Analysis of the role of fimbriae in the virulence of *Salmonella enterica* in poultry

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By

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Abstract

Salmonella is a Gram-negative bacterium that consists of two species; *S. enterica* and *S. bongori.* The species *S. enterica* can be further divided into 6 subspecies and subspecies I is predominantly associated with disease in warm blooded animals and contains over 2,500 antigenically distinct serovars. Each serovar is >90% identical at the DNA level but can infect a different range of hosts and cause different diseases. Poultry are an important reservoir of entry of *Salmonella* into the human food chain owing to the contamination of their eggs and meat. The molecular mechanisms underlying colonisation of food producing animals with *Salmonella* are unknown. Fimbrial genes encode proteinaceous surface exposed appendages which have been shown to mediate adhesion of bacterial cells but the precise role for fimbriae in the carriage and virulence of *Salmonella* is poorly defined.

The purpose of this study was to annotate and characterise the fimbrial genes of the poultry-associated *S. enterica* serovars Enteritidis and Gallinarum and relate this role to host-specificity. The availability of the genome sequences of several strains of *S. enterica* allowed a comparison of the sequence, location and repertoire of fimbrial genes and although no unique fimbrial genes were identified all serovars possessed a unique repertoire. The host-specific serovars contain a higher number of pseudogenes within fimbrial operons than the ubiquitous serovars and the rate of attrition of fimbrial genes was 3-4 fold higher than the genomic mean. Such gene decay may partially explain the narrowing of host-range of the host-restricted and host-specific serovars. Polymorphisms that may alter transcription were identified along with targets that may be associated with phase variation of the fimbrial genes.

Lambda red-mediated homologous recombination was used to construct a panel of *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 strains lacking major fimbrial subunit

genes which were examined *in vitro* and *in vivo*. Several fimbrial subunits played a role in the adherence to and invasion of different cell lines in different growth conditions and the role appeared to be serovar-specific. A mutation in the *steA* gene impaired interactions with different cell lines *in vitro* but this phenotype was found to be due to a polar effect on genes downstream of *steA*. The majority of fimbrial subunits played no significant role in the colonisation of the alimentary tract in an established chicken model. Mutation of the *stcA* gene resulted in the greatest degree of attenuation *in vivo* of all of the fimbrial mutants examined. This phenotype was *trans*-complemented and was not the result of a polar or second-site defect thereby fulfilling molecular Koch's postulates. The *stcA* genes therefore play a significant role in the colonisation of the chicken caeca.

Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

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Declaration

I, Debra Jayne Clayton declare that the work presented herein represents my own work, except where acknowledged and has not been previously submitted for a higher degree at any university. I agree to grant access and to permit copies to be made for other libraries or individuals without my specific authorisation.

Signed ……..

Publications

Work from this thesis has been previously presented as follows:

1. D.J. Taylor, M Watson, M. Stevens, E. Morgan, N. Thomson, P. Barrow, M. Jones and M Woodward. Analysis of the repertoire of fimbrial genes in *Salmonella* and *Escherichia coli*. Poster presented at the I3S Conference, St. Malo, May 2005.

2. D.J. Clayton. Analysis of the repertoire and function of fimbrial operons in *Salmonella*, presented at the Society of General Microbiology conference, York, September 2006.

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List of Abbreviations

%	Percent
ºC	Degrees Celsius
α	Alpha
β	Beta
λ	Lambda
Ω	Ohms
ACT	Artemis comparison tool
A600	Absorbance at 600 nm
BCIP/NBT	5-bromo-4-chloro-3-indolylphosphate nitro blue tetrazolium
bcf	Bovine colonisation factor
bp	Base pair
BLAST	Basic local alignment sequencing tool
BSA	Bovine serum albumin
cfu	Colony forming units
CKC	Chick kidney cells
CO_2	Carbon dioxide
CTAB	Cetyl trimethylammonium bromide
DC	Dendritic cells
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucelotides
dpi	Days post-infection
EDTA	ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
ELISA	Enzyme linked immunosorbent assay
FAE	Follicle associated epithelium
FRT	Flippase recombinase target
G	Gram
g	Gravity
GALT	Gut-associated lymphoid tissue
h	Hours
HEp-2	Human epithelial cells
HMM	Hidden markov models

IAH	Institute for Animal Health
IFN	Interferon
IL	Interleukin
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kb	Kilo bases
kDa	Kilo Dalton
LB	Luria-bertani
LD_{50}	50 % lethal dose
lpf	Long polar fimbriae
LPS	Lipopolysaccharide
M	Molar
Min	Minutes
MHC	Major histocompatibility complex
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Millilitre
MLEE	Multilocus enzyme electrophoresis
mm	Millimetre
mM	Millimolar
MRHA	Mannose resistant haemagglutination
MSHA	Mannose sensitive haemagglutination
nm	Nanometres
NaCl	Sodium chloride
NNPP	Neural network promoter prediction
NK	Natural killer cells
P	Pseudogenes
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
pef	Plasmid-encoded fimbriae
PM	*Necropsy*
PMN	Polymorphonuclear leukocytes
Rpm	Revolutions per minute
RNA	Ribonucleic acid

rRNA	Ribosomal ribonucleic acid
RT-PCR	Real-time PCR
s	Seconds
SCV	*Salmonella*-containing vacuole
SDS	Sodium dodecyl sulphate
sef	*Salmonella*-encoded fimbriae
SEF	*Salmonella* Enteritidis fimbriae
SEM	Standard error of the mean
SPI	*Salmonella* pathogenicity island
SSC	Saline sodium citrate
SPF	Specific pathogen free
STM	Signature tagged mutagenesis
T3SS	Type three secretion system
TAE	Tris acetate and EDTA buffer
tcf	Typhi colonisation factor
TE	Tris/ EDTA buffer
Thyb	Hybridisation temperature
TLR	Toll like receptor
Tm	Melting temperature
TNF	Tumour necrosis factor
TSAP	Thermosenstitive alkaline phosphatase
µg	Microgram
µl	Micro litre
µM	Micrometre
UV	Ultra violet
V	Volts
VLA	Veterinary Laboratories Agency

Chapter 1

Introduction

1.1.1. General introduction

Salmonella are Gram-negative, rod-shaped, facultative anaerobic bacteria that belong to the *Enterobacteriaceae* family and cause a variety of diseases in a range of hosts. *Salmonella* infections are a significant cause of morbidity and mortality in livestock and humans and a greater understanding of the molecular mechanisms needed to cause disease is required.

1.1.2. Classification

The genus *Salmonella* consists of two species: *S. bongori,* which causes disease mainly in cold-blooded animals and *S. enterica,* which infects warm-blooded animals. It is believed that *Escherichia coli* and *Salmonella* originally diverged from a common ancestor 120-160 million years ago (Ochman and Wilson, 1987). *S. bongori* is the most divergent of *Salmonella* when attempting to group in evolutionary terms and appears to have evolved separately from *Salmonella enterica*. Different techniques have been used to define the phylogenetic relationships between *Salmonella*, such as multi-locus enzyme electrophoresis (MLEE), DNA hybridisation and micro-array analysis, and suggests that *S. bongori* is more closely related to *E. coli* (Boyd et al., 1996, Christensen et al., 1998, Reeves et al., 1989, Porwollik et al., 2002). Phylogenetic relationships based on rRNA sequences place *S. bongori* evolutionary closer to *E. coli* when examining 16s rRNA and with *S. enterica* when examining 23s rRNA (Christensen et al., 1998). *S. bongori* is thought to be the ancestral species from which *S. enterica* evolved and it is rarely associated with human disease. *S. bongori and S. enterica* both possess *Salmonella* pathogenicity island (SPI)-1, but (SPI)-2 is not present in *S. bongori* and it is believed that the addition of SPI-2 allowed *Salmonella* to cause systemic

disease in a range of hosts (Ochman and Groisman, 1996, Ochman et al., 1996), (reviewed in (Hensel, 2000).

S. enterica consists of seven subspecies: I *enterica,* II *salamae,* IIIa *arizonae*, IIIb *diarizonae,* IV *houteriae*, VI *indica* and subspecies VII (unnamed) (Boyd et al., 1996, Reeves et al., 1989). *S. enterica* are further subdivided into over 2,400 serovars and the majority (60 %) belong to subspecies I with only a small number of these serovars being responsible for 99 % of *Salmonella* infections in humans and warm-blooded animals (Popoff et al., 2004, Chan et al., 2003). The serovars are characterised by their antigenic properties using the Kaufmann-White scheme (LeMinor and Popoff, 1987, Popoff and LeMinor, 2001) which is maintained and updated by the World Health Organisation (WHO). *Salmonella* are classified by somatic lipopolysaccharide (O) antigens and then by the presence of specific flagellar (H) antigens, followed by the phase of the capsular polysaccharide (Vi) antigen. The Vi antigen is a virulence-associated capsular polysaccharide (Looney and Steigbigel, 1986) and is present in a limited number of *Salmonella enterica* serovars including *S.* Dublin, *S.* Typhi and *S.* Paratyphi C.

1.1.3. Host specificity

The *S. enterica* serovars of subspecies I can be further divided into three broad groups based on host-range and the type of disease they produce in healthy, outbred, adult individuals of a given species (reviewed in (Uzzau et al., 2000). The serovars may be host-specific, causing a severe systemic disease in one healthy adult host; host-restricted, causing a systemic disease in a limited range of hosts; or ubiquitous, causing disease ranging from mild enteritis to a severe systemic infection in a wide range of

hosts. The severity of the disease is dependent upon both the serovar and the host, and identification of the genetic factors influencing host adaptation would provide an insight into the mechanisms of host-specificity.

1.1.4. Host-specific serovars

Host-specific serovars are able to cause a severe often fatal systemic disease in only one host. *S. enterica* serovar Typhi is a host-specific serovar that causes typhoid fever in humans resulting in an estimated 21.6 million cases globally every year with a 1 % fata rate (Crump et al., 2004). *S.* Typhi has no natural animal reservoir and spreads by human-to-human transmission resulting in bloody diarrhoea, fever and abdominal cramps for 4 to 7 days (reviewed in (Pang et al., 1995). *S.* Typhimurium infection of inbred mice results in murine typhoid fever and is most commonly used as a model for typhoid fever.

Host-specific serovars are also associated with severe disease in animals, for example *Salmonella enterica* serovar Gallinarum is a non-motile bacterium that only infects avian hosts causing Fowl Typhoid, a septicaemic disease with a mortality rate as high as 90 % in two-week-old birds (Barrow et al., 1987b, Smith, 1955). Chicks infected with *S.* Gallinarum by the oral route within a few days of hatch rarely survive, symptoms include: poor growth, weakness, laboured breathing and the yolk sac will often be found to be colonised. In mature birds, symptoms include a decrease in food consumption, droopy and ruffled feathers, shrunken combs and green and yellow diarrhoea. *Necropsy* examinations reveal lesions in the lung, heart and gizzard, hepatomegaly and splenomegaly and the liver and spleen appear green or bronzed (reviwed in (Pomeroy, 1991). The factors that dictate the host-specificity of these

serovars are currently undefined but are believed to be due to the ability of the *Salmonella* to multiply in tissues particularly those of the reticuloendothelial system at least in chickens and mice (Barrow et al., 1994).

Several vaccines have been developed for the protection of chickens from Fowl typhoid. The most widely used and commercially available is the live attenuated 9R vaccine, developed in the 1950s (Smith, 1956). The attenuation has not been fully characterized and is believed to be due in part to the semi-rough nature of this strain. The 9R vaccine can reduce mortality from 95-100 % down to 0-5 % (Lee et al., 2005). Plasmid cured derivatives of *S*. Gallinarum 9 also were able to reduce mortality to 0 % but only for the large virulence plasmid, the same effect was not seen with other smaller plasmids (Barrow et al., 1987b). A crp deletion in *S*. Gallinarum transduced from *S*. Typhimurium resulted in the complete attenuation of *S*. Gallinarum in ileal loop models and in chickens (Rosu et al., 2007).

1.1.5. Host-restricted serovars

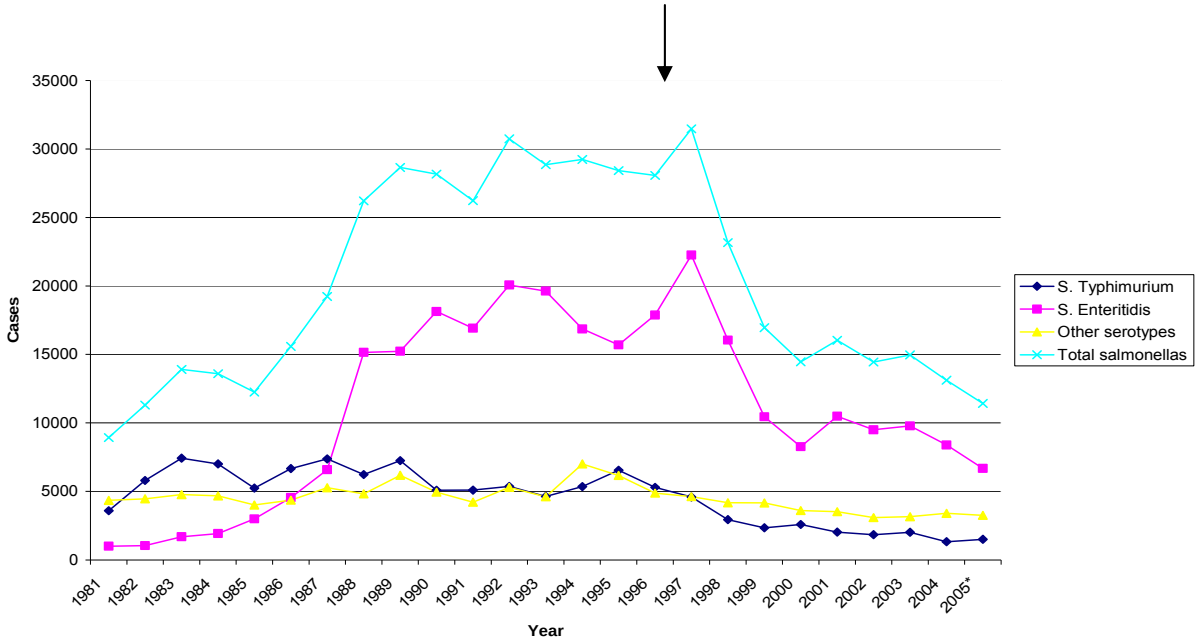
Host-restricted serovars preferentially cause disease in one host but can occasionally cause infection in other hosts. *S. enterica* serovar Choleraesuis primarily infects pigs causing a systemic infection (swine paratyphoid), but occasionally infects humans. Pigs infected with *S.* Choleraesuis develop a range of symptoms; the lungs, spleen and liver increase in size, septicaemia and severe pneumonia may develop and there is an acute inflammatory response (Wilcock et al., 1976). In humans, *S*. Choleraesuis causes fever, gastroenteritis, bacteraemia and extra-intestinal localised infections in many organs but is only fatal in those people with an underlying condition or immuno-compromised individuals (Cheng-Hsun et al., 2006, Lee et al., 2002, Chiu et al., 2004).

1.1.6. Ubiquitous serovars

Ubiquitous serovars of *Salmonella* are able to cause disease in a range of hosts and the severity of the disease depends upon host parameters including species, genetics, age at challenge and imunity. *Salmonella enterica* serovar Enteritidis usually causes an enteric infection in man and during 1988-1998 *S.* Enteritidis resulted in over 25,000 reported cases of acute diarrhoel illness in humans per year in England and Wales with symptoms including fever, diarrhoea and abdominal cramps (Health Protection Agency, 2005). Although *S.* Enteritidis usually causes acute self-limiting gastroenteritis it can be fatal in people with underlying conditions due to kidney failure or septicaemia (Shibusawa et al., 1997). At the time of writing *S.* Enteritidis is the predominant cause of non-typhoidal salmonellosis in England and Wales as shown in Figure 1.1. It is believed, that many cases go unreported and that Figure 1.1 represents an underestimate of the actual number of cases of *Salmonella* infections.

Figure 1.1. Non-typhoidal *Salmonella* cases in humans in England and Wales

Data obtained from the Health Protection Agency, 2005.



The number of cases of *S.* Enteritidis infections in humans increased dramatically during the late 1980s however in the late 1990s the number of cases started to decrease dramatically due to the introduction of the Lion Quality Code of Practice as denoted by the arrow in Figure 1.1. All laying hens were vaccinated against *Salmonella* and all eggs now have a best before date stamped on them. It is not acceptable to vaccinate broiler chickens as the may introduce live *Salmonella* into the human food chain. The decrease in the the number of cases of *Salmonella* from broiler chickens may be due to an increased awareness of *Salmonella*, improved husbandry, better care in the handling, preparation and cooking of chickens or periodically strains emerge causing epidemics and other strains disappear often for unknown reasons.

The broad host range of the ubiquitous serovars frequently results in their transmission between animals and from animals to humans either by direct or indirect contact via the food chain. As *S.* Enteritidis is the most commonly isolated *Salmonella*

from humans, the route of infection through the food chain would appear to be from chickens or eggs. The Food Standards Agency (FSA) reported that during 2003, 0.3 % of eggs in the U.K. were infected with *Salmonella* mostly due to the importation of eggs, and 78 % of these were infected with *S*. Enteritidis (FSA, 2004). Infected adult chickens can often be without symptoms but *S.* Enteritidis can still be isolated from the liver and spleen and a lifelong infection can be established in the reproductive tissue which is easily transmitted to the rest of the flock (reviewed in (Saeed, 1999, Hopper and Mawer, 1988).

Young chicks are more severely affected by *S*. Enteritidis infections than adult chickens and symptoms may include pericarditis, necrosis of the liver, anorexia, depression, drowsiness, dehydration and white diarrhoea (Gorham et al., 1994, McIlroy et al., 1989). During *necropsy* examination, chicks often have an enlarged liver and spleen, infected and firm yolk sac, dilation of the pericardial sac and microscopic or gross lesions (Gorham et al., 1994). Morbidity and mortality are common within the first 24 hours of life (McIlroy et al., 1989).

S. Enteritidis is the *Salmonella enterica* serovar most commonly isolated from eggs, yet the virulence factors that allow *S*. Enteritidis to colonise eggs at a higher frequency than other serovars are currently unknown but have been proposed to be in the ability of S. Enteritidis to survive low pH, low temperatures and low availability of iron (Kang et al., 2006). It has been identified that the majority (92%) of contaminated eggs in the UK contain a 38 MDa plasmid that is present at a much lowere frequency in *S.* Enteritidis strains identified fom humans or chickens (Threlfall et al., 1994), whereas the presence of a 54 kb plasmid appeared to play no role in egg colonisation of PT4 strains (Halavatkar and Barrow, 1993). In part this may be due to its ability to survive in albumin better than other serovars (Clavijo et al., 2006, Keller et al., 1997).

Contamination of the egg contents is thought to occur through infected ovaries or contact with faeces (Henzler et al., 1994). The precise mechanisms are still unclear but it has been suggested that trans-ovarian infection may occur prior to shell formation (reviewed in (Pang et al., 1995, Saeed, 1999, Keller et al., 1997). *S.* Enteritidis was identified in the albumin and yolk in comparable numbers (Gast and Beard, 1990) despite it being reported that *S.* Enteritidis is unable to multiply as well in the egg albumin (Cogan et al., 2004, Gast and Holt, 2000b). Differences in infection rate of eggs were observed with different strains of *S.* Enteritidis (Gast and Beard, 1990). A signature-tagged mutagenesis (STM) screen carried out in *S.* Enteritidis identified several genes required for survival in albumin and indicated a requirement for iron acquisition. However of the genes impicated in survival in egg albumin only two of these were unique to *S.* Enteritidis (Clavijo et al., 2006).

S. enterica serovar Typhimurium is also a ubiquitous serovar. Most studies carried out on the pathogenesis of *Salmonella* infections use *S.* Typhimurium in mice as a model for typhoid fever in humans, a systemic and often fatal infection (reviewed in (Zhang et al., 2003). In healthy adult mice infected with *S.* Typhimurium acute colitis develops (Harrington et al., 2007). Most mice develop a systemic infection similar to typhoid fever in humans including typhoid nodules and deep tissue colonisation occurs (Collins, 1974, Collins et al., 1966) and in immuno-compromised mice can develop meningitis and neurological problems (Wickham et al., 2007). In calves and pigs, *S.* Typhimurium infection is localised to the intestine and mesenteric lymph nodes and results in diarrhoea that may be severe and life threatening if untreated (reviewed in (Wallis and Barrow, 2005).

In humans, *S.* Typhimurium infection results in gastroenteritis, abdominal cramps and fever but is not usually fatal. *S.* Typhimurium can also be isolated from chickens

and in experimentally inoculated day-old-chicks a systemic disease may be seen that results in a high rate of mortality. In adult birds, *S.* Typhimurium mostly causes asymptomatic infections although shedding is persistent (Barrow et al., 1988, Barrow et al., 1987a). *S.* Typhimurium is the most prevalent serovar isolated from livestock particularly pigs whereas the incidence of *Salmonella* from cattle and sheep is comparatively low (Davies and Wray, 1996, Davies et al., 2004).

1.2.1. *Salmonella* genome sequences

The genome sequences of several strains of *Salmonella* ranging in host-specificity are now available. These include the ubiquitous serovar Typhimurium, strain LT2 (McClelland et al., 2001), the host-restricted serovar Choleraesuis, strain SC-B67 (Chiu et al., 2005), and Typhi strains CT18 (Parkhill et al., 2001) and Ty2 (Deng et al., 2003). At the time of writing, complete but unpublished genomes are also available for serovar Typhimurium strains SL1344 and DT104, Enteritidis phage type 4 strain P125109, Gallinarum strain 287/91 and *S. bongori* (http://www.sanger.ac.uk/Projects/Salmonella).

The genome sequences of some *Salmonella* serovars have been available for several years but linking genotype to phenotype is a difficult and labour intensive task. A comparison of the genome sequences of the two *S.* Typhi strains, CT18 and Ty2 has shown that both contain over 200 pseudogenes but differ in prophages, insertions, deletions and distribution of pseudogenes (Parkhill et al., 2001, Deng et al., 2003). By comparison the ubiquitous serovar *S.* Typhimurium LT2 possesses only 39 pseudogenes (McClelland et al., 2001). The loss of genes may be the key to the reduction of the number of niches or hosts available but the virulence factors relating to host-specificity are currently unknown.

1.3.1. Control and prevention of *Salmonella* infections in poultry

Controlling *Salmonella* infections in the avian host is difficult due to the large number of *Salmonella* serovars able to colonise poultry, the size of industrial chicken flocks and the range of niches that some of these serovars can be isolated from including, feed, litter, environment and nest boxes (Bains and MacKenzie, 1974). The task can be made more difficult by the fact that chick fluff can remain contaminated up to four years after an outbreak (Miura et al., 1964). Despite this, the avian-specific serovar *S.* Gallinarum has been eradicated from chickens in the U.K. and countries with a developed poultry industry via a test-and-slaughter policy (Rabsch et al., 2000).

Poultry infections with *S*. Enteritidis and other serovars remains a problem and several control strategies have been developed. Husbandry plays a key role in controlling *Salmonella* numbers in chickens. The cleansing and disinfecting of poultry units decreased the number of *Salmonella* isolated but did not eliminate it, largely due to transfer via rodents, over dilution and inconsistent application of disinfectants (Davies and Wray, 1996).

The vaccination of chicken flocks is mandatory in several countries but is not always successful if good husbandry is not present and occurrence of *Salmonella* can still be as high as 63 % (Davison et al., 1999, Davies and Breslin, 2004). Several vaccines are currently in production that are either killed or non-characterised live-attenuated vaccines. There are only a few licensed live vaccines that are available including Nobilis SG9R an attenuated derivative of *S*. Gallinarum 9 that provides cross-immunity to *S.* Enteritidis, TALOVAC or TADSvacE and TADSvacT which are produced by chemical mutagenesis (Lohmann Animal Health)(Gantois et al., 2006) and offer protection against *S*. Enteritidis and an avirulent live delta cya delta crp *S*. Typhimurium strain showed long term protection against *Salmonell*a (Hassan and Curtiss, 1997) .

Several inactivated vaccines have also ben developed including Nobilis Salenvac T protects against both *S*. Enteritidis and *S*. Typhimurium. An *aroA* and a separate hilA mutants of *S*. Enteritidis has successfully been used to provide significant protection against an oral infection in chickens (Cooper et al., 1992, Bohez et al., 2008). Salenvac, an inactivated iron-restricted *S*. Enteritidis vaccine also showed a significant decrease in the numbers of *Salmonella* isolated from experimentally infected laying hens (Woodward et al., 2002) and other vaccines have been developed against *S*. Typhimurium in the avian host and are decreasing colonisation numbers of *Salmonella* but not eradicating it (Clifton-Hadley et al., 2002). It has been proposed that avirulent strains produce a cellular immunity whereas killed vaccines provide an humoral response which is more beneficial but may have a shorter effect (Curtiss et al., 1993). However other groups have found a long term protective effect of live vaccines (Hassan and Curtiss, 1997).

The number of *Salmonellae* isolated from the avian host can also be reduced by a change in diet as the feed can have anticoccidial and antimicrobial properties (Bailey et al., 1988). Medium chain fatty acids act as an antibacterial agent against *Salmonella* and fermented feed improves the barrier of the crop and gizzard by increasing the concentration of lactic and acetic acid resulting in a decrease in *Salmonella* isolated from the anterior parts of the gastrointestinal tract. Mannose-oligonucleotides in the feed also decrease *Salmonella* numbers in the caeca (Heres et al., 2003, Van Immerseel et al., 2006, Fernandez et al., 2002). Colonisation of the chicken gut may be inhibited by gut microflora, a phenomenon known as competitive exclusion which can include obligate and facultative anaerobic bacteria. The absence of gut microflora allows *Salmonella* to colonise at any point along the gastrointestinal tract (Soerjadi et al., 1982). The caecal contents from an uninfected adult chicken can be given orally, to

day-old-chicks which results in decreased numbers of *Salmonella* colonising the caeca (Soerjadi et al., 1982). A mixture of organisms in gut microflora provides better protection against *S*. Typhimurium in 2-day-old-chicks than gut microflora composed of either *E. coli* or *Lactobacillus* alone (Baba et al., 1991). Several studies have been carried out to examine the presence of other bacteria competing with *S.* Enteritidis including *Bacillus subtilis* (La Ragione and Woodward, 2003) and *Lactobacillus salivarius* (Zhang et al., 2007). Competitive exclusion can reduce *Salmonella* shedding and prevalence in the environment as well as reduce contamination of processing plants (Davies and Breslin, 2003). Yogurt has also been shown to promote an earlier immune response to *Salmonella* than other microbiota resulting in lower numbers of S. Enteritidis in the livers and spleens (Tayeb et al., 2007, Avila et al., 2006). Several commercial competive exclusion agents are now available with varying degrees of success (Ferreira et al., 2003).

Bacteriophages can be included in the gut content to target and kill bacteria without the use of antibiotics. Bacteriophages have been shown to reduce but not eliminate the number of *S*. Enteritidis PT4 and *S*. Typhimurium in chickens (Atterbury et al., 2007). *Salmonella* can become resistant to bacteriophages and higher doses of phage resulte in a higher rate of resistance (Fiorentin et al., 2005, Atterbury et al., 2007). Treatment with *Salmonella*-specific bacteriophages resulted in a decrease of *Salmonella* in the caeca in experimentally inoculated chickens. A 4 log decrease was observed with *S*. Enteritidis and a 2 log difference with *S*. Typhimurium (Atterbury et al., 2007). Treatment of the chicken skin with bacteriophages also resulted in a decrease in *Salmonella* numbers and was correlated with the multiplicity of infection of the bacteriophage applied (Goode et al., 2003). The use of certain antibiotics, whilst no

longer allowed in many countries, has been shown to actually increase the severity of *S.* Enteritidis infections in chickens (Manning et al., 1994).

In addition to these strategies breeding for heritable resistance to *Salmonella* may be feasible. In-bred lines of birds exhibiting heritable differences in resistance to several different *Salmonella* serovars have been defined and understanding the factors that mediate protection will be key to developing a generation of *Salmonella*-resistant chickens (Bumstead and Barrow, 1993).

1.4.1. Immunity and avian host resistance

Salmonella enter the host and pass through the epithelial cell layer which acts as a barrier and an initiator of the innate immune response. Lymphocyte populations in the gut are dependent upon age, genetics and diet and consist of NK cells, B cells and heterophils all of which are consistently present in low numbers (reviewed in (Beal et al., 2006b). Lymphoid tissues are poorly organised and characterised in the chicken, distinct Peyer's patches similar to those in mammals become visible after hatch and increase in numbers (Befus et al., 1980). Within the avian Peyer's patch lies lymphoepithelium with M cells. M cells are an efficient site for entry of microorganisms and often possess irregular microvilli distinguishing them from other epithelial cells. The M cells possess vacuoles that maybe involved in pinocytosis (Befus et al., 1980). M cells more efficiently transfer antigens from the lumen than other absorptive cells (Bockman et al., 1983).

Salmonella are first detected in the host by polymorphonuclear leukocytes (PMN) such as monocytes or heterophils which immediately engulf the bacteria (Henderson et al., 1999). Heterophil numbers increase rapidly in response to challenge with invasive

S. Enteritidis strains but not with non-invasive strains (Ziprin, 1997). Heterophils are efficient bacteriocidal cells which express a range of toll-like receptors (TLR) and are able to kill non-opsonised cells (Kogut et al., 2005b). Heterophils are also capable of producing rapid increases in Th1 cytokines after phagocytosis (Stabler et al., 1994). The role of NK cells and macrophages is reviewed elsewhere (Beal et al., 2006b).

Salmonella are detected by receptors for pathogen-associated molecular patterns (PAMPs) such as TLRs that most cells possess. Activation of TLRs induces a cascade of events leading to a pro-inflammatory response e.g. IL-1, IL-6 and IL-8 (Kogut et al., 2005c, Stabler et al., 1994) and the production of cytokines (reviewed in (Kaiser et al., 2005). Chickens constitutively express multiple TLRs which may be activated by lipopolysaccharide (LPS), peptidoglycan, flagella or CpG and it is likely that other factors are involved (Kogut et al., 2005a, Higgs et al., 2006). A different collection of TLRs exist in the avian host compared to the mammalian host.

Experimental *S*. Enteritidis and *S*. Typhimurium infections in CKCs produce a strong inflammatory response, in contrast *S.* Gallinarum does not produce an inflammatory response and results in systemic disease, possible arriving at the systemic sites by stealth (Kaiser et al., 2000). *S*. Gallinarum lacks flagella and is thought to be related to the ability of *S*. Gallinarum to avoid a TLR5-induced proinflammatory response (Iqbal et al., 2005).

The clearance of *S*. Typhimurium from the gut has been shown to be age-dependent and relies upon the development of a Th1 T cell response and IFN γ and an increased expression of CD4+ and CD8+ T cells (Withanage et al., 2004, Beal et al., 2004a). Gallinacins are antimicrobial peptides that increase in response to *S*. Enteritidis infection and in older birds may contribute to the host immune response (Yoshimura et al., 2006).

A primary infection with *S.* Typhimurium or *S.* Enteritidis induces a strong lymphocyte proliferation and an increase in the production of antibodies of the IgM, IgG and IgA class (Beal et al., 2006c). B cells are produced by the avian host in the Bursa of Fabricius and an antibody response occurs in chicks as young as 2-weeks-old. Older birds have a much stronger and more rapid antibody response than young chicks. However surgical bursectomy during embryo development resulting in B cell deficient birds has indicated that B cells are not essential for the clearance of a *Salmonella* infection or enhanced clearance after secondary challenge, despite the fact that infection induces high titres of specific antibody (Beal et al., 2006a, Beal et al., 2005). The clearance may be due to a general increase of the entire immune response including the enteric T cell response and not just due to B cells (Beal et al., 2004b). The onset of sexual maturity has been implicated in the increase of *S.* Pullorum in the reproductive tract and is correlated with a fall in T cell responses during a short period of around 3 weeks (Wigley et al., 2005).

Dendritic cells (DC) are a direct link between the innate and the adaptive immune systems. DCs are professional antigen-presenting cells which present bacterial antigens to T cells. *S.* Typhimurium avoids lysosomal degradation and thus prevents activation of the adaptive immune response by DCs (Tobar et al., 2006). *In vitro,* IFNγ-activated macrophages use alternative pathways to present *Salmonella* to CD8+ cells by the loading of an antigenic peptide to major histocompatibility complex class I (MHC-I) molecules on macrophage cell surfaces. *Salmonella* engulfed into B cells cannot use this pathway and this provides a survival advantage (Rosales-Reyes et al., 2005).

Inbred lines of birds show variability in their susceptibility to *Salmonella* and this has been correlated with the production of cytokines and chemokines. The macrophages in chicken lines that are resistant to *Salmonella* produce more chemokines and cytokines

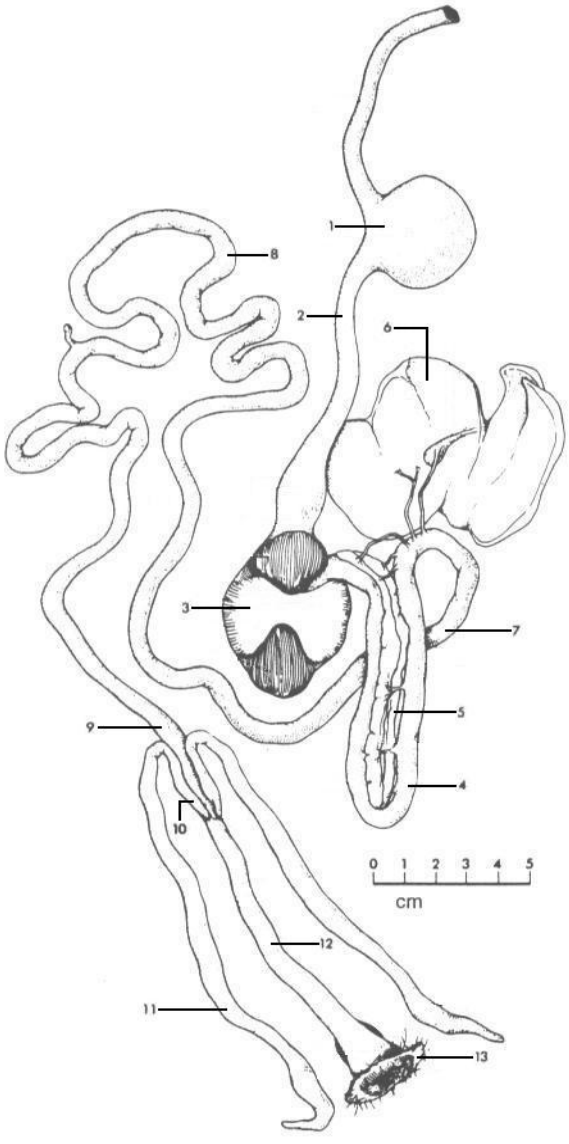
than those that are susceptible in both *S*. Typhimurium and *S*. Gallinarum infections (Wigley et al., 2006). The genetic differences between the birds is currently poorly understood (reviewed in (Wigley, 2004), but it has been proposed that it is due to variation in specific TLRs (Leveque et al., 2003).

1.4.2. Avian gastrointestinal tract

The avian digestive system extends from the beak to the external orifice of the cloaca and consists of the buccal cavity, pharynx, oesophagus, crop, stomach consisting of two components; proventriculus and gizzard, intestine, caeca and cloaca and *Salmonella* passes through these if it gains entry via the oral route (Figure 1.2).

Figure 1.2. Digestive tract of a 12-week-old chicken.

1, crop; 2, esophagus; 3, gizzard; 4, duodenum; 5, pancreas; 6, liver; 7, jejenum; 8, ileum; 9, colon; 10, caecal tonsils; 11, caeca; 12, rectum; 13, cloaca. Scale is in centimeters. (Denbow, 2000). Gastrointestinal anatomy and physiology", in *Sturkie's Avian Physiology*, fifth edition, edited by G. C. Whittow, p. 300.



The avian digestive tract is shorter than that in mammals, food retention time is lower and nutrients are less efficiently absorbed (Turk, 1982, Carlson, 1982). The mouth consists of the beak, tongue, salivary glands and pharynx. The oesophagus is a

muscular tube that draws the bird's food further into the body and ends in a specialised storage organ called the crop (Ramel, 2006). The crop leads into the stomach, which can be divided into two sections, the proventriculus and the gizzard. The proventriculus consists of a thickened mucosa, and circular and longitudinal muscle layers. It is sometimes called the glandular stomach and produces a relatively large volume of digestive juices e.g. pepsin and hydrochloric acid (Ramel, 2006). A valve, the pylorus, is located between the gizzard and the duodenum and controls the passage of food further into the digestive tract (Ramel, 2006). The proximal portion of the intestine is U shaped around the pancreas and is called the duodenum. The portion of the intestine distal to the duodenum is the jejunum and anterior to the caeca is the ileum (Turk, 1982). From the upper duodenum to the lower ileum the mucosa decreases in thickness, the villi become smaller and the crypts increase in depth. The interior surface of the intestine is folded to increase the surface area and goblet cells are scattered over the surface of the villi and produce a mucus secretion (Turk, 1982). Immediately post-hatch, at the end of the jejunum is the yolk sac or Merkels diverticulum.

The end of the ileum is marked by a circular ring of muscle or sphincter projecting into the lumen, the ileo-caecal valve (Turk, 1982). The caeca are a pair of blind-ended tubular structures that lie along the ileum and are folded at midpoint. They are approximately 12-16 cm long in adult chickens and the proximal end of the caeca possess large numbers of mucus producing goblet cells (Turk, 1982). The caeca is the site of highest colonisation and *Salmonella* persist here longer than any other part in the gut (Fanelli et al., 1971). The caecal tonsils occupy the first 4-10 mm of the proximal region the caeca and are lymphomyeloid tissue that appear as an enlargement of the caeca and have germinal centres (Glick et al., 1978). Peyer's patches are located at the distal end of the caecal tonsil (Jeurissen et al., 1994). The bursa is located dorsally to

the cloaca as a shallow sac and is a central lymphoid organ for B cell proliferation (Jeurissen et al., 1994). The large intestine or colon is short, (5-8 cm) and leads from the ileo-caecal junction to the cloaca, a posterior opening that is also known as the vent.

1.4.3. Avian reproductive tract

Egg contamination by *Salmonella* is primarily due to *S.* Enteritidis and occasionally *S.* Typhimurium (Keller et al., 1997). *S.* Enteritidis is currently the only serovar that causes frequent human infection from contaminated eggs. *S.* Enteritidis has the advantage over other serovars in its ability to colonise the vaginal tissue of hens and the reproductive tract including the ovaries and oviduct (Okamura et al., 2001a, Okamura et al., 2001b). The mechanisms underlying egg colonisation of *Salmonella* are poorly understood and several methods have been proposed.

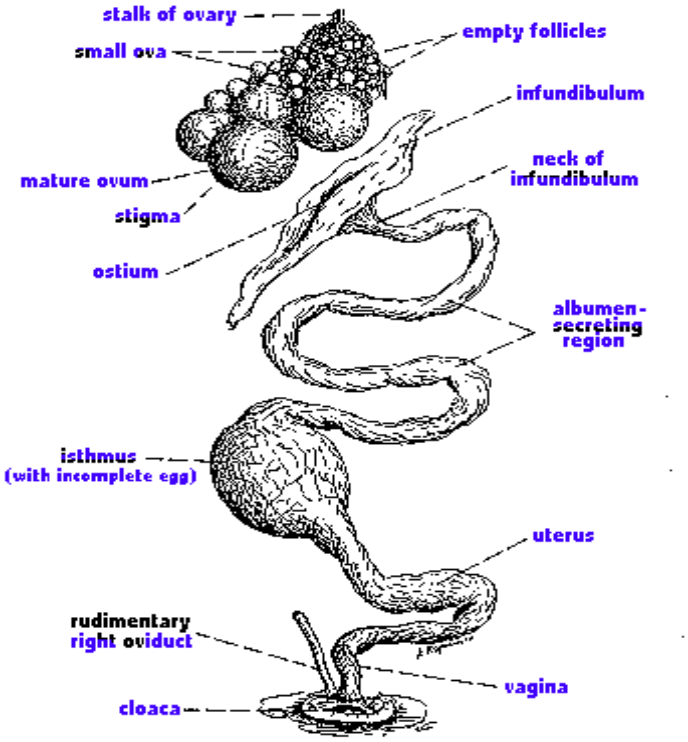
The infection of the eggs can occur via a descending infection from the ovarian tissue or an ascending infection from the cloaca (Keller et al., 1995). *S.* Enteritidis infection of the ovary and oviduct tissue can lead to contamination of the egg prior to the formation of the shell via vertical transmission to the egg or albumin from the reproductive tract (reviewed (Guard-Petter, 2001), Figure 1.3 shows the close proximity of the cloaca and the vagina (Snoeyenbos et al., 1969). It has been suggested that factors within the egg control the pathogen (Keller et al., 1995).

Alternatively the outside of the egg becomes contaminated with faecal matter and as it cools a vacuum forms, pulling in the bacteria (Keller et al., 1995). It has also been proposed that semen may serve as a vehicle for transmission of *S.* Enteritidis to egg samples (Reiber et al., 1995). *In vitro* analysis of egg colonisation indicated *S.* Enteritidis colonises the preovulatory follicles at different stages of development by

interacting with the ovarian granulose cells (Thiagarajan et al., 1994). In several reports the albumin is usually colonised rather than the yolk (Humphrey, 1994) but other studies have found the opposite (Gast and Holt, 2000a).

Figure 1.3. The female avian reproductive tract.

(http://www.iacuc.arizona.edu/training/poultry/species.html#body)



1.5.1. Establishment of *Salmonella* infection in the avian host

Salmonellae usually enter the avian host by the faecal-oral route and colonise the gastro-intestinal tract. Following oral inoculation *Salmonella* isolation is highest from the caeca and it persists here longer than at any other part of the intestine in both young and adult chickens (Barrow et al., 1988, Williams, 1978, Fanelli et al., 1971). Bacteria must adhere to the epithelial cells if they are to avoid clearance by the slow continuous

flow of the intestinal contents as the caeca empty 2-4 times per day. The host and bacterial factors mediating adherence are ill-defined however, glycosphingolipids (GSL) in the plasma membrane of avian cells are used by the SEF21 *Salmonella* fimbriae (discussed later) as receptors on the epithelial surfaces of the chicken intestine (Li et al., 2003b, Li et al., 2003a), other mechanisms or receptors for adherence may also exist.

S. Enteritidis has been visualized in the intestinal mucosa following experimental infection of 1-day-old chickens and the uptake of *Salmonella* was associated with evaginations or membrane ruffles on the epithelial cell surface and the bacteria were enclosed within membrane-bound vacuoles (Turnbull and Richmond, 1978). These lysosomal vacuoles appear abnormal but rupture releasing *Salmonella* back into the lumen (Popiel and Turnbull, 1985).

S. Gallinarum causes a systemic disease in the avian host and preferentially invades the caecal tonsils via the gut-associated lymphoid tissue (GALT) during the early stages of infection rather than through non-phagocytic cells and high bacterial counts are found in the caecal tonsils (Chadfield et al., 2003). However the caecal tonsils do not mature in the chick until two to three weeks post hatch, therefore *S*. Gallinarum is likely to use more than one route of infection (Bar-Shira et al., 2003). *S*. Gallinarum has no advantage over other serovars in terms of survival or replication but is able to cause a systemic disease in chickens (Chadfield et al., 2003). *S*. Typhimurium has been demonstrated to be more invasive than *S*. Gallinarum which does not reflect the disease (Chadfield et al., 2003). The host-specific serovars are able to colonise the distal alimentary canal and the differences between the host-specific and ubiquitous serovars is in their ability to translocate, survive and replicate in specific organs such as the liver and spleen (Barrow et al., 1994). The ubiquitous serovars induce a pro-inflammatory

response by increasing the production of IL-6, a cytokine whereas *S*. Gallinarum does not induce an inflammatory response or increase levels of IL-6 (Kaiser et al., 2000).

1.5.2. Establishment of *Salmonella* infections in the mammalian host

The study of *Salmonella* infections has primarily taken place in mammalian hosts. Screening of signature-tagged transposon mutants of *S*. Typhimurium in cattle, pigs and mice revealed that different factors were required for colonisation in different hosts (Morgan et al., 2004, Carnell et al., 2007, Tsolis et al., 1999). Therefore, observations made in one species cannot necessarily be extrapolated to other hosts.

Visualisation of the interaction of *S.* Typhimurium with cultured epithelial cells was undertaken and the transient induction of surface-expressed appendages was seen which appear to be required for internalisation of *Salmonella* but rapidly disappear (Ginocchio et al., 1994). The presence of these appendages was associated with the *inv* operon and mutations within this operon resulted in their absence (Ginocchio et al., 1994). The production of these appendages has been associated with induced fluid secretion both in the presence and absence of mucosal damage.

The mammalian intestinal epithelium is interspersed with specialised antigen-sampling cells or M cells, which are clustered within Peyer's patches. In the mouse model, the primary route of infection for *S.* Typhimurium is via the M cells and is often accompanied by M cell destruction (Jones et al., 1994, Clark et al., 1998). Type three secretion system (T3SS)-1 promotes the invasion of M cells and enterocytes by inducing polymerised actin cytoskeleton rearrangements or membrane ruffles leading to uptake (Clark et al., 1994, Watson et al., 1995). *S*. Typhi and *S*. Typhimurium are capable of entering the murines intestinal epithelium via M cells, however *S*. Typhi does

not destroy the epithelium like *S.* Typhimurium and *S.* Gallinarum enters the murine Peyer's patch epithelium at a much lower frequency than other serovars proposing that other routes of infection exist (Pascopella et al., 1995).

In the bovine intestines, *S.* Typhimurium interacts with the lymphoid follicle-associated epithelium (FAE) within 5 minutes and M cells can be observed to form ruffles that engulf the bacteria. Within 15 minutes the bacteria were seen adhering to enterocytes and were taken up in vacuoles, after 60 minutes there was no further interaction between the FAE and the bacteria, with most bacteria residing in an ill-defined cell population in the *lamina propria* but are no longer interacting within the enterocytes after 60 minutes (Frost et al., 1997).

In the bovine ligated ileal loop model no evidence was found to support the presence of Peyer's patches or M cells as a mode of dissemination to distal organs as enteric and systemic serovars invaded M cells and enterocytes to the same extent (Bolton et al., 1999, Paulin et al., 2002). Few bacteria that enter the epithelium progress beyond the *lamina propria.* *Salmonella* serovars that are able to cause a systemic infection such as *S.* Dublin in calves and *S.* Choleraesuis in pigs are able to persist and translocate from the ileal lymph nodes to systemic sites via the efferent lymphatics unlike *Salmonella* serovars that result in enteric infections (Paulin et al., 2002, Pullinger et al., 2007). The net replication of *S.* Choleraesuis in pigs was lower than that of *S.* Typhimurium in the intestinal mucosa and *S.* Choleraesuis induced expression of lower amounts of pro-inflammatory cytokines e.g. IL-8. IL-18, TNFα than *S.* Typhimurium (Paulin et al., 2007). *S.* Typhimurium is confined to the intestines, possibly because it replicates rapidly and induces a stronger host immune response than *S.* Choleraesuis suggesting that systemic serovars may disseminate via a strategy of stealth (Paulin et al., 2007, Paulin et al., 2002).

The ability of enteropathogenic *Salmonella* to recruit inflammatory cells and induce secretory responses is one of the main features of enteritis. The interaction between *Salmonella* and the epithelial cells trigger an inflammatory response resulting in fluid secretion. This is an indication of innate immunity as a direct or indirect consequence of the deployment of T3SS-1 to invade enterocytes and M cells (Wallis et al., 1986, Jones et al., 1998). Invasive *Salmonella* can also directly penetrate the epithelial layer in a process that is independent of T3SS-1 and is believed to be mediated by DC and M cells. The DCs are capable of opening up tight junctions allowing further influx of *Salmonella* (Vazquez-Torres et al., 1999, Watson et al., 1998, Niess and Reinecker, 2005).

1.6.1. Virulence factors

The symptoms of a *Salmonella* infection vary depending on the host and bacterial virulence factors. Many *Salmonella* virulence factors have only recently been identified and include virulence plasmids, flagella and fimbriae, which are briefly discussed below (reviewed in (van Asten and van Dijk, 2005). Attempts have been made to identify virulence genes by targeted and genome wide mutagenesis and screening for attenuation in animal models (Lawley et al., 2006, Morgan et al., 2004, Carnell et al., 2007, Hensel et al., 1995, Shea et al., 1996, Tsolis et al., 1999, Turner et al., 1998). These screens have reinforced the roles for the virulence factors discussed below.

1.6.2. Plasmids

Many of the serovars of *S. enterica* subspecies I carry a low copy number virulence plasmid which varies in size from 50 to 100 kb (Rychlik et al., 2006, Rotger and

Casadesus, 1999). All *S. enterica* virulence plasmids contain a 7.8 kb conserved region consisting of 5 genes within the *spv* operon, a recent acquisition to the *Salmonella* genome probably after *Salmonella* speciation (Boyd and Hartl, 1998). This operon is required for bacterial multiplication within the reticuloendothelial system and to trigger systemic disease by non-typhoidal *Salmonellae* in susceptible hosts (Rotger and Casadesus, 1999, Haneda et al., 2001). The virulence plasmids also contain a range of other genes such as *tra,* fimbrial genes and *rck* genes which maybe involved in various stages of infection (Rotger and Casadesus, 1999).

The virulence plasmids of *S.* Gallinarum and *S.* Pullorum are able to restore full virulence in plasmid-cured strains of the same *Salmonella* serovars (Barrow and Lovell, 1989). The removal of this plasmid from *S.* Pullorum results in reduction in mortality in chickens from 70 % to 0 % (Barrow and Lovell, 1988) and the removal of the large plasmid in *S.* Gallinarum prevented Fowl Typhoid, suggesting that the plasmids may be a key virulence factor (Williams Smith and Tucker, 1980, Barrow et al., 1987b). *S.* Dublin strains that carry a virulence plasmid are highly virulent in calves resulting in a systemic and fatal disease whereas plasmid-free strains only cause mild diarrhoea. The *S.* Dublin virulence plasmid has been shown to mediate persistence at systemic sites in cattle (Wallis et al., 1995).

The virulence plasmids of some *Salmonella* carry fimbrial genes (plasmid-encoded fimbriae *pef)*. In *S.* Typhimurium, a 90 kb virulence plasmid has been shown to confer increased virulence in mice by promoting the spread of infection after invasion of the intestinal epithelium (Friedrich et al., 1993). In *S.* Gallinarum *pef* is not present on the virulence plasmid instead it has 3 genes with homology to the K88 fimbrial genes of *E. coli* (Rychlik et al., 2006).

1.6.3. Flagella

A flagellum is a filamentous projection composed of subunits of a single protein that is helically arranged and rotates, driving motility. Mutations within the flagella genes of *S.* Typhimurium result in reduced virulence in mice and decreased survival in macrophages (Schmitt et al., 2001, Carsiotis et al., 1984). The flagella are a major component in triggering a pro-inflammatory response in *S.* Typhimurium (Zeng et al., 2003, Iqbal et al., 2005).

Flagella mutants exhibit reduced enteropathogenicity in a bovine ligated ileal loop model of infection (Schmitt et al., 2001). The expression of the flagella may enable bacteria to penetrate the host epithelial cells and provide an advantage in invasion and colonisation (Carsiotis et al., 1984). Flagella have been implicated in chemotaxis, however these characteristics are not required for full virulence in some serovars since *S.* Gallinarum and *S.* Pullorum do not possess flagella and are non-motile but are still able to cause a systemic disease in the avian host (Iqbal et al., 2005, Barrow and Lovell, 1989).

TLR-5-mediated detection of flagellin is believed to be important in the control of Salmonellosis in chickens and it has been proposed that an absence of flagellin may allow strains to evade the innate immune response at mucosal surfaces. The significance of flagella in bacterial pathogenesis of *Salmonella* is reviewed elsewhere (Penn and Luke, 1992). Aflagellate *S.* Enteritidis strains have a decreased invasion rate and produce fewer ruffles than the wild-type in both avian and human cell lines (La Ragione et al., 2003).

1.6.4. *Salmonella* Pathogenicity Islands (SPI)

Pathogenicity islands are found in Gram-negative and Gram-positive bacteria. SPIs are defined regions of the *Salmonella* chromosome that contain a collection of genes whose roles have been implicated in pathogenicity and have often been obtained though horizontal gene transfer indicated by the differing % G+C content to the rest of the genome (Groisman and Ochman, 1996). SPI's are often near to tRNA or insertion sequences representing target sites for integration (Hansen-Wester and Hensel, 2001, Hacker et al., 1997). At the time of writing 14 pathogenicity islands have been identified in *S. enterica* and the distribution is variable across the serovars (McClelland et al., 2001, Parkhill et al., 2001, Morgan, 2007). Many SPIs are capable of acting synergistically for example effector proteins encoded within SPI-5 are translocated by T3SS-1 encoded by SPI-1 and SPI-2 (Knodler et al., 2002, Hansen-Wester and Hensel, 2001).

A 40 kb region of the chromosome consisting of invasion genes was identified as *Salmonella* pathogenicity island 1 (SPI-1) and encodes a T3SS-1 (Mills et al., 1995). In *S.* Typhimurium the % G+C content of SPI-1 is 45.9 %, much lower than the genomic average for *S.* Typhimurium LT2 of 52 % (McClelland et al., 2001). Invasion of intestinal epithelial cells is dependent on a functional T3SS-1 of SPI-1 causing the cytoskeleton rearrangements (Zhou and Galan, 2001). Mutation of *S.* Typhimurium SPI-1 results in a reduction in systemic virulence in 1-week-old birds and 4-week-old calves but only a moderate reduction in virulence of mice (Morgan et al., 2004, Tsolis et al., 1999). SPI-1 is not essential in *S.* Gallinarum or *S.* Pullorum infections in chickens (Jones et al., 2001, Wigley et al., 2002, Tsolis et al., 1999). However, SPI-1 mutations decrease the bacterial levels both in the gastrointestinal tract and at systemic sites in 2-

week-old-birds infected with *S.* Typhimurium, these mutants were later shown to be instable (Jones et al., 2001, Jones et al., 2007).

A second pathogenicity island has been identified; SPI-2, a 40 kb region which has a low % G+C content indicating that it too has been acquired through horizontal gene transfer and its function is reviewed in detail elsewhere (Waterman and Holden, 2003). SPI-2 is not present in *S. bongori* and may have been acquired after *S. enterica* and *S. bongori* diverged (Ochman and Groisman, 1996). SPI-2 encodes a second T3SS-2 that is functionally distinct from the T3SS-1 of SPI-1. (Shea et al., 1996, Yap et al., 2001, Hensel, 2000, Chakravortty et al., 2005). *S.* Gallinarum in the avian host requires a functional SPI-2 to possess full virulence, to survive within macrophages and to translocate to systemic sites (Jones et al., 2001). SPI-2 was also required for systemic and enteric infection of *S.* Dublin in calves and *S.* Typhimurium in mice (Shea et al., 1996, Bispham et al., 2001).

SPI-1 and SPI-2 are the most widely studied pathogenicity islands and whilst the screening of signature-tagged mutants of *S.* Typhimurium has indicated pivotal roles for SPI-1 and SPI-2 encoded T3SSs in the colonisation of cattle (Morgan et al., 2004) and pigs (Carnell et al., 2007), the same mutations had only a minor effect on the colonisation of chickens. Further studies have identified a role for SPI-1 and SPI-2 in systemic virulence of *S.* Typhimurium in chicks but is age dependent (Jones et al., 2007). There is little information is known about the other 12 pathogenicity islands but a brief description of the islands is given below (reviewed in (Morgan, 2007).

SPI-3 was originally identified through examination of the *selC* locus and was later extended to cover a 34 kb mosaic region (Blanc-Potard and Groisman, 1997, McClelland et al., 2001). Four genes within SPI-3 are required for colonisation of

either chickens, calves or both (Morgan et al., 2004). SPI-4 was defined through genome sequencing of *S*. Typhimurium LT2 (Wong et al., 1998) and the sequence was confirmed by Morgan et al*.,* 2004 and consists of six ORFs, *siiA-F* (McClelland et al., 2001). SPI-4 encodes a Type 1 secretion system which is required for colonisation of calves but not chickens or pigs by *S.* Typhimurium (Morgan et al., 2007, Carnell et al., 2007). The expression of SPI-4 genes is co-regulated with SPI-1 invasion genes by a global regulator (Gerlach et al., 2007a). SPI-5 is a small pathogenicity island and consists of 5 novel genes that contribute to enteric but not systemic infections in calves (Wood et al., 1998). SPI-6 contains the *saf* fimbrial operon which has been implicated with the colonisation of the porcine intestines but not calves or chickens (Carnell et al., 2007). The typhi colonisation fimbriae (*tcf)* is also found on SPI-6 in *S*. Typhi (Morgan et al., 2004, Carnell et al., 2007, Parkhill et al., 2001). The deletion of the SPI-6 region resulted in a decrease in the ability of *S*. Typhimurium to enter eukaryotic cells but no difference in virulence in mice was detected (Folkesson et al., 2002). SPI-7 of *S.* Typhi encodes the Vi antigen and a type IV pilus for attachment to epithelial cells (Zhang et al., 2000). It consists of a mosaic structure and has inserted in the tRNA ^{pheU} site (Parkhill et al., 2001, Pickard et al., 2003). SPI-8 and SPI-9 were identified from the genome sequencing of *S*. Typhi CT18 and whilst little is known on the function of SPI-8 other than it encodes for bacteriocins, SPI-9 has a similar genetic organisation to SPI-4 and contains 4 genes exhibiting homology to genes in SPI-4 (Parkhill et al., 2001). SPI-10 was first identified through the genome sequencing of *S*. Typhi and contains the *sef* fimbrial operon (Parkhill et al., 2001), which influences virulence in mice and internalisation into macrophages (Edwards et al., 2000). SPI-11 and SPI-12 were both identified from the complete genome sequencing of *S*. Choleraesuis SC-B67 (Chiu et

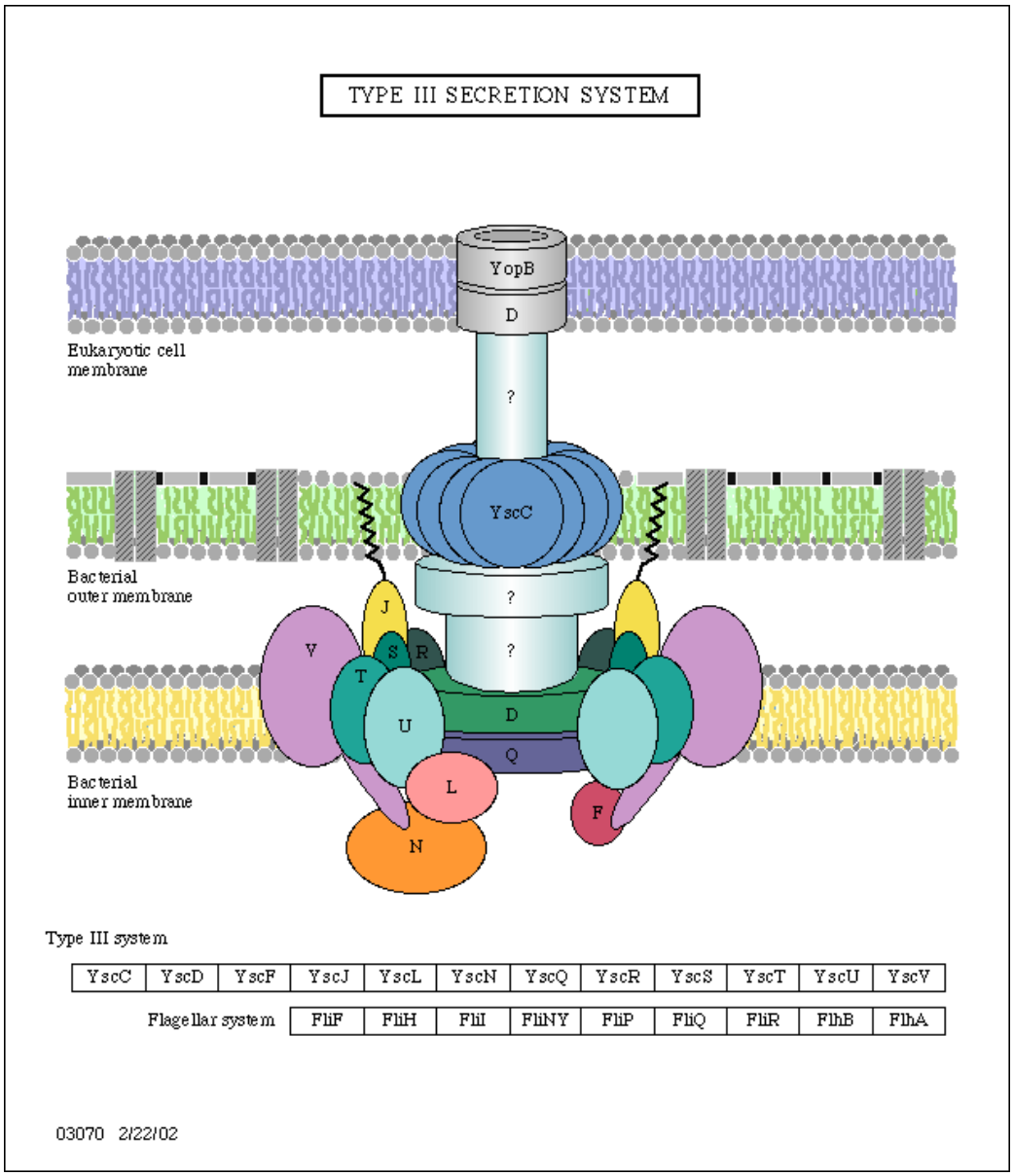
al., 2005) and SPI-13 and SPI-14 were identified at the same time and play a role in colonisation of day-old chicks with *S.* Gallinarum (Shah et al., 2005).

The acquisition of discrete genomic islands each conferring novel properties may have contributed to the evolution of virulence in *Salmonella.* The high proportion of attenuating mutations in SPI's indicates that they may play a role in determining the outcome of infection (Hensel et al., 1995, Carnell et al., 2007, Lawley et al., 2006, Morgan et al., 2004, Shea et al., 1996, Tsolis et al., 1999).

1.6.5. Type three secretion systems (T3SS)

T3SS often play key roles in the virulence of Gram-negative pathogens of humans, animals and plants and are encoded on pathogenicity islands reviewed in (Hansen-Wester and Hensel, 2001). The invasion of *S.* Typhimurium into the intestinal epithelial cells is mediated by T3SS and the expression of T3SS proteins is tightly regulated and is often influenced by environmental conditions such as iron (Ellermeier and Slauch, 2008). They consist of a multi-protein complex to translocate proteins from the bacterial cytoplasm into the host-cell (reviewed in (Galan and Wolf-Watz, 2006). The translocation of proteins into the host cell occus via a needle complex and may consist of over 20 proteins, summarised in Figure 1.4. The structure of the T3SS-1 needle complex has been defined (Marlovits et al., 2004).

Figure 1.4. KEGG Diagram for Type III secretion system



Once inside the cell, these proteins inhibit or activate cellular processes for the benefit of the bacteria, the proteins have been identified to function in different ways including inner membrane proteins (InvA, SpaP, SpaQ, SpaR), outer membrane proteins (InvG, PrgH, PrgK), energy transduction (InvC), regulation (InvF, HilA), transcription factors and secretory proteins (InvJ, EaeB) reviewed in (Suarez and Russmann, 1998). T3SS-1 is required for invasion by injecting effector proteins that reorganise the actin cytoskeleton inducing membrane ruffles whereas T3SS-2 influences intracellular survival by modulating the trafficking of *Salmonella* containing vacuoles.

Although they function in different stages of infection the molecular mechanisms are very similar and it is presumably the differences in the repertoire and function of the substrates of each system that dictates their respective roles (reviewed (Hansen-Wester and Hensel, 2001, Hueck, 1998). It has been proposed that mutations in genes encoding for one T3SS can directly impact on a different T3SS (Deiwick et al., 1998).

T3SS-1 plays a key role in the induction of inflammatory responses for the penetration of the bovine intestinal mucosa (Watson et al., 1995, Jones et al., 1998). T3SS-1 also plays a role in the colonisation of *S*. Typhimurium in pigs and calves but not in chickens (Carnell et al., 2007, Morgan et al., 2004).

1.6.6. Lipopolysaccharide (LPS)

The LPS is an essential part of the outer membrane of Gram-negative bacteria. The outermost hydrophilic portion of the LPS, the O side chain, is composed of repeating oligosaccharides, the type, order and number of which differ between different serovars. The core region connects the O polysaccharide with the hydrophobic lipid A antigen in the outer leaflet of the bacterial outer membrane. The O antigen is the major heat-stable antigen and lipid A is the primary agent responsible for the endotoxicity of Gram-negative bacteria (Murray, 1986). The LPS is a major virulence determinant and is associated with complement resistance, induction of immune responses and resistance to macrophage engulfment, and may play a role in bacterial adherence to epithelial cells (Craven, 1994, Turner et al., 1998). In *S*. Enteritidis, the chain length and glycosylation of the O antigen structure plays a major role in invasion and colonisation of the hen ovaries and oviduct and enhances the persistence of *Salmonella* in the avian intestinal

tract (Guard-Petter et al., 1996), however it is difficult to separate the role of LPS *per se* from its role in insertion, folding and stability of membrane proteins.

S. Typhimurium LPS directly inoculated into 3-week-old broiler chickens resulted in an increase in temperature and a decrease in the body weight. The liver weight increased whilst the bursa of Fabricius decreased in weight indicating that LPS has a profound effect on the chicken (Xie et al., 2000). The LPS plays a role in colonisation of chickens but the role is less important in young birds (Carroll et al., 2004). An LPS defective mutant of *S.* Typhimurium did not persist in the caeca to the same extent as the wild-type in day old chicks (Craven, 1994). Screening of random transposon mutants of *S.* Typhimurium F98 in chickens revealed a role for 9 genes in the colonisation of 3-week-old chicks that lacked a component of the LPS (Turner et al., 1998). Screening of STM revealed that LPS also contributes to colonisation of the intestines of calves, chickens, pigs and mice (Morgan et al., 2004, Craven, 1994, Carnell et al., 2007, Hensel et al., 1995, Lawley et al., 2006, Tsolis et al., 1999).

1.7.1. Fimbriae

Fimbriae are surface-expressed filamentous appendages of bacteria that are composed primarily of protein subunits called fimbrins and are present in all members of the *Enterobacteriaceae* family (reviewed in (Clegg and Gerlach, 1987, Thorns, 1995). In total approximately 20 fimbrial operons have been identified in the genome sequences of *Salmonella* (Chiu et al., 2005, McClelland et al., 2001, Parkhill et al., 2001). It has been proposed that multiple insertions of fimbrial operons contribute to the ability of *Salmonella* to cause disease in a range of hosts and fimbriae may determine the cell type invaded (Baumler et al., 1996a). Fimbriae have been proposed

to mediate adhesion to intestinal epithelial surfaces (reviewed in (Baumler et al., 1996a, Ernst et al., 1990, Wilson et al., 2000). In *S.* Enteritidis, virulent strains isolated from different sources were able to adhere to glass and formed visible filaments, whereas the avirulent strains did not adhere or produce filaments, indicating that virulence may be associated with the production of filaments (Solano et al., 1998).

1.7.2. Fimbrial operons and structure

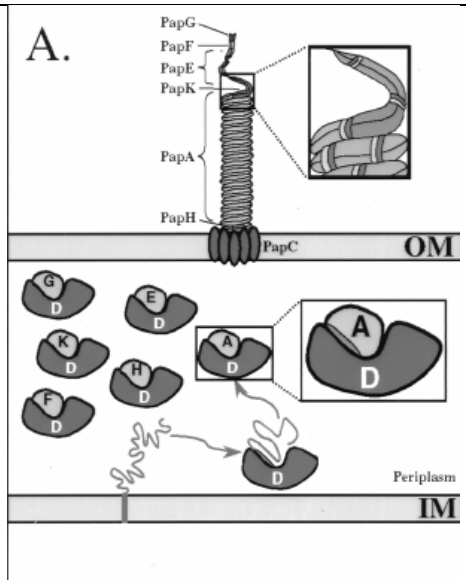
Fimbriae are composed of repeats of the major subunit (fimbrin) that translocates to the cell surface in a process requiring several accessory proteins (Fernandez and Berenguer, 2000, Thanassi and Hultgren, 2000). Fimbriae can polymerise by hydrophobic or hydrophilic interactions to form either thick rigid structures, thin flexible filaments or a combination of both (Collinson et al., 1996). Different systems exist in Gram-positive bacteria which will not be covered here (reviewed in (Telford et al., 2006, Scott and Zahner, 2006). The expression and adhesion of fimbriae is dependent on motility and temperature (Jones and Richardson, 1981, Old et al., 1986).

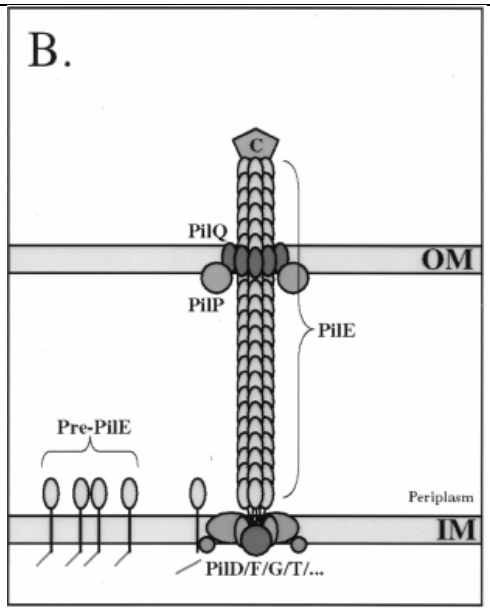
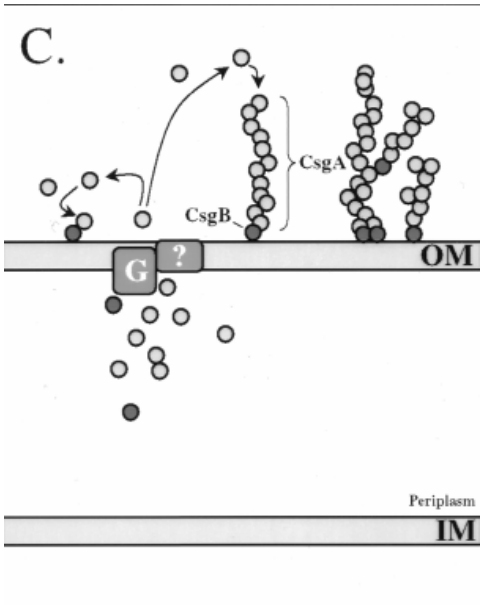
The surface-exposed fimbrial subunits are diverse and many, although not all fimbrial operons contain a predicted major fimbrial subunit protein domain within them which is more variable than the chaperone and usher protein domains (Choudhury et al., 1999, Edwards et al., 2002, Sauer et al., 1999). Several fimbrial operons contain minor fimbrial subunits and these can often be adhesive molecules such as FimH (Kisiela et al., 2006) or secondary fimbrial structures such as SefD (Clouthier et al., 1994). In all fimbrial operons except *csg,* a major fimbrial subunit is located at the 5′ end and only in some fimbrial operons do multiples of the fimbrial subunit exist which are often referred to as minor fimbrial subunits.

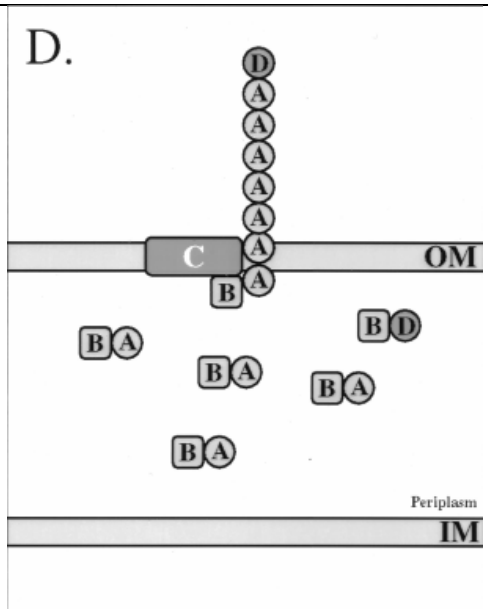
Fimbrial assembly has been separated into four distinct pathways (Table 1.1)(reviewed in (Soto and Hultgren, 1999). *Salmonella* fimbriae are known to utilise two of these pathways; the Csg encoded curli fimbriae are assembled via the extra cellular-nucleation pathway (Hultgren et al., 1991).

Table 1.1. Fimbrial assembly pathways

Reviewed in (Soto and Hultgren, 1999)

Pathway	Description	Diagram
Chaperone-usher	Requires two specialised classes of protein, a periplasmic immunoglobulin like chaperone and an outer membrane usher. The majority of fimbriae are assembled in this way (Thanassi et al., 1998). Diagram shows the assembly of P pili from *E. coli.*	

General secretion	Requires the interaction of several proteins (Hobbs and Mattick, 1993). Prepilin is processed by the peptidase which cleaves the leader peptide from the N-terminus of the subunits. The mature subunit is assembled by the inner membrane assembly complex. The adhesion is incorporated at the end of the tip. Diagram shows type IV pilus assembly in *N. gonorrhoeae.*	
Extra cellular Nucleation-Precipitation	The main component of curli is secreted across the outer membrane (Hammar et al., 1996) Diagram shows the assembly of curli from *E. coli.*	

Alternative Pathway	Requires a specialised set of periplasmic chaperones distinct from those of the chaperone-usher pathway (Soto and Hultgren, 1999). The chaperone forms complexes with the main components of the pilus. Diagram shows assembly of CS1 pili from *E. coli*.	

The cell surface-bound nucleator primes the polymerisation of secreted curlin by a nucleator-presenting cell and is extended by addition of monomers to the free end (Hammar et al., 1996). All other fimbriae are assembled by the chaperone-usher pathway which allows the association of adhesive multi-subunit fibers on the bacterial cell surface.

The usher protein facilitates the translocation of the subunits across the outer membrane to the surface. The immunoglobulin-like periplasmic chaperone stabilises the subunit by donating a strand to complete a fold within the substrate via a mechanism termed donor strand complementation (Sauer et al., 1999, Choudhury et al., 1999). The complementary beta strand is then replaced by an N-terminal extension of the next incoming subunit (Remaut et al., 2006). The chaperone consists of two conserved protein domains or Pfam domains, an N- and C-terminal protein domain and the usher contains a single Pfam domain. These domains are highly conserved in ushers and chaperones in all *Enterobacteriaceae.*

The expression of many fimbriae has not widely been studied. In *S.* Typhimurium, FimA was the only fimbrial subunit expressed at detectable levels in static growth in LB but the BcfA, LpfA, PefA, StbA, StcA, StdA, StfA and StiA fimbriae were all expressed in the bovine ileal ligated loop model as determined by a measurement of fimbrial antigens by flow cytometry (Humphries et al., 2003).

1.7.3. Biofilms

Fimbriae have been associated with the production of biofilms, a mucous layer formed on inanimate surfaces and certain cell lines. Mutations in *Salmonella csg* and *lpf* operons of *S.* Typhimurium reduced biofilm production and a mutation in the *bcf* operon increased biofilm production (Ledeboer et al., 2006, Austin et al., 1998). The production of biofilms is under the regulatory control of *csgD* (Gerstel and Romling, 2003). Different allelic variations of the same fimbriae can alter biofilm production (Boddicker et al., 2002). The Csg fimbriae may also function to stabilise the biofilm (Austin et al., 1998).

1.7.4. Fimbriae typing

The different types of fimbriae are grouped on the basis of their size, appearance, ability to agglutinate erythrocytes and the sensitivity of this process to mannose and tannic acid (Duguid et al., 1966). Fimbriae that produce a mannose-sensitive haemagglutination (MSHA) result are more adhesive than those that produce a mannose-resistant haemagglutination (MRHA) test (Tavendale et al., 1983). The differences between types can be due to a single base pair substitution. The typing

system does not reflect any sequence differences or similarities and will not be referred to in this study.

1.7.5. *S.* Enteritidis fimbriae

S. Enteritidis produces fimbriae that are morphologically indistinguishable from the fimbriae of all other *Enterobacteriaceae* but the fimbrial proteins are named after the protein subunit size. They share homology with fimbriae from other *Salmonella* serovars which have a different nomenclature (Muller et al., 1991, Feutrier et al., 1986). Primarily SEF14, SEF17 and SEF21 have been studied.

The SEF14 gene cluster consists of 3 genes *sefABC* and are composed of repeating protein subunits of 14 kDa and has only relatively recently been acquired (Thorns et al., 1990). The structural gene (*sefA*) encoding for SEF14 has been identified and sequenced and has a limited distribution across *Salmonella* (Turcotte and Woodward, 1993). SEF17 comprises protein subunits of 17 kDa with a unique N-terminal sequence and are compromised of polymerised CsgA proteins. There is a high degree of similarity between SEF17 and CsgA of *E. coli* (Collinson et al., 1996). SEF21 refers to the product of the *fimA* gene (Muller et al., 1991). The FimA protein of *S.* Enteritidis in the rat was distributed throughout the gut mainly in the large intestine and lumina contents. Purified FimA was isolated in the gut in a similar pattern to that found with whole *Salmonella* (Naughton et al., 2001).

Throughout this thesis, the *Salmonella* fimbrial genes will be referred to by name rather than the size of the protein formed. The best characterised fimbrial loci in *Salmonella* are the *fim, lpf, csg*, and *sef* operons. The genetics, assembly and role of fimbriae encoded by these operons are reviewed below.

1.7.6 *E. coli* fimbriae

The complete genome sequence of *E. coli* K-12 MG1655 has been available for several years and different types of fimbriae have been identified and in some cases a role has been determined for adhesion and colonisation of chickens (Blattner et al., 1997).

The *stg* fimbriae from avian pathogenic *E. coli* (APEC) play a significant role in the colonisation of the avian respiratory tract (Lymberopoulos et al., 2006). The type 1 fimbriae have been shown to play an important role in the colonisation of avain pathogenic *E. coli* in breeders whereas the P and S fimbriae were not required for bacterial adherence. The the absence of P fimbriae promotes bacterial adhesion (Mellata et al., 2003),(Monroy et al., 2005). The presence of fimbriae and flagellar on the cell surface of *E. coli* O87:K80 play a major role in colonisation, invasion and persistence in chickens (La Ragione et al., 2000).

Several other bacterial species also possess fimbriae including P pili, type 1 fimbriae, tpe IV pili, curli and CS1 pili. These different type of fimbriae have been mainly characterised in different strains of *E. Coli* but are also present in several other species of the *Enterobactericeae* family including *Neisseria, Klebsiella pneumonia* and *Yersinia enterocolitica* reviewed (Soto 1999).

1.7.7. The *fim* operon

The *fim* operon consists of 9 genes in two operons, *fimA, fimI, fimC, fimD, fimH,* and *fimF* in one operon and *fimZ*, *fimY* and *fimW* in a convergently transcribed second operon (McClelland et al., 2001). Downstream of *fimA* is the *fimI* gene, which also encodes a fimbrin protein. There is a similar genetic organisation between *fimA* and *fimI* and the *fimI* gene may have originated from a duplication of *fimA* (Rossolini et al.,

1993). The *fimA* gene is adjacent to the *folD* gene and the *fimA-folD* intergenic region of *Salmonella* encompasses a junctional site of genetic rearrangement probably due to chromosomal relocation, suggesting that the *fim* operon was gained through horizontal gene transfer. The *fim* operon does not have regulatory genes directly upstream as in *E. coli* (Rossolini et al., 1994). In *Salmonella* the *fim* operon is in a different chromosomal location to the homolog in *E. coli* and the *fimWYZ* regulatory operon has a lower % G+C content than the genomic mean (Boyd and Hartl, 1999). The *fimA* promoter is always orientated so that transcription can occur (Clegg et al., 1996), in marked contrast to the location of the *fimA* promoter to an invertible element subject to recombinase-mediated inversion and thus phase-variable expression of Type 1 fimbriae in *E. coli* (Abraham et al., 1985).

The *fimC* gene encodes a 26 kDa polypeptide which contains a chaperone domain similar to the chaperone *papD* in *E. coli*. The chaperones are highly conserved and may fold and function in a similar way (Bonci et al., 1997). The *fimD* gene encodes an usher and in *E. coli* the protein was found to be located in the outer membrane and when produced on its own has deleterious effects on growth (Klemm and Christiansen, 1990). A *fimD* mutation in *S.* Enteritidis resulted in no adherence *in vitro* or in chickens and resulted in prolonged bacteraemia and reduced egg shell contamination, it was also incapable of adhering to the isthmal secretions which are involved in generating fibers of the egg shell membrane (De Buck et al., 2004, De Buck et al., 2003). The FimD protein is involved in the export and assembly of FimA (De Buck et al., 2004). The *fimH* gene encodes an adhesin (Jones et al., 1995). The FimD, FimC and FimH proteins form a complex to protect the usher from degradation *in vivo* due to a conformational change in the usher (Saulino et al., 1998). The cloning and sequencing of the *fimH* gene from different *S.* Typhimurium strains resulted in a range of products being

characterised that differed in their ability to adhere to HEp-2 cells (Boddicker et al., 2002). A mutation in *fimH* can produce non-fimbriate, non-adhesive *Salmonella* and it appears that the adhesive properties of Fim are due to both the fimbrial shaft and the fimbrial tip (Thankavel et al., 1999, Duncan et al., 2005, Hancox et al., 1997). Recombinant FimH adhesins of type 1 fimbriae of *S*. Gallinarum and *S*. Pullorum do not bind to mannose oligosaccharides or to HT29 human colon carcinoma cells due to a single base pair mutation of FimH at position 78; resulting in a isoleucine to threonine substitution (Kisiela et al., 2005). Differences seen between the low and high adhesive properties of the type 1 fimbriae of two serovars, *S*. Enteritidis and *S*. Typhimurium, are partially due to only 4 amino acid substitutions in FimH and the differences in adherence between two strains of *S*. Typhimurium was due to only 2 amino acid substitutions (Kisiela et al., 2006). FimH is also required for efficient interactions with DCs and mutations in *fimH* result in an impaired ability of *S*. Typhimurium to bind and be internalised (Guo et al., 2007).

The *fim* operon has 4 regulatory genes downstream of the first *fim* operon; *fimZ, fimY, fimU* and *fimW* and the expression of FimA is controlled at the transcriptional level by the products of *fimZ,* a transcriptional activator which works in co-operation with *fimY*, a positive regulator (Tinker et al., 2001, Yeh et al., 1995, Tinker and Clegg, 2000). FimZ may be a DNA-binding protein and plays a role in its own expression along with FimY (Yeh et al., 2002). The increased expression of FimZ results in a hyper-fimbriated non-motile *Salmonella* in soft agar (Clegg and Hughes, 2002). FimZ is the molecular connection between flagella and fimbriae (Clegg and Hughes, 2002). The *fimW* gene is a negative regulator of Type 1 fimbriae expression and mutations in this gene result in a 4-8 fold increase in the number of fimbriae produced (Tinker et al., 2001). The *fimW* gene is initiated from its own promoter and the encoded protein may

function by interfering with FimZ activation of FimA expression (Tinker et al., 2001). The FimU is an activator of FimY and a mutation in the *fimU* gene results in an afimbriate phenotype (Tinker and Clegg, 2001). A *fimU* mutant in *S.* Enteritidis resulted in a decrease in expression of both FimA and SefA (Clouthier et al., 1998b). Chickens infected with *S.* Enteritidis not expressing *fimA* were less intensively colonised and had less faecal shedding than the wild-type in laying hens (Thiagarajan et al., 1996) but the same mutation appears to play no role in the colonisation of non-laying chickens (Rajashekara et al., 2000).

Mutations in *fim* of *S.* Enteritidis resulted in a decrease in adherence and invasion of cultured epithelial cells (Dibb-Fuller et al., 1999) and in *S.* Typhimurium a decreased attachment of HeLa cells (Baumler et al., 1996a). *In vivo* in combination with other fimbrial genes *fim* of *S.* Typhimurium plays a role in the colonisation of mice and production of murine typhoid fever (van der Velden et al., 1998, Lockman and Curtiss, 1992).

1.7.8. Long polar fimbriae (*lpf)*

In *S.* Typhimurium, the *lpf* operon contains 5 genes *lpfA, lpfB, lpfC, lpfD* and *lpfE* and the operon has entered the *Salmonella* genomes early as it has a scattered phylogeny and has been lost from many serovars (Baumler and Heffron, 1995, Baumler et al., 1997a). The genes flanking the *lpf* operon in *S.* Typhimurium have homology to the flanking genes in enterohaemorrhagic *Escherichia coli* (EHEC), and the *lpf* operon has inserted into a similar chromosomal position (Torres et al., 2002). EHEC has 2 *lpfC* genes, encoding proteins of 40.2 kDa and 17.8 kDa respectively and *S.* Typhimurium has 1 *lpfC* gene encoding a protein of 94.4 kDa suggesting a merge of two genes in

Salmonella or a separation in EHEC (Torres et al., 2002). The EHEC *lpf* operon is 60 % identical to that of *S.* Typhimurium. The *lpfA* and *lpfE* genes in *E. coli* are translationally coupled as are the *lpfA* and *lpfC* genes, it is unknown if this translational coupling occurs in *Salmonella* (Torres et al., 2002).

A mutation in the *lpfC* gene of *S.* Typhimurium resulted in a 5-fold increase in the median lethal dose of mice, a decrease in the numbers of bacteria isolated from the Peyer's patches, mesenteric lymph nodes, liver and spleen as well as impaired destruction of M cells (Baumler et al., 1996b). The *lpf* operon of *S*. Typhimurium was also required for adherence to and invasion of HEp-2 cells (Baumler et al., 1996a). Multiple fimbrial mutations including the *lpf* operon resulted in an 26-fold increase in the LD_{50} of orally inoculated mice with *S*. Typhimurium implying a synergistic effect (van der Velden et al., 1998). Genome-wide and targeted mutagenesis have implicated Lpf fimbriae in *Salmonella* pathogenesis in mice and the major fimbrial subunit is up-regulated in *S*. Typhimurium in a ligated bovine ileal ligated loops, suggesting a role in virulence or colonisation (Humphries et al., 2003, van der Velden et al., 1998, Weening et al., 2005, Lawley et al., 2006).

1.7.9. Csg fimbriae

The Csg fimbriae are also known as the Agf fimbriae or thin aggregative fimbriae (tafi) and in *S*. Enteritidis are known as SEF17. Throughout this thesis they will be referred to as Csg or curli fimbriae. The *csg* fimbrial operon contains 7 genes in two operons, *csgB, csgA* and *csgC* in one operon and *csgD, csgE, csgF* and *csgG* in a divergently transcribed operon. The *csg* operon is believed to be the oldest fimbriae as it is present and highly conserved in *E. coli* (Baumler et al., 1997a, Romling et al.,

1998a). The Csg fimbriae are assembled via the nucleator-dependent assembly pathway and are the only *Salmonella* fimbriae currently identified that assemble in this way (Hammar et al., 1996). The expression of the curli fimbriae is temperature and pH dependent, the majority of strains of *S*. Enteritidis examined express curli when grown at 18-30 ºC, however for some strains of *S*. Enteritidis expression only occurs at 37 ºC or 42 ºC (Dibb-Fuller et al., 1997). Csg were also produced by some strains at 20 ºC but not at 37 ºC and only at pH 6.18 or above (Walker et al., 1999).

The *csgBAC* operon encodes thin aggregative fimbriae which are fibrous and polymeric and are composed of repeats of the major subunit CsgA (White et al., 2001). The CsgA is the subunit and CsgB is the surface-exposed nucleator protein (Gerstel and Romling, 2003). CsgA and CsgB share 51 % amino acid sequence and dimers form between CsgA-CsgA, CsgB-CsgB and CsgA-CsgB producing a highly rigid multicellular stable structure which is controlled at the level of transcription by *csgD* (Romling et al., 1998b);(White et al., 2001). The CsgA fimbrin is a pathogen-associated molecular pattern (PAMP) and triggers the hosts innate immune response causing inflammation (Tukel et al., 2005). A mutation in *csgBA* of *S*. Typhimurium resulted in an inflammatory response, a decrease in the fluid accumulation, and a decrease in the IL-8 production in macrophages in streptomycin-pretreated mice (Tukel et al., 2005). The curli of *S*. Typhimurium can also bind flagellin and the bound flagella may modulate immune responses in this manner (Rochon and Romling, 2006).

The expression of the curlin protein is dependent on the starvation-induced CsgD protein, a positive transcriptional activator of the LuxR family. CsgD has an N-terminal receiver domain, a helix-turn-helix motif and a DNA-binding motif at the C-terminus (Gerstel et al., 2003). The expression of the CsgD protein results in altered transcription of 24 novel genes and the *csgD* gene is itself controlled by a variety of regulators,

including a transcriptional activator upstream (Brombacher et al., 2006, Gerstel and Romling, 2003). The *csgD* gene regulates two distinct pathways, both of which contribute to multicellular morphology (Romling et al., 2000).

The CsgC protein is localised to the periplasm and a mutation of *csgC* results in the production of fibers of 20 nm, much larger than the usual curli which are usually 5-7 nm which results in an increase in the surface hydrophobicity. The *csgC* and *csgE* genes are important for extracellular assembly of curli fibers (Gibson et al., 2007). CsgG is an outer membrane lipoprotein and is required for the secretion of CsgA and CsgB (Loferer et al., 1997). In *E. coli* the *csgE, csgF* and *csgG* each play a role in the formation of *csgA* (Hammar et al., 1995) and in *S.* Typimurium the *csgEFG* function as accessory genes, although their precise role is unclear, they appear to contribute to long-term systemic infection in mice (Lawley et al., 2006, Gerstel et al., 2003).

Multiple fimbrial mutations including the *csg* operon resulted in an 26-fold increase in the LD_{50} of orally inoculated mice with *S.* Typhimurium and implies that the Csg fimbriae have a synergistic effect (van der Velden et al., 1998). Curli-deficient *S.* Enteritidis strains were able to reproduce more rapidly inside less eggs than the curli-expressing *S.* Enteritidis (Cogan et al., 2004). Mutations in curli of *S.* Enteritidis delay the colonisation of the spleen and liver of chickens for the first 24 hours post-infection (Dibb-Fuller and Woodward, 2000).

1.7.10. Sef fimbriae

The *Salmonella*-encoded fimbriae or *sef* operon consists of five genes four co-transcribed genes (*sefA, sefB, sefC* and *sefD)* and a regulatory gene *sefR* (or *sefE*) which is homologous to an *araC*-like positive transcriptional activator (Edwards et al., 2001,

Collighan and Woodward, 2001, Clouthier et al., 1993). The SefA protein assembles to form a thin filamentous fimbrin structure, SefB is a chaperone, SefC is an outer membrane usher and SefD is the tip-located adhesin (Clouthier et al., 1993, Edwards et al., 2001). SefB and SefC cannot be expressed in the absence of SefA due to translational coupling and SefC has a predicted signal sequence (Clouthier et al., 1993). The *sef* operon has a much lower % G+C content than the genomic mean and it has a scattered presence across the *Salmonella* serovars implying that it may have been acquired through horizontal gene transfer (Edwards et al., 2001). Further to support this is the presence of an insertion like element adjacent to the operon (Collighan et al., 2000).

Expression of Sef was optimal during growth in late exponential phase and was repressed during stationary phase (Edwards et al., 2001). Sef expression appears to be regulated by *fimU* (Clouthier et al., 1998b). The Sef protein of *S*. Enteritidis mediate adhesion to inanimate objects at low temperatures but the adhesion is lost at 37 ºC (Woodward et al., 2000), however Sef expression was detected by ELISA only at 37 ºC and at pH 4.77 and above, showing strain-to-strain variation (Walker et al., 1999).

S. Enteritidis contains the *sef* fimbrial operon which influences virulence in mice and internalisation into macrophages (Edwards et al., 2000). Polar mutations affecting the entire *sef* operon of *S*. Enteritidis resulted in a decrease in virulence in mice of 1000-fold. A non-polar mutation affecting only *sefA* had no effect on virulence but a non-polar *sefD* mutant resulted in a severe virulence defect in mice. The product of *sefD* influences efficient uptake by macrophages (Edwards et al., 2000). A mutation in the *sef* operon of *S*. Enteritidis results in lower numbers of bacteria being isolated from the livers and a faster clearance rate from the spleen in chickens (Rajashekara et al., 2000). The SefA protein has potential vaccine properties as a recombinant plasmid containing

SefA given orally to day-old-chickens reduced the colonisation of an experimental challenge of *S*. Enteritidis (Lopes et al., 2006).

The SefA protein is insoluble in most detergents and in SDS can depolymerise into monomers, dimers and other multimers. SefA fimbrins multimerise through the N-terminus and undergo changes before assembling into fibers. Once the fibers are formed the subunits are held in contact with each other by hydrophobic interactions (Clouthier et al., 1998a).

1.7.11. Bcf fimbriae

The bovine colonisation fimbriae are encoded by the *bcf* operon which consists of eight genes in one large polycistronic unit *bcfA, bcfB, bcfC, bcfD, bcfE, bcfF, bcfG* and *bcfH*. It is located between the *dnaJ* and *nhaA* in *S.* Typhi and between *uvrB* and *yphK* in *S.* Typhimurium on a 30 kb region absent from *E. coli* K-12 (Townsend et al., 2001). The BcfA fimbriae of *S.* Typhimurium has been found to be up-regulated in the bovine ileal ligated loop model as detected by flow cytometry (Humphries et al., 2003).

Genome-wide and targeted mutagenesis have implicated the *bcf* genes in *S.* Typhimurium pathogenesis in mice (van der Velden et al., 1998, Weening et al., 2005, Lawley et al., 2006). However, although the *bcf* operon was identified as being required for colonisation in mice it appears to play no role in calves and the nomenclature may therefore be inappropriate (Tsolis et al., 1999).

1.7.12. Saf fimbriae

Salmonella atypical fimbriae (saf) comprise a non-fimbrial adhesin and are encoded by four genes (*safA, safB, safC* and *safD)*. In *S*. Typhi *safE* also exists downstream of

safA (Parkhill et al., 2001). The SafB protein is a chaperone, SafC an usher and SafD is an adhesin that is similar to AFA adhesins of *E. coli* (Folkesson et al., 1999). The *saf* operon is located on a large region that is absent from *E. coli* K-12 (Folkesson et al., 1999, Folkesson et al., 2002).

The SafB and SafD proteins form a complex fimbrial adhesin, which is sensitive to low pH and enzymatic degradation and can induce different immune responses in mice depending on the route of administration (Strindelius et al., 2004). The *safA* gene has a more heterogeneous sequence than the *safB* and *safC* genes. A transposon mutation in the *saf* operon of *S.* Typhimurium resulted in a decrease in colonisation of pigs but not in chickens or calves (Carnell et al., 2007, Morgan et al., 2004).

1.7.13. Other fimbriae

With the completion of the genome sequences of *S.* Typhimurium LT2 and *S.* Typhi CT18 (McClelland et al., 2001, Parkhill et al., 2001) several other fimbrial loci were identified for the first time. These have been designated st followed by a letter from a-g and little is known of their role or the assembly process.

The *sta* operon has orthologues in *E. coli* K-12 *(yadCKLM),* as does the *stc* operon (*yehDCBA).* The *stb, stg, std, ste* and *sth* operons are all absent in *E. coli* K-12 (Townsend et al., 2001). The *stf*-encoded fimbriae have been identified in *S.* Typhimurium and are absent from *S.* Typhi and *S. bongori*. The *stf* operon is flanked by *fhuB* and *hemL* whereas in *E. coli* these genes are adjacent to each other indicating that it is likely a genomic insertion (Emmerth et al., 1999). The *stg* fimbrial operon is encoded in the *glmS-pstS* intergenic region in *S.* Typhi and in some *E. coli* strains. In *S.* Typhi, the *stgC* gene contains a pseudogene but it appears that the gene products of the

stg operon are important as in its absence a decrease in adherence and an increase in macrophage uptake is seen (Forest et al., 2007). The *stj* fimbrial operon has been identified in *S.* Typhimurium LT2 but consists of only 2 genes with homology to a chaperone and an usher, it is unknown if it is functional (McClelland et al., 2001). The *tcf* fimbrial operon is present in *S.* Typhi and has similarity to the *coo* operon encoding the CS1 fimbrial adhesin in *E. coli* and the operon consists of 4 genes *tcfA, tcfB, tcfC* and *tcfD* (Folkesson et al., 1999). The *tcf* operon is not unique to *S.* Typhi as was once believed (Townsend et al., 2001).

The role of the st(a-g) fimbriae is poorly defined and it has been proposed that they may carry out several subtle roles at different points during infection (reviewed in (Baumler et al., 1997b). The *stc* and *sth* operons of *S.* Typhimurium have been implicated in contributing to gastrointestinal colonisation and long-term systemic disease in mice (Lawley et al., 2006), the *stb, stc, std* and *sth* of *S.* Typhimurium are required for intestinal persistence in mice (Weening et al., 2005) and the *stbB* and *sthC* genes are required for colonisation in chickens but not calves (Morgan et al., 2004).

1.8.1. Phase Variation

Phase variation refers to the reversible switch between all-or-nothing expression of a factor and is different from gene regulation as the change is heritable and reversible in a given population and often occurs at a rate of 1 change per 10^3 generations (reviewed in (van der Woude and Baumler, 2004, Casadesus and Low, 2006). In other Gram-negative bacteria phase variation is known to be mediated by recombination (e.g. FimBE-mediated inversion of a 314 bp segment containing the *fimA* promoter in *E. coli* (Abraham et al., 1985, Klemm, 1986), epigenetic regulation dependent on Dam

methylation (e.g. control of Pap pili in uropathogenic *E. coli* (Blyn et al., 1990)) or slipped-strand mispairing between homo- or hetero-polymeric tracts during DNA replication (e.g. assembly and maturation of Neisserial pilin (reviewed in (Meyer and van Putten, 1989, van Belkum et al., 1998).

In *Salmonella* evidence exists for phase variable expression of Type I fimbriae which is not the same as the phase variation that exist for Type I fimbriae of *E. coli* (Abraham et al., 1985, Old and Duguid, 1970, Swenson and Clegg, 1992) and long polar fimbriae (Norris et al., 1998). Further, epigenetic regulation of the expression of *pef* fimbrial genes in *S.* Typhimurium by Dam methylation has been described (Nicholson and Low, 2000) and transcription of *std* fimbrial genes have been observed to be repressed in a *S.* Typhimurium Dam methylase mutant (Balbontin et al., 2006).

Phase variation may be sensitive to environmental stimulus, acid conditions have been reported to stimulate Dam methylation of the GATC sites in *Salmonella* (Nicholson and Low, 2000), and in *E. coli* temperature and growth media have been implicated in altering phase variation (Gally et al., 1993, Clegg et al., 1996). The change in response to the environment may allow a proportion of the bacterial population to survive sudden changes in the environment (Norris et al., 1998).

Phase variation of the *lpf* operon has also been proposed to be a mechanism to evade cross immunity between serotypes by changing the antigenic properties of the cell surface. If a host has encountered the Lpf fimbriae of *S.* Typhimurium it will select against *S.* Enteritidis that is also expressing the Lpf fimbriae, but if Lpf expression can be switched off then *S.* Enteritidis can colonise the same host, indicting that Lpf cannot be essential for colonisation (Nicholson and Baumler, 2001, Norris and Baumler, 1999).

Most *Salmonella* cells are richly fimbriated but a proportion of the cells are always in the off phase. After passage of *S*. Typhimurium in LB broth for 120 generations, 96 % of the cells were carrying *lpf* in the on-phase; however after 500 generations on an LB solid media plate only 2 % were in the on phase. The differences were due to changes in the on-to-off and off-to-on rate of phase variation and the starting culture (Kingsley et al., 2002). The genetic mechanisms underlying such regulation are ill-defined and it remains unclear if other *Salmonella* fimbriae are subject to phase variable expression.

1.9.1. Rationale for the project

Given the important role of fimbriae in adherence and colonisation of different hosts and given that *Salmonella* serovars vary both in virulence and tissue tropism it is a reasonable hypothesis that these differences are due to fimbriae.

The repertoire of fimbrial operons in different *Salmonella* serovars has been examined using different techniques. Micro-array hybridisation studies were used to separate core regions of the genome using decision trees (Anjum et al., 2005), comparative genomic hybridisation to micro-arrays and Southern hybridisation have produced results that contain ambiguous data (Porwollik et al., 2004, van Asten and van Dijk, 2005) and analysis of strain variation of the same serovars indicated differences (Boyd et al., 2003). The availability of several whole genome sequences of *Salmonella* strains has made it possible to carry out the first large-scale genome comparison of the sequence and repertoire of *Salmonella enterica* fimbrial operons in host-specific and ubiquitous serovars in fast and efficient manner (Chiu et al., 2005, McClelland et al., 2001, Parkhill et al., 2001). With the recent sequencing of the genome of an *S*. Enteritidis PT4 strain and given the importance of such strains in human health, this

project will include a comprehensive analysis of its fimbriae *in vitro* and *in vivo,* to attempt to link genotype to phenotype.

Thesis Aims

- To analyse the repertoire sequence and chromosomal location of fimbrial genes within the genome sequences of several strains of *Salmonella* using *in silico* techniques.
- To mutate all chromosomal fimbriae in *S. enterica* serovars Enteritidis and Gallinarum
- To characterise the fimbrial mutants *in vitro* using cell adherence and invasion assays, microscopy and protein expression analysis.
- To characterise the fimbrial mutants *in vivo* in the avian host.
- To verify the phenotype/s produced using *trans*-complementation techniques to fulfill molecular Kochs postulates.

Chapter 2

Materials and Methods

2.1. Strains, plasmids, reagents and media

2.1.1. Strains

Forty *Salmonella enterica* strains isolated from a range of hosts were used in this study and are listed in Table 2.1. *S.* Enteritidis S1400 was used in the initial stages of mutant construction and *Escherichia coli* K-12 strain MG1655 was used as a non-invasive control.

Table 2.1. Bacterial strains used in this study

Serovar and strain	Host	Reference or source
Arizona KMS	Turkey	IAH field isolate, 1977
Arizona S1489	Turkey	1977 VLA, Weybridge, Surrey, UK.
Dublin 2229	Bovine	(Baird *et al.*, 1985).
Dublin S1326	Bovine	VLA, Weybridge, Surrey, UK, 1973.
Dublin Curry	Bovine	IAH, field isolate.
Enteritidis 1714-03 PT11	Horse	VLA, Weybridge, Surrey, UK.
Enteritidis 4247	Poultry	Public Health Laboratory, Colindale 1991.
Enteritidis Ex Ross PT4	Poultry	Public Health Laboratory, Colindale 1988.
Enteritidis P121779 PT4	Poultry	Public Health Laboratory, Colindale 1988.
Enteritidis P125109 PT4 [a]	Poultry	Public Health Laboratory, Colindale 1988.
Enteritidis S1400 PT4 [a]	Poultry	VLA, Weybridge, Surrey, UK.
Enteritidis P125588 PT4	Poultry	Public Health Laboratory, Colindale 1988.
Enteritidis P125592 PT4	Poultry	Public Health Laboratory, Colindale 1988.
Enteritidis PT6	Poultry	(Evans *et al.*, 1998).
Enteritidis PT8	Poultry	University of Pennsylvania, C Bowen.
Enteritidis S-2334-03 PT9B	Duck	VLA, Weybridge, Surrey, UK.
Enteritidis S-2693-03 PT12	Chicken	VLA, Weybridge, Surrey, UK.
Enteritidis S-3405-04	Chicken	VLA, Weybridge, Surrey, UK.
Enteritidis S-4687-03	Pheasant	VLA, Weybridge, Surrey, UK.
Enteritidis S-1810-03	Chicken	VLA, Weybridge, Surrey, UK.

Enteritidis S-3688-04	Chicken	VLA, Weybridge, Surrey, UK.
Enteritidis S-523-04	Chicken	VLA, Weybridge, Surrey, UK.
Enteritidis S-7016-03	Pig	VLA, Weybridge, Surrey, UK.
Enteritidis S-4850-04	Bovine	VLA, Weybridge, Surrey, UK.
Gallinarum 1026	Chicken	Athens, 1979.
Gallinarum 287/91 [a]	Poultry	Angelo Berchiere, Brazil.
Gallinarum 72/80	Poultry	University of Nairobi, Tony Harris, 1986.
Gallinarum 8338/1	Chicken	Christensen/Olsen, Denmark, 1993.
Gallinarum 95/80	Chicken	University of Nairobi, Tony Harris, 1986.
Gallinarum 9	Poultry	(Barrow and Lovell, 1989).
Pullorum 2249	Chicken	IAH, field isolate.
Pullorum 449/87	Chicken	VLA, Weybridge, Surrey, UK.
Pullorum 5078	Chicken	(Li *et al.*, 1993).
Typhimurium 333 PT49	Human	Public Health Laboratory, London, 1985.
Typhimurium SL1344	Bovine	(Wray and Sojka, 1978).
Typhimurium 68/67 PT56	Bovine	IAH, field isolate, 1967.
Typhimurium bangor PT44	Bovine	Public Health Laboratory, Colindale, 1978
Typhimurium F98 PT14	Chicken	(Barrow and Lovell, 1989)
Typhimurium S1622 PT104	Chicken	VLA, Weybridge, Surrey, UK, 1984.
Typhi *aroCD*	Lab. strain	Steve Chadfield, 1999.
E. coli K-12 MG1655	Standard laboratory strain due to suitablitly for genetic rearrangements (Zinder and Lederberg, 1952)	

[a] These strains are those used to make fimbrial mutants. All other strains were used in dot blot analysis.

All strains were confirmed at the outset using anti-*Salmonella* serum O9 for *S.* Enteritidis, *S.* Dublin, *S.* Pullorum and *S.* Gallinarum, O4 for *S.* Typhimurium and H antigens were used to distinguish between the different O9 groups according to the Kauffmann-white scheme. All *Salmonella* serovars were confirmed to possess an intact LPS by the use of acriflavine-HCl as detailed in section 2.4.4.

2.1.2. Plasmids

Several plasmids used in this study for the construction of fimbrial mutants and for *trans*-complementation are listed in Table 2.2.

Table 2.2. Plasmids used in this study

Plasmid	Details/ Description	Source	Reference
pKD46	Temperature-sensitive origin of replication. Ampicillin resistance *gam, bet* and *exo* genes under arabinose-inducible promoter.	Barry Wanner	(Datsenko and Wanner, 2000)
pKD3	Derivative of pSC140, used as PCR template to amplify FRT flanked chloramphenicol resistance cassette for mutant construction.	Barry Wanner	(Datsenko and Wanner, 2000)
pACYC177	Derivative of P15A, cloning vector, ampicillin and kanamycin resistance	New England Biolabs (NEB)	(Chang and Cohen, 1978)
pCP20	Temperature-sensitive origin of replication. Ampicillin and chloramphenicol resistance. Expresses FLP recombinase.	Barry Wanner	(Cherepanov and Wackernagel, 1995)
pCR®4Blunt-TOPO	Vector for topoisomerase-mediated cloning of blunt-ended amplicons or fragments, ampicillin and kanamycin resistant.	Invitrogen	

2.1.3. Media and reagents

All media were obtained from Microbiological Services, IAH (supplied by Sigma, Difco or Oxoid) and were sterilised by autoclaving prior to use. Unless otherwise stated all strains were cultivated in Luria-Bertani (LB) broth in a Unitron INFORS HT incubator at 37 ºC and 130 revolutions per minute (rpm).

LB broth: 25 g LB broth base (Miller # 244610) comprising 10 g peptone, 140.5 g yeast extract and 10 g NaCl dissolved in 1 litre of sterilised water.

LB agar: 25g LB broth base (Miller # 244510) and 15 g bacto agar dissolved in 1 litre of sterilised water.

Antibiotic supplements: LB agar or broth was supplemented with antibiotics (Sigma) to a final concentration of chloramphenicol cat. # C0378 (25 µg/ml), ampicillin cat # A9518 (100 µg/ml), novobiocin cat # N1628 (1 µg/ml) and nalidixic acid cat # N4382 (20 µg/ml) where appropriate. Typhi *aroCD* mutant was grown in LB broth supplemented with 10 mg/ml tyrosine and 1 mg/ml each of tryptophan, phenylalanine, p-aminobenzoic acid (PABA) and di-hydroxybenzoic acid (DAB).

Brilliant green agar: 58.0 g Brilliant green agar base (Difco #228530) comprising 10 g protease peptone, 3 g yeast extract, 10 g lactose, 10 g saccharose, 5 g NaCl, 20 g agar, 12.5 mg brilliant green and 0.08 g phenol red dissolved in 1 litre of sterilised water.

SOC: 31 g SOC powder (Q-BIO gene # 3031-012) comprising 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 5 g $MgSO_4.7H_20$ and 20 mM glucose dissolved in 1 litre of SQW water.

DMEM: Dulbecco's minimal essential medium supplemented with 3 g/litre of sodium bicarbonate with L-glutamine(Sigma # D7777).

EMEM: Eagles minimal essential medium supplemented with 2 g/litre of sodium bicarbonate with L-glutamine (Sigma # M0268)

RPMI 1640: Supplemented with 2 g/litre of sodium bicarbonate with L-glutamine (Sigma # R6504).

Phenol:chloroform:isoamyl alcohol 24:1: (Sigma # P3803)

CTAB: 10 % (w/v) hexa-decyl-trimethyl ammonium bromide in sterilised water (Sigma # 83935).

Isopropanol: (VWR #133)

Ethanol: (VWR #101077Y)

EDTA: Ethylenediaminetetraacetic acid disodium salt (Sigma # E7889)

Proteinase K: (Sigma #P6556)

RNase: (#R4875)

Paraformaldehyde: (Sigma # 158127)

Vectashield: (Vector labs #H1400)

Formic acid: (VWR #83634)

5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT): (Sigma # B5655)

Zero blunt TOPO PCR cloning kit: – Salt solution (1.2 M sodium chloride and 0.06 M magnesium chloride) and pCR4Blunt TOPO vector (Invitrogen #K286020)

Herculase: (Stratagene #600310) comprising *pfu* DNA polymerase, Archae Maxx polymerase and *Taq* 2000.

TOP10 *E. coli* cells: (Invitrogen # C664-11)

IPTG – isopropyl-beta-D-thiogalactopyranoside: (Invitrogen #15520019)

Ligafast T4 DNA ligase: (Promega # 9PIM822)

Agarose: (Sigma # A9539)

Ethidium bromide: 10 mg/ml (Biorad #161-0433)

1 kb DNA ladder: (Invitrogen # 10787-026)

Hydrochloric acid: (VWR # 190686W)

Sodium hydroxide: (Sigma # 30620)

Sodium chloride: (Sigma # S7653)

Tri-sodium citrate: (Sigma #32320)

Trizma hydrochloride: (Sigma # T5941)

dNTPs: (Promega #U1240)

Sodium dodecyl sulphate: (Sigma # L4390)

DIG Easy Hyb granules: (Roche # 11796895001)

DIG Wash and block buffer set: (Roche # 11585762001)

Tween 20: (Sigma # P9046)

Anti-digiocigenin-AP: (Roche # 11093274910)

PCR DIG labelling kit: (Roche # 11585550910)

CSPD disodium-3 (4-methoxyspiro-[1,2-dioxetane-3,2(5'chloro)tricycle[3.3.1.1]decan]-4-yl: (Roche # 11755633001)

Foetal bovine serum: (Autogen #318A)

Triton 100: (Sigma # 234729)

Haemacolour stains: (Merck # 1.11661.0001)

***ClaI, DpnI, HindIII*:** (NEB # RO197L, #RO176L, #RO104L)

Saline: 0.9 % (w/v) sodium chloride in sterilised water.

TE buffer: 10 mM Tris-Cl (pH 7.5) and 1 mM EDTA in sterilised water (made in house).

SSC: 3 M NaCl and 0.3 M sodium citrate in sterilised water (Invitrogen #15557044).

TAE: 400 mM Tris-acetate and 10 mM EDTA in sterilised water (Invitrogen #15558-042). Sodium acetate

Loading buffer: 250 mg bromophenol blue in 33 ml of 150 mM Tris (pH 7.6), glycerol and 7 ml of water.

PCR Taq polymerase: – comprising 10x PCR buffer, 1.5 mM Magnesium chloride, ***Taq* DNA polymerase**: (Invitrogen #10342-020)

Anti-Salmonella O serum: Wellcome, diagnostics Dartford UK

Anti-rabbit-Ig Alexa^{568}: (Invitrogen # A10037)

Phalloidin flouroscein-isothiocyanate-conjugate: (Invitrogen # F432)

Gentamycin: 50 mg/ml (Sigma #G1397)

Permanent stocks of all strains and plasmids were maintained as stationary phase cultures in LB broth supplemented with 20 % (v/v) glycerol and stored at -70 ºC. For growth, the strains were streaked from permanent stocks to single colonies on LB agar plates supplemented with antibiotics where appropriate and incubated for 16-18 h at 37 °C statically. Liquid cultures were inoculated using a streak of colonies and incubated as described above. All *Salmonella* strains were confirmed by the use of serology testing as described in Section 2.4.4.

2.2. *In silico* tools

2.2.1. Source of sequences

The complete genome sequences of *Salmonella enterica* serovar Enteritidis PT4 strain P125109, *S.* Gallinarum strain 287/91, *S.* Typhimurium SL1344, *S.* Typhimurium DT104, *S.* Typhi CT18 and *S. bongori* 12419 were produced by the Pathogen Sequencing Unit at the Wellcome Trust Sanger Institute, UK [http://www.sanger.ac.uk/Projects/Salmonella/]. Published genome sequences were obtained from the National Center for Biotechnology Information (NCBI). The genome sequences are described with their RefSeq curated accession numbers; *S.* Typhi Ty2 NC_004631 (Deng *et al.*, 2003), *S.* Choleraesuis SC-B67 NC_006905 (Chiu *et al.*, 2005), *S.* Typhimurium LT2 NC_003197 (McClelland *et al.*, 2001), *Escherichia coli* K-12 MG1655 NC_000913 (Riley *et al.*, 2006) and *Escherichia coli* O157:H7 EDL933 NC_002695 (Hayashi *et al.*, 2001).

2.2.2. Glimmer

The genome sequence of *S.* Gallinarum 287/91 was unannotated and Glimmer was used to predict coding regions in the genome sequence (Delcher *et al.*, 1999). Glimmer uses an input training set of sequences with known genes in this case from *E. coli* and other sequenced strains of *Salmonella* and selects the putative gene predictions based on this training set.

2.2.3. ClustalW

ClustalW 1.83 [http://www.ebi.ac.uk/clustalw](Pearson and Lipman, 1988, Thompson et al., 1994) was used to align the protein sequences of the known fimbrial

proteins and identify polymorphisms. Alignments for the ushers, chaperones, and regulatory proteins as identified from the literature were generated for each group.

2.2.4. Pfam

Pfam version 17.0 (Bateman *et al.*, 2000) was used to search the translated genome sequence in all 6 reading frames of *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 to identify conserved domains within putative fimbrial proteins.

2.2.5. HMMER

HMMER version 3.3.2 (http://hmmer.wustl.edu) was used to generate a Hidden Markov Model (HMM) from a ClustalW alignment of known fimbrial protein sequences. The HMM allows variation, so at any one position the amino acid could be one of a selection if it has occurred within the training set, rather than fixed. The HMM was used to search the genome sequence of *S.* Enteritidis P125109 for fimbriae-associated Pfam domains.

2.2.6. BLAST

Basic Local Alignment Sequencing Tool (BLASTp version 2.2.11 (http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul et al., 1997) compared protein sequences with the Swiss-Prot version 5.2 database and Uniprot (Bairoch and Apweiler, 1996, Bairoch et al., 2005). BLAST allows partial alignments at the beginning, middle or end of a sequence and takes into consideration the length of the sequence being searched. All putative fimbrial gene products were confirmed using this search tool.

The closer the E-value or output value to zero the less likely the match occurred by chance. Default parameters were used.

2.2.7. Artemis/ACT

Artemis is a DNA sequence visualisation and annotation tool and was used to visualise genome sequences used in this study (Rutherford *et al*., 2000). Sequences were viewed in a graphical and interactive format and multiple lines of information were presented in a single context. Artemis can use annotation directly from EMBL (Baker *et al.*, 2000) and GenBank (Benson *et al.*, 2000).

ACT (Artemis Comparison Tool) is an extension of the Artemis program and allows comparison of two or more genomes simultaneously (Carver *et al.*, 2005). The *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 genome sequences were compared with the published genome sequences using ACT version 4 (Rutherford *et al.*, 2000).

2.2.8. Perl

Perl and Bioperl [http://www.bioperl.org] were used to write a script to visualise sections of the genome (Appendix 2.1). The script was written to use Artemis and extract gene features of a specific region, Pfam domains and % G+C of the extracted region.

2.2.9. Neural Network Promoter Prediction (NNPP)

The NNPP v 2.2 (Reese, 2001) identifies promoters by training a neural network on known promoters in a training set from *E. coli.* The program was used to search for

promoters within the genome sequence of *S*. Enteritidis P125109. A threshold level of 0.8 was set allowing 60 % of promoters to be identified with a 0.4 % false positive hit rate.

2.2.10. Tandem Repeats Finder

The tandem repeats finder program (Benson, 1999) was used to analyse DNA repeat sequences in the genome sequence of *S*. Enteritidis P125109. A detection component finds candidate tandem repeats and the analysis component produced alignments of these candidates. This programme is unique in its ability to find tandem repeats without the need to specify the pattern or size. All default parameters were used to identify homopolymeric tracts in *S.* Enteritidis P125109.

2.3. Molecular techniques

2.3.1. Extraction of genomic DNA

A 1.5 ml aliquot of a 10 ml overnight culture of *Salmonella* was harvested by centrifugation at approximately 10,000 *g* for 15 minutes (min) at room temperature and the pellet was resuspended in 567 µl of TE buffer. To this 30 µl of 10 % (w/v) sodium dodecly sulphate (SDS) and 3 µl of 20 mg/ml of proteinase K was added and mixed by inversion before being incubated for 1-2 h at 37 °C statically to lyse the bacteria. The cell wall debris, protein and polysaccharides were bound by the addition of 100 µl of 5 M sodium chloride and 80 µl of CTAB and incubated statically for 10 min at 65 °C, 750 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was then added, mixed by inversion and the suspension centrifuged at 10,000 *g* for 40 min at room temperature. The aqueous phase was removed and the process repeated three times. The DNA was precipitated by the addition of 450 µl of isopropanol and harvested by centrifugation at approximately 10,000 *g* for 10 min at room temperature. The DNA was purified by the addition of 400 µl of 70 % (v/v) ethanol and the DNA was harvested by centrifugation at approximately 10,000 *g* for 5 min at room temperature. The pellet was air-dried and resuspended in 50 µl distilled sterile water. Any contaminating RNA was removed by the addition of RNase at a final concentration of 10 µg/ml. The DNA was stored at -20 ºC (Manfioletti and Schneider, 1988).

2.3.2. Isolation of plasmid DNA

All plasmids were extracted using a QIAGEN Plasmid Midi kit, following the manufacturers' instructions using a modified alklaine lysis method (version July 2004). Briefly bacterial cells were harvested and lysed under alkaline conditions and

neutralised. The DNA was adsorbed onto silica in the presence of high salt, salts and endonucleases were washed and removed. The plasmid was eluted and the DNA was purified by isopropanol precipitation and ethanol washing.

2.3.3. Restriction endonuclease digestion of genomic DNA

The concentration of genomic DNA was measured at 260 nm using a DNA spectrophotometer (Ultrospec 2100 pro, Amersham) and the concentration (mg/ml) calculated (A_{260} x 50 x dilution factor). DNA (20 µg) was cleaved in 20 µl of the appropriate enzyme buffer and 2 µl of enzyme at 10 units/µl, in a total volume of 200 µl of water at 37 ºC for 16-18 h. The digest was cleaned using 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1), centrifuged at 10,000 *g* for 3 min at room temperature, followed by recovery of the aqueous layer. One-tenth final volume of 3 M sodium acetate (pH 5.2) was added along with at least 2 volumes of ice-cold absolute ethanol to precipitate the DNA. The samples were incubated at -20 ºC for a minimum of 15 min and the DNA harvested by centrifuguation at approximately 10,000 *g* for 15 min at room temperature. The DNA was washed in 500 µl of 70 % ethanol (v/v) and re-suspended in 12 µl of water and 2 µl of 10 x loading buffer (250 mg bromophenol blue in 33 ml of 150 mM Tris (pH 7.6), 60 ml glycerol and 7 ml water);(Sambrook *et al.*, 1989).

2.3.4. Agarose gel electrophoresis

PCR products and DNA digests were separated and visualised using horizontal slab gel electrophoresis with a 0.8 % (w/v) agarose gel unless otherwise stated. For a 0.8 % gel, 0.8 g of agarose was added to 100 ml of 1 x TAE buffer (Sigma) and heated until

the agarose had dissolved. Once cooled, ethidium bromide (BioRad) was added at a final concentration of 1 μg/ml and the gel was poured into a gel tray with a comb and allowed to set. DNA samples were loaded onto the gel using a 1 x loading buffer. The DNA was separated by applying a constant voltage of 80 V and visualised under UV light. The image was captured using a UVP gel documentation system (Sony). A 1 kb DNA ladder (Promega) was always included on the gel as a reference of size.

2.3.5. Southern blotting

DNA fragments were separated on a 1 % agarose gel as described in Section 2.3.4 and the DNA on the gel was depurinated in 250 mM hydrochloric acid for 10 min. The DNA was denatured in a mixture of 0.5 M sodium hydroxide and 1.5 M sodium chloride for 15 min then rinsed in sterile water and the denaturation step was repeated. The DNA was neutralised in a mixture of 1.5 M sodium chloride and 0.5 M Tris (pH 7.5) for 15 min at room temperature then rinsed with water and repeated. The DNA was equilibrated in 2 x SSC (Invitrogen) for 10 min before being transferred using a membrane pump system (Biometra^{R}) to Hybond-N membrane according to the manufacturers' instructions (Amersham). Briefly, the gel was placed on top of the Hybond-N membrane which sat on top of Whatmann paper pre-soaked in 2 x SSC. A vacuum was applied to draw the DNA across the gel and onto the membrane. The DNA was fixed to the membrane by exposure to UV light for 3 min whilst the membrane was still wet (Southern, 1975).

2.3.6. Dot blotting

Genomic DNA (5 μg) was spotted directly onto Hybond-N membrane and allowed to dry at room temperature. The membrane was placed DNA side up on Whatmann paper soaked in a mixture of 1.5 M NaCl and 0.5 M NaOH for 5 min to denature the DNA. The membrane was then placed on Whatmann paper soaked in 0.5 M Tris (pH 7.4) for 30 seconds followed by 5 min on Whatmann paper soaked in a mixture of 1.5 M NaCl and 0.5 M Tris (pH 7.4). The membrane was allowed to dry for 30 min before the DNA was fixed by exposure to UV light for 3 min.

2.3.7. Primer design for amplification of major fimbrial subunits and chloramphenicol gene probes

The genome sequence of *S.* Enteritidis P125109 was used as the template for designing primers and the sequences of *S.* Typhimurium LT2 and *S.* Typhi CT18 were used when genes were not present in *S.* Enteritidis P125109 and are shown in Table 2.3.

Table 2.3. Primer sequences for amplification of major fimbrial subunit and chloramphenicol cassette gene probes

Gene	Name	Sequence (5′-3′)
csgA	*csgA* F	TTCGCAGCAATCGTAGTTTC
	*csgA*R	TAATACTGGTTAGCCGTGGCGT
lpfA	*lpfA* F	TTTTGCTCTGTCTGCTCTCGCTG
	lpfA R	AAGTCCACTTCTGCGTTACCGTAACCA
stfA	*stfA* F	GTTGCTGCCGCACTGGTTATGGGTG
	stfA R	ACAGATAGCTGATCGTGAAGTTTACGGTGC
sefA	*sefA* F	GCGTAAATCAGCATCT
	sefA R	CTGAACGTAGAAGGTCGCAGT
stbA	*stbA* F	ATGATCACAGGCTCGCTTCTTGTCTC
	stbA R	ACGAAACGGCGTATTGTAGGGTGGCA
stcA	*stcA* F	CGTTCACTTATTGCTGCTTCTG
	stcA R	CCGTCATCGTCAGTACAGATTC
fimA	*fimA* F	ATGACCTCTACTATTGCGAGTCTGA
	fimA R	TTATTCGTATTTCATGATAAAGGTG
bcfA	*bcfA* F	AAAAGCCTGTACTAGCATTAATGGT
	bcfA R	TCAGGAATAAACCATGCTAAATGTC
steA	*steA* F	ATGAAGTCATCTCATTTTTGTAAAC
	steA R	TTACAGGTAAGAGATAGTGACGTTG
safA	*safA* F	GTGGTTATTCAAATGAAAAGCATAA
	safA R	TTAAGGCTGATATCCCACTACGTCT
stdA	*stdA* F	GTGCTTCGTTTAACACCAGGCGTTT
	stdA R	TCACAGGTATTTCAGGGTGTAGGTG
sthA	*sthA* F	ATGTTTAATAAGAAAATTATCATC
	sthA R	ACGAAACGGTATACGTAACCTGAGT
stiA	*stiA* F	ATGAAACTCTCCTTAAAAACACTC
	stiA R	TCAGTTATATTGCAGATAGAATGTT
tcfA	*tcfA* F	AATTTTAAAGATACTCTTCCCGGGGT
	tcfA R	TTACTTTCCGGCTGCTGTTAAATCCA
staA	*staA* F	ATGAAAAAAGCGATTTTAGCTGCCG
	staA R	TTACTGGTAAGTAAAGGTATACATT
stjB	*stjB* F	GTGAAGTATTTAAAACTGCCGCTAT
	stjB R	TCATTGACAGACTCCCTTTGCCGTT
stgA	*stgA* F	AAACTGAATTTAATTGCCAGCGCTCT
	stgA R	TTATTTTTGGTATTCGACAGTGAAC
Chl	Chl F	TTCAGCTGGATATTACGG
cassette	Chl R	ATCGCAGTACTGTTG

The genome sequences were visualised in Artemis and primer sequences were selected of approximately 20 base pairs (bp) in length either directly at the 5′ and 3′ ends of the gene of interest or 5 bp into the downstream of the start and stop codons and were obtained from Sigma-Aldrich. The primer sequences for the chloramphenicol cassette were obtained from (Datsenko and Wanner, 2000). All oligonucleotides were resuspended in sterile water at a concentration of 100 mM and maintained at –20 ºC. The start and stop codons are underlined in Table 2.3.

2.3.8. Digoxigenin (DIG) labelled probes

The primers listed in Table 2.3 were used in a PCR reaction incorporating DIG-labelled dUTP to prepare probes to each fimbrial gene and the chloramphenicol cassette using *S.* Enteritidis P125109, *S.* Typhi aroCD, *S.* Typhimurium SL1344 or pKD3 as a template. This was carried out according to the manufacturers' instructions (June 2006, PCR DIG probe synthesis kit, Roche Applied Science, U.K.). The PCR mixture consisted of the following; final concentrations 1 x PCR buffer (Invitrogen), 1.5 mM magnesium chloride (Invitrogen), 0.2 mM of each dNTP including dUTP (Roche Applied Science, U.K.), 0.5 µM of each primer (Sigma), template genomic DNA (2 µg), *Taq* DNA polymerase 0.5 µl and made up to 50 µl with distilled water.

The reaction was set at

1. Initial denaturation 95 ^{o}C for 2 min

Followed by 30 cycles of :-

2. Denaturation at 95 ^{o}C denaturation for 30 seconds
3. Annealing at 55 ^{o}C for 1 min
4. Elongation at 72 ^{o}C for 1 min
5. One final elongation step at 72 ºC for 7 min

PCRs were carried out in a GeneAmp PCR system 2700 thermocycler from Applied Biosystems and the PCR products were analysed using agarose gel electrophoresis as described in Section 2.3.4.

2.3.9. Hybridisation of fimbrial probe to membrane

The optimal hybridisation temperatures were calculated for each of the probes and are shown in Table 2.4. The hybridisation temperature (Thyb) is dependent upon the melting temperature of the probe (Tm) and is set 20-25 ºC lower.

The melting temperatures of the probe can be calculated;

Tm = 49.82 + 0.41 x (%G+C) - 600/l where l is the length of the probe and % G+C is the GC content of the probe. Thyb = Tm-(20-25 ºC)

Table 2.4. Hybridisation temperatures of DIG-labelled probes

Probe target	Hybridisation temperature (^{o}C)
staA	47
stbA	45
stcA	44
stdA	46
steA	47
stfA	46
stgA	47
sthA	47
stiA	48
stjA	47
lpfA	44
tcfA	46
fimA	49
safA	43
sefA	48
csgA	47
bcfA	47
Chloramphenicol cassette	41

The DIG Easy Hyb Granules (Roche Applied Science, U.K.) were dissolved in sterile water as described in manufacturers' guidelines (version 1 July 2003), and pre-heated to the appropriate hybridisation temperature as calculated in Table 2.4. The membrane was incubated at the appropriate temperature with 20 ml of the hybridisation solution for 15-30 min with agitation. The labelled probe was denatured by heating to 100 ^{o}C for 5 min and cooling on ice and 10 µl was then added to 20 ml of the dissolved DIG Easy Hyb Granules. The pre-hybridisation solution was replaced with the probe and hybridisation solution and the membrane was incubated for a minimum of 6 h with constant agitation at the appropriate temperature specified in Table 2.4.

2.3.10. Detection of DIG-labelled probes

The hybridisation solution was replaced with low stringency buffer (2 x SSC and 0.1 % (w/v) SDS) and incubated for 2 x 15 min at room temperature with agitation. Two high stringency washes (0.5 x SSC and 0.1 % (w/v) SDS) were carried out for 15 min each with agitation at 68 °C. The membrane was washed for 15 min with washing buffer (0.1 M maleic acid and 3-5 % Tween 20) at room temperature and then incubated with blocking buffer (10 % blocking solution provided within the kit and 0.1 M maleic acid) for 30 min with agitation at room temperature (All reagents from DIG wash and block kit, Roche, Applied Science, U.K.). Anti-digoxigenin-AP (Roche, UK), was added to 20 ml of fresh blocking solution and the membrane was incubated for 30 min at room temperature with agitation. The membrane was washed and equilibriated in detection buffer (1 M NaCl and Tris-HCl (pH 9.5)). The membrane was placed DNA side up in a developing folder and disodium-3(4-methoxyspiro-[1,2-dioxetane-3,2-(5'chloro)tricyclo[3.3.1.1]decan]-4-yl) phenyl phosphate (CSPD; Roche) was added dropwise following the manufacturers' guidelines (version 3, June 2000) and was incubated for 5 min at room temperature. The excess CSPD was squeezed out and the developing folder was sealed and incubated for 10 min at 37 °C. The membrane was exposed to a X-ray film for 5-15 min and the image captured using a X-ograph machine X4 Compact, from Imaging Systems (as described in manufacturers' manual version 1, March 2003).

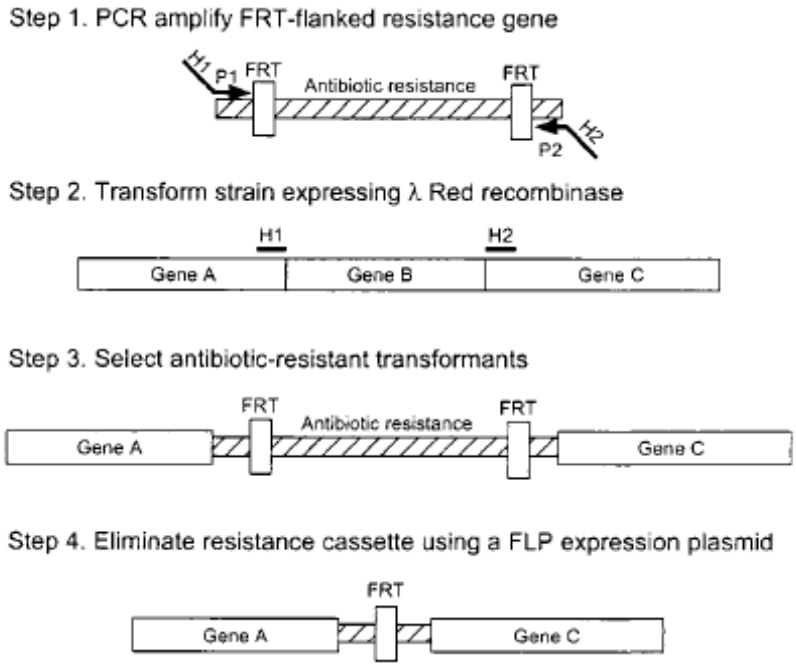
2.4. Construction and confirmation of fimbrial mutants

2.4.1. Construction of fimbrial gene mutants by λ Red mutagenesis

This method allows for mutagenesis of chromosomal genes in a single step by homologous recombination of linear PCR products harbouring an insertion of an antibiotic resistance cassette in the gene of interest. Recombination requires the pKD46 helper plasmid which encodes the bacteriophage λ, *gam, bet* and *exo* recombinase functions that are under the control of an arabinose inducible promoter. The helper plasmid can be selected for with ampicillin and cured after recombination by virtue of its temperature-sensitive origin of replication. Once the helper plasmid is present, the linear PCR products containing the chloroamphenicol cassette can recombine with the gene of interest resulting in its inactivation. Where polar effects are anticipated the antibiotic resistance gene inserted in the gene of interest may be excised by transient expression of flippase (FLP) recombinase which catalyses the reaction between flippase recognition target (FRT) sites flanking the cassette leaving an in frame 84 nucleotide 'scar'. The process is summarised in Figure 2.1.

Figure 2.1. λ Red recombinase-mediated integration of linear PCR products to disrupt chromosomal genes

Image taken from (Datsenko and Wanner, 2000).



2.4.2. Preparation of electrocompetent cells

LB broth was inoculated with the strain of interest and incubated as appropriate. The culture was diluted 1:100 into 100 ml of LB broth and grown at 130 rpm (standard grwoth condition to aerate the bacteria) at the appropriate temperature until the A_{600nm} was approximately 0.5, where the pKD46 plasmid was present 10 mM L-arabinose was added to induce expression of the bacteriophage λ genes. The bacterial cells were harvested by centrifugation at approximately 2000 *g* for 15 min at 4 ^{o}C and the pellet was washed twice in 20 ml ice-cold sterile water and once in 20 ml of 10 % (v/v)

glycerol. The bacterial cells were resuspended in 500 µl of 10 % (v/v) glycerol (Sambrook *et al.*, 1989).

2.4.3. Transformation of electocompetent cells

The pKD46 plamid was extracted as described in Section 2.3.2. A range of volumes of the plasmid or linear PCR products (Section 2.4.5) were mixed with electrocompetent bacterial cells (50 µl) (Section 2.4.2) and incubated on ice for 10 min. The electroporation was carried out in a 0.2 mm cuvette using a Biorad gene pulser and pulse controller at 200 Ω, 25 µF, 2.5 kV, with a time constant of approximately 4. The bacteria were recovered in 1 ml of SOC with appropriate antibiotic and incubated at 37 °C unless the plasmid is temperature-sensitive. The recovered bacteria (100 µl) were plated on LB agar plates with appropriate antibiotics and incubated for 16-18 h at 37 °C, unless the plasmid is temperature sensistive.

2.4.4. Verification of transformants

All colonies were checked for the presence of intact lipopolysaccharide (LPS), during electroporation bacteria lacking LPS take up plasmid DNA at a higher frequency than those with intact LPS. A colony was resuspended in 5 µl PBS and 2 µl acriflavin, HCl 5 % (w/v) was added; aggregation indicates the absence of the LPS. To confirm the colonies were *S*. Enteritidis or *S*. Gallinarum, the process was repeated but anti-*Salmonella* O9 serum was added instead of acriflavin HCl and the anti-H serum was used to distinguish between *S.* Enteritidis and *S*. Gallinarum. Agglutination indicates the presence of specific O or H antigens. The presence of the plasmid was confirmed by the re-isolation and digestion of the plasmid as described in Sections 2.3.2 and 2.3.3.

2.4.5. Generation of amplicons for mutagenesis of major fimbrial subunits

Forward and reverse primers were designed with 40 bp homology extensions to the major fimbrial subunit gene of *S.* Enteritidis P125109 (40 bp 3′ of start codon and 5′ of stop codon, underlined) some primers were designed 1-5 bp downsteam of the start or stop codon.

Table 2.5. Forward and reverse primers for lambda Red mutagenesis

Primer Name	Primer sequence (5′- 3′)
stbAFmut	ATGTCTATGAAAAAATATTTAGCAATGATCACAGGCTCGCTGTGTAGGCTGGAGCTGCTTCG
stbARmut	TTATTTATACGAAACGGCGTATTGTAGGGTGGCAGCGACTCATATGAATATCCTCCTTA
stcAFmut	ATGAAACGTTCACTTATTGCTGCTTCTGTATTGTCTGCTGTGTGTAGGCTGGAGCTGCTTCG **GCTGCTGATGAAGATATGGGGGAATTAAAAATAAACGGTGTGTAGGCTGGAGCTGCTTCG**
stcARmut	TTAATCAGTTAATACCGTCATCGTCAGTACAGATTCAACACATATGAATATCCTCCTTA **AGTATTTTCAACGGCGGCATAGCGGGCAGAAAAGTTCAGGGTCATATGAATATCCTCCTT**
stdAFmut	GTGCTTCGTTTAACACCAGGCGTTTATTATTCATACGAATTGTGTAGGCTGGAGCTGCTTCG
stdARmut	TCACAGGTATTTCAGGGTGTAGGTGACGGATGCGTTGAAGCATATGAATATCCTCCTAA
steAFmut	ATGAAGTCATCTCATTTTTGTAAACTGGCAGTAACTGCATGTGTAGGCTGGAGCTGCTTCG
steARmut	TTACAGGTAAGAGATAGTGACGTTGGCGGCGCTGCTGAACATATGAATATCCTCCTTA
stfAFmut	ATGAATACAGCAGTAAAAGCTGCGGTTGCTGCCGCACTGGTGTGTAGGCTGGAGCTGCTTCG
stfARmut	TTACAGATAGCTGATCGTGAAGTTTACGGTGCTGCTGAATCATATGAATATCCTCCTTA
sthAFmut	ATGTTTAATAAGAAAATTATCATCCTGGCAATGTTAACTTGTGTAGGCTGGAGCTGCTTCG
sthARmut	TTACTGATACGAAACGGTATACGTAACCTGAGTGCTAACACATATGAATATCCTCCTTA
stiAFmut	ATGAAACTCTCCTTAAAAACACTCACTGTGGCACTGCCGTGTGTAGGCTGGAGCTGCTTCG
stiARmut	TCAGTTATATTGCAGATAGAATGTTGCGGTTGCATCGACCCATATGAATATCCTCCTTA
bcfAFmut	ATGAAAAAGCCTGTACTAGCATTAATGGTCTCTGCCATTGTGTGTAGGCTGGAGCTGCTTC
bcfARmut	TCAGGAATAAACCATGCTAAATGTCGCCGTCGCGGTAACCATATGAATATCCTCCTTA
csgAFmut	ATGAAACTTTTAAAAGTGGCAGCATTCGCAGCAATCGTAGTTGTGTAGGCTGGAGCTGCTTCG **GACTCAACGTTGAGCATTTATCAGTACGGTTCCGCTAACGCTGTGTAGGCTGGAGCTGCTTCG**
csgARmut	TTAATACTGGTTAGCCGTGGCGTTGTTGCCAAAACCAACCCATATGAATATCCTCCTTA **AAAACCAACCTGACGCACCATTACGCTGGAATCAGATGCCATATGAATATCCTCCTT**
lpfAFmut	ATGGAGTTTTTAATGAAAAAGGTTGTTTTTGCTCTGTCTGTGTGTAGGCTGGAGCTGCTTCG
lpfARmut	TTATTCGTAGGACAGGTTGAAGTCACTTCTGCGTTACCGCATATGAATATCCTCCTTA
fimAFmut	ACCTCTACTATTGCGAGTCTGATGTTTGTCGCTGGCGCATGTGTAGGCTGGAGCTGCTTCG
fimARmut	TTATTCGTATTTCATGATAAAGGTGGCGTCGGCATTAGCCTGCATATGAATATCCTCCTTA
sefAFmut	ATGCGTAAATCAGCATCTGCAGTAGCAGTTCTTGCTTTAATGTGTAGGCTGGAGCTGCTTCG
sefARmut	GTTTTGATACTGCTGAACGTAGAAGGTCGCAGTGGGTCCATTTCATATGAATATCCTCCTTA

safAFmut	GTGGTTATTCAAATGAAAAGCATAAAAAAATTGATTATCGTGTGTAGGCTGGAGCTGCTTCG
	TGAAAAGCATAAAAAAATTGATTATCGCAAGTGCGTTGAGTGTGTAGGCTGGAGCTGCTT
safARmut	TTAAGGCTGATATCCCACTACGTCTACAGTTATTGGGTACCATATGAATATCCTCCTTA
	CACTACGTCTAAAGTTATTGGGTACGTGTCAGCTGTGACATTCTGCATATGAATGTCCTCCTT

NB: **bold** In some cases alternative primers were designed to *S*. Gallinarum 287/91

The pKD3 plasmid containing the chloramphenicol resistant cassette was used as a template for PCR and a 20 bp region homologous sequence to the pKD3 plasmid was inluded in the primer design to permit amplification of the chloramphenicol resistant cassette. The PCR was carried as described in Section 2.3.8 except the initial denaturation step was for 5 min and the dNTPs were unlabelled. All oligonucleotides were resuspended in sterile water at a concentration of 100 mM and maintained at –20 ºC.

2.4.6. Confirmation of fimbrial mutations position

Primers were designed to regions 50 bp upstream or downstream of the targeted mutation and these were used in combination with primers designed within the chloramphenicol resistant cassette in the putative mutants (Table 2.6). The PCR was carried as described in Section 2.3.8 except the initial denaturation step was for 5 min and the dNTPs were unlabelled. All PCR products were visualised on a 0.8 % agarose gel as described in Section 2.3.4.

Table 2.6. Primer sequences for confirmation of the location of fimbrial mutants

Primer combination	Predicted size of PCR product (bp)	Sequence (5′-3′)
bcfAFOR + C1	633	TGCACTATCCGCAACGATATATTT
bcfAREV + C2	507	TAAAATACGCTTTCGCGATCGGTCGGT
csgAFOR + C2	173	CAAGGAGCAATAAAGTATGCATAATTT
csgAREV + C1	302	CAGCAGTTGTAGTGCAGAAACAGTCGCATA
lpfAFOR + C2	867	TTAGTTACGCGCTGTGTCAA
lpfAREV + C1	288	ATCCAATACCCACCTCTATACACTCCA
fimAFOR +C1	807	AACCTCAGATCGCACCTGCTGC
fimAREV + C2	429	ATGCCGACATGACGCCAGACC
sefAFOR + C1	373	CTATTAATGGGGATGTTGTGTAA
sefAREV + C2	946	CTAATAATCTCTTATAATTTC
safAFOR + C1	701	TGAGACTCTCTCATTGGAGCGCT
safAREV + C2	597	AATTGAGGTCAAGGGTCGCGCC
stbAFOR + C2	887	TTAATGGTGGGGGACATCGTA
stbAREV + C1	295	TTATTTTTACCACTCCATAAGCACGAA
stcAFOR + C2	179	CACAAGCCAGGCATAATGCAATCATC
stcAREV + C1	377	ACATTGCGATAACTTCCTGTCTATGAGAA
stdAFOR + C2	587	GCTGTACCGTACCTGACTGTC
stdAREV + C1	714	TGTTTTTAAATTTCATCCGCGAAG
steAFOR + C1	739	TACGACAACGCCTATATAATA
steAREV + C2	600	AGCAGCGTGGAGTGTCCCAGGTCAGC
stfAFOR + C1	283	CATATAAACATGGGGTATTGATGA
stfAREV + C2	155	GGCTGGCATCATCTTTAACA
sthAFOR + C1	584	GCGTTGATTTTGTTAATGC
sthAREV + C2	704	GAAAGCTCACGATTTGAGATCAAC
stiAFOR + C2	385	TTTGGCCGACAACACACTATG
stiAREV + C1	661	GTAAATCAGCTTAAATTCCG
C1	-	TTATACGCAAGGCGACAAGG
C2	-	GATCTTCCGTCACAGGTAGG

The primers were designed using the genome sequence of *S*. Enteritidis P125109 and were obtained from Sigma. All oligonucleotides were resuspended in sterile water at a concentration of 100 mM and maintained at –20 ºC.

2.4.7. Transduction of mutations between strains using bacteriophage P22/int

Bacteriophage P22 packages approximately 40 kb of the hosts DNA and injects this foreign DNA into a new bacterial cell (Zinder and Lederberg, 1952, Casjens and Hayden, 1988). This provides an efficient means of transduction of marked mutations between bacteria and reduces the likelihood that any secondary defects that may have occurred will explain observed phenotypes. Each of the fimbrial mutants was used as a donor strain and grown in 10 ml LB broth statically for 16-18 h at 37 ºC. Phage P22 lysate was added at an approximate multiplicity of infection of 1:1 and the bacteria were incubated further at 130 rpm for 16 h at 37 ºC. The bacteria were centrifuged at 2000 *g* for 15 min at room temperature and the supernatant was syringe-filtered through a 0.45 µm membrane to remove all bacteria, leaving a sterile filtrate containing the phage. The number of plaque-forming units of the phage were counted by flooding an LB agar plate with a wild-type recipient strain, which was dried statically for 2 h at 37 ºC, and 20 µl of 10 x serial dilutions of the phage were plated and incubated for 18 h at 37 ºC. The plaques were counted and plaque-forming units per millilitre were calculated.

The recipient strain or archived strain was grown at 130 rpm for 16-18 h at 37 °C and harvested at approximately 2000 *g* for 15 min at room temperature. The bacteria were resuspended with transducing phage at a multiplicity of infection of approximately 0.8 and incubated statically for 25 min at 37 ºC. Colonies were selected on LB agar plates with chloramphenicol (25 µg/ml) and were purified by examining single colonies for

signs of phage such as jagged edges and selecting those that did not appear to possess phage. Each colony was streaked on LB agar plates with chloramphenicol (25 μg/ml) to single colonies and incubated for 16 h at 37 ºC. This was repeated a minimum of five times until no phage-infected bacteria were seen. All colonies were also screened with the primers in Table 2.6 as described in Section 2.4.6 and for the presence of LPS as described in Section 2.4.4.

2.5. *In vitro* analysis of fimbrial mutants

2.5.1. Growth kinetics

To ensure that the growth rate of the *Salmonella* strains was not affected by the introduction of a mutation, the growth rates of wild-type and mutant strains were measured. The strains were grown overnight and diluted 1:1000 in LB broth. The growth rate was assessed over a period of 18 h at 37 ºC with readings every 30 min using a BioscreenC, real-time spectrophotometer from Thermo®. For each wild-type or mutants strain 3 replicates were carried out on 3 individual days.

2.5.2. Adhesion and invasion assay of fimbrial mutants

Adherence and invasion assays were carried out essentially as described (La Ragione et al., 2000, Clark et al., 1998). *Salmonella* wild-type and fimbrial mutant strains and *E. coli* K-12 were grown in 10 ml LB broth at 130 rpm for 16-18 h at 37 ºC and statically for 16-18 h at 25 °C. The shaking cultures were diluted 1:100 in 10 ml of LB broth and were grown at 130 rpm for a further 4 h at 37 ºC to grow the bacteria to log phase; the statically grown cultures were used immediately. The absorbance of the cultures was measured at A_{600} nm using a ThermoSpectronic Helios gamma (Thermo^{R}) and appropriate volumes were added to 5 ml of pre-warmed media to give a multiplicity of infection (MOI) of approximately 5:1 and an MOE of 3:1.

Optical density	0.16-0.20 add 0.70 ml
	0.21-0.25 add 0.65 ml
	0.26-0.30 add 0.60 ml
	0.31-0.35 add 0.55 ml
	0.36-0.40 add 0.50 ml
	0.41-0.45 add 0.45 ml
	0.46-0.50 add 0.425 ml
	0.51-0.55 add 0.40 ml
	0.56-0.60 add 0.375 ml

Typically the A_{600} of the mutants and respective parent in a given experiment were comparable, however *S*. Gallinarum and *S*. Enteritidis grown for the same duration often exhibited markedly different A_{600}. Serial dilutions of the 5 ml cultures were carried out and 100 µl of a range of dilutions was plated on LB agar plates. This was done in triplicate and the plates were incubated statically for 16-18 h at 37 ºC. The colonies were counted and the number of colony forming units (cfu) per millilitre was calculated to ensure that the dilutions resulted in comparable numbers of viable bacteria being used.

Three cell lines were used; chick kidney cells (CKC), human epithelial cells (HEp-2) and the chicken macrophage-like cell line HD11. All cells were obtained from the Microbiological services department at the IAH and were seeded at $5x10^5$ cells/well in appropriate growth media containing 5 % foetal bovine serum albumin and non-essential amino acids. Prior to infection, the cell media was replaced with 900 µl fresh pre-warmed media.

A 1:10 dilution of the 5 ml cultures was added to each well and the cells were returned for 15 min to a 5 % CO_2, 37 ºC incubator. As *S*. Gallinarum bacteria are non-motile the plates were centrifuged at approximately 100 *g* for 3-5 min at room temperature before being incubated.

The inoculum was removed and for the adhesion assay the cells were washed 6 times in 1 ml pre-warmed phosphate-buffered saline (PBS). For the invasion assay gentamicin was added at a final concentration of 100 µg/ml and the cells were incubated for 1 h at 37 ºC, 5 % CO_2 and then washed 3 times with pre-warmed PBS. In both assays, 1 % (v/v) Triton was added for 15 min to disrupt the monolayer. Ten-fold serial dilutions were carried out and 100 µl was plated onto LB agar plates that were then incubated for 16-18 h at 37 °C. The colonies were counted and the number of colony forming units per millilitre calculated (cfu/ml).

2.5.3. Validation of adherence assay

All cell lines were grown in wells on coverslips, (1 cm diameter, poly-L-lysine coated to attract cells). The same methods were used as described in Section 2.5.2, except at the point where Triton-X-100 was added, 1 ml of 4 % (w/v) paraformaldehyde in PBS was added to fix the cells. The coverslip was removed from the well with a hypodermic needle and fine forceps and was stained using Haemacolor™ reagents (Merck); the coverslip was placed in fixant solution for 5 s, red staining eosin solution for 3 s and in methylene blue solution for 6 s. The excess dye was removed by rinsing in PBS and excess fluid was removed by blotting on a paper towel. The coverslips were placed cell side down on mounting media on a slide and 20 fields of view per slide were

examined, ensuring that views were taken from the edges and the middle of the coverslip. The numbers of bacteria attached to cells or to the coverslips were counted.

2.5.4. Confocal microscopy

Alternatively after paraformaldehyde treatment as described in Section 2.5.3, the cells were rinsed twice in PBS and permeabilised with 0.5 % Triton-X-100 for 15 min. Non-specific binding sites were blocked with 0.5 % (w/v) bovine serum albumin (BSA). The coverslips were then covered with 50 µl of a primary antibody, anti-*Salmonella* O9 serum 1:200 in BSA for 1 h at 37 ºC and then rinsed in PBS. The secondary antibody, anti-rabbit-Ig Alexa^{568} was diluted 1:100 in BSA and 50 µl was added to the coverslip for a further 1 h at 37 °C and rinsed. Phalloidin fluorescein-isothiocyanate-conjugate was added and incubated at room temperature for 15 min to stain F-actin and the coverslip was rinsed with PBS before mounting on a slide with Vectashield®. The slides were viewed and the image captured using a confocal microscope and PC.

2.5.5. Confirmation of mutants by antibody-mediated detection of fimbrial proteins

To confirm that selected mutations abolished expression of the targeted protein, antibody-mediated detection of fimbrial proteins was used to detect differences between the wild-type strain and mutant strains under identical growth conditions used in the *in vitro* assays. Antibodies specific to *S.* Enteritidis FimA, SefA and CsgA were a kind gift from R.M LaRagione, Veterinary Laboratories Agency, Weybridge. These antibodies were raised against a purified fimbrial subunit in mice. Due to the auto-

aggregative nature of selected fimbrial proteins, a dot blot approach was taken to analyse expression of the protein.

The fimbrial protein preparation was carried out as previously described (Collinson *et al.*, 1993). Briefly, a 10 ml culture of *S.* Enteritidis wild-type or fimbrial mutant was grown at 130 rpm for 24 h at 37 ºC or statically for 36 h at 25 ºC. For detection of FimA and SefA, the 10 ml culture was pelleted by centrifugation at 4000 *g* for 15 min at room temperature and resuspended in 1 ml of PBS. The culture was washed in sterile water by centrifugation at 4000 *g* for 15 m at room temperature. The washed pellet was resuspended in 100 µl of PBS and boiled for 10 min. To detect the expression of the CsgA protein, the bacteria were harvested, washed twice in water and resuspended in 90 % (v/v) formic acid. The insoluble CsgA was clarified and resuspended in loading buffer supplemented with 0.2 M glycine (pH 2).

Hybond-ECL membrane (Amersham) was used and 15 µl of the final culture was spotted directly onto the membrane and allowed to air dry for 1 h. The membrane was blocked for 1 h in a mixture of 1 % (w/v) skimmed milk and 0.1 % (v/v) Tween 20 in PBS (blocking solution) at room temperature. The membrane was washed for 5 min, 3 times in PBS and 0.1 % (v/v) Tween 20 (washing buffer). The primary antibody for the fimbrial subunit protein was diluted 1:100 in blocking solution and added onto the membrane for 1 h at room temperature and a second wash step was carried out. The secondary antibody anti-mouse Ig conjugated to alkaline phosphatase and produced in goat was diluted 1:10,000 in blocking solution and incubated with the membrane for 1 h and a third wash step was carried out as before. The developing solution 5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium (BCIP/NBT) (Sigma) was added until the antibody was developed and the reaction was stopped by the addition of water. The membrane was allowed to air dry and a digital image collected.

2.5.6. Removal of the antibiotic cassette from fimbrial mutants

The pCP20 plasmid was extracted from *E. coli* DH5α using a QIAGEN Midi kit as described in Section 2.3.2. This plasmid encodes FLP recombinase which is thermally induced and promotes recombination between the FRT sites flanking the pKD3-derived chloramphenicol cassette (Figure 2.1, step 4). The plasmid was electroporated into the mutant strains with selection for ampicillin resistance at 30 ºC for 16-18 h (described in Sections 2.4.2, 2.4.3 and 2.4.4). The colonies were grown in 10 ml of LB broth at 43 ºC for 16-18 h to induce FLP and plated on LB agar plates for a further 16-18 h to select for bacterial cells that have lost both the chloramphenicol cassette and the pCP20 plasmid.

Colonies were streaked on LB agar plates to create a master plate and were simultaneously plated on LB agar plates with ampicillin (100 μg/ml) and LB agar plates with chloramphenicol (25 μg/ml). Colonies that did not grow on chloramphenicol and ampicillin supplemented agar plates were selected for further analysis. Primers flanking the mutation were used to screen for the absence of the chloramphenicol cassette and fimbrial gene, resulting in a smaller fragment size (Datsenko and Wanner, 2000).

2.5.7. *Trans*-complementation of fimbrial mutants

Primers were designed to the gene or operon of interest and when required sites for specific restriction enzymes were introduced (Table 2.7).

Table 2.7. Primer sequences for cloning

Name	Primer sequence (5′-3′)
steoperonfor	ATGAAGTCATCTCATTTTTGT
steoperonrev	TTACTGATATTCAAAACTCACTGT
stcAfor	ATATAT**ATCGAT**AAGGGTTAATAACTCTTAACAA
stcArev	ATATAT**ATCGAT**GATTGTTAATCAGTTAATA

The bold section highlights the *Cla*I restriction enzyme site. The start and stop codon are underlined. the stcA primers were designed downstream of the start and stop codons. All primers were designed using the genome sequence of S. Enteritidis P125109 and were obtained from Sigma. All oligonucleotides were resuspended in sterile water at a concentration of 100 mM and maintained at –20 ºC.

The cloning of the *steA* gene was carried out using primers in Table 2.3 and PCR conditions as described in Section 2.3.8 without labelled dNTPs. Due to the large size of the amplicons (for the *ste* operon), a long-range PCR reaction was carried out. Hercules (Stratagene) uses a mix of polymerases, *pfu* DNA polymerase, Archae Maxx polymerase and *Taq* 2000. For long-range PCR, the reaction conditions were as described in Section 2.3.8 but the elongation stage was increased to 1 min per kilobase of region to be amplified and 35 cycles were carried out.

The PCR product for *stcA* was amplified using a proof reading enzyme *pfu* (Promega) and all PCR reactions were carried out in a GeneAmp PCR system 2700 thermocycler from Applied Biosystems. PCR products were visualised on a 0.8 % agarose gel as described in Section 2.3.4.

The PCR products were cloned using a Zero Blunt® TOPO® PCR cloning kit (Invitrogen), according to the manufacturers' instructions. Briefly, 4 µl of the PCR products, 1 µl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$) and 1 µl of pCR4Blunt-TOPO vector were mixed together for 5 min at room temperature and then stored on ice.

2.5.8. Transformation of chemically-competent cells

Two microlitres of the cloning reactions generated in Section 2.5.6 were added to chemically-competent one-shot TOP10 *E. coli* cells (Invitrogen), which had been slowly thawed on ice. The mixture was incubated on ice for 5-30 min. The cells were heat-shocked for 30 s at 42 ºC statically and immediately transferred to ice. To this 250 µl of room temperature SOC medium was added and incubated at 130 rpm for 1 h at 37 ºC, and 100 µl of the transformants were plated on LB agar plates with selection for the plasmid-encoded antibiotic resistance and incubated at 37 °C for 18 h. Up to 10 colonies were analysed further.

2.5.9. Analysis of recombinant plasmids

The plasmids containing the inserts were extracted as described in Section 2.3.2 and digested in a 50 µl restriction digest for 1 h at 37 ºC that included 1 unit of enzyme, 1 x buffer and 10 µg DNA. Plasmids with the inserts were electroporated into the strain of interest as described in Section 2.4.2, 2.4.3 and 2.4.4.

2.5.10. *Trans*-complementation *in vitro*

pCR4Blunt-steA with inserts in the sense (forward) and antisense (reverse) orientation were used in the *in vitro* adherence and invasion assay as described in Section 2.5.2. Wild-type, mutant, and *trans*-complemented strains were supplemented with isopropyl-beta-D-thiogalactopyranoside 0.5 mM (IPTG) (Invitrogen) to induce the expression of cloned genes which are under control of the *lac* operon. The LB broth was supplemented where appropriate with antibiotics to ensure the plasmid was maintained during growth.

2.6. *In vivo* analysis of fimbrial mutants

2.6.1. Experimental inoculation of chickens

Day-old specific pathogen-free (SPF) Rhode Island Red chickens were obtained from the Poultry Production Unit at IAH Compton. All animal experiments were carried out under project license number 30/1998 with the approval of the local ethical review committee. Fifteen birds were housed in each cage with water and food provided *ad libitum*. Four cages were used in each experiment and incorporated a wild-type control as it was not possible to evaluate all fimbrial mutants simultaneously owing to the constraints on space and time. The cages were separated by plastic sheeting to ensure faeces from one cage did not drop onto another.

All birds were dosed using a gavage needle with 0.1 ml of adult gut flora on day of hatch. The gut flora was prepared by the extraction and culturing of the caecal contents from an SPF adult Light Sussex chicken in LB broth in stationary conditions. The broth was confirmed to be free of *E. coli, Salmonella* or *Campylobacter* by plating on MacConkey plates, brilliant green plates and Campy blood-free agar plates respectively for 16-18 h at 37 ºC and for *Campylobacter* 48 h at 37 ºC in microaerophilic conditions (10 % CO_2 and 5 % O_2) in nitrogen. Each gut flora was sub-cultured and incubated at 37 ºC statically for 16-18 h before use. The same batch of gut flora was used for the *S.* Enteritidis P125109, a different batch was used for *S.* Enteritidis S1400 experiments and another batch was used for the *trans*-complementation studies, to use gut flora that was no older than 3 months.

At 18-days-old, each bird was dosed with a gavage needle and 0.3 ml of a culture of either *S.* Enteritidis S1400 or *S.* Enteritidis P125109 wild-type or mutant strains that had been grown for 16-18 h at 37 ºC, 130 rpm. All wild-type and mutant strains were

nalidixic acid resistant and this was used to aid recovery. Ten-fold serial dilutions of the inoculum were carried out and 20 µl of a range of dilutions was plated on brilliant green agar plates supplemented with nalidixic acid (20 µg/ml) and novobiocin (1 µg/ml) *S*. Enteritidis P125109 and *S*. Gallinarum 287/91 were also novobiocin resistant. This was done in triplicate and the plates were incubated for 16-18 h at 37 ºC. The pink colonies were counted and the number of colony-forming units per millilitre was calculated. One caeca from each group of 5 wild-type birds was checked for *Salmonella* using O9 anti-*Salmonella* serum as described in Section 2.4.4 and was also plated on LB plates supplemented with chloramphenicol to confirm hte absence of any mutant contamination.

At 3, 7 and 10 days post-infection groups of 5 birds from each cage were killed by cervical dislocation of the neck. A pilot experiment had shown that this number of birds was sufficient to give an accurate and reliable assessment of bacterial counts whilst allowing for bird to bird variation. Samples of the spleen, liver, caecal contents, caecal wall, ileal contents and ileal wall were taken at *necropsy* examination with sterile forceps and scissors. The samples were weighed and a 1:10 dilution with saline was made. The samples were homogenised with a rotary blade, which was sterilised with 70 % (v/v) ethanol between samples. Bacterial counts were determined by serial dilutions on brilliant green agar and the number of colony-forming units per gram of tissue was calculated. Samples that contained bacteria below the limits of detection were enriched by addition of selenite broth 1x for 16-18 h at 37 ºC and then 100 µl was plated on brilliant green agar to produce a positive or negative result.

2.6.2. *Trans*-complementation of a fimbrial mutant *in vivo*

The recombinant pCR4Blunt plasmid harbouring the *stcA* gene obtained in Sections 2.5.6 and the pACYC177 plasmid was purified from *E. coli* K-12 ER2420 (NEB) as described in Section 2.3.2 were digested for 2 h at 37 ºC with *Cla*I. The two products were ligated for 5 min at room temperature with 1 µl of LigaFast T4 DNA ligase (Promega) and 5 µl of 2 x DNA ligase buffer to catalyse the formation of phosphodiester bonds. Different ratios of vector and insert were used. The product was transformed into chemically-competent *E. coli* as described in Section 2.5.7 and the orientation was confirmed by a restriction digest as described in Section 2.5.8.

The plasmids containing the confirmed inserts were extracted as described in Section 2.3.2 and electroporated into the *S.* Enteritidis P125109 strain that were mutated and lacked the chloramphenicol resistance cassette (Section 2.4.2, 2.4.3 and 2.4.4). *S.* Enteritidis P125109 wild-type, Δ*stcA::cat*, Δ*stcA* and the *trans*-complemented strains were screened *in vivo* as described in Section 2.6.1.

2.7.1. Statistics

Statistical analysis was carried out on both the *in vitro* and *in vivo* data in the same manner using the Statistical Analysis System SAS, version 9 (Newnan and Lavelle, 1998). The data were $\log_{10}$ transformed and a generalised linear model was constructed using the least square means. Significant differences were measured by using an F-test analysis with data taken as repeated measurements and the standard error of the mean was calculated, P values of less than 0.05 were considered significant.

Chapter 3

In silico analysis of *Salmonella* fimbrial loci

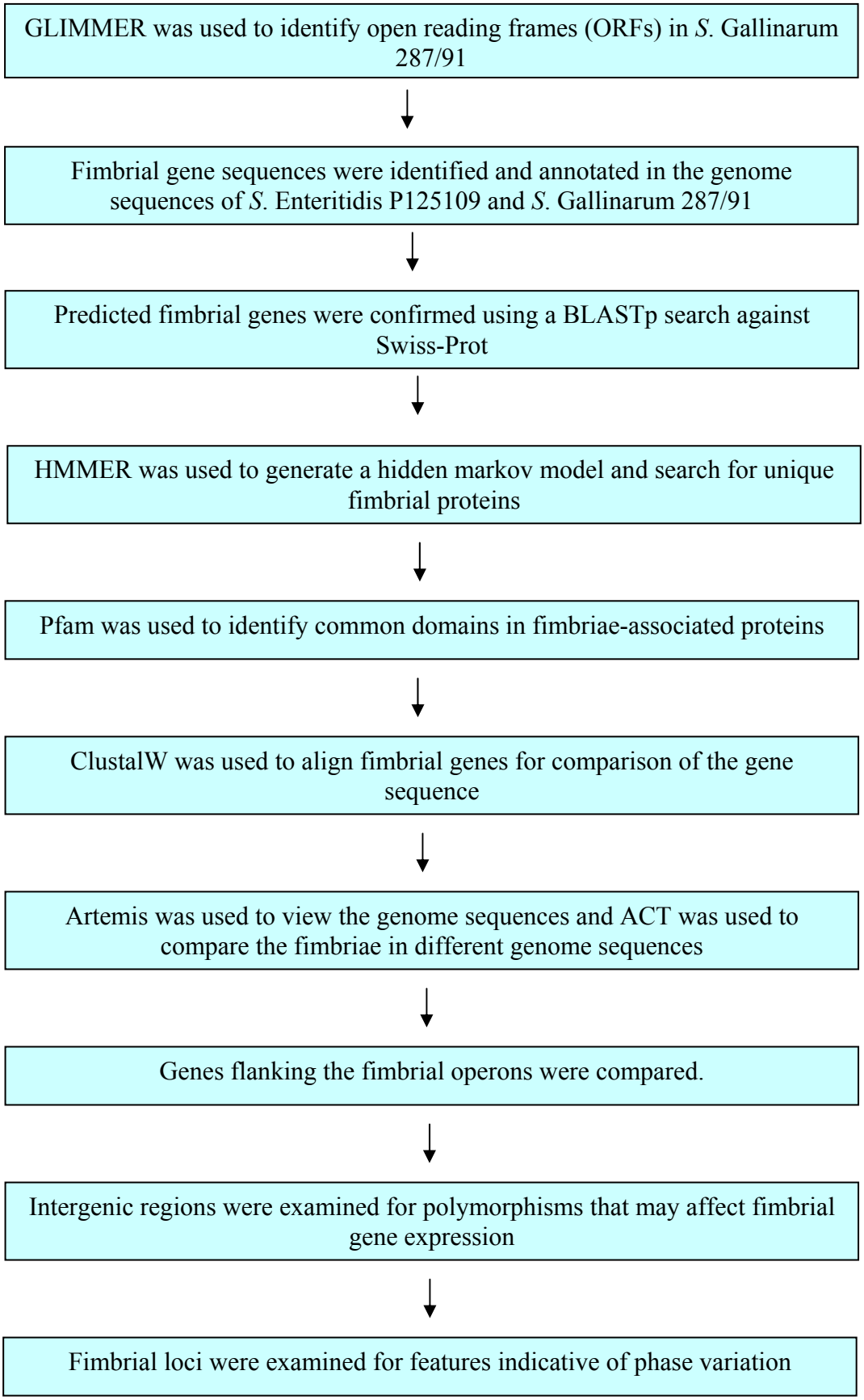
3.1. Introduction

Salmonella enterica subspecies I is a facultative intracellular pathogen of animals and humans and as yet, the genetic basis of the differential virulence, tissue tropism and host range of *S. enterica* serovars is poorly defined. The genome sequences of several strains of *Salmonella* are available including *S.* Enteritidis P125109, *S.* Gallinarum 287/91, *S.* Typhimurium LT2, *S.* Typhi CT18, *S.* Choleraesuis SC-B67*, S. Bongori* 12419*, E. coli* O157:H7 EDL933 and *E. coli* K-12 MG1655. Comparative analysis has indicated that the narrowing of host range of *S.* Typhi is associated with gene decay (Deng *et al.*, 2003) as has been described for other host-restricted pathogens including *Burkholderia mallei* (Holden et al., 2004, Nierman et al., 2004) and *Mycobacterium leprae* (Cole *et al.*, 2001), compared to their broad host-range counterparts. The precise genetic basis of host-restriction and the role of residual genes in colonisation and pathogenesis are not understood.

Fimbriae are proteinaceous surface-exposed structures that can adhere to abiotic and biotic surfaces and interact with host cells *in vitro* and *in vivo* (Boddicker et al., 2002, Woodward et al., 2000, Rajashekara et al., 2000, Edwards et al., 2000, Dibb-Fuller and Woodward, 2000, Baumler et al., 1996a, Thiagarajan et al., 1996). Several fimbrial loci have been implicated in *Salmonella* pathogenesis by genome-wide and targeted mutagenesis as described in Chapter 1. Thus, I sought to determine if variations in the repertoire, sequence or organisation of fimbrial operons among *S. enterica* serovars could be correlated with host-specificity using a range of *in silico* approaches. At the time of writing, there is no literature available that compares the complete fimbrial repertoire of *Salmonella* strains and by defining the repertoire, sequence and organisation of fimbrial operons and considering their role in colonisation and

pathogenesis, it may be possible to partially explain the host-specificity of *S. enterica* serovars.

Figure 3.1. A flow diagram describing the methodology used to identify and analyse *Salmonella* fimbrial loci *in silico*



3.2. Aims

- To identify and annotate all fimbrial loci in sequenced strains of *Salmonella enterica.*
- To determine if the repertoire and/or sequence of fimbrial loci correlates with host-specificity.
- To examine the genomic insertion sites of fimbrial loci and relate this to the phylogeny of the strains.
- To identify conservation or polymorphisms in the coding and non-coding regions of the fimbrial loci that may affect their function or expression.
- To identify features indicative of phase variation.
- To identify targets for mutagenesis to probe the role of fimbrial loci in *Salmonella* pathogenesis.

3.3. Identification and comparative analysis of fimbrial loci

3.3.1. Identification of fimbrial genes

At the outset of this project the genome sequence of *S*. Gallinarum 287/91 was in a raw format consisting of 2 contigs, chromosome and plasmid, and no predicted genes. Glimmer was used to predict coding regions in the entire genome of *S*. Gallinarum 287/91. The fimbrial loci of *S*. Enteritidis P125109 and *S*. Gallinarum 287/91 were identified by BLASTp searches against SwissProt/Uni-prot. The Artemis Comparison Tool (ACT) was used to compare the genome sequences of *S*. Enteritidis P125109 and *S*. Gallinarum 287/91 to the fully annotated genome sequences of *S.* Typhimurium LT2, *S.* Typhi CT18, *S.* Choleraesuis SC-B67*, S. Bongori* 12419*, E. coli* O157:H7 EDL933 and *E. coli* K-12 MG1655. The relative chromosomal positions of fimbrial operons in the *Salmonella* strains are shown in Figure 3.2. This is the first time a large-scale comparison has been carried out on the repertoire of fimbrial genes at the genomic level.

The analysis revealed differences in the number of fimbrial loci but no single locus directly correlated with host-specificity. The *sta, stg* and *tcf* operons were specific to serovars causing human systemic illness, however they are absent in serovars Enteritidis and Typhimurium, which infect humans and induce enteritis. The *bcf, sth* and *csg* operons are present in all the *Salmonella* strains examined, although the genes within the *csg* operon of both *S.* Gallinarum 287/91 and *S.* Enteritidis P125109 are part of a large region that has undergone an inversion relative to the other *Salmonella* strains (Figure 3.2 large arrows). In *E. coli* O157:H7 and *S. bongori* 12419*,* the *lpf* operon exists in duplicate compared to the single copy in the other *Salmonella* strains, yet it is absent in *E coli* K-12. Interestingly, the *fim* operon of *Salmonella* also shares structural, chromosomal and sequence identity with the *sfm* operon of *E. coli* K-12 and O157, not

the *fim* operon of these strains. The *ste* operon is missing from *S*. Typhimurium but the remnants and flanking regions of *ste* are present implying that the *ste* operon has been lost.

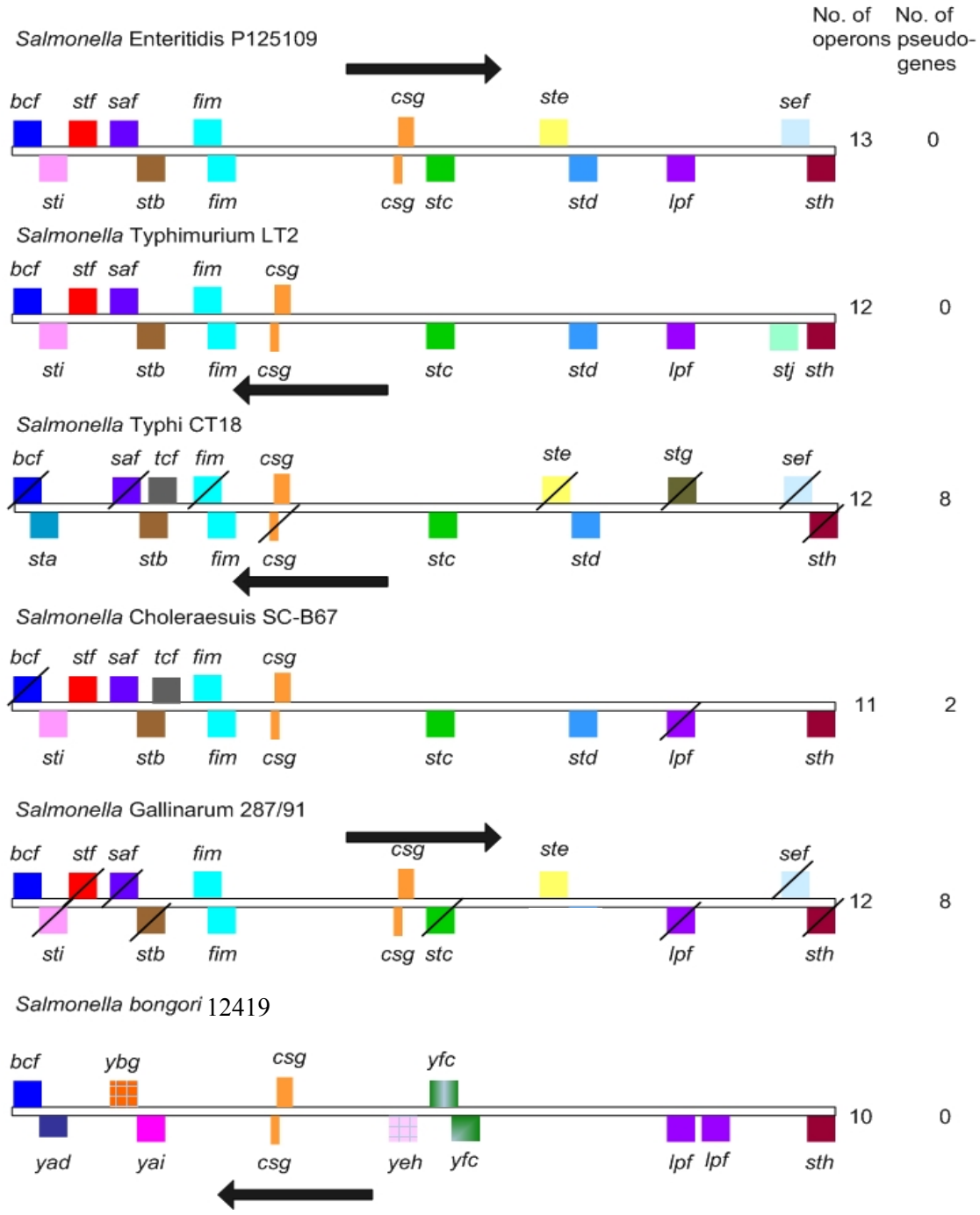
3.3.2. Molecular detection of fimbrial loci

Molecular techniques were used to examine the full repertoire of fimbrial operons in 40 different strains of *Salmonella.* Despite several attempts to detect conservation by DNA hybridisation (Southern blots and dot blots) all were unsuccessful. The results were unreliable, ambiguous and produced false positives, owing to the high degree of homology between fimbrial loci. As the complete genome sequences were available for some of the strains used it it easy to identify false positives, primarily *staA*. Studies *in silico* identified *staA* as present in *S*. Typhi but both Southern and dot blots showed it to be present in *S*. Enteritidis P125109. This introduces an element of doubt over all of the data and reliable conclusions can not be made. Other groups have attempted to detect the entire repertoire of fimbrial operons using comparative genomic hybridisation to microarrays has also been reported to be unreliable for detecting the full complement of fimbriae in different strains of *Salmonella* as ambiguous data was generated (Porwollik et al., 2004, van Asten and van Dijk, 2005).

Hybridisation to the chaperone or usher will always result in cross-hybridisation to other fimbriae dur to the very large highly conserved Pfam domain contained within the chaperone and usher. The najor fimbrial subunit in many cases also contains a Pfam domain but it is not as conserved as the chaperone and usher but it some cases may still be allowing cross-hybridisation to occur.

Figure 3.2. Schematic representation of the repertoire and relative genomic location of the fimbrial operons in strains representing different *S. enterica* serovars

Each coloured block represents a distinct fimbrial operon encoded in the sense (top) or anti-sense (bottom) orientation. Boxes of the same colour represent divergently transcribed operons. A diagonal line through the block indicates that at least one gene in the operon is a predicted pseudogene. All genomes are aligned relative to their predicted origin (not to scale).



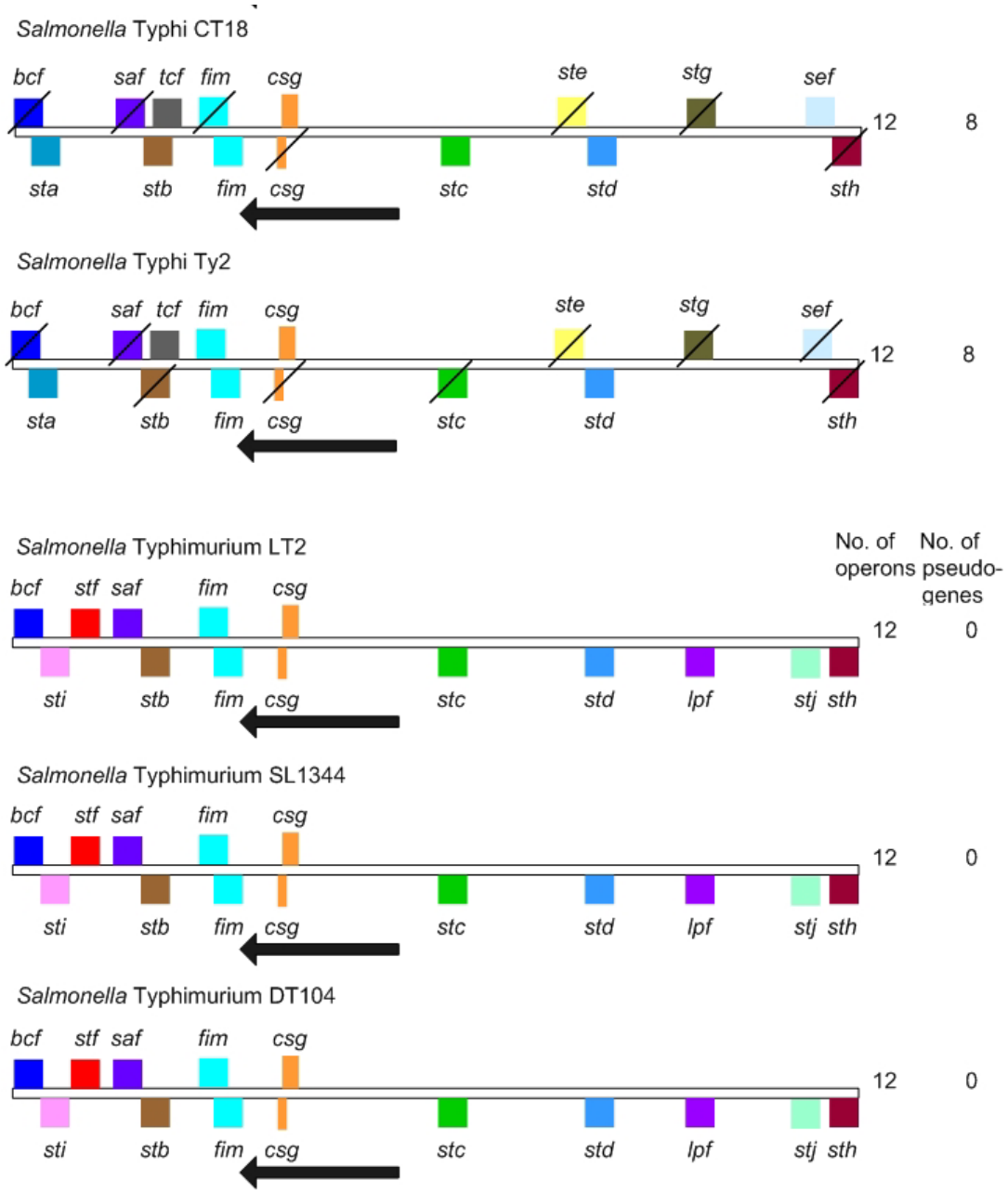
3.3.3. Conservation of the repertoire of fimbrial loci in the same serovars

To confirm the strains are representative of the serovars in respect of their complement of predicted fimbrial loci, ACT was used to compare the genome sequences of *S*. Typhi strains, CT18 and Ty2 and three strains of *S*. Typhimurium; LT2, SL1344 and DT104, (the two latter strains were un-annotated). The presence or absence of fimbrial loci was determined and summarised in Figure 3.3.

These comparisons revealed striking conservation of repertoire and sequence of the fimbrial genes (Figure 3.3). In *S*. Typhimurium, all fimbrial genes are 99 % identical to each other and the repertoire of the three sequenced strains is identical. In *S*. Typhi CT18 and Ty2, the repertoire of operons is identical but the occurrence of pseudogenes is different. In *S*. Typhi Ty2, the *fimI* gene is intact whereas *stcC* and *stbC* genes are pseudogenes in addition to those pseudogenes predicted in CT18. The *stj* operon is present in all 3 strains of *S*. Typhimurium but not in any other serovars and appears truncated, as it only encodes a putative chaperone and usher proteins. No other *Salmonella* strain examined possessed a fimbrial operon at the same chromosomal position but a putative fimbrial operon was identified in *E. coli* K-12 in the same position.

Figure 3.3. Schematic representation of the repertoire and relative genomic location of the fimbrial operons in strains of the same *S. enterica* serovars

Each coloured block represents a distinct fimbrial operon encoded in the sense (top) or anti-sense (bottom) orientation. Boxes of the same colour on either strand represent divergently transcribed operons. A diagonal line through the block indicates that at least one gene in the operon is a predicted pseudogene. All genomes are aligned relative to their predicted origin (not to scale).



3.3.4. Conservation of fimbrial genes

The conservation of the fimbrial loci amongst the *S. enterica* strains examined in Figure 3.2 was compared by ACT. At the nucleic acid level, 56 out of 71 fimbrial genes examined possessed ≥95 % sequence identity as shown in Table 3.1. These include all of the genes of the fimbrial operons *sti, stb, fim, csg* and *lpf,* implying that their function may also be conserved.

The host-specific strains of *S.* Typhi CT18 and *S.* Gallinarum 287/91 possessed the highest number of predicted fimbrial pseudogenes 11 out of 68 fimbrial genes (based on the presence of at least one stop codon in the predicted gene co-ordinates). In *S.* Typhi CT18 8 out of 56 fimbrial genes were predicted to be pseudogenes including *sefD, sefR, sefA, bcfC, fimI, steA, sthC, csgD*, *bcfG* and *sthE* contained internal stop codons, as did *stiC, stfF*, *safC, stbC, stcC, lpfC, sefD, sefC*, *sthB, sthC* and *sthE* in *S.* Gallinarum 287/91. In *S.* Choleraesuis SC-B67, only *bcfC* and *lpfC* contain internal stop codons and there were no predicted pseudogenes in the fimbrial operons of the other serovars examined. Where a pseudogene exists in more than one strain, there was no evidence that the same residue was always mutated.

The percentage of fimbrial genes that are pseudogenes in *S.* Typhi CT18, Ty2 and *S.* Gallinarum 287/91 was found to be 14 %, 16 % and 16 % respectively compared to the total genomic mean of 4 % of predicted genes in *S.* Typhi CT18 and Ty2 and 7 % in *S.* Gallinarum 287/91. This may imply that selection has occurred for loss of fimbrial function in host-restricted serovars, or that mutations have accumulated as the fimbriae are no longer required in the niche occupied. The pseudogenes are merely predicted and it remains possible that rest of the operon may still function and contribute to the assembly or expression of distally-encoded fimbriae (Forest et al., 2007).

Table 3.1. Conservation of the nucleotide sequences of *S.* Enteritidis strain P125109 fimbrial genes across strains representing other *S. enterica* serovars

	SE	***SG***		***STm***		***ST***		***SC***	
Gene	n	n	%	n	%	n	%	n	%
bcfA	543	543	99	543	99.63	543	98.89	543	99.63
bcfB	687	688	97.2	687	99.85	687	98.98	687	79.18
bcfC	2622	2622	99	2622	99.24	**2622p**	**98.36**	**2622p**	**99.05**
bcfD	1008	1008	100	1008	99.31	1008	98.12	1008	98.71
bcfE	546	546	100	546	98.35	546	97.62	546	98.90
bcfF	519	519	99	519	99.81	519	97.88	519	99.23
bcfG	732	732	100	732	99.32	705	95.63	732	99.45
bcfH	846	846	100	846	99.76	846	100	846	99.53
stiA	540	540	99	540	99.23	**X**	**X**	540	99.63
stiB	684	684	97	684	98.97	**X**	**X**	684	98.97
stiC	2547	**2547p**	**99.8**	2547	99.72	**X**	**X**	2547	99.37
stiH	1080	1080	99	1080	99.35	**X**	**X**	1080	99.17
stfA	561	561	99	561	98.57	**X**	**X**	561	99.15
stfC	2658	2658	99	2658	99.59	**X**	**X**	2658	99.47
stfD	753	753	99	753	99.47	**X**	**X**	753	99.47
stfE	513	513	98	513	99.02	**X**	**X**	513	98.83
stfF	477	**477p**	**85**	477	99.58	**X**	**X**	477	92.66
stfG	531	531	98	531	96.99	**X**	**X**	531	91.34
safA	510	495	51.2	513	81.48	495	68.63	513	81.18
safB	738	741	83.5	738	87.12	741	81.10	738	95.94
safC	2511	**2482p**	**50.8**	2511	98.85	2511	98.49	2511	98.73
safD	471	471	93.6	471	96.39	471	96.60	471	95.54
stbA	537	537	100	537	98.88	537	98.69	537	99.26
stbB	762	762	99	762	99.21	762	98.03	762	98.95
stbC	2562	**2559p**	**99**	2562	99.22	2562	98.83	2562	99.53
stbD	1326	1326	99	1326	99.02	1326	98.87	1326	99.25
stbE	759	756	99	759	97.76	759	98.95	760	98.82
fimA	558	556	98.6	558	98.75	555	97.83	555	99.95
fimI	534	534	100	534	98.32	**534p**	**99.06**	**534p**	**98.50**
fimC	693	693	98	693	98.99	693	98.70	**X**	**X**
fimD	2613	2613	99	2613	99.00	2613	98.81	2619	99.00
fimH	1008	1008	99	1008	98.41	1008	97.62	1008	98.81
fimF	519	519	100	519	99.23	519	98.07	519	99.81
fimZ	633	633	98	633	100	633	98.74	634	99.53
fimY	723	723	98	723	98.20	723	98.20	723	97.79
fimW	597	596	99	597	98.49	597	98.32	597	98.83
csgC	327	327	100	327	100	327	99.69	327	100
csgB	456	456	99	456	99.56	456	99.34	456	99.78
csgA	456	456	99	456	98.90	456	98.68	456	100
csgD	651	651	99	651	99.69	627	95.39	651	100
csgE	396	396	99	396	100	396	100	396	100
csgF	417	417	99	417	100	417	99.52	417	99.52

csgG	834	834	100	834	99.73	834	99.33	834	99.73
stcA	534	534	100	531	66.85	531	66.85	531	67.23
stcB	681	684	98	684	64.51	684	64.51	684	64.51
stcC	2487	**2488p**	**99**	2490	66.25	2490	66.25	2490	66.25
stcD	1023	1023	98	1008	58.14	1008	58.14	1008	58.14
stdA	711	**X**	**X**	711	89.31	708	95.48	711	91.14
stdB	2484	**X**	**X**	2490	99.52	2490	97.63	2490	99.44
stdC	744	**X**	**X**	744	99.06	744	97.31	744	98.25
steA	588	588	100	**X**	**X**	**588p**	**98.13**	**X**	**X**
steB	2700	2700	99	**X**	**X**	2700	99.23	**X**	**X**
steC	774	774	99	**X**	**X**	774	98.32	**X**	**X**
steD	507	504	99	**X**	**X**	507	99.21	**X**	**X**
steE	471	471	100	**X**	**X**	471	98.31	**X**	**X**
steF	537	537	100	**X**	**X**	537	85.66	**X**	**X**
lpfA	537	537	99	537	99.81	**X**	**X**	537	99.81
lpfB	699	699	100	699	99.86	**X**	**X**	699	100
lpfC	2529	**2528p**	**99**	2529	99.20	**X**	**X**	**2528p**	**99.64**
lpfD	1080	1080	99	1080	99.54	**X**	**X**	1080	99.54
lpfE	528	528	99	528	99.81	**X**	**X**	528	97.35
sefA	537	537	92.7	**X**	**X**	**536p**	**99.32**	**X**	**X**
sefB	741	740	91.9	**X**	**X**	753	97.98	**X**	**X**
sefC	2445	**2445p**	**98**	**X**	**X**	2517	99.79	**X**	**X**
sefD	453	**452p**	**100**	**X**	**X**	**443p**	**97.79**	**X**	**X**
sefR	837	837	53	**X**	**X**	**812p**	**95.10**	**X**	**X**
sthA	546	546	99	546	98.35	546	98.71	546	98.72
sthB	684	**684p**	**99**	684	99.71	684	97.91	684	98.90
sthC	2538	**2454p**	**93.1**	2538	99.68	**2534p**	**98.70**	2538	99.09
sthD	558	558	100	558	99.13	558	97.31	558	98.75
sthE	1086	**1047p**	**90**	1086	99.36	**1085p**	**97.88**	1086	98.98
pefA	519	X	X	519	81.31	X	X	519	82.08
pefB	300	X	X	300	97.67	X	X	300	98.6
pefC	2409	X	X	2409	96.60	X	X	2409	98.5
pefD	681	X	X	681	98.97	X	X	681	99.41
pefI	213	X	X	213	90.14	X	X	X	X
Orf5	558	X	X	558	73.47	X	X	X	X
Orf6	351	X	X	351	96.58	X	X	X	X

Percent identity to the *S.* Enteritidis P125109 gene was calculated using BLASTn. Abbreviations: n, length in nucleotides; X, gene absent; SE, *S.* Enteritidis P125109; SG, *S.* Gallinarum 287/91; STm, *S.* Typhimurium LT2; ST, *S.* Typhi CT18; SC, *S.* Choleraesuis SC-B67; **P**, pseudogene. The shading reflects the divergence of the *safA* gene and the existence of two variants of *stc*.

The *safA* fimbrial gene exhibits the greatest variation between the serovars (Table 3.1) which has been implicated in the colonisation of the porcine intestines by *S.* Typhimurium (Carnell et al., 2007). Screening of the same *safA* mutant in calves and chickens did not reveal any attenuating effects (Morgan *et al.*, 2004), implying that *Salmonella* atypical fimbriae may play a host-specific role in colonisation. The *saf* operon appears intact in serovars able to colonise pigs, but contains pseudogenes in the avian- and human-specific serovars. Polymorphisms were also identified in the *safA-B* intergenic region in the avian- and human-specific strains (below) and this may reflect the fact that a porcine-specific colonisation factor is no longer needed. The *safA* gene contains a conserved N- and C-terminus but the region in between is highly variable. The remainder of the *saf* operon is highly conserved.

The *stc* fimbrial operon exists in two forms in identical chromosomal positions but each possesses different sequences with limited sequence identity (highlighted in Table 3.1); one is highly conserved in *S.* Enteritidis P125109 and *S*. Gallinarum 287/91, and a different variant exists in *S.* Typhi CT18, *S*. Typhimurium LT2 and *S*. Choleraesuis SC-B67.

3.3.5. Operon organisation

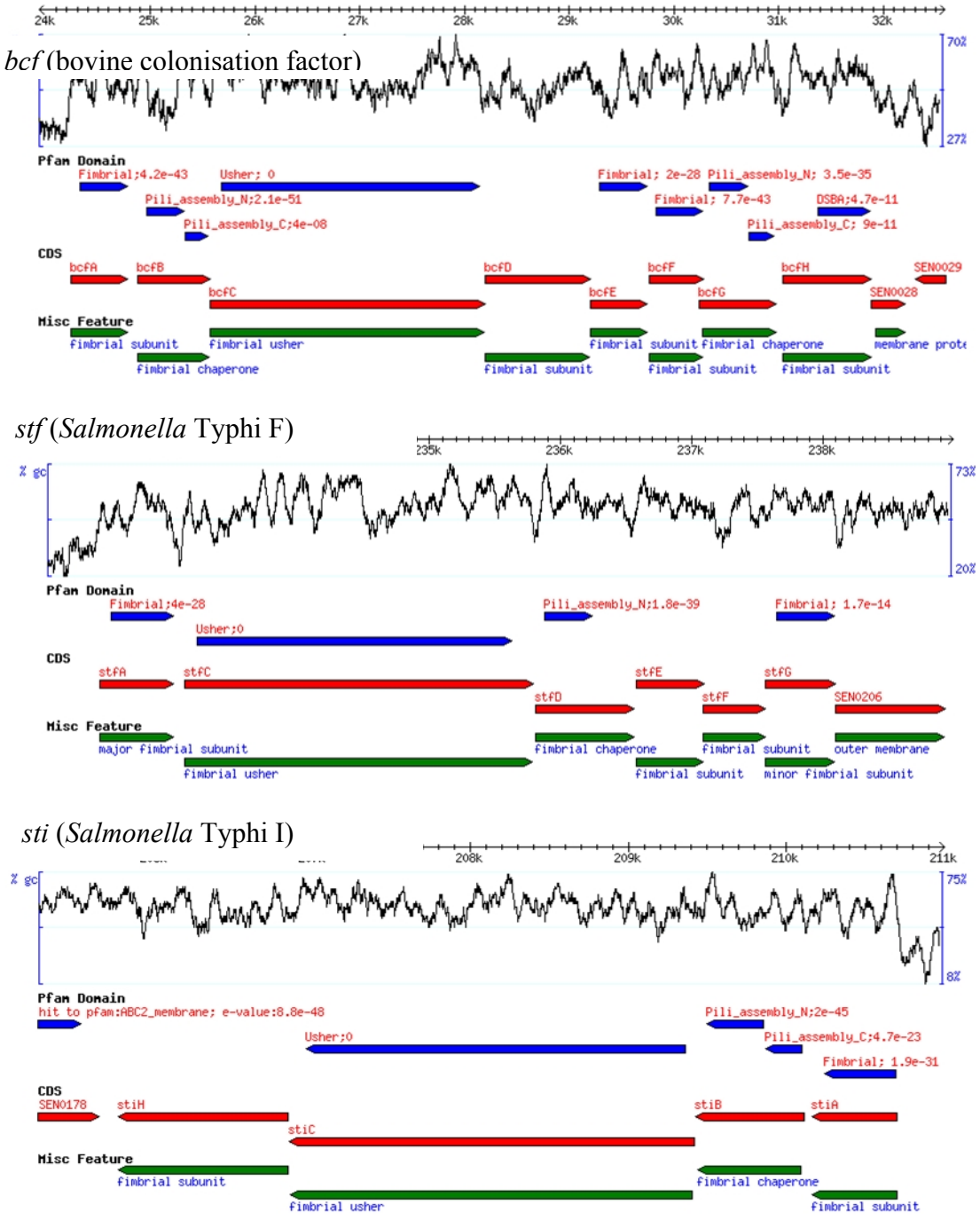
A BioPerl script (Appendix 2.1) was written to visually display the organisation of the complete repertoire of fimbrial loci in *S*. Enteritidis P125109. The size and number of genes together with the positioning of Pfam domains is shown in Figure 3.4.

The operon diagrams demonstrate the variation that exists between the fimbrial operons both in the number of genes within the operon and the size of the genes. The gene number and order are conserved across all of the strains examined in which the

operons appear. The *bcf* operon comprises 8 genes and the *fim* operon comprises 9 genes including the regulatory genes, yet the *std* operon contains only 3 genes. The function of the additional genes is unknown but implies that some loci may not encode functional fimbriae *per se* but rely on distally-encoded genes for assembly. The *saf, stb, stc, std, ste, sth, bcf* and *sef* operons are present in regions of the *S*. Enteritidis P125109 chromosome that are absent in *E. coli* K-12 (Townsend *et al.*, 2001). Only the *sef* fimbrial operon is a recent acquisition by the serogroup D *Salmonella* strains which is supported by the fact that this operon has a much lower % G+C content than the rest of the genome (Figure 3.4) (Turcotte and Woodward, 1993). The other fimbrial operons have no major deviations in % G+C content; however a reproducibly lower % G+C region was detected in the region 5′ of the operon in the direction of transcription (Figure 3.3).

Figure 3.4. Organisation of the fimbrial operons of *S.* Enteritidis P125109

Arrows denote the direction of transcription. Blue arrows denote the location of predicted Pfam domains, red arrows show predicted co-ordinates and green arrows show miscellaneous features (to scale).

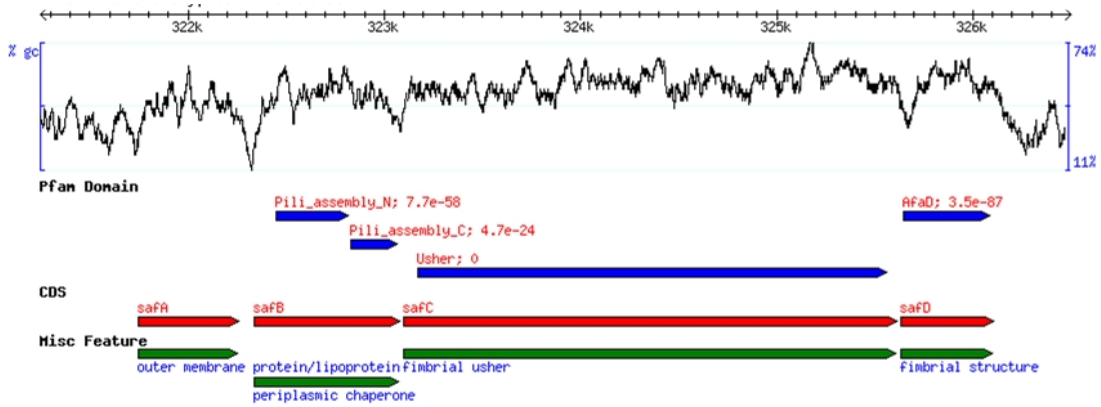


bcf (bovine colonisation factor)

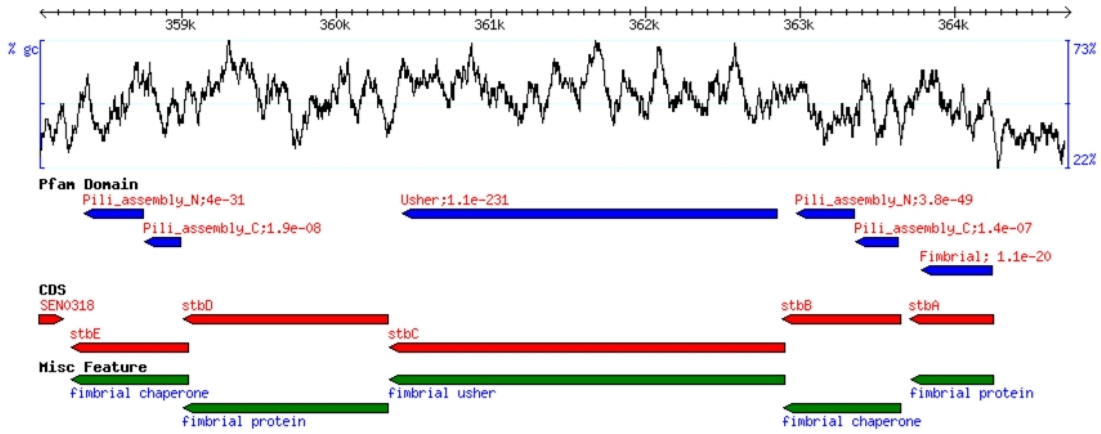
stf (*Salmonella* Typhi F)

sti (*Salmonella* Typhi I)

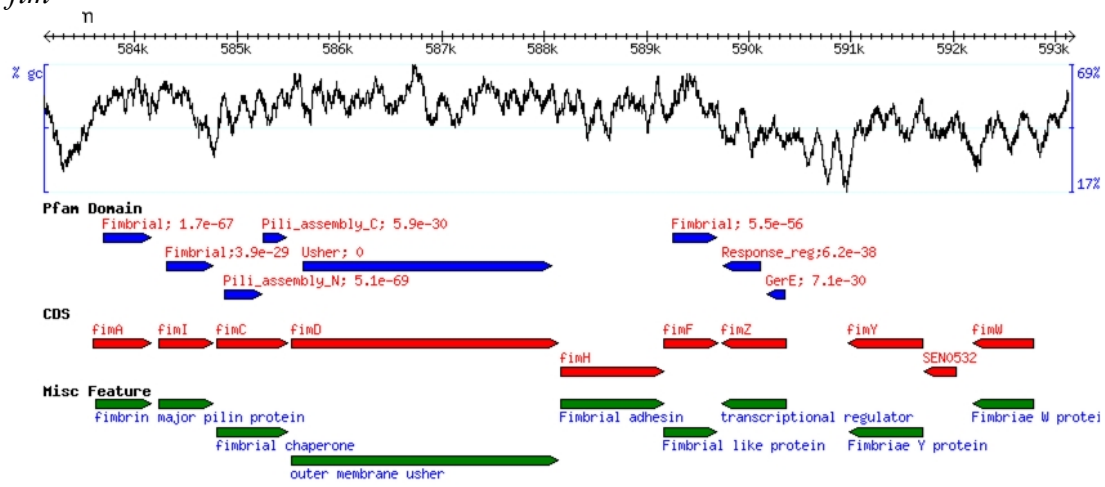
saf (*Salmonella* atypical fimbriae)



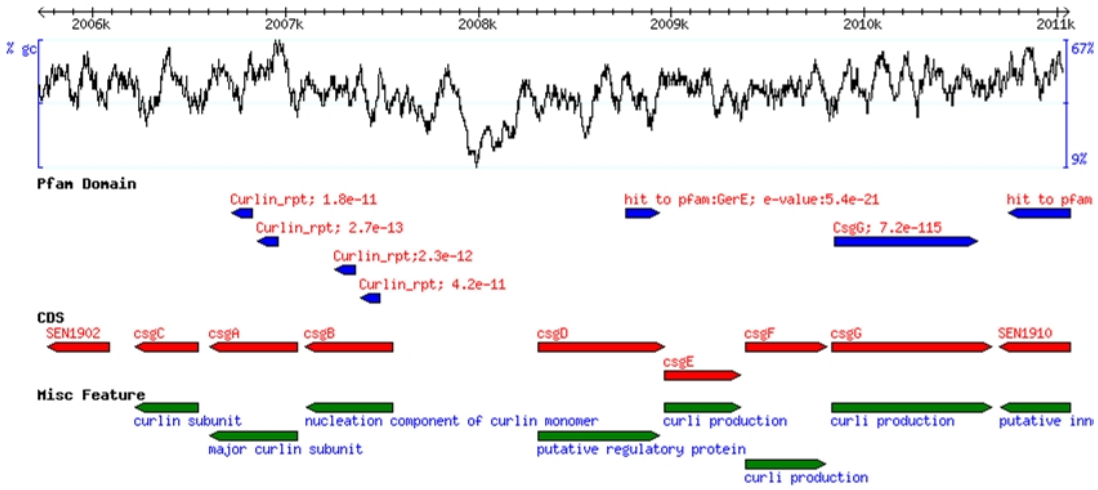
stb (*Salmonella* Typhi B)



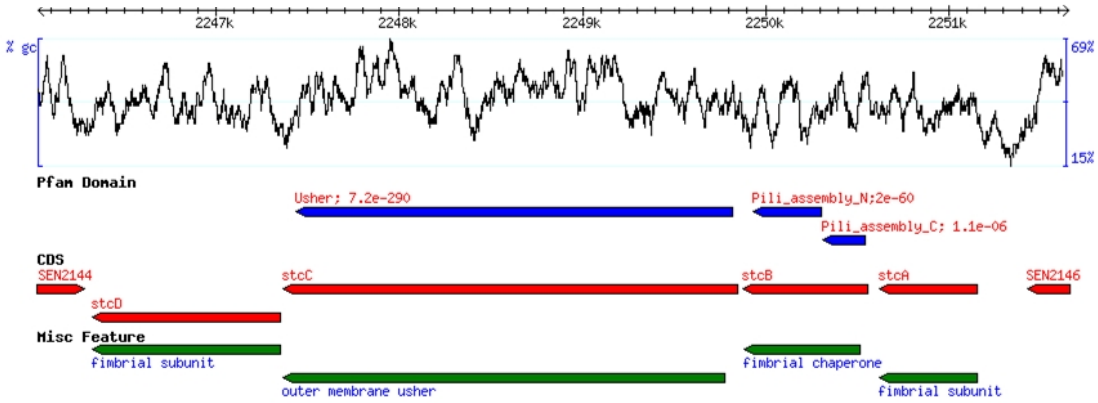
fim



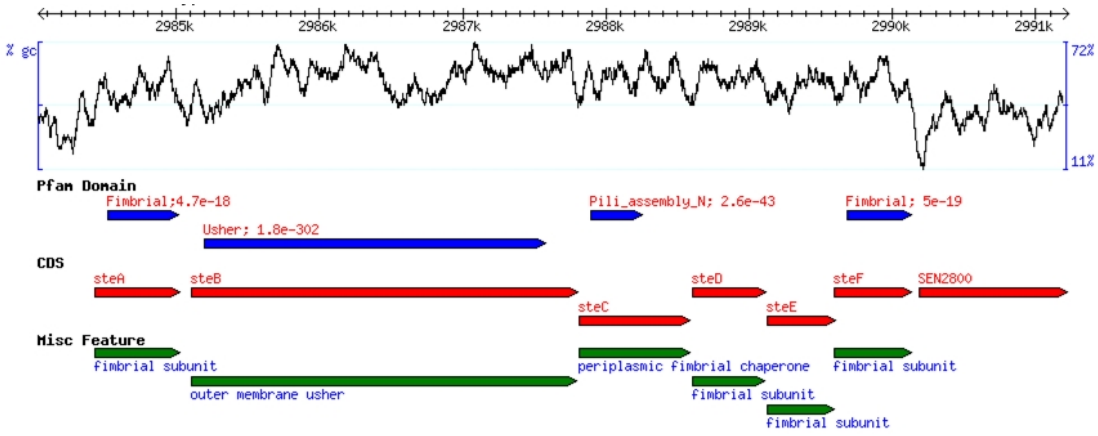
csg



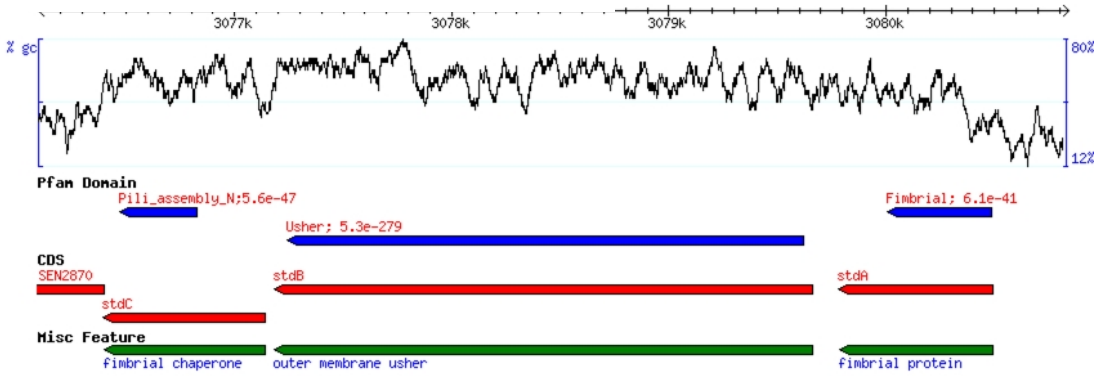
stc (*Salmonella* Typhi C)

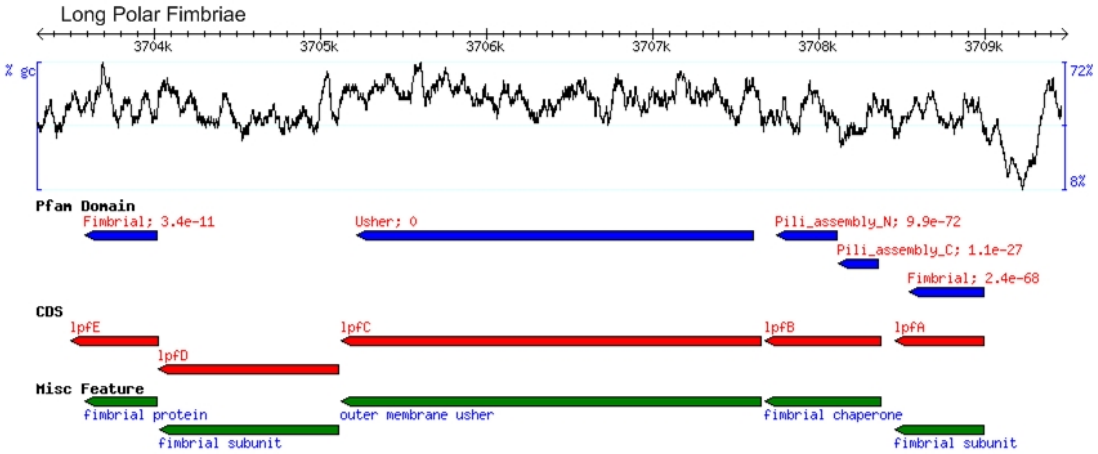


ste (*Salmonella* Typhi E)

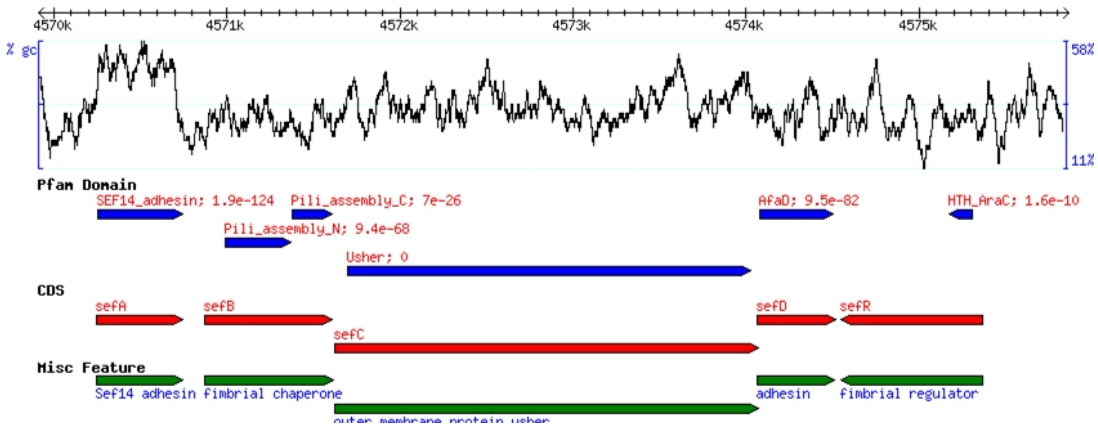


std (*Salmonella* Typhi D)

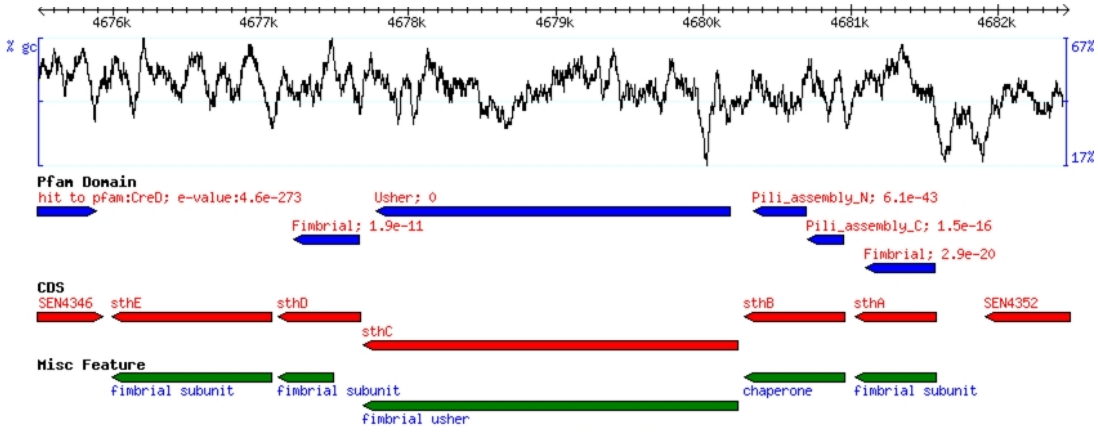




sef (*Salmonella* Enteritidis fimbriae)



sth (*Salmonella* Typhi H)



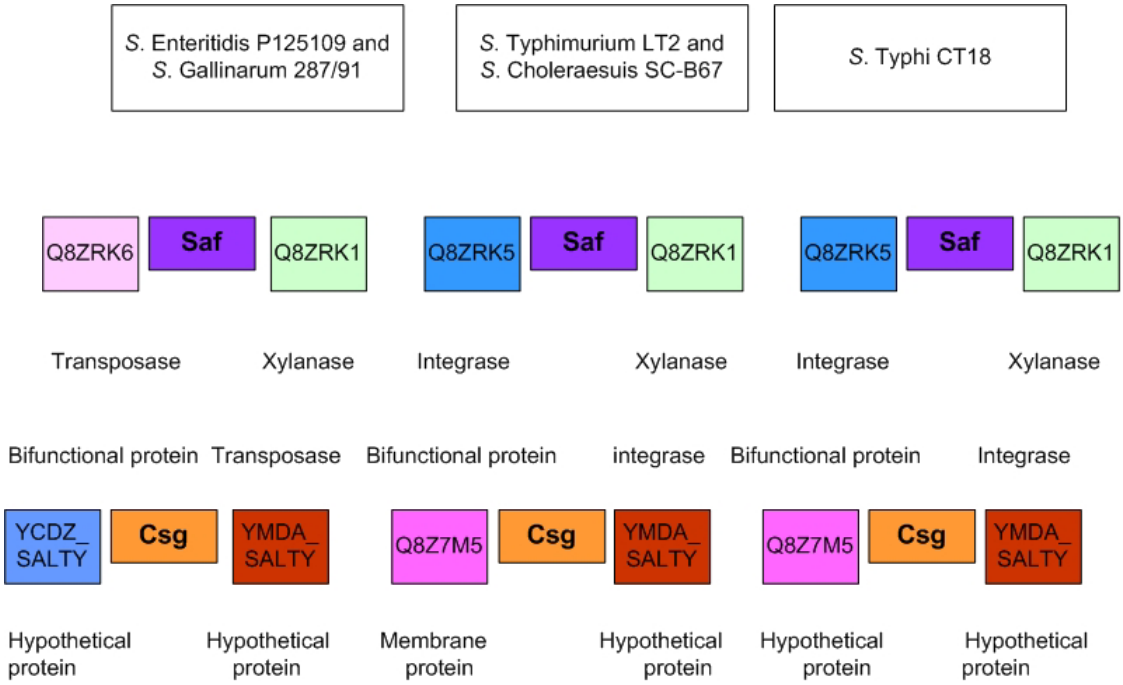
3.3.6. Comparisons of flanking regions

The regions flanking predicted fimbrial operons were examined to determine if the fimbrial genes were acquired individually, as part of the operon or larger region, or were maintained from a common ancestor. The first coding regions 5` and 3` of each fimbrial operon were identified and compared using ACT. The *bcf, sti, stf, stb, stc, ste, std, lpf, sef* and *sth* fimbrial operons show complete conservation of their flanking genes between the strains examined, implying that these operons may have been maintained from a common ancestor.

The *saf* operon has a 3′ flanking gene encoding a putative xylanase (Figure 3.4) in all serovars examined. The region 5′ of *safA* consists of two variants *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 possess one variant, a putative transposase 5′ of *safA*, and all of the other serovars encode a putative integrase at this position, suggesting that the *saf* genes may have been acquired or rearranged differently (Figure 3.5). *S.* Enteritidis P125109, *S.* Gallinarum 287/91 and *S.* Typhi CT18 possess a gene encoding a hypothetical protein 5′ of the *csg* operon, whereas *S.* Choleraesuis SC-B67 and *S.* Typhimurium LT2 possess a gene encoding a membrane protein (Figure 3.5). Interestingly, only *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 have an inverted *csg* operon relative to all other *Salmonella* serovars and *E. coli* strains examined.

Figure 3.5. Schematic representation of the flanking gene regions of predicted fimbrial operons

Only those with differences are shown, all are 5´ to 3´.



3.3.7. Comparison of intergenic regions

The non-coding sequences of the fimbrial operons were aligned using ClustalW to detect polymorphisms that may affect the transcription or translation of fimbrial genes. The majority of differences existed between rather than within the *S. enterica* serovars examined implying that the fimbrial operons were likely acquired as whole operons at a similar time in evolution or were maintained from a common ancestor.

The region between the *safA* and *safB* genes varies between serovars; *S.* Typhi CT18 has an extra gene in the *saf* operon, (*safE)* at this position that is not present in any other serovars examined. The regions between *safE* and *safB* in *S.* Typhi CT18 and the region between *safA* and *safB* in *S.* Gallinarum 287/91 are both 25 bp in length and

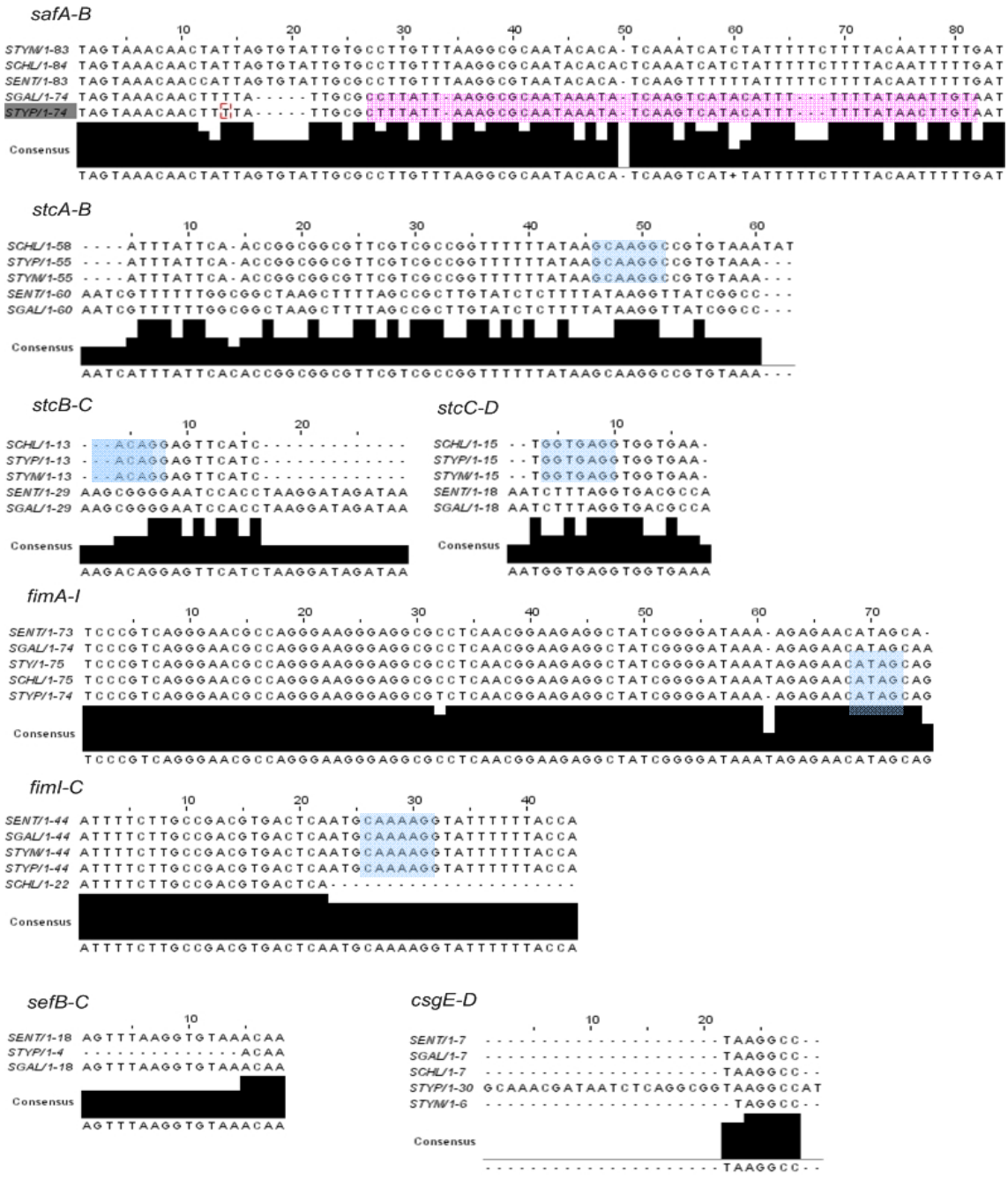
identical bar 2 bases. The *safA-safB* intergenic region contains a putative promoter in *S.* Gallinarum 287/91 and *S.* Typhi CT18 and it is unclear if this is required for expression of the downstream genes (Figure 3.6). Interestingly, *S.* Typhimurium LT2, *S.* Enteritidis P125109 and *S.* Choleraesuis SC-B67 also have an identical region between *safA* and *safB* but it differs from that in *S.* Gallinarum 287/91 and *S.* Typhi CT18.

The non-coding sequence of the *stc* operon has two variants (Figure 3.6). In each, *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 share the same *stc* intergenic sequences whereas the other serovars possess a different sequence, implying divergent evolution. Several putative ribosomal binding sites have been predicted in the intergenic regions in *S.* Choleraesuis SC-B67, *S.* Typhimurium LT2 and *S.* Typhi CT18 as highlighted in Figure 3.6 but are absent in *S.* Enteritidis P125109 and *S.* Gallinarum 287/91. The impact of such polymorphisms on translation of *stc* genes is unknown.

An insertion is present in the *fimA-I* intergenic region of *S.* Typhimurium LT2 and *S.* Choleraesuis SC-B67 which is not present in *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 (Figure 3.6). A ribosomal binding site has been predicted in this region of *S.* Typhimurium LT2, *S.* Choleraesuis SC-B67 and *S.* Typhi CT18 genome sequences. The *fimI-C* intergenic region is truncated and missing a ribosomal binding site in *S.* Choleraesuis SC-B67.

The *fimZ-Y* and the *fimY-W* intergenic regions were not identical between serovars due to point mutations. In *S.* Choleraesuis SC-B67, the *fimY-W* intergenic region has a completely different sequence and it does not contain an additional coding region as in all of the other serovars examined, identified as *fimU*. *S.* Typhi CT18 has more variation in the *csgE-D*, *steA-B* and the *sefB-C* intergenic regions than the other serovars, which may be due to the high degree of genome decay that has occurred in *S.* Typhi relative to the other genomes examined.

Figure 3.6. Polymorphisms detected in the intergenic regions in fimbrial operons of *S. enterica* serovars



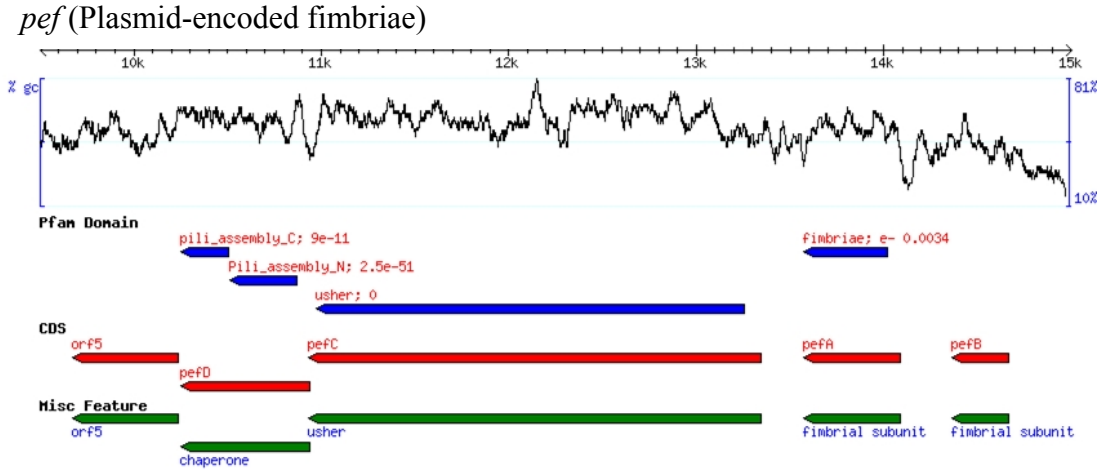
The hyphen shows a gap in the alignment. Putative promoter regions predicted using NNPP shown in pink. Predicted ribosomal binding sites are shown in blue. The entire intergenic region is shown. STYM = *S.* Typhimurium LT2, SCHL = *S.* Choleraesuis SC-B67, SENT = *S.* Enteritidis P125109, SGAL = *S.* Gallinarum 287/91 and STYP = *S.* Typhi CT18.

3.3.8. Plasmid-encoded fimbriae

The *pef* operon consists of 5 genes *pefA, pefB, pefC, pefD* and *pefR* (*pefI*) and is identical in *S*. Enteritidis P125109, *S*. Typhimurium LT2 and *S*. Choleraesuis SC-B67 (Figure 3.7). The *pef* operon is absent in *S*. Gallinarum 287/91; instead, 3 genes with homology to fimbrial genes in *E*. *coli* K88 were identified (*faeH* and *faeI*, predicted to encode minor fimbrial subunits and *faeJ*). In *E*. *coli,* this operon consists of 9 genes (*faeB-J),* suggesting that these 3 genes will not form a functional fimbrial operon but may have an accessory role.

Whilst the *pefI* gene is absent from the *pef* locus in the sequenced plasmid of *S*. Choleraesuis SC-B67, a *pefI* homologue is present 3' of the chromosomal *sef* operon in *S*. Enteritidis P125109 and *S*. Choleraesuis SC-B67. The *pefI* gene is also present 3' of *sef* in *S*. Typhi CT18 chromosome genome sequence despite it lacking a virulence plasmid and it has been reported that other *pef* remnants exist at this chromosomal locus (Bishop *et al.*, 2005), however BLASTp analysis revealed negligible homology. The promoter region of chromosomal and plasmid-encoded *pefI* differ entirely (Collighan and Woodward, 2001), however the impact of this is unknown.

Figure 3.7. Gene organisation and %G+C content of the plasmid-encoded fimbriae



3.3.9. Identification of Pfam domains in the fimbrial genes of *S.* Enteritidis P125109 and *S.* Gallinarum 287/91

A Pfam search on the fimbrial genes identified four domains within putative fimbrial proteins; a major fimbrial subunit (PF00419), an usher (PF00577) and chaperone C-(PF02753) and N-(PF00345) terminal domains in most of the operons examined.

The *csg* operon does not contain an usher or chaperone domain, consistent with the finding that it is the only fimbriae predicted to be assembled via the nucleator-dependent pathway and it does not have a major fimbrial subunit gene, instead it encodes for curlin rather than fimbrin (Hammar *et al.*, 1996).

The major fimbrial subunit Pfam domain is missing from the predicted products of the *sef, saf* and *stc* operons based on a Pfam search, however BLAST searches identified putative outer membrane fimbrial protein in all of the serovars examined. The *saf* operon encodes a predicted adhesin and *sef* and *stc* may be able to function in a different way either by using ancillary genes from other fimbrial operons or by allowing adherence to different receptors.

Several fimbriae contain two or more fimbrial subunit domains, including the *bcf* and *fim* operons which each contain 3 fimbrial domains, and the *ste, sth* and *lpf* operons contain 2 fimbrial domains. The *bcf* and *stb* operons also code for two putative chaperone proteins each with both an N-terminal and C-terminal chaperone domain. The function of the duplicated putative proteins is unknown and implies that some loci may not encode functional fimbriae on their own but rely on distally-encoded proteins for assembly.

3.3.10. Analysis of fimbrial loci for traits associated with phase variation

Fimbrial genes in some bacteria are subject to phase variable (on-off) expression at the transcriptional level (Brinton, 1959, van der Woude and Baumler, 2004, Casadesus and Low, 2006). The mechanisms behind phase variation in *Salmonella* are poorly characterised. During the annotation of *S*. Enteritidis fimbrial operons and genome sequence comparisons, no genes with homology to known recombinases were detected within or proximal to fimbrial loci. Putative transposase and integrase genes associated with DNA mobility were observed proximal to the *saf, sef* and *fim* loci. Direct or inverted repeat sequences that may serve as substrates for recombination were not detected in any of the fimbrial operons examined using the tandem repeats finder.

A pattern matching search was carried out for the Dam methylase target sequence GATC within and proximal to the fimbrial operons of *S*. Enteritidis P125109. This identified hundreds of potential targets, however those present from -500 to +200 bp relative to the predicted translation initiation codon of fimbrial genes are listed in Table 3.2. This search identified the same sites in the *pef* gene cluster as predicted to be methylated in *S*. Typhimurium (Nicholson and Low, 2000). Whereas *S*. Typhimurium

strains LT2 and SL1344 possess GATC sites at -98, -110 and -212 relative to the start of *pefB, S*. Enteritidis P125109 possessed only the sites at -110 and -212 but an additional site at +47 in *pefB* that is absent in the two strains of *S*. Typhimurium. Three potential Dam methylation target sites were also identified upstream of the *std* operon (-88, -97 and -110) in *S*. Enteritidis P125109 and *S*. Typhimurium LT2. This density of GATC sites is higher than would be predicted due to random distribution and further studies will be required to determine the role of these in Dam-dependent repression of the *std* genes as detected by microarray analysis (Balbontin *et al.*, 2006). Predicted Dam methylase targets were also identified upstream of the *sef, sti* and *stf* operon in *S*. Enteritidis P125109, which require further investigation (Table 3.2).

Searches were also performed to detect stretches of a repeated nucleotide homo-polymeric tract or those with a repeating unit containing different nucleotides (hetero-polymeric tracts) that may be subject to slipped-strand mispairing to introduce frame-shift mutations (van Belkum *et al.*, 1998). Mispairing between such tracts occurs during DNA replication and can result in production of a non-sense or truncated protein genes e.g. in assembly and maturation of Neisserial pilin (Meyer and van Putten, 1989). Homo-polymeric tracts consisting of repeats of A's or C's were searched on both strands using the pattern matching function within ACT and those identified within 200 bp of the predicted translation initiation codon of a fimbrial gene are listed in Appendix 3.1. Several conserved hetero-polymeric tracts were identified using a variable tandem repeat pattern finder (Davison *et al.*, 1999), however only one potential tract was identified within the *stdA* gene, the tract was a 6-mer GACCAT repeated 10 times. Variation in the number of such repeats is not predicted to alter the open reading frame resulting in premature termination; rather it may reduce the number of codons and thus the primary sequence of the encoded product StdA.

Table 3.2. Putative Dam methylation sites in the fimbrial loci of *S.* Enteritidis

GATC genome location	Relative location	Fimbrial gene location
24391-24394	156 bases into *bcfA*	*bcfA* 24235-24777
25564-25567	Overlapping start of *bcfC*	*bcfC* 25569-28190
232155-232158	330 upstream of *stf* operon	*stfA* 232485-233045
232259-232262	226 upstream of *stf* operon	*stfA* 232485-233045
233275-233278	144 bases into *stfC*	*stfC* 233131-235788
235893-235896	87 bases into *stfD*	*stfD* 235806-236558
321303-321306	444 bases upstream of *safA*	*safA* 321747-322256
2007363-2007366	190 bases into *csgB*	*csgB* 2007101-2007556c
2007481-2007484	75 bases into *csgB*	*csgB* 2007101-2007556c
2008355-2008358	44 bases into *csgD*	*csgD* 2008311-2008961
2984272-2984275	163 bases upstream of *steA*	*steA* 2984435-2985022
2984588-2984591	153 bases into *steA*	*steA* 2984435-2985022
4569956-4569959	245 bases upstream of *sefA*	*sefA* 4570250-4570747
4570097-4570100	153 bases upstream of *sefA*	*sefA* 4570250-4570747
4570145-4570148	105 bases upstream of *sefA*	*sefA* 4570250-4570747
4570397-4570400	147 bases into *sefA*	*sefA* 4570250-4570747
583667-583670	69 bases into *fimA*	*fimA* 583598-584155
589175-589178	2 bases into *fimF*	*fimF* 589173-589691
2987904-2987907	90 bases into *steC*	*steC* 2987814-2988587
4677536-4677539	135 bases into *sthD*	*sthD* 4677117-4677674c
4677600-4677603	74 bases into *sthD*	*sthD* 4677117-4677674c
4680139-4680142	87 bases into *sthC*	*sthC* 4677692-4680229c
4681823-4681826	249 bases upstream of *sthA*	*sthA* 4681029-4681574c
4681472-4681475	99 bases into *sthA*	*sthA* 4681029-4681574c
3080578-3080581	88 bases upstream of *stdA*	*stdA* 3079783-3080490c
3080587-3080590	97 bases upstream of *stdA*	*stdA* 3079783-3080490c
3080600-3080603	110 bases upstream of *stdA*	*stdA* 3079783-3080490c
3080295-3080298	195 bases into *stdA*	*stdA* 3079783-3080490c
364215-364218	29 bases into *stbA*	*stbA* 363708-364244c
364336-364339	92 bases upstream of *stbA*	*stbA* 363708-364244c
362854-362857	4 bases upstream of *stbC*	*stbC* 360340-362901c

210707-210710	8 bases upstream of *stiA*	*stiA* 210160-210699c
211099-211102	400 bases upstream of *stiA*	*stiA* 210160-210699c
210991-210994	292 bases upstream of *stiA*	*stiA* 210160-210699c
14610-14613	47 bases within *pefB*	*pefB* 14358-14657c
14767-14770	110 bases upstream of *pefB*	*pefB* 14358-14657c
14869-14872	212 bases upstream of *pefB*	*pefB* 14358-14657c

The location of the GATC site is noted below along with the fimbrial gene in close proximity.

This analysis provides merely a starting point for identifying traits associated with phase variation by known mechanisms. It is possible that fimbrial phase variation will occur via another unknown mechanism, which cannot be predicted.

3.3.11. Correlation of fimbrial repertoire and virulence of the strains examined

The virulence of *S*. Typhimurium SL1344, *S*. Enteritidis P125109 and *S*. Gallinarum 287/91 has been compared in a streptomycin-pre-treated mouse model. Each strain was observed to induce enterocolitis at comparable levels (Suar *et al.*, 2006). The fimbrial loci *bcf*, *lpf*, *stb*, *stc*, *std* and *sth* have been reported to influence colonisation of the murine intestines by *S*. Typhimurium (van der Velden et al., 1998, Weening et al., 2005, Lawley et al., 2006). *S*. Gallinarum 287/91 lacks the *std* operon and has pseudogenes in *lpf*, *stb*, *stc* and *sth* relative to *S*. Typhimurium SL1344, indicating that either these fimbriae are not essential for enterocolitis in mice or their absence is compensated for by other fimbriae. Interestingly, *S*. Gallinarum 287/91 was observed to invade polarised *trans*-immortalized IEC cells (derived from the small intestines of a transgenic mouse) at lower levels than *S*. Typhimurium SL1344 and *S*. Enteritidis P125109. It can be hypothesised that this may reflect polymorphisms in the repertoire and function of

fimbrial operons between the strains, though the differences may be explained by other traits.

3.4. Discussion

A comparative analysis of fimbrial operons in complete *Salmonella* genome sequences available at the time of writing was performed. Fimbriae have been implicated in virulence and tissue tropism, however no single fimbrial locus directly correlated with the host range of the *S. enterica* serovars examined. Several of the strains examined (*S.* Typhimurium SL1344, *S.* Enteritidis P125109 and *S.* Gallinarum 287/91) are of well-defined virulence in murine models (Suar *et al.*, 2006) and food-producing hosts (Carnell et al., 2007, Morgan et al., 2004), however variations in the repertoire of fimbrial loci possessed by such strains could not be correlated with virulence. Previous studies have indicated that fimbriae may act in concert (van der Velden *et al.*, 1998) and it remains to be seen if such synergy is important in host-specificity.

Host-specific *S. enterica* serovars contained a higher number of predicted pseudogenes in fimbrial loci than host-restricted serovars, and in turn ubiquitous serovars. Gene decay is a feature of the genome of *S.* Typhi in general, which possesses over 200 pseudogenes relative to *S.* Typhimurium (Deng *et al.*, 2003), as well as other host-restricted pathogens including *B. mallei* (Holden et al., 2004, Nierman et al., 2004, Sebaihia et al., 2006) and *M. leprae* (Cole *et al.*, 2001). The decay of genomes appears to correlate with a reduced ability to survive in the environment and in different hosts (Nierman et al., 2004, Holden et al., 2004). However, the observed frequency of pseudogenes among fimbrial genes in host-specific serovars was 2-4 folds higher than

the genomic mean. Such genetic attrition may contribute to the inability of host-restricted and -specific *S. enterica* serovars to persist in diverse animal hosts.

Salmonella has maintained a large repertoire of fimbriae suggesting their role may be important in colonisation of diverse niches. The majority of fimbrial genes across the serovars examined show >95% homology at the nucleic acid level when compared to the fimbrial genes of *S.* Enteritidis P125109. The high conservation of the genes should imply that their function is also conserved. However this is not always the case, in *S.* Typhi CT18 the *sefA* gene is 99% identical to the *sefA* gene in *S.* Enteritidis P125109 but it is a pseudogene due the presence of a stop codon. *S.* Gallinarum has lost its mannose-specific binding properties due to a single base pair mutation in the *fimH* gene resulting in the translated adhesin being formed with a point mutation at position 78; isoleucine to threonine (Kisiela et al., 2005, Kisiela et al., 2006).

The number of genes encoded in each fimbrial locus varies, however each locus is highly conserved across the serovars. For loci that lack a fimbrial subunit Pfam domain but encode a predicted usher and chaperone (e.g. *saf* and *stc*) it remains unclear if distally-encoded genes are required for the complete assembly or function of fimbriae. The *csg* operon also has no fimbrial subunit domain, however in this case it is known to promote fimbriae assembly via a distinct nucleator-dependent pathway which occurs extracellularly through a self-assembly process (Hammar *et al.*, 1996).

The differences that occur within the flanking genes of the fimbrial operons as well as in the intergenic regions of these operons may play a crucial role in the expression of the fimbriae. These areas may assist with phase variation or efficient transcription and/or translation of the gene. Promoter sequences are required to enable a gene to be transcribed and the ribosomal binding sites promote efficient and accurate translation of mRNA. The variation that occurs in these sites in the *saf, stc* and *fim* operons could

have huge consequences on the downstream regions. Further examination of the expression profiles would be needed to determine the effects of these polymorphisms e.g. detection of proteins by antibody mediated methods and transcripts by hybridisation or RT-PCR.

Polymorphisms were identified that have the potential to affect the regulation of fimbrial genes and transcriptomic and proteomic analysis of different *S. enterica* serovars is needed to determine the impact of these. Further research is also required to dissect the role in virulence of the fimbriae loci identified herein and to understand the regulatory cross-talk that may co-ordinate their expression (Encheva et al., 2007).

Phase variation of fimbrial genes is common and has been described in several bacterial species including *E. coli* (Abraham et al., 1985, Klemm, 1986, Blyn et al., 1990) and in Neisseria (Meyer and van Putten, 1989, van Belkum et al., 1998)and some of the *Salmonella* fimbrial loci including *lpf* (Norris and Baumler, 1999). Whether this phase variation occurs across all fimbrial operons is currently unknown and if it does occur in all fimbriae, the mechanisms behind this may vary. The mechanisms behind phase variation are not fully understood in *Salmonella.*

The strains examined have evolved at different times and cause disease in different hosts, however, given the limited number of each of the serovars examined only limited conclusions regarding the conservation of these operons can be made as differences have been identified amongst different strains of the same serovar (Boyd et al., 2003, Deng et al., 2003). The remaining studies were focused on defining a role for fimbriae through targeted mutagenesis of the predicted major fimbrial subunits and analysis of these mutants *in vitro* and *in vivo.*

Chapter 4

Construction and characterisation of fimbrial mutants

4.1. Introduction

To understand the function of a gene it is a standard method to inactivate the gene and identify any resulting phenotype. This may involve random mutagenesis of bacterial genomes using transposons, followed by screening of individual mutants or signature-tagged mutagenesis (STM) to produce a library of tagged mutants that can be screened simultaneously (Hensel *et al.*, 1995). Screening of random transposon mutants of *S.* Typhimurium F98 in chickens revealed a role for 18 genes in the colonisation of 3-day-old chicks, of these 9 were rough (lacked LPS) and 9 contained mutations in other genes including *rfaY, dksA, clpB, hupA,* and *sipC* (Turner *et al.*, 1998). Screening of a library of signature-tagged transposon mutants has shown a role for *S.* Typhimurium 4/74 *stbC, fimZ, csgD* and *sthB* in chickens, but not in calves (Morgan *et al.*, 2004). A role for *saf* fimbriae in *S.* Typhimurium 4/74 has been identified in pigs but not in other animal models (Carnell et al., 2007). Transposon insertions may have polar effects on the expression of proximal genes and multiple insertions may occur. Therefore, a need exists to confirm the phenotype of such mutants by construction of defined non-polar mutants. This can be accomplished by using targeted mutagenesis techniques such as lambda Red recombinase-mediated homologous recombination. This permits the replacement of the target gene with an antibiotic resistance gene flanked by a target site for flippase recombinase (FRT) (Datsenko and Wanner, 2000). Alternatively, mutants may be constructed by introducing a defective gene on a suicide plasmid into a wild-type strain. Some suicide replicons permit a second round of homologous recombination by positive-selection to excise the vector. This method is commonly used but has been reported to result in a high frequency of second-site defects (Johnson *et al.*, 2003).

The λ red mutagenesis method was used as described in Section 2.4 to determine a phenotype of individual mutants each lacking a single major fimbrial subunit both *in vitro* and *in vivo*. Lambda Red mutagenesis has the advantage over other targeted mutagenesis methods in that chromosomal genes can be mutated in a single step using linear PCR products rather than by cloning, inactivation and re-introduction of the gene on a suicide plasmid. The chances of second site defects was reduced using bacteroiphage P22/int mediated transduction into an archived strain. The unique putative major fimbrial subunit of each fimbrial operon was chosen for mutation as identified by Pfam and BLAST searches, described in Chapter 3.

4.2. Aims

- To mutate the putative major fimbrial subunit of all chromosomally located fimbrial operons in *S*. Enteritidis P125109 and *S*. Gallinarum 287/91.
- To confirm the positions of all fimbrial mutations following transduction where possible.
- To confirm the mutations had no affect on growth.
- To reduce the chances of second-site defects contributing a phenotype.

4.3. Lambda Red mutagenesis

The lambda Red method of mutagenesis promotes homologous recombination between a linear PCR fragment and a targeted gene of interest and was used here to separately introduce an antibiotic resistance cassette into each major fimbrial subunit gene of two poultry-associated *S. enterica* strains. The helper plasmid, pKD46 contains 3 genes from bacteriophage lambda λ: *exo, bet* and *gam* which promote homologous recombination. The *gam* gene inhibits the bacterial exonuclease activity so that the proteins encoded by *bet* and *exo* can gain access to the DNA and promote recombination. In pilot experiments, pKD46 failed to promote integration of a range of linear amplicons including *steA*::chl^R in *S*. Enteritidis P125109 despite the presence of pKD46 being confirmed both by PCR amplification of regions of the plasmid and by re-extracting the plasmid (data not shown). The reasons behind this are unknown and it could be speculated that the presence of the plasmid has a lethal or inhibitory effect on *S*. Enteritidis P125109 or *S*. Enteritidis P125109 contains an element in its genome that prevents the lambda genes of pKD46 from functioning. The same stock of pKD46 has successfully been used in *S.* Gallinarum 287/91 and *S*. Typhimurium F98 to integrate the same linear amplicons. Therefore, an alternative strain, *S*. Enteritidis S1400 was used to construct the mutants in the first instance. Other laboratories have had similar strain specific problems but the reasons behind this are currently unknown. Transformants containing pKD46 were selected at 30 ºC on LB agar plates supplemented with ampicillin (100 µg/ml).

A PCR reaction was carried out using pKD3 plasmid DNA (GenBank, AY048742) as a template to produce linear DNA for recombination using the primers listed in Table 2.5. Each PCR reaction was carried out 12 times to provide 600 µl of each PCR product. The PCR products were concentrated using a PCR clean up kit from QIAGEN

and were cleaved by *Dpn*I (2 µl) in 1 x buffer in a total 100 µl reaction with water to remove unmethylated template DNA. The reaction was incubated statically for 3 h at 37 ºC. The digests were separated on a 1 % agarose gel as described in Section 2.3.4 and the DNA fragments excised from the gel with a clean sharp scalpel. The DNA was purified from the agarose using a gel extraction kit from QIAGEN. An example of the location of the primers for the *stcA* major fimbrial subunit is shown in Figure 4.1.

Figure 4.1. The sequence and location of primers targeting the major fimbrial subunit of *stcA*

5′**ATG**AAACGTTCACTTATTGCTGCTTCTGTATTGTCTGCTGTATTTATGAGCGCTGGG
GTTTTTGCTGCTGATGAAGATATGGGGGAATTAAAAATAAACGGAGAAGTGGTGGGAAC
ATCCTGTACTTTCGAAGGTGCAAATAGCGCGACTATTGAATTATCCCAGGTAGGTGTTG
ATAGATTGACTGACTTAAACCCTGGCGATATATATACAGGATACACTAGCCCAGAAGCG
ATTTTAAAAGTAAGATGTACGAATACAGCTAATCCACGAATTAGTTTTAACCGTTCTCA
ATTTGTGGATAACATGCAAATCACCAAAAATAATGCTACTAATAATGGTGCGGGCTTCG
CTGTTTATCTTGATGGTATTCAGGTGAAACCGGATGAGGCGGGGAATTACACTCTGAAT
TCAAGTAAGTTTGAAAATGGTGTATATACCCTGAACTTTTCTGCCCGCTATGCCGCCGT
TGAAAATACTGTAACACCAGGTTCTG**TTGAATCTGTACTGACGATGACGGTATTAACTG**
ATTAA -3′

NB The entire gene is shown and the primers are highlighted in blue. The start and stop codons are underlined

The 3′ end of each primer contains a 20 nucleotide sequence to permit annealing to the pKD3 antibiotic resistance cassette 5′ TGTGTAGGCTGGAGCTGCTTCG and 3′ ATTCCTCCTATAAGTATAC, to replace the internal portion of the gene with a chloramphenicol resistance cassette and leave a 40 bp region either side of the insertion behind. The cleaned and digested PCR products were electroporated into electro-competent *S.* Enteritidis S1400 or *S.* Gallinarum 287/91 carrying the pKD46 plasmid.

The strains were grown at 30 ºC and supplemented with 10 mM L-arabinose to promote recombinase expression as described in Sections 2.4.2-2.4.4.

4.4. Selection and confirmation of fimbrial mutants

Mutants were selected on LB agar plates supplemented with chloramphenicol (25 µg/ml) and cured of pKD46 by growth at 37 °C in the absence of ampicillin. Integration of the antibiotic resistance cassette at the expected chromosomal location was confirmed by two colony PCR reactions, using primers annealing to the resistance cassette in combination with primers designed adjacent to the fimbrial gene as shown in Figures 4.2 and the sequence is shown in Figure 4.3. The primer sequences for each fimbrial mutant are listed in Table 2.6.

Figure 4.2. Location of primers for PCR validation of mutant strains

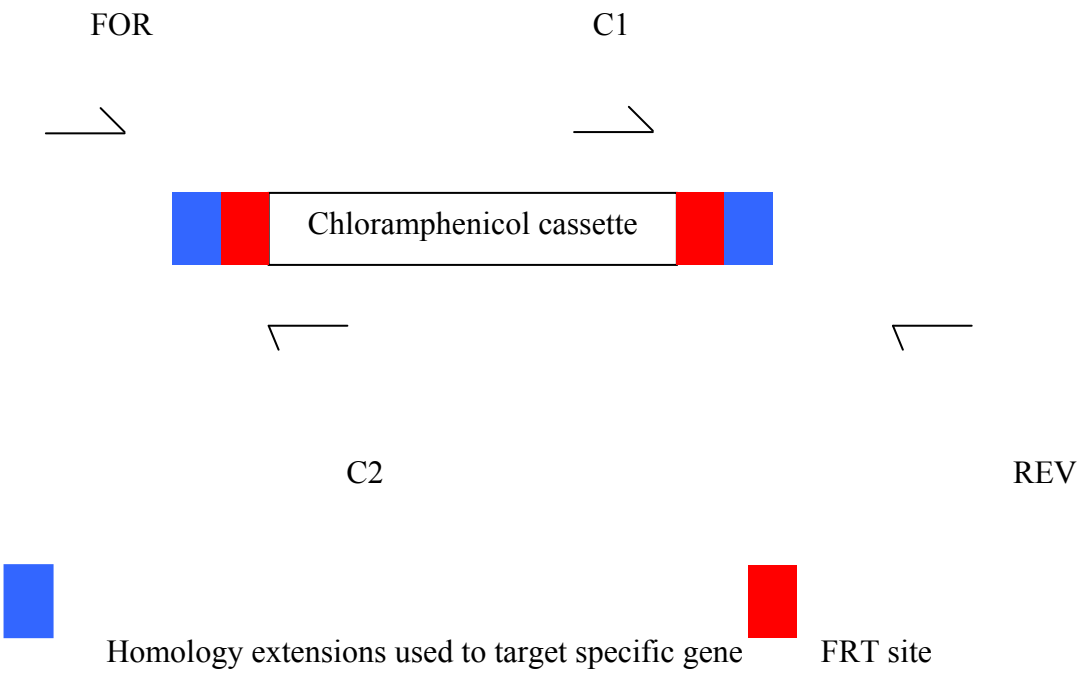


Table 4.1. Predicted sizes of PCR products for *S.* Enteritidis S1400 and *S.* Gallinarum 287/91 fimbrial mutants

Primer	PCR product size (base	PCR product size (base
bcfAFOR + C1	633	633
bcfAREV + C2	507	507
csgAFOR + C2	173	173
csgAREV + C1	302	302
lpfAFOR + C2	867	867
lpfAREV + C1	288	288
fimAFOR +C1	807	807
fimAREV + C2	429	429
sefAFOR + C1	373	373
sefAREV + C2	946	946
safAFOR + C1	701	701
safAREV + C2	597	597
stbAFOR + C2	887	887
stbAREV + C1	295	295
stcAFOR + C2	179	179
stcAREV + C1	377	377
stdAFOR + C2	587	-
stdAREV + C1	714	-
steAFOR + C1	739	739
steAREV + C2	600	600
stfAFOR + C1	283	283
stfAREV + C2	155	155
sthAFOR + C1	584	584
sthAREV + C2	704	704
stiAFOR + C2	385	385
stiAREV + C1	661	661

All amplicons from respective mutant strains were approximately of the sizes predicted. The stdA gene was not examined as it is not present in the genome sequence of *S.* Gallinarum 287/91. PCR reactions were performed using the following primer pairs: FOR and C2, and REV and C1. Some fimbrial mutants have the chloramphenicol resistant cassette in the opposite orientation (Table 4.1).

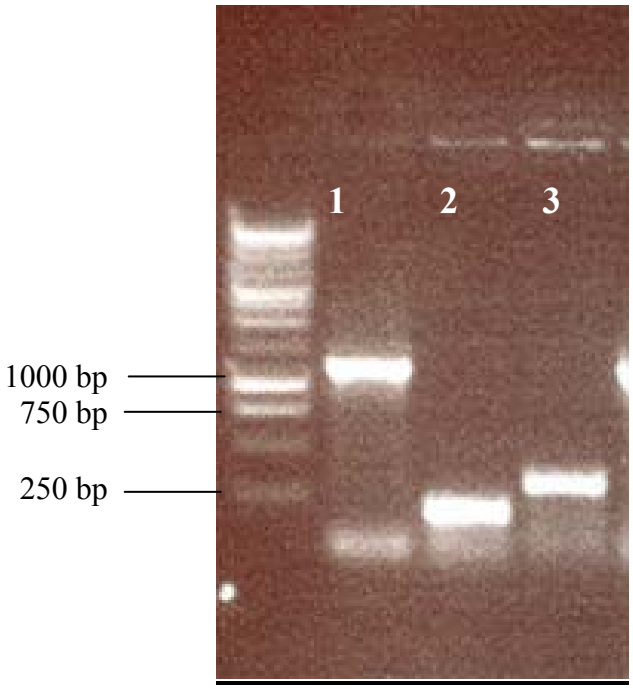
Figure 4.3. Predicted sequence of the insertion of the pKD3-derived chloramphenicol resistance cassette in *stcA*

5′-CAACAAAACATCGTGTTTACATTGCGATAACTTCCTGTCTATGAGAATTTTCGTTG
CAAGGGTTAATAACTCTTAACAAATAGAAATTACTTCATTAAGGAAGAGATTATGAAAC
GTTCACTTATTGCTGCTTCTGTATTGTCTGCTGTGTGTAGGCTGGAGCTGCTTCGAAGT
TCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCATTTAAATGGCGCGCC
TTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTATTCATTAAGCATCTGCCGACA
TGGAAGCCATCACAAACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTC
GCCTTGCGTATAATATTTGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGG
CCACGTTTAAATCAAAACTGGTGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATA
TTCTCAATAAACCCTTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTG
CGAATATATGTGTAGAAACTGCCGGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAA
ACGTTTCAGTTTGCTCATGGAAAACGGTGTAACAAGGGTGAACACTATCCCATATCACC
AGCTCACCGTCTTTCATTGCCATACGTAATTCCGGATGAGCATTCATCAGGCGGGCAAG
AATGTGAATAAAGGCCGGATAAAACTTGTGCTTATTTTTCTTTACGGTCTTTAAAAAGG
CCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGCAACTGACTGAAATGCC
TCAAAATGTTCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTT
TTTCTCCATTTTAGCTTCCTTAGCTCCTGAAAATCTCGACAACTCAAAAAATACGCCCG
GTAGTGATCTTATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCT
CATTTTCGCCAAAAGTTGGCCCAGGGCTTCCCGGTATCAACAGGGACACCAGGATTTAT
TTATTCTGCGAAGTGATCTTCCGTCACAGGTAGGCGCGCCGAAGTTCCTATACTTTCTA
GAGAATAGGAACTTCGGAATAGGAACATTCCTCCTATAAGTATACTGTTGAATCTGTAC
TGACGATGACGGTATTAACTGATTAACAATCGTTTTTTGGCGGCTAAGCTTTTAGCCGC
TTGTATCTCTTTTATAAGGTTATCGGCCATGAAATGGATGATTGCATTATGCCTGGCTT
GTGCCGCTATGCCTGCGTGGAGCGGCATTTATATATAT-3′

The primers for constructing the mutant are shown in blue and are positioned at the 5′ and 3′ ends of the *stcA* gene, the start and stop codons of the *stcA* gene are underlined. The pink font is the chloramphenicol resistant cassette. The red text denotes the primers within the chloramphenicol resistance cassette used to confirm the location of the mutation in combination with primers flanking the gene shown in green. The black bold text denotes the flippase recombinase target (FRT) sites and the yellow sequences are the primers for amplification of pKD3 chloramphenicol resistant cassette.

The location of all fimbrial mutants constructed in both *S.* Enteritidis S1400 and *S.* Gallinarum 287/91 and the transduced fimbrial mutants were confirmed by the use of PCR using primers in the combinations listed in Table 4.1 and Figure 4.2 and the forward and reverse primers together as indicated in Figure 4.2. The PCR product sizes were predicted from the genome sequences of *S.* Enteritidis P125109 and for all mutants were approximately as predicted, Figure 4.4.

Figure 4.4. Construction and validation of a Δ*stcA*::cat fimbrial mutant of *S.* Enteritidis P125109



Lane 1 is stcFOR and stcREV, lane 2 is stcFOR and C2 and lane 3 is stcREV and C1. Product sizes are predicted from Figure 4.3 to be 1280, 179 and 377 bp respectively.

4.5. Transduction of mutations using bacteriophage P22HT/int

It is possible during homologous recombination that secondary unwanted recombination events may have occurred (Datsenko and Wanner, 2000). To avoid such second-site defects major fimbrial subunit mutations were transduced into the archived strain or an alternative strain by bacteriophage P22 HT/int transduction as described in Section 2.4.7. General transduction was first discovered in *S.* Typhimurium, genetic material from pLT-22 could be transferred to *S.* Typhimurium LT2, (pLT-22 is now known as P22) (Zinder and Lederberg, 1952). Generalised transduction relies on the endonuclease activity of bacteriophage P22 and the accidental packaging of linear DNA

fragments into phage capsids instead of approximately 40 kb of phage genome. Infection of a susceptible *Salmonella* strain by such particles leads to insertion of linear fragments into the bacterial cell, which may then replace endogenous sequences by homologous recombination.

Within the two serovars examined, *S.* Gallinarum and *S.* Enteritidis, 25 fimbrial mutations were constructed and confirmed and 20 of the mutations were transduced into clean or archived strains. The fimbrial mutants that were made in *S.* Enteritidis S1400 were transduced into *S.* Enteritidis P125109 and those made in *S.* Gallinarum 287/91 were transduced into the archived stock of this strain. Five fimbrial mutants could not be transduced despite multiple attempts. Interestingly, of these 5 fimbrial mutations there are only 3 genes involved *fimA, steA* and *safA*. The *fimA, steA* and *safA* could not be transduced between the two strains of *S.* Enteritidis, and the *fimA* and *safA* mutants of *S.* Gallinarum 287/91 could not be transduced into the archived strain. The reasons for this are unclear. All transduced mutants were confirmed by PCR as described in Section 4.4 and were shown to possess intact LPS as described in Section 2.4.4.

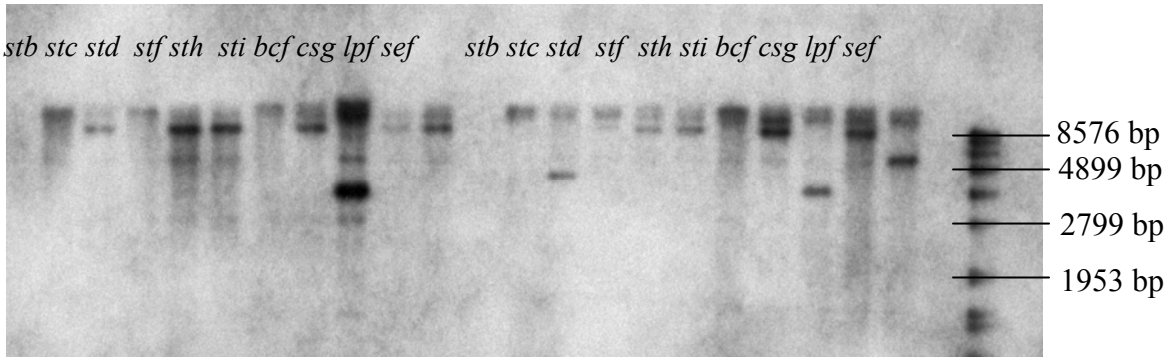
4.6. Verification of insertion mutants by Southern blotting

Bacteriophage P22 transduction is capable of transducing approximately 40 kb of chromosomal DNA (Vander Byl and Kropinski, 2000, Casjens and Hayden, 1988) which may potentially include the fimbrial mutation and approximately 40 kb 5′ or 3′. The transferred region may contain sequences that are only present in one strain of *S.* Enteritidis and not in another since the sequence flanking the *S.* Enteritidis S1400 fimbrial loci is unknown. The introduction of these unique regions may alter the phenotype of the recipient and therefore DNA was extracted from the fimbrial mutants

of both strains of *S.* Enteritidis used (the three that would not transduce were omitted) as described in Section 2.3.1. Genomic DNA digests were carried with *Hind*III and the digests were resolved on a 1 % agarose gel. The DNA was denatured before being transferred to a membrane by vacuum transfer for 1 h as described in Section 2.3.5.

A DIG-labelled probe targeted to the chloramphenicol resistance cassette was amplified by PCR using pKD3 as the template DNA and incorporating DIG-labelled dNTPs. The primer sequences are listed in Table 2.3 and PCR conditions are as described in Section 2.3.8. The membrane was hybridised with the DIG-labelled probe and detected as described in Sections 2.3.9 and 2.3.10 to determine if the chloramphenicol cassette localised to *Hind*III fragments of the expected size in the *S.* Enteritidis S1400 λ Red mutants and the *S.* Enteritidis P125109 transductants.

Figure 4.5. Southern blot analysis of *Hind*III fragments from fimbrial mutants of *S.* Enteritidis using the chloramphenicol cassette as a probe



The DIG-labelled probe was observed to hybridise in part to a high molecular weight product migrating at a similar position to intact genomic DNA despite having being digested for 16-18 h. In the case of *stbA, bcfA* and *stdA* the expected fragment size is > 16 kb and it is not possible this as it can not be separated from intact DNA. Despite the large size of the *bcf* fragment it appears to have separated from the intact DNA as a second band is visible but it is too large to accurately size. The *stcA, stfA, sthA, stiA, csgA, lpfA* and *sefA* fragment sizes were predicted by NEB cutter to be in a sizeable range (Table 4.2). The fragment sizes for *S.* Enteritidis P125109 *stcA, stfA, sthA, csgA, lpfA* and *sefA* are approximately as expected Table 4.2. The size of the hybridising species for *stiA* was much too large and this may be due to incomplete digestion. In several cases, the prominent hybridising fragment in the two strains examined was approximately the same size for each fimbrial mutant. The exceptions are *stc* and *sef* that hybridise to different size fragments in the two strains, suggesting strain-strain genetic variation in or adjacent to the fimbrial genes resulting in different fragment sizes. However, the predicted fragment sizes for *stc* and *sef* are as predicted in *S.* Enteritidis P125109.

Table 4.2. Predicted sizes of the *Hind*III restriction fragments of *S.* Enteritidis P125109 fimbrial mutants containing the chloramphenicol resistance cassette

Mutant	*Hind*III size of fragment (bp)	
	Predicted size	Actual size
stbA	19495	>8576
stcA	4533	3639-4899
stdA	16236	>8576
stfA	8572	~8576
sthA	8509	~8576
stiA	3227	>8576
bcfA	41270	>8576
csgA	3045	2799-3639
lpfA	5858	4899-6106
sefA	5012	4899-6106

Fragment size predictions were made using NEB cutter (Vincze *et al.*, 2003) and the genome sequence of *S.* Enteritidis P125109. Actual sizes are from the known size of markers that migrate to a similar position.

4.7. Antibody mediated detection of fimbrial proteins

To confirm that the mutation of the fimbrial genes abolished expression of the major fimbrial subunit as expected, Western blots were attempted on three of the fimbrial mutants SefA, CsgA and FimA owing to the availability of subunit-specific antibodies. Due to the auto-aggregative nature of some fimbrial subunits (Eisenstein et al., 1983, Collinson et al., 1991, Humphries et al., 2005) conventional Western blot analysis proved extremely difficult as the Csg fimbrial subunits would not enter the SDS-PAGE gel as previously reported (Collinson et al., 1991, Humphries et al., 2005). Therefore, a dot blot approach was undertaken. The protein was prepared from LB-grown cultures amplified at two different temperatures as detailed in Section 2.5.4, and 15 µl was

applied directly onto Hybond-ECL membrane (Amersham) and dried for 1 h at room temperature. The membrane was blocked (1 % (w/v) skimmed milk and 0.1 % (v/v) Tween 20 in PBS) and detected as for a conventional Western blot as described in Section 2.5.4. The results are shown in Figure 4.6. Despite repeated attempts, the SefA antibody did not detect any protein in *S*. Enteritidis P125109, *S*. Enteritidis S1400 or *S.* Gallinarum 287/91 wild-type strains despite having been reported to be capable of doing so (Woodward et al., 2000, Walker et al., 1999, Collinson et al., 1993). As the Δ*fimA::cat* mutant could not be transduced into *S*. Enteritidis P125109, the phenotype of this mutant was examined in the validated S1400 strain. The FimA antibody detected protein in wild-type *S.* Enteritidis S1400 grown at 25 ºC and at 37 ºC here and in other strains of *Salmonella* (Humphries et al., 2003). No FimA protein was detected in the S1400 Δ*fimA::cat* strain as expected or in *S*. Gallinarum 287/91 wild-type. The CsgA antibody detected protein only at 37 ºC in both *S*. Gallinarum 287/91 wild-type and *S*. Enteritidis P125109 wild-type but no protein was detected at 25 ºC. This may be due to temperature-sensitive regulation of expression of the CsgA protein as indicated in other studies (Dibb-Fuller et al., 1997, Walker et al., 1999, Woodward et al., 2000). The Δ*csgA::cat* mutant did not express the CsgA protein as expected under the two conditions studied. Expression of many fimbriae has not been detected *in vitro* but expression is induced *in vivo* which may depend upon host factors (Humphries et al., 2003).

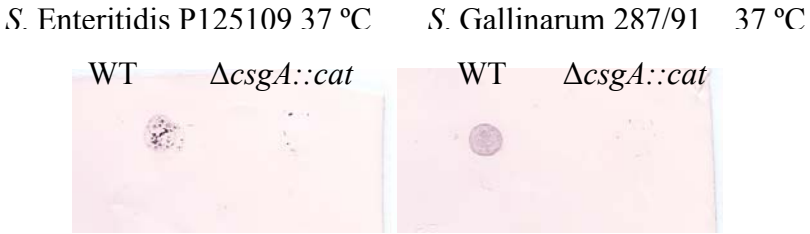
The expression of fimbriae in the wild-type strains was not examined either by electron microscopy due to the lack of specific antibodies for specific fimbriae (which would have made it extremely difficult to identify individual fimbriae) nor by haemagglutination which was have tols us that type 1 fimbriae were being expressed, a phenomenon that does not occur in *S*. Gallinaurum (Kisiela et al., 2006). Only 2 strains

of *S.* Enteritidis were examined and 1 strain of *S.* Gallinarum and their fimbrial expression may not be typical of all strains of that serovar.

Figure 4.6. Antibody-mediated detection of FimA in *S.* Enteritidis S1400 wild-type and Δ*fimA::cat* mutant strains

In *S.* Gallinarum 287/91 no protein was detected (not shown).

Figure 4.7. Antibody-mediated detection of CsgA in *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 wild-type and Δ*csgA::cat* mutant strains



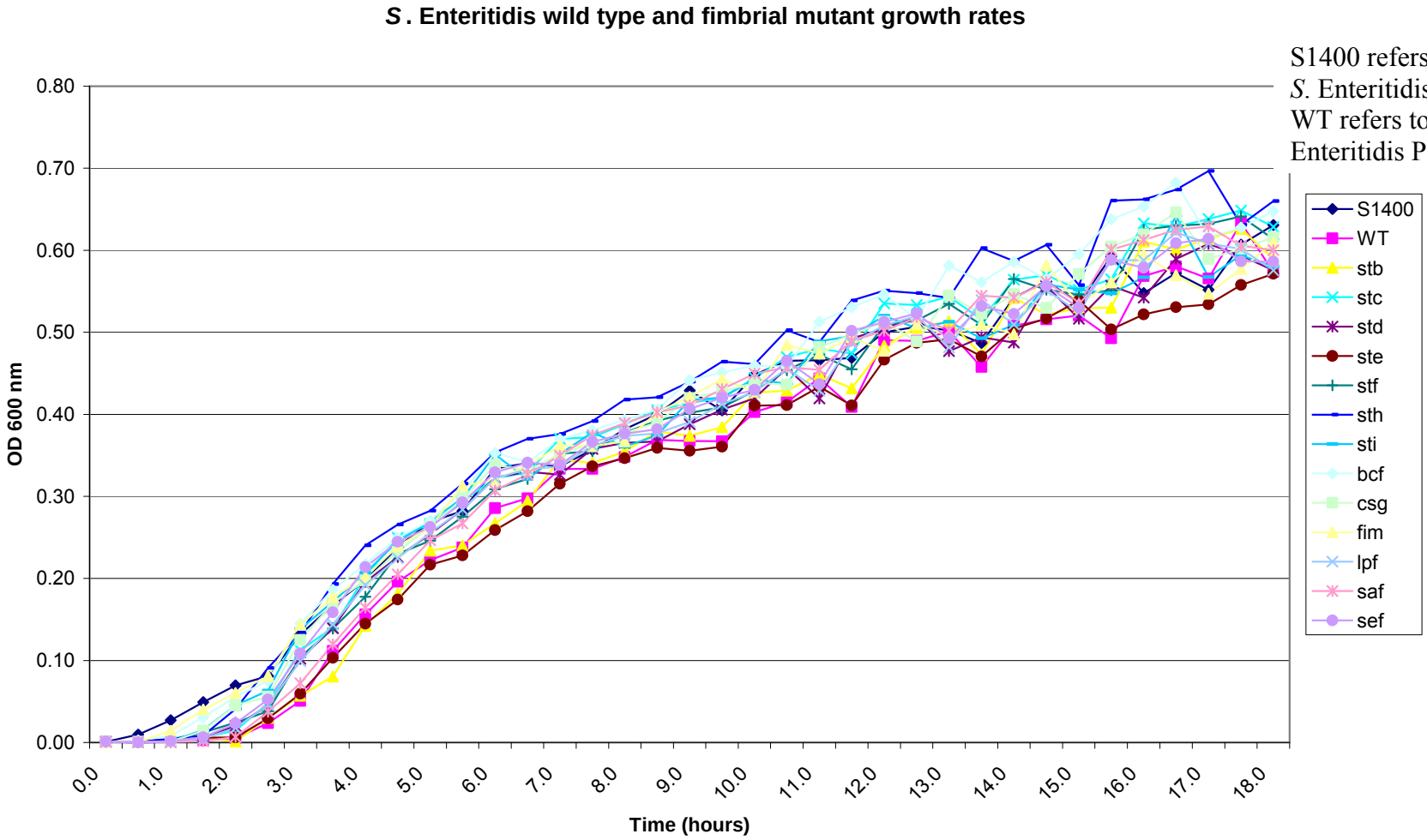
At 25 ºC, CsgA was not detected in either *S.* Enteritidis P125109 or *S.* Gallinarum 287/91 wild-type strains (not shown).

4.8. Growth kinetics

To ensure that the introduction of a chloramphenicol resistance cassette into the major fimbrial subunit genes did not have an adverse effect on bacterial growth, replication kinetics of all fimbrial mutants were compared with the parent strain.

The growth curves of the fimbrial mutants in *S.* Enteritidis P125109 and S1400 are comparable to the respective wild-type strain in LB at 37 ºC. The *steA, safA and fimA* mutants are compared to *S.* Enteritidis S1400 as they could not be transduced into *S.* Enteritidis P125109, all other fimbrial mutants are compared to *S.* Enteritidis P125109 wild-type strain. Although minor variation occurs the same general kinetics of replication are seen across the mutants, which all reach approximately the same density after 18 hours.

Figure 4.8. Growth curves of *S.* Enteritidis P125109 and S1400 wild-type and fimbrial mutant strains in LB at 37 ºC



S1400 refers to wild-type *S.* Enteritidis S1400 and WT refers to wild-type *S.* Enteritidis P125109

Using a BioscreenC real-time spectrophotometer to record the A_{600} at 30 min intervals during growth in LB at 37 ºC as described in Section 2.5.1. The results are for the average of three wells on three independent days, only minor variation occurred. The error bars are not shown for ease of read of the graph. The cultures were

4.9. Removal of chloramphenicol resistance cassette

The chloramphenicol resistance cassette was removed from the fimbrial mutation of Δ*steA:cat* and Δ*stcA:cat*, to confirm the phenotype was due to the loss of the major fimbrial subunit and not due to polar effects on the expression of downstream genes caused by the presence of the chloramphenicol resistance cassette. This removal was carried out as described in Section 2.5.5, by transient expression of flippase recombinase from a temperature-sensitive plasmid. The predicted sequences of the Δ*steA:cat* region with and without the chloramphenicol resistance cassette are shown in Figures 4.9 and 4.10 (Datsenko and Wanner, 2000).

Figure 4.9. The predicted sequence of the Δ*steA::cat* fimbrial mutant

5′-CGTATTATTCTTAACCATTCACGCACAGAGATACTACGACAACGCCTATATAATAA
AATATATTGTTAACAGGCGTTGAATGCTACCTTTCCCGTATAACTTTAAAATTATTAAT
CGATACACAACAATATAATATATCATATAACTAATTTATTAAAAAACAGATATTTGCAC
CAGGCATTATAAATAACATCAATTAAGTAAAAAAATTATGCAAACACTATAAGCCTCCC
CCCCCCAAAGAGCCTTCCCTTTCAAAAAAAATAAATTATTTCACTTTTACGGAAAAACG
AGTAGCATGCACGCCAGTTTAATATTCAAAAAAGACCGCTTTTTTGTTTTTTCTGGAAC
ATACATGAAATAAATATATTGAAAGTATTACATATAATATTCTATGAGCAGGTACGATC
ATTCAGCTCATAAGAATATACTCATAAAAATGTAGAAATATAATATTTTTATTATGACC
TATTTTTTACCCAAAGCCCGCAGCATGGCTCTATGCCGCATATCCCTTGGTATACGCGT
ACAAAATGTTGCCATAAAACACGTTAATTAAGGATAACACGATGAAGTCATCTCATTTT
TGTAAACTGGCAGTAACTGCATGTGTAGGCTGGAGCTGCTTCAAGTTCCTATACTTTCT
AGAGAATAGGAACTTCGGAATAGGAACTTCATTTAAATGGCGCGCCTTACGCCCCGCCC
TGCCACTCATCGCAGTACTGTTGTATTCATTAAGCATCTGCCGACATGGAAGCCATCAC
AAACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAA
TATTTGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGGCCACGTTTAAATC
AAAACTGGTGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAACC
CTTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGT
AGAAACTGCCGGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTG
CTCATGGAAAACGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTT
TCATTGCCATACGTAATTCCGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAG
GCCGGATAAAACTTGTGCTTATTTTTCTTTACGGTCTTTAAAAAGGCCGTAATATCCAG
CTGAACGGTCTGGTTATAGGTACATTGAGCAACTGACTGAAATGCCTCAAAATGTTCTT
TACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTTTA
GCTTCCTTAGCTCCTGAAAATCTCGACAACTCAAAAAATACGCCCGGTAGTGATCTTAT
TTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAACGTCTCATTTTCGCCAAA
AGTTGGCCCAGGGCTTCCCGGTATCAACAGGGACACCAGGATTTATTTATTCTGCGAAG
TGATCTTCCGTCACAGGTAGGCGCGCCGAAGTTCCTATACTTTCTAGAGAATAGGAACT
TCGGAATAGGTAAGGAGGATATTCATATGTTCAGCAGCGCCGCCAACGTCACTATCTCT
TACCTGTAATTCAGTTAACAGAGTCACGACGATAATACCCTGCCGCTTTAGGTAGGGTA
TTCCTGTTGAGTTTTATTGCGGAATATATTTGATGAATAACACATGGAAAAGTGTTCTT
TGCCCAATAGCGTGCGGAGTGGGAATGCTTCTAAGCCTCTCCCCTTATAGCGCGTCAGG
CAAAGACATCGAATTTAATACCGATTTCCTCGATGTAAAAAATCGCGATAACGTTAACA

TTGCACAGTTTTCTCGTAAGGGTTTTATTCTGCCAGGCGTCTACCTTTTACAAATTAAA
ATTAACGGACAGACTCTGCCGCAGGAATTTCCTGTTAACTGGGTTATTCCAGAACATGA
TCCACAAGGAAGTGAGGTTTGCGCAGAACCAGAATTAGTTACGCAATTGGGTATAAAGC
CGGAACTCGCGGAAAAACTCGTCTGGATAACGCACGGTGAACGACAATGTCTGGCGCCA
GATTCACTGAAAGGCATGGATTTTCAG**GCTGACCTGGGACACTCCACGCTGCT**G- 3′

The green sequences indicate the primers flanking the gene of interest (Table 2.6), the blue and orange sequences were used to construct the original mutant at the 5′ and 3′ ends of the gene of interest, the orange sequence was used to amplify the pKD3-derived chloramphenicol cassette and the blue has homology to the *steA* gene for homologous recombination (Table 2.3). The start and stop codons of the gene are underlined. The bold font shows the flippase recombinase target (FRT) site and the pink sequence is the chloramphenicol resistance cassette.

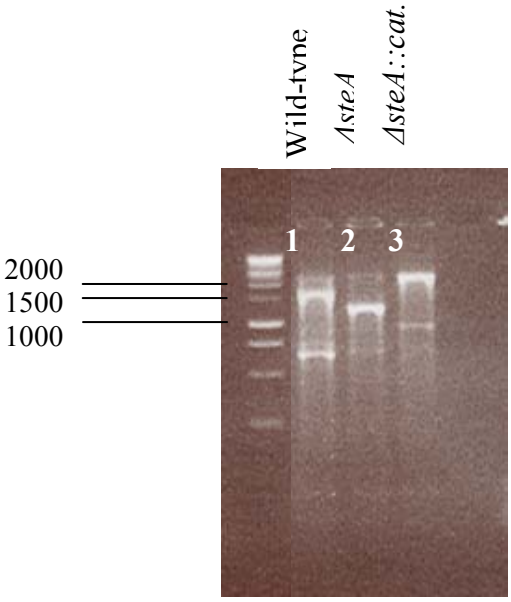
Figure 4.10. The predicted sequence of the Δ*steA* fimbrial locus after FLP-mediated excision of the chloramphenicol resistance cassette

5′ -CGTATTATTCTTAACCATTCACGCACAGAGATACTACGACAACGCCTATATAATA
AAATATATTGTTAACAGGCGTTGAATGCTACCTTTCCCGTATAACTTTAAAATTATTAA
TCGATACACAACAATATAATATATCATATAACTAATTTATTAAAAAACAGATATTTGCA
CCAGGCATTATAAATAACATCAATTAAGTAAAAAAATTATGCAAACACTATAAGCCTCC
CCCCCCCAAAGAGCCTTCCCTTTCAAAAAAAATAAATTATTTCACTTTTACGGAAAAAC
GAGTAGCATGCACGCCAGTTTAATATTCAAAAAAGACCGCTTTTTTGTTTTTTCTGGAA
CATACATGAAATAAATATATTGAAAGTATTACATATAATATTCTATGAGCAGGTACGAT
CATTCAGCTCATAAGAATATACTCATAAAAATGTAGAAATATAATATTTTTATTATGAC
CTATTTTTTACCCAAAGCCCGCAGCATGGCTCTATGCCGCATATCCCTTGGTATACGCG
TACAAAATGTTGCCATAAAACACGTTAATTAAGGATAACACGATGAAGTCATCTCATTT
TTGTAAACTGGCAGTAACTGCATGTGTAGGCTGGAGCTGCTTCAAGTTCCTATACTTTC
TAGAGAATAGGAACTTCGGAATAGGTAAGGAGGATATTCATATGTTCAGCAGCGCCGCC
AACGTCACTATCTCTTACCTGTAATTCAGTTAACAGAGTCACGACGATAATACCCTGCC
GCTTTAGGTAGGGTATTCCTGTTGAGTTTTATTGCGGAATATATTTGATGAATAACACA
TGGAAAAGTGTTCTTTGCCCAATAGCGTGCGGAGTGGGAATGCTTCTAAGCCTCTCCCC
TTATAGCGCGTCAGGCAAAGACATCGAATTTAATACCGATTTCCTCGATGTAAAAAATC
GCGATAACGTTAACATTGCACAGTTTTCTCGTAAGGGTTTTATTCTGCCAGGCGTCTAC
CTTTTACAAATTAAAATTAACGGACAGACTCTGCCGCAGGAATTTCCTGTTAACTGGGT
TATTCCAGAACATGATCCACAAGGAAGTGAGGTTTGCGCAGAACCAGAATTAGTTACGC
AATTGGGTATAAAGCCGGAACTCGCGGAAAAACTCGTCTGGATAACGCACGGTGAACGA
CAATGTCTGGCGCCAGATTCACTGAAAGGCATGGATTTTCAGGCTGACCTGGGACACTC
CACGCTGCTG-3′

The green sequences indicate the primers flanking the gene of interest (Table 2.6), the blue and orange sequences were used to construct the original mutant at the 5′ and 3′ ends of the gene of interest, the orange sequence was used to amplify the pKD3 derived chloramphenicol cassette and the blue has homology to the *steA* gene for homologous recombination (Table 2.3). The start and stop codons of the gene are underlined. The bold font shows the flippase recombinase target (FRT) site.

The removal of the chloramphenicol resistance cassette was confirmed by negative selection on LB agar plates supplemented with chloramphenicol and by PCR using the primers in Table 2.6 flanking the gene of interest (Figure 4.10). The removal of the chloramphenicol resistance cassette leaves an 81 nucleotide scar.

Figure 4.11. PCR confirmation of FLP-mediated excision of the chloramphenicol resistance cassette



Lane 1 is wild-type *S.* Enteritidis S1400, Lane 2 is the *steA* mutant with the chloramphenicol resistance cassette removed (Δ*steA)* and Lane 3 is the *steA* mutant with chloramphenicol resistance cassette present (Δ*steA::cat)*. Although some non-specific amplification has occurred the most intense band is approximately the same size as predicted. The sizes of the products were predicted from Figures 4.9 and 4.10 and the genome sequence of *S.* Enteritidis P125109 wild-type, wild-type (1639 bp), Δ*steA* (1233 bp) and Δ*steA::cat* (2163 bp).

4.10. *Trans*-complementation of the fimbrial mutation of *S*. Enteritidis P125109

For *in vitro trans*-complementation the entire *steA-E* operon was cloned using the primers in Table 2.7 designed to the 5′ and 3′ ends of the *steA-E* operon and a Hercules kit was used to carry out a long-range PCR due to the large size of the operon (Stratagene). A PCR reaction was carried out as described in Section 2.5.6 to amplify the whole operon and the PCR product was ligated with the TOPO pCR4Blunt cloning vector and transformed into *E. coli* strain TOP10f by heat-shock as described in Sections 2.5.7 and 2.5.8. Plasmids were obtained and restriction digests confirmed the orientation of the inserts. Two plasmids were selected, one with the operon transcribed from the *lac* promoter and the other not expressed. These were both electroporated into the Δ*steA::cat* mutant and the *in vitro* assays for adhesion and invasion were repeated. All cultures were supplemented with 0.5 mM of IPTG to induce operon expression during growth and in the cell media and the strains carrying the plasmid were grown with ampicillin (100 μg/ml) to maintain the plasmid.

For *in vivo trans*-complementation studies the *stcA* gene was amplified by PCR from *S*. Enteritidis P125109 genomic DNA using *pfu,* proof-reading DNA polymerase and the stcAfor and stcArev primers listed in Table 2.7. Each of the primers has a *Cla*I restriction enzyme site (shown in bold) and as many restriction enzymes will not cut efficiently close to the end of DNA fragments, the site was preceded by a 5′ spacer region. The primers target the ORF of the entire gene and do not include any flanking regions. The amplified PCR product was cloned into pCR-4Blunt as described in Section 2.5.6 and transformed into chemically-competent *E. coli* TOP10f by heat shock at 42 ºC for 30 seconds. The presence of an insert of the expected size was confirmed by PCR using the primers stcAF and stcAR listed in Table 2.3. Recombinant plasmids were verified by digestion with *Cla*I. The *Cla*I insert was then separated on a 1 %

agarose gel and extracted from the gel using a QIAGEN Gel extraction kit for subcloning.

The pACYC177 plasmid was chosen for *in vivo trans*-complementation studies as it has been shown to have a negligible impact on invasion of cultured cells and the virulence of *S.* Typhimurium compared to other cloning vectors (Knodler et al., 2005). The pACYC177 plasmid was purified from *E. coli* K-12 ER2420 as described in Section 2.3.2 and digested with *Cla*I and 1.5 µl of Thermosensitive alkaline phosphatase (TSAP) to prevent self-ligation of the plasmid. All restriction enzymes and TSAP were heat-inactivated at 74 ºC for 15 min. The digested plasmid and insert were ligated using a 5:1 ratio of vector to insert and T4 DNase ligase and then transformed into TOP10 chemically-competent *E. coli* (Invitrogen). The orientation of the insert was confirmed by a restriction digest using the *Sph*I restriction enzyme. Plasmids with inserts in the sense orientations will be expressed from the promoter whereas plasmids with the insert in the antisense orientation are predicted not to express the *stcA* gene. Plasmids with the insert in both orientations were electroporated into *S.* Enteritidis P125109 Δ*stcA::cat*.

4.11. Discussion

Despite repeated attempts, the fimbrial genes within the sequenced strain of *S.* Enteritidis P125109 could not be directly mutated by λ Red mutagenesis. The helper plasmid, pKD46 failed to mediate integration of a range of linear amplicons despite its presence being confirmed both by PCR and plasmid extraction. The reasons for this are unclear though other laboratories have reported strain-, gene- and serovar-specific difficulties of this kind with the same system. *S.* Enteritidis S1400 was therefore chosen as a surrogate host strain as it has already been shown to be an efficient coloniser of the chicken alimentary tract (Robertson et al., 2003, Allen-Vercoe et al., 1999).

All of the chromosomally-located fimbrial operons of *S.* Enteritidis S1400 and *S.* Gallinarum 287/91 were mutated, and 20 out of 25 mutants were transduced into clean genetic backgrounds, summarised in Table 4.3.

Table 4.3 Fimbrial mutations constructed and transduced for use in this study.

S. Enteritidis S1400	*S.* Enteritidis P125109	*S.* Gallinarum 287/91
safA	*stbA*	*stbA*
fimA	*stcA*	*stcA*
steA	*stdA*	*stfA*
	stfA	*sthA*
	sthA	*stiA*
	bcfA	*bcfA*
	csgA	*csgA*
	lpfA	*lpfA*
	sefA	*sefA*
	stiA	
		safA
		fimA
		steA

The highlighted *safA, fimA,* and *steA* of *S.* Gallinarum 287/91 were not transduced into an archived strain of *S.* Gallinarum 287/91 but were still compared to that wild-type. The *safA, fimA* and *steA* of *S.* Enteritidis S1400 were not transduced into *S.* Enteritidis P125109 and throughout this study were compared to their respective wild-type strains.

It is unknown at this stage whether these strains are good expressors of fimbriae they wee chosen solely based on the availability of the complete genome sequences. The bacteriophage P22 HT/int targets the O antigen on the cell surface (Gemski and Stocker, 1967). Bacteria lacking an O antigen are described as rough and are resistant to P22 transduction, along with those bacteria described as semi-rough which contain most of the LPS but may be missing portions (Gemski and Stocker, 1967). However all fimbrial mutants and archived strains were checked for the presence of intact LPS and confirmed to be smooth. The effectiveness of P22 transduction has also been linked with motility and non-motile strains are much less effective at being transduced. However, some motile strains were also shown to be less effective and the reasons are unknown (Old and Duguid, 1971). In this study all fimbrial mutants were in one of two

strains and both strains were capable of being transduced. The reasons behind the lack of transduction for specific genes are unknown.

All 25 fimbrial mutations were confirmed by PCR and the fimbrial mutants of *S.* Enteritidis have no obvious effect on the growth rate during batch culture in rich medium. Analysis of the expression of the fimbrial proteins (where antibodies were available) could not detect the FimA protein from wild-type *S.* Gallinarum 287/91. This may be due to the growth conditions being unsuitable for expression in this strain or only small amounts of the protein may be produced which are below the limits of detection for this technique. Alternatively the antibody maybe specific for *S.* Enteritidis despite the *fimA* gene of *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 being highly conserved (98 %) as shown in Table 3.1 and FimA was detected in *S.* Enteritidis S1400 wild-type.

The results for the CsgA protein showed that both *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 expressed the protein at 37 ºC but not at 25 ºC. It has been shown that CsgA is expressed at 25 ºC in certain strains of *S.* Enteritidis but not others (Clouthier et al., 1998b) and it is likely that there may be inter-serovar variation in the expression of CsgA (Dibb-Fuller et al., 1997, Walker et al., 1999). At the time of writing, there is limited literature regarding the expression of fimbriae in *S.* Gallinarum and this is the first report of CsgA being expressed by *S.* Gallinarum 287/91 under these conditions. Antibodies were also obtained for SefA but this protein could not be detected in either wild-type strain under the two growth conditions, which is likely to be due to the sensitivity of the method or could be indicative of strain variation. SefA has been detected in a different strain of *S.* Enteritidis at 37 ºC but not at 25 ºC (Clouthier et al., 1998b). Expression of fimbriae has also been shown to be depedent upon pH (Walker et al., 1999) and on host-pathogen interactions (Humphries et al., 2003).

Southern blots were used to identify differences in the two *S*. Enteritidis strains used in this study and to determine if genomic rearrangements had occurred proximal to the insertions. Southern blots were not conclusive in this respect. A single point mutation anywhere in the genome sequence could cause different restriction digest patterns that would result in an increase or decrease in the size of the fragment containing the fimbrial genes. Alternatively, insertions in the genome may have occurred that do not alter the restriction digest patterns but do result in the transfer of unique sequences. Mutations in the *stc* and *sef* operons produced different fragment sizes patterns but the correct fragment size was obtained in 6 out of 10 fimbrial mutants in the sequenced strain of *S*. Enteritidis P125109 including *stc* and *sef*. The differences in the size of the fragment obtained may be due to differences in this region in *S*. Enteritidis S1400. In *S*. Enteritidis S1400, the P22 fragment may have included only one of the restriction enzyme sites and the other enzyme site is absent in *S*. Enteritidis S1400 resulting in different size fragments. The role of the major fimbrial subunits mutated herein in adherence to cultured cells and intestinal colonisation in chickens will be discussed in the following chapters.

Chapter 5

Characterisation of fimbrial mutants *in vitro*

5.1. Introduction

Salmonellae usually enter the avian host by the faecal-oral route and colonise the gastro-intestinal tract. Adherence to and colonisation of the gut epithelium is key to the survival and invasion of *Salmonella* if it is to avoid clearance by the intestinal flow. The *S.* Enteritidis FimA fimbrial subunit has been shown to adhere to specific glycosphingolipid (GSL) receptors on the epithelial surface of chicken oviductal mucosal cells and it is possible that other fimbriae may adhere to receptors at other sites (Li et al., 2003b, Li et al., 2003a). The role of fimbriae in adherence has been studied using several *in vitro* techniques such as immortalised cell lines, primary cell lines and explant models and have indicated a role for several fimbriae in adherence.

Mutations in the *lpfC* fimbrial gene of *S.* Typhimurium 4252 resulted in the reduction of adherence to and invasion of HEp-2 cells. A *fimA* fimbrial mutant of *S.* Typhimurium resulted in no difference in adherence to or invasion of HEp-2 cells but was deficient in the ability to attach to and invade HeLa cells (Baumler et al., 1996a). This study suggests that adherence is a pre-requisite for invasion and implies that different fimbriae target different cells. Mutations affecting the *csgA* and the *fimA* fimbrial genes of different strains of *S.* Enteritidis decreased the association with and invasion of cultured epithelial cells including Int-407 and Caco-2 cells and histological examination of the *csgA* mutants indicated a role in localised aggregated adhesion (Dibb-Fuller et al., 1999). Mutations of the *sefA* fimbrial gene did not result in any difference in adherence to or invasion of Int-407, Caco-2 cells, HEp-2 or HeLa cells (Ogunniyi et al., 1997, Thorns et al., 1996, Dibb-Fuller et al., 1999).

The adherence to certain cell lines such as HEp-2 cells and HeLa cells has been shown to vary not only between different fimbriae but also between different allelic variations of *fimH* in *S.* Typhimurium (Boddicker et al., 2002, Hancox et al., 1997). *S.*

Gallinarum and *S.* Pullorum expressing FimA from *S.* Typhimurium exhibited an increase in adherence to HEp-2 cells of 10-20 fold and an increase in invasion by 20-60 fold (Wilson et al., 2000). Invasion of HEp-2 cells by *S.* Typhimurium is also increased in anaerobic growth conditions (Ernst et al., 1990).

Mutations in *pefA, lpfA, fimA, csgA* and *sefA* genes of *S.* Enteritidis S1400 played no role in adherence to chick gut explants (Allen-Vercoe and Woodward, 1999b). In an organ culture model the *lpfC* fimbrial mutant of *S.* Typhimurium adhered in much lower numbers to murine Peyers patches than the wild-type but there was no difference in the association with the villous intestine epithelium in an organ culture model (Baumler et al., 1996b).

Salmonella have also been shown to adhere to abiotic surfaces and only virulent strains of *S.* Enteritidis adhered to glass tubes and formed visible filaments (Solano et al., 1998). *S.* Enteritidis can adhere to inanimate surfaces at both 37 ºC and 25 ºC. The expression of fimbriae is dependent on the growth phase, the growth media and the temperature of growth as detected by ELISA and flow cytometry, (Edwards et al., 2001, Dibb-Fuller et al., 1997, Woodward et al., 2000, Humphries et al., 2003).

The choice of *in vitro* model, the strain, serovar and growth conditions all appear to be important in characterising fimbriae and the adhesive properties of many of the fimbriae have not been studied *in vitro.*

5.2. Aims

- To investigate the role of *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 fimbriae in adherence to and invasion of cultured cell lines
- To microscopically confirm the adherence and invasion of cells by *Salmonella*
- To determine if fimbriae play conserved roles between serovar and cell types
- To confirm selected mutant phenotypes by *trans*-complementation

5.3.1. Adhesion to and invasion of chick kidney cells (CKC)

In chickens, *S*. Gallinarum 287/91 causes a systemic infection whereas *S*. Enteritidis P125109 is mostly restricted to the alimentary tract causing an enteric infection. It was of interest to determine if the difference in disease outcome of these strains depends upon their ability to adhere to and subsequently invade different cell lines. Chick kidney epithelial cells (CKC), a primary cell line that was used to provide a model for adherence and invasion and was always grown to a confluent monolayer. Transposon mutants of *S*. Typhimurium SR11 that were shown to exhibit a decrease in adherence and invasion in this model were later shown to be attenuated in a chick oral inoculation model (Lee et al., 1996). The CKCs were obtained from uninfected Rhose Island Red outbred chickens as a primary cell line so the entire kidney was removed from a 2-3 week old bird and the kidneys were trypsonised (Kaiser et al., 2000). The primary cell line will be a mixture of kidney cells and were not characterised.

During the construction and bacteriophage transduction of the fimbrial mutants (Chapter 4), the Δ*safA::cat,* Δ*fimA::cat* and Δ*steA::cat* fimbrial mutants could not be transduced between the two *S*. Enteritidis strains used, therefore throughout this study all fimbrial mutants were compared to their respective parent wild-type strain, either P125109 or S1400. All wild-type and fimbrial mutant strains were cultured under two growth conditions, 25 ºC static and 37 ºC with agitation to enable a comparison of different condtions across all of the cell lines examined and all assays were carried out at 37 ºC. It has been shown that in *S*. Enteritidis expression of CsgA, FimA and SefA fimbriae is dependent on temperature (Woodward et al., 2000, Walker et al., 1999, Humphries et al., 2003). Differences in the expression of *S*. Enteritidis CsgA fimbriae were also detected at 42 ºC (Dibb-Fuller et al., 1997). This was not examined herein

despite it being the clocal temperature for a chicken model as it would not have been possible to culture and maintain mammalian cell lines.

The adhesion and invasion assays were performed as described in Section 2.5.2, mannose was not used in any assay in this study. Briefly, the optical density of the culture of the wild-type or mutant strains was measured and bacterial numbers were adjusted accordingly to give an MOI of 5:1 and an MOE of 3:1. Each mutant and wild-type strain was separately added to three wells on 3 individual days and the assay was carried out in two replica plates. Bacterial counts were taken of the inocula to confirm the number of bacteria used. After 15 minutes, gentamicin was added to one plate to kill all non-internalised bacteria and the other plate was untreated to enable a count of the number of bacteria that were cell-associated. Triton 1 % (v/v) was added to both plates and ten-fold serial dilutions were carried out on LB agar plates to calculate the number of bacteria cell-associated. The data for all mutants, cell lines and growth conditions are shown separately in the following sections but will be summarised later in Table 5.7.

A non-invasive control strain, *E. coli* K-12 MG1655 was included and did not invade CKCs or did so below the limit of detection of 100 viable bacteria. Mutation of fimbrial genes in *S*. Enteritidis had only subtle effects on adherence and invasion of CKC and significant differences were only seen when the bacteria were grown at 25 ºC statically. The *S*. Enteritidis fimbrial mutants, Δ*steA::cat* (P=0.021), Δ*lpfA::cat* (P=0.037), Δ*safA::cat* (P=0.014) and Δ*sefA::cat* (P=0.002) invaded in significantly lower numbers than the wild-type and the Δ*stbA::cat* (P=0.004), Δ*steA::cat* (P=0.007), and Δ*stiA::cat* (P=0.005) mutants adhered in significantly lower numbers than the wild-type. The adherence and invasion results for all *S*. Enteritidis fimbrial mutants are shown in Table 5.1.

Table 5.1. The log_{10} values of bacterial counts of adherence and invasion of *S.* Enteritidis wild-type and fimbrial mutant strains to CKC

	Grown statically at 25 ºC				Grown with shaking at 37 ºC			
Strain	Invasion	P value	Adher-ence	P value	Invasion	P value	Adher-ence	P value
WT	2.89		4.10		3.46		3.87	
K-12	0	<0.0001	2.76	<0.0001	0	<0.0001	2.98	0.227
stbA	2.93	0.933	3.47	0.004	3.86	0.531	4.36	0.504
stcA	3.24	0.279	3.84	0.200	4.45	0.132	4.73	0.245
stdA	2.99	0.762	3.86	0.236	4.12	0.306	4.47	0.416
stfA	2.93	0.921	3.89	0.315	4.18	0.271	4.62	0.306
sthA	2.62	0.362	3.77	0.106	4.5	0.116	4.28	0.575
stiA	2.75	0.625	3.49	0.005	3.49	0.962	3.56	0.673
bcfA	2.68	0.477	3.83	0.185	4.47	0.125	4.74	0.239
csgA	2.49	0.191	3.95	0.459	3.26	0.757	3.72	0.839
sefA	2.39	0.002	3.73	0.074	4.2	0.255	4.78	0.217
lpfA	2.23	0.037	4.04	0.563	3.44	0.384	4.74	0.250
S1400	3.07		4.16		4.07		3.89	
fimA	2.96	0.705	3.79	0.135	4.39	0.157	4.63	0.299
steA	2.33	0.021	3.39	0.0007	3.24	0.253	4.11	0.759
safA	2.27	0.014	3.81	0.103	3.51	0.441	4.84	0.196
SEM	0.217		0.144		0.452		0.512	

F-test analysis was carried out using SAS, P values below 0.05 were considered significant and are highlighted. In Table 5.3 and Figure 5.2, WT refers to *S.* Enteritidis P125109 wild-type, S1400 refers to *S.* Enteritidis S1400 and K-12 refers to *E. coli* K-12 MG1655. SEM indicates the standard error of the mean as all results were the mean of triplicate experiments. *fimA, steA* and *safA* were compared to *S.* Enteritidis S1400 wild-type as they could not be transduced into *S.* Enteritidis P125109. All other mutants were compared with *S.* Enteritidis P125109.

The fimbrial mutants of *S*. Gallinarum 287/91 were examined using the same assay as for *S.* Enteritidis except as *S*. Gallinarum 287/91 is non-motile, the cells were centrifuged after the addition of the inoculum at 100 *g* for 3 min at room temperature. The *S*. Gallinarum 287/91 fimbrial mutants played a more significant role in adherence to and invasion of CKCs than *S*. Enteritidis. At 25 ºC, the *S*. Gallinarum Δ*steA::cat,* Δ*lpfA::cat* and Δ*safA::cat* mutants did not display a significant reduction in invasion compared to the wild-type unlike the results for *S*. Enteritidis. Interestingly, the mutations of Δ*sefA::cat* ($P<0.0001$) and Δ*sthA::cat* ($P<0.0001$) of *S*. Gallinarum 287/91 reduced invasion to undetectable levels (Table 5.2).

At 37 ºC, the Δ*sefA::cat* ($P<0.0001$) fimbrial mutant also invaded in numbers below the limit of detection, surprisingly the Δ*stbA::cat* ($P=0.045$), Δ*stcA::cat* ($P=0.036$) and Δ*bcfA::cat* ($P=0.037$) fimbrial mutants showed a significant increase in the number of bacteria invading CKC. The Δ*fimA::cat,* Δ*stiA::cat* and Δ*lpfA::cat* mutations also increased invasion of CKCs but not significantly but the same fimbriae produced a significant increase in adherence compared to the wild-type ($P<0.001$ for both) (Table 5.2). The Δ*safA::cat* and Δ*steA::cat* mutants of *S*. Gallinarum 287/91 did not show any significant differences in adhesion or invasion in either growth condition compared to the wild-type unlike in *S*. Enteritidis.

Table 5.2. The log_{10} values of bacterial counts of adherence and invasion of *S.* Gallinarum 287/91 wild-type and fimbrial mutant strains to CKC

	Grown statically at 25 ºC				Grown with shaking at 37 ºC			
Strain	Invasion	P value	Adher-ence	P value	Invasion	P value	Adher-ence	P value
Gal WT	2.38		2.53		2.51		2.24	
stbA	3.11	0.419	3.12	0.362	4.13	0.045	3.86	<0.0001
stcA	3.07	0.405	3.25	0.273	4.21	0.036	3.33	0.002
steA	2.30	0.935	2.90	0.564	2.56	0.925	2.48	0.454
stfA	2.22	0.875	2.63	0.895	3.05	0.528	2.31	0.827
sthA	0	<0.0001	2.30	0.787	2.64	0.858	2.56	0.325
stiA	3.68	0.166	3.01	0.464	3.86	0.089	4.28	<0.0001
bcfA	3.07	0.444	3.15	0.389	4.19	0.037	4.14	<0.0001
csgA	2.45	0.993	2.48	0.926	2.45	0.947	2.63	0.392
fimA	2.36	0.981	2.42	0.873	4.03	0.058	3.14	0.0091
lpfA	3.11	0.419	2.63	0.896	4.08	0.058	4.58	<0.0001
safA	2.64	0.773	2.37	0.795	2.00	0.639	2.60	0.434
sefA	0	<0.0001	2.69	0.850	0	<0.0001	2.52	0.387
K-12	0	<0.0001	2.77	0.718	0	<0.0001	2.98	0.029
SEM	0.419		0.402		0.534		0.226	

F-test analysis was carried out using SAS, P values below 0.05 were considered significant and are highlighted. Gal WT refers to *S.* Gallinarum 287/91 and K-12 refers to *E. coli* K-12 MG1655. SEM indicates the standard error of the mean and all results are the mean of triplicate experiments.

5.3.2. Adhesion to and invasion of human epithelial cells (HEp-2)

Salmonella gains entry into the host via epithelial cells as discussed in Chapter 1 and the invasion and replication of *Salmonella* and other species has been characterised using HEp-2 human lung carcinoma cells *in vitro* (Small et al., 1987, Lee et al., 1992, Ledeboer et al., 2006, La Ragione et al., 2003, Boddicker et al., 2002). The role of fimbriae in adherence to HEp-2 cells has been correlated with attenuation detected *in vivo* and it was predicted that using HEp-2 cells may also allow the role of other fimbriae to be determined and provide targets for *in vivo* analysis (Thorns et al., 1996, Baumler et al., 1996a). HEp-2 cells were first established in the 1950s (Moore et al., 1955) from tumors produced in irradiated cortisonised rats after injection with epidermoid cancer tissue from the larynx of a human (Toolan, 1954).

The *in vitro* adhesion and invasion assays were carried out as described in Section 2.5.2 at 37 ºC in a 5 % CO_2 incubator. The majority of *S.* Enteritidis fimbrial mutants showed no significant difference in invasion of or adherence to HEp-2 cells (Table 5.3). During growth at 25 ºC, the Δ*steA::cat* ($P<0.0001$), Δ*fimA::cat* ($P<0.0001$) and Δ*safA::cat* ($P<0.0001$) mutants of *S.* Enteritidis showed a significant decrease in ability to invade HEp-2 cells and the Δ*fimA::cat* ($P<0.0001$) and Δ*safA::cat* ($P=0.020$) fimbrial mutants also showed a significant decrease in adherence to HEp-2 cells. The Δ*fimA::cat* mutant strain could not be obtained at detectable limits at 25 ºC from adhesion or invasion assays but could be detected at 37 ºC (Table 5.3). At 37 ºC, the Δ*safA::cat* ($P=0.006$) and Δ*steA::cat* ($P= 0.004$) fimbrial mutations of *S.* Enteritidis S1400 resulted in a significant increase in adherence compared to the wild-type. Interestingly, all three of these mutants were in *S.* Enteritidis S1400 and could not be transduced into clean backgrounds.

Table 5.3. The log_{10} values of bacterial counts of adherence and invasion of *S.* Enteritidis wild-type and fimbrial mutant strains to HEp-2 cells

	Grown statically at 25 ºC				Grown with shaking at 37 ºC			
Strain	Invasion	P value	Adher-ence	P value	Invasion	P value	Adher-ence	P value
WT	3.58		3.81		4.19		4.69	
K-12	2.30	0.01	2.11	0.003	0	<0.0001	2.71	<0.0001
stbA	3.52	0.897	4.58	0.262	4.16	0.952	4.76	0.868
stcA	3.88	0.353	4.31	0.299	4.42	0.601	5.02	0.369
stdA	3.95	0.265	4.30	0.311	4.45	0.561	5.07	0.299
stfA	3.51	0.881	4.44	0.353	4.17	0.953	4.68	0.974
sthA	3.49	0.784	3.41	0.404	3.93	0.559	4.66	0.904
stiA	3.45	0.709	4.25	0.365	4.26	0.880	4.84	0.688
bcfA	3.89	0.341	4.57	0.118	4.67	0.288	5.00	0.392
csgA	3.91	0.317	4.14	0.499	4.39	0.655	4.64	0.869
sefA	3.43	0.658	4.07	0.582	4.30	0.809	4.77	0.839
lpfA	3.75	0.606	4.47	0.176	4.11	0.847	4.66	0.904
S1400	4.13		4.14		2.99		4.02	
fimA	0	<0.0001	0	<0.0001	3.02	0.949	3.96	0.979
steA	2.58	<0.0001	3.21	0.080	3.79	0.065	5.02	0.004
safA	2.59	<0.0001	2.91	0.020	3.51	0.222	4.96	0.006
SEM	0.230		0.334		0.312		0.246	

F-test analysis was carried out using SAS, P values below 0.05 were considered significant and are highlighted. In Table 5.3, WT refers to *S.* Enteritidis P125109 wild-type, S1400 refers to *S.* Enteritidis S1400 and K-12 refers to *E. coli* K-12 MG1655. SEM indicates the standard error of the mean as all results were the mean of triplicate experiments. *fimA, steA* and *safA* were compared to *S.* Enteritidis S1400 wild-type as they could not be transduced into *S.* Enteritidis P125109. All other mutants were compared with *S.* Enteritidis P125109.

In *S.* Gallinarum 287/91, the Δ*steA::cat* and Δ*fimA::cat* fimbrial operons played no significant role in adherence or invasion of HEp-2 cells, in contrast to their roles in *S.* Enteritidis. The Δ*safA::cat* (P=0.008) mutant when grown in static growth conditions at 25 ºC, was significantly decreased in its ability to adhere to HEp-2 cells but not at 37 ºC as shown in Table 5.4. At 37 ºC, the Δ*lpfA::cat (*P=0.030) and Δ*bcfA::cat* (P=0.039) fimbrial mutations resulted in a significant increase in their ability to invade HEp-2 cells and a slight but insignificant increase in adherence (Table 5.4).

Table 5.4. The log_{10} values of bacterial counts of adherence and invasion of *S.* Gallinarum 287/91 wild-type and fimbrial mutant strains to HEp-2 cells

	Grown statically at 25 ºC				Grown with shaking at 37 ºC			
Strain	Invasion	P value	Adher-ence	P value	Invasion	P value	Adhe rence	P value
Gal WT	2.75		3.74		2.30		4.05	
K-12	0	<0.0001	2.11	0.023	0	<0.0001	2.66	0.081
stbA	3.18	0.309	3.86	0.840	3.31	0.253	3.81	0.620
stcA	2.26	0.204	3.67	0.905	3.38	0.224	3.54	0.305
steA	2.42	0.419	3.69	0.936	3.32	0.293	3.52	0.279
stfA	2.00	0.079	2.81	0.133	2.91	0.492	3.71	0.484
sthA	3.04	0.482	3.34	0.479	2.83	0.519	3.65	0.388
stiA	2.36	0.304	3.99	0.691	3.47	0.188	4.72	0.185
bcfA	3.00	0.495	3.58	0.794	4.18	0.039	4.91	0.09
csgA	2.32	0.299	3.07	0.280	2.81	0.558	4.04	0.983
fimA	2.11	0.132	3.26	0.433	1.66	0.465	3.74	0.527
lpfA	2.31	0.245	3.55	0.757	4.29	0.030	4.77	0.153
safA	2.81	0.872	2.00	0.008	2.33	0.980	3.43	0.214
sefA	0	<0.0001	2.69	0.130	2.56	0.789	3.19	0.125
SEM	0.285		0.426		0.548		0.345	

F-test analysis was carried out using SAS, P values below 0.05 were considered significant and are highlighted. Gal WT refers to *S.* Gallinarum 287/91 and K-12 refers to *E. coli* K-12 MG1655. SEM indicates the standard error of the mean as all results were the mean of triplicate experiments.

5.3.3. Adhesion to and invasion of HD11 cells

HD11 cells are a chicken-derived macrophage-like cell line, they were first derived from chicken hematopoietic cells that had been transformed *in vitro* and *in vivo* with 7 different strains of a replicons deficient avain leukaemia viruses which resulted in the production of a cell line with macrophage like properties (Beug et al., 1979). It was relevant to use these cells because in the avian host *S*. Enteritidis infection has been seen to involve macrophage uptake in the caecal lumen (Popiel and Turnbull, 1985). A reduction in uptake into HD11 cells has been correlated with a decrease in virulence in the avian host in early colonisation and this has been proposed to be mediated by a reduction in macrophage cell death and a faster clearance rate (Bohez et al., 2006). The relative importance of epithelial cell attachment and invasion versus macrophage-uptake in the colonisation of the avian host are unknown. Several papers have reported differences in uptake of *Salmonella* into macrophages due to various specific mutations which have then correlated with an attenuated effect *in vivo* (Bohez et al., 2006, Adriaensen et al., 2007, Amy et al., 2004). The results obtained here showed only subtle effects of a single fimbrial gene mutation. The non-invasive *E. coli* K-12 control was internalised due to the phagocytic nature of HD11 cells. It is not possible to separate the number of bacteria present due to invasion and those present due to engulfment by the HD11 cells. It is also difficult to capture *Salmonella* only adhering to the surfaces without being engulfed, therefore only uptake data is shown. The values obtained for uptake are also likely to be an underestimate due to HD11 cells being capable of engulfing gentamycin and therefore killing some internalised bacteria. The *S*. Enteritidis fimbrial mutants showed no significant difference when compared to the wild-type in the uptake into HD11 cells as shown in Table 5.5.

Table 5.5. The log_{10} values of bacterial counts of uptake of *S.* Enteritidis wild-type and fimbrial mutant strains into HD11 cells

	Grown statically at 25 ºC		Grown with shaking at 37 ºC	
Strain	Invasion	P value	Invasion	P value
WT	5.19		5.84	
K-12	5.03	0.506	3.69	0.020
stbA	5.19	0.984	5.80	0.821
stcA	5.03	0.506	5.49	0.163
stdA	4.94	0.296	5.59	0.172
stfA	5.23	0.897	5.85	0.887
sthA	4.86	0.178	5.49	0.228
stiA	4.98	0.377	5.78	0.272
bcfA	5.24	0.869	5.69	0.686
csgA	5.26	0.782	5.09	0.176
sefA	4.78	0.105	5.84	0.413
lpfA	5.19	0.983	5.88	0.881
SEM	0.174		0.238	
S1400	3.79		5.77	
fimA	4.63	0.080	5.69	0.352
steA	4.98	0.828	5.76	0.523
safA	4.67	0.121	5.59	0.345

F-test analysis was carried out using SAS, P values below 0.05 were considered significant and are highlighted. WT refers to *S.* Enteritidis P125109 wild-type, S1400 refers to S. Enteritidis S1400 and K-12 refers to *E. coli* K-12 MG1655. SEM indicates the standard error of the mean and all results were from triplicate experiments. *fimA, steA* and *safA* were compared to *S.* Enteritidis S1400 wild-type as they could not be transduced into *S.* Enteritidis P125109. All other mutants were compared with *S.* Enteritidis P125109.

In *S.* Gallinarum 287/91, at 25 ºC the Δ*bcfA::cat* fimbrial mutants showed an increase in invasion and the Δ*fimA::cat* fimbrial mutant showed a decrease in invasion but no significant differences were seen at 37 ºC from any fimbrial mutant (Table 5.6).

Table 5.6. The log_{10} values of bacterial counts of invasion of *S.* Gallinarum 287/91 wild-type and fimbrial mutants to HD11 cells

	Grown statically at 25 ºC		Grown shaking at 37 ºC	
Strain	Invasion	P value	Invasion	P value
Gal WT	4.08		4.78	
K-12	3.79	0.674	3.68	0.252
stbA	4.77	0.319	5.08	0.757
stcA	4.90	0.239	6.06	0.186
steA	4.22	0.831	5.54	0.426
stfA	4.26	0.793	3.40	0.156
sthA	5.00	0.185	5.16	0.670
stiA	5.30	0.083	4.38	0.669
bcfA	5.53	0.040	6.02	0.202
csgA	4.13	0.943	5.61	0.388
fimA	2.23	0.014	5.06	0.773
lpfA	4.96	0.209	5.18	0.678
safA	5.33	0.078	6.37	0.103
sefA	5.05	0.214	5.61	0.388
SEM	0.482		0.668	

P values below 0.05 are considered significant and are highlighted. Gal WT refers to *S.* Gallinarum 287/91 and K-12 refers to *E. coli* K-12 MG1655. Standard error of the mean was included, as all results were from triplicate experiments.

Table 5.7. Summary of the phenotype of fimbrial subunit mutants in assays for adherence to and invasion of CKC, HEp-2 and HD11 cells

	CKC cells				HEp-2 cells				HD11 cells	
	25 ºC		37 ºC		25 ºC		37 ºC		25 ºC	37 ºC
Mutant	A	I	A	I	A	I	A	I	I	I
stbA	↓		↑	↑						
stcA			↑	↑						
stdA										
steA	↓	↓				↓	↑			
stfA										
sthA		↓								
stiA	↓		↑							
bcfA			↑	↑				↑	↑	
csgA										
fimA			↑		↓	↓			↓	
lpfA		↓	↑					↑		
safA		↓			↓ ↓	↓	↑			
sefA		↓ ↓		↓		↓				

Black arrows – *S.* Enteritidis, green arrows- *S.* Gallinarum, no arrows indicates no difference when compared to the wild-type.

A- adherence, I –invasion

The Δ*stdA::cat,* Δ*stfA::cat* and Δ*csgA::cat* fimbrial mutants produced no significant diffrerences in adhesion to or invasion of any of the cell lines examined in either serovar.

5.4. Validation of adherence and invasion assay

5.4.1 Quantitative confirmation of cell association

Confirmation of bacterial association with cells was carried out to confirm that the bacterial counts obtained in the adherence and invasion assays were not due to bacteria adhering to the plastic surfaces used for cell culture, as fimbriae have been shown to mediate adherence to abiotic surfaces (Woodward et al., 2000).

It is considered unlikely that the bacteria adhered to plastic as differences were seen between cell lines despite the fact that they were cultured in the same plastic trays, implying that the differences may be due to the different cell lines used. However, to confirm this and that the washes were successful in removing non-specifically associated bacteria, haemacolour staining of infected CKC monolayers was carried out. The three wild-type strains used in this study were used in the adherence and invasion assays as previously described in Section 2.5.2 but the CKC cells were grown in the plastic trays on a poly-L-lysine coverslip. The cells and bacteria on the coverslips were stained using haemacolour staining as described in Section 2.5.3 and the number of cell-associated and non-cell associated bacteria were counted in 100 fields of view from 3 independent experiments and the mean was calculated, shown in Table 5.8. The quantification of the *Salmonella* showed that ≥75% of bacteria were cell-associated and that the washes had removed the majority of non-cell-associated bacteria. The remaining bacteria were seen floating and were not assocated with the plastic trays. Under these assay conditions relatively few bacteria were detected by microscopy and therefore only the wild-type strains were examined.

Table 5.8. Quantification of *Salmonella* cell-association with CKCs

Wild-type strain	Average number of bacteria cell-associated	Average number of bacteria not cell-associated	Percentage cell-associated	P value
S. Enteritidis P125109	38	12	76	0.001
S. Enteritidis S1400	45	10	81	0.0061
S. Gallinarum 287/91	48	13	78	0.0006

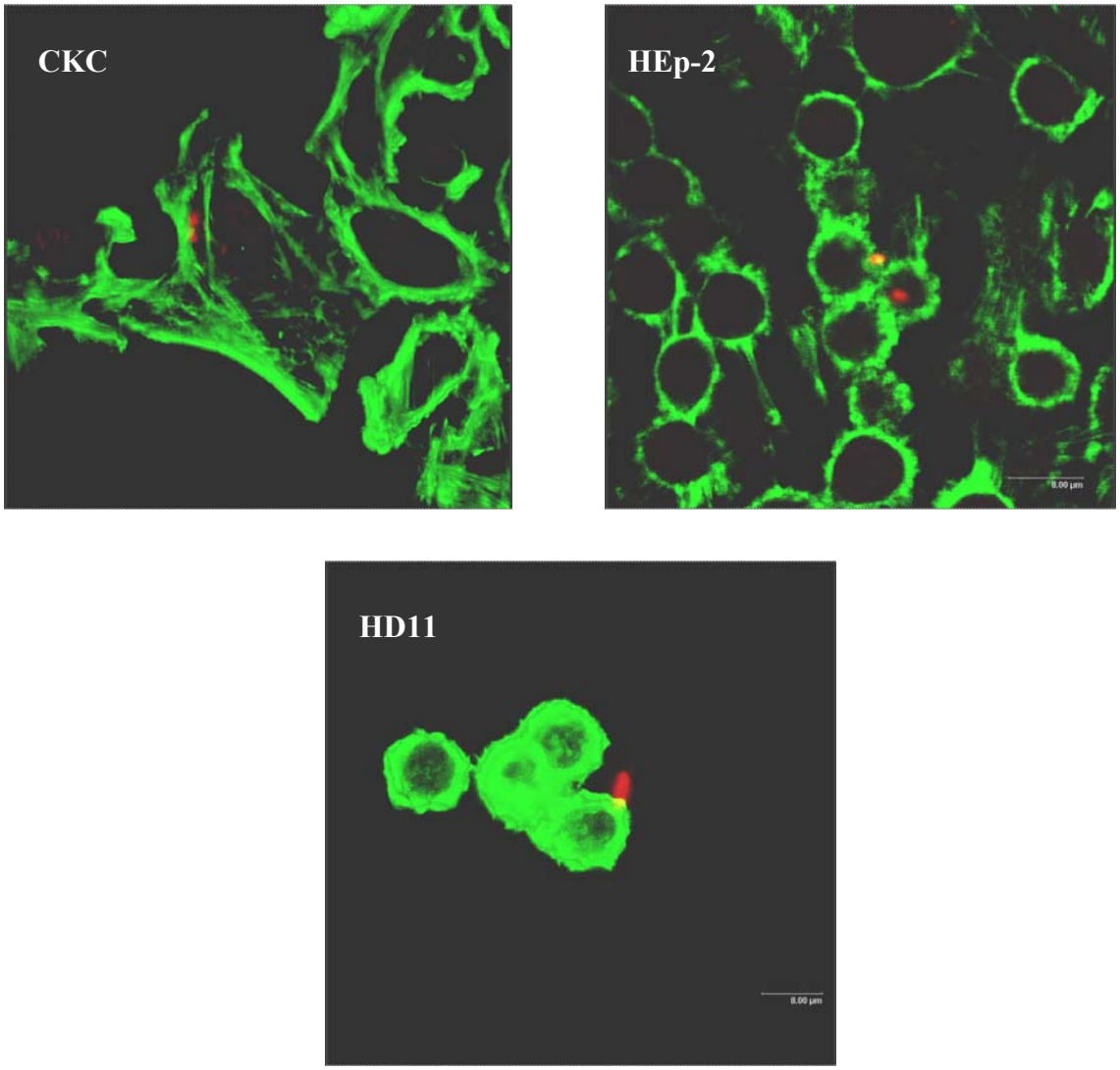
The number of bacteria cell-associated or not cell-associated was determined by visual inspection 100 hundred fields of view, the counts are the mean of 3 independent experiments per strain.

5.4.2. Visual confirmation of cell association by confocal laser scanning microscopy

To visually capture the counts in Section 5.4.1, *S*. Enteritidis P125109 wild-type was used to infect CKC, HEp-2 and HD11 cells as in the adherence and invasion assays at 37 ºC. The coverslips were treated as described in Section 2.5.3 for examination by confocal laser scanning microscopy. The bacterial numbers were low in relation to the number of cells, but the majority of bacteria were cell-associated, despite the monolayer not being confluent after treatments with bacteria, trypsine, several washed and abitbodies. A 0.25 µm optical section was collected which indicated that the bacteria were in contact with or surrounded by the cellular structures shown in Figure 5.1 (f-actin, green). By scanning vertically through cells in the Z plane it was possible to find bacteria that were apically located. Each of the cell lines were examined to rule out differences between the cells and these images are shown in Figures 5.1. It is possible that not all bacteria are captured by the antibody as internalised bacteria may have been

missed. Differences may also be seen between different serovars/strains but this was not examined here.

Figure 5.1. Confocal microscopy showing adherence of *S.* Enteritidis P125109 wild-type to different cells



The cells are shown in green and the *Salmonella* are shown in red, the black area is where there are no cells and no *Salmonella*. Giemsa staining was not done to enumerate all bacteria and it is possible that internalised bacteria are not recognised by the primary antibody. These images are representative of the low number of bacteria and cells that were captured after examining 3 coverslips from 3 independent experiments.

These experiments suggest that the assay predominantly reports on the association of *Salmonella* strains with cells as opposed to abiotic surfaces. Too few events can be captured by these methods to permit a robust statistical analysis of the role of fimbrial subunits in this process in a time-effective manner.

5.5.1. Confirmation of the phenotype of fimbrial mutants

Only some of the fimbrial mutations resulted in altered phenotypes as summarised in Table 5.7. The *steA* fimbrial mutant Δ*steA:cat,* was of particular interest since it exhibited a defect in both adherence to and invasion of CKC and HEp-2 cells. *Trans*-complementation of the *S*. Enteritidis Δ*steA:cat* was therefore undertaken.

5.5.2 Confirmation of the Δ*steA::*cat fimbrial mutant phenotype

The *S*. Enteritidis S1400 wild-type, Δ*steA::cat* and Δ*steA* were used in the *in vitro* adhesion and invasion assays as described in Section 2.5.2. In CKC only growth at 25 ºC was examined as this was the growth condition that permitted a phenotype to be identified whereas HEp-2 cells were examined in both conditions. The wild-type phenotype was restored by the removal of the chloramphenicol resistance cassette indicating that the phenotype of Δ*steA::cat* was likely the result of a polar effect of the chloramphenicol resistance cassette on a downstream gene (Table 5.9 and 5.10).

Table 5.9 Effect of the removal of the chloramphenicol resistance cassette from *S.* Enteritidis Δ*steA::cat* mutant on adherence to and invasion of CKC cells

	Grown statically at 25 ºC			
Mutant	Invasion	P value	Adherence	P value
WT	3.23		4.22	
Δ*steA::cat*	2.54	0.001	2.56	0.0004
Δ*steA*	3.60	0.02	4.29	0.8225
SEM	0.09		0.258	

F-test was carried out using SAS, P values below 0.05 are considered significant and are highlighted. WT refers to *S.* Enteritidis S1400, Δ*steA::cat* is the original mutant and Δ*steA* is the mutant without the chloramphenicol resistance cassette. SEM indicates the standard error of the mean.

In the original experiment, the Δ*steA::cat* fimbrial mutant adhered and invaded CKCs to a significantly lower level than the wild-type strain at 25 ºC which was successfully repeated here.

Table 5.10. Effect of the removal of the chloramphenicol resistance cassette from *S.* Enteritidis Δ*steA::cat* fimbrial mutant on adherence to and invasion of HEp-2 cells

	Grown statically at 25 ºC				Grown with shaking at 37 ºC			
Mutant	Invasion	P value	Adhe-rence	P value	Invasion	P value	Adher-ence	P value
WT	2.93		4.22		3.70		3.41	
Δ*steA::cat*	2.46	0.2247	3.04	0.02	3.87	0.828	4.56	0.05
Δ*steA*	3.14	0.5224	3.79	0.38	4.36	0.411	4.09	0.2254
SEM	0.738		0.312		0.551		0.384	

F-test was carried out using SAS, P values below 0.05 are significant and are highlighted. WT refers to *S.* Enteritidis S1400, Δ*steA::cat* is the original mutant and Δ*steA* is the mutant without the chloramphenicol resistance cassette. SEM indicates the standard error of the mean.

During the original experiment at 25 ºC the Δ*steA::cat* showed a significant difference in adherence and invasion of HEp-2 cells and whilst this pattern was repeated here, it was only significant for adherence. At 37 ºC, adherence and invasion were increased compared to the wild-type in the original experiment and here a significant increase in adherence was seen but not in invasion (Table 5.9).

5.5.3. *Trans*-complementation of the entire *ste* operon

The region between the *steA* gene and the *steB* gene is 83 bp and it is considered unlikely that the effect of the chloramphenicol cassette insertion may be to interrupt the stop-start codon translation of *steA* and *steB* owing to translational coupling. It remains feasible that the insertion impaired the transcription of genes downstream of *steA* and therefore the entire operon was used for *trans*-complementation.

Table 5.11. *Trans*-complementation of *S.* Enteritidis S1400 Δ*steA::cat* using the *ste* operon in CKC adherence and invasion assay

	Grown statically at 25 ºC			
Mutant	Invasion	P value	Adherence	P value
WT	3.65		5.3	
Δ *steA::cat*	2.71	0.089	4.07	0.032
Δ *steA::cat* [p^{steA-E} fwd]	3.42	0.651	4.95	0.491
Δ *steA::cat* [p^{steA-E} rev]	3.43	0.660	4.90	0.441
SEM	0.3411		0.3398	

F-test was carried out using SAS, P values below 0.05 are considered significant and are highlighted. WT is *S.* Enteritidis S1400 wild-type, Δ*steA::cat* is the original fimbrial mutant, Δ*steA::cat* [psteA-E fwd] is the mutant with a plasmid carrying the fimbrial operon in the on orientation and Δ*steA::cat* [psteA-E rev] is with the operon in the off orientation. SEM indicates the standard error of the mean as all experiments were carried out in triplicate.

The Δ*steA::cat* fimbrial mutant again showed a decrease in invasion and adherence of CKC at 25 ºC as shown in Table 5.11. The presence of the *steA-E* operon on a TOPO pCR4Blunt plasmid in either orientation partially restored adherence and invasion to wild-type levels. It is therefore likely that the expression of the *steA-E* operon does not strictly require the *lac* promoter of the cloning vector and that the genes may be transcribed from internal operon promoter/s or cryptic plasmid promoters. The data confirmed the adherence and invasion defect of the original Δ*steA::cat* mutant but suggests that genes encoded downstream of *steA* rather than *steA* alone are required for the full effect.

5.6. Discussion

The purpose of this study was to characterise a role for fimbrial subunits *in vitro* in relation to adherence or invasion of different cell lines. Several fimbrial subunits in *S.* Enteritidis showed no role at all as evidenced by the phenotypes of the Δ*stcA::cat,* Δ*stdA::cat,* Δ*stfA::cat,* Δ*sthA::cat,* Δ*bcfA::cat* and Δ*csgA::cat* mutants. The Δ*stdA::cat*, Δ*stfA::cat* and Δ*csgA::cat* encoded fimbrial subunits played no significant role in either *S.* Gallinarum or *S.* Enteritidis. One cannot preclude the possibility that these fimbriae may play a role in other growth conditions, at different temperatures, different cell lines or may require *in vivo* host conditions to be expressed as has been previously indicated (Humphries et al., 2003). The expression of the fimbriae in cell culture media was not examined and may play an important role but a comparison to the wild-type strain was always carried out. Therefore any differences observed would be due to the differences in phenotype of the strain which may or may not be due to the expression of fimbriae in different media or at a different temperature. *Salmonella* were only in cell culture media for 15 minutes and would not have time for one complete round of replication, making it unlikely that the cell culure media or change in temperature will alter fimbriae expression. Differences in fimbrial expression under different growth conditions has been shown previously for SefA, FimA and CsgA and both temperature and pH play a role in their production (Walker et al., 1999, Woodward et al., 2000, Dibb-Fuller et al., 1997). The expression of these 3 fimbriae are known in some strains to occur under the conditions examined herein, however for many fimbriae the conditions under which expression occurs is unknown and appears to vary greatly from *in vitro* to *in vivo* (Dibb-Fuller et al., 1997, Humphries et al., 2003, Woodward et al., 2000, Walker et al., 1999).

Using CKC in the *in vitro* assays showed that the Δ*lpfA::cat,* Δ*steA::cat,* Δ*safA::cat* and Δ*sefA::cat* fimbrial mutants of *S.* Enteritidis were impaired in their ability to invade at 25 ºC and the Δ*steA::cat,* Δ*stbA::cat* and Δ*stiA::cat* were impaired in their adherence to CKC at 25 ºC but no significant differences were seen at 37 ºC. As CKC are a primary chicken epithelial cell, it was anticipated that fimbriae would play a more significant role at the higher temperature, as the core temperature of the avian host is 42 ºC. It may be that other environmental conditions that would be present in the natural host are missing and this may be masking the full phenotype of the fimbrial mutants.

The role of *S.* Gallinarum fimbriae are poorly characterised and limited information exists in the literature on the role of its fimbriae *in vitro* or *in vivo*. In CKC cells, the Δ*sthA::cat* and Δ*sefA::cat* fimbrial mutants of *S.* Gallinarum were significantly decreased in their ability to invade at 25 ºC. At 37 ºC the Δ*stbA::cat,* Δ*stcA::cat,* Δ*stiA::cat,* Δ*bcfA::cat,* Δ*fimA::cat* and Δ*lpfA::cat* fimbrial subunit mutations all showed significant increases in adherence and the Δ*stbA::cat,* Δ*stcA::cat* and Δ*bcfA::cat* fimbrial mutations all resulted in an increase in invasion. The Δ*sefA::cat* fimbrial mutant showed a decrease in invasion at both temperatures. Interestingly the *stb, sti* and *lpf* fimbrial operons of *S.* Gallinarum 287/91 contain frame-shift mutations in their putative usher gene. Mutation of the predicted fimbrial subunits of these operons still produces a phenotype despite this. Therefore the pseudogene may not be required for assembly of the fimbriae and other fimbrial genes may substitute for their function. The loss of a functional major fimbrial subunit may result in single or multiple fimbriae to function in its place and a mutation in the major fimbrial subunit of one operon, may still allow other components of the same operon to be produced. The precise mechanisms behind such proposed compensation systems are unclear and may vary between the fimbrial operons. It is also possible that a fimbrial mutation results in the

up-regulation of non-fimbrial adhesins, such as SiiE whose expression is related to activation of *Salmonella* invasion genes (Gerlach et al., 2007b).

In *S*. Enteritidis, the Δ*steA::cat* and Δ*safA::cat* fimbrial mutations increased adherence at 37 ºC to HEp-2 cells. At 25 ºC, the Δ*fimA::cat* and Δ*safA::cat* fimbrial mutants exhibited decreased adhesion to HEp-2 cells and Δ*steA::cat,* Δ*fimA::cat* and Δ*safA::cat* mutations resulted in a decrease in the number of bacteria invading HEp-2 cells. Interestingly, all of these mutants were made in *S*. Enteritidis S1400 and could not be transduced and the phenotype may be the result of secondary recombination events, but was not seen in CKC.

In *S*. Gallinarum, the Δ*safA::cat* fimbrial mutant exhibited a decrease in adherence to HEp-2 cells at 25 ºC. The *saf* operon contains a frame-shift mutation in the usher gene, suggesting it uses a different usher or ancillary genes to function fully. This has also been demonstrated in the *stg* fimbriae found in *S*. Typhi, the *stg* usher is a predicted pseudogene yet the *stg* operon still functions (Forest et al., 2007).

A *fimA* mutant of *S*. Typhimurium was previously shown to adhere to HEp-2 cells in comparable numbers to the wild-type (Baumler et al., 1996a)Rajasheakera and at 37 ºC this concurred with the data obtained here. However, at 25 ºC in *S*. Enteritidis, the *fimA* mutant showed a significant decrease in adherence and invasion which has not previously been identified (Baumler et al., 1996a). It has been previously shown that *sefA* fimbrial mutations do not affect the adherence or invasion of HEp-2 cells (Ogunniyi et al., 1997, Thorns et al., 1996) as was also identified from the results herein. Mutations of the *lpfC* gene reduce the invasion and adherence of *S*. Typhimurium which was not seen in these studies possible due to the fact that in literature the *lpfC* gene was mutated not the major subunit (Baumler et al., 1996a).

Despite the phagocytic nature of HD11 cells, the Δ*bcfA::cat* and Δ*lpfA::cat* fimbrial mutants of *S.* Gallinarum 287/91 showed an increase in invasion of HD11 cells but no difference was seen with *S.* Enteritidis fimbrial mutants, concurring with earlier observations (Rajashekara et al., 2000). However, disrupting genes downstream of the *sefA* major fimbrial subunit in a polar manner has been shown to decrease the bacterial uptake of *S.* Typhi into macrophages (Edwards et al., 2000). During the assays the centrifugation of the bacteria to the cells may have resulted in serovar differences becoming more apparent in one serovar than another.

Both haemacolour staining and quantitation along with confocal imaging were used to visually confirm the adherence of bacteria to the cells and to confirm that the washes were effective and that the majority (>75%) of bacteria were associated with cells and remained behind after washing to be counted.

The phenotype of Δ*steA::cat* mutant of *S.* Enteritidis was independently confirmed but further studies showed that the phenotype was an indirect effect caused by the presence of the chloramphenicol resistance cassette since FLP-mediated excision of the cassette reversed the effect. It is likely that the chloramphenicol resistance cassette had a polar effect on the *ste* operon affecting the *steB, steC, steD* and *steE* genes. The *steA* and *steB* genes are separated by 83 bp and are unlikely to be translationally coupled. It is unclear whether all of the phenotypes identified were a result of the mutation of the major fimbrial subunits or a consequence of polar effects on other genes within the fimbrial operon or other genes. It has been reported in the literature that a *sefA* non-polar mutation had no effect on virulence whereas a polar mutant showed reduced virulence (Edwards et al., 2000). To confirm the phenotypes of all other fimbrial mutants a non-polar mutation would have to be constructed using the Flippase

recombinase. The effects of polar mutations could also be examined using RT-PCR or where possible Western blots to determine which genes are expressed.

Complementing the *S.* Enteritidis S1400 Δ*steA::cat* polar mutation with the *steA-E* operon resulted in the partial return of wild-type characteristics. However, the plasmid that possessed the operon in the antisense orientation still partially restored cell-association to the same extent as the plasmid with the operon in the sense orientation. This implies that the operon may be expressed from internal promoters or transcribed from extraneous plasmid promoters.

The differences in adherence to different cells, in different growth conditions has been demonstrated for *S.* Typhimurium and *S.* Enteritidis but is not reported in *S.* Gallinarum (Ernst et al., 1990, Woodward et al., 2000, Dibb-Fuller et al., 1997). *S.* Gallinarum showed a role for some fimbriae that was not seen in *S.* Enteritidis and may be because of serovar-specific traits related to the genomic attrition that was seen in the host-specific serovars, resulting in reduced redundancy in the repertoire of fimbriae (Chapter 3). There are less functional genes in the host-specific serovars than in the ubiquitous serovars and therefore less compensation systems exist and the phenotype of any one gene is more pronounced. A mucous secreting cell line HT29 has also been used to study the role of fimbriae in adhesion in *E. Coli* and *Salmonella* (La Ragione et al., 2000, Rajashekara et al., 2000) and is sensitive enough to detect differences that occur as a result of single base pair substituitions (Kisiela et al., 2006).

It has also been proposed that fimbrial adherence is mediated by allelic variation or point mutations within fimbriae which could help in part to explain serovar differences (Kisiela et al., 2005, Boddicker et al., 2002, Hancox et al., 1997). The *stbA* and *stcA* genes are identical in *S.* Gallinarum and *S.* Enteritidis, but the phenotype of mutants in

these genes are different which may be due to variation in the sequence of the rest of the operon or other ancillary genes (Table 3.1).

This chapter highlights the potential for strain-, serovar- and host cell-specific effects in the role of fimbriae during adherence and invasion. To better define their role emphasis was next placed on the role of fimbrial subunits of *S.* Enteritidis in colonisation of the alimentary tract of chickens.

Chapter 6

Characterisation of fimbrial mutants *in vivo*

6.1. Introduction

Targeted and genome-wide mutagenesis of *Salmonella* has revealed roles for selected fimbriae in the colonisation of different hosts. The *lpf, fim, bcf, stb, stc, std, sth* and *csg* fimbriae of *S.* Typhimurium are required for long-term systemic carriage in mice (van der Velden et al., 1998, Weening et al., 2005, Lawley et al., 2006, Edwards et al., 2000, Tsolis et al., 1999). The *saf* fimbriae have been shown to play a role in colonisation of pigs (Carnell et al., 2007) and in the avian host the *stbC, csgD* and *sthB* fimbrial genes of *S.* Typhimurium have been implicated in colonisation (Morgan et al., 2004, Turner et al., 1998). The majority of studies involving *S.* Enteritidis fimbriae in chickens has shown a weak or undetectable role for those fimbriae examined (Allen-Vercoe and Woodward, 1999a, Rajashekara et al., 2000). A *fimD* mutant of *S.* Enteritidis produced prolonged bacteremia and decreased egg shell colonisation but exhibited no difference in gut colonisation (De Buck et al., 2003).

The majority of studies to date have involved the use of *S.* Typhimurium despite *S.* Enteritidis being the prevalent non-typhoidal *Salmonella* isolated from humans in the England and Wales (Figure 1.1). To define the role of *S.* Enteritidis fimbriae in colonisation of the avian alimentary tract, an established chicken colonisation model was used. In chickens, *S.* Enteritidis, was shown to preferentially colonise the caeca and was primarily isolated from the lumen of the caeca (Fanelli et al., 1971, Barrow et al., 1988). During initial infection *S.* Enteritidis can be isolated from the liver, spleen and caeca at 1-week post-infection but by 4-weeks post-infection only the caeca is still colonised (Gast and Holt, 1998, Bohez et al., 2006) and our own studies have indicated that the caeca is most commonly colonised (personal communication). Obviously the length of persistence and type of colonisation can depend on several factors and therefore a pilot experiment was to be carried out. Although *in vitro* assays are useful in

predicting the roles of certain genes it is not possible to mimic the natural hosts' *in vivo* conditions. An out-bred line of Rhode Island Red chickens were used to provide a model that was as close to natural populations as possible.

6.2. Aims

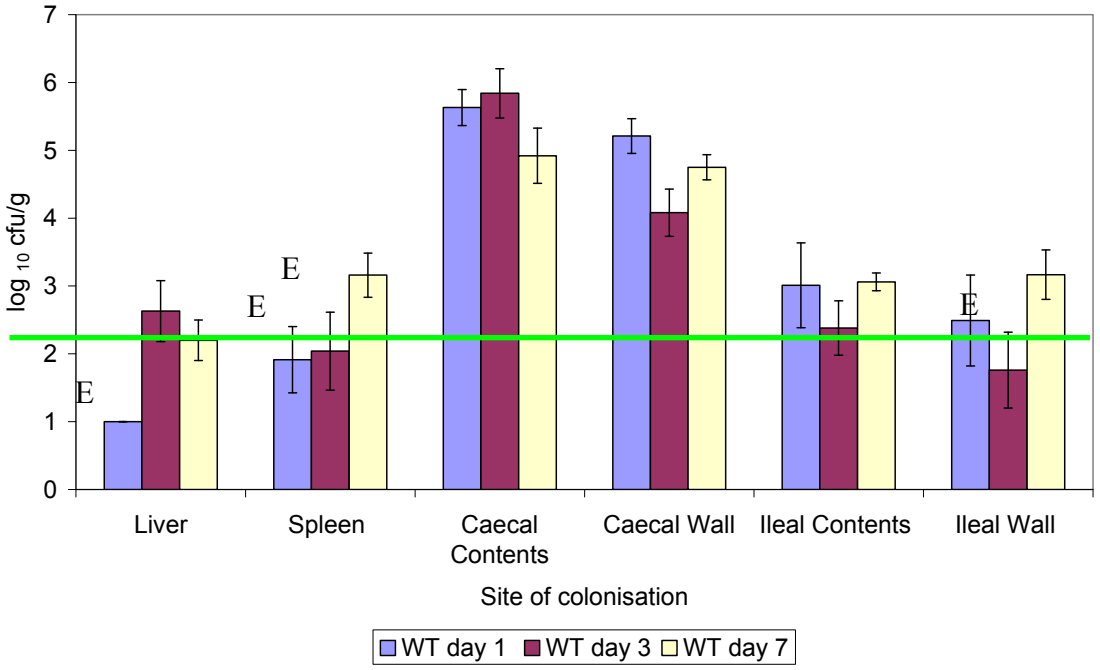
- To produce a valid model to study the role of *S*. Enteritidis fimbriae *in vivo*
- To characterise the role of *S*. Enteritidis fimbrial subunit genes in the colonisation of chickens
- To confirme any phenotype identified by *trans*-complementation

6.3. Pilot experiment

The design and execution of experimental infection studies with *Salmonella* wild-type and mutant strains is described in Section 2.6. A pilot experiment was carried out, as although the colonisation of *S.* Enteritidis in chickens is widely reported in the literature there is limited information on the colonisation of chickens with the sequenced strain of *S.* Enteritidis P125109 (Atterbury et al., 2007) and at the ouset of this study data was only available for colonisation of this strain in mice (Suar et al., 2006). The pilot experiment aimed to define the kinetics (magnitude and duration) of intestinal colonisation and systemic translocation (if any) and to gain an assessment of variance between birds. This experiment allowed time intervals to be identified at which adequate numbers of bacteria can be recovered to permit a robust statistical analysis of the effect of fimbrial subunit mutations. Groups of 15 SPF birds were gavage dosed with 0.1 ml of adult gut flora to standardise the gut contents at day of hatch. At 18-days-old each bird was orally dosed with 0.3 ml of approximately $5x10^8$ cfu/ml wild-type or fimbrial mutant as used in other experiments (Bohez et al., 2006). Five birds from each group were killed by cervical dislocation of the neck and *necropsy* examinations were performed at 1, 3 and 7 days post-infection (dpi) to enable assessment of early adhesion in colonisation of the chicken. The liver (caudate lobe), spleen, caecal contents, caecal wall, ileal contents and ileal (prior to the ileo-caecal junction) were recovered aseptically and diluted 1:10 in saline for homogenisation. A rotary blade was used to homogenise the samples and ten-fold serial dilutions were carried out. As each sample was diluted 1:10 for homogenisation and 20 µl of this was plated in triplicate, the theoretical limits of detection are $\log_{10}$ 2.2 colony forming units per gram (cfu/g).

For some samples bacterial counts were below the limits of detection and therefore enrichment was used. The entire sample was grown overnight at 37 ºC in 1 x selenite broth and then plated on brilliant green agar plates supplemented with nalidixic acid (20 μg/ml) and novobiocin (1 μg/ml) (*S.* Enteritidis P125109 and *S.* Gallianrum 287/91 were both nalidixic acid and novabiocin resistant, *S.* Enteritidis S1400 was only nalidixic acid resistant). This results in a qualitative rather than a quantitative count but was given an arbitrary figure of $\log_{10}1$ as the sample diluted 10^{-1} must have contained at least one viable organism. The enrichments may have been improved by increasing the time of enrichment or the temeperature (Huhtanen and Naghski, 1972, June et al., 1995).

Figure 6.1. Kinetics of intestinal colonisation and systemic translocation of *S.* Enteritidis P125109 wild-type in 18-day-old Rhode Island Red chickens at intervals post-oral inoculation



The green line indicates the limit of detection (log10 2.2). Where counts are reported below the limits of detection this is due to either enrichment or the recovery of counts above the limits of detection in some but not all birds in a cohort such that the average appears below the limits of detection. The error bars show the standard error of the mean and provide a measure of bird-to-bird variation. As early liver, spleen and ileum samples yielded bacterial numbers approaching the limits of detection, subsequent samples were taken from 3 dpi instead of 24 hours post-infection and at 7 and 10 dpi.

The caecal mucosa and contents were colonised with $\geq 10^4$ cfu/g for the duration of the experiment peaking in the caecal contents at almost 10^6 cfu/g. This compares to the lower loads in the ileum and in systemic sites as expected as most studies identify the caeca as containing the highest bacterial load (Gast and Holt, 1998, Fanelli et al., 1971, Barrow et al., 1988).

The magnitude and duration of colonisation of enteric and systemic sites by *S.* Enteritidis S1400 in chickens has been widely reported in the literature and was

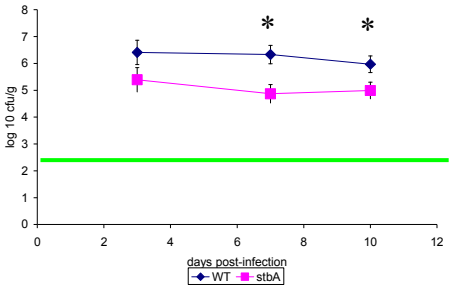
therefore not examined here (Carroll et al., 2004, Allen-Vercoe and Woodward, 1999a, Woodward et al., 1996, Cooper et al., 1992).

6.4. Caecal colonisation by *S*. Enteritidis fimbrial subunit mutants

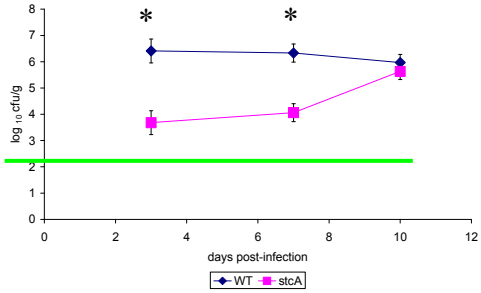
In studies to define the role of *S*. Enteritidis fimbriae, four groups of fifteen birds were used. One group was always a control and was dosed with the wild-type strain and three different fimbrial mutants were separately used to inoculate the 3 other groups. Each of the fimbrial mutants was compared to their respective wild-type strain. Each group of unsexed SPF birds were housed in separate cages and provided with food and water *ab litium* and were gavage dosed at 18 days old (Section 2.6). This would ensure that the chickens would develop an immune response as B cell responses are capable at 14 days old and caecal tonsils are mature (Beal 2006, Bar-Shira 2003). At 3, 7 and 10 dpi, 5 birds from each group were killed. The liver (caudate lobe), spleen, caecal contents, caecal wall, ileal contents and ileal (prior to the ileo-caecal junction) were taken as described in Section 2.6 and homogenised. Since attenuation of defined and random mutants has been detected by examining bacterial numbers in the caeca, data on the role of *S*. Enteritidis fimbriae in intestinal colonisation are evaluated in the following sections against this criterion. Owing to the difficulty in physically separating contents from mucosa bacterial counts from the caecal wall and caecal counts were combined to give a total caecal load; these are displayed in Figures 6.2-6.3. Each tissue sample was homogenised, serially diluted and plated on brilliant green agar plates supplemented with nalidixic acid (20 μg/ml) and novobiocin (1 μg/ml) in triplicate. Values represent the mean ± the standard error of the mean and an F-test statistical analysis was carried out. P values < 0.05 were considered significant and are marked on the figures with an asterix.

Figure 6.2. Total caecal load of *S.* Enteritidis P125109 wild-type and fimbrial mutant strains at intervals after oral inoculation of 18-day-old Rhode Island Red chickens

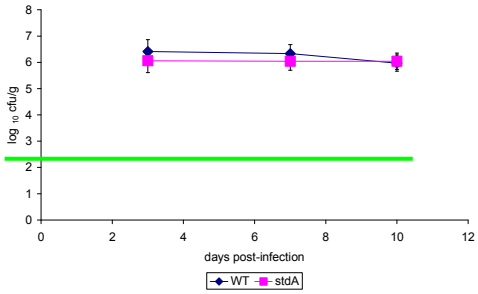
Δ*stbA::cat* P values at 7 dpi = 0.0081 and at 10 dpi = 0.03



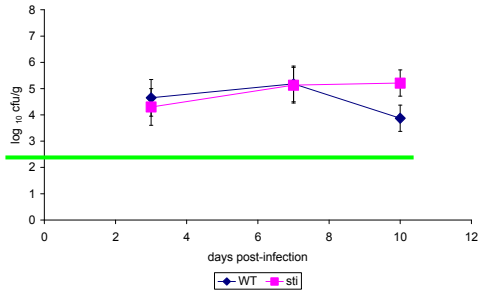
Δ*stcA::cat* P values at 3 dpi = 0.0006 and at 7 dpi = 0.0002



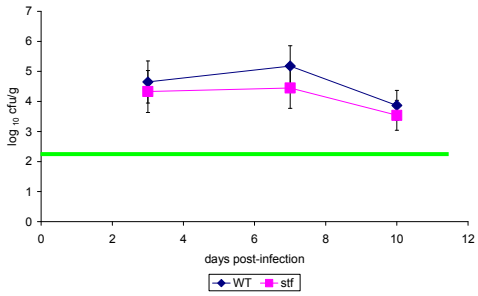
Δ*stdA::cat*



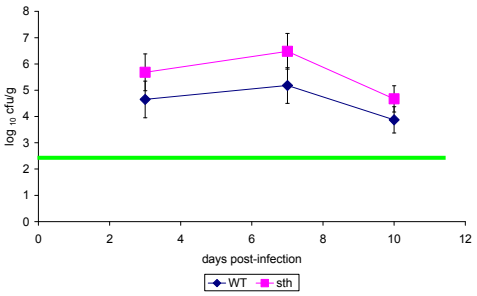
Δ*stiA::cat*



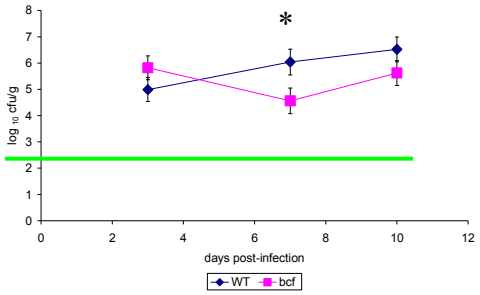
Δ*stfA::cat*



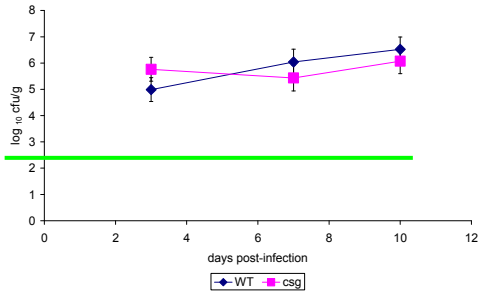
Δ*sthA::cat*



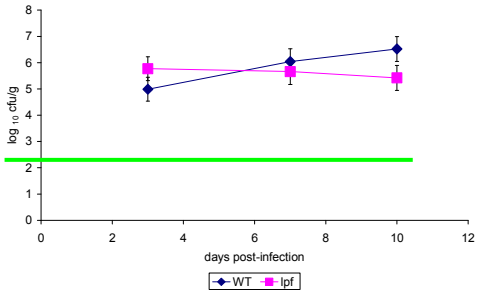
Δ*bcfA::cat* P values at 7 dpi = 0.04



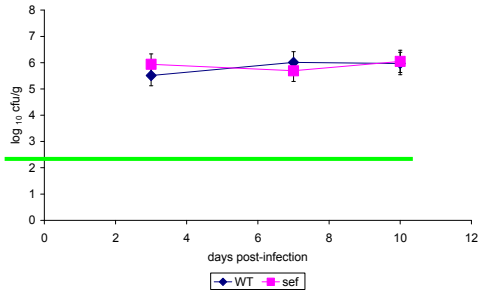
Δ*csgA::cat*



Δ*lpfA::cat*



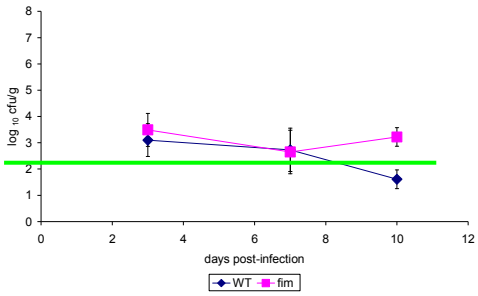
Δ*sefA::cat*



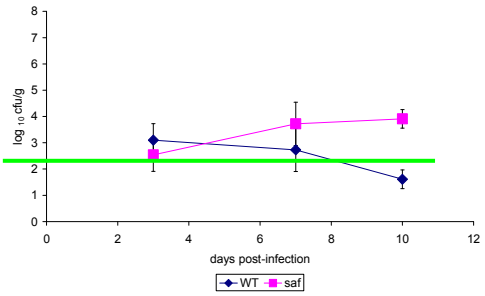
The green line indicates the limit of detection; all samples below this line were obtained via enrichment. The data reflect the total bacterial count from the caecal wall and caecal contents taken from five birds at each time point for each mutant. The error bars denote the standard error of the mean (SEM). An F-test analysis was carried out and P values below 0.05 were considered significant and are marked with an astrix.

Figure 6.3. Total caecal load of *S.* Enteritidis S1400 wild-type, Δ*fimA::cat*, Δ*steA::cat* and Δ*safA::cat* mutant strains at intervals after oral inoculation of 18-day-old Rhode Island Red chickens

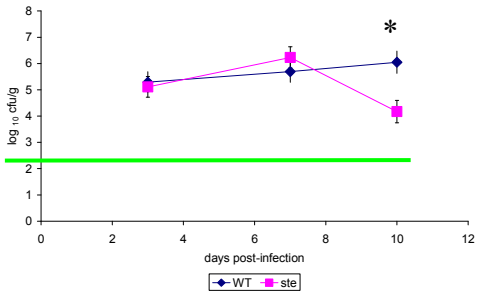
Δ*fimA::cat*



Δ*safA::cat*



Δ*steA::cat* P value at 10 dpi = 0.0034



S. Enteritidis S1400 was used in this experiment because these mutations could not be transduced into *S.* Enteritidis P125109. The examination of Δ*fimA::cat* and Δ*safA::cat* mutations were carried out in the same experiment whereas the Δ*steA::cat* mutant was examined in a different experiment but still in comparison with age matched birds inoculated with the wild-type at the same time.

Figures 6.2-6.3 show the limited variation that occurs between the fimbrial mutants and the respective wild-type strain. The *csgA, sefA* and *fimA* have previously shown no role in the colonisation of the chicken and data obtained here agrees with this (Rajashekara et al., 2000). In Figure 6.2, the Δ*stbA::cat* fimbrial mutant was reliably recovered from the chicken caeca at lower levels than the wild-type although the difference only became significant at 7 and 10 dpi with P values of 0.0081 and 0.03

respectively. The Δ*stcA::cat* fimbrial mutant showed a significant difference in colonisation of the caeca at 3 and 7 dpi with P values of 0.0006 and 0.0002 respectively, implying that the *stcA* gene may play a role in the initial stages of infection and *stbA* in later stages of infection. The Δ*bcfA::cat* fimbrial mutant resulted in statistically lower numbers of bacteria being recovered in the caeca only at 7 dpi with a P value of 0.04.

In Figures 6.3, it is of interest to note the lower colonisation rates of the caeca of *S.* Enteritidis S1400 wild-type compared with *S.* Enteritidis P125109 wild-type. This difference may have arisen due to the composition of gut-flora which was different between the cohorts or due to strain variation and the data reinforce the need to use a wild-type strain in all groups of challenged birds. In Figure 6.3, it can be seen that the Δ*steA::cat* fimbrial mutant is recovered in lower numbers from the caeca only at 10 dpi compared to the wild-type (P = 0.0034). The Δ*fimA::cat* and Δ*safA::cat* fimbrial mutants persisted in the caeca in greater numbers than *S.* Enteritidis S1400 wild-type at 10 dpi. However, the numbers of wild-type bacteria in the caeca are below the limits of detection and it would be unsafe to draw conclusions on the role of *fimA* and *safA* in the absence of further experiments.

During each experiment the liver, spleen, and ileum were also processed and bacterial counts were determined, an F-test analysis was carried out on all data and the P values are shown in Table 6.1. All bacterial counts are recorded in Appendix 6.1. Although several significant differences were identified at sites other than the caeca, the majority were below the limits of detection as indicated by E in Table 6.1 and therefore cannot be reliably used for comparison. Several fimbrial subunit mutants displayed significant differences to the wild-type only at one organ at one time point e.g. Δ*stfA::cat* and Δ*sthA::cat* or at only one site across different times e.g. Δ*steA::cat* in the

ileum, whereas other fimbrial mutants' were attenuated at more than one organ or at more than one time point (Table 6.1).

Both the Δ*bcfA::cat* and the Δ*lpfA::cat* fimbrial mutant strains were recovered in the ileum below the limits of detection at 3 and 7 dpi, despite all other sites being colonised in comparable numbers to the wild-type. The Δ*sefA::cat* mutation resulted in a decrease in the numbers of bacteria recovered from the ileal wall and contents at both 3 and 10 dpi and from the liver and spleen at 7 dpi. The Δ*safA::cat* mutant was reduced in the spleen at all time points and in the ileal contents at 3 dpi and the Δ*fimA::cat* was lower in the spleen at 3 and 7 dpi and in the ileal contents at 3 dpi. The Δ*stcA::cat* mutant was recovered from the spleen at 10 dpi at significantly lower numbers than the wild-type and the Δ*stdA::cat* fimbrial mutant at 7 dpi was found in significantly greater numbers in the ileal wall and ileal contents with P values of 0.05 and 0.03, respectively. The Δ*stiA::cat* produced significant differences to the wild-type at 3 and 10 dpi but not at 7 dpi.

The Δ*stbA::cat* and Δ*csgA::cat* mutant phenotypes are not shown in Table 6.1 as there were no significant differences in the P values at any of the sites examined. Despite a significant difference occurring for the Δ*stbA::cat* mutant in the caeca.

Table 6.1. P values obtained from an F-test analysis of bacterial counts of *S.* Enteritidis fimbrial mutant strains from the liver, spleen and ileum at various time points post-oral inoculation

The E represents those samples which were obtained via enrichment. The bacterial counts are shown in Appendix 6.1.

Day 3	Liver	Spleen	Ileal contents	Ileal wall
sefA	0.063	0.638	E 0.001	E 0.02
steA	1	0.599	E 0.0004	0.125
stcA	0.091	0.893	0.061	0.111
stdA	0.178	0.319	0.976	0.961
sthA	0.291	E 0.035	0.280	0.054
stiA	0.670	0.020	0.240	E0.046
stfA	0.600	0.910	0.010	0.849
bcfA	0.670	0.190	E 0.010	E 0.020
lpfA	0.590	0.780	E 0.005	E 0.030
fimA	0.640	E 0.030	E 0.026	0.390
safA	0.350	E 0.019	E 0.007	0.350

Day 7	Liver	Spleen	Ileal contents	Ileal wall
sefA	E <0.0001	E 0.0003	0.541	0.821
steA	E 0.0012	0.153	E 0.0058	E 0.0006
stcA	0.661	0.922	0.248	0.881
stdA	0.622	0.311	0.052	0.031
sthA	0.840	0.449	0.890	0.828
stiA	0.410	0.101	0.750	0.687
stfA	0.070	0.269	0.930	0.674
bcfA	0.384	E 0.015	E 0.002	E 0.001
lpfA	0.384	0.082	E 0.014	0.115
fimA	0.550	E 0.002	0.690	0.115
safA	0.120	E 0.010	0.240	1.000

Day 10	Liver	Spleen	Ileal contents	Ileal wall
sefA	0.981	0.0071	0.0017	0.0162
steA	0.169	0.951	E 0.0064	0.287
stcA	0.091	0.031	0.116	0.015
stdA	0.178	0.877	0.692	0.841
sthA	0.960	0.610	0.360	0.402
stiA	0.010	0.870	0.660	0.580
stfA	0.770	0.316	0.550	0.145
bcfA	0.709	0.850	0.760	0.770
lpfA	0.676	0.550	0.175	0.180
fimA	0.440	0.212	0.370	0.270
safA	E 0.660	E 0.020	0.520	E 0.007

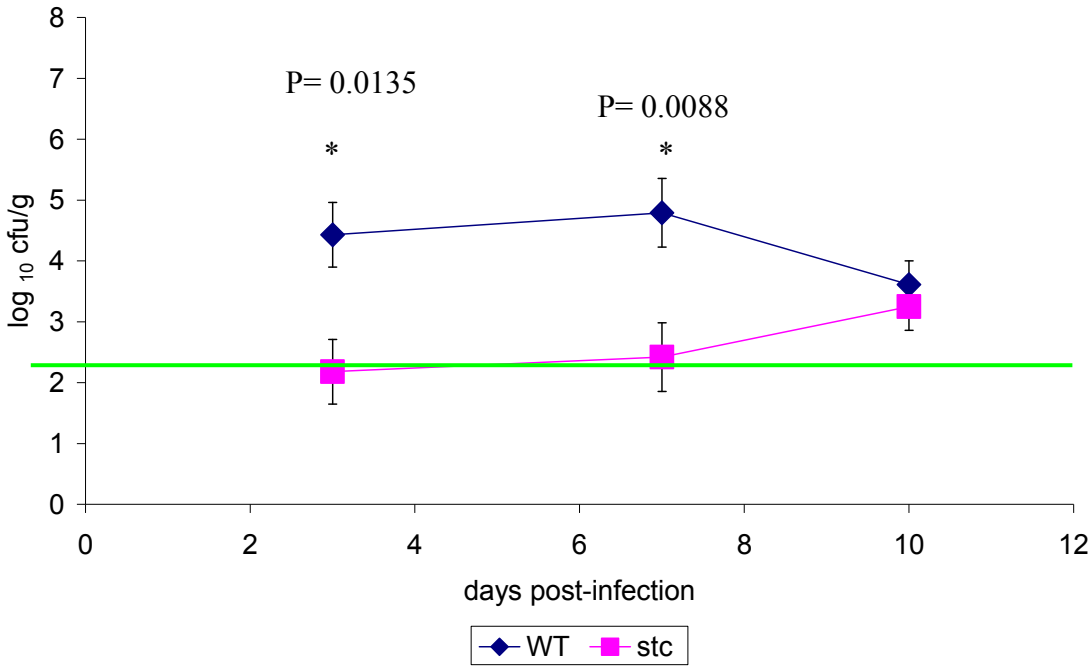
The phenotypes obtained *in vivo* do not correlate with the results obtained *in vitro* in Chapter 5. *In vivo,* the Δ*stcA::cat* and Δ*stbA::cat* mutants are attenuated in their ability to colonise the caeca. *In vitro* the Δ*stbA::cat* mutation resulted in a decrease in the adherence to CKC only 25 ºC but interestingly, no attenuating phenotype was identified for the Δ*stcA::cat* mutant *in vitro.*

Whilst phenotypes were detected for fimbrial mutants in respect of adherence (Δ*steA::cat,* Δ*stiA::cat,* Δ*fimA::cat* Δ*safA::cat,* Δ*stbA::cat)* and invasion *(*Δ*lpfA::cat,* Δ*safA::cat,* Δ*sefA::cat,* Δ*fimA::cat,* Δ*steA::cat),* the same mutants did not exhibit attenutation in chickens.

6.5. Confirmation of the phenotype of an *S*. Enteritidis P125109 Δ*stcA::cat* mutant phenotype

The Δ*stcA::cat* fimbrial mutant of *S.* Enteritidis P125109 produced the strongest phenotype in terms of magnitude and the time intervals at which attenuation was detected and was therefore chosen for further analysis. To confirm that the *stcA* contributes to colonisation, an independent Δ*stcA::cat* fimbrial mutant of *S.* Enteritidis S1400 was screened using the same experimental design as above relative to the parent strain as described in Section 6.3. The total bacterial load in the caeca is shown in Figure 6.4.

Figure 6.4. Total caecal load of *S.* Enteritidis S1400 wild-type and Δ*stcA::cat* strains at intervals after oral inoculation of 18-day-old Rhode Island Red chickens



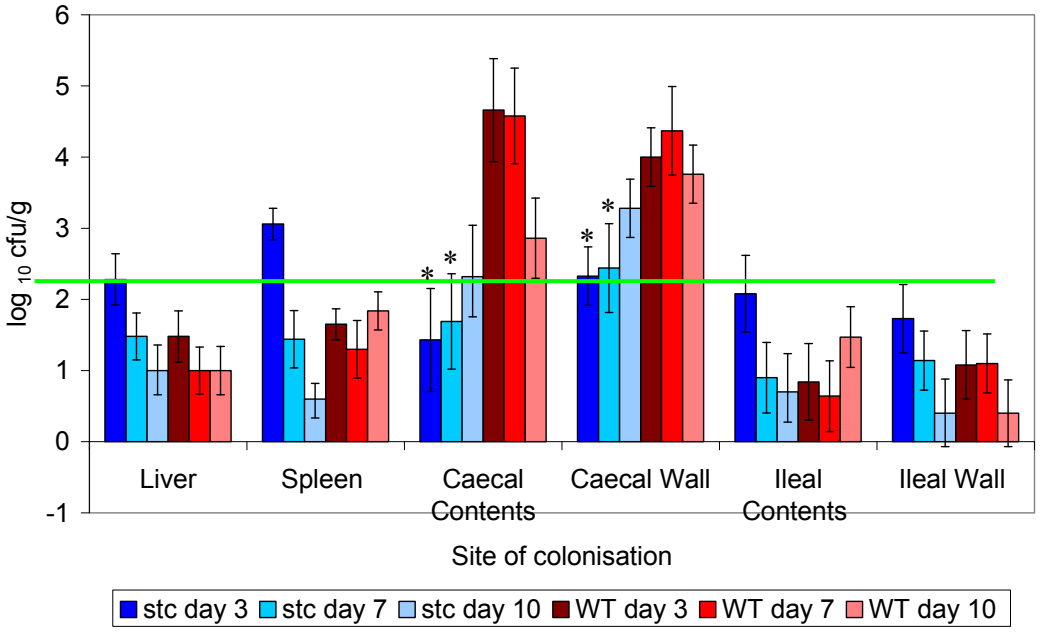
Despite the lower number of bacteria in the caeca of birds infected with *S.* Enteritidis S1400 wild-type compared with *S.* Enteritidis P125109 wild-type, the Δ*stcA::cat* of *S.* Enteritidis S1400 was recovered at approximately 2 logs cfu/g lower than the wild-type strains at 3 and 7 dpi ($P<0.05$), however caecal loads were comparable for the wild-type and Δ*stcA::cat* mutant at 10 dpi.

6.6. Comparison of the colonisation of other enteric and systemic sites by the Δ*stcA::cat* mutants of *S.* Enteritidis P125109 and S1400

As a significant difference was seen with the Δ*stcA::cat* mutant in the caeca, bacterial counts from other sites were examined and are shown in Figure 6.5. With *S.* Enteritidis S1400 wild-type and Δ*stcA::cat* mutant strains bacterial recoveries from the liver, spleen, ileal contents and ileal wall bacterial counts approached the limits of detection by direct plating, indicated by the green line in Figure 6.5a.

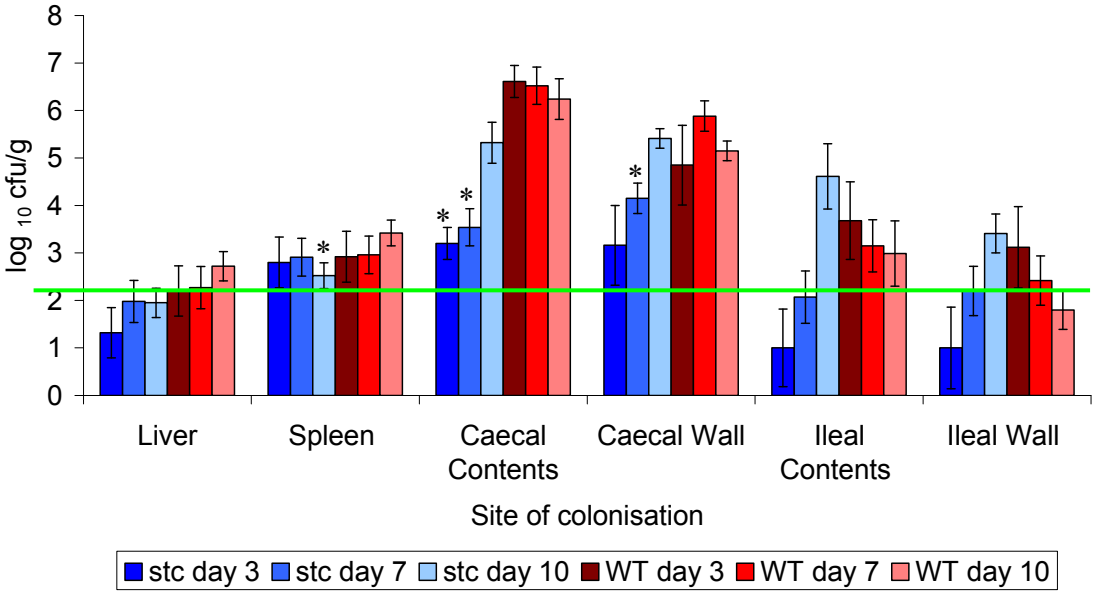
For the *S.* Enteritidis P125109 wild-type and Δ*stcA::cat* mutant, there is little difference between the bacterial counts in the liver, spleen and ileal contents Figure 6.5b. In this background an estimate of viable bacteria per gram of tissue could be derived by direct plating and therefore may be considered a more reliable indicator of bacterial load. Recoveries of the *S.* Enteritidis P125109 Δ*stcA::cat* mutant from the ileal contents and wall at 3 dpi were higher than the wild-type (P values of 0.06 and 0.1 respectively) but by 7 and 10 dpi comparable numbers of bacteria could be recovered from this site.

Figure 6.5a. Colonisation of enteric and systemic sites by *S.* Enteritidis S1400 wild-type and Δ*stcA::cat* fimbrial mutant strains at intervals after oral inoculation of 18-day-old Rhode Island Red chickens



The green line indicates the limit of detection; all samples below this line were obtained via enrichment. N = 5. The error bars are standard error of the mean SEM. F test analysis was carried out and P values below 0.05 were considered significant and are marked with an astrix. At 3 dpi in the caecal contents the P = 0.0075 and at 7 dpi P = 0.0078 and in the caecal wall at 3 dpi P = 0.0078 and at 7 dpi in the caecal contents the P value is 0.045.

Figure 6.5b. Colonisation of enteric and systemic sites by *S.* Enteritidis P125109 wild-type and Δ*stcA::cat* fimbrial mutant strains at intervals after oral inoculation of 18-day-old Rhode Island Red chickens



The green line indicates the limit of detection; all samples below this line were obtained via enrichment. N= 5. The error bars are standard error of the mean SEM. F test analysis was carried out and P values below 0.05 were considered significant and are marked with an astrix. At 10 dpi in the spleen P = 0.03, at 7 dpi in the caecal contents P = <0.0001 and in the caecal wall P = 0.0015 and at 3 dpi in the caecal contents the P value is <0.0001.

6.7. Removal of the chloramphenicol resistant cassette.

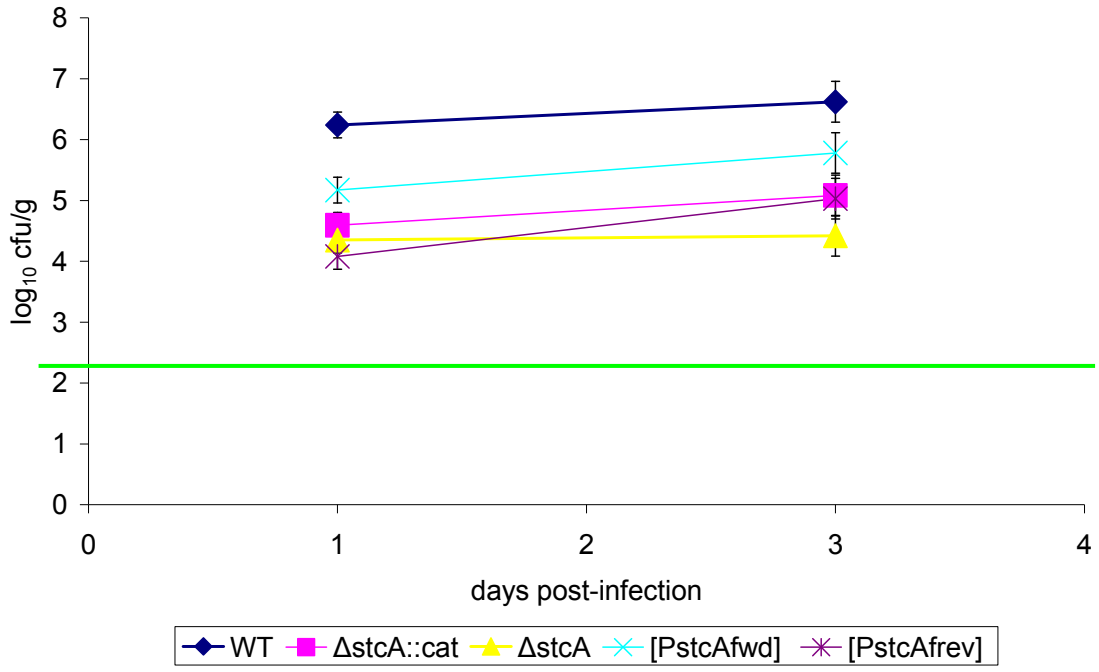
The chloramphenicol resistance cassette was removed from the Δ*stcA::cat* fimbrial mutant to investigate if the phenotype was due to the mutation of the major fimbrial subunit or due to polar effects on the expression of downstream genes. This was carried out by transient expression of the flippase recombinase from plasmid pCP20 as described in Section 2.5.5 and Chapter 4, Figure 4.3 (Datsenko and Wanner, 2000).

The removal of the chloramphenicol resistance cassette was confirmed by negative selection on LB agar supplemented with chloramphenicol and by PCR using the stcAFOR and stcAREV primers flanking the gene of interest.

6.8. *Trans*-complementation of the *S.* Enteritidis P125109 Δ*stcA::cat* fimbrial mutant

The *stcA* gene was cloned on to the pACYC177 plasmid in both orientations, on and off and both were electroporated into *S.* Enteritidis P125109 Δ*stcA::cat. S.* Enteritidis P125109 wild-type, Δ*stcA::cat* mutant, Δ*stcA* mutant, Δ*stcA::cat* [pstcAfwd] and Δ*stcA::cat* [pstcArev] were cultured for 16-18 hours at 37 ºC and those strains carrying the plasmid were supplemented with ampicillin to maintain the plasmid during growth. These cultures were used to orally inoculate 18-day-old Rhode Island Red chickens, with approximately $5x10^9$ cfu/ml. *Necropsy* examinations were performed at 1 and 3 dpi as the Δ*stcA::cat* phenotype was most pronounced at early time points and plasmid stability may be an issue owing to the absence of antibiotic selection *in vivo.*

Figure 6.6. *Trans*-complementation of the Δ*stcA*::cat fimbrial mutant



WT is the *S*. Enteritidis P125109 wild-type, the green line indicates the limit of detection via direct plating. The error bars represent the standard error of the mean. Each point is the average of 5 birds per group.

The removal of the chloramphenicol resistance cassette from *S*. Enteritidis P125109 Δ*stcA::cat* mutant did not significantly alter the total caecal load at either time point (P values were 0.27 and 0.648). The caecal load of both the Δ*stcA::cat* and Δ*stcA* were approximately two orders of magnitude lower than the parent strain at both time points indicating the original phenotype is not likely due to polar effects of the insertion. The Δ*stcA::cat* mutant exhibited the same degree of attenuation as observed in previous experiments. At 1 dpi P = <0.0001 and at 3 dpi P = 0.0002 (Figure 6.2 and 6.5).

Introduction of pstcArev into the Δ*stcA::cat* mutant resulted in total caecal counts that that were comparable to the Δ*stcA* fimbrial mutants both with and without the chloramphenicol resistance cassette and was significantly different to the wild-type at 1

dpi (P= 0.0005) and 3 dpi (P= 0.024). Introduction of the pACYC177-derived plasmid containing the Δ*stcA::cat* [pstcAfwd] partially restored the ability of the mutant strain to colonise the caeca at both time points. The inability of the pstcAfwd replicon to fully restore colonisation to wild-type levels may reflect differences in the expression level of the fimbrial subunit and/or the fitness cost of maintaining the plasmid since the pstcArev exerted a slightly inhibitory effect at least at 1 dpi. The stability of the plasmid was not examined *in vivo* herein but has previously been examined in mice with no effect on the virulence of the strain examined (Knodler et al., 2005).

6.9. Discussion

There are limited studies available that use *S.* Enteritidis P125109 to examine the colonisation of chickens (Atterbury et al., 2007) and the role of candidate virulence factors and it was used here to link phenotype to genotype. Therefore, it was important that the pilot experiment was carried out to confirm that this strain of *S.* Enteritidis was able to colonise birds of the age and breed used in this study at a level that permited a robust statistical analysis. Chapter 3, highlighted differences that can occur between two strains of the same serovar and it cannot be assumed that because one strain of *S.* Enteritidis colonises the avian alimentary tract that all strains will be able to do so at a comparable level.

S. Enteritidis P125109 colonised the caeca at levels that were anticipated for *S.* Enteritidis in outbred birds of this age (Atterbury et al., 2007) and *S.* Enteritidis S1400 colonised at lower levels than P125109 but these were consistent with the levels reported in the literature (Carroll et al., 2004). Swabbing was not undertaken as an initial colonisation rate was to be assessed not the rate of clearance as would have been measured with swabbing.

The role of several major fimbrial subunit genes (*fimA, sefA* and *csgA)*, has been examined *in vivo* and the data obtained here cocncurred with previous studies as no differences were seen in the colonisation of the caeca (Rajashekara et al., 2000). In previous studies a mutation in the *sefA* gene of *S.* Enteritidis resulted in reduced numbers of bacteria in the liver and a faster clearance rate from the spleen. This phenotype was not observed here however bacterial recoveries from these sites were close to the limits of detection by direct plating (Rajashekara et al., 2000).

In STM screens, a role for the *stbC, sthB* and *csgD* fimbrial genes was inferred from the attenuation of *S.* Typhimurium miniTn5Km2 mutants in 14-day-old Light Sussex chickens (Morgan et al., 2004). However, single defined mutants were not made for these genes to confirm the phenotype and no role for the *sthA* or *csgA* genes was identified in this study. The role of the remaining fimbrial genes annotated herein in the avian host has not been previously described in the literature.

The Δ*stbA::cat* mutant of *S.* Enteritidis P125109 exhibited a significant reduction in the total caecal load at 7 and 10 dpi compared to the parent strain (P = 0.0081 and 0.03 respectively). The *stbA* gene is unlikely to be involved in the initial colonisation but may be required for long term carriage of *Salmonella.* Further studies using non-polar mutations and *trans*-complementation strains are required to confidently establish a role for Stb encoded fimbriae in the avian host.

The Δ*stcA::cat* mutant exhibited a significant reduction in bacterial numbers at 3 and 7 dpi (P = 0.0006 and 0.0002). Several fimbrial mutants exhibited a reduction in colonisation compared with the wild-type at sites other than the caeca, however in many cases the bacterial counts were below the limits of detection by direct plating making it difficult to assess a phenotype with confidence.

Several studies have suggested that fimbriae play a role in the adhesion and invasion of specific cells and *in vitro* studies within this project (Chapter 5) have indicated that the *lpfA, steA, safA, sefA, stiA* and *fimA* genes influence adherence to or invasion of specific cell lines. The differences identified *in vitro* did not correlate with differences identified *in vivo.* Thus, mutants that exhibited attenuation in chickens *stbA, stcA* and *bcfA* showed wild-type levels of adhesion and invasion in CKC, HEp-2 and HD11 cells. This highlights the importance of assessing mutant phenotypes in relevant animal models wherever feasible.

The majority of fimbrial subunits played either a very subtle role or no role at all in the colonisation of the caeca (P values greater than 0.05). However, more statistically significant differences may have been identified if bird-to-bird variation was decreased. All chickens were orally given 0.1 ml of adult gut-flora on the day of hatch to provide comparable microbiota and competitition in the gut for adherence sites as would be seen in a natural infection. The microfloras composition was not confirmed by FISH or DGGE but with two different gut microflora and two different strains of *S*. Enteritidis the same attenuating phenotype was seen.

Inbred chicken lines of defined heritable resistance or susceptibility to *Salmonella* could be used in future studies; however it was considered that out-bred birds provided an industry-relevant model in which to evaluate the role of fimbriae. The use of bicarbonate could also be used to neutralise the stomach acid and allow a higher proportion of bacteria to enter the host and subtle differences may become pronounced, unfortunately the side effct of this is that some chickens can receive an extremely high dose of *Salmonella* (personnal communication R.M. LaRagione).

In the mouse model, it has been proposed that a compensation mechanism exists, whereby a single fimbriae may compensate for loss of another. Deletions in the *lpf, pef, fim* and *csg* operons individually only moderately impair mouse virulence, but a quadruple mutation resulted in a 26-fold increase in the LD_{50} and a reduced ability to colonise the intestinal lumen for *S*. Typhimurium (van der Velden et al., 1998). Given the repertoire of *S*. Enteritidis P125109 fimbrial operons there is no reason no believe that such functional redundancy does not also apply to the chicken model and further studies with strains harbouring multiple mutations may be warranted. The use of microarray analysis of to examine the expression of genes *in vivo* in a fimbrial mutated

strain may indicate areas of compensation provided probes are used to discriminate between fimbrial loci in the absence of cross-hybridisation.

A mutation in the *stcA* fimbrial subunit gene produced the most attenuating phenotype of all of the *S.* Enteritidis fimbrial mutants examined *in vivo,* yet no phenotype was identified *in vitro* for the same mutant. As the same pattern of colonisation of the caeca was seen with both strains of *S.* Enteritidis it is unlikely that secondary mutations have occurred that produced a comparable phenotype in two independently constructed mutants. It should be noted that the region flanking the Δ*stcA::cat* mutation yielded two different restriction fragment sizes in *S.* Enteritidis S1400 and *S.* Enteritidis P125109 in Southern blots (Chapter 4). The predicted size was based on the genome sequence of *S.* Enteritidis P125109 and the actual fragment size seen in this strain was approximately the same size. The removal of the chloramphenicol resistance cassette did not alter the phenotype seen *in vivo* and it is unlikely that a polar effect on the expression of downstream genes has occurred. Introduction of *stcA* on a plasmid in an orientation that permits its expression partially restored the colonisation phenotype of the *S.* Enteritidis P125109 Δ*stcA::cat* mutant, fulfilling molecular Kochs postulate.

Interestingly, the *stc* operon nucleotide sequence exists as 2 different variants consisting of different DNA sequences (Table 3.1). *S.* Enteritidis P125109 and *S.* Gallinarum 287/91 possess one sequence which is highly conserved between these two serovars with all genes being >99% identical. *S.* Typhi CT18, *S.* Typhimurium LT2 and *S.* Choleraesuis SC-B67 possess a different nucleotide sequence that is also highly conserved >99%. However, there is limited identity (58-66 %) between these two variants. *S.* Gallinarum 287/91 also possesses a predicted pseudogene in the chaperone of this operon yet all other serovars appear to maintain a functional gene. It would be of

interest to examine the *S*. Gallinarum 287/91 *stcA* gene to determine if the operon plays a role in virulence and mediates an important phenotype even with a putative pseudogene. It would also be of interest to examine a serovar possessing the different sequence or to clone the different sequence into *S*. Enteritidis P125109 to confirm if the phenotype is due to the *stc* operon sequence or whether the variation in sequence produces the same or different effects, as point mutations have been shown to play significant roles *in vitro* (Boddicker et al., 2002). *S*. Typhimurium strains carrying deletions in the *stc* operon were recovered at significantly reduced numbers from the faeces of mice (Weening et al., 2005) but have not been studied in chickens.

Chapter 7

General discussion

The aim of this study was to annotate and characterise fimbrial genes of the poultry associated ubiquitous *Salmonella enterica* serovars Enteritidis and host-specific *S.* Gallinarum. This enabled the entire genome sequence to be analysed and allowed the potential for a link between phenotype and genotype to be established. This was done to determine whether the repertoire or sequence of the fimbrial operons influences the outcome of infection and may help explain the differential tissue and host tropisms of serovars of *S. enterica.* Three approaches were used; *in silico* analysis of genome sequences available at the time of writing, *in vitro* analysis of defined wild-type, fimbrial mutant and *trans*-complemented strains and *in vivo* analysis of the phenotype of such strains in a chicken model of infection. In this way, this study compares and correlates genotype to phenotype and the two strains used were selected solely for this purpose as the strains were previously uncharacterised.

The *in silico* analysis compared the genome sequences of strains representing ubiquitous, host-restricted and host-specific *Salmonella* serovars. The *in silico* analysis included mainly one strain from each serovar and it is uknown if these strains are representative of a particular serovar. It must be considered that conclusions made from the genome sequences are limited.

Using a range of bioinformatic tools, primarily ACT and Artemis thirteen fimbrial operons were identified in *S.* Enteritidis P125109 and twelve in *S.* Gallinarum 287/91. Although all of the fimbrial loci in *S.* Enteritidis P125109 were predicted to encode intact genes, eight of the identified operons in *S.* Gallinarum were predicted to contain pseudogenes. Whilst no single fimbrial locus was associated with host-specificity, the mutational attrition seen in the fimbrial operons may account for the restricted host range available to host-restricted and host-specific serovars. The genomes sequences of *S.* Typhi CT18 and *S.* Gallinarum 287/91 contain a large number of pseudogenes

throughout accounting for 4-7 % of the genome whilst in fimbrial operons this increases to 14-16 % and is unlikely to be the result of a random distribution of pseudogenes. Genome decay has been associated with the host restriction of agents other than *Salmonella* and it is possible that the loss of functional fimbriae may partially restrict the niches that may be colonised (Holden et al., 2004, Nierman et al., 2004, Sebaihia et al., 2006).

Having predicted putative major fimbrial subunit genes in both *S*. Enteritdis P125109 and *S*. Gallinarum 287/91 a total of 25 defined mutants were constructed using the lambda Red system for homologous recombination in these two strains. (Datsenko and Wanner, 2000). The location of all fimbrial mutations was confirmed by PCR using primers flanking the targeted gene. Where possible, mutations were transduced using bacteriophage P22 HT/int into an archived strain to eliminate the effects of any rare second-site defects. Five fimbrial mutations could not be transduced, therefore *fimA, safA,* and *steA* remained in *S*. Enteritidis S1400 and were compared to this wild-type strain throughout this study. The bacteriophage P22 HT/int can also move 40 kb of DNA and this may vary between the two strains of *S*. Enteritidis used.

All working strains were maintained on LB agar slopes whereas archive strains were stored in LB glycerol stocks at -70 ºC. This ensures no cross-contamination and no changes such as spontaneous mutations will have occurred to the bacteria at this temperature for long periods of time. The growth kinetics of the fimbrial mutants were also analysed and the insertion of the chloramphenicol resistance cassette was not found to adversely affect growth.

The validated fimbrial mutants were used for adherence and invasion assays with CKC, HEp-2 and HD11 cell lines. These *in vitro* assays indicated that the *S*. Enteritidis *stbA, steA, stiA, lpfA, safA* and *sefA* encoded fimbrial subunits play a role in adherence

to and invasion of CKC only at 25 ºC and the *steA, fimA* and *safA* fimbrial subunits play a role in adherence to and invasion of HEp-2 cells. In *S*. Gallainrum more fimbriae played a significant role than in *S*. Enteritidis; the *stbA, stcA, stiA, bcfA, sthA, fimA, lpfA* and *sefA* genes influenced adherence to and invasion of CKC, *safA* and *sefA* influenced adherence to and invasion of HEp-2 cells and *bcfA, fimA* and *lpfA* played a role in the uptake into HD11 cells.

In previous studies, the *sefA, csgA* and *fimA* genes have been widely studied *in vitro* in *S*. Enteritidis and *S*. Typhimurium. In *S*. Enteritidis, *fimA* was not required for adherence to chick gut explant, or adherence to HEp-2 cells (Allen-Vercoe and Woodward, 1999b, Baumler et al., 1996a) but in *S*. Typhimurium *fimA* was involved in adherence to and invasion of HeLa cells, HEp-2 cells and porcine enterocytes (Baumler et al., 1996a, Baumler et al., 1997b, Dibb-Fuller et al., 1999, Ernst et al., 1990). In this study, *fimA* played a role in *S*. Enteritidis infection of HEp-2 cells and in *S.* Gallinarum infection of CKC, indicating that strain-, serovar- and cell-line specific effects may exist. The *sefA* encoded fimbrial subunit of *S*. Enteritidis has previously been reported not to be required for adherence to chick gut explant or cultured epithelial cells (Dibb-Fuller et al., 1999, Allen-Vercoe and Woodward, 1999b), in contrast to the findings here where it played a significant role in adherence to CKC in both serovars examined and in *S*. Gallinarum *sefA* also played a role in adherence or invasion of HEp-2 cells. The differences may be due to the cell-line examined. The *csgA* fimbrial subunit of *S*. Enteritidis played no role in adherence to chick gut explant or invasion of cultured epithelial cells (Allen-Vercoe and Woodward, 1999b, Dibb-Fuller et al., 1999) which agrees with this study. The remainder of the fimbrial loci have not previously been assigned roles in the litereature *in vitro* adherence or invasion and this report is likely to be the first to do so.

These studies identified a role for the *S*. Enteritidis *ste* operon in adherence and invasion. The removal of the chloramphenicol resistance cassette from the Δ*steA::cat* mutant resulted in the restoration of the wild-type characteristics which indicated that the phenotype was due to polar effects on the genes downstream of the *steA* gene. The entire *ste* operon was used to *trans*-complement the Δ*steA::cat* mutant and resulted in the partial restoration of the wild-type, therefore fulfilling molecular Kochs postulates.

Mutations in the same fimbrial operon in the host-specific and the ubiquitous serovars influenced adherence and invasion to differing extents when compared to their respective wild-type strains. Despite many of the *S*. Gallinarum fimbrial operons containing predicted pseudogenes, significant decreases and increases in adherence and invasion were still apparent compared with the wild-type (Table 5.7). This suggests that the presence of a pseudogene does not always affect the functionality of the other genes encoded in the same operon and has been previously shown for the *stg* fimbriae of *S*. Typhi (Forest et al., 2007). This may indicate that the products encoded by such loci contribute to the assembly of fimbriae partially encoded by other distal loci or may induce the expression of other genes (Gerlach et al., 2007b).

It is unclear why differences exist between the role of the same fimbriae of *S*. Gallinarum 287/91 and *S*. Enteritidis P125109, but maybe due to the method used or point mutations between the fimbriae as has been previously demonstrated (Boddicker et al., 2002). The expression of *fimA* from *S*. Typhimurium in *S*. Gallinarum increased adherence to mammalian cells (Wilson et al., 2000). The serovar differences could be due to different co-operation systems existing because of a difference in the repertoire of fimbrial operons. A fimbrial mutation in one serovar results in the up-regulation of several fimbrial operons or other genes. It has been previously shown that *fimU* of *S*. Enteritidis is capable of regulating the expression of two fimbrial subunits in distinct

regions of the genome, FimA and SefA (Clouthier et al., 1998b) and the CsgD promoter in *S.* Typhimurium is under the control of a global regulator which can have wider implications for the expression of a range of genes, including the production of O antigens and components of T3SS (Gerstel et al., 2003, Gibson et al., 2006).

Differences observed *in vitro* may have been due to the growth conditions. The expression of *S.* Enteritidis and *S.* Gallinarum 287/91 curli, Fim and Sef fimbriae has been shown in this study to depend upon the growth conditions and the regulation may subtley differ between strains and serovars. There is no published data on fimbriae expression in *S.* Gallinarum (Edwards et al., 2001, Dibb-Fuller et al., 1997, Walker et al., 1999, Humphries et al., 2003, Woodward et al., 2000).

It is possible that during bacteriophage transduction unwanted traits were transferred as whilst the *S.* Gallinarum 287/91 archived strain could be used, two different *S.* Enteritidis strains were used as recipients of the mutagenic cassette at the λ Red and transduction stages. Therefore the phenotypes seen in *S.* Enteritidis P125109 could be due to additional genomic material being moved between the two strains. During transduction the timing was limited so that only one round of bacterial replication could occur. This ensures that only one insert is encorporated into the bacteria. The P22 phage were grown prior to use in *S.* Enteritidis or *S.* Gallinarum wild-type cultures to ensure that they contained only components of the *Salmonella* genome.

However, the most significant phenotype both *in vitro* and *in vivo* was successfully *trans*-complemented and this would need to be carried out to confirm all fimbrial mutant phenotypes. The *in vitro* analysis was carried out at 37 ºC not at 42 ºC (the clocal temperature of a chicken) and it is therefore clear that the cell culture assays will not mimic this aspect of the host environment. It would not have been possible to

compare the HEp-2 cell lines with the CKC and HD11 as the human epithelial cell line will not tolerate a high temperature.

Colonisation of chickens with the fimbrial mutants and their cognate wild-type strains showed that the *stbA* and *stcA* fimbrial mutants of *S.* Enteritidis were significantly attenuated in colonisation of the chicken caeca. This suggests that *stcA* plays a role in the initial colonisation of the chicken and *stbA* may be important during later stages of infection. Interestingly, *stcA* was identified by *in silico* analysis using ClustalW as occurring in two different variations at the nucleotide level in different serovars and it may be that variation in sequence that influences the *in vivo* phenotype. The Δ*stcA::cat* was not found to be important for adherence or invasion of any of the cell lines examined. This shows that the data obtained *in vitro* cannot be used to give an accurate indication as to the events *in vivo* in contrast to other reports (Baumler et al., 1996a, Baumler et al., 1996b). The expression of fimbriae has been previously shown to vary considerably *in vitro* to *in vivo* (Humphries et al., 2003) and this may partially explain the differences. One cannot preclude the possibility that the fimbrial mutations elicit subtle effects on colonisation that cannot be detected owing to animal-animal variability and the sensitivity of bacterial detection. One way to reduce variation may be to examine mutant phenotypes in co-infection studies and to derive a competive index relative to the parent strain and/or to construct multiple mutants. It would be of interest to examine other *stc* variants e.g. in *S.* Typhimurium (Turner et al., 1998, Morgan et al., 2004). Competitive index (CI) studies were considered where both the wild-type and the mutant strain are given to the chicken and they compete against each other. This idea was dismissed due to several problems that have been highlighted in our laboratory and others. For competitive index studies to be successful high doses of bacteria are required along with a high number of animals to reduce the variation that

occurs in order to produce an accurate CI value. It is possible that in vivo the mutant strain maybe complemented by the wild-type, reviewed (Beuzon and Holden, 2001).

None of the *S.* Enteritidis fimbrial mutants showed any difference in their growth kinetics *in vitro* in LB, implying that the differences seen in the caeca are likely due to differences in their ability to colonise and survive in the alimentary tract as opposed to defects in fitness. However, the different rates of growth were not measured in different media e.g. minimal media supplemented with or without appropriate nutrients. A growth curve in these conditions may have produced different results as LB is nutrient rich. Bacteria in the gut are likely to be exposed at some point to a lack of nutrients and the different fimbrial mutations may have produced different growth rates. This may also have shown a direct or indirect role for fimbriae in the utilisation of specific nutrients.

Those fimbriae expressed *in vitro* and not expressed *in vivo* may be phase variable and this has been proposed as a mechanism to explain the degree of functional redundancy that occurs in the fimbrial operons of *Salmonella* (Humphries et al., 2001). In *S.* Typhimurium the *lpf* operon undergoes phase variation to avoid cross immunity. Different serovars of *Salmonella* express the same fimbriae which would prevent them colonising the same host therefore one fimbriae is switched off and others are switched on or up-regulated (Nicholson and Baumler, 2001). The differences in the role of fimbriae *in vivo* compared with *in vitro* may be partially due to phase variation dicatated by the environmental conditions *in vivo* (Norris et al., 1998).

Several *in vivo* screens have implicated fimbriae in the colonisation of mice. The *stcC*, *stcD* and *fimY* of *S.* Typhimurium have been implicated in a microarray-based negative selection screen as being involved in the colonisation of mice along with *bcfD, safB, fimI* and *csgA* in long-term infections (Lawley et al., 2006). The *lpf and fim*

operons of *S*. Typhimurium have been implicated in mouse colonisation and several fimbriae appear to act in a synergistic manner (van der Velden et al., 1998) as well as the *lpf, bcf, stb,stc, std* and *sth* fimbrial operons (Weening et al., 2005). In chickens much fewer fimbriae have been proposed to be involved in colonisation. In a STM screen of *S*. Typhimurium *in vivo* the *stbC, csgD* and *sthB* were implicated in colonisation of chickens (Morgan et al., 2004) although single defined mutants or *trans*-complementation were not carried out. Several *in vivo* screens using random mutants of *S*. Typhimurium did not implicate any of the major fimbrial subunits as playing a role in the colonisation of the chicken as was identified in this study using targeted mutants (Morgan et al., 2004, Turner et al., 1998). It is important to note however that some phenotypes may be age-dependent. In several reports *fimA* plays no role in the colonisation of chickens by *S*. Enteritidis (Allen-Vercoe et al., 1999, Rajashekara et al., 2000, Thorns et al., 1996) however in laying hens, *fimA* appears to have an effect on colonisation of the gut (Thiagarajan et al., 1996). For *S*. Pullorum infections it has been shown that a drop in immunity occurs at sexual maturity (Wigley et al., 2005)The differences maybe due to the fact in older birds there will be a more developed gut flora to compete with fimbriae for specific receptors, the birds may have been pre-exposed to *Salmonella* and/or specific fimbriae and the birds immune system will be more developed (Beal et al., 2005, Beal et al., 2006b).

Further potential exists for strain-specific effects as studies of *S*. Enteritidis in mice found that in one strain *sefA* played a role in colonisation and yet in a different strain of the same serovar *sefA* did not play any role (Ogunniyi et al., 1997).

The fimbrial proteins, in particular the StcA protein may be a target for vaccine development as it could generate a significant immune response and is surface expressed. *S*. Typhimurium fimbriae serve as antigens during infection in mice

(Humphries et al., 2005) and the SefA fimbrial proteins has elicited an immune response against *S.* Enteritidis in chickens (Lopes et al., 2006). *Salmonella* fimbriae may have a potential role in future vaccine development (Thorns, 1995).

Although this study indicated a role for the *stcA* gene in colonisation of chickens experimental infections are artificial, involving a one-off high does which is unlikely to occur in nature. Chickens are more likely to be exposed to lower doses of *Salmonella* over a longer period of time and although the oral route of infection is believed to mimic the natural infection, it would be of interest to examine if other fimbriae play a more significant role in colonisation if administered via other routes of infection e.g. aerosol transmission or intra-cloacally particularly in relation to oviduct and egg colonisation where it is a challenge to examine oviduct colonisation due to the rare occurrence in artificial models.

For at least one of the fimbrial genes (*stcA)* I have defined a significant role in the colonisation of chickens and a role for the *ste* operon has been identified *in vitro.* Both of these phenotypes have been partially restored by the presence of the gene or operon on a plasmid there by confirming the phenotype. This project has combined sequence and function analysis which is an important step in the post-genomic era.

The next step within this project would to mutate more than one fimbrial operon in a strain and to carry out a systematic approach to examine different combinations of fimbriae, in different aged birds and different species. Although several parts of the gut were examined only the caeca proved to be reliably colonised at a level that permits a robust statistical analysis to detect attenuation. Analysis of the *in vivo* expression levels with the fimbrial mutants either singularly or with multiple mutants would provide an insight into the compensation systems, regulatory systems and the degree of cross-talk that exists within fimbrial operons. Microarray analysis of *S.* Typhimurium gene

expression in macrophages has indicated that *fimA* expression is downregulated whilst *csgA* is upregulated, the relevance of this *in vivo* is currently unknown (Eriksson et al., 2003). Further research into the role of fimbriae would also benefit from the production of a complete set of antibodies for all fimbrial subunits which would allow detection of fimbriae via Western blots, confocal microscopy and electron microscopy to examine their morphology. Significant potential also exists for analysis of the large commendium of microarray data that exists at Instiute for Food Research relating to *Salmonella* gene expression under many different environmental conditions and in the absence of several global regulators. This may then allow for analysis of components that are involved in the regulation of fimbriae expression. To develop further the idea that fimbriae could be used as vaccines we would need to examine the host immune response triggered by purified fimbrial subunits or possible other components of fimbriae.

Chapter 8

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Chapter 9

Appendices

Appendix 2.1. Perl Script to extract and draw specific regions of the genome with CDS, Pfam domains and miscellaneous features labelled

```
use lib "/usr/local/bioperl-1.4";
use Bio::SeqIO;
use Bio::Graphics;

my $gbk   = $ARGV[0];
my $start = $ARGV[1];
my $stop  = $ARGV[2];

my $in    = Bio::SeqIO->new(-file => "$gbk", -format => "embl");
my $seq   = $in->next_seq;

my @features = $seq->all_SeqFeatures;
my @GENES = grep [$_->primary_tag eq 'gene'] @features;
my @CDS      = grep [$_->primary_tag eq 'CDS']  @features;
my @MF       = grep [$_->primary_tag eq 'misc_feature']  @features;

my $panel = &get_panel($start,$stop);

print $panel->png();

sub get_panel {
      my $start = shift;
      my $stop  = shift;

      my $panel = Bio::Graphics::Panel->new(-key_style => between,
                                            -offset    => $start,
                                            -length    => $stop - $start + 1,
                                            -width     => 800
                                            );
      # draw the genome arrow
```

```
my $full_length = Bio::SeqFeature::Generic->new(-start  => $start,
                                                -end    => $stop,
                                               );
$panel->add_track($full_length,
                  -glyph       => 'arrow',
                  -label       => 1,
                  -tick        => 2,
                  -fgcolor     => 'black',
                  -double      => 1,
                  -label       => 1,
                  -font        => 'gdSmallFont',
                  );

# draw the gene features
$panel->add_track(\@GENES,
                  -glyph       => 'transcript2',
                  -description => undef,
                  -bump        =>  1,
                  -font        => 'gdSmallFont',
                  -fontcolor   => 'red',
                  -font2color  => 'blue',
                  -bgcolor     => 'blue',
                  -fgcolor     => 'black',
                  -key         => "Genes",
                  -label       => \&gene_label,
                  );

# draw the CDS features
$panel->add_track(\@CDS,
                  -glyph       => 'transcript2',
                  -description => undef,
                  -bump        =>  1,
                  -font        => 'gdSmallFont',
                  -fontcolor   => 'red',
```

```
                        -font2color  => 'blue',
                        -bgcolor     => 'red',
                        -fgcolor     => 'black',
                        -key         => "CDS",
                        -label       => \&gene_label,
                        );

    # draw the misc_features
    $panel->add_track(\@MF,
                        -glyph       => 'transcript2',
                        -bump        =>  1,
                        -font        => 'gdSmallFont',
                        -fontcolor   => 'red',
                        -font2color  => 'blue',
                        -bgcolor     => 'green',
                        -fgcolor     => 'black',
                        -key         => "Misc Feature",
                        -description => \&mf_label,
                        );
    return $panel;
}
sub gene_label {
  my $feature = shift;
  my @notes;
  foreach (qw(gene locus_tag)) {
    next unless $feature->has_tag($_);
    @notes = $feature->each_tag_value($_);
    last;
  }
  $notes[0];
}

sub mf_label {
  my $feature = shift;
```

```
  my @notes;
  foreach (qw(note)) {
    next unless $feature->has_tag($_);
    @notes = $feature->each_tag_value($_);
    last;
  }
  $notes[0];
}
```

Appendix 3.1. Putative homo-polymeric tracts in the *S.* Enteritidis P125109 genome sequence

Homo-polymeric repeats non-divisible by 3 at the beginning of a fimbrial gene were noted. Only A's and C's were searched for as obviously homo-polymeric tracts consisting of T's or G's will complement those identified from A's and C's. The region of the homo-polymeric tracts were noted along with the gene they were present in.

Repeats	Location	Gene
5A	24238-24242	*bcfA* 24235-24777
4A	24339-24342	*bcfA* 24235-24777
8A	24881-24888	*bcfB* 24878-25564
4A	24897-24900	*bcfB* 24878-25564
7A	25047-25053	*bcfB* 24878-25564
4C	25627-25630	*bcfC* 25569-28190
4C	25664-25667	*bcfC* 25569-28190
4A	25582-25585	*bcfC* 25569-28190
4A	25675-25678	*bcfC* 25569-28190
4A	25737-25740	*bcfC* 25569-28190
4A	28194-28197	*bcfD* 28191-29198
4A	28204-28207	*bcfD* 28191-29198
5A	28266-28270	*bcfD* 28191-29198
4A	28327-28330	*bcfD* 28191-29198
6A	28357-28362	*bcfD* 28191-29198
5A	29221-29225	*bcfE* 29199-29744
4A	29887-29890	*bcfF* 29760-30278
4A	29928-29931	*bcfF* 29760-30278
4A	29963-29966	*bcfF* 29760-30278
4C	30380-30383	*bcfG* 30244-30975
5A	30283-30287	*bcfG* 30244-30975
4A	30443-30446	*bcfG* 30244-30975
4A	31056-31059	*bcfH* 31039-31884
5A	31231-31235	*bcfH* 31039-31884
4C	31130-31133	*bcfH* 31039-31884
4A	232499-232502	*stfA* 232485-233045
4A	232580-232583	*stfA* 232485-233045
4A	232903-232906	*stfA* 232485-233045
6A	233143-233148	*stfC* 233131-235788
4A	233204-233207	*stfC* 233131-235788

4A	233287-233290	*stfC* 233131-235788
4C	233313-233317	*stfC* 233131-235788
4C	236087-236090	*stfD* 235806-236558
5C	236111-236115	*stfD* 235806-236558
4A	235817-235820	*stfD* 235806-236558
4A	235840-235843	*stfD* 235806-236558
4A	235849-235852	*stfD* 235806-236558
5A	235947-235951	*stfD* 235806-236558
4C	236766-236769	*stfE* 236577-237089
5C	237115-237119	*stfF* 237086-237562
7A	237089-237095	*stfF* 237086-237562
4A	237242-237245	*stfF* 237086-237562
4A	237568-237571	*stfG* 237562-238092
5A	237651-237655	*stfG* 237562-238092
5A	237720-237724	*stfG* 237562-238092
4C	237610-237613	*stfG* 237562-238092
4A	321762-321765	*safA* 321747-322256
7A	321770-321776	*safA* 321747-322256
5A	321831-321835	*safA* 321747-322256
4A	321886-321889	*safA* 321747-322256
4A	322343-322346	*safB* 322340-323077
4A	322354-322357	*safB* 322340-323077
4A	322359-322362	*safB* 322340-323077
4A	322536-322539	*safB* 322340-323077
4C	323209-323212	*safC* 323101-325611
5C	323235-323239	*safC* 323101-325611
4A	325642-325645	*safD* 325633-326103
4A	325657-325660	*safD* 325633-326103
5A	325727-325731	*safD* 325633-326103
4C	325753-325756	*safD* 325633-326103
4C	210492-210495	*stiA* 210160-210699 c
5C	210643-210647	*stiA* 210160-210699 c
4A	210646-210467	*stiA* 210160-210699 c
4A	210518-210522	*stiA* 210160-210699 c
5A	210564-210568	*stiA* 210160-210699 c
4A	210581-210584	*stiA* 210160-210699 c
5A	210681-210685	*stiA* 210160-210699 c
5A	209876-209880	*stiB* 209429-210112 c
5A	210014-210018	*stiB* 209429-210112 c
4C	209917-209920	*stiB* 209429-210112 c

4A	209225-209228	*stiC* 206856-209414 c
4A	209281-209284	*stiC* 206856-209414 c
4A	209375-209378	*stiC* 206856-209414 c
4A	209393-209396	*stiC* 206856-209414 c
4A	206676-206679	*stiH* 205768-206847 c
6A	206726-206731	*stiH* 205768-206847 c
4A	206808-206811	*stiH* 205768-206847 c
4C	206721-206724	*stiH* 205768-206847 c
5A	358925-358929	*stbE* 358286-359044 c
4A	359019-359022	*stbE* 358286-359044 c
4A	360199-360202	*stbD* 359010-360335 c
4A	360219-360222	*stbD* 359010-360335 c
5A	360327-360331	*stbD* 359010-360335 c
4C	360429-360432	*stbD* 359010-360335 c
5C	362787-362791	*stbC* 360340-362901 c
4A	362758-362761	*stbC* 360340-362901 c
4A	362771-362774	*stbC* 360340-362901 c
4A	362809-362812	*stbC* 360340-362901 c
6A	363492-363497	*stbB* 362885-363646 c
4A	363549-363552	*stbB* 362885-363646 c
5A	363599-363603	*stbB* 362885-363646 c
4A	363640-363643	*stbB* 362885-363646 c
4C	364035-364038	*stbA* 363708-364244 c
4C	364148-364151	*stbA* 363708-364244 c
4A	364043-364046	*stbA* 363708-364244 c
6A	364233-364238	*stbA* 363708-364244 c
4C	584255-584258	*fimI* 584229-584762
4A	584263-584266	*fimI* 584229-584762
4A	584820-584823	*fimC* 584806-584762
4A	584993-584996	*fimC* 584806-584762
6A	585014-585019	*fimC* 584806-584762
7C	584877-584883	*fimC* 584806-584762
5A	585658-585662	*fimD* 585529-588141
5A	585704-585708	*fimD* 585529-588141
5A	588198-588202	*fimH* 588156-589163
4A	588261-588264	*fimH* 588156-589163
4A	588280-588283	*fimH* 588156-589163
5A	588328-588332	*fimH* 588156-589163
4C	588185-588188	*fimH* 588156-589163
4A	589189-589192	*fimF* 589173-589691

4A	589210-589213	*fimF* 589173-589691
4A	589286-589289	*fimF* 589173-589691
4A	589249-589352	*fimF* 589173-589691
4A	590267-590270	*fimZ* 589737-590369 c
5A	590293-590297	*fimZ* 589737-590369 c
4A	590305-590308	*fimZ* 589737-590369 c
4A	591477-591480	*fimY* 590973-591695 c
4A	591485-591488	*fimY* 590973-591695 c
4A	592690-592693	*fimW* 592187-592783 c
4A	592729-592732	*fimW* 592187-592783 c
4A	592755-592758	*fimW* 592187-592783 c
5C	2006388-2006392	*csgC* 2006216-2006542 c
5A	2006366-2006370	*csgC* 2006216-2006542 c
4A	2007045-2007048	*csgA* 2006604-2007059 c
4C	2006980-2006983	*csgA* 2006604-2007059 c
4A	2007362-2007365	*csgB* 2007101-2007556 c
4A	2007461-2007464	*csgB* 2007101-2007556 c
5A	2007552-2007556	*csgB* 2007101-2007556 c
4A	2008424-2008427	*csgD* 2008311-2008961
4A	2008477-2008480	*csgD* 2008311-2008961
6A	2009422-2009427	*csgF* 2009388-2009804
5A	2009505-2009509	*csgF* 2009388-2009804
4C	2009489-2009492	*csgF* 2009388-2009804
4C	2009546-2009549	*csgF* 2009388-2009804
4C	2009886-2009889	*csgG* 2009831-2010664
4C	2009921-2009924	*csgG* 2009831-2010664
4C	2010287-2010290	*csgG* 2009831-2010664
4A	2009847-2009850	*csgG* 2009831-2010664
4A	2009859-2009862	*csgG* 2009831-2010664
4A	2010041-2010044	*csgG* 2009831-2010664
5A	2247332-2247336	*stcD* 2246326-2247348 c
4A	2247342-2247345	*stcD* 2246326-2247348 c
4A	2249732-2249735	*stcC* 2247364-2249850 c
5A	2249818-2249822	*stcC* 2247364-2249850 c
4A	2249843-2249846	*stcC* 2247364-2249850 c
5A	2250365-2250369	*stcB* 2249879-2250559 c
6A	2250458-2250463	*stcB* 2249879-2250559 c
5C	2251070-2251074	*stcA* 2250621-2251154 c
4C	2251097-2251100	*stcA* 2250621-2251154 c
5A	2251061-2251065	*stcA* 2250621-2251154 c

4A	2251092-2251095	*stcA* 2250621-2251154 c
5A	2984449-2984453	*steA* 2984435-2985022
5C	2984559-2984563	*steA* 2984435-2985022
4C	2985172-2985175	*steB* 2985102-2987801
4A	2985120-2985123	*steB* 2985102-2987801
6A	2985227-2985232	*steB* 2985102-2987801
4A	2985258-2985261	*steB* 2985102-2987801
4A	2985275-2985278	*steB* 2985102-2987801
4A	2985295-2985298	*steB* 2985102-2987801
4A	2985306-2985309	*steB* 2985102-2987801
4A	2987831-2987834	*steC* 2987814-2988587
5A	2987923-2987927	*steC* 2987814-2988587
4A	2987935-2987938	*steC* 2987814-2988587
5A	2987961-2987965	*steC* 2987814-2988587
4A	2988631-2988634	*steD* 2988607-2989113
5A	2988687-2988692	*steD* 2988607-2989113
4A	2989131-2989134	*steE* 2989128-2989598
4A	2989153-2989156	*steE* 2989128-2989598
4A	2989256-2989259	*steE* 2989128-2989598
4A	3076986-3076990	*stdC* 3076396-3077139 c
4A	3077109-3077113	*stdC* 3076396-3077139 c
4A	3077111-3077114	*stdC* 3076396-3077139 c
5A	3077132-3077136	*stdC* 3076396-3077139 c
4C	3079526-3079529	*stdB* 3077180-3079663 c
4A	3079621-3079624	*stdB* 3077180-3079663 c
4A	3079657-3079660	*stdB* 3077180-3079663 c
4A	3080259-3080262	*stdA* 3079783-3080490 c
4A	3080352-3080355	*stdA* 3079783-3080490 c
4A	3080397-3080400	*stdA* 3079783-3080490 c
4A	3080414-3080417	*stdA* 3079783-3080490 c
4A	3080446-3080449	*stdA* 3079783-3080490 c
5C	3080227-3080232	*stdA* 3079783-3080490 c
5A	3703808-3703812	*lpfE* 3703495-3704022 c
4A	3703834-3703837	*lpfE* 3703495-3704022 c
4A	3703886-3703889	*lpfE* 3703495-3704022 c
4A	3704969-3704972	*lpfD* 3704028-3705107 c
4A	3705024-3705027	*lpfD* 3704028-3705107 c
4A	3705053-3705056	*lpfD* 3704028-3705107 c
5A	3705097-3705102	*lpfD* 3704028-3705107 c
4C	3705048-5705051	*lpfD* 3704028-3705107 c

4C	3707567-3707570	*lpfC* 3705125-3707653 c
5A	3708169-3708173	*lpfB* 3707676-3708374 c
4A	3708227-3708230	*lpfB* 3707676-3708374 c
4A	3708309-3708312	*lpfB* 3707676-3708374 c
4A	3708316-3708319	*lpfB* 3707676-3708374 c
5A	3708811-3708815	*lpfA* 3708459-3708995 c
5A	3708966-3708970	*lpfA* 3708459-3708995 c
5A	3708976-3708980	*lpfA* 3708459-3708995 c
5A	3708985-3708989	*lpfA* 3708459-3708995 c
4A	4570876-4570880	*sefB* 4570869-4571609
5A	4570908-4570912	*sefB* 4570869-4571609
7A	4570914-4570920	*sefB* 4570869-4571609
7A	4570950-4570956	*sefB* 4570869-4571609
5A	4570978-4570982	*sefB* 4570869-4571609
4A	4571632-4571635	*sefC* 4571626-4574070
4A	4571654-4571657	*sefC* 4571626-4574070
4A	4571684-4571684	*sefC* 4571626-4574070
4A	4571748-4571751	*sefC* 4571626-4574070
4A	4571814-4571817	*sefC* 4571626-4574070
4C	4574231-4574234	*sefD* 4574067-4574513
5A	4574110-4574114	*sefD* 4574067-4574513
6A	4574129-4574134	*sefD* 4574067-4574513
4A	4574175-4574178	*sefD* 4574067-4574513
6A	4574191-4574196	*sefD* 4574067-4574513
4A	4574220-4574223	*sefD* 4574067-4574513
8A	4574255-4574261	*sefD* 4574067-4574513
7A	4575220-4575226	*sefR* 4574550-4575362 c
5A	4575254-4575258	*sefR* 4574550-4575362 c
5A	4575301-4575305	*sefR* 4574550-4575362 c
4A	4575316-4575319	*sefR* 4574550-4575362 c
4A	4575336-4575339	*sefR* 4574550-4575362 c
5A	4575341-4575345	*sefR* 4574550-4575362 c
8A	4575349-4575356	*sefR* 4574550-4575362 c
4C	4676973-4676976	*sthE* 4675991-4677076 c
4C	4677516-4677519	*sthD* 4677117-4677674 c
4A	4680069-4680072	*sthC* 4677692-4680229 c
4A	4680078-4680081	*sthC* 4677692-4680229 c
4A	4680114-4680117	*sthC* 4677692-4680229 c
4A	4680134-4680137	*sthC* 4677692-4680229 c
4A	4680158-4680161	*sthC* 4677692-4680229 c

7C	4680062-4680068	*sthC* 4677692-4680229 c
4C	4680205-4680208	*sthC* 4677692-4680229 c
4C	4680775-4680778	*sthB* 4680275-4680958 c
4A	4680867-4680870	*sthB* 4680275-4680958 c
5A	4681389-4681393	*sthA* 4681029-4681574 c
4A	4681481-4681484	*sthA* 4681029-4681574 c
4A	4681559-4684562	*sthA* 4681029-4681574 c

Appendix 6.1. Bacterial counts of *S.* Enteritidis wild-type and fimbrial mutants 3, 7 and 10 days post oral-inoculation of 18-day-old Rhode Island Red chickens

Values below $\log_{10}$ 2.2 are below the levels of detection by direct plating

	Liver	Spleen	Caecal Contents	Caecal Wall	Ileal Contents	Ileal Wall
WT day 3	2.2	2.92	6.61	4.85	3.68	3.118
WT day 7	2.27	2.96	6.52	5.88	3.15	2.42
WT day 10	2.72	3.42	6.24	5.15	2.99	1.8
stdA day 3	2.14	2.14	6.25	5.5	3.71	3.06
stdA day 7	1.96	3.54	6.29	5.41	P 0.05 4.78	P 0.03 4.3
stdA day 10	2.11	3.49	6.23	5.46	2.61	1.68
stbA day 3	1.4	2.25	P 0.0075 5.13	4.82	2.56	3.03
stbA day 7	2.46	3.12	P 0.002 4.48	5.01	2.84	2.37
stbA day 10	1.85	3.39	P 0.05 4.98	4.76	1.41	2.11
stcA day 3	1.32	2.8	P <0.0001 3.2	3.16	1	1
stcA day 7	1.98	2.91	P <0.0001 3.54	P 0.0015 4.15	2.07	2.2
stcA day 10	1.95	P 0.03 2.52	5.32	5.41	4.61	3.41
WT day 3	1.370	2.940	6.180	4.170	1.530	3.130
WT day 7	2.380	3.090	5.180	4.960	2.010	2.430
WT day 10	1.640	3.420	3.770	3.840	1.560	1.000
sthA day 3	2.040	P 0.03 1.750	5.620	5.400	1.000	1.420
sthA day 7	2.480	3.370	6.130	5.470	1.890	2.650
sthA day 10	1.670	3.160	4.550	P 0.005 4.720	1.000	1.850
stiA day 3	1.640	P 0.02 2.870	P 0.03 4.180	3.370	1.000	P0.04 1.000
stiA day 7	1.930	2.470	5.290	4.590	1.740	2.060
stiA day 10	P 0.01 3.680	3.340	P 0.0037 5.330	P 0.001 4.950	1.300	1.470
stfA day 3	1.700	1.610	P 0.04 4.390	4.210	P 0.01 2.830	3.280
stfA day 7	1.380	2.650	4.470	4.050	2.080	2.070
stfA day 10	1.420	2.910	4.500	4.220	1.920	2.340

WT day 3	1.44	1.14	5.14	4.62	3.38	2.84
WT day 7	1.34	2.8	6.22	4.628	4.13	3.51
WT day 10	1.139	2.08	6.67	5.84	2.18	2.28
bcfA day 3	1.68	2.2	5.96	5.26	P 0.01 1.33	P 0.02 1.03
bcfA day 7	1	P 0.015 1	4.7	P 0.04 5.68	P 0.002 0.61	P 0.0014 0.4
bcfA day 10	1.34	2.19	5.8	5.31	2.54	2
csgA day 3	1	1.82	5.92	5.27	4.34	3.92
csgA day 7	1.68	2.92	5.15	4.29	3.91	2.62
csgA day 10	1.34	3.27	6.26	5.67	2.07	1.5
lpfA day 3	1.14	1.36	5.87	5.58	P 0.005 0.98	P 0.03 1.24
lpfA day 7	1	4.03	5.34	5.19	1.6	P 0.014 2.16
lpfA day 10	1.37	2.44	5.51	5.21	3.89	3.62
WT day 3	0.4	1.78	5.73	4.94	2.74	1.88
WT day 7	0.8	1.96	6.15	5.32	1.77	1.31
WT day 10	1.72	2.19	6.22	5.13	1.52	0.2
sefA day 3	1	2.14	6.14	5.35	P 0.001 0.6	P 0.02 0.2
sefA day 7	P <0.0001 2.59	P 0.0003 3.2	5.96	4.64	2.38	1.16
sefA day 10	1.71	P 0.0071 3.82	6.29	5.29	P 0.017 4.48	P 0.016 2.69
S1400 day 3	0.4	1.04	5.32	4.92	3.23	4.37
S1400 day 7	0.2	0.6	5.97	5.51	3.92	3.79
S1400 day 10	1	1.24	6.48	5.53	4.37	3.26
steA day 3	0.4	0.64	5.04	4.87	P 0.0004 0.84	1.9
steA day 7	1.24	1	6.39	5.83	P 0.006 0.8	P0.0006 0.84
steA day 10	0.4	1.21	P 0.0035 3.96	P 0.038 3.94	P 0.006 1.9	2.23
S1400 day 3	1.86	2.39	2.14	3.19	3.07	1.97
S1400 day 7	1.36	1.93	2.77	2.17	0.9838	0.8
S1400 day 10	1.42	1	1.24	1.58	1.2074	0.5037
fimA day 3	2.13	1	3.33	2.59	P 0.02 1.2	1.18
fimA day 7	1	P 0.002 1	2.36	2.6	1.24	0.2
fimA day 10	1	1.76	2.09	P 0.002 3.45	1.207	0
safA day 3	1.3	0.8	2.38	2.06	P 0.006 0.7	1.1
safA day 7	2.35	P 0.01 1.45	3.56	3.08	0.2	0.8
safA day 10	1.66	P 0.02 2.42	P 0.008 3.43	P 0.0002 4.11	1.68	1.88

S1400 day 3	1.48	1.65	4.66	4	0.84	1.08
S1400 day 7	1	1.3	4.58	4.37	0.64	1.1
S1400 day 10	1	1.84	2.86	3.76	1.47	0.4
stcA day 3	2.28	3.06	P 0.0075 1.43	P 0.0126 2.327	2.08	1.73
stcA day 7	1.48	1.44	P 0.0078 1.69	P 0.044 2.44	0.9	1.14
stcA day 10	P 0.0048 1	0.6	2.32	3.28	0.7	0.4