ANTIBIOTIC RESISTANCE AND CELL SURFACE COMPONENTS OF *SALMONELLA*

by

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(Under the Direction of JINRU CHEN)

ABSTRACT

Certain *Salmonella* isolates from poultry meat and our laboratory collection were resistant to multiple antibiotics. Some antibiotic resistance genes in the *Salmonella* were transferable to *Escherichia coli* during conjugation. Subsequent incubation of *E. coli* transconjugants at elevated temperature caused the loss of acquired antibiotic resistance genes but not transferred plasmids, suggesting a possible involvement of mechanisms other than plasmid-mediated antibiotic resistance gene transfer. The *Salmonella* isolates were further tested for their ability to express cellulose and thin aggregative fimbriae, and their ability to attach to and form biofilm on polystyrene and glass surfaces. Cells of *Salmonella* expressing thin aggregative fimbriae alone or in combination with cellulose attached in higher numbers or formed more biofilm compared to those that synthesized only cellulose or neither surface component. Salt and glucose supplementation inhibited attachment and biofilm formation. Cell-surface contact time and properties of contact surface also had influence on attachment and biofilm formation.

INDEX WORDS: *Salmonella*, antibiotic resistance, thin aggregative fimbriae, cellulose, attachment, biofilm, gene transfer and conjugation.

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DEDICATION

To Dad, Mom and my loving family

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CHAPTER 1

INTRODUCTION

Salmonella is an important foodborne pathogen in developing as well as developed countries and is responsible for considerable morbidity and some deaths (Tauxe, 1991). *Salmonella* infection in humans can lead to several clinical conditions such as enteric (typhoid) fever, uncomplicated enterocolitis, or systemic infections. The infection is usually self-limiting, antibiotic treatment may, however, be required if the illness persists or the symptoms are severe (Cabrera *et al.* 2004).

Salmonella may have to live for a considerable length of time, during their life cycle, outside human or animal hosts where conditions may not always be optimal. Low temperature, nutrient starvation, and low osmolarity induce the expression of certain cell surface structures including thin aggregative fimbriae and cellulose and these cell surface structures lead to the formation of biofilm and the persistence of *Salmonella* in the environment (Solano *et al.* 2002). The thin aggregative fimbriae promote cell adherence to surface (Romling *et al.* 1998) while cellulose mediates cell to cell interaction and imparts cells the ability to resist disinfection by chlorine sanitizers (Solano *et al.* 2002).

The widespread use of antibiotics in agriculture and animal husbandry causes the release of antibiotic residues to the environment. Residual antibiotics in the systems of food animals present another surviving obstacle for pathogens like *Salmonella*. The natural response of *Salmonella* to such a condition is to develop resistance towards a wide range of antibiotics. The development of antibiotic resistance by bacteria in general

is mediated by several mechanisms, including changes in bacterial cell wall permeability, energy-dependent removal of antimicrobials via membrane-bound efflux pumps, modification of the site of drug action, as well as destruction and inactivation of antimicrobials (Schwarz and Chaslus-Dancala, 2001). A bacterial cell can acquire antibiotic resistance genes through three different approaches: transformation, conjugation, and transduction. The development of antibiotic resistance in *Salmonella* raises a serious public health concern because it reduces the therapeutic options available for the treatment of *Salmonella* infections.

This study was undertaken to determine the incidences of *Salmonella* contamination on poultry meats sold in Griffin and Peachtree City, GA area and to characterize the cell surface and antibiotic resistance properties of the isolated strains. The objectives of the present study are:

1. To determine the prevalence of *Salmonella* contamination in retail poultry meat in the sampling area and evaluate the antibiotic resistance profiles as well as the expression of thin aggregative fimbriae and cellulose by the isolated *Salmonella* strains,
2. Quantify the thin aggregative fimbriae and cellulose produced by *Salmonella* and evaluate the role of the two cell surface components in attachment and biofilm formation on certain abiotic surfaces,
3. Determine whether the antibiotic resistance genes in the isolated *Salmonella* can be transferred to *E. coli,* and whether the acquired antibiotic resistance genes can be eliminated from the recipient strains under certain environmental conditions.

CHAPTER 2

LITERATURE REVIEW

1. *SALMONELLA:* GENERAL MICROBIOLOGICAL CHARACTERISTICS

First described in a contemporary work by Salmon and Smith in 1885 as the causative agent of hog cholera and termed *Bacillus cholerae-suis*, *Salmonella* is now a very well recognized human and animal pathogen in the family *Enterobacteriaceae* (Tauxe, 1991). *Salmonella* cells are facultatively anaerobic, Gram-negative, and rod-shaped with an average size of 0.3 to 1.0 X 1.0 to 6.0 μm. The identification of *Salmonella* is based on several distinct biochemical characteristics, including the production of oxidase and catalase, utilization of citrate, production of hydrogen sulfide, decarboxylation of lysine, and ornithination of urea (D'Aoust and Purvis, 1998). *Salmonella* cells are chemoorganotrophic and can metabolize nutrients by respiratory as well as fermentative pathways. The optimum growth temperature for *Salmonella* is 37°C. Some serotypes can grow at temperatures up to 54°C, while others can grow at 2 to 4°C (D'Aoust, 1997). The optimum growth pH range for *Salmonella* is 6.5-7.5 but survival in the pH range 4.1-9.5 has been reported. Certain *Salmonella* isolates can grow at a pH level as low as 3.99 at 22°C (Jay, 2000). Water activity levels lower than 0.94 inhibit the growth of *Salmonella* cells. Salt concentration of 3-4% is inhibitory however, the salt tolerance of *Salmonella* increases with increasing temperature in the range of 10-30°C (D'Aoust, 1997). Brine above 9% is reported to be lethal to *Salmonella* (Jay, 2000).

Classification

Solely for epidemiological purposes, *Salmonella* can be placed into three groups. In group 1, are those *Salmonella* that infect only humans and are agents of typhoid and para-typhoid fever. They include *S*. Typhi, *S*. Paratyphi A and *S*. Paratyphi C. Group 2 consists of host adapted serovars some of which are human pathogens and may be contracted from foods. This includes *S*. Gallinarum, *S*. Dublin, *S*. Abortus-equi, *S*. Abortus-ovis, and *S*. Choleraesuis. *Salmonella* belonging to group 3 are un-adapted serovars which have no host preference and are pathogenic for humans and other animals. Most foodborne *Salmonella* fall into this category (Jay, 2000).

For taxonomic classification, *Salmonella* genus is divided into 2 species: *S. enterica* and *S. bongori. S. enterica* contains 5 subspecies (I, II, III, IV, and VI) while *S. bongori* was previously designated as subspecies V in *S. enterica.* In total, there are 2,463 serovars in these two species: 2,443 in *S. enterica* and 20 in *S. bongori.* The species and subspecies can be differentiated biochemically or by genomic relatedness (Brenner *et al.* 2000). The biochemical identification is usually coupled with serological confirmation, which types the strains based on their somatic (O), lipopolysaccharides (LPS) on the external surface of the bacterial outer membrane, flagellar (H) antigens associated with peritrichous flagella, or the capsular (Vi) antigens. Most of the human *Salmonella* isolates belong to subspecies I (*S. enterica* subspecies *enterica*) while most strains in subsp. II, III, IV, and VI are isolated from cold-blooded animals or the environment and seldom contain human isolates. Most strains of *S. bongori* are environmental isolates.

A number of molecular typing methods have been used in recent years to improve the identification and to differentiate *Salmonella* strains. Molecular typing methods such as ribotyping, IS-200 typing, pulse-field gel electrophoresis (PFGE) and PCR-based typing are more discriminatory than serotyping (Laconcha *et al.* 2000). For ribotyping, restriction digestion of chromosomal DNA is followed by southern blotting with rRNA probes in order to generate DNA banding patterns which allow subtype differentiation of bacterial isolates beyond the species and subspecies levels. PFGE is used for the subtyping of foodborne pathogens, namely non-typhoidal *Salmonella* serotypes, *E. coli* O157:H7, *Listeria monocytogenes*, and *Shigella* (Swaminathan *et al.* 2001). It uses endonucleases to cut DNA at rare restriction sites and generate large fragments. The DNA fragments are then subject to electrophoresis under alternating electric fields through a flat gel matrix of agarose. PFGE is useful in epidemiological investigation of *Salmonella* serovars and provides an improved level of discrimination over plasmid typing and ribotyping (Laconcha *et al.* 2000).

The Disease: Salmonellosis

Foodborne *Salmonella* infections are a major problem in most industrialized countries. In the United States, *Salmonella* is one of the most common pathogens implicated in foodborne illness (Logue *et al.* 2003). It is an almost universally accepted dogma that human salmonellosis is a zoonosis (D'Aoust, 1997).

Salmonella infection in humans can lead to several clinical conditions such as enteric (typhoid) fever, uncomplicated enterocolitis, or systemic infections by non-typhoidal salmonellas. The symptoms of non-typhoid *Salmonella* infection usually appear within 6-72 h of exposure to the causative agent. Enteritis is the most common

form of salmonellosis, with symptoms that include nausea, vomiting, abdominal pain, headache, chills, and diarrhea. These symptoms persist for 2-3 days and are usually accompanied by prostration, muscular weakness, faintness, moderate fever, restlessness, and drowsiness. Most *Salmonella* infections are self-limited, antibiotic treatment may, however, be required if the illness persists for a long time or if the symptoms are severe (Cabrera *et al.* 2004). The diagnosis of the disease relies on the isolation of the infective agent from blood or urine samples in the early stages of the disease or from stools after the onset of clinical symptoms (D'Aoust *et al.* 2001).

Pathogenesis and Virulence Factors

The virulence factors of *Salmonella* include toxin production, lipopolysaccharide (LPS) O-side chain, adhesion and invasion of host cells, virulence plasmids, survival in phagocytes, siderophores, and serum resistance (Rabsch, 1987). Some virulence factors are host specific and may appear only in a limited number of serovars or sometimes even strains (Fluit, 2005). *Salmonella* enterotoxin is a thermolabile protein with a molecular mass of 90 to 110 kDa. The toxin is encoded by a 6.3 kb chromosomal gene *(stx)* which regulates the synthesis of three proteins of 45, 26, and 12 kDa (Chopra *et al.* 1987). *Salmonella* strains also produce a heat labile cytotoxin that inhibits protein synthesis and causes lysis of host cells. The lipid A component of LPS is an endotoxin which causes an induced inflammatory response during invasion of the mucosal cells causing abdominal pain and fever associated with gastroenteritis. The damage to the mucosal cells reduces their water absorption and water holding capacity causing a net efflux of water and electrolytes into the intestinal lumen manifested as diarrhea (D'Aoust, 1991).

Salmonella cells force the host cells to engulf them which cause the host cell membrane to be deformed or ruffled. The invasion of mammalian cells by the bacteria is an integral aspect of *Salmonella* pathogenesis (Park, 1997). *Salmonella* possesses an invasin operon (*inv* A-H) which encodes for factors that regulate their entry into host cells (D'Aoust, 1991).

Salmonella enterica contains plasmids varying in size from 2 to more than 200 kb (Rychlik *et al.* 2006). Virulence plasmids ranging in size from 50-100 kb are among the most extensively studied. These plasmids are present in serovars Enteritidis, Typhimurium, Dublin, Cholerae-suis, Gallinarum, Pullorum, and Abortus-ovis. The intra-macrophage survival of strains within these serotypes is encoded by a *Salmonella* plasmid virulence (*spv*) operon, which is an 8 kb region of the virulence plasmid and consists of *spv*R, spvA, *spvB, spvC,* and *spvD* (Rychlik *et al.* 2006). The transfer of this operon upon plasmid transfer from a wild-type to a plasmid-cured strain restores the virulence of the recipient.

Microorganisms invading the host tissue encounter the first line of host defense in the form of phagocytes. *Salmonella* can resist phagocytic invasion due to the capsular (Vi) cell surface antigen, which provides resistance to superoxide radicals and defensins (the toxic peptides that kill bacteria) (D'Aoust, 1991). In the bacterial host, iron is bound to transferrin and lactoferrin in body fluids. One of the mechanisms for the acquisition of iron by bacteria is the secretion of iron specific chelators, termed siderophores, the function of which is to sequester and transport iron via specific transport proteins into the cell. *Salmonella* cells have two types of siderophores; enterochelin or enterobactin and aerobactin to meet their iron requirements (Rabsch, 1987).

As *Salmonella* cells invade the body, they encounter the complement system. The ability to resist the complement system is thus an important bacterial virulence factor. The O antigen polysaccharide of the LPS is a well recognized defense against complement. Longer O side chains activate complement via the alternative pathway distant from the cell to prevent membrane attack complex (MAC) insertion into the outer membrane (Joiner *et al.* 1982).

Reservoirs of *Salmonella*

Salmonella are widespread in the environment. Their primary habitat is the gastrointestinal tract of humans and animals (especially those on farms), birds, reptiles (both wild and domestic), and occasionally insects (Jay, 2000). They may also be found in body parts such as the spleen, liver, bile, mesenteric and portal lymph nodes, diaphragm, and pillar of slaughtered animals (Jay, 2000). In the environment, contaminated water, soil, and manure are the reservoirs of *Salmonella.*

The persistence of *Salmonella* in slaughterhouses and meat processing facilities continues due to the exposure of livestock to environmental sources of contamination, contaminated feeds, and the parental transmission of infections (D'Aoust, 1997). The feces of infected humans and animals contaminate water sources, which subsequently infect farm animals that contaminate meat during slaughter, and subsequently infect humans repeating the transmission cycle (Jay, 2000). This cycle is further augmented by the international shipping of live animals, animal products, and feed (Jay, 2000). Increased frequency of international travel from industrially developed countries to countries where public health prevention measures such as purification of water supplies, sewage control, treatment of chronic carriers, and sanitary and hygiene education,

especially among food handlers, are not prevalent has contributed to the global spread of *Salmonella* infections (Kubota *et al.* 2005).

Different parts of the world are predominated by different serovars of *Salmonella*. Serovars such as *S.* Typhimurium and *S.* Enteritidis are found throughout the world, whereas, other serovars can be found with high frequency for short periods of time and then disappear suddenly (D'Aoust *et al.* 2001).

Foods Associated with *Salmonella*

Salmonella has been implicated in human illness worldwide by the consumption of a wide variety of contaminated food products of animal as well as plant origin. Farm animals such as swine, poultry, and cattle often carry *Salmonella* which contaminates meat, eggs, and dairy products (Cabrera *et al.* 2004). Poultry and eggs are the predominant reservoirs of *Salmonella*, but other meats such as beef, pork, and mutton could also be a source of infection (D'Aoust *et al.* 2001). Various *Salmonella* outbreaks have been associated with the consumption of raw and pasteurized milk, chocolate milk, milk powder, ice cream, and cheddar cheese (D'Aoust *et al.* 2001). The largest outbreak of salmonellosis in the United States occurred in 1985, which caused more than 16,000 confirmed cases of illness (Lecos, 1986). Cross contamination between raw and pasteurized milk was determined as the probable cause of the outbreak.

Fruits and vegetables have also gained notoriety in recent years as vehicles of human salmonellosis. The situation has developed due to the export of fruits and vegetables from subtropical and tropical climate regions where production, harvesting, and distribution of these products do not meet the minimum hygiene standards. Human salmonellosis outbreaks have been associated with the consumption of tomatoes,

cantaloupes, paprika, alfalfa and mung bean sprouts, spices, chocolate, orange juice, and peanut sauce (D'Aoust *et al.* 2001).

Despite the general perception that meat and egg products are the primary sources of human *Salmonella* infections, many outbreaks in recent years have been associated with the consumption of fresh fruits and vegetables, spices, chocolate, and milk products (D'Aoust *et al.* 2001). This emphasizes the importance of sanitary practices during the harvesting, processing and distribution of all raw foods and food ingredients.

Poultry and *Salmonella*

A considerable number of human salmonellosis outbreaks have been associated with the consumption of contaminated or undercooked poultry (Bryan and Doyle, 1995). Poultry and poultry products are the frequent vehicles in *Salmonella* transmission and dominate other foods of animal origin as a potential source of the pathogen, thereby overshadowing the importance of other meats, such as pork, beef, and mutton as potential sources of infection (Dominguez *et al.* 2002). Carraminana *et al.* (1997) sampled poultry carcasses in Spanish slaughter houses and reported *Salmonella* contamination ranging from 20-70% with an average of 35%. In the U.S. a prevalence rate of 10% was reported in Ohio (Bokanyi, 1990). Another large, three-year study carried out in the U.S., reported a *Salmonella* prevalence rate of 20% in broilers, 44.6% in ground chicken, and 49.9% in ground turkey (Rose *et al.* 2001). *S.* Enteritidis, *S.* Virchow, and *S.* Hadar are normally associated with chicken and chicken products whereas, serotype Hadar is predominantly turkey related (Threlfall, 2002).

A recent survey estimates 1 in every 20,000 eggs to be contaminated with *Salmonella* (Ebel and Schosser, 2002). The most common *Salmonella* serotype

contaminating eggs is *S.* Enteritidis (Humphrey *et al.* 1995). It infects the ovarian tissues, principally the upper oviduct of healthy hens (Humphrey, 1994). *S.* Enteritidis can be deposited in the interior of a developing egg prior to shell deposition (Humphrey *et al.* 1995). *S.* Enteritidis cells can cross the shell even after the eggs are laid (Chen *et al.* 1996). The viability of internalized cells remains unaffected by the egg surface sanitizing practices currently used by the egg processing industry. Strains of *S.* Enteritidis phage type 4 are noted for their increased virulence and invasiveness in young broiler chicks and for their greater resistance to heat (Humphrey *et al.* 1995). *S.* Enteritidis phage type 4 in Europe and phage type 8 in North America associated with the consumption of foods containing raw or lightly cooked eggs are a continuing cause of disease (D'Aoust *et al.* 2001).

Epidemiology

The Centers for Disease Control and Prevention (CDC) estimated that in 1997, foodborne infections caused approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths (Mead *et al.* 1999). Among the 14 million cases of illness caused by the known pathogens, *Salmonella* infections alone were responsible for 1.3 million or 9.7% of the total cases of disease (Mead *et al.* 1999). These infections cause 15,608 hospitalizations (25.6% of total hospitalizations due to foodborne diseases) and 553 deaths each year (30.6% of total foodborne deaths). The estimated range of overall patient-related costs due to salmonellosis is between $275 million and $1.1 billion per annum in the U.S. (Tauxe, 1991). According to the Economic Research Service of the United States Department of Agriculture the estimated annual economic costs due to foodborne *Salmonella* infections are $3 billion (USDA, 2006). *Salmonella* infections

accounted for the largest number of outbreaks and cases of foodborne disease between the years 1993 and 1997 in the U.S. (CDC, 2000). The FoodNet surveillance system which covers 15.2% of the U.S. population and gathers data from 10 sites, identified a total of 15,806 laboratory-diagnosed cases of infections caused by nine foodborne pathogens in the year 2004 (CDC, 2005). Out of all the reported cases of foodborne illness, 6,464 were due to *Salmonella* infection making it the leading cause of foodborne illness in the year 2004. Among the 5,942 (92%) *Salmonella* isolates serotyped, the five serotypes which accounted for 56% of the total infections were *S*. Typhimurium (1,170, 20%), *S*. Enteritidis (865, 15%), *S*. Newport (585, 10%), *S*. Javiana (406, 7%), and *S*. Heidelberg (304, 5%) (CDC, 2005). The estimated incidence of overall *Salmonella* infections dropped by 8% from the year 1996 to 2004 (CDC, 2005). Although there was an overall decrease in *Salmonella* infections, an actual decrease was seen only in the incidence of *S.* Typhimurium (41%). The incidence of *S*. Enteritidis and *S*. Heidelberg did not change significantly whereas, the incidence of *S.* Newport and *S.* Javiana increased 41% and 167%, respectively. The overall incidence of *Salmonella*-related illness in 2004 was 14.7 per 100,000 persons (CDC, 2005).

Several important food safety initiatives and education efforts at the farm as well as at the processing and distribution levels have contributed to the decrease in incidences of *Salmonella* infection. The implementation of HACCP programs in the poultry and beef processing industry and the adoption of new microbiological standards for egg quality are examples of such initiatives. Baseline studies conducted prior to the implementation of the "Final rule on pathogen reduction and hazard analysis and critical control points (HACCP) in meat and poultry industry" indicated a *Salmonella*

contamination of 24% in U. S. broiler chicken. During the first three years of the mandatory implementation of the HACCP system, the rate of contamination had been reduced to approximately 11% (D'Aoust *et al.* 2001). Similar reductions were also observed with other meat animals. Coordinated efforts from all sectors of the meat industry towards the implementation of strict control measures to reduce contamination at the farm, processing, distribution, and retail levels will help to further reduce the frequency of *Salmonella* infections.

2. ANTIBIOTIC USE IN ANIMAL HUSBANDRY

The use of antimicrobials in food animals has become an integral part of animal husbandry. Food animals, like humans, are very likely to be treated with antibiotics at some point during their lives. The U.S. Food and Drug Administration (FDA), Center for Veterinary Medicine has approved the use of antibiotics in animal and poultry farming for (i) therapeutic use to treat infected animals; (ii) prophylactic use to avoid the spread of infection in the herd if one animal starts to show symptoms of an infectious disease; and (iii) nutritive use at subtherapeutic levels to induce growth promotion (Teuber, 1999). The commonly used antimicrobial agents in food animals belong to one of the five major classes: β-lactams, tetracyclines, aminoglycosides, macrolides, and sulfonamides (White and McDermott, 2001). Most sub-therapeutically used antibiotics in poultry farming are broad spectrum and can inhibit gram-positive as well as gram-negative bacteria. The U.S. poultry industry currently uses bacitracin, virginiamycin, bambermycin, and lincomycin for growth promotion (National Research Council, 1999). Cephalosporin-ceftiofur and aminoglycoside gentamicin are given to "day of hatch" chicks after vaccination against viral pathogens to prevent secondary bacterial abscesses (White *et al.*

2001b). Until the mid 1990's, tetracyclines were the only drug class allowed to treat *Escherichia coli*-associated infections in poultry however, the introduction of nalidixic acid-derived synthetic fluoroquinolones, enrofloxacin, and sarafloxacin have replaced the use of tetracyclines (White *et al.* 2001b). Neomycin, novobiocin, penicillin, streptomycin, the sulfonamides, the macrolides, and ionophores such as monensin and salinomycin have also been approved by the FDA for use in chickens and turkeys (National Research Council, 1999).

Aquaculture industry employs high density farming conditions to maximize yields and to satisfy the growing demand for fish and other marine products. Many of these aquacultural products originate from Asian, African, or South American countries where raw meat scraps and offal used as feed, are frequently contaminated with *Salmonella* (D'Aoust, 1994). Rearing of fish in earthen ponds or other unprotected facilities continuously expose them to environmental contamination. Bacterial contamination of rearing facilities causes severe economic losses due to poor growth or death of aquacultural populations. Antibiotics such as ampicillin, chloramphenicol, sulfa drugs, and quinolones are therefore used extensively to protect the health of farmed fish and shellfish (D'Aoust, 1994).

The use of antibiotics in animal feed, aquaculture, and agriculture may lead to the release of antibiotic residues into the environment which may exert selective pressure for the selection of antibiotic resistant bacteria. Antibiotics given to animals and humans for therapeutic purposes can exert selective pressure on their target bacteria (Angulo *et al.* 2000). Opposing views exist about the use of antibiotics in agriculture and animal husbandry. The proponents of the use of antibiotics as growth promoters in animals

attest to the benefits of antibiotic use by citing advantages such as an overall improvement in animal health and decreased feed costs. The opponents of antibiotic use in agriculture and animal husbandry argue that the widespread use of prophylactic doses of clinically important drugs in animal farming increase the human health risks associated with the handling and consumption of contaminated meat products (D'Aoust, 1994).

Antibiotics and Associated Risks

Antibiotics have become invaluable tools in decreasing morbidity and mortality associated with bacterial infections in humans. Animal health and productivity have also improved significantly since the introduction of antibiotics in veterinary medicine (National Research Council, 1999). However, antibiotic use has not come without serious repercussions, manifested in the form of development of resistance towards antibiotics in bacteria. The use of antimicrobial drugs in any ecosystem may select for antimicrobial-resistant bacteria (O'Brien, 2002). This issue first came under light in Britain in 1968 when a rise in *S.* Typhimurium DT29 infections in calves was observed during the years 1964-1966 (Anderson, 1968). The extensive use of antimicrobial agents in human and veterinary medicine has caused an increase in the frequency of isolation of antibiotic resistant bacteria (Bronzwaer, 2002). Additionally, antimicrobial resistant bacterial pathogens in animals pose a risk not only to animal health but also to humans by possible transmission as foodborne pathogens (Fey *et al.* 2000). Antibiotic use in agriculture increases the frequency of isolation of antibiotic resistant *Salmonella* (Smith *et al.* 2002). The rate of isolation of multidrug resistant *Salmonella* from food products sold in the U.S. and other countries has increased in recent years (Glynn *et al.* 1998; Guerra *et al.* 2001; Chiu *et al.* 2002; Antunes *et al.* 2005). The pentaresistant *S. enterica*

serotype Typhimurium DT104, with resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (R-type ACSSuT), has emerged as a global health problem due to its involvement in animal and human diseases (Antunes *et al.* 2005).

The major public health concern with the development of antibiotic resistance in *Salmonella* is their acquired resistance to antibiotics such as ampicillin, chloramphenicol, sulfa drugs, and quinolones which are used to cure acute cases of *Salmonella* infection in humans (Fey *et al.* 2000). The emergence of resistance to fluoroquinolones among non-typhoid *Salmonella* is even more important because this class of antimicrobials is the drug of choice for treating potentially life-threatening infections caused by multiple-antibiotic resistant *Salmonella* strains (Angulo *et al.* 2000).

Antimicrobial resistance in bacteria can spread by multiple routes such as (i) translocation of a resistance gene from one place in the bacterial genome (plasmid or chromosome) to another; (ii) horizontal spread of resistance genes from one individual bacterium to another of the same species or over species; (iii) spread of resistant bacteria from animal to animal and from animal to the environment; (iv) spread from animal to human by direct contact or via food; (v) global spread by export / import of live animals and products; (vi) nosocomial spread of antibiotic resistant bacteria in healthcare settings; and; (vii) community transmissions by food, domestic animals, or person-to-person contact (Teuber, 1999).

Antibiotic resistance varies among bacteria isolated from different countries; therefore, continued surveillance of antimicrobial resistance is essential for providing

information on the magnitude and trends in resistance and for monitoring the effect of intervention strategies.

Evolution and Transfer of Antibiotic Resistance

Tolerance is the ability of the bacteria to survive in the presence of antibiotics, but not to undergo cell division. Antibiotic resistance on the other hand, refers to the ability of the bacteria to survive as well as multiply in the presence of antibiotics. When placed under environmental stress by an antibiotic or disinfectant, bacteria have two choices; adaptation or death. Bacteria are extremely adaptable, so their innate response is to become resistant (Cohen and Tauxe, 1992). Bacterial resistance to antimicrobials is due to one or more of the following mechanisms: (i) changes in bacterial cell wall permeability, (ii) energy-dependent removal of antimicrobials via membrane-bound efflux pumps, (iii) modification of the site of drug action, and/or (iv) destruction or inactivation of antimicrobials (Schwarz and Chaslus-Dancala, 2001).

Bacterial resistance to antibiotics can be intrinsic or acquired. Intrinsic resistance is the natural tendency of bacteria to resist an antibiotic without modification of the target site of the antimicrobial or without the acquisition of resistance genes. Cells of the bacteria belonging to *Mycoplasma* species lack cell walls. The absence of cell walls in these microorganisms makes them intrinsically resistant to β-lactams as well as all other antibiotics which target the bacterial cell wall (Bebear and Pereyre, 2005). *Pseudomonas aeruginosa* have an intrinsic capability to resist the effect of macrolides due to the presence of multidrug efflux pumps (Hancock and Speert, 2000). Acquired antibiotic resistance is a result of bacteria sharing the genetic information to survive in the presence

of antibiotics. It is usually a result of chromosomal mutations or gene transfer by transduction, transformation, or conjugation (Thomas and Nielsen, 2005).

Generalized transduction in *S.* Typhimurium as a means of gene transfer was discovered by Zinder and Lederberg in 1952, who noticed that strain LT22 could transfer genetic material to recipient cells of strain LT2 by means of a temperate bacteriophage, P22. Schmieger and Schicklmaier (1999) reported that bacteriophage ES18 could successfully transfer resistance to ampicillin, chloramphenicol, streptomycin, and tetracycline from a resistant *S.* Typhimurium DT104 strain DT17 to a susceptible strain, DT16. The genes *amp, sul,* and *str* which encode for resistance towards ampicillin, sulfonamides, and streptomycin could be transferred by a bacteriophage (Ridley and Threlfall, 1998). Bacteriophage AP-151 isolated from *P. aeruginosa* has the ability to transduce multiple antibiotic resistance gene cassettes encoding resistance to imipenem, ceftazidime, and cefotaxime from a resistant to antibiotic-susceptible strain of *P. aeruginosa* (Blahova *et al.* 2000).

Transformation involves the uptake of DNA by the bacterial cells from the surrounding environment. It is the most efficient *in vitro* technique of introducing plasmids into bacteria for gene cloning purposes, but has a very limited role in gene transfers *in vivo* (Davison, 1999). Some bacteria are competent and have a natural capability to uptake DNA from the environment whereas others have to be made so by treatment with calcium chloride in the early log phase of their growth (Thomas and Nielsen, 2005). *Campylobacter coli, C. jejuni*, and *Streptococcus mutans* are naturally competent and are transformable either with homologous or foreign DNA (Wiesner *et al.* 2003).

Conjugation, unlike transduction and transformation requires direct cell-to-cell contact of two bacterial cells for the transfer of DNA (Gebreyes and Altier 2002). The spread of antibiotic resistance genes via conjugation involves genetic elements that can effectively capture and disseminate resistance determinants in gram-negative and gram-positive bacterial species (Salyers *et al.* 1995). Plasmids, transposons, and integrons are such vehicles (Schwarz and Chaslus-Dancala, 2001). Plasmids are double-stranded linear or circular, extra-chromosomal elements capable of replicating independently of the host chromosome. Transposons are mobile DNA elements that can move from one location to another within a genome while integrons are small DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, by site-specific recombination (Schwarz and Chaslus-Dancala, 2001).

Some believe conjugation to be a dominant mode for the transfer of genetic material between bacterial cells and for the development of antibiotic resistant bacteria (Wolska, 2003). Numerous reports relate the transfer of antibiotic resistance to plasmid transfer by the process of conjugation. Galimand *et al.* (1997) reported the transfer of plasmids carrying the resistance genes for ampicillin, chloramphenicol, kanamycin, streptomycin-spectinomycin, sulfonamides, and tetracycline-minocycline from a resistant *Yersinia pestis* strain 17/95 to a susceptible *Y. pestis* strain 6/69cN. It is widely known that *Salmonella* could transfer drug resistance to susceptible *E. coli* by conjugation (Gebreyes and Altier, 2002; Robertson *et al.* 2003; Chen *et al.* 2004; Gebreyes and Thakur, 2005). Gebreyes and Altier (2002) reported the transfer of plasmids which carried genes for resistance to ampicillin, amikacin, kanamycin, tetracycline, streptomycin, and suflamethoxazole from *Salmonella*. Resistance to amoxixillin-

clavulanic acid, cefoxitin, chloramphenicol, tetracycline, ceftriaxone, cephalothin, kanamycin, streptomycin, and sulfamethoxazole was transferred from various *Salmonella* strains to *E. coli* upon conjugation (Chen *et al.* 2004). The transfer of the antimicrobial genes in *E. coli* was confirmed by PCR using gene specific primers. Pai *et al.* (2003) found that strains of *S. enterica* Typhi could also transfer resistance against ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol, tetracycline, streptomycin, gentamicin, and tobramycin upon conjugation with *E. coli* J53.

The integrons found in clinical isolates of some Gram-negative bacteria contain one or more integrated cassettes, each including an antibiotic resistance gene (Recchia and Hall, 1995). A larger number of gene cassettes containing different resistance genes have been identified (Recchia and Hall, 1995). The *dfrA14* gene in pHCM1 is an integron gene cassette in *S.* Typhi (Partridge *et al.* 2001). Pai *et al.* (2003) reported the presence of gene cassettes with *aacA4b, catB8, aadA1, dfrA1, aac (6')-IIa,* and *blaP2* resistance genes in *S.* Typhi which could be transferred to *E. coli*. *S*. Derby, *S*. Muenchen, and *S*. Worthington carry the gene cassette *aadA* which confers resistance to aminoglycosides (Gebreyes *et al.* 2004).

Antibiotic Resistance in Poultry Related *Salmonella*

Poultry contaminated with *Salmonella* has gained considerable attention recently due to the increased incidence of antimicrobial-resistance and the changes in the level and type of resistance observed in poultry isolates. Logue *et al.* (2003) observed that 50% and 35% of *Salmonella* isolated from freshly-processed poultry were resistant to tetracycline and streptomycin, respectively. Antunes *et al.* (2003) reported that 39% of the *Salmonella* isolates from raw poultry products were resistant to streptomycin, 36% to

tetracycline, and 19% to penicillin. A resistance rate of 30-50% against doxycycline, josamicin/trimethoprim, sulfisoxazole/trimethoprim, and tetracycline was found in *Salmonella* isolated from ducks in Taiwan (Tsai and Hsiang, 2005). Oliveira *et al.* (2005) found 91% and 86% of the *Salmonella* isolated from broiler carcasses to be resistant to sulfonamides and nitrofurantoins. Carraminana *et al.* (2004) isolated *Salmonella* from a poultry slaughterhouse in Spain and found that 96% of the isolates were resistant to sulfadiazine, 53% to neomycin, 22% to tetracycline, and 11% to streptomycin. Among the *Salmonella* isolated from raw, chilled, retail poultry in the U. K., 52% of the isolates were resistant to sulfonamides, 26% to streptomycin, 22% to tetracycline, and 17% to ampicillin (Wilson, 2004).

Multidrug resistance (MDR), defined as resistance to two or more antimicrobial agents, is a common phenomenon in *Salmonella* isolated from poultry. Larkin *et al.* (2004) reported that 42% of the *Salmonella* isolated from chicken were MDR. Chung *et al.* (2004) observed MDR in 65% of the *Salmonella* isolates obtained from broiler carcasses while Robertson *et al.* (2003) reported even higher values of 87% of isolates being MDR.

III. BACTERIAL ATTACHMENT AND BIOFILM FORMATION ON ABIOTIC SURFACES

Bacteria generally enter a developmental pathway in response to nutrient limitation which can be considered as a collective defense to environmental stresses (Costerton *et al.* 1987). Adhesion to solid surfaces is part of the bacterial response to survival under stressful conditions. Bacteria adhered to a solid surface tend to clump together in order to maximize nutrient acquisition and stress tolerance (Otto and

Hermansson, 2004). As a result, cells living in a community on a solid surface have greater survivability than their free-living counterparts.

Attachment

Bacterial attachment to a surface is the first and essential stage in the formation of a biofilm. The surface characteristics of both the bacteria and the surface influence the rate of bacterial attachment. Cell attachment to a surface is generally described as a two-step process; an initial, reversible attachment step followed by an irreversible and permanent attachment step (Marshall *et al.* 1971). Reversible attachment is an immediate, weak interaction between the bacterial cells and the surface, and involves van der Waals and electrostatic forces along with weak hydrophobic interactions (Herwald *et al.* 1998). The removal of attached bacteria in this step is easy and can be achieved by the application of a mild shear force. Irreversible bacterial attachment takes place when the cells anchor themselves to the surface by utilizing their surface components such as pili, flagella, and adhesin proteins (Pratt and Kolter, 1998). The irreversible attachment usually occurs within hours of initial contact of the bacterium with the attachment surface (Hood and Zottola, 1997).

Biofilm Formation

Biofilms are ubiquitous in the environment and are formed when a solid surface is in contact with bacteria in an aqueous environment. Lehner *et al.* (2005) defined biofilm as a structured community of microorganisms enclosed in self-produced, hydrated, polymeric matrices attached to surfaces. Various cell surface components such as flagella, fimbriae, outer membrane proteins, and extracellular polymeric substances are involved in biofilm formation (Pratt and Kolter, 1998). Cellulose, along with thin

aggregative fimbriae, is particularly important in the formation of biofilm by *Salmonella* (Zogaj *et al.* 2001). Biofilm formation on solid surfaces takes place in four sequential steps: (i) transport of the microorganisms; (ii) initial microbial adhesion; (iii) attachment by cell surface structures; and (iv) colonization by growth of firmly attached organisms (van Loosdrecht *et al.* 1990).

The bacterial biomass and surrounding polymer matrix exist as a columnar structure and are surrounded by water-filled spaces in a biofilm. The channels and pillars in biofilms permit the exchange of nutrients and wastes. Bacterial cells which are embedded in a biofilm exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002). In the natural environment bacteria can undergo transition from free-living unicellular organisms to sessile multi-cellular communities (Costerton *et al.* 1995).

Factors Affecting Bacterial Cell Attachment and Biofilm Formation

Bacterial cell surface components

The surface of a Gram-negative bacterium is heterogeneous as well as three-dimensional. It has a complex chemical composition, predominated by various proteins or carbohydrates. The proteinaceous components include outer membrane proteins, fimbriae, and flagella, while the carbohydrate-rich components are extracellular polysaccharides (EPS), cellulose, and lipopolysaccharides. These cell surface components assist bacterial cells to overcome the electrostatic repulsive forces between themselves and the surface to which they may attach.

Thin aggregative fimbriae

Thin aggregative fimbriae were first characterized in an enterotoxigenic *S.* Enteritidis isolate (Collinson *et al.* 1991, 1996) and were found to be homologous to the curli fibers expressed by *E. coli* (Doran *et al.* 1993). They appear as thin (2 to 4 nm) or thick (7-8 nm), flexible, thread-like structures on the outer membrane of *Salmonella* cells (Collinson *et al.* 1996). The fimbriae are remarkably stable requiring treatment with 90% formic acid to depolymerize (Collinson *et al.* 1991). They are comprised of AgfA and AgfB protein subunits; the AgfA is the fimbrin protein, and the AgfB functions as a surface-exposed nucleator for AgfA (White *et al.* 2001a). Assembly of AgfA takes place via the extracellular nucleation-precipitation pathway. The secreted AgfA undergoes conformational alteration upon interaction with AgfB during polymerization and assembly (Bian and Normark, 1997). The biosynthesis of thin aggregative fimbriae is controlled by two divergently transcribed operons, *agfDEFG* and *agfBA(C)*. The two operons are separated by a 521 bp intergenic region (Romling *et al.* 1998). The *agfD* acts as a positive transcriptional regulator for the *agfBA(C)* operon (Hammar *et al.* 1995). .

The expression of thin aggregative fimbriae is regulated by growth temperature as well as the nutritional composition and osmolarity of the growth media (Romling *et al.* 1998). The optimum expression of thin aggregative fimbriae takes place in the stationary growth phase and generally no expression occurs in the logarithmic phase of growth (Gerstel and Romling, 2001). Presence of phosphate, nitrogen, and iron in the growth media inhibits the synthesis of thin aggregative fimbriae (Romling *et al.* 1998). Northern blot analysis has shown that the transcription levels for the *agfD* and *agfA* under conditions of iron depletion doubled in comparison to their levels in growth medium

supplemented with iron (Romling *et al.* 1998). Most *S.* Typhimurium and *S.* Enteritidis strains express thin aggregative fimbriae at 28°C but not at 37°C (Woodward *et al.* 2000). The expression of thin aggregative fimbriae is abolished under conditions of high osmolarity as the transcription of *agfD* is switched off under these conditions (Romling *et al.* 1998). The outer membrane protein R (OmpR) is a cytoplasmic protein which regulates the transcription of *agfD*. The deletion of the gene encoding OmpR in *S.* Typhimurium abolishes transcription of *agfD*, thereby inhibiting the expression of thin aggregative fimbriae (Romling *et al.* 1998).

Cellulose

Cellulose is the most abundant organic polymer found in nature. Bacteria such as *Rhizobium, Agrobacterium tumefaciens,* and *Acetobacter xylinus* produce cellulose to facilitate attachment to their host tissues (Ross *et al.* 1991). Cellulose produced by *S.* Enteritidis and Typhimurium mediates their survival on solid surfaces (Solano *et al.* 2002) and protects them from mechanical and chemical damage (Solano *et al.* 2002).

Bacterial cellulose or biocellulose has a chemical structure similar to the plant cellulose, which is a long polysaccharide polymer of beta glucose subunits. The linear polymerization of β(1-4) linked D- glucopyranosyl rings build a structural network which is a major component of biofilm formed by *Salmonella* and *E. coli* (Ross *et al.* 1991; Zogaj *et al.* 2001). Although sharing similar structures, biocellulose has different physical and chemical properties in comparison to cellulose produced by plant cells.

Four proteins essential for cellulose biosynthesis, the cellulose synthase, the cylic-di-GMP binding protein, BcsC, and BcsD, are encoded by the bacterial cellulose synthesis operon, *bcs* (Ross *et al.* 1991). The precursor of cellulose, UDP-glucose is

polymerized to cellulose by the cellulose synthase complex in the bacterial membrane. The activator cylic-di-GMP binds to the c-di-GMP binding protein, which is structurally associated with cellulose synthase and regulates its activity. Although *bcs* genes are constitutively transcribed; cellulose synthesis only takes place when *adrA* (*agf*D-dependent regulator), a gene encoding a putative transmembrane protein regulated by *agfD*, is expressed (Zogaj *et al.* 2001). The AgfD stimulates the transcription of AdrA, which activates cellulose production by direct interaction with the *bcs* operons (Zogaj *et al.* 2001; Solano *et al.* 2002). The addition of glucose to culture media has a repressive effect on the activity of *agfD* (Gerstel and Romling, 2001), therefore reducing the expression of cellulose.

Expression of thin aggregative fimbriae and cellulose by *Salmonella*

Salmonella cells grown on LB no-salt agar supplemented with Congo red and Coomassie brilliant blue exhibit four different colony morphotypes depending on their ability to produce thin aggregative fimbriae and /or cellulose. The colonies of cells that produce the thin aggregative fimbriae appear red or brown, while those that do not produce the fimbriae form pink or colorless colonies, depending upon the ability of the cells to produce or not to produce cellulose. Expression of thin aggregative fimbriae alone leads to a brown, dry, and rough (bdar) colony morphotype whereas the expression of cellulose alone leads to a pink, dry, and rough (pdar) colony morphotype. Co-expression of thin aggregative fimbriae and cellulose leads to a red, dry, rough (rdar) colony morphotype, while the expression of neither of the two cell surface components leads to a smooth and white (saw) colony morphotype (Romling *et al.* 1998).

Influence of thin aggregative fimbriae and cellulose on attachment and biofilm formation

The formation of biofilm by *Salmonella* requires the participation of both thin aggregative fimbriae and cellulose (Romling *et al.* 2000; Zogaj *et al.* 2001). Cells with thin aggregative fimbriae have a better ability to attach to glass and polystyrene surfaces (Vidal *et al.*1998). Cells expressing the rdar and bdar colony morphotype form more biofilm on abiotic surfaces compared to the cells that express the pdar colony morphotype (Romling *et al.* 1998; Solano *et al.* 2002). Very low levels of attachment and biofilm formation are characteristic of the *Salmonella* cells expressing the saw morphotype. These findings suggest that thin aggregative fimbriae impart an extraordinary attachment and biofilm-forming ability to *Salmonella* and their co-expression with cellulose enhances the biofilm forming ability of the cells.

Other factors influencing attachment and biofilm formation

Flagella

Before they can attach, microorganisms must reach a surface by their own ability to move or being transported by other means such as water or food. Flagella are cell appendages which facilitate the movement of bacteria. The motility provided by flagella enables the bacterium to reach the attachment site thereby playing an important role in facilitating the attachment of bacteria to solid surfaces (Piette and Idziak, 1991). Allen-Vercoe and Woodward (1999) compared the adherence of a wild-type with that of an isogenic non-fimbriatic strain of *S.* Enteritidis using an *in vitro* gut adherence assay. It was noticed that the adhesion of the non-flagellated mutant strains on the chicken gut explants was significantly lower than was the adhesion of the flagellated strains.

Vatanyoopaisarn *et al.* (2000) found that the flagellated cells of *L. monocytogenes* were 10 times more likely to attach to the surface of stainless steel compared to the non-flagellated mutants. A comparison between the attachment of flagellated and mechanically deflagellated cells of *P. fluorescens* on meat tendon slices showed that more flagellated cells attached to the meat surfaces than did the deflagellated cells (Piette and Idziak, 1991). Removal of flagella by mechanical means or by treatment with specific antiflagella serum reduced the attachment of *S.* Choleraesuis to beef muscle by 90% (Bouttier *et al.* 1997). The reduced attachment of bacterial cells to the beef muscle was believed to be caused by the loss of cell mobility. Non-motile cells could not readily reach the surface during the contact period, therefore significantly reducing the rate of their attachment.

The flagella-mediated bacterial motility is also important in biofilm formation by bacteria. During biofilm formation by *E. coli* in LB broth, flagella were instrumental in establishing the cell-surface contacts (Pratt and Kolter, 1998). The non-motile strains of *P. aeruginosa* and *P. fluorescens* have reduced biofilm formation compared to the motile strains (Pratt and Kolter, 1999).

Bacterial surface charge and hydrophobicity

The cells of a bacterium can gain a surface charge by virtue of ionization of surface molecules and adsorption of ions from the aqueous environment surrounding them. The bacterial cell wall and membranes contain numerous charged molecules such as proteins, lipid molecules, teichoic acids, and lipopolysaccharides, which impart a characteristic charge to the cell surface (Szumski *et al.* 2005). These charged groups attract oppositely charged groups from the environment and form an electric double

layer. The difference between the charge on the surface of bacterial cells and the surrounding medium is known as the zeta electric potential. Busscher *et al.* (1997) observed that the bacterial cells with the most negative zeta potentials had the poorest ability to adhere to the negatively charged silicone rubber. The surface tension of a bacterial cell influences its ability to adhere to various surfaces. Bacterial cells having higher surface tension than that of the surrounding medium are more likely to attach to the surface of polymers compared to the cells whose surface tension is lower than the surrounding medium (Absolom, 1988).

Bacterial hydrophobicity, defined as the tendency for a microbial cell to associate with similar cells or molecules as opposed to water, has been evaluated by the use of a contact angle of a water drop on a lawn of bacterial cells (Wan *et al.* 1994). Bacteria having a hydrophobic surface have a relatively larger contact angle than do bacteria with a hydrophilic surface. Wan *et al.* (1994) found that the contact angle of a water drop on a relatively hydrophobic bacterial culture was 77.1° while the same angle for a relatively hydrophilic bacterial culture was 24.1°. Liu *et al.* (2004) found that bacterial adhesion is highly facilitated when both bacterial and support surfaces are hydrophobic, however, the adhesion would proceed with difficulty if both the bacterial and support surfaces are hydrophilic. They also observed that increased cell surface hydrophobicity would increase cell adhesion on hydrophilic surfaces.

Material properties

Surface topography of the attachment surface is an important determinant for bacterial adhesion. Roughness and irregularity in the surface increase the area of contact between bacteria and the surface, making it easy for bacterial cells to deposit on the

material (Alava *et al.* 2005). Higher levels of attachment by *P. aeruginosa* cells was observed on the surface of contact lenses which had an irregular and relatively rougher surface than the ones which had a smooth and regular surface (Alava *et al.* 2005).

Polymers such as polystyrene, rubber, and Teflon are hydrophobic and possess less surface energy and charge, whereas, metals usually carry a positive or neutral charge on their surface. Non-polymeric synthetic materials such as glass and stainless steel are hydrophilic, have a negative surface charge, and possess high surface energy (Fletcher and Loeb, 1979). Preferential bacterial adhesion takes place on hydrophobic surfaces with little or no surface charge while very few bacterial cells attach to hydrophilic negatively charged surfaces. Henriques *et al.* (2005) observed that high numbers of *P. aeruginosa* and *Staphylococcus epidermidis* cells attached to the hydrophobic silicone-hydrogel contact lenses compared to the hydrophilic lenses made of the same material. Lehner *et al.* (2005) observed that approximately 59% of the *Enterobacter sakazakii* strains examined in their study were able to attach to the hydrophobic polyvinylchloride (PVC) surface while only 43% of the tested strains were able to attach to the hydrophilic surface of glass. Conditioning of silicone rubber with a biosurfactant obtained from *Streptococcus thermophilus* decreased the water contact angle from 109° to 58° thus, increasing the surface hydrophilicity. This increase in surface hydrophilicity decreased the rates of initial deposition and subsequent attachment by the cells of *S. aureus* and *Rothia dentocariosa* (Rodrigues *et al.* 2006).

The chemical composition of the surface influences the bacterial adhesion to the surface and the formation of biofilm by the microorganisms coming in contact with the surface (Alava *et al.* 2005). Copper-alloying of stainless steel slowed down the adhesion

of microorganisms (Kielemoes and Verstraete, 2001). Lopes *et al.* (2005) reported that more cells of *Desulfovibrio desulfuricans* adhered to the surface of nickel coupons compared to the surfaces of stainless steel and polymethylmetacrylate coupons. The higher levels of attachment were partly due to the positive effect of nickel on the growth of *D. desulfuricans.*

Environmental conditions

Bacterial attachment and biofilm formation on any surface are largely governed by the environmental conditions of the attachment system. The strain of bacteria, culturing method, and the concentration of the cells in the adhesion medium influence the rate of bacterial adhesion (Piette and Idziak, 1992). Physical factors such as the properties of attachment surface, contact time between the bacterial cells and surface, temperature, pH, concentration of mono and divalent ions, and presence of nitrogenous compounds in the medium are the additional determinants of bacterial attachment to a surface (Piette and Idziak, 1992). Similar factors also regulate the formation of biofilm by bacteria (Stepanovic *et al.* 2003).

The optimum temperature for biofilm formation is different for bacteria of different species. Stepanovic *et al.* (2003) reported that *Salmonella* cells formed more biofilm after incubation at 30°C for 24 h compared to those incubated at 37°C for the same length of time. Norwood and Gilmour (2001) reported an optimum temperature of 18°C for biofilm formation by the cells of *L. monocytogenes*. When the osmolarity of the culture media was increased by the addition of glucose, the amount of biofilm formed by the cells of *E. coli, Citrobacter freundii, Klebsiella pneumoniae*, and *S.* Typhimurium were reduced by about 50-75% (Jackson *et al.* 2002). Likewise, supplementing salt in

the culture media reduced the ability of the *Salmonella* cells to form biofilms (Romling *et al.* 1998). *Salmonella* cells grown in CO_2 or under microaerophilic atmosphere formed more biofilm compared to those grown under aerobic conditions (Gerstel and Romling, 2001). The nutrient content of the medium also influenced the amount of biofilm produced. Stepanovic *et al.* (2004) found that *Salmonella* cells produced the maximum amount of biofilm in 1/20 diluted TSB, followed by TSB, brain heart infusion (BHI) broth, and meat broth (MB). Interestingly, *L. monocytogenes* cells produced the maximum amount of biofilm in BHI, followed by TSB, MB, and 1/20-TSB.

4. SIGNIFICANCE OF ATTACHED BACTERIA AND BIOFILMS IN FOOD PROCESSING SYSTEMS

The prevention of contamination caused by pathogenic microorganisms during the manufacture, processing, and packaging of food is a major issue for the food industry. The equipment surfaces in the food industry are in constant contact with the various nutrients in foods which may be deposited on these surfaces, forming a layer also known as conditioning film (Frank, 2001). The conditioning film is rich in nutrients and favors bacterial growth. Some microorganisms present in the food which have the ability to attach to surfaces may colonize this layer and a population of attached microorganisms may be developed. Bacteria attached to the surface or embedded in a biofilm may later detach from the surface as part of their normal life cycle and cause product contamination. Austin and Bergeron (1995) noticed microbial biofilms on rubber and Teflon gaskets in a dairy processing line after the routine cleaning and sanitization procedures were carried out. Bacterial cells embedded in biofilm could contaminate the milk passing through the same processing line. Arnold and Silvers (2000) found that

bacteria can attach to the surfaces of stainless steel, polyethylene, and rubber equipment used in a poultry processing plant.

Research has shown that the cells of *Salmonella* can form biofilms on commonly used food contact surfaces such as stainless steel (Hood and Zottola, 1997), glass (Gerstel and Romling, 2001), and polystyrene (Romling *et al.* 2000). *L. monocytogenes* and *E. coli* can also form biofilms on polystyrene, stainless steel, metals, and glass (Frank, 2001; Stepanovic *et al.* 2004). *E. sakazakii* can attach and form biofilm on silicon, latex, polycarbonate, PVC, and stainless steel (Lehner *et al.* 2005).

The food industry uses sanitizing agents such as chlorine and iodine sanitizers, as well as quaternary ammonium compounds, etc. on a day-to-day basis. The concentration of sanitizing agents frequently used in the food industry may not be able to kill all spoilage or disease causing bacteria which are attached or embedded in a biofilm on the surface (Solano *et al.* 2002). The attached cells may escape killing by sanitizers because even the most effective sanitizers for suspended cells are not necessarily able to kill the attached cells (Joseph *et al.* 2001). Attached cells have higher resistance to the inactivation treatments (Stepanovic *et al.* 2003). The protective effect of biofilm in protecting the cells from UV light, osmotic stress, heat, starvation, acids, detergents, antibiotics, phagocytes, antibiotics, and bacteriophages has been reported earlier (O'Toole *et al.* 2000).

The detrimental effects of biofilms in the food industry are not just confined to endangering food safety. Biofilms lead to reduced efficiency of heat exchangers, unexpected corrosion of stainless steel, premature destruction of mineral materials, and clogging of industrial pipes (Coetser and Cloete, 2005).

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CHAPTER 3

ANTIBIOTIC RESISTANCE PROFILES AND CELL SURFACE COMPONENTS OF *SALMONELLA* ISOLATED FROM RETAIL CHICKEN MEATS OR SELECTED FROM LABORATORY COLLECTION

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ABSTRACT

Salmonellae were isolated from raw, chilled, retail poultry meats (n=100) using the procedures outlined in the *Bacteriological Analytical Manual* and *Microbiology Laboratory Guidebook.* Sixteen poultry isolates, along with 36 *Salmonella* strains from our laboratory culture collection were tested for their resistance to 12 different antibiotics, as well as their ability to produce thin aggregative fimbriae and/or cellulose, two of the most important surface components influencing the ability of cells to attach and form biofilm. The sensitivity of the salmonellae to the antibiotics was determined with a disc diffusion assay. Of 52 *Salmonella* isolates, 25 (48.0%) were resistant to one antibiotic, 5 (9.6%) to two, 4 (7.7%) to three, 6 (11.5%) to four, and 5 (9.6%) to five antibiotics. Additionally, 2 (3.8%) isolates were resistant to nine of the antibiotics tested. Fifty-one (98%) isolates were resistant to novobiocin, 18 (34.6%) to streptomycin, 14 (26.9%) to tetracycline and 14 (26.9%) to oxytetracycline. In separate experiments, the isolates were grown on Luria-Bertani (LB) no salt agar supplemented with Congo red (40 μg/ml) and Coomassie brilliant blue (20 μg/ml) or Calcofluor (200 μg/ml) in order to determine whether they produced thin aggregative fimbriae and/or cellulose. Of the total 52 *Salmonella* isolates, 25 expressed only thin aggregative fimbriae, and 1 synthesized only cellulose. An additional 10 produced both thin aggregative fimbriae and cellulose while the remaining 16 expressed neither surface structure. This study reveals a prevalence of *Salmonella* on raw poultry products retailed in Griffin and Peachtree City, GA. It also suggests that salmonellae have the abilities to develop resistance to multiple antibiotics and synthesize the cell surface components that help them to survive in hostile or suboptimal environments.

Key words: *Salmonella*, poultry, thin aggregative fimbriae, cellulose, and antibiotic resistance

Salmonella is the second most common cause of human enteric infections in the developed world and is responsible for considerable illness as well as some deaths *(2)*. The pathogen affects about 1.4 million people each year in the U.S. with approximately 20,000 hospitalizations and 500 deaths annually *(27)*. Various epidemiological reports have shown that foods of animal origin are the major vehicle of foodborne illness *(31)*. Poultry and poultry products are the frequent vehicles in transmission of *Salmonella* species *(14)* and dominate other foods of animal origin as a potential source of infection *(13)*.

The distribution of *Salmonella* on raw poultry meat products vary depending on the geological areas where the surveys are conducted. The prevalence of the pathogen ranges from 8% in Albania *(7)*, 23-34% in Belgium *(43, 44)*, 25% in the United Kingdom *(33)*, to 26% in Malaysia *(38)*. In the U.S., a prevalence rate of 43% was reported in Columbus, OH *(9)*. Another large, three-year study carried out in the U.S. had a *Salmonella* prevalence rate of 20% in broilers, 44.6% in ground chicken, and 49.9% in ground turkey *(37)*. The incidence of *Salmonella* poultry contamination is reportedly higher in other countries, i.e., 60% in Spain *(11)*, 61-69% in Canada *(22)*, and 69% in Greece *(4)*.

The development of antimicrobial resistance among pathogenic bacteria has emerged as a major public health concern in the recent years *(29)*, which to bacterial cells, is a strategy to survive in hostile and sub-optimal environments. Dupont and Steele *(15)* have suggested that the increased use of antimicrobials in veterinary and medical situations has led to the development of antibiotic-resistant bacteria in the agricultural environment. The evolution of antimicrobial-resistant bacteria in the environment has led

to the emergence of new strains of foodborne pathogens displaying increased resistance to certain antimicrobials. Food animals subjected to sub-therapeutic doses of antimicrobials on a regular basis may serve as reservoirs for resistant bacteria that may spread to human populations and therefore, limit the medical value of some antimicrobial classes *(1, 18)*. Antimicrobial resistance in *Salmonella*, first reported in the early 1960's *(10)* is mediated by several mechanisms, including changes in bacterial cell wall permeability, energy-dependent removal of antimicrobials via membrane-bound efflux pumps, modification of the site of drug action, as well as destruction and inactivation of antimicrobials *(6, 39)*.

Many bacteria including *Salmonella* enter a developmental program in response to nutrient limitation, which can be considered as a collective defense under environmental stress *(12)*. The bacteria cells, under starvation conditions tend to change cellular morphology and physiology, including the expression of thin aggregative fimbriae and cellulose; both of which are involved in the formation of a highly resistant extracellular matrix *(41)*. In addition to its role in forming the extracellular matrix, cellulose also imparts cells the ability to resist disinfection by chlorine sanitizers *(41)*. *Salmonella* cells expressing the thin aggregative fimbriae adhere to plastic and glass surfaces *(36)* and eventually form biofilms. Biofilm formed in the food processing environment is of special importance as it has the potential to act as the chronic source of microbial contamination that may lead to food spoilage or transmission of diseases. Bacterial cells embedded in biofilms often exhibit enhanced resistance to cleaning and sanitation *(21)*.

The objectives of this study were to determine the prevalence of *Salmonella* contamination in poultry meat retailed in Griffin and Peachtree City of Georgia, to determine the antibiotic resistance profiles of the *Salmonella* isolated from retail poultry as well as from our laboratory collection, and to characterize the expression of thin aggregative fimbriae and/or cellulose by the *Salmonella* strains.

MATERIALS AND METHODS

Sample collection. One hundred samples of chicken meat (25 samples of thighs, 15 of ground, 10 of breasts, 10 of liver and gizzards, 10 of wings, and 5 each of livers, wing drummets, drum sticks, tenders, legs, and split breast with ribs) were purchased from eight retail outlets in Griffin and Peachtree City, GA. The samples obtained had been processed and packaged at 3 different poultry-processing plants in the U.S. The samples were stored at 4°C while being transported to the laboratory and then processed within 4 h of collection.

Salmonellae were isolated from the meat samples using methods described in the *Bacteriological Analytical Manual* (BAM) *(16)* and *Microbiology Laboratory Guidebook (42)* with slight modifications. For isolation of *Salmonella* using the BAM procedure, 25 g of meat sample were aseptically placed in a stomacher bag with 225 ml lactose broth being subsequently added. The samples were stomached (Seward Stomacher Lab System 400, England) for 2 min at normal speed and then allowed to stand at room temperature for 1 h. The pH of the meat homogenates was adjusted to 6.8±0.2 with 0.1 N NaOH / 0.1 N HCl and 2.25 ml of Triton- X 100 was subsequently added. The samples were then incubated at 37°C for 24 h. A 0.1 ml portion of the resulting culture was transferred to

10 ml Rappaport-Vassiliadis (RV) broth and another 1 ml portion of the culture was transferred to 10 ml tetrathionate (TT) broth. Both RV and TT broth were incubated at 42°C for 24 h. One loopful of RV and TT broth culture was streaked separately on bismuth sulfite agar (BSA). The inoculated plates were then incubated at 37°C for 24 h. Presumptively *Salmonella* colonies were selected and inoculated on MacConkey agar and the inoculated plates were incubated at 37°C for 24 h. Selected colorless colonies on MacConkey agar were confirmed biochemically with triple sugar iron (TSI) agar and lysine iron agar (LIA), and serologically using "O" agglutinating antisera (Difco *Salmonella* O Antiserum Poly A-I and Vi, Becton Dickinson and Company, Sparks, MD).

For isolation using the USDA procedure, a 25 g sample along with 225 ml of lactose broth was aseptically placed in a stomacher bag and stomached for 2 min at normal speed (Seward Stomacher Lab System 400, England). The stomached sample was incubated at 37°C for 24 h. A 0.5 ml of the resulting culture was transferred to 10 ml TT broth and another 0.1 ml of the culture was transferred to 10 ml RV broth. The inoculated RV and TT broth were then incubated at 42°C for 24 h. A loopful of RV and TT broth culture was inoculated on xylose lysine tergitol 4 (XLT 4) agar and incubated at 37°C for 24 h. Presumptive *Salmonella* colonies were inoculated on MacConkey agar and incubated at 37°C for 24 h. Selected colorless colonies on MacConkey agar were confirmed as described above. All media and reagents used in the study were obtained from Becton, Dickinson and Co., Sparks, MD unless otherwise stated.

Antimicrobial susceptibility testing. *Salmonella* isolates were tested for resistance to antibiotics using the standard disc diffusion assay following the National

Committee for Clinical Laboratory Standards (NCCLS) guidelines M2- A6 *(28)*. Briefly, the cultures were grown on tryptic soy agar (TSA; Becton, Dickinson and Co., Sparks, MD) plates for 18 h at 37°C. A single colony of the culture was transferred to tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD) and incubated at 37°C until the optical density (O.D.) reached 0.08-0.10 at a wavelength of 600 nm (Novaspec II Visible Spectrophotometer, Pharmacia Biotech, Cambridge, England). The culture was inoculated on fresh Mueller-Hinton agar (Becton, Dickinson and Company, Sparks, MD) plates using a sterile swab and allowed to dry for 5 min. Four antibiotic discs (BD-BBL Sensi-Disc Antimicrobial Susceptibility Test Discs, Becton, Dickinson and Company, Sparks, MD) were placed on each plate. Each culture was tested for its sensitivity to 12 antimicrobials, namely, ampicillin (AM; 10 μg), cephalothin (CF; 30 μg), chloramphenicol (C;30 μg), gentamicin (GM;10 μg), kanamycin (K;30 μg), nalidixic acid (NA; 30 μg), neomycin (N;30 μg;), novobiocin (NB; 30 μg), oxytetracycline (T; 30 μg), streptomycin (S;10 μg), sulfisoxazole (G; 0.25 mg), and tetracycline (TE; 30 μg). The plates were incubated at 37°C for 16-18 h. The diameter of the zone of inhibition was measured to the nearest whole mm. Isolates were classified as sensitive, resistant, or intermediate according to the NCCLS guidelines or the criteria provided by the supplier of the antibiotic discs.

Colony morphology and expression of extracellular components. Isolates of *Salmonella* were inoculated on Luria-Bertani (LB) no salt agar, supplemented with Congo red (40 μg/ml; Sigma Chemical Company, St. Louis, MO) and Coomassie brilliant blue G (20 μg/ml; Sigma Chemical Company, St. Louis, MO) *(36).* The inoculated plates were incubated at 28°C for 48-72 h, and the morphologies of the

Salmonella colonies were examined both visually and by light microscope (Nikon Eclipse TS 100, Japan). Additionally, scanning electron micrographs were taken in order to confirm the expression of thin aggregative fimbriae and/or cellulose by the *Salmonella* cells. For scanning electron microscopy, the cells were grown on LB no salt agar plates at 28°C for 72 h. The cells were collected and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB; pH 7.4) at room temperature for 90 min. The fixed cells were washed 3 times with SCB, each for 10 min. Lipids on the *Salmonella* cells were fixed with 1% osmium tetroxide at room temperature for 1 h. The samples were then washed twice with SCB and dehydrated serially with 50, 70, 80, 90, and 100% ethanol, each for 15 min. The samples were stored at 4°C in 100% ethanol and were dried at critical point temperature with liquid CO_2 using a critical point drier (Samdri model 780-A, Tousimis, Rockville, MD). The samples were mounted on aluminum stubs and the cells were coated with gold in a sputter coater (Structure Probe, Inc., West Chester, PA). Samples were loaded and the cells were visualized using a scanning electron microscope (LEO Electron Microscopy, Inc. Thornwood, NY).

Expression of cellulose. Cellulose production was determined by growing the *Salmonella* cultures on LB no salt agar plates supplemented with 200 μg/ ml Calcofluor white stain (fluorescent brightener no.28, ENG Scientific Inc., Clifton, NJ) *(41)*. After incubation at 28°C for 48 h, fluorescent stain bound to the cellulose produced by *Salmonella* cells was observed under UV light with an excitation wavelength of 365-395 nm and an emission wavelength of 420 nm in a multi-purpose imaging system; using the Kodak Digital Science Image Station 440 CF (Eastman Kodak Company, Rochester, NY).

Formation of pellicle. Pellicle formation by the *Salmonella* isolates was evaluated by growing the cell cultures in 5 ml of LB no salt broth at 28°C for 72 h with no agitation *(35).* Pellicle formation by the tested cultures at the air-liquid interface was examined visually.

RESULTS AND DISCUSSION

***Salmonella* prevalence in poultry.** Salmonellae were isolated from 16.0% of the raw chicken meat tested in this study. This value is relatively lower than the incidences reported by some of the previous studies which were 43.0% *(9),* 38.8% *(14)*, 35.0% *(11)* and 20.0% for whole raw broilers, and 44.6% for ground chicken *(37)*. However, lower incidences have been reported in other studies, i.e., 4.2% in chicken meats *(46)*, and 8.2% in turkey meats *(7)*. Logue et al. reported an overall incidence of *Salmonella* contamination of 16.7% on pre-chilled and post-chilled carcasses in mid-western U.S. processing plants *(24)*, which was similar to the incidence observed in the present study.

Many factors contribute to carcass contamination with salmonellae *(30)*. Chicken feed and drinking water could be significant sources of salmonellae *(26)* and may, therefore, be contributing factors in the contamination of entire flocks of birds. Conditions on farms and the stress associated with transport *(45)* as well as the general contamination that occurs during processing, such as that associated with chilling and other handling procedures *(8)* are some other factors which might be responsible for higher levels of *Salmonella* contamination in poultry meats. Increased pathogen loads in fecal shedding may also occur during times of stress, such as high population housing or transport; as a consequence, cross contamination between birds in transit may take place.

This bird-to-bird transmission may result in increased incidence of contamination in live birds and enhance the risk of contamination to chicken carcasses *(19)*.

Two different methods, the BAM and USDA, were employed for recovering *Salmonella* from poultry meat. The USDA method is slightly more sensitive than is the BAM method. Of the total sixteen isolates, eleven were obtained using the USDA method and the remaining five were obtained using the BAM method. The mechanism that causes the difference in the *Salmonella* isolation rates by the two methods have not been thoroughly investigated, but the same phenomena have been observed by other investigators *(40)*.

Antibiotic sensitivity of *Salmonella* isolates. Incidence of antimicrobial resistance in strains of *Salmonella* is of importance. Our findings show that *Salmonella* have acquired resistance to novobiocin, oxytetracycline, tetracycline, and streptomycin. These results are similar to ones described in previous studies showing that *Salmonella* isolates in retail meats are commonly resistant to multiple antimicrobials, including tetracycline, sulfimethoxazole, and streptomycin *(24)*. Our study presented 11 different antibiotic resistance patterns (Table 3.1) for 52 *Salmonella* isolates. Fifty-one (98%) isolates were resistant to novobiocin, while 13 (25%) isolates were resistant to oxytetracycline. Resistance to novobiocin and oxytetracycline may originate from their use in broiler feed as these two are among the 32 antimicrobial compounds permitted in broiler feed in the U.S. without veterinary prescription *(20)*. The widespread use of these antimicrobial agents at sub-therapeutic levels in chicken feed and drinking water might be the reason for the development of antibiotic resistant strains. Neomycin resistance was uncommon and was found only in one (1.9%) isolate. Tetracycline resistance was

observed in 12 (23.0%) isolates, while 18 (34.6%) isolates were resistant to streptomycin. Resistance to tetracycline and streptomycin has been attributed to their use at subtherapeutic levels in feed for growth promotion in U.S. and Canada *(23).* Four (7.7%) isolates were resistant to gentamicin. Gentamicin resistance is usually found in chicken isolates in the U.S. due to the practice of routinely injecting chicken eggs with gentamicin before they hatch *(23)*. In Europe, gentamicin is not used for such purposes, and gentamicin-resistant *Salmonella* isolates from humans and poultry are rare *(25).* Multi-drug resistance (MDR), defined as resistance to 2 or more antimicrobial agents was associated with 21 (40.4%) isolates. Furthermore, among the 21 multi-drug resistant isolates, 9 (42.8%) were resistant to novobiocin, streptomycin, tetracycline, and oxytetracycline. *Salmonella* isolates from retail market poultry meat were resistant to at least one antimicrobial agent with 75.0% of the isolates being classified as MDR, however, among the isolates from our laboratory culture collection, 25.0% were MDR and 72.0% showed resistance only to novobiocin.

Antimicrobial resistance genes may be located on bacterial chromosomes or on transmissible plasmids. If resistance is chromosomally mediated, drug resistance is passed on to the cell's progeny via cell division; while plasmid associated resistance may be transferred to other species or stains by means of R factor plasmid vectors *(3, 32)*. The prevalent antimicrobial resistance among *Salmonella* isolates observed in this study was not all that unexpected, given the use of antibiotics in intensive poultry production. The uncontrolled use of antimicrobial agents given to poultry as prophylaxis, growth promoters, and treatment may have contributed to the selection of drug resistant *Salmonella* strains.

Colony morphology and expression of extracellular components. Cells producing only thin aggregative fimbriae formed brown, dry, and rough colonies (bdar morphotype; Fig 3.1A) while those producing both thin aggregative fimbriae and cellulose formed red, dry, and rough colonies (rdar morphotype; Fig 3.1B). Cells producing only cellulose formed pink, dry, and rough colonies (pdar morphotype; Fig 3.1 C), and those producing neither thin aggregative fimbriae nor cellulose formed smooth and white colonies (saw morphotype; Fig 3.1D) on LB no salt agar supplemented with Congo red and Coomassie brilliant blue G (*34, 36)*. The appearances of the *Salmonella* colonies with the bdar, rdar, pdar, and saw morphotype under a light microscope (magnification power of 4 X) are shown in Fig 3.2A, 3.2B, 3.2C, and 3.2D, respectively. Of the 52 cultures analyzed in the present study, 25 had the bdar morphotype, 16 had the saw morphotype, 10 had the rdar morphotype, and 1 had the pdar morphotype (Table 3.2). The correlation between colony morphotype and expression of thin aggregative fimbriae and/or cellulose was further established by scanning electron microscopy (Fig 3.3A, 3.3B, 3.3C, and 3.3D). Two divergently transcribed operons, *agfDEFG* and *agfBA(C)*, are needed for the biogenesis of thin aggregative fimbriae *(17, 36)*, where *agfD* is the positive transcriptional regulator of the *agfBA(C)* operon *(17).* Expression of thin aggregative fimbriae occurs under conditions of low temperature, as well as low osmolarity *(36)*, and requires transcriptional regulator *ompR* and the stationary sigma factor σ^S encoded by *rpoS*. The latter is a protein of 41.5 kDa that controls a regulon of at least 30 genes which are expressed during starvation and during the transition into the stationary phase *(36).* A deletion in *agfA*, which encodes a subunit of thin aggregative fimbriae, abolishes the rdar morphotype and results in a pdar morphotype which retains

some aspects of intercellular interactions and adhesive behavior *(5, 36).* An insertional mutation on *adrA,* a gene encoding a putative transmembrane protein, converts the rdar morphotype to bdar morphotype *(41).* Cells expressing the bdar morphotype have thin aggregative fimbriae with all binding characteristics, but do not synthesize cellulose. This indicates that in addition to thin aggregative fimbriae, cellulose is also necessary for the expression of rdar morphotype *(34, 41).* Deletion of *agfD* , which is a gene that encodes the putative response regulation of thin aggregative fimbriae expression *(35)* converts the bdar to the saw morphotype, which is devoid of both, thin aggregative fimbriae as well as cellulose *(34, 36)* and thereby lacks all forms of multicellular behavior *(41).*

Cellulose production by *Salmonella.* Cellulose produced by *Salmonella* cells on LB no salt agar supplemented with calcofluor binds the fluorescent dye, which when viewed under UV light at 366 nm emits fluorescence *(41)*. The calcofluor dye was chosen in the present study for its ability to bind with the β (1→4) - D-glucopyranosyl units of polysaccharide such as cellulose *(47)*. The intensity of the fluorescence is directly proportional to the amount of cellulose produced by the *Salmonella* cells. Cells of *Salmonella* exhibiting pdar morphotype produced an extraordinary amount of cellulose per number of cells; hence, emitting a high level of fluorescence (Fig 3.4A). Ten *Salmonella* isolates, exhibiting rdar morphotype produced less cellulose on Calcofluor plate and subsequently had weaker fluorescence signals (Fig 3.4B). Cells displaying bdar and saw morphotype did not synthesize cellulose and, therefore, emitted no fluorescence (Fig 3.4C and 3.4D).

Pellicle formation by *Salmonella* cultures. Different morphotypes of *Salmonella* formed distinct pellicle patterns upon undisturbed growth at 28°C in LB no salt broth. Cells of *Salmonella* expressing rdar morphotype formed a rigid pellicle at the air-liquid interface which could not be dispersed by gentle shaking (Fig 3.5A). The strength of the pellicle formed by the rdar strain is due to a tight mat of cells at the air-liquid interface and also due to the strength imparted by cellulose to the extracellular matrix *(41).* The pellicle formed by the cells expressing bdar morphotype appeared in the form of a ring and had a fragile appearance at the air-liquid interface and could easily be disrupted by gentle shaking (Fig 3.5B). The fragile nature of the pellicle is due to the absence of cellulose in the bacterial mat formed by thin aggregative fimbriae *(35, 47).* The cells expressing pdar morphotype produce cellulose but due to their inability to synthesize thin aggregative fimbriae were unable to form a pellicle. The crystallization of relatively larger amount cellulose in the aqueous environment makes the cells settle at the bottom of the test tube as seen in Fig 3.5C *(34, 36).* Cells expressing saw morphotype lack the ability to synthesize thin aggregative fimbriae and cellulose therefore, were neither able to form a pellicle nor settle at the bottom of the test tube and remained suspended in the broth (Fig 3.5D).

It was found that retail poultry meat sold in the supermarkets of two cities in Georgia, from where the samples are collected is sometimes contaminated with *Salmonella*, and may serve as vehicles for transmitting foodborne diseases. To diminish *Salmonella* contamination in retail meats, it is critical that risk reduction strategies are implemented throughout all phases of poultry production. These strategies may include on-farm practices that reduce pathogen carriage, increased hygiene at both the slaughter

and meat processing facilities, continued implementation of HACCP systems, and increased consumer education efforts. A considerable number of *Salmonella* isolates tested in the present study exhibited the ability to resist antimicrobial agents. The incidences of substantial multiresistance in foodborne *Salmonella* isolates is a major public health concern and suggests the need for more prudent use of antibiotics by farmers, veterinarians, and physicians. The expression of cell surface components such as thin aggregative fimbriae and cellulose are two important means of survival in hostile or suboptimal environments, rendering cells the ability to form biofilm on food contact surfaces. The identification and characterization of factors responsible for biofilm development will lead to design and implementation of effective measures to prevent or minimize biofilm formation in the poultry processing environments.

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Table 3.1 Antimicrobial resistance patterns of *Salmonella* isolated from retail poultry meats and selected from laboratory collection

Resistance pattern[a]	No. of isolates (%)
None	1 (1.9)
NB	30 (57.7)
AM, NB	2 (3.8)
AM, CF, NB	1(1.9)
GM, NB, S	3(5.8)
G, GM, NB, S	1(1.9)
NB, S, T, TE	9(17.3)
AM, NB, S, T, TE	1(1.9)
G, NB, S, T, TE	2(3.8)
AM, C, G, K, N, NB, S, T, TE	1(1.9)
AM, CF, K, NA, NB, S, T, TE	1(1.9)

[a] AM, ampicillin; CF, cephalothin; C, chloramphenicol; GM, gentamicin; K, kanamycin; NA, nalidixic acid; N, neomycin; NB, novobiocin; T, oxytetracycline; S, streptomycin; G, sulfisoxazole; TE, tetracycline.

Table 3.2 Expression of thin aggregative fimbriae and cellulose by *Salmonella* isolated from retail poultry meats and selected form laboratory collection

Culture[a]	*Salmonella* serotype	Colony morphotype[b]	Calcofluor binding[c]
S4-1	Enteritidis	rdar	+
S4-2	Heidelberg	bdar	-
S4-12	Panama	bdar	-
S4-24	Hadar	saw	-
S4-25	Rubislaw	saw	-
S4-27	Johannesburg	rdar	+
S4-28	Typhimurium	pdar	++
S4-29	Heidelberg	bdar	-
S4-30	Saintpaul	bdar	-
S4-33	Agona	bdar	-
S4-34	Indiana	bdar	-
S4-35	Brandenburg	bdar	-
S4-36	Reading	rdar	+
S4-37	Enteritidis	bdar	-
S4-38	Berta	bdar	-
S4-39	Dublin	bdar	-
S4-40	Sendai	saw	-
S4-41	Infantis	bdar	-
S4-42	Thompson	bdar	-
S4-43	Mbandaka	bdar	-
S4-44	Braenderup	bdar	-
S4-45	Ohio	bdar	-
S4-46	Oranienburg	bdar	-
S4-47	Tennessee	bdar	-
S4-48	Newport	bdar	-
S4-49	Haardt	bdar	-
S4-50	Hadar	saw	-
S4-51	Urbana	rdar	+
S4-52	Johannesburg	rdar	+
S4-53	Choleraesuis	bdar	-
S4-54	Anatum	bdar	-
S4-56	Enteritidis	saw	-
S4-69	Enteritidis	bdar	-
S4-70	Enteritidis	rdar	+
S4-71	Senftenberg	rdar	+
S4-72	Litchfield	bdar	-
33.3	n.a.[d]	bdar	-
67.1	n.a.	saw	-
69.1	n.a.	saw	-
96.2	Mbandaka	bdar	-
98.1	n.a.	rdar	+
13.1	Arizona	bdar	-

15.1	Arizona	bdar	-
51.3	n.a.	saw	-
59.1	n.a.	bdar	-
66.3	Arizona	saw	-
67.1	n.a.	saw	-
68.1	Arizona	saw	-
68.4	Arizona	saw	-
69.1	Arizona	saw	-
70.1	Arizona	saw	-
99.1	n.a.	rdar	+

[a] *Salmonella* cultures from our laboratory collection or isolated from retail poultry meats.

[b] Morphotypes of *Salmonella* colonies on Congo red and Coomassie brilliant blue agar plates at 28°C after 48-72 h; rdar: red, dry, and rough; bdar: brown, dry, and rough; pdar, pink, dry, and rough; saw: smooth and white.

[c] The relative level of Calcofluor binding was assayed qualitatively; (-) no fluorescence, (+) less fluorescence, (++) more fluorescence.

[d] Serotyping not attempted.

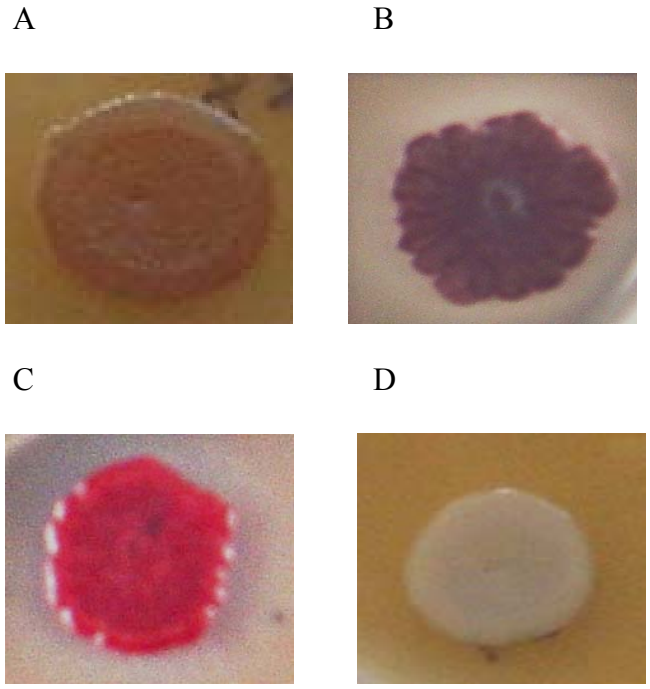

FIGURE 3.1 *Different colony morphotypes of* Salmonella *cells grown on Congo red and Coomassie brilliant blue supplemented LB no salt agar plates. S4-69: bdar morphotype (A), S4-1: rdar morphotype (B), S4-28: pdar morphotype (C), and 67.1: saw morphotype (D). All cultures were grown at 28℃ for 72 h.*

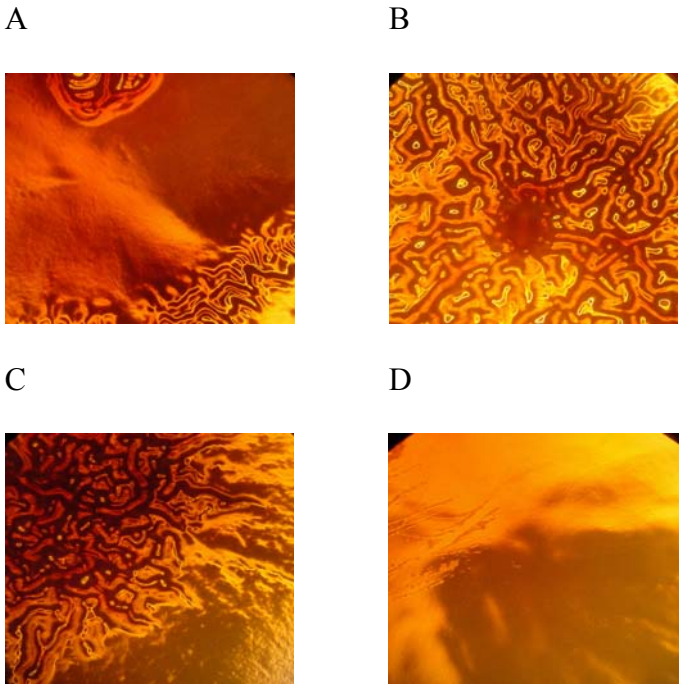

FIGURE 3.2 *Salmonella colony morphotypes viewed under a light microscope. S4-69: bdar morphotype (A), S4-1: rdar morphotype (B), S4-28: pdar morphotype (C), and 67.1: saw morphotype (D). All cultures were grown at 28℃ for 72 h on Congo red and Coomassie brilliant blue supplemented LB no salt agar plates. The magnification power was 4X. The right bottom corners of the phones show the edges, while the left top corners of the photos show the centers of* Salmonella *colonies with different morphotypes.*

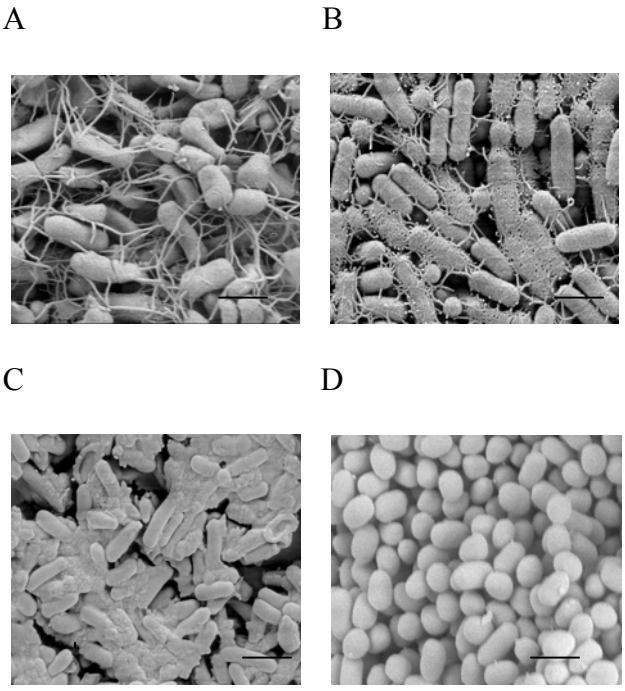

FIGURE 3.3 *Scanning electron micrographs of* Salmonella *cells grown on LB no salt agar plates. S4-69 exhibits thin aggregative fimbriae which are seen on the surfaces of the* Salmonella *cells (A), S4-1 exhibits the matrix structure of rdar morphotype (B), S4-28 exhibits a densely packed unstructured matrix composed of cellulose in direct contact to the bacterial cells and between cells (C), and 67.1 exhibits saw morphotype which does not express thin aggregative fimbriae and cellulose (D). All cultures were grown at 28℃ for 72 h. Bars represent 1 μm.*

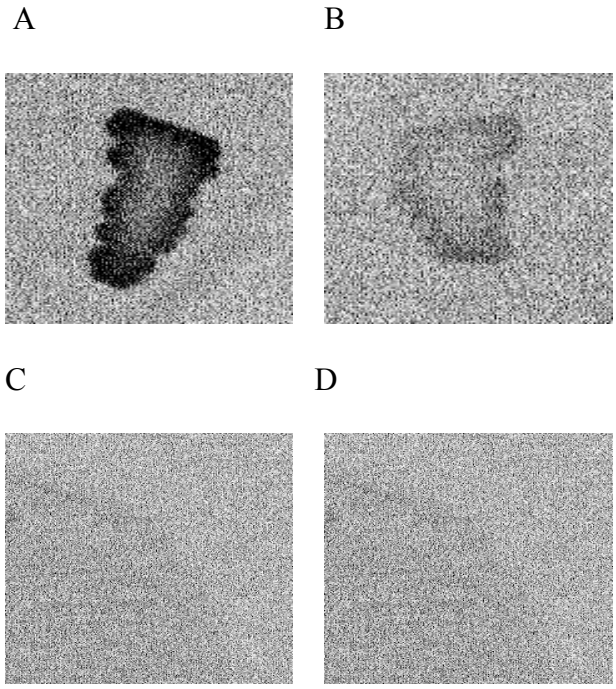

FIGURE 3.4 *Salmonella cultures of various morphotypes grown on LB no salt agar plates supplemented with Calcofluor. S4-28: pdar morphotype which emitted a high level of fluorescence (A), S4-1: rdar morphotype which had weaker fluorescence signal (B). S4-69, bdar morphotype (C) and 67.1, saw morphotype (D) did not synthesize cellulose and therefore emitted no fluorescence. All cultures were grown at 28℃ for 72 h.*

A B C D

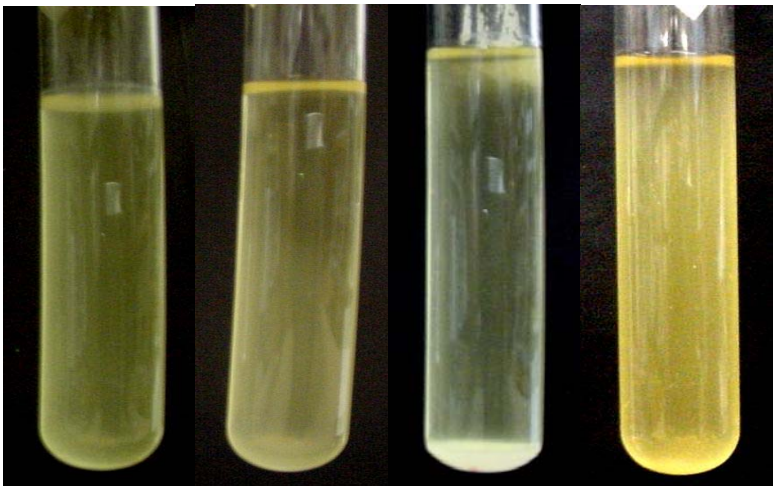

FIGURE 3.5 *Formation of pellicle by* Salmonella *cultures of various morphotypes grown in LB no salt broth at 28℃ for 72 h. S4-1 expressing rdar morphotype formed a tight pellicle at the air-liquid interface (A). A fragile pellicle was formed by S4-69 which expresses bdar morphotype (B). S4-28, which expresses pdar morphotype deposited at the bottom of the test tube (C) and a turbid cell culture was formed by 67.1, which has a saw morphotype (D).*

CHAPTER 4

ATTACHMENT AND BIOFILM FORMATION BY *SALMONELLA* AS INFLUENCED BY CELLULOSE PRODUCTION AND THIN AGGREGATIVE FIMBRIAE BIOSYNTHESIS

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ABSTRACT

Aims: To quantify thin aggregative fimbriae and cellulose produced by *Salmonella* and evaluate their roles in attachment and biofilm formation on abiotic surfaces.

Methods and Results: Thin aggregative fimbriae and cellulose produced by four wild type and two isogenic pairs of *Salmonella*, representing four different colony morphotypes (rdar, pdar, bdar, and saw), were quantified using the Congo red binding and a colorimetric assay, respectively. The ability of these cultures in attaching and forming biofilm on polystyrene and glass surface were evaluated in LB broth with or without salt (0.5%) or glucose (2%) at 28^{o}C during a 7 d period. The cells expressing rdar or pdar colony morphotypes produced the highest amount of thin aggregative fimbriae and cellulose, respectively. The rdar cultures attached in higher numbers and formed more biofilm than did the pdar culture on the test surfaces. The variants of the isogenic pairs expressing the fimbriae attached more efficiently and formed more biofilm on the tested surfaces than their counterparts. In general, the addition of salt and glucose to the media proved to be detrimental to the attachment and biofilm formation, but not without exceptions. Higher levels of attachment and biofilm formation were associated with polystyrene surface and increased lengths of incubation time.

Conclusions: Thin aggregative fimbriae may impart attachment ability to *Salmonella*, and upon co-expression with cellulose enhance biofilm formation on certain abiotic surfaces. Medium composition, cell-surface contact time, and properties of the abiotic surface may influence the levels of attachment and biofilm formation.

Significance and Impact of the Study: This study emphasizes the role of thin aggregative fimbriae and cellulose in assisting the cells of *Salmonella* to attach and form

biofilms on abiotic surfaces. Knowledge about the factors influencing attachment and biofilm formation would help develop better cleaning strategies for food processing equipment.

Keywords: attachment, biofilm, cellulose, *Salmonella*, thin aggregative fimbriae, polystyrene and glass.

INTRODUCTION

Salmonella is one of the most important bacterial pathogens worldwide, and in the United States more than 95% of the cases of *Salmonella* infections are foodborne. According to the Centers for Disease Control and Prevention, foodborne salmonellosis is responsible for an estimated 1.3 million illnesses, 15,000 hospitalizations, and 553 deaths each year (Mead *et al.* 1999), and accounts for approximately 30% of the deaths resulting from all foodborne diseases (Hohmann, 2001). Food of animal origin, particularly meat and poultry, is the main vehicle in transmitting *Salmonella* to humans (Dominguiz *et al.* 2002). During meat and poultry processing, pathogens such as *Salmonella* may attach and subsequently form biofilm on a variety of surfaces including stainless steel, aluminium, glass, Buna N rubber, Teflon, and Nylon materials that are widely used in food processing environments (Kumar and Anand, 1998).

A biofilm is a population of microbial cells growing on a surface and enclosed in an amorphous, extracellular matrix (Donlan, 2002). For *Salmonella,* the formation of the matrix involves at least two of the cell surface components; namely, thin aggregative fimbriae and cellulose (Romling *et al.* 2000; Zogaj *et al.* 2001). The thin aggregative fimbriae are expressed in response to nutrient limitation under conditions of low osmolarity, low growth temperature, and in the stationary phase of growth (Romling *et al.* 1998a). Although the isolates that express the thin aggregative fimbriae are often capable of causing diseases in humans, a definite role of the fimbriae in *Salmonella* pathogenesis has not yet been established (Collinson *et al.* 1991, 1996; Grund and Weber, 1998). The function of these fibers in adhesion to solid surfaces has however, been documented (Vidal *et al.* 1998), and cells with the thin aggregative fimbriae have been

shown to have a better ability to interact with their contact surfaces. Bacteria generally produce cellulose as an extracellular component for mechanical and chemical protection (Solano *et al.* 2002). Cellulose produced by *Salmonella* plays a structural role by conferring mechanical strength to biofilm (Solano *et al.* 2002). When thin aggregative fimbriae and cellulose are co-expressed by *Salmonella,* a matrix of tightly packed cells is covered in a well-structured hydrophobic network (Zogaj *et al.* 2001; Gerstel and Romling, 2003). This network is extremely important in biofilm formation and the persistence of *Salmonella* on various surfaces.

Bacterial attachment and biofilm formation on food contact surfaces is of significance for the overall quality of food because over time, cells attached to a surface will detach and migrate to a new growth site (Bar-Or, 1990). Bacterial cells sloughing from a biofilm could be a source of sporadic contamination of food products coming in direct contact with the surface on which a biofilm has formed. The contaminated food may spoil or be linked to foodborne diseases (Joseph *et al.* 2001).

Attachment and biofilm formation by *Salmonella* on plastic, metal, glass or rubber surfaces has been reported in earlier studies (Helke et al. 1993;Austin *et al.* 1998; Sinde and Carballo, 2000), but the influence of thin aggregative fimbriae and cellulose produced by *Salmonella* on their ability to attach and form biofilms has not yet been investigated. The objectives of this study were to quantify the thin aggregative fimbriae and cellulose produced by *Salmonella* isolated from poultry as well as other sources, and to determine the influence of these two cell surface components on the ability of *Salmonella* to attach and form biofilm on polystyrene and glass surfaces.

MATERIAL AND METHODS

Salmonella strains and their abilities in expressing cellulose and thin aggregative fimbriae

Qualification of thin aggregative fimbriae was carried out by growing the *Salmonella* cultures at 28°C for 72 h on Luria-Bertani (LB; 0.1% Bacto tryptone, 0.5% Bacto yeast extract, and 0.5% NaCl) no salt agar supplemented with 40 μg ml^{-1} Congo red (Sigma Aldrich Co., St. Louis, MO, USA) and 20 μg ml^{-1} Coomassie brilliant blue (Sigma Aldrich Co.) (Hammar *et al.* 1995). The colonies of cells that produce the thin aggregative fimbriae appeared red or brown while those cells that do not produce the fimbriae formed pink or colorless colonies, depending upon their ability or inability to produce cellulose.

Qualitative assessment of cellulose produced by the *Salmonella* cells was done by growing the *Salmonella* cultures for 72 h at 28°C on LB no salt agar containing 200 μg ml^{-1} Calcofluor (ENG Scientific Inc., Clifton, NJ, USA). The cellulose binds with the Calcofluor dye which, when excited by a UV light (365-395 nm), emits the fluorescence with a wavelength of 420 nm.

Six *Salmonella* strains, S4-1, S4-28, S4-69, S4-70, P-67, and P-96, either isolated from poultry meats or selected from our laboratory collection, were used in this study. Cells of P-67 and P-96 formed both thin aggregative fimbriae expressing and non thin aggregative fimbriae expressing colonies on LB no salt agar supplemented with Congo red and Coomassie brilliant blue. The two variants were purified and used as separate cultures in the attachment and biofilm studies. The thin aggregative fimbriae expressing variant of P-67 was designated as $P67F^{+}$ and the non-thin aggregative fimbriae

expressing variant as P67F$^-$. Similarly, the thin aggregative fimbriae expressing and non-thin aggregative fimbriae variant of P-96 were named P96F$^+$ and P96F$^-$, respectively.

These eight aforementioned *Salmonella* strains/variants have 4 distinct colony morphotypes (Table 4.1). Cells of S4-1 and S4-70 synthesize both thin aggregative fimbriae and cellulose, and the colonies of these cells have a red, dry, and rough (rdar) appearance on LB no salt agar supplemented with Congo red and Coomassie brilliant blue. Colonies of S4-69, P67F$^+$, and P96F$^+$ are however, brown, dry, and rough (bdar), and cells of these three *Salmonella* cultures synthesize abundant thin aggregative fimbriae while producing no detectable cellulose. Colonies of S4-28 cells appear to be pink, dry, and rough (pdar), which is an indication of cellulose production but lack of thin aggregative fimbriae biosynthesis. Cells of P67F$^-$ and P96F$^-$ produced neither surface structure and the colonies of which have a smooth and white (saw) appearance on LB no salt agar supplemented with Congo red and Coomassie brilliant blue.

Quantification of thin aggregative fimbriae expressed by *Salmonella* cells

Quantification of thin aggregative fimbriae expressed by *Salmonella* cells was carried out using the procedures of Gophna *et al.* (2001) and Pawar and Chen (2005) with some modifications. The *Salmonella* cultures were grown on LB no salt agar for 72 h at 28°C. The cells were collected in 0.85% saline, and the optical densities of the cell suspensions were adjusted to 1.00 ± 0.03 at 600 nm. One and a half ml of this suspension was centrifuged at 16,000 *g* for 10 min (Eppendorf Centrifuge 5415 C, Westbury, NY, USA). The supernatant was discarded and the cell pellets were suspended in 1.5 ml 0.002% Congo red solution for 10 min. The cell suspension was re-centrifuged under the same conditions, and the quantities of unbound Congo red were determined by measuring the

absorbance of the supernatant at 500 nm (A_{500}) using a spectrophotometer (Novaspec II, Pharmacia Biotech, Cambridge, England). To ascertain that an equal number of cells of the tested cultures would be used in the quantification of thin aggregative fimbriae, appropriate dilutions of the *Salmonella* cell suspensions were plated on tryptic soy agar (TSA; Difco Laboratories, Sparks, MD, USA) using an Autoplate® 4000 (Spiral Biotech Inc., Bethesda, MA, USA). The inoculated plates were then incubated for 24 h at 37°C and colony counts were determined using an automatic colony counter (Q Count®, Spiral Biotech, Norwood, MD, USA).

Quantification of cellulose produced by *Salmonella* cells

Quantification of cellulose produced by *Salmonella* cells was carried out by a colorimetric method developed by Updegraff (1969) with some modifications. Briefly, *Salmonella* cultures were grown in LB no salt broth for 72 h at 28°C. The cultures were centrifuged at 2,100 *g* for 5 min (Beckman GS 6-R centrifuge, Beckman Instruments Inc., Palo Alta, CA, USA). The cell pellet of each culture was collected after the supernatant fluids were discarded. Three ml of an acetic-nitric reagent (150 ml 80% acetic acid and 15 ml conc. nitric acid) were added to the cell pellet of each culture in a glass centrifuge tube (17x118 mm; Fisher Scientific, Fair Lawn, NJ, USA) and mixed properly. The tubes were covered with aluminum foil and placed in a boiling water bath for 30 min. The contents of the tube were re-centrifuged at 2,100 *g* for 5 min. Ten ml of 67% sulfuric acid was then added in three installments with intermittent mixings and the mix was allowed to stand for 1 h at room temperature. One ml of the mix was diluted with 4 ml distilled water. The centrifuge tubes that contained the mixtures were placed in an ice bath and into each tube was added 10 ml of cold anthrone reagent (0.2 g anthrone in 100

ml conc. H_2SO_4) (Acros Organics, New Jersey, NJ, USA). The centrifuge tubes were inverted gently and then placed in a boiling water bath for 16 min after which they were placed in an ice bath. The absorbance of each sample at 620 nm (A_{620}) was recorded using the Novaspec II Spectrophotometer. A standard curve of absorbance as a function of cellulose concentration was prepared. The quantities of cellulose produced by each *Salmonella* culture were calculated by comparing the absorbance values of the standard with the values of the tested sample. All reagents used in the quantification of cellulose were purchased from Fisher Scientific unless otherwise specified.

Preparation of *Salmonella* cultures for attachment and biofilm formation

A colony of each *Salmonella* culture on LB no salt agar supplemented with Congo red and Coomassie brilliant blue was transferred into 9 ml LB no salt broth. The inoculated broth was incubated at 28°C for 18 h. The cultures were diluted (1:40) in LB, LB plus 2% (w/v) glucose (LBG), LB no salt (LBNS), or LB no salt plus 2% (w/v) glucose (LBNSG). The diluted cultures were used for attachment and biofilm formation (1-7 d at 28°C) on glass vials and polystyrene tissue culture plates specified below. The LB, LBG, LBNS, and LBNSG not inoculated with *Salmonella* were used as negative controls throughout the study.

Salmonella cultures used in the attachment and biofilm studies were serially diluted and appropriate dilutions were plated using the methods described above to ensure that approximately an equal population of cells of each *Salmonella* culture would be used in the studies.

Abiotic surfaces

Abiotic surfaces, made of polystyrene and glass were used for attachment and biofilm formation. Twenty-four well polystyrene tissue culture plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and SepCap glass vials (4.5 x 1.4 cm; Fisher Scientific) were used as polystyrene and glass surface, respectively. The SepCap glass vials were washed with an alkaline detergent, TergA-zyme (Alconox, Inc., White Plains, NY, USA), rinsed thoroughly with deionized water, and air dried before being autoclaved at 121°C for 30 min. The tissue culture plates were sterile and individually wrapped upon purchase.

Attachment and biofilm formation on polystyrene and glass surface

The diluted *Salmonella* cultures described above were placed into the glass vials and the wells of polystyrene tissue culture plates. The *Salmonella* cells in broth cultures were allowed to attach and form biofilm on the surface of polystyrene and glass for 1 to 7 d, respectively at 28°C. Lids were placed on the glass vials and polystyrene tissue culture plates to prevent evaporation of the broth. At the end of each day *Salmonella* cells attached to or embedded in biofilms on the test surfaces were quantified using a crystal violet binding assay previously described by Sonak and Bhosle (1995) and Pawar and Chen (2005) with some modifications. Briefly, the broth cultures were withdrawn at the end of each day. The polystyrene wells and glass vials were rinsed twice with 2 ml fresh sterile broth to remove loosely attached cells. Both the plates and the vials were then air dried at 60°C for 2 h. The *Salmonella* cells attached to the polystyrene and glass surfaces were fixed by passing the plates and glass vials over the flame of a Bunsen burner (Fisher Scientific) several times. The fixed cells on the surfaces were stained with 2 ml of 1%

crystal violet (Fisher Scientific) for 15 min. Excess stain was rinsed off by placing the tissue culture plates and glass vials under running tap water until the wash water contained no visible stains. The wells and glass vials were dried at 60°C for 2 h and cooled to room temperature. Two ml of an ethanol-acetone (80:20) mixture was added to the glass vials and polystyrene tissue culture plate wells to extract the bound crystal violet from the stained *Salmonella* cells. The concentrations of the dye extracted from the cells bound to the surfaces were determined by measuring the absorbance of the ethanol-acetone mixture at a wavelength of 550 nm (A_{550}) using the Novaspec II Spectrophotometer. The A_{550} of the negative controls was subtracted from the absorbance values of the tested samples before the data was analyzed statistically.

Statistical analysis

Two replicates of each experiment were performed and each experiment in an individual trial had duplicate samples. A randomized complete block design was used to set up the experiments and the data collected was analyzed using the general linear model of the Statistical Analysis Software (SAS Institute Inc., Cary, NC, USA). The means were compared using Fisher's least significant difference at 95% confidence level.

RESULTS

Quantification of thin aggregative fimbriae

The cells of S4-70 synthesized the highest amount of thin aggregative fimbriae among the eight strains/variants tested in the study whereas, the cells of S4-28, P67F$^-$, and P96F$^-$ bound much less Congo red compared to S4-70 (Table 4.2). The A_{500} values from the

two members of the *Salmonella* pairs, P67F$^+$/ P67F$^-$ and P96F$^+$/P96F$^-$ were significantly different except when these cultures were grown at 28°C for 24 h *(P*≤ 0.05) (Table 4.2).

The increasing expression of thin aggregative fimbriae with the advancement of the incubation period was most evident in S4-70, S4-69, and P67F$^+$ (Table 4.2). The amounts of the fimbriae expressed by the cells of these three cultures at each time interval differed significantly from the amounts of fimbriae expressed at other lengths of incubation *(P*≤ 0.05). Cells of S4-1 expressed significantly less fimbriae than those of the other rdar strain, S4-70. The level of expression at 24 h of incubation was also significantly different from the levels of expression at 48 and 72 h of incubation *(P*≤ 0.05). Variants P67F$^-$ and P96F$^-$ lack the ability to synthesize thin aggregative fimbriae and therefore showed little or no significant difference in A_{500} values at various lengths of incubation time.

Quantification of cellulose produced by *Salmonella* cells

Salmonella cells of S4-28, which form colonies of pdar morphotype on LB no salt agar supplemented with Congo red and Coomassie brilliant blue produced approximately 8.57 ± 0.06 μg cellulose per 10^9 cells, which is the maximum amount of cellulose produced by the eight strains/variants tested in the study (Table 4.2). Cells of S4-1 and S4-70 produced relatively less cellulose than did the cells of S4-28. The amounts of cellulose produced by the cells of S4-1 and S4-70 were 2.47 ± 0.06 μg and 4.87 ± 0.21 μg per 10^9 cells, respectively. Although the cells of S4-70 and S4-1 both formed rdar colonies, S4-70 produced almost twice as much cellulose as did S4-1. The remaining six strains/variants with either bdar or saw colony morphotype produced an average of 0.02 μg cellulose per 10^9 cells, which is about 430 times less than the amount of cellulose

produced by the cells of S4-28 and 125 -240 times less than the cells of S4-1 and S4-70 (Table 4.2).

Attachment and biofilm formation on polystyrene surface

The results of the attachment and biofilm study involving the polystyrene surface show that the cells that have the ability to produce thin aggregative fimbriae alone or thin aggregative fimbriae in conjunction with cellulose had significantly greater abilities in attachment and biofilm formation than did the cells that do not synthesize the fimbriae *(P*<0.05) (Table 4.3). S4-70 had the maximum attachment and biofilm forming potentials among the cultures tested in the study (Tables 4.3 and 4.5). Although the cells of S4-1 and S4-70 both formed rdar colonies on LB no salt agar plates supplemented with Congo red and Coomassie brilliant blue, the overall amount of cells of S4-70 attaching to the polystyrene surface at the end of day 1 were almost double in comparison to the amount of cells of S4-1 attached to the surface (Table 4.3). The amount of biofilm formation by S4-70 was also higher in comparison to S4-1 at all the sampling times used in the study (Table 4.3). The differences in the amounts of thin aggregative fimbriae and cellulose produced by the two strains might be among the factors influencing their attachment and biofilm forming potentials. Variants P67F^{+} and P96F^{+} had significantly better attachment and biofilm forming abilities as compared to their non thin aggregative fimbriae synthesizing counterparts P67F^{-} and P96F^{-} *(P*<0.05) (Table 4.3). Cells of S4-28 and P67F^{-} attached in least numbers and formed the least amount of biofilm on the test surfaces.

Attachment and biofilm formation on glass surface

The results of the attachment and biofilm formation study indicated that S4-70 had the maximum, while S4-28 and P67F$^-$ had the least ability to attach and form biofilms on the glass surface among the strains/variants tested in the study (Table 4.4). Thin aggregative fimbriae producing strains, S4-69, as well as thin aggregative fimbriae and cellulose producing strains, S4-1 and S4-70, attached more efficiently to, and formed more biofilm on the glass surface than did the non-thin aggregative fimbriae expressing strain, S4-28 (Table 4.4). These findings are consistent with those observed on the polystyrene surface (Table 4.3). Significantly higher levels of attachment and biofilm formation were observed with the thin aggregative fimbriae producing variants P67F$^+$ and P96F$^+$ compared to their non-thin aggregative fimbriae expressing counterparts over the 7 d period ($P<0.05$) (Table 4.4). These results suggest that thin aggregative fimbriae and cellulose are the cell surface components that facilitate the attachment and biofilm formation by *Salmonella* cells to/on abiotic surfaces.

Influence of the type of contact surface on the efficiency of attachment and biofilm formation

Cells of all the *Salmonella* cultures used in this study formed significantly more biofilm on the polystyrene as compared to the glass surface ($P<0.05$) with the exception of S4-28 which formed similar amounts of biofilm on both the surfaces (Table 4.5). The amounts of biofilms formed by S4-70, S4-69, and P96F$^+$ on the polystyrene surface were approximately 40-70% higher compared to those on the surface of glass.

Influence of medium composition on the efficiency of attachment and biofilm formation

The inclusion of salt in the culture media seemed to have some effects on the attachment and biofilm formation of *Salmonella*. Cells of the tested *Salmonella* formed more biofilm on the polystyrene and glass surfaces in LBNS compared to LB ($P<0.05$) except for 4-28 on polystyrene surfaces (Tables 4.3 and 4.4). Similar effect was observed with the tested cultures on glass surface in LBG compared to LBNSG except for the culture of S4-69, and on the surface of polystyrene with the cultures of $P96F^+$ and $P96F^-$.

The addition of glucose to the media significantly reduced the attachment and biofilm formation by the tested *Salmonella* ($P<0.05$) with the exception of $P67F^+$ and $P67F^-$ which, when placed in LB and LBG, showed no significant difference in biofilm formation on polystyrene surfaces (Table 4.3). Similar repressive effects of glucose on biofilm formation by S4-1, S4-70, S4-69, $P96F^+$, and $P96F^-$ were observed on glass surface in LBNSG compared to LBNS (Tables 4.3 and 4.4). The amounts of biofilm formed by S4-28 and P67F- on glass surface were also influenced by the addition of glucose in LB. The difference in the amounts of biofilm formed by these two strains in LB compared to LBG was however, not statistically significant. Interestingly, the amount of biofilm formed by $P67F^+$ on the glass surface was higher ($P<0.05$) in LBG compared to LB (Table 4.4).

Influence of cell-surface contact time on attachment and biofilm formation

The cell surface contact time positively influenced *Salmonella* attachment and biofilm formation to the polystyrene and glass surface (Tables 4.3 and 4.4). The amounts of

biofilm formed steadily increased with increasing length of contact between the examined cells and the tested surfaces.

DISCUSSION

The cells of the *Salmonella* strains/variants used in this study had four different colony morphotypes: rdar, pdar, bdar, and saw (Table 4.1). The ability of these strains/variants to produce thin aggregative fimbriae and cellulose varied and was influenced by environmental conditions (Table 4.2). Thin aggregative fimbriae of *Salmonella* are amyloid proteins, and have a special affinity to Congo red dye. The Congo red has a linear configuration, and the azo and amine groups of which can form hydrogen bonds with the similarly spaced carbohydrate hydroxyl radicals of the amyloid proteins. The binding of thin aggregative fimbriae to Congo red causes a decrease in the concentrations of the free dye in the supernatants, which in turn causes a decrease in absorption at a wavelength of 500 nm. The amount of dye bound by the thin aggregative fimbriae is an indirect measure of the amount of fimbriae produced by *Salmonella* cells. Gophna ***et al.*** (2001) reported that curli-expressing cells of *Escherichia coli* bound 10 times more Congo red than did the non-curli expressing cells and that a greater amount of dye was bound by samples having a higher population of cells. Since curli and thin aggregative fimbriae are homologous fibers expressed by *Salmonella* and *E. coli* respectively (Doran ***et al.*** 1993), the methods developed by Gophna ***et al.*** (2001) and Pawar and Chen (2005) were adopted in the present study to quantify the thin aggregative fimbriae expressed by *Salmonella* cells. The results indicated that a larger quantity of Congo red bound to the cells that produce more thin aggregative fimbriae (Table 4.2). In order to negate the

effect of the population of cells on binding of Congo red, approximately similar numbers of cells of each culture were used. The changes in the absorption of Congo red solution were therefore, entirely due to the differences in the levels of thin aggregative fimbriae synthesized by the *Salmonella* cells. Statistical differences were observed in the expression of thin aggregative fimbriae by the two members of the isogenic pairs, $P67F^-$ /$P67F^+$ and $P96F^-$/$P96F^+$. Variants $P67F^+$ and $P96F^+$ produced significantly higher ($P<0.05$) amounts of thin aggregative fimbriae than did their non-thin aggregative fimbriae expressing counterparts (Table 4.2). Significant differences were also observed in the amount of thin aggregative fimbriae produced by the two rdar strains, S4-1 and S4-70, with S4-70 producing significantly greater amounts ($P<0.05$) of thin aggregative fimbriae than S4-1 after 48 and 72 h of incubation at 28°C. The amounts of the fimbriae produced by the two strains at 24 h of incubation were however, not statistically different (Table 4.2). In addition to this study, other researchers have also observed the effect of incubation time on the expression of thin aggregative fimbriae (Gerstel and Romling, 2001).

The results of cellulose estimation revealed that *Salmonella* cells expressing rdar or pdar colony morphotype produced more cellulose than did the cells expressing bdar or saw colony morphotype (Tables 4.1 and 4.2). The cellulose estimation procedure involved incubating *Salmonella* cells in an acetic-nitric reagent, which destroys all polysaccharide material with the exception of crystalline cellulose (Updegraff, 1969). A subsequent addition of an anthrone reagent produces a green color in the presence of cellulose. The intensity of the color is directly proportional to the amount of cellulose produced by the cells. Comparing the absorbance of the tested samples with the

absorbance of the standard cellulose solution of known concentrations gives an indirect measure of the amount of cellulose present in the sample. Strain S4-28 produced a significantly higher amount of cellulose than all other cultures tested in the study *(P*<0.05) (Table 4.2). Although strains S4-70 and S4-1 both expressed rdar colony morphotype (Table 4.1), S4-70 produced a significantly higher amount of cellulose than did S4-1 *(P*<0.05) (Table 4.2).

The quantitative analysis of thin aggregative fimbriae and cellulose suggests that the inclusion of both wild type strains and isogenic variants of *Salmonella* in the study concerning attachment and biofilm formation is necessary. Use of wild type strains alone can sometimes be problematic because they often have more variations than just synthesizing different amounts of cellulose and thin aggregative fimbriae. The isogenic variants of P67 and P96 used in the present study differed from each other only by their ability to produce or not produce thin aggregative fimbriae and therefore, were the appropriate choice for study of the effects of thin aggregative fimbriae on adhesion and biofilm formation.

The crystal violet binding assay adopted in the present study uses an indirect approach to measure the number of bacteria cells attached to the polystyrene and glass surfaces as well as embedded in biofilm. When the bacterial cell walls are disrupted by a destaining solution, ethanol: acetone mixture, the dye bound to the *Salmonella* cell wall is released. Measurement of the absorption of the dye, leaked from *Salmonella* cells gives an indirect indication of the number of cells attached or in biofilm. The amount of dye bound by the *Salmonella* cell wall is a critical parameter of the experiment; care was therefore taken to thoroughly dry the bacteria before staining and to keep a consistent

timing of staining and de-staining procedures. Djordjevic *et al.* (2002) obtained more consistent results upon measuring biofilm formation using crystal violet binding assay compared to direct microscopy. Heat fixing the cells attached to the surfaces before staining was found to be critical for obtaining reproducible results in the crystal violet assay (Genevaux *et al.* 1996). A heat fixing step was hence incorporated in this particular procedure of the present study. A similar method employing crystal violet binding was also used by O'Toole and Kolter (1998) who evaluated the impact of various growth conditions and environmental signals on biofilm formation.

The sole expression of cellulose by S4-28 leads to elastic cell-cell interactions and reduced biofilm formation on polystyrene surfaces. Scanning electronic microscopy performed in our laboratory (Jain and Chen, Unpublished) has shown that the cells of S4-28 were densely packed in an unstructured matrix of cellulose, which may prevented the cells from attaching and forming biofilm on certain contact surfaces ($P<0.05$) (Table 4.5).

The two non-thin aggregative fimbriae expressing variants $P67F^-$ and $P96F^-$ showed significantly less attachment ($P<0.05$) and formed significantly less biofilm ($P<0.05$) (Tables 4.3 and 4.4) on the polystyrene and glass surfaces than did their thin aggregative fimbriae expressing counterparts, $P67F^+$ and $P96F^+$. S4-69 had the ability to synthesize thin aggregative fimbriae and attached in higher numbers and formed more biofilm than the cells of S4-1, S4-28, $P67F^-$, $P96F^-$ and $P96F^+$ on polystyrene, and S4-28 and $P96F^-$ on glass surface ($P<0.05$) (Tables 4.3 and 4.4).

The concurrent expression of thin aggregative fimbriae and cellulose by *Salmonella* cells produces a hydrophobic extracellular matrix which is a major determinant of cell-cell interactions and is responsible for cell adherence to hydrophilic

as well as hydrophobic surfaces (Zogaj *et al.* 2003). Simm *et al.* (2004) found that *Salmonella* cells which express thin aggregative fimbriae have greater attachment ability, while those expressing thin aggregative fimbriae in combination with cellulose have more biofilm forming potential than the cultures which lack the expression of the former or both of the aforementioned cell surface components. It was found in the present study that strain S4-70 had better adherence ability during the initial 24 h period ($P<0.05$) (Tables 4.3 and 4.4) than all other cultures, and formed significantly more biofilm ($P<0.05$) (Table 4.5) than the other cultures used in this study. The role of thin aggregative fimbriae in adhesion and its role in combination with cellulose towards biofilm formation are even more evident upon comparing the levels of these two cell surface components produced by strains S4-70 and S4-1. Strain S4-70 produces significantly higher levels of both of these cell surface components ($P<0.05$) (Table 4.2) and as a result had better attachment and biofilm forming ability than did strain S4-1 ($P<0.05$) (Tables 4.3 and 4.4). Austin *et al.* (1998) described thin aggregative fimbriae of *Salmonella* as major structures required for cell to surface attachment. Cellulose is known to play a structural role in biofilm formation once the initial attachment to the surface has been initiated by thin aggregative fimbriae. When the biofilm formed by cellulose producing *S.* Enteritidis was treated with the enzyme cellulase, the rigid structure of biofilm was disrupted underlining the structural role of cellulose in biofilm formation (Solano *et al.* 2002).

The cell-surface contact time had a significant influence on biofilm formation by *Salmonella* on polystyrene and glass surfaces (Tables 4.3 and 4.4). In general, the biofilm formation by *Salmonella* cells on the test surfaces increased with the increase of

cell-surface contact time *(P*<0.05). Pawar and Chen (2005) noticed that as attachment time increased, the rate of attachment of enterohaemorrhagic *E. coli* on abiotic surfaces also increased. Takeuchi *et al.* (2001) reported an increased attachment with increase in contact time when studying the attachment of *E. coli* O157:H7 to lettuce. Longer contact time perhaps allowed bacterial cells more opportunities to interact with their contact surface through the function of thin aggregative fimbriae.

The composition of culture medium significantly affected the adhesion of *Pseudomonas fragi, Listeria monocytogenes*, and *S.* Typhimurium to stainless steel surfaces (Hood and Zottola, 1997). In the present study, the attachment and biofilm-froming behavior of *Salmonella* cells on polystyrene and glass surfaces were affected by the composition of the culture media (Tables 4.3 and 4.4). Previous work also showed that supplementation of glucose to media was inhibitory to biofilm formation by *E. coli, S.* Typhimurium (Jackson *et al.* 2002), and *Bacillus subtilis* (Stanley *et al.* 2003). Bacteria generally form biofilms under conditions of nutrient limitation. Glucose is a rapidly metabolizable carbon and energy source and its addition to growth media makes nutrients readily available to bacterial cells thereby repressing biofilm formation. The decrease in biofilm formation in *E. coli* in the presence of glucose is due to the catabolite repression system mediated in part by cyclic AMP (cAMP) and the cAMP receptor protein (Jackson *et al.* 2002). In *Salmonella,* the biosynthesis of thin aggregative fimbriae is controlled by the *agf* operons. The AgfD enables the expression of thin aggregative fimbriae by transcriptional activation of the *agfBAC* operon, which are the structural genes for thin aggregative fimbriae (Romling *et al.* 1998a). The AgfD also stimulates the transcription of AdrA (*agfD*-dependent regulator) which activates cellulose

production by direct interaction with the genes of bacterial cellulose synthesis operons *bcsABZC* and *bcsEFG* (Zogaj *et al.* 2001; Solano *et al.* 2002) or by production of a cyclic nucleotide which acts as an activator of cellulose biosynthesis (Romling, 2002). The transcription of AgfD is dependent on environmental factors such as the addition of glucose (Gerstel and Romling, 2001). Glucose has a repressive effect on the activity of *agf*D (Gerstel and Romling, 2001). A reduced transcription of AgfD leads to decreased biosynthesis of thin aggregative fimbriae as well as cellulose.

A previous report indicates that the expression of thin aggregative fimbriae by *S.* Typhimurium is abolished when the *Salmonella* cells were cultured under conditions of high osmolarity. This inhibition was most likely caused by the negative regulation of salt on the expression of *agfD* (Romling *et al.* 1998b). The low osmolarity condition in LBNS provided an optimum environment for *Salmonella* cells to express thin aggregative fimbriae which in turn enhanced the ability of cells to attach and subsequently form more biofilm on abiotic surfaces (Tables 4.3 and 4.4).

Higher levels of attachment and biofilm formation by *Salmonella* cells occurred on polystyrene compared to glass surface with only a few exceptions. This might be partly due to the surface properties of bacterial cells, as well as the surfaces to which the bacteria were allowed to adhere. Polymers, particularly polystyrene are electrostatic, hydrophobic, and possess low surface energy while glass surface is hydrophilic, and possess a negative surface charge (Sinde and Carballo, 2000). The interactions between bacterial cells and abiotic surfaces differ for adhesion onto hydrophobic or hydrophilic surfaces (Helke *et al.* 1993; Sommer *et al.* 1999). Generally, hydrophobic cells are more adherent than are hydrophilic cells (Van Loosdrecht *et al.* 1987). In the present study,

Salmonella cells attached more efficiently and formed more biofilm on the hydrophobic polystyrene surface than on the hydrophilic glass surface (Tables 4.3, 4.4, and 4.5). Sinde and Carballo (2000) also reported that *Salmonella* cells used in their study adhered in higher numbers to hydrophobic surfaces such as polytetrafluorethylene than to rubber and stainless steel. Other reports indicated preferential bacterial adhesion to hydrophobic surfaces such as polystyrene and rubber compared to hydrophilic materials such as glass and stainless steel (Fletcher and Loeb, 1979).

In summary, a number of factors such as bacterial cell surface components, the nature and degree of roughness of cell-contact surface, the temperature of the environment, nutrient availability, and the time available for cell and surface interaction may affect bacterial attachment and biofilm formation. Thin aggregative fimbriae may impart an extraordinary ability to *Salmonella* to attach to polystyrene and glass surfaces and upon co-expression with cellulose enhance biofilm formation on certain abiotic surfaces. Polystyrene is often used by the food processing industry for fabrication of pipelines, tanks, machinery, and working surfaces such as cutting boards whereas, glass is a very common packaging material which is also used to fabricate see-through pipelines, and watch glasses for milk or other liquid storage tanks. The attachment of bacteria and subsequent biofilm formation on these surfaces is a primary food safety concern because the attached bacteria can evade routine cleaning and sanitation steps. Therefore, cleaning operations specifically targeting surface associated microflora are extremely important. A cleaning product or a mix of different cleaning agents that could enzymatically degrade the proteinaceous thin aggregative fimbriae and polysaccharide cellulose or disrupt the matrix materials will ensure an effective removal of attached

Salmonella from food contact surfaces. A disinfection step following the treatment by such a cleaning agent will leave the food contact surface free of harmful bacteria. The knowledge acquired by studies such as this could be used to develop novel cleaning agents and cleaning protocols thereby ensuring food safety and the safety of consumers.

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Table 4.1 *Salmonella* strains used in the study

Laboratory designation	Identity	Source	Colony morphotype[a]	Cellulose production[b]	Thin aggregative fimbriae biosynthesis[c]
S4-1	*S.* Enteritidis	Poultry	rdar	+	+
S4-70	*S.* Enteritidis	Unknown	rdar	+	+
S4-69	*S.* Enteritidis	Unknown	bdar	-	+
S4-28	*S.* Typhimurium	Human	pdar	++	-
P67F^{-}	*S.* Rubislaw	Poultry	saw	-	-
P67F^{+}	*S.* Rubislaw	Poultry	bdar	-	+
P96F^{-}	*S.* Mbandaka	Poultry	saw	-	-
P96F^{+}	*S.* Mbandaka	Poultry	bdar	-	+

[a] Colony morphotypes of *Salmonella* cells grown at 28°C for 72 h on LB no salt agar supplemented with Congo red (40 μg ml^{-1}) and Coomassie brilliant blue (20 μg ml^{-1}).

[b] Determined by examination of fluorescence (420 nm), emitted by Calcofluor after its binding with cellulose produced by *Salmonella* cells, using a long wavelength UV light (365-395 nm).

[c] Determined by the colors of the colonies on LB no salt agar supplemented with Congo red (40 μg ml^{-1}) and Coomassie brilliant blue (20 μg ml^{-1}) after incubation for 72 h at 28°C.

Table 4.2 Quantification of thin aggregative fimbriae and cellulose produced by *Salmonella* cells

Culture age (h)	*Salmonella* cultures							
	S4-1	S4-70	S4-69	S4-28	P67F$^-$	P67F$^+$	P96F$^-$	P96F$^+$
Thin aggregative fimbriae biosynthesis (A_{500})								
24	0.91 a A	0.89 a A	0.92 a A	0.95 a A	0.95 a A	0.92 a A	0.94 a A	0.88 a A
48	0.79 d B	0.66 e B	0.84 b B	0.94 a B	0.94 a A	0.79 cd B	0.93 a A	0.83 bc A
72	0.74 b B	0.59 d C	0.67 c C	0.92 a B	0.92 a B	0.71 bc C	0.93 a A	0.67 c B
Cellulose production (μg 10^{-9} cells)								
72	2.473 b	4.865 c	0.016 a	8.574 d	0.026 a	0.028 a	0.027 a	0.034 a

The values for thin aggregative fimbriae biosynthesis are the mean absorbance, at a wavelength of 500 nm (A_{500}), of the Congo red absorbed by *Salmonella* cells.

The values for cellulose production are the average amounts of cellulose synthesized by *Salmonella* cells.

The values in column not followed by the same upper case letter are significantly different with respect to incubation time ($P \leq 0.05$).

The values in rows not followed by the same lower case letter are significantly different with respect to cultures ($P \leq 0.05$).

Table 4.3 Attachment and biofilm formation by *Salmonella* cells on polystyrene surface as influenced by the time of incubation and composition of culture media

	Salmonella cultures							
Variables	S4-1	S4-70	S4-69	S4-28	P67F$^-$	P67F$^+$	P96F$^-$	P96F$^+$
Incubation time (d)								
1	0.17 bc C	0.31 a B	0.26 ab D	0.13 c C	0.12 c D	0.26 ab BC	0.15 bc E	0.22 abc D
2	0.17 b C	0.39 a B	0.46 a C	0.16 b BC	0.14 b D	0.18 b C	0.23 b D	0.41 a C
3	0.39 c AB	0.74 a A	0.60 b BC	0.26 d A	0.31 cd A	0.40 c A	0.37 cd B	0.53 b D
4	0.34 cd B	0.68 a A	0.67 a AB	0.18 e B	0.27 d B	0.39 c AB	0.37 c AB	0.54 b B
5	0.36 c AB	0.63 a A	0.55 ab BC	0.15 e BC	0.24 d C	0.35 c AB	0.31 cd C	0.53 b B
6	0.34 c B	0.63 b A	0.79 a A	0.18 d B	0.25 d BC	0.39 c AB	0.39 c AB	0.64 b A
7	0.42 c A	0.78 a A	0.74 a A	0.26 d A	0.32 d A	0.40 c A	0.41 c A	0.49 b BC
Average	0.32 c	0.60 a	0.58 a	0.19 d	0.23 d	0.34 c	0.32 c	0.48 b
Media								
LB	0.20 d B	0.57 a B	0.55 a B	0.23 d A	0.13 e B	0.19 d B	0.28 c B	0.39 b B
LBG	0.19 b BC	0.16 c C	0.18 b C	0.13 d B	0.14 d B	0.20 b B	0.18 bc C	0.26 a C
LBNS	0.72 c A	1.50 a A	1.37 a A	0.26 e A	0.55 d A	0.82 c A	0.70 cd A	1.12 b A
LBNSG	0.16 b C	0.15 b C	0.21 a C	0.14 c B	0.12 d B	0.15 bc B	0.12 d D	0.16 b D
Average	0.32 c	0.60 a	0.58 a	0.19 d	0.24 d	0.34 c	0.32 c	0.48 b

The values are the mean absorbance of the crystal violet extracted from *Salmonella* cells at wavelength 550 nm (A_{550}).
The values in the same columns not followed by the same upper case letter are significantly different with respect to incubation time or media ($P \leq 0.05$).
The values in the same row not followed by the same lower case letter are significantly different with respect to culture ($P \leq 0.05$).

Table 4.4 Attachment and biofilm formation by *Salmonella* cells on glass surface as influenced by the time of incubation and composition of culture media

	Salmonella cultures							
Variables	S4-1	S4-70	S4-69	S4-28	P67F$^-$	P67F$^+$	P96F$^-$	P96F$^+$
Incubation time (d)								
1	0.11 bc E	0.16 a F	0.11 bc E	0.10 cd E	0.13 b C	0.15 a E	0.10 d E	0.10 d D
2	0.13 d E	0.21 b E	0.22 b D	0.15 cd D	0.13 d C	0.17 c E	0.20 b D	0.30 a C
3	0.26 cd D	0.36 a D	0.27 c C	0.20 f C	0.20 f B	0.24 de D	0.24 e C	0.32 b C
4	0.30 c C	0.44 a C	0.36 b B	0.23 d B	0.24 d A	0.27 cd D	0.26 d B	0.38 b B
5	0.32 c BC	0.57 a A	0.37 b B	0.24 d AB	0.25 d A	0.31 c C	0.29 cd B	0.40 b B
6	0.33 bc B	0.51 a B	0.49 a A	0.24 d AB	0.25 d A	0.37 b B	0.32 c A	0.48 a A
7	0.36 d A	0.52 a AB	0.52 ab A	0.26 e A	0.24 e A	0.43 c A	0.34 d A	0.48 b A
Average	0.26 cd	0.40 a	0.34 b	0.21 e	0.21 e	0.28 c	0.25 d	0.35 b
Media								
LB	0.21 e B	0.42 a B	0.36 b B	0.17 f C	0.14 f C	0.16 f D	0.25 d B	0.32 c B
LBG	0.16 b C	0.30 a C	0.18 b C	0.12 d C	0.13 d C	0.19 b C	0.18 b C	0.18 b C
LBNS	0.52 d A	0.73 b A	0.61 c A	0.33 g A	0.38 f A	0.52 d A	0.44 e A	0.76 a A
LBNSG	0.14 d D	0.14 d D	0.19 bc C	0.20 b B	0.18 c B	0.24 a B	0.13 d D	0.15 d D
Average	0.26 cd	0.40 a	0.34 b	0.21 e	0.21 e	0.28 c	0.25 d	0.35 b

The values are the mean absorbance of the crystal violet extracted from *Salmonella* cells at wavelength 550 nm (A_{550}).
The values in the same columns that are not followed by the same upper case letter are significantly different with respect to incubation time or media ($P \leq 0.05$).
The values in the same row that are not followed by the same lower case letter are significantly different with respect to culture ($P \leq 0.05$).

Table 4.5 Overall statistical analyses on the attachment and biofilm formation by *Salmonella* on abiotic surfaces

	Salmonella cultures							
	S4-1	S4-70	S4-69	S4-28	P67F$^-$	P67F$^+$	P96F$^-$	P96F$^+$
Time*media*polystyrene	0.32 c A	0.60 a A	0.58 a A	0.19 d A	0.23 d A	0.34 c A	0.32 c A	0.48 b A
Time*media*glass	0.26 cd B	0.40 a B	0.34 b B	0.21 e A	0.21 e B	0.28 c B	0.25 d B	0.35 b B
Time*media*surface	0.29 c	0.50 a	0.46 ab	0.20 d	0.22 d	0.31 c	0.28 c	0.42 b

The values are the mean absorbance of the crystal violet extracted from *Salmonella* cells at wavelength 550 nm (A_{550}).
The values in the same column not followed by the same upper case letters are significantly different with respect to surface *(*$P \leq 0.05$).
The values in the same row not followed by the same lower case letter are significantly different with respect to culture *(*$P \leq 0.05$).

CHAPTER 5

TRANSFER OF ANTIBIOTIC RESISTANCE GENES FROM *SALMONELLA ENTERICA* TO *ESCHERICHIA COLI*[1]

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[1] Jain, Sudeep and Jinru Chen. To be submitted to International Journal of Food Microbiology.

Abstract

Foodborne pathogens demonstrating a resistance to antibiotics is a primary public health concern. Infections caused by such bacteria may have fatal consequences because an antibiotic regimen used in modern medicine would be ineffective in killing these pathogens. This study was undertaken to evaluate whether antibiotic resistance genes of *Salmonella enterica* from various sources can be transferred to *Escherichia coli*, and if the acquired antibiotic resistance genes can be eliminated from the recipient strain under certain environmental conditions. Eleven *Salmonella* donors were allowed to mate with *E. coli* K-12 MG 1655, respectively in tryptic soy broth (TSB) at 37^{o}C for 90 min before transconjugants were selected on TSA containing appropriate antibiotics at 37^{o}C for 24 h. The plasmid profiles and antibiotic resistance properties of the donors, recipient, and transconjugants were determined. The liquid cultures of the transconjugants were then placed in TSB at 42^{o}C for an extended period of time in order to cure the acquired antibiotic resistance genes. The plasmid profiles and antibiotic resistance properties of the cured derivatives were examined and compared with those of the donors, recipient, and transconjugants. The results indicated that resistance to oxytetracycline, tetracycline, and streptomycin was transferable by six *Salmonella* donors while one other donor only transferred the gene(s) encoding ampicillin resistance. The conjugation efficiency ranged from 7.60 X 10^{-12} to 1.53 X 10^{-10} transconjugant per recipient cell. Incubation at 42^{o}C did not cure the transferred plasmid(s) but did successfully eliminate the acquired resistance to oxytetracycline, tetracycline, and streptomycin. Plasmid profiling indicated that the resistance to the antibiotics in the *Salmonella* strains may not be plasmid-encoded. The study shows that antibiotic resistance is transferable to bacteria of different species. It

suggests that food of animal origin may serve as a source for dissemination of antimicrobial resistant *Salmonella* and for interspecies antibiotic resistance gene transfer.

Keywords: Antibiotic resistance, Gene transfer, Conjugation, *Salmonella enterica, Escherichia coli*

1. Introduction

Salmonella is a zoonotic pathogen and can be transmitted to humans through the consumption of food, especially meat and poultry (Dominguez *et al.* 2002). The duration of human *Salmonella* infection ranges from 4 to 7 d and the symptoms of the illness are usually self-limiting. However, treatment with antibiotics, such as chloramphenicol, ampicillin, tetracycline, or trimethoprim-sulfisoxazole, may be necessary if the illness persists or the symptoms are severe (Cabrera *et al.* 2004). *Salmonella* infections can normally be cured by antibiotic therapy however, in recent years, reports of patients not responding to antibiotic treatment have surfaced (Molbak, 2004). The occurrence of antibiotic resistance in *Salmonella* is a major obstacle to successful therapeutic treatment for salmonellosis as well as salmonellosis-related systemic infections (Molbak, 2004).

Antibiotic resistance in bacteria, including *Salmonella* may be due to changes in bacterial cell wall permeability, removal of antibiotics by efflux pumps, destruction or inactivation of the antibiotics, or modification of the site of action (Schwarz and Chaslus-Dancla, 2001). Most of the antibiotics used in human and animal therapy can be inactivated or blocked by one or more of these mechanisms. A bacterial cell can acquire antibiotic resistance genes through three different approaches, which include transformation, conjugation, and transduction. Some believe that conjugation is the dominant mode of gene transfer between bacterial cells (Wolska, 2003) and most antibiotic resistant bacteria are developed by this approach (Gebreyes and Altier, 2002). Others however, are convinced that gene transfer by other means such as transduction is just as important as the transfer by conjugation in the environment (Zeph *et al.*, 1988).

Plasmids are generally the molecular elements that mediate the transfer of antibiotic resistance genes between bacterial cells. Genes responsible for resistance towards commonly used antibiotics such as chloramphenicol, ampicillin, tetracycline, and trimethoprim-sulfimethoxazole are often encoded by conjugative plasmids (Datta *et al.* 1981). Some broad host range plasmids, such as RSF 1010 and pBP1 in *Salmonella* (Pezzella *et al.* 2004), and RK2 in *E. coli* and *Pseudomonas aeruginosa* (Kowalczyk *et al.* 2005) are equipped with systems that enable them to transfer and stably maintain the transferred genes to/in closely related bacterial species (Mandal *et al.* 2003). Such interspecies transfer of antibiotic resistance genes may take place between *Salmonella* and *E. coli* (Winokur *et al.* 2001; O'Brian, 2002). Bischoff *et al.* (2004) demonstrated that *Salmonella* Kinshasa transferred the genes for chloramphenicol, streptomycin, sulphamethoxazole, and tetracycline resistance along with the gene for ampicillin resistance through conjugation to *E. coli* JM 109. Similarly, Mandal *et al.* (2003) reported the transfer of genes encoding resistance to ampicillin, chloramphenicol, cotrimoxazole, and tetracycline from *S.* Typhi to *E. coli* C600 through conjugation.

In addition to plasmid, mobile DNA elements such as integrons and transposons can mediate the transfer of antibiotic resistance genes between cells of the same or closely related bacterial species (Recchia and Hall, 1995). Temperate phages capable of generalized transduction are a common genetic element in *Salmonella* (Schicklmaier and Schmieger, 1995). These bacteriophages are an important vehicle for delivering antibiotic resistance genes between *Salmonella*, and from *Salmonella* to other closely related bacterial species (Brabban *et al.* 2005).

The objectives of this study are to evaluate whether antibiotic resistance genes of *Salmonella enterica* can be transferred to *E. coli* K-12 MG1655 and if the acquired antimicrobial resistance genes by the recipient strains can be eliminated under certain environmental conditions.

2. Materials and methods

2.1. Bacterial strains

A total of eleven *Salmonella* strains were used as the donors for antibiotic resistance genes in the study. Five strains of various sources, S4-12, S4-24, S4-28, S4-50, and S4-71 were selected from our laboratory culture collection. Six other strains, P59, P66, P67, P68, P69, and P70 were recently isolated from raw, retail poultry meat (Jain and Chen, Unpublished) using the methods described in the Bacteriological Analytical Manual (FDA, 2003) and Microbiology Laboratory Guidebook (USDA, 1998). The isolates were biochemically identified using the API 20E system (Biomerieux, Marcy I' Etoile, France), and serologically confirmed at the Poultry Diagnostic Research Center (Athens, GA, USA) and National Veterinary Services Laboratories (Ames, IA, USA). The recipient strain, a spontaneous nalidixic acid resistant derivative of *Escherichia coli* K-12 MG1655 (strain CA32), was generously provided by Wondwossen A. Gebreyes of North Carolina State University, Raleigh, NC, USA.

2.2. Antimicrobial susceptibility testing

The antibiotic resistance profiles of all tested strains were determined using the standard disk diffusion assay following the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2003). Briefly, the cultures were grown on tryptic soy agar (TSA; Difco Laboratories, Sparks, MD, USA) plates for 18 h at 37°C. A single colony of each culture was transferred to tryptic soy broth (TSB) and incubated at 37°C until the optical density (O.D.) reached 0.08-0.10 at a wavelength of 600 nm (Novaspec II Visible Spectrophotometer, Pharmacia Biotech, Cambridge, England). The culture was inoculated on Mueller-Hinton agar (Becton, Dickinson and Company, Sparks, MD, USA) plates using a sterile swab and allowed to dry for 5 min. Four antibiotic discs (BD-BBL Sensi-Disc Antimicrobial Susceptibility Test Discs, Becton, Dickinson and Company, Sparks, MD, USA) were placed on each plate. Each culture was tested for its sensitivity to 12 antibiotics, namely ampicillin (AM; 10 μg), cephalothin (CF; 30 μg), chloramphenicol (C;30 μg), gentamicin (GM;10 μg), kanamycin (K;30 μg), nalidixic acid (NA; 30 μg), neomycin (N;30 μg;), novobiocin (NB; 30 μg), oxytetracycline (T; 30 μg), streptomycin (S;10 μg), sulfisoxazole (G; 0.25 mg), and tetracycline (TE; 30 μg). Susceptibility or resistance of the cultures to the antibiotics was determined according to the guidelines of NCCLS or the instructions from the suppliers of the antibiotic discs.

2.3. Conjugation experiment

The donor and recipient cultures were inoculated on TSA and TSA supplemented with 100 μg /ml nalidixic acid, respectively and incubated for 24 h at 37°C. A single

colony of each of the cultures was inoculated in TSB or TSB supplemented with nalidixic acid and incubated under the conditions described above. One ml of the recipient culture was mixed with 100 μl of the donor cultures, respectively in a sterile microfuge tube and the mixtures were incubated at 37°C without shaking for 90 min for mating to take place. One hundred microliters of each mixture was then inoculated on the TSA plate which contained the antibiotics for selecting both the donor and the recipient and incubated for 24 h at 37°C. Five putative transconjugant colonies were selected from each conjugation experiment and confirmed whether they were *E. coli* on MacConkey agar (Difco) plates supplemented with appropriate antibiotics. The antibiotic combinations included nalidixic acid (100 μg/ml) and one of the following six antibiotics: ampicillin (100 μg/ml), chloramphenicol (50 μg/ml), kanamycin (50 μg/ml), oxytetracycline (30 μg/ml), streptomycin (100 μg/ml), and tetracycline (25 μg/ml). The antibiotic resistance patterns of the transconjugants were determined by the disc diffusion assay described above.

2.4. Determination of conjugation efficiency

One ml of the recipient culture was mixed with 100 μl of donor cultures, respectively in a sterile microfuge tube and incubated at 37°C without shaking for 90 min. One ml of each of the conjugation mixtures was inoculated on MacConkey agar plates, containing nalidixic acid (100 μg/ ml) and one of the antibiotics listed above. The inoculated plates were then incubated for 24 h at 37°C. Lactose fermenting colonies indicative of *E. coli* transconjugants were enumerated. The numbers of the recipient cells per ml of the culture were determined by plating appropriate dilutions of *E. coli* MG1655

culture on TSA. Conjugation efficiencies were expressed as the ratio of the number of transconjugants to the number of recipients (Chen *et al.* 2004).

2.5. Curing of antibiotic resistance genes

The transconjugants were cured of antibiotic resistance genes using the method of Asheshov (1966). Briefly, the transconjugants from different *Salmonella* donors were grown in TSB without any antibiotics at 42°C for 7 d. At the end of the incubation period, the cultures were plated on MacConkey agar (Difco) supplemented with 100 µg/ ml nalidixic acid and incubated at 37°C for 24 h. Fifteen randomly selected bacterial colonies from each sample were transferred to MacConkey agar supplemented with one of the six antibiotics described above, in addition to 100 µg/ ml nalidixic acid. The inoculated plates were incubated at 37°C for 24 h. Colonies which failed to grow on the MacConkey agar plate supplemented with nalidixic acid and one of the six antibiotics were regarded as the cured derivatives. Cultures of the cured derivatives were then recovered from the MacConkey agar plate supplemented with nalidixic acid and stored in TSB containing 15% glycerol at -30^{o}C for future analysis.

2.6. Isolation of plasmids

Plasmid DNA was isolated from the *Salmonella* donors, *E. coli* recipient, transconjugants, and the cured derivatives by alkaline lysis as described by Sambrook *et al.* (1989). Briefly, cell cultures were grown in 10 ml of Luria-Bertani (LB) broth at

37°C for 18 h with continuous shaking at 250 rpm. Cell pellets obtained by centrifuging the liquid cultures at 4,000 *g* for 10 min were re-suspended in 200 µl suspension buffer (50 mM glucose, 25 mM Tris-Cl, and 10 mM EDTA, pH 8.0) supplemented with 2 mg/ml of lysozyme. Each cell suspension was lysed with 400 µl of the lysis solution (0.2 N NaOH and 1% SDS) and the DNA was re-natured with 300 µl of neutralizing solution (5 M acetate and 3 M potassium). DNA containing supernatant of the lysate was collected after centrifugation at 12,000 *g* for 5 min. The DNA was purified, first with an equal volume of phenol followed by a phenol:chloroform mixture, and finally by chloroform alone. Between each purification step, centrifugation was repeated for 2 min and the aqueous phase was transferred to a fresh microfuge tube. The plasmid DNA was precipitated with ethanol (95%) and centrifuged at the same conditions for 5 min. The DNA pellets were rinsed with ice-cold 70% ethanol, air dried, and then re-suspended in 100 µl of Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8.0) containing 2 µg/ ml RNase (Boehringer Mannheim, Germany)

Electrophoresis was performed using the Midicell Primo electrophoretic gel system (E-C Apparatus Corporation, Holbrook, NY), 0.8% agarose gel, and 1 X TBE running buffer. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ ml) and de-stained using deionized water before being photographed under UV light with the Gel Doc 2000 system (Bio-Rad Laboratories, Segrate, Italy).

2.7. Restriction analysis of plasmid DNA

Plasmid DNA isolated from the donors, recipient, transconjugants, and cured derivatives were digested with restriction endonuclease *EcoRI*. The reaction mix containing 8 µl DNA, 1 µl of *EcoRI*, and 1 µl of 10X reaction buffer, was incubated for 24 h at 37°C. Electrophoresis of the digested plasmid DNA was performed as described above. The 1 kb DNA ladder from Promega (Madison, WI, USA) was used as the standard for the digested plasmid DNA.

3. Results

3.1. Antimicrobial susceptibility of Salmonella*,* E. coli*, and transconjugants*

All *Salmonella* isolates used in this study were multidrug resistant and resisted at least 4 different antibiotics (Table 5.1). All of the isolates were resistant to oxytetracycline, tetracycline, and streptomycin while ten of them were also resistant to novobiocin. Sulfonamide resistance was found in 4 isolates and 3 isolates were resistant to the β-lactam antibiotic, ampicillin (Table 5.1). Isolates P59 and S4-28 were the most drug resistant, each resisting 9 different antibiotics (Table 5.1). Recipient *E. coli* K-12 strain MG1655 (strain CA32) was resistant to nalidixic acid as well as novobiocin.

3.2. Conjugation

The antibiotic resistance profile of the transconjugants show that certain antibiotic resistance genes from the *Salmonella* donor cells were transferable to the *E. coli* recipient cells upon conjugation (Table 5.1). Out of the eleven *Salmonella* donors examined in this study, seven were able to transfer antibiotic resistant genes to the *E. coli* recipient. Six donor strains, P59, P66, P67, P68, P69, and P70, successfully transferred the genes for resistance to oxytetracycline, tetracycline and streptomycin, while one other donor, S4-28 only transferred the genes for resistance to ampicillin. No antibiotic resistance was transferred from strains S4-12, S4-24, S4-50, and S4-71 to *E. coli* under the conjugation conditions used in this study.

The conjugation efficiency ranged from 7.60×10^{-12} to 1.53×10^{-10} transconjugant per recipient cell (Table 5.2). The highest conjugation efficiency of 1.53×10^{-10} was observed with P59 for the transfer of tetracycline resistance while the same strain had the lowest conjugation efficiency of 3.80×10^{-12} for the transfer of streptomycin resistance.

3.3. Curing of antibiotic resistance genes

Fifteen transconjugant colonies from each conjugation experiment were screened for the possible loss of antibiotic resistance after the 7 d incubation at 42^{o}C. The transconjugants of P59, P67, and P68 lost the acquired resistance to oxytetracycline, tetracycline, and streptomycin but a similar loss of resistance was not observed with the

transconjugants of P66, P69, and P70 (Table 5.1). Additionally, the transconjugant of S4-28 remained to be resistant to ampicillin after the 7 d incubation at 42^{o}C. The results indicated that the incubation conditions used for curing were able to eliminate the acquired antibiotic resistance genes from some, but not all transconjugants (Table 5.1).

3.4. Plasmid DNA analysis

Analysis of the undigested plasmid DNA isolated from the cells of the donors, recipient, transconjugants, and cured derivatives indicated that the cured derivatives of P59, P67, and P68, although becoming sensitive to oxytetracycline, streptomycin, and tetracycline upon curing, did not lose the acquired plasmid(s) (Fig. 5.1). This suggests the possible involvement of a mechanism other than plasmid-mediated resistance gene transfer.

Analysis of the digested plasmid DNA revealed some degrees of similarity among the restriction patterns of the donors and their corresponding transconjugants (Fig. 5.2). The *EcoR* I restricted plasmid DNA appeared to have four distinct patterns, one pattern belonged to the donors (Lane 2-7, Fig. 5.2) and another pattern belonged to the transconjugants (Lane 10-15, Fig. 5.2) that were resistant to oxytetracycline, streptomycin, and tetracycline. The third pattern belonged to the donor (Lane 8, Fig. 5.2) and the forth pattern belonged to the transconjugants (Lane 16, Fig. 5.2) that were resistant to ampicillin. The restricted plasmid DNA of the six donor strains recently isolated from poultry meat (Lane 2-7, Fig. 5.2) shared remarkable similarities with their corresponding transconjugants (Lane 10-15, Fig. 5.2). The transconjugant of S4-28 had a

unique restriction pattern (Lane 16, Fig. 5.2) which seemed to share some degree of similarity with the pattern of its donor strain (Lane 8, Fig. 5.2).

The cured derivatives of P67 and P68 (Lane 18 and 19, Fig. 5.2) appeared to share a similar restriction pattern with their corresponding transconjugants in Lane 12 and 13 (Fig. 5.2), suggesting that the genes for resistance to oxytetracycline, tetracycline, and streptomycin may not be located on plasmid. Lane 17 (Fig. 5.2) shows a unique restriction pattern of a cured derivative of a transconjugant resulting from the conjugation between *Salmonella* P59 (Lane 10, Fig. 5.2) and the *E. coli* recipient. It was noticed that a DNA fragment with a size slightly greater than 5 Kb was missing from the restriction pattern (Fig. 5.2). It is not clear at the present time whether the missing DNA fragment has contributed to the change in the antibiotic resistance property of the cured derivative. Future work is needed in order to determine the possible link between these two observations.

4. Discussion

The majority of the *Salmonella* strains used in this study were of poultry meat origin. The antibiotic resistance demonstrated by these strains is similar to those documented in the early studies showing that isolates from retail meats are often resistant to antibiotics (Guerra *et al.* 2000; Gebreyes and Altier, 2002; Chen *et al.* 2004). The *Salmonella* strains selected for the conjugation assay had different antibiotic resistance profiles and were resistant to multiple antibiotics (Jain and Chen, Unpublished).

Previous research has shown that the gene CMY-2, which encodes resistance to β–lactam antibiotics could be transferred between closely related enteric bacteria such as *Salmonella* and *E. coli* (Winokur *et al.* 2001). The results of the present study provide additional evidence to support the notion that horizontal gene transfer contributes to the spread of antibiotic resistance from *Salmonella* to *E. coli* as six out of the eleven *Salmonella* donors used in the study were able to transfer antibiotic resistance to the *E. coli* recipient.

Chen *et al.* (2004) reported a conjugation efficiency of 6.0×10^{-8} to 2.4×10^{-4} from *Salmonella* to *E. coli* which is much higher than the conjugation efficiency of 7.60×10^{-12} to 1.53×10^{-10} observed in this study (Table 5.2). The comparison of the conjugation efficiencies obtained by these two studies is somewhat difficult due to the differences in the conjugation procedures adopted by the two research groups. The conjugation time used by our laboratory was 90 min at 37°C while Chen *et al.* (2004) incubated the donor-recipient mixture at 37°C for 24 h. Longer incubation time could have caused an increase in the population of transconjugants, thereby resulting in relatively higher conjugation efficiencies.

Transconjugants of some donor strains used in the present study, namely S4-12, S4-24, S4-50, and S4-71, were not recovered. Low efficiency of conjugation is a probable cause because only a limited number of potential transconjugants were screened. Selecting a larger number of colonies for potential transconjugants might be able to increase the chances of recovering the transconjugants of these strains.

Various physical and chemical agents such as heat, U.V. radiation, acridine orange, ethidium bromide, sodium dodecyl sulfate, phenothiazines, sarkomycin, and

methylene blue can be used to cure antibiotic resistance genes (Stanisich, 1988). The transconjugants were incubated at 42°C for 7 d in the present study because heat curing requires incubation of the conjugation mixture at 5-10°C above the optimal growth temperature (Stanisich, 1988). At the elevated incubation temperature, heat interferes with plasmid replication due to which the plasmid failed to replicate at the same rate as the bacterial cells (Stadler and Adelberg, 1972). During growth in an antibiotic free medium, the cells do not need resistance plasmid for survival which is eventually lost from the cells. In this study, the plasmid(s) transferred to the transconjugants were not lost upon curing (Fig. 5.1 and 5.2), even though the acquired antibiotic resistance was successfully eliminated (Table 5.1). A similar observation was reported by Guerra *et al.* (2000), after going through the curing procedure, the transconjugants of *S.* Typhimurium LSP31/93 (DT104) lost the acquired resistance to ampicillin, chloramphenicol, gentamicin, streptomycin, and imipenem but continued to carry the transferred plasmids. These results suggest a putative involvement of a gene transfer mechanism other than plasmid-mediated transfer. It is possible that other mobile genetic elements such as integrons, transposons, or temperate bacteriophages might have been involved in the transfer of the antibiotic resistance genes.

Integrons have the ability to capture genes, notably antibiotic resistance genes in gram-negative bacteria. Three classes of integrons that encode antimicrobial resistance genes have been described (Hall and Collis, 1998). Class I integrons are most commonly found in the clinical isolates of Gram-negative bacteria and more than 60 different gene cassettes have been identified thus far (Hall and Collis, 1998). Antibiotic resistance in *Salmonella* Keurmassar has been found to be encoded by 3 different gene cassettes

located on a Class I integron. The *aadA2* cassette encodes resistance to spectinomycin and streptomycin, the *aac(6')-IIc* cassette confers resistance to gentamicin, netilmicin, and tobramycin, and the *ereA2* cassette encodes resistance to erythromycin (Gassama-Sow *et al.* 2004). Working with *S.* Typhimurium, Guerri *et al.* (2004) noticed the involvement of *flor, tetG, oxa1* and *aadA2* genes located on Class I integrons in conferring resistance to chloramphenicol, tetracycline, β-lactams, streptomycin, and spectinomycin, respectively. In *E. coli,* ten gene cassettes were detected on different class I integrons, among which nine, were related to antibiotic resistance. Gene cassette *dfrA1, dfrA5, dfrA7, dfrA12,* and *dfrA17* encode resistance to trimethoprim, cassette *aadA1, aadA2,* and *aadA5* encode resistance to aminoglycosides, and cassette *blaP1* confers resistance to β-lactam antibiotics (Kang *et al.* 2005).

Antibiotic resistance has been linked to integrons located on transposons. Resistance to trimethoprim, kanamycin, sulfonamides, and streptomycin in *Salmonella* serotype Agona, Bredeney, Enteritidis, Heidelberg, and Typhimurium is carried on *Tn21*-associated integrons (Pezzella *et al.* 2004). The *S.* Enteritidis and Agona strains in the same study also carry, on transposon *Tn5393, strA,* and *strB* genes which encode resistance to streptomycin. The transfer of integrons through plasmids and transposons has been shown to be an important aspect of dissemination of antimicrobial resistance genes.

There is a possibility that the transfer of antibiotic resistance genes from the donor to the recipient was mediated by bacteriophage because temperate bacteriophages have been associated with the dissemination of antibiotic resistance genes (Schmieger and Schicklmaier, 1999). Research has shown that bacteriophages ES18 and PDT 17

transduced antibiotic resistance genes in *S.* Typhimurium DT104 *in vitro* and potentially *in vivo* (Schmieger and Schicklmaier, 1999). Antibiotic resistance to imipenem, ceftazidime, and aztreonam in *Pseudomonas aeruginosa* was transduced by a bacteriophage, AP-151, released from a lysogenic strain (Blahova *et al.* 2000). The dissemination of erythromycin resistance in *Streptococcus pyogenes* has been shown to be mediated by bacteriophage transduction (Giovannetti *et al.* 2005).

5. Conclusion

Salmonella may disseminate antibiotic resistance genes conferring resistance to ampicillin, tetracycline, oxytetracycline, and streptomycin to *E. coli.* The similar plasmid profiles shared by the transconjugants and their cured derivatives indicate a possible involvement of a gene transfer mechanism other than plasmid-mediated antibiotic resistance gene transfer. Because the resistance to multiple antibiotics is common in *Salmonella* and intra- and inter-species transfer of antibiotic resistance genes is possible, a judicious use of antibiotics in agriculture and animal husbandry is recommended in order to control the spread of antibiotic-resistant bacteria in the environment.

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Table 5.1 Bacterial strains used in the study and their antimicrobial resistance profiles

Laboratory designation	Identity	Source	Resistance pattern of the strains	Resistance pattern of transconjugants	Resistance pattern of cured derivatives
Donors					
P59	n.a.[a]	Poultry	AM, C, CF, K, NA, NB, T, S, TE [b]	NA, NB, T, S, TE	NA, NB
P66	*S.* Arizona[c]	Poultry	NB, T, S, TE	NA, NB, T, S, TE	NA, NB, T, S, TE
P67	*S.* Rubislaw	Poultry	NB, T, S, TE	NA, NB, T, S, TE	NA, NB
P68	*S.* Arizona	Poultry	NB, T, S, TE	NA, NB, T, S, TE	NA, NB
P69	*S.* Arizonae	Poultry	NB, T, S, TE	NA, NB, T, S, TE	NA, NB, T, S, TE
P70	*S.* Arizonae	Poultry	NB, T, S, TE	NA, NB, T, S, TE	NA, NB, T, S, TE
S4-12	*S.* Panama	Unknown	NB, T, S, G, TE	-[d]	-
S4-24	*S.* Hadar	Unknown	AM, T, S, G, TE	-	-
S4-28	*S.* Typhimurium	Human	AM, C, K, N, NB, T, S, G, TE	AM, NA, NB	AM, NA, NB
S4-50	*S.* Hadar	Human	NB, T, S, TE	-	-
S4-71	*S.* Senftenberg	Unknown	NB, T, S, G, TE	-	-
Recipient					
	E. coli K-12 MG1655	W. A. Gebreyes N.C. State Univ.	NA, NB		

[a] Serotyping was not attempted.
[b] AM, ampicillin; CF, cephalothin; C, chloramphenicol; GM, gentamicin; K, kanamycin; NA, nalidixic acid; N, neomycin; NB, novobiocin; T, oxytetracycline; S, streptomycin; G, sulfisoxazole; TE, tetracycline.
[c] *Salmonella* strains isolated from poultry or other sources.
[d] Conjugant not obtained.

Table 5.2 Efficiency of conjugation between donor *Salmonella* and recipient *E. coli* K-12 MG 1655

	Conjugation efficiency					
Donors	Ampicillin	Chloramphenicol	Kanamycin	Oxytetracycline	Streptomycin	Tetracycline
P59	-[a]	-	-	9.20×10^{-11}	3.80×10^{-12}	1.53×10^{-10}
P66	-	-	-	6.53×10^{-11}	1.53×10^{-11}	1.15×10^{-11}
P67	-	-	-	1.00×10^{-10}	1.92×10^{-11}	3.46×10^{-11}
P68	-	-	-	1.07×10^{-10}	7.60×10^{-12}	4.61×10^{-11}
P69	-	-	-	7.60×10^{-12}	7.60×10^{-12}	7.60×10^{-12}
P70	-	-	-	1.30×10^{-10}	7.69×10^{-12}	7.69×10^{-12}
S4-12	-	-	-	-	-	-
S4-24	-	-	-	-	-	-
S4-28	1.15×10^{-8}	-	-	-	-	-
S4-50	-	-	-	-	-	-
S4-71	-	-	-	-	-	-

[a] Conjugation efficiency could not be determined because no transconjugants were obtained.

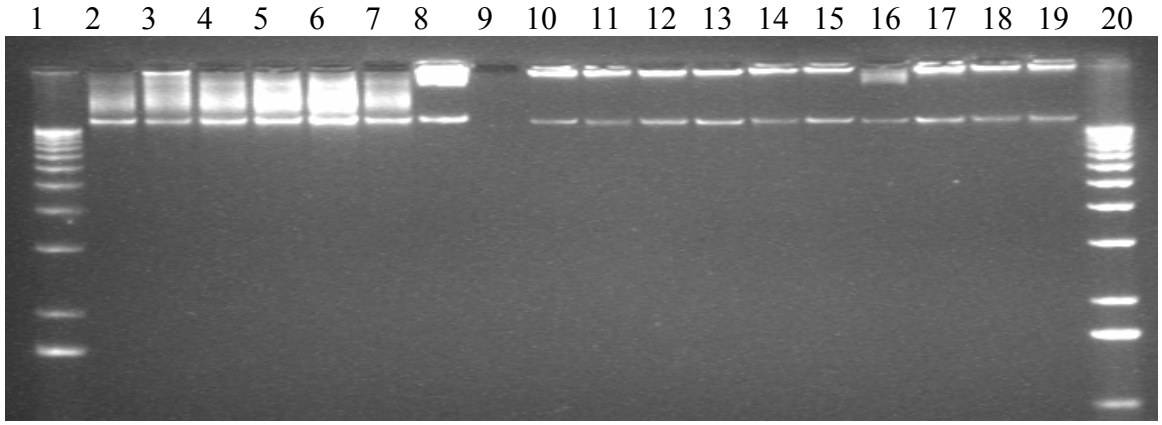

Figure 5.1 Undigested plasmid DNA isolated from donor *Salmonella*, recipient *E. coli* K-12 MG 1655, *E. coli* transconjugants, and cured derivatives. Lanes 1 and 20: 1 kb DNA ladder. Lanes 2-7: P59, P66, P67, P68, P69, and P70. Lane 8: S4-28. Lane 9: *E. coli* K-12 MG 1655. Lane 10-15: transconjugants of P59, P66, P67, P68, P69, and P70. Lane 16: transconjugant of S4-28. Lane 17-19: cured derivatives of P59, P67, and P68.

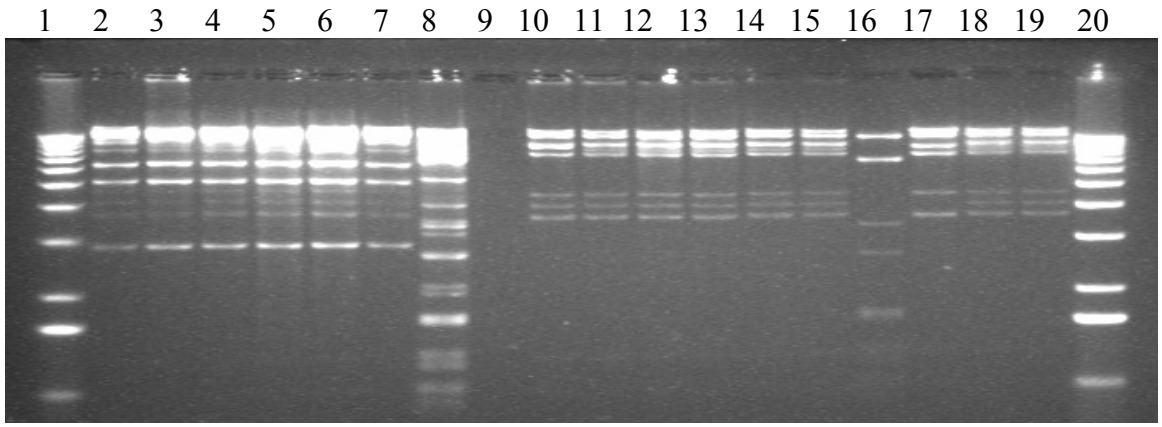

Figure 5.2 *EcoRI*-digested plasmid DNA of donor *Salmonella,* recipient *E. coli* K-12 MG 1655, *E. coli* transconjugants, and cured derivatives. Lanes 1 and 20: 1 kb DNA ladder. Lane 2-7: P59, P66, P67, P68, P69, and P70. Lane 8: S4-28. Lane 9: *E. coli* K-12 MG 1655. Lane 10-15: transconjugants of P59, P66, P67, P68, P69, and P70. Lane 16: transconjugant of S4-28. Lane 17-19: cured derivatives of P59, P67, and P68.

CHAPTER 6

CONCLUSIONS

The following conclusions are drawn from the studies described in Chapter 3, 4, and 5:

1. The retail poultry meat sold in the sampled area had a *Salmonella* contamination rate of 16% suggesting that raw poultry meat could be a potential source of *Salmonella* infection in humans. A considerable number of *Salmonella* strains isolated in the present study exhibited the ability to resist antibiotics. Out of the 52 *Salmonella* isolates tested, 25 (48.0%) were resistant to one antibiotic, 5 (9.6%) to two, 4 (7.7%) to three, 6 (11.5%) to four, and 5 (9.6%) to five antibiotics. Two (3.8%) isolates were resistant to up to nine of the antibiotics tested. The study of the expression of cell surface components by the *Salmonella* strains showed that among the 52 *Salmonella* isolates, 25 expressed only thin aggregative fimbriae and formed brown, dry, and rough (bdar) colonies while 1 expressed only cellulose and formed pink, dry, and rough (pdar) colonies. Ten isolates produced both thin aggregative fimbriae and cellulose and formed red, dry, and rough (rdar) colonies while the remaining 16 expressed neither of the two surface structures and formed smooth and white (saw) colonies on Luria-Bertani no-salt agar supplemented with Congo red and Coomassie brilliant blue dyes. Scanning electron micrographs and pellicle formation study further confirmed the expression of thin aggregative fimbriae and/or cellulose by the *Salmonella* cells. The first part of the study shows that *Salmonella* cells have the abilities to develop resistance to multiple antibiotics and

synthesize the cell surface components that help them to survive in hostile or suboptimal environments.

2. Four wild type *Salmonella* strains producing thin aggregative fimbriae and/or cellulose (S4-1, S4-70, S4-69, and S4-28) and the isogenic pairs of two *Salmonella* strains differing in their ability to produce or not to produce thin aggregative fimbriae ($P67F^+$, $P67F^-$, $P96F^+$, and $P96F^-$) were used in the second part of the study. The cells of rdar strain S4-70 synthesized the highest amount of thin aggregative fimbriae while the highest amount of cellulose was produced by the pdar strain S4-28. Cells expressing thin aggregative fimbriae alone or in combination with cellulose attached in higher numbers to the surfaces of polystyrene as well as glass compared to the cells which expressed only cellulose or none of the two surface components. More biofilm was formed on the test surfaces by S4-70 and the least amount of biofilm was formed by $P67F^-$. Addition of salt to the culture media decreased the tendency of the cells to attach to the surfaces but had no pronounced effect on their ability to form biofilms. Glucose supplementation of the culture media was however, detrimental to both the attachment and biofilm-forming ability of the cells. Cell-surface attachment to both the test surfaces increased with an increase in contact time.
3. Certain antibiotic resistance genes from *Salmonella* donor cells were transferable to the *E. coli* recipient cells upon conjugation. Six donor strains used in the third part of this study, P59, P66, P67, P68, P69, and P70, successfully transferred the genes for resistance to oxytetracycline, tetracycline, and streptomycin, while one other donor, S4-28 only transferred the genes for resistance to ampicillin. The conjugation efficiency ranged from

7.60×10^{-12} to 1.53×10^{-10} transconjugant per recipient cell. Upon incubation at 42°C for an extended period of time, three transconjugants lost the acquired antibiotic resistance genes. Analysis of the undigested plasmid DNA isolated from the cells of the donors, recipient, transconjugants, and cured derivatives indicated that the cured derivatives of P59, P67, and P68, although becoming sensitive to oxytetracycline, streptomycin, and tetracycline upon curing, did not lose the acquired plasmid(s). This suggests the possible involvement of a mechanism other than plasmid-mediated transfer of the antibiotic resistance genes between the *Salmonella* donors and the *E. coli* recipient.