ABSTRACT

Drake, Stephenie Lynn. Characterization of the response of *Vibrio vulnificus* to sublethal stresses during oyster handling and processing (Under the direction of Dr. Lee-Ann Jaykus)

Vibrio vulnificus, a naturally occurring marine bacterium, causes severe disease in at-risk individuals consuming contaminated raw shellfish. The organism can be difficult to discriminate from natural microflora present in the product, complicating the evaluation of process control efficacy. The purpose of this study was to construct a strain of *V. vulnificus* expressing green fluorescent protein (*Vv*-GFP-K) which could be readily distinguished from background flora. Once constructed, the objectives were to compare the physiological characteristics of *Vv*-GFP-K to the wild-type parent (*Vv*-WT); to use *Vv*-GFP-K to evaluate survival of the bacterium under various environmental stresses relevant to food processing; and to assess the effect of sodium pyruvate media supplementation on recovery efficiency, with particular reference to sublethally injured cells.

V. vulnificus strain ATCC 27562 was engineered to express GFP and kanamycin resistance using methods of conjugation. Comparisons were made between *Vv*-GFP-K and *Vv*-WT with respect to growth characteristics, heat tolerance (45^{o}C), freeze/thaw tolerance (-20^{o} and -80^{o}C), acid tolerance (pH 5.0, 4.0, and 3.5), cold storage (5^{o}C), cold adaptation (15^{o}C) and starvation. Recoveries were evaluated using non-selective [tryptic soy agar-2%NaCl, ($TSAN_2$)] medium with and without sodium pyruvate supplementation. To represent the food matrix, seeding studies were done with either shellstock or shucked oysters and the survival of *Vv*-GFP-K in the food matrix was evaluated after exposure to cold and acid stress. Specifically, cooling regimens

designated (i) rapid cooling (iced); (ii) conventional cooling (5^{o}C); and (iii) mild abusive cooling (temperature dropped to 5^{o}C over 8 hr) were evaluated. Acetic and citric acids at pH values ranging between 3.5 to 5.0 were evaluated in the acid studies.

In most cases, *Vv*-GFP-K was comparable to *Vv*-WT with respect to growth, survival, thermal inactivation, and freeze thaw survival. There were differences between *Vv*-WT and *Vv*-GFP-K with respect to acid tolerance, although these differences disappeared with sodium pyruvate supplementation of media. In broth studies dealing with organic acids, *Vv*-GFP-K was rapidly inactivated with acetic acid. Similar, but not as dramatic results were seen for citric acid. As pH values declined, the positive impact of pyruvate supplementation on cell recovery disappeared.

In the refrigeration studies done in the matrix, there were no apparent differences in *Vv*-GFP-K levels for all three treatments within the first few days of storage. In all cases, levels dropped 1 $\log_{10}$ after 8 days refrigerated storage. By the end of the study (21 d), *Vv*-GFP-K levels were nondetectable for both iced and conventionally cooled product, however mild abusively cooled oysters still had levels approximating 10^3 CFU/oyster. *Vv*-GFP-K levels remained stable for up to 24 hrs within the oyster meat under acidic conditions at various pH values. The oyster meat provided a protective environment that prevented inactivation of *Vv*-GFP-K.

Similarities between *Vv*-GFP-K and *Vv*-Wt with respect to growth and survival suggest that it may be an appropriate surrogate for evaluating processing stress tolerance. Sodium pyruvate supplementation of media may aid in the recovery of *V. vulnificus* cells sublethally injured by exposure to food processing-related stresses, although the efficacy of pyruvate supplementation was highly dependent upon the specific stress. Cooling

alone cannot be relied upon to eliminate *V. vulnificus*. Furthermore, specific cooling methods or organic acids appear to make little difference in the survival of *V. vulnificus* during extended refrigerated storage of whole (shellstock or shucked) oysters.

CHARACTERIZATION OF THE RESPONSE OF *VIBRIO VULNIFICUS* TO SUBLETHAL STRESSES DURING OYSTER HANDLING AND PROCESSING

By

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BIOGRAPHY

Stephenie Lynn Drake was born February 6, 1979 in Sunnyside, Washington. She grew up in the city of Wenatchee in the foothills of the Cascade Mountains. She graduated from Wenatchee High School in 1998, where she was very active in FFA and 4-H. She then moved to Starkville, Mississippi to escape shoveling snow and to start her undergraduate college education at Mississippi State University. While at MSU, she was very active in the Food Science club especially as president for 3 years. She completed an underdergraduate research project under the direction of Dr. Marshall. She received second place in the undergraduate research competition at the Institute of Food Technologists/ Meeting in June, 2002. She obtained her Bachelor of Science degree in Food Science in 2002.

The confidence gained during her undergraduate research project was invaluable when it came time for her to begin her own graduate research under the direction of Dr. Jaykus. She received second place for her oral presentation in the Food Microbiology Division at the Institute of Food Technologists' Metting in July, 2003.

After graduating, Stephenie will pursue her doctoral degree here at North Carolina State.

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CHAPTER 1

Literature Review

Introduction

In the U.S., contaminated seafood is responsible for 26.5% of all foodborne disease outbreaks (Mead et al., 1999). The majority of these seafood-related human illnesses are associated with the consumption of raw bivalve molluscan shellfish such as oysters, clams, and mussels (Cook, 1991). Bivalves are filter feeding organisms that pump seawater through their digestive systems to obtain oxygen and food. In this process they accumulate and concentrate microorganisms in their digestive tracts. These include harmless environmental organisms as well as pathogens, the most significant of which are the human enteric viruses and the pathogenic *Vibrio* species. Since shellfish are frequently consumed whole and raw, they can serve as passive carriers of foodborne disease agents.

Classification

The bacterial genus *Vibrio* is in the family *Vibrionaceae*, which also includes the genera *Aeromonas*, *Plesiomonas*, and *Photobacterium* (Atlas, 1997). All Vibrios are environmentally ubiquitous in the marine environment. *V. cholerae* O1 and *V. mimicus* are the only *Vibrio* species that do not grow in media with added sodium chloride. There are 30 species in the genus *Vibrio.* Thirteen of these species are known to be pathogenic to human and fish, including *V. cholerae* O1, *V. cholerae* non-O1, *V. mimicus*, *V. fluvialis*, *V. parahaemolyticus*, *V. alginolyticus*, *V. cincinnatiensis*, *V. hollisae*, *V. vulnificus*, *V. furnissii*, *V. damsela*, *V. metshnikovii*, and *V. carchariae*. All pathogenic

Vibrios have been reported to cause foodborne disease; however, *V. cholerae* O1, *V. parahaemolyticus,* and *V. vulnifiucus* are the most significant agents. Members of the *Vibrio* genus are straight or curved Gram negative, non-spore forming rods, 0.5 to 0.8 μm in width and 1.4 to 2.6 μm in length (McLaughlin, 1995). However, when they are grown in the laboratory, they frequently revert to a straight rod morphology (Atlas, 1997). 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 (McLaughlin, 1995). *V. vulnificus* is similar phenotypically to *V. parahaemolyticus* (Oliver, 1989). The two most distinctive reactions of *V. vulnificus* are fermentation of lactose and production of B-D-galactosidase and these tests can be used to distinguish it from the related *Vibrio parahaemolyticus* (Hollis et al., 1976).

Habitat

Vibrio species are environmentally ubiquitous to estuarine waters and can frequently be isolated in high numbers from bivalves, sediment, and plankton. *V. vulnificus* can be isolated directly from seawater and a variety of other marine foods as well (Kelly, 1982: Oliver et al., 1983; Tamplin et al., 1982; Oliver et al., 1983; Oliver, 1989; O'Neil et al., 1992; Ruple and Cook, 1992; Tamplin and Capers, 1992; Cook, 1994; DePaola et al., 1994; Wright et al. 1996). The numbers of *V. vulnificus* in Gulf Coast oysters peak during the summer followed by a gradual reduction in the winter (Motes et al., 1998). Higher densities of *V. vulnificus* are found in oyster digestive tissue (DePaola et al., 1997) as compared to muscle tissue.

Although *V. vulnificus* levels are higher in the estuarine environment during the warm summer months as compared to the winter, the organism remains persistent throughout the year. There are several theories to explain this apparent persistence. Some

have found that *V. vulnificus* survives in plankton and the organism has in fact been found in plankton, suggesting that this bacterium inhabits similar niches to *V. cholera* and *V. parahaemolyticus* (Wright et al, 1996 and Vanoy et al.,1992). Others believe that *V. vulnificus* lives in sediment. For instance, Vanoy et al. (1992) found *V. vulnificus* during the months of January to March in marine sediment, suggesting winter survival in the floc zone at the sediment interface. It may be possible that *V. vulnificus* survives in the sediment interface during the winter and when conditions are more conducive for growth (summer months), *V. vulnificus* will go on to colonize plankton.

Distribution

V. vulnificus has been isolated from waters as far north as the Great Bay of Maine (O'Neil et al., 1992). Amongst the U.S. areas where *V. vulnificus* has been naturally isolated, the majority are found in Mid-Atlantic, Cheaspeake Bay, and Gulf of Mexico waters, while some strains are found in Pacific, Canadian, and North Atlantic waters. Levels of *V. vulnificus* are also similar in these earlier three sites. Virulent strains of *V. vulnificus* have been found on the West Coast although not as frequently or at as high levels as from Gulf and Atlantic Coast waters. Furthermore, *V. vulnificus* has not been isolated as frequently from shellfish harvested from West Coast sites as compared to East Coast locations (Kaysner et al., 1987). Clams harvested from the northeastern U.S. coast and all U.S. west coast waters had the lowest levels of *V. vulnificus* (Brenton et al., 2001), perhaps due to the lower mean temperatures of these waters. In a survey conducted by Cook et al. (2002), levels of *V. vulnificus* were below the detection limit of 0.2 MPN/g oyster for shellfish harvested from North Atlantic, Pacific, and Canadian coastal waters. However, *V. vulnificus* and *V. parahaemolyticus* densities exceeded 10,000 MPN/g in

shellfish samples collected from Mid-Atlantic waters. *V. vulnificus* accounted for 8-10% of the total aerobic bacteria isolated from Chesapeake Bay oysters, suggesting that levels of *V. vulnificus* in Chesapeake waters may be comparable to those in Gulf of Mexico waters (Wright et al., 1996).

Pathogenicity

There are virulent and avirulent strains of *V. vulnificus*. Many virulence factors have been reported for *V. vulnificus* and include (i) the presence of a polysaccharide capsule; (ii) various extracellular enzymes; (iii) the ability to obtain iron from transferrin; and (iv) the absence of estrogen in the host (Linkous and Oliver, 1999).

The presence of a capsule, which is also related to colony opacity, is probably the best known virulence factor. The transformation of encapsulated isolates to the non-encapsulated form is dependent on growth phase and other variables in the environment, which in turn affect bacterial cell morphology. Encapsulated isolates have an opaque colony morphology but can undergo a reversible phase variation to a translucent colony phenotype that is correlated with reduced capsular polysaccharide (CPS). Translucent strains are less virulent than opaque strains. For instance, Moreno and Landgraf (1998) found that translucent colonies of *V. vulnificus* were avirulent in mice and Wright et al. (1990) reported that nonencapulated strains produced by transposon mutagenesis had a lethal dose over four times higher than that of the encapsulated strains (Wright et al., 1990).

There is evidence that several extracellular enzymes play a role in *V. vulnificus* pathogenicity. Moreno and Landgraf (1998) found that the enzymes lecithinase, lipase, caesinolytic protease and DNase were present in >90% *V. vulnificus* strains. The

protease of *V. vulnificus* may be of extreme importance to the pathogenicity of this bacterium (Linkous and Oliver 1999). A metalloprotease containing a zinc atom, this enzyme degrades a number of biologically important proteins including elastin, fibrinogen, and plasma protease inhibitors (Miyoshi et al., 1995). The most dramatic pathological action of the metalloprotease is its vascular permeability-enhancing action (Shinoda and Miyoshi, 2000). *V. vulnificus* also produces both hydroxymate and phenolate (catechol) siderophores which have been shown through production of isogenic mutants to be associated with enhanced virulence (Strom and Paranjpye, 2000).

Extracellular proteins produced by *V. vulnificus* are also important in the organism's ability to survive in the estuarine environment and perhaps cause disease in infected hosts. For example, *V. vulnificus* exports a chitinase that may be used by the bacterium to colonize and adhere to the chitin exoskeletons of zooplankton. The production of the metalloprotease and a hemolysin may allow the organism to colonize and multiply in molluscan shellfish by breaking down tissue at the site of colonization, promoting release of necessary nutrients (Strom and Paranjpye, 2000).

The amount of iron available in the host is an important factor influencing the lethality of both virulent and avirulent strains of *V. vulnificus*. Stelma et al. (1992) found that an iron overloaded mouse was killed at doses of <100 CFU for a virulent strain of *V. vulnificus* and at a dose >4000 CFU for an avirulent strain. On the other hand, Reyes et al. (1987) showed a lethal oral infective dose of *V. vulnificus* to be $<10^5$ CFU/ml for virulent strains and $>10^9$ CFU/ml for avirulent strains when mice were not iron overloaded, although route of transmission was an important mitigating factor. The increased saturation of transferrin, either through an excess of iron or a relative decrease

in the amount of transferrin, may be associated with the pathogenesis of *V. vulnificus* infection (Morris et al., 1987 and Brennt et al., 1991). Transferrin is an iron transport protein, and because free iron is virtually absent in the human body, pathogenic bacteria like *V. vulnificus* may have evolved a mechanisms to scavenge iron from the iron transport proteins (Strom and Paranjpye, 2000).

Recent studies suggest that men are more susceptible to *V. vulnificus* infection than are women, and it appears that estrogen may be protective against infection. According to a study conducted by Shapiro et al. (1998), 86% of the reported cases of *V. vulnificus* infection occurred in men. Although this may be due to the fact that men are more likely to consume raw oysters, or that men are more likely to have underlying liver disease, a recent study by Linkous and Oliver (1999) offers an alternative explanation. This investigation showed that male rats injected with *V. vulnificus* LPS had an 82% fatality rate, whereas, normal female rats treated identically had a fatality rate of only 21%. When these female rats that were ovariectomized, which lowered their estrogen levels, fatality rates increased to 75%. Estrogen may protect women by reducing iron availability. Women have a tendency to be anemic, which reduces overall iron availability in this gender.

Epidemiology

General

There are three major clinical manifestations of *V. vulnificus* infection: wound infection, primary septicemia, and gastroenteritis. The risk factors for *V. vulnificus* infection include pre-existing health conditions, seasonality, and seafood consumption (Hlady and Klontz, 1996). Liver disease is a strong predictor for fatal outcomes of *V.*

vulnificus infection, with 80% of those who die from the infection having underlying liver dysfunction (Strom and Paranjpye, 2000).

In one of the first of its kind, Desenclos et al. (1991) used the case control study design to show an estimated annual incidence of *Vibrio* infectious at 95.4 per million for raw oyster consumers with liver disease, 9.2 per million for raw oyster consumers without liver disease, and 2.2 per million for those who do not consume raw oysters. Another case control study conducted by Hlady and Klontz (1996) reported disease manifestation proportions of 51%, 24%, and 17% for gastroenteritis, wound infection, and septicemia, respectively. Fatality rates were only 1% for gastroenteritis, 5% for wound infection, and 44% for septic disease. Sixty-eight percent of gastroenteritis and 83% of primary septicemia cases were associated with raw oyster consumption. Ninety-one percent 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 infection

The majority of *V. vulnificus* wound infections are associated with fisherman and seafood handlers. Wound infections are defined as those cases where a patient incurred a wound before or during exposure to seawater or seafood drippings and from which *V. vulnificus* was subsequently cultured from that wound, blood, or an otherwise normally sterile site (Strom and Paranjpye, 2000). In a study conducted by Strom and Paranjpye (2000), 69% of wound infections appeared to be related to occupational exposures among oyster shuckers and commercial fisherman. In the case control study of Hlady and

Klontz (1996), it was reported that other *Vibrio* species can also be responsible for wound infections.

Primary septicemia

Primary septicemia is defined as a systemic illness characterized by fever and shock and for which *V. vulnificus* is isolated from blood or an otherwise sterile sites (Strom and Paranjpye, 2000). The consumption of contaminated raw oysters is the cause of this disease in almost all cases. Desenclos et al. (1991) showed that raw oyster consumers were 10 times more likely to develop primary septicemia than non-consumers. Hlady and Klontz (1996) showed that *V. vulnificus, V. cholera* non O1, and *V. parahaemolyticus* can all cause septic disease. Liver disease due to cirrhosis, alcoholism, or hepatitis is the most important risk factor for fatality from *V. vulnificus* infections, especially primary septicemia (Shapiro et al., 1998).

Gastroenteritis

When *V. vulnificus* is isolated from stool alone, it is characterized as causing gastroenteritis (Strom and Paranjpye, 2000). Gastroenteritis caused by *V. vulnificus* may go unreported since it is not life threatening and symptoms are rarely severe enough to warrant medical attention. In a study conducted by Hlady and Klontz (1996), *V. parahaemolyticus*, *V. cholera*, and *V. hollisae*, *V. miniass*, and *V. fluvialis* as well as *V. vulnificus* could all cause the gastroenteritis syndrome. Koo et al. (2000b) found that *V. vulnificus* was resistant to bile, meaning that it may be capable of proliferating in the small intestines.

Enumeration and Detection Methods

There are several different methods recommended for the detection and/or enumeration of *V. vulnificus*. The FDA Bacteriological Analytical Manual (1998) cites the standard procedures for recovery of *V. vulnificus* (Figure 1). In this method, phosphate buffered saline (PBS) dilutions are followed by alkaline peptone water (APW) enrichments in a MPN format. PBS enrichment broth can be used in parallel, and in both cases, enrichments are incubated at 35-37^{0}C for 16-18 hrs. MPN tubes having turbidity are streaked onto modified Cellobiose Polymysin B Colstrin (mCPC), with the PBS enrichment broth also streaked onto mCPC agar plates as a control. The mCPC plates streaked from the PBS enrichment broth serves as a control in case APW tubes lack turbidity. Modified CPC plates are incubated for 18-24hr at 39-40^{0}C after which they are examined for typical *V. vulnificus* colonies which are flat and yellow with opaque centers and translucent peripheries, about 2mm in diameter. These presumptive colonies are then transferred to APW for the monoclonal antibody-Enzyme Immunoassay (EIA) test for rapid identification of *V. vulnificus*. For biochemical identification, three or more typical colonies are selected from mCPC, streaked onto 1% trypton, 1% NaCl (T_1N_1) agar, and also inoculated into gelatin agar (GA) and gelatin salt (GS) agar, followed by incubation for 18-24 hr at 35-37^{0}C. Other preliminary biochemical tests for *V. vulnificus* include oxidase, motility, arginine-glucose slant (AGS), triple sugar iron (TSI) agar, O/129 Vibriostat sensitivity, and the ONPG test. The different reactions for *V. vulnificus* and *V. parahaemolyticus* to the preliminary biochemical tests are outlined in Table 1.

Even though the FDA-BAM method is the "gold standard," there has been considerable debate about the best method to detect wild-type *V. vulnificus* in shellfish.

For instance, Azanza et al. (1996) found that peptone (0.1%) solution containing 3% NaCl was a better diluent than was phosphate buffered saline. Hagen et al. (1994) found that alkaline peptone water (APW) was better at recovering *V. parahaemolyticus* and *V. vulnificus* than was salt-polymyxin B broth (SPB). Alam et al. (2001) suggested using nutrient agar with 2% NaCl as the primary culture medium followed by transfer to a selective medium such as Thiosulfate-citrate-bile salt sucrose (TCBS), since this two-step method recovered significantly higher numbers of *V. parahaemolyticus* and other Vibrios from seawater samples. Hoi et al. (1998) found that pre-enrichments followed by selective plating on cellobiose-colistin (CC) agar were more effective in increasing and enumerating *V. vulnificus* than were cellobiose-polymyxin B-colistin (CPC), modified cellobiose-polymyxin B-colistin (mCPC), and thiosulfate-citrate-bile salt sucrose (TCBS) agars. However, TCBS was the only agar for which these recovery differences were significant. It was observed in this research that levels of colistin and polymyxin B retard the growth of *V. vulnificus*. TCBS agar was generally a good medium for cultivating *V. vulnificus*; however, it was not good at differentiating *V. vulnificus* from other sucrose negative *Vibrios* and CPC agar outperformed in this respect (Oliver et al., 1992).

It has proven difficult to discriminate *V. vulnificus* from the natural background microflora in oysters. Micelli et al. (1993) developed and evaluated an alternative method for direct isolution of *V. vulnificus* from oyster homogenates, using the so-called *V. vulnificus* enumeration (VVE) medium. These investigators were able to detect as few as 10 culturable cells in a 100g sample using this method, with only a 3.2% false positive rate and a 0.4% false negative rate. The procedure produced higher counts than did the APW and MPN methods, and could be completed within 2 to 4 days rather than the

traditional 5 to 7 days. Alam et al. (2001) found that replicate plating produced better cell recovery and higher counts than did the MPN method.

It is known that low temperature induces membrane damage that tends to make cells more sensitive to salt, in which case salt levels generally found in marine and estuarine media can be lethal to stressed cells. Both *V. vulnificus* and *V. parahaemolyticus* require a minimum of 0.5% NaCl, but large amounts of salt can negatively affect the recovery of these organisms. Oliver (1981) found that TCBS, estuarine salts (ES), and brain heart infusion (BHI) agars all produced comparable results for *V. vulnificus* and *V. parahaemolyticus* as plating media on survival assays, especially after cold shock. However, the addition of 3% salt to BHI greatly affected cell viability, with *V. vulnificus* killed after 30 min and *V. parahaemolyticus* after 24 hours.

The development of DNA based methods for the detection of *V. vulnificus* has aided in both the identification and discrimination of *Vibrio* species from one another. Wright et al. (1993) developed an oligonucleotide DNA probe (VVAP) to identify *V. vulnificus*, which could effectively differentiate *V. vulnificus* from other *Vibrio* species. Gooch et al. (2001) found that direct plating colony lifts followed by hybridization using probes specific for *V. vulnificus* and *V. parahaemolyticus* produced results similar to MPN methods. In their study, the differences between direct plating followed by colony lift hybridization and MPN methods occurred only at lower densities which may imply different detection sensitivities; MPN methods were more sensitive (3 MPN/g for a 0.1g) than direct plating methods (10 CFU/g for 0.1g sample). The hydrophobic grid membrane filtration (HGMF) technique has been used to enumerate and differentiate between *V. parahaemolyticus* and *V. vulnificus* within 4 days. In this method, filters are

placed on tryptic soy magnesium sulfate-sodium chloride (TSAMS) agar for 3 hours at 35^0C and then transferred to *Vibrio parahaemolyticus* sucrose agar (VPS) and incubated at 42^0C overnight. Blue to blue-green colonies are counted and differentiation is done using a TDH-specific gene probe for *V. parahaemolyticus* and a gene probe specific for the cytotoxin-hemolysin gene of *V. vulnificus* (Kaysner, 1994).

PCR has also been used to identify *V. vulnificus*. Brauns et al. (1991) detected both culturable and non-culturable *V. vulnificus* by PCR amplification using primers flanking a 340-bp fragment of the cytotoxin-hemolysin gene. As little as 72 pg of DNA from culturable cells and 31 ng of DNA from nonculturable cells could be detected. Brasher et al. (1998) designed a multiplex PCR assay to simultaneously detect several foodborne pathogens, including *V. vulnificus, V. parahaemolyticus,* and *V. cholerae.* When applied to artificially inoculated oyster homogenate, they were able to detect $<10^1$-10^2 cells/g. Amplification targets were *invA*, *ctx*, and *tl* genes for *Vibrio vulnificus*, *V. cholerae*, and *V. parahaemolyticus,* respectively. In addition, Wang et al. (1997) developed a different PCR method to detect 13 species of foodborne pathogens, including *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. Detection limits per PCR reactions for *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are 40, 4, and 100 cells, respectively, and no interference was observed using the PCR assay when food samples were artificially inoculated with other competing bacterial species.

Environmental Factors

Temperature

Temperature plays an important role in the proliferation of *V. vulnificus*. Growth of *V. vulnificus* is favored by relatively high temperatures and the organism has an

optimum growth temperature of 37^0C (Kelly, 1982). *V. vulnificus* was rarely isolated from surface water samples collected from the Gulf of Mexico in January through March, when water temperatures were below 20^0C. Peak recovery occurred in September indicating seasonal variation in organism prevalence (Kelly, 1982). The inability to culture *V. vulnificus* from cold waters is probably due to the entry of cells into a "viable but not culturable (VBNC)" state (Wolf and Oliver, 1992; Oliver, 1993; 1995; Oliver et al., 1995). In a study conducted by Kaspar and Tamplin (1993), *V. vulnificus* grew quite well in the temperature range of 13^0C to 22^0C, but did not grow well below 8.5^0C. Wright et al. (1996) were able to retrieve *V. vulnificus* from estuarine waters of the Chesapeake Bay at temperatures as low as 8^0C, however, *V. vulnificus* could not be recovered at temperatures lower than 12.5^0C from the Gulf of Mexico waters (Simonson and Siebeling, 1986).

Salinity

Salinity also seems to play an important role on the prevalence of *V. vulnificus*. Tamplin et al. (1982) found that *V. vulnificus* could not be isolated during winter months but could be isolated at high densities during summer months. These researchers concluded that both temperature and salinity play important and interrelated roles, as *V. vulnificus* was not isolated until temperatures of 17^0C and salt content of 17 ppt. were reached, and higher levels of *V. vulnificus* were found when the water temperature exceeded $>29^0$C and salt content was greater than 23 ppt. Further research by Kaspar and Tamplin (1993) demonstrated that at salinities between 5 and 25 ppt., *V. vulnificus* levels increased; however, when salinities were 30, 35, and 38 ppt, *V. vulnificus* levels decreased by 58, 88, and 83%, respectively. The same trend was reported by Motes et al.

(1998) who observed lower numbers of *V. vulnificus* cells at salinities above 28 ppt, which is typical of Atlantic coastal sites. This high salinity may explain in part why *V. vulnificus* could not be routinely isolated from oysters harvested from waters off of North or South Carolina. High *V. vulnificus* levels, however, were found in oysters harvested from intermediate salinities between 5 and 25 ppt (Motes et al., 1998).

pH

There are two strategies for bacteria to survive low pH conditions. One mechanism is the *rpoS* gene that encodes for the alternative sigma factor. A sigma factor is a subunit of bacterial RNA. The sigma factor attaches to the bacterial RNA polymerase which in turn attaches to a DNA promoter, signaling the transcription of a particular gene (Lewin, 1997). The RpoS alternative sigma factor regulates at least 30 proteins, including the expression of genes that control thermotolerrance, starvation survival, osmotic stress response, and acid response (Jay, 2000).

The acid tolerance response is another mechanism for survival of *V. vulnificus*. In many instances, when bacteria are exposed to a pH <5.9, the acid tolerance response is induced and this enables cells to survive at pH values as low as 3.3 (Waterman and Small, 1996). It has been suggested that this response may also reduce the infectious dose of certain bacteria (Waldor and Mekalanos, 1996), theoretically because the cells may be "conditioned" to survive at low pH, promoting their survival through the GI tract.

There are a wide variety of resilient and adaptable strains of bacteria even in the family *Vibrionaceae*. Wong et al. (1998) found in a broth model that when a pH of 7.5 was shifted down to 5.0, *V. parahaemolyticus* was more acid tolerant. It seems that *Aeromonas hydrophila* exhibits an adaptive acid tolerance response capable of protecting

cells from a pH as low as 3.5. Just like *V. vulnificus,* when *A. hydrophila* in broth was shifted from pH 7.2 to 5.0 then to 3.5, the cells were able to survive in culture (Karem et al., 1994). This study showed that proteins synthesized during adaptation to pH 5.0 were involved in the subsequent survival during exposure to pH 3.5. Koo et al. (2000) reported strain to strain differences in acid tolerance, although at pH 2.0 there was no variability in reduction between strains suggesting that after a certain pH all strains were killed. It appears that low temperature offers protection against the anitibacterial effects of both pH and salinity. Hijarrubia et al. (1996) observed a more rapid inactivation of *V. vulnificus* cells kept at low pH and salinity at 20^0C as compared to storage under the same conditions at 5^0C.

Cold Stress

Some organisms such as *V. vulnificus* are able to adapt and endure at very low temperatures, when previously conditioned by exposure to less cold temperature. *V. vulnificus* is more susceptible to low temperatures when in its logarithmic growth phase as compared to its stationary phase (Oliver et al., 1991). Kim and Dunn (1997) found that lactic acid bacteria freeze-thaw survivability improved when cells were cold shocked at 10^0C for 2 hrs prior to freezing at -20^0C, and that a gene homologous to the major cold shock protein of *E. coli* and *B. subtilus* appeared responsible. Bryan et al. (1999) suggested that cold adaptive "protective" proteins in *V. vulnificus* may enhance survival and tolerance to cold temperatures and that iron plays a role in adaptation at cold temperature, since the removal of iron from the growth medium prior to cold adaptation reduced viability by 2 logs. However, when comparing Western blots between *E. coli* and *V. vulnificus, V. vulnificus* does not show a major cold shock protein homologous to

that of *E. coli.* Nonetheless, even though overall protein synthesis for *V. vulnificus* showed a sharp decline, forty unique proteins were synthesized at higher levels during the cold stress response (McGovern and Oliver, 1995). When *V. vulnificus* cells are cold shocked, their numbers initially decline but after a brief lag, cell growth resumes at a slower rate.

V. vulnificus's entry into the VBNC state can be slowed by exposure to a slightly colder temperature prior to the final cold temperature, this slower entry into the VBNC state may be attributed to an adaptative response. In a study conducted by Bryan et al. (1999), *V. vulnificus* entered the so-called VBNC state when the temperature shifted from 35^0C to 6^0C; however, when the culture was subjected to 15^0C prior to further temperature downshift, the cells remained culturable, showing an adaptive response to cold temperature. The culture was also able to survive better during freezing at -78^0C when freezing was preceded by cold shock. In a study conducted by Bang and Drake (2002), cold stress had no effect on *V. vulnificus* heat resistance or freeze/thaw resistance, but cold adaptation improved cold temperature tolerance.

Starvation

The removal of nutrients from the environment has been reported to affect the survival and culturability of *Vibro* species. Marden et al. (1985) observed the initial phases of starvation in marine bacteria and found an initial increase of activity followed by a decrease. For instance, cells that were starved of nitrogen or phosphorus showed an initial increased oxygen uptake, followed by a delayed and more pronounced decline in respiratory activity. These investigators hypothesized that starvation induces an initial energy dependent reorganization which involves both increased endogenous respiration

and decreased rate of recovery. This leads to a rapid loss of available poly-B-hydroybutyrate that in turn results in the formation of outer membrane vesicles that are related to the continuous size reduction that occurs during starvation survival.

In a study conducted by Nystrom et al. (1990) using *Vibrio* spp. strain S14, which resembles *Vibrio proteolyticus*, the investigators described long-term multiple nutrient starvation as a three-phase process. The first step was the accumulation of guanosine5'-diphosphate (ppGpp) and a decrease in RNA and protein synthesis. The second phase was characterized by a temporary increase in the rates of RNA and protein synthesis, lasting for 1-3 hr and paralleling a decrease in the ppGpp pool. Finally, a gradual decline in macromolecular synthesis occurred. A total of 66 proteins were identified as starvation inducible (Sti), and these were temporally expressed throughout the three phases of starvation. Chronologically, the early class of starvation proteins was the most essential for long-term survival. When strain S14 was prestarved (1 hr.) for glucose, amino acids, ammonium, or phosphate and then briefly (1hr.) exposed to $CdCl_2$, the strain showed an enhanced survival during a subsequent multiple-nutrient starvation. Exposure to $CdCl_2$ during single nutrient starvation allowed cross protection to occur in the cell during multiple starvation. In general, the total number of proteins induced by single nutrient starvation less than that of a multiple starvation.

In comparing single and multiple nutrient starvation there are apparently different proteins that are produced during each starvation. Nystrom et al. (1990b) found that while most proteins induced during single nutrient starvation conditions were also induced during multiple nutrient starvation, four of the five phosphorus starvation specific proteins were not significantly induced during multiple nutrient starvation. In

addition, 14 polypeptides were found to be exclusively expressed during multiple nutrient starvation, which indicates that other signals may be involved when cells are starved of several different nutrients at the same time.

VBNC

The term viable but nonculturable (VBNC) was introduced in 1985 to describe bacterial cells that do not form colonies on high nutrient solid media, but are considered alive because metabolic activity can still be detected (Oliver, 2000; Gauthier, 2000). The VBNC state can be contrasted to cell injury in that injured cells lose their ability to grow on selective media, but can still be cultured on nutrient-rich media; VBNC cells cannot be cultured at all. The VBNC state is a mechanism for bacteria to survive adverse conditions. There appear to be several environmental factors that induce the VBNC state, including cold temperature, starvation, and suboptimal pH (Gauthier, 2000).

There are many changes observed in bacteria upon entry into the VBNC state. Morphologically, *V. vulnificus* cells in the VBNC state are small cocci (0.3μm), whereas after resuscitation the cells become rod shaped (3um length and 0.7 width) (Linder and Oliver, 1989, and Nisson et al., 1991). In another study, Holmquist and Kjelleberg (1993) found that carbon and multiple-nutrient starvation resulted in small cells, whereas all starved for nitrogen and phosphorous became filamentous or swollen large rods. VBNC cells also clump, suggesting the production of exopolysaccharides resulting in an outer membrane that is "blebbed" (Johnston and Brown, 2002). Membrane blebbing is a modification to the outer membrane that is frequently associated with bacterial resistance mechanisms (Jones et al., 1989). Light microscopy shows that *V. vulnificus* cells converted from the coccoid shape to normal rod shape when the cells were culturable

again. Oliver and Colwell (1973) summarized that as temperature decreased, there was a switch to more unsaturated fatty acids in the cell membrane. Indeed, the fatty acid content of palmitic (C_{16}) and palmitoleic ($C_{16:1}$) was decreased by 57%, whereas, short chain fatty acid content increased from 5.4% to 29.0% while cells were entering the VBNC state (Linder and Oliver, 1989).

It appears that starvation combined with cold stress play a role in the induction of the VBNC state. For instance, Linder and Oliver (1989) found that *V. vulnificus* become nonculturable after 23 days in artificial seawater (ASW) at 5^0C, while Nilsson et al. (1991) found *V. vulnificus* became nonculturable after 27 days at 5^0C in nutrient limited ASW. Regardless, *V. vulnificus* was able to be revived from the VBNC state within 3 days after a temperature shift to 21^0C. Nilsson found that chloramphenicol and ampicillin inhibited VBNC cell resuscitation for 24 hrs., however after 72 hrs. the effects were minimal, suggesting that active protein and peptidoglycan synthesis are ongoing during all stages of resuscitation.. According to Oliver et al. (1991), *V. vulnificus* prestarved for 24 hr. at room temperature before exposure to 5^0C did not enter the VBNC state, whereas cells starved for the same amount of time while at 5^0C entered the VBNC state. When cells were starved for only 1, 2, and 4 hrs before exposure to 5^0C, the cells entered the VBNC state but at a slower rate. Consistent with this observation, *V. vulnificus* inoculated into natural estuarine waters during the winter months has been observed to enter the VBNC state, and VBNC cells of *V. vulnificus* inoculated into natural estuarine waters during summer months rapidly become culturable (Oliver et al., 1995). Whiteside and Oliver (1997) found that *V. vulnificus* in the VBNC state was not able to be resuscitated using nutrient rich broth such as Brain Heart Infusion (BHI), but

could be resuscitated in artificial seawater (ASW) with an increase in temperature to 22^0C. Yet, VBNC cells incubated in BHI for 12 hrs., followed by incubation in ASW for 12 hrs, could be readily resuscitated. The resuscitation of cells from the VBNC state appear not to be possible when cells are not pre-exposed to nutrients. Oliver and Wanucha (1989) showed that *V. vulnificus* cells readily took up and metabolized amino acids at 13^0C, with cells held at 2^0C and 9^0C showing a reduced rate of uptake. This study suggesting that *V. vulnificus* survives at reduced temperatures, however with reduced rates of activity.

The relationship between VBNC state and virulence is of great interest. In a study done by Colwell et al. (1985), VBNC cells of *V. cholerae,* at a concentration of 5×10^6 cells/ml by direct viable count, were fed to human volunteers and caused fecal shedding at levels of 2.9×10^3 cfu/g. Another study by Linkous and Oliver (1989) suggested that VBNC cells of *V. vulnificus* lost virulence in mice, however a low level of inoculum (5×10^4 cells) was used in these experiments. Most recently, Oliver and Bockian (1995) showed that injection of a population as low as < 0.1 cfu/ml of VBNC cells of *V. vulnificus* into mice resulted in lethality.

There are contrasting theories to explain why culturability can be restored after removal of the stress that induced the VBNC state. One camp believes that in essence, the VBNC state does not really exist, but instead some viable cells remain and when the sample is subjected again to a more favorable environment, those residual viable cells replicate and become detectable on microbiological media. The other camp believes that VBNC cells are indeed alive and can resuscitate and grow after restoration of optimal conditions.

Historically, media supplemented with sodium pyruvate or catalase improves the recovery of injured cells. It is believed that calatase is produced by most aerobic microorganisms for the degradation of toxic hydrogen peroxide. Standard recovery procedures are based on aerobic incubation of selective media, which requires the cell to function under a respiratory metabolism involving the reduction of oygen to water. This process dictates the need for detoxifying enzymes which degrade hydrogen peroxide (Calabrese et al., 1990). Unlike catalase, which most likely acts to degrade peroxides in and around the cell surface, pyruvate may be absorbed into the cell and degrade endogenous sources of H_2O_2 (Rayman et al., 1978). When bacteria are transferred from one environment to another, the process may challenge the adaptive resources of the bacteria. Because the bacteria do not have time to adapt, a resulting metabolic imbalance occurs causing rapid production of superoxide and free radicals which cells cannot detoxify, resulting in cell death (Bloomfield et al., 1998).

The beneficial effect of pyruvate was first described by Baird-Parker and Davenport (1965) who demostrated that pyruvate was responsible for optimal growth of stressed *Staphylococcus aureus* cells. Mizunoe et al. (1999) found that nonculturable *E. coli* O157:H7 when plated on media supplemented with 0.1% of catalase, pyruvate, α-ketoglutaric acid or 3,3'-thidipropionic acid; cells regained culturability on all plates. Futhermore, Calabrese and Bissonnette (1990) showed that *E. coli* exhibited significant reduction in catalase activity following exposure to acid mine water, but the exogenous addition of catalase (500-2000U) or sodium pyruvate (0.05-5%) to a nonselective recovery medium resulted in enhanced detection (10 to >465 fold).

Bogosian et al. (2000) were the first to use catalase or pyruvate supplemented media with *V. vulnificus.* These investigators forced *V. vulnificus* cells into the VBNC state by starving them in sterile seawater stored at 5^{o}C. While they noted a logarithmic decline in cell numbers over time when plating on nutrient-rich medium. Higher culturable cell counts, were observed using plates containing catalase or sodium pyruvate. They proposed that at least a proportion of VBNC cells may be hydrogen peroxide-sensitive culturable cells, and that the use of sodium pyruvate in the nutrient rich media eliminated hydrogen peroxides produced during autoclaving. Bogosian et al. (2000) also showed that warming samples of hydrogen peroxide-sensitive cells to room temperature led to the growth of these residual culturable cells, using nutrients provided by the nonculturable cells. However, when the hydrogen peroxide-sensitive culturable cells had declined to undetectable levels and only nonculturable cells were present, warming had no effect. Evaluation of different concentrations of sodium pyruvate (20, 40, 80, 160, and 320mg) showed no higher cell recovery than with 40mg of sodium pyruvate (Bogosian et al., 2000). Throughout these studies different levels of catalase and sodium pyruvate have been used; however, the optimal level of pyruvate may vary based on the type of injury to which the microorganisms have been exposed (Lee and Hartman, 1989). The use of hydrogen peroxide degrading compounds may help to bridge the gap between the contrasting theories about the nature of the VBNC state.

Techniques to eliminate *V. vulnificus* from oysters

Currently, shellfish harvesting waters are classified using the fecal coliform index. Unfortunately, since *V. vulnificus* is ubiquitous to the marine environment, its levels do not correlate with the fecal coliform index and this classification is therefore irrelevant

for control of this organism (Tamplin et al., 1982). Hood et al. (1983) confirmed that fecal coliform levels do not correlate with the levels of *V. cholerae* and *V. parahaemolyticus* in fresh oysters, although total bacterial levels correlated with levels of total *Vibrio* spp. after 7 days. This correlation did not, however, hold up after extended (14 days) refrigerated storage of oysters. O'Neil et al. (1992) also found no correlation between fecal coliforms levels and *V. vulnificus* levels. Most recently, Ruple and Cook (1992) suggested a correlation between the fecal coliform level and *V. vulnificus* during the warm weather months (May-Sept) but not in the cooler months of the year.

Commercial Heat Shock

Currently, the commercial heat shock process is used at processing facilities to facilitate the shucking of shellstock oysters (Hesselman et al., 1999). The process involves removing bushel sacks of oysters from a primary cooler into a staging cooler, emptying these bushels into one-half bushel size wire baskets, and submerging each basket in a heat-shock tank containing approximately 850 liters of potable water at a temperature of 67^0C. Approximately 70 oysters are contained in a basket. Exposure time in the heat-shock tanks varies but is usually around 5 min, depending on oyster size and relative oyster condition. After heat shocking, the oysters are sprayed for 1 min with potable water to provide cooling prior to delivery to the shuckers. The shuckers open the oysters by hand and place the meats into pint-sized bowls for delivery to the packing room. Upon delivery to the packing room, the oyster meats are chilled in an ice slurry, washed in a skimmer, and packed into gallon-size containers. Hesselman et al. (1999) found that the commercial heat shock process reduces *V. vulnificus* levels from 1 to 4 logs in the finished product.

Early work by Hood et al. (1983, 1984) demonstrated conflicting data regarding the persistence of *Vibrio* species in shucked versus shellstock oysters. In general, these researchers found that processing reduced the overall microbial load in oysters, but the individual *Vibrio* species examined remained statistically the same throughout processing. Later work demonstrated that in artificially contaminated shellstock and shucked oysters, *V. vulnificus,* survived for 14 days at 2^0C and could be isolated from the drip exuded from the shellstock oyster (Kaysner et al., 1989). Research by Ruple and Cook (1992) showed that commercial heat shock processing of oysters does not reduce the levels of *V. vulnificus* per se, but immediate storage on ice did reduce the level of *V. vulnificus* by a 1 to 2 logs. Murphy and Oliver (1992) found that the levels of *V. vulnificus* in shellstock oysters stored at temperatures ranging from 0.5^0C to 22^0C declined, suggesting that temperature abuse may not be as important a factor in increasing the public health risk of *V. vulnificus* as previously believed. Overall, the commercial heat shock process cannot be relied upon to eliminate *V. vulnificus* from oysters.

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 (Groubert and Oliver, 1994). Disinfectants commonly used in depuration waters are chlorine, ozone, and ultraviolent light. According to the work of Groubert and Oliver (1994), oysters allowed to filter feed in waters artificially contaminated with *V. vulnificus* were able to eliminate all of the accumulated *V. vulnificus* within 48 hours of subsequent depuration; however, the level of naturally occurring *V. vulnificus* in these oysters was

not reduced by depuration. It may be that *V. vulnificus* becomes part of the normal microflora or that it attaches to the oyster gut tissue quite tenaciously. Eyles and Davey (1984) also confirmed that depuration did not reduce *V. parahaemolyticus* nor *V. cholera* levels in shellfish, concluding that depuration is not effective in eliminating naturally occurring *Vibrio* species in oysters. Tamplin and Capers (1992) found that recirculation through 60 W of UV light did not effectively decontaminate depuration waters held at elevated temperatures (>21^{0}C). It was hypothesized that *V. vulnificus* multipled in the oyster tissue rapidly at this elevated temperature, being excreted into the waters at higher rates than the bactericidal activity of UV light. At lower temperatures (15^{0}C), *V. vulnificus* levels in depuration waters were, however, effectively reduced by this same treatment (Tamplin and Capers, 1992).

Relaying is another controlled purification method that involves moving shellfish from a restricted harvesting area to an open area where natural cleansing can occur. Cook and Ellender (1986) found that the temperature of the relaying water and its microbiological quality had an impact on the length of time needed to hold oysters in relay locations. Optimal relaying time appears to be 16 to 17 days. Oysters that were physiologically stressed took longer to cleanse than did unstressed oysters, presumably due to slower metabolic activity. While pathogens such as *Salmonella* can be eliminated within 5 days by relaying (Cook and Ellender, 1986), Motes and DePaola (1996) demonstrated that relaying decreased *V. vulnificus* levels from 10^3 CFU/ml to <10 MPN/g within 17 to 49 days, a considerably lengthier time period. Again, normal relaying conditions cannot be relied upon to eliminate *V. vulnificus* from shellfish.

GRAS compounds

As a possible aid in controlling *V. vulnificus* contamination in shellfish, investigators have also examined FDA approved food preservatives. Several Generally Recognized as Safe (GRAS) compounds have been found to have antimicrobial properties, and some have been applied to control *Vibrio* contamination in shellfish. Ascorbic acid has been shown to have antibacterial effects on some bacteria such as *E. coli*, presumably due to the formation of diketones during autooxidation (Myrvik et al., 1954). The antimicrobial effect of diacetyl has been shown to be most effective on Gram-negative bacteria, with *Pseudomonas* species being the most sensitive (100ug/ml) (Jay, 1982). The greater sensitivity of Gram-negative bacteria to α-dicarbonyl inhibitors appears to be due to their capacity to inactivate amino acid binding proteins in the periplasm of the cell, especially the arginine binding proteins. Sun et al. (1994) found that diacetyl at a concentration of 0.05% was lethal to *V. vulnificus* strains occurring naturally in oysters, while in comparative experiments, lactic acid and BHA compounds did not appear to decrease the level of *V. vulnificus*. Interestingly, the antimicrobial activity of diacetyl did not have the same effect on the overall microflora in the oysters, suggesting that diacetyl has an enhanced effect on *V. vulnificus*. Diacetyl appears to affects the permeability of cell membranes and accumulates in the membrane lipid bilayer (Johnson and Steele, 2001).

There are also naturally occurring compounds in oysters that help reduce the levels of *V. vulnificus.* Oysters contain hemocytes which have been found to produce a toxic compound that can inactivate *V. vulnificus*. However, there is conflicting evidence to support this statement. For instance, Genthner et al. (1999) reported that oyster

hemocytes did not have a significant lethal effect on either opaque or translucent strains of *V. vulnificus*, although the opaque strains of *V. parahaemolyticus* were more resistant to the hemocytes than were the translucent strains. Unfortunately, most oysters (*C. virginica*) found in mid-Atlantic and Gulf Coast waters are heavily infected with *Perkinsus marinus*, an oyster pathogen responsible for severe oyster population losses throughout this region. *P. marinus* produces a serine protease which is capable of digesting oyster connective tissues by degrading extracellular matrix proteins. Tall et al. (1999) found oyster hemocytes treated with serine protease produced by *Perkinsus marinus* were not as effective at lowering the level of naturally occurring *V. vulnificus* compared to untreated oysters hemocytes. The presence of the protease secreting oyster pathogen *P. marinus* appears to suppress the natural ability of oyster hemocytes to eliminate *V. vulnificus.*

Ionizing Irradiation

Gamma irradiation can eliminate *V. vulnificus* from both shellstock and shucked oysters. Novak et al. (1966) found that a 0.2 Mrad dose of gamma radiation could be applied for pasteurization of oyster meat without causing changes in organoleptic quality. After this treatment, total bacterial counts decreased by 99%. After storage on ice for 7, 14, and 21 days, these oysters had better flavor, odor, and appearance compared to untreated oysters. In fact, irradiated oysters were found to be acceptable after 21 days of refrigerated storage whereas untreated oysters had spoiled by that time. Matches and Liston (1971) found that in most cases, *V. parahaemolyticus* was reduced 4-6 $\log_{10}$ using a pasteurizing irradiation dose of 30-40 Krad. *Vibrio* species have in fact been found to be among the most radiation sensitive bacteria; both *V. cholera* and *V. vulnificus* can be

eliminated with doses less than 0.1 kGy of ^{60}Co (Mallett et al., 1991). Interestingly, little additional work on irradiation control strategies has been completed in recent years.

Thermoradiation

The combination of heat and radiation is known as thermoradiation and this technique appears to have excellent potential to eliminate *V. vulnificus*. *V. vulnificus* cells are more sensitive to thermoradiation than to heating or radiation alone (Ama et al., 1994), having been eliminated from oysters using treatments of 40^0C plus 0.480 or 0.875 kGy. Low initial numbers of *V. vulnificus* cells (10^4 CFU/ml) in pH 7.0 buffer were more rapidly inactivated by thermoradiation (40^0C with 0.048 kGy) compared to higher cell numbers (10^7 cfu/ml). It is possible that higher cell numbers allow the cells to clump together which in turn protected the interior cells, resulting in the need for a higher dose in order to achieve an equivalent degree of inactivation.

Refrigeration

Refrigeration is an excellent method to control the multiplication of *V. vulnificus* in oysters. Cook (1994) observed that *V. vulnificus* did not multiply in oysters stored a $<13^0$C; however, significant growth did occur in oysters stored at 18^0C or above. Cook (1997) later observed that the largest increase in numbers of *V. vulnificus* in freshly harvested shellstock oysters occurred during the first 3.5 to 5 hours after harvest. The growth of *V. vulnificus* is rapid in unrefrigerated oysters and peaks after only 12 hours. Although refrigeration controls growth, Cook and Ruple (1992) observed it took 14 to 21 days of refrigerated storage to reduce *V. vulnificus* in shellfish to undetectable levels.

In 1993, the National Shellfish Sanitation Program stipulated the first refrigeration guidelines for shellfish, stating that shellstock not intended for wet storage

or depuration must be placed under temperature control within 20 hours of harvest (April-November) and within 36 hours for product harvested from December through March. Once placed under temperature control, shellstock must be iced or the storage area or conveyance otherwise continuously maintained at 7.2^0C or below until final sale to the consumer. In 1995, time controls were changed so that after harvesting, shellstock were required to be placed under temperature control within 12-14 hours depending on the average monthly maximum water temperature (Cook, 1997). Also in 1995, the Interstate Shellfish Sanitation Conference (ISSC) adopted an additional control plan for states that had been confirmed as the originating site of shellstock products associated with two or more *V. vulnificus* illnesses. In this case, if water temperature was between 18^0C and 23^0C, shellstock was required to be placed under temperature control within 14 hrs; if greater than 23^0C and less than 28 ^{0}C, the time limit was less than 12 hrs; and if the water temperature was greater than 28^0C, the time limit was less than 6 hrs (Associated Press, 1996). However, in more recently approved shellfish harvesting and handling guidelines, shellfish may remain unrefrigerated for as long as 10 hours after harvest, even when water temperatures exceed 27^0C (U.S. Department of Health and Human Services, 1999).

Freezing and Vacuum packaging

Cook and Ruple (1992) reported that freezing reduces the levels of *V. vulnificus* although it does not eliminate the organism, even after frozen storage for up to 12 weeks. Temperatures of -20^0C were more effective in killing *V. vulnificus* than were temperatures of 0^0C. At -80^0C, *V. vulnificus* and *V. parahaemolyticus* cell numbers dropped by 1 log during the freezing process and remained stable thereafter for 35 days

(Boutin et al., 1985). Dombroski et al. (1999) showed that freezing inactivated 95 to 99% of *V. vulnificus* in oyster homogenates irregardless of the type of freezing conditions (conventional freezing (-20^{o}C), cold blast freezing (-85^{o}C), and cryogenic freezing (liquid nitrogen). .Johnston et al. (2002) showed that the total cell numbers were the same for freshly cultured *V. vulnificus, V. cholerae,* and *V. parahaemolyticus* both before and after freezing (-20^{0}C), with thawing providing a maximum reduction of 1.5 logs. Similar results were obtained from VBNC cells. In a study conducted by Parker et al. (1994), the combination of vacuum packaging and freezing decreased *V. vulnificus* levels in oysters by 3 to 4 logs within 7 days post-freezing, and levels continued to drop throughout frozen storage up to day 70 although, complete elimination was never achieved. The combination of vacuum packaging and freezing controlled *V. vulnificus* levels more effectively than did freezing with conventional packaging (Parker et al., 1994).

Hydrostatic pressure

Most microorganisms are baroduric, meaning they can survive high pressures but normally grow best at atmospheric pressures. The use of high pressure has been a promising emerging technology to control pathogens in certain foods. When using pressure to inactivate microorganisms, treatment will depend on both the intensity of the pressure and the length of exposure (Hoover et al. 1989). It appears that high hydrostatic pressure causes a change in cell morphology (Hoover et al., 1989). For example, *Escherichia coli* exposed to a pressure to 400 atm. demonstrated an 10-100 μm increase in length compared to normal size cells (1-2 μm). Hoover et al. (1989) showed that bacterial cells are more sensitive to pressure when the pH of the suspending menstrum is acidic. In general, *Vibrio* spp. are exquisitely sensitive to high pressure. Styles et al.

(1991) demonstrated that *V. parahaemolyticus* is rapidly reduced to non-detectable levels at pressures higher than 1,700 atm. when suspendended in clam juice. Later research uses the MPa in place of the atm, the relationship between the two being 10:1 (atm:Mpa). Research shows that treatment with hydrostatic pressure of 250 MPa for 10 min at 25^0C provided a 5 log reduction of *V. vulnificus* in culture to undetectable levels, again in clam juice (Styles et al. 1991). However, cells of *V. vulnificus* in the viable but noncultural (VBNC) state appear to be more resistant to the lethal effects of high hydrostatic pressure (Berlin et al. 1999).

Heat Treatment

Heat is a very effective means to eliminate *V. vulnificus*. While not inactivated rapidly at 40^0C, *V. vulnificus* cells are rapidly and exponentially inactivated at 50^0C or higher (Ama et al., 1994). Cook and Ruple (1992) found that naturally occurring numbers of *V. vulnificus* could be reduced to non-detectable levels at a temperature of 50^0C for 10 min in oysters. Cultures of *V. vulnificus, V. cholera* and *V. parahaemolyticus* showed D-values 12 sec, 22.5 sec, and 1.75 min respectively at 55^0C, and all three organisms, were suspended in broth, were reduced by >7 $\log_{10}$ when treated at 70^0C for 2 min (Johnston & Brown, 2002). In broth, *V. parahaemolyticus* was found to be more resistant to heat inactivation at 47^0C when it was previously heat shocked at 42^0C for 30 min, yet unconditioned *V. parahaemolyticus* cells were readily inactivated at 47^0C (Wong et al., 2002). The combination of a low pH (5.5) and heating (50^0C) effectively inactivated *V. vulnificus* by 7 $\log_{10}$ in a broth model (Wong et al., 2002). *V. vulnificus* was more resistant to heating when suspended in oyster homogenates than in buffers, presumably due to the protective effects of this suspending matrix (Ama et al., 1994).

According to Kim et al. (1997), the *V. vulnificus* morphotype influences thermal-death times such that opaque strains have higher D and Z values than translucent strains, indicating that they are more resistant. The range of D-values for opaque colonies was 3.44-3.66 min and for translucent colonies was 3.18-3.38 min at 47^0C; the range of Z-values for opaque colonies was 2.45-2.51 ^{0}C while that for translucent colonies was 1.89-2.07^{0}C.

Summary

V. vulnificus infections, with fatality rates in septic patients approaching 60%, are associated with the consumption of raw seafood, particularly oysters, where this bacterium becomes concentrated during the natural process of filter feeding. The occurrence of this bacterium in oysters is a significant concern to both the shellfish industry and public health agencies. Yet, conflicting evidence exists regarding the efficacy of proposed processing strategies to control *V. vulnificus.* The methods of heat, ionizing radiation and the combination of these methods known as thermoradiation appears to work well to reduce levels or even eliminate *V. vulnificus* from shellfish; however, the organoleptic quality of the product may be negatively impacted by these processes. While refrigeration may prevent the growth of *V. vulnificus* in oysters, it does not eliminate this bacterium. Likewise, relaying and depuration do not eliminate *V. vulnificus* cells that have become part of the digestive tract of the oyster and these methods can be time consuming and expensive. Furthermore, there is conflicting evidence regarding the ability of *V. vulnificus* to withstand a variety of freeze inactivation strategies. Although there are many alternative processing controls that can be used to reduce *V. vulnificus* levels in shellfish, there are difficulties in evaluating the efficacy of

these processes due to the viable but non- culturable (VBNC) state. Furthermore, cross-protection provided by a previous exposure to processing stresses such as reduced pH or low temperatures, may result in *V. vulnificus* cells that are more resistant to these processing technologies.

The purpose of this study is to characterize the effects of starvation, cold stress, acid stress, and cold adaption on *V. vulnificus* in a model food system. Oysters have their own natural microflora of *Vibrio* species, so there is also a need to differeniate between natural microflora in artificially-contaminated shellfish, particularly if enumeration data is sought.

Figure 1.1: Schematic of oyster

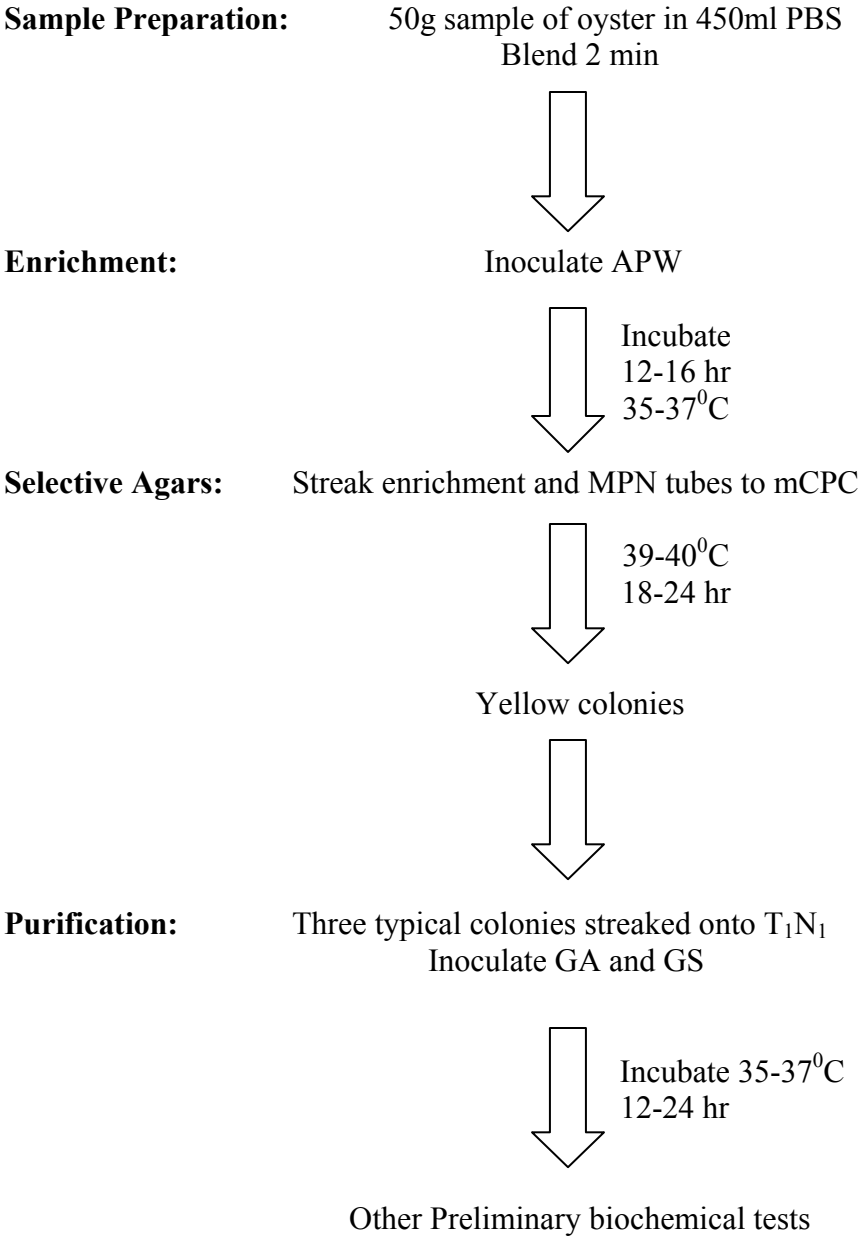

Table 1.1: Preliminary Biochemical Tests

Test	***V. parahaemolyticus***	***V. vulnificus***
Oxidase	+	+
Motility	Motile	Motile
AGS	K/A--	K/A--
TSI	K/A--	K(A rare)/A--
O/129 (10µg)	Resistant	Sensitive
O/129(150µg)	Sensitive	Sensitive
ONPG	-	+

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CHAPTER 2

Comparison of Growth and Survival Characteristics of *Vibrio vulnificus* Expressing Green Fluorescent Protein to the Parent Strain

ABSTRACT

Vibrio vulnificus, a naturally occurring marine bacterium, causes severe disease in at-risk individuals consuming contaminated raw shellfish. Control of the organism in shellstock oysters is of interest, however the bacterium is often difficult to discriminate from natural background microflora. The objectives of this study were to construct a strain of *V. vulnificus* expressing green fluorescent protein (*Vv*-GFP-K), to compare its physiological characteristics to the wild type parent (*Vv*-WT), and to assess the effect of sodium pyruvate media supplementation on recovery efficiency, with particular reference to sublethally injured cells. *V. vulnificus* strain ATCC 27562 was engineered to express GFP and Kanamycin resistance using methods of conjugation. Comparisons were made between the GFP strain (*Vv*-GFP-K) and the parent stain (*Vv*-WT) with respect to growth characteristics, heat tolerance (45^{o}), freeze/thaw tolerance (-20^{o} and -80^{o}C), acid tolerance (pH 5.0, 4.0, and 3.5), cold storage (5^{o}C), cold adaptation (15^{o}C) and starvation. In most cases, *Vv*-GFP-K was comparable to *Vv*-WT with respect to growth, survival, thermal inactivation, and freeze thaw survival. There were differences between *Vv*-WT and *Vv*-GFP-K with respect to acid tolerance, although these differences disappeared with sodium pyruvate supplementation of media. The data suggests that media

supplementation with sodium pyruvate may aid in the recovery of *V. vulnificus* cells sublethally injured by exposure to food processing-related stresses.

Introduction

Overall, seafood is responsible for 26.5% of all foodborne disease outbreaks occurring in the U.S (Mead et al., 1999) and the majority of these are associated with the consumption of raw bivalve mollusks (Cook, 1991). Bivalves are filter feeders that concentrate microorganisms in their digestive tracts, and therefore can serve as passive carriers for foodborne pathogens, especially since they are often consumed whole and raw. *Vibrio vulnificus* is an environmentally ubiquitous marine bacterium which is frequently isolated in high numbers from bivalve molluscan shellfish (McLaughlin, 1995). The organism is known to cause three types of infections; i.e., a primary septicemia, a wound infection syndrome, and gastroenteristis. The first of these occurs sporadically in at-risk shellfish consumers and is frequently fatal.

There are a wide variety of resilient and adaptable strains of bacteria, even in the family *Vibrionaceae.* In fact, bacteria in food are often exposed to a number of sublethal stresses, including pH, temperature, and reduced water activity. In the *Vibrionaceae* family, the acid stress response has been studied. For instance, Wong et al. (1998) reported that a pH downshift from 7.5 to 5.0 resulted in an increased acid tolerance of *V. parahaemolyticus,* in a model broth system. Similarly, *Aeromonas hydrophila* exhibited an adaptive acid tolerance response capable of protecting cells from pH as low as 3.5 (Karem et al., 1994). Proteins specifically synthesized during adaptation to pH 5.0 were involved in subsequent survival during exposure to pH 3.5.

Another well studied stress response is upon exposure to cold temperature. Bryan et al. (1999) suggested that cold adaptive "protective" proteins in *V. vulnificus* may enhance the organism's survival and tolerance to cold temperatures. For instance, *V.*

vulnificus strain ATCC 27562 showed increased survival when subjected to 15°C for 4 h and then shifted to 5°C, compared to those cells that were directly subjected to 5°C without prior conditioning (Bang and Drake, 2001).

An interesting phenomenon observed for members of the *Vibrionaceae* family is the ability to enter the so-called viable but nonculturable (VBNC) state. The term VBNC was introduced in 1985 to describe bacterial cells that do not form colonies on any media, but are nonetheless considered alive because they remain metabolically active (Oliver, 2000; Gauthier, 2000). Presumably, the VBNC state allows bacteria to survive under adverse conditions, however it differs from the injured state because injured cells only lose their ability to be cultured on selective media, while VBNC cells cannot be cultured at all, even on highly nutritive non-selective media. Many studies report restoration of culturability from the VBNC state, but there are contrasting theories to explain this phenomenon. Some believe that the VBNC state does not exist, but instead a small number viable cells remain and when the sample is subjected to more favorable conditions, the residual cells grow and become detectable on microbiological media. Others believe that VBNC cells are indeed alive and can resuscitate and grow after restoration of more favorable conditions (Kell, 1998).

It has long been known that sublethally injured cells have an increased sensitivity to hydrogen peroxide. Since peroxide is a common by-product of media preparation, this may exacerbate detection of injured cells using normal cultural methods. To this end, investigators have supplemented microbiological media with either catalase or sodium pyruvate, which apparently degrades the toxic by-product hydrogen peroxide (Baird-Parker and Davenport, 1965). Bogosian et al. (2000) demonstrated that while *V.*

vulnificus cells in the VBNC state showed a logarithmic decline in cell numbers over time when recovered on nutrient-rich medium, higher culturable cell counts were observed when this media was supplemented with catalase or sodium pyruvate. These authors suggested that the use of hydrogen peroxide degrading compounds may help to bridge the gap between the contrasting theories about the nature of the VBNC state.

The Interstate Shellfish Sanitation Conference (ISSC) has proposed that *V. vulnificus* infections be reduced by 60% by 2007. To achieve this reduction, ISSC suggests that oysters contain <30 *V. vulnficicus* cells per gram of oyster meat (U.S. Department of Health and Human Services, 1999). Although recommended detection methods exist for foods, the fact remains that it is easier to isolate and identify *V. vulnificus* from clinical samples than from environmental samples (Anono et al., 1997; Oliver et al., 1992; Vanoy et al., 1992). One of the reasons for this is that environmental members of the *Vibrio* genus comprise >50% of the bacterial isolates from marine waters, but most of these marine *Vibrios* have not been characterized phenotpically, and are easily confused with *V. vulnificus*. In short, *V. vulnificus* is difficult to discriminate from natural microflora in shellfish and coupled with the VBNC issues, it has been challenging to study the response of this organism to stresses that may be encountered in the processing and storage of oysters. With this in mind, our objectives were to construct a strain of *V. vulnificus* expressing green fluorescent protein (GFP) that could be readily differentiated from natural microflora that might be associated with the oyster matrix. In further experiments, we compared the growth and survival characteristics of the engineered strain to the parent to assure that it would be a useful surrogate for stress

response studies. These growth and survival characteristics were evaluated in nutrient-rich medium both with and without sodium pyruvate supplementation

Materials and Methods

Bacteriological media

Most of the media used in this work have been previously described in the FDA Bacteriological Analytical Manual (BAM, 2001). All bacteriological media were obtained from Difco Laboratories (Detroit, MI) unless otherwise stated and were prepared according to manufacturer's recommendations, or altered accordingly based on requirements of the experimental design. In most cases, incubations were done at 37^{o}C for 24 h on non-selective media and at 40^{o}C for 24 h for selective media unless otherwised stated. Trypticase Soy Broth supplemented with 2% NaCl ($TSBN_2$) was used as a diluent and Trypticase Soy Agar supplemented with 2% NaCl ($TSAN_2$) was used for enumeration. Selective media included modified Cellobiose-Polymyxin B-Colistin (mCPC). Since the green fluorescent protein expressing strain (*Vv*-GFP-K) was designed to also express kanamycin resistance, media were supplemented with the antibiotic (0.05g/L) ($TSAN_2K$ and mCPCK), which assured the stability of the green fluorescent protein and prevented the growth of competitive microflora associated with the food matrix. Kanamycin supplementation was not used when recovering the wild type *V. vulnificus* (*Vv*-WT) strain. To promote recovery of injured cells in some experiments, $TSAN_2$ was supplemented with sodium pyruvate (Sigma Chemical Co., St. Louis, M.O.) at a concentration 80 mg/ml. All experiments except for growth rate were conducted with and without sodium pyruvate supplementation.

Stock strains of *Vv*-WT and *Vv*-GFP-K were maintained at room temperature on $TSAN_2$ and $TSAN_2$ K slants, respectively with sterilized mineral oil overlays (Sigma Chemical Co., St. Louis, M.O.). *V. vulnificus* stock strains were transferred monthly. Three consecutive transfers of each strain at 37°C for 12 h were done before experimental inoculations.

Development of mutant (*Vv*-GFP-K) of *V. vulnificus*

Based on previous studies by Bang and Drake (2002) and unpublished work by this same group, strain ATCC 27562 was engineered to express GFP and kanamycin resistance by Elhanafi. Selection of this strain was considered appropriate because comparatively speaking, ATCC 27562 exhibited better survival when exposed to long term refrigeration temperatures and acidic conditions than were strains MO6-24 and 304C. The plasmid pNKBOR system, an oriR6K-based suicide vector that permits the random insertion of a mini-transposon into the chromosome of Gram-negative bacteria (Rossignol et al., 2001) was used for conjugation and transposition. This mini-transposon contains a conditional R6K plasmid origin of replication (Fig. 1), a kanamycin resistance gene and unique nuclease restriction sites, specifically *Pst*I and *BamH*I. pNKBOR can be propagated by replication in *E. coli* strains containing the R6K replicase π protein, in this case *E. coli* S17-1 λpir (obtained courtesy of Dr. Izallalen, University of Laval, Canada). Efficient NKBOR transposition is ensured by expression of an adjacent Tn10 transposase gene. To allow the entry of plasmid pNKBOR into *Vibrio vulnificus*, we cloned an 850bp *Pst*I fragment containing the origin of transfer "oriT" sequence from plasmid pCON-1 (Li and Kathariou, 2003) in the *Pst*I site of pNKBOR, yielding plasmid pMAD278. OriT allows the transfer of pNKBOR from S17-1 λpir to *Vibrio vulnificus* by

conjugation. The *gfp* gene was isolated from plasmid pGFP from which it is over expressed by the lactose operon promoter (CLONTECH Laboratories, Palo Alto, CA). Plasmid pGFP was digested with restriction endonucleases *Ase*I (New England Biolabs, Baverly, MA) and *EcoR*I ((Promega, Madison, WI), producing a Plac-*gfp* DNA fragment, the extremities of which were filled-in with Klenow enzyme (Promega, Madison, WI). This was then inserted in the *BamH*I site of the transposon in plasmid pMAD278, generating the plasmid pMAD281 in which the *gfp* gene was over expressed by the Plac promoter present inside the transposon NKBOR. To isolate a Plac::*gfp* insertion in the chromosome of *V. vulnificus*, an aliquot of 8 hr culture of S17-1 λpir/pMAD281 was spotted on $TSAN_2$ and dried, overlaid with an aliquot of 8 hr culture of *V. vulnificus,* dried, and the plate incubated at 37^{o}C for 12-18 h. After incubation, the mixed culture was spread on $TSAN_2K$. *V. vulnificus* strains with chromosomal insert of the Plac::*gfp* transposon were easily identified as large fluorescent colonies displaying kanamycin resistance by way of rapid growth, as compared to strain *E. coli* S17-1 λpir which grew very slowly and formed very small colonies on $TSAN_2K$.

Comparison of Growth and Survival Characteristics of *Vv*-GFP and wildtype *Vv*-WT

Recovery of *Vv*-GFP-K in Oyster matrix: Twenty-five gram samples of shucked oysters were homogenized and then inoculated with 12 hr cultures of *Vv*-WT or *Vv*-GFP-K, to achieve an inoculum level of approximately 10^5-10^6 CFU/g. To compare plating and recovery efficiency, homogenates were serially diluted and plated on $TSAN_2$, $TSAN_2K$, mCPC, and mCPCK.

Growth Curves: Twelve hr cultures of *Vv*-WT and *Vv*-GFP-K were inoculated into 100 mL of fresh $TSBN_2$ and $TSBN_2K$ broth, respectively to achieve initial inoculum of 10^2 CFU/ml. Freshly inoculated cultures were incubated at 37°C and aliquots were removed at 2 h intervals, for a total of 24 h. Absorbance of each aliquot was read at 600 nm using Beckman, Du 530 spectrophotometer (Fullerton, CA). Simultaneously, each aliquot was also serially diluted and plated for enumeration on $TSAN_2$, $TSAN_2K$, mCPC, and mCPCK

Refrigerated Storage and Starvation: Both cold temperature storage and cold temperature adaptation were studied in these experiments. In both cases, a 12 hr culture of *Vv*-WT and *Vv*-GFP-K was transferred to 99 ml sterile $TSBN_2$ to reach an initial population of approximately 10^6 CFU/ml. For cold storage studies, $TSBN_2$ was prechilled to 5°C before addition of cells; thereafter, samples were stored at 5°C and plated daily for recovery on $TSAN_2$ and $TSAN_2K$ with and without sodium pyruvate supplemention for a total of 10 days. For cold temperature adaptation studies, *Vv*-WT and *Vv*-GFP-K cells were stored at 15°C for 4 h as described by Bryan et al. (1999) before inoculating into prechilled (5°C) $TSBN_2$ and extended storage for 15 days with daily sampling and plating on $TSAN_2$ and $TSAN_2K$ with and without sodium pyruvate supplementation.

For starvation studies, one mL of 12 hr culture of *Vv*-WT and *Vv*-GFP-K were transferred to 99 mL of prechilled sterile artificial seawater to achieve an initial concentration of 10^6 CFU/ml. Inoculated seawater samples were stored at 5°C and subsamples taken daily (for up to 25 days) for enumeration on $TSAN_2$ and $TSAN_2K$ with and without pyruvate supplementation for 25 days.

Frozen Storage: Two different types of freezing conditions were chosen, one to evaluate the survival of *V. vulnificus* during long term, low temperature (-80^{o}C) storage, and another to evaluate survival after repetitive freeze-thaw cycling. In both cases, one mL 12 hr culture of *Vv*-WT and *Vv*-GFP-K were centrifuged (11950 g, 2 min) and resuspended in fresh $TSBN_2$ prior to beginning experiments, with recoveries done on both $TSAN_2$ and $TSAN_2K$with and without sodium pyruvate supplementation.

For long temperature frozen storage survival studies, resuspended cultures (approx. 10^5-10^6 CFU/ml), were aliquoted (5 mL) into plastic tubes and stored at -80 ^{o}C. A subsample was taken every 5 days for a total of 3 months, thawed at room temperature on benchtop (approximately 5 min) and plated for recovery on $TSAN_2$ and $TSAN_2K$ with and without sodium pyruvate. For freeze-thaw (-20^{o}C) survival, 10 mL aliquots of cultures were stored at -20^{o}C. One freeze-thaw cycle consisted of freezing at -20^{o}C for 24 h, followed by thawing at 23^{o}C for 30 min on benchtop (Leenanon & Drake, 2001). Multiple freeze-thaw cycles on a single sample were performed daily for 5 days. After each cycle, duplicate thawed samples were serially diluted and plated for recovery $TSAN_2$ and $TSAN_2K$ with and without sodium pyruvate.

Thermal Inactivation: Overnight suspensions of *Vv*-WT and *Vv*-GFP-K cultured in $TSBN_2$ were heat inactivated using the capillary tube method described by Foegeding and Leasor (1990). Briefly, capillary tubes were filled to a volume of 50 µL (approx. 1.0 x 10^6 CFU total), heat sealed, and submerged into a water bath (45^{o}C), then removed at designated time intervals and placed in an ice slurry. Specifically, capillary tubes were sampled prior to heat treatment, and at 10 min intervals when heated at 45^{o}C. Immediately after cooling, the tubes were sanitized by dipping in sodium hypochlorite

(500ppm, pH 6.5), crushed, serially diluted and spread plated on $TSAN_2$ and $TSAN_2K$ with and without sodium pyruvate.

Acid Exposure: Based on previous work in our location (data not shown), acid tolerance of *Vv*-GFP-K and *Vv*-WT was evaluated at pH values of 5.0, 4.0, and 3.5. One ml of overnight culture was transferred to 99 ml of fresh $TSBN_2$ (final concentration of approximately 10^6 CFU/ml), and acidified to the target pH using 1N HCl. Cells were held at 23°C on benchtop and withdrawn immediately (time 0 control) and 1, 3, 5 and 10 h for pH 5.0; after 10, 20, 30, 40, 50 and 60 min for pH 4.0; and after 5, 10, 15, 20, 25 and 30 min for pH 3.5. Samples at each timepoint were serially diluted and plated for recovery on $TSAN_2$ and $TSAN_2K$ with and without sodium pyruvate.

Statistical Analysis

For all experiments, three replications of each treatment were performed for each strain and on all recovery media. D-values were calculated as the time (in days or minutes) required for a 1-log reduction of the population using regression analysis (PROC REG). Statistical comparison of D-values were done by ANOVA (PROC MIXED), and the least-squares' method was used to determine significant differences ($p<0.05$) (SAS Statistical Analysis Software, version 8.0, SAS Institute, Cary, N.C.).

Results

In preliminary studies, a thorough comparison of the physiological characteristics of *Vv*-Wt and *Vv*-GFP-K was done. Physiological characteristics included Gram reaction gelatin agar, gelatin agar supplemented with 2% NaCl, motility, o-nitrophenyl-β-D-glactosidase, triple sugar iron slant supplemented with 2% NaCl, and fermention of cellobiose on mCPC. *Vv*-WT colonies appeared more translucent and slightly smaller on

$TSAN_2$ than did *Vv*-GFP-K, with *Vv*-GFP-K colonies clearly fluorescing upon exposure to ultraviolet light (data not shown). On mCPC, *Vv*-GFP-K did not show fluorescence after exposure to ultraviolet light, but this could be due to the natural color of mCPC (dark green). However, *Vv*-GFP-K appeared as small, flat yellow colonies with a halo, whereas *Vv*-WT colonies had the same morphology but lacked the halo (data not shown). In regards to biochemical tests, both the *Vv*-WT and *Vv*-GFP-K were identical showing motility and positive results for o-nitrophenyl-β-D-glactosidase, triple sugar iron slant supplemented with 2% NaCl, and fermention of cellobiose on mCPC (data not shown).

To evaluate the ability of *Vv*-GFP-K to be consistently recovered from kanamycin supplemented media, and to evaluate the ability of kanamycin to suppress the growth of competitive microflora, a plating comparison was undertaken in matrix format. In this experiment, 12 h cultures of both *Vv*-GFP-K and *Vv*-WT were plated on mCPC and $TSAN_2$ both with and without kanamycin supplementation. Both *Vv*-WT and *Vv*-GFP-K were detected similarly (approximately 10^7 CFU/ml) on $TSAN_2$ without kanamycin supplementation. *Vv*-WT was not recovered at all on media supplemented with kanamycin although titers of 10^7 /ml were obtained for *Vv*-GFP-K on $TSAN_2K$. Likewise on selective medium (mCPC) without the addition of kanamycin, both *Vv*-WT and *Vv*-GFP-K were detected similarly (at approximately1 x 10^7 CFU/ml) while the kanamycin supplemented media suppressed the growth of *Vv*-WT. *Vv*-GFP-K could be clearly discriminated from background microflora by visualization using ultraviolet illumination on $TSAN_2$, and under irredescent light for mCPC (data not shown). Supplementation of media with kanamycin effectively reduced background microflora in oyster homogenates by 1-2 $\log_{10}$ and suppressed the growth of *Vv*-WT without impacting

the recovery of *Vv*-GFP-K. Non-selective medium ($TSAN_2$ and $TSAN_2K$) was chosen for subsequent experiments to facilitate the recovery of sublethally injured cells of *Vv*-WT and *Vv*-GFP-K, respectively.

There were no statistically significant differences in the growth rates of both strains at 37°C on non-selective medium (Fig. 2.2a), with exponential growth rates (EGR) of 2.17 h $\pm$ 0.52 and 1.38 h $\pm$ 0.11, for *Vv*-Wt and *Vv*-GFP-K, respectively. Both strains reached a maximum population densities of approximately 8 $\log_{10}$ CFU/ml within 12 hr. Similar EGRs for *Vv*-GFP-K and *Vv*-Wt were observed when plating on selective medium (mCPC) (2.42 $\pm$ 0.36 and 1.66 $\pm$ 0.12, respectively) (Fig. 2.2b). As with non-selective media, both strains reached a maximum population density of approximately 8 $\log_{10}$ CFU/ml within 12 hr on mCPC. *Vv*-GFP-K did not lose its selective antibiotic resistance marker, nor its ability to be cultured on selective medium after numerous (>10) passages (data not shown).

Experiments to evaluate survival upon exposure to sublethal stresses were done on $TSAN_2$ both with and without sodium pyruvate supplemented. Bogosian et al. (2000) investigated the effects of different concentrations of sodium pyruvate (ranging from 0-320 mg) on the recovery of *V. vulnificus* when plated on Brain Heart Infusion agar. These authors found maximum recovery in *V. vulnificus* cells on media supplemented with 40 mg of sodium pyruvate, with higher levels of supplementation failing to improve recoveries further, but also not negatively impacting overall recovery. We also looked at different concentrations of sodium pyruvate (0-320 mg) (data not shown), finding that 40 mg or more of pyruvate improved recovery, but choosing supplementation with 80 mg to be consistent with Bogosian et al. (2000).

When *Vv*-WT and *Vv*-GFP-K were stored at 5°C (Fig. 2.3a), there was a statistically significant difference in D-values (5.64 ± 2.95 and 2.15 ± 0.18 days, respectively) between the strains when evaluated on media without pyruvate supplementation. However using supplemented media, this difference disappeared (6.54 ± 0.55 and 4.91 ± 0.13 days, for *Vv*-WT and *Vv*-GFP-K, respectively). In cold adaptation studies (Fig. 2.3b), there was a statistically significant difference between *Vv*-Wt and *Vv*-GFP-K D-values using both non-supplemented (7.44 ± 1.24 and 2.84 ± 0.28 days, respectively) and supplemented media (6.07 ± 0.48 and 9.74 ± 1.44 days, respectively). There were no significant differences between recoveries on supplemented versus non-supplemented media for *Vv*-WT. The opposite was the case for *Vv*-GFP-K.

For cells starved in artificial seawater, (Fig. 2.3c) there was a statistically significant difference between *Vv*-Wt and *Vv*-GFP-K on non-supplemented media (D-values 2.83 ± 0.04 and 7.52 ± 0.86 days, respectively). A similar significant difference was observed between *Vv*-Wt and *Vv*-GFP-K on supplemented media (D-values 2.64 ± 0.05 and 11.64 ± 1.54 days, respectively). Improved recovery was observed with pyruvate supplementation for both *Vv*-Wt and *Vv*-GFP-K, however this was only statistically significant for *Vv*-GFP-K. For instance, *Vv*-GFP-K reached nondetectable limits within 21 days on non-supplemented media, whereas, nondetectable limits were not reached on sodium pyruvate supplemented media until day 33.

In terms of frozen storage (-80°C), both *Vv*-GFP-K and *Vv*-Wt populations dropped approximately 1 $\log_{10}$ within the first day, however, thereafter both populations remained stable for more than 50 days (Fig. 2.4a), regardless of media supplementation. Survival of *V. vulnificus* was quite different under conditions of repetitive freeze/thaw.

The freeze/thaw survival of *Vv*-GFP-K and *Vv*-Wt were practically identical using either supplemented or non-supplemented media (Fig. 2.4b).

Likewise for thermal inactivation at 45^{o}C (Fig. 2.5), for which there was no statistically significant difference in D-values for *Vv*-WT and *Vv*-GFP-K (10.18 $\pm$ 0.31 and 10.12 $\pm$ 0.50 min, respectively) recovered on media without pyruvate supplemention, nor was there a significant difference between *Vv*-WT and *Vv*-GFP-K D-values (13.70 $\pm$ 0.90 and 15.08 + 1.44 min, respectively) when using pyruvate supplemented media. However, when comparing non-supplemented and pyruvate supplemented media, there were statistically significant differences for both *Vv*-WT (10.18 $\pm$ 0.31 and 13.70 $\pm$ 0.90 min, respectively) and *Vv*-GFP-K (10.12 $\pm$ 0.50 and 15.08 + 1.44 min, respectively).

At pH 5.0, both *Vv*-GFP-K and *Vv*-Wt populations remained constant for 20 h on both supplemented and non-supplemented media (Fig. 2.6a). However, *Vv*-Wt was more resistant to pH 4.0 than was *Vv*-GFP-K, having a D-value of 35.41 $\pm$ 6.84 min versus 26.31 $\pm$ 2.31 min on non-supplemented media. When using supplemented media, this difference disappeared, with both *Vv*-Wt and *Vv*-GFP-K sharing D-values of approximately 50 min (Fig. 2.6b). At pH 3.5 on non-supplemented media, *Vv*-GFP-K was more resistant than *Vv*-Wt, (D-values of 8.13 $\pm$ 1.25 and 5.20 $\pm$ 0.24 min, respectively). Again, using supplemented media, there was no statistically significant difference between *Vv*-Wt *an*d *Vv*-GFP-K with respect to acid tolerance (D-values of 18.83 $\pm$ 6.25 and 25.08 $\pm$ 6.57 min, respectively) (Fig. 2.6c).

Discussion

There are many methods to construct bacterial strains to facilitate their differentiation from background microflora or otherwise monitor biocontrol in the food

matrix. One method is the development of antibiotic resistant strains. For instance, Fairchild and Foegeding (1993) constructed a spontaneous antibiotic resistant strain of *L. innocua*, to be used as a surrogate for *L. monocytogenes*, by selective enrichment on gradient plates with increasing concentrations of streptomycin and rifampin. A similar approach was undertaken by Dombroski et al. (1999) in their development of a nalidixic acid resistant strain of *V. vulnificus* to be used in evaluating freeze and heat tolerance of the organism in oyster homogenates. Others have produced antibiotic resistance by electrotransformation of a plasmid encoding for resistance and the subsequent use of these strains to evaluate survival in different processing environments. Foegeding and Stanley (1991) suggested that *L. innocua* containing a plasmid (pGK12) coding for chloramphenicol and erythromycin-resistance might be a conservative surrogate to evaluate thermal processes with respect of *L. monocytogenes*, since this strain was more heat resistant than *L. monocytogenes.* In addition, Foegeding et al. (1992) constructed five strains of *L. monocytogenes* by electroporation of a plasmid pGK12 to evaluate survival of *L. monocytogenes* in dry fermented sausage. In all cases, the low probability that indigenous microflora would possess the same resistance as the engineered strain(s) permitted selection of target bacterial strains from a mixed background microflora and subsequent monitoring of the survival and growth of the target population.

Another popular method for labeling bacteria involves incorporating the green fluorescent protein (*gfp*) gene. In some cases, the gene is integrated by electroporation of a plasmid, and in other cases, by chromosomal gene insertion. The *gfp*-labeled strain has then been used to detect target bacteria in food matrices and monitor metabolic activity or VBNC (Lowder et al., 2000; Lowder and Oliver, 2001;Dykes et al., 2001; Gandhi et al.,

2001; Kenney and Beuchat, 2002). GFP-tagged strains of *E. coli* O157:H7 and *Salmonella muenchen* have been used to evaluate the efficacy of commercial cleaners on the surface of apples (Kenney and Beuchat, 2002). Additionally, GFP-labeled strains of *E. coli* O157:H7 and *Salmonella* have been used to evaluate survival in chilled-vacuum or carbon dioxide packaged primal beef cuts (Dykes et al., 2001). Also, a *Salmonella stanley* strain in which the GFP gene was inserted has been used to evaluate survival, spatial location, and pathogen control in alfalfa sprouts (Gandhi et al., 2001).

In our study, a *V. vulnificus* strain was engineered through conjugation and transposition, to express both kanamycin resistance and green fluorescent protein. This strain was then evaluated as a potential surrogate to follow *V. vulnificus* survival upon exposure to sublethal stresses encountered in oyster processing. In past studies, researchers have encountered difficulties when attempting to isolate and identify *V. vulnificus* in shellfish because of the high background levels of indigenous microflora (Aono et al., 1997). The use of the kanamycin resistant and GFP-expressing strain of *V. vulnificus* allowed easy discrimination of the organism from the natural oyster microflora when used in seeding studies. Stability of chromosomal insertion of the *gfp* gene could be monitored since the kanamycin resistance sequence was linked to the insert. In this case, the chromosomal insertion was very stable over time and multiple passage.

In order to effectively use a tagged strain as a surrogate for a wild-type bacterial strain, the two must be comparable in key biochemical, physiological, growth, survival, and inactivation characteristics. Interestingly, many previous reports provide inconsistent comparison of strain comparability. In some cases, no comparisons to parental strains have been conducted (Gandhi et al., 2001; Qazi et al., 2001; Sun and Oliver, 1995). In a

few cases, limited comparisons were done. For instance, Fratamico et al. (1997) reported that a GFP-tagged *E. coli* O157:H7 strain was indistinguishable from the parent with regards to biochemical identification and detection by immunological and PCR assay. The importance of comparisons is illustrated by Foegeding and Stanley (1991) who found that plasmid-encoded antibiotic resistance resulted in slower growth rates for transformed *L. monocytogenes* compared to the wild-type strain, presumably due to issues associated with the maintenance of the plasmid. Although Sun and Oliver (1995) used a kanamycin resistant strain to evaluate the effects of Tabasco or horseradish-based cocktail sauces on the survival of *V. vulnificus* in oyster meat, this strain was not compared to the wild-type parent. In perhaps the most comprehensive comparison study, Dombroski et al. (1999) found that their spontaneous nalidixic acid resistant *V. vulnificus* strain was comparable to the wild-type strain in regards to physiological and biochemical characteristics, but did indeed show differences in freeze tolerance, with the resistant *V. vulnificus* strain offering a more conservative estimate of total inactivation.

In our study, *Vv*-GFP-K and *Vv*-WT were frequently comparable with respect to physiological and biochemical characteristics. For instance, no statistically significant differences were observed between the two strains in regards to growth, -80^{o}C survival, repetitive freeze/thaw cycling (-20^{o}C), and thermal inactivation, regardless of sodium pyruvate supplementation. In a few instances, differences in survival were noted, but these generally disappeared when recovery media was supplemented with sodium pyruvate, as was the case for acid tolerance. In the few cases where statistically significant differences were observed between *Vv*-WT and *Vv*-GFP-K strains on media both with and without pyruvate supplementation (e.g., 5^{o}C, cold adaptation, and

starvation), the use of sodium pyruvate resulted in a more conservative estimate of survival when comparing *Vv*-GFP-K to the wild type parent. On the basis of these observations, we conclude that *Vv*-GFP-K survival upon exposure to processing-related stress is equivalent to, or better than, the wild type parent as long as recovery media is supplemented with sodium pyruvate.

By and large, pyruvate supplementation made a difference when cells were exposed to acidic conditions, starvation, or long term storage at refrigeration temperatures. It has been suggested that injury and perhaps VBNC may be manifested by increased sensitivity to hydrogen peroxide. For instance, Bogosian et al. (2000) proposed that at least a proportion of VBNC cells obtained by starvation may in fact be hydrogen peroxide-sensitive culturable cells. These investigators observed recovery of *V. vulnificus* on pyruvate supplemented media even after they entered the so-called VBNC state. Our hypothesis was that pyruvate supplementation would indeed promote the recovery of sublethally injured cells, and similar to Bogosian et al. (2000), we also observed better recovery, sometimes statistically significant and sometimes not, for both *Vv*-WT and *Vv*-GFP when using sodium pyruvate supplemented media. Interestingly, the degree to which pyruvate supplementation enhanced recovery of sublethally injured cells appeared to be greater for *Vv*-GFP-K as compared to *Vv*-WT. This may be due to the exact location of the chromosomal insertion, although this remains speculative. The fact that pyruvate supplementation was at times effective, and at other times unnecessary, suggests that cell injury is not an all or nothing phenomenon. Indeed, we hypothesize that a "spectrum" of injury may occur, and the degree to which cells are injured may well depend on the type of sublethal stress to which they are exposed.

Within the family *Vibrionaceae*, cold and acid stress responses have been the best characterized. Oliver et al. (1991) reported that *V. vulnificus* is more susceptible to low temperatures when in its logarithmic growth phase as compared to its stationary phase. Bryan et al. (1999) suggested that cold adaptive "protective" proteins in *V. vulnificus* may enhance survival and tolerance to cold temperatures and that iron plays a role in adaptation at cold temperature, since the removal of iron from the growth medium prior to cold adaptation reduced viability by 2 $\log_{10}$. In a study by Bang and Drake (2002), cold stress had no effect on *V. vulnificus* thermal resistance or freeze/thaw tolerance, but conditioning the cells by exposure to 15^{o}C prior to temperature shift to 5^{o}C resulted in improved long term survival at refrigeration temperatures. Our data is consistent with that of Bang and Drake (2002), particularly with respect to the survival and recovery of cold adapted *Vv*-GFP-K, especially when using sodium pyruvate supplemented medium.

It is generally recognized that when many bacterial species are exposed to sublethal pH (usually <5.9), an acid tolerance response is induced that enables the cells to survive at even lower pH values (Waterman and Small, 1996). In a broth model, Wong et al. (1998) found that conditioning cells to pH 5.0 prior to further downshift to pH 3.5 resulted in better survival of *V. parahaemolyticus* when compared to immediate exposure to pH 3.5 without prior conditioning. The same phenomenon has been observed for *Aeromonas hydrophila*, in which case the investigators showed that proteins synthesized during adaptation to pH 5.0 were involved in the subsequent improved survival during exposure to pH 3.5 (Karem et al., 1994).

In this study, we constructed a GFP-expressing strain of *V. vulnificus* to facilitate detection in the shellfish matrix, specifically with reference to evaluating the survival of the organism upon exposure to sublethal stresses that might be encountered in food processing. The kanamycin resistant GFP-expressing strain was relatively comparable to the wild type parent with respect to survival upon exposure to a number of processing associated stresses, and could easily be discriminated from background micorflora. Keeping in mind that there are strain to strain differences in survival of *V. vulnificus* (Bang and Drake, 2002; Koo et al, 2000a; Koo et al., 2000b), our strain did appear to be a somewhat conservative surrogate. In the next chapter, we describe the use of the *Vv*-GFP strain in studies designed to evaluate survival of the organism within the oyster matrix when exposed to storage and preparation conditions commonly used for this commodity.

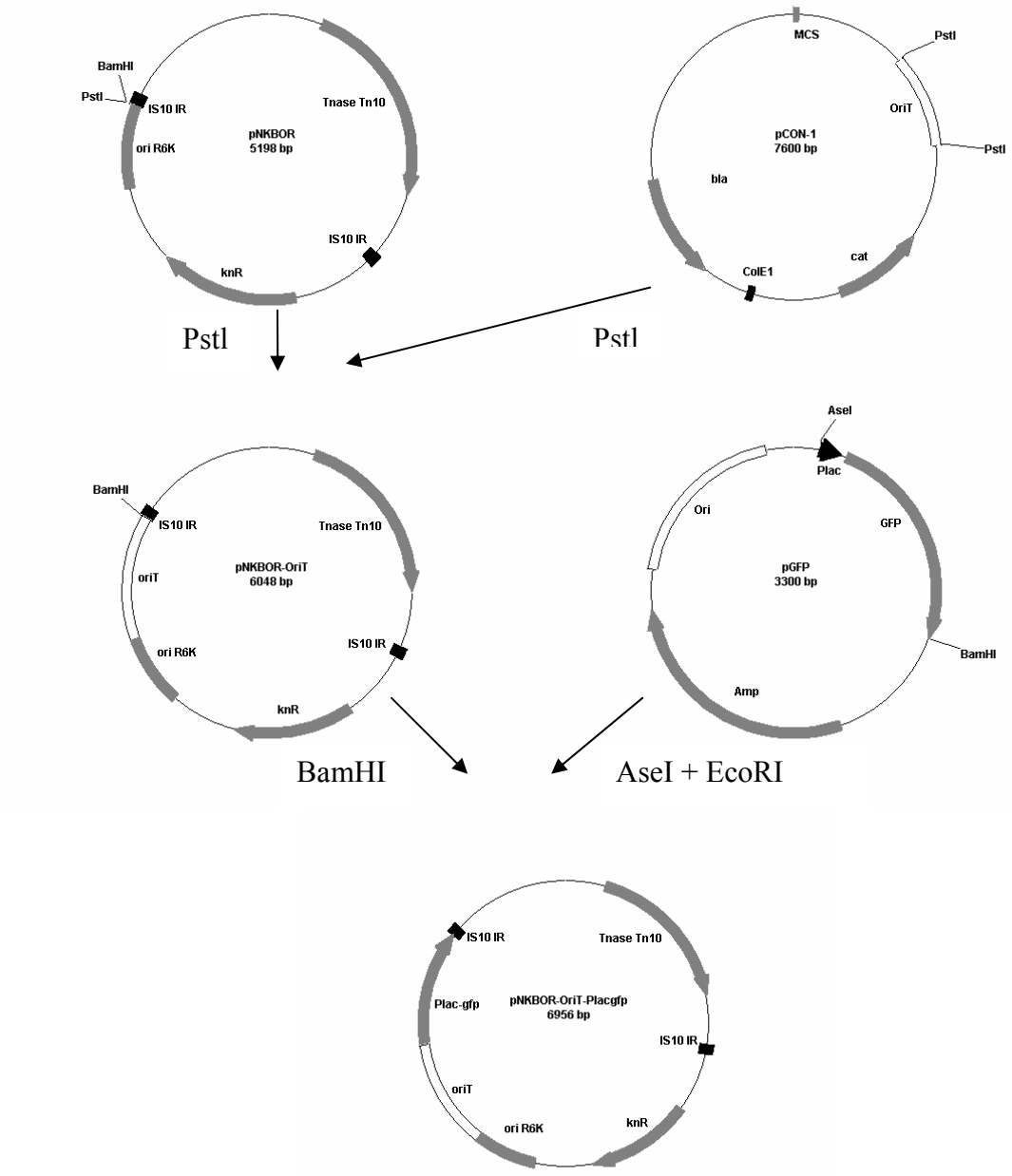
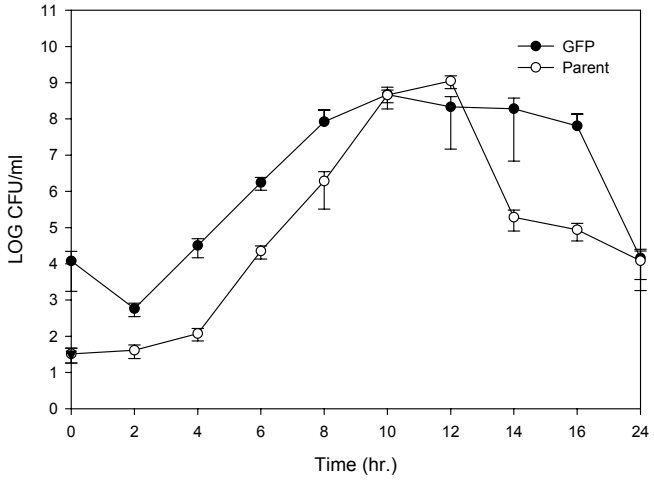


Figure 2.1: Schematic representation of the procedure for construction of plasmid pNKBOR-OriT-Plac::gfp, to achieve transposition in *V. vulnificus* (see materials and methods section for details). Each plasmid structure shows relevant endonucleases restriction sites, important elements and genes. Each gene is represented by an arrow indicating the transcription direction. The abbreviations used: Amp, ampicilin resistance determinant; Bla, ampicilin resistance determinant; Cat, Chloramphenicol resistance determinant; ColE1, plasmidic origine of replication; *gfp*, green fluorescent protein determinant; IS10 IR, insertion sequence important for transposition; KnR, kanamycin resistance determinant; MCS, multi-cloning site; Ori R6K, conditional origin of replication; OriT, origin of transfer allowing the plasmid to be conjugated from *E. coli* into *V. vulnificus;* Plac, promoter of the *E. coli* lactose operon; Tnase Tn10, *Tn*10 transposase gene.

A.



B.

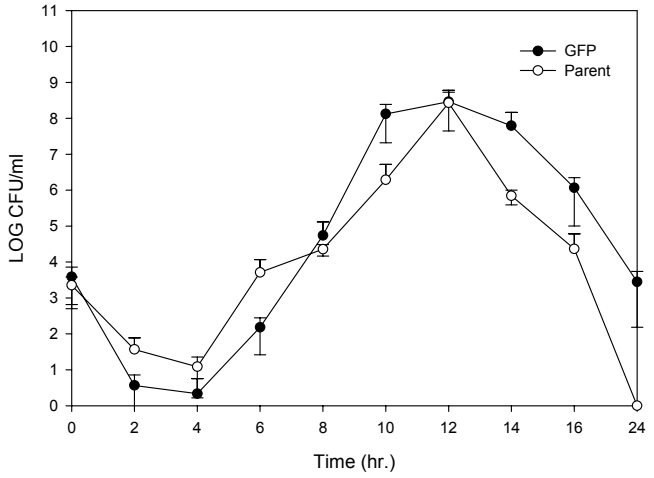
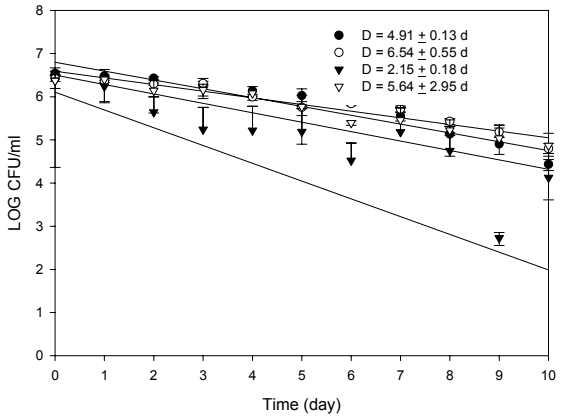
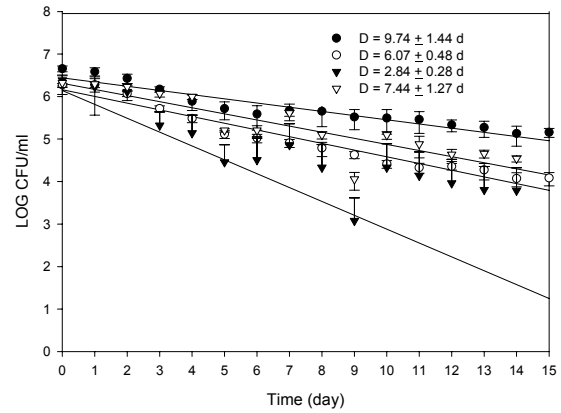


FIG 2.2. Growth Curves at 37^{o}C of GFP-K and parent strains of *V. vulnificus* (A) on non-selective medium ($TSAN_2$) and (B) on selective medium (mCPC). (●) *Vv*-GFP and (○)*Vv*-WT.

A.



B.



C.

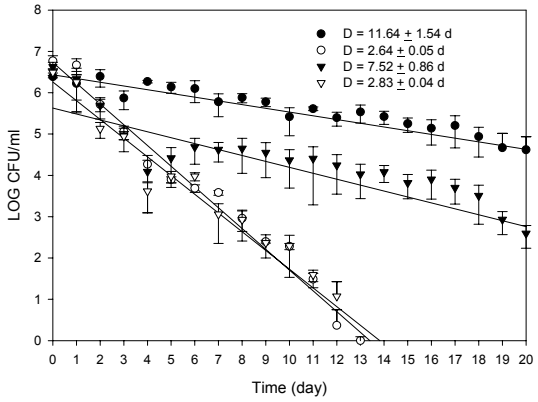
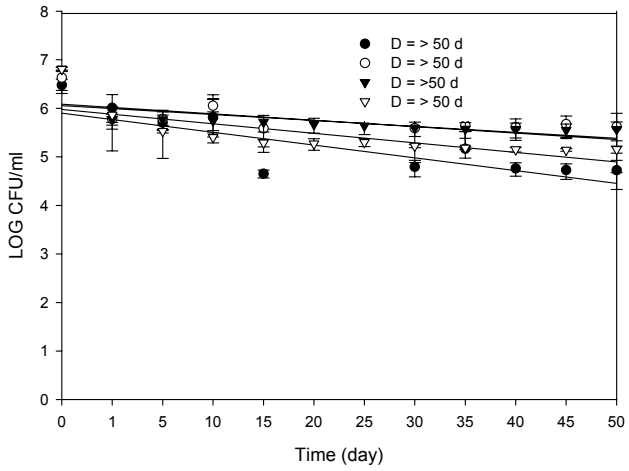


FIG 2.3. Survival of GFP-K and parent strain of *V. vulnificus* at various conditions (A) 5°C storage in $TSBN_2$ (B) stored at 15°C for 4 h and then moved to 5°C, and (C) 5°C in ASW(●) *Vv*-GFP with sodium pyruvate, (○)*Vv*-WT with sodium pyruvate, (▼)*Vv*-GFP without sodium pyruvate and (▽) *Vv*-WT without sodium pyruvate.

A.



B.

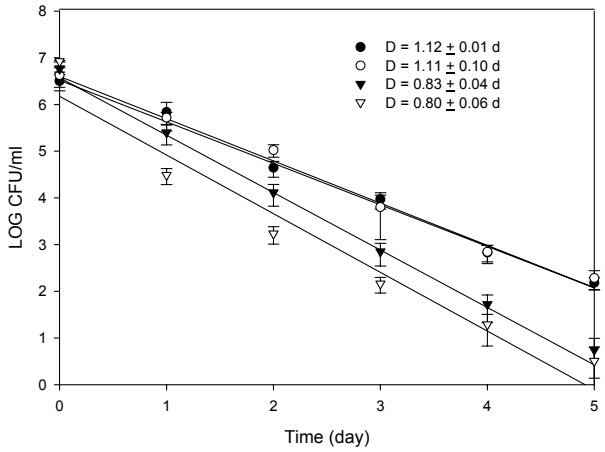


FIG 2.4. Survival of GFP-K and parent strain of *V. vulnificus* during different freezing temperatures (A) -80^{o}C storage and (B) -20^{o}C freeze/thaw. (●) *Vv*-GFP with sodium pyruvate, (○)*Vv*-WT with sodium pyruvate, (▼)*Vv*-GFP without sodium pyruvate and (▽) *Vv*-WT without sodium pyruvate.

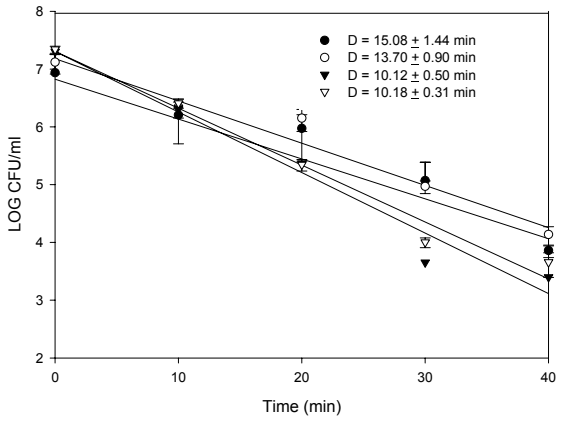
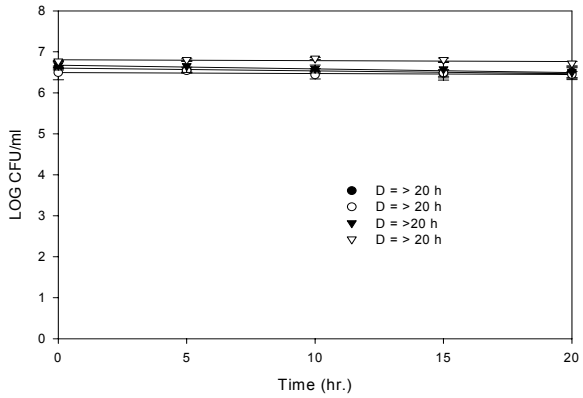


FIG 2.5. Survival of GFP-K and parent strain of *V. vulnificus* during thermal inactivation at 45^{o}C (○)*Vv*-WT with sodium pyruvate, (▼)*Vv*-GFP without sodium pyruvate and (▽) *Vv*-WT without sodium pyruvate.

A.



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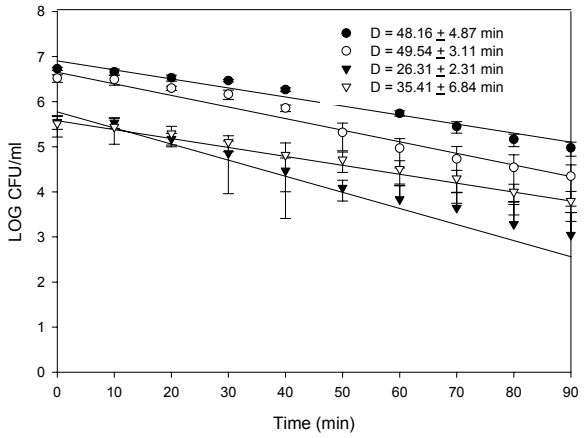

C.

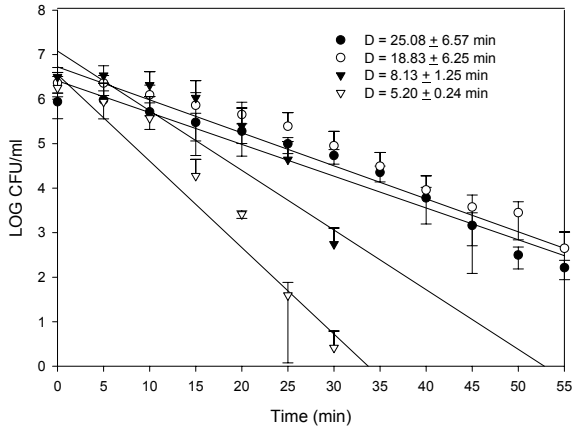

FIG 2.6. Survival of GFP-K and parent strain of *V. vulnificus* under various acidic conditions (A) pH 5.0, (B) pH 4.0, and (C) pH 3.5. (●) *Vv*-GFP with sodium pyruvate, (○)*Vv*-WT with sodium pyruvate, (▼)*Vv*-GFP without sodium pyruvate and (Δ) *Vv*-WT without sodium pyruvate.

Table 2.1: Comparison of *Vv*-WT to *Vv*-GFP-K on pyruvate supplemented and non-supplement media

	***Vv*-WT + Pyruvate**	***Vv*-GFP-K + Pyruvate**	***Vv*-WT**	***Vv*-GFP-K**
5°C Storage (d)	6.54 ± 0.55 a	4.91 ± 0.13 a	5.64 ± 2.95 c	2.15 ± 0.18 d
Cold Adaptation (d)	6.07 ± 0.48 a	9.74 ± 1.44 b	7.44 ± 1.27 c	2.84 ± 0.28 d
Starvation (d)	2.64 ± 0.05 a	11.64 ± 1.54 b	2.83 ± 0.04 c	7.52 ± 0.86 d
-80°C Storage (d)	>50 a	>50 a	>50 c	>50 c
-20°C Freeze/thaw (d)	1.11 ± 0.10 a	1.12 ± 0.01 a	0.80 ± 0.06 c	0.83 ± 0.04 c
45°C (min)	10.18 +0.31 a	10.12 ± 0.50 a	13.70 ± 0.90 c	15.08 ± 1.44 c
pH 5.0 (h)	>20 a	>20 a	>20 c	>20 c
pH 4.0 (min)	49.54 ± 3.11 a	48.16 ± 4.87 a	35.41 ± 6.84 c	26.31 ± 2.31 c
pH 3.5 (min)	18.83 ± 6.25 a	25.08 ± 6.57 a	5.20 ± 0.24 c	8.13 ± 1.25 c

Table 2.2: Comparison of pyruvate supplemented and non-supplement media with *Vv*-WT and *Vv*-GFP-K

	***Vv*-WT + Pyruvate**	***Vv*-WT**	***Vv*-GFP-K + Pyruvate**	***Vv*-GFP-K**
5°C Storage (d)	6.54 ± 0.55 e	5.64 ± 2.95 e	4.91 ± 0.13 g	2.15 ± 0.18 h
Cold Adaptation (d)	6.07 ± 0.48 e	7.44 ± 1.27 e	9.74 ± 1.44 g	2.84 ± 0.28 h
Starvation (d)	2.64 ± 0.05 e	2.83 ± 0.04 e	11.64 ± 1.54 g	7.52 ± 0.86 h
-80°C Storage (d)	>50 e	>50 e	>50 g	>50 g
-20°C Freeze/thaw (d)	1.11 ± 0.10 e	0.80 ± 0.06 f	1.12 ± 0.01 g	0.83 ± 0.04 h
45°C (min)	10.18 +0.31 e	13.70 ± 0.90 f	10.12 ± 0.50 g	15.08 ± 1.44 h
pH 5.0 (h)	>20 e	>20 e	>20 g	>20 g
pH 4.0 (min)	49.54 ± 3.11 e	35.41 ± 6.84 f	48.16 ± 4.87 g	26.31 ± 2.31 h
pH 3.5 (min)	18.83 ± 6.25 e	5.20 ± 0.24 f	25.08 ± 6.57 g	8.13 ± 1.25 h

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CHAPTER 3

Using a green fluorescent protein strain of *Vibrio vulnificus* to evaluate microbial stress in the oyster matrix

ABSTRACT

The objective of this study was to monitor the survival of *V. vulnificus* at sublethal (refrigeration) temperatures and under acidic conditions relevant to those that might be encountered from harvest to consumption of shellstock oysters. For cold temperature storage studies, shellstock oysters *(Crassostrea virginica)* were equilibrated to 25^{o}C and inoculated (by drilling) with approximately 10^{6} CFU/oyster specimen with a previously constructed *V. vulnificus* strain genetically engineered to contain green fluorescent protein (GFP) and kanamycin resistance genes (*Vv*-GFP-K). After sealing with epoxy, inoculated oysters were submitted to 3 different cooling regimens designated (i) rapid cooling (iced); (ii) conventional cooling (5^{o}C within 4 hrs.); and (iii) mild abusive cooling (temperature dropped to 5^{o}C over 8 hrs). Samples were taken at various time points through a 21 d refrigerated product shelf life. For acid studies, shellstock oysters were shucked and inoculated with *Vv*-GFP-K to a level of approximately 10^{6} CFU/oyster and then submerged in trypticase soy broth with 2% NaCl acidified with either acetic or citric acids to achieve pH values of 5.0, 4.0, and 3.5 followed by storage for up to 24 hrs. In all experiments, *Vv*-GFP-K recovery was evaluated by direct plating on trypticase soy agar plus 2% NaCl and kanamycin ($TSAN_2K$) with and without sodium pyruvate supplementation. In the refrigeration studies, there were no apparent differences

in *Vv*-GFP-K levels for all three treatments within the first few days of storage. In all cases, levels dropped 1 $\log_{10}$ after 8 days refrigerated storage. By the end of the study (21 d), *Vv*-GFP-K levels were nondetectable for both iced and conventionally cooled product, however mild abusively cooled oysters still had levels approximately 10^3 CFU/g. *Vv*-GFP-K levels remained stable for up to 24 hrs within the oyster meat under acidic conditions at various pH's. These results support previous findings that cooling alone cannot be relied upon to eliminate *V. vulnificus* and that acidic condiments do not inactivate *V. vulnificus* cells entrapped within the oyster meat.

Introduction

In the U.S., contaminated seafood is responsible for 26.5% of all foodborne disease outbreaks (Mead et al., 1999), and many more cases, with the majority of these being associated with the consumption of raw bivalve molluscan shellfish such as oysters, clams, and mussels (Cook, 1991). Bivalves are filter feeders that concentrate microorganisms in their digestive tracts. These include harmless environmental and commensal microorganisms as well as pathogens, the most significant of which are the human enteric viruses and the pathogenic members of the *Vibrionaceae* family. *Vibrio vulnificus* is of particular concern as the organism produces severe, life-threatening disease in at-risk shellfish consumers, and has been particularly difficult to control at the pre-harvest level.

There are a variety of adaptable strains of bacteria, even in the family *Vibrionaceae.* Bacteria in food can be exposed to many different sublethal stresses, including pH, temperature, and reduced water activity. Molluscan shellfish intended for raw consumption are minimally processed foods (Abee and Wouters, 1999) and are hence exposed to few processing controls, with the notable exception of low temperature storage. It has been suggested that low temperature control could stimulate adaptation. Indeed, Bryan et al. (1999) suggested that cold adaptive "protective" proteins in *V. vulnificus* may enhance survival of the organism to cold temperatures. A subsequent study also demonstrated improved survival when some *V. vulnificus* strains were conditioned to 15^{o}C for 4 h and then shifted to 5^{o}C, compared to those cells that were directly subjected to 5^{o}C without prior conditioning (Bang and Drake, 2001).

Raw molluscan shellfish are frequently consumed with acidic condiments such as Tabasco or cocktail sauces, and/or lemon juice. Some investigators have reported a similar adaptation response when members of the family *Vibrionaceae* were exposed to sublethal acidic conditions. For instance, Wong et al. (1998) reported that pH downshift from 7.5 to 5.0 resulted in an improved acid tolerance of *V. parahaemolyticus* in broth. Similarly, *Aeromonas hydrophila* exhibited an adaptive acid tolerance response capable of protecting cells at pH values as low as 3.5 (Karem et al., 1994 and Isonhood et al., 2002). These investigators showed that proteins synthesized during adaptation to pH 5.0 were involved in subsequent survival during exposure to pH 3.5. Sun and Oliver (1995) used a kanamycin resistant strain of *V. vulnificus* to evaluate the effects of Tabasco or horseradish-based cocktail sauces on the levels of *V. vulnificus* in the oyster matrix, finding a reduction in the *V. vulnificus* levels on the surface of oysters, but not within the oyster meat.

The Interstate Shellfish Sanitation Conference (ISSC) has proposed that *V. vulnificus* infections be reduced by 60% by 2007. To achieve this reduction, ISSC suggests that oysters contain <30 *V. vulnificus* cells per gram (U.S. Department of Health and Human Services, 1999). To achieve these endpoints, more stringent temperature controls, such as immediate on-board icing, has been suggested (FAO/WHO *Vibiro vulnificus* risk assessment, 2001). Furthermore, there remains interest in how well organic acid-based "natural" control strategies (such as lemon juice), might impact *V. vulnificus* survival in the oyster matrix. In both cases, the potential for exposure to sublethal stress (low temperature and low pH) to induce a stress tolerance response that might result in improved survival, must be considered. Using a previously constructed

strain of *V. vulnificus* that has been genetically engineered to produce green fluorescent protein (*Vv*-GFP-K), our objectives were to study the effect of different cooling treatments of the survival of *V. vulnificus* in the oyster matrix; and to observe the effects of different pH levels and organic acids on the survival of *V. vulnificus* in the oyster matrix.

Materials and Methods

Strain

Vv-GFP-K was constructed from *V. vulnificus* ATCC 27562 by chromosomal insertion using the methods of conjugation and transposition. The *Vv*-GFP-K strain expresses both green fluorescent protein and resistance to kanamycin. See chapter 2 for further details.

Bacteriological media

Most of the media used in this work have been previously described in the FDA Bacteriological Analytical Manual (BAM, 1998). All bacteriological media were obtained from Difco Laboratories (Detroit, MI) unless otherwise stated and were prepared according to manufacturer's recommendations, or altered accordingly based on requirements of the experimental design. All incubations were done at 37^{o}C for 48 hrs unless otherwised stated. Non-selective media included Trypticase Soy Agar with 2% NaCl plus kanamycin ($TSAN_2K$) (Sigma Chemical Co., St. Louis, M.O.). Phosphate buffered saline (PBS) was used a diluent for homogenates and alkaline peptone water (APW) was used for subsequent serial dilutions. To promote recovery of injured cells, $TSAN_2K$ was supplemented with sodium pyruvate (Sigma Chemical Co., St. Louis,

M.O.) at a concentration 80 mg/ml. All experiments were done both with and without sodium pyruvate supplementation.

Refrigerated Storage

Shellstock oysters (*Crassostrea virginica*) from the U.S. Gulf coast were obtained from local commercial sources. Oysters were washed with cold water to remove debris, and then shells were drilled into and injected with 50 µl 12 hr culture (resulting in approximately 1 x 10^6 CFU/oyster) of *Vv*-GFP-K and sealed with epoxy resin (Kaysner et al., 1989). Three shellstock oysters were placed in a Whirl-pak bag with holes to allow breathing for each sampling time point, and stored in accordance with three treatments: i) immediately placed on ice (ice cooling) until internal temperature of 5^{o}C was reached, then placed under refrigerated storage at 5^{o}C; ii) immediately placed in refrigerator at 5^{o}C (conventional cooling); and iii) allowed to drop from ambient (25^{o}C) to 5^{o}C slowly (over 8 hrs) (mild abusive cooling), followed by extended refrigerated storage at 5^{o}C. Thermocouples (TMQSS-032U-6, OMEGA Engineering Inc. Stamford, CT), were placed in the oyster meat of six oysters to monitor internal temperature during the cool-down phase (Appendix D). For all treatments, subsamples were taken on day 0 both immediately after inoculation and when internal temperature reached 5^{o}C; day1 (early and late); daily for days 2-8; and then every other day through day 22.

Each subsample (consisting of three oysters) was processed for enumeration of *Vv*-GFP-K by shucking, rinsing, homogenizing and serially diluted in APW, with plating for recovery on $TSAN_2K$ with and without sodium pyruvate supplementation.

Acid Exposure

Based on previous work at our location (data not shown), we chose acetic and citric acid (Sigma Chemical Co. St. Louis, M.O.) for the acid tolerance studies. Stock solutions of acetic acid (5M) was used to adjust the pH of $TSBN_2$ to 5.0, 4.5 and 4.0. $TSBN_2$ was adjusted to pH 5.0, 4.5, 4.0, and 3.5 using 1M stock solution of citric acid. One ml of 12 hr culture was transferred to 99 ml of fresh $TSBN_2$ (final concentration of approximately 10^6 CFU/ml) which was previously acidified to the target pH. Cells were held at 23^{o}C on benchtop and withdrawn immediately following exposure after 1, 2, 3, 4, and 5 h for acetic acid at pH 5.0; after 15, 30, 45, 60, 75, and 90 min for acetic acid at pH 4.5; and after 2, 4, 6, 8, and 10 min for acetic acid at pH 4.0. The timpoints for citric acid were as follows: after 2, 4, 6, 8, and 10 h for pH 5.0; after 1, 2, 3, 4, and 5 h for pH 4.5; after 15, 30, 45, 60, 75, 90, 105, and 120 min for pH 4.0; and after 10, 20, 30, 40, and 50 min for pH 3.5. Samples at each timepoint were serially diluted and plated for recovery on $TSAN_2K$ with and without sodium pyruvate supplementation.

We also looked at the survival of *Vv*-GFP-K when present in the oyster matrix subjected to acidic conditions. For these experiments, shellstock oysters were washed, shucked and warmed to room temperature (23^{o}C). Shucked oysters were inoculated with 50 µl of overnight culture (resulting in approximately 1 x 10^6 CFU/oyster) of *Vv*-GFP-K. Two shucked oysters per timepoint were placed in whirl pak bags with 10 mL of fresh $TSBN_2$ acidified to the target pH using stock solutions of 5M acetic acid or 1M citric acid (pH 5.0 and 4.0 for acetic acid and pH 5.0 and 3.5 for citric acid). During the experiments, oysters were maintained at 23^{o}C on benchtop, so that any decrease in the survival of *Vv*-GFP-K was due to acidic conditions alone. Subsamples were plated for

recovery on $TSAN_2K$ with and without sodium pyruvate supplementation immediately following exposure (time 0) and after 4, 8, 12, 16, 20, and 24 h for $TSBN_2$ adjusted to pH 5.0 using either acetic and citric acids, and after 2, 4, 6, and 8 h for $TSBN_2$ adjusted to pH 4.0 with acetic acid or pH 3.5 with citric acid.

Joint Effects of Refrigeration and Acidic Storage

In an effort to evaluate the joint effects of low temperature storage and exposure to acidic pH on the survival of *Vv*-GFP-K in the oyster matrix, washed and shucked shellstock oysters were equilibrated to room temperature and inoculated with 50 µl of a 12 hr culture (resulting in approximately 1 x 10^6 CFU/oyster) of *Vv*-GFP-K. Two shucked oysters per timepoint were placed in whirl pak bags with 10 mL of pre-chilled (5^oC) fresh $TSBN_2$ acidified to the target pH using 5M acetic acid or 1M citric acid (pH 5.0 and 4.0 for acetic acid and pH 5.0 and 3.5 for citric acid). During the experiments, oysters were refrigerated at 5^oC. Subsamples were withdrawn at time 0 and following exposure for 4, 8, 12, 16, 20, and 24 h for pH 5.0 acetic and citric acids; and after 2, 4, 6, and 8 h for pH 4.0 and 3.5 acetic and citric acids, respectively. Samples were serially diluted and plated for recovery using $TSAN_2K$ with and with sodium pyruvate supplementation.

Statistical Analysis

For all experiments, three replications of each treatment were performed on all recovery media. D-values were calculated as the time (in days, hours, or minutes) required for a 1-log reduction of the population using regression analysis (PROC REG). Statistical comparison of D-values was done by ANOVA (PROC MIXED), and the least-

squares' method was used to determine significant differences ($p<0.05$) (SAS Statistical Analysis Software, version 8.0, SAS Institute, Cary, N.C.).

Results

Experiments to evaluate survival upon exposure to sublethal stress were done on $TSAN_2K$ both with and without sodium pyruvate supplementation. When *Vv*-GFP-K was subjected to three different cooling treatments (mild abusive, conventional, and iced cooling) and plated on sodium pyruvate supplemented media (Fig.3.1a) there were no statistically significant differences in D-values (5.29 ± 1.54, 4.51 ± 0.63, and 5.10 ± 0.96 d, respectively) over time between the three different refrigeration treatments. Likewise when plating on non-supplemented media (Fig. 3.1b), there were no statistically significant differences in D-values (4.95 ± 1.71, 4.51 ± 1.08, and 4.76 ± 0.51 d, respectively). Pyruvate supplementation of media did not impact overall recoveries and subsequent D-values. In all cases, counts dropped 2 $\log_{10}$ after 11-12 d refrigerated storage. Steeper population declines after 12 d refrigerated storage were observed for both conventionally cooled and iced cooled treatments as compared to the mild abusively cooled product. By the end of the study (22 d), the counts of *Vv*-GFP-K for conventionally cooled and iced cooled treatments were at nondetectable levels, but mild abusively cooled oysters still had approximately 3 $\log_{10}$/g. The shelf-life for fresh oysters at the retail outlet is 21 days.

When *Vv*-GFP-K was subjected to broth acidified to pH 5.0 (0.04 M) with acetic acid (Fig. 3.2a), there was a statistically significant difference in D-values when recovery was done on sodium pyruvate supplemented versus non-supplemented media (D-values 2.64 ± 0.91 and 1.41 ± 0.16 h, respectively). Likewise, at pH 4.5 (0.08 M) (Fig. 2b),

there was a statistically significant difference between D-values comparing sodium pyruvate supplemented to non-supplemented media (D-values 19.99 $\pm$ 0.18 and 17.35 $\pm$ 0.53 min, respectively). At pH 4.0 (0.21 M) (Fig. 3.2c) although D-values could not be calculated due to biphasic inactivation kinetics, there appeared to be little difference in survival when comparing sodium pyruvate supplemented to non-supplemented media. In both cases, cells reached nondetectable levels within 10 min.

For cells subjected to broth acidified to pH 5.0 (0.02 M) with citric acid (Fig. 3.3a), *Vv*-GFP-K populations remained constant for 10 h. At pH 4.5(0.02 M)(Fig. 3.3b), D-values ranged from 1.2-1.4 hr, and there was no statistically significant difference in D-values when enumeration was done using pyruvate supplemented or non-supplemented media (D-values 1.38 $\pm$ 0.24 and 1.24 $\pm$ 0.21 h, respectively). Similar results were observed for pH 4.0 (0.033M) (Fig. 3.3c), with shorter D-values (approximately 30 min). At pH 3.5(0.06 M) (Fig. 3.3d), cells died rapidly, reaching nondetectable levels within 50 min.

When *Vv*-GFP-K was inoculated directly into shucked oysters which were then exposed to acidic conditions and held at room temperature, populations for both acids and all pH values tested, irrespective of pyruvate supplementation remained stable (Fig. 3.4a,b,c,d). When a similar experiment was done at refrigeration temperatures, *Vv*-GFP-K levels were stable for 24 hr in acetic or citric acids (Fig. 3.5a and c) at pH 5.0, and for 8 hr when stored in acetic acid at pH 4.0 (Fig. 3.5b) or citric acid at pH 3.5 (Fig. 3.5d). Again, no difference was observed between recoveries on pyruvate supplemented or non-supplemented media.

Discussion

There is concern for increased survival of pathogens due to cold and/or acid adaptation. In 1995, there was salmonellosis outbreak associated with the consumption of unpasteurized orange juice contaminated with *Salmonella enterica* serovars. Hartford, Gaminara, and Rubislaw (Parish, 1998). Later, another large outbreak associated with unpasteurized apple juice contaminated with *Escherichia coli* O157:H7 (Cody et al., 1999) was reported. Historically, unpasteurized juice products were considered unlikely vehicles for foodborne pathogen transmission because the low pH (high organic acid content) was thought to inactivate these Gram negative pathogens. However, acid adaptation of *Salmonella enterica* serovar. Typhimurium has been studied in-depth. For instance, Leyer and Johnson (1992) found that acid adapted serovar. Typhimurium had better survival capabilities than non-adapted cells during milk fermentation and curing of various cheeses. Fermentation places other stresses on bacteria as well, including exposure to hydrogen peroxide, increased salt concentration, decreased water activity, and various antimicrobial agents such as bacteriocins, lysozyme and lactoperoxidase. Leyer and Johnson (1993) also reported that acid adapatation in serovar. Typhimurium induced cross-protection against some of these other environmental stresses.

Acid and cold adaptation have been well studies in *E. coli* O157:H7, too. In general, *E.coli* O157:H7 survives and may even grow in products of low pH (3.6-4.0) that are also stored at refrigeration temperatures. For instance, Zhao et al. (1993) reported survival of *E. coli* O157:H7 in cider lacking intentionally added preservatives (e.g., sodium benzoate or potassium sorbate) for 10 to 31 days and 2 to 3 days at 8 and 25^{o}C, respectively. In a similar study, *E. coli* O157:H7 declined to undetectable levels within 7

days at 25°C in unpreserved cider, while slight growth was observed during 20 days of storage at 8°C (Besser et al., 1993). After 21 days of storage at 4°C, Miller and Kaspar (1994) recovered up to 98% of *E. coli* O157:H7 in unpreserved cider. The addition of preservatives reportedly decreased survival of *E. coli* O157:H7 in cider. Weagant et al. (1994) looked at the survival of three strains *E. coli* O157:H7 in four mayonnaise-based sauces stored at either 5 or 25°C. All *E. coli* O157:H7 strains reached nondetectable limits within 72 hrs in all four sauces held at 25°C; whereas levels of *E. coli* O157:H7 in contaminated product held at 5°C decreased at a much slower rate, with viable cells detected after as long as 35 days of refrigerated storage.

E. coli O157:H7 shows enhanced survival in acidified food products, and its acid adaptation response is well characterized. Leyer et al. (1995) reported that *E. coli* O157:H7 cells pre-conditioned by exposure to moderately low pH (5.0) had better survival than non-adapted cells during sausage and hard salami fermentation and in apple cider. Massa et al. (1997) observed that non-adapted *E. coli* O157:H7 inoculated into yogurt mix only decreased 2 $\log_{10}$ CFU/ml during the traditional yogurt fermentation process. In this case, pH values dropped from 6.6 to 4.4 over 5 hours, suggesting that there is enough time for adaptation to occur during fermentation itself. In a broth model study, Ryu et al. (2001) demonstrated that various organic acids differ in their inhibitory or lethal activity against acid-adapted and non-adapted *E. coli* O157:H7 cells. For instance, both adapted and non-adapted *E. coli* O157:H7 cells were able to grow after 4 hrs of exposure to malic and citric acid (pH 4.5), whereas populations remained stable for 24 hrs in broth acidified (pH 3.9 or pH 3.4) with the same two organic acids, regardless of prior conditioning. In this same study, the acid adaptation response was seen when *E.*

coli O157:H7 was exposed to lactic or acetic acid at pH values of 3.9 or 3.4, with conditioned cells demonstrating about a one $\log_{10}$ improvement in survival at room temperature, when compared to non-adapted cells.

In broth studies, *V. vulnificus* has been shown to have similar adaptative responses to cold and acid. For instance, Bryan et al. (1999) reported that *V. vulnificus* entered the so-called VBNC state when the temperature was shifted from 35^0C to 6^0C; however, when the culture was subjected to 15^0C prior to further temperature downshift, the cells remained culturable, showing an adaptive response to cold temperature. McGovern and Oliver (1995) demonstrated that forty unique proteins were synthesized by *V. vulnificus* during this same temperature conditioning phase. Likewise, Bang and Drake (2001) showed improved survival for some *V. vulnificus* strains were conditioned to 15^oC for 4 h and then shifted to 5^oC, compared to those cells that were directly subjected to 5^oC without prior conditioning.

Although we did not look at acid adaptation per se, we did look at survival of *V. vulnificus* cells in different organic acids at different pH values. In broth studies, after 5 hrs of exposure to acetic acid (pH 5.0), significant inactivation occurred when recovering cells on media without sodium pyruvate supplementation, with a 2 $\log_{10}$ improvement in recovery using supplemented media. Similar, but not as dramatic results were seen for citric acid (pH 4.5). As pH values declined, the positive impact of pyruvate supplementation on cell recovery disappeared. A different story was observed with regards to survival of *Vv*-GFP-K strain in the oyster matrix at 23^oC for 24 hrs. Contrary to the broth model, there was no reduction in *Vv*-GFP-K levels within the oyster meat between 8 to 24 hrs, regardless of pH or organic acid type. The oyster meat provided a

protective environment that prevented inactivation of *Vv*-GFP-K, and although not evaluated in this study, may provide an opportunity for acid adaptation to occur in the matrix.

Refrigeration is an excellent method to control the multiplication of *V. vulnificus* in oysters. Cook (1994) observed that *V. vulnificus* did not multiply in oysters stored a $<13^0$C; however, significant growth did occur in oysters stored at 18^0C or above. Cook (1997) later observed that the largest increase in numbers of *V. vulnificus* in freshly harvested shellstock oysters occurred during the first 3.5 to 5 hours after harvest. The growth of *V. vulnificus* is rapid in unrefrigerated oysters and peaks after only 12 hours. Although refrigeration controls growth, Cook and Ruple (1992) observed that it took 14 to 21 days of refrigerated storage to reduce *V. vulnificus* in shellfish to undetectable levels.

Our cold temperature storage study results were similar to those of Cook and Ruple (1992) in that *Vv*-GFP-K levels in artificially contaminated shellstock that were conventionally cooled or ice cooled oysters reached nondetectable levels after 22 days of refrigerated storage. However, mild abusively cooled oysters still had approximately 10^3 CFU/oyster at the end of 22 days. During the 8 hours in which it took the mild abusively cooled oysters to reach 5^oC, there is the possibility for cold adaptation, which may be responsible for the improved survival of *Vv*-GFP-K under long-term refrigerated storage.

When evaluating *V. vulnificus* survival in oysters subjected to the joint effects of cold storage and acidic pH, there was no decrease in *Vv*-GFP-K levels over the entire 24 hour storage period at 5^oC. Again, the oyster meat was protective of inactivation even

when the two stressful conditions were combined. This would likely occur for naturally contaminated shellstock as well.

In this study, we used a GFP-expessing strain of *V. vulnificus* to monitor the survival of *V. vulnificus* after exposure to different processing controls that oyster might encounter from harvest to consumption. Cold temperature studies demonstrated that cooling methods alone cannot be relied upon to elimatinate *V. vulnificus*. Furthermore, organic acids (acetic or citric) had no affect on the survival of *V. vulnificus* within the oyster meat. The oyster meat appeared to provide a protective environment that prevented inactivation of *Vv*-GFP-K under these conditions.

CONCLUSIONS

In this work, we describe a method to develop a readily identifiable strain of *V. vulnificus* by inserting two selective markers, one coding for the production of green fluorescent protein and the other for kanamycin resistance. This provided an engineered strain that could be used to evaluate the survival of the organism under conditions of sublethal stress both in the broth model and in the oyster matrix. Initial experiments sought to compare the growth and survival of *Vv*-GFP-Kcompared with the wild-type parent. In our study, *Vv*-GFP-K and *Vv*-WT were frequently comparable with respect to physiological and biochemical characteristics. A few differences in survival were observed, particularly with respect to acid tolerance (i.e., through acidification with inorganic acids). These differences in acid tolerance may be due to the location of the gene insert, although this has not been confirmed. Interestingly, these differences frequently disappeared when recovery media was supplemented with sodium pyruvate, suggesting that the cells were indeed sublethally injured and hence sensitive to hydrogen peroxide buildup in the media. Even when statistically significant differences were observed between *Vv*-WT and *Vv*-GFP-Kon media both with and without pyruvate supplementation, the use of sodium pyruvate resulted in a more conservative estimate of survival when comparing *Vv*-GFP-K to *Vv*-WT. *Vv*-GFP-K was therefore deemed a good surrogate for *Vv*-WT to evaluate survival upon exposure to processing-related stress, provided that sodium pyruvate supplemented recovery media was used.

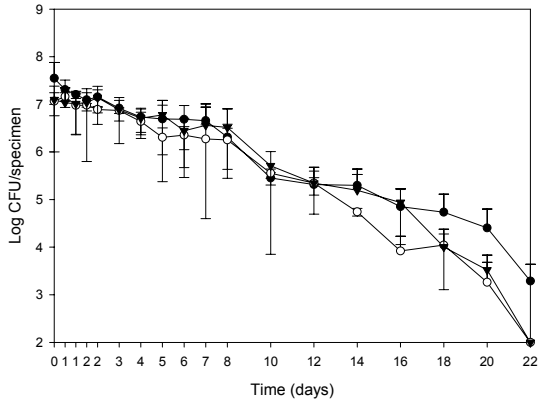
Cold temperature storage studies done in the oyster matrix showed that *Vv*-GFP-K reached nondetectable levels after 22 days of refrigeration in conventionally cooled or ice cooled oysters. However, mild abusively cooled oysters still had approximately 10^3

CFU/oyster at the end of 22 days. Since the levels of *Vv*-GFP-K did not increase during the cool-down period, these data suggest that in mild abusively cooled oysters, the organism did indeed adapt to the cooler storage temperatures, facilitating its long-term survival at refrigeration temperatures. Furthermore, we can conclude that cooling alone cannot be relied upon to eliminate *V. vulnificus*. Organic acids (acetic or citric acid) had no affect on the survival of *V. vulnificus* within the oyster meat, a result that was completely different from model broth studies. It is clear that the oyster matrix is protective of the effects of acidification, and therefore it is unlikely that the use of acidic condiments with raw oysters will control disease risks associated with *V. vulnificus*. Overall, we can conclude that *Vv*-GFP-K was an effective surrogate in this application, and design of similarly marked strains of other foodborne pathogens may be an effective strategy to be used when evaluating microbial behavior in other complex sample matrices.

Although we developed, validated, and tested the *Vv*-GFP-K strain in this study, there are other potential uses for this strain. For instance, *Vv*-GFP-K could be used to evaluate other processing techniques such as high pressure, specifically in the oyster matrix. Furthermore, *Vv*-GFP could be used to monitor entrance into the VBNC state by measuring total fluorescence as a function of metabolic activity. Another possibility would be to use the strain to monitor level of contamination from harvest through processing, retail, and up to consumption. Since it is well known that oysters contain a variety of *Vibrio* species and specific strains, a similar selective marker approach could be used to develop multiple strains of gfp/kanamycin *V. vulnificus* and/or *V. parahaemolyticus* which could be used in combination.

With regulatory agencies seeking to reduce *V. vulnificus* infections by 60% by 2007, effective control of *V. vulnificus* in raw shellfish is vital. It is apparent that refrigeration and organic acids do not eliminate *V. vulnificus*. Currently, heating, irradiation, and high pressure are the only methods to effectively control this organism, but all of these can impact organoleptic quality. It is our hope that this surrogate may provide an additional tool as we seek to understand the ecology of *V. vulnificus* in the environment and in shellfish, with the ultimate goal of reaching the ISSC mandate as soon as possible.

A.



B.

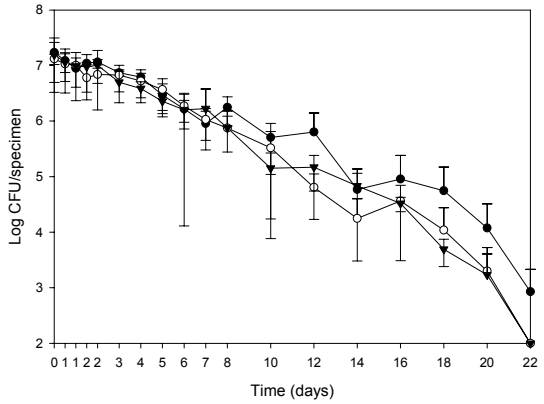
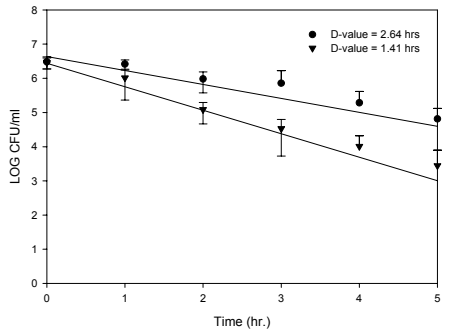


FIG. 3.1 Survival of *Vv*-GFP-K in shellstock oysters plated with and without sodium pyruvate supplementation (A) $TSAN_2K$ with sodium pyruvate (B) $TSAN_2K$ without sodium pyruvate (●) dropped from 25°C to 5°C over 8 hrs, (○) placed directly into 5°C, and (▼) immediately iced.

A.



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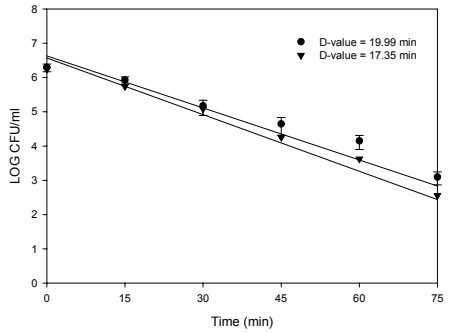

C.

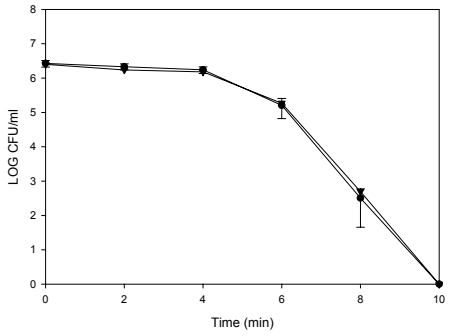

FIG 3.2. Survival of *Vv*-GFP-K in acidified $TSBN_2$ with acetic acid plated with and without sodium pyruvate supplementation (A) pH 5.0, (B) pH 4.5, and (C) pH 4.0 (●)$TSAN_2K$ with sodium pyruvate and (▼)$TSAN_2K$ without sodium pyruvate.

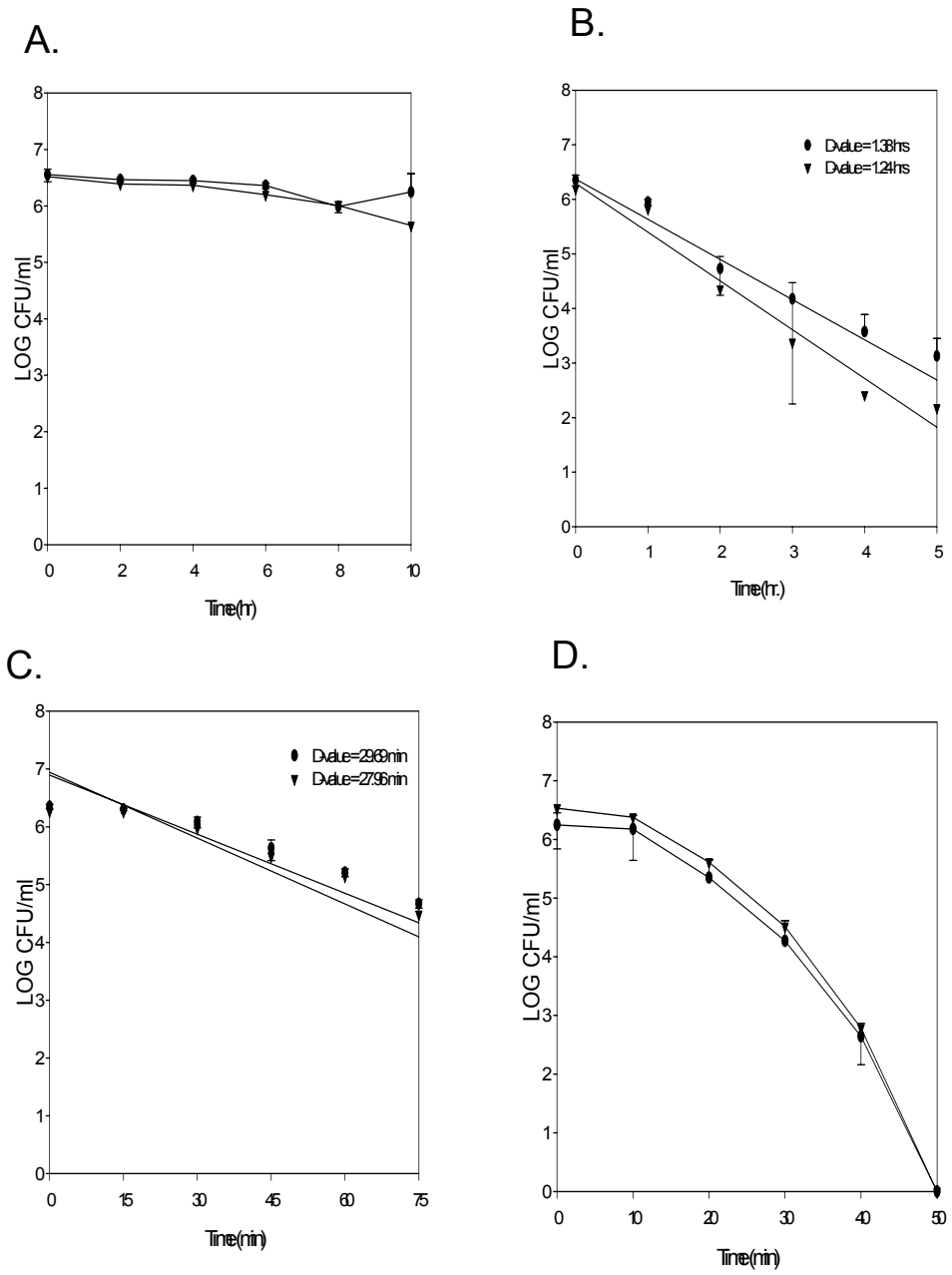

FIG 3.3. Survival of *Vv*-GFP-K in acidified $TSBN_2$ with citric acid plated with and without sodium pyruvate supplementation (A) pH 5.0, (B) pH 4.5, (C) pH 4.0, and (D) pH 3.5 (●)$TSAN_2K$ with sodium pyruvate and (▼) $TSAN_2K$ without sodium pyruvate.

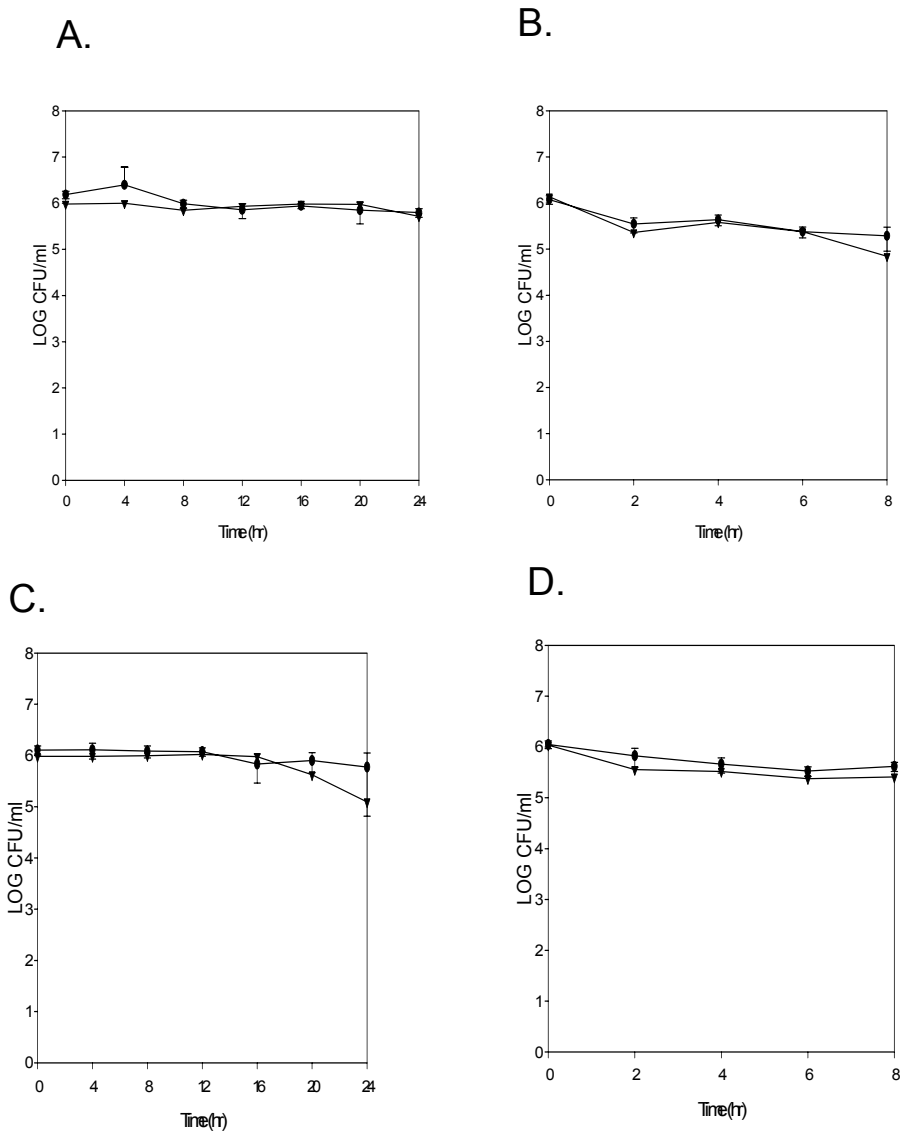

FIG 3.4. Survival of *Vv*-GFP-K in shucked oysters and acidified $TSBN_2$ held at 23°C and plated with and without sodium pyruvate supplementation (A) pH 5.0 with acetic acid, (B) pH 4.0 with acetic acid, (C) pH 5.0 with citric acid, and (D) pH 3.5 with citric acid (●)$TSAN_2K$ with sodium pyruvate and (▼)$TSAN_2K$ without sodium pyruvate

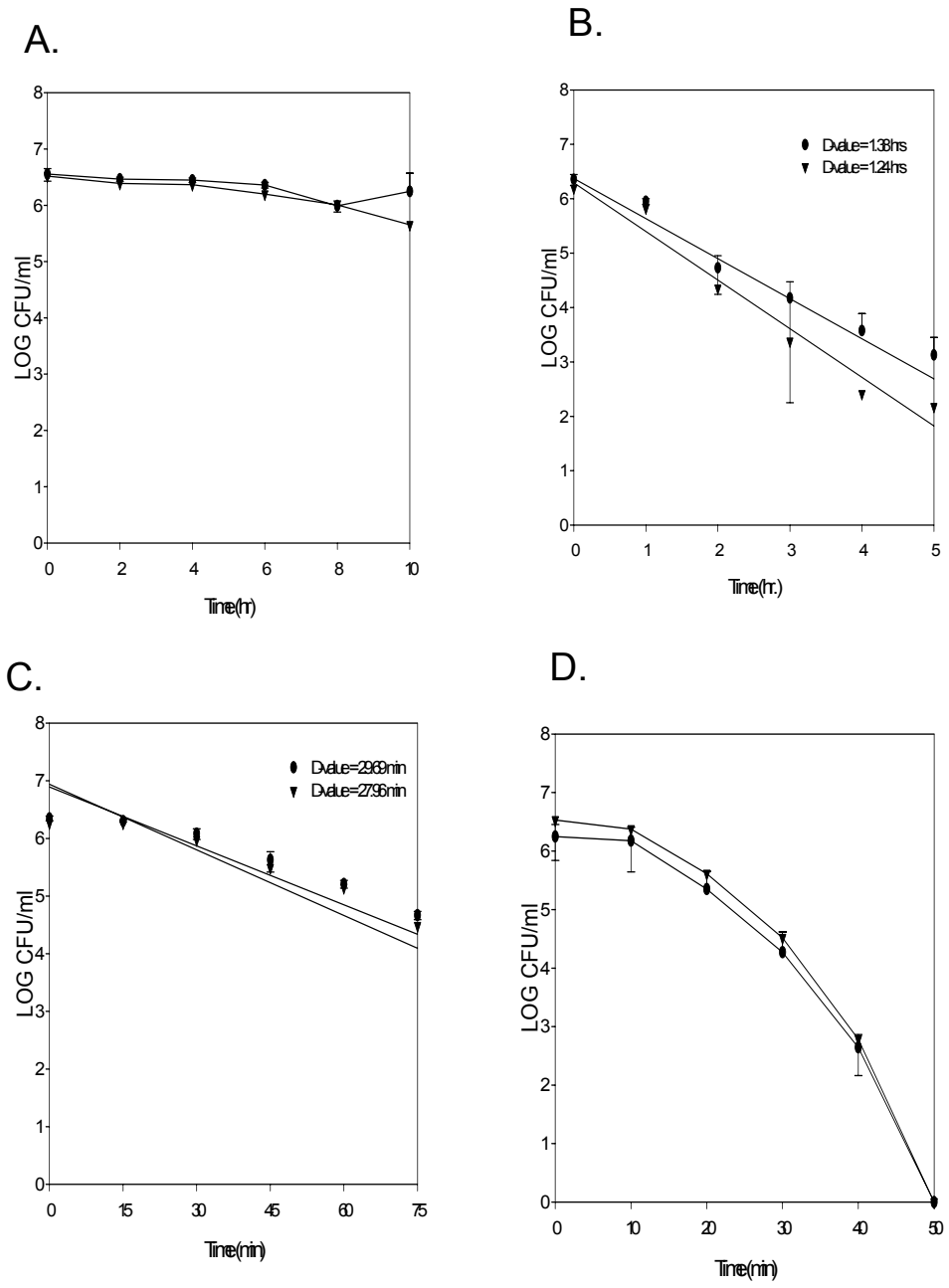

FIG 3.5. Survival of *Vv*-GFP-K in acidified $TSBN_2$ with citric acid plated with and without sodium pyruvate supplementation (A) pH 5.0, (B) pH 4.5, (C) pH 4.0, and (D) pH 3.5 (●)$TSAN_2K$ with sodium pyruvate and (▼) $TSAN_2K$ without sodium pyruvate.

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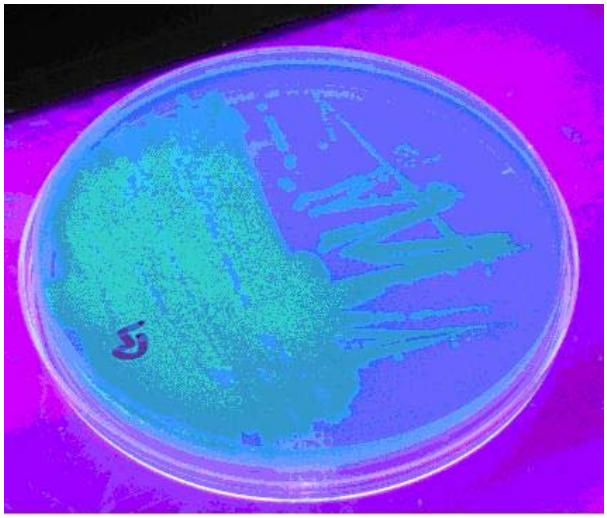
APPENDICES

APPENDIX A: Appearance of *Vv*-GFP-K expressing fluorescence under ultraviolet light on $TSAN_2$ compared to *Vv*-WT.

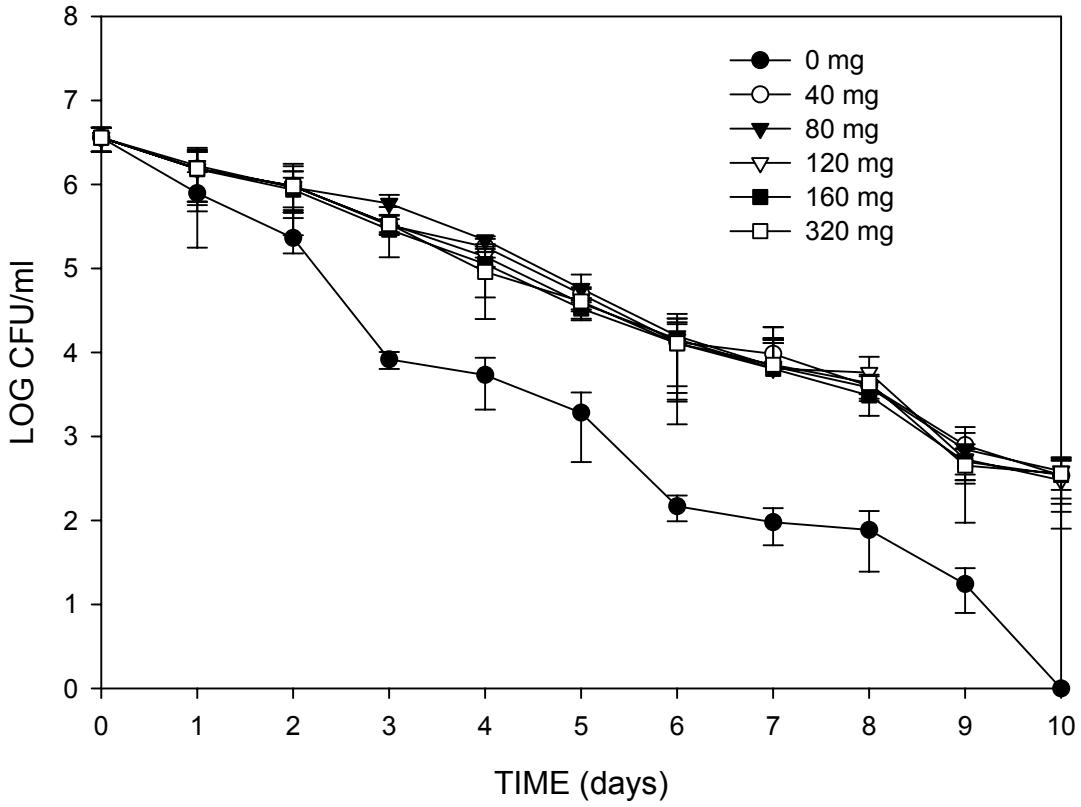


APPENDIX B: Morphological and biochemical comparisons between *Vv*-WT and *Vv*-GFP-K.

Media for Morphologic Comparison	***Vv*-WT**	***Vv*-GFP-K**
Tryticase Soy Agar supplemented with 2% NaCl ($TSAN_2$)	Round opaque colonies	Round opaque colonies
Gelating Agar (GA)	No Growth	No Growth
Gelating Agar supplemented with 2% NaCl (GS)	Growth	Growth

Tests for Biochemical Comparison	***Vv*-WT**	***Vv*-GFP-K**
Motality Test Medium supplemented with 2% NaCL	Motile	Motile
o-nitrophenyl-B-D-galactosidase Test (ONPG)	Positive	Positive
Triple Sugar Iron Slant supplemented with 2% NaCl (TSI)	Positive	Positive
Fermentation of cellobiose	Positive	Positive

APPENDIX C: Recovery of injured cells due to starvation at 5^{o}C using different concentrations of sodium pyruvate (0-320mg).



APPENDIX D: Cooling curves for cold temperature studies at mild abusive, normal, and iced cooling methods