GROWTH AND VIRULENCE RESPONSE OF *Salmonella* Typhimurium TO SOLUBLE MAILLARD REACTION PRODUCTS

A Thesis

by

MEGAN M. KUNDINGER

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

MASTER OF SCIENCE

May 2004

Major Subject: Poultry Science

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Approved as to style and content by:

Steven C. Ricke (Chair of Committee)	Luc Berghman (Member)
J. Allen Byrd (Member)	Randle Moore (Member)

Alan Sams
(Head of Department)

May 2004

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ABSTRACT

Growth and Virulence Response of *Salmonella* Typhimurium to Soluble Maillard Reaction Products. (May 2004)

Megan M. Kundinger, B.S.,

University of Wisconsin-River Falls

Chair of Advisory Committee: Dr. Steven C. Ricke

In order to determine the effects that Maillard Reaction Products (MRP) have on *Salmonella* Typhimurium, growth rates and virulence expression, in the presence of Maillard reaction products, were observed, using the β–galactosidase Miller Assay and Reverse Transcription Polymerase Chain Reaction (RT-PCR).

The presence of MRP compounds in liquid media caused no negative effect on the growth rate of *Salmonella* cells. However, the addition of MRP compounds at a 1% level in the media caused a significant increase in *hilA* expression in *Salmonella* Typhimurium, and the highest induction levels were observed in media supplemented with arginine and histidine-MRP compounds. There was no effect on the induction of *hilA* with the 0.5% addition of the MRP compounds in the amended media as shown by the Miller Assay. However, there was an effect seen when using the Real Time RT-PCR assay that resulted in the same levels of significance seen at 1.0% additions of MRPs being seen at the 0.5% level as well.

Since *rsmC* was shown to be a constitutive gene that had continuous levels of expression in *Salmonella* based on cell number, Real-Time PCR was then used to assess the *hilA* expression of *Salmonella* Typhimurium under different oxygen, pH levels, and osmolarity conditions. The results under low oxygen indicate that the combination of low osmolarity and high pH have the highest inducing effect on *hilA* expression. The *hilA* response under the same media conditions and a high oxygen environment showed the same pattern of expression as those bacteria grown under a non-aerobic environment. The media with a pH of 8 and low osmolarity conditions had the greatest effect on the induction of *hilA* with none of the other media showing any significant effect. The relative expression of *hilA* did decrease for those bacteria grown under aerobic conditions versus those grown under low oxygen conditions.

DEDICATION

This thesis is dedicated to my husband, Daniel H. Kundinger, for all of his support over the many years we have been together. You have always encouraged me to go above and beyond what I ever thought possible. I hope I can do the same for you in the many years to come.

To our baby, knowing you were coming made this all easier to get through. I hope the rest of your life is not as stressful as your first few months of your existence.

To Bailey, thanks for always being there, ready with a hug for all the times that this thesis became discouraging.

To my parents, thank you for always driving us to everything and making sure we always did our best. I will always remember you love me no matter what.

I would also like to thank all of my family for their encouragement; it really helped me to accomplish all of this. Thank you to the many friends I have met over the years; you have all inspired me.

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An extreme appreciation goes to all of my fellow graduate students in our lab who have worked with me in the past few years: Dr. Irene Zabala Díaz, Dr. Xin Li, Kristin Medvedev, Angela Kelley, Zoe Howard, Jeff Nutt, Cliff Froehlich, Lisa Donalson, Rebecca Hardin, and Jennifer Golbach. I would especially like to thank Angela Kelley and Dr. Randy Moore who helped me to adjust to life in Texas and always served as my sounding board. I would also like to thank Cassie Woodward, our laboratory technician, for her ideas and assistance in planning my research studies. Also thank you to my fellow Biotechnology majors at the University of Wisconsin-River Falls who encouraged me to pursue more.

Last but by no means least, I would like to thank my friends who have been with me long before I even started college. They were always there to remind me I will be back in Wisconsin soon enough. There was always a “Yah There Hey” to brighten my day.

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CHAPTER I

INTRODUCTION

Foodborne illness is an ever-increasing concern among the consumers about the food supply. With the multitude of foodborne illness occurring every year it is understandable that consumers may become concerned about consuming potentially dangerous food products. For example, there was a 9.6% increase in laboratory confirmed bacterial infective diarrheal cases in 1997 when compared to 1996 (FoodNet, 1998). This shows that there is obvious cause for concern; cases of illness where a food source was identified as the vehicle for infection have increased. Along with the increase of foodborne illness the inevitable consequences of these diseases also occur. It is believed that foodborne diseases cause roughly 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths each year in the United States (Kennedy et al., 2000). This fails to mention the amount of money needed to cover (lost wages and medical treatment) all of these cases that are the consequence of foodborne illnesses. The estimated cost of foodborne *Salmonella* infection is more than 2 billion dollars a year (Frenzen et al., 1999). A multitude of different bacteria are responsible for these diseases with one type of bacteria in particular being responsible for roughly 27.4% of all foodborne infections (FoodNet, 1998). *Salmonella* is the second most often isolated bacteria associated with food (Figure 1.1.; Alketruse et al., 1999).

This thesis follows the style of Poultry Science.

Salmonella is believed to be the source of between 2 and 4 million cases of foodborne illness each year (CFSAN, 1996; Texas Dept of Health, 1998). Due to the amount of money spent on *Salmonella* alone each year the quest to better understand this pathogen greatly increases.

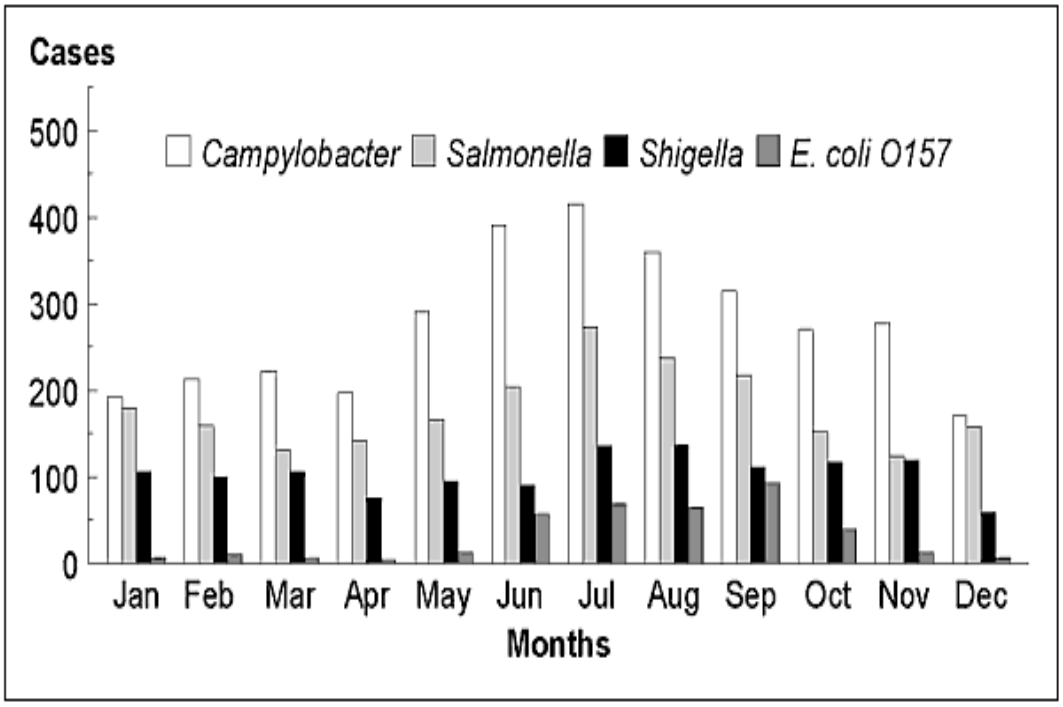

Figure 1.1. Cases of foodborne infections by month of specimen collection (Centers for Disease Control and Prevention/U.S. Department of Agriculture/ Food and Drug Administration Collaborating Sites Foodborne Disease Active Surveillance Network, 1996; Altekruse et al., 1999).

CHAPTER II

LITERATURE REVIEW

Salmonella

Salmonella is a gram-negative rod shaped foodborne pathogen responsible for causing severe gastroenteritis. Over 2400 different serotypes of *Salmonella* have been identified (Humphrey, 2000). It is believed to be the source of between 2 and 4 million cases of foodborne illness each year in the US with 600 of these cases resulting in death annually (Mead, 2000). The main symptoms of *Salmonella* consist of diarrhea, fever, nausea, vomiting, headache, and abdominal pain (CFSAN, 1996). The onset of these symptoms is usually between six to forty-eight hours after infection and usually lasts for one or two days depending on initial infective dose as well as strain type. The most susceptible human populations continue to be the young, old, and immunosuppressed. People who suffer from an autoimmune deficiency syndrome have been observed to exhibit a twenty-fold greater frequency of clinical salmonellosis than the rest of the general public (CFSAN, 1996).

Salmonella is commonly found in the gastrointestinal tracts of animals, and has been commonly associated with foods such as raw meat, poultry, eggs, and dairy products. Poultry and products derived from poultry are believed to make up roughly 50% of the total vehicle of transmission when dealing with *Salmonella* (Texas Dept. of Health, 1998). *Salmonella* is usually thought to be

spread by the fecal-oral route. The commercial industry has a common turn over rate of forty two days in a broiler setting which can lead to a number of possible contamination sites such as litter, air, or feed (Humphrey, 2000). Birds are also raised in close proximity with other birds, which increases the likelihood of horizontal transfer. Since *Salmonella* has several opportunities to be introduced to the host, there is an increased risk of *Salmonella* reaching the consumer. It is believed that chickens have a chronic carrier state similar to that of Typhoid Mary. Typhoid Mary was a cook in New York City back in the 1900's, who killed her employers by contaminating their food with *Salmonella* (Salyers and Whitt, 2002). The actual mechanism of this chronic carrier state is still unknown; however, if there is a large incidence of these carriers in the poultry industry, this could also be a large source of contamination. Due to the number of foodborne illness cases evolving from poultry and poultry products, it is important to understand how the bacteria are invading the product and ultimately causing illness in humans.

Epidemiology of *Salmonella* in the United States

Foodborne illness is an ever-increasing concern among the consumers about the food supply. Surveillance of the *Salmonella* outbreaks was activated in 1962 by the Council of State and Territorial Epidemiologists, the Association of Public Health Laboratories, and the Centers for Disease Control and Prevention (CDC). The main objectives in starting this investigation were to

"define endemic patterns of salmonellosis, to identify trends in disease transmission, to detect outbreaks, and to monitor control efforts" (Olsen et al., 2001). There was a 9.6% increase in laboratory confirmed bacterial infective diarrheal cases in 1997 when compared to 1996 (FoodNet, 1998). However, since 1997 there has been a nineteen percent decline in bacterial foodborne infections (Kennedy et al., 2000). *Salmonella* cases however increased in 1999 and were accompanied by decreases in *Campylobacter jejuni, Shigella, and E. coli* O157:H7 (Kennedy, et al., 2000). Cases of illness where a food source was identified as the vehicle for infection have increased. It is believed that foodborne diseases cause roughly 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths each year in the United States alone (Kennedy et al., 2000; CDC 2001). *Salmonella* is the second most often isolated bacteria associated with food at roughly 27.4% of all foodborne infections (FoodNet, 1998). The *Salmonella* surveillance system has shown that from 1987 to 1997 44,863 isolates were imparted to the CDC (Olsen et al., 2001) (Table 2.1 and Figure 2.1). Human disease can be caused by about 2000 serotypes of *Salmonella.* The main symptoms of *Salmonella* consist of diarrhea, fever, nausea, vomiting, headache, and abdominal pain (CFSAN, 1996).

The onset of these symptoms is usually between six to forty eight hours after infection and usually last for one or two days depending on initial infective dose as well as strain type. *Salmonella* infections may not be limited to the digestive tract and can lead to septic arthritis due to localized infection, or sepsis. The most susceptible populations continue to be the young, old, and immunosuppressed. Estimates of medical costs and lost productivity due to foodborne *Salmonella* infections range from $464 million to $2.329 billion (Frenzen 1999). In 1999, twenty two percent of all culture confirmed *Salmonella* individuals were hospitalized (Kennedy, et al., 2000). Very low infectious doses are often associated with high fat foods (Portillo 2000). *Salmonella* has shown a higher heat resistance in foods with a high fat content. The infectious dose for humans may be as low as 1-10 *Salmonella* cells, though typically a dose of 10^4 to 10^6 *Salmonella* cells is necessary. *Salmonella* has also been commonly associated with foods such as raw meat, poultry, eggs, and dairy products.

Table 2.1. Annual number of reported *Salmonella* isolates from humans and their ranking in the United States, 1987-1997. (Adapted from Olsen et al., 2001).

Serotype	1992	1993	1994	1995	1996	1997	Total
Typhimurium	7,950	8,743	8,365	9,702	9,501	9,116	53,377
Enteriditis	6,578	8,071	9,866	10,200	9,570	7,924	52,209
Heidelberg	2,528	2,457	1,825	2,095	1,998	2,104	13,007
Newport	1,481	1,487	1,673	2,566	1,985	1,584	10,776
Agona	750	651	752	683	605	739	4,180
Montevideo	559	789	631	685	1,227	718	4,609
Thompson	690	576	549	625	586	695	3,721
Aviana	648	641	540	758	749	675	4,011
Infantis	499	568	520	521	503	651	3,262
Hadar	1,532	1,298	1,001	812	658	643	5,944
Oranienberg	597	522	62	595	690	623	3,089
Braenderup	477	381	426	588	531	559	2,962
Muenchen	449	657	559	754	595	543	3,557
Saintpaul	529	380	479	467	562	436	2,853
Typhi	449	472	507	442	440	349	2,659
Other	6,836	7,554	775	8,674	8,162	6,867	38,868
Unknown	2,136	1,649	1,469	952	382	382	6,970
Total	**34,688**	**36,917**	**3,522**	**41,222**	**39,035**	**34,608**	**189,992**

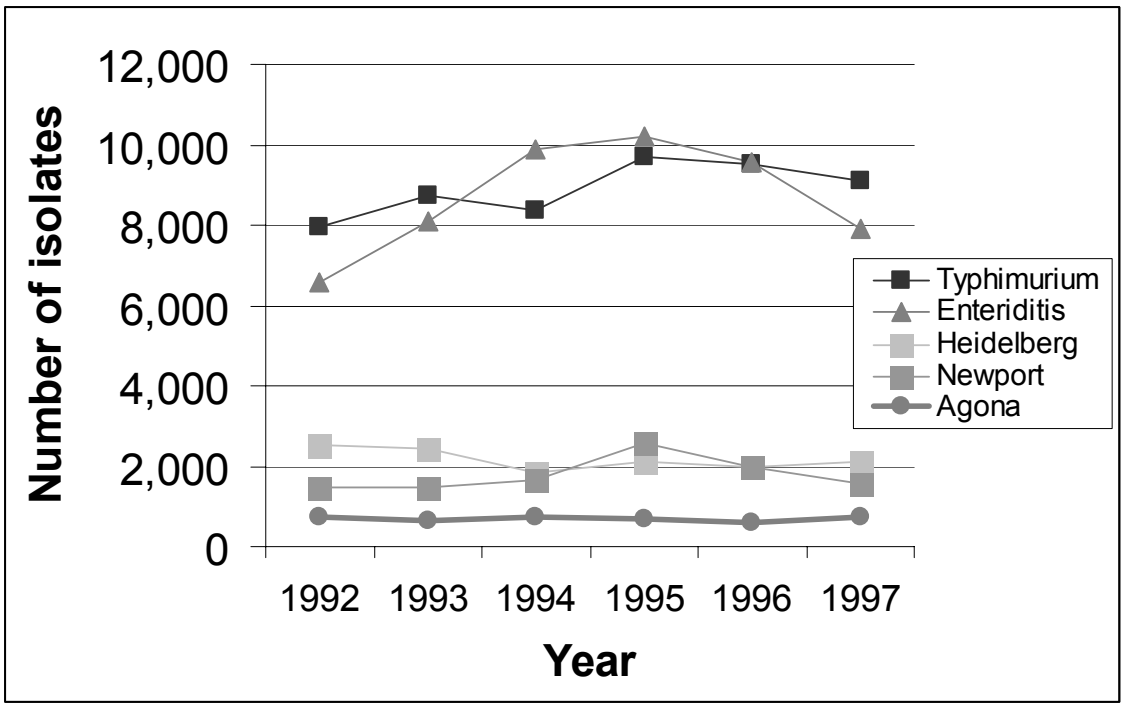

Figure 2.1. *Salmonella* isolates from humans: five most frequent serotypes in the United States, 1992-1997. (Adapted from Olsen et al., 2001).

Prevalence of *Salmonella* in Poultry and Animal Feeds

Animal feeds have long been considered a major player in the transmission of *Salmonella* to poultry (Williams, 1981; McChesney, 1995; Ricke et al., 1998). Even though feed is widely accepted as a source of possible contamination, the incidence of outbreaks being attributed to feed is very low (Davies and Hinton, 2000) as well as the argument that serotypes that are obtained from feed do not match those isolated from the contaminated poultry flocks (Brunton, 1991). *Salmonella* has been isolated from feed and or feed ingredients in a number of studies (Williams, 1981; Jones et al., 1982; Cox et al., 1983; Stuart, 1984; Davies, 1992; Veldman et al., 1995; Whyte et al., 2003). Levels of contamination in feed and animal by products have been estimated at five and thirty-one percent respectively (Allred et al., 1967; Williams, 1981). In a study by Whyte and coworkers (2003), Salmonella was recovered from preheat (18.8%) and postheat (22.6%) areas in the feed mill. Whyte and coworkers (2003) also found that 11.8 percent of feed ingredients and 33.3 percent of dust samples taken from preheat areas of the feed mills were found to be positive for *Salmonella*. Feed delivery vehicles were also found to have a high rate of contamination with 57.1 percent of them testing positive for *Salmonella* (Whyte et al., 2003). Since the level of feed contamination may be a problem, the potential method of transfer to the poultry farm becomes a concern. Henken and coworkers (1992) found that farms who obtained *Salmonella* contaminated

feeds had a 5.3 greater likelihood of producing a positive *Salmonella* flock than facilities who did not obtain contaminated feed.

With the increased risk of producing a *Salmonella* positive flock through the feeding of contaminated feed identified, an additional question is how long can *Salmonella* organisms actually survive in the feed. Ha and coworkers (1998) found that between 5.16 and 5.52 log colony forming units of *Salmonella* per gram of feed were recovered from *Salmonella* Typhimurium marker strain spiked poultry mash containing either meat and bone meal or soybean meal after fifty-six days storage at room temperature. This level of survivability was found to be consistent with Davies and Wray (1996) who recovered *Salmonella* after three months storage time and with the report by Williams and Benson (1978) that *Salmonella* may survive up to sixteen months in feed stored at 25ºC.

Poultry and products derived from poultry are believed to make up roughly 50% of the total vehicle of transmission when dealing with *Salmonella* (Texas Dept. of Health, 1998). Waldroup (1996) reports that *Salmonella* was present at low levels (1-30 cells per carcass) on an estimated 30-50% of poultry carcasses. *Salmonella* has been isolated from the skin, feathers, and feces of broilers; these organisms can then be spread to other animals either on the same poultry farm or at the slaughter facility (Nisbet and Ziprin, 2001). When *Salmonella* infection occurs it can result in two forms: the asymptomatic and the symptomatic form. Animals that are asymptomatic can continue to contaminate the food supply because they in a sense carry the disease with no signs of

illness (Nisbet and Ziprin, 2001). The two major health concerns when dealing with asymptomatic carrier states lie in the contamination of *Salmonella* on carcasses and shell eggs. Even those individuals whose infection is symptomatic may only have acute symptoms and may never be associated to a *Salmonella* infection itself.

Salmonella Pathogenesis

Today, there are three different types of disease considered to be associated with *Salmonella,* which are caused by different strains: typhoid fever, gastroenteritis, and an invasive form. The two most widely known types are typhoid fever (caused by *Salmonella* Typhi) and the gastroenteritis form most often caused by *Salmonella enteriditis* Typhimurium or *Salmonella enteriditis* Enteriditis. However, *S*. Typhimurium has also been linked to typhoid like disease in mice (Kaufman et al., 2001). For the purposes of this discussion, the gastroenteritis form of disease caused by S. Typhimurium will be used as the model to discuss the mechanisms of pathogenesis and the corresponding immune response.

In order to invade the host, *Salmonella* must undergo an infectious disease cycle. The conventional infectious cycle consists of: entry of the pathogen, establishment and multiplication, avoidance of host defenses, and finally damage and exit (Donneburg, 2000). *Salmonella* may undergo all of these steps when it invades the host. Due to the prevalence of *Salmonella*

contamination on a number of different food products, it can easily gain access to and colonize the intestinal system of the host. Therefore, introduction to the host is not the problem for *Salmonella.* The problem lies in how it adapts to the condition of the gastrointestinal system via its virulence mechanisms located on the *Salmonella* pathogenicity islands.

Salmonella Pathogenicity Islands

Salmonella has five different pathogenicity islands that encode the majority of the virulence genes used for invasion and evasion in the host by *Salmonella*. Pathogenicity islands is a term used to describe a set of genes encoding for virulence that are located on a particular loci in the bacterial genome, but are absent from nonvirulent strains of the same species (Donneburg, 2000). Blanc-Potard and Groisman (1997), stated that "pathogenicity islands constitute a major driving force in the evolution of bacterial pathogens because their acquisition often determines the virulence properties of a microorganism."

The most often discussed group of genes involved in pathogenicity is *Salmonella* Pathogenicity Island 1 (SPI1), which is located at the 63 centisome (Wood et al., 1998). *Salmonella* Pathogenicity Island 1 is the most widely known island in that it encodes multiple genes required for *Salmonella* invasion and survival in the host system. It has been determined that more than 28 genes are responsible for encoding a type III secretion system (*spa*, *inv*, *prg*,

and *org*), secretory proteins (*sip*, or *ssp*; *spt*) and regulatory mechanisms (*invF* and *hilA*), all of which aid in the ability of *Salmonella* to invade the host cell (Deiwick et al., 1998). Mutants with mutations in the *sip* gene have been shown to decrease the amount of fluid buildup in the ileum which shows that the expression of this gene is required to induce diarrhea (Zhang et al., 2003). *hilA* (Hyper Invasive Locus) is a transcriptional activator encoded on SPI1 (Bajaj, et al., 1995). The *hilA* encoding protein was believed to be roughly 531 to 553 amino acids in length (Bajaj, et al., 1995). It was later proven by Rodriguez and co-workers (2002) that the *hilA* gene encodes for a protein that is 553 amino acids (63kDa) in length. *hilA* is also thought to be a requirement for *Salmonella* invasion due to its transcriptional properties (Bajaj, et al., 1995). *hilA* has also been shown to trigger the rendering of *lacZY* infusion genes where it is in a gene fusion (Bajaj et al, 1996). This allows for experiments to be conducted that look into what environmental stimuli cause the induction of *hilA* in *Salmonella* in order to determine the virulence response upon induction. SPI1 also encodes one of the two type III secretion systems of *Salmonella.* The type III secretion system encoded on SPI1 is a key component in piercing through the epithelial layer of the intestine and delivering proteins needed for virulence mechanisms (Deiwick et al,. 1998).

The second *Salmonella* Pathogenicity Island (SPI2) is located at the 30 centisome (Wood et al., 1998). SPI2 encodes the components of the second type III secretion system of *Salmonella* (Wood et al., 1998). This secretion

system is more homogenous to the secretion system of Enteropathogenic *Escherichia coli* (EPEC) than to the secretion system encoded on SPI1 (Sukhan, 2000). The type III secretion system encoded on SPI2 allows *Salmonella* to reproduce in the spleen upon infection of the host system (Deiwick et al., 1998). SPI2 has also been shown to be required for the expansion of systemic infection (Shea et. al, 1996). It was discovered that SPI2 also has genes for a two component regulatory system (Ochman et al., 1996; Shea et al., 1996; Diewick et al., 1998). SPI2 genes have additionally been shown to be expressed only when entry into the mammalian cell has occurred (Cirillo et al., 1998).

Researchers are just beginning to understand the role of the last three *Salmonella* pathogenicity islands in the ability of *Salmonella* to cause infection and circumvent the immune response of the host.

Salmonella Pathogenicity Island 3 (SPI3) is located at the 82 centisome (Wood et al., 1998). SPI3 encodes genes essential for the survival of *Salmonella* in macrophages namely *mtgC* and *mtgB* (Blanc-Potard and Groisman, 1997).

Salmonella Pathogenicity Island 4 (SPI4) is located at the 92 centisome (Wong et al., 1998). This Pathogenicity Island is flanked by *ssb* on one side and *soxSR* on the other. SPI4 is believed to encode genes involved in toxin secretion and perhaps even a type I secretion system, due to the homology between the open reading frames (ORFs) of SPI4 when compared to known

proteins involved in toxin secretion (Wong et al., 1998). A type I secretion system is used to form a pore between both inner and outer membranes to transport proteins across to the host (Salyers and Whitt, 2002). SPI4 also was the first pathogenicity island to be completely sequenced owing to its short length, roughly 25kb (Wong et al., 1998).

Salmonella Pathogenicity Island 5 is located at roughly the 20 centisome (Wood et al., 1998). SPI5 has been discovered to contain four pathogenicity island- encoded proteins, as well as *sopB* and *orfX*, which are believed to be linked to enteropathogenicity (Wood et al., 1998). One of these proteins, *sopB*, has been shown to instigate cellular responses, causing an influx of polymorphonuclear leukocytes (PMNs), thereby establishing fluid secretion in the intestinal epithelium (Wood et al., 1998).

Invasion

Salmonella also has genes that encode for a number of different virulence factors such as fimbriae, acid survival, and iron acquisition systems, but these genes are not as well understood as the genes encoded for on the pathogenicity islands. All of these genes and pathogenicity islands which encode them, are key to the survival of *Salmonella* within the host not to mention its ability to colonize and invade; thereby ultimately leading to infection. The specifics of how these characteristics of *Salmonella* help to accomplish the

infection will be discussed further along with the systematic route to infection of the host.

The clinical beginning to a *Salmonella* infection lies in the initial contact of *Salmonella* with the epithelium of the gastrointestinal system of the particular host such as humans or chickens. The bacteria that have survived long enough for attachment and penetration of the epithelium had to overcome such hazards such as low pH in the gastrointestinal system as well as any antimicrobial and/or physical barriers. It is believed that the appendages such as fimbraie, seen only when in contact with the epithelial cells help mediate this survival (Ginochio et al., 1994). These structures are no longer observed upon bacterial entry into the cell. The next challenge facing the invading bacteria involves the colonization of the intestine through attachment (Lucas and Lee, 2000).

Salmonella cells must also be able to undergo proliferation as well as evade any further barriers, such as pH or acid shock that may try to hamper their colonization (Lucas and Lee, 2000). The preferred port of entry for *Salmonella* is the Peyer's patches in the distal ileum (Jones, 1997).

Intestinal antigens are sampled by the Peyer's patches, which are made up of specialized lymphoid (Slauch et al., 1997). Peyer's patches are made up of twenty-eight percent T cells with a CD4: CD8 ratio of 3.7:1 (Hathaway and Kraehenbuhl, 2000). M cells form a cover over the Peyer's patches. M cells are responsible for the consumption of antigens found in the lumen (Hathaway and Kraehenbuhl, 2000; Slauch et al., 1997) and M cells are believed to be the

route of invasion for *Salmonella* (Clark et al., 1998; Slauch et al., 1997). The invasion of M cells is also associated with their destruction as well as the destruction of follicle associated epithelium (Slauch et al., 1997). When M cells take up the antigen, they send it to the immune system cells (Hathaway and Kraehenbuhl, 2000). After passage through the Peyer's patch, *Salmonella* enters the follicle dome, which is home to the host lymphocytes and macrophages (Jones and Falkow, 1996). Antigen presenting cells seize the antigen, which upon processing, will be taken to the T cells. This process initiates the production of IgA specific B cells (Hathaway and Kraehenbuhl, 2000). The B and T cells are relocated to the lymph nodes (lymph "accumulations" in birds) and ultimately the blood system. This is the process by which the common mucosal immune system (CMIS) is initiated (Hathaway and Kraehenbuhl, 2000). This allows IgA to be released at mucosal effector sites (Hathaway and Kraehenbuhl, 2000). Dendritic cells (DCs) have recently been associated with non-invasive *Salmonella* movement without M cells (Rescigno et al., 2001). DCs have also been shown to be involved in presentation of peptides to MHC-II and MHC-I (Major Histocompatability Complex) in dealing with *Salmonella* infection (Wick, 2003). MHC is a genetic locus which encodes for proteins required for antigen processing (Abbas et al., 2000).

Once colonization of the intestine occurs, *Salmonella* is able to move through and colonize other cells by inducing them to uptake the bacteria (Lucas

and Lee, 2000). In most cases, this is where the type III secretion (contact dependent) system comes into play. The type III secretion system is used by the *Salmonella* bacteria cells to move proteins across the host cell membrane in order to induce physiological changes that will eventually lead to *Salmonella* entering the host cell. The type III secretion system is commonly referred to as a "need-like" export system (Hayward et al., 2000). One of these physiological changes is termed membrane ruffling which is an alteration in the membrane allowing the bacteria to be internalized. The membrane essentially moves in an outward direction and surrounds the bacteria (Jones and Falkow, 1996; Collazo and Galan, 1997). The type III secretion system is made up of structural proteins such as ATPase, outermembrane proteins, and proteins with homology to known flagellar export proteins (Sukhan, 2000). The type III secretion system has been shown to initiate chemokine production as well as recruitment of polymorphonuclear leukocytes (PMNs), which ultimately leads to inflammatory diarrhea in the host. It has also been shown to induce apoptosis in macrophages and has characteristics of chromosomal fragmentation, and presence of apoptotic bodies in the cytosol of the cell (Sukhan et al., 2000). It is believed that this is not a host response but more of a way to promote salmonellosis (Boise and Collins, 2001). "Features common to all type III secretion systems include:

1. The absence of a cleavable amino-terminal signal sequence in the secreted protein.

2. The requirement of specific chaperones for the secretion of many of the effector proteins.

3. The requirement of an inducing signal (typically contact with the host cell) for full activation of the system.

4. The ability to deliver the secreted proteins to the cytosol of host cells (Sukhan, 2000) ".

The type III secretion system is a needed commodity in order for the *Salmonella* bacteria to be taken up within the host cell.

Once the *Salmonella* have been taken up into the cell, they rely on other mechanisms for survival. In the cell, *Salmonella* has evolved ways to work around the targeting of these internal bacteria to the phagosome-lysosome fusion pathway. *S*. Typhimurium has been shown to need an acidic pH in order to induce reproduction and survival within the cells (Rathman et al., 1996). It is believed to take up residence in the membrane bound vacuole of phagocytic and non-phagocytic cells (Finlay and Falkow, 1997). When *Salmonella* enters the vacuole, the presence of lysosomal glycoproteins and removal of the surface marker assists with changes to the vacuole (Finlay and Falkow, 1997). The type III secretion system causes subsequent neutrophil (heterophil in birds) and fluid accretion in the ileum (Zhang et al., 2003). The neutrophil addition causes necrosis of the surrounding tissue and ultimately diarrhea thereby bringing about the symptoms of disease (Zhang et al., 2003).

Genetics of *hilA*

The *hilA* (Hyper Invasive Locus) gene is a transcriptional activator encoded on *Salmonella* Pathogenicity Island 1 that is part of the OmpR/ToxR family (Bajaj, et al., 1995). See Figure 2.2 for figure depicting *lacZY* fusion strain used. The determination of hyperinvasive mutants occurred through insertions of constitutive neo promoters into the *Salmonella* Typhimurium genome (Lee et al., 1992). *hilA* has been found to map at minute 59.5 in between srl and mutS genes (Lee et al., 1992). The *hilA* gene encodes for a protein that was believed to be between 531 to 553 amino acids in length (Bajaj, et al., 1995; 1996; Darwin and Miller, 1999; Lucas et al., 2000; Lostroh and Lee, 2001; Lucas and Lee, 2001; Baxter et al., 2003; Boddicker et al., 2003). It was later proven by Rodriguez and co-workers (2002) that the *hilA* gene encodes for a protein that is 553 amino acids (63kDa) in length. According to Bajaj and coworkers (1995; 1996) HilA is believed to be a binding protein for DNA. The *hilA* gene is also thought to be a requirement for *Salmonella* invasion due to its transcriptional properties. *hilA* provides a key step in invasion gene regulation while *Salmonella* mounts an infection against its host (Bajaj et al., 1995) and is required for the access of *Salmonella* into epithelial cells (Lee et al., 1992). In order for invasion to be observed SirA is required as well for the greatest extent of *prgH*, *hilA*, and other SPI1 gene expression (Johnston et al., 1996).

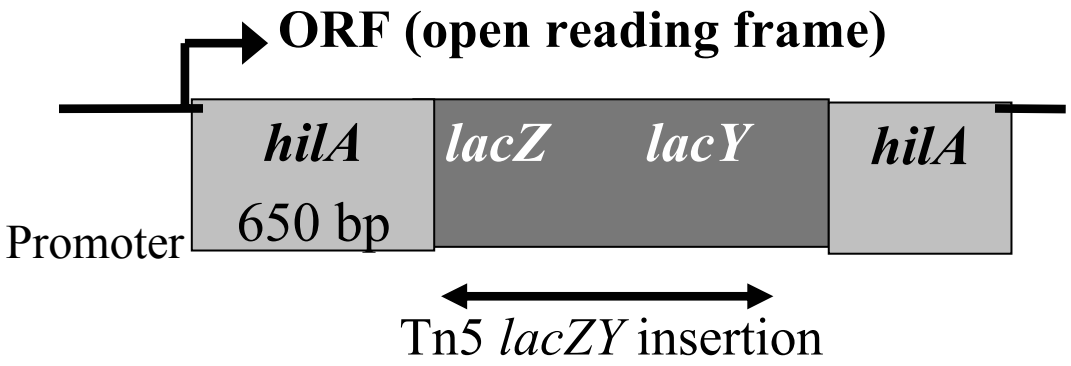

Figure 2.2. *Salmonella* Typhimurium strain EE658 carrying a *hilA:lac ZY* fusion. (Adapted from Bajaj et al., 1996).

The expression of *hilA* and other invasion genes is dependent on environmental signals, oxygen levels, osmolarity, pH, and growth phase (Galan and Curtiss, 1989; Ernst et al., 1990; Galan and Curtiss, 1990; Lee and Falkow, 1990; Schiemann and Shope, 1991; Lee et al., 1992; Behlau and Miller, 1993; MacBeth and Lee, 1993; Jones et al., 1994; Pegues et al., 1995; Bajaj et al., 1996; Vescovi et al., 1996; Gunn et al., 1996; LeClerc et al., 1998; Rakeman, 1999; Fahlen et al., 2000; Fahlen et al., 2001; Boddicker et al., 2003). *hilA* expression can be inhibited by high oxygen,osmolarity, *pho-24*, or and disturbance in the *sirA* or *barA* genes(Bajaj et al., 1996; Johnston et al., 1996; Lundberg et al., 1999). This inhibition requires the specific region of -39 to-314 upstream of the *hilA* start site (Schechter et al. 1999). The expression of hilA as well as other SPI1 genes has been shown to be transcriptionally regulated by PhoP and SirA. This can be seen when as a result of PhoP phosphorylation by PhoQ, PhoP is activated and thus suppresses *hilA* and SPI1 gene transcription (Bajaj et al., 1996; Behlau and Miller, 1993; Gunn et al., 1996; and Pegues et al., 1995). PhoQ sensor kinase phosphorylates PhoP when extracellular cation levels are low (Vescovi et al., 1996). *hilA* seems to directly activate transcription of SPI1 genes encoding for specific elements needed for the mechanism of the type III secretion system to work properly (Bajaj et al., 1995; 1996). Expression of invasion genes and *hilA* has been shown to be decreased when disruptions have occurred in *sirA, barA*, and *phoP* (Ahmer et al., 1999; Altier et al.,2000; and Johnston et al., 1996; Lucas et al., 2000). BarA is involved in the

regulation of SirA under certain environmental conditions, when SirA is activated, it activates the expression of *hilA* as well as other invasion genes whether directly or indirectly is still unknown (Lucas et al., 2000). SPI1 genes that are believed to be responsible for transcription regulation are *sprB, hilC, hilD, hilA,* and *invF* (Lostroh et al., 2000, Akbar et al., 2003). Lundberg and co-workers (1999) found that *hilA* expression was needed for the expression of prgH, but *sipB, invA*, and *invF*, could be expressed without the presence of *hilA*. InvF and HilA, regulation genes encoded on SPI1 have an influence on the transcription of SPI1 genes such as *orgA*, *prgH, invF, sspC* as well as other genes required for invasion and the type three secretion systems (Rakeman,1999). InvF is also a regulator of transcription for AraC (Rakeman, 1999). *hilC* and *hilD* encode AraC like transcriptional regulators that can inhibit hilA repressors under certain environmental conditions (Eichelberg and Galan, 1999, Rakeman et al., 1999, Schechter et al., 1999). *hilC* mutants have been shown to have a slight decrease in *hilA* expression while *hilD* mutants experience dramatic reductions in levels of *hilA* expression (Rakeman et al., 1999; Schechter et al., 1999).

Reverse Transcriptase Polymerase Chain Reaction for Studying Gene Response

There are currently many methods used to further research the invasion of *Salmonella* and the immune response it induces. Tissue culture has been

popular for a long time, and research could be conducted using cell lines comparable to dendritic cells and even macrophages. However, due to the time consuming tasks and difficulty of maintaining those cell lines, current research has started to move away from tissue culture in favor of methods with a more molecular base. Assays such as *in vivo* expression technology (IVET) and signature tag mutagenesis (STM), as well as differential fluorescence induction (DFI), rely on the induction of genes within the host as the basis for experimental results. These methods are used to discover which genes are expressed by the bacteria under certain conditions meant to mimic either the host environment or the bacterial environment within the host. IVET is achieved by creating biosynthetic gene mutants that lessen virulence which allows the researcher to specifically choose for bacterial mutants that are expressed in the host (Mahan et al., 1993). Julio et al., (1998) describes the creation of a chromosomal deletion mutant with defined endpoints and no size limitations to solve problems of previous IVET strategies. DFI involves differential fluorescence as an identification of gene expression which is activated when the bacteria come in contact with host cells (Valdivia and Falkow, 1997). A benefit of this method is that the expression of genes is not dependent on any chemical or antibiotic (Valdivia and Falkow, 1997). STM is transposon mutagenesis that uses inserted transposons where each mutant carries its own DNA sequence tag; this process was created to isolate genes required for virulence (Hensel et al., 1995). The tags of mutants are recovered using amplification, radiolabeling,

and hybridization analysis. Mutants with an avirulent phenotype can then be identified because they will be in the inoculum given to mice, but they will not be in the bacteria recovered out of the mice. Mouse models are still popular; although the samples taken from them are used for modern molecular techniques such as RT-PCR and ELISA. RT-PCR is a more current method that is used to determine what genes are being expressed by looking at the mRNA (messenger RNA) that is extracted from the bacteria.

Real Time Reverse Transcriptase Polymerase Chain Reaction for Studying Gene Response

Currently there are a number of techniques used to evaluate the amount of mRNA expression including Northern Blotting, cDNA arrays, *in situ* hybridization, RNase protection assays, and reverse transcription polymerase chain reaction (RT-PCR) (Giulietti et al., 2001). Reverse transcription along with the polymerase chain reaction has proven to be a powerful method to quantify gene expression (Murphy et al., 1990; Noonan et al., 1990; Horikoshi, 1992). Of these methods RT-PCR is the method for quantification known to be the most discerning and faultless in its use (Giulietti et al., 2001). Real time RT-PCR is rapidly becoming the new method for determining mRNA expression (Bustin, 2000; Orlando et al., 1998; Pierson et al., 2003) due to its capacity to use up to 1000 times less RNA than other known methods (Hashimoto et al., 2004).

RT-PCR measures the buildup of the product during the exponential phase of the reaction which can be observed on an amplification plot (Giulietti et al., 2001). Since results of real time RT-PCR can be seen while the amplification plot itself is being made and therefore no other reactions or reagents are needed to determine the results. That means no more detection of PCR products by agarose gel electrophoresis and ethidium bromide staining which can be tedious and require the use of hazardous chemicals. SYBR Green is a fluorescent dye with a high affinity for double stranded DNA that shows its fluorescence when bound to double stranded cDNA (Li, et al., 2003; Dhar et al., 2002). See Figure 2.3. The accumulation of fluorescence is read at the end of every cycle via the target amplification. The threshold cycle values are determined by the cycle at which the fluoresecent emission rises above the threshold (Guiletti et al., 2001). Since SYBR green is not sequence specific, melting curves can be used to analyze different products. By this method primer dimers can be distinguished by their lower melting temperature (Tm) (Wittwer et al., 2001; Ririe et al., 1997; Wittwer et al., 1997). The cycle at which the threshold was overcome (C_T) decreases as the amount of target increases (Guiletti et al., 2001). A method for determining relative quantification is the

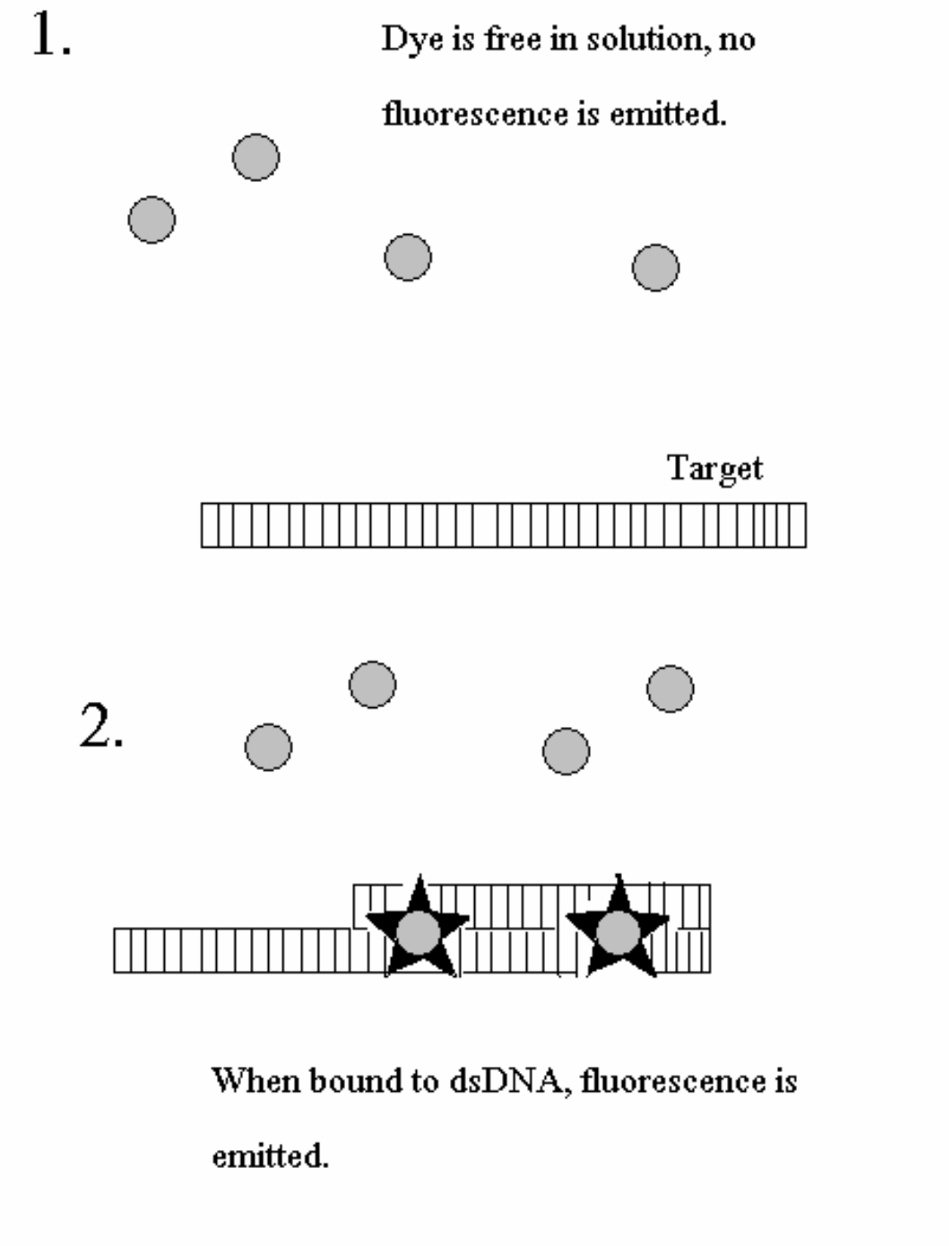

Figure 2.3. SYBR Green DNA binding dye. 1. The dye is free in solution. 2. The dye has been bound to the dsDNA target. (Figure adapted from Giulietti etal., 2001).

comparative C_T method. This method does not require the construction of cDNA plasmids nor does it depend on techniques where the variations in transcription efficiency can not be controlled. The comparative C_T method is determined by normalizing the target to a housekeeping gene which is then compared to the non treated sample or control. The determination of the relative expression that a treatment causes can be sufficient to assess its effects and the absolute expression level is not necessarily needed (Schmittgen, 2001). Relative expression is determined by $2^{-\Delta\Delta C}{}_T$, $\Delta\Delta C_T = \Delta C_T$ (sample)-ΔC_T (control) whereas ΔC_T is calculated by subtracting the C_T of the target gene from the C_T of the housekeeping gene (Guiletti et al., 2001; Livak and Schmittgen, 2001; Lehman and Kriepe, 2001). By using a housekeeping gene, minor variations in amount of RNA used or differences in reverse transcription efficiency can be overcome. The use of a housekeeping gene is at present the most approved method for this form of RT-PCR. The perfect housekeeping gene should be always expressed at a stable level without being affected by the treatment groups.

Overview of the Maillard Reaction

The Maillard reaction was first characterized by Louis Maillard in 1912. Louis Camille Maillard was born on February 4th in 1878. His work was on six topics in particular those being urinary indoxyle, metabolism of nitrogenized substances, metabolism of sulphur, synthesis of peptides, and the titanium in

the environment (LCMA, 2003). His name has been linked to such things as the Maillard coefficient and the Maillard Reaction. The first mention of the Maillard Reaction came in a note by Maillard presented by Armand Gautier at the Academy of Science meetings (LCMA, 2003). In this note, Maillard states that ”the consequences of these facts appear as many to me as interesting in various fields of science: not only in physiology and human pathology, but also in vegetable physiology (cyclic alkaloids, etc.), in agronomy (maturation of manure, humus, various industries), and in geology (minerals fuels, etc)” (LCMA, 2003). Little did he know that this reaction would indeed be used for a multitude of procedures in food, medicine, and nutrition not to mention many other applications.

The Maillard Reaction is a browning reaction that occurs frequently in such processes as bread and cereal production and is commonly found to take place during heating, processing, and storage of food products (Marounek et al., 1995). The Maillard Reaction is a non-enzymatic reaction that involves free amino groups of the amino acids and the reducing sugar’s carbonyl group to produce a Schiff base that is subsequently transformed into the Amadori products (Jing and Kitts, 2002). Further reactions and rearrangements take place in order to achieve the final pigmented nitrogenous polymer products known as melanoidins (Martins et al., 2001). The first step in this reaction is to form an N-glycoside from a reducing sugar. Once this N-glycoside is formed, immonium forms and undergoes Amardori rearrangement to form ketosamines.

Dehydration of these products leads to production of reductones and short chain hydrolytic products. These dehydration products then undergo Strecker degradation. See Figure 2.4. The Maillard Reaction has been found to occur when conditions are at lower temperatures and higher concentrations than carmelization (UBC, 2003). These Maillard Reaction products are used to enhance the aroma, color, flavor, and nutritional value of the food products being processed (Kitts et al., 1993; Martins et al., 2001). The Maillard Reaction can occur in food products such as in roasted meats and bakery products.

Maillard reaction products (MRP) have been linked to antioxidative, antibacterial, and cytotoxic effects (Jing and Kitts, 2000; Einarsson, 1987). The effect of an MRP depends on bacterial strain, concentration of the compound, as well as the type of amino acid, which is used to create the MRP (Einarsson et al, 1983). Einarsson et al. (1988) observed a decrease in the usage of serine, glucose, and oxygen in the presence of MRPs by the bacterial cells. *Listeria monocytogenes* growth is inhibited by a product generated during the heat sterilization of Brain Heart Infusion media (BHI), and the greater the duration of treatment with the BHI byproduct the greater the suppression of *Listeria* growth (Sheikh-Zeinoddin et al., 2000). Maillard Reaction products have also been shown to bind metal ions under certain dietary conditions and this activity can independently contribute to the inhibitory effect that MRPs have on microorganisms (O'Brien and Morrissey, 1997). In addition, antioxidant effects have been related to the changes in pigment color and have been shown

to be affected by the production of melanoidins, the characteristics of the system, and the food preparation conditions (Manzocco et al., 2001).

For the purposes of this study lysine, arginine and histidine were chosen as the amino acids to be used in conjunction with glucose to generate the MRPs. Lysine, arginine, and histidine are also all basic amino acids that are sensitive to the heating process and possess an extra amino group, which allows for Maillard reactions to be produced readily. All three amino acids are nutritional requirements for poultry diets and considered essential amino acids (Phalke and Annison, 1995). Lysine is also considered a limiting amino acid in poultry feeds meaning that growth of the animal is dependent on the amount of the specific amino acid in the diet, thus giving it more reason to be looked at in this study since it is a required commodity in processed poultry feeds. At zero to six weeks Leghorn-type chickens require 1.0%, 0.26%, and 0.85% of arginine, histidine and lysine respectively (NRC, 1994). These levels of required amino acids will increase and decrease as the birds age depending on their nutritional needs.

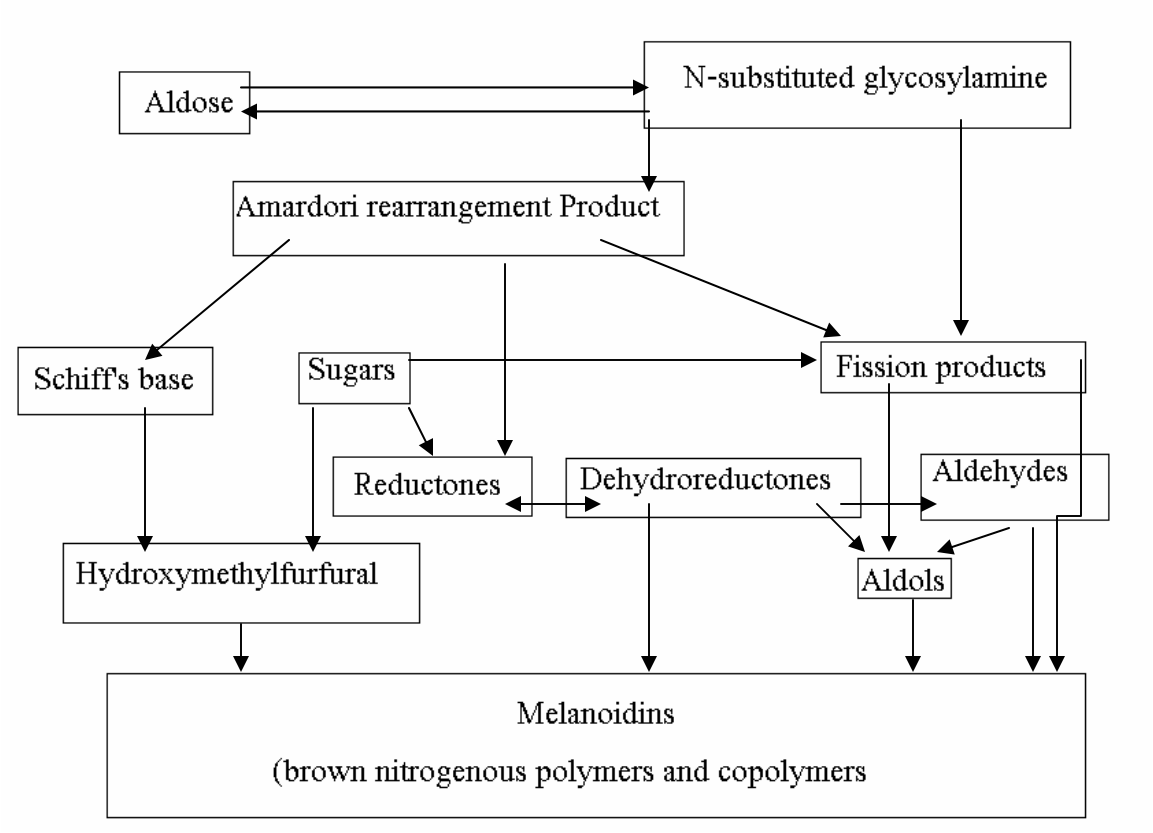

Figure 2.4. Steps involved in the Maillard Reaction. (Information adapted from UBC, 2003).

Rationale and Significance

The occurrences of foodborne illnesses have increased over the years. *Salmonella* is believed to be the source of between 2 and 4 million cases of food borne illness each year (CFSAN, 1996). The bacteria that cause these illnesses become virulent when exposed to different factors ranging from pH, oxygen availability, and osmolarity (Bajaj et al., 1996; Figure 2.5). All of these factors can occur in the gastrointestinal system of both the chicken and humans. It is important to understand the mechanisms by which these factors contribute to the increase in virulence in *Salmonella*. This understanding will allow for new developments to be made so that products do not cause stressful conditions to the bacteria thus increasing their virulence and ultimately disease via foodborne outbreaks.

There have been many new advancements in the fight against foodborne bacteria. New techniques and treatments are being developed continuously to stop microbial populations from causing disease in their host. The genetic aspect of these organisms gives insight into how infection is first established and then proliferates into a full blown disease. By understanding what specific genes activate establishment and how they are indeed activated, the establishment of infection can either be stopped or suppressed. Since *Salmonella* is exposed to a number of by-products from different production aspects both in feed and food product production, there are many instances in which virulent activity could be activated. *hilA,* a gene that has been found to be

a requirement for infection can be used as an indicator of virulence induction when exposed to certain environmental conditions. The overall focus of this research is to develop a more sensitive and rapid method to detect virulence gene induction in bacteria when they are exposed to certain environmental conditions. The specific goals of this research are to (1) use a *Salmonella* Typhimurium *hilA:lacZY* fusion strain to assess virulence gene expression when exposed to Maillard Reaction Products using B-galactosidase assay (2) develop reverse transcription and real time- polymerase chain reactions to detect *hilA* gene expression in *Salmonella* Typhimurium (3) use a *Salmonella* Typhimurium wild type strain to assess *hilA* gene expression when exposed to Maillard Reaction Products using RT-PCR to compare gene expression results to the B-galactosidase Assay.

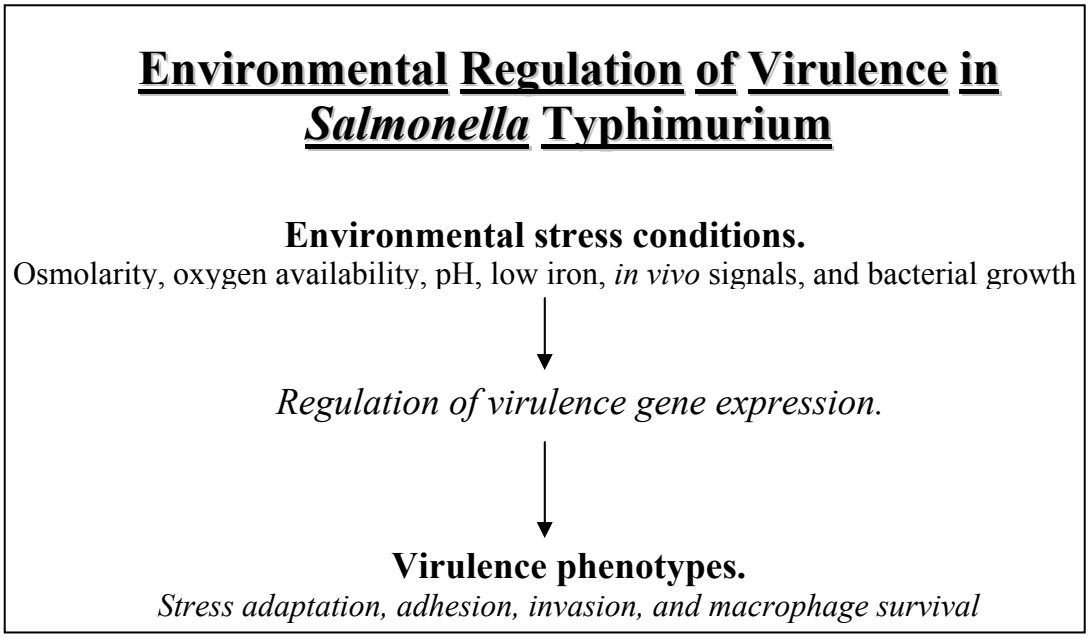

Figure 2.5. Environmental regulation of virulence in *Salmonella* Typhimurium. (Adapted from Bajaj et al., 1996).

CHAPTER III

DEVELOPMENT OF A QUANTITATIVE GENE EXPRESSION ASSAY FOR *Salmonella* Typhimurium GENE *hilA*

Synopsis

Salmonella is an important human enteric pathogen responsible for causing severe gastroenteritis. *Salmonella* is believed to be the source between 2 and 4 million cases of food borne illness each year (CFSAN, 1996). Bacterial virulence is regulated in response to a combination of environmental stimuli and involves a coordinated genetic response; therefore, it is important to understand the relevant features of the host that affect development of virulence *in vivo*. The Hyper Invasive Locus (*hilA*) gene is involved in the expression of virulence in *Salmonella* Typhimurium (ST). It is a transcriptional activator encoded on *Salmonella* pathogenicity island 1 (SPI1), which has been found to be a required gene component for the intestinal phase of infection.

The *hilA* gene is a transcriptional activator encoded on *Salmonella* Pathogenicity Island 1 (Bajaj et al., 1995). The *hilA* gene encodes for a protein that was believed to be between 531 to 553 amino acids in length (Bajaj et al., 1995). It was later proven by Rodriguez and co-workers (2002) that the *hilA* gene encodes for a protein that is 553 amino acids (63kDa) in length. The *hilA*

gene is also thought to be a requirement for *Salmonella* invasion due to its transcriptional properties.

Introduction

Currently there are a number of techniques used to evaluate the amount of mRNA expression including Northern Blotting, cDNA arrays, *in situ* hybridization, RNase protection assays, and reverse transcription polymerase chain reaction (RT-PCR) (Giulietti et al., 2001). Reverse transcription along with the polymerase chain reaction has proven to be a powerful method to quantify gene expression (Murphy et al., 1990; Noonan et al., 1990; Horikoshi, 1992).Of these methods RT-PCR is the method for quantification known to be the most discerning and faultless in its use (Giulietti et al., 2001). Real time RT-PCR is rapidly becoming the new method for determining mRNA expression (Bustin et al., 2000; Orlando et al., 1998; Pierson et al., 2003) due to its capacity to use up to 1000 times less RNA than other known methods (Hashimoto et al., 2004).

RT-PCR measures the buildup of the product during the exponential phase of the reaction which can be observed on an amplification plot (Giulietti et al., 2001). Since results of real time RT-PCR can be seen while the amplification plot itself is being made and therefore no other reactions or reagents are needed to determine the results. This alleviates the need for detection of PCR products by agarose gel electrophoresis and ethidium bromide

staining which can be tedious and require the use of hazardous chemicals. SYBR Green is a fluorescent dye with a high affinity for double stranded DNA that exhibits its fluorescence when bound to double stranded cDNA (Li et al., 2003; Dhar et al., 2002). The accumulation of fluorescence is read at the end of every cycle via the target amplification. The threshold cycle (C_T) values are determined by the cycle at which the fluoresecent emission rises above the threshold (Guiletti et al., 2001). Since SYBR green is not sequence specific, melting curves can be used to analyze different products. By this method primer dimers can be distinguished by their lower melting temperature (Tm) (Wittwer et al., 2001; Ririe et al., 1997). The C_T decreases as the amount of target increases (Guiletti et al., 2001). A method for determining relative quantification is the comparative C_T method. This method does not require the construction of cDNA plasmids nor does it depend on techniques where the variations in transcription efficiency can not be controlled. The comparative C_T method is determined by normalizing the target to a housekeeping gene which is then compared to the non treated sample or control. The determination of the relative expression that a treatment causes can be sufficient to assess its effects and the absolute expression level is not necessarily needed (Schmittgen, 2001). Relative expression is determined by $2^{-\Delta\Delta CT}$, $\Delta\Delta C_T = \Delta C_T$ (sample)-ΔC_T (control) whereas ΔC_T is calculated by subtracting the C_T of the target gene from the C_T of the housekeeping gene (Guiletti et al., 2001; Livak and Schmittgen, 2001; Lehman et al., 2001). By using a housekeeping gene,

minor variations in amount of RNA used or differences in reverse transcription efficiency can be overcome. The use of a housekeeping gene is at present the most common method for this form of RT-PCR (Guiletti et al., 2001). The perfect housekeeping gene should be always expressed at a stable level (constitutively expressed) without being affected by the treatment groups.

Materials and Methods

Inoculation for Growth Rate Determinations

Frozen aliquots of *Salmonella* Typhimurium 14028 were used to inoculate 6 mL of Brain Heart Infusion (BHI) media. The culture was grown for 18 hours at 37˚C in a shaking water bath with gentle agitation (Lab-line, Melrose Park, Il., U.S.A.). After incubation, a 0.25 mL aliquot of the culture was added to 8ml of BHI or *Salmonella* spent media (SP) and the culture was allowed to grow in the shaking water bath and readings were taken at 0.5, 1, 2, 3, 4, and 5 hour intervals at 600nm using a Spectronic 20 spectrophotometer (Milton Roy 20 D, Rockford, Il., U.S.A.). Growth rates were determined by the slope of the natural log of optical density versus time (Ricke and Schaefer, 1991). Aliquots were also taken at each interval and either plated on BHI agar plates or placed in RNA Protect for subsequent RNA extraction.

Inoculation for RT-PCR

Two different growth conditions were used to evaluate the *hilA* expression of *Salmonella* Typhimurium 14028 using RT-PCR as described by Lee et al. (1992). High oxygen cultures were obtained by inoculating Brain Heart Infusion media (2ml) with *Salmonella* Typhimurium strain ATCC 14028, which was allowed to grow overnight for twenty hours on a rotator drum. From this overnight culture 100ul was inoculated into 1ml BHI broth and allowed to grow for two hours on the rotator drum. Cultures that were grown in the presence of low oxygen were obtained by inoculating 5ml of BHI with *Salmonella* Typhimurium 14028. These tubes were incubated at 37°C in an incubator without agitation overnight. Aliquots (200ul) were then taken from both the high and low oxygen tubes to inoculate 2ml of the appropriate test media (Luria Bertani (LB) pH 6, LB pH8, LB No NaCl pH6, LB No NaCl pH8, LB 1% NaCl pH 6, LB 1%NaCl pH 8). Two aliquots of 400ul each were taken from each sample at fifteen minutes for RNA extraction.

RNA Extraction

After the different media were inoculated with the bacterial suspension following the inoculation phase, the samples were placed in a shaking water bath at 37˚C for the desired duration. At these specific time points 400ul of the bacterial suspension were removed and placed in a microcentrifuge tube with 800ul of RNAprotect (Qiagen, Valencia, CA). Samples were stored at -4˚C

overnight prior to RNA extraction. RNA was extracted from *Salmonella* Typhimurium cultures according to the RNeasy Mini Kit from Qiagen.

Primer Design

Primers were designed using sequences obtained from the GenBank website for the *hilA* and 16srRNA gene sequences and then primers were designed using Primer Express 1.0 Software from Perkin-Elmer Applied Biosystems (Foster City, CA). Sequences were then blasted on the NCBI website in order to determine if they were compatible with any other species of bacteria. Primers used in this study are listed in Table 3.1.

Reverse Transcription Reactions

Reverse Transcription Reactions were performed using the reagents from the QIAGEN® OneStep RT-PCR Kit. Each reaction contained 5X QIAGEN OneStep RT-PCR Buffer, 400 µM of each dNTP Mix , 5U/reaction RNase Inhibitor, 2.0ul of QIAGEN® OneStep RT-PCR Enzyme Mix, 200 ng of RNA, 6.0ul of each Primer (0.6uM), and RNase-free water to a final volume of 20 µl. A positive RT reaction was run in order to ensure that the procedure was working properly. The positive reaction contained all of the same components except that in place of a RNA sample template a DNA sample was used as the template. In order to determine if RNA samples were contaminated with DNA, two (-) RT reactions were run on each RNA sample. One (-) RT reaction

contained the same components as the (+) RT reactions, except it lacked the RNA sample template and contained more water to ensure that the final concentration of the remaining components remains the same. The other (-) RT reaction did not contain the RT enzyme to ensure that there was no DNA contamination. All one step RT reactions were performed on a Gene Amp PCR System from Perkin Elmer (Wellesley, MA) under the following conditions: reverse transcription at 30 minutes at 50˚C, initial PCR activation at 15 minutes at 95˚C, three step cycling 1 minute at 94˚C, 1 minute at 53˚C and 1 minute at 72˚C for 40 cycles, followed by a final extension period of 10 minutes at 72˚C. The samples were then held at 4˚C until the samples could be removed. Three RT reactions were performed, one for each RNA sample. Samples were then run on 1.5% agarose gel in 1X TAE buffer for 30 minutes at 100V. The predicted RT-PCR products of *Salmonella* Typhimurium genes *rsmC* and *hilA* are shown in Figure 3.1.

Table 3.1. Primer sequences used in RT-PCR reactions for the quantitation of gene expression.

hilA FWD	**5' tatcgcagtatgcgccctt 3'**
hilA REV	**3' tcgtaatggtcaccggcag 5'**
rsmC FWD	**5' tgcgcgaccaggctaaa3 '**
rsmC REV	**3' ttcaccactggcaggtattaagc 5'**

Real-time PCR Reactions

Real-time PCR reactions were performed on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). Each 20 µl SYBR Green PCR reaction contained 2µl RNA, 0.2µl (5µM) of each primer, 10µl 1X SYBR® Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA), 0.1µl MultiScribe Reverse Transcriptase (Perkin-Elmer Applied Biosystems), 0.4µl RNase Inhibitor (Perkin-Elmer Applied Biosystems), and water. Thermal cycling conditions were as follows: 48˚C for 30 minutes, 95˚C for 10 minutes, and 40 repeats of 95˚ C for 15 seconds and 60˚C for 1 minute. A dissociation curve was run following the real-time reaction to determine if the primers used in each reaction generated a specific product. The same (+) and (-) RT reactions were run for the Real Time PCR as for RT reactions.

Data Analysis

Real-time PCR reactions were run to analyze the expression of two genes, each gene was analyzed in replicate three times for each sample. Each sample was run in triplicate for each trial. Data were analyzed using the C_T value for each sample, or the cycle at which each samples amplification curve crosses a specified threshold.

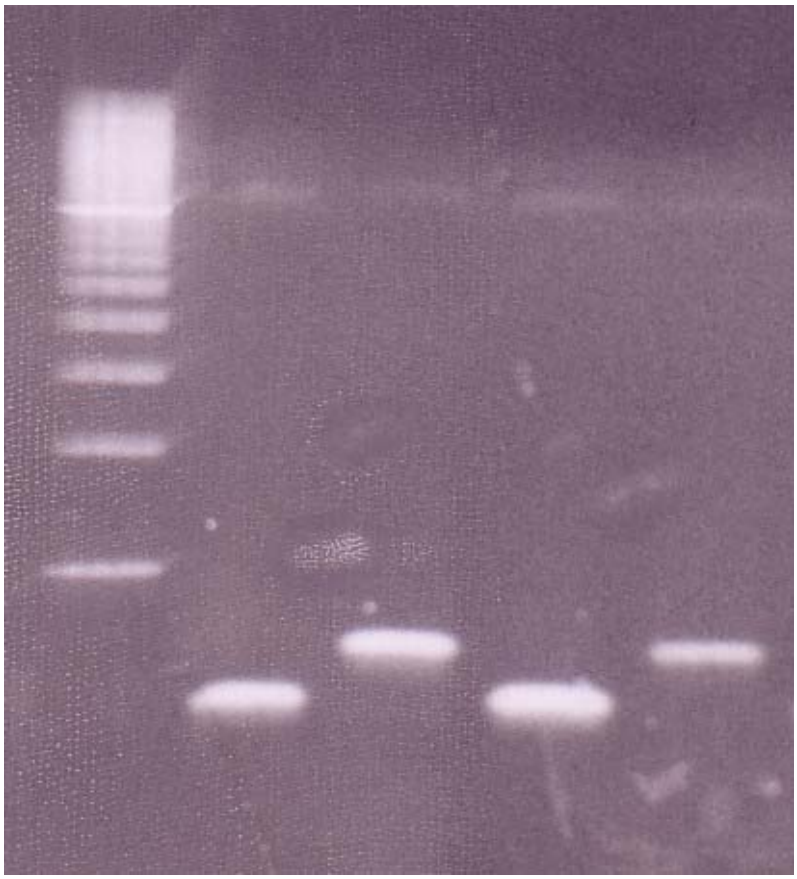

Figure 3.1. Detection of RT-PCR products with OneStep RT-PCR for the validation of RT-PCR for the quantitation of gene expression. Lanes (1)100bp Ladder, (2) Pre-Treatment sample at an OD600 of 0.5 for *hilA* gene; (3) Pre-Treatment sample at an OD_{600nm} of 0.5 for *rsmC* gene; (4) Sample treated for 15 minutes in Spent Media for the *hilA* gene; (5) Sample treated for 15 minutes in Spent Media for the *rsmC* gene. Samples were run on 1.5% agarose gel in 1X TAE buffer for 30 minutes at 100V.

First, the C_T of the replicated samples for each gene was averaged. The relative induction of each target gene of the three samples was then determined using the Comparative C_T ($\Delta\Delta C_T$) method. The Δ C_T value of the target gene (*hilA*) was determined by normalizing to the endogenous control gene (*rsmC).* The gene rsmC was chosen since the gene product it encodes for, 16S RNA, is constitutively expressed by the bacterium. These samples were then subtracted from the baseline sample which in this case was the LB pH6 high oxygen sample. The resulting $\Delta\Delta$ C_T was then used to calculate relative expression using the formula 2^-$\Delta\Delta$ C_T. Dissociation curves were also run to determine gene melting temperatures as shown in Figure 3.2. Amplification curves were also run to determine the cycle at which the threshold was overcome and corresponds to the C_T value as shown in Figure 3.3.

Statistical Analysis

Relative expression values were analyzed by the least square means separation of the GLM procedure in the SAS Statistical Analysis Software (SAS Institute, Cary, NC). Significance determination for all statistical analyses was calculated at $p<0.05$. Significantly different means were further separated using the Tukey test.

Results and Discussion

The addition of spent media although altering the growth curve of *Salmonella* Typhimurium (Figure 3.4), it does not affect the expression of *rsmC* when compared to that of *Salmonella* Typhimurium expression in BHI media (Figure 3.5). The cycle number at which *rsmC* expression crossed the threshold was more variable with a lower number of CFU/ml in both BHI media and spent media. As the number of CFU/ml increased the variability in the cycle of which the threshold was surpassed decreased so that both spent media and BHI exhibited overlapping C_T levels for the same CFU/ml level of bacteria (Figure 3.6). By comparing the Log of *Salmonella* to the C_T value at that time point an inverse relationship is observed for both BHI media and spent media (Figures 3.7 and 3.8). As the log number of bacteria increases the C_T value at which the threshold was overcome decreases. This shows that the C_T value corresponds to the number of viable bacteria.

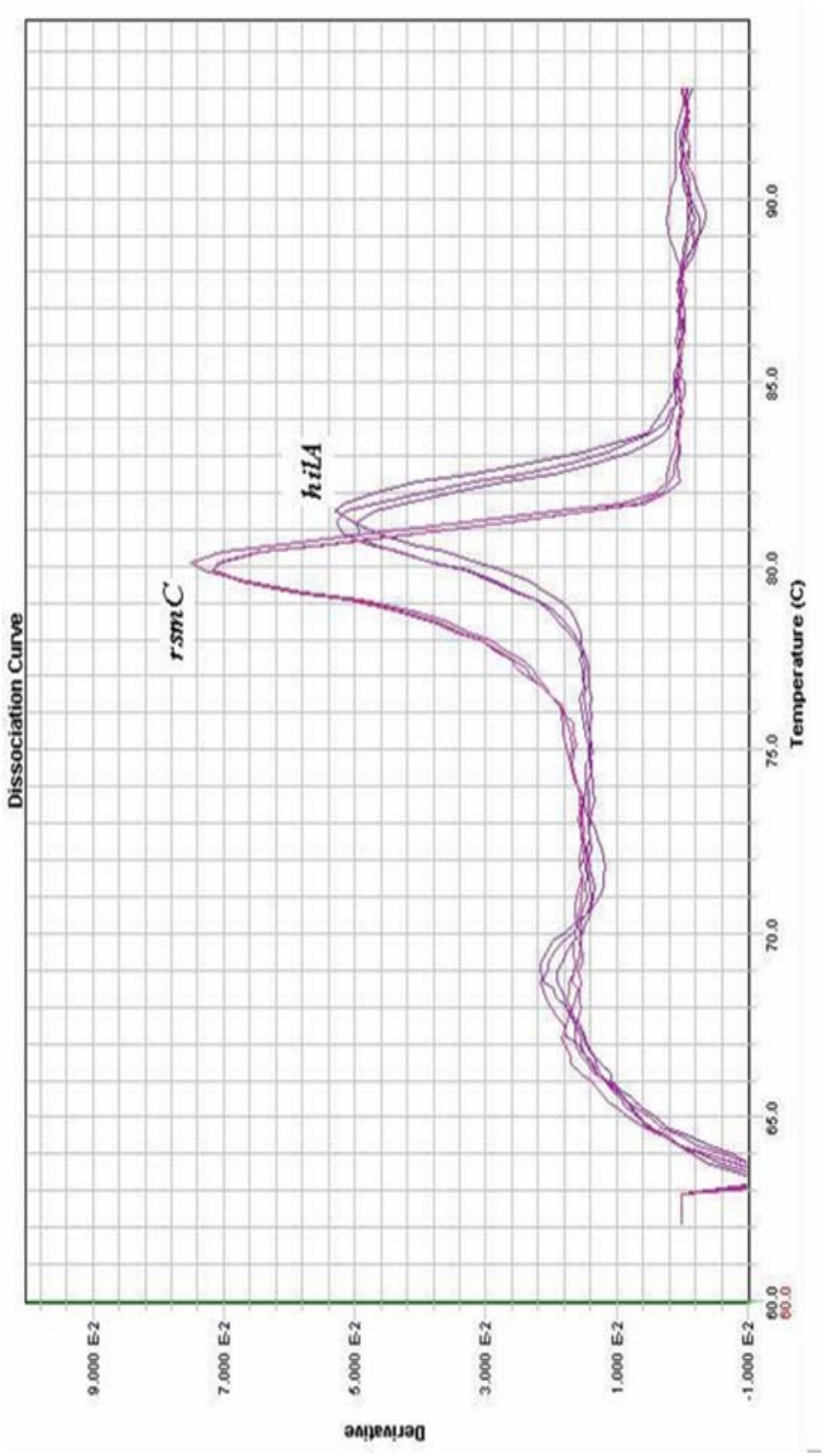

Figure 3.2. Dissociation curve of RT-PCR products showing melting temperatures (Tm) of *hilA* and *rsmC* for the quantiation of gene expression The pink and purple lines correspond to the replicates for the *rsmC* and *hilA* genes, respectively.

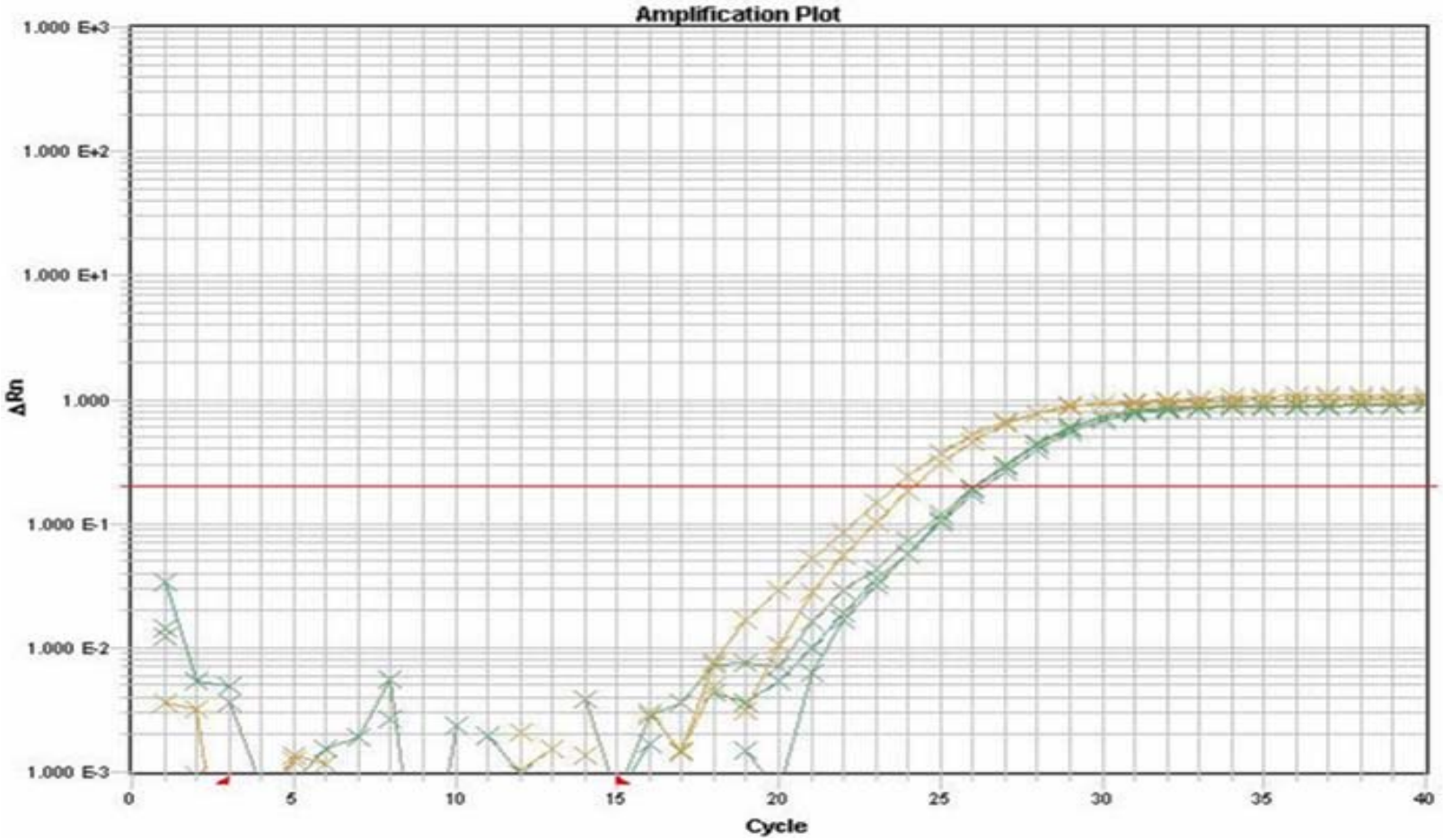

Figure 3.3. Amplification curve of RT-PCR products showing the cycle at which the threshold was overcome (C_T) for the quantitation of gene expression. The teal and brown lines indicate treatment groups of Luria Bertani under high oxygen at pH of 6 and 8, respectively.

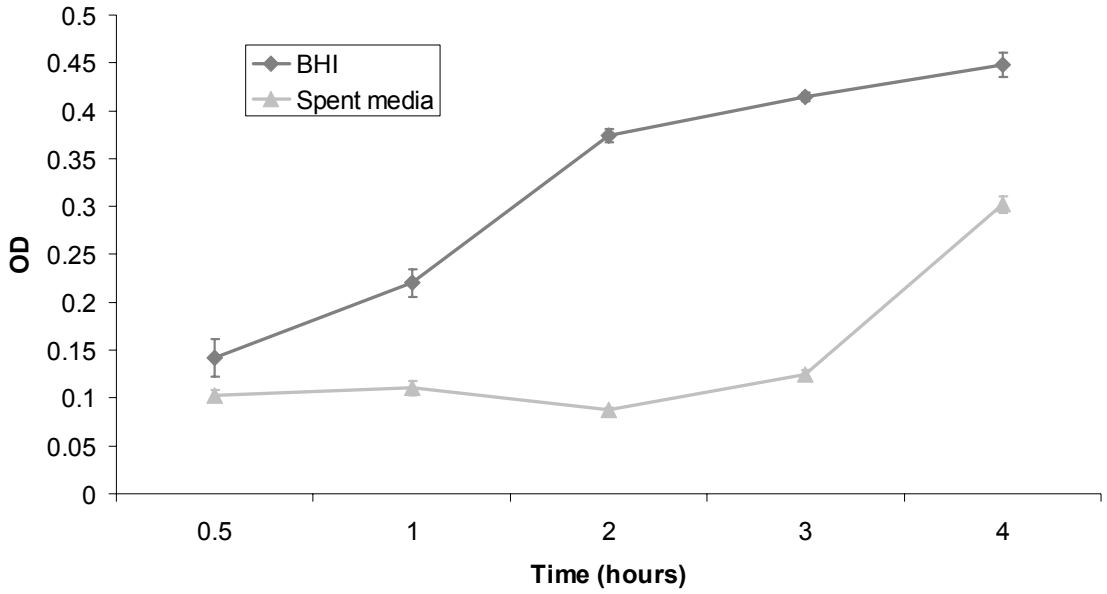

Figure 3.4. *Salmonella* Typhimurium growth curve by optical density in Brain Heart Infusion media (BHI) and spent media.

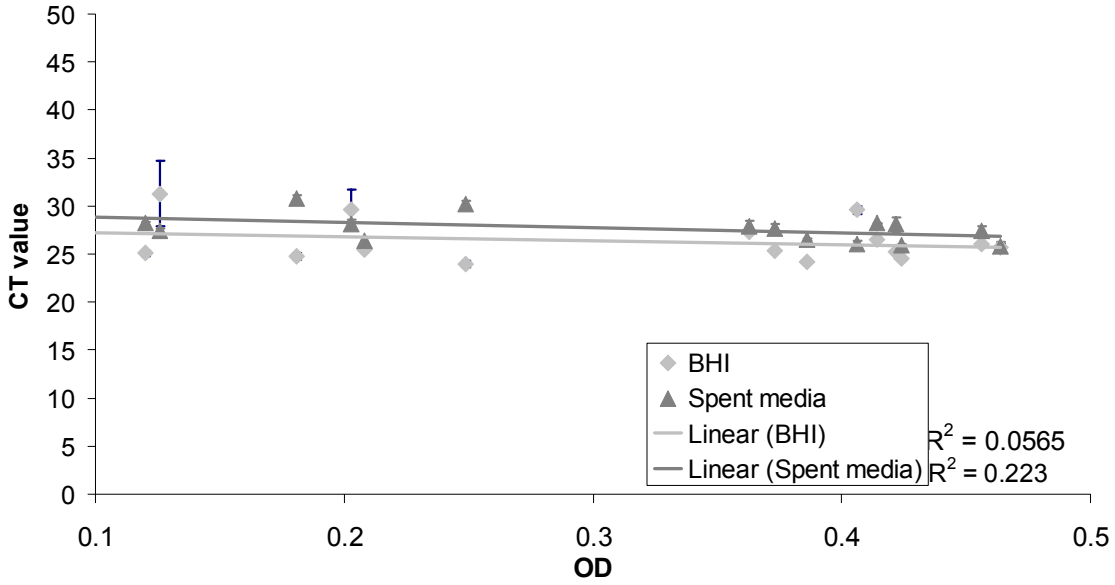

Figure 3.5. Cycle number where *Salmonella* Typhimurium *rsmC* expression in Brain Heart Infusion media (BHI) and spent media crosses the threshold value.

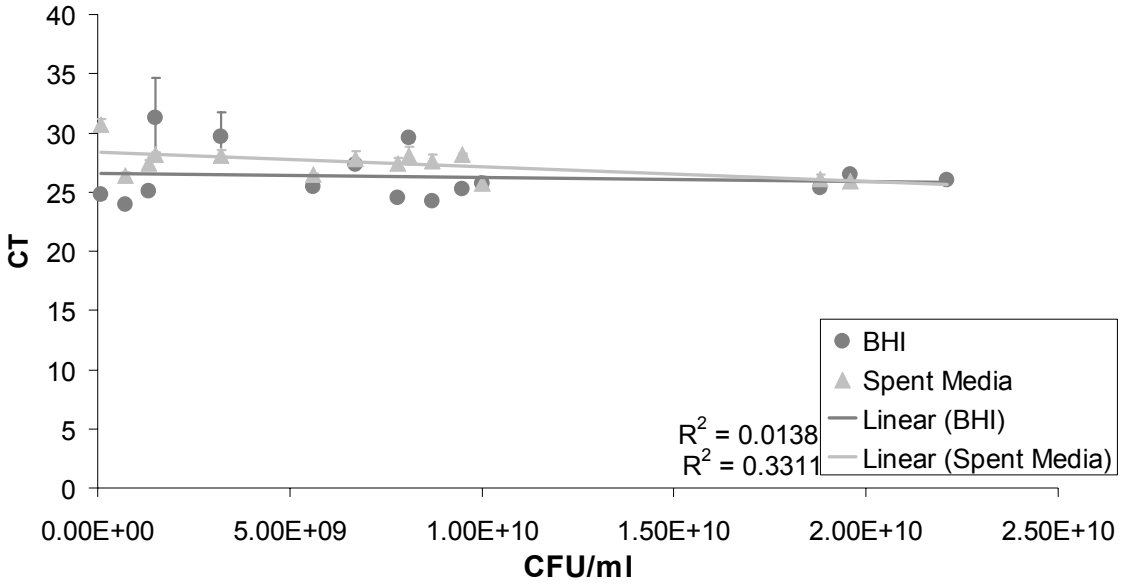

Figure 3.6. *Salmonella* Typhimurium CFU/ml isolated in Brain Heart Infusion media (BHI) and spent media corresponding to the cycle at which *rsmC* expression crossed the threshold.

Since *rsmC* was shown to be a constitutive gene that had continuous levels of expression in *Salmonella* based on cell number, Real-Time PCR was then used to assess the *hilA* expression of *Salmonella* Typhimurium under different oxygen, pH levels, and osmolarity conditions. The results under low oxygen indicate that the combination of low osmolarity and high pH have the highest inducing effect on *hilA* expression (Figures 3.9 and 3.10). This is consistent with Bajaj and coworkers (1996), who showed that low osmolarity has an increased effect on *hilA* expression. Bajaj and coworkers (1996), also showed that pH 8 has an increased effect on *hilA* expression when compared to the *hilA* expression of those bacteria grown under pH 6 conditions. The *hilA* response under the same media conditions only under a high oxygen environment showed the same pattern of expression as those bacteria grown under a non-aerobic environment (Figures 3.11 and 3.12). The media with a pH of 8 and low osmolarity conditions had the greatest effect on the induction of *hilA* with none of the other media showing any significant effect. The relative expression of *hilA* did decrease for those bacteria grown under aerobic conditions versus those grown under non-aerobic conditions as shown in Figures 3.13, 3.14, and 3.15. As shown in Figure 3.15, the highest inducing condtions remains to be the low osmolarity, low oxygen, pH 8 treated sample. Growing *Salmonella* under low aerobic conditions versus aerobic conditions was found to increase *hilA* expression by Bajaj and coworkers (1996) as well.

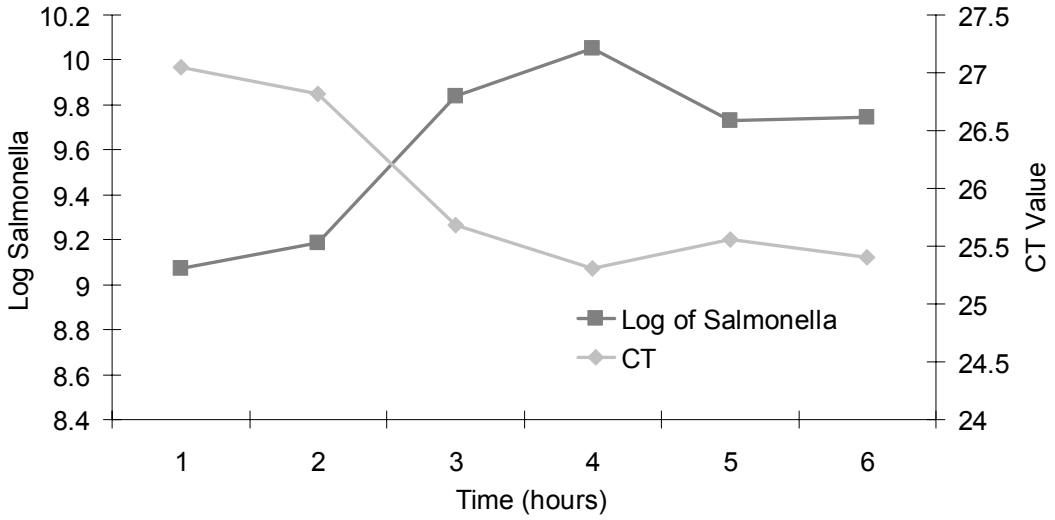

Figure 3.7. *Salmonella* Typhimurium C_T value versus log *Salmonella* in Brain Heart Infusion media (BHI).

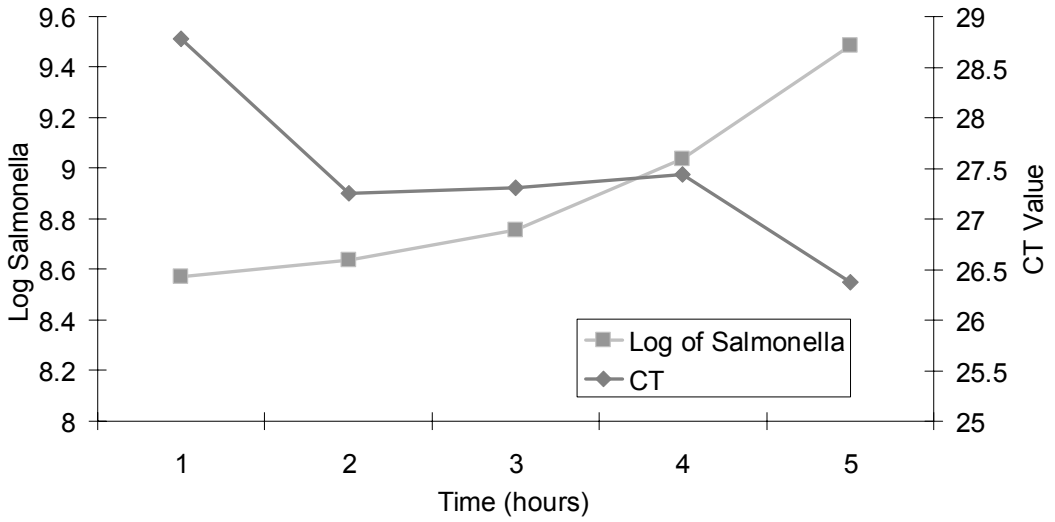

Figure 3.8. *Salmonella* Typhimurium C_T value versus log *Salmonella* in spent media.

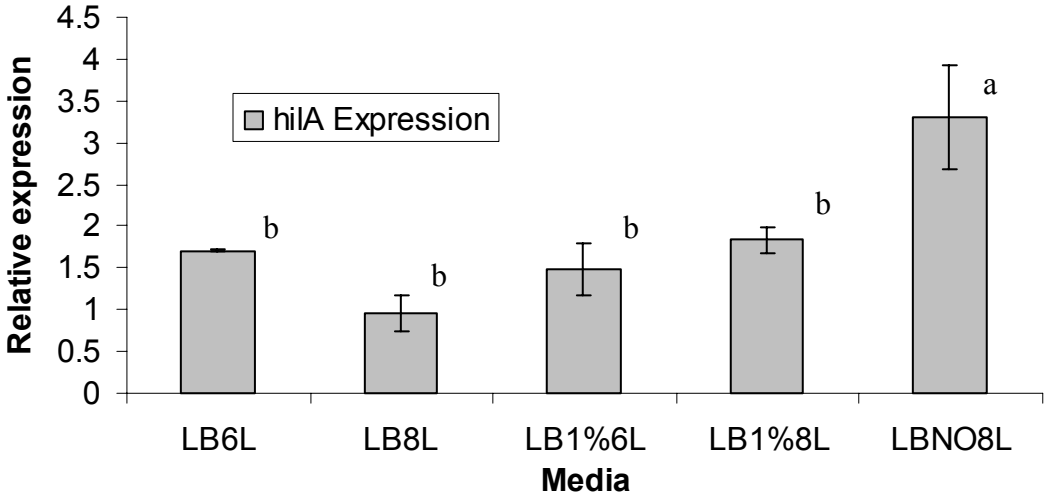

Figure 3.9. *Salmonella* Typhimurium *hilA* response to different media conditions under low oxygen (Trial 1). LB6L= Luria Bertani media, pH6, low oxygen; LB8L= Luria Bertani media, pH8, low oxygen; LB1%6L= Luria Bertani media with 1% NaCl, pH6, low oxygen; LB1%8L= Luria Bertani media with 1% NaCl, pH8, low oxygen; and LBNO8L= Luria Bertani media without NaCL, pH8, low oxygen. Values with different letter are significantly different ($p<0.05$).

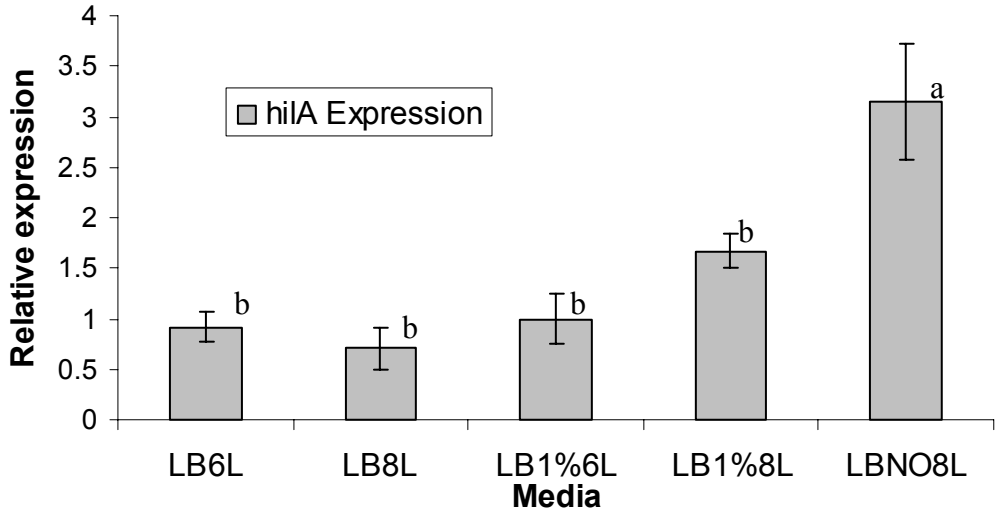

Figure 3.10. *Salmonella* Typhimurium *hilA* response to different media conditions under low oxygen (Trial 2). LB6L= Luria Bertani media, pH6, low oxygen; LB8L= Luria Bertani media, pH8, low oxygen; LB1%6L= Luria Bertani media with 1% NaCl, pH6, low oxygen; LB1%8L= Luria Bertani media with 1% NaCl, pH8, low oxygen; and LBNO8L= Luria Bertani media without NaCL, pH8, low oxygen. Values with different letter are significantly different ($p<0.05$).

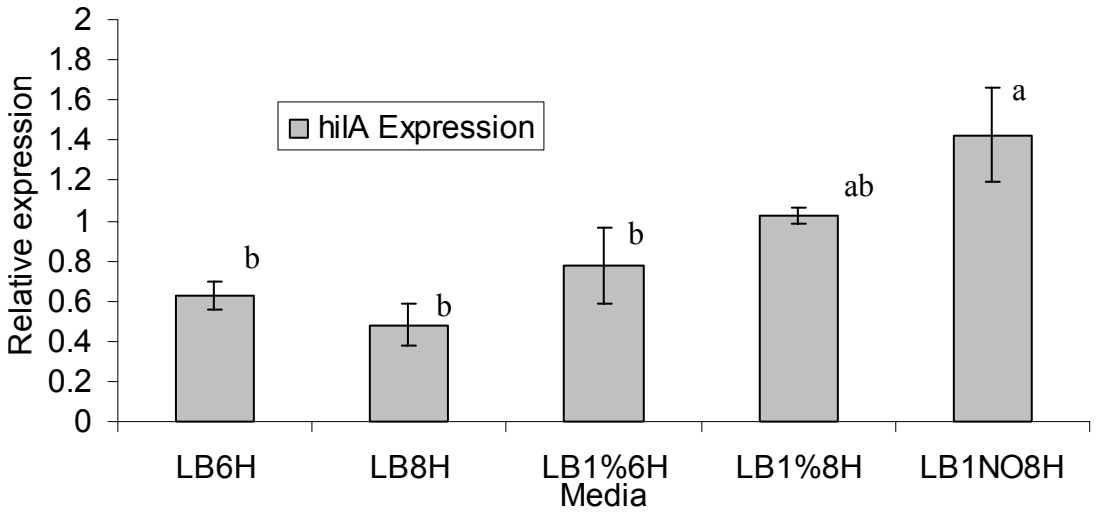

Figure 3.11. *Salmonella* Typhimurium *hilA* response to different media conditions under high oxygen (Trial 1). LB6H= Luria Bertani media, pH6, high oxygen; LB8H= Luria Bertani media, pH8, high oxygen; LB1%6H= Luria Bertani media with 1% NaCl, pH6, high oxygen; LB1%8H= Luria Bertani media with 1% NaCl, pH8, high oxygen; and LBNO8H= Luria Bertani media without NaCL, pH8, high oxygen. Values with different letter are significantly different ($p<0.05$).

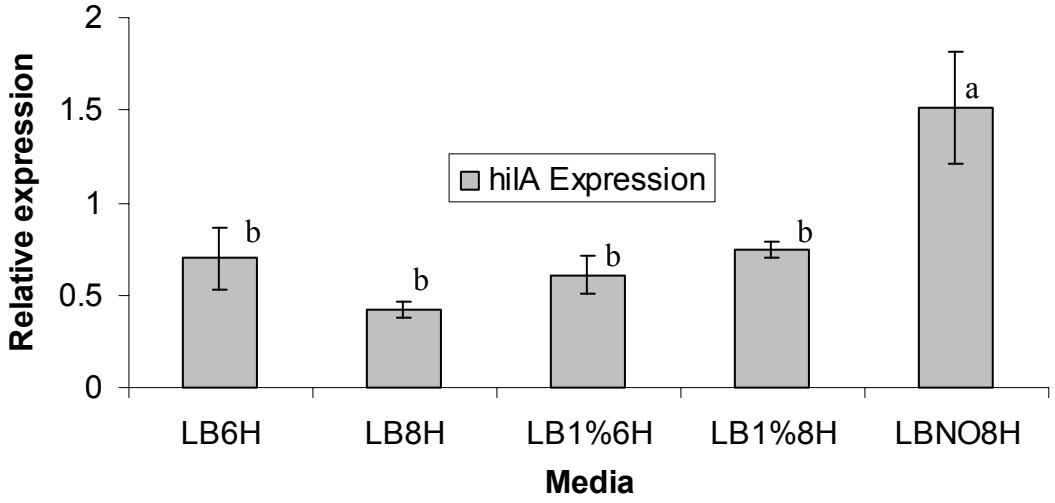

Figure 3.12 *Salmonella* Typhimurium *hilA* response to different media conditions under high oxygen (Trial 2). LB6H= Luria Bertani media, pH6, high oxygen; LB8H= Luria Bertani media, pH8, high oxygen; LB1%6H= Luria Bertani media with 1% NaCl, pH6, high oxygen; LB1%8H= Luria Bertani media with 1% NaCl, pH8, high oxygen; and LBNO8H= Luria Bertani media without NaCL, pH8, high oxygen. Values with different letter are significantly different ($p<0.05$).

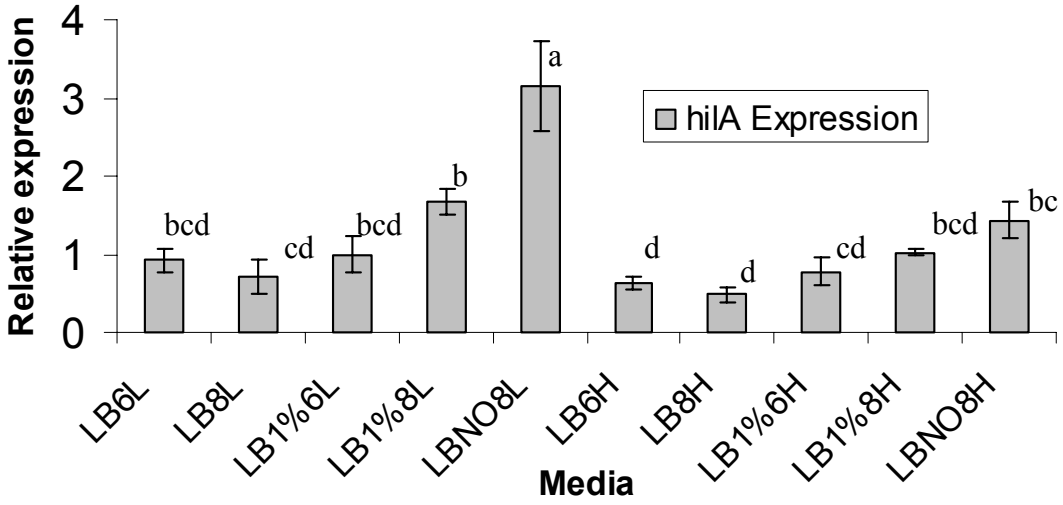

Figure 3.13. *Salmonella* Typhimurium *hilA* response to different media conditions under low and high oxygen (Trial 1). LB6L= Luria Bertani media, pH6, low oxygen; LB8L= Luria Bertani media, pH8, low oxygen; LB1%6L= Luria Bertani media with 1% NaCl, pH6, low oxygen; LB1%8L= Luria Bertani media with 1% NaCl, pH8, low oxygen; LBNO8L= Luria Bertani media without NaCL, pH8, low oxygen; LB6H= Luria Bertani media, pH6, high oxygen; LB8H= Luria Bertani media, pH8, high oxygen; LB1%6H= Luria Bertani media with 1% NaCl, pH6, high oxygen; LB1%8H= Luria Bertani media with 1% NaCl, pH8, high oxygen; and LBNO8H= Luria Bertani media without NaCL, pH8, high oxygen. Values with different letter are significantly different ($p<0.05$).

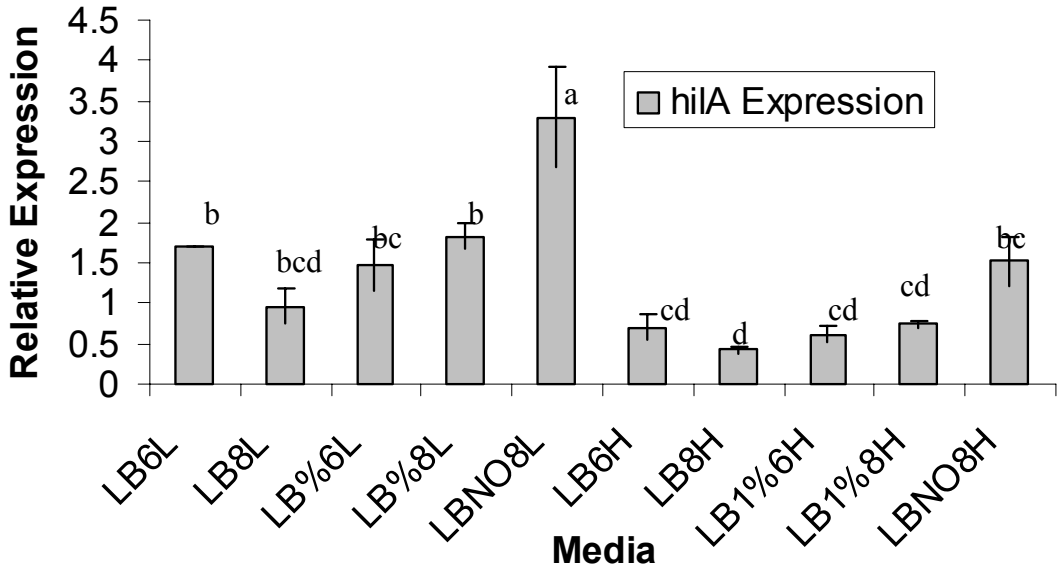

Figure 3.14. *Salmonella* Typhimurium *hilA* response to different media conditions under low and high oxygen (Trial 2). LB6L= Luria Bertani media, pH6, low oxygen; LB8L= Luria Bertani media, pH8, low oxygen; LB1%6L= Luria Bertani media with 1% NaCl, pH6, low oxygen; LB1%8L= Luria Bertani media with 1% NaCl, pH8, low oxygen; LBNO8L= Luria Bertani media without NaCL, pH8, low oxygen; LB6H= Luria Bertani media, pH6, high oxygen; LB8H= Luria Bertani media, pH8, high oxygen; LB1%6H= Luria Bertani media with 1% NaCl, pH6, high oxygen; LB1%8H= Luria Bertani media with 1% NaCl, pH8, high oxygen; and LBNO8H= Luria Bertani media without NaCL, pH8, high oxygen. Values with different letter are significantly different ($p<0.05$).

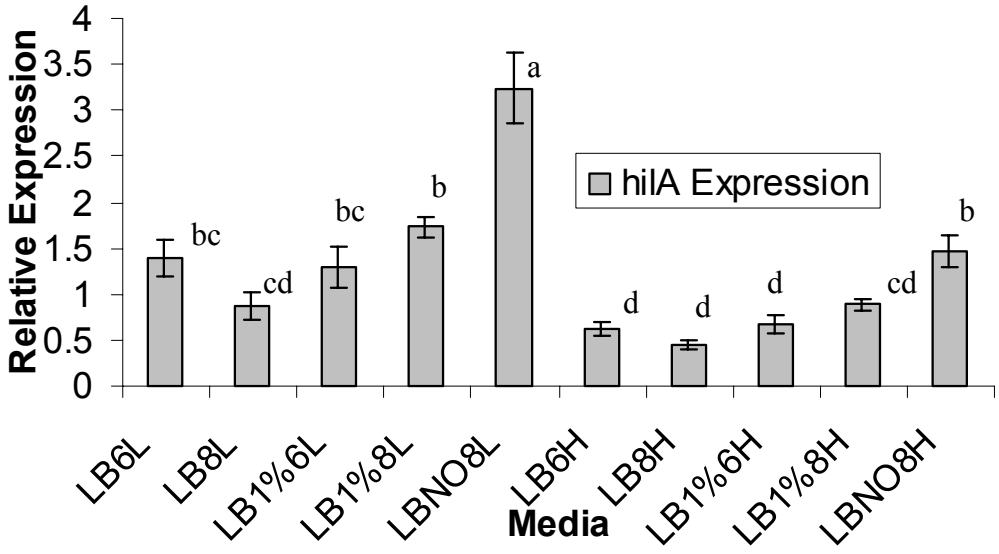

Figure 3.15. Overall *Salmonella* Typhimurium *hilA* response to different media conditions under low and high oxygen. LB6L= Luria Bertani media, pH6, low oxygen; LB8L= Luria Bertani media, pH8, low oxygen; LB1%6L= Luria Bertani media with 1% NaCl, pH6, low oxygen; LB1%8L= Luria Bertani media with 1% NaCl, pH8, low oxygen; LBNO8L= Luria Bertani media without NaCL, pH8, low oxygen; LB6H= Luria Bertani media, pH6, high oxygen; LB8H= Luria Bertani media, pH8, high oxygen; LB1%6H= Luria Bertani media with 1% NaCl, pH6, high oxygen; LB1%8H= Luria Bertani media with 1% NaCl, pH8, high oxygen; and LBNO8H= Luria Bertani media without NaCL, pH8, high oxygen. Values with different letter are significantly different ($p<0.05$).

CHAPTER IV

THE EFFECTS OF MAILLARD REACTION PRODUCTS ON *hilA* EXPRESSION IN *Salmonella* Typhimurium

Synopsis

Salmonella is an important human enteric pathogen responsible for causing severe gastroenteritis. *Salmonella* is believed to be the source between 2 and 4 million cases of food borne illness each year (CFSAN, 1996). It is believed to be the source of between 2 and 4 million cases of foodborne illness each year in the US. Bacterial virulence is regulated in response to a combination of environmental stimuli and involves a coordinated genetic response; therefore, it is important to understand the relevant features of the host that affect development of virulence *in vivo*. The Hyper Invasive Locus (*hilA*) gene is involved in the expression of virulence in *Salmonella* Typhimurium (ST). It is a transcriptional activator encoded on *Salmonella* pathogenicity island 1 (SPI1), which has been found to be a required gene component for the intestinal phase of infection. The Maillard reaction (MR) is a browning reaction common to the food and poultry industry found to take place during heating, processing, and storage of food products as well as processed poultry feeds. These Maillard reaction products (MRP) are one of the major contributors to the volatile content, which compile the aroma and taste normally associated with roasted poultry products and processed feeds. The objective of this research is

to study the effect of MRP on growth and virulence expression in ST. Maillard Reaction products were prepared by adding equimolar concentrations of D-glucose and L-amino acid (arginine, histidine, or lysine) in distilled water. These mixtures were then aliquoted into glass bottles and autoclaved at 121°C for three hours. These MRPs were then used to assess growth rates and *hilA* expression response of *hilA*: lacZY fusion strain. To quantitively detect ST EE658 *hilA* virulence gene response of the *Salmonella* fusion strain, a β-galactosidase enzyme assay was conducted in microtiter plates. MRP glucose derivatives of arginine, histidine, or lysine did not significantly affect the growth rates of ST at 0.5 or 1.0% v/v (0.36±0.00 to 0.44±0.01 h^{-1}). However, 1.0% histidine and arginine MRP derivatives increased expression of ST*hilA* (six fold and seven fold respectively) while lysine MRP complexes did not.

Introduction

The Maillard Reaction is a browning reaction that occurs frequently in such processes as bread and cereal production and is commonly found to take place during heating, processing, and storage of food products (Marounek et al., 1995). The Maillard Reaction is a non-enzymatic reaction that involves the free amino groups of the amino acids and the reducing sugar's carbonyl group to produce a Schiff base that is then transformed into the Amadori products (Jing and Kitts, 2002). Further reactions and rearrangements take place in order to achieve the final pigmented nitrogenous polymer products known as melanoidins (Martins et al., 2001). These Maillard Reaction products are used

to enhance the aroma, color, flavor, and nutritional value of the food products being processed (Kitts et al., 1993; Martins et al., 2001). The Maillard Reaction can occur in these food products such as in roasted meats and bakery products.

In general, Maillard reaction products (MRP) have also been linked to antioxidative, antibacterial, and cytotoxic effects (Jing and Kitts, 2000; Einarsson, 1987). The effect of an MRP depends on bacterial strain, concentration of the compound, as well as the type of amino acid, which was used to create the MRP (Einarsson et al., 1983). Einarsson et al. (1988) observed a decrease in the usage of serine, glucose, and oxygen in the presence of MRPs by the bacterial cells. *Listeria monocytogenes* growth is inhibited by a product generated during the heat sterilization of Brain Heart Infusion media (BHI), and the greater the duration of treatment with the BHI by-product the greater the suppression of *Listeria* growth (Sheikh-Zeinoddin et al., 2000). Maillard Reaction products have also been shown to bind metal ions under certain dietary conditions and this activity can independently contribute to the inhibitory effect that MRPs have on microorganisms (O'Brien et al., 1997). In addition to this, antioxidant effects have been related to the changes in pigment color and have been shown to be affected by the production of melanoidins, the characteristics of the system, and the food preparation conditions (Manzocco et al., 2001).

Salmonella is believed to be the source of between 2 and 4 million cases of food borne illness each year (CFSAN, 1996). *Salmonella* is a gram-negative

rod shaped foodborne pathogen; it has been commonly associated with foods such as raw meat, poultry, eggs, and dairy products. The *hilA* (Hyper Invasive Locus) gene is a transcriptional activator encoded on *Salmonella* Pathogenicity Island 1 (Bajaj et al., 1995). The *hilA* gene is also thought to be a requirement for *Salmonella* invasion due to its transcriptional properties. The *hilA* gene encodes for a protein that was believed to be between 531 to 553 amino acids in length (Bajaj et al., 1995). It was later proven by Rodriguez and co-workers (2002) that the *hilA* gene encodes for a protein that is 553 amino acids (63kDa) in length.

The Maillard Reaction may have some effect on the virulence of *Salmonella* bacterial population in contaminated food products. It is very important to understand the characteristics of *Salmonella* as well as what can trigger virulent invasiveness in order to make the food supply safer for public consumption. The purpose of this study was to determine the effects of MRPs on the growth and virulence of *Salmonella* Typhimurium using expression of *hilA* has an indicator.

Materials and Methods

Bacterial Strain

Salmonella Typhimurium strain EE658 carrying a *hilA: lac ZY* (St. hilA; Bajaj et al., 1996), was used to determine the growth rate and *hilA* expression

via the Miller Assay (1972). The St. *hilA* strain was maintained on BHI agar plates (DIFCO) containing X–gal (50 mg/mL) and blue colonies were selected each time. For purposes of *hilA* expression measurements, using Reverse Transcription Polymerase Chain Reaction, the wild type *Salmonella* Typhimurium strain ATCC 14028 was used.

Media and Growth Conditions

Maillard Reaction products were prepared by adding equimolar concentrations of D-glucose and L-amino acid (arginine, histidine, or lysine) in distilled water. These mixtures were then aliquoted into glass bottles and autoclaved at 121°C for three hours, as described by Li and Ricke (2002). Glucose minimal medium M9 (Sambrook et al., 1989) was used for the determination of *hilA* expression and growth rates. For the higher concentrations of MRPs in the M9 media only the liquid portion of each resulting MRP was used in the assay. Lysine, arginine, and histidine MRPs were added to M9 in order to create final concentrations of 0.5% and 1.0% (v/v).

Inoculation

A 24-hour blue St. *hilA* colony was collected from a BHI plate, and was used to inoculate 6ml of BHI media. The culture was grown for 18 hours at 37˚C in a shaking water bath with gentle agitation (Lab-line, Melrose Park, Il., U.S.A.). After incubation a 0.25ml aliquot of the culture was added to 6ml of

M9 broth media, and the culture was allowed to grow to an absorbency of 0.5 at 600nm using a Spectronic 20 spectrophotometer (Milton Roy 20 D, Rockford, Il., U.S.A.). Culture suspension was then used to inoculate the different MRP amended media used in the growth rate and β-galactosidase assay. For the RT-PCR experiments, frozen aliquots of all three strains were used to inoculate the 6mL of BHI and then the same inoculation procedure was followed.

Growth Rate

Glucose minimal mineral medium M9 supplemented with different MRP products at various concentrations was used to determine growth rates. Different media were inoculated with St. *hilA*, and 200µl aliquots were then transferred to microtiter plates. The M9-MRP amended media were prepared at 1, 3, and 5% final concentrations (v/v) of each amino acid MRP. The plates were then incubated at 37˚C with moderate shaking (180-200 rpm). The growth rate was determined by reading absorbance at 600nm every half hour for a period of six hours. Growth rates were determined by the slope of the natural log of optical density versus time (Ricke and Schaefer, 1991).

Expression of hilA *via β-galactosidase Assay*

Aliquots of 200ul of the desired MRP supplemented M9 media were placed into a 96-well microtiter plate with v-shaped wells (Nalge Nunc International, Rochester, NY, U.S.A.). The assay was initiated by adding 20µl

of the inoculum to the appropriate microtiter wells (Nutt et al., 2003). The microtiter plate was allowed to incubate for 2 hours at 37ºC with gentle shaking using a microtiter incubator (Boekel Industries, Philadelphia, PA, U.S.A.) at level 3. Microtiter plates were centrifuged at 4000rpm at 4˚C and the supernatant was discarded (Eppendorf model 5804R, Westbury, NY, U.S.A.). β-galactosidase activity was determined according to Miller (1972), using a Tecan Spectrophotometer (Tecan U.S., Inc., Research Triangle Park, N.C., U.S.A.) as described by Srivastava et al., (1997). Miller unit (MU) values to determine the amount of expression were calculated using the following formula: 100 X [Optical Density $(OD)_{420}$ – (1.75 x OD_{550})] / (T x V x OD_{600}); where T is equal to the amount of time it takes to observe the reaction, and V is equal to the volume of cell suspension used in the assay in ml amounts. For the purpose of this assay, 0.1ml was used as a constant for V. Optical densities for MUs were taken using the Tecan spectrophotometer at 420 and 550nm.

RNA Extraction

After the MRP amended media were inoculated with the bacterial suspension following the inoculation phase, the samples were placed in a shaking water bath at 37˚C for the desired duration. At these specific time points, 400ul of the bacterial suspension were removed and placed in a microcentrifuge tube with 800µl of RNAprotect (Qiagen, Valencia, CA). Samples were stored at -20˚C overnight prior to RNA extraction. RNA was

extracted from *Salmonella* Typhimirium cultures according to the RNeasy Mini Kit from Qiagen.

Primer Design

Primers were designed using sequences obtained from the GenBank website for the *hilA* and *rsmC* gene sequences, and then primers were designed using Primer Express 1.0 Software from Perkin-Elmer Appplied Biosystems (Foster City, CA). Sequences were then blasted on the NCBI website in order to determine if they were compatible with any other species of bacteria. Primers used in this study are listed in Table 4.1.

Reverse Transcription Reactions

Reverse Transcription Reactions were performed using the reagents from the QIAGEN® OneStep RT-PCR Kit. Each reaction contained 5X QIAGEN OneStep RT-PCR Buffer, 400 µM of each dNTP Mix , 5U/reaction RNase Inhibitor, 2.0µl of QIAGEN OneStep RT-PCR Enzyme Mix, 200 ng of RNA, 6.0µl of each Primer (0.6µM), and RNase-free water to a final volume of 20 µl. A positive RT reaction was run in order to ensure that the procedure was working properly. The positive reaction contained all of the same components except that in place of a RNA sample template a DNA sample was used as the template. In order to determine if RNA samples were contaminated with DNA, two (-) RT reactions were run on each RNA sample. One (-) RT reaction

contained the same components as the (+) RT reactions, except it lacked the RNA sample template and contained more water to ensure that the final concentration of the remaining components remained the same. The other (-) RT reaction did not contain the RT enzyme to ensure that there was no DNA contamination. All one step RT reactions were performed on a Gene Amp PCR System from Perkin Elmer (Wellesley, MA) under the following conditions: reverse transcription at 30 minutes at 50˚C, initial PCR activation at 15 minutes at 95˚C, three step cycling 1 minute at 94˚C 4, 1 minute at 53˚C and 1 minute at 72˚C for 40 cycles, followed by a final extension period of 10 minutes at 72˚C.The samples were then held at 4˚C until the samples could be removed. Three RT reactions were performed, one for each RNA sample. Samples were then run on 1.5% agarose gel in 1X TAE buffer for 30 minutes at 100V. The predicted RT-PCR products of Salmonella Typhimurium genes *rsmC* and *hilA* are shown in Figure 4.1.

Real-time RT-PCR Reactions

Real-time PCR reactions were performed on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). Each 20 µl SYBR Green PCR reaction contained 2µl RNA, 0.2µl (5µM) of each primer, 10µl 1X SYBR® Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA), 0.1µl MultiScribe Reverse Transcriptase (Perkin-

Table 4.1. Primer sequences used in RT-PCR reactions to determine the effects of Maillard Reaction Products on *Salmonella* Typhimurium.

hilA FWD	**5' tatcgcagtatgcgccctt 3'**
hilA REV	**3' tcgtaatggtcaccggcag 5'**
rsmC FWD	**5' tgcgcgaccaggctaaa3 '**
rsmC REV	**3' ttcaccactggcaggtattaagc 5'**

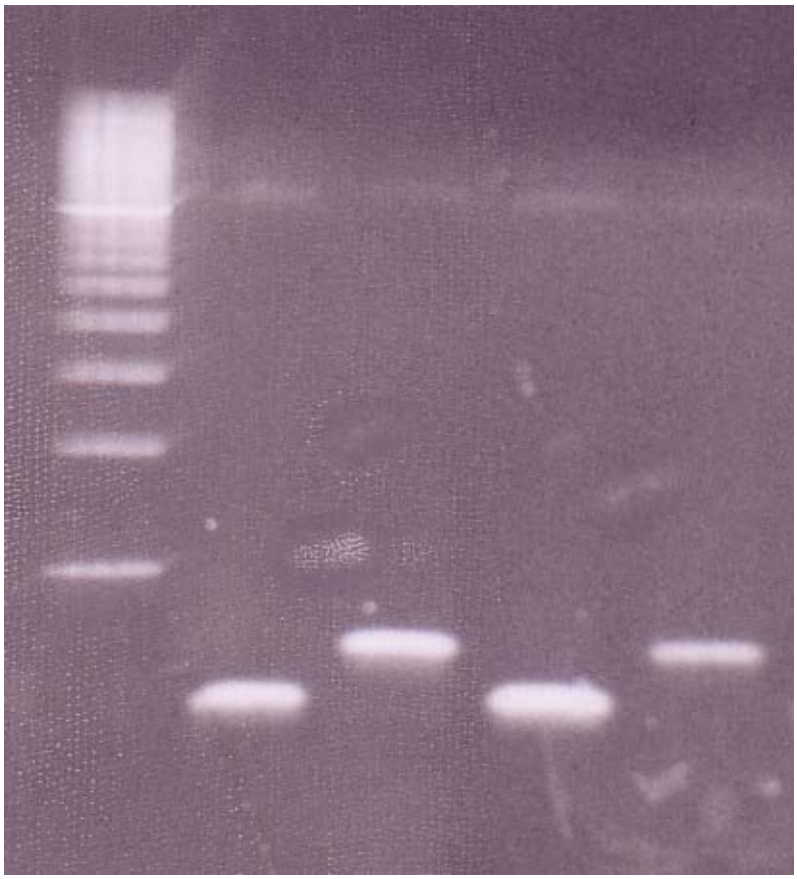

Figure 4.1. Detection of RT-PCR products with OneStep RT-PCR to determine the effects of Maillard Reaction Products on *Salmonella* Typhimurium. Lanes (1)100bp Ladder, (2) Pre-Treatment sample at an OD600 of 0.5 for *hilA* gene; (3) Pre-Treatment sample at an OD_{600nm} of 0.5 for *rsmC* gene; (4) Sample treated for 15 minutes in Spent Media for the *hilA* gene; (5) Sample treated for 15 minutes in Spent Media for the *rsmC* gene**.** Samples were run on 1.5% agarose gel in 1X TAE buffer for 30 minutes at 100V.

Elmer Applied Biosystems), 0.4µl RNase Inhibitor (Perkin-Elmer Applied Biosystems), and water. Thermal cycling conditions were as follows: 48˚C for 30 minutes, 95˚C for 10 minutes, and 40 repeats of 95˚ C for 15 seconds and 60˚C for 1 minute. A dissociation curve was run following the real-time reaction to determine if the primers used in each reaction generated a specific product. The same (+) and (-) RT reactions were run for the Real Time PCR as for Reverse Transcription reactions.

Data Analysis

Real-time PCR reactions were run to analyze the expression of two genes, each gene was analyzed in replicate three times for each sample tested. Data were analyzed using the C_T value for each sample, or the cycle at which each sample's amplification curve crosses a specified threshold. First, the C_T of the replicated samples was averaged. The average C_T of the three samples was then averaged for the three *Salmonella* strains. Finally, this C_T value was averaged for the three real-time PCR runs. The fold induction of each target gene of the three strains was then determined using the Comparative C_T ($\Delta\Delta C_T$) method. The Δ C_T value of the target gene (*hilA*) was determined by normalizing to the endogenous control gene (*rsmC).* Dissociation curves were also run to determine gene melting temperatures as shown in Figure 4.2. Amplification curves were also run to determine the cycle at which the threshold was overcome and corresponds to the C_T value as shown in Figure 4.3.

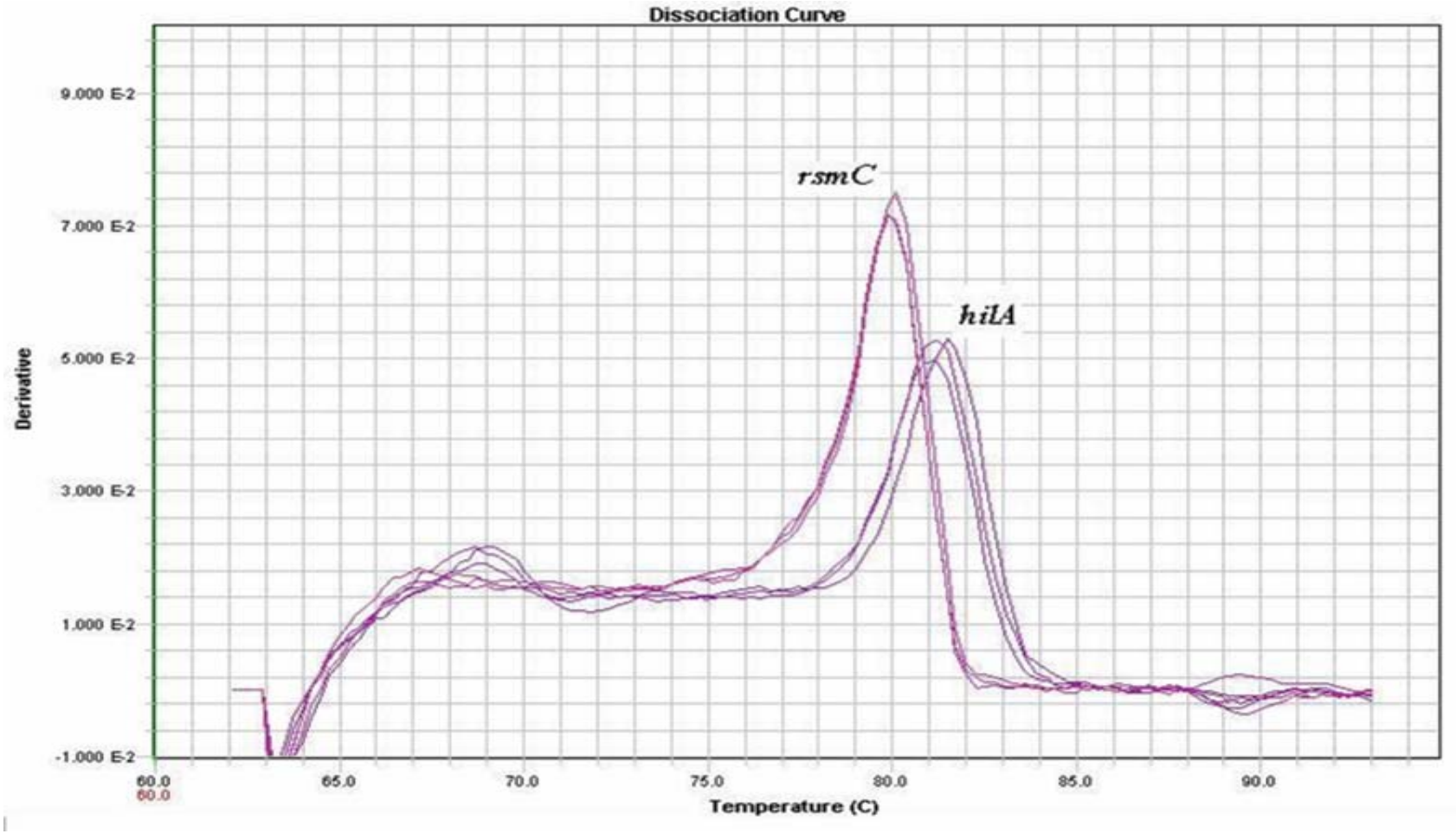

Figure 4.2. Dissociation curve of RT-PCR products showing melting temperatures (Tm) of *hilA* and *rsmC* to determine the effects of Maillard Reaction Products on *Salmonella* Typhimurium. The pink and purple lines correspond to the replicates for the *rsmC* and *hilA* genes, respectively.

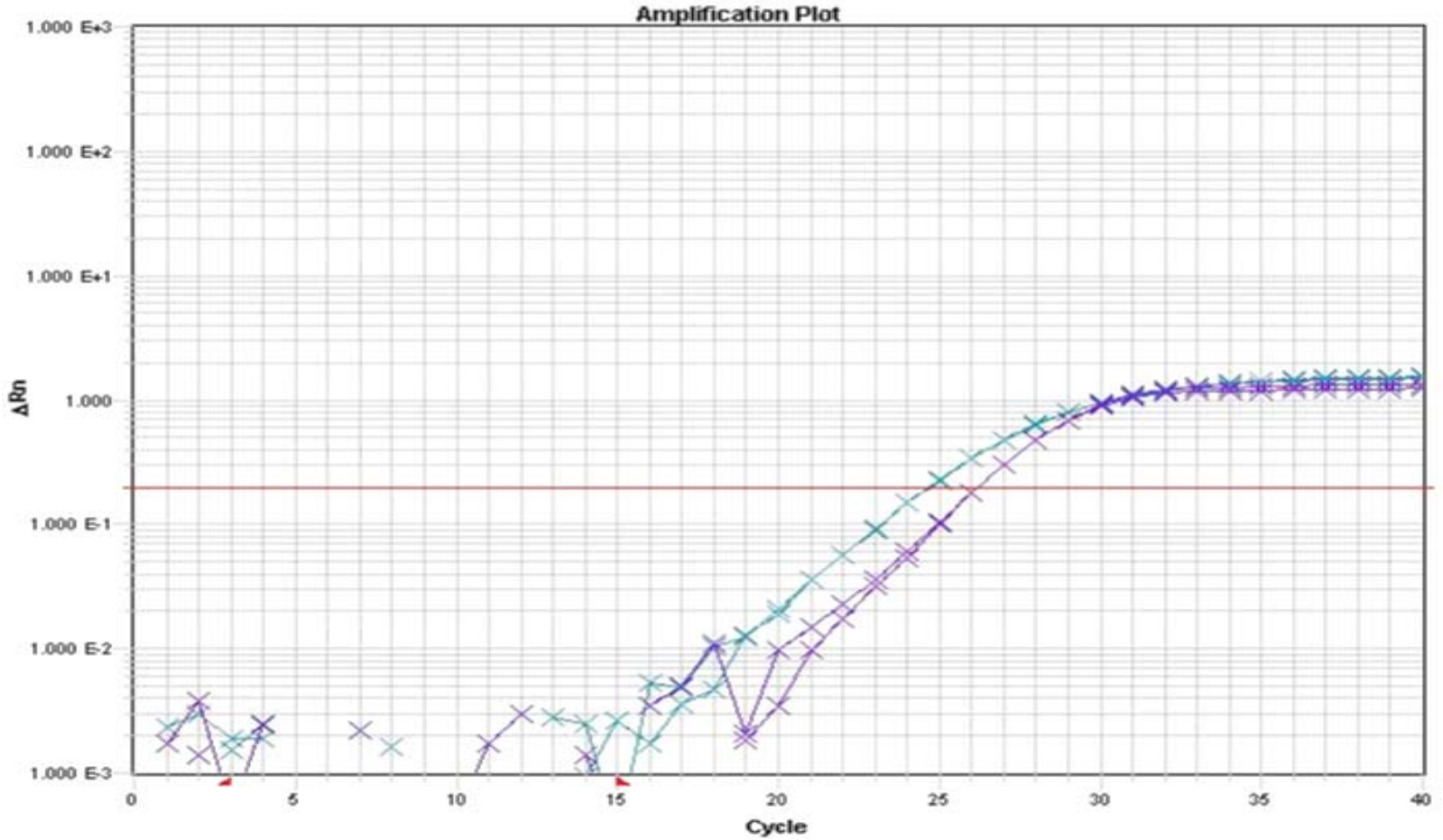

Figure 4.3. Amplification curve of RT-PCR products showing the cycle at which the threshold was overcome (C_T) to determine the effects of Maillard Reaction Products on *Salmonella* Typhimurium. The purple and teal lines indicate treatment groups of Minimal M9 media and M9 with 1.0% Argnine-MRP, respectively.

Statistical Analysis

Miller unit means for relative induction and growth rates were obtained for two separate trials, with 176 wells for 0.5% and 440 wells for 1.0%, and analyzed by the least square means separation of the GLM procedure in the SAS Statistical Analysis Software (SAS Institute, Cary, NC). Significance determination for all statistical analyses was calculated at $P<0.05$. Significantly different means were further separated using the Tukey test.

Results and Discussion

The addition of MRP components to media did not significantly alter growth rates of St. *hilA*, but at the 1.0% level, there was a numerical decrease in growth rate (Table 4.2). In contrast, the addition of MRPs at the concentrations of 0.5% and 1.0% increased the induction of *hilA* in ST, as indicated by the increase in β -galactosidease activity (Table 4.3). In particular, the addition of arginine and histidine based MRP compounds caused a significant increase in β-galactosidease activity when added at both the 0.5% and 1.0% concentrations. M9 broth supplemented with 0.5% arginine MRP resulted in a MU average of 308.5 while M9 supplemented with 0.5% histidine MRP had a MU average of 327.1, significantly different ($P<0.05$) from that of the minimal M9 with no MRP supplementation, which had a MU average of 183.83. When the concentration of MRP was increased to 1.0% (v/v), the arginine and

Table 4.2. Effect of Maillard Reaction Products (MRP) on growth rate of *Salmonella* Typhimurium.

Treatment	**0.5%**	**1.0%**
Control (no MRP)	0.44 ± 0.00	0.36 ± 0.00
Lysine MRP	0.43 ± 0.01	0.40 ± 0.02
Arginine MRP	0.43 ± 0.00	0.39 ± 0.01
Histidine MRP	0.44 ± 0.01	0.37 ± 0.02

No significant differences in growth rate were observed. ($P < 0.05$).

Table 4.3. Effect of Maillard Reaction Products (MRP) on the induction of *hilA* in *Salmonella* Typhimurium via the β-galactosidase assay.

Treatment	**0.5% MRP**	**1.0% MRP**
Control (no MRP)	183.8 ± 25.3 ^{a,x}	183.7 ± 21.7 ^{c,x}
Lysine MRP	184.0 ± 18.5 ^{a,x}	186.3 ± 107.5 ^{c,x}
Arginine MRP	308.5 ± 32.7 ^{a,y}	1254.0 ± 100.8 ^{a,x}
Histidine MRP	327.1 ± 16.6 ^{a,y}	1054.1 ± 158.2 ^{b,x}

a-c =The statistical groupings within concentrations of MRP ($P < 0.05$).

x-y= The statistical groupings within MRP additive treatments at differing concentrations ($P < 0.05$).

histidine MRP treatments again showed significantly higher MU averages than that of the minimal M9 alone (Table 4.3). In addition to the difference from M9 broth, there was also a significant difference between arginine and histidine MRP treatments ($P<0.05$). In the 1% concentrations, the media to which arginine MRP treatment was added had a MU average of 1254.0 while the media to which histidine MRP treatment was added had an average of 1054.0 (Table 4.2). The results of the effect of MRPs on Salmonella Typhimurium was also determine through the use of Real Time RT-PCR (Figures 4.4, 4.5, 4.6, and 4.7). By using this technique, induction of MRP treatments was observed to be the highest when using the arginine MRP treatment, followed by the histidine MRP treatment, and finally by the lysine MRP treatment. Using RT-PCR, differences between the lysine MRP treatment and controls are seen where they were not seen by just using the β-galactosidase assay. At 0.5% MRP additives to M9 media arginine MRP treatment has the highest induction, testing significantly different from that of lysine and histidine MRP treatments which tested significantly different from the controls as shown in Figures 4.4 and 4.5. This significance was not seen when using the β-galactosidase assay indicating that Real Time RT-PCR is more sensitive. At 1.0% MRP additives to M9 media arginine MRP treatment has the highest induction, testing significantly different from that of lysine and histidine MRP treatments which tested significantly different from the controls as shown in Figures 4.6 and 4.7. This coincides with the results seen while using the β-galactosidase assay for

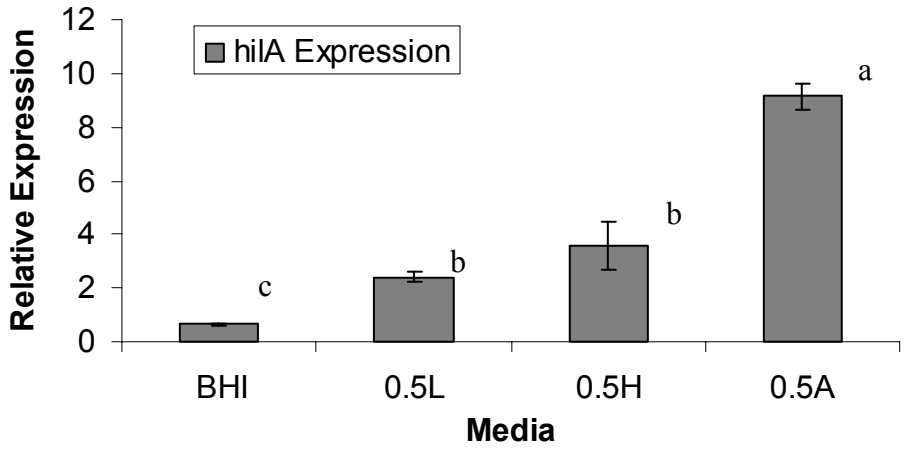

Figure 4.4. *Salmonella* Typhimurium *hilA* response to different media conditions at 0.5% Maillard Reaction Products in M9 (Trial 1). BHI= Brain Heart Infusion Media; 0.5L= 0.5% lysine MRP added to M9 media; 0.5H= 0.5% histidine MRP added to M9 media; and 0.5A= 0.5% arginine MRP added to M9 media. Values with different letter are significantly different ($p<0.05$).

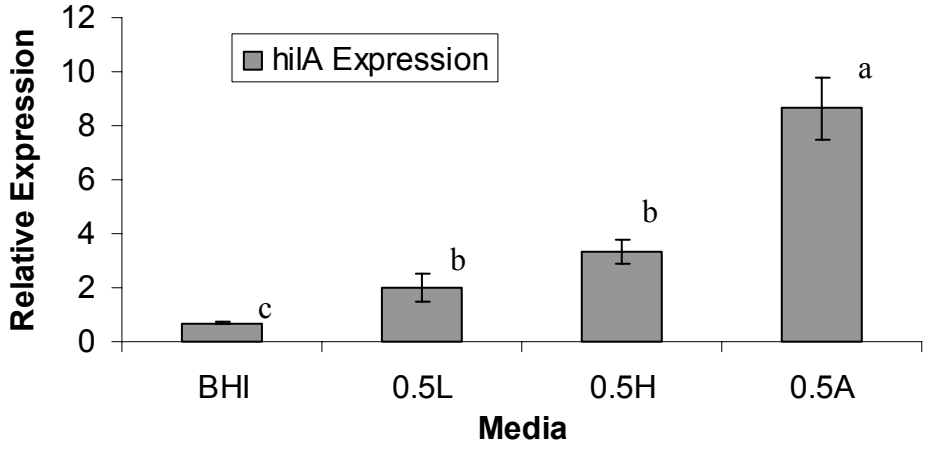

Figure 4.5. *Salmonella* Typhimurium *hilA* response to different media conditions at 0.5% Maillard Reaction Products in M9 (Trial 2). BHI= Brain Heart Infusion Media; 0.5L= 0.5% lysine MRP added to M9 media; 0.5H= 0.5% histidine MRP added to M9 media; and 0.5A= 0.5% arginine MRP added to M9 media. Values with different letter are significantly different ($p<0.05$).

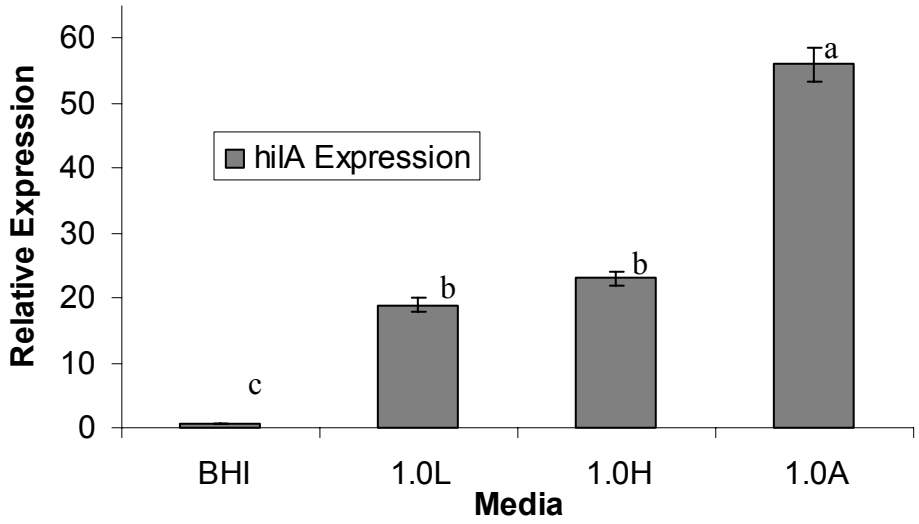

Figure 4.6. *Salmonella* Typhimurium *hilA* response to different media conditions at 1.0% Maillard Reaction Products in M9 (Trial 1). BHI= Brain Heart Infusion Media; 1.0L= 0.5% lysine MRP added to M9 media; 1.0H= 0.5% histidine MRP added to M9 media; and 1.0A= 0.5% arginine MRP added to M9 media. Values with different letter are significantly different ($p<0.05$).

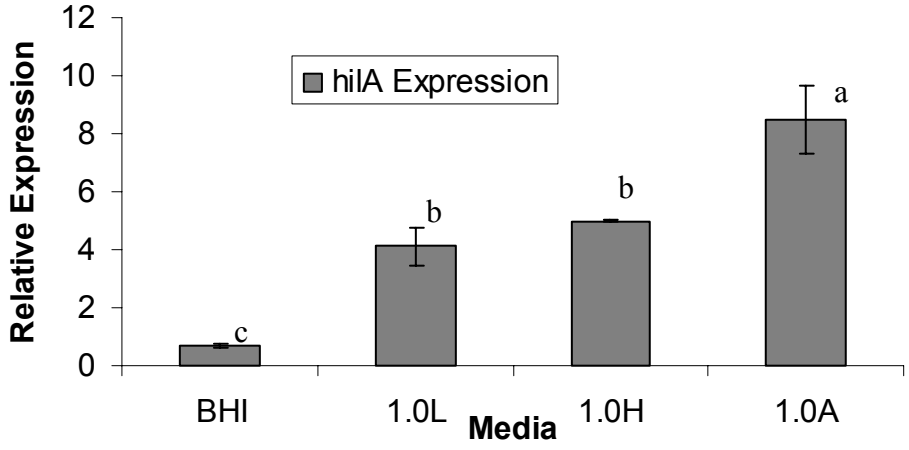

Figure 4.7. *Salmonella* Typhimurium *hilA* response to different media conditions at 1.0% Maillard Reaction Products in M9 (Trial 2). BHI= Brain Heart Infusion Media; 1.0L= 0.5% lysine MRP added to M9 media; 1.0H= 0.5% histidine MRP added to M9 media; and 1.0A= 0.5% arginine MRP added to M9 media. Values with different letter are significantly different ($p<0.05$).

arginine and histidine MRP treatments, however, the lysine MRP treatment did not test significantly different from the control whereas under the use of Real Time RT-PCR the lysine MRP treatment tests significantly different from the control and the same as the histidine MRP treatment.

The addition of MRPs was shown to have no significant effect on the growth rate of St.hilA in comparison to the M9 media. This is in accordance with the findings of Einarsson et al. (1983), who observed that the lag phase may be prolonged by MRP exposure, although the maximum growth rate and final bacterial concentration did not appear to be greatly affected in their study of foodborne pathogens. Einarsson et al. (1987) also determined that arginine and histidine Maillard Reaction compounds showed the highest inhibitory effect. This relates well to our finding that arginine and histidine had the greatest effect on St.hilA expression resulting in higher MU values when the bacteria were exposed to media with these additives. In this case, if arginine and histidine have been shown to have the greatest effect on *Salmonella* it could be speculated that this inhibition is causing the *Salmonella* to become more virulent to survive in its new environment, thus increasing the MU values. The MU values have been shown to be associated with the level of virulence expression the bacterial cells are emitting (Nutt et al., 2002). In this study by Nutt and coworkers (2002), the *hilA* gene was used to assess the virulence response of *Salmonella* Typhimurium.

If this scenario were applied to a food product situation, it could be speculated that the more arginine and histidine MRPs in a product which is exposed to *Salmonella* after processing could potentially create the right environment which may lead to the *Salmonella* to become more virulent, thereby resulting in a larger epidemic with more severe cases of salmonellosis occurring. Food processors may wish to look into methods of food preparation that do not produce MRPs from these specific amino acids.

CHAPTER IV

CONCLUSIONS

The development of a Real-Time Reverse Transcriptase Polymerase Chain Reaction for the determination of the relative expression of *hilA* in *Salmonella* Typhimurium was presented. The effect of Maillard Reaction Products on the *hilA* expression of *Salmonella* Typhimurium was also presented. Maillard Reaction Products are the byproduct of an important reaction for the development of flavors and colors in the food industry. The consequences caused by these byproducts is an important concern for the general public. The effects of these products on the virulence of bacteria is important when considering the prevalence of foodborne illnesses in today's society.

The objectives of this study were to 1) use a *Salmonella* Typhimurium *hilA:lacZY* fusion strain to assess virulence gene expression when exposed to Maillard Reaction Products using the β-galactosidase assay 2)develop reverse transcription and Real-Time reverse transcriptase polymerase chain reactions to detect *hilA* expression in *Salmonella* Typhimurium 3) use a *Salmonella* Typhimurium wild type strain to assess *hilA* gene expression when exposed to Maillard Reaction Products using RT-PCR to compare gene expression result to the β-galactosidase.

The presence of MRP compounds in liquid media caused no negative effect on the growth rate of *Salmonella* cells. However, the addition of MRP compounds at a 1% level in the media caused a significant increase in *hilA* expression in *Salmonella* Typhimurium, and the highest induction levels were observed in media supplemented with arginine and histidine-MRP compounds. There was no effect on the induction of *hilA* with the 0.5% addition of the MRP compounds in the amended media as seen by the Miller Assay however there was an effect seen when using the Real Time RT-PCR assay that resulted in the same levels of significance seen at 1.0% additions of MRPs being seen at the 0.5% level as well.

Since *rsmC* was shown to be a constitutive gene that had continuous levels of expression in *Salmonella* based on cell number Real-Time PCR was then used to assess the *hilA* expression of *Salmonella* Typhimurium under different oxygen, pH levels, and osmolarity conditions. The results under low oxygen indicate that the combination of low osmolarity and high pH have the highest inducing effect on *hilA* expression. The *hilA* response under the same media conditions only under a high oxygen environment showed the same pattern of expression as those bacteria grown under a non-aerobic environment. The media with a pH of 8 and low osmolarity conditions had the greatest effect on the induction of *hilA* with none of the other media showing any significant effect. The relative expression of *hilA* did decrease for those bacteria

grown under aerobic conditions versus those grown under low oxygen conditions.

Through the development of a Real-Time Reverse Transcriptase Polymerase Chain Reaction, the gene expression of *Salmonella* Typhimurium can be determined under a wide variety of environmental and laboratory conditions thus enabling a faster and more efficient turnover of results from this assay.

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SYMPOSIUM PRESENTATIONS

1) **M.M. Kundinger,** I.B. Zabala-Díaz, and S.C. Ricke. 2002. Effects of Maillard Reaction Products on *Salmonella* Typhimurium *hil A*. UW-RF Food Microbiology Symposium & Workshop - Current Concepts in Foodborne Pathogens and Rapid and Automated Methods in Food Microbiology, River Falls, Wisconsin.
2) **M.M. Kundinger,** R.W. Moore, I.B. Zabala-Díaz, J.A. Byrd, D.J. Nisbet, and S.C. Ricke. 2003. Effect of Spent Media Collected from a PREEMPT™ Continuous Flow Culture on *Salmonella* Typhimurium Virulence Expression. International Poultry Scientific Forum, Atlanta, Georgia.
3) **M.M. Kundinger**, I.B. Zabala Díaz, R. W. Moore, and S.C. Ricke. 2003. *Salmonella* Typhimurium Growth and Virulence Response to Maillard Reaction Products. Poultry Science Association Annual Meeting, Madison, Wisconsin.
4) Z. R. Howard, R. W. Moore, I. B. Zabala-Diaz, K. L. Medvedev, **M. M. Kundinger**, S. G. Birkhold, S. C. Ricke, L. F. Kubena, J. A. Byrd, and D. J. Nisbet. 2003. Effect of storage time on the growth of *Salmonella* Enteritidis in egg components. Poultry Science Association Annual Meeting, Madison, Wisconsin.

PROFESSIONAL/ ACADEMIC HONORS

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