MOLECULAR CHARACTERISATION OF
SALMONELLA ENTERICA SEROVAR SOFIA
IN AUSTRALIA

A thesis submitted in fulfilment of the requirements for the degree of Doctor
of Philosophy

by

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DECLARATION

The work described in this thesis was carried out while I was an enrolled student for the degree of doctor of Philosophy in the School of Applied Sciences at Royal Melbourne Institute of Technology (RMIT) University. To the best of my knowledge, all work and research performed by others, published or unpublished has been dully acknowledged in this thesis. This work has also not been submitted previously in whole or in part to qualify for any other academic award.

Name:……………………………

Signature:………………………….

Date:……………………………..
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“I may not have gone where I intended to go, but I think I have ended up where I intended to be – Douglas Adams”
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ABBREVIATIONS

Ω: ohms
AR: analytical grade
Amp’: ampicillin resistant
bp: base pairs
°C: Celsius
cm/nm: centimeter/nanometer
CDC: Centers for Disease Control and Prevention
CFU: colony forming units
cDNA: complimentary DNA
DNA: deoxyribonucleic acid
DIG: Digoxigenin
DMEM: Dulbecco’s modification of Eagle’s medium
EPEC: enteropathogenic Escherichia coli
HEPES: N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
h: hour(s)
IPTG: isopropylthio-β-D-galactosidase
Kan’: kanamycin resistant
kB: kilobases
kDa: kilodaltons
kg/g/mg/µg/ng/pg: kilogram/gram/miligram/microgram/nanogram/picogram
L: liter
mL/µL: milliliter/microliter
min: minute(s)
M/mM/µM: molar/milimolar/micromolar
MOPS: 3-[N-morpholino] propanesulfonic acid
NBT/BCIP:
O.D.: optical density
ONPG: o-nitrophenyl-β-D-galactopyranosidase
PCI: phenol/chloroform/isoamylalcohol (25:24:1)
PCR: polymerase chain reaction
pH: negative logarithm of hydrogen ion concentration
PT: phage type
RNA: ribonucleic acid
RT: reverse transcriptase
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
s: second(s)
ss/dsDNA: single-/double-stranded DNA
ssRNA: single-stranded RNA
spp.: species (plural)
TEM: transmission electron microscopy
Tris: Tris(hydroxymethyl)aminomethane
V: volts
UV: ultraviolet light
v/v: volume for volume
w/v: weight for volume
WHO: World Health Organization
X-gal: 5-bromo-4-chloro-3-indoyl-β-D-galactose
XLD: xylose-lysine-deoxycholate agar
SUMMARY

First isolated in Australia in the 1970s, *Salmonella* II Sofia is currently the most prevalent *Salmonella* serovar isolated from Australian poultry flocks, accounting for at least half of the *Salmonella* strains isolated annually from chickens and chicken products in Australia. Despite its high frequency in chickens, *S*. II Sofia is rarely associated with animals or human salmonellosis as this serovar is avirulent in nature. The reason for the persistence of *S*. II Sofia (belonging to the *Salmonella* subspecies group II, which is more commonly associated with cold-blooded animals) in chickens but its avirulence is unknown as very few studies have been conducted on the epidemiology and pathogenicity of this strain. This study details the various experimental methods utilised to characterise the genetic relatedness of Australian *S*. II Sofia isolates and to investigate the molecular mechanisms involved in *S*. II Sofia pathogenesis.

The first part of this study was to investigate the epidemiology of *S*. II Sofia isolates using 2 molecular fingerprinting methods: PFGE (*Xba*I and *Spe*I) and Rep-PCR (using REP1 element). The previous problem of DNAse activity from *S*. II Sofia strains causing DNA degradation was solved by modifying the lysis solution used to treat the bacterial plugs (addition of SDS and increasing sarkosyl and proteinase K concentration), allowing *S*. II Sofia to be subtyped using PFGE. A total of 84 Australian *S*. II Sofia isolates from various states in Australia were subtyped using these 2 fingerprinting methods – resulting in generation of 8 *Xba*I, 6 *Spe*I and 5 REP1 pattern subtypes although no correlation could be found between any of the fingerprint typing methods. Assessment of the individual typing methods with the Simpson’s Index of Diversity showed low index values (less than 0.5), indicating the poor discriminatory ability of the methods. However, the combination of the typing methods was able to improve the discrimination of the
isolates, further dividing them into 16 subtypes and raising the index value to 0.721. The Australian S. II Sofia isolates were found to show limited genetic diversity and probably share a clonal relationship. A majority of the S. II Sofia isolates were not geographically restricted with the predominant pattern subtype spread out among the isolates from various states – however, the analysis of more isolates is still required before any firm conclusions could be drawn.

The second focus of this study was to examine the distribution and variation of the SPI-associated virulence genes within S. II Sofia. Southern hybridisation detecting the SPI1 to SPI5 regions showed that while differences were observed between the Typhimurium and Sofia serovars, all the S. II Sofia isolates tested shared the same SPI pattern. Based on RFLP and sequencing analysis, most of the differences observed in SPI1 to SPI5 of S. II Sofia could be attributed to a loss or gain of restriction cleavage sites within these regions. Most of the SPI genes were predicted to encode proteins sharing 87% to 99% similarity with corresponding SPI proteins from S. Typhimurium LT2 (reference strain). However, a number of genes in SPI1 (orgB, prgl, sptP, sipD and invJ), SPI2 (ssaP, ssal and ssaE), SPI3 (misL, marT and slsA) and SPI5 (ORF) were found to have accumulated mutations that could have affected gene transcription and/or protein translation – these genes have been shown to be involved in different aspects of the virulence process. Further, the deletion of avrA (from SPI1), sugR-rhuM (from SPI3) and sopB-pipA (from SPI5) and the insertion of an identical 1.2 kB transposase in SPI1 and SPI3 were observed. The effects of these mutations, insertions and deletions probably contributed to the reduced pathogenicity in S. II Sofia and thus, avirulence of this serovar is not the result of a single genetic change but rather a series of alterations to a large number of its virulence-associated genes.
Plasmid-mediated virulence was also assessed in S. Il Sofia isolates. Isolation and analysis of plasmids from S. Il Sofia isolates showed the presence a large plasmid along with several smaller plasmids (~ 1 to 100 kB in size). Southern hybridisation with 2 DNA probes (spvRABCD and traYALE) derived from the virulence plasmid of S. Typhimurium failed to detect the presence of both these regions in S. Il Sofia isolates, indicating either the total absence of the virulence plasmid or the presence of a virulence plasmid containing major deletions. Clones constructed with the missing spv operon using 2 different cloning vectors (high-copy pCR®2.1 and low-copy pWSK29) were inserted into S. Il Sofia Bt6 and the adherence, invasion (INT407 and CEF-DF1 cells) and intracellular survival (J774 macrophages) of the mutant strain was evaluated in vitro. The presence of spvRABCD was shown to have no effect on intracellular survival and replication in murine macrophages. Although the cloning of spv with pCR®2.1 was observed to significantly increase invasiveness of S. Il Sofia in INT407 cells, but it was not capable of restoring the invasive ability of S. Il Sofia to the level of S. Typhimurium 82/6915. On the other hand, the uneven adherence and invasion ability of the other mutant strains appeared to be linked to the presence of pWSK29 and this observation is further supported by RT-PCR analysis of the clones – indicating that perhaps pWSK29 is not a suitable vector for this study.

The assessment of the pathogenic potential of Australian S. Il Sofia isolates was done based on the epidemiology and molecular data obtained in this study. Wild-type S. Il Sofia isolates are unlikely to regain full pathogenicity because of the numerous mutations in many important virulence genes: even the chance acquisition of a virulence factor (e.g. spvRABCD) is not sufficient to completely restore S. Il Sofia virulence. Therefore, S. Il Sofia should not be considered similar to other Salmonella spp. when monitoring Salmonellae in food samples.
CHAPTER 1: INTRODUCTION

1.1 Salmonella spp.

Salmonella spp. is one of the most widespread disease-causing organisms isolated from animals and humans worldwide. First isolated from infected pigs in 1885, the bacterium was described by Theobald Smith and Damon Elder Salmon in the 1886 paper Investigations on Swine Plague, published in the Second Annual Report of Bureau of Animal Industry (Enerson, 2007). However, it was the French bacteriologist, Joseph Léon Marcel Lignières who, in 1900, made the suggestion of adopting the name Salmonella (in honour of D.E. Salmon) to encompass this whole genus, as we know it today (Enerson, 2007).

1.1.1 Nomenclature

The issue of taxonomy of Salmonella spp. is complex and has been the subject of debate for some time now (Brenner et al., 2000; Ezéby, 1999; Ezaki et al., 2000a; Ezaki et al., 2000b; Grimont et al., 2000; Le Minor and Popoff, 1987; Tindall et al., 2005). The nomenclature used by the CDC and WHO is chosen as the nomenclature for this thesis. According to this system, Salmonella spp. is divided into 3 species – S. enterica, which contains six subspecies (I: enterica, II: salamae, IIIa: arizonae, IIIb: diarizonae, IV: houtenae and VI: indica), S. bongori, formerly known as subspecies V (Brenner et al., 2000) and S. subterranea (Shelobolina et al., 2004). To simplify matters, the name of Salmonella spp. will be written as outlined: e.g. Salmonella Typhimurium (S. Typhimurium) and Salmonella II Sofia (S. II Sofia), instead of the full designation,
*Salmonella enterica* subspecies *enterica* serovar Typhimurium and *Salmonella enterica* subspecies *salamae* serovar Sofia, respectively.

1.1.2 Morphology

*Salmonella* spp. are non-spore forming rod shaped bacteria belonging to the family *Enterobacteriaceae*. They are Gram negative due to the higher lipid content and thinner bacterial cell wall and most are motile with peritrichous flagella (Chapin, 2007; Grimont *et al.*., 2000). Like all enterobacteria, *Salmonella* spp. is able to ferment glucose and other sugars (producing gas), can grow in the presence and absence of oxygen, reduce nitrate to nitrite and are oxidase negative and catalase positive (Ewing, 1986; Farmer III *et al.*., 2007). Most *Salmonella* spp. are also able to utilise citrate as a carbon source, produce hydrogen sulphide from inorganic sulphur, decarboxylate ornithine and lysine (Ewing, 1986; Farmer III *et al.*., 2007; Grimont *et al.*., 2000). In addition, most *Salmonella* strains cannot produce indole and beta-galactosidase, deaminate tryptophan and phenylalanine, hydrolyse urea or utilise malonate (Ewing, 1986; Farmer III *et al.*., 2007; Grimont *et al.*., 2000). It is these properties that allow *Salmonella* spp. to be biochemically identified and the serovars differentiated from one another (see Section 1.2.1).

1.2 Typing of *Salmonella* spp.

1.2.1 Biotyping

*Salmonella* spp. can be differentiated based on their biochemical reactions via biotyping. For example, the test for utilisation of sodium malonate can differentiate subspecies I (negative) from subspecies II (positive) (Ewing, 1986). Biotyping have been used to study *Salmonella* isolates in several instances (Crichton and Old, 1990;
Millemann et al., 1995; Old et al., 1999). However, the choice of tests for biotyping has often been empirical (Grimont et al., 2000). This typing method is also not discriminatory enough to separate strains within the same subspecies or serovar.

1.2.2 Serotyping

*Salmonella* isolates are serotyped based on the antigenic properties of 3 surface structures from the lipopolysaccharide (LPS) layer of the cell wall – the O (somatic), H (flagellar) and Vi (capsular) antigens (Ewing, 1986; Nataro et al., 2007). Salmonella serotyping has been invaluable in epidemiological investigations of disease outbreaks (Ethelberg et al., 2004; Uesugi et al., 2007). Since salmonella serotyping is done worldwide, this has assisted in the surveillance and identification of international outbreaks (Nataro et al., 2007). However, full serotype determination is beyond the expertise of many routine laboratories, limiting this method to larger reference laboratories (Nataro et al., 2007).

1.2.3 Phage Typing

This typing method is based on the susceptibility or resistance of *Salmonella* spp. to a set of lysogenic and temperate bacteriophages. Phage typing has become a useful tool in epidemiological investigations – detecting and following salmonellosis outbreaks, tracing the source of infections and future disease control and prevention strategies (Ethelberg et al., 2004; Gudmundsdottir et al., 2003; Matsumoto et al., 2006; McNeil et al., 1999; Stafford et al., 2002; Usera et al., 1998; Ward et al., 2005). Due to the stability of the phage type (in most cases), this method is also employed in the long-term surveillance of the spread of a particular *Salmonella* spp. serotype within a single or different host populations (Maré et al., 2000; Nastasi and Mammina, 2000; Pang et al., 2005; Rabatsky-Ehr et al., 2004; Rabsch et al., 2002; Rabsch et al., 2007; Sullivan et al., 2005; ...
Although salmonella phage typing is sensitive, cheap, rapid and requires no specialised equipment, smaller laboratories may encounter problems in maintaining expertise in this method (Demczuk et al., 2003; Grimont et al., 2000). This would generally limit phage typing of strains to larger reference laboratories.

1.2.4 Plasmid Profile Typing

A majority of *Salmonella* strains carry plasmids between 2 to 150 kB in size and the frequency, size and distribution of plasmids vary from serovar to serovar (Olsen, 2000). It is this variation that forms the basis of this typing method. Plasmid profiling has been employed to investigate the clonality of *Salmonella* strains (Bakeri et al., 2003; Liebisch and Schwarz, 1996; Malorny et al., 2001; Mare et al., 2001; Ridley et al., 1998), elucidate routes of transmission of *Salmonella* spp. (Langvad et al., 2006; Nayak et al., 2004) and in epidemiological surveillance (Holmberg et al., 1984; Liebana et al., 2001; Nastasi and Mammina, 2000; Pang et al., 2005; Tsen et al., 2000; Tsen and Lin, 2001). However, this method does have its disadvantages. *Salmonella* strains without plasmids cannot be typed using this method. Moreover, plasmid typing alone is not discriminatory enough to analyse the strains (Baquar et al., 1994; Mare et al., 2001; Millemann et al., 1995). Therefore, plasmid typing and its restriction analysis is usually performed together with other typing methods.

1.2.5 Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis involves all techniques that utilise probes to investigate the polymorphic nature within DNA regions of bacterial isolates (Olive and Bean, 1999; Olsen, 2000). This method is carried out by digesting chromosomal DNA, transferring the fragments on to hybridisation membranes and probing said membranes with specific
probes. RFLP is based on the observation that the locations of various restriction enzymes (RE) sites differ from serovar to serovar, resulting in DNA band patterns that differ between unlike strains (Olive and Bean, 1999). Two examples of this method are ribotyping and IS200 typing.

1.2.5.1 Ribotyping

Ribotyping utilises DNA probes derived from the 16S and 23S rRNA genes (Olive and Bean, 1999; Olsen, 2000). This typing method has been widely used to study the relationships within and between Salmonella serovars (Baquar et al., 1994; Gruner et al., 1994; Millemann et al., 1995; Old et al., 1999; 2000). Ribotyping usually results in a small number of bands, which makes data interpretation simple. However, this also lowers the discriminatory ability of the method to differentiate between closely related strains (Liebisch and Schwarz, 1996; Millemann et al., 1995; Thong et al., 1995; Usera et al., 1998). It is for this reason that ribotyping is not commonly used to investigate disease outbreaks (Olsen, 2000).

1.2.5.2 IS200 Typing

Lam and Roth (Lam and Roth, 1983) discovered a 708 bp insertion sequence in S. Typhimurium LT2, which they identified as IS200. This insertion sequence (IS) element appears to be found in a majority of the Salmonella strains examined and is absent from other enterobacteria, making it a good candidate for use in Salmonella spp. typing (Lam and Roth, 1983). In some cases, IS200 typing alone is discriminatory enough to distinguish between strains. Threlfall and his colleagues (Threlfall et al., 1993) discovered that it was possible to differentiate between drug-sensitive and drug-resistant Salmonella serovar Typhi of Vi-phage types E1 and M1 based on their IS200 profiles.
IS200 typing has since been applied to the typing of a wide variety of *Salmonella* serovars (Baquar *et al.*, 1994; Millemann *et al.*, 1995; Old *et al.*, 1999; 2000).

1.2.6 Pulsed-Field Gel Electrophoresis (PFGE)

This particular typing method is often considered the “gold standard” of molecular typing methods. Whole bacterial cells are embedded in agarose plugs and treated with a detergent-lysis solution to break down cell walls and proteins (Olive and Bean, 1999; Olsen, 2000). The treated plugs containing whole bacterial DNA are then digested with a rare cutting restriction enzyme (e.g. *Xba*I) before being subjected to pulsed field electrophoresis. This allows large fragments of DNA, ranging from 10 to 800 kB, to be separated for analysis (Olive and Bean, 1999).

With the advent of computerised gel scanning and analysis software, it has become feasible to create reference databases of PFGE patterns for all disease-causing organisms like *Salmonella* spp. (Olive and Bean, 1999). This has allowed the comparison of any new strain isolated from an outbreak to the strains available in the database, enabling phylogenetic identification of said strain (Olive and Bean, 1999). An example of one such database is PulseNet. Established in 1996 and maintained by the CDC, PulseNet has utilised PFGE analysis to rapidly identify incidents of food-borne illness in the United States (Hunter *et al.*, 2005). However, PFGE method is time-consuming and labour-intensive, usually taking 2-3 days to complete (Olive and Bean, 1999; Olsen, 2000). This can reduce and limit the number of strains that can be processed by a laboratory. There is a great need to develop alternative, shorter PFGE protocols, like the one devised by Gautom (Gautom, 1997).

1.2.7 PCR-Based Typing

1.2.7.1 *Salmonella*-specific PCR

This typing method utilises gene-specific primers to amplify and examine variation between strains. Target sequences that have been used include the IS200 element (Millemann *et al.*, 2000) and the *fljB* gene (Kardos *et al.*, 2007). Using a set of 6 primers, Alvarez and his coworkers (Alvarez *et al.*, 2004) developed a multiplex PCR assay to detect and type *Salmonella* serotypes, which is both sensitive and reproducible. This PCR typing method has the advantage in that it is specific to *Salmonella* spp. and will not detect any other bacterial isolates (thereby giving false positive results).
1.2.7.2 Rapid Amplified Polymorphic DNA (RAPD)

RAPD assay (also known as arbitrary primed PCR) is based on the use of short random PCR primers (between 9-10 bases in length) to subtype Salmonella isolates (Olive and Bean, 1999). The number and location of random primer sites within the bacterial genome differ from strain to strain and thus, amplification of the primers at low annealing temperatures will result in DNA band patterns characteristic of the particular strain (Olive and Bean, 1999). RAPD typing has been successfully used to investigate Salmonella isolates in several studies (Chansiripornchai et al., 2000; Hilton et al., 1996; Hudson et al., 2001; Kerouanton et al., 1996; Lin et al., 1996; Löfström et al., 2006; Malorny et al., 2001; Millemann et al., 1996; Millemann et al., 2000). However, reproducibility and standardisation of this method has been reported to be problematic (Olive and Bean, 1999; Olsen, 2000; Seo et al., 2006; Tsen et al., 2000).

1.2.7.3 Repetitive PCR (Rep-PCR)

Short, interspersed repetitive DNA sequences are found throughout the genome of all organisms (Lupski and Weinstock, 1992; Versalovic et al., 1991, Versalovic et al., 1994). They are conserved and the number and distribution of repeat DNA elements present in the bacterial genome differs in different species, strains and serovars (Olsen, 2000). Using specific primers and PCR to detect and amplify these short sequences, a set of band patterns can be obtained for analysis and this forms the basis for this typing method. Examples of such repeat sequences are BOX element (Koeuth et al., 1995; Martin et al., 1992; Versalovic et al., 1991; Versalovic et al., 1994), the enterobacterial repetitive intergenic consensus (ERIC) sequence (Hulton et al., 1991; Versalovic et al., 1991; Versalovic et al., 1994) and the repetitive extragenic palindrome (REP) element (Gilson et al., 1984; 1990; Stern et al., 1984; Versalovic et al., 1991; 1994).
First discovered in the Gram positive bacterium *Streptococcus pneumoniae*, BOX element is composed of 3 subunits – boxA, box B and boxC (Martin et al., 1992; Versalovic et al., 1994). Koeuth and his colleagues (Koeuth et al., 1995) discovered that out of the 3 subunits, only boxA appears to be conserved among diverse bacteria and sequences similar to boxB and boxC were only found in *S. pneumoniae*. The ERIC sequence is 126 bp in length and consists of a central core inverted repeat located in extragenic regions of the bacterial genome (Hulton et al., 1991; Lupski and Weinstock, 1992; Versalovic et al., 1991; Versalovic et al., 1994). REP elements are 38 bp palindrome units, which contain 6 degenerate sites and a 5 bp variable loop able to form stem-loop structures (Gilson et al., 1984; Lupski and Weinstock, 1992; Stern et al., 1984; Versalovic et al., 1991; Versalovic et al., 1994).

Rep- PCR is fast becoming one of the most frequently used DNA typing method to investigate strains of *Salmonella* (Beyer et al., 1998; Foley et al., 2006; Johnson et al., 2001; Maré et al., 2001; Millemann et al., 2000; Mills-Robertson et al., 2003; Rasschaert et al., 2005; Saxena et al., 2002; Weigel et al., 2004; Woo and Lee, 2006). This typing method is economical and simple to carry out, which allows a large number of samples to be processed at once (Olive and Bean, 1999). However, there have been problems regarding the reproducibility and discriminatory ability of this method (Bessa et al., 2007; Burr et al., 1998; Gebreyes et al., 2006; Kerouanton et al., 1996; Millemann et al., 1996).

### 1.2.7.4 Amplified Fragment Length Polymorphism (AFLP)

First developed in the 1990s, AFLP is based on the selective PCR amplification of DNA fragments generated from restriction enzyme digestion (Vos et al., 1995). The typing method is carried out in 3 steps – digestion of genomic DNA and ligation to adapters containing restriction sites followed by PCR amplification with selective primers.
and gel analysis of the amplified products (Olive and Bean, 1999; Vos et al., 1995). AFLP has been successfully applied to subtype Salmonella isolates in several studies (Aarts et al., 1998; Gebreyes et al., 2006; Lan et al., 2007; Ross and Heuzenroeder, 2005b; Torpdahl and Ahrens, 2004). Although AFLP typing is reproducible and has a high discriminatory ability, the setup cost for equipment and expertise is high (Olive and Bean, 1999).

1.2.8 Other Typing Methods

Other typing methods that have been used to investigate and study Salmonella strains include the use of antibiotic resistance genes as epidemiological markers (Bessa et al., 2007; Cardinale et al., 2005; Foley et al., 2006; Malorny et al., 2001; Matsumoto et al., 2006; Nayak et al., 2004; Rabatsky-Ehr et al., 2004; Ward et al., 2005), multilocus sequence typing, which analyses the DNA sequences of a series of housekeeping, ribosomal and/or virulent genes (Foley et al., 2006, Kotetishvili et al., 2002), multilocus variable-number tandem repeats analysis, which makes use of short sequence repeat motifs as a target to type isolates (Lindstedt et al., 2004; Lindsedt, 2005) and multiple amplification of phage typing locus, which detects presence/absence of a range of bacteriophage in the genome (Ross and Heuzenroeder, 2005a; 2008; Ross et al., 2008).

1.3 Pathogenicity of Salmonella spp.

Non-typhoidal Salmonella spp. have the ability to cause infection in 2 ways – gastroenteritis and systemic infection (Jones and Falkow, 1996; Marcus et al., 2000; Ohl and Miller, 2001). Salmonella pathogenesis begins with the ingestion or uptake of the bacteria into the host. After encountering and surviving the acidic pH environment of the stomach (Foster and Hall, 1990; Garcia-del-Portillo et al., 1993a; Giannella et al., 1972),
the bacteria migrate to the intestines, where it attaches and invades the intestinal epithelial cells (Ohl and Miller, 2001). In *Salmonella* spp. associated with gastroenteritis, the adhesion and invasion of the intestinal cells results in a series of inflammatory and secretory responses in the host, causing disease (Hansen-Wester and Hensel, 2001; Ohl and Miller, 2001).

On the other hand, *Salmonella* serotypes that cause systemic disease establish infection through their ability to survive and replicate in phagocytic macrophage cells (Ohl and Miller, 2001). After invading M cells of the Peyer’s patches in the intestine (Jones *et al.*, 1994), the bacteria migrate to the local mesenteric lymph nodes and then to the spleen and liver via phagocytic cells, resulting in the spread of the disease (Hansen-Wester and Hensel, 2001; Jones and Falkow, 1996; Marcus *et al.*, 2000; Ohl and Miller, 2001). In order for *Salmonella* spp. to initiate both gastroenteritis and systemic infection, a wide variety of virulence determinants are required. These virulence factors and their complex interactions with one another are outlined in detail below.

1.3.1 *Salmonella* Pathogenicity Island (SPI)

A pathogenicity island is a genetic element that occurs as a distinct and separate unit in the bacterial chromosome (Hentschel and Hacker, 2001; Hensel, 2004). All pathogenicity islands share a few common features: (a) they are not found in a closely related, nonpathogenic reference species or strain, (b) they often encompass large DNA regions (10-200 kB) and contains genes that often confer virulence to a bacteria, (c) they have a GC content lower than the rest of the bacterial genome (d) they are inserted into tRNA sites, (e) they are often flanked by short sequences (direct repeats), and (f) they
are usually associated with elements like inverted repeats, transposases, integrases, IS elements and origin of replications (Hacker and Kaper, 2000; Hensel, 2004; Hentschel and Hacker, 2001; Marcus et al., 2000; van Asten and van Dijk, 2005). First described in pathogenic *E. coli* (Hacker et al., 1983; Low et al., 1984), pathogenicity islands are now found in a wide variety of bacterial pathogens, including *Salmonella* spp.

### 1.3.1.1 SPI1

Galán and Curtiss III (Galán and Curtiss III, 1989) were the first to identify a genetic locus called *inv* that gives *S. Typhimurium* its ability to invade tissue culture cells. The *inv* locus discovered is located on the pathogenicity island termed SPI1 (see Figure 1.1A). SPI1 is a 40 kB region in the *Salmonella* genome, located at centisome 63 and flanked by the *fhlA* and *mutS* genes (Mills et al., 1995). It is thought that SPI1 is acquired very early on in the evolution of *Salmonella* spp. – at the divergence of *Salmonella* and *Escherichia* spp. from a common ancestor (Bäumler, 1997; Boyd et al., 1996; Galán, 1999; Li et al., 1995). In order for *Salmonella* spp. to cause disease, the presence of an intact and functional SPI1 is required. Ginnocchio and her coworkers (Ginnocchio et al., 1997) have found that environmental isolates of *Salmonella* carrying naturally occurring deletions in the SPI1 region lack the ability to invade mammalian cells. SPI1 contains genes that encode a type III secretion (TTS) system that gives *Salmonella* isolates the ability to colonise and invade intestinal epithelial cells and transport virulent effector proteins from the bacteria into the host cell cytosol (Galán, 1999; Hansen-Wester and Hensel, 2001; Hensel, 2004; Marcus et al., 2000; Ohl and Miller, 2001).
Figure 1.1  Genetic organisation of Salmonella pathogenicity island (SPI), SPI1 and SPI2 (Amavisit et al., 2003)
1.3.1.1.1 SPI1-Associated TTS Apparatus

Several genes in SPI1 (see Figure 1.1) encode for structural components that form a supramolecular structure called the needle complex, which spans both the outer and inner membranes of the bacteria (Kubori et al., 1998). Using electron microscopy, Kubori and his colleagues (Kubori et al., 1998) have shown that this needle apparatus is multi-ringed with a cylindrical base that is anchored to both the inner and outer membranes and a slender, hollow, needle-like barrel projecting outward from the outer membrane. For the proper assembly of a functional needle complex, a variety of genes are required – prg, org, spa and inv operons have been shown to be involved in the formation of the needle complex (Eichelberg et al., 1994; Kaniga et al., 1994; Kimbrough and Miller, 2000; Klein et al., 2000; Kubori et al., 1998; Kubori et al., 2000; Sukhan et al., 2001; Watson et al., 1998).

The entire prg operon (prgHIJK) is required for the assembly of a functional needle apparatus (Kimbrough and Miller, 2000). The proteins prgH, prgK and invG have been identified as components of the base of the needle structure and prgI and prgJ as part of the external needle substructure (Kimbrough and Miller, 2000; Kubori et al., 1998; Kubori et al., 2000; Sukhan et al., 2001; Sukhan et al., 2003). The length of the needle structure is controlled by another TTS associated protein invJ, whose secretion is stimulated by contact with cultured epithelial cells and thus, is required for cell invasion (Chen et al., 1996; Collazo et al., 1995; Kubori et al., 2000, Sukhan et al., 2001; Zierler and Galán, 1995). A loss of function of the invJ gene has resulted in the formation of abnormally long needle structures and the complete absence of TTS system (Kubori et al., 2000, Sukhan et al., 2001).
Kubori and his coworkers (Kubori et al., 2000) have reported that mutations in either invA or invC have resulted in the complete absence of the needle substructure, highlighting the importance of these genes in the full assembly of the needle complex. It has been observed that the InvC protein shared significant sequence similarity with the F_0F_1 ATPase family of proteins, leading to the suggestion that the translocation of effector proteins via the TTS apparatus may be driven by ATPase activity of this gene (Eichelberg et al., 1994). On the other hand, Salmonella strains carrying mutations in invI were unable to secrete the protein Prgl and lacked needle substructures, dismissing the initial theory that InvI is a chaperone of invJ since its mutation did not lead to a similar phenotype observed in invJ mutation (Collazo et al., 1995; Sukhan et al., 2001).

While not involved in the needle complex assembly, invH increases the efficiency of the process (Kubori et al., 1998; Kubori et al., 2000; Sukhan et al., 2001) and a mutation in the gene not only reduces the number of needle complexes observed (Sukhan et al., 2001) it also appears to hinder the normal secretion of several proteins including prgH, prgK, prgI, invG and sipC (Sukhan et al., 2001; Watson et al., 1998).

Another operon found to be involved with the TTS apparatus is the orgABC. Originally identified as a single operon (orgA) during a screening of oxygen-regulated genes required for invasion (Jones and Falkow, 1994), Klein and her colleagues (Klein et al., 2000), reported an error in the published DNA sequence, splitting this region into 2 ORFs – orgA and orgB along with the identification of another ORF overlapping the orgB gene (orgC). The proteins orgA and orgB have been demonstrated to be required for invasion and secretion (e.g. protein prgI) while orgC does not appear to be essential for the invasive phenotype (Klein et al., 2000; Sukhan et al., 2001). The spa locus (spaOPQR) has also been shown to be part of the TTS apparatus and is essential for host invasion and secretion of proteins like InvJ, SipB and SipC (Collazo and Galán,
1996). Further evidence of this has been demonstrated by Sukhan and his coworkers (Sukhan et al., 2001) through electron microscopy and biochemical analysis of needle complexes from spa mutants.

1.3.1.1.2 SPI-Associated Effector Proteins

After assembly of the TTS system, Salmonella strains are able to transport a range of substrate proteins (encoded by genes within and outside of SPI1) into the host cells to initiate invasion (Cherayil et al., 2000; Collazo and Galán, 1997b; Fu and Galán, 1998b; Galyov et al., 1997; Hardt and Galán, 1997; Wood et al., 1996). An example of translocated effectors encoded by SPI1 are the Sip proteins, encoded by the genes sipABCD. First characterised by Kaniga and his coworkers (Kaniga et al., 1995a; 1995b), these proteins share high sequence homology to the Ipa proteins, which are required by Shigella spp. for invasion. Although SipD is not internalised into the host cells, it does interact with SipB and SipC and is involved in the translocation process of effector proteins (Collazo and Galán, 1997b; Darwin and Miller, 1999b). It has been suggested that SipD, along with the proteins sipB and SipC form a translocation complex that delivers effector proteins into the host cells (Cherayil et al., 2000; Darwin and Miller, 1999b; Zhang et al., 2003).

SipB and SipC in particular appear to have dual roles in acting as translocases and as effectors proteins (Darwin and Miller, 1999b; Hayward et al., 2000; Hayward and Koronakis, 1999; Zhang et al., 2003). SipB is able to induce cell cytotoxicity and apoptosis via a mechanism dependent on the proapoptotic protease caspase-1 (Hersh et al., 1999; van der Velden et al., 2003). The ability of SipC to form dimers and multimers to elicit actin nucleation and condensation (“bundling”) allows Salmonella spp. to rearrange host cytoskeletal structure, contributing to bacterial invasion (Chang et al.,
SipC-mediated actin polymerisation is demonstrated to be enhanced by sipA, which binds directly to actin, reducing its critical concentration and inhibiting depolymerisation of its filaments, thereby inducing membrane ruffling and bacterial internalisation (Lilic et al., 2003; McGhie et al., 2001; Zhou et al., 1999). In addition, Brawn and her colleagues (Brawn et al., 2007) have recently found that during infection, sipA cooperates with the SPI2 effector sifA to modulate phagosome maturation and intracellular replication.

Another substrate protein translocated by the TTS system in a Sip-dependent manner is the tyrosine phosphatase protein SptP (Fu and Galán, 1998b; Kaniga et al., 1996). SptP functions by disrupting the actin cytoskeleton of the cell, possibly by targeting filament proteins like vimentin, which has been shown to be recruited to the Salmonella-induced membrane ruffling process (Fu and Galán, 1998b; Murli et al., 2001). Although not required for bacterial internalisation (Chen et al., 1996; Kaniga et al., 1996), SptP is still needed for virulence in vivo, since sptP mutants lack the ability to colonise the spleens of orally infected mice (Kaniga et al., 1996). Hardt and Galán (Hardt and Galán, 1997) identified a novel Salmonella protein, AvrA which is encoded by the avrA gene and translocated by the SPI1 TTS system. Secretion of AvrA is not dependent on sipA, sipB or sipC but requires sipD for translocation and strains carrying mutations in invG, invJ and spaO are unable to secrete this protein (Hardt and Galán, 1997). It has been shown that AvrA inhibits activation of the proinflammatory anti-apoptotic NF-κB transcription factor, initiating and enhancing cell apoptosis (Collier-Hyams et al., 2002; Ye et al., 2007).

The TTS system also translocates effector proteins that are encoded by the genes located outside of the SPI1 region. These include proteins like sopA (Wood et al., 2000),
sopB/sigD (Galyov et al., 1997; Hong and Miller, 1998), sopD (Jones et al., 1998), sopE (Wood et al., 1996) and sopE2 (Stender et al., 2000). It has been demonstrated that these proteins interact and cooperate with one another to cause a range of responses in the host such as cytoskeleton rearrangements, membrane ruffling, leading to bacterial internalisation and invasion (Bakowski et al., 2007; Mirold et al., 2001; Raffatellu et al., 2005; Zhou et al., 2001), fluid accumulation and intestinal inflammation (Jones et al., 1998; Zhang et al., 2002), stimulation of Rho family of GTPases and disruption of intestinal epithelial tight junctions and function (Boyle et al., 2006; Zhou et al., 2001).

1.3.1.1.3 SPI1-Associated Accessory/Chaperone Proteins

SPI1 of Salmonella spp. also contains genes that encode a group of proteins that functions as accessory or cognate chaperones of effector proteins. The type III secretion chaperones share a few common features like being small acidic proteins (15–20 kDa) with a putative alpha-helical structure in the C-terminal portion and a signal portion in the N-termini region of the corresponding effector protein (Cornelis and van Gijsegem, 2000; Galán and Wolf-Watz, 2006; Parsot et al., 2003; Wattiau et al., 1996). Chaperone proteins perform a wide range of functions, from prevention of protein degradation, premature interactions, maintenance of optimal conformation for export to conferring TTSS-pathway specificity to effector proteins (Collazo and Galán, 1997a; Galán and Wolf-Watz, 2006; Lee and Galán, 2004; Parsot et al., 2003; Stebbins and Galán, 2001). One example would be the protein invB, part of the inv genetic locus in SPI1 (Eichelberg et al., 1994). InvB has been shown to act as a chaperone for range of substrate proteins, including sipA (Bronstein et al., 2000), sopA (Ehrbar et al., 2004; Higashide and Zhou, 2006), sopE (Ehrbar et al., 2003; Lee and Galán, 2003) and sopE2 (Ehrbar et al., 2003).
Fu and Galán (Fu and Galán, 1998a) identified and characterised the 13 kDa protein sicP as a specific chaperone for SptP. Expression levels of SptP was observed to be drastically reduced in sicP mutant strains and although the sptP expression itself was not hindered, there was an increased in SptP degradation (Fu and Galán, 1998a). SicA encodes a protein that functions as a putative chaperone for the TTS-associated Sip proteins (Kaniga et al., 1995b; Tucker and Galán, 2000). SicA functions by binding to both SipB and sipC, thereby partitioning and stabilising the proteins and degradation of both these proteins have been observed in sicA mutants (Tucker and Galán, 2000). Tucker and Galán (Tucker and Galán, 2000) also show that SicA has an indirect influence on the expression of SopE, which is also a TTS system effector protein. In addition, the chaperone SicA also acts in concert with transcriptional regulator InvF to activate the expression of several Salmonella virulence genes (see Section 1.3.1.1.4) (Darwin and Miller, 2000).

The iagB gene, located within SPI1 region, is predicted to encode a 160 amino acids polypeptide that shares sequence homology to the ipgF (a glycosidase) from the virulence plasmid of Shigella flexneri (Miras et al., 1995). Due to this similarity, it has been suggested that this protein has peptidoglycan hydrolysing activity and is involved in the passage of the needle complex through the bacterial peptidoglycan layer (Sukhan, 2000) However, a later study has shown that it is not involved in the TTS-associated apparatus since a mutation in this gene does not seem to have any effect on the secretion of invG and Prg proteins or on the structure of the needle complex (Sukhan et al., 2000). Its precise function has not been elucidated yet. In addition to identifying sipD and sipA genes, Kaniga and his colleagues (Kaniga et al., 1995a) also found an ORF immediately downstream from sipA, predicted to encode a 9 kDa protein homologous to the acyl carrier proteins. As such proteins are involved in the biosynthesis of lipids
(Kaniga et al., 1995a), it has been suggested that this protein (IacP) could be involved in the modification of effectors before translocation (Collazo and Galán, 1997a; Kaniga et al., 1995a). However, the actual function and nature of this gene has yet to be elucidated.

1.3.1.1.4 SPI1-Associated Regulators

Salmonella spp. is able to detect environmental stimuli in its surroundings and activate/repress virulence-associated genes accordingly. SPI1 genes are optimally expressed under the following conditions: temperature of 37°C, low or limited oxygen, high osmolarity, neutral to slightly basic pH and during late log to stationary phase of bacterial growth (Bajaj et al., 1996; Ernst et al., 1990; Jones and Falkow, 1994; Lee and Falkow, 1990; Tartera and Metcalf, 1993). The expression of all the SPI1-associated genes is controlled by a series of transcriptional activators and regulators, which are detailed below. For example, although not essential for assembly of the needle complex (Sukhan et al., 2001), SPI1 TTS system protein InvE controls translocation process by regulating and interacting with the SipB-SipC-SicA protein complex (Kubori and Galán, 2002).

The hilA gene encodes a novel transcriptional regulator whose polypeptide sequence shares significant homology with the ompR/toxR family of transcriptional activators (Bajaj et al., 1995; Lee and Falkow, 1992). Epithelial cell invasion, apoptosis of macrophages and long term colonisation of chicken caeca have been demonstrated to be dependent on hilA expression (Bajaj et al., 1995; Bohez et al., 2006; Monack et al., 1996). The hilA gene is considered a central regulator of SPI1, binding to promoters and activating TTS-associated genes like prg/org and inv/spa operons (Ahmer et al., 1999; Bajaj et al., 1995; Lee and Falkow, 1992; Lostroh and Lee, 2001). Following activation of
inv/spa, a small amount of transcriptional read-through from this operon continues through to the sic/sip genes (Darwin and Miller, 1999a; Eichelberg and Galán, 1999; Ellermeier and Slauch, 2007; Lostroh and Lee, 2001). However, optimal expression of sic/sip operon is dependent on invF, a homolog of the AraC family of proteins and part the inv genetic locus of SPI1 (Darwin and Miller, 1999a; Eichelberg and Galán, 1999; Kaniga et al., 1994). InvF and its putative chaperone protein, SicA (along with HilA) is responsible for activating and controlling the expression of TTS-associated proteins encoded within and outside of SPI1 (Darwin and Miller, 1999a; Darwin and Miller, 2000; Darwin and Miller, 2001a; Eichelberg and Galán, 1999).

A variety of environmental and regulatory factors affect hilA expression, which in turn affects the invasion phenotype of Salmonella strains. An upstream repressing sequence (URS) has been mapped and identified in the hilA promoter region and it has been hypothesised that an unidentified repressor protein binds to this sequence to stop hilA expression under conditions unfavourable for bacterial invasion (Schechter et al., 1999). Schechter and her coworkers (Schechter et al., 1999) also identified 2 SPI1-associated genes, hilC and hilD, that can counteract repression of hilA. The proteins of HilC and HilD are similar to the members of AraC/XylS family of transcriptional regulators and have been demonstrated to derepress hilA expression by binding directly to the URS in the hilA promoter region (Schechter et al., 1999; Schechter and Lee, 2001). While a mutation in hilC only modestly affected bacterial invasion, it has been shown that the presence of a functional hilD is essential for the expression of hilA, highlighting the minor and major role these genes play in SPI1 regulation (Akbar et al., 2003; Boddicker et al., 2003; Ellermeier et al., 2005; Lucas and Lee, 2001; Schechter et al., 1999).
Expression of *hilA* is also controlled by RtsA, another member of the AraC/XylS family of proteins (Ellermeier and Slauch, 2003). Although not encoded within SPI1, *rtsA* is able to increase *hilA* expression (independent of both *hilC* and *hilD*) and induce the expression of *hilC*, *hilD* and *invF* genes (Ellermeier and Slauch, 2003). Ellermeier and his colleagues (Ellermeier *et al.*, 2005) recently showed that HilC, HilD and RtsA can each individually activate *hilA* and that these 3 proteins can act together in a feed-forward loop that controls the expression of *hilA* and thus, the SPI1 TTS system. It has also been demonstrated that the HilC and HilD proteins are capable of activating the regulator *invF* and in turn, a subset of SPI1 genes, independently of *hilA* (Akbar *et al.*, 2003).

In addition to regulators encoded within the island itself, SPI1 TTS system is also regulated by a series of global regulators, which are not found in the SPI1 region. One such regulatory system is the two-component EnvZ/OmpR, which affects the expression of *hilC*, possibly in a *hilD*-mediated manner (Ellermeier *et al.*, 2005; Lucas and Lee, 2001). A response regulator, SirA and its corresponding sensor kinase, BarA regulates SPI expression by interacting with *hilA* (in the presence of *hilD*) in response to unknown environmental conditions (Ahmer *et al.*, 1999; Altier *et al.*, 2000b; Ellermeier *et al.*, 2005; Ellermeier and Slauch, 2007). The PhoP/PhoQ regulatory system has been determined to be required for virulence *in vivo* (Miller *et al.*, 1989). It has an opposing regulatory function in SPI1 - shown to repress *prg* operon and *hilA* expression and also able to induce the *orgBC* operon (Aguirre *et al.*, 2006; Behlau and Miller, 1993; Pegues *et al.*, 1995).

Ellermeier and Slauch (Ellermeier and Slauch, 2007) have recently shown that a ferric uptake regulator (Fur), a major iron regulator in *Salmonella*, is able to mediate *hilA*
transcription (and SPI expression) in a \textit{hilD}-dependent manner. The \textit{hilE} protein is another transcriptional regulator that is capable of negatively regulating \textit{hilA} \cite{Baxter2003, Ellermeier2007}. Baxter and his colleagues \cite{Baxter2003} characterised the \textit{Salmonella}-specific \textit{hilE} and found that the presence of this gene super-represses expression of \textit{hilA} through its interaction with \textit{hilD}. Examples of other genes that affect SPI1 gene expression by regulating \textit{hilA} transcription include \textit{fis} \cite{Wilson2001}, \textit{csrAB} \cite{Altier2000a, Altier2000b}, \textit{phoB}, \textit{fadD} and \textit{fliZ} \cite{Lucas2000}, \textit{hha} \cite{Fahlen2001}, \textit{lon} \cite{Takaya2002, Takaya2005}, \textit{ams} and \textit{pag} \cite{Fahlen2000}. In conclusion, SPI1 expression is a complex series of interactions between different genes in response to the host environment, allowing \textit{Salmonella} spp. to initiate invasion and bacterial internalisation.

\subsection*{1.3.1.2 SPI2}

The SPI2 locus is another 40 kB pathogenicity island that encodes a second TTS system in \textit{Salmonella} spp. \cite{Hensel1995, Ochman1996, Shea1996}. This virulence element is located between centisome 30.5 and 31 and inserted adjacent to the \textit{valV} tRNA gene \cite{Hensel1997a, Shea1996}. The SPI2 region is composed of 2 segments – a larger 25.3 kB portion containing more than 30 genes required for virulence of SPI2 and a 14.5 kB portion harbouring a cluster of 5 \textit{ttr} genes (involved in anaerobic tetrathionate reduction) and 7 ORFs of unknown function \cite{Hensel1999a, Hensel1999b}. Hensel and his coworkers \cite{Hensel1999b} have shown that the 14.5 kB portion of the SPI2 region does not contribute as significantly to systemic infection in mice compared to the larger 25.3 kB portion. The mosaic structure of SPI2 suggests that each portion may have been the result of distinct and separate horizontal transfer events \cite{Hensel1999b}. 
Although SPI2 is conserved among and specific to *Salmonella* spp., it is not present in all of the *Salmonella* subspecies groups (Hensel *et al*., 1997a; 1997b; Ochman and Groisman, 1996; Shea *et al*., 1996). Hybridisation studies have demonstrated that while SPI1 is found in all *Salmonella* spp., the larger 25.3 kB segment of SPI2 is absent from *S. bongori*, suggesting that SPI2 was probably acquired by *Salmonella enterica* after its divergence from *S. bongori* (Hensel *et al*., 1997a; 1997b; Hensel *et al*., 1999b; Ochman and Groisman, 1996). While SPI1 is necessary for bacterial invasion and internalisation, SPI2 is required for establishment of systemic infection and intracellular replication (Cirillo *et al*., 1998; Hensel *et al*., 1998; Hensel *et al*., 1999b; Ochman *et al*., 1996; Ohl and Miller, 2001; Paesold *et al*., 2002). DNA sequence analysis of the SPI2 region resulted in the identification of about 42 ORFs (see Figure 1.1), encoding components of a TTS apparatus (*ssa*), effector proteins and their cognate chaperones (*sse* and *ssc*) and regulatory proteins (*ssr*) (Hensel *et al*., 1997a; Hensel, 2000; Shea *et al*., 1996).

### 1.3.1.2.1 Intracellular Replication Mechanism of *Salmonella* spp.

Upon bacterial invasion, *Salmonella* bacterium is enclosed within unique membrane-bound vesicles known as *Salmonella*-containing vacuoles (SCV) (Finlay *et al*., 1988; Richter-Dahlfors *et al*., 1997; Salcedo *et al*., 2001). The SCVs then migrate to a perinuclear position close to the Golgi apparatus, where the SCV undergoes maturation via selective interaction with different components from the endocytic pathway of the host cell (Garcia-del Portillo *et al*., 1993; Garcia-del Portillo and Finlay, 1995; Méresse *et al*., 1999; Rathman *et al*., 1997; Salcedo and Holden, 2003; Steele-Mortimer *et al*., 1999; Stein *et al*., 1996). The formation of endosomes containing lysosomal glycoproteins (*lgp*) occurs at the final maturation step of the SCV (Garcia-del Portillo *et al*., 1993b). The presence of *Salmonella* isolate induces the formation of *lgp*-
tubules (Sif formation) that interconnect with the SCVs, resulting in eventual intracellular replication (Garcia-del Portillo et al., 1993b; Garcia-del Portillo and Finlay, 1995; Stein et al., 1996). The maturation, maintenance and precise positioning of the SCV is dependent on the SPI2 TTS system and its effector proteins, which are outlined in detail below.

1.3.1.2.2 SPI2-Associated TTS Apparatus

The TTS system encoded by SPI2 is structurally and functionally different from that of SPI1 – components from SPI2 are not able to compensate for mutations in components of SPI1 within the same bacterium (Chakravortty et al., 2005; Hensel et al., 1997b; Marcus et al., 2000). A majority of the ssa genes show significant homology to the lcr/ysc genes from Yersinia spp. and only ssaL, ssaM and ssaP seem to be specific to Salmonella spp. (Hensel et al., 1997b). Mutations in any of these genes have been shown to affect the secretion of SPI1-encoded protein SipC (Hensel et al., 1997b), hinder the ability of Salmonella spp. to accumulate in spleen cells (Shea et al., 1999), restricts movement of the SCVs (Ramsden et al., 2007), prevents the formation of the SPI2 needle appendages and stop the secretion and translocation of SPI2 effector proteins (Chakravortty et al., 2005; Coombes et al., 2004; Klein and Jones, 2001; Nikolaus et al., 2001; Yu et al., 2004).

Using resolution field emission scanning and TEM, Chakravortty and his coworkers (Chakravortty et al., 2005) have shown that the SPI2 needle appendages are cylindrical structures with an irregular surface appearance, indicating the distribution of secreted proteins on the surface of these structures. The ssaV, ssaC and ssaH genes have been shown to be essential in the formation of the needle structures as the appendages were absent in Salmonella strains carrying these mutations (Chakravortty et al., 2005). The
SsaC protein is predicted to form the outer membrane ring of the SPI2 TTS system while ssaV and ssaT are predicted to be part of the TTS apparatus in the inner membrane of the bacteria (Freeman et al., 2002). A functional TTS apparatus also requires the presence of intact ssaL, ssaR, ssaM, ssaG and ssal genes as such Salmonella mutant strains are unable to replicate intracellularly and are defective in their translocation and secretion process (Chakravortty et al., 2005; Coombes et al., 2004; Yu et al., 2004).

Coombes and his colleagues (Coombes et al., 2004) demonstrated that SsaL is only required for the secretion and translocation of SPI2-encoded effectors and a mutation in ssaL has no effect on the secretion of SPI2 effectors encoded outside of this pathogenicity island. Another TTS component, SsaM has been shown to interact with ssaB/SpiC and it was proposed that these 2 proteins form a complex able to distinguish between translocators and effector proteins and thus, control their ordered secretion through the SPI2 TTS system (Yu et al., 2004). Analysis of the combination of an aroC and ssaV mutation in S. Typhi Ty2 and S. Typhimurium reveal the potential of utilising TTS system mutants for the development of live vaccines in humans (Hindle et al., 2002). However, the precise function of a number of the ssa genes in SPI2 have yet to be elucidated and more work on this region needs to be carried out.

Several studies on the SPI2-encoded proteins SseBCD show that they are localised primarily to the cell membrane of Salmonella spp. (Beuzón et al., 1999; Chakravortty et al., 2005; Klein and Jones, 2001; Nikolaus et al., 2001). These proteins share weak but significant homology to the Esp proteins from enteropathogenic E. coli (EPEC) and their mutants were severely attenuated in virulence and intramacrophage replication (Hensel et al., 1998). Chakravortty and his coworkers (Chakravortty et al., 2005) have reported that the irregular appearance of the needle structures is due to the
distribution of SseC and SseD on its surface. On the other hand, SseB loosely associates with the needle appendage and a mutation in sseB results in formation of appendages lacking its irregular form (Chakravortty et al., 2005). Nikolaus and his colleagues (Nikolaus et al., 2001) have determined that sseB, sseC and sseD interact with each other after secretion to form a complex for the translocation of effector proteins. It has also been shown that sseC is not essential for the secretion of sseD and vice versa (Chakravortty et al., 2005; Nikolaus et al., 2001). Although not required for the formation of the needle appendages, sseB is needed for the assembly of SseCD translocon (Chakravortty et al., 2005; Nikolaus et al., 2001).

1.3.1.2.3 SPI2-Associated Effector Proteins

The protein ssaB/SpiC was the first SPI2-encoded effector protein to be identified (Uchiya et al., 1999). It has been reported that ssaB does not show sequence homology to any known proteins and is translocated via the SPI2 TTS system into the macrophage cytosol (Hansen-Wester and Hensel, 2001; Uchiya et al., 1999). SsaB is able to interfere with normal intracellular trafficking and inhibit the fusion of phagosome-lysosome vesicles (Uchiya et al., 1999). Freeman and his coworkers (Freeman et al., 2002) have also shown that SsaB is required for the secretion and translocation of the components SseB and SseC to the bacterial surface. In addition, ssaB interacts with ssaM to control the secretion process of translocators and effectors (Yu et al., 2004).

Located downstream of the sseBCD cluster (see Figure 1.1), sseF and sseG have been identified as genes encoding SPI2 effector proteins (Hansen-Wester et al., 2002; Kuhle and Hensel, 2002). Both these genes are not required for the assembly of the needle appendages and their mutations have been shown to only modestly affect salmonella virulence (Chakravortty et al., 2005; Hensel et al., 1998). Like ssaB, SseF
and SseG are proteins specific to *Salmonella* spp. and require a functional SPI2 TTS system for secretion (Hensel *et al.*, 1998; Kuhle and Hensel, 2002). Work conducted by 2 different research groups (Guy *et al.*, 2000; Kuhle and Hensel, 2002) have indicated that SseF and SseG contribute to *Salmonella*-induced aggregation of host endosomal compartments. Guy and his colleagues (Guy *et al.*, 2000) also show that in addition to SseFG; SsaB, SsaJ, SsaL, SsaM, SsaV, and SsaP is needed for formation of lgp-tubules (see Section 1.3.1.2.1). SseG is characterised as a Golgi-targeting protein while SseF affects the distribution of the motor protein dynein (required for the formation of perinuclear microcolonies of *Salmonella* spp. in host cells) to the SCV (Abrahams *et al.*, 2006; Salcedo and Holden, 2003). Both these proteins are thought to form a complex that interacts with the host cells either by connecting the SCVs to the Golgi network and/or by modulating the recruitment of microtubule motor proteins kinesin and dynein, which in turn controls the vacuole membrane dynamics (Abrahams *et al.*, 2006; Deiwick *et al.*, 2006; Ramsden *et al.*, 2007).

Another SPI2 effector protein that is involved in the association of SCV with the Golgi network is SifA (Brumell *et al.*, 2001; Deiwick *et al.*, 2006; Stein *et al.*, 1996). Although not encoded by SPI2, SifA is involved in lgp-tubule formation, recruitment of kinesin and maintenance of SCV and is thereby required for intracellular survival and replication and systemic infection (Beuzón *et al.*, 2000; Boucrot *et al.*, 2005; Brumell *et al.*, 2001; Brumell *et al.*, 2002; Stein *et al.*, 1996). The precise intracellular positioning of the SCV in the host cell is the result of the concerted effort between 3 SPI2 effectors – SseF, SseG and SifA (Deiwick *et al.*, 2006; Salcedo and Holden, 2003). SifB, which shares a conserved N-terminal domain with SifA, is localised to the SCV in infected cells and its trafficking away from the SCV along Sifs requires SifA (Freeman *et al.*, 2003; Miao and Miller, 2000). However, SifB is not involved in *Salmonella* virulence as its
mutants are not attenuated in their intracellular replication (Freeman et al., 2003; Ruiz-Albert et al., 2002).

On the other hand, SseJ is required for intracellular replication of Salmonella spp. and it has been suggested that the combined actions of SseJ and SifA regulate the vacuole dynamics in infected host cells (Freeman et al., 2003; Ohlson et al., 2005; Miao and Miller, 2000; Ruiz-Albert et al., 2002). More recently, Ohlson and his colleagues (Ohlson et al., 2005) have demonstrated that SseJ is an enzyme with deacylase activity that is important for salmonella pathogenesis. SspH2 has been identified as a SPI2 translocated effector protein that contains leucine-rich repeats and shares the same conserved amino terminal as SifA, SifB and SseJ (Miao et al., 1999; Miao and Miller, 2000). The transcription and translocation of SspH2 is specific to SPI2 (requires SseBCD translocon) and sspH2 mutants lack the ability to cause lethal infection in calves (Miao et al., 1999; Nikolaus et al., 2001). Brumell and his coworkers (Brumell et al., 2003) described SopD2, a novel SPI2-associated TTS effector required for mouse virulence, that targets late endocytic compartments in host cells. Examples of other non SPI2-encoded proteins that affect salmonella virulence through the SPI2 TTS system include SseI (Miao and Miller, 2000; Miao et al., 2003), SseK1 and SseK2 (Kujat-Choy et al., 2004) and SseL (Coombes et al., 2007).

1.3.1.2.4 SPI2-Associated Chaperones

Based on sequence analysis, SPI2 region has been predicted to contain genes encoding proteins that function as chaperones (Hensel et al., 1998). Coombes and his colleagues (Coombes et al., 2003) have reported that a SPI2-encoded protein, SseA, is required for intracellular survival and replication in macrophage and epithelial cells. The virulence defect in the sseA mutant show a phenotype similar to that of a SPI2 TTS
apparatus mutant and it was shown that such mutants are attenuated due to their inability to translocate effector proteins via the SPI2 TTS system (Coombes et al., 2003). Two different research groups (Ruiz-Albert et al., 2003; Zurawski and Stein, 2003) separately demonstrate that SseA functions as a chaperone for the translocon components SseB and SseD and absence of sseA leads to the failure in the assembly of a proper SPI2 translocon complex. The presence of a C-terminal SseB and SseD domain is required for SseA binding and the N-terminus of this protein has been shown to be essential for export of SseB to cell surface (Zurawski and Stein, 2004). SPI2 also encodes the chaperone genes sscA and sscB (see Figure 1.1) – sscA mutants have been shown to be unable to secrete SseC while SscB acts as a chaperone for SseF by modulating its secretion, function and stability (Dai and Zhou, 2004; Hensel et al., 1998; Nikolaus et al., 2001).

1.3.1.2.5 SPI2-Associated Regulators

SPI2 gene expression appears to be induced when Salmonella spp. are located intracellularly in infected host cells (Deiwick et al., 1999; Valdivia and Falkow, 1997). It has been proposed that detection of these intracellular environmental conditions by the bacteria leads to activation/repression of SPI2 genes. Analysis of the SPI2 region by Deiwick and his coworkers (Deiwick et al., 1999) have shown that conditions that activate SPI1 gene expression (see Section 1.3.1.1.4) are not able to induce expression of SPI2 genes and vice versa. This particular observation has led to suggestions that the TTS systems of SPI1 and SPI2 are inversely regulated – both these regions response to different environmental signals and therefore are not likely to be induced simultaneously (Deiwick et al., 1999). Deprivation of divalent cations like Mg$^{2+}$ and phosphate starvation, which probably mirrors the conditions encountered by intracellular Salmonella isolates, have been identified as signals that induce SPI2 gene expression (Deiwick et al., 1999).
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An acidic pH environment inside the SCVs appears to be another physiological stimulus essential for SPI2 mediated expression. A low pH (pH 5.0) has been implicated in a wide variety of SPI2-associated functions: macrophage-induction of SPI2 genes (Cirillo et al., 1998), secretion of the translocon components SseBCD (Coombes et al., 2004; Nikolaus et al., 2001), functional assembly of SPI2 TTS system (Rappl et al., 2003), increasing expression levels of SsaC and oligomerisation of its subunits (Rappl et al., 2003) and secretion of formation of needle appendages (Chakravortty et al., 2005).

The 2 component regulon encoded in SPI2, SsrAB (See Figure 1.1) (Deiwick et al., 1999; Ochman et al., 1996) is observed to be stimulated by an acidic pH condition (Lee et al., 2000; Miao et al., 2002).

SsrA is a putative sensor kinase and SsrB has been identified as a transcriptional activator that shares sequence homology with proteins like UvrY of E. coli and SirA of S.Typhimurium (Deiwick et al., 1999; Ochman et al., 1996). Together, both these proteins are upregulated intracellularly although studies on their expression in response to different environmental signals have yielded mixed results (Deiwick et al., 1999; Lee et al., 2000; Miao et al., 2002; Worley et al., 2000). Deiwick and his colleagues (Deiwick et al., 1999) reported that induction of SPI2 gene expression is modulated by SsrAB and PhoPQ under Mg$^{2+}$ and phosphate limited conditions. This conclusion was contradicted by 2 research groups (Lee et al., 2000; Miao et al., 2002) which presented data indicating that transcription of SsrAB is independent of Mg$^{2+}$ concentration and PhoPQ regulator.

However, SsrAB is essential for salmonella pathogenesis as they have been shown to regulate the expression of proteins encoded within and outside of the SPI2 region (Chakravortty et al., 2005; Cirillo et al., 1998; Coombes et al., 2004; Coombes et
al., 2007; Deiwick et al., 1999; Hensel et al., 1998; Julio et al., 2000; Kujat-Choy et al., 2004; Miao et al., 2002; Worley et al., 2000). Transcription of SsrA and SsrB is uncoupled and overexpression of ssrB in the absence of SsrA is sufficient to activate SPI2 gene expression (Feng et al., 2003; 2004; Walthers et al., 2007). SsrA has been recently shown to promote reproductive tract (but not intestinal) colonization of chickens by *S. Enteritidis* (Bohez et al., 2008). SsrB is autoregulated and appears to have a dual function - activation of SPI2-associated genes by binding to their promoters and to counteract gene silencing by the H-NS protein (Feng et al., 2003; 2004; Walthers et al., 2007).

It has been suggested that SPI2 expression is positively modulated by PhoP/PhoQ through the regulation of the SPI2-encoded SsrA-SsrB regulon, further supporting the theory that SPI1 and SPI2 are inversely regulated since SPI1 is repressed by PhoP/PhoQ (Behlau and Miller, 1993; Bijlsma et al., 2005; Deiwick et al., 1999; Garmendia et al., 2003; Pegues et al., 1995; Worley et al., 2000). Furthermore, it has been demonstrated that the *in vitro* accumulation of the SPI2 translocated effectors SseK1 and SseK2 required intact *ssrB* and *phoP/phoQ* genes (Kujat-Choy et al., 2004). However, other investigators have reported that the transcription and expression of SPI2 genes does not require PhoP/PhoQ regulation (Kim and Falkow, 2004; Lee et al., 2000; Miao et al., 2002). These contradictory observations and findings highlight the need for more extensive research to be carried out on this regulator to elucidate its exact role in SPI2 gene expression and regulation.

Another 2-component global regulator, OmpR/EnvZ, has been shown to regulate expression of SPI2 via SsrAB (Feng et al., 2003; 2004; Garmendia et al., 2003; Kim and Falkow, 2004; Lee et al., 2000; Worley et al., 2000). Upon entry into the macrophages,
OmpR binds directly to ssrA promoter region, activating its transcription (Feng et al., 2003; Lee et al., 2000) and an overlap of binding sites at the ssrA and ssrB regions have been shown to occur between SsrB and OmpR (Feng et al., 2003; 2004). OmpR activation of SsrAB requires the presence of its cognate sensor kinase EnvZ (involved in the phosphorylation of OmpR and thereby converting it into its active form, OmpR-P) and mutations in envZ have resulted in the elimination of SPI2 promoter activity and gene expression (Feng et al., 2003; 2004; Kim and Falkow, 2004). The transcription factor protein SlyA has been shown to contribute to Salmonella virulence by affecting expression of SPI2 genes like ssrA-ssrB (Feng et al., 2004; Linehan et al., 2005). Examples of other proteins that are involved in regulation of SPI2 genes include the nucleoid-associated protein H-NS (Walthers et al., 2007), Hha and YdgT (Coombes et al., 2005; Silphaduang et al., 2007) and Fis (Kelly et al., 2004; Lim et al., 2006).

1.3.1.3 SPI3

Blanc-Potard and Groisman (Blanc-Potard and Groisman, 1997) identified the presence of a 17 kB DNA region at the selC tRNA locus in S. Typhimurium, which is also the insertional site of pathogenicity islands in E. coli (Blum et al., 1994, McDaniel et al., 1997). This pathogenicity island termed SPI3 is specific to Salmonella spp., with a G+C content of 47.5% and found to contain 10 ORFs organised in 6 transcriptional units (see Figure 1.2) (Blanc-Potard and Groisman, 1997; Blanc-Potard et al., 1999). One such transcriptional unit is the mgtCB operon, which is essential for salmonella virulence and required for intramacrophage survival and growth in low Mg\(^{2+}\) conditions (Blanc-Potard and Groisman, 1997). Southern hybridisation experiments have revealed that while the right end of SPI3 (including slsA and mgtBC) is present in all Salmonella subspecies, the central region (including mbA, fidL, and marT) is only found in Salmonella subspecies I, II and V (S. bongori) and is surrounded by remnants of insertion sequences.
Figure 1.2  Genetic organisation of SPI3 (Amavisit et al., 2003), SPI4 (schematic drawn based on Gerlach et al., 2007) and SPI5 (Amavisit et al., 2003)
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(Blanc-Potard et al., 1999). Such a mosaic structure indicates that this island appeared to have evolved in a mutlistep process (Blanc-Potard et al., 1999).

The *misL* gene encodes a 955 amino acid polypeptide that shares sequence homology with the immunoglobulin A1 protease family of autotransported proteins (Blanc-Potard et al., 1999). The C-terminus of this protein also shares similarity with the C-terminal region of the AIDAI protein from enteropathogenic *E. coli* and VirG from *Shigella flexneri*, which have been shown to be involved in diffuse adherence to HeLa cells (Benz and Schmidt, 1992) and cell-to-cell spreading (Goldberg et al., 1993), respectively (Blanc-Potard et al., 1999). This would suggest that MisL protein functions as an autotransporter in some host-pathogen virulence capacity. Although it has been reported that *misL* is not essential for virulence (Blanc-Potard et al., 1999), screening of transposon mutants for attenuation in animal models by Morgan and his colleagues (Morgan et al., 2004) have revealed a *misL* mutant is defective in its ability to colonise chicks. It was discovered that MisL functions as a surface adhesin, able to bind to extracellular matrix protein fibronectin and is required for the long-term intestinal colonisation in mice (Dorsey et al., 2005). The MarT protein shares sequence similarity with 2 different proteins – the homology of its N-terminal region to the ToxR protein from *Vibrio cholerae*, a transmembrane regulatory protein required for the synthesis of cholera toxin; and of its central region with the putative DNA binding domain of CadC, a transcriptional activator of *E. coli* K-12 (Blanc-Potard et al., 1999). The regulatory function of MarT was recently confirmed from work carried out by Tükel and his colleagues (Tükel et al., 2007) which identified MarT as a transcriptional activator of the *misL* promoter.
As stated earlier, *mgtCB* operon is required for salmonella virulence (Blanc-Potard and Groisman, 1997). The *mgtC* gene, which encodes a 22.5 kDa hydrophobic protein, is cotranscribed with *mgtB* and has been shown to be essential for lethal infection in mice and long-term intramacrophage survival (Blanc-Potard and Groisman, 1997; Snavely *et al.*, 1991). While MgtC shows no similarity to any other known protein sequence, the 102 kDa MgtB protein contains 10 transmembrane domains and shares significant homology with the eukaryotic Ca\(^{2+}\)-type ATPase, a part of the cation transporter family of proteins (Smith and Maguire, 1993; Snavely *et al.*, 1991). In contrast to other Ca\(^{2+}\)-type ATPases, which mediates efflux of cations, MgtB is involved in the influx of cations (like Mg\(^{2+}\)) into the cell (Snavely *et al.*, 1991). Mutation of *mgtB* caused only a mild defect in virulence (Blanc-Potard and Groisman, 1997) and its role during salmonella infection is still unclear (Blanc-Potard and Groisman, 1997; Moncrief and Maguire, 1998; Smith *et al.*, 1998).

Initially thought to contribute to Mg\(^{2+}\) transport (due to the defect in growth of *mgtC* mutants in Mg\(^{2+}\) limited conditions) (Blanc-Potard-Groisman, 1997; Lawley *et al.*, 2006; Snavely *et al.*, 1991; Soncini *et al.*, 1996), studies have revealed that MgtC is not a Mg\(^{2+}\) transporter, is not produced or unstable in the presence of MgtB and does not mediate the transport of any other cations like H\(^{+}\), Ca\(^{2+}\) and Ni\(^{2+}\) (Alix and Blanc-Potard, 2007; Günkel *et al.*, 2006; Moncrief and Maguire, 1998; Smith *et al.*, 1998). Recent work conducted by Günkel and his colleagues (Günkel *et al.*, 2006) have lead to suggestions that MgtC may be involved in the modulation of cellular ion homeostasis and thus, membrane potential through the activation of the host cell Na\(^{+}\) K\(^{-}\) -ATPase. The exact function of MgtC in pathogenesis is still as yet unknown. In a genome-wide screening of genes from *Salmonella* spp. required for long-term systemic infection in mice, Lawley and his coworkers (Lawley *et al.*, 2006) were able to identify *cigR*, *rhuM*, *rmbA* and *fidL*.
(along with mgtC and marT) as SPI3 genes that could potentially be involved in the systemic disease process. This observation supports the work done by Morgan and his colleagues (Morgan et al., 2004) on the identification of host-specific colonisation factors: the SPI3 gene rmbA is required for colonisation of calves while slsA is needed for colonisation in both calves and chicks. However, more research needs to be carried out on this pathogenicity island in order to elucidate the roles played by these genes.

Magnesium concentration levels have been shown to control the expression of PhoP regulon and in turn, the transcriptional of PhoP-regulated genes, which includes the mgtCB operon (Soncini et al., 1996). MgtC is only expressed in wild-type Salmonella strains in low Mg\(^{2+}\) conditions and phoP mutant strains are unable to induce the expression of mgtC, indicating that the SPI-3 carried gene mgtC is regulated by magnesium levels and PhoP/PhoQ (Soncini et al., 1996). This is further confirmed by other investigators (Moncrief and Maguire, 1998; Smith et al., 1998): the increase in transcription levels of mgtCB operon is influenced by Mg\(^{2+}\) deprivation and invasion of epithelial cells by Salmonella spp. And unlike SPI2 genes, mgtCB does not require a low pH for activation and expression (Moncrief and Maguire, 1998; Smith et al., 1998). Currently, the induction and regulation of the other SPI3 genes are still not known.

1.3.1.4 SPI4

SPI4 is a 27 kB pathogenicity island located at centisome 92 and flanked by the ssb and soxSR loci (Wong et al., 1998). This island has a mosaic structure, is found to be conserved among the most common serovars of S. enterica and is likely to carry genes encoding a Type I Secretion System (T1SS) (Marcus et al., 2000; Wong et al., 1998). A previously identified locus shown to be required for intramacrophage survival (Bäumler et al., 1994) was also mapped to the SPI4 region, leading to suggestions that
SPI4 may be needed for intramacrophage survival (Wong et al., 1998). Initially predicted to contain 18 ORFs (Wong et al., 1998), an improved analysis on the genome sequence of \textit{S. Typhimurium} have reorganised the operons into 6 ORFs, renamed \textit{siiABCDEF} (see Figure 1.2) (McCelland et al., 2004; Morgan et al., 2004). SPI4 mutants have been shown to be defective in the intestinal colonization of calves (Morgan et al., 2004) and attenuated in long-term persistence in mice (Lawley et al., 2006). Furthermore, work conducted on SPI4 region have revealed: it is not required for invasion and persistence in epithelial and macrophage cells, causes gastrointestinal inflammation but not systemic infection in mice, does not contribute to enteropathogenic responses, needed for adhesion to epithelial cells and has no effect on the expression and secretion of SPI1 effector proteins (Gerlach et al., 2007b; Morgan et al., 2004).

Detailed sequence and functional analysis have determined that SPI4 encodes a very large novel secreted protein (SiiE), components of a T1SS (SiiDCF) and 2 other putative secreted proteins (SiiAB) (Gerlach et al., 2007b; Morgan et al., 2004; 2007). Sequence analysis indicate that all six genes form an operon and are most probably cotranscribed together as a large mRNA (Gerlach et al., 2007a; 2007b; Morgan et al., 2004; 2007). SiiE is a highly repetitive ~600 kDa non-fimbrial adhesin protein that mediates contact-dependent adhesion of \textit{Salmonella} to epithelial cell surfaces (Gerlach et al., 2007b; Morgan et al., 2007). This protein is secreted through a cognate T1SS complex, composed of the proteins SiiCDF, which is predicted to be a putative outer membrane component, a putative fusion protein spanning the inner and outer membranes and a ATP-binding cassette component, respectively (Gerlach et al., 2007b; Morgan et al., 2007). In addition, Morgan and his coworkers (Morgan et al., 2007) have confirmed that the intestinal colonisation of cattle is influenced by the genes \textit{siiE} and \textit{siiF}. On the other hand, \textit{siiA} and \textit{siiB} show no homology to any known DNA or protein
sequences (Morgan et al., 2004). Both SiiA and SiiB are not secreted into the culture medium and are not part of the T1SS, leading to the hypothesis that these 2 proteins may have a regulatory or chaperone function (Gerlach et al., 2007b).

The discovery of insertions within the SPI4 region during a screening of random lacZY fusions for regulation by sirA and hilA dependence indicated a possible link between the regulation of SPI1 and SPI4 genes (Ahmer et al., 1999). Expression of SiiE has been shown to be regulated by HilA, a central regulator of SPI1 genes (Ahmer et al., 1999; Bajaj et al., 1995; Morgan et al., 2007). This finding is further confirmed through a recent study carried out by Gerlach and his colleagues (Gerlach et al., 2007a) whose data demonstrate that expression of SPI4 genes is coregulated with SPI1 invasion genes by the global regulator SirA and that both SirA and HilA is essential for the secretion of SiiE and SPI4-mediated adhesion. The fact that SPI4 gene expression is controlled by HilA, which is regulated by SirA (Ahmer et al., 1999), also indicates that SPI4 genes are activated under SPI1-inducing conditions – highest activity level was detected when grown at pH 7.4 and during late log/early stationary phase of growth (Gerlach et al., 2007a). Other than HilA and SirA, the various other regulatory proteins (InvF, PhoPQ, OmpR/EnvZ, SlyA, SsrB, RpoS and RpoE) have little to no effect on the expression of SPI4 genes (Gerlach et al., 2007a).

1.3.1.5 SPI5

SPI5 is a 7.6 kB Salmonella-specific DNA region found in serovars like Dublin and Typhimurium (Hong and Miller, 1998; Wood et al., 1998). SPI5 is located at centisome 25 and flanked by serT on one end and copS/copR on the other (Hong and Miller, 1998; Wood et al., 1998). Sequence analysis revealed that this region contains 6 novel genes – pipD, orfX, sopB/sigD, pipC/sigE, pipB and pipA (see Figure 1.2) (Wood et al., 1998).
SPI5-encoded genes have been shown to be involved in intestinal secretory and inflammation responses (but not systemic infection) and mutations in these genes have resulted in attenuation and reduced virulence (Norris et al., 1998; Pfeifer et al., 1999; Wood et al., 1998).

The SPI5-encoded protein, SopB/SigD shares sequence homology with IpgD of *Shigella* spp. and is essential for salmonella enteropathogenicity (Galyov et al., 1997; Hong and Miller, 1998; Norris et al., 1998). SopB is an inositol phosphate phosphatase whose enzyme activity mediates chloride secretion in infected epithelial cells; leading to fluid influx, neutrophil accumulation in the gut and intestinal inflammation; all of which cause diarrhoea (Galyov et al., 1997; Norris et al., 1998). SopB has been shown to be translocated into the host cytosol in a Sip-dependent pathway, indicating that the SPI1 TTS system is responsible for its delivery into host cells (Galyov et al., 1997; Hong and Miller, 1998; Zhang et al., 2002). In addition, the transcriptional organisation of *sopB/sigD-pipC/sigE* suggests that these 2 genes are likely to form an operon and it has been demonstrated that pipC functions as a chaperone for sopB and is able to interact with itself and SopB (Darwin et al., 2001b; Wood et al., 1998).

On the other hand, PipB is translocated by the SPI2 TTS system to both the SCVs and Sifs but is not involved in the formation and maintenance of either of these structures (Knodler et al., 2002). Southern hybridisation studies show that while the left segment of SPI5 (including *sopB*) is found in all *Salmonella* spp. (Mirold et al., 2001), the left segment (including *pipB* and *pipA*) was much less conserved; indicating the mosaic structure and acquisition of SPI5 by horizontal gene transfer (Knodler et al., 2002). Insertional mutations in *pipB* are attenuated in the bovine ligated ileal loop model (Wood et al., 1998) and chicks (Morgan et al., 2004). However, their attenuation in the murine
model have yielded mixed results with some investigators reporting full virulence of mutants (Wood et al., 1998) and others a reduction in virulence (Pfeifer et al. 1999). Knodler and his colleagues (Knodler et al., 2002) have shown the possibility that the reduction in virulence for the pipB mutant could have resulted from polar effects on the pipA gene. This observation would mean that pipBA is likely to be cotranscribed and that it is PipA (and not PipB) that contributes to the development of systemic infection and virulence (Knodler et al., 2002). However, the exact function of PipB and PipA is currently still unknown.

SPI5 encoded effector proteins are able to interact with other virulence effectors to perform a range of virulence-associated functions. SopB has been shown to interact with SPI1 translocated effectors like SipA, SopA, SopD, SopE and SopE2 to induce host cell invasion (Mirold et al., 2001; Raffatellu et al., 2005) and enteropathogenicity (Jones et al., 1998; Zhang et al., 2002), to disrupt tight junction structure and function (Boyle et al., 2006) and mediate actin cytoskeletal rearrangements (Zhou et al., 2001). SipB along with SipB2 associate with the detergent-resistant microdomains (DRM) present on the membranes of SCVs and Sifs, leading to suggestions that the interaction between DRMs and effectors modulate Salmonella-induced signal transduction events in host cells (Knodler et al., 2003).

As SPI5 effectors are translocated by different TTS systems, it is reasonable to assume that the effectors are regulated by the same conditions that control the particular TTS system. As SopB is translocated by the SPI1 TTS system, it has been shown to be expressed upon bacterial invasion and during late log/early stationary phase and is dependent on the global regulator SirA (Hong and Miller, 1998; Pepezeva et al., 2007; Pfeifer et al., 1999). In contrast, PipB is induced intracellularly, during stationary phase
of bacterial growth and is regulated by SsrAB of SPI2 (Knodler et al., 2002; Papezova et al., 2007; Pfeifer et al., 1999).

1.3.1.6 Other SPIs

Salmonella Chromosomal Island (SCI) or SPI6 is a 59 kB region located adjacent to the aspV tRNA gene and contains the saf fimbrial operon, pagN, which encodes an invasin and other genes encoding proteins sharing homology with virulence-associated proteins from other Gram negative bacteria (Folkesson et al., 2002; Hensel, 2004; Parkhill et al., 2001). SPI6 is present in all S. enterica subspecies I but only portions of this region was detected in isolates of subspecies IIIb and IV (Folkesson et al., 2002; Hensel, 2004). SPI7 is a very large genetic locus (134 kB in size) inserted adjacent to tRNA pheU (Hansen-Wester and Hensel, 2002; Pickard et al., 2003; Parkhill et al., 2001). This pathogenicity island appears to be specific to serovars Typhi, Dublin and Paratyphi C and encodes virulence factors like the ViaB proteins, necessary for the synthesis and export of the Vi polysaccharide antigen; and SopE (Hansen-Wester and Hensel, 2002; Hensel, 2004; Pickard et al., 2003). Examples of other SPIs include the S. Typhi-specific SPI8, SPI9 and SPI10 – 6.8, 16.3 and 32.8 kB genetic regions, respectively (Hensel, 2004; Parkhill et al., 2001).

1.3.2 Virulence Plasmids

Although a majority of the Salmonella spp. carry plasmid(s), not all of these plasmids confer a virulence phenotype to the strain (Boyd and Hartl, 1998). Such virulence plasmids have a low-copy number, vary in size (50 to 95 kB) (Akiba et al., 1999; Barrow et al., 1987b; Chu et al., 1999; Haneda et al., 2001) and appear to be limited to S. enterica subspecies I serovars like Typhimurium, Enteritidis, Dublin,
CHAPTER 1

Choleraesius, Gallinarum, Pullorum and Abortusovis (Barrow and Lovell, 1988; Barrow et al., 1987a; 1987b; Guiney et al., 1994; Montenegro et al., 1991; Popoff et al., 1984; Rotger and Casadesús, 1999; Rychlik et al., 2006; Woodward et al., 1989). Virulence plasmids have been shown to be involved in the expression of salmonella virulence (Akiba et al., 1999; Barrow et al., 1987b; 1988; Danbara et al., 1992; Gulig and Curtiss III, 1987; Gulig and Doyle, 1993; Guilloteau et al., 1996; Jones et al., 1982; Fierer et al., 1992 Kawahara et al., 1988; Pardon et al., 1986; Uzzau et al., 2000; Wallis et al., 1995).

All virulence plasmid-containing Salmonella spp. share a highly conserved 8 kbp operon encompassing 5 genes, designated spvRABCD (Guiney et al., 1994; Gulig et al., 1993; Rychlik et al., 2006). It has been demonstrated that this operon encodes the major virulence phenotype of the plasmid as the presence of this 8 kbp region is sufficient to restore wild-type virulence to plasmid-cured strains in the murine model (Guiney et al., 1994; Gulig et al., 1992; Krause et al., 1991; Williamson et al., 1988). While SpvABCD show no significant homology with any known proteins, the 33 kDa SpvR protein shares sequence similarity with the MetR/LysR family of bacterial transcriptional activators (Caldwell and Gulig, 1991; Gulig et al., 1992; Gulig et al., 1993). SpvR is able to activate its own expression and the expression of SpvABCD by binding to a promoter region upstream of spvA (Coynault et al., 1992; Grob and Guiney, 1996; Krause et al., 1992; Krause et al., 1995). SpvR is required for pathogenesis as spvR mutants are attenuated and unable to proliferate in macrophages (Gulig et al., 1992; Krause et al., 1995; Libby et al., 1997; 2000). This is probably due to the fact loss of function in spvR results in the nonactivation of the downstream spvABCD.

Insertional mutations in the individual genes (spvA, spvB, spvC and spvD) have resulted in reduction of virulence and attenuation (Gulig et al., 1992). Localisation
studies by El-Gedaily and his coworkers (El-Gedaily et al., 1997) have shown that SpvA was present in the outer membrane, SpvB in the cytoplasm and inner membrane, SpvC in the cytoplasm and SpvD is not only secreted into the supernatant but also associated with the cytoplasm and membranes. SpvB has been identified as a mono (ADP-ribosyl) transferase that mediates virulence functions like actin degradation and apoptotic cell death and whose translocation is independent of any TTS system (Gotoh et al., 2003; Kurita et al., 2003; Otto et al., 2000; Tezcan-Merdol et al., 2005). By examining a series of spv mutations and cloned spv sequences, Matsui and his colleagues (Matsui et al., 2001) have shown that spvBC was sufficient to replace the spv operon, as well as the entire virulence plasmid, of S. Typhimurium for causing systemic disease in mice after subcutaneous, but not oral inoculation.

It has been suggested that SpvC is required for the functional activity of SpvB and also interacts with spvD for its function, since an overexpression of SpvC can compensate for the absence of spvD (Matsui et al., 2001). This observation seems to be supported by the work carried out by Valone and his coworkers (Valone et al., 1993) who show that a mutation in spvC resulted in loss of expression of all the Spv proteins. Furthermore, it has been demonstrated that none of the Spv proteins were expressed in a spvA mutant and that a spvB mutant only expressed SpvA, indicating that the spv genes interact with one another and may be transcribed as an operon (Valone et al., 1993).

Regulation of spv gene expression is dependent on a number of conditions: genes are induced intracellularly (Fierer et al., 1993; Rhen et al., 1993; Wilson et al., 1997), during stationary growth phase (Coynault et al., 1992; El-Gedaily et al., 1997; Krause et al., 1992; Spink et al., 1994; Valone et al., 1993; Wilson et al., 1997; Wilson and Gulig,
1998), in nutrient limited condition like glucose starvation and low pH (Valone et al., 1993) and in low iron concentrations (Gotoh et al., 2003; Spink et al., 1994; Valone et al., 1993). Valone and his colleagues (Valone et al., 1993) also show that when S. Dublin was grown to late log phase in the absence of glucose, application of heat shock treatment strongly induced expression of SpvA. SpvR is a positive regulator of spvABCD operon and of its own expression (Coynault et al., 1992; Gulig et al., 1993; Krause et al., 1992; Spink et al., 1994). In addition, SpvA appear to regulate SpvR expression, providing a negative feedback mechanism for this gene (Heiskanen et al., 1994; Spink et al., 1994).

The rpoS or katF gene, which encodes the alternative sigma factor RpoS, regulates the stationary phase expression of a wide variety of genes, including the spv operon (Chen et al., 1995; Fang et al., 1992; Heiskanen et al., 1994; Kowarz et al., 1994; Nickerson and Curtiss III, 1997; Norel et al., 1992; Wilson et al., 1997). RpoS mediates expression of spvRABCD by controlling the transcription of spvR, and rpoS/katF mutants have been shown to be less virulent and more susceptible to a range of environmental stresses like nutrient limitation, DNA damage, oxidative and acid stress (Fang et al., 1992; Kowarz et al., 1994; Nickerson and Curtiss III, 1997; Wilson et al., 1997). The PhoP/PhoQ regulatory system also controls rpoS/katF (and thereby spv expression) via the stabilisation of RpoS in low Mg²⁺ concentrations (Tu et al., 2006). Other proteins that have been found to regulate spv expression include polynucleotide phosphorylase (PNPase), integration host factor (IHF) and leucine-responsive regulatory protein (Lrp) (Marshall et al., 1999; Ygberg et al., 2006).

The spv genes are responsible for promoting intracellular survival and replication in the host (i.e. macrophage cytopathology, leading to cell detachment and apoptosis),
thereby leading to development of systemic infection (Guiney et al., 1994; Gulig et al., 1998; Libby et al., 1997; 2000; Paesold et al., 2002). However, this observation has been contradicted by other investigators who have found no correlation between \textit{spv} gene expression and intracellular survival (Gulig and Curtiss III, 1987; Guilloteau et al., 1996; Rhen et al., 1993; Riikonen et al., 1992; Wilson et al., 1997). Therefore, \textit{spv} function in salmonella pathogenesis appears to be more complex than initially thought and requires further study.

Friedrich and his colleagues (Friedrich et al., 1993) analysed a 13.9 kB segment of the 90 kB virulence plasmid from \textit{S. Typhimurium} and reported a 7 kB region containing the \textit{pef} operon, which encodes components of a fimbriae/pili structure (see Section 1.3.3), and \textit{rck}, which mediates serum resistance in \textit{Salmonella} spp. Rck is a 17 kDa OMP that shares homology with virulence-associated proteins like PagC from \textit{Salmonella} spp. and Ail from \textit{Yersinia enterocolitica} (Heffernan et al., 1992a). Rck confers high level resistance to the bactericidal activity of human serum by interfering with the polymerisation of tubular membrane attack complexes on the bacterial surface (Hackett et al., 1987; Heffernan et al., 1992a; 1992b). Rck has also been shown to be important in cell adhesion/invasion (Heffernan et al., 1994). Examples of other plasmid-encoded loci conferring serum resistance include the \textit{traT} and \textit{rsk} genes (Rhen and Sukupolvi, 1988; Vandenbosch et al., 1989a; 1989b).

1.3.3 Bacterial Fimbriae

Bacterial fimbriae are defined as thin, filamentous appendages localised on the cell surface that mediate the attachment of bacteria to host cells (Anon, 1998). To date, 13 fimbrial operons have been identified in the \textit{Salmonella} genome: \textit{agf} (\textit{csf}), \textit{fim}, \textit{bcf}, \textit{lpf},
pef, saf, stb, std, stc, stf, sti and stj (Bäumler and Heffron, 1995; Clegg et al., 1987; Clouthier et al., 1993; Collinson et al., 1996; Doran et al., 1993; Folkesson et al., 1999; Friedrich et al., 1993; McCelland et al., 2001; Müller et al., 1991; Townsend et al., 2001). Genetic analysis have shown that a series of horizontal transfer and deletion events have resulted in the highly variable distribution pattern of fimbrial operons observed among the *Salmonella* serovars (Bäumler et al., 1997; Folkesson et al., 1999). *Salmonella* spp. fimbrial genes have been implicated in virulence *in vitro* and *in vivo* (Althouse et al., 2003; Bäumler et al., 1996a; 1996b; Boddicker et al., 2002; Cogan et al., 2004; De Buck et al., 2004; Dibb-Fuller et al., 1999; Forest et al., 2007; Guo et al., 2007; Humphries et al., 2003; Ledeboer et al., 2006; van der Velden et al., 1998; Weening et al., 2005). Furthermore, several studies have shown that a large number of fimbrial operons often act in concert to elicit various virulence-associated functions (Ledeboer et al., 2006; van der Velden et al., 1998; Weening et al., 2005).

### 1.3.4 Toxins

*Salmonella* spp. is capable of producing both endotoxins and exotoxins (van Asten and van Dijk, 2005). The lipopolysaccharide (LPS) endotoxin is a major component of the outer membrane of many enterobacteria, including *Salmonella* spp. (Hitchcock et al., 1986; Raetz and Whitfield, 2002). Structural analysis of LPS has revealed that it is composed of 3 regions: the core polysaccharide, the O-specific (somatic) antigen polysaccharide side chain and the hydrophobic lipid A (Hitchcock et al., 1986; Raetz and Whitfield, 2002; Todar et al., 2002). Although the core and Lipid A region is conserved among the *Salmonella* genus, great variation exist with the O-antigen chain, forming the basis for the Kaufmann-White serotyping scheme (Fierer and Guiney, 2001; Todar, 2002). LPS forms a layer around the bacterial cell and functions as a permeability
barrier, selectively allowing and excluding substances (Todar, 2002). This enables the Salmonella bacterium to protect itself against the environment (e.g. acid and bile salts in the gastrointestinal tract) and substances (e.g. lysozyme and antimicrobial) found inside the host cell (Fierer and Guiney, 2001; Todar, 2002). Since it is a surface structure, LPS also mediates host-pathogen interaction, making it important in the virulence process (Todar, 2002).

Lipid A is the active portion of LPS and its interaction with the Toll-like receptors during infection activates the host innate immune system and results in the production of substances necessary for inflammatory responses, which at high levels, leads to the development of sepsis and septic shock (Ernst et al., 2001; Khan et al., 1998; Morrison and Ryan, 1987; Raetz, 1993; Raetz and Whitfield, 2002; Todar, 2002). Biosynthesis and assembly of this molecule involves a number of genes, including lpx, pag and pmr (Ernst et al., 2001; Raetz and Whitfield, 2002). Formation of the core region involves the waa operon (Raetz and Whitfield, 2002; Yethon et al., 1998). The rfb and wzz locus is responsible for the synthesis and assembly of monosaccharide units into the O-antigen chain and controlling the length of the polysaccharide chain, respectively (Batchelor et al., 1992; Fierer and Guiney, 2001). Mutations in any of genetic loci above have led to a loss in virulence and/or reduction in complement resistance (Craven, 1994; Licht et al., 1996; Mintz and Deibel, 1983; Murray et al., 2003; 2005; 2006; Nevola et al., 1985; 1987; Shaio and Rowland, 1985; Yethon et al., 2000). Although Salmonella spp. are able to secrete exotoxins – cytotoxins and enterotoxins, not much is known about its exact role and function in pathogenesis (van Asten and van Dijk, 2005).
1.4 Variation in Salmonella Pathogenicity

Although a majority of *Salmonella* strains are pathogenic to humans and animals, their virulence appears to vary from serovar to serovar. *Salmonella* serotypes usually cause gastroenteritis, with a few (e.g. *S. Typhi* and *S. Typhimurium*) able to cause enteric fever and serovars like Dublin and Pullorum more likely to cause bacteraemia to target animals (Fierer and Guiney, 2001; Wallis, 2006). *Salmonella* spp. also have a wide host range: from the ubiquitous serovars like Typhimurium and Enteritidis, to host-restricted (e.g. *S. Dublin* and *S. Choleraesuis*, both capable of causing infection in cattle, pigs and humans) and host-specific ones (e.g. *S. Typhi* and *S. Pullorum*, which are fully adapted to humans and chickens, respectively) (Fierer and Guiney, 2001; Wallis, 2006). As pathogenicity of a serotype is determined by various virulence genes, this diversity can be attributed to genetic variation or polymorphisms in virulence genes.

Since infection is initiated by contact between the host and pathogen, it has been suggested that surface structures such as LPS and fimbriae can contribute to the pathogenic diversity of a strain (Fierer and Guiney, 2001; Wallis, 2006). *Salmonella* serotypes differ structurally in the monosaccharide units that make up the O-antigen chain of the LPS and this is due to the highly polymorphic nature of the *rfb* region, which encodes genes responsible for the biosynthesis of the polysaccharide chain (Fierer and Guiney, 2001; Liu *et al.*, 1991; Verma *et al.*, 1988; Wang *et al.*, 1992; 2002). Genetic variation is also observed with the fimbrial operons of *Salmonella* spp. While the *agf* and *fim* fimbrial cluster is found in all *Salmonella* serotypes, some have been shown to be present in only a small number of serovars (e.g. *pef* and *sef*, whose presence correlate with serotypes frequently isolated from common domesticated animals) and others appear to be serovar-specific e.g. the *S. Typhi* fimbrial operon *tcf* (Bäumler *et al.*, 1997;
Folkesson et al., 1999; Porwollik et al., 2002). Both LPS and fimbriae are located on the bacterial surface and therefore, subjected to selection pressure from the host immune system, leading to the development of genetic variation (Fierer and Guiney, 2001).

Salmonella pathogenicity is affected by large chromosomal islands, termed SPIs that confer virulence to a serotype. SPIs are the result of a single or a series of horizontal gene transfer events and mediated by mobile genetic elements such as integrons, transposons, IS elements and origin of replications (Gal-Mor and Finlay, 2006; Hacker and Kaper, 2000; Hensel, 2004; Hentschel and Hacker, 2001; Marcus et al., 2000). Once inside the bacteria, the SPI regions might undergo genetic modifications (insertion and deletion events, mutations, etc), generating the pathogenic diversity observed in Salmonella spp. Environmental isolates of Salmonella with naturally occurring deletions in the SPI1 region are unable to invade mammalian cells (Ginnocchio et al., 1997). S. bongori, a commensal usually found in cold-blooded animals, rarely causes infections in humans and this can be attributed to the absence of SPI2-TTSS and effector genes from its genome, which renders the serovar unable to cause systemic infection (Boyd et al., 1996; Hansen-Wester et al., 2004; Ochman and Groisman, 1996; Porwollik et al., 2002).

Analysis and comparison across the entire SPI1 to SPI5 region of various Salmonella serotypes have revealed that a range of insertions and deletions were found in the strains (Amavisit et al., 2003; Porwollik et al., 2002). While SPI2 and SPI4 appear to be relatively conserved, great variation is observed with SPI1, SPI3 and SPI5 – a number of genes appear to be absent or divergent amongst the different Salmonella subspecies (Amavisit et al., 2003; Porwollik et al., 2002). It has been reported that only Salmonella subspecies I contain the full complement of genes of all 5 SPIs and that the
variation seen in the SPs is conserved within a serovar (Amavisit et al., 2003; Porwollik et al., 2002). This finding could explain why the subspecies I strains are more able to effectively cause infections in a wide range of hosts (especially warm-blooded animals) in comparison to the other Salmonella subspecies.

Insertion and deletion of various genes is mediated by mobile elements like transposons and IS elements (Dobrindt et al., 2004; Steinert et al., 2000). While a transposon contains both functional genes and transposase, the IS element is a small DNA fragment that encode only the transposase gene (Dobrindt et al., 2004; Steinert et al., 2000). These “jumping genes” are capable of changing chromosomal location, inserting or excising genes as they “jump”. By inserting itself into the sequence of virulence genes, the IS element can inactivate the gene by causing a frameshift, which disrupts gene transcription (Steinert et al., 2000). IS elements have also been associated with the rfb region in several Salmonella serovars, showing that the presence of such mobile genes can indicate genetic polymorphisms in a specific region (Wang et al., 2002). Changes in the genetic sequence such as base insertion, deletion or substitution can also seriously affect expression of virulence-associated genes (Anon, 2008). Such mutations can result in the creation of a stop codon, truncating the protein (nonsense mutation) or translation of a different amino acid (misense mutation), which may alter function of the protein (Anon, 2008).

The virulence plasmid of Salmonella spp. is sometimes referred to as a serovar-specific plasmid (Rotger and Casadesús, 1999). They are usually found in S. enterica subspecies I strains showing host adaptation, implying a possible correlation between the presence of the plasmid and host-specific virulence (Guiney et al., 1994; Rotger and Casadesús, 1999; Woodward et al., 1989). Ahmer and his colleagues (Ahmer et al.,
have determined that the 90 kB virulence plasmid of S. Typhimurium is self-transmissible, allowing for the possibility of new salmonella virulence gene combinations. While the virulence-associated spv operon is located within the virulence plasmid of Salmonella subspecies I, this region is encoded on the chromosome in subspecies II, IIIa and IV (Boyd and Hartl, 1998). It is possible that the presence of spv operon on the chromosome either confers a selective advantage to these subspecies or causes a disadvantage by affecting virulence, in comparison to S. enterica subspecies I (Boyd and Hartl, 1998). A single point mutation leading to the substitution of amino acid glycine for aspartic acid in Rck, another virulence plasmid protein has been shown to reduce the Rck-mediated serum resistance property of Salmonella isolates (Cirillo et al., 1996). Overall, although virulence plasmid are particularly associated with host adapted serovars and may contribute to pathogenic diversity of Salmonella spp., more research needs to be done in order to gain further knowledge regarding the association between the two factors.

1.5 Nontyphoidal Salmonella spp. Associated with Human Hosts

1.5.1 Source

Transmission of Salmonella spp. occurs either via the ingestion of substances contaminated by faeces from human or animal sources or directly from an infected person or animal (Bell and Kyriakides, 2002; Stevenson and Hughes, 1988). The most common way for people to acquire salmonellosis is through the consumption of contaminated food. This usually happens when there is insufficient cooking of raw foods, improper storage of food items and cross-contamination of cooked foods from contact
with surface and utensils used for raw food products (Humphrey, 2000; 2006; Stevenson and Hughes, 1988; Wagner, 2007). High protein foods like meat (e.g. beef, pork, poultry and seafood), eggs and milk are more likely to become Salmonella-contaminated (Stevenson and Hughes, 1988; Wagner, 2007). Salmonella infection can also be acquired through contact with an infected animal, e.g. turtle-associated salmonellosis in the United States (CDC, 2007b).

1.5.2 Disease Symptoms and Treatment

After an incubation period of 6-72 hours (depending on the host and dosage), infected patients generally manifest symptoms like diarrhoea, abdominal pain and cramping, nausea, vomiting and fever (Bell and Kyriakides, 2002; Hohmann, 2001; Humphrey, 2000; Nataro et al., 2007; Stevenson and Hughes, 1988). This is the most common form of salmonellosis – development of gastroenteritis. Occasionally, salmonella infection can occur in the bloodstream, leading to bacteraemia, whose onset results in prolonged spiking fever, malaise, chills, chest and abdominal pains and anorexia (Bell and Kyriakides, 2002; Stevenson and Hughes, 1988). Focal infection of certain tissues (brain, heart valves, etc) can also occur, with local inflammation and abscesses, leading to conditions such as pneumonia, meningitis, endocarditis and osteoarthritis (Bell and Kyriakides, 2002; Stevenson and Hughes, 1988).

Treatment for gastroenteritis usually involves lots of rest, rehydration and electrolyte replacement (Bell and Kyriakides, 2002; Stevenson and Hughes, 1988). Upon recovery, a patient may excrete Salmonella for a period of time – anywhere from the normal (up to 3 months) to the chronic carrier status (persistent shedding of Salmonella, making the individual a great risk to food safety and public health) (Bell and Kyriakides,
2002). Antibiotic treatment is only applied in cases of severe gastroenteritis or for bacteraemia and in focal infections (Bell and Kyriakides, 2002; Hohmann, 2001; Stevenson and Hughes, 1988). However, the issue of treatment of salmonella infection with antibiotic therapy is becoming fairly controversial as use of antibiotics can prolong excretion time of *Salmonella* spp. and due to the emerging problem of antibiotic-resistant *Salmonella* strains (Bell and Kyriakides, 2002; Hohmann, 2001; Stevenson and Hughes, 1988).

### 1.5.3 Epidemiology

Young children, the elderly and patients with chronic illnesses or immunocompromised systems are particularly susceptible to salmonellosis (Bell and Kyriakides, 2002). Infective dose of *Salmonella* bacterium required to overcome host defenses and cause disease varies, usually about $10^5$ to $10^8$ CFU is needed (Humphrey, 2000; 2006). It has been reported that lower numbers of *S. enterica* may be capable of causing outbreaks, especially in cases involving foods with a high fat content (Bell and Kyriakides, 2002; Humphrey, 2000; 2006; Jay *et al.*, 2003). In the United States (USA) alone, an estimated 1.4 million cases of salmonellosis is thought to occur annually, of which about 200 000 cases are reported to the CDC (Lynch *et al.*, 2002; Voetsch *et al.*, 2004). *Salmonella* infection accounts for ~30% (about 400 yearly) of deaths resulting from foodborne illnesses in USA and the most commonly isolated serovars are Typhimurium and Enteritidis (CDC, 2007a; Mead *et al.*, 1999).

Incidence of salmonella infections in the United Kingdom (which have salmonellosis patterns typical of much of the rest of Western Europe) have increased from about 10 000 in 1981 to 33 000 cases in 1997; with the serovars Enteritidis,
followed by Typhimurium and Virchow being identified as the most frequent causative agent of illnesses (Humphrey, 2006). On the other hand, Australian salmonellosis cases have been reported to be largely caused by serotypes Typhimurium (PT9, 135 and 170), followed by Saintpaul and Virchow (Miller et al., 2005; Murray, 1994; Yohannes et al., 2004). Approximately 7000-8000 cases of salmonella infections (about 30-35% of all Australian foodborne illnesses) are reported in Australia annually, and it is estimated to cost as much as 1.2 billion dollars for disease control and treatment (Dalton et al., 2004; Miller et al., 2005; OzFoodNet Working Group, 2003; 2004; 2005; 2006). Such economic losses highlight the importance of improved surveillance and intervention to prevent *Salmonella* spp. infections and outbreaks.

1.6 *Salmonella* spp. Associated with Domesticated Animal Hosts

1.6.1 *Salmonella* spp. in Cattle and Sheep

Salmonellosis affects cattle worldwide and is most commonly associated with the serovars Dublin and Typhimurium (Murray, 1994; Stevenson and Hughes, 1988; Wallis, 2006). S. Typhimurium is considered a broad-range host and hence, is frequently isolated from both cattle and humans. While S. Dublin is capable of causing serious disease in humans, it is a host adapted serovar – it commonly causes gastroenteritis and/or septicaemia in cattle but is rarely isolated from humans (Murray, 1994; Stevenson and Hughes, 1988; Wallis, 2006). Cattle are useful as a model for studying salmonella enteropathogenesis: they are a natural target species for *Salmonella* bacteria and its virulence mechanisms are similar to that in humans (Wallis and Galyov, 2000; Zhang et al., 2003). Several studies conducted on the virulence factors involved in the intestinal
colonisation and inflammation (Jones et al., 1998; Libby et al., 1997; Morgan et al., 2004; 2007; Wallis et al., 1995; Wallis, 2006; Watson et al., 1998; Zhang et al., 2002), and development of systemic disease (Libby et al., 1997; Wallis et al., 1995; Wallis, 2006) in cattle have allowed researchers to gain a better understanding of salmonella pathogenesis in animals and humans.

Ovine salmonellosis has been associated with *Salmonella* serovars Typhumurium, Dublin and Abortusovis (Murray, 1994; Pardon et al., 1988; Uzzau et al., 2001). Serovar Abortusovis is highly specific to sheep, known to cause spontaneous abortions through a mechanism that is not yet fully understood (Pardon et al., 1988; Rubino et al., 1993; Uzzau et al., 2001). It has been reported that *S. Abortusovis* is able to invade ovine intestinal mucosa in a SPI1-dependent manner but does not cause enteritis, leading to the suggestion of the serovar being involved in dissemination and persistence at systemic disease sites (Uzzau et al., 2001). In addition, mutations in the virulence factors of this serovar have shown a reduced virulence in the mouse model (Rubino et al., 1993; Uzzau et al., 2000).

### 1.6.2 *Salmonella* spp. in Pigs

The *Salmonella* serovars most commonly associated with porcine salmonellosis include the ubiquitous *S. Typhimurium* and the host-restricted *S. Choleraesius* (Murray, 1994; Stevenson and Hughes, 1988; Wallis, 2006). *Salmonella* spp. infections in pigs usually results in either enteritis, caused by *S. Typhimurium* or septicaemia, typically caused by *S. Choleraesuis* (Wallis, 2006; Watson et al., 2000). *S. Typhimurium* is able to induce enteritis through adherence and invasion of intestinal epithelial cells (Althouse et al., 2003; Boyen et al., 2006b; Isaacson and Kinsel, 1992) and it has been shown that
the tonsil and lung tissue appear to be important sites for bacterial invasion and dissemination in pigs (Fedorka-Cray et al., 1995; Wood et al., 1989). In contrast, septicaemia appears to be related to the ability of S. Choleraesuis to cause systemic infection (Danbara et al., 1992; Watson et al., 2000). A variety of virulence genes within Salmonella spp. have been determined to be important for virulence in pigs (Althouse et al., 2003; Boyen et al., 2006a; 2006b; Brumme et al., 2007; Carnell et al., 2007; Danbara et al., 1992; Huang et al., 2007; Kawahara et al., 1988; Ku et al., 2005; Lichtensteiger and Vimr, 2003; Uthe et al., 2007).

1.6.3 Salmonella spp. in Horses

Horses of all ages can be affected by salmonellosis and serovars known to cause infection in horses include Typhimurium, Bovismorbificans, Newport and Abortusequi (Akiba et al., 1999; Stevenson and Hughes, 1988). Knowledge on the virulence mechanisms involved in equine salmonellosis is restricted by the limited number of studies investigating these virulence factors in relation to infections in horses. However, Akiba and his colleagues (Akiba et al., 1999) have reported the presence of a single 95 kB plasmid in S. Abortusequi strains, which contain spv genes and confers virulence to this serovar in mice.

1.6.4 Salmonella spp. in Cats and Dogs

Although Salmonella spp. can be isolated from the faeces of dogs and cats, clinical salmonellosis is rare in both these animals (Stevenson and Hughes, 1988). However, there have been reports that domestic pets can acts as a reservoir for Salmonella
strains, facilitating its transmission to humans and other animals, resulting in disease (Cherry et al., 2004; Sato et al., 2000; Van Immerseel et al., 2004; Willard et al., 1987).

1.6.5 *Salmonella* spp. in Poultry

*Salmonella* infections occurring in poultry worldwide have mainly been associated with the serotypes Typhimurium, Enteritidis, Gallinarum and Pullorum (Murray, 1994; Stevenson and Hughes, 1988; Wallis, 2006). Although subspecies II Sofia is the most prevalent serotype amongst Australian poultry, it is avirulent in both chickens and humans and this situation appears to be unique in Australia (see Section 1.7) (Harrington et al., 1991; Heuzenroder et al., 2001; Murray, 1994). The ubiquitous *S. Typhimurium* is able to invade and colonise intestinal tissue and can cause severe systemic infection in young chicks (Barrow et al., 1987a; 1994; Henderson et al., 1999; Kaiser et al., 2000; Wallis, 2006). While *S. Enteritidis* (another broad host range serovar) shares the same pathogenic mechanisms as serovar Typhimurium (Kaiser et al., 2000; Wallis, 2006); this serovar has also been shown to be very efficient in the colonisation of reproductive organs of chickens, which may explain the frequent contamination of eggs by *S. Enteritidis* (Mizumoto et al., 2005; Okamura et al., 2001; Saeed et al., 2006). On the other hand, serotypes Gallinarum and Pullorum are avian-specific and most commonly associated with the ability to produce systemic disease in poultry (Barrow et al., 1994; Buchholz and Fairbrother, 1992; Henderson et al., 1999; Kaiser et al., 2000; Roy et al., 2001; Stevenson and Hughes, 1988; Wallis, 2006).

It has been shown that the genes within SPI1 and SPI2 are involved in both gastrointestinal colonisation and systemic infection of serovars Typhimurium and Enteritidis in chickens (Bohez et al., 2006; Jones et al., 2007; Porter and Curtiss III,
1997; Turner et al., 1998). On the other hand, both S. Gallinarum and S. Pullorum require SPI2 for virulence and persistence in chickens and SPI1 appears to contribute to, but is not essential for virulence or survival in the host (Jones et al., 2001; Wigley et al., 2002). In addition, other virulence factors have also been reported to be important for Salmonella virulence in poultry – virulence plasmid (Bakshi et al., 2003; Barrow et al., 1987b; 1988), fimbriae (Cogan et al., 2004; De Buck et al., 2004; Lee et al., 1996) and LPS (Caroll et al., 2004; Craven, 1994; Turner et al., 1998; Zhao et al., 2002).

1.7 Salmonella Serovar II Sofia

Salmonella II Sofia, which belongs to the S. enterica subspecies II, was first isolated in Australia in the 1970s, where its isolation incidence has continued to increase over the past 3 decades (Harrington et al., 1991; Heuzenroder et al., 2001). The S. II Sofia situation in Australia is unique for a number of reasons: it is the predominant serovar isolated from Australian chickens (see Table 1.1) – around 50 to 60% of Salmonella chicken isolates belong to this group (Heuzenroder et al., 2001). There has been no reported spread to other domesticated animal hosts nor has there been a parallel increase in the isolation of S. II Sofia from humans (Harrington et al., 1991). This high frequency of S. II Sofia in chickens and the very low incidence in humans (<0.2-0.3% of total Salmonella isolates) does not appear to be prevalent elsewhere, although S. II Sofia has been reported in Israel (Harrington et al., 1991; Murray, 1994; Ross et al., 2003). S. II Sofia is not widely as researched compared to other Salmonella serotypes: from its identification in the 1970s until the present day, only a handful of articles have been published, detailing studies conducted on this strain (Harrington et al., 1991; Heuzenroder et al., 2001; Rickard, 1998; Ross et al., 2003). The epidemiological study
Table 1.1 Distribution of *Salmonella* serovars isolated from chickens in Australia from 1987-1992 and 1997-1999.

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Year</th>
<th>Percentage (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Il Sofia</td>
<td>1987-1992</td>
<td>35.7</td>
<td>Murray, 1994</td>
</tr>
<tr>
<td>Typhimurium</td>
<td></td>
<td>19.9</td>
<td></td>
</tr>
<tr>
<td>Anatum</td>
<td></td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Infantis</td>
<td></td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Singapore</td>
<td></td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Il Sofia</td>
<td>1997-1999</td>
<td>55.5</td>
<td>Heuzenroeder <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>Typhimurium</td>
<td></td>
<td>16.9</td>
<td></td>
</tr>
<tr>
<td>4, 12:d:-</td>
<td></td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Kiambu</td>
<td></td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Virchow</td>
<td></td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Singapore</td>
<td></td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Ohio</td>
<td></td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>
carried out by Harrington and her colleagues (Harrington et al., 1991) have shown significant differences exist between the Australian and Israeli S. II Sofia strains.

Harrington and her coworkers (Harrington et al., 1991) also suggested that the Israeli chicken strains appear to be more pathogenic to humans compared to the Australian chicken strains, but were unable to detect the presence of any potential virulence factors (e.g. pili or outer membrane proteins) to support this observation. It is possible that these Israeli strains may have acquired virulence genes but have been unable to maintain them in the genome for long. A more likely explanation would be that the S. II Sofia is avirulent and masking the presence of the “real” pathogen, which would be present in lower numbers due to the low infective dose. Using fluorescent AFLP, Ross and his colleagues (Ross et al., 2003) have shown that while S. II Sofia is genetically variable, clonal groupings persist over time and no dominant clone could be found in the Australian isolates that would explain its persistence and isolation from poultry.

Despite widespread colonisation of Australian poultry, S. II Sofia is avirulent and does not cause disease in either humans or poultry (Harrington et al., 1991; Heuzenroder et al., 2001). The efficient colonisation of a warm-blooded host like the chicken is puzzling as S. enterica subspecies II strains are usually associated with cold-blooded animals such as reptiles (Heuzenroeder et al., 2001). This colonisation property has led to the idea that S. II Sofia can act as a competitive exclusion agent, capable of excluding more pathogenic Salmonella variants from the intestinal tract (Heuzenroeder et al., 2001). However, studies conducted by Heuzenroeder and his coworkers (Heuzenroeder et al., 2001) have found no evidence that S. II Sofia is able to exclude or reduce numbers of pathogenic S. Typhimurium.
Very little is known or understood about pathogenicity of S. II Sofia at a molecular level. It has been shown that while S. II Sofia is able to adhere and invade cultured epithelial cells, the strain is unable to survive intracellularly (Rickard, 1998). However, Rickard (Rickard, 1998) did show that a dosage of $10^7$ bacteria was sufficient to cause systemic infection, leading to mortality (at levels comparable to that of pathogenic S. Typhimurium 82/6915) in 70-95% of the chicken embryos. This finding indicates that S. II Sofia infection is only able to cause systemic disease in hosts with immature or immunocompromised systems (Rickard, 1998). DNA sequence analysis have revealed that S. II Sofia is incomplete within the inv region of SPI1 (lacking invH as well as adjacent genes in the inv operon) and also missing the plasmid borne spv and pef genes (Heuzenroeder et al., 2001). As these genes have been shown to be required for Salmonella pathogenesis (Bäumler et al., 1996a; Darwin and Miller, 1999b; Fierer et al., 1992; Galán and Curtiss III, 1989; Guiney et al., 1994; Gulig et al., 1992; Kubori et al., 1998; Kubori et al., 2000; Sukhan et al., 2001; Watson et al., 1998), their absence could explain the avirulent nature of S. II Sofia. However, more research into this area needs to be done before any conclusions can be made.
1.8 Aims

This research project was designed to study the molecular pathogenesis of the Australian isolates of *Salmonella* serovar II Sofia and has 3 main objectives:

1) To investigate the genetic relationship between S. II Sofia isolates from various locations around Australia using molecular typing methods.

2) To examine the genetic variation and distribution of *Salmonella* pathogenicity islands (SPI1 to SPI5) and virulence plasmid in S. II Sofia in comparison to S. Typhimurium 82/6915.

3) To assess the pathogenic potential of S. II Sofia isolates in Australian poultry.
CHAPTER 2: MATERIALS AND METHODS

2.1 General Procedures

All chemicals used were of analytical and molecular reagent grade. Solutions were prepared in deionised water (dH$_2$O) delivered from a Millipore Milli-Q® water system, unless otherwise specified. Solutions were dispensed using Finnpipette micropipettes (Pathtech Pty Ltd., Australia) with the following volume ranges: 200 µL to 1 mL, 20 µL to 200 µL, 5 µL to 50 µL, 0.5 µL to 10 µL and 0.1 µL to 2 µL.

Glassware was washed using Pyroneg detergent, rinsed in tap water, with a final rinse in dH$_2$O. All glassware, micropipette tips, plasticware, media and solutions used for bacterial, DNA and molecular work was sterilised by autoclaving at 121°C for 20 min, unless stated otherwise. General chemicals and equipment used throughout this study is listed in Appendix I.

2.2 Bacteriological Methods

2.2.1 Media and Antibiotics

2.2.1.1 Antibiotics

Ampicillin and Kanamycin were dissolved in sterile dH$_2$O at a concentration of 100 mg/mL and 50 mg/mL, respectively. Gentamicin sulfate was prepared by dissolving the antibiotic in sterile dH$_2$O to a concentration of 400 mg/mL. All antibiotic stock solutions were sterilised by filtration through a 0.2 µm membrane filter and stored at -20°C.
2.2.1.2 **Luria Bertani Agar (LBA)**

Tryptone (1.0% w/v), yeast extract (0.5% w/v), NaCl (0.5% w/v) and bacteriological agar (1.0% w/v) was dissolved in dH2O and autoclaved under standard conditions. After cooling to 50°C, the agar was poured into Petri dishes and allowed to set. LBA plates were stored at 4°C.

2.2.1.3 **LBA Containing Ampicillin**

LBA was prepared as stated above. After cooling agar to 50°C, ampicillin was added to a final concentration of 100 µg/mL before pouring into Petri dishes. Plates were stored at 4°C.

2.2.1.4 **Luria Bertani Broth (LBB)**

Tryptone (1.0% w/v), yeast extract (0.5% w/v) and NaCl (0.5% w/v) was dissolved in dH2O and autoclaved under standard conditions.

2.2.1.5 **LBB Containing Ampicillin**

LB broth was prepared as stated above. Before use, ampicillin was added at a final concentration of 100 µg/mL.

2.2.1.6 **Malonate Broth**

The following ingredients: (NH4)2SO4 (0.2% w/v), K2HPO4 (0.06% w/v), KH2PO4 (0.04% w/v), NaCl (0.2% w/v), sodium malonate (0.3% w/v), DL-phenylalanine (0.2% w/v), yeast extract (0.1% w/v) and NaCl (0.5% w/v) were dissolved in dH2O by heating. Media was filtered before the addition of bromothymol blue (0.0025% w/v) and autoclaved at 115°C for 20 min.
2.2.1.7 N Minimal Medium

The following ingredients: KCl (5 mM), (NH₄)₂SO₄ (7.5 mM), K₂SO₄ (0.5 mM), KH₂PO₄ (1 mM), 0.1 mM Tris-HCl (pH 7.4), MgCl₂ (10mM) were dissolved in dH₂O and autoclaved under standard conditions. After cooling to room temperature, the required amount of filter-sterilised glucose (0.2% w/v) was added to the solution.

2.2.1.8 ONPG Broth

This broth was made up of 2 components: ONPG solution and peptone water. ONPG (6% w/v) was dissolved in Na₂HPO₄ (0.01 M) at pH 7.5, filter and stored wrapped in foil at 4°C. Bacteriological peptone (0.1% w/v) and NaCl (0.5% w/v) was dissolved in dH₂O, adjusted to pH 7.2 and autoclaved. ONPG solution was aseptically added to the peptone water at a ratio of 1:3 before use.

2.2.1.9 SOC Broth

The following ingredients: tryptone (2.0% w/v), yeast extract (0.5% w/v), NaCl (0.05% w/v), KCl (5 mM), MgSO₄ (10 mM), MgCl₂ (10 mM) and glucose (20 mM) were dissolved in dH₂O, adjusted to pH 7.0 with NaOH and autoclaved under standard conditions. After the addition of glucose, the broth was filter-sterilised and stored at room temperature in aliquots.

2.2.1.10 X-gal Agar

Using LBA plates supplemented with the appropriate antibiotics, 40 µL of both 24 mg/mL IPTG and 40 mg/mL X-gal was spread evenly onto the surface of the agar just before use. These plates were incubated wrapped in foil as X-gal is light sensitive.
2.2.1.11 XLD Agar

XLD agar was prepared by mixing XLD agar powder (5.3 % w/v) with dH₂O and heating the mixture in a microwave oven until completely dissolved. Agar mixture was allowed to cool to 50°C before pouring into Petri dishes. Before use, XLD plates are dried at 37°C for 30 min.

2.2.2 Bacterial Isolates

2.2.2.1 Bacterial Strains Used

The majority of *S. II Sofia* isolates used in this study were supplied by Inghams Enterprises Pty Ltd and are listed in Table 2.1. The *S. II Sofia* strains were isolated from chicken flocks from different states around Australia. Other *Salmonella* spp. used in this study are listed in Table 2.2. The *Escherichia coli* strain DH5α was utilised as a host for pCR2.1 and pWSK29 (see Section 2.3.8.4 and 2.3.8.5). *Salmonella* constructs were first passaged into *S. Typhimurium* LT2 9121 (to stabilise constructs and for restriction modification) before being inserted into *S. II Sofia* Bt6 (refer to Section 2.3.8.4 and 2.3.8.5).

2.2.2.2 Bacterial Culture Conditions

All bacteria were grown on LBA or in LBB and incubated aerobically at 37°C overnight (16-18 h), unless specified otherwise. Cultures grown in LBB were placed on a rotary shaker for appropriate periods. All bacterial analysis was carried out on culture no older than 8 passages.
Table 2.1  *Salmonella* II Sofia isolates used in this study.

<table>
<thead>
<tr>
<th>Strain Identification</th>
<th>Location/Origin</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bt1-3, 6, 8, 13-16 and 18</td>
<td>Victoria</td>
<td>Biotechnology Laboratory, RMIT University, Melbourne, VIC</td>
</tr>
<tr>
<td>FSAW 3451-3455</td>
<td>Victoria</td>
<td>Dr. Alvin Lee, Food Science Australia, Werribee, VIC</td>
</tr>
<tr>
<td>826V, 838V, 842V, 846V, 856V, 858V, 9VCA, 10VCA, 985VCA, 987VCA, 992VCA, 620MSC, 627MSC, 629SMC, 630MSC, 632MSC, 633MSC, 637MSC</td>
<td>Victoria</td>
<td>Inghams Enterprises Pty Ltd., Springwood, QLD</td>
</tr>
<tr>
<td>539NC, 561NC, 566NC, 568NC, 537NCA, 538NCA, 541NCA, 554NCA, 561NCA, 566NCA, 567NCA, 569NCA, 768XTA</td>
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<td>Inghams Enterprises Pty Ltd., Springwood, QLD</td>
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<td>786CL, 963Q, 965Q, 985Q, 986Q, 988Q, 336QC, 339QC, 348QC, 360QC, 362QC, 365QC, 366QC, 403QCA, 404QCA</td>
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<td>Inghams Enterprises Pty Ltd., Springwood, QLD</td>
</tr>
<tr>
<td>146TC, 147TC, 148TC, 150TC, 140TCA, 146TCA, 147TCA, 148TCA, 150TCA</td>
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<td>Inghams Enterprises Pty Ltd., Springwood, QLD</td>
</tr>
<tr>
<td>131</td>
<td>Unknown</td>
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</tbody>
</table>
Table 2.2 Other *Salmonella* spp. isolates used in this study.

<table>
<thead>
<tr>
<th>Isolate Serotype</th>
<th>Strain Identification</th>
<th>Location/Origin</th>
<th>Source</th>
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<td>FSAW 3416, 3463</td>
<td>-</td>
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</tr>
<tr>
<td>Cholaerasius</td>
<td>Braenderup H9812 (ATCC BAA664)</td>
<td>-</td>
<td>Libby Grabsch, Department of Microbiology, Austin Health, VIC</td>
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<tr>
<td>Enteritidis</td>
<td>446302</td>
<td>-</td>
<td>Biotechnology Laboratory, RMIT University, Melbourne, VIC</td>
</tr>
<tr>
<td>Hadar</td>
<td>P12</td>
<td>-</td>
<td>Biotechnology Laboratory, RMIT University, Melbourne, VIC</td>
</tr>
<tr>
<td>Heidelberg</td>
<td>-</td>
<td>-</td>
<td>Biotechnology Laboratory, RMIT University, Melbourne, VIC</td>
</tr>
<tr>
<td>Infantis</td>
<td>999S</td>
<td>South Australia</td>
<td>Inghams Enterprises Pty Ltd., Springwood, QLD</td>
</tr>
<tr>
<td>Kiambu</td>
<td>343W, 345W</td>
<td>West Australia</td>
<td>Inghams Enterprises Pty Ltd., Springwood, QLD</td>
</tr>
<tr>
<td>Ohio</td>
<td>213N</td>
<td>New South Wales</td>
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<td>Typhimurium</td>
<td>82/6915</td>
<td>-</td>
<td>Biotechnology Laboratory, RMIT University, Melbourne, VIC</td>
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<tr>
<td></td>
<td>LT2 9121</td>
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<td></td>
<td>ATCC14028</td>
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<td>Inghams Enterprises Pty Ltd., Springwood, QLD</td>
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<tr>
<td></td>
<td>370QC</td>
<td>Queensland</td>
<td>Inghams Enterprises Pty Ltd., Springwood, QLD</td>
</tr>
</tbody>
</table>
2.2.2.3 Storage of Stock Cultures

Overnight bacterial cultures were mixed with sterile 100% glycerol at a ratio of 1:1, placed into cyrovials and stored frozen at -70°C. Bacterial stocks were reconstituted by scraping the surface of frozen culture with a heated loop and streaking onto a LBA plate followed by overnight incubation at 37°C.

2.2.2.4 Estimation of Bacterial Cell Concentration

*Salmonella* cell culture densities were estimated by measuring absorbance \(A_{600}\) at 600 nm and calculating CFU. The suspension solution (without any bacterial cells) was used as a blank for absorbance readings. Concentrations were confirmed by serial dilutions of bacteria and plating onto LBA plates.

2.2.3 Bacterial Profiles

Biochemical testing was carried out to confirm the identity of *S. Il* Sofia isolates used in this study. The appropriate bacteria were used as negative/positive controls throughout the testing. All strains were streaked onto XLD agar and incubated overnight at 37°C. Biochemical tests for malonate and ONPG were performed as described in Cowan and Steel's Manual for Identification of Medical Bacteria (Shaw and Clarke, 1955; Lowe, 1962). The results are listed in Appendix II and confirm that all the bacteria received were *S. Il* Sofia isolates. The bacterial isolates were also confirmed as *Salmonella* spp. and serogrouped with mono- and polyvalent *Salmonella* agglutinating sera, according to the manufacturer's instruction. The results are listed in Appendix II.
2.3 DNA Methods

2.3.1 Buffers and Reagents

2.3.1.1 Anti-DIG Antibody Solution

Anti-DIG antibody (1:5000 Dilution) in 1% blocking solution.

2.3.1.2 Blocking Reagent Stock Solution

Blocking reagent (10% w/v) was dissolved in 1x maleic acid buffer (pH 7.5) and heated at low heat for 15 min with constant stirring. Once completely dissolved, the mixture was autoclaved and stored at 4°C.

2.3.1.3 1% Blocking Solution

10% Blocking reagent stock solution diluted with 1x maleic acid buffer (pH 7.5).

2.3.1.4 Chloroform/Isoamyl Alcohol (24:1)

Chloroform was mixed with isoamyl alcohol at a ration of 24:1.

2.3.1.5 CTAB Solution

NaCl (0.7 M) and CTAB (10% w/v) was mixed in sterile dH₂O with constant heating and stirring until completely dissolved.

2.3.1.6 Detection Buffer (pH 9.5)

100 mM Tris-HCl and 100 mM NaCl dissolved in dH₂O and adjusted to pH 9.5 with 20 M NaOH.
2.3.1.7 Denaturation Solution

NaOH (0.5 M) and NaCl (1.5 M) dissolved in dH₂O.

2.3.1.8 Depurination Solution

HCl (0.25 M) diluted in dH₂O.

2.3.1.9 70 % Ethanol

Ethanol (100% v/v) diluted in sterile dH₂O.

2.3.1.10 E buffer (pH 7.9)

Tris-acetate (40 mM) and EDTA (2 mM) was dissolved in dH₂O, adjusted to pH 7.9 with glacial acetic acid and autoclaved.

2.3.1.11 0.5 M EDTA

EDTA (0.5 M) was dissolved in dH₂O, adjusted to the appropriate pH with NaOH and autoclaved.

2.3.1.12 ESP Solution (PFGE Lysis Buffer)

EDTA (0.5 M, pH 9), sarkosyl (1% w/v) and proteinase K (100 µg/mL) dissolved in sterile dH₂O and stored at -20°C.

2.3.1.13 Frozen Storage Buffer (FSB)

- MnCl₂·4H₂O 45 mM
- CaCl₂·2H₂O 10 mM
KCl 100 mM
Hexaminecobalt chloride 3 mM
Potassium acetate 10 mM
Glycerol 10% v/v

All ingredients were dissolved in sterile dH2O, adjusted to pH 6.4, filter sterilise and stored at 4°C.

2.3.1.14 HEPES Running Buffer

HEPES-NaOH (16 mM), sodium acetate (16 mM) and 0.8 mM EDTA (pH 7.5) was dissolved in dH2O and autoclaved.

2.3.1.15 IPTG Stock Solution

IPTG (24 mg/mL) was dissolved in sterile dH2O, filter sterilised and stored at -20°C.

2.3.1.16 λDNA (PstI and HindIII) marker

λDNA 20 µg
RE buffer 1x
PstI 100U
sterile dH2O variable

All ingredients were mixed and incubated overnight at 37°C. A volume of 40 µL of sterile dH2O and 20 µL of 11x loading dye was added into the mixture before being stored at -20°C in aliquots. This was used as a molecular weight marker at a final concentration of 100 ng/µL (Appendix IV).
2.3.1.17 11x Loading Buffer

Ficol-400 (10% v/v), Orange G dye (0.5% w/v), SDS (1% w/v), glycerol (50% w/v), 50 mM Tris-HCl (pH 8.0) and EDTA (10 mM) were dissolved in sterile dH₂O and stored at room temperature.

2.3.1.18 Lysis Solution (Plasmid Extraction)

Tris-base (50 mM) was dissolved in dH₂O, adjusted to pH 12.6 and autoclaved. SDS (3% w/v) was added to the solution.

2.3.1.19 10× Maleic Acid Buffer Stock solution

Maleic acid (1 M) and NaCl (1.5 M) dissolved in dH₂O, adjusted to pH 7.5 with NaOH. This was diluted to 1× and autoclaved before use.

2.3.1.20 Maleic Acid Wash Buffer

Tween® 20 (0.3% w/v) in 1× maleic acid buffer.

2.3.1.21 Neutralisation Solution

Tris-HCl (0.5 M, pH 7.5) and NaCl (3 M) dissolved in dH₂O.

2.3.1.22 1× PBS

To obtain a final concentration of 1×, one PBS tablet was dissolved in 100 mL dH₂O and autoclaved.
2.3.1.23 Phenol/Chloroform/Isoamyl Alcohol (25:24:1)

Phenol and Chloroform/Isoamyl alcohol was mixed at a ration of 1:1 in a sterile bottle or plastic tube (wrapped in aluminium foil to keep out light) and stored at 4°C.

2.3.1.24 PIV Buffer

Tris-base (10 mM) and NaCl (1 M) dissolved in deionised H₂O, adjusted to pH 8.0, and autoclaved.

2.3.1.25 Proteinase K Stock Solution

Proteinase K (20 mg/mL) was dissolved in sterile dH₂O in a sterile microcentrifuge tube and stored at -20°C.

2.3.1.26 RNase Stock Solution (DNase-free)

Bovine pancreatic RNase (10 mg/mL) was dissolved in sterile dH₂O and boiled for 5 min to denature any contaminating DNAses. The stock solution was then stored at -20°C.

2.3.1.27 Standard Hybridisation Buffer (Pre-hybridisation Buffer)

5× SSC, sarkosyl (0.1% w/v), SDS (0.02% w/v) and blocking solution (1% w/v) were diluted in sterile dH₂O and stored at -20°C.

2.3.1.28 3M Sodium Acetate (pH 4.6)

Sodium acetate (3 M) was dissolved in dH₂O, adjusted to pH 4.6 with glacial acetic acid and autoclaved.
2.3.1.29 5 M NaCl
NaCl (5 M) was prepared with dH₂O and autoclaved.

2.3.1.30 20X SSC
NaCl (3 M) and sodium citrate (0.3 M) dissolved in dH₂O, adjusted to pH 7.0 and autoclaved.

2.3.1.31 2X SSC Wash Buffer
SSC (2X) and 0.1% SDS diluted in sterile dH₂O.

2.3.1.32 0.1X SSC Wash Buffer
SSC (0.1X) and 0.1% SDS diluted in sterile dH₂O.

2.3.1.33 Solution I
Glucose 50 mM  
Tris-HCl 25 mM  
EDTA 10 mM  
All ingredients were dissolved in dH₂O, autoclaved and stored at 4°C.

2.3.1.34 Solution II
NaOH (0.2 M) and SDS (1% w/v) dissolved in sterile dH₂O. This solution is prepared fresh before every use.
2.3.1.35 Solution III

Potassium acetate (5 M), 11.5 mL of glacial acetic acid were dissolved in 28.5 mL of dH₂O, autoclaved and stored at 4°C.

2.3.1.36 Substrate Solution

NBT/BCIP substrate solution (200 µL) diluted in 10 mL of detection buffer.

2.3.1.37 50× TAE Buffer

Tris-base (24.2% w/v), glacial acetic acid (5.17% w/v) and EDTA (1.86% w/v) were dissolved in deionised water. This was diluted to 1X before use.

2.3.1.38 5× TBE Buffer

Tris-base (0.445 M), boric Acid (0.445 M) and EDTA (12.5 mM, pH 8.0), dissolved in H₂O and autoclaved. This was diluted to 0.5× before use.

2.3.1.39 TE buffer

Tris-base (10 mM) and EDTA (1 mM) was dissolved in water. The pH was adjusted as required with HCl and solution was then autoclaved.

2.3.1.40 X-gal Stock Solution

X-gal (40 mg/mL) was dissolved in DMF and stored at –20°C.
2.3.2 DNA Isolation Techniques

2.3.2.1 Chromosomal DNA Extraction

Bacterial chromosomal DNA was isolated using the CTAB method as described in Current Protocols In Molecular Biology (Volume 1) (Reichardt and Rogers, 1994). Cultures were grown overnight in 5 to 10 mL LBB at 37°C. Bacterial cells were obtained by centrifugation at 4000 × g for 10 min. Cell pellets were resuspended in 565 µL of TE (pH 8.0) in a sterile microcentrifuge tube. Lysis of cells was achieved by the addition of SDS and proteinase K to a final concentration of 0.5% and 100 µg/mL, respectively. Tubes were mixed by inversion and incubated at 37°C for 1h. 100 µL of 5 M NaCl and 80 µL of CTAB was added and after mixing thoroughly, the tubes were incubated at 65°C for 15 min.

An approximately equal volume of chloroform/isoamyl alcohol was added and the mixture was centrifuged. The aqueous phase was recovered and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (PCI). In both cases, phase separation was achieved by centrifugation at 10 000 × g for 10 min. DNA was precipitated by the addition of 0.6 volumes of isopropanol. Precipitated DNA was collected by centrifugation at 10 000 × g for 10 min. DNA pellets were washed in 70% ethanol before being resuspended in 20-50 µL of TE or sterile dH2O. All isolated chromosomal DNA was stored frozen at -20°C.

2.3.2.2 Plasmid Miniprep

Plasmid miniprep was performed using the method described by Sambrook and Russell (Sambrook and Russell, 2001). Cultures were grown overnight in 3 mL LBB at 37°C. Bacterial cells were obtained by centrifuging 1.5 mL of the overnight culture at 4000 × g for 10 min. Cells were washed with 500 µL of sterile 1× PBS and after centrifugation, the cell pellets were
resuspended in 100 µL of ice-cold Solution I. To this, 200 µL of ice-cold Solution II was added and after mixing the tubes by inversion, the mixture was incubated on ice for 5 min. A 150 µL volume of Solution III was added and the samples were mixed by inversion and incubated on ice for another 5 min.

Cell debris and proteins were pelleted by centrifugation at 16,100 × g for 5 min at room temperature. The supernatant was removed and extracted with an equal volume of PCI. Phase separation after the addition of PCI was achieved by centrifugation as stated above. Plasmid DNA was precipitated by the addition of 2 volumes of 100 % ethanol and 0.1 volumes of sodium acetate (pH 4.6). The samples were mixed, incubated at room temperature for 2 min and then centrifuged as stated above. DNA pellets were rinsed with 1 mL of 70% ethanol, air dried for 5-10 min and resuspended in 20-50 µL of sterile dH2O. All plasmid DNA isolated were stored at -20°C.

2.3.2.3 Plasmid Midiprep

To obtain larger amounts of DNA from larger volumes of culture, plasmid midiprep was carried out according to the miniprep method described above with the following scaled-up volumes:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultures</td>
<td>10 mL LBB</td>
</tr>
<tr>
<td>Sterile 1× PBS</td>
<td>1 mL</td>
</tr>
<tr>
<td>Solution I</td>
<td>200 µL</td>
</tr>
<tr>
<td>Solution II</td>
<td>400 µL</td>
</tr>
<tr>
<td>Solution III</td>
<td>350 µL</td>
</tr>
</tbody>
</table>
Cell debris and proteins were pelleted by centrifugation at 16 100 × g for 10 min at room temperature. The supernatant (600 µL) was removed and extracted with an equal volume of PCI. Phase separation was achieved by centrifugation as stated above. Plasmid DNA was precipitated by the addition of an equal volume of isopropanol and 0.1 volumes of sodium acetate (pH 4.6). The samples were mixed, incubated at room temperature for 2 min and then centrifuged as stated above. DNA pellets were rinsed with 1 mL of 70% ethanol, air dried for 5-10 min and resuspended in 20-50 µL of sterile dH₂O. All plasmid DNA isolated were stored at -20°C.

2.3.2.4 Plasmid Extraction (Kado and Liu Method)

Isolation of large plasmids was carried out as described by Kado and Liu (Kado and Liu, 1981) with some modifications. Cultures were grown overnight at 37°C in 10 mL LBB. A 5 mL volume of culture was centrifuged at 4000 × g for 10 min and the pellet was resuspended in 200 µL of E buffer. Lysis of cells was achieved by the addition of 400 µL of lysis solution and incubation at 60°C for 1 hr. Sample was then extracted with 900 µL of PCI and cell debris and proteins were pelleted by centrifugation at 16 100 × g for 6 min. The aqueous phase was recovered and PCI extraction was repeated until no protein precipitate occurred at the phase interface. A 500 µL volume of chloroform was added and the sample was centrifuged as stated above. The upper aqueous phase containing the plasmid DNA was collected and stored at -20°C.

2.3.2.5 Purification of DNA from Agarose Gel

Purification of DNA from agarose gel was performed with the Geneclean kit by following the manual provided by the manufacturer. Briefly, the desired DNA band was excised from the
gel and weighed. Three gel volumes of sodium iodide was added to the gel slice and the mixture was incubated at 55°C until the agarose was melted. An appropriate amount of Glassmilk suspension was mixed with the sample and allowed to stand at room temperature for 5-15 min, with frequent agitation. The Glassmilk was centrifuged at 16,100 × g for 30s and the supernatant was discarded. The Glassmilk was washed twice with New Wash solution provided in the kit and recovered by centrifugation at 16,100 × g for 30s. Glassmilk pellet was then dried at 55°C for 10 min. DNA was eluted from Glassmilk by resuspending pellet with 10-50 µL of sterile dH₂O and collecting the dH₂O after centrifugation at 16,100 × g for 1 min. All purified DNA samples were stored frozen at -20°C.

2.3.2.6 Estimation of DNA Concentration

Concentration of isolated DNA was determined using a spectrophotometer to measure absorbance at 260 and 280 nm. The DNA concentration was calculated by using the ratio of 1 O.D. to 50 µg/mL of dsDNA and taking the dilution factor into account. Alternatively, amount of DNA was measured by running the sample on an agarose gel and comparing band intensity with the intensity of DNA marker.

2.3.3 PCR

2.3.3.1 Design of Primers

Primers were designed with the Clone Manager 7 software using the normal criteria for primer design. When RE sites were incorporated, an additional 4 bp were added to the 5‘-end of the primer sequence to allow the binding of the RE.
2.3.3.2 General PCR Amplification

PCR was carried out according to the protocol below, unless specified otherwise:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× PCR buffer</td>
<td>1×</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>200 µM</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.25 µM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.25 µM</td>
</tr>
<tr>
<td>DNA template</td>
<td>100 ng</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.25U</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>to a total volume of 25 µL</td>
</tr>
</tbody>
</table>

The reaction mixture was subjected to the following cycling conditions, unless stated otherwise: 1 cycle of 95°C for 5 min followed by 35 cycles of 94°C for 45 s, annealing temperature for 1 min and 72°C for 1 min, with a final extension time of 72°C for 10 min. All PCR products were stored at either 4°C (short term) or -20°C (long term).

2.3.3.3 General Expand Amplification

PCR was carried out according to the protocol below, unless specified otherwise:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× Expand Buffer 3</td>
<td>1×</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>350 µM</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>DNA template</td>
<td>200-400 ng</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.25U</td>
</tr>
</tbody>
</table>
STERILE DH₂O TO A TOTAL VOLUME OF 50 µL

The reaction mixture was subjected to the following cycling conditions (Amavisit et al., 2003):

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>92°C</td>
<td>3 min</td>
</tr>
<tr>
<td>92°C</td>
<td>10s</td>
</tr>
<tr>
<td>Annealing Temperature</td>
<td>30 s</td>
</tr>
<tr>
<td>68°C</td>
<td>Variable</td>
</tr>
<tr>
<td>92°C</td>
<td>10 s</td>
</tr>
<tr>
<td>Annealing Temperature</td>
<td>30 s</td>
</tr>
<tr>
<td>68°C</td>
<td>Variable (+ 10 s per cycle)</td>
</tr>
<tr>
<td>68°C</td>
<td>7 min</td>
</tr>
</tbody>
</table>

All PCR products were stored at either 4°C (short term) or -20°C (long term).

2.3.3.4 DNA Sequencing Reactions

DNA sequencing was carried out according to the protocol below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing mix</td>
<td>2 µL</td>
</tr>
<tr>
<td>5x Dilution buffer</td>
<td>3 µL</td>
</tr>
<tr>
<td>Forward primer (3 µM)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Reverse primer (3 µM)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Purified DNA template</td>
<td>100-1000 ng</td>
</tr>
<tr>
<td>Sterile DH₂O</td>
<td>to a total volume of 20 µL</td>
</tr>
</tbody>
</table>

The reaction mixture was subjected to the following cycling conditions: 1 cycle of 96°C for 1 min followed by 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min.
Dye-labeled DNA was precipitated by the addition of 0.1 volumes of sodium acetate (pH 4.6) and 2.5 volumes of 100% ethanol. Tubes were vortexed, allowed to stand at room temperature for 10 min. and centrifuged at 16100× g for 20 min. After discarding the supernatant, pellets were rinsed twice with 250 µL of 70% ethanol and air dried before being sent for sequencing analysis at the Micromon facility at Monash University, Melbourne, Australia.

2.3.4 Agarose Gel Electrophoresis

2.3.4.1 Agarose Gel Preparation

Agarose gels for electrophoresis of DNA samples were prepared by boiling required amount of agarose in 1× TAE (50 mL for minigels, 100 mL for midigels and 300 mL for maxigels) until fully dissolved. After cooling to 50°C, the mixture was poured into a casting tray and allowed to set.

2.3.4.2 Sample Preparation

Prior to running the gel, a 1/10 volume of 11× loading dye was added to the sample.

2.3.4.3 Electrophoresis

Samples were mixed with dye and loaded into wells of the gel. Electrophoresis was carried out using the power supply units at the required voltage and appropriate time periods.
2.3.4.4  **Staining of Agarose Gels**

All DNA gels were stained after electrophoresis in a 1 mg/mL ethidium bromide solution for about 5-10 min and detained by washing in running tap water.

2.3.4.5  **Visualisation of DNA**

DNA gels were visualized with the UV transilluminator and photographed using Gel-Doc System.

2.3.5  **Restriction Enzyme (RE) Digestion**

All RE digestions were carried out with the following reagents, unless specified otherwise:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× RE buffer</td>
<td>1×</td>
</tr>
<tr>
<td>BSA</td>
<td>100 ng/µL</td>
</tr>
<tr>
<td>DNA</td>
<td>Variable</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>Variable</td>
</tr>
<tr>
<td>RE (10-12U/µL)</td>
<td>5-10U</td>
</tr>
</tbody>
</table>

The digest reaction was incubated for 2-16 h at 37°C and stored at -20°C.

2.3.6  **Pulsed-Field Gel Electrophoresis (PFGE)**

**2.3.6.1  Conventional PFGE**

**2.3.6.1.1  Preparation of bacterial plugs**

PFGE protocol used in this study was adapted from the method of Smith and Cantor (Smith and Cantor, 1987). One colony from LBA was inoculated into 5 mL LBB and incubated
with shaking at 37°C for about 3 h. Cells were harvested by centrifugation at 4000 × g for 10 min and washed in 1 mL of TE (pH8.0). Cells were centrifuged again and resuspended in 400 µL of PIV buffer on ice. The suspension was mixed with an equal amount of 2% low melting point agarose (dissolved in PIV) and pipetted into perspex moulds. Plugs were allowed to set at 4°C for 20 min before being placed in 2 mL of ESP solution and incubated overnight at 50°C. After overnight lysis, plugs were incubated in PMSF-TE solution (made fresh before very use) for 2 h at 37°C. Plugs were then placed in 2-3 mL of TE and incubated for 1 h with gentle shaking at room temperature. This is followed by 3 additional washes in TE of 20 min each. All plugs were stored in TE buffer at 4°C for up to 6 months.

2.3.6.1.2 Restriction digests of plugs and PFGE

A 1 mm portion of DNA plug was cut and placed in 100 µL of RE solution, containing the appropriate RE (30U of XbaI or 20U of SpeI), 1× RE buffer, BSA (100 ng/µL) and sterile dH₂O. DNA was digested 6 h at 37°C. Following RE digestion, the DNA plugs were inserted into the wells of 1.2% agarose midigel (dissolved in 0.5× TBE) and sealed with molten agarose. The gel was placed in the electrophoretic chamber with 2.5 L of 0.5× TBE buffer and subjected to electrophoresis (Bio-rad CHEF DR II system), with PFGE λ ladder as a reference marker (see Appendix III). The electrophoretic conditions for XbaI digestion: initial switch time of 2.2 s, final switch time of 54.1 s and run time of 22 h (Gautom, 1997). The electrophoretic conditions for SpeI digestion: pulse ramped time of 4 to 40 s for duration of 24 h (Tsen et al., 2001). Both PFGE conditions were performed at 14°C and a constant voltage of 6.0 V/cm. PFGE gel was stained and visualized as outlined in Section 2.3.4.5.
2.3.6.2 Formaldehyde Treatment of Cells

Inactivation of possible DNase activity in *S. II Sofia* isolates was performed as described by Gibson and colleagues (Gibson et al., 1994) with some minor modifications. After harvesting the cells, cell pellets were resuspended in 100 µL of formaldehyde and incubated on ice for 1 h. Cell suspension was then centrifuged and washed 3 times with PBS to remove excess formaldehyde. Formalin-treated cells were then processed according to the PFGE protocol stated above.

2.3.6.3 Modifications to PFGE Running Buffer

Two methods involving alterations to the running buffer to prevent DNA degradation in PFGE was tested and in both cases, PFGE plugs were prepared as outlined above. Addition of 100 µM thiourea to 0.5× TBE buffer was carried out according to method of Leisegang and Tschäpe (Leisegang and Tschäpe, 2002). Replacement of 0.5× TBE with HEPES as a running buffer was performed as described by Koort and colleagues (Koort et al., 2002).

2.3.7 DNA-DNA Hybridisation

2.3.7.1 Probe Synthesis, Labelling and Estimation

All DNA probes were synthesized from *S. Typhimurium* SL1344 by general or expand PCR (genes used as probes are outlined in the methods section of the relevant chapters). DNA was labeled using the random primed method according to the manufacturer’s protocol. Briefly, 3 µg of DNA was diluted to 15 µL with sterile dH2O, heat-denatured at 100°C for 10 min and quickly chilled on ice. Hexanucleotide mix (2 µL), DIG labeling mix (2 µL) and Klenow enzyme (1 µL) was added and the reaction was incubated overnight at 37°C. Labeling reaction was terminated by the addition of 2 µL of EDTA.
Yield of DIG-labeled DNA was estimated with a spot test using DIG-labeled controls. The DIG-labeled control DNA was diluted to 1 ng/µL. From this stock, five 10-fold serial dilutions were prepared, creating a set of standards ranging from 1 ng/µL to 0.1 pg/µL. Using the table from the manufacturer’s manual, the expected yield of labeled probes was obtained. The experimental probe was then diluted to approximately 1 ng/µL and from this stock, a series of dilutions were made.

Labeled controls and experimental probe dilutions were spotted parallel to one another on a piece of nylon membrane. The DNA was fixed to the membrane by cross-linking with UV light for 5 min. Membrane was then washed briefly in washing buffer before being incubated in 1% blocking solution, followed by anti-DIG antibody solution for 30 min each. The membrane was washed twice for 15 min each with washing buffer at room temperature (to remove any unbound antibody). The membrane was equilibrated in detection buffer for 2 min and then incubated in NBT/BCIP substrate in the dark to develop the colour. Substrate reaction was stopped by washing the membrane with sterile dH₂O. Spot intensities of the control and experimental dilutions were compared to determine probe concentration.

2.3.7.2 Southern Blot Procedure

About 15-20 µg of DNA was digested with 10U of the appropriate REs and subjected to electrophoresis in a 1% midigel for 2 hrs at 70V. Following electrophoresis, the gel was depurinated in 0.25 M HCl for 10 min and then rinsed in water (to make fragments larger than 10 kb easier to transfer into the membrane). The gel was incubated in denaturation solution twice for 15 min each, followed by two 15 min washes in neutralisation solution. Capillary transfer reaction was employed to blot DNA from the gel onto the membrane, using 20× SSC
buffer. This reaction was allowed to proceed for approximately 16-18 h to ensure efficient transfer of DNA.

The DNA was cross-linked to the membrane as specified above. The blot was placed in pre-hybridisation buffer and incubated for 2 h at 65°C. The labeled probe was diluted in standard hybridisation buffer to a concentration of 20 ng of DIG-labeled probe per mL of buffer. The probe solution was denatured by heating at 95°C for 10 min before being added to the membrane and incubated overnight at 65°C. The membrane was washed twice with 2×SSC wash buffer for 5 min each at room temperature, followed by two 15 min washes in 0.5× SSC wash buffer at 68°C (0.1× SSC was used when more stringent wash conditions was required). After equilibration in maleic acid wash buffer, the membrane was incubated in 1% blocking solution for 1 h at room temperature. This was then replaced with anti-DIG antibody solution and incubation was continue for another 30 min. Unbound antibody was removed by washing the membrane twice in maleic acid wash buffer for 15 min each. The blot was equilibrated in detection buffer before finally, being incubated in the dark with substrate solution for colour development.

2.3.8 Recombinant DNA Techniques

2.3.8.1 Synthesis and Cloning of PCR Products with pCR®2.1

The gene(s) of interest was amplified using Expand PCR system and DNA from S. Typhimurium 82/6915 (details of the experiment are outlined in Chapter 5). The PCR product was then analysed by electrophoresis and the concentration was estimated. The gene(s) was inserted into the plasmid pCR®2.1 according to the manufacturer’s manual. Briefly, the following ligation reaction was set up: fresh PCR product (~100-300 ng), 10× Ligation buffer (1×),
pCR®2.1 vector (50 ng), T4 DNA ligase (2 Weiss U) and sterile dH₂O to a total volume of 10 µL. The ligation mix was incubated overnight at 16°C and transformed into competent *E. coli* cells, as outlined below.

### 2.3.8.2 Restriction Enzyme Digestion

The appropriate RE digest of vector and/or insert was prepared as stated in Section 2.3.5. The digest reaction was incubated at 37°C for at least 2 h before being subjected to gel electrophoresis and purified by extraction.

### 2.3.8.3 Ligation of DNA

DNA ligation was prepared according to the protocol outlined below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested plasmid DNA</td>
<td>100 ng</td>
</tr>
<tr>
<td>Digested insert DNA</td>
<td>Variable</td>
</tr>
<tr>
<td>10× Ligation buffer</td>
<td>1×</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>3 Weiss U</td>
</tr>
<tr>
<td>Sterile dH₂O</td>
<td>to a total volume of 10 µL</td>
</tr>
</tbody>
</table>

Ligation was carried out overnight (16-18 h) at 16°C and stored at -20°C. The amount of insert DNA required for each ligation reaction was estimated by using the formula below:

\[
\frac{\text{(ng of vector DNA) (size of insert in kb)}}{\text{size of vector (kb)}} \times \frac{3}{1} = \text{ng of insert DNA}
\]
2.3.8.4 Competent Cells (E. coli DH5α, S. Typhimurium 9121 and S. II Sofia Bt6)

2.3.8.4.1 Electrocompetent cells

A 2 mL volume of fresh overnight culture was inoculated into 200 mL of LBB. Cells were grown at 37°C with shaking until A₆₀₀ of 0.4 to 0.6 is reached. Culture was then incubated on ice for 1 h and harvested by centrifugation at 5445 × g for 15 min at 4°C. The resulting cell pellet was washed twice in 100 mL of chilled sterile dH₂O, once in 4 mL of ice cold sterile 10% glycerol before being resuspended in 800 µL of 10% glycerol. The cell suspension was divided into 100 µL aliquots and stored at -70°C for up to 8 months.

2.3.8.4.2 Chemically competent cells

Chemically competent cells were prepared according to the Hanahan method described in Sambrook and Russell (Sambrook and Russell, 2001). A 2 mL volume of fresh overnight culture was inoculated into 200 mL of LBB. Cells were grown at 37°C with shaking until A₆₀₀ of 0.4 to 0.6 is reached. Culture was then incubated on ice for 10 min and recovered by centrifugation at 5445 × g for 15 min at 4°C. Cells were then washed in 20 mL of ice cold FSB solution (see Section 2.3.1.13) and allowed to stand in ice for 10 min. After centrifugation at 5445 × g for 15 min, cell pellet was resuspended in 2 mL of FSB, dispensed into 200 µL aliquots and stored at -70°C.

2.3.3.1 Transformation of Plasmid into Cells

2.3.3.1.1 Electroporation

Frozen cells were gently thawed on ice, mixed with 1-2 µL of ligation mix and allowed to sit on ice for 2 min. The suspension was transferred to a pre-chilled electroporation cuvette with
a 0.2 cm gap. A charge of 2.5 V, 25 µF and 200 Ω was pulsed through the cuvette. Electroporation with time constants greater than or equal to 4.0 were immediately resuspended in 1 mL of SOC broth and incubated at 37°C for 1 h to maximise recovery of transformants. Cells were then plated out onto X-gal agar (see Section 2.2.1.12) and incubated at 37°C wrapped in foil.

2.3.3.1.2 Chemical Transformation

Frozen aliquots of cells were thawed on ice, mixed with up to 10 µL of ligation mix and incubated on ice for 30 min. Cells were transformed by “heat-shocking” the mix at 42°C for 1.5 min. Tubes were incubated on ice for 2 min, and then 800µL of SOC broth was added to each tube. Transformants were allowed to recover at 37°C for 1 h before 100 µL of each reaction is plated onto X-gal agar (see Section 2.2.1.12).

2.4 Tissue Culture Methods

All tissue culture related work was carried out in a biological safety cabinet class II to minimise risk of contamination. Glassware used for storage of media and solutions was washed with Pyroneg detergent, rinsed twice in dH2O and then soaked overnight in dH2O. All glassware was autoclaved before being used to make and store tissue culture media. Solutions and dH2O (for tissue culture media) were made in sterilised bottles before being re-autoclaved.
2.4.1 Tissue Culture Cell Lines Used

A human intestine epithelial cell line (INT407), chicken embryonic fibroblasts (CEF-DF1) and a mouse macrophage cell line (J774) were utilised to carry out the assays outlined in Sections 2.4.3.4 and 2.4.3.5.

2.4.2 Media and Solutions

2.4.2.1 1× DMEM (For INT407 and J774)

The following reagents were filter-sterilised into 372 mL of sterilised dH₂O: 100 mL 5× DMEM, 13.5 mL of 7.5% of sodium bicarbonate, 10 mL of HEPES Buffer and 4.5 mL of L-glutamine. Media was stored at 4°C and aliquots were supplemented with heat-inactivated NCS (10% v/v) before use (DMEM/NCS).

2.4.2.2 1× DMEM (For CEF-DF1)

The following reagents were filter-sterilised into 320 mL of sterilised dH₂O: 100 mL 5× DMEM, 10 mL of 7.5% of sodium bicarbonate, 10 mL of HEPES Buffer, 10 mL of L-glutamine and 50 mL of 45 g/L glucose. Media was stored at 4°C and aliquots were supplemented with heat-inactivated NCS (10% v/v) before use (DMEM/NCS).

2.4.2.3 1× PBS

Standard PBS was used when working with INT407 and J774 cells while Dulbecco’s 1× PBS (without Mg or Ca) was utilised for all CEF-DF1 related work.
2.4.2.4 **Trypsin-EDTA Solution**

Trypsin-EDTA stock was mixed with 1× PBS at a ration of 1:1 and stored at -20°C. This solution was used to dissociate INT407 cells from the flask.

2.4.2.5 **Trypsin/Versene Solution**

Versene (0.1% v/v), trypsin (2.5% v/v) and Dulbecco’s 1× PBS were mixed and stored at -20°C. This solution was used to dissociate CEF-DF1 cells from the flask.

2.4.2.6 **Gentamicin Solution**

The required concentration of gentamicin sulphate in DMEM/NCS.

2.4.2.7 **0.25% Triton X-100**

The detergent Triton X-100 was diluted in sterile dH₂O to a concentration of 0.25% v/v. Solution was incubated at 37°C for 30 min, filter-sterilised and stored at room temperature.

2.4.3 **Tissue Culture Techniques**

2.4.3.1 **Tissue Culture Growth Conditions**

Frozen tissue culture cells were resuscitated from liquid nitrogen, thawed and grown in their respective media in a 37°C CO₂ incubator. Cells were grown until an 80-95% confluency was reached before being split and transferred to a new flask or used in an assay. Tissue culture cells were only passaged between 5-10 times before a new stock of cells were resuscitated.
2.4.3.2 Splitting, Recovery and Banking of Cells

Old media was removed from the flask and the cells washed twice with 1× PBS. To dissociate INT407 and CEF-DF1 cells, an appropriate amount of trypsin-EDTA solution was added and the flask was incubated at 37°C for 10-15 min. J774 cells were dissociated by gently scraping the flask interior with a cell scraper. The dissociated cells were washed twice in the appropriate media and collected each time by centrifugation at 2000 × g for 5 min. Cells were resuspended in 1 mL of DMEM/NCS and an appropriate amount was used to seed a new tissue culture flask.

Cells were banked down for storage as outlined above but resuspended in media supplemented with 10% DMSO, which acts as a cryoprotectant. The cell suspensions were placed into cryovials and stored at -70°C for 2-3 days before being transferred to liquid nitrogen for long term storage.

2.4.3.3 Seeding of Tissue Culture Trays

Tissue culture cells were prepared according to the protocol for splitting the cells, as outlined above. A 10-fold and 100-fold dilution of cells was made with PBS (to a total volume of 100 μL). A volume of 92 μL of each dilution was mixed with 8 μL of 0.25% trypan blue and incubated at room temperature for 5 min to allow dead cells to be stained. Each dilution was then transferred (10 μL) to a grid on a cell counting chamber and covered with a cover slip. Viable cells were counted (blue cells were excluded) and the number of cells required to seed each well was calculated as outlined in Appendix XI.
The required amount of cells was mixed with the appropriate DMEM/NCS and 500 µL of this suspension was dispensed into each well in a 24-well tissue culture tray. This was then incubated at 37°C in 5% CO₂ for 16-18 h before being used in an assay (see Section 2.4.3.4 and 2.4.3.5).

2.4.3.4 Adhesion/Invasion Assay

Adhesion and invasion of *Salmonella* strains in INT407 and CEF-DF1 cells was carried out based on the method previously described by Elsinghorst (Elsinghorst, 1994) and modified by Jason Chang, RMIT University (Chang, 2002). Briefly, cells were cultured, harvested and counted as stated in Section 2.4.3.2. Both cell lines were seeded at a concentration of 3 × 10⁵ cells per well and incubated overnight. Before commencing the assay, media was aspirated from each well and the cells washed 3 times with PBS. To each well, 100 µL of ~ 10⁹ bacterial cells (MOI~ 50-100 bacteria per tissue culture cell) along with 400 µL of DMEM/NCS was added and the culture trays were incubated at 37°C in 5% CO₂. Each assay was done in triplicates and repeated three times on different days.

For the adherence assay, tissue culture trays were incubated for 1 h as described above. Wells were then washed 3 times with PBS. tissue culture cells were lysed by the addition of 100 µL of 0.25% Triton X-100 into each well and incubation at 37°C for 15 min. After pipetting 800 µL of PBS into each well, 100 µL was subjected to a serial 10-fold dilution before being plated out on LBA plates (supplemented with Amp where appropriate). Plates were incubated overnight at 37°C and bacteria enumerated.

For the invasion assay, tissue culture trays were incubated for 2 h instead of 1 h. Contents of each well was washed 3 times with PBS before 500 µL of 400 µg/mL of gentamicin solution
(see Section 2.4.2.6) was added. Culture trays were incubated at 37°C in 5% CO2 for 1 h to kill any extracellular bacteria. Wells were again washed thrice with PBS and intracellular bacteria released with Triton X-100 as mentioned above. Dilutions were plated and counted as outlined above.

2.4.3.5 Intracellular Survival Assay

The ability of *Salmonella* isolates to survive intracellularly within macrophages was measured based on the method outlined by Buchmeier and Heffron (Buchmeier and Heffron, 1989) with some modifications. Briefly, 4 × 10⁵ macrophages were seeded into each well in a 24-well tissue culture tray and incubated overnight. Bacterial cells were opsonised in 10% mouse serum at 37°C for 30 min before being added to the wells at a ratio of 10 to 20 bacteria per macrophage. Culture trays were centrifuged at 250 × g for 5 min to allow the enhancement and synchronisation of infection. Phagocytosis was allowed to proceed for 20 min and wells were washed thrice with PBS before 100 µg/mL of gentamicin solution (see Section 2.4.2.6) was added. To measure the adherence of bacteria to the macrophages, tissue culture cells were lysed in 0.25% Triton X-100 after the washing step (without the addition of any antibiotics) and plated out.

After the addition of gentamicin, incubation was continued for another hour. Wells were once again rinsed 3 times with PBS and 10 µg/mL of gentamicin solution was added (time point 0 h). Wells sampled at 0 h were lysed without any further addition of antibiotics. The other wells were sampled at 1, 4 and 24 h after infection by removing the media, washing the wells with PBS and then lysing the macrophages. Dilutions were plated out and counted as outlined in Section 2.4.3.4. All samples were carried out in triplicates and repeated 3 times.
2.5 RNA Methods

All RNA related work was performed in a biological safety cabinet class II to minimise contamination from environmental RNAses. A 20 min. UV sterilisation was applied in the biohazard cabinet prior to any RNA work. Glassware to be used was treated with diethylpyrocarbonate (DEPC) before sterilisation. All solutions were prepared using RNase-free water and stored in DEPC-treated glassware and sterile RNase-free plastic ware. Only sterile barrier pipette tips and RNase-free plasticware were used for the RNA work.

2.5.1 Reagents and Buffers

2.5.1.1 RNAse-free water

Ultrapure™ water was used for work involving volumes less than 100 mL. For larger volumes (e.g. see Section 2.5.1.3), DEPC-treated water was used. This was prepared by incubating dH₂O in 0.1% DEPC at 37°C overnight and autoclaving the solution under standard conditions (which removes residual DEPC).

2.5.1.2 RNAse-free 75% Ethanol

100% ethanol (AR grade) from a new unopened bottle was opened, decanted into a DEPC treated bottle and diluted to 75% with DEPC treated water in a biohazard cabinet class II. Solution was stored at -20°C.
2.5.1.3 10× Formaldehyde Gel Running Buffer

MOPS (0.2 M), sodium acetate (0.05 M) and EDTA (0.01 M) was dissolved in RNase-free water and adjusted to pH 7.0 with NaOH. Buffer stock was diluted to 1× with DEPC-treated water and mixed with formaldehyde (0.74% v/v) before use.

2.5.1.4 5× RNA Loading Buffer

The following reagents were mixed together: 16 µL saturated Orange G, 80 µL of 500 mM EDTA (pH 8.0), 720 µL of 37% formaldehyde, 2 mL of 100% glycerol, 3.038 mL of formamide and 4 mL of 10 x Formaldehyde Gel Running Buffer. Dye was stored in aliquots at 4°C.

2.5.2 RNA Techniques

2.5.2.1 RNA Isolation

_Salmonella_ spp. RNA was extracted using the RNAgents® Total RNA Isolation System kit as per the manufacturer’s instructions with some modifications. After incubating bacteria at the required conditions, the cell suspensions were immediately mixed with RNAprotect reagent at a ratio of 1:1. This was incubated for 10 min at room temperature to stabilise mRNA expression. Cells were then pelleted by centrifugation at 16 000 × g for 5 min. Stabilised cell pellets may now be stored frozen at -70°C for a later extraction.

Cell pellets were resuspended in 300 µL of chilled RNAgents® denaturing solution and mixed well to break up any cell clumps. After the addition of 30 µL of sodium acetate, the mixture was extracted with 300 µL of room temperature PCI by shaking the tube vigorously for 10 s. After incubating on ice for 15 min, phase separation was achieved by centrifugation at 16 000 × g for 20 min. The upper aqueous phase was retained and RNA was precipitated by
adding an equal volume of isopropanol and incubating the sample at -20°C for 30 min. Precipitated RNA was recovered by centrifugation at 10 000 × g for 10 min. RNA pellet was then washed in 1 mL of 75% ethanol, air dried and resuspended in 10-20 µL of RNase-free water. All isolated RNA samples were stored at -70°C.

2.5.2.2 Estimation of RNA concentration

A 250-fold dilution of RNA samples were made with RNase-free water. Concentration of isolated RNA was determined using spectrophotometer to measure absorbance at 260 and 280 nm. The RNA concentration was calculated by using the ratio of 1 O.D. to 40 µg/mL of ssRNA and taking the dilution factor into account.

2.5.2.3 Formaldehyde Gel Electrophoresis

All RNA samples were subjected to electrophoresis to check the integrity and purity of the isolation and to ensure that the RNA was in linear form. All gel apparatus (tank, tray and comb) were first cleaned with 0.5% SDS, rinsed with water, then dried with 70% ethanol and treated with 3% H₂O₂ for 10 min at room temperature to remove contaminating RNases. After this treatment, the apparatus was rinsed thoroughly with DEPC-treated water and placed in a fume hood.

Prior to running the gel, a 1/5 volume of 5× RNA loading dye was added to the samples. Samples were heated at 65°C for 5 min to further denature the RNA. The samples were then loaded with barrier tips into a pre-equilibrated 1.2% agarose gel prepared with 1× formaldehyde running buffer containing 100 µg/mL of ethidium bromide. Electrophoresis was carried out at 90
V for about 2 h. Gel was then visualised with the transilluminator and photographed using the Gel-Doc system.

2.5.2.4 DNAse Treatment of RNA Samples

To remove any contaminating genomic DNA, RNA samples were treated with RQ1 RNase-Free DNase kit according to the manufacturer’s manual outlined below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA stocks (420 ng/μL)</td>
<td>43 μL</td>
</tr>
<tr>
<td>RQ1 DNase buffer (10×)</td>
<td>5 μL</td>
</tr>
<tr>
<td>RQ1 DNase</td>
<td>2 μL</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>50 μL</strong></td>
</tr>
</tbody>
</table>

The reaction was prepared on ice and incubated at 37°C for 30 min to allow DNA digestion. After the addition of 1 μL of RQ1 stop solution to each reaction (on ice), the DNase was inactivated at 65°C for 10 min. All treated RNA samples (final concentration 400 ng/μL) were stored at -70°C.

2.5.3 RT-PCR

2.5.3.1 Reverse Transcription (RT)

The mRNA present in samples was converted to cDNA through RT using the Improm-II Reverse Transcription kit as per manufacturer’s instructions. Briefly, 1 μg of RNA was mixed with 0.6-1.0 μM of primer and RNase-free water to a total volume of 11 μL on an ice water bath. The mixture was denatured at 70°C for 5 min and placed on ice. The RT reaction was prepared by mixing the reagents below in the following order:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5× RT buffer</td>
<td>1×</td>
</tr>
</tbody>
</table>
25 mM MgCl₂ 2.5 mM

dNTP mix 200 µM

RNasin RNase inhibitor 20U

Reverse transcriptase (RTase) 1U

Total volume 9 µL

The production of cDNA was achieved by mixing together the denaturation and RT reactions and incubating them at 25°C for 5 min, 42°C for 1 h, followed by a RTase inactivation step at 70°C for 15 min. All cDNA samples were stored at -20°C and used as a starting template for the PCR step below, where cDNA is converted into dsDNA.

2.5.3.2 Gene-Specific RT-PCR

The addition of formamide to reduce non-specific bands in a PCR was performed as described by Sarkar and colleagues (Sarkar et al., 1990). It was discovered that the addition of Q solution to the reaction mix greatly improved the specificity and quality of the PCR compared to the addition of formamide (data not shown).

Therefore, PCR amplification was carried out according to the protocol outlined in Section 2.3.3.2 with the modifications below:

Template cDNA 5 µL

5× Q solution (QIAGEN) 5 µL

The PCR reactions were subjected to the following cycling conditions: 1 cycle of 94°C for 3 min followed by 40 cycles of 94°C for 30 s, annealing temperature for 1 min and 72°C for 1 min, with a final extension time of 72°C for 10 min. All PCR products were stored at -20°C.
CHAPTER 3: MOLECULAR TYPING OF SALMONELLA

III SOFIA

3.1 Introduction

Molecular fingerprinting methods are commonly employed to characterise and study the genetic relatedness of various bacterial isolates, including Salmonella spp. PFGE is one such method that uses the separation of infrequently digested genomic DNA to subtype bacterial isolates (Olive and Bean, 1999; Olsen, 2000). Considered by some to be the “gold standard” of molecular typing methods, PFGE is accurate, reliable, reproducible, highly discriminatory and relatively easy to perform (Olive and Bean, 1999; Olsen, 2000). PFGE typing is one of the most commonly used methods to characterise and group Salmonella isolates from various sources (Bakeri et al., 2003, Bessa et al., 2007; Cardinale et al., 2005; Ethelberg et al., 2004; Langvad et al., 2006; Matsumoto et al., 2006; Old et al., 1999; Olsen et al., 1994; Pang et al., 2005; Seo et al., 2006; Tsen et al., 2000; Tsen and Lin, 2001; Uesugi et al., 2007; Usera et al., 1998; Ward et al., 2005).

On the other hand, amplification fingerprinting utilises PCR to identify and subtype bacterial isolates (Olive and Bean, 1999). One such method makes use of short repetitive DNA sequences to generate banding patterns for analysis. An example of this fingerprinting method is rep-PCR, which uses REP elements present in bacterial genomes to characterise and subtype bacterial isolates (Gilson et al., 1984; 1990; Stern et al., 1984; Versalovic et al., 1991; 1994). Rep-PCR is frequently used to subtype and analyse Salmonella isolates as the method
is easy to perform, reproducible, sensitive and discriminatory (Beyer et al., 1998; Foley et al., 2006; Johnson et al., 2001; Maré et al., 2001; Millemann et al., 2000; Mills-Robertson et al., 2003; Rasschaert et al., 2005; Saxena et al., 2002; Weigel et al., 2004; Woo and Lee, 2006).

Although Salmonella serovar II Sofia has consistently been isolated from Australian poultry since the 1970s, surprisingly few studies of these isolates using genotyping methods have been carried out. So far, only two papers regarding the molecular fingerprinting of S. II Sofia isolates have been published (Harrington et al., 1991; Ross et al., 2003). Harrington and her colleagues (Harrington et al., 1991) compared S. II Sofia isolates from Australian and Israeli chickens and humans through SDS-PAGE of the OMPs, plasmid profile and RFLP analysis. Although plasmid profiling identified a 6.4 kB plasmid of unknown function, this method was not sufficiently discriminatory to characterise the isolates (Harrington et al., 1991). No reproducible differences could be obtained from the analysis of the OMPs from strains from either location (Harrington et al., 1991). However, RFLP analysis was able to successfully group S. II Sofia and divided the isolates into 7 groups and showed that significant differences exist between the Australian and Israeli S. II Sofia strains (Harrington et al., 1991). However, only a limited number of strains were used in the RFLP analysis and this result should be treated with caution – perhaps more samples should have been analysed.

Fluorescent AFLP has also been shown to be a useful method in the molecular fingerprinting of S. II Sofia isolates (Ross et al., 2003). The typing of 68 Australian S. II Sofia strains has revealed that most isolates were clustered into 4 groups (70-90% similarity) and that S. II Sofia is genetically variable with certain clonal groupings persisting over time (Ross et al., 2003). However, no relationship could be established between a particular group and their
geographical location, source or host (Ross et al., 2003). No major differences were reported between the Australian and Israeli S. II Sofia isolates tested - no specific clonal group corresponding to Israeli isolates (Ross et al., 2003). In addition, Ross and his colleagues (Ross et al., 2003) reported that S. II Sofia cannot be typed with PFGE due to DNase activity, despite the application of anti-DNase treatments such as formaldehyde (Gibson et al., 1994) and thiourea (Leisegang and Tschäpe, 2002).

A total of 84 S. II Sofia isolates from various locations around Australia were subtyped using PFGE (XbaI and SpeI) and Rep-PCR (with REP DNA sequence). All resulting electrophoretic banding patterns were analysed both through visual analysis and with computer software - GelCompar® II (Applied Maths, Belgium).

The aims of the experimental methods carried out in this chapter were to:

(i) minimise DNA degradation in isolates by devising a DNase inactivation step,
(ii) investigate the genetic and clonal relationship of Australian S. II Sofia isolates using Rep-PCR, the modified PFGE protocol and GelCompar® II,
(iii) compare the discriminatory ability of each typing system using Simpson’s Index of Diversity (D).

3.2 Methods and Results

3.2.1 Inactivation of DNase Activity in S. II Sofia

S. II Sofia DNA was prepared as described previously in Chapter 2 (see Section 2.3.6.1). Attempts to analyse S. II Sofia samples through PFGE were unsuccessful, resulting in smears on the gel (data not shown). Neither the formaldehyde treatment of cells (see Section 2.3.6.2)
nor modifications to the PFGE running buffer (see Section 2.3.6.3) could protect the DNA of S. II Sofia isolates from degradation. Modification of protocol conditions such as variation in incubation time (during cell lysis and RE digestion), varying the concentration of Proteinase K and sarkosyl in ESP solution (see Section 2.3.1.12) and preincubation with lysozyme prior to cell lysis did not improve the generation of banding patterns of the DNase-sensitive isolates (data not shown).

Further modifications were carried out on the ingredients of the ESP lysis solution. It was discovered that the addition of the detergent SDS had the potential to stop DNA degradation of isolates. A series of ESP solutions (ESP1 to 36) were created by varying the concentrations of EDTA, pH 9 (0.5, 0.9 and 1.2 M), sarkosyl (1%, 3% and 5% w/v), Proteinase K (100, 200 and 500 µg/mL) and SDS (1%, 3% and 5% w/v). Each ESP solution was used to lyse S. II Sofia cells and PFGE was conducted to assess the efficiency of each combination in protecting DNA from degradation. The lysis solutions ESP31, 34 and 35 were found to stop DNA degradation, resulting in the production of visible banding patterns (data not shown). ESP34 (0.5 M EDTA, 5% sarkosyl, 200 µg/mL Proteinase K and 5% SDS) was chosen as the PFGE lysis solution for processing S. II Sofia isolates.

DNA degradation observed after PFGE is due to the presence of DNase in the S. II Sofia isolates. To observe the effect of DNase activity on PFGE of digested DNA fragments, the following Salmonella strains were used: S. Typhimurium 82/6915, S. II Sofia strains Bt8 and 786CL. Bacterial plugs were made with each of these isolates and by combining 82/6915 with Bt8 and 82/6915 with 786CL (bacterial suspensions were mixed at a ratio of 1:1 before being set in low-melt agarose). PFGE patterns of each sample were compared after treatment with
Figure 3.1  PFGE patterns showing a comparison between normal (N) and modified (S) ESP lysis treatment. Lane M, PFGE λ ladder; Lane 1, S. Typhimurium 82/6915 (N); Lane 2a, S. II Sofia Bt8 (N); Lane 2b, S. II Sofia 786CL (N); Lane 3a, 82/6915 and Bt8 (N); Lane 3b, 82/6915 and 768CL (N); Lane 4, 82/6915 (S); Lane 5a, Bt8 (S); Lane 5b, 786CL (S); Lane 6a, 82/6915 and Bt8 (S); Lane 6b, 82/6915 and 768CL (S).
Figure 3.2  PFGE patterns showing a comparison between Treatment 1, 2 and 3. Lane M, PFGE λ ladder; Lane 1a-1c, Treatment 1 of *S. Typhimurium* 9, *S. Enteritidis* 446302 and *S. II Sofia Bt1* respectively; Lane 2a-2c, Treatment 2 of Bt1, 446302 and 9 respectively; Lane 3a-3c, Treatment 3 of 9, 446302 and Bt1 respectively.
normal and modified lysis solution (Figure 3.1). S. Typhimurium 82/6915 was not affected by either of the lysis solutions, producing identical PFGE patterns after both treatments. Normal treatment of the S. II Sofia and mixed plugs resulted in total degradation and partial smearing of DNA bands, respectively (Figure 3.1). In contrast, PFGE bands produced after incubation with ESP34 lysis solution were visible and undegraded.

The PFGE protocol used to type S. II Sofia can be hazardous and labour-intensive (see Section 2.3.6.1), limiting the number of samples that can be processed simultaneously. Therefore, a series of treatments involving modifications to the lysis incubation time and washing steps were devised:

(i) Treatment 1 – as described by Nayak and his colleagues (Nayak et al., 2004) with modifications (1% SDS in plugs, overnight incubation in ESP34, wash once with preheated [50°C] dH₂O and 4× with preheated TE buffer, 20 min for each wash),

(ii) Treatment 2 – as described by Nayak and his colleagues (Nayak et al., 2004) (1% SDS in plugs, 2 h incubation in ESP34, washes as outlined in Treatment 1),

(iii) Treatment 3 – PFGE protocol with ESP34 lysis solution (see Section 2.3.6.1).

All 3 treatment systems were applied individually to S. Typhimurium 9, S. Enteritidis 446302 and S. II Sofia Bt1. Each experiment was repeated 3 times on different days. The addition of 1% SDS into bacterial plugs was also assessed for improvement to banding patterns.

All resulting PFGE patterns from the different treatments were compared (Figure 3.2). No differences in quality of the PFGE bands were observed with the serovars Typhimurium and Enteritidis between each treatment. Generation of clear S. II Sofia bands were uneven with Treatment 1 and 2, producing visible bands once (Treatment 1) and twice (Treatment 2) out of
the 3 repeats. PFGE patterns that were produced were observed to have partial smearing (data not shown). Only Treatment 3 was able to protect Bt1 DNA from degradation, producing visible patterns for each repeat (Figure 3.2). Addition of SDS to bacterial plugs did not improve the quality of PFGE patterns of any of the *Salmonella* isolates.

### 3.2.2 PFGE Profile

Bacterial plugs of all 84 *S. II Sofia* isolates were prepared using the modified PFGE protocol, containing ESP34 lysis solution. PFGE was carried out as described previously (see Section 2.3.6.1), with the restriction enzymes *Xba*I and *Spe*I. Preparation of plugs and PFGE was repeated 3 times with the same conditions on different occasions. Commercial PFGE λ ladder was loaded onto all gels. PFGE profiles of all isolates were compared visually and DNA patterns differing by one or more bands were assigned a profile type: X(n) for *Xba*I and S(n) for *Spe*I, with n = profile/group number. Several isolates representing each profile were selected and subjected to PFGE on 1% SeaKem® Gold agarose for 18 h (*Xba*I) and 22 h (*Spe*I). This high-strength agarose is routinely utilised in methods that require the separation of large DNA fragments (PFGE). Gels were stained, visualised and the final images used during computer analysis of isolates (see Section 3.2.5).

Each PFGE profile obtained was compared to a specific PFGE profile, which is arbitrarily assigned as the reference profile. In this case, all patterns obtained were compared to X1 and S1 for *Xba*- and *Spe*-digested DNA, respectively. In addition, the Dice coefficient, *F*, for each PFGE profile was calculated as described by Tsen and his coworkers (Tsen *et al.*, 2000). This value expresses the proportion of shared DNA bands in 2 patterns and is calculated using the formula: 

\[ F = \frac{2n_{xy}}{n_x + n_y}, \]

where *n*<sub>x</sub> and *n*<sub>y</sub> is the total number of bands in pattern X and Y,
respectively and \( n_{xy} \) is the number of bands shared by both patterns. When \( F = 1.0 \), both PFGE patterns are identical and when \( F = 0 \), the two patterns are completely dissimilar (Tsen et al., 2000).

### 3.2.2.1  XbaI

The PFGE analysis of XbaI-digested DNA from 84 S. II Sofia isolates gave 13 to 22 visible, stable and reproducible DNA fragments in the size range of 40 to 825 kB (Figure 3.3). Based on the PFGE patterns, all isolates can be divided into 8 distinct groups – X1 to X8. A representative of these 8 PFGE profiles is shown in Figure 3.3 and is summarised in Table 3.2. The most common PFGE profile obtained was X1 (Figure 3.3), with 73.8% of S. II Sofia isolates showing this profile (62 strains). This was then followed by X6 (9 isolates), X3 (4 isolates), X4 and X5 (3 isolates each), and X2, X7 and X8 (1 isolate each). Xbal PFGE profiles are related, differing from one another by 1-7 bands with \( F \) values ranging from 0.44 to 0.96 (Table 3.1).

Distributions of each Xbal profile (X1 to X8) across the various locations around Australia were compared. Pattern X1 was observed to be the most common PFGE profile amongst the isolates from each location (Victoria, New South Wales, South Australia, Queensland and Tasmania). S. II Sofia isolates from Victoria were found to exhibit the most variation, with 5 different patterns (X1, X3, X4, X5 and X6) obtained from the typing of 34 isolates. Four different patterns were present among the 13 and 15 isolates from New South Wales (X1, X2, X3 and X6) and Queensland (X1, X3, X6 and X7), respectively. While only profile X1 and X2 were found among the 11 South Australian isolates, all 10 Tasmanian S. II Sofia strains were shown to have only one Xbal pattern, X1. It was also observed that S. II Sofia 131 was the only isolate to show profile X8 (origin of this isolate is unknown).
Figure 3.3  Representations of XbaI-digested PFGE pattern profiles X1 to X8 from S. II Sofia isolates (PFGE pattern of each individual isolate is listed and summarised in Table 3.2).
3.2.2.2 Spel

Digestion of all 84 S. II Sofia isolates with SpeI yielded 16 to 24 stable and reproducible DNA fragments of 40 to 1000 kb in size (Figure 3.4). Six distinct PFGE profiles were seen among the isolates (S1 to S6) and a representative of these profiles is shown in Figure 3.4 and summarised in Table 3.2. S1 was the most common pattern observed, representing 88.1% (74 isolates) of the 84 isolates typed (Figure 3.4). This was then followed by pattern S3 (3 isolates), S4 (2 isolates) and S5 and S6 (1 isolate each). For SpeI, PFGE patterns obtained were closely related, differing from one another by only 1-3 bands and exhibiting $F$ values in the range of 0.72 to 0.98 (Table 3.1).

The PFGE patterns between the Victorian, South Australian, New South Wales, Queensland and Tasmanian S. II Sofia isolates were compared. SpeI pattern S1 was observed to be the major profile at each location. Three different profiles were found among the 34 Victoria (S1, S4 and S5), 15 Queensland (S1, S3 and S4) and 13 New South Wales (S1, S2 and S3) isolates. South Australian isolates were found to have 2 different profiles, either S1 or S2 (11 isolates). Only one SpeI profile was seen among all 10 isolates from Tasmania (pattern S1). The isolate S. II Sofia 131 (the origin of isolate is unknown), was observed to be the only strain to exhibit pattern S6.
Figure 3.4  Representations of SpeI-digested PFGE pattern profiles S1 to S6 from S. II Sofia isolates (PFGE pattern of each individual isolate is listed and summarised in Table 3.2).
Table 3.1  Coefficient, $F$ values of each typing profile for the 84 S. II Sofia isolates

<table>
<thead>
<tr>
<th>PFGE pattern</th>
<th>Rep-PCR pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_{bal}$</td>
<td>$Spel$</td>
</tr>
<tr>
<td>X1 (1.0)</td>
<td>S1 (1.0)</td>
</tr>
<tr>
<td>X2 (0.78)</td>
<td>S2 (0.72)</td>
</tr>
<tr>
<td>X3 (0.89)</td>
<td>S3 (0.98)</td>
</tr>
<tr>
<td>X4 (0.89)</td>
<td>S4 (0.95)</td>
</tr>
<tr>
<td>X5 (0.92)</td>
<td>S5 (0.90)</td>
</tr>
<tr>
<td>X6 (0.96)</td>
<td>S6 (0.72)</td>
</tr>
<tr>
<td>X7 (0.71)</td>
<td></td>
</tr>
<tr>
<td>X8 (0.44)</td>
<td></td>
</tr>
</tbody>
</table>

$F$ values are shown in parentheses.

Definition and calculation of $F$ values is as described in Section 3.2.2.
3.2.3 Rep-PCR Profile

Genomic DNA was extracted from all 84 S. II Sofia isolates using the CTAB method as described previously in Chapter 2 (see Section 2.3.2.1). The extracted DNA was subjected to repetitive PCR using BoxA1R (Koeuth et al., 1995), ERIC2, REP1R-I and REP2-I (Versalovic et al., 1991) primers. PCR ribotyping with the combination of P1 and P2 primers (see Appendix V) was also performed according to the method by Lagatolla and her coworkers (Lagatolla et al., 1996). PCR amplification with each primer set was carried out on a selected number of S. II Sofia isolates (for primer sequence and PCR conditions, see Appendix VI). All PCR amplification products were subjected to electrophoresis (1.5% agarose gels) and gels were stained and visualised. The ability of each PCR typing method to differentiate the S. II Sofia isolates was evaluated.

Molecular patterns generated from PCR carried out using either the ERIC2 and BoxA1R primers were not discriminatory enough to differentiate the isolates – amplification resulted in either a single DNA pattern or patterns that differ by only one band (data not shown). The same observation was also seen with the PCR ribotyping method using the primers P1 and P2. Amplification with REP1R-I yielded PCR patterns that were more varied and complex (but still distinct enough for analysis) compared to the patterns obtained with REP2-I (data not shown). The use of both REP primers together for typing was also evaluated and it was found that the PCR patterns generated were too numerous and complex, making analysis difficult (data not shown). Therefore, rep-PCR typing of S. II Sofia isolates was carried out using REP1R-I. All PCR products were loaded on 1.5% maxigels (see Section 2.3.4) for electrophoresis (50V for 16-18 h) and gels were stained and visualised.
Figure 3.5  Representations of REP1 pattern profiles R1 to R5 from S. II Sofia isolates (REP1 pattern of each individual isolate is listed and summarised in Table 3.2).
Visual comparison of all REP1 profiles was conducted and profile types were assigned based on differing DNA banding patterns: R(n), with n = profile/group number. Each REP1 profile obtained was compared to a specific REP1 profile, in this case, all patterns obtained were compared to R1. The $F$ value for each REP1 pattern was also calculated (for the calculation formula, see Section 3.2.2). REP1 amplification of the 84 S. II Sofia isolates yielded 5 different profile patterns (R1 to R5), consisting of about 13 to 22 visible, stable and reproducible DNA fragments (0.5 to 4.5 kB in size). A summary of these profiles is listed in Figure 3.5 and Table 3.2.

Profile R1 was observed to be the major pattern among the isolates, with 73.8% (62 isolates) of isolates exhibiting this pattern (Figure 3.5). The next most predominant profile is pattern R3 (12 isolates) and this was followed by R2 (8 isolates), R4 and R5 (1 isolate each). REP1 patterns were found to differ from one another by 1-7 bands with $F$ values in the range of 0.65 to 0.97 (Table 3.1). R1 is the dominant S. II Sofia pattern obtained at each location (34 Victorian, 13 New South Wales, 11 South Australian, 15 Queensland and 10 Tasmanian isolates). Victorian isolates showed the most variation, with 4 different REP1 profiles (R1, R2, R3 and R5) and S. II Sofia isolates from New South Wales and South Australia exhibited 3 (R1, R2 and R4) and 2 patterns (R1 and R5), respectively. On the other hand, Queensland and Tasmanian isolates were found to have only one REP1 pattern, R1. In contrast to its PFGE profiles, S. II Sofia 131 did not have a unique REP1 pattern (R2).
Table 3.2  Summary of molecular fingerprinting data for the 84 S. II Sofia isolates

<table>
<thead>
<tr>
<th>S. II Sofia isolates</th>
<th>Profile pattern</th>
<th>Number of isolates showing this pattern combination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PFGE</td>
<td>PCR</td>
</tr>
<tr>
<td></td>
<td>( X_{\text{bal}} ) (X)</td>
<td>( S_{\text{pel}} ) (S)</td>
</tr>
<tr>
<td>( B_{t1}, 2, 3, 6, 7, 8, 12, 13, 14, 15, 16, 18 )</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>( 644SC, 569NCA, 636SC, 656SC, 656SCA )</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>( 786CL, 768XTA )</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>( 642SC )</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>( 554NCA )</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>( 403QCA, 539NC, 566NCA )</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>( 630MSC )</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>( 987VCA, 10VCA )</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>( 858V )</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>( 3452 )</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>( 620MSC, 838V )</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>( 985VCA, 992VCA, 664SCA, 671SC, 671SCA, 9VCA, 568NC, 963Q )</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>( 856V )</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>( 988Q )</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>( 131 )</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total number of isolates:</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.4 Combination of PFGE and Rep-PCR Profiles

When all three molecular fingerprinting profiles (PFGE and REP1 patterns) were combined, a total of 16 pattern combinations were obtained (Table 3.2). Pattern combination X1S1R1 was found to be the most common profile among S. II Sofia isolates, with 50% (42 isolates) of all strains showing this pattern combination. It was shown that 42 of the 62 X1 strains (the predominant XbaI profile) belong to this profile (X1S1R1; Table 3.2). The other combinations observed in X1 strains include X1S1R3 (12 isolates), X1S1R2 (5 isolates), X1S3R1 (2 isolates) and X1S3R2 (1 isolate). Distribution of different profiles between the different locations: Victoria (9 combinations), New South Wales (5 combinations), South Australia and Queensland (4 combinations). All Tasmanian isolates were found to exhibit only one profile, which is the major pattern combination X1S1R1 (Table 3.2).

3.2.5 Analysis of Banding Patterns with GelCompar® II

After manual analysis of both PFGE and REP profiles, the computer software GelCompar® II was used to analyse the banding patterns and to generate dendograms. All analysis using this software was done at the Department of Microbiology at Austin Health, Melbourne, Victoria. S. Braenderup H9812 was used as the reference strain in PFGE for the determination of size standards (see Appendix III) and for the normalisation of gel images for analysis (Hunter et al., 2005). This was accomplished by placing this strain in certain positions on each gel - on the gel, S. Braenderup H9812 was inserted between every 3 isolates of S. II Sofia. For REP1 analysis, the λDNA (PstI) marker was used as a reference. Using the software, genetic similarity was calculated with Dice coefficient and DNA profiles clustered using the Unweighed Paired Group Arithmetic Average (UPGMA) method, creating dendograms for the isolates (Figures 3.6 to 3.8). The UPGMA method assumes a constant evolutionary rate occurs
between the different lineages and identifies patterns in order of similarity, allowing the tree to be constructed in a step-wise manner (Opperdoes, 1997). A position tolerance of 1.0% was chosen for generation of all dendograms as the analysis of multiple runs of S. Braenderup H9812 (PFGE) and λDNA (PstI) marker (REP1) with GelCompar® II showed similarity of 100%. This parameter indicates the extent of positional deviation between 2 identical bands run in different lanes and on different gels (Gerner-Smidt et al., 1998). As GelCompar® II was able to accurately align the reference strain, which was run in different lanes and gels, the default position tolerance of 1.0% was chosen.

Using the fingerprint patterns from selected S. II Sofia isolates representing each profile type, three separate dendograms were constructed (XbaI, SpeI and REP1; Figure 3.6, 3.7 and 3.8, respectively). Software analysis of the fingerprint patterns have matched and confirmed with the visual comparison of patterns. The dendogram for XbaI showed an overall similarity of between 44 to 100% and was divided to 4 clusters (I to IV; Figure 3.6) with the first cluster (73.8% of the isolates) containing 5 profiles (X1 and X3 to X6) and cluster II, III and IV consisting of only 1 profile each. The genetic relatedness of SpeI PFGE patterns ranged from 66 to 100%, with the patterns being categorised into 3 clusters (I to III; Figure 3.7). While the first cluster (96.4% of isolates) consisted of 4 profiles (S1 and S3 to S5), the second and third cluster contained only one profile. REP1 patterns were divided into 2 major clusters (I and II) that showed an overall similarity of 70 to 100% on the dendogram (Figure 3.8): the first (98.8% of the isolates) comprised of 4 profiles (R1 to R3 and R5); and the second of only one profile, R4.
Figure 3.6  Dendogram showing cluster analysis of selected S. II Sofia isolates representing the different XbaI patterns. Eight profiles were obtained (see Table 3.2) and divided into 4 major clusters (I, II, III and IV). Similarity was determined using Dice coefficient and profiles clustered with the UPGMA method.
Figure 3.7  Dendogram showing cluster analysis of selected S. II Sofia isolates representing the different SpeI patterns.

Six profiles (see Table 3.2) were observed and categorized into 3 major clusters (I, II and III). Similarity was calculated using Dice coefficient and profiles clustered with the UPGMA method.
Figure 3.8  Dendogram showing cluster analysis of selected S. II Sofia isolates representing the different REP1 patterns. Five profiles (see Table 3.2) were obtained and divided into 2 major clusters (I and II). Similarity was determined using Dice coefficient and dendograms generated with the UPGMA method.
3.2.6 Simpson’s Index of Diversity (D)

The discriminatory ability of the molecular typing systems (used individually or in combination) was evaluated by calculating the Simpson’s Index of Diversity as described by Hunter and Gaston (Hunter and Gaston, 1988). This discrimination index calculates the probability of two unrelated strains being characterized into different typing groups and formula is as listed below:

\[
D = 1 - \frac{1}{N(N - 1)} \sum_{j=1}^{S} n_j (n_j - 1)
\]

where \(N\) is total number of strains in sample population, \(S\) is total number of types described and \(n_j\) is the number of isolates belonging to \(j\)th type. All \(D\) values were evaluated as indicated - the higher the index value (the closer the \(D\) value gets to 1.0), the better the discriminatory ability of the typing method.

A summary of the discrimination indices and the number of profiles generated by each molecular typing method(s) is listed in Table 3.3. Restriction analysis with \(XbaI\) was shown to be better at discriminating \(S.\) II Sofia isolates compared to \(SpeI\). The \(REP1\) typing system has better discrimination than \(PFGE\) with \(SpeI\), despite the latter being able to differentiate more profile types and containing a higher proportion of isolates in the predominant profile type (Table 3.2). The various combinations of typing systems were found to have higher discriminatory power compared to the individual typing methods. Overall, the 2 most discriminatory molecular typing methods for the analysis of \(S.\) II Sofia are the \(XbaI/Spec/REP1\) and \(XbaI/REP1\) systems, with index values of 0.721 and 0.693, respectively (Table 3.2).
**Table 3.3**  Discrimination indices for *S. II Sofia* molecular fingerprinting methods

<table>
<thead>
<tr>
<th>Molecular typing method</th>
<th>Number of profiles</th>
<th>Size (%) in the dominant profile type</th>
<th>Simpson’s index of discrimination ($D$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XbaI</td>
<td>8</td>
<td>73.8</td>
<td>0.444</td>
</tr>
<tr>
<td>SpeI</td>
<td>6</td>
<td>89.3</td>
<td>0.225</td>
</tr>
<tr>
<td>REP1</td>
<td>5</td>
<td>73.8</td>
<td>0.431</td>
</tr>
<tr>
<td>XbaI/SpeI</td>
<td>12</td>
<td>70.2</td>
<td>0.498</td>
</tr>
<tr>
<td>XbaI/REP1</td>
<td>12</td>
<td>52.4</td>
<td>0.693</td>
</tr>
<tr>
<td>SpeI/REP1</td>
<td>10</td>
<td>65.5</td>
<td>0.548</td>
</tr>
<tr>
<td>XbaI/SpeI/REP1</td>
<td>16</td>
<td>50.0</td>
<td>0.721</td>
</tr>
</tbody>
</table>

*D* values were calculated as described in Section 3.2.5
3.3 Discussion

All previous attempts to subtype S. II Sofia strains through PFGE have been unsuccessful, resulting in smears indicative of DNA degradation (Ross et al., 2003). Ross and his coworkers (Ross et al., 2003) have reported that about 60% of the S. II Sofia isolates used in their study were non-typeable by PFGE. DNA degradation can occur for various reasons and some bacterial strains appear more sensitive to DNA degradation than others. DNase-positive bacterial strains (e.g. Campylobacter jejuni Lior biotype II) have been shown to degrade their genomic DNA during the preparation stage before PFGE (Gibson et al., 1994). However, formalin fixation of cells was sufficient to inactivate DNase produced by these strains, allowing them to be typed by PFGE (Gibson et al., 1994). Other strains appear to be more sensitive to the Tris radicals generated during the electrophoresis process (Ray et al., 1995), which are capable of breaking down DNA (Corkill et al., 2000; Koort et al., 2002; Liesegang and Tschäpe, 2002; Lyytikäinen et al., 2000; Zhang et al., 2004). However, the reason for the sensitivity of certain strains to Tris radicals over others is unknown. Modifications to the running buffer such as addition of thiourea or use of HEPES buffer have been shown to counteract this activity (Corkill et al., 2000; Koort et al., 2002; Liesegang and Tschäpe, 2002; Zhang et al., 2004).

However, any efforts to protect S. II Sofia DNA from degradation before and during PFGE through modifications to the protocol (see Section 3.2.1) and/or use of established anti-DNase treatments (formaldehyde, thiourea and HEPES), in the previous (Ross et al., 2003) and current study have been unsuccessful. An evaluation on whether altering contents of the ESP lysis solution would improve typeability of S. II Sofia isolates was conducted since DNA degradation is most likely to occur at this stage (lysis of cells and release of genomic DNA in the plugs). The addition of SDS into bacterial plugs (presumably to improve lysis of cells) has been utilised in
several PFGE protocols (Fakhr et al., 2006; Nayak et al., 2004; Ribot et al., 2001). It is included in the CDC PulseNet PFGE protocol used in the United States to process food-borne pathogens (Hunter et al., 2005; Swaminathan et al., 2001). By adding SDS into the lysis solution, which already contains sarkosyl, the potential of the combined strength of both these detergents in the inactivation of S. II Sofia DNase activity was investigated.

It was discovered that addition of 5% SDS (w/v) and the increase of sarkosyl and Proteinase K concentration (from 1 to 5%, w/v and 100 to 200 µg/mL, respectively) in the lysis solution (ESP34) was sufficient to inactivate nuclease activity in S. II Sofia, leading to the generation of visible bands. This observation was confirmed by the normal and modified ESP lysis treatment of bacterial plugs (Figure 3.1). The DNase activity of S. II Sofia isolates is evident in the S. II Sofia and mixed plugs lysed with normal ESP solution, resulting in partial to total smearing on the gel. It is also evident that the presence SDS is able to stop this nuclease activity, producing bands that can be used for further analysis (Figure 3.1). Moreover, PFGE quality of S. Typhimurium 82/6915 appears to be unaffected by the use of ESP34, which means that this lysis solution can be used to process DNase-negative strains, making pattern comparison between S. II Sofia and other Salmonella serovars possible.

The conventional PFGE protocol used in this chapter is easy to perform but labour-intensive, taking 3 days (from preparation of plugs to restriction digest to PFGE) to complete (see Section 2.3.6.1). The protocol also requires the use of PMSF during the washing stage, a chemical which can be hazardous as it is known to be destructive to the mucous membranes of the respiratory tract, skin and eyes (Sambrook and Russell, 2001). Development of newer PFGE protocols have removed the use of PMSF, where bacterial plugs were washed several
times in preheated (50-55°C) dH2O and/or TE buffer instead (Fakhr et al., 2006; Liebana et al., 2001; Nayak et al., 2004; Ribot et al., 2001; Swaminathan et al., 2001; Thong et al., 1995). Furthermore, reduction of cell lysis incubation time has been carried out without affecting the generation of PFGE bands (Fakhr et al., 2006; Nayak et al., 2004; Ribot et al., 2001; Swaminathan et al., 2001). This can be observed with the PFGE patterns of the Typhimurium and Enteritidis serovars, where application of any one of the 3 treatment systems did not affect the generation or quality of DNA bands (Figure 3.2). Neither of these serotypes is known to produce DNase, making PFGE typing easy to conduct as both these isolates were not prone to DNA degradation.

However, the same cannot be said regarding the S. II Sofia isolate (Figure 3.2), which seems only to consistently produce visible bands with no smearing using Treatment 3. Overnight incubation with SDS is probably required in order to properly inactivate DNase in S. II Sofia, which would explain the inconsistent generation of bands from Treatment 2 (2 h incubation with ESP). Since S. II Sofia DNA appear to be more sensitive to degradation compared to other Salmonella serovars, it is possible that the change in temperature (from RT to 50 °C) during the washing steps could have encouraged the DNA degradation process. PMSF can function as a protein inhibitor and thus, is used in the first step of plug washing to inactivate Proteinase K and any other bacterial proteins not digested after overnight lysis incubation. The omission of PMSF from plug preparation (Treatment 1 and 2) could have lowered the effectiveness of the washing step in removing proteins and cell debris after lysis. This could increase the chances for DNA breakdown, resulting in the inconsistent production of visible patterns. Therefore, Treatment 3 is deemed the best protocol for preparation of S. II Sofia DNA for PFGE.
A collection of S. II Sofia strains (84 strains) isolated from poultry flocks around Australia was studied using 2 different molecular subtyping methods: PFGE (with XbaI and SpeI) and rep-PCR (with REP1 element). Restriction analysis of the isolates resulted in generation of banding patterns and a number of profile types: 8 XbaI (X1 to X8) and 6 SpeI patterns (S1 to S6) (Figures 3.3 and 3.4). The XbaI restriction patterns are more easily discernable compared to the SpeI patterns, which are more numerous and closely packed together, making analysis of banding pattern more difficult. Molecular fingerprinting through the detection of a number of repetitive sequences (BOX, ERIC and REP) was also carried out on the S. II Sofia isolates. Only REP elements were varied enough to be used for typing since all the other repetitive elements gave a single pattern (indicating that the number and location of BOX and ERIC sequences in S. II Sofia strains were constant). There does not seem to be a correlation between the typing methods as overlap and diversity can be obtained within each profile (in other words, no single pattern type is associated with a specific profile type).

The overall difference between a majority of the PFGE and REP profiles were small, with only S. II Sofia 131 and 988Q (PFGE), and 554NCA (PFGE and REP1) displaying greater band differences (compared to the rest of the 84 II Sofia isolates). It appears that a majority of the patterns are grouped within a single profile type, with the other types encountered consisting of a few isolates each (Table 3.2). This limited genetic diversity is confirmed by the Dice coefficient \( (F) \) values calculated manually and with computer software (Table 3.1 and Figures 3.6 to 3.8). Dice coefficient is defined as a correlation coefficient for discrete events and is used to measure the genetic similarity in 2 isolates (with the assumption that bands of identical size are, with high probability, genetically homologous) (Anon, 2004). Most of the strains are found to be closely related (with \( F \) values in the range of 0.89 to 0.98), with the exception of isolate 131, 988Q and
554NCA (Table 3.1). This genetic relatedness can also be seen from the dendograms generated using with GelCompar® II, with a majority of the S. II Sofia strains being grouped into Cluster I in each dendogram and showing similarity of 80 to 100% (Figures 3.6 to 3.8).

However, the estimate of genetic relatedness of S. II Sofia isolates varies, depending on the typing method used. XbaI restriction digest analysis appears to be the most discriminatory method of the three, with 8 different profile types generated (Figure 3.3). Compared to SpeI and REP1 patterns, XbaI profiles have a wider diversity range, showing an overall similarity of between 44 to 100% (Figure 3.6). Although the discriminatory ability of the typing methods used here was comparable to the RFLP typing conducted previously (Harrington et al., 1991), neither the previous nor current typing methods were as highly discriminatory as the fluorescent AFLP method (Ross et al., 2003) for the study of Australian S. II Sofia isolates. AFLP tends to generate a large number of DNA fragments (in comparison to other typing methods such as PFGE), which means that subtle changes in the bacterial genome are more likely to be detected, contributing to the number of pattern types (Ross et al., 2003). While this typing method is highly discriminatory, the high setup cost (for equipment and expertise) is a disadvantage, compared to cheaper methods such as PFGE or PCR typing.

The discriminatory ability of XbaI PFGE is also reflected in the Simpson’s Index values (Table 3.3). Initially used to evaluate species diversity within an ecological habitat (Hunter and Gaston, 1988), the Simpson’s Index of Diversity has been employed to evaluate the typing systems used to characterise bacterial strains such as Salmonella spp. (Malorny et al., 2001; Pang et al., 2005; Woo and Lee, 2006). Individually, the different degrees of polymorphism generated by each typing system are ranked in the descending order: PFGE (XbaI, 8 types, $D =$
0.444), followed by REP1 (5 types, $D = 0.431$) and PFGE ($Spel$, 6 types, $D = 0.225$). Overall, the discriminatory power of each typing system is rather poor. The close association of a portion of the fragments produced from restriction analysis with $Spel$ (bands were situated close to one another and occasionally appear to be “fused” together, see Figure 3.4) could have made scoring of bands difficult, lowering the discrimination power of the method. In the case of PCR typing, a generated pattern typically consists of dominant intense bands, that are easy to score, and faint or less intense bands, which introduces uncertainty during the development of profile types (Burr et al., 1998). It is possible that this, along with other factors (slight variation between the different PCR amplifications, presence of PCR artifacts, etc) could have decreased the ability of REP1 typing to differentiate S. II Sofia isolates. The high genetic similarity shared by the S. II Sofia strains could have contributed to the poor discriminatory power of the individual typing methods – a limited genetic diversity makes it more difficult to find typing methods that are capable of detecting the minor genome changes present in the isolates.

Therefore, a single typing method usually cannot be relied on to properly characterise and discriminate strains. It has been shown that a combination of typing methods has been employed to successfully study various Salmonella spp. (Gudmundsdottir et al., 2003; Liebana et al., 2001; Lindqvist et al., 2002; Malorny et al., 2001; Nayak et al., 2004; Pang et al., 2005; Ridley et al., 1998). The use of more than one RE has also been shown to increase the ability of PFGE to discriminate Salmonella isolates (Cardinale et al., 2005; Lindqvist et al., 2002; Malorny et al., 2001; Murase et al., 1995; Pang et al., 2005; Seo et al., 2006; Thong et al., 1995; Tsen et al., 2000; Tsen and Lin, 2001). However, the high genetic similarity exhibited by S. II Sofia isolates have made it difficult to type the strains and only 2 fingerprint methods were chosen: PFGE ($XbaI$ and $Spel$) and rep-PCR (REP1). By combining the typing systems, the
discrimination of S. II Sofia isolates could be improved: e.g. the dominant SpeI pattern (S1), which comprised of 89.3% of the isolates, can be further divided into 8 pattern combinations with XbaI and REP1 analysis – X1S1R1, X1S1R3, X1S1R2, X3S1R1, X3S1R2, X4S1R1, X5S1R2 and X6S1R1. When all 3 methods are combined for typing, the 84 isolates could be divided into 16 subtypes, increasing the discriminatory index to 0.721 (Tables 3.2 and 3.3). An index of 0.721 indicates that if 2 strains were randomly sampled from the population, on 72.1% of the occasions, they would fall into different groups. This improvement in differentiation is reflected not only in the increase in index value but also in the decrease of sample size in the predominant profile type over a single measure (indicating that the capability of the typing methods in further dividing the strains) (Table 3.3).

Although the high pattern similarity exhibited by the Australian S. II Sofia isolates could limit the value of typing systems used here as a tool for epidemiological analysis, it can also be an indication of the clonal nature of the strains. This limited clonal diversity is reflected in the Dice coefficient values and dendograms (Table 3.1 and Figures 3.6 to 3.8), which show that a large portion of DNA bands (about 70 to 90%) observed in the patterns from the strains, were probably identical. The limited diversity of BOX, ERIC and ribotyping pattern obtained via PCR also reflects on the clonal nature of the isolates. Only the REP sequences were varied enough to be used for typing. Further, as ribotyping usually results in a small number of bands, this tends to hinder differentiation among closely related strains (as is probably the case in S. II Sofia). The poor discrimination indices from each individual typing system supports the theory regarding the clonal nature of the isolates, making it necessary to combine fingerprinting methods to increase discrimination of said isolates.
The clonality observed in the current study is also supported by previous epidemiological studies of *S. II Sofia* (Harrington *et al.*, 1991; Ross *et al.*, 2003). Harrington and her colleagues (Harrington *et al.*, 1991) found that the Australian *S. II Sofia* isolates studied belonged to a single clonal group. Characterisation of *S. II Sofia* using fluorescent AFLP have shown that isolates share >70% similarity, with certain groups persisting over time (Ross *et al.*, 2003). The limited genetic diversity observed here indicates that the isolates were possibly derived from a single clone. The spread of *S. II Sofia* throughout Australia meant that the strains were subjected to selective pressure from a variety of sources: different farm management practices, different environments and food, use of antibiotics, etc. Such selection is sufficient to cause minor genetic changes (gain or loss of restriction site), leading to the variation seen in the 84 isolates.

There was little correlation between the profile types and geographical location. A majority of the *S. II Sofia* isolates were not geographically restricted: the predominant pattern combination (X1S1R1) was found to be spread among the isolates from various locations (Table 3.2). Victorian *S. II Sofia* isolates were found to exhibit the most diverse pattern types (9 combinations) compared to isolates from other states. However, this could be due to the larger sample population: 34 Victorian *S. II Sofia* strains were analysed compared to 10-15 isolates from the other states. In addition, all 10 Tasmanian isolates appear to belong to a single pattern type, which is the major pattern X1S1R1 (Table 3.2).

Two *S. II Sofia* strains were observed to show unique profile pattern – 554NCA and 131. The XbaI, SpeI and REP1 pattern of isolate 554NCA (New South Wales) was not obtained from the typing of the other 83 isolates (all isolates obtained were confirmed as *S. II Sofia* through
malonate and ONPG biochemical tests [see Appendix II] before being subjected to further analysis). The reason for the particular pattern combination is unknown and more samples would need to be typed to ascertain whether such a pattern is restricted to a certain location (New South Wales). On the other hand, while S. II Sofia 131 had unique PFGE profiles (X8 and S6), PCR typing has shown it has the second most common REP1 pattern R2. XbaI restriction analysis appears to be most efficient at discriminating 131, producing a pattern with an overall similarity of 44% (Figure 3.6). The origin of 131 is unknown (the strain has been in the RMIT collection) and since no correlation could be found between profile type and location, there is no way to determine where the isolate is from. Any conclusion regarding the relationship of profile patterns and geographical locations can only be made after more isolates (from Queensland, New South Wales, Tasmania and South Australia) are collected and analysed.

A DNAse inactivation step to minimise DNA degradation was successfully devised, allowing S. II Sofia to be subtyped using PFGE. A total of 84 Australian S. II Sofia isolates were typed using PFGE (XbaI and SpeI) and PCR typing (REP1) – resulting in generation of 8 XbaI, 6 SpeI and 5 REP1 profile types. The discriminatory ability of the individual typing systems was found to be poor, with low diversity indices. By combining the typing systems, isolates could be further discriminated into 16 profile types with an index diversity of 0.721. No link could be established between any of the fingerprint typing methods. There was no correlation between a majority of the profile types and geographical location – the analysis of more samples is still required before any conclusions could be drawn. Molecular fingerprinting of the isolates and its subsequent analysis indicates that Australian S. II Sofia strains have limited genetic diversity and are probably clonally related. PFGE has potential as an epidemiological tool for the future
study of S. Il Sofia and further experimental work is needed to develop the method e.g. the use of additional restriction enzymes in subtyping.
CHAPTER 4: MOLECULAR ANALYSIS OF SPI1 TO SPI5 IN SALMONELLA II SOFIA

4.1 Introduction

Most Salmonella species are capable of invading a host and causing gastrointestinal and/or systemic disease (Jones and Falkow, 1996; Marcus et al., 2000; Ohl and Miller, 2001). The pathogenic ability of Salmonella spp. is dependent upon the presence of virulence determinants that can induce a variety of functions once within the host cell, such as cell invasion, cytoskeletal rearrangements, fluid accumulation, intracellular survival and replication, etc. A large proportion of virulence factors are found clustered together in distinct regions of bacterial chromosomes, which are termed pathogenicity islands (Hentschel and Hacker, 2001; Hensel, 2004). Pathogenicity islands are found in a wide variety of bacterial pathogens, including Salmonella spp. and an example would be the Salmonella Pathogenicity Island (SPI). A number of SPIs can be found within the Salmonella spp. genome (depending on the serovar) but the most common and best studied are SPI1 to SPI5.

SPI1 is a 40 kB region that encompasses a large number of genes encoding components of a TTSS and its effector proteins. This region is responsible for enabling Salmonella spp. to colonise and invade intestinal epithelial cells and transport virulence effector proteins from the bacteria into the host cell cytosol (Galán, 1999; Hansen-Wester and Hensel, 2001; Hensel, 2004; Marcus et al., 2000; Ohl and Miller, 2001). Several genes in this region (prg, org, spa and inv operons) have been shown to be
involved in the formation of the needle complex of the SPI1 TTS system (Eichelberg et al., 1994; Kaniga et al., 1994; Kimbrough and Miller, 2000; Klein et al., 2000; Kubori et al., 1998; 2000; Sukhan et al., 2001; Watson et al., 1998). This structure acts like a needle, piercing the host cell membrane and allowing effector proteins to be delivered into the host. Furthermore, SPI1 contains genes encoding effector (sip, sptP and avrA), accessory (invB, sicP, sicA and iagB) and regulatory (invF, hilA, hilC and hilD) proteins. Once inside the host cells, the virulent effector proteins are involved in a variety of functions such as aiding translocation of proteins, cytoskeletal rearrangements, membrane ruffling and cell apoptosis, all of which leads to bacterial invasion (Chang et al., 2007; Cherayil et al., 2000; Collier-Hyams et al., 2002; Darwin and Miller, 1999b; Fu and Galán, 1998b; Hayward and Koronakis, 1999; Ye et al., 2007; Zhang et al., 2003).

Another large virulence region is the 40 kB SPI2, which encodes a second TTS system that is structurally and functionally different from the TTSS encoded by SPI1 (Chakravortty et al., 2005; Hensel et al., 1995; 1997b; Marcus et al., 2000; Ochman et al., 1996; Shea et al., 1996). In addition to genes encoding components of the TTSS (ssa and sseBCD), this region also consists of ORFs that encode effector proteins, their cognate chaperones (sse and ssc) and regulatory proteins (ssr) (Chakravortty et al., 2005; Hensel et al., 1997a; 2000; Shea et al., 1996). SPI2 effector proteins are responsible for virulence functions such as aiding the translocation of effector proteins, intracellular trafficking of SCVs (maturation, maintenance and positioning) and intracellular survival and replication (Guy et al., 2000; Kuhle and Hensel, 2002; Nikolaus et al., 2001; Uchiya et al., 1999; Yu et al., 2004). Through the effects of proteins encoded by this region, Salmonella spp. is able to accumulate in the liver and spleen cells, leading to the development of systemic disease.
Two other pathogenicity islands, SPI3 and SPI4 (a 17 kB and 27 kB region, respectively) are present in *Salmonella* spp. (Blanc-Potard and Groisman, 1997; Wong et al., 1998). Compared to SPI1 and SPI2, these two virulence regions are not as well studied and thus, the role of some of the genes is still unknown. SPI3 is comprised of 10 ORFs organised into 6 transcriptional units and include *mgtCB* (required for intramacrophage survival), *misL* (a surface adhesin that is required for intestinal colonisation) and *marT* (a transcriptional activator of the *misL* promoter) (Blanc-Potard and Groisman, 1997; Dorsey et al., 2005; Morgan et al., 2004). Further, it has been shown that the genes *cigR*, *rhuM*, *rmbA* and *fidL* could potentially be involved in the systemic disease process (Lawley et al., 2006). However, the exact function played by the proteins encode by these genes is not known. Analysis of the SPI4 region have revealed that it contains 6 genes (*siiABCDEF*) that form an operon and are most probably co-transcribed together as a large mRNA (Gerlach et al., 2007a; Gerlach et al., 2007b; Morgan et al., 2004; Morgan et al., 2007). This operon encodes components for a T1SS (*siiCDF*), a large non-fimbrial adhesin protein that mediates contact-dependent adhesion of *Salmonella* to epithelial cell surfaces (*siiE*) and 2 other proteins of unknown function (*siiAB*) (Gerlach et al., 2007b; Morgan et al., 2004; 2007).

The smallest of the 5 SPIs, SPI5 is a 7.6 kB region found in *Salmonella* serovars such as Dublin and Typhimurium (Hong and Miller, 1998; Wood et al., 1998). Sequence analysis revealed that this region contains 6 novel genes – *pipD*, *orfX*, *sopB*, *pipC*, *pipB* and *pipA* (Wood et al., 1998). SPI5 is responsible for eliciting intestinal secretory and inflammatory responses in the host by mediating chloride secretion in infected epithelial cells (leading to fluid influx), accumulation of neutrophils in the gut and intestinal inflammation (Galyov et al., 1997; Norris et al., 1998; Pfeifer et al., 1999; Wood et al., 1998). Unlike the other SPIs, SPI5 does not seem to encode its own secretion system.
and effector proteins are transported by the TTSS of SPI1 and SPI2. SopB (and its chaperone, PipC) has been shown to be translocated in a Sip-dependent pathway, indicating that the SPI1 TTS system is responsible for its delivery into host cells (Galyov et al., 1997; Hong and Miller, 1998; Zhang et al., 2002). In contrast, the protein PipB is translocated by the SPI2 TTS system to the SCVs; however, its exact function in the virulence process is still unclear (Knodler et al., 2002).

Although a majority of *Salmonella* spp. are pathogenic, their virulence (disease and host range) seem to vary from serovar to serovar. As pathogenicity of a serovar is dependent on the effects of various virulence proteins, this diversity can be attributed to genetic polymorphisms in virulence genes encoding such proteins. *Salmonella* spp. with naturally occurring deletions in the SPI regions are less virulent and unable to invade the host or cause systemic disease (Boyd et al., 1996; Ginnocchio et al., 1997; Porwollik et al., 2002). Analysis of SPI1 to SPI5 regions in various *Salmonella* serovars have revealed that a range of insertions and deletions were found in the strains, which could explain the pathogenic diversity of *Salmonella* spp. (Amavisit et al., 2003; Porwollik et al., 2002). Such insertions and deletions are mediated by mobile elements such as transposons and IS elements; and by changes in the genetic sequence - base insertion, deletion or substitution (Anon, 2008b; Dobrindt et al., 2004; Steinert et al., 2000).

*Salmonella* II Sofia is an example of *Salmonella* serovar that exhibits low virulence in humans and chickens. It is an efficient coloniser of chickens and therefore, is frequently isolated from poultry flocks (Harrington et al., 1991; Heuzenroeder et al., 2001). Although S. II Sofia is able to adhere to and invade cultured epithelial cells, the strain is unable to survive intracellularly (Rickard, 1998). Further, this serovar only appears capable of causing systemic disease in hosts with immature or
immunocompromised systems (Rickard, 1998). As very few studies have been conducted on the pathogenicity of S. II Sofia, little is known about its virulence process. However, Heuzenroeder and his colleagues (Heuzenroeder et al., 2001) have shown that S. II Sofia is incomplete within the inv region of SPI1. The inv operon is required for TTSS of SPI1 (Eichelberg et al., 1994; Kaniga et al., 1994; Kimbrough and Miller, 2000; Kubori et al., 2000; Sukhan et al., 2001) and its absence could be one reason for the avirulent nature of S. II Sofia. However, more research on SPI1 and the other SPIs needs to be carried out before any conclusions can be made.

In this study, investigation into the pathogenicity of S. II Sofia was undertaken through molecular analysis of Salmonella Pathogenicity Islands (SPI) 1 to 5. A series of Southern hybridisation, PCR and sequencing experiments (using probes and/or primers detecting gene(s) of interest) was carried out on the isolate S. II Sofia Bt8. The aims of the experimental methods conducted for this chapter were to:

(i) examine the genetic variation and distribution of genes across the entire length of SPI1 to SPI5 in S. II Sofia using RFLP and sequencing analysis,
(ii) construct a genetic map of the SPIs in S. II Sofia using all data and compare it to S. Typhimurium 82/6915 (a known virulent strain),
(iii) investigate whether the distribution of genes in the SPIs is able to explain the avirulent nature of S. II Sofia.

4.2 Methods and Results

4.2.1 Southern Hybridisation Analysis of SPI Regions

The majority of the primers used for the amplification of DNA probes were designed using the Clone Manager 7 software as described previously (see Section
2.3.3.1), with the SPI sequences of S. Typhimurium LT2 as a reference. The GeneBank accession numbers of the LT2 SPI sequences are listed in Appendix VI. All primers used here were designed from LT2 reference sequence data except for primers in the amplification of a part of SPI1 (1C) and the entire SPI3, which were described previously (Amavisit et al., 2003). DNA probes used in Southern blot detection were prepared by expand amplification (see Section 2.3.3.3) of one or more fragments encompassing the entire length of each SPI (see Appendix VI for details on primer sequence, length, elongation time and annealing temperature of probes). Probes were produced using genomic DNA extracted from S. Typhimurium SL1344 and restriction analysis was carried out on each probe (using the REs BglII, EcoRV and HindIII) to verify that the probe produced was accurate. Labeling and estimation of probe concentration was conducted as outlined in Chapter 2 (see Section 2.3.7.1).

Genomic DNA from selected S. II Sofia isolates, including strain Bt8 (Table 2.1) and S. Typhimurium isolates SL1344, LT2 and 82/6915 (Table 2.2) was digested with 10U of REs BglII, EcoRV or HindIII or with 10U of a combination of the stated REs (BglII and EcoRV or HindIII and EcoRV) (Amavisit et al., 2003). All resulting DNA fragments were subjected to electrophoresis before being transferred onto nylon membranes for Southern hybridisation (see Section 2.3.7.2). S. Typhimurium SL1344 was included in every blot as a reference strain. The bands obtained within each SPI region after Southern hybridisation were compared for presence/absence and differences in restriction pattern (by using SL1344 as a reference). Any differences observed were subjected to further analysis through PCR and sequencing experiments.

Two methods of obtaining genomic DNA for Southern blotting were evaluated: DNA extraction and DNA agarose plugs. Genomic DNA was extracted from Salmonella
strains using the CTAB method as outlined in Chapter 2 (see Section 2.3.2.1). DNA plugs were prepared as described previously (see Section 2.3.6.1.1) with a minor modifications – doubling the amount of bacterial cells used for making the agarose plugs. For DNA extraction, approximately 20-25 µg of DNA was used for restriction digest (see Section 2.3.7.2). DNA plugs were digested as described previously (see Section 2.3.6.1.2) and plugs were inserted into wells of the gel and sealed with molten agarose. Southern blotting was conducted using DNA prepared from both methods and the efficiency of each method was evaluated (data not shown). The agarose plug method was found to be more efficient compared to DNA extraction. A small portion of the plug (~ 2 mm) contained a sufficient amount of DNA for Southern hybridisation. In contrast, DNA extraction had to be carried out a number of times in order to obtain an equivalent amount (data not shown). Therefore, all subsequent Southern blotting experiments were done by detecting digested fragments from DNA plugs.

4.2.2 PCR Analysis of SPI Regions

After comparing the restriction pattern between S. Typhimurium SL1344 and S. II Sofia Bt8, the regions of difference observed in each SPI region was subjected to further analysis by PCR and restriction digest. PCR (general and expand amplification) was carried out according to the method detailed in Chapter 2 (see Section 2.3.3.2 and 2.3.3.3). The oligonucleotide sequences of primers (along with the expected product size and annealing temperatures) for all PCR amplifications are listed in Appendix VII. Restriction cleavage of PCR products was conducted as described previously (see Section 2.3.5) using the appropriate REs, which are mentioned in the relevant sections (see Section 4.2.5 to 4.2.10). All resulting PCR amplification and restriction digestion was analysed by ethidium bromide agarose gel electrophoresis (see Section 2.3.4).
4.2.3 RT-PCR Analysis of SPI Regions

After incubation in appropriate conditions (see Sections 4.2.6 and 4.2.8), RNA from S. Typhimurium 82/6915 and S. II Sofia Bt8 was extracted and analysed as described previously (see Section 2.5.2). RT-PCR was carried out on selected genes (listed in greater detail in the relevant sections) within the SPI1 and SPI3 region in order to study the expression of said genes. The reverse transcription of all samples (with different primers) was prepared in pairs: with and without RTase (see Section 2.5.3.1) in order to verify that the PCR band produced is the result of mRNA transcription and not genomic DNA contamination during the extraction process. RT-PCR analysis was conducted as outlined previously (see Section 2.5.3) and primers used (along with expected product size and annealing temperature) are listed in Appendix VII.

4.2.4 Sequence Analysis of SPI Regions

DNA sequencing (see Section 2.3.3.4) was carried out on SPI1 to SPI5 of S. II Sofia Bt8. Using S. II Sofia Bt8 DNA, the SPI regions were amplified with the primers for DNA probes. The PCR products were purified (see Section 2.3.2.5) and used as a template for sequencing via the primer walking method. Briefly, each SPI region is sequenced with the corresponding primers, which were used to amplify the region. The sequence from the end of the fragment is then used to design a primer for sequencing the next portion of the long DNA region: in short, the sequence “walks” along the entire DNA region. All resulting sequences were assembled together (using the multiple alignment function of Clone Manager 7 software) to produce a full length SPI, which was further analysed with the same software. The DNA sequence of each S. II Sofia Bt8 SPI region was compared to the sequence of the corresponding S. Typhimurium LT2 SPI sequence. The restriction and sequencing data was used to construct a map of SPI1 to
SPI5 of isolate Bt8. For the construction of the map, fragments smaller than 0.1 kB were excluded as they cannot be detected through Southern hybridisation or restriction digest of PCR products (even though such fragments were detected in the sequencing data). In addition, the putative protein sequence of each SPI gene was obtained (using the software to translate DNA sequence) for comparison with LT2 SPI proteins (see relevant sections for further details).

4.2.5 Variation of SPI1 to SPI5 within each serovar

S. Typhimurium strains 1344, LT2 and 82/6915 were shown to share identical restriction cleavage patterns with each of the SPI DNA probes and thus, SL1344 was used as a reference in all the blots. However, differences were observed with the Southern hybridisation patterns (SPI1 to SPI5) between the Typhimurium and II Sofia serovars (results not shown). The selected S. II Sofia isolates (Bt8, Bt15, FSAW3452, 987VCA, 131, 988Q, 786CL, 554NCA, 140TCA and 642SC) shared the same SPI pattern with one another (Figure 4.1). This pattern similarity supports the theory that the S. II Sofia isolates are clonally related (see Chapter 3) and thus, the SPI regions of isolate Bt8 can be used as a reference for sequencing and map construction. An example of pattern similarity within each serovar and variation between the serovars can be seen in the Southern hybridisation pattern obtained after digestion using HindIII with EcoRV and detection with probe 1A and 4A (see Figure 4.1, Sections 4.2.6, 4.2.9 and Appendix VI).
Figure 4.1  Southern hybridisation pattern of the *Salmonella* serovars Typhimurium and II Sofia digested with *Hind*I and *EcoRV*. The fragment sizes of S. Typhimurium SL1344 are indicated at the side of each blot. Lane 1a and 2a, S. Typhimurium SL1344; Lane 1b and 2b, S. Typhimurium 82/6915; Lane 1c and 2c, S. II Sofia Bt8; Lane 1d and 2d, S. II Sofia Bt15; Lane 1e and 2e, S. II Sofia 988Q; Lane 1f and 2f, S. II Sofia 642SC; Lane 2g, S. II Sofia 554NCA. A) Probe 1A (13.8 kB). B) Probe 4A (15.5 kB).
4.2.6 Variation of SPI1 between S. Typhimurium and S. II Sofia

The SPI1 region is 40 kB in length, making it difficult to be utilised as a single probe. Therefore, amplification of DNA probes for this region was divided into 3 parts: 1A (13.8 kB), 1B (15.1 kB) and 1C (10.8 kB) (Figure 4.2 and Appendix VI). All 3 probes were used in the Southern blotting of Salmonella strains. Overall, variation in the restriction cleavage data can be observed between Salmonella isolates SL1344 and Bt8 with all 3 probes. However, attempts to amplify 1A and 1B in S. II Sofia isolates, including strain Bt8 have been unsuccessful and only amplification of 1C was possible, producing a 10 kB fragment (data not shown).

A series of primers amplifying shorter segments of the 1A and 1B region was devised in order to amplify the rest of SPI1 (Appendix VII). The following primer pairs were able to produce fragments (3.7 to 8.4 kB in size) within the particular SPI1 region: fhlA/F and sitD/R, sprB/F and prgH/R, prgH/F and hilA/R, iagB/F and sipB/R, sipB/F and spaR/R (see Figures 4.2 to 4.4 and Appendix VII). These products, along with 1C were subjected to RFLP and sequencing analysis as outlined (see Sections 4.2.2 and 4.2.4). The final map showing restriction sites of the various REs (BglII, EcoRV and HindIII) and genes within SPI1 of S. II Sofia Bt8 can be seen in Figure 4.2. After comparison between RFLP and sequencing data, a majority of the differences observed can be attributed to a series of base substitutions, resulting in the loss or gain of restriction sites (Figure 4.2). However, several segments within SPI1 seem to have accumulated changes that could affect gene expression and these areas were subjected to further analysis.
Figure 4.2 Variation of SPI1 between S. Typhimurium SL1344 and S. II Sofia Bt8 as detected by Southern hybridisation, PCR restriction digest and sequencing analysis. A) Schematic representation of SPI genes and their direction of transcription (adapted from Amavisit et al., 2003). DNA probes (size and extent of each probe) used for Southern hybridisation are indicated above the schematic diagram. B) BglII, EcoRV and HindIII restriction maps for S. II Sofia Bt8. The numbers indicate the sizes of detected fragments in kB. The maps are estimation of the sizes of the restricted DNA fragments and are not drawn to scale. C) Comparison of EcoRV bands between SL1344 and Bt8. +, presence of a fragment of the same size as that observed in S. II Sofia Bt8; -, absence of fragment. Blue boxes and numbers indicate the extent of and size (in kB) of the EcoRV fragment.
Figure 4.3  Comparison of RFLP analysis encompassing the 1A region of SPI1 in S. Typhimurium SL1344 and S. Il Sofia Bt8. M, λDNA marker digested with PstI. A) fhlA-sitD (3.79 kB). Lane 1a, 1c and 1e, fhlA-sitD amplified using SL1344 DNA and digested with BglII, EcoRV and BglII+EcoRV, respectively; Lane 1b, 1d and 1f, fhlA-sitD amplified using Bt8 DNA and digested with BglII, EcoRV and BglII+EcoRV, respectively. B) sprB-prgH (6.36 kB). Lane 2a, 2c and 2e, sprB-prgH amplified using SL1344 DNA and digested with HindIII, EcoRV and HindIII+EcoRV, respectively; Lane 2b, 2d and 2f, sprB-prgH amplified using Bt8 DNA and digested with HindIII, EcoRV and HindIII+EcoRV, respectively. C) prgH-hilA (4.6 kB). Lane 3a-3c, prgH-hilA amplified using SL1344 DNA and digested with BglII, EcoRV and HindIII, respectively; Lane 3d-3f, prgH-hilA amplified using Bt8 DNA and digested with BglII, EcoRV and HindIII, respectively.
Figure 4.4  Comparison of RFLP analysis encompassing the 1B and 1C region of SPI1 in S. Typhimurium SL1344 and S. II Sofia Bt8. M, λDNA marker digested with *Pst*I. A) *iagB-sipB* (8.27 kB). Lane 1a, 1c and 1e, amplified using SL1344 DNA and digested with *Hind*III, *EcoRV* and *Hind*III + *EcoRV*, respectively; Lane 1b, 1d and 1f, amplified using Bt8 DNA and digested with *Hind*III, *EcoRV* and *Hind*III + *EcoRV*, respectively. B) *sipB-spaR* (3.72 kB). Lane 2a, 2c and 2e, *sipB-spaR* amplified using SL1344 DNA and digested with *Hind*III, *EcoRV* and *Bgl*II, respectively; Lane 2b, 2d and 2f, *sipB-spaR* amplified using Bt8 DNA and digested with *Hind*III, *EcoRV* and *Bgl*II, respectively. C) 1C (10.8 kB). Note that this image is visualised in a negative background to make visible comparison of bands easier. Lane 3a, 3c and 3e, 1C amplified using SL1344 DNA and digested with *Bgl*II, *EcoRV* and *Hind*III, respectively; Lane 3b, 3d and 3f, 1C amplified using Bt8 DNA and digested with *Bgl*II, *EcoRV* and *Hind*III, respectively.
It was observed that all PCR amplifications of S. II Sofia Bt8 involving primer sets containing one primer designed to detect avrA e.g. sitD/F and avrA/R, avrA/F and hilC/R, etc (Appendix VII) were unable to produce any products (data not shown). The primer pair sitD/F and sprB/R (see Appendix VII) was designed to amplify the genes adjacent to avrA (see Figure 4.2), producing a fragment encompassing the avrA gene. Amplification of the sitD-sprB region was carried out on selected S. II Sofia isolates and other Salmonella serovars and the resulting PCR products were subjected to electrophoresis (Figure 4.5). The products encompassing sitD to sprB from the Salmonella serovars Typhimurium, Enteritidis, Infantis, Virchow and Agona were the expected 2.3 kB fragment (Figure 4.5). However, sitD-sprB product obtained from all the S. II Sofia isolates, and serovars Kiambu and Ohio were 1.4 kB in size (Figure 4.5).

Sequencing analysis of sitD-sprB from strain Bt8 has revealed the replacement of the missing 0.9 kB fragment between this region (that would encode avrA) with a 0.28 kB fragment bordered by sitD and sprB (results not shown). A BLAST search (from the PubMed website) was performed on this sequence in order to identify other DNA sequences in the GeneBank database that may share similarity with the fragment. This fragment was found to share 98% sequence similarity with the 200 bp genetic element located in the sitD-sprB region of SPI1 from S. Typhi and S. Choleraesius (Appendix VIII). Further, this fragment harboured an ORF encoding a protein of unknown function (results not shown). Although the sequence data shows that avrA is absent from its expected location within SPI1, Southern hybridisation and PCR amplification with avrA alone showed that this gene is still present in the S. II Sofia genome - however, Southern hybridisation and PCR bands detected/produced were weaker compared to S. Typhimurium SL1344 (Figure 4.5).
Figure 4.5  PCR and Southern hybridisation analysis of avrA within SPI1. M, λDNA marker digested with PstI. A) PCR amplification of sitD-sprB region from various Salmonella serovars. Lane 1a, Typhimurium 82/6915; Lane 1b, Kiambu 343W; Lane 1c, Enteritidis 446302; Lane 1d, Ohio 213N; Lane 1e, Infantis 999S; Lane 1f, Virchow 845V; Lane 1g, Agona FSAW3416; Lane 1h-1m, II Sofia (Bt8, 630MSC, 988Q, 554NCA, 150TC and 664SCA, respectively). B) Southern blot detection of avrA. Lane 2a, Typhimurium SL1344; Lane 2b-2d, II Sofia (Bt8, 404QCA and 131, respectively). C) PCR amplification of avrA from various Salmonella serovars. Lane 3a, Typhimurium 82/6915; Lane 3b, Kiambu 343W; Lane 3c, Enteritidis 446302; Lane 3d, Virchow 845V; Lane 3e, Infantis 999S; Lane 3f, Ohio 213N; Lane 3g, Agona FSAW3416; Lane 3h-3k, II Sofia (Bt8, 988Q, 554NCA, 671SC, respectively).
Sequencing analysis (through primer walking) of the 1B region (iagB-sipB) of SPI1 revealed the presence of a 1.2 kB fragment between iagB and sptP, which is not found in S. Typhimurium SL1344 (Figure 4.2). Translation of this putative ORF predicted a protein that is 400 amino acids in length (results not shown). A BLAST search was performed on this protein in order to identify other polypeptide sequences in the GeneBank database that may share similarity with this protein, enabling the elucidation of the putative function of the protein. The BLAST results indicate that this protein shares sequence similarity with the transposase from the mutator family; with 52%, 55%, 62%, 68%, 68-74%, 91% and 96% match to the transposase from Bacteroides thetaiotaomicron, Rickettsiella grylli, E. coli CTF073, Psychrobacter spp., Shewanella spp. (serovars oneidensis, putrefaciens, denitrificans and baltica), Yersinia enterocolitica and S. Weltevreden; respectively (see Figure 4.6 for amino acid alignment).

However, this DNA fragment (termed an insertion sequence or IS – see Figure 4.2) appears to be restricted to S. II Sofia. PCR amplification using primer pair iagB/F and sptP/R, which detects the genes flanking the IS was performed on selected Salmonella serovars and 18 S. II Sofia isolates (Figure 4.7). Amplification of all the other Salmonella serovars (Typhimurium, Hadar, Heidelberg, Enteritidis, Agona, Ohio, Infantis, Virchow and Kiambu) resulted in a 0.69 kB PCR product (Figure 4.7). In contrast, the amplified product from S. II Sofia isolates was much larger (1.99 kB), indicating the presence of an additional 1.2 kB ORF between iagB and sptP (Figure 4.7).
Figure 4.6  Alignment of the transposase from S. II Sofia Bt8 (IS) and other bacteria species (Salmonella Weltevreden, Y. enterocolitica, Shewanella spp., R. grylli, B. thetaotaomicron, Psychrobacter spp. and E. coli CFT073, respectively) using Clone Manager 7 software. Identical residues are shaded in pink and non-identical residues are not shaded. Numbering on the right side of the residues refers to the position of each amino acid in the polypeptide sequence. A list with both the single- and three-letter amino acid codes is shown in Appendix IX.
Figure 4.7  PCR amplification of iagB-sptP region from various Salmonella serovars. M, λDNA marker digested with PstI. Lane 1 and 15, PCR negative control; Lane 2 and 3, Typhimurium (SL1344 and 82/6915, respectively); Lane 4, Hadar P12; Lane 5, Enteritidis 446302; Lane 6, Agona FSAW3463; Lane 7, Ohio 213N; Lane 8, Infantis 999S; Lane 9, Virchow 370QC; Lane 10, Kiambu 345W; Lane 11-14 and 16-29, Il Sofia (Bt8, 988Q, 554NCA, FSAW3452, 826V, 630MSC, 147TC, 147TCA, 150TC, 150TCA, 537NCA, 566NC, 664SCA, 659SCA, 642SC, 985Q, 391QCA and 348QC, respectively).
Overall, a majority of the proteins encoded by the genes in SPI1 of S. II Sofia Bt8 were highly similar (in length and amino acid content) to the proteins from the corresponding genes in SPI1 of S. Typhimurium LT2, with most S. II Sofia Bt8 SPI1-associated proteins showing a 87 to 99% match to its LT2 SPI1-encoded counterparts (see Appendix VIII for further details). However, a number of SPI1 genes (orgB, prgI, sptP, sipD and invJ) from strain Bt8 appear to have accumulated changes in their DNA sequence, which in turn alters the sequence of the predicted proteins (Appendix VIII). The orgB gene contains an additional 10 bp sequence (ATCGTAAAAT) near the end of the gene, which (due to an in-frame stop codon) resulted in a protein missing 3 amino acids from the end of the polypeptide sequence when translated with Clone Manager 7 – valine, isoleucine and arginine. PrgI from S. II Sofia Bt8 was observed to contain an extra threonine residue at position 3 due to the insertion of CCA after the start codon of the gene (Appendix VIII). Although the InvJ protein from strain Bt8 was shown to be highly similar to InvJ from LT2 (98% match, see Appendix VIII), a single base substitution of adenine with guanine (A with G: ATG to GTG) at the start codon of invJ from strain Bt8 resulted in first amino acid of the polypeptide being changed from methionine to valine.

From the sequencing data, the two SPI1 genes (and thus, their putative proteins) observed to have the most changes are sptP and sipD. Multiple changes could be observed in the genetic sequence of sptP such as insertion and deletion of sequences and base substitutions, with a majority of these changes being base substitutions (data not shown). After translation of the DNA sequence, the predicted SptP obtained was found to contain 511 amino acids as opposed to the expected 543 amino acids – there was an absence of 32 amino acids from the polypeptide sequence (Figure 4.8 and Appendix VIII). In addition, an alignment performed between SptP from strains LT2 and
Bt8 showed that the multiple alterations in the gene led to changes in the amino acid composition of the protein – a 72% similarity was obtained (Figure 4.8). Multiple sequence changes could also be observed in the *sipD* from *S. II Sofia Bt8* (Figure 4.9). The *sipD* gene (from LT2) is expected to encode a 343-amino acid protein. However, a number of sequence changes (insertion, deletion and substitution events) towards the end portion of the gene (from base 790 to 820) seemed to have altered the predicted protein sequence of *sipD* from isolate Bt8 (Figure 4.9). This resulted in the translation of a truncated 260-amino acid protein.

The absence of *avrA* and the genetic changes observed in *sptP* and *sipD* within SPI1 from *S. II Sofia Bt8* could imply that these genes are not expressed during bacterial invasion (when the SPI1 region is most active). All 3 genes were then subjected to conditions known to activate SPI1 in order to investigate the expression of the individual genes. Induction of SPI1 expression was carried out as described previously with some minor modifications (Miao and Miller, 2000). Briefly, overnight cultures of *S. Typhimurium* 82/6915 and *S. II Sofia Bt8* were diluted 1:30 in 5 mL of fresh LBB and incubated at 37°C for 5 h with aeration before RNA isolation (see Section 4.2.3). RT-PCR analysis was performed on all RNA samples and the expression of the 3 genes between isolates 82/6915 and Bt8 was compared. The expression of *avrA*, *sipD* and *sptP* from Typhimurium 82/6915 was detected, producing a single band of 0.67, 0.79 and 0.95 kB in size, respectively (Figure 4.10). However, no products were obtained from *S. II Sofia Bt8* after RT-PCR, indicating the possibility that *avrA*, *sptP* and *sipD* are not expressed by this strain (Figure 4.10).
Figure 4.8  Amino acid alignment of the SptP protein from S. Typhimurium LT2 with the putative SptP from S. II Sofia Bt8 using Clone Manager 7 software. Identical residues are shaded in pink and non-identical residues are not shaded. Numbering on the right side of the residues refers to the position of each amino acid in the polypeptide sequence. The SptP proteins share 72% match in amino acid composition. A list with both the single- and three-letter amino acid codes is shown in Appendix IX.
Figure 4.9 DNA sequence alignment of sipD from S. Typhimurium LT2 with sipD from S. II Sofia Bt8 using Clone Manager 7 software. Identical bases for sipD sequence from LT2 and Bt8 are shaded in green and non-identical bases are unshaded. Numbering on the right side of the sequence refers to the position of each base in the DNA sequence. The sipD genes share an 80% sequence similarity.
Figure 4.10  RT-PCR analysis of avrA, sipD and sptP from S. Typhimurium 82/6915 and S. II Sofia Bt8. M, λDNA marker digested with PstI. Lane 1, 3, 5, 7, 9 and 11 are samples prepared without RTase (negative control) and Lane 2, 4, 6, 8, and 12 are samples with added RTase. Lane 1 and 2, avrA from 82/6915; Lane 3 and 4, avrA from Bt8; Lane 5 and 6, sptP from 82/6915; Lane 7 and 8, sptP from Bt8; Lane 9 and 10; sipD from 82/6915; Lane 11 and 12, sipD from Bt8; Lane 13, no sample.
4.2.7 Variation of SPI2 between S. Typhimurium and S. II Sofia

The SPI2 region was divided into 3 parts to make detection by Southern hybridisation easier to perform. Three individual DNA probes were derived from the SPI2 of S. Typhimurium SL1344 and used to probe Salmonella spp. genomic DNA after restriction digest with the appropriate REs: 2A (13.6 kB), 2B (13.5 kB) and 2C (12.9 kB) (Figure 4.10 and Appendix VI). All 3 probes detected variation in the hybridisation patterns between strains SL1344 and Bt8 (results not shown). Further, the Southern hybridisation data was found to match with both the RFLP and sequencing data – thus, all the data were used to construct the restriction maps of SPI2 (Figures 4.11 and 4.12). A large proportion of the differences found in the SPI2 from isolate Bt8 were attributable to the loss or gain of restriction sites (Figure 4.11) and most possibly did not hinder gene expression. These predicted proteins were highly similar to their S. Typhimurium LT2 counterparts with an 83% to 98% identity between proteins (Appendix VIII).

However, insertion and deletion events were observed in 3 of the S. II Sofia Bt8 SPI2-associated genes – ssaP, ssal and ssaE (Appendix VIII). The SsaP is missing one amino acid (proline) from its polypeptide sequence due to the deletion of three bases from the gene – CCT (Appendix VIII). In contrast, ssal contains an insertion of AGA, making the Ssal protein longer by one amino acid, with arginine being the extra residue (Appendix VIII). A deletion of approximately 200 bp was observed at the junction between ssea and ssaE of isolate Bt8 (indicated by * in the table of fragments obtained from EcoRV digestion in Figure 4.11). A single base substitution of T to C in ssaE probably interfered with the stop codon of this protein, resulting in 5 extra residues at the end of the polypeptide chain of SsaE: glutamine-lysine-serine-leucine-serine (Appendix VIII).
Figure 4.11  Variation of SPI2 between S. Typhimurium SL1344 and S. II Sofia Bt8 as detected by Southern hybridisation, PCR restriction digest and sequencing analysis. A) Schematic representation of SPI genes and their direction of transcription (adapted from Amavisit et al., 2003). DNA probes (size and extent of each probe) used for Southern hybridisation are indicated above the schematic diagram. B) BglII, EcoRV and HindIII restriction maps for S. II Sofia Bt8. The numbers indicate the sizes of detected fragments in kB. The maps are estimation of the sizes of restricted DNA fragments and are not drawn to scale. C) Comparison of EcoRV bands between SL1344 and Bt8. +, presence of a fragment of the same size as that observed in S. II Sofia Bt8; -, absence of fragment. *, shows the missing 230 bp between sseA and ssaE from Bt8. Blue boxes and numbers indicate the extent of and size (in kB) of the EcoRV fragment.
Figure 4.12 Comparison of RFLP analysis encompassing the 2A, 2B and 2C region of SPI2 in S. Typhimurium SL1344 and S. II Sofia Bt8. M, λDNA marker digested with *Pst*I. A) 2A (13.6 kB). Lane 1a, 1c, 1e, 1g and 1i, 2A amplified using SL1344 DNA and digested with *Hind*III, *Eco*RV, *Bgl*II, *Bgl*II + *Eco*RV and *Hind*III + *Eco*RV, respectively; Lane 1b, 1d, 1f, 1h and 1j, 2A amplified using Bt8 DNA and digested with *Hind*III, *Eco*RV, *Bgl*II, *Bgl*II + *Eco*RV and *Hind*III + *Eco*RV, respectively. B) 2B (13.5 kB). Lane 2a, 2c, 2e, 2g and 2i, 2B amplified using SL1344 DNA and digested with *Hind*III, *Eco*RV, *Bgl*II, *Bgl*II + *Eco*RV and *Hind*III + *Eco*RV, respectively; Lane 2b, 2d, 2f, 2h and 2j, 2B amplified using Bt8 DNA and digested with *Hind*III, *Eco*RV, *Bgl*II, *Bgl*II + *Eco*RV and *Hind*III + *Eco*RV, respectively. C) 2C (10.9 kB). Lane 3a, 3c, 3e, 3g and 3i, 2C amplified using SL1344 DNA and digested with *Hind*III, *Eco*RV, *Bgl*II, *Bgl*II + *Eco*RV and *Hind*III + *Eco*RV, respectively; Lane 3b, 3d, 3f, 3h and 3j, 2C amplified using Bt8 DNA and digested with *Hind*III, *Eco*RV, *Bgl*II, *Bgl*II + *Eco*RV and *Hind*III + *Eco*RV, respectively.
4.2.8 Variation of SPI3 between S. Typhimurium and S. II Sofia

A single 17 kB SPI3 probe was derived from the genomic DNA of S. Typhimurium SL1344 for use in Southern hybridisation. A comparison of the Southern blotting data obtained from strains SL1344 and Bt8 revealed a variation in the SPI3 hybridisation pattern (results not shown) and this was confirmed by the restriction digest and sequencing of SPI3 (Figures 4.13 and 4.14). Most of the RFLP pattern variation observed can be attributed to a series of loss/gain of restriction sites (Figure 4.13). However, several genes within SPI3 were observed to carry mutations that could affect protein sequences and these areas were subjected to further analysis.

The left hand portion of SPI3, which contains the genes sugR and rhuM, was found to be absent in the corresponding region of S. II Sofia Bt8. PCR amplification of genomic DNA from Salmonella serovars Typhimurium and II Sofia with the primer pair 3/F3 and rhuM/R, showed that only the Typhimurium strains (SL1344, LT2 and 82/6915) yielded the specific 3.43 kB fragment containing both the genes (Figure 4.15). The missing sugR-rhuM region was further confirmed by amplification of another 10 S. II Sofia isolates with the same primer pair, by Southern hybridisation and by sequencing of SPI3 from isolate Bt8 (results not shown). Analysis of the sequence located in the missing sugR-rhuM region have revealed that it contains a 135 bp nucleotide sequence sharing 99% similarity with the putative IS1351 transposase (pseudogene) from S. Typhi CT18 (data not shown).
Figure 4.13  Variation of SPI3 between S. Typhimurium SL1344 and S. II Sofia Bt8 as detected by Southern hybridisation, PCR restriction digest and sequencing analysis. A) Schematic representation of SPI genes and their direction of transcription (adapted from Amavisit et al., 2003). The absence of sugR-rhuM and presence of the ORF (IS) is indicated as an unshaded block and green shaded box, respectively. DNA probes (size and extent of each probe) used for Southern hybridisation are indicated above the schematic diagram. B) BglII, EcoRV and HindIII restriction maps for S. II Sofia Bt8. The numbers indicate the sizes of detected fragments in kB. The maps are estimation of the sizes of restricted DNA fragments and are not drawn to scale. C) Comparison of EcoRV bands between SL1344 and Bt8. +, presence of a fragment of the same size as that observed in S. II Sofia Bt8; -, absence of fragment. Blue boxes and numbers indicate the extent of and size (in kB) of the EcoRV fragment.
Figure 4.14  Comparison of RFLP analysis of the SPI3 region in S. Typhimurium SL1344 and S. II Sofia Bt8. M, λDNA marker digested with PstI. Lane 1, 3, 5, 7 and 9, probe 3 amplified using SL1344 DNA and digested with HindIII, EcoRV, BglII, BglII+ EcoRV and HindIII +EcoRV, respectively; Lane 2, 4, 6, 8 and 10, probe 3 amplified using Bt8 DNA and digested with HindIII, EcoRV, BglII, BglII+ EcoRV and HindIII +EcoRV, respectively.
Figure 4.15  PCR analysis of sugR-rhuM and slsA-cigR regions within SPI3. M, λDNA marker digested with PstI. A) PCR amplification of sugR-rhuM region (with primer pair 3/F3 and rhuM/R) from various Salmonella serovars. Lane 1, Typhimurium SL1344; Lane 2, Typhimurium LT2 9121; Lane 3, Typhimurium 82/6915; Lane 4-7, II Sofia (Bt8, 988Q 664SCA and 554NCA, respectively). B) PCR amplification of slsA-cigR region (with primer pair 3/F12 and 3/R8) from various Salmonella serovars. Lane 1, Typhimurium 82/6915; Lane 2, Hadar P12; Lane 3, Heidelberg; Lane 4, Enteritidis 446302; Lane 5, Agona FSAW3416; Lane 6, Ohio 213N; Lane 7, Infantis 999S; Lane 8, Virchow 370 QC; Lane 9, Kiambu 343W; Lane 10-29, II Sofia (Bt8, FSAW3452, 826V, 630MSC, 988Q, 348QC, 404QCA, 985Q, 554NCA, 561NC, 566NC, 537NCA, 140TCA, 147TC, 150TC, 150TCA, 659SCA, 671SCA, 636SC and 642SC, respectively).
Sequencing analysis has also revealed the presence of a 1.2 kB fragment between slsA and cigR, which is not found in S. Typhimurium SL1344 (Figure 4.13). This ORF (IS) was found to encode a 400-amino acid protein that is identical to the protein from the putative ORF located between iagB and sptP of SPI1 from strain Bt8 (see Section 4.2.6). Further, this sequence is also restricted to S. II Sofia: using the primer pair 3/F12 and 3/R8 (Appendix VII), it has been shown that only the amplification of S. II Sofia isolates generated PCR products containing this ORF; resulting in a 1.6 kB fragment instead of the expected 0.42 kB seen in all the other Salmonella serovars (Figure 4.15).

Sequencing analysis revealed that out of the 8 genes (rmbA, misL, fidL, marT, slsA, cigR, mgtC and mgtB) in SPI3 of S. II Sofia Bt8, 3 of these genes (misL, marT and slsA) were found to contain changes that affected the translation of its proteins; with the rest of the SPI3-associated proteins showing a 95-98% identity when compared with the corresponding SPI3 proteins from strain LT2 (Appendix VIII). A 15 bp deletion (TCCCTGACCCTGTCG) from the middle of misL from S. II Sofia resulted in the translation of a protein missing 5 amino acid residues from the middle portion polypeptide sequence: valine-proline-aspartic acid-proline-valine (Appendix VIII). On the other hand, 23 residues were absent from the start of the MarT protein due to the insertion of the 10 bp sequence (ACCGGGAGAAA), which caused a frameshift within the gene (see Appendix VIII). The presence of the IS sequence in the slsA-cigR region of strain Bt8 appeared to have interfered with the expression of slsA. From the sequencing data, it was shown that the 1.2 kB IS element was inserted towards the end of the slsA gene, causing a frameshift which disrupted the stop codon for this protein (data not shown). The predicted SlsA (translated using Clone Manager 7) had an additional 25 amino acids at the end of the polypeptide sequence (Appendix VIII).
The expression of SPI3 genes marT and slsA were analysed through RT-PCR (see Section 4.2.3). Cultures of S. Typhimurium 82/6915 and S. Il Sofia Bt8 were incubated as described previously (see Section 4.2.6) before being subjected to RNA isolation and RT-PCR. No expression of slsA was detected from either S. Typhimurium 82/6915 or S. Il Sofia Bt8: no RT-dependent PCR product could be amplified from RNA of either strain (Figure 4.16). However, RT-PCR analysis of marT from strains 82/6915 and Bt8 yielded the expected 0.42 kB fragment (Figure 4.16).

4.2.9 Variation of SPI4 between S. Typhimurium and S. Il Sofia

Two probes (4A and 4B, see Appendix VI for details) were used to investigate the variation between S. Typhimurium SL1344 and S. Il Sofia Bt8 in SPI4. The variation in the Southern hybridisation pattern obtained was confirmed by restriction digest and sequencing analysis and all data was used in the construction of the SPI4 restriction maps of Bt8 (Figures 4.17 and 4.18). Analysis of the sequencing data obtained (using Clone Manager 7) has revealed that the S. Il Sofia Bt8-associated SPI4 proteins were highly similar to the S. Typhimurium LT2-associated SPI4 proteins, exhibiting a 91-98% match (Appendix VIII). Thus, the differences observed in the restriction pattern between the 2 serovars could be attributed to the loss or gain of restriction sites, not to any insertions or deletions.
Figure 4.16  RT-PCR analysis \textit{marT} and \textit{slsA} from S. Typhimurium 82/6915 and S. II Sofia Bt8. M, \textit{\lambda}DNA marker digested with \textit{PstI}. Lane 1, 3, 5 and 7 are samples prepared without RTase (negative control) and Lane 2, 4, 6 and 8 are samples with added RTase. Lane 1 and 2, \textit{slsA} from 82/6915; Lane 3 and 4, \textit{slsA} from Bt8; Lane 5 and 6, \textit{marT} from 82/6915; Lane 7 and 8, \textit{marT} from Bt8.
Figure 4.17  Variation of SPI4 between S. Typhimurium SL1344 and S. II Sofia Bt8 as detected by Southern hybridisation, PCR restriction digest and sequencing analysis. A) Schematic representation of SPI genes and their direction of transcription (adapted from Gerlach et al., 2007). DNA probes (size and extent of each probe) used for Southern hybridisation are indicated above the schematic diagram. B) BglII, EcoRV and HindIII restriction maps for S. II Sofia Bt8. The numbers indicate the sizes of detected fragments in kB. The maps are estimation of the sizes of restricted DNA fragments and are not drawn to scale. C) Comparison of EcoRV bands between SL1344 and Bt8. +, presence of a fragment of the same size as that observed in S. II Sofia Bt8; -, absence of fragment. Blue boxes and numbers indicate the extent of and size (in kB) of the EcoRV fragment.
Figure 4.18  Comparison of RFLP analysis encompassing the 4A and 4B region of SPI4 in S. Typhimurium SL1344 and S. II Sofia Bt8. M, λDNA marker digested with PstI. Lane 1, 3 and 5, 4A amplified using SL1344 DNA and digested with BglII, EcoRV and HindIII respectively; Lane 2, 4 and 6, 4A amplified using Bt8 DNA and digested with BglII, EcoRV and HindIII, respectively; Lane 7, 9 and 11, 4B amplified using SL1344 DNA and digested with BglII, EcoRV and HindIII, respectively; Lane 8, 10 and 12, 4B amplified using Bt8 DNA and digested with BglII, EcoRV and HindIII, respectively.
4.2.10 Variation of SPI5 between S. Typhimurium and S. II Sofia

For the Southern hybridisation of SPI5, chromosomal DNA was digested with 10U of *DraI* or *EcoRV* or with 10U of a combination of both REs as described previously (Amavisit *et al.*, 2003). Southern blotting of digested genomic DNA (serovars Typhimurium SL1344 and II Sofia Bt8) was carried out with DNA probe 5 (6.23 kB, see Appendix VII). The Southern hybridisation pattern obtained from *S. II Sofia* Bt8 was observed to be different compared to that of Typhimurium SL1344 (results not shown). However, all attempts to amplify this region from strain Bt8 (with the primer pair 5/F and 5/R) were unsuccessful. Therefore, a series of primers encompassing different areas within SPI5 were designed in an attempt to amplify this region within *S. II Sofia* Bt8 (Appendix VII). Only the region containing *copR*, *copS*, *pipD* and *orfX* was successfully amplified and the SPI5 of isolate Bt8 appeared to be missing the 4.5 kB region containing *sopB*, *pipC*, *pipB* and *pipA* – all PCR amplifications of this particular region did not yield any product despite numerous attempts (data not shown).

The total absence of this 4.5 kB region was further confirmed by the Southern hybridisation (carried out in low and high stringency conditions) of *Salmonella* serovars Typhimurium and II Sofia with the probe *sopB-pipA* (Figure 4.19). The map of SPI5 of *S. II Sofia* Bt8 was then constructed based on Southern hybridisation, restriction digest and sequencing data (Figures 4.19 and 4.20). Overall, the deduced proteins encoded by the SPI5-associated genes of strain Bt8 share 95 to 100% polypeptide match when compared to the corresponding SPI5 proteins of strain LT2 (Appendix VIII). However, a base substitution (G with T) was observed at the start of the ORF (Appendix VIII) from Bt8 and this caused a missense mutation in the start codon of this gene (ATG to ATT) – protein encoded by this gene is probably not translated.
Figure 4.19  Southern hybridisation and restriction digest analysis of the SPI5 region from S. Typhimurium and S. II Sofia serovars. The fragment sizes of S. Typhimurium SL1344 were indicated at the side for each blot (A and B). A) Southern blot detection of sopB-pipA (4.5 kB) under high stringency conditions (hybridisation temperature 65°C, ~90% identity between genes). Genomic DNA was digested with XbaI. Lane 1, Typhimurium SL1344; Lane 2-4, II Sofia (Bt8, 986Q and 554NCA, respectively). B) Southern blot detection of sopB-pipA (4.5 kB) under low stringency conditions (hybridisation temperature 50°C, ~65% identity between genes). Genomic DNA was digested with EcoRV. Lane 1 and 2, Typhimurium (SL1344 and 82/6915, respectively); Lane 3-7, II Sofia (Bt8, 659SCA, 391QCA, 242T and 554NCA, respectively). C) RFLP analysis of copR-orfX (4.0 kB) from 1344 and Bt8. M, λDNA marker digested with PstI. Lane 1 and 3, SL1344 digested with HindIII and EcoRI, respectively; Lane 2 and 4, Bt8 digested with HindIII and EcoRI, respectively.
Figure 4.20  Variation of SPI5 between S. Typhimurium SL1344 and S. II Sofia Bt8 as detected by Southern hybridisation, PCR restriction digest and sequencing analysis. A) Schematic representation of SPI genes and their direction of transcription (adapted from Amavisit et al., 2003). The absence of sopB-pipA region is indicated by unshaded block. DNA probes (size and extent of each probe) used for Southern hybridisation are indicated above the schematic diagram. B) DraI, EcoRV and HindIII restriction maps for S. II Sofia Bt8. The numbers indicate the sizes of detected fragments in kB. The absence of sopB-pipA region is indicated by dashed lines on the restriction maps. The maps are estimation of the sizes of restricted DNA fragments and are not drawn to scale. C) Comparison of EcoRV bands between SL1344 and Bt8. +, presence of a fragment of the same size as that observed in S. II Sofia Bt8; -, absence of fragment. Blue boxes and numbers indicate the extent of and size (in kB) of the EcoRV fragment.
4.3 Discussion

To evaluate the RE digests of S. Il Sofia DNA, a relatively large amount of genomic DNA (approximately 20-25 µg) was required for the subsequent Southern blotting experiments to ensure that all bands were detected after probe hybridisation and detection. The intensity of the bands detected (with the ANTI-DIG system, see Section 2.3.7) was dependent on the amount of digested chromosomal DNA present (higher DNA concentration produce more intense bands) and size of the fragment binding to the probe (smaller fragments tend to bind less efficiently compared to larger fragments – using more DNA increases the intensity of such bands). Therefore, by using 20-25 µg of DNA for Southern hybridisation studies, membrane blots with visible and clear bands were produced (Figure 4.1).

The preparation of agarose plugs was found to be a more useful method for obtaining high concentrations of genomic DNA than the extraction method. DNA extraction usually involves a series of steps that extract, purify and concentrate the chromosomal DNA from the cells and at every step; a small amount of DNA is lost - e.g. after PCI extraction, a small amount of the aqueous phase (which contains the DNA) is not removed in order to avoid disturbing the interface where the contaminants are precipitated (see Section 2.3.2.1). The loss from each step ultimately affects the final yield of DNA, evident as the extraction process had to be carried out a few times in order to obtain the amount of DNA required. In contrast, plugs prevent the loss of DNA as the bacterial cells are embedded in agarose before being subjected to lysis and washing steps (see Section 2.3.6.1.1): a small slice of the plug (~ 2 mm out of 6 mm plug) was found to contain sufficient DNA for Southern hybridisation.
The Southern hybridisation patterns (SPI1 to SPI5) obtained from S. II Sofia isolates was observed to be identical to one another and different from those of S. Typhimurium isolates. Amavisit and his colleagues (Amavisit et al., 2003) carried out and compared Southern hybridisation patterns across the 5 SPIs from various *Salmonella* serovars and found that all strains within the same serovar shared the same SPI1 to SPI5 pattern. While a majority of differences observed in the SPI pattern from various *Salmonella* serovars were attributable to a series of loss and gain of restriction sites, insertion and deletions were found in the SPI1, SPI3 and SPI5 regions (Amavisit et al., 2003). This was also the case with the S. II Sofia isolates. An example of the altered restriction sites can be seen from the comparison of *EcoRV* restriction maps between S. Typhimurium LT2 and S. II Sofia Bt8 (see part C in Figures 4.2, 4.11, 4.13, 4.17 and 4.20). When compared to SPI1 to SPI5 of strain LT2, the SPI regions of isolate Bt8 were found to contain point mutations that caused either a gain of a new (creating a 2-3 smaller fragments) or loss of *EcoRV* site (creating a larger fragment). The major genetic variation in S. II Sofia Bt8 was also found to be contained within SPI1, SPI3 and SPI5.

The point mutations (base substitutions) throughout the SPIs of strain Bt8 not only changed the restriction patterns of the strain but also the amino acid composition of the predicted proteins (when the base substitution changes the codon encoding an amino acid). A majority of the predicted S. II Sofia SPI-associated proteins had such changes in their polypeptide sequence, sharing 87% to 99% identity with corresponding SPI proteins from S. Typhimurium LT2 (Appendix VII). The assumption that the S. II Sofia proteins are functional is reasonable due to the high sequence similarity between a large proportion of the SPI proteins from strains LT2 and Bt8. But it is possible that the translation of a different amino acid (missense mutation) may have an effect on protein function (Anon, 2008b). In addition, base substitution could cause nonsense mutations.
by mutating start and stop codon or creating a termination codon in the middle of a gene (Anon, 2008b) and this has been observed within the SPI regions of S. II Sofia Bt8. A number of S. II Sofia SPI genes also appeared to have accumulated changes (other than base substitutions) such as insertions and deletions of bases or sequences, which have been shown (using computer software) to alter protein translation process (Appendix VIII).

Two major insertion and deletion events were found in the SPI1 of S. II Sofia Bt8: insertion of a 1.2 kB transposase ORF and deletion of avrA (Figures 4.5 and 4.6). The absence of avrA between sitD-sprB in SPI1 is not unique to S. II Sofia isolates – deletion of avrA was also detected within the Salmonella serovars Kiambu and Ohio (Figure 4.5) and other Salmonella serovars, which was confirmed by previous studies (Amavisit et al., 2003; Hardt and Galán, 1997). These previous studies revealed that the 0.9 kB fragment from the avrA region was replaced with ~200 bp sequence, which contained an ORF encoding a 69-amino acid protein of unknown function (Amavisit et al., 2003; Hardt and Galán, 1997). This sequence replacement was also observed in the sitD-sprB SPI1 region of S. II Sofia Bt8 (Appendix VIII).

Further, avrA may be missing from SPI1 but the gene is still present in the S. II Sofia genome, as can be seen from Figure 4.5. This would indicate that at some point, an insertion and excision (mediated by genetic elements such as a phage or IS) or horizontal transfer event (Amavisit et al., 2003) probably occurred within SPI1 of strain Bt8 that resulted in the transfer of avrA to another location in the S. II Sofia genome. It is possible that the ORF found in the deleted region was transferred there as a result of genetic recombination between avrA and the ORF, leading to the exchange of genetic locations. This transfer event could also have altered the sequence of the gene, resulting
in the weaker bands detected by Southern blot and PCR (Figure 4.5). Since \textit{avrA} is no longer influenced by SPI1, the resulting protein is probably not expressed because secretion of \textit{avrA} requires a number of SPI1 genes (Hardt and Galán, 1997). RT-PCR analysis of the expression of \textit{avrA} seemed to support this view: while this gene was expressed from \textit{S. Typhimurium} 82/6915, expression of \textit{avrA} from \textit{S. II Sofia} Bt8 was not detected (Figure 4.9). The AvrA protein has been shown to be involved in the initiation and enhancement of cell apoptosis (Collier-Hyams \textit{et al.}, 2002; Ye \textit{et al.}, 2007) and thus, its absence in \textit{S. II Sofia} may affect the virulence of the strain.

The insertion of a 1.2 kB sequence (between \textit{iagB} and \textit{sptP}) was observed within the SPI1 of \textit{S. II Sofia} (Figure 4.6). Sequence analysis of this fragment revealed that it contains a putative ORF encoding a 400-amino acid protein with sequence similarity to transposase proteins from the mutator family. This family of proteins function as mobile DNA elements, inserting and excising into various locations in the bacterial genome and thereby, causing and promoting mutations (Pacurar \textit{et al.}, 2006). As this ORF is only 1.2 kB in size and encodes no other function other than the one involved in its mobility, the ORF is probably an IS element. Although found in a number of bacterial species (e.g. \textit{Yersinia enterocolitica}, \textit{E. coli}, \textit{Shewanella} spp., etc), the transposase was observed to be restricted to the \textit{Salmonella} serovars Weltevreden (Figure 4.6) and II Sofia (Figure 4.7). The presence of this IS element in \textit{S. II Sofia} could have potential as a marker to differentiate this serovar from other \textit{Salmonella} spp. As \textit{S. II Sofia} is so prevalent among Australian poultry (but is avirulent), the development of a rapid identification method would be useful in the screening of \textit{S. II Sofia} from the other more pathogenic \textit{Salmonella} strains that are present in the poultry flocks. Although \textit{S. Weltevreden} is currently the most common cause of non-typhoidal salmonellosis in the Southeast Asia region (e.g. Malaysia and Thailand), it is rarely isolated from Australian poultry (Galanis
et al., 2006; Heuzenroeder et al., 2001; Miller et al., 2005; Murray, 1994; Yohannes et al., 2004).

Insertions were observed in the sequence of SPI1-associated orgB and prgI from strain Bt8, resulting in translation of proteins with changes in the polypeptide chain length. While the insertion in orgB appeared to have caused a frameshift (resulting in translation of a protein missing 3 residues from the end of the chain), PrgI contained an extra amino acid as the insertion of the CCA codon was in frame with the ORF encoding this protein (Appendix VIII). The changes to the polypeptide chain could also have altered the function of the proteins. PrgI and OrgB have been shown to be involved with the TTS apparatus of SPI1 (Kimbrough and Miller, 2000; Klein et al., 2000; Sukhan et al., 2001). Therefore, both these proteins are required for invasion and secretion of effector proteins and any mutations would affect the virulence.

A point mutation at the start codon of invJ from isolate Bt8 resulted in the first amino acid of the polypeptide being changed from methionine to valine, which could affect its function. Although the start codon for most proteins is ATG (methionine), occasionally GTG (valine) and TTG (leucine) are used by bacteria as a start codon e.g. when using the normal ATG would cause the increase in expression and impair normal mechanism of gene expression (Masaru, 2008). Examples of SPI genes with alternative start codon include orgC, invA, ttrS, siiB (start codon of GTG) and sicP (start codon of TTG). It is possible that the change in the start codon could have impaired function of invJ - translation may not occur or be reduced due to the change from normal ATG to GTG. As this protein controls the length of the needle complex in the SPI1 TTS system, a loss of function in invJ would have an effect on the bacterial invasion process – it has been shown that invJ mutants are unable to form a functional TTS system (Chen et al.,
Another point mutation that altered the protein start codon was observed in the sequence of \textit{sptP} (Appendix VIII). Unlike the mutation in \textit{invJ}, this base substitution caused a nonsense mutation in the codon – the predicted SptP protein is missing 32 amino acids from the beginning of its polypeptide sequence (Figure 4.8). This gene was also observed to contain a number of other mutations including the insertion and deletion of DNA sequences, which caused multiple changes observed in the amino acid residues (Appendix VIII and Figure 4.8). The SPI1-associated \textit{sipD} contained multiple changes in its DNA sequence (Figure 4.9), resulting in the truncation of SipD protein due to the creation of a termination codon in the middle of the gene. Analysis of the nucleotide sequence revealed that this protein truncation could not be attributed to a single mutation event – the accumulation of a number of genetic mutations (from base 790 to 820, see Figure 4.9) changes the protein sequence.

Both these genes could be potential pseudogenes. Pseudogenes are defined as ORFs of known genes that are truncated or contain a number of mutations that have disrupted a sizeable portion of the coding sequence (Lerat and Ochman, 2005; Liu \textit{et al.}, 2004; Mira and Pushker, 2005). They are a common feature in many genomes, including the bacterial species (especially those undergoing processes such as niche changes and host specialisation) (Lerat and Ochman, 2005; Liu \textit{et al.}, 2004; Mira and Pushker, 2005). A nonsense or missense mutation probably occurred in \textit{sipD} and \textit{sptP} genes of \textit{S. II Sofia}, disrupting the gene transcription/protein translation. Without selective pressure (proper function of the gene) on the gene, mutations are likely to occur more frequently within the ORF, as can be observed with both these genes. This accumulation
of genetic changes further disrupts transcription/translation process – the gene then becomes a pseudogene due to loss of function.

The SipD and SptP proteins have been shown to be required for the invasion process of *Salmonella* spp. (Collazo and Galán, 1997b; Kaniga *et al.*, 1995a; 1996). SipD appears to be part of a protein complex (with the SipBC proteins) that delivers effector proteins into the host cells (Cherayil *et al.*, 2000; Collazo and Galán, 1997b; Darwin and Miller, 1999b; Zhang *et al.*, 2003) and SptP is a SPI1-encoded effector protein, which is translocated in a Sip-dependent manner and is involved in disruption of the cell cytoskeleton (Fu and Galán, 1998b; Murli *et al.*, 2001). Therefore, mutations within these genes would probably alter the function of their encoded proteins. It is also possible that *sptP* and *sipD* from *S. II Sofia* are non-functional (i.e. not expressed) due to these mutations (alteration of start codon and creation of an extra stop codon, respectively). This observation appears to be supported by the RT-PCR analysis on the expression of *sptP* and *sipD*: no band was amplified from the samples of strain Bt8, indicating the possibility that both these genes are not expressed by the SPI1 of *S. II Sofia* (Figure 4.10). However, the low levels of expression obtained in strain 82/6915 (as seen from the faint bands in Figure 4.10) could mean that mRNA from *S. II Sofia* was produced at levels too low to be detected by RT-PCR. This low expression level could also be the result of the gene mutations observed, which may have affected the mRNA transcription of *sptP* and *sipD* and thereby, the translation of sufficient amounts of protein to mediate the bacterial invasion process.

Comparison of SPI2 from *S. Typhimurium* and *S. II Sofia* has revealed that this region is relatively conserved between the 2 strains, with the putative proteins sharing an 83 to 98% identity (Figure 4.11 and Appendix VIII). However, three *S. II Sofia* SPI2
genes (ssaP, ssaI and ssaE) appeared to have acquired mutations that could affect the encoded proteins. An insertion in ssaI and deletion in ssaP (in both cases, the sequence in question was a codon) resulted in translation of a protein with an extra or missing residue, respectively (Appendix VIII). A point mutation in ssaE removed the original termination codon of this ORF: translation of predicted SsaE with 5 additional amino acids in its polypeptide sequence (Appendix VIII). The mutation in ssaE may be linked to the 200 bp deletion found between ssea and ssaE. It is possible that the deletion of this 200 bp sequence was mediated by the insertion and excision of an IS element and this event could have caused the mutation in the stop codon of ssaE, which was located close to the deletion point (Appendix VIII).

All 3 SPI2 genes encode components of the SPI2 TTS system and ssaI mutants have been shown to be defective in intracellular replication and in the translocation and secretion process (Chakravortty et al., 2005). Therefore, the mutations observed in the SPI2 could have had an effect on pathogenicity of S. II Sofia. The SPI4 region of S. II Sofia Bt8 was found to contain only minor genetic variation (when compared to S. Typhimurium LT2): the loss or gain of restriction sites and translation of predicted proteins sharing 91-98% identity with their S. Typhimurium counterparts (Figure 4.17 and Appendix VIII). This region is probably functional in S. II Sofia – however, research on expression of the SPI4 proteins needs to be carried out to confirm the functionality of this region.

Major genetic variation was detected within the SPI3 region of S. II Sofia Bt8, including the deletion of 2 genes (sugR and rhuM) and insertion of an IS element within the slsA-cigR region, which appeared to have affected slsA (Figures 4.13 and 4.15 and Appendix VIII). This deletion was observed in other Salmonella spp., including serovars
Ratchaburi, Virchow, Infantis, Bovismorbificans and Zanzibar (Amavisit et al., 2003). The selC region, which is next to the left hand portion of the SPI3 region (and contains the genes sugR and rhuM) has been shown to be the integration site for pathogenicity islands in *Salmonella* spp. and *E. coli* (Amavisit et al., 2003; Blanc-Potard et al., 1997; 1999). Thus, this portion of SPI3 is probably more prone to genetic changes (insertions and deletions) and found to be less conserved compared to the central region, which contains the genes rmbA, misL, fidL and marT (Amavisit et al., 2003; Blanc-Potard et al., 1999). The deletion of sugR and rhuM could possibly be related to the detection of an ORF with homology with putative IS1351 transposase pseudogene (Appendix VIII). This 135 bp fragment appears to be a gene remnant of an insertion sequence (it is non-functional; hence the term pseudogene) (Parkhill et al., 2001), which was probably left behind after the insertion and excision of the IS element, leading to the deletion of sugR and rhuM as well. The exact function of both these genes is still unknown – however, rhuM has been identified as one of the SPI3 genes that could potentially be required for systemic disease (Lawley et al., 2006). Therefore, its absence may affect *S. II Sofia* pathogenicity.

The insertion of a 1.2 kB ORF was detected between SPI3 genes *slsA* and *cigR* in *S. II Sofia* (Figures 4.13 and 4.15). This ORF was shown to encode a putative transposase of the mutator type that is identical to the transposase encoded by the IS element located between SPI1 genes *iagB* and *sptP* in strain Bt8 (Appendix VIII). The presence of more than one IS element (of the same type) in the bacterial genome is not an uncommon occurrence: e.g. IS200, which has been successfully utilised for the typing of *Salmonella* spp. (Baquar et al., 1994; Lam and Roth, 1983; Millemann et al., 1995; Old et al., 1999; 2000; Threlfall et al., 1993). The insertion of this sequence alters the predicted expression of *slsA* – a frameshift was detected, which disrupted the
termination codon of the gene and resulted in the translation of a protein with an additional 25 residues (Appendix VIII). This alteration may also have affected transcription of the gene, as can be observed in the RT-PCR analysis of slsA (Figure 4.16). However, the absence of any RT-PCR product could be due to a number of other reasons: expression levels were too low to be detected by RT-PCR, isolates were not incubated in conditions that induce gene expression, etc. The exact function of slsA is unclear but it has been shown to be needed for colonisation in cattle and chickens (Morgan et al., 2004).

An insertion and deletion in misL and marT respectively has resulted in predicted proteins with altered polypeptide chain length (Appendix VIII). These changes to the protein sequence may have an effect on function. While the MarT protein has been identified as a transcriptional activator of the misL promoter (Tükel et al., 2007), MisL functions as a surface adhesin and is required for intestinal colonisation in mice and chickens (Dorsey et al., 2005; Morgan et al., 2004). The mutations within slsA, marT and misL could have altered the functionality of the proteins encoded by the genes, which may have affected the virulence ability of S. II Sofia. It is also possible that the extra residues in SlsA and MarT could have altered their function in a different manner – by increasing the colonisation function of these proteins, which would explain the persistence of S. II Sofia in poultry flocks.

A single base substitution in the SPI5 gene ORF (Figure 4.20) appeared to have caused a mutation in the start codon of this gene – the putative protein was probably not translated. This ORF was predicted to encode a putative cytoplasmic protein (although its exact function is unclear) and therefore, a mutation could affect protein function (especially when the mutation renders the gene non-functional). However, the biggest
change observed in SPI5 of S. II Sofia Bt8 is the deletion of the right half of this region, which comprised the genes sopB, pipC, pipB and pipA (Figures 4.19 and 4.20). The detection of this major deletion was confirmed by Southern hybridisation experiments carried out in both low and high stringency conditions (Figure 4.19). By carrying out the blotting experiments under both these conditions, it can be concluded that not only is this 4.5 kB region absent from SPI5 of S. II Sofia, the possibility that relatively similar regions (~65% sequence similarity) could be present (which would bind with the probe at lower stringency conditions – e.g. lower hybridisation temperature) was also excluded.

The genes in the deleted region have been shown to be involved in intestinal secretory and inflammation responses and mutations in these genes have resulted in attenuation and reduced virulence (Galyov et al., 1997; Morgan et al., 2004; Norris et al., 1998; Pfeifer et al., 1999; Wood et al., 1998). Further, the proteins encoded by these genes are able to interact with other virulence effectors (from outside the SPI5 region) to perform a range of virulence-associated functions such as host invasion, enteropathogenicity and disruption of tight junction structure and actin cytoskeletal rearrangements (Boyle et al., 2006; Jones et al., 1998; Mirolid et al., 2001; Raffatellu et al., 2005; Zhang et al., 2002; Zhou et al., 2001). The absence of these genes could be one of the reasons that S. II Sofia is unable to cause disease in poultry or humans – S. II Sofia isolates are probably unable to induce intestinal inflammation and secretion, which is required to cause gastroenteritis.

It would appear the avirulence of S. II Sofia is not due to a single mutation or deletion but rather a series of mutations in the genes within the SPI regions. These mutations have been observed to consist of base substitutions, insertion and deletion of sequences, which have either altered the codons or caused frameshifts within SPI
genes, resulting in truncation of encoded proteins or translation of putative proteins with extra/missing amino acid residues (Appendix VIII). Genes could become non-functional due to the mutations - the gene transcription process does not occur. The mutations could have altered the level of gene expression (and thereby, translation of sufficient amounts of protein to mediate the bacterial virulence) or interfered with protein translation. It is also possible that transcription and translation occurred but the structure and function of the protein produced was impaired as a result of the mutations. In addition, the deletion of genes from SPI regions has been observed and this would definitely affect the pathogenicity of S. II Sofia.

The mutations observed in SPI1 to SPI3, and SPI5 genes (see Figures 4.2, 4.5, 4.7 to 4.9, 4.13, 4.15, 4.19 and 4.20; along with Appendix VIII) could be the reason for the avirulent nature of S. II Sofia. SPI1 and SPI2 have been shown to be required for bacterial invasion and intracellular replication, respectively (Galán, 1999; Hensel et al., 1998; Marcus et al., 2000; Ohl and Miller, 2001) - mutations of genes within these regions would affect virulence ability of a Salmonella strain. This can be seen from the pathogenesis studies done by Rickard (Rickard, 1998): although S. II Sofia is still able to adhere and invade cultured epithelial cells, the level of adherence and invasion is greatly reduced compared to the pathogenic S. Typhimurium 82/6915. S. II Sofia isolates also do not replicate intracellularly and can only cause systemic disease in hosts with immature or immunocompromised systems (Rickard, 1998).

The SPI1 mutations discovered in the present study differs from the previous studies on this pathogenicity island (Heuzenroeder et al., 2001): other than the point mutation in invJ, the rest of the inv region appears to be intact (Appendix VIII). It could be that the distribution of genes in the inv operon is not the same within all S. II Sofia
isolates. In addition, the study done by Heuzenroeder and his coworkers (Heuzenroeder et al., 2001) employed PCR amplification to detect inv genes, which is not as sensitive as a Southern hybridisation method. Further studies would have to be carried out to properly investigate the distribution pattern of this operon amongst S. II Sofia isolates.

The avirulent nature of S. II Sofia could also be caused by the major deletion in SPI5 as these genes are necessary for gastroenteritis disease process (Norris et al., 1998; Pfeifer et al., 1999; Wood et al., 1998). On the other hand, the SPI3 region appears to contain mainly genes encoding host-specific colonisation factors – the genes have been shown to be involved in the colonisation of a variety of animals such as cattle, chickens and mice (Dorsey et al., 2005; Lawley et al., 2006; Morgan et al., 2004). It is possible that the mutations observed in SPI3 genes of S. II Sofia could have increased the colonisation ability of the strain, which would explain its persistence in Australian poultry flocks.

The SPI1 to SPI5 regions of S. II Sofia were observed to exhibit variation in the RFLP pattern when compared to S. Typhimurium and these differences could be attributed to loss or gain of restriction sites within these regions. A number of SPI genes in S. II Sofia were found to have accumulated mutations that could have affected gene transcription and/or protein translation. Further, the insertion of transposase and deletion of genes within the SPI regions of S. II Sofia were observed (particularly in SPI1, SPI3 and SPI5). The effects of these mutations, insertions and deletions probably contributed to the reduced pathogenicity in S. II Sofia and thus, avirulence of this serovar is not the result of a single genetic change but rather a series of alterations to a large number of its virulence-associated genes. Further studies on the expression of these genes (and the
functionality of their encoded proteins) may reveal the extent of the effect the mutations might have had on virulence process.
CHAPTER 5: MOLECULAR ANALYSIS OF
VIRULENCE PLASMID AND CHARACTERISATION
OF spv OPERON IN SALMONELLA II SOFIA

5.1 Introduction

A majority of Salmonella strains carry plasmids between 2 to 150 kB in size and
the frequency, size and distribution of plasmids vary from serovar to serovar (Olsen,
2000). Some of these plasmids have been shown to contain genes that confer virulence
phenotype to the strain, as can be seen in virulence plasmids harbouring Salmonella
serovars Typhimurium, Dublin, Enteritidis, Choleraesius, Gallinarum, Pullorum and
Abortusovis (Akiba et al., 1999; Barrow and Lovell, 1988; Barrow et al., 1987a; 1987b
Haneda et al., 2001; Rotger and Casadesús, 1999; Rychlik et al., 2006; Woodward et
al., 1989). These virulence plasmid share a few common properties, including a low
copy number, large size (50 to 95 kB) and appear to be limited to S. enterica subspecies
I serovars, which are mentioned above (Chu et al., 1999; Guiney et al., 1994;
Montenegro et al., 1991; Popoff et al., 1984; Rotger and Casadesú, 1999; Rychlik et
al., 2006; Woodward et al., 1989).

All virulence plasmid-containing Salmonella isolates share a highly conserved 8 kB
operon designated spv region (Guiney et al., 1994; Gulig et al., 1993; Rychlik et
al., 2006). This operon has been shown to confer virulence phenotype to a strain and the
insertion of this 8 kB region on a low copy number vector is sufficient to restore wild-type
virulence to plasmid-cured Salmonella strains (Guiney et al., 1994; Gulig et al., 1992;
The spv operon encompasses 5 genes, (spvRABCD), which interact with one another and is probably transcribed in the same direction as a single operon (Gulig et al., 1993; Valone et al., 1993). spvR functions as a transcriptional activator, activating its own expression and that of spvABCD (Coynault et al., 1992; Grob and Guiney, 1996; Krause et al., 1992; 1995). Localisation studies on the SpvABCD proteins have shown their presence in cytoplasm, supernatant, outer and inner membrane, probably indicating the varied roles these proteins have in the virulence process (El-Gedaily et al., 1997). The protein encoded by spvB acts as a mono (ADP-ribosyl) transferase and such enzymes are responsible for mediating virulence functions like actin degradation and apoptotic cell death (Gotoh et al., 2003; Kurita et al., 2003; Otto et al., 2000; Tezcan-Merdol et al., 2005). The rest of the spv-encoded proteins have also been demonstrated to interact with one another and with SpvR and SpvB proteins (Matsui et al., 2001; Valone et al., 1993).

The spv region is essential for Salmonella spp. virulence: insertional mutations in the individual gene have resulted in reduction of virulence and attenuation (Gulig et al., 1992; Krause et al., 1995; Libby et al., 1997; 2000). Further, it has been shown through a series of spv mutations and cloned spv sequences that spvBC was sufficient to replace the spv operon, as well as the entire virulence plasmid, of S. Typhimurium (Matsui et al., 2001). However, the exact role of these genes in virulence is rather contradictory, with some investigators reporting that they promote intracellular survival and replication in the host (i.e. macrophage cytopathology, leading to cell detachment and apoptosis), thereby leading to development of systemic infection (Guiney et al., 1994; Gulig et al., 1998; Libby et al., 1997; 2000; Paesold et al., 2002) and others finding no such correlation between spv gene expression and intracellular survival (Gulig and Curtiss III, 1987; Guilloteau et al., 1996; Rhen et al., 1993; Riikonen et al., 1992; Wilson
et al., 1997). Therefore, spv function in pathogenesis of Salmonella spp. appears to be more complex than initially thought and requires further study.

A number of conditions have been reported to induce and regulate the expression of spv operon, including being induced intracellularly (Fierer et al., 1993; Rhen et al., 1993; Wilson et al., 1997), during stationary growth phase (Coynault et al., 1992; El-Gedaily et al., 1997; Krause et al., 1992; Spink et al., 1994; Valone et al., 1993; Wilson et al., 1997; Wilson and Gulig, 1998), in nutrient limited condition like glucose starvation and low pH (Valone et al., 1993) and in low iron concentrations (Gotoh et al., 2003; Spink et al., 1994; Valone et al., 1993). The genes themselves contribute to this regulation process as well: SpvR is a positive regulator of spvABCD operon and of its own expression (Coynault et al., 1992; Gulig et al., 1993; Krause et al., 1992; Spink et al., 1994) and SpvA has been shown to regulate SpvR expression, providing a negative feedback mechanism for this gene (Heiskanen et al., 1994; Spink et al., 1994).

The spv gene expression is also regulated by regulatory proteins encoded elsewhere in the Salmonella genome – e.g. the PhoP/PhoQ regulatory system, polynucleotide phosphorylase (PNPase), integration host factor (IHF) and leucine-responsive regulatory protein (Lrp) (Marshall et al., 1999; Tu et al., 2006; Ygberg et al., 2006). The rpoS or katF gene, which encodes the alternative sigma factor RpoS, has been shown to regulate the stationary phase expression of a wide variety of genes, including the spv operon (Chen et al., 1995; Fang et al., 1992; Heiskanen et al., 1994; Kowarz et al., 1994; Nickerson and Curtiss III, 1997; Norel et al., 1992; Wilson et al., 1997). By controlling the transcription of spvR, the RpoS protein is able to mediate the expression of the downstream spvABCD (Fang et al., 1992; Kowarz et al., 1994; Nickerson and Curtiss III, 1997; Wilson et al., 1997). This regulatory protein is required
for full expression of spv region as rpoS/katF mutants have been shown to be less virulent and more susceptible to a range of environmental stresses like nutrient limitation, DNA damage, oxidative and acid stress (Fang et al., 1992; Kowarz et al., 1994; Nickerson and Curtiss III, 1997; Wilson et al., 1997).

Other virulence-associated genes have also been discovered in the virulence plasmid of Salmonella serovars. An example would be the 7 kB pef operon encoding components of a fimbriae/pili structure, which has been shown to mediate adhesion to small intestine of mice (Bäumler et al., 1996a; Friedrich et al., 1993). Another example would be the plasmid-encoded rck, which confers serum resistance to Salmonella serovars by interfering with the polymerisation of tubular membrane attack complexes on the bacterial surface (Hackett et al., 1987; Heffernan et al., 1992a; 1992b). Rck has also been shown to be important in cell adhesion/invasion (Heffernan et al., 1994). The plasmid-encoded loci of traT and rsk have also been shown to have a role in serum resistance of Salmonella spp. (Rhen and Sukupolvi, 1988; Vandenbosch et al., 1989a; 1989b).

However, very little is known about plasmids (and their genes) from S. II Sofia, despite the amount of research that has been carried out on the large plasmids of Salmonella serovars and their virulence factors. Harrington and coworkers (Harrington et al., 1991) have identified a small 6.4 kB plasmid in their epidemiological study of Australian and Israeli S. II Sofia isolates but have been unable to discern the genes or their function within this plasmid. Further, PCR and Southern hybridisation analysis have revealed that S. II Sofia strains appear to be missing the plasmid borne spv region (Heuzenroeder et al., 2001) - its absence would contribute to the avirulence property of this particular Salmonella serovar.
This chapter will focus on the detection of the large virulence plasmid (and spv cluster of genes) among the S. II Sofia isolates and to investigate the effect the spv operon may have on the virulence phenotype of S. II Sofia.

The aims of the research methods carried out in this study were to:

(i) examine the distribution of the large virulence plasmid and to confirm the absence of spv genes in the S. II Sofia isolates,
(ii) create mutants via the insertion of spv operon (cloned on a high-copy and low-copy number vector) into S. II Sofia Bt6,
(iii) investigate the effect of this insertion on the pathogenicity of S. II Sofia through adhesion, invasion and intracellular survival assays.

5.2 Methods and Results

5.2.1 Presence of Virulence Plasmids and spv Operon in S. II Sofia Isolates

Isolation of the plasmids was carried out as described by Kado and Liu (Kado and Liu, 1981) with some modifications (see Section 2.3.2.4). Plasmid extraction was performed on 26 selected S. II Sofia and 2 S. Typhimurium isolates (82/6915 and ATCC14028). The presence or absence of any plasmid was then determined by subjecting the samples to agarose gel electrophoresis and ethidium bromide staining as described in Section 2.3.4 (0.7% gel, 60V for approximately 3 h) (Figure 5.1). Plasmid analysis was repeated 3 times on different days in order to confirm accuracy and reproducibility of data.
Figure 5.1  Plasmid content of S. II Sofia isolates. M, λDNA marker digested with HindIII. Lane 1 3, and 15, S. Typhimurium ATCC 14028; Lane 2, S. Typhimurium 82/6915; Lane 4-14 and 16-24, S. II Sofia isolates (Bt2, Bt8, 131, FSAW3452, 988Q, 554NCA, 360QC, 365QCA, 665SCA, 242T, 768XTA, 146TCA, 539NC, 566NC, 10VCA, 838V, 403QCA, 662SCA, 632MSC and 642SC, respectively). White smears (indicated by the blue boxes) observed on the gel are from chromosomal DNA contamination.
Both the *S. Typhimurium* strains (82/6915 and ATCC 14028) studied possessed the 90 kB large plasmid (Figure 5.1). Multiple extractions of *S. Typhimurium* ATCC14028 have shown that only a single band (corresponding to the 90 kB virulence plasmid) was produced. Thus, this strain was used as a reference strain in the extraction of the *S. II Sofia* isolates – to verify that the extraction procedure was successfully carried out each time. Plasmid analysis of the *S. II Sofia* isolates showed that almost all the isolates carried a plasmid slightly larger than the 90 kB plasmid obtained from *S. Typhimurium* strains (Figure 5.1). Further, a number of *S. II Sofia* isolates were observed to contain plasmids of various sizes: in the range of ~1.0 to 23 kB (Figure 5.1). Only 2 *S. II Sofia* isolates showed a slightly different plasmid profile – 131 (single plasmid of about 90 kB) and 242T (absence of any plasmids) (Figure 5.1). The plasmids extracted were not subjected to restriction digest analysis due to the presence of chromosomal DNA in the samples: smearing was obtained when the samples were digested (results not shown). This contamination of the samples could not be removed despite modifications to the protocol (increase in lysis temperature, increase of lysis incubation time and additional PCI extraction steps, see Section 2.3.2.4) and careful extraction process (data not shown).

Two different DNA probes were used in the detection of a virulence plasmid in *S. II Sofia* isolates: one probe to detect the *spv* region and the other encompassing *traY-traE* region of the *S. Typhimurium* LT2 virulence plasmid pSLT (GeneBank accession number NC_003277). Probes were PCR-amplified (from *S. Typhimurium* SL1344 genomic DNA) and analysed through restriction digest (to verify that the probe produced was accurate) before being labeled and estimated for Southern hybridisation experiments (see Section 2.3.7.1). The following primers were used for the expand amplification (see Section 2.3.3.3): *spvRABCD* (6.6 kB), 5’–GGTTTACAGGGATCTTGCTA–3’ and 5’–
CAGGTCACCGCCATCCTGTTTT-3' (annealing temperature of 55°C and elongation time of 5 min); and \( \text{traY-traE} (14.1\,\text{kB}) \), \( 5'–\text{TTCGTCTGAGGGATCATCTG}-3' \) and \( 5'–\text{CCGTAAGCGGCAATAATGAG}-3' \) (annealing temperature of 55°C and elongation time of 13 min). Genomic DNA (in agarose plugs, see Section 2.3.6.1.1 and 4.2.1) from \( S.\, \text{Typhimurium} \) (SL1344 and 82/6915) and selected \( S.\, \text{II Sofia} \) isolates were digested with 10U of \( \text{EcoRV} \), prepared by electrophoresis for capillary transfer onto nylon membranes before being subjected to Southern hybridisation (see Section 2.3.7.2). Probe hybridisation was carried out under 2 conditions: low (hybridisation temperature 50°C) and high stringency (hybridisation temperature 65°C), which identifies genes sharing ~65% and ~90% sequence similarity, respectively

Southern blotting of \( \text{Salmonella} \) isolates revealed that only the \( S.\, \text{Typhimurium} \) strains hybridised to both the \( spv \) (Figure 5.2) and \( \text{traY-traE} \) (data not shown) DNA probes. None of the \( S.\, \text{II Sofia} \) isolates tested hybridised to either one of the DNA probes in both low and high stringency conditions, indicating the possible absence of large virulence plasmid in these isolates (for \( spv \) detection, see Figure 5.2). The absence of these regions (and thereby, the virulence plasmid) was further confirmed by direct expand amplification of these regions from \( S.\, \text{II Sofia} \) genomic DNA (results not shown).
Figure 5.2 Southern blot detection of *spvRABCD* (probe hybridisation temperature 65°C). The fragment sizes of *S. Typhimurium* SL1344 were indicated at the side of each blot. Lane 1 and 9, *S. Typhimurium* SL1344; Lane 2, *S. Typhimurium* 82/6915; Lane 3-8 and 10-17; *S. II Sofia* isolates (Bt6, Bt16, 985Q, 554NCA, 147TC, FSAW3455, 131, 664SCA, 636SC, 987VCA, 630MSC, 362QC, 541NCA and 148TCA, respectively).
5.2.2 Construction of spv Mutant in S. II Sofia Bt6

To investigate the effect of the spv operon on the pathogenic ability of S. II Sofia, a mutant was constructed by inserting the complete spv cluster of genes into the isolate S. II Sofia Bt6. Genomic DNA from the pathogenic S. Typhimurium 82/6915 was isolated (see Section 2.3.2.1) and used as a template for the expand amplification of the spv operon (see Section 2.3.3.3). The following primer pair was designed for this purpose (annealing temperature of 60°C and elongation time of 8 min):

5’–TTCGCTCGAGGCGCTACAAATAGCTTCG-3’ (spvR-XhoI/F)
5’–CAAGTCTAGAGCAGGCGAGATCGCTGC-3’ (spvD-XbaI/R)

Restriction cleavage sites XhoI and XbaI (indicated by the underlined bases above) were incorporated into the primers for directional cloning into the plasmid pWSK29 (Wang and Kushner, 1991). The expected product of this amplification is an 8.2 kB DNA fragment encompassing the entire spv operon, along with the ORFs encoding a putative integrase (located next to spvR) and transposase (located next to spvD) protein (Figure 5.3). After amplification, PCR product was subjected to restriction digest analysis in order to confirm that the DNA fragment produced was accurate (Figure 5.3). The resulting fragment was then cloned into 2 different plasmids: the high copy pCR®2.1 (Amp’ and Kan’) and low copy number pWSK29 (Amp’) (see Appendix X) before being transformed into S. II Sofia Bt6.
Figure 5.3  Expand PCR amplification of spvRABCD (8.2 kB). A) Schematic diagram showing the genetic map of the DNA fragment (containing spv operon) used for mutant construction. Map was constructed using Clone Manager 7 software. B) RFLP analysis of spvRABCD. M, λDNA marker digested with PstI; Lane 1-3 contains spvRABCD digested with a different RE. Lane 1, BamHI; Lane 2, EcoRV; Lane 3, HindIII; Lane 4, PstI.
After amplification and RFLP analysis, \textit{spvRABCD} was then ligated to the pCR®2.1 plasmid according to the manufacturer’s manual (see Section 2.3.8.1). The ligation mix was then transformed into electrocompetent \textit{Escherichia coli} DH5α cells (see Sections 2.3.8.4.1 and 2.3.8.5.1). Potential positive transformants were detected by blue/white screening – white colonies were selected from the agar plate and subcultured in LBB broth (overnight at 37°C) before being subjected to plasmid miniprep extraction (see Section 2.3.2.2). All the miniprep extraction samples were analysed by restriction digest to detect and identify the clone containing the insert. The clone was further confirmed through sequencing analysis (see Section 2.3.3.4). RFLP analysis of the plasmid miniprep samples have identified several positive clones and clone pCspv8 was chosen (Figure 5.4). Identity of the positive clone chosen was further confirmed through sequencing analysis (data not shown).

Before inserting pCspv8 into S. II Sofia Bt6, the clone was first inserted into S. Typhimurium 9121. This transformation modifies the plasmid and prevents its degradation by endogenous enzymes when placed into S. II Sofia Bt6. Electrocompetent S. Typhimurium and S. II Sofia cells were prepared as described previously (see Section 2.3.8.4.1). The pCspv8 was transformed into S. Typhimurium 9121 and positive transformants selected by blue/white screening and miniprep assay. The modified plasmid was analysed by restriction digest (Figure 5.4) before being inserted into S. II Sofia Bt6. Positive clones from S. II Sofia were also confirmed through RFLP analysis before \textit{in vitro} assessment (Figure 5.4).
Figure 5.4A  Construction of S. II Sofia mutant containing spvRABCD (cloned on high copy number plasmid pCR®2.1). A) RFLP analysis of the clone pCspv8. M, λDNA marker digested with PstI; Lane 1, miniprep assay of pCspv8; Lane 2-4, pCspv8 (isolated from E. coli DH5α) digested with different REs (BamHI, EcoRV and a combination of XbaI and XhoI, respectively), where blue box indicates the DNA fragment containing the spvRABCD insert; Lane 5-6, pCR®2.1 digested with BamHI and EcoRV, respectively; Lane 7-8; pCspv8 (isolated from S. Typhimurium 9121) digested with BamHI and EcoRV, respectively; Lane 9-10; pCspv8 (isolated from S. II Sofia Bt6) digested with BamHI and EcoRV, respectively; B) Schematic diagram representing the genetic map of plasmid pCspv8 (in the same orientation as lacZ) constructed using Clone Manager 7. Arrows indicate the position of the genes (red for plasmid genes and blue arrows for spv genes).
To construct the spv clone in the low copy number plasmid pWSK29, pCspv8 was digested with the REs XbaI and XhoI (see Section 2.3.8.2) to obtain the spvRABCD insert from the clone. Plasmid pWSK29 was also digested with this enzyme combination to generate compatible sticky ends, which will facilitate the ligation of the digested insert to the plasmid. After gel purification, an overnight ligation reaction was carried out with the 2 samples (see Section 2.3.8.3). It was discovered that electroporation was not as efficient compared to chemical transformation (data not shown). Thus, all subsequent transformations were carried out chemically (see Section 2.3.8.5.2). Chemically competent E. coli DH5α, S. Typhimurium 9121 and S. Il Sofia Bt6 cells were prepared as described in Chapter 2 (see Section 2.3.8.4.2). Ligation product was then chemically transformed in DH5α cells, potential positive clones selected via blue/white screening and analysed by midiprep isolation (see Section 2.3.2.3) and RFLP analysis (Figure 5.5). Clone pWSPV1 was identified as positive construct containing the spvRABCD insert (Figure 5.5). This clone was then transformed into S. Typhimurium 9121 before finally being inserted in isolated S. Il Sofia Bt6.
Figure 5.4B  Construction of S. II Sofia mutant containing spvRABCD (cloned on low copy number plasmid pWSK29). A) RFLP analysis of the clone pWSPV1 (isolated from E. coli DH5α). M, λDNA marker digested with PstI; Lane 1-3, pWSPV1 digested with different REs (BglII, EcoRI and EcoRV, respectively); Lane 4-5, pWSPV1 digested with a combination of REs (XbaI/XhoI and Spel/NotI, respectively), where blue box indicates the DNA fragment containing the spvRABCD insert. B) Schematic diagram representing the genetic map of plasmid pWSPV1 constructed using Clone Manager 7. Arrows indicate the position of the genes (red for plasmid genes and blue arrows for spv genes).
However, all attempts to analyse midiprep samples isolated after insertion in *S. Typhimurium* 9121 and *S. II Sofia Bt6* by restriction digest were unsuccessful. The modified pWSPV1 appeared to be extremely sensitive to DNA degradation and all attempts at RFLP analysis resulted in smears on the gel, despite variation in the digest conditions such as RE concentration, addition of bovine serum albumin (BSA), amount of DNA, duration of digestion and incubation temperature (results not shown). As intact positive clones could be extracted via midiprep (data not shown), detection and confirmation of the mutant was performed by dot blotting. In this method (which is a slight variation of the Southern hybridisation technique), samples are spotted directly onto the membrane instead of being transferred via capillary action after restriction digest. All samples were denatured with NaOH (final concentration of 0.2 M) at 37°C for 15 min before 1 µL of each sample was spotted on the nylon membrane. Membrane was then crosslinked with UV light and subjected to Southern blotting (see Section 2.3.7.2).

The *spv* DNA probe was prepared for use in the dot blot detection as described previously (see Section 5.2.1) with some modifications: probe was digested with *EcoRI*, producing 3 fragments (*spvR, spvABC* and *spvD*), which were individually labeled and used separately in the Southern hybridisation. The *spv* insert (used in *S. II Sofia mutant construction*) and clone pCspv8 was used as positive controls and were spotted onto each membrane. Dot blot analysis of serovars Typhimurium and II Sofia midiprep samples confirmed that *spvRABCD* was successfully cloned in pWSK29 and inserted in isolate *S. II Sofia Bt6*, indicated by the hybridisation of all 3 probes to the samples containing the insert – *spvRABCD*, midiprep and CTAB isolation (see Section 2.3.2.1) of pCspv8 and pWSPV1 clones (Figure 5.5).
Figure 5.5  Dot blot analysis of pWSPV1 clones. Each sample was spotted in the exact same location on each membrane. Spot 1, spvRABCD insert; Spot 2, Miniprep of plasmid pCR®2.1; Spot 3, Midiprep of plasmid pWSK29; Spot 4, pCspv8 clone; Spot 5, midiprep of pWSPV1 from S. Typhimurium 9121; Spot 6, CTAB isolation of pWSPV1 from S. Typhimurium 9121; Spot 7, midiprep of pWSPV1 from S. II Sofia Bt6; Spot 8, CTAB isolation of pWSPV1 from S. II Sofia Bt6.
5.2.3 In Vitro Assays

Pathogenic ability of the constructed S. II Sofia mutants was assessed in 3 ways: its adherence and invasion of INT407 and CEF-DF1 cells and its ability to survive and replicate within murine J774 macrophages. All growth, maintenance and the preparation of monolayers (for in vitro assay) was conducted as described in Chapter 2 (see Section 2.4.3.1 to 2.4.3.3). Along with the S. II Sofia mutants Bt6/pCspv8 and Bt6/pWSPV1, the following controls were included in each in vitro assessment: E. coli DH5α (negative control), S. Typhimurium 82/6915 (positive control), S. II Sofia Bt6 (isolate in its original form), Bt6/pCR®2.1 (S. II Sofia Bt6 containing plasmid pCR®2.1 without any insert) and Bt6/pWSK29 (S. II Sofia Bt6 containing plasmid pWSK29 without any insert). Since it has been shown that spv genes are expressed at the late-log or early stationary growth phase (Valone et al., 1993), incubation of the mutant and control strains were carried out as described before being assessed in vitro: overnight cultures of the bacterial strains were diluted 1:30 in 5 mL of fresh LBB and incubated at 37°C for 7 h with aeration. Adhesion, invasion and intracellular survival assays were performed as described previously (see Sections 2.4.3.4 and 2.4.3.5).

5.2.3.1 Statistical Analysis

The CFU counts were expressed as means ± standard errors of the values. Statistical analysis was performed using Microsoft Excel program. All data was analysed using one-way analysis of variance or ANOVA, which is used in the comparison of 3 or more treatments with the assumption that all treatment of samples have similar variance (Deacon, 2006). A post hoc analysis of the experimental data was also conducted (after ANOVA analysis) using the Dunnett’s test, which compares the means of all treatments against a control (Lane, 2008). Only the CFU value of S. II Sofia Bt6 was compared to
that of *S. Typhimurium* 82/6915 – all other experimental values (pCR®2.1, pWSK29, pCspv8 and pWSPV1) were compared to mean CFU of *S. II Sofia Bt6*, which is designated as the control group for the statistical analysis.

### 5.2.3.2 INT407 and CEF-DF1 Cell Adherence

The adhesion ability of each bacterial sample was expressed as a percentage of the number of adhered bacteria (mean value of CFU count) over the number in the inoculum (CFU/mL) and is listed in Table 5.1. All statistically significant results have a *p* value of less than *p* = 0.01. The isolate *S. II Sofia Bt6* was significantly less adherent compared to the pathogenic *S. Typhimurium* 82/6915 in both cell lines (Table 5.1). No significant difference was observed in the INT407 adhesion values between *S. II Sofia Bt6* and the pCspv8 and pWSPV1 mutants (Table 5.1). However, insertion of the plasmid pCR®2.1, pWSK29 and pCspv8 in *S. II Sofia Bt6* appeared to have significantly decreased the adhesion ability of this isolate in CEF-DF1 cells (Table 5.1). Only the adherent property of pWSPV1 was not affected by the insertion of *spvRABCD* (Table 5.1).
Table 5.1  Effect of spvRABCD on the adherence abilities of bacterial strains to INT407 and CEF-DF1 cells (compared to S. Typhimurium 82/6915). Data values that are significantly altered (ANOVA and Dunnett’s post hoc analysis) compared to that of 82/6915 (for Bt6) and Bt6 (all other samples) are indicated by * and **, respectively ($p < 0.01$).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Strain</th>
<th>% Adherence$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT407</td>
<td>DH5α</td>
<td>1.49 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>82/6915</td>
<td>9.73 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>Bt6</td>
<td>0.57 ± 0.09*</td>
</tr>
<tr>
<td></td>
<td>Bt6/pCR®2.1</td>
<td>0.71 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Bt6/pCspv8</td>
<td>0.33 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Bt6/pWSK29</td>
<td>0.77 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>Bt6/pWSPV1</td>
<td>0.68 ± 0.08</td>
</tr>
<tr>
<td>CEF-DF1</td>
<td>DH5α</td>
<td>0.72 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>82/6915</td>
<td>12.27 ± 1.48</td>
</tr>
<tr>
<td></td>
<td>Bt6</td>
<td>1.00 ± 0.11*</td>
</tr>
<tr>
<td></td>
<td>Bt6/pCR®2.1</td>
<td>0.48 ± 0.06**</td>
</tr>
<tr>
<td></td>
<td>Bt6/pCspv8</td>
<td>0.27 ± 0.03**</td>
</tr>
<tr>
<td></td>
<td>Bt6/pWSK29</td>
<td>0.58 ± 0.07**</td>
</tr>
<tr>
<td></td>
<td>Bt6/pWSPV1</td>
<td>0.98 ± 0.13</td>
</tr>
</tbody>
</table>

$^a$ Percentage of adherent bacteria expressed as described in Section 5.2.3.2 (mean ± standard error, $n = 9$)
5.2.3.3 INT407 and CEF-DF1 Cell Invasion

The invasive ability of each bacterial sample was expressed as the percentage of percentage of invasive bacteria shown relative to that of the pathogenic S. Typhimurium 82/6915 (set to 100%), which is calculated using the formula below:

\[
\text{% invasion of sample} = \frac{\text{number of invasive bacteria over number in inoculum}}{\text{number of invasive 82/6915 over number in inoculum}}
\]

All statistically significant results have a \( p \) value of less than \( p = 0.01 \) and can be seen in Figures 5.6 and 5.7.

The isolate S. II Sofia Bt6 was significantly less invasive compared to S. Typhimurium 82/6915 in both cell lines (Figure 5.6). When the data was compared relative to the invasion of S. Typhimurium 82/6915 (set to 100%), a significant difference was observed in the invasion of INT407 by Bt6/pCspv8, Bt6/pWSK29 and Bt6/pWSPV1 (Figure 5.6). No significant change was seen in the 3 bacterial samples above when assessed with CEF-DF1 cells. However, the presence of pCR®2.1 in isolate Bt6 significantly hindered the invasive ability of S. II Sofia Bt6 in CEF-DF1 (Figure 5.6).
Invasion of INT-407 and CEF-DF1 Cells

**Figure 5.6** Effect of *spvRABCD* on the invasive abilities of *S. II Sofia* in INT407 and CEF-DF1 cells (compared to *S. Typhimurium* 82/6915). Invasion of the plasmid-containing strains was shown relative to that of *S. Typhimurium* 82/6915 (set to 100%). Data point of *S. Typhimurium* 82/66915 (100%) was not included here to enable visible comparison of other values. Data values that are significantly altered (ANOVA and Dunnett’s post hoc analysis) compared to that of parent strain *S. II Sofia* Bt6 are indicated by * (p < 0.01).

Data values calculated as outlined in formula given in Section 5.2.3.3
5.2.3.4 Intracellular Survival Assay (with J774 Macrophages)

The viable counts of each bacterial sample over a 24 h period was recorded and expressed as the total number of surviving bacteria in CFU/mL (Figure 5.7). Viable counts for the virulent *S. Typhimurium* 82/6915 were observed to decrease initially at 1 h post-infection before increasing by 4-fold of the level of 0 h post-infection numbers (data not shown). This pattern of initial decline followed by the increase in bacterial CFU was also shown in each mutant and the parent strain (from which the mutants were derived): however, only a 1- to 1.5-fold increase was observed (Figure 5.7). The insertion of plasmid (with and without *spv* insert) did not appear to have affected the survival and replication ability of *S. II Sofia* Bt6, except for Bt6/pCspv8 – the presence of *spvRABCD* in pCR®2.1 decreased the level of *S. II Sofia* numbers at 24 h post-infection by approximately 1-fold, compared to the 24 h viable counts of the other *S. II Sofia* strains (Figure 5.7).

The adhesion and invasive ability of each strain was also assessed (expressed as the percentage of adherent/invasive bacteria over the number in the inoculum) (Figure 5.8). *S. Typhimurium* 82/6915 was observed to be the most adherent strain to the macrophages (Figure 5.8). Although the presence of the plasmids caused a change in the adhesion of the strains (an increase with pCspv8 and pWSK29 and a decrease with pWSPV1), only the change in Bt6/pWSK29 was significant (Figure 5.8). Despite the different adhesion abilities observed, the amount of each *Salmonella* isolate in the macrophages after initial invasion (uptake or phagocytosis of *Salmonella* spp.) was approximately the same – the only exception was Bt6/pWSK29, which showed a significant increase in its invasive ability (Figure 5.8). Neither the parent nor mutant *S. II* Sofia strains showed any significant change in its survival and replication ability in J774 cells, with a 1.2- to 1.5-fold increase observed for all the strains (Figure 5.9).
Figure 5.7 Survival of *E. coli* DH5α and *Salmonella* strains in murine J774 macrophages. Viable CFU per 10^5 macrophage cells in each well (y axis) and time after addition of gentamicin (x axis) are indicated. Data points of *S. Typhimurium* 82/6915 were not included here to enable visible comparison of the other values. All values are the means ± standard errors of 3 different experiments done in triplicates.
Adherence and invasion of J774 macrophages

<table>
<thead>
<tr>
<th>Strains</th>
<th>% Adherence and Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>82/6915</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Bt6</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Bt6/pCR2.1Bt6</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Bt6/pCspv8</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Bt6/pWSK29</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Bt6/pWSPV1</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

Data values expressed as percentage of the number of adherent/invasive bacteria over the total number in the original inoculum (mean ± standard error, n = 9).

Figure 5.8 Effect of spvRABCD on the adhesion and invasive abilities of S. II Sofia in J774 macrophages (compared to S. Typhimurium 82/6915). Data values of mutant strains that are significantly altered (ANOVA and Dunnett’s post hoc analysis) compared to that of parent strain S. II Sofia Bt6 are indicated by * (p < 0.01).
Survival and Replication in J774 Macrophages

![Bar chart showing replication of different strains](Image)

<table>
<thead>
<tr>
<th>Strains</th>
<th>Fold replication $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>82/6915</td>
<td>1</td>
</tr>
<tr>
<td>Bt6/pCR®2.1</td>
<td>2</td>
</tr>
<tr>
<td>Bt6/pCspv8</td>
<td>2</td>
</tr>
<tr>
<td>Bt6/pWSK29</td>
<td>2</td>
</tr>
<tr>
<td>Bt6/pWSPV1</td>
<td>2</td>
</tr>
</tbody>
</table>

$^d$ Data values is shown as the number of CFU at 24 h post-infection over the number of CFU at 0 h post-infection (mean ± standard error, $n = 9$)

**Figure 5.9**  Effect of $spvRABCD$ on the survival and replication of S. II Sofia in J774 macrophages (compared to S. Typhimurium 82/6915) over 24 h. Data values of mutant strains that are significantly altered (ANOVA and Dunnett’s post hoc analysis) compared to that of parent strain S. II Sofia Bt6 are indicated by * ($p < 0.01$).
5.2.4 RT-PCR Analysis of \textit{spvRABCD} expression

The expression of each individual gene in the \textit{spv} cluster from the clones pCspv8 and pWSPV1 was evaluated. Induction of \textit{spv} expression was carried out as described previously (see Section 5.2.3) before RNA isolation of the samples (see Section 2.5.2). RNA extraction was performed on all bacterial strains employed in the \textit{in vitro} assay (DH5\textalpha{}, 82/6915, Bt6, Bt6/pCR®2.1, Bt6/pCspv8, Bt6/pWSK29 and pWSPV1). The expression of the individual \textit{spv} was compared through RT-PCR analysis (see Section 2.5.3). Primers used (along with the annealing temperatures) are listed in Appendix XII. Along with each \textit{spv} gene, the expression of the alternative sigma factor or \textit{katF} was also assessed.

PCR amplification carried out on \textit{S. Typhimurium} 82/6915 and 15 \textit{S. II Sofia} isolates to detect \textit{katF} yielded the specific 0.7 kB product, indicating the presence of this gene in the \textit{S. II Sofia} genome (results not shown). \textit{katF} expression was observed in all the bacterial samples evaluated (Figure 5.10). RT-PCR analysis showed that the \textit{spvRABCD} genes were expressed in Bt6/pCspv8, indicating that the clone constructed was functional in \textit{S. II Sofia} Bt6 (Figures 5.10 and 5.11). However, \textit{spvA}, \textit{spvB} and \textit{spvC} were not expressed in Bt6/pWSPV1, showing the possibility that these genes were not induced during \textit{in vitro} assay (Figures 5.10 and 5.11). No \textit{spv} expression was detected in any of the control strains, except \textit{S. Typhimurium} 82/6915, which is designated as the positive control (Figures 5.10 and 5.11).
Figure 5.10  Expression of katF, spvR and spvA in the control strains and Salmonella mutant strains. M, λDNA marker digested with PstI. Lane 1, 3, 5, 7, 9, 11 and 13 are samples prepared without RTase (negative control) and Lane 2, 4, 6, 8, 12 and 14 are samples with added RTase. Lane 1 and 2, E.coli DH5α; Lane 3 and 4, S. Typhimurium 82/6915; Lane 5 and 6, Parent stain S. II Sofia Bt6; Lane 7 and 8, Bt6/pCR®2.1; Lane 9 and 10; Bt6/pCspv8; Lane 11 and 12, Bt6/pWSK29; Lane 13 and 14, Bt6/pWSPV1.
Figure 5.11  Expression of spvB, spvC and spvD in the control strains and Salmonella mutant strains. M, λDNA marker digested with PstI. Lane 1, 3, 5, 7, 9, 11 and 13 are samples prepared without RTase (negative control) and Lane 2, 4, 6, 8, 12 and 14 are samples with added RTase. Lane 1 and 2, E.coli DH5α; Lane 3 and 4, S. Typhimurium 82/6915; Lane 5 and 6, Parent stain S. II Sofia Bt6; Lane 7 and 8, Bt6/pCR®2.1; Lane 9 and 10; Bt6/pCspv8; Lane 11 and 12, Bt6/pWSK29; Lane 13 and 14, Bt6/pWSPV1.
5.3 Discussion

Plasmid analysis showed that a majority of the S. II Sofia isolates appear to contain more than one plasmid, ranging in size from approximately 1 kB to >90kB (Figure 5.1). This observation contradicts the previous studies carried out on the plasmid content of S. II Sofia strains – identification of a single small 6.4 kB plasmid of unknown function (Harrington et al., 1991). A number of S. II Sofia isolates studied here were observed to contain a plasmid of approximately 6.4 kB (see Lanes 10, 11, 13 and 14 in Figure 5.1). However, no isolate was shown to contain a single plasmid of 6.4 kB (Figure 5.1). The work of Harrington and colleagues (Harrington et al., 1991) has suggested that S. II Sofia isolates of the same clone (probably from the same geographical area) were used in the study, resulting in the isolation of the same 6.4 kB plasmid in all the Australian strains but not the Israeli strains. It is possible that the distribution of plasmids amongst the S. II Sofia strains from various locations is different and more isolates would have to be analysed for their plasmid content before any conclusions can be drawn.

However, the presence of more than one band in the gel could also be due to the plasmid taking more than one conformation. Plasmid DNA can appear in more than one form, which will run at different rates during gel electrophoresis; resulting in more than one band: supercoiled (conformation with the most compact form as the plasmid DNA is wound around proteins and thereby, migrates the fastest on the gel), nicked-circular (plasmid with a single strand cut, which releases the supercoiling and migrates the slowest on a gel), relaxed circular (intact plasmid with no supercoiling) and linear (plasmid with cuts in both strands, which on a gel usually runs between the supercoiled and nicked forms) (Dellis, 2008; Rogers, 2008). Further, 2 or more plasmids have been
known to fuse together under certain conditions to form dimers or multimers – these plasmids are much larger than the individual plasmid and thereby, migrate very slowly regardless of their conformation (Dellis, 2008; Rogers, 2008). This could explain the presence of the much larger plasmid in a majority of the S. II Sofia samples (Figure 5.1). The various bands seen in the agarose gels in Figure 5.1 could be from the different conformations of a plasmid.

All non-linear conformations of the same plasmid will disappear once digested with a RE that is known to cut once in the plasmid, resulting in a single band corresponding to the linear form of the plasmid. However, attempts to analyse the plasmids from S. II Sofia through restriction digest (using numerous REs since the genetic content and therefore, restriction cleavage sites of this sequence is unknown) have been unsuccessful as smearing was obtained after RFLP and electrophoresis analysis (results not shown). This is most probably due to contamination of the samples with chromosomal DNA, which is also cleaved along with the plasmid DNA during digestion (resulting in smearing). Although the plasmid isolation method by Kado and Liu (Kado and Liu, 1981) showed successful isolation of plasmids that can be directly used for restriction digestion, transformation and nick translation; the removal of chromosomal DNA from the samples in this study could not be achieved, despite modifications to the protocol. Therefore, the plasmid samples could not be analysed through RFLP. Perhaps another plasmid isolation method should be utilised in order to obtain sufficient amounts of relatively pure plasmid DNA for analysis - the cesium chloride/ethidium bromide density gradient plasmid prep method (Rogers, 2008; Sambrook and Russell, 2001).

Southern hybridisation analysis showed that the S. II Sofia isolates may harbour a number of plasmids of various sizes but not the virulence plasmid found in S.
Typhimurium. Two DNA probes detecting 2 different regions of the virulence plasmid failed to hybridise to any of the 15 S. II Sofia strains tested (for spv detection, see Figure 5.2). This observation was also confirmed through attempts to amplify these 2 regions from S. II Sofia DNA, which were unsuccessful (data not shown). The 14 kB probe contains 4 genes (traY, traA, traL and traE) that encode components involved in the conjugation of the plasmid (Rotger and Casadesús, 1999; Rychlik et al., 2006). The absence of these 4 genes could mean that the virulence plasmid is not present in S. II Sofia. However, the absence of the virulence plasmid cannot be assumed from failure to detect these tra genes as major deletions in the tra operon have been observed in the virulence plasmid of various Salmonella serovars (Rotger and Casadesús, 1999; Rychlik et al., 2006).

On the other hand, the spv cluster of genes is highly conserved among the Salmonella spp. and its absence could indicate that S. II Sofia does not contain the large virulence plasmids common in the other Salmonella serovars (Guiney et al., 1994; Gulig et al., 1993; Rychlik et al., 2006). The absence of spv genes could also be one of the many reasons contributing to the avirulence nature of S. II Sofia as this operon is involved in the virulence process (Guiney et al., 1994; Gulig et al., 1992; Krause et al., 1995; Libby et al., 1997; 2000; Williamson et al., 1988). It is possible that the plasmid(s) present in S. II Sofia does encode for functions other than virulence e.g. antibiotic-resistance or bacteriocins (Anon, 2008a). Further, it has been shown that S. II Sofia is missing pefA, a plasmid-encoded fimbriae gene (Heuzenroeder et al., 2001). Another explanation would be that the virulence plasmid is present in S. II Sofia genome but has undergone major mutations, including the deletion of spv and pef operon and portions of the tra region. This is a reasonable assumption as other virulence-associated regions in
the S. II Sofia genome such as SPIs have been observed to contain major insertions and deletions (see Chapter 4).

The spv operon was successfully cloned with pCR®2.1 and pWSK29 (creating pCspv8 and pWSPV1) and inserted into S. II Sofia Bt6 (Figures 5.3, 5.4 and 5.5). The effect exerted by the spv operon on S. II Sofia was then evaluated through the strain’s ability to adhere, invade and replicate intracellularly in host cells. The ability of each mutant to adhere, invade and replicate was compared to the pathogenic S. Typhimurium 82/6915 and parent strain S. II Sofia Bt6. Two other control strains (containing the empty plasmid pCR®2.1 and pWSK29, respectively) was also included in this study to differentiate between the effects caused by spv genes and that caused by the presence of the plasmid itself. The adherence ability of the S. II Sofia mutant strains was dependent on the cell line used to assess its adherence (Table 5.1). While the insertion of the plasmid (with and without spv insert) had no effect on the adherence of S. II Sofia to INT407 cells, it reduced the adhesion ability of S. II Sofia to CEF-DF1 cells and had a mixed effect on the adherence of mutant strains to J774 macrophages – increasing adherence in Bt6/pCspv8 and Bt6/pWSK29 and decreasing it in Bt6/pWSPV1 (Table 5.1 and Figure 5.9).

The adhesion of Bt6/pCR®2.1 and Bt6/pWSK29 in CEF-DF1 was observed to decline by approximately 2-fold and the presence of spvRABCD in Bt6/pCspv8 further decreased the adherence by another 2-fold (when compared to that of parent strain Bt6) (Table 5.1). This decrease in the adhesion could be due to the metabolic burden placed by the plasmid on the bacterial strain: the adherent ability of Bt6/pCR®2.1 is more affected compared to that of Bt6/pWSK29 since pCR®2.1 is a high-copy number plasmid and thus, are present in greater numbers in the cell, increasing its metabolic
load (Table 5.1). This can further be observed from Bt6/pCspv8 as the presence of both pCR®2.1 and spvRABCD caused a 4-fold decline due to the additional burden placed on the strain by the insert (Table 5.1). The decrease in adherence could also be due to the interaction of the plasmids with an unknown factor within the S. II Sofia genome, resulting in the negative effect observed. Further, it appears that the presence of spv insert with low-copy number pWSK29 in S. II Sofia Bt6 was able to restore the adherent ability of the strain to the levels of the parent strain, indicating the possibility that spvRABCD may be involve in the adherence function of Salmonella spp. (Table 5.1).

On the other hand, the opposite was observed in adherence of Bt6/pCspv8, Bt6/pWSK29 and Bt6/pWSPV1 to J774 cells (Figure 5.8). It would seem that the adherent ability of the mutant strains (with and without the spv insert) is dependent upon the type of mammalian host cells the strains come into contact with. Statistical analysis showed that a significant difference exist in the adhesion ability of the Salmonella strains between the 3 different cell lines ($p < 0.001$). The empty plasmid or clones may not only have interacted with the S. II Sofia genome but also with the host cells, resulting in the different adherence abilities obtained (Table 5.1 and Figure 5.8). While INT407 cells are of human origin, CEF-DF1 and J774 cells are of animal origin (chicken and mouse, respectively) and this might play a part in the different adherent abilities of the Bt6 strains. It is possible that spvRABCD could play a role in host-specific virulence – the virulence plasmids of Salmonella spp. are also known as serovar-specific plasmids as they are frequently isolated from Salmonella strains showing host adaptation (Guiney et al., 1994; Rotger and Casadesús, 1999; Rychlik et al., 2006). The effect of spvRABCD on the adhesion ability of a strain may be a function that is unique to S. II Sofia and should be assessed by inserting this operon into another low-copy plasmid to ascertain if the same effect was obtained.
The presence of \textit{spvRABCD} in high-copy pCR®2.1 significantly increased the bacterial invasion (compared to the invasion of Bt6 and Bt6/pCR®2.1) in the INT407 cell line but had no effect in the CEF-DF1 cells (Figure 5.6). Since the empty plasmid pCR®2.1 did not appear to affect the invasive ability of S. II Sofia Bt6 in INT407 cells, the 2.7-fold increase observed in Bt6/pCspv8 could be attributed to the presence of the \textit{spv} insert – indicating the possibility that \textit{spv} operon may be involved in the bacterial invasion process (Figure 5.6). However, the presence of the \textit{spv} insert was not able to restore the invasiveness of S. II Sofia Bt6 to wild type levels observed in S. Typhimurium 82/6915 (data not shown). The results also suggest that the presence of plasmid vectors may have had an effect on the invasive ability of the S. II Sofia mutant strains, as evident from the invasiveness of Bt6/pWSK29 and Bt6/pWSPV1 in INT407 cells (Figure 5.6).

Bacterial sample Bt6/pWSK29 only contains the empty plasmid and therefore, should not show any increase in invasion without the presence of any virulence factors. However, a 2-fold increase in invasion was observed in this mutant strain – higher than the invasiveness observed in Bt6/pWSPV1, indicating that the increase was not from the effect of \textit{spv} genes but from the plasmid itself (Figure 5.6). It has been shown that certain cloning vectors (plasmids and fluorescent protein reporter systems) used in the study of bacterial pathogenesis can affect the ability of S. Typhimurium to productively infect either cultured mammalian cells or mice (Knodler \textit{et al.}, 2005). One of the vectors investigated was pWSK29 and the presence of the vector in S. Typhimurium appeared to cause an increase in the invasive ability of the strain in HeLa and RAW264.7 cells (although the increase observed was not significant) (Knodler \textit{et al.}, 2005). Insertion of the pWSK29 plasmid (with or without insert) could have caused an increase in bacterial invasion due to the interaction of the plasmid with an unknown factor in the S. II Sofia genome – this could also explain the improved adherent and invasion ability of
Bt6/pWSK29 to J774 cells (Figure 5.8). No significant differences were observed in the invasiveness of the other strains in either CEF-DF1 cells or J774 macrophages, indicating that invasion ability (like adherence) is also dependent on the type of cultured mammalian cells the strains come into contact with (Figures 5.6 and 5.8).

Despite starting with approximately equal invasion rates, differences were observed in the subsequent survival and replication of the *Salmonella* strains in the J774 macrophages (Figures 5.7 and 5.9). A 3- to 4-fold difference was shown in the levels of 24 h post-infection numbers between *S*. Typhimurium 82/6915 and *S*. Il Sofia strains (with and without insert), indicating that serovar Typhimurium has a survival and replication advantage in J774 cells. This observation correlates well with previous studies, in which *S*. Typhimurium strains displayed enhanced survival ability in murine macrophage cells (Alpuche-Aranda *et al*., 1995; Buchmeier and Heffron, 1989; Schwan and Kopecko, 1997; Schwan *et al*., 1997). The poor survival and replication of parent *S*. Il Sofia Bt6 in J774 cells could explain why this strain was not able to cause disease in humans and chickens – intracellular survival in macrophages has been shown to be essential for *Salmonella* virulence (Alpuche-Aranda *et al*., 1995; Fields *et al*., 1986; Schwan *et al*., 1997).

The presence of *spvRABCD* did not significantly affect the survival and replication of *S*. Il Sofia Bt6 in J774 macrophages - except in Bt6/pCspv8, in which the slight decrease in 24 h post-infection viable counts could be attributed to the metabolic burden placed on the strain by the presence of insert in a high-copy number plasmid (Figures 5.7 and 5.9). This indicates that it is probable that *spv* operon is not responsible for intracellular survival of *Salmonella* spp., adding to the contradictory studies regarding the role of *spv* genes in virulence. The exact function of the *spv* genes has been in
contention for some time, with some investigators reporting their involvement in intracellular survival and replication (Libby et al., 1997; 2000; Paesold et al., 2002) and others finding no such link (Gulig and Curtiss III, 1987; Guilloteau et al., 1996; Rhen et al., 1993; Riikonen et al., 1992; Wilson et al., 1997). The contradictory results obtained could be due to the different in vitro and/or in vivo models used to study the spv genes.

The research showing no established relationship between spv operon and intracellular survival (Gulig and Curtiss III, 1987; Guilloteau et al., 1996; Rhen et al., 1993; Riikonen et al., 1992; Wilson et al., 1997) were all carried out using murine cultured cells or animal model. On the other hand, the assessment of the spv genes by Libby and his coworkers (Libby et al., 1997; 2000) and by Paesold and his colleagues (Paesold et al., 2002) were done using other host systems – bovine (Libby et al., 1997) and cultured mammalian cells of human origin, respectively (Libby et al., 2000; Paesold et al., 2002). This difference may indicate the host-specificity of the spv operon and that the murine host system is not a suitable model for investigating function of the spv genes. The S II Sofia mutant strains should be evaluated again using a different macrophage cell line e.g. human monocyte-derived macrophages as employed by Libby and his colleagues (Libby et al., 2000). Further, an in vivo assay (using chicken animal model) should be conducted to further investigate the effect of spv operon on virulence as it is possible that some virulence processes do not manifest unless present in a live host (Garmony et al., 2002).

The transcription of the individual spv genes was investigated via RT-PCR to verify that the clones constructed were expressed within S. Il Sofia Bt6. The expression of the alternative sigma factor gene katF was also analysed as it has been shown this protein controls and regulates this operon (Chen et al., 1995; Fang et al., 1992; Heiskanen et al.,
PCR and RT-PCR analysis revealed that not only is katF present in S. II Sofia; the gene appears to be functional (Figure 5.10). A number of bands observed in RT-PCR detection of katF from the E. coli DH5α RNA sample could be attributed to the presence of katF in E. coli genome. The katF gene is present in enteric bacteria (such as E. coli and Salmonella spp.) and it mediates adaptation of the bacterial strain to stress conditions – low temperature, nutrient limitation and growth to stationary phase (Hirsch and Elliot, 2005). Sequence comparison between the katF of E. coli and S. Typhimurium demonstrate a high degree of conservation (Prince et al., 1994) and this shared similarity would enable amplification of product from DH5α sample (Figure 5.10).

The expression of the spv operon from Bt6/pCspv8 (and corresponding non-expression from samples without spv genes) show that this clone is probably functional after insertion into S. II Sofia Bt6 (Figures 5.10 and 5.11). Thus, the increase in invasive ability of this mutant (Figure 5.6) could be caused by this operon. On the contrary, only the expression of spvR and spvD was detected from Bt6/pWSPV1, which could indicate that spvABC was not functional during in vitro assay (Figures 5.10 and 5.11). However, there are other factors that could account for this non-expression. pCR®2.1 is a high-copy number vector (200-500 copies per cell) and the cloning of spvRABCD with this vector would lead to high levels of gene expression, making it easy to detect via RT-PCR (Figures 5.10 and 5.11). In contrast, pWSK29 is only present at 6-8 copies per cell (Wang and Kushner, 1991) and the low-copy number of the vector could have resulted in spvABC expression levels too low to be detected by RT-PCR – although this was not the case for spvR and spvD (Figures 5.10 and 5.11). It is also possible that transcription of spvABC was transient or only induced intracellularly, making it difficult to detect when grown in LBB broth. However, the most probable reason for this non-expression could
be the pWSK29 plasmid is an unsuitable cloning vector in this study – plasmid could have interacted with an unknown factor in the S. II Sofia genome, which impaired only part of the operon expression, leading to the uneven gene transcription observed (Figures 5.10 and 5.11).

Although *spvRABCD* has been shown to be of importance in *Salmonella* virulence (Guilloteau *et al.*, 1996; Gulig and Curtiss III, 1987; Gulig *et al.*, 1992; 1998; Krause *et al.*, 1995; Libby *et al.*, 1997; 2000; Paesold *et al.*, 2002), so far it has only been implicated in intracellular survival and replication (and thereby, the development of systemic disease) and not in the bacterial adherence and invasion. The *spv* insert was cloned into 2 different plasmid vectors: the high-copy pCR®2.1 (to ensure maximum gene expression) and low-copy pWSK29 (to mimic the low-copy number conditions of *spv* operon in the virulence plasmid). The increase in invasive ability of Bt6/pCspv8 (Figure 5.6) could be caused by the overexpression of Spv proteins due to the high-copy nature of pCR®2.1 cloning vector (200-500 copies per cell). It is possible that at such a high level of gene expression, the Spv proteins can play a role in the bacterial invasion process.

The uneven adherence and invasion ability as well as gene transcription exhibited by some of the other mutant strains appeared to be linked to the presence of pWSK29 plasmid (Table 5.1 and Figures 5.6, 5.8, 5.10 and 5.11). This cloning vector has been shown to alter the ability of *Salmonella* spp. to invade and replicate in epithelial and macrophage-like cell lines (Knodler *et al.*, 2005) and this property could have influenced the in vitro assessment of the mutants. The use of another low-copy number cloning vector should be considered such as pACYC177 (Chang and Cohen, 1978), pLG338 and pLG339 (Stoker *et al.*, 1982). In addition, in vivo studies with animal models e.g.
chickens should be carried out with the mutant strains as it has been shown that in vitro experiments may not be an accurate representation of in vivo processes (Garmony et al., 2002) and therefore, both in vitro and in vivo assessment should be used to investigate the effect of the presence of spvRABCD to the pathogenicity of S. II Sofia.

Analysis of the plasmid content of S. II Sofia isolates revealed the presence of a large plasmid (> 90 kB) along with several smaller plasmids in the range of 1.0 to 23 kB in size. The presence of more than one plasmid could be attributed to either the existence of multiple plasmids in S. II Sofia genome or the different conformations exhibited by a plasmid. However, attempts to further investigate the plasmids through RFLP analysis have been unsuccessful due to contamination of plasmid samples with chromosomal DNA, which could not be removed despite protocol modifications and careful extraction. Southern blot detection using DNA probes from different regions of virulence plasmid (including the virulence-associated spvRABCD) failed to detect the presence of both these regions, indicating either the absence of the entire virulence plasmid or a virulence plasmid containing major deletions in the S. II Sofia isolates. Cloning of the spv genes (into high-copy pCR®2.1 and low-copy pWSK29) and insertion of constructs into S. II Sofia Bt6 was performed.

The presence of spv operon in pCR®2.1 (clone pCspv8) appeared to significantly increase the invasive ability of S. II Sofia Bt6 – overexpression of Spv proteins may have a positive effect on bacterial invasion. The uneven adherence and invasion ability of the other mutant strains can be explained by the plasmid compromising bacterial fitness or interacting with an unknown factor in bacterial genome, leading to decrease and increase of adherence and invasion, respectively. The spv operon does not seem to affect the survival and replication of S. II Sofia in macrophages in this study. RT-PCR
analysis indicates that while the pCspv8 is fully functional, spvABC is not expressed in pWSPV1, which further supports the theory that pWSK29 is able to affect the interaction between S. II Sofia and host cells. However, more studies on the function of plasmids (native and clones) in S. II Sofia are required before any conclusions can be made.
CHAPTER 6: GENERAL DISCUSSION

Although *Salmonella* spp. is one of the most widespread disease-causing organisms worldwide, its pathogenicity appears to vary depending on factors such as serotype and host range. An example of this pathogenic variation can be seen from the *Salmonella* spp. isolated from Australian poultry flocks. The most common pathogenic *Salmonella* strains isolated from Australian chickens include the serovars Typhimurium, Saintpaul and Virchow (Miller *et al.*, 2005; Murray, 2001; Yohannes, 2004). However, the most common and prevalent *Salmonella* serovar amongst Australian poultry is the subspecies group II *S.* Sofia, accounting for 50-60% of the *Salmonella* chicken isolates (Heuzenroder *et al.*, 2001). This particular serovar is persistent in the chicken flocks but is avirulent in nature and thus, is rarely associated with diseases in animals and humans. The avirulence of *S.* II Sofia and its high frequency in chickens is a situation that is unique to Australia although the reason for this observation is unknown. Surprisingly few studies have been done on *S.* II Sofia despite the serovar being around since the 1970s (Harrington *et al.*, 1991; Heuzenroder *et al.*, 2001).

A major focus of this research is to study the genetic variation of the Australian *S.* II Sofia isolates and this includes investigating the epidemiology and genetic distribution of virulence genes within the strains. This work is important as little is known about the regarding the molecular pathogenesis of this serovar and its genetic relationship with one another. The few studies that have been done by other investigators (Harrington *et al.*, 1991; Heuzenroder *et al.*, 2001; Rickard, 1998; Ross *et al.*, 2003) have shown that the Australian *S.* II Sofia isolates may share clonal relationship, is only avirulent within
individuals with a mature and healthy immune system and this avirulence may be attributed to missing virulence-associated genes such as inv and spv regions. By further characterising the clonal relationship of the strains and investigating the virulence factors important to pathogenesis (e.g. SPI-associated genes), a greater understanding of the virulence and colonisation mechanisms of S. II Sofia may be obtained and its future pathogenic potential towards chickens and humans assessed.

So far, S. II Sofia isolates have only been subtyped using RFLP and AFLP analysis (Harrington et al., 1991; Ross et al., 2003). This study characterised S. II Sofia isolates from various locations around Australia using 2 molecular fingerprinting methods commonly used to subtype Salmonella strains: pulsed-field gel electrophoresis (PFGE) and repetitive PCR typing. Previous attempts to analyse S. II Sofia isolates through PFGE have been unsuccessful due to DNAse activity of the strains (leading to DNA degradation), which could not be removed despite protocol modifications and use of conventional anti-DNAse treatments (Ross et al., 2003). The addition of detergent SDS into the lysis solution (used to break open cells and release genomic DNA in the plugs) was found to stop DNAse activity of S. II Sofia, allowing the production of clear and visible PFGE banding patterns. The combined strength of the 2 different detergents in the lysis solution (sarkosyl and SDS) was probably capable of inactivating nuclease activity and thus, protected the S. II Sofia genomic DNA from degradation. Further, the use of this modified lysis solution on other Salmonella strains did not affect its PFGE quality (visibility of bands and PFGE pattern), which means that this lysis solution can be used to process DNase-negative strains - making pattern comparison between S. II Sofia and other Salmonella serovars possible.
A number of repetitive sequences (BOX, ERIC and REP and PCR ribotyping) were evaluated in the typing of S. II Sofia and it was discovered that only the REP sequence was discriminatory enough to be used for typing since all the other repetitive elements showed very limited pattern diversity. Therefore, PFGE (XbaI and SpeI) and REP-PCR (with REP1R primer) was carried out on 84 different S. II Sofia isolates, resulting in the generation of 8 XbaI, 6 SpeI and 5 REP1 profile patterns. No correlation was obtained between the typing methods as there was overlap and diversity within each profile. A majority of the patterns were observed to be grouped within a single profile type, with the other types encountered consisting of a few isolates each. This limited genetic diversity is reflected by the limited pattern diversity obtained with the other repetitive sequences, relatively high Dice coefficient ($F$) values, dendograms and low Simpson's discrimination indices ($< 0.5$). Combination of the typing methods was able to improve the discrimination of samples, dividing the 84 isolates into 16 subtypes, with a discriminatory index of 0.721.

All these factors indicate that the Australian S. II Sofia isolates are probably clonally related, supporting previous epidemiological studies of S. II Sofia (Harrington et al., 1991; Ross et al., 2003) and the minor genetic variation observed could be caused by selective pressure on the strains as they spread throughout Australia. In addition, a majority of the S. II Sofia isolates were not geographically restricted with the predominant pattern combination spread out among the isolates from various locations. Victorian S. II Sofia isolates were found to exhibit the most diverse pattern combinations compared to isolates from other states, probably due to the larger sample population: 33 Victorian S. II Sofia strains were analysed compared to 10-15 isolates from the other states. The restriction of all Tasmanian isolates to a single pattern type could also be from the small sample size. Two S. II Sofia strains were observed to show unique profile
pattern – isolate 554NCA and 131 (although the reason for the particular pattern combination is unknown). More S. II Sofia isolates (especially those from stated other than Victoria) could have to be collected and analysed before any conclusion regarding the relationship of profile patterns and geographical locations can be made.

The clonal nature of the S. II Sofia isolates would probably also be reflected in the distribution and variation of SPI-associated virulence genes – the genetic content of these pathogenicity islands amongst S. II Sofia isolates should be identical. This is further confirmed from the identical Southern hybridisation patterns of the S. II Sofia isolates obtained using DNA probes encompassing the entire length of the major SPIs (SPI1 to SPI5). The SPI1 to SPI5 regions of S. II Sofia were observed to exhibit variation in the RFLP pattern when compared to S. Typhimurium and through Southern hybridisation, RFLP and sequencing analysis; a majority of the genetic variation detected within the 5 SPIs can be attributed to the loss or gain of restriction cleavage sites. While SPI2 and SPI4 were found to be relatively conserved, major insertions and deletions were observed in SPI1, SPI3 and SPI5 of S. II Sofia.

The major insertion and deletion events observed in SPI1, SPI3 and SPI5 is most likely due to the insertion and excision of IS elements. This is reflected by the deletion of \textit{avrA}, \textit{sugR-rhuM} and \textit{sopB-pipA} regions from SPI1, SPI3 and SPI5, respectively and also by the presence of 1.2 kB ORF encoding a transposase in SPI1 and SPI3 (transposase found in both these region were shown to be identical). Although found in a number of bacterial species such as \textit{Yersinia enterocolitica}, this transposase was observed to be restricted to S. II Sofia (in this study) – there is potential for the development of this IS element as a marker to differentiate this serovar from other \textit{Salmonella} spp. in Australian poultry flocks. In addition, a number of base and sequence
alterations (insertion, deletion and substitution events) were shown in SPI1 (orgB, prgI, sptP, sipD and invJ), SPI2 (ssaP, ssal and ssaE), SPI3 (misL, marT and slsA) and SPI5 (ORF) genes, resulting in the predicted translation of proteins with altered polypeptide sequences – altered amino acid start residue, truncated proteins from formation of a extra termination codon, proteins with missing or additional residues due to genetic frameshifts.

Such mutations are able to affect the transcription and/or translation process of the SPI genes, which in turn probably altered protein function. Therefore, the avirulent nature of S. II Sofia is not caused by a single mutation or deletion event but rather a series of mutations in the genes within the SPI regions. The mutations observed in SPI1 and SPI2 include genes required for the formation of the needle complex structure, which is required to translocate effector proteins into the host cells (Chakravottry et al., 2005; Chen et al., 1996; Cherayil et al., 2000; Collazo et al., 1995; Darwin and Miller, 1999b; Kimbrough and Miller, 2000; Klein et al., 2000; Kubori et al., 2000, Sukhan et al., 2001; Zhang et al., 2003). The mutations also affected the 2 SPI1-encoded effector proteins SptP and AvrA, shown to be involved in cell cytoskeleton disruptions and apoptosis, respectively (Collier-Hyams et al., 2002; Fu and Galán, 1998b; Murli et al., 2001; Ye et al., 2007).

On the other hand, the mutations in SPI3 involve genes encoding host-specific colonisation factors in mice, cattle and chickens (Dorsey et al., 2005; Lawley et al., 2006; Morgan et al., 2004), indicating the possibility that the mutations may have increased the colonisation ability of the strain, which would explain its persistence in Australian poultry flocks. The major deletion in SPI5 includes genes necessary for the fluid accumulation and inflammation in the intestinal system, which is required for the gastroenteritis
disease process (Norris et al., 1998; Pfeifer et al., 1999; Wood et al., 1998). Therefore, the reduction in the pathogenicity of S. II Sofia probably resulted from the many changes in essential SPI genes. However, further studies targeting actual SPI gene and protein expression is required in order to gain more in-depth knowledge on the effect of these mutations on S. II Sofia virulence.

Salmonella spp. virulence (for certain serovars such as Typhimurium, Dublin, etc) has also been shown to be dependent on the presence of large plasmids containing virulence factors (Akiba et al., 1999; Barrow and Lovell, 1988; Barrow et al., 1987a; 1987b Haneda et al., 2001; Rotger and Casadesús, 1999; Rychlik et al., 2006; Woodward et al., 1989). Analysis of plasmid content of S. II Sofia isolates revealed the presence of a large plasmid along with several smaller plasmids, all in the range of approximately 1 to 100 kB in size. However, failure of the S. II Sofia isolates to hybridise to the DNA probes detecting regions of the virulence plasmid (including the highly conserved virulence-associated spvRABCD) during Southern hybridisation experiments was observed. This could indicate 2 things: the total absence of the virulence plasmid or the presence of virulence plasmid containing major deletions, either of which would contribute to the avirulence of S. II Sofia.

Investigation on the effect of restoring spvRABCD into S. II Sofia was carried out by cloning this operon into high-copy pCR®2.1 and low-copy pWSK29 (producing clones pCspv8 and pWSPV1, respectively) and assessing its pathogenicity in vitro. The presence of pCspv8 was observed to significantly increase invasiveness of S. II Sofia in INT407 cells: the high-copy nature of the cloning vector probably lead to the overexpression of Spv proteins, which may have had an effect on the invasion process. A number of mutant strains containing empty plasmids were shown to exhibit uneven
adherence and invasion ability in the cell lines tested. This observation could reflect the ability of the cloning vector to affect the fitness of host bacteria through interaction of the vector with an unknown factor in bacterial genome, leading to either increase or decrease in adherent and invasion ability. This is clearly seen in the apparent increase in invasiveness of S. II Sofia Bt6 containing empty pWSK29 in INT407 and J774 cells. The pWSK29 plasmid may not be a suitable cloning vector in this study – perhaps the use of another low-copy vector such as pACYC177 should be considered.

Further, this cluster of genes was observed to have no effect on the survival and replication ability of S. II Sofia in J774 macrophages. Although a few studies have shown a positive link between spv operon and intracellular survival, these were carried out using different in vitro and in vivo systems (Libby et al., 1997; 2000; Paesold et al., 2002). In contrast, all studies showing no established relationship between spv operon and intracellular were all carried out using murine cultured cells or animal model (Gulig and Curtiss III, 1987; Guilloteau et al., 1996; Rhen et al., 1993; Riikonen et al., 1992; Wilson et al., 1997). The in vitro and in vivo evaluation of the constructed clones with a non-murine macrophage cell line or animal model should be considered. Animal studies should particularly be considered for the analysis of the clones in order to confirm that any effects seen in vitro are valid and to assess any virulence effects that do not develop unless present in a live host. Finally, RT-PCR assessment of the clones showed while the pCspv8 is fully functional, spvABC is not expressed in pWSPV1, which further supports the theory that pWSK29 is able to affect the interaction of S. II Sofia with the host cells.

In conclusion, the Australian S. II Sofia isolates showed limited genetic diversity and are probably clonally related. The avirulent nature of S. II Sofia is most likely caused
by the accumulative effects of mutations in a number of virulence-associated genes such as the genes in SPI1 to SPI5 and virulence plasmid. The final aim of this work was to assess the pathogenic potential of S. II Sofia in Australia. Based on the study of SPI-and plasmid-associated virulence genes, it is most likely that the wild-type S. II Sofia strains are unable to regain full pathogenicity (comparable to the pathogenicity of S. Typhimurium 82/6915) due to the mutations observed in a large portion of these genes.

This is evident from the in vitro assessment on the effect of spv operon in S. II Sofia Bt6. Although the cloning of spv genes with a high-copy vector was able to increase the invasive ability of S. II Sofia, the increase was not enough to restore invasiveness to the level of S. Typhimurium 82/6915. Restoration of virulence is likely to be a very complex and multifactorial process and thus, insertion of just one virulence factor back within S. II Sofia is not sufficient to restore virulence levels to those of wild-type S. Typhimurium. In other words, the numerous mutations observed in the virulence genes of S. II Sofia would probably inhibit this serovar from regaining pathogenicity in the future, rendering this serovar harmless to both poultry and humans.
REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES

the Outer Membrane Protein Genes rck from Salmonella typhimurium and ail from Yersinia enterocolitica. Infect Immun 62, 5183-5186.


REFERENCES


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REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Zurawski, D. V. and Stein, M. A. (2004). The SPI2-encoded SseA chaperone has discrete domains required for SseB stabilization and export, and binds within the C-terminus of SseB and SseD. Microbiology 150, 2055-2068.
Appendix I: General Chemicals and Equipment

**Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>5X DMEM Media</td>
<td>Thermo Trace Ltd., Australia</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>Invitrogen, USA</td>
</tr>
<tr>
<td>10X PCR Buffer (GeneAmp®)</td>
<td>Applied Biosystems, USA</td>
</tr>
<tr>
<td>10X Ligase Buffer</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>Applied Biosystems, USA</td>
</tr>
<tr>
<td>100 bp DNA Ladder</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>Acetic Acid, Glacial Agarose (DNA Grade)</td>
<td>BDH Chemicals, Australia</td>
</tr>
<tr>
<td>Ammonium Sulfate, (NH₄)₂SO₄</td>
<td>Bioline Pty Ltd., Australia</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Ajax Chemicals Ltd, Australia</td>
</tr>
<tr>
<td>Anti DIG-AP (Fab Fragments)</td>
<td>Roche Applied Sciences, Germany</td>
</tr>
<tr>
<td>Bacteriological Agar (No. 1)</td>
<td>Oxoid Australia Pty. Ltd.</td>
</tr>
<tr>
<td>Bacteriological Peptone Balance</td>
<td>Oxoid Australia Pty. Ltd.</td>
</tr>
<tr>
<td>Balance</td>
<td></td>
</tr>
<tr>
<td>(i) Analytical Balance</td>
<td>Sartorius GMBH, Germany</td>
</tr>
<tr>
<td>(ii) Balance (0.1-500g)</td>
<td>U-Lab, Australia</td>
</tr>
<tr>
<td>Barrier micropipette plastic tips</td>
<td>Mirella Research Pty Ltd., Australia</td>
</tr>
<tr>
<td>Biological Safety Cabinet Class II</td>
<td>LAF Technologies, Australia</td>
</tr>
<tr>
<td>Blocking Reagent</td>
<td>Roche Applied Sciences, Germany</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>BDH Chemicals, Australia</td>
</tr>
<tr>
<td>Bovine serum albumin, 10 µg/µL (BSA)</td>
<td>Promega, USA</td>
</tr>
<tr>
<td>Bromothymol Blue</td>
<td>Chem-Suppy, Australia</td>
</tr>
<tr>
<td>Calcium Chloride, CaCl₂</td>
<td>Merck, USA</td>
</tr>
<tr>
<td>Cell Counter (Neubauer, double ruled)</td>
<td>Propper MFG Co., USA</td>
</tr>
<tr>
<td>Cell scrapers (25 cm)</td>
<td>Interpath Pty Ltd., Australia</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>(i) Microcentrifuge</td>
<td>Beckman, USA</td>
</tr>
<tr>
<td>(ii) Bench Top Centrifuge</td>
<td></td>
</tr>
<tr>
<td>Centrifuge Tubes</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>(i) 1.5 mL Microcentrifuge Tubes</td>
<td>Greiner Bio-One, Germany</td>
</tr>
<tr>
<td>(ii) 10 mL Centrifuge Tubes</td>
<td>Greiner Bio-One, Germany</td>
</tr>
<tr>
<td>(iii) 15 mL Centrifuge Tubes</td>
<td>Greiner Bio-One, Germany</td>
</tr>
<tr>
<td>(iv) 50 mL Centrifuge Tubes</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>CHEF-DR II System (PFGE)</td>
<td>Merck, USA</td>
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<tr>
<td>Chloroform</td>
<td>Mediglass, Australia</td>
</tr>
<tr>
<td>Coverslips</td>
<td>Nalgene Company, USA</td>
</tr>
<tr>
<td>Cryovials (1.8 mL)</td>
<td>Cryo Biological Systems, USA</td>
</tr>
<tr>
<td>Cryogenic tank (liquid nitrogen tank)</td>
<td>Roche Applied Sciences, Germany</td>
</tr>
<tr>
<td>Deoxynucleoside triphosphates (dNTPs)</td>
<td>Sigma-Aldrich Pty. Ltd., USA</td>
</tr>
<tr>
<td>DEPC (97% NMR)</td>
<td>Roche Applied Sciences, Germany</td>
</tr>
<tr>
<td>DIG DNA Labelling and Detection Kit</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td></td>
</tr>
</tbody>
</table>
APPENDICES

Dimethylformamide (DMF) BDH Chemicals, Australia
Dimethylsulfoxide (DMSO) BDH Chemicals, Australia
DNase agar Oxoid Australia Pty. Ltd.
DNA Ligase (T4) Promega, USA
DNA Polymerase
   (i) TAQ Polymerase Invitrogen, USA
   (ii) Platinum Invitrogen, USA
   (iii) AmpliTAQ Gold® Applied Biosystems, USA
Dulbecco’s 1× PBS (without Mg or Ca) Invitrogen (Gibco®), USA
Dye-terminator Sequencing Mix V3.1 Monash University, Australia
Electrophoresis Power Supply
   (i) EPS 500/400 Pharmacia LKB, Sweden
   (ii) EPS600 Pharmacia LKB, Sweden
   (iii) PAC300 Bio-Rad Laboratories, USA
   (iv) PPBasic Bio-Rad Laboratories, USA
Electrophoresis Units
   (i) DNA Bio-Rad Laboratories, USA
      a) Mini-gel Bio-Rad Laboratories, USA
      b) Midi-gel Bio-Rad Laboratories, USA
      c) Maxi-gel Pharmacia LKB, Sweden
   (ii) RNA Bio-Rad Laboratories, USA
      a) Mini-gel Bio-Rad Laboratories, USA
      b) Midi-gel Bio-Rad Laboratories, USA
Electroporation Cuvettes (0.2 cm) Molecular Bio Products, USA
ELISA Plate Reader (MR7000) Dynatech
Ethylene diamine tetra acetic acid (EDTA) BDH Chemicals, Australia
Ethanol BDH Chemicals, Australia
Ethidium Bromide Roche Applied Sciences, Germany
Expand Long Template PCR System Roche Applied Sciences, Germany
Ficol-400 BDH Chemicals, Australia
Filter (Acrodisc 0.2 µm, 0.45 µm) Gelman Sciences USA
Formaldehyde (37%) Sigma-Aldrich Pty. Ltd., USA
Gel-Doc System Bio-Rad Laboratories, USA
Gentamicin sulfate Sigma-Aldrich Pty. Ltd., USA
D-Glucose BDH Chemicals, Australia
L-glutamine (200 mM) Thermo Trace Ltd., Australia
Geneclean® Kit Bio101® Systems, USA
Glycerol BDH Chemicals, Australia
Glycine BDH Chemicals, Australia
HEPES Sigma-Aldrich Pty. Ltd., USA
HEPES Buffer (1 M) ThermoTrace Ltd., Australia
Hexamminecobalt Chloride Sigma-Aldrich Pty. Ltd., USA
Hexadecyltrimethyl ammonium bromide (CTAB) Sigma-Aldrich Pty. Ltd., USA
Hexanucleotide mix Roche Applied Sciences, Germany
Hydrochloric Acid, HCl (32%) Eficell Products P/L, Australia
Hydrogen Peroxide, H2O2 (30%) BDH Chemicals, Australia
ImProm-II™ Reverse Transcriptase Promega, USA
Incubator, for tissue culture (5% CO2) Forma Scientific, USA
IPTG Sigma-Aldrich Pty. Ltd., USA
Isoamyl alcohol BDH Chemicals, Australia
Isopropanol BDH Chemicals, Australia
Kanamycin
Klenow Enzyme
Kim Wipes
Lambda DNA, λDNA
DL-Leucine
Low Melting Point Agarose
Lysozyme
Magnesium Chloride, MgCl₂
Magnesium Sulfate, MgSO₄
Maleic Acid
Manganese(II) Chloride, MnCl₂.4H₂O
Micropipette plastic tips (Blue and Yellow)
Micropipette plastic tips (5 mL and 10 mL)
Microscopes
  (i) Light Microscope
  (ii) Phase Contrast Microscope
Microscope Slides
Microtitre Plate (96-well, flat bottom)
MOPS
Mouse Serum
NBT/BCIP substrate solution
Newborn Calf Serum (NCS)
N-Lauroyl Sarcosine (Sarkosyl)
Nylon Membrane (Hybond-N)
ONPG
Orange G Dye
Parafilm
Petri Dish
PFGE λ ladder (48.5 to 630.5 kB)
PFGE Plug Moulds
pH Meter
DL-Phenylalanine
Phenol, Saturated
Phenol/Chloroform/Isoamyl Alcohol
Phenylmethylsulfonylfluoride, PMSF
Phosphate Buffered Saline, PBS
Plastic Cuvettes
Potassium Acetate
Potassium Chloride, KCl
Potassium dihydrogen orthophosphate
di-Potassium hydrogen orthophosphate
Potassium Sulfate
Proteinase K
QIAGEN TAQ PCR Core Kit
QIAGEN Miniprep Kit
Recombinant RNasin® Ribonuclease Inhibitor
Restriction Enzymes (RE)
  BamHⅠ, BglⅡ, DraⅠ, EcoRV,
  HindⅢ, NotⅠ, PstⅠ, SpeⅠ, XhoⅠ, XbaⅠ
Sigma-Aldrich Pty. Ltd., USA
Roche Applied Sciences, Germany
Kimberly Clarke
Promega, USA
BDH Chemicals, Australia
Progen Industries, Australia
Roche Diagnostics, Germany
Perkin Elmer, USA
Merck, USA
BDH Chemicals, Australia
BDH Chemicals, Australia
Greiner Bio-One, Germany
Pathtech Pty Ltd., Australia
Olympus Optical
Nikon Kogaku KK, Japan
LOMB Scientific Co., Australia
Nunc, Denmark
Sigma-Aldrich Pty. Ltd., USA
Chemicon International Inc., USA
Roche Diagnostics, Germany
Thermo Trace Ltd., Australia
BDH Chemicals, Australia
Amersham, USA
Sigma-Aldrich Pty. Ltd., USA
Sigma-Aldrich Pty. Ltd., USA
Pechiney Plastic Packaging Inc.,
Nunc, Denmark
Bio-Rad Laboratories, USA
Bio-Rad Laboratories, USA
Radiometer, Denmark
BDH Chemicals, Australia
BDH Chemicals, Australia
BDH Chemicals, Australia
Sigma-Aldrich Pty. Ltd., USA
Oxoid Australia Pty. Ltd.
Eppendorf, Germany
BDH Chemicals, Australia
BDH Chemicals, Australia
Merck, USA
BDH Chemicals, Australia
BDH Chemicals, Australia
Roche Diagnostics, Germany
Promega, USA
Promega, USA
Promega, USA
Promega, USA
Promega, USA
Promega, USA
Promega, USA
RNAgents® Total RNA Isolation System
Promega, USA

RNAprotect Bacterial Reagent
Qiagen, USA

RNase
Roche Diagnostics, Germany

RQ1 RNase-Free DNase
Promega, USA

Salmonella O Agglutinating Sera, including
Remel, Europe

Polyvalent O (Group A-S) and monosera
(4-O, 6,7-O, 8-O, 9-O, 3,10,15,19-O)

SeaKem® Gold Agarose
Cambrex BioScience Inc., USA

Sodium Acetate
Amresco, USA

Sodium Bicarbonate (7.5%)
Thermo Trace Ltd., Australia

Sodium Chloride, NaCl
BDH Chemicals, Australia

Sodium Citrate
BDH Chemicals, Australia

Sodium Dodecyl Sulphate, SDS
BDH Chemicals, Australia

di-Sodium hydrogen orthophosphate
BDH Chemicals, Australia

Sodium Hydroxide, NaOH
Sigma-Aldrich Pty. Ltd., USA

Sigma-Aldrich Pty. Ltd., Australia

Sodium Malonate
Invitrogen, USA

Spectrophotometer (Biophotometer)
Fluka Chemika, Switzerland

Syringe (5 mL, 10 mL, 20 mL, 50 mL)
Greneir Bio-One, Germany

TA Cloning® Kit
Greneir Bio-One, Germany

Transilluminator (UV)
Bio-Rad laboratories, USA

Tris-Base
Roche Diagnostics, Germany

Tris-HCl
Roche Diagnostics, Germany

Triton-X 100
Sigma-Aldrich Pty. Ltd., USA

Trypan Blue
Sigma-Aldrich Pty. Ltd., USA

Trypsin EDTA (1:250)
Thermo Trace Ltd., Australia

Trypsin (4%)
Invitrogen (Gibco®), USA

Tryptone
Oxoid Australia Pty. Ltd.

Tweezers
Invitrogen, USA

UltraPure™ Water (RNase-/DNase-free)
Invitrogen, USA

Versene (1:5000, 0.53 mM EDTA)
Invitrogen, USA

Vortex mixer
Invitrogen (Gibco®), USA

Ratek Instruments, Australia

Whatman paper
Whatman, England

X-Gal
Sigma-Aldrich Pty. Ltd., USA

XLD Medium
Oxoid Australia Pty. Ltd.

Yeast Extract
Oxoid Australia Pty. Ltd.
Appendix II: Biochemical profiles of *Salmonella* Sofia and other bacteria isolates used in this study

Table II(A) Malonate and ONPG results of *Salmonella* spp.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Strain identification</th>
<th>ONPG*</th>
<th>Malonate**</th>
</tr>
</thead>
<tbody>
<tr>
<td>II Sofia</td>
<td>554NCA, 858V (colourless to light yellow)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>131</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>All others strains listed in Table 2.1 in Section 2.2.2.1</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Typhimurium</td>
<td>82/6915</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* (ONPG) +: colourless to bright yellow; -: no colour change  
** (Malonate) +: green to blue; -: no colour change

Table II(B) Serogroup profiles of S. II Sofia isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Strain identification</th>
<th>Serogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhimurium</td>
<td>82/6915</td>
<td>B</td>
</tr>
<tr>
<td>II Sofia</td>
<td>All strains listed in Table 2.1 in Section 2.2.2.1</td>
<td>B</td>
</tr>
</tbody>
</table>

Appendix III: PFGE λ ladder and size standards for *S. Braenderup* H9812
The size of the λ ladder starts at 48.5 kB and increases 48.5 kB with each successively larger band to approximately 1018.5 kB.

Approximate band sizes (in kB) of S. Braenderup H9812 digested with XbaI and subjected to PFGE (conditions described in Section 2.3.6.1) (Hunter et al., 2005):

**Appendix IV: DNA (PstI and HindIII, respectively) markers**
## Appendix V: Primer sequences and amplification conditions of PCR typing (see Section 3.2.3)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>PCR conditions</th>
<th>Amplification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>TTGTACACACCGCCCGTCA</td>
<td>1× PCR buffer, 3 mM MgCl₂, 1× Q solution, 200 µM dNTPs, 0.5 µM primer (each), 100 ng DNA and 2.5U Taq</td>
<td>35 cycles of 94°C (1 min), 55°C (1 min) and 72°C (1 min)</td>
<td>Lagatolla et al., 1996</td>
</tr>
<tr>
<td>P2</td>
<td>GGTACTTAGATGTTTCAGTTC</td>
<td>1× PCR buffer, 2.5 mM MgCl₂, 1× Q solution, 500 µM dNTPs, 0.5 µM primer, 50 ng DNA and 2.5U Taq</td>
<td>1 cycle of 95°C (7 min), 30 cycles of 90°C (30 s), 53°C (1 min) and 65°C (8 min), final extension of 65°C (16 min)</td>
<td>Koeuth et al., 1995</td>
</tr>
<tr>
<td>BOXA1R</td>
<td>CTACGGCAAGGCACGCTGACG</td>
<td>1× PCR buffer, 3.5 mM MgCl₂, 1× Q solution, 500 µM dNTPs, 1.0 µM primer, 100 ng DNA and 2.5U Taq</td>
<td>Same as above</td>
<td>Versalovic et al., 1991</td>
</tr>
<tr>
<td>ERIC2</td>
<td>AAGTAAGTGACTGGGGTGAGCG</td>
<td>1× PCR buffer, 3.0 mM MgCl₂, 1× Q solution, 500 µM dNTPs, 0.5 µM primer, 20 ng DNA and 2.5U Taq</td>
<td>1 cycle of 94°C (7 min), 35 cycles of 94°C (30 s), 46°C (1 min) and 68°C (8 min), final extension of 68°C (15 min)</td>
<td>Versalovic et al., 1991</td>
</tr>
</tbody>
</table>

* “I” in the REP1R oligonucleotide sequence represent inosine
### Appendix VI: Primers for amplification of DNA probes for Southern blot detection of SPI1 to SPI5 (see Section 4.2.1)

<table>
<thead>
<tr>
<th>Probes</th>
<th>Primer sequence*</th>
<th>PCR annealing temperature (°C)</th>
<th>PCR elongation time (min)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A (13.8 kB)</td>
<td>1A/F – CACGCTTAGCAGCATGGAATGG &lt;br&gt; 1A/R – CAGGAGGTTATGAGCAGTATC</td>
<td>53.0</td>
<td>13</td>
<td>This study</td>
</tr>
<tr>
<td>1B (15.1 kB)</td>
<td>1B/F – TCATCGTTCCTCTGAGAATG &lt;br&gt; 1B/R – GCCTCGCATTGCTGTTTCTAT</td>
<td>53.0</td>
<td>13</td>
<td>This study</td>
</tr>
<tr>
<td>1C (10.8 kB)</td>
<td>1C/F – GTCTCGGCTCTCTCTTCCATACTGACG &lt;br&gt; 1C/R – CTCTCATGAGCCAGCAAGTAACGCTCG</td>
<td>62.0</td>
<td>13</td>
<td>Amavisit et al., 2003</td>
</tr>
<tr>
<td>2A (13.6 kB)</td>
<td>2A/F – CGCAGTGGCCAGAAGAAGCATA &lt;br&gt; 2A/R – ATGCCTCAATGAGTGAGATTGC</td>
<td>62.0</td>
<td>13</td>
<td>This study</td>
</tr>
<tr>
<td>2B (13.5 kB)</td>
<td>2B/F – TACTTGGCCGTGACGGAATA &lt;br&gt; 2B/R – GGGCAGAAGCTGAAGTTGGA</td>
<td>60.0</td>
<td>13</td>
<td>This study</td>
</tr>
<tr>
<td>2C (12.9 kB)</td>
<td>2C/F – GCCTGCACGATCCGGTACTA &lt;br&gt; 2C/R – TCCAGGTCAGTGCCGATGTTT</td>
<td>63.0</td>
<td>13</td>
<td>This study</td>
</tr>
<tr>
<td>3 (17 kB)</td>
<td>3/F – ACACACCTTCCTCTCATGCCACCTATT &lt;br&gt; 3/R – ACAGACCATATCGCGCATATTTAAGCAA</td>
<td>62.0</td>
<td>13</td>
<td>Amavisit et al., 2003</td>
</tr>
<tr>
<td>4A (15.5 kB)</td>
<td>4A/F – AGGGCGGTAGGCTTCAGTT &lt;br&gt; 4A/R – ATATCGCTACGCTCCATG</td>
<td>60.0</td>
<td>13</td>
<td>This study</td>
</tr>
<tr>
<td>4B (8.2 kB)</td>
<td>4B/F – AGCGGTGATAGCGATGTAGA &lt;br&gt; 4B/R – CACCTGATAACAGCGACAAG</td>
<td>56.6</td>
<td>7</td>
<td>This study</td>
</tr>
<tr>
<td>5 (6.3 kB)</td>
<td>5/F – AATGGTGGCGTAGTACGACTT &lt;br&gt; 5/R – AAGTGTGGCCGGACCGTGATA</td>
<td>60.0</td>
<td>5</td>
<td>This study</td>
</tr>
<tr>
<td>sopB-pipA (4.5 kB)</td>
<td>sopB/F – CGGAATGCAGATTCCTTCAG &lt;br&gt; pipA/R – CCCGAAGGTTAGTCAGAGTT</td>
<td>57</td>
<td>4</td>
<td>This study</td>
</tr>
</tbody>
</table>

Primers for 1A, 1B and 1C were designed from SPI1 (GeneBank accession number NC_003197); 2A, 2B and 2C from SPI2 (GeneBank accession numbers X99944, Y09357, AJ224892, U51927, Z95891, AJ224978 and X99945); 4A and 4B from SPI4 (GeneBank accession number AF060869); and 5 and sopB-pipA from SPI5 (GeneBank accession number AE008747)

All DNA probes are amplified as described in Section 2.3.3.3

*Primer name, F: forward primer and R: reverse primer
### Appendix VII: PCR primers for analysis of SPI regions (see Sections 4.2.2 to 4.2.4)

#### Table VII(A) Primers for PCR analysis of SPI1 to SPI5 (see Sections 4.2.6 to 4.2.10)

<table>
<thead>
<tr>
<th>SPI</th>
<th>PCR product</th>
<th>Primer sequence**</th>
<th>Primer annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fhlA-sitD (3.8 kB)</td>
<td>fhlA/F – CCCTGCTGTCGCGTATGAAG sitD/R – GGGCATAATGCGATGTTG</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>sitD-avrA (1.8 kB)</td>
<td>sitD/F – ATTCCTTCGCGGCTTACTGTG avrA/R – CGGTGCAAGGACATCATGT</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>avrA-hilC (2.7 kB)</td>
<td>avrA/F – GTTGTGCGGCTTGGATGTTG hilC/R – GGAGTTCATGCGACGATCTG</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>avrA (0.7 kB)</td>
<td>avrA/F – GTTGTGCGGCTTGGATGTTG avrA/R – CGGTGCAAGGACATCATGT</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>sitD-sprB (2.3 kB)</td>
<td>sitD/F – ATTCCTTCGCGGCTTACTGTG sprB/R – CCGTGCGCTCGTCTAGTGATGCT</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>sprB-prgH (6.4 kB)*</td>
<td>sprB/F – AGCGATACGGGTAAGGTCTG prgH/R – TTGCTGACAGGCCGCAACT</td>
<td>60 (5 min)</td>
<td></td>
</tr>
<tr>
<td>prgH-hilA (4.6 kB)*</td>
<td>prgH/F – AGCCGAAGAACGGGTTACG hilA/R – GGTAAGGCAAGCGTACTATT</td>
<td>56 (4 min)</td>
<td></td>
</tr>
<tr>
<td>hila-iaqB (2.1 kB)</td>
<td>hila/F – GCTCAAACCGGCTGAGTATG iaqB/R – AGCCGGCTCCAGGATACAACC</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>iaqB-sipP (1.9 kB)*</td>
<td>iaqB/F – GATGCTTCACACGCTTACTTGG sipP/R – CGAGCCGACATTAGGGGATGT</td>
<td>60 (7 min)</td>
<td></td>
</tr>
<tr>
<td>sipP-spaR (3.7 kB)</td>
<td>sipP/F – GACCAACATTACCGGAACGTC spaR/R – GCCCTCTGTTAGGGTTATC</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>spaS-spaO (2.7 kB)</td>
<td>spaS/F – ACAGGTACGGCCACACCTTCTC spaO/R – GAGTCTGTAATTGGGGAAT</td>
<td>56</td>
<td></td>
</tr>
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</table>

1C (10.8 kB) | See Appendix VI

2A (13.6 kB) | See Appendix VI

#### Table VII(B) Primers for PCR analysis of SPI6 (see Section 4.2.11)

<table>
<thead>
<tr>
<th>SPI</th>
<th>PCR product</th>
<th>Primer sequence**</th>
<th>Primer annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssaQ-ssaO (1.0 kB)</td>
<td>2A/F6 – AGGTGGCGGAGGATAAATCT 2A/R8 – AATGGGGCCGGCCCACTTCACTC</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>ssaL (3.7 kB)</td>
<td>2A/F13 – TGCCCATCAAGGTGGGAGRAGC 2A/R3 – AAAAAACCAACCACTTCAAAAC</td>
<td>58</td>
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</tr>
</tbody>
</table>
| 2B (13.5 kB) | See Appendix VI

#### Table VII(C) Primers for PCR analysis of SPI7 (see Section 4.2.12)

<table>
<thead>
<tr>
<th>SPI</th>
<th>PCR product</th>
<th>Primer sequence**</th>
<th>Primer annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sugR-rhuM (3.9 kB)</td>
<td>3/F3 – ACATCAACCCGCACTTACC rhuM/R – TAAAGGCGGCTTGTTGCTTACAG</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>sra-cigR (1.6 kB)</td>
<td>3/F12 – TGTCAACCGGCTTATGCTGAG 3/R8 – GGTGTTGACCACCCATATTA</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>rmbA-sla (7.6 kB)</td>
<td>3/F3 – ACATCAACCCGCACTTACC 3/R9 – TGGCAATGGCCACATCAGCAG</td>
<td>58</td>
<td></td>
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</table>

#### Table VII(D) Primers for PCR analysis of SPI8 (see Section 4.2.13)

<table>
<thead>
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<th>SPI</th>
<th>PCR product</th>
<th>Primer sequence**</th>
<th>Primer annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>copR-orfX (4.0 kB)</td>
<td>copR/F – CTCAACCAAGGCCGATATGTA GCAAGTCATGGTGAATAGGAA</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>orfX-pipB (3.1 kB)</td>
<td>orfX/F – TCATCGGTCCGCTTATTGTGCC pipB/R – ATACCCGGTGGTGGAAGTGGTGC</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>pipC-pipA (2.7 kB)</td>
<td>pipC/F – ATGGCTAAGGTTGGTGCAAGGT pipA/R – CCGGAAAGGTGTTTCCAGAGGT</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

All PCR products ≤ 4.0 kB were amplified as described in Section 2.3.3.2

*The following PCR products were amplified as described in Section 2.3.3.3 (PCR elongation times indicated with the annealing temperature in parentheses)

** Primer name, F: forward primer and R: reverse primer
Table VII(B) Primers for sequencing analysis of SPI1 to SPI5 (see Section 4.2.3)

<table>
<thead>
<tr>
<th>SPI</th>
<th>PCR product</th>
<th>Primer sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPI1</td>
<td>fhlA-sitD</td>
<td>fhlA/F – CCCTGCTGTCGCCGATGATAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fhlA/F2 – ACCAGGCATCTGCTCTCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sitB/F – CCGAGTTTTTGGCAGTATACG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sitB/R – CATCCACGCAGGTAAAGGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sitB/R2 – GGAACCTTTAGCCCTACCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sitD/R – GGGCATAATCGAGTAGTG</td>
</tr>
<tr>
<td></td>
<td>sitD-sprB</td>
<td>sitD/F – ATTTCCCTGCGGCTTACTGTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sprB/R – CCGTGCGCTGCTACGATGCT</td>
</tr>
<tr>
<td></td>
<td>avrA</td>
<td>avrA/F – GTTTGCGCCTGAGTATG</td>
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<tr>
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<td></td>
<td>avrA/R – CCGTGCGAGGCTATCAGT</td>
</tr>
<tr>
<td></td>
<td>sprB-prgH</td>
<td>sprB/F – AGCCGATAGGCTGAGAGTCT</td>
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<td>hilC/F – CGATGAACTCCACAAATTGC</td>
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<tr>
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<td></td>
<td>hilC/R – GGAAGTTTCTCAGAGATCTG</td>
</tr>
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<td></td>
<td>orgC/R – TCTCCTGATCAGAGGTGCT</td>
</tr>
<tr>
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<td></td>
<td>orgC/R2 – AGCCGAAAACAGTCTGTTC</td>
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<tr>
<td></td>
<td></td>
<td>orgA/F – CCTTGCAGATGGGCTGCTCA</td>
</tr>
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<td></td>
<td>orgA/R – CCGCATGGCCGCTTAAGAAC</td>
</tr>
<tr>
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<td></td>
<td>prgK/F – AGCGGCGAAGACSGCTTTCA</td>
</tr>
<tr>
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<td></td>
<td>prgK/R – GACCCGAAACAGCTAATTAG</td>
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<td>prgJ/F – CCGTGATCCAGCGGACAA</td>
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<td>prgH/F2 – AGGCCAGAAAACGGGTATTAG</td>
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<tr>
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<td></td>
<td>prgH/R – TTTCGACAGGCGAAACTC</td>
</tr>
<tr>
<td></td>
<td>prgH-hilA</td>
<td>prgH/F – AGCCGAAACGCGGTATTAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hilD/F – TTTCGACAGGCGAAACTC</td>
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<tr>
<td></td>
<td></td>
<td>hilD/R – GGAAGTTTCTCAGAGATCTG</td>
</tr>
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<td></td>
<td></td>
<td>hilD/R2 – AGCCGAGGCTATCAGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hilD/R3 – GGCGGCACTGCGGAAACTC</td>
</tr>
<tr>
<td></td>
<td>hila-lagB</td>
<td>hila/F – GCTCAACCTGACCGTTAGTA</td>
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<tr>
<td></td>
<td></td>
<td>hila/R – AGGCCGCACTCAGGAAACTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lagR/B – AGCGGCCGCTCCAGAACACT</td>
</tr>
<tr>
<td></td>
<td>lagB-sipB</td>
<td>lagB/F – GATGGTTCAACGGAGCTCGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lagB/F2 – ATGGCCCGGACGCTACCCGA</td>
</tr>
<tr>
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<td></td>
<td>lagB/F3 – AGCCAAAAGTTTCGCGCCTTC</td>
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<tr>
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<td></td>
<td>sipT/F – CGTATCGTCTCCAGATTACT</td>
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<td></td>
<td>sipT/R – AACCGCGGACGACCGATG</td>
</tr>
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<td></td>
<td>sipT/R2 – GCCGATCCAGACCGGTATC</td>
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<td></td>
<td></td>
<td>sicP/F – TGGGGCATCTCCAGAACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sicP/R – TACCCGTCACTTTTGGACG</td>
</tr>
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<td></td>
<td>iacP/F – CAGGCGTAAAGATCCTCACCC</td>
</tr>
<tr>
<td></td>
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<td>iacP/R – GACGCGACAAAGATCCTCACCC</td>
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<td>sipA/F – CGGCGGATGTTGTGAATTG</td>
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<td></td>
<td>sipA/R – GACGCGGATGTTGTGAATTG</td>
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<td></td>
<td>sipC/F – CAGGACCGCATTAAACTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sipC/R – CTGTGCGCTGCTGCTGCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sipC/F2 – CTGTGCGCTGCTGCTGCTG</td>
</tr>
<tr>
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<td></td>
<td>sipC/R2 – CAGGACCGCATTAAACTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sipB/F – GACGGCATTACGGCAGTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sipB/F2 – CTCAATACGTCCGCTCCATA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spaS/F – ACAGGGTACGCAACCTCCTC</td>
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<td></td>
<td>spaS/R – GGGGCAACGCGGCTACCAAAG</td>
</tr>
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<td></td>
<td></td>
<td>spaR/R – GCCGCCGCTTCAAATGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spaR/R2 – AAACCCGCTGCTGCAGATG</td>
</tr>
</tbody>
</table>
### APPENDICES

<table>
<thead>
<tr>
<th>Region</th>
<th>Oligonucleotides</th>
</tr>
</thead>
</table>
| **spas-spao**| spaS/F – ACAGGTACGCCAACCTTCTC  
spaS/F – ACAGGTACGCCAACCTTCTC  
spaQ/F – CGCCCGTAAAGAGAAACAAAC  
spaO/R – GAGTCTGGTAAATGGGGGAAAT |
| **1C**       | 1C/F – GTCTCGGCTCCTTCTCCATACTGACG  
1C/F2 – TGCTCAGCCAGTCCCTGTT  
1C/F3 – GGCTCTCGCAAGAGATCAAAC  
1C/F4 – ATTCGCCCCGTAATGTCCTCG  
1C/F5 – GCACGTCCAGTGCAGGATAAAC  
1C/F6 – GAGGAAGTACTGCGCAAGG  
1C/F7 – GGCCTACGTCTTTGTCCAC  
1C/R – CTTCATGGGCCAGCAAGTAGCTGCTG  
1C/R2 – AGAGAAAGGCCCAACGC9AA  
1C/R3 – TACAACTGGGCTAGTTTTGA  
1C/R4 – AGATACCCCTGCAGGCAAGTC  
1C/R5 – GTGCGCGCAATTTCCTGTACC |
| **2A**       | 2A/F – CGCGATGCGCTGAAGAAGCATA  
2A/F2 – ACAGTACGCTACCGCCGAAG  
2A/F3 – CAATCTGTTGTCGGACCGAT  
2A/F4 – AGGGAACCGCGCAAAACAC  
2A/F5 – GCTTCTACGTCCTCCTCAGA  
2A/F6 – AGGTGTTGGCCAGGATAATCT  
2A/F7 – ACCCAAGGCTTCCCTCAACT  
2A/F8 – GCCAACGGGCTCATTACATAC  
2A/F9 – CACTCTCCTTGCGCTAAGCTG  
2A/F10 – CTGCTCACCCCAACCCACTAGC  
2A/F11 – CGGCAATCGATGTTCTCTTTA  
2A/F12 – GAACGCATTTGGGCAGTACAG  
2A/F13 – TGCGGATCAAGTTGGGAGAAG  
2A/R – ATCGCTCATGGAGTGAGATCG  
2A/R2 – GCCCAATGGCCCCAGTGAAG  
2A/R3 – AAAAGTGCGCCGGAGTAATC  
2A/R4 – TTTCCGCGACGCTAGTTGAG  
2A/R5 – TCAAACTGCTGCGCTATGG  
2A/R6 – ATCCGAGACCGCTATCGTTA  
2A/R7 – GAAATAGGAATGCGCTGAG |
| **ssaQ-ssaO**| 2A/F6 – AGGTGTTGGCCAGGATAATCT  |
| **SPI2**     | ssaG-ssaL (3.7 kB)  
2A/R3 – AAAAGTGCGCCGGAGTAATC  
2A/R5 – TCAAACTGCTGCGCTATGG  |
|              | ssaQ-ssaO (1.0 kB)  
2A/F6 – AGGTGTTGGCCAGGATAATCT  |
| **2B**       | 2B/F – TACTTTGCAGCTGACGGAAATA  
2B/F2 – CGCGGAAAGTCCCGAAGGAAG  
2B/F3 – GGCSCGCCCGAGCATACAT  
2B/F4 – CCGGCGCTTCCGCTCATATT  
2B/F5 – CGCGCGTATCGCTATTACCTG  
2B/F6 – CACGGTCAGCCGTGCTCTTTG  
2B/F7 – GATTGCTGGCAGATCTGT  
2B/F8 – ATTTGCCCGGCACAATAAGA  
2B/F9 – CCCGTAGTGATGAGTAGGATCAAG  
2B/R – GGGCGCGAAGCTGAAAGTTGTA  
2B/R2 – TTTCGCGGCCGACGTAGCCT  
2B/R3 – TGTACACCTTTTGCCTGATCC  
2B/R4 – ATTCGCCCCGTCAGGTAATAAGG  
2B/R5 – GCCAGGGTCACTAGGGATTCC  
2B/R6 – TCACAGAAACCGCATACAGG  
2B/R7 – GGCCTGCAATTTGGGAGGACC  
2B/R8 – GGATACATGCCTGCGACTT  
2B/R9 – GCAGATATCCCCGCGTATTAT  |
|              | sseB-ssaB (3.9 kB)  
2B/F6 – CACGGTCAGCCGTGCTCTTTG  
2B/R8 – GGGATACATGCCTGCGACTT  |
| **2C**       | 2C/F – GGCGGAGTCCGGGTATCA  
2C/F2 – CGCGGAATTACGCGAGGTAAG  
2C/F3 – CACATCTCGCGGTACAGTTC  |
<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
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<tbody>
<tr>
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<td>GCGTTAGTGACCGCCGTATC</td>
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<td>2C/F5</td>
<td>CCAGCAGCATGACCCAAATA</td>
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<td>2C/F6</td>
<td>ACCCGGTAAACCCGGAGATGA</td>
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<td>2C/F7</td>
<td>TCACAACAAACGCGGGTGATG</td>
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<tr>
<td>2C/F8</td>
<td>GTACAGGCGTGGCTCTTACGA</td>
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<tr>
<td>2C/F9</td>
<td>CGCTCTACAGGGATTCTCTGG</td>
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<td>2C/F10</td>
<td>ACCCTGGTTCTGAGAAGACAC</td>
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<td>2C/F11</td>
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<tr>
<td>2C/F12</td>
<td>CGCCTTAAGCCGTGATTTC</td>
</tr>
<tr>
<td>2C/R</td>
<td>TCCAGTGCGATGGCGATGTT</td>
</tr>
<tr>
<td>2C/R2</td>
<td>GCAGCAAGCTGAGCCGGATAG</td>
</tr>
<tr>
<td>2C/R3a</td>
<td>GAGGAGACTGCGCGGATAG</td>
</tr>
<tr>
<td>2C/R3b</td>
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<td>2C/R4</td>
<td>GAACATGAACGGCTTCC</td>
</tr>
<tr>
<td>2C/R5</td>
<td>ATCAAAGCGCCAGAAGGCG</td>
</tr>
<tr>
<td>2C/R6</td>
<td>CCGCCCTCAGCGCAACTATAC</td>
</tr>
<tr>
<td>2C/R7</td>
<td>AGCGGAGGGCATATTGATAG</td>
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<tr>
<td>2C/R8</td>
<td>GCCTAGAATCAGGAAAGATAG</td>
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<tr>
<td>3/F</td>
<td>ACATCCTCTCTCTCTATGCACCCACTT</td>
</tr>
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<td>3/F2</td>
<td>CCTGCCGCAAAGAAGGGGATAA</td>
</tr>
<tr>
<td>3/F3</td>
<td>ACATCACACGCGATGCTTACC</td>
</tr>
<tr>
<td>3/F4</td>
<td>TCACTACATCCACCCCGTAC</td>
</tr>
<tr>
<td>3/F5</td>
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</tr>
<tr>
<td>3/F6</td>
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<td>3/F7</td>
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<tr>
<td>3/F8</td>
<td>GGTACAGCTCGTACCTAG</td>
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<tr>
<td>3/F9</td>
<td>GCAGCCGTCATTTTCTCTGGA</td>
</tr>
<tr>
<td>3/F10</td>
<td>GAGATCTTGCGCCATATAA</td>
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<td>3/F11</td>
<td>AGCAGACACTGTGATGAG</td>
</tr>
<tr>
<td>3/F12</td>
<td>TTGCAAGCGACCGGAAATCG</td>
</tr>
<tr>
<td>3/F13</td>
<td>CCAAGTGCAGAGCGCCCTTGG</td>
</tr>
<tr>
<td>3/F14</td>
<td>CAGCTACGCCTGTGACTCC</td>
</tr>
<tr>
<td>3/R</td>
<td>ACAGAGCTCATCGGCCGGGATATAGCA</td>
</tr>
<tr>
<td>3/R2</td>
<td>TTACGTGCGCGCTACCTGG</td>
</tr>
<tr>
<td>3/R3</td>
<td>TGCGAGAAACACACACCC</td>
</tr>
<tr>
<td>3/R4</td>
<td>TCCGCTCGTCCGCGGAATCC</td>
</tr>
<tr>
<td>3/R5</td>
<td>TTTGTACTGCGCCGGGTATC</td>
</tr>
<tr>
<td>3/R6</td>
<td>CTCACTGTGCTTGAGGAAG</td>
</tr>
<tr>
<td>3/R7</td>
<td>TGTTATCCACATCGAAGG</td>
</tr>
<tr>
<td>3/R8</td>
<td>GTGTTGTACAGCGGCGATTATTA</td>
</tr>
<tr>
<td>3/F5</td>
<td>TTTGTACGTGCGCGCGGTATC</td>
</tr>
<tr>
<td>3/F7</td>
<td>CAGGTCTCCGGCAACTCTTCC</td>
</tr>
<tr>
<td>3/F10</td>
<td>GAGATCTCGCGCTGATAA</td>
</tr>
<tr>
<td>mil/F</td>
<td>TCTGCTACCAGTACACTACC</td>
</tr>
<tr>
<td>4A/F</td>
<td>AGGGCGGTAGCGCTTCACCTT</td>
</tr>
<tr>
<td>4A/F2</td>
<td>GTGTCTACCGCGGGTACAA</td>
</tr>
<tr>
<td>4A/F3</td>
<td>GCAGACGTGGAGGCAATAG</td>
</tr>
<tr>
<td>4A/F4</td>
<td>TAGTGCTCAGAGGGTGTG</td>
</tr>
<tr>
<td>4A/F5</td>
<td>GAGCAGAGGTTACCTAGAG</td>
</tr>
<tr>
<td>4A/F6</td>
<td>CTCTAGTGTGTGACGTGACGT</td>
</tr>
<tr>
<td>4A/F7</td>
<td>ACCCGGCTCTAAAAATCTCGG</td>
</tr>
<tr>
<td>4A/F8</td>
<td>AGGGCTTAACGCGGACCTTG</td>
</tr>
<tr>
<td>4A/F9</td>
<td>TTCGCGAGCTTCTTCTGGAG</td>
</tr>
<tr>
<td>4A/F10</td>
<td>GATGCGTCTACGACGTAAAC</td>
</tr>
<tr>
<td>4A/F11</td>
<td>AGCAGCAACACTGATGTTTC</td>
</tr>
<tr>
<td>4A/F12</td>
<td>TGTCAGAGCCGGGTATACGT</td>
</tr>
<tr>
<td>4A/F13</td>
<td>GACAAAGCGGGGAACACCC</td>
</tr>
<tr>
<td>4A/R</td>
<td>ATATCCACGGGCGCATCCCT</td>
</tr>
<tr>
<td>4A/R2</td>
<td>CAGCGGGAACCTGCAATTCTG</td>
</tr>
<tr>
<td>4A/R3</td>
<td>TGCTGCTCCTCCTCGCATATC</td>
</tr>
<tr>
<td>4A/R4</td>
<td>CGTTATCCGTTACTCTAC</td>
</tr>
</tbody>
</table>
### APPENDICES

<table>
<thead>
<tr>
<th>SPI</th>
<th>PCR product</th>
<th>Primer sequence*</th>
<th>PCR annealing temperature (°C)</th>
</tr>
</thead>
</table>
| SPI1 | avrA (0.67 kB) | avrA/F – GGCGGATCCGGCCGAGTGTA 
avrA/R – ACCTCACGCGGGGAGCTAAC | 61 |
|      | sipD (0.79 kB) | sipD/F – TGCCGGACGTCGCTGTATAGT 
sipD/R – ATATACGGCACGGCGACGTCTAG | 58 |
|      | sptP (0.95 kB) | sptP/F – GGTACCTGCTCCACCGAGTCTC 
sptP/R – CGGAGAAACTTCAGCGACGAGA | 56 |
| SPI3 | marT (0.42 kB) | marT/F – TGCTGGAAGGGGAAATAGG 
marT/R – ACCTAACGGCGGGAAATG | 57 |
|      | slsA (0.54 kB) | slsA/F – ACGGCCTGGCCCATTGATC 
slsA/R – ACGTACGCTGGCACGCTCAG | 55 |

All RT-PCR reactions were carried out as described in Section 2.5
* Primer name, F: forward primer and R: reverse primer
**Appendix VIII:** Analysis of SPI1 to SPI5 genes of S. II Sofia Bt8 and their predicted protein sequences

<table>
<thead>
<tr>
<th>SPI</th>
<th>Gene</th>
<th>Analysis of the genes and predicted proteins from S. II Sofia Bt8 (using Clone Manager 7) in comparison to S. Typhimurium LT2 SPI genes and proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sitA-invH</td>
<td>SPI1 from LT2 (base 1 to 39600)</td>
</tr>
<tr>
<td></td>
<td>sitA</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (98-99% sequence similarity)</td>
</tr>
<tr>
<td></td>
<td>sitB</td>
<td>Missing from this region (confirmed by Southern hybridisation, PCR and sequencing analysis). Replaced with a 200 bp sequence harbouring an ORF encoding a protein of unknown function; sharing 98% sequence similarity with the 200 bp genetic element located in the sitD-sprB region of SPI1 from S. Typhi (GeneBank accession no. AF013575) and S. Choleraesuis (GeneBank accession no. AF013574)</td>
</tr>
<tr>
<td></td>
<td>sitC</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 94%, 95% and 92% sequence similarity, respectively)</td>
</tr>
<tr>
<td></td>
<td>sitD</td>
<td>Addition of ATCGTAAAAT at base 7149 to 7158 (between base 8257 and 8258 in SPI1 of LT2). Translated protein (98% match to OrgB from LT2) is missing 3 amino acids from the end of the protein: V, I and R</td>
</tr>
<tr>
<td></td>
<td>avrA</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 94%, 95% and 92% sequence similarity, respectively)</td>
</tr>
<tr>
<td></td>
<td>sprB</td>
<td>Addition of CCA at base 9670 to 9672 (between base 11038 and 11039 in SPI1 of LT2). Translated protein has an extra threonine (T) residue at position 3: PrgI (LT2): MA_TPW------QNFR PrgI (Bt8): MPTTPW------QNFR (87% sequence similarity)</td>
</tr>
<tr>
<td></td>
<td>hilC</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97%, 93%, 95% and 93% sequence similarity, respectively)</td>
</tr>
<tr>
<td></td>
<td>orgC</td>
<td>Gene is 1.2 kB and translated protein is 400-amino acids in length, showing sequence similarity to transposase from the mutator family of proteins (Figure 4.6). These include transposase from S. Welterveden (ZP_02834589), Yersinia enterocolitica (AAO39032), Bacteroides thetaiotaomicron (NP_809193), Rickettsiella grylli (ZP_02061857), E. coli (NP_757024) and Shewanella spp. (NP_717888, ZP_0170506, YP_563181 and YP_001557092). This ORF is not found in any other Salmonella serovar, including Typhimurium (Figure 4.7)</td>
</tr>
<tr>
<td></td>
<td>orgB</td>
<td>Additions of ATCGTAAAAT at base 7149 to 7158 (between base 8257 and 8258 in SPI1 of LT2). Translated protein (98% match to OrgB from LT2) is missing 3 amino acids from the end of the protein: V, I and R</td>
</tr>
<tr>
<td></td>
<td>orgA</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 93%, 99% and 98% sequence similarity, respectively)</td>
</tr>
<tr>
<td></td>
<td>prgK</td>
<td>Addition of CCA at base 9670 to 9672 (between base 11038 and 11039 in SPI1 of LT2). Translated protein has an extra threonine (T) residue at position 3: PrgI (LT2): MA_TPW------QNFR PrgI (Bt8): MPTTPW------QNFR (87% sequence similarity)</td>
</tr>
<tr>
<td></td>
<td>prgJ</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97%, 93%, 95% and 93% sequence similarity, respectively)</td>
</tr>
<tr>
<td></td>
<td>prgI</td>
<td>Change of T to C, which altered start codon of sptP (ATG to ACG). If translated, predicted protein is missing the first 32 amino acid residues</td>
</tr>
<tr>
<td>IS</td>
<td>sptP</td>
<td>Multiple changes in the gene sequence. A majority of the changes are base substitutions. Insertion of TGG at base 17858 to 17860 (between base 17928 and 17929 in SPI1 of LT2). Deletion of 39 bp sequence between base 17929 and 17930 (corresponds to base 17997 to 18035 in SPI1 of LT2). Change of T to C, which altered start codon of sptP (ATG to ACG). If translated, predicted protein is missing the first 32 amino acid residues</td>
</tr>
</tbody>
</table>
and starts with valine (V) instead of methionine (M) – see Figure 4.8

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>sicP</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 85%, 90% and 79% sequence similarity, respectively)</td>
</tr>
<tr>
<td>iacP</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 85%, 90% and 79% sequence similarity, respectively)</td>
</tr>
<tr>
<td>sipA</td>
<td>Multiple changes in the gene (Figure 4.9), resulting in nonsense mutation and creating a termination codon in the middle of the gene. SipD is truncated after translation of 260 amino acids.</td>
</tr>
<tr>
<td>sipD</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 91%, 97% and 99% sequence similarity, respectively)</td>
</tr>
<tr>
<td>sipC</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 91%, 97% and 99% sequence similarity, respectively)</td>
</tr>
<tr>
<td>sipB</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 98% sequence similarity)</td>
</tr>
<tr>
<td>sicA</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 98% sequence similarity)</td>
</tr>
<tr>
<td>spaS</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 98% sequence similarity)</td>
</tr>
<tr>
<td>spaQ</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100% sequence similarity)</td>
</tr>
<tr>
<td>spaP</td>
<td>Translated protein is identical to InvI from LT2</td>
</tr>
<tr>
<td>spaO</td>
<td>Change of A to G at base 30866 (corresponds to base 30980 in SPI1 of LT2), which altered the start codon of invJ (ATG to GTG). Translated protein starts with valine (V) instead of methionine (M)</td>
</tr>
<tr>
<td>invJ/spaN</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 99% and 97% sequence similarity, respectively)</td>
</tr>
<tr>
<td>invI/spaM</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 99% and 97% sequence similarity, respectively)</td>
</tr>
<tr>
<td>invC</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 99% and 97% sequence similarity, respectively)</td>
</tr>
<tr>
<td>invB</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 99% and 97% sequence similarity, respectively)</td>
</tr>
<tr>
<td>invA</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 99% sequence similarity)</td>
</tr>
<tr>
<td>invE</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 99% sequence similarity)</td>
</tr>
<tr>
<td>invG</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 99% sequence similarity)</td>
</tr>
<tr>
<td>invF</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 99% sequence similarity)</td>
</tr>
<tr>
<td>invH</td>
<td>Deletion of CCT between base 3999 and 4000 (corresponds to base 5998 to 6000 in SPI1 of LT2). Translated protein is missing proline (P) residue at position 46 (SsaP of LT2 is 124 amino acids in length)</td>
</tr>
<tr>
<td>ssaUorf48</td>
<td>SPI2 from LT2 (base 1 to 41744) SPI2 from Bt8 (base 1 to 39513)</td>
</tr>
<tr>
<td>ssaU</td>
<td>Deletion of CCT between base 3999 and 4000 (corresponds to base 5998 to 6000 in SPI1 of LT2). Translated protein is missing proline (P) residue at position 46 (SsaP of LT2 is 124 amino acids in length)</td>
</tr>
<tr>
<td>ssaT</td>
<td>Deletion of CCT between base 3999 and 4000 (corresponds to base 5998 to 6000 in SPI1 of LT2). Translated protein is missing proline (P) residue at position 46 (SsaP of LT2 is 124 amino acids in length)</td>
</tr>
<tr>
<td>ssaS</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100%, 98% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaR</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100%, 98% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaQ</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100%, 98% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaP</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100%, 98% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaO</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100%, 98% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaN</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100%, 98% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaV</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100%, 98% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaM</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100%, 98% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaL</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100%, 98% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaK</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 100%, 98% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>putative</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97% and 96% sequence similarity, respectively)</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>ssaJ</td>
<td>Addition of ACA at base 11446 and 11448 (between base 13446 and 13447 in SPI2 of LT2). Translated protein has extra arginine (R) residue at position 3 (SsaJ of LT2 is 82 amino acids in length)</td>
</tr>
<tr>
<td>ssaI</td>
<td>SsaI from LT2 and Bt8 share 75% sequence similarity</td>
</tr>
<tr>
<td>ssaH</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97%, 96% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaG</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 94%, 95% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>sseF</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 90%, 96%, 97% and 91% sequence similarity, respectively)</td>
</tr>
<tr>
<td>sscB</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 83%, 92%, 94% and 93% sequence similarity, respectively)</td>
</tr>
<tr>
<td>sseE</td>
<td>Missing approximately 234 bp at the junction between sseA and ssaE. Change from T to C at base 17939 (corresponds to base 20149 in SPI2 of LT2), which altered the stop codon of ssaE (TAA to CAA). Translated protein contains 5 extra residues at the end of the polypeptide chain (QKSLS)</td>
</tr>
<tr>
<td>ssaD</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 95%, 96%, and 94% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaC</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 91%, 97% and 95% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssaB</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 91%, 97% and 95% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssrA</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 91%, 97% and 95% sequence similarity, respectively)</td>
</tr>
<tr>
<td>ssrB</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 91%, 97% and 95% sequence similarity, respectively)</td>
</tr>
<tr>
<td>orf242</td>
<td>Translated protein is the same length as Orf242 from LT2 (showing 97% sequence similarity)</td>
</tr>
<tr>
<td>orf319</td>
<td>Translated protein is the same length as Orf319 from LT2 (showing 97% sequence similarity)</td>
</tr>
<tr>
<td>orf70</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 98% sequence similarity)</td>
</tr>
<tr>
<td>trrS</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 96%, 99% and 98% sequence similarity, respectively)</td>
</tr>
<tr>
<td>trrB</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 96%, 99% and 98% sequence similarity, respectively)</td>
</tr>
<tr>
<td>trrC</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 96%, 99% and 98% sequence similarity, respectively)</td>
</tr>
<tr>
<td>trrA</td>
<td>Translated protein is the same length as TrrA from LT2 (showing 97% sequence similarity)</td>
</tr>
<tr>
<td>orf408</td>
<td>Translated protein is the same length as Orf408 from LT2 (showing 96% sequence similarity)</td>
</tr>
<tr>
<td>orf245</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 98% sequence similarity)</td>
</tr>
<tr>
<td>orf32</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 98% sequence similarity)</td>
</tr>
<tr>
<td>orf48</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 98% sequence similarity)</td>
</tr>
<tr>
<td>sugR-mgtC</td>
<td>SPI3 from LT2 (base 1 to 17039) SPI3 from Bt8 (base 1 to 14387)</td>
</tr>
<tr>
<td>sugR and rhuM</td>
<td>Sequence in this region was found to harbour an ORF showing 98% homology to gene remnant encoding putative IS1351 transposase pseudogene from S. Typhi CT18 (GeneBank accession no. AL627280)</td>
</tr>
<tr>
<td>rmbA</td>
<td>Translated protein is the same length as RmbA from LT2 (showing 95% sequence similarity)</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>misL</td>
<td>Deletion of 15 bp sequence between base 4051 and 4052 (corresponds to base 7829 to 7845 in SPI3 of LT2), causing a frameshift in translation of misL. Translated protein is missing 5 residues (VPVPD) from position 578 and 582 (MisL of LT2 is 955 amino acids in length). MisL from LT2 and Bt8 share 97% sequence similarity.</td>
</tr>
<tr>
<td>fidL</td>
<td>Translated protein is the same length as FidL from LT2 (showing 96% sequence similarity).</td>
</tr>
<tr>
<td>marT</td>
<td>Deletion of 10 bp sequence at base 6585 to 6593 (between base 10380 and 10381 in SPI3 of LT2), causing a frameshift in translation of marT. Translated protein is missing the first 23 amino acids (MSCYAATASDYTQWCLLTLSNYV). MarT from LT2 and Bt8 share 90% sequence similarity.</td>
</tr>
<tr>
<td>slsA</td>
<td>Insertion of IS element from base 8091 to 9392 (between base 11861 and 11862 in SPI3 of LT2). As this ORF is from base 7420 to 9402, insertion of IS causes a frameshift in translation of slsA. Translated protein has 25 extra residues at the end of the polypeptide chain.</td>
</tr>
<tr>
<td>IS</td>
<td>Gene is 1.2 kB and translated protein is 400-amino acids in length and identical to the IS protein from SPI1. ORF is not found in any other Salmonella serovar, including Typhimurium (Figure 4.15).</td>
</tr>
<tr>
<td>cigR</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 98%, 96% and 97% sequence similarity, respectively).</td>
</tr>
<tr>
<td>mgtB</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 98%, 96% and 97% sequence similarity, respectively).</td>
</tr>
<tr>
<td>mgtC</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97%, 91% and 98% sequence similarity, respectively).</td>
</tr>
<tr>
<td>siiABCDEF</td>
<td>SPI4 from LT2 (base 1 to 23411) SPI4 from Bt8 (base 1 to 23460).</td>
</tr>
<tr>
<td>siiA</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 96%, 95% and 98% sequence similarity, respectively).</td>
</tr>
<tr>
<td>siiB</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97%, 91% and 98% sequence similarity, respectively).</td>
</tr>
<tr>
<td>siiC</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97%, 95% and 97% sequence similarity, respectively).</td>
</tr>
<tr>
<td>siiD</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97%, 91% and 98% sequence similarity, respectively).</td>
</tr>
<tr>
<td>siiE</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97%, 91% and 98% sequence similarity, respectively).</td>
</tr>
<tr>
<td>siiF</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97%, 91% and 98% sequence similarity, respectively).</td>
</tr>
<tr>
<td>copR-pipA</td>
<td>SPI5 from LT2 (base 1 to 9069) SPI5 from Bt8 (base 1 to 3941).</td>
</tr>
<tr>
<td>copR</td>
<td>Translated proteins are the same length as the corresponding proteins from LT2 (showing 97% and 95% sequence similarity, respectively).</td>
</tr>
<tr>
<td>copS</td>
<td>Translated protein is the same length as SipD from LT2 (showing 97% sequence similarity).</td>
</tr>
<tr>
<td>pipD</td>
<td>Change of G to T at base 3698 (corresponds to base 3864 in SPI5 of LT2), which altered the start codon of protein (ATG to ATT). Protein is probably not translated.</td>
</tr>
<tr>
<td>ORF</td>
<td>Translated protein is identical as that from LT2.</td>
</tr>
<tr>
<td>sopB, pipC, putative, pipB and pipA</td>
<td>Missing from this region (confirmed by Southern hybridisation).</td>
</tr>
</tbody>
</table>
**Appendix IX: Single- and three-letter codes for amino acid residues**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Three-letter code</th>
<th>Single-letter code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine (ATG)</td>
<td>Met</td>
<td>M</td>
</tr>
<tr>
<td>Glycine (GGT, GGC, GGA, GGG)</td>
<td>Gly</td>
<td>G</td>
</tr>
<tr>
<td>Alanine (GCT, GCC, GCA, GCG)</td>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td>Leucine (TTA, TTG, CTT, CTC, CTA, CTG)</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td>Phenylalanine (TTT, TTC)</td>
<td>Phe</td>
<td>F</td>
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<tr>
<td>Tryptophan (TGG)</td>
<td>Trp</td>
<td>W</td>
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<tr>
<td>Lysine (AAA, AAG)</td>
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<td>K</td>
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<tr>
<td>Glutamine (CAA, CAG)</td>
<td>Gln</td>
<td>Q</td>
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<tr>
<td>Glutamic Acid (GAA, GAG)</td>
<td>Glu</td>
<td>E</td>
</tr>
<tr>
<td>Serine (TCT, TCC, TCA, TCG)</td>
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</tr>
<tr>
<td>Proline (CCT, CCC, CCA, CCG)</td>
<td>Pro</td>
<td>P</td>
</tr>
<tr>
<td>Valine (GTT, GTC, GTA, GTG)</td>
<td>Val</td>
<td>V</td>
</tr>
<tr>
<td>Isoleucine (ATT, ATC, ATA)</td>
<td>Ile</td>
<td>I</td>
</tr>
<tr>
<td>Cysteine (TGT, TGC)</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td>Tyrosine (TAT, TAC)</td>
<td>Tyr</td>
<td>Y</td>
</tr>
<tr>
<td>Histidine (CAT, CAC)</td>
<td>His</td>
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</tr>
<tr>
<td>Arginine (AGA, AGG)</td>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td>Asparagine (AAT, AAC)</td>
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<td>N</td>
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<tr>
<td>Aspartic Acid (GAT, GAC)</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>Threonine (ACT, ACC, ACA, ACG)</td>
<td>Thr</td>
<td>T</td>
</tr>
<tr>
<td>Termination (TAA, TAG, TGA)</td>
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<td>-</td>
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</table>
Appendix X: Cloning vectors used in this study (see Chapter 5)
**Appendix XI:** Formula for calculation of the required amount of tissue culture cells per well (see Section 2.4.3.3)

Percentage of viable cells = \( \frac{\text{Average number of viable cells}}{\text{Total number of cells}} \) \times 100

Total cell number per mL = Average number of viable cells \times \text{dilution factor} \times 10^4

Volume that contains \( 3 \times 10^5 \) cells (A) = \( \frac{3 \times 10^5 \text{ cells}}{\text{Total number of cells per mL}} \) \times 1 mL

Total volume required to seed \( n \) wells (B) = A \times (n + 1)

Make up volume B to a total volume \( (0.5 \text{ mL} \times [n + 1]) \) with DMEM media.
**Appendix XII:** Primers for RT-PCR analysis of *katF* and *spvRABCD* in clone pCspv8 and pWSPV1 (see Section 5.2.4)

<table>
<thead>
<tr>
<th>PCR product</th>
<th>Primer sequence*</th>
<th>PCR annealing temperature (°C)</th>
</tr>
</thead>
</table>
| *katF* (0.7 kB) | katF/F – TGATAACGACCTGGCTGAAG  
katF/R – CATATCCCAGCAGACCGAAA | 56 |
| *spvR* (0.48 kB) | spvR/F – GGACCTACGGGTAAACGAA  
spvR/R – TCTGACGGGGATGAGTAACA | 55 |
| *spvA* (0.6 kB) | spvA/F – ATTTGTCCGTCAGACCGTAA  
spvA/R – CTAACTGTGGGGCAAAAGGTA | 55 |
| *spvB* (0.86 kB) | spvB/F – GCGCAATGGCTGGTGTAGGA  
spvB/R – TCAGGCCCCGGTATACGACT | 61 |
| *spvC* (0.63kB) | spvC/F – GGGGCGGAAATACCATCTAC  
spvC/R – CCTCACTTAACGCTGTCGCT | 57 |
| *spvD* (0.61 kB) | spvD/F – TTTCTGTATGCGGTGTCATCC  
spvD/R – CCCCTGATGATGAGAAGTGT | 55 |

All RT-PCR reactions were carried out as described in Section 2.5

* Primer name, F: forward primer and R: reverse prime