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Phenotypic and Functional Genomics Analyses of Salmonella for Food Safety Applications

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Phenotypic and Functional Genomics Analyses of *Salmonella* for Food Safety Applications

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By

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Abstract

Non-typhoidal *Salmonella* species have been major foodborne zoonotic pathogens causing serious problems in public health and food industry for several decades. Numerous *Salmonella* species have frequently been associated with different food commodities mainly poultry meat, eggs, and their products. This dissertation begins with a literature reviews discussing many aspects of *Salmonella* generally; and subsequently focused on two serotypes, *Salmonella* Enteritidis and *S*. Typhimurium as they are at the top of all other serovars responsible for most illness cases and outbreaks. In addition, some *Salmonella* strains have exhibited their ability to tolerate and survive many food processing treatments. We can divide this dissertation into two major sections. The first section is focused on *S*. Enteritidis. Since this serovar is the most foodborne routinely isolated from eggs and its products, we aimed to compare the ability of some strains in invading ovarian follicles of laying hens as a route of contaminating eggs (**Chapter 2**). As an important step in identifying gene function using high-throughput screening, we developed a mutant library of *S*. Enteritidis strain selected from the previous chapter using transposon mutagenesis (**Chapter 3**). This library will be a source for numerous future research projects to identify essential genes for survival and virulence in *S*. Enteritidis serving as potential targets to develop advance technology in controlling *Salmonella*. In the second section, mutants of *S*. Typhimurium were utilized to better understand its ability to grow in temperatures associated with human and poultry body temperatures in comparison to their wild type strain through phenotype microarray screening. In addition, we tested the same mutants in surviving some heat treatments practically applied in food processing systems (**Chapter 4**). In the last part (**Chapter 5**), a mutant library of *S*. Typhimurium was screened using Tn-seq technique to identify conditionally essential genes for surviving cold temperature on chicken carcass. All

chapters of this dissertation including Literature Reviews (**Chapter 1**) have been written in journal formats to which they have been published or in preparation to be submitted.

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Dedication

This dissertation is dedicated to my loving and caring parents, *Mohammed Dawoud* and *Aljoharah Dawoud*, for all the hard work in raising me in the right way, encouragement and devotion in making me what I am today. Although I am many miles far away from you, you have always been in my heart and my mind. I would also like to dedicate this precious piece of work to my wife, *Ohoud Al-Tolassi* in recognition of all the unwavering supports she has always provided over the years. Furthermore, I would not forget the supports of my kids, *Adwa*, *Mohammad*, and the last member joined the family during the work of this dissertation, *Aljoharah*, I love you my Angels and I promise you to make your life better. Whether I was moving along well or experiencing an unpleasant time, you and your mother were always there for me to keep working hard and accomplish this dissertation. I would like to express my gratitude to my sister, *Athari*, and my brothers, *Sultan* and *Khaled Dawoud* who has strongly encouraged and supported me during my graduate degrees.

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List of Published Papers

Chapter 2 (Poultry Science, 90 (5), 1134-1137, 2011, Impact Factor: 1.516)

Title: *In vitro* Invasion of Laying Hen Ovarian Follicles by *Salmonella* Enteritidis strains.

Chapter 3 (Molecular Biotechnology. 56.11 (2014): 1004-1010, Impact Factor: 2.275)

Title: Improving the Efficiency of Transposon Mutagenesis in *Salmonella* Enteritidis by Overcoming Host-Restriction Barriers.

Chapter 4 (in preparation)

Title: Heat Survival and Phenotype Microarray Profiling of *Salmonella enterica* serovar Typhimurium Mutants.

Chapter 5 (in preparation)

Title: Genome-wide Identification of *Salmonella* Typhimurium Genes Essential for Cold Temperature Survival on a Chicken Carcass Using a Tn-seq Method.

Introduction

Foodborne agents can cause numerous infectious diseases as a result of ingestion of contaminated foods. This contamination can take place at multiple points during all stages of food production and subsequent processing and retail (Crandall et al, 2013; Koo et al, 2012, 2013; Crandall et al, 2011; Finstad et al, 2012; Howard et al, 2012; Hanning et al, 2009; Park et al, 2008). Contamination by foodborne pathogens is considered a major concern to the food industry since they have been estimated to cause approximately 48 million illnesses in the U.S. with 128,000 hospitalizations and over 3,000 deaths, which means that approximately 15% of the total U.S. population will annually experience a foodborne infection (Scallan et al, 2011). Contaminated food and water with various pathogens has been the main vehicle of infection for diarrheal diseases worldwide (Cairncross et al., 2010; Nath et al, 2006; O'Ryan et al, 2005; Podewils et al, 2004; Marino, 2007; Sheth and Dwivedi, 2006; Santosham et al, 2010; Schmidt and Cairncross, 2009; Schmidt et al., 2009; Wilson, 2005; Zwane and Kremer, 2007). Diarrheal diseases are the second most prominent cause of deaths of children under 5 years old accounting for 11% of children deaths (Chopra et al, 2013; Farthing et al., 2013; WHO and UNICEF, 2013).

A historical date that marked the beginning of new era of food Safety in the last 70 years occurred when the FDA **F**ood **S**afety **M**odernization **A**ct (**FSMA**) became a law on January 4th, 2011. This new law provided the U.S. FDA (U.S. Food and Drug Administration) with the right to work with other government and private agencies to develop an innovative comprehensive food safety system to prevent any problems associated with food that can lead to infection of people prior to actual incidences. The rules and standards of prevention have long been a component of both FDA and USDA (U.S. Department of Agriculture) activities, but the new law gave the FDA the support and responsibility to protect the entire national food system from

farm-to-table and also provided oversight of imported foods to the U.S. (Taylor, 2011; Cavallaro et al, 2011; Kheradia and Warriner, 2013).

Chapter 1

Literature Review

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1. Introduction:

Non-typhoid *Salmonella* species are among the prominent foodborne bacterial pathogens that cause human infections and are considered a critical public health problem in the United States and worldwide. According to the Centers of Diseases Control and Prevention through the national collaborative surveillance program, FoodNet, laboratory-confirmed infections in 2012 revealed that *Salmonella* was ranked as one of the top occurring foodborne pathogens. It caused a total of 7800 cases of salmonellosis, 16.42 incidences and 29% hospitalization with an insignificant increase of 3% from previous reported years. The incidences were 11.26 for age group 10 to 19 years, 12.15 for 20 to 64 years, 17.22 for 65 years and over, 19.33 for age group 5 to 9 years, and 63.49 for children under 5 years old. Among *Salmonella* infections, approximately 44% was reported for the top three serotypes, S. Enteritidis with 18%, S. Typhimurium with 13%, and S. Newport with 13% (CDC, 2013).

2. Characterizations of *Salmonella*:

2.1. General concepts

Salmonella is Gram negative, rod-shaped, non spore-forming and facultative anaerobic bacteria. It is widely distributed in numerous environments and found in human and animal gastrointestinal tracts (Foley and Lynne, 2008; Foley et al, 2008, 2011, 2013; Finstad et al, 2012; Hanning et al, 2009; Howard et al, 2012; Lynch et al, 2009; Park et al, 2008; 2013). This microorganism nomenclature has been through several controversial and complex iterations with the classification of salmonellae being based correspondingly on several points of references including geographical places, animal hosts, clinical aspects, antigenic patterns, and biochemical reactions.

2.2 Historical development of salmonellosis

Salmonella infection has been encountered and described frequently since the beginning of the 19th century, but before that typhoid fever disease certainly existed even before the 18th century probably as a disease characterized under a large group referred to as *Hippocratic typhus* with the common symptoms of prolonged fever and partial unconsciousness. Symptoms of the disease reported at that time were only fever without any other visible symptoms. As a result, those diseases were classified under one large group with several subgroups according to the length of fever time as short, long, or continuous. The *Hippocratic typhus* was classified under those characterized fevers and from this classification emerged the confusion of typhoid fever being associated with other diseases (Paul, 1930). Historically, there has been considerable uncertainty linked to the typhoid fever that is caused by *Salmonella* Typhi. The first source of dispute occurred between the two types of infection, namely, typhus and typhoid. Initial observations of typhoid fever were made by Thomas Willis in 1659 (Newsom, 2007; White, 2008). An early attempt to distinguish these two infections was made by John Huxham in 1737 by respectively referring to typhoid fever as a slow nervous fever, and the typhus fever as a putrid malignant fever (Bechah et al, 2008; McCrae, 1907). In 1810, von Hildenbrand distinguished between typhus and typhoid fever as simple regular typhus and irregular typhus. By 1824, Nathan Smith provided a clear and accurate description of Typhous (Typhoid) Fever (Smith, 1914; Smith, 1981). During the 1820s, Pierre Bretonneau with other French physicians made two observations. The first observation originated from those who died from typhoid fever being characterized as swelling, inflamed victims, with Peyers' Patches ulceration and enlarged lymph nodes, and the second occurred in people who recovered from typhoid infection that were subsequently immunized from further typhoid infections (Paterson, 1949; Shulman, 2004). In

1829, Pierre Charles Alexandre Louis, a French physician, was the first to propose the name "Typhoid Fever". Although numerous observations were reported to distinguish between the typhus and typhoid fever, some confusion remained. Two reports made a significant contribution towards differentiating the typhus and typhoid forms of the disease and the confusion associated with these two diseases. In 1837, William Wood Gerhard made a clear distinction between typhus and typhoid fever (Cirillo, 2000; Ober, 1976). By the late 1840s, William Jenner delineated the differences between typhus and typhoid fever based on the distinctive infection symptoms and epidemiology he personally experienced after infection by both diseases (Bechah et al, 2008; Cook, 2001; Seddon, 2004). William Budd made some remarkable findings as his observations on several outbreaks of typhoid fever occurring between 1839 and 1873 were reported in a series of published papers and ultimately his comprehensive work on "Typhoid Fever". The most important observation by Budd was the conclusion that typhoid fever was spread by an unknown agent mostly transmitted through contaminated water thus introducing the concept of a fecal-oral route (Moorhead, 2002; Parry, 2006).

The second primary point of confusion occurred between malaria and typhoid fever by associating them together with the name, "typhomalarial fever". This name was given by the U.S. Army doctor Joseph J. Woodward in 1862 during the American Civil War (Bynum, 2002; Sulaiman, 2006; Cunha and Cunha, 2008; Cunha et al, 2013). This association was made due to the difficulty faced by many physicians at that time to report a clear diagnostic conclusion on the causative agent of both infections. In 1892, William Osler was able to distinguish between typhoid and malaria based on their clinical features without conducting any laboratory tests (Bryan, 1996). Currently, those observations on the symptoms of both diseases are still applicable and practical to modern day physicians (Cunha, 2007; Cunha and Cunha, 2008;

Pradhan, 2011). One of the main clinical signs was the acute fever that Osler noted clearly exhibited a differential pattern between the two diseases. In malaria, fever becomes elevated rapidly and remains high. However, the fever gradually rises through the early few days post-typhoid infection up to the second or third week (Cunha, 2004, 2005; Uneke, 2008). It was previously described by Jhaveri and colleagues (1995) that the Widal reaction may give misleading results as false positive with cross-reactions occurring between typhoidal, non-typhodial *Salmonella*, and malaria (Jhaveri et al, 1995; Shanthi et al, 2012). The Widal reaction is a specific agglutination test that has been used in the diagnosis of typhoid bacilli and afterward for other *Salmonella* strains based on a reference strain mixed with a titrated serum of the patient.

2.3 Isolation and characterization of *Salmonella*

The first reported observation of this bacillus was in 1880 by Karl (Carl) Joseph Eberth from specimens of typhoid victims. This bacterium was called *Eberthella typhosa*, the typhoid bacillus and is now known as *Salmonella* Typhi. By the mid 1880s, Georg Gaffky was the first to isolate this typhoid bacilli bacterium, and a year later, Theobald Smith who worked under Daniel E. Salmon's supervision, isolated *Salmonella choleraesuis* (now known as the species, *Salmonella enterica*) from pigs and named it "the hog-cholera" which was originally thought to be the cause of swine fever, but later proved to be a secondary infection agent (Collins and Petts, 2011).

In 1888, Gäertner isolated *Bacterium enteritidis*, known now as *Salmonella* Enteritidis, and this was considered the first confirmed laboratory case connecting human salmonellosis outbreak to a consumed food. In 1896, four studies reached the same conclusion from two

different sources. A study by Pfeiffer and Koller (Bazin, 2011; Gröschel and Hornick, 1981) and another study by Gruber and Durham (Wright and Semple, 1897; Wilson, 1909; Bensted, 1951; Sansonetti, 2011) recognized that serological responses to typhoid bacillus could be demonstrated when using the serum from immunized animals by typhoid bacillus. Likewise, a study by G. F. Widal, a French physician, and another one by Grünbaum & Durham reached the same conclusion that typhoid bacillus could be agglutinated by the typhoid patient serum (Fison, 1897; Waller, 2002; Chart et al, 2007; Shanthi et al, 2012 ; Tan and Linskey, 2012). Widal called this test the "sero-diagnosis" test, and subsequently used this approach on samples isolated from patients with typhoid symptoms (Gupta and Rao, 1981). Although some results were negative, these were later identified as a new species named *Bacille paratyphique*, now known as *Salmonella* Paratyphoid. In 1897, Smith and Stewart came to the conclusion that all previously identified organisms could be classified under one large group since they shared numerous morphological and biochemical relatedness (Hornick, 1974).

In 1900, Joseph Léon Marcel Lignieres, a French bacteriologist, proposed the genus name, *Salmonella*, named after D. E. Salmon for this bacterial group which had become generally acknowledged by 1933. In 1925, a new classification was initiated by P. B. White using serological diagnostics method through the (H) antigen. By 1930, Kauffman set up the basis for the existing serological analysis now known as Kauffman-White classification system using (O- somatic or lipopolysaccharide) and (H-flagellar) antigens. A few years later, 44 serotypes were listed in the first publication using the serotyping Kauffman-White Scheme. F. Kauffman had been leading the *Salmonella* International Centre for three decades and by 1964, a total of 958 serovars were identified. In 1965, L. Le Minor took over responsibility of supervising the *Salmonella* Centre until retiring in 1989. When he left the office, there were 2267

serovars of *Salmonella*. Grimont and Weill in a World Health Organization publication "Antigenic Formulae of *Salmonella* Serovars" proposed renaming the serological scheme that had historically been known as Kauffman-White Scheme to the White-Kauffman-Le Minor Scheme in honor of L. Le Minor efforts to characterize the numerous *Salmonella* serotypes (Grimont and Weill, 2007; Guibourdenche et al, 2010; Wattiau et al, 2011).

In the early 1960s, the identified serotypes were considered as species and were commonly given the names regarding their isolated geographical sites such as *Salmonella panama* and *Salmonella london*. Because of the confusion caused by this classification and lack of differentiation of the serovars with biochemical analysis, many proposals had been suggested to resolve this matter. In 1944, Borman and colleagues proposed three species, *Salmonella choleraesuis, Salmonella typhosa,* and *Salmonella kauffmannii* as newly proposed species names. In 1952, F. Kauffmann and P. R. Edwards proposed having three species as well, *Salmonella choleraesuis, Salmonella typhosa,* and *Salmonella enterica*. Ten years later, W. H. Ewing suggested *Salmonella choleraesuis, Salmonella typhi*, and *Salmonella enteritidis*. All proposed species belonged to one species excluding *Salmonella choleraesuis, Salmonella typhi* (previously *S. typhos*a) (Agbaje et al, 2011; Evangelopoulou et al, 2010; Ewing, 1972; Su and Chiu, 2007). In 1966, Kauffmann divided *Salmonella* to four subgenera recognized by Roman numbers (I-IV) which were considered later as species. These subgenera were *Salmonella kauffmannii* (I), *S. salamae* (II), *S. arizonae* (III), and *S. houtenae* (IV) (Boyd et al, 1996; Brenner et al, 2000; Le Minor and Popoff, 1987). A significant and historical publication for this bacterium nomenclature was generated by Skerman et al in 1980 for the Approved Lists of Bacterial Names that included five *Salmonella* species: *S. arizonae, S. choleraesuis, S. enteritidis, S. typhi*, and *S. typhimurium* (Euzéby, 1999; Ezaki et al, 2000).

Currently, the genus *Salmonella* are grouped in the Gammaproteobacteria class under Enterobacteriaceae family. *Salmonella* consists of two species, *S. enterica* and *S. bongori*, as developed in 1987 by Le Minor and Popoff and proposed by Reeves et al at late 1980s (Tindall et al, 2005; Su and Chiu, 2007). *Salmonella enterica* has been further divided into six subspecies, *enterica* (subspecies I), *salamae* (subspecies II), *arizonae* (subspecies IIIa) *diarizonae* (subspecies IIIb), *houtenae* (subspecies IV), and *indica* (subspecies VI). These two species are now divided into more than 2,575 serotypes (serovars) using serological methods introduced by White and Kauffmann in the late 1920s with classification now depending on the cell-antigens, mainly lipopolysaccharide (O antigens), flagella (H) that were discovered in 1918 by Weil and Felix and less frequently the capsular antigens (Vi) (Grimont and Weill, 2007; Guibourdenche et al, 2010; Hardy, 2015; Mølbak et al, 2006). The Vi antigen is uniquely produced by *Salmonella* Typhi, Paratyphi C, and some strains of *S*. Dublin. In 2004, a new species was proposed, *S. subterranean* (Shelobolina et al, 2004). With all the controversy and complexity associated with *Salmonella* nomenclature over the years, this proposed new species name was announced to be effectively valid, but not for taxonomic purposes (Euzéby, 2005); however in 2010, it was confirmed that this species is primarily correlated to *Escherichia hermannii* (Canals et al., 2011; Parte, 2014; http://www.bacterio.net/salmonella.html#subterranea).

2.4 *Salmonella* Bacteriophage and Further Classification

Some serotypes have been divided into Phage Types (PTs) in their connection to specific bacteriophages (Baggesen et al, 2010). Furthermore, they are placed into serogroups based on their similarities of one or more of the somatic (O antigens), and most of these serotypes belong to the species *S. enterica*. *Salmonella* serovars were first considered as species and were given

their name either according to the diseases, the animals from which they were isolated, or in the case of a few with the person who isolated them or the place where they were initially isolated from (Su and Chiu, 2007).

During the first half of the 20th century, *Salmonella* species, *S.* Typhimurium received considerable attention compared to other *Salmonella* species both generally as well as for genetic-based studies. To further distinguish this species, phage typing was introduced by Lilleengen in 1948 to characterize as many as 24 isolated strains. This Lilleengen typing (LT) was given in recognition of the most studied Typhimurium serovar namely, Typhimurium LT2 (Stanley et al, 1993). In late 1950s, another phage typing system was reported by B. R. Callow after developing the original system by Felix and Callow to differentiate 34 typing phages followed by Anderson efforts to discriminate 195 phage types and eventually at least 300 definitive phage types have been distinguished using this system (Ghilardi et al, 2006; Rabsch et al, 2002). For additional differentiation based on phage typing, biotyping can be performed that depends on the fermentative characteristics originally applied by Edwards as well as tests developed by Kristensen and others along with improvements made by Hansen and Harhoff to recognize 21 biotypes (Duguid et al, 1975). In the early 1970s, Alfredsson et al (1972) made a modification in the Kristensen fermentation test that improved its result. This approach was further improved by Duguid et al (1975) to contain 32 primary biotyping and 144 sub-typing thus achieving full bio-typing (Duguid et al, 1975; Pang et al, 2012; Platt et al., 1987; Rabsch, 2007, Rabsch et al, 2011).

3. *Salmonella* spp. and Strain Epidemiology

3.1 Historical perspectives

Salmonella became first known when it was discovered in the late 1870s and the species now known as *S.* Typhi was identified as the cause of the enteric fever transmitted by water and milk. Foodborne salmonellosis was linked to the consumption of beef meat in the late 1880s by Gärtner who isolated this bacillus. In the early second half of 20th century, *Salmonella* Agona appeared in the association with Latin American fishmeal formulated as an ingredient in poultry feeds (Clark et al, 1973, Fleming et al, 2000; Wright et al, 2011) and in the mid 1970s the appearance of *Salmonella* Hadar in turkey flocks and subsequently in chicken broiler flocks was reported (Rowe et al, 1980; O'Brien, 2013). *Salmonella* Enteritidis has been a major problem with high frequency of infections associated with eggs for over 3 decades (St. Louis et al, 1988; Threlfall et al., 2014; Velge et al., 2005). It has been suggested that the appearance of *S.* Enteritidis in chicken flocks by 1960s was a direct consequence of the control measures taken toward eradicating *S.* Gallinarum and *S.* Pullorum (Bäumler et al, 2000; Hitchener, 2004).

Salmonella Typhimurium and *Salmonella* Enteritidis are the most common serotypes of *Salmonella* that cause foodborne illness in humans worldwide as well as in the U.S. and are typically associated with the consumption of poultry, beef, lamb, seafood, vegetables, fruits and their food products (Brands et al., 2005; Davies et al , 2004; de Freitas et al, 2010, Heinitz et al, 2000, Rajashekara et al, 2000, Martinez-Urtaza et al, 2004, Heaton and Jones, 2008; Perelle et al, 2004; Mor-Mur and Yuste, 2010; St. Louis et al, 1988; White et al, 2001; Lynch et al, 2009; Hanning et al , 2009). They cause gastroenteritis in humans and a typhoid-like disease in mice in contrast to symptoms attributed to *Salmonella* Typhi infections. It is not difficult to minimize *Salmonella* infections by hygiene practices and strategies (Cox et al, 2011). However, the rapid

emergence of antibiotic-resistance of some *Salmonella* strains has complicated the treatment of their infection (Chen et al, 2004; van den Bogaard and Stobberingh, 2000; Fey et al, 2000; Randall et al, 2004; Threlfall, 2002; Threlfall et al., 2000; Velge et al, 2005; White et al, 2001).

Non-typhodial *Salmonella* (NTS) species are responsible for human gastroenteritis and bacteraemia cases with annual estimated global illnesses of 1.3 billion and 3 million deaths. Also, *Salmonella* Typhi causes typhoid fever with an estimated 21.6 million and 200,000 deaths occurring annually (Smith et al, 2011). This serovar is mostly transmitted through the oral route from contaminated food or water with people feces or urine. An annual average of 9.4 million incidents was caused by 31 major foodborne pathogens. Non-typhoid *Salmonella* species were responsible for 11% (1, 027,561 cases) of those incidents as the second main pathogenic agent (Scallan et al, 2011). In the U.S., *Salmonella* as a predominant bacterial agent was responsible for 44% of laboratory confirmed bacterial foodborne infections in 2009. A more recent report by CDC in 2014 revealed that the incidence of laboratory confirmed non-typhodial *Salmonella* infections in the U.S. for 2013 decreased by 9% in comparison to the period 2010 to 2012 and exhibited no changes when compared to the period of 2006 to 2008. In both comparisons, the incidence rate of 15.19 in 2013 is still above the national health objectives of 11.4 cases per 100,000 persons for 2020 (Crim et al, 2014).

3.2 *Salmonella* Enteritidis as a Rodenticide

Salmonella infections have knowingly been a primary public health concern for the past few centuries as "Typhoid fever" even before identifying that *Salmonella* Typhi specifically was the cause of the infection. In the 19th century, an outbreak of *Salmonella* Typhimurium was connected to meat contamination as a result of using strains of this serotype as well as

Salmonella Enteritidis as rodenticides that turned out also to be associated with illness and death among humans. Consequently, the incorporation of this serotype in rodenticides was terminated by the beginning of the last century. However, *Salmonella enterica* serotype Enteritidis was still being used in rodenticides by some European countries until the late 1950s. It was not until the mid 1960s that the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) suggested ending the use of *Salmonella*-based rodenticides since it was considered a public health hazard (MacNicoll, 2007; Painter et al, 2004). Despite this mandate, many Latin-American and Asian countries still make use of a rodenticide product, which employs a strain of *S.* Enteritidis claimed to infect only animals versus *S.* Enteritidis strains generally known to infect humans with life-threatening diseases (Friedman et al,1996; Mendoza et al., 2010; Painter et al, 2004; Velge et al, 2005). However, *Salmonella* infections among rodents, mice and rats, remain a clinical and environmental concern since they serve as reservoir hosts on farms and food processing plants. Rodents can transmit *Salmonella* by contaminating the feed, water, food or the environment surrounding them which in turn can lead to human infections (Endepols, 2002; Meerburg and Kijlstra, 2007; Meerburg et al, 2009).

3.3 Current Epidemiology Issues

The infections of *Salmonella* cost the U.S. economy annually an estimated average of 11.39 billion U.S. dollars exceeding the annual cost of other bacterial foodborne infections making non-typhoidal *Salmonella* the costliest foodborne pathogen in terms of health outcome losses with approximately 3.7 billion dollars (Batz et al., 2014; ERS-USDA, 2014; Hoffmann and Anekwe, 2013; Scharff, 2012; Byrd-Bredbenner et al, 2013). In 2013, a 9% decrease of *Salmonella* infection incidences was reported in comparison to the period of 2010 to 2012 of

other bacterial foodborne pathogens; of those 90% were serotyped isolates. Among them were *S.* Enteritidis with 19%, *S.* Typhimurium with 14% and *S.* Newport with 10%. Recently, a study reported that between 1998 and 2008 approximately 34% of all single serotype *Salmonella* outbreaks were linked to a particular food, of those 66% were caused by the top four serotypes, *S.* Enteritidis with 36%, *S.* Typhimurium with 14%, *S.* Newport with 10%, and *S.* Heidelberg with 6%. Poultry and poultry products have accounted for 54% of *Salmonella* outbreaks connected to a single identified product with eggs, chickens, turkeys as the most frequent foods of origin (Gould et al, 2013; Jackson et al, 2013; Painter et al, 2013) and responsible for the majority of deaths along with *Listeria*. In addition, outbreaks of *Salmonella* in association with fresh produce are approximately 50% (Strawn et al., 2014).

A program initiated by WHO in 2000 as a part of the global Salmonella surveillance (Salm-Surv) system "**E**xternal **Q**uality **A**ssurance **S**ystem" (EQAS) for *Salmonella* species serotyping is considered the largest surveillance system worldwide and is operated through a web-based self evaluating system. To enhance and facilitate the capability of the central laboratories in many countries to serotype *Salmonella* species with high accuracy, WHO conducted an EQAS *Salmonella* serotyping annual comparative series of studies between 2000 and 2007 with 249 participating laboratories from 97 nations using serological titration. Participating laboratories average at all processes was 76% with the accuracy of detecting all serotypes at 82 and 94% for *Salmonella* Enteritidis. The goal of reaching high accuracy for reporting *Salmonella* serotypes increased by about 20% from 2000 to 2007 although the incorrect reporting of some serotypes was observed during the same period with lowest percentage of 3.6% in 2007 and the highest reported in 2006 of 41% (Chaitram et al, 2003; Petersen et al, 2002; Hendriksen et al, 2009a).

The sensitivity of identifying *Salmonella* serovars using a phase two flagellar antigen (H-antigen) detection approach accounted for the high percentage error when reporting serotypes results mostly due to the loss of the phase two flagellar antigen (Hopkins et al, 2010). More importantly, the occurrence of laboratory errors was influenced by several issues. Approximately 30% of the participating laboratories lacked high quality antigenic detection materials, which caused difficulties of detecting uncommon serotypes in 26% of the overall laboratories as false positives or false negatives (Galanis et al, 2006; Hendriksen et al, 2009a). These two factors are more likely to occur in regions of Africa, Central Asia, the Middle East, Russia, and the Caribbean. Problems with identifying the correct serotype can in turn lead to major delays in tracing back the main source of numerous *Salmonella* infections (Galanis et al, 2006; Hendriksen et al, 2009a).

The WHO Global Salm-Surv (GSS) system had been focusing solely on the surveillance of *Salmonella* species and recently had been expanded to include food-borne and other enteric pathogens. This system is now known as **G**lobal **F**ood-borne Infections **N**etwork (**GFN**). This system has two subsystems, EQAS and **C**ountry **D**ata**b**ank (**CDB**), the first system was established to assess the quality of *Salmonella* serotyping and currently is employed for foodborne pathogens serotyping and antimicrobial susceptibility worldwide. The **CDB** has been established to report the data annually for the 15 main frequently identified *Salmonella* serotypes as a global passive surveillance system from members of national laboratories (Hendriksen et al, 2009b; 2011).

A study by Hendriksen and others (2011) reported the global distribution of the 15 major consistent *Salmonella* serotypes identified from human clinical specimens as a part of the WHO GFN system between 2001 and 2007. Two specific serovars out of all the serovars that had been

reported were predominant in most regions, *Salmonella* Enteritidis ranked first and *Salmonella* Typhimurium as second. This pattern of predomination is in contrast to the regions of North America and Oceania (Australia, and New Zealand). These reported serotypes were isolated from both human and non-human (animals, food, feed, and environment) sources (Hendriksen et al, 2011; Vieira et al, 2009). The data were collected from the Country Databank (CDB) uploaded by Public Health Laboratories of 37 countries within 6 geographical regions (18 sub-regions). The European region was divided to 5 sub-regions, Northern Europe, Western Europe, Central Europe, Southern Europe, and Eastern Europe. *Salmonella* Enteritidis was the most common serovar isolated from human in all sub-regions ranging from 56 to 89.9% followed by *S.* Typhimurium ranging from 5.4 to 21.6%. For non-human sources, *S.* Enteritidis was the most common isolated serovar in Central Europe, Southern Europe, and Eastern Europe ranging from 30.9 to 49.6% followed by *S.* Typhimurium in Central Europe with 27.7% and Eastern Europe with 8.1%. Other serovars isolated from non-human sources that ranked as second were *S.* Gallinarum in Southern Europe with 13.8% and *S.* Infantis in Northern Europe with 9.8%. The data for non-human sources was not reported from Western Europe. In North America, the most frequent isolated serovar from both human and non-human sources was *S.* Typhimurium with 29.4% and 23.1% respectively, followed by *S.* Enteritidis isolated from humans with 23.6% and *S.* Heidelberg originating from non-human sources at 14%. In recent years, the U.S. most frequently reported *Salmonella* serovars from human infections reported to CDC (2012) are *S.* Enteritidis followed by *S.* Typhimurium. Also, the most frequent serovars from analyzed samples by FSIS-USDA (2013) for broilers' young chicken are *S.* Kentucky and *S.* Enteritidis. Latin America was partitioned into 3 sub-regions, Central America, South America, and the Caribbean. The most frequently isolated serovars from humans were *S.* Typhimurium with 31.5 and 24.4%

followed by *S.* Enteritidis with 30.5 and 17.1% in Central America and the Caribbean respectively. In Central America, *Salmonella* II 1,4,12,27 with 25.3% and *S.* Heidelberg with 16.8% were the dominant isolated serotypes. While in the Caribbean, *S.* Kentucky and *S.* Typhimurium with 42.7 and 9.8% were the leading serovars respectively. In South America, from human and non-human sources, *S.* Enteritidis occurred at 48.3 and 34.1% and *S.* Typhimurium at 18 and 9.8% correspondingly.

The African continental region was divided to 2 sub-regions, North Africa and Sub-Saharan Africa. *Salmonella* Enteritidis was the major serovar isolated from humans with 30% occurring in North Africa, 28.3% in Sub-Saharan Africa and 32.9% from non-human sources in North Africa. *Salmonella* Typhi was the second most frequently isolated serovar from humans in Sub-Saharan Africa. *S.* Kentucky with 13% was the most frequently isolated serovar from non-humans in Sub-Saharan Africa. Rare serovars have also been isolated and reported as the second main serovar, *S.* Livingstone from humans with a frequency of 15.6%, *S.* Anatum with 17.9% from non-human sources in North Africa, and *S.* Bredeney with 8.5% from non-human sources in Sub-Saharan Africa. In the Middle East, East Asia, Central Asia, South and Southwest Asia, *S.* Enteritidis was the predominant serovar isolated from humans, while in Australia and New Zealand, *S.* Typhimurium was leading with 59.3% of all isolated serovars. The data was collected from participating countries using the WHO GFN system. Eighty three countries reported data with 1.5 million human, and 360,000 of non-human *Salmonella* isolations and 307 distinct serovars. The general conclusion based on the reported data is that *S.* Enteritidis and *S.* Typhimurium accounted for 78.8% of human isolates and 37.9% of non-human isolates (Hendriksen et al, 2011; Vieira et al, 2009). In the period from 2009 to 2010, *S.* Enteritidis accounted for 34% of all *Salmonella* serotype reported outbreaks with a total of 39 outbreaks in

2009 and 37 outbreaks in 2010 (CDC, 2013). According to the U.S., FoodNet surveillance system, *S*. Enteritidis has leaded other serovars with laboratory-confirmed *Salmonella* infections for the period from 2007 to 2012 followed by *S*. Typhimurium. *S*. Enteritidis and *S*. Typhimurium accounted for approximately 16% and 12% respectively in 2012 (CDC, 2014). In 2013, *S*. Enteritidis was accountable for 19% of *Salmonella* cases and 14% for *S*. Typhimurium. In comparison to the period of 2010 to 2012, *Salmonella* incidences for some serotype were substantially very low with a 14% decrease for *S*. Enteritidis and an insignificant change occurring for *S*. Typhimurium (Crim et al., 2014).

3.4 *Salmonella* Etiology

Salmonella infection in general develops into one of the following; the systematic infection referred to as enteric fever, an intestinal infection such as gastroenteritis, or becomes a blood infection in humans referred to as bacteremia. The process of the salmonellosis starts with the intake of *Salmonella* cells that must resist the acidity of stomach with a pH range from 1 to 2 during digestion (Foster and Spector, 1995; Smith, 2003; Bearson et al, 2006; Álvarez-Ordóñez et al, 2009, 2011) and subsequently leads to colonization in the small intestine with the possibility of causing systemic infection. Many factors play roles in susceptibility to *Salmonella* infections such as age, health conditions (immuno-compromised patients), and other circumstances. The transmission of *Salmonella* species is usually through a fecal to oral route with the consumption of contaminated food or water with feces and/or urine from infected humans or animals (Chen et al, 2013; Monack, 2012; Raffatellu et al, 2008; Sterzenbach et al, 2013; Tsolis et al 2011).

4. *Salmonella*: Poultry- and Poultry Products-Associated Pathogens

The sources of *Salmonella* infection are relatively wide in range, but one of the primary sources is poultry and poultry products. The association of poultry and poultry products has long been documented (Buncic and Sofos, 2012; Chittick et al, 2006; Currie et al, 2005; Foley et al, 2011; 2013; Finstad et al, 2012; Liljebjelke et al, 2005; Singh et al, 2010; Smith et al, 2008; Vandeplas et al, 2010; Yildirim et al, 2011). Throughout the last decade, approximately 80% of all *Salmonella* diseases outbreaks were linked to a specific *Salmonella* serovar; of those 34% (403 outbreaks) had been traced to a particular food product. Of those outbreaks, *S.* Enteritidis caused 35.7% with 65% were linked to egg-associated outbreaks and 13% to chicken-associated outbreaks. *S.* Typhimurium was associated with 14.4% of all outbreaks, 26% of those were chicken related infections and only 7% was connected to egg-borne outbreaks. Another serotype, *S.* Newport, was responsible for 10% of all outbreaks caused by a single food product. This serotype had a similar percentage, 13%, to *S.* Enteritidis in association with chicken outbreaks which did not involve egg association outbreaks. *S.* Heidelberg was associated with a particular food product with roughly 6%, among them, 42% outbreaks associated with eggs and 33% with chicken (Jackson et al, 2013). These serotypes have been the primary serovars isolated from human and other non-human sources (Vieira et al, 2009; Painter et al, 2013). During the last decade, the food products and processing regulated by USDA and FDA that have been linked mostly with *Salmonella* infections were poultry and poultry products, with 458 outbreaks for meat and 125 outbreaks were connected to eggs and its related dishes (DeWaal and Glassman, 2013).

4.1 Poultry Meat Products

Globally, sources of meat such broiler chicken and other poultry as well as turkey and eggs are among the most highly consumed foods (Harmon, 2013; Kearney, 2010; Rask and Rask, 2011). The average U.S. consumption of chicken per capita is approximately 86 pounds annually (MacDonald, 2008) and by 2010 it surpassed beef (Bentley, 2012). The U.S. is one of the largest producers of poultry and its products primarily include chickens, turkey meats, and eggs (Harvey, 2012). The production of broiler meat by the end of November 2013 was to be 34.7 billion pounds (Mathews, 2014a). The U.S. broiler shipments in November 2013 were 639.6 million pounds, and the amount held in domestic cold storage was approximately 700 million pounds by the end of 2013 with an increase of 7.5% from the same period of 2012 and a major increase (87%) of whole birds stocks of 22 million pounds by the end of November 2013 (Mathews, 2014a). In 2014, broiler meat production up to the end of October was totaled to be 32.3 billion pounds with an estimate broiler meat production for the fourth quarter of 2014 of 9.75 billion pounds and totaled of 38.5 billion pounds for 2014 (Mathews and Haley, 2014).

Turkey meat is the second most prevalent avian meat product in the U.S. with 1.47 billion pounds by the end of the third quarter of 2014 (Mathews, 2014b). A total of 390 million pounds of turkey meat products were in cold storage holdings with whole bird stocks totaling 112 million pounds by the end of October 2014 (Mathews and Haley, 2014). The U.S. turkey shipments are expected to be 807 million pounds by the end of 2014 and 820 million pounds for 2015 (Mathews, 2014b). The U.S. per capita consumption of table eggs was approximately 255.1 eggs in 2013 and is expected to be 261.1 eggs by the end of 2014 and 264.6 in 2015 (Mathews, 2014b). The U.S. table egg production by the end of October 2014 was a total of 70.8 billion eggs and is expected to be totaled 22.08 billion eggs for the last quarter of 2014 (Mathews and

Haley, 2014). It is expected the total production for the year of 2015 to be 87.24 billion and the egg and egg product shipments totaled of 4.3 billion by the end of 2014 (Mathews, 2014b).

In European Union countries, 26.3 billion pounds of poultry meat were produced in 2011; of those approximately 80.7% consisted of broiler chicken meat and 15.7% turkey meat (AVEC, 2012). In 2012, the production of poultry meat increased by 259.4 million pounds. Moreover, broiler chicken meat per capita consumption was approximately 38.8 pounds in 2011 and 7.5 pounds per person of turkey meat was consumed. The total consumption of poultry meat was 25.9 billion pounds with 51.6 pounds of poultry meat consumed per capita in 2011. Approximately 135.5 million additional pounds of poultry meat were consumed in 2012. Furthermore, world production of poultry meat was approximately 225.31 billion pounds and the total poultry meats consumed was 225.25 billion pounds (AVEC, 2012). In 2014, the U.S is accounted for 20% of the world broiler meat production and 17% the world broiler meat consumption. In the condition of turkey meat, the U.S. is accounted for the world production and consumption with 49% and 45% respectively in 2014 (AVEC, 2014). This high quantity of demand and production will continue to require advanced food safety standards since several foodborne pathogens such as *Salmonella* and *Campylobacter* can contaminate poultry meat and eggs at any stage of processing and/or storage of food products (Carrasco et al, 2012; Luber, 2009; Mor-Mur and Yuste, 2010; Newell et al, 2010; Park et al, 2008; Todd et al, 2010; Zhu et al, 2005).

4.2 *Salmonella* association with poultry and its products

Most common non-typhoidal *Salmonella* (NTS) isolated from human are also associated with poultry mainly chicken, turkey, their meat products, eggs and eggs products (EFSA, 2010; de

Freitas Neto et al, 2010; Hoelzer et al, 2011; Sandt et al, 2013). According to a study by CSPI (2013) for foodborne outbreaks caused solely by meat, poultry, or their products in the US between 1998 and 2010, chicken is in the top category of the risk pyramid with 452 outbreaks and the recalling of 127 million pounds of chicken and its corresponding products. Turkey was the second highest category with 130 outbreaks and 33 million pounds of turkey meat being recalled. In general, *Salmonella* spp were associated with animal products, meat and poultry as one of two pathogens responsible for 30% of infections and 27.2% of hospitalization cases (DeWaal and Glassman, 2013; CSPI, 2013). Another report for the top ten riskiest foods regulated by FDA, eggs and egg-related products were ranked the second highest food source with 352 outbreaks and 11,163 cases of infection (Klein et al., 2009). In European countries, approximately 1426 reported outbreaks were foodborne enteric diseases; of those 20% had been associated with the poultry consumption (Kessel et al, 2001). Chickens were linked in approximately 75% of the outbreaks whereas turkey accounted for about 20%. The prevalence of *Salmonella* spp in those outbreaks was approximately 30%.

The association of *Salmonella* with fowl species has a long history of documentation. In the late nineteenth century, the two avian-adapted *Salmonella enterica* subspecies *enterica* serotypes Gallinarum and Pullorum were first identified as being responsible for fowl typhoid and pullorum disease respectively. During the early last century, these two serovars were widespread in the poultry flocks of the United States and Europe, mainly chickens and turkeys (Bullis, 1977; Rabsch, et al., 2000; Shivaprasad, 2000; Barrow and Neto, 2011). Because of the increased mortality associated with poultry, and the significant economic losses from these serovars, monitoring programs were initiated to control and eradicate these diseases (Bullis, 1977; Bäumler et al, 2000; Hitchner, 2004; Shivaprasad, 2000). In the U. S., two serovars were

prevalent causing pullorum disease and fowl typhoid by *Salmonella* Pullorum and *S*. Gallinarum respectively, and the diseases identified with these species were controlled and eventually eradicated by the mid 1960s (Bäumler et al, 2000; Barrow and Neto 2011, Shivaprasad, 2000). The eradication of *S*. Gallinarum and *S*. Pullorum was followed by the emergence of *S*. Enteritidis in chicken and becoming the most likely culprit with eggs between early 1980s and mid 1990s with the highest reported year, 1994, due to an ice cream outbreak in the U.S. (Braden, 2006; Olsen et al, 2000, 2001). This serovar declined in chicken, but remained linked to chicken eggs. Another serovar, *S*. Heidelberg has become more predominant since 1997 with highest percentage reported at the beginning of last decade comprising over 50% of all *Salmonella* isolations from chickens (Foley et al, 2008). The increase of *S*. Heidelberg in chickens has been possibly connected to the measurements taken to control and reduce *S*. Enteritidis by the National Poultry Improvement Plan (NPIP)-USDA (Foley et al, 2008; 2011). In addition, several studies have shown the capability of *S*. Heidelberg to infect the reproductive tract of laying hen and penetrate into the internal contents of eggs (Gast et al, 2004; Gast et al, 2007; Okamura et al, 2001).

4.3 *Salmonella* Infection and Poultry

As *Salmonella* Gallinarum and Pullorum declined in prevalence, a new serotype, *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S*. Enteritidis), took over and became the most chicken egg associated pathogen (Bäumler et al., 2000; Rabsch et al., 2000). By late 1970s, *S*. Enteritidis started to receive more attention as an important food safety concern and by the mid 1990s it was the most frequent *Salmonella* serovar accounted for cases reported in the United States (CDC, 2000; Olsen et al, 2000, 2001; Rabsch et al., 2001). Vertical transmission from

parent to offspring via infected hatching eggs is the primary transmission modes in birds (Lutful Kabir, 2010), and horizontal transmission can possibly occur via bird-to-bird contact as a result of shedding *Salmonella* in feces by infected birds and inhalation by birds in close proximity. Another route is ingesting *Salmonella* -contaminated materials such as feed and/or contact with other environmental surfaces (Byrd et al, 1998; Liljebjelke et al, 2005; Thakur et al, 2013).

Humans become ill with salmonellosis by consuming raw or lightly cooked eggs (Braden, 2006; Hope et al, 2002; Kimura et al, 2004; Patrick et al, 2004; St. Louis et al, 1988). Between the mid and late 1980s, 82% of the reported human infections of *S*. Enteritidis in the United States were linked to chicken egg consumption (Dhillon et al, 2001; Seo et al, 2003; Kimura et al, 2004).

Salmonella Enteritidis represents a major threat to the poultry industry because not only does it contaminate table eggs, but it also transmits vertically from infected breeder flocks. With broiler breeders, this can lead to infection of broilers and the contamination of carcasses (Rasschaert et al, 2007; Van Immerseel et al., 2009). The antigenic attributes and pathogenesis characteristics of *S*. Enteritidis may in part play a role in its increased frequencies and persistence since they share similarities with the restricted avian-host pathogens, *S*. Gallinarum and *S*. Pullorum (Foley et al., 2011; Thomson et al., 2008; Uzzau et al., 2000). In some experimental studies, laying hens showed significant reduction of egg production after *S*. Enteritidis infection even though *S*. Enteritidis does not cause severe pathological infections in laying hen flocks or any visible illness (Gast et al, 2002). Because *S*. Enteritidis has multiple reservoirs and vectors including mice and other rodents' populations, minimizing its infections in hen flocks requires a complex effort (Garber et al, 2003; Mollenhorst et al, 2005; Carrique-Mas and Davies, 2008; Carrique-Mas et al, 2009).

The incidence of *S.* Enteritidis infected hens are influenced by two critical aspects, the microbial traits and the management systems in the US commercial eggs industries. Cyclic timely collection of contaminated eggs produced by laying hens may be related to acquired-stress from specific procedures such as molting (Holt, 2003). Historically, induced molting using feed withdrawal was the primary method used in the layer industry to stimulate numerous egg-laying cycles in hens (Dunkley et al, 2009; Golden et al, 2008; Mazzuco et al, 2011; Patwardhan and King, 2011; Ricke, 2003, 2014; Ricke et al, 2013). The stress associated with feed withdrawal unfortunately was identified with an increased susceptibility to *S.* Enteritidis infection, with typical increases in intestinal shedding and spreading of *S.* Enteritidis to internal organs such as the liver, spleen and ovary (Dunkley et al., 2007; Durant et al, 1999; Kubena et al, 2005); also, feed withdrawal increased the invasion susceptibility of tissues such as the crop, ilea, ceca to the invasion of *S.* Enteritidis (Moore and Holt, 2006). A study by Holt (1995) demonstrated that during 48 hours after molting procedures, systematic invasion of *S.* Enteritidis occurred rapidly and they recovered significantly more *S.* Enteritidis from the livers and spleens of molted hens compared to the un-molted ones. This susceptibility has also been shown to lead to increased horizontal distribution of *S.* Enteritidis infection to molted hens in nearby cages and more eggs contaminated with the bacterium (Holt, 1995; Holt et al., 1995, 1998). Moreover, recent studies showed that horizontal transmission can take place between a group of experimentally infected hens and a susceptible group of laying hens in connection to the housing system with a higher risk in systems with continued fecal contact, as alternative housing systems, due to the contact of chicken manure contaminated with *Salmonella* species (De Vylder et al, 2011) although alternative housing systems such as the furnished cages, and birds in the aviaries, exhibited decreased shedding level, the colonization was less frequently in spleen and

substantially less in liver with no differences in the reproductive tissues, ovary and oviduct (De Vylder et al, 2009).

4.3.1. *Salmonella* and egg contamination

The main egg contamination mechanism appears to be due to *S.* Enteritidis invasion of the reproductive tract organs after a disseminated infection (Gantois et al, 2008; Gast et al, 2009; Upadhyaya et al, 2013, 2014). Before eggshell formation and during the formation of eggs, it is possible that formed eggs exposed to either descending infections from *S.* Enteritidis colonized ovarian tissue, and infection from upper-oviduct tissues, or ascending infections of vaginal and cloacal tissues colonization (Keller et al., 1995; Bichler et al, 1996; Miyamoto et al, 1997; Gast et al, 2004).

The colonization of the reproductive tract tissues by *S.* Enteritidis is certainly a primary factor for contamination of egg contents and may be responsible for the persistence of *S.* Enteritidis and other *Salmonella* serovars in mature laying flocks (Barnhart et al., 1991; Berchieri Jr et al., 2001; Davies and Breslin, 2003a, b; Gast et al, 2005; Guard et al, 2010; Van Immerseel, 2010). *S.* Enteritidis can be isolated from reproductive tissues and organs of naturally infected hens without prior intestinal colonization and some strains can be isolated from oviduct tissue and other tissues within an hour of experimentally infected laying hens and crop inoculation (Humphrey et al., 1993; Nassar et al, 1994); also, the isolation rate was drastically higher when extending the incubation time (Humphrey and Whitehead, 1993). The virulence capabilities of *S.* Enteritidis and its connection to potential tissue tropism for reproductive tissues in laying hens have been a major concern that needs to be understood since several phenotypic evidence suggested genetic association (Clavijo et al., 2006; Gantois et al., 2008, 2009; Kang et

al., 2006; Lu et al., 2003; Parker et al., 2002; Raspoet et al., 2011; Shah, 2014; Shah et al., 2012a, b; Van Immerseel, 2010). A *S*. Enteritidis PT4 isolate that displayed wrinkled appearance colonies on plates and more tolerance to heat, acid and hydrogen peroxide stresses was also more invasive to reproductive tissue (Humphrey et al. 1996). Some studies reported that infection with *S*. Enteritidis can induce regression of the reproductive tract (Guard-Bouldin et al, 2004, 2006; Parker et al, 2001, 2002).

The prospective mechanism by which *S.* Enteritidis colonizes the reproductive tissues and causes transovarian infection may definitely raise some questions on what does distinguish this serovar from others to routinely contaminate eggs? What are the genes required for adherence and invasion of reproductive tissues? Although both *S.* Enteritidis and *S.* Typhiumurium are capable of colonizing chicken reproductive tissues only *S.* Enteritidis can be isolated from the contents of intact eggs after being laid and able to survive within the intact chicken eggs (Keller et al., 1995; Lu et al, 2003; Clavijo et al, 2006; Gantois et al, 2008, Shah et al, 2012a, b; De Vylder et al, 2013). This provides evidence that *S.* Enteritidis has distinctive genetic factors compared to other *Salmonella* serovars (Silva et al, 2012; Porwollik et al, 2005).

Another characteristic of *S.* Enteritidis pathogenesis that makes it unique for laying hens is the frequent passage and isolation of *S*. Enteritidis from the ovary and the oviducts dramatically increases its ability to contaminate the eggs internally without any changes in the invasiveness or colonization of the reproductive organs (Gast et al., 2003; Gast et al, 2009). *S.* Enteritidis has specificity for the hen's reproductive tissue in general and can cause transovarian infection (Braden, 2006; Foley et al., 2011; Gantois et al., 2009; Galiş et al., 2013; Howard et al., 2012; Rabsch et al., 2001; Threlfall et al., 2014**).** This serovar is known to colonize and infect the

ovary and *S.* Enteritidis has been commonly isolated from the ovary interstitial tissue surrounding the yolk rather than the follicle yolk (Barrow and Lovell, 1991; Keller et al, 1995).

Chicken ovarian follicles are present at various stages of maturation primarily differing in the yellow yolk quantity. A mature hen will lay eggs when ovarian follicles become fully developed. Follicles maturity goes into three major stages (Howard et al, 2012; Klein-Hessling, 2007). In stage one, follicles grow very slowly and this stage may last for several months with no significant amounts of yolk. These follicles are known as small white follicles. In the second stage, a few follicles will develop more and subsequently achieve yolk deposition. These follicles will appear yellow and are known as small yellow follicles. In the last stage, follicles from small yellow follicles pool are selected and go into a rapid growth stage, leading to the mature follicles within 7 days. These follicles go through the oviduct during ovulation and egg formation will initiate (Recheis et al, 2000). Generally, the immature follicles exist with multiple granulosa cell layers; however, the granulosa cells can be seen in the lone layer of the higher mature large yellow follicles or F follicles (Howard et al, 2012; Lovell et al, 2003). *S.* Enteritidis has displayed attachment to both developing and mature follicular granulosa cells (Thiagarajan et al., 1994).

A study was performed *in vitro* to compare the ability of *Salmonella* in invading ovarian follicles of laying hens at various maturation phases (Howard et al., 2005). By comparing the two most common non-typhodial *Salmonella* serovars, *S.* Typhimurium and S. Enteritidis, they demonstrated that invading intact follicles by *S.* Typhimurium was higher for the immature follicles, small white follicles and were less invasive as follicles developed to mature, small yellow and then large yellow type (Howard et al, 2005). They concluded that *S.* Enteritidis may be possibly use different mechanisms to engage with different follicle patterns while the follicles

developed. The involvement of attachment and invasion mechanisms into *S.* Enteritidis infection of ovarian follicles may require the ability of two separate genetic properties that should be recognized and understood.

5. *Salmonella*: Genetics of Pathogenesis

As with other pathogens, *Salmonella* species require virulence factors that contribute to infection and result in a disease in addition to the characteristics such as adherence and invasion of host tissues (Bäumler et al, 1998; Cox, 1995). Previous studies demonstrated that most genes contribute to *Salmonella* virulence and/ or adaptations are located among genes clusters encoded by ***S****almonella* **P**athogenicity **I**slands (SPIs) that are believed to have occurred mainly via the **h**orizontal **g**ene **t**ransfer (HGT) (Hacker et al, 1997; Ochman et al, 2000; Vernikos et al, 2006). Approximately 21 *Salmonella* Pathogenicity Islands have been discovered (Sabbagh et al, 2010; Wisner et al, 2012) designated SPI-1 to SPI-21 that differentiate between subspecies and serotypes of *Salmonella* in size, genetic composition, function, and dissemination. The Pathogenicity Islands, SPI-1 to SPI-5, are common in the subspecies *Salmonella enterica* serovars (Ochman and Groisman, 1996; Amavisit et al, 2003; Hensel, 2004).

Salmonella Pathogenicity Island 1 (SPI-1) is a 40 kb cassette on the *S.* Typhimurium chromosomal DNA (Mills et al, 1995) and 40.2 kb cassette on the *S.* Enteritidis chromosomal DNA (Thomson et al, 2008). Its function is to initiate the infection into the non-phagocytic cells through the invasion and internalization into the gastrointestinal epithelial cells and play a role in fecal shedding throughout localized enteric infection. The virulence-associated region of SPI-1 contains 41 genes that encode a needle complex by structural genes set (*invACEGHIJ*, *spaOPQRS*, *prgHIJK*, and *orgABC*), translocon genes (*sipBCD*), secreted effector genes (*sipA*,

sptP, and *avrA*), chaperone genes (*invB* and *sicAP*), transcriptional regulator genes (*hilACD*, *invF*, and *sprB*), metal transport system genes, an iron uptake system (*sitABCD*) and an association of uncharacterized genes with unknown function (*iagB*, *SEN2743*, and *SEN 2744*) (Ellermeier and Slauch, 2007; Fàbrega and Vila, 2013; Wisner et al, 2012)

Salmonella Pathogenicity Island 2 (SPI-2) is a 40 kb locus on the *S*. Typhimurium chromosomal DNA (Shea et al, 1996) and 39.8 kb locus on the *S*. Enteritidis chromosomal DNA (Thomson et al, 2008). It functions in intracellular pathogenesis, for *Salmonella* survival, replication, and systemic infection. The SPI-2 contains 44 genes that encode structural genes (*ssaBCDEGHIJKLMNOPQRSTUV*), transcriptional regulatory genes (*ssrAB*), translocon genes (*sseBCD*), chaperons genes (*sscAB*), secreted effectors genes (*sseAEFG*), uncharacterized genes (*SEN1635*, *orf242*, *orf319*, *orf70*,*orf408*, *orf245*, *orf32*, *orf48*), tetrathionate reductase complex genes (*ttrABC*) and its two component regulatory system (*ttrRS*) (Hensel et al, 1999; Hensel, 2000; Hansen-Wester and Hensel, 2001; Fàbrega and Vila, 2013; Wisner et al, 2012).

Salmonella Pathogenicity Island 3 (SPI-3) is a 36 kb cassette on the *S*. Typhimurium chromosomal DNA (Sabbagh et al, 2010) and 16.6 kb cassette on the *S*. Enteritidis chromosomal DNA (Thomson et al, 2008). The function of this pathogenicity island is the survival of *Salmonella* inside the macrophage and the growth in magnesium limited environment such as phagosomes. This SPI contains 13 ORFs with functions not compatible to each other, 10 of which are without identified functions (*3752*, *sugR*, *3754*, *rhuM*, *rmbA*, *fidL*, *3760*, *slsA*, *cigR*, and *yicL*) and 3 that encode genes with magnesium transporter proteins (*mgtBC*), a transcriptional regulator (*marT*), and a dual functional gene (*misL*), a structural gene and as a secreted effectors gene (Marcus et al, 2000; Morgan et al, 2004; Fàbrega and Vila, 2013).

Salmonella Pathogenicity Island 4 (SPI-4) is a 25 kb island on the *S*. Typhimurium and *S*. Enteritidis chromosomal DNA (Wong et al, 1998; Thomson et al, 2008). The main function of this SPI is the adherence to the intestinal cells and subsequent survival while in the macrophage (Wong et al, 1998; van Asten and Dijk, 2005; Schmidt and Hensel, 2004). It encodes 6 genes with secretion structural genes (*siiCDF*), secreted effector gene (*siiE*), and uncharacterized genes (*siiAB*), and it is co-regulated with SPI-1 (Main-Hester et al, 2008; Gerlach et al, 2008).

Salmonella Pathogenicity Island 5 (SPI-5) is approximately a 7 kb locus in *S*. Typhimurium chromosomal DNA (Sabbagh et al, 2010; Schmidt and Hensel, 2004), and a 6.6 kb island in the *S*. Enteritidis chromosomal DNA (Thomson et al, 2008). The function of this SPI is to encode effectors for both SPI-1 and SPI-2 systems. This SPI encodes 8 genes with secreted effectors (*pipABD*, and *sigD*), a chaperone (*sigE*), and unknown functional genes (*orfX*, *1089*, *1093*) (Hong and Miller, 1998; Wood et al, 1998; Darwin and Miller, 2000, 2001; Fàbrega and Vila, 2013). Other *Salmonella* Pathogenicity Islands are more serovar specific and will be referred to accordingly in the text.

The primary functions associated with SPI are the secretion systems and there are three types of secretion systems, type III secretion system (T3SS), type I secretion system (T1SS), and type VI secretion system (T6SS). The main secretion systems are those of SPI-1 and SPI-2, type III secretion systems (T3SS). Since there is two T3SS associated with these pathogenicity islands, they can be distinguished as T3SS-1 and T3SS-2 for SPI-1 and SPI-2 respectively, as each T3SS functions differently. Type III Secretion System acts as a delivery system injecting the secreted effectors directly into the infected host cells. Although many bacteria either pathogenic or non-pathogenic can contain T3SS that is utilized for their reactive specific functions, these secreted systems are phylogenetically related and can be distributed to five

groups. *Salmonella* SPI-1 T3SS belongs to group (4) four, the Inv/Mxi/Spa group and *Salmonella* SPI-2 T3SS can be placed with group (5) five, the Esa/Ssa group (He et al, 2004). As stated previously, SPI-1 functions at the initial stages of infection to colonize the gastrointestinal tract and invade the intestinal epithelial cells. A study by Galán and Curtiss III (1989) using a strain of *Salmonella* Typhimurium with *invC* (ATPase) mutation showed that this strain is more lethal to mice by 50% than its corresponding wild type, but was comparable with the wild type when introduced to mice within the peritoneal cavity, which verified that the genes in SPI-1 do not play a role in the systemic stage of infection for mice.

The efficiency of *Salmonella* pathogenesis depends on the regulation of the genes to coordinate the expression of the virulence genes to act at the right time, condition, and site of infection. Numerous regulatory proteins have the influence and control of the SPIs expression since these islands contain the most essential elements for virulence. The major regulators of SPI are encoded within the SPI with only a few encoded outside those regions. The hyperinvasion locus (*hil*) was one of the first regulators identified and is now known as *hilA* (Lee et al, 1992). HilA is a transcriptional regulator and a member of the OmpR/ToxR family (Bajaj et al, 1995). This regulator is encoded within SPI-1 and transcriptional activates SPI-1, SPI-4, and SPI-5, and represses SPI-2. The importance of *hilA* is its central role in high degree of SPI-1 T3SS expression leading to high invasiveness. It was previously shown that a single deletion mutant of *hilA* has the same phenotype characteristic when deleting the entire SPI-1 (Ellermeier et al, 2005). For T3SS to be functional, the HilA protein binds directly to the promoter of *inv/spa* and *prg/org* operons and activates them (Bajaj et al, 1995; Bajaj et al, 1996; Lostroh and Lee, 2001; Jones, 2005). An investigation by Thijs et al (2007) demonstrated a more precise direct role for the HilA regulon using genome-wide and transcriptional analysis. HilA positively regulates

genes encoding T3SS effectors secreted proteins, SigD/SopB, SopE, and SopA, located in SPI-5 and the SiiA of SPI-4, and negatively regulates SsaH and SseL of SPI-2 to enhance the influence for invasion stage of infection (Eichelberg and Galán, 1999).

Another regulator encoded within SPI-1 is InvF and it belongs to the AraC/XylS family (Kaniga et al, 1994). It transcriptionally activates the *sic/sip* operons containing *sicA* (Darwin and Miller, 2000, 2001; Tucker and Galán, 2000). This regulator can be activated in two different ways, either dependent on or independent of HilA. As stated previously HilA activates the transcription of the operon *inv/spa* that includes *invF*. The transcriptional activation of *inv/spa* operon can be HilA-independent to an extent that is significantly less than that of the HilA-dependent through the induction of HilD, HilC, and RtsA (Rakeman et al, 1999; Akbar et al, 2003; Ellermeier et al, 2005). Thijs et al. (2007) demonstrated that HilA and InvF share the role of expressing the genes of SPI-1 secreted effectors proteins in different manner to cause invasion. Han et al (2012) observed that a deletion of *invF* increased the expression of *sseA*, a gene of SPI-2.

HilD, HilC, and RtsA. These three regulators are members of the AraC/XylS family. HilD and HilC are both located and encoded within SPI-1 (Schechter et al, 1999), while RtsA is encoded and located on a 15 kb island outside the SPI-1 in the *Salmonella* chromosome (Ellermeier and Slauch, 2003; Golubeva et al , 2012). These activating regulators have the potential to auto-induce the expression of themselves and each other. An environmental signal will induce *hilD* gene first and then both *hilC* and *rtsA* will be activated. As a result, the combined transcriptional expression of these genes will form a loop and intensify the signal to transcriptionally activate HilA. It has been shown that RtsA can solely activate two effectors genes, *slrP* (*Salmonella* leucine-rich repeat protein) and *dsbA* (disulphide bond) or in

combination with HilD and HilC to express the periplasmic protein required by SPI-1 T3SS. A mutation in SlrP showed a 6-fold down-regulation of *Salmonella* strain virulence after oral inoculation of mice (Bernal-Bayard and Ramos-Morales, 2009). Also, diminished *Salmonella* virulence with significant invasion reduction was associated with improper function of SPI-1 T3SS due to the instability of InvH as a result of a *dsbA* gene deletion (Ellermeier and Slauch, 2004; Miki et al., 2004; Lin et al, 2008; Heras et al, 2009). Miki et al (2004) previously demonstrated that *dsbA* gene is involved a role for both SPI-1 and SPI-2 T3SS. An outer membrane protein of SPI-2 T3SS encoded by *spiA* gene, also known as *ssaC*, serves as a DsbA substrate into SPI-2 T3SS and a substrate for SrgA, a protein encoded in the virulence plasmid and functions similar to DsbA as disulfide oxidoreductase, but with less efficiency. A single mutation of *dsbA, srgA*, and a double mutation of both genes was tested using a mouse infection model. The double mutation of both genes showed a significant attenuation in comparison to a single gene mutation of either *dsbA* or *srgA* genes. This resulted in no detectable expression of SpiA protein leading to considerable reduction in the systemic infection and decline of its ability to survive in the *Salmonella*-Containing Vacuole (SCV) (Lasica et al, 2007; Miki et al, 2004). Previous studies showed that HilD is involved with the transcriptional regulation of SPI-1, SPI-2, SPI-4 expression depending on the infection condition phase (Bustamante et al, 2008; Main-Hester et al, 2008). HilD instantly binds to the ssrAB operon in SPI-2. In the crosstalk with SPI-4, *siiE* gene expression requires the positive transcriptional activation of *hilA, hilC, hilD* combined.

HilE. This main regulator was identified by Fahlen et al (2000), and has a negative effect on *hilA* gene expression by interacting with HilD, the predominant activator of SPI-1. It also represses *invF* in a manner independent of HilA activation (Baxter et al, 2003). A study by

Boddicker et al (2003) showed that either in the presence or absence of the negative regulator, *hilA* transcriptional activation requires *hilD*. It was demonstrated that a deletion of *hilE* gene significantly improved the *siiE* expression of SPI-4 as a consequence of increased *hilA* expression with the combination of other SPI-1 transcriptional regulatory genes (Main-Hester et al, 2008).

Salmonella species similar to other microorganisms also use systems known as two component regulatory systems, to sense and respond to the surrounding environmental changes. Basically, these systems serve as an incentive mechanism that respond through a signal transduction to environmental conditions or stresses either physical or chemical such as nutrient limitation, osmolarity, temperature, pH, desiccation, and other conditions (Beier and Gross, 2006; Groisman, 2001; Gunn and Miller, 1996; Miller et al, 1989; Soncini and Groisman, 1996; Mouslim and Groisman, 2003). Typically, these signal transduction systems are formed from a histidine protein kinase (HPK) in concert with a response regulator substrate. The histidine kinase is a trans-membrane protein with extracellular sensing receptor and the response regulator is a protein with a receiver domain. The signals move from the histidine kinase auto-phosphorylated domain to be transferred to the aspartate phosphorylation site at the response regulator receiver domain. Subsequently, the receiver domain acts as a transcriptional regulator that causes cellular mechanisms to respond as physiological changes through gene expression to adapt, tolerate, resist the milieu modifications. In bacteria, the coding genes for these two-component systems are mainly located in one locus (West and Stock, 2001; Wolanin et al, 2002; Laub and Goulian, 2007). Several two-component systems play a role in the *Salmonella* pathogenicity. For instance, PhoQ-PhoP, SsrA-SsrB, and EnvZ-OmpR represent some of these systems.

SPI-1 T3SS expression. It is controlled by several genetic regulators and environmental stresses. Most of the primary environmental conditions that are known to regulate invasiveness include pH, bile, oxygen limitation, cation concentration (Mg^{2+} and Fe^{2+}), osmolarity and short chain fatty acids (Durant et al., 2000a, b; Jones, 2005; Altier, 2005; Ibarra et al, 2010). *Salmonella* invasion is stimulated after the SPI-1 expression when the pH is near neutral, the osmolarity reaches a high level, and the oxygen level is limited (Bajaj *et al.*, 1996).

When a cation such as iron is at a high concentration, the ferric uptake regulator (Fur) interacts with HilD, an important regulator of SPI-1, resulting in the initiation of SPI-1 expression and this interaction by Fur is dependent on the iron availability similar to the conditions inside *Salmonella* containing vacuole (Teixidó et al, 2011; Ellermeier and Slauch, 2008).

As in all microbial infections, there are two groups of factors; one belongs to the microorganism, the invader, and the other belongs to the infected host responding to the infection. Factors accounted with the chicken may play a role in the infection of ovarian tissue as host responses. A study by Subedi et al (2007) involved the expression of different gallinacin genes (GALs) types throughout ovarian follicular development in laying hens. Gallinacin is the beta format member of the defensin antimicrobial peptides family. It is the first line of defense and a part of the innate immunity against pathogenic microorganisms. It was found that the expression of some gallinacin genes were down-regulated while others were up-regulated at the two cell layers forming the ovarian follicles wall, granulosa and theca layers when using a cell wall component, lipopolysaccharide (LPS) to simulate an infection of pathogenic bacteria. The cause of the down regulation of some genes expression in responding to the mimic infection was not identified. A recent study by Chen (2013) had identified a novel transcriptional regulator,

STM0029 in *Salmonella* that is involved in the role of *Salmonella* survival within the cell(s) and resistance in opposition to the cell defense mechanisms by the host, mainly antimicrobial peptides (AMP) of which β-defensin (Chen, 2013).

6. *Salmonella* and foods: stress, tolerance and response

6.1 General aspects of stresses in food chain production.

The objective of using specific preservation factors or processing methods for some foods depend on the food kinds and to better have reduction or elimination of foodborne pathogens and spoilage microorganisms to ensure the food safety and extend the shelf life of the product supply. Foods in general contain various microorganisms at different levels, but not all are undesirable microorganisms (Gálvez et al, 2008; Raybaudi-Massilia et al, 2009; Settanni and Corsetti, 2008; Calo-Mata et al, 2008). The microorganisms' responses to food composition and to treatments throughout foods chain processing as sub-lethal stress vary from one microorganism to another with cell metabolic changes when sensing external environmental changes to create protection of certain stress and/or form cross-protection for other stresses (Archer, 1996; Pichereau et al, 2000; Skandamis et al, 2008; Dodd et al, 2007; Sirsat et al, 2011). The stress severity and the cell properties of microorganisms are two important factors that result in different effects. These effects can be a variety of responses such as inhibiting growth, causing death to microbial cells, or forming spores (Wesche et al., 2009; Storz and Hengge; 2010).

Preservation and processing has long been performed to foods for the purpose of extending the shelf life using drying (Ayensu, 1997; Karathanos and Belessiotis, 1999; Midilli et al, 2002; Ratti, 2001; Sobukola et al., 2007; Vega-Mercado et al, 2001), salting (Albarracín et al, 2011; Martínez-Alvarez et al , 2005; Turan et al, 2007), cooking (roasting, grilling, and

smoking), fermentation (McGovern et al, 2004; Hui et al, 2004; Motarjemi, 2002), canning, pasteurization (Holsinger, 1997; Hotchkiss, 2001; Steele, 2000), and cooling/freezing (Barbosa-Cánovas et al, 2005; Dincer and Kanoglu, 2011; FSIS-USDA, 2010; Singh and Heldman, 2014). These methods were used either individually or in combinations before even realizing that microorganism can play a role in spoilage and foodborne diseases. Preservation by drying can commonly cause dehydration as water is removed from food. This method was used in geographical regions where hot climates exist and can generally be applied to meat, grains, and fruits. Salting was used with drying meat and adding sugar to dried fruits to achieve a longer preservation time became known as curing (Duea, 2011)

Smoking has been also used with salt to preserved meat with some slight level of cooking. In addition, a preserved method should not make the food inconsumable as other factors may cause undesirable changes to the final products. However, in cold and frozen climates, some places were used for the purpose of food preservation (Flandrin and Montanari, 2013). For instance, historically, caves and ice-cold streams were used for these purposes. In other areas, underground food cellars with clean blocks of ice cut from frozen rivers or lakes during the winter season were used initially to store root crops (vegetables and fruits) and subsequently for storing other foods. These cellars were placed in locations away from sunlight exposure and typically were located near cold streams. The first report of using a designated ice space occurred in narratives on Chinese, Greek and Roman cultural food descriptions (Archer, 2004; Hulse, 2004; Whitman et al., 2012).

In food industry systems, foods undergo production, processing, storage, transportation, distribution and marketing. These steps offer numerous challenges and stresses for pathogens and spoilage microorganisms. However, it may possibly be beneficial for some microorganisms

to retain their growth and thus leading to more resistance as biofilms. Stresses encountered by foodborne microorganisms can occur at any point of food chain in either pre-harvest, post-harvest or both stages. Generally, pre-harvest stresses are weather- and/or environmental-related. While post-harvest stresses are caused by factors during food chain production as processing, storage, distribution, preparation for consumption, and finally inside the host throughout the digestive system. In pre-harvest level, food or its source are stressed by heat and UV radiation through the exposure to sunshine. Another environmental stress is the low pH milieu "acidity" that is caused by acid rain, irrigation systems, fermentation and/or decomposition of products as in silage processing, fruit juices and plant saps fermentation, and in animals stressed muscles (Francis et al., 2012, Gil et al., 2015; Leifert et al., 2008; Yousef and Courtney, 2003). Salinity of soil and limited to non-nutrient ecosystems can also cause stress conditions (de Souza Silva and Fay, 2012; Egamberdieva, 2012).

Typically, food processing can go through food production and processing steps such as harvesting, soaking and washing, sorting and grading, peeling, coring, cutting, trimming and shredding; others are expected to be an important step of some preservation techniques such as blanching, pasteurization, sterilization, cooking, drying, evaporation, salting, smoking, concentrating, and baking (Alexandre et al, 2014; Sivasankar, 2002; Ramaswamy and Marcotte, 2006).

6.2 Thermal (Heat) Stress:

Foodborne pathogen contamination of a wide range of foods has been a primary concern of public health, food safety authorities, and food industries in the United States and worldwide (Beier et al, 2004; Braden and Tauxe, 2013; CDC, 2013; Finstad et al., 2012; Gillis et al, 2011;

Hajmeer and Crozier-Dodson, 2012; Hanning et al., 2009; Imanishi et al., 2014; Jackson et al., 2013; Jacob et al, 2012; Jahid and Ha, 2012; Jay-Russell, 2014; Masuku et al, 2012; Morris, 2011; Murphree et al., 2012; Newell et al, 2010; Painter et al., 2013; Pires, 2013; Rostagno and Ebner, 2012; Van Loo et al, 2012a, 2012b).

Salmonella species are present in various foods including fresh produce (frequently alfalfa sprouts, baby spinach, basil, cantaloupe, lettuce, peppers, and tomatoes), with poultry meat and eggs being considered some of the more common sources of salmonellosis (Finstad et al, 2012; Franz and van Bruggen, 2008; Hanning et al, 2009; Howard et al, 2012; Lynch et al , 2009; Nayak et al, 2012). Foods can be contaminated at any point of food chain starting from production, through processing, distribution, preparation and consumption. *Salmonella* spp. were identified with 17% of fresh produce outbreaks for the period between 1998 and 2007 (Olaimat and Holley, 2012). It was estimated by Scharff (2010) that produce either fresh or processed was the cause of 27% of *Salmonella* outbreaks (Olaimat and Holley, 2012). The Food Safety and Inspection Service of the U.S. Department of Agriculture reported positive *Slamonella* samples in 18% of ground chicken meat, 15% of ground turkey meat, 2.3% of turkey, and 3.9% of broiler chicken (FSIS-USDA, 2013).

Numerous strategies have been investigated and/or applied throughout food processing. Several of these methodologies are routinely used to decontaminate foods from pathogenic and spoilage microorganisms and as well to extend the food life shelf as preservation methods for the last two decades (Chen et al, 2012; Gil et al, 2015; Ricke et al, 2005; Sirsat et al., 2009; Vandeplas et al., 2010; Wheeler et al, 2014). These intervention treatments can be categorized as either thermal or non-thermal. Each category is divided to sub-group with thermal including heat (water and steam air) or thermal radiation (infrared microwave), and non-thermal including

chemical, physical, or biological treatments (Warriner, 2011). All of these intervention strategies have been conducted to control and decontaminate products during pre-harvesting or post-harvesting such as meat, poultry, and fresh produce (Aymerich et al., 2008; Beuchat et al., 1998; Chaine et al., 2013; Chen et al., 2012; Dinçer and Baysal, 2004; Huffman, 2002; Hugas and Tsigarida, 2008; James et al., 2007; Loretz et al., 2010, 2011a,b; Otto et al., 2011; Parish et al., 2003; Rajkovic et al., 2010; Vandeplas et al., 2010).

Heat treatment is considered as one of the more effective food processing techniques to eliminate *Salmonella* and other foodborne pathogens from food products (Bermúdez-Aguirre and Corradini, 2012; Silva and Gibbs, 2012; Sun, 2014). Some *Salmonella* strains are capable of growing at temperatures as high as 54^{o}C and after contaminating different food products can thus survive their processing (Droffner and Yamamoto, 1991; Finstad et al, 2012; Hanning et al, 2009; Howard et al, 2012; Lynch et al, 2009; Nayak et al, 2012; Montville and Matthews, 2008; Park et al, 2014).

Pasteurization is a heat process between 55^{o}C and below 100^{o}C for a period of time and may not entirely eliminate all microorganisms (Gould, 2000). It is a long established and a perceived acceptable thermal food treatment when evaluated by the consumers according to a study conducted by Wright et al (2007). This method has been the traditional and the primary procedure in food processing and preservation (Charles-Rodríguez et al., 2007; Lado and Yousef, 2002). The produced heat on the food outer surface is transmitted to the food interior; however it possibly causes unfavorable food changes (Silva and Gibbs, 2012). Steam pasteurization processing was approved in the mid 1990s by FDA as an effective preservation method for meat and poultry carcasses combining elevated temperature and pressure for a short exposure time followed by a chilled water treatment to minimize the destructive effects on

carcasses (Chen et al., 2012; Wheeler et al., 2014). It was demonstrated that *S.* Typhimurium on beef carcass was exposed to steam pasteurization and achieved effective reduction of greater than 1.0 log CFU/cm^2 at approximately 6 seconds with a temperature of 93.3^oC (Retzlaff et al, 2004). Park and Kang (2014) investigated the outcome of using steam pasteurization to inactivate the biofilm of *Salmonella* and other foodborne pathogens. Biofilms were formed on polyvinyl chloride coupons with three strains of *Salmonella* after an incubation period of six days. Subsequently, the coupons were exposed to 75^oC and 85^oC, each for 50 seconds and a 2.04 and 3.01 log CFU/coupon reduction respectively were observed suggesting a promising control intervention for difficult and resistance forms of bacteria.

Hot water and steam interventions have been applied to poultry carcasses to be decontaminated from *Salmonella* up to 2.1 and 3.8 log units respectively. A temperature and exposure time combination has a *vice versa* relation as increasing both may improve the efficiency of sanitizing the food surfaces, but such combinations can affect the food product quality and appearance (Buncic and Sofos, 2012).

Many studies have examined the heat inactivation and thermal resistance of several *Salmonella* strains. A valid heat treatment for *Salmonella* requires a mixture of several serotypes and strains. A reduction of 6.5 log from meat and meat products, and a reduction of 7 logs from poultry and their products with minimal process time has been established by USDA (FSIS-USDA, 2012). Thermal treatments using steam and hot water can possibly decontaminate carcasses from *Salmonella* species by approximately 100 to 1000 times in comparison to other interventions (Hald, 2013; Montville and Matthews, 2008). *Salmonella* was reduced by immersing chicken carcasses in hot water as the result of elevating the carcass surface temperature (Morrison and Fleet, 1985). In addition, several researchers have achieved

reductions with pathogens and spoilage microorganisms between 2 and 3.7 log CFUs with temperatures between 70^{o}C and 95^{o}C, but those treatments for poultry exhibited either a cooked appearance or caused tearing of the chicken skin (Corry et al, 2007; Dorsa et al, 1997, 1998; Purnell et al, 2004).

Since using hot water or steam as heating treatment with temperatures over 70^{o}C may cause undesirable changes for food in its characteristics such as appearance, the color, the taste, mild heating has been used in combination with other treatments to effectively inactivate pathogens and other spoilage bacteria. Milillo and colleagues concluded that combining multiple treatments can possibly and effectively reduce *Salmonella* in a short time. They applied mild thermal treatments and acidified organic acid salt solutions for an exposure of one minute. Sodium propionate with heating has shown to be the most significantly effective in causing viability loss of *Salmonella* tested strains (Milillo and Ricke, 2010; Milillo et al, 2011). O'Bryan with others reviewed the heat resistance of *Salmonella* species and other foodborne pathogens associated with meat and poultry. They found that many factors and parameters are involved in the heat resistance and inactivation of those pathogens and spoilage microorganisms. Strains of the same microorganisms can react differently to the same treatments. Also, the stages of the growth, the culture age, and the conditions of the growth have yielded various outcomes regarding heat inactivation or destruction of microorganisms (O'Bryan et al, 2006).

Some *Salmonella* strains are known to survive food processing and resist thermal treatment due to their prior growth and stress conditions. Microorganisms tested against heat exhibited different response in regard to their growth conditions with stationary phase cells to be more resistance to heat than log phase cells (Humphrey et al, 1995; Mackey and Derrick, 1990; Wilde et al, 2000). In addition, the growth temperature of an organism above the average growth

range, heat shocked cells, growth on limited carbon sources or going through starvation stress prior to the heat treatments have exhibited more thermal tolerance (Bunning et al, 1990; Foster and Spector, 1995; Ng et al, 1969; Xavier and Ingham, 1997). Furthermore, *Salmonella* attached to food surfaces are more resistance to heat than their swimming form or detached throughout food processing (Humphrey et al, 1997; Orta-Ramirez et al, 2005; Mogollon et al, 2009; Tuntivanich et al, 2008; Velasquez et al, 2010).

Nielsen and colleagues compared two different growth states of *S*. Typhimurium cells, immobilized and planktonic. *Salmonella* cells growth is planktonic when growing in liquid media whereas versus growing in immobilized states within food products. Under each form, the cells have different molecular mechanisms and levels of gene expression including their stress responses. In their study, *S*. Typhimurium cells that were heat treated responded differently to heat shock at 45^{o}C for 30 minutes. The results revealed that 538 genes were expressed differently. Genes up-regulated in the immobilized state were mainly flagella and virulence genes, and the cells were more invasive in HeLa cells after heat stress treatment, but S. Typhimurium planktonic cells were less invasive. This study showed that inadequate cooking and heat treatments during food processing may increase the survival and resistance of *Salmonella* and other foodborne pathogenic (Knudsen et al., 2012; Nielsen et al, 2013).

A study conducted by Sirsat and co-workers (2011) examined the effect of heat stress on *S*. Typhimurium gene expression using transcriptional profiling. This profiling was applied to identify the thermal stress response of *S*. Typhimurium at sub-lethal temperature of 42^{o}C; as a result, the expression of several genes was observed. In response to the sublethal heat stress, 144 genes exhibited up-regulation and 167 genes exhibited down-regulation expression. These genes belong to various functional categories, but primarily among them general stress response sigma

factor S (RpoS) and heat shock proteins, sigma factors H and E (RpoH and RpoE), and HtrA (high temperature requirement A). The latter protein has been shown to be critical for virulence of numerous pathogens. Generally, genes associated with stress and energy metabolism are the first response of the cells to tolerate the heat stress. These genes may possibly give the pathogen cross-resistance to other stresses and become more virulent. This study is considered the first to report that sub-lethal heat stress influenced *Salmonella* interaction with Caco-2 cells through the expression of fimbriae associated genes. Genes of two *Salmonella* Pathogenicity Islands, SPI-2 and SPI-5, were up-regulated resulting in improved adhesion and survival in the host while genes of pathogenicity island, SPI-1, was down-regulated.

Some studies concluded that the modifications of the membrane fatty acid composition of *Salmonella* strains had a connection to its ability to resist thermal treatment mainly for those cells with less fluidity of their membrane (Álvarez-Ordóñez et al, 2008, 2009; Balamurugan, 2010; Sampathkumar et al, 2004).

6.2.1 Thermal Stress Response:

High temperature stress over the optimal growth range of a microorganism just as with any other environmental harsh stress conditions, initially affects the cytoplasm and the extra-cytoplasmic (cell envelope) structures of bacterial cells (Silhavy et al., 2010). Responses differ mainly by their regulation and possibly involve numerous genes. *Salmonella* cells may face thermal stress at several points in food production/ processing/preservation including internally in avian host body with a normal body temperature of $42^{o}C$; consequently, they generally express two sigma factors, a cytoplasmic thermal stress response regulated by heat-shock sigma typical factor, σ^{H} or σ^{32}, and an extra-cytoplasmic thermal stress response regulated by the extra-

cytoplasmic function sigma factor (ECF), σ^{E} or $\sigma^{24,}$ also known as extreme heat stress sigma factor (Ades, 2008; Bashyam and Hasnain; 2004; Heimann, 2002; Morimoto, 1998; Raivio and Silhavy, 2001). Throughout or after the exposure to a high temperature and as a protective reaction, mis-folded and unfolded proteins are present in considerable numbers in the cytoplasmic membrane and the outer membrane "cell envelope", subsequent to initiating the expression of heat shock proteins (HSPs) through the regulation of the heat shock factor σ^{H}.encoded by the *rpoH* and *rpoE* genes (Arsène et al., 2000; Gruber and Gross, 2003; Guisbert et al., 2008; Nonaka et al., 2006; Yura and Nakahigashi, 1999).

High temperatures induce heat shock proteins formation, mainly chaperones, proteases, and small heat shock proteins (s-HSPs) that play a role in protecting, refolding salvaged proteins and /or removing damaged proteins, and repairing and/or degrading protein aggregation (Dougan et al, 2002; Hayes and Dice, 1996; Lim and Gross, 2011; Robinson, 2013; Turgay, 2011; Tyedmers et al., 2010).

6.2.2 Sigma factors and thermal stress regulation:

Sigma factors are a very large group comprised of genes expressing proteins with significant mechanisms associated with RNA polymerase holoenzyme complex (RNAP) that function as guidance for core RNA polymerases to recognize their promoters and initiate transcription. Primarily, the sigma factors are divided into two categories, sigma factor 70 family (σ^{70}) that coordinates the transcriptional activities in various stress responses, also known as σ^{A} in *Bacillus subtilis* and some other bacterial species (de la Fuente et al, 2012; Kazmierczak et al, 2005; Kumar and Moran, 2008; Kumar et al, 2004), and a second identified family of sigma factors encoded by rpo^{N}, is known as sigma factor 54 ($\sigma^{54/N}$) (Bang et al., 2005; Doucleff et al.,

2007; Treviño-Quintanilla et al., 2013). It has been identified in *Campylobacter jejuni*, *Enterococcus faecalis*, *Listeria monocytogenes*, and *Pseudomonas* spp (Boor, 2006; Buck et al, 2000; Gruber and Gross, 2003; Merrick, 1993).

The σ^{70} family includes several sub-groups including the primary sigma factor $\sigma^{70/D}$ encoded by *rpoD* gene and the transcriptional activity is mainly for housekeeping genes at exponential growth. The second significant sigma factor subgroup is the general stress regulator; $\sigma^{38/S}$ encoded by *rpoS* gene that is commonly expressed during stationary growth phase and in response to numerous other stresses. It has been identified in *E. coli*, *S* Typhimurium, *S.* Typhi, and *Pseudomonas aeruginosa* (Cheville et al, 1996; Dong and Schellhorn, 2009a, b, 2010; Dong et al, 2008; Fang et al, 1992; Schuster et al, 2004; Suh et al, 1999; Venturi, 2003; Vijayakumar et al, 2004). A similar types in functions, $\sigma^{B/37}$ and σ^{F}, have been described for mainly Gram positive bacteria such as *Bacillus subtilis; Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* (Becker et al, 1998; Benson and Haldenwang, 1993; Chan et al, 1998; Ferreira et al, 2001; Kazmierczak et al, 2005; Kies et al, 2001; Kullik, and Giachino, 1997; Manganelli et al., 2004; Wu et al, 1996).

The heat shock responses are regulated by the alternative sigma factors $\sigma^{32/H}$ and $\sigma^{24/E}$. These two make up the third and fourth sub-groups of sigma factor encoded by *rpoH* and *rpoE* genes respectively (Kültz et al, 2005; Paget and Helmann, 2003). The transcriptional activity of *rpoH* gene is a specific heat shock response and the gene regulation takes place at the translational stage. When temperature is at the optimal growth range, the translation of *rpoH* gene is blocked. The stem III and I of the *rpoH* mRNA secondary structure is liberated with rising temperatures (42^{o}C) facilitating the ribosomal binding and enhances the efficiency of translation (Morita et al., 1999; Yura et al., 1999; 2000; Narberhaus et al., 2006; Narberhaus,

2010). Sigma factors associated with heat stress response have been demonstrated to regulate over a hundred genes; of those sigma factor $\sigma^{32/H}$ controls more than 30 proteins most of which are associated chaperones and proteases (Guisbert et al, 2008; Lund 2001; Österberg et al, 2011; Nonaka et al, 2006; Wade et al, 2006; Zhao et al, 2005). A recent study by Lim and colleagues made it clear that $\sigma^{32/H}$ is not just localized at the bacterial cytoplasm, it is also found in the inner membrane through a direct interaction with the signal recognition particle (SRP) and its receptor (SR) (Lim et al, 2013; Robinson, 2013). Proteases expressed by sigma factor $\sigma^{32/H}$ can control and decrease the expression of the membrane heat shock proteins to a level as needed by the cell to withstand the environmental stresses. For instance, FtsH is one of the ATP-dependent proteases, which possesses numerous cellular functions. FtsH has been demonstrated to be very critical to *E. coli* viability (Akiyama et al, 1994; Ogura et al, 1999; Tomoyasu et al, 1993; Katz and Ron, 2008). In addition, FtsH functions as a protein qualifying protease and has a role in membrane proteins degradations primarily those with SsrA-tagged cytoplasmic proteins at their carboxy- terminal (Herman et al, 1998; Lies and Maurizi, 2008). FtsH degrades MgtC, a membranous protein with five trans-membrane domains with the contribution of MgtR. A virulence factor, MgtC, has been identified to be required for survival inside macrophages (Alix and Blanc-Potard, 2008). Katz and Ron (2008) demonstrated a maintenance role of FtsH for lipopolysaccharide biosynthesis with a shielding permeability function (Delcour, 2009; Helander and Mattila-Sandholm, 2000; Katz and Ron, 2008; Nikaido, 2003). Although $\sigma^{32/H}$ and $\sigma^{24/E}$ are alternative sigma factors, $\sigma^{32/H}$ regulates heat shock proteins (HSPs) for the cytoplasmic components and $\sigma^{24/E}$ regulates the extra-cytoplasmic (cell envelope) proteins in response to high temperatures and other envelop stress factors (Ades, 2004, 2008; Alba and Gross, 2004; Duguay and Silhavy, 2004; Hayden and Ades, 2008; Raivio, 2005; Rowley et al, 2006). An interesting

finding is that one of the four promoters of *rpoH* gene expression are regulated by $\sigma^{24/E}$ for additional coordination of thermal response requiring both cytoplasmic and extra-cytoplasmic components (Bury-Moné et al, 2009; De Las Peñas et al, 1997; Erickson and Gross, 1989; Wang and Kaguni, 1989; Hiratsu et al, 1995; Missiakas and Raina, 1998).

A large number of proteins form the family of small heat shock proteins (s-HSP) that consists of proteins with up to 50 amino acids. Typically, they are considered energy free and universally found in numerous microorganisms with a diverse group and variable molecular weights. They have chaperone-like function commonly maintaining protein homeostasis as proteins are their main substrates. The sHSP are active primarily during stresses to stabilize cell proteins at diverse cellular activities (metabolism, translation, transcription and others). They bind unfolded proteins forming a complex blocking non-specific irreversible aggregation (Haslbeck et al, 2005; Lindquist and Craig, 1988; Nakamoto and Vígh, 2007; Sun and MacRae, 2005).

6.2.3 Heat shock proteins and virulence:

Numerous chaperones and proteases regulated by the alternative heat shock factors, $\sigma^{H/32}$, $\sigma^{E/24}$, and others have been notably involved in bacterial virulence. Several studies have linked these proteins to *Salmonella* and *E. coli* virulence factors (Ensgraber and Loos, 1992; Humphreys et al., 1999; Kazmierczak et al., 2005; Lewis et al., 2009; McMeechan et al., 2007; Raspoet et al., 2014). Although both $\sigma^{H/32}$ and $\sigma^{E/24}$ are regulators for heat shock stress, their initiation of molecular mechanisms are not similar.

Several studies have demonstrated the role of the heat shock factors in bacterial pathogenesis and virulence. A loss of *rpoE* gene activity, a virulence regulator, exhibited a defect

in cell viability of *E. coli* and increased cell envelope stress (Alba and Gross, 2004; Button et al, 2007; De Las Peñas et al, 1997; Hayden and Ades, 2008). In *Salmonella*, the mutants can be isolated that are less responsive to heat shock temperatures. They exhibit an intracellular defect in the survivability within a macrophage and become avirulent in a mouse infection model (Cano et al, 2001; Humphreys et al., 1999; Miticka et al, 2003; Testerrman et al, 2002). In addition, *rpoE* gene has been shown to be essential in response to starvation stress (Kenyon et al, 2002), oxidative stress (Bang et al, 2005), antimicrobial peptides resistance (Crouch et al, 2005), osmotic and cold stress (McMeechan et al, 2007).

Another thermo-sensing gene known as *htrA*, ***h***igh ***t***emperature ***r***equirement A, is a member of the serine proteases group within the endoproteases family and is regulated by sigma factor E (Alba and Gross, 2004; Clausen et al, 2002; Kazmierczak et al, 2005; Pallen and Wren, 1997; Raivio, 2005). It is a highly conserved gene in numerous microorganisms and was first discovered in *E coli* as *degP*. At low temperature, the protein HtrA (DegP) functions as a chaperone in the outer membrane; however, at high temperature, it acts as a protease to degrade mis-folded proteins with ATP-independent activity and other co-factors (Schumann, 2007, 2012). An early study linked the activity of this gene to its sensitivity to thermal stress (Skorko-Glonek et al, 1995). A strain with a mutation in this gene exhibited an inability to grow at high temperature and/or the lack of degrading unfolded proteins in the periplasmic space. *Salmonella* Typhimurium was less affected by the sigma factor E mutation than *E. coli* (Spiess et al, 1999; Ingmer and Brøndsted, 2009; Frees et al., 2013)

Lewis and colleagues (2009) demonstrated that *Salmonella* possesses two additional promoters for *htrA* that were possibly regulated by other sigma factor(s) of sigma factor 70. Furthermore, they discovered that both functions of *htrA*, proteases and chaperones act during

infection with the protease function of the proteases being most critical inside the host (Lewis et al, 2009). A recent study verified that HtrA protein activity is critical for *Salmonella* Enteritidis to persist in egg whites at 42^{o}C (chicken body temperature) (Raspoet et al, 2014).

FkpA, an FKBP-type ***p***eriplasmic ***p***eptidyl-prolyl cis/trans ***i***somerase (PPIase) expressed by a gene regulated by sigma factor E and is involved in heat tolerance (Duguay and Silhavy, 2004). The protein is a comparable to other proteins known as macrophage infectivity potentiator (MIP) in other pathogenic bacteria and exhibits a characteristic of improving the survivability and proliferation inside the macrophages and epithelial cells (Arié et al, 2001). Horne et al (1997) demonstrated that a mutation in *fkpA* cause the *Salmonella* strain to become avirulent; however, Humphreys and co-workers (2003) argued that a single mutant deletion of *fkpA* was not enough to reach that conclusion. They observed that only when combining that mutation with one of the other σ^{E} regulated genes, *surA* or *htrA*, the virulence of *S.* Typhimurium would be then disrupted (Humphreys et al, 2003; Henderson et al, 2006). In a recent study by Weski and Ehrmann (2012), they conducted a genetic analysis of some chaperones and proteases of the *E. coli* associated with the cell envelope. They tested single mutant deletions and double mutant deletions under different growth conditions. The *fkpA* mutation was examined at 37^{o}C and 42^{o}C using rich medium agar plates with and without 0.5 M NaCl and the corresponding mutants did not exhibit any defect under any of the conditions. However, when combining this strain with another mutation at *dsbA*, disulfide bond formation A, the strain displayed weak growth in the hyperosmolar media when incubated at 37^{o}C and no sign of growth at the hyperosmolar media when incubated at 42^{o}C with a minimal growth of *dsbA* single mutants at the latter condition (Łasica and Jagusztyn-Krynicka, 2007; Weski and Ehrmann, 2012).

SurA, survival protein A, is a PPIase. It is regulated by σ^E and exhibits thermo-tolerance fitness. This protein participates in the outer membrane proteins (OMPs) development, assembly and plays a role in their folding generally transportation channels, known as porins (Bos and Tommassen 2004; Bos et al., 2007; Geitner et al, 2013; Lazar and Kolter, 1996; Sklar et al, 2007; Ünal and Steinert, 2014). A recent study by Sklar and others revealed that *surA* role starts from the initial phases of OMP biosynthesis (Sklar et al, 2011). A study by Tormo and others (1990) demonstrated that *surA* is critical to *E. coli* for survival in stationary growth stage. Tamae and co-workers (2008) screened approximately 4000 single mutant deletions, among them Δ*surA* that exhibited chemical sensitivity to tested drugs and detergents (Tamae et al, 2008). A mutation of *surA* in *S.* Typhimurium led to extensive attenuation when introduced to mice orally and/or intravenously (Sydenham et al, 2000). It has been demonstrated in several studies that *surA* is a critical factor for outer membrane protein transport and associated with virulence of uro-pathogenic *Escherichia coli* and *Salmonella* species (Behrens-Kneip, 2010; Ünal and Steinert, 2014). Using a high-throughput technique, Tn-seq, to screen the entire genome, Khatiwara et al. (2012) identified numerous genes in *S.* Typhimurium associated with high temperature and among them, *surA* as a gene associated with growth at 42^oC (avian body temperature).

6.2.4 Other heat stress response:

Diverse mechanisms have been recognized in bacterial systems as thermosensors to conform to the temperature fluctuations of their milieus. The genes involved in these mechanisms are regulated at different genetic stages beginning from transcription, through translation to post-translational level (Hurme and Rhen, 1998; Konkel and Tilly, 2000).

Numerous studies have associated virulence factors with thermal changes that mediate DNA topology. These modifications can be helical conformation "supercoiling", the degree of helical twists and coiling (Dorman, 1991, 2006; Hurme and Rhen, 1998; Marko and Siggia, 1994; Tse-Dinh et al, 1997; Travers and Muskhelishvili, 2005) and/or alteration in the chromosomal or plasmid DNA as a specific-sequence curvature (Browning et al, 2010; Dorman and Deighan; 2003; Owen-Hughes et al, 1992; Pérez-Martín et al, 1994). Positive supercoiling of DNA after heat exposure causes DNA to be twisted in a right-handed fashion until it causes a knot, which is mainly seen in plasmid DNA. This transformation is controlled by DNA gyrase and topoisomerase I. A normal supercoiling form is regulated by the action of DNA gyrase, and other associated proteins. Some studies demonstrated that DNA topology plays a role in *Salmonella* pathogenicity (Adamčík et al, 2002; López-García and Forterre, 2000). Changes at the level of DNA supercoiling trigger SPI-1 genes and initiate the intestinal invasion. Once inside the host cells, the DNA changes its form and as a result SPI-2 genes are induced (Cameron et al, 2011, 2012).

The second mechanism is through a recognized bending DNA sequence "promoter-curvature". Commonly, this bending DNA region is an AT-rich sequence that has been primarily identified in the 5'-end upstream of the promoter region influencing RNA polymerase binding as a silencing factor. Initially, thermal stress induces some alterations in the DNA topology as bends in the AT-rich sequence regions on the transcriptional level. This will influence the interaction between RNA polymerase and the promoter region altering the gene expression (Browning et al, 2010; Dorman and Deighan; 2003). A well-known gene encodes for nucleotide-associated protein (H-NS), **h**istone-like **n**ucleotide-**s**tructuring protein, has been associated with virulence factors as a temperature-dependent phenotype (Amit et al, 2003; Bolshoy and Nevo,

2000; Dorman and Kane, 2009; Gourse et al, 2000; Jáuregui et al, 2003; Lucchini 2006). This protein is considered a common regulator and widely related to the transcription regulation level induced by thermal changes. At low temperature, H-NS binds to an AT-rich sequence and forms a complex. When temperature rises to 37^{o}C (host body temperature), the binding capacity is reduced until dissociation occurs leading to virulence gene expression. This mechanism was demonstrated in *E. coli* K-12 to control over 60% of the genes regulated by temperature including virulence factors (White-Ziegler and Davis, 2009). The association of H-NS with virulence had been verified in other pathogens such as *Salmonella* (Baños et al, 2009; Lucchini et al, 2006; Olekhnovich and Kadner, 2007; Martínez et al, 2014), *Shigella* , *Yersinia enterocolitica* and *Y.* pseudotuberculosis *(*Baños et al, 2008; Ellison, 2006; Heroven et al, 2004).

Two studies were conducted to identify the mechanism of H-NS in *Salmonella*. The first study was performed on *S*. Typhimurium LT2 (Lucchini et al, 2006). They found that H-NS negatively regulated approximately 254 genes. The second study was carried out on *S.* Typhimurium 14028 (Navarre et al, 2006). They discovered 265 unique *Salmonella* genes were negatively associated with H-NS and contained low GC content. In both studies, among the discovered genes were *Salmonella* Pathogenicity Islands (SPI 1, 2, 3, and 5) (Duong et al, 2007; Shapiro and Cowen, 2012; Steinmann and Dersch, 2013).

Another thermosensor belongs to the RNA family that generally is located at the un-translated region (5'-UTR). This temperature responsive RNA element contains a stretch of four uridine nucleotide within the ribosomal binding site pairing with a sequence of AGGA. This element is known as fourU; initially discovered in *S.* Typhimurium as the small heat shock gene *agsA*, aggregation suppression A (Cimdins et al, 2013; Kortmann and Narberhaus, 2012; Narberhaus, 2010, 2012; Rinnenthal et al, 2010, 2011; Waldminghaus et al, 2007). Afterward, a

similar RNA thermometer was also confirmed to be associated with *Yersinia* virulence through the induction of the transcriptional activator *lcrF* (Böhme et al, 2012; Kortmann and Narberhaus, 2012).

TlpA, TIR-like protein A, is considered one of the first reported proteins of a thermo-sensor gene regulator activity to the high temperature response (HTR) encoded by *Salmonella* enteric virulence plasmid, pSLT (Hurme et al, 1997; Hurme and Rhen, 1998). It is a robust homolog to a eukaryotic protein family known as, tropomyosin, and the structure of TlpA is a dimer form with unusual long alpha-helical coiled coil structure (Mäkelä et al, 1993). It consists of an N-terminal with DNA binding domain and transcriptional auto-regulatory repression activity. At temperatures below 30^{o}C, the transcription of *tlpA* is low and the TlpA repression activity is high. The TlpA is present in two forms, as a dimeric α- helical (folded) coiled coil oligomer at low temperature (28^{o}C) and an unfolded (non-functional) monomer at high temperature (37^{o}C) that leads to increased transcription (Clements et al, 2001; Hurme and Rhen, 1998; Storz, 1999). Although the function of this protein is still unidentified, it was demonstrated that this transcriptional regulator was not essential for virulence of *Salmonella* using a mouse infection model (Gal-Mor et al, 2006).

6.3 Cold Stress

Foods such as fresh produce, animal carcasses and their products are required to be chilled to low temperatures throughout food processing mainly during storage, transportation and distribution to lengthen the shelf- life of food and limit microbial growth (Archer, 2004; Buncic and Sofos, 2012; FSIS-USDA, 2010; Galiş et al., 2013; Guillard et al., 2010; McDonald and Sun, 2000; Russell, 2002).

Some procedures are performed at cold temperature, cooling and/or freezing, as preservation processes to effectively reduce the bacterial burden of contaminated food (Dinçer and Baysal, 2004; Loretz et al, 2010). These preservation procedures can lead to some concerns in regards to understanding the cold adaptation mechanisms of food associated microorganisms predominantly spoilage microorganisms and foodborne pathogens to better apply more control measures (Abee and Wouters, 1999; Alzamora et al, 1998; Beales, 2004; Berry and Foegeding, 1997). Due to the limited understanding of pathogen biology, there is a need to develop new and improved current control strategies to increase food safety by possibly recognizing conditionally essential bacterial traits. Among those pathogens, *Salmonella* required features to grow or survive food processing/ preservation (farm to table chain) (Bower and Daeschel, 1999; Davidson and Harrison, 2002; Ricke et al, 2005).

Freezing is a common method of food preservation by lowering the temperature of food products affecting several functional mechanisms of microorganisms including metabolism. When food begins to freeze, water within the food is converted to ice lowering the water activity along with an increase in solutes concentration and other changes in food properties. Numerous extrinsic and intrinsic factors are involved in accomplishing an optimal level of controlling foodborne pathogens and spoilage microorganisms. These factors belong to food matrix characteristics (texture, pH, chemicals, cryoprotectants, water activity), food processing (process rate, holding time, temperature, freezing type), or microbial characteristics (strain, growth rate, growth phase).

Numerous studies of *Salmonella* survival involving freezing and chilling (refrigeration) have been conducted. A simulation study of commercial freezing used beef trimmings as the food matrix and three *Salmonella* serotypes (*S.* Brandenberg, *S.* Dublin and *S.* Typhimurium) for

exposures to slow freezing (-18^{o}C) rate and rapid freezing (-35^{o}C) rates reaching a temperature in between -17^{o}C and -22^{o}C within 24 hours incubation for slow freezing. The rate exhibited by rapid freezing was 1.8 times faster. Subsequently, all treated beef trimmings were stored at -18^{o}C for nine months. Monthly samples were collected after thawing and refreezing, plated on selective and nonselective media, and enumerated *Salmonella* strains. Beef trimmings samples were partially thawed and refrozen at -18^{o}C for 24 hours to evaluate the abusive procedure on the *Salmonella* serotypes. The survival difference of all strains was not significant as expected during the time period of storage (Dykes and Moorhead, 2001). Their explanation was that the inoculated strains were rapidly frozen compared to the meat. In addition, no significant sublethal cell injuries were determined after comparing cell counts on selective and non-selective media.

Dominguez and Schaffner (2009) conducted a study on *Salmonella* survival in processed chicken products stored under frozen conditions. A cocktail of *Salmonella* strains, *S*. Kentucky and *S*. Typhimurium, originally isolated from chicken, with and without antibiotic resistance, were inoculated in fully cooked chicken nuggets and uncooked (raw) chicken strips; subsequently, the inoculated products were stored in a laboratory freezer (-20^{o}C) for 16 weeks with a weekly sampling collection. Samples were analyzed and plated in minimal, selective and non-selective media. After incubation for 24 hours at 37^{o}C, colonies were enumerated to determine the survival of the bacterial cell population. The results demonstrated that *Salmonella* strains are capable of surviving freezing food processing for long periods of storage time when using frozen processed chicken products (Dominguez and Schaffner, 2009).

A recent research by Müller and colleagues (2012) investigated the ability of some *Salmonella* clones from stationary- and exponential- phases to survive and grow after exposure to freezing stress (mimicking a meat processing chain) for up to 48 weeks in minced pork meat.

Salmonella strains were selected using a mathematical model for epidemiological studies, and characterized the selected isolates to be successful or non-successful clones. Twenty six *Salmonella* isolates were selected with different antimicrobial resistance characteristics belonging to 6 serovars (14 strains *S*. Typhimurium, 4 strains *S*. Derby, 2 strains *S*. Newport, 2 strains *S*. Infantis, 2 strains *S*. Saintpaul, and 2 strains *S*. Virchow) from human and animal sources. The study concluded that up to 1 log reduction of cells at stationary phase of all strains was observed after 1 year of frozen storage while more than 1 log decrease of cells occurred at exponential phase for two strains of *S*. Typhimurium that showed the same reduction in 49 days of freezing stress indicating that exponential phase cells have more sensitivity to the same stress (Müller et al, 2012). They evaluated the recovery time needed by observing the growth in lag phase after the freezing stress for stationary and exponential phases of *S*. Typhimurium strains. The initiation of growth acquired an average of 102 minutes for stationary phase cells and shorter than that for cells of exponential phase (Müller et al, 2012). Some food contents can play a role as protective factors for pathogens. For instance, the amount of fat in meat products can influence the survival rate of pathogenic cells (Ahmed et al., 1995; Asselt and Zwietering, 2006; Beuchat et al., 2013; Finn et al., 2013; Juneja and Eblen, 2000; Moorhead and Dykes, 2002; Murphy et al., 2000; Orta-Ramirez et al., 2005; Waterman and Small, 1998).

Chaves et al. (2011) conducted a study to determine the survival of *S.* Typhimurium artificially inoculated on the surface of raw poultry products, skinless chicken breasts and chicken thighs with skin, treated with crust freezing (−85°C for 20 min). Late exponential-phase cultures were cold-shocked at 4^{o}C incubated for 10 days, and non-cold shocked cultures were used to inoculate skinless chicken breasts and chicken thighs with skin. For crust freezing treatment, samples were divided into two groups. One group was crust frozen at -85^{o}C for 20

minutes and the other group was frozen at -85^{o}C for 60 minutes. Subsequently, all samples were refrigerated for 20 hours and were recovered by rinsing with 50 ml sterile Bacto Peptone water. The collected solution was serially diluted and bacteria was enumerated on selective media, tryptic soy agar (TSA) and brilliant green Agar (BGA) with nalidixic acid. The results exhibited no significant reduction in any of the treatments with reductions of less than 1 $\log_{10}$ cfu/ml (Chaves et al, 2011).

Phillips et al (1998) investigated the effect of chilling on two *S.* Enteritidis PT4 strains, E and I with strain E being more tolerant and pathogenic. These strains are different in heat- and acid- tolerant with ability to survive on surfaces, and pathogenicity in animal models. For both strains, stationary phase cells were diluted to correspond to 5 x 10^{5} ml^{-1} and were used for chilling at 4^{o}C for 12 days. The results indicated that strain I was consistent at all time periods and did not show any significant reductions whereas strain E showed significant reduction at day 12. Xylose Lysine Desoxycholate (XLD) media was used to recover stressed cells for enumeration. At the end of the treatment, strain E exhibited a sub-lethal injury (metabolic and structural injury) of 93% while 29% was exhibited strain I (Phillips et al, 1998). Even strains from the same species can possibly respond to stresses differently.

Salmonella Typhimurium DT104, a multidrug-resistant strain, was used to analyze its growth on chicken meat under cold stress storage since it can be enumerated in the presence of other microorganisms and because it was previously isolated from chicken (Oscar, 2014). Chicken breasts, thighs, and skin were inoculated by spotting the surface with *S.* Typhimurium DT104. Subsequently, they were stored at cold storage from -8 to 16 °C for 0 to 8 days. Results indicated that when samples were inappropriately refrigerated at 12 to 16 °C, *S.* Typhimurium

DT104 proliferated at the highest level on thighs, followed by the skin, and breast meat (Oscar, 2014).

Microorganisms differ in responding to a sudden shift or decrease to low temperature affecting their metabolic processes and causing some physiological modifications mainly during lag phase of growth (Wouters et al, 2000; Beales, 2004; Schumann, 2007, 2012). Bacteria can encounter unexpected down-shifts of temperature in the environment that will require them to generate cellular physical and biochemical modifications in response to gene expression regulation. This will include maintaining cell membrane fluidity, DNA supercoiling modifications, solutes uptake and synthesis regulation, cold-shock proteins, mRNA secondary structure modulation and other different responses depending on the cold shock level and exposure time.

6.3.1 The cold shock responses (CSRs):

6.3.1.1 Cell membrane modification

The microbial cell membrane is the first cellular barrier of the external environment. A sudden temperature down-shift leads to decrease membrane fluidity and as a result disrupting its function. The membrane fluidity converts from its liquid phase state to a gel phase state that can be reversed back and retain fluidity. In general, the membrane consists mainly of fatty acids that adjust and maintain membrane fluidity. Marr and Ingraham (1962) observed in *E. coli* that was exposed to low temperature an increase of unsaturated fatty acids synthesis in cell membrane with fatty acid isomerisation modifications as cells in the stationary phase exhibited great mass of cyclopropane fatty acids. In another study by Ng et al (1962), cells growing at a temperature range between 10 to 15^{o}C and subsequently shifted to 37^{o}C exhibited some reduction in growth

rate and acquired at least one generation to recover and grow. The content of unsaturated fatty acids is synthesized through three enzymes FabA, FabB, and FabF, of which *fabF* is the main gene encoding a beta-ketoacyl-acyl carrier protein synthase II with increased activity of Fabf at low temperature (Mansilla et al, 2004). Sinensky (1974) also documented this mechanism in *E. coli* and suggested a homeostatic process that regulates viscosity of membrane phospholipids (Chattopadhyay, 2006; Los and Murata, 2004).

6.3.1.2 DNA topology

DNA characteristics (structure and shape) have an impact on DNA functions. DNA supercoiling is the shape of DNA packed inside living cells in a very high DNA helix coiled with interwound supercoiling in prokaryotic organisms. The state of DNA supercoiling can be either positive "overtwist" or negative "unwind" (Mirkin, 2001). DNA supercoiling has been shown to play many roles in genome functions. It assures that DNA is not damaged through the integration of DNA chains as a requirement for replication initiation, transcription and recombination (Mirkin, 2001). DNA topoisomerases are the enzymes with functions in all DNA associated topological states including DNA supercoiling. Negative DNA supercoiling state is regulated through DNA gyrase and topoisomerase IV while positive DNA supercoiling is regulated by topoisomerases I and III (López-García, 1999; Terekhova et al 2012). In each DNA supercoiling state, the enzymes function to relax negative to positive supercoiling and *vice versa* (Champoux, 2001). The association of DNA negative supercoiling and cold temperature has been previously demonstrated (Prakash et al, 2009; Shivaji and Prakash, 2010). When DNA supercoiling increases in its negative state, it indicates that DNA gyrase and topoisomerase IV were induced

at high levels to effectively maintain the cell DNA replication, transcription and recombination (Mizushima et al, 1997; Shapiro and Cowen, 2012).

6.3.1.3 Protein synthesis and responses

At cold temperature, transcriptional and translation processes become obstructed causing the ribosomal to be functionally ineffective. As a result, this leads to inadequate protein folding, decline in cellular protein biosynthesis, and eventually affecting the growth rate (Chattopadhyay, 2006; Ermolenko and Makhatadze, 2002; Phadtare, 2004). In general, bacterial cells exposed to cold or low temperature depending on the microorganism's optimal growth temperature, goes through three stages (Thieringer et al, 1998; Weber and Marahiel, 2003). The first stage is the transient response immediately after the exposure known as acclimatization post-shock phase and the time duration for this response may vary with regards to growth rate reduction and the gene expression of proteins for cold survival response. Subsequently, the cells enter the second stage known as a recovery phase, with bacterial cells growing more rapidly and gradually resuming cellular protein biosynthesis. In the last stage, the cells become permanently adapted to cold temperature with gene expression modification and this occurs when the cells reach stationary growth phase. When cells are shifted to cold temperatures, numerous proteins are up-regulated in response to the cold shock. They are designated as cold-shock proteins (CSPs), cold-induced proteins (CIPs), and cold acclimatization proteins (CAPs). The first two groups can accumulate and become associated with most of the housekeeping genes being repressed following the cold temperature exposure. It was suggested that CSPs are small expressed proteins with sizes less than 10 kDa and CSPs proteins larger than that should fall within CIP group. However, CAPs are proteins characterized by very high synthesis occurring

primarly during extended exposure and subsequent growth at cold temperature (Hébraud and Potier, 1999; Neuhaus et al, 2000; Panoff et al, 1998; Phadtare, 2004; Polissi et al, 2003).

6.3.1.4 Cold shock protein synthesis

CSPs are small response proteins involved in several molecular functional mechanisms such as DNA replication, transcription, translation, DNA dynamics RNA processing, and other mechanisms yet to be identified (Golovlev, 2003). It has been shown that these proteins are conserved in numerous Gram-positive and –negative bacteria sharing a similarity of more than 45% with CSP families with some consisting of up to nine members. In some bacteria, these *csp* genes are organized in chromosomal clusters (Neuhaus et aI., 1999; Wouters et al, 1998; Yamanaka et aI., 1998; Yang et al, 2009). Other cold associated proteins, CIPs, vary in numbers from bacterial species to another; for instance, 18 proteins have been identified in *E. coli* playing a role in numerous cellular mechanisms.

6.3.2. Cold-induced proteins

6.3.2. 1 CspA: the major cold shock protein

This protein is considered the most studied of all CSPs, mainly in *E. coli*. It binds DNA acting as a gene expression regulator and has transcriptional and translational enhancer properties. Also, it has the ability to bind mRNA acting as a chaperone. Two genes, *gyrA* and *hns*, are transcriptionally activated by CspA by stabilizing the RNA polymerase process. GyrA and H-NS have previously been demonstrated to be involved in the negative DNA supercoiling (Gualerzi et al, 2003; Stella et al, 2006; Phadtare and Severinov, 2010).

6.3.2. 2 The CspA proteins family

CspA is a member of a family of nine homologous proteins, CspABCDEFGHI. Of all the members, only four, CspA, CspB, CspG, and CspI, have been demonstrated to be strict cold shock proteins with overlapping functions among them. Following the cold temperature exposure, numerous cold-shock proteins are expressed at early stages including CspA, CspB, CsdA, and NusA. During the late acclimation phase, other proteins are expressed such as GyrA, H-NS, IF-2, and TF. At least 26 cold-shock genes have been verified previously (Phadtare and Severinov, 2010).

CsdA. Cold-shock DEAD box protein A, previously known as "DeaD", is one of five members of the DEAD-box family, a branch of the helicases superfamily 2 (Kaberdin and Bläsi, 2013). Toone and others (1991) were the first to isolate this gene from *E. coli* with ATP-dependent RNA helicases functioning in suppressing mutations in the gene, *rpsB*, which encodes for ribosomal protein S2 and also restores the protein S1 of 30S ribosomal subunit (Iost et al, 2013; Phadtare and Severinov, 2010). This protein is involved in several cellular mechanisms, including ribosome biosynthesis, translational initiation, and mRNA decay by stabilizing the *cspA* mRNA for the major cold shock protein A (CspA). During cold shock conditions, it binds the RNA degradosome with RNase E and is required for ribo-regulation of *rpoS* mRNA (Horn et al, 2007; Kaczanowska and Rydén-Aulin, 2007; Peil et al, 2008; Shajani et al, 2011; Weber and Marahiel, 2003).

NusA. This protein is a component of an anti-termination complex and induced earlier at DNA transcription to bind RNA polymerase and influences pausing and/or termination of transcription. It also influences transcriptional anti-termination and stabilizes the RNA

polymerase process. It was identified to be induced under cold temperature (Bae et al, 2000; Mah et al, 2000).

DnaA. A cold shock protein plays a central role in the initiation of chromosomal and mini-chromosomal DNA replication on *oriC*. It also auto-regulates the *dnaA* gene and influences cell membrane structural properties (Altlung and Hansen, 1999; Atlung et al, 1985; Braun et al, 1985; Kaguni, 2006; Messer and Weigel, 1997; Wegrzyn et al, 1999; Wegrzyn and Wegrzyn, 2002).

RecA. A cold shock protein is involved in the recombination and the SOS response for DNA repair. The SOS response occurs when RecA inactivates LexA and as a result over 31 genes are up-regulated. In addition, the elevated amount of active RecA in the cytoplasm can associate them with the membrane (Han and Lee, 2006; Lee and Lee, 2003).

TF. This is a molecular chaperone known as a trigger factor. It is expressed by the *tig* gene and induced by multiple stresses and is involved in ribosome binding. It is induced under cold shock condition and has a role in improving the cellular viability of *E. coli* when temperature falls between 4^{o}C and 16^{o}C where the level of TF protein increases significantly (Kandror and Goldberg, 1997). Also, the protein is involved in co-translational proteins folding and sustains the exportation of proteins in a structurally efficient state through the support of the cold-damaged proteins refolding (Barria et al, 2013; Han and Lee, 2006; Phadtare and Severinov, 2010).

RbfA, Ribosome-binding factor A and a cold-shock protein, is essential for efficient translation (16S rRNA processing and 30S ribosomal subunit) and cell growth at cold temperature. The *rbfA* mRNA has a section containing an A/T rich sequence downstream where the start codon is known as a translation enhancing element. In cold-shock mRNAs, it has been

recognized as a translation initiation enhancement factor (Barria et al, 2013; Kaczanowska and Rydén-Aulin, 2007; Phadtare and Severinov, 2010; Qing et al, 2004; Shajani et al, 2011).

PNPase. This enzyme, **p**oly**n**ucleotide **p**hosphorylase, is encoded by the *pnp* gene. It is a major *E. coli* degradosome element with a 3′-to-5′ exonuclease mainly involved in RNA metabolism. PNPase activity has been demonstrated to be significantly essential at cold induced conditions for cell survival and growth (Haddad et al., 2009; Hu et al., 2014; Mathy et al., 2001). In addition, it is induced at a post-transcriptional stage and is auto-regulated with a role in inhibiting translation and stabilizing mRNA. Furthermore, it suppress the CSPs family production at the end of the acclimation phase (Barria et al, 2013; Iost et al, 2013; Kaberdin and Bläsi, 2013; Phadtare and Severinov, 2010).

KsgA. This protein is a dimethyl adenosine transferase (a 16S rRNA adenine methyltransferase in *E. coli*). The *ksgA* gene is critical at cold induced temperatures for cell growth rate, and is a regulator of ribosome biogenesis (Kaczanowska and Rydén-Aulin, 2007; Shajani et al, 2011; Zhang-Akiyama et al, 2009).

SrmB. This protein is a member of the DEAD-box family of the helicases superfamily 2 (Kaberdin and Bläsi, 2013). It was first isolated by Nishi and others (Nishi and Schnier, 1986; Nishi et al, 1988). It plays a role in ribosome biogenesis mainly for the assembly of the 50S ribosomal subunit. It has been shown that SrmB is involved at the ribosomal biogenesis in advance of CsdA. At cold temperatures, it causes a defect in cell growth when deleted and was over-expressed in the wild type strain of *E. coli*. In addition, the *srmB* mutant exhibited a major increase of un-processed RNA with the most of the increase occurring in 40S ribosomal. In addition, it was proposed that this protein possibly operates as ATP-independent RNA chaperones (without the energy source of ATP hydrolysis) and interacts with 23S ribosomal

RNA subunit (Iost et al, 2013; Kaczanowska and Rydén-Aulin, 2007; Phadtare and Severinov, 2010; Shajani et al, 2011).

IF2. It is the initiation factor 2, encoded by *infB* gene and is involved in the initiation of bacterial translation with GTPase activity (Jones et al, 1987; Laursen et al, 2005). This protein in concert with two other factors, IF1 and IF3, directs the selection of the 30S subunit of initiator tRNA and mRNA translation initiation region to form the "30S pre-initiation complex" and initiates the process. A mutation in IF2 results in the cold sensitive growth phenotype of *E. coli* (Laursen et al, 2003; Victor, 2010).

Other proteins. Several transcriptional analysis (gene expression) studies of the *E. coli* cold shock responses have been conducted and leading to the detection of the primary CSPs and other genes that are involved in this response coming from different categories of functional groups such as motility associated genes (flagellar coding genes), proteins of sugar metabolism and transport, along with some heat shock proteins induced under cold shock. The cold shock proteins CspA, CspB, CspG, and CspE are mainly expressed at the acclimation phase. A study by White-Ziegler et al (2008) conducted microarray analysis on *E. coli* K-12 MC4100 and demonstrated that approximately 7% of the genome (297 genes) exhibited increased expression at low temperature (23^{o}C) in comparison to their optimal growth temperature of 37^{o}C. Of those genes, 122 genes (41%) are under the regulation of the general stress response *rpoS*. Proteins expressed by the genes, *otsA* and *otsB*, revealed in this study synthesized the osmoprotectant, trehalose that plays a role in improving cell viability at cold shock conditions. In particular, 107 genes (36%) are not specifically related to any COG functional group and roughly 50% (149 genes) of the increased expressed genes at low temperature were either hypothetical or with

unknown functions signifying the need of more research to understand the adaptation of microorganisms to low/cold temperature.

6.3.3. Cold shock/stress responses and virulence.

Bacterial adaptation to cold shock/stress produces some cellular changes at different levels from the cell membrane that serves as an initial sensing barrier to the DNA encoding the genetic information (Panoff et al, 1998; Phadtare, 2004; Shapiro and Cowen, 2012). One of earliest studies conducted in evaluating the pathogenicity after freezing was by Sorrells and colleagues (1970). They used a *S.* Gallinarum cell suspension and froze it at -75^{o}C in a bath of dry ice acetone followed by storage at -20^{o}C for 1 day. This treatment yielded three kinds of cells, dead, metabolically injured, and unscathed cells. They compared freezing the cell suspension at -75^{o}C as the sole condition and after storage at -20^{o}C for 1 day. They found that the freezing condition of -75^{o}C resulted in 86% cell death and 29% of survivors were injured cells whereas storing the cell suspension at -20^{o}C for 1 day after freezing at -75^{o}C increased the death rate by 2% (total of 88%) and resulted in 13% additional of injured cells (total of 42%) of the survivors. Furthermore, they evaluated the pathogenicity of un-injured and freezing sub-lethally injured cells by injecting a 1 ml of cell suspension into peritoneum (the body cavity) of 6 week old chicks (180 chicks, 18 groups, 10 chicks in each group). They concluded that the differences between the two treatments were not significant. This demonstrated that metabolically injured cells after freezing preservation were capable of recovering when conditions are favorable and potentially causing infections (Sorrells et al, 1970).

During food processing, foodborne pathogens are exposed to many stresses that possibly have major influence in stress cross-protection. It has been confirmed in numerous studies the

improvement in tolerance and resistance of a microorganism when it is exposed to other subsequent stresses (Rangel, 2011). Broadbent and Lin (1999) demonstrated that the cold shock treatment at 10°C for 2 hours enhanced the resistance of *Lactococcus lactis* species to freezing at −60°C for 24 hours and freeze-drying (lyophilization) (Broadbent and Lin, 1999). A study by Xu and others (2008) evaluated the cross-protective of acid-adapted *S. enterica* subsp. *enterica* serovar Enteritidis strain to resist cold stress (Xu et al, 2008), and concluded that acid shocked cells for a period of 2 hours were more resistance to cold stress than a longer acid shock for 7 hours. Also, they found that acid-adapted *S. enterica* cells can be present in the viable non-culturable (VBNC) state that requires the resuscitation for the transition to the culturable state.

A more recent study by Shah and others (2013) simulated the condition by which *Salmonella* cells are exposed to cold temperatures used in storing foods and subsequently consumed followed by exposure to gastric acidic conditions. They tested the response of *S.* Typhimurium LT2 strain for various stresses (peroxide, osmotic, and acid (pH 5.3) for a time period of 30 minutes (shock) and 5 hours (stress). They observed that only peroxide shock critically decreased cellular survival indicating potential capacity of this foodborne pathogen to endure harsh environmental stresses for several hours mainly during transit and inside the host. Proteomic profiles indicated that 104 proteins were expressed during exposure to cold stress. They were divided into three categories as information storage and processing, cellular processing, and metabolism.

The main and significant genes expressed at cold stress exposure were *cspA*, *cspB, cspC, cspD,* and *cspE*, those of the cold shock proteins (CSPs). Fifty seven ribosomal associated proteins were detected in the profile analysis after the cold stress, 13 proteins (23%) declined and 44 (77%) of the expressed proteins persisted throughout the cold stress treatment. The genes

recA and *ssB* that are involved in recombination, SOS response and repair, were among the genes significantly expressed at the exposure of cold stress. They regulate the expression of numerous genes of membrane biogenesis and transcription. Other genes significantly induced due to cold stress belonged to various functional groups such as oxidative stress, amino acids transport and metabolism, TCA cycle, and complex I (NADH dehydrogenase of Electron Transfer Chain) (Shah et al, 2013).

The process of causing an infection is complicated and involves many steps in bacterial pathogens. Critical steps of infection are adherence and invasion to host cells. Two main bacterial adhesions are fimbriae (pili) (type 1, P, and S fimbriae) and afimbrial adhesion. Afimbrial adhesins are proteins that play a role in colonization as adherence factors, but differ in not forming a long structure such as fimbrial adhesions (Wilson et al., 2002). Cold stress has been shown to enhance the association between *S.* Typhimurium and Caco-2 epithelial cells through adhesion and invasion (Shah et al, 2014). Cold stress induced the gene expression of numerous genes associated with virulence such as Type III Secretion Systems and their effectors for SPI-1 and SPI-2. Other induced genes belonged to cell processes (pathogenesis and DNA transformation), prophage functions, plasmid functions, protein secretion and trafficking, DNA replication, recombination and repair, purine ribonucleotide biosynthesis, and RNA degradation. The SPI-1 and SPI-2 that encode for Type III Secretion Systems (T3SS) are the main factors of *Salmonella* pathogenesis. Some genes associated with SPI-2 T3SS are located outside SPI-2 in SPI-5 are necessary for intracellular replication of *Salmonella* during enteric infection. A set of genes that creates part of the Type IV Secretion System (T4SS), are expressed in response to cold stress. The function of these genes are essential as part of plasmid function and conjugal DNA transformation.

Because of stresses encountered by *Salmonella* during infection, the major modifications required by *Salmonella* to withstand those stresses are metabolism regulatory mechanisms. *Salmonella* switches its aerobic metabolism during the exposure to cold stress to fermentation. This was demonstrated by the detection of the products of the fermented pyruvate, the short chain fatty acids in the medium and on epithelial cells infections with major elevated concentrations as these products triggered the invasion into the epithelial cells of the intestine of host (Shah et al, 2011).

6.4 Molecular Approaches:

6.4.1 Genomics Analysis.

Genome is a term refers to the complete set of genes and chromosomes in an organism. Generally, most genomes are of the nucleic acid, DNA, with a few exceptions of some viruses containing RNA as genomes (Gorbalenya et al, 1989). By mid 1980s, the term "genomics" emerged to describe a new scientific field of sequencing, mapping and analyzing the genomes (Hieter and Boguski, 1997; McKusick, 1997). The path to the genomics era was paved by numerous discoveries by the turn of second half of 20^{th} century. After Watson and Crick revealed the structure of DNA, it opened the way for many scientists in molecular biology with breakthroughs determining the molecular fundamental foundation of the gene functions through gene structure, gene expression, DNA replication, protein synthesis, and other mechanisms (Choudhuri, 2003; Dahm, 2008).

In the middle of 1960s, Robert Holley, an American biochemist, with his group was the first to sequence and determine the structure of alanine tRNA from the yeast, *Saccharomyces cerevisiae*. This finding was the first yet to determine the sequence of a ribonucleic acid (Holley,

1965, 1966, 1968). At the same path, Frederick Sanger, a pioneer scientist for nucleic acids sequencing, with his group were able to sequence the 5S ribosomal RNA from *E. coli* in 1967 and 1968 (Brownlee et al, 1967, 1968). In 1972, Fiers group was able to determine the nucleotide sequence of a gene coding for a protein from the bacteriophage MS2; and subsequently, they reported the first complete genomic sequence of all genes of the RNA Bacteriophage MS2 containing 3569 nucleotide sequence (Jou et al, 1972; Fiers et al, 1976).

In 1973, Sanger published a paper describing the procedure of using DNA polymerase I to determine the DNA nucleotide sequence of phage fl (Sanger et al, 1973). Not long after, Sanger and Coulson (1975) reported a simple and rapid method known as "plus and minus" to verify DNA sequences with DNA polymerase I from *E. coli* and T4 DNA polymerase from bacteriophage (Sanger and Coulson, 1975). It was not long after that Sanger and colleagues published two papers. One paper was to report the first complete sequence of DNA genome, and the second to document the method by which they utilized for DNA sequencing with chain-terminating inhibitors (Brenner, 2014; Sanger et al, 1977; Roe, 2014). In 1978, Sanger and Coulson developed a method that made an impact on visualizing the DNA sequencing with a better rate and resolution of separating DNA fragment bands using thin acrylamide gels (Sanger and Coulson, 1978). The mitochondrial DNA of human cells was also sequenced in 1981 by the Sanger group (Anderson et al., 1981).

At early 1980s, another innovation was developing that revolutionized the field of molecular biology. Kary Mullis developed a simple polymerase chain reaction using DNA polymerase from *E coli* to amplify the desired DNA fragment(s) (Saiki et al, 1985). His method had one limitation that the heating step required to denature the DNA was also inactivating the DNA polymerase which made it necessary to add DNA polymerase after each cycle (Bartlett and

Stirling, 2003). The drawback of this technique was later overcome by using thermo-stable polymerase known as Taq DNA polymerase that was first isolated from the extreme thermophile *Thermus aquaticus* by Chen and others (1976) (Saiki et al, 1988). In 1984, another groundbreaking discovery was published by Francis Collins. A technique known as "chromosome jumping" was employed for DNA fragments positional cloning as a method for disease gene identification with unknown function (Collins et al, 1984).

By the mid of 1980s, the interest of sequencing the human genome began to mature mainly driven by the U.S. Department of Energy (DOE)/ the Office of Health and Environmental Research (OHER). In 1983, GenBank was established as the database of DNA sequences, and by 1990, the basic local alignment search tool (BLAST) was introduced to search the database for query sequence homologous. By 1986, the DOE initiated the Human Genome Sequencing Project (Choudhuri, 2003). Two years later, the National institute of Health (NIH) joined DOE collaborating in Human Genome Sequencing Research, and by 1989, NIH named their Human Genome Research office as the National Center for Human Genome Research (NCHGR). In October 1990, the Human Genome Project (HGP) led by the U.S. was a collaborative enterprise performed through the International Human Genome Consortium in many research centers from 5 countries beside the U.S., which included the United Kingdom, France, China, Japan, and Germany. In 1997, NCHGR became National Human Genome Research Institute (NHGRI), and DOE established the Joint Genome Institute (JGI) (Choudhuri, 2006).

Another human genome sequencing project was initiated in parallel with HGP by Celera Genomics, a private company in 1998 to complete sequencing the human genome ahead of the HGP targets within a 3 year timeline (Venter et al, 2001). In 2003, the Human Genome Project announced the completion of the project. Throughout the path to the completion of HGP, several

species have had their genomes sequenced in an effort to understand some of the species-specific biology, molecular mechanisms and to identify the possible conserved genes that cross various species (Collins et al, 2003). In 1995, the whole genome of *Haemophilus influenza* was the first completed sequenced of free living organisms, and by 2009 approximately 250,000 organisms had their genomes sequenced and publicly available at National Center for Biotechnology Information (NCBI) (Bickel et al, 2009).

Numerous genome sequencing projects have been either finished or in the process of being sequenced for many model organisms. With this massive quantity of data, researchers turned from just studying several genes to more comprehensive systematic studies of the interactions of biological systems structure and functions. In late 2003, this transition necessitated the establishment of the **ENC**yclopedia **O**f **D**NA **E**lements (**ENCODE**) Project, a large scale international project consortium. The aim of this project is to identify the functional characteristics in genomics analysis (Ecker et al., 2012; The ENCODE Project Consortium, 2004).

The vast database of sequenced microbial genomes paved the way for a new transition with the emphasis on functional genomics studies. The analysis required the evaluation of gene function in the basis of genome-wide screening by the combination of high-throughput techniques with bioinformatics analysis. This expanded the scope of the microbial investigations to identify the roles of all genes simultaneously under certain conditions during the life cycle of microorganisms. For example, approximately 33% of the coding genes in *E. coli* are associated with unknown functions or are still be hypothetical (Gagarinova and Emili, 2012). Numerous methods have been developed for investigating bacterial gene functions such as improved cloning methods (McKinnell and Di Berardino, 1999), DNA microarray (Heller, 2002), serial

analysis of gene expression (SAGE) (Velculescu et al., 1995), and the more recent next-generation sequencing (NGS) (Schuster, 2008) approaches.

At the beginning of 21st century, new platforms with sophisticated capability were developed for newer more advanced technologies. They are known as massively parallel sequencing or Next-generation DNA sequencing (NGS) (Metzker, 2010). These massively parallel sequencing platforms have been commercialized by different companies (Ku and Roukos, 2013; Metzker, 2010; Reis-Filho, 2009; Schuster, 2008). They differ in their template preparation and sample(s) processing. The amplification step in 454 FLX Roche system and ABI SOLiD system is an Emulsion PCR (emPCR) bead-based enrichment method with one DNA molecule per bead and clonal amplification occurs in micro-reactors in an emulsion, but are different in sequencing strategy. The 454 FLX Roche system uses pyro-sequencing techniques that are sequenced by synthesis whereas ABI SOLiD system utilizes ligation sequencing procedures. The llumina genome analyzer utilized Bridge amplification step and reverse terminator sequencing strategy with one DNA molecule per cluster. A system known as Helicos Heliscope with no amplification used the sequencing strategy with a single molecule sequence. Numerous NGS systems are now in the process of development to enter implementation for routine use that started in 2004. These technologies have rapidly progressed from next-generation sequencing or second-generation sequencing platforms to third-generation sequencing and to fourth-generation sequencing, The main factors for improving and developing new high-throughput sequencing systems include sequencing rate, read lengths, simple sample preparation, and lower analysis cost (Ku and Roukos, 2013; Perkel, 2011).

6.4.1.1 Mutagenesis-based techniques

For functional genomics screening of a microorganism, an efficient and comprehensive library of random transposon insertions is utilized by combining transposon mutagenesis and high-throughput parallel sequencing through NGS. This molecular approach can assist in determining the essential genes for survival and/or growth in any particular conditions. Transposon mutagenesis is an advanced molecular genetics method that has been utilized to study prokaryotes and eukaryotes (yeasts) with emphasis on microbial pathogenesis. Different kinds of transposon have been introduced and applied since it was first introduced by Kleckner and colleagues (Kleckner et al, 1977; Kleckner, 1981).

6.4.1.1.1 Signature-tagged mutagenesis (STM)

Signature-tagged mutagenesis is a technique that was developed to identify virulence factors of *S.* Typhimurium in mouse BALB/c systemic infection model as the first negative selection screening duo to loss of function (Hensel et al., 1995). A follow up study by Shea and others (1996) using STM identified the SPI-2 containing the second T3SS. Tsolis et al (1999) conducted a genome screening for *S.* Typhimurium as a comparative study using the mouse BALB/c and Calf infection models. This mutagenesis method utilizes the insertion of random transposon sequences tagged with a specific defined DNA sequences (barcodes). These barcodes serve as a mean for determining the presence of individual mutants within a complex DNA input pool of transposon mutants and missing transposon mutants in the output pool (Hensel et al., 1995). In general, the identification of essential genes in specific conditions using STM requires two major steps, mutants' library construction and *in vivo* screening.

The mutants library can be constructed through four various approaches. The first way is the original STM where tagged oligonucleotides are synthesized randomly and subsequently cloned into the pUT mini-Tn5. They in turn are transformed into a recipient to form pools of tagged mutants for screening of specific tags and finally stocked into a micro-titer plate (De Lorenzo et al., 1990; Hensel et al., 1995). The second method is an STM with the synthesized tags ligated to short random genomic library of DNA fragments to create a library of tagged plasmid either without a transposon using Insertion-duplication mutagenesis (Polissi et al., 1998) or shuttle mutagenesis (Claus et al., 1998). This library can be used for the insertion mutagenesis through homologous recombination, followed by transformation and creation of pools. The pools can be screened for specific tags before generating the master STM clone plate. A third STM library process involves 96 specific tags from clones selected after hybridization and supsequently transformed to generate pools with 96 tagged mutants (Mei et al., 1997). The last modification of STM is the incorporation of PCR-based avoiding the use of hybridization. In this process, 21 nucleotide sequences with the variable 3' end defining 12 specific tags are applied in separate PCR reactions to integrate each individual tag in the amplified DNA fragments. After conjugation, twelve mutant libraries with variable tags are formed and for screening each pool contains 12 tagged mutants (Lehoux et al., 1999).

The second major stage is *in vivo* screening of the generated STM library. This stage can be processed either through hybridization-based or PCR-based STM. In the hybridization-based STM, the *in vitro* culture is formed by pooling mutants to create the input pool, used for *in vivo* passage through animal or plant models, and recovery of the output pool. Subsequently, genomic DNA from both pools are extracted and used as templates for preparation of probes for Dot-blot hybridization. In PCR-based STM, genomic DNA of the *in vitro* pool (input pool) and *in vivo*

pool (output pool) are used in comparative PCR through gel electrophoresis. These variations of STM have been applied to comprehensively identify and characterize genes in numerous microorganisms (Autret and Charbit, 2005; Dziva et al., 2004; Grant et al., 2005; Hendrixson et al., 2004; Kavermann et al., 2003; Kelly et al., 2006; Merrell et al., 2002; Mundy et al., 2003; van Diemen et al., 2005). It has been extensively utilized to identify *Salmonella* genes necessary for colonization of calves, chicken, and swine that are the primary sources of contaminated meat, poultry, and their products (Carnell et al., 2007; Ku et al., 2005; Lichtensteiger et al., 2003; Morgan et al., 2004; Pullinger et al., 2007; Santiviago et al., 2009; Shah *et al.*, 2005). This technique unites the advantages of transposon mutagenesis with the ability to practically screen numerous mutants using fewer animals with less intensive lab and less time.

6.4.1.1.2 *Mariner*-based mutagenesis

Mariner transposons are one of the best-studied transposons and are insect-derived transposons include Mariner-Like Elements (*MLEs*) (*Hyalophora cecropia*, *Caenorhabditis elegans* and others), *Mar1* (*Dugesia tigrina*), *p19* (*Drosophila erecta*), and *Himar 1*(*Drosophila mauritiana* and the horn fly, *Haematobia irritans*). They are members of the large super-family, Tc1/*mariner* DNA transposable elements that range from approximately 1.3 kbp to 2.4 kbp in length with approximately 30-bp in two flanking inverted repeat sequences encoding for the transposase (Plasterk et al., 1999). A mariner element was initially discovered in *Drosophila mauritiana* (Jacobson et al., 1986; Robertson, 1993; Robertson and Lampe, 1995). It has been applied successfully to Gram-negative and Gram-positive bacteria for random mutagenesis as they do not require species-specific host factors for adequate transposition. Also, its derivatives are commonly employed for random mutagenesis in both prokaryotes and eukaryotes (Hayes,

2003). Numerous studies have applied this mariner-based mutagenesis in various bacteria. Akerley and others (1998) applied *Himar 1* mutagenesis to *Haemophilus influenzae* and *Streptococcus pneumonia*. They identified some loci and assigned functions to them through genetic footprinting screening (Akerley et al., 1998). A mariner-based transposon was developed for the pathogen *Listeria monocytogenes* and demonstrated that *prsA2* is critical for virulence (Zemansky et al., 2009). The mariner was also incorporated in STM for some studies including *Campylobacter, Neisseria meningitides* (Pelicic et al., 2000), *Streptococcus sanguinis* (Paik et al., 2005). A constructed random mutagenesis library was successfully employed using the new derivative *HimarBP* in *Burkholderia pseudomallei* (Rholl et al., 2008). Another derivative, *MAR2x T7*, has been applied in *Pseudomonas aeruginosa* PA14 and over 300 putative essential genes were identified.

6.4.1.1.3 Phage Mu mutagenesis

Bacteriophage Mu is one of the temperate phages with higher mutation rates. It is considered the first to be described of the known prokaryotic transposable elements (Toussaint and Resibois, 1983). An earlier demonstration of Mu is that it is randomly inserted into single bacterial genes at several sites (Bukhari and Zipser, 1972). In general, phages infecting a host cell enter one of the following states, the lytic cycle or the lysogenic state. In the lytic cycle, the phage injects its genetic material into the bacterial host cell, reproduces more phage copies and gets released after destroying the cell. In the lysogenic state, the viral genetic material gets integrated into the cell chromosome. Unlike other phages, Mu uses both states and their genetic material integrates randomly in the host genomic DNA inactivating the gene(s) in which they get inserted into. The procedure involves going through a process of nicking and joining the 3'-ends

of the transposon to the target (Chaconas and Harshey, 2002; Paolozzi and Ghelardini, 2005). This phage has the ability to replicate by transposition (Shapiro, 1979; Symonds et al., 1987) with high frequency and a low transposition bias in target site selection. This advantage of insertions makes recovery of mutants from chromosome fairly straight forward for non-essential genes (Groisman, 1991; Rice, 2005).

The Mu phage contains approximately a 38 kb linear DNA molecule of which 36 kb consists of Mu DNA, 1.65 kb of host DNA with 51% GC contents. Although Mu is a transposon, it ends are not inverted repeats. For transposition, phage Mu requires two trans-acting proteins, MuA and MuB. Also, it maintains MuL, the left end and MuR, the right end sequences of the Mu genome required to act in cis mode (Harshey and Bukhari, 1981; Pato, 1989). Several studies have applied Mu transportation in *Salmonella* species. Bowe and others (1998) demonstrated that approximately 4% of the *S.* Typhimurium genome is necessary for virulence as the genes exhibited attenuation in mice infection (Bowe et al., 1998). In another study, an effective phage Mu DNA transposon mutagenesis was developed and tested in *S.* Typhimurium to generate an efficiency of 10^4 to 10^6 CFU/ μg (Lamberg et al., 2002). Frye and colleagues (2006) isolated some mutants of derivative Mu insertions that directed to the identification of some *S.* Typhimurium novel flagellar genes. A high-throughput study was conducted by Chaudhuri and others (2009) using a quantitative screening by combining transposon mutagenesis that included custom Mu transposon and a developed microarray, Transposon-Mediated Differential Hybridisation (TMDH). They were able to comprehensively identified genes essential for mice infection.

6.4.1.1.4 Tn: A Transposon Family

This is a distinct class of transposons (Tn) and is also known as bacteria-derived transposons belongs to class II (DNA transposons) of transposable elements (Muñoz-López and García-Pérez, 2010; Pray, 2008; Vizváryová and Valková, 2004). Numerous random integrated transposon studies have been applied in bacteria (Anriany et al., 2006; Bertram et al., 2005; Clavijo et al., 2006; de Los Santos et al., 2005; Dorsey et al., 2002; Filiatrault et al., 2006; Fricke et al., 2009; Hartmann et al., 2010). A two-step transposition mechanism is generally shared by all transposons of this class. It includes Tn*3*, Tn*5*, Tn*10*, Tn*552*, the modified Tn*7*, and others.

Two genes are encoded; for instance, *tnpA* gene and the *tnpR* gene of Tn*3*, or *tnpB* gene of Tn*552*. Both gene products play a role in the transposition final products formation. The *tnpA* gene encodes a transposase and the other gene (*tnpR* or *tnpB*) encodes a site-specific recombinase. These transposons have sizes of at least 5 kb flanked with long terminal inverted repeats of related sequence (35 to 40 bp). Alongside their transposition function, in most cases they encode for genetic markers such as antibiotic resistance or heavy metal resistance included within the transposing segment. The Tn*3* and Tn*3*-like transposons, Tn*917* and Tn*4430*, use a replicative transposition mechanism with 5-bp target site duplication. In Tn*5* and Tn*10*, the transposition mechanism is via a cut-and-paste procedure with a 9-bp target site duplication with Tn*5* utilized for random mutagenesis and the Tn*10* requires a unique sequence in target (Choi and Kim, 2009).

6.4.1.1.4.1 Tn5-based Transposon

A Tn5-based transposon was originally identified as transposition element in lambda bacteriophage by Berg and other (1975) encoding for kanamycin resistance. Subsequent to its

recognition, several studies have shown the basic requirments of this transposon for transposition (Boucher et al., 1985; De Bruijn et al., 1984; De Lorenzo et al., 1990; Kwon and Ricke, 2000; Kwon et al., 2003a, b; Park et al, 2002; Simon, 1984). Since then, Tn5 system has become a very useful tool for the study of molecular mechanisms through the utilization of transposition. It has identical inverted repeat sequences, IS*50*L and IS*50*R with a set of three resistance genes encoding for kanamycin, bleomycin, and streptomycin in the Tn5 central region. The entire transposition process is catalyzed by the Tnp protein, the transposase protein (cis-acting protein) encoded by the inverted sequence, IS*50*R. The transposase protein acts by binding immediately to the unique 19-bp nucleotide sequences at both ends of its recognized transposable elements (Goryshin and Reznikoff, 1998; Reznikoff, 2003, 2008; Vizváryová and Valková, 2004).

6.4.1.1.4.2 Transposome

The first report of using *in vitro* transposition in a cell-free reaction system was by Mizuuchi in 1983 for bacteriophage Mu. It was identified as a protein-DNA complex and later was named "transpososomes" (Mizuuchi, 1983; Mizuuchi et al., 1992; Savilahti et al., 1995; Surette et al., 1987). Transposition in general is a process that includes deletion, insertion, inversion, or chromosome fusion (Biel and Berg, 1984; Goryshin and Reznikoff, 1998; Kleckner, 1981). In the late 1990s, Goryshin and Reznikoff (1998) developed a system for *in vitro* transposition with a modified Tn5 transposase. Furthermore, other *in vitro* mutagenesis systems have been developed for Tn*7*, Mu, *Himar1*, and Ty*1* (Akerley e al., 1998; Devine and Boeke, 1994; Gwinn et al., 1997; Haapa et al., 1999). In the Tn5 system, the transposome is a transposon-transposase complex formed between the Tn5 transposon and Tn5 transposase recognizing the hyperactive 19 bp Mosaic End (ME). This complex is formed in the ratio of

1:2:1 for Tn5 transposon, transposase, and 100% glycerol (Goryshin et al., 2000). When this complex is introduced to the target bacteria, the transposase is activated by Mg^{+2} in the host's cell resulting in random insertion of the transposon into the genomic DNA of the bacterial cells and the plasmid.

6.5 High-throughput profiling (massively parallel sequencing)

The mid 1990s marked the completion of the first genomic DNA sequencing of the free living organism, *Haemophilus influenzae* Rd, by Fleischmann and others (1995) that opened the path for other microbial genomes to be sequenced. Although numerous completed genomes deposited in the public database, we still do not yet know or fully understand the functions of numerous genes. Simultaneously, the advancement that have been made in the use of transposon mutagenesis and combining it with the high-throughput microarray and afterward with NSG assisted in verifying gene functions (Chan et al., 2005; Chaudhuri et al., 2009; Gresham et al., 2008; Groh et al., 2005; Lamichhane et al., 2006; Salama et al., 2004; Sassetti et al., 2001; Winterberg et al., 2005).

In 2004, new techniques of high-throughput sequencing were commercialized and later were used by many labs. This advanced technology initiated a new era of screening transposon mutagenesis libraries by massively parallel sequencing. Four groups introduced their developed methods for microbial system analysis in 2009. All four techniques depend on the combination of the next generation sequencing and transposon mutagenesis and became known as transposon insertion sequencing. Due to the important of the "fragmentation" step in all methods, we are dividing them to restrictive and non-restrictive transposon insertion sequencing. In restrictive methods, specific restriction enzymes are utilized for DNA fragmentation of the genomic DNA

from a transposon mutant library during the preparation of the genomic DNA for sequencing. In contrast, mechanical shearing, sonication, and other physical methods are applied to fragment the genomic DNA of transposon library.

6.5.1 Restrictive transposon insertion sequencing

Goodman et al. (2009) developed **in**sertion-**seq**uencing (**INSeq**) and later refined the technique (2011) by adding a purification step for genomic regions with mariner transposon-junctions as they were amplified by linear PCR with a biotinylated primer. Subsequently, the biotinylated fragments attached to the magnetic beads and were digested with *MmeI*. These digested fragments were subsequently indexed with sample-specific barcoded adapters (Goodman et al., 2009, 2011). In the main INSeq study, Goodman et al. (2009) applied the two power methods, transposon mutagenesis and the massively parallel sequencing to the human gut bacterium, *Bacteroides thetaiotaomicron*, using a mariner transposon to generate two libraries of approximately 35,000. They investigated the genetic requirements of this bacterium under *in vitro* growth conditions as well as *in vivo* conditions within a murine model gut to evaluate the influence of other microbial communities in the ability of *B. thetaiotaomicron* to adapt and establish a symbiotic relationship with their host.

van Opijnen et al. (2009) developed a similar technique around the same time. They called it as **Tn-seq** short for transposon insertion sequencing, a genome screening method for the quantitative determination of fitness of the Gram-positive bacterium *Streptococcus pneumonia*. They used the mariner *Himar1* transposon derivative *magellan6* with *MmeI* recognition site at both inverted repeats. Six libraries were generated each with approximately 25,000 insertions and screened for genes that were important for basal growth, mainly for transcriptional

regulation and carbohydrate transport (van Opijnen et al., 2009; van Opijnen and Camilli, 2010). In 2012, Khatiwara and others developed a new version of Tn-seq using a modified transposon of the commercialized transposon (Tn5) for transposon mutagenesis (EZ: Tn*5*). They used *S.* Typhimurium to generate a library of approximately 16,000 transpoon insertion mutants. The transposon insertions contained a single-end recognition site of type IIS enzyme BsmFI in one mosaic end (ME, inverted repeat). They used the constructed library to screen three conditions mimicking stresses encountered by the pathogen inside the host (human and/or chicken). This was the first study that simultaneously screened and pooled the Tn5 insertion samples from multiple conditions for subsequent sequencing by next generation sequencing. Barcoded primers were used to differentiate DNA samples from each condition. They were able to identify 105 conditionally essential genes that could be considered protein-coding genes (Khatiwara et al., 2012).

6.5.2 Non- restrictive transposon insertion sequencing

Gawronski et al. (2009) developed a technique they called **h**igh throughput **i**nsertion **t**racking by deep **s**equencing (**HITS**) for genome wide screening of *H. influenza*. In this method, a mariner-based transposon mutagenesis library was generated resulting in approximately 75,000 transposon insertions. This genomic approach was applied to identify essential genes that allow *H. influenza* to persist and survive in lung using a murine pulmonary model (Gawronski et al., 2009).

Langridge et al. (2009) constructed one of the largest libraries yet with approximately 1.1 million Tn5 transposon mutants to screen *Salmonella* Typhi genome. They developed a method termed **tra**nsposon **d**irected **i**nsertion-site **s**equencing (**TraDIS**) to conduct genome screening for

essential genes for growth under laboratory and bile tolerance conditions (Langridge et al., 2009). In another study, TraDIS was applied to a library of approximately 1800 insertions of STM in *E. coli* O157:H7 (EHEC) as a demonstration of previously identified genes in screening of calves inoculated with this microorganism (Eckert et al., 2011).

Christen et al. (2011) conducted their study on the bacterium, *Caulobacter crescentus*. They screened a Tn5 mutants library of approximately 800,000 insertions with 428,735 being inserted in unique sites. The aim was to analyze the association of non-coding RNAs (ncRNAs), other non-coding regions to *C. crescentus* fitness and recognized essential promoter requirements (Christen et al., 2011). A study conducted by Zhang and colleagues (2012) to identify genes, domains and other numerous non-coding regions such as regulators and non-coding RNAs in *Mycobacterium tuberculosis*. They scanned two libraries each with approximately 100,000 mutant insertions, of which 36,488 represented unique inserted sites. In 2013, Pickard et al. conducted a study on *Salmonella* Typhi screening a Tn5 mutant library of 1.1 million; of which 370,000 were in unique inserted sites. The screen was to identify genes associated with ViII bacteriophage killing activity during phage infection using TraDIS (Pickard et al., 2013).

A modification to the original Tn-seq resulted in a straight forward technique that is applicable to numerous restricted species. This method is termed **TnLE-seq,** transposon liquid enrichment sequencing. TnLE-seq was applied to the strictly anaerobic soil bacterium *Desulfovibrio vulgaris* Hildenborough. Since this bacterium is an obligate anaerobe, the use of solid media would possibly expose it to oxygen resulting in a very low growth. This restriction along with the low recovered mutants from electro-transformation supported the use of the conjucation transformation as a naturally occurring process between numerous bacteria. This was followed with the recovery step by the dilution in liquid media that minimized the exposure to

oxygen and is considered time and labor saving with the high efficiency growth of many bacteria. More selection steps were added for the final pool of transposon-junction sequences. Since the donor strain requires diaminopimelic acid to grow, this was used to control the growth of the donor strain during enrichment step. To remove the extracellular DNA, the lysis step of recovered mutants was processed using DNase exonuclease digestion to eliminate donor plasmid DNA. Two PCR steps were carried out to enrich for the Tn unique insertion sites and give the final size fragments with the Tn-junction sequences for the high-throughput sequencing (Fels et al., 2013).

7. Aims and Objectives

The long-term goal of this dissertation is to understand the molecular mechanisms utilized by *S.* Enteriditis and Typhimurium as the two most primary serovars in causing human infections through contaminated food. The immediate goals of this dissertation project are:

1. To compare the capability of some *S.* Enteriditis selected strains in invading laying hen ovarian follicles as a vertical route of contaminating intact eggs before forming the egg shell. *S.* Enteriditis is the most routinely isolated strain from contaminated egg and causing egg-borne infections. We used a previously tested method by Howard et al. (2005) to conduct our study with some modifications.
2. To generate a mutant library of *S.* Enteriditis strain from first objective using transposon mutagenesis with enhanced transformation efficiency. This library will be a source of several projects in discovering gene associated with numerous mechanisms of virulence and pathogenicity. As a future project, the generated library will be used to screen the genome of *S.* Enteriditis for the required genes for *in vitro* association and invasion of

laying hen ovarian follicles. Commercialized transposon construction vector, EZ-Tn5^{TM} pMOD-6 <KAN-2/ MCS>, will be used to construct a transposon-transposase complex and subsequently transformed into the *S.* Enteriditis strain.

3. To examine some *S.* Typhimurium mutants for surviving thermal stress and growing in temperatures associated with human and poultry body temperatures using Phenotype Microarray. The mutants were selected from a previous study by Khatiwara et al. (2012) and exhibited deficiency under temperatures of 37^{o}C and 42^{o}C.
4. To screen the genome of *S.* Typhimurium and identify essential genes for survival under cold temperature storage in association with chicken carcass using a transposon insertion sequencing (Tn-seq) method. A constructed library will be used to inoculate chicken carcasses in triplicate trials and recover transformants will be compared to their input pool to identify the defective phenotypes. Subsequently, the identified phenotypes will be single mutants deleted and tested for survival in competition assay mixing each one with the wild type to verify the gene function at cold temperatures.

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Chapter 2

In vitro Invasion of Laying Hen Ovarian Follicles by *Salmonella* Enteritidis strains

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Short Title: *Salmonella* Enteritidis Ovarian Follicles Invasion

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ABSTRACT

Salmonella is the major foodborne bacterial pathogen worldwide. Among numerous serotypes, *Salmonella* Enteritidis (SE) is one of the most common *Salmonella* serotypes responsible for human infections in the United States. The main source of SE outbreaks is foods associated with raw or undercooked chicken eggs. SE is the only serotype that routinely contaminates eggs. The transovarian transmission of SE and subsequent contamination of the eggs before egg shell formation is considered to be the main route of egg-contamination by SE. To evaluate if invasion of ovarian follicles is an important step during the production of eggs contaminated by SE, we used an *in vitro* invasion assay to determine ovarian follicle invasion by five SE strains. After inoculating the freshly collected ovarian follicles, all five SE strains were able to invade into the follicles after 2 hrs incubation at 37^{o}C. The mean percentage of SE invasion ranged from 0.016 -0.034% and there was no significant difference among the SE strains. For *Escherichia coli* (EC) K12 strain, which was used as a negative control, the mean percentage of invasion was 0.0003%. The *in vitro* follicle invasion by SE strains demonstrated in this study may reflect their ability to invade ovarian follicles in laying hens once SE cells reach ovaries through various routes.

Key words: *Salmonella* Enteritidis, laying hen, egg, ovarian follicle, invasion

INTRODUCTION

Human infections by foodborne bacterial pathogens are a critical public health problem. Among them are non-typhoid *Salmonella* serotypes, one of the major causes of human foodborne illnesses in the United States and worldwide (Guard-Petter, 2001). According to the Centers for Disease Control and Prevention (CDC), the annual number of salmonellosis cases in the United States was estimated to be 1.4 million (Mead et al, 1999). The transmission vehicles of *Salmonella* are wide-ranging sources, but most incidences of this foodborne illness have been traced to the consumption of the poultry meat, eggs, and their food products (Andrews and Bäumler, 2005; Rabsch et al., 2001). The fact that these pathogens infect the host animals without observable sickness contributes to high numbers of human infections (Angulo and Swendlow, 1999; Patrick et al, 2004; Poppe, 1999).

Among the more than 2,500 serotypes of *Salmonella*, the serotype Enteritidis (SE) is one of the most common serotypes implicated in human illness. This serotype has been accountable for 14% of all foodborne human cases of salmonellosis in the United States (Braden, 2006), making it the second most common *Salmonella* serotype after *S.* Typhimurium. In the European Union, SE is responsible for 62.5 % of human salmonellosis (Gantois et al, 2009). SE has distinct ecological characteristics in association with chicken shell eggs, which raises unique concerns about public safety. Before the 1950s, there was no reported incidence of SE due to the consumption of raw or undercooked chicken eggs or their products in the United States (Andrews and Bäumler, 2005; Bäumler et al, 2000; Rabsch et al, 2001). From the late 1970s to the mid 1990s, the incident of SE increased rapidly, reaching pandemic proportions, with most outbreaks associated with the consumption of raw or lightly-cooked hens' eggs (Velge et al, 2005; Ward et al, 2000). In fact, among more than 2,500 serotypes SE is the only serotype that

routinely contaminates chicken eggs, suggesting that SE possesses unique genetic and phenotypic characteristics that enable routine egg contamination by this serotype.

The modes of transmission are considered to be through both vertical (transovarian) and horizontal (shell egg penetration) routes. The vertical route of transmission is a result of the infection in reproductive tract that causes the contamination of the eggs by SE before egg shell is formed (Gast and Beard, 1990; Okamura et al, 2001). In the horizontal route, SE penetrates the egg shell and multiplies within the egg after the shell is contaminated with SE from the external environment (Messens et al, 2006; Cox et al, 2000). Although there is experimental evidence supporting transovarian transmission through the vertical route, it still remains uncertain which steps in the process are associated with the unique ability of SE to contaminate eggs routinely (Gast et al, 2004). One possible mechanism is that SE cells once transmitted to reproductive tract, invade into ovarian follicles at different stages of development before egg shell formation.

In this study, we investigated the ability of SE strains to invade laying hens' ovarian follicles by *in vitro* invasion assays in an effort to evaluate the importance of follicle invasion in the process leading to the production of SE-contaminated eggs.

MATERIAL AND METHODS

Bacterial strains and Preparation of Bacterial cultures

Five different strains of SE and *Escherichia coli* (EC) K-12 strain were used in this study (Table 1). All frozen bacterial cultures were resuscitated by transfer into Luria-Bertani (LB) broth media (Difco, Sparks, MD) for two successive cycles of 24 hours incubation at 37^{o}C. All SE strains and the EC strain were maintained and cultured in LB media. The bacterial cultures

for ovarian follicle invasion assay were prepared by incubating the cultures until optical densities at 600 nm (OD_{600}) reached 0.2, which corresponds to a mid-log phase.

Collection and Preparation of Ovarian Follicles

Ovarian follicles were collected from a flock of Single Comb White Leghorn hens maintained in the Poultry Science research facility at the University of Arkansas and other local farms. Birds used for follicle collection were from between 60 and 80 weeks of age that had not been subjected to a forced molting. After being euthanized, ovarian follicles were removed from the ovaries of laying hens aseptically. All of the follicles obtained from each hen were rinsed with sterile 1X Phosphate Buffered Saline (PBS), and distributed evenly into 6 groups with follicles in different stages of maturation in each group. The follicles in each group were subsequently placed in 90 ml sterile culture media-Hank's Balanced Saline Solution (1X HBSS) supplemented with sodium bicarbonate (EMD Chemicals Inc., Gibbstown, NJ) to a final concentration of 4.2 mM.

Ovarian Follicle Invasion Assay

The ovarian follicle invasion assay previously devised by Howard et al. (2005) was used in our study with some modifications. After ovarian follicles were prepared as described above, bacterial cultures were added to 1X HBSS cell medium containing the collected ovarian follicles to a final concentration of 10^6 Colony Forming Units (CFUs)/ml. Immediately after inoculation, an aliquot of the medium was collected and used to determine the total CFUs in the medium at t_0.

Follicles were then incubated with bacteria for 2 hours at 37^{o}C. The follicles were subsequently removed from the inoculated medium, rinsed with sterile 1X PBS, and then placed in sterile 100 ml 1X HBSS supplemented with gentamicin sulfate (Omnipur, EMD Chemicals Inc., Gibbstown, NJ) at a final concentration of 500 µg/ml. The follicles were incubated for an

additional 4 hours at 37^{o}C. Treatment with gentamicin was to kill all bacteria present in the culture media that had not invaded into the ovarian follicles. Subsequent to 4 hours incubation with gentamicin, each group of follicles were removed from the culture media, rinsed, and stomached separately with 10 ml of sterile 1X PBS. These stomached samples were then used to determine the total CFUs in the follicles at t_{inv}.

For enumeration of bacterial cells at t_0 and t_{inv}, each sample was serially diluted into 1X PBS and plated in triplicate sets onto Brilliant Green Agar (Difco, Sparks, MD) plates supplemented with Novobiocin (25 µg/ml) or LB agar (Difco, Sparks, MD) plates supplemented with Novobiocin (25 µg/ml) for enumeration of SE and EC strains, respectively. Inoculated plates were incubated for 24 hours at 37^{o}C, and CFUs were counted. The average CFUs of t_o and t_{inv} samples from triplicate sets were used for calculation of the percent invasion using the following equation: % invasion = [CFUs at t_{inv} / CFUs at t_0] x 100, where CFUs at t_0 = the total CFUs in the media post-inoculation of bacteria and CFUs at t_{inv} = the total CFUs recovered from the ovarian follicles contents. Four separate trials of ovarian follicle invasion assays were performed for each strain.

Statistical Analysis

Each trial consisted of 6 groups, and each group was inoculated with a different bacterial strain (5 SE and 1 EC). The percent invasion from four separate trials was analyzed using JMP 7 statistical software. The analysis of variance test for significance was used with the statistical significance set at a probability value of $P < 0.05$.

RESULTS AND DISCUSSION

After incubating the ovarian follicles with bacterial strains, all strains were able to penetrate into the ovarian follicles during the 2 hours incubation at 37^{o}C. The mean percent invasion of the ovarian follicles by SE and EC strains determine

d in this study is shown in Figure 1. The mean percent invasion of the SE strains ranged from 0.016 to 0.034%, whereas it was 0.0003% for EC, which was used as the negative control. The percent invasion was closely similar among the five SE strains. The percent invasion of SE13076 (0.016%), which showed lowest invasion among all 5 SE strains, was 53-fold higher than that of EC K-12 strain (0.0003%). However, there was no significant ($p>0.05$) difference in percent invasion among all strains tested.

The concentration of gentamicin sulfate used in this study (500 μg/ml) was determined previously to ensure the killing of all bacteria in the media while bacterial counts inside the follicles are not affected (Howard et al, 2005). As expected, when we plated culture media after 4 hours incubation with gentamicin sulfate at this final concentration, no viable bacteria were detected (data now shown).

The goal of this study was to determine and compare the capability of different SE strains to invade laying hens' ovarian follicles. Our study showed through the *in vitro* invasion assay that all tested SE strains were able to penetrate the ovarian follicles under the assay condition used in this study. Also, the result indicated that all SE strains invaded the follicles consistently without significant variation among all strains.

Developing eggs have been found to be contaminated with SE in the reproductive tract of laying hens after both natural and experimental infections (Bichler et al, 1996; Humphrey et al, 1989). Transovarian transmission of SE has been confirmed by others following experimental

infection of SE-free laying hens. In the study by Gast et al (2004), approximately 66.7% of ovary and oviduct samples from experimentally infected hens were contaminated with SE. In another study, SE was recovered from 71% of egg contents following experimental infection of laying hens (Braden, 2006).

In the study by Howard et al, (2005), developing small white follicles were invaded more efficiently by SE strain PT 19A as compared to the follicles at different stages of maturity. The attachment of SE to ovarian follicles was observed in follicular granulosa cells and the level of attachment varied among the ovarian follicles at different developmental stages (Thiagarajan et al, 1994). Mizumoto et al (2005) also showed that SE was associated with follicular explants. Our study demonstrated that different SE strains have the ability to invade ovarian follicles with insignificant variation.

Our results, combined with those from previous studies, suggest that SE strains can efficiently adhere to and penetrate hens' ovarian follicles in a manner dependent on follicle maturity. The ovarian colonization by SE may lead to invasion of ovarian follicles, contributing to the production of contaminated eggs. However, the factors responsible for SE's higher prevalence in egg-borne infections vis-à-vis other *Salmonella* serotypes are still unclear. Future work should compare follicle invasion capacity between SE strains and that of other *Salmonella* serotypes.

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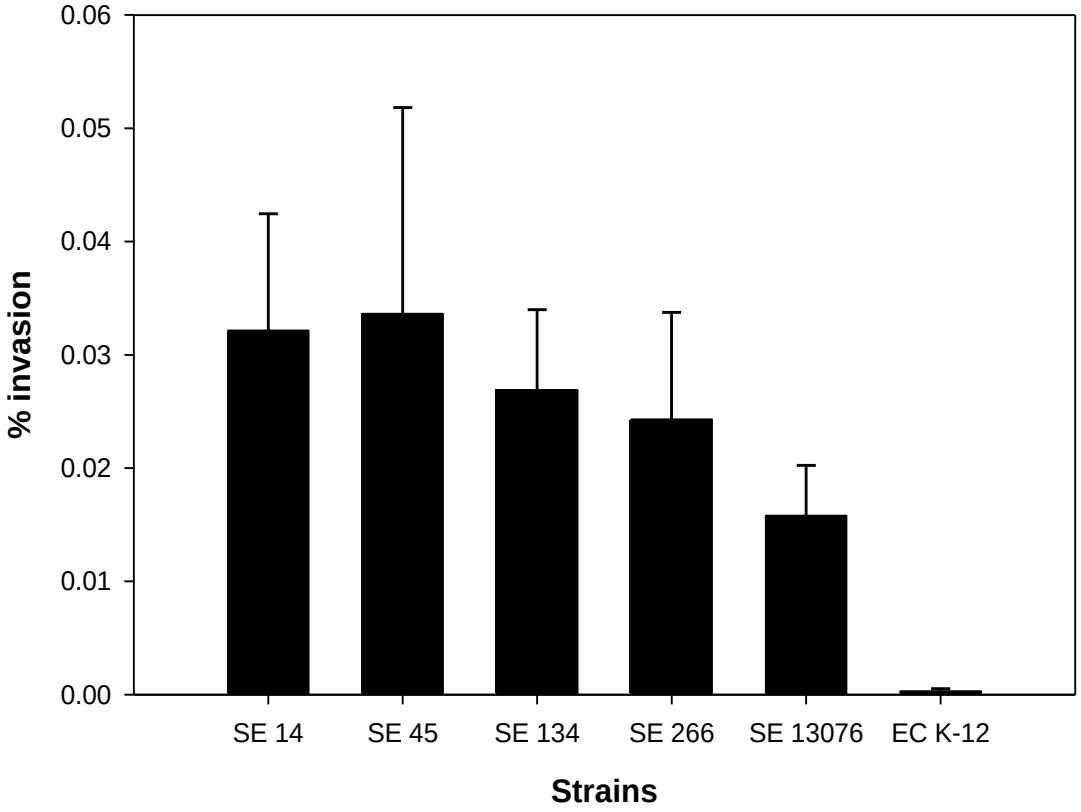
Table 1. Bacterial strains used in this study

Strain Designation	**Strain**	**Reference or source**
SE14	*S.* Enteritidis LK5 PT 8	Edwards et al. 2000
SE45	*S.* Enteritidis PT 13A	National Veterinary Services Laboratories, Ames, IA
SE134	*S.* Enteritidis PT 4 NCTC13349	NCTC (National collection of type cultures)
SE266	*S.* Enteritidis BM4246	Dr. Billy Hargis (Univ. of Arkansas)
SE13076	*S.* Enteritidis ATCC 13076	ATCC (American Type Culture Collection**,** Manassas, VA, USA)
EC-K12	*Escherichia coli* K-12	The Coli Genetic Stock Center (CGSC) (Yale University New Haven, CT).

Figure 1. The mean percentage invasion of *Salmonella* Enteritidis (SE) strains and *Escherichia coli* (EC) K-12 strain into ovarian follicles.


0.06
0.05
0.04
0.03
0.02
0.01
0.00
% invasion
SE 14
SE 45
SE 134
SE 266
SE 13076
EC K-12
Strains

Appendix

Chapter 2 Publication by Journal

In vitro invasion of laying hen ovarian follicles by *Salmonella* Enteritidis strains

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ABSTRACT *Salmonella* is the major foodborne bacterial pathogen worldwide. Among numerous serotypes, *Salmonella* Enteritidis (SE) is one of the most common *Salmonella* serotypes responsible for human infections in the United States. The main source of SE outbreaks is foods associated with raw or undercooked chicken eggs. *Salmonella* Enteritidis is the only serotype that routinely contaminates eggs. The transovarian transmission of SE and subsequent contamination of the eggs before egg shell formation is considered to be the main route of egg contamination by SE. To evaluate whether invasion of ovarian follicles is an important step during the production of eggs contaminated by SE, we used an in vitro invasion assay to determine ovarian follicle invasion by 5 SE strains. After inoculating the freshly collected ovarian follicles, all 5 SE strains were able to invade into the follicles after 2 h of incubation at 37°C. The mean percentage of SE invasion ranged from 0.016 to 0.054% and no significant difference was found among the SE strains. For *Escherichia coli* K-12 strain, which was used as a negative control, the mean percentage of invasion was 0.0003%. The in vitro follicle invasion by SE strains demonstrated in this study may reflect the ability of the strains to invade ovarian follicles in laying hens once SE cells reach ovaries through various routes.

Key words: *Salmonella* Enteritidis, laying hen, ovarian follicle, invasion, egg

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INTRODUCTION

Human infections by foodborne bacterial pathogens are a critical public health problem. Among the foodborne bacterial pathogens are nontyphoid *Salmonella* serotypes, one of the major causes of human foodborne illnesses in the United States and worldwide (Guard-Petter, 2001). A recent study estimated that an average of 9.4 million incidents of foodborne illness from 31 major pathogens occur in the United States and that, of those, 11% are caused by nontyphoid *Salmonella* species as the second main causative pathogen. Furthermore, nontyphoid *Salmonella* species caused approximately 35% of the annual hospitalizations cases and 28% of deaths related to foodborne illness, leading other foodborne pathogens (Scallan et al., 2011).

The transmission sources of *Salmonella* are wide ranging, but most incidences of this foodborne illness have been traced to the consumption of poultry meat, eggs, and their food products (Rabsch et al., 2001; Andrews and Baumler, 2005). The fact that these pathogens infect the host animals without observable sickness contributes to high numbers of human infections (Angulo and Swerdlow, 1999; Poppe, 1999; Patrick et al., 2004).

Among *Salmonella enterica* serotypes, the serotype Enteritidis is one of the most common serotypes implicated in human illness. This serotype has been accountable for 14% of all foodborne human cases of salmonellosis in the United States (Braden, 2006), making it the second most common *Salmonella* serotype after *Salmonella* Typhimurium. In the European Union, *Salmonella* Enteritidis (SE) is responsible for 62.5% of human salmonellosis (Gantois et al., 2009). *Salmonella* Enteritidis has distinct ecological characteristics in association with chicken shell eggs, which raise unique concerns about public safety. Before the 1950s, there was no reported incidence of SE resulting from the consumption of raw or undercooked chicken eggs or their products in the United States (Bäumler et al., 2000; Rabsch et al., 2001; Andrews and Bäumler, 2005). From the late 1970s to the mid 1990s, the incidence of SE increased rapidly, reaching pandemic proportions, with most outbreaks associated with the consumption of raw or light-

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Chapter 3

Improving the Efficiency of Transposon Mutagenesis in *Salmonella* Enteritidis by Overcoming Host-Restriction Barriers

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Short running head: Transposon mutagenesis of *Salmonella* Enteritidis.

Abstract

Transposon mutagenesis using the transposome complex is a powerful method for functional genomics analysis in diverse bacteria by creating a large number of random mutants to prepare a genome-saturating mutant library. However, strong host restriction barriers can lead to limitations with species- or strain-specific restriction modification systems. The purpose of this study was to enhance the transposon mutagenesis efficiency of *Salmonella* Enteritidis to generate a larger number of random insertion mutants. Host-adapted Tn5 DNA was used to form a transposome complex, and this simple approach significantly and consistently improved the efficiency of transposon mutagenesis, resulting in a 46-fold increase in the efficiency as compared to non-adapted transposon DNA fragments. Random nature of Tn5 insertions was confirmed by high-throughput sequencing of the Tn5-junction sequences. The result based on *Salmonella* Enteritidis in this study should find broad applications in preparing a comprehensive mutant library of other species using transposome complex.

Keywords: Transposon mutagenesis, *Salmonella* Enteritidis, host restriction, host adapted DNA, Tn-seq.

Introduction

Transposon mutagenesis using a transposon-transposase complex (transposome) has been used in diverse bacterial species to create random transposon mutants [***1-14***]. The mutant libraries thus prepared are a critical resource for functional genomics analysis of bacteria for gene identification. When the goal is to screen the mutant library for phenotypes of interest, it is desirable to create a comprehensive library that covers the entire genome. This usually means thousands to hundreds of thousands of independent random insertions, depending on the genome size [***11, 15***]. However, the efficiency of mutagenesis is often inhibited by the host restriction modification system in the bacterial cells [***16-19***]. When EZ:Tn5^{TM} Transposome complex (Epicentre Biotechnologies, Madison, WI, USA) is used to transform *Salmonella* Typhimurium (ST) strains, we routinely obtain 10^{4} to 10^{5} mutants per electroporation. However, with *Salmonella* Enteritidis (SE) strains, the efficiency of mutagenesis drops by greater than 100-fold and only 10^{2} transformants are produced per electroporation.

S. Enteritidis is one of the top five non-typhodial serovars causing human salmonellosis infections with wide-ranging reservoirs and the serovar most commonly associated with eggs and egg products. Many reported human infection cases of salmonellosis were frequently linked to *S.* Enteritidis. In the European Union (EU), S. Enteritidis was the cause of 60% of *Salmonella* outbreaks in 2009 [***20***]. In the United States, *S.* Enteritidis was responsible of almost one fifth of all *Salmonella* reported cases in 2009, and in 2010, a half billion eggs were recalled as the result of an outbreak caused by *S.* Enteritidis [***21***]. Our knowledge on the genetic *tropism* of this serovar as the distinctive predominance on eggs is limited [***20***, ***21***, ***22***]. To identify genes essential for *S.* Enteritidis to be associated with poultry and their products, specifically eggs, we

are initially required to generate a comprehensive random insertions library to screen the whole genome.

An earlier attempt to overcome the restriction of transforming plasmids DNA to *Salmonella* strains was made by Tsai and others by using a *S.* Typhimurium strain with defective restriction system (JR501) to modify plasmids for *Salmonella* compatibility [***23***]. Afterward, the strain JR501 and similarly constructed strains has been used into various genetic studies of *Salmonella* [***24-35***].

Edwards et al. reported a simple method to overcome host restriction barriers to protect the exogenous DNA introduced into *S.* Enteritidis against host restriction systems, thus increasing transformation efficiency [***36***]. This technique involves heat treatment during preparation of the competent cells to temporarily inactivate or reduce the activity of the DNA restriction systems within the host cells and enable successful transformation. In this study, we used host-adapted transposon DNA to improve the efficiency of transposon mutagenesis and compared the result with that obtained with heat inactivation method for temporary inactivation of the host restriction barrier of *S.* Enteritidis using the EZ: Tn5^{TM} Transposome complex (Epicentre Biotechnologies).

In a study by Colegio et al, a host-adapted Tn552 fragment in *Campylobacter jejuni* was used to form a complex with transposase [***37***]. When the complex was used to transform *C. jejuni*, it efficiently increased transposition efficiency 30 to 80 times as compared with that without host adaptation and readily generated 2.9 x 10^3 to 7.7 x 10^3 mutants/ electroporation. Therefore, we hypothesized that a similar strategy can increase transformation efficiency when transposon mutagenesis is performed for *S.* Enteritidis strain using EZ: Tn5^{TM} Transposome complex.

Materials and Methods

Bacterial strains and culture conditions

EZ-Tn5^{TM} pMOD-6 <KAN-2/ MCS> transposon construction vector (Epicentre Biotechnologies, Madison, WI, USA) was maintained in TOP10 *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA) in Luria-Bertani (LB) broth with 100% glycerol at -80^{o}C. *Salmonella* Enteritidis PT13A (National Veterinary Services Laboratories, Ames, IA) was used for transposon mutagenesis library construction. Luria-Bertani agar media (BD Difco, Sparks, MD) was first used for growth of all frozen bacterial cultures with 24 hours of incubation at 37°C. SOC media (**S**uper **o**ptimal broth (SOB) with carbon **c**atabolite repression, generally glucose) (Invitrogen, Carlsbad, CA, USA) was used immediately for recovering the cells after electro-transformation. As suitable, the following antibiotics were added to the media, novobiocin (25 µg/ml) (No) and kanamycin (50 µg/ml) (Km).

Electrotransformation

S. Enteritidis PT13A had been prepared for electroporation according to a previously described protocol with few modifications [***38***]. Briefly, a single colony of bacterial culture from LB agar was inoculated into 10 ml of 2X YT broth (BD Difco, Sparks, MD) with novobiocin and grown at 37°C overnight with vigorous shaking. Subsequently, 100 µl of overnight culture was re-inoculated into 15 ml fresh 2X YT broth without salt (16 g/L tryptone, 10 g/L yeast extract) and incubated at 37°C for 3 to 4 hours to reach mid-log corresponding to an approximate OD_{600} of 0.6 (0.5 to 0.7). Instantly, cultures were chilled on ice followed by centrifugation and pellets were kept on ice throughout the procedures for electrocompetent cell preparation. Cells were washed five times in ice-cold sterile ddH_2O water and finally re-suspended in 70 µl of ice-cold

sterile 10% glycerol. The prepared electrocompetent cells were used instantaneously, and were mixed with DNA. After incubation in ice for 10 minutes, the cells were then electroporated at 2450 kV with 2 mm cuvettes for 5 to 6 ms using ECM 399 Electroporation System (Harvard Apparatus, Holliston, MA), subsequently incubated in SOC for 1 to 1.5 hour at 37°C and plated on LB media with appropriate antibiotics.

Heat inactivation method

Briefly, the above preparation protocol of electrocompetent cells was used with slight modification. Mid-log cultures of *S.* Enteritidis PT13A were incubated at 50ºC for 25 min and then kept on ice throughout the preparation of electro-competent cells [***38***]. Subsequently, cells were immediately used for electroporation with 2 µl of EZ: Tn5^{TM} Transposome complex. To recover the transposon mutants after performing each electro-transformation, we immediately added 500 µl of pre-warmed SOC medium to the reaction and incubated it at 37°C for 1 hour with vigorous shaking and then enumerated on LB agar plates supplemented with kanamycin (50 µg/ ml). After incubation at 37^{o}C for 18 hours, the colony forming units (number of mutants) were enumerated.

Host-adapted DNA method

A strategy to overcome the host restriction barrier is to adapt the exogenous transposon DNA to the host cells before the introduction as demonstrated previously with slight modification [***37***]. To test the hypothesis, we performed transposon mutagenesis of *S.* Enteritidis PT13A strain using EZ: Tn5^{TM} transposon that had been replicated in *S.* Enteritidis PT13A wild type (adapted DNA) or *Escherichia coli* Top10 (non-adapted DNA; negative control). Initially,

we used 1 µl of EZ-Tn5^{TM} pMOD-6 <KAN-2/ MCS> transposon construction vector (Epicentre Biotechnologies, Madison, WI, USA) to transform *S.* Enteritidis PT13A (adapted transposon DNA) or *E. coli* Top10 (non-adapted transposon DNA, as control) by electroporation. The transformants were inoculated on Luria-Bertani (LB) agar plates supplemented with kanamycin (50 µg/ ml) for each of *S.* Enteritidis PT13A or *E. coli* Top10 and incubated overnight at 37ºC.

One transformant colony resistant to kanamycin from each *S.* Enteritidis PT13A or *E. coli* Top10 was inoculated into Luria-Bertani (LB) broth containing 50 µg/ ml kanamycin, and incubated overnight at 37ºC. Plasmid DNA was purified using QIAprep Miniprep kit (Qiagen, Valencia, CA) from the overnight cultures, and the concentration and purity were checked with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Restriction enzyme digestion of plasmid DNA was performed with PvuII-HF following the manufacture instruction (New England Biolabs, Ipswich, MA) to release EZ: Tn5 fragments of 1,117 bp long.

These fragments were carefully extracted from gels using a QIAquick gel extraction kit (Qiagen, Valencia, CA) without exposing the transposon DNA to ultraviolet light during visualization. Afterward, the purified transposon DNA was checked for concentration and purity as indicated above. The Tn5 transposome complex was produced by mixing 2 ul of the Tn5 transposon DNA with concentration of approximately 200 ng/µl, 4 ul EZ-Tn5^{TM} Transposase (Epicentre Biotechnologies, Madison, WI, USA), and 2 ul 100% sterile glycerol in magnesium minus milieu as directed by the manufacture manual. Subsequently, the reaction was incubated at room temperature for 30 min, then incubated at 4^{o}C overnight, and stored at -20ºC [***39***, ***40***, ***41***]. Two microliters of the Tn5 transposome complex were used for electroporation into *S.* Enteritidis PT13A as described above. Kanamycin resistance colonies were selected on LB plates supplemented with kanamycin (50 µg/ ml) and subsequently collected to form a complex

library of EZ-Tn5 mutants. The library was stocked at -80^{o}C in 30% glycerol. .Electroporation using the transposome complex in both methods was performed in three independent trials.

Illumina sequencing sample preparation

Genomic DNA was extracted using QIAamp DNA Mini kit (Qiagen, Valencia, CA) from *S.* Enteritidis PT13A EZ:Tn5 mutant library, and the concentration and purity were checked with a NanoDrop 1000 spectrophotometer (Thermo Scientific). Subsequently, the Tn5-junctions of the extracted genomic DNA were specifically amplified using the following protocol: Tn5-junction sequences were enriched from the genomic DNA of the mutant library using a single primer extension [***42***] with EZ: Tn5 specific primer for right end (RE) and cloned *pfu* DNA polymerase (Agilent Technologies, La Jolla, CA) resulting in fragments with variable lengths. The PCR reaction was then purified with MinElute PCR purification kit (Qiagen, Valencia, CA) and eluted in 10 µl EB buffer as directed by the manufacturer's guide prior to the addition of deoxycytosine homopolymer tail (C- tail) using Terminal Transferase (TdT) following a previous protocol [***43***].

After heat inactivation of TdT, the reaction was subsequently used as a template in a two steps exponential PCR reactions using ExTaq DNA polymerase (Takara Bio Inc.). The first exponential PCR reaction was performed with short forward Tn5 primer (IR-W) and C-tail linker primer for 20 cycles. The PCR product from this first reaction step was amplified in a second exponential PCR reaction using ExTaq DNA polymerase (Takara Bio Inc.) with long forward barcoded primer (IR2+IS) and HTM primer for 25 cycles to add an adapter containing Illumina sequence at both endss (Table 3). The PCR product in the range of 200 to 300 bp from last reaction was PAGE-purified, dissolved in ultra pure water. DNA concentration and purity was

measured with the Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA) using Qubit® Assay Kits (dsDNA BR Assay) following the manufacturer's manual. The gel-purified PCR amplicons containing Tn5-junction sequences were sequenced (being spiked into other unrelated samples) using an Illumina MiSeq sequencer for 100 cycles at the Center for Food Safety in the Department of Food Science at the University of Arkansas, Fayetteville, AR.

Illumina sequencing data analysis

Custom Pearl script was used to analyze the data. Briefly, the sequence reads of 100 bp from Illumina sequencing were analyzed to find and retain the reads that contain 34 bp region corresponding to the Tn5 sequences. The 60 bp region subsequently corresponding to Tn5-flanking region was extracted and used in the next step. A local blast database of the complete *S.* Enteritidis P125109 genome (accession no. NC_011294) [***44***] was created and used for genome mapping of 60 bp the Tn5-junction sequences. The final output data obtained by this script contained the information on the Tn5--junction sequence and genomic coordinate corresponding to EZ: Tn5 insertion site. The output data was processed using JMP Pro11 (SAS, Cary, NC). The length of the transposon-junction sequences extracted by this method is long enough for unambiguous identification of the genomic locations from which the insertions were originated.

Statistical analysis

Each method was carried out using the transposome complex three times independently. The comparison of improved mutagenesis efficiency was analyzed using JMP Pro 11 statistical software (SAS Institute, Cary, NC). The Student's t-test for significance was used, with the statistical significance set at $P < 0.05$.

Results

Our goal was to form a comprehensive saturated mutant library of *S.* Enteritidis as a stock to be used in various functional genomics analyses. Initially, we applied the heat inactivation method by Edward et al [***36***] for temporarily inactivating *S.* Enteritidis restriction-modification systems. As a result, the mutagenesis efficiency exhibited an increase of only about 12-fold which is statistically significant ($P < 0.05$) (Table 1).

Since the increase of mutagenesis was not adequate to achieve a saturated complex library, we examined other methods by which we could improve the efficiency. We chose the method by Colegio et al. [***37***] using adapted DNA to improve transposon mutagenesis of *S.* Enteritidis. The result presented in Table 2 clearly demonstrates the increase in the number of transposon mutants per electroporation. There was an approximately 46-fold increase in the efficiency of transposon mutagenesis compared to non-adapted transposon DNA, resulting in an average of 12,626 colonies per electroporation reaction with a transformation efficiency of 1.58x 10^5 cfu/µg after the addition of pre-warmed SOC medium. This increase is significantly different when comparing the results from Table 2 and also when comparing the results with Table 1.

The data obtained using illumina sequencing after analysis revealed that there were 95,780 reads containing Tn5 sequences, among which 30,594 reads were mapped perfectly on *Salmonella* Enteritidis genome (accession no. NC_011294). From the Illumina sequencing reads result, there were 30,594 unique insertions located on the genome (100%). These transposon insertion sites were confirmed using nucleotide local BLAST program and exhibited a random distribution pattern throughout the genome of *S.* Enteritidis with the global view through JMP Pro 11 system (SAS, Cary, NC) (Figure 1). Although the distribution pattern showed coverage throughout the genome, there is still one large gap of approximately 37,103 bp that falls in

between the locations 2018270 bp and 2055373 bp of *S*. Enteritidis reference genome. We identified the genes of that region in the reference genome and determined that they mainly belong to phage and phage related products. This accurately reflects the genomic region uniquely present in the reference genome (phage type 4), but absent in *S*. Enteritidis strain (phage type 13A) used in our study.

Discussion

Numerous strategies have been developed to enhance and/or overcome the problem(s) that negatively influence the transformation efficiency. Even among different serovars of *Salmonella*, some methods work ideally with some bacterial strains, while other methods fail to yield any improvements. Heat was used to increase the DNA transformation efficiency either among different strains (intra-species) or different species (inter-species) by temporarily inactivating the restriction barriers during the preparation of electro-competent cells [***36***]. Plasmid DNA from *Escherichia coli* was electro-transformed into heat pre-treated *S.* Enteritidis competent cells and resulted in enhanced transformation efficiency by recovering about 2,600 colonies [***36***].

Transposon mutagenesis has broad application in functional analysis of bacterial genes by generating random insertional mutations. For convenient and efficient transposon mutagenesis in diverse bacterial species, an *in vivo* transposon mutagenesis was developed by Goryshin et al. [***39***]. In this system, a pre-formed transposon-transposase complex (transposome complex) is electro-transformed into the host bacterial cells resulting in high and efficient transposition frequency. We used this method in attempt to construct a comprehensive library of random transposon insertions, but were faced by the obstacle of a host restriction system that

significantly reduced the number of random mutants. As a result, we examined other techniques in combination with the *in vivo* transposon mutagenesis to improve the efficiency of random transposon mutagenesis.

The result from Table 1 suggests that inactivation of the host restriction system by heat treatment was only moderately effective for transposon mutagenesis. By recovering a host-modified vector from *S.* Enteritidis, we were able to construct a transposome complex that can be used for transposon mutagenesis with significantly increased transformation efficiency (Table 2). Most genetic screening studies with *Salmonella* serotypes have been performed with *Salmonella* Typhimurium partially due to the limitations associated with other serotypes in performing genetic studies. A study by Shah and others identified virulence genes essential for chicken infection using a small library of the serotype Gallinarum constructed through PCR and signature-tagged mutagenesis (STM) [***45***]. A Tn-seq method was developed previously in our lab with a modified EZ: Tn5 transposon and successfully used to identify genetic determinants of S. Typhimurium that are conditionally essential for growth or survival under various conditions [***15***]. The disadvantage of this version was the short sequence of Tn5-junctions that resulted in ambiguous mapping of a large portion of the reads to multiple genomic locations on the genome. The method described in this study to amplify Tn5-junctions overcame the limitation with a long Tn5-junction sequence that can be mapped solely to single locations of genomic DNA.

Although *S.* Enteritidis has been a major public health issue for the last 3 decades, not many genomes of this serovar strains have been sequenced. A recent publication by Timme reported the draft of genome sequenced for 21 strains of *S.* Enteritidis [***46***]. *S.* Enteritidis strain P125109 [***44***] and, more recently, LA5 strain [***47***] are the only strains with completed genome sequences, but more genome completed sequences will support in-depth genetic analysis of *S.*

Enteritidis and potentially the identification of more host-specific restriction-modification systems in *Salmonella* that are unique to the species or even strains-specific systems [***48-51***].

Overcoming the restriction system barriers of bacteria has been a major obstacle for various routine genetic manipulations in bacteria. It is not clear yet the reasons that some serotypes of *Salmonella* are less efficient in transformation than other serotypes. Other alternative approaches or combinations of those may help improve the transformation efficiency and transposon mutagenesis. We believe that the simple strategy tested in this study using host-adapted EZ: Tn5 transposon can be applied to other species to create more comprehensive Tn5 insertion libraries and facilitate functional genomics studies.

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Table 1. Effect of heat inactivation step on the efficiency of Tn5 mutagenesis in *S.* Enteritidis*

Condition	Average cfu/µg DNA	Standard Error
No heat	4.65E+03	± 9.60E+02
Heat	5.62E+04	±3.95E+03

*This experiment was performed in three independent trials.

Table 2. Effect of host-adaptation on the efficiency of Tn5 mutagenesis in *S.* Enteritidis*

Condition	Average cfu/µg DNA	Standard Error
No adaptation	3.38E+03	±1.88E+02
Host adaptation	1.58E+05	± 4.13E+03

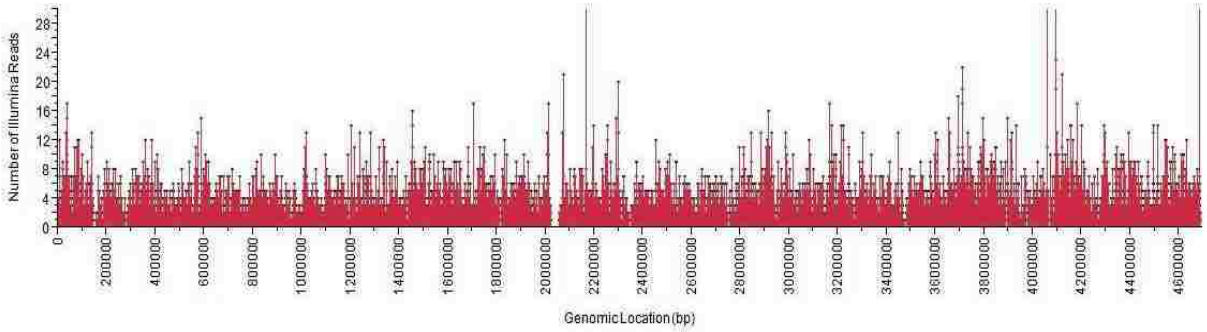
*This experiment was performed in three independent trials.

Table 3. Oligonucleotides used in Tn-seq method in this study.

Oligonucleotide	Sequence (5'→3')
EZ: Tn5 Primer RE	5-CTAGCCAACAAGAGCTTCAGGGT-3
IR2 Primer + IS	5-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCT ACACGACGCTCTTCCGATCTNNNNAGXXXXXX TCAGGGTTGAGATGTGTATAAGGGACAG-3
HTM-Primer	5-CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT GGGGGGGGGGGGGGG- 3
IR-W	5-TCAGGGTTGAGATGTGTATAAGAGACAG-3

Note:
1. NNNN: random sequence for efficient cluster analysis.
2. XXXXXX: 6 nt barcode sequence. This barcode was designed to allow sorting of the sequence reads, but this feature was not used in this study.

Figure 1. Global view of Tn5 insertion sites on the *S*. Enteritidis genome.



Appendix

Chapter 2 Publication by Journal

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RESEARCH

Improving the Efficiency of Transposon Mutagenesis in *Salmonella* Enteritidis by Overcoming Host-Restriction Barriers

Tarek M. Dawoud · Tiezhan Jiang · Rabindra K. Mandal · Steven C. Ricke · Young Min Kwon

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Abstract Transposon mutagenesis using transposome complex is a powerful method for functional genomics analysis in diverse bacteria by creating a large number of random mutants to prepare a genome-saturating mutant library. However, strong host restriction barriers can lead to limitations with species- or strain-specific restriction-modification systems. The purpose of this study was to enhance the transposon mutagenesis efficiency of *Salmonella* Enteritidis to generate a larger number of random insertion mutants. Host-adapted Tn*5* DNA was used to form a transposome complex, and this simple approach significantly and consistently improved the efficiency of transposon mutagenesis, resulting in a 46-fold increase in the efficiency as compared to non-adapted transposon DNA fragments. Random nature of Tn*5* insertions was confirmed by high-throughput sequencing of the Tn*5*-junction sequences. The result based on *S. Enteritidis* in this study should find broad applications in preparing a comprehensive mutant library of other species using transposome complex.

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Keywords Transposon mutagenesis · *Salmonella* Enteritidis · Host restriction · Host-adapted DNA · Tn*5*

Introduction

Transposon mutagenesis using a transposase–transposase complex (transposome) has been used in diverse bacterial species to create random transposon mutants [1–14]. The mutant libraries thus prepared are a critical resource for functional genomics analysis of bacteria for gene identification. When the goal is to screen the mutant library for phenotypes of interest, it is desirable to create a comprehensive library that covers the entire genome. This usually means thousands to hundreds of thousands of independent random insertions, depending on the genome size [11, 15]. However, the efficiency of mutagenesis is often inhibited by the host restriction-modification system in the bacterial cells [16–19]. When EZ-Tn*5*™ Transposome complex (Epicentre Biotechnologies, Madison, WI, USA) is used to transform *Salmonella* Typhimurium (ST) strains, we routinely obtain 10^4–10^5 mutants per electroporation. However, with *Salmonella* Enteritidis (SE) strains, the efficiency of mutagenesis drops by greater than 100-fold and only 10^2 transformants are produced per electroporation.

S. Enteritidis is one of the top five non-typhoidal serovars causing human salmonellosis infections with wide-ranging reservoirs and the serovar most commonly associated with eggs and egg products. Many reported human infection cases of salmonellosis were frequently linked to SE in the European Union. SE was the cause of 60 % of *Salmonella* outbreaks in 2009 [20]. In the United States, SE was responsible of almost one fifth of all *Salmonella* reported cases in 2009, and in 2010, a half billion eggs were recalled as the result of an outbreak caused by SE

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Chapter 4

Heat Survival and Phenotype Microarray Profiling of *Salmonella enterica* serovar Typhimurium Mutants

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Abstract

Food contamination with pathogenic or spoilage bacteria has been a major public health and food industry problem. One of the leading foodborne pathogens are serovars of non-typhoidal *Salmonella* that have been associated with a wide range of outbreaks from various foods. Numerous methods have been used and introduced for treatment of fresh food to eliminate pathogenic microorganisms as well as extend the shelf life of food products. Heat treatment has been applied in a variety of forms for retaining shelf life of food products. However, under certain circumstances pathogens such as *Salmonella* are known to survive such treatments. In this study, we tested mild heating temperatures against *Salmonella* Typimurium strains (wild type, Δ*recD*, ΔSTM14_5307, and Δ*aroD*) to understand its ability to survive and resist these treatments. We demonstrated that Δ*aroD* has the highest sensitivity alongside all heating temperature followed by Δ*recD*, ΔSTM14_5307 with significant differences compared to the wild type. We also characterized these strains using high throughput phenotypic profiling in response to two different growth temperatures, 37^{o}C (human body temperature) and 42^{o}C (poultry body temperature). Understanding the molecular mechanisms of heat survival may be helpful in developing more effective strategies to control *Salmonella* in food products by thermal treatment.

Keywords: *Salmonella* Typhimurium, thermal, phenotype, heat survival

Introduction

Contamination of a wide range of foods by foodborne pathogens has been a primary concern of public health and food safety authorities in the United States and worldwide (Beier et al, 2004; Newell et al, 2010; Masuku et al, 2012; Gillis et al, 2011; CDC, 2013; Jacob et al, 2012; Rostagno and Ebner, 2012; Van Loo et al, 2012a, 2012b). *Salmonella* strains are one of the primary pathogenic bacteria responsible for numerous foodborne diseases outbreaks with the highest incidence of all culture-confirmed foodborne bacterial cases with 15.19 and hospitalization of 28% (Crim et al, 2014). The economical loss for the US is approximately 15 billion dollars (Nayak et al, 2012). *Salmonella* can contaminate various foods with leafy greens, poultry meat, and eggs considered the more frequent sources of salmonellosis (Finstad et al, 2012; Franz and van Bruggen, 2008; Hanning et al, 2009; Howard et al, 2012; Lynch et al , 2009; Nayak et al, 2012). A recent report by Food Safety and Inspection Service indicated that samples were *Salmonella* positive in 18% of ground chicken meat, 15% of ground turkey meat, 2.3% of turkey, and 3.9% of broiler chicken (FSIS-USDA, 2013).

Many intervention strategies have been tested or applied throughout food processing for several decades to decontaminate foods from pathogenic and spoilage microorganisms and also to extend the food life shelf as preservation methods (Ricke, 2003; Ricke et al, 2005; Sirsat et al., 2009). These intervention treatments can be categorized as chemical, physical, or biological treatments and have been conducted to control and/or decontaminate products during pre-harvesting or post-harvesting such as meat, poultry, and fresh produce (Aymerich et al., 2008; Beuchat et al., 1998; Bolder, 1997; Chaine et al., 2013; Chen et al., 2012; Dinçer and Baysal, 2004; Huffman, 2002; Hugas and Tsigarida, 2008; James et al., 2007; Loretz et al., 2010, 2011; Otto et al., 2011; Parish et al., 2003; Rajkovic et al., 2010; Vandeplas et al., 2010).

Heat treatment has been one of the most effective modes to eliminate *Salmonella* and other foodborne pathogens from food products (Bermúdez-Aguirre and Corradini, 2012; Silva and Gibbs, 2012; Sun, 2012); however, due to the negative effects of this method on the foods properties for heat sensitive foods, the food industries have been using this approach to a lesser degree. Food characteristics such as color, flavor, and nutrition, can be influenced particularly as causes of Maillard reaction product (MRP) formation as a result of food processing and storage. Mild heat treatments have been one of the major methods used in numerous food processing. They are practically employed in processing fruits, vegetables, juices, dairy products, fish, meat, and poultry carcasses decontamination (Allende et al., 2006; Berrang et al., 2000; Godden, 2007; Heinz and Hautzinger, 2007; James et al., 2007; Li,et al., 2002; McCann et al., 2006; McDonald et al., 2005; Morrison and Fleet, 1985; Northcutt et al., 2005; Patras et al., 2011; Purnell et al., 2004; Rastogi , 2012; Rosnes et al., 2011; Roy et al., 2007; Rawson et al., 2011).

Pasteurization has been the traditional and the main universal procedure in producing microbial-safe foods (Charles-Rodríguez et al., 2007; Lado and Yousef, 2002) by generating heat in the food outer surface that transmit to the food interior, but it has an adverse impact on food characteristics or formulation (Silva and Gibbs, 2012). Some *Salmonella* strains can survive food processing and resist heat due to their prior growth and stress conditions. Cells of organism tested against heat showed different response depending on their growth conditions as stationary phase cells are more resistance to heat than log phase cells (Humphrey et al, 1995; Mackey and Derrick, 1990; Wilde et al, 2000). In addition, the growth temperature of an organism above the average growth range, heat shocked cells, growth on limited carbon sources or undergoing starvation stress prior to the heat treatments have exhibited more thermal tolerance (Bunning et al, 1990; Foster and Spector, 1995; Ng et al, 1969; Xavier and Ingham, 1997). Furthermore,

Salmonella attached to food surfaces (sessile or immobile) are more resistance to heat than their dispersed or unattached (planktonic) throughout food processing (Garrett et al., 2008; Humphrey et al, 1997; Orta-Ramirez et al, 2005; Mogollon et al, 2009; Tuntivanich et al, 2008; Velasquez et al, 2010).

Phenotype microarray (PM) analysis was employed to identify the effects of gene deletions for loss and/or gain of the gene function(s), and to measure cell metabolism changes over time incubated at two different temperatures, 37^{o}C for human body and 42^{o}C for avian body. This high-throughput screening method was developed in the term of a comprehensive and quantitative profile with possible simultaneous monitoring of approximately 2000 assays (Bochner et al., 2001; Bochner, 2009). These assays are performed in wells of 96-well micro-plates testing the metabolic activity for carbon, nitrogen, sulfur, phosphate substances, as well as varying concentrations of inhibitory compounds. Each well contains a 100 µl culture (a media with a particular substrate) and tetrazolium violet dye. With the aid of the latter colorimetric reagent, the cellular respiration is possibly determined by developing a quantitative purple color according to the respiration of inoculated microbial cells (Bochner et al., 2001).

In this study, we compared different phenotype strains of *Salmonella* Typhimurium 14028 in their ability to survive under various mild heating treatments mimicking some food processing. In addition, we obtained results using a phenotype profiling to give a deeper insight into the cellular physiological variations that possibly play a role in the growth deficiency of these mutants in comparison to their wild type parental strain. It had been found to exhibit asscociation with high temperature in a previous study from our lab (Kahtiwara et al., 2012). Mutants were evaluated for thermo-tolerance defects under an *in vitro* growth condition mimicking poultry body temperature (42^{o}C).

Materials and Methods

Bacterial Strains and Growth Conditions

Four frozen (-80^{o}C) glycerol bacterial culture stocks of *Salmonella* Typhimurium ATCC 14028 strains were resuscitated by streaking onto Luria-Bertani (LB) agar plates (Difco, Sparks, MD) supplemented with novobiocin antibiotic (NO 25 µg/ml) for two successive cycles of 24 hours incubation at 37^{o}C (one wild type with nalidixic acid (NA) resistance, and three mutants with kanamycin (Km) resistance Δ*recD*, ΔSTM14_5307, and Δ*aroD*). A single colony from each strain was inoculated into 5 ml of Luria-Bertani (LB) broth media (Difco, Sparks, MD) supplemented with the appropriate antibiotic (NA 25 µg/ml or Km 50 µg/ml) and incubated overnight at 37^{o}C with vigorous shaking.

Growth measurement

For growth response determination, the wild type and mutant strains were inoculated onto LB agar media. One colony of each strain was inoculated in 5 ml of Luria Bertani (LB) broth and incubated for 16 hrs at 37°C with vigorous shaking. Subsequently, 1% (100 ul) of each *Salmonella* overnight culture was inoculated in 10 ml of fresh Luria Bertani (LB) broth and incubated for 12 hrs at 37°C or 42°C with 200 rpm shaking using a C76 Water Bath Shaker (New Brunswick Scientific, Edison, NJ, USA). Optical density (OD) was measured at 600 nm every 10 minutes over 5 hrs using a Spectronic 20D+ spectrophotometer (Thermo Electron Scientific, Madison, WI, USA). The natural log of each strain OD was calculated and plotted versus time. Afterward, the exponentional phase was verified to be used to demonstrate the slope and the R^2. The slope used to calculate the doubling time in the equation: $t_d = \ln 2/\mu$, where t_d is the doubling time, µ is the demonstrated slope, and ln 2 is the natural log of 2.

Inocula preparation and heat stress treatment

Overnight cultures from the previous step were used to inoculate 2 ml of LB broth and incubated at 37^{o}C for 18 hours with vigorous shaking. These cultures were used for heat stress treatments in water baths set at 50, 55, and 60^{o}C. The cultures were centrifuged at 6500 rpm for 5 minutes, and subsequently washed twice by re-suspension into 2 ml of sterile 1X PBS. A final re-suspension of 2 ml sterile 1X PBS was used. One hundred µL of each suspension was used for serial dilution of pre-treatment culture enumeration. Subsequently, the tubes were placed into the water bath set at 50, 55, and 60^{o}C with gentle shaking. Time points for each temperature of 3 hours, 15 minutes, and 3 minutes were used for each temperature, respectively. The tubes of tested strains were removed immediately, serially diluted and plated for colony forming unit (CFU) enumeration from LB plates. Three independent trials of heat stress treatments were performed for each strain. The survival percentage was calculated to compare all tested samples under different heat stress treatments.

Phenotype characterization

The Phenotype Microarray^{TM} (PM) service was purchased from the manufactures (PM Services, Biolog, Inc., Hayward, CA, USA) to perform full array testing of 20 PM micro-plates, each with 96 wells. For each strain, 20 plates (PM1 to PM20) were inoculated in duplicate with the bacterial cell suspension, and were incubated at 37°C or 42°C, typically for 24 to 48 hours. The metabolic activity of cells was measured every 15 min for up to 48 hours through the reduction of tetrazolium dye depending on the respiration of bacteria (Bochner et al., 2001).

The data of PM was captured every 15 minutes and saved as a digital image storing the color changes into quantitative values in the OmniLog® PM system. Subsequently, they were

displayed in the form of kinetic graphs and compared the mutant to the wild type (reference strain) to determine the phenotypic differences. For result normalization, wells containing the Omnilog™ growth medium with no substrate were inoculated to serve as negative control (Bochner et al., 2001; Bochner, 2009).

Single mutant deletion construction

Salmonella Typhimurium ATCC 14028 mutants with a single deletion in *recD*, and STM14_5307 were constructed previously by Khatiwara et al (2012). To construct *aroD* mutant, we used the method of λ Red recombination system as described previously (Cox et al, 2007), with pKD4 (Datsenko and Wanner, 2000) to amplify the Km resistance cassette using the oligonucleotide shown in Table 1. The deletion of *aroD* was confirmed by DNA sequencing and then used to transfer to a fresh *S.* Typhimurium ATCC 14028 wild type background using P22 transduction. The transductions were subsequently inoculated onto selective LB agar plates supplemented with kanamycin antibiotic (Km). Colonies from LB plates with Km were further selected into Evan's Blue Uranine (EBU) plates to be purified before using in an additional assay.

Statistical analysis

Statistical analysis was performed using JMP® Pro 11 (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance was used, with mean comparison using Student's t-test and Tukey–Kramer honestly significant difference, to compare mean survival percentage values and generation time. P values ≤ 0.05 reflected data that was statistically significant.

Results and discussion

Overview of genes

The gene *recD* is a subunit with exonuclease V activity of the complex RecBCD. This enzyme of the complex required for homologous recombination, DNA repair, and plays a role in *Salmonella* virulence through the recombination pathway during systemic infection and the growth within macrophages (Amundsen et al, 1986, 2000; Biek and Cohen, 1986; Cano et al, 2002; Miesel and Roth, 1996; Zahrt et al, 1997). A mutation in recD of *Salmonella* strain cells will enhance the transduction frequency, the exchanged DNA length, and influence the plasmid maintenance in comparison to the wild type. Some studies exhibited that *recD* is regulated significantly through the extreme heat stress sigma factor, sigma factor E (σ^E) (Bury-Moné et al., 2009; Rhodius et al., 2010).

A quantitative screening study using a developed microarray revealed the attenuation of *recD* mutant in a murine infection model. González-Soltero and collegues (2006) demonstrated that a mutation in *recD* can exclude the required product function of *recA* for the Heat-induced replication (HIR). A study exhibited the requirement of a specific recombination function for the repair of DNA damage by the activity of bile. The loss of the exonuclease activity of *recD* is not essential to resist the bile stress in *Salmonella enterica* (Prieto et al., 2006). A double mutation with *recD* and *recJ* exhibited bile sensitivity although each mutation is bile resistant. Cano et al. (2002) demonstrated that a *recD* and *recJ* double mutation strain displayed attenuation in mice and is incapable of surviving inside macrophages. A study by Amundsen et al. (2000) exhibited the inhibition role of *recD* in DNA repair for strains with loss nuclease activity due to a *recB* nuclease mutation.

The second gene is STM14_5307, a putative transcriptional regulator that has shown to be associated with the temperature 42^{o}C (Khatiwara et al, 2012). It is known as *iolR* (STM4417) in *Salmonella* strain LT2 as regulating negatively the genes involving in *myo*-inositol metabolism. This carbon source is rich in soil and numerous Gram positive enteric bacteria can utilize it such as *Bacillus subtilis*, *Clostridium perfringens*, *Lactobacillus casei*, *Corynebacterium glutamicum* (Yoshida et al., 2004, 2008; Yebra et al., 2007; Greiner and Konietzny, 2006; Kawsar et al., 2004; Krings et al., 2006). Kröger and others (2009, 2010, 2011) conducted several studies of *myo*-inositol utilized by *Salmonella* Typhimurium 14028. One of the characteristics of this strain is the long lag phase when growing on *myo*-inositol as the sole carbon and energy source that in average takes approximately 50 hours. As demonstrated, a mutation in *iolR* had significantly cut down the lag phase time by approximately in average 15 hours (Kröger et al., 2011). Several *in vivo* studies had associated the *myo*-inositol, *iol* genes with *Salmonella* Typhimurium virulence. Carnell et al. (2007) demonstrated using signature –tagged transposon mutagenesis (STM) the role of *iol* genes in *Salmonella* Typhimurium to colonize the pig intestinal. Chaudhuri et al (2013) using a *Salmonella* Typhimurium library of 1045 STM conducted a study to compare the results from screening several food-producing animals (chicken, calves, and pig) and mouse model. They identified genes associated with intestinal colonization with *iol* mutant being attenuated in all screened animals. Lawley and others (2006) screened a complex library of transposon in *S*. Typhimurium using microarray to identify genes with long-term systemic infection role and selected 118 genes after 28 days post-injecting mice from spleen, among them *iol* gene. A recent study by Cordero-Alba et al. (2012) indicated that *iolR* (STM14_5307 or STM4417) in *S*. Typhimurium plays a role in regulating negatively *srfJ*, a *Salmonella* T3SS effector for SPI-2 and associated *iolR* with virulence.

The last gene is *aroD*, encodes the enzyme 3-dehydroquinate dehydratase and is involved in aromatic amino acid biosynthesis. The *aroD* mutation is auxotrophic and has sensitivity to the innate immune response, a deficiency in outer membrane and cell wall unity, and an inability to form biofilm (Cano et al, 2003; Malcova et al, 2009; Sebkova et al, 2008). This gene is associated with microaerophilic respiration and essential in the stationary phase (Rychlik and Barrow, 2005). A mutation in *aroD* will result in non-suppressive growth phenotype that cannot compete, overcome the growth of the wild type strain, and colonize the host gut (Nógrády et al., 2003). In *S*. Enteritidis, *aroD* mutation was attenuated in porcine blood serum and this is applied to *S*. Typhimurium (Sebkova et al., 2008). In addition, using microarray to study gene expression for genes of *aroD* mutant strain resulted in the identification of 50% of genes negatively modulated by *aroD* mutant strain that were expressed from virulence plasmids. One of the suppressed genes is *dps* encoding for a virulence-related protein. Cano and co-workers (2003) demonstrated that a strain of *aroD* mutation exhibited higher intracellular persistence in the cell culture, fibroblasts more than the wild type parental strain. Furthermore, they observed a slow growth rate under aerobic conditions without any association of nutrient limitation for *aroD* mutant strain.

Growth response determination

At first, we determined the growth response and linear-regression of all strains under 37^{o}C and 42^{o}C using a natural log of the optical density readings of wild type and the mutants (Figures 2 and 3) (Table 2 and 3). The calculated doubling time for WT, Δ*recD*, ΔSTM14_5307, and Δ*aroD* at 37^{o}C was 19.3, 21.01, 20.4, and 22.4 minutes respectively. At 42^{o}C, a delay of doubling time was noted as 19.8, 21.7, 20.4, 25.7 minutes for WT, Δ*recD*, ΔSTM14_5307, and

Δ*aroD* respectively (Figure 1). At 37°C, the linear regression R^2 value for the wild type, Δ*recD*, ΔSTM14_5307 and Δ*aroD* were 0.998, 0.997, 0.998 and 0.992 respectively. In comparison at 42°C, the linear regression R^2 was 0.999, 0.995, 0.998, and 0.962 for the wild type, Δ*recD*, ΔSTM14_5307 and Δ*aroD* respectively (Table 4).

Survival of heat treatment

All *Salmonella* strains grew similarly and were processed for heat treatment responded equally. The survival percentage of heat treated strains at 50, 55, and 60°C was calculated from three independent trials with the greatest reductions observed from the Δ*aroD* strain with only 0.05, 0.13, and 0.02 % survival respectively. The reductions for both Δ*recD* and ΔSTM14_5307 strains with survival percentage were 1.12 and 1.22 % at 50°C, 2.62 and 2.58% at 55°C with 1.22 and 1.32 % at 60°C . In contrast, the wild type exhibited a survival percentage of 11.84, 14.39, and 10.50 % correspondingly at 50, 55, and 60°C (Figure 3).

Phenotype microarray profiling

The results of each mutants compared to the wild type (parental strain) were from two temperatures (37°C and 42°C). Reproducibility analysis was performed using two biological replicates for each mutant, and all mutants passed this test. In general, all mutants exhibited resistance to kanamycin and some other aminoglycoside antibiotics such as kanamycin served as the selective marker for constructing the single deletion mutant. For *recD* mutant, the metabolic tested panels did not exhibit any phenotypes differences. In addition, testing this mutant for the chemical compounds sensitivity, it exhibited a high defect of phenotype for lomefloxacxin, an inhibitor of DNA gyrase. At 42°C, Δ*recD* strain exhibited no differences except a sensitive

phenotype for the chemical compound, 2-phenylphenol and small deficient degree for enoxacin, a gyrase inhibitor.

For STM14_5307 mutant and at 37^{o}C, the mutant particularly does not use ammonia as a nitrogen source and exhibited a defect in sulfur metabolism with negative utilization of most sulfur compounds. This mutant lost some phenotype for numerous amino acid metabolism pathways and a few other nitrogen sources. In addition, there were lost phenotypes for peptides that frequently include the amino acids glycine or aspartate; and also many lost phenotypes for phosphorus sources. Numerous lost phenotypes were exhibited for sulfur perhaps with the exception of certain methionine containing sulfur sources. Sensitivity for some chemical compounds was observed for a few inhibitors. At 42^{o}C, the mutant exhibited the greatest metabolic phenotype for utilizing myo-inositol as a sole carbon source. In addition, neither the wild type nor STM14_5307 mutant used ammonia as a nitrogen source. There were a large phenotype lost for cytidine 5`-monophosphate, as an organic source of phosphorus and moderate magnitude lost for citric acid as sole carbon source.

In the conditions for *aroD* mutant, at both 37^{o}C and 42^{o}C, the mutant is largely incapable of dye reduction on nitrogen utilization assays, phosphorus and sulfur utilization assays, biosynthetic pathway and nutrient stimulation assays. It is unclear why this mutant exhibited this malfunction, but it is possibly either related to its auxotrophy or some unidentified problem. At 37^{o}C, lost phenotypes were exhibited for various carbohydrates and carboxylic acids, as well as a small number of other carbon substrates. This mutant has a notable growing defect at high pH and exhibited considerable sensitivity to chelators, membrane targeting agents, and toxic ions. At 42^{o}C, numerous phenotypes lost for carboxylic acids and a few other carbon sources. Measurable sensitivity was observed for chelators and a few toxic ions.

As a measurement for ensuring food safety, thermal treatments are one of the more effective physical methods for controling food-borne pathogens on food handling practices (Leistner, 2000; Gould, 2000; Aymerich et al, 2008). *Salmonella* Typhimurium is known to be able to survive such conditions including high heating temperature treatment (Doyle and Mazzotta, 2000; de Jong et al, 2012; Humphrey et al, 2001; Palumbo et al, 1995).

In our study, we tested the ability of *Salmonella* Typhimurium 14028 wild type and three constructed single deletion mutants (Δ*recD*, ΔSTM14_5307, and Δ*aroD*) as these mutants previously exhibited a growth deficiency as temperature-related phenotypes for 42^{o}C (Khatiwara et al, 2012) referring to the essentiality of these genes for *Salmonella* Typhimurium inside the avian body. Testing the ability of those mutants to survive mild thermal treatment mimicking thermal food processing will help in understanding *Salmonella* adaptation. Furthermore, distinguishing the metabolic requirements and the sensitivity of each mutant to numerous chemical conditions and compounds at both 37^{o}C and 42^{o}C will assist in providing a clear prospective in developing strategies to control *Salmonella* in human and chicken in association with food safety.

Some *Salmonella* strains are able to grow at temperatures as low as 2^{o}C and as high as 54^{o}C make this microorganism capable of contaminating various food products and survive the processing (Droffner and Yamamoto, 1991; Finstad et al, 2012; Hanning et al, 2009; Howard et al, 2012; Lynch et al, 2009; Nayak et al, 2012; Montville and Matthews, 2008; Park et al, 2014). In general, thermal (heat) has been the most applied preservative treatment in processing foods. Thermal treatment of foods can take place either before or after packaging. Pasteurization is mild heat process between 55^{o}C and below 100^{o}C for a period of time and may not completely eradicate all microorganisms (Gould, 2000). Hot water and steam interventions have been

applied to poultry carcasses to be decontaminated of *Salmonella* by up to 2.1 and 3.8 log units respectively. A temperature and exposure time combination has a *vice versa* relation as increasing both may improve the efficiency of sanitizing the food surfaces; however, it will affect the food product quality and appearance (Buncic and Sofos, 2012). Steam pasteurization processing received approval as an effective preservation method for meat and poultry carcasses, combining elevated temperature and pressure for a short exposure time followed by chilled water treatment to minimize the destructive effects (Chen et al., 2012; Wheeler et al., 2014). Retzlaff et al (2004) demonstrated that *Salmonella* Typhimurium on beef carcass when exposed to steam pasteurization achieved an effective reduction of greater than 1.0 log CFU/cm^2 at approximately 6 seconds with a temperature of 93.3^oC. A study verified a 1.5 log CFU/cm^2 for *Salmonella* Typhimurium at steam temperature of 83^oC for a treatment of 15 seconds (McCann et al., 2006). Park and Kang (2014) investigated the outcome of using steam pasteurization to inactivate the biofilm of *Salmonella* and other foodborne pathogens. Biofilms were formed on polyvinyl chloride coupons with three strains of *Salmonella* after incubation of six days. Subsequently, the coupons were exposed to 75^oC and 85^oC, each for 50 seconds and a 2.04 and 3.01 log CFU/coupon reduction was observed respectively suggesting a promising control intervention for hardly treated form of cells.

In fresh produce the condition is totally different; hot water is used to limit the loss of fruits and vegetables by controlling pests and other plant pathogens causing spoilage to post-harvest products. Fresh produce that are heat treated have been considered by the FDA as not fresh (FDA-CFR, 2013). Thermal treatment for fresh produce may not be as effective as it should be in reducing *Salmonella* and other foodborne pathogens especially when followed by immediate chilling process in contaminated immersion tanks; in contrast, it may facilitate the

pathogen to internalize into the tissue of fruits or vegetables. An outbreak of *Salmonella* Newport was associated with the consumption of Brazilian imported mangos. As an extra measurement for mangos exported to the U.S., mangos undergo mild heat treatment to get rid of fruit flies using hot water at 46.7 to 47^{o}C for approximately 90 minutes and subsequently cooled down with low temperature water of 21^{o}C for 10 minutes in a water tanks considered to be chlorinated to control any contamination. This cooling step believed to be an important and possible infiltration factor that assists *Salmonella* to cross the fruit surface (Berger et al., 2010; Levantesi et al., 2012; Parish et al., 2003; Penteado et al., 2004; Sivakumar et al., 2011; Sivapalasingam et al., 2003).

Many studies have examined the heat inactivation and thermal resistance of several *Salmonella* strains. A valid heat treatment for *Salmonella* requires a cocktail of several serotypes and strains. A reduction of 6.5 logs from meat and meat products, and a reduction of 7 logs from poultry and their products with minimal process time is the regulatory required by USDA (FSIS-USDA, 2012). Thermal treatment using steam and hot water decontaminates carcasses from *Salmonella* by approximately 100 to 1000 times (Hald, 2013; Montville and Matthews, 2008). In a study by Morrison and Fleet (1985), *Salmonella* was reduced by immersing chicken carcasses in hot water as the result of elevating the carcass surface temperature. In addition, several researchers have achieved reductions with pathogens and spoilage microorganisms between 2 and 3.7 log CFUs with temperatures between 70^{o}C and 95^{o}C, but those treatments for poultry exhibited either a cooked appearance or caused tearing of the chicken skin (Avens et al., 2002; Corry et al., 2007; Göksoy et al., 2001; James et al., 2007; Purnell et al, 2004; Whyte et al., 2003).

Since using hot water or steam as a heating treatment with temperatures over 70^{o}C may be undesirable for food by changing its characteristics such as appearance, the color, the taste, mild heating has been used in combination with other treatments to effectively inactivate pathogens and other spoiling bacteria. A study by Milillo and others concluded that combining multiple treatments can possibly and effectively reduce *Salmonella* in a short time. They used mild thermal treatments and acidified organic acid salt solutions for an exposure of one minutes. Sodium propionate with heating has shown to be the most significantly effective in causing viability loss of *Salmonella* tested strains (Milillo and Ricke, 2010; Milillo et al, 2011).

O'Bryan et al. (2006) reviewed the heat resistance of *Salmonella* species and other foodborne pathogens associated with meat and poultry. They found that many factors and parameters are involved in the heat resistance and inactivation of those pathogens and spoilage microorganisms. Strains of the same microorganisms can react differently to the same treatments. Also, the stage of the growth, the culture age, and the conditions of the growth will yield various outcomes regarding heat inactivation or destruction of microorganisms. Although it is used to mimic the actual food production chain, it is still not fairly accurate for the commercial processing conditions (O'Bryan et al, 2006). Some studies have concluded that the modifications of the membrane fatty acid composition of *Salmonella* strains had a connection to its ability to resist thermal treatment mainly for those cells with less fluidity of their membrane (Álvarez-Ordóñez et al, 2008; Álvarez-Ordóñez et al, 2009; Balamurugan, 2010; Sampathkumar et al, 2004).

Phenotype Microarray profiling has been applied to over 300 bacterial strains, among them approximately 20 publications on *Salmonella* species (Biolog, Inc., personal communication request). Testing strains can be profiled for metabolic and/or sensitivity with 20

plates with different substrates. Metabolic profile includes 8 plates of utilization assays for carbon, nitrogen, phosphorus, sulfur, nutrient stimulation, and biosynthetic pathway. The carbon metabolism is profiled in 2 micro-plates with 192 assays including 2 controls. Nitrogen sources are utilized in 4 plates with 384 assays, phosphorus and sulfur metabolic profile is performed in one plate with 96 assays, and 96 assays for nutrient stimulation with biosynthetic pathway. For sensitivity profiling, 12 plates can be tested for osmotic and ionic response, pH response, and chemical sensitivity assays. The osmotic and ionic response are profiled in 1 plate with 96 assays, the pH response in 1 plate with 96 assays, and the chemical sensitivity are tested in 10 plates for 960 assays including 3 plates of antimicrobial compounds (Bochner, 2003, 2009; Bochner et al., 2008).

As a major foodborne pathogen, numerous *Salmonella* strains were profiled using a phenotype microarray (PM). The mutants that exhibited important and major phenotypes with regard to viability, survival, virulence, pathogenicity are discussed here. Each of the wild type and the three mutant strains was screened 1920 assays at the temperatures, 37^{o}C (human body temperature) or 42^{o}C (poultry body temperature) with 3840 assays per strain for both temperatures. All strains were tested for a total of 7680 assays at each temperature with a total of 15360 assays for all strains (wild type and 3 mutants) at both temperatures.

In STM 14_5307 as we previously stated in the result section, it is a homolog to STM4417 from S. Typhimurium LT2, also known as *iolR* gene. This gene is a negative regulator of *iol* genes in the utilization of myo-inositol as a sole carbon source (Kröger and Fuchs, 2009; Kröger et al., 2010, 2011). Surprisingly, the complete genes set of this genomic island (*iol*) containing the gene (STM14_5307 or *iolR*) is not present in all *Salmonella* species. It was identified in the genomes of some strains of *S*. Typhimurium (LT2, 14028, SL1344), *S*. Saintpaul

SARA23, *S.* Weltevreden HI_N05-537, and *S.* Paratyphi B strain SPB7. This genomic island is lacking some *iol* genes in several *Salmonella* species such as *S.* Typhi CT18/Ty2, *S.* Paratyphi A/C, *S.* Enteritidis, *S.* Dublin, *S.* Gallinarum, and *S.* Diarizonae (Kröger and Fuchs, 2009). *S.* Typhimurium growth on myo-inositol exhibited some characteristics mainly with a lag phase that may last to approximately 2 ½ days.

Myo-inositol (inositol) is an essential carbon source and substance for phospholipid phosphatidylinositol (PI) synthesis. Pathogens need either to acquire the inositol from the environment, the host or synthesize it to produce phospholipid phosphatidylinositol that mainly involves as a membrane structural component and other lipd molecules (Reynolds, 2009; Staib and Fuchs, 2014). The inositol is also used by numerous eubacterial pathogens to synthesize compounds serve as de-toxification and cell protection from oxidative damage. Inositol transporters are used by numerous pathogens to import (strip) the inositol and subsequently transport it through the membrane ion gradients into pathogen cells. One main milieu during infection is the bloodstream of mammalian and other animal hosts as a source for numerous pathogens for inositol (Reynolds, 2009; Staib and Fuchs, 2014). For growth and/or virulence of the pathogen, phospholipid phosphatidylinositol is utilized to produce phosphatidylinositol mannoside (PIM), lipomannan (LM) and lipoarabinomannan (LAM). In some eubacterial pathogens, the first two products are necessitated for cell viability and the latter product is involved in regulating the immune response as a virulence factor by blocking the phagosome from becoming mature (Reynolds, 2009; Staib and Fuchs, 2014). Some genes in some *Salmonella* species such as *sopB* from *S.* Dublin that encoded in *Salmonella* Pathogenicity Island 5 (SPI-5) is an inositol phosphate phosphatase and the gene *sigD*, an *sopB* homologue is encoded in *S.* Typhimurium (Brumell et al., 1999).

A critical phenotype lost as a result of the STM14_5307 mutation is the incapability to utilize cytidine 5`-monophosphate (CMP, cytidylate), a ribonucleotides and one of the structural units of RNAs. This substrate, CMP, is phosphorylated to cytidine diphosphate (CDP) through the catalyzation of CMP kinase in the pyrimidine biosynthesis pathway by adenosine triphosphate (ATP) or guanosine triphosphate (GTP). Since the mutant strain could not utilize CMP as a phosphorus source, it is feasible to conclude that CMP kinase was directly or indirectly inactivated as an effect of STM14_5307 mutation. CMP kinase function has been recognized to play vital roles in numerous bacterial strains. Fricke et al (1995) observed a growth reduction in *E. coli* strain with inactivated *cmk* gene with a decline in DNA replication rate. Yu and other (2003) reported the essential function of CMP kinase in *Streptococcus pneumonia* viability. Walker et al (2012) demonstrated that CMP kinase is crucial for virulence in *Yersinia pseudotuberculosis*, viability, and *cmk* mutant exbited attenuation in mice infection model.

In conclusion, we conducted two studies in this chapter. We used *S*. Typhimurium for heat survival under 50^{o}C, 55^{o}C, and 60^{o}C. Also, Phenotype Microarray screening was performed for 3 mutants (Δ*recD*, ΔSTM14_5307, and Δ*aroD*) as formerly exhibited temperature-association growth defect in comparison to their wild type parental strain. The heat survival result was significant as Δ*aroD* exhibited the highest defect follow by the Δ*recD* and ΔSTM14_5307. For phenotype microarray profiling, Δ*recD* exhibited no differences from the wild type, and Δ*aroD* could not reduce the dye that differentiated from the wild type for metabolic profile remains unclear. In the case of ΔSTM14_5307, two important phenotypes were noted. The first one was the phenotype being able to utilize myo-inositol as a sole carbon source and in the second phenotype; the mutant exhibited a lost ability to utilize cytidine

monophosphate as a substrate for phosphorus source. Both phenotypes have shown to play a role in virulence, viability, and/or pathogenicity of *Salmonella* and/or other bacterial pathogens.

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Table 1. Oligonucleotides used in aroD mutant construction.

Oligonucleotide	Sequence (5'→3')	Reference
aroD-Up F	CGCATATGACAAAGTTATTGCAG	This
aroD-Up R	GAAGCAGCTCCAGCCTACACACTTCGCCAACCACGAGAT	study
	To Add P1-seq↑	
	To Add P2-seq↓ (Reverse Complimentary)	
aroD-Dn F	ACTAAGGAGGATATTCATATGCCGATCTGCGTACCGTATTA	This
aroD-Dn R	CCATTAATGCGTGATGGCTTA	study
aroD-Seq-F	ACCAAAGTTGGCATGAAACC	This
aroD-Seq-R	GTGGTGACTGGGTTGTTTGA	study
pKD4-P1	TGTGTAGGCTGGAGCTGCTTC	Datsenko and
pKD4-P2	CATATGAATATCCTCCTTAGT	Wanner (2000)

Table 2. *Salmonella* Typhimurium growth at 37°C

Strain	WT		Δ*recD*		ΔSTM14_5307		Δ*aroD*	
Time (min)	OD_{600}	Ln (OD_{600})	OD_{600}	Ln (OD_{600})	OD_{600}	Ln (OD_{600})	OD_{600}	Ln (OD_{600})
0	0.068	-2.68825	0.068	-2.688248	0.067	-2.703063	0.097	-2.333044
10	0.106	-2.24432	0.097	-2.333044	0.092	-2.385967	0.099	-2.312635
20	0.14	-1.96611	0.137	-1.987774	0.126	-2.071473	0.133	-2.017406
30	0.193	-1.64507	0.187	-1.676647	0.18	-1.714798	0.195	-1.634756
40	0.281	-1.2694	0.251	-1.382302	0.245	-1.406497	0.26	-1.347074
50	0.425	-0.85567	0.38	-0.967584	0.38	-0.967584	0.382	-0.962335
60	0.65	-0.43078	0.57	-0.562119	0.535	-0.625489	0.52	-0.653926
70	0.865	-0.14503	0.75	-0.287682	0.68	-0.385662	0.74	-0.301105
80	1.19	0.173953	0.985	-0.015114	0.93	-0.072571	0.935	-0.067209
90	1.7	0.530628	1.3	0.262364	1.11	0.10436	1.07	0.0676586
100	1.99	0.688135	1.8	0.587787	1.5	0.4054651	1.22	0.1988509
110	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.38	0.3220835
120	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.44	0.3646431
130	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.46	0.3784364
140	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.56	0.4446858
150	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.72	0.5423243
160	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.8	0.5877867
170	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.99	0.6881346

Table 3. *Salmonella* Typhimurium growth at 42°C

Strain	WT		Δ*recD*		ΔSTM14_5307		Δ*aroD*	
Time (min)	OD_{600}	Ln (OD_{600})	OD_{600}	Ln (OD_{600})	OD_{600}	Ln (OD_{600})	OD_{600}	Ln (OD_{600})
0	0.098	-2.32279	0.06	-2.813411	0.097	-2.333044	0.067	-2.703063
10	0.124	-2.08747	0.135	-2.002481	0.133	-2.017406	0.126	-2.071473
20	0.183	-1.69827	0.194	-1.639897	0.192	-1.65026	0.173	-1.754464
30	0.26	-1.34707	0.26	-1.347074	0.269	-1.313044	0.25	-1.386294
40	0.355	-1.03564	0.375	-0.980829	0.38	-0.967584	0.34	-1.07881
50	0.51	-0.67334	0.542	-0.612489	0.545	-0.606969	0.48	-0.733969
60	0.755	-0.28104	0.795	-0.229413	0.78	-0.248461	0.71	-0.34249
70	0.995	-0.00501	0.995	-0.005013	0.96	-0.040822	0.815	-0.204567
80	1.25	0.223144	1.25	0.223144	1.04	0.0392207	0.915	-0.088831
90	1.6	0.470004	1.7	0.530628	1.3	0.2623643	0.98	-0.020203
100	1.99	0.688135	1.99	0.688135	1.8	0.5877867	1.07	0.0676586
110	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.1	0.0953102
120	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.14	0.1310283
130	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.19	0.1739533
140	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.21	0.1906204
150	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.27	0.2390169
160	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.3	0.2623643
170	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.38	0.3220835
180	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.45	0.3715636
190	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.5	0.4054651
200	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.57	0.4510756
210	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.63	0.48858
220	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.69	0.5247285
230	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.75	0.5596158
240	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.81	0.5933268
250	1.99	0.688135	1.99	0.688135	1.99	0.6881346	1.99	0.6881346

Table 4. *Salmonella* Typhimurium phenotypes growth rate and linear regression

Strain	Doubling Time (min)	R^2
WT (37^{o}C)	19.3	0.9977
Δ*recD* (37^{o}C)	21.01	0.9967
ΔSTM14_5307 (37^{o}C)	20.4	0.9978
Δ*aroD* (37^{o}C)	22.4	0.992
WT (42^{o}C)	19.8	0.9988
Δ*recD* (42^{o}C)	21.7	0.9946
ΔSTM14_5307 (42^{o}C)	20.4	0.9967
Δ*aroD* (42^{o}C)	25.7	0.9617

Figure 1. Doubling Time for growth at different temperatures

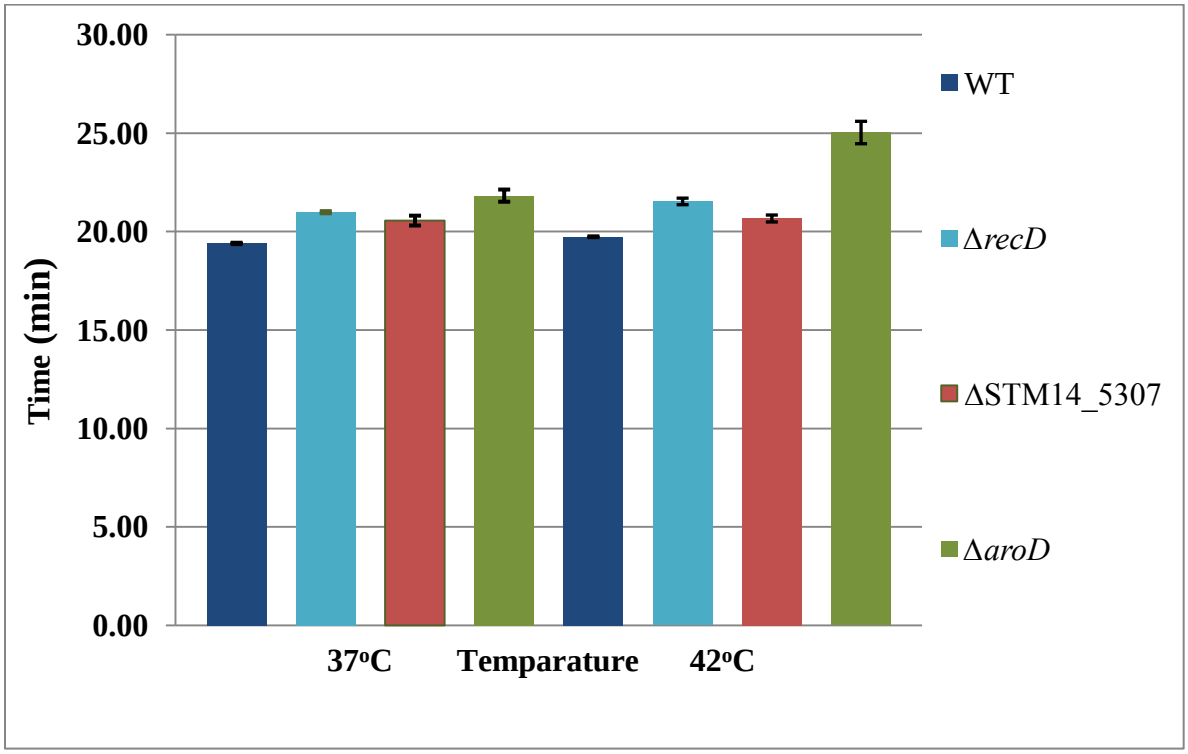


Figure 2. Growth rate curve of *S.* Typhimurium 14028 Phenotypes at 37°C

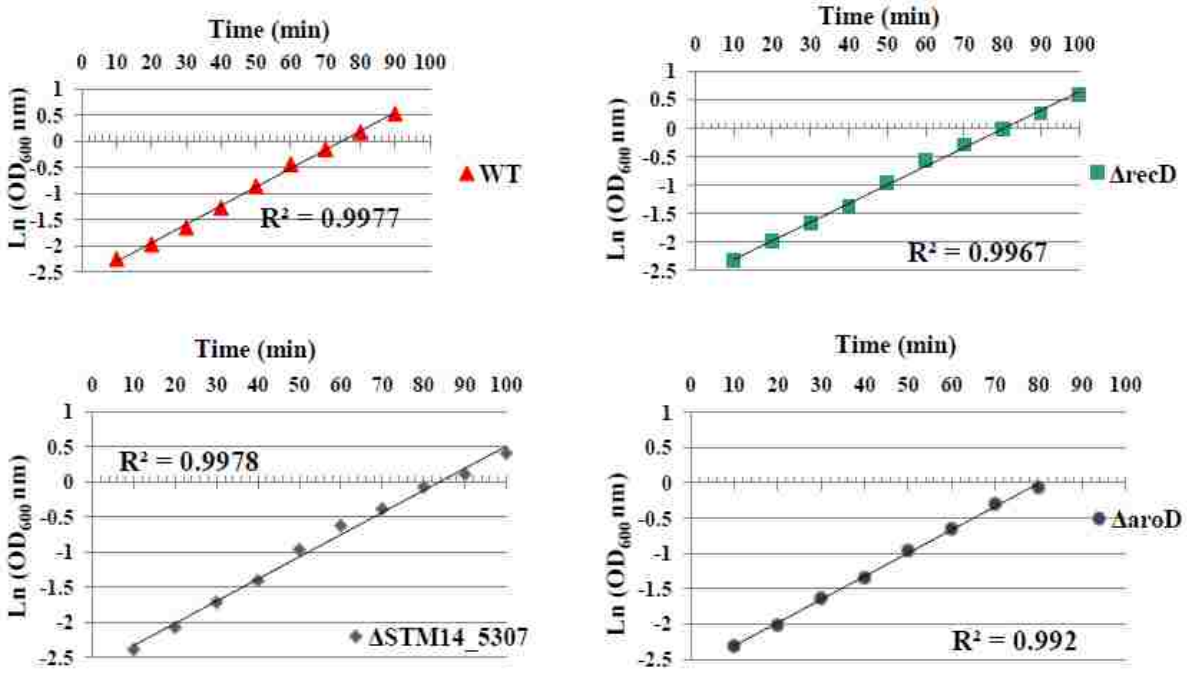


Figure 3. Growth rate curve of *S.* Typhimurium 14028 Phenotypes at 42°C

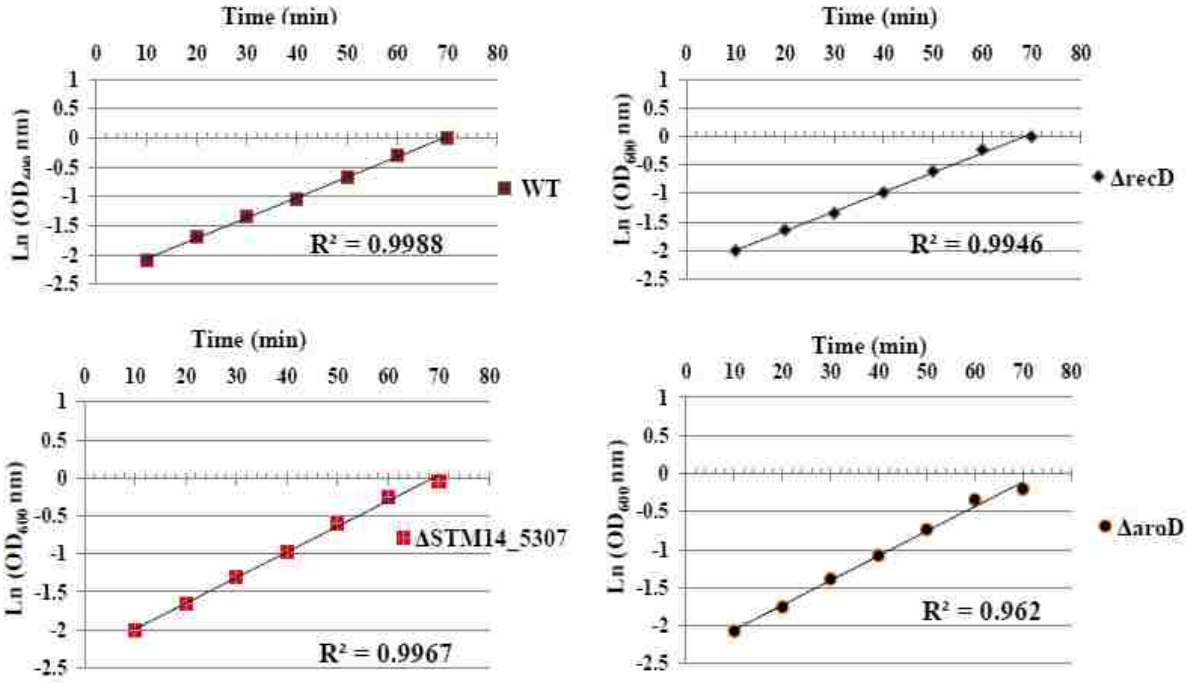
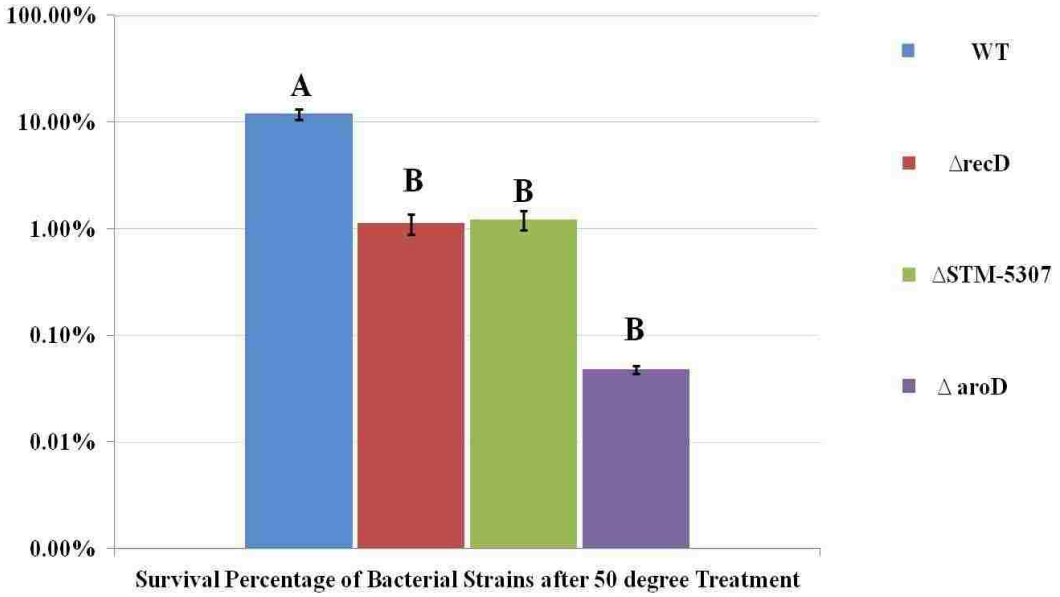
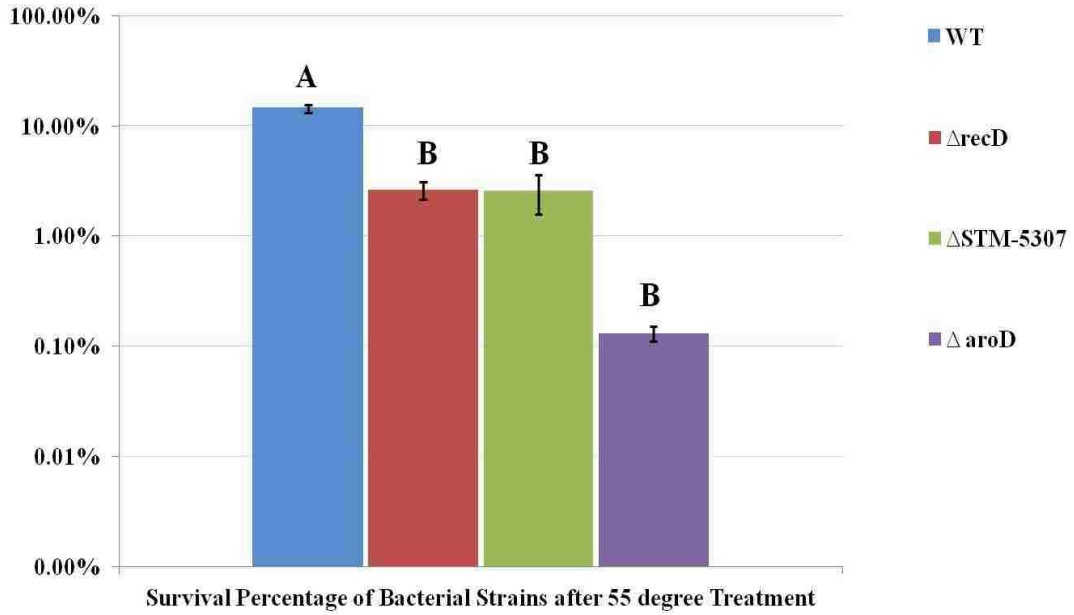


Figure 4. Survival phenotype of *S.* Typhimurium 14028 mutants after heat treatments

A) At 50°C for 3 hours



B) At 55 °C for 15 minutes



C) At 60 °C for 3 minutes

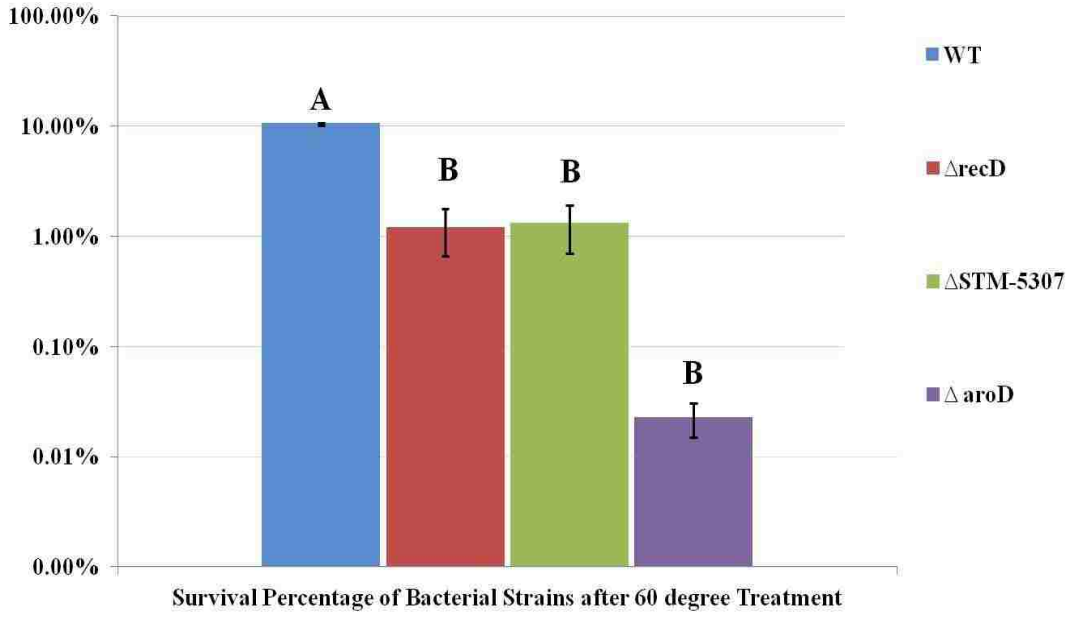


Table 2. Results of comparing phenotypes expressed by three mutants and ATCC 14028 (Wild Type) at two temperatures[a]

Phenotype MicroArray (PM)[a]			Substrate(s)[b]	Mode of action
No.	37°C	42°C		
Phenotypes by Δ*recD* relative to ATCC 14028				
PM12B	Gained	Gained	Paromomycin	protein synthesis, 30S ribosomal subunit, aminoglycoside
PM11C	Gained	Gained	Kanamycin	protein synthesis, 30S ribosomal subunit, aminoglycoside
PM13B	Gained	Gained	Geneticin disulfate (G418)	protein synthesis, 30S ribosomal subunit, aminoglycoside
PM11C	Gained	Gained	Neomycin	protein synthesis, 30S ribosomal subunit, aminoglycoside
PM11C	Lost	No difference	Lomefloxacin	DNA topoisomerase
PM18C	No difference	Lost	2- Phenylphenol	DNA intercalator
PM11C	No difference	Lost	Enoxacin	DNA topoisomerase
Phenotypes by ΔSTM14_5307 relative to ATCC 14028				
PM12B	Gained	Gained	Paromomycin	protein synthesis, 30S ribosomal subunit, aminoglycoside
PM11C	Gained	Gained	Kanamycin	protein synthesis, 30S ribosomal subunit, aminoglycoside
PM11C	Gained	Gained	Neomycin	protein synthesis, 30S ribosomal subunit, aminoglycoside
PM13B	No difference	Gained	Geneticin disulfate (G418)	protein synthesis, 30S ribosomal subunit, aminoglycoside
PM01	No difference	Gained	*m*-Inositol	C-Source, carbohydrate
PM04A	No difference	Lost	Cytidine 5'-Monophosphate	P-Source, organic
Phenotypes by Δ*aroD* relative to ATCC 14028 (Sensitivity Profile Only).				
PM11C	Gained	No difference	Colistin	membrane, cyclic peptide
PM14A	Gained	No difference	Furaltadone	nitro compound, oxidizing agent, DNA damage
PM12B	Gained	Gained	Paromomycin	protein synthesis, 30S ribosomal subunit, aminoglycoside
PM11C	Gained	Gained	Kanamycin	protein synthesis, 30S ribosomal subunit, aminoglycoside

Table 2. Continue..

Phenotype MicroArray (PM)[a]			Substrate(s)[b]	Mode of action
No.	37°C	42°C		
PM13B	No difference	Gained	Geneticin disulfate (G418)	protein synthesis, 30S ribosomal subunit, aminoglycoside
PM18C	No difference	Gained	Semicarbazide hydrochloride	amine oxidase inhibitor, carcinogen
PM16A	No difference	Gained	Dichlofluanid	fungicide, phenylsulphamide
PM15B	No difference	Gained	Alexidine	membrane, electron transport, biguanide

[a] The OmniLog-PM software generates time course curves for respiration or growth and calculates differences in the areas under the curve for mutant versus parent cells. Gained indicates that the mutant showed greater rates of respiration or growth than the parent strain. Lost indicates that the mutant showed lower rates of respiration or growth than the parent strain.

[b] Chemicals were tested in 96-well PMs.

[c] Rest of the results will be in the supplement file (PM Reports, Biolog, Inc.).

[d] See text for explanation.

Appendix

Biological Materials Transfer Agreement

ATCC™
Office of Intellectual Property and Licensing

A global bioscience nonprofit organization dedicated to biological standards and biodiversity

10801 University Boulevard
Manassas, Virginia 20110-2209 USA
Telephone: (703) 365-2700
Facsimile: (703) 334-2932
Email: licensing@atcc.org

April 15, 2014

Dr. Michael Ziman
Biolog, Inc.
21124 Cabot Blvd.
Hayward, CA 94545

Dear Dr. Ziman,

The ATCC authorizes the one-time transfer of ATCC® 14028™ and three single deletion mutants ("Biological Materials") that was acquired from the ATCC so that Biolog, Inc. can perform the following service:

Comparing ATCC® 14028™ to the three deletion mutant cell lines provided by the University of Arkansas. After the service is complete, Biolog must return or destroy the Biological Materials. If you agree to the terms of the authorization letter, please indicate your acceptance by signing a copy of this letter and returning it to my attention via fax to: 703-334-2932 or email to licensing@atcc.org.

Sincerely,

Stewart N. Davis
Director, Intellectual Property and Licensing

AGREED TO AND ACCEPTED:
Biolog, Inc.

Name: Michael Ziman

Title: Dir. PM Services

Date: 4/16/2014

Chapter 5

Genome-wide Identification of *Salmonella* Typhimurium Genes Essential for Cold Temperature Survival on a Chicken Carcass Using a Tn-seq Method

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Abstract

Non-typhodial salmonellae are one of the more prominent foodborne pathogenic bacteria responsible for serious public health problems both in the United States as well as worldwide. Although *Salmonella* contamination is associated with a wide range of foods including meat, poultry, eggs, and their products, poultry meat products are often a primary source of human salmonellosis, and *Salmonella* Typhimurium is one of the most common serotypes associated with human illness outbreaks. To extend the shelf- life of chicken carcasses by limiting microbial growth, poultry carcasses are required to be chilled to low temperatures throughout transportation and distribution. However, despite the administration of a variety of anitmicrobials during poultry processing and the subsequent chilled temperature exposure, foodborne disease causing *Salmonella* still persists in processed raw chicken. To develop and improve food intervention strategies, a better understanding of *Salmonella* survival under cold temperatures and response to such stresses is needed. In this study, we screened a genome-saturating library of *S.* Typhimurium Tn*5* mutants for survival on a chicken carcass stored at different temperatures [room temperature (RT, reference sample), refrigeration (4^{o}C), and freezing (-20^{o}C)] using a Tn-seq method. Bioinformatics analysis of the Illumina sequencing data revealed more than 100 candidate genes that could be identified as essential specifically for cold temperature survival on a chicken carcass. Six single deletion mutants' Δ*fur*, Δ*trkA*, and Δ*rfbI* for skin attachment, Δ*topA*, ΔSTM14_1047, and ΔSTM14_3311 for cold temperature only associated with chicken carcasses were selected. They were tested individually in competition assays mixed with wild type and the results demonstrated the predicted phenotypes by Tn-seq data and their role under cold temperature. This is the first study of its kind to identify genetic factors important for *Salmonella* survival on a chicken carcass. These results will

provide valuable insights for the development of more effective measures to reduce the pathogen in poultry products under these types of environmental conditions.

Keywords: *Salmonella*, chicken carcass, cold temperature, survival, Tn-seq.

Introduction

Salmonella species are one of the main public health concerns in the United States and Worldwide. *Salmonella* is the second leading cause of food-borne illnesses resulting in hospitalized cases with 35% and 28% of deaths in the United States (Scallan et al, 2011). It is estimated that the total economic cost of non-typhoidal *Salmonella* illness in the United States to be an average of 11.39 billion dollars (Scharff, 2012; Byrd-Bredbenner et al, 2013).

The sources of infection are wide range, but the association of poultry and poultry products has long been documented (Braden, 2006; Bäumler et al., 2000; Cogan and Humphrey, 2003; Foley et al., 2011, 2013; Guard-Petter, 2001; Howard et al., 2012). *S.* Typhimurium has been along with *S.* Enteritidis leading serovars isolated from human and other non-human sources, mainly broiler and broiler products in the United States (Foley et al., 2011, 2013; Jackson et al., 2013). During last decade, *Salmonella* infections have been associated with poultry and poultry products as poultry meat is regulated by USDA (U.S. Department of Agriculture) and eggs are regulated by the U.S. FDA (U.S. Food and Drug Administration). *Salmonella* was linked to 458 outbreaks for meat and 125 outbreaks were connected to eggs and its related dishes (DeWaal and Glassman, 2013). It has been previously concluded that approximately 35% of human infections by *Salmonella* were associated with poultry consumption (Finstad et al, 2012).

Globally, chicken and other poultry are the most consumed and popular food source of meats (Harmon, 2013; Kearney, 2010). The average American consumption of chicken per capita is 83.4 pounds annually in 2014 and expected to be 85.5 in 2015 (Mathews and Haley, 2014) and by 2010 it surpassed the beef. The United States is one of the largest producers of poultry and its products are mainly chickens, turkey meats, and eggs. With this high demand of meat consumption domestically and internationally, it is required to increase the safety of poultry

products. It was estimated that the production of broiler meat by the end of March 2013 to be 9.16 billion pounds. This high quantity of production will require proper storage to control the growth of foodborne pathogens, namely *Salmonella* species and other bacterial species. The U.S. shipments of broiler in February 2013 were 588.4 million pounds, and the domestic cold storage was approximately 61.5 million pounds by the end of February 2013 with an increase of 8.3% from the same period of 2012 (Johnson, 2013).

Salmonella species can contaminate the chickens' carcasses and their meat products at any stage of processing, storage of food products, and distribution system (Corry et al., 2002; Cox et al., 2011; Heyndrickx et al., 2002; Wilson, 2002). As a method of preservation, poultry is stored under cold-temperature storage into refrigeration ($0^{o}C$-$5^{o}C$) or freezing ($\geq$ -$18^{o}C$) (Buffo and Holley, 2006; Buncic and Sofos, 2012; James et al., 2006) to extend shelf life and improve poultry and its product safety (Smadi et al., 2012; Sofos, 2008; Zhou et al., 2010). Although this method is used to control and minimize the growth of spoilage and foodborne pathogenic bacteria in general (Bolder, 2007; Dave and Ghaly, 2011), recent attention has been given to understand the mechanisms by which *Salmonella* adapt to survive and/or grow at low temperature conditions in association with poultry and eggs. Mainly, microorganisms differ in responding to sudden shift or decrease to low temperature affecting their metabolic processes and causing some physiological modifications during lag stage (Wouters et al, 2000). These changes include DNA supercoiling modifications, maintaining cell membrane fluidity, solutes uptake and synthesis regulation, improper protein folding, cold-shock response involves protein production, mRNA secondary structure modulation, macromolecules, such as ribosomes, structural integrity maintenance, and an increase in the concentration of sugars synthesis and transport systems (Wouters et al, 2000; Beales, 2003; Schumann, 2007, 2012).

Most studies focused on *Salmonella* stress response have been limited to understanding its survival mechanism to heat and acid stresses (Álvarez-Ordóñez et al, 2008, 2009, 2010, 2012; Doyle and Mazzotta, 2000; Foster and Spector 1995; Mani-López et al, 2012; Milillo et al, 2011; Milillo and Ricke, 2010; Muller et al, 2009; Ricke, 2003; Ricke et al, 2005; Rychlik and Barrow, 2005; Spector and Kenyon, 2012; Wouters et al, 2000). It is feasible to say that microorganisms were thought to not be able to grow and/or survive cold temperature. In addition, the cells at cold temperature are not active which make it impossible to yield enough expressed protein. In our study, we conducted a high throughput screening approach to identify the genes that play a role in the adaptation of *Salmonella* Typhimurium under cold storage temperature (refrigeration and freezing) that might yield applications for poultry industry, especially with respect to broiler meat production.

2. Materials and Methods

Transposon mutant library Construction.

We previously used a QuikChange Site-directed mutagenesis kit (Agilent Technologies La Jolla, CA) to modify one nucleotide of the EZ-Tn5^{TM} pMOD^{TM}-6 <KAN-2/ MCS> Transposon Construction Vector (Epicentre BioTechnologies, Madison, WI) in one of the mosaic end (ME) sequences as performed by Khatiwara et al (2012) to introduce the recognition sequence of BsmFI, a TypeIIS enzyme, in one ME sequence. The nucleotide changes was confirmed previously (Khatiwara et al, 2012) through DNA sequencing. We used the modified plasmid, pMOD-6-BsmFI for transposon mutagenesis of the wild type *Salmonella* Typhimurium ATCC 14028. Digestion of this modified plasmid was done using PvuII-HF enzyme (New England Biolabs, Ipswich, MA) to get a fragment of 1117 bp of the modified transposon (EZ:Tn5-BsmFI). QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) was used to extract this

fragment from agarose gel without being exposed to the ultraviolet light. The DNA concentration and purity of the purified fragment were checked with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) with a DNA concentration of 190 ng /µl for the Transposome complex formation. Subsequently, the purified DNA fragment was incubated in a reaction with EZ-Tn5^{TM} Transposase to form a transposon-transposase complex according to the manufacture's manual (Epicentre Biotechnologies, Madison, WI). To construct the transposon complex library, two µl of the EZ-Tn5-BsmFI transposome complex were electro-transformed into *S.* Typhimurium 14028 wild type competent cells. Transformants were inoculated into LB agar plates supplemented with 50 µg/ mL kanamycin. Mutants' colonies were combined to form the complex mutants' library of EZ: Tn5-BsmFI with approximately 1.6 x 10^4 mutants and 30% glycerol stored at -80^{o}C.

Transposon mutant library: *in vitro* selection.

The EZ: Tn5-BsmFI mutant library was subjected to selection on chicken carcasses placed under the following 3 different conditions: room temperature (control), refrigeration (4^{o}C), and freezing (-20^{o}C) with the following procedure:

Inoculum preparation

The inoculum was prepared from the *S.* Typhimurium ATCC 14028 Tn5-BsmFI complex library glycerol stock (-80^{o}C). The inocula stock vials (approximately 1 ml), were combined with 9 ml of Luria Broth (LB) media and the resulting 10 ml combination incubated at 37^{o}C for an hour with vigorous shaking. After incubation, the cells was centrifuged and washed three (3) times with sterile 1X PBS to remove any remaining glycerol. Subsequently, the cell suspension was gradually diluted with sterile 1X PBS to reach the optical density (Optical density, OD600)

approximately 1.0 which corresponded to approximately 10^7 colony forming units (cfu) per ml. For a more accurate enumeration of the cell density, 100 µl of the diluted library was serially diluted and plated on LB with kanamycin (50 µg/ ml). The calculated cell density was approximately 2.83 x 10^7 cfu/ ml. One hundred ml of this library was used to inoculate each chicken carcass. Aliquots of 2 ml of the last 100 ml inocula were centrifuged and pellets were stored at -20^{o}C to be used for the input pool (INP).

Whole chicken carcass preparation:

Cornish hen carcasses (n=9) with similar size and weight were obtained from a local retail store. The carcasses were thawed overnight at 4^{o}C and then aseptically placed into 1 gallon sterile Ziploc bags and treated with 100 ml of the inocula as described in the previous section. Carcasses were inoculated by placing carcasses individually with ST-Tn5-BsmFI inocula followed by vigorous manual shaking for 5 to 10 minutes to insure that the inocula thoroughly came into contact with the carcass prior to removing the carcass and placing it over a sterile beaker inside a new 2.5 gallon sterile Ziploc bag. The inoculated carcasses were placed on top of 500 ml beaker to avoid direct contact of the surface at the bottom. All carcasses were kept at room temperature for one hour (1 hr) to slowly remove the extra inocula dripping inside the beaker (Figure 1). Subsequently, two groups of three (3) carcasses were incubated at 4^{o}C (first group) and -20^{o}C (second group) for three days. The last group of three (3) carcasses was used without further incubation to collect the first output pool for room temperature as the control condition.

Carcass sample collection:

After the treatment, the carcasses were each subjected to individual rinsing according to USDA guidelines for whole bird carcass rinses with some modifications involving the addition of 100 ml of sterile 1X PBS followed by manual inversion and rubbing for 10 minutes to recover a high level of collected mutant cells (Bauermeister et al., 2008). The rinse collected from each carcass was serially diluted and then enumerated on LB plates supplemented with kanamycin (50 µg/ ml). LB agar plates were subsequently incubated at 37^{o}C for 18 hrs and enumerated to determine the cfu/ml for each carcass rinse. A 10^{-1} dilution from the remaining suspension was used to inoculate 200 µl without dilution into LB plates supplemented with kanamycin (50 µg /ml) with 3 plates for each carcass. After an overnight incubation at 37^{o}C, cells from each carcass plates were pooled together. A mixture of recovered mutants was made by combining the pools from the same temperature condition together to generate the output pools. Aliquots of 2 ml of the last 100 ml inoculum were centrifuged and pellets were stored at -20^{o}C to be used for the input pool (INP). After vortexing thoroughly the recovered mixture of mutant pools, an aliquot of 500 µl for each temperature was used to extract the genomic DNA for the molecular process.

Illumina sequencing: sample preparation

After extracting the genomic DNA from all samples, the DNA was subsequently digested with BsmFI restriction enzyme (New England BioLabs, Ipswich, MA) at 65°C for 3 hours followed by heat inactivation at 80°C for 20 minutes. To prevent self-ligation of the digested DNA at the ligation reaction, the DNA was treated with calf intestinal alkaline phosphatase (CIP) (New England BioLabs, Ipswich, MA) at 37°C for 1 hour. DNA was extracted with phenol-chloroform, followed by ethanol precipitation and dissolved in 10 µl sterile ultrapure

water (H_2O). The digested DNA was then diluted ten times and ligated to a 10 µM Tn-seq linker by incubating the reaction at 16^{o}C for 12 hours. The Tn-seq linker was formed by annealing an equal amount of Tn-seq linker 1 and 2 oligonucleotides at final concentration of 100 µM (Table 1). The ligated samples were subsequently used as templates in separate PCR reactions using cloned *Pfu* DNA polymerase (Agilent Technologies, Santa Clara, CA) using one barcoded Tn5 primer and Tn-seq linker primer (Table 1). The PCR cycles consisted of initial denaturation at 94°C for 2 min, and 30 cycles of 94°C for 30 sec, 62°C for 1 min, and 72°C for 30 sec followed by final extension at 72°C for 10 min. The 129 bp long amplicon was visualized on a PAGE gel, purified and dissolved in 10 mM Tris-HCl pH 8.5. The 4 DNA samples tagged with different barcodes were mixed at the same amount of the DNA concentration confirmed by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Five µl of the mixture sample were used for cloning using a pGEM-T Vector system as instructed by the manufacture (Promega Corporation, Madison, WI). Colonies were picked and inoculated into LB broth media supplemented with ampicillin (100 µg/ml), and then incubated overnight at 37^{o}C with vigorous shaking. Plasmids were extracted with illustra plasmidPrep Mini Spin Kit (GE Healthcare Biosciences, Pittsburgh, PA) and DNA sequenced to confirm the present of 12 bp Tn5-junction sequences matched to *S*. Typhimurium 14028 genome. The final mixed sample was analyzed using Affimetrix BioAnalyzer to check the DNA quantity and quality. The sample was subsequently sequenced using an Illumina Genome Analyzer II at the Institute for Integrative Genome Biology at the University of California at Riverside.

Illumina sequencing: data analysis.

A Python programming language, a computer program, was used to perform the initial analysis for the raw data obtained by Illumina sequencing. First, the sequence reads were first sorted for the reads that contained the precise 19 bp modified ME sequence. Subsequently, the filtered sequences were sorted according to the 6 bp barcode sequences with an exact match to one of the 4 barcodes used. The transposon-junction sequences were then extracted from the filtered reads and the junction sequences of 11 to 13 bp long were selected for additional analysis. These selected transposon-junction sequences were subsequently mapped to the complete genome of *S.* Typhimurium 14028 (Chromosome CP001363, Plasmid CP001362) to select the reads that precisely mapped to the genome. Additional filtering was performed to select the reads that map to the genome only in one locus. The final output data obtained by the Python script contained all the information needed to be further processed by JMP.

From this step, the output data for chromosome and plasmid were processed separately using JMP10 Pro software (SAS, Cary, NC). For additional filtering, normalization, the sequence read numbers for each insertion in each output pool, the insertions in a gene with less than 3 insertion sites, and the numbers of all normalized sequence reads within each gene were processed according to Khatiwara et al (2012) with some modifications. For the chicken carcass attachment, the fitness index value was calculated for each insertion in every gene by dividing the total number of sequence reads in the output pool by those in the input pool unless the total number of sequence reads in the output pool equal to zero (0), then the calculation was 1/ the total number of sequence reads in the input pool. To distinguish those genes for cold temperature only from genes associated with carcass attachment, we used the normalized room temperature

pool as our input and 4^{o}C with -20^{o}C as output pools, and applied for all further data processing steps as described in the previous section.

Statistical Analysis

Chromosomal and plasmid data were analyzed individually using JMP Pro 10 software (SAS, Cary, North Carolina). The mean of fitness of room temperature (RT), refrigeration (4^{o}C) and freezing (-20^{o}C) conditions were calculated along with the t-test and *P* value. For genes associated with attachment, the genes considered essential and required for each particular condition were identified applying a *P* value <0.05 followed by further refinement using a *P* value <0.01 to represent a strict reduction in relative abundance throughout the data process. For genes related to cold temperature, the genes considered essential and involved into each distinct condition were identified applying *P* value <0.05 subtracting those required for attachment using the room temperature data as our reference for data processing.

Construction of deletion mutants

S. Typhimurium 14028 single deletion mutants harboring a mutation in *fur*, *trkA*, *rfbI* were previously constructed using Lamba Red recombination system and P22 transduction with kanamycin resistance gene cassette representing genes predicted by Tn-seq for chicken carcass attachment (Khatiwara et al., 2012; Kim and Kwon, 2013). We constructed single deletion mutants with a mutation in *topA*, STM14_1047, and STM14_3311, using Lamba Red recombination system and P22 transduction with kanamycin resistance gene cassette from pKD4 amplified using the primes listed in Table 1 (Datsenko and Wanner, 2000) representing genes predicted by Tn-seq for cold temperature only.

3. Results

The method used herein was previously developed as a modified version of the original Tn-seq and was tested to identify conditionally essential genes of *S.* Typhimurium 14028 (Khatiwara et al, 2012).

Transposon mutant library selection

By using this high-throughput method, we were able to identify 100 and 113 candidate genes of *Salmonella* Typhimurium 14028 under low temperature conditions recovered from chicken carcasses that possibly could be considered essential for cellular processes for carcass attachment and cold temperature only respectively. Based on deep profiling of Tn5-junction sequences using Illumina sequencing, we utilized a new version method developed in our lab to a genome-saturating *S.* Typhimurium mutant complex library to identify *S.* Typhimurium genetic factors important for survival on chicken carcass at different temperatures (Room Temperature, 4^{o}C, -20^{o}C). The EZ:Tn5-BsmFI mutant complex library we used consists of approximately 1.6 x 10^4 different mutants, and the 1 ml inoculum contained approximately 10^7 cells and an average of $2.83x10^7$ cfu/ml in the final inoculum used for chicken carcasses inoculation, demonstrating approximately greater than 4000 cells for each mutant in the library. We also included inoculated chicken carcasses incubated at room temperature as a reference condition to identify the genes that are essential for fitness under this condition. We recovered an average of $5.62x10^6$ cfu/ml (RT), 1.46x 10^7 cfu/ml (4^{o}C), and 3.46x 10^6 cfu/ml (-20^{o}C). We were concerned that the genes were distinctively essential under the low temperature conditions thus the genes were identified in the reference condition so that they could be removed from those identified under other selective conditions.

Illumina sequencing: data analysis.

A total of 134,602,006 sequence reads obtained from a single flowcell lane, and about 82,005,007 reads (61%) contained the complete 19 bp ME sequence. Among them, 80,318,864 reads (98%) contained the complete 6 bp barcode sequences perfectly matching one of the 4 barcodes. When these reads were sorted according to the barcode, the distribution across the different barcodes were as follows: 28,314,312 reads (CGATGT; INP), 18,103,539 (TTAGGC; RT), 14,719,260 (TGGTCA; 4^{o}C), 18,436,748 (ACAGTG; -20^{o}C). The transposon-junction sequences were subsequently extracted from the reads for each barcode. As expected the majority (approximately 94%) of the sequence reads were 11 to 13 bp long. These 11 to 13 bp sequence reads for each barcode was further filtered for those that map to the genome at only one genomic locus. Finally, we obtained 12,982,374;, 7,355,491; 6,610,185; 8,789,658 reads respectively for each barcode, which corresponds to approximately 49% of the total number of the transposon-junction sequences of 11~13bp for each barcode (Table 2).

The normalization factors for the output pools for attachment were 1.55 (RT), 1.72 (4^{o}C), and 1.32 (-20 ^{o}C). For cold temperature only, the normalization factors for the output pools were 1.02 (4^{o}C) and 0.78 (-20 ^{o}C). To obtain a more robust and reliable result for both analysis conditions, the genes that contained less than 3 insertions were removed.

Essential genes identification

The conditionally essential genes for carcass attachment under low temperature were first selected by a cut-off fitness index of ≤ 0.05, which indicates at least a 20 -fold reduction in relative abundance during selection. The selected genes are expected to exhibit a strong fitness defect under low temperature condition. In total, 477 genes conditionally essential under either

one of the selective conditions were identified, and were identified as exclusively chromosomal genes (Figure 1). Since there are many genes conditionally essential for fitness under low temperature condition and many genes were also shown to be essential for general fitness under room temperature condition, another selection was made to narrow down the number of genes by a cut-off fitness index of ≤ 0.01 (Figure 2). A total of 99 genes conditionally essential under either one of the selective conditions were identified with all being identified as chromosomal genes except one gene from a plasmid which yielded the same cut-off. For essential genes associated with cold temperature only, selection was made by a cut-off fitness index of ≤ 0.05, which indicates at least a 20 -fold reduction in relative abundance during selection, and the obtained genes were expected to exhibit considerable fitness defect under the chosen low temperature condition (Figure 3).

Phenotypic characterization of deletion mutants was further verified by choosing genes showing consistent deficiency under low temperature only and not under the reference condition, RT. The selected genes for chicken skin attachment were *fur*, *trkA*, and *rfbI*. For cold temperature only, we selected *topA*, STM14_1047, and STM14_3311.

4. Discussion

Salmonella illness prevalence has remained consistent during last decade and considered the most common bacterial pathogen caused by food as vehicle in the majority of cases (Franz and van Bruggen, 2008; Hald, 2013; Hopkins et al, 2010; Majowicz et al, 2010; Wright et al, 2011). The practices taken by food safety personnel to decrease the contamination of poultry products by *Salmonella* involve a wide range of interventions including chemical and physical handling of poultry products during processing (Cox et al, 2011; Cox and Pavic, 2010; Higgins

et al, 2005; Joerger, 2003; Ricke, 2003; Ricke et al, 2005). These intervention strategies are effective in decreasing the bacterial load to some extent but often without defined molecular targets. Consequently, currently applied approaches to eradicate bacteria from the poultry products remain somewhat inconsistent and at times ineffective. To develop more consistently effective intervention strategies, it requires a more in-depth understanding of *Salmonella* biology under these particular environmental conditions. Thus discovering unique molecular targets required for pathogens to grow or survive during poultry processing and cold storage may provide novel targets that would result in more predictable and effective intervention strategies.

To control and prevent the contamination of foods by microorganisms, numerous interventions have been used by food industries, and among them cooling and freezing. These forms of preservation cause the microorganism, namely bacteria, a stressful condition that suppresses the microbial growth or its destruction. However, some microorganisms have developed exceptional mechanisms to tolerate or resist the environmental stresses specifically, preservative procedures used during food processing (Bower and Daeschel, 1999; Davidson and Harrison, 2002; Ricke et al, 2005). In the poultry industry, the poultry and poultry products safety have been demanded mainly through the post-harvest handling stage of production by either killing or inhibiting the bacterial pathogens. During food processing, some procedures are performed at low temperatures and hygienic restrictions to effectively reduce the bacterial burden of contaminated poultry (Dinçer and Baysal, 2004; Loretz et al, 2010).

Designing and acquiring strategies to effectively target specific pathways of *Salmonella* is important to prevent their persistence in poultry and poultry products processing, handling, storing and distribution systems (Ricke et al., 2013; Ricke, 2014). By understanding the conditionally essential genetic distinctive components of *Salmonella* to withstand specific

stresses throughout the poultry processing production, there is a need to develop measures to handle this pathogen using the identified pathways as targets. Our knowledge and understanding of the genetics basis of bacterial pathogens have been delayed due to limited understanding of gene identification and functionally. However, lately the advances of high-throughput applications in microbial genomics fields have assisted in determining the functions of genes and the cellular pathways essentially involved in numerous particular conditions (Kwon et al, 2010). These genes and pathways can serve as promising targets to develop novel antimicrobials agents. The molecular mechanisms of foodborne bacterial pathogens in general and associated with *Salmonella* specifically for surviving and persisting in stressful environments throughout the stages of pre-harvest and post-harvest food processing (poultry products), transportation, storing and distribution is fundamental for developing effective procedures to diminish the food-borne pathogens in the final food products for consumption (Park et al., 2008; Dunkley et al., 2009; Kwon et al, 2010; Foley et al., 2011; 2013; Waldner et al, 2012).

Functional genomics approaches which have been used to study various bacterial pathogens in the host-pathogen interactions, offer a possible approach make these to increase understanding of foodborne stress adaptation (Kwon et al, 2010). Evaluating and identifying the genetic factors by combining transposon mutagenesis to form a complex transposon mutant library with the massively parallel sequencing technologies will be of use to identify essential genetic pathways of *S.* Typhimurium to survive and persist correlating with chicken carcasses in poultry production system and marketing (Christen et al, 2011; Gallagher et al, 2011; Gawronski et al, 2009; Goodman et al, 2009; Langridge et al, 2009; van Opijnen et al., 2009; Khatiwara et al, 2012; Fels et al, 2013)

Low temperature stress leads to the induction of a myriad of responses as attempts to survive the harsh condition. When microorganisms are exposed to low temperature, their adaptation mechanisms initially rely on sensing followed by up-regulation of genes associated with cold adaptation for generation of specific proteins. These proteins known as cold-induced proteins (CIPs) or cold-shock proteins (CSPs) differ in their quantity from bacterial strains to another (Phadtare and Severinov, 2010; Weber and Marahiel, 2003). It is significant to examine and understand the response of pathogens to the cold shock condition due to the common use of refrigeration and freezing in food processing system with the case of foodborne pathogens such as *Salmonella* species.

A genome-wide high-throughput screening technique for functional genomics has recently been developed by Khatiwara et al (2012) to examine the entire *Salmonella* genome for the conditionally essential genes under selective conditions. The method, Tn-seq, was successfully performed for a genome-wide screening to identify genes essential for optimal growth in several stress conditions in connection with chicken infection as low nutrient condition (starvation), bile salt-rich condition throughout the gastrointestinal tract (pH), and at 42°C for chicken body temperature mimicking the host stresses (Khatiwara *et al.*, 2012). In the current study, we applied the same Tn-seq technique to study *Salmonella* conditionally essential genes as a means to comprehensively identify *Salmonella* genetic systems required for survival and persistence in cold storage conditions refrigeration (4°C) and freezing (-20°C). The reduction of *Salmonella* in poultry carcasses and poultry products has been a primary concern of many studies, yet little is known about the genetic mechanisms employed by *Salmonella* to survive and thrive in the environments against diverse stress conditions and antimicrobial treatments. The approach herein is utilized to identify specific pathways as unique targets in order to develop

novel antimicrobial compounds or vaccines as an efficient control or prevention of any resistance development (Clatworthy et al., 2007, Davies and Davies, 2010). By elucidating *Salmonella* genes required for growth and survival in foods, and the nature of the food processing stress conditions, this has the potential for focusing on future intervention targets,.

In the current study, we attempted to identify conditionally genes that are essential during food storage at 4°C and -20°C using the new version of Tn-seq method by Khatiwara and others (2012). These conditional genes may be potential new molecular targets candidates for future antimicrobials development (Figure 4 and 5). In general, microorganism cells go through many physiological changes in response to temperature (Beales, 2004; Georlette et al., 2004; Gounot, 1991; Jones and Inouye, 1994; Panoff et al., 1998; Phadtare, 2004; Smith et al., 1994). When downshifting the temperature to a temperature below their optimal growth temperature , several proteins known as cold shock proteins (CSPs) are induced at the bacterial cells to oppose the harmful effects of this condition (Beales, 2004; Phadtare, 2004; Wouters et al 2000). Genes encoding cold shock proteins possess insertions that were detected in our first data analysis; however, none of them were considered as essential in our conditional study for not meeting our more restrictive standard of *P* value <0.01.

In this study, a number of those genes were identified in response to the cold temperature stress on chicken carcasses. For instance, the major and most studied cold shock protein, CspA and its family homologous proteins, CspB, CspC, and CspD were identified in response to cold temperature shock (Criag et al, 1998; Jeffreys et al, 1998; Horton et al, 2000; Kim et al, 2001). Alternative sigma factors, *rpoE* and *rpoS* are activated in S. Typhimurium in cold shock (Abee and Wouters, 1999; McMeechan et al, 2007; Miticka, et al., 2003; Robbe-Saule et al, 2003), *otsA* and *otsB* genes encoding proteins of trehalose-6-phosphate synthase and trehalose-6-phosphate

phosphatase respectively (Howells et al, 2002; Balaji et al, 2005), and many other encoding genes have been formerly identified with cold stress in *Escherichia coli* and other bacteria (Phadtare, 2004; Polissi et al, 2003)**.** In this study, we were able to identify 100 (P <0.01) (Figure 6) and 113 (P<0.05) conditionally essential genes associated with chicken carcasses for attachment and cold temperature respectively. For attachment conditionally genes, out of 100 genes, 14 genes were common to all conditions (RT, 4^{o}C, and -20^{o}C), 13 genes were common to RT and 4^{o}C, 8 genes were common to RT and -20^{o}C, 7 genes were common to 4^{o}C and -20^{o}C, and 18, 18, and 21 genes were conditionally essential for RT, 4^{o}C, -20^{o}C respectively. In association with cold temperature only, out of 113 conditionally essential genes identified, 12 genes were common to both conditions (4^{o}C and -20^{o}C), 43 genes were essential for 4^{o}C, and 58 genes were essential for -20^{o}C. The genes encoding for iron uptake system (*fur*), a potassium transport system (*trkA*), and a Na+-transporting NADH:ubiquinone oxidoreductase (*rfbI*) were identified in our study as conditionally essential genes, indicating the importance of ions in the skin attachment and survival of *Salmonella* at cold temperatures. In addition, the association of DNA negative supercoiling and cold temperature has been previously demonstrated (Prakash et al, 2009; Shivaji and Prakash, 2010). DNA supercoiling increases in its negative state, which leads to a conclusion that enzymes regulating this state of DNA supercoiling, namely DNA gyrase and topoisomerase IV are induced in high levels directing DNA replication, transcription and recombination promptly (Mizushima et al, 1997; Shapiro and Cowen, 2012). The deletion of *topA* gene, the topoisomerase I, will cause the DNA to be over-supercoilied, which will delay and make it challenging to DNA gyrase and topoisomerase IV to keep up with DNA replication (Richardson et al., 1984), transcription and recombination. The gene STM14_1047 is a putative nucleoside-diphosphate-sugar epimerase. Searching for its association with cold temperature, it

was only found that it belongs to a group of proteins involved in cellular pathways like photosynthesis, carbohydrate and lipid metabolism, and secondary metabolite synthesis. It is up-regulated in the Siberian spruce, *Picea obovata* to tolerate extreme freezing climate (Kjellsen et al., 2010). The last gene is STM14_3311, a putative cytoplasmic protein and hypothetical protein in other *Salmonella* strains. Based on these findings, further studies to find natural products that would block the ions pathway or deplete iron, potassium, and/or sodium ions could be an efficient means to reduce *Salmonella* from food during cold storage. The phenotypic study results obtained from the mutants (Δ*fur*, Δ*trkA*, Δ*rfbI*, Δ*topA*, ΔSTM14_1047, and ΔSTM14_3311) exhibited correlation to the findings of the Tn-seq method (Figure 7).

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Table 1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'→3')
Tn-seq method[a]	
Tn-seq linker 1	NNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT (5' phosphorylation)
Tn-seq linker 2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
Tn-seq linker primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCC CTACACGACGCTCTTCCGATCT
Tn5 primer Barcode 1	CAAGCAGAAGACGGCATACGAGCTCTTCCGATC T**ACATCG**AGATGTGTATAAGGGACAG
Tn5 primer Barcode 2	CAAGCAGAAGACGGCATACGAGCTCTTCCGATC T**GCCTAA**AGATGTGTATAAGGGACAG
Tn5 primer Barcode 3	CAAGCAGAAGACGGCATACGAGCTCTTCCGATC T**TGACCA**AGATGTGTATAAGGGACAG
Tn5 primer Barcode 4	CAAGCAGAAGACGGCATACGAGCTCTTCCGATC T**CACTGT**AGATGTGTATAAGGGACAG
Mutant construction[b]	
topA-Up-P1	ATCAACGTGCGACGCATTCCTGGAAGAATCAACTTAG GTAAAGGTGAA**TTGTGTAGGCTGGAGCTGCTTC**
topA-Dn-P2	AAAGGGCCGCTTACGCGGCCCGCTTGACTATAGT GACGACAGGCAGGGG**CATATGAATATCCTCCTTAGT**
STM14_1047-Up-P1	AATTCACGGAAGAATGCGCACGACGGTTTATTAAAC ACAGTGGATTGAT**TGTGTAGGCTGGAGCTGCTTC**
STM14_1047-Dn-P2	CCGAAACGCAGGCGATTGCCGAAGCATTACTGGAGAAG TACGGCCAGGA**CATATGAATATCCTCCTTAGT**
STM14_3311-Up-P1	AATGAACAGCCTCCGTGATATCACCGGTGTTTCAGAC TGGATATCTGGA**TGTGTAGGCTGGAGCTGCTTC**
STM14_3311-Dn-P2	TTCGGTATTGAAACGGTGTATTTCGCTATTTTTTTTT GAATGTAATTTA**CATATGAATATCCTCCTTAGT**
topA-seqF	CTGGCAACAGAATTGCTTGA
topA-seqR	TAACGATGTGACTGGGGTGA
STM14_1047-seqF	TAGCTTCACGCTGCACGATA
STM14_1047-seqR	ACGGTGCTTGCGTTACTCTC
STM14_3311-seqF	CATGTTGCTTGGCAAAAAGA
STM14_3311-seqR	CGATGAGCTTGCAGAATTGA

a Khatiwara et al. (2012).
b This Study.

Table 2. Summary of Illumina sequencing data analysis.

Sample	**Total No. of reads**	**No. of reads with 19bp modified ME sequence**	**No. of reads with 6bp barcode**	**No. of 11-13bp Transposon-junction sequence**	**Authentic insertions**		**After removal of insertions with < 10 reads in the input pool**	
					No. of reads	**No. of insertion sites**	**No. of reads**	**No. of insertion sites**
Total	134,602,006	82,005,007 (61%)	80,318,864 (98%)	75,793,893 (94%)	36,076,567 (48%)			
Input			28,314,312	27,383,736	12,982,374	86,462	4,724,786	18,843
RT			18,103,539	16, 333, 359	7,355,491	77,765	2,464,999	15,463
4^{o}C			14,719,260	13,954,949	6,610,185	75,399	2,289,577	15,691
-20^{o}C			18,436,748	17,518,733	8,789,658	85,623	2,967,987	16,177

Figure 1. Venn diagram for selected genes with *P* value < 0.05 for carcass skin attachment.

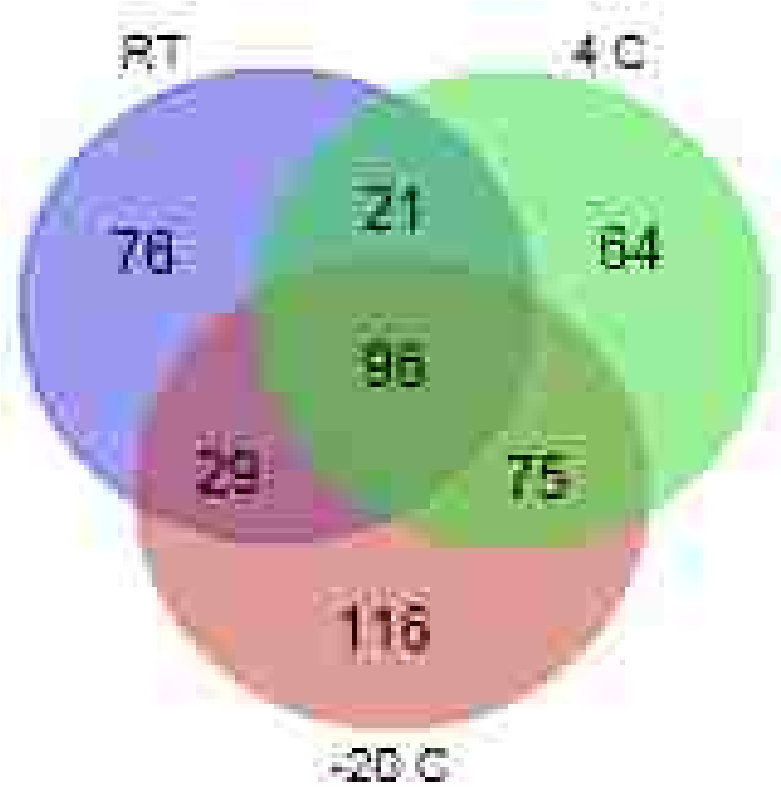


Figure 2. Venn diagram for selected genes with *P* value < 0.01 for carcass skin attachment.

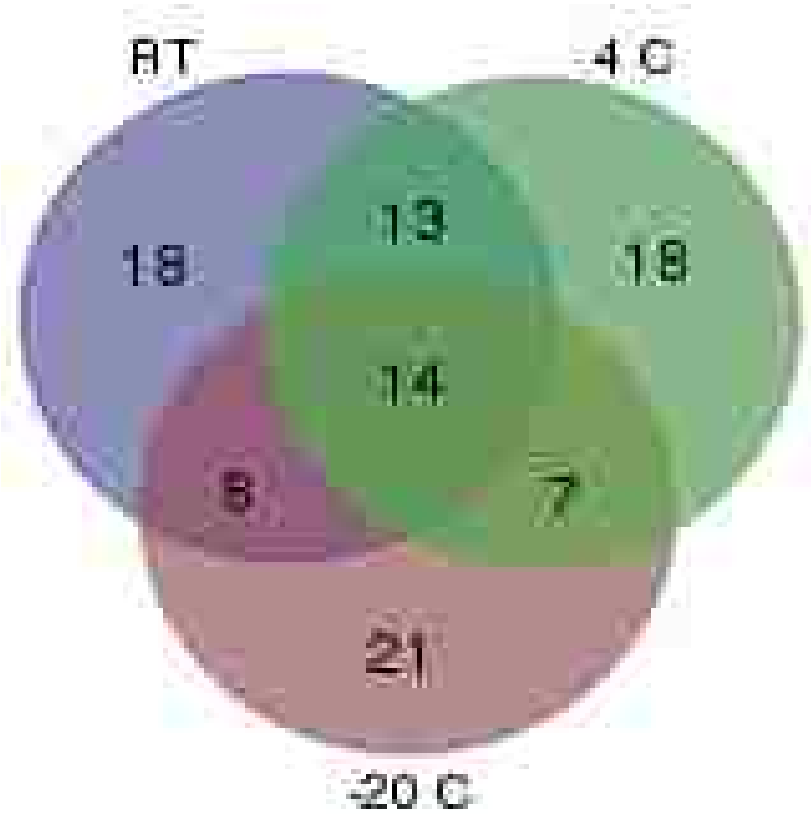


Figure 3. Venn diagram for selected genes with *P* value < 0.05 for cold temperature only.

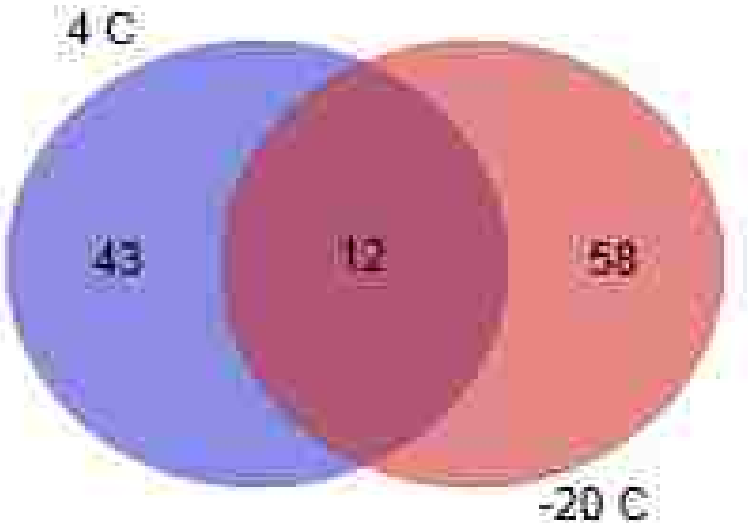


Figure 4. Functional classification of the identified conditionally essential genes for attachment and cold temperatures.

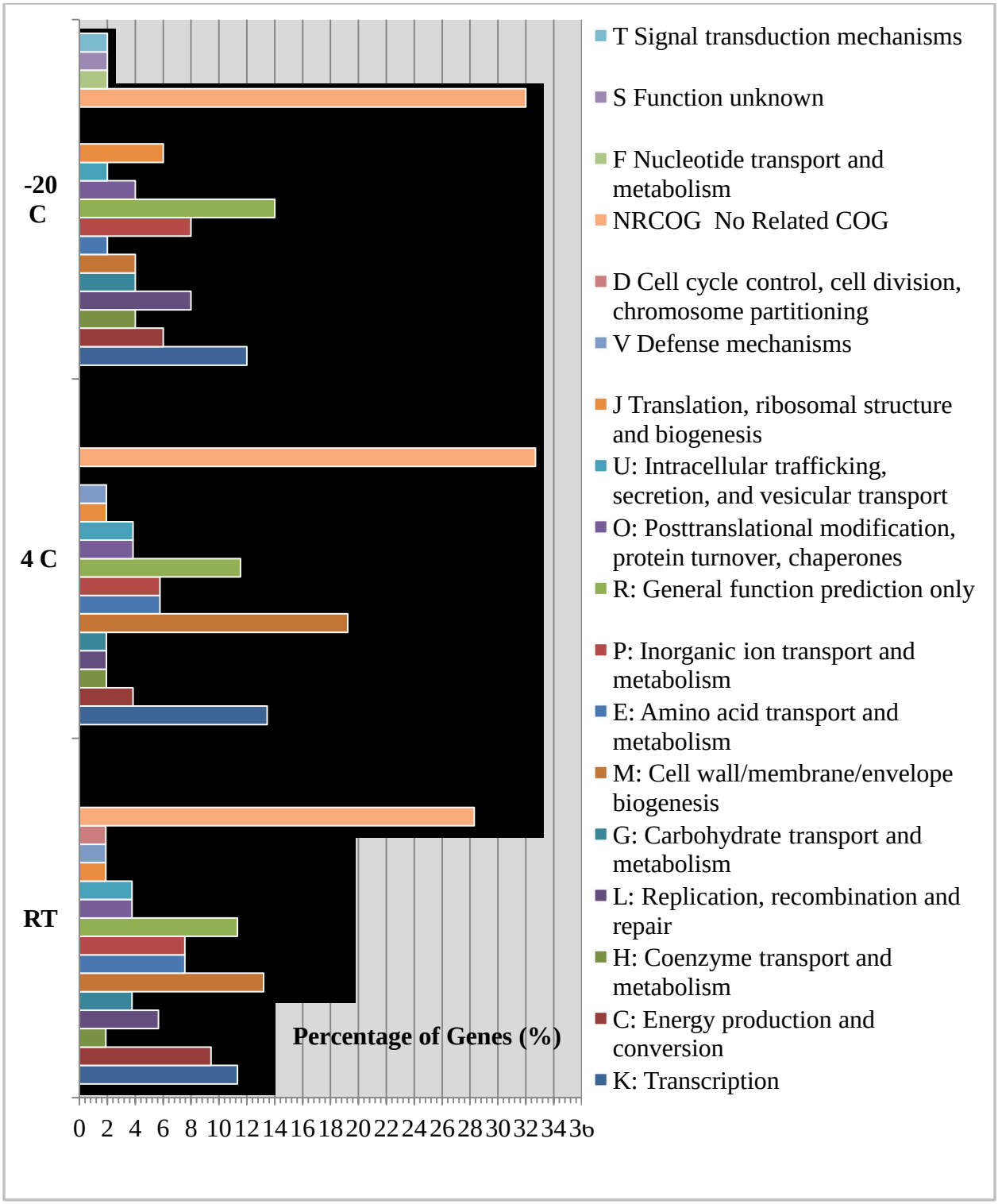


Figure 5. Functional classification of the identified conditionally essential genes for cold temperatures only.

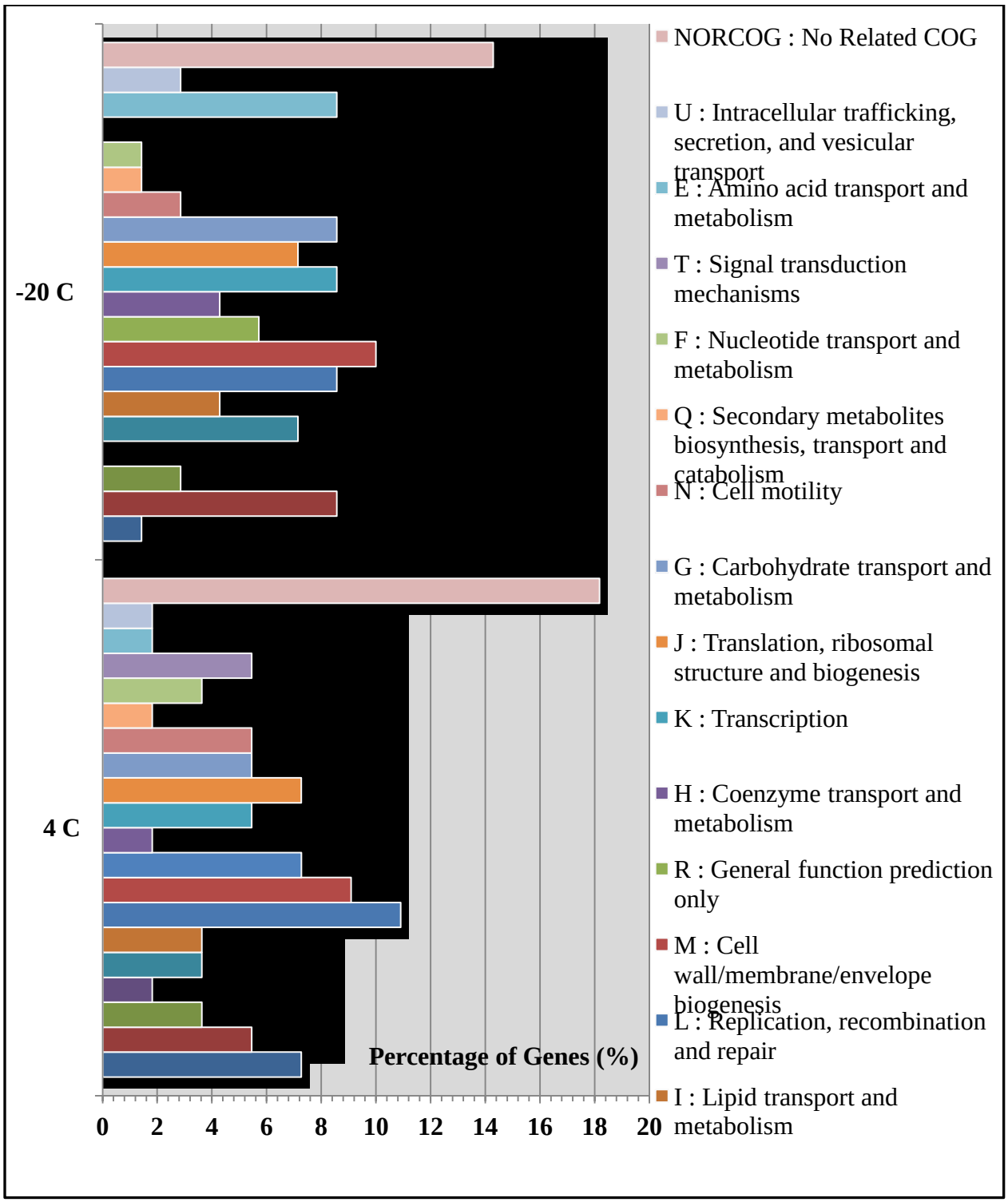
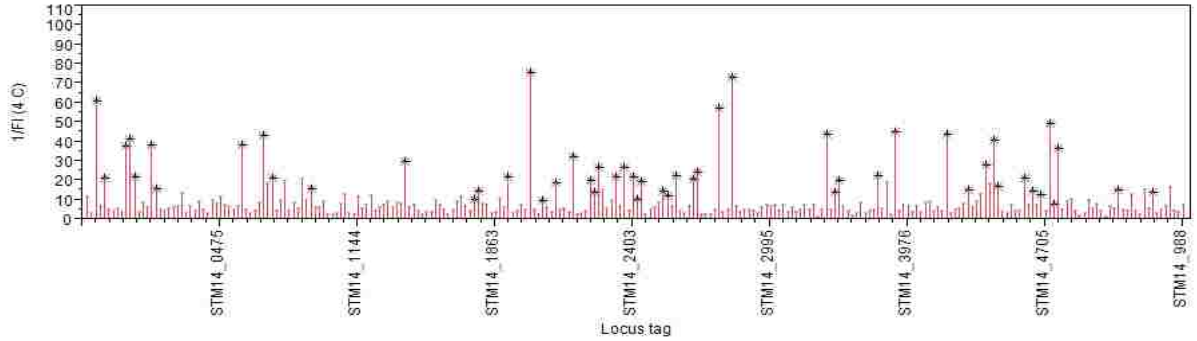


Figure 6. Identification of the genes conditionally essential for growth or survival on chicken carcass under 3 different temperatures: room temperature, refrigeration (4^{o}C), and freezing (-20^{o}C). Genome-wide view of the "inverted fitness index" (total read counts in the input pool/ total read counts in the normalized output pool) for each gene obtained under the 3 different selective conditions. Inverted Fitness index was used for Y-axis to show fitness defect as indicated by the high peaks for better visualization of significant mutants. Genes negatively selected under each condition (Student t-test; $p < 0.05$) are shown below wiith red peaks, and the genes significant at $p < 0.01$ are indicated with "*".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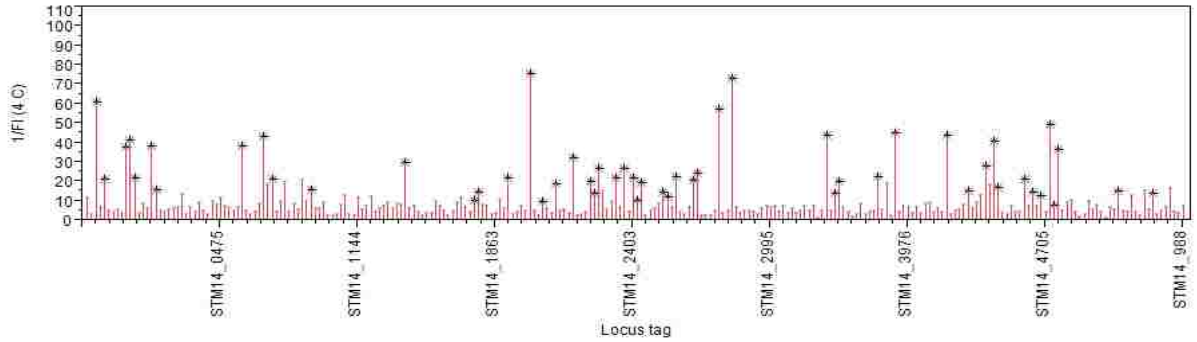
Locus tag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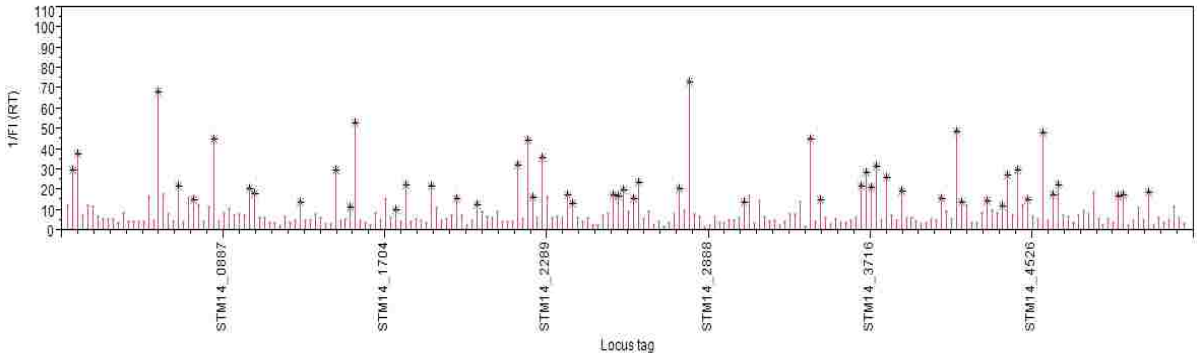
Locus tag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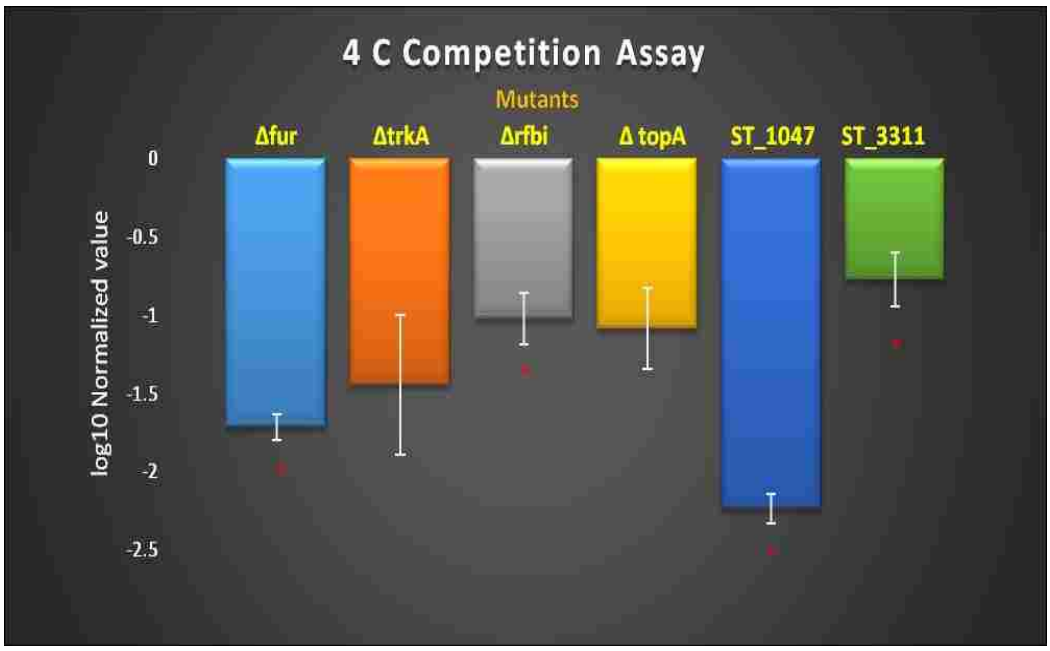
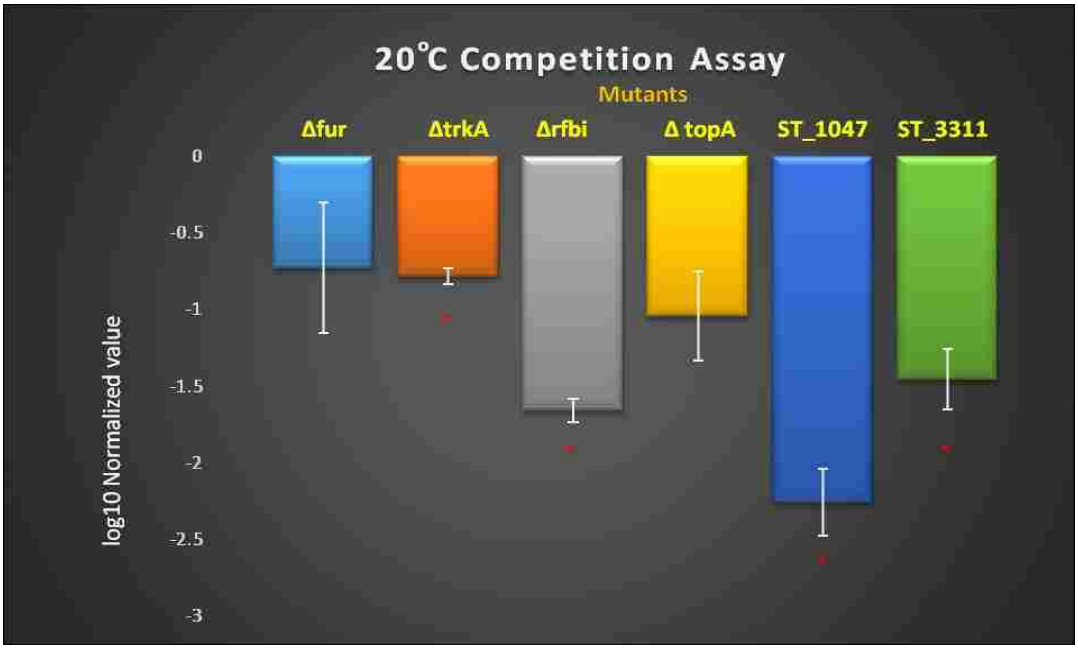
STM14_2289
STM14_2888
STM14_3716
STM14_4526
Locus tag

Figure 7. S. Typhimurium strains (wild type and each mutant) in competition assay on chicken carcasss skin at A) 4^{o}C and B) -20^{o}C. Normalized competitive index is converted logarithmically to base 10 and then means ± standard error are calculated. Red asterisk below error bar shows that mutants survival are significantly impaired ($p < 0.05$) as compared to wild type using student's t test.

A) 4°C Competition Assay.



B) -20 °C Competition Assay.



Chapter 6

Conclusions

Conclusions

Salmonella as a foodborne agent has the ability to cause numerous infections after consumption of contaminated food. The tracking of the origin of contamination is very complex as it requires screening of all stages of food production and subsequent processing and retail. *Salmonella* species has been leading other bacteria in the number of illness cases, hospitalization, and deaths which makes it a major public health problem and can cause serious loss for food industries and the U.S economy in general. This will require more investigation in understanding and evaluating this foodborne pathogen.

We conducted four studies in order to recognize the phenotypes of *Salmonella* using strains from the two top serovars, *S.* Enteritidis and Typhimurium, responsible for major part of *Salmonella* infections. We first compared several strains of *S.* Enteritidis in invading ovarian follicles of laying hens. Our results exhibited no significant statistical differences between tested *S.* Enteritidis strains. In our next study, we selected a *S.* Enteritidis strain from the previous study to generate a genome saturating random insertion mutant library for several future projects. To overcome the host-restriction barriers, we performed a straightforward technique that resulted in a 46-fold increase efficiency for transposon mutagenesis using adapted Tn5 DNA to form transposome complex for electro-transformation of *S.* Enteritidis. We confirmed that Tn5 insertions were randomly inserted by sequencing Tn5-junctions with Miseq Illumina sequencing system.

In the next two studies, we used *S.* Typhimurium for heat survival under 50^{o}C, 55^{o}C, and 60^{o}C. Also, Phenotype Microarray screening was carried out for 3 mutants (Δ*recD*, ΔSTM14_5307, and Δ*aroD*) previously exhibited temperature-association growth defect in comparison to their parental strain (wild type). The result of the heat survival was significant as

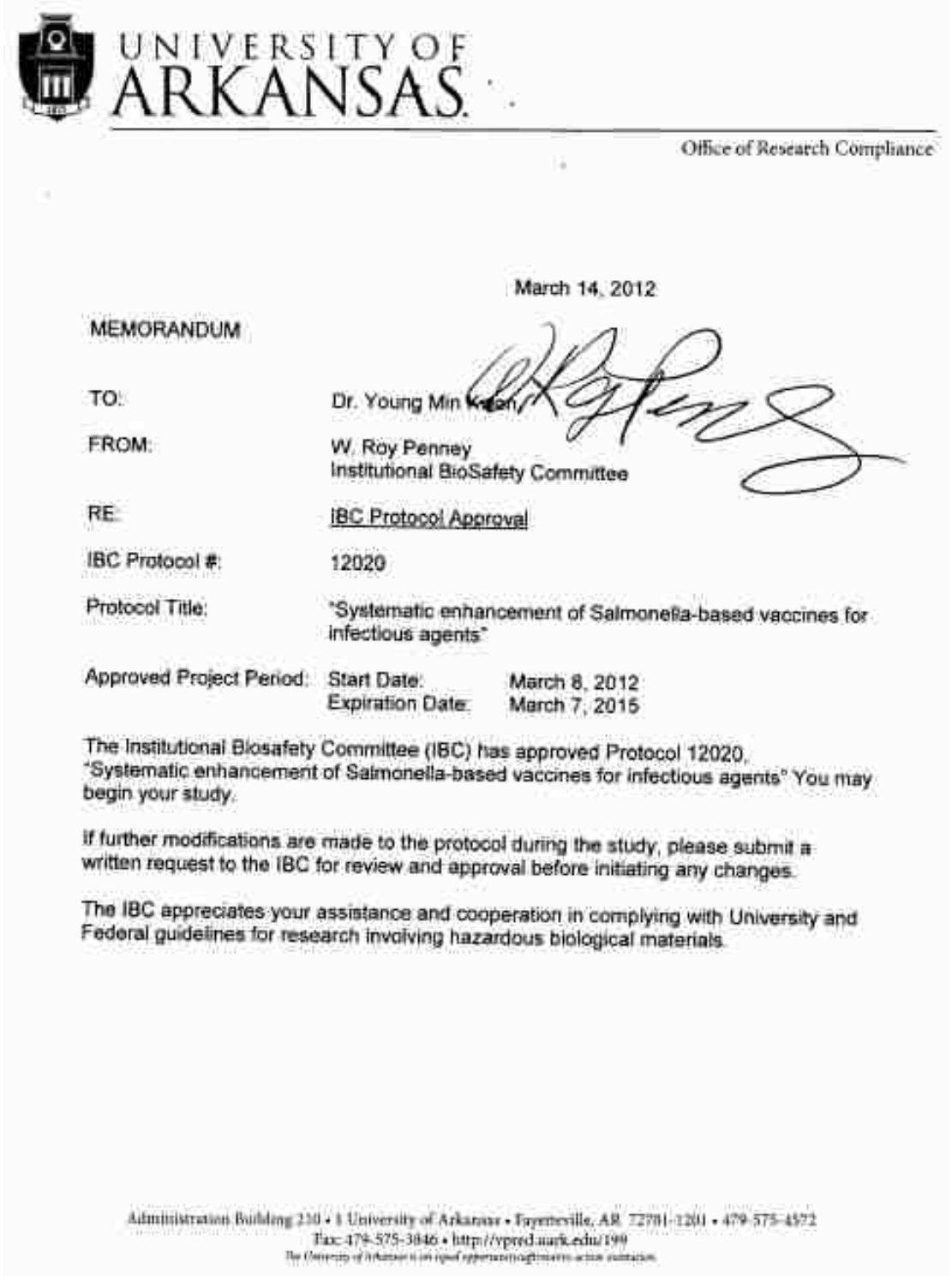
Δ*aroD* exhibited the highest defect follow by the Δ*recD* and ΔSTM14_5307. For the phenotype microarray, Δ*recD* exhibited no differences from the wild type, and Δ*aroD* could not reduce the dye that differentiated from the wild type for metabolic profile remains unclear. In the case of ΔSTM14_5307, two important phenotypes were noted. The first one was the phenotype being able to utilize myo-inositol as a sole carbon source and in the second phenotype; the mutant exhibited a lost ability to utilize cytidine monophosphate as a substrate for phosphorus source. Both phenotypes have shown to be involved directly or indirectly in virulence, viability, and/or pathogenicity of *Salmonella* and/or other bacterial pathogens.

In the last study, we conducted a study in identifying gene conditionally essential for cold temperature on chicken carcass using high-throughput sequencing and analyzed the collected data by a bioinformatics method. Some genes from the bioinformatics analysis were selected for testing on chicken skin assay. Three genes were associated with chicken skin attachment and the others were for cold temperature only. Unexpectedly, some gene showed a different defect phenotype at 4^{o}C and -20^{o}C in comparison to wild type parental strain. At both 4^{o}C and -20^{o}C, ΔSTM14_1047 exhibited highest survival defect. At 4^{o}C, Δ*fur* and Δ*trkA* exhibited more deficiency of attaching chicken skin than when incubated at -20^{o}C. Δ*rfbI* exhibited less deficiency of chicken skin attachment than Δ*fur* and Δ*trkA* at 4^{o}C and higher deficiency at -20^{o}C. The lowest defect at 4^{o}C was for ΔSTM14_3311, a gene associated with cold temperature only. At -20^{o}C, both Δ*rfbI* and ΔSTM14_3311 exhibited over 50% more deficient phenotype than at 4^{o}C in chicken skin attachment and cold temperature only respectively. At both 4^{o}C and -20^{o}C, Δ*topA* exhibited no changes of deficiency of cold temperature in chicken skin.

In conclusion, *S*. Enteritidis has been associated routinely with eggs and its products starting from the second half of last century. The need to understand this serovar mechanism that makes it unique in contaminating egg is of major demand for poultry industries and public health. Furthermore, the need to understand the capability that serovars of *Salmonella* are able to survive food processing treatments is crucial. We have developed innovative techniques to identify important genes to that could potentially serve as targets for antimicrobial agents and vaccines in order to fight *Salmonella* and control it.

Appendix

1 . Institutional Biosafety Committee (IBC) Number



UNIVERSITY OF ARKANSAS

Office of Research Compliance

March 14, 2012

MEMORANDUM

TO: Dr. Young Min Kwon

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 12020

Protocol Title: "Systematic enhancement of Salmonella-based vaccines for infectious agents"

Approved Project Period: Start Date: March 8, 2012
Expiration Date: March 7, 2015

The Institutional Biosafety Committee (IBC) has approved Protocol 12020, "Systematic enhancement of Salmonella-based vaccines for infectious agents" You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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2. Institutional Animal Care and Use Committee (IACUC)

UNIVERSITY OF ARKANSAS.

Office of Research Compliance

MEMORANDUM

TO: Young Min Kwon

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: April 10, 2012

SUBJECT: IACUC PROTOCOL **APPROVAL**
Expiration date : **April 14, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#12032-** ": **SYSTEMATIC ENHANCEMENT OF SALMONELLA-BASED VACCINES USING CHICKEN INFECTION MODEL** ". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes in the protocol during the research, please notify the IACUC in writing [Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **04-14-2015**, you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cmc/car

cc: Animal Welfare Veterinarian

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