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ALTERATION IN SENSITIVITY OF STRESS-ADAPTED LISTERIA INNOCUA TO THE CHEMICAL SANITIZER CETRIMIDE

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ALTERATION IN SENSITIVITY OF STRESS-ADAPTED LISTERIA INNOCUA TO THE CHEMICAL SANITIZER CETRIMIDE

By

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ABSTRACT

ALTERATION IN SENSITIVITY OF STRESS-ADAPTED LISTERIA INNOCUA TO THE CHEMICAL SANITIZER CETRIMIDE

By

Mark A. Moorman

Experts in food hygiene have long struggled to eliminate microorganisms established within the food-manufacturing environment. Such environments may be contaminated with pathogenic or spoilage organisms that evade sanitation and contaminate food. It was hypothesized that sensitivity of *L. innocua* to the quaternary ammonium compound sanitizer cetrimide is altered following adaptation to acid, starvation, cold and heat stress, stressors commonly found within the food manufacturing environment and this relates to altered cell hydrophobicity and membrane fluidity. This research demonstrated that exposure of *L. innocua* to acid and starvation stress diminishes sensitivity to 10 ppm cetrimide while exposure to cold and heat stress enhance sensitivity. Furthermore, acid and starvation stress increased net cell hydrophobicity and reduced cell membrane fluidity. In contrast, decreased hydrophobicity and increased membrane fluidity were observed in cold adapted *L. innocua*. No significant changes in hydrophobicity or indicators of membrane fluidity, aside from increased C-18 unsaturated fatty acids, were detected in heat adapted *L. innocua*.

That certain environmental conditions within food manufacturing facilities such as acid and starvation could diminish cellular sensitivity to industrial sanitizers suggest the physiological stress response not only diminishes sensitivity to the stress, but also

enables persistence upon exposure to low levels of quaternary ammonium compound sanitizers. Conversely, that other modifications of the environment, such as cold temperature, would stress-adapt and concurrently enhance sensitivity of *L. innocua* to quaternary ammonium compounds suggest interventions exist that enhance sanitation efficacy. The potential exists therefore, for the application of stress conditions to equipment or manufacturing sites persistently testing positive for problematic microorganisms, and thereby diminish the ability the microorganisms to survive sanitizer exposure.

This dissertation is dedicated to my close friend and mentor Dr. John Silliker. While the long lunches at Olympia Fields Country Club covered innumerable topics better left undocumented, the many on the discipline of food microbiology coupled with his unending encouragement, provided me the confidence and thirst necessary to complete this dissertation. I will forever be grateful to Dr. Silliker for his friendship and interest in teaching me about the fascinating field of food microbiology.

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CHAPTER I. LITERATURE REVIEW

INTRODUCTION

The purpose of this introduction is to review the current literature concerning 1) the effect of the chemical sanitizers chlorine and quaternary ammonium containing compounds on microorganisms 2) the response of microorganisms to sub-lethal heat and 3) the adaptation of microorganisms to acidic cold and starvation environments.

CHEMICAL SANITATION

Cleaning of food manufacturing equipment is performed to remove accumulated soils (carbohydrate, proteins, fats, and minerals) and to purge the resident microorganisms. In general terms, sanitation is comprised of cleaning to remove soils, and chemical sanitizer application to destroy microorganisms remaining after cleaning. During the chemical cleaning and sanitizing process microorganisms are exposed to a) alkali or acid conditions from cleaning compounds b) heat during equipment rinse and cleaner application and c) chemical sanitizers. The two basic classes of cleaning compounds used in the food industry are alkaline and acid cleaners (Juneja, Foglia et al. 1998). Most cleaners are alkaline while acids are used to remove highly insoluble mineral deposits on equipment (Dychdala 2001).

The basic functions of the alkali cleaners are peptidization of proteins and emulsification and saponification of fat (Phan-Thanh and Gormon 1995; Phan-Thanh and Gormon 1997). In chlorinated alkali cleaners, used in clean-in-place (CIP) systems,

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The basic functions of the alkali cleaners are peptidization of proteins and emulsification and saponification of fat (Phan-Thanh and Gormon 1995; Phan-Thanh and Gormon 1997). In chlorinated alkali cleaners, used in clean-in-place (CIP) systems, chlorine serves not as a sanitizer but to increase the peptizing efficiency of the alkaline compounds (Dychdala 2001).

The soil rinse (35 - 43°C) and detergent application (46 – 48.9 °C) temperatures are sub-lethal for most microorganisms and are ideal for the induction of heat shock proteins (HSP) in *Saccharomyces cerevisiae* (Piper 1996), *Listeria monocytogenes* (Farber and Brown 1990), *E. coli* (Arsene, Tomoyasu et al. 2000), and *Campylobacter jejuni* (Konkel, Kim et al. 1998). Production of heat shock proteins permits survival of the heat-induced microorganism at higher temperatures that would otherwise be lethal. The adaptation of *L. monocytogenes* to heat conditions encountered in inadequately cleaned and sanitized environments may induce thermotolerance in this microbe and promote its survival in foods receiving mild heat treatment (Taormina and Beuchat 2001). Interestingly, alkali exposure (pH 12 for 45 minutes), similar to conditions encountered with alkali cleaners, induces thermotolerance in *L. monocytogenes* (Taormina and Beuchat 2001). It is unknown if alkali-induced thermotolerance is a result of heat shock protein expression.

Since chemical sanitizers are the principal means by which the food industry controls microorganisms in wet manufacturing environments, understanding the impact of microbial adaptation on sanitizer efficacy is critical for design of interventions that may improve effectiveness of food plant sanitation.

CHLORINE SANITIZERS

The three most common classes of sanitizers contain chlorine, quaternary ammonium compounds and iodine as active ingredients. Among the sanitizer subclasses, the hypochlorites are the oldest and most widely used (Dychdala 2001). Hypochlorite germicidal efficacy diminishes with increasing pH and with the presence of organic matter, but is unaffected by water hardness and increases with temperature and concentration (Dychdala 2001).

The bactericidal mechanism for hypochlorite (HOCl) is still incompletely understood. Early theories proposed that chlorine destroys bacteria by combining with proteins of cell membranes (Baker, 1926). Later work confirmed the irreversible oxidation of chlorine on sulfhydyl groups of vital enzymes (Knox, 1948) including ATPase within the cytoplasmic membrane which catalyzes respiration linked phosphoanhydride bond formation between ADP + Pi (Barrette, Hannum et al. 1989). Cells that are exposed to lethal concentrations of HOCl are unable to maintain the necessary levels of cytoplasmic ATP. The net loss of the cytoplasmic pool of ATP results from oxidative damage to as few as two amino acids within subunits of the F_1-ATPase complex (Hannum, Barrette et al. 1995).

Increasing membrane permeability might also increase the germicidal activity of chlorine compounds. EDTA increased permeability of the cellular membrane of

Salmonella Typhimurium and increased HOCl lethality (Leyer and Johnson 1997). Although the bactericidal mechanism of free chlorine is debated, the bactericidal activity of lethal concentrations of free chlorine is nevertheless established.

Microorganisms have evolved systems to resist and adapt to oxidative compounds such as superoxides, peroxides and hypochlorous acid within neutrophils and macrophage. It is notable that sub-lethal HOCl concentrations (0.2 mg/liter) induce the alternative sigma factor (σ^{32}) heat shock promoter rpoH in *E. coli* (Dukan, Dadon et al. 1996) and heat shock proteins in *E. coli* O157:H7. σ^{32} regulates the transcription of *DnaK* and other heat-shock genes (Lindquist and Craig 1988). Heat shock proteins reside within the cytoplasm and function to fold translated proteins. Thus, thermotolerance may be induced in microorganisms within the food manufacturing environment that are exposed to sub-lethal HOCl concentrations.

QUATERNARY AMMONIUM COMPOUNDS (QAC)

Quaternary ammonium compounds primarily act by disrupting cellular permeability of the cytoplasmic membrane (Merianos 2001). The net negative charge of the cell membrane renders the cell impermeable to polar, charged and hydrophobic molecules. This bacterial cytoplasmic membrane assumes the functions of all internal membranes of eukaryotic organisms including ATP synthesis by F_1ATPase, electron transport and photosynthesis, chromosome segregation, synthesis of membrane proteins, secretory proteins, lipids and the cell wall. QAC below 12 carbons in length interfere at receptor sites for many biologically active compounds (Merianos 2001). When length exceeds 12 carbons, these compounds become surface active and antimicrobial. As polycationic surface-active agents, they reduce the surface tension of electrochemically

repulsed materials, including the negatively charged microorganism, thus permitting interaction of substances that are otherwise repulsed. QAC disrupt the cytoplasmic membrane after 1) adsorption of compound on the bacterial cell surface, 2) diffusion through the cell wall, 3) binding to cytoplasmic membrane, 4) disruption of cytoplasmic membrane, 5) release of potassium ions and other cytoplasmic constituents and 6) precipitation of cell contents and death of the cell (Merianos 2001). Microorganisms can modify the fatty acid composition of their membrane lipid bilayer in response to changes in their environment. *Pseudomonas aeruginosa* is known to be resistant to antibiotics and disinfectants and can modify its fatty acid composition when grown in the presence of two quaternary ammonium compounds. (Guerin-Mechin, Dubois-Brissonnet et al. 1999).

BACTERIAL MEMBRANES

The microbial response to stress involves changes in protein and membrane lipid composition that collectively enable the microorganism to persist. The bacterial membrane serves as an interface between the external environment and the cellular cytoplasm, the composition of which it helps to regulate. The membrane performs vital functions, such as maintenance of the proton-motive force and the uptake of nutrients (Russell, Evans et al. 1995). The membrane is composed of lipids that are united not by a common structural feature, but by the common physical property of being insoluble in water and soluble in organic solvents (Voet and Voet 1995). The primary lipids of a biological membrane are phospholipids, a group of molecules with a structure related to triglycerides (Fig. 2). The 3-carbon glycerol molecule is esterified to fatty acids at two carbons while the third links to a bridging phosphate group often bound to a nitrogen

containing alcohol. The amphipathic phospholipid naturally orients the hydrophobic tail within the inside of the membrane exposing the hydrophilic moiety to the external aqueous environment (Fig 3). We believe it is likely the bacterial stress response induces protein and fatty acid compositional changes which collectively alter a cells sensitivity to chemical sanitizers.

BACTERIAL LIPID COMPOSITION AND MEMBRANE FLUIDITY

The fluidity of biological membranes is one of their important physiological attributes since it permits their embedded proteins to interact. As the cell membrane of Gram-positive bacteria is comprised of 75% protein and 25% lipid (Voet and Voet 1995), the movement or fluidity of these proteins is critical for cellular vitality. The primary way that bacteria maintain constant membrane fluidity at different growth temperatures is by adjusting their fatty acid composition (Russell, Evans et al. 1995). The ability of bacteria to change their membrane fluidities determines to some extent how well a bacterium tolerates certain environmental stresses (Li, Chikindas et al. 2002) and is based in part upon the fatty acid composition. Saturated fatty acid membranes are tightly packed and non-fluid while branched fatty acid membranes have increased fluidity. The cell will modify the fatty acid composition (chain length, branched, saturated/unsaturated) of the lipids in the membrane to sustain viability in diverse and stressful environments. Membranes are fluid when composed of unsaturated and branched fatty acids and relatively non-fluid when containing saturated or tightly packed fatty acids. High melting point fatty acids (saturated long and normal chain) decrease membrane fluidity whereas low melting point fatty acids (unsaturated and branched chain) increase membrane fluidity (Table 1) (Russell, Evans et al. 1995).

Hydrophobicity of the cell is based upon the cells protein and fatty acid composition. Protein and fatty acid hydrophobicity is influenced by amino acid and fatty acid (acyl chain length, the degree of saturation and branch position) composition (Li, Chikindas et al. 2002). The interaction of the cell with the surrounding environment, and molecules that may affect the microorganism, may be influenced by the net hydrophobicity of the cell. The lipid bilayer of the microbial cell is extraordinarily impermeable to ionic and polar substances (Voet and Voet 1995). Changes in fatty acid composition likely influence the net hydrophobicity of the cell and the ability of bioactive molecules, such as quaternary ammonium compounds, to affect the cell. The complex change in proteins and fatty acid composition in response to stress may influence the cells net hydrophobicity.

FATTY ACID CHAIN LENGTH AND SATURATION

Lipids within a bacterial membrane have a transition temperature, below that temperature the lipids are in an orderly array or gel-like solid. This orderly array decreases membrane fluidity and cellular function. The transition temperature of the lipid increases with the chain length and the degree of saturation of its component fatty acid residues (Voet and Voet 1995). The membrane becomes more gel-like as the chain length increases or becomes more saturated. Fatty acid composition changes in three ways in response to temperature fluctuation: acyl chain length, the degree of saturation, and the branch position of the fatty acids. In *L. monocytogenes,* the major cell membrane response to temperature changes is alteration of the fatty acid component of the membrane's lipids; changes in the head group composition are generally minor (Li, Chikindas et al. 2002). *L. monocytogenes* membrane fluidity is maintained upon

temperature reduction by an increase in C15 and decrease in C17 (increased ratio) and an increase in C-18:1 (Chihib, Ribeiro da Silva et al. 2003) (Russell, Evans et al. 1995). Shortening fatty acid chain length or increasing unsaturation results in low melting point fatty acids (Annous, Becker et al. 1997). These changes in membrane composition are reversible and may occur in a few hours (Li, Chikindas et al. 2002).

BRANCHED CHAIN (ANTEISO/ISO) FATTY ACIDS

Branched chain fatty acids in microorganisms typically have a methyl group in the *iso*-methyl (branch point on the penultimate carbon *i.e.* one from the end) or anteiso-methyl (branch point on the *ante*-penultimate carbon atom *i.e.* two from the end) (Christie 2004). Anteiso fatty acids have lower melting points than iso fatty acids and contribute to increased membrane fluidity. Lower melting point fatty acids are less likely to be in the gel-like or non-fluid state than higher melting point fatty acids. As the anteiso number increases, the cell is lowering its melting point. Anteiso C-15 in *L. monocytogenes*, coupled with unusually low levels of straight-chain saturated fatty acids plays a critical role in providing an appropriate degree of membrane fluidity for growth at low temperatures (Annous, Becker et al. 1997).

The susceptibility of *L. monocytogenes* to the antimicrobial activity of nisin illustrates the relationship of membrane lipid composition to bioactive molecules. Nisin, a 34 amino acid-containing protein produced by a *Lactococcus lactis* ssp. *lactis*, has antimicrobial activity principally against gram-positive bacteria (Davidson and Harrison 2003). Nisin has GRAS status and is used in heat processed and low pH foods (Register 1999). The bactericidal activity of nisin is due to pore formation in the bacterial membrane, which occurs through a four-step process of binding to the anionic (net

negative charge) phospholipids of the cell membrane, insertion into the membrane and pore formation (Davidson and Harrison 2003). The cell's sensitivity to nisin is influenced by the membrane's lipid composition, which might act on any of the fours steps. Nisin-resistant strains of *L. monocytogenes* had altered phospholipid compositions resulting in a decreased net negative charge that hindered binding of the cationic (net positive charge) nisin compound (Davidson and Harrison 2003). These nisin resistant strains had increased long chain fatty acids and reduced ratios of C15/C17 fatty acids resulting in reduced membrane fluidity (Mazzotta and Montville 1997). Mildly acidic conditions (pH 5.5) diminished nisin sensitivity of *L. monocytogenes* and are related to increases in C-14:0 and C-16:0 and decreases in C-18:0 fatty acids (van Schaik, Gahan et al. 1999). Predicting the effect of altered hydrophobicity on the ability of the amphipathic sanitizer cetrimide to interact with the cell membrane is difficult.

GROWTH AND SURVIVAL WITHIN ACIDIC ENVIRONMENTS

Undoubtedly the ability of a microorganism to induce stress-response proteins and consequently adapt to a hostile environment plays a major role in the ubiquity of microorganisms in diverse environments. One such hostile environment frequently encountered by microorganisms is acidity. The acid tolerance response (ATR) describes the phenomenon whereby bacteria that have been exposed to mildly acidic conditions acquire the ability to survive at normally lethal pH values (Hill, O'Driscoll et al. 1995). Key issues are how acid adaptation increases microbe virulence and induces protection against stress encountered in food and food manufacturing environments.

ACID TYPES AND EMERGENCY PH HOMEOSTASIS

The outer cell membrane is intrinsically impermeable to polar, charged or hydrophobic molecules including the protons liberated by acidic compounds. Hence inorganic acids are largely incapable of penetrating the cell membrane and acidifying the cytoplasm. Undissociated lipid permeable weak acids such as acetic and citric are able to pass through the membrane with relative ease and may dissociate to liberate protons in the cytoplasm (Hill, O'Driscoll et al. 1995). Reducing cytoplasmic (intracellular) pH acidifies the menstrum that permits all vital biochemical reactions. This acidification can lead to the denaturation of acid sensitive proteins and cell death (Hill, O'Driscoll et al. 1995).

A cell can maintain intracellular pH near neutrality via passive and active mechanisms (Hill, O'Driscoll et al. 1995). Passive mechanisms include cell membrane impermeability to protons and the inherent buffering capacity of the protein-rich cytoplasm (Hill, O'Driscoll et al. 1995). Active mechanisms require energy (ATP) to transport H^+ ions out of the cell while importing potassium ions. In *Enterobacteriaceae*, an emergency pH homeostasis system has been described that functions to keep intracellular pH (pH_i) above 5 as the organism encounters severe acid outside of the cell (pH_o). Within the cell are pools of positively charged amino acids such as lysine, histidine and arginine. Upon acid shock, inducible amino acid decarboxylases act to remove carbon dioxide from these amino acids and in turn consume a proton. One such system, lysine decarboxylase, consumes lysine and a proton generating cadaverine which is transported outside of the cell by a lysine-cadaverine antiporter (Bearson, Bearson et al. 1997). Removing protons raises the intracellular pH of the cytoplasm.

ATR MECHANISM

The inducible ATR of microorganisms is highly dependent upon the growth phase of the organism. *L. monocytogenes* is tolerant to acid stress at stationary phase, but this tolerance is rapidly lost during exponential growth (Hill, O'Driscoll et al. 1995). Mid-exponential cells are most sensitive to low pH. In *Enterobacteriaceae* the alternate sigma factor-38 (σ^{38}), encoded by *RpoS* is a critical regulator of stationary phase physiology and general stress resistance (Bearson, Bearson et al. 1997). This factor binds to RNA polymerase and enhances transcription of select genes during stationary phase. σ^{38} itself is an acid shock protein and controls the expression of eight other acid shock proteins (Lee, Lin et al. 1995). The pH-inducible ATR has been described as pre-acid (log phase) acid shock response (induced at pH 5.8) or post-acid (stationary phase) acid shock response (induced at or below pH 4.0). Pre-acid phase inducible ATR in *Salmonella* triggers the synthesis of 43 acid shock proteins while the post-acid phase ATR induces the synthesis of 15 acid shock proteins (Lee, Lin et al. 1995). Both phases of the ATR are necessary for maximal protection against low pH. Reduction in pH during growth will induce both phases of the ATR, however, transfer of cells from pH 7 to pH 3.5 bypasses the pre-acid ATR resulting in death of the cell.

STRESS-INDUCED PROTEINS

Two dimensional electrophoretic gels of acid-adapted and acid stressed *L. monocytogenes* have revealed that the initial response to moderate stress (pH 5.5) is to increase the synthesis of predominately constitutive proteins. A second category of novel proteins, not synthesized at neutral pH, is synthesized upon acid adaptation (pH 5.5) and acid stress (pH 3.5) conditions (Phan-Thanh and Mahouin 1999). Protein bands that

appeared in large quantities on 2-D gels were tryptic digested to peptides and analyzed by mass spectrometry. Masses of these peptide sequences was compared against the masses of peptides for microorganisms with known genomes using NCBI database at the University of California at San Francisco. This analysis revealed that a number of these proteins including dehydrogenases, quinones, oxydoreductases and a subunit of ATP synthase function in pumping protons out of the cell (Phan-Thanh and Mahouin 1999). Interestingly, two proteins identified as chaperonins, one similar to GroEL, are induced upon sub-lethal heat shock in a number of organisms. This might explain why acid adaptation in *S.* Typhimurium also induces thermotolerance (Leyer and Johnson 1993). Biotinylation of proteins from SDS-PAGE gels demonstrated that acid adaptation alters the outer membrane structure through the synthesis of specific outer membrane proteins (Leyer and Johnson 1993).

Most ATR proteins of *S.* Typhimurium are membrane associated (Foster and Hall 1990). While thermotolerance can be rapidly induced (approximately 1 minute) upon heat shock, full acid tolerance in *L. monocytogenes* can take up to 60 minutes (Davis, Coote et al. 1996). Since more time is required for full acid tolerance, major changes must occur in cellular composition rather than simple up-regulation of endogenous proteins within the cell (Davis, Coote et al. 1996). It might be predicted these acid-induced changes in the cytoplasmic membrane would affect permeability of chemical sanitizers altering their ability to destroy the cell.

CROSS-PROTECTION AGAINST MULTIPLE STRESSES

Many investigators have sought to determine whether adaptation to a stress induces the cell to survive other types of stress. This cross-protection permits the stress-

adapted cell to survive a myriad of stressful conditions (Lou and Yousef 1997). For example, acid-adapted *S.* Typhimurium has increased tolerance towards heat, salt, the activated lactoperoxidase system, crystal violet and polymyxin B (Leyer and Johnson 1993). The later two stressors exert their action on the cytoplasmic membrane confirming that acid adaptation and the ATR involve changes in the outer membrane structure. These acid-adapted *Salmonella* are more hydrophobic and more resistant to surface active agents (Leyer and Johnson 1993).

Maldi-mass spectrophotometric analysis of peptide sequences from acid-adapted *L. monocytogenes* demonstrated that chaperonin GroEL was produced. This protein serves to maintain, fold or transport damaged or denatured proteins (Phan-Thanh and Mahouin 1999). Using this strain the authors demonstrated that acid-adapted *L. monocytogenes* had increased resistance to heat shock (52 °C), osmotic shock (25 – 30% NaCl) and alcohol stress (15%). In this study, heat-adapted *Listeria* (50°C for 45 minutes) also displayed increased resistance to acid shock. Chaperonins are produced in *L. monocytogenes* upon acid and heat adaptation and provide cross-protection against acid and heat stress. However the synthesis of heat shock proteins by heat-shocked *Salmonella* did not increase acid resistance (Leyer and Johnson 1993), indicating that induction pathways for acid and heat are different (Lee, Lin et al. 1995).

Acid-adapted but not acid-shocked *E. coli* O157:H7 cells in low pH fruit juices exhibit enhanced heat tolerance in orange juice at 52 °C (Ryu and Beuchat 1998). Interestingly, acid adaptation to pH 5.0 to 5.8 for one to two cell doublings markedly increased sensitivity of *S.* Typhimurium to halogen sanitizers (hypochlorite and iodine based disinfectant) (Leyer and Johnson 1997). Changes in the cytoplasmic membrane

that result from acid-adaptation may increase penetration or exposure of the cell to the lethal activity of hypochlorite.

VIRULENCE

Since acid shock induces the cell to survive other stresses, this ATR may serve as an important signal for inducing general stress resistance. Indeed these cells may become "hardened" to innate gastric acid exposure - one of the first stresses encountered - and within macrophage. Any bacterial cell product that enhances survival in a host can be thought of as a virulence factor (Abee and Wouters 1999). Lee and Lin (Lee, Lin et al. 1995) discovered that virulence of *S.* Typhimurium is dependent on a sustained induction of the ATR and that this induction is dependent on the "*RpoS* status" of the cell. The *RpoS* gene product is the alternate sigma factor 38 (σ^{38}) responsible for inducing transcription of stationary phase stress-response proteins. A *L. monocytogenes* mutant incapable of inducing an ATR was less virulent again suggesting that ATR response contributes to in-vivo survival of *L. monocytogenes* (Marron, Emerson et al. 1997). Four test strains of *L. monocytogenes* grown at 4°C were more virulent (recovery of *L. monocytogenes* from spleens and livers) in mice (Czuprynski, Brown et al. 1989). Therefore, the adaptive mechanisms discussed in this review (acid and temperature) may be considered virulence properties.

STARVATION

Most free-living heterotrophs are thought to lead a "feast and famine" existence, with famine the more habitual state (Koch 1971). The principal changes in response to nutrient deprivation in bacteria relate to nutrient scavenging systems with glucose uptake

being the best characterized. (Ferenci 1996). The *RpoS* independent starvation response in *E. coli* regulates outer membrane proteins and transporters (porins) involved in nutrient scavenging (Ferenci 1996). These membrane proteins selectively transport carbohydrates and other select molecules across the cell membrane. Bacteria have developed a Starvation Survival Response (SSR) to permit survival during lengthy periods of nutrient limitation (Herbert and Foster, 2001). The SSR in *L. monocytogenes* involves both protein and cell wall biosynthesis permitting survival during starvation and cross-protection to several environmental stresses (Herbert and Foster 2001). Glucose deprived *L. monocytogenes* cultures decreased up to three logs over the first two days yet persisted with only one additional log decrease over the remaining 18 days (Herbert and Foster 2001). Interestingly, addition of penicillin G and chloramphenicol, inhibitors of cell wall and protein synthesis respectively, to starved *L. monocytogenes* cultures followed by enumeration demonstrated that cell wall biosynthesis stopped after 7 days while protein synthesis stopped 8 hours after starvation. This suggests that proteins produced during the SSR response occur early upon carbon deprivation and that the cell quickly senses nutrient changes within the environment. SSR of *L. monocytogenes* may enable environmental and food persistence and enhance the organisms ability to establish infection. It is plausible the *L. innocua* starvation response results in protein and cell wall biosynthesis that alters cetrimide sensitivity.

This starvation response enhances nutrient scavenging capability but also induces resistance to various environmental stressors. The D_{56C} value of nutrient-starved *L. monocytogenes* increased 13-fold during 163 hours of starvation at 30°C and significantly increased the heat resistance (56°C) of two of three strains of *E. coli* O157:H7 (Rowe and

Kirk 2000). Interestingly, when starved *E. coli* culture is supplemented with glucose, cells lose their elevated levels of DnaK, H_2O_2 resistance, and thermotolerance (Rockabrand, Arthur et al. 1995) suggesting that increased environmental resistance is related to the starvation response. Heat and freeze-thaw resistance of *E. coli* O157:H7 and nonpathogenic *E. coli* is enhanced after acid adaptation and starvation (Leenanon and Drake 2001). Heat, acid and freeze-thaw resistance of *Vibrio parahaemolyticus* adapted to starvation with or without low salinity were higher than non-adapted controls (Wong, Chang et al. 2004).

As the starvation response induces cell membrane proteins to scavenge nutrients, the acid tolerance response likewise results in production of proteins that predominantly are membrane associated. The *E. coli* O157:H7 starvation response characteristically involves an increased resistance to chlorine and to deoxycholate, a membrane-active detergent (Lisle, Broadaway et al. 1998). Conversely, adaptation of *L. monocytogenes* to environmental stress conditions (ethanol (5%), acid (pH 4.5 to 5.0), H_2O_2 (500 ppm) or salt (7% wt/vol)) did not enhance starvation survival (Lou and Yousef 1997). Habituation of *Salmonella* at 0.95 A_w resulted in increased heat tolerance at 54°C (Mattick, Jorgensen et al. 2000). While habituation at low A_w is distinct from starvation, its relevance to starvation relates to the potential mechanism for increased heat tolerance during nutrient deprivation. During habituation at low A_w, the microorganism lacks a solvent to solubilize surrounding excess nutrients. Three solutes (glucose-fructose, NaCl and glycerol) at the same A_w induce different inactivation rates at 54°C (Mattick, Jorgensen et al. 2000). This thermotolerance is independent of protein synthesis suggesting *L. monocytogenes* is reacting to more than A_w. These solutes would cause

substantial osmotic stress leading to the accumulation of compatible solutes. Taken together, these data suggest that cellular targets of heat activation can be protected via scavenger proteins or by accumulated solutes.

COLD ADAPTATION

Temperature downshift causes the production of cold shock proteins in *E. coli* (Abee and Wouters 1999) and *L. monocytogenes* (Bayles, Annous et al. 1996) concurrent with severe inhibition of general protein synthesis and cell growth arrest (Phadtare 2004). A reduction in temperature from 37°C (optimum) to 10°C in *E. coli* results in a 4-hour lag period followed by growth with a generation time of 24 hours (Jones and Inouye 1994). During the lag period many physiological changes occur, including modification of the fatty acid composition of the membrane bilayer, and an inhibition of DNA, RNA and protein synthesis. Membrane lipid bilayer saturated fatty acid composition is reduced with an increase in unsaturated fatty acids (Russell 1990). Such lipid change will modulate membrane fluidity and the activity of intrinsic proteins that perform functions such as electron transport, ion pumps and nutrient uptake (Russell 1990).

Similar to heat shock, temperature downshift (37°C to 10°C) induces 15 proteins in *E.coli* (Abee and Wouters 1999) and 12 proteins in *L. monocytogenes* (Bayles, Annous et al. 1996). These proteins are produced at concentrations 2 to 10 times greater than at 37°C and function at the level of transcription and translation (Jones and Inouye 1994). The cold shock protein CspA has the highest induction level reaching up to 13% of total cellular protein synthesis and may be the general activator of the cold shock regulon. Membrane bilayer modifications that affect permeability and cold shock protein

induction that impact metabolism, transcription, translation and protein folding might alter the effectiveness of chemical sanitizers.

SUB-LETHAL HEAT

The primary structure of a polypeptide dictates the final three-dimensional structure of a protein. Upon ribosomal translation of mRNA, the unfolded protein must pass through biological membranes (eukaryotes) and then properly fold to form a functional protein. Protein miss-folding will disrupt its secondary structure thereby rendering it non-functional. To maintain and shield unfolded newly synthesized proteins, the cell produces a set of proteins called "chaperones". These chaperones affect newly synthesized proteins by a) preventing miss-folding or aggregation, b) allowing them to traverse biological membranes, and c) facilitating their proper folding (Ang, Liberek et al. 1991).

All stresses to some extent cause protein denaturation and increase the concentration of unfolded proteins (Juneja, Foglia et al. 1998). As microorganisms encounter changes in their environmental temperatures, they sense the temperature change mainly at the level of the cell membrane, nucleic acids and ribosomes (Phadtare 2004). The cellular response to heat results in a dramatic increase in chaperone proteins called heat shock proteins (HSP) that function to prevent stress-induced accumulation of unfolded proteins. This heat stress (shock) response has been described in every organism investigated, from microorganisms to plants and animals, and represents the most highly conserved genetic system known (Lindquist and Craig 1988). This stress response system permits the cell to rapidly adapt to heat and survive under conditions that would otherwise be fatal. These heat shock proteins have been thoroughly described in

E. coli, *Salmonella* and *L. monocytogenes.* In both *L. monocytogenes* and *L. innocua*, heat and cold shock turns off roughly half the number of proteins synthesized at normal (25°C) temperatures (Phan-Thanh and Gormon 1995).

Cells exposed to mild heat modify their cell membrane by increasing the saturation and length of the fatty acids in order to maintain optimal fluidity of the membrane and activity of intrinsic proteins (Abee and Wouters 1999). While most compounds traverse the cytoplasmic membrane through porins (Smith 1997), modifying the fluidity of the cell membrane may alter the ability of compounds to penetrate the lipid bilayer. Acid, cold, heat and starvation-induced changes in the membrane lipid bilayer may also alter permeability of chemical sanitizers which in turn affect their ability to destroy the cell.

HEAT SHOCK PROTEINS

Heat shock proteins function as chaperones and proteases that act together to maintain quality control of cellular proteins (Abee and Wouters 1999). The HSP 70 family of heat shock proteins is conserved across species and is named DnaK in *E. coli*. In response to stress, pathogenic microorganisms accumulate levels of heat shock proteins that may represent up to 20% of total cellular protein (Lindquist 1992). *Salmonella* also induces the DnaK protein in response to oxidative stress within macrophage, one of the most hostile environments encountered by an invading microorganism (Buchmeier and Heffron 1990). The large concentration of heat shock proteins during infection coupled, with the high sequence homology of mammalian and bacterial heat shock proteins, suggests that heat shock proteins play a role in autoimmune disease – i.e., the immune response against stress-induced pathogens may result in "self"

immune humoral and cell mediated response. Heat shock protein immunity is implicated in several autoimmune pathologies, including insulin-dependent diabetes mellitus (IDDM), trachoma, systemic lupus erythematosus, Graves disease and both reactive and rheumatoid arthritis (Lindquist 1992).

RELEVANCE OF HEAT SHOCK PROTEINS TO THE FOOD INDUSTRY

Heat-shocked pathogens are induced to produce heat shock proteins that may permit survival under lethal heat conditions. While heat-shocked pathogens are less resistant to heat than endospore forming organisms (e.g. *Bacillus*), they might survive in foods receiving a minimal heat process. Additionally these heat-shocked organisms might reside for longer periods of time in the manufacturing environment surrounding high-heat manufacturing processes (e.g. surrounding oven areas). A 3 to 20-fold increase in the time necessary to reduce numbers of *S.* Typhimurium 7 logs occurred following sub-lethal heating at 48°C (Mackey and Derrick 1986). An average 2.4-fold increase in the $D_{64}{}^{\circ}C$ value occurred when *L. monocytogenes* inoculated fermented meat was held at 48°C followed by lethal heating at 68°C (Farber and Brown 1990). Since the authors did not state the pH of this fermented meat, it is unknown if the acidity further induced thermotolerance.

ALTERNATE MECHANISMS FOR ALTERED CETRIMIDE SENSITIVITY

That gram-positive cytoplasmic membranes are comprised of 70% protein and 25% lipid (Voet and Voet 1995) suggest alterations in sanitizer sensitivity in stress-adapted *L. innocua* may be due to fatty acid and/or protein compositional changes in the cell membrane. The response to acid stress in *L. monocytogenes* and *Salmonella* results

in increased expression of acid tolerance proteins of which many are membrane associated (Foster and Hall 1990; Phan-Thanh and Mahouin 1999). Interestingly, upon acid-adaptation *L. monocytogenes* increased expression of Fo/F1 ATPase containing hydrophobic proteolipid Co residues (Phan-Thanh and Mahouin 1999). These hydrophobic proteolipid residues span the membrane and potentially alter net hydrophobicity. It is plausible the cells response to acid may inadvertently alter the cells sensitivity to amphipathic molecules such as quaternary ammonium compounds.

RATIONALE AND SIGNIFICANCE

Stress adaptation of microorganisms permits their survival under hostile conditions that would otherwise be lethal. In some instances the response to one stress leads to resistance to a myriad of stresses. Because chemical sanitizers are the principal means by which the food industry eliminates microorganisms from the food environment during wet sanitation, it is critical to understand their efficacy when the microorganisms adapt to those environments. This research will determine the effect of chemical sanitizers on stress-adapted *L. innocua* (Objective 1). *L. innocua* exposed to acid, starvation, cold and heat conditions will be exposed to the quaternary ammonium compound cetrimide followed by enumeration on trypticase soy agar at 35°C for 48 hours. This work will also seek to understand the relationship of net cell hydrophobicity and fatty acid profiles of stress-adapted *L. innocua* (Objective 2) to sanitizer sensitivity differentials noted in objective 1. This work will determine if changes in cell membrane fatty acid composition are related to cetrimide sensitivity in stress exposed *L. innocua*. Microorganisms normally exist in a stressed state, yet chemical sanitizer efficacy studies

are rarely performed using microorganisms cultured under stress conditions. This research will evaluate chemical sanitizer efficacy using stress-adapted microorganisms.

The development of interventions by the food industry to increase sanitation effectiveness and reduce cross-contamination is critical to reducing post-process contamination. Sponge sampling of beef hide, feces and carcass immediately prior and after slaughter demonstrated the presence of *E.coli* O157:H7 on carcass not detected on the beef cattle hide or within feces (Elder, Keen et al. 2000) illustrating the possible presence of a pathogenic microorganism within the manufacturing environment contaminating processed food.

The December 1998 Listeriosis outbreak linked to hot dogs and sliced luncheon meats resulted in a CDC reported 101 illness, 15 adult deaths and 6 still births or miscarriages (USDA 2001). The isolate responsible for the outbreak was purported to reside in the air conditioning unit cooling air over cooked product (Perl 2000) again illustrating contamination of processed food from pathogens persisting within the manufacturing environment.

Processed foods likely become contaminated with microorganisms that have adapted the environment of food processing, distribution and retail facilities. While commercially produced foods that contain pathogenic microorganisms may have been under processed, we would predict post-process contamination by pathogens in the manufacturing environment is responsible for adulteration of most foods classified by the USDA-FSIS as Ready To Eat (RTE). The draft *L. monocytogenes* risk assessment published by the joint effort of the USDA, FDA and CDC requests new strategies to

"decrease the rates of recontamination during the manufacturing and marketing of ready-to-eat foods" (Buchanan 2001).

AIMS

This research will determine whether sensitivity to the quaternary ammonium compound sanitizer cetrimide is altered when *L. innocua* is adapted to acid, starvation, cold and heat stress, conditions commonly found within food manufacturing environments. This research will investigate whether altered sanitizer sensitivity in stress exposed *L. innocua* is related to cell membrane changes in fatty acid composition. This research strives to further our understanding of the behavior of *L. innocua* within the food manufacturing environment and lead to the development of sanitation interventions providing a useful tool to the food industry and to U.S. Agriculture.

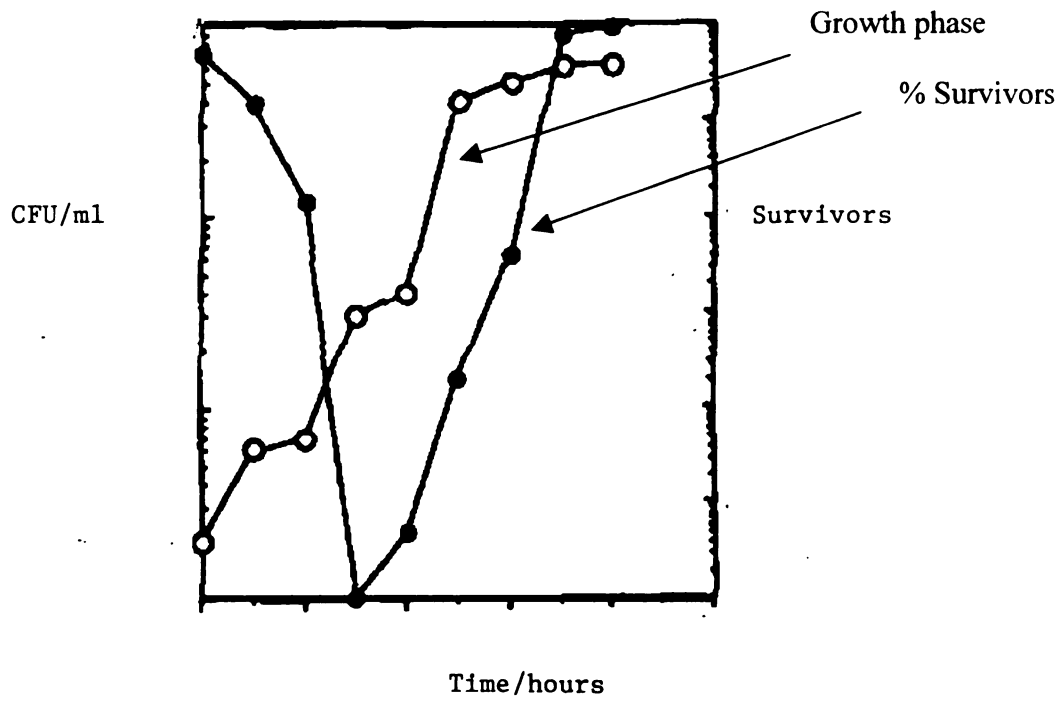


Figure 1. Effect of growth phase on resistance to pH 3.5. The growth curve is represented by the open circles, while the % survivors after exposure to pH 3.5 for 60 min is indicated by the closed circles **(Hill, O'Driscoll et al. 1995)**.

R' and R" = fatty acid

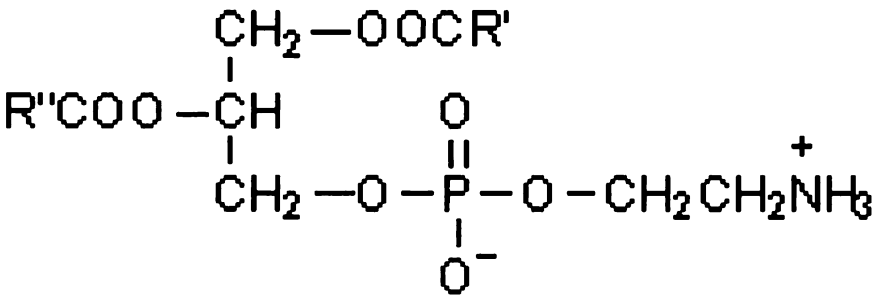


FIGURE 2. PHOSPHOLIPID MOLECULE (CHRISTIE 2005)

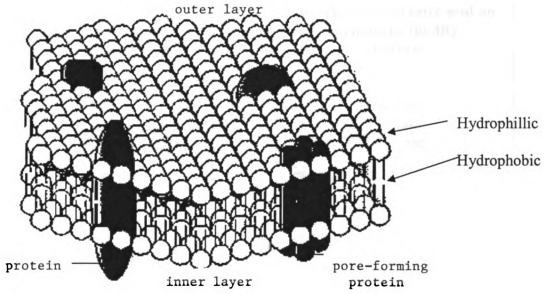


FIGURE 3. LIPID BILAYER (KAISER 2005).

TABLE 1. EFFECT OF FATTY ACID MODIFICATION ON MEMBRANE FLUIDITY

Fatty Acid	**Effect of increased concentration of fatty acid on membrane fluidity**
Saturation	Decrease
Methyl-branched	
Iso	Decrease
Anteiso	Increase
acyl chain length	Decrease

CHAPTER II. ALTERED SENSITIVITY TO A QUATERNARY AMMONIUM SANITIZER IN STRESSED LISTERIA INNOCUA

SUMMARY

Chemical sanitizers are commonly used to inactivate *Listeria monocytogenes* and other *Listeria* spp. that persist in food processing environments after cleaning. In this study, sensitivity of *L. innocua* to the quaternary ammonium compound cetrimide was assessed following exposure to acid, heat, cold and starvation stress. Unstressed and stressed cultures were exposed to cetrimide for three minutes, neutralized and plated on Tryptic Soy Agar with yeast extract to determine the percent survivors. Relative to controls, *L. innocua* demonstrated diminished cetrimide sensitivity when exposed to acid and starvation conditions, whereas heat and cold stress increased cetrimide sensitivity ($P<0.05$). Diminished sensitivity of acid and starvation-exposed *L. innocua* to cetrimide suggests that these stressors might increase the persistence of this organism within food manufacturing facilities. In contrast, enhanced *L. innocua* sensitivity to cetrimide following heat and cold suggests that these interventions might increase sanitation efficacy.

INTRODUCTION

Research over the past two decades has determined that microorganisms have multiple genetic and physiological mechanisms to respond to adverse or stressful conditions (Lindquist and Craig 1988). Stressed microorganisms may exhibit changes ranging from minor metabolic alterations to more extreme modifications in cell structure (Johnson 2003). Physiological or structural changes resulting from exposure to moderate

or sub-lethal stress might permit the organism to survive greater amounts of stress than an organism grown under optimal conditions (Johnson 2003).

In food manufacturing facilities where conditions are maintained to minimize growth of pathogens and spoilage agents, microorganisms may be sub-lethally stressed by exposure to acid, heat, cold, or nutrient depletion. These conditions retard growth of microorganisms yet might trigger stress-induced cellular changes that enable the organism to persist within these environments. "Controlling the presence and growth of *Listeria species* has proven to be very difficult for the food industry and this is attributed in part to the microorganisms ability to grow under refrigeration conditions. Control of *L. monocytogenes* in the food manufacturing environment has been a challenge despite being sensitive to commonly used chemicals such as acid anionic, quaternary ammonium compound, iodine, and chlorine based chemical sanitizers (Lopes 1986). Sanitizer efficacy is generally determined with microorganisms cultured under ideal conditions (Grab and Bennett 2001). However, there is scant information on sanitizer efficacy under conditions of stress. The known existence of stress-hardened organisms (Lou and Yousef 1997) raises questions about the applicability of lethality studies conducted when the microorganism is cultured under ideal conditions.

The objective of this research was to determine efficacy of the quaternary ammonium compound cetrimide on *L. innocua* that was sub-lethally stressed by exposure to acid, elevated temperature, cold and starvation conditions. *L. innocua* was selected as the model challenge organism because this species has proven to be very difficult to eliminate in food manufacturing environments following sanitation (Tompkin 2002) and

it serves as a safe and scientifically appropriate laboratory surrogate for the pathogen *L. monocytogenes*.

MATERIALS AND METHODS

L. innocua ATCC strain 33090 (Microbiologics, Saint Cloud, MN), maintained at –80 °C, was used in all experiments, and cultured in Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA) (BBL™, Sparks, MD). Acid and heat stress induction was determined by exposing the culture to modest acid or heat stress, and then enumerating survivors following exposure of adapted and control cultures to the lethal stress (Buchanan and Edelson 1996; Lou and Yousef 1997). Cold and starvation stress was induced following established protocols (Rowe and Kirk 2000; Leenanon and Drake 2001). Following stress exposure, treated and control cultures were exposed to the quaternary ammonium compound cetrimide for 3 minutes. Survivors were enumerated by pour plating in TSA containing 0.6% yeast extract (TSA-YE). All plates were counted following 48 h incubation at 37°C.

The acid adaptation method of Buchanan and Edelson (Buchanan and Edelson 1996) was used. Briefly, *L. innocua* was inoculated into TSB supplemented with 1% (w/v) glucose (EM Science, Gibbstown, NJ) (TSB+G). Following 18-20 hours of incubation, the pH decreased from pH 5.5 to pH 4.7 at which point *L. innocua* was classified as acid-adapted. A non-adapted control culture (pH 7.0) was generated by adding 0.15 ml of 0.25M Butterfield's Phosphate Water (BPW) (Food and Drug Administration 1998) to 1 ml of the acid-adapted culture and incubating for 1 h at 37°C .

The ability of acid-adapted *L. innocua* to survive lethal acid was evaluated by exposing 0.1 ml of the acid-adapted and control cultures to 9 ml of Brain Heart Infusion

broth (adjusted to pH 2.5 with HCl) for 2 h at 37°C (Buchanan and Edelson 1996). After 2 h of exposure, *L. innocua* was enumerated on TSA-YE.

The heat adaptation method of Lou and Yousef was used (Lou and Yousef 1997). Briefly, heat exposure was conducted using log phase (8 h) cultures grown at 37°C in TSB supplemented with 0.6% yeast extract (TSB-YE). Cultures (5 ml containing approximately 10^6 CFU/ml) were centrifuged (5,000 x g) twice for 10 minutes at room temperature and resuspended in 5 ml BPW. TSB-YE (0.5 ml) was added to 1 ml of washed cells, followed by 1 h of incubation at 45°C in a static waterbath (heat-adapted) or at room temperature (22°C) (control).

Heat tolerance was evaluated by adding 0.1 ml of heat-adapted and control cultures to 55°C (lethal heat) tempered BPW (9 ml) supplemented with 0.5 ml TSB-YE. After 1 h, these cultures were rapidly cooled to 10°C and enumerated on TSA-YE.

The method of Leenanon and Drake (Leenanon and Drake 2001) was used for cold exposure. Overnight (18-22 h) TSB-YE cultures of *L. innocua* were centrifuged (5,000 x g) twice for 10 minutes and resuspended in 1 ml of TSB-YE (1 ml containing approximately 10^8 CFU/ml) (Leenanon and Drake 2001). Cells were incubated in TSB-YE for 5 days at 10°C (cold culture). The control culture was similarly prepared on the day of cetrimide exposure by suspending a twice washed overnight *L. innocua* (10^8 CFU/ml) culture in 1 ml of TSB-YE.

Nutrient starvation was carried out as described by Rowe and Kirk (Rowe and Kirk 2000). Overnight TSB-YE cultures (9 ml) of *L. innocua* were centrifuged (5,000 x g) twice for 10 minutes and resuspended in 1 ml of TSB-YE (approximately 10^8 CFU/ml) after which 0.2 ml was added to 20 ml of sterile distilled water. These cells were starved

for 24 h at 37°C (Rowe and Kirk 2000). The control culture was similarly prepared on the day of cetrimide exposure by suspending 0.2 ml of twice washed (5,000 x g for 10 min.) overnight culture (TSB-YE) in 20 ml of sterile distilled water.

Sanitizer sensitivity of stress-exposed and control cultures was evaluated by exposing the cultures to 10 ppm cetrimide (J.T. Baker, Phillipsburg, NJ) prepared in distilled water. Cultures (0.1 ml) were added to 10 ml of double-distilled water containing 10-ppm cetrimide. This cetrimide concentration reduced *L. innocua* numbers yet was sufficiently low to discern differences in sensitivity between adapted and control groups. Initially and after 0.5, 1.5 and 3 minutes of exposure, the sanitizer-exposed culture (50 µl) was neutralized in 0.2 ml Letheen broth (Food and Drug Administration 1998) in the first column of wells in a 96 well microtiter plate (Corning, Corning, NY). Neutralized cultures (50 ul) were serially diluted in 0.2 ml BPW to permit quantification. Aliquots (0.1 ml) were plated in TSA-YE and incubated for 48 h at 37°C.

Tolerance induction and sanitizer sensitivity experiments were run in duplicate or triplicate and replicated twice. Logarithms of initial and subsequent populations at the various time points were calculated. The differences between these paired sets were analyzed using analysis of variance with culture as one factor and time of analysis as a second factor.

RESULTS

Following exposure to sub-lethal acid (pH 4.7) and heat (45°C), *L. innocua* was exposed to lethal acid (pH 2.5) and heat (55°C). The lethal conditions were designed to eliminate the population at a rapid yet quantifiable rate. A 7-fold decrease in sensitivity to lethal acid was observed in acid-adapted *L. innocua* relative to the control (Table. 2).

The control culture was generated in this experiment by raising the pH of the acid-adapted culture to pH 7.0 using 0.25 M BPW. To determine if this elevated BPW ion concentration per se affected acid tolerance, acid-adapted *L. innocua* was exposed to BPW with pH reduced from 7.0 to pH 4.7 with 1 M HCL. Acid-tolerance of the raised ion concentration culture was unaffected by addition of BPW, indicating that decreased acid sensitivity in acid-adapted *L. innocua* is related to acid tolerance but not to elevated buffer ion concentration (data not shown).

A 276-fold decrease in sensitivity to lethal heat was observed in heat-adapted *L. innocua* relative to the non-adapted control (Table 2). While the non-adapted control *L. innocua* decreased 3 logs during 1 hr min of heating at 55°C, heat-adapted *L. innocua* was unaffected.

Cetrimide at 10 ppm reduced the *L. innocua* control by 4 log within the first 30 sec. Acid-adapted *L. innocua* was less sensitive to cetrimide than the control ($P<0.05$) (Table 3). For example, after 1.5 min of cetrimide exposure, the acid-adapted and control cultures decreased 0.8 and 4.6 logs, respectively.

Cetrimide reduction of heat-adapted *L. innocua* exceeded that seen in the control population at all time points. After 1.5 min of cetrimide exposure, the heat-adapted and control cultures decreased 5.5 and 2.4 log, respectively (Table 3).

Cold-exposed *L. innocua* cells were also more sensitive to 10 ppm cetrimide than control cultures (Table 3). Cold-exposed *L. innocua* was nearly eliminated (3.7 log reduction) by 30 sec cetrimide exposure, while the respective control population was reduced by less than 1 log.

Cetrimide reduced starvation-exposed *L. innocua* just over 1 log after 30 sec. (Table 3), whereas the starvation control decreased 3.4 log. The starvation-exposed and control populations were reduced to the same level (approximately 3.5 log) after 3 min exposure.

DISCUSSION

The general stress response induces multiple physiological changes in the cell including multiple stress resistance (Lou and Yousef 1997). These stress-induced changes have the potential to alter a microbe's sensitivity to cetrimide.

The major findings of this study were that acid and starvation stress diminished sensitivity of *L. innocua* when exposed to the quaternary ammonium sanitizer cetrimide, whereas heat and cold stress enhanced survival. Relative differences in cetrimide sensitivity between stress-exposed and control cultures are summarized in Fig. 4 as the differential (log reduction) between stress-exposed and control cultures. It should be noted that cetrimide sensitivity might be influenced by the cell preparation method and the suspending medium (Grab and Bennett 2001). The suspending media were identical for each stress and its respective control but varied between acid, heat, cold, and starvation experiments. These factors preclude direct statistical comparisons of cetrimide sensitivity across stress types within time periods. Further variability in sanitizer sensitivity may exist across *Listeria* species as this research was conducted using a single ATCC sourced strain.

Quaternary ammonium compounds such as cetrimide act primarily by disrupting cellular permeability of the cytoplasmic membrane (Merianos 2001). The sequence of

events leading to cell death are as follows: (a) adsorption of the compound on the bacterial cell surface, (b) diffusion through the cell wall, (c) binding to the cytoplasmic membrane, (d) disruption of the cytoplasmic membrane, (e) release of potassium ions and other cytoplasmic constituents and (f) precipitation of cell contents and death of the cell. Microorganisms can modify the fatty acid composition of their membrane lipid bilayer in response to changes in their environment. For example, *Pseudomonas aeruginosa* modifies its fatty acid composition when grown in the presence of two quaternary ammonium compounds (Guerin-Mechin, Dubois-Brissonnet et al. 1999). *Pseudomonas aeruginosa* resistance to QAC is attributed to an increase in content of cellular fatty acids resulting in decreased penetration of sanitizer (QAC) through the cell wall. Susceptibility to QAC may be related to the appearance of phospholipids and neutral lipids in the outer layer of the outer membrane (Sakagami, Yokoyama et al. 1989). Recent research demonstrated that *Pseudomonas aeruginosa* QAC sensitivity is related to an outer membrane associated protein (OprR) with homology to lipoproteins of other bacterial species (Tabata, Nagamune et al. 2003). OprR knockout *Pseudomonas aeruginosa* exhibits increased sensitivity to QAC relative to the wild type strain.

Heat-adapted *L. innocua* exhibited greater sensitivity to cetrimide than control cultures. The cellular response to heat results in a dramatic increase in chaperone proteins called heat shock proteins (HSP) that function to prevent stress-induced accumulation of unfolded proteins (Lindquist and Craig 1988). This heat stress (shock) response has been described in every organism investigated, from microorganisms to plants and animals, and represents the most highly conserved genetic system known (Kaufmann 1990). This stress response system permits the cell to rapidly adapt to heat and survive under

conditions that would otherwise be fatal. These heat shock proteins have been thoroughly described in *E. coli*, *Salmonella* and *L. monocytogenes*. In both *L. monocytogenes* and *L. innocua*, heat and cold shock turns off roughly half the number of proteins synthesized at normal (25°C) temperatures (Phan-Thanh and Gormon 1995). Down-regulation of specific proteins during heat and cold stress may sensitize the cell to cetrimide.

Cold and heat stress induce divergent changes in membrane lipid composition. Organisms cultured under cold conditions respond by increasing the ratio of unsaturated to saturated fatty acids within the cellular membrane while organisms cultured under heat conditions decrease unsaturated relative to saturated fatty acids. These changes in membrane fatty acid composition presumably occur to maintain fluidity and to increase the efficiency of solute uptake at low temperatures (Rowe and Kirk 2000). For example, *L. monocytogenes* cells grown at 10°C were more sensitive to the bacteriocin nisin than cells grown at 30°C (Li, Chikindas et al. 2002). Cells grown at 10°C relative to 30°C had cell membranes with increased amounts of shorter, branched-chain fatty acids and increased fluidity. The short (1 h) duration of heat employed here may be insufficient time to affect a compositional change in membrane lipids.

Increased resistance of *L. innocua* to cetrimide following exposure to acid is consistent with the diminished sensitivity of acid-adapted *Salmonella* Typhimurium and *Vibrio parahemolyticus* to surface-active agents. Acid adaptation induces cross-protection against heat, crystal violet, bile and deoxycholic acid in *V. parahemolyticus* (Koga, Sakamoto et al. 1999). Acid-adapted *S. typhimurium* (Leyer and Johnson 1993) and *L. monocytogenes (Lou and Yousef 1996)* exhibited increased surface hydrophobicity. The diminished lethality of surface-active agents and increased cell

surface hydrophobicity in acid-adapted organisms suggests that the cell membrane directly or indirectly affects the ability of antimicrobials to destroy the cell. Many of the 37 known proteins induced upon acid-adaptation in *L. monocytogenes* are membrane-associated and function by pumping protons out of the cell (Phan-Thanh, Mahouin et al. 2000). Most proteins induced upon acid-adaptation in *S. typhimurium* are membrane-associated (Foster and Hall 1990) and likely alter outer membrane structure (Leyer and Johnson 1993). Heat shock proteins serve as chaperones within the cell protecting intracellular proteins from heat denaturation. These chaperone proteins protect the cell by protecting proteins within the cytoplasm, while the acid tolerance response proteins principally function at the cytoplasmic membrane. Diminished sensitivity of acid-adapted and enhanced sensitivity of heat-adapted *L. innocua* following cetrimide exposure might be explained by protein-induced alterations of the cell membrane in acid-adapted, but not heat-adapted *L. innocua*.

Consistent with data reported here, *L. monocytogenes* starved in a low nutrient medium and PBS demonstrated decreased QAC sensitivity (Ren and Frank 1993). *L. monocytogenes* in a low nutrient medium was less sensitive to QAC than in PBS (Ren and Frank 1993). Starved cells have decreased membrane fluidity and permeability and increased surface hydrophobicity (Lou and Yousef 1996). The observation that microorganisms held in low nutrient and even starved conditions are less sensitive to QAC has important industrial implications. Cleaning programs aim to remove nutrients, thereby preventing unacceptable levels of microbial growth. Microorganisms within food manufacturing environments typically starved for nutrients and these studies suggest they are less likely to be destroyed upon exposure to low concentrations of QAC. The

levels of QAC used in this study are 20 times less than the 200-ppm maximum level permissible on food contact surfaces without rinsing (Federal Register 2002). While it is unlikely that acid and starvation stress encountered within the food-manufacturing environment induces survival upon exposure to 200 ppm QAC, sanitizers applied in wet environments are often diluted by water used during manufacturing to levels less than 10 ppm in which acid and starvation-induced *L. innocua* might survive.

Taken together, this research demonstrated decreased cetrimide sensitivity in acid-adapted and starvation-exposed *L. innocua,* and increased sensitivity in cultures exposed to heat and cold conditions. Alteration of sanitizer sensitivity in *L. innocua* exposed to stress conditions suggests that the manufacturing environment may influence the sanitarians ability to purge the manufacturing environment of spoilage or pathogenic microorganisms. Evidence that acid and starvation conditions diminish cetrimide sensitivity suggests that certain conditions may enable microorganisms to persist and establish growth niches. It may be necessary to increase sanitizer concentration in environments that select for these persistent strains. Conversely, heat and cold conditions enhanced cetrimide sensitivity suggesting that sanitation efficacy might be increased by the sanitarian through application of sub-lethal stress conditions.

TABLE 2. HEAT AND ACID TOLERANCE OF RESPECTIVE ADAPTED AND NON-ACID-ADAPTED *L.INNOCUA*.

	Log reduction [1]	
	Acid [2]	Heat [3]
Adapted	0.30 (0.01) [a]	0.01 (0.20) [a]
Control	2.13 (0.15) [b]	2.99 (0.39) [b]

[1] Log reduction of acid or heat adapted and non-adapted (control) *L. innocua* following exposure to respective lethal condition. Values represent mean log reduction +/- SEM. Columns with the same letters are not significantly different ($P>0.05$; N=6). Statistical comparisons of cetrimide sensitivity across stress types were not determined.

[2] Acid-adapted (overnight TSB+G) and control (pH 7.0 adjusted) cultures were exposed to lethal acid (pH 2.5 BHI for 2 h at 37°C) followed by dilution plating in TSA-YE. Colony forming units (CFU) were enumerated after 48 h at 37°C.

[3] Heat-adapted (1 h at 45°C) and control (1 h at room temp.) were exposed to lethal heat (55°C) for 1 h. CFU were enumerated after 48 h incubation at 37°C.

TABLE 3. LOG REDUCTION OF ADAPTED AND NON-ACID-ADAPTED *L. INNOCUA* DURING A 3 MIN EXPOSURE TO 10 PPM CETRIMIDE.[1]

	Acid		**Heat**		**Cold**		**Starvation**	
Cetrimide exposure (min.)[2]	Adapted	Control	Adapted	Control	Adapted	Control	Adapted	Control
0.5	0.19 (0.04) [a]	4.06 (0.47) [b]	2.28 (0.66) [a]	1.24 (0.11) [b]	3.74 (0.27) [a]	0.91 (0.11) [b]	1.03 (0.21) [a]	3.36 (0.20) [b]
1.5	0.75 (0.12) [a]	4.60 (0.46) [b]	3.24 (0.84) [a]	1.68 (0.21) [b]	4.09 (0.41) [a]	2.68 (0.39) [b]	2.21 (0.22) [a]	3.34 (0.11) [b]
3.0	1.49 (0.24) [a]	4.42 (0.45) [b]	4.24 (0.41) [a]	2.57 (0.50) [b]	3.89 (0.17) [a]	3.81 (0.43) [a]	3.38 (0.00) [a]	3.76 (0.00) [a]

[1] Log reduction of adapted and non-adapted (control) *L. innocua* following exposure to respective 10 ppm cetrimide. Values represent mean log reduction +/- SEM. Columns with the same letters within time periods are not significantly different ($P>0.05$; N=6). Statistical comparisons of cetrimide sensitivity across stress types were not determined.

[2] Adapted and control cultures were exposed to 10 ppm cetrimide followed by lecithin neutralization and dilution plating in TSA-YE. Colony forming units (CFU) were enumerated after 48 h at 37°C.

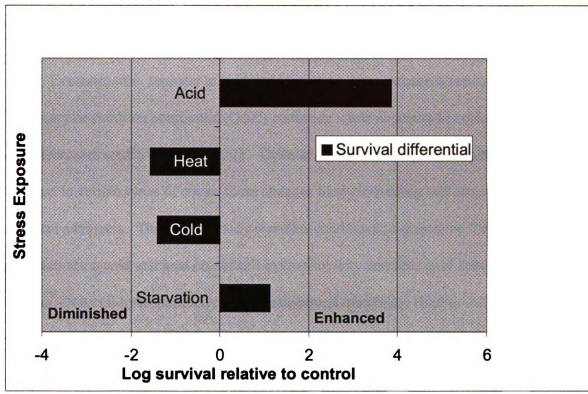


FIGURE 4. Comparative effects of different stressors between stressed cetrimide-induced reduction after 1.5 minutes. 1

[1] Differential calculated as reduction (control - stress exposed).

CHAPTER III. ALTERED HYDROPHOBICITY AND MEMBRANE COMPOSITION IN STRESS-ADAPTED *LISTERIA INNOCUA*

SUMMARY

Exposure of *L. innocua* to acid and starvation stress enhances resistance to the quaternary ammonium compound (QAC) cetrimide while exposure to cold and heat stress increases sensitivity (chapter 2). Cellular changes in membrane lipids occur in response to certain types of stress, these changes likely impacting cell sensitivity to chemical sanitizers. The present study was thus conducted to determine the effect of acid, starvation cold and heat on net cell hydrophobicity and fatty acid composition in *L. innocua*. Net cell hydrophobicity was increased and membrane fluidity decreased upon exposure to acid and starvation stress, whereas hydrophobicity was decreased and membrane fluidity increased upon cold exposure. Increased hydrophobicity and decreased membrane fluidity correlated with as indicated by reductions in anteiso fatty acids and the C-15/C-17 and anteiso/iso ratios. Conversely, cold adapted populations exhibited decreased net cell hydrophobicity and increased membrane fluidity which correspond with increased C-15/C-17 and anteiso/iso ratios and increased C-18 unsaturated fatty acids. In contrast, significant change in net cell hydrophobicity or membrane fluidity were not observed with heat-adapted cells that had enhanced sensitivity to cetrimide, suggesting that another mechanism was involved with altered cellular sensitivity. These results suggest modification of the environment may enhance or diminish sanitation efficacy.

INTRODUCTION

Surface-active agents (surfactants) are amphipathic molecules comprised of hydrophilic "water loving" and hydrophobic "oil loving" moieties. The interfacial tension of two immiscible liquids is reduced upon addition of a surface-active compound as the molecules polar and non-polar moieties orient themselves into the respective solutions. Benzalkonium chloride (cetrimide) is comprised of four carbon atoms covalently linked to a nitrogen atom. The functional part of the molecule is this positively charged cation and it is electrovalently bonded to a chloride molecule to form the salt. One of the four carbon molecules attached to the nitrogen atom is a 12 to 16 carbon alkyl chain (Merianos 2001). The positively charged cation is attracted to the net negative charge of the bacterial cell.

Stress encountered by microorganisms within the food-manufacturing environment may induce cellular changes that result in altered sensitivity upon exposure to chemical sanitizers commonly employed by food manufacturers. We have previously demonstrated that sensitivity of *L. innocua* following exposure to cetrimide was enhanced by prior exposure to acid and starvation and diminished by cold and heat (chapter 2). To maintain intracellular homeostasis, bacteria respond to stress by changing fatty acid and protein composition (Leyer and Johnson 1993; Russell, Evans et al. 1995; Annous, Becker et al. 1997; Juneja, Foglia et al. 1998; Briandet, Meylheuc et al. 1999; Li, Chikindas et al. 2002; Chihib, Ribeiro da Silva et al. 2003; Yuk and Marshall 2003). And such changes might alter a microbes resistance to cetrimide.

The biocidal mode of action of cetrimide and other QACs follows a six step process of 1) adsorption of the molecule to the cell surface, 2) diffusion through the cell wall, 3) binding to the cytoplasmic membrane, 4) disruption of the cytoplasmic membrane, 5) release of potassium ions and other cytroplasmic consistutents and 6) precipitation of cell contents and death of the cell (Merianos 2001). Although QACs act on all bacteria, gram-negative organisms are more sensitive (Ikeda, Yamaguchi et al. 1990). The critical factor for biocidal efficacy is the hydrophilic-lipophilic balance of the QAC (Merianos 2001). Cell membrane fatty acid and protein composition changes in response to stress, with these factors likely influencing the ability of cetrimide to bind and disrupt the cytoplasmic membrane.

Membrane hydrophobicity affects bacterial attachment to surfaces (van Loosdrecht, Lyklema et al. 1987; Briandet, Meylheuc et al. 1999) and the ability of metabolites to transit the cellular membrane. Net hydrophobicity of a microbial cell can be altered by fatty acid and protein compositional changes that occur in response to stress (Leyer and Johnson 1993; Briandet, Meylheuc et al. 1999). Net cell hydrophobicity decreases with increases in short chain "hydrophilic" fatty acids, and conversely increases with decreases in short chain "hydrophobic" fatty acids. Net hydrophobicity in *L. innocua* might be influenced by fatty acid composition changes induced by adaptation to stress, and that these changes in net hydrophobicity would relate to the ability of bioactive molecules, such as quaternary ammonium compounds, to affect the cell.

The fatty acids of bacterial membranes, typically part of phospholipid molecules, can change in length, saturation and branching in response to stress, and these changes alter membrane fluidity and the cells interaction with the surrounding environment (Voet

and Voet 1995). For example, the fatty acid composition changes in three ways in response to temperature change: acyl chain length, the degree of saturation, and the branch position of the fatty acids (Li, Chikindas et al. 2002). The major cell membrane response to temperature changes for *L. monocytogenes*, for instance, is the alteration in the fatty acid component of the membrane's lipids while changes in the head group composition are generally minor (Li, Chikindas et al. 2002). The transition temperature of a bilayer increases with the chain length and the degree of saturation of its component fatty acid residues (Voet and Voet 1995) such that higher temperatures are required for mobility of saturated membranes. Changes in cell hydrophobicity and/or fatty acid composition likely influence the ability of compounds in the surrounding environment to interact or penetrate the cell membrane.

A membrane comprised of high melting point saturated long (C-17) chain fatty acids is tightly packed and non-fluid. Conversely, membranes comprised of lower melting point (C-15) unsaturated fatty acids are fluid (Voet and Voet 1995; Juneja, Foglia et al. 1998; Yuk and Marshall 2003). The ratio of short to long chain fatty acids (C-15/C-17) is used as an indicator of membrane fluidity (Juneja, Foglia et al. 1998). Membrane fluidity increases with increased short chain and unsaturated fatty acids and decreases with increased long chain fatty acids. The terms anteiso and iso refer to the branching in the methyl end of the fatty acid. Anteiso fatty acids have lower melting points than iso fatty acids. An increase in the anteiso number increases it indicates that the cell is lowering its melting point and increasing membrane fluidity.

The long term goal of this research seeks to understand the role of membrane lipids in sensitivity of *L. innocua* to the sanitizer cetrimide. We hypothesize that

hydrophobicity and cell membrane fluidity changes in response to stress are related to sensitivity upon cetrimide exposure. The objective of this research was to determine net hydrophobicity and lipid compositional changes in *L. innocua* in response to acid, starvation, cold and heat, stress.

MATERIALS AND METHODS

L. innocua ATCC strain 33090 (Microbiologics, Saint Cloud, MN), maintained at –80oC, was used in all experiments, and cultured in Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA) (BBLTM, Sparks, MD).

The acid adaptation method of Buchanan and Edelson (Buchanan and Edelson 1996) was used. Briefly, *L. innocua* was inoculated into TSB supplemented with 1% (w/v) glucose (EM Science, Gibbstown, NJ) (TSB+G). Following 18-20 hours of incubation, the pH decreased from pH 5.5 to pH 4.7 at which point *L. innocua* was classified as acid-adapted. A non-adapted control culture (pH 7.0) was generated by adding 0.15 ml of 0.25M Butterfield's Phosphate Water (BPW) (Food and Drug Administration 1998) to 1 ml of the acid-adapted culture and incubating for 1 h at 37oC.

Nutrient starvation was carried out as described by Rowe and Kirk (Rowe and Kirk 2000). Overnight TSB-YE cultures (9 ml) of *L. innocua* were centrifuged (5,000 x g) twice for 10 minutes and resuspended in 1 ml of TSB-YE (approximately 108 CFU/ml) after which 0.2 ml was added to 20 ml of sterile distilled water. These cells were starved for 24 h at 37oC (Rowe and Kirk 2000). The control culture was similarly prepared on the day of cetrimide exposure by suspending 0.2 ml of twice washed (5,000 x g for 10 min.) overnight culture (TSB-YE) in 20 ml of sterile distilled water.

The method of Leenanon and Drake (Leenanon and Drake 2001) was used for cold exposure. Overnight (18-22 h) TSB-YE cultures of L. innocua were centrifuged (5,000 x g) twice for 10 minutes and resuspended in 1 ml of TSB-YE (1 ml containing approximately 108 CFU/ml) (Leenanon and Drake 2001). Cells were incubated in TSB-YE for 5 days at 10 °C (cold culture). The control culture was similarly prepared on the day of cetrimide exposure by suspending a twice washed overnight *L. innocua* (108 CFU/ml) culture in 1 ml of TSB-YE.

The heat adaptation method of Lou and Yousef was used (Lou and Yousef 1997). Briefly, heat exposure was conducted using log phase (8 h) cultures grown at 37°C in TSB supplemented with 0.6% yeast extract (TSB-YE). Cultures (5 ml containing approximately 106 CFU/ml) were centrifuged (5,000 x g) twice for 10 minutes at room temperature and resuspended in 5 ml BPW (Food and Drug Administration 1998). TSB-YE (0.5 ml) was added to 1 ml of washed cells, followed by 1 h of incubation at 45°C in a static waterbath (heat-adapted) or at room temperature (22°C) (control).

Net cell hydrophobicity was determined by measuring the absorbance of a cell suspension after partitioning with the hydrocarbon n-hexadecane (Aldrich Chemical) (Leyer and Johnson 1993), (Rosenberg, Gutnick et al. 1980). Following culture preparation an equal volume of n-hexadecane was added to adapted and control cultures after which the tubes were vortexed for 20 sec and allowed to partition for 10 minutes at 22 °C. The cell suspension partitioned into the polar (water) or nonpolar (n-hexadecane) phase. Absorbance (650 nm) of the aqueous phase was determined before (Abs. To) and after (Abs. TF) addition of n-hexadecane. Net cell hydrophobicity = {1-(Abs. TF/Abs. To)} x 100.

Cells grown under the different conditions were centrifuged (10,000 x g, 10 min. at 22°C) and washed twice with BPW. Total lipids were extracted by a modified Folch method (Folch, Lees et al. 1957). Briefly, the cell pellet was first extracted using 2 ml of chloroform, following by 2 ml of methanol and 1 ml of double distilled water containing sodium chloride. The lipid extract was centrifuged and the bottom chloroform layer transferred to another test tube. The top methanol/water layer was extracted again with chloroform, centrifuged and the bottom chloroform layer combined with the previous chloroform extract. Chloroform was removed from the lipid extract under a stream of nitrogen. Lipids were then dissolved in 1 ml of heptane and 2 ml of 7% (w/v) boron trifluoride in methanol and the test tube was flushed with nitrogen and tightly capped. Tubes were incubated at 10 °C for 45 min and subsequently cooled. Water (2 ml) was added and the top heptane layer containing fatty acid methyl esters was concentrated under nitrogen.

Fatty acid methyl esters were separated and quantified using an HP 5890 Gas Chromatograph equipped with an FID detector and a DB-1 column (DB-1, 30m x 0.32 mm ID, film thickness 1.0 um, J & D Scientific). Helium was used as the carrier gas and the inlet and FID detector temperatures were 250 and 280 C respectively. The GC oven conditions were: initial temperature 150 C, held for 4 min, then increased to 250 C at 4 C/min, final holding time was 18 min. Fatty acid methyl esters were identified using authentic standards obtained from Metreya Inc (Pleasant Gap, PA).

STATISTICAL ANALYSIS

Hydrophobicity and fatty acid experiments were run in duplicate or triplicate and repeated three times. Differences between these paired sets were analyzed using mixed

model analysis of variance with culture as a fixed factor and date of analysis as a random factor ($P<0.05$). Average test and control values were calculated using-least squares means which adjusts the means for unbalanced data when either the control or test cells had an unequal number of observations on the same day.

RESULTS

Net cell hydrophobicity and fatty acid profiles were determined for control and stress adapted L. innocua. Control hydrophobicity and fatty acid percentages varied between treatments since culture conditions differed (culture preparation protocol, ionic and nutrient concentration) across the four controls (Tables 4 and 5). These factors precluded direct statistical comparison of hydrophobicity and fatty acid profile across control and stress-adapted cell types.

Net cell hydrophobicity increased upon exposure to acid and starvation and decreased upon exposure to cold (Table 4). The fatty acids detected (Table 5) comprised the majority of fatty acids (50-70%) detected and were typical of the genus Listeria (Russell, Evans et al. 1995; Annous, Becker et al. 1997; Mazzotta and Montville 1997; Juneja, Foglia et al. 1998; Li, Chikindas et al. 2002; Chihib, Ribeiro da Silva et al. 2003). The C-18 fatty acids consisted of stearic acid (C-18:0), and the C-18 unsaturated oleic (C-18:1 9c, C-18:1 9t) and linoleic acids (C-18:2 9c, 12c).

Acid conditions significantly reduced C-15, C-17 and the C-15/C-17 ratio (Table 5). The anteiso/iso ratios were also significantly decreased by acid exposure. These changes in fatty acids suggest decreased membrane fluidity in acid-adapted L. innocua. Starvation conditions likewise decreased the anteiso/iso ratio, although the C-15/C-17 ratio was not significantly lowered.

Cold conditions tended to increase C-15 and C-18 unsaturated fatty acids, increase the C-15/C-17 ratio as well as increase the anteiso/iso ratio. Heat conditions decreased C-15 and C17 fatty acids without influencing the C-15/C-17 ratio. Likewise the anteiso/iso ratio was unchanged by heat.

DISCUSSION

The ability of bacteria to change their membrane fluidity influences how well a bacterium tolerates certain environmental stresses (Li, Chikindas et al. 2002). The primary way bacteria maintain constant membrane fluidity at different growth temperatures is by adjusting their fatty acid composition (Li, Chikindas et al. 2002), a process termed "homeoviscous adaptation" (Yuk and Marshall 2003). This study was conducted to determine whether changes in net cell hydrophobicity and membrane fluidity are associated with survival of *L. innocua* subjected to various stressors and then exposed to cetrimide. Prior research demonstrated diminished sensitivity of *L. innocua* to cetrimide when adapted to acid and starvation and enhanced sensitivity to cetrimide upon exposure to cold and heat conditions (Moorman, 2005). The main findings of this research were that net cell hydrophobicity was increased and membrane fluidity decreased upon exposure of *L. innocua* to acid and starvation, conditions that diminished sensitivity of *L. innocua* to cetrimide. Conversely, net hydrophobicity was decreased and membrane fluidity increased upon exposure of L. innocua to cold, conditions that enhanced sensitivity of the organism to cetrimide. This models the effect of these conditions on adapted populations following cetrimide exposure suggesting hydrophobicity and membrane fluidity is related to cellular survival upon cetrimide exposure. Adaptation of *L. innocua* to heat failed to influence hydrophobicity and

modestly increased membrane fluidity even though sensitivity to cetrimide exposure was diminished by heat adaptation.

The sensitivity of *L. innocua* adapted to acid, starvation and cold and subsequently exposed to the sanitizer cetrimide relates to net cellular hydrophobicity under these conditions. This hydrophobicity reflects the aggregate charge of the fatty acid and protein compositional changes occurring at the cellular membrane. We postulate the cells net hydrophobicity affects the ability of cetrimide to adhere and intercalate into the cell membrane. Furthermore the membrane fluidity is changing in response to stress, a vital cellular process as it affects critical membrane functions such as biochemical reactions, transport systems, and protein secretion (Yuk and Marshall 2003).

The hydrophobicity of the cellular membrane affects bacterial attachment to surfaces (Briandet, Meylheuc et al. 1999) and the ability of metabolites to transit the cellular membrane. Net hydrophobicity of *L. innocua* adapted to acid was higher (Table 4) and the membrane was more fluid as indicated by decreased C-15, C-17, anteiso fatty acids, and the C-15/C-17 and anteiso/iso ratios (Table 5). *L. monocytogenes* Scott A similarly had increased hydrophobicity upon pH reduction when cultivated in TSYE supplemented with either glucose or lactic acid (Mafu, Roy et al. 1991). Acid adapted *Salmonella* Typhimurium also displays increased cell surface hydrophobicity and is more resistant to certain surface-active agents (Leyer and Johnson 1993). Increased hydrophobicity and/or diminished membrane fluidity upon acid adaptation might diminish the ability of cetrimide to adhere or intercalate into the cellular membrane.

While an increase in short chain fatty acids lowers the melting point and should decrease the hydrophobicity, this research demonstrated increased hydrophobicity in

acid-adapted *L. innocua*. Gram-positive cytoplasmic membranes are comprised of 70% protein and 25% lipid (Voet and Voet 1995) suggesting that alterations in hydrophobicity in stress-adapted *L. innocua* may be due to protein compositional changes in the cell membrane.

Acid stress in *L. monocytogenes* and *Salmonella* results in increased expression of acid tolerance proteins of which many are membrane associated (Foster and Hall 1990; Leyer and Johnson 1993; Phan-Thanh and Mahouin 1999). Of particular interest in L. monocytogenes is the increased expression of Fo/F1 ATPase containing hydrophobic proteolipid Co residues (Phan-Thanh and Mahouin 1999). These hydrophobic proteolipid residues span the membrane and potentially alter net hydrophobicity. Perhaps the increased hydrophobicity in acid-adapted populations is related to increased Fo/F1 ATPase containing the hydrophobic proteolipid Co residues. It is plausible the cells response to acid may inadvertently alter the cells sensitivity to amphipathic molecules such as quaternary ammonium compounds.

Starvation likewise induced an increase in net cell hydrophobicity and lowered membrane fluidity as indicated by a significant reduction in anteiso fatty acids and the A/I ratio. Similar to prior results demonstrating increased survival upon starvation conditions, E. coli O157:H7 subjected to starvation exhibited increased resistance to chlorine and deoxycholate, a membrane-active detergent (Lisle, Broadaway et al. 1998). Similar to the acid tolerance response, the starvation response induces cell membrane proteins to scavenge nutrients (Ferenci 1996). We hypothesize that these cell membrane proteins could similarly alter net cell hydrophobicity and influence the ability of cetrimide to bind and interact with the cellular membrane.

In contrast to responses of acid and starvation adapted *L. innocua*, cold adapted populations had decreased hydrophobicity (Table 4) and increased membrane fluidity as indicated by increased C-18 unsaturated fatty acids and C15/C17 ratio (Table 5). The increase in C15 and decrease in C17 (increased ratio) is also the primary response of *L. monocytogenes* to lower temperatures (Chihib, Ribeiro da Silva et al. 2003). Thus the cold population is likely maintaining membrane fluidity by shifting to shorter chain fatty acids with lower melting or transition points. Increased cell membrane fluidity may enhance chemical sanitizer affinity or ability to intercalate into the cell membrane. This is consistent with the mechanism proposed for nisin sensitivity of cold-adapted *L. monocytogenes*. The bactericidal activity of nisin is due to pore formation in the bacterial membrane, which occurs through a four-step process of binding, insertion, aggregation, and pore formation (Demel, Peelen et al. 1996). Cold-adapted *L. monocytogenes* exhibited diminished survival when exposed to nisin (Li, Chikindas et al. 2002), similar to the diminished survival of cold-adapted *L. innocua* exposed to cetrimide (chapter 2).

Prior research demonstrated diminished survival of heat adapted *L. innocua* exposed to cetrimide (chapter 2). However no significant changes in cell hydrophobicity or indicators of membrane fluidity (C15/C17 or A/I ratios) were observed except for the increase in C-18 unsaturated fatty acids in heat adapted *L. innocua*. Survival of heat adapted *L. innocua* in contrast to acid, starvation, and cold adapted *L. innocua* following exposure to cetrimide is poorly correlated with changes in cell hydrophobicity or membrane fluidity. The microbial heat shock response results in a large increase in intracellular heat shock proteins that are not membrane associated. That neither net cell

hydrophobicity nor membrane fluidity was altered upon heat adaptation suggests that another mechanism, including possible changes in heat shock proteins, is responsible for enhanced sensitivity to cetrimide upon heat adaptation.

CONCLUSIONS

Acid and starvation stress increases net cell hydrophobicity and reduces cell membrane fluidity and these changes relate to enhanced survival of stress-adapted *L. innocua* upon exposure to cetrimide. Decreased hydrophobicity and increased membrane fluidity in cold adapted *L. innocua* are associated with diminished survival of *L. innocua* to cetrimide and supports a role of hydrophobicity in cetrimide sensitivity. No significant change in net cell hydrophobicity or indicators of membrane fluidity, aside from increased C-18 unsaturated fatty acids, were detected suggesting mechanisms other than hydrophobicity and membrane fluidity are responsible for increased sensitivity in heat adapted *L. innocua*.

Analysis of bacterial strains implicated in food borne disease outbreaks reveals the event is typically caused by one to a few strains of the pathogenic microorganism. Furthermore, food manufacturing environments with systemic contamination of pathogenic or spoilage organisms are often similarly limited in the number of persistent strains. That certain environmental conditions within food manufacturing facilities could diminish cellular sensitivity to industrial sanitizers suggests their physiological stress response not only diminishes sensitivity to the stress but also enables persistence upon exposure to low levels of a quaternary ammonium compound sanitizer. Experts in food hygiene have struggled to eliminate microorganisms established within the food-manufacturing environment and these data suggest the intrinsic stress adaptation

mechanisms may enable the microorganism to evade sanitation and persist. Conversely, that modification of the environment would stress-adapt and concurrently enhance sensitivity of the microorganism to quaternary ammonium compounds suggest that interventions exist that will enhance sanitation efficacy.

TABLE 4: NET CELL HYDROPHOBICITY OF CONTROL AND STRESS-ADAPTED *L. INNOCUA*.

Condition	Control (%)	Adapted (%)	Change (%) [b]
Acid	32.6 ± 1.8	36.4 ± 1.9 [a]	3.8
Starvation	9.7 ± 1.1	18.3 ± 6.2 [a]	8.6
Cold	6.3 ± 1.6	3.1 ± 0.5 [a]	-3.2
Heat	2.9 ± 0.4	4.3 ± 0.5	1.4

[a] Control and adapted populations within the same condition with different letters are significantly different ($p<0.05$).

[b] Change (%) = hydrophobicity stress adapted – hydrophobicity control

TABLE 5: CHANGES IN FATTY ACID COMPOSITION IN STRESS-ADAPTED *L. INNOCUA*

	Acid			Starvation	
Fatty Acid	Control (%) [b]	Adapted (%)	Fatty Acid	Control (%)	Adapted (%)
C:15	37.4 ± 1.3	32.6 ± 2.7 * [c]	C:15	36.7 ± 2.8	33.4 ± 1.8
C:17	23.4 ± 0.9	21.8 ± 1.7	C:17	22.1 ± 1.3	20.6 ± 0.7
C:18:0	3.6 ± 0.5	4.3 ± 0.9	C:18:0	3.5 ± 0.8	1.9 ± 0.2 *
C:18 unsaturated	8.4 ± 1.3	8.7 ± 2.1	C:18 unsaturated	7.2 ± 1.5	6.8 ± 0.8
Anteiso	44.4 ± 1.5	38.9 ± 3.5 *	*Anteiso*	40.9 ± 2.9	32.0 ± 2.7 *
Iso	18.8 ± 0.8	18.4 ± 1.2	*Iso*	23.6 ± 1.4	23.5 ± 1.1
Fatty Acid ratio					
15/17	1.6 ± 0.0	1.5 ± 0.0 *	15/17	1.6 ± 0.1	1.6 ± 0.1
A/I	2.4 ± 0.1	1.9 ± 0.2 *	A/I	1.7 ± 0.1	1.3 ± 0.1 *
	Cold			**Heat**	
Fatty Acid	Control (%) [b]	Adapted (%)	Fatty Acid	Control (%)	Adapted (%)
C:15	36.0 ± 3.8	38.2 ± 2.9	C:15	40.0 ± 1.9	36.8 ± 2.4 *
C:17	24.0 ± 1.7	20.9 ± 1.4 *	C:17	26.1 ± 1.8	22.8 ± 0.9 *
C:18:0	2.4 ± 0.4	1.3 ± 0.1 *	C:18:0	4.3 ± 1.2	5.5 ± 1.4 *
C:18 unsaturated	4.1 ± 1.8	6.2 ± 2.3 *	C:18 unsaturated	1.9 ± 0.4	2.6 ± 0.7 *
Anteiso	38.8 ± 3.5	40.4 ± 3.0	*Anteiso*	42.7 ± 2.7	40.1 ± 2.9 *
Iso	24.1 ± 2.1	22.7 ± 1.7	*Iso*	25.6 ± 0.3	23.8 ± 0.5 *
Fatty Acid ratio					
15/17	1.4 ± 0.1	1.8 ± 0.0 *	15/17	1.6 ± 0.0	1.6 ± 0.0
A/I	1.6 ± 0.1	1.8 ± 0.0 *	A/I	1.7 ± 0.1	1.7 ± 0.1

(a) Fatty acid compositions were determined for stress-adapted and control *L. innocua*.

(b) Fatty acid values represent percentage of total cellular fatty acids for principal fatty acids of the bacterial membrane

(c) * Indicates significantly different from respective adapted population ($P<0.05$)

CHAPTER IV. SUMMARY AND RESEARCH CONCLUSIONS

Research presented here demonstrated acid and starvation stress increases net cell hydrophobicity and reduces cell membrane fluidity and that these changes relate to diminished sensitivity of stress-adapted *L. innocua* upon exposure to cetrimide. Decreased hydrophobicity and increased membrane fluidity in cold adapted *L. innocua* are associated with enhanced sensitivity of *L. innocua* to cetrimide and supports the roles of net cell hydrophobicity and membrane fluidity in cetrimide sensitivity. No significant change in hydrophobicity or indicators of membrane fluidity, aside from increased C-18 unsaturated fatty acids, were detected in heat adapted *L. innocua* suggesting mechanisms other than hydrophobicity and membrane fluidity are responsible for enhanced sensitivity of heat-adapted *L. innocua* exposed to cetrimide.

SIGNIFICANCE

Analysis of bacterial strains implicated in food-borne disease outbreaks reveals the event is typically caused by one to a few strains of the pathogenic microorganism. Furthermore, food manufacturing environments with systemic contamination of pathogenic or spoilage organisms is often similarly limited in number of persistent strains. That certain environmental conditions within food manufacturing facilities could diminish cellular sensitivity to industrial sanitizers suggest the physiological stress response not only enhances sensitivity to the stress, but also enables persistence upon exposure to low levels of a quaternary ammonium compound sanitizer. It is important to note the concentration of sanitizer used in this research is 20 times less than the 200 ppm concentration typically applied to food contact surfaces post sanitation. These research

findings unlikely relate to microorganisms that persist on food contact surfaces after cleaning, but rather to those in surrounding areas that evade insufficient cleaning, and are exposed to sanitizer diluted by process water.

Experts in food hygiene have struggled to eliminate microorganisms established within the food manufacturing environment. These data indicate intrinsic stress adaptation mechanisms may enable the microorganism to evade sanitation and persist. Conversely, that modification of the environment would stress-adapt and concurrently diminish survival of the microorganism to quaternary ammonium compounds suggest interventions exist that enhance sanitation efficacy. The potential exists, therefore, for exposure of difficult to clean equipment or sites persistently testing positive for problematic microorganisms to conditions that adapt the microorganism to stress and enhance sensitivity to sanitizer.

MECHANISM AND ALTERNATE EXPLANATION

This research demonstrated a relationship of net cell hydrophobicity, and fatty acid compositional indicators of membrane fluidity, to cetrimide sensitivity in stress-adapted *L. innocua*. It is important to note the stress response of *L. innocua* also involves the production of proteins that could affect cellular sensitivity to cetrimide. The acid tolerance response (ATR) and starvation survival response (SSR) result in production of proteins that ultimately act at the cellular membrane. Conversely, cold and heat shock proteins protect intracellular proteins and mRNA and are not known to act at the cell membrane. That acid and starvation enhanced cetrimide survival, while cold and heat enhanced sensitivity to cetrimide, suggests a relationship between stress response protein production and their location. I would predict the effect of stress-adaptation proteins on

cetrimide sensitivity in *L. innocua* would be greatest when these proteins reside at the cellular membrane and impede, directly or through alteration of total cellular hydrophobicity, cetrimide's ability to affect the cell membrane.

POSSIBLE FUTURE STUDIES

While this research advances our understanding of cetrimide sensitivity of stress-adapted *L. innocua*, further research is necessary to fully characterize the mechanisms responsible for altered sensitivity. I would propose two areas of research that would (1) determine if differences exist in levels of cetrimide bound to stress adapted and control *L. innocua*; and (2) determine changes in net cell hydrophobicity and cetrimide sensitivity of *L. innocua* genetically modified through knockout of FoF1 ATPase gene.

For the first research area, I would determine if altered sensitivity in stress-adapted *L. innocua* can be explained by differences in the amount of cetrimide bound to the cell following exposure of stress-adapted and control populations to cetrimide. To test this hypothesis I envision exposing stress-adapted and control populations of *L. innocua* to 10 ppm cetrimide for 1.5 minutes followed by filtration and subsequent washing to remove unbound cetrimide. The 1.5 minute cetrimide exposure period is proposed as this duration provided the greatest difference in survival between stress-adapted and control populations. Following filtration, cells would be chemically lysed with phospholipase to liberate bound cetrimide. The cetrimide level would be quantified in cell extracts and membrane filtrates using gas chromatography with mass spectrophotometry. The level of cetrimide recovered from cell extracts and filtrates would be normalized to the cell density exposed to cetrimide and expressed as ug cetrimide/10^6 *L. innocua* for both cell extracts and filtrates. I would predict the level of

cetrimide in cell extracts from acid and starvation adapted populations (diminished cetrimide sensitivity) would be lower than levels recovered from cold and heat adapted populations (enhanced cetrimide sensitivity). I base this prediction on the expectation that populations with increased net cell hydrophobicity diminish the ability of the amphipathic cetrimide molecule to adhere and disrupt the cytoplasmic membrane.

That gram-positive cytoplasmic membranes are comprised of 70% protein and 25% lipid (Voet and Voet 1995) suggests that changes in proteins may contribute to alterations in sanitizer sensitivity in stress-adapted *L. innocua*. The response to acid stress in *L. monocytogenes* and starvation stress in *E. coli* results in increased expression of membrane associated proteins (Ferenci 1996; Phan-Thanh and Mahouin 1999). Of particular interest in acid adapted L. monocytogenes is the increased expression of Fo/F1 ATPase containing hydrophobic proteolipid residues (Phan-Thanh and Mahouin 1999). This multimeric protein displaces cytoplasmic hydrogen ions (increasing intracellular pH) and concurrently generates ATP. The hydrophobic proteolipid residues span the membrane and potentially alter net hydrophobicity. It is plausible the cells response to acid may inadvertently alter the cells sensitivity to amphipathic molecules such as quaternary ammonium compounds. Regarding the second possible research area, I would predict that genetic attenuation of Fo/F1 ATPase gene(s) in *L. innocua* would reduce net cell hydrophobicity of acid-adapted *L. innocua* supporting the role of this protein in net cell hydrophobicity and cetrimide sensitivity. Conclusions drawn from experiments involving attenuation of genes normally expressed during growth must be cautiously interpreted as the loss of the gene product may affect the experiment in ways neither predicted nor experimentally controlled.

APPENDICES

APPENDIX A ACID TOLERANCE INDUCTION PROTOCOL

Date: October 21, 2001
Notebook page: 100 (book 1 and 1 book 2)
Objective: 1
Experiment: 2
Study Purpose:
To evaluate acid tolerance induction in *Listeria innocua* cultured under acid conditions. These experiments address objective 1, experiment 2. This experiment is based upon the 8/28 protocol (pg 88). In this protocol 100 ul culture was added more precisely using a micropipette rather than in 8/28 study where a 2-ml pipette was employed.
Media
Used protocol of (Buchanan and Edelson 1996) for acid induction. TSB was supplemented with 1% (8 g/800 ml media) glucose to create TSB-G.
Prepared BHI at pH 2.5 with 37% HCL
Culture
Listeria innocua B culture inoculated to TSB-G from –70 C frozen culture. After overnight (stationary phase) growth culture was split to two 1.5 ml vials (1 ml/vial) label Acid and Control (pH 4.7). Performed in triplicate.
To raise pH of Control to neutral (pH 7.0), 150 ul of Butterfields Phosphate Buffer (BPW) stock was added and vortexed. Both cultures were incubated 1 hour at 37 C. Culture pH verified after 1 hour.
Microbiologics *Listeria innocua* culture 814113
Catalog # PML08145 (33090) exp. Jan. 2002.

Acid Tolerance Determination
Culture: Per Buchanan added 100 ul of culture (used microtiter pipette) to 9 ml BHI (pH 2.5). Immediately removed 100 ul to BPW dilution blank (1 dilution) for time zero determination. Incubated BHI tubes additional 2 and 4 hours at 35 °C.
After incubation, 100 ul of both Acid and Control BHI tubes were removed and dispensed to BPW blank (zero dilution). Plated 1-4 dilutions.

Acid Tolerance Results

Temperature	**0**	**Hour 2**
Control-1	232,000	7,100
Control-2	246,000	5,100
Control-3	300,000	2,700
Control Mean	259,333	4,967
Acid-Adapted - 1	159,000	78,000
Acid-Adapted - 2	181,000	63,000
Acid-Adapted - 3	166,000	84,000
Acid-Adapted Mean	168,667	75,000
Temperature	**0**	**Hour 2**
Control	259,333.33	4,966.67
Acid-adapted	168,666.67	75,000.00
Temperature	**0**	**Hour 2**
Control	5.41	3.70
Acid-adapted	5.23	4.88
Percent Survival		
Temperature	**0**	**Hour 2**
Control	100.00	68.27
Acid-adapted	100.00	93.27

Huge induction in acid tolerance. Submit for statistical evaluation.

APPENDIX B SANITIZER SENSITIVITY PROTOCOL

Date: September 13, 2002

Notebook page: 57

Study Purpose:
To develop method permitting D-value determination upon exposure of *Listeria innocua* to QAC exposure.

Culture
Blue loop of *Listeria innocua* B culture stored in –80 C transferred to TSB-G broth (1% glucose) and incubated overnight at 37 C.
The pH of overnight culture is 4.7.

Cetrimonium Bromide (Cetrimide) exposure.
Hexadecyl-trimethylammonium Bromide from J.T. Baker (Lot V18H10) N130-07.
Diluted 1 gram/Liter.

C. Cetrimide protocol
Protocol
100 ul of culture added to 10 ml of double distilled water (pH 5.0) in screw cap test tube rinsed with double distilled water.
After vortex, 50 ul culture removed from conical and placed in first column of microtiter well containing 200 ul letheen broth.
After setting time to 3 minutes, 100 ul of 1 gram/liter cetrimide was added to the 10 ml culture. Culture was vortexed and the gently inverted across 3 minutes.
After 30 seconds, 1.5 and 3 minutes, 50 ul of cetrimide exposed culture was removed and added to 200 ul letheen in first column of microtiter well.
Using micropipetter, 50 ul of letheen neutralized culture in column 1 was diluted into 200 ul BPW diluent in column 2. After mixing 6 times with micropippeter, 50 ul culture was subsequently diluted into 200 ul BPW through column 6.

Results

1. See Will notebook page 56

Column	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Dilution	5	5	5	5	5	5	5	5	5
Cummulative Dilution	**5**	**25**	**125**	**625**	**3,125**	**15,625**	**78,125**	**390,625**	**1,953,125**

	Duration of QAC exposure							
Culture	0	0.5	1	1.5	2	3	4	5
Culture	103	228	120	28	107	6	1	1

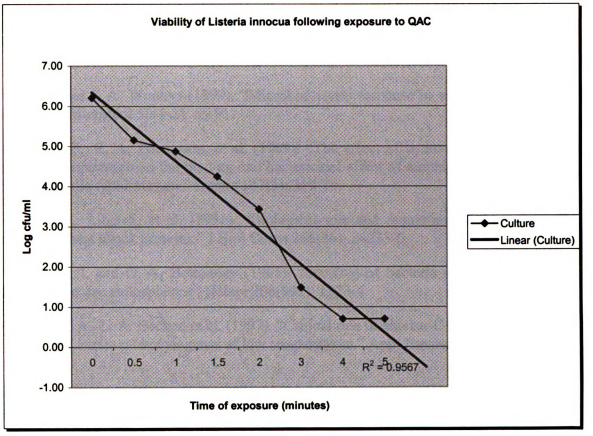
Dilution counted 10 ppm water	0	0.5	1	1.5	2	3	4	5
	6	4	4	4	2	1	0	0

Sensitivity of Listeria innocua to QAC.

	Duration of QAC exposure							
Culture	0	0.5	1	1.5	2	3	4	5
Culture	1,609,375	142,500	75,000	17,500	2,675	30	5	5

Sensitivity of Listeria innocua to QAC

	Duration of QAC exposure							
Culture	0	0.5	1	1.5	2	3	4	5
Culture	6.21	5.15	4.88	4.24	3.43	1.48	0.70	0.70


Viability of Listeria innocua following exposure to QAC
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
Log cfu/ml
0
0.5
1
1.5
2
3
4
5
R^2 = 0.9567
Time of exposure (minutes)
Culture
Linear (Culture)

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