SELECTED GENOMIC AND PHENOTYPIC RESPONSES OF *Salmonella* SEROVARS TO CHLORINE, CHLORINE DIOXIDE, AND CETYLPYRIDINIUM CHLORIDE

A Dissertation

by

GRIHALAKSHMI KAKANI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Food Science and Technology

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May 2011

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ABSTRACT

Selected Genomic and Phenotypic Responses of *Salmonella* Serovars to Chlorine, Chlorine Dioxide, and Cetylpyridinium Chloride. (May 2011)

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Non-typhoidal *Salmonella enterica* serovars continue to be the leading cause of foodborne illnesses in United States. Chlorine, chlorine related, and quaternary compounds are generally used for disinfecting carcasses and equipment in processing industries.

The current study was aimed at understanding the inactivation kinetics of four *Salmonella* serovars to chlorine, chlorine dioxide and cetylpyridinium chloride (CPC). The transcriptomic responses to oxidative stress was investigated in stationary and log phase cells of *S.* Typhimurium. The study was also aimed at understanding the effect of the chemicals on the expression of virulence genes associated with the *Salmonella* Pathogenecity Island 1 (SPI1). The possible induction of the viable but nonculturable (VBNC) state in *Salmonella* due to CPC was also investigated.

The inactivation parameters for each serovar and the chemical were estimated based on the Hom's model, $\ln (N/N_0) = -k\ C^n\ T^m$ and it appeared that while disinfectant contact time was significant, biocide concentration in the overall disinfection was insignificant. This was true especially for chlorine and CPC with subtle differences observed between the serovars. The inactivation efficacy was, however, dependent on both concentration and the exposure time for chlorine dioxide.

The highest degree of inactivation was obtained with chlorine followed by chlorine dioxide and CPC. Transcriptomic responses of *S.* Typhimurium revealed significant downregulation of several metabolic processes such as tricarboxylic acid cycle, oxidative phosphorylation, and amino acid biosynthesis in both log and stationary phase cells. Several stress related genes such as *usp*, *rpoS* and *ompR* were upregulated in the stationary phase cells. Majority of the virulence genes associated with the SPI1 were found to be downregulated for all the treatments. While treatment with chlorine and CPC caused downregulation of all the virulence genes, treatment with chlorine dioxide caused significant upregulation of few (*hilC*, *invC*, *sipA* and *sipB*) genes associated with the SPI1. Finally, the induction of VBNC state was not concluded as a result of treatment with CPC. However, significant percentage of cells (45%) with intact membrane was established based on the BacLight assay™.

DEDICATION

This work is dedicated to my friend, philosopher and guide.

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Many people both directly and indirectly were involved in the successful completion of this project. I would like to express my gratitude and sincere thanks to my supervisor, Dr. Suresh Pillai, who gave me an opportunity to work with him on this project. He has been a constant source of inspiration and encouragement throughout my program. I sincerely thank Drs. Jimmy Keeton, Allen Byrd, Alejandro Castillo, and Marcos Sanchez-Plata for willing to serve on my committee. I thank them for their constructive criticism, comments, and valuable suggestions. I am thankful to Fred Mussari (BCR Environmental, FL) for providing me the protocol and the necessary materials for the preparation of chlorine dioxide. I am thankful to Dr. Linda Talley (Department of Nutrition and Food Science) for being very supportive in every possible way over the years. I thank Denise Caldwell (USDA-ARS), for her time in assisting me work through the project.

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CHAPTER I

INTRODUCTION

One of the most difficult problems encountered today in the fields of public health and animal foods is increasing resistance of microorganisms to antimicrobials. Pathogens of concern are generally controlled by application of biocides in food industry. Resistance of microorganisms to chemicals in food processing environments has been a cause of concern for the last few decades. Other concerns such as reduced efficacy of the chemicals in controlling the pathogens and possible cross protection to antibiotics have been expressed recently. Despite such discouraging results, the use of chemicals in the food industry has not been reduced.

The precise mechanisms conferring resistance to microorganisms are yet to be elucidated and research in this area is very limited. Factors that contribute to the limited efficacy of chemicals may possibly have a role in conferring resistance in the microorganisms. These factors are not completely known and need to be thoroughly investigated. A comprehensive analysis of the response of microorganisms to chemicals may help in gaining a better understanding of the complex molecular mechanisms involved.

This study was aimed at understanding the response of *Salmonella* to various chemicals that are generally used in various processing operations.

The dissertation follows the style of Applied and Environmental Microbiology.

The chemicals under investigation have been approved for use, but research determining the true efficacy of these has been very limited. The literature on the effectiveness of these biocides is still unsettled. Previous data is not very reliable and could be attributed to experimental flaws including imprecise estimation of biocide concentrations, use of biocides containing large concentrations of uncharacterized “inert” ingredients and improper experimental controls.

This research was aimed at gaining a comprehensive understanding of the stress response of *Salmonella* to different biocides. The overall objective of this study was to analyze the effect of different biocides on inactivation and virulence expression in *Salmonella*. The specific objectives include:

1) To compare the efficacy of biocides - chlorine, chlorine-dioxide, and cetylpyridinium chloride (CPC) in inactivating *Salmonella* spp.
2) To determine the effect of biocide applications in development of “Viable but nonculturable” (VBNC) condition in *Salmonella enterica* serovars upon exposure to CPC.
3) To characterize the genes expressed in *S.* Typhimurium upon treatment with free chlorine.
4) To investigate the effect of biocides on expression of virulence genes in *S.* Typhimurium.

CHAPTER II

LITERATURE REVIEW

Modern food production systems have evolved to become dynamic and complex processes. To meet the ever increasing demand of consumers for safe and quality food (primarily meat and other animal products) animal (especially beef, poultry and pork) production practices have changed dramatically over the past half century. Increased use of antibiotics for preventing and controlling diseases, use of biocides and sanitizers for preventing microbial contamination caused by microorganisms, have become a common place all around the world (80).

In the United States, foods are regulated by federal agencies, primarily by USDA - FSIS (United States Department of Agriculture, Food Safety Inspection Services) and FDA (Food and Drug Administration). The USDA-FSIS oversees meat and poultry production and the processing in the United States. This agency is responsible for stipulating performance standards with the goal of reducing pathogen contamination during production and processing of meat and meat products. USDA-FSIS encourages poultry processing plants to reduce pathogens especially *Salmonella* on carcasses during poultry processing operations. Plants are expected to meet the established standards (20% positives, n = 51, max positives = 12) for *Salmonella* (USDA/FSIS 1996). This standard is currently under revision. New performance standards require plants to have only 7.5% of broiler carcasses testing positive for *Salmonella* (195).

Poultry processing plants have been using biocides/chemicals such as chlorine, chlorine dioxide, and acidified sodium chlorite for decades for controlling pathogens. The USDA-FSIS stipulates the use of free available chlorine at concentration ≤ 50 μg/ml, residual

chlorine dioxide at ≤ 3 µg/ml in the chiller tank and cetylpyridinium chloride in solution at concentration of the biocide not exceeding 0.8% by weight of carcass either prior to or after chiller immersion (196).

Mechanisms of action of biocides on bacterial cells

Irrespective of the mode of action, biocides commonly target the cell wall, cytoplasmic membrane and cytoplasmic constituents of a bacterial cell (95). Bacterial cell membranes are composed of lipids, carbohydrates and proteins (175) in addition to enzymes that are involved in respiration such as succinate dehydrogenase, and acid phosphotase (94)**.** Any damage caused to the membrane will cause changes in the permeability resulting in leakage of the metabolites ultimately leading to either inhibition of metabolic processes or loss of viability.

The interaction of the biocide with the cell is thought to occur in two stages: step # 1 a primary step involving adsorption of the biocide onto the surface of the cell. Four patterns of adsorption (S, L, H and C) have been described (76)**.** The step # 2 involves several processes that eventually cause either inhibition of the metabolic processes (bacteriostatic) or even death of the cell (bactericidal). A bacteriostatic effect includes changes in the cell permeability and leakage of metabolites. Damage to the membrane was studied at the biochemical level and it was demonstrated that compounds containing nitrogen and phosphorus leaked in *Staphylococcus* when exposed to QAC (quaternary ammonium compound) and the antibiotic tyrocidin (90). A bactericidal effect involves complete lysis of the cell and this can occur in several ways. Certain compounds such as sodium hypochlorite, phenols and mercuric chloride at sub-lethal concentrations caused the lysis of certain bacterial species (158) and this was attributed to the activation of lytic enzymes present in the

cell wall. Other forms of secondary interactions include denaturation of proteins and nucleic acids (43), disruption in the general metabolism of the cell (inactivation of the enzyme succinate dehydrogenase (183), inhibition of oxidative phosphorylation by nitrophenols (182), and inhibition of respiration (12, 13).

Mechanism of action of chlorine related compounds

Chlorine and chlorine related compounds such as chlorine dioxide are chemically oxidizing agents that cause irreversible damage to the bacterial cell membrane (111). As a disinfectant, chlorine is effective in removing protein residues and carbohydrate materials from surfaces. The bactericidal activity is high when the pH is between 6.0 and 7.0. The effectiveness is reduced in the presence of organic material. The efficacy of the chemical decreases with increasing pH and there is little or no hypochlorous acid formed at pH 7.0 or above (32).

Another chlorine compound that is being extensively used in food industry is chlorine dioxide. Chlorine dioxide reacts very rapidly, achieving a higher kill of viable bacterial at low concentrations (3-5 ppm) when compared to chlorine (32). Studies conducted demonstrated that chlorine caused sub-lethal injury and survival curves for gram-negative organisms showed a linear inactivation initially followed by zero-slope tailing indicating that the efficacy of the antimicrobial occurs only in the initial phase of the application (202). Several studies indicated that the effectiveness of chlorine as an antimicrobial depends on several factors that include type of microorganism, treating medium (water), heat, temperature, pH, composition of the food matrix and presence of other inhibitory substances such as organic material. Studies demonstrated the role of cellular structures in addition to the presence of organic material as being responsible in protecting the microorganisms (202).

Chlorine is generally considered as a nonselective oxidant that reacts with cell structures affecting metabolic processes (178). The principle target cell component involved in bacterial inactivation is cell membrane (200). Changes and damage to the membrane permeability has been demonstrated in several gram negative microorganisms and the extent of damage is largely affected by the presence of organic material (little no damage observed in presence of large amount of organic material) (202).

Membrane damage causes leakage of cellular material causing an irreparable damage to the bacterial cells. However, membrane damage alone has not been attributed as a key event in causing cell death, because permeability of the membrane to substances has been demonstrated even at high concentrations of chlorine (50 ppm) which typically is several folds higher than the concentration that causes the actual death of the microorganism (202). Results from various other studies suggest the stabilization of the cell membrane by the organic material retarding/preventing the penetration of chlorine into the cell (132). In addition, an external layer (outer membrane) in gram-negative organisms acts as a barrier preventing permeability of antimicrobial compounds. Also, divalent cations such as calcium and magnesium stabilize the outer membrane by neutralizing the electrostatic repulsion between adjacent lipopolysaccharide molecules (197).

Other antimicrobial activities of chlorine in *Salmonella* include inactivation of enzymes primarily targeting iron sulfur clusters and disruption of DNA synthesis (135). Chloramine that is formed upon reaction with ammonium has been demonstrated to induce lesions in DNA and single strand breakage in DNA (180). Furthermore, HOCl reacts with peroxide to form singlet oxygen that is known to cause damage to DNA (114). As indicated earlier the efficacy of chlorine as an antimicrobial depends on the target organism. It has

been demonstrated that the resistance of *Salmonella* to HOCl is due to a combination of physiological adaptations. Studies with resistant strains of *Salmonella* indicated lower levels of superoxide dismutase (SOD) and higher levels of catalase upon exposure to HOCl (142). This suggests that resistant strains have the capacity to degrade hydrogen peroxide and therefore the production of singlet oxygen by reaction with HOCl is retarded. Exposure of bacterial cells to HOCl was shown to affect the enzymes of the cell membrane. Enzymes glyceraldehyde-6-phosphate dehydrogenase (G6PD), malate dehydrogenase (MDH) and lactate dehydrogenase(LDH) were significantly reduced in resistant strains upon exposure to HOCl (142). This illustrates that the amount of superoxide produced due to leakage of electrons in the respiratory metabolism is reduced when the enzymes are inhibited/retarded by either glucose or oxidants (142)**.**

The lower levels of SOD in resistant strains of *Salmonella* indicates that the need for SOD was lower because of the lowered production of superoxide radical which is due to reduced electron transport rate. The corresponding increase in catalase levels is suggested to be due to inhibition of SOD (117). The oxidative stress caused by hydrogen peroxide and superoxide results in stimulation of several enzymes that are under the control of *oxyR* and *soxRS* regulons respectively (122, 128). It has been suggested that resistance of *Salmonella* Typhimurium to hydrogen peroxide is due to initiation of 30 H_2O_2 inducible proteins and 21 superoxide stress proteins respectively (143). Studies have also demonstrated that exposure to HOCl increased the levels and activity of endonuclease on the chloramine sensitized DNA (180). Other studies reported mutations in recombination repair genes (*recA* and *recB*) causing decreased resistance to HOCl (54). Other factors that promote resistance to HOCl include presence of complete O-antigen side chain that limits the permeability of HOCl

molecule because of very low fluidity of lipopolysaccharide monolayer, and reduced uptake of oxygen and phosphate (83, 99). Reduced uptake of oxygen is suggested to be due to inhibition of enzymes that catalyse the oxidation of the substrate (malate, succinate, and lactate). Complete termination of phosphorylation was suggested to be due to inactivation of trans-phosphorylases and was thought to be due to decoupling action of chlorine. Zeta potential and oxidative phosphorylation of cell membrane are suggested to be affected by exposure to chlorine. Constant leakage of RNA and proteins has also been reported (200).

Inhibition of protein synthesis is suggested to be the primary mode of lethal action of chlorine dioxide on bacterial cells (21). The antimicrobial effect is related to protein synthesis rather than inactivation of the enzyme system. This can be achieved in several ways: **A)** inhibition of formation of aminoacyl adenylate, or inhibition of amino acid activation and can be represented as follows:

Amino acid + adenosine triphosphate -→ adenosine monophosphate.amino acid and soluble ribonucleic acid (sRNA)

B) ribosomal level sRNA . amino acid + ribosomes -→ protein

C) Inactivation of mRNA preventing translation of coded information.

D) Destruction of the ribosome by chlorine dioxide

Previous studies with chlorine dioxide did not demonstrate any significant alteration of the dehydrogenase enzymes or any lethal effect on the DNA (135). A study with *E. coli* did not demonstrate any cellular damage and subsequent leakage of contents. However, it was observed that respiration was inhibited at sub-lethal concentrations and there was extensive efflux of potassium (22). The study also demonstrated that there was no significant loss of macromolecules even at higher concentrations (10 mg/l) of the chemical, but,

membrane damage causing loss of intracellular potassium was significant eventually leading to the destruction of transmembrane ingredient. At low concentrations, respiration was inhibited, but was reversible. Other studies reported that chlorine dioxide caused significant alterations of proteins at the molecular level that subsequently cause changes in outer membrane permeability. It is suggested that loss of membrane related functions eventually causes lethal inhibition of metabolic processes in the cells (22).

Mechanism of action of quaternary ammonium compounds (QAC's)

Adsorption and subsequent inhibition of the several metabolic and reproductive processes causing cell lysis is considered to be the primary mode of action of several QAC's (95). QAC's typically dissociate conjugated proteins and lipoprotein conjugate of the cell membrane causing extensive damage to the cell (121). This phenomenon of extensive damage to the cell with QAC's is often compared to that of hemolytic damage (177).

Leakage of several nitrogen and phosphorus containing compounds indicating an extensive and rapid membrane damage at the biochemical level from *Staphylococcus* upon treatment with QAC has been reported earlier (90).

The cationic compounds were found to inhibit respiration in several gram-positive and gram-negative organisms at a concentration of 333µg/ml (12, 13)**.** Inhibition of metabolism in several of these microorganisms has also been reported in the same study. Another study reported that the cytochrome system in *E. coli* was susceptible to CPC (152).

Resistance to biocides

With stringent regulatory requirements in place, the poultry industry is hard pressed for meeting the performance standards. Several studies reported mixed results with regards to the efficacy of biocides in reducing *Salmonella* contamination in poultry (7, 194, 209). In

addition to being ineffective, concerns such as employee safety, costs, environmental issues and negative impact on sensory characteristics have also been implicated with the use of biocides in poultry processing (25, 62). Resistance to biocides in food processing environments has been observed for the past several decades (172, 185, 186). The mechanisms of bacterial resistance to antibiotics have long been established, however, studies involving the determination of the underlying mechanisms for biocidal resistance have been minimal and largely restricted to studies involving clinically relevant microorganisms (169). Previous studies found few similarities between bacterial resistance to biocides and to antibiotics (170). A few *in vitro* studies also exemplified possible relationships between bacterial resistance to biocides and antibiotics especially when bacterial cultures were exposed to sub-lethal concentrations of biocides (23, 27). However, these studies were largely confined to clinical settings.

A study conducted by Solveig (185) in food and food processing industry reported cross-resistance in bacteria between antibiotics and biocides with possible genetic linkage. Currently, it is speculated that continuous exposure of microorganisms to sub-lethal concentrations of biocides eventually leads to acquisition and expression of resistance features causing reduced susceptibility to biocides. It is also hypothesized that bacteria surviving the selective pressure persist longer in the environment thereby increasing the chances of accumulating mutations/plasmids with high level of antibiotic resistance (74, 147). There is no proof thus far to support this hypothesis.

Resistance in gram-negative bacteria is generally considered to be intrinsic (controlled by the chromosome of the cell) as against the acquired phenomenon (transfer of plasmid, transposons) that is more prevalent in gram-positive bacteria (135). The cell

membrane is suggested to be the primary barrier restricting the entry of chemicals into the cell in bacteria (8, 75, 82). The LPS (lipopolysaccharide) composition as well as Mg^{+2} content of the outer membrane are thought to contribute to the resistance of gram-negative bacteria (31, 153).

Literature also suggests two other resistance mechanisms: the first being the accumulation of the lipid by the bacterial cell and second being the ability to breakdown the biocide. Studies involving several gram negative bacteria showed a marked change and increase in cellular lipid composition that equaled a simultaneous increase in resistance (198). Degradation of the biocide by the microorganism has been reported in *Pseudomonas aeruginosa* (96).

This phenomenon of resistance in bacteria is very complex and is just not confined to the food environment. Resistance to multiple antibiotics in clinical settings is already a cause of concern all around the world. This is attributed to misuse and overuse of drugs both in human and veterinary medicine. Continuous use of biocides/antibiotics at sub-lethal concentrations in food industry (in production and processing settings) may trigger similar challenges. Persistent and increased use of biocides eventually may lead to selection of bacteria that are less susceptible to biocides. These resistant strains establish in the environment due to selective pressure. Resistant strains of *Salmonella* are already being reported and isolated in poultry and majority of these strains are found to be resistant to several antibiotics and treating infections caused by these resistant strains is getting increasingly difficult with none of the existing drugs being effective.

Biocides are being extensively used in the industry; however, data quantifying the use and the presence of residues of these biocides on the product and in the environment is very

limited. In addition, molecular mechanisms contributing to the resistance in bacteria are not known or very well understood. This requires extensive research to prevent future catastrophic events.

Viable but nonculturable (VBNC) state in bacterial cells

Most inactivation studies involving biocides typically report log reductions (CFU/ml) based on the colony counts obtained on a routine agar media. Such studies also assume and conclude that cells are dead or killed which is not always true.

The viable but nonculturable (VBNC) is defined as a state where a bacterial cell fails to grow on normal culture media (150) that is otherwise considered appropriate for the organism. VBNC is a physiological state observed in several gram negative and gram positive bacteria wherein the cells fail to grow on nutrient media but retain features such as respiration and substrate uptake (116, 211). It has been suggested that cells after exposure to stress (depletion of nutrients, changes in osmotic concentration, extreme pH and temperature conditions and exposure to disinfectants such as chlorine) enter into a dormant state (a physiological state where the cell exhibits reduced metabolic activity). This state has been extensively reported in *Campylobacter* wherein the cells assume a coccoid shape upon exposure to temperature stress (159).

Phenomenon associated with VBNC

Other studies reported several other phenomena associated with the VBNC state. These include inhibition of DNA synthesis resulting in the loss of cell membrane (35, 36), reduction in potassium content internally associated with a lowered membrane potential in *Campylobacter* cells (193), shrinkage of the cytoplasm (159), reduction in respiration, and nutrient transport (149, 157), production of shock proteins (137, 145), increased ATP activity

(24, 64), modification of the fatty acid composition of the cytoplasmic membrane (48), increase in cross-linking of peptidoglycan (181), and genetic changes (204).

Methods to evaluate VBNC

Several methods have been suggested for determining the VBNC state. Some of these include examination of metabolic activity in the cell (hydrolysis of CTC, reduction of INT (166, 211), presence of intact cell membrane by the Baclight method (136, 150), measurement of enzyme activity and membrane potential (157), use of molecular methods such as analysis of degradation of RNA and DNA (206).

Understanding VBNC

Despite extensive studies in understanding VBNC, the subject still remains controversial. VBNC is suggested to be a survival strategy employed temporarily by microorganisms to overcome stress. If this indeed is true then cells should resuscitate from the dormant state after removal of the stress. Several studies have tried to investigate the resuscitation of the stressed cells, but proved in vain because it has been difficult to prove if re-growth has been due to presence of few culturable cells present after stress exposure or due to true resuscitation of VBNC cells (53, 207). Studies fail to discriminate nonculturable from culturable cells in a population and assays proving biochemical activities with culturability are not valid (47, 113). Moreover, it has been observed that significant number of nonculturable cells retain metabolic activity (53) hence, measurements determining the metabolic activity cannot be concluded as viability assays. Also, determining the metabolic activity in the cell and concluding that the cell is viable is a complete contradiction of the definition of VBNC (dormant state). Dormancy is defined as a physiological state where there is low to negligible metabolic activity in the cell. The cell in this state is still considered

to be culturable. A VBNC on the other hand is a physiological state where the cell is nonculturable but has metabolic activity. To avoid any controversy in reporting this phenomenon, it has been suggested that a positive result (metabolic activity) would be reported as the cell being active rather than being classified as viable (109, 110).

In addition, disinfection assays reporting VBNC state in the organism are not always valid. The stressed population in any assay is never homogeneous and has a mix of injured and dormant cells. It is very difficult to differentiate the contribution of each to a colony forming unit because the activity of the cells is not being tracked (53).

The controversy however doesn't make the colony count method as the only appropriate procedure of reporting viability of cells. A drop in colony count doesn't necessarily imply a certain death of the cell. It may reflect a "VBNC related phenomenon" which is still of practical significance for *in vitro* studies.

Last but not least, the retention of virulence in VBNC is a subject of public health concern. This has been clearly illustrated with infectious diseases such as cholera and campylobacteriosis where there was no recovery of pathogens in known definite reservoirs exemplifying the presence of nonculturable cells (29, 155). However, metabolic activity has been reported in these nonculturable cells. Cells in a VBNC state are not considered to be infective since they are considered to be dormant, but they are thought to retain the virulence characteristics (15). Several studies investigated this phenomenon and the conclusions were found to be conflicting (112, 191). *Helicobacter pyroli* cells transitioned from rods to coccoid forms (VBNC state) when exposed to fresh water (1) and were found to produce DNA transcripts even after 26 h after entry into VBNC state. In addition, it was also observed that cells in VBNC state were found to be resistant to antibiotics that are routinely

used to treat ulcers. This indicates that persistent recurrence of infection is due to resuscitation of VBNC cells (17).

Retention of infectivity in VBNC cells

Resuscitation of VBNC cells in *S*. Typhimurium and *S*. Oranienburg was reported (163, 164) and further investigation revealed that invasion rates were different for different subpopulations (105). Although not conclusive it is suggested that VBNC cells retain infectivity and therefore considered to be a cause of concern.

Retention of virulence traits in VBNC state has been reported in *Shigella dysenteriae* Type 1 (161). The presence of virulence gene Shiga toxin (*stx*) in the VBNC state was confirmed using a PCR amplification method. The biologically active toxin (ShT) was maintained and monitored using the enzyme-linked immunosorbent assay (ELISA) using mouse monoclonal antibodies against the rabbit polyclonal antibodies and the virulence property was determined by evaluating the extent of adherence to the intestinal epithelial cells and it was found that cells of *Shigella* lost their ability to invade the Henle 407 cells in VBNC state.

VBNC state has been extensively reported in *Vibrio cholerae* O1 and it was found that the cells get back to the culturable state in human intestine indicating that the pathogenic features are retained in the VBNC state (41). A significant inhibition of virulence traits was however observed in *Vibrio vulnificus* in VBNC state (151). The cells were found to resuscitate in the peritoneal cavities of mice and were capable of causing infection. However, the pathogenic features were found to be retained only for three days after entering into the VBNC state.

Salmonella pathogenicity island 1 (SPI1) and virulence expression

The ability of microorganisms to survive and multiply in a host of stressful environments (pH, temperature, and osmolarity fluctuations, depletion of nutrients, exposure to disinfectants) is considered to be very intriguing. Several strategies are being employed, but the most important being the complex signal transduction systems that regulate the expression of several stress related genes. Genes that are typically expressed in *S.* Typhimurium during starvation (low carbon, nitrogen and phosphate) include *rpoS, sitA, sitB* and *sitC.* The *rpoS* is also known to confer protection to the pathogen against other stresses such as osmotic, heat and oxidative stress. Genes implicated in overcoming the osmotic stress include *ompR, ompC* and *ompF* and these genes are also considered to impact the virulence properties of the pathogen (52).

The pathogen not only survives, but is also known to retain its unique feature of causing infection and in fact, several studies proved enhancement of the virulence properties (77, 140, 179) in *Salmonella*.

Virulence in *Salmonella* is characterized by presence of pathogenicity islands in the chromosome and SPI1 (*Salmonella* pathogenecity island 1) is a large genetic determinant that is about 40kb in length comprising of 25 genes. SPI1 is considered to be involved in regulating invasion (70) of the pathogen into the epithelial cells and it is suggested that 13 genes are associated with the invasion of the mammalian epithelial cells.

Type III secretory system

The most important and critical function associated with SPI1 is type III secretion system (TTS) that transports virulent factors known as effectors (93). The phenomenon of pathogenesis is observed during the interaction of the effectors with the host proteins. The

TTS apparatus consists of a multi-ringed basal body with a needle like barrel projecting out from the center of the apparatus (120). The system contains proteins such as PrgH, PrgI, PrgK and InvG (119, 120) and it has been suggested that simultaneous expression of *prgH* and *prgK* results in a ring structure that resembles the base of the needle complex.

The proteins associated with the TTS are classified into two groups: a) structural components associated with the inner and outer membrane, regulatory proteins, proteins associated with the energy transduction and chaperones, b) proteins associated with either secretion or effector functions inside the host (189).

The genes present on SPI1 encode for apparatus of a secretory system that gets activated only upon contact with the host cell (38, 70, 71)**.** Proteins associated with the inner membrane include InvA, SpaP, SpaQ, SpaR and SpaS (38, 86) and proteins belonging to the Spa family are considered to be the structural components that aid in the translocation process (39). The outer membrane consists of InvG, PrgH and PrgK proteins. PrgH and PrgK are lipoproteins while InvG (107) is suggested to be a multimeric protein that has been associated with the outer membrane channel and considered to play an important role in bacterial uptake.

The protein InvC is known to have ATPase activity suggesting that it may be associated with energizing the apparatus, and this protein is also considered to be a component of the inner membrane (58). Chaperones (proteins that assist in maintaining the confirmation of the effector proteins) associated with the TTS include InvJ and SicA and SicA is known to be associated with the secretion of Sip proteins namely SipA, SipB and SipC.

The secretory system is regulated by two proteins HilA and InvF and InvF belongs to the AraC family of transcriptional activators (107). The protein HilA (141) belongs to OmpR/ToxR family and is considered to regulate three genes *orgA*, *sipC* and *invF* and the gene *orgA* is expressed in a low oxygen environment (103).

In addition to HilA and InvF, other transcriptional regulators associated with the SPI1 include HilC, HilD and SprB (46, 57, 162). HilA is directly involved in the expression of TTS structural genes and *invF* and transcription initiated at the promoter region of *invF* results in expression of *sicA*, and *sipABCD* genes (46, 57).

Other proteins that are secreted and that are involved with the TTS include SipA, SipB, SipC, SipD, SpaO, InvJ, SptP and AvrA (39, 40, 84, 106, 108). Proteins InvJ and SpaO play a very critical role in the secretion of other target proteins involved in the system (39, 126).

Secreted proteins that have a putative effector role in the host include SipA, SipB and SipC (106, 108). SipB, SipC and SipD are considered to play an important role in translocating other effector proteins into host cells (38, 84). SipB and SipC are also known to be associated with the plasma membrane of the host cell and SipB and SipD are considered to be involved in associating SipC to the host cell membrane (176).

The TTS system gets activated upon contact with the epithelial cells and is regulated by the transcriptional activators InvF and HilA. In addition to the regulation of the genes encoded on the SPI1, the secretory system is influenced by other mechanisms such as PhoQ-PhoP, a two component system and RpoS sigma factor (19, 77, 156).

Expression of virulence genes

An ordered expression of virulence genes associated with SPI1 has been reported (154) and majority of the genes were found to be expressed during transition from the exponential to the stationary phase of growth in Luria-Betani (LB) medium. The expression of regulatory genes *hilD*, *hilA* and *hilC* associated with the regulation of the secretory apparatus is followed by the expression of chaperone gene *sicA*. Other genes *sopA*, *sopB*, and *sopE*2 associated with the secretion of proteins are expressed soon after. Based on the order of expression of the genes it is speculated that *S*. Typhimurium has the highest expression of *sopA* gene that primarily affects the mitochondrial function (123). This is followed by the dephosphorylation of the phosphate in the lipid membrane by the *sopB* gene (55, 148) with eventual increase in the expression of *sopE*2 that is associated with membrane ruffling (187). This results in the engulfment of the pathogen into the epithelial cells. Genes associated with the intracellular survival (*phoP*, *ssrA*, *ssab, ssaG, sifA, sifB and pipB*) were expressed in stationary phase in LB and minimal (M9) medium and it is speculated that *phoP* and *ssrA* are expressed inside the gut.

Environmental and genetic factors that influence invasion

Invasion of the pathogen into the epithelial cells is regulated by the transcriptional regulators. The regulators activate and control the expression of genes contained within and outside the pathogenicity island 1. The gene *hilA* belonging to the ToxR/OmpR family is considered to be the critical regulator of the secretory apparatus. The gene activates the *sip* operon and the *inv/spa* operons that encode the components of the TTS system (10, 46). *hilA* is also considered to activate *invF*, another transcriptional regulator that belongs to the AraC family inducing the expression of several secreted proteins associated with the *sip* operon

(46, 107)**.** *invF* controls the expression of an secreted effector, *sopB* and this gene is encoded outside of the SPI1 (57).

The regulators of SPI1 are in turn controlled by other genes that are located outside of the island 1. *invF* is regulated by other regulators that are not regulated by *hilA* (5, 162). It is therefore assumed that environmental factors that incite invasion stimulate the expression of several regulators that in turn aide in the activation of several components involved with the secretory system. The expression of *hilA* is controlled by two other genes, *hilC* and *hilD* (102) that are located at a region upstream of the *hilA* promoter (28, 130). While it is considered that the expression of *hilD* is of absolute importance for the invasion (57), the role of *hilC* is not well understood.

A two-component regulator BarA/SirA located outside of SPI1 is known to control the invasion of the pathogen. The expression of *hilA* and other genes associated with the SPI1 is considered to be controlled by BarA (6, 102). The BarA/SirA system also controls a secondary regulatory system; *csr* system. The system consists of CsrA, a small protein that is known to bind to the ribosome binding site and varying the expression (168) of the target. The gene *csrA* is considered to be essential for the expression of SPI1 gene, however, overexpression of this was found to inhibit the expression of invasion genes (5). The BarA/SirA also controls the expression of CsrB and CsrC (untranslated RNA molecules) and the expression of these two molecules is known to activate invasion. Other genes that are known to regulate the gene expression of SPI1 include *rtsA* which in turn induces the expression of *dbsA*, a gene that encodes for a periplasmic disulfide bond isomerase (60). The gene *filZ* associated with the flagellar regulation is also known to regulate the expression of *hilA* (131).

The expression of invasion genes is controlled and this leads to the expression of TTS apparatus at the place of infection where the virulence is considered to be effective. The point of infection in the small intestine is the ileum where *Salmonella* is known to associate with the M cells (104). The environment in the small intestine

(microaerophilic in the brush border of the ileum) is an important factor that is thought to be responsible for the stimulation of the invasion gene expression. Most of the SPI1 genes expressed through HilA are thought to be expressed under reduced oxygen (11, 104) and high osmolarity conditions (67). In addition, the neutral pH of the small intestine also contributes to the expression of *hilA* (11).

The expression of genes associated with the SPI1 is very tightly controlled and the factors involved in the invasion are expressed only at appropriate time points of the infection process. The expression of SPI1 genes is under stringent control of several global regulators indicating that the system controlling the invasion is incorporated into several other processes. This integrated control is considered to result in ordered expression of virulence genes promoting the invasion of the intestinal epithelial cells by *Salmonella* (4).

CHAPTER III

INACTIVATION, TRANSCRIPTOMIC AND VIRULENCE GENE EXPRESSION RESPONSES OF *Salmonella* TO CHLORINE

Introduction

Chlorine has been the most popular disinfectant used for several decades to treat water in United States. It is also widely used by the poultry industry to disinfect broiler carcasses. Typical disinfection of poultry carcasses involves use of a sodium hypochlorite solution. The concentration of hypochlorite is expressed as free available chlorine by "determining the electrochemical equivalent amount of Cl_2 to that compound" (81). Free available chlorine refers to the sum concentrations of chlorine (Cl_2), hypochlorous acid (HOCl) and hypochlorite ion (OCl^-) and HOCl is a weak acid that dissociates to hypochlorite (144). Hypochlorite anion is a weak disinfectant when compared to HOCl and antimicrobial efficacy of chlorine is highly dependent on pH of the system.

The efficacy of chlorine as a disinfectant is highly dependent on presence of organic material and free chlorine reacts with other dissolved substances to form chlorides and organic and inorganic chloramines (34). Presence of chlorine demand (organic material) in the system will reduce free available chlorine reducing its disinfectant capacity. Only excess chlorine present (after the reaction with organic material forming chlorinated organic products (208) will produce hypochlorous acid and nitrogen gas or monochloramine and dichloramine (171).

Most kinetic studies with chlorine investigated the efficacy of the chemical in inactivating viruses, parasites such as *Giardia* and *Cryptosporidium* in waste water and coliform bacteria in drinking water (26, 61). The disinfectant's efficacy is typically assessed

using kinetic models and is dependent on several factors such as pH, temperature, and type of microorganism being inactivated.

Chlorine is an oxidizing agent and studies evaluating the disinfection efficacy reported lethal action of the chemical on several cellular constituents of the microorganism. Some of these include impact on the bacterial DNA resulting in the formation of chlorinated products of nucleotide bases (49, 54), inhibition of oxidative phosphorylation (16), and disruption in the membrane related activity (33).

Most kinetic studies involving chlorine were largely restricted to investigating only specific indicator organisms such as coliforms and *E. coli* commonly found in drinking water. Studies evaluating the disinfection kinetics of *Salmonella* are very limited. This study is aimed at understanding the inactivation kinetics of *Salmonella* serovars to chlorine at varying levels of concentration and contact time. In addition, the transcriptomic responses of *S.* Typhimurium at different time points both in fresh and overnight cultures were also evaluated. The study was also aimed at understanding the differential expression of virulence genes in overnight culture of *S.* Typhimurium. The overall hypothesis of the study is: Chlorine is ineffective and causes significant upregulation of several metabolic and virulence genes in *Salmonella*.

Materials and methods

Bacterial strains and culture conditions

Salmonella strains (*S.* Typhimurium, *S.* Enteritidis, *S.* Heidelberg and *S.* 4,5,[12]:i) were obtained from USDA – ARS (College Station, TX). The strains were stored at -80ºC prior to use. Each strain (a loopful of the initial culture) was cultured in 10 ml of Tryptic Soy Broth (TSB; Difco, Detroit, MI) thrice successively with incubation at 37 ºC for 18-24 h. After two consecutive transfers, cultures were streaked on Tryptic Soy Agar plates (TSB;

Difco, Detroit, MI) and incubated at 37ºC for 18-24 h. Plates with individual colonies were stored at 4 ºC for experimental studies.

Preparation of Salmonella cultures

A day before the actual experiment a single colony of each strain was inoculated into 10 ml of TSB and incubated at 37ºC for 18-24 h. Overnight cultures were washed twice with 10 ml chlorine demand free phosphate buffered saline solution by centrifugation at 3313 x g for 10 min at room temperature. Harvested cells were suspended in 10 ml of chlorine demand free phosphate buffered saline solution.

Chlorine standardization

Laboratory experiments with free available chlorine were conducted using chlorine solution (50-75 mg/L) (HACH). The volume of chlorine solution required to achieve free chlorine concentrations of 0.1, 0.5, and 1 ppm in the experimental buffer solution was standardized.

Inactivation of Salmonella with chlorine

Laboratory experiments were conducted in 20 ml of chlorine demand free phosphate buffer solution (pH adjusted to 7). Washed cells (100 µl of cell suspension) were exposed to free chlorine concentrations of 0.1, 0.5, and 1 ppm for 0.5, 2, and 4 min. The chemical was neutralized with 1 ml of sodium thiosulfate (20% w/v) at the end of contact time.

Transcriptomic responses of S. Typhimurium

The genomic responses of cells present in log (fresh culture) and stationary phases (overnight culture) of growth of *S.* Typhimurium were compared after exposing the cells to chlorine at a concentration of 10 ppm for 0.5 min.

Preparation of overnight culture

A day before the actual experiment a single colony of *S.* Typhimurium was inoculated into 10 ml of TSB and incubated at 37ºC for 18-24 h. Cell suspension was washed twice (3,313 x g for 10 min at room temperature) and resuspended in 10 ml of chlorine demand free phosphate buffer solution.

Preparation of fresh culture

On the day of actual experiment a single colony of *S.* Typhimurium was inoculated into 10 ml of TSB and incubated at 37 ºC for 3.5 h. Cell suspension was washed twice (3,313 x g for 10 min at room temperature) and resuspended in 10 ml of chlorine demand free phosphate buffer solution.

Treatment of cultures with chlorine

Laboratory experiments were conducted in 5 ml of chlorine demand free buffer solution. The cell density in the buffer for both the treatments was 5 x 10^8 CFU/ml. Cell suspensions in buffer solutions were exposed to chlorine at a concentration of 10 ppm for 0.5 min. The chemical was neutralized with 1 ml of sodium thiosulfate (20% w/v). Treated sample and the control (5 ml of each immediately after the treatment) were stabilized in RNAprotect™ (Qiagen, CA) immediately.

Incubation of cells for 30 min

After the treatment, 5 ml of the sample (both treatment and control) was transferred into a 25 ml conical flask containing 5 ml of 2x TSB and incubated at 37 ºC for 30 min. Incubated samples (5 ml of treatment and control) was stabilized in RNAprotect™ (Qiagen, CA) immediately.

RNA extraction

Total RNA from all the samples (0 and 30 min) was extracted using an RNeasy™ kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNase-free DNase set (Qiagen) was used for on-column DNA digestion to remove residual genomic DNA. The quantity and the quality of the total RNA extracted was verified using the Nanodrop (ND-1000) spectrophotometer (Nanodrop ® Technologies, Palo Alto, CA), and the Bioanalyzer ™ (Agilent Technologies, Santa Clara, CA), respectively.

cDNA synthesis, labelling and hybridization

Total RNA (5 μg) was used for synthesizing cDNA using a random primer for reverse transcription (Invitrogen, Carlsbad, CA). The standard operating protocols (# M007 and M008) of the J. Craig Venter Institute (JCVI, formerly The Institute for Genomic Research) was followed with few simple variations. cDNA synthesized was purified and labelled with Cy-3 mono-Reactive Dye and Cy-5 MONO-Reactive Dye (GE Health Care Biosciences Corp, Piscataway, NJ) and was processed using a dye-swapping design. Labelled cDNA's were purified using a QIAquick™ PCR purification kit (Qiagen, Valencia, CA). Equal amounts of labelled cDNA from the treatment and the control were hybridized on *S.* Typhimurium genome microarrays (version 8 arrays developed by JCVI) provided by the Pathogen Functional Genome Resource Center (PFGRC) of the National Institute of Health (NIH). Hybridization was done overnight in a water bath at 42 °C using Corning hybridization chamber. After hybridization, the slides were washed and scanned using a GenePix 4100A scanner (Molecular Devices, Sunnyvale, CA) at 532 nm (Cy3 channel) and 635 nm (Cy5 channel), and the images were stored for further analysis.

Determining the differential gene expression of virulence factors

The differential expression of virulence genes in overnight culture of *S.* Typhimurium was investigated using RT-PCR method. The gene expression was quantified using a comparative Ct method.

The total RNA extracted earlier from overnight culture of *S.* Typhimurium was used for synthesizing cDNA for the RT-PCR study.

cDNA synthesis

Total extracted and pooled RNA was used for cDNA synthesis. cDNA was synthesized using Gene Amp RNA PCR kit (Applied Biosystems, Branchburg, NJ). The reaction mixture contained the following: 1 µl of RNA template and 19 µl of master mix consisting of 4 µl of 25 mM $MgCl_2$ solution, 2 µl of DEPC treated DI water, 2 µl each of dATP, dTTP, dGTP, and dCTP, 1 µl of RNase inhibitor, 1 µl of MuLV reverse transcriptase enzyme, and 1 µl of random hexamer. The reaction mixture was incubated for 10 min at room temperature before placing in the thermocycler. First strand of cDNA was synthesized using single Reverse-Transcriptase polymerase chain reaction (RT-PCR). The RT-PCR reaction conditions were 60 min at 42°C for annealing and elongation followed by 5 min at 99 °C for inactivating the enzyme. All RT-PCR cycles were run in a programmable thermocycler (Applied Biosystems, Gene Amp PCR system 2700). The quantity of cDNA synthesized was measured using the Nanodrop (ND-1000) spectrophotometer (Nanodrop ® Technologies, Palo Alto, CA) and stored at -20 °C for experimental studies.

Real time PCR amplification

Appropriate primer sequences (See Appendix) for the specific targets were used for gene expression studies. The 384 well reaction plate (Applied Biosystems, Foster City, CA)

was filled with 1 µl of cDNA and 19 µl of master mix consisting of 10 µl of SYBR® GREEN PCR mix (Applied Biosystems, Warrington, UK), primers (0.6 µl each of forward and reverse primers (10 µmol) adjusted with DEPC treated deionized water. The negative controls consisted of DEPC – treated DI water. The plate was sealed with optical adhesive covers (Applied Biosystems, Foster City, CA) and placed in a thermocycler (Applied Biosystems, Unit – Abi – prism, 7000 HT, Foster City, CA) that was programmed for relative quantification to obtain a Ct value.

Results

Inactivation of Salmonella

The initial cell density in the test solution was 1 x 10^7 CFU/ml. Inactivation due to chlorine was very rapid with a 5.7 log inactivation observed after 0.5 min of contact time at concentrations 0.1 and 0.5 ppm with few or no survivors detected at chlorine concentration of 1 ppm. Inactivation of *S.* Typhimurium at varying concentrations of chlorine is presented in Fig. 3.1.

No colony forming units were detected with further increase in contact time (2 and 4 min) for all the concentration levels for *S.* Typhimurium. A 4.0 and 5.2 log inactivation was obtained for *S.* Enteritidis after 0.5 min of exposure time to disinfectant at concentrations of 0.1 and 0.5 ppm respectively. Log inactivation of *S.* Enteritidis is presented in Fig. 3.2.

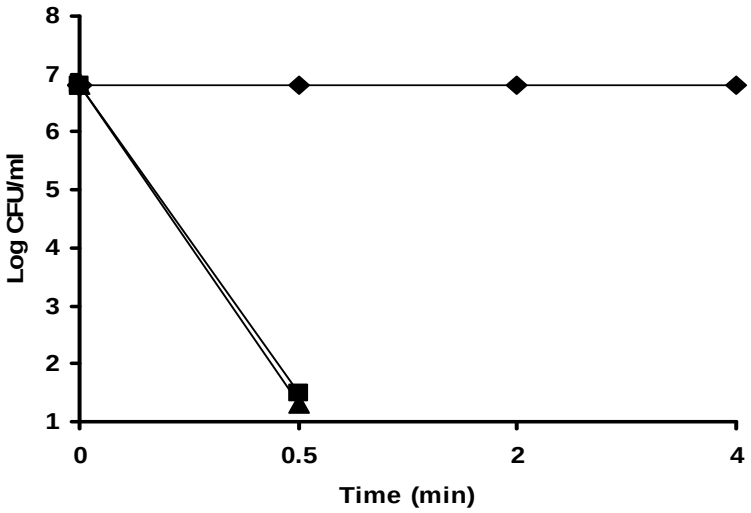

FIG. 3.1. Inactivation of *S.* Typhimurium after treatment with chlorine for 0.5, 2, and 4 min. Symbols: ▼, 0 ppm chlorine; ■, 0.1 ppm chlorine; ▲, 0.5 ppm chlorine; ◊, 1 ppm chlorine. No survivors detected at chlorine concentrations of 0.1, and 0.5 ppm at 2 and 4 min. No survivors detected at chlorine concentration of 1 ppm at 0.5, 2, and 4 min. Detection limit: 1 CFU/ml = 0 log CFU/ml.

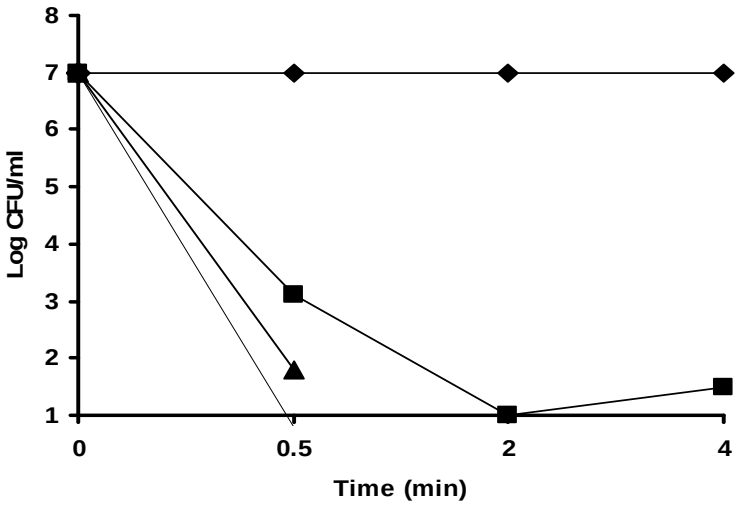

FIG. 3.2. Inactivation of *S.* Enteritidis after treatment with chlorine for 0.5, 2, and 4 min. Symbols: ▼, 0 ppm chlorine; ■, 0.1 ppm chlorine; ▲, 0.5 ppm chlorine; ◊, 1 ppm chlorine. No survivors detected at chlorine concentrations of 0.5 and 1 ppm at 2 and 4 min. Detection limit: 1 CFU/ml = 0 log CFU/ml.

A 6.0 log inactivation was obtained at a chlorine concentration of 0.1 ppm after 2 and 4 min of contact time. No colony forming units were detected for concentrations 0.5 and 1 ppm at 2 and 4 min of exposure time. Inactivation of *S.* Heidelberg for different concentration levels of chlorine is presented in Fig. 3.3.

Log inactivation of 5.0 and 5.6 was obtained for *S.* Heidelberg at concentrations of 0.1 and 0.5 ppm respectively for a contact time of 0.5 min. Very few to no colony forming units (< 1.0 log) were detected at 1 ppm.

The inactivation obtained for *S.* 4,5,[12]:i is presented in Fig. 3.4. For *S.* 4,5,[12]:i, log inactivation of 5.0, 6.0, and 6.5 was achieved at concentrations of 0.1, 0.5 and 1.0 ppm respectively for a contact time of 0.5 min. The detectable survivors were few to none at all concentration levels for time periods 2 and 4 min.

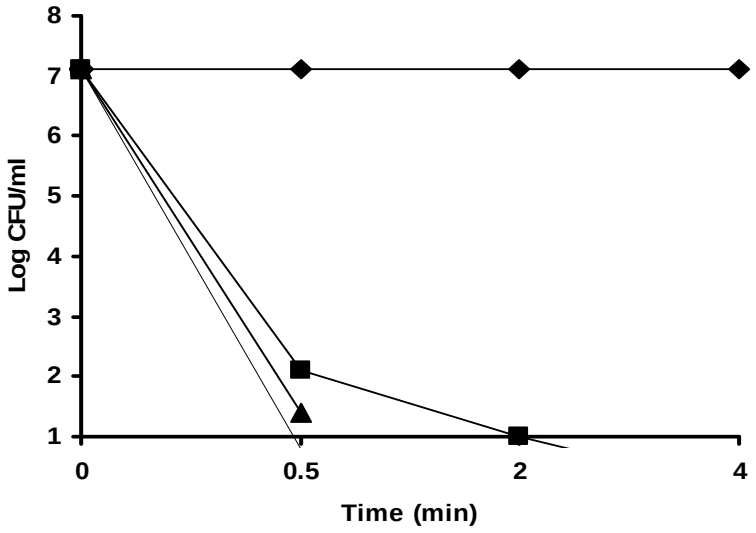

FIG. 3.3. Inactivation of *S.* Heidelberg after treatment with chlorine for 0.5, 2, and 4 min. Symbols: ▼, 0 ppm chlorine; ■, 0.1 ppm chlorine; ▲, 0.5 ppm chlorine; ◊, 1 ppm chlorine. No survivors detected at chlorine concentrations of 0.5 and 1 ppm at 2 and 4 min. Detection limit: 1 CFU/ml = 0 log CFU/ml.

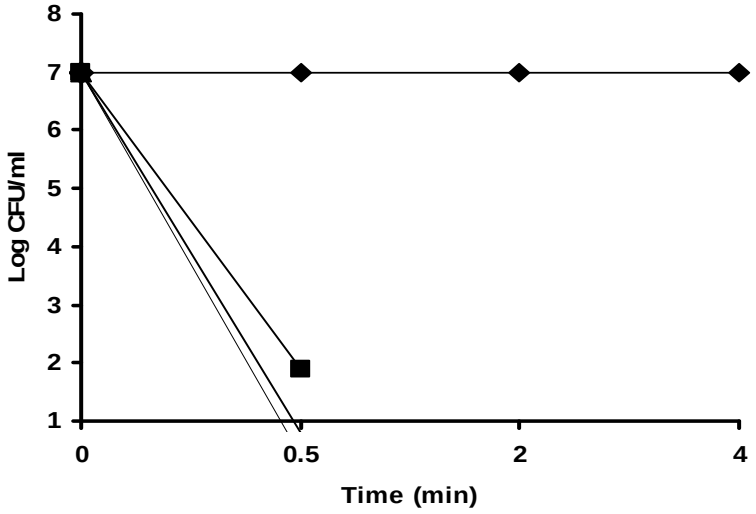

FIG. 3.4. Inactivation of *S.* 4,5,[12]:i after treatment with chlorine for 0.5, 2 and 4 min. Symbols: ▼, 0 ppm chlorine; ■, 0.1 ppm chlorine; ▲, 0.5 ppm chlorine; ◊, 1 ppm chlorine. No survivors detected at chlorine concentrations of 0.1, 0.5 and 1 ppm at 2 and 4 min. Detection limit: 1 CFU/ml = 0 log CFU/ml.

Transcriptomic responses of S. Typhimurium to chlorine

The signal intensity of each gene was globally normalized using LOWESS within the R (R.2.5.1) statistics package (210). The normalized data was analyzed using SAS 9.1.3 (SAS Institute Inc. Cary, NC). An approximate F test on least square means was used to estimate the significant difference ($p \leq 0.05$) for each gene. The DAVID Gene Functional classification tool was used for functional clustering of differentially expressed genes ((91, 92).

The global gene expression profile of *S.* Typhimurium was evaluated for two experimental conditions namely fresh and overnight culture to gain a better understanding of the molecular mechanisms affecting the actively dividing cells (log phase) to that of stationary phase cells. Overall, 136 genes (2.3%) were differentially expressed in fresh

culture at 0 min. Genes are grouped into clusters based on the function and the underlying mechanism involved using DAVID gene Analysis software. Accordingly, the genes were grouped into seven clusters based on the enrichment score. An enrichment score of 2.0 and above has been used in functional classification of the differentially expressed genes. Differential expression of genes in *S.* Typhimurium in fresh culture immediately after treatment with chlorine at 10 ppm is presented in Table 3.1.

TABLE 3.1. Differential expression of genes in *S.* Typhimurium in fresh culture immediately after treatment with chlorine at 10 ppm for 0.5 min.

Cluster	Gene	Annotation	Fold change
Two component system	*frdB*	Fumarate reductase iron-sulfur subunit	3.3
	hydG	Transcriptional regulatory protein ZraR	-2.2
	pgtC	Phosphoglycerate transport regulatory protein precursor	-1.8
	narG	Nitrate reductase 1 alpha subunit	-1.7
	trpA	Tryptophan synthase subunit alpha	-1.6
Carboxylic acid metabolism	*argA*	N-acetylglutamate synthase	8.7
	proS	Prolyl-tRNA synthetase	2.8
	aspA	Aspartate ammonia-lyase	-7.8
	glyS	Glycyl-tRNA synthetase subunit beta	-2.8
	prpD	2-methylcitrate dehydratase	-2.7
	bioD	Putative dithiobiotin synthetase	-2.6
Chromosome partition	*ftsK*	DNA translocase FtsK	2.3
	mukB	Cell division protein MukB	-3.3
	xerC	Site-specific tyrosine recombinase XerC	-2.1
Tetrapyrrole biosynthesis	*yggW*	Coproporphyrinogen III oxidase	-2.6
	bioD	Putative dithiobiotin synthetase	-2.6
Amine metabolism	*argA*	N-acetylglutamate synthase	8.7
	pros	Propyl-tRNA synthetase	2.8
	murB	UDP-N-acetylenolpyruvoylglucosamine reductase	1.5
	aspA	Aspartate ammonia-lyase	-7.8
	glyS	Glycyl-tRNA synthetase subunit beta	2.8

A majority of the genes that were expressed are commonly associated with the general metabolic processes and virulence. All the genes were down regulated except *frdB* (two component system) and related to stress response, *argA*, *proS* (carboxylic acid metabolism), *ftsK* (chromosome partition), *argA, murB, pros* (amine metabolism) were up regulated.

Incubation of stressed cells for 30 min in TSB caused significant upregulation of several molecular mechanisms in *Salmonella*. Differentially expressed genes were grouped into more than 30 clusters based on the function, however, only the top 5 clusters are being represented in Table 3.2. Also, the percentage differential gene expression at 30 min was higher (12%) as compared to the expression at 0 min. The two-component system and the citric acid cycle are the common mechanisms exhibited by log cells both at 0 and 30 min. In specific, the *phoQ* gene, a sensor protein was downregulated at 30 min.

TABLE 3.2. Differential gene expression in fresh culture of *S.* Typhimurium after treatment with chlorine at 10 ppm and subsequent incubation for 30 min.

Cluster	Gene	Annotation	Fold change
Two component system	*glnL*	Nitrogen regulation protein NR(II)	14.7
	acnB	Bifunctional aconitate hydratase	11.8
	glnA	Glutamine synthetase	9.8
	hydG	Transcriptional regulatory protein ZraA	3.0
	yegO	Multidrug efflux system subunit MdtC	2.1
	yqeF	Acetyl-CoA acetyltransferase	2.4
	fdnG	Formate dehydrogenase-N alpha subunit	-87.3
	narJ	Nitrate reductase 1 delta subunit	-75.4
	citD	Citrate lyase subunit gamma	-50.0
	phoQ	Sensor protein PhoQ	-10.0
Citrate Cycle	*sdhD*	Succinate dehydrogenase cytochrome b556 small membrane	116.4
	sdhB	Succinate dehydrogenase iron-sulfur	55.6

TABLE 3.2. continued

Cluster	Gene	Annotation	Fold change
	gltA	Type-II citrate synthase	66.0
	sucD	Succinyl-CoA synthetase subunit alpha	11.0
	citD	Citrate lyase subunit gamma	-50.0
	citF	Citrate lyase alpha chain	-32.9
Nitrogen metabolism	*asnA*	Asparagine synthetase AsnA	105.5
	argT	Lysine/arginine/ornithine transport protein	25.2
			-75.4
	narJ	Nitrate reductase 1 delta subunit	-58.4
	nirD	Nitrite reductase small subunit	-28.9
	narL	Nitrate reductase 1 gamma subunit	
ABC transporters	*rbsA*	D-ribose transporter ATP binding protein	157.7
	rbsD	D-ribose pyranase	152.0
	yliB	Putative ABC transporter periplasmic binding protein	1.6
	gltI	Glutamate and aspartate transporter subunit	35.0
	livG	Leucine/isoleucine/valine transporter ATP binding subunit	-3.4
Purine metabolism	*purD*	Phosphoribosylamine-glycine lygase	45.6
	purM	Phosphoribosylaminoimidazole synthetase	5.2
	nudF	ADP-ribose pyrophosphatase NudF	-5.2
	dnaX	DNA-polymerase III subunits gamma and tau	-3.7
	rpoB	DNA-directed RNA polymerase subunit beta	-1.4

The *phoP* locus in *Salmonella* contains two genes *phoP* and *phoQ* that respond to environmental stress. This two component system is known to control the virulence in *S.* Typhimurium and mutations in the *phoP* locus was found to reduce the virulence of the pathogen in mice (138)**.** The gene (*phoQ*) associated with the two-component system was

downregulated in the current study, hence it can be concluded that the virulence of the microorganism was reduced.

Overnight culture

Differential gene expression of stationary cells in *S*. Typhimurium after exposure to chlorine at a concentration of 10 ppm for 0.5 min is presented in Table 3.3. Of the 5,918 genes globally expressed by *Salmonella*, 13% of the genes were differentially expressed in stationary cells at time 0. The top five molecular mechanisms that were affected as a result of chlorine exposure were glycerolipid metabolism, ABC transporters, glycerophospholipid metabolism, ketone metabolism and two-component system with majority of the genes being downregulated in all the mechanisms.

The number of differentially expressed genes were 810 (14%) of the 5,918 global genes expressed in *S.* Typhimurium at 30 min in overnight culture. The molecular mechanisms involved were clustered into 53 groups, however only the top five mechanisms are presented in Table 3.4.

The top mechanisms affected include ribosomal protein, oxidative phosphorylation, flagellar assembly, and amino acyl tRNA synthetase activity and the enrichment score for ribosomal protein was 60 indicating that this molecular mechanism was greatly affected with the chlorine treatment. Several proteins associated with both 50S and 30S subunits of the ribosome were downregulated. Genes encoding for several proteins associated with the oxidative phosphorylation pathway are down-regulated. Of particular importance are genes encoding for ATP synthase (*atpABCEFGH*), and NADH dehydrogenase (*nuoBEFGIJKMN)*.

TABLE 3.3. Differential gene expression in overnight culture of *S.* Typhimurium after treatment with chlorine at a concentration of 10 ppm for 0.5 min.

Cluster	Gene	Annotation	Fold change
Glycerolipid metabolism	*gldA*	Glycerol dehydrogenase	-1.8
	dgkA	Diacylglycerol kinase	-1.7
	plsB	Glycerol-3-phosphate acyltransferase	-1.5
ABC transporters	*dppB*	Dipeptide transporter permease DppB	1.7
	rbsB	D-ribose transporter subunit RbsB	1.4
	sitA	Putative periplasmic binding protein	-4.2
	sfbA	Putative ABC-type transport system ATPase component	-3.2
	ftsE	Cell division protein FtsE	-2.1
Glycero-phospholipid metabolism	*pgsA*	Phosphatidylglycerophosphate synthetase	4.1
	pgpB	Phosphatidylglycerophosphatase B	1.4
	dgkA	Diacylglycerol kinase	-1.7
	plsB	Glycerol-3-phosphate acyltransferase	-1.5
Cellular ketone metabolism	*ilvL*	ilvG operon leader peptide	2.1
	asnB	Asparagine synthetase B	-7.1
	dkgA	2,5-diketo-D-gluconate reductase A	-4.8
	prpD	2-methylcitrate dehydratase	-4.0
	sdaA	L-serine deaminase I/L-thronine deaminase 1	-3.4
Two- component system	*rcsF*	Outer membrane lipoprotein	1.7
	torR	DNA-binding transcriptional regulator TorR	1.3
	ompR	Osmolarity response regulator	-2.8

TABLE 3.4. Differential gene expression in overnight culture of *S.* Typhimurium exposed to chlorine at a concentration of 10 ppm for 0.5 min followed by incubation for 30 min.

Cluster	Gene	Annotation	Fold Change
Ribosomal Protein	*rpmJ*	50S ribosomal protein L36	974.9
	rpsV	30S ribosomal subunit S22	33.2
	rpsS	30S ribosomal protein S19	-702.8
	rpsG	30S ribosomal protein S7	-686.8
	rpsN	30S ribosomal subunit protein	-38.4
Oxidative Phosphorylation	*sdhD*	Succinate dehydrogenase cytochrome b556 small membrane subunit	101.7
	sdhC	Succinate dehydrogenase cytochrome b556 large membrane subunit	74.1
	sdhA	Succinate dehydrogenase flavoprotein subunit	9.2
	nuoM	NADH dehydrogenase subunit M	-38.1
	nuoN	NADH dehydrogenase subunit N	-33.1
Flagellum	*motA*	Flagellar motor protein MotA	4.5
	flgN	Flagellar FlgK/FlgL export chaperone	4.5
	flgB	Flagellar basal body rod protein FlgB	-642.4
	flgD	Flagellar basal body rod modification protein	-284.1
	fliH	Flagellar assembly protein	-273.0
	fliE	Flagellar hook-basal body protein FliE	-96.1
Aminocyl-tRNA synthetase	*glyQ*	Glycyl-tRNA synthetase subunit alpha	-55.0
	argS	Arginyl-tRNA synthetase	-40.9
	glyS	Glycyl-tRNA synthetase subunit beta	-38.6
	trpS	Tryptophanyl-tRNA synthetase	-37.5
	asnC	Asparaginyl-tRNA synthetase	-36.2
Oxoacid metabolic process	*aceA*	Isocitrate lyase	2714.6
	aceB	Malate Synthase	14315.5
	guaA	GMP synthase	-282.5
	accB	Acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	-58.1
	glyQ	Glycyl-tRNA synthetase subunit alpha	-55.0

The genes *flgBCDEFGHIN* associated with the flagellum basal body operon were downregulated and other genes associated with the biosynthesis of the flagellum were also inhibited except for the gene (*motA*) encoding for motor protein of the flagella. Aminocyl – tRNA synthetase is an enzyme involved in esterification of the amino acid to tRNA and several genes encoding for aromatic amino acids such as phenylalanine (*pheST*), tryptophan (*trpS*) and tyrosine (*tyrS*) were down-regulated. In addition, genes involved in biosynthesis of other aminoacids such as glycine, alanine, histidine, and leucine were also greatly inhibited. Other mechanisms that were significantly involved include TCA cycle (significant upregulation of majority of the genes, *gltA, mdH, pckA, sdhACD, sucABC*), purine and pyrimidine metabolism (downregulated), ABC transporters (mix of upregulated and downregulated genes), pentose phosphate pathway (downregulated), bacterial secretion system associated with virulence (downregulated), fatty acid biosynthesis (downregulated), glycolysis/gluconeogenesis (downregulated), and fatty acid metabolism (upregulated).

Differrential gene expression of virulence factors in S. Typhimurium

The change in gene expression was calculated using the formula Treatment 1/Treatment 2 = $2^{\Delta\Delta C}{}_T$, where $\Delta\Delta C_T = \Delta C_T$ for treatment 1 - ΔC_T for treatment 2 and ΔCt will be the difference between the Ct value of the target gene and the normalization gene (16s). A two-tail t-test with combined standard deviation was used to identify differential expression of the target gene.

No significant change in gene expression of virulence factors was observed in log phase cells at time 0 min. However, few genes associated with SPI1 such as *orgAB, sicP, sipD,* and *invAGI* were found to be downregulated in log phase cells at 30 min.

The differential gene expression of virulence factors in overnight culture of *S.* Typhimurium immediately after exposure to chlorine at a concentration of 10 ppm for 0.5 min is presented in Fig 3.5. Microarray analysis revealed only three genes that were differentially expressed namely *sitA, sipC* and *sipB* and all of these were found to be downregulated. However, a qt-PCR analysis showed differential expression of all the genes investigated. Most of the genes associated with the needle complex of the TTS system and invasion were downregulated in stationary phase cells at 0 min. No significant change in gene expression of virulence factors was observed in log phase cells at time 0 min. However, few genes associated with SPI1 such as *orgAB, sicP, sipD,* and *invAGI* were found to be downregulated in log phase cells at 30 min.

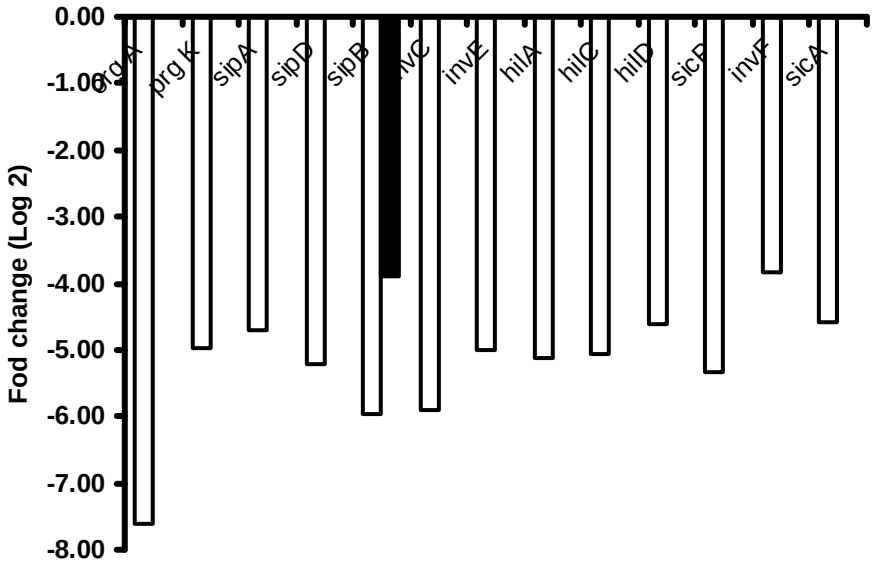

FIG. 3.5. Comparison of differential expression of virulence factors in overnight culture of *S.* Typhimurium after exposure to chlorine at a concentration of 10 ppm for 0.5 min by microarray and RT-PCR. The fold changes were converted to $\log_2$ values. Symbols: □, RT-PCR ; ■, Microarray.

Differential gene expression of virulence factors in stationary cells of *S.* Typhimurium after incubation for 30 min is presented in Fig 3.6. All the genes investigated were found to be downregulated.

FIG. 3.6. Comparison of differential expression of virulence factors by microarray and RT-PCR in overnight culture of *S.* Typhimurium after treatment with chlorine at a concentration of 10 ppm for 0.5 min with subsequent incubation for 30 min. The fold changes were converted into $\log_2$ values. Symbols: □, Microarray ; ■, RT-PCR.

Discussion

Inactivation kinetics of Salmonella

At the basic level the disinfection equation (37) is stated as $\ln (N/N_0) = k^*t$

N = number of microorganisms at time t

N_0 = number of microorganisms at time 0

k^* = reaction rate constant

t = contact time

This equation was further modified as empirical logarithmic function (205) to relate reaction rate constant to the concentration of the disinfectant $k^* = kC^n$ where

k = reaction rate constant (specific for given set of conditions)

C = concentration of the disinfectant

n = coefficient of dilution

In most cases the value of n = 1 giving a fixed *Ct* (product of concentration and contact time) value for a fixed level of inactivation. This equation was further modified (89) to fit inactivation data that does not fit Chick-Watson equation. This is given as $\ln (N/N_0) = -k'' C^n t^m$ where m is an empirical constant. While m<1 depicts a tailing pattern, m>1 gives a shoulder. When n = 1, the degree of inactivation is given as product of concentration and time. The degree of inactivation is dependent on the concentration of the disinfectant when n > 1, for compounds where n < 1 there is reduced affect of the concentration on inactivation. Inactivation kinetics for chlorine for different *Salmonella* serovars was modelled using empirical equation $\ln (N/N_0) = -k C^n t^m$ (Figs. 3.7, 3.8, 3.9 and 3.10).

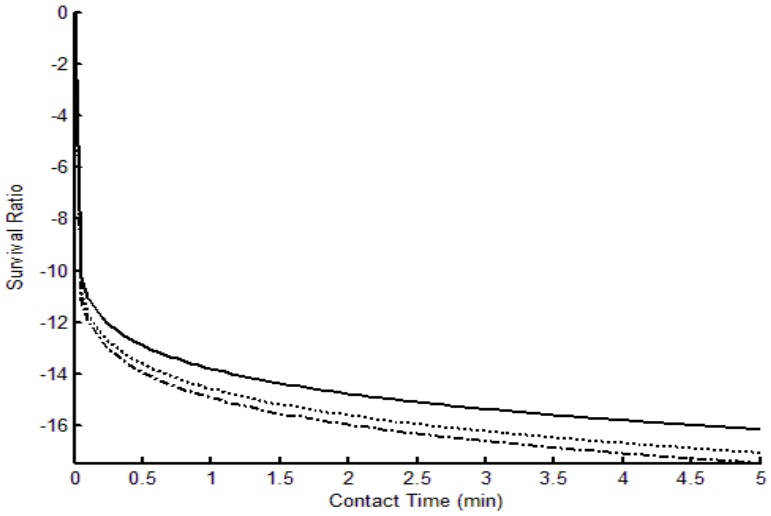

FIG. 3.7. Inactivation kinetics of *S.* Typhimurium after treatment with chlorine. Symbols: —(solid line), 0.1 ppm of chlorine; … (dot), 0.5 ppm of chlorine; -.-.-.-.(dot-line), 1 ppm of chlorine.

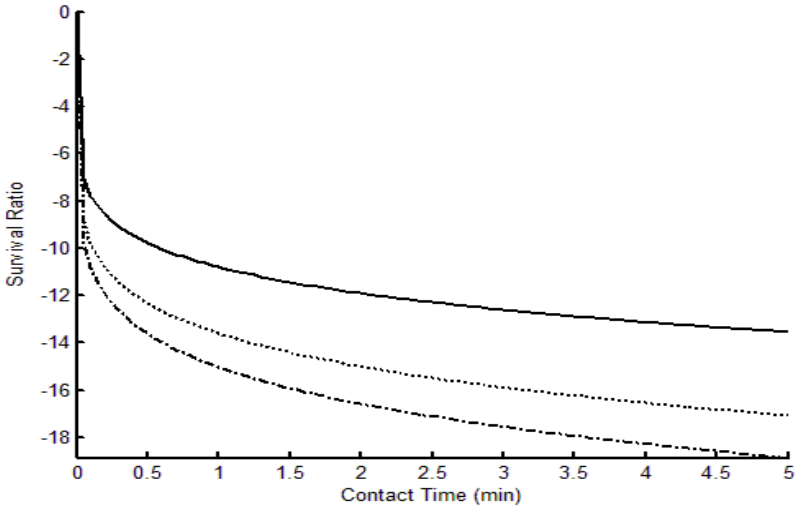

FIG. 3.8. Inactivation kinetics of *S.* Enteritidis after treatment with chlorine. Symbols: —(solid line), 0.1 ppm of chlorine; …(dot), 0.5 ppm of chlorine; -.-.-.-.(dot-line), 1 ppm of chlorine.

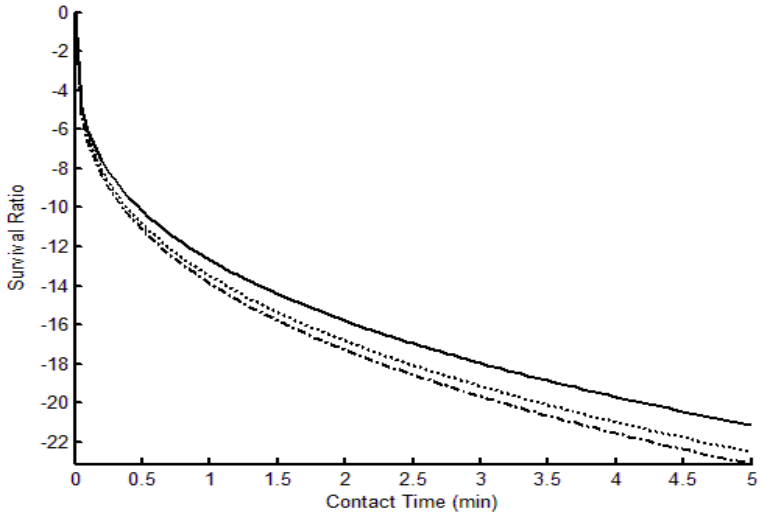

FIG. 3.9. Inactivation kinetics of *S.* Heidelberg after treatment with chlorine. Symbols: — (solid line), 0.1 ppm of chlorine; …(dot), 0.5 ppm of chlorine; -.-.-.-(dot-line), 1 ppm of chlorine.

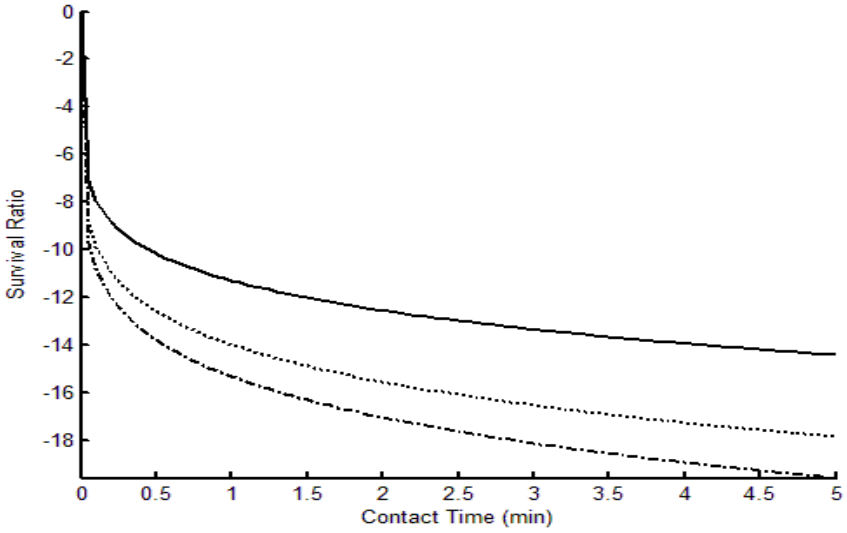

FIG. 3.10. Inactivation kinetics of *S.* 4,5,[12]:i after treatment with chlorine. Symbols: — (solid line), 0.1 ppm of chlorine; …(dot), 0.5 ppm of chlorine; -.-.-.-(dot-line), 1 ppm of chlorine.

The data obtained from the inactivation experiments for chlorine concentrations (0.1, 0.5, and 1 ppm) at contact time (0.5, 2, and 4 min) were fitted into the model. The exponents (n and m) and reaction constant k for individual strains was obtained by means of multiple linear regression. The inactivation equations obtained for serovars are as follows: *S.* T – ln $(N/N_0) = -2.7\ C^{0.03}\ T^{0.1}$

S. E - $\ln (N/N_0) = -2.7\ C^{0.14}\ T^{0.14}$

S. H - $\ln (N/N_0) = -2.6\ C^{0.04}\ T^{0.3}$

S. 4,5,[12]:i - $\ln (N/N_0) = -2.7\ C^{0.13}\ T^{0.2}$

The n value obtained for all the serovars is less than 1 indicating that the disinfection efficiency is independent of the concentration of the biocide and dependent on the exposure time. This is quite true when observed with the data obtained (log inactivation was almost equal for all concentration levels at similar contact time with higher inactivation achieved after prolonged exposure time).

Also, the m value obtained is less than 1 indicating that there is tailing affect. Although close, the n value obtained for *S.* Enteritidis and *S.* 4,5,[12]:i were higher when compared to *S.* Typhimurium and *S.* Heidelberg indicating that the strains Enteritidis and 4,5,[12]:i are resistant to the disinfectant and there was a concentration effect.

Several studies evaluated the inactivation kinetics of chlorine in drinking water. Inactivation kinetics generally followed a linear pattern for coliforms with the degree of inactivation being directly dependent on the concentration of the chemical. A study (125) investigating the disinfection efficacy of the chemical reported no significant antimicrobial activity of the chemical after 15 min of contact time. This particular study evaluated the effect of the chemical at different concentrations (0.1, 0.5, 1, 3 and 5 ppm) for a contact time

of 0.5 min. Log reductions in the order of 0.2, 0.91, 0.95, 1.00 and 1.05 respectively, were reported for different concentration levels indicating that the degree of inactivation achieved was directly dependent on the concentration of the chemical and the concentration is inversely related to the contact time. A 4.0 log reduction in coliforms was reported after a contact time of 95.0 min at a concentration of 1 ppm, whereas the same level of reduction was achieved in 2.75 min at a concentration of 5 ppm (125). The disinfection efficacy of chlorine in the presence and absence of organic material was investigated (202) and it has been reported that organic material significantly increased the resistance of all the organisms to the chemical and it was concluded that this was due to the stabilization of the membrane resulting in reduced contact of the chemical with the cell. The degree of inactivation achieved was higher for gram-negative organisms (*Escherichia coli* and *Yersinia enterocolitica*) when compared to gram–positive organisms (*Bacillus subtilis* and *Listeria monocytogenes*) in absence of organic material. Also, the inactivation was achieved at a much lower concentration (0.8 ppm for *E. coli*) as against 1.4 ppm for *B. subtilis*. However, addition of organic material resulted in significant differences in the inactivation pattern of the microorganisms. A concentration of 30 ppm was required to inactivate *E. coli* as against 15 ppm for *B. subtilis*.

A 6.5 log reduction was obtained at a concentration of 0.1 ppm in the current study and the differences observed in the log reductions between the current study and the earlier studies are due to inherent differences in the experimental design. The current study used phosphate buffer solutions at a pH of 7.0 with free available chlorine in the range of 50-75 ppm as against use of distilled water with hypochlorite solutions (15%) used in the previous studies. Also, the microorganisms tested were different (coliforms and *E. coli* vs *Salmonella*).

Transcriptomic responses of S. Typhimurium

Fresh culture

The two-component system and the citric acid cycle are the common mechanisms exhibited by log cells both at 0 and 30 min. In specific, the *phoQ* gene, a sensor protein was downregulated at 30 min. The *phoP* locus in *Salmonella* contains two genes *phoP* and *phoQ* that respond to environmental stress. This two component system is known to control the virulence in *S.* Typhimurium and mutations in the *phoP* locus was found to reduce the virulence of the pathogen in mice (139).

phoQ has two transmembrane domains creating periplasmic and cytoplasmic domains. The periplasmic part of *phoQ* is involved in sensing external signals such as in macrophages. *phoQ* encodes a amino-acid protein that is similar to sensor kinase protein that autophosphorylates at histidine residue and transfers phosphate to *phoP* (138) a transcriptional activator protein. The citrate cycle was significantly upregulated in log cells at 30 min, however, most genes of the cycle were downregulated at 0 min. This was quite obvious, because at 0 min, the actively dividing cells are injured or exposed to stress resulting in reduced respiration. This phenomenon of reduced respiration was reported earlier (12, 13) and the results obtained in this study also reflect the same phenomenon. Upregulation of majority of the genes involved in tricarboxylic acid has been observed at 30 min and this perhaps can be attributed to the recovery of cells in nutrient media. This recovery also highlights the fact that the injury to the cells is sub-lethal and resuscitation to normal metabolic state is quite rapid. The genes encoding for the enzymes required for amine and aromatic amino acid metabolism are downregulated in cells at 0 min and this also explains the reduced virulence phenomenon exhibited by *Salmonella* (66).

ABC transporters are present in all the species and in bacteria they are primarily involved in translocating the molecules across the cytoplasmic membrane (87, 129). These transporters utilize ATP, hence referred to as ATP binding cassette and the proteins contained in the cassette are ABC transporters. The domains that bind ATP are highly conserved. Bacterial ABC importers contain periplasmic protein that binds to the incoming substrate and delivers it to the complex in the inner membrane. Another subfamily of ABC transporters comprises of ABC exporters with a conserved ATP binding domain (63). In the current study, it was observed that cells in the log phase at 30 min consists of significantly upregulated genes such as *glnH, glnP, glnQ, gltL, hisJ, hisQ,* and *rbsA* associated with ABC transporters. In contrast, genes such as *livG, btuC,*and *btuD* were found to be downregulated. Purine metabolism was significantly enhanced in cells at 30 min with upregulation of several genes (*cyaA, nrdA, nude, nudF, purC, purD, purH, purM and yfeJ*).

Overnight culture

The ABC transporters are down regulated, similar to that seen in log cells (fresh culture) at time 30 min, however the genes regulating the mechanism are quite different indicating that although similar systems seem to be affected with the treatment, the response is different with different genes encoding different proteins. In addition, critical metabolic processes such as glycerolysis and glycerophopholipid metabolism are being impacted by the chlorine treatment in stationary cells at time 0. The gene *gldA* encoding for the enzyme glycerol dehydrogenase in glycerolipid metabolism known to be induced only in anaerobic conditions was down regulated. Of particular interest is the down regulation of the gene *sitA* in ABC transporters. This is associated with iron transport system in *Salmonella* and is located on SPI1 (*Salmonella* pathogenecity Island 1). The *sitABCD* is induced *in vitro* only in

iron deficiency conditions (100)**.** A down regulation of this gene also implies reduced virulence properties in *S.* Typhimurium. Another important gene expressed was *ompR,* an osmolarity reponse regulator. This is an acid induced response regulator typically required in stationary phase for acid tolerance and is known to regulate the expression of acid induced virulence operon ssrAB in *Salmonella* (14)**.** This gene was down regulated as that of *sitA***.**

Genes encoding for several proteins associated with the oxidative phosphorylation pathway are downregulated (at 30 min). Of particular importance are genes encoding for ATP synthase (*atpABCEFGH*), and NADH dehydrogenase (*nuoBEFGIJKMN)*. Downregulation of enzymes associated with ATP indicates that energy production is greatly inhibited in the cell. The genes associated with succinate dehydrogenase such as *sdhACD* were however upregulated which was quite interesting. Inhibition of the oxidative phosphorylation pathway has been reported earlier (16) and this study confirms this result.

Results indicate that several metabolic processes in the cell are greatly inhibited in both log and stationary phase cells and this is primarily attributed to depletion of ATP. Mechanisms involved in log cells at 0 min were comparatively low as compared to that of 30 min with significant upregulation of genes associated with citrate cycle, nitrogen metabolism and amino acid metabolism at 30 min. This indicates rapid recovery of the cells in nutrient rich media.

The impact of the chlorine treatment on cells in the stationary phase was very profound when compared to that of log cells and this was quite expected. Cells in the stationary phase are already exposed to a stressful environment and further application of chlorine would have an increased inhibitory impact on the basic metabolic processes. This was very evident in the gene expression profile, wherein several genes associated with the

oxidative phosphorylation, citric acid cycle and amino acid biosynthesis were downregulated. The recovery of the cells after 30 min of incubation was rather minimal or slow when compared to that of fresh culture. Based on this it is speculated that the cell is perhaps in a dormant state (may be VBNC) with reduced metabolic activity.

Pathways involved in the biosynthesis of amino acids are downregulated indicating that the protein synthesis within the cell is very minimal. Inhibition of ABC transporters in both log and stationary phase cells indicates significant disruption of cellular membrane activity. Recovery or regrowth of stressed cells was observed both in log phase and stationary phase cells. However, the time required to resuscitate was comparatively less in fresh culture as against overnight culture.

Differential expression of virulence genes

Downregulation of virulence determinants has been reported earlier (124, 203) in *S.* Typhimurium and it has been suggested that the chlorine stress results in the inhibition of the biosynthesis of the apparatus associated with the TTS system in *Salmonella*. This reduced activity is attributed to reduced ATP levels in the stressed cell. In addition, several of the other metabolic processes that are known to regulate the expression of virulence factors such as flagellar biosynthesis, and two-component system were downregulated. The exact underlying molecular mechanisms that play a role in controlling the expression of virulence factors by these metabolic processes are not known at this time.

Typically, bacterial cells are known to adapt when exposed to stress conditions such as acid, and heat (88) and studies tried to evaluate the affect of stress exposure in expression of virulence factors. A study evaluating the effect of acid tolerant stationary phase cells of *Salmonella enterica* observed that there was no significant increase in virulence potential of

the acid tolerant stationary cells to that of acid sensitive log phase cells (97). Similar phenomenon was also observed in *Listeria monocytogenes* wherein an increase in acid tolerance did not show a concomitant change in the virulence potential of the pathogen. However, other studies (45, 188) demonstrated increased virulence of refrigerated *L. monocytogenes* cells in mice. The authors concluded that factors such as changes in cell physiology, and expression of cold shock and virulence genes significantly influence the virulence potential of the stressed cells and these changes are considered to be induced and temporary.

Studies have shown that acid tolerance of *S.* Typhimurium is regulated by *rpoS* (alternative sigma factor), two-component regulator (PhoP/Q) and Fur (69). Similar phenomenon was observed in the current study, wherein the alternative sigma factor was regulated in stationary phase cells at 30 min and PhoQ was downregulated in log phase cells at 30 min when exposed to chlorine at a concentration of 10 ppm. These factors however were not expressed at 0 min of both stationary and log phase cells. In fact, there was no differential expression of virulence genes in fresh culture at 0 min indicating that chlorine does not have any impact on the expression of virulence factors in log phase cells at 0 min. However, downregulation of several virulence genes (*invA*, *invG*, *invI*, *orgA*, *orgB*, *sicP*) in log phase cells was observed after 30 min of incubation. However, gene associated with stress (*rpoS*) was not expressed in fresh culture at 30 min and the two-component system (*phoQ*) was not expressed in stationary culture at 30 min indicating that there are other mechanisms that are involved in the regulation of expression of virulence factors. This needs further analysis and investigation.

The current study also confirms the fact that genes related to stress are expressed only in stationary phase cells and not in log phase cells. Also, the upregulation of several stress related genes (*rpoS* and *uspA*) was observed only after 30 min of incubation of both log and stationary phase cells indicating an adaptive response of the pathogen to the chemical treatment.

The alternative sigma factor is known to control the expression of several virulence factors including the *spv* operon (85). The two-component system (PhoP/Q) controls the expression of several genes associated with the induction of the system (*pagS*) and genes associated with the repression of the system (*prgS*) and interference in the expression of specific *pagS* and *prgS* genes is known to inhibit the expression of virulence factors in *S.* Typhimurium (69). Also, it was demonstrated that the two-component system is in turn regulated by pH conditions and low pH was found to induce acid tolerance in *S.* Typhimurium (18). This indicates that the virulence expression is controlled by other environmental stress factors (69).

Studies evaluating the effect of chemicals on virulence expression of *Salmonella* are very limited. Most studies evaluated the effect of other stress factors such as heat and acid on virulence expression. The expression of certain genes such as *rpoS* and *phoQ* indicates that mechanisms affecting the virulence expression after exposure to chlorine stress are similar to that of acid induced virulence expression. The current study did not investigate the expression of virulence factors in cell lines (both *in vitro* and *in vivo*). Future studies can be oriented in this direction.

CHAPTER IV

PHENOTYPIC AND GENE EXPRESSION RESPONSE OF *Salmonella* TO CHLORINE DIOXIDE

Introduction

The practice of disinfecting drinking water with chlorine has been in vogue in United States for several decades. However, formation of carcinogenic trihalomethanes (THM) with chlorine has warranted research for finding alternatives to chlorine. Chlorine dioxide was being used in Europe since 19th century for treating water, however, data proving its bactericidal efficacy was not known until early 20th century (20). Although chlorine dioxide was known to be a safe disinfectant, its use in treating drinking water was not common in US because of problems associated with the production of chlorine free chlorine dioxide (73).

Formation of THMs with chlorine is primarily attributed to the presence of humic and fulvic acids in water and treatment with chlorine dioxide although did not produce THMs was however found to release other disinfection by-products such as chlorite (ClO_2^-) and chlorate (ClO_3^-) ions. Chlorate and chlorine dioxide were quickly converted to chlorite ions in humans and these by-products were found to cause hemolytic anemia (42). However, none of these products were found to be carcinogenic in rats in spite of chronic exposure (73).

Chlorine dioxide cannot be used as gas due to compression and storage issues (2). Hence, it has to be manufactured onsite and liquid form of the chemical is highly explosive at -40°C. The chemical cannot be generated continuously; hence its use in large scale disinfection operations has been limited.

The disinfectant's efficacy is modeled based on the following equation:

$\ln (N/N_0) = -k\ C^n\ t^m$ (89) and is dependent on several factors such as temperature, pH and type of the pathogen inactivated.

The efficacy of the chemical as a germicide has been reviewed quite extensively. Reports of its bactericidal activity have been reported as early as 1940 (134), with inactivation of polio virus reported back in 1949. A comparative study analyzing several disinfectants (chlorine, chlorine dioxide, ozone and monochloramine) on *Cryptosporidium* oocysts showed that chlorine dioxide was a better chemical in inactivating the parasite (118).

Chlorine dioxide is an oxidizing agent and used in the poultry industry because of its inherent advantages when compared to chlorine. The disinfectant's action is not affected by the pH of the system (can work in a pH range of 7-12) and is also considered to be unaffected by the presence of organic material. However, the bactericidal efficacy of the chemical was observed to be lowered in disinfecting secondary effluent (9).

Chlorine dioxide is considered to primarily affect the protein synthesis in the target microorganism (21). However, other studies claimed that the primary target was not the protein machinery in the cell; it was rather the loss of integrity of the outer membrane resulting in efflux of potassium (167) and decrease in respiration.

The chemical has been approved for use at a concentration of 3 ppm in chiller tank during poultry processing. Most studies involving disinfection kinetics were restricted to coliforms and *E. coli* in drinking water and waste water. Studies evaluating the disinfection kinetics for *Salmonella* are very limited. The following studies were aimed at understanding the disinfection efficiency of the chemical against *Salmonella* serovars. The differential expression of virulence factors as a result of chemical exposure was also investigated. The

central hypothesis of this study is: Chlorine dioxide is ineffective and causes significant upregulation of virulence genes in *Salmonella*.

Materials and methods

Bacterial strains and culture conditions

Salmonella strains (*S*. Typhimurium, *S*. Enteritidis, *S*. Heidelberg and *S*. 4,5,[12]:i) were obtained from USDA – ARS (College Station, TX). The strains were stored at -80ºC until use. Each strain (a loopful of the initial culture) was cultured in 10 ml of Tryptic Soy Broth (TSB; Difco, Detroit, MI) twice successively with incubation at 37 ºC for 18-24 h. After two consecutive transfers, cultures were streaked on Tryptic Soy Agar plates (TSA; Difco, Detroit, MI) and incubated at 37ºC for 18-24 h. Plates with individual colonies were stored at 4ºC for experimental studies.

Preparation of Salmonella cultures

A day before the actual experiment a single colony of each strain was inoculated into 10 ml of TSB and incubated at 37ºC for 18-24 h. Overnight cultures were washed twice with 10 ml chlorine demand free phosphate buffered saline solution by centrifugation at 4,000 x g for 10 min at room temperature. Harvested cells were suspended in 10 ml of chlorine demand free phosphate buffered saline solution. The preparation of chlorine demand free PBS is provided in the Appendix.

Preparation of chlorine dioxide

Chlorine dioxide was prepared on-site based on the following reaction:

$$4NaClO_2 + 2H_2SO_4 \longrightarrow 2ClO_2 + HCl + HClO_3 + 2Na_2SO_4 + H_2O$$

The steps involved in the preparation of the chemical are provided in the Appendix. The concentration of the chemical prepared was verified using digital spectrophotometer

(HACH) and accordingly volumes needed to achieve concentrations of 0.1, 0.5 and 1.0 ppm in the buffer solution were estimated.

Inactivating Salmonella with chlorine dioxide

Experiments were conducted in 20 ml of chlorine demand free phosphate buffer solution (pH adjusted to 7). Washed cells (100 µl of cell suspension) were exposed to chlorine dioxide at concentrations 0.1, 0.5 and 1 ppm for 0.5, 2 and 4 min. The chemical was neutralized with 1 ml of sodium thiosulfate (20% w/v) at the end of contact time.

Determination of virulence gene expression in S. Typhimurium

The expression of virulence genes in overnight night culture (16-18 h) of *S*. Typhimurium was evaluated using RT-PCR method. Reverse transcription along with PCR was used for quantifying gene expression by the comparative Ct method.

Preparation of overnight culture

A day before the actual experiment a single colony of *S*. Typhimurium was inoculated into 10 ml of TSB and incubated at 37ºC for 18-24 h. Cell suspensions were washed twice (3313 x g) for 10 min at room temperature and resuspended in 10 ml of chlorine demand free buffer solution.

Treating cells with chlorine dioxide

Laboratory experiments were conducted in 5 ml of chlorine demand free buffer solution. A prepared cell suspension (5 ml) of overnight culture in buffer solution was exposed to chlorine dioxide at a concentration of 2 ppm for 0.5 min. The biocide was neutralized with 1 ml of sodium thiosulfate (20% w/v). Treated sample and the control (5 ml of each at 0 min) were stabilized in RNAprotect™ (Qiagen, Valencia, CA) immediately.

Incubation of treated cells

After the treatment, 5 ml of the sample (both treatment and control) was transferred into a 25 ml conical flask containing 5 ml of 2x TSB and incubated at 37ºC for 30 min. Incubated samples (5 ml of treated and control at 30 min) were stabilized in RNAprotect™ (Qiagen) immediately.

RNA extraction

Total RNA from all the samples (0 and 30 min) was extracted using an RNeasy™ kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNase-free DNase set (Qiagen) was used for on-column DNA digestion to remove residual genomic DNA. The quantity and the quality of the total RNA extracted was measured using the Nanodrop (ND-1000) spectrophotometer (Nanodrop ® Technologies, Palo Alto, CA), and the Bioanalyzer ™ (Agilent Technologies, Santa Clara, CA), respectively.

cDNA synthesis

Total extracted and pooled RNA was used for cDNA synthesis. cDNA was synthesized using GeneAmp RNA PCR kit (Applied Biosystems, Branchburg, NJ). The reaction mixture was as follows: 1 µl of RNA template and 19 µl of master mix consisting of 4 µl of 25 mM $MgCl_2$ solution, 2 µl of DEPC treated DI water, 2 µl each of dATP, dTTP, dGTP, and dCTP, 1 µl of RNase inhibitor, 1 µl of MuLV reverse transcriptase enzyme, and 1 µl of random hexamer. The reaction mixture was incubated for 10 min at room temperature before placing in the thermocycler. First strand of cDNA was synthesized using single Reverse-Transcriptase polymerase chain reaction (RT-PCR). The RT-PCR reaction conditions were 60 min at 42°C for annealing and elongation followed by 5 min at 99 °C for inactivating the enzyme. All RT-PCR cycles were run in a programmable thermocycler

(Applied Biosystems, Gene Amp* PCR system 2700). The quantity of cDNA synthesized was measured using the Nanodrop (ND-1000) spectrophotometer (Nanodrop ® Technologies, Palo Alto, CA) and stored at -20 °C for further studies.

Real time PCR amplification

Appropriate primer sequences (See Appendix) for the specific targets were used for gene expression studies. The 384 well reaction plate (Applied Biosystems) was filled with 1 µl of cDNA and 19 µl of master mix consisting of 10 µl of SYBR® GREEN PCR mix (Applied Biosystems, Warrington, UK), primers (0.6 µl each of forward and reverse primers (10 µmol)) adjusted with DEPC treated deionized water. The negative controls consisted of DEPC – treated DI water. The plate was sealed with optical adhesive covers (Applied Biosystems, Foster City, CA) and placed in a thermocycler (Applied Biosystems, Unit – Abi – prism, 7000 HT, Foster City, CA) that was programmed for relative quantification to obtain a Ct value.

Results

Inactivation of Salmonella

Inactivation due to chlorine dioxide was rather gradual as compared to chlorine with only 0.4 log reduction observed at 0.5 min at a concentration of 0.1 ppm for *S*. Typhimurium. Further inactivation of approximately 2.0 and 4.6 logs was observed at concentrations 0.5 and 1 ppm respectively. No colony forming units were detected at concentrations 0.5 and 1 ppm at time intervals 2 and 4 min. Log reductions of 3.0 and 4.0 were obtained at 2 and 4 min at a concentration of 0.1 ppm. The inactivation model for *S*. Typhimurium is presented in Fig. 4.1.

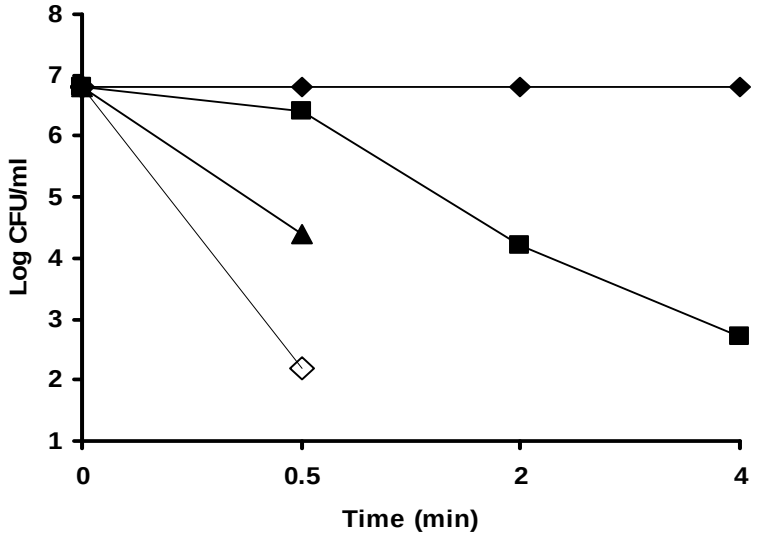

FIG. 4.1. Inactivation of *S*. Typhimurium after treatment with chlorine dioxide for 0.5, 2, and 4 min. Symbols: ▼, 0 ppm chlorine dioxide; ■, 0.1 ppm chlorine dioxide; ▲, 0.5 ppm chlorine dioxide; ◊, 1 ppm chlorine dioxide. No survivors detected at chlorine dioxide concentrations of 0.5 and 1 ppm at 2 and 4 min. Detection limit: 1 CFU/ml = 0 log CFU/ml.

No significant reduction in microbial population was observed at a concentration of 0.1 ppm at 0.5 min of contact time for *S*. Enteritidis. Further reductions of about 2.4 and 4.2 logs were obtained at concentrations 0.5 and 1.0 ppm at 0.5 min when compared to control. Log inactivation of *S*. Enteritidis at different concentrations and contact time points is presented in Fig. 4.2.

Log reductions of approximately 3.0 and 5.0 logs were obtained at a concentration of 0.1 ppm at 2 and 4 min of contact time with few to none significant colony forming units detected at concentrations 0.5 and 1.0 ppm.

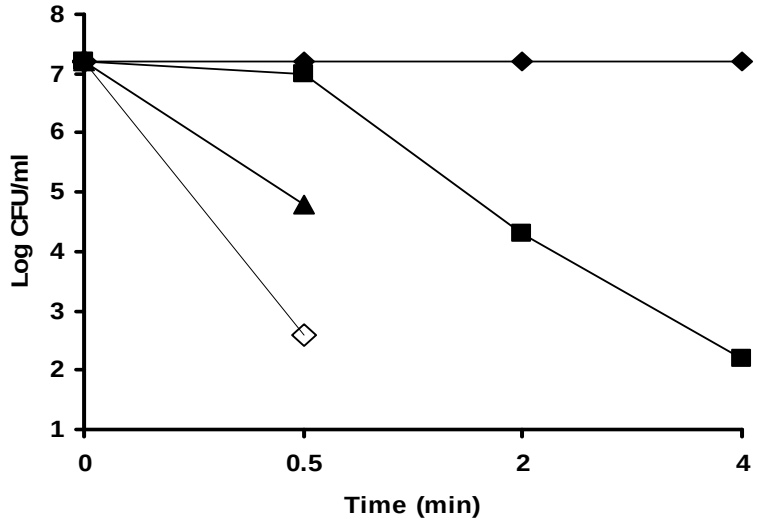

FIG. 4.2. Inactivation of *S*. Enteritidis after treatment with chlorine dioxide for 0.5, 2, and 4 min. Symbols: ▼, 0 ppm chlorine dioxide; ■, 0.1 ppm chlorine dioxide; ▲, 0.5 ppm chlorine dioxide; ◊, 1 ppm chlorine dioxide. No survivors detected at chlorine dioxide concentrations of 0.5 and 1 ppm at 2 and 4 min. Detection limit: 1 CFU/ml = 0 log CFU/ml.

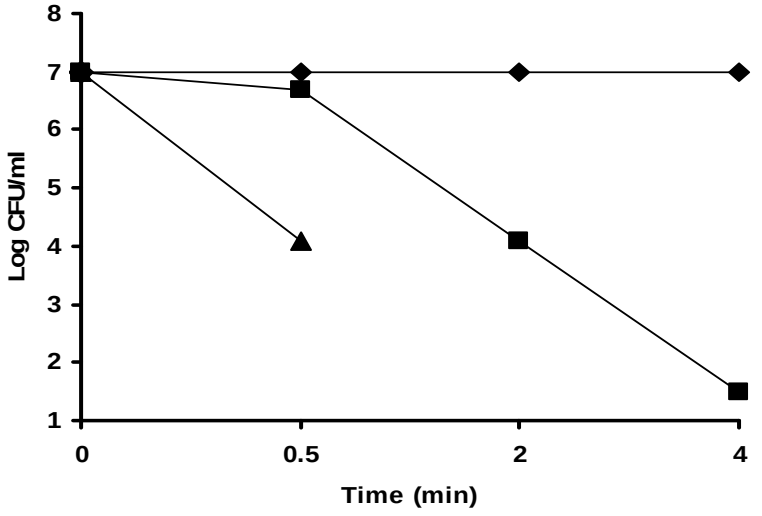

FIG. 4.3. Inactivation of *S*. Heidelberg after treatment with chlorine dioxide for 0.5, 2 and 4 min. Symbols: ▼, 0 ppm chlorine dioxide; ■, 0.1 ppm chlorine dioxide; ▲, 0.5 ppm chlorine dioxide; ◊, 1 ppm chlorine dioxide. No survivors detected at chlorine dioxide concentrations of 0.5 ppm at 2 and 4 min and for 1 ppm at 0.5, 2, and 4 min. Detection limit: 1 CFU/ml = 0 log CFU/ml.

Inactivation obtained for *S*. Heidelberg at varying concentration levels and contact time is presented in Fig. 4.3. A 0.3 log reduction was obtained at a concentration of 0.1 ppm at 0.5 min with further log reductions of 2.6 and 6.7 obtained at concentrations 0.5 and 1 ppm respectively. Log reductions of approximately 3.0 and 5.5 were obtained at a concentration of 0.1 ppm at time intervals of 2 and 4 min with few to no colony forming units detected at concentrations 0.5 and 1.0 ppm .

Inactivation model for *S*. 4,5,[12]:i is presented in Fig. 4.4. A reduction of 0.3, 2.3 and 6.2 logs was obtained at concentrations 0.1, 0.5 and 1.0 ppm at 0.5 min of contact time.

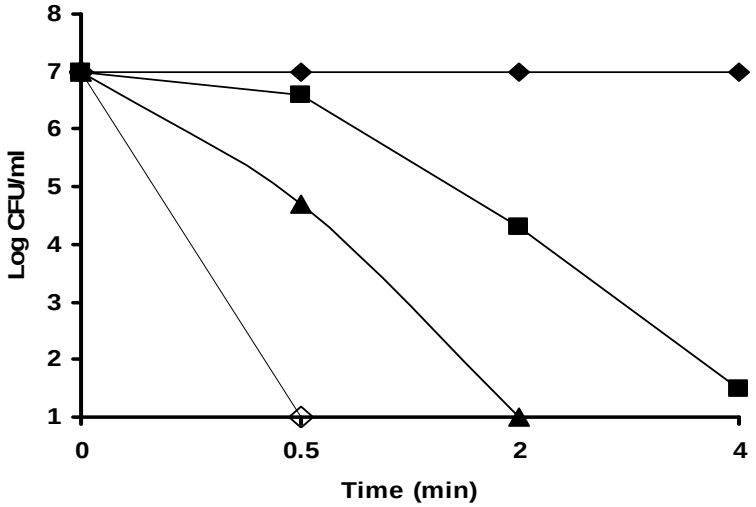

FIG. 4.4. Inactivation of *S*. 4,5,[12]:i after treatment with chlorine dioxide for 0.5, 2, and 4 min. Symbols: ▼, 0 ppm chlorine dioxide; ■, 0.1 ppm chlorine dioxide; ▲, 0.5 ppm chlorine dioxide; ◊, 1 ppm chlorine dioxide. No survivors detected at chlorine dioxide concentrations of 0.5 ppm at 4 min and for1 ppm at 2 and 4 min. Detection limit: 1 CFU/ml = 0 log CFU/ml.

Log reductions of 2.4 and 4.8 were obtained at a concentration of 0.1 ppm at 2 and 4 min respectively with no significant colonies detected at concentrations 0.5 and 1.0 ppm at 4 min.

An interesting phenomenon that was observed for all the serovars in the laboratory was delayed appearance of colony forming units especially at concentrations 0.1 and 0.5 ppm for all the time intervals (0.5, 2 and 4 min). These colonies were detected only after 48 – 72 h of incubation suggesting either bacterial injury/repair or an altered growth rate.

Virulence gene expression analysis

The differential expression of virulence genes after exposure to chlorine dioxide at a concentration of 2 ppm for 0.5 min is presented in Table 4.1.

TABLE 4.1. Differential expression of virulence genes in overnight culture of *S.* Typhimurium immediately after exposure to chlorine dioxide at a concentration of 2 ppm for 0.5 min.

Gene	Fold change	p - value
hilD	-1.1	0.86
invF	4.8	0.17
orgA	-1.6	0.30
sicA	1.2	0.60
prgK	1.6	0.5
invE	4.9	0.14
sicP	3.0	0.17
sipD	18.5	0.06
hilA	-1.1	0.49
hilC	14.6	0.02
sipA	37.88	0.01
sipB	41.74	0.06
invC	35.96	0.06

Only two genes namely *hilC* and *sipA* ($p < 0.05$) associated with SPI1 were found to be upregulated at 0 min. Two other genes (*sipB* and *invC* ($p = 0.06$)) were also upregulated. The differential expression of virulence genes in stationary phase cells of *S*. Typhimurium after treatment with 2 ppm of chlorine dioxide for 0.5 min followed by incubation for 30 min is presented in Table 4.2. None of the virulence factors were differentially expressed at 30 min.

TABLE 4.2. Differential expression of virulence genes in overnight culture of *S*. Typhimurium after treatment with 2 ppm of chlorine dioxide and followed by incubation for 30 min.

Gene	**Fold change**	**p - value**
hilD	-4.3	0.35
invF	-11.92	0.26
orgA	-16.17	0.30
sicA	-1.64	0.60
prgK	-6.54	0.5
invE	1.04	0.97
sicP	3.0	0.17
sipD	-7.11	0.06
hilA	-3.2	0.49
hilC	-1.1	0.9
sipA	-5.03	0.4
sipB	-8.46	0.3
invC	-9.75	0.28

Discussion

Inactivation kinetics

The inactivation equations were obtained for all the serovars based on the following equation $\ln (N/N_0) = -k\, mC^n t^m$ which was modified as following

$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2$ where $k = -\exp(\beta_0)$, $n = \exp(\beta_1)$, $m = \exp(\beta_2)$, $X_1 = \ln C$ (concentration), $X_2 = \ln t$ and $Y = \ln(\ln (N/N_0))$. The inactivation curve obtained for *S.* Typhimurium is presented in Fig. 4.5.

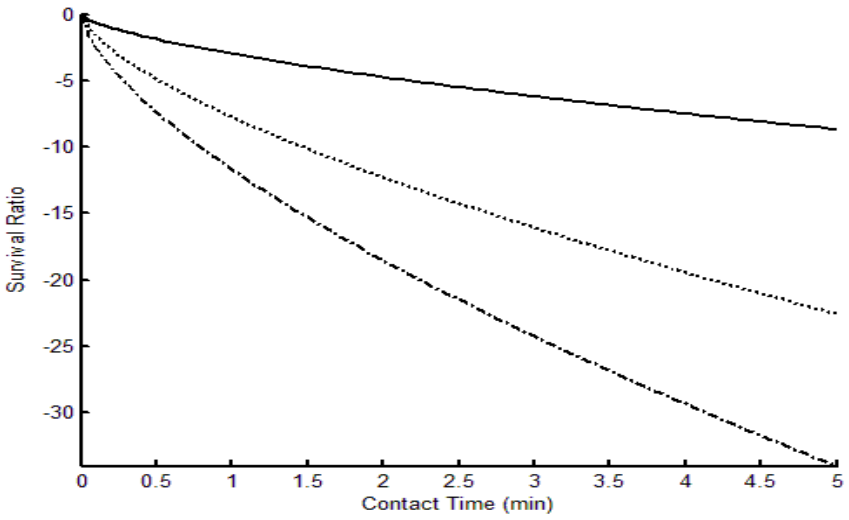

FIG. 4.5. Inactivation kinetics of *S.* Typhimurium after treatment with chlorine dioxide. Symbols: —(solid line), 0.1 ppm of chlorine dioxide; …(dot), 0.5 ppm of chlorine dioxide; -.-.-.-(dot-line), 1 ppm of chlorine dioxide.

The inactivation equations obtained for the *Salmonella* serovars were as follows

S. T – $\ln (N/N_0) = -11.71\ C^{0.6} T^{0.7}$

S. E - $\ln (N/N_0) = -13.8\ C^{0.7} T^{0.8}$

S. H - $\ln (N/N_0) = -16.9\ C^{0.8} T^{0.8}$

S. 4,5,[12]:i - ln $(N/N_0) = -13.9\ C^{0.7}\ T^{0.7}$

Inactivation curves obtained for *S.* Enteritidis is presented in Fig. 4.6.

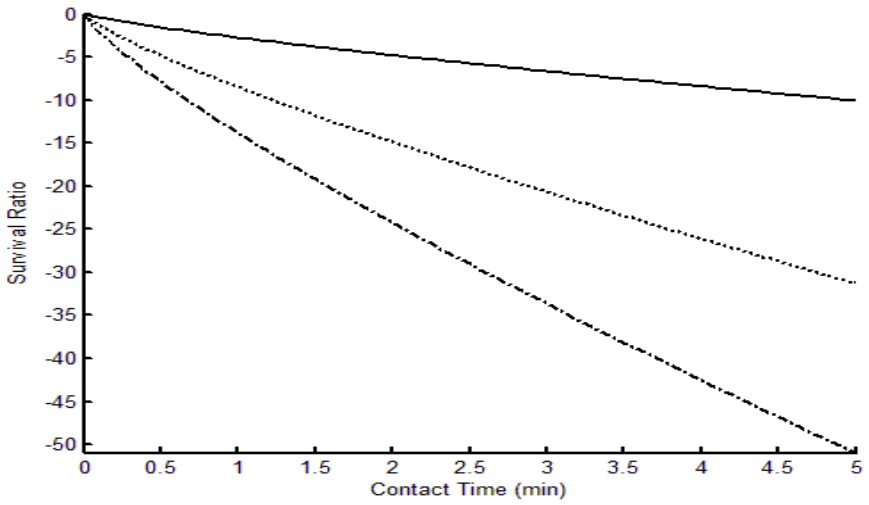

FIG. 4.6. Inactivation kinetics of *S.* Enteritidis after treatment with chlorine dioxide. Symbols: —(solid line), 0.1 ppm of chlorine dioxide; …(dot), 0.5 ppm of chlorine dioxide; -.-.-.-(dot-line), 1 ppm of chlorine dioxide.

Although the value of n was not equal to 1, it was close. Based on this, it can be assumed that the product of a given concentration and time will give an approximate degree of inactivation. Inactivation kinetics of *S.* Heidelberg is presented in Fig. 4.7.

The m value is close to 1 indicating that the inactivation follows a linear pattern and this is quite evident from the curves obtained. Inactivation curves obtained for *S.* 4,5,[12]:i obtained for chlorine dioxide concentrations of 0.1, 0.5 and 1.0 ppm is presented in Fig. 4.8.

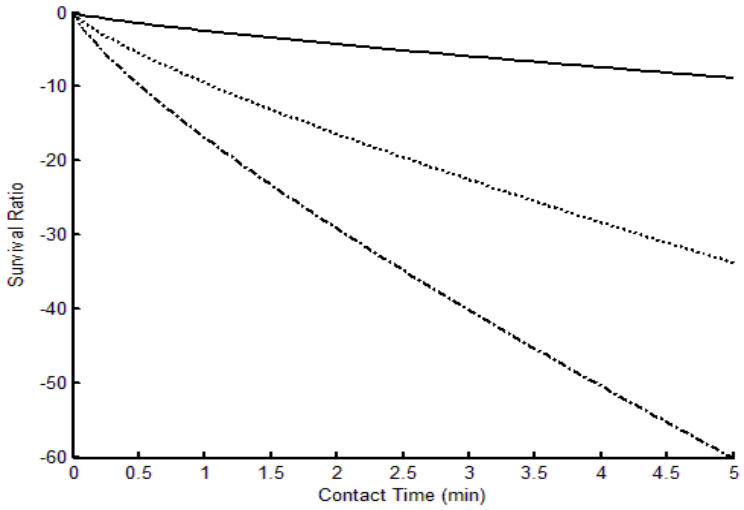

FIG. 4.7. Inactivation kinetics of *S*. Heidelberg after treatment with chlorine dioxide. Symbols: —(solid line), 0.1 ppm of chlorine dioxide; …(dot), 0.5 ppm of chlorine dioxide; -.-.-.-(dot-line), 1 ppm of chlorine dioxide.

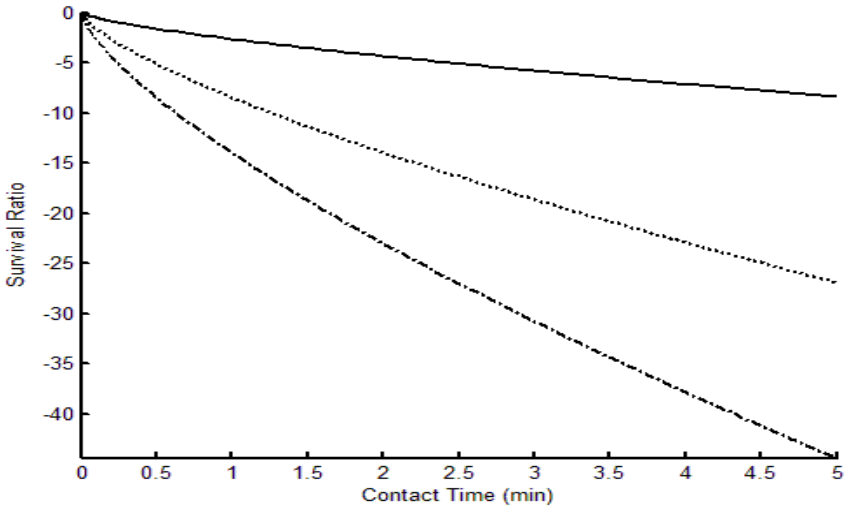

FIG. 4.8. Inactivation kinetics of *S*. 4,5,[12]:i after treatment with chlorine dioxide. Symbols: —(solid line), 0.1 ppm of chlorine dioxide; …(dot), 0.5 ppm of chlorine dioxide; -.-.-.-(dot-line), 1 ppm of chlorine dioxide.

Studies evaluating the inactivation kinetics for chlorine dioxide and especially with *Salmonella* are very limited. Previous studies reported that the chemical is efficient as a disinfectant in presence of low organic content (134), however, the efficacy was suggested to be lower than that of chlorine at higher levels of organic material. Another study also reported bactericidal effect of chlorine dioxide, but the efficacy was found comparable to that of chlorine (165). However, data obtained in these studies are considered to be questionable because the methods employed in estimating the precise concentration of the chemical used in disinfection were questionable or not standardized (20).

Another study investigating the bactericidal efficacy of chlorine dioxide reported that both chlorine and chlorine dioxide were efficient in inactivating *E*. coli in sewage effluent at a concentration of 0.75 ppm with higher kill (2 logs) obtained at a concentration of 0.25 ppm in 15 s for chlorine dioxide at a pH of 8.5. Chlorine on the other hand produced this level of reduction only after 300 s of exposure time (20). The same study also concluded that chlorine dioxide was more efficient as a disinfectant as compared to chlorine and the efficacy of chlorine dioxide was not found to be affected by pH changes. Also, evaluating the disinfection efficacy based on residuals is considered to be invalid because the actual disinfection is considered to be occurring only in the first few seconds/minutes of contact time (20). Similar results were obtained in the current study wherein a concentration of 0.5 ppm for a contact time of 0.5 min resulted in 3 log reduction of *Salmonella.*

Another study modeled the inactivation kinetics for *E. coli* taking into consideration three variables: concentration, contact time and temperature (21). The study used nonlinear least square analysis for estimating the values of k and n. The study also reported disruption

of cellular membrane as the primary mode of action of the chemical. Inhibition of protein synthesis was also demonstrated (21) in the same study.

The antimicrobial efficacy of the chemical in reducing microbial contamination has been extensively investigated in poultry. Chlorine dioxide applied at a concentration of 1.39 ppm in a chiller tank was shown to result in significant reduction (< 1 log) of coliforms, and psychrotrophs with *Salmonella* not being detected in the system. The efficacy was however found to be reduced with only < 0.5 log reduction reported on broiler breast skin (192). Another study evaluating the effect of chlorine dioxide in reducing microbial contamination on poultry reported increased disinfection efficacy of chlorine dioxide as compared to chlorine (127).

The results and conclusions provided in the previous studies are however debatable and cannot be compared to the current study because the composition of the chemical employed, methods used for determining the exact concentrations of the chemical and type of system (phosphate buffer vs chiller tank) used for testing the disinfection efficacy differ significantly. A buffer system (current study) produced a 6 log reduction of *Salmonella* at a concentration of 1 ppm as against 1 log reduction reported at a concentration of 3 ppm in the chiller (127). Also, the concentration used in the chiller tank has been reported as 3 ppm which in actuality is the measurement of the residuals in the system and this concentration actually may have less disinfection efficacy as compared to the initial concentration used.

Virulence gene expression analysis

The change in gene expression was calculated using the formula Treatment 1/Treatment 2 = $2^{\Delta\Delta C}{}_{T}$, where $\Delta\Delta C_T = \Delta C_T$ for treatment 1 - ΔC_T for treatment 2 and ΔCt is the difference between the Ct value of the target gene and the normalization gene (16s). A

two-tail t-test with pooled standard deviation was used to identify differential expression of the target gene.

The gene *sipA* encodes an effector protein, *sipB* encodes a protein translocase, *invC* encodes for ATPase and *hilC* is involved in overall regulation of the TTS. Upregulation of these genes is a cause of concern since it enables the pathogen to invade and cause infection in host cells. The exact molecular mechanisms involved in upregulation of the virulence factors due to chlorine dioxide injury is not known at this time, however, it is speculated that upregulation is a direct outcome of available energy in the system and the gene involved with ATP in SPI1 is *invC*. None of the virulence factors were differentially expressed at 30 min. This phenomenon perhaps may result in reduced expression of virulence genes implying that the ability of the pathogen in causing infection in the host cells is greatly reduced.

Many of the virulence determinants responsible for causing infection in the host are not expressed under laboratory environment and may need unique conditions such as the host environment for expression (146, 190, 199). Several studies investigated the essential gene regulatory functions that control the growth of *Salmonella* and it was determined that few of the regulatory circuits are associated with the switching of the house-keeping genes while majority were found to be involved in functioning as global gene regulators that affect both house-keeping and virulence functions (133).

Salmonella possesses a catabolite repressor system that responds to "small-molecular signal" in the form of cyclic adenosine monophosphate (cAMP) and it was demonstrated that starvation (depletion of nutrients) results in increased concentration of cAMP (133). "The gene regulatory catabolite repressor protein (CRP)" binds to cAMP and the cAMP-CRP complex controls the gene expression to offset starving conditions. The response of CRP is

known to regulate the regulation of virulence gene expression: a gene regulatory protein is associated, the activity of the regulatory protein is altered by a signal substance, the response of CRP is vital for the expression of virulence in *Salmonella* (44) and the repression of catabolite system exemplifies the capability of the pathogen in responding to the environmental stresses. Other protein families that respond to environmental stresses include LysR/MetR (regulatory protein), AraC, Fur (iron response regulator), and the two-component systems.

The intracellular growth of *Salmonella* is controlled by SpvR and this belongs to the LysR/MetR family (78, 79). Proteins such as InvF and SprA associated with the SPI1 belong to the AraC family. The sensor domain of the two-component system is located in the periplasmic membrane and is known to phosphorylate the regulatory domain located in the cytoplasm. Alterations in osmolarity and concentration of Ca^{+2}/Mg^{+2} result in the induction of OmpR/EnvZ and PhoP/PhoQ systems and these are known to be critical for virulence expression in *Salmonella* (51, 65). The gene *hilA*, the overall transcriptional regulator of TTS system belongs to the families OmpR/EnvZ and PhoP/PhoQ. The protein SprB encoded by the SPI1 shares similar homology as that of LuxR that is involved in quorum sensing (59).

The regulon (*phoP*/*phoQ*) controls the invasion of the pathogen into the epithelial cells (65, 139) and the regulon activates the expression of *pagS* (adaptation of the pathogen to the intracellular environment) with a concomitant repression of *prgS* (essential for invasion) (72, 156). The gene *hilA* encoded by SPI1 induces the expression of several invasion genes (102, 162) and the expression of *hilA* is in turn regulated SirA.

The current study demonstrated an upregulation of few virulence genes (*sipA*, *sipB*, *sipD*, *invC* and *hilC*) at 0 min with no significant expression at 30 min. The exact

mechanisms are not known, however, it is speculated that mechanisms (PhoP/PhoQ) similar to that expressed with acid and thermal stress may have been involved. Also, *in vitro* investigation with cell lines will further help in understanding the virulence expression.

CHAPTER V

INACTIVATION, VIABLE BUT NON-CULTURABLE, AND VIRULENCE RESPONSE OF *Salmonella* TO CETYLPYRIDINIUM CHLORIDE

Introduction

Cetylpyridinium chloride is a quaternary ammonium compound that is soluble in water. It is a surfactant and contains both hydrophilic and hydrophobic groups. Surfactants are classified into the following categories namely cationic, anionic, nonionic and amphoteric based on the presence or absence of charge on the hydrophilic group (135). The antimicrobial properties are exhibited only by cationic compounds and are commonly referred to as quaternary ammonium compounds (68).

The antimicrobial action of the chemical is dependent on several factors such as pH, protein content and temperature with increased germicidal activity observed at low pH and protein content at room temperature (160).

The antimicrobial action of the disinfectant is considered to occur as sequence of events – adsorption to the outer membrane and subsequent interaction with the constituents of the cell membrane resulting in disruption and eventual leakage of the contents causing death (174).

The phenomenon of electrophoresis in bacterial cells (migration of cells in an electric field based on the electric charge) was first demonstrated with this compound (56). The movement of the cells towards cathode decreased and eventually reversed with increasing concentration of the chemical. This phenomenon of reversed mobility explains the apparent antimicrobial action of the disinfectant on the outer membrane of the cell.

The chemical is extensively used in the poultry processing industry at a concentration of 0.8% by weight of the carcasses. However, studies evaluating the kinetics of the chemical are very limited. In addition to understanding the disinfection kinetics of the chemical, other aspects such as induction of VBNC state and differential gene expression of virulence factors were also investigated. The central hypothesis of the study is: CPC is ineffective and induces VBNC state with significant upregulation of virulence genes in *Salmonella*.

Materials and methods

Bacterial strains and culture conditions

Salmonella strains (*S*. Typhimurium, *S*. Enteritidis, *S*. Heidelberg and *S*. 4,5,[12]:i) were obtained from USDA – ARS (College Station, TX). The strains were stored at -80ºC until further use. Each strain (a loopful of the initial culture) was cultured in 10 ml of Tryptic Soy Broth (TSB; Difco, Detroit, MI) twice successively with incubation at 37 ºC for 18-24 h. After two consecutive transfers, cultures were streaked on Tryptic Soy Agar plates (TSB; Difco, Detroit, MI) and incubated at 37 ºC for 18-24 h. Plates with individual colonies were stored at 4 ºC for experimental studies.

Preparation of Salmonella cultures

A day before the actual experiment a single colony of each strain was inoculated into 10 ml of TSB and incubated at 37 ºC for 18-24 h. Overnight cultures were washed twice with 10 ml chlorine demand free phosphate buffered saline solution by centrifugation at 3313 x g for 10 min at room temperature. Harvested cells were suspended in 10 ml of chlorine demand free phosphate buffered saline solution.

Inactivation of cells with CPC

A stock solution of CPC was prepared by dissolving 1 g in 10 ml of distilled water (w/v). Experiments were conducted in 1 ml of chlorine demand free phosphate buffer

solution. Washed cells were treated with CPC at concentrations 100, 1000, and 10000 ppm for 0.5, 15, and 30 min. The chemical was neutralized with 32 ml of 1x letheen broth. All the treatments were decimally diluted in buffered peptone water and spread plated on TSA. Plates were incubated at 37 ºC for 18-24 h.

Determination of VBNC state in Salmonella

The VBNC state in *Salmonella* was determined using the BacLight^{TM} assay. Overnight TSB cultures (10 ml) of individual strains were washed (3313 x g for 10 min) at room temperature and resuspended in 10 ml of chlorine demand free phosphate buffered saline solution. Cells were exposed to CPC at a concentration of 10000 ppm for 0.5 min and subsequently neutralized with 32 ml of 1x Letheen broth. Test solution (100 µl) was dispensed and mixed thoroughly with 0.6 µl of stain mixture (SYTO 9 green- fluorescent nucleic acid stain and propidium iodide red-fluorescent nucleic acid stain, (Invitrogen) in a 96 well plate. The plate was incubated at room temperature for 15 min. The excitation/emission wavelengths are 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide were used for measuring the number of live and dead cells. The ratio of live to dead cells (G/R) was estimated using the standard curve (See Appendix).

Evaluating differential expression of virulence factors in S. Typhimurium

The expression of virulence genes associated with SPI1 was evaluated using RT-PCR. Relative quantitation based on comparative Ct method was used for quantifying the gene expression. The gene expression was evaluated in over night culture (16-18 h incubation of cell culture) of *S.* Typhimurium.

Culture conditions of S. Typhimurium

A day before the actual experiment a single colony of *S*. Typhimurium was inoculated into 10 ml of TSB and incubated at 37 ºC for 18-24 h. Cell suspensions were washed twice (3313 x g for 10 min at room temperature) and resuspended in 10 ml of chlorine demand free phosphate buffer solution.

CPC treatment of stationary phase cells

Laboratory experiments were conducted in 1ml of chlorine demand free buffer solution. The cell density in the buffer for the treatment was 1 x 10^8 CFU/ml. Cell suspension (100 µ) in buffer solution was exposed to CPC at a concentration of 10000 ppm for 0.5 min. The biocide was neutralized with 32 ml of 1x Letheen broth. Treated sample and the control (5 ml of each at 0 min) were stabilized in RNAprotect™ (Qiagen) immediately.

Incubation of cells for 30 min

The treated cells along with the control were incubated in nutrient rich medium for 30 min for determining the genetic response (recovery) of the stressed cells. After the treatment, 5 ml of the sample (both treatment and control) was transferred into a 25 ml conical flask containing 5 ml of 2x TSB and incubated at 37ºC for 30 min. Incubated samples (5 ml of treated and control at 30 min) were stabilized in RNAprotect™ (Qiagen) immediately.

RNA extraction

Total RNA from all the samples (immediately after the treatment and after 30 min of incubation) was extracted using an RNeasy™ kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNase-free DNase set (Qiagen) was used for on-column DNA digestion to remove residual genomic DNA. The quantity and the quality of the total RNA extracted was verified using the Nanodrop (ND-1000) spectrophotometer (Nanodrop ®

Technologies, Palo Alto, CA), and the Bioanalyzer ™ (Agilent Technologies, Santa Clara, CA), respectively.

cDNA synthesis

Total extracted and pooled RNA was used for cDNA synthesis. cDNA was synthesized using GeneAmp RNA PCR kit (Applied Biosystems, Branchburg, NJ). The reaction mixture contained the following: 1 µl of RNA template and 19 µl of master mix consisting of 4 µl of 25 mM $MgCl_2$ solution, 2 µl of DEPC treated DI water, 2 µl each of dATP, dTTP, dGTP, and dCTP, 1 µl of RNase inhibitor, 1 µl of MuLV reverse transcriptase enzyme, and 1 µl of random hexamer. The reaction mixture was incubated for 10 min at room temperature before placing in the thermocycler. First strand of cDNA was synthesized using single Reverse-Transcriptase polymerase chain reaction (RT-PCR). The RT-PCR reaction conditions were 60 min at 42 °C for annealing and elongation followed by 5 min at 99 °C for inactivating the enzyme. All RT-PCR cycles were run in a programmable thermocycler (Applied Biosystems, Gene Amp PCR system 2700). The quantity of cDNA synthesized was measured using the Nanodrop (ND-1000) spectrophotometer (Nanodrop ® Technologies, Palo Alto, CA) and stored at -20 °C for further studies.

Real time PCR amplification

Appropriate primer sequences (See Appendix) for the specific targets were used for gene expression studies. The 384 well reaction plate (Applied Biosystems, Foster City, CA) was filled with 1 µl of cDNA and 19 µl of master mix consisting of 10 µl of SYBR® GREEN PCR mix (Applied Biosystems, Warrington, UK), primers (0.6 µl each of forward and reverse primers (10 µmol)) adjusted with DEPC treated deionized water. The negative controls consisted of DEPC – treated DI water. The plate was sealed with optical adhesive

covers (Applied Biosystems, Foster City, CA) and placed in a thermocycler (Applied Biosystems, Unit – Abi – prism, 7000 HT, Foster City, CA) that was programmed for relative quantification to obtain a Ct value.

Results

Inactivation of Salmonella

Log populations of *S.* Typhimurium obtained after treatment with CPC at varying concentrations and at different exposure times are presented in Fig. 5.1. Log reductions of approximately 1.2, 4.2 and 4.3 were obtained at a concentration of 100 ppm for contact time periods of 0.5, 15 and 30 min respectively.

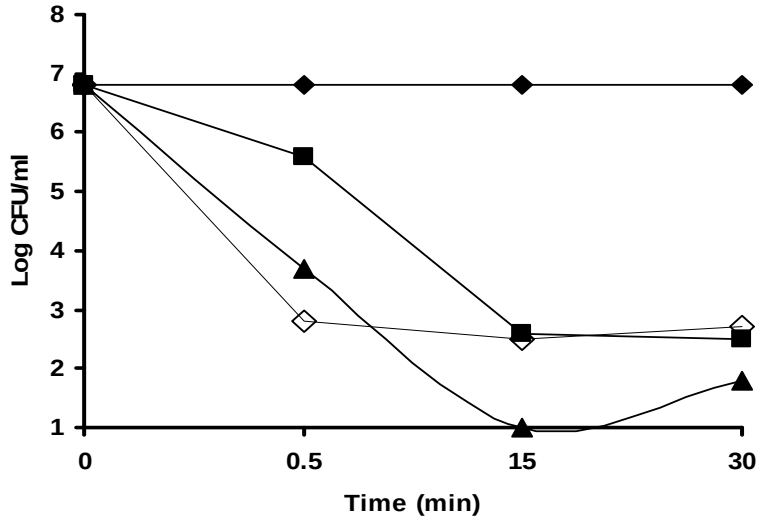

FIG. 5.1. Inactivation of *S.* Typhimurium after treatment with CPC for 0.5, 15, and 30 min. Symbols: ▼, 0 ppm CPC; ■, 100 ppm CPC; ▲, 1000 ppm CPC; ◊, 10000 ppm CPC. Detection limit: 1 CFU/ml = 0 log CFU/ml.

A 3.0 log reduction was obtained at a concentration of 1000 ppm at 0.5 min with reductions of 5.4 and 4.3 logs observed at 15 and 30 min respectively. Further increase in

concentration to 10,000 ppm resulted in log reductions of 3.8, 4.1 and 3.9 at 0.5, 15 and 30 min respectively.

For *S*. Enteritidis, a log reduction of 2.7 was obtained at 0.5 min at a concentration of 100 ppm. Log inactivation of *S*. Enteritidis obtained at different concentrations is presented in Fig. 5.2.

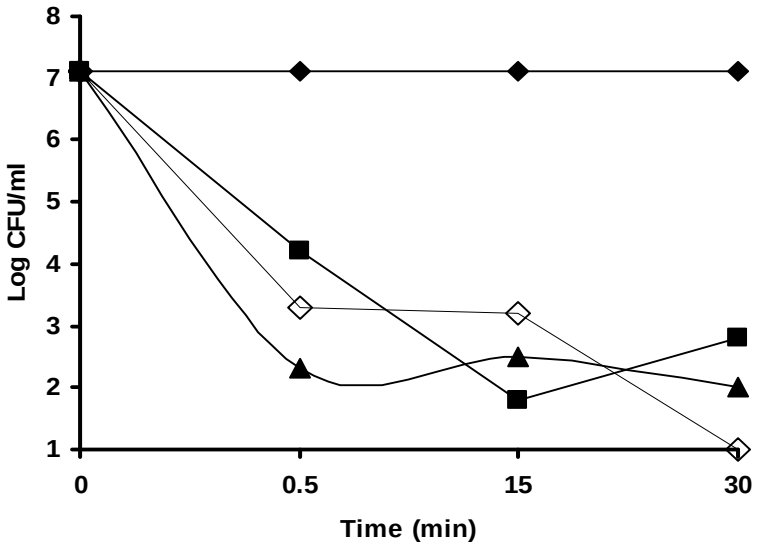

FIG. 5.2. Inactivation of *S*. Enteritidis after treatment with CPC for 0.5, 15, and 30 min. Symbols: ▼, 0 ppm CPC; ■, 100 ppm CPC; ▲, 1000 ppm CPC; ◊, 10000 ppm CPC. Detection limit: 1 CFU/ml = 0 log CFU/ml.

Further reductions of 5.1 and 4.1 were obtained for contact times of 15 and 30 min respectively. Log reductions of 3.7, 4.3 and 3.9 were obtained for contact time periods of 0.5, 15 and 30 min respectively at a concentration of 1000 ppm. A concentration of 10000 ppm resulted in log reductions of 3.6, 4.1 and 3.9 at 0.5, 15 and 30 min respectively.

Log reductions of 2.3, 4.3 and 3.2 were obtained for *S*. Heidelberg at 0.5, 15 and 30 min, respectively, at a concentration of 100 ppm. Inactivation of *S*. Heidelberg at varying concentration levels and exposure times is presented in Fig. 5.3.

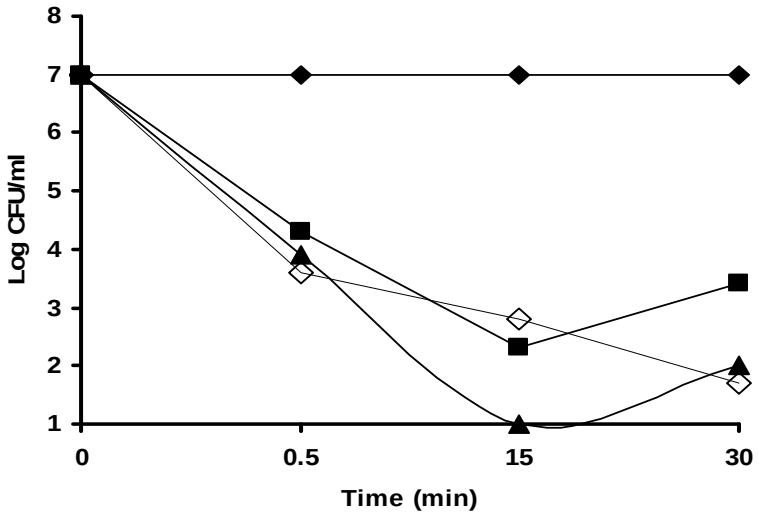

FIG. 5.3. Inactivation of *S*. Heidelberg after treatment with CPC for 0.5, 15, and 30 min. Symbols: ▼, 0 ppm CPC; ■, 100 ppm CPC; ▲, 1000 ppm CPC; ◊, 10000 ppm CPC. Detection limit: 1 CFU/ml = 0 log CFU/ml.

Further increases in concentration to 1000 ppm resulted in 3.8, 4.4, and 4.5 log reductions at 0.5, 15, and 30 min, respectively. Log reductions of 3.7, 3.7, and 5.0 were obtained at 0.5, 15 and 30 min, respectively, at a concentration of 10000 ppm.

For *S*. 4,5,[12]:i, a log reduction of 1.9 was obtained at 0.5 min at a concentration of 100 ppm. Log reductions of 4.9 and 3.4 were obtained at 15 and 30 min, respectively, at the same concentration level. An increase in concentration to 1000 ppm resulted in further reductions of 2.2, 3.7, and 3.7 logs at 0.5, 15, and 30 min, respectively. Inactivation obtained is presented in Fig 5.4. A concentration of 10000 pm resulted in inactivation of 3.3, 5.4, and 4.4 logs at 0.5, 15, and 30 min, respectively.

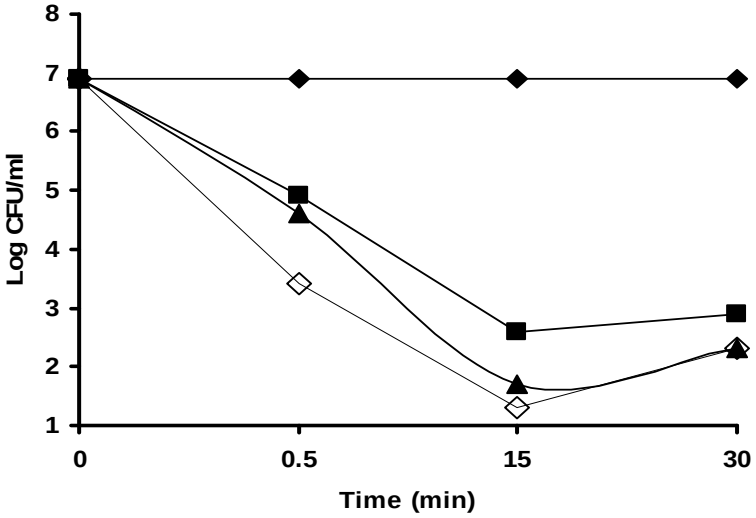

FIG. 5.4. Inactivation of *S*. 4,5,[12]:i after treatment with CPC for 0.5, 15, and 30 min. Symbols: ▼, 0 ppm CPC; ■, 100 ppm CPC; ▲, 1000 ppm CPC; ◊, 10000 ppm CPC. Detection limit: 1 CFU/ml = 0 log CFU/ml.

VBNC study

Results (G/R ratio, Table 5.1) obtained indicate no significant difference between the strains of *Salmonella* and a plot of the ratio on the standard curve (Fig. 5.5) indicates that approximately 45% of the cells were alive in the treated samples for all the serovars.

Table 5.1. Percentage of live (green) cells determined in overnight culture of *Salmonella* serovars after treatment with CPC at a concentration of 10000 ppm for 0.5 min using BacLight dye by Fluorescence spectroscopy and plate count method.

Strain	**% live cells using Baclight assay**	**% live cells using plate count**
S. Typhimurium	45%	< 1 %
S. Enteritidis	46%	< 1 %
S. Heidelberg	46%	< 1 %
S. 4,5,[12]:i	46%	< 1 %

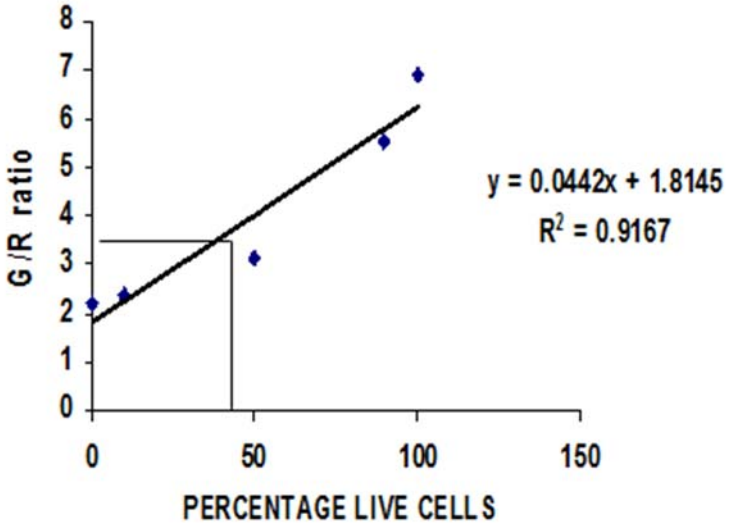

FIG. 5.5. Percentage live cells estimated using standard curve at CPC concentration of 10000 ppm for a contact time of 0.5 min.

RT-PCR study

The differential expression of virulence genes at two time points; immediately after the exposure to CPC at a concentration of 10000 ppm for 0.5 min and after incubation for 30 min are presented in Tables 5.2 and 5.3.

The change in gene expression was calculated using the formula Treatment 1/Treatment 2 = $2^{\Delta\Delta C}{}_T$, where $\Delta\Delta C_T = \Delta C_T$ for treatment 1 - ΔC_T for treatment 2 and ΔCt is the difference between the Ct value of the target gene and the normalization gene (16s). A two-tail t-test with pooled standard deviation was used to identify differential expression of the target gene. All the virulence factors with exception of two (*orgA* and *hilA*) were found to be downregulated at 0 min. However, these two genes were not significantly different compared to the control ($p > 0.05$). Three factors, *sipA* (effector protein), *sipD* (translocase

protein) and *invE* (invasion protein) with a fold change of < 2.0 were significantly upregulated ($p < 0.05$) at 30 min.

TABLE 5.2. Differential expression of virulence factors in overnight culture of *S.* Typhimurium after exposure to CPC at a concentration of 10000 ppm for 0.5 min.

Gene	**Fold change**	**p - value**
hilD	-4.1	0.01
invF	-8.9	0.02
orgA	16.7	0.2
sicA	-18.7	0.00
prgK	-27.1	0.00
invE	-1.9	0.03
sicP	-7.6	0.00
sipD	-4.4	0.01
hilA	16.3	0.5
hilC	-2.6	0.02
sipA	-16.8	0.00
sipB	-13.4	0.00
invC	-2.8	0.01

TABLE 5.3. Differential expression of virulence genes in overnight culture of *S.* Typhimurium after exposure to CPC at a concentration of 10000 ppm for 0.5 min and subsequent incubation for 30 min.

Gene	Fold change	p - value
hilD	1.3	0.2
invF	1.4	0.22
orgA	1.7	0.2
sicA	1.6	0.06
prgK	1.3	0.20
invE	1.9	0.03
sicP	1.5	0.06
sipD	1.8	0.04
hilA	0.9	0.7
hilC	1.4	0.2
sipA	1.7	0.05
sipB	1.6	0.08
invC	1.1	0.7

Discussion

Inactivation kinetics

The inactivation curves obtained for *S.* Typhimurium, *S.* Enteritidis, *S.* Heidelberg, and *S.* 4,5,[12]:I are presented in Figs. 5.6, 5.7, 5.8 and 5.9 respectively. Inactivation curves obtained for all the serovars were comparable.

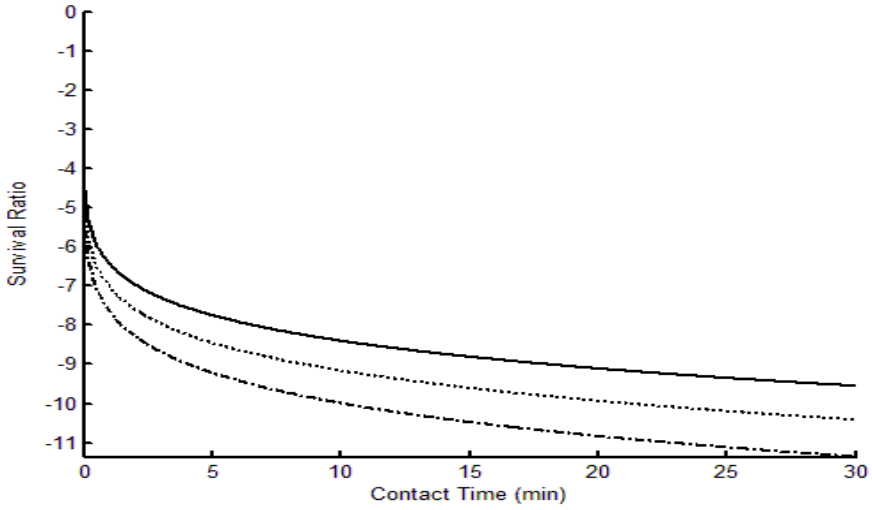

FIG. 5.6. Inactivation kinetics of *S*. Typhimurium after treatment with CPC. Symbols: — (solid line), 100 ppm of CPC; …(dot), 1000 ppm of CPC; -.-.-.-(dot-line), 10000 ppm of CPC.

The inactivation equations were based on Hom's model – $\ln (N/N_0) = -k\, C^n\, t^m$ and given as follows:

S. T – $\ln (N/N_0) = -5.4\, C^{0.04}\, T^{0.12}$

S. E - $\ln (N/N_0) = -6.1\, C^{0.03}\, T^{0.1}$

S. H - $\ln (N/N_0) = -5.8\, C^{0.04}\, T^{0.1}$

S. 4,5,[12]:i - $\ln (N/N_0) = -5.9\, C^{0.03}\, T^{0.1}$

The curves depict a tailing effect with $m < 1$. Also, in general, $n < 1$ for all the serovars indicates that the degree of inactivation is independent of the concentration level. The observed log reductions obtained were similar for all the concentration levels and based on the equations it can be concluded that inactivation is dependent on the exposure time. However, although subtle, two strains, *S*. Typhimurium and *S*. Heidelberg exhibited increased inactivation with higher concentration levels indicating the significant contribution of the variable (concentration) in inactivating the pathogen.

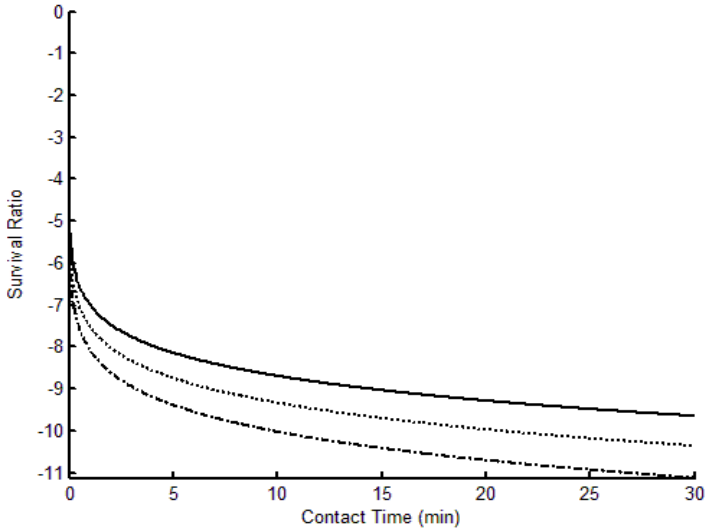

FIG. 5.7. Inactivation kinetics of *S*. Enteritidis after treatment with CPC. Symbols: —(solid line), 100 ppm of CPC; …(dot), 1000 ppm of CPC; -.-.-.-(dot-line), 10000 ppm of CPC.

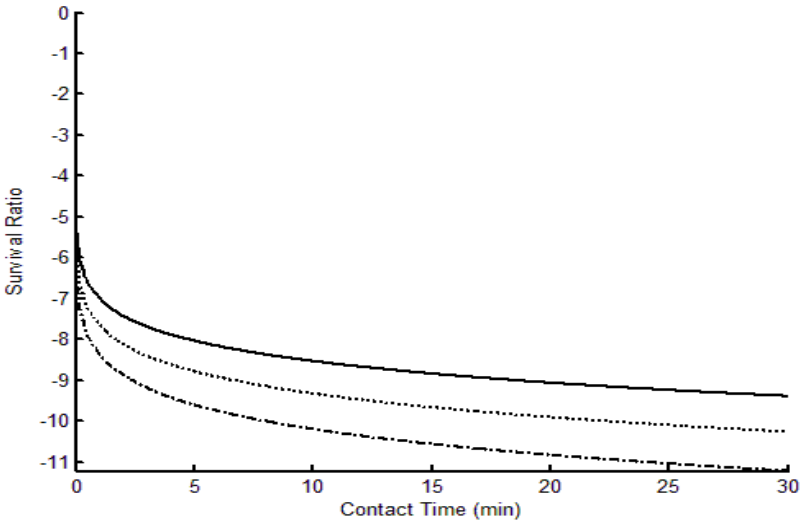

FIG. 5.8. Inactivation kinetics of *S*. Heidelberg after treatment with CPC. Symbols: —(solid line), 100 ppm of CPC; …(dot), 1000 ppm of CPC; -.-.-.-(dot-line), 10000 ppm of CPC.

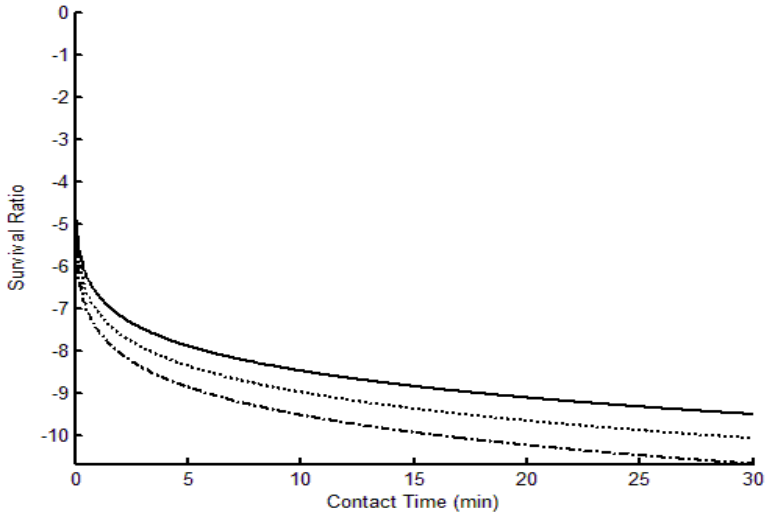

FIG. 5.9. Inactivation kinetics of *S*. 4,5,[12]:i after treatment with CPC. Symbols: —(solid line), 100 ppm of CPC; …(dot), 1000 ppm of CPC; -.-.-.-(dot-line), 10000 ppm of CPC.

In general, results of this study indicate significant resistance of *Salmonella* to the biocide both at higher concentrations and at different exposure time points (15 and 30 min). Log populations of 2.5 were obtained for *S*. Typhimurium and *S*. 4,5,[12]:i at CPC concentrations of 1000 and 10000 ppm after a contact time of 30 min indicating reduced antimicrobial efficacy of the biocide. Previous studies investigating the inactivation kinetics for *Salmonella* with CPC are very limited. The critical killing dilution (CKD – is the highest dilution of biocide that will result in death of the organism at 10 min but not in five) for several microorganisms both in presence and absence of protein was evaluated. It has been reported that the CKD's for several gram-negative organisms was in the range of 1: 34000 to 1:68000 in the presence of serum as against a CKD range of 1:1000 to 1:2700 in the absence (160).

Other studies evaluated the effect of pH on the bactericidal properties of the QAC's. Results obtained in these studies suggest that QAC's such as alkyl dimethyl ethyl benzyl

ammonium chloride and alkyl dimethyl benzyl ammonium chloride were efficient in destroying *Escherichia coli* and *Pseudomonas aeruginosa* in acidic conditions (173, 184). The same study has concluded that the type of buffer system used for maintaining the pH of the chemical has a significant impact on the bactericidal properties of the biocide. The result obtained in the current study also confirms this aspect. The pH of the buffer solution used in the current study was in the range of 7.0 – 7.2. The efficacy of the biocide was found to be reduced significantly with increasing resistance exhibited by the organism at a concentration as high as 10000 ppm.

The chemical CPC is widely used in the poultry processing industry for disinfecting carcasses either prior to after chiller immersion. Several studies investigated the antimicrobial efficacy of the chemical on poultry carcasses. The chemical was reported to severely inhibit attachment of *S*. Typhimurium to the skin tissue in chicken. The same study also reported a 65% reduction of *Salmonella* in human buccal epithelial cells (HBE) at a concentration of 10 ppm (30). Another study reported log reduction of *Salmonella* on chicken skin in the range of 0.9–1.7 units after treatment with CPC at a concentration of 0.1% (101).

Results obtained in the current study however differ from that reported in the other studies. The current study did not test the efficacy of the chemical in poultry, however, a comparison of log reductions reveals that a concentration as high as 10000 ppm for a contact time of 15 min produced a 4.0 log reduction of *Salmonella* in a buffer solution. Other studies reported log reductions only in the range of 1–1.2 logs at a much lower concentration. The chemical used other studies is a commercial product with unknown composition and typically used in the poultry industry whereas the chemical tested in the current study is 99% pure with presence of no inert material. Hence, the results reported by other studies do not

reflect the precise efficacy of the chemical and the data obtained in these studies is definitely questionable.

Analysis of VBNC state in Salmonella

Results indicate that approximately 45% of the treated cells were estimated to be viable. However, this does not imply presence of VBNC state because treatment at a concentration of 10000 ppm for 0.5 min did result in detectable survivors; hence presence of nonculturable cells was not established. Also, presence of intact membrane was established by BacLight assay. Based on this, it is speculated that cells in the test solution perhaps exist in different physiological states such as injured, dead, dormant and non-injured.

Studies investigating the presence of VBNC state in *Salmonella* after exposure to biocides are very limited. Studies evaluating this phenomenon have had drawbacks such as lack of quantitative estimation of recovery and growth of nonculturable cells (3, 98). Hence, a definite existence of VBNC state cannot be concluded, because the resuscitation reported in these studies might have been from the regrowth of cells that were already in a culturable state. Induction of VBNC state in *Salmonella* due to biocides has not been evaluated previously.

RT-PCR analysis of differential gene expression of virulence factors

Three factors, *sipA* (effector protein), *sipD* (translocase protein) and *invE* (invasion protein) with a fold change of < 2.0 were upregulated at 30 min. Previous research indicates significant downregulation of invasive genes associated with SPI1 of *S*. Typhimurium upon treatment with QAC's containing formaldehyde and glutaraldehyde (115). The same study suggested that overexpression of antibiotic resistance determinants such as efflux pumps has resulted in impairment of virulence factors. Another study concluded upregulation of

virulence genes in *Listeria monocytogenes* upon exposure to sub-lethal concentrations of QAC (201).

Another study evaluated the effect of four disinfectants namely sodium hypochlorite, benzalkonium chloride, hydrogen peroxide (HP) and triclosan on virulence gene expression of *L. monocytogenes* and *Salmonella enterica* (50). Biofilms of three strains of each bacterial species were treated with the disinfectants and the resultant stress response of each strain was evaluated. Differences in the susceptibility to the disinfectants were observed and the chemical triclosan was found to be the least effective amongst the disinfectants tested and sodium hypochlorite had the lowest minimum biofilm eradication concentration (MBEC) values (3.125-12.5 μg/ml).

Stress response of *Salmonella* was noticed only for one strain (NCTC 13349) after disinfection with hydrogen peroxide with no significant expression of *rpoS* gene in *Salmonella* strain CC when treated with sodium hypochlorite (SH) and benzalkonium chloride (BAC). Cells surviving in the biofilms treated with all the disinfectants except for sodium hypochlorite lead to significant increase in expression of virulence gene *avrA*. Triclosan treated cells had the highest expression of virulence gene followed by HP and BAC in that order.

Of all the disinfectants tested, SH was found to be the most effective and triclosan to be the least effective against both bacterial species. The differences in susceptibility are attributed to the differences in mode of action of the different chemicals and the differences in resistance mechanisms of the bacterial species to the antimicrobial agents. Strains of both the bacterial species were susceptible to BAC, a QAC and the concentration at which the chemical was found to be effective was 200 μg/ml. *Listeria* was found to be more susceptible

to this disinfectant as compared to *Salmonella* and one strain (CC) of *Salmonella* species was very resistant to this chemical.

While significant increase in expression of stress response gene (*cplC*) was observed with threefold upregulation for SH and HP and two fold increase for BAC in *L.* monocytogenes, the expression of *rpoS* gene in *Salmonella* species was reported only in cells treated with HP. The virulence gene *prfA* was not expressed in *L. monocytogenes* in any of the test conditions used in this study. However, significant upregulation of virulence gene *avrA* in *Salmonella* has been reported with the highest fold change (six fold) observed in cells disinfected with triclosan. Biofilms of *Salmonella* treated with SH however, did not express the virulence factor (50).

Salmonella serovars used in the current study were found to be resistant to CPC at concentrations as high as 10000 ppm. It was interesting to observe a significant down regulation of all the virulence factors except *hilA* and *orgA* at 0 min. Downregulation of virulence factors at 0 min in the current study is attributed to lack of sufficient ATP that is required for activating the system. Other precise molecular mechanisms that contribute to this downregulation are not known at this time and needs further investigation. Upregulation of virulence genes *sipA*, *sipD*, *invE*, *sicP*, and *sicA* was observed at 30 min; however, the fold change obtained was very low ≤ 2.0. Also, the expression of the virulence factors has not been tested in cell lines. Hence, the potential public health risk associated with the upregulation of virulence genes cannot be definitely concluded at this time. The results obtained in this study are preliminary and further studies are needed to understand the precise mechanisms involved in expression of virulence factors.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Numerous studies evaluated the efficacy of several biocides that are frequently used in the food industry; however methods employed in assessing the efficacy of these were always debatable. For instance, studies with chlorine rarely take into consideration the organic demand in the system; hence the quantity of free available chlorine available for microbial decontamination is never estimated accurately. In addition, the chemical composition of the biocides used in such studies is rarely known or understood, so the concentration of the actual ingredient in the product is never estimated accurately. Also, the presence of inert material (composition unknown) in the product may contribute to other unknown chemical interactions leading to overestimation of the efficacy of the chemical which may not be always true. Residues of the chemical on the food product are rarely accounted for and are a cause of concern to human health. Besides these, another critical issue that always gets overlooked is the neutralization of the chemical. The residual activity of the chemical is rarely accounted for resulting in overestimation of the efficacy. Hence, data obtained in a majority of these studies may not always be completely valid. The current study was undertaken to address some of these issues.

All experiments were conducted in chlorine demand free buffer solutions (no organic demand for chlorine) and all the biocides evaluated were 99% pure in composition with presence of no inert material. Also, all the biocides evaluated in this study were neutralized appropriately and tests were conducted to evaluate the toxicity and the efficacy of all the neutralizers used in this study.

Analysis of inactivation kinetics

The data obtained by plate counts is presented in Table 6.1. Amongst the biocides tested, chlorine was found to be the most effective followed by chlorine dioxide and serovars were found to be resistant to CPC.

TABLE 6.1. Comparison of *Salmonella* populations (Log CFU/ml) after treatment with chlorine, chlorine dioxide at concentrations 0.1, 0.5, and 1 ppm for 0.5, 2, and 4 min and CPC at concentrations 100, 1000, and 10000 ppm for 0.5, 15, and 30 min.

Biocide(ppm)/Time(min)	***S*. Typhimurium**	***S*. Enteritidis**	***S*. Heidelberg**	***S*. 4,5,[12]:i**
Chlorine (0.1,0.5,1.0)				
0.5 min	1.5, 1.3, ND*	3.1,1.8,0.8	2.1,1.4,0.8	1.9,0.8,0.4
2 min	ND,ND,ND	1,ND,ND	1,ND,ND	ND,ND,ND
4 min	ND,ND,ND	1.5,ND,ND	ND,ND,ND	ND,ND,ND
Chlorine dioxide (0.1,0.5,1.0)				
0.5 min	6.4,4.4,2.2	7.0,4.8,2.6	6.7,4.1,ND	6.6,4.7,1.0
2 min	4.2,ND,ND	4.3,ND,ND	4.1,ND,ND	4.3,1.0,ND
4 min	2.7,ND,ND	2.2,ND,ND	1.5,ND,ND	1.5,ND,ND
CPC (100,1000,10000)				
0.5 min	5.6,3.7,2.8	4.2,3.2,3.3	4.3,3.9, 3.6	4.9, 4.6, 3.4
15 min	2.6,1.0,2.5	1.8,2.5,3.2	2.3,1.0, 2.8	2.6, 1.7, 1.3
30 min	2.5,1.8,2.7	2.8,2.0,1.0	3.4,2.0, 1.7	2.9, 2.3, 2.3

* ND – No survivors detected

Overall, the inactivation due to chlorine was very rapid with few to no survivors detected at 0.5 min and none were detected at 2 and 4 min at all concentration levels indicating that the degree of inactivation is dependent on contact time rather than concentration ($n < 1$). There were however two exceptions to this. *S.* Enteritidis and *S.* 4,5,[12]:i were found to be inactivated at higher concentrations (0.5 and 1.0 ppm) rather than at 0.1 ppm. The inactivation curves depicted a tailing effect with $m < 1$. Inactivation due to

chlorine dioxide was however gradual with maximum reduction obtained at concentrations 0.5 and 1.0 ppm at 2 and 4 min. Inactivation at 0.1 ppm was very minimal with no significant reduction obtained at 0.5 min. The inactivation curves were close to linear (n = 1) so, a given degree of inactivation can be obtained as product of concentration and time. All the serovars exhibited similar inactivation pattern. The behavior of the serovars to CPC was however different. The highest degree of inactivation was obtained at 0. 5 min for all concentration levels and the inactivation curves obtained closely resembled to that obtained for chlorine with $n < 1$ (inactivation is independent of concentration) and $m < 1$ (tailing effect). The serovars were resistant (contact time of 15 and 30 min as against 2 and 4 min for chlorine) to the biocide at higher concentrations (1000 and 10,000 ppm as against 0.5 and 1.0 ppm for other chemicals) and colony forming units (~ 2 logs) were detected after 30 min of exposure.

The data obtained was fitted into a kinetic model for predicting the combination of concentration of the biocide and the contact time to achieve a specific level of inactivation. The observed survival ratio (plate counts) to the predicted ratio (based on the fitted kinetic model) was close for all the biocides and presented in Tables 6.2, 6.3 and 6.4. Based on this it can be concluded that the model was robust in predicting the coefficients of concentration and contact time for a given set of conditions (temperature, type of microorganism, pH, and the type of disinfectant).

TABLE 6.2. Comparison of survival ratio of *S.* Typhimurium after treatment with chlorine at concentrations 0.1, 0.5, and 1 ppm for 0.5, 2, and 4 min.

C(ppm)	T(min)	Observed	Predicted
0.1	0.5	-9.3	-9.8
0.1	2	-13.4	-11.9
0.1	4	-12.9	-13.1
0.5	0.5	-12.2	-12.3
0.5	2	-16.4	-15.0
0.5	4	-16.4	-16.5
1	0.5	-14.4	-13.6
1	2	-16.4	-16.6
1	4	-16.4	-18.3

survival ratio calculated – ln (N/N_0)
observed – based on plate counts
predicted – based on the fitted model

TABLE 6.3. Comparison of survival ratio of *S.* Typhimurium after treatment with chlorine dioxide at concentrations 0.1, 0.5, and 1 ppm for 0.5, 2, and 4 min.

C(ppm)	T(min)	Observed	Predicted
0.1	0.5	-0.4	-1.6
0.1	2.0	-6.6	-4.8
0.1	4.0	-11.5	-8.4
0.5	0.5	-5.5	-4.8
0.5	2.0	-14.3	-14.9
0.5	4.0	-14.7	-26.1
1.0	0.5	-10.6	-7.9
1.0	2.0	-16.6	-24.2
1.0	4.0	-16.6	-42.5

survival ratio calculated – ln (N/N_0)
observed – based on plate counts
predicted – based on the fitted model

TABLE 6.4. Comparison of survival ratio of *S.* Typhimurium after treatment with CPC at concentrations 100, 1000, and 10000 ppm for 0.5, 15, and 30 min.

C(ppm)	T(min)	Observed	Predicted
100	0.5	-5.7	-6.5
100	15	-10.6	-9.0
100	30	-8.8	-9.6
1000	0.5	-7.9	-7.0
1000	15	-10.6	-9.7
1000	30	-10.8	-10.4
10000	0.5	-7.9	-7.5
10000	15	-9.0	-10.4
10000	30	-11.4	-11.1

survival ratio calculated – ln (N/N_0)
observed – based on plate counts
predicted – based on the fitted model

Analysis of VBNC state in *Salmonella* after exposure to biocides

The study sought to investigate the presence of VBNC state in *Salmonella* using BacLight assay. The presence was not established conclusively due to inherent constraints in the experimental design (low cell density, possible interference from the phosphate buffered saline and failure in analyzing the regrowth of nonculturable cells). The assay used in addition to microscopic study could help determine the presence of intact membrane. Based on this, it is speculated that cells treated with biocides perhaps undergo several changes and assume various physiological states namely injured, dead, ABNC, dormant and noninjured.

Transcriptomic responses of *S*. Typhimurium to chlorine stress

The global gene expression profiles of *S.* Typhimurium at different phases of growth (log and stationary) were compared. The molecular mechanisms being impacted immediately

after the chemical exposure (0 min) to that at 30 min (after incubation of the treated cells) were analyzed.

Results indicate that several metabolic processes in the cell were greatly inhibited in both log and stationary phase cells and this is primarily attributed to depletion of ATP. Mechanisms involved in log cells at 0 min were comparatively low as compared to that of 30 min with significant upregulation of genes associated with citrate cycle, nitrogen metabolism and amino acid metabolism at 30 min. This indicates rapid recovery of the cells after incubation in nutrient rich media.

The impact of the chlorine treatment on cells in the stationary phase was very profound when compared to that of log cells and this was quite expected. Cells in the stationary phase are already exposed to a stressful environment and further application of chlorine would have an increased inhibitory impact on the basic metabolic processes. This was very evident in the gene expression profile, wherein several genes associated with the oxidative phosphorylation, citiric acid cycle and amino acid biosynthesis were downregulated. The recovery of the cells after 30 min of incubation was rather minimal or slow when compared to that of fresh culture. Based on this it can be speculated that the cell is perhaps in a dormant state (may be VBNC) with reduced metabolic activity.

Pathways involved in the biosynthesis of amino acids are downregulated indicating that the protein synthesis within the cell is very minimal. Inhibition of ABC transporters in both log and stationary phase cells indicates significant disruption of cellular membrane activity. Recovery or regrowth of stressed cells was observed both in log phase and stationary phase cells, however the time required for resuscitation was comparatively less in fresh culture as against overnight culture.

Differential expression of virulence genes in *S*. Typhimurium

Majority of the virulence genes (with few exceptions) associated with the SPI1 were found to be downregulated for all the treatments. While treatment with chlorine caused downregulation of all the virulence genes both 0 and 30 min, treatment with chlorine dioxide caused significant upregulation of few (*hilC*, *invC*, *sipA* and *sipB*) genes that are associated with the invasion and overall regulation of the TTS system. This indicates that the TTS system associated with the SPI1 is induced/activated in response to the stress. While other molecular mechanisms being impacted as a result of chlorine dioxide stress are unknown at this time, it is speculated that the injury may have been sub-lethal. All the virulence determinants were found to be downregulated in response to CPC at 0 min with upregulation (< 2.0 fold) observed only for three genes (*sipA*, *sipD* and *invC*) at 30 min.

Implications

Biocides are used in the processing industries in United States for containing the microbial population on carcasses. Chlorine and chlorine dioxide are approved to be used in the chiller in poultry processing, but CPC is used either before or after chiller immersion. The reduced efficacy of biocides, especially chlorine and chlorine dioxide is attributed to presence of enormous amount of organic material (blood, ingesta) in the chiller. Other factors such as pH, and temperature also contribute to the limited efficiency. In addition to being inefficient, other factors such as worker safety, environmental pollution, toxic residues, and degradation of the organoleptic properties of the product limit the application of these chemicals causing only sub-lethal injury to the microorganisms. This perhaps is leading to the emergence of resistant bacteria in the food environment which is a serious concern.

The current study proved that chlorine and chlorine dioxide at a concentration of 1 ppm gave an approximate 6 log reduction while CPC at a concentration of 10000 ppm gave only 4 log reduction in chlorine demand free conditions. In fact, chlorine dioxide although claimed to be unaffected by organic material is required at a concentration of 80 ppm for achieving a 6 log reduction (current study, data not presented) in presence of organic material (buffered peptone water). Further studies evaluating the efficacy of the chemicals used in poultry processing are very essential. This study did not prove the presence of VBNC state but, its presence is not completely ruled out and studies evaluating this phenomenon *in vivo* are very limited. Further research is this area is essential in understanding this phenomenon.

Transcriptomic analysis revealed downregulation of majority of the metabolic processes; however the analysis was done in absence of organic material. Hence, the response to stress *in vivo* is not known and studies elucidating the molecular mechanisms associated with the injury are very few. Majority of the virulence genes associated with the pathogenicity were downregulated in response to chlorine and CPC treatments. However, upregulation of genes associated with invasion and regulation was observed with chlorine dioxide. Virulence response of *Salmonella* upon exposure to acid and thermal stresses was investigated in the past and the mechanisms involved in the virulence expression of *S.* Typhimurium upon exposure to chemicals are thought to be similar to that observed with acid and heat. Expression of virulence factors *in vivo* has been investigated in the past and enhancement of pathogenic characteristics has been reported by majority of these studies. The current study did not investigate this aspect; however future research will be directed in understanding this phenomenon in poultry.

CHAPTER VII

FUTURE RESEARCH

This project was conducted with the goal of determining the efficacy of biocides *in vitro* that are generally used in the poultry industry. In addition to phenotypic response, genetic changes were also investigated to gain an understanding of the molecular mechanisms involved with the stress. The objectives of the project were achieved partially and further research in this area is needed for gaining an in depth perspective.

- Inactivation studies (both *in vitro* and *in vivo*) with other strains (*Listeria*, *Campylobacter*, *Escherichia coli*) is strongly suggested for developing kinetic models that would serve in better prediction of degree of inactivation.
- Phenotypic response of strains in presence of organic material (simulating industry) needs to be investigated to have a better understanding of the impact of organic material on the degree of inactivation.
- Comparison of transcriptomic responses both in presence and absence of organic material for different chemicals.
- Transcriptomic responses (*in vitro* and *in vivo*) of *Salmonella* to chlorine dioxide and CPC need investigation. This helps in comparing the molecular mechanisms being impacted with different chemicals and better explains the mode of action of the biocides.
- Microscopic studies to understand the changes in the bacterial cell membrane after exposure to chemicals.

REFERENCES

1. **Adams, B. L., T. C. Bates, and J. D. Oliver.** 2003. Survival of Helicobacter pylori in a natural freshwater environment. Appl. Environ. Microbiol. **69:**7462-7466.

2. **Aieta, E. M., and J. D. Berg.** 1986. A review of chlorine dioxide in drinking water treatment. Amer. Water Works Assoc. **78:**62-72.

3. **Allen-Austin, D., B. Austin, and R. R. Colwell.** 1984. Survival of *Aeromonas salmonicida* in river waters. FEMS Microbiol. Lett. **21:**143-146.

4. **Altier, C.** 2005. Genetic and environmental control of *Salmonella* invasion. Microbiol. **43:**85-92.

5. **Altier, C., M. Suyemoto, and S. D. Lawhon.** 2000a. Regulation of *Salmonella enterica* serovar *typhimurium* invasion genes by csrA. Infect. Immun. **68:**6790-6797.

6. **Altier, C. M., M. Suyemoto, A. I. Ruiz, K. D. Burnham, and R. Maurer.** 2000b. Characterization of two novel regulatory genes affecting *Salmonella* invasion gene expression. Mol. Microbiol. **35:**635-646.

7. **Arritt, F. M., J. D. Eifert, M. D. Pierson, and S. S. Sumner.** 2002. Efficacy of antimicrobials against *Campylobacter jejuni* on chicken breast skin. J. Appl. Poultry. Res. **11:**358-366.

8. **Ayers, H., J. R. Furr, and A. D. Russell.** 1999. A rapid method of evaluating permeabilizing activity against *Pseudomonas aeruginosa*. Lett. Appl. Microbiol. **17:**149-151.

9. **Ayyildiz, O., B. Ileri, and S. Sanik.** 2009. Impacts of water organic load on chlorine dioxide disinfection efficacy. J. Hazardous. Mater. **168:**1092-1097.

10. **Bajaj, V., C. Hwang, and C. A. Lee.** 1995. *hilA* is a novel *ompR/toxR* family member that activates the expression of *Salmonella typhimurium* invasion genes. Mol. Microbiol. **18:**715-727.

11. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol. **22:**703-714.

12. **Baker, Z., R. W. Harrison, and B. F. Miller.** 1941a. Action of synthetic detergents on the metabolism of bacteria. J. Exp. Med. **74:**249-271.

13. **Baker, Z., R. W. Harrison, B. F. Miller and with the technical assistance of R. Wexler.** 1941b. The bactericidal action of synthetic detergents. J. Exp. Med. **74:**611-620.

14. **Bang, I. S., J. P. Audia, Y. K. Park, and J. W. Foster.** 2002. Autoinduction of the *ompR* response regulator by acid schock and control of the *Salmonella enterica* acid tolerance response. Mol. Microbiol. **44:**1235-1250.

15. **Banin, E., T. Israely, A. Kushmaro, Y. Loga, E. Orr, and E. Rosenber.** 2000. Penetration of the coral-bleacing bacterium *Vibrio shiloi* into *Oculina patagonica*. Appl. Environ. Microbiol. **66:**3031-3036.

16. **Barrette, W. C., Jr., D. M. Hannum, W. D. Wheeler, and J. K. Hurst.** 1989. General mechanism for the bacterial toxicity of hypochlorous acid: Abolition of ATP production. Biochemistry. **28:**9172-9178.

17. **Bates, T., B. Adams, and J. D. Oliver.** 2003. Survival of VBNC *Helicobacter pylori* cocci following antibiotic treatment., Intern. Conf. on *Helicobacter pylori*., Aarhus, Denmark.

18. **Bearson, B. L., L. Wilson, and J. W. Foster.** 1997. A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic stress. J. Bacteriol. **180:**2409-2417.

19. **Behlau, I., and S. J. Miller.** 1993. A Pho-P repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol. **175:**4475-4484.

20. **Benarde, M. A., B. M. Israel, V. P. Oliveri, and M. L. Granstrom.** 1965. Efficiency of chlorine dioxide as a bactericide. Appl. Microbiol. **1965:**776-780.

21. **Benarde, M. A., W. B. Snow, V. P. Oliveri, and B. Davidson.** 1967. Kinetics and mechanism of bacterial disinfection by chlorine dioxide. Appl. Microbiol. **15:**257-265.

22. **Berg, J. D., P. V. Roberts, and A. Matin.** 1986. Effect of chlorine dioxide on selected membrane functions of *Escherichia coli.* Appl. Bacteriol. **60:**213-220.

23. **Beumer, R., S. F. Bloomfield, M. Exner, G. Fara, and E. A. Scott.** 1999. The need for a home hygiene policy and guidelines on home hygiene. Ann. Ing. **11:**11-26.

24. **Beumer, R. R., J. de Vries, and F. M. Rombouts.** 1992. *Campylobacter jejuni* non-culturable coccoid cells. Internl. J. Food Microbiol. **15:**153-163.

25. **Bilgili, S. F., D. E. Conner, J. L. Pinion and K. C. Tamblyn.** 1998. Broiler skin color as affected by organic acids: Influence of concentration and method of application. Poultry. Sci. **77:**751-757.

26. **Bitton, G. (ed.).** 1994. Wastewater microbiology. Ann Arbor Science, New York.

27. **Bloomfield, S. F.** 2002. Significance of biocide usage and antimicrobial resistance in domiciliary environments. J. Appl. Microbiol (Suppl). **92:**144S-157S.

28. **Boddicker, J. D., B. M. Knosp, and B. D. Jones.** 2003. Transcription of the *Salmonella* invasion gene activator, *hilA*, required HilD activation in the absence of negative regulators. J. Bacteriol. **185:**525-533.

29. **Brayton, P. R., M. L. Tamplin, A. Huq, and R. R. Colwell.** 1987. Enumeration of *Vibrio cholerae* O1 in Bangladesh waters by fluorescent antibody direct viable count. Appl. Environ. Microbiol. **53:**2862-2865.

30. **Breen, P. J., C. M. Compadre, E. K. Fifer, H. Salari, D. C. Serbus, and D. L. Lattin.** 1995. Quaternary ammonium compounds inhibit and reduce the attachment of viable *Salmonella typhimurium* to poultry tissues. J. Food Sci. **60:**1191-1196.

31. **Brown, M. R. W. (ed.).** 1975. The role of the cell envelope in resistance. John Wiley and Sons, Ltd, Chichester, England.

32. **Byrd, J. A. (ed.).** 2005. Improving slaughter and processing technologies. CRC, Boca Raton, FL.

33. **Camper, A. K., and G. A. McFeters.** 1979. Chlorine injury and the enumeration of waterborne coliform bacteria. Appl. Environ. Microbiol. **37:**633-641.

34. **Chang, S.** 1971. Modern concept of disinfection. ASCE J. Sant. Eng. Div. **97:**689.

35. **Chaveerach, P., A. Keuzenkamp, H. A. Urlings, L. J. Lipman, and F. van Knapen.** 2002. In vitro study on the effect of organic matter and *Campylobacter jejuni/coli* populations in mixtures of water and feed. Poultry Sci. **81:**621-628.

36. **Cherrington, C., M. Hinto, G. R. Pearson, and I. Chopro.** 1991. Short-chain organic acids at pH 5.0 kill *Escherichia coli* and *Salmonella* spp. without causing membrane perturbation. J. Appl. Bacteriol. **70:**161-165.

37. **Chick, H.** 1908. An investigation of the laws of disinfection. J. Hyg. **8:**92-158.

38. **Collazo, C. M., and J. E. Galan.** 1997. The invasion-associated type III protein secretion in *Salmonella*. Gene. **192:**51-59.

39. **Collazo, C. M., and J. E. Galan.** 1996. Requirement for exported in secretion through the invasion-associated type III of *Salmonella* Typhimurium. Infect. Immun. **64:**3524-3531.

40. **Collazo, C. M., M. K. Zierler, and J. E. Galan.** 1995. Functional analyses of the Salmonella typhimurium invasion genes *invI* and *invJ* and identification of a target of the protein secretion apparatus encoded in the *inv* locus. Mol. Microbiol. **15:**25-38.

41. **Colwell, R. R., P. Brayton, D. Herrington, B. Tall, A. Huq, and M. M. Levine.** 1996. Viable but nonculturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. World Microbiol. Technol. **12:**28-31.

42. **Condie, L. W.** 1986. Toxicological problems associated with chlorine dioxide. Amer. Water Works Assoc. **78:**73-78.

43. **Cooper, E. A.** 1912. On the relationship of phenol and m-cresol to proteins; a contribution to our knowledge to the mechanism of disinfection. J. Biochem. **6:**362-387.

44. **Curtiss, R. I., and S. M. Kelly.** 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. **55:**3035-3043.

45. **Czuprynski, C. J., J. F. Brown, and J. T. Roll.** 1989. Growth at reduced temperatures increases the virulence of *Listeria monocytogenes* for inrtavenously but not intragastrically inoculated mice. Microb. Pathog. **7:**213-223.

46. **Darwin, K. H., and V. L. Miller.** 1999. InvF is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus in *Salmonella typhimurium*. J. Bacteriol. **181:**4949-4954.

47. **Davey, H. M., and D. B. Kell.** 1996. Flow cytometry and cell sorting of heterogeneous microbial populations: The importance of single cell analysis. Microbiol. Rev. **60:**641-696.

48. **Day, A. P., and J. D. Oliver.** 2004. Changes in membrane fatty acid composition during entry of *Vibrio vulnificus* into the viable but nonculturable state. J. Microbiol. **42:**69-73.

49. **Dennis, W. H., V. P. Olivieri, and C. W. Kruse.** 1979. The reaction of nucleotides with aqueous hypochlorous acid. Water Res. **13:**357-362.

50. **Diana, R., N. Cerca, P. Teixeira, R. Oliveira, H. Ceri, and J. Azeredo.** 2011. *Listeria monocytogenes* and *Salmonella enterica* Enteritidis biofilms susceptibility to different disinfectants and stress-response and virulence gene expression of surviving cells. Microbiol. Drug Res. **00:**1-9.

51. **Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan.** 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium: ompR* mutants are attenuated in vivo. Infect. Immun. **57:**2136-2140.

52. **Douglas, L. A.** 1996. Preservation microbiology and safety: Evidence that stress enhances virulence and triggers adaptive mutations. Trends Food Sci. Tech. **7:**91-95.

53. **Douglass, B. K., A. S. Kaprelyants, D. H. Weichart, C. R. Harwood, and M. R. Barer.** 1998. Viability and activity in readily culturable bacteria: A review and discussion of the practical issues. Antoni Van Leeuwenhoek **73:**169-187.

54. **Dukan, S., and D. Touati.** 1996. Hypochlorous acid stress in *Escherichia coli*: Resistance, DNA damage, and comparison with hydrogen peroxide stress. J. Bacteriol. **178:**6145-6150.

55. **Dukes, J. D., H. Lee, R. Hagen, B. J. Reaves, A. N. Layton, E. E. Galyov, and P. Whitley.** 2006. The secreted *Salmonella dublin* phosphoinositide phosphotase, SopB, localizes to PtdIns(3)P-containing endosomes and perturbs normal endosome to lysosome trafficking. Biochem. **395:**239-247.

56. **Dyar, M. T., and E. J. Ordale.** 1946. Electrokinetic syudies of bacterial surfaces. I. The effects of surface-active agents on the electrophoretic mobilities of bacteria. J. Bacteriol. **51:**149-167.

57. **Eichelberg, K., and J. E. Galan.** 1999. Differential regulation of *Salmonella typhimurium* type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and HilA. Infect. Immun. **67:**4099-4105.

58. **Eichelberg, K., C. Ginocchio, and J.E. Galan.** 1994. Molecular and functional characterization of the *Salmonella typhimurium* invasion genes *invB* and *invC*: Homology of InvC to the $F_0 F_1$ ATPase family of proteins. J. Bacteriol. **176:**4501-4510.

59. **Eichelberg, K., W. D. Hardt, and J. E. Galan.** 1999. Characterization of SprA, an AraC-like transcriptional regulator encoded within the *Salmonella typhimurium* pathogenecity island 1. Mol. Microbiol. **33:**139-152.

60. **Ellermier, C. D., and J. M. Slauch.** 2004. RtsA coordinately regulates DsbA and the *Salmonella* pathogenecity island I type III secretion system. J. Bacteriol. **186:**68-79.

61. **EPA.** 1991. Guidance manual for compliance with the filtration and disinfection requirement for public water, p. 141-152, AWWA., Denver.

62. **Farkas, J.** 1998. Irradiation as a method for decontaminating food: A review. Int. J. Food Microbiol. **44:**189-204.

63. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: Bacterial exporters. Microbiol Rev. **57:**995-1017.

64. **Federighi, M., J. L. Tholozan, J. M. Cappelier, J. P. Tissier, and J. L. Jouve.** 1998. Evidence of non-coccoid viable but non-culturable *Campylobacter jejuni* cells in microcosm water by direct viable count, CTC-DAPI double staining, and electron microscopy. Food Microbiol. **15:**539-550.

65. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A *Salmonella* locus that controls resistance to antibacterial proteins from phagocytic cells. Science. **85:**5189-5193.

66. **Finlay, B. B., and S. Falkow.** 1988. Comparison of the invasion strategies used by *S. choleraesuis*, *Shigella flexneri*, and *Yersinia enterocolitica* to enter cultured animal cells: Endosome acidification is not required for bacterial invasion or intracellular replication. Biochimie. **70:**1089-1099.

67. **Fordtran, J. S., and F. J. Ingelfinger. (ed.).** 1968. Absorption of water, electrolytes, and sugars from the human gut. Waverly Press, Baltimore, MD.

68. **Frier, M. (ed.).** 1971. Derivatives of 4-amino-quinaldinium and 8-hydroxyquinoline. Academic Press Ltd, London, England.

69. **Gahan, C. G. M., and H. Colin.** 1999. The relationship between acid stress responses and virulence in *Salmonella typhimurium* and *Listeria monocytogenes*. Intl. Food Microbiol. **50:**93-100.

70. **Galan, J. E.** 1996. Molecular genetic bases of *Salmonella* entry into host cells. Mol. Microbiol. **20:**263-271.

71. **Galan, J. E., and J. B. Bliska.** 1996. Cross-talk between bacterial pathogens and their host cells. Annu. Rev. Cell. Dev. Biol. **12:**221-255.

72. **Garcia Vescovi E., F. C. S., and E. A. Groisman.** 1996. Mg^{+2} as an extracellular signal: Environmental regulation of *Salmonella* virulence. Cell. **84:**165-174.

73. **Gates, D.** 1998. The chlorine dioxide handbook. Amer Water Works Assoc., Denver.

74. **George, A. M.** 1996. Multiresistance in enteric and other gram-negative bacteria. FEMS Microbiol. Lett. **139:**1-10.

75. **Gilbert, P. (ed.).** 1988. Microbial resistance to preservative systems. Ellis Horwood, Chichester, England.

76. **Giles, C. H., T. H. MacEwan., S. N. Nakhura., and D. Smith.** 1960. Studies in adsorption. Part XI. A system of classification of solution adsorption isotherms and its use in the diagnosis of adsorption mechanisms and in measurements of specific surface areas of solids. J. Chem. Soc. **3973:**3973-3993.

77. **Groisman, E. A.** 1997. How *Salmonella* became a pathogen. Trends Microbiol. **5:**343-349.

78. **Guiney, D. G., S. Libby, F. Fang, M. Krause, and J. Fierer.** 1995. Growth-phase regulation of plasmid virulence genes in *Salmonella*. Trends Microbiol. **3:**275-279.

79. **Gulig, P. A., H. Danbara, D. G. Guiney, A. J. Lax, F. Norel, and M. Rhen.** 1993. Molecular analysis of the *spv* virulence genes of the *Salmonella* virulence plasmids. Mol. Microbiol. **7:**825-830.

80. **Gustafson, R. H., R.E. Bowen.** 1997. Antibitic use in animal agriculture. J. Appl. Microbiol. **83:**531-541.

81. **Haas, C. N., and S. B. Karra.** 1984. Kinetics of microbial inactivation by chlorine. A review of results in demand-free systems. Water. Res. **18:**1443-1449.

82. **Hammond, S. M., P. A. Lambert, and A. N. Rycroft. (ed.).** 1984. The bacterial cell surface. Croom Helm, London, England.

83. **Hancock, R. E. W.** 1991. Bacterial outer membrane: Evolving concepts. Ameri. Soc. Microbiol. **57:**175-182.

84. **Hardt, W. D., and J. E. Galan.** 1997. A secreted *Salmonella* protein with homology to an avirulence determinant of plant pathogenic bacteria. Proc. Natl. Acad. Sci. **94**:9887-9892.

85. **Heiskanen, P., S. Taira, and M. Rhen.** 1994. Role of *rpoS* in the regulation of *Salmonella* plasmid virulence (*spv*) genes. FEMS. Microbiol. Lett. **123:**125-130.

86. **Hensel, M., J. E. Shea, A. J. Baumler, C. Gleeson, F. Blattner, and D. W. Holden.** 1997. Analysis of the boundaries of *Salmonella* pathogenecity island 2 and the correponding chromosomal region of *Escherichia coli* K-12. J. Bacteriol. **179:**1105-1111.

87. **Higgins, C. F.** 1992. ABC transporters: From microrganisms to man. Annu. Rev. Cell Biol. **8:**67-113.

88. **Hill, C., B. O' Driscoll, and I. Booth.** 1995. Acid adaptation and food poisoning microorganisms. Intl. J Food Microbiol. **28:**245-254.

89. **Hom, L. W.** 1972. Kinetics of chlorine disinfection in an ecosystem. J. Sanit. Eng. Div. **98:**183-193.

90. **Hotchkiss, R. D.** 1944. Gramicidin, tyrocidin and tyrothricin. Adv. Enzymol. **4:**153-199.

91. **Huang, D. W., B. T. Sherman, and R. A. Lempicki.** 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. **37:**1-13.

92. **Huang, D. W., B. T. Sherman, and R. A. Lempicki.** 2009. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nature. **4:**44-57.

93. **Hueck, C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62:**379-433.

94. **Hughes, D. E.** 1962. The bacterial cytoplasmic membrane. J. Gen. Microbiol. **29:**39-46.

95. **Hugo, W. B.** 1967. The mode of action of antibacterial agents. J. Appl. Bact. **30:**17-50.

96. **Hugo, W. B., and J. H. S. Foster.** 1964. Growth of *Pseudomonos aeruginosa* in solutions of esters of *p*-hydroxybenzoic acid. J. Pharm. Pharmacol. **16:**209.

97. **Humphrey, T. J., A. Williams, K. McAlpine, F. Jorgensen, and C. O'Byrne.** 1998. Pathogenecity in isolates of *Salmonella enterica* serotype Enteritidis PT4 which differ in RpoS expression: Effects of growth phase and low temperature. Eipdemiol. Infect. **121:**295-301.

98. **Hussong, D., R. R. Colwell, M. Obrien, E. Weiss, A. D. Pearson, R. M. Weiner, and W. D. Burge.** 1987. Viable *Legionella pneumophila* not detectable by culture on agar media. Bio/Technol. **5:**947-950.

99. **Jacques, M.** 1996. Role of lipopolysaccharides in bacterial adherence. Trends Microbiol. **4:**408-410.

100. **Janakiraman, A., and J. M. Slauch.** 2002. The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of *Salmonella typhimurium*. Mol. Microbiol. **35:**1146-1155.

101. **Jeong-Weon, K., and M. F. Slavik.** 1995. Cetylyridinium chloride (CPC) treatment on poultry skin to reduce attached *Salmonella*. J. Food Prot. **59:**322-326.

102. **Johnston, C., D. A. Pegues, C. J. Hueck, A. Lee, and S. I. Miller.** 1996. Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response regulator superfamily. Mol. Microbiol. **22:**715-727.

103. **Jones, B. D., and S. Falkow.** 1994. Identification and characterization of a *Salmonella* typhimurium oxygen-regulated gene required for bacterial internalization. Infect. Immun. **62:**3745-3752.

104. **Jones, B. D., N. Ghori, and S. Falkow.** 1994. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J. Exp. Med. **180:**15-23.

105. **Julien, P., G. Patrice, D. Sam, and M. Patrick.** 2009. Respective roles of culturable and viable-but-nonculturable cells in the heterogenecity of *Salmonella enterica* serovar Typhimurium invasiveness. Appl. Environ. Microbiol. **75:**5179-5185.

106. **Kaniga, K., D. Trollinger, and J. E. Galan.** 1995. Identification of two targets of the type III secretion system encoded in the *inv* and *spa* loci of *Salmonella typhimurium* that share homology to IpaD and IpaA proteins. J. Bacteriol. **177:**7078-7708.

107. **Kaniga, K., J. C. Bossio, and J. E. Galan.** 1994. The *Salmonella typhimurium* invasion genes *invF* and *invG* encode homologues to PulD and AraC family of proteins. Mol. Microbiol. **13:**555-568.

108. **Kaniga, K., S. C. Tucker, D. Trollinger, and J. E. Galan.** 1995. Homologues of the *Shigella* invasins IpaB and IpaC are required for *Salmonella typhimurium* entry into cultured cells. J. Bacteriol. **177:**3965-3971.

109. **Kaprelyants, A. S., and D. B. Kell.** 1993. Dormancy in stationary-phase cultures of *Micrococcus luteus*: Flow cytometric analysis of starvation and resuscitation. Appl. Environ. Microbiol. **59:**3187-3196.

110. **Kaprelyants, A. S., and D. B. Kell.** 1992. Rapid assessment of bacterial viability and vitality using rhodamine 123 and flow cytometry. J. Appl. Bacteriol. **72:**410-422.

111. **Keeton, T. J., and S. M. Eddy. (ed.).** 2004. Chemical methods for decontamination of meat and poultry. Blackwell, Ames, IA, USA.

112. **Kell, D. B., A.S. Kapreylants, D. H. Weichart, C. L. Harwood, and M. R. Barer.** 1998. Viability and activity in readily culturable bacteria: A review and discussion of the practical issues. Antoni Van Leeuwenhoek. **73:**169-187.

113. **Kell, D. B., H. M. Ryder, A. S. Kaprelyants, and H. V. Westerhoff.** 1991. Quantifying heterogenecity - flow cytometry of bacterial cultures. Antonie Van Leeuwenhoek **60:**145-158.

114. **Khan, A. U., and M. Kasha.** 1994. Singlet molecular oxygen evolution upon simple acidification of aqueous hypochlorite: Application to studies on the deleterious health effects of chlorinated drinking water. Proc. Natl. Acad. Sci. **12:**362-364.

115. **Kimon, A. G. K., M. A. Webber, F. Jorgensen, M. J. Woodward, L. J. V. Piddock, and T. J. Humphrey.** 2007. Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. J. Antimicrobiol. Chemo. **60:**947-955.

116. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting live marine bacteria. Can. J. Microbiol. **25:**415-420.

117. **Kono, Y., and I. Fridovich.** 1982. Superoxide radical inhibits catalase. J. Biol. Chem. **257:**5751-5754.

118. **Korich, D. G., J. R. Mead, M. S. Madore, N. A. Sinclair, and C. R. Sterling.** 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol. **56:**1423-1428.

119. **Kubori, T., A. Sukhan, S. I. Aizawa, and J. E. Galan.** 2000. Molecular characterization and assembly of the needle complex of the *Salmonella* Typhimurium type III protein secretion system. Proc. Natl. Acad. Sci. **97:**10225-10230.

120. **Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan, and S. I. Aizawa.** 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. Science. **280:**602-605.

121. **Kuhn, R., and H. J. Bielig.** 1940. Uber invertseifen. I ; die Einwirkung vun Invertseifen auf Eiweins-Stoffe. Ber. dt. chem. Ges. **73:**1080.

122. **Kullik, I., M. B. Toledano, L.A. Tartaglia, and G. Storz.** 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyT: Regions important for oxidation and transcriptional activation. J. Bacteriol. **177:**1275-1284.

123. **Layton, A. N., P. J. Brown, and E. E. Galyov.** 2005. The *Salmonella* translocated effector SopA is targeted to the mitochondria of infected cells. J. Bacteriol. **2005:**3565-3571.

124. **LeChevallier, M. W., A. Singh, D. A. Schiemann, and G. A. McFeters.** 1985. Changes in virulence of waterborne enteropathogens with chlorine injury. Appl. Environ. Microbiol. **50:**412-419.

125. **Lee, Y-J., and S-H. Nam.** 2002. Reflection on kinetic models to the chlorine disinfection for drinking water production. J Microbiol. **40:**119-124.

126. **Li, J., H. Ochman, E. A. Groisman, E. F. Boyd, F. Salomon, K. Nelson, R. K. Selander.** 1995. Relationship between evolutionary rate and cellular location among the *inv/spa* invasion proteins of *Salmonella enterica*. Proc. Natl. Acad. Sci. **92:**7252-7256.

127. **Lillard, H. S.** 1979. Levels of chlorine and chlorine dioxide of equivalent bactericidal effect in poultry processing water. J. Food Sci. **44:**1594-1597.

128. **Liochev, S. I., and I. Fridovich.** 1992. Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the soxRS regulon. Proc. Natl. Acad. Sci. **89:**5892-5896.

129. **Lory, S.** 1992. Determinants of extracellular protein secretion in gram-negative bacteria. J. Bacteriol. **174:**3423-3428.

130. **Lucas, R. L., and C. A. Lee.** 2001. Roles of *hilC* and *hilD* in regulation of *hilA* expression in *Salmonella enterica* serovar Typhimurium. J. Bactriol. **182:**2733-2745.

131. **Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M . P. Spector, B. L. Wanner, and C. A. Lee.** 2000. Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar *typhimurium.* J. Bacteriol. **182:**1872-1882.

132. **Manas, P., R. Pagan, F. J. Salan, and S. Condon.** 2001. Low molecular weight milk whey component protect *Salmonella* senftenberg 775W against heat by a mechanism involving divalent cations. J. Appl. Microbiol. **91:**871-877.

133. **Mark, C., E. Sofia, T-M. Dilek, and D. H. Jay.** 2001. Virulence gene regulation in *Salmonella enterica*. Ann. Med. **33:**178-185.

134. **McCarthy, J. A.** 1944. Bromine and chlorine as water disinfectants. J. New. Engl. Water Works Assoc. **58:**55-65.

135. **McDonnell, G., and A. D. Russell.** 1999. Antiseptics and disinfectants: Activity, action and resistance. Clin Microbiol Rev **12:**147-179.

136. **McFeters, G. A., F. P. Yu, B. H. Pyle, and P. S. Stewart.** 1995. Physiological assessment of bacteria using fluorochromes. J. Microbiol. Meth. **21:**1-13.

137. **McGovern, V. P., and J. D. Oliver.** 1995. Induction of cold responsive proteins in *Vibrio vulnificus*. J. Bacteriol. **177:**4131-4133.

138. **Miller, S. I.** 1991. phoP/phoQ: macrophage-specific modulators of *Salmonella* virulence? Mol. Microbiol. **5:**2073-2078.

139. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. **86:**5054-5058.

140. **Mills, D. M., V. Bajaj, and C. A. Lee.** 1995. A 40 kilobase chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome. Mol. Microbiol. **15:**749-759.

141. **Miras, I., D. Hermant, N. Arricau, and M.Y. Popoff.** 1995. Nucleotide sequence of *iagA* and *iagB* genes involved in invasion of HeLa cells by *Salmonella enterica* subsp. *enterica* ser. *typhi*. Res. Microbiol. **146:**17-20.

142. **Mokgatla, R. M., P. A. Gouws, and V. S. Brozel.** 2002. Mechanisms contributing to hypochlorous acid resistance of a *Salmonella* isolate from a poultry-processing plant. J. Appl. Microbiol. **92:**566-573.

143. **Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz, and B. Ames.** 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc. Natl. Acad. Sci. **83:**8059-8063.

144. **Morris, J.** 1966. The acid ionization constant of HOCl from 5 C to 35 C. J. Phys. Chem. **70:**3798.

145. **Morton, D., and J. D. Oliver.** 1994. Induction of carbon starvation proteins in *Vibrio vulnificus.* Appl. Environ. Microbiol. **60:**3653-3659.

146. **Neidhardt, F. C. (ed.).** 1999. *Escherichia coli* and *Salmonella*: Cellular and molecular biology., Am. Soc. Microbiol., Washington, DC.

147. **Nikadio, H.** 1998. Antibiotic resistance caused by gram-negative multidrug efflux pumps. Clin. Infect. Dis. **27:**S32-S41.

148. **Norris, F. A., M. P. Wilson, T. S. Wallis, E. E. Galyov, and P. W. Majerus.** 1998. SopB, a protein required for virulence of *Salmonella dublin* in an inositol phosphate phosphatase. Proc. Natl. Acad. Sci. **95:**14057-14059.

149. **Oliver, J. D. (ed.).** 2000a. Problems in detecting dormant (VBNC) cells and the role of DNA elements in this response. Landes, Biosciences, Georgetown, TX.

150. **Oliver, J. D. (ed.).** 2000b. The public health significance of viable but nonculturable bacteria. American Society for Microbiology, Washington, D. C.

151. **Oliver, J. D., and R. Bockian.** 1995. In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus.* Appl. Environ. Microbiol. **61:**2620-2623.

152. **Ordal, E. J., and A. F. Borg.** 1942. Effect of surface active agents on the oxidation of lactate by bacteria. Proc. Soc. Exp. Biol. Med. **50:**332-336.

153. **Pallent, L. J., W. B. Hugo, D. J. W. Grant and A. Davies.** 1983. *Pseudomonas cepacia* and infections. J. Hosp. Infect. **4:**9-13.

154. **Papezova, K., D. Gregorova, J. Jonischies, and I. Rychlik.** 2005. Ordered expression of virulence genes in *Salmonella enterica* serovar Typhimurium. Folia Microbiol. **52:**107-114.

155. **Pearson, A. D., M. Greenwood, T. D. Healing, D. Rollins, M. Shahamat, J. Donaldson, and R. R. Colwell.** 1993. Colonization of broiler chickens by waterborne *Campylobacter jejuni*. Appl. Environ. Microbiol. **59:**987-996.

156. **Pegues, D. A., M. J. Hantman, I. Behlau, and S. I. Miller.** 1995. PhoP/PhoQ transcriptional repression of *Salmonella* typhimurium invasion genes: Evidence for a role in protein secretion. Mol. Microbiol. **17:**169-181.

157. **Porter, J., C. Edwards, and R. W. Pickup.** 1995. Rapid assessment of physiological status in *Escherichia coli* using fluorescent probes. J. Appl. Bacteriol. **4:**399-408.

158. **Pulvertaft, J. V., and C. D. Lumb.** 1948. Bacterial lysis and antiseptics. J. Hyg. **46:**62-65.

159. **Pussadee, T., C. Suganya, K. Prapaporn, and M. W. Griffiths.** 2006. Morphological and physiological responses of *Campylobacter jejuni* to stress. J. Food Prot. **69:**2747-2753.

160. **Quisno, R., and M. J. Foter.** 1946. Cetylpyridinium chloride. J. Bacteriol. **52:**111-117.

161. **Rahman, I., M. Shahamat, M. A. R. Chowdhury, and R. R. Colwell.** 1996. Potential virulence of viable but nonculturable *Shigella dysenteriae* Type 1. Appl. Environ. Microbiol. **62:**115-120.

162. **Rakeman, J. L., H. R. Bonifield, and S. I. Miller.** 1999. A HilA-independent pathway to *Salmonella typhimurium* invasion gene transcription. J. Bacteriol. **181:**3096-3104.

163. **Reissbordt, R., I. Rienaecker, J. M. Romanova, P. P. E. Freestone, R. D. Haigh, M. Lyte, H. Tschape, and P. H. Williams.** 2002. Resuscitation of *Salmonella enterica* serovar Typhimurium and enterohemorrhagic *Escherichia coli* form the viable but nonculturable state by heat-stable enterobacterial autoinducer. Appl. Environ. Microbiol. **68:**4788-4794.

164. **Reissbrodt, R., H. Heier, H. Tschape, R. A. Kingsley, and P. H. Williams.** 2000. Resuscitation by ferrioxamine E of stressed *Salmonella enterica* serovar Typhimurium from soil and water microcosms. Appl. Environ. Microbiol. **66:**4128-4130.

165. **Ridenour, G. M., and R. S. Ingols.** 1947. Bactericidal properties of chlorine dioxide. J. Ameri. Water Works Assoc. **39:**561-567.

166. **Rodriguez, G. G., D. Phipps, K. Ishiguro, and H. F. Ridgway.** 1992. Use of fluorescent redox probe for direct visualization of actively respiring bacteria. Appl. Environ. Microbiol. **58:**1801-1808.

167. **Roller, S. D., V. P. Oliveri, K. Kawata.** 1980. Mode of inactivation by chlorine dioxide. Water Res. **14:**635-641.

168. **Romeo, T.** 1998. Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. Mol. Microbiol. **29:**1321-1330.

169. **Russell, A. D.** 1998. Bacterial resistance to disinfectants: Present knowledge and future problems. (Suppl) J. Hospital. Infect. **43:**S57-S68.

170. **Russell, A. D.** 2000. Do biocides select for antibiotic resistance. J. Pharm. Pharmacol. **52:**227-233.

171. **Saguinsin, J. L. S., and J. C. Morris. (ed.).** 1975. The chemistry of aqueous nitrogen trichloride. Ann Arbor, MI.

172. **Sakagami, Y., H. Yokoyama, H. Nishimura, Y. Ose, T. Tashima.** 1989. Mechanism of resistance to benzalkonium chloride by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **55:**2036-2040.

173. **Salton, M. R. J.** 1950. The bactericidal properties of certain cationic detergents. Aust. J. Sci. Research B. **3:**45-60.

174. **Salton, M. R. J.** 1968. Lytic agents, cell permeability and monolayer penetrability. J. Gen. Physiol. **52:**252S-277S.

175. **Salton, M. R. J., and J. H. Freer.** 1965. Composition of the membranes isolated from several gram-positive species. Biochim. Biophys. Acta. **107:**531-538.

176. **Scherer, C. A., M. J. Hantman, and S. I. Miller.** 1997. *Salmonella* invasion and delivery of protein effectors to maintain cell cytoplasm. Trends Microbiol. **5:**127-129.

177. **Schulman, J. H., and E. K. Rideal.** 1937. Molecular interaction in monolayers. I. Complexes between large molecules. Proc. R. Soc. **B122:**29-45.

178. **Shang, C., and E. R. Blatchley III.** 1999. Differentiation and quantification of free chlorine and inorganic chloramines in aqueous solution by MIMS. Environ. Sci. Technol. **33:**2218-2223.

179. **Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden.** 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. **93:**2593-2597.

180. **Shih, K. L., and J. Lederberg.** 1976. Effects of chloramine on *Bacillus subtilis* deoxyribonucleic acid. J. Bacteriol. **125:**934-945.

181. **Signoretto, C., M. M. Lleo, and P. Canepari.** 2002. Modification of the peptidoglycan of *Escherichia coli* in the viable but nonculturable state. Curr. Microbiol. **44:**125-131.

182. **Simon, E. W.** 1953c. Mechanisms of dinitrophenol toxicity. Biol. Rev. **28:**453-478.

183. **Skyes, G.** 1939. The influence of germicides on the dehydrogenases of *Bact coli*. J. Hyg. **39:**463-469.

184. **Soike, K. F., D. D. Miller, and P. R. Elliker.** 1952. Effect of pH of solution on germicidal activity of quaternary ammonium compounds. J. Dairy. Sci. **35:**764-771.

185. **Solveig, L., M. S. Sidhu, E. Heir and A. L. Holck.** 2003. Bacterial disinfection resistance – a challenge for the food industry. Intl. Biodet. Biodeg. **51:**283-290.

186. **Stecchini, M., M. Manzano, and I. Sarais.** 1992. Antibiotic and disinfectant susceptibility in Enterobacteriaceae isolated from minced meat. Intl. J. Food Microbiol. **16:**79-85.

187. **Stender, S., A. Friebel, S. Linder, M. Rohde, S. Mirold, and W. D. Hardt.** 2000. Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc-42 of the host cell. Mol. Microbiol. **36:**1206-1221.

188. **Stephens, J. C., I. S. Roberts, D. Jones, and P. W. Andrew.** 1991. Effect of growth temperatures on virulence of strains of *Listeria monocytogenes* in the mouse: Evidence of a dose dependence. J. Appl. Bacteriol. **70:**239-244.

189. **Suarez, M., and R. Holger.** 1998. Molecular mechanisms of *Salmonella* invasion: The type III secretion system of the pathogenecity island 1. Intl. Microbiol. **1:**197-204.

190. **Sukupolvi, S., R. G. Lorenz, J. I. Gordon, Z. Bian, J. D. Pfeifer, and S. J. Normark.** 1997. Expression of thin aggregrative fimbriae promotes interaction of *Salmonella typhimurium* SR-11 with mouse intestinal epithelial cells. Infect. Immun. **65:**5320-5325.

191. **Sylvester, D. M., R. Taylor, and T. R. LaHann.** 2001. Viable but nonculturable bacteria: A public health threat? Infect. Dis. Rev. **3:**70-82.

192. **Thiessen, G. P., W. R. Usborne, and H. L. Orr.** 1983. The efficacy of chlorine dioxide in controlling *Salmonella* contamination and its effect on product quality of chicken broiler carcasses. Poultry Sci. **63:**647-653.

193. **Tholazan, J. L., J. M. Cappelier, J. P. Tissier, G. Delattre, and M. Federighi.** 1999. Physiological characterization of viable-but-non-culturable *Campylocater jejuni* cells. Appl. Environ. Microbiol. **65:**1110-1116.

194. **Tsai, L., J.E. Scahde, and T. Molyneux.** 1992. Chlorination of poultry process water: Chlorine demand and disinfection efficiency. J. Poultry Sci. **71:**188-196.

195. **USDA-FSIS.** 2010. Compliance Guideline for controlling *Salmonella* and *Campylobacter* in Poultry. **3rd Edition**., Beltsville, MD.

196. **USDA-FSIS.** 2010. Safe and Suitable Ingredients Used in the Production of Meat and Poultry Products. Beltsville, MD

197. **Vaara, M.** 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. **56:**395-411.

198. **Vaczi, L., J. Szita, V. Cieleszky.** 1957. The role of lipids in induced chloramphenicol resistance of bacteria. Acta . Microbiol. **4:**437-445.

199. **Van der Velden, A. W., A. J. Baulmer, R. M. Tsolis, and F. Heffron.** 1998. Multiple fimbrial adhesins are required for full virulence of *Salmonella typhimurium* in mice. Infect. Immun. **66:**2803-2808.

200. **Venkobacher, C., L. Iyengar, and A. V. S. P. Rao.** 1997. Mechanism of disinfection: Effect of chlorine on cell membrane functions. Water Res. **11:**727-729.

201. **Vicky, G. K., M. H. Larsen, L. Gram, and H. Ingmer.** 2010. Influence of sublethal concentrations of common disinfectants on expression of virulence genes in *Listeria monocytogenes*. Appl. Environ. Microbiol. **76:**303-309.

202. **Virto, R., P. Manas, I. Alvarez, S. Condon, and J. Raso.** 2005. Membrane damage and microbial inactivation by chlorine in the absence and presence of a chlorine-demanding substrate. Appl. Environ. Microbiol. **71:**5022-5028.

203. **Wang, S., A. M. Phillippy, K. Deng, X. Rui, Z. Li, M. L. Tortorello, and W. Zheng.** 2010. Transcriptomic responses of *Salmonella enterica* serovars Enteritidis and Typhimurium to chlorine-based oxidative stress. Appl. Environ. Microbiol. **76:**5013-5024.

204. **Warner, J. M., and J. D. Oliver.** 1998. Randomly amplified polymorphic DNA analysis of starved and viable but nonculturable *Vibrio vulnificus* cells. Appl. Environ. Microbiol. **64:**3025-3028.

205. **Watson, H. E.** 1908. A note on the variation of the rate of disinfection with change in the concentration of the disinfection. J. Hyg. **8:**536-542.

206. **Weichart, D., D. McDougald, D. Jacobs, S. Kjelleberg.** 1997. In situ analysis of nuclic acids in cold-induced nonculturable *Vibrio vulnificus*. Appl. Environ. Microbiol. **63:**2754-2758.

207. **Whitesides, M. D., J. D. Oliver.** 1997. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Appl. Environ. Microbiol. **63:**1002-1005.

208. **Wojtowicz, J. A. (ed.).** 1979. Chlorine monoxide, hypochlorous acid, and hypochlorites. John Wiley, New York.

209. **Yang, H., Y. Li, and M. G. Johnson.** 2001. Survival and death of *Salmonella* Typhimurium and *Campylobacter jejuni* in processing water and on chicken skin during poultry scalding and chilling. J. Food Prot. **64:**770-776.

210. **Yang, Y. H., D. Sandrine, L. Percy, L. M. David, P. Vivian, N. John, and S. P. Terence.** 2002. Normalization for cDNA microarray data: A robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. **30:**e15.

211. **Zimmerman, R., R. Iturriaga, and J. Becker-Birck.** 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ. Microbiol. **36:**926-935.

APPENDIX

Preparation of chlorine demand free buffer (CDF buffer)

- Prepare 1M stock solution of sodium monobasic and sodium dibasic solutions and filter sterile.
- Glass material (bottles) used for preparing the buffer solutions are thoroughly rinsed in distilled water and autoclaved at 121°C for 15 min.
- Prepare 0.1M buffer solution by adding 58 ml of dibasic and 42 ml of monobasic solutions made up to 1 L.
- Add ~ 5 ml of chlorine standard solution and check for concentration of chlorine in the solution immediately and after 15 min. Note the reading
- Add another 10 ml of chlorine standard solution and check for the concentration immediately and after 15 min. Take the reading.
- Store the buffer solutions in dark at room temperature.
- Autoclave the solution at 121°C for 15 min.
- Check the chlorine concentration and make sure it is < 0.1 ppm

Preparation of Chlorine dioxide

- Prepare 50% H_2SO_4 – 5 ml of H_2SO_4 in 5 ml of DI water
- Prepare 15% sodium chlorite – 1.5 g dissolved in 10 ml of DI water
- Add 1.2 ml of H_2SO_4 followed by 1.2 ml of sodium chlorite into the syringe that is fastened to the bottle
- Leave the contents in the syringe for 1 min and mix the contents in 200 ml of DI water.
- Check the concentration of the chemical prepared using spectrophotometer.

Procedure for Standard curve

- Overnight culture (25 ml) of *S.* Typhimurium grown to late log phase was concentrated by centrifugation at 4000 x g for 10 min.
- The supernatant was decanted and the pellet was resuspended in 10 ml of 0.85% Nacl solution.
- The resuspended culture was centrifuged again at 4000 x g for 10 min, supernatant decanted and resuspended in 2 ml of 0.85% Nacl solution.
- The cell suspension (1 ml) was dispensed into 50 ml centrifuge tubes containing either 20 ml of chlorine demand free buffer solution (live cells) or 20 ml of 70% isopropyl alcohol (dead cells).
- The samples were incubated at room temperature for 1 h with intermittent mixing. Incubated samples were centrifuged at 4000 x g for 10 min and pellets resuspended in 20 ml of 0.85% Nacl solution.
- The cell suspensions were centrifuged again at 4000 x g for 10 min and resuspended in 10 ml of 0.85% Nacl solution.
- The O.D of the cell suspensions were adjusted to a final cell density of 2×10^8 cells/ml. The two different cell suspensions were mixed in five different combinations for a volume of 2 ml for each combination.
- Equal proportions of component A and component B (6 µl) were mixed in 2 ml of filter sterile water and 100 µl of the stain mixture was added into a 96 well plate and mixed thoroughly with the bacterial cell suspension (100 µl).

- The plate was incubated at room temperature for 15 min and read at the excitation/emission wavelengths of 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide and the ratio of green/red intensity was recorded.

Primer sequences for RT-PCR study

Oligo Name	Sequence 5' to 3'	bp
invC-F	TCGGTCGATCGCTGCAA	17
invC-R	CCCTGGTCGCGAAAATATTC	20
invE-F	CGAATGACGCCAGCTGTTC	19
invE-R	TGCGTCAGGCGTCGTAAA	18
invF-F	TGAAAGCCGACACAATGAAAAT	22
invF-R	GCCTGCTCGCAAAAAAGC	18
hilA-F	CTGCAGAAATGGGCGAAAGTA	21
hilA-R	TCGGGAGTTTGCTATTCAAACA	22
hilC-F	GCTGAGGTGGCAGGAAAGC	19
hilC-R	CCTCTTCAGCGGCCAGTTT	19
hilD-F	CGACTTGGCGCTCTCTATGC	20
hilD-R	TCTCTGTGGGTACCGCCATT	20
orgA-F	AGGCAGGGAGCCTTGCTT	18
orgA-R	CCCTGATGCATTGCCAAAA	19
prgK-F	GGGTGGAAATAGCGCAGATG	20
prgK-R	TCAGCTCGCGGAGACGATA	19
sicA-F	GATGAGTCTCTGCGGGCAAA	20
sicA-R	GCTCTGTCTCCGCCGTTTT	19
sicP-F	AGATGATATCTGGTTATTGAACGGTATG	28
sicP-R	CTGCCGCCAGATAGAATCG	19
sipA-F	GTCTTCGCCTCAGGAGAATCA	21
sipA-R	TTGCCGGGCTCTTTCGT	17
sipB-F	GGCGCGCTGCTAACCAT	17
sipB-R	TCGCCCCACCGGTAAAA	17
sipD-F	ACAGAACATCGCGGTACAGATATC	24
sipD-R	GCTGTGCCTGGTGGATTTTAG	21

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