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# A Comparison of Salmonella enterica Serovars: Are Prevalence, Virulence and Responses to Environmental Conditions Serovar or Strain Dependent?

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To the Graduate Council:

I am submitting herewith a thesis written by Nan Zhang entitled "A Comparison of Salmonella enterica Serovars: Are Prevalence, Virulence and Responses to Environmental Conditions Serovar or Strain Dependent?." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

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# A Comparison of *Salmonella enterica* Serovars: Are Prevalence, Virulence and Responses to Environmental Conditions Serovar or Strain Dependent?

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# Abstract

*Salmonella* are considered the leading cause of foodborne illnesses with frequent outbreaks in United States. While more than 2500 serovars have been identified, *Salmonella* Typhimurium and *Salmonella* Enteritidis are primarily responsible for most human infections while *Salmonella* Kentucky and *Salmonella* Heidelburg are currently the most prevalent serovars associated with poultry contamination. The relationship between virulence and prevalence is not fully understood, but may be a result of environmental stress exposure such as acid stress. In this research, organic acid (acetate) and inorganic acid (hydrogen chloride) were applied to 15 different serovars isolated from human infections or poultry. The growth curves and pH changes were graphed over time. In addition, the virulence gene *hilA* which regulates the pathogenesis of *Salmonella* were evaluated using real-time PCR at four different time points, 0, 2, 4, and 24 hours after *Salmonella* was exposed to two different acids individually. We found: 1) growth of *Salmonella* and the changes of pH were inversely related; 2) the greatest changes of *hilA* gene expression occurred between 0 and 2 hours; 3) the expression of *hilA* was serovar and strain dependent; and 4) hydrogen chloride had a greater impact on *hilA* gene expression than acetate did. Our study gives a better understanding of the *hilA* pathway used by *Salmonella*. At the same time, the effect of environmental stimuli on each serovar was revealed. Differences between serovars were unveiled by comparing the responses to acids as well.

# Table of Contents

iv

# List of Tables

## List of Figures

**Chapter I**

**Literature Review**

**Introduction**

*General Characteristics*

Named after an American scientist Daniel E. Salmon, *Salmonella* was discovered in the late 1800s. Since its discovery, *Salmonella* was identified as one of the major pathogens resulting in numerous foodborne diseases prevalent in a global range. The increasing food safety problems necessitate exploring the pathogens' characteristics.

*Salmonellae* are rod-shaped, Gram-negative, non-spore-forming foodborne pathogens with a 0.7-1.5 micrometer diameter and 2-5 micrometer length. *Salmonellae* are predominantly motile enterobacteria possessing 6-8 peritrichous flagella (Aldridge and Hughes, 2002). The flagella are directly associated with the pathogen invasion process. It was found that flagella increased pathogenesis by promoting the invasiveness to the host cells (Jones *et al.*, 1992). However, overexpression of flagella exerted an attenuation effect on the pathogenesis of *Salmonellae* (Schmitt *et al.*, 2001; Yang *et al.*, 2012).

*Salmonellae* have a very broad host range from cold-blooded animals to warm-blooded animals. However, poultry products are the most frequent vehicles for transmission of the *Salmonellae*, dominating other foods of animal origin as potential sources of infection (Bryan and Doyle, 1995; D'Aoust, 1997). Recently, even products with low water activity, like peanut butter and pepper, were found to be contaminated with *Salmonella* (Maki, 2009).

In general, *Salmonella* is an enteric pathogen that causes gastroenteritis and typhoid fever. However, the severity of infection varies depending on the host species, ranging from severe disease to asymptomatic carriage (Morgan, 2004). It has also been clearly demonstrated that different serovars of *Salmonella* induce different diseases with different symptoms (Coburn, 2007).

Two species, *Salmonella bongor*i and *Salmonella enterica*, divide the genera based on a genetic linear relationship (Darwin and Miller, 1999; Schechter and Lee, 2000; Selander *et al.*, 1997). However, serotype is needed to further differentiate host specification and statistical analytics to understand foodborne diseases. *Salmonella* is facultative anaerobes. Central metabolic pathways can be shifted along with oxygen ratio changes. By possessing mechanisms by which to adjust to the environments, *Salmonellae* can survive and replicate throughout their whole life cycle within both aerobic and anaerobic environments (Encheva *et al.*, 2009).

*Salmonella spp.* has been reported to cause illnesses over the past 100 years. Among the 9.4 million foodborne illnesses annually in the U.S., salmonellosis contributes approximately 11%, or about 1.02 million human cases (Scallan *et al.*, 2011). Of these cases, 35% result in hospitalization. In addition, it is estimated that for every one laboratory confirmed case, 29 or more milder cases are not reported to Centers for Disease Control and Prevention (CDC) meaning that more than 30 million persons are likely affected by Salmonellosis annually (Nyachuba, 2010).

Unlike other foodborne diseases *Salmonella* outbreaks have not declined (Maki, 2009). With three, nine, eleven and twelve investigations were completed in the years of

2009, 2010, 2011, 2012, respectively by the CDC. Many kinds of contaminated food have caused *Salmonella* infections, including mangoes, cantaloupe, live poultry, and ground beef (Hanning et al., 2009). In 2012, one of the larger outbreaks involved contaminated raw ground tuna products. Between January and July 2012, *Salmonella* serotype Bareilly and Nchanga were detected from 425 people in 28 states and the District of Columbia. A total of 55 patients were hospitalized but no deaths were reported (CDC, 2012).

*Serovars*

    *Salmonella* are subdivided into thousands of serotypes that may be food product dependent. In general, *Salmonella* Typhimurium, Enteritidis and Newport are the most common serotypes existing in food products and are responsible for 50% of Salmonellosis (Porwollik, 2004). The first difference between these two species is pathogen island 2 (SPI-2), which *S. enteric*a has and *S. bongori* does not. (Capra and Laub, 2012). In addition, *S. bongori* is rarely isolated from clinical specimens while *S. enterica* accounts for most human illness.

    *S. enterica* is further divided into six subspecies, including enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV), and indica (VI) (Fierer and Guiney, 2001; Brenner, 2000). While *S. enterica* subspecies II, IIIa, IIIb, IV, VI, and VII are mainly associated with cold-blooded vertebrates, members of *S. enterica* subspecies I are frequently isolated from avian and mammalian hosts which are also the leading cause of *Salmonella* infections (Popoff and Minor, 1992).

3

The Kauffman-White scheme based on the O (somatic) and the H (flagellar) surface antigens characterize over 2500 serovars of *S. enterica* (Brenner et al., 1998). *S. enterica* serovars are genetically nearly identical in both nucleotide sequences and amino acid sequences. It was demonstrated that divergence in the nucleotide sequence of orthologous genes ranges from 3.8 to 4.6% while amino acid sequences difference range from 0.7 and 1.3% (Selander *et al.*, 1994). Common ancestors of various serotypes would have been present about 25 to 40 million years ago (Selander *et al.*, 1994). However non-genetic differences between serovars exist as well. Each serovar has their specific range of host and their compatibility with a host is very different. Some common hosts are listed in table 1 with their most commonly isolated serotypes (Miller, 1995; Joy, 1990; Rice, 1997; Buxton, 1977; Pardon, 1988; Singh, 1971; Carter, 1974).

Traditionally, *S. enterica* serotype Typhimurium has been thought of as the prototypical broad-host-range serotype, since it is frequently associated with disease in numerous species as table 1 shows (Rabsch *et al*., 2002). However, serotypes with narrow adaption to the host tend to have more virulence and higher mortality rates (Bäumler et al., 1998). While *Salmonella* serovars have a preference for hosts, the disease manifestation also depends on both host health and the infectious serovar.

In humans, typhoid is caused by *S. enterica* Serovar Typhi, Paratyphi and Sendai (Coburn, 2007). Most understanding about typhoid comes from mouse models infected with *Salmonella* Typhimurium. The primary treatment for typhoid is fluoroquinolones, although nalidixic acid and other antimicrobial agents are also used (Parry *et al*., 2002). Typhimurium*,* Enteriditis and most serovars cause enterocolitis/diarrhea. In animal models, after *S. enterica* infects the host, it typically colonizes in the gastrointestinal

tract. Then the pathogen is taken up to the apical epithelium and induces an invasion-associated virulence apparatus. Inflammation and acute onset occur between 6 and 72h after consumption. Diarrhea and vomiting are common symptoms. If the patients do not get any medical treatment, symptoms usually last for 5-7 days and may be resolved by themselves (Coburn et al., 2007). However, some cases can be more serious requiring hospitalization and antibiotic treatment. Several serovars including Choleraesuis and Dublin are more commonly associated with bacteremia in humans (Fierer and Guiney, 2001)

The prevalence of *Salmonella* outbreaks is serovar dependent as well. According to the report from the CDC in 2009, in humans, only three of the thousands of *Salmonella* serovars are responsible for approximately 45% of *Salmonella* infections, which are Typhimurium (15%), Enteritidis (17.5%), and Newport (9.3%). The 12 most prevalent *Salmonella* serovars cause more than 70% of all human *Salmonella* infections (http://www.cdc.gov/ncezid/dfwed/PDFs/SalmonellaAnnualSummaryTables2009.pdf). Therefore, only a few of the thousands of serovars frequently cause infection in humans and domestic animals (Porwollik *et al*., 2004).

### *Salmonella* Virulence

*Genes and Pathways*

**General Information**

The process of *Salmonella* invasion of the host challenges *Salmonella* because passage through the gastrointestinal tract (GIT) presents hostile environments. Therefore *Salmonella* needs to regulate its genes to survive passage through the GIT and traverse

the intestinal mucous layer in order to gain access to the underlying epithelium, which are the main target cells for *Salmonella*. In addition, it is required for *Salmonella* to synthesize some specific proteins and factors to protect themselves against the stressful environments, including the acidic environment in the stomach, the anaerobic environment in the GIT, innate immune responses, antimicrobial defenses, and competition with other bacteria present in GIT for nutrition, space, and other resources.

As far as we know, the complete process of invasion occurs mainly using two specific pathogen islands: *Salmonella* Pathogen Island I (SPI-1), and II (SPI-2). Two distinct virulence-associated Type III Secretion Systems (T3SS) within SPI-1 and SPI-2 are essential to *Salmonella* pathogenesis and colonization. The T3SS helps transfer bacterial virulence proteins from the bacterial cell into host-cell cytoplasm (Haraga *et al*. 2008). Some two-component regulatory systems also show an important global regulation of *Salmonella* virulence, including PhoPQ (Soncini *et al.*, 1996). Some factors encoded by SPI-1, including HilA and InvF, act as transcriptional regulators to regulate genes of both SPI-1 and SPI-2. Other mechanisms are also discussed next to provide some new ideas such as concerns about gene regulation and vaccine production.

**SPI-1, SPI-2 and Type-III Secretion System**

Generally speaking, SPI-1 is mainly responsible for *Salmonella* invasion while SPI-2 accounts for systemic infections and intracellular accumulation. Both SPI-1 and SPI-2 contain a large number of genes encoding type III secretion systems (T3SSs) to translocate virulence proteins or effectors into eukaryotic target cells. This T3SSs system is a complex system involving over 25 proteins, some of which assemble into a

macroscopic complex (Ginocchio *et al.*, 1994; Hueck, 1998; Kubori *et al.*, 1998). Although both of the T3SSs play an important role in pathogenesis, experiments conducted on chicken models confirm that SPI-2 has a dominant effect on systemic infection and gastrointestinal colonization and functions of T3SSs seem to be location dependent (Jones *et al.*, 2007). T3SSs are not unique to *Salmonella*. Actually, they are found in a large number of Gram-negative pathogens of various hosts, including humans animals and plants (Hueck, 1998).

SPI-1 is 40kb in size and located on the 63$^{rd}$ centisome of *Salmonella*'s chromosomes. More than 25 invasion genes cluster on SPI-1 (Galan, 1996). However, not all genes on SPI-1 are related to the invasion process. It was demonstrated that an iron uptake system is encoded by genes on SPI-1 (Zhou *et al.*, 1999). A total of 44 open reading frames (ORFs) were identified on a 25kb size of SPI-2. It is similar between SPI-1 and SPI-2 that only portions of the genes are responsible for systemic infections. T3SSs are structurally and functionally related to the flagella assembly systems, which help *Salmonella* secrete substrate proteins. These substrate proteins might be required to allow the translocation of further substrate proteins or effector proteins interacting with host cells (Hansen-Wester and Hensel, 2001).

Expression of genes located on SPI-1 and SPI-2 are environmental factor dependent, and different environmental factors induce SPIs. Conditions with high osmolarity and oxygen limitation induce SPI-1 gene expression (Bajaj *et al.*, 1995; 1996; Jones *et al.*, 1994; Galan *et al.*, 1990). The local transcriptional activator HilA has a major regulation potential on SPI-1 gene expression. It was found that SPI-2 genes could be induced by Mg2+ deprivation and phosphate starvation, which indicates that the

global regulatory system PhoPQ has an induction effect on SPI-2 genes (Deiwick *et al.*, 1999).

SPI-1 and SPI-2 mutants were constructed by mutations in the genes *spaS* and *ssaU*, which encode major structural components of the SPI-1 and SPI-2 TTSSs, respectively （Jones et al., 1998, 2001; Wood et al., 2000; Wigley et al., 2002). It was found that a vaccine made from a SPI-1 mutant proved to be more effective in protecting poultry from *Salmonella* infection compared to a SPI-2 mutant. However vaccination with the SPI-2 mutant stimulated a slightly higher antibody production (M. Matulova 2012).

**Pho-PQ**

It is common for bacteria to respond to environmental stimuli, such as nitrogen limitation, phosphate limitation, sugar transport, by using two-component regulators, (Miller *et al.*, 1989; Ronson *et al.*, 1987).  As a two-component regulator, the PhoPQ system not only regulates acidic environment tolerance, but also has a great influence on virulence of *Salmonella* as a global regulation factor.

The PhoPQ system has two components: PhoP and PhoQ. PhoQ is a histine kinase capable of phosphorylation of PhoP to form the activated PhoPQ system (Capra *et al.*, 2012). It has been shown that the PhoPQ system is able to sense the concentration of both $Mg^{2+}$ and $H^+$ as environmental stimuli. However, $Mg^{2+}$ transportation was not essential for acid tolerance. The PhoPQ system is used primarily against inorganic acid stress versus organic acid stress in which case the RpoS system is used (Bearson *et al.*,

1998).

It has been demonstrated that strains with *phoP* or *phoQ* mutations are avirulent and have reduced survival rates in cultured mouse macrophages. However, these mutants are still capable of colonizing the mouse intestinal tract and confer substantial protective immunity (Miller *et al.*, 1989).

The induction of *phoPQ* by low pH also has important implications in terms of virulence (Bearson *et al.*, 1998). Although acid can damage cells by intracellular accumulation of $H^+$ and restricted growth, it can activate the PhoPQ system which induces pathogenesis in advance. This would be important as *Salmonella* would be exposed to both inorganic acid in the stomach (HCl) and organic acids (acetic acid) in the intestine.

### *hilA* Gene

HilA is a DNA binding protein of the OmpR/ToxR family encoded by the gene *hilA* on SPI-1. Based on the *hilA* Open Reading Frame (ORF), the HilA amino acid sequence was predicted to be a protein containing 531 or 553 amino acids with a molecular weight of 60400 or 62986 Da with an initial methionine codon. As a transcriptional activator, HilA is required for the expression of genes encoding proteins secreted by SPI-1 and essential for *Salmonella* entry into epithelial cells (Bajaj, 1995). Furthermore, HilA is a major regulator that co-ordinates with environmental factors to regulate gene pathways.

Three promoters located upstream of *prgH*, *orgA* and *invF* on SPI-1 control

invasion operon expression. HilA has the potential to bind all of these promoters and regulate invasion genes in a direct way (Bajaj, 1996). Therefore, the *hilA* gene plays a central regulatory role in *Salmonella* invasion and virulence.

A large number of effectors have been identified which exert either positive or negative regulation on *hilA* gene expression. Based on previous research, *hilC/sirC/sprA* (Eichelberg *et al.*, 1999; Rakeman *et al.*, 1999; Schechter *et al.*, 1999) *hilD* (Schechter *et al.*, 1999) *sirA/barA* (Altier *et al.*, 2000; Johnston *et al.*, 1996) *fis* (Wilson *et al.*, 2001) *csrAB* (Altier *et al.*, 2000) and *phoB*, *fadD*, and *fliZ* (Lucas *et al.*, 2000) have a positive modulation on *hilA* gene expression. Some of these factors, including FadD, FliZ, PhoB, and EnvZ/OmpR, activate *hilA* transcription by binding to the upstream regulatory sequence of *hilA* (Lucas *et al.*, 2001; Olekhnovich *et al.*, 2002; Schechter *et al.*, 2001). Some other factors may have an ability to repress *hilA*-negative regulators, like HilD. Negative regulators on *hilA* have also been found. The genes *ams*, *hilE*, *pag*, and *hha* were found to repress *hilA* expression and the *Salmonella* invasive phenotype (Fahlen *et al.*, 2000; Baxter *et al.*, 2003).

**DNA Methylation**

DNA methylation is a major regulation mechanism in epigenetics, a method of regulating gene expression without changing the DNA sequence. Methylation has little impact on the regulation of genetic pathways, however it does play an important role in determining gene expression. Most DNA methylation occurs in cytosine and adenine, which are two major bases in nucleotides. DNA cytosine methylase (Dcm) and DNA

adenine methylase (Dam) are two enzymes that account for DNA methylation (Handy et al., 2011).

Experiments were conducted to test the relationship between methylation and *Salmonella* pathogenesis. Dcm and Dam mutants together with a control wild type strain were applied to BALB/c mice and lethal doses required to kill 50% of the animals (LD50) were measured. It turned out that LD50 of the Dam- mutant in BALB/c mice was 10,000 times more than that of the wild type while no difference was shown between the Dcm⁻ and wild type. The authors concluded that it was methylation of adenine but not cytosine residues impacted *Salmonella* pathogenesis (Heithoff *et al.*, 1999). Furthermore, it was also demonstrated that Dam had a great impact on virulence genes expression, including *hilA, hilC, hilD* and *invF* (Lopez-Garrido *et al.*, 2009).

In addition, a Dam⁻ vaccine was developed based on the previous fact that Dam⁻ mutants had an attenuated virulence. The result showed that all the Dam⁻ immunized mice survived the challenge while all the non-immunized mice died after the challenge. Therefore, Dam⁻ live strains are potentially excellent targets for vaccine development against *Salmonella* (Heithoff *et al.*, 1999).

*Regulation Factors*

**General Information**

After entering the gastrointestinal tract (GIT) through oral consumption of contaminated foods, *Salmonella* confronts many challenging conditions. *Salmonella* is sensitive to environmental factors, including pH, oxygen availability, and osmolarity.

However, *Salmonella* is able to sense and respond to these physiological inputs by modulating genes expression and phenotypes. The transcription regulator responds to physical cues which can control a wide range of genes and pathways, including invasion genes and two component regulatory systems. However, different regulation mechanisms exist for each parameter.

The mechanism by which *Salmonella* adapts to environments is not fully known. Four hypotheses were suggested by Bajaj et al. (1996). First, there may be one single sensor responsible for processing environmental cues and transducing signals to a transcriptional regulator, which affect invasion genes. Second, *Salmonella* may have separate systems with many sensory pathways for each environmental condition. Third, a regulatory cascade in which one regulator controls invasion gene expression indirectly by controlling the expression of another regulator constituted the separate regulatory system. The last hypothesis described a system equipped with multiple sensors and one single transcriptional regulator of invasion genes.

**pH**

*Salmonella* are neutrophilic bacteria. However, *Salmonella* do encounter a variety of potentially lethal acid stress conditions (Foster *et al.*, 1995). Acid can be up-taken by *Salmonella*. In a severe acidic environment, the rate of uptake of $H^+$ is faster than that of removal of the $H^+$ leading to a broken homeostasis with acid accumulation in *Salmonella*. Intracellular acidification can damage and interrupt most of the biochemical processes and have a lethal effect on *Salmonella*. But even mild acidic environments caused by

organic acid have a great impact on *Salmonella* because the protonated forms of acid can dissociate in the cells and cause an intracellular pH drop.

Acid stress is not a simple phenomenon. It involves organic and inorganic acids or combinations of both. *Salmonella* have separate systems to overcome different kinds of acid stresses. *PhoPQ*, is typically used mostly against inorganic acid stress, while another global regulatory system termed the RpoS-dependent system, is utilized to defend against organic acid stresses (Bearson *et al.*, 1998).

In the *PhoPQ* system, PhoQ is a membrane-bound sensor kinase and is primarily responsive to mammalian phagosome environments. It was found that the PhoQ sensor domain can change its conformation in response to pH changes. Furthermore, it was found that acidic growth medium in millimolar concentrations of divalent cations had the potential to activate PhoQ directly (Prost *et al.*, 2007). After the activation of *PhoPQ*, a subset of acid shock proteins (ASPs) is induced to help *Salmonella* survive acidic environments (Bearson et al., 1998; Adams et al., 2001).

**Other Factors**

*Salmonella* is sensitive to various physical stimuli. The invasion potential of *Salmonella* changes along with conditions. Based on the previous studies, several environmental conditions are known to regulate invasion, including oxygen tension, osmolarity, growth states, and short chain fatty acids (Altier, 2004). Some environmental factors influence gene expression in an independent way, while others may overlap in the regulation of *Salmonella* genes ( Tartera and Metcalf, 1993).

Oxygen concentration has been shown to be a major regulator of invasion gene expression. Ernst (1990) reported the role of anaerobiosis as a possible controlling factor in bacterial invasion of HEp-2 epithelial cells. It was indicated that anaerobic growth of *S.* Typhimurium resulted in increased invasiveness (Ernst *et al.*, 1990). The mechanism of oxygen regulation was not known until interactions between oxygen and the *orgA* gene was found. Under the *hilA* gene control, the *orgA* gene, located on SPI1, encodes a protein that is involved in the formation of the Type III secretion apparatus. It was demonstrated that oxygen had a repressive effect on the *orgA* gene at the mRNA level during the logarithmic growth phase (Russell *et al.*, 2004). Under low oxygen conditions, SPI1 genes were maximally expressed through HilA regulation (Jones and Falkow, 1994; Bajaj *et al.*, 1996; Russell *et al.*, 2004).

Osmolarity is another probable signal for *Salmonella* invasion. It was demonstrated that high osmolarity, a condition known to increase DNA superhelicity, could induce higher expression levels of invasion genes, such as *invA* (Galan and Curtiss, 1990). The high osmolarity of the small intestine, greater than 300 mOsm, provided *Salmonella* with an inducible condition for its invasion (Fordtran and Ingelfinger, 1968).

In addition, growth state plays an important role in invasion potential. Previous studies measured the ability of *Salmonella* to adhere to and enter cultured mammalian cells. It was found that *Salmonella* attenuated their invasiveness in the stationary phase while invasiveness was varied during logarithmic and late logarithmic phases (Lee and Falkow, 1990).

Acetate is a short chain fatty acid commonly produced in the large intestine and condensed in the distal ileum, the primary site of *Salmonella* invasion, with a 15-30 mM concentration (Argenzio et al., 1974; Argenzio and Southworth, 1975). It was demonstrated that acetate at this concentration could induce invasion and the expression of SPI1 (Durant et al., 2000; Lawhon et al., 2002). The *sirA* gene was also involved in this process. However, some short chain fatty acids produce a contrary effect on invasion. Propionate and butyrate were found to repress SPI1 gene expression (Lawhon et al., 2002).

The investigations of *Salmonella* invasion responding to environmental stimuli suggest a substantial portion of regulatory capacity is directed to control invasion. More than 20 genes were found in the regulation system while most genes are located on SPI1. The mechanisms involved with gene interactions need to be further studied.

*Salmonella and Poultry*

**Introduction and Brief Overview**

The incidence of *Salmonella* infection and the number of related outbreaks in the United States has increased dramatically in poultry. It has been shown that poultry consumption has been increased to 60 pounds per capita per year, which is a 6.0 fold increase from 1910 in the United States (Buzby and Farah, 2006). Broilers are generally reared in large housing operations consisting of 6000 to 40,000 birds per housing unit. The majority of broilers are generally raised cage-free in barns on litter, with the stocking density ranging from 6.5 to 8.5 lb/ft$^2$ (0.27 to 0.36 kg/m$^2$) depending on the size of the birds (National Chicken Council, 2010). In 2012, over 9 billion broilers were hatched,

raised and processed and over 80.5 billion table eggs were produced in the United States according to a USDA report (U.S. Department of Agriculture, 1998).

A significant proportion of human *Salmonella* outbreaks have been traced to the consumption of contaminated eggs and egg products, which are a result of infected laying hens (Gast, 1994). Three serotypes, *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Newport, account for approximately 50% of the *Salmonella* outbreaks in the US and can be isolated from chicken ovaries and feces (Schoeni et al., 1995).

Transmission is an essential stage of a pathogen's life cycle. The mechanism by which *Salmonella* contaminates eggs was proposed as shell penetration and transovarian transmission. External contamination of the shell by *S.* Enteritidis occurs during the egg passage through the hen cloaca. The ability of *Salmonella* penetration is greatly influenced by temperature. Penetration potential of the 3 major serotypes of *Salmonella* were examined. It was revealed that all three strains existing in feces could penetrate the shell and could be isolated from egg contents at $25^{\circ}$C. However, when the temperature dropped to $4^{\circ}$C, *S.* Typhimurium could only penetrate into the membrane but not the contents, while *S.* Enteritidis and *S.* Heidelberg could not even penetrate the shells (Schoeni et al., 1995).

The hypothesis that *Salmonella* might be transmitted directly to the internal contents of the egg prior to the laying of the egg which is termed transovarian transmission was supported by the US Department of Agriculture (USDA). To confirm the transovarian transmission, pathogen-free birds were orally inoculated with *S.* Enteritidis for 3 weeks. From the experiments, 71% of sampled eggs were contaminated

which supported the possibility of direct contamination without shell penetration before egg laying (Forsythe *et al.*, 1967; Gast *et al.*, 1990).

The frequency of *Salmonella* present in egg content has been evaluated and was very low, 1 in 20,000 eggs on average (Gast and Beard, 1992; Ebel and Schlosser, 2000). After one day under $25^{\circ}$C storage, an increase of 3 to 5 logs in the numbers of *S.* Enteritidis, *S.* Typhimurium and *S.* Heidelberg occurred in both the yolk and albumen. The number of pathogens continued to increase as storage prolonged under room temperature. Low temperature tests evaluating survival and growth of *Salmonella* were designed. It was reported that growth of *S.* Enteritidis, and *S.* Typhimurium were not observed at $4^{\circ}$C or $8^{\circ}$C (Humphrey *et al.*, 1989; Humphrey 1990). However, *Salmonella* has been observe to grow between $5^{\circ}$C and $45^{\circ}$C (Doyle and Cliver, 1990). Therefore, early and sustained refrigeration of shell eggs was one of the key interventions for preventing contamination (Braden, 2006).

**Colonization Process**

Contaminated food or water is a major source of *Salmonella* infections. After consumption, the pathogen must survive the stressful gastrointestinal tract before arriving in the small intestine. By coordinating with environmental stimuli, gene regulatory systems of *Salmonella* facilitate this colonization and invasion processes.

After entering the small intestine, *Salmonella* traverse the intestinal mucous layer in order to get access to the underlying epithelium. *Salmonella* can invade the non-phagocytic enterocytes of the intestinal epithelium, which are targets of *Salmonella*

invasion. By using various fimbrial adhesins, adherence to the apical surface of the cells is achieved. Adherence of *Salmonella* results in the disruption of the epithelial brush border and ruffles of the membrane, by which organisms are engulfed into host cells. Microfold (M) cells are the preferred target of *Salmonella*. Symptoms such as inflammation and diarrhea may appear after invasion (Haraga *et al.*, 2008).

The farm environment can be a main foodborne reservoir (Guard-Petter, 2001). The physical conditions, and feed constitute major reservoirs in which *Salmonella* can survive for extended periods of time (Jarquin *et al.*, 2009). Serotypes are diverse existing around the farm, including various serotypes isolated from insects, rodents and wild birds. Chickens provide the possibility of efficient transmission of *Salmonella*. Infections can spread among chickens in flocks through direct contact with infected birds and with the contaminated environment (Jarquin *et al.*, 2009). Thus the poultry farm is a key place for *Salmonella* prevention.

**Chicken Lines**

Great improvement and intense genetic selections of specific biological traits, including body weight, growth rate and meat or egg-yield have continuously increased in the past of decades. Marketing age, which is the time a broiler reaches 2000g, is reduced by 1 day every generation per year (Havenstein *et al.*, 1994a, b). Therefore, while it took 52 days before the chickens achieved market weight 10 years ago, it only takes 42 days currently.

However, as the preferred physical properties improve, the fitness of the modern broiler chickens shows a serious reduction (Emmerson 1997, Julian 1998, Marks 1996). The health issues of the new line of chickens are various, including physiological disorders, such as obesity, ascites, sudden death syndrome and leg problems, and even some deficiencies in the immune system. The causes of these health issues were hypothesized as imbalances among body mass, internal organs, the vascular systems, and the skeletal system (Dunnington and Siegel, 1996; Katanbaf *et al.*, 1988).

Poultry and poultry products are major sources of human disease caused by *Salmonella* infections (Rabsch *et al.*, 2001). There is a need to identify *Salmonella*-resistant chickens and turkeys as economic losses due to *Salmonella* amount to approximately US $64 million to US $114 million annually to the poultry industry in the United States (Bryan and Doyle, 1995). Thus, research to identify *Salmonella* susceptibility between chicken lines is being conducted and effective methods are being sought to develop methods of preventing *Salmonella*.

Bumstead and Barrow (1993) identified an interesting mechanism of resistance existing in chicken inbred lines. They showed that the chicken lines which possessed the potential to resist *S.* Typhimurium, were resistant to the serovars Gallinarum, Pullorum and Enteritidis (Bumstead and Barrow, 1993). Studies in the chickens have also revealed that broilers have a marked difference with layers on *Salmonella* susceptibility. Four chicken lines, L2, B13, PA1(layer-type) and Y11 (broiler-type) were challenged by *S.* Enteritidis strain 1009 and colonies were numbered from ceca samples of four chicken lines. The results showed that L2 and B13 lines presented more numerous *S.* Enteritidis infected eggs and a higher level of infection that lasted longer than the PA1 and Y11 lines.

The Y11 chicken line showed the most resistance to *S*. Enteritidis cecal colonization (Duchet-Suchaux *et al.*, 1997). There is not only a significant difference between layers and broilers with respect to *Salmonella* susceptibility, but also between fast growing chickens and slow growing chickens.

A number of studies have demonstrated that the resistance to systemic salmonellosis in the chicken is regulated by multiple genes (Lamont, 1998; Kramaer *et al.*, 2003a; Malek and Lamont, 2003; Malek *et al.*, 2004; Swaggerty *et al.*, 2004). Gene expression response to *Salmonella* infection in the chicken intestine differs between fast growing chicken lines and slow growing chicken lines. The genes that are able to affect T-cell activation were activated when fast growing lines encountered *Salmonella* infection while macrophage activation occurred when slow growing lines met the same situation (Hemert *et al.*, 2006).

*Conclusions*

*Salmonella* contamination is a worldwide food safety issue. The complexity of the process of *Salmonella* leading to illnesses is well characterized. First, the differences exist in the 2500 serovars of *Salmonella* that have been identified and outbreaks of foodborne diseases may be caused by different serovars. Secondly, although poultry are primarily responsible for *Salmonella* infection, various food products are vehicles that contribute to *Salmonella* transmission. Third, the environmental stimuli have a great impact on regulation of *Salmonella* pathogenesis. Finally, *Salmonella* has an organized and sensitive genomic system, including two-component regulatory systems, which respond to different environments, including the environments in the gastrointestinal tract.

Poultry is a major transmission vehicle for *Salmonella*. Chickens provide pathogens an optimal environment to maintain and grow. Although a low rate of contamination was identified in eggs, the storage temperature plays an important role in preventing contamination. Furthermore, different chicken lines have different susceptibilities to *Salmonella*. Compared to layers, broilers show more resistance to *Salmonella*. More research is needed to identify chicken lines with resistance to *Salmonella* while maintaining healthy and hardy animals.

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# Appendix

Table 1. Examples of *Salmonella enterica* adaption to host specification

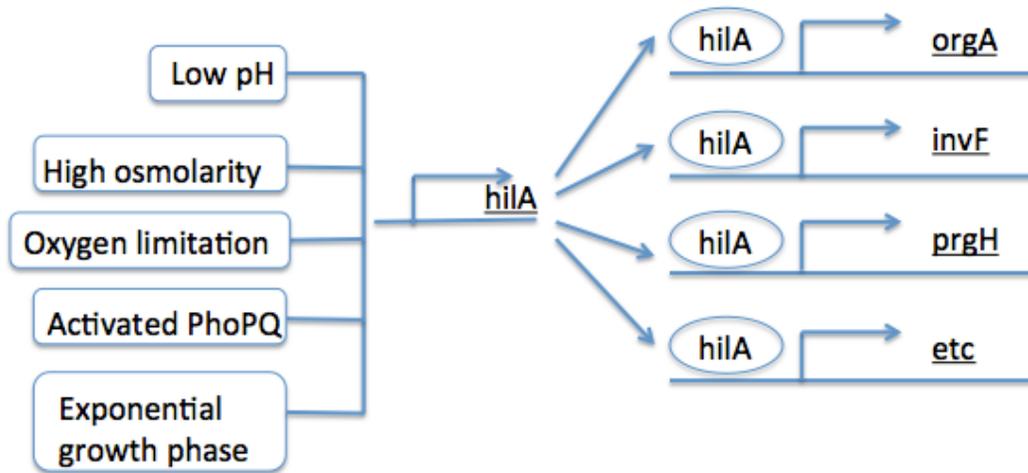| Host | Serovars |
|------|----------|
| Humans | Typhimurium, Enteritidis, Typhi,  Sendai, and Paratyphi A, B, and C |
| Cattle | Typhimurium, Dublin |
| Poultry | Pullorum, Gallinarum, Enteritidis, Typhimurium |
| Sheep | Abortusovis, Typhimurium |
| Pigs | Choleraesuis, Typhimurium, Typhisuis |
| Horses | Abortusequi, Typhimurium |
| Wild rodents | Typhimurium, Enteritidis |

Figure 1. Primary regulation of *hilA* in response to environmental stimuli. Genes *orgA*, *invF*, and *prgH* are located in SPI-1 and play an important role in invasion process.

# Chapter II

# Expression of *hilA* in Response to Mild Acid Stress in *Salmonella enterica* is Serovar

# and Strain Dependent

# Abstract

*Salmonella enterica* is the leading cause of foodborne illness with poultry and poultry products being primary sources of infection. The 2 most common *S. enterica* serovars associated with human infection are Typhimurium and Enteritidis. However, Kentucky and Heidelburg and the 2 most prevalent serovars isolated from poultry environments. Given the prevalence of other serovars in poultry products and environments, research is needed to understand virulence modulation in response to stress in serovars other than Typhimurium and Enteritidis. Thus, the objective of this research was to compare *hilA* gene expression (a master regulator of the virulence pathogenicity island) in response to acid stress among different strains and serovars of *Salmonella*. A total of 11 serovars consisting of 15 strains of *S. enterica* were utilized for these experiments. Cultures were suspended in tryptic soy broth (TSB) adjusted to pH 7.2, 6.2, or 5.5 with HCl or acetic acid. Total RNA was extracted from cultures at specific time points (0, 2, 4, and 24 h). Gene expression of *hilA* was measured with quantitative reverse transcriptase real time PCR (qRT-PCR). Growth and pH were measured throughout the 24 h time frame. Regulation of *hilA* in response to acid stress varied by serovar and strain and type of acid. The results of these experiments indicate that *hilA* regulation may have some impact on virulence and colonization of *S. enterica*. However, these results warrant further research to more fully understand the significance of *hilA* regulation in response to mild acid stress in *S. enterica*.

## Practical Applications

In the industry, some treatments or marinades used for poultry meat utilize a low pH that may also kill bacteria. However, if bacteria survive these treatments, they can endure further stress because genes to survive stresses are activated and may remain activated. In this research, we show that a virulence gene (*hilA*) is regulated in response to acid stress in *Salmonella.* If upregulation is sustained, this may have an effect on the virulence of *Salmonella* of the bacteria. Thus, we show that some acid based treatments could have an effect on the potential virulence of *S. enterica.*

**Introduction**

*Salmonella enterica* is responsible for approximately 1.028 million cases of foodborne illness in the United States annually (Scallan and others 2011). The majority of these infections have been sourced to poultry and poultry products (Braden 2006). The gastrointestinal tract of poultry can be colonized with *Salmonella* without causing harm or reducing growth and production parameters of the flock (Bryan and Doyle 1995; Limawongpranee and others 1999). Thus if intestinal contents are released during processing, contamination of the carcasses with *Salmonella* can occur (Byrd and others 2002). Consuming raw or undercooked poultry meat or mishandling of contaminated products may result in human illnesses (Tencate and Stafford 2001; Kimura and others 2004).

For the species *S. enterica*, approximately 2500 serovars have been described. Of these serovars, *S.* Typhimurium (ST), and *S.* Enteritidis (SE) are responsible for the majority of foodborne salmonellosis infections (CDC 2010). However, the most prevalent serovars isolated from poultry farm environments and poultry products are *S.* Kentucky and *S.* Heidelburg. The exact reason for the emergence of these 2 serovars is unknown; however, it has been suggested that targeting of ST and SE for control by the Natl. Poultry Improvement Program (NPIP) has resulted in the opening of a *Salmonella* niche that *S.* Kentucky and *S.* Heidelburg have begun to fill (Foley and others 2011).

Because SE and ST are the most prevalent *Salmonella* serovars associated with human illness, research concerning virulence of *Salmonella* has primarily utilized these 2 serovars. However, given the emergence of other serovars on poultry farms and in

poultry products, more research is needed to understand virulence regulation in serovars other than Typhiumurium and Enteritidis. Some *Salmonella* research has been conducted concerning acid stress and any correlation with virulence (Durant and others 2000; Lawhon and others 2002; Van Immerseel and others 2004, 2006; Gantois and others 2006) and it appears that virulence can be activated by pH through the expression of the master regulatory gene *hilA*. Measurement of the expression of the *hilA* gene has been proposed as an indicator of the potential virulence of *Salmonella* in the GI tract (Durant and others 2000; Nutt and others 2003; Bohez and others 2006; Park and others 2011). However, because most of this research has been conducted with SE and ST, little is known concerning the effects of these same environmental stimuli, including pH, on the expression of *hilA* in serovars other than SE and ST. Therefore, the purpose of these experiments was to evaluate any differences in expression of the virulence gene *hilA* in *S. enterica* serovars in response to acid stress.

**Materials and Methods**

*Bacteria and Culturing Conditions*

In these studies, a total of 11 serovars consisting of 15 strains of *S. enterica* were utilized (Table 2.1). All *Salmonella* strains were initially cultured on tryptic soy agar (TSA) and incubated at 37 °C for 24 h. After incubation, a loop of bacteria was inoculated into tryptic soy broth (TSB; pH 7.2) and incubated in a shaking water bath at 37 °C for 3 h. The cultures then were split into 2 equal aliquots and centrifuged at 8000 × $g$ for 5 min. The supernatant was discarded and the pellets were resuspended in TSB at pH 7.2 (control), 6.2, or 5.5. The pH of the TSB was adjusted with either HCl or acetic

acid (Sigma Aldrich, St. Louis, Mo., U.S.A.). The pellet was resuspended by vortexing and 1 mL aliquots of this preparation were placed into separate wells of a 12-well plate. The remaining suspensions were utilized to measure pH changes and to conduct growth curve measurements over the course of the experiment. The pH of the suspensions was measured using a pH meter (Denver Instruments, Bohemia, N.Y., U.S.A.) at specific time points (0, 2, 4, 6, 8, 12, 16, 20, and 24 h). For growth curves, triplicate 200 μL aliquots of the cell suspensions were placed into the wells of a 96 well flat bottom plate. The optical density of the suspensions was determined using a plate reader (ELX 800 Universal Plate Reader; Bio-Tek Instruments, Winooski, Vt., U.S.A.) every hour for a 24-h time period at 590 nm. At the conclusion of the 24 h period, viability of the cultures was evaluated by culturing aliquots of the cell suspension on tryptic soy agar (TSA).

*RNA Preparation*

At specific time points (0, 2, 4, and 24 h) an equal volume of RNA protect bacterial reagent (Qiagen, Valenica, Calif., U.S.A.) was added to the wells of the 12-well plate containing the *Salmonella* suspensions. The entire sample was collected into a 2-mL microfuge tube and allowed to stand at room temperature for 5 min. Subsequently, total RNA was extracted from the samples with the RNeasy mini kit (Qiagen) as directed by the manufacturer. After extraction, the RNA samples were subjected to a DNase treatment utilizing the Qiagen DNase kit (Qiagen) as directed by the manufacturer. Prior to use in the Real-Time PCR assay, all samples were quantified using spectrophotometry (Nanodrop ND-1000, ThermoScientific, Pittsburgh, Pa., U.S.A.).

*Quantitative Reverse Transcriptase Real Time PCR (qRT-PCR)*

All qRT-PCR reactions were performed using the ABI 7100 (Applied Biosystems, Carlsbad, Calif., U.S.A.). Primer sets for the *hilA* analysis were previously published by Weir and others (2008). For each reaction, a 20-μL total volume consisted of 10 μL of EXPRESS SYBR Green ERTM qPCR SuperMix with Premixed ROX (Invitrogen, Carlsbad, Calif., U.S.A.), 0.5 μL of EXPRESS SuperScript Mix for One-Step SYBR Green ER (Invitrogen), 500 nM of each primer, 100 ng of RNA template, and water to volume. All primers were synthesized by Integrated DNA technologies (Coralville, Iowa, U.S.A.). The qRT-PCR reaction was optimized to the conditions of 50 °C for 5 min for the initial reverse transcriptase step. This was followed by 40 cycles of 95°C for 15s, 55°C for 15s, and 68°C for 20s with fluorescence being measured during the extension phase. Melting curves were conducted subsequently and consisted of 95 °C for 15 s, 60 °C for 20 min increasing by 0.5 °C per min to a final temperature of 95 °C. All reactions were performed independently and in triplicate.

*Analysis of Gene Expression*

Samples were normalized using the 16S rRNA gene as an internal standard. The relative changes (n-fold) in *hilA* expression between the treated and nontreated samples were calculated us- ing the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001).

## Results

The pH of the *Salmonella* suspensions were measured every 2 for an 8 h period and subsequently measured every 4 h until the conclusion of the experiment at 24 h (Figure 2). This was done to determine if pH varied by serovar and thus may have had some impact on the consistency of the experiments. All serovars at all pH values in both acids followed the same general pattern during the 24 h period with 3 exceptions. For strain ST 23595 at pH 6.2 in acetic acid, this strain had a lower pH value (4.5) at 8 h, but prior to and after this time point the pH values for this strain were very similar to all other strains. Strains *S.* Javiana and SE 13076 deviated from the general pattern at pH 7.2 and pH 6.2 in HCl, respectively. These strains did not return to the general pattern after deviation.

The optical density was also measured every hour to evaluate any differences in growth by serovar and strain (Figure 2). Like the pH values, the growth curves were all very similar among the strains for each pH and acid used. These curves then were plotted on the same chart and revealed that pH and growth were inversely related. This is most likely because *S. enterica* ferments sugars during metabolism and growth, which results in a more acidic pH. Thus, when growth of the bacteria increased, the pH values became lower. After exhausting the supply of sugar, the bacteria will decarboxylate lysine, which increases the pH. However, this increase in pH was not apparent at all pH values tested within the time course of these experiments. At the neutral pH (7.2) *hilA* was down regulated at time point 0 for all serovars and strains with the exception of both strains of Enteritidis (Table 2.2). After 2 h, *hilA* expression increased relative to the 0 h time point in all serovars and strains with the exception of both strains of Enteritidis. This change in

regulation varied by strain in serovar and ranged widely from 0.4 to 24.18 fold change. After 4 h, there was some change in *hilA* expression in all strains and serovars and the change in regulation ranged from 0.07 to 4.15 fold change. At the final time point of 24 h, the range of change in expression varied from 0.03 to 3.69 fold change. Overall, the greatest change in expression occurred between the 0 and 2 h time points in strains ST 23595, ST 14028, *S.* Seftenburg, *S.* Heidelburg, *S.* Mbandanka, *S.* Montevideo, and *S.* Infantis. These changes in expression ranged from 5.23 to 25 fold increase in *hilA* expression.

Similar to the neutral pH, the effect of treatment with HCl on *hilA* gene expression varied by strain and by serovar (table 2.2). Like the neutral pH, the greatest change in regulation occurred between the 0 and 2 h time points for both pH 6.2 and 5.5 (0.55 to 34.59, respectively) for most for the strains. It should be noted, that at pH value 7.2 and at both pH values adjusted with HCl, the TSB became more basic or changed very little between the 0 and 2 h time points and no growth occurred (Figure 2A,C,E).

Similar to the HCl acid, the change in gene expression from time point 0 to 2 h in acetic acid for pH 6.2 and pH 5.5 ranged widely from 0.11 to 20.48 and from 0.11 to 10.02, respectively (Table 2.2). However, there was little change in gene expression from the 2 to 4h time point and from the 4h to 24h time point for most of the *S. enterica* strains in acetic acid, which was also similar to the data collected from HCl acid.

**Discussion**

Acid stress would be encountered by *Salmonella* in several locations within the chicken gastrointestinal tract including the stomach and lower intestinal tract. In the stomach, HCl acid is secreted to initiate the digestion of food. In the cecum, acetic acid is the primary acid found and is a metabolic byproduct from the bacteria that colonize this organ (Van Immersaal and others 2006). The stomach pH is typically low, but can be buffered when food is ingested (Duke 1986). The cecum is a primary location of colonization for *Salmonella*, which has a pH range that is typically between 5.5 and 6 (van der Weilin and others 2000). Thus, these experiments were conducted with both HCl and acetic acid to assess any differences in the expression of *hilA* gene response due to the acids found in the stomach and ceca. Mildly acidic pH values (6.2 and 5.5) were utilized, so that a time study of 24 h could be conducted. Preliminary experiments as well as published reports (Foster 1991) determined that non-acid adapted *Salmonella* rapidly dies at pH values of 4 or lower and thus sufficient RNA would not be available for analysis.

The transcriptional activator *hilA*, regulates the expression of invasion genes in *Salmonella* in response to environmental conditions, including stress and low nutrient concentrations (Bajaj and others 1996; Durant and others 2000). Lawhon and others (2002) demonstrated that acetate increased *hilA* expression in ST. Our study also agreed with these findings in some ST strains. Additionally, our data demonstrates that regulation of *hilA* in acetic acid is serovar and strain dependent. Thus, this study supports the idea that acid can regulate the invasive phenotype of *S. enterica* (Van Immersaal and

others 2006). Additionally, this study demonstrates that the invasive phenotype varies by *S. enterica* serovar and strain and is also dependent on acid type (organic versus inorganic) and pH.

In *Salmonella*, the acid stress response is dependent on the type of acid. Organic acid stress involves both RpoS and PhoPQ, while inorganic acid stress involves only RpoS (Bearson and others 1998). Furthermore, it appears that the regulation of virulence is dependent on the type of acid. The pathway of virulence induction, described subsequently, is well known for organic acids, but not as well-known for inorganic acids. At pH 5.5 and above, acetic acid converts to acetate and *Salmonella* converts acetate to acetyl- phosphate by acetate kinase (ackA), which phosphorylates *BarA-SirA* (a 2-component regulator of *hilA*) leading to the expression of *hilA* (Altier and others 2000; Lawhon and others 2002). When exposed to HCl, the intracellular pH of *S. enterica* is decreased to maintain homestatis. A change in regulation of *hilA* also occurs, but the mechanisms of activation of *hilA* are not fully understood for HCl.

It was not surprising to find that *hilA* regulation was dependent on the type of acid utilized because others have demonstrated this in ST and SE (Durant and others 2000; Lawhon and others 2002; Van Immerseel and others 2004). Lawhon and others (2002) demonstrated that butyrate and proprinate decreased *hilA* expression while acetate increased expression in ST. Van Immerseel and others (2004) demonstrated that a 4 h pre-treatment of SE with butyrate decreased cell invasion while acetate pre-treatment in-creased epithelial cell invasion. The present study indicated that expression of *hilA* was down regulated at time 0 or up regulated less than 1-fold. However, after 2 h in the acidic medium, the *hilA* gene was up regulated relative to the 0 time point in nearly all the

strains within all pH values, which agrees with the published studies (Durant and others 2000; Lawhon and others 2002; Van Immerseel and others 2004).

The data presented in this study indicated that *hilA* gene expression changed over time and, for most strains, increased expression of *hilA* occurred relative to the 0 h time point. This observation was most obvious when examining the change in *hilA* regulation in HCl between the 0 and 2 h time points. The fact that time was a factor in *hilA* expression is further supported by examining the pH data of the mediums in relation to *hilA* expression. For example, the pH of the medium adjusted to 7.2 reached a pH of 6.2 at 4 h. When comparing the *hilA* expression at this time point with the *hilA* expression of cells cultured in 6.2 adjusted with HCl at time point 0, the expression levels of *hilA* differed even though the pH of the medium was the same. This also occurred for pH 6.2, which reached a pH of 5.5 at time point 4 h. This can be seen when comparing *hilA* expression levels of this time point with expression of *hilA* in cultures initially cultured at pH 5.5.

In the United States, the most common *S. enterica* serovars isolated from human illness cases are Typhimurium, Enteritidis, Newport, and Heidelberg (CDC 2010). However, in recent years *S.* Kentucky has become the most prevalent serovar isolated from poultry and poultry farm environments (USDA 2010), but is not considered a major foodborne pathogen because it is responsible for less than 1% of human salmonellosis cases (1999 to 2009; CDC 2010). Thus, *S.* Kentucky most likely lacks genes necessary to cause human disease, but possesses traits that enable it to be a competitive colonizer of the chicken cecum (Pedroso and others 2007; Joeger and others 2009). Joeger and others (2009) evaluated 88 *S. enterica* isolates and reported that *S.* Kentucky isolates were

highly susceptible to low pH conditions (2.5) but as sensitive as other serovars to mildly acidic pH conditions (5.5) compared to the other serovars evaluated. The researchers speculated *S.* Kentucky may allocate metabolic efforts towards growth rather than acid adaption, which could give these isolates an advantage during colonization in the crop and cecum, which are mildly acidic (6.2 to 5.5). Our data did not indicate dramatic changes in *hilA* in *S.* Kentucky compared to other serovars and strains that may support the idea that efforts of *S.* Kentucky are aimed at growth rather than virulence expression.

## Conclusions

This study determined that the regulation of *hilA* was serovar and strain dependent and also dependent on the type of acid. A limitation of this study is the fact that only 1 strain of 8 serovars was evaluated. We did evaluate several strains of *S.* Kentucky, *S.* Eneridtis, and *S.* Typhiumurium and differences in *hilA* expression were noted between some of these strains. Thus, additional strains of each serovars should be evaluated to investigate further understand any differences by strain of *S. enterica*.

## Acknowledgement

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# Appendix

Table 2.1. A list of the *S. enterica* serovars, the source of the strains and references describing characteristics of these strains utilized in other research.

| *S. enterica serovar* | Source | Reference |
| --- | --- | --- |
| *S.* Typhimurium DT104 | Human infection | Threlfall (2000) |
| *S.* Typhimurium ATCC 23595 (LT2) | Laboratory strain | Swords and others (1997) |
| *S.* Typhimurium ATCC 14028 | Laboratory strain | None |
| *S.* Enteritidis (WT) | Human infection | None |
| *S.* Enteritidis ATCC 13076 | Human infection | None |
| *S.* Kentucky[a] | Poultry carcass | Clement and others (2010) |
| *S.* Kentucky[a] | Poultry carcass | Clement and others (2010) |
| *S.* Seftenburg | Poultry farm | Rodriguez and others (2006) |
| *S.* Heidelburg | Poultry farm | Rodriguez and others (2006) |
| *S.* Mbandanka | Poultry carcass | Melendez and others (2010) |
| *S.* Newport | Poultry carcass | Melendez and others (2010) |
| *S.* Bairely | Poultry carcass | Melendez and others (2010) |
| *S.* Javiana | Poultry Farm | Rodriguez and others (2006) |
| *S.* Montevideo | Swine farm | Rodriguez and others (2006) |
| *S.* Infantis | Poultry Farm | Rodriguez and others (2006) |
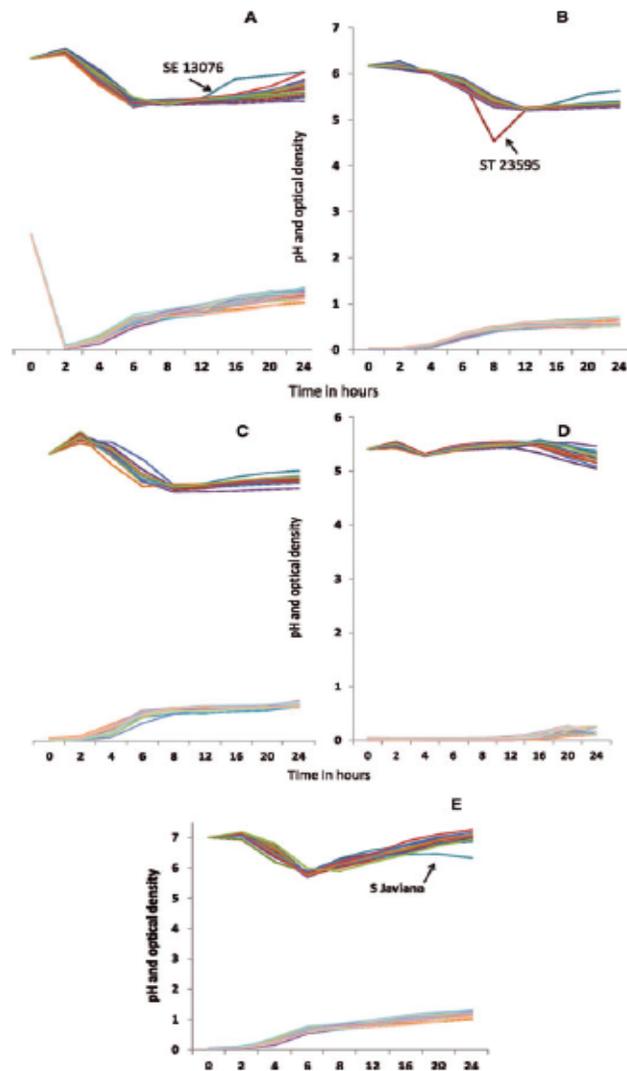
Figure 2. Line graphs representing the pH changes and growth of the *Salmonella* suspensions over the time course of the experiments measured at times 0, 1, 2, 4, 6, 8, 12, 16, 20, and 24 h and every hour, respectively. The top curves represent changes in pH value over time and the bottom curves represent changes in optical density over time. (A) pH 6.2 with HCl acid, (B) pH 6.2 with acetic acid, (C) pH 5.5 with HCl acid, (D) pH 5.5 with acetic acid, and (E) pH 7.2 no acid. Those serovars that deviated from the consensus curve pattern are indicated on each chart.

Table 2.2. The relative fold change in gene expression of *hilA* of *S. enterica* serovars in response to 3 pH values, 7.2, 6.2, and 5.5. pH values 6.2 and 5.5 were created by adjusting with HCl and acetic acid.

| | HCl acid | | | | | | | | | | | |
| | pH 7.2 | | | | pH 6.2 | | | | pH 5.5 | | | |
| | Time in hours (h) | | | | | | | | | | | |
| *S. enterica* strain | 0 | 2 | 4 | 24 | 0 | 2 | 4 | 24 | 0 | 2 | 4 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S. Typhimurium DT104 | −2.88 | 0.48 | 0.55 | −1.84 | 0.73 | 0.17 | −13.7 | −1.36 | 0.79 | 1.10 | −2.51 | 0.49 |
| S. Typhimurium ATCC 23595 | −13.3 | 0.7 | 0.33 | 0.30 | −27.0 | 0.40 | 0.44 | 0.20 | −28.5 | 0.14 | −1.33 | 0.20 |
| S. Typhmurium ATCC 14028 | −24.8 | 0.62 | 0.50 | 0.16 | −34.3 | 0.29 | 0.18 | 0.57 | −59.6 | 0.17 | 0.30 | 0.35 |
| S. Enteritidis (WT) | 0.58 | −2.89 | 1.26 | 0.57 | −8.12 | 0.25 | −1.05 | 0.59 | 0.56 | 0.68 | −1.41 | −2.09 |
| S. Enteritidis ATCC 13076 | 0.75 | −1.73 | −1.63 | 0.47 | 0.8 | −1.88 | −1.03 | 0.64 | −1.45 | −1.91 | 0.58 | 0.65 |
| S. Kentucky | −1.76 | 0.32 | 0.60 | −3.09 | −1.95 | 0.86 | 0.97 | 0.64 | −1.45 | 0.31 | 1.35 | −1.68 |
| S. Kentucky | −1.65 | 0.91 | −1.07 | 0.63 | −1.64 | −1.16 | 0.88 | 0.61 | −5.24 | 0.38 | 0.23 | −3.75 |
| S. Seftenburg | −7.5 | 0.58 | 0.36 | 0.75 | −6.63 | 0.42 | 1.02 | 0.40 | −6.31 | 0.60 | 0.41 | 0.80 |
| S. Heidelburg | −4.72 | 0.51 | −2.95 | 0.18 | 0.73 | −1.26 | −6.35 | 0.23 | −1.38 | 0.33 | −10.6 | 0.26 |
| S. Mbandanka | −11.8 | 0.35 | 0.44 | 0.65 | −16.3 | 0.15 | 0.42 | −1.07 | −8.96 | 0.24 | −1.39 | 0.33 |
| S. Newport | −2.01 | −1.61 | −1.13 | 0.29 | −7.90 | −1.19 | 0.75 | 0.14 | −3.71 | 0.41 | 0.04 | −14.5 |
| S. Bairely | −2.18 | 0.85 | 0.58 | −1.15 | −16.7 | −2.13 | 0.29 | 0.17 | −14.6 | 0.83 | 0.47 | 0.18 |
| S. Javiana | −1.62 | −1.24 | 1.21 | 0.53 | −2.27 | 1.16 | −2.36 | 0.19 | −1.82 | 0.48 | −3.16 | 0.39 |
| S. Montevideo | −22.5 | 0.60 | 0.23 | 0.45 | −30.9 | 0.44 | 0.27 | 0.45 | −15.6 | 1.76 | 0.59 | 0.07 |
| S. Infantis | −6.3 | 0.62 | 0.19 | −1.37 | −7.27 | 0.15 | −9.62 | 0.10 | −7.24 | 0.06 | 0.22 | −9.18 |

| | Acetic acid | | | | | | | |
| | pH 6.2 | | | | pH 5.5 | | | |
| | Time in hours (h) | | | | | | | |
| *S. enterica* strain | 0 | 2 | 4 | 24 | 0 | 2 | 4 | 24 |
|---|---|---|---|---|---|---|---|---|
| S. Typhimurium DT104 | 0.85 | −2.10 | 1.32 | 0.47 | −5.94 | −3.64 | 0.63 | 0.25 |
| S. Typhimurium ATCC 23595 | −9.52 | 0.65 | 0.71 | 0.23 | −3.36 | 0.13 | −1.63 | −1.55 |
| S. Typhmurium ATCC 14028 | 0.31 | −1.38 | −1.39 | −1.77 | 0.80 | 0.90 | −1.2 | −1.15 |
| S. Enteritidis (WT) | −5.98 | 0.52 | 0.57 | 0.57 | −1.31 | 0.41 | 0.55 | −3.54 |
| S. Enteritidis ATCC 13076 | 0.78 | 0.89 | −2.10 | 0.86 | 0.49 | 0.38 | 0.60 | 0.72 |
| S. Kentucky (A) | −1.04 | −1.69 | 0.79 | 0.73 | −3.26 | −2.49 | 1.06 | 0.14 |
| S. Kentucky (F) | 0.80 | −1.88 | −1.03 | 0.65 | −1.45 | −1.91 | 0.58 | 0.65 |
| S. Seftenburg | −1.12 | −1.67 | −1.39 | 0.40 | 0.44 | −1.79 | −1.98 | −2.82 |
| S. Heidelburg | −1.24 | 0.42 | −2.10 | 0.94 | −2.48 | 0.67 | 0.61 | 1.05 |
| S. Mbandanka | −1.86 | −1.96 | 0.57 | −4.19 | 0.42 | −1.58 | −1.05 | −1.57 |
| S. Newport | 0.44 | −1.95 | 0.77 | −1.86 | 0.23 | 0.80 | −2.86 | −1.93 |
| S. Bairely | −1.87 | −1.01 | 0.76 | 0.74 | 1.26 | −1.75 | 0.97 | 0.84 |
| S. Javiana | −2.7 | 0.84 | −1.03 | 0.44 | −1.33 | −1.06 | −1.18 | 0.75 |
| S. Montevideo | −20.3 | 0.18 | 0.45 | 0.66 | −9.80 | 0.22 | −1.08 | 0.45 |
| S. Infantis | 0.66 | 0.42 | −3.04 | −1.20 | −1.61 | −1.33 | −1.21 | 0.42 |

## Vita

Nan Zhang was born in Shijiazhuang, China. She attended Wuhan University from 2007 to 2011, where she received her B.S. degree in Life Sciences and Technology while working under Professor Ling Zheng. After graduation, she enrolled as a graduate student in the Department of Food Science and Technology at the University of Tennessee, Knoxville. She joined Professor Irene Hanning's research group in 2011 and worked on food safety. Nan Zhang received a Mater's Degree in Food Science and Technology from the University of Tennessee in August, 2013.