each temperature point coinciding with the microarray samples shows a clear visual correlation in the gene regulation pattern throughout the cold shock event as shown in Figure 12. The *cspA* and *cspB* genes were shown to be those undergoing major induction in all time points in both the microarray and PCR reactions. The exception to this occurred in the 10.5°C samples. Microarray analysis showed a slight induction of the *cspB* gene while the Real-Time RT-PCR results showed a strong repression. Another exception which also occurred in the 10.5°C samples was in the VV1_2532 cold shock gene. This gene was repressed in the microarray data while it was strongly induced in the PCR reactions. All cold shock gene patterns are shown in Figure 13. While other genes also displayed conflicting expression

levels at this temperature, the remaining temperatures had a strong parallel in gene expression in both the PCR and microarray data. The correlation between the microarray and Real-Time RT-PCR results can be seen in Figure 14. The discrepancy observed between microarray and RT-PCR results at 10.5°C was responsible for a low correlation between both methods (R^2 value of 0.3944) when 10.5°C values were included. However, without the 10.5°C expression values, both techniques showed a good correlation (R^2 valued of 0.7364). In addition, the Pearson Product Moment Correlation Coefficient (PPMCC) was calculated between the two experiment data matrices and gave an *r* value of 0.63 when the 10.5°C samples were included and 0.86 when removed.

Discussion

Microarray analysis has proven to be a useful and reliable tool for the discovery of gene expression patterns *(31, 58, 69, 72, 99)*. In each of these studies, the microarray technique was used to both identify genes and to quantify gene expression. Although other quantitative gene expression methods such as RT-PCR have gained tremendous momentum in the past few years, microarrays have the potential of simultaneously screening all genes present in a genome. Therefore, microarray technology is a powerful tool in detecting stimulons or groups of transcript units that are differentially expressed in response to environmental perturbation. In the present study, we have identified genes that are differentially expressed in *V. vulnificus* when subjected to cold shock following the oyster industry refrigeration practices.

The first thing that should be noted when analyzing the *V. vulnificus* cold shock stimulon is that only a small percent of genes (165 out of 4,488) greatly (twofold) changed their expression profile during this drastic transition from 35°C to 4°C. In a similar study conducted with *V. parahaemolyticus* more than 1,000 genes were noted to have their expression profiled altered by more than twofold when the bacterium was cold shocked *(183)*. Similarly, *Shewanella oneidensis* reportedly has 785 genes that show significant differential expression during cold shock *(58)*. On the contrary, only 20 genes having a twofold or greater variation during cold shock have been reported in *Escherichia coli (140)*. It is interesting that *V. vulnificus* exhibited a simpler, more similar response to *E. coli* than to the phylogenetically close *V. parahaemolyticus*. However, extreme caution needs to be employed when comparing results from different microarrays studies since, beyond the intrinsic variability of the experiment based on the bacteria tested and the specific cold shock conditions, microarray data could be affected by the type of analysis performed *(2)*.

During the initial temperature downshift from 35-14°C all main classes of genes exhibited a reduction in expression. Eighteen of 20 genes involved in genetic information processing, including those coding for ribosomal proteins, and transcriptional regulators were repressed at a rate of twofold or higher. The remaining two genes *cspA* and *cspB*, were each induced with *cspA* having the highest induction. This is similar to results found in previous studies that showed a similar increase in expression of this cold shock gene occurring at 15°C *(100)*. The categories of cellular processes and signaling, environmental information processing, energy, carbohydrate, amino acid, and various other metabolisms, along with several other uncategorized proteins were repressed. The

only exception occurred within the cellular processes and signaling category. The gene VV1_0956, coding for a permease of the major facilitator superfamily was induced by 2.13 fold. This protein as described by Law *(95)*, controls the movement of substrates across the cell membrane and is a necessary component for the long term stability of the cell. In the next temperature range of 14-10.5°C up to 38 genes showed significant regulation with only 2 being up regulated. The gene coding for an adenylosuccinate lyase-like protein was induced 2.21 fold. This protein has been characterized as playing a critical role in the de novo purine biosynthetic pathway in which it affects both cellular replication and metabolism *(133, 167)*. The other upregulated gene coded for an acetyltransferase. Phosphate acetyltransferase is used in the conversion of acetyl-CoA to acetate. During this process ATP is formed via substrate level phosphorylation *(20)*. An intermediate in the reaction is acetyl phosphate. Because acetyl phosphate functions as the phosphoryl donor of response regulator proteins of two-component systems as shown in *E. coli*, it has been suggested that its presence acts as a global regulatory signal *(111, 176)*. This may be shown to be true by the regulation profile in the next thermal range.

After temperature dropped below 10°C, an overall increase in gene expression was observed and 11 genes showed significant upregulation. One of them coded for Universal Stress Protein B (UspB). This protein has been shown to be an integral membrane protein with application to thermotolerance, osmotolerance, and oxidative stress *(50)*. The next temperature range (7.5-6°C) was also characterized by an induction in 56 of the 65 genes showing differential expression, and included all major gene classes covered in this study. These included genes involved in cellular growth and motility, environmental and genetic information processing, amino acid, carbohydrate, energy, and

nucleotide metabolism, in addition to various uncategorized genes. Repressions in this range consisted mainly of hypothetical proteins with no known function and thus will not be discussed here. Finally, when cells were cooled down below 6°C an increase of gene expression in 7 genes, which included a gene coding for a ribosomal protein and another coding for a cold shock-like protein, was observed. There were no repressions at this point indicating that all cellular functions were still being carrying out. These may be the critical functions needed to ensure the viability of the culture during cold stress and which allow the organism to be recovered at a later time as previously shown *(100)*. The large amount of activity at this low temperature range may also be the beginning stages of what has been termed the viable but nonculturable (VBNC) state described in many previous papers *(128, 129, 157, 177, 178)* on cold shock and its effect on *V. vulnificus*.

The RT-PCR assays confirmed the reproducibility of the genetic response of the bacterium during cold shock, as a similar expression pattern was shown between the microarray and RT-PCR experiments. This was true for all time points except for samples taken at 10.5°C and has yet to be explained. This temperature could represent a critical junction where many important shifts in expression take place and as such, make it difficult to accurately and reproducibly capture the same profile twice. It should be noted that the cultures for the microarray and Real-Time RT PCR were grown and processed at different times and each experiment maintains this highly variable expression pattern at this particular temperature. Further investigation is indeed necessary to ascertain the cause and effect of this variability.

This study investigated the global effects on gene regulation in *V. vulnificus* during a cold shock event. Our data showed *cspA* as the key element in triggering the

cold-shock response. This gene was highly induced within 17 minutes after the cooling process started until the culture reached 14°C, behaving as a typical class I cold shock gene. However, the *cspA* expression profile changed over time as the temperature decreased. It was not detected as differentially expressed at temperatures 10.5°C and 4°C but it had a twofold increase at 7.5°C. Interestingly, the *cspA* homolog gene *cspB* showed a complementary expression pattern to *cspA*, being upregulated at the temperatures in which *cspA* was not (10.5°C and 4°C). It has been shown in *E. coli* that RNA chaperones such as CspA and its homologs are needed during the acclimation phase of cold shock *(139)*. At low temperature, the secondary structures of the RNA stabilize, which slows down translation. CspA homologs are thought to play an important role during the first phase of the acclimation period and after the cells are acclimated they are not longer needed. However, our data showed that *cspA* or *cspB* were positively regulated at any given temperature. This may indicate that in *V. vulnificus* csp class I genes are continuously upregulated during all stages of cold shock or that under our conditions the cells were not allowed to acclimate to cold shock since we performed an abrupt shift from 35°C down to 4°C. Previous studies done by our group and others have shown that when *V. vulnificus* is allowed to adapt to cold temperatures slowly (15°C for a few hours) the bacterium not only survives at 7°C but can multiply under those conditions *(23, 100)*. Our microarray and RT-PCR data highlighted a remarkable shift in the gene expression pattern occurring at 10.5°C in where the majority of the differently expressed genes were repressed. It is clear that during the transition from optimal temperature down to 14.5°C the cells start the cold shock response by inducing the expression of the main cold shock gene *cspA* as well as repressing genes involved in genetic information processes. We

hypothesize that if *V. vulnificus* cells are allowed to acclimate at that suboptimal temperature for hours, expression of *cspA* would likely return to basal levels like it has been proved in *E. coli*. However, if cells are not allowed to acclimate and temperature continues to drop, *cspA* homologous may be required to maintain RNA stability at lower temperatures and secure a minimum level of translation.

This study has generated the transcriptome of *V. vulnificus* during cold shock and has identified potential critical temperature/time relationships, which could lead to modifications in refrigeration practices by the industry.

Table 4. Genes used in Real-Time RT-PCR

Gene Number	Product	Expression	°C
VV2_0503	Cold shock protein CspA	Induction	35-14
VV1_2757	Cold shock protein CspB	Induction	35-15
VV2_0399	Acetyltransferase	Induction	14-10.5
VV2_1620	Parvulin-like peptidyl-prolyl isomerase ppi1	Induction	14-10.5
VV1_0794	Putative periplasmic protein pmp1	Induction	10.5-7.5
VV1_0978	Protoporphyrinogen oxidase	Induction	10.5-7.5
VV1_0750	50S ribosomal protein L24 RplX	Induction	7.5-6
VV1_1019	ATP synthase subunit A	Induction	7.5-6
VV1_0823	50S ribosomal protein L33 RpmG	Induction	6-4
VV1_2119	Cold shock-like protein CspD	Induction	6-4
VV2_1117	Delta 1-pyrroline-5-carboxylate dehydrogenase	Repression	35-14
VV1_0453	Peroxiredoxin por1	Repression	35-15
VV2_0491	enoyl-CoA hydratase FadJ	Repression	14-10.5
VV1_1117	universal stress protein UspB	Repression	14-10.5
VV2_1319	FOG: GGDEF domain	Repression	10.5-7.5
VV1_0345	Predicted esterase	Repression	10.5-7.5
VV2_0839	chorismate mutase AroQ	Repression	7.5-6
VV2_0167	NADH:ubiquinone oxidoreductase subunit 2	Repression	7.5-6
VV2_0253	Phosphotransferase system IIA component	Repression	6-4
VV2_0352	AraC-type DNA-binding domain-containing protein	Repression	6-4
VV2_0519	Cold shock protein	None	None
VV1_2532	Cold shock domain family protein	None	None
VV1_0485	DNA uptake lipoprotein	None	None
VV1_R06	16S rRNA	N/A	N/A

Table 5: Genes identified by microarray analysis to be up/down regulated ≥ 2 fold during cold shock

Temp Range/ Gene Category/ Accession Number	Product	Fold Change	
35-14°C			
Cellular Processes and Signaling; Transporters			
VV1_0956	Permease of the major facilitator superfamily	2.13	↑
VV2_0132	Cytochrome c553	2.06	↓
Environmental Information Processing; Membrane Transport			
VV1_1724	Nucleoside permease	2.97	↓
VV1_2896	Nitrate/TMAO reductase, membrane-bound tetraheme cytochrome c subunit	2.22	↓
VV1_0211	Phosphoenolpyruvate-protein phosphotransferase	2.17	↓
VV1_0357	Molecular chaperone DnaK	2.05	↓
VV1_1723	Nucleoside permease	2.02	↓
Genetic Information Processing			
VV2_0503	Cold shock protein, CspA	8.05	↑
VV1_2757	Cold shock protein, CspD	2.78	↑
VV1_0453	Peroxiredoxin	3.00	↓
VV1_0693	Ribosome-associated protein Y	2.98	↓
VV1_2120	ATP-dependent Clp protease adaptor protein ClpS	2.94	↓
VV1_2923	DNA-binding protein H-NS	2.71	↓
VV1_0024	Trigger factor	2.63	↓
VV1_1336	30S ribosomal protein S12	2.56	↓
VV1_1615	30S ribosomal protein S16	2.52	↓
VV1_0751	50S ribosomal protein L14	2.44	↓
VV1_3013	50S ribosomal protein L32	2.41	↓
VV1_3058	Elongation factor P	2.28	↓
VV1_0019	Bacterial nucleoid DNA-binding protein	2.17	↓
VV1_2119	Cold shock-like protein CspD	2.14	↓
VV2_0846	Glutathione synthase	2.13	↓
VV1_2399	50S ribosomal protein L35	2.05	↓
VV1_0371	SsrA-binding protein	2.04	↓

VV2_1135	Co-chaperonin GroES	2.02	↓
VV1_2850	50S ribosomal protein L25	2.02	↓
VV2_1282	Transcriptional regulator	2.00	↓
Amino Acid Metabolism			
VV2_1117	Delta 1-pyrroline-5-carboxylate dehydrogenase	4.76	↓
VV2_1118	Delta 1-pyrroline-5-carboxylate dehydrogenase	2.87	↓
VV1_3050	Glutamate decarboxylase	2.07	↓
VV1_2357	Biosynthetic arginine decarboxylase	2.03	↓
VV1_1594	Aspartate kinase	2.01	↓
Carbohydrate Metabolism.			
VV1_0161	Succinate dehydrogenase cytochrome b556 large membrane subunit	2.79	↓
VV2_0552	Transaldolase	2.63	↓
VV1_3140	Glyceraldehyde-3-phosphate dehydrogenase	2.47	↓
VV1_2221	Acetate/propionate kinase	2.36	↓
VV1_0212	Glucose-specific PTS system enzyme IIA component	2.24	↓
VV1_2098	Formate acetyltransferase	2.22	↓
VV1_1537	Transketolase	2.16	↓
VV1_3111	Alcohol dehydrogenase	2.06	↓
Energy Metabolism			
VV1_2618	Cbb3-type cytochrome oxidase, subunit 3	2.81	↓
VV1_0209	Cysteine synthase A	2.70	↓
VV1_0597	Ubiquinol-cytochrome c reductase, iron-sulfur subunit	2.22	↓
VV1_1016	ATP synthase subunit C	2.14	↓
VV1_1019	ATP synthase subunit A	2.03	↓
Other Metabolism			
VV1_0023	ATP-dependent Clp protease proteolytic subunit	2.73	↓
VV1_1465	Aspartate carbamoyltransferase catalytic subunit	2.24	↓
VV1_1986	3-oxoacyl-(acyl-carrier-protein) synthase	2.15	↓
Miscellaneous Functions			
VV2_0021	Hypothetical protein	2.04	↑

VV2_0281	Hypothetical protein	2.87	↓
VV1_2250	Hypothetical protein	2.75	↓
VV2_1205	Hypothetical protein	2.66	↓
VV1_0542	Acid-induced glycyl radical enzyme	2.64	↓
VV2_0240	Hypothetical protein	2.47	↓
VV1_1117	Universal stress protein UspB	2.37	↓
VV1_2222	Hypothetical protein	2.36	↓
VV1_1818	Lipoprotein	2.25	↓
VV2_1315	Hypothetical protein	2.22	↓
VV2_0495	Predicted transcriptional regulator	2.18	↓
VV1_3149	Lipoprotein-related protein	2.16	↓
VV1_2376	Hypothetical protein	2.16	↓
VV1_2384	Hypothetical protein	2.15	↓
VV2_0720	Hypothetical protein	2.14	↓
VV1_2171	Peptidoglycan-associated lipoprotein	2.13	↓
VV2_0279	Hypothetical protein	2.08	↓
VV2_0974	Zinc metalloprotease	2.07	↓
VV1_3049	Saccharopine dehydrogenase	2.07	↓

14-10.5°C

Cellular Processes; Cell Motility

VV1_2108	Methyl-accepting chemotaxis protein	2.36	↓
VV1_1927	Flagellar protein FlaG	2.21	↓
VV1_0226	Flagellar basal body protein	2.11	↓

Environmental Information Processing; Membrane Transport

VV1_1724	Nucleoside permease	2.36	↓
VV1_0028	TRAP-type C4-dicarboxylate transport system, large permease component	2.15	↓
VV1_1723	Nucleoside permease	2.10	↓

Genetic Information Processing

VV1_0750	50S ribosomal protein L24	2.54	↓
VV1_0453	Peroxiredoxin	2.30	↓
VV1_1210	50S ribosomal protein L7/L12	2.18	↓
VV1_0371	SsrA-binding protein	2.14	↓
VV1_1207	50S ribosomal protein L11	2.12	↓
VV1_0788	Ribosomal S7-like protein	2.10	↓
VV1_1208	50S ribosomal protein L1	2.06	↓
VV1_2757	Cold shock protein, CspD	2.00	↓

Carbohydrate Metabolism

VV1_2731	Citrate synthase	2.42	↓
VV1_0154	Succinyl-CoA synthetase alpha subunit	2.10	↓
VV1_2728	AMP-(fatty) acid ligase	2.06	↓
VV2_0470	Pyruvate/2-oxoglutarate dehydrogenase complex, dehydrogenase component, beta subunit	2.05	↓

Other Metabolism

VV2_0491	Enoyl-CoA hydratase	2.93	↓
VV2_0494	Acetyl-CoA acetyltransferase	2.33	↓
VV2_0490	Enoyl-CoA hydratase/carnithine racemase	2.14	↓
VV2_0493	NAD-dependent aldehyde dehydrogenase	2.13	↓
VV2_0560	Protoheme IX farnesyltransferase	2.08	↓
VV2_0561	Uncharacterized protein required for cytochrome oxidase assembly	2.05	↓

Miscellaneous Functions

VV1_0784	Adenylosuccinate lyase-like protein	2.21	↑
VV2_0399	Acetyltransferase	2.00	↑
VV1_0766	Hypothetical protein	3.37	↓
VV1_2518	Hypothetical protein	3.01	↓
VV1_1117	Universal stress protein UspB	2.78	↓
VV1_0789	Hypothetical protein	2.57	↓
VV1_0787	Hypothetical protein	2.56	↓
VV1_2939	Hypothetical protein	2.52	↓
VV1_0790	Hypothetical protein	2.42	↓
VV2_0126	Hypothetical protein	2.36	↓
VV1_3199	Transposase and inactivated derivatives	2.22	↓
VV1_0794	Putative periplasmic protein	2.13	↓
VV2_1545	Murein lipoprotein	2.05	↓
VV1_2295	Hypothetical protein	2.02	↓

10.5-7.5°C

Genetic Information Processing

VV2_0113	Predicted transcriptional regulator	2.03	↑

Metabolism of Cofactors and Vitamins

VV1_0978	Protoporphyrinogen oxidase	2.13	↑

Miscellaneous Functions

VV2_0845	Hypothetical protein	2.55	↑
VV1_0766	Hypothetical protein	2.52	↑
VV1_1250	Protein affecting phage T7 exclusion by the F plasmid	2.37	↑
VV1_0794	Putative periplasmic protein	2.22	↑
VV1_1117	Universal stress protein UspB	2.17	↑
VV1_0168	Hypothetical protein	2.06	↑
VV2_0009	Hypothetical protein	2.04	↑
VV1_0244	Hypothetical protein	2.04	↑
VV1_2893	Hypothetical protein	2.03	↑

7.5-6°C

Cell Growth

VV1_0573	Cell division protein FtsZ	2.35	↑

Cell Motility

VV1_0221	Flagellar basal body rod protein FlgG	2.44	↑
VV1_0219	Flagellar P-ring protein precursor	2.28	↑
VV1_1952	Flagellar biosynthesis sigma factor FliA	2.26	↑
VV1_1957	SOJ-like and chromosome partitioning protein	2.05	↑
VV1_1951	Flagellar biosynthesis MinD-related protein	2.04	↑

Environmental Information Processing

VV2_1163	Methylase of chemotaxis methyl-accepting protein	2.21	↑
VV1_1956	Chemotaxis-specific methylesterase	2.13	↑
VV1_2895	Anaerobic dehydrogenase	2.07	↑

Genetic Information Processing

VV1_0750	50S ribosomal protein L24	3.01	↑
VV1_0743	30S ribosomal protein S5	2.63	↑
VV2_0503	Cold shock protein CspA	2.57	↑
VV2_0147	Superfamily II DNA and RNA helicase	2.34	↑
VV1_0760	50S ribosomal protein L23	2.31	↑
VV1_1737	Translation elongation factor G	2.30	↑
VV1_0763	30S ribosomal protein S10	2.30	↑
VV1_1336	30S ribosomal protein S12	2.24	↑
VV1_1615	30S ribosomal protein S16	2.23	↑

VV1_0742	50S ribosomal protein L30	2.22	↑
VV1_1616	16S rRNA-processing protein	2.20	↑
VV1_0756	30S ribosomal protein S3	2.20	↑
VV1_1338	Elongation factor EF-2	2.18	↑
VV1_2371	Phenylalanyl-tRNA synthetase beta subunit	2.16	↑
VV1_0758	30S ribosomal protein S19	2.16	↑
VV1_1696	Translation initiation factor IF-2	2.13	↑
VV1_2399	50S ribosomal protein L35	2.08	↑
VV1_1695	Transcription elongation factor NusA	2.07	↑
VV1_0761	50S ribosomal protein L4	2.03	↑
VV1_2122	ATP-dependent Clp protease	2.01	↑
Carbohydrate Metabolism			
VV1_2732	PEP phosphonomutase	2.36	↑
VV1_0156	Dihydrolipoamide acetyltransferase	2.12	↑
VV1_2731	Citrate synthase	2.04	↑
Energy Metabolism			
VV1_1019	ATP synthase subunit A	2.70	↑
VV1_1021	ATP synthase subunit B	2.49	↑
VV1_1017	ATP synthase subunit B	2.49	↑
VV1_2619	Cbb3-type cytochrome oxidase, cytochrome c subunit	2.22	↑
VV1_2618	Cbb3-type cytochrome oxidase, subunit 3	2.22	↑
VV1_1018	ATP synthase subunit D	2.21	↑
VV1_1015	ATP synthase subunit A	2.16	↑
Nucleotide Metabolism			
VV1_1212	DNA-directed RNA polymerase beta' subunit	2.28	↑
VV1_1211	DNA-directed RNA polymerase beta subunit	2.25	↑
VV1_0565	Carbamoyl-phosphate synthase large subunit	2.09	↑
VV1_0736	DNA-directed RNA polymerase alpha subunit	2.02	↑
Other Metabolism			
VV2_0492	Acyl-CoA dehydrogenase	2.01	↑
VV2_0496	Acyl-CoA dehydrogenase	2.01	↑
VV2_0839	Chorismate mutase	2.23	↓
Miscellaneous Functions			
VV1_1822	Na(+)-translocating NADH-quinone reductase subunit B	2.45	↑

VV1_2729	Hypothetical protein	2.33	↑
VV1_1823	Na(+)-translocating NADH-quinone reductase subunit C	2.24	↑
VV1_1315	NAD-dependent aldehyde dehydrogenase	2.22	↑
VV1_1566	GTP-binding protein Era	2.16	↑
VV1_1080	Membrane-fusion protein	2.13	↑
VV1_1752	Hypothetical protein	2.12	↑
VV1_1824	NADH-ubiquinone oxidoreductase	2.10	↑
VV1_0439	Predicted transcriptional regulator	2.07	↑
VV1_1039	Hypothetical protein	2.43	↓
VV1_1480	Hypothetical protein	2.24	↓
VV2_0845	Hypothetical protein	2.20	↓
VV2_0584	Hypothetical protein	2.16	↓
VV1_0168	Hypothetical protein	2.14	↓
VV1_2596	Hypothetical protein	2.05	↓
VV2_0167	NADH:ubiquinone oxidoreductase subunit 2	2.04	↓
VV2_0009	Hypothetical protein	2.00	↓

6-4°C

Genetic Information Processing

VV1_0823	50S ribosomal protein L33	2.30	↑

Miscellaneous Functions

VV1_1040	Hypothetical protein	2.69	↑
VV1_0787	Hypothetical protein	2.53	↑
VV1_2680	Hypothetical protein	2.17	↑
VV1_2119	Cold shock-like protein CspD	2.06	↑
VV1_1619	Hypothetical protein	2.06	↑
VV2_1545	Murein lipoprotein	2.01	↑

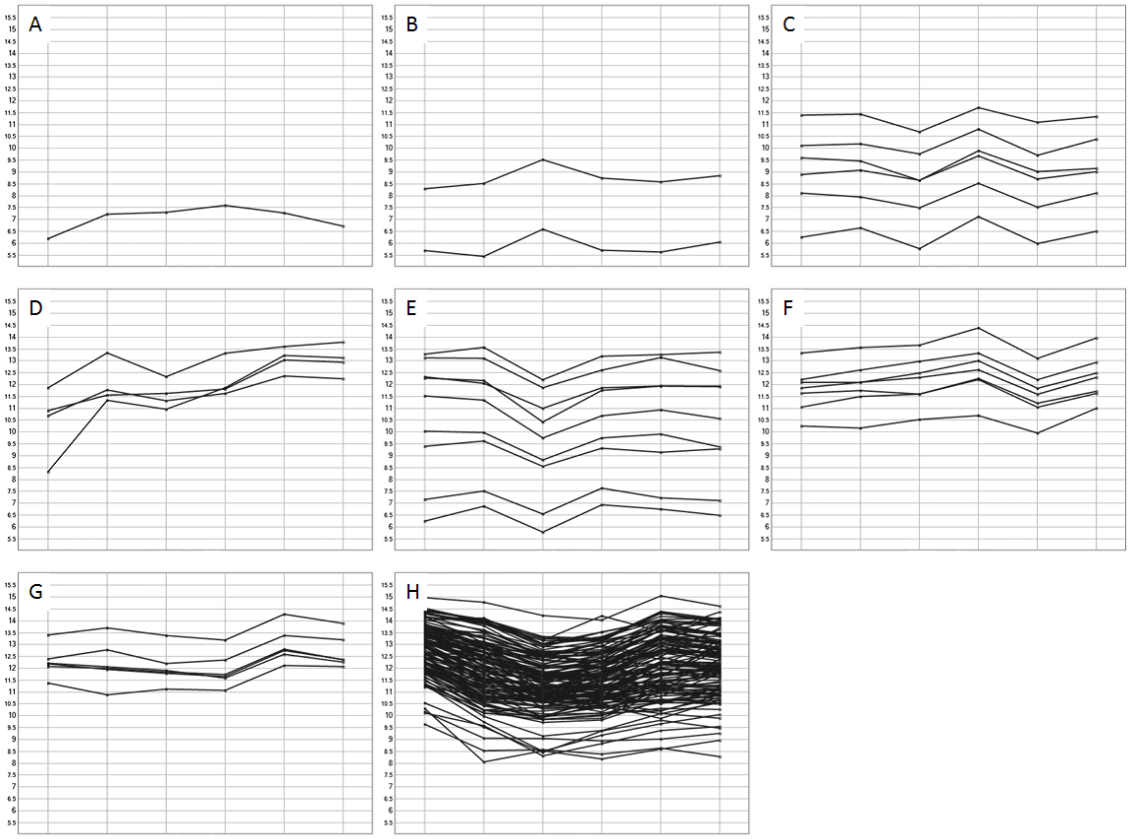

Figure 11. Clustering of microarray data. Eight gene clusters were made using standard Pearson Correlation Coefficient to measure the differences in trends between genes and expression levels. The X-axis represents the different temperature points during cold-shock and the Y-axis represents expression values. Each cluster contains A) 1, B) 2, C) 6, D) 4, E) 9, F) 7, G) 6, H) 130 genes.

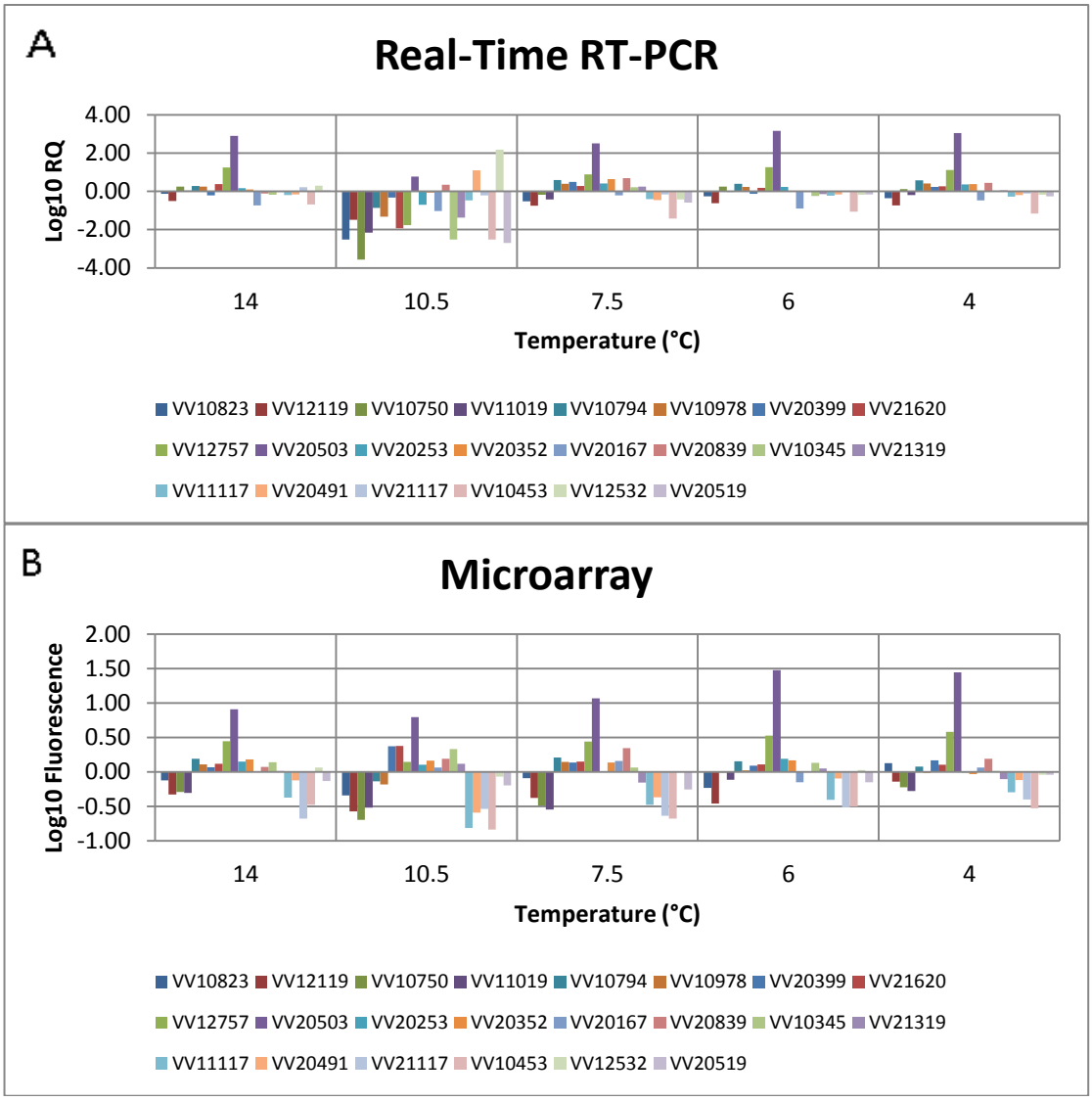

Figure 12. Panel A, Real-Time RT-PCR expression patterns. Panel B, microarray expression patterns of the 24 selected genes at 5 of the 6 temperature points used for the microarray. The 35°C temperature was used as a normalization standard for the other temperatures.

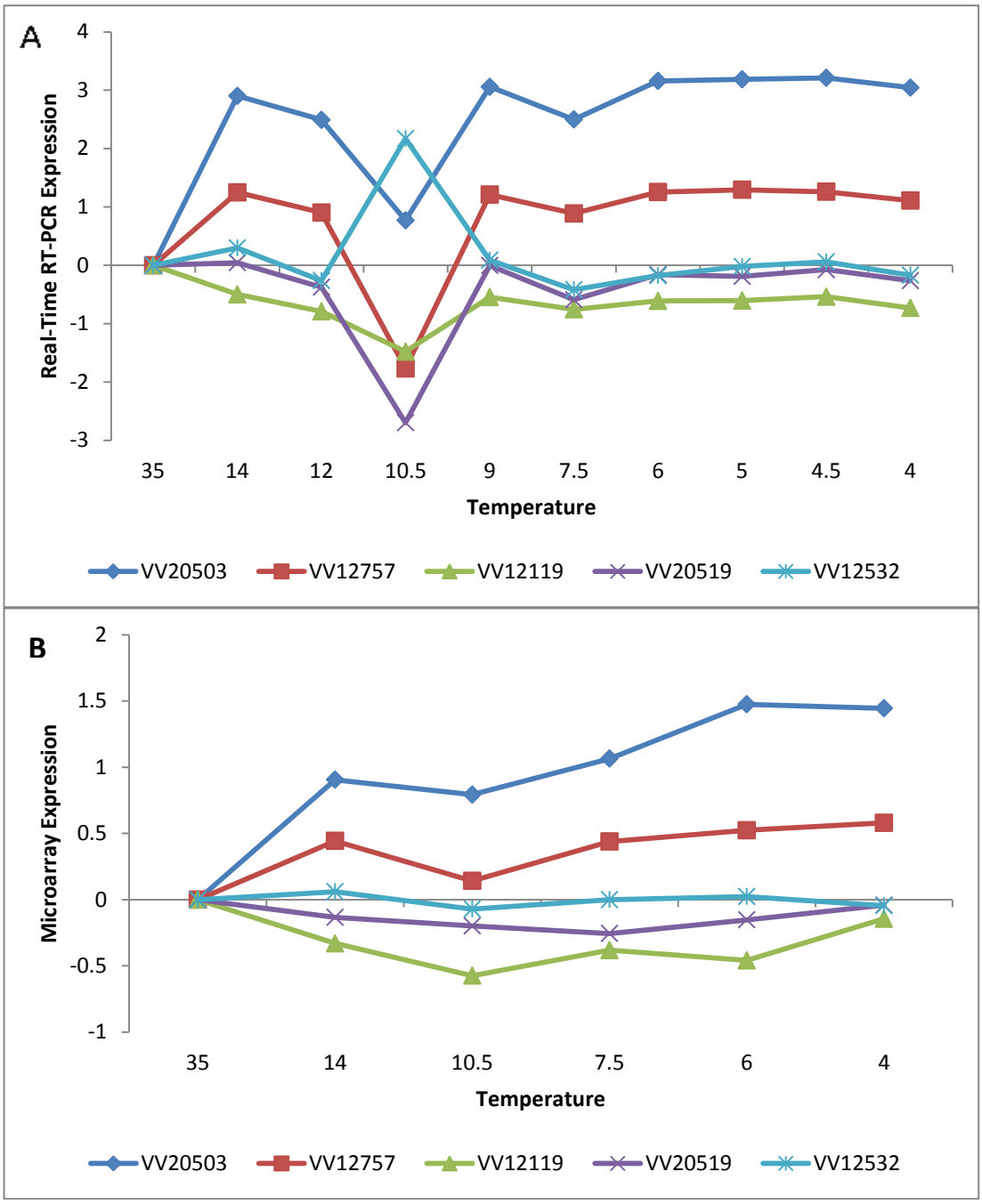

Figure 13. Time-course expression patterns of the five cold-shock genes using all available temperature data points as detected by Real-Time RT-PCR (panel A) and Microarray analysis (panel B).

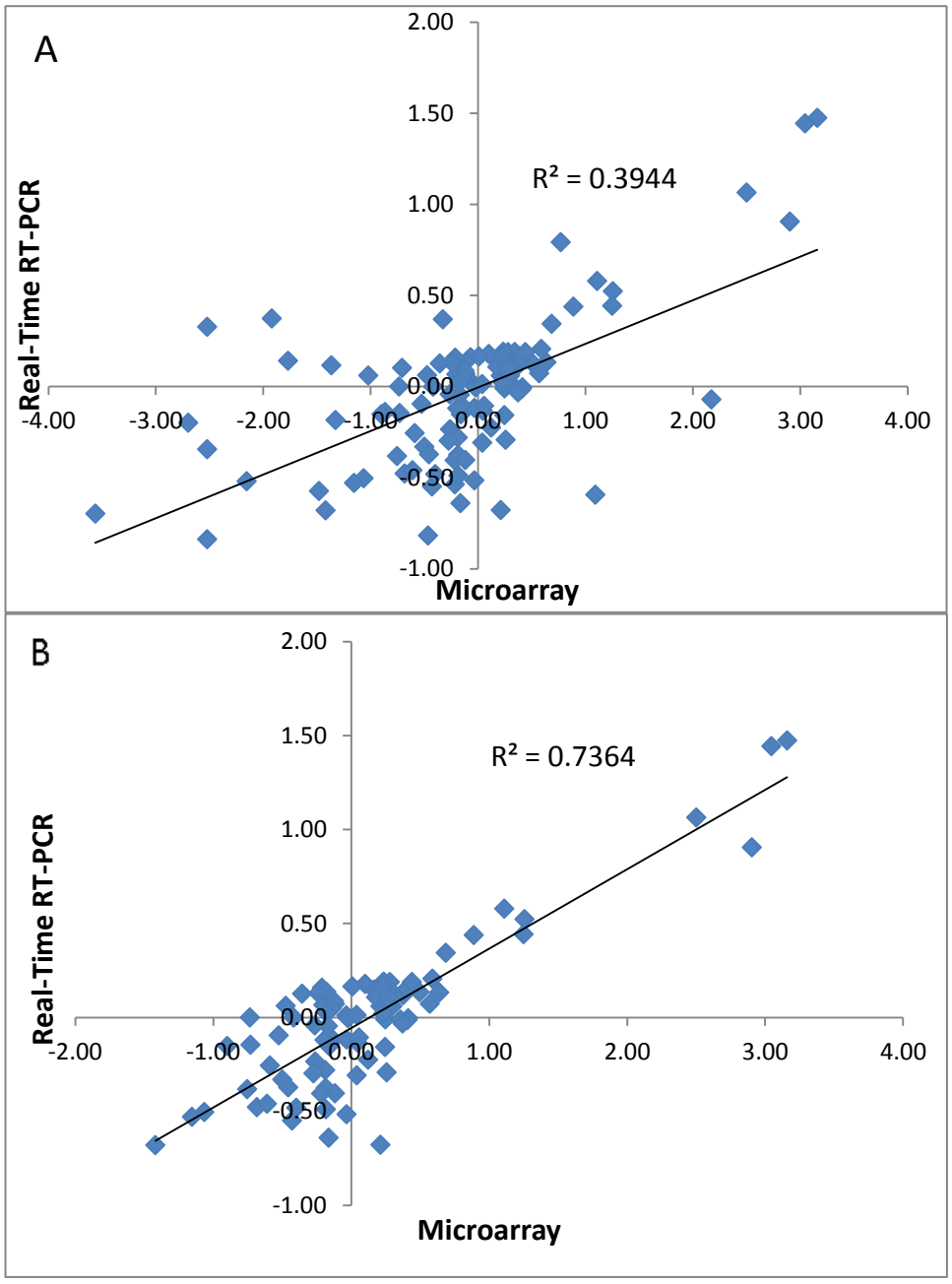

Figure 14. Correlation between RT-PCR and Microarray analysis data. The $\log_{10}$ values of the Real-Time RT-PCR were plotted against the Microarray analysis $\log_{10}$ values. The expression levels for the 24 selected genes are shown. Panel A includes all temperature points while panel B show correlation when 10.5°C temperature data points were excluded from the analysis.

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