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Molecular Basis of Salmonella Enterica Serovar Kentucky Attachment to Broiler Skin

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Molecular basis of *Salmonella enterica* serovar Kentucky attachment to broiler skin

By

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The presence of *Salmonella enterica* throughout the production and processing continuum is a concern in broiler industry. While federal regulations have lowered the acceptable level of *Salmonella* contamination on broiler carcasses, the mechanisms that contribute to pathogen attachment are not fully understood. *Salmonella* Kentucky has become the predominant *Salmonella* serovar isolated from broilers carcasses at the end of the immersion chill tank. In Europe and Africa this serovar has been shown to acquire antibiotic resistance genes that may lead this non-typhoidal serotype to become a potential public health concern. To investigate the genes that are involved in colonization of the bacteria to broiler skin, a mutant library of the bioluminescent strain of *S.* Kentucky was constructed.

According to the chicken attachment assay, it was concluded that attachment is a multifactorial process with the following elements contributing: i), flagella, ii), LPS structure, iii), amino acid metabolism, iv), TCA cycle pathway; v), conjugative transfer system, vi), multidrug resistant protein, vii), signaling and transportation system, viii),

metabolism, ix), different enzymes, x), phage tail fiber protein H, xi), fimbrial export usher proteins, xii), membrane proteins xiii), and several unnamed proteins. The role of flagella between all of these contributing elements appeared to be the most significant. The flagella motor gene, filament sub-units and hook associated protein were deleted by using the λ red recombination method. The mutants' ability to colonize broiler skin was compared to their parental strain, and the motility and flagellin main sub-unit (FliC) were recognized as the key factors contributing to bacterial attachment. Using Caco-2 cell lines as a cell model to assess adhesion and invasion capacity of flagella mutants, similar results were observed. Based on the result of the experiments conducted in this study, it appears that the active flagella FiC sub-unit plays an important role in colonization of epithelial cells outside and inside of the broilers.

DEDICATION

I wish to dedicate this work to my dear parents, beloved husband, Sasan, and my beautiful daughter, Amanda. Without the encouragement and support of my family through difficult times, none of this would have been possible. I'm forever grateful to them.

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CHAPTER I

LITERATURE REVIEW

Salmonella

Salmonella is a gram-negative rod shape bacteria that belongs to the family of entrobacteriaceae. There are two species in this genus: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae, S.* enterica subsp. *diarizonae*, *S. enterica* subsp. *Houtenae*, and *S. enterica* subsp. *indica*. More than 2400 *Salmonella* serotypes have been identified. The majority of these serotypes belong to the *S. enterica* subsp. *enterica*. Strains in this group cause 99% of *Salmonella* infections in humans and warm-blooded animals (Brenner et al., 2000).

Of all *Salmonella enterica* subsp. *enterica* serotypes, *Salmonella enterica* serovar typhi and paratyphi cause typhoid and paratyphoid fever and are called typhoidal *Salmonella*, which colonize and infect humans. The rest of serovars are referred to as non-typhoidal *Salmonella* serotypes that have a broad host range (Feasey et al., 2012). Non-typhoidal *Salmonella* are believed to cause zoonotic infections that are transmitted from healthy carrier animals to humans, especially through contaminated foods. Meat, meat products, eggs and egg products are the most common vehicles of transmission that contain *Salmonella enterica* spp (Wegener et al., 2003).

Foodborne illness in the United States

According to a survey in 2011, out of 9.4 million foodborne illnesses in the United States, 5.5 million were caused by viruses, 3.6 million by bacteria, and 0.2 million by parasites. Non-typhoidal *Salmonella* spp. with one million cases per year is the leading cause of bacterial foodborne illnesses. These serotypes are also the leading cause of foodborne hospitalizations (35%) and deaths (28%) in the United States (Scallan et al., 2011). Poultry has been frequently associated with human cases of salmonellosis (Foley et al., 2011). The origin of *Salmonella* contamination has been associated with the farm and processing plants, which lead to the presence of *Salmonella* in retail products. Cross-contamination in the processing plant can be due to bacteria remaining on improperly cleaned equipment following cleaning. However, the level of *Salmonella* on carcasses has been shown to decrease through the processing (Berrang et al., 2009).

Different *Salmonella* serotypes have been isolated from various poultry farms. Most of these serotypes have the potential to be pathogenic for humans. Result of a research sampling of carcasses from 20 different processing plants in 13 different states has revealed the three most prevalent serotypes to be *S*. Kentucky, Heidelberg, and Typhimurium (Berrang et al., 2009).

According to the United States Department of Agriculture (USDA), *Salmonella enterica* serovar Kentucky had the highest percentage of isolation from broilers in 2011. Table 1.1 shows quarterly results of the USDA *Salmonella* serotyping from whole carcass rinses.

Table 1.1 Quarterly results of the USDA *Salmonella* serotyping from whole carcass rinses

Quarter 1			
Serotypes	No. of Isolates	Percent of Total Positive	Percent of Analyzed Samples
Kentucky	40	47.06	3.17
Enteritidis	27	31.76	2.14
4,5,12:i:-	3	3.53	0.24
Heidelberg	3	3.53	0.24
Brandenburg	2	2.35	0.16
Typhimurium	2	2.35	0.16
Berta	1	1.18	0.08
Montevideo	1	1.18	0.08
Thompson	1	1.18	0.08
Typhimurium var 5-	1	1.18	0.08
Worthington	1	1.18	0.08
TOTAL	82	96.47	6.50

Quarter 2			
Serotypes	No. of Isolates	Percent of Total Positive	Percent of Analyzed Samples
Kentucky	27	69.23	4.72
Enteriditis	8	20.51	1.40
Typhimurium	2	5.13	0.35
8,20:-:Z6	1	2.56	0.17
Heidelberg	1	2.56	0.17
TOTAL	39	100.00	6.82

January–June 2011
Source: http://www.fsis.usda.gov/science/Q1-2_2011_Salmonella_Serotype_Tables/index.asp

Shift of *Salmonella* serotypes in poultry

Various *Salmonella* serovars have been isolated from poultry products. However, due to different reasons, the prevalence of serotypes changes from time to time. In the early 1900s, *Salmonella* serovars Pullorum (pullorum disease) and Gallinarum (fowltyphoid) caused widespread diseases in poultry in the United States (Shivaprasad, 2000). These two serovars were eradicated in the mid-1970s (Bäumler et al., 2000). The space created by removal of these organisms from the poultry intestinal microbiota was eventually filled with the emergence of *S*. Enteritidis (Bäumler et al., 2000). Although this serotype is still common in poultry diseases, it declined in incidence in the 1990s (Shivaprasad, 2000) and Heidelberg was more prevalent than Enteritidis from 1996 to 2005 (CDC, 2005). In 2007, *S.* Kentucky was the most commonly isolated serovar

(Berrang et al., 2009). One reason for the emergence of *S*. Kentucky could be the acquisition of the virulence plasmid from avian pathogenic *Escherichia coli* (APEC) which appears to be important in the colonization in poultry (Johnson et al., 2010). Another reason could be the increasing immunity of poultry to *S*. Enteritidis. It has been proven that *S*. Heidelberg has some common surface antigens with *S*. Enteritidis that *S*. Kentucky does not have. This fact may explain the increasing numbers of *S.* Kentucky in recent years (Foley et al., 2011).

The origin of antibiotic resistant strain of *S*. Kentucky

United States poultry is not the only area that has been affected by *Salmonella* serovar Kentucky. National *Salmonella* surveillance from several European countries, as well as the United States, has recognized an emergence of a multidrug resistant isolate of *Salmonella enterica* serovar Kentucky. This strain has a high level of resistance to ciprofloxacin. Fluroquinolones, such as ciprofloxacin, are the most efficient drug of choice for *salmonella* infections and resistance to this drug can increase the rate of morbidity and mortality in humans. The origin of this strain is from a single clone, which is sequence type STI98 and a genomic island 1 variant SGI1-K. This strain, according to recent investigations, originated from Egypt and has spread into Africa and the Middle East. In Europe and North America, contamination is the result of imported food or secondary contamination. *Salmonella* Kentucky was first isolated from a chick in the US in the early part of the last century (Edwards, 1938), but this strain (ST198-98K) was different from the pathogenic strain that was identified from Africa (ST198-X1 CIP ^{R}) (Le Hello et al., 2011). ST198-98K was prevalent in the poultry industry, but was not pathogenic in humans (Joerger et al., 2009). This new antibiotic-resistant pathogenic

strain has been mainly identified in chickens and turkeys in African countries, which routinely use ciprofloxacin and other fluoroquinolons in poultry production, believed to be the origin of the resistance (Alo & Ojo, 2007). There are other speculations on the source of this genetic variation (Le Hello et al., 2011). This pathogenic strain first appeared in Egypt in the 1990s. Furthermore, Egypt provides 80% of farmed fish products to African countries (El-Sayed, 2007). There are some data on harboring SGI1 in some aquatic bacterial strains in these fish farms. Data shows that the acquisition of CIP^R by ST198 *Salmonella enterica* serovar Kentucky, could have happened on the farms with integrated aquaculture (Le Hello et al., 2011). In these farms, poultry manure is usually used as a fertilizer for the fish ponds, and pond waste is used as a poultry food (Suloma & Ogata, 2006). The above research shows the potential for global spread of this antibiotic-resistant *Salmonella* serotype, especially in poultry and poultry plants (Le Hello et al., 2011).

Possible route of contamination throughout the processing plant

Salmonella serotypes are one of the main contaminants of broilers in processing plants. The route of contamination is not completely understood, but there are some possible pathways for consideration. At the poultry processing plant, the common steps of processing includes stunning, killing, scalding, defeathering, eviscerating, washing, cooling, and packaging (Keener et al., 2004). Given that *Salmonella* can be present on the surface as well as gastrointestinal track, it can easily spread through the processing.

Scalding

During the scalding step, carcasses are immersed in long water-filled tanks heated to 50-60°C (Barbut, 2002). The purpose of the scalding procedure is to facilitate the removal of the feathers from feather follicles. If *Salmonella* is present on the exterior of the bird, this procedure has the potential to allow cross-contamination during feather-picking. The reason for contamination is that the feather follicles may stay open throughout processing until chilling. When the follicles close, the microorganisms may be retained inside (Bailey et al., 1987)

Defeathering

In modern processing plants, mechanical pickers with rubber fingers remove the feathers from the carcass. In a continuous operation, hanging birds move forward between two or three sets of drums or disks covered with rubber fingers. Depending of the work and the speed of the machine, the length and elasticity of fingers might be varied (Barbut, 2002). While removing the feathers from the bird, the rubber finger-like projections may get contaminated and cross-contaminate the rest of the birds (Wempe et al., 1983).

Evisceration

During evisceration, a circle cutting blade cuts the body cavity and removes the viscera. It is very important not to cut the viscera during this procedure as it may spread the bacterial content to other parts, such as muscles, skins, etc. (Barbut, 2002).

Chicken skin has been shown to harbor and support bacteria (Lee et al., 1998). Berrang et al. (2001) reported high levels of contamination on the muscles of the broilers.

These results suggested the crop content may be an important source of contamination. Byrd et al. (1998) found the crop to be a source of *Campylobacter jejuni, Salmonella*, *E. coli* and other coliform bacteria.

Carcass washers

In the poultry processing plants, there are different washers with different parameters of efficiency. These parameters include wash water temperature, water pressure, nozzle type, nozzle arrangement, flow rate, line speed, and surfactant agents used. Despite the large volume of water used in chicken plants, there are still problems in removing the fecal bacteria from the surface of the broilers. Water surface tension might be a possible reason for this deficiency (Keener et al., 2004).

Chillers

To prevent bacterial growth, poultry carcasses need to be rapidly cooled. The most common chilling methods include water-immersion, air-chilling and spray-chilling. During immersion-chilling, large tanks of cold water with a counter-flow direction to the broiler flow is used to reduce the carcasses temperature to about 4-5°C within 30-5 minutes. The chiller tanks contain paddles that move the carcasses forward to the next step (Barbut, 2002).

Chlorination of the chiller tanks can have the maximum of 50 ppm of free chlorine. The bactericidal effect of chlorine is affected by the organic load of the water (Goresline et al., 1951).

There are different chemicals that have also been used in poultry plants to help reduce the bacterial surface contamination. Among these are various organic acids, such

as acetic acid, lactic acid, citric acid, and succinic acid. These acids kill the bacteria, especially *Salmonella,* as they have the ability to penetrate the bacterial membrane and disrupt it. Comparing the effects of acid, heat treatment, and chlorine on *Salmonella* deactivation on the surface of poultry has shown a 50% reduction of *Salmonella* by utilizing 1% of succinic acid alone (Thomson et al., 1976). Also, addition of 0.1% of acetic acid to the scalding water resulted in the reduction of *Salmonella* Typhimurium from 0.5 to 1.5 $\log_{10}$ CFU/ml (Okrend et al., 1986).

Other chemicals that have been used in poultry plants to control contamination include chlorine dioxide, trisodium phosphate, and acidified sodium chlorite. Regardless of the multiple steps used in processing broilers*, e.g.,* scalding, defeathering, washing, and immersion-chilling, *Salmonella* can still persist through these different stages. In order to remove the bacteria from the surface of the broilers, it is necessary to investigate the mechanism of attachment, which is a complex process.

Bacterial surface determinants of attachment

Various surface molecules and structures are involved in the attachment of bacteria to surfaces. The role of bacterial flagella, motility, and chemotaxis has been investigated in a number of studies. In a recent study, it was demonstrated that flagella are able to reach into crevices, access additional surface area, and produce a dense, fibrous network. According to this study, although motility plays an important role in the attachment, flagella structure can help the bacteria overcome harsh surface structures (Friedlander et al., 2013). Fimbriae have also been shown to be associated with bacterial attachment. Type 1 fimbriae are the most common type of adhesions in commensal bacteria. They are 7 nm wide and approximately 1 μm long rod organelles (van Houdt &

Michiels, 2005). Other forms of cell structures are rod-shaped proteins that are called pili. They are made of single sub-units, pilin. Pilin sub-units pack in a helical pattern into a cylindrical structure. Pili make contact with other bacteria and cell surfaces. The tip of the pilus usually attaches to carbohydrate residues on the surface of the cells. Pilin sub-units and the tip are secreted into periplasmic spaces and then across the membrane. Some bacteria have cell surface proteins that promote attachment to surfaces. Extra polysaccharide slime is another bacterial attachment factor that acts like a glue and attaches the bacteria together and to different surfaces (Salyers & Whitt, 2002).

Flagella and its structure

Flagella help bacteria move in the environment so it can get closer to attractants and escape from repellents. The number of flagella and their arrangement on bacterial surfaces are different, but they are all composed of several substructures: a basal body, a transmembrane motor, and a hook that links the motor and the filament (Bonifield & Hughes, 2003). The *Salmonella* filament is 10 μm in length and composed of either FliC or FljB flegellin sub-units (Macnab, 1999). More than 50 genes are expressed for assembly and function of flagella. The promoters of flagella regulon are classified in three classes according to the order of expression, which is parallel with flagella structure assembly. The class I promoter directs the transcription of the *flhDC* master operon that contains six transcriptional start sites. Expression of class II promoters requires the function of class I and are responsible for hook-basal body assembly. *flgA, flgB, flhB, fliA, fliE, fliF and fliL* are categorized in class II. Class III operons express the final step of filament assembly and the function of flagellum. *flgK, fliD, fliC, motA,* and *tar* are

class III promoters and their expressions need the function of class II operon (Kutsukake et al., 1990). Figure 1.1 shows the schematic image of flagella.

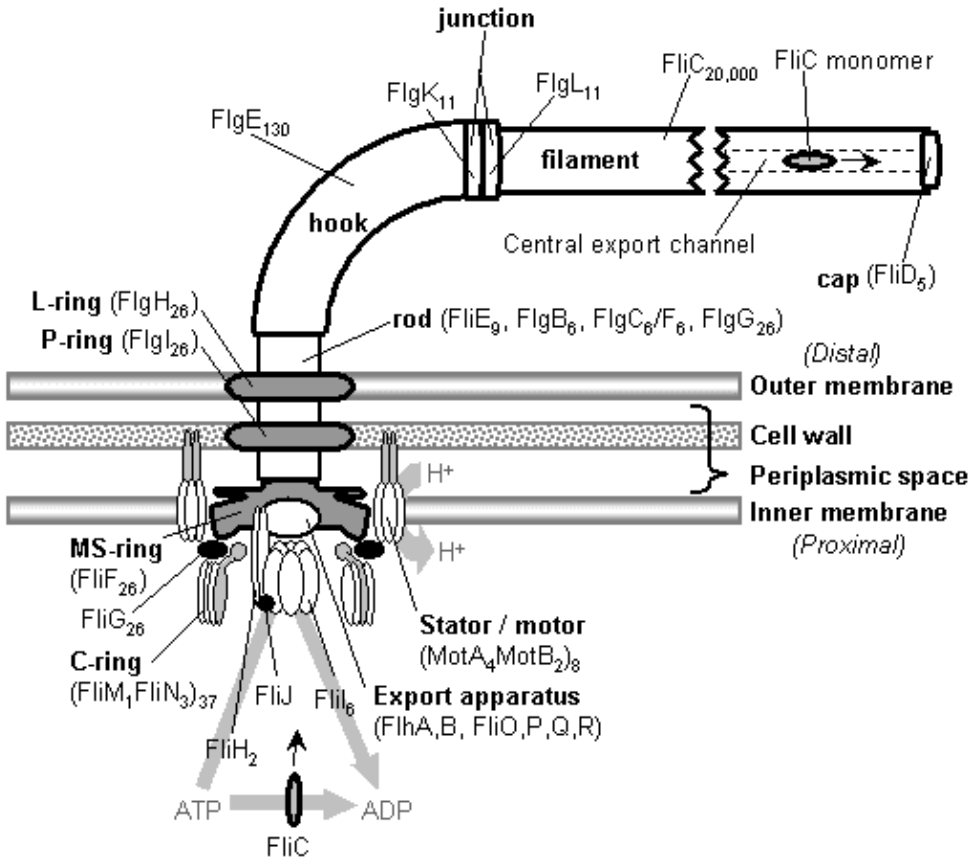

Figure 1.1 Schematic image of flagella

http://agnophilo.xanga.com/728670894/evolution-and-irreducible-complexity/

Flagella phase variation in *Salmonella enterica*

Salmonella enterica alternately express two different flagellin filament proteins (FliC, FljB). This mechanism is called flagellar phase variation. Although it is suggested that flagellar phase variation is important in the pathogenicity of microorganisms due to its provision of a mechanism to temporarily escape cell immunity, the exact role of phase variation in *Salmonella enterica* is not clear (Ikeda et al., 2001). The molecular basis of

phase variation is DNA inversion in the chromosome. The *fljB* promoter is flanked by the recombination sites *hixL* and *hixR*. In order to invert the promoter, the *Hin* recombinase and the recombination enhancer proteins Fis (factor for inversion stimulation) and HU (factor that stimulates *in vitro* Salmonella *fljB* flagellar phase variation reaction) attach together to promote a reversible recombination reaction between the *hix* sites. This leads to the inversion of the DNA that contains the *fljBA* promoter. Conversion of DNA in one side would block the transcription and in the other side would promote the transcription of *fljBA* and production of the FljB protein. The *fljA* gene is located downstream of *fljB* and is simultaneously transcribed. FljB plays a role as the *fliC* inhibitor (Kutsukake & Iino, 1980). Recent studies have shown that FljA is not just a transcriptional inhibitor, but also a translational inhibitor (Bonifield & Hughes, 2003). The alternate orientation of the *fljBA* promoter blocks the operon and leads to the transcription of the *fliC* gene. Figure 1.2 shows the schematic process of phase variation.

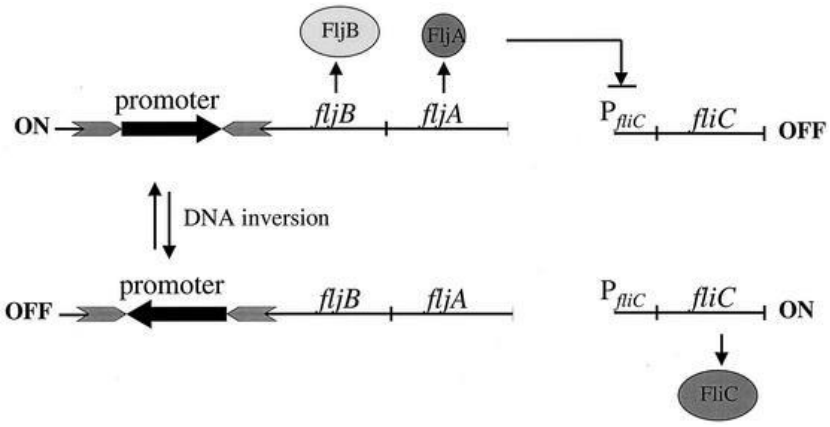

Figure 1.2 Schematic process of phase variation of flagella sub-units in *Salmonella* http://www.ncbi.nlm.nih.gov/pmc/articles/PMC156219/figure/f1/

Flagelline protein structures are similar in both FliC and FljB. The first 71 amino acids and last 46 amino acids are the same, but different amino acids are present in the middle that are surface-exposed and lead to distinct antigenic characteristics (Namba, 2001).

Flagella motor of Salmonella enterica

Flagellar motion is the result of the electrochemical potential gradient of protons across the membrane. The basal body, which is implanted in the cell membrane, works like a bi-directional rotary motor that is powered by electrochemical power. The hook and filament spread out of the membrane and the filament moves like a helical propeller. The hook attaches the basal body to the filament so it can transfer the power of the motor to the filament. A counter-clockwise rotation of the motor causes the cell to swim and a clockwise rotation changes the direction to a favorable location (Minamino et al., 2008).

In *Salmonella enterica*, five flagellar proteins, MotA, MotB, FliG, FliM and FliN, are responsible for torque generation. MotA and MotB are transmembrane proteins that form the stator complex of the motor. The complex consists of four copies of MotA and two copies of MotB. It forms a channel for inward proton flow, which generates the torque and in fact converts the motive force from proton flow into the mechanical work required for flagellar motion. Two highly conserved amino acids, Pro-173 in MotA and Asp-33 in MotB, are involved in the energy coupling mechanism. FliG, FliM and FliN are located on the MS ring and are responsible for torque generation and changing the direction of the flagellar rotation. Torque generation is the result of sequential electrostatic interactions between MotA and FliG (Berg, 2003). This is demonstrated in Figure 1.3.

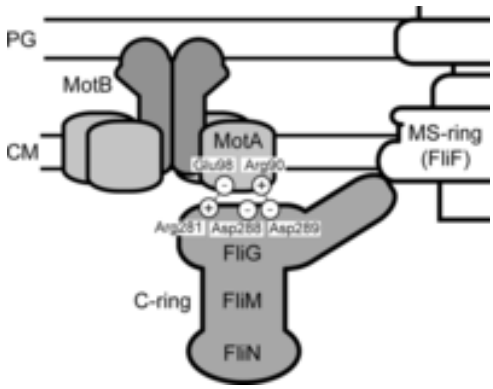

Figure 1.3 Schematic figure of electrostatic interactions between MotA and FliG

http://jb.asm.org/content/early/2012/11/12/JB.01971-12.full.pdf

Mechanism of *Salmonella* attachment to broiler skin

Having in mind the general idea of bacterial attachment to various surfaces, the aim of this study was to investigate the factors that are involved in the attachment of *Salmonella* to chicken skin. There are conflicting opinions on the mechanism of *Salmonella* attachment to chicken skin. According to Thomas and McMeekin (1981), attachment is a function of bacterial retention in the network of fibers that forms with the immersion of chicken muscle fascia in water. Using confocal scanning laser microscopy, Kim et al. (1996) located *Salmonella* in the cervices and feather follicles of chicken carcasses, suggesting that *Salmonella* can become entrapped in water inside the follicles. However, Firstenberg-Eden et al. (1978) reported that kinetics of attachment is dependent on bacterial strains. Lillard (1985, 1986) reported that attachment is not influenced by fimbriae or pili, and motility of the bacteria does not significantly affect attachment, though earlier works indicated the contrary (Notermans & Kampelmacher, 1974). Dickson and Koohmaraie (1989) demonstrated that cell charge is considered an important factor in attachment. However, Kim et al. (1996) stated that a difference in cell charge

did not affect the attachment rate. Lillard (1985) proposed that attachment is a time dependent process; however, McMeekin and Thomas (1978) reported that time did not influence the attachment, but the bacterial population did. Later work by Conner and Bilgili (1994) indicated that bacterial concentration and inoculation time are not important in the attachment of bacteria to chicken skin.

Finally, it was reported that adhesion is involved in the attachment of bacteria to chicken fascia, and there should be some receptors involved. It has been determined that *Salmonella* most frequently attach to chicken fascia, which is composed of collagen and elastin inter-spread in the glycosaminoglycan (GAG) matrix. *Salmonella* mostly attaches to matrix rather than the fibers. The GAG components are hyaluronan and chondroitin sulphate. Hyaluronan is the part of matrix that interacts with *Salmonella.* It was reported that the attachment of *Salmonella* to hyaluronan is not due to ionic strength or hydrophilic interaction, since it was not removed by a strong ionic compound. According to this study, using SDS-PAGE, several proteins on the surface of *Salmonella* could bind to hyaluronan (Sanderson et al., 1991).

In our laboratory, we developed a real-time bioluminescent monitoring system to monitor *Salmonella* attachment to chicken skin. For this purpose, a bacterial *lux* operon was introduced and stabilized in 12 different *Salmonella* serotypes that were isolated from various points along the broiler processing continuum. A skin assay model with labeled *Salmonella* serotypes showed different attachment properties after washing in an agitated water bath. Based upon this research, we formulated that different *Salmonella* serotypes show different attachment properties to chicken skin (Karsi et al., 2008).

It is not completely clear if *Salmonella* follow the same mechanism in attachment to epithelial cells on the surface of the broiler skin and the intestinal epithelial cells. However, the following discussion shows there is a considerable body of knowledge that supports the role of surface structures in attachment to broiler skin.

Role of surface structures in adhesion of *Salmonella* to epithelial cells

Early bacterial-epithelial cell interactions are necessary before a complete attachment and invasion of *Salmonella* to epithelial cells can form. To date, the nature of these interactions is not clear, but it seems that surface structures, such as flagella, fimbriae, pili, capsule, and lipopolysaccharide play an important role in the process (Bravo et al., 2011).

Bishop et al. (2008) demonstrated that production of the virulence capsule, in the *Salmonella enterica* serovar Typhi reduced adhesion, but showed a significant increase in invasion. Also, according to this study, mutation in fimbriae genes (*staA* and *tcfA*) did not affect the invasion or adhesion of this bacteria to Caco-2 cells in the presence or absence of Vi capsule. In another study, investigating the role of *Salmonella* Enteritidis fimbriae in adherence to chicken gut explant, it was concluded that mutants unable to express SEF14, SEF17, and SEF21 fimbriae structures adhere to the chicken gut explant as well as the wild type strain (La Ragione et al., 2000). However, Dibb-Fuller et al. (1999) indicated that naturally occurring regulatory defective strains of *Salmonella* Enteritidis, which do not express SEF17 or SEF21 (different fimbriae structures), do not have the ability to adhere to cultured epithelial cells as well as the wild type strain does. This result was confirmed in other studies through the evaluation of adhesion by mutants of

Salmonella Enteritidis that lacked any kind of fimbriae to attach to chicken gut and rat ileal explants (Allen-Vercoe & Woodward, 1999; Robertson et al., 2000).

Zhang et al. (2000) studied the role of type IVB pili on the attachment and invasion of *Salmonella enterica* serovar Typhi to intestinal epithelial cells. In this research, an insertion mutation in the *pilS* gene, as well as mutants lacking the whole *pil* operon, were constructed. These genes are responsible for the expression of the putative type IVB structural prepilin. Results indicated lower level of attachment and invasion in mutants lacking pili, when compared to positive controls. However, Bishop et al. (2008) demonstrated that a mutation to pilS did not cause a reduction in adhesion and invasion to cultured epithelial cells.

Although, the role of LPS in *Salmonella* virulence has been established (Freudenberg et al., 2001), the role of LPS components in attachment and invasion of epithelial cells has not been completely defined. In a study on colonization of S. Typhimurium to mouse intestine, it was indicated that the mutant strain with uncompleted LPS core showed a lower level of colonization, when compared to the wild type (Nevola et al., 1985). Using signature-tagged mutagenesis of S. Typhimurium, Morgan et al. (2004) demonstrated that mutation in O antigen biosynthesis impaired the ability of the mutants to colonize chicken and calf intestines. In a conflicting study, it was indicated that mutation in O antigen is not required for cell invasion (Kihlström & Edebo, 1976). In a recent study by Bravo et al. (2011) on the role of LPS in early interactions of S. Typhi and Typhimurium with epithelial cells, it was indicated that O antigen is not required for attachment and invasion, but the sugar residues of the LPS outer core are necessary for attachment

Use of flagella and motility for Caco-2 cells colonization

The role of flagella in adhesion and pathogenicity of *Salmonella enterica* serovars is complicated, but there is a growing body of knowledge that supports their role in adhesion and invasion. However, there are two different opinions concerning early interaction of bacteria with the target host cells: one involves the role of motility as a facilitating factor; the other is the structural presence of the flagella.

Girón et al. (2002), studied the attachment of enteropathogenic *Escherichia coli* to epithelial cells. They concluded that the flagella are potential adhesive structures. The data did not explain the mechanism of binding by the flagella, but it was suggested that there is a binding area on the non-conserved part of the H6 flagellin that is exposed to the surface of the filament. The H6 flagella were purified from EPEC and attachment to HeLa cells was demonstrated. It was indicated that EPEC, with a mutation in *fliC* gene, was not able to attach to the cells, but a *motB* mutant was still able to adhere to cultured cells. These authors also determined that motility is not necessary for attachment, but the flagella structure is (Girón et al., 2002). In a more recent work on the attachment of bacteria in biofilm formation, Friedlander et al. (2013) concluded that, in addition to motility, flagella act as structural elements that enable the bacteria to access the unfavorable surfaces.

An early study by Jones et al. (1981) suggested that, motility only facilitates the interaction of bacteria with the host cells and leads to a reversible and irreversible attachment. There have also been numerous *in vivo* and *in vitro* studies on the role of un-flagellated and paralyzed strains of *Salmonella enterica* serovars in adhesion to the cells. Comparison of an aflagellated (fla^-) strain of *Salmonella* Enteritidis with aflagellated, but

non-motile (mot$^-$) strain, in adhesion to rat ileal explants cultured cells indicate that both motility and flagellar structure are necessary in the specific attachment to epithelial cells (Robertson et al., 2000). In a similar study comparing a non-flagellated mutant of *Salmonella* Enteritidis (lacking FliC) and a paralyzed strain (mutation in *motAB*) in adhering to chick gut explants, both mutants were significantly less adherent than the wild type strain (Allen-Vercoe & Woodward, 1999).

In several experiments, adhesion with paralyzed and aflagellated *Salmonella* strains have been investigated separately. Dibb-Fuller et al. (1999) demonstrated that an aflagellated strain of *Salmonella* Enteritidis showed less association with cultured epithelial cells. A study by Forbes et al. (2008) using a paralyzed strain of *Salmonella* Typhimurium, in which its motility was inhibited by a secretory IgA (SIgA) antibody directed against the O-antigen of lipopolysaccharide (LPS), indicated significantly less colonizing ability to epithelial cell. In a conflicting study, Yim et al. (2011) designed a PCR screening tool to recognize the point mutation of *motA* in *S*. Enteritidis strains that were isolated from nationwide epidemics in Uruguay. Mutants were classified according to the source of infection into three categories: food, egg, and humans. Results indicated that the presence of paralyzed flagella strains may impair the ability of bacteria to cause disease in humans, but it does not prevent them from attaching to chicken intestine and egg.

There have also been numerous investigations, indicating the fact that the flagella do not play a role in the adhesion to epithelial cells, but take part in invasion. To investigate the role of flagella and fimbriae in early stages of infection with *Salmonella* Enteritidis in the rat, insertionally inactivated mutants were constructed. These mutants

were unable to express the fimbriae (fim^-/fla^+), flagella (fim+/fla-), or fimbriae and flagella (fim-/fla-) genes. According to the results of this study, all mutants were able to colonize the gut, but less aflagellated mutants were found in the liver and spleen (Robertson et al., 2003). In another study, the invasion assessment of mutant strains of S. Typhimurium revealed that mutation in *fliCfljB*, which leads to an aflagellated strain with a flagellar secretory apparatus and mutation in flagella export apparatus (*flhD*), are important for the ability of the organism to invade cultured cells. However, the presence of the flagella is not necessary in cell colonization (Schmitt et al., 2001). Furthermore, mutation of *fliC* in S*almonella* Entertidis caused fewer invasions and membrane ruffling in human and avian cell lines, but revealed a different result on cell colonization. According to these data, comparing the *fliC* mutant and wild type strain did not show any difference in cell colonization in the cell culture assay, but microscopic methods showed reduction of cell colonization with the *fliC* mutant in comparison to the wild type. The same *fliC* mutant in *S*. Enteritidis showed a huge decrease in Caco-2 cells invasion, while bacterial adherence to these cells was unaffected (van Asten et al., 2000).

In a similar study, adhesion characteristics of two different mutants of *S*. Enteritidis to Caco-2 cells were compared with wild type. Infection of differentiated Caco-2 cells with *fliC* and *motA* mutants demonstrated that flagella negative mutants and flagellated, but non-motile, mutants exhibited similar adhesion when compared to the wild type strain. In the invasion assay, motile flagella showed a higher level of internalization compared to flagellum itself, and it was concluded that motility is not required for invasion, but it will accelerate the process (van Asten et al., 2004).

In conclusion, despite the considerable body of work on the role of flagella and its motility in adhesion and invasion of epithelial cells, there are still gaps in the knowledge base concerning the mechanisms for adhesion and attachment.

Use of Bioluminescence in food safety

Bioluminescence has been used as a tracking method to investigate the attachment of *Salmonlla* Kentucky on the surface of broilers. Recently, bioluminescent reporter systems have been widely used in food safety research. In a study by Hakkila et al. (2002), performance of several different reporter genes from firefly luciferase (Photinus pyralis lucFF), bacterial luciferase operon (Photorhabdus luminescens luxCDABE), green fluorescent protein (Aequorea victoria gfp), and red fluorescent protein (Discosoma sp. dsred) in whole-cell bacterial sensors were compared. Their results indicated that the luxCDABE had the fastest response with the lowest detectable concentration of analyte, demonstrating that luciferases are the best indicators in whole-cell sensor bacteria (Hakkila et al., 2002). The reaction in which the firefly produces light is listed below (Meighen & Dunlapi, 1993):

$$ATP + D\text{-luciferin} + O2 \leftrightarrow CO2 + AMP + PPi + oxyluciferin + light\ (560\ nm) \quad (1.1)$$

The bacterial luxCDABE genes of the luciferase operon originate from the marine bacteria *Vibrio fischeri* and *Vibrio harveyi* or *Photorhabdus luminescens.* This operon expresses the enzyme that catalyzes the oxidase reaction of a reduced riboflavin phosphate and a long chain fatty aldehyde (Meighen & Dunlapi, 1993):

$$FMNH2 + O2 + RCHO \leftrightarrow FMN + RCOOH + H2O + light\ (490\ nm) \quad (1.2)$$

The advantage of bacterial luciferin over the firefly is that there is no need to add a substrate (D-luciferin) (Meighen & Dunlapi, 1993).

Bioluminescence is a useful reporter system which is a real-time, sensitive and non-destructive method. Bioluminescence producing power directly depends on the viability of the cells since only metabolically active cells have this reducing power. This characteristic, allows us to use bioluminescent bacteria to assess the effect of different biological and chemical signals. This method can be used for the assessment of sterilization techniques, and detect the position of bacteria on food surfaces (Alloush et al., 2006).

Howe et al. (2010) used a Tn7-based transposon system to clone luxCDABE genes into the chromosome of several *Salmonella enterica* serotypes. *Salmonella* has been the subject of a number of bioluminescent studies with the majority conducted on poultry. Bautista et al. (1998) used bioluminescent *Salmonella* Hedar to monitor the effect of acid and temperature on cell survival on poultry carcasses. In a study by Moza et al. (2009), bioluminescent *Salmonella* Enteritidis was applied to determine the depth of penetration of the bacteria in marinated chicken breast fillets. Bioluminescent imaging can be applied to monitor biofilm formation, bacterial pathogenesis, and attachment.

Application of transposon in bacteria

In addition to bioluminescence, constructing a transposon mutant library of bacteria of interest is another way to analyze the function of different genes that are involved in virulence, attachment, or other biological reactions.

Chromosome manipulation can be divided to three categories: 1) homologous recombination; 2) site specific recombination; 3) transposon mediated integration.

Transposons are mobile genetic segments that are also called jumping genes since they move from one position in the genome to another and induce a mutation. According to site specificity of the transposon, it can insert in a specific chromosomal site, or just make a knockout mutant, which, in the case of the nonspecific insertion, random mutagenesis can be constructed. The former situation contains integration of the conjugative transposon into a target site of a conserved AT-rich sequence by site-specific recombination. An example of a site-specific transposon is Tn7, which inserts at attTn7, downstream of the *glmS* gene. Mariner, Mu, Tn3 derivatives, Tn5, Tn10, and Tn552 are examples of non-specific transposons. Randomly inserted transposons have been used for different applications, such as genetic foot-printing, gene transcriptional and translation fusion, signature-tagged mutagenesis, DNA or cDNA sequencing, and transposon site hybridization (Choi & Kim, 2009). Transposition can happen via one of two different mechanisms: cut-and-paste transposition or replicative transposition. Two enzymes are involved in these mechanisms: transposase and resolvase. These enzymes can either make a copy on the target site or two copies on both the donor and target DNA (Shapiro, 1979). Most transposons don't need a homologous sequence. Therefore, they can be used to create random mutations, which can be applied to characterizing the essentiality and functions of the interested genes. A small ratio of transposons that are site-specific usually apply for integration of a specific gene into the genome of bacteria. This specific gene can be a reporter gene, such as luciferase (*lux*) (Choi & Kim, 2009).

Drosophila-derived transposons*; mariner*

Mariner transposons have been used to create a mutant library in this research. The *mariner* family of transposons was originally found in *Drosophila mauritiana.* This

transposon is 1300 bp in length and contains 30 bp of inverted terminal repeats. *Mariner* transposon does not require a species-specific host factor for transposition and, therefore, can be used in a broad range of prokaryotes and eukaryotes for creation of random mutagenesis. Transposition occurs by a cut-and-paste process and generates TA dinucleotide at the insertion target site. It can be used in both Gram-positive and Gram-negative bacteria (Hayes, 2003).The *MAR2xT7* transposon, a derivative of *Himar1*, has been used in the creation of a mutant library. This transposon has minimal insertion site specificity that leads to the construction of a non-redundant mutant library. The *Himar1* transposase is located outside the *MAR2xT7* coding sequence on the suicide vector to prevent the transposition of the transposon to a secondary site (Liberati et al., 2006).

Bioluminescence-Based Mutant Screening Strategy (BLMS)

Karsi et al. (2009) constructed a mutant library of bioluminescent bacteria of interest that makes it possible to utilize a bioluminescent-based mutant screening assay. This method is a combination of a transposon mutant library and a bioluminescent reporter system. It allows real-time monitoring of mutant characteristics with different mutated genes at multiple time points. Viability and attachment characteristics of the mutants can be measured since they directly correlate with bioluminescent activity. Bioluminescence-based mutant screening strategy was used in a study to identify the virulence genes of specific bacteria. For this purpose, a mutant library tagged with bioluminescent, using a Tn7-based transposon system, was constructed and encountered with host serum. Bioluminescence was used to detect mutants that were more susceptible to killing by host factors (Karsi et al., 2009).

Mutants that proved to play a role in the reaction of interest with the BLMS assay were further characterized to identify the gene that has been interrupted with the transposon.

Characterization of transposon insertion site

There are a variety of techniques that can be used to characterize the DNA that flanks the region with a known sequence. One of these methods is single-primer PCR. The procedure involves just one transposon specific primer and a single PCR with three rounds of amplification. The first round is designed to amplify the transposon site as the template and is carried out at 50°C, which is a standard annealing temperature. The annealing temperature of the second round of PCR is as low as 30°C. The reason for the low temperature is to amplify the region adjacent to the transposon insertion site. At 30°C, the primer binds to sites with limited complementarity sequences on the opposite strand of DNA in which the specific primer binding sites are located. These sites are usually close enough to transposon insertion sites that it can be amplified with PCR. The third round of PCR amplifies the product from the second round and has a standard annealing temperature (Karlyshev et al., 2000). Figure 1.4 outlines the procedure for single-primer PCR.

After recognizing the genes that have been interrupted with transposon insertion, the role of these genes are further investigated with a specific in-frame mutation to confirm the obtained results.

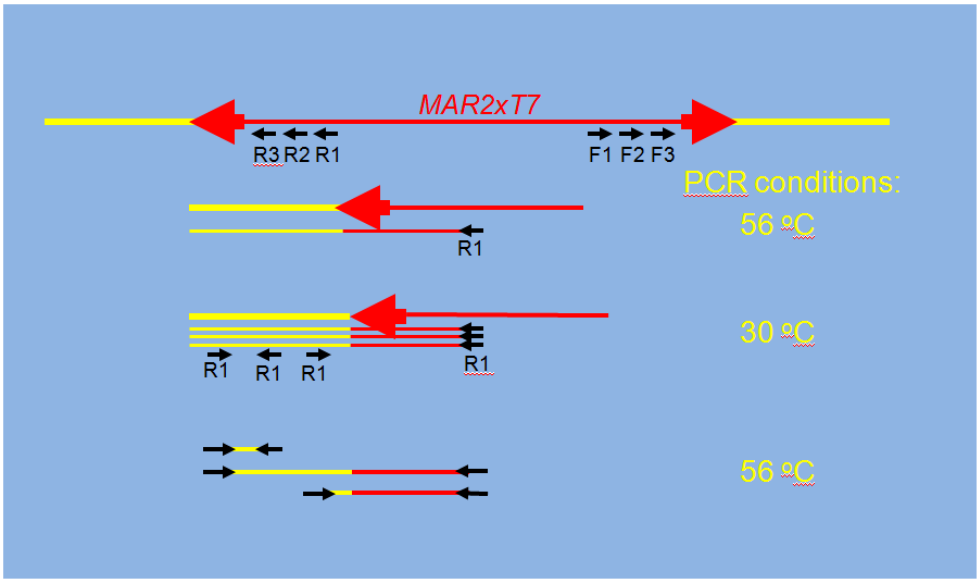

Figure 1.4 Procedure for single-primer PCR to amplify the transposon insertion site (Attila Karsi)

Making deletions using λ Red recombination system

Making in-frame mutations is one of the best ways to analyze the function of specific genes. An easy, fast, and efficient method is needed to make in-frame deletions in some flagella genes and to determine how these deletions affect the attachment rate to chicken skin and Caco-2 cells.

Although there are several allelic exchange methods that can be used for this purpose, all require the creation of a gene disruption on a plasmid prior to recombining it to the chromosome (Hamilton et al., 1989). However, it is possible to make a direct gene disruption with a linear DNA product in *Saccharomyces cervisiae*. For this purpose, a PCR product with an antibiotic resistance marker with at least 35 nt of flanking homologous section with the DNA would be transferred to the yeast (Baudin et al., 1993). However, this method was limited to yeast and a few bacteria because of the

presence of intracellular exonuclease that degrades the linear DNA (Lorenz & Wackernagel, 1994). Mutation in exonuclease V of RecBCD or *recD* of the host cell made this recombination possible (Biek & Cohen, 1986). Using a λ Red (λ, Ɓ, *exo)* homologouse recombination system that has been encoded in bacteriophages enhances the linear DNA recombination rate as compared to other methods (Murphy, 1998), although it has not been successful in making gene disruption when short homology extension was used.

The new λ Red recombination system is a very fast and efficient method. It is based on the replacement of the target gene with an antibiotic resistance gene from a linear DNA. Linear DNA is a PCR product, which is flanked by FRT region and is made with homology extension primers. Recombination is mediated with a λ Red system in homology extension regions. After gene replacement, the antibiotic resistance gene is eliminated by FLP recombinase, which is expressed by a helper plasmid. The λ Red recombination and helper plasmid are temperature-sensitive plasmids and can be cured by growing at 37°C (Datsenko & Wanner, 2000).

Another advantage of using this method is the fact that it creates a nonpolar deletion. Elimination of the antibiotic resistance gene will leave behind an 82-85-nt scar in place of deleted genes that have stop codons in all six reading frames. This scar has a ribosomal binding site and start codon for downstream genes that can create a nonpolar deletion of a gene in an operon (Datsenko & Wanner, 2000).

Using Caco-2 cells as a model

Caco-2 cell lines can be applied as a model to investigate the interaction of mutants and intestinal cells to analyze the function of the gene that has been mutated.

Originally obtained from a human colon adenocarcinoma, the human intestinal Caco-2 cell line has been widely used as a model for intestinal barriers over the last twenty years. The spontaneous growth and differentiation of the cells leads to the formation of a monolayer of cells with different morphological and functional characteristics that are similar to mature cells (Sambuy et al., 2005).

In the 1970s, several different cell lines of gastrointestinal tumors were collected to further investigate the cancer mechanisms. Due to difficulties in obtaining a differentiated intestinal cell line from a normal cell culture, scientists became interested in some properties of the tumor cell lines. They were successful in partially differentiating the cell lines using synthetic and biological factors that were added to the culture (Pinto, 1983). However, between all the tumor cell lines collected, Caco-2 cell lines showed spontaneous differentiation over the period of time in culture.

According to the studies, the Caco-2 cells' differentiations change the biochemical and morphological characteristics of the cells in a way that is similar to the small intestine (Pinto, 1983). These cells form a monolayer of polarized cells that have a tight junction with each other and microvilli on the apical side. They produce several small intestine enzymes on the apical side, although these enzymes more resemble human fetal enzymes rather than adult ileal enterocytes (Sambuy et al., 2005).

There have also been some polarized membrane growth factor receptors and transport activities on membranes that make the cells even more similar to enterocytes. One study suggested that there are transient mosaic patterns of functional intestinal patterns on Caco-2 cell lines that proliferate and differentiate separately (Vachon et al.,

1996). These findings support the idea that morphological and functional differentiation happen gradually and were not present in parental cell lines.

References

Allen-Vercoe, E., & Woodward, M. J. (1999). The Role of Flagella, but Not Fimbriae, in the Adherence of Salmonella Enterica Serotype Enteritidis to Chick Gut Explant. *Journal of Medical Microbiology, 48*(8), 771-780.

Alloush, H. M., Lewis, R. J., & Salisbury, V. C. (2006). Bacterial Bioluminescent Biosensors: Applications in Food and Environmental Monitoring. *Analytical Letters, 39*(8), 1517-1526.

Alo, O. S., & Ojo, O. (2007). Use of Antibiotics in Food Animals: A Case Study of a Major Veterinary Outlet in Ekiti-State, Nigeria. *Nigerian Veterinary Journal, 28*(1), 80-82.

Bailey, J. S., Thomson, J. E., & Cox, N. A. (1987). Contamination of Poultry During Processing. *The Microbiology of Poultry Products*, 193-211.

Barbut, S. (2002). *Poultry Products Processing: An Industry Guide*. Boca Raton, FL: CRC Press.

Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., & Cullin, C. (1993). A Simple and Efficient Method for Direct Gene Deletion in Saccharomyces Cerevisiae. *Nucleic Acids Research, 21*(14), 3329–3330.

Bäumler, Andreas J., Hargis, Billy M., & Tsolis, Renee M. (2000). Tracing the Origins of Salmonella Outbreaks. *Science, 287*(5450), 50-52.

Bautista, D. A., Chen, J., Barbut, S., & Griffiths, M. W. (1998). Use of an Autobioluminescent Salmonella Hadar to Monitor the Effects of Acid and Temperature Treatments on Cell Survival and Viability on Lactic Acid-Treated Poultry Carcasses. *Journal of Food Protection, 61*(11), 1439-1445.

Berg, H. C. (2003). The Rotary Motor of Bacterial Flagella. *Biochemistry, 72*(1), 19.

Berrang, M. E., Bailey, J. S., Altekruse, S. F., Shaw, W. K., Patel, B. L., Meinersmann, R. J., & Fedorka-Cray, P. J. (2009). Prevalence, Serotype, and Antimicrobial Resistance of Salmonella on Broiler Carcasses Postpick and Postchill in 20 Us Processing Plants. *Journal of Food Protection, 72*(8), 1610-1615.

Berrang, M. E., Ladely, S. R., & Buhr, R. J. (2001). Presence and Level of Campylobacter, Coliforms, Escherichia Coli, and Total Aerobic Bacteria Recovered from Broiler Parts with and without Skin. *Journal of Food Protection, 64*(2), 184-188.

Biek, D. P., & Cohen, S. N. (1986). Identification and Characterization of Recd, a Gene Affecting Plasmid Maintenance and Recombination in Escherichia Coli. *Journal of Bacteriology, 167*(2), 594-603.

Bishop, A., House, D., Perkins, T., Baker, S., Kingsley, R. A., & Dougan, G. (2008). Interaction of Salmonella Enterica Serovar Typhi with Cultured Epithelial Cells: Roles of Surface Structures in Adhesion and Invasion. *Microbiology, 154*(7), 1914-1926.

Bonifield, H. R., & Hughes, K. T. (2003). Flagellar Phase Variation in Salmonella Enterica Is Mediated by a Posttranscriptional Control Mechanism. *Journal of Bacteriology, 185*(12), 3567-3574.

Bravo, D., Hoare, A., Silipo, A., Valenzuela, C., Salinas, C., Álvarez, S. A., Molinaro, A., Valvano, M. A., & Contreras, I. (2011). Different Sugar Residues of the Lipopolysaccharide Outer Core Are Required for Early Interactions of< I> Salmonella Enterica</I> Serovars Typhi and Typhimurium with Epithelial Cells. *Microbial Pathogenesis, 50*(2), 70-80.

Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R., & Swaminathan, B. (2000). Salmonella Nomenclature. *Journal of Clinical Microbiology, 38*(7), 2465-2467.

Byrd, J. A., Corrier, D. E., Hume, M. E., Bailey, R. H., Stanker, L. H., & Hargis, B. M. (1998). Incidence of Campylobacter in Crops of Preharvest Market-Age Broiler Chickens. *Poultry Science, 77*(9), 1303-1305.

CDC. (2005). Preliminary Foodnet Data on the Incidence of Infection with Pathogens Transmitted Commonly through Food (Report). Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5514a2.htm?s_cid=mm5514a2_e.

Choi, K.-H., & Kim, K.-J. (2009). Applications of Transposon-Based Gene Delivery System in Bacteria. *Journal of Microbiology and Biotechnology, 19*(3), 217-228.

Conner, D. E., & Bilgili, S. F. (1994). Skin Attachment Model for Improved Laboratory Evaluation of Potential Carcass Disinfectants for Their Efficacy against Salmonella Attached to Broiler Skin. *Journal of Food Protection, 57*(8), 684-688.

Datsenko, K. A., & Wanner, B. L. (2000). One-Step Inactivation of Chromosomal Genes in Escherichia Coli K-12 Using Pcr Products. *Proceedings of the National Academy of Sciences, 97*(12), 6640-6645.

Dibb-Fuller, M. P., Allen-Vercoe, E., Thorns, C. J., & Woodward, M. J. (1999). Fimbriae-and Flagella-Mediated Association with and Invasion of Cultured Epithelial Cells by Salmonella Enteritidis. *Microbiology, 145*(5), 1023-1031.

Dickson, J. S., & Koohmaraie, M. (1989). Cell Surface Charge Characteristics and Their Relationship to Bacterial Attachment to Meat Surfaces. *Applied and Environmental Microbiology, 55*(4), 832-836.

Edwards, P. R. (1938). A New Salmonella Type: Salmonella Kentucky. *The Journal of hygiene, 38*(3), 306-308.

El-Sayed, A. (2007). Analysis of Feeds and Fertilizers for Sustainable Aquaculture Development in Egypt. *FAO Fisheries Technical Paper, 497*, 401.

Feasey, N. A., Dougan, G., Kingsley, R. A., Heyderman, R. S., & Gordon, M. A. (2012). Invasive Non-Typhoidal Salmonella Disease: An Emerging and Neglected Tropical Disease in Africa. *The Lancet, 379*(9835), 2489–2499.

Firstenberg-Eden, R., Notermans, S., & Schothorst, M. (1978). Attachment of Certain Bacterial Strains to Chicken and Beef Meat. *Journal of Food Safety, 1*(3), 217-228.

Foley, S. L., Nayak, R., Hanning, I. B., Johnson, T. J., Han, J., & Ricke, S. C. (2011). Population Dynamics of Salmonella Enterica Serotypes in Commercial Egg and Poultry Production. *Applied and Environmental Microbiology, 77*(13), 4273-4279.

Forbes, S. J., Eschmann, M., & Mantis, N. J. (2008). Inhibition of Salmonella Enterica Serovar Typhimurium Motility and Entry into Epithelial Cells by a Protective Antilipopolysaccharide Monoclonal Immunoglobulin a Antibody. *Infection and Immunity, 76*(9), 4137-4144.

Freudenberg, M. A., Merlin, T., Gumenscheimer, M., Kalis, C., Landmann, R., & Galanos, C. (2001). Role of Lipopolysaccharide Susceptibility in the Innate Immune Response to Salmonella Typhimurium Infection: Lps, a Primary Target for Recognition of Gram-Negative Bacteria. *Microbes and Infection, 3*(14-15), 1213.

Friedlander, R. S., Vlamakis, H., Kim, P., Khan, M., Kolter, R., & Aizenberg, J. (2013). Bacterial Flagella Explore Microscale Hummocks and Hollows to Increase Adhesion. *Proceedings of the National Academy of Sciences, 110*(14), 5624-5629.

Girón, J. A., Torres, A. G., Freer, E., & Kaper, J. B. (2002). The Flagella of Enteropathogenic Escherichia Coli Mediate Adherence to Epithelial Cells. *Molecular Microbiology, 44*(2), 361-379.

Goresline, H. E., Howe, M. A., Baush, E. R., & Gunderson, M. F. (1951). In-Plant Chlorination Does a 3-Way Job. *The U.S. Egg and Poultry Magazine, 57*, 12-31.

Hakkila, K., Maksimow, M., Karp, M., & Virta, M. (2002). Reporter Genes Lucff, Luxcdabe, Gfp, and Dsred Have Different Characteristics in Whole-Cell Bacterial Sensors. *Analytical Biochemistry, 301*(2), 235-242.

Hamilton, C. M., Aldea, M., Washburn, B. K., Babitzke, P., & Kushner, S. R. (1989). New Method for Generating Deletions and Gene Replacements in Escherichia Coli. *Journal of Bacteriology, 171*(9), 4617-4622.

Hayes, F. (2003). Transposon-Based Strategies for Microbial Functional Genomics and Proteomics. *Annual Review of Genetics, 37*(1), 3-29.

Howe, K., Karsi, A., Germon, P., Wills, R. W., Lawrence, M. L., & Bailey, R. H. (2010). Development of Stable Reporter System Cloning Luxcdabe Genes into Chromosome of Salmonella Enterica Serotypes Using Tn7 Transposon. *BMC Microbiology, 10*(1), 197.

Ikeda, J. S., Schmitt, C. K., Darnell, S. C., Watson, P. R., Bispham, J., Wallis, T. S., Weinstein, D. L., Metcalf, E. S., Adams, P., & O'Connor, C. D. (2001). Flagellar Phase Variation of Salmonella Enterica Serovar Typhimurium Contributes to Virulence in the Murine Typhoid Infection Model but Does Not Influence Salmonella-Induced Enteropathogenesis. *Infection and Immunity, 69*(5), 3021-3030.

Joerger, R. D., Sartori, C. A., & Kniel, K. E. (2009). Comparison of Genetic and Physiological Properties of Salmonella Enterica Isolates from Chickens Reveals One Major Difference between Serovar Kentucky and Other Serovars: Response to Acid. *Foodborne Pathogens and Disease, 6*(4), 503-512.

Johnson, T. J., Thorsness, J. L., Anderson, C. P., Lynne, A. M., Foley, S. L., Han, J., Fricke, W. F., McDermott, P. F., White, D. G., & Khatri, M. (2010). Horizontal Gene Transfer of a Colv Plasmid Has Resulted in a Dominant Avian Clonal Type of Salmonella Enterica Serovar Kentucky. *PLOS ONE, 5*(12), e15524.

Jones, G. W., Richardson, L. A., & Uhlman, D. (1981). The Invasion of Hela Cells by Salmonella Typhimurium: Reversible and Irreversible Bacterial Attachment and the Role of Bacterial Motility. *Journal of General Microbiology, 127*(2), 351-360.

Karlyshev, A. V., Pallen, M. J., & Wren, B. W. (2000). Single-Primer Pcr Procedure for Rapid Identification of Transposon Insertion Sites. *Biotechniques, 28*(6), 1078, 1080, 1082.

Karsi, A., Gülsoy, N., Corb, E., Dumpala, P. R., & Lawrence, M. L. (2009). High-Throughput Bioluminescence-Based Mutant Screening Strategy for Identification of Bacterial Virulence Genes. *Applied and Environmental Microbiology, 75*(7), 2166-2175.

Karsi, A., Howe, K., Kirkpatrick, T. B., Wills, R. W., Bailey, R. H., & Lawrence, M. L. (2008). Development of Bioluminescent Salmonella Strains for Use in Food Safety. *BMC Microbiology, 8*(1), 10.

Keener, K. M., Bashor, M. P., Curtis, P. A., Sheldon, B. W., & Kathariou, S. (2004). Comprehensive Review of Campylobacter and Poultry Processing. *Comprehensive Reviews in Food Science and Food Safety, 3*(2), 105-116.

Kihlström, E., & Edebo, L. (1976). Association of Viable and Inactivated Salmonella Typhimurium 395 Ms and Mr 10 with Hela Cells. *Infection and Immunity, 14*(4), 851-857.

Kim, K. Y., Frank, J. F., & Craven, S. E. (1996). Three-Dimensional Visualization of Salmonella Attachment to Poultry Skin Using Confocal Scanning Laser Microscopy. *Letters in Applied Microbiology, 22*(4), 280-282.

Kim, K. Y., Lillard, H. S., Frank, J. F., & Craven, S. E. (1996). Attachment of Salmonella Typhimurium to Poultry Skin as Related to Cell Viability. *Journal of Food Science, 61*(2), 439-441.

Kutsukake, K., & Iino, T. (1980). Inversions of Specific DNA Segments in Flagellar Phase Variation of Salmonella and Inversion Systems of Bacteriophages P1 and Mu. *Proceedings of the National Academy of Sciences, 77*(12), 7338-7341.

Kutsukake, K., Ohya, Y., & Iino, T. (1990). Transcriptional Analysis of the Flagellar Regulon of Salmonella Typhimurium. *Journal of Bacteriology, 172*(2), 741-747.

La Ragione, R. M., Cooley, W. A., & Woodward, M. J. (2000). The Role of Fimbriae and Flagella in the Adherence of Avian Strains of Escherichia Coli O78: K80 to Tissue Culture Cells and Tracheal and Gut Explants. *Journal of Medical Microbiology, 49*(4), 327-338.

Le Hello, S., Hendriksen, R. S., Doublet, B., Fisher, I., Nielsen, E. M., Whichard, J. M., Bouchrif, B., Fashae, K., Granier, S. A., & Jourdan-Da Silva, N. (2011). International Spread of an Epidemic Population of Salmonella Enterica Serotype Kentucky St198 Resistant to Ciprofloxacin. *Journal of Infectious Diseases, 204*(5), 675-684.

Lee, A., Smith, S. C., & Coloe, P. J. (1998). Survival and Growth of Campylobacter Jejuni after Artificial Inoculation onto Chicken Skin as a Function of Temperature and Packaging Conditions. *Journal of Food Protection, 61*(12), 1609-1614.

Liberati, N. T., Urbach, J. M., Miyata, S., Lee, D. G., Drenkard, E., Wu, G., Villanueva, J., Wei, T., & Ausubel, F. M. (2006). An Ordered, Nonredundant Library of Pseudomonas Aeruginosa Strain Pa14 Transposon Insertion Mutants. *Proceedings of the National Academy of Sciences, 103*(8), 2833-2838.

Lillard, H. S. (1985). Bacterial Cell Characteristics and Conditions Influencing Their Adhesion to Poultry Skin. *Journal of Food Protection, 48*(9), 803-807.

Lillard, H. S. (1986). Role of Fimbriae and Flagella in the Attachment of Salmonella Typhimurium to Poultry Skin. *Journal of Food Science, 51*(1), 54-56.

Lorenz, M. G., & Wackernagel, W. (1994). Bacterial Gene Transfer by Natural Genetic Transformation in the Environment. *Microbiological Reviews, 58*(3), 563.

Macnab, R. M. (1999). The Bacterial Flagellum: Reversible Rotary Propellor and Type Iii Export Apparatus. *Journal of Bacteriology, 181*(23), 7149-7153.

McMeekin, T. A., & Thomas, C. J. (1978). Retention of Bacteria on Chicken Skin after Immersion in Bacterial Suspensions. *Journal of Applied Microbiology, 45*(3), 383-387.

Meighen, E. A., & Dunlapi, P. V. (1993). Physiological, Biochemical and Genetic Control. *Advances in Microbial Physiology, 34*, 1.

Minamino, T., Imada, K., & Namba, K. (2008). Molecular Motors of the Bacterial Flagella. *Current Opinion in Structural Biology, 18*(6), 693-701.

Morgan, E., Campbell, J. D., Rowe, S. C., Bispham, J., Stevens, M. P., Bowen, A. J., Barrow, P. A., Maskell, D. J., & Wallis, T. S. (2004). Identification of Host-Specific Colonization Factors of Salmonella Enterica Serovar Typhimurium. *Molecular Microbiology, 54*(4), 994-1010.

Moza, L. F., Griffiths, M. W., & Barbut, S. (2009). Use of Bioluminescent Salmonella Enterica Serovar Enteriditis to Determine Penetration in Tumbled and Hand-Tumbled Marinated Chicken Breast Fillets. *Journal of Applied Poultry Research, 18*(2), 269-273.

Murphy, K. C. (1998). Use of Bacteriophage Λ Recombination Functions to Promote Gene Replacement in Escherichia Coli. *Journal of Bacteriology, 180*(8), 2063-2071.

Namba, K. (2001). Roles of Partly Unfolded Conformations in Macromolecular Self-Assembly. *Genes to Cells, 6*(1), 1-12.

Nevola, J. J., Stocker, B. A., Laux, D. C., & Cohen, P. S. (1985). Colonization of the Mouse Intestine by an Avirulent Salmonella Typhimurium Strain and Its Lipopolysaccharide-Defective Mutants. *Infection and Immunity, 50*(1), 152-159.

Notermans, S., & Kampelmacher, E. H. (1974). Attachment of Some Bacterial Strains to the Skin of Broiler Chickens. *British Poultry Science, 15*(6), 573-585.

Okrend, A. J., Johnston, R. W., & Moran, A. B. (1986). Effect of Acetic Acid on the Death Rates of 52 Degrees C of Salmonella Newport, Salmonella Typhimurium and Campylobacter Jejuni in Poultry Scald Water. *Journal of Food Protection, 49*(7), 500-503.

Pinto, M. (1983). Enterocyte-Like Differentiation and Polarization of the Human Colon Carcinoma Cell Line Caco-2 in Culture. *Biology of the Cell, 47*, 323-330.

Robertson, J. M. C., Grant, G., Allen-Vercoe, E., Woodward, M. J., Pusztai, A., & Flint, H. J. (2000). Adhesion of Salmonella Enterica Var Enteritidis Strains Lacking Fimbriae and Flagella to Rat Ileal Explants Cultured at the Air Interface or Submerged in Tissue Culture Medium. *Journal of Medical Microbiology, 49*(8), 691-696.

Robertson, J. M. C., McKenzie, N. H., Duncan, M., Allen-Vercoe, E., Woodward, M. J., Flint, H. J., & Grant, G. (2003). Lack of Flagella Disadvantages Salmonella Enterica Serovar Enteritidis During the Early Stages of Infection in the Rat. *Journal of Medical Microbiology, 52*(1), 91-99.

Salyers, A. A., & Whitt, D. D. (2002). *Bacterial Pathogenesis: A Molecular Approach* (2 ed.). Washington, D.C.: ASM Press.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A., & Zucco, F. (2005). The Caco-2 Cell Line as a Model of the Intestinal Barrier: Influence of Cell and Culture-Related Factors on Caco-2 Cell Functional Characteristics. *Cell Biology and Toxicology, 21*(1), 1-26.

Sanderson, K., Thomas, C. J., & McMeekin, T. A. (1991). Molecular Basis of the Adhesion of Salmonella Serotypes to Chicken Muscle Fascia. *Biofouling, 5*(1-2), 89-101.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Diseases, 17*(1), 7.

Schmitt, C. K., Ikeda, J. S., Darnell, S. C., Watson, P. R., Bispham, J., Wallis, T. S., Weinstein, D. L., Metcalf, E. S., & O'Brien, A. D. (2001). Absence of All Components of the Flagellar Export and Synthesis Machinery Differentially Alters Virulence of Salmonella Enterica Serovar Typhimurium in Models of Typhoid Fever, Survival in Macrophages, Tissue Culture Invasiveness, and Calf Enterocolitis. *Infection and Immunity, 69*(9), 5619-5625.

Shapiro, J. A. (1979). Molecular Model for the Transposition and Replication of Bacteriophage Mu and Other Transposable Elements. *Proceedings of the National Academy of Sciences, 76*(4), 1933-1937.

Shivaprasad, H. L. (2000). Fowl Typhoid and Pullorum Disease. *Revue Scientifique et Technique (International Office of Epizootics), 19*(2), 405.

Suloma, A., & Ogata, H. Y. (2006). Future of Rice-Fish Culture, Desert Aquaculture and Feed Development in Africa: The Case of Egypt as the Leading Country in Africa. *Japan Agricultural Research Quarterly, 40*(4), 351.

Thomas, C. J., & McMeekin, T. A. (1981). Attachment of Salmonella Spp. To Chicken Muscle Surfaces. *Applied and Environmental Microbiology, 42*(1), 130-134.

Thomson, J. E., Cox, N. A., & Bailey, J. S. (1976). Chlorine, Acid, and Heat Treatments to Eliminate Salmonella on Broiler Carcasses. *Poultry Science, 55*(4), 1513-1517.

Vachon, P. H., Perreault, N., Magny, P., & Beaulieu, J.-F. (1996). Uncoordinated, Transient Mosaic Patterns of Intestinal Hydrolase Expression in Differentiating Human Enterocytes. *Journal of Cellular Physiology, 166*(1), 198-207.

van Asten, F. J. A. M., Hendriks, H. G. C. J. M., Koninkx, J. F. J. G., van der Zeijst, B. A. M., & Gaastra, W. (2000). Inactivation of the Flagellin Gene of Salmonella Enterica Serotype Enteritidis Strongly Reduces Invasion into Differentiated Caco-2 Cells. *FEMS Microbiology Letters, 185*(2), 175-179.

van Asten, F. J. A. M., Hendriks, H. G. C. J. M., Koninkx, J. F. J. G., & van Dijk, J. E. (2004). Flagella-Mediated Bacterial Motility Accelerates but Is Not Required for Salmonella Serotype Enteritidis Invasion of Differentiated Caco-2 Cells. *International Journal of Medical Microbiology, 294*(6), 395-399.

van Houdt, R., & Michiels, C. W. (2005). Role of Bacterial Cell Surface Structures in Escherichia Coli Biofilm Formation. *Research in Microbiology, 156*(5), 626-633.

Wegener, H. C., Hald, T., Wong, L. F., Madsen, M., Korsgaard, H., Bager, F., Gerner-Smidt, P., & Mølbak, K. (2003). Salmonella Control Programs in Denmark. *Emerging Infectious Diseases, 9*(7), 774.

Wempe, J. M., Genigeorgis, C. A., Farver, T. B., & Yusufu, H. I. (1983). Prevalence of Campylobacter Jejuni in Two California Chicken Processing Plants. *Applied and Environmental Microbiology, 45*(2), 355-359.

Yim, L., Betancor, L., Martínez, A., Bryant, C., Maskell, D. J., & Chabalgoity, J. A. (2011). Naturally Occurring Motility-Defective Mutants of Salmonella Enterica Serovar Enteritidis Isolated Preferentially from Nonhuman Rather Than Human Sources. *Applied and Environmental Microbiology, 77*(21), 7740-7748.

Zhang, X.-L., Tsui, I. S. M., Yip, C. M. C., Fung, A. W. Y., Wong, D. K.-H., Dai, X., Yang, Y., Hackett, J., & Morris, C. (2000). Salmonella Enterica Serovar Typhi Uses Type Ivb Pili to Enter Human Intestinal Epithelial Cells. *Infection and Immunity, 68*(6), 3067-3073.

CHAPTER II

CONSTRUCTION AND SCREENING OF ATTACHMENT-IMPAIRED *SALMONELLA ENTERICA* SEROVAR KENTUCKY MUTANTS

Abstract

To gain an understanding of the mechanism of attachment of *Salmonella* to broiler skin, a bioluminescent-based mutant screening assay was used. *Salmonella* with a transposon insertion in the relevant genes showed an attenuated attachment. Thus, a random mutant library of a field-isolated bioluminescent strain of *Salmonella* Kentucky was constructed. Mutants' attachment to chicken skin was assessed in 96-well plates containing a uniform 6 mm diameter piece of circular chicken skin. After washing steps, mutants with reduced bioluminescence were selected and transposon insertion sites were identified.

Attachment attenuation in transposon-inserted genes that encodes flagella, lipopolysaccharide core biosynthesis protein, tryptophan biosynthesis and amino acid catabolism pathway, TCA cycle, conjugative transfer system, multidrug resistant protein, and ABC transporter system were seen. According to our results, mutations in flagella genes (*flgA*, *flgC*, *flgK*, *flhB* and *flgJ*) of *S.* Kentucky led to the weakest attachment. This study indicated that attachment of *Salmonella* to broiler skin surfaces could be a multifactorial process, in which flagella play an important role.

Introduction

Salmonella contamination is an important food safety concern in poultry processing plants. This organism persists in all stages of chicken processing regardless of the hygienic steps taken. While poultry intestines are considered the most probable origin of contamination, abundant bacteria have been detected on the surface of the broilers. Recently, *Salmonella enterica* serovar Kentucky has been recognized as one of the most prominent *Salmonella* serovar in poultry processing and appears to be resistant to all washing steps (Berrang et al., 2009). According to the National Antimicrobial Resistance Monitoring System (NARMS), the prevalence of *S*. Kentucky isolates from broiler chicken has elevated from 25% in 1997 to 50% in 2007 (Foley et al., 2011). This new emerging multi-drug-resistant bacterium may not be considered a major source of human disease, but it carries a virulence plasmid from avian pathogenic *Escherichia coli* that gives the bacteria an enhanced ability to colonize the chicken intestine by enabling the bacteria to survive the intestinal environment (Fricke et al., 2009).

Salmonella attachment to chicken skin is a complicated process. Thomas and McMeekin (1981) suggested that attachment is a result of bacterial retention in a network of fibers that forms when chicken muscle fascia is immersed in water. Kim et al. (1996b) isolated *Salmonella* from cervices and feather follicles, suggesting that *Salmonella* can be entrapped in water inside the follicles. While cell charge was considered as an important attachment factor (Dickson & Koohmaraie, 1989), Kim et al. (1996) indicated that cell charge did not affect the attachment rate. Bacterial concentration and inoculation time are other aspects that have been suggested as contributors to the attachment of bacteria to chicken skin (Kim, Frank, et al., 1996a). There also has been conflicting information on

the role of some surface structures (e.g., fimbriae, pili and flagella) on the attachment of the bacteria to the broiler skin surface (Lillard, 1985, 1986; Notermans & Kampelmacher, 1974).

Molecular studies have indicated that adhesion is involved in the attachment of bacteria to chicken fascia, and there should be some receptors involved in this process. *Salmonella* mostly attach to chicken fascia, which is composed of collagen and elastin interspread in the glycosaminoglycan (GAG) matrix. The GAG components are hyaluronan and chondroitin sulphate. Hyaluronan is the part of matrix that interacts with *Salmonella*. Several proteins on the surface of *Salmonella* could bind hyaluronan (Sanderson et al., 1991). However, the exact characteristics and properties of these binding sites are not completely understood. Recently, emergence of a persistent serovar of *Salmonella* in broiler production plants highlights the need for a better understanding of the molecular basis of attachment for this bacterium.

The purpose of this research is to test the hypothesis that *S.* Kentucky must possess gene products that mediate the bacterial attachment to chicken skin and the mutation of these genes will prohibit or limit the attachment. The rationale of this research was to expand our knowledge of the attachment mechanism in *S.* Kentucky and provide scientifically valid information to assist the broiler processing industry in meeting regulatory concerns for pre- and post-harvest food safety.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Escherichia coli SM10 *λpir* (Karsi et al., 2009) was used as the donor strain in conjugations for transfer of p*MAR2xT7* (Karsi et al., 2009) into bioluminescent *S.*

Kentucky carrying pBEN276 (Karsi et al., 2008). This strain was originally isolated from a broiler processing plant. Bioluminescent *E. coli* DH5α and wild type *S.* Kentucky carrying pBEN276 were used as negative and positive controls. *E. coli* and *Salmonella* strains were grown on Luria- Bertani broth and agar plates with selective antibiotics.at 37°C.

MAR2xT7, a derivative of the mariner family transposon *Himar1* was used to make a mutant library. This is not a site-specific transposon and can transpose in both prokaryotic and eukaryotic genomes. It shows minimal insertion-site specificity and its transposase is located outside the transposon, which leads to single transposon insertion and prevents secondary insertions. There are T7 promoters at both ends of transposon and gentamycin resistance gene in between the transposon ends (Liberati et al., 2006). Figure 2.1 shows the map of p*MAR2xT7*.

Bioluminescent *S.* Kentucky strain was established using the plasmid pBEN276. This plasmid expresses a site-specific recombination system due to the presence of the bacterial transposon Tn7. The Tn7 system inserts at the *attTn7* site and integrates in such a way that the right end of Tn7 is adjacent to the 3' end of the *glmS* gene. This is a temperature-sensitive plasmid and can be cured after gene transfer by culturing at 42°C. The *luxCDABE* operon is inserted between the transposon arms and owns the *E. coli frr* promoter. This promoter controls the expression of a house keeping gene, which leads to continuous expression of the *Lux* operon. The advantage of using this system is that the insertion of the *Lux* operon to the chromosome is site-specific and does not interrupt the function of any genes (Howe et al., 2010).

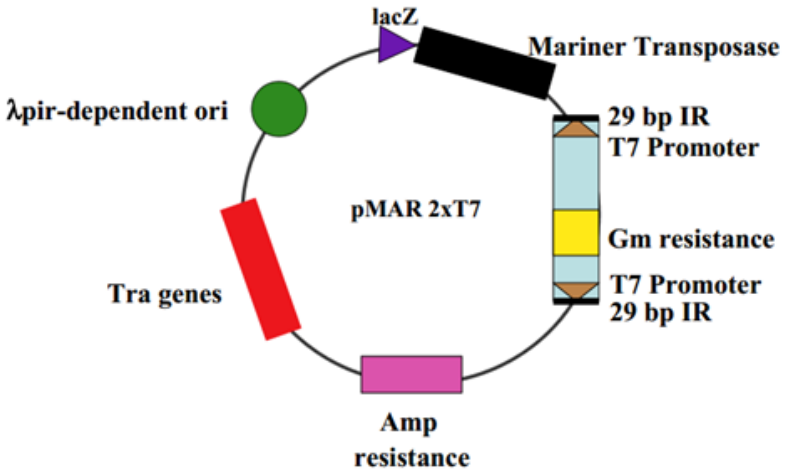

Figure 2.1 Plasmid p*MAR 2xT7* vector

http://ausubellab.mgh.harvard.edu/pa14/downloads/MAR2xT7_Map.pdf

Insertion of bacterial *lux* operon in to *Salmonella* chromosome

Insertion of the *luxCDAB* operon to the *S*. Kentucky chromosome has been previously described by Howe et al. (2010). Briefly, pBEN276 carrying the *lux* operon was transferred into the competent *S*. Kentucky by electroporation. First, *S*. Kentucky was washed with 10% ice-cold glycerol four times. Following the last wash, the glycerol was removed and the residual glycerol was kept to make a slurry of the washed cells. In preparation for electroporation, 25 µl of competent cells were mixed with 2 µl of plasmid and transferred to a 0.1 mm electroporation cuvette. Electroporation was performed using the following parameters: 1.8 kV, 25 µF, and 400 Ω. Cells were recovered in SOC media at 30°C for 1 h at 200 rpm. Transformants were selected on LB media with ampicillin (100 µg/ml) at 30°C. Several colonies were picked and cultured in LB broth that was supplemented with arabinose overnight to induce transposition. The cultured cells were streaked on LB agar at 42°C to cure the plasmid (Howe et al., 2010).

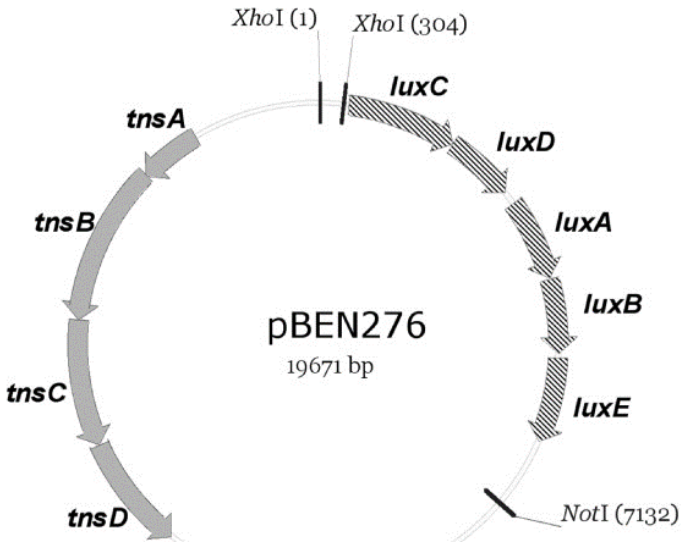

Figure 2.2 Plasmid pBEN276 vector

http://www.biomedcentral.com/1471-2180/10/197/figure/F1

Antibiotic sensitivity test

In order to differentiate the recipient from the donor cells in the final stage of conjugation, an antibiotic sensitivity test with more than 15 different antibiotic disks was conducted. A specific antibiotic that recipient was resistant to with removed donor strain was identified. To study the minimum inhibitory concentrations (MICs) of the identified antibiotic, the concentrations of 2, 5, 10, 25, 50 and 100 µg/ml of the selected antibiotic was tested for both donor and recipient strain. The final concentration of the antibiotic was applied for further selection of the recipient cells.

Preparation of transposon insertion library

p*MAR2xT7* was transferred from *E. coli* SM10 λ *pir* into bioluminescent *S.* Kentucky by conjugal mating (Karsi et al., 2009). Briefly, a colony of *E. coli* SM10 *λpir* and *S.* Kentucky were inoculated in 5 ml of LB broth at 37°C overnight at 200 rpm.

Equal amount of overnight culture of donor and recipient (1.5 ml) was pelleted separately by centrifugation, washed three times with LB broth, and resuspended in 1 ml of LB broth. Donor and recipient strains were mixed with different ratios of 1:3, 1:4 and 1:5 (with a starting point of 20 µl of donor with 60 µl of recipients). The mixture was centrifuged at 12,100 x g for 2 min. The harvested cells were diluted in 10 µl of LB broth and transferred to 0.45 µm sterile filter, which was placed on LB agar and incubated at 37°C for 18 h. The filter was washed with 5 ml LB broth and 50 µl washed bacteria was plated onto LB agar containing gentamicin and streptomycin (Karsi & Lawrence, 2007). A batch of gentamycin-resistant colonies was tested for the presence of transposon insertion using single-primer PCR and sequencing. After this, more than 2000 colonies of mutant *S*. Kentucky were picked using a pipette tip and inoculated in 150 µl of LB plus gentamycin broth in 96-well plates and incubated in an incubator shaker overnight. Later, 50 µl of glycerol was added to the wells and plates were sealed and stored at -80°C (Karsi et al., 2009).

Chicken skin attachment assay

In order to reactivate the mutants kept in the freezer, a clear-bottomed 96-well black cell culture plates containing 100 µl of LB broth were prepared. Each well was inoculated with 5 µl of mutant bacteria from each well of frozen stock (one replicate per mutant). Each 96-well plate contained four replicates of positive and negative controls (wild type strain of *S*. Kentucky pBEN276 and *E. coli* DH5α pBEN276 consequently). Plates were covered with Breath-Easy film (Diversified Biotech, Boston, MA) and grown at 37°C overnight at 250 rpm on a shaker incubator. After verifying the mutants' growth, a second 96-well plate was prepared by inoculating with 5 µl of overnight bacteria and

incubated at 37°C for 2 h to grow to log phase in order to reach the highest possible bioluminescent activity.

Cleaned, flat, and frozen chicken skins were cut into uniform, circular sections by 6 mm skin biopsy punch (Figure 2.3) and introduced into each well. Plates were incubated at room temperature (25°C) for 1 h to allow bacterial attachment to the broiler skin (Figure 2.4).

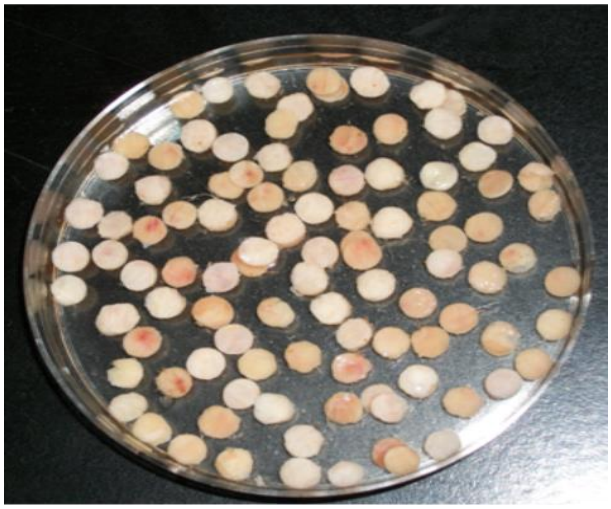

Figure 2.3 6 mm chicken skin cuts

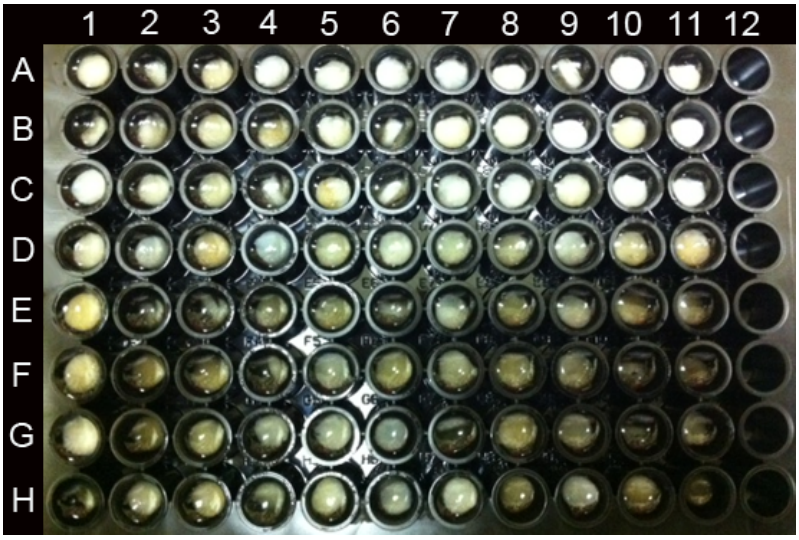

Figure 2.4 96-well plate containing chicken skin cuts

Following incubation, the suspensions were removed by suction, and the wells were washed with 200 µl of distilled water by pipetting twice. The water was removed by suction, which also removed unattached bacteria. Plates were incubated at 37°C for 10 min and bioluminescent imaging was recorded for 15 s of exposure at 37°C using an IVIS Imaging System 100 series (Figure 2.5) and bioluminescence was quantified with Living Image software (Howe et al., 2010).

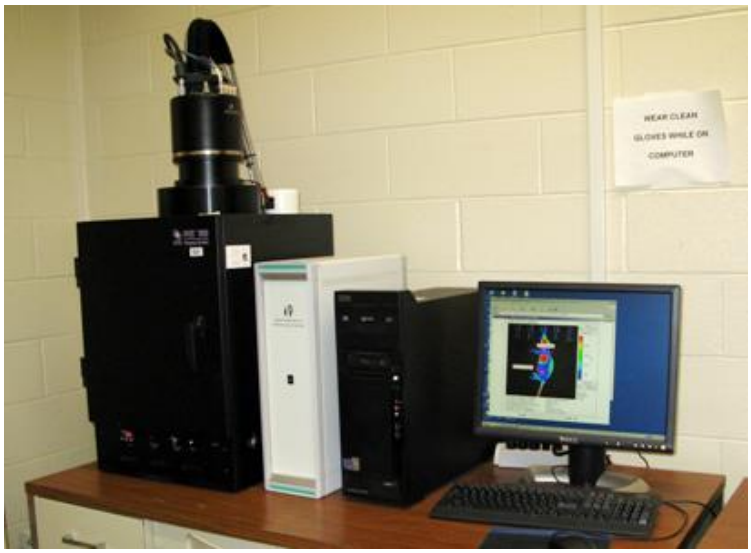

Figure 2.5 Bioluminescent imaging was recorded with IVIS imaging system

To determine the effect of washing on mutants attachment properties, plates were filled with 200 µl of water and placed in a rotating platform incubator at 700 rpm for 1 h. After removal of excess solution, bioluminescence on skin sections was measured and recorded for 15 s of exposure.

Determination of mutants with attenuated attachment potential

The change in bioluminescence originating from the number of attached bacteria in each well, before and after the washing step with the positive and negative controls, is indicative of bacterial attachment properties. After initial screening, mutants that had a high reduction in bioluminescence were tested again in quadruplicate and the average bioluminescence was used to calculate the differences. Candidates with high percentage of detachment rate were chosen for transposon end mapping.

Identification of transposon insertion site

Transposon insertion sites for the mutants with reduced attachment to chicken skin were identified by using single-primer PCR (Karsi et al., 2009). Briefly, genomic DNA was prepared from overnight cultures using a Wizard Genomic DNA Purification kit (Promega, Madison, WI). Each 25 µl of PCR reaction contained 0.2 µM forward or reverse transposon specific primer, 0.2 mM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$ and 1.25 U of *Taq* polymerase (Promega, Madison, WI). PCR was performed using a PTC-100 thermocycler (MJ Research, Water town, MA) with the following cycling steps : initial denaturation (2 min at 94°C); followed by 25 cycles of denaturation (30 s at 94°C); annealing (30 s at 55°C) and elongation (3 min at 72°C), followed by 30 cycles of denaturation (30 s at 94°C); annealing (30 s at 30°C) and elongation (2 min at 72°C), followed by 30 cycle of denaturation (30 s at 94°C); annealing (30 s at 55°C) and elongation (2 min at 72°C) and the final extension (10 min at 72°C).The PCR product was cleaned with ExoSAP-IT enzyme mix (USB Corp. Cleveland, Ohio). Sequencing was conducted using BigDye Terminator v1.1 with 2 µl of cleaned DNA product as a template, 0.5 µM of a nested transposon specific primer and 8.0 µl of Terminator Ready Reaction Mix. The transposon end was trimmed and remaining sequences were checked against the protein database of the NCBI using the BLAST program which revealed the transposon insertion location (Karsi et al., 2009).

Results

Transposon insertion library

Ampicillin-resistant bioluminescent colonies were obtained successfully (300-500 transposants out of a 1 ml conjugation reaction). Using this bioluminescent recipient and

MAR2xT7 transposon, a total of 2112 colonies with transposon insertion were obtained. The best donor to recipient ratio was 1:3 with 20 and 60 µl of each. According to the antibiotic sensitivity test, 50 µg ml^{-1} of gentamicin and 50 µg ml^{-1} of streptomycin were appropriate for selection of the mutants and removal of the donor. Considering occurrence of random insertion, this number means half of the *Salmonella* genes were represented in the library.

Identification of mutants with reduced skin attachment properties

A library of 2112 *S.* Kentucky mutants was generated. Comparing bioluminescence measurements before (Figure 2.6) and after washing steps (Figure 2.7) resulted in 264 mutants with decreased bioluminescence compared to wild-type *S.* Kentucky (positive control). Of these 264 mutants, 88 had a dramatic reduction in bioluminescence before starting the washing step and 176 mutants displayed bioluminescence reduction after washing with agitation.

A second screening of selected mutants (total of 264), done in quadruplicate, resulted in 66 mutants with attenuated attachment. Wild-type *S.* Kentucky was not removed from chicken skin after an hour of washing while *E. Coli* DH5α was completely removed (Figures 2.6 and 2.7 last column first four wells are positive and the last four wells are negative controls). A total of 66 mutants showing complete or reduced attachment were chosen for insertion mapping.

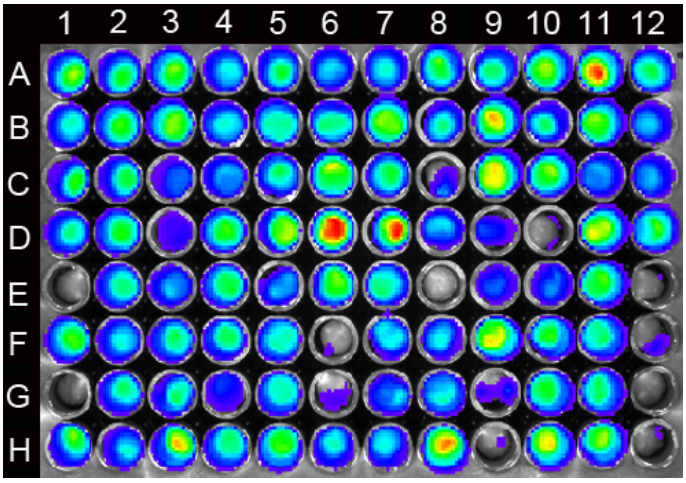

Figure 2.6 Bioluminescence imaging of 96-well plate at time zero

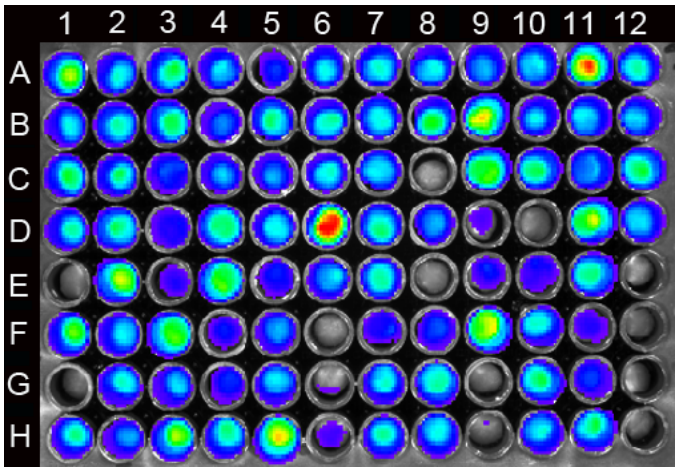

Figure 2.7 Bioluminescence image of 96-well plate after washing step

The first four wells in the last column (A12, B12, C12, D12) are wild type *S.* Kentucky (positive control) and the same column last four wells (E12, F12, G12, H12) are *E. Coli* (negative control). The rest of the wells represent individual mutants that have been repeated in 4 plates.

Identification of Transposon insertions in *S.* Kentucky genome

Transposon inserted genes for 66 mutants with attenuated attachment characteristics were identified using single-primer PCR. Of these 66 mutants, those with

non-attached characteristics (removed before washing step) mostly had various mutations in different flagella structure genes (Table 2.2). Other mutants with attenuated attachment properties showed insertions in different genes: LPS structure, amino acid catabolism, TCA cycle pathway, conjugative transfer system, signaling and transportation system, metabolism, different enzymes phage tail fiber protein H, fimbrial export usher protein, membrane proteins, and several unnamed proteins (Table 2.1).

Table 2.1 List of attachment attenuated mutants and their associated protein function that were removed after washing with agitation

	SEQ#	MUTANT	GI	FUNCTION
		Plate 1		
1	1	P02F10-P01G01	363549433	3-dehydroquinate dehydratase
2	2	P04G08-P01E02	161617071	hypothetical protein SPAB_04904
3	3	P07D05-P01C06	213586960	major outer membrane lipoprotein
4	4	P07G06-P01E06	213417570	dihydrolipoamide acetyltransferase
5	5	P07G09-P01F06	353667979	Dihydrolipoamide succinyltransferase
6	6	P08F01-P01A08	213852747	polynucleotide phosphorylase/polyadenylase
7	7	P08C05-P01B08	167994734	type IV conjugative transfer system coupling protein TraD
8	8	P09B04-P01D09	353657416	Lipopolysaccharide biosynthesis protein RffA, partial
9	9	P09E05-P01F09	363552450	ADP-heptose:LPS heptosyltransferase II
10	10	P10H10-P01D10	16765937	antiterminator-like protein
11	37	P10D11-P01G11	374980070	DNA helicase IV
12	38	P04H01-P01F02	16767450	phage tail fiber protein H
13	39	P06F05-P01H04	197248508	precorrin-4 C11-methyltransferase
14	40	P10F06-P01B11	16767420	NADH pyrophosphatase
15	41	P10D07-P01G10	392752129	conserved protein with nucleoside triphosphate hydrolase domain
16	42	P08F09-P01H07	224586194	Oligoribonuclease
		Plate 2		
1	22	P12F08-P02D02	213028242	glutamyl-Q tRNA(Asp) synthetase
2	23	P13H05-P02A03	321222757	Chaperone protein HscA
3	24	P14B06-P02H03	213861459	chaperone protein HscA
4	25	P16D03-P02A05	353564343	Shikimate 5-dehydrogenase AroDI gamma
5	26	P16H02-P02C05	213162217	multifunctional fatty acid oxidation complex sub-unit alpha
6	27	P17C05-P02A07	5410518	SppA
7	28	P18H08-P02C07	161502533	NADH dehydrogenase sub-unit H
8	29	P23E10-P02E10	16766771	ribulose-phosphate 3-epimerase
9	30	P25D03-P02C11	51449510	tryptophan synthase beta sub-unit
10	31	P15C06-P02G04	289807180	ATP-dependent RNA helicase DeaD
11	32	P11H11-P02B02	323200048	putative regulatory protein
12	33	P15C03-P02H04	213583420	glutathione reductase
13	34	P21E02-P02C09	16767287	aldolase
14	35	P12C04-P02F02	357959410	putative sodium/sulfate transporter, partial
15	36	P18C07-P02F07	16767274	GTP-binding protein
16	59	P23F01-P02D10	289826303	ornithine decarboxylase
17	60	P16E01-P02B05	16759660	hypothetical protein STY0758
18	61	P19F07-P02H08	161353580	oxidoreductase
19	62	P25G02-P02F11	16766811	intramembrane serine protease GlpG
20	63	P19A07-P02A08	381293021	fimbrial outer membrane usher protein
21	64	P23C06-P02B10	213618564	pyruvate dehydrogenase sub-unit E1
22	65	P20C11-P02B09	16764020	alpha ribazole-5'-P phosphatase
23	67	P18D02-P02G07	353600411	4-hydroxythreonine-4-phosphate dehydrogenase
24	68	P22G01-P02A10	353617221	permease protein SitC
25	69	P13B03-P02E03	392612905	putative sensor kinase protein
26	70	P10C09-P02C01	392811833	exoribonuclease II
27	71	P22E10-P02G09	297521596	phosphopyruvate hydratase
28	72	P19H03-P02B08	205352403	high-affinity zinc transporter periplasmic protein

Mutants are listed in each plate according to the degree of attachment attenuation

Table 2.2 List of attachment attenuated mutants removed before the washing step

	SEQ#	MUTANT	GI	FUNCTION
1	11	P09G05-P03F06	353571650	Flagellar basal-body P-ring formation protein FlgA, partial
2	12	P03G04-P03G01	353605658	Flagellar basal-body rod protein FlgC
3	13	P24B04-P03D10	62180543	unnamed protein product
4	14	P21C09-P03G09	213420820	flagellar biosynthesis protein FlhB
5	15	P05D08-P03D03	353073575	multidrug resistance protein, SMR family
6	16	P05H05-P03C03	213584956	cysteine/glutathione ABC transporter membrane/ATP-binding component
7	17	P09H05-P03G06	213421346	lipopolysaccharide core biosynthesis protein
8	18	P15B11-P03C08	353663511	Flagellar hook-associated protein Flgk
9	19	P22D04-P03H09	213615933	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA
10	20	P25E10-P03E10	16764538	flagellar rod assembly protein/muramidase FlgJ
11	43	P16F02-P03F8	353593388	DamX protein
12	44	P13F05-P03G07	353577876	Nitrogen regulation protein NR2, partial
13	45	P05E06-P03F02	213029736	dimethyladenosine transferase
14	46	P17E05-P03D09	16761949	1-acyl-glycerol-3-phosphate acyltransferase
15	47	P12H05-P03F07	285803803	Chain A, Dna-Binding Transcriptional Repressor Acrr
16	49	P25F11-PO3C11	289804587	cystathionine beta-lyase
17	50	P08C02-P03E05	16764529	flagellar basal body P-ring biosynthesis protein FlgA
18	51	P13C07-P03H07	379699155	parB gene product
19	52	P02E02-P03B01	353611155	two-component sensor kinase SsrA
20	53	P16F04-P03G08	395983535	heme lyase sub-unit NrfE
21	54	P06D02-P03F04	205357693	hypothetical protein SeI_A3977
22	56	P05F11-P03H02	353651358	Membrane protein, suppressor for copper-sensitivity ScsD, partial

Mutants are listed according to the degree of attachment attenuation

Discussion

In this study, we used random transposon mutagenesis on bioluminescent *Salmonella*. Kentucky, to identify the genes involved in attachment to chicken skin. Our results showed that *S*. Kentucky attachment to broiler skin is a multifactorial process and requires the expression of many genes. We recognized two different groups of mutants with distinguished phenotypes according to attachment attenuation pattern. First group (plate number 3) showed the most attachment reduction with no attachment to chicken skin. The second group of mutants (plate numbers 1 and 2), with less attachment attenuation, were removed from the chicken skin after one hour of washing with agitation. The non-attached group had various mutations in different genes, but

transposon insertion in six different flagella genes highlights the role of flagella in attachment compared to other mutants in this group. These six mutants (P09G05-P01F06 and P08C02-P03E05, P03G04-P01G01, P15B11-P01C08, P25E10-P03E10 and P21C09-P01G09) harbored transposon insertion in *flgA* (two different mutations), *flgC, flgK, flgJ* and *flhB*). *flgA* encodes flagellar basal-body p-ring formation protein. The *flgC* encodes flagellar basal-body rod protein. The *flgK* encodes flagellar hook-associated protein, *flgJ* encodes a rod assembly protein and *flhB* encodes flagellar biosynthesis protein. According to these results, flagella seems to contribute to the attachment of *S*. Kentucky to chicken skin. Previous studies indicated conflicting results on the role of flagella on attachment of *Salmonella* to broiler skin. In a study by Notermans and Kampelmacher (1974) using several different bacteria, attachment to broiler skin was dependent on the presence of flagella. In later studies by Lillard (1985) he concluded that under controlled conditions, non-flagellated bacteria attach as willingly as flagellated bacteria. In his later experimentation, Lillard (1986) stated that attachment is a complex reaction, in which fimbria and flagella might not play an important role in attachment, but they do have a contribution to the process. Flagella have an important role in adherence of *Salmonella enterica* serovar Enterica to chicken intestine according to Allen-Vercoe and Woodward (1999). Their results indicated that non-flagellated mutants are less adherent when compared to wild type and a flagellated, but non-motile mutant, is less adherent. However, this depends on assay conditions

The second phonotypic group with less attachment attenuation mostly had transposon insertion in LPS structure, biosynthesis of aromatic amino acids, TCA cycle pathway, conjugative transfer system, signaling and transportation system, metabolism,

different enzymes, phage tail fiber protein H, fimbrial export usher protein, membrane proteins, and several unnamed proteins.

Attachment reduction in phage tail fiber protein H and type IV secretion-like conjugative transfer system coupling protein mutants may be due to existence of functional and structural similarities between these two filamentous structures (Blocker et al., 2003). It has been suggested that substrate of a type III secretion system is encoded by cluster of phage tail and tail fiber protein (Hardt et al., 1998). A mutation in the phage tail region would inactivate the type III secretion system and its flagella like needle complex consequently. A similar case is the mutation in the type IV secretion system having a sex pilus for initiating physical contact between the donor and recipient cells (Gomis-Ruth et al., 2004). All these secretion systems have one structure in common, an extracellular filamentous surface appendage that might be a possible reason for their involvement in the attachment process.

Furthermore, mutation in transporter and signaling systems, which have higher expression in attached bacteria, make these mutants more susceptible to environmental conditions. It has been suggested that these transporter systems work as an efflux pump to help the bacteria resist the stress (Svensson et al., 2008).

Furthermore, other parts of the cell structure and metabolites have also been shown to contribute to the attachment of *Salmonella* on broiler skin. P09H05-P01G06 has an insertion in the waaG gene, which encodes the lipopolysaccharide (LPS) core biosynthesis protein. In *E. coli* LPS is known to contribute to attachment. Some mutations in *E. coli* genes that involved lipopolysaccharide core biosynthesis showed a decreased adhesion to solid surfaces (Genevaux et al., 1999). Moreover, Otto and Silhavy

(2002) studied the role of membrane lipoprotein on bacterial attachment and suggested that under specific environmental conditions, an outer membrane lipoprotein, NIpE, might sense and generate an adhesion signal to the Cpx pathway that leads to stable adhesion. In this study, in addition to waaG, three other mutants had insertions in a part of the membrane. P07D05-P01C06 was mutated in lpp which encodes the major outer membrane lipoprotein, and P09B04-P01D09 was mutated in rffA that encodes lipopolysaccharide biosynthesis protein, and P09E05-P01F09 was mutated in rfaF which encodes ADP-heptose:LPS heptosyltransferase II that contributes to synthesis of the inner core backbone of the membrane.

P25D03-P01C11 has an insertion in trpB, which encodes tryptophan beta sub-unit synthase. Tryptophan is a major factor in forming *Salmonella enterica* serovar Typhimurium biofilms on food surfaces. Studies have shown that amino acids metabolites, especially tryptophan biosynthesis, have been up-regulated at early stages of attachment. Although attachment of *Salmonella* to broiler skin is not considered a biofilm formation process, it is comparable with bacterial attachment to solid surfaces at the early stage of biofilm formation. The over-expression of tryptophan and its precursor, Indole, can be explained by signaling characteristics of Indole in *E. coli*. Indole prepares the cells for the nutrient-poor environments and increases catabolism of amino acids. Indole also up-regulates detoxifying genes (e.g., drug exporters) to make the bacterium more resistant to the toxic environments and compounds and increase bacterial adherence to surfaces (Hamilton et al., 2009). These characteristics can be vital in adhering *Salmonella* to chicken skin. Also, P16D03-P01A05 and P02F10-P01G01 had insertions in aroDI and aroD that encodes for 5-dehydrogenase gamma and 3-dehydroquinate dehydratase,

respectively. These compounds are both enzymes in the Shikimate pathway and are involved in the biosynthesis of aromatic amino acids. These mutations also emphasize the importance of tryptophan in the attachment process.

Overall, the results of this study indicate that the attachment of *S*. Kentucky to broiler skin is a complex process that results from the combination of effects of several different cell structure factors and cell metabolites. We discovered that the high incidence of flagella mutated genes in non-attached group of mutants can be the indicative of the important role of flagella in attachment of *S*. Kentucky to broiler skin. Further investigations, especially in flagella structure and motion genes, can lead to a better understanding of the exact molecular mechanism of *Salmonella* attachment to poultry skin.

References

Allen-Vercoe, E., & Woodward, M. J. (1999). The Role of Flagella, but Not Fimbriae, in the Adherence of Salmonella Enterica Serotype Enteritidis to Chick Gut Explant. *Journal of Medical Microbiology, 48*(8), 771-780.

Berrang, M. E., Bailey, J. S., Altekruse, S. F., Shaw, W. K., Patel, B. L., Meinersmann, R. J., & Fedorka-Cray, P. J. (2009). Prevalence, Serotype, and Antimicrobial Resistance of Salmonella on Broiler Carcasses Postpick and Postchill in 20 Us Processing Plants. *Journal of Food Protection, 72*(8), 1610-1615.

Blocker, A., Komoriya, K., & Aizawa, S.-I. (2003). Type Iii Secretion Systems and Bacterial Flagella: Insights into Their Function from Structural Similarities. *Proceedings of the National Academy of Sciences, 100*(6), 3027-3030.

Dickson, J. S., & Koohmaraie, M. (1989). Cell Surface Charge Characteristics and Their Relationship to Bacterial Attachment to Meat Surfaces. *Applied and Environmental Microbiology, 55*(4), 832-836.

Foley, S. L., Nayak, R., Hanning, I. B., Johnson, T. J., Han, J., & Ricke, S. C. (2011). Population Dynamics of Salmonella Enterica Serotypes in Commercial Egg and Poultry Production. *Applied and Environmental Microbiology, 77*(13), 4273-4279.

Fricke, W. F., McDermott, P. F., Mammel, M. K., Zhao, S., Johnson, T. J., Rasko, D. A., Fedorka-Cray, P. J., Pedroso, A., Whichard, J. M., LeClerc, J. E., White, D. G., Cebula, T. A., & Ravel, J. (2009). Antimicrobial Resistance-Conferring Plasmids with Similarity to Virulence Plasmids from Avian Pathogenic Escherichia Coli Strains in Salmonella Enterica Serovar Kentucky Isolates from Poultry. *Applied and Environmental Microbiology, 75*(18), 5963-5971. doi: 10.1128/aem.00786-09

Genevaux, P., Bauda, P., DuBow, M. S., & Oudega, B. (1999). Identification of Tn10 Insertions in the Rfag, Rfap, and Galu Genes Involved in Lipopolysaccharide Core Biosynthesis That Affect Escherichia Coli Adhesion. *Archives of Microbiology, 172*(1), 1-8.

Gomis-Ruth, F. X., Sola, M., & Coll, M. (2004). Coupling Factors in Macromolecular Type-Iv Secretion Machineries. *Current Pharmaceutical Design, 10*(13), 1551-1565.

Hamilton, S., Bongaerts, R., Mulholland, F., Cochrane, B., Porter, J., Lucchini, S., Lappin-Scott, H., & Hinton, J. (2009). The Transcriptional Programme of Salmonella Enterica Serovar Typhimurium Reveals a Key Role for Tryptophan Metabolism in Biofilms. *BMC Genomics, 10*(1), 599.

Hardt, W.-D., Urlaub, H., & Galán, J. E. (1998). A Substrate of the Centisome 63 Type Iii Protein Secretion System of Salmonella Typhimurium Is Encoded by a Cryptic Bacteriophage. *Proceedings of the National Academy of Sciences, 95*(5), 2574-2579.

Howe, K., Karsi, A., Germon, P., Wills, R. W., Lawrence, M. L., & Bailey, R. H. (2010). Development of Stable Reporter System Cloning Luxcdabe Genes into Chromosome of Salmonella Enterica Serotypes Using Tn7 Transposon. *BMC Microbiology, 10*(1), 197.

Karsi, A., Gülsoy, N., Corb, E., Dumpala, P. R., & Lawrence, M. L. (2009). High-Throughput Bioluminescence-Based Mutant Screening Strategy for Identification of Bacterial Virulence Genes. *Applied and Environmental Microbiology, 75*(7), 2166-2175.

Karsi, A., Howe, K., Kirkpatrick, T. B., Wills, R. W., Bailey, R. H., & Lawrence, M. L. (2008). Development of Bioluminescent Salmonella Strains for Use in Food Safety. *BMC Microbiology, 8*(1), 10.

Karsi, A., & Lawrence, M. L. (2007). Broad Host Range Fluorescence and Bioluminescence Expression Vectors for Gram-Negative Bacteria. *Plasmid, 57*(3), 286-295.

Kim, K. Y., Frank, J. F., & Craven, S. E. (1996a). Attachment of Salmonella on Modified Poultry Skin Surface. *Journal of Food Science, 61*(2), 442-444.

Kim, K. Y., Frank, J. F., & Craven, S. E. (1996b). Three-Dimensional Visualization of Salmonella Attachment to Poultry Skin Using Confocal Scanning Laser Microscopy. *Letters in Applied Microbiology, 22*(4), 280-282.

Kim, K. Y., Lillard, H. S., Frank, J. F., & Craven, S. E. (1996). Attachment of Salmonella Typhimurium to Poultry Skin as Related to Cell Viability. *Journal of Food Science, 61*(2), 439-441.

Liberati, N. T., Urbach, J. M., Miyata, S., Lee, D. G., Drenkard, E., Wu, G., Villanueva, J., Wei, T., & Ausubel, F. M. (2006). An Ordered, Nonredundant Library of Pseudomonas Aeruginosa Strain Pa14 Transposon Insertion Mutants. *Proceedings of the National Academy of Sciences, 103*(8), 2833-2838.

Lillard, H. S. (1985). Bacterial Cell Characteristics and Conditions Influencing Their Adhesion to Poultry Skin. *Journal of Food Protection, 48*(9), 803-807.

Lillard, H. S. (1986). Role of Fimbriae and Flagella in the Attachment of Salmonella Typhimurium to Poultry Skin. *Journal of Food Science, 51*(1), 54-56.

Notermans, S., & Kampelmacher, E. H. (1974). Attachment of Some Bacterial Strains to the Skin of Broiler Chickens. *British Poultry Science, 15*(6), 573-585.

Otto, K., & Silhavy, T. J. (2002). Surface Sensing and Adhesion of Escherichia Coli Controlled by the Cpx-Signaling Pathway. *Proceedings of the National Academy of Sciences, 99*(4), 2287-2292.

Sanderson, K., Thomas, C. J., & McMeekin, T. A. (1991). Molecular Basis of the Adhesion of Salmonella Serotypes to Chicken Muscle Fascia. *Biofouling, 5*(1-2), 89-101.

Svensson, S. L., Frirdich, E., & Gaynor, E. C. (2008). Survival Strategies of Campylobacter Jejuni: Stress Responses, the Viable but Nonculturable State, and Biofilms *Campylobacter* (3 ed., pp. 571-590). Washington, DC: ASM Press.

Thomas, C. J., & McMeekin, T. A. (1981). Attachment of Salmonella Spp. To Chicken Muscle Surfaces. *Applied and Environmental Microbiology, 42*(1), 130-134.

CHAPTER III

ROLE OF FLAGELLA IN ATTACHMENT OF *SALMONELLA ENTERICA* SEROVAR KENTUCKY TO BROILER SKIN

Abstract

Non-typhoidal *Salmonella* are the main source of bacterial contamination in the poultry industry. Recently, *Salmonella enterica* serovar Kentucky has been recognized as the most prominent *Salmonella* serovar in poultry processing plants. High prevalence of this *Salmonella* serovar can be correlated to its high ability to attach to broiler surfaces, which makes this bacterial strain a suitable representative for investigation of bacterial attachment to poultry surfaces. Based on the literature and our previous study, flagella seem to be one of the main factors that contribute to bacterial attachment to broiler skin. In this current study, we made deletions in several flagella structural sub-units, *flgK, fliC and fljB*, and flagella motor force (*motA*) to differentiate the role of flagella and its motility in colonization. Our results demonstrated that the deletion of the *flgK* gene caused the inability of the bacteria to assemble the flagella, which made it significantly less adherent than the wild type. Furthermore, the presence of the motile flagella sub-unit (FliC) is necessary for bacterial attachment to the broiler skin. Although, to our best knowledge, the expression of the alternative flagella sub-unit (FljB) leaves the bacteria motile, the presence of this active flagella sub-unit is not adequate to make a tight attachment.

Introduction

It is estimated that, non-typhoidal *Salmonella* is the leading cause of foodborn hospitalization in the United States. The most important route of contamination is consuming undercooked or contaminated food. In recent years, there has been a rising trend in the consumption of poultry in the United States. Given that processed poultry has been associated with the presence of *Salmonella,* the risk of exposure to *Salmonella enterica* may have been increased. Recent studies concerning *Salmonella* serotypes' contamination of poultry have shown the emergence of a multi-drug-resistant strain of *Salmonella* enterica serotype Kentucky in several European and African countries. In the US, this serotype has been reported to be the most prominent *Salmonella* serotype in broiler processing. However, so far, there has been no report of the antibiotic-resistant strain or evidence of it causing human disease. *Salmonella* has been shown to colonize on broiler surfaces and persist in all stages of chicken processing regardless of the hygienic steps that may have been taken.

Cell surface structures are the determining factor for bacterial attachment to surfaces (van Houdt & Michiels, 2005). Non-flagellated bacteria rarely attach to broiler skin (Notermans & Kampelmacher, 1974). A biofilm formation study has shown that some of the flagella defective mutants of *Salmonella enterica* serovar Typhimurium have lost their ability to form a biofilm (Elhadidy & Hashem, 2012). Also, non-flagellated and paralyzed mutants of *Salmonella enterica* serotype Enteritidis were less adherent to chick gut explant than the wild type strain (Allen-Vercoe & Woodward, 1999). However, conflicting data has suggested that motility of bacteria has a negligible role in bacterial attachment when compared to bacterial density (McMeekin & Thomas, 1978; Thomas &

McMeekin, 1981). Furthermore, in an adhesion study, paralyzed flagella were shown not to prevent *Salmonella enterica* serovar Enteritidis from colonizing chicken and egg (Yim et al., 2011).

Previous work in our lab indicated that flagella may play an important role in colonizing *Salmonella* Kentucky on broiler skin. In this study, *Salmonella enterica* serovar Kentucky was chosen due to the fact that epidemiological investigations of *S.* Kentucky in the US as well as several European countries have shown an increase in the incidence of this serotype. The focus of this study was to differentiate the role of motility and flagella on the attachment of bacteria to broiler skin. For this purpose, several mutations in structural sub-units and the flagellar motor gene were generated in the bioluminescent strain of *S.* Kentucky. The attachment of non-flagellated mutants, non-motile strains, and those with one of the flagellin sub-units, were compared with that of the wild type in a series of established broiler skin attachment assays with the use of bioluminescence as a tracking tool.

Materials and Methods

Bacteria, Plasmids and Media:

Salmonella enterica serovar Kentucky was made bioluminescent by transposition of the *lux* operon in to the chromosome by plasmid pBEN276 (Howe et al., 2010). *E. coli* DH5α carrying pBEN276 was used as a negative control for the chicken skin attachment assay. Plasmids: pCP20, pKD46, pKD3, and pKD4 were used to generate genetic deletions and were provided by Yale University (Datsenko & Wanner, 2000). Table 3.1 shows the list of bacterial strains and plasmids that have been used in the current study.

Transformants were selected from LB agar containing ampicillin and chloramphenicol with respective concentrations of 100 and 10µg/ml.

Table 3.1 Bacterial strains and plasmids used in this study

Name of bacteria	Characteristics	Source or Reference
Salmonella enterica serovar Kentucky	Wild type *::luxCDAB*	Laboratory collection
E.coli DH5α	Contained *Lux* gene	Laboratory collection
*S.*KΔ*fliC*	Mutation in alternative flagellin sub-unit, ApS, Cm^S	This study
*S.*KΔ*fljB*	Mutation in alternative flagellin sub-unit, ApS, Cm^S	This study
*S.*KΔ*motA*	Mutation in motion gene, ApS, GmS	This study
*S.*KΔ*flgK*	Mutation in hook associated protein, ApS, GmS	This study
*S.*KΔ*fliCfljB*	Double mutation in both flagellin sub-units, ApS^S, GmS	This study
*S.*KΔ*flhB*-t	Transposon mutation in flagella export apparatus, GmR	Previous study
*S.*KΔ*flgA*-t	Transposon mutation in basal-body p-ring protein, GmR	Previous study
*S.*KΔ*flgK*-t	Transposon mutation in hook associated protein, GmR	Previous study
*S.*KΔ*flgJ*-t	Transposon mutation in rod assembly protein, GmR	Previous study
*S.*KΔ*flgC*-t	Transposon mutation in basal-body rod protein, GmR	Previous study
*S.*KΔ*motAC*	Complement strain of *S.*KΔ*motA,* ApR	This study
*S.*KΔ*flgK*C	Complement strain of *S.*KΔ*flgK,* ApR	This study
*S.*KΔ*fliCfljBC*	Complement strain of *S.*KΔ*fliCfljB,* ApR^R	This study
E.coli K-12(BW25141/pKD3)	*(lacIq rrnBT14 ΔlacZWJ16 ΔphoBR580 hsdR514 ΔaraBADAH33 ΔrhaBADLD78 galU95 endABT333 uidA(ΔMluI)::pir+ recA1)*	Yale University (Source of strain B.L. Wanner).
E.coli K-12(BW25141/pKD4)	*(lacIq rrnBT14 ΔlacZWJ16 ΔphoBR580 hsdR514 ΔaraBADAH33 ΔrhaBADLD78 galU95 endABT333 uidA(ΔMluI)::pir+ recA1)*	Yale University (Source of strain B.L. Wanner).
E.coli DH5α/pCP20	*ΔargF-lac)169 φ80dlacZ58 glnV44 λ-rfbC1 gyrA96 recA1 spoT1 thi-1 hsdR17*	Yale University (Source of strain B.L. Wanner).
E.coli K-12 (BW25113)/pKD46 ($lacI^q$ $rrnB_{T14}$ Δ$lacZ_{WJ16}$ *hsdR514* Δ$araBAD_{AH33}$ Δ$rhaBAD_{LD78}$)		Yale University (Source of strain B.L. Wanner)

Plasmids	Characteristics	Reference
pCP20	*Ts-rep, [c857](lambda)ts ,bla(*ApR*), cat, [*FLP*]*	Yale University (Source of strain B. L. Wanner)
pKD46	*repA101(ts), araBp-gam-bet-exo, oriR101, bla(*ApR*)*	Yale University (Source of strain B. L. Wanner)
pKD3	*oriR6Kγ, bla(*ApR*), rgnB(Ter), cat*	Yale University (Source of strain B. L. Wanner)
pKD4	*oriR6Kγ, bla(ApR), rgnB(Ter), kan*	Yale University (Source of strain B. L. Wanner)
pBEN276	*mTn7::MC, ApR, luxCDABE*	Howe et al.
pBBR1MCS-4	Expression vector; *ApR*	Kovach et al

PCR reaction and primers

PCR was performed (50 µl reaction) using the 2720 thermal cycler AB Applied Biosystem with the following cycling protocol: initial denaturation (for 5 min at 94°C); followed by 30 cycles of denaturation (for 30 s at 94°C); annealing (for 30 s at 56°C) and elongation (for 1 min at 72°C), and final extension (for 10 min at 72°C).

Table 3.2 List of primers used to generate and verify the deletions and complementation of the mutants

Primers	Sequence (5′-3′)
flhC	TGTGGATGCGGTGATTAAAG
motB	GCGCATATACGGCTCAACTT
sekA_A1494	CAGGCAAGACTCAGGGAGTT
sekA_A1497	TGGACGCCACGGTAGACTTA
flgL	TGCTGGGTAACACTTTTCTCG
flgJ	ACTGGCCAGCGAACAAAG
sekA_A2274	TACCGTATGCGTTATTCAGCA
sekA_A2276	AAGGGTTCGCTCGACAATTA
c1	TTATACGCAAGGCGACAAGG
c2	GATCTTCCGTCACAGGTAGG
*motA*H1P1F1	GTGCTTATCTTATTAGGTTACCTGGTGGTTATCGGTACAGGTGTAGGCTGGAGCTGCTTC
*motA*H2P2 R1	TCATGCTTCCTCAGTCGTCTGCTGCTGGTTTGGGTTTCTCCATATGAATATCCTCCTTA
*motA*H1P1F2	TGTCATAGTCAACAGCGGAAGGATGATGTCGTGCTTATCTTATTAGGTTAGTGTAGGCTGGAGCTGCTTC
*motA*H2P2 R2	TTACGACGACAATGGGATGAGCCTGATTTTTCATGCTTCCTCAGTCGTCTCATATGAATATCCTCCTTA
*fliC*H2p2	ATTGTGTACCACGTGTCGGTGAATCAATCGCCGGACATATGAATATCCTCCTTA
*fliC*H1p1	AATAACATCAAGTTGTAATTGATAAGGAAAAGATCGTGTAGGCTGGAGCTGCTTC
*flgK*H2p2	CATCTGGGTACTGATACGCATGTCATCCTTCTCCTCATATGAATATCCTCCTTA
*flgK*H1p1	TAACAACGAGTATTGAAGGATTAAAAGGAACCATCGTGTAGGCTGGAGCTGCTTC
*fljB*H1p1	TTGCTTTATCAAAAACCTTCCAAAAGGAAAATTTTGTGTAGGCTGGAGCTGCTTC
*fljB*H2p2	CCCCGGATTCACGGGGCTGAATAAAACAAAATAAACATATGAATATCCTCCTTA
*flgK*Fc	[1]AA**GAGCTC**GCGAATCTCGACAATCT
*flgK*Rc	[2]AA**CCCGGG**ATACGCATGTCATCCTT
*fliC*Fc	[1]AA**GAGCTC**CCTTGATTGTGTACCAC
*fliC*Rc	[2]AA**CCCGGG**GAAATTCAGGTGCCGA
*motA*Fc	[1]AA**GAGCTC**CTCACGCTATCACCTCG
*motB*Rc	[2]AA**CCCGGG**GAAACGGTGTGGACAA

[1] Bold letters represent *SacI* restriction site added to 5′ end of primer sequence.[2] Bold letters represent S*maI* restriction site added to 5′ end of primer sequence. AA nucleotides were added to the end of each restriction site to increase the enzyme efficiency.
Underlined letters stand for homology extensions added to 3′ end of primer sequence.

To mutate the *motA* gene, a series of forward and reverse primers was designed with different homology extension length and location. The first set of primers had 40 nucleotides with a homology extension that was complementary to the inside *motA* gene. The homology extension of the second set of primers was 50 nt in length and consisted of 30 nt outside the gene and 20 nt inside the gene and both primers had a 20 nucleotide at 3′ end that was complementary to template plasmid pKD3. To mutate *fliC*, *flgK* and *fljB* genes, primers had 35nt of homology extension that was complementary to adjacent sides of the genes of interest. Primer sequences are listed in Table 3.2.

Gene Disruption:

Deletion in phase 1 and 2 flagellin genes (*fliC*) and (*fljB*) hook associated protein (*flgK*), and flagella motor gene (*motA*) of *Salmonella* Kentucky was achieved using the λ red recombinase system as described previously by Datsenko and Wanner (2000). Briefly, *Salmonella* Kentucky competent cells were transformed with Red helper plasmid (pKD46), an ampicillin-resistant and temperature-sensitive plasmid that contains an arabinose-induced λ Red recombinasing system.

Competent cells were made by washing the *Salmonella* Kentucky cells with 10% ice-cold glycerol water. In brief, the overnight culture of bacteria was aliquoted equally into four 50 mL centrifuge bottles and was centrifuged at 10,000 rpm for 10 min at 4°C. Cells were washed three times with cold sterilized glycerol water (10% glycerol). After the third spin, the supernatant was poured off and the pellets from the four tubes were combined into one tube with 25 mL glycerol water. The tube was washed once more and the supernatant was poured off and the bacteria were resuspended in the remaining liquid (This step also has been performed with a bench top centrifuge as described above and

had the same result with a lesser yield). Electroporation was performed in a 1 mm cuvette using the following parameters: 1.8 kV, 25 µF, and 400 Ω. Cells were recovered in SOC media at 30°C for 1 h at 200 rpm. Transformants were selected on LB media with ampicillin (100 µg/ml) at 30°C. After 24 h, successful transformants were selected.

Cells carrying pKD46 were grown overnight in LB broth with ampicillin (100µg/ml) at 30°C and 1 ml was transferred to 100 ml of LB broth supplemented with ampicillin and L-arabinose (10 mM) at 30°C for 3-4 h to reach the O.D. $_{(600)}$ of 0.6. Cells were made competent and transformed with PCR products, which were amplified previously. The PCR product consisted of the chloramphenicol gene (cm^r) flanked by flippase recognition target (FRT) sites, which were amplified from pKD3 and primers with homology extension from 5′ and 3′ end of target gene. PCR products were digested with 1 µl *Dpn*I (Promega, Madison, WI) enzyme added to 50 µl PCR product at 37°C for 1 h and gel purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

In preparation for electroporation, 40 µl competent cells were mixed with 2-5 µl of PCR product with a final concentration of 20-50 ng/µl and transferred to a 1 mm electroporation cuvette. The transformed cells were recovered in SOC media at 30°C for 3-4 h at 200 rpm. A portion of the cell suspension (100-200 µl) was spread on LB media with 10 µg/ml chloramphenicol and incubated at 37°C to select successful transformants. After 24 h, colonies were picked and sub-cultured at 42°C to cure plasmid pKD46.

PCR verification

In order to verify that the mutants have the right structure, several primers were designed about 400 to 500 base pairs upstream and downstream of the gene of interest (Table 3.3). These site specific primers consisted of: *flgJ* and *flgL*, *sekA_A1497* and

sekA_A1494, *motB* and *flhC*, *sekA_A*2274 and *sekA_A*2276. Two test primers for chloramphenicol (c1 and c2) were used in combination with the site-specific primers in PCR reactions to verify that new junctions were created after recombination. PCR products were sequenced for confirmation. Primer sequences are listed in Table 3.2.

Table 3.3 Primers that have been applied for junction confirmation of constructed mutants

Deleted gene	Up-stream chloramphenicol gene junction	Down-stream chloramphenicol gene junction	Up –stream, down-stream gene
flgK (A)	C2, flgL (A1)	A2: C1, flgJ (A2)	flgL, flgJ (A3)
fliC (B)	C2, sekA-A1494 (B1)	C1, sekA-A1497 (B2)	sekA-A1494, sekA-A1497 (B3)
motA (C)	C2, motB (C1)	C1, flhC (C2)	motB, flhC (C3)
fljB (D)	C2, sekA-A2274 (D1)	C1, sekA-A2276 (D2)	sekA-A2274, sekA-A2276 (D3)

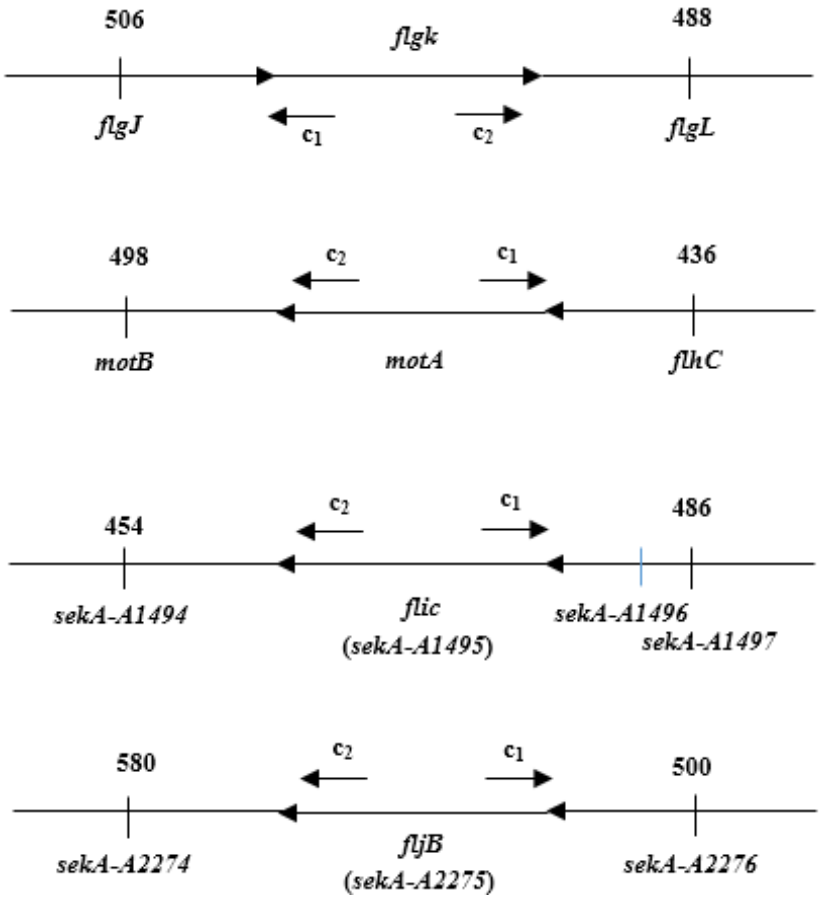

Figure 3.1 The location of designed primers for the junction verification of the mutants

Removing Antibiotic resistance gene:

In order to remove the antibiotic cassette after recombination, mutated cells were transformed with pCP20 using the electroporator with similar parameters mentioned above. Transformed cells were recovered for 1 h at 30°C in SOC and spread on an ampicillin plate. Amp^r colonies were selected after 48 hours and plated on LB agar at 43°C and were tested for loss of all antibiotic resistances. Non-resistance colonies were also PCR-verified with Cm primers to make sure the antibiotic has been removed.

Construction of complement strains

The flgKFc, flgKRc, fliCFc, fliCRc, motAFc and motBRc primers were designed at 50 bp upstream of the *flgK, fliC* and *motA* genes flanked by *SmaI* and *SacI* restriction site sequences in order to amplify the whole genes with their operons. Genomic DNA was isolated from *Salmonella* Kentucky WT using a Wizard Genomic DNA Kit (Promega, Madison, WI). Genes and their related operon were amplified using *S.* Kentucky WT genomic DNA as the template in a 50 µl PCR reaction containing 0.2 mM dNTP mix, 1.5 mM MgCl2, 0.2 mM primers, and 1.25U Taq DNA polymerase (Promega, Madison, WI). In the case of *motA*, a long fragment consisting of both *motA* and *motB* genes was amplified since *motA* and *motB* share several nucleotides and mutation in *motA* can interfere with *motB* expression as well. Amplified fragment length for *motAmotB*, *fliC*, and *flgK* were 1,813bp, 1,487bp, and 1,661bp, respectively. PCR products were purified using a PCR Clean-Up Kit (Promega, Madison, WI) and were digested with *SmaI* and *SacI* restriction enzymes (Promega, Madison, WI) and gel-purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Cloning vector, pBBR1MCS-4, was isolated from an over-night *E. coli* culture by a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA n), cut with *Sac*I and *Sma*I restriction enzymes, and the gel was purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). PCR fragments were ligated into the cloning vector with T4 DNA ligase (Promega, Madison, WI) at 4°C over-night. Purified plasmids were size-checked and those with larger sizes were cut with *SmaI* and *SacI* restriction enzymes and the presence and size of inserts were confirmed on 1% agarose gel. Further, plasmids were sequenced for confirmations,

which were then electroporated into each mutant. Preparation of electrocompetent *S.* Kentucky mutants and transformation were achieved as described previously.

Motility test

The motility assay was performed by stabbing the motility agar with a bacterial strain and the movement of bacteria from the inoculation line was assessed after incubation at 37°C for 18 h. Motility agar contained 10 g of trypton and 5 g of NaCl per 1 liter plus 0.35% (wt/vol) agar, pH 7.4 (Schmitt et al., 2001).

Scanning electron microscopy

Poly-L-Lysine coated glass cover slips were placed in 6-well cell culture plates containing LB broth. Each well was inoculated with a flagella mutant with, a lambda red and MAR2xT7 system. Plates were placed in a shaker incubator and incubated at 37°C at 250 rpm overnight. Cover slips were removed from each well, air-dried, prepared according to Merritt et al. (2010), and viewed with a field emission scanning electron microscope (JEOL JSM-6500F).

Chicken skin attachment assay

Chicken skin attachment properties of all the flagella mutants and the complement strains were compared with positive and negative controls in the following assay. The clear-bottomed 96-well black cell culture plates containing 100 µl of LB broth were prepared. Each row (containing eight wells) was inoculated with 5 µl of flagella mutant bacteria that were constructed with Lambda Red in-frame mutations and their complement strains as well as transposon insertion mutations described in Chapter 2. Each 96-well plate contained eight replicates of positive and negative controls (wild type

strain of *S.* Kentucky pBEN276 and *E. coli* DH5α pBEN276). The plate was covered with Breath-Easy film (Diversified Biotech, 1208 V.F.W Parkway, Boston, MA 02132) and grown overnight at 37°C in a shaker-incubator at 250 rpm. A second 96-well plate was prepared by inoculating with 5 µl of overnight bacteria and incubated for 2 h to grow to log phase to reach the highest bioluminescent activity possible. Uniform circular chicken skin sections were made using a 6-mm skin biopsy punch and placed into each well. Plates were incubated at room temperature (25°C) for 1 h to allow bacterial attachment. Following incubation, the suspensions were removed by suction, and wells were washed with 200 µl of distilled water by pipetting twice. The extra water was suctioned to remove unattached bacteria.

Bioluminescent imaging was recorded for 15 s of exposure at 37°C using an IVIS Imaging System 100 series and the number of bacteria was quantified with Living Image software. To determine the effect of washing on mutants' attachment properties, wells were filled with 200 µl of water and were shaken on a plate shaker at 700 rpm for 1 h. After removal of excess solution, bioluminescence on skin sections was measured and recorded following 15 s of exposure.

Determination of mutants with attenuated attachment

The comparison of the average mean of attached bacteria (the total light) for each row (containing eight wells) after the washing step with the positive and negative controls can be indicative of the mutants' detachment properties. Loss of attachment property in the mutants reveals the vital role of the gene of interest in chicken skin colonization.

Statistics

Mutants' adhesion assay to broiler skin was analyzed with completely randomized design in eight replications. The mean value of bioluminescence (P/S) for each strain was compared with ANOVA test (SPSS 21.0 software program) and Tukey's multiple comparisons post-test to investigate the difference in mutants' adhesion. The P value of ≤ 0.05 was considered statistically significant.

Results

Construction of Δ*fliC*, Δ*fljB*, Δ*fliCfljB*, Δ*flgK* and Δ*motA* mutants

Confirmation PCR reaction with chloramphenicol and site-specific primers on several mutant representatives revealed that all mutants had the correct junction (Figure 3.2). The Δ*fljB* was not shown here as it was deleted later after construction of *ΔfliC*.

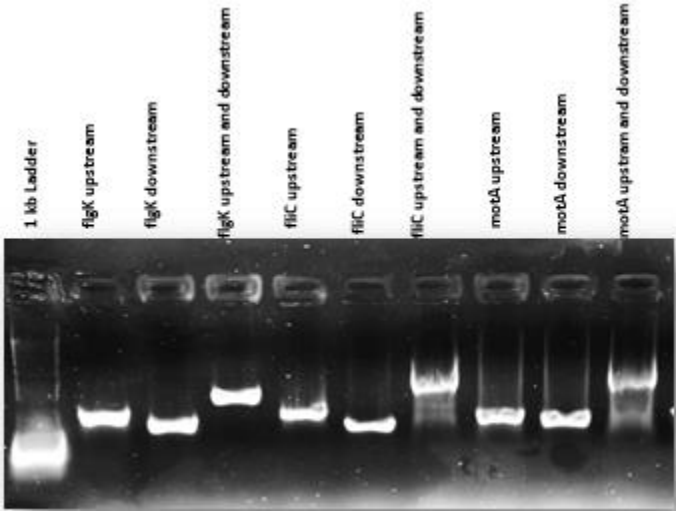

Figure 3.2 PCR confirmation for the correct mutant junction

Bands from left to right: 1 Kb plus Ladder, (A1) upstream junction of Δ*flgK* with chloramphenicol and *flgL*, (A2) downstream junction of Δ*flgK* with chloramphenicol and *flgJ*, (A3) A fragment containing upstream and downstream of Δ*flgK*, (B1) upstream junction of Δ*fliC* with chloramphenicol and *sekA-A1494*, (B2) downstream junction of Δ*fliC* with chloramphenicol and *sekA-A1497*, (B3) A fragment containing upstream and downstream of Δ*fliC, (C1)*) upstream junction of Δ*motA* with chloramphenicol and *motB*, (A2) downstream junction of Δ*motA* with chloramphenicol and *flhC*, (A3) A fragment containing upstream and downstream of Δ*motA*.

Upon elimination of the antibiotic resistant gene, the resulting PCR fragment with site-specific primers had the correct expected length. The mutants were chloramphenicol sensitive and PCR reaction with chloramphenicol specific primers did not yield any product.

The motility test revealed that all the mutants except for the Δ*fliC* and Δ*fljB* mutants that were mutated in one of the alternate flagellin sub-units are non-motile. The Δ*motA* was mutated in the flagella motor genes. Therefore, regardless of the mutant having flagella, it was non-motile (Figure 3.3).

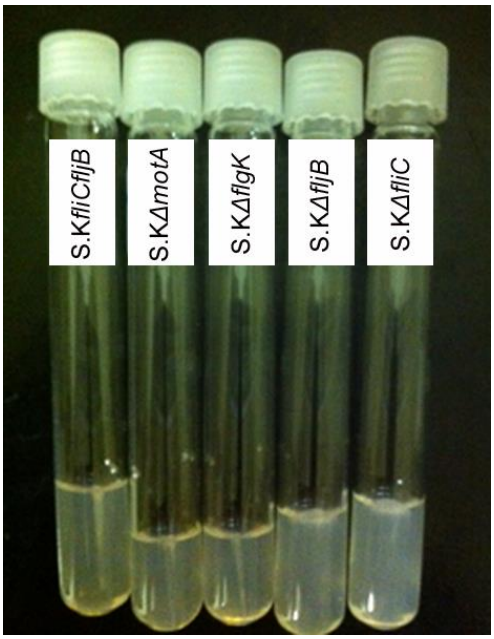

Figure 3.3 Motility test results after 18h

From left to right: *S*.K*fliCfljB*, *S*.KΔ*motA*, *S*.KΔ*flgK*, *S*.KΔ*fljB*, *S*.KΔfliC

The scanning electron microscopy images (Figure 3.4) on both flagella transposon mutants from a previous study and in-frame deletion from the current study for non-motile mutants showed no trace of flagella with the exception of Δ*motA* mutant (Figure 3.4, Image E) that had paralyzed flagella. Flagella were observed in *ΔfliC* and *ΔfljB* mutants (Figure 3.4, Images B and C), although in a different shape and location.

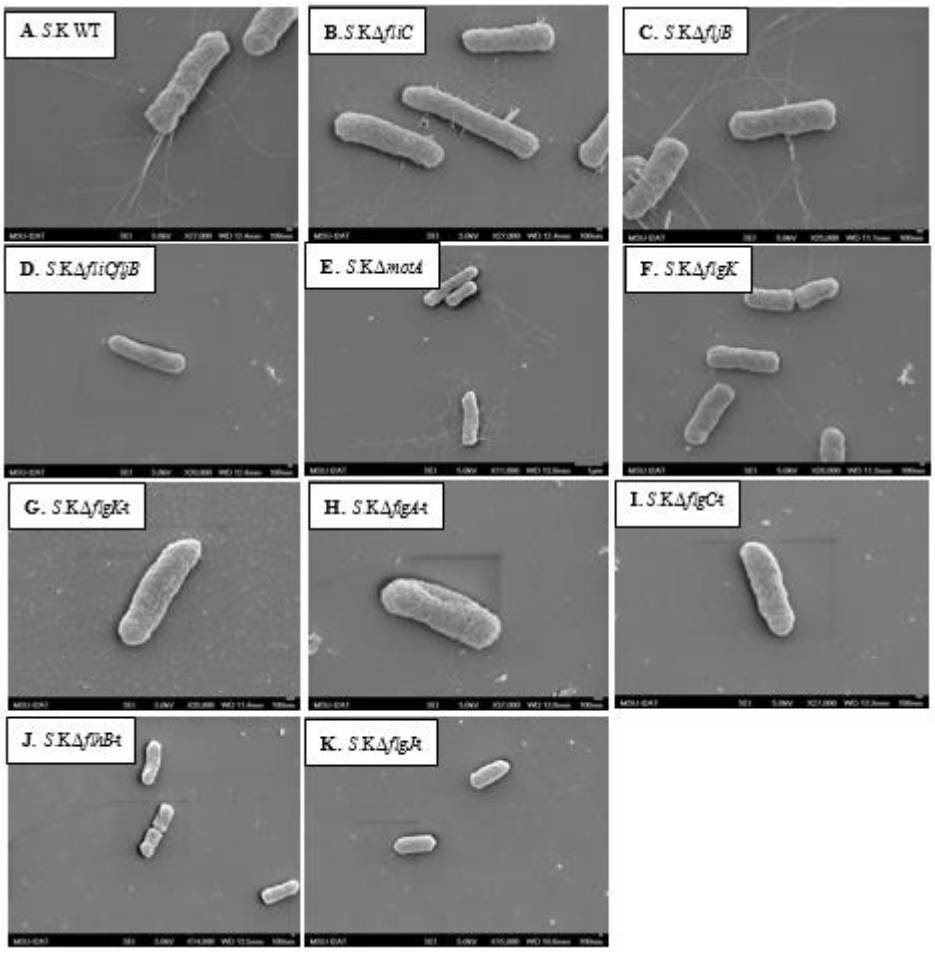

Figure 3.4 SEM images of the mutants made with lambda red system and transposon insertion.

A. *S*. Kentucky WT, B. *S*.KΔ*fliC*, C. *S*.KΔ*fljB*, D. *S*.KΔ*fliCfljB*, E. *S*.KΔ*motA*, F. *S*.KΔ*flgK*, G. *S*.KΔ*flgK*-t, H. *S*.KΔ*flgA*-t, I. *S*.KΔ*flgC*-t, J. *S*.KΔ*flhB*-t, K. *S*.KΔ*flgJ*-t.

Sequence analysis results of the mutants confirmed the deletion of the gene immediately after the homology extension nucleotides (Figures 3.5, 3.6, 3.7, 3.8). All of the deletions are considered non-polar mutations as elimination of the antibiotic-resistant gene created a scar containing the stop codon and also a ribosomal binding site and start codon for the downstream gene expression (Datsenko & Wanner, 2000).

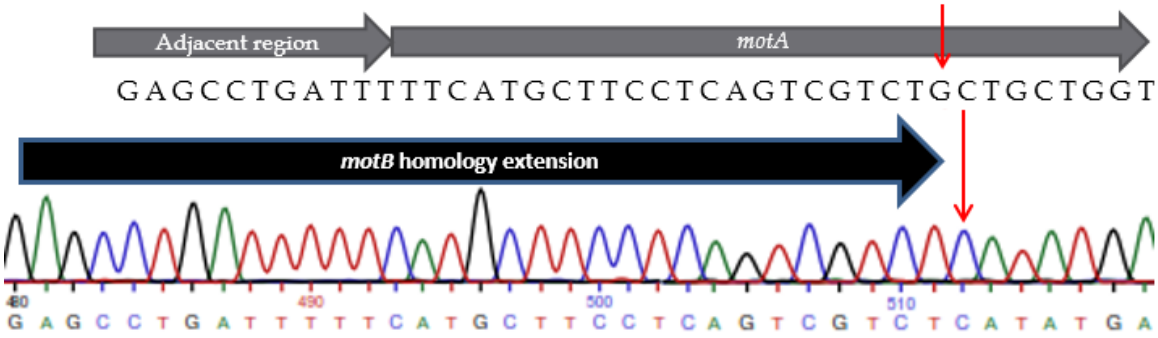

Figure 3.5 The chromatogram illustrating the *motA* gene deletion

The arrows on top show the sequence of the gene and its adjacent area in parental strain. The homology extension in case of *motA* deletion consists of adjacent area and part of the target gene.

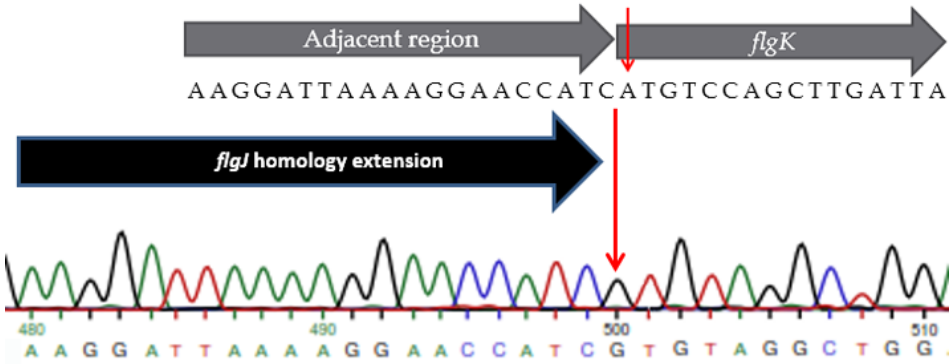

Figure 3.6 The chromatogram illustrating the *flgK* gene deletion

The arrows on top show the sequence of the gene and its adjacent area in parental strain. The homology extension sequence consists of the adjacent area of target gene.

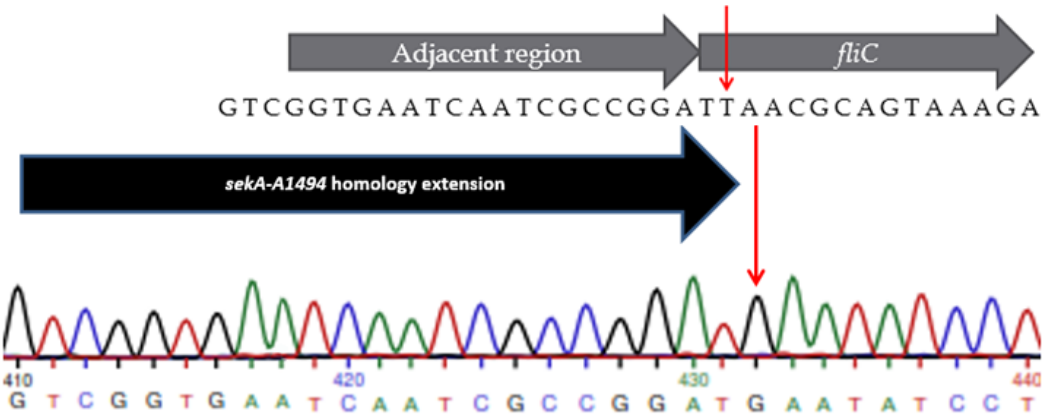

Figure 3.7 The chromatogram illustrating the *fliC* gene deletion.

The arrows on top show the sequence of the gene and its adjacent area in parental strain and the homology extension sequence consists of the adjacent area of target gene.

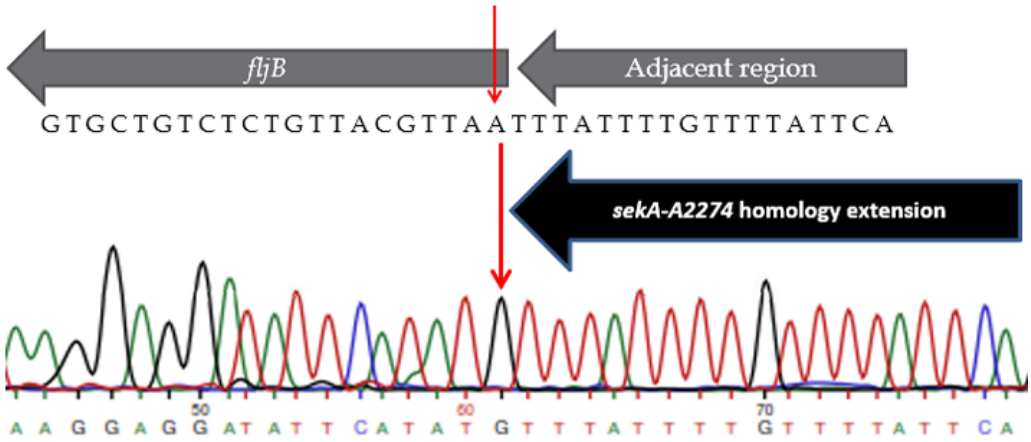

Figure 3.8 The chromatogram illustrating the *fljB* gene deletion.

The arrows on top show the sequence of the gene and its adjacent area in parental strain and the homology extension sequence consists of the adjacent area of target gene.
The black arrows show the homology extension of the adjacent region of the deleted gene. The red arrow shows the deletion spot in parental and mutant strain. The sequence after the red arrow is the PCR-amplified segment of pKD3, which carries the FRT and the antibiotic-resistance gene, which was substituted for the deleted gene. Later this segment was eliminated with pCP20 plasmid coding the FLP.

Genetic complementation of non-motile strains

Cloning of the deleted gene with its operon in non-motile mutant strains of Δ*fliCfljB*, Δ*flgK* and Δ*motA* restored motility in non-motile mutants. In the case of the Δ*fliCfljB* mutant, cloning of one of the flagellin sub-units (the *fliC* gene was used here) was enough to make the non-motile strain motile again. The Δ*motA* mutant was complemented with a parental copy of both *motA* and *motB* genes

Attachment characteristics of mutant strains to chicken skin

Attachment characteristics of all the flagella mutants, from pervious and current studies, have been compared with the wild type *S*. Kentucky to determine the effect of deletion on several different flagella genes. The photon count of each well after washing on the plate shaker, which correlate with the number of bacteria attached to broiler skin, were compared with that of the wild-type parent. According to the results, all transposon and in-frame flagella mutants showed adhesion reduction to broiler skin (Figure 3.9), except for *S*.KΔ*fljB* (wells on the 4th column), which did not show significant difference compared to the wild type *S*. Kentucky ($P > 0.05$) (Figure 3.10).

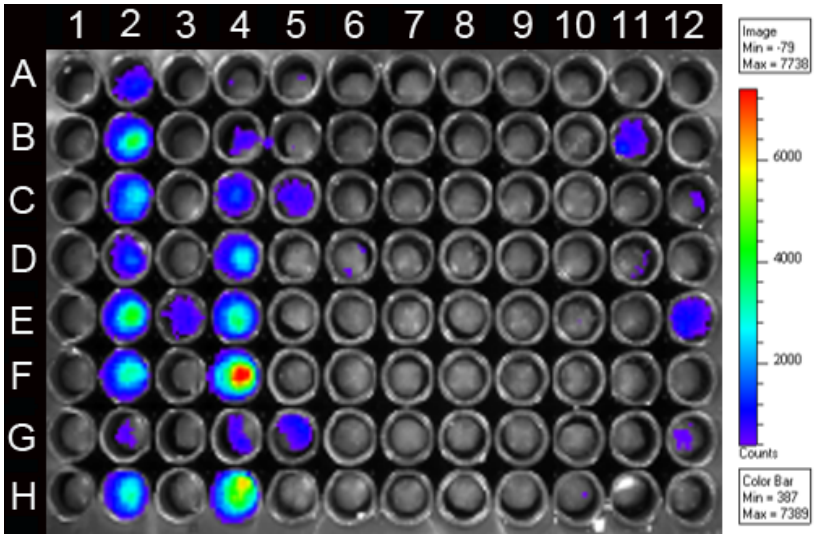

Figure 3.9 Bioluminescence image of flagella mutants' attachment to chicken skin after washing with agitation.

Rows from the left are as follow: Column 1. *E.coli* (Negative control), Column 2. *S.* Kentucky WT (Positive control), Column 3. *S.*K*ΔfliC*, Column 4. *S.*K*ΔfljB*, Column 5 *S.*K*fliCfljB*, Column 6. *S.*K*ΔflgK*, Column 7. *S.*K*ΔmotA*, Column 8. *S.*K*ΔflgA*, Column 9. *S.*K*ΔflgK*-t, Column 10. *S.*K*ΔflgJ*-t, Column 11. *S.*K*ΔflgC*-t, Column 12. *S.*K*ΔflhB*-t

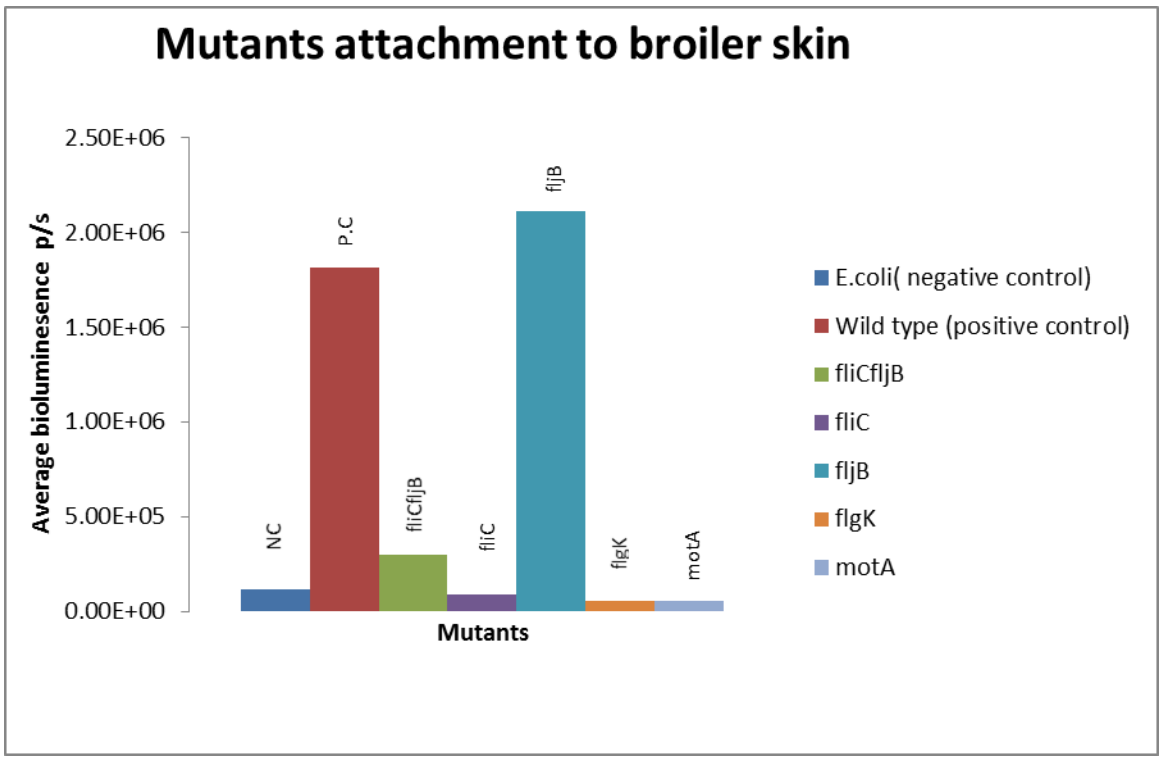

Figure 3.10 Average bioluminescence (p/s) of each mutant on the broiler surface shows the attachment properties of the mutants

Between the mutants constructed with in-frame deletion, *S*.KΔ*flgK* which is unable to assemble the flagella, attachment was reduced ($P < 0.001$). The *S*.KΔ*fliCfljB*, which lacks both flagellar sub-units, encoded by *fliC* and *fljB* genes, displayed attachment reduction ($P < 0.001$). Individual deletion in *fliC* reduced the attachment rate ($P < 0.001$), but *fljB* deletion did not cause any difference in broiler skin colonization compared to parent strain ($P > 0.05$). Furthermore, mutation of *motA* gene encoding flagellar motor force reduced broiler skin colonization compared to the wild-type parent strain ($P < 0.001$). All the mutants made by transposon insertion, which had insertions in the hook associated protein, basal body p-ring, flagella export apparatus, rod assembly protein, and in the basal-body rod protein led to reduction in broiler skin attachment ($P < 0.001$).

In complementation experiments (Figures 3.11 and 3.12), restoration of the *motA* gene in *S*.KΔ*motA* (wells in the 7th column) complemented the colonization defect and there was no difference between the *S*.KΔ*motA*C (wells in the 8th column) and the parent strain (P > 0.05). Also expression of *fliC* in *S*.KΔ*fliCfljB (S*.KΔ*fliCfljB* wells are in the 5th and *S*.KΔ*fliCfljB*C are in the 6th column) and *flgK* in *S*.KΔ*flgK (S*.KΔ*flgK, S*.KΔ*flgK*t and *S*.KΔ*flgK*C are located in column 9th 10th and 11th*)* made these mutants attach to broiler skin as well as the wild type strain (P > 0.05).

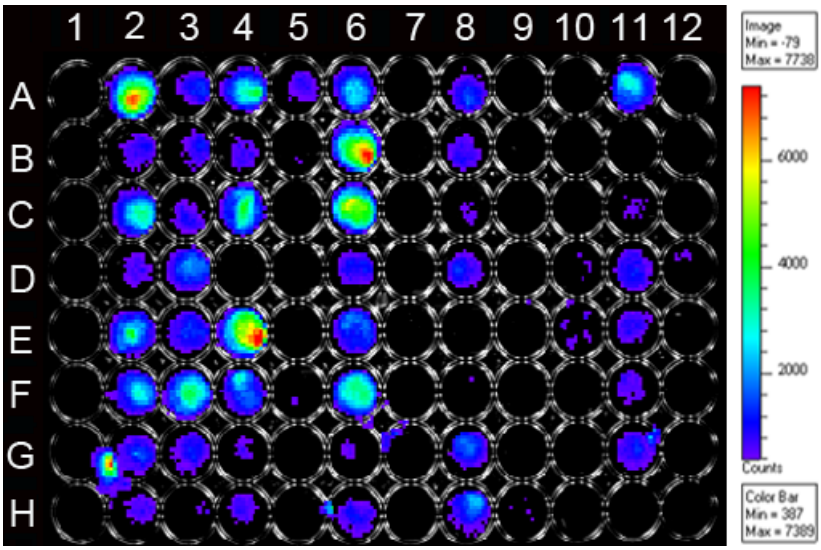

Figure 3.11 Bioluminescence image of mutants' attachment to broiler skin and their associated complement strains after washing with agitation

Rows from left to right: Column 1: *E.coli* (negative control), Column 2*: S.* Kentucky WT (Positive control), Column 3*: S*.KΔ*fliC*, Column 4 *S*.KΔ*fljB*, Column 5*: S*.KΔ*fliCfljB*, Column 6*: S*.KΔ*fliCfljB* complement, Column 7*: S*.KΔ*motA*, Column 8*: S*.KΔ*motA* complement, Column 9*: S*.KΔ*flgK*, Column 10*: S*.KΔ*flgK*-t, Column 11*: S*.KΔ*flgK* complement.

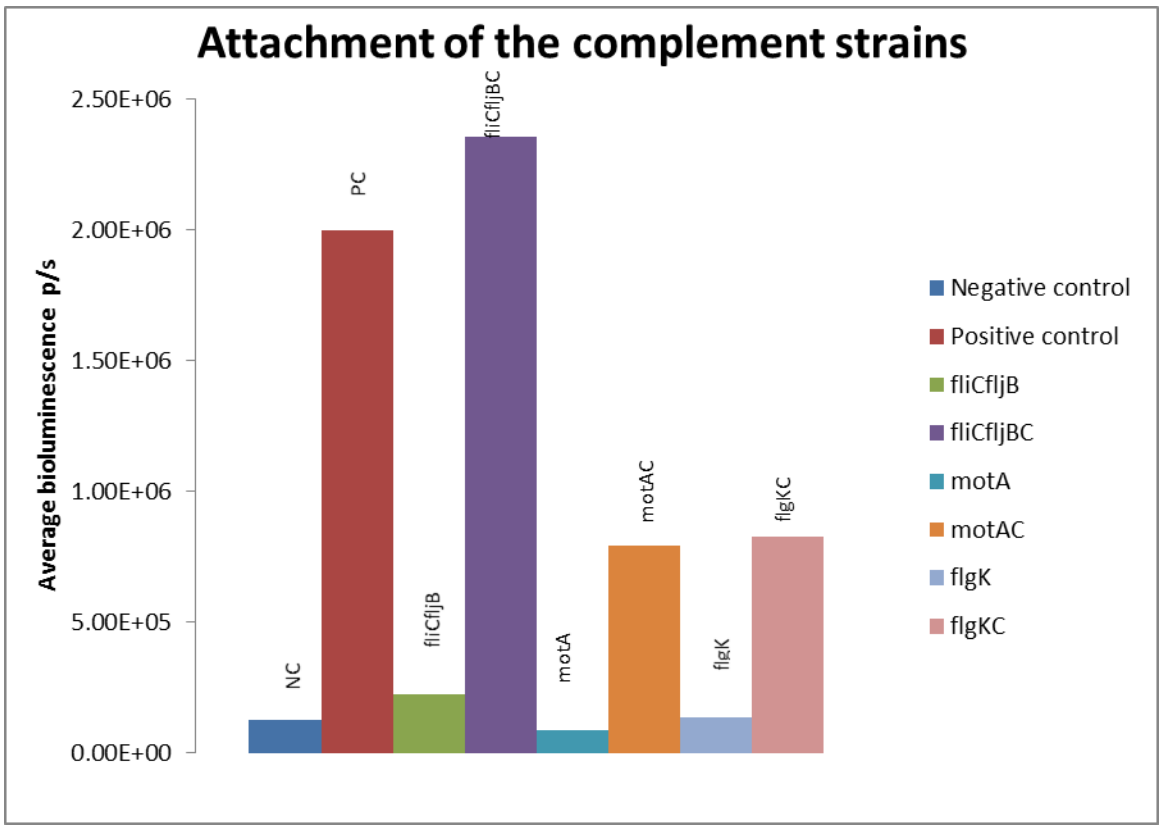

Figure 3.12 Average bioluminescence (p/S) of each mutant and its correlated complement strain on the surface of broiler

Discussion

Bacterial attachment to broiler surfaces helps them survive broiler production and processing procedure (Trachoo et al., 2002). It has been suggested that attached bacteria would be less susceptible to detergents and other antibacterial treatments (Northcutt et al., 2005). Also, broiler skin can protect the attached bacteria from oxidative stress (Yang et al., 2001). Proteomic studies on sessile and planktonic bacteria revealed some physical changes in the protein expression of these bacteria. According to these experiments, the higher expression of stress genes in sessile bacteria can be another reason for the persistence of attached bacteria (Kalmokoff et al., 2006). The mechanism of bacteria

attachment to broiler skin is not fully understood. There have been several conflicting reports on bacterial colonization on broiler surfaces and different bacterial properties have been suggested to contribute to colonization. In our previous research, we concluded that attachment is a multifactorial process. According to our results, several bacterial structures and metabolites, such as flagella, lipopolysaccharide core biosynthesis protein, amino acid metabolites, as well as different secretion and signaling systems can contribute to bacterial attachment on broiler skin. Of these factors, flagella demonstrated the highest potential for broiler colonization. However, it was not clear whether flagella structure or its related motility is the cause of attachment. For this reason, several in-frame deletions were constructed in several specific flagella sub-units and motor genes and attachment comparison were made with their parental wild-type strain. Due to the high frequency of *Salmonella* Kentucky contamination in broiler processing plants, this serovar was selected as the bacterial representative for these experiments. The high prevalence of *S.* Kentucky can be correlated with its high ability to attach to broiler surfaces (Humphrey et al., 2001). Current studies in our laboratory demonstrated that the presence of active flagellar sub-unit, FliC, is the key for the successful broiler skin colonization of *Salmonella*. Deletion of the *fliC* gene results in the expression of the *fljB* flagellar sub-unit and leaves the bacteria motile with significantly less attachment properties. However, mutants with *fljB* deletion were as adherent as the wild type. Furthermore, the presence of paralyzed flagella significantly reduced the bacterial colonization to broiler skin, which indicates the presence of FliC is not enough for attachment and it needs to be motile. This explains the reason why none of the un-flagellated transposon mutants showed any attachment to chicken skin.

Scanning electron microscopy images and the positive motility test confirmed the existence of short active flagella in *ΔfliC* mutants, suggesting that motility is not the only limiting factor in broiler skin attachment. Flagella structure is also an important contributor. Although attachment of bacteria to chicken surface is not long enough to be considered a biofilm formation process, it is comparable to the early stage of biofilm formation and bacterial attachment to surfaces. In several biofilm studies, the role of flagella in bacterial attachment has been investigated. When comparing the ability of several different *Salmonella* serovar strains to form biofilm in poultry processing plants, it was concluded that attachment of bacteria to different surfaces is strain-dependent and bacterial cell surfaces such as cell wall proteins, flagella and lipopolysaccharide all contribute to biofilm formation (Chia et al., 2009). Furthermore, *Salmonella* Typhimurium biofilm formation on food and food processing surfaces demonstrated that transposon mutagenesis in flagellar hook associated protein and lipopolysaccharide production would severely alter bacterial attachment to meat and poultry and biofilm formation on different surfaces (Kim & Wei, 2009). In more recent biofilm formation studies on cholesterol surfaces, several mutations in *motA*, *fliC*, *fljB* and *flicfljB* genes of *Salmonella* Typhimurium were made. From this work, it was concluded that flagella motility does not affect the biofilm formation on cholesterol surfaces, but the *fliC* expression does (Crawford et al., 2010). Elsewhere, transposon mutagenesis in several motility genes as well as flagella filament sub-units in *E. coli*, hindered biofilm formation. This work suggested that cells either with paralyzed flagella or lacking the flagella are effective at forming biofilm (van Houdt & Michiels, 2005; Wood et al., 2006).

The comparison of in-frame deletion and transposon insertion mutation was made with the evidence that a similar gene (*flgK*) was mutated in both methods and showed similar results according to the broiler skin assay. This confirms that the reason for deactivation of *flgK* is due to the mutation in this gene and not the polar effect of the mutation on adjacent regions. Moreover, complementation of the deleted genes with the wild type copy verified the lack of polar effect.

In conclusion, this present study suggests that FliC, one of the flagella filament sub-units, plays an important role in the colonization of *Salmonella* Kentucky to broiler skin. Furthermore, reduction of broiler skin attachment in motor force deleted mutants implies the necessity of an active flagella sub-unit for successful colonization. Future studies on the role of flagella in adherence of *Salmonella* to epithelial cells are required to fully understand the attachment model. Understanding the details of the mechanism of attachment can lead us to beneficial control strategies to eliminate or reduce bacterial contamination in food systems.

References

Allen-Vercoe, E., & Woodward, M. J. (1999). The Role of Flagella, but Not Fimbriae, in the Adherence of Salmonella Enterica Serotype Enteritidis to Chick Gut Explant. *Journal of Medical Microbiology, 48*(8), 771-780.

Chia, T. W. R., Goulter, R. M., McMeekin, T., Dykes, G. A., & Fegan, N. (2009). Attachment of Different Salmonella Serovars to Materials Commonly Used in a Poultry Processing Plant. *Food Microbiology, 26*(8), 853-859.

Crawford, R. W., Reeve, K. E., & Gunn, J. S. (2010). Flagellated but Not Hyperfimbriated Salmonella Enterica Serovar Typhimurium Attaches to and Forms Biofilms on Cholesterol-Coated Surfaces. *Journal of Bacteriology, 192*(12), 2981-2990.

Datsenko, K. A., & Wanner, B. L. (2000). One-Step Inactivation of Chromosomal Genes in Escherichia Coli K-12 Using Pcr Products. *Proceedings of the National Academy of Sciences, 97*(12), 6640-6645.

Elhadidy, M., & Hashem, E. A. (2012). Identification of Two Flagellar Genes Required for Biofilm Formation in a Salmonella Enterica Serovar Typhimurium. *Annals of Microbiology, 62*(2), 533-537.

Howe, K., Karsi, A., Germon, P., Wills, R. W., Lawrence, M. L., & Bailey, R. H. (2010). Development of Stable Reporter System Cloning Luxcdabe Genes into Chromosome of Salmonella Enterica Serotypes Using Tn7 Transposon. *BMC Microbiology, 10*(1), 197.

Humphrey, T. J., Martin, K. W., Slader, J., & Durham, K. (2001). Campylobacter Spp. In the Kitchen: Spread and Persistence. *Journal of Applied Microbiology, 90*(S6), 115S-120S.

Kalmokoff, M., Lanthier, P., Tremblay, T.-L., Foss, M., Lau, P. C., Sanders, G., Austin, J., Kelly, J., & Szymanski, C. M. (2006). Proteomic Analysis of Campylobacter Jejuni 11168 Biofilms Reveals a Role for the Motility Complex in Biofilm Formation. *Journal of Bacteriology, 188*(12), 4312-4320.

Kim, S.-H., & Wei, C.-I. (2009). Molecular Characterization of Biofilm Formation and Attachment of Salmonella Enterica Serovar Typhimurium Dt104 on Food Contact Surfaces. *Journal of Food Protection, 72*(9), 1841-1847.

McMeekin, T. A., & Thomas, C. J. (1978). Retention of Bacteria on Chicken Skin after Immersion in Bacterial Suspensions. *Journal of Applied Microbiology, 45*(3), 383-387.

Merritt, M. E., Lawrence, A. M., & Donaldson, J. R. (2010). Comparative Study of the Effect of Bile on the Listeria Monocytogenes Virulent Strain Egd-E and Avirulent Strain Hcc23. *Archives of Clinical Microbiology, 1*(1).

Northcutt, J. K., Smith, D. P., Musgrove, M. T., Ingram, K. D., & Hinton, A. (2005). Microbiological Impact of Spray Washing Broiler Carcasses Using Different Chlorine Concentrations and Water Temperatures. *Poultry Science, 84*(10), 1648-1652.

Notermans, S., & Kampelmacher, E. H. (1974). Attachment of Some Bacterial Strains to the Skin of Broiler Chickens. *British Poultry Science, 15*(6), 573-585.

Schmitt, C. K., Ikeda, J. S., Darnell, S. C., Watson, P. R., Bispham, J., Wallis, T. S., Weinstein, D. L., Metcalf, E. S., & O'Brien, A. D. (2001). Absence of All Components of the Flagellar Export and Synthesis Machinery Differentially Alters Virulence of Salmonella Enterica Serovar Typhimurium in Models of Typhoid Fever, Survival in Macrophages, Tissue Culture Invasiveness, and Calf Enterocolitis. *Infection and Immunity, 69*(9), 5619-5625.

Thomas, C. J., & McMeekin, T. A. (1981). Attachment of Salmonella Spp. To Chicken Muscle Surfaces. *Applied and Environmental Microbiology, 42*(1), 130-134.

Trachoo, N., Frank, J. F., & Stern, N. J. (2002). Survival of Campylobacter Jejuni in Biofilms Isolated from Chicken Houses. *Journal of Food Protection, 65*(7), 1110-1116.

van Houdt, R., & Michiels, C. W. (2005). Role of Bacterial Cell Surface Structures in Escherichia Coli Biofilm Formation. *Research in Microbiology, 156*(5), 626-633.

Wood, T. K., Barrios, A. F. G., Herzberg, M., & Lee, J. (2006). Motility Influences Biofilm Architecture in Escherichia Coli. *Applied Microbiology and Biotechnology, 72*(2), 361-367.

Yang, H., Li, Y., & Johnson, M. G. (2001). Survival and Death of Salmonella Typhimurium and Campylobacter Jejuni in Processing Water and on Chicken Skin During Poultry Scalding and Chilling. *Journal of Food Protection, 64*(6), 770-776.

Yim, L., Betancor, L., Martínez, A., Bryant, C., Maskell, D. J., & Chabalgoity, J. A. (2011). Naturally Occurring Motility-Defective Mutants of Salmonella Enterica Serovar Enteritidis Isolated Preferentially from Nonhuman Rather Than Human Sources. *Applied and Environmental Microbiology, 77*(21), 7740-7748.

CHAPTER IV

REMOVING FLAGELLA STRUCTURAL AND MOTION GENES REDUCES ADHESION AND INVASION OF SALMONELLA SEROVAR KENTUCKY TO CACO-2 CELL LINES

Abstract

Flagella and its related motility contribute important roles in the colonization of *Salmonella* Kentucky on broiler surfaces. *Salmonella* phase variation allows the bacteria to express two different flagella sub-units (FliC and FljB), but only one of them can be expressed at one time. According to our previous studies, the FliC sub-unit seems to be the main contributor to *Salmonella* Kentucky attachment to broiler skin and not the FljB. In spite of the fact that *Salmonella* Kentucky has been identified as the most prevalent serovar on broilers as they exit the chiller tank of the processing plants, little is known of its adhesion mechanism or possible route of invasion into intestinal epithelial cells. In the present study, we show that a flagellated and non-motile strains of *S*. Kentucky with deletion in both *fliC* and *fljB* genes and *flgK*, as well as a flagellated but paralyzed flagella exhibit less adhesion and invasion characteristics, but *fljB* mutant is as adherent and invasive as the wild type strain. These results suggest that an active FliC sub-unit is the significant part of the flagella that contributes in adhesion and invasion of Caco-2 cell lines.

Introduction

Salmonella enterica serovar Kentucky has been recognized as the most prevalent *Salmonella* serotype in poultry processing plants in the US and some European countries (Le Hello et al., 2011). The ciprofloxacin resistance gene has been found in some strains of this serovar (Le Hello et al., 2011), which, interestingly, has rarely been reported in non-typhodidal *Salmonella* in humans (NCCLS, 2008). This fact makes this serovar a new potential risk from the foodborne illness and public health point of view. According to recent studies (Foley et al., 2011; Le Hello et al., 2011), *S.* Kentucky potentially can be the new emerging *Salmonella* serotype that can develop into a more prominent human pathogen. Early bacterial epithelial cell interaction with the broiler skin and possibly the intestinal cells is the first route of contamination or probable pathogenesis. Previous work in our laboratory concerning the attachment characteristics of *S.* Kentucky to broiler skin demonstrated the active role of flagella in broiler skin colonization. Further studies in our laboratory on mutated flagella sub-units and motility genes indicated a strong reduction in the attachment rate in both mutants. This finding emphasized the presence of motility as well as the flagella structure was necessary for *S.* Kentucky to attach and colonize broiler skin. Moreover, flagella and motility are suggested to play an important role in *Salmonella* virulence (Stecher et al., 2008; Stecher et al., 2004). Mutation in *S.* Enterica flagella and fimbriae genes showed less association and invasion in non-flagellated strains, but the deletion of the fimbriae did not show any reduction in attachment or invasion (Dibb-Fuller et al., 1999). Other *in vivo* parallel studies on rat ileal and chick intestines indicated that paralyzed strains of *S.* Enteritidis were significantly less adherent than the wild-type strain, so it was concluded that an active flagella is needed for early

stages of infection (Allen-Vercoe & Woodward, 1999; Robertson et al., 2000). However, naturally occurring motility defective mutants of *Salmonella enterica* serovar Enteritidis indicated that paralyzing flagella does not affect chicken and egg colonization, but rather reduced the ability of the organism to cause human disease (Yim et al., 2011). Furthermore, a non-flagellated mutant strain of *S.* Typhimurium was able to attach to cultured intestinal epithelial cells, but was impaired in its ability to invade the cells (Schmitt et al., 2001). Similarly, a non-flagellated mutant of *S.* Enteritidis was 50-fold less invasive to Caco-2 cell lines even if the adhesion rate was similar to a flagellated strain (van Asten et al., 2000) However, in a comparison of paralyzed with non-flagellated mutant of *S.* Enteritidis, flagella-mediated motility was suggested to accelerate the invasion rather than being essential (van Asten et al., 2004)

In spite of the significant body of knowledge on the role of flagella in different pathogenic *Salmonella* serovars, there are major gaps in knowledge concerning the mechanism of adhesion of *S.* Kentucky to the intestine or the possible route of invasion. The purpose of this study was to use flagella mutants to recognize the role of flagella sub-units and its associated motility in adhesion and possible invasion of intestinal epithelial cells. The molecular basis of adhesion to Caco-2 cell lines as a model can lead to recognition of the mechanism of adhesion to the intestinal and other epithelial cells.

Materials and Methods

Bacterial strains, plasmids and cultural conditions

The characteristics of *S.* Kentucky WT, non-motile *S*KΔ*motA*, non-flagellated *S*KΔ*flgK*, SKΔ*fliCfljB*, and semi-flagellated SKΔ*fliC* and SKΔ*fljB* have been described previously in Chapter 3. *E. coli* DH5α and pBBR1MCS-4 were used for cloning and

E.coli K-12 and pKD3, pKD46 and pCP20 were used for deletion purposes. Bacteria were grown in Luria-Bertani (LB) broth and agar at 37°C, with ampicillin (100 µg/ml), and chloramphenicol (10 µg/ml) added when necessary. Bacterial motility was tested with stabbing motility agar that contained 10 g of trypton and 5 g of NaCl per 1 liter plus 0.35% (wt/vol) agar, pH 7.4 (Schmitt et al., 2001).

PCR reaction and primers

The 50 µl PCR reaction containing 0.2 mM dNTP mix, 1.5 mM MgCl2, 0.2 mM primers and 1.25 U Taq DNA polymerase (Promega, Madison, WI) was performed using a 2720 thermal cycler AB (Applied Biosystems). Conditions were initial denaturation (5 min at 94°C); followed by 30 cycles of denaturation (30 s at 94°C); annealing (30 s at 56°C) and elongation (1 min at 72°C), and final extension (10 min at 72°C). Primers sequences are listed in Table 3.2.

Construction of *S*KΔ*motA*, *S*KΔ*flgK*, SKΔ*fliCfljB*, SKΔ*fliC* and SKΔ*fljB*

Deletions in flagella su-units and the motion gene were performed using a λ red recombinant system as previously described by Datsenko and Wanner (2000). Briefly, the PCR products were amplified using pKD3 as the template. The Primers were 55-70 nucleotides in length and consisted of adjacent regions of the target genes and 20 nt of complementary region of template plasmid pKD3 (Table 4.1). The wild type strain of *Salmonella* Kentucky was transformed with the ampicillin resistant plasmid, pKD46, expressing arabinose inducer prompter in the λ red recombinant system. Transformed cells were grown in LB broth containing ampicillin and 10 mM arabinose at 30°C to reach the OD_{600} of 0.6 and were made electrocompotent by washing with 10% glycerol at

a tabletop centrifuge. The PCR products were digested with 1 µl of *Dpn*I (Promega, Madison, WI) added to 50 µl PCR product at 37°C for 1 h and gel purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR product (20-50 ng/µl) was electroporated (1.8 kV, 25 µF, and 400 Ω) into *S*. Kentucky containing λ red recombinant system using a 0.1 cm chambers. Cells were recovered in SOC media at 30°C for 3-4 h at 200 rpm. A portion of the cell suspension (100-200 µl) was spread on LB media with chloramphenicol (10 µg/ml) and incubated at 37°C. Colonies were selected after 24 h and sub cultured at 42°C to cure the plasmid pKD46. In order to confirm the correct structure of the mutants, a PCR reaction was performed using an antibiotic primer with another site-specific primer down or upstream of the target gene to test the new junction. Later, mutants were transformed with pCP20 containing FLP synthesis to eliminate the antibiotic-resistant gene. Transformants were selected after 48 h on LB agar containing ampicillin at 30°C. Several colonies were selected and purified at 42°C to cure the plasmid and were tested for loss of antibiotic resistance.

Table 4.1 Mutants with their homology extensions used for gene disruption

Mutation	**Homology extensions***
Δ*fliC*, *sekA-A1495*	35nt; H1:1427041C; H2; 1428530
Δ*fljB*, *sekA-A2275*	35nt; H1: 2217399C; H2; 2218903
Δ*motA*1+	40nt; H1; 1404061C; H2:1400252
Δ*motA*2+	50nt; H1: 1401080C; H2: 1400233
Δ*flgK*	35nt; H1: 576035C; H2: 574372

*extension lengths are followed by 3´ nucleotide of the extension of *Salmonella* Kentucky genome shotgun sequence (GenBank: ABAK02000001.1). C, complement; H1, homology 1; H2, homology 2 (Datsenko & Wanner, 2000)
+*motA* mutations were made with 2 different primers with different homology extensions

Construction of complement strains

The fragments carrying both *motA* and the *mot*B genes as well as *fliC* and *flgK* and their operons were PCR amplified using *Salmonella* Kentucky WT as the template. The *motA*Fc, *motB*Rc, *flgK*Fc, *flgK*Rc, *fliC*Fc and *fliC*Rc, primers flanking by *SmaI* and *SacI* restriction sites were used (Table 3.2). PCR products were digested and purified with a PCR Clean-Up Kit (Promega, Madison, WI). Cloning vector, pBBR1MCS-4, was isolated from over-night *E. coli* culture using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), cut with *Sac*I and *Sma*I restriction enzymes, and gel purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). PCR fragments were ligated into the cloning vector with T4 DNA ligase (Promega, Madison, WI) overnight at 4°C. Ampicillin resistant colonies were picked and the cloned plasmids were sequenced for further confirmation. Each mutant was transformed with associated cloned plasmid and colonies harboring the plasmids were tested for motility and sequenced for verification.

Cell Culture media and growth condition

Caco-2 cells (ATCC: HTB-37) were maintained in Eagle's Modified Essential Media plus sterilized L-glutamine (EMEM, ATCC: 30-2003), which was supplemented with 10% Fetal Bovine serum (FBS was added to EMEM and filter sterilized) in 200 ml cell culture flasks. Cells were incubated at 37°C with 5% CO_2.

Association and Invasion assay

The association and invasion assays were performed as described previously (Dibb-Fuller et al., 1999). Briefly, to reach a confluent layer of Caco-2 cell lines, 5×10^5 cells were seeded in 24-well cell culture plates. For the adhesion assay, the overnight

culture of the wild-type strain and mutants were adjusted to an OD_{600} of 1.0 and diluted to 10^7/ml bacteria prior to addition to the cells to yield an MOI of 100:1. Cells were washed twice with PBS and fresh pre-warmed media prior to inoculation. Plates were centrifuged for 1 min at 800 rpm and incubated at 37°C in 5% CO_2 for 45 min to allow for adhesion. Inoculums were removed and plates were washed with PBS five times to remove the unattached bacteria. The Cells were then lysed with cold 0.1% of Triton X-100 to release the bacteria. The number of associated bacteria (total of invaded and attached) was calculated by plating the appropriate dilution on LB agar and results were calculated as the percent adherence (number of bacteria after wash/number of inoculated bacteria × 100).

For the invasion assay, after adding the bacteria to the cells and centrifugation, plates were incubated for 1 h to allow the invasion. The medium was changed to the fresh medium containing 100 µg/ml of gentamycin for 2 h. The resulted suspension was aspirated and plated to confirm the removal of all extracellular bacteria. Cells were washed twice with PBS, lysed with 0.1% cold Triton X-100 and plated to count the bacteria and were calculated as the percent Invasion (number of intra cellular bacteria/ number of inoculated bacteria × 100). Each test strain was repeated in four wells of a plate (Yim et al., 2011)

Statistics

The mutants' association and invasion assay to broiler skin was performed in two sets of quadruplicate. The percentage of intra cellular bacterial count was used in ANOVA test (SPSS 21.0 software program) with Tukey's multiple comparisons post-test to analyze the difference in mutants' invasion. The association assay performed on two

different days and a generalized randomized block design with four replicates was used for comparison of the percentage of associated bacterial count between mutants and parent strain. The P value of ≤ 0.05 was considered statistically significant.

Results

Adhesion and Invasion of Caco-2 cell-lines

Mutants with deletion in flagella structural and motion genes were compared with their parental strain for their invasion and adhesion ability to Caco-2 cell lines. Mutants with double mutation in both flagellin sub-units (Δ*fliCfljB)* were less invasive and adherent than the wild type strain ($P < 0.001$). However, individual deletion in each alternate sub-unit had a different outcome. Deletion of the *fliC* gene reduced cell invasion and adhesion ($P < 0.001$) but, the *fljB* mutant was as invasive and adherent as the parent strain ($P > 0.05$). The mutation in *flgK* lacking the ability to assemble the flagella made this mutant less invasive and adherent ($P < 0.001$). The paralyzed flagella, which had the deletion in *motA* gene, demonstrated a reduction in cell adhesion and invasion ($P < 0.001$).

The comparison between the complement strains and their correlated mutants invasion into intestinal epithelial cells showed that the restoration of a wild-type copy of deleted genes in the mutants made them invasive again. Expression of *fliC* in Δ*fliCfljB* and *flgK* in Δ*flgK* and *motA* in Δ*motA* made these mutants as invasive as the parental strain ($P > 0.05$) (Figure 4.1)

In the adhesion assay, restoration of deleted genes made the complements partially adherent. The complement strains were more adherent than the mutants and less adherent than the wild-type strain although this difference was not statistically

significant. The mean percentage of adhesion of the mutants and their correlated complements is shown in Figure 4.1.

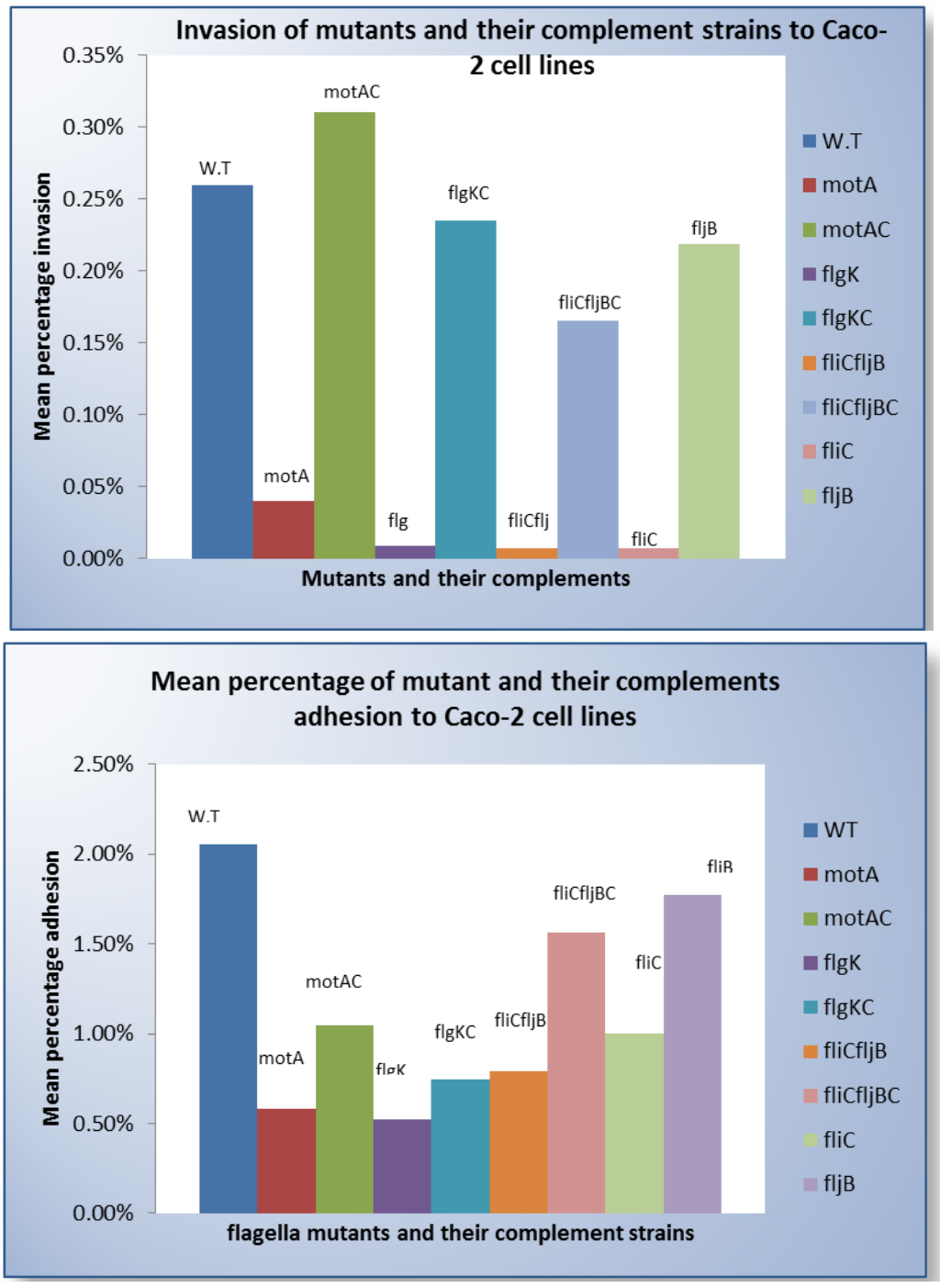

Figure 4.1 Mean percentage invasion (A) and adhesion (B) of the mutants and their complement strains

Discussion

Salmonella enterica serovar Kentucky has been recognized as the most prevalent *Salmonella* serovar in broilers sampled at the end of the immersion chill, which marks the end of processing where antimicrobial means may be exerted. The high prevalence of the bacteria can be correlated to its high ability to attach to broiler skin. While attachment of bacteria to broiler surfaces can extend the bacterial survival during food processing (Trachoo et al., 2002), the attachment mechanism has not been clearly elucidated. In a previous study in our laboratory, we found that multiple factors contributed to the colonization of *Salmonella* Kentucky on broiler skin surfaces. Of all the elements studied, flagella seemed to play the most important role in bacterial colonization. Additional studies by our group on flagella and its correlated motility clarified that the main flagella sub-unit (FliC) and motility can strongly interfere with bacterial attachment to broiler skin. Previous studies using avian and human epithelial cell lines revealed similar results in *Salmonella* adhesion and invasion (La Ragione et al., 2003). Therefore, in the current study, human intestinal epithelia cells (Caco-2) were used as a model for bacterial attachment to broiler skin. For this purpose, several flagella mutant strains of *S.* Kentucky with deletions in *fliC*, *fljB*, *fliCfljB*, *motA* and *flgK,* produced in a previous study were tested for their capacity to adhere and invade the Caco-2 cell lines. These assays allowed for the comparison of the role of flagella in adhesion inside and outside the broilers.

In both adhesion and invasion assays, aflagellated mutants, *ΔfliCfljB* and *ΔflgK* and flagellated non-motile mutant, Δ*motA*, were less adherent and invasive than the wild type strain. Deletion of individual flagellar sub-units resulted in two motile mutants with different adhesion characteristics. The *fliC* deletion, which leads to expression of the *fljB*

gene, made the bacteria less adherent and invasive than the wild-type strain; however, the *ΔfljB* mutant with the FliC flagellar sub-unit was as adherent and invasive as the wild type. Complementation of the deleted genes made for a partial recovery of the cells in adherence and a complete recovery in invasion. A possible explanation for the partial adherence recovery can be the absence of cis acting gene regulation systems on the complemented fragment that are necessary for adhesion, but not invasion.

Previous results on the role of flagella on adhesion, invasion, and membrane ruffling of the *ΔfliC* mutant of *S.* Enteritidis to human (HEp-2) and avian (Div-1) cell lines with microscopic methods demonstrated less adhesion to the either cell lines. Yet, the same study with cell culture and biological counting did not show the difference in adhesion between mutants and the wild type. Invasion and ruffling in both cell lines was reduced in aflagellated mutants (La Ragione et al., 2003).

Flagellar sub-units of *Salmonella* consist of two antigenically distinct proteins, FliC and FljB. The expression of each of these flagellin sub-units is controlled by a mechanism that is called phase variation (Ikeda et al., 2001). There are different theories as to the role of flagellin sub-units in adherence and invasion of epithelial cells. According to Ikeda et al. (2001), adherence and invasion of wild-type *S. enterica* serovar Typhimurium with the phase-locked mutants *(ΔfliC* and *ΔfljB*) to murine intestinal epithelial cell lines did not result in any differences, but FliC was more effective in virulence. However, at the same time, Schmitt et al. (2001) demonstrated that aflagellated mutants of *S. enterica* serovar Typhimurium with mutation in *fliCfljB* did not exhibit a difference in pathogenicity but were less invasive to Caco-2 cells.

Most of flagella motility related studies on *Salmonella* cell adhesion and invasion characteristics were more focused on comparison between aflagellated mutants and flagellated non-motile mutants without considering the role of flagella sub-units separately. In an *in vitro* study on the role of flagella and its related motility on adhesion and invasion of Caco-2 cell lines, van Asten et al. (2004) suggested that mutation in the *fliC* gene of flagellin sub-unit will lead to an aflagellated strain of *S.* Enteritidis that together with *motA* mutation will severely impair the bacterial invasion, but not adhesion. Yim et al. (2011) compared several naturally occurring *motA* mutants of *Salmonella* Enteritidis with non-flagellated mutant strains (*fliC* mutant) and determined that they exhibited less invasiveness to Caco-2 cell lines. It was concluded that a paralyzed flagella induced a higher pro-inflammatory response than the aflagellated strain, suggesting that presence of a paralyzed flagella is more effective than a non-flagellated one (Yim et al., 2011). In the current research reported here, the role of each flagella sub-unit and flagella motor force in adhesion and invasion of Caco-2 cell lines have been studied individually. We were able to make a deletion in the *flgK* gene, which encodes the hook associated protein. Mutants lacking this protein were unable to assemble the flagella and are non-motile, but can still secrete the flagellin sub-units. The comparison of this mutant with *fliCfljB* mutant (a non flagellin expressing mutant) did not show a difference in invasiveness. Both of these mutants were less invasive than the wild type. These results are consistent with findings of previous studies on several poultry associated isolates of *Salmonella* Enterica with mutations in *flgK*, *flgL* and *fljB* genes, which exhibited invasion reduction in Caco-2 cell lines when compared to the parental strain (Shah et al., 2011).

In conclusion, the fact that deletion of *fliC* leaves the bacteria motile but makes them less adherent and invasive, and the presence of paralyzed flagella diminishes the cell invasion and adhesion suggest that the presence of an active FliC sub-unit is the key factor for adhesion and invasiveness of *S.* Kentucky to Caco-2 cell lines. Future studies on protein expression of different flagellar mutants can lead to a better understanding of the molecular changes that the bacteria undergoes when attaching or invading the cells.

References

Allen-Vercoe, E., & Woodward, M. J. (1999). The Role of Flagella, but Not Fimbriae, in the Adherence of Salmonella Enterica Serotype Enteritidis to Chick Gut Explant. *Journal of Medical Microbiology, 48*(8), 771-780.

Datsenko, K. A., & Wanner, B. L. (2000). One-Step Inactivation of Chromosomal Genes in Escherichia Coli K-12 Using Pcr Products. *Proceedings of the National Academy of Sciences, 97*(12), 6640-6645.

Dibb-Fuller, M. P., Allen-Vercoe, E., Thorns, C. J., & Woodward, M. J. (1999). Fimbriae-and Flagella-Mediated Association with and Invasion of Cultured Epithelial Cells by Salmonella Enteritidis. *Microbiology, 145*(5), 1023-1031.

Foley, S. L., Nayak, R., Hanning, I. B., Johnson, T. J., Han, J., & Ricke, S. C. (2011). Population Dynamics of Salmonella Enterica Serotypes in Commercial Egg and Poultry Production. *Applied and Environmental Microbiology, 77*(13), 4273-4279.

Ikeda, J. S., Schmitt, C. K., Darnell, S. C., Watson, P. R., Bispham, J., Wallis, T. S., Weinstein, D. L., Metcalf, E. S., Adams, P., & O'Connor, C. D. (2001). Flagellar Phase Variation of Salmonella Enterica Serovar Typhimurium Contributes to Virulence in the Murine Typhoid Infection Model but Does Not Influence Salmonella-Induced Enteropathogenesis. *Infection and Immunity, 69*(5), 3021-3030.

La Ragione, R. M., Cooley, W. A., Velge, P., Jepson, M. A., & Woodward, M. J. (2003). Membrane Ruffling and Invasion of Human and Avian Cell Lines Is Reduced for Aflagellate Mutants of Salmonella Enterica Serotype Enteritidis. *International Journal of Medical Microbiology, 293*(4), 261-272.

Le Hello, S., Hendriksen, R. S., Doublet, B., Fisher, I., Nielsen, E. M., Whichard, J. M., Bouchrif, B., Fashae, K., Granier, S. A., & Jourdan-Da Silva, N. (2011). International Spread of an Epidemic Population of Salmonella Enterica Serotype Kentucky St198 Resistant to Ciprofloxacin. *Journal of Infectious Diseases, 204*(5), 675-684.

NCCLS. (2008). Performance Standards for Antimicrobial Susceptibility Testing: Eighteenth Informational Supplement. Wayne, PA: National Committee for Clinical Laboratory Standards.

Robertson, J. M. C., Grant, G., Allen-Vercoe, E., Woodward, M. J., Pusztai, A., & Flint, H. J. (2000). Adhesion of Salmonella Enterica Var Enteritidis Strains Lacking Fimbriae and Flagella to Rat Ileal Explants Cultured at the Air Interface or Submerged in Tissue Culture Medium. *Journal of Medical Microbiology, 49*(8), 691-696.

Schmitt, C. K., Ikeda, J. S., Darnell, S. C., Watson, P. R., Bispham, J., Wallis, T. S., Weinstein, D. L., Metcalf, E. S., & O'Brien, A. D. (2001). Absence of All Components of the Flagellar Export and Synthesis Machinery Differentially Alters Virulence of Salmonella Enterica Serovar Typhimurium in Models of Typhoid Fever, Survival in Macrophages, Tissue Culture Invasiveness, and Calf Enterocolitis. *Infection and Immunity, 69*(9), 5619-5625.

Shah, D. H., Zhou, X., Addwebi, T., Davis, M. A., Orfe, L., Call, D. R., Guard, J., & Besser, T. E. (2011). Cell Invasion of Poultry-Associated Salmonella Enterica Serovar Enteritidis Isolates Is Associated with Pathogenicity, Motility and Proteins Secreted by the Type Iii Secretion System. *Microbiology, 157*(5), 1428-1445.

Stecher, B., Barthel, M., Schlumberger, M. C., Haberli, L., Rabsch, W., Kremer, M., & Hardt, W.-D. (2008). Motility Allows S. Typhimurium to Benefit from the Mucosal Defence. *Cellular Microbiology, 10*(5), 1166-1180.

Stecher, B., Hapfelmeier, S., Müller, C., Kremer, M., Stallmach, T., & Hardt, W.-D. (2004). Flagella and Chemotaxis Are Required for Efficient Induction of Salmonella Enterica Serovar Typhimurium Colitis in Streptomycin-Pretreated Mice. *Infection and Immunity, 72*(7), 4138-4150.

Trachoo, N., Frank, J. F., & Stern, N. J. (2002). Survival of Campylobacter Jejuni in Biofilms Isolated from Chicken Houses. *Journal of Food Protection, 65*(7), 1110-1116.

van Asten, F. J. A. M., Hendriks, H. G. C. J. M., Koninkx, J. F. J. G., van der Zeijst, B. A. M., & Gaastra, W. (2000). Inactivation of the Flagellin Gene of Salmonella Enterica Serotype Enteritidis Strongly Reduces Invasion into Differentiated Caco-2 Cells. *FEMS Microbiology Letters, 185*(2), 175-179.

van Asten, F. J. A. M., Hendriks, H. G. C. J. M., Koninkx, J. F. J. G., & van Dijk, J. E. (2004). Flagella-Mediated Bacterial Motility Accelerates but Is Not Required for Salmonella Serotype Enteritidis Invasion of Differentiated Caco-2 Cells. *International Journal of Medical Microbiology, 294*(6), 395-399.

Yim, L., Betancor, L., Martínez, A., Bryant, C., Maskell, D. J., & Chabalgoity, J. A. (2011). Naturally Occurring Motility-Defective Mutants of Salmonella Enterica Serovar Enteritidis Isolated Preferentially from Nonhuman Rather Than Human Sources. *Applied and Environmental Microbiology, 77*(21), 7740-7748.

CHAPTER V

CONCLUSION

It has been estimated that non-typhoidal *Salmonella* are the leading cause of foodborne illness related hospitalizations and deaths in the United States. Poultry products have been associated with the presence of *Salmonella*. With the increase in per capita consumption of poultry in the US when compared to red meat, the potential risk of human exposure to *Salmonella* serovars could be elevated. Recently, *Salmonella* Kentucky has been recognized as the most prevalent *Salmonella* serovar in broiler processing, when the carcasses exit the immersion chill tank. This serovar is not listed as a common human pathogen. However, there has been an increased frequency of this serovar acquiring the antibiotic resistance gene in some countries outside the US. This fact could heighten interests for this serovar as a public health concern. Among all the antibiotics, ciprofloxacin is the drug of choice for serious cases of gastroenteritis in humans and *S*. Kentucky is one of the rare non-typhoidal *Salmonella* strains resistant to the antibiotic. The high prevalence of *S*. Kentucky that is present on the broilers at the end of processing phase might correlate with its ability to attach to the broiler skin. Attached bacteria are more resistant to antibacterial treatments and oxidative stress, which helps them survive through the slaughter and processing cycle. Discovery of the attachment mechanism would be beneficial for improved control strategies to decrease bacterial contamination of broiler carcasses.

In the current study, a library of more than 2000 mutant strains of bioluminescent *S.* Kentucky was constructed. Applying high throughput bioluminescence mutant screening (BLMS), several mutants with attenuated attachment properties to chicken skin were selected for further investigation. According to the chicken attachment assay, it was concluded that attachment is a multifactorial process with the following elements contributing: i), flagella, ii), LPS structure, iii), amino acid metabolism, iv), TCA cycle pathway; v), conjugative transfer system, vi), multidrug resistant protein, vii), signaling and transportation system, viii), metabolism, ix), different enzymes, x), phage tail fiber protein H, xi), fimbrial export usher protein, xii), membrane proteins xiii), and several unnamed proteins. Most of these elements can be categorized in bacterial cell surface structure. It can also be concluded that some signaling and transportation, amino acid metabolism and energy production genes contribute to tolerance procedures that are intended to have a higher expression in sessile bacteria when compared to planktonic ones.

The high frequency of mutations in flagella genes among the attachment attenuated mutants highlighted the importance of flagella in the process of bacterial attachment. Work in our laboratory, using the λ red recombination system, deleted several flagella structural and motion genes to clarify the contribution of flagella and its motility in colonizing the broiler skin. Deletion in *flgK* and *fliCfljB* resulted in aflagellated non-motile strains and deletion in the *motA* gene led to paralyze flagella. However, deletion of each of the flagella sub-units (FliC and FljB) that can be expressed separately in a process known as phase variation result in a motile strain with a specific individual sub-unit. Lack of attachment ability in aflagellated and paralyzed mutants

emphasized the role of both flagella and motility on attachment. Moreover, comparison of two motile flagella sub-units in *ΔfliC* and *ΔfljB* mutants lead to the lack of attachment ability in *ΔfliC* mutant and no difference in *ΔfljB*. This stresses the importance of the FliC sub-unit as the main factor for *Salmonella* Kentucky colonizing on the broiler skin.

In the next step of the experimental process, the adhesion and invasion abilities of constructed flagella mutants to Caco-2 cell lines were evaluated as a model. Results confirmed the active role of motile FliC in both invasion and adhesion to the intestinal epithelial cell.

In conclusion, attachment of *S*. Kentucky to broiler epithelial cells (inside and outside of the bird) can be a multifactorial process. This process can be affected by several bacterial surface structures and various gene expression profiles. However, among all of the factors that were tested, the effect of the active flagella main sub-unit (FliC) is the most significant contributor to attachment.