The Distribution of Large Molecular Weight Plasmids and Plasmid Genomics in *Salmonella*

by

Karen Lyons

(Under the Direction of Margie D. Lee)

ABSTRACT

Salmonella remains one of the leading causes of foodborne illnesses; however information regarding the distribution and genomics of plasmids other than the virulence plasmid is limited. In this study, we characterized the diversity of large molecular weight plasmids in *Salmonella* isolated from a diverse group of vertebrate hosts to determine if distribution of large molecular weight plasmids is affected by the carriage of the virulence plasmid. Few plasmids other than the virulence plasmid were detected among a diverse group of Typhimurium isolates, although novel plasmids were detected among other serotypes. DNA sequencing of novel plasmids isolated from *Salmonella* groups C1 and H revealed genetic similarities to two newly characterized *Salmonella* plasmids and identified novel conjugation systems. While transposons and colicin genes were identified, no antibiotic or heavy metal resistance genes were identified. While some serotypes may not exhibit the propensity to harbor multiple plasmids within their genome, other *Salmonella* isolates have acquired unique plasmids from unknown bacterial hosts. These results indicate that there are unknown genetic barriers to plasmid acquisition in some salmonellae.

INDEX WORDS: *Salmonella* Typhimurium, plasmid distribution, antibiotic resistance

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B.S., Saint Francis University, 2003

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GA

2009

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December 2009

DEDICATION

This thesis is dedicated to my parents and sister for their never-ending love and support throughout my journey in life.

AKNOWLEDGEMENTS

First and foremost I would like to thank Dr. Adriana Pedroso who provided both assistance and guidance in making decisions regarding the routine experiments for my research. She always answered my copious amounts of questions with a smile, and attempted to keep me sane during stressful time periods.

I would also like to thank Dr. Margie D. Lee for giving me this opportunity. Her persistent 'push' to think more scientifically, and mindset of 'never relying on kits and computer software when you have your brain' challenged me in many ways. While I originally did not favor her love of numbers and biochemistry, I now know and see the benefits of working out calculations and understanding the biochemistry basics before an experiment, in order to achieve good-quality results. I cannot thank her enough for her expert advice and knowledge, as it has made me both a better student and scientist then when arrived in Georgia two years ago.

I would like to thank Dr. Anne Summers and Joy Wireman for teaching me the basics of the plasmid isolation technique that became the foundation of my research. To the remaining members of my committee, Dr. Susan Sanchez and Dr. John J. Maurer, I would like to say thank you for their continuous guidance and support throughout my master's program.

I would also like to thank my undergraduate research students: Katherine Miller, William Waters, and Iliane Miranda. Mentoring these individuals posed a new challenge of identifying a balance between 'teacher' and 'friend', but also brought the reward of instilling knowledge in another individual.

I would also like to thank my parents, sister, and friends for always believing in me. Without their constant encouragement, motivation and love, I would not have made it this far!

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INTRODUCTION

Salmonella is one of the leading causes of foodborne illnesses. Of 16, 614 cases of foodborne disease, *Salmonella* was associated with 6, 471 cases followed by *Campylobacter* (5, 655), *Shigella* (2, 078), and *Cryptosporidium* (1, 313) (68). Virulence of *Salmonella* is mediated by genes encoded by pathogenicity islands; however some serotypes of *Salmonella,* including Typhimurium, Dublin, Enteritidis, Choleraesuis, Abortus-ovis, and Gallinarum-Pullorum harbor large-molecular weight plasmids that contribute to virulence and occasionally antibiotic resistance (30, 73). The virulence plasmids are heterogeneous in size, ranging from 50-258 kb resulting from deletions in specific regions; however, a 7.8 kb *Salmonella* plasmid virulence locus is conserved among the serovars (71, 73).

The literature suggests that virulence plasmid containing serotypes tend not to contain other large molecular weight plasmids, including resistance plasmids. For example, *Salmonella* Typhimurium DT104, which contained the virulence plasmid, expressed resistance to five different classes of antibiotics, rather than acquiring an R-plasmid; the resistance genes were all located on a transposon within the chromosome. Gebreyes and Altier described two pentaresistant phenotypes in their study of 484 *S.* Typhimurium isolates. One of the phenotypes, which comprised about half of the isolates was DT104. The second pentaresistant phenotype composed of a group of isolates which contained plasmids ranging from 70-140 kb (8). Only two isolates had more than one plasmid. Restriction enzyme maps appeared to indicate that these plasmids were different from the virulence plasmid. These results suggest that the virulence plasmid may affect acquisition of antibiotic resistance.

Antibiotic resistance genes may also become incorporated into the virulence plasmid. Some *Salmonella* Typhimurium isolates have been shown to harbor a self-transferable virulence/resistance plasmid accountable for multi-drug resistance (35). Isolates were shown to harbor a large molecular weight plasmid of approximately 140 kb, composed of 95 kb virulence plasmid with an insertion resulting from integration of a multidrug resistance plasmid. It is hypothesized that these types of virulence/resistance plasmids found in other *Salmonella* serotypes such as Choleraesuis are the result of a recombination-transposition process between the virulence and resistance plasmids (18, 35). As a result, the typical virulence plasmid sizes of Typhimurium and Choleraesuis become larger in size due to the insertion of a large transposable element containing the resistance locus.

It is unclear why the virulence plasmid affects the acquisition of other large molecular weight plasmids if they are of another incompatibility group. As there is limited information regarding the diversity and distribution of plasmids in *Salmonella,* the purpose of this study is to detect and characterize the diversity of large molecular weight plasmids present within serovars from diverse animal hosts., as well as to determine if the distribution of large molecular weight plasmids is affected by the carriage of the virulence plasmid when using Typhimurium as a test strain.

Chapter 1: *Salmonella*

Salmonella is one of the leading foodborne pathogens, causing roughly 1.4 million foodborne illnesses annually (21). With 40,000 reported cases each year in the United States alone (21), the number of *Salmonella* outbreaks has continued to increase significantly over time. This escalation is the result of several factors, including agricultural and food distribution methods, an increase in the consumption of raw and slightly cooked foods, as well as a decline in the public health infrastructure (22). Between the years 1998-2002, *Salmonella* was accountable for the largest portion of outbreaks reported to the CDC, followed by *E.coli*, *Shigella, Clostridium*, and *S. aureus* (52). In 2005, the most prevalent *Salmonella* serotypes from human sources consisted of Typhimurium (19.3%), Enteritidis (18.6%), and Newport (9.1%) (92).

Salmonella are gram-negative, facultative, motile rod shaped bacteria belonging to the *Enterobacteriaceae* family (21). Identified and cultured in the late 1880s, *Salmonella* strains were first distinguished and differentiated based on serologic identification of lipopolysaccharide somatic (O) and flagellar (H) antigens. With each serotype considered to be a separate species of the bacteria, researchers were faced with approximately 2,500 species of *Salmonella* (12). With the progression of DNA-DNA hybridization and small subunit RNA gene sequencing, scientists created a new taxonomy consisting of only two species: *S. enterica* and *S. bongori*. The first species is further divided into six subspecies on the basis of genomic relatedness and biochemical differences. Subspecies are further

characterized into serotypes distinguished by the antigenic formula of O antigens and the presence of both or either phase of H antigens (21).

The bacterial species are adept in causing a variety of diseases and symptoms including gastroenteritis, enteric fever and bacteremia or septicemia. Gastroenteritis is the most common of *Salmonella* infections, and can be caused by approximately 2,000 various serotypes of the bacteria. However, during a specific period of time, roughly ten serotypes may account for the bulk of infections and habitually include Typhimurium, Heidelberg and Enteritidis (68). Transmission of the bacteria to humans may occur in an assortment of manners including consumption of contaminated food, animal-to-human or human-to-human transmission. The most common sources of foodborne *Salmonella* in 2005 involved restaurant food, with poultry being the most common food source (60) Although substantial outbreaks of the bacteria have also been caused by produce milk, marijuana, and most recently, peanut butter. For example, in 1985, Illinois and surrounding states' residents suffered from one of the largest outbreaks of a *S.* Typhimurium strain capable of surviving the high temperatures of pasteurization. Surveys and hospital logs reported over 200,000 affected individuals, yielding one of the largest recorded outbreaks of the bacteria in the United States (72).

Pathogenic strains of *S.* Typhimurium inject proteins into the cells lining the intestinal wall via a Type III secretion system; this disturbance triggers an inflammatory response which signals for polymorphonuclear leukocytes (PMNs) to the area. The neutrophils, eosinophils and basophils release a compound that increases the adenylate cyclase activity within the mucosal cells, causing an inhibition of sodium uptake and an increase of chlorine

secretion (21). The metabolic changes are related to a loss of water from the cells and increase of water in the lumen of the intestine, a typical trademark of diarrhea. Other symptoms of gastroenteritis include abdominal pain, vomiting, headache, fever and muscle aches (21).

Enteric fever, also referred to as typhoid fever, is the result of host-adapted *Salmonella* serotypes. Examples may include Dublin in cattle, Pullorum in birds, as well as Typhi and Paratyphi in humans (4). After initial ingestion of the bacteria, *Salmonella* will invade lymphoid tissue within the intestine and replicate within the macrophages for an extended incubation period ranging from one week to month. After initiating an inflammatory response in the lymph nodes and intestinal tissues, the bacterial cells proliferate within the spleen and liver and bacteria are then released into the bloodstream. As the bacteria travels from the spleen to the gall bladder where cells are shed in bile back into the intestine, individuals may experience characteristic symptoms including a high fever, anorexia, chills, and convulsions. Individuals affected by typhoid fever may die from the result of infection or become severely ill and remain colonized after recovery; however others may be infected and never experience symptoms (4).

Bacteremia, or septicemia, are common forms of *Salmonella* disease associated with severe gastroenteritis. Septicemica, defined as viable bacterial cells proliferating within the blood, is most often caused by the swine-adapted *S.* Choleraesuis and bovine-adapted *S.* Dublin (74). This form of pathogenesis is often lethal due to bacterial endotoxins being released into the bloodstream, resulting in septic shock. Observed symptoms include a high

spiking fever, a rapid heart rate, a lowered blood pressure and difficulties in blood clotting leading to red marks on the skin (74).

Prior to invasion of the host cell, *Salmonella* bacteria must first encounter and attach to one or more of the various cell types within the intestinal tissue. This form of tropism entails a number of fimbriae and pili including type I (Fim), plasmid-encoded (PE), thin aggregative (curli), and long polar (LP) fimbriae (21). Type I fimbriae are classified as peritrichous, ranging in size up to 1.1μm long and are encoded by the *fimAICDHF* operon encoded on the chromosome (21, 49). While the tissue binding specificity has not been determined, type I fimbriae expressly bind to α-D-mannose receptors on eukaryotic host cells (63). Plasmid-encoded fimbriae genes are located on the virulence plasmids of four *Salmonella* serotypes: Typhimurium, Choleraesuis, Enteritidis, and Paratyphi C (21). While other genes located on the plasmids, including *spv* and *rck* are involved in the virulence of the bacteria, experiments have demonstrated that mutants unable to produce plasmid encoded fimbriae are capable of adhering to and invading intestinal cells as efficiently as the wild type strain. For example, comparison of an *S.* Typhimurium wild type strain and a mutational inactivated *pefC* strain administered intragastrically in mice resulted in only a two-fold difference in LD_{50} indicating that *pef*-mediated adhesion was not fundamental in the bacteria's virulence in a mouse typhoid model (5)

Thin aggregative fimbriae, SE17, were identified from an *S.* Enteritidis strain and described as flexible fimbriae of 3-4nm in diameter. While the appendages morphologically resemble curli and thin fimbriae located on other bacteria such as *E.coli* (22). Expression of the *agf* genes, located within the locus encoding Typhimurium curli, influences the colonial

morphology of the bacteria to form a rigid multicellular network. This phenotype may be observed when *Salmonella* bacteria are grown under stressful environmental conditions, including low temperature or osmolarity, or starvation (21). The *Salmonella* thin fimbriae may aid in the attachment of the pathogen to the villi of enterocytes, but also cause the bacteria to attach to one another in a phenomenon referred to as autoaggregation (44). While the exact purpose of this occurrence has yet to be determined, researchers hypothesize that the autoaggregation may aid in the survival of *Salmonella* cells encountering hostile environmental conditions or host physiological barriers (21).

The long polar fimbriae *lpfABCDE* operon in *S.* Typhimurium mediates the adhesion and colonization of bacterial cells to the Peyer's patches of the small intestine in typhoid (5, 21). *In vivo* experiments have demonstrated that mutations in the long polar fimbriae locus, specifically *lpfC* results in a decrease in the number of bacterial cells present in the Peyer's patches when compared to the *S.* Typhimurium wild type strain (5). This data suggests that the *lpf* mutation causes a deficiency early on during an infection. Homologs of *lpf* have also been identified in *E.coli* K12 signaling the possibility of horizontal gene transfer of the locus. *lpf* genes have not been identified in enteropathogenic or enterotoxigenic strains of *E.coli* and have also appeared to either been lost or not acquired in other *Salmonella* serotypes including Typhi and Arizona (21).

Invasion of host epithelial cells by pathogenic *Salmonella* occurs in a phagocytic manner termed "ruffling" (5). The bacteria trigger actin rearrangements in the epithelial cells of the intestine, as well as macrophages. This reorganization forms pseudopods that ultimately engulf the bacterial cells. While the cell surface of the epithelial cells are able to recover

from the invasion of *S.* Typhimurium, macrophage cells undergo apoptosis and are destroyed shortly after coming into contact with the bacteria (5).

Apart from the virulence plasmid large portion of *Salmonella's* virulence genes are encoded by chromosomal pathogenicity islands. The best characterized pathogenicity island of *Salmonella* is SPI-1, a 40 kb region that contains approximately 33 genes that aid in the ability of the bacteria to invade host epithelial cells (21, 33, 63). *inv, spa, prg,* and *org* genes located within SPI-1 are involved with the production of a type III secretion system, in which a needle apparatus secretes effector proteins that stimulate the engulfment of the bacteria by the host cell. Studies have shown that strains harboring mutations in genes encoding for the type III secretion system or effector proteins result in diminished invasion and attenuation (21, 33).

Also located on SPI-1 are secreted effector proteins and their chaperonins. Genes include *sptP*, encoding a protein tyrosine phosphatase that is translocated into the host's cell cytoplasm where the enzyme aids in altering the response of the mucosal cells to outside stimuli (21). Assisting in the translocation of SptP are putative chaperones encoded by genes *sicP, sipB*, *sipC*, and *sipD* which are also injected by the type III secretion system in *Salmonella*. Injected proteins SopE and SipA are considered to be involved in the ruffling phenomenon, as *sopE* mutants did not produce similar changes in the host cell surface as the wild-type *Salmonella* strain (21). An additional property of SPI-1 proteins, such as SipB, includes stimulation of apoptosis in infected macrophages (21, 33).

SPI-1 also carries regulator genes for the virulence of *Salmonella*. While little is known regarding the mechanism by which the transcriptional activators SirA and HilA regulate

invF, researchers have suggested the InvF protein regulates the expression of the translocation *sip* genes. The HilA protein is also responsible for the expression of the type III secretion system of SPI-1, and is therefore involved in the invasion of the bacteria into host cells (21). The gene is activated by SirA, a *Salmonella* invasion regulator located outside the pathogenicity island. Expression of *hilA* is also affected by the two-component regulatory system encoded by *phoPQ*. From both *in vitro* and *in vivo* studies, researchers have concluded the genes located on SPI-1 to be involved in the early stages of pathogenic infection as mutations eliminating the expression of SPI-1 genes had no effect on the lethal dosage in mice (33).

Salmonella enterica serotypes, but not *Salmonella bongori*, also harbor a second 40 kb pathogenicity island, deemed SPI-2, which is required for survival of the bacterial cells within macrophages (63). The genes are organized into four operons based on regulatory, structural and effector/chaperon functions (85). Similar to SPI-1, the second pathogenicity island also contains genes for a type III secretion system, designated Spi-Ssa; however, this system does not operate by injecting proteins into the host cell, but instead is used by the bacteria to prevent phagosome-lysosome fusion (32). Genes including *sseE, sseF,* and *sseG* have been hypothesized to encode effectors of the Spi-Spa system, as sequence similarity to known effectors of the type III secretion system present in pathogenic *E.coli* strains have been shown (32). Strains harboring mutations within *sseF* and *sseG* exhibit only modest defects in virulence in typhoid models; however mutations in these specific genes have demonstrated to be defective in Sif production in host epithelial cells indicating that the genes are not a requirement for virulence, but instead may play a role in membrane dynamics (40, 85).

The SPI-2 pathogenicity island also encodes for a putative two-component system, SsrA-SsrB, which regulates the type III secretion system of this pathogenicity island. The first gene of the SpiR operon, *ssrA,* contains two predicted transmembrane domains hypothesized to be involved in sensing host signals. *ssrB*, the second gene within the two component regulatory system, is a response regulator with an identified receiver domain (28, 51). The expression of the SsrA-SsrB operon is regulated by a second two-component system, OmpR-EnvZ, as OmpR binds directly to the *ssrAB* promoter region (28, 51). Mutations within the *ompR* gene result in an avirulent strain within the typhoid model, as the absence of the protein does not allow *Salmonella* to survive and replicate within the macrophages. Studies have shown that several environmental factors, including alterations in pH and osmolarity, induce *in vitro* expression of SPI-2 genes regulated by the two-component systems in both *E.coli* and *Salmonella* species (28, 51).While further experimental testing is required, one model predicts that there are two levels of *ssrA* regulation: EnvZ senses the variations of nutrients within the vacuole environment, to which OmpR responds in order to regulate the second two-component system. SsrA may then act as a second detection system of environmental stimuli within the cell, allowing *Salmonella* to quickly respond to changes within its surroundings (51).

Among the first effector genes identified and characterized from the SPI-2 locus was *spiC*, a gene encoding a small acidic protein. *In vitro* experimental evidence has demonstrated the protein's role in inhibiting endosome fusion; a strain containing a mutation of *spiC* led to a greater percentage of vesicles undergoing fusion, as well as a decline in virulence and survival of *Salmonella* within macrophages (32, 40, 85). A second function of the protein is to inhibit normal cellular trafficking of internalized molecules, which may be

identified as proteolysis of an internalized ligand. In one particular study, macrophages infected by wild-type *Salmonella* showed strong trafficking inhibition, whereas heat-killed bacterial cells produced no inhibition (84).

In contrast to the abundance of information regarding the genes located on SPI-1 and SPI-2, the virulence functions of the remaining *Salmonella* pathogenicity islands are less well characterized. A third pathogenicity island approximately 17 kb in size, SPI-3, carries the *mgtCB* operon encoding for the putative virulence protein MgtC, which regulates the adaptation to low Mg2+ environments. The *mtg*C gene is cotranscribed with a high-affinity Mg2+ transporter gene, *mgtB*, by the PhoP-PhoQ regulatory system (6). In addition to the *mgtCB* operon, SPI-3 encodes for at least 10 genes. MarT is a predicted outer membrane protein that has homology to an *E.coli* K12 protein, and similarity in its N-terminal domain to ToxR, transmembrane regulatory protein required for the synthesis of toxin in *V. cholera.* The MisL protein consists of an N-terminal signal sequence required for translocating the protein across the inner membrane and domains responsible for membrane insertion and secretion (6). The remaining predicted membrane proteins showed no sequence similarity to proteins of known function in the database.

SPI-4 is a 24 kb pathogenicity island which harbors six genes encoding for a type I secretion system, in addition to a large repetitive protein, SiiE. Studies have demonstrated that this particular protein is involved in intestinal colonization of calves (31, 53). Secretion of the protein SiiE requires SiiC, a TolC homolog, the periplasmic adaptor SiiD, and finally SiiF an inner membrane ATPase (53). SPI-4 genes are activated through a complex system involving SPI-1 genes *sirA, hildD, hilC*, and *hilA.* Binding upstream of *siiA*, HilA promotes

the expression of SPI-4 genes through xenogeneic silencing with H-NS, a nucleoid-associated protein that represses sequences horizontally acquired by the cell (53).

The fifth pathogenicity island is 7.6kb in size and encodes effector proteins for both of the type III secretion systems observed in SPI-1 and SPI-2 (65). Specifically, SPI-5 contains the translocated effector SigD/SopB for the SPI-1 secretion system, which promotes 'ruffling' and invasion of the host cell (48). A second protein, PipB identified on SPI-5 is a translocated protein for the TTSS of SPI-2 which induces the production of filaments and is essential in the bacteria's ability to survive and replicate within macrophages. As with the previous five pathogenicity islands, the G+C composition of SPI-5 is considerably lower when compared to the genome of *S.* Enterica; this in combination with the finding that many of the pathogenicity islands reside near tRNA genes, leads researchers to hypothesize that the pathogenicity islands are the result of horizontal gene transfer throughout the evolution of *Salmonella* (33, 41, 48).

Salmonella species also contain the lysogenic phages, Gifsy-1 and Gifsy-2 that promote and facilitate virulence (7). Located within Gifsy-1 are genes *gipA* and *gogB,* which are required for the proliferation and survival of bacterial cells within the Peyer's patches. Experiments with Typhimurium have demonstrated strains with a deletion of Gifsy-1 showed slight attenuation in mice models; however the effects were dependent not only the strain background, but also on the means of inoculation. These results strongly differ from experiments in which Gifsy-2 was deleted; in this circumstance, strains became significantly attenuated in both oral and intraperitoneal modes of administration in the mouse model (7).The second lysogenic phage contains two *sod* genes that code for a superoxide dismutase,

which aids and protects bacterial cells from the oxidative burst of macrophages by, converting toxic superoxide molecules into the less toxic peroxide (7). While experiments in which *sodC1* was either mutated or deleted led to results of attenuation in mouse models, studies have revealed strains harboring a *sodC2* deletion to be unaffected in their virulence and ability to infect the host (7). Also present on the Gifsy-2 phage is an antivirulence gene, *grvA*; disruption of this particular locus causes the serotype Typhimurium to become more virulent (42).

Salmonella, like other bacterial species, must face a number of various obstacles and hindrances both in its natural environment and within the host. After being shed in the feces, *Salmonella* cells must overcome a temperature downshift, as well as fluctuations in pH and osmolarity (25). Once the bacteria have re-entered a host through consumption of contaminated food or water, the *Salmonella* species must survive the low pH of the stomach. Traveling into the intestine, bacterial cells face not only low oxygen levels and volatile fatty acids, but also an increased osmolarity, intestinal bile salts and competition from the normal microflora for nutrients and space (25). In order to combat such challenges and increase the chance of survival, *Salmonella* species have acquired and maintained genes and regulation circuitry for stress-response systems.

Acidic pH is one of the most common stress conditions faced by the neutralophilic *Salmonella* organism. To survive exposure to varying pH levels within the host animal's stomach, intestine and phagocytic cells, *Salmonella* has developed several mechanisms including inducible systems for acid resistance, as well as an adaptive acid tolerance response (ATR) (25, 90). Experiments have demonstrated two types of ATR's within the bacteria; the

first, a log phase ATR, may be demonstrated in cells whose growth is interrupted by a two-step transition in pH. Proteins induced after the initial shift to a pH of 5.8, referred to as a pre-acid shock, function to stimulate a homeostatis that maintains an internal pH when housekeeping homeostasis systems fall short. The second change to a pH less than or equal to 4.5 provokes the generation of approximately 43 proteins whose primary roles are to prevent and repair acid damage to cells (90). The second ATR involves stationary-phase cells experiencing a pH shift to 4.3; in this instance, only 15 proteins are synthesized to provide resistance to an acidic environment (25, 90). The alternate sigma factor, σ^S appears to play an essential role within the log-phase ATR in *Salmonella* as mutants were capable of inducing a slight acid tolerance response; however, the mutants were unable to produce the level of ATR achieved by the wild-type that expressed *rpoS* (25). Also dependent on σ^S is the ability of acid-shocked cells to produce a cross-protection against other stresses including oxidative, osmotic and thermal.

A second frequent stress encountered by *Salmonella* species is carbon, nitrogen, and phosphate starvation. The starvation-stress response, or SSR, refers to the physiologically and genetic changes the bacterial cells undergo upon reduction of an energy or nutrient source (25, 79). The SSR may be divided into individual stress responses for each of the three nutrients, which involves both unique and overlapping sets of genes. Starvation-inducible loci (*sti)*, refer to genes induced by two or more of the starvation conditions, whereas genes stimulated solely by a single starvation are given specific terms, such as carbon starvation induced (25). The regulation of the SSR involves a complex system involving the primary regulators cAMP receptor protein, CRP and the alternate sigma factor, σ^S (79). Regulation control by CRP may be either positive or negative, depending upon the

specific condition. For instance, the complex positively regulates phase 1 genes including two transport proteins (*csiA, csiG*), and carbon compound catabolic enzymes (*aldB, gabD, fadF*). It has been suggested the positive regulation of such proteins may be involved in a starvation stress avoidance tactic, in which the bacterial cells scavenge and utilize all available nutrients (79). The alternative negative regulation of survival genes, such as *stiA, stiB,* and *stiC,* by CRP is hypothesized to be essential for long-term starvation survival. The second component in the regulatory system, σ^S, is crucial not only for long-term survival methods, but also once more for cross-resistance to other environmental stresses. RpoS positively regulates the starvation survival genes *stiA* and *stiC*, as well as the virulence operon *spvABCD* during nutrient starvation. However, negative regulation of genes *csiG, csiM*, and *stiB* may be the result of the synthesis of a repressor protein as cells transition into the starvation-induced stationary phase (25). During carbon and nitrogen starvation, *sti* and *csi* genes trigger a *relA*-dependent production of guanosine tetraphosphate (ppGpp), as well as a stringent-like response. Studies have shown that ppGpp serves as a positive regulator and is involved with σ^S expression of genes (25, 79).

Salmonella species, as well as other pathogenic bacteria, may encounter a third obstacle of oxidative stress from aerobic metabolism, environmental agents, or the oxidative burst of phagocytes. These reactive oxygen species have been shown to cause damage not only to DNA and RNA, but also proteins and lipids (23, 25). The bacteria's ability to combat oxidative stress relies on two distinct stimulons: peroxide stress response and superoxide stress response. When exposed to lethal doses of peroxide, the cellular concentrations of approximately 30 proteins becomes elevated (23). A trivial number of these genes, roughly nine in Typhimurium, are positively regulated by the OxyR locus. OxyR acts as a

transcriptional activator of genes including *katG*, a peroxide-destroying enzyme catalase, a glutathione reductase, *gorA*, and a NADPH-dependent alkyl hydroperoxidase, *ahpFC* (25). The role of *katG*, along with *katE*, is to convert hydrogen peroxide to water and oxygen, while researchers predict *gorA* prevents oxidative stress by maintaining a reduced state of cellular proteins. *ahpFC* operates by converting lipid hydroperoxides into their corresponding nontoxic alcohols (25). To sense, and therefore regulate the peroxide stress response, OxyR binds to specific sites near the target genes and senses stress by reversible oxidation. Once the regulator becomes oxidized, OxyR undergoes a conformational change which is assumed to reposition a region of the protein to stimulate RNA polymerase; this in turn transcriptionally activates *katG* and *ahp*. (25). Similarly, when bacterial cells are challenged with high levels of superoxides, a second stimulon with distinct proteins is invoked. Several of the genes involved with the superoxide stress response are regulated by the products of two specific genes *soxR* and *soxS* (23). The protein SoxR perceives oxidative stress signals and activates the transcription of *soxS*. The increased levels of SoxS stimulate the transcription of several SoxRS regulon genes including *sodA,* a manganese-containing superoxide dismutase, and the DNA-repair enzyme endonuclease IV, *nfo*. Additional genes regulated by SoxRS consist of a glucose-6-phosphate dehydrogenase, *zwf,* and *micF*, an antisense RNA that suppresses the synthesis of the OmpF outer membrane protein (25). This particular response is crucial to the survival of *Salmonella* cells as macrophages produce nitric oxide as one mechanism of killing pathogens.

The fourth common stress encountered both outside and within the host is osmotic. Variations in osmolarity triggers an increase in either the inward pressure on the cell membrane causing swelling and ultimately bursting of the cell, or outward pressure leading

to plasmolysis and dehydration(25, 45). In order for the cell to maintain both its shape and positive turgor pressure, bacteria transport compatible solutes, such as potassium, glycine-betaine, and glutamate into the cell. Proline and glycine-betaine uptake in *Salmonella* is regulated by the *proU* operon consisting of genes *proV, proW* and *proX*. While a great deal of information is still required to understand this system, studies have shown *proU* to be regulated by other previously mentioned stress factors, such as pH, and may be involved in DNA supercoiling (25). Genes encoding for the nonspecific porins OmpC and OmpF are inversely regulated in response to differences in osmolarity. OmpC synthesis levels increase during hypertonic conditions, while OmpF predominates under hypotonic conditions (25, 45). Regulation of this system is arbitrated by the aforementioned two –component system OmpR-EnvZ, in which EnvZ senses changes in the osmotic pressure and phosphorylates OmpR. The inverse environments in which one particular porin demonstrates an advantage allows *Salmonella* bacteria to transition between different environments (25).

In order to survive environmental stresses, including iron-deprivation and heat-shock, experienced within different environments, *Salmonella* cells must utilize the numerous and diverse virulence loci encoded in the pathogenicity islands and lysogenic bacteriophages. Collectively, the genes provide the bacteria with adhesion and invasion mechanisms, as well as defenses that allow the pathogen to proliferate within the targeted areas of the host.

Chapter 2: Plasmids

A plasmid is circular autonomously replicating DNA separate molecule (36, 38). Genetic indications of the existence of bacterial plasmids occurred in the late 1950s by the studies of J. Lederberg and W. Hayes (31). Plasmids are generally circular molecules that are smaller than the chromosome and range in size from 1.0 kb to over 500 kb (14, 44). Plasmids may be present within a single bacterial cell in either low copy numbers, in which there are only one to two per cell, or a high copy number with up to one hundred copies per cell (14, 26). The control number of plasmids is regulated by the rate of initiation of DNA synthesis. A plasmid-encoded repressor, dependent upon concentration, will decline when cells enlarge; as a result, inactive monomers form and the number of plasmid DNA molecules doubles (26). Via protein synthesis, the amount of repressor genes and the concentration will increase twofold, resulting in the formation of active monomers and the impediment of replication (26). In high copy number circumstances, a larger concentration of monomers is required before the active repressor is formed (26).

Plasmids are characterized on the basis of their form of existence and distribution mechanisms (10, 14, 38, 62). Conjugative plasmids possess the ability to promote transfer by means of pili between two bacterial cells (10, 14, 38, 62). The fertility factor, or F, plasmid was first discovered in an *Escherichia coli* K-12 strain and encodes genes responsible for plasmid transfer among bacterial cells during conjugation (10, 37, 38, 62). The F plasmid is an approximately 100 kb plasmid that may exist in bacterial cells in three distinctive forms (10, 38, 62, 66). Bacterial cells may contain autonomously replicating F plasmids which are

efficient in transferring the plasmid to a suitable recipient cell, but rarely transfer donor chromosomal DNA (66). A second form of the F plasmid is able to integrate into the donor cell chromosome to form high frequency donor cells, deemed Hfr (10, 37, 38, 62). In this circumstance, the Hfr strain is able to transfer chromosomal genes from the donor cells at a high frequency to recipient cells by conjugation (10, 37, 38, 62). F-prime plasmids may also arise from Hfr strains in a variety of methods including recombination between an insertion element flanking the integrated F plasmid, recombination between homologous chromosomal sequences flanking the F plasmid, or recombination in which there is no known mechanistic basis (10, 38, 62). The 100 kb plasmid consists of four discrete regions: two segments, *inc* and *rep* are involved in the replication of the plasmid (10, 38, 62). A third region of the plasmid is regarded as a silent region, as very little genetic functions have been demonstrated to reside in this particular area (10, 38, 62). Approximately 35 kb of the plasmid entails the *tra* genes, which allow the fertility plasmid to be self transmissible, and also encompasses the *oriT* site (10, 38, 62).

The 463 bp *oriT* region was first sequenced by Thompson et al in the early 1980s (27, 83). This locus is the site by which 'nicking' of the DNA occurs, in order to initiate transfer (27). The TraI transferase protein 'nicks' the plasmid DNA by covalently attaching the 5' end of the DNA of the plasmid, in response to an unidentified signal transpiring between genes involved with the mating pair formation and those encoding for DNA transfer (27). Succeeding steps of DNA transfer consisting of the completion of the nicking reaction, unwinding of the DNA and transfer of a single strand of DNA into a recipient cell, entails at least four *tra* genes (27).

Nicking of the plasmid DNA at the *oriT* site requires the regulatory TraJ protein, in addition to the products of *traY* and *traZ* (27). The 15.2 kDa protein encoded by *traY* is located within the cell envelope, approximately 30 kb from *traZ* protein found within the cytoplasm (34). The TraY protein has been proven to be essential not only for pilus expression, but also in the transfer of DNA as studies have demonstrated an insertional inactivation of *traY* will result in both a deficiency in pilus expression, while a substitution in the protein's promoter produces a decrease in DNA transfer to the recipient cell (26). The traY protein affects the expression of a second *tra* gene, *traM,* by two mechanisms as experimental evidence has proven that a lack of the TraY protein leads to an increase in transcription of the *traM* promoter, but a decrease in the production of the TraM protein (26). Although this specific protein is not a requisite for pilus synthesis, formation of the mating pairs or nicking of the DNA, *traM* is hypothesized to be involved with signaling for DNA transfer to begin (27, 34).

Once nicking of the intended DNA strand for transfer has occurred, a third *tra* gene aids in catalyzing a reaction in which the 5' end of the DNA covalently binds to the TraI protein (27). The highly characterized protein has been located within the cytoplasm and exhibits a DNA-dependent ATPase activity (27, 34). Before unwinding of the double strands may occur, the TraI protein must first bind to a substrate of single stranded DNA of approximately 200 nucleotides long; unwinding may then proceed in a 5' to 3' fashion with an estimated rate of 1,200bp per second (34). TraD, located within the inner membrane of F+ cells, is suggested to only play a role in pilus assembly and retraction (27); however, the gene product is not required for nicking of the DNA at the origin of transfer nor for the unwinding of the DNA (64).

The *tra* operon is positively regulated by the 27 kDa cytoplasmic protein TraJ and is transcribed from the P_Y promoter (27, 64). While the specific mechanism and requirement for the protein in expression of the *traY* promoter is vague, the t*raJ* gene is negatively regulated by the two-component inhibition system of *finO* and *finP* (2, 27, 64). Approximately 79 nucleotides long, the antisense RNA FinP is believed to block ribosomal entry by folding into two stem-loop structures, with the first stem being complementary to the initial 211 bp of the *traJ* gene (2, 27). The pairing, or coupling, of FinP and *traJ* occurs due to the formation of 'kissing complexes' between the complementary loops in the two RNAs and is stabilized by the cytoplasmic FinO protein (2, 27, 64). The RNA-binding protein recognizes duplex stems with 5' and 3' single-stranded tails at one end, and is capable of binding to several of these structures in both FinP and *traJ*, increasing the duplex formation between the two RNAs by 10-fold (2).

Transcription from the P_Y promoter not only depends on initiation by *traJ*, but also on *sera/arc*, a member of a second two-component regulation system (27). Collectively, *traJ* and *sera* open a 30 kb *traY-Z* gene block, while the product of *arc* is essential in regulating gene expression when cells become anaerobic (27). The chromosomal gene *copra* is also involved in *tra* gene expression; however the specific host cell functions remain unclear as researchers only speculate the gene to control sera activity (27).

The initial step of conjugation entails donor cells forming specific contacts and attaching to suitable recipient cells by means of an extracellular filament extending from the bacterial cell, referred to as the F pilus (27, 88). F donor cells may possess one to three flexible pili, with each consisting of a single subunit ranging in size from 1-20μm (27). While the primary

role of the pili is cell to cell interaction and DNA transfer, the filaments also provide circumstances for the attachment sites for pilus-specific bacteriophages, recognition of surface exclusion proteins leading to the disengagement of mating pairs with closely related plasmids, and the generation of signals that may trigger DNA metabolism (27, 88). The F-pilus is assembled from an inner membrane pool of 11 *tra* and several *trb* proteins (54). Assembly of the pilus tip structure is encoded by TraA, -L, -E, -K, -C, and –G, while TraB, -V, -W, -F, and –H are involved with pilus extension (54). The extended pilus structure is stabilized by TraP, whereas TrbI initiates pilus retraction once DNA has been transferred to the recipient cell (54).

Production of the F pilus itself requires the products of three *tra* genes: *traA, traQ,* and *traX* (27). The 13 kDa *traA* product, originally identified by Minkley et al in 1976, is comprised of the pilin subunit, as well as a 121 amino acid propilin polypeptide signal sequence (27, 54, 56). Mutations in *traA* affecting both the F pilus synthesis and functions, such as the addition or deletion of charged residues to the hydrophobic molecule, have been identified in the signal sequence and the coding region (27). Manchak and colleagues identified one particular 'class' of mutations which affected the stability of the F pilin by influencing the insertion of the propilin in the inner membrane, while a second class of mutations decreases mating stability by affecting the pilus tip's assembly (54). TraQ, a 94 amino acid inner membrane protein, is essential for pilin maturation and is required for the accurate insertion of propilin into the membrane (27, 54). Bacterial cells with mutations in the *traQ* gene are not only unable to synthesize pilin, but also elaborate F pili resulting in transfer deficiency (27). *TraQ* also encodes for a pilin-specific chaperone, which prevents the accumulation of pilin in the inner membrane of the cell (54). The highly conserved *traX*

sequence among F-like plasmids is predicted to encode for a 248 amino acid integral inner membrane protein that is responsible for the acetylation of the propilin at the N-terminus (27).

To limit, or reduce, conjugation among cells carrying identical or closely related plasmids, a 'barrier' known as surface exclusion occurs (27, 88). Five surface exclusion groups have been identified in IncF plasmids with examples including F, R100, ColB4, and R124 (24). Surface exclusion is dependent on the presence of two *tra* proteins: TraT and TraS, and the specificity for each exclusion group may be due to partial functional differences in TraT as well as the noninterchangeability of the TraS productions (27, 88). The mechanism of action of the cytoplasmic membrane protein, TraS, is to block a signal transduced from the recipient to the donor cell in order to prevent donor DNA synthesis and transfer (27). The precise role of the 25 kDa outer membrane TraT protein in surface exclusion is somewhat unclear; however the general perception of the gene product's function is to reduce or block the initial steps in mating-pair formation (88). The TraT gene product also has been demonstrated to increase serum resistance in bacteria and therefore contribute to the virulence of the organism (27). For instance, Rhen and colleagues in 1988 investigated this hypothesis within the mouse liver and discovered that the TraT protein increased serum resistance in *S. typhimurium*. (27, 69, 80).

The occurrence of surface exclusion is quite distinct from a second plasmid control mechanism, referred to as plasmid incompatibility. Plasmid inheritance in a bacterial cell depends not only on the effective synchrony with particular host functions, but also in the operation and execution of a set of plasmid-coded regulatory elements regulating replication

and partitioning (61). While it is possible for different types of plasmids or episomes, a portion of genetic material capable of existing either independently or integrating into the chromosome, to coexist within a bacterial cell, plasmid incompatibility occurs when closely related plasmids cannot be stably inherited and maintained (27, 61). Identical or similar plasmids are recognized by the proteins that regulate plasmid replication. Once one of the plasmids has been replicated, the regulatory system prevents further replication rounds and the second plasmid, deemed incompatible, is eliminated from the cell line (61). The second instance in which a plasmid is lost from a cell line involves accurate partitioning of plasmids to the daughter cells. Again, if two plasmids are identified as incompatible, only one plasmid will be equally divided between the daughter cells, while the second plasmid will be randomly segregated into the daughter cells (61). Incompatibility may therefore be either symmetric, in which either of the co-residing plasmids is lost with equal probability, or vectorial, in which one plasmid is lost completely or with a higher probability than the other (61).

Plasmids may also encode a diverse array of virulence factors, including toxins, pili and type III secretion systems, which allow the bacterial host to survive in adverse environments and compete more successfully with other microorganisms (20, 22). Virulence plasmids have been identified in a number of bacteria, including enteroinvasive *E.coli*, *Salmonella*, *Yersinia* and *Staphylococcus*. Virulence plasmids often tend to possess a modular structure, with clusters of virulence genes, replication genes and transfer-related genes differing in G+C content from the neighboring cluster (20). These types of plasmids frequently contain the remnants of small mobile elements including insertion sequences and transposons, as well as a large percentage of open reading frames encoding putative proteins with unknown

functions (20). While the precise origin of virulence plasmids remains a mystery, there are several theories regarding how plasmids acquire virulence genes. For instance, it is likely that insertion sequences and transposons facilitated the acquisition of the virulence genes from chromosomal loci, or that various virulence genes were introduced into plasmids by means of co-integration between plasmids and lysogenic phages carrying the genes. An additional mechanism by which plasmids may obtain virulence genes includes lysogenic phages losing the ability to integrate and are therefore maintained as a plasmid (20).

A second type of plasmid capable of spreading by conjugation harbors genes that code for enzymes capable of destroying or modifying antibiotics (10, 37, 38, 62). The drug-resistance plasmid, or R plasmid, was first identified from *Shigella dysenteriae,* but resistance plasmids have been found in many different bacterial species (26). Generally, resistance plasmids consist of genes that regulate DNA replication and copy number, frequently transfer genes as well, and the R determinant, which encodes the genes for antibiotic resistance against such drugs as β-lactams, aminoglycosides, chloramphenicol, tetracyclines and sulfonamides (10, 26, 38, 62). R plasmids may contain multiple resistance genes, which may be located within a transposon or integron (26). Once cells acquire an R plasmid, the plasmid may be transferred to other bacterial cells rapidly through various means including conjugation, transduction and transformation (10, 37, 38, 62). Thus a significant portion of bacterial populations can become resistant to the same antibiotics through acquisition of the same plasmid. The use of antibiotics can impose a strong selective pressure on bacterial populations (10, 38, 45, 62). Thus despite the efforts to control the emergence and spread of multi-drug resistant bacteria, the situation continues to worsen as it

appears that extensive drug treatment, both in humans and animals promotes the development and spread of antibiotic resistant strains (10, 38, 62, 77).

Colicinogenic, or Col, plasmids encode bacteriocidal proteins referred to as colicins, (13). The low molecular weight Col plasmids carries mobilization factors that allow them to transfer during conjugation regulated by high molecular weight plasmids (13, 26). The toxic proteins encoded by Col plasmids, are produced by *E.coli* and other related bacteria to prevent the growth of a closely related strain that does not contain a Col plasmid (10, 26, 38, 62). Colicins share a distinct genetic structure comprised of three genes: a colicin, a lysis, and an immunity gene (10, 38, 62). The colicin protein is organized into four functional domains, with the translocation domain at the N-terminus and immunity binding domain at the C-terminus (13). The central domain, comprising approximately 50% of the protein, is involved in receptor binding and the killing domain (13). Several types of colicins exist, with each having a particular mode of inhibiting susceptible cells. For instance, colicin B and Ib damage the cytoplasmic membrane of sensitive cells, while colicin E2 degrades DNA (10). The lysis proteins of the colicin assist in the release of the toxins through the cytoplasmic membrane (13). While this protein is primarily a main component of the colicin protein, several colicins including Ia, Ib, and M contain no lysis-like protein, while others such as colicin V contain two lysis proteins aiding in the export of the protein (86). The third gene of the 'colicin cluster', the immunity protein is more divergent in amino acid sequences than both the colicin and lysis proteins (13). However, the function of conferring specific immunity to the corresponding colicin remains constant, and occurs by means of high affinity binding of the immunity protein to the C-terminus of the colicin protein (13).

The existence of plasmids, whether self-transmissible through conjugation systems or dependent on other elements for transmission, play an important role in bacteria. Plasmids may harbor genes that benefit the bacteria by destroying other bacterial cells by means of bacteriocins, or providing more pathogenicity to invade and resist host defenses. Still the acquisition of other plasmid genes give bacterial strains a competitive advantage against antimicrobial agents, or the ability to degrade metabolic substances including toluene or sugars.

Chapter 3: *Salmonella* Plasmids

Among *Salmonella* species, plasmids have been found ranging in size from 2 to larger than 200 kb (17, 73). While some serovars such as Typhi, Paratyphi, and Infantis tend to lack plasmids, some such as Typhimurium, Enteritidis, Choleraesuis, Gallinarum, Sendai, and Abortus-ovis contain a virulence plasmid, which varies in size among the serovars (17). For instance, Choleraesuis and Enteritidis harbor plasmids of 50 and 60 kb respectively, while pSLT of Typhimurium is 94.7 kb and outweighed by the 285 kb pSSV for *S.* Sendai (17).

In spite of the differences in sizes, three main regions contribute to the replication and stable maintenance of the virulence plasmids in *Salmonella*: *parAB*, IncFIB, and IncFIIA (73). The *parVP* locus is comprised of two partitioning systems; one requiring the proteins ParA and ParB with the *parS* locus, while the second system entails the *incR* locus and ParA only (71). The *parVP* locus assists in the control of plasmid segregation during cell division, as well as incompatibility functions in the virulence plasmids of *S.* Typhimurium, *S.* Enteritidis, and *S.* Choleraesuis (71, 73). Nucleotide sequences of the IncFIIA replication protein, RepA, demonstrate a 98-99% similarity among the serovars Dublin, Typhimurium and Choleraesuis (17, 71). The *repC* replicon of pSEV in Enteritidis shares a 98% DNA similarity with the IncFIB of the Typhimurium virulence plasmid pSLT, and comprises a set of interons which implement stringent control of the plasmids' copy number (71). Interons within the *incC* locus of the IncFIB replicon in Typhimurium and Enteritidis correspond to the *rsk* sequence, a gene whose expression imparts serum resistance for the bacteria (71, 73).

Salmonella plasmids confer the virulence phenotype by harboring a 7.8 kb region, called *spv* (71). The *spv* operon is well conserved among the serovars and consists of a positive transcriptional regulator, *spvR*, and four structural genes *spvABCD* (17, 24, 71, 73). The *spvR* gene is located upstream of the *spvABCD* genes and binds to the operator region of *spvA* in a two-step manner to activate transcription of the operon (36, 71). Expression of the *spv* locus is also regulated by the *rpoS* gene that encodes for the alternative sigma factor σ^s (17, 71). The alternative sigma factor is induced during stationary phase, and activates the transcription of a number of genes involved in survival and resistance to environmental stresses such as low pH and oxidation (36). During early exponential growth, little to no expression of the *spvABCD* genes are detected, and synthesis of the 28 kDA outer membrane protein SpvA occurs during the transition from late-logarithmic to early stationary phase (36, 71). The remaining Spv proteins are detected later on, with full expression occurring shortly before growth stops completely (30). Expression of the *spv* locus is also dependent upon nutrient limitations, such as carbon starvation, nitrogen, phosphate, or iron limitations, and an increase in temperature (17, 36, 71).

Although *Salmonella* virulence plasmids were identified as non-conjugative, a recent study determined that the Typhimurium plasmid pSLT was self-transmissible in both liquid matings and filter-matings (1). The virulence plasmids of Typhimurium, Enteritidis, and Gallinarum-Pullorm harbor an F or F-like origin of transfer, which allows mobilization by an F or F-like conjugative plasmid (17, 71). Some virulence plasmids seem to contain a more or less complete *tra* operon, while others including those harbored by Enteritidis, Dublin, and Choleraesuis have suffered major deletions (71). The pSEV differs from the virulence plasmid of Typhimurium by two deletions; the most significant, a 22 kb deletion including

*tra*B, -K, -E, -L, -Y, -J, and –M (71). It has been suggested that the presence of the *tra* operon, whether fully complete or partial in the virulence plasmids signifies that a *Salmonella* ancestor acquired the virulence plasmid through conjugation, and throughout evolution, divergence has occurred among the serovars (17, 71).

Based on sequence similarity of the previously mentioned genes among the virulence plasmids of *S.* Typhimurium, *S.* Choleraesuis, *S.* Enteritidis, and *S.* Gallinarum, Rylick and colleagues have conceived a theory that the ancestral virulence plasmid was the IncFIIA replicon with the *spv* locus and a carbonic anhydrase, *mig-5* as all *Salmonella* virulence plasmids contain such genes. *Tra* genes located on the ancestral virulence plasmid allowed for conjugation, which ultimately led to fusion with a second replicon, IncFIB (73). However, the insertion of the IncFIB occurred only in plasmids currently identified in the serovars Typhimurium, Enteritidis and Choleraesuis, as *S*. Dublin and *S.* Gallinarum do not harbor the origin of replication for IncFIB (73). Throughout evolution, *tra* genes and the *rck* locus were lost in several serovars' plasmids to produce the current virulence plasmids of *Salmonella.*

Large multidrug resistance plasmids, such as that found in *S*. Newport, are hypothesized to possess mobile antibiotic resistance genes that transfer between the plasmids and the chromosome (73). However, in *Salmonella* serotypes, such as Typhimurium, these high molecular weight plasmids are found less frequently in virulence plasmid-containing isolates. If Typhimurium isolates contain the serovar-specific 95 kb virulence plasmid, studies have shown that the isolate may harbor additional low molecular weight plasmids of up to 20 kb (73). In 1991, Rivera et al demonstrated that 11 of 18 Typhimurium strains that

contained the serovar specific virulence plasmid also contained several low molecular weight plasmids carrying antibiotic resistance genes. Plasmid profiles of the isolates showed similar plasmids of 1.6 kb in four isolates, as well as a plasmid of 3.0 kb in four of the isolates. Other low molecular weight plasmids isolated from the strains ranged in size of 0.86 kb, 0.92 kb, 4.1 kb, and 6.1 kb (70). *S.* Enteritidis strains containing the virulence plasmid of 60 kb also harbored small multi-drug resistance plasmids (70). In this particular study, only one Enteritidis isolate out of the 219 tested carried a conjugative R plasmids of 104 kb in addition to the virulence plasmid.

Salmonella Typhimurium isolates have also been shown to harbor a self-transferable virulence/resistance plasmid accountable for multi-drug resistance such as ampicillin, chloramphenicol-florfenicol, streptomycin, sulfonamides and tetracycline (35). Isolates were shown to harbor a large molecular weight plasmid of approximately 140 kb, as well as several small plasmids. However, the serotype specific 95 kb plasmid was not shown (35). Researchers have hypothesized that this plasmid, as well as several other types of virulence/resistance plasmids found in *Salmonella* Choleraesuis are the result of a recombination-transposition process between the virulence and resistance plasmid (18, 35). As a result, the typical 90 kb or 60 kb virulence plasmids become larger in size due to the insertion of a large transposable element containing the resistance locus. It appears that the antibiotic resistance genes in virulence plasmid-containing serotypes are located either on low molecular weight plasmids, or may be inserted into the chromosome or virulence plasmid (18, 35).

Salmonella serotypes, including Newport, which do not contain a virulence plasmid, appear to carry antibiotic resistance genes on both large and small molecular weight plasmids. In 2005, Poppe and colleagues revealed multi-drug resistance Newport isolates harboring either a 133 kb or 150 kb plasmid, in addition to smaller plasmids. Several of the Newport isolates also contained a second high molecular weight plasmid, ranging in sizes of 66 kb to 120 kb (67). This phenomenon was also illustrated in *S.* Virchow, with high molecular weight plasmids of 120 kb and 33 kb, as well as a 3.0 kb plasmid harboring resistance genes to streptomycin, kanamycin, chloramphenicol and trimethoprim-sulfamethoxazole. Experiments have also shown a strain of *S.* Bredeney, resistant to eight different antibiotics, to harbor three large molecular weight plasmids [125, 90, and 30 kb], in addition to four lower molecular weight plasmids [6.5, 1.95, 1.45, and 1.06 kb] (70). Therefore, it seems probable that the *Salmonella* virulence plasmid excludes the maintenance of additional large molecular weight plasmids carrying antibiotic resistance genes.

In addition to virulence and antibiotic resistance, *Salmonella* can also harbor plasmids that confer lactose fermentation, changes in serotype, and changes in phage susceptibility. For example, an H1 plasmid containing a lactose-positive marker, as well as resistance genes to chloramphenicol, streptomycin, sulfonamides, and tetracycline was isolated in veal cows (82). In addition a plasmid isolated from *S.* Borreze encoding a functional O-antigen biosynthetic gene cluster showed nucleotide and protein similarity to the *E.coli* ColE1 plasmid (46). Due to the complete gene cluster, mobilization of the plasmid by a broad host range conjugative plasmid pRK2013 was sufficient in altering O-serotype specificity. Furthermore, a recent study demonstrated phage type conversion when a conjugative plasmid

belonging to the incompatibility group X was transferred into Enteritidis isolates of various phage types (11).

Chapter 4: Materials and Methods

Bacterial Isolates

Salmonella isolates were acquired from culture collections archived at University of Georgia. These included *Salmonella* cultured from clinical cases by Athens Veterinary Diagnostic Lab, isolates cultured as part of surveillance efforts, and isolates acquired from colleagues at Louisiana State University and North Dakota State University. Isolates were confirmed as *Salmonella* biochemically and by *invA* PCR. (81).

Avian *Salmonella* Typhimurium (strain 934, rifampicin resistant) and *E.coli* were acquired from culturing feces of birds from an *in vivo* gene transfer study in broiler chickens at The University of Georgia. Two day old chicks were administered orally 10^6 CFU of *S.* Typhimurium 934. On day 1, one group was administered a subcutaneous injection of ceftiofur (0.1mg/chick) on the day of hatch. At day 14, several groups were administered antibiotics in their drinking water for 3 consecutive days: 200mg/gallon streptomycin or 1.4g/gallon oxytetracycline. Fresh fecal droppings were collected from the pens on a weekly basis. Before each sampling, 2' by 3' sheets of paper were laid down in each pen, seeded with chicken feed and weighed down. Papers were collected the following day in order to acquire fecal droppings. Three pools of 10 fecal samples were taken from each paper and placed into 50 ml centrifuge tubes. Fecal samples were diluted 1/10 freezer stock solution (1% peptone and 15% glycerol in water) and stored at -80C. Samples were serially diluted and antimicrobial resistant bacteria were isolated by plating onto selective media. *Salmonella* were isolated from XLT4 plates containing either 10μg/ml tetracycline, 32μg/ml

streptomycin, 25μg/ml ampicillin, or 32μg/ml florfenicol by screening plates for black, H2-S positive colonies. *Salmonella* recipient strain was enumerated by plating onto XLT containing 64μg/ml rifampin, used as a selectable marker for the strain. One hundred and ninety-four *Salmonella* were isolated and stocked. Resistant coliforms were enumerated by plating onto MacConkey agar containing either 25μg/ml ampicillin, 32μg/ml streptomycin, 10μg/ml tetracycline, 32μg/ml florfenicol by recording pink, lactose-fermenting colonies. Plates were incubated at 37C for 18-22 hours and counts were recorded. Thirteen *E.coli* isolates were used for further study.

In addition, 10 *S.* Typhimurium or *S.* monophasic Typhimurium, 4 [5], 12:i:- isolates cultured from bovine, nondomestic birds and poultry were obtained (91). *Salmonella* Typhimurium 4, [5], 12:i:- isolates were collected from several different poultry companies and dairy farms in the state of Georgia (91).

Plasmid Isolation

Cultures were grown in glass tubes to an OD_{600nm} of 0.8-1.0 in 3mL Luria Bertani broth at 37C with 220rpm shaking. Plasmid isolation, was carried out as described by Williams (89) using the CosMC Prep Tube Protocol for High and Low Copy Plasmid Purification (Agencourt Bioscience, Beverly, MA). Plasmids were separated on a 0.5% Seakem Gold agarose (Lonza; Rockland, ME) gel by electrophoresis at 5.14V/cm for 18 hours at room temperature. 10μl of BAC-Tracker Supercoiled DNA ladder (Epicenter Biotech, Madison, WS) was used as the molecular weight markers. Gels were stained with SYBR green I nucleic acid gel stain (Lonza; Rockland, ME) for 60 minutes.

invA and *spvC* detection

Isolates were tested for the presence of two virulence loci, *invA* and *spvC* using PCR primers and conditions described in *Swamy, 1996* (81). Positive control for *invA* PCR was Typhimurium isolate, 98A-3397 (43). Positive control for *spvC* PCR was *S.* Typhimurium SR11. spvC – positive PCR results were confirmed by DNA: DNA hybridization as described by *Swamy* 1996. *Salmonella* Typhimurium SR11 was used to generate the probe by amplification of *spvC* by PCR using digoxigenin-labeled nucleotides. Positive control for the hybridization was Typhimurium isolate, 98A-3397, (*Hudson, 2000)*. Negative control was *E .coli* LE392.

Restriction enzyme digest of plasmids

Salmonella plasmids were separated on a 0.5% Seakem Gold agarose gel and visualized after Sybr Green staining. Agarose bands containing plasmid of interest were excised, washed in cold 1:10 strength TE buffer pH 8.0 (10mM Tris HCl, 1mM EDTA) at 4C. The agarose band was washed with 100µl NEBuffer 4 (50mM potassium acetate, 20mM Tris-acetate, 10mM magnesium acetate, 1mM DTT), or SuRE/Cut Buffer B (10mM Tris HCl, 10mM Mg Cl_2, 100mM NaCl, 1M 2-Mercaptoethanol), and then replaced with fresh enzyme buffer with 20U of restriction enzyme *AccI* (New England Biolabs, Ipswich MA) or 1U of restriction enzyme *Hind*III (Roche; Indianapolis, IN) and incubated at 37C for 2 hours. Fragments were separated on a 0.75% Seakem Gold agarose gel (1x TAE) by gel electrophoresis at 45V for 18 hours at room temperature. 1 kb DNA Ladder Plus (Fermentas, Glen Burnie, MD), *Hind* III digest of λ DNA, and *AccI* digest of λ DNA were used as the molecular weight markers. Gels were stained with Sybr Green for 60 minutes. DNA fragment sizes were then determined using the genotyping

and DNA fragment analysis computer software Gene Profiler 4.05 by Scanalytics, Inc. (Fairfax, VA).

Antibiotic Susceptibility Testing

S. Typhimurium isolates (strain 934) from the chicken *in vivo* gene transfer study were tested for antimicrobial susceptibility to a panel of seventeen antibiotics (amikacin, amoxicillin, ampicillin, cefazoilin, cefoxitin, cefpodoxime, ceftiofur, cephalothin, chloramphenicol, enrofloxacin, gentamicin, impenem, marbofloxacin, orbifloxacin, rifampicin, tetracycline, ticarcillin, clavulanic acid, and trimethoprim) using COMEQ3F SENSITITRE plates (Trek Diagnostic Systems, England) as described by the Clinical Laboratory standards (15). Isolates were first grown on XLT4 plates and incubated over night at 37C. A single black isolated colony was chosen and re-streaked onto Blood Agar plates containing 5% sheep's blood. Bacterial cultures were suspended in ddH_20 and 10µl of suspension was transferred to 11 ml of Mueller Hinton broth. 50µl of the Mueller Hinton broth was distributed to the wells of the 96 well plate containing varying concentrations of antibiotic powder. Samples were incubated at 37C and MIC results were recorded at 18 hours

Salmonella Typhimurium and 4,[5], 12:i:- isolates were tested for antibiotic sensitivity using the Kirby-Bauer disk diffusion method. Isolates were streaked onto Mueller Hinton II Agar (BBL, MD). Antibiotic disks of ampicillin 1µg, chloramphenicol 20µg, tetracycline 30µg, streptomycin 10µg and of kanamycin 30µg (BBL, MD) were placed onto each plate. To test disk accuracy, positive control of antibiotic resistant *Salmonella* Newport and negative control of *E. coli* HB101 were used. Samples were incubated at 37C overnight and zones of inhibition were measured the following morning as described by the Clinical Laboratory standards (16)

DNA sequencing and analysis

Plasmid DNA was submitted to SymBio Corporation (Menlo Park, CA) for sequencing. Standard Sanger dideoxy sequencing conditions were used, and sequences were trimmed to Q20 quality score (99% accuracy). Contiguous sequences were assembled using the public domain sequence assembler, Phrap (www.phrap.com). Returned contiguous sequences were analyzed for both amino acid and protein similarities to known sequences on GenBank at the nucleotide and amino acid level. Sequences were aligned using Sequencher 4.5 (Gene Codes Corporation Ann Arbor, MI). Graphical images of *Salmonella* Heidelberg pSL674_91 (GenBank: CP001118) and *Salmonella* Typhi pED208 (GenBank: AF411480.1) were created using the sequence analysis program BioEdit Sequence Alignment Editor 7.0.0 (Ibis Therapeutics Carlsbad, CA) and the plasmid drawing software BVTech Plasmid (BV Tech, Inc Bellevue, WA). Sequence alignment and genetic distance of relaxase components were performed using MEGA 4 (www.megasoftware.net).

Chapter 5: Results

One of the objectives of this research was to expand the available knowledge regarding plasmid distribution and genomics, by determining the diversity of plasmids in *Salmonella* isolates from a diverse population of vertebrate hosts. In order to pursue this objective, sixty-five *Salmonella* isolates were examined, which included those cultured from avian hosts (25), reptile hosts (33), and wildlife (7). These included *S.* enterica subspecies Typhimurium, *S.* enterica subspecies arizonae, Java, Alabama, *S.* enterica subspecies houtenae, Orientalis, Thompson, and Fresno and *Salmonella* serogroups B, C1, D, E1, F, H, I, U, O and P. Thirty-five percent of the isolates (23/65) harbored at least one plasmid (Table 1). Thirty-six percent of the Typhimurium isolates harbored a single plasmid of 95 kb, identified as the virulence plasmid due to the detection of *spvC* (81). Two large molecular weight plasmids equaling 120 kb and 95 kb were present among seven isolates derived from reptile hosts belonging to group H (71%) and an unknown serogroup. The 95 kb plasmid in these isolates did not contain *spvC* virulence gene, indicating that they did not harbor the virulence plasmid commonly present in *Salmonlla* serovars Enteritidis and Typhimurium (17). Forty percent of the tested *S.* enterica subspecies arizonae isolates harbored a high molecular weight plasmid ranging in size of 120-165 kb, and one isolate contained additional plasmids of 51 kb and 17 kb. *Salmonella* serotypes Java and Rubislaw each contained a single 58 kb plasmid. Two low molecular weight plasmids <8 kb in size were detected in *Salmonella* Aderlard and Fresno. Twenty-five percent of the isolates belonging to Salmonella serogroups C1 and E1 harbored a 95 kb plasmid which did not contain *spvC*. Plasmids were

not detected in *S.* enterica subspecies houtenae (group U), or *S.* enterica subspecies I serovars Bangkolk (group P), Thompson and Lohbreogge.

Restriction enzyme giestion of isolated plasmids was used to determine genetic relatedness of plasmids using the resctriction enzymes *AccI* and *HindIII*. As seen in Figure 1, Salmonella isolated from reptile hosts contained two plasmids of 95 kb and 55 kb in size. Restriction enzyme analysis showed identical band patterns for each of the plasmids, indicating that the isolates harbored the same plasmids. Another Salmonella isolated from a reptile harbored two different plasmids, 120 kb and 95 kb as determined by RLFP analysis. Digestion of the 58 kb plasmid harbored by *S.* Java 96A-29192 produced a band pattern that was also dissimilar from the other plasmids identified. To evaluate the relatedness of these plasmids to those which have already been archived on GenBank, we downloaded sequences of *Salmonella* plasmids and determined their *AccI* restriction fragment sizes in silico. None of the plasmids harbored by our isolates showed similarity to fragment patterns of known plasmids, including Typhimurium plasmids pTPqnrS-1a (GenBank: AM746977), R64 (GenBank: AP005147), pSC101 (GenBank: X01654), Heidelberg plasmid pSL946_91 (GenBank: CP001118) and Choleraesuis plasmids pOU7519 (EU219534) and pSC138 (AY509003).

To determine the prevalence of multiple large molecular weight plasmids in Typhimurium, 14 isolates from a diverse selection of sources including bovine, poultry, and nondomestic birds were screened for the presence of plasmids (Table 1) (Figure 3). Fifty-seven percent of the isolates examined harbored a large molecular weight plasmid. Fifty percent of the Typhimurium isolates harbored the 95 kb virulence plasmid. Among these

isolates, 50% carried an additional high molecular weight plasmid. Typhimurium isolates SARA14 and 181231' CNT 1A contained a 111 kb plasmid. A plasmid of 80 kb in size was observed in Typhimurium isolate SARB66 and Monophasic isolate 124831. These results indicate that of the isolates examined, the prevalence of multiple large molecular weight plasmids within *Salmonella* isolates is low.

In order to determine whether Typhimurium would acquire additional high molecular weight plasmids, a strain was used to colonize chickens which were administered antibiotics. Table 2 shows that the average phenotype was ESBL. The parent strain, Typhimurium 934, was shown to contain a single plasmid approximately 95 kb in size The presence of a 99 kb and 95 kb were observed in 15% of isolates in Day 13, 15 (50%), and 20 (25%). Because Day 15 contained a significantly larger portion of isolates with two plasmids, these isolates became the focus of this particular study. As seen in Figure 2, the two high molecular weight plasmids harbored within the isolates occurs as a 'doublet'. Twenty percent of the isolates were obtained from chickens that received no antibiotic treatment in the *in vivo* study, while the largest percent of these isolates, 28% were obtained from chickens that received ceftiofur treatment. Animals that received tetracycline and streptomycin yielded 20% and 8% of the isolates containing two large molecular weight plasmids (Table 2).

In order to determine if the plasmids were a gel artifact, two large molecular weight plasmids, or the virulence plasmid with additions, restriction digest using the *HindIII* enzyme was performed (Figure 2). In order to investigate the potential of gel artifact, λ was digested in a plug and separated on a gel. The control revealed some artifactual waviness of the bands and broadening of the 23 kb band, which appeared to be a doublet band. The 9.4, 6.5, and 4.3 kb bands were appropriately detected; however the 2.3 and 2.0 kb bands were not.

Typhimurium pSLT exhibited the 37.8, 15.3, 12.7, 9.4 and 7.3 kb bands. The 3.7 and 3.1 kb bands were not detected. However, similar to the doublet artifact seen in the λ control, a 50 kb band was also detected and a number of more faintly stained bands were seen in the 9 kb range. These findings indicate that additional bands present in isolates, when compared to the control pSLT may be a gel artifact. When this experiment was repeated twice, similar results were found. However, all but one of the isolates tested demonstrated the same band fragments as pSLT. One of the ESBL isolates exhibited an additional 5 kb band. These results suggest that the doublet was not two large molecular weight plasmids. ESBL resistance appeared to be encoded on an element not detected by our system, which may not have been capable of detecting a 2 kb insert in the large molecular weight fragment of the virulence plasmid or in a plasmid smaller than 2 kb. However, the resistance could have been on an element inserted in the chromosome.

In order to determine whether there was a diverse population of plasmids among the microbiota, plasmid distribution among *E.coli* was observed from isolates obtained from the same chickens of the *in vivo* gene transfer study. *E.coli* isolates harbored a greater diversity of plasmids, (Figure 4) The *E.coli* isolates expressed resistance to tetracycline (78%) and ampicillin (22%), and harbored two large molecular weight plasmids of sizes 126 kb and 95 kb. Isolates resistant to streptomycin (33%) carried a large molecular weight plasmid of 126 kb, and additional low molecular weight plasmids of approximately 10 kb and 6 kb. Of the 13 isolates examined, one harbored a plasmid greater than 165 kb in size, as well as a low molecular weight plasmid of approximately 4 kb. These findings indicate that a diverse population of plasmids was present within this *E. coli* population.

In order to obtain a greater understanding of *Salmonella* plasmid genomics, DNA of the unique plasmids harbored in Salmonella reptile isolates #40 and #14 were sequenced. For the 95 kb and 55 kb plasmids harbored by isolate #40, 4799 sequences were acquired. Of these sequences, 410 were excluded due to poor quality. An additional 614 sequences were excluded as a result of BlastN showing cloning vector or bacterial host sequence. Due to the theory that the plasmids might have sequences in common with the vector, SymBio Corporation excluded only the vector sequence between the end of the sequencing primer and the point of insertion. Of the 3774 sequences remaining, with a mean 616bp length $\pm$ 266, 92.3% paired and 64.5% assembled into a contiguous sequence. Forty-nine contiguous sequences were produced, varying in lengths between 9219 bp and 1355 bp. The total amount of plasmid DNA from isolate #40 equaled 150 kb (plasmid sizes were 95 kb and 55 kb); however, the total contiguous sequence length was approximately 175 kb, indicating that non- plasmid sequences were present. Bacteriophage may account for a certain amount of the DNA as seven contiguous sequences showed similarity to bacteriophages phiK02 and PY54, as well as bacteriophage N15 (Figure 5). Twenty contiguous sequences aligned with a recently characterized *Salmonella* Heidelberg plasmid, pSL476_91 (GenBank: CP001118) with 94-99% similarity (Figure 5). Approximately 75% of the 91 kb pSL476_91 was represented, including an SOS inhibition gene, UV protection gene, conjugal transfer genes, and pilus genes involved in mating pair formation. Restriction enzyme analysis by means of *AccI* revealed variation in the restriction fragments between the 95 kb plasmid of isolate #40 and pSL476_91, indicating that the #40 plasmid exhibited some heterogenicity. Twenty-five percent of the contiguous sequences with DNA similarity to the Heidelberg plasmid pSL476_91 identified genes located in the conjugal transfer region. Gene arrangement of the

conjugation system of pSL476_91 shows similarity to previously characterized conjugation systems of plasmids on GenBank (Figure 7). Plasmid pSL476_91 from Heidelberg carries 18 *tra* genes within its conjugation system; in comparison, the sequenced conjugation systems of plasmids including *S*. flexneri R100 (GenBank: AP000342), Typhimurium R64 (GenBank: AP005137.1) and the F plasmid (GenBank: AP001918.1) carry up to 28 various *tra* genes. These results indicate that although the arrangements of conjugation system in plasmids appear similar, conjugal transfer genes may have been lost by some plasmids during evolution as a result of selection pressure.

Contiguous sequences from the plasmids of isolate #40 also showed DNA similarity to genes located within the type IV pilus operon. Sequence 40-48 had 98% similarity to *pilT*; contiguous sequences 4-24 and 40-4 had showed 99% similarity to *pilO* and *pilL* respectively. The gene arrangement of the type IV pilus operon of the Heidelberg plasmid pSL476_91 was compared to those already archived on GenBank to evaluate relatedness of the systems (Figure 9). The pilus operon of pSL476_91 is different from other described pilus operons. These results indicate again the possible effects of selection and/or environmental pressure that can occur during plasmid evolution.

Transmissible plasmids may be classified on the basis of the MOB machinery that allows them to be transmitted to other bacterial cells (28). The essential component of the MOB machinery is the relaxase, a large protein containing two or more domains that initiates and terminates the conjugative DNA process. The protein contains a relaxase domain located at the N-terminus of the protein, while the C-terminus may contain either a DNA helicase, DNA primase, or other domain of an unknown function (28). Based on amino acid sequence similarity of the relaxase domain, conjugation systems of plasmids may be divided into six

families. Among the MOB_F family is the *trwC* gene of *E. coli* plasmid R388, and the *traI* gene from plasmids R46, R1, pED208 and the F plasmid. Included within the MOB_H , family are the *traI* genes of plasmids R27, R478, R391. The *traA* genes encoded within plasmids including pTic58, p42d, and pIP501 have been identified as belonging to the MOB_Q family. MOB_P family members include the *traI* of plasmids RP4 and R751 and the *nikB* of plasmids R64, R721 and R387. Prototype relaxases among the MOB_V family include MobM of plasmid pMV158, the MobA of transposons Tn*4555*, as well as the Mob identified in plasmid pBR1. The MOB_C family includes the *traX* gene of plasmid pAD1, as well as the MobC of the mobilizable plasmid CloDF13 (28). Sequence analyses using the Conserved Domain Blast (NCBI), components of the relaxase protein were identified in two contiguous sequences from the plasmids of #40. 40-46 showed a 96% similarity at the nucleotide level to the *nikB* of plasmid R64, indicating that the contiguous sequence represents a plasmid in the MOB_P family (28). The nature of the 55 kb is likely to be revealed by the remaining non-phage sequences. The plasmid appears to be colicin encoding and contains a novel conjugation system with no DNA similarity to any other system, as well as a novel type IV pilus system.

For the 120 kb and 95 kb plasmids of isolate #14, 4,032 sequences were acquired. 236 sequences were excluded due to poor quality sequences; 40 were excluded as a result of BlastN exhibiting cloning vector or bacterial host sequence. Of the remaining 3,756 sequences averaging 768bp in length $\pm$ 224bp, 93.6% paired and 70.6% assembled into a contiguous sequence. Roughly 215 kb plasmid DNA is present within isolate #14; however the total contiguous sequence length produced was 261 kb. This difference indicates that DNA other than plasmid was present. Forty-four contiguous sequences were produced,

ranging in lengths of 14955bp to 758bp. Four contiguous sequences from isolate #14 showed DNA similarity of 93-97% with the plasmid transfer region of Typhi plasmid pED208 (GenBank: AF411480.1) (Figure 10). The 9246bp contiguous sequence 14-0 showed 95% similarity to *tra* genes, *-N, F, H,* and *G*. *traD* and nearly full sequence of the *traI* of plasmid pED208 were identified with 94% DNA similarity to contiguous sequence 14-1. Contiguous sequence 14-28, 4761bp, showed a 95% DNA similarity to a segment of the *traI* sequence of Typhi plasmid pED208. Sequence alignment by Sequencher 4.5 (Gene Codes Corporation) identified an overlap in contiguous sequences 14-1 and 14-28.

Similar to the findings in isolate #40, we detected conjugation system genes that exhibited low or no DNA similarity to known conjugation systems. This is likely to represent the second plasmid isolated in #14. Among the remaining sequences, seven transposases were identified including the ISL3 family, IS200, YhgA-like transposase, IS66, and the Tn3 family. A fimbrial usher protein with DNA similarity to the FimD protein of a *S.* Javiana strain was detected, as well as the type IV pilus protein PilQ in *Erwinia tasmaniensis.*

An interesting finding in the contiguous sequences of the plasmids from isolate #14 was the similarity of two sequences to copper/silver efflux proteins; however it is not known which of the two plasmids contains these genes (Figure 6). Contiguous sequence 14-14 showed a 69% identity similarity to the outer membrane protein CusC of *E.coli* plasmid APEC 01 (GenBank: YP_001481465.1) Contiguous sequence 14-15 had a 87% similarity and E. value of 0.0 to CusA and SilB proteins found in the chromosome of *E. coli* strain 55989 (GenBank: CU928145). Experimental evidence in 2000 identified *cusC* as a chromosomal gene whose expression is induced by copper involving the two component signal transduction system *cusRS*. CusS was identified as the membrane-bound histidine

kinase and CusR as the cytoplasmic response regulator (57). Downstream of the *cusC* gene are two large open reading frames *cusBA*; the integral membrane proteins CusA and CusB are thought to form a two-channel pump for the export of metal ions and concurrent import of protons (57). This two-channel pump has also been identified in the CusBA homolog, CzcA, to which contiguous sequence 14-15 showed 82% similarity to in *E.coli* ATCC 8739 (GenBank: ACA79039.1). The organization of the copper-responsive regulatory system CusRS has been shown to be similar to those of other operons known to encode metal efflux systems, including the *sil* locus of *S.* Typhimurium (57). The two contiguous sequences show only a slight overlap in sequence alignment when the minimum match percent is 85 and the minimum overlap is 20, indicating that the *cusC*, *cusA* and *silB* genes identified may not belong in the same operon. The contiguous sequence of 14-14 is also interesting as BlastN was unable to identify similarity with the sequence region identifying with the *cusC* gene. This infers the possibility of a novel gene within the copper/silver resistance system.

Chpater 6: Discussion

The purpose of this study was to determine how readily *Salmonella* acquires plasmids when using Typhimurium in poultry as a test system. In this study we examined isolates from an *in vivo* gene transfer study, in which chickens received various antibiotic treatments. We detected a narrow range of antibiotic resistance in isolates cultured from all groups. Because we only detected resistance to β-lactam, the prevalence of resistance and phenotype did not correlate with the antibiotics administered to the chickens. The 2006 National Antibiotic Resistance Monitoring System reports that 20% of human *Salmonella* are resistant to at least one antibiotic, with the most common antibiotic resistance to tetracycline (58). Among *Salmonella* isolated from chickens and turkeys, 42% and 71% respectively were resistant to at least one antibiotic, with tetracycline again being the most common (59). *Salmonella* frequently exhibit antibiotic resistance, indicating that resistance gene acquisition occurs at a high rate. Virulence plasmid containing Enteritidis are infrequently resistant, with a prevalence of 4% of human isolates and 1.6% in chickens (58, 59). Previous studies have shown that unlike some serotypes such as *Salmonella* Newport, Typhimurium, and Choleraesuis, tend not to harbor additional large multidrug resistant plasmids. Instead, in these serotypes antibiotic resistance genes tend to integrate into the chromosome, virulence plasmid, or small plasmids.

Our results demonstrate a lack of diversity of plasmids among *S* .Typhimurium isolates. However, novel plasmids were detected in non-virulence plasmid containing *Salmonella* serotypes. Each of these plasmids contained a conjugation system and multiple

large molecular weight plasmids coexisted in a single isolate. With one exception, this was never seen in Typhimurium. These findings suggest that some genetic feature of the virulence plasmid may suppress acquisition or maintenance of R plasmids. Such features can include plasmid incompatibility and surface exclusion, which have been shown to affect the acquisition and maintenance of plasmids. The majority of multidrug resistant plasmids are typically not of the F incompatibility group. Therefore, incompatibility is not likely to play a large role in the suppression of maintenance of antibiotic resistance plasmids in *Salmonella*. Soni et al showed that several serotypes including Typhimurium and Enteritidis were able to acquire the Newport multidrug resistance plasmid by conjugation and acquisition rates were similar among virulence and non virulence plasmid containing serotypes (78). These findings indicate that the F plasmid did not prevent acquisition by conjugation but it is unclear whether they were maintained as separate plasmids or whether the Newport plasmid had co-integrated within the virulence plasmid.

The genetic basis behind plasmid exclusion in virulence plasmid containing *Salmonella* is still unknown. *In vitro* studies have shown that additional plasmids can be acquired and maintained; however, *in vivo* and environmental factors may affect the fitness cost of maintaining additional large molecular weight plasmids and the virulence plasmid. It is also unclear why the virulence plasmid is stably maintained in serotypes such as Typhimurium when it has not been shown to be important in the colonization of the gastrointestinal system. A better understanding of its role in *Salmonella*'s lifestyle may reveal the genetic factors which underlie the acquisition of antibiotic resistance gene-encoding high molecular weight plasmids.

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Table 1: *Salmonella* isolates used in this study and their genotypes.

Isolate	Animal Host	Serotype and/ or group	Number of plasmids	Plasmid sizes [kb]	spvC
SARA 14	Unknown	Typhimurium	3	111, 95, 29	+
SARB 66	Unknown	Typhimurium	3	95, 80, 31	+
124831	Unknown	O4,5,12:i:-	3	95, 80, 31	+
99A-39744-26	Reptile	Typhimurium B	2	160, 95	-
181231'	Unknown	B O4,5,12:i:-	2	111, 95	+
SARA 4	Unknown	Typhimurium	2	95, 12	+
98A-3397	Senegal parrot	Typhimurium	1	95	+
98A-3398	Lorikeet	Typhimurium	1	95	-
97A-17878	Emu	Typhimurium B	1	95	+
98A-17535	Laughing gull	Typhimurium B	1	95	-
98A-24966-2	Cowbird	Typhimurium B	1	95	+
174904	Unknown	B O4,12:i:-	1	95	+
176833	Unknown	B O4,5,12:i:-	1	95	-
196596	Unknown	B O4:i:-	1	95	-
F32	Bovine	Typhimurium B	1	75	+
192500	Unknown	B O4:i:-	0	N/A	+
SARA 16	Unknown	Typhimurium	0	N/A	-
96-37518	Cowbird	Typhimurium	0	N/A	Not Done
96A-38567	Owl	B O4,5,12:i:-	0	N/A	Not Done
97A-9840	Parakeet	Typhimurium B	0	N/A	Not Done
97A-11201	Heron	B O4,5,12:i:-	0	N/A	Not Done
98A-7582	English sparrow	Typhimurium	0	N/A	Not Done
98A-9551	Coturnix Quail	Typhimurium B	0	N/A	Not Done
98A-12202	English sparrow	Typhimurium	0	N/A	Not Done
98A-29239	Cow bird	Typhimurium B	0	N/A	Not Done
98-33516	Goldfinch	Typhimurium	0	N/A	Not Done

#10	Frilled lizard	H	2	120, 95	Not Done
#14	Reptile	H	2	120, 95	Not Done
#15	Frilled lizard	H	2	120, 95	Not Done
#17	Frilled lizard	H	2	120, 95	Not Done
#30	Frilled lizard	H	2	120, 95	Not Done
39137	Gecko	E1	3	95, <8	Not Done
#5	Rattlesnake	E1	0	N/A	Not Done
#21	Death adder	E1	0	N/A	Not Done
2350	Key Deer	E1	0	N/A	Not Done
99A-7661	Bobcat	E1	0	N/A	Not Done
99A-7661	Bobcat	E	0	N/A	Not Done
AAVLD	Chicken	D	0	N/A	Not Done
#40	Iguana	C1	2	95, 55	-
AO-29174	Lemur	C1	0	N/A	Not Done
37947	Gecko	Thompson C1	0	N/A	Not Done
96A-24660	Swine	C	0	N/A	Not Done
96A-29192	Turkey	Java B	1	58	-
33576	Goldfinch	B	0	N/A	Not Done
47570	Parrot	B	0	N/A	Not Done
7993	Iguana	B	0	N/A	Not Done
7994	Iguana	B	0	N/A	Not Done
96A-35703	Emu	B	0	N/A	Not Done
AO5053	Laughing gull	B	0	N/A	Not Done
AO-26035	Avian	B	0	N/A	Not Done
39833	Gecko	Aderlard O	2	<8	Not Done
7992	Red Tail Hawk	Alabama D	3	95, 93, 90	-
3016	Gecko	Arizona	3	120, 51, 17	Not Done
3000	Snake	Arizona	1	165	Not Done
3010	Rattlesnake	Arizona	0	N/A	Not Done
3011	Salamander	Arizona	0	N/A	Not Done
LSU 5404	Boa	Arizona	0	N/A	Not Done
98A-31777	Emu	Anatum	0	N/A	Not Done
37849	Gecko	Bangkolk P	0	N/A	Not Done
37838	Gecko	Fresno D	2	<8	Not Done
39367	Gecko	Houten U	0	N/A	Not Done
39062	Gecko	Lohbreogge	0	N/A	Not Done
37852	Gecko	Orientalis I	0	N/A	Not Done
39360	Gecko	Rubislaw F	0	N/A	Not Done
#42	Reptile	Unknown	2	95, 55	-
9240	Rabbit	Unknown	0	N/A	Not Done

96A-21096	Boa	Unknown	0	N/A	Not Done
99A-9465	Lemur	Unknown	0	N/A	Not Done
LSU 832	Snake	Unknown	0	N/A	Not Done
LSU 7360	Lizard	Unknown	0	N/A	Not Done
AO-25624-1292	Reptile	Unknown	0	N/A	Not Done

Table 2: Typhimurium 934 isolates (n=194) from chicken colonization study

Treatment	Day	Resistance	Plasmid sizes (kb)
None (n = 48)	13 (n = 20)	Extended-Spectrum Beta-Lactamases	95 (100%); 99 (50%)
		None	95 (100%) ; 99 (7%)
	15 (n = 12)	Extended-Spectrum Beta-Lactamases	95 (100%); 99 (80%)
		None	95 (100%)
	20 (n = 16)	Extended-Spectrum Beta-Lactamases	95 (100%); 99(50%)
		None	95 (100)
Ceftiofur (n = 52)	13 (n = 16)	Extended-Spectrum Beta-Lactamases	95 (100%); 99 (27%)
		None	95 (100%); 99 (100%)
	15 (n = 16)	Extended-Spectrum Beta-Lactamases	95 (100%); 99 (54%)
		None	95 (100%)
	20 (n = 20)	Extended-Spectrum Beta-Lactamases	95 (100%); 99(38%)
		None	95 (100%); 99 (100%)
Streptomycin (n = 40)	13 (n = 14)	Extended-Spectrum Beta-Lactamases	95 (100%)
		None	95 (100%)
	15 (n = 5)	Extended-Spectrum Beta-Lactamases	95 (100%); 99(50%)
		None	95 (100%)
	20 (n = 21)	Extended-Spectrum Beta-Lactamases	95 (100%)
		Gentamicin	95 (100%); 99 (100%)
		None	95 (100%); 99 (20%)
Tetracycline (n = 54)	13 (n = 15)	Extended-Spectrum Beta-Lactamases	95 (100%); 99(11%)
		None	95 (100%)
	15 (n = 18)	Extended-Spectrum Beta-Lactamases	95 (100%); 99 (67%)
		None	95 (100%); 99(11%)
	20 (n = 21)	Extended-Spectrum Beta-Lactamases	95 (100%); 99(45%)
		None	95 (100%); 99 (10%)

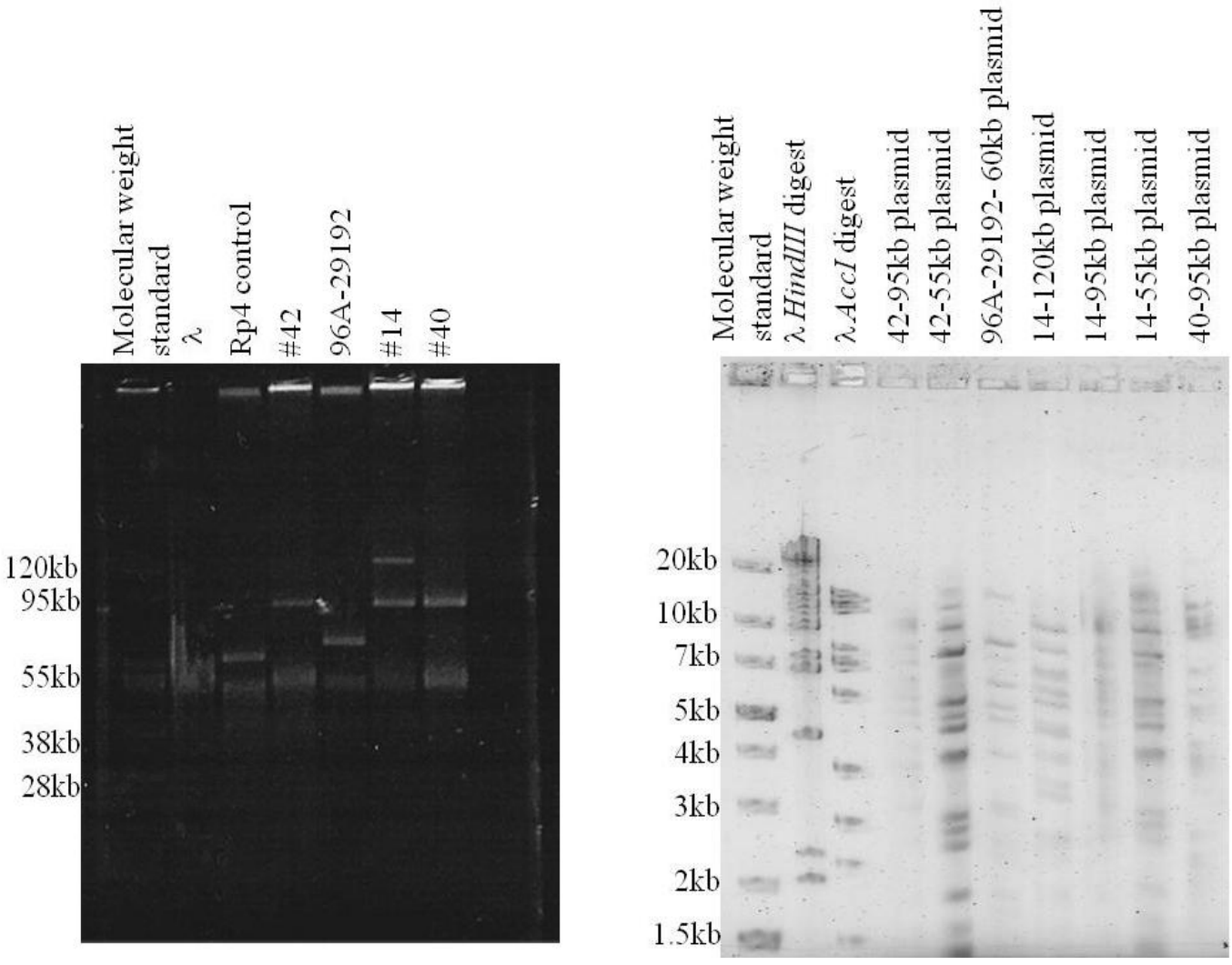
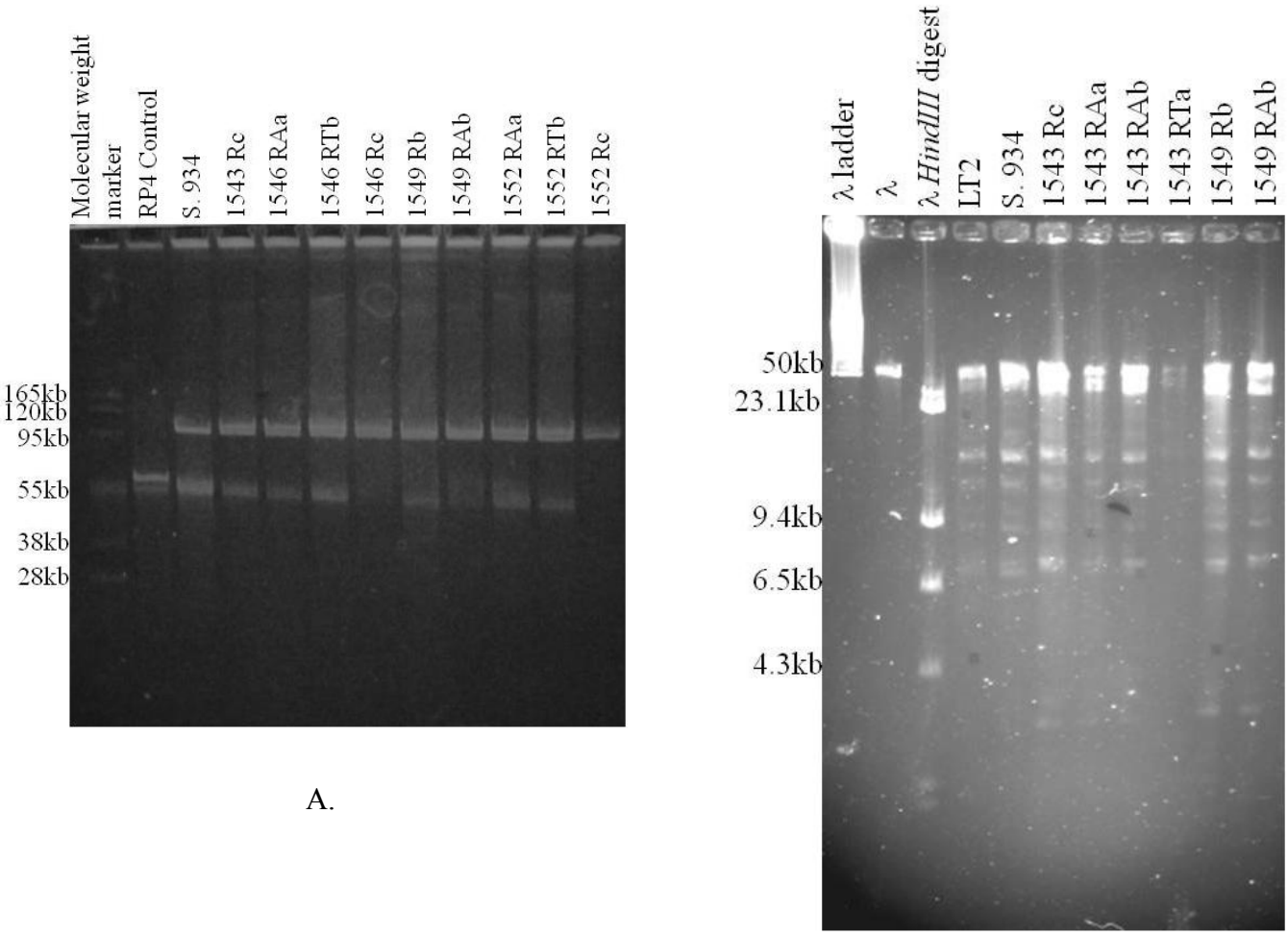


Figure 1: Unique large molecular weight plasmids detected from *Salmonella* isolates. (panel A). Restriction enzyme digestion using *Acc*I was used to determine genetic relatedness of plasmids (panel B). Two plasmids [95, 55kb] plasmids were identified in isolate #42 (panel A lane 4). Restriction digestion band pattern for the 95kb plasmid is similar to band pattern for isolate #40 (panel B lanes 4 and 10) but different from the 95kb isolated from #14 (panel B lanes 4 and 8). Restriction digestion band patterns for plasmids isolated from #14 (panel B lanes 7 and 8) were different, indicating the presence of two plasmids.



A.

B.

Figure 2: Detection of two high molecular weight plasmids (99, 95kb) harbored within Typhimurium isolates from a chicken colonization study (panel A). Characterization of acquired plasmids by restriction enzyme digestion analysis using *Hind*III enzyme (panel B). 194 isolates from the colonization study were screened; although some isolates showed a single plasmid band that was the same size as the virulence plasmid, a large majority exhibited a second band approximately 99kb (panel A lanes 4-9). 934 control (lane 5) and isolates from colonization experiment (lanes 6-11) exhibited the same restriction digestion band pattern as the virulence plasmid (lane 4), indicating a second plasmid was not present.

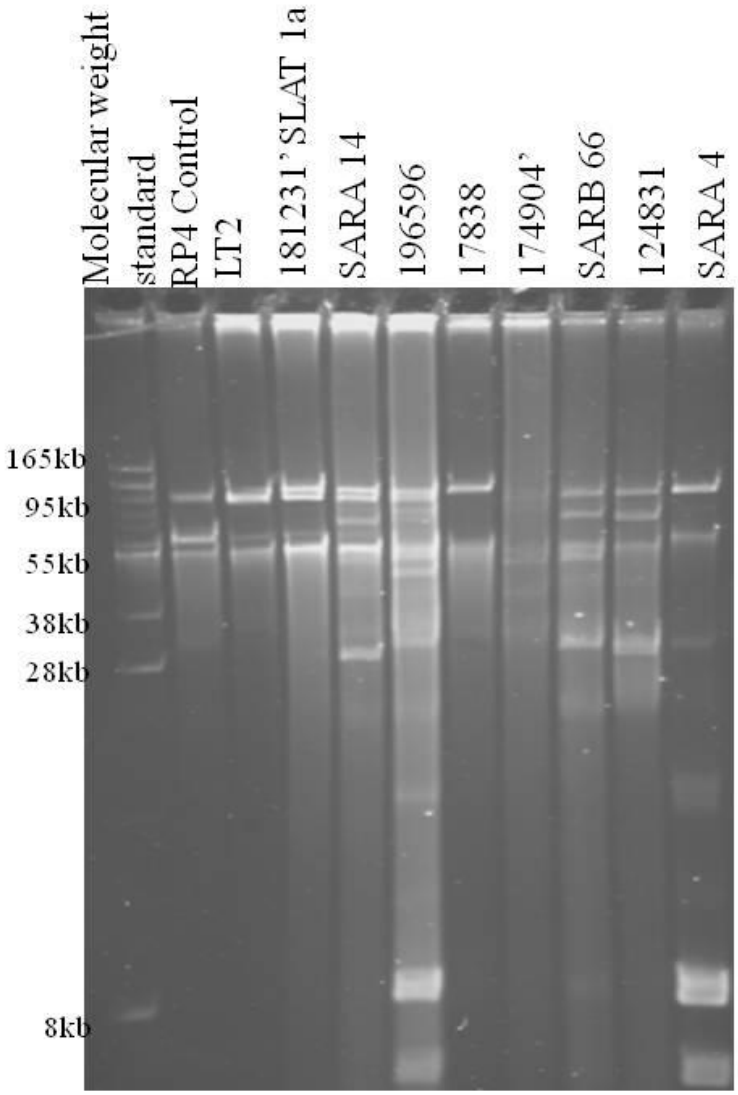


Figure 3: Distribution of plasmids detected in *Salmonella* Typhimurium isolates from diverse hosts. Fifty percent of isolates harbored the 95kb virulence plasmid and among these, 50% carried an additional high molecular weight band.

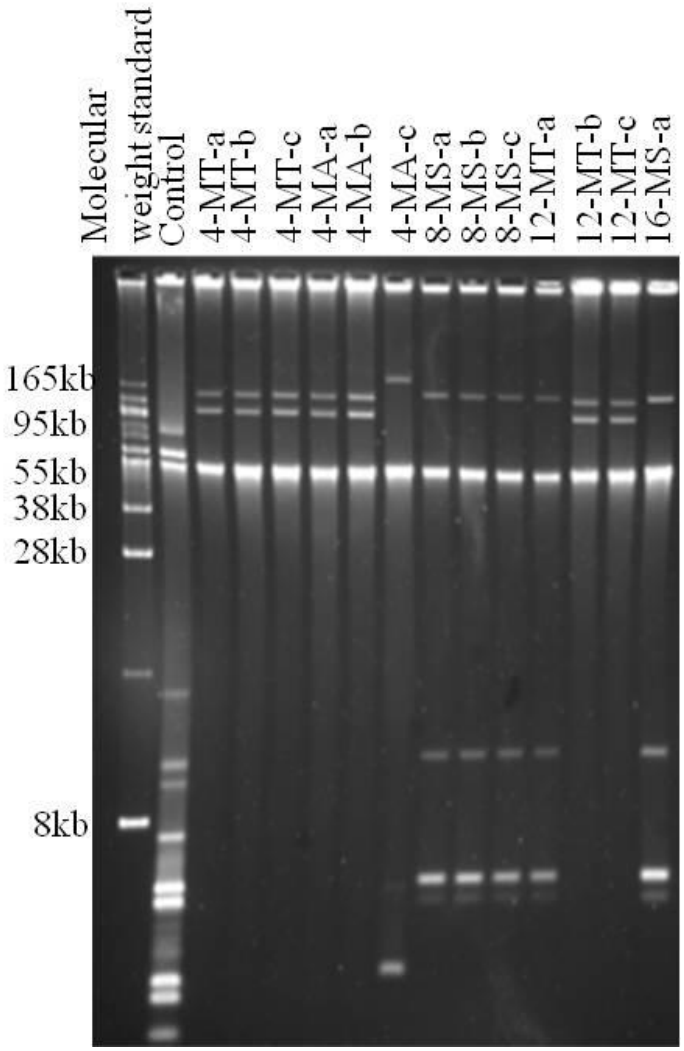


Figure 4: Plasmids detected in *E. coli* isolates acquired from chicken colonization study to determine presence of a diverse population of plasmids among the microbiota. Large molecular weight plasmids of sizes 126kb and 95kb were observed in *E. coli* isolates expressing resistance to tetracycline and ampicillin (lanes 3-7; 13-14). Isolates resistant to streptomycin carried a large plasmid of 126kb in size and additional low molecular weight plasmids of 10kb and 6kb (lanes 9-12).

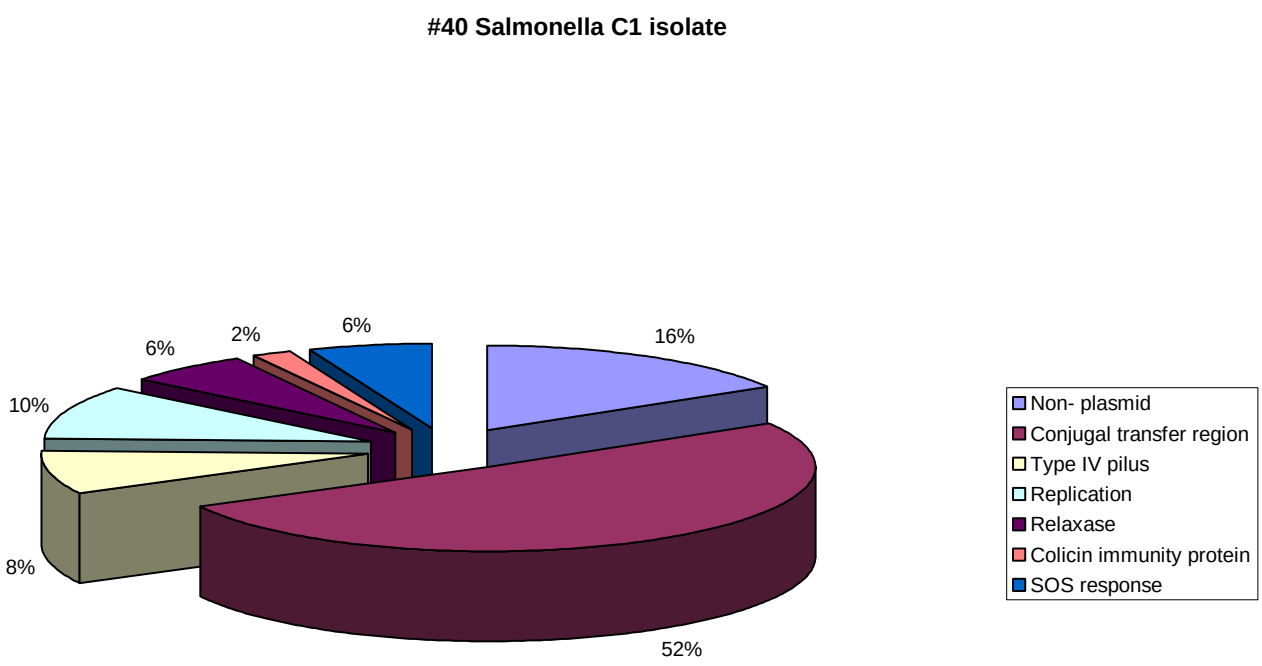


Figure 5: Annotation of DNA sequences of plasmids from #40 *Salmonella* C1 isolate from an iguana. Forty-none contiguous sequences were produced varying in lengths between 9219bp and 1355bp. The total amount of plasmid DNA equaled 150kb (plasmid sizes of 95kb and 55kb); however the total contiguous sequence length was 175kb, indicating that non-plasmid sequences were present.

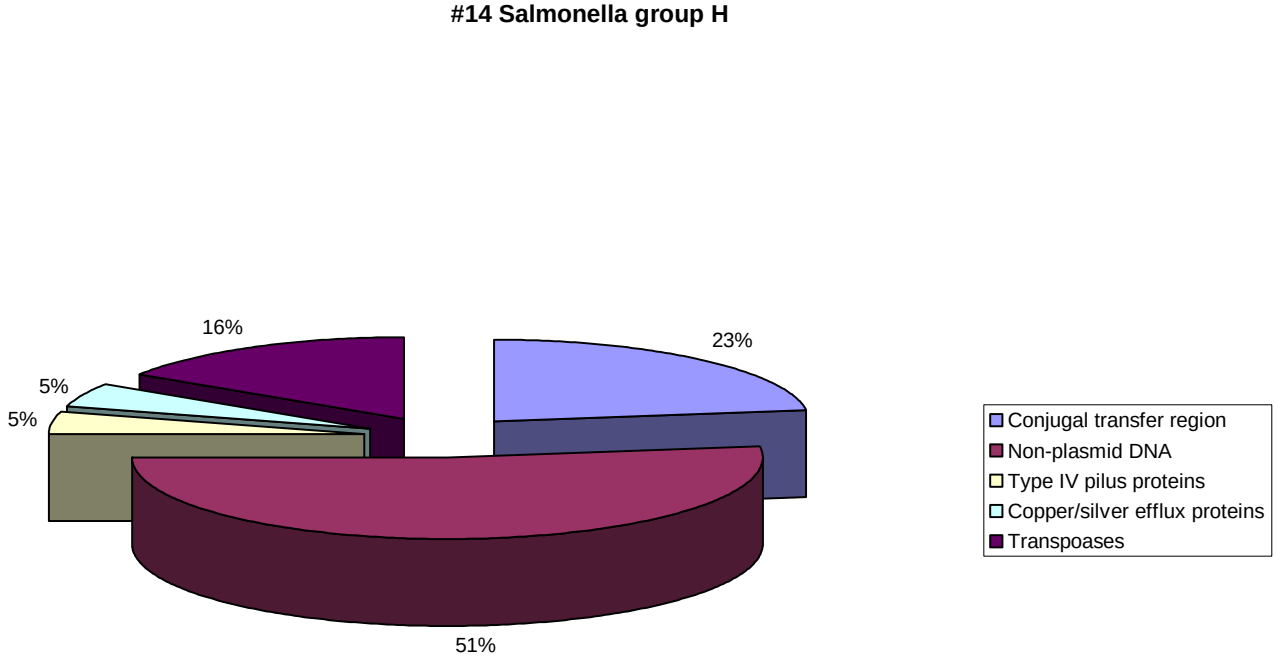


Figure 6: Annotation of DNA sequence of plasmids from #14 *Salmonella* group H isolate from a reptile. Forty-four contiguous sequences were produced, averaging in length of 768bp ± 224bp. The total contiguous sequence length produced was 261kb with plasmid DNA accounting for roughly 215kb, indicating a small amount of non-plasmid DNA.

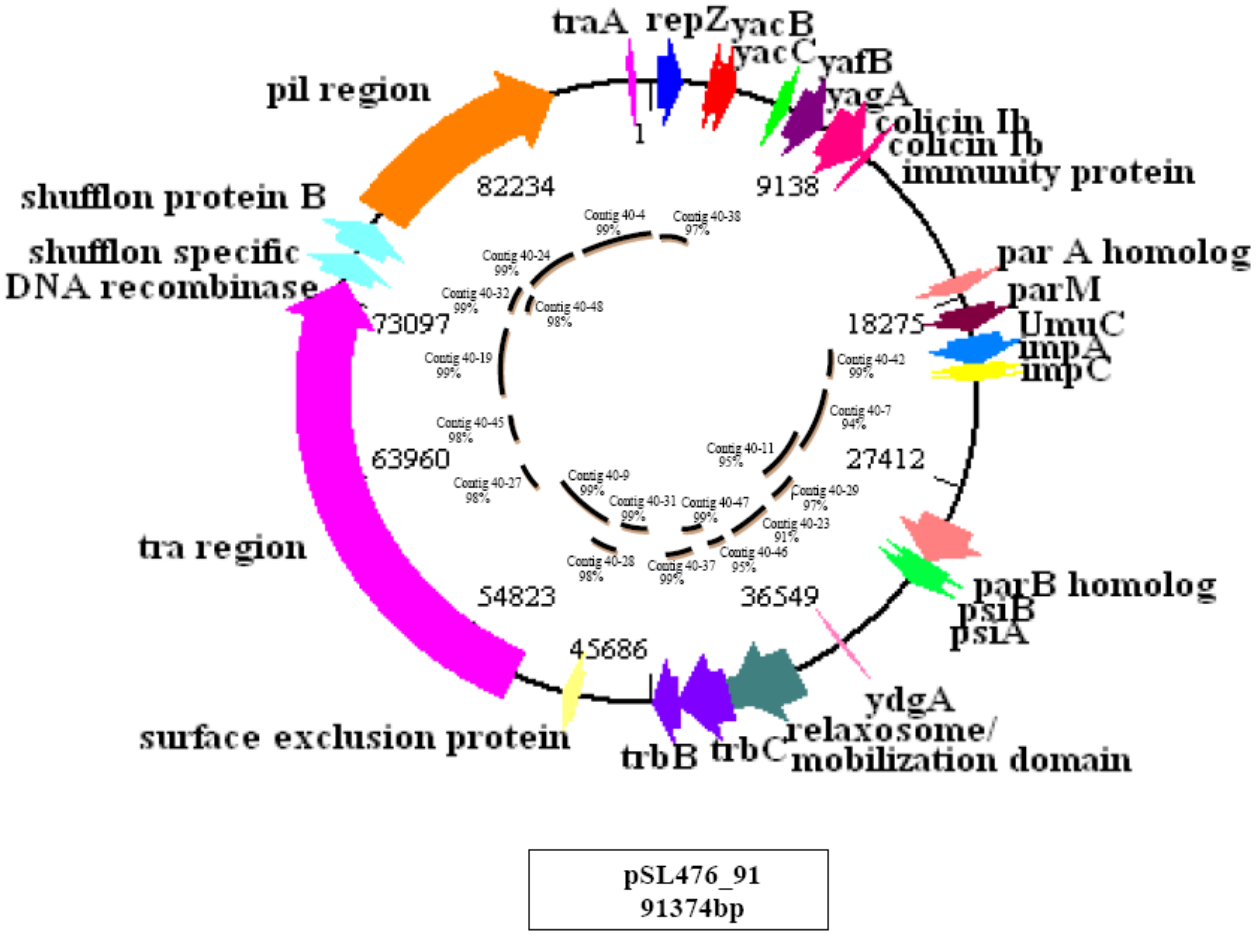


Figure 7: DNA similarity of a plasmid isolated from C1 isolate #40 to *Salmonella Heidelberg* pSL476_91. A large portion of the contiguous sequences represented the *tra* genes which are responsible for transmission of the plasmid to other bacterial cells by conjugation. This includes genes encoding the pilus which mediates attachment to bacterial cells for plasmid transfer.

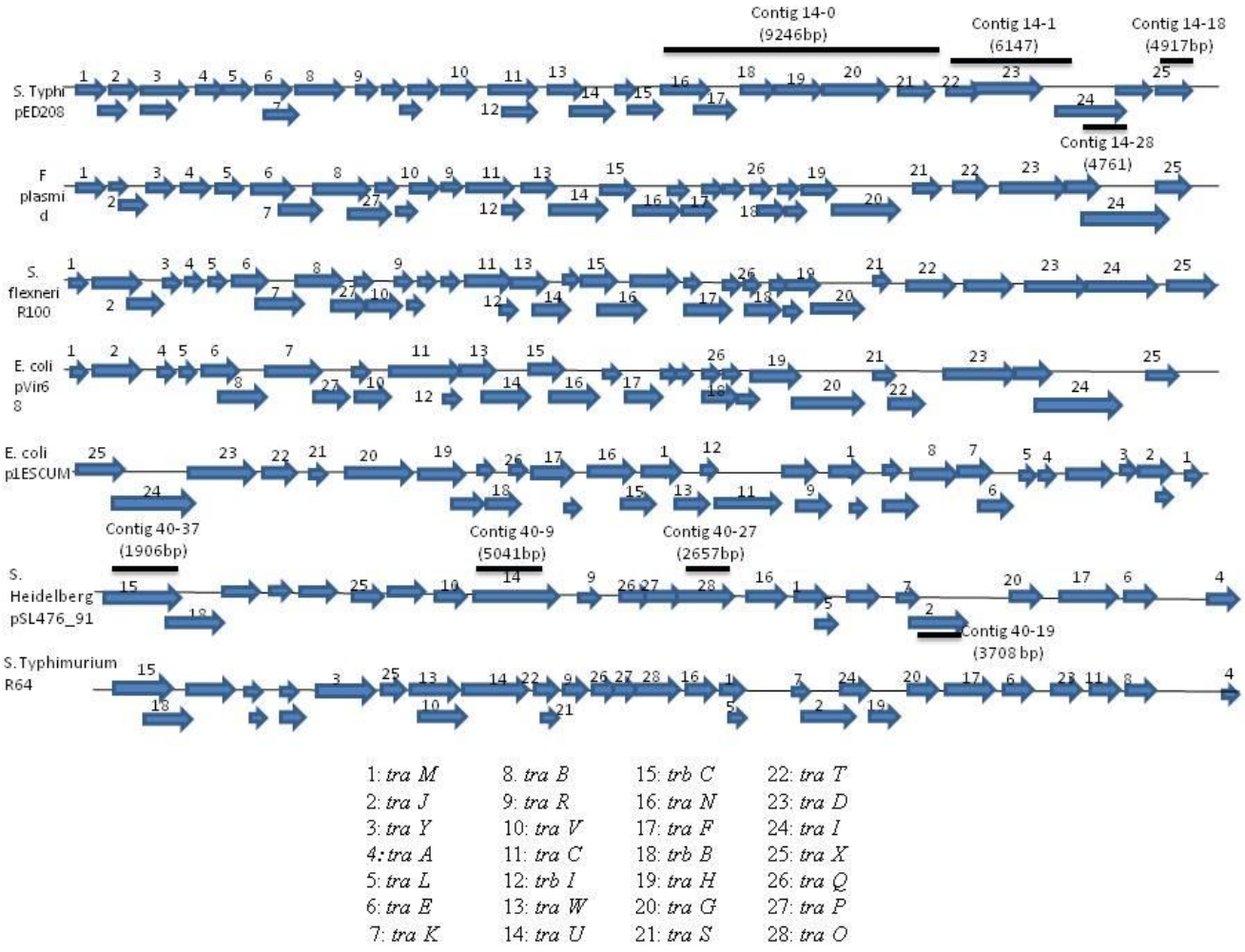
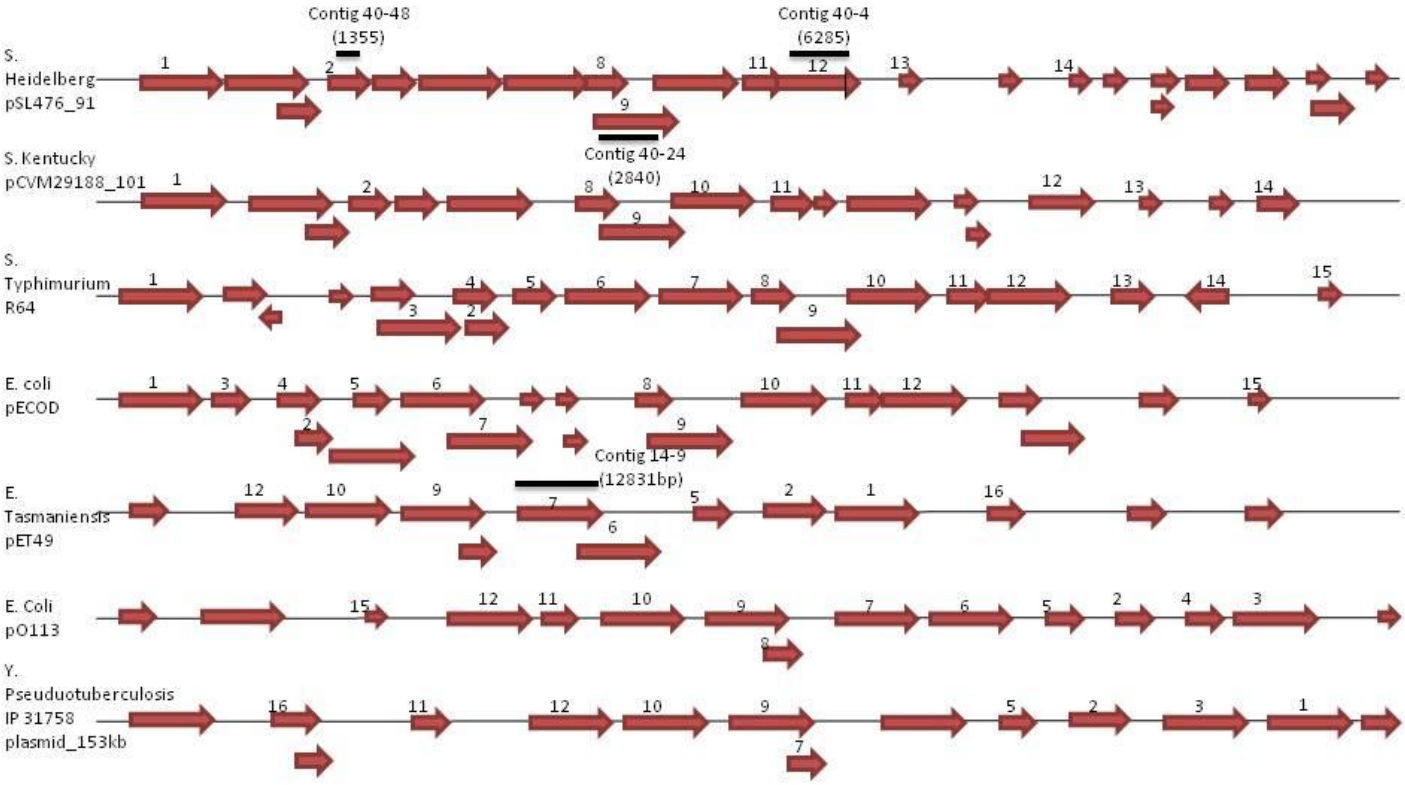


Figure 8: Gene arrangements of plasmid conjugation systems illustrating loss of *tra* genes during plasmid evolution. Four contiguous sequences obtained from the plasmid harbored by isolate #14 produced approximately 25kb of contiguous similarity to the tra region of Typhi plasmid pED208. In contrast, four sequences obtained from the plasmid harbored by isolate #40 consisted of approximately 13kb of noncontiguous similarity to the Heidelberg plasmid pSL476.



1: shufflon protein
2: *pil T*
3: *pil V*
4: *pil U*
5: *pil S*
6: *pil R*
7: *pil Q*
8: *pil P*
9: *pil O*
10: *pil N*
11: *pil M*
12: *pil L*
13: *pil K*
14: *pil J*
15: *pil I*
16: *pil D*

Figure 9: Gene arrangements of type IV pilus operons characterized among bacteria. Three contiguous sequences obtained from the plasmid harbored by isolate #40 contained similarity to the *pilT, O,* and *L* loci of Heidelberg while one large contiguous sequence from isolate #14 contained similarity to *pilQ* from *Erwinia*.

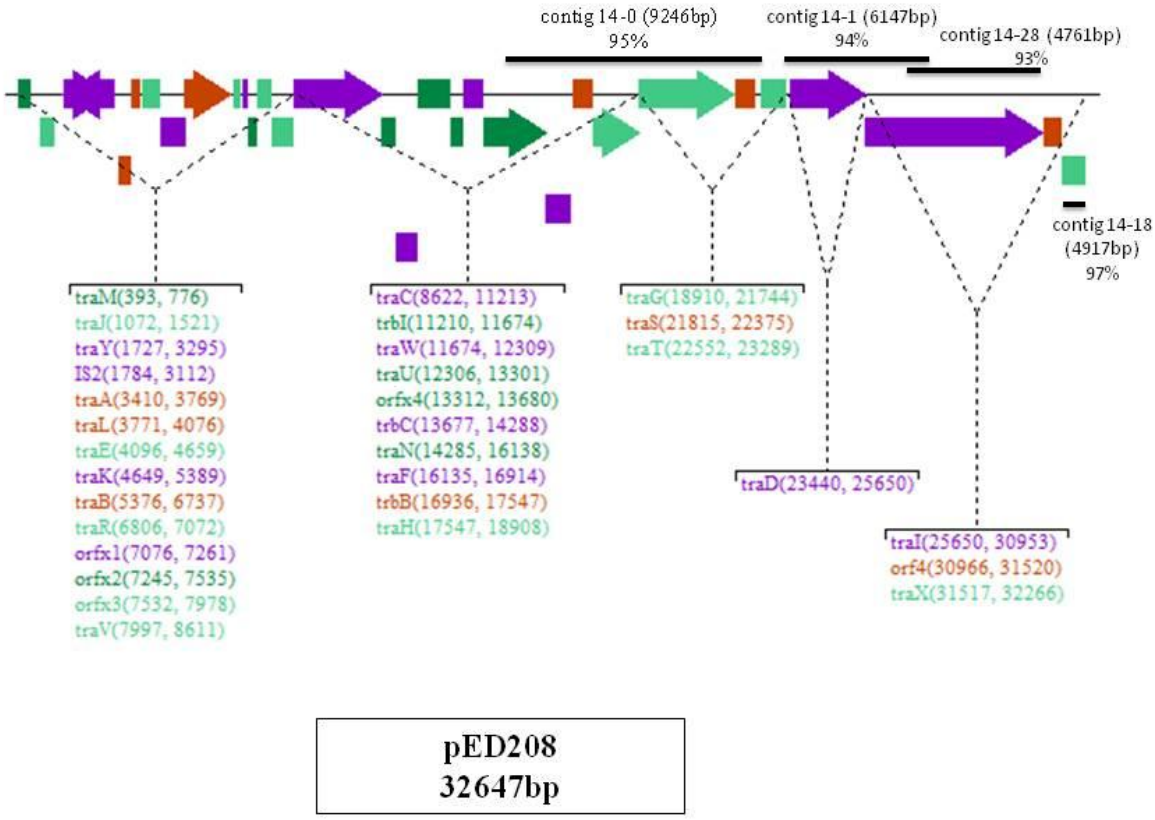


Figure 10: DNA similarity of a plasmid isolated from serogroup H isolate #14 mapped to a 25kb region of *tra* genes within the *Salmonella* Typhi pED208.