The contribution of SEF14, SEF17, SEF21 and flagella to the pathogenesis of *Salmonella enteritidis* in poultry.

By

Michael P. Dibb-Fuller BSc.

A thesis presented for the degree of Doctor of Philosophy

University of Leicester

2000

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Dedication

This thesis is dedicated to my mother, Susan Dibb-Fuller, who lives with me in spirit and to my grandmother, Claris Smith, whose words of encouragement have kept me going through some very difficult times.

Declaration

I hereby declare that this thesis has been composed by myself and that it has not been presented or accepted in any previous application for a degree. It is a record of any work carried out by myself unless stated otherwise and all sources of information have been acknowledged.

Michael P. Dibb-Fuller

Acknowledgements

This work was supported by the Ministry of Agriculture, Fisheries and Food (MAFF), UK (Project OZ0128) and was performed at the Veterinary Laboratory Agency (VLA), Weybridge, Surrey as part of a Foodborne Zoonosis Research Programme. I would like to acknowledge MAFF and VLA for providing the funding and facilities, respectively, for this study.

I would personally like to thank my original supervisor, Chris Thorns, for encouraging me to register for a higher degree and also Martin Woodward for agreeing to help me see it through. Both have been extremely helpful, supportive and patient. In addition, I have had many useful discussions with my external supervisor, Julian Ketley, to whom I am extremely grateful.

I would also like to say a big "Thankyou" to Emma Allen-Vercoe for providing the mutants used in this study, Peter Gilmore for his expert animal handling and advice, Robin Sayers for providing statistical analyses and, in particular, to Marciana Sojka for her friendship, continual encouragement as well as expert help and advice on protein purification. I would also like to thank Philippe Velge, INRA, France, for his invitation, hospitalilty and a wonderful week in his laboratory.

Finally, I owe the biggest thanks to my two wonderful children, Ben (5) and Megan (8) for keeping me focused on the most important things in life, and Rebecca my wife for all her love and support.

Publications

Dibb-Fuller, M.P., Allen-Vercoe, E., Woodward, M.J. & Thorns, C.J. (1997) Expression of SEF17 fimbriae *by Salmonella enteritidis*. *Lett Appl Microbiol* **25**; 447-452

Dibb-Fuller, M.P., Allen-Vercoe, E., Thorns, C.J. & Woodward, M.J. (1999). Fimbriae- and flagella-mediated association with and invasion of cultured epithelial cells by *Salmonella enteritidis*. *Microbiol* **145**; 1023-1031

Dibb-Fuller, M.P. & Woodward, M.J. (2000) The contribution of fimbriae and flagella of *Salmonella enteritidis* in colonisation and invasion of chicks. *Avian Pathol* **29**, 295-304

Contributions to publications:

Allen-Vercoe, E., Dibb-Fuller, M.P., Thorns, C.J. & Woodward, M.J. (1997). SEF17 fimbriae are essential for the convoluted colonial morphology of *Salmonella enteritidis*. *FEMS Microbiol Letts* **153**. 33-42.

Walker, S.L., Sojka, M., Dibb-Fuller, M.P. & Woodward, M.J. (1999). Effect of pH, temperature and surface contact on the elaboration of fimbriae and flagella by *Salmonella* serotype Enteritidis. *J Med Microbiol* **48**, 253-261

Contents

Chapter 2

Materials and methods

Chapter 3

Purification of SEF14, SEF17, SEF21 and flagella and the production and characterisation of specific immune reagents to these surface appendages.

Chapter 4

Expression and phenotypic characteristics of SEF17 fimbriae of *S. enteritidis*.

Chapter 5

Fimbrial and flagella-mediated association with and invasion of cultured epithelial cells by *S. enteritidis*.

Chapter 6

The contribution of fimbriae and flagella of *S. enteritidis* to colonisation, invasion, persistence and lateral transfer in chicks.

Index of Tables

Index of Figures

Abbreviations

ANOVA Analyses of variance
BCIP 5-bromo-4 chloro-3 indophosphate
BGA Brilliant green agar
CFA Colonisation factor antigen
CFU Colony forming units
ChFla Chicken antisera raised to flagella
ChSEF14 Chicken antisera raised to SEF14
ChSEF17 Chicken antisera raised to SEF17
ChSEF21 Chicken antisera raised to SEF21
DNA Deoxyribose nucleic acid
dNTPs Deoxyribose nucleotide triphosphates
DA Drigalski agar
EMEM Eagles minimal essential media
FCA Freund's complete adjuvant
FIA Freund's incomplete adjuvant
FITC Fluorescein isothiocyanate
fla$^-$ Flagella mutant
g Gravitational force
GACh Goat anti-chicken
GAM Goat anti-mouse
HBSS Hanks balanced salt solution
HIB Heart infusion broth
IEM Immuno-electron microscopy
LB Luria-Bertami
M Molar
Mab Monoclonal antibody
NA Nutrient agar
NB Nutrient broth
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
PBS/T Phosphate buffered saline + tween
PCR Polymerase chain reaction
PEG Polyethylene glycol
PI Post inoculation
PO Alkaline phosphatase
PPB Potassium phosphate buffer
PW Peptone water
Px Peroxidase
RACh Rabbit anti-chicken
SA Sensitest agar
SDS Sodium dodecyl sulphate
SEM Scanning electron microscopy
SPF Specific pathogen free
TBS Tris buffered saline
TBS/T Tris buffered saline + tween
TEM Transmission electron microscopy
TW Tryptone water
14$^-$ SEF14 mutant
17$^-$ SEF17 mutant
21$^-$ SEF21 mutant

Summary

The contribution of SEF14, SEF17, SEF21 and flagella to the pathogenesis of *Salmonella enteritidis* in poultry.

Michael P. Dibb-Fuller BSc.

Salmonella enteritidis continues to be a major cause of foodbourne infections in the United Kingdom, the rest of Europe, and the USA with poultry implicated as a major source. To understand the virulence mechanisms of bacterial pathogens it is necessary to study directly host-bacterial interactions. For *E. coli* it is considered that adherence to enterocytes is a prerequisite to infection and the adhesive function of certain fimbriae have been described (Parry & Rooke, 1985). However, for *Salmonella spp.*the role of surface appendages is poorly understood.

S. enteritidis express flagella and several tightly regulated fimbriae including SEF14 and SEF21 (type 1) that are morphologically and antigenically distinct. In addition, Collinson *et al.* (1991) demonstrated elaboration of aggregative SEF17 fimbriae by *S. enteritidis* 27655R, although conditions neccesary for expression of this surface appendage, other than strain 27655R, were unknown. In this study specific immunological reagents were raised to SEF17 and used to investigate the expression of SEF17 fimbriae by cultured strains of *S. enteritidis*. Elaboration of SEF17 was dependant upon temperature and media with optimal expression at 18°C on CFA agar. However, two wild type strains produced SEF17 when also grown at 30°C to 42°C. In addition, fine fimbriae produced by a strain *of S. typhimurium* and *E. coli* were specifically and strongly labelled by SEF17 monoclonal and polyclonal antibodies, indicating considerable antigenic conservation.

A panel of mutants prepared in three strains of *S. enteritidis* defective for the elaboration of fimbrial types SEF14, SEF17, SEF21 and flagella were utilised to determine the role of these surface appendages in association with and invasion of cultured epithelial cells. In all assays, the naturally occuring regulatory defective strain 27655R associated to tissue culture cells significantly greater than LA5 and S1400. Compared with wild type progenitor strains, SEF14 mutants had no effect on association and invasion, whereas for both association and invasion, SEF17, SEF21 and aflagellate mutants showed significant reductions.

Isogenic mutants prepared in a chicken isolate, *S. enteritidis* strain LA5, were used to study the contribution of these organelles in the colonisation, invasion, persistence and lateral transfer in young chicks. The caecum, liver and spleen were colonised within 24 hours following oral inoculation of day old chicks with 10^5 wild type *S. enteritidis*. SEF17, SEF21 and flagella contributed to a delay in colonisation of the spleen as did SEF21 and flagella mutants in the liver. Lower numbers of bacteria were recovered from the caecum with mutants deficient for SEF21 elaboration. LA5 and EAV40 (14^-, 17^-, 21^-, fla^-) persisted for six weeks in the caecum and to a lesser extent the liver and spleen of birds. In addition, sentinel birds were colonised by LA5 or EAV40 directly from the environment within two days, although, a slight delay was observed with the multiple mutant.

Overall, data from this study suggests that SEF17, SEF21 and flagella, but not SEF14, play a role in the early stages of colonisation and invasion of young chicks by *S. enteritidis*, but are unnecessary for *S. enteritidis* to persist for up to six weeks or colonise birds from their immediate environment.

Chapter 1

Introduction

1.1 General introduction

The first member of the *Salmonella* genus to be studied was the typhoid bacillus which was originally observed in human tissues by Eberth in 1880 and cultured by Gaffkey in 1884. Soon afterwards, Salmon and Smith isolated an organism from diseased pigs, *Bacillus cholerae-suis*, and within the following years similar isolations were made from a wide variety of human and animal sources (Buxton & Fraser, 1977). The genus *Salmonella* was named after Salmon in recognition of his early work on these organisms which now comprises of a large group of more than 2000 serotypes (Turner *et al.,* 1998).

Salmonella is a member of a family of micro-organisms collectively known as Enterobacteriaceae that are gram negative, rod-shaped bacteria which can grow rapidly by respiratory metabolism in the presence of oxygen or by fermentation in its absence. Enterobacteriaceae is a large and diverse group numbering at least 29 genera and 157 species. Many species, including *Klebsiella spp.* and *Proteus spp.,* are free living in nature and other genera such as *Escherichia spp.* and *Enterobacter spp.* are noted for their close association with humans and vertebrates in general, either as part of their normal flora or as pathogens such as *Salmonella spp.*, *Shigella spp.* and *Yersinia spp.* causing potentially life threatening diseases. *Klebsiella spp.*, *Citrobacter spp.* and *Serratia spp.* live outside of animals but may cause opportunist infections in humans (Brenner, 1992).

1.2 Isolation and classification of *Salmonella*

Salmonella can be isolated from a variety of sources including the environment or the infected host. Faecal samples are taken from patients suffering from gastroenteritis, whereas, in systemic disease a blood sample is collected. Samples are placed in enrichment media

such as Selenite F, tetrathionate or gram negative broth or directly onto one or more selective media including, MacConkey agar, XLD agar, Hektoen enteric media and Brilliant Green agar (Dwight & Zee, 1998).

Following isolation, *Salmonella* are biochemically confirmed and serotyped by agglutination tests. Detailed examination is required to distinguish *Salmonella spp.* and other closely related Enterobacteria.

Biochemical properties of *Salmonella*

With few exceptions *Salmonella* ferment glucose, maltose, mannitol, dulcitol, and dextrin with the production of acid and gas, but do not ferment lactose, sucrose or salicin. Additionally, *Salmonella* do not breakdown urea, liquify gelatin or produce indol but reduce nitrates to nitrites and produce H_2S. (Bergey, 1975).

***Salmonella* serotyping**

The surface of the bacterial cell is composed of lipopolysaccharide (LPS) which constitute the somatic "O" antigens. In addition, most *Salmonella* possess flagella which are composed of repeating flagellin protein subunits that contain the flagellar "H" antigen. Due to the variety of these antigens, *Salmonella* isolates can be classified serologically based on possession of certain combinations of "O" and "H" antigens. This method of classification established in 1929 by White and Kauffmann and continues to be used today (Kauffmann, 1964). For example, *Salmonella enteritidis* express somatic antigens 1, 9, 12 and flagellar antigens g, m and is classified in serogroup D1.

Phage typing

In the UK the most prevalent serotypes, *S. typhimurium* and *S. enteritidis*, are sub-typed in accordance with Central Public Laboratory phage typing schemes (Guinee & van Leeuwen, 1978). Cultures seeded onto agar plates and exposed to a specific set of phages, develop a unique pattern of lysis. Thus, a phage type (PT) can be assigned to *S. enteritidis* or a definitive type (DT) attributed to *S. typhimurium* isolates. Hinton *et al.* (1990), suggested that *Salmonella* isolates may have become more virulent in recent years and Barrow (1991) speculated that this could be attributed to certain phage types of *S. enteritidis.*

1.3 Hosts of *Salmonella* serotypes

A striking feature about the natural distribution of *Salmonella* is the host specificity of a few serotypes and the broad host range of the majority and, therefore, in terms of pathogenesis the genus can be divided into two major groups. Host-specific *Salmonella* produce systemic disease and are rarely involved in human food poisoning whereas *Salmonella* serotypes which produce the symptoms of gastro-enteritis rarely produce systemic disease and colonise a variety of hosts (D'Aoust *et al*, 1990).

1.3.1 *Salmonella* serotypes causing systemic infection

The small number of *Salmonella* serotypes that characteristically produce systemic infections involving the reticuloendothelial system include amongst others *S. typhi* in man, *S. typhimurium*, *S. gallinarum* and *S. pullorum* in poultry, *S. dublin* in cattle and *S. cholera-suis* in pigs. Experimental evidence in animal models and clinical evidence in typhoid patients suggests the pathogenesis of the systemic disease is similar in all of these hosts (D'Aoust,

1991) and, therefore, the infections of *S. typhimurium* in mice has been studied extensively as a model for human typhoid.

Infection with host adapted *Salmonella* is normally via the oral route, although the organisms do not necessarily multiply extensively in the alimentary tract. The bacteria are highly invasive and have been observed microscopically within enterocytes and lymphoid tissues (Takeuchi, 1967). In mice, *Salmonella* pass from the sub-mucosa to lymph nodes where some bacteria become trapped in the lymphoid tissue whilst others enter the circulatory system and are transported within host phagocytic cells to the liver and spleen (Dunlap *et al.*, 1991). Bacterial multiplication is followed by cell death which in earlier stages of infection is contained within the reticuloendothelium, but eventually causes a bacteraemia. The lipid A component of lipopolysaccharide activates host macrophages and release factors that exert biological effects such as fever and endotoxic shock. Bacteria can re-enter the gut via the gall bladder and the organisms are shed in the faeces, although the numbers are generally low (D'Aoust, 1991).

1.3.2 *Salmonella* serotypes causing gastro-enteritis

Human salmonellosis from non-typhoid strains of *Salmonella* generally occurs as a self-limiting gastroenteritis lasting up to five days. Following an incubation period ranging from eight to seventy-two hours, symptoms include abdominal pain, nausea, watery diarrhoea with occasional mucus and traces of blood in the stools (Johnson *et al.*, 1983). A fever lasting less than 48 hours may accompany the diarrhoeal illness. Susceptibility to infection is highest in infants, elderly people and the immunocompromised hosts. Clinical management of the disease is fluid and electrolyte replacement (D'Aoust, 1991).

Enterotoxin is a major bacterial virulence factor in human salmonellosis which is a generic trait commonly found in *Salmonella spp.* (Kaura *et al.*, 1982) and is both functionally and immunologically related to cholera toxin and the thermolabile toxin of *E. coli* (D'Aoust *et al.*, 1989). The enterotoxin is released into the intestinal lumen and in the cytoplasm of host cells during epithelial invasion. The profuse loss of intestinal fluid is due to toxin-mediated activation of adenylate cyclase localized in the cytoplasmic membrane of the host epithelial cells (Peterson *et al.,* 1983). Mucosal inflammation contributes to the fluid secretion through stimulation of enteric nerves in the ileum wall (Brunsson,1987) and activation of adenylate cyclase by host tissue prostaglandins (Duebbert *et al.,* 1985). Fluid exorpsion arises from a net secretion of chloride ions in the crypt region and depressed sodium ion absorption at the intestinal villi (Fromm *et al.*, 1974).

In addition to the enterotoxin, most strains of *Salmonella* produce a cytotoxic factor that is related to the shiga neurotoxin in it's effects on animal models and tissue culture cells (Ashkenazi *et al.,* 1988) and is inactivated by *Shigella dysenteriae* 1 antitoxin (Ketyi *et al.*, 1979). *Salmonella* cytotoxin is a thermolabile protein which is bound to the outer membrane of the bacterial envelope (Reitmeyer *et al.,* 1986) and inhibits protein synthesis in host cells (Koo *et al.*, 1983)

1.4 *Salmonella* as a zoonotic foodbourne pathogen

Since the early 1980's the incidence of *S. enteritidis* has increased in United Kingdom, United States and throughout Europe (Hopper and Mawer, 1988; Dreesen *et al.*, 1992) with an increase in the number of sporadic cases and cases associated with recognised outbreaks of food poisoning due to *S. enteritidis* infections (Coyle *et al.*, 1988; Rodrigue *et al.,* 1990).

Salmonella are common pathogens of many species of mammals and fowls and prior to the 1980's *S. enteritidis* was rarely isolated from poultry (Snoeyenbos *et al.*, 1969) and most isolates may have been derived from contaminated feed (Williams *et al.*, 1988). However, the recent increase of *S. enteritidis*-mediated gastro-enteritis in humans has been attributed to the consumption of contaminated poultry meat, eggs or egg products (Hedberg *et al.*, 1993; St. Louis *et al.*, 1988).

1.4.1 The emergence of *S. enteritidis* as a dominant serotype

Reports of human *Salmonella* infection to the Public Health Laboratory Service (PHLS) in England and Wales rose from approximately 12,000 in 1982 to over 31,000 in 1992 and this was largely attributed to an increase of reports of *S. enteritidis* (Roberts & Sockett, 1994). In 1982, *S. enteritidis* accounted for 9% of all *Salmonella* reports, but isolates of this serotype rose to 64% by 1992 (Roberts & Sockett, 1994). Since 1988, *S. enteritidis* PT 4 has continued to be the most prevalent *Salmonella* serotype isolated from human patients suffering from gastroenteritis in England and Wales. Government reports and epidemiological investigations have identified fresh shell egg and poultry as important vehicles of these infections (Advisory Committee on Microbiological Safety of Food, 1993; Coyle *et al.*, 1988; Cowden, *et al.* 1989). In addition, the isolation rate for *S. enteritidis* in humans in the United States of America increased considerably between 1976 and 1995 and *S. enteritidis* was the most prevalent serotype reported in the USA in 1990, 1994 and 1995. In a majority of regions PT 8 was the most common phage type, however, PT 4 has recently emerged in the egg industry in the Western USA (Hogue *et al.*, 1997).

Preceding 1981, *Salmonella* was the most prevalent bacterial foodbourne pathogen isolated from patients suffering from acute gastroenteritis in England and Wales. However the

incidence of *Campylobacter spp.* has steadily increased since 1977 and is presently the most common cause of bacterial gastroenteritidis in humans in England and Wales (Anon., 1999).

1.4.2 *S. enteritidis* in chickens

It is generally recognised that non-typhoid serotypes of *Salmonella* such *as S. enteritidis* and *S. typhimurium* which are responsible for food poisoning in humans cause symptomless intestinal infections in a wide range of domestic and wild birds. The response to *S. enteritidis* in hens is different to that of chicks, with the infection seldom causing mortality in birds more than one month old (Lister *et al.* 1988). In addition, the resistance to *Salmonella* increases with age and it is likely that the development of normal flora in the intestine and the maturation of the host immune system are contributary factors (Ziprin *et al.* 1989).

Chickens from broiler breeders and layer flocks naturally infected with *S. enteritidis* show no obvious clinical abnormalities, increase in mortality or decrease in egg production (Cooper *et al.* 1989; Hopper *et al.* 1988), although the body condition of some may be poor (Lister *et al.* 1988). At *post mortem*, pathological changes in infected chickens can reveal deformed, shrunken, discoloured ovaries and ovules with shrunken or malformed follicles with fluid filled cysts attached, soft shelled eggs or egg peritonitis, indicating transovarian infection of *S. enteritidis* (Lister *et al.*, 1988). *S. enteritidis* can be recovered from the blood, heart, liver, spleen, yolk sac, ovaries, oviduct, peritoneum, egg, alimentary tract and faeces of infected birds (Suzuki, 1994). Birds can carry large numbers of *S. enteritidis* in their caeca (Hopper, 1988) and therefore, symptomless carriers can spread the infection within the flock.

1.5 Infection of eggs by *S. enteritidis*

Contamination of egg contents with *S. enteritidis* may occur by penetration of the bacteria through the shell (Coyle *et al.*, 1988) or by transovarian infection (Williams *et al.*, 1988; Timoney *et al.*, 1989). *S. enteritidis* shed in faeces from infected chickens persists for long periods in the environment. This will increase egg soiling with infected droppings and may assist penetration of the organism. Keller *et al.* (1995) investigated the colonisation of the reproductive tract by *S. enteritidis* and reported that prior to egg shell deposition, forming eggs were subject to descending infections from colonised ovarian tissue, ascending infections from colonised vaginal and cloacal tissues and lateral infections from upper oviduct tissues. Thiagarajan *et al.* (1996a; b) reported the potential of transovarian transmission and demonstrated the *in vitro* colonisation of granulosa cells derived from the mature and developing follicles of the hen ovary by *S. enteritidis*. Importantly, it has been demonstrated that ovarian infections by *S. enteritidis* may result in the laying of contaminated eggs or infected progeny from the contaminated eggs (Hopper & Mawer, 1988; Lister, 1988; Holt & Porter, 1993).

1.6 Incidence of *S. enteritidis*

Incidence of human gastro-enteritis caused by *S. enteritidis* is seasonal, with the greatest number of isolates reported during the summer and the lowest numbers during the winter months. *S. enteritidis* PT 4 continues to be the most prevalent phage type isolated in England and Wales with 72% of all isolates of this serotype in 1997 (Anon., 1998a). However, by 1999 the incidence of *S. enteritidis* PT 6 infection increased by 70% compared to the previous year, thus making PT 6 the second most commonly isolated *S. enteritidis* phage type from humans in England and Wales (Anon., 1998b). Concurrently, an increase in isolation of this

phage type from poultry was reported with incidents mainly traced to broiler flocks (Anon., 1997).

Interestingly, *S. typhimurium* is the most prevalent serotype in many farmed animals including cattle, sheep, pigs and also in other avian species including turkeys, ducks and geese (Anon., 1997). The most prevalent definitive type of *S. typhimurium* is DT 104 which in recent years has been a subject of concern due to reports of multiple resistance to antimicrobials commonly used in human and veterinary practice (Evans, 1996).

1.7 Transmission of *S. enteritidis* amongst chickens

Egg contamination with *S. enteritidis* can occur by vertical transmission with invasion of the chicken reproductive tissues which can result in infected progeny (Gast & Beard, 1990; Humphrey *et al.*, 1991; Holt & Porter, 1993). *Salmonella* are only isolated in small numbers from eggs laid by infected hens and this is probably due to the complex membranes, anti-bacterial components in the albumen and immunoglobulins found in the yolk (Board *et al.*, 1974; Dadrast *et al.*, 1990).

The process of natural infection and propagation during hatching is difficult to study because of the low numbers of naturally occurring hatching eggs (Humphrey *et al.*, 1991). However, following inoculation of fertile hatching eggs by immersion in physiological saline containing *S. typhimurium,* Cason *et al.* (1994) demonstrated that inoculated eggs hatched at a high rate despite contamination with *S. typhimurium* which indicated that chicks from eggs infected with *Salmonella* were likely to hatch and contaminate chicks in the same hatcher.

In addition to vertical transmission, *S. enteritidis* is considered to be transmitted laterally through contact with infected birds by ingestion of faecally contaminated materials or aerosols (Holt *et al.* 1998). Commercial broiler and breeder hatcheries have been shown to be important reservoirs of *Salmonella* (Cox *et al.*, 1996). Indeed, Cox *et al.* (1996) showed that day-of-hatch chicks were intestinally colonised with inhaled doses of 20 colony forming units (CFU), oral doses of 100 CFU and intracloacal doses of two CFU of *S. typhimurium* which is indicative of how vulnerable chicks are to colonisation by *Salmonella* during hatching and early stages of development. Holt (1993) reported that stress factors such as disease and food deprivation exacerbated the horizontal transmission of the organism through the flock. For example, the removal of feed from ageing hens to induce a molt and extend the active egg-laying life of a flock is commonly practiced in the United States (Bell, 1987) and molted birds have a higher susceptibility to infection by *S. enteritidis* (Holt, 1993; Holt & Porter, 1993).

1.8 Protective immune responses to *Salmonella*

Innate and aquired avian immune responses have been demonstrated to have a protective role against *Salmonella* infections. It has been reported that cell-mediated responses (Lee *et al.*, 1981;1982; Desmidt *et al.,* 1998) and phagocytes (Kogut *et al.*, 1993; 1994; 1998) assist in the clearance of *Salmonella* from deeper tissues and that immune exclusion by secretory IgA is an important factor in intestinal clearance by preventing attachment to the intestinal surfaces (Desmidt *et al.,* 1998).

1.8.1 Innate response

Following invasion, heterophil responses in chickens are regarded as important in resistance to *S. enteritidis* and *S. typhimurium*. Kogut *et al.* (1993; 1994) described the treatment of birds with 5-fluorouracil to specifically reduce the avian polymorphonuclear leukocyte populations without affecting mononuclear cells and results suggested that birds had a greater susceptibility to parenteral infection by *Salmonella* and to lesser extent to oral infection.

1.8.2 Aquired immune response in chickens

Humoral and cell mediated immune responses of poultry have been demonstrated to have a protective role against *Salmonella* infections. However, the humoral response has been studied more extensively. Chickens have at least three classes of immunoglobulin (Ig). IgM is structurally and functionally similar to mammalian IgM (Sharma, 1991) and is the first immunoglobulin to be induced after primary challenge with an antigen. IgM can be detected in bile and intestinal washings, but in general, has a limited role in the intestinal immune response (Schatt & Myers, 1991). Avian IgA is similar to that of the mammalian orthologue and is the major immunoglobulin in bile and intestinal secretions (Schatt & Myers, 1991). IgY is structurally different from, but functionally analogous to mammalian IgG and is commonly referred to as IgG (Leslie & Clem, 1969). The passively acquired antibody in the developing chicken is IgG, which is actively transported from the circulation of the hen into the developing ova and subsequently from the yolk into the circulation of the chick (Kowalczyk *et al.*, 1985).

In one study, Hassan *et al.* (1991) orally infected day-old chicks with *S. typhimurium* resulting in faecal excretion persisting for at least ten weeks and *Salmonella* could be isolated

from deeper tissues for several weeks. In the serum, high titres of IgM, IgA and IgG specific for LPS, flagella and outer membrane proteins were detected as early as one week post infection. Within nine weeks titres of serum IgM and IgA fell to low levels, whereas high titres of IgG were detected for up to nine months post infection. In intestinal washings and bile, high titres of secretory IgA were present after nine weeks.

The role of avian humoral immunity in the clearance of *S. enteritidis* has recently been investigated by bursectomy (Desmidt *et al.,* 1998). Faecal excretion of *S. enteritidis* in control birds were significantly lower than bursectomised birds within 13 days of oral inoculation which suggested a role for humoral immunity in clearance of *S. enteritidis* within the intestine. However, numbers of *S. enteritidis* in the spleen and liver, were reduced at two and six weeks post inoculation, respectively, in bursectomised and control birds. These results were in agreement with Lee *et al.* (1981, 1982) and implied that cell mediated responses were responsible for tissue clearance, whereas secretory IgA assisted intestinal clearance. In addition, Withanage *et al.* (1999) recently reported that a significant local immune response to *Salmonella* with elevated levels of IgA antibodies were responsible for partial clearance of *Salmonella* from the oviducts.

Evidence of a protective cell mediated immune response by poultry against organ invasion by *S. enteritidis* was provided by Kogut *et al.* (1998) and Genovese *et al.* (1998). These researchers demonstrated a protective role of the chicken and turkey cell mediated defence and in particular a heterophilic inflammatory response using a prophylactic administration of lymphokines derived from T cell supernatants from *S. enteritidis* immune birds or a transformed chicken T cell line. Genovese *et al.* (1998) reported a significant peripheral blood heterophilia at 4 hr post injection of an immune lymphokine preparation, but not in the PBS-

injected control group. The lyphokine-induced heterophilia was correlated with a reduction in organ invasion by *S. enteritidis*. T cells isolated and virally transformed from non-immune chicks did not produce factors that protected chicks from *S. enteritidis* organ invasion, whereas secretions of *S. enteritidis* activated T cells were protective. These results were in agreement with those of Ziprin *et al.* (1989) who demonstrated that the treatment of newly hatched chicks with the T cell-specific, immunosuppresive agent, cyclosporin were more susceptible to *S. enteritidis* infections. Results indicated that within 5 days, treatment with cyclosporin reduced *in vitro* T cell responses to T cell-specific mitogens, suppressed *in vivo* delayed-type hypersensitivity, and impaired the development of native resistance to *Salmonella* infections.

1.9 Control of *Salmonella* in chickens

In many countries *S. enteritidis* has replaced *S. typhimurium* as the most prevalent serotype in both human disease and in poultry (Rodrigue *et al.,* 1990) and therefore several intervention strategies have been investigated with the overall aim of reducing the incidence of *Salmonella* infections in poultry. These have included the development of specific and sensitive diagnostic assays for determining the status of flocks, strategies to reduce colonisation and carriage in the avian intestinal tract, vaccination and studies investigating the effective cleansing and disinfection of infected premises.

1.9.1 Screening and improved diagnostics

In the United Kingdom, *Salmonella* is a Notifiable Disease and under the Zoonoses Order 1989 the Ministry of Agriculture, Fisheries and Food are to be informed of any isolations. In addition to the statutory programme of testing under the Poultry Breeding Flocks Order 1993

and Hatcheries Order 1993 in the UK, an increasing number of commercial companies are reported to operate voluntary monitoring, in particular, terminal broiler and egg laying flocks (Zamora *et al.*,1999). The necessity for rapidly obtainable information on the infection state of flocks has led to the development of serological enzyme-linked immunosorbent assays (ELISA) or chemiluminescent immunoassays (CLIA) for the detection of specific circulating immunoglobulins which are produced by invasive serotypes of *Salmonella.* Advantages of ELISA include, the ability to cope with large numbers of samples of either serum or egg yolk, the availability of high quality reagents and mechanisation (Barrow, 1992; 1994; Zamora *et al.*,1999). A variety of detecting antigens have been used including LPS (Hassen *et al.*, 1990; Barrow *et al.*, 1992; 1994), whole flagella (Timoney *et al.*,1990), recombinant flagellin protein containing the serotype specific flagellin fragment (Baay *et al.*, 1993), outer membrane proteins (Kim *et al.*, 1991), disrupted whole bacterial cell proteins (Hassen *et al.*, 1990), native or recombinant fimbrial antigens (Thorns *et al.*, 1990; 1993b; Rajashekara *et al.*, 1998) or a combination of flagella and fimbrial antigens (Zamora *et al.*, 1999). European Union legislation (Council Directive 92/117/EEC, 1992) recognises the use of certain serological tests for screening of flocks. However, *Salmonella* must be isolated as confirmation of infection. .

1.9.2 Exclusion of *Salmonella* by dietary sugars

Several investigators have reported the inhibitory effect that carbohydrates exert on adherence of bacteria to epithelial cells (Swanson, 1973; Jones & Freter, 1976; Oyofo *et al.*, 1988). Oyofo *et al.* (1989) showed that strains of *S. typhimurium* expressing type 1 fimbriae adhered to chick gut explants in significantly greater numbers than non-fimbriate strains, and the addition of mannose and lactose to the drinking water of day-old broiler chicks followed by an oral inoculation of *S. typhimurium* at three days of age resulted in a significant reduction in

colonisation by *Salmonella* (Oyofo *et al.*,1989). In combination, these results implied a significant role for mannose receptors and potentially type 1 fimbriae as the bacterial ligand in the early stages of colonisation of young chicks, although Corrier *et al.* (1990) reported that dietary lactose exerted a protective effect by lowering the intestinal pH by increasing the concentration of volatile fatty acids. However, Barnhart *et al.* (1999) provided evidence that 2.5% lactose in the drinking water during the last 5 to 11 days growout prior to slaughter was ineffective for *Salmonella* control under commercial conditions.

By-products of plants such as beet pulp, cereal bran, and palm kernal meal (PKM) which contain considerable quantities of structural carbohydrates including arabinans, galactans, glucans, mannans and pectins when included in the diet may serve as substrates for enzymatic production of oligosaccharides or monosaccharides. Studies indicated that inclusion of PKM in the diet of chicks reduced the extent to which the intestine was contaminated with *Salmonella (*Allen *et al.*, 1997).

1.9.3 Competitive exclusion and probiotics

Nurmi & Rantala (1973) first introduced the concept of competitive exclusion to reduce *Salmonella* infections. Studies have indicated that the introduction of an adult chicken intestinal flora into a day-old chick increased the resistance of chicks to *Salmonella* colonisation (Pivnick & Nurmi, 1982). Stavric *et al.* (1991) demonstrated that undefined cultures were more protective than defined probiotic treatments, although the addition of lactose to a defined culture increased effectiveness (Nisbet *et al.*, 1993). The most effective competitive exclusion agents were derived directly from chicken intestines (Cameron & Carter, 1992; Hoszowski *et al.*, 1997), although, Spencer *et al.* (1998) reported the

effectiveness of exclusion cultures produced by earthworms which had been fed fresh chicken faeces.

Recently, defined competitive exclusion cultures have been reported to give protection against low and moderate *Salmonella* challenges (Hume *et al.*, 1998) and in addition, spray vaccination with a combination of *Lactobacillus acidophilus*, *Streptococcus faecium* and specific antibodies to *S. typhimurium* have proved beneficial in reducing colonisation in market aged broilers (Promsopone *et al.,* 1998).

Live micro-organisms with beneficial effects for the host, or probiotics, may offer an alternative to conventional antimicrobials in the treatment and prevention of enteric infections. For example, Mack *et al.*, (1999) demonstrated that *Lactobacillus plantarum* and *Lactobacillus rhamnosus* inhibited the adherence of Enteropathogenic *E. coli* to mucin-secreting HT-29 intestinal epithelial cells but did not inhibit adherence to non-intestinal Hep-2 cells. Inhibition of adherence to epithelial cells was mediated by increased expression of intestinal mucins by *Lactobacillus spp.*

1.9.4 Chemotherapeutic agents

The administration of enroflaxacin followed by a gut floral preparation has been shown to be effective in eliminating *S. enteritidis* from naturally infected flocks (Barrow *et al,* 1998; Reynolds *et al.*, 1997) and advocated for use *in ovo* injections to produce *Salmonella*-free eggs from which clean breeding stock could be raised. However, there is concern that excessive use of antimicrobial agents lead to the development of resistance and Barrow *et al.* (1998) reported the rapid development of resistance to enrofloxacin by commensal *E. coli*

population during an experiment to examine the effect of this antimicrobial on shedding of *Salmonella* by chickens.

1.9.5 Vaccination

Immunological research on the control of intestinal and tissue colonisation of poultry by virulent *Salmonella* has focused mainly upon the development of live attenuated vaccines. Smith (1955; 1956) first investigated the use of live avirulent *Salmonella* to immunise chickens following the isolation of a rough mutant of *S. gallinarum* which did not colonise well but gave good protection following parental immunisation. The *S. gallinarum* strain 9R is currently used to control infections in chickens.

The development of rationally-attenuated *Salmonella* vaccines for use in man (Dougan, 1987) and farm animals (Cooper *et al.*, 1990; 1994; Hassan & Curtiss, 1997) have been reported. They have several potential advantages over killed vaccines including good cellular immune responses (Eisenstein *et al.*, 1984), ease of administration to large numbers of animals and the generation of mucosal response in the gut (Karem *et al.*, 1995). The primary aim of developing live attenuated vaccines in poultry is to protect colonisation of the reproductive tract and prevent vertical transmission (Timoney *et al.*, 1989). Cooper *et al.* (1994, 1996) developed a model to simulate lateral transfer of *S. enteritidis* infection in chickens. Newly hatched birds were vaccinated orally with an attenuated *S. enteritidis aro*A strain and at three weeks of age brought into contact with "seeder" birds infected with wild type *S. enteritidis*. Vaccinated birds were protected against colonisation of the gut by wild type strain when compared to unvaccinated controls. Additionally, following booster doses at two, 16 and 18 weeks of age, there was a significant reduction in the numbers of bacteria recovered from the

spleen, liver, ovaries and caeca of chicks that were challenged intravenously with wild type *S. enteritidis* at 23 weeks of age.

In addition, Curtiss & Hassan (1996), evaluated a live avirulent *S. typhimurium* vaccine strain which had been attenuated by deletion mutations in genes encoding adenylate cyclase (*cya*) and the cAMP receptor protein (*crp*). Curtiss & Hassan, (1996) claimed that immunisation induced significant cross-protection against various *Salmonella* serotypes and protected laying hens from transmission of *Salmonella* in or on eggs following challenge with *S. enteritidis* or *S. typhimurium*. Laying hens boosted at 16-18 weeks of age led to maternal transfer of immunity to chicks and also, oral vaccination at two and four weeks of age induced protection against intestinal tract colonisation, visceral and reproductive tract and egg contamination for up to 11 months following challenge with *S. enteritidis* or *S. typhimurium.*

1.9.6 Cleansing and disinfection of premises infected with *Salmonella*

Salmonella are capable of prolonged survival outside the host with survival times of over two years in dried bovine or avian faeces reported (Morse *et al.,* 1974). Davies & Wray (1995) performed a study on the persistence of *S. enteritidis* in poultry units and poultry food and reported that organisms persisted for at least one year in an empty trial house in which naturally-infected broiler breeder birds had previously been housed. *S. enteritidis* were also found surviving in small pockets of litter and fan dust which had been left after cleansing and disinfection of the site and also in wild bird droppings. In addition, *S. enteritidis* survived for 26 months in artificially contaminated poultry food.

Results from a further study by Davies *et al.* (1996) showed that following cleansing and disinfection of three broiler breeder houses infected with *S. enteritidis*, non of the farms

achieved total elimination of the bacterial pathogen, and in each case the failure to eliminate a mouse population infected with *S. enteritidis* was likely to be the most important hazard for the new flock. These studies provided evidence that thorough cleansing and disinfection of infected premises is essential to avoid reinfection.

1.9.7 Resistance to colonisation of *Salmonella* in chickens

Differences in susceptability of chicken lines to *Salmonella* have been investigated to consider a genetic approach to their reduction (Lindell *et al.*, 1994, Guillot *et al.,* 1995; Beaumont *et al.*, 1999). Duchet-Suchaux *et al.* (1997) tested four chicken lines commonly used as laying hens or meat production for experimental carrier state of *S. enteritidis*. Following oral inoculation of week old chicks with *S. enteritidis*, liver, spleen, ovaries and caecum were examined weekly for levels of colonisation for up to twelve weeks. In agreement with other experimental trials very few ovaries were colonised, and numbers of positive livers and spleens did not differ between chicken lines. However, there was significant differences in the numbers of caeca colonised, numbers of *Salmonella* recovered and duration of carriage between the four chicken lines. Horizontal transmission, eggshell contamination, carcass contamination at slaughter and potentially egg contamination by ascending route (Keller *et al.*, 1995) may originate from the caeca. Duchet-Suchaux *et al.* (1997) suggested that resistance and susceptibility could be in part genetic, and therefore, selection of chicken lines may contribute to control of *S. enteritidis*.

1.10 Mechanisms involved in colonisation and penetration by *Salmonella*

The intestinal tract is a highly specialised system which perform digestive and absorptive functions, but also provide a protective barrier between the external and internal organs. Mucosal surfaces have many mechanisms of defence against bacterial colonisation and penetration (Befus *et al*, 1980; Schaechter *et al.*, 1989; Forstner & Forstner, 1994; Finlay, 1995) which include the sweeping action of ciliated epithelium, mucus, and the peristaltic actions of the stomach and intestines that propel bacteria through and out of the gastrointestinal tract, thereby decreasing the interactions of the bacteria with the epithelial linings. Normal flora may prevent adherence by occupying common receptors, compete for nutrients or produce antimicrobial metabolites, thus preventing establishment of incoming pathogens.

The host has a variety of other defences to protect the mucosal surface, including cellular defences such as resident macrophages and polymorphonuclear leukocytes, neutrophils in mammals and heterophils in avians. The host may secrete antibacterial factors such as secretory immunoglobulin A, lysozyme and lactoferrin and other antimicrobial peptides. Many pathogens have developed strategies for avoiding such factors, which include the production of molecules that bind lactoferrin, thus obtaining essential iron, or neutralizing the host immune system by producing immunoglobulin A proteases (Klauser *et al.*, 1993) .

1.10.1 The intestinal barrier

The intestinal barrier is composed of columnar absorptive cells, goblet cells which secrete mucus to coat the epithelial surface and Paneth cells which are phagocytic cells found in the

bottom of the crypts of the small intestine that secrete lysozyme and other antimicrobial products (Keren, 1992). Columnar epithelial cells are closely linked to neighbouring cells by tight junctions (Rodriguez-Boulan & Nelson, 1989). The apical or luminal surface of an intestinal epithelial cell contains well defined microvilli which are finger-like projections supported by actin filaments. *Salmonella* denude the epithelial microvilli, resulting in a "bulging" or "ruffling" (Finlay & Falkow, 1990; Francis *et al.*, 1992), and this makes the cell more susceptible to invasion. Unlike *Salmonella, Yersinia spp.* and *Shigella spp.* are unable to adhere to the apical surface of polarized monolayers but interact specifically with the basolateral surface (Mounier *et al.*, 1992).

1.10.2 M cells and Peyer's patches

Host protection from the immune system requires the passage of macromolecules and particulate matter from the gut lumen to pass through the mucosal barrier in order to reach the gut associated lymphoid tissue which consists of Peyer's patches, isolated lymphoid follicles, the appendix and the mesenteric lymph nodes (Keren, 1992). Peyer's patches are aggregates of subepithelial lymphoid follicles that are present throughout the small intestines of mammals and avian species (Befus *et al.*,1980; Burns, 1982) but are most prominent in the ileum. The luminal "domed" covering of epithelial cells is distinct to that of absorptive cells whereby an estimated 10% of the epithelium in humans and mice (Wolf, 1984) consist of a unique cell type called M cells. M cells first described by Owen & Jones, (1974) were named membranous epithelial cells because of their morphology and are interspersed with absorptive columnar epithelial cells and attached to adjacent cells by tight junctions. M cells contain short, sparse and irregular microvilli and the surface is invaginated to form an intracellular central pocket into which macrophages and small lymphocytes migrate (Sneller & Strober, 1986). Foreign material attach to and are internalised by M cells (Neutra & Kraehenbuhl,

1993). Pathogens including *Yersinia spp.* (Grutzkau *et al.,* 1990), *Shigella flexneri* (Wassef *et al.*, 1989), and *S. typhimurium* (Jones *et al.*, 1994) use M cells as a route of penetrating the hosts intestinal barrier.

Scattered along the avian intestinal tract lies several defined immunological structures such as bursa of Fabricus (Cloaca), cecal tonsils, Meckel's diverticulum, and Peyer's patches in the small and large intestines (Befus *et al.*, 1980; Burns, 1982). The numbers of Peyer's patches in birds can vary with age, but in 12 week old birds up to five or six can be found in the intestine, each approximately 5mm in diameter. Avian Peyer's patches resemble those of mammals for the villi are thick, the epithelium of the villi are flat and, in addition, contain pinocytic, microfold (M) cells and lack goblet cells (Befus *et al.*, 1980; Burns, 1982).

The lamina propria of day-old chicks contains little stroma containing capillaries, lacteals, muscle fibres and few lymphocytes, however, with increased age, more stromal cells are present, often accompanied by an increase in mononuclear cells (Yasson *et al.*, 1987). Avian B and T lymphocytes reside within the epithelium and the lamina propria. The B cells being positive for IgA and IgG (Jeurissen *et al.*, 1989) and a majority of the T cells are $CD4^+$ (Bucy *et al.*, 1988).

1.10.3 Mucus penetration and adherence to epithelial cells by *Salmonella*

Molecular and immunohistological techniques have demonstrated that under normal conditions the mammalian intestinal epithelial cell surface is free of adherent bacteria (Swidinski *et al.*,1998). Mucosal surfaces are coated with a thick covering of mucus which is composed of many carbohydrates (Forstner & Forstner, 1994). Little is known about how colonising pathogens penetrate the mucus barrier, although, motility has been reported to be

involved in *Vibrio cholerae* colonisation (Freter *et al.,* 1981), and also *S. typhimurium* (Schmitt *et al.,* 1994). However, other pathogens, such as *Shigella spp.* and *Yersinia spp.* penetrate the mucosal layer but are non-motile. In addition, M cells have very little mucus coating their apical surfaces and organisms penetrate through this cell type which suggests that the thick layer of mucus on columnar epithelial cells plays a major role in preventing adherence.

The mechanisms used for adherence for many pathogens are still poorly understood, but bacterial surface appendages such as fimbriae have been implicated for binding to host cell surface receptors (Smyth *et al.*, 1994). However, bacteria express non-fimbrial adhesins that can mediate adherence including, for example, the filamentous haemagglutinin of *Bordetella bronchiseptica* (Cotter *et al.*, 1998). In addition, bacterial adhesins mediate bacterium-bacterium contact that result in the formation of microcolonies, such as Enteropathogenic *E. coli* (EPEC) (Jerse *et al.*, 1990) and *Vibrio cholerae* (Iredell & Manning, 1994). This "intraspecies" adherence may aid colonisation where host receptor sites are limited.

1.10.4 Invasion of epithelial cells by *Salmonella*

Once adherent on mucosal surfaces pathogens may either localize without invasion or may breach the host barrier. Those that penetrate the surfaces can do so by passing through the cells (transcellular) or between cells (pericellular) often disrupting the intercellular junctions. Eukaryotic models, such as polarised Caco-2 cells have been used for studying bacterial interactions with host cells (Finlay & Falkow, 1990). In such models, *S. typhimurium* has been shown to cause disruption of the apical surface of the epithelium on bacterium-host interaction. There is cytoskeletal rearrangement of actin, alpha-actinin, talin, and ezrin in the host cells, whereas other cytoskeletal components such as keratin, vinculin and vimentin are

unaffected (Finlay *et al.*, 1991). Functional actin filaments are neccessary for bacterial invasion for cytochalesin D blocks internalisation (Finlay & Falkow, 1990).

1.10.4.1 Cytoskeletal rearrangement and signal transduction

S. typhimurium triggers signals in epithelial cells to initiate actin and other apical surface rearrangements and bacterial uptake. Some of the signals that are generated by host cells by invasive organisms have been identified (Bliska *et al.*, 1993). For example, *S. typhimurium* can trigger intracellular Ca^{2+} influxes in HeLa cells and chelation of intracellular Ca^{2+} blocks bacterial uptake (Ruschowski *et al.*, 1992). Also, Ginnochio *et al.* (1993) reported that mutants in the invasion loci of *S. typhimurium* that were unable to trigger Ca^{2+} release were non-invasive.

S. typhimurium also triggers the release of inositol triphosphate (IP_3) in infected epithelial cells (Rushkowski *et al.*, 1992). This event is correlated with invasion as avirulent mutants do not trigger the release of IP_3. Another signalling mechanism in eukaryotic cells is the activation of tyrosine kinase which results in tyrosine phosphorylation of specific proteins which then propagate various signals. Interestingly, Rosenshine *et al.* (1992) reported that tyrosine kinase inhibitors were capable of blocking invasion in EPEC but not in *S. typhimurium.* Additional signals that are triggered by *S. typhimurium* in epithelial cells include, activation of epidermal growth factor, stimulation of protein kinase and the involvement of leukotriene D_4 (Pace *et al.*, 1993; Galàn, 1996).

1.10.4.2 Salmonella Pathogenicity Island 1 and Type III Secretory system

A 40kb region of DNA, referred to as "*Salmonella* pathogenicity island 1" (SPI1), encompassing a contiguous region of the *S. typhimurium* chromosome at centrisome 63

encode determinants required for entry (Mills *et al.*, 1995). The following genes have been shown by the appropriate genetic and functional analysis to be necessary for entry: *inv*H, *inv*F, *inv*G, *inv*E, *inv*A, *inv*C, *inv*L, *inv*J (Galán *et al.*, 1992; Altmeyer *et al.*, 1993; Ginocchio *et al.*, 1993; Kaniga *et al.*, 1994; Eichelberg *et al.*, 1994; Collazo *et al.*, 1995), *spa*O, *spa*P, *spa*Q, *spa*R, *spa*S (Groisman & Ockman 1993; Collazo & Galán, 1996), *sic*A, *sip*B, *sip*C, *sip*D (Kaniga *et al.*, 1995a; b) and *org*A (Jones & Falkow, 1994). In addition, a polar mutation in *prg*H rendered *S. typhimurium* entry defective (Pegues *et al.*, 1995) with the mutation affecting the expression of PrgI, PrgJ and PrgK proteins. Only mutations in *inv*H had any measurable effect on the ability of *S. typhimurium* to attach to cultured cells, indicating that attachment and entry were largely independent events in *Salmonella*.

A type III protein secretion system has been identified in animal pathogens such as *Salmonella spp., Shigella spp., Yersinia spp.* and enteropathogenic *E. coli* as well as in plant pathogens such as *Pseudomonas spp., Erwinia spp.* and *Xanthomonas.spp* (Galán, 1996). The type III secretion system differs from type I or type II for it is a contact dependent secretory pathway (Galán, 1996). Interestingly, Coburn & Frank (1999) demonstrated that macrophages and epithelial cells responded differently to the type III secretion system of *Pseudomonas aeruginosa* for macrophages were efficiently killed whereas, the viability of lung epithelial cells was unaffected, which suggested that the target cells were important.

Of the 30 proteins encoded by SPI1, at least 16 constitute the type III secretion system. The mechanisms by which the different invasion proteins are exported through the two membranes of the bacterium are not well understood, however, SpaP, SpaQ and SpaR are localised within the cytoplasmic membrane, and InvG in the outer membranes. These proteins form a channel or assist with translocation of exported proteins. In addition, other

proteins such as InvC may play a role in energising the translocation machinery (Eichelberg *et al.*, 1994). Interestingly, interspecies complementation studies have indicated homology of *inv*A and *spa*P genes of *S. typhimurium* with *mxi*A and *spa*24 of *Shigella flexneri* genes, respectively (Ginocchio & Galán, 1995). In addition, Rosquist *et al.* (1995), showed that *S. typhimurium* could secrete the *Yersinia* YopE protein when expressed with its own chaperone.

Contact with host cells leads to the stimulation of secretion of *Salmonella* invasion proteins (Zierer & Galán, 1995) and Ginocchio *et al.* (1994) reported that the interaction of *Salmonella* with cultured cells lead to the assembly of surface structures called invasomes. Both required an intact type III secretion system but not *de novo* protein synthesis.

Salmonella entry of cells is subject to regulation at several levels. A number of environmental stimuli such as osmolarity (Galán & Curtiss III, 1989), oxygen tension (Ernst *et al.*, 1990) and growth phase (Lee & Falkow, 1990) significantly influenced the invasion phenotype. The mechanisms by which these different cues influence invasion are poorly understood, but, Galán & Curtiss III (1990) suggested that changes in degree of DNA superhelicity dramatically affected *inv* gene expression and the invasion phenotype. Two regulatory genes *hil*A (Miras *et al.*, 1995) and *inv*F (Kaniga et al., 1994) have been identified, but regulatory targets of both remain unknown. Another locus involved in the regulation of the invasion phenotype is the *pho*P/*pho*Q two component system. These genes have been shown to influence the expression of *prg* gene which is negatively regulated by the *pho*P/*pho*Q system (Pegues *et al.*, 1995). That the *pho*P/*pho*Q locus has been shown to regulate survival within macrophages (Miller, 1991) suggests that bacterial entry and intracellular survival are co-ordinately regulated.

1.11 The cell envelope of *Salmonella.*

Enteric bacteria have a complex molecular structure that balances the need for protection and survival with necessity for rapid proliferation.

1.11.1 The cell envelope

The cytoplasmic membrane is the boundary between the cytoplasm and the environment and is involved in a variety of cellular functions, including energy generation and conservation (Ingledew & Poole, 1984), regulated transport of nutrients and metabolic products (Kaback *et al.,* 1993), translocation of macromolecules (Sasaki *et al.*, 1990), and transmembrane signaling (Parkinson, 1993).

The rigid shape of the bacteria is determined by the cell wall and is composed of peptidoglycan (Weidel & Pelzer, 1964) which is common to all gram negative bacteria as well as a few gram positive rods (Schlelfer & Kandler, 1972) and is necessary to preserve the integrity of the cytoplasmic membrane from rupture in environments of low osmolality (Cooper, 1988).

The periplasmic space lies between the inner and outer membranes of gram-negative bacteria. The molecules within the periplasm create a microenvironment that buffers the cell from changes that occur in surroundings. Periplasmic enzymes promote biogenesis of components for incorporation into the peptidoglycan and outer membrane, including LPS and proteinaceous appendages, such as fimbriae (Bardwell, *et al.* 1991). A large family of periplasmic chaperones are required for the assembly of fimbriae. Most genes encoding fimbriae-specific chaperones are located in fimbrial operons that are contained on

chromosomal segments or plasmids (Jones *et al.*, 1993). Unlike cytoplasmic chaperones which recognise their substrates in unfolded states, fimbrial-specific chaperones maintain their substates in a conformation similar to that of their native state (Kuehn *et al.,* 1991). The fimbrial-specific chaperones cap interactive surfaces and prevent aggregation and degradation of the proteins within the periplasm (Soto *et al.,* 1998). Chaperones also possess an effector function by targeting the subunits to an outer membrane protein that controls their assembly (Hultgren & Normark, 1991).

The outer membranes of Enterobacteriaceae contain lipopolysaccharide (LPS), and phospholipids, as well as several proteins. LPS is composed of the proximal, hydrophobic, lipid A region, the distal, hydrophilic, O-antigen polysaccharide region that protrudes into the external environment; and the core oligosaccharide region that connects the two. Studies by Vaara, (1993) with mutants that produced incomplete LPS molecules suggested that the loss of O-antigen resulted in reduced virulence, whereas loss of the the proximal part of the core rendered bacteria sensitive to a wide range of hydrophobic dyes, antibiotics, bile salts and detergents which implied that this region acts as a barrier for the membrane (Vaara, 1992).

The proteins of the outer membrane include murein lipoprotein (Braun & Wu, 1993) and OmpA proteins (Schweizer *et al.*, 1978) which assist in stabilising the membrane (Hirota, *et al.* 1977; Nikaido & Vaara, 1985). In addition, porins produce non-specific channels that allow the passage of small hydrophilic molecules across the outer membrane (Benz, 1988). Porins of *S. typhimurium* have been studied extensively and are finely regulated by environmental stimuli. For example, synthesis of OmpF, which forms a porin with a large channel is repressed by high osmolarity and high temperatures (Nikaido, 1992). By switching channels the permeability to nutrients would not be reduced significantly, whereas

permeability towards larger, more hydrophobic or negatively charged compounds would be dramatically reduced (Nikaido, 1992; Madeiros *et al.*, 1987). Conversely, the bacterium would benefit from the OmpF porin when out of the host, for example, in rivers and ponds, and the wider channel would allow more efficient uptake of nutrients from a very dilute environment. The *omp*R gene encode elements of the two-component regulatory system OmpR-EnvZ which responds to changes in osmolarity and regulates expression of the porin proteins OmpF and OmpC in *E .coli* and *Salmonella* (Pratt & Silhavy, 1995). Interestingly, in *Salmonella*, the OmpR-EnvZ system also regulates other genes including *sif*A which encodes *Salmonella*-induced filaments in Hela cells (Mills *et al.,* 1998) and *aas* which encodes for 2-acylglycerolphosphoethanolamine acyltransferase which is induced under acidic conditions and inside macrophages (Valdivia & Falkow, 1996).

1.11.2 Flagella and Motility

Most *Salmonella* serotypes are motile, moving through liquid media and undergoing directional change. When observed by electron microscopy *Salmonella* possess thin, helical appendages named flagellar filaments. Flagella vary in number, but protrude randomly around the cell surface known as peritrichous flagellation. The bacterial flagellum has a diameter of approximately 20nm and consists of subunits of just one protein (Macnab,1992). The flagellum is driven by a motor at its base (Berg,1974) which has a switch and can operate in a counterclockwise or clockwise direction, and the energy source is the proton motive force (Manson *et al.*, 1977). The flagellum has a long helical filament (Namba *et al.* 1989), a short curved structure called the hook and a basal body (DePamphilis & Adler, 1971a), which consists of a central rod and several rings. The filament and hook are external to the cell,

whereas the basal body is embedded in the cytoplasmic membrane (DePamphilis & Adler, 1971b).

Rings act as a mounting plate for components necessary for motor switching and rotation; the rod is a shaft that transmits torque from the motor to the external hook and filament, and a static ring pair act as a bushing through which the rod passes preventing damage to the bacterial envelope from lateral shear forces.

1.11.3 Fimbriae

Fimbriae are proteinaceous, hair-like appendages, ranging from 2 to 8 nm in diameter projecting from the surface of bacteria (Duguid *et al.*, 1955) and are composed of protein subunits termed fimbrins that range in size from 14 to 30 kDa from Enterobacteria (Thorns, 1995). Fimbriae of *E. coli* have been studied extensively (Smyth *et al.*, 1994) and in particular those of Enterotoxigenic *E. coli* such as F4 and F5, referred to as K88 and K99 respectively, that are responsible for the attachment of the bacterium to villous epithelial of the small intestine of domestic animals (Thorns *et al.*, 1989). However fimbriae are not restricted to Enterobacteria. For example, Hamada *et al.* (1998) reviewed the importance of fimbriae in virulence of oral bacteria and suggested that fimbriae from gram-negative bacteria such as *P. gingivitis* exhibited a wide variety of biological activities including immunogenicity, binding to various host proteins and stimulation of cytokine production, whereas, those from *Prevotella loescheii* were found to cause coaggregation with other bacteria, including *Actinomyces viscosus*. In addition, fimbriae from Gram-positive oral bacteria such as *Actinomyces* and *Streptococci spp.* were responsible for co-aggregation, binding to saliva-coated hydroxyapetite or glycoproteins of oral epithelial cells.

1.11.3.1 Classification of fimbriae

E. coli and *Salmonella spp.* express a wide variety of different fimbriae and single bacterial isolates can express more than one type simultaneously. Historically, *E. coli* and *Salmonella* fimbriae have been classified based on their structure, phenotypic traits or adhesive properties. For example, the "F type" classification of *E. coli* described by Orskov & Orskov (1990) was based upon primary amino acid sequences of major fimbrial subunit proteins. However, this classification was restrictive for many fimbrial operons also code for minor fimbrial subunits, which may or may not share similarities with the major subunit (Hansom & Brinton, 1988).

The adhesive properties of bacteria have also been used for classification and is the most commonly used method for classifying *Salmonella* fimbriae (Thorns, 1995). Duguid *et al.* (1955) first noticed a correlation between expression of fimbriae and the ability to agglutinate certain species of erythrocytes by *E. coli* and *Shigella spp*, and later in *Salmonella spp., Klebsiella spp.* and *Proteus spp.* (Duguid & Gilles, 1958). Duguid *et al.* (1966) reported that the addition of mannose-containing carbohydrates interfered with the agglutination reaction and a study of 1400 *Salmonella* strains classified fimbriae on their ability to mediate erythrocyte agglutination in the presence and absence of D-mannose. Currently, adhesive properties in combination with the fimbrial diameter forms the basis for classification of *Salmonella* fimbriae.

Type 1 fimbriae are rigid structures, approximately 7nm in diameter that mediate mannose sensitive haemagglutination (MSHA). Type 2 fimbriae are morphologically similar to type 1 but lack the ability to agglutinate erythrocytes. They were first described on strains of *S.*

gallinarum and *S. pullorum* (Duguid & Gilles, 1958) and later on strains of *S. paratyphi* B and *S. dublin* (Duguid *et al.,* 1966). A close antigenic relationship exists between these fimbriae and Clegg & Gerlach, (1987) suggested that type 2 fimbriae were non-agglutinating variants of type 1 and Minion *et al.* (1986) demonstrated that separate genes encoded structural and haemagglutinating components of *E. coli* by the construction of recombinant plasmids. These results were also confirmed by Hanson & Brinton, (1988).

Type 3 fimbriae are thinner, more flexible structures with a diameter of 3-5 nm which mediate the agglutination of tannic acid treated erythrocytes in the presence of ?-D- mannose (Duguid *et al.*, 1966). Type 3 fimbriae were designated mannose resistant haemagglutination (MRHA) as opposed to mannose sensitive haemagglutination (MSHA) conferred by type 1 fimbriae . A strong antigenic homology exists between type 3 fimbriae expressed by *Salmonella spp.*, *Klebsiella spp.* and *Yersinia spp.* when examined by immunoelectron microscopy (Adegbola *et al.,* 1983; Old & Adegbola., 1985).

Type 4 fimbriae were originally described as thin flexible fimbriae of approximately 4 nm in diameter, that mediate agglutination of erythrocytes in the presence of mannose (MRHA), and have been described on a strain of *S. typhimurium* isolated from a pigeon (Grund & Weber, 1988; Grund & Seiler, 1993). More recently, two other fimbriae elaborated by *S. enteritidis* have been described which do not fit into the classification because of their size and inability to haemagglutinate erythrocytes. These have been designated SEF14 (Thorns *et al.*, 1990) and SEF17 (Collinson *et al.*, 1991).

Hansom & Brinton, (1988) have shown that the adhesin of type 1 fimbriae from *E. coli* is different from the major fimbrial subunit, however, the major fimbrial subunit K88 and K99

fimbriae of enterotoxigenic *E. coli* is reported to contain the adhesin (de Graff *et al.*, 1984; Bakker *et al.*, 1992). Therefore, identification of the adhesin binding characteristics does not necessarily describe the major fimbrial subunits themselves, and this can only be determined by DNA and amino acid sequencing.

1.11.3.2 Fimbriae of *S. enteritidis*

SEF14

SEF14 were first described on strains of *S. enteritidis* (Thorns *et al.*, 1990; Müller *et al.*, 1991), but was later found to be unique to certain serotypes in serogroup D, including all strains of *S. enteritidis* and a proportion of *S. dublin* strains (Thorns *et al.*, 1992). The fimbrial structure is <3 nm in diameter and composed of repeating fimbrin subunits 14.3 kDa in size. The structural gene, *sef*A, has been cloned and sequenced and shown to be limited in distribution to serotypes in group D. However, *S. gallinarum*, *S. pullorum* and *S. typhi* possess the *sef*A gene but do not express SEF14 fimbriae (Turcotte & Woodward, 1993).

The genes that encode the expression of SEF14 fimbriae have been fully characterised. The *sef*ABCDE genes make up part of the *sef* operon responsible for SEF14 assembly and expression by *S. enteritidis* (Clouthier *et al.*, 1993; 1994). The *sef*A gene encodes the SEF14 structural fimbrin subunit, *sef*B gene encodes the subunit chaperones, which are homologous to *E. coli* and *Klebsiella pneumoniae* fimbrial periplasmic chaperone proteins. The *sef*C gene encodes the fimbrial usher proteins located on the outer membrane that are thought to be responsible for the organisation and assembly of subunits into the polymeric fimbriae. The *sef*D gene appeared to encode another fimbrial type named SEF18 (Clouthier, 1994), but the phenotype has not been expressed *in vitro* nor confirmed by other workers. Collighan & Woodward (unpublished observations) showed the presence of *sef*E and knock out mutants

made in this gene failed to express SEF14 fimbriae which support the role of *sef*E gene product in regulation of expression.

SEF17

SEF17 fimbriae are 3 to 4 nm in diameter, extremely hydrophobic structures composed of repeating subunits of 17 kDa molecular mass, which are reported to mediate binding to tissue matrix proteins (Collinson *et al.*, 1991; 1993). The operon encoding SEF17 has been characterised and comprises two co-located but distinct gene clusters *agf*BAC (Collinson *et al.*, 1996a; b) and *agf*DEFG (Römling *et al.*, 1998a; b). The *agfA* gene encodes the fimbrin subunit AgfA, which is genetically related to curli fimbriae of *E. coli* (Collinson *et al.*, 1992). The role of AgfB is unknown, but resembles AgfA in size and primary amino acid sequence which suggest that it is a minor fimbrin-like protein (Collinson *et al.*, 1996a). The third open reading frame, *agf*C, also belongs to this operon since no transcriptional start site between *agf*A and *agf*C was found. However, an *agf*C transcript has not been detected (Collinson *et al.*, 1996a). The same group of researchers have speculated that *agf*C may contribute to the stabilisation of the *agf*BA transcript and the individual *agf*A transcripts. The *agf*BAC operon does not encode proteins characteristic of chaperone or usher proteins typical for fimbrial biosynthesis (Hultgren & Normark, 1991)

Doran *et al.* (1993) reported that a DNA probe which targeted the structural gene *agf*A strongly hybridised with 603 of 604 clinical isolates of *Salmonella* of various serotypes which suggested that the gene was conserved across the *Salmonella* genus, however, little is known about the *in vitro* conditions for expression of SEF17, since most research has focused on the single *S. enteritidis* strain 27655R.

Regulation of aggregative fimbriae of *Salmonella* and curli of *E. coli* is complex and similarities have been described between *S. typhimurium* and *E. coli* for the biogenesis of thin aggregative fibres. Hammer *et al.* (1995) demonstrated the role of two co-located but distinct gene clusters, *csg*DEFG and *csg*BAC, which were transcribed in opposite orientations for the regulation and expression of curli in *E. coli*. A similar gene arrangement, *agf*DEFG and *afg*BAC, was recently described for *S. typhimurium* by Römling *et al.* (1998a). Also, expression of thin aggregative fimbriae and curli by *S. typhimurium* and *E. coli* strains, respectively, required the stationary sigma factor σ^s, encoded by the global regulator *rpo*S (Olsen *et al.*, 1993; Römling *et al.*, 1998b) and transcriptional regulator *omp*R. Interestingly, Olsen *et al.*, (1993), reported that mutations in the *hns* gene encoding the histone-like protein H-NS, relieved the necessity for *rpo*S in curli production in *E. coli*.

SEF21

Type 1 fimbriae of *S. enteritidis*, referred to as SEF21 (Müller *et al.*, 1991), are rigid structures, approximately 7nm in diameter and up to 100nm in length. They consist of identical protein subunits of approximately 21 kDa (Korhonen *et al.*, 1980), that are non-covalently linked around a hollow core, which gives a channelled appearance when viewed by electron microscopy (Müller *et al.*, 1991).

A 13.7 kb chromosomal region of *S. typhimurium* encodes the entire type 1 gene cluster (Clegg *et al.*, 1987) and nucleotide sequence analyses has identified nine open reading frames (Swenson *et al.*, 1992; 1994). The structure of the operon is similar to that encoding type 1 fimbriae of *E. coli* apart from haemagglutinin and regulatory genes. The *fim*A gene encodes the 21 kDa protein which is the major structural gene. Extensive cross-reactive studies with polyclonal antisera (Müller *et al.*, 1991) and monoclonal antibodies (Sojka *et al.*, 1996) raised

to SEF21 have shown phenotypic conservation amongst *Salmonella*. The *fim*I gene encodes a 16 kDa protein which shares 65% homology with FimA but the role of this protein is unclear but may be a minor component of the mature fimbrial structure (Rossilini *et al.,* 1993). Genes *fim*C and *fim*D encode proteins of 25 and 82 kDa respectively, which perform chaperone and usher functions for fimbrial synthesis (Hultgren *et al.*, 1991), whilst *fim*F encodes a minor protein which may have a role in initiation of fimbrial synthesis (Russell & Irndorff, 1992). The *fim*H gene encodes a 34 kDa protein which is related to the SfaS protein of *E. coli* which probably confers mannose sensitive haemagglutination properties of *Salmonella* (Krogfelt *et al.,* 1990). *fim*ICDHF are transcribed polycistronically from a single promotor (Swenson *et al.*, 1992), whereas three genes encoding regulatory functions, *fim*ZYW are transcribed in the opposite direction (Yeh *et al.*, 1995).

PEF and LPF

Genetic evidence suggests that *S. enteritidis* may elaborate a further two fimbriae, Plasmid encoded fimbriae (PEF) and Long Polar Fimbriae (LPF). The virulence plasmid of *Salmonella* referred to as "Serotype Associated Plasmid" or SAP (Browning & Platt, 1995) has been shown to contribute to the systemic phase of *S. typhimurium* infection in murine model (Pardon *et al.*, 1986) and *S. dublin* in bovine (Wallis *et al.*, 1995). The genetic loci, *spv*, has been described by Gulig *et al.* (1993) and transposon mutants produced in a region distinct from *spv* (Sizemore *et al.,* 1991) resulted in reduced invasiveness of *S. typhimurium* in the mouse model and the site of the mutation was demonstrated to encode PEF (Friedrich *et al.,* 1993). Woodward *et al.* (1996), demonstrated the presence of *pef* sequences encoded by the SAP's of *S. enteritidis*, *S. cholerae-suis* and *S. bovis-mobificans*. In addition, evidence has emerged that *S. enteritidis* encode *lpf*-like sequences (Baumler & Heffron, 1995),

however, evidence to demonstrate that LPF or PEF may be expressed in *S. enteritidis* has not been gained.

1.11.3.3 Fimbriae as diagnostic antigens

The first diagnostic tests based on fimbrial antigens were developed for the detection of enterotoxigenic *E. coli (ETEC)* (Sojka *et al.*, 1971; Orskov *et al.*, 1975; Guinee *et al.*, 1976). Fimbrial adhesins such as F4 and F5 are essential for *E. coli* to cause disease in calves and piglets and, therefore, their detection on isolates from clinical cases of diarrhoea were utilised as a diagnostic antigens. Macroscopic agglutination tests were developed for the detection of fimbriae expressed by ETEC by coating latex beads with monoclonal antibodies specific for these bacterial adhesins and mixing with stool samples or isolated bacteria (Thorns *et al.* 1989a; b). Using the same technique Thorns *et al.* (1990) developed a latex agglutination test for the specific detection of *S. enteritidis* which was based on the the SEF14 fimbrial antigen and extensive evaluations demonstrated the test to be an accurate, presumptive test for *S. enteritidis* (McLaren *et al.*, 1992, Thorns *et al.*, 1993a; b). In addition, Thorns *et al.* (1996) reported that chickens infected with *S. enteritidis* produced a serological response to SEF14 within 10 days of infection and that the IgG response persisted for at least 4 weeks. These results suggest that the detection of SEF14 antibodies in chicken serum could be used as a diagnostic tool, or alternatively, the fimbrial structure could form the basis of a serodiagnostic test *for S. dublin* in cattle (Thorns, 1995).

Surface antigens such as LPS, flagellin and OMP have been used as the basis of *Salmonella* genus tests in ELISA (Kerr, 1992; Feldsene *et al.*, 1992; Wyatt *et al.*, 1993) and agglutination tests (Clark *et al.*, 1989; Manafi & Sommer, 1992). However, Doran *et al.* (1993; 1996) developed a fimbrial gene probe that was used as a genus-specific diagnostic tool and

reported that 99.8% of *Salmonella* isolates react with a DNA based test which targeted the *agf*A structural gene of SEF17 whereas 11.6% of other members of the Enterobacteriaceae bound, albeit weakly.

1.11.6.4 Fimbriae as virulence determinants of *Salmonella*.

Many studies have reported that fimbriae of *Salmonella* mediate either attachment to or invasion of mammalian tissue culture cell lines or isolated enterocytes (Tavendale *et al.*, 1983; Lindquist *et al.*, 1987; Ernst *et al.*, 1990; Craven *et al.*, 1992; Isaacson & Kinsel, 1992). Specifically, Kukkonen (1993) reported that type 1 fimbriae of *S. enteritidis, S. typhimurium* and *E .coli* specifically bound to oligomannoside chains of the laminin network in basement membranes, whereas type 3 fimbriae of *S. typhimurium* bound to human type V collagen (Tarkanen *et al.*, 1990), which implicated potential mechanisms for host colonisation of intestinal epithelium.

However, few investigations have been performed using animal models to study the role of *Salmonella* fimbriae in the infective process and most of the earlier experiments have been performed using poorly defined variants rather than defined well characterised mutants. Early studies revealed differences in infectivity between fimbriated (fim^+) and non-fimbriated (fim^-) strains of *S. typhimurium* isolated from pigs or dogs following oral inoculation of mice (Darekar & Duguid 1972; Duguid *et al.*, 1976; Tanaka *et al.*, 1977; Tanaka & Katsube, 1978; Tanaka *et al.*,1981).

Many *Salmonella* serotypes, including *S. enteritidis, S. typhimurium* and *S. dublin* are able to establish persistent carriage in their host by colonising specific niches and selective trapping may aid the bacteria in this process. Eisenstein, (1987) reported that *Salmonella* could exhibit

phase variation between phenotypically fimbriated and non-fimbriated states, which was influenced by the environment. These results have recently been supported by Lim *et al.* (1998) who demonstrated *in vivo* phase variation of *E. coli* type 1 fimbrial genes in women with urinary tract infections. Also, in one study in the murine typhoid model non-fimbriated *S. typhimurium* were more prevalent in the blood, whereas fimbriated bacteria were most commonly found in the spleen and liver of mice which had been orally infected with a mixed population of fimbriated and non-fimbriated bacteria (Lockman & Curtiss, 1992a). Similarly, Leunk *et al.* (1992) reported that fimbriated strains of *S. typhimurium* were cleared more rapidly from the blood of inbred mice than non-fimbriated bacteria, and that endothelial and Kuppfer cells of the liver and phagocytic cells of the spleen and kidneys were observed to trap fimbriated bacteria. In addition, Keith *et al.* (1990) demonstrated that attachment and internalisation in phagocytes was mediated by type 1 fimbriae of *E. coli* and *S. typhimurium* and suggested that type I fimbriae decreased the virulence of *Salmonella* in this model by reducing bacteraemia and increasing the rate of clearance. However, Lockman & Curtiss (1992b) reported that a double mutant of *S. typhimurium* deficient in the expression of flagella and type I fimbriae exhibited a 50% lethal dose of 10^7 CFU's in orally infected mice, whereas the wild type and single mutants exhibited a 50% lethal dose of $2x10^4$ which offers evidence of a synergistic effect between surface appendages of *Salmonella* in the pathogenic process.

Although indigenous bacteria intimately colonise the intestinal mucosa, under normal conditions the intestinal epithelia is free of adherent bacteria. However, Hendrickson *et al.*, (1999) reported that a 7,500-fold increase in the numbers of commensal *E. coli* had adhered to the caecum following starvation of mice. Immmunostaining and electron micrographs revealed that type 1 fimbrial expression were more abundant in the commensal *E. coli*

harvested from the caeca of stressed mice which implicated a role for type 1 fimbriae in the adherence of *E. coli* to the caeca of mice.

In addition, Mulvey *et al.*, (1998) demonstrated that type 1 fimbriae of Uropathogenic *E. coli* were responsible for induction and evasion of host defences. High-resolution electron microscopy showed that type 1 fimbriae of *E. coli* interacted directly with the lumenal surface of the mouse bladder. Bacterial attachment resulted in exfoliation of host bladder epithelial cells as part of an innate host defence system, however, *E. coli* resisted clearance within the bladder by invading into the epithelium.

Expression of LPF by *S. typhimurium* has been shown to mediate adherence to murine Peyer's patches (Baumler *et al.*, 1995) and a PEF insertion mutant was demonstrated by Baumler *et al.* (1996) to associate in lower numbers with murine small intestine epithelial cells than wild type.

Enteropathogenic *E. coli* (EPEC) that exhibit a localised adherence phenotype have been shown to express "bundle forming pili" (BFP) encoded by the *bfp* gene cluster (Donnenberg *et al.,* 1991) which mediate bacterium-to-bacterium contact that results in the formation of micro-colonies and may aid colonisation where host receptor sites are limited (Jerse *et al.*, 1990).

1.12 Aims of the project

Although the expression of fimbriae by certain strains of *Salmonella* was first described over 40 years ago (Duguid & Gilles, 1958), until recently little was known of the variety of

Salmonella fimbriae and their functions. Within the last decade, the rapid spread of *S. enteritidis* through the poultry population and subsequent increase in human food poisoning cases caused by this serotype has influenced a renewed interest in the molecular and antigenic characterisation and functions of surface antigens. There are numerous examples of fimbriae as key components in cell-to-surface and cell-to-cell adherence in various bacteria, however, the role of these surface appendages of *S. enteritidis* in colonisation and invasion of the avian host remains unknown.

S. enteritidis strains have been shown to elaborate SEF14, SEF17, SEF21 and flagella. However, little information is available on expression of SEF17 fimbriae on *S. enteritidis* strains other than 27655R (Collinson *et al.*, 1991). Therefore, the aim of this study was to evaluate the elaboration of SEF17 in response to environmental stimuli and relative phase of growth, and to compare with *in vitro* expression of SEF14, SEF21 and flagella by *S. enteritidis* strains. In addition, an *in vitro* model will be developed and used to study fimbrial and flagella-mediated interaction with tissue culture cells. Finally, the contribution of SEF14, SEF17, SEF21 and flagella of *S. enteritidis* in colonisation and invasion of chicks, persistence in young birds, and their role in lateral transfer will be investigated.

The thesis contains four results chapters (chapters 3, 4, 5 and 6) and in each of these chapters the tables and figures follow the results sections. All materials and methods are described in chapter 2.

Chapter 2

Materials and methods

2.1 Bacterial strains and cultural conditions.

2.1.1 Bacterial isolates

S. enteritidis strain 27655R and 27655S were isolated from a human patient with diarrhoea (Baloda, 1988) but kindly donated by Professor T. Wadström, Lund, Sweden. *S. enteritidis* strains E, I and C6a were obtained from Professor T. Humphrey, PHLS, Exeter, Devon, UK. The human *E. coli* strain NG7C (Collinson *et al.*, 1992) and pigeon isolate *S. typhimurium var. copenhagen* strain Mö8 (Grund & Weber, 1988) were supplied by Dr. L. Emödy, Budapest, Hungary. All other *S. enteritidis* strains were obtained from the reference collection at the Veterinary Laboratory Agency, Weybridge, UK. A full list of strains are presented in table 2.1 on page 64.

2.1.2 Isogenic fimbrial and flagella mutants

The single and multiple defined mutants of *S. enteritidis* strains LA5, S1400 and 27655R have been produced recently by Emma Allen-Vercoe in this laboratory (Allen-Vercoe *et al.*, 1999). Initially, single mutations were prepared in *S. enteritidis* strain S1400 and mutations were transduced into strains LA5 and 27655R by P22-mediated transduction. All methods used for the construction of mutants are described in appendix 5.

2.1.3 Bacterial culture

For studying the expression of fimbriae and flagella the bacteria were cultured on solid agar and planktonic growth. Agar media used included Colonisation Factor Antigen (CFA) agar with or without Congo red, Drigalski agar, Luria-Bertami agar, MINCA agar, Nutrient agar, Sensitest agar and Brilliant Green agar. Liquid media included Heart Infusion broth (HIB), Nutrient broth, CFA broth, Luria-Bertami broth, Peptone water, Tryptone water, and Eagles

Minimal Essential Media (EMEM) supplemented with 0.29 g/l L-glutamine. Medium composition is described in Appendix 2.

Bacteria were grown in an aerobic environment unless otherwise stated. However, anaerobic growth conditions was generated with a Gas Generating Kit (Anaerobic system, BR38, Oxoid) in a gas jar, and carbon dioxide (CO_2) enriched air conditions in a 37°C incubator supplemented with 5% CO_2. Cultures were incubated at various temperatures including 18°, 25°, 30°, 37° or 42°C.

To test for aggregative colonies bacteria were grown on CFA agar for 48 hours at various temperatures and colonies were recorded as non-aggregative, weakly aggregative or strongly aggregative according to how difficult the bacteria were to remove from the agar surface with an inoculating loop. To test for the formation of "lacy" colony bacteria were grown on CFA agar for 48 hours at 25°. Bacteria were grown on CFA agar containing 0.01% congo red for 48 hours at various temperatures to test for the uptake of the hydrophobic dye.

2.1.4 Growth curves of mutants

S. enteritidis wild type and mutants cultured in Nutrient broth (NB) overnight at 37°C were shaken in fresh broth for 30 mins before placing in 100ml of pre-warmed NB at a density of approximately $5x10^4$ bacteria per ml media. Cultures were incubated statically at 37°C and colony forming units (CFU) estimated by serial dilution's at times 0, 1, 2, 3, 5, 7, 8, 24, 48 and 72 hrs. Dilution's were plated onto Brilliant Green agar.

2.2 Detection, purification, analyses and characterisation of surface appendages

2.2.1 Latex agglutination for detection of SEF14

SEFEX is a commercially available latex agglutination kit (VLA) for the detection of *S. enteritidis*. The diagnostic kit is specific for SEF14 fimbriae and was performed in accordance to the kit instructions. To induce SEF14 expression *S. enteritidis* strains were inoculated onto Sensitest agar plates and incubated at 37°C overnight. For the test, 50µl glycine buffer was added to duplicate circles marked on the latex agglutination card and a sweep of colonies was resuspended in each. To one bacterial suspension was added 50µl test latex, which contained Mab passively adsorbed to latex beads, and control latex coated containing normal mouse sera adsorbed to latex beads, was added to the other. Suspensions were mixed thoroughly on an orbital shaker at 30 revolutions per minute, for two minutes, and macroscopic agglutination was recorded. Agglutination with test latex only, indicated expression of SEF14 by bacteria, whereas, agglutination with test and control latex suggested autoagglutination, as demonstrated by figure 2.1.

2.2.2 Mannose sensitive haemagglutination (MSHA)

S. enteritidis strains were grown in 10 ml of HIB and incubated at 37°C for 48 hours. Bacteria were pelleted by centrifugation at 2500 g for 10 mins and resuspended in one ml 0.1M PBS, pH7.2. For the test, 50µl of PBS was added to a well of a ceramic tile and 50µl of 3% mannose in PBS to a duplicate well. To each well was added 50µl of bacterial suspension and 50µl of glutaraldehyde stabilised horse red blood cells (Sigma) containing a 3%

haematocrat (v/v). The tile was rotated on an orbital shaker at 60 revolutions per min for 2 mins and haemagglutination was recorded as shown in figure 2.2.

2.2.3 Transmission electron microscopy (TEM)

Cultured bacteria were washed by centrifugation at 2,500 g for ten minutes and a turbid bacterial suspension containing approximately 10^{10} bacteria per ml was prepared in 0.1M PBS, pH 7.2. Carbon-formvar coated grids were floated on top of a 50 µl drop of cell suspension for 15 minutes, blotted dry, and counterstained with 1% (w/v) phosphotungstic acid for 15 seconds. After blotting dry grids were viewed by TEM.

2.2.4 Purification of SEF14 fimbriae

SEF14 was purified using the method described by Thorns *et al.* (1990). *S. enteritidis* strain LA5 was grown on 50 Sensitest agar plates overnight at 37°C. Bacteria were harvested into 20 ml 0.1M phosphate buffered saline, pH 7.2 and washed by centrifugation (2,500 g for 10 mins). Fimbriae were then removed from the surface of the bacteria by agitation whilst heating the suspension to 60°C in a sonicating water bath for 30 mins. The cell free supernatant was then ultracentrifuged at 50,000 g for 2 hrs and the sedimented pellet discarded. The supernatant was applied to a size exclusion chromatography column (Bio-Sep Sec 4000 HPLC, Phenomenex) at a flow rate of 2 ml per min.. Fractions (2 ml) were collected and examined for antigenic activity in the direct-binding ELISA. Fractions containing SEF14 were pooled, concentrated by ultrafiltration using a centrifugal concentrator (Amicon) with a 10,000 Da cut-off membrane and the purity of the preparation was determined by SDS-PAGE using 12.0% gels.

2.2.5 Purification of SEF17 fimbriae

SEF17 fimbriae was purified from *S. enteritidis* strain 27655R by the method described by Collinson *et al.* (1991). Strain 27655R and a spontaneous variant 27655S deficient for expression of SEF17 were grown on 30 CFA agar plates containing 100μg congo red, for 48 hours at 30°C and the bacteria harvested in 30ml 10mM Tris HCL, pH 8.0. The cells were broken by sonication and digested at 37°C for 20 mins with 0.1μg/ml RNase (Sigma), DNase (Sigma) and 10mM magnesium chloride (Sigma) with gentle agitation. Ten mg of lysozyme (Sigma) per ml was added to the digest and incubated for a further 30 mins before adding 10mg sodium dodecyl sulphate (Lauryl Sulphate, Sigma) per ml. Following an incubation for 40 mins at 37°C the insoluble material was pelleted by centrifugation and resuspended in 10mM Tris HCl buffer. The digestion was repeated, the pellet washed in deionised water, resuspended in sample buffer and boiled for 5 mins before running on SDS-PAGE. The insoluble material not entering the stacking gel was removed with a pipette, sonicated to break up the clumps, and boiled in 0.2M glycine buffer pH 1.5. The insoluble fimbriae was pre-treated with 90% formic acid , and the acid removed by drying the preparation on a Gyrovap (Howe) for 4 h at 45°C. The pellet was resuspended in sample buffer and run on SDS-PAGE.

2.2.6 Purification of SEF21 fimbriae

SEF21 was purified from *S. enteritidis* strain LA5 using the method described by Sojka *et al.* (1996). LA5 was inoculated into 10 mls of HIB and grown in static culture at 37°C for 48 hrs. The whole culture was then transferred to one litre of HIB, pre-warm to 37°C, and incubated for a further 48 hrs. The cells were harvested at 10,000 g for 30 mins, resuspended in 25 mls of 0.1M potassium phosphate buffer containing 1mM EGTA and 0.5mM $MgCl_2$

(Utsunomiyia, 1988). The fimbriae were detached by blending for 10 mins at 20, 000 rpm on ice using a Omni 1000 homogeniser (Camlab) and then centrifuged for 20 mins at 10,000 g. The supernatant was ultracentrifuged at 100,000 g at 15°C for 1 hr and sedimented flagella and cell fragments were discarded and supernatant containing fimbriae was concentrated by ultrafiltration using a centrifugal concentrator (Amicon) with a 10,000 Da cut-off membrane. The concentrated preparation was further purified by gel filtration on a Bio-Sep Sec 4000 HPLC column (Phenomenex) and equilibrated with 0.1M sodium phosphate buffer, pH 6.8. The crude SEF21 preparation was separated at one ml per minute flow rate and fractions were dissociated into subunits by mixing 1:1 with low pH (1.8) sample buffer containing 0.2M glycine, boiling for 5 mins and resolving on SDS-PAGE with a 12.0% gel. Fractions containing semi-pure SEF21 fimbriae were boiled for 5 mins in 5% 2-mercaptoethanol (Sigma) and pelleted by ultracentrifugation at 200,000 g for 4 hrs. The pellet was resuspended in deionised water and analysed for SEF21 fimbriae as described above.

2.2.7 Purification of flagella

The method used for purification of flagella was amended from that described by Kondoh and Hotani (1974). Briefly, 10 mls of Nutrient broth was inoculated with *S. enteritidis* strain LA5 and shaken aerobically overnight at 37°C and transferred to one litre of pre-warmed Nutrient broth. The culture was again aerobically shaken overnight at 37°C before sedimenting the bacteria by centrifigation at 8000 g for ten mins. The pellet was resuspended in 0.1M potassium phosphate buffer (PPB) pH 6.8, blended for 3 mins on ice at 20, 000 rpm using an Omni 1000 homogeniser (Camlab) and then centrifuged for 20 mins at 10,000 g. The supernatant was then ultracentrifuged at 50,000 g for 2 hrs and the sedimented pellet containing the flagella reconstituted into 2 mls of PPB. The flagella preparation was

depolymerised by boiling in sample buffer for 5 mins before resolving on a SDS-PAGE with a 15% separating gel.

2.2.8 SDS-PAGE and Coomassie blue staining

Gels were prepared as described in appendix 4 and SDS-PAGE was performed according to the method described by Laemmli, (1970) in 12.0 or 15.0% polyacrylamide resolving gels, 5% stacking gel with a discontinuous Tris/HCl buffer system (Biorad). Gels were immersed in electrophoresis running buffer and prior to electrophoretic separation samples were boiled for 5 mins in equal volumes of sample buffer. 10 µl of test sample or 5µl protein standards were loaded per well and the gel run at 200 volts for 40 mins. Gels were washed twice in deionised water with gentle agitation for 5 mins per wash and stained with Coomassie blue using Gelcode Blue Stain (Pierce). After staining for one hr, gels were immersed in water to remove excess stain.

2.2.9 Total protein estimation

Total protein concentration was estimated using the Bicinchononic acid colorimetric method (Pierce) according to the manufacturers instructions.

2.2.10 Fibronectin binding immunoassay

The ELISA was performed as described for the direct-binding assay (section 2.3.4). Bacteria were washed and coated to microwells in carbonate buffer and dried onto the plates overnight. After blocking with 3% dried milk, 1µg human fibronectin (Sigma) was added per well. Bound fibronectin was detected with rabbit anti-human fibronectin antibody (Sigma), and this complex detected with goat anti-rabbit IgG conjugated with horseradish peroxidase

(Sigma). TMB substrate was used to develop the assay and the reaction stopped with sulphuric acid.

2.3 Production and analyses of immune reagents

2.3.1 Production of monoclonal antibody (Mabs)

Two female BALB/c mice (6-8 weeks old) were injected subcutaneously with 100 μg of each purified antigen in FCA, separately. Booster injections were given (50 μg) with FIA adjuvant on days 28, 42 and 56 after the first injection. The mice were bled five days after each booster injection and the serum used to assess the antibody response by Western blot or ELISA. Spleens were removed 3 days after the final boost (day 45 for flagella and day 59 for SEF17), and the splenocytes fused with NS0/1 myeloma using PEG 4000 at ratio of 2:1 by the method described by Köhler & Milstein (1975). Hybridomas were selected in media containing 5mM hypoxanthine, 0.02 mM aminopterin and 0.8mM thymidine (HAT, GIBCO) and supernatants were screened for specific Mab secretion by ELISA in a direct-binding immunoassay using purified flagella, or whole cells (*S. enteritidis* strains 27655R and 27655S) for SEF17. Hybridomas from selected wells were cloned twice by limiting dilution, expanded and secured in liquid nitrogen . The isotype of Mabs were determined by Ouchterlony immunodiffusion technique using a Mab typing kit (ICN Biomedicals Inc.) in accordance to the manufacturers instructions.

2.3.1.1 Screening of Mabs for SEF17

Tissue culture supernatants were screened for presence of Mab specific for SEF17 by a selective whole cell ELISA. *S. enteritidis* strain 27655R and 27655S, a naturally occurring variant of 27655R (Baloda *et al.*, 1988) unable to express SEF17, were cultured on CFA agar

at 30°C for 48 hrs. Bacteria were resuspended in PBS, pH 7.2, washed by centrifugation at 2500 g for 10 mins, resuspended in 0.1M carbonate buffer, pH 9.6, coated to microtitre wells and a direct-binding ELISA was performed as described in Chapter 2. Supernatants from each well were screened at least twice, however, fusion plates were left untouched for at least 48 hrs between screening to allow antibody levels to build-up in the fresh media.

2.3.1.2 Screening of Mabs in flagella fusion

Purified flagella derived from *S. enteritidis* strain LA5 was diluted to 10 μg per ml in 0.1M carbonate buffer, pH9.6, coated to Maxisorb microtitre plates (NUNC), covered with a lid and incubated overnight at 4°C. Wells were washed in PBS/T and a direct-binding ELISA was performed as described in the 2.3.4 (page 52).

2.3.1.3 Production and semi-purification of Mabs

Cloned hybridomas were adapted to growth in low serum medium containing 5% (v/v) SRC (Tissue Culture Services Biologicals Ltd., Buckinghamshire, U.K.) in RPMI 1640 with 1mM Glutamax-1 (Gibco), and supplemented with 0.5 to 1% foetal calf serum (Gibco) as neccessary. Each cell line was grown to 2 litre volumes in roller flasks. Tissue culture supernatants were clarified and ultraconcentrated by tangential flow filtration using 0.45 μm and 30 kDa cut-off filters (Sartorius), respectively. Concentrated Mabs were precipitated with equal volumes of saturated ammonium sulphate, stirred for one hr at room temperature and centrifuged at 5,000 g for one hr. Sedimented antibody was resuspended in 0.1M PBS, pH7.2, dialysed extensively against the same buffer, filtered through a 0.22μm cut-off filter and stored frozen at -20°C. Antibody concentrations were estimated by isotype specific radial immuno-diffusion agar plates (The Binding Site) using the manufacturers instructions.

2.3.2 Production of chicken polyclonal antisera

Inoculations and exanguination techniques used for chicken polyclonal sera production were performed by Mike Bell, VLA, using the purified antigens produced in this study. Eighteen-week-old White Leghorn hens were injected intramuscularly with 0.5ml purified antigen (50μg SEF14, SEF17, SEF21 or flagella) emulsified with 0.5ml of Freund's Complete Adjuvant (FCA). On days 28 and 42 the process was repeated with FCA replaced by Freund's Incomplete Adjuvant. Hens were exanguinated under general anaesthesia on day 49 and whole blood was incubated at 37°C for one hour, followed by one hr at 4°C. The clot was separated by centrifugation at 1000 g for 10 mins and the serum stored frozen at -20°C.

2.3.2.1 Adsorption of antisera raised to fimbriae and flagella

S. enteritidis strain LA5 (27655R for adsorption of SEF17 sera) was cultured under conditions permissive of expression of individual fimbriae or flagella, washed by centrifugation at 2,500 g for 10 mins and resuspended in 0.1M PBS, pH 7.2, to form a turbid suspension containing approximately 10^{10} bacteria per ml. Antisera diluted 1/10 in PBS was mixed with pelleted bacteria and agitated for 24 hrs at 37°C. Bacteria were pelleted again, and antisera decanted onto freshly pelleted cells and incubated again. Each antisera was adsorbed 5 times, filtered through a 0.2μm membrane, aliquoted and frozen at -20°C.

2.3.3 Western blotting

For Western blot analysis, the antigens resolved by SDS-PAGE were transferred electrophoretically onto nitrocellulose membranes (Sartorius) for one hour at 300 mA, and blocked with 3% skimmed milk in 20mM Tris buffer, pH 7.5, containing 0.5M NaCl (TBS).

The membranes were then washed 3 times, mins per wash in TBS containing 0.05% Tween 20 (TBS/T), and gently agitated for one hr at 37°C with primary antibody diluted in the same buffer. The nitrocellulose membranes were washed 3 times with TBS/T before adding the secondary antibody, anti-mouse Ig or anti-chicken IgM or IgG, conjugated to alkaline phosphatase (GACh/IgM/PO, Sigma; RACh/IgG/PO, Nordic Immunological Laboratories, respectively). Following 3 washes in TBS/T and once in TBS the blot was developed with 5-bromo-4 chloro-3 indophosphate (BCIP, Sigma) for 10 mins. The reaction was stopped by immersing the membrane in deionised water.

2.3.4 Direct-binding ELISAs

Cultures were grown under conditions permissive of expression of surface appendage. Bacteria from agar plates were suspended in PBS, whereas broth cultures were sedimented by centrifugation and resuspended in PBS. All bacterial suspensions were re-sedimented by centrifugation and resuspended in carbonate buffer, pH 9.6. Bacterial suspensions were adjusted to 0.8 optical density at 540nm, equivalent to approximately 10^9 bacteria per ml, and titrated two fold in microtitre wells (Maxisorb, Nunc) in carbonate buffer to determine optimal coating concentrations. Wells coated with 100µl bacterial suspension were dried overnight at 37°C and washed twice in PBS containing 0.05% (v/v) Tween 20 (PBST). The washing step was repeated after each incubation step and all subsequent incubations were performed at 37°C for one hr except for the substrate which was performed at room temperature for 5 mins. The free binding sites were blocked with 200µl 3% (w/v) dried milk (Marvel) in PBS. All other ELISA reagents were added at 100µl per well. Monoclonal antibody was added and then goat anti-mouse horseradish peroxidase conjugated antibody (A-0412, Sigma). Tetramethyl benzidine (TMB, Cambridge Veterinary Sciences) substrate

was added and the reaction stopped with 10% sulphuric acid (v/v) and the absorbances were determined at 450nm.

In chicken polyclonal assays, Mab was replaced with adsorbed chicken sera diluted as in table 3.2 (page 75), followed by the addition of rabbit anti-chicken horseradish peroxidase conjugated antibody (Sigma). The reaction was developed with TMB.

2.3.5 Immunoelectron microscopy (IEM)

For immunogold labelling, bacteria were coated to Carbon-formvar grids as described for TEM (2.2.3, page 45) in chapter 2. Grids were incubated with antibody for 30 mins at room temperature, washed 3 times in PBS/T (5 mins per wash), incubated with goat anti-mouse Ig Auroprobe or rabbit anti-chicken Ig immunogold reagent labelled with 5nm gold particles (RPN 430, Amersham; EM.RCHL5, British Biocell International, respectively) diluted 1/10 in PBS for 30 mins, washed a further 3 times, counterstained with phosphotungstic acid and viewed as described for TEM.

2.4 Gene Probing

2.4.1 Amplification of *agf*A region by PCR

DNA sequences encoding the AgfA gene were amplified by PCR following the method of Saiki *et al.* (1988) with primers designed on the *agf* gene cluster using the sequence data of Collinson *et al.* (1996) and were:

3'-ATG ATG TTG ACA ATA CTG GGT-5' (forward)

3'-TGA TAA ATG CAG TGA TTG TCC-5' (reverse)

PCR reactions contained 5µl Thermo DNA polymerase reaction buffer (Promega), 200µM dNTP's (Pharmacia), 1.5mM $MgCl^2$, 2.5 units of thermo stable Taq polymerase (Promega), 10pmols of each primer (Oswel), 1ng of total genomic DNA and brought to a final volume of 50µl with sterile distilled water. Reactions were overlaid with 50µl of mineral oil (Sigma) and cycling was carried out in a thermo-cycler (Biometra) using the following program: 5 mins initial denaturation at 95°C followed by 30 cycles of 2 mins denaturing at 95°C, 1.5 mins annealing at 56°C, 2 mins extension at 72°C, followed by a final extension of 10 mins at 72°C. PCR reactions were mixed 1:1 with sample buffer, 10µl was loaded onto a 0.8% agarose gel and were run for 45 mins on at 120 volts. The gel was stained with ethidium bromide and visualised under UV light. PCR products of predicted size (1.3kb) were purified by Sephaglass^R (Pharmacia), the DNA concentration estimated and stored at 4°C until required.

2.4.2 Radiolabelling of probe

The DNA probe produced by PCR was heated to 95°C for 3 mins and kept denatured by placing directly on ice. The DNA was adjusted to a concentration of 30ng/µl and 28µl was denatured at 95°C for 5 mins and then placed immediately onto ice for 2 mins. The denatured probe was added to a ready to go dCTP tube (Amersham) containing 20µl cooled water. To this was added 2µl P^{32} radioactivity (Amersham) and the tube was vortexed and placed at 37°C for 30 mins.

2.4.3 Colony lysis

A square piece of nylon membrane (Hybond) with a suitably marked grid was placed on a square Nutrient agar plate which had been air dried for one hr at 37°C. Colonies were spotted

onto the nylon membrane and the plates grown overnight at 37°C. The membrane containing the visible colonies were removed from the agar plate and placed faced-up on blotting paper containing 5% SDS for 5 mins and then in denaturing solution for 7 mins and neutralising solution for 10 mins. The membrane was gently agitated for 5 mins in 2x SSC solution and the cell debri was removed by placing face down on a piece of blotting paper. The membrane was air dried and placed face down over a UV transilluminator for 5 mins to fix the DNA to the membrane.

2.4.4 Hybridisation

Nylon membranes containing fixed DNA from bacterial colonies were placed in hybridisation tubes (Hybaid) with 5mls Rapid hybridisation buffer (Amersham) and heated to 65°C in a hybridisation oven. The radiolabelled DNA was then heated to 95°C for 5 mins, added to a hybridisation tube containing the filter and buffer, placed in a pre-heated hybridisation oven and incubated for a further 2 hrs in a Hybaid rotisserie oven at a constant speed.

The radioactive probe was discarded into a liquid discard pot and 50ml ambient temperature 2x SSC was added to the hybridisation tube containing the membrane and rotated at 65°C for 20 mins. The first wash was poured into a radioactive liquid discard pot and replaced with 50ml of 2x SSC containing 0.1% SDS which had been pre-warmed to 65°C. The tube was rotated for a further 20 mins at 65°C and the wash discarded down the sink before adding 50ml of pre-warmed (65°C) 0.2x SSC containing 0.1% SDS and rotating for a further 20 mins at 65°C. The final wash was discarded and the membrane was removed from the tube and allowed to dry in air. Once dry the membrane was placed onto blotting paper, wrapped in SaranWrap^{R} and placed in a radiography cassette. The membrane was then exposed to an

autoradiography film (Fujifilm) and left for 2-24 hrs before developing for 2 mins (Photosol CD18 X-ray developer) and then fixing for 1 min (Photosol CF40).

2.5 Tissue culture models

2.5.1 Preparation of confluent monolayers of cell lines

Tissue culture cell lines INT-407 (ECACC, Salisbury, UK), CACO-2 (ECACC), DIV-1 (Institute Nationale de la Recherche Agronomique, Tours, France) and HT-29 cl 16E (Pasteur Institute, Paris) were maintained in $75cm^2$ tissue culture flasks (Bibby Sterilin) in logarithmic growth phase by passaging every 3-4 days in Eagles Modified Essential Media (EMEM, Sigma) supplemented with 10% heat inactivated foetal calf serum (Sigma), 2mM L-glutamine (Sigma) and 50μg/ml gentamicin (Sigma).

Three ml of pre-warmed Trypsin-EDTA was added to the cells, removed and replaced with a further 3 ml and incubated for 10 mins at 37°C (5% CO_2). The disrupted cells were gently agitated to obtain a homogeneous suspension and adjusted to $2x10^5$ cells per ml before adding one ml to each well of a 24 well plate. Plates were incubated for 48 hrs at 37°C (5% CO_2) to obtain a confluent monolayer containing $4\text{-}6x10^5$ cells per well. HT-29 cells were left for 5-7 days post confluence to secrete sufficient mucins.

2.5.2 Association and Invasion assays

Confluent monolayers of epithelial cells in 24 well tissue culture plates (Bibby Sterilin) were washed twice in Hank's Balanced Salt Solution (HBSS, Sigma) prior to association and invasion assays. Duplicate plates were set up for association and invasion assays and 3 wells containing confluent monolayers were used for each test.

For the association assay, cells were infected at a ratio of 1:100 with pre-washed bacteria diluted in one ml incomplete medium (EMEM and 2mM L-glutamine), and incubated for 2 hrs at 37°C (5% CO_2). Monolayers were washed 6 times by aspirating the contents of the wells and replacing with fresh HBSS. Cells were disrupted with one ml (1% v/v) Triton (Sigma) diluted in sterile PBS. Colony forming units (CFU) were determined by serial dilution's which were plated onto thoroughly dried Brilliant Green agar plates. Association was reported as a combination of bacteria that had adhered to and invaded the tissue culture cells.

In the duplicate 24 well plates the number of bacteria which had invaded was determined. The monolayers were infected with bacteria and incubated for 2 hrs as for the association assay. Wells were washed 3 times before adding 2 mls incomplete media containing 100 μg per ml of gentamicin (Sigma). Plates were incubated at 37°C (5% CO_2) for 2 hr, washed twice with HBSS, the monolayers disrupted with Triton and CFU determined, as described for the association assay.

Each association and invasion assay was performed at least twice (3 wells per test). For statistical analyses of association with and invasion of epithelial cells, counts were transformed to their logarithm to base ten, and analyses of variance (ANOVA) performed. When the F-tests were significant at the 5% level ($p<0.05$) further comparisons were made between the means using t-tests.

2.5.3 Blocking assays

Mannose, adsorbed polyclonal antisera (1/1000 dilution) and Mab (100μg/ml) were added to the bacterial suspension and the purified fimbriae (100μg/ml), flagella (100μg/ml), LPS (100μg/ml) and fibronectin (100μg/ml) added to the tissue culture cells diluted in incomplete media. All reactions were incubated for 15 mins at 37°C (5% CO_2) with gentle agitation, prior to infection of the monolayer. Counts were statistically compared by one-way ANOVA.

2.5.4 Histological staining of infected INT-407 cells

Confluent monolayers prepared in 6 well dishes (Bibby Sterilin) on 18mm diameter round coverslips were washed twice in HBSS and infected with $2.5x10^8$ bacteria in 5 mls of incomplete media. After incubating for 2 hrs at 37°C (5% CO_2) monolayers were washed 6 times in HBSS, fixed for 10 mins with cold methanol (70%) and stained with 10% Giemsa stain (Sigma) for one hr. Coverslips were washed 4 times in deionised water and differentiated in 1% acetic acid for 1 min, washed again, air dried and mounted onto slides with DPX mountant (Sigma). Slides were examined by light microscopy under oil immersion (1000x magnification). Adherent cells were visualised with a Zeiss light microscope (Axiovert 25) equipped with a Contax 167MT camera.

2.5.5 Immunofluorescence on infected tissue culture monolayers

Epithelial cells suspended in complete media were seeded into 6 well tissue culture plates (Bibby Sterilin) containing 18mm diameter glass slides and were incubated at 37°C (5% CO_2) to form a confluent monolayer. Monolayers were washed twice with HBSS, infected with approximately 10^8 bacteria diluted in incomplete media and incubated for 2 hrs at 37°C (5% CO_2). Wells were washed 6 times with pre-warmed HBSS, fixed in 70% methanol for 15

mins at room temperature, washed once in sterile 0.1M PBS and blocked with 3% (w/v) dried milk (Marvel) for 30 mins at room temperature. The fixed monolayers were washed in PBS/T and incubated in primary antibody diluted in PBS/T (Table 3.2) for a further 30 mins incubation at room temperature. After washing 3 times in PBS/T, goat anti-mouse Ig conjugated with FITC (Sigma) diluted 1:200 in PBS/T was added for 30 mins at room temperature. The monolayers were washed again 3 times in PBS/T and once in PBS and the coverslip mounted in 1:1 glycerol and water and viewed by fluorescence microscopy at a magnification of x1000.

2.6 *In vivo* pathogenesis studies in chickens

2.6.1 Chick inocula preparation

LA5 and mutants derived from this strain were aerobically shaken overnight at 37°C in Nutrient broth, washed and resuspended in PBS. Bacterial suspensions were adjusted to 0.8 O.D. at 540nm, which was estimated as $1x10^9$ bacteria per ml. Inocula were prepared by serial dilution's in PBS and orally administered to the crop by gavage in 0.1ml volumes per chick. Colony forming units for each inocula were determined by serial dilution's plated onto Brilliant Green agar (BGA).

2.6.2 Colonisation and invasion experiments

2.6.2.1 Colonisation in day-old chicks

Groups of White Leghorn "Specific Pathogen Free" chicks (SPF, Wickhams) aged between 18 and 24 hrs were orally inoculated with *S. enteritidis* LA5 and mutants derived from this strain, with doses ranging between 10^3 and 10^7 bacteria. Infected chicks were housed in isolators and feed and water were provided *ad libitum.* Chicks were killed and organs

removed at *post mortem* after 6, 24, 48 and 72 hrs post inoculation (PI) and presence of *Salmonellae* determined from organ homogenates. Analyses of variance were performed on bacterial counts using the GenStat 5 software program. In the chick colonisation and invasion studies the analyses accounted for the detection of *S. enteritidis* by enrichment but treated such data as "censored", with 500 as the upper limit. Statistical significance was assessed by F-tests, and further comparisons for effects significant at $p<0.05$ were made using t-tests. Additionally, the percentage of chicks colonised were analysed using Fisher's Exact Test.

2.6.2.2 Persistence of *Salmonellae* in organs

One hundred and twenty white leghorn SPF chicks were divided into 4 equal groups and housed in separate isolators. Groups of 30 chicks were orally inoculated with 10^5 *S. enteritidis* strain LA5 or EAV40 (14^-, 17^-, 21^- & fla^-) at one and 7 days of age. Each bird was cloacal swabbed and bled weekly and at weeks 2, 4 and 6 PI, ten birds from each group were killed and *Salmonellae* isolated from organ homogenates. Statistical analyses was performed as described in section 2.6.2.1.

2.6.2.3 Lateral transfer of *Salmonellae*

Fifty white leghorn SPF chicks were divided into 2 groups and housed in separate isolators. At 7 days of age, 5 "seeder" birds from each group were orally inoculated with 10^5 *S. enteritidis* strain LA5 or EAV40 (14^-, 17^-, 21^- & fla^-). Ten uninfected "sentinel" birds were added to each isolator and cloacal swabbed daily. All birds were killed after 7 days and *Salmonellae* isolated from organ homogenates. Both isolators were left uninhabited and untouched for 14 days before the introduction of a further 10 "sentinel" birds, 28 days of age. Again, birds were cloacal swabbed daily for 7 days, killed and *Salmonellae* isolated from organ homogenates. Analyses of variance on counts were performed using the Statistica

software package. Statistical significance was assessed by F-tests, and further comparisons for effects significant at $p<0.05$ were made using t-tests.

2.6.2.4 Colonisation and invasion by *S. enteritidis* following oral inoculation of chicks with a mixed inoculum containing wild type and mutants

Seven day old chicks were dosed orally with a mixed inoculum containing $5x10^4$ wild type *S. enteritis* strain LA5 and equal numbers of B214 (14^-), EAV42 (14^-, 17^-), EAV21 (14^-, 17^-, 21^-) or EAV40 (14^-, 17^-, 21^-, fla^-). Groups of birds were housed in separate isolators and on days 1, 7 and 14 PI birds were killed and *Salmonella* isolated from liver, spleen and caecum. Numbers of mutant and wild type strains were estimated by plating directly onto BGA, in the presence or absence of kanamicin, respectively.

2.6.3 Isolation and enumeration of *Salmonella*

2.6.3.1 Isolation of *Salmonella* from tissues and swabbings

Birds were killed by cervical dislocation and spleen, liver and caecum aseptically removed at *post mortem* and placed in 10 ml of sterile deionised water. Each tissue was homogenised (Ystral) for one min and viable counts determined by plating serial dilution's prepared in sterile PBS onto BGA. Single and multiple mutants were selected on BGA containing 0.5μg kanamicin per ml media. Additionally, organ homogenates and cloacal swabbings were selectively enriched in selenite F broth (Oxoid) with incubation overnight at 42°C, followed by incubation at room temperature for up to 7 days. *Salmonellae* were isolated from selenite F broth by subculture onto BGA. All *Salmonellae* were confirmed by slide agglutination using 09 rabbit typing sera (PL 6015, VLA, Weybridge, Surrey).

2.6.3.2 Semi-quantitative scoring of cloacal swabbings

Cloacal swabs from birds were streaked with 5 horizontal strokes across the top of a BGA plate. The plate was turned 90° and with a fresh disposable loop streaked across the top of the BGA plate. The plate was turned a further 90° and again streaked across the top of the BGA plate. After incubation overnight at 37°C the shedding was interpreted as:

heavy shedding = confluent colonies across top of BGA plate

medium shedding = semi-confluent across the top of BGA plate

low shedding = few colonies across top of BGA plate

no shedding = no colonies

2.7 Serological assays

0.1µg purified fimbriae, flagella and *S. enteritidis* LPS (Sigma) diluted in carbonate buffer, pH 9.6 were coated to microtitre wells (Maxisorb, NUNC), covered, incubated overnight at 4°C and washed 4 times in 0.1M PBS containing 0.05% (v/v) Tween 20 (PBST). The washing step was repeated after each incubation and all subsequent incubations were performed at 37°C for one hr except for the substrate which was performed at room temperature for 5 mins. The free binding sites were blocked with 200µl 3% (w/v) dried milk (Marvel) in PBS. All other ELISA reagents were added at 100µl. Test chicken serum was added (1/500) dilution and then rabbit anti-chicken Ig horseradish peroxidase conjugated antibody (RACh/IgPx) (Sigma). Tetramethyl benzidene (TMB, Cambridge Veterinary Sciences) substrate was added and the reaction stopped with 10% sulphuric acid (v/v) and the absorbances recorded at 450nm.

For isotype specific assays, RACh/IgPx was replaced with goat anti-chicken IgG or IgM (Nordic Immunological Laboratories, Netherlands), followed by rabbit anti-goat IgG peroxidase conjugated antibody (Sigma) and then finally the substrate.

Table 2.1 Characteristics of a) wild type bacteria and b) mutants used in this study

a)

Serotype	Strain	Phage type	Source	Reference
S. enteritidis	27655R	nd	Human	Baloda, (1988)
S. enteritidis	27655S	nd	Human	Baloda, (1988)
S. enteritidis	LA5	4	Chicken	Cooper *et al.*, (1989)
S. enteritidis	S1400	4	Chicken	Allen-Vercoe *et al.*, (1997)
S. enteritidis	I	4	Chicken	Humphrey *et al.*, (1996)
S. enteritidis	E	4	Chicken	Humphrey *et al.*, (1996)
S. enteritidis	C6B	4	Chicken	Humphrey *et al.*, (1996)
S. enteritidis	S1900	6	Chicken	VLA reference collection
S. enteritidis	10360	4	Chicken	VLA reference collection
S. enteritidis	12804	8	Turkey	VLA reference collection
S. enteritidis	8524	4	Chicken	VLA reference collection
S. typhimurium	Mö8	nd	Pigeon	Grund & Weber, (1988)
E. coli	NG7C	nd	Human	Collinson *et al.*, (1992)

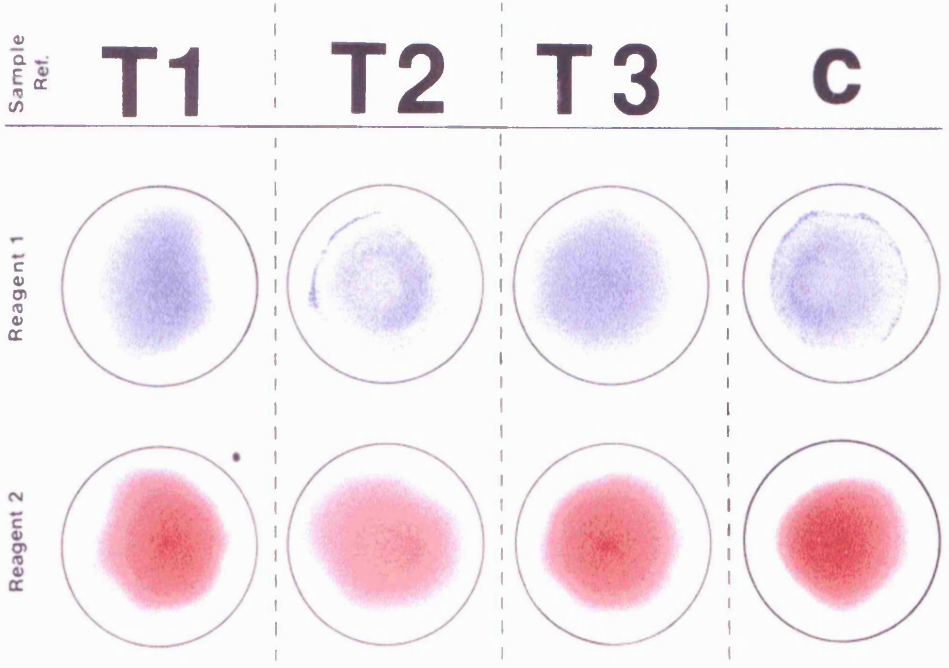
nd Not phage typed

b)

		Potential phenotype			
Strain	**Reference name**	**SEF14**	**SEF17**	**SEF21**	**Flagella**
LA5	B214	-	+	+	+
"	EAV12	+	-	+	+
"	EAV3	+	+	-	+
"	EAV10	+	+	+	-
"	EAV42	-	-	+	+
"	EAV21	-	-	-	+
"	EAV40	-	-	-	-
S1400	EAV13	-	+	+	+
"	EAV11	+	-	+	+
"	EAV1	+	+	-	+
"	EAV9	+	+	+	-
27655R	EAV28	-	+	+	+
"	EAV30	+	-	+	+
"	EAV29	+	+	-	+

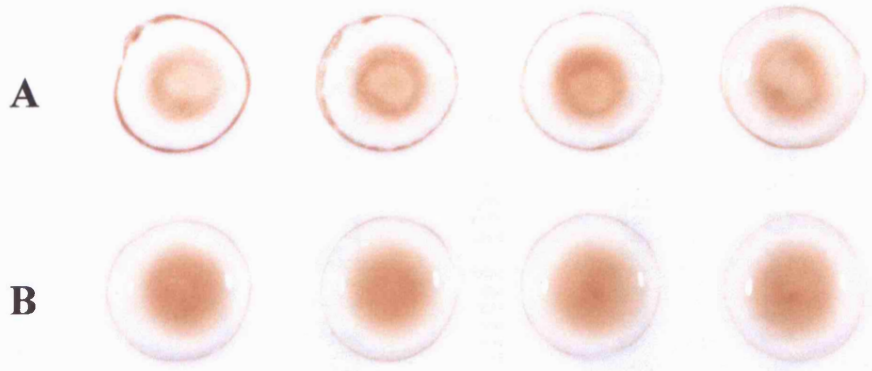
SEF *Salmonella enteritidis* fimbriae

Figure 2.1 Detection of SEF14 fimbriae by latex agglutination.



Samples of bacterial suspensions (T1, T2 & T3) were mixed with SEF14 antibody-coated latex beads (reagent 1) or with uncoated, negative control beads (reagent 2). *Salmonella* in columns T1 and T3 are negative for SEF14 elaboration, whereas *S. enteritidis* in column T2 was positive. The test was validated with a positive control antigen (C) containing a cell free suspension of SEF14 fimbriae.

Figure 2.2 Mannose Sensitive Haemagglutination to detect SEF21 expression.



Doubling dilutions of bacterial suspension were prepared in duplicate from left to right and equal volumes of **A)** PBS or **B)** 3% mannose in PBS were added. Finally horse red blood cells were added to each well and the mixture rocked for 2 mins. SEF21-mediated haemagglutination was inhibited in the presence of mannose

Chapter 3

Purification of SEF14, SEF17, SEF21 and flagella and the production and characterisation of specific immune reagents to these surface appendages.

3.1 Introduction

S. enteritidis elaborate flagella and have been reported to express SEF14 (Thorns *et al.*, 1990), SEF17 (Collinson *et al.,* 1990) and SEF21 (Müller *et al.*, 1991) which are antigenically and morphologically unique fimbrial structures. In order to detect the elaboration of these surface appendages *in vitro* and *in vivo* it was necessary to produce purified antigens and raise specific immune reagents to these organelles. Monoclonal antibodies would primarily be utilised for the development of specific immuno-assays for the detection of surface appendages on whole bacteria, whereas the purified antigens and polyclonal sera would be used to develop serological assays to detect seroconversion to fimbriae and flagella following oral inoculation of chicks. In addition, the purified antigens and antibodies would be useful as reagents for blocking or neutralising specific interaction between surface appendages and tissue culture cells. Mabs specific to SEF14 and SEF21 have already been produced and characterised in this laboratory (Thorns *et al.*, 1992; Sojka *et al.*, 1996), but specific reagents for SEF17 and flagella were unavailable. Thus, the aims were 1) to purify SEF14, SEF17, SEF21 and flagella, 2) to raise a panel of specific polyclonal antibodies to these antigens and Mabs to SEF17 and flagella, and 3) to characterise and evaluate the immune reagents for use in assays for specific detection of these surface appendages.

3.2 Results

3.2.1 Purification of fimbriae and flagella

3.2.1.1 Purification of SEF17

SEF17 was purified as described in section 2.2.5 from strains 27655R and the SEF17 negative control strain 27655S. With strain 27655R a white insoluble pellet remained in the well following SDS-PAGE, whilst no pellet was observed from 27655S. It was assumed that the insoluble pellet was SEF17 and this was therefore removed by pipette and boiled in low pH glycine buffer, depolymerised in formic acid and lyophilised. Following the addition of sample buffer, proteins were resolved by electrophoresis (Figure 3.1) and stained with Coomassie blue which revealed a visually pure band with a molecular mass of approximately 17 kDa. Interestingly, the general procedure of boiling the sample for 5 mins in sample buffer prior to electrophoresis (Laemli *et al.,* 1970) gave a lower yield than non-boiled sample (Figure 3.1). This suggested that the fimbrin subunits may have become weakened following such harsh treatment and that further boiling had caused degeneration of the fragile subunits. Following the SEF17 purification procedure described above, approximately 800μg of purified protein was obtained from 30 agar plates.

3.2.1.2 Purification of flagella

Flagella were purified by the method performed by Kondoh and Hotani (1974) and is described in full in section 2.2.7. Preliminary electron microscopy studies suggested that large quantities of peritrichously arranged flagella were expressed by *S. enteritidis* when grown aerobically in Nutrient broth with shaking at 37°C for 24 hrs (Figure 3.2). Therefore strain LA5 was cultured in Nutrient broth under these conditions. Whole flagella were

removed from the bacterial surface by homogenisation and purified from the cell extract by differential centrifugation. Following the purification procedure SDS-PAGE analysis indicated a single band of depolymerised flagellin subunits of approximately 50 kDa (Figure 3.3) and further purification was unnecessary. Approximately 5mg of purified flagella had been produced from one litre of bacterial culture.

3.2.1.3 Purification of SEF14 fimbriae

Purification of SEF14 fimbriae was performed by the method of Thorns *et al.* (1992) and is described in section 2.2.4. Briefly, *S. enteritidis* strain LA5 was cultured on Sensitest agar for 24 hours at 37°C and the presence of SEF14 fimbriae was confirmed by latex agglutination as described in chapter 2.2.1 and shown in figure 2.1. Fimbriae were detached from bacteria by a combination of heating at 60°C and agitation in a sonicating water bath. Bacteria were harvested by centrifugation and the fimbrial suspension was purified by size exclusion chromatography. The purity of the SEF14 preparation was analysed by SDS-PAGE (Figure 3.4). Following this procedure 2.5 mg of SEF14 fimbriae were purified from bacteria grown on 50 Sensitest agar plates.

3.2.1.4 Purification of SEF21 fimbriae

The method used for the purification of SEF21 fimbriae was that performed by Sojka *et al.* (1996) and is described in section 2.2.6. Surface appendages were detached from the bacteria by blending with a homogeniser. Crude fimbriae were prepared by differential centrifugation and purified by gel filtration. To dissociate the fimbrial fibres into fimbrin subunits the

purified preparation was first boiled in low pH sample buffer containing 0.2M glycine (Figure 3.5). Following this procedure 2 mg of SEF21 fimbriae were purified from bacteria grown in 2 litres of Heart Infusion broth.

3.2.2 Production and characterisation of antibodies to fimbriae and flagella

3.2.2.1 Antibodies to SEF17

Monoclonal antibodies

Monoclonal antibodies were raised to SEF17 following the method described by Köhler and Milstein, (1975) and full details are described in section 2.3.1. The differential screening identified a single well containing hybridomas. Cells were cloned twice and secured in liquid nitrogen and the cell line designated M117. The Mab was identified as IgM isotype by Ouchterlony immunodiffusion technique using a Mab typing kit (ICN Biomedicals Inc.).

Hybridoma cell line M117 was adapted to growth in low serum media and 100 mls of supernatant was produced in tissue culture flasks. Spent tissue culture media was clarified by centrifugation and semi-purified Mab was prepared by ammonium sulphate precipitation. A total yield of 5 milligrams of Mab was aliquoted and stored at -20°C (Table 3.2).

Mab M117 reacted with the purified 17 kDa protein in a Western blot (Figure 3.6), which indicated the antibody recognised a linear epitope of the fimbrin subunit. The Mab failed to react with strain 27655S (which did not express thin aggregative fimbriae), purified SEF14, SEF21 or flagella (data not shown). In addition, the Mab specifically labelled cell-associated and cell free SEF17 fimbriae as shown by immuno-electron microscopy (Figures 3.7 A and B), albeit rather sparsely. Some apparently non-specific labelling may be accounted for by the

labelling of detached pieces of fimbriae or alternatively non-specific binding by the Mab. That strain 27655S was unlabelled with antibody and the background was clear, suggested specific binding to detached fimbriae.

A direct binding ELISA using Mab M117 was developed for the detection of SEF17 fimbriae on whole bacteria. A checkerboard titration was performed to determine optimal dilution's of bacteria and monoclonal antibody. Used at 0.1μg/ml (1/2000 dilution) M117 Mab detected SEF17 expression on $1.0x10^5$ *Salmonella* which had been cultured on CFA agar for 48 hours at 37°C (Figure 3.8 A).

Polyclonal sera.

Chicken SEF17 antibodies were produced in eighteen week-old White Leghorn hens as described in section 2.3.2. Evaluation of polyclonal anti-sera raised to SEF17 (ChSEF17) by immuno-electron microscopy indicated the presence of antibodies which reacted with 27655R and 27655S. Anti-sera was adsorbed with strain 27655S to remove common antibodies and further evaluation of adsorbed ChSEF17 showed specific labelling of SEF17 fimbriae by immuno-electron microscopy (Figure 3.7 C and D). The extracellular matted fimbrial material was heavily and evenly labelled with gold particles, whereas flagella and bacterial surfaces not elaborating fimbriae were unlabelled (Figure 3.7 C). In addition, depolymerised fimbrin subunits of SEF17 were illuminated in Western blots following reactivity with ChSEF17, whereas SEF14, SEF21 and flagellin subunits were not (data not shown). Adsorbed ChSEF17 was suitable for use in the direct binding ELISA at a dilution of 1/5000 (Figure 3.9).

3.2.2.2 Antibodies to flagella

A panel of monoclonal antibodies and chicken polyclonal sera (ChFla) were raised to flagella of *S. enteritidis* strain LA5 as described in section 2.3.1.2. Tissue culture supernatants were evaluated in the screening ELISA on days 10 and 14 post fusion and, in total, ten hybridomas derived from six wells were cloned and secured in liquid nitrogen. All Mabs were IgG isotype and eight from ten reacted with the flagellin subunit in Western blots (Figure 3.10), although, interestingly none labelled flagella by immuno-electron microscopy. Each Mab was reacted in a direct binding ELISA to a panel of *Salmonella* from several serogroups and expressing a range of defined "H" antigens (Table 3.1). Eight from ten Mabs bound to each *Salmonella* tested, except M138/2 clones which did not bind *S. kedougu, S. virchow* or EAV9 and EAV10, defined aflagellate mutants produced in *S. enteritidis* strains LA5 and S1400 (Table 3.1). Mabs reacted with defined mutants of SEF14, SEF17 and SEF21 (data not shown). M138/4/1 and M138/4/2 weakly reacted with purified flagella but not with *Salmonella* . Most Mabs had a similar reactivity pattern as ChFla, and in general, all antibodies reacted stronger with *S. enteritidis*, *S. typhimurium* and *S. dublin*, than *S. virchow* and *S. kedougu*.

Mab M38/8/1 was chosen for the development of a flagella specific direct binding ELISA for it appeared to have a higher affinity than the other Mabs by giving a slightly stronger absorbance when compared at the same concentration. When probed with Mab at 0.2μg/ml (1/5000 dilution) flagella were detected on less than $1x10^5$ bacteria (Figure 3.8 B). In addition, adsorbed ChFla was specific for flagella in the direct binding ELISA (Figure 3.9),

bound to depolymerised flagellin subunits in Western blots but failed to label flagella in immuno-electron microscopy (data not shown).

3.2.2.3 SEF14 and SEF21 antibodies

Panels of Mabs for SEF14 and SEF21 were prepared previously in this laboratory. SEF14 Mabs reacted with dissociated fimbrin subunits in Western blots (Thorns *et al.,* 1992) and evenly labelled SEF14 in immuno-electron microscopy (Figure 3.11 A). Interestingly, SEF14 was observed as a structure encapsulating the bacterium which was difficult to visualise by transmission electron microscopy when unlabelled for the individual fimbrial structure was not visible. In addition, SEF21 Mabs labelled fimbriae in immuno-electron microscopy (Figure 3.11 B), but did not label dissociated SEF21 fimbrin subunits in Western blot which suggested that the antibodies recognised conformational epitopes.

In direct binding ELISA's the SEF14 Mab M69/25/1 and SEF21 Mab M87/4/3 detected fimbrial expression of $1x10^6$ bacteria (Figures 3.8 C & D, respectively) when cultured on Sensitest agar for 24 hours at 37°C or HIB for 48 hours at 37°C, respectively. Adsorbed ChSEF14 and adsorbed ChSEF21 were suitable for use at 1/5000 dilution in a whole cell assay (Figure 3.9).

Table 3.1 Reactivity of flagella Mabs to purified antigen and *Salmonella* serotypes in direct binding ELISA.

Serotypes	Strain	†H Ag	Mabs										Chofla
			138/2/1	138/2/3	138/3/1	138/8/1	138/4/2	138/3/2	138/5/1	138/5/2	138/8/1	138/11/1	
Isotype			IgG2a	IgG1	IgG1	IgG2a	IgG2a	IgG1	IgG1	IgG1	IgG1	IgG1	IgM,IgG,IgA
Purified flagella		g.m.	+++	+++	++	+	+	++	++	+++	+++	+++	++
S. enteritidis	LA5	g.m.	+++	+++	+++	-	-	+++	+++	+++	+++	++	+++
"	*EAV10	-	-	-	-	-	-	-	-	-	-	-	-
"	S1400	g.m.	+++	++	+++	-	-	+++	+++	++	+++	++	+++
"	*EAV9	-	-	-	-	-	-	-	-	-	-	-	-
S. typhimurium	S3688/93	i, 1,2	++	++	++	-	-	++	++	++	+++	+	++
"	S133/95	i, 1,2	+++	+++	+++	-	-	++	+++	+++	+++	++	+++
S. dublin	1065/94	g.p.	+++	+++	++	-	-	++	++	++	+++	++	+++
"	S76/94	g.p.	++	++	++	-	-	++	++	++	++	+	++
S. virchow	S4148/95	r, 1,2	-	-	+	-	-	+	+	+	++	+	++
S. kedougu	S7262	i, l.w.	-	-	+	-	-	+	+	+	+	+	+
"	S2972	i, l.w.	+	+	+	-	-	+	+	+	++	+	++

- \- 0-0.2 Absorbance at 450nm in direct binding ELISA
- \+ 0.2-0.5 "
- ++ 0.5-1.0 "
- +++ >1.0 "

*EAV10 LA5 (fla$^-$)
*EAV9 S1400 (fla$^-$)
†Flagella antigens (Kauffmann, 1964)

Purified flagella were coated at 0.1µg/well and bacteria were grown aerobically in Nutrient broth with shaking at 37°C for 24 hours and $1x10^7$ *Salmonella* were coated to microtitre wells. Mabs were added at 1µg/ml and Chfla at 1/5000 dilution. Bound primary antibody were detected with peroxidase-labelled anti-species secondary Ig antibody and the assays developed with TMB substrate. The reactions were stopped with sulphuric acid and absorbances read at 450nm.

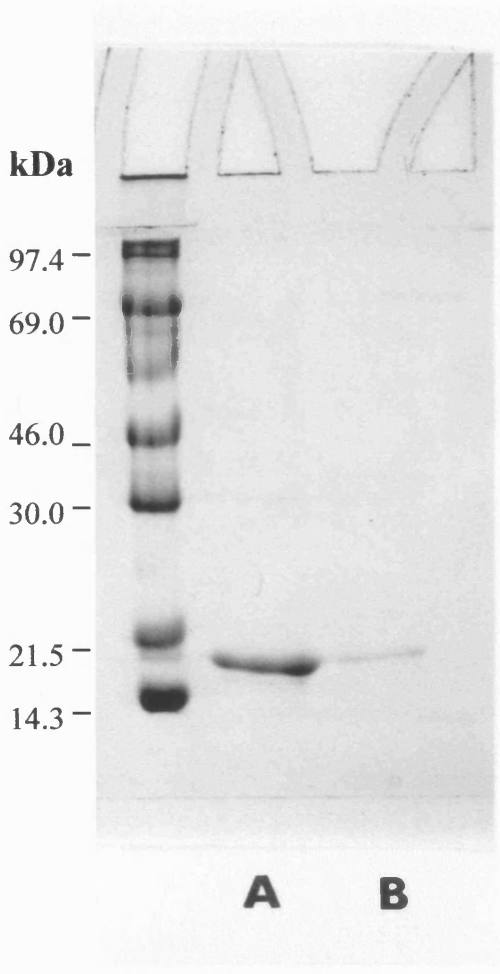
Table 3.2 Summary of antibodies used in this study.

Antibody reference	Antibody specificity	Monoclonal (Mab) or polyclonal (p/c)	Isotype	Concentration mg/ml	Immuno-electron microscopy	Western blot analyses	Titre used in direct binding ELISA/IF
M69/25	SEF14	Mab	IgG1	13.6	✓	✓	1/5000
*†ChSEF14	"	p/c	NA	NA	✓	✓	1/5000
*M117/1	SEF17	Mab	IgM	0.25	✓	✓	1/2000
*†ChSEF17	"	p/c	NA	NA	✓	✓	1/5000
M87/4/3	SEF21	Mab	IgG3	4.0	✓	-	1/1000
*†ChSEF21	"	p/c	NA	NA	✓	✓	1/5000
*M138/3/1	Flagella	Mab	IgG1	1.1	-	✓	1/5000
*†ChFla	"	p/c	NA	NA	-	✓	1/5000

* Antibodies prepared in this study
† Adsorbed sera
NA Not Applicable
IF Immunofluorescence

✓ Suitable for use
- Unsuitable for use

Figure 3.1 SDS-PAGE of purified SEF17 fimbriae extracted from *S. enteritidis* strain 27655R.



Following whole cell enzymic digestion the fimbrial preparation was boiled in low pH glycine buffer and depolymerised in formic acid. The dried preparation was dissolved in sample buffer and lane **A)** added directly, or lane **B)** boiled for 5 minutes prior to SDS-PAGE. The first lane contained molecular mass markers (Amersham).

Figure 3.2 An electron micrograph showing elaboration of flagella by *S. enteritidis* **strain LA5 following growth in Nutrient broth for 24 hours at 37°C with shaking.** Bar represent 1 micron.

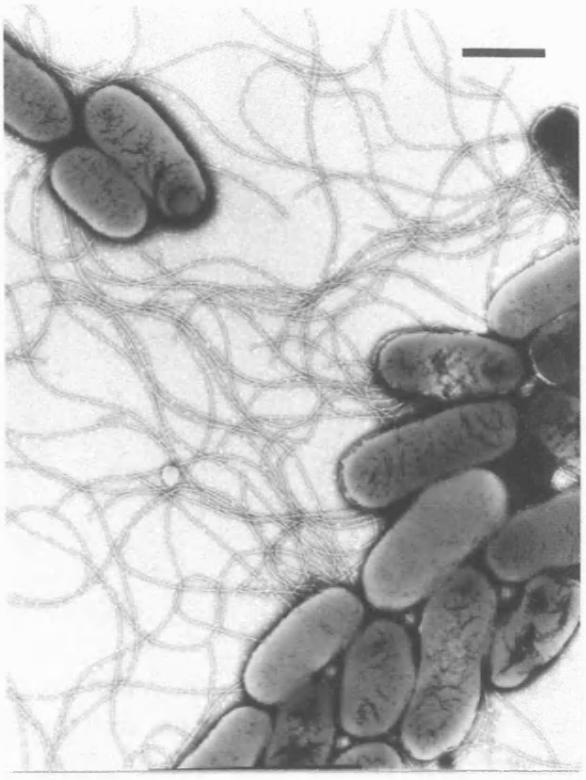
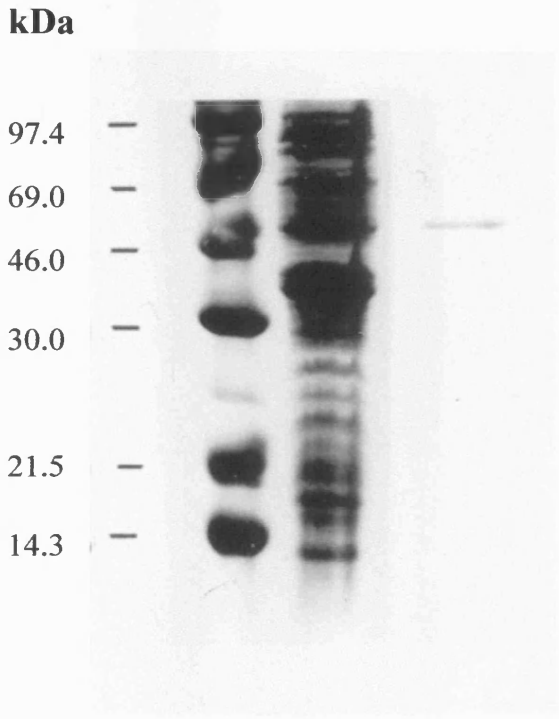
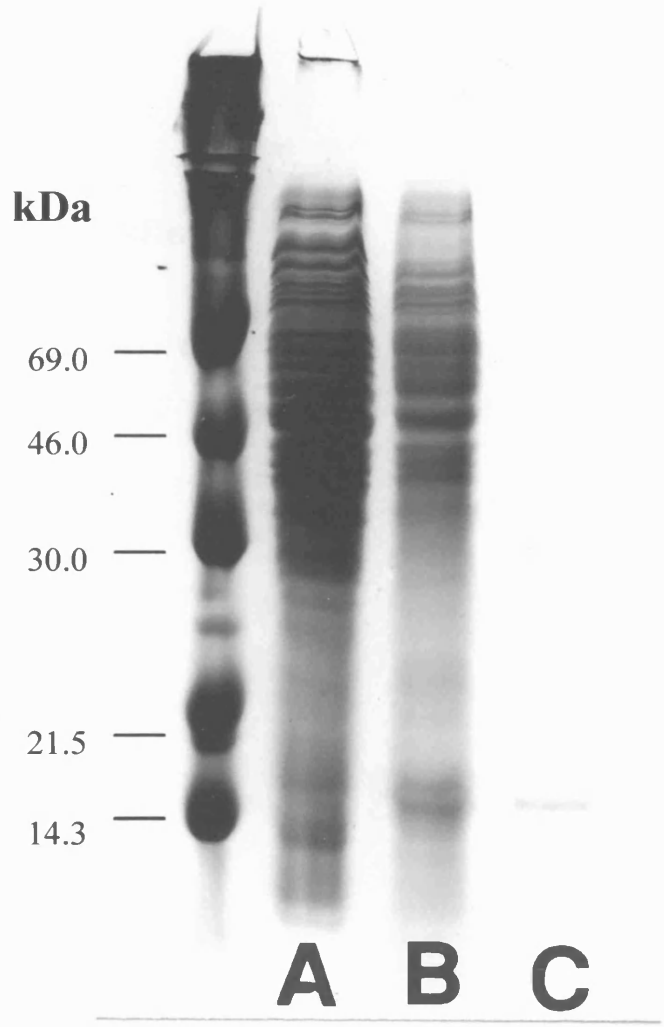


Figure 3.3 SDS-PAGE of purified flagella extracted from *S. enteritidis* strain LA5.



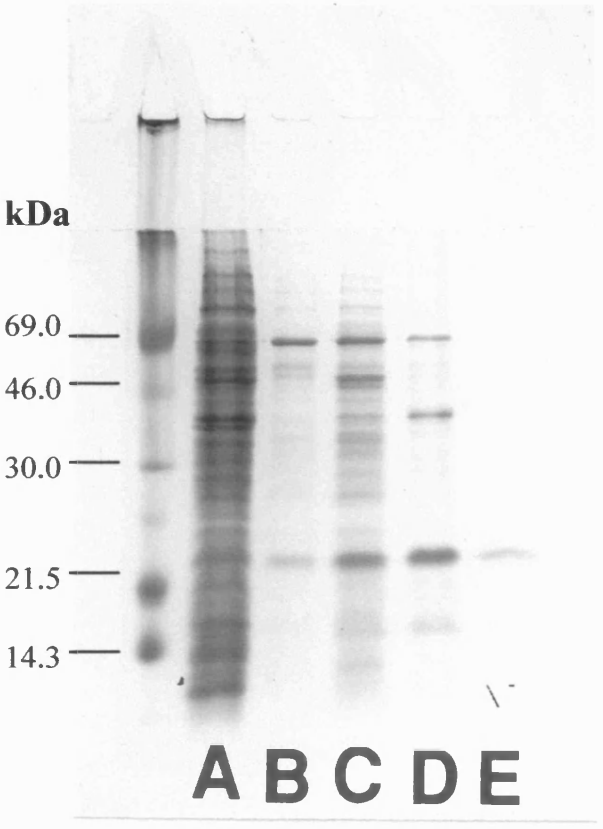
Whole flagella were removed from the bacterial surface by homogenisation and purified from the cell extract by differential centrifugation. Purified flagella were depolymerised into flagellin subunits by boiling in sample buffer and run on a 15% separating gel (5% stacking gel). The protein was visualised by staining with Coomassie blue. The first lane contains molecular mass markers (Amersham), lane **A)** contains whole cell extract, whereas, lane **B)** shows purified flagellin subunits.

Figure 3.4 SDS-PAGE separation of purified SEF14 fimbriae extracted from *S. enteritidis* strain LA5.



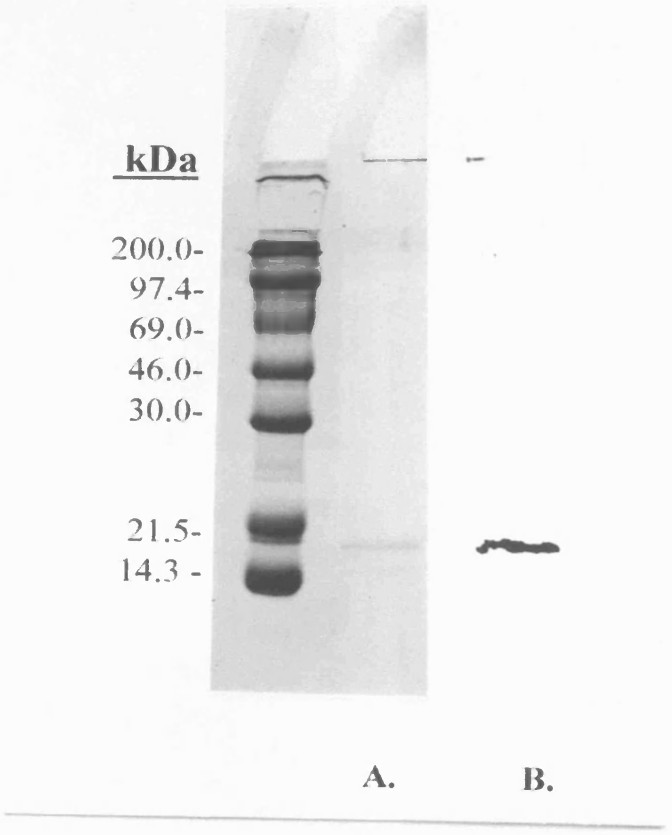
Bacteria were grown under conditions permissive for elaboration of fimbriae. SEF14 were removed from the bacterial surface by a combination of heating (60°C) and agitation. Purified fimbriae were obtained from cell free supernatant by size exclusion chromatography and dissociated into fimbrin subunits in neutral sample buffer. The first lane contains molecular mass markers, lane **A)** whole cell extract, lane **B)** cell free supernatant and lane **C)** purified fimbriae.

Figure 3.5 SDS-PAGE separation of purified SEF21 fimbriae.



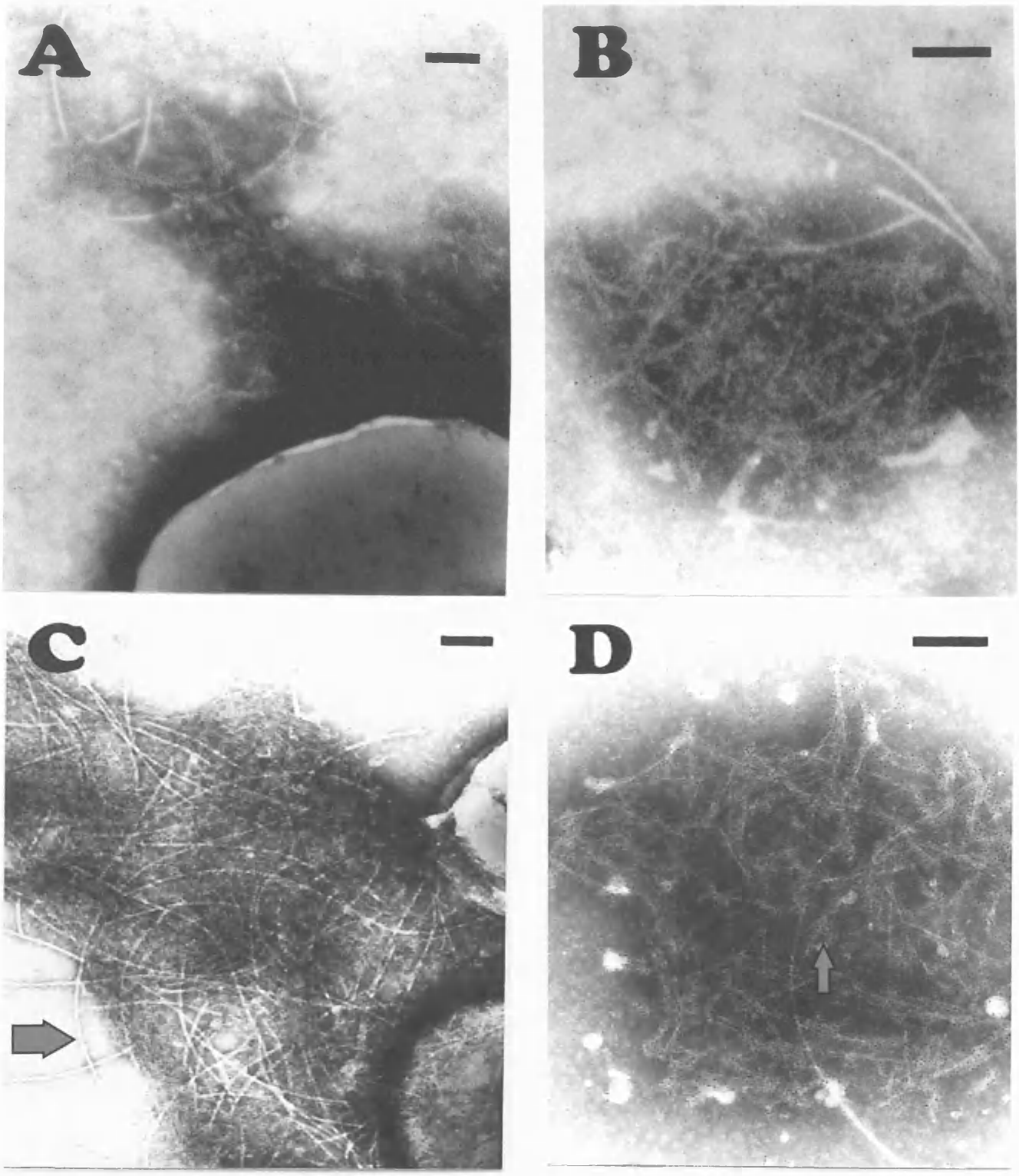
SEF21 were detached from bacteria by homogenisation. Cell fragments were removed by centrifugation and the fimbrial preparation was separated by gel filtration chromatography, boiled in sample buffer and pelleted by ultracentrifugation. To dissociate into fimbrin subunits the fimbrial preparation was boiled in low pH sample buffer prior to electrophoresis. The first lane contains molecular mass markers, lane **A)** homogenised whole cell, lane **B)** cell free supernatant, lane **C)** concentrated supernatant, lane **D)** concentrated size exclusion chromatography fractions, and lane **E)** purified fimbrial preparation.

Figure 3.6 A) SDS-PAGE of purified SEF17 fimbriae extracted from *S. enteritidis* strain 27655R and B) Western blot of SEF17 probed with Mab M117.



S. enteritidis strain 27655R was digested with lysozyme, RNase, DNase and sodium dodecyl sulphate and the extract run on a SDS-PAGE gel. The precipitate not entering the gel was removed and depolymerised with formic acid (A). The purified protein was transferred onto nitocellulose and probed with Mab M117. The primary antibody was detected with anti-mouse IgPx conjugate and visualised by the addition of AEC substrate (B). The first lane contains molecular mass markers (Amersham).

Figure 3.7 Electron micrographs of immuno-gold labelled fimbriae from *S. enteritidis* strain 27655R probed with monoclonal antibody (A and B) and ChSEF17 (C and D).



Frames **A)** and **C)** show fimbrial material associated with the bacterium amongst unlabelled flagella and **B)** and **D)** highlights matted clumps of gold-labelled fimbrial material detached from the bacterium. Thicker arrow show flagella, whereas thinner arrow indicates SEF17 fimbriae. Bars represent 200nm.

Figure 3.8 The development of a whole cell direct binding immunoassay for the detection of fimbriae and flagella.

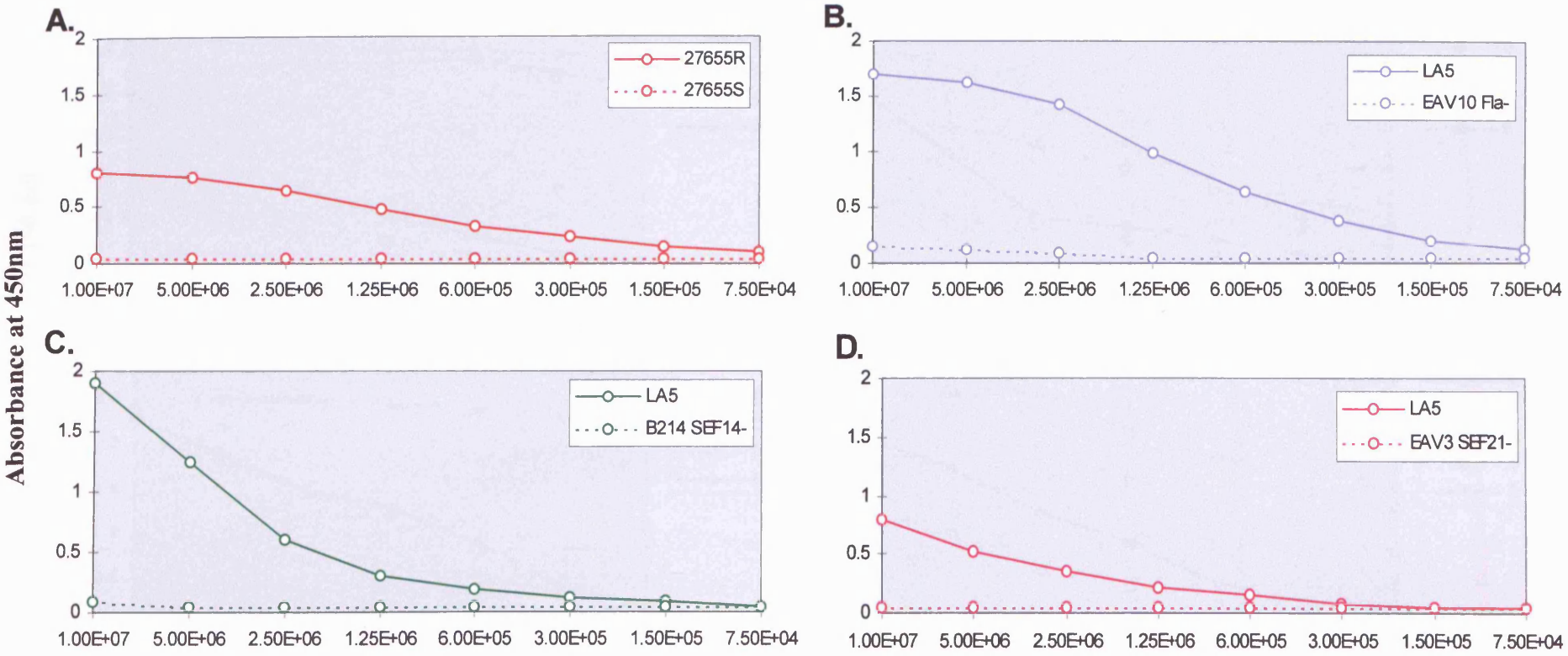
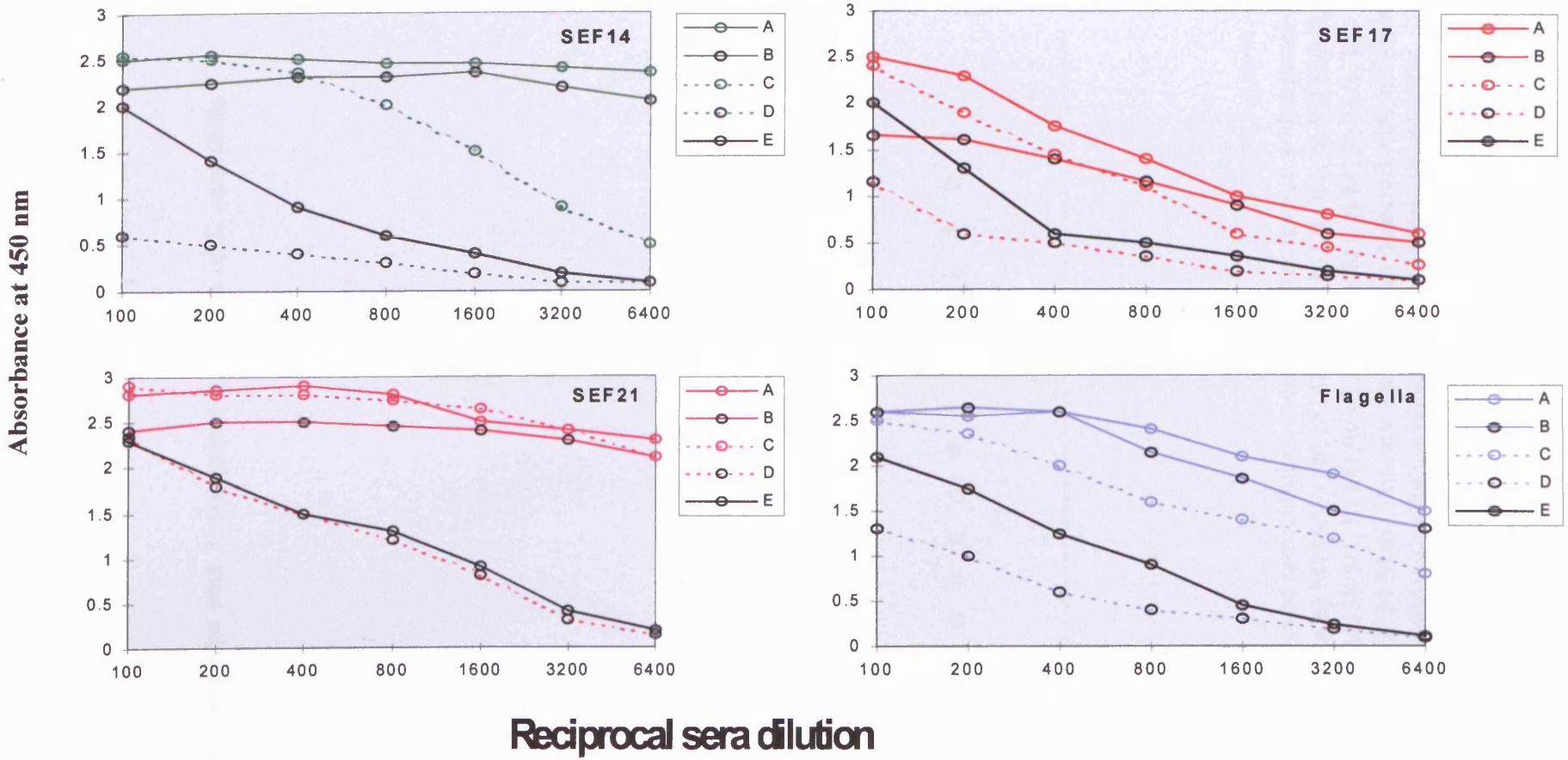


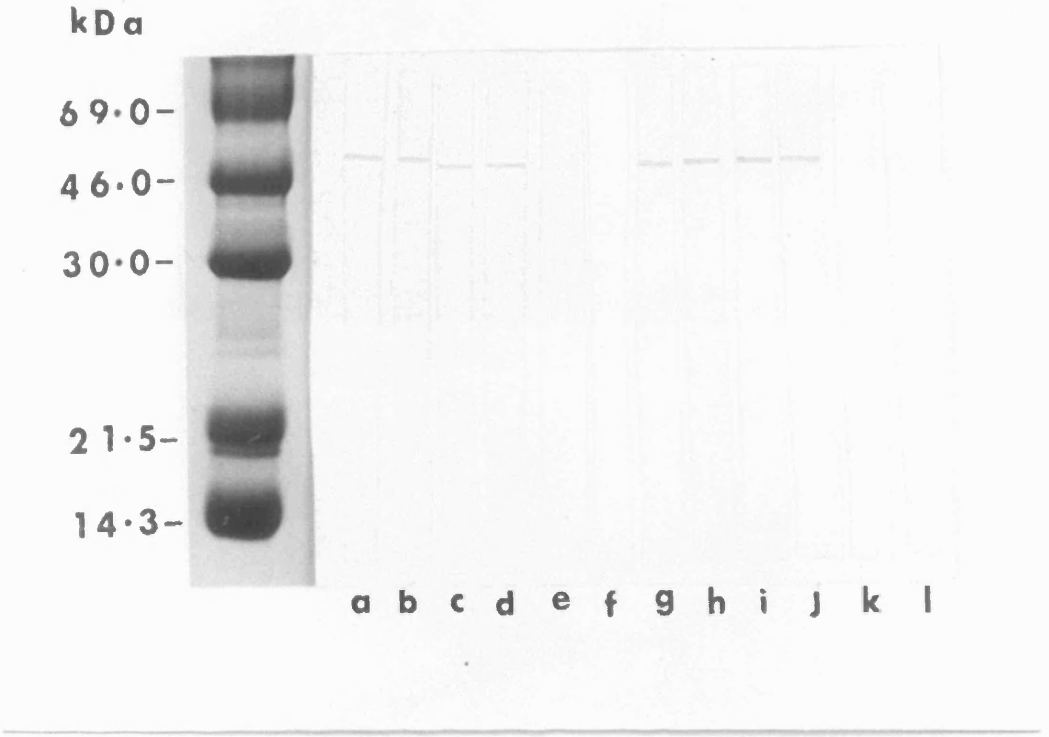
Figure **A)** shows SEF17 expression using Mab M117. *S. enteritidis* strain 27655R and the naturally occuring variant 27655S defective for SEF17 expression, were prepared by culturing on CFA agar for 48 hours at 37°C. Figure **B)** shows flagella expression using Mab M138/8/1. *S. enteritidis* strain LA5 and isogenic flagella mutant EAV10 were prepared by culturing in Nutrient broth and shaking aerobically for 24 hours at 37°C before coating to microtitre wells. Figure **C)** shows the detection of SEF14 expression using Mab M69/25/1. *S. enteritidis* strain LA5 and B214 (SEF14- mutant) were prepared by culturing on Sensitest agar and incubating for 24 hours at 37°C. Figure **D)** shows the detection of SEF21 expression using Mab M87/4/3. *S. enteritidis* strain LA5 and EAV3, an isogenic mutant defective in SEF21 elaboration, were prepared by culturing in Heart Infusion broth for 48 hours at 37°C before coating to microtitre wells. Bacteria were coated to microtitre wells and probed with Mabs (1mg/ml). Bound Mabs were detected with anti-mouse Ig peroxidase conjugate and developed with TMB substrate and the reaction stopped with sulphuric acid. Absorbances were read at 450nm.

Figure 3.9 Comparison of adsorbed and non-adsorbed chicken sera raised to fimbriae and flagella.



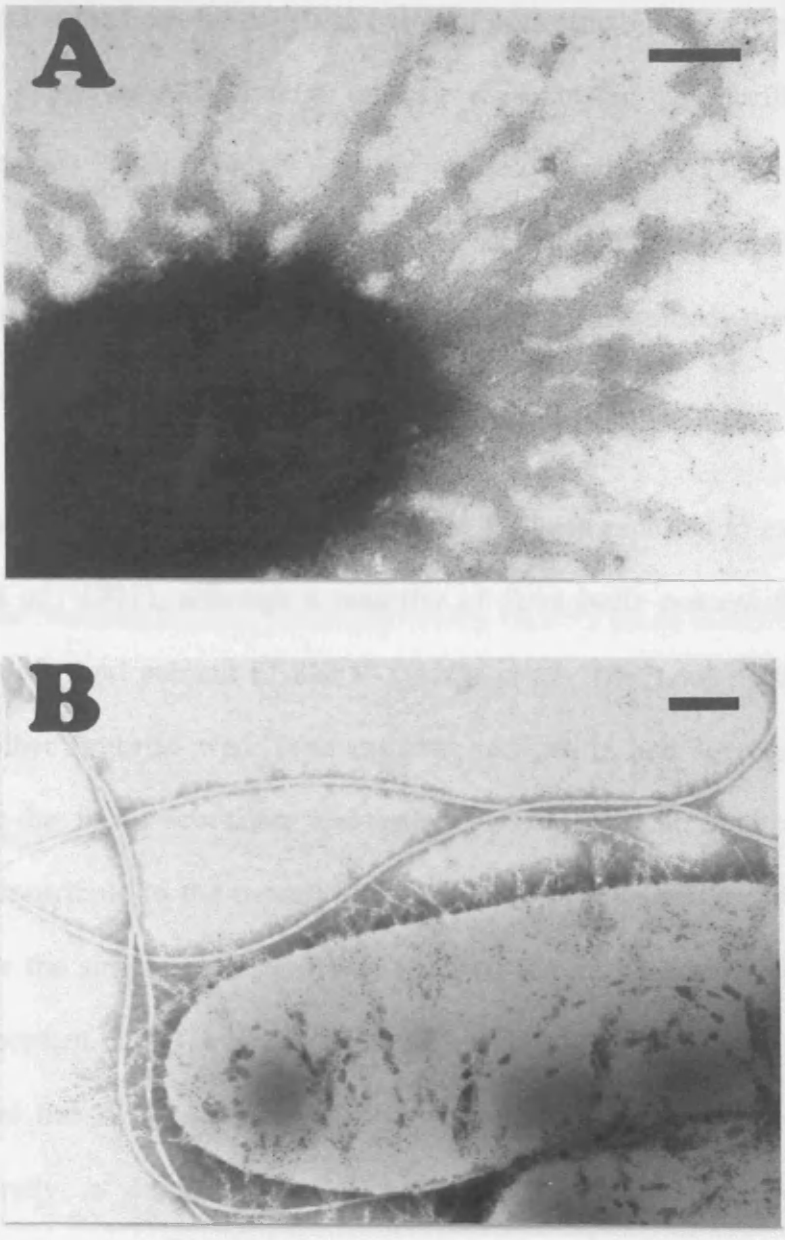
Sera raised to SEF14, SEF17, SEF21 and flagella were adsorbed with isogenic mutants B214 (14$^-$), EAV12 (17$^-$), EAV3 (SEF21$^-$) and EAV10 (Fla$^-$). *S. enteritidis* strains and their isogenic mutants were grown under conditions permissive for expression of fimbriae and flagella. LA5 and B214 (14$^-$) were grown on Sensitest agar for 24 hours at 37°C, 27655R and EAV12 (17$^-$) were grown on CFA agar for 48 hours at 37°C, LA5 and EAV3 (21$^-$) were grown in Heart Infusion broth for 48 hours at 37°C and LA5 and EAV10 (Fla$^-$) in Nutrient broth shaken aerobically for 24 hours at 37°C, for elaboration of SEF14, SEF17, SEF21 and flagella, respectively. All bacteria were coated to microtitre wells at 1x10^6 *Salmonella* per well. Wild type *Salmonella* was reacted with serial dilutions of **A)** unadsorbed chicken immune sera **B)** adsorbed chicken sera, whereas isogenic mutants were reacted with serial dilutions of **C)** unadsorbed chicken immune sera and **D)** adsorbed chicken sera. In addition, wild type *Salmonella* was reacted with serial dilutions of **E)** normal chicken sera. Primary antibody was detected with anti-chicken immunoglobulin labelled with peroxidase and developed with TMB substrate. Absorbances were recorded at 450nm after stopping with sulphuric acid.

Figure 3.10 Western blot with Mabs raised to flagella of *S. enteritidis*.



Purified flagella transferred onto nitrocellulose membrane was reacted with a panel of monoclonal antibodies: lanes **a)** M138/2/1, **b)** M138/2/3, **c)** M138/3/1, **d)** M138/3/2, **e)** M138/4/1, **f)** M138/4/2, **g)** M138/5/1, **h)** M138/5/2, **i)** M138/8/1, **j)** M138/11/1, **k)** normal mouse sera and **l)** SEF14 Mab. Primary antibody was detected with anti-mouse secondary antibodies conjugated with alkaline phosphatase and visualised by the addition of AEC insoluble substrate.

Figure 3.11 Immunogold-labelling of A) SEF14 and B) SEF21 fimbriae of *S. enteritidis* strain LA5 using Mabs M69/25/1 and M87/4/3, respectively.



Bacteria were passively bound to Carbon-Formvar coated grids and reacted with Mab. Grids were incubated with anti-mouse immuno-gold labelled antibody, counterstained with phosphotungstic acid and viewed under transmission electron-microscope. Bars represent 200 nm.

3.3 Discussion

Fimbriae and flagella of *S. enteritidis* are biochemically, antigenically and morphologically distinct. Specific methods for the purification of each structure were employed to extract and purify these appendages to produce immune reagents for the specific detection of these surface structures. Panels of Mabs specific for SEF14 and SEF21 have been raised in this laboratory prior to the commencement of this project and therefore, the primary focus was upon the purification of SEF17 and flagella and subsequent antibody production.

A single strain of *S. enteritidis,* 27655R, has so far been reported to express SEF17 *in vitro* (Collinson *et al.*, 1991), although a majority of *Salmonella* possess the gene *agf*A, which encodes the structural subunit of SEF17 (Doran *et al.*, 1993). A feature which set SEF17 apart from other fimbriae were their extreme insolubility and aggregative nature which is thought to be due to the secondary and tertiary structures of the fimbrin and its polymerised form which contribute to the overall hydrophobicity of the surface appendage (Collinson *et al.*, 1991) for the structural subunit was reported not have an unusually high hydrophobic amino acid content. This was not confirmed in this study, however, several observations suggested that the 17 kDa protein purified from strain 27655R was derived from SEF17 fimbriae. Firstly, as described by Collinson *et al.* (1991) an extracellular matted fimbrial material was observed from 27655R, but not 27655S, by electron microscopy prior to purification, and secondly, the purified protein had not entered the preparative SDS-PAGE gel but had resolved following brief treatment with formic acid at approximately 17 kDa.

For SEF17 purification the extreme insolubility of the molecule was utilised, since a majority of other cellular macromolecules were removed by enzymic digestion of sonicated cells, solubilised with SDS and contaminants separated by preparative SDS-PAGE. Interestingly, SEF21 fimbriae required mild acidic treatment for dissociation into fimbrial subunits, by first boiling in low pH (1.8) sample buffer containing 0.2M glycine (Sojka *et al.*, 1996). This method was unsuitable for SEF17 for it failed to depolymerise the thin aggregative fimbriae although brief treatment with formic acid was successful.

Methods used routinely for releasing fimbriae from whole cells can include blending (Aleksic *et al.*, 1979; de Graaf & Roorda, 1982; Feutrier *et al.*, 1988; Guerina et al., 1985) or by moderate heat treatment of cells (Baloda, 1988; Gaastra & de Graaf, Thorns *et al.*, 1992). Normally fimbriae released by either method remain in the supernatant after the cells were removed by centrifugation. Conventional purification protocols would not result in the detection of similar aggregative fimbriae from other bacteria, for such organelles would be removed by centrifugation following the initial blending step. Indeed, it is plausible that Baloda *et al.* (1988) mis-interpreted the fimbrial type responsible for fibronectin binding for they reported that a 14 kDa molecule was detected using conventional methods. However, it was possible that SEF14 and SEF17 were concurrently expressed by *S. enteritidis* strain 27655R and that SEF14 was depolymerised and resolved by SDS-PAGE, whereas, SEF17 fimbriae remained intact and importantly, unidentified by this method.

Following the harsh formic acid treatment of SEF17 fimbriae there was evidence that the fimbrin structures had been weakened for the purified preparation was extremely unstable in

electrophoresis sample buffer. Rather than giving a sharp, pure 17 kDa band the preparation became smeared if not used immediately and, in addition, boiling the sample prior to loading the gel resulted in a lower yield of SEF17. This was not observed with purified SEF14, SEF21 or flagella.

SEF17 was considered a poorly immunogenic molecule in mice and chickens because a poor immune response was evoked with the primary and secondary inoculation, but this study was successful in producing one Mab and mono-specific ChSEF17. The specificity of the antibodies were confirmed by Western blot and in immuno-electron microscopy analysis antibodies bound to SEF17 of *S. enteritidis* strain 27655R. Interestingly, Collinson *et al.*, (1991) reported that SEF17 was only found attached to the bacterium, but electron microscopy observations revealed cell-associated and copious quantities of cell-free SEF17 fimbriae.

A single Mab of the IgM isotype from two fusions offers further evidence of the poorly immunogenic nature of SEF17, indicating a dominant IgM B blast population of cells in the mouse spleen. In contrast, a single flagella fusion yielded ten monoclonal antibodies of IgG isotype and these results were consistent with fusions previously performed in this laboratory for SEF14 (Thorns *et al.*, 1992) and SEF21 (Sojka *et al.*, 1996). In addition, the hybridoma secreting SEF17 Mab contained a genetically unstable karyotype even after several clonings. Therefore, it was not possible to prepare large quantities of Mab by continuous culture. A seed stock of twenty vials was established and stored in liquid nitrogen and from each

ampoule approximately 5 mg of Mab could be produced in 100 ml of tissue culture supernatant.

In addition, purified SEF17 was used for the production of polyclonal sera in chickens which was to be the experimental animal used later in this study. The specificity of the antibody was evaluated in a whole cell ELISA and was found necessary to adsorb the sera with the isogenic SEF17 mutant of strain 27655R to remove non-SEF17 antibodies. The specificity of adsorbed sera was confirmed in immuno-electron microscopy and it was observed that anti-SEF17 sera labelled SEF17 fimbriae more densely than the Mab, although they both labelled specifically. It is likely that ChSEF17 antibodies were raised to many SEF17 epitopes, whereas the Mab recognised a single epitope.

In general, to attain a multivalent chicken sera specific for fimbriae and flagella it was necessary to adsorb with the appropriate isogenic mutants, although for anti-SEF14 sera it was unnecessary, This is likely due to a combination of the purity of the fimbrial preparation and the immunogenicity of the SEF14 molecule. Indeed, Thorns *et al.* (1992) reported SEF14 to be highly immunogenic in mice and rabbits and a serological response was observed in chickens following infection with *S. enteritidis* (Thorns *et al.,* 1993; 1996).

The Mabs raised to flagella reacted similarly to ChFla sera in the whole cell direct binding ELISA (Table 2.1). In the Kauffmann-White scheme (1964), eight major subfactors (*f, g, m, p, q, s, t and u*) of the *Salmonella* phase-1 *g* antigen were described and it has been shown that

factor *g* itself is a complex composed of two or more of at least five subfactors, *g*1 to *g*5 (Yamaguchi & Iino, 1968). Chemical and immunological analyses on *g* flagellin by Parish *et al.* (1969) demonstrated that all the antigenic specificities reside in the central region of the flagellin polypeptide and from a genetic approach, Iino, (1977) provided additional evidence that the central area of the molecule contained the antigenically variable regions. A more detailed comparison of *Salmonella fli*C genes by Maston & Joys, (1993) encoding phase-1 flagellins from members of the "g" series showed complete amino acid homology in the N terminal (regions I, II and III) and C-terminal (segments VIII) segments of the protein, whereas, differences in amino acid composition were found throughout regions IV, V, VI and VII, the central portion of the flagellins. In this study the ELISA results indicated that the Mabs recognised non-variable flagellin epitopes. These results are supported by Western blot analyses, whereby, all Mabs and Chfla bound to depolymerised flagellin subunits indicating that the antibodies recognised linear epitopes, whereas, Maston & Joys (1993) have suggested that the *g* series are conformational for no specific subfactor epitopes could be identified in regions IV, V, VI and VII.

Common flagellin epitopes flank the polypeptide chain and these have been reported to be incorporated within the hollow core of the intact flagellin whereas the specific epitopes, for *S. enteritidis g* and *m*, are exposed (Wei & Joys, 1985). This may explain the lack of labelling by Mabs when viewed by electron microscopy, but it was surprising that the ChFla failed to label. It is plausible that common epitopes were immunodominant when presented by the antigen presenting cells of the chicken and mouse immune systems.

In summary, monoclonal antibodies to SEF17 and flagella and chicken antibodies to all four surface structures have been raised to the purified reagents. The specificity of immune reagents have been thoroughly evaluated in Western blots and immuno-electron microscopy and the results are summarised in table 3.2. Specific direct binding ELISA's have been developed for each appendage and should prove valuable in chapter 4, in which phenotypic expression of surface appendages will be studied in response to environmental stimuli.

Chapter 4

Expression and phenotypic characteristics of SEF17 fimbriae of *S. enteritidis*.

4.1 Introduction

Little information is available on expression of SEF17 either *in vitro* or *in vivo*. The *agf*A gene encodes the major fimbrin subunit AgfA, which is related to *E. coli* curli fimbriae (Collinson *et al.*, 1992) and thin aggregative fimbriae of *S. typhimurium* (Stolpe *et al.*, 1994). Doran *et al.* (1993) reported that a DNA probe targeting the *agf*A structural gene hybridised strongly with 603 of 604 isolates tested suggesting the gene is conserved across the *Salmonella* genus. However, preliminary experiments in this study indicated that SEF17 fimbriae was not expressed on *S. enteritidis* strains other than 27655R (Collinson *et al.*, 1991) when cultured under similar conditions. Therefore, it was likely that the conditions for elaboration were non-permissive in all strains except 27655R.

It has been reported that SEF14 (Thorns *et al.*, 1990; 1993), SEF21 fimbriae (Sojka *et al.*, 1996; 1998) and flagella (Walker *et al.*, 1999) were elaborated in response to specific environmental stimuli and relative phase of growth. Therefore, in order to establish whether SEF17 could be expressed *in vitro* by strains other than 27655R, the effect of media composition, gaseous phase and temperature on SEF17 elaboration were to be determined. Specifically, the aims were 1) to demonstrate that SEF14, SEF21 and flagella ELISA's may be used to develop model systems to evaluate elaboration of surface antigens in response to known environmental stimuli, 2) to compare and contrast some environmental factors affecting expression of these surface organelles, 3) to apply this model to establish whether SEF17 may be expressed *in vitro* by *S. enteritidis* strains and 4) to characterise phenotypic traits that have been reported to be associated with SEF17 expression.

4.2 Results

4.2.1 Development of a model system

Monoclonal antibody-based direct binding ELISA's developed and evaluated in chapter 3 were utilised to determine the effect of growth media, gaseous phase and temperature on the expression of SEF14, SEF21 and flagella by *S. enteritidis* as a model system. *S. enteritidis* strains LA5, 27655R and 8524 were grown statically for 48 hours in Heart Infusion broth, CFA broth, Luria broth, Peptone Water, Tryptone Water, Minimal Essential Media, Sensitest agar, Nutrient agar, Drigalski agar or CFA agar (Table 4.1), under aerobic, anaerobic or CO_2 enriched air environments (Table 4.3) or at 18°C, 25°C, 37°C or 42°C (Table 4.4) and samples were assayed at known time points up to 72 hours (Table 4.2). Bacteria were washed, serial diluted in microtitre plates and direct-binding ELISA's performed as described in the methods section. Absorbances corresponding to wells coated with 10^7 bacteria are summarised in tables 4.1, 4.2, 4.3 & 4.4.

SEF14 expression

SEF14 fimbriae was expressed by *S. enteritidis* strains LA5, 27655R and 8524 grown in all liquid and agar media tested (Table 4.1), albeit in varying amounts. High or moderate elaboration by all three strains was observed on CFA broth, Peptone water, Sensitest agar and Nutrient agar, whereas Drigalski agar and CFA agar only supported low expression. ELISA results correlated with a latex agglutination test (Thorns *et al.*, 1994) for the specific detection of SEF14 (Figure 2.1), which utilised the same Mab. High expression of SEF14 was maintained by static growth in CFA broth at 37°C over time as assessed by sampling up to 72 hours post inoculation (Table 4.2).

LA5, 27655R and 8524 were grown for 48 hours in CFA broth at 37°C in aerobic, anaerobic or CO_2 enriched air (5% CO_2) environments. All three strains expressed high levels of SEF14 when grown aerobically and low levels after anaerobic growth (Table 4.3). Following growth in $C0_2$ enriched air strains LA5 and 8524 expressed very high quantities of SEF14 fimbriae, whereas 27655R expressed low levels.

The effect of growth temperature was evaluated by culturing LA5, 27655R and 8524 statically in CFA broth for 48 hours at 18°C, 25°C, 37°C or 42°C. All three strains expressed high levels of SEF14 at 37°C and above, but did not elaborate detectable levels of fimbriae at 25°C or below (Table 4.4).

SEF21 expression

SEF21 was expressed by strain 27655R in all media following growth at 37°C for 48 hours and all planktonic growth by strains LA5 and 8524 (Table 4.1). For all three strains moderate or high expression of SEF21 fimbriae was observed in Heart Infusion broth whereas CFA broth and Luria broth supported moderate and high expression, respectively, by strain 27655R. SEF21 elaboration was not detected on strains LA5 or 8524 on Sensitest agar, Nutrient agar, Drigalski agar or CFA agar, whereas low expression was observed on these media by 27655R. Heart Infusion broth supported fimbrial elaboration in all three strains, therefore, this media was used for expression of SEF21 in subsequent studies.

Mannose Sensitive Haemagglutination (MSHA) assays were performed and a good correlation was observed between ELISA results and MSHA (Figure 2.2). For example, a strong MSHA was observed and high ELISA absorbance was recorded with strain 27655R following growth in Heart Infusion broth for 48 hours at 37°C indicating high SEF21

expression, whereas LA5 grown under the same conditions gave lower absorbances in ELISA and a weaker agglutination reaction. In addition, LA5 grown on all agar was negative in both tests.

SEF21 expression was not detected by ELISA on strains LA5 and 8524 until 48 hours aerobic growth in Heart Infusion broth at 37°C, whereas fimbriae were elaborated on 27655R within 24 hours (Table 4.2). Anaerobic growth suppressed fimbrial expression, whereas CO_2 enriched air growth enhanced elaboration (Table 4.3). For all three strains, SEF21 expression was observed at 18°C, 25°C, 37°C and 42°C and, in general, expression was enhanced with increased temperature (Table 4.4).

Flagella expression

Flagella were expressed by all *S. enteritidis* strains on all media (Table 4.1). Growth of *Salmonella* in CFA broth, Luria broth, Sensitest agar, Nutrient agar and CFA agar encouraged high or moderate elaboration of flagella whereas, Tryptone water consistently expressed low levels of flagella by all three strains. ELISA results were confirmed by transmission electron microscopy (Figure 4.1).

All three strains grown in CFA broth at 37°C for 48 hours in aerobic, anaerobic or CO_2 enriched air environments produced moderate or high levels of flagella (Table 4.3), whilst temperature appeared to have little effect on expression (Table 4.4).

4.2.2 Expression of SEF17 fimbriae

Detailed analysis of the growth conditions of three *S. enteritidis* strains confirmed that media composition, gaseous phase, temperature and growth phase influenced elaboration of SEF14 and SEF21 and to a lesser extent flagella. ELISA results showed good correlation with latex agglutination, haemagglutination and electron microscopy such that confidence was gained that ELISA was an appropriate method for detecting the elaboration of surface appendages. Therefore, the conditions described in section 4.3.1 were subsequently applied to the study of SEF17 expression.

4.2.2.1 Growth conditions affecting the elaboration of SEF17

Elaboration of SEF17 was restricted to strain 27655R when grown at 37°C for 48 hours irrespective of media (Figure 4.1). Interestingly, CFA agar supported maximal elaboration by strain 27655R and it was on this media that thin aggregative fimbriae (Baloda *et al.*, 1988) and curli (Olsen *et al.*, 1989) were initially observed. Low expression of SEF17 by strain 27655R was observed on minimal essential media and Heart Infusion broth with moderate fimbrial elaboration on all other media tested (Table 4.1). Thus, bacteria were grown on CFA agar for ensuing experiments.

At 37°C, high expression of SEF17 was observed on strain 27655R between 24 and 72 hours, but not LA5 or 8524 (Table 4.2). After growth for 48 hours, an anaerobic environment induced low SEF17 expression in strain 27655R whereas high fimbrial expression was observed after aerobic or CO_2 enriched air incubation of this strain (Table 4.3). LA5 and 8524 failed to express SEF17 fimbriae when grown under these conditions whereas after aerobic growth of bacteria on CFA agar for 48 hours at 25°C or below, SEF17 fimbriae was expressed by all three *S. enteritidis* strains (Table 4.4). Interestingly, strain 27655R expressed

high levels of fimbriae at all temperatures tested, whereas, strains LA5 and 8524 both elaborated low levels of SEF17 at 25°C but greater quantities at 18°C. These results indicated that lower temperatures induced SEF17 expression on strains LA5 and 8524, whereas elaboration was not tightly regulated on 27655R.

A panel of fifteen clinical or avian bacterial isolates were tested to determine whether expression of SEF17 was a common trait amongst *Salmonella* and an *E. coli* strain, which was reported to express curli at 37°C (Collinson *et al.*, 1992). *S. enteritidis* strains 27655R and S1900 expressed SEF17 equally well at temperatures between 18°C to 42°C (Table 4.5), whereas, the remaining wild type *S. enteritidis* strains tested produced SEF17 at temperatures ranging from 18°C to 30°C with highest expression observed at 18°C. Elaboration was not detected at 37°C. *S. typhimurium* strain Mö8, which was reported to express a highly hydrophobic and thin (3nm in diameter) fimbriae (Grund & Webber, 1988), bound strongly to the Mab when cultured at all temperatures examined, whereas *E. coli* strain NG7C, which expressed curli fimbriae, bound the SEF17 Mab at 25°C and above (Table 4.5).

For expression in different media the bacteria were grown aerobically at 25°C for 48 hours on various agar. *S. enteritidis* strains 27655R and S1900 and *S. typhimurium* Mö8 bound the SEF17 Mab when grown in all media, whereas the other wild type *S. enteritidis* strains bound Mab only when grown on CFA, Luria-Bertani, MINCA and Nutrient agars (Table 4.6). None of the defined mutants of *S. enteritidis*, the naturally occurring mutants, 27655S or I, bound the SEF17 Mab after growth on any media. The *E. coli* curli-producing strain NG7C only bound SEF17 Mab when cultured on CFA and Luria agars.

4.2.2.2 Expression of thin aggregative fimbriae amongst Enterobacteriaceae

Doran *et al.* (1993) reported that an *agf*A DNA fragment, encoding the SEF17 fimbrin structural gene, hybridised strongly to 603 of 604 *Salmonella* isolates albeit very weakly to 31, but not at all with 235 of 266 other members of the family Enterobacteriaceae. This implied that the gene was conserved across the *Salmonella* genus, although phenotypic expression had not been confirmed. It was decided to apply the growth conditions determined in this study which were permissive for SEF17 elaboration to ascertain the extent of expression of thin aggregative fimbriae amongst the *Salmonella* genus. Therefore, a total of 57 *Salmonella* isolates from ten serogroups and 17 other strains from the Enterobacteriaceae family were probed with an *agf*A DNA probe. Each *Salmonella* strain hybridised (Table 4.7), as did two *Citrobacter freundii* strains and one *Enterobacter tarde* (Table 4.8). The panel of strains was grown on CFA agar for 72 hours at 18°C and tested with the SEF17 Mab and the adsorbed chicken polyclonal sera (ChSEF17) in the direct binding ELISA's. Fifty three *Salmonella* isolates from 57 reacted in SEF17 ELISA's, 49 reacted with both Mab and ChSEF17 and two isolates reacted with either Mab or ChSEF17 alone. The *Citrobacter freundii* strains and *Enterobacter tarde* reacted with Mab and ChSEF17 (Table 4.8). Expression of a morphologically similar fimbriae was confirmed by electron microscopy on these strains (Figure 4.2). *Citrobacter spp* and *Salmonella spp.* are antigenically similar and are commonly mis-serotyped, and therefore, the *Citrobacter* strains were confirmed biochemically by API 20E system.

4.2.3 Phenotypic characteristics of SEF17 fimbriae

4.2.3.1 Correlation with fibronectin binding

SEF17 and similar fimbriae of *S. typhimurium* and *E. coli* have been implicated in mediating-fibronectin binding (Collinson *et al.*, 1993). To test this a panel of wild type and mutant *S.*

enteritidis, *S. typhimurium* strain Mö8 and the curli expressing *E. coli* strain NG7C were grown on CFA agar at 37°C or 18°C (strain NG7C at 25°C) for 48 and 72 hours, respectively. Bacteria were probed separately with monoclonal and polyclonal antibodies to SEF17, and also soluble fibronectin in direct-binding ELISA's. A complete correlation was observed between the binding of soluble fibronectin and SEF17 elaboration (Figure 4.3). After incubation at 37°C *S. enteritidis* strains 27655R and S1900, *S. typhimurium* strain Mö8 and *E. coli* NG7C bound fibronectin, ChSEF17 and Mab strongly, whereas all other strains were negative in both assays. Following growth at 18°C strains 27655R, S1400, LA5, E, C6B, S1900, 10360, 12804, 8524 and Mö8 bound fibronectin, SEF17 Mab and ChSEF17, whereas the defined SEF17 mutants EAV11 and EAV12 derived from strains S1400 and LA5, respectively, and strain 27655S did not (Figure 4.3). *E. coli* strain NG7C and strain I bound ChSEF17 and fibronectin weakly but did not bind the Mab.

4.2.3.2 Formation of "lacy" colonies

The production of a convoluted morphotype by some strains of *S. enteritidis* has been linked with the ability of isolates to invade reproductive tracts of laying hens (Humphrey *et al.*, 1996) and increased virulence in chicks (Guard-Petter *et al.*, 1996). A correlation was observed between a variety of convoluted colony formations, generically referred to as "lacy" colonies (Figure 4.4), and SEF17 expression. Following growth on CFA agar for 48 hours at various temperatures, "lacy" colonies were generated by *S. enteritidis* strains 27655R, S1900 and *S. typhimurium* strain Mö8 between 25°C and 37°C (Table 4.9). Strains S1400, E, C6B and 8524 formed "lacy" colonies at 25°C and 30°C, whereas the convoluted phenotype was observed on strains LA5, 10360,12804 and *E. coli* strain NG7C at 25°C only. After seven days at 18°C strains LA5, E and C6B showed evidence of "laciness", whereas the SEF17 mutants 27655S, I, EAV11 and EAV12 did not form "lacy" colonies at any temperature.

In addition, the formation of "lacy" colonies was dependent upon growth media. Following growth at 25°C for 48 hours (Table 4.10) a majority of SEF17 expressing strains produced the convoluted morphotype on CFA and Luria-Bertani agars alone, whereas, laciness was observed on Nutrient and Sensitest agars by *S. enteritidis* strains 27655R and S1900 and *S. typhimurium* strain Mö8. Drigalski and MINCA agars did not support "lacy" colony formation.

4.2.3.3 Auto-aggregative phenotype and the uptake of congo red

Austin *et al.* (1998) have reported that wild type *S. enteritidis* strain 27655R readily adhered to inanimate surfaces forming thick cell aggregates and implicated the involvement of SEF17 in cell-cell interactions during biofilm formation. In this study good correlation was observed between SEF17 expression and the ability of colonies to auto-aggregate and adhere to agar following growth on CFA agar for 48 hours at various temperatures (Table 4.11). Aggregative bacterial colonies were difficult to scrape from the agar surface without disrupting the agar. A majority of SEF17 elaborating strains displayed the auto-aggregative phenotype at 18°C, 25°C and 30°C, but was not observed on cultures grown at 42°C. SEF17 mutants, strain 12804 and *E. coli* NG7C were non-aggregative following growth at any temperature.

A correlation has been reported between SEF17 expression and binding of the hydrophobic dye Congo red (Collinson *et al.*, 1993). Therefore, early on in this study, and before specific antibodies were available for the detection of SEF17, a panel of *S. enteritidis* isolates were serially cultured at 37°C on CFA agar containing Congo red which Collinson *et al.* (1993) reported provided a simple and rapid test for screening colonies which harboured SEF17 or related fimbriae. Upon serial passage, strain 27655R consistently adsorbed the hydrophobic

dye, whereas 27655S did not. Following the third serial culture, a few red colonies were visible amongst cream colonies by strain S1925/93. These colonies remained red on subsequent passages but the bacteria were not auto-aggregative (Collinson *et al.*, 1991; 1993; Römling *et al.*, 1998b) and when examined by electron microscopy did not elaborate SEF17-like fimbriae (data not shown). Later in the study after the conditions necessary for SEF17 elaboration had been established, *Salmonella* were grown for 48 hours on CFA agar containing 100μg/ml Congo red at temperatures between 18°C and 37°C. Strains 27655R, S1900 and Mö8 bound the red dye strongly at all temperatures tested, whereas the SEF17 deficient mutants did not (Figure 4.5). Interestingly, strains which only produced low levels of SEF17 fimbriae, as detected by ELISA, could not be distinguished from the mutants and, additionally, if incubated for longer periods of time many SEF17 producing strains and non-SEF17 producing strains bound the dye to varying degrees. Therefore, Congo red binding was deemed an insensitive and non-specific indication of SEF17 expression.

Table 4.1 ELISA to show the effect of growth media on expression of SEF14, SEF17, SEF21 and flagella *in vitro.*

Organelle	Strains	Media: Heart Infusion broth	CFA broth	Luria broth	Peptone water	Tryptone water	Minimal essential media	Sensitest agar	Nutrient agar	Drigalski agar	CFA agar
SEF14	LA5	+	+++	+	+++	++	+	++	++	+	+
	27655R	++	+++	+++	+++	+++	++	++	++	+	+
	8524	+	+++	+	++	++	++	+++	++	+	+
SEF17	LA5	-	-	-	-	-	-	-	-	-	-
	27655R	+	++	++	++	++	+	++	++	++	+++
	8524	-	-	-	-	-	-	-	-	-	-
SEF21	LA5	++	+	+	+	+	+	-	-	-	-
	27655R	+++	++	+++	+	+	+	+	+	+	+
	8524	++	+	+	+	+	+	-	-	-	-
Flagella	LA5	+++	+++	+++	+++	+	++	++	++	++	++
	27655R	+	++	++	+	+	+	+++	++	+	++
	8524	+++	+++	+++	+	+	++	+++	+++	++	+++

- \- No expression (0.0-0.1 absorbance at 450 nm)
- \+ Low expression (0.1-0.5 absorbance at 450 nm)
- ++ Moderate expression (0.5-1.0 absorbance at 450 nm)
- +++ High expression (>1.0 absorbance at 450 nm)

Prior to direct-binding ELISA bacteria were grown for 48 hours at 37°C in each media. Bacteria were coated to microtitre plates, blocked and probed with M69/25, M117, M87/4/3 and M138/3/1Mabs to detect SEF14, SEF17, SEF21 and flagella, respectively. Primary antibody was detected with anti-mouse Ig -peroxidase conjugate and and the assay was developed with TMB substrate. Absorbances corresponded to wells coated with 10^7 *Salmonella.* Results represent the mean absorbances of 2 assays.

Table 4.2 Direct-binding ELISAs to determine the effect of fimbrial and flagella expression following static growth for up to 72 hours at 37°C.

Organelle	Strain	Hours of growth		
		24	48	72
SEF14	LA5	+++	+++	+++
	27655R	++	++	++
	8524	+++	+++	+++
SEF17	LA5	-	-	-
	27655R	+++	+++	+++
	8524	-	-	-
SEF21	LA5	-	++	++
	27655R	++	+++	+++
	8524	-	++	++
Flagella	LA5	+++	+++	+++
	27655R	++	++	++
	8524	+++	+++	+++

-	No expression	(0.0-0.1 absorbance at 450nm)
+	Low expression	(0.1-0.5 absorbance at 450nm)
++	Moderate expression	(0.5-1.0 absorbance at 450nm)
+++	High expression	(1.0-1.5 absorbance 450nm)

Salmonella were grown in different media to express each appendage: CFA broth for expression of SEF14 and flagella, CFA agar for SEF17 elaboration and Heart Infusion broth for SEF21 expression. Bacteria were coated to microtitre plates, blocked and probed with M69/25, M117, M87/4/3 and M138/3/1 Mabs to detect SEF14, SEF17, SEF21 and flagella, respectively. Primary antibody were detected with anti-mouse Ig - peroxidase conjugate and and the assay was developed with TMB substrate. Absorbances corresponded to wells coated with 10^7 *Salmonella* and represent the mean absorbances from 2 assays..

Table 4.3 Direct-binding ELISA to determine the effect of growth in aerobic, anaerobic and air enriched with CO_2 on the *in vitro* expression of fimbriae and flagella by *S enteritidis*.

Organelle	Strain	Aerobic	Anaerobic	$C0_2$ enriched air (5% CO_2)
SEF14	LA5	+++	+	++++
	27655R	+++	+	+
	8524	+++	+	++++
SEF17	LA5	-	-	-
	27655R	+++	+	+++
	8524	-	-	-
SEF21	LA5	++	+	+++
	27655R	+++	+	+++
	8524	++	+	+++
Flagella	LA5	+++	+++	++
	27655R	++	++	++
	8524	+++	+++	++

- No expression (0.0-0.1 absorbance at 450nm)
+ Low expression (0.1-0.5 absorbance at 450nm)
++ Moderate expression (0.5-1.0 absorbance at 450nm)
+++ High expression (1.0-1.5 absorbance 450nm)
++++ Very high expression (>1.5 absorbance 450nm)

Salmonella were cultured for 48 hours at 37°C in optimal media for elaboration of each appendage in the various gaseous environments. Bacteria were coated to microtitre plates, blocked and probed with M69/25, M117, M87/4/3 and M138/3/1 Mabs to detect SEF14, SEF17, SEF21 and flagella, respectively. Primary antibody were detected with anti-mouse Ig - peroxidase conjugate and and the assay was developed with TMB substrate. Absorbances corresponded to wells coated with 10^7 *Salmonella* and represent the mean absorbances from 2 assays.

Table 4.4 Direct-binding ELISA to determine the effect of growth temperature on *in vitro* expression of fimbriae and flagella by *S. enteritidis*.

Organelle	Strain	18°C	25°C	37°C	42°C
SEF14	LA5	-	-	+++	+++
	27655R	-	-	+++	+++
	8524	-	-	+++	+++
SEF17	LA5	++	+	-	-
	27655R	+++	+++	+++	+++
	8524	++	+	-	-
SEF21	LA5	+	++	++	++
	27655R	+	++	+++	+++
	8524	+	+	++	++
Flagella	LA5	+++	+++	+++	+++
	27655R	++	++	++	++
	8524	+++	+++	+++	+++

- No expression (0.0-0.1 absorbance at 450nm)
+ Low expression (0.1-0.5 absorbance at 450nm)
++ Moderate expression (0.5-1.0 absorbance at 450nm)
+++ High expression (1.0-1.5 absorbance at 450nm)
++++ Very high expression (>1.5 absorbance at 450nm)

Salmonella were grown aerobically for 48 hours in optimal media for elaboration of each appendage at 18°C, 25°C, 37°C or 42°C. Bacteria were coated to microtitre plates, blocked and probed with M69/25, M117, M87/4/3 and M138/3/1 Mabs to detect SEF14, SEF17, SEF21 and flagella, respectively. Primary antibody were detected with anti-mouse Ig - peroxidase conjugate and and the assay was developed with TMB substrate. Absorbances corresponded to wells coated with approximately 10^7 *Salmonella* and represent the mean absorbances from 2 assays.

Table 4.5 Direct binding of SEF17 monoclonal antibody to strains of *Salmonella* and *E. coli* grown on CFA agar for 48 hours at different temperatures.

Bacteria	Strain	Temperature				
		18°	25°	30°	37°	42°
S enteritidis	27655R	++	++	++	++	++
S. enteritidis	27655S	-	-	-	-	-
S. enteritidis	S1400	+	+	+	-	-
S. enteritidis	EAV11	-	-	-	-	-
S. enteritidis	LA5	+	+	-	-	-
S. enteritidis	EAV12	-	-	-	-	-
S. enteritidis	I	-	-	-	-	-
S. enteritidis	E	++	+	+	-	-
S. enteritidis	C6B	+	+	+	-	-
S. enteritidis	S1900	++	++	++	++	++
S. enteritidis	10360	+	+	+	-	-
S. enteritidis	12804	+	+	-	-	-
S. enteritidis	8524	++	+	+	-	-
S. typhimurium	Mö8	++	++	++	++	ND
E. coli	NG7C	-	++	++	++	+

ND not done
- absorbance in direct binding ELISA <0.1
+ absorbance in direct binding ELISA between 0.1 and 0.5
++ absorbance in direct binding ELISA >0.5
EAV11 S1400 *agf*A$^-$
EAV12 LA5 *agf*A$^-$
Results shown represent the mean absorbances from 2 assays.

Table 4.6 Direct binding of SEF17 monoclonal antibody to strains of *Salmonella* and *E. coli* grown for 48 hours at 25°C on various media.

		MEDIA					
Bacteria	**Strain**	CFA agar	Drigalski agar	Luria agar	MINCA agar	Nutrient agar	Sensitest agar
S. enteritidis	27655R	++	++	++	++	++	++
S. enteritidis	27655S	-	-	-	-	-	-
S. enteritidis	S1400	+	-	+	+	+	-
S. enteritidis	EAV11	-	-	-	-	-	-
S. enteritidis	LA5	+	-	+	+	+	-
S. enteritidis	EAV12	-	-	-	-	-	-
S. enteritidis	I	-	-	-	-	-	-
S. enteritidis	E	+	-	+	+	+	-
S. enteritidis	C6B	+	-	+	+	+	-
S. enteritidis	S1900	++	++	++	++	++	+
S. enteritidis	10360	+	-	+	+	+	-
S. enteritidis	12804	+	-	+	+	+	+
S. enteritidis	8524	+	-	+	+	+	-
S. typhimurium	Mö8	+	++	+	++	++	+
E.coli	NG7C	++	-	+	-	-	-

- absorbance in direct binding ELISA < 0.1
+ absorbance in direct binding ELISA between 0.1 and 0.5
++ absorbance in direct binding ELISA >0.5

EAV11 S1400 *agf*A$^-$
EAV12 LA5 *agf*A$^-$
Results shown represent the mean absorbances from 2 assays.

continued:

Serotype	Strain	Sero-group	Phage type	*agf*A probe*	Mab †	ChSEF17†
S. enteritidis	S6462	D1	14b	+	+++	+++
S. enteritidis	S893	D1	24	+	+++	+++
S. enteritidis	S5812	D1	29	+	+++	+++
S. enteritidis	S6632	D1	30	+	+	+
S. enteritidis	27655R	D1	nd	+	++	++
S. enteritidis	27655S	D1	nd	+	-	-
S. enteritidis	S1400	D1	4	+	++	++
S. enteritidis	LA5	D1	4	+	++	++
S. enteritidis	S8524	D1	4	+	++	++
S. dublin	S4129	D1	nd	+	-	-
S. dublin	S262	D1	nd	+	++	+
S. freono	S169	D2	nd	+	++	++
S meleagridis	S29	E1	nd	+	++	++
S. drypool	S365	E2	nd	+	++	++
S. aberdeen	S129	F	nd	+	+	++
S. grumpensis	S331	G1	nd	+	+	+
S. caracas	S1435	H	nd	+	++	-
S chameleon	S258	I	nd	+	+	++
S. minnesota	S357	L	nd	+	++	++
S. omifisan	S2576	R	nd	+	++	++

†ELISA
- 0.0-0.1 absorbance at 450 nm
+ 0.1-0.5 "
++ 0.5-1.0 "
+++ >1.0 "

nd Not phage typed

*Probe
- negative
+ positive

For detection of SEF17 expression by ELISA bacteria were grown on CFA agar for 48-72 hours at 18°C. ELISAs were performed twice and results shown represent the mean absorbances from 2 assays.

Table 4.7 The distribution of *agf*A gene and direct binding of SEF17 Mab and polyclonal sera (ChSEF17) amongst *Salmonella* serotypes.

Serotype	Strain	TYPE		*agf*A probe*	ELISA	
		Sero-group	Phage type		Mab †	ChSEF17 †
S. stanleyville	S359	B	nd	+	+	++
S. agona	S38	B	nd	+	+	++
S. agona	S1	B	nd	+	+	++
S. derby	S8930	B	nd	+	+	++
S. typhimurium	S10769	B	4	+	-	++
S. typhimurium	S5091	B	4	+	++	+++
S. typhimurium	S2316	B	12	+	+	++
S. typhimurium	S210	B	12	+	+	++
S. typhimurium	S3382	B	49	+	++	++
S. typhimurium	S4784	B	146	+	-	-
S. typhimurium	S2387	B	146	+	+	++
S. typhimurium	S1702	B	160	+	+	++
S. typhimurium	S1369	B	170	+	+	+
S. typhimurium	S1497	B	170	+	+	+
S. typhimurium	S1735	B	170	+	+	+
S. typhimurium	S2504	B	193	+	++	++
S. typhimurium	S3073	B	193	+	+	++
S. typhimurium	S889	B	204	+	++	+
S. typhimurium	S2522	B	204	+	+	+++
S. typhimurium	S1068	B	204c	+	+	++
S. typhimurium	S4850	B	204c	+	+	++
S. typhimurium	Mö8	B	nd	+	++	++
S. muenchen	S584	D1	nd	+	+	++
S. rostock	S696	D1	nd	+	-	-
S. dublin	S9	D1	nd	+	+	++
S. enteritidis	S1461	D1	4	+	++	++
S. enteritidis	S1362	D1	4	+	+	++
S. enteritidis	S9672	D1	9b	+	+++	+++
S. enteritidis	S3950	D1	9b	+	++	++
S. enteritidis	S4832	D1	1	+	++	++
S. enteritidis	S2694	D1	1a	+	++	++
S. enteritidis	S2869	D1	5	+	-	++
S. enteritidis	S3655	D1	7	+	++	++
S. enteritidis	S6489	D1	7a	+	+	++
S. enteritidis	S3451	D1	6	+	++	++
S. enteritidis	S6648	D1	8	+	+	++
S. enteritidis	S6958	D1	11	+	++	+++

Table 4.8 The distribution of *agf*A gene and the direct binding of SEF17 Mab and ChSEF17 to other Enterobacteriaceae when grown for 48-72 hours at 18°C on CFA agar.

			ELISA	
Species	**Strain**	***agf*A probe***	**Mab †**	**ChSEF17 †**
E. coli	NG7C	-	+	+
E. coli	K12	-	+	++
E. coli	7/94	-	++	+++
Citrobacter freundi	2884/93	+	+++	+++
"	1740/93	+/-	+	+
Klebsiella	C236	-	-	-
Yersinia enterolitica	CB56	-	-	-
Providencia stuartis	CB52	-	-	-
"	CB24	-	-	-
Enterobacter agglomera	4542/93	-	-	-
Enterobacter cloacae	6621/92	-	-	-
Enterobacter fergusonni	3584/93	-	-	-
Enterobacter hafnia	CB17	-	-	-
Enterobacter tarde	CB12	+/-	+	+
Enterobacter omnigenis	5114/93	-	-	-
Serratia nebidae	CB36	-	-	-
Serratia marascens	CB24	-	-	-

†ELISA
- 0.0-0.1 absorbance at 450 nm
+ 0.1-0.5 "
++ 0.5-1.0 "
+++ >1.0 "

*Probe
- negative
+/- very weak positive
+ positive

ELISAs were performed twice and results shown represent the mean absorbances from 2 assays.

Table 4.9 The effect of growth temperature on the formation of "lacy" colonies by *Salmonella* following growth on CFA agar for 48 hours as shown in figure 4.5.

		Temperature				
Bacteria	**Strain**	18°	25°	30°	37°	42°
S. enteritidis	27655R	-	+	+/-	+/-	-
S. enteritidis	27655S	-	-	-	-	-
S. enteritidis	1400	-	+	+	-	-
S. enteritidis	EAV11	ND	-	-	-	-
S. enteritidis	LA5	-*	+	-	-	-
S. enteritidis	EAV12	-	-	-	-	-
S. enteritidis	I	-	-	-	-	-
S. enteritidis	E	-*	+	+	-	-
S. enteritidis	C6B	-*	+	+	-	-
S. enteritidis	S1900	-	+	+	+/-	-
S. enteritidis	10360	-	+	-	-	-
S. enteritidis	12804	-	+	-	-	-
S. enteritidis	8524	-	+	+	-	-
S. typhimurium	Mö8	ND	+/-	+/-	+/-	-
E. coli	NG7C	-	-	-	-	-

ND not done (insufficient growth)
- Non-lacy
+/- Partial laciness
\+ Full laciness
* "lacy" after 7 days

EAV11 (1400 *agf*A⁻)
EAV12 (LA5 *agf*A⁻)

Table 4.10 The formation of "lacy" colonies by *Salmonella* after growth at 25°C for 48 hours on various media.

		MEDIA					
Bacteria	**Strain**	CFA agar	Drigalski agar	Luria agar	MINCA agar	Nutrient agar	Sensitest agar
S. enteritidis	27655R	+	-	+	-	+	+
S. enteritidis	27655S	-	-	-	-	-	-
S. enteritidis	S1400 wt	+	-	+	-	-	-
S. enteritidis	*EAV11	-	-	-	-	-	-
S. enteritidis	LA5 wt	+	-	+	-	-	-
S. enteritidis	*EAV12	-	-	-	-	-	-
S. enteritidis	I	-	-	-	-	-	-
S. enteritidis	E	+	-	+	-	-	-
S. enteritidis	C6B	+	-	+	-	-	-
S. enteritidis	S1900	+	-	+	-	+	+
S. enteritidis	10360	+/-	-	+/-	-	-	-
S. enteritidis	12804	+	-	+	-	-	-
S. enteritidis	8524	+	-	+	-	-	-
S. typhimurium	Mö8	+/-	-	+	-	+/-	+/-
E. coli	NG7C	-	-	-	-	-	-

- non lacy
+/- partial laciness
+ full laciness

*EAV11 (S1400 *agfA*⁻)
EAV12 (LA5 *agfA*⁻)

Table 4.11 Auto-aggregative phenotype of strains of *Salmonella* grown on CFA agar for 48 hours at different temperatures.

Bacteria	Strain	Temperature 18°	25°	30°	37°	42°
S. enteritidis	27655R	+	++	++	+	-
S. enteritidis	27655S	-	-	-	-	-
S. enteritidis	S1400/94	+	++	++	-	-
S. enteritidis	*EAV11	-	-	-	-	-
S. enteritidis	LA5	+	++	-	-	-
S. enteritidis	*EAV12	-	-	-	-	-
S. enteritidis	I	-	-	-	-	-
S. enteritidis	E	++	+	+	-	-
S. enteritidis	C6B	+	+	+	-	-
S. enteritidis	S1900	+	+	+	+	-
S. enteritidis	10360	-	-	+	-	-
S. enteritidis	12804	-	-	-	-	-
S. enteritidis	8524	+	++	++	-	-
S. typhimurium	Mö8	ND	+	+	+	-
E. coli	NG7C	-	-	-	-	-

ND not done (insufficient growth)
\- Non-aggregative
\+ Weakly aggregative
++ Strongly aggregative

*EAV11 (S1400 *agf*A$^-$)
EAV12 (LA5 *agf*A$^-$)

Auto-aggregation was determine by the degree of adherence of the bacteria to the agar surface.

Figure 4.1 Expression of flagella by *S. enteritidis* strain LA5 following aerobic growth in A) Heart Infusion broth, or B) Tryptone water for 48 hours at 37°C. Bars, 200 nm

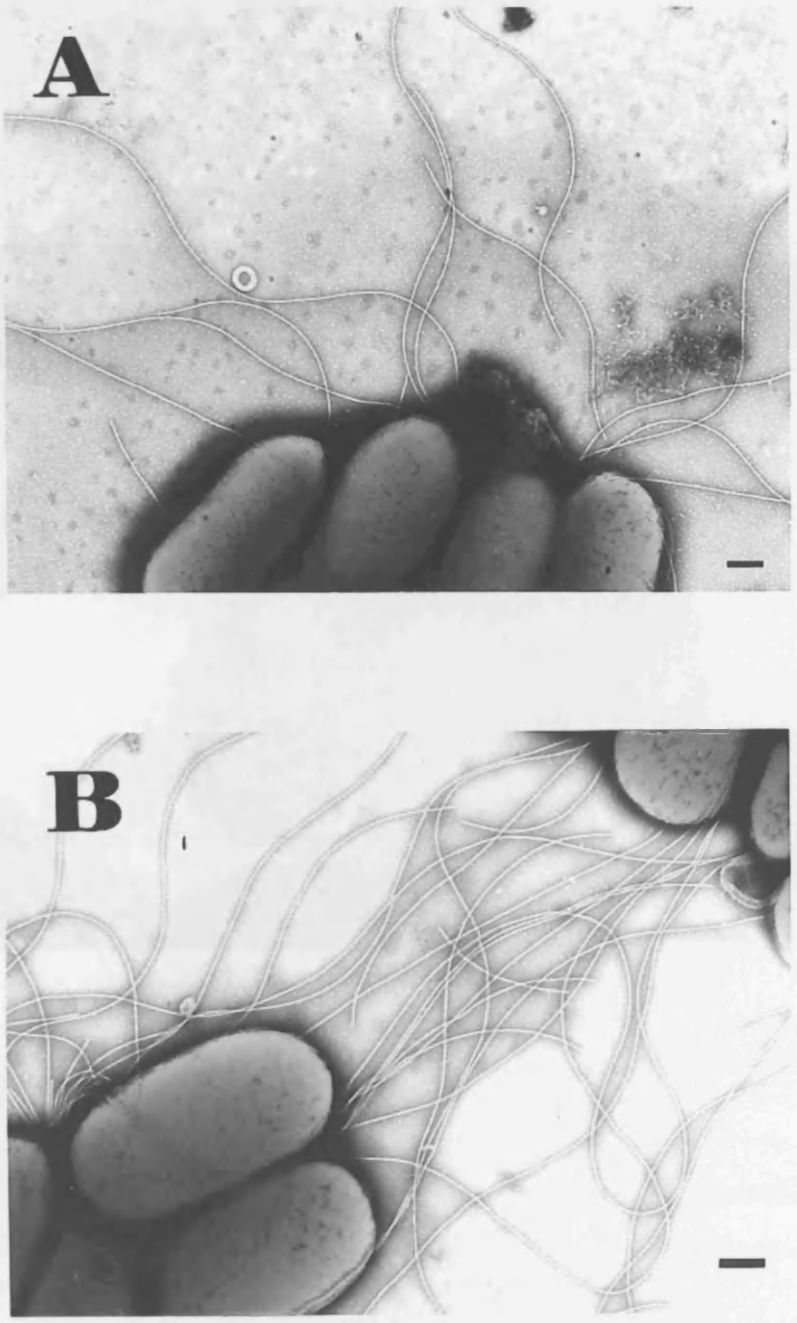


Figure 4.2 Electron micrograph of a SEF17- like fimbriae expressed by *Citrobacter freundii*. **Bar, 200 nm.**

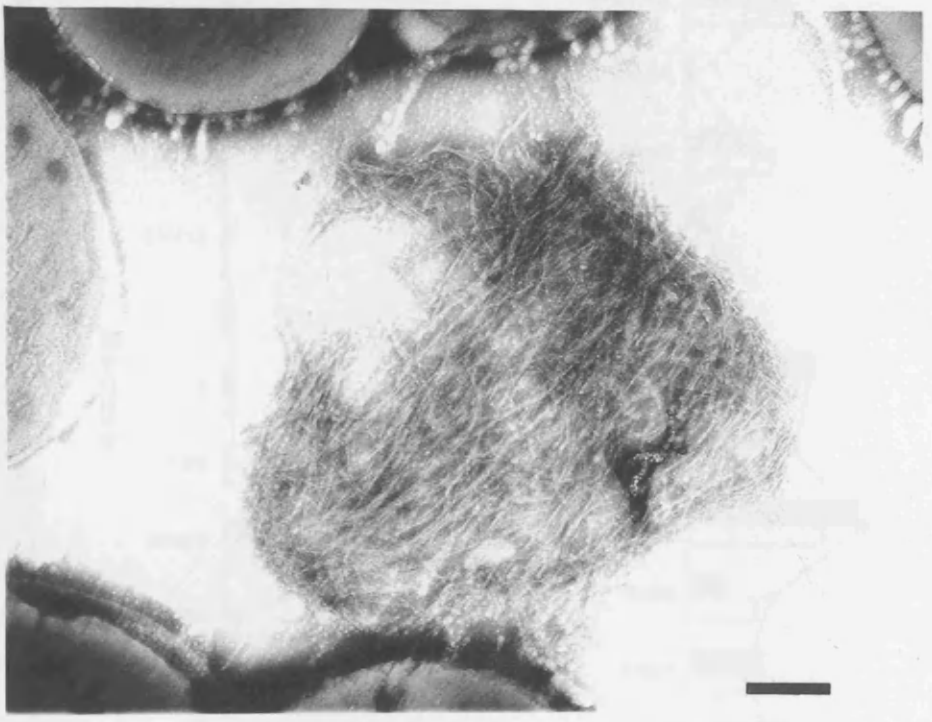
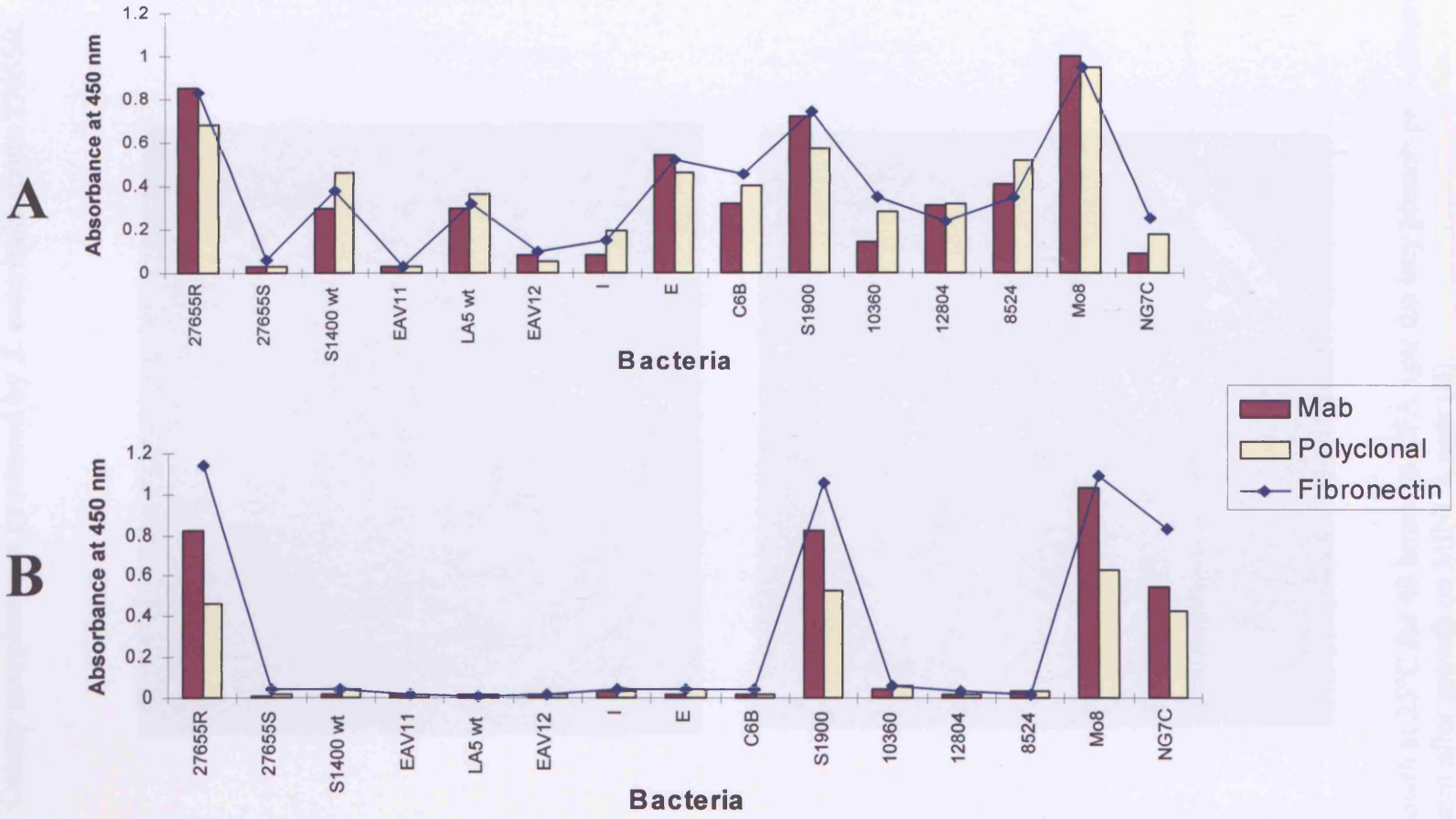
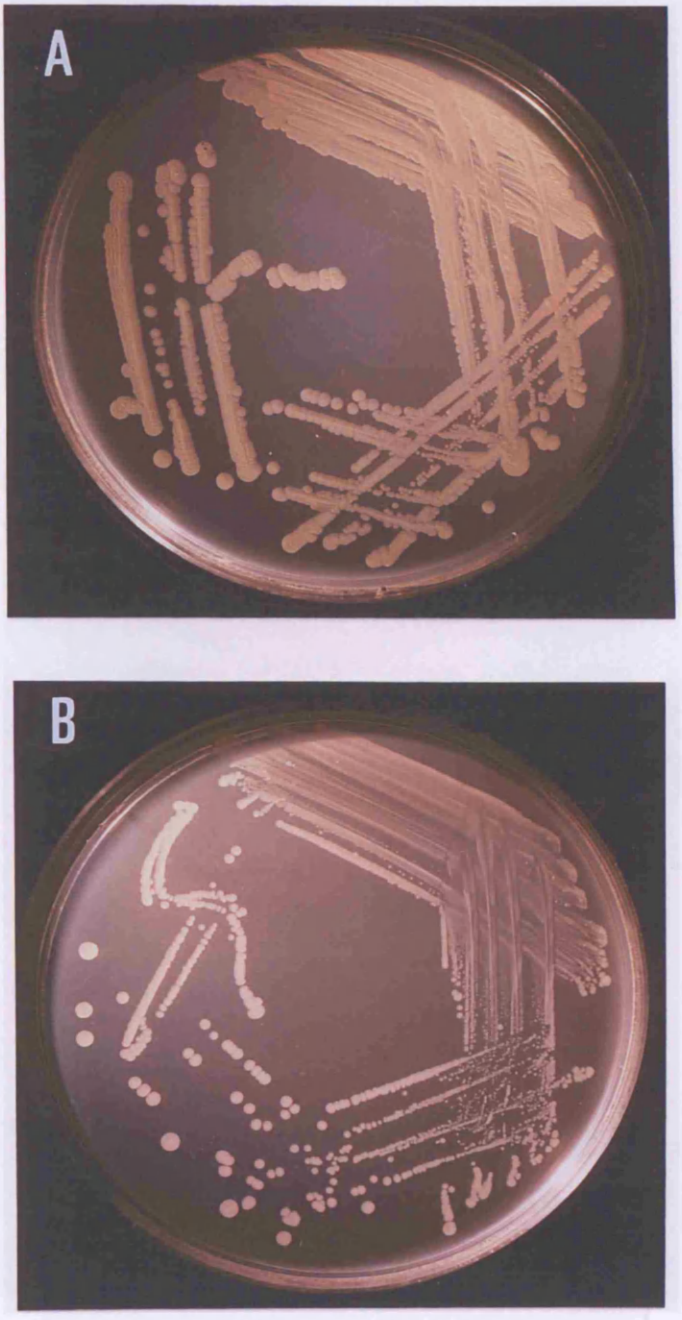


Figure 4.3 The correlation between direct binding of SEF17 Mab, ChSEF17 and soluble fibronectin to strains of *Salmonella* and *E. coli*.



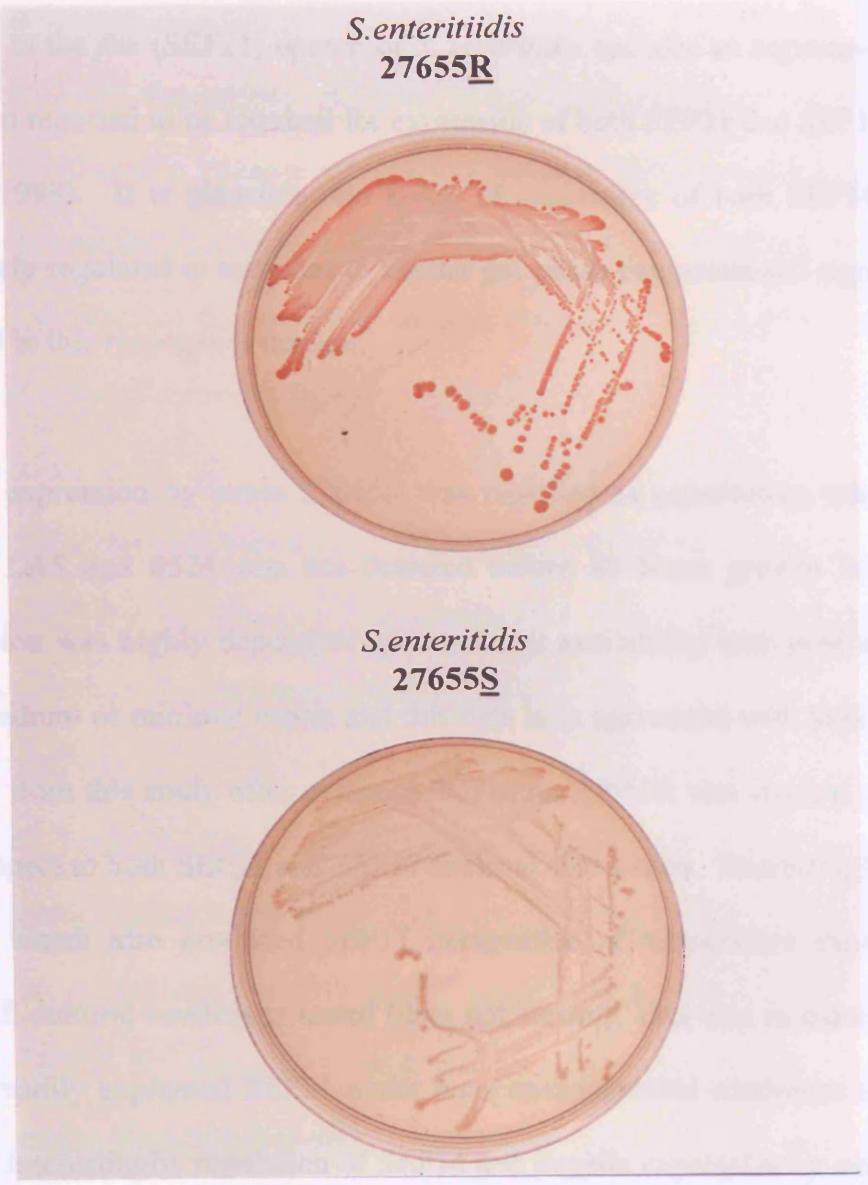
Bacteria were grown on CFA agar for 48-72 hours at **A)** 18°C, and **B)** 37°C and reacted with fibronectin, SEF17 Mab and ChSEF17 in direct binding assays. Bound Mab was detected with anti-mouse peroxidase conjugate, ChSEF17 with anti-chicken peroxidase conjugate and fibronectin with goat anti-human fibronectin antibody followed by anti-goat immunoglobulin peroxidase conjugate. All reactions were developed with TMB substrate. The experiment was performed twice and data shown is the mean from the two experiments.

Figure 4.4 Colony morphotypes produced by *S. enteritidis* strain 27655R.



- Following growth at 25°C for 48 hours on CFA agar the lacy phenotype is observed (**A**) but is absent after growth on MINCA agar (**B**).

Figure 4.5 Binding of congo red by *S. enteritidis* strains 27655R and 27655S following incubation at 30°C for 48 hours on CFA agar containing 0.01% congo red.



4.3 Discussion

The effect of environmental stimuli upon the expression of SEF14, SEF21 and flagella by strains of *S. enteritidis* were studied using direct-binding ELISA's as a preliminary model for studying SEF17 elaboration. Results indicated that SEF14 and SEF21 expression were enhanced by CO_2 enriched air growth but suppressed by anaerobic growth. The *fim*U gene, located in the *fim* (SEF21) operon of *S. enteritidis* encodes an arginine-specific tRNA which has been reported to be required for expression of both SEF21 and SEF14 fimbriae (Clouthier *et al.*, 1998). It is plausible that levels of expression of both SEF14 and SEF21 are co-ordinately regulated in response to similar gas phase environmental signals, although the role of *fim*U in this response is unclear.

SEF21 expression by strain 27655R was regarded as constitutive, whereas, elaboration by strains LA5 and 8524 was not detected before 48 hours growth in broth. In addition, expression was highly dependent upon nutrient availability with poor expression in nutrient poor medium or minimal media and this data is in agreement with Sojka *et al.* (1996; 1998). Results from this study offer evidence that strain 27655R was atypical amongst *S. enteritidis* with respect to both SEF21 and SEF17 fimbrial elaboration. Interestingly, *S. enteritidis* strain S1900, which also produced SEF17 irrespective of temperature expressed SEF21 poorly under all cultural conditions tested (data not shown). This was in contrast to strain 27655R, which readily expressed SEF21 under most environmental conditions and phases of growth tested. Interestingly, regulation of SEF14 and flagella expression by strain 27655R appeared to be similar to strains LA5 and 8524, which suggested a regulatory defect affecting SEF17 and SEF21, but not SEF14. Here are further examples of the complex multifactoral regulation of fimbrial expression.

Detection by ELISA of wild type SEF21 fimbriae was made between 48-72 hours following growth at 37°C. As for all ELISA's, expression may have preceded detection but was not observed due to the lack of sensitivity of the assay. Reporter gene systems may be a more appropriate means of detecting expression but suitable constructs were not available for this study. It is plausible that nutrient depletion or a build-up of toxic metabolites may have triggered SEF21 expression. All three *S. enteritidis* strains grown for up to seven days at 18°C or in anaerobic phase elaborated low levels of SEF21, which implied that anaerobiosis and low temperature suppressed SEF21 expression. These results are in contrast to Ernst *et al.* (1990) who reported that type 1 fimbrial expression of *Salmonella* was enhanced following growth in anaerobic environments.

Expression of flagella was not dependant upon temperature, whereas SEF14 fimbriae was produced at 30°C and above but expression was suppressed below this threshold, which is consistent with results reported by Thorns *et al.* (1990).

Initial data suggested that SEF14 and SEF21, at least, were elaborated in response to growth media, gaseous phase, temperature and phase of growth. Therefore, a similar approach was undertaken to establish whether SEF17 may be expressed *in vitro* by strains other than 27655R, which by comparison with other *S. enteritidis* isolates was probably atypical. ELISA results indicated that most strains of *S. enteritidis* produced SEF17 but expression, like SEF14 and SEF21, was dependent upon specific environmental signals including temperature and nutrient availability. In contrast to SEF14, SEF17 was expressed at temperatures below 30°C and, in general, restricted to growth on nutrient rich media such as

CFA, Luria and Nutrient agars and not on nutrient poor media, such as Sensitest and Drigalski agars.

Interestingly, *S. typhimurium* Mö8 strain that expressed a morphologically similar fimbrial antigen to SEF17 (Grund & Weber, 1988) and the curli-producing *E. coli* (Collinson *et al.*, 1992) also reacted with the SEF17 antibodies in the direct-binding ELISA's which confirmed that SEF17, thin aggregative fimbriae and curli were indeed antigenically similar. Doran *et al.* (1993; 1996) reported that the *agf*A gene encoding the SEF17 structural monomer was conserved across the *Salmonella* genus and this study confirmed the elaboration of a morphologically and antigenically similar thin aggregative fimbriae following growth at ambient temperature. These results were in agreement with those of Sukopolvi *et al.* (1997) who reported expression of temperature-dependent strains of *S. typhimurium* following growth at 28°C, but not 37°C. Of particular relevance, Römling *et al.* (1998b) reported that depletion of iron in agar medium led to the expression of significant amounts of thin aggregative fimbriae by *S. typhimurium* at 37°C. In addition, it was also demonstrated in this laboratory by direct-binding ELISA that *S. enteritidis* isolates previously shown to produce SEF17 at 25°C or below, could elaborate SEF17 at 37°C in broth culture for a brief period during late log growth phase (Personal communication, Dr S. Walker). Although these results have not been confirmed by electron microscopy, here was evidence that regulation of fimbrial elaboration was multifactoral whereby, for example, expression may be induced in response to one stimulus such as temperature in media that did not generally support expression. This may have important implications for the role of this family of surface appendage in pathogenesis.

Two strains of *S. enteritidis*, namely, 27655R and S1900, one of *S typhimurium* strain, Mö8, and curli expressing *E. coli* strain NG7C all expressed aggregative fimbriae at 37°C which was undoubtedly an important factor in their original identification (Baloda, 1988; Grund & Weber, 1988; Collinson *et al.*, 1992), for each group of researchers cultured bacteria at 37°C to express fimbriae. These strains elaborated aggregative fimbriae at most temperatures and for *Salmonella,* in particular, in all media tested albeit in varying amounts. Results confirmed initial observations that expression of aggregative fimbriae, were not as tightly regulated in these strains as wild type. Interestingly, *S. enteritidis* strain S1900 was a randomly chosen poultry isolate, and, therefore, it is plausible that temperature-independent expression of thin aggregative fimbriae were not uncommon amongst *Salmonella.* Assuming these atypical isolates possessed defective regulatory gene, they would make ideal test candidates to analyse the global basis of expression. Complementation studies with a *S. enteritidis* gene library may identify controlling regulatory genes.

Of interest, Römling *et al.*, (1998b) reported that upon repeated subculturing, a temperature-independent mutant was derived from a temperature-dependent *S typhimurium strain* SR-11 (Sukupolvi *et al.*, 1997) which resembled the phenotype of *S. enteritidis* strain 27655R. SR-11 -like variants were reported to arise spontaneously at a frequency of about 1:1000 and was stable following several subcultures at 37°C. Therefore, the mutants appeared to have been locked within a fimbriate phase. Interestingly, storage of *S. typhimurium* strains at 4°C led to a return to wild type phenotype (Römling *et al.*, 1998b). In contrast, results from this study suggested that temperature-independent strains were not readily derived from repeated sub-culturing of the *S. enteritidis* strains tested.

The production of convoluted colonies was associated with SEF17 expression and these results are in full agreement with those of Allen-Vercoe *et al.*, (1997), although SEF17 fimbriae could be elaborated without "lacy" colony formation. For example, certain strains of *Salmonella* elaborated SEF17 on Drigalski agar and MINCA agar but did not produce the convoluted colonies and in addition, this phenotype was rarely observed at 18°C. These results suggest that SEF17 fimbriae are an integral component of the extracellular matrix responsible for this morphotype. It is likely that the "lacy" phenotype observed was due to O-antigen variation and the production of high molecular weight lipopolysaccharide (Guard-Petter *et al.*, 1996) and that SEF17 fimbriae may act as a scaffolding on which to lay down the exopolysaccharides. It appears that exopolysaccharide production at lower temperatures is not restricted to the *Salmonella* genus for Gamar-Nourani *et al.*, (1998) have described an enhanced exopolysaccharide production in *Lactobacillus rhamnosus* following a temperature shift from 37°C to 25°C.

The "lacy" morphotype has been observed with certain strains of *S. enteritidis* which has been reported to be associated with the ability of isolates to invade the reproductive tract of laying hens (Humphrey *et al.*, 1996) and increased virulence of the organism in chicks (Guard-Petter *et al.*, 1996). Whether the extracellular matrix is produced by *S. enteritidis* during infection is unknown, but it is plausible that a combination of auto-aggregation, hydrophobicity and the production of the extracellular material may protect the bacteria from harsh environments or interaction with the host immune system such as complement assisted phagocytosis. Indeed, Humphrey *et al.* (1996) reported that *Salmonella* isolates could be divided into two distinct populations on the basis of tolerance to hostile conditions, of which tolerant isolates showed enhanced pathogenicity in mice and chicks. Those that were more tolerant to heat, acid and hydrogen peroxide became "lacy" in appearance, whereas colonies which were sensitive to

these stresses remained smooth. Strain E used in this study was more tolerant and expressed SEF17, auto-aggregated and produced "lacy" colonies, whereas in contrast, strain "I" was sensitive to heat, acid and hydrogen peroxide and did not develop any of these characteristic phenotypic traits under any conditions tested. Strain "I" has subsequently been assigned as an *rpo*S mutant (Allen-Vercoe *et al.*, 1998).

Results confirmed that SEF17 fimbriae mediate the binding of soluble fibronectin to *S. enteritidis* as previously suggested (Baloda, 1988; Baloda *et al*, 1988; Collinson *et al.*, 1993). There was complete correlation between aggregative fimbrial production when measured by ELISA and fibronectin binding by all *S. enteritidis* and *S. typhimurium* strains. In addition, the *E. coli* curli strain also bound fibronectin. Congo red binding and auto-aggregation have also been associated with SEF17 production (Collinson *et al.*, 1991; 1993), although with prolonged incubation non-SEF17 producing strains could bind the hydrophobic dye, and in addition, organisms could produce SEF17 without binding Congo red or auto-aggregating, therefore, these traits were deemed to be insensitive indicators of SEF17 elaboration.

These studies have confirmed that expression of flagella, and in particular, fimbriae of *S. enteritidis* are tightly regulated and expression is affected by phase of growth and environmental stimuli, including nutrient availability, gaseous phase and temperature. It is therefore a reasonable hypothesis that *Salmonella* are advantaged by fimbriate and non-fimbriate phases. It is plausible that these surface appendages may act in synergy, complementing or compensating for each other depending on environmental conditions. In addition, results from this study have highlighted that some strains of *S. enteritidis,* and indeed *S. typhimurium* and *E. coli* strains, are atypical with respect to fimbrial expression and this should be considered for it may have important implications for pathogenesis studies.

Chapter 5

Fimbrial and flagella-mediated association with and invasion of cultured epithelial cells by *S. enteritidis*.

5.1 Introduction

Understanding the virulence mechanisms of bacterial pathogens depends upon the ability to study directly host-bacterial interactions. For pathogenic *E. coli* it is considered that adherence to enterocytes is a prerequisite to infection and the adhesive function of certain fimbriae have been described, in particular for Enterotoxigenic *E. coli* and those that cause urinary tract infections (Parry & Rooke, 1985). However, for *S. enteritidis* the role of fimbriae and flagella is poorly understood although it is commonly thought that they contribute to bacterial survival and persistence in the host and its environment (Thorns, 1995).

Tissue culture models have proved valuable for studying bacterial interactions with epithelial cells. For example, cultured epithelial cells have been demonstrated by electron microscopy to develop characteristics that have been observed *in vivo,* including the development of attaching and effacing lesions and pedestal formation following interaction with Enteropathogenic and Enterohaemorrhagic *E. coli* (Canil *et al.*, 1993; Agin *et al.*, 1997). Similarly, *S. typhimurium* infection of the apical surface of polarised monolayers caused microvilli rearrangements and epithelial ruffling resembling those seen in infected animals (Finlay & Falkow, 1990). In addition, previous studies have suggested that *Salmonella* isolates from clinically affected birds could be distinguished from those of apparently healthy birds by their high degree of invasion of (Nolan *et al.*, 1991) and survival within (Kottom *et al.*, 1995) tissue culture cells.

Prior to *in vivo* experiments, the role of fimbriae and flagella in the interaction between *S. enteritidis* and cultured epithelial cells was studied. Avian primary cell lines or immortalised

epithelial cells were unavailable for this study. Therefore commonly used transformed cell lines of human origin were evaluated for association with and invasion by *S. enteritidis*. CACO-2 (Pinto *et al.*, 1983), Hep-2 (Black *et al.*, 1956) and INT-407 (Henle & Dienhardt, 1956) have been utilised extensively for studying the interactions between pathogen and epithelial cells which have included, *Salmonella spp.* (Gahring *et al.*, 1990; Leung & Finlay, 1991; Kottom *et al.*, 1995; Burns-Keliher *et al.*, 1998), *Pseudomonas spp.* (Zanetti *et al.*, 1995), *E. coli* (Jallet *et al.*, 1994; Benjamin *et al.*, 1995; Agin *et al.*, 1997), *Campylobacter spp.* (Everest *et al.*, 1992; Doig *et al.*, 1996), *Providencia spp.* (Albert *et al.*, 1995), *Neisseria spp.* (Virji *et al.*, 1993), *Helicobacter spp.* (Gold *et al.*, 1995; Osaki *et al.*,1998), *Aeromonas spp.* (Kirov *et al.*, 1998), *Haemophilus spp.* (Totten *et al.*, 1994), *Listeria spp.* (Conte *et al.*, 1994), *Vibrio spp.* (Benítez *et al.*, 1997), *Shigella spp.*(Watarai *et al.*, 1995) and *Cryptosoridia spp.* (Guarino *et al.*, 1995).

Numerous tissue culture models have been developed to study interactions such as adherence, invasion and translocation of bacteria through monolayers and these have employed a wide variety of parameters reported to affect interaction (Jackson *et al.*, 1998). Such variables included, the use of confluent or partially-confluent monolayers (Jallet *et al*, 1994), the use of pre-fixed or native cells (Zepeda-Lopez & Gonzalez-Lugo, 1995), a wide range of inocula ranging from one to 1000 bacteria per tissue culture cell (Pessina *et al.*, 1995), a variety of incubation times (Douce *et al.*, 1991; Pessina *et al.*, 1995), a range of culture media (Zanetti *et al.*, 1995), bacterial growth phase (Tartera & Metcalf, 1993; Ernst *et al.*, 1990) and the use of centrifugation to facilitate contact of bacteria with monolayers (Gahring *et al.*, 1990; Albert *et al.*, 1995). In addition, several methods for quantitative determination of bacterial association have been described including total counts by light (Barrow & Lovell, 1989; Pessina *et al.*, 1995) or fluorescent microscopy (Gahring *et al.*, 1990), detection of bound

radiolabelled bacteria (Everest *et al.*, 1992) or an estimate of viable counts by determining colony forming units (CFU) (Stone *et al.*, 1992).

The aims of this investigation were 1) to develop an *in vitro* model to study the interaction of *S. enteritidis* with tissue culture cells and study the effect of environmental factors upon association and invasion of epithelial cells, and 2) to evaluate the effects of fimbrial and flagella-mediated association and invasion by *S. enteritidis* using a panel of defined isogenic mutants.

5.2 Results

5.2.1 Development of an *in vitro* model to study the interaction of *S. enteritidis* with tissue culture cells

For this study INT-407 cells were evaluated using overnight cultures of *S. enteritidis* strain LA5 as the test strain. In addition, a *S. typhimurium* isolate as invasive control strain and *E. coli* strain K12 as non-invasive control strain were used as described by Barrow & Lovell (1989). Confluent, non-polarised monolayers were seeded in tissue culture wells and infected with $5x10^6$, $5x10^7$ or $5x10^8$ bacteria, a ratio of 1:10, 1:100 and 1:1000 tissue culture cell to bacteria, respectively. Duplicate plates were infected with bacteria and incubated for 2 hours at 37°C (5% CO_2) in order to discriminate between the bacteria which had associated with the tissue culture cells (total number of bacteria adhering to and invading tissue culture cells) and those which had invaded . At an inoculum ratio of 100 bacteria to one tissue culture cell up to 10 bacteria associated per tissue culture cell within 2 hours and this ratio was used for future experiments (Table 5.1). At a ratio of 10 bacteria to one tissue culture cell very few bacteria had associated, whereas at 1000:1 large portions of the monolayer were destroyed. The number of *Salmonella* that invaded the tissue culture cells ranged between one and 10 percent of the total associated bacteria whereas, *E. coli* K12 failed to invade (Table 5.1).

A comparative study indicated that a greater number of bacteria associated with and invaded INT-407 tissue culture cells than either Hep-2 or CACO-2 cells following infection of confluent epithelial monolayers at a ratio of 100 bacteria per tissue culture cell (data not shown). Therefore, INT-407 monolayers were chosen for subsequent experiments. There was an additional preference towards INT-407 cells or CACO-2 cells for these cell lines were originally derived from human intestinal tissue (Henle & Deinhardt, 1957; Pinto *et al.*, 1983,

respectively), whereas Hep-2 cells originated from the human upper respiratory tract (Black *et al.*, 1954).

5.2.1.1 Association with and invasion of INT-407 monolayers by strains of *S. enteritidis*

A panel of *S. enteritidis* isolates were evaluated for association with and invasion of INT-407 monolayers (Figure 5.1). The inoculum were prepared by culture of *Salmonella* isolates on CFA agar for 24 hours at 37°C as described by Baloda *et al.* (1988). *S. enteritidis* strains 27655R and S1900 associated and invaded in much higher numbers than the other isolates. In general, two levels of interaction were observed with these strains which associated with approximately 10^8 CFU per well, whereas the other *S. enteritidis* strains interacted between 10^6 and 10^7 CFU per well. Statistical analyses was performed using one-way analyses of variance and revealed significant differences between strains LA5, 10360 and 12804 for total number of *Salmonella* associated with INT-407 cells ($p<0.001$ for each comparison) whereas, no significant difference was observed between strains 27655S, LA5 and S1400. For invasion, there were highly significant differences between strains 27655S, 10360 and 12804 ($p<0.001$ for each comparison), whereas, there were no significant differences between strains 27655S, S1400, LA5 and 8524.

5.2.1.2 The effect of bacterial growth conditions on association with and invasion of epithelial cells

Results obtained from the development of the tissue culture model implied that growth conditions of the inoculum influenced the interaction between bacteria and epithelial cells, and therefore, the effect of growth media and gaseous phase were investigated.

Association and invasion assays were performed on three *S. enteritidis* strains cultured in various media (Figure 5.2). Inocula were prepared by static incubation of *S. enteritidis* strains LA5, 27655R and S8524 for 48 hours at 37°C in Heart Infusion broth (HIB), Colonisation Factor Antigen broth (CFA), Luria broth (LB), Peptone water (PW), Tryptone water (TW) or Minimal Essential Media (MEM). *Salmonella* were pelleted by centrifugation, resuspended in PBS and diluted to $5x10^7$ bacteria per ml in MEM before infecting monolayers. One-way analyses of variance followed by multiple t-tests were used to compare the effect of growth media upon association with and invasion of INT-407 monolayers.

Following growth in Peptone water and Tryptone water *S. enteritidis* strain LA5 associated with INT-407 cells in greater numbers ($p<0.13$ & $p<0.062$, respectively) than any other media tested (Figure 5.2). Consistent with other assays, strain 27655R associated with tissue culture cells in greater numbers than strains LA5 and S8524. Similar to LA5, strain 27655R associated with INT-407 cells in greater numbers following growth in Peptone water ($p<0.001$) or Tryptone water ($p<0.062$), whereas strain 8524 grown in CFA broth associated in significantly lower numbers ($p<0.015$) than the rest. In all three strains, a higher number of *Salmonella* were internalised following growth in Luria broth ($p<0.057$), with the exception of HIB, whereas, in general bacteria grown in MEM invaded least (LA5, $p<0.49$; 27655R, $p<0.001$; 8524, $p<0.60$).

Inocula were prepared by culture of *S. enteritidis* strains LA5, 27655R and 8524 in CFA broth for 48 hours at 37°C in aerobic, anaerobic or CO_2 enriched air environments prior to infection of INT-407 monolayers (Table 5.2). Two-way analyses of variance were performed on CFU counts to test for the effect of gaseous phase and the strains. Following anaerobic growth, LA5 associated in greater numbers ($p<0.29$) than other gaseous phases, whereas strain 27655R

associated less well ($p<0.001$), and for strain S1400 no significant difference between culture conditions and adherence were observed. For invasion the effects of the gaseous phase were similar for each strain. Significantly higher numbers ($p<0.001$) of *Salmonella* grown in an anaerobic environment invaded compared with those in CO_2 enriched air conditions, which in turn were higher than those following aerobic growth ($p<0.105$).

5.2.2 Evaluation of single and multiple mutants in the INT-407 model

To evaluate the role of fimbriae and flagella-mediated association with and invasion of cultured epithelial cells a panel of isogenic mutants prepared in *S. enteritidis* strains LA5, S1400 and 27655R (Allen-Vercoe *et al.*, 1999) were investigated. Single and multiple mutants constructed by Emma Allen-Vercoe in strains S1400, LA5 and 27655R are listed in table 5.3.

5.2.2.1 Phenotypic expression of mutants

Prior to *in vitro* assays all transductants were quantitatively assayed for the expression of SEF14, SEF17, SEF21 and flagella by direct binding ELISA (Table 5.3). Wild type *S. enteritidis* and mutant derivatives were grown under conditions permissive of expression of each respective surface appendage. Expression of SEF14, SEF17, SEF21 and flagella and were induced by growth in Sensitest agar for 24 hours at 37°C, Colonisation Factor Antigen agar for 72 hours at 18°C, Heart Infusion broth for 48 hours at 37°C and 24 hours in Nutrient broth, respectively, and direct-binding ELISA's were performed on whole cells as described in section 2.3.4. Results indicated that mutant strains failed to elaborate the appendage encoded by the insertionally inactivated gene, and additionally, no evidence was gained that mutation of any one locus altered expression of any other surface appendage.

5.2.2.2 Growth curves of mutants

S. enteritidis wild type LA5 and mutants cultured in Nutrient broth overnight at 37°C were shaken in 10 ml fresh broth for 30 minutes before placing in 100ml of pre-warmed Nutrient broth at a density of approximately $5x10^4$ bacteria/ml media. Cultures were incubated statically at 37°C and colony forming units (CFU) estimated by serial dilutions at times 0, 1, 2, 3, 5, 7, 8, 24, 48 and 72 hours. Dilutions were plated onto Brilliant Green Agar. There was no evidence that single or multiple mutations in strain LA5 affected growth kinetics or that they differed from *S. enteritidis* wild types 27655R and S1400 (Figure 5.3).

5.2.2.3 Fimbrial and flagella-mediated association with and invasion of INT-407 monolayers by single mutants

Evidence was gained in section 5.2.1 that the cultural conditions of the inocula affected bacterial-cell interaction, and therefore, for studying the effect of fimbriae and flagella within tissue culture models bacteria were grown under cultural conditions permissive for elaboration of each appendage (Figure 5.4). In all three $SEF14^-$ strains there was no significant reduction in association or invasion of INT-407 cells compared with wild type. However, the wild types associated in greater numbers than the isogenic SEF17 mutants ($p<0.001$), whilst the differences for bacterial invasion were not significant for S1400 ($p>0.05$), significant for LA5 ($p<0.02$) and highly significant for 27655R ($p<0.001$). The wild types associated ($p<0.001$) and invaded ($p<0.001$) in significantly higher numbers than the isogenic SEF21 mutants of all three strains. Aflagellate mutants of strains LA5 and S1400 associated and invaded significantly less ($p<0.001$) than their respective wild types. In all experiments, strain 27655R associated in significantly greater numbers than LA5 and S1400 (Figure 5.4).

5.2.2.4 Detection of fimbrial elaboration by immunofluorescence

To test whether adherent bacteria elaborated fimbriae, confluent monolayers were prepared on glass coverslips and infected with *S. enteritidis* wild type or single mutants as control. All *Salmonella* were grown under conditions permissive of expression of SEF17, SEF21 and flagella. Following incubation non-bound bacteria were removed from the monolayer by washing and the bound bacteria were probed with antibodies to SEF14, SEF17, SEF21 and flagella. Bound antibody was detected with anti-species immunoglobulin labelled with FITC. Coverslips were mounted onto slides and observed by fluorescence microscopy. Results, summarised in table 5.4, showed that *S. enteritidis* strain 27655R bound in high numbers and bacteria elaborated SEF14, SEF17 and SEF21. LA5 grown to elaborate SEF17, bound SEF17 and SEF21 Mab, whereas LA5 cultured to express SEF21, bound SEF21 Mab and also SEF14 Mab weakly. Each mutant was negative when tested with its corresponding Mab. In addition, flagella was not detected by monoclonal or polyclonal antibodies.

5.2.2.5 Blocking of fimbrial and flagella mediated association and invasion

To demonstrate the specific interactions of fimbriae and flagella of *S. enteritidis* with epithelial cells, purified antigens and specific antibodies were incorporated into the association and invasion assays. Mannose (1%), polyclonal antisera (1/1000 dilution) and Mab (100μg/ml) were added to the bacterial suspension and 100μg of the purified fimbriae, flagella, LPS and fibronectin was added to one ml media covering the tissue culture cells. All reactions were incubated for 15 minutes at 37°C (5% CO_2) with gentle agitation, prior to infection of the monolayer. Counts were compared statistically by one-way ANOVA.

The addition of polyclonal antisera and purified SEF17 fimbriae significantly reduced association ($p<0.001$). The addition of LPS reduced adherence significantly ($p<0.021$), but adherence was unaffected by the addition of soluble fibronectin. Polyclonal sera significantly reduced bacterial invasion ($p<0.001$), whereas the other treatments had no effect (Table 5.5). Morphological observations suggested that tissue culture cells were unaffected by the addition of reagents.

SEF21 mediated association was significantly reduced by the addition of D-mannose and monoclonal antibody M8732 ($p<0.001$) whereas invasion was unaffected by any of the reagents added (Table 5.6). Interestingly, both Mabs M8732 and M8743 partially inhibited Mannose Sensitive Haemagglutination, whereas the other Mabs tested or polyclonal sera had no inhibitory effect (Table 5.7).

In addition to blocking agents the effect of centrifugation to facilitate contact of bacteria with the monolayer was tested (Table 5.8). Centrifugation of wild type *S. enteritidis* or EAV10 (aflagellate mutant) onto the monolayer increased bacterial association by a numerically small, but significant amount ($p<0.001$). Also a significant increase in invasion by the aflagellate mutant ($p<0.001$) was observed after centrifugation. The addition of purified flagella or polyclonal sera had no effect.

5.2.2.6 Histological examination of infected monolayers

To determine adherence patterns of *S. enteritidis* mediated by fimbriae, confluent monolayers were prepared in 6 well dishes on round coverslips and were infected with bacteria cultured to induce expression of SEF17 or SEF21. After incubation for three hours at 37°C (5% CO_2) monolayers were washed thoroughly, fixed with cold methanol (70%) and stained with

Giemsa stain. Washed coverslips were differentiated in acetic acid, washed again, air dried and mounted onto slides. Examination by light microscopy indicated that wild type 27655R adherence was localised and often aggregative (Figure 5.5 A) whereas wild type LA5 cultured to express SEF21 colonised the INT-407 cells in a random, diffuse pattern (Figure 5.5 C). EAV12 (27655R, SEF17$^-$) and EAV3 (LA5, SEF21$^-$) associated visibly less than wild types (Figure 5.5 B & D, respectively).

5.2.2.7 Adhesion of *S. enteritidis* strain LA5 to mucin-secreting HT-29 cells

The mucin-secreting cell line HT-29 cl 16E (Lesuffleur *et al.*, 1991), kindly received from Dr. Lesuffleur, INSERM, Villejuif, France, was utilised for studying the role of flagella in crossing a mucus layer. A monolayer of HT29, grown for ten days post confluence were infected with *S. enteritidis* strain LA5 and EAV10 an aflagellate mutant derived from this strain. After two hours incubation, the infected monolayers were thoroughly washed, but the mucin layer remained intact over a majority of the monolayer. Therefore, it was not possible to accurately assess the number of wild type *Salmonella* or mutants that had associated with the tissue culture cells. However, after staining with Giemsa stain, light microscopy revealed that where the mucin layer had been removed from the cell surface by washing, fewer aflagellate mutants had translocated across the mucus layer and attached to the underlying cells when compared to wild type (Figure 5.6). In addition, a large proportion of both wild type and mutant bacteria appeared to be trapped within the mucus layer.

5.2.2.8 Association with and invasion of INT-407, CACO-2 and DIV-1 cell lines by single and multiple mutants of *S. enteritidis* strain LA5

Single and multiple mutants prepared in LA5 were tested in the INT-407 and CACO-2 models. In addition, an avian epithelial cell line DIV-1, was evaluated whilst visiting another

laboratory [Institute Nationale de la Recherche Agronomique, Tours, France] (Figure 5.7). Counts following infection of tissue culture cells with LA5 wild type were compared statistically with single and multiple mutants using one-way ANOVA.

Association of the wild type in the INT-407 was significantly higher than all the mutants except for B214 (SEF14$^-$), with the significance probability for EAV12 (SEF17$^-$) of $p<0.025$, and the remaining differences being $p<0.001$. EAV10 (fla$^-$) and EAV40 (SEF14$^-$, 17$^-$, 21$^-$, fla$^-$) exhibited significant reductions in invasion ($p<0.001$) compared to wild type.

For CACO-2 cells there was no significant difference in the total number of associated bacteria between wild type and B214 (14$^-$), although there were highly significant differences ($p<0.001$) between wild type and EAV12 (17$^-$), EAV3 (21$^-$) or EAV10 (fla$^-$). There were no significant differences in association between EAV10 (fla$^-$) or EAV40 (14$^-$, 17$^-$, 21$^-$, fla$^-$). For invasion, there were significant reductions between wild type and EAV3 (21$^-$) and EAV21 (14$^-$, 17$^-$, 21$^-$) with probabilities of $p<0.004$ and $p<0.037$, respectively, and highly significant differences ($p<0.001$) between wild type and EAV10 (fla$^-$) or EAV40 (14$^-$, 17$^-$, 21$^-$, fla$^-$).

The trend of association and invasion of single and multiple mutants with DIV-1 cells was very similar to INT-407 and Caco-2 cells, although there was no significant difference between association of EAV12 (SEF17$^-$) and wild type LA5. For association, highly significant differences ($p<0.001$) were observed between wild type and EAV3 (21$^-$), EAV10 (fla$^-$), EAV21 (14$^-$, 17$^-$, 21$^-$) or EAV40 (14$^-$, 17$^-$, 21$^-$, fla$^-$), whereas, a highly significant reductions in the number of *Salmonella* invading tissue culture cells ($p<0.001$) were observed between wild type and EAV10 (fla$^-$) or EAV40 (14$^-$, 17$^-$, 21$^-$, fla$^-$). There were no significant

differences in invasion between LA5 wild type and B214 (14^-), EAV3 (21^-) or EAV21 (14^-, 17^-, 21^-).

Table 5.1 Association and invasion of INT-407 monolayers by a) *S. typhimurium* strain 85577, b) *S. enteritidis* strain LA5 or c) *E. coli* strain K12, following an inoculation ratio of 1:1000, 1:100 or 1:10 tissue culture cell to bacteria. Results expressed as CFU per well ± SE.

a)		*S. typhimurium* strain 85577	
	*Ratio	Association	Invasion
	1:1000	†$9.1 \times 10^7 \pm 0.54$	†$1.1 \times 10^6 \pm 0.32$
	1:100	$1.75 \times 10^6 \pm 0.15$	$1.95 \times 10^4 \pm 0.40$
	1:10	$1.6 \times 10^5 \pm 0.36$	$4.1 \times 10^3 \pm 1.12$

b)		*S. enteritidis* strain LA5	
	*Ratio	Association	Invasion
	1:1000	†$2.2 \times 10^7 \pm 0.23$	†$1.25 \times 10^6 \pm 0.30$
	1:100	$1.75 \times 10^6 \pm 0.54$	$1.9 \times 10^4 \pm 0.62$
	1:10	$2.2 \times 10^4 \pm 0.32$	$5.0 \times 10^2 \pm 0.73$

c)		*E.coli* K12	
	*Ratio	Association	Invasion
	1:1000	$6.5 \times 10^6 \pm 0.23$	-
	1:100	$7.5 \times 10^5 \pm 0.42$	-
	1:10	$9.1 \times 10^4 \pm 0.58$	-

* ratio of tissue culture cells to bacteria
† parts of monolayer destroyed
- no colonies recovered
The tissue culture assay was performed twice. Results from a single representative assay are shown.

Table 5.2a Association and invasion of INT-407 monolayers by *Salmonella* following growth in CFA broth for 48 hours at 37°C in aerobic, anaerobic or CO_2 enriched air.

Strain	Cultural conditions	TISSUE CULTURE		
		*Inoculum	†Associated bacteria	†Invaded bacteria
LA5	aerobic	$6.6x10^7$	$1.0x10^6 \pm 0.13$	$5.5x10^4 \pm 0.93$
	anaerobic	$4.3x10^7$	$1.6x10^6 \pm 0.02$	$4.8x10^5 \pm 0.41$
	5% CO_2 in air	$4.6x10^7$	$1.2x10^6 \pm 0.09$	$8.0x10^4 \pm 1.06$
27655R	aerobic	$4.3x10^7$	$4.5x10^7 \pm 0.93$	$5.2x10^5 \pm 3.20$
	anaerobic	$5.4x10^7$	$1.0x10^7 \pm 0.13$	$9.7x10^6 \pm 1.25$
	5% CO_2 in air	$6.8x10^7$	$7.9x10^7 \pm 0.54$	$8.6x10^5 \pm 1.50$
S1400	aerobic	$4.0x10^7$	$1.6x10^6 \pm 0.26$	$6.7x10^4 \pm 1.3$
	anaerobic	$4.0x10^7$	$1.7x10^6 \pm 0.21$	$9.4x10^5 \pm 1.6$
	5% CO_2 in air	$5.5x10^7$	$1.5x10^6 \pm 0.12$	$1.5x10^5 \pm 0.4$

* CFU per ml

† CFU per well ± SE

The tissue culture assay was performed twice. Results from a single representative assay are shown.

Table 5.3 Direct binding ELISA using specific monoclonal antibodies to determine expression of fimbriae and flagella by single and multiple mutants of *S. enteritidis*, when grown under permissive conditions.

Strain	Reference name	Genotype	Antibiotic marker	Phenotype			
				SEF14	SEF17	SEF21	Flagella
LA5	Wild -type	-	-	+++	+	+	++
"	B214	*sef*A	kan	-	+	+	++
"	EAV12	*agf*A	bla	+++	-	+	++
"	EAV3	*fim*D	tet	+++	+	-	++
"	EAV10	*fli*C	cam	+++	+	+	-
"	EAV42	*sef*A, *agf*A	kan, bla	-	-	+	++
"	EAV21	*sef*A, *agf*A, *fim*D	kan, bla, tet	-	-	-	++
"	EAV40	*sef*A, *agf*A, *fim*D, *fli*C	kan, bla, tet, cam	-	-	-	-
S1400	Wild -type	-	-	+++	+	+	++
"	EAV13	*sef*A	kan	-	+	+	++
"	EAV11	*agf*A	bla	+++	-	+	++
"	EAV1	*fim*D	tet	+++	+	-	++
"	EAV9	*fli*C	cam	+++	+	+	-
27655R	Wild -type	-	-	+++	++	+	++
"	EAV28	*sef*A	kan	-	++	+	++
"	EAV30	*agf*A	bla	+++	-	+	++
"	EAV29	*fim*D	tet	+++	++	-	++

Abbreviations:
kan, kanamycin
bla, ampicillin
tet, tetracycline
cam, chloramphenicol
+++, optical density in direct binding ELISA >1.0
++, optical density in direct binding ELISA >0.5
+, optical density in direct binding ELISA >0.1
-, optical density in direct binding ELISA <0.1

Table 5.4 Detection of fimbrial and flagella elaboration by immunofluorescence of *S. enteritidis* associated to INT-407 cells.

S. enteritidis strain 27655R

a) Antibody	Wild type	Mutant
SEF14	+	-
SEF17	++	-
SEF21	++	-
flagella	-	-

S. enteritidis strain LA5

b) Antibody	Wild type	Mutant
SEF14	-	-
SEF17	+	-
SEF21	+	-
flagella	-	-

S. enteritidis strain LA5

c) Antibody	Wild type	Mutant
SEF14	+	-
SEF17	-	-
SEF21	++	-
flagella	-	-

- No fluorescence
+ Weak fluorescence
++ High fluorescence

Prior to infection of the monolayers, **a)** 27655R was cultured on CFA agar for 24 hours at 30°C, **b)** LA5 grown on CFA agar for 72 hours at 18°C and **c)** LA5 grown in HIB for 72 hours at 37°C (5% CO_2). Assays were performed twice.

Table 5.5b: Multiple t-tests for the association and invasion of INT-407 monolayers by *S. enteritidis* strain 27655R after blocking with neutralising antibodies and purified surface antigens.

	Strains/Blocking agents					
	Association					
	27655R	**antisera**	**fibronectin**	**SEF17 fimbriae**	**LPS**	**EAV30**
27655R						
antisera	<0.001					
fibronectin	0.203	ND				
SEF17 fimbriae	<0.001	ND	ND			
LPS	0.153	ND	ND	ND		
EAV30	<0.001	ND	ND	ND	ND	
	Invasion					
	27655R	**antisera**	**fibronectin**	**SEF17 fimbriae**	**LPS**	**EAV30**
27655R						
antisera	<0.001					
fibronectin	0.451	ND				
SEF17 fimbriae	0.105	ND	ND			
LPS	0.890	ND	ND	ND		
EAV30	<0.001	ND	ND	ND	ND	

ND Not Done

For statistical analyses of association with and invasion of epithelial cells, one-way analyses of variance was performed followed by t-tests. Results expressed as "p" values.

Table 5.5a Blocking of SEF17 mediated association and invasion of INT-407 monolayers using neutralising antibodies and purified surface antigens.

Strain	Blocking agent	CFU per well ± SE	
		Association	Invasion
27655R	none	$8.75x10^7 \pm 0.38$	$1.55x10^6 \pm 0.09$
"	SEF17 polyclonal antisera (chicken)	$1.75x10^7 \pm 0.21$ †	$9.0x10^5 \pm 1.79$ †
"	100 µg soluble fibronectin	$7.5x10^7 \pm 1.30$ §	$1.45x10^6 \pm 0.20$ §
"	100 µg purified SEF17 fimbriae	$1.65x10^7 \pm 1.20$ †	$1.3x10^6 \pm 0.16$ §
"	100 µg *S. enteritidis* LPS	$7.25x10^7 \pm 1.0$ §	$1.55x10^6 \pm 0.15$ §
EAV30 (17-)	none	$1.2x10^7 \pm 0.16$ †	$6.5x10^4 \pm 1.2$ †

§ No significant difference from wild type
† Significant difference from wild type ($p<0.001$)

Bacteria were prepared by culturing in CFA agar for 24 hours at 30°C. Polyclonal antibodies and fibronectin were pre-incubated with the bacteria, whilst purified surface antigens were added to the cells, prior to infection of monolayers. The tissue culture assay was performed twice. Results from a single representative assay are shown. Counts were compared statistically by ANOVA.

Table 5.6b: T-tests for the association and invasion of INT-407 monolayers by *S. enteritidis* strain LA5 after blocking with neutralising antibodies and purified surface antigens.

	LA5	
	Association	**Invasion**
Mannose	<0.001	0.020
M8614Mab	0.512	0.075
M8642 Mab	0.007	0.032
M8732 Mab	<0.001	0.060
M8743 Mab	0.020	0.080
SEF21 antisera	0.092	0.092
S. enteritidis LPS	0.231	0.045
EAV3 (21-)	<0.001	0.103

For statistical analyses of association with and invasion of epithelial cells, one-way analyses of variance was performed followed by t-tests. Results expressed as "p" values.

Table 5.6a Blocking of SEF21 mediated association and invasion of INT-407 monolayers using D-mannose, neutralising antibodies and purified surface antigens.

Strain	Blocking agent	CFU's per well ± SE	
		Association	Invasion
LA5	none	$1.6x10^6 \pm 0.14$	$1.9x10^5 \pm 0.42$
"	1% D-mannose	$2.05x10^5 \pm 0.21$ †	$1.3x10^5 \pm 0.24$
"	100µg M8614 (Mab)	$1.7x10^6 \pm 0.11$	$9.9x10^4 \pm 1.3$
"	100µg M8642 (Mab)	$1.9x10^6 \pm 0.15$	$8.1x10^4 \pm 3.5$
"	100µg M8732 (Mab)	$2.05x10^5 \pm 0.23$ †	$9.9x10^4 \pm 1.5$
"	100µg M8743 (Mab)	$1.85x10^6 \pm 0.13$	$1.0x10^5 \pm 0.10$
"	SEF21 polyclonal antisera (chicken)	$2.1x10^6 \pm 0.20$	$1.3x10^5 \pm 0.2$
"	100 µg purified SEF21 fimbriae	$1.7x10^6 \pm 0.19$	$1.22x10^5 \pm 0.14$
"	100 µg *S. enteritidis* LPS	$1.65x10^6 \pm 0.14$	$1.15x10^5 \pm 0.21$
EAV3 (21-)	none	$2.15x10^5 \pm 0.01$ †	$1.3x10^5 \pm 0.20$

† Significant difference from wild type ($p<0.001$)

Bacteria were cultured in HIB for 48 hours at 37°C (5% CO_2). Antibodies and mannose were pre-incubated with the bacteria, whilst purified surface antigens were added to the cells, prior to infection of monolayers. The tissue culture assay was performed twice. Results from a single representative assay are shown. Counts were compared statistically by ANOVA.

Table 5.7 Haemagglutination blocking assay using SEF21 antibodies.

	Reciprocal dilution of bacteria				
Blocking agent	N	2	4	8	16
PBS	+	+	+	+/-	-
3% D-mannose	-	-	-	-	-
*M8732	+	+	+/-	-	-
*M8642	+	+	+	+/-	-
*M8614	+	+	+	+/-	-
*M8743	+	+/-	-	-	-
†ChSEF21	-	-	-	-	-

\+ Haemagglutination
+/- Partial haemagglutination
\- No haemagglutination

* Mabs added at 100μg/ml
† adsorbed sera added at 1/500 dilution

Blocking agents were added to serial dilutions of *S. enteritidis* strain LA5 which had been grown to express SEF21. Horse erythrocytes were added and the suspension was rotated for 2 minutes before the results were read. The assay was performed twice.

Table 5.8b: T-tests for the association and invasion of INT-407 monolayers by *S. enteritidis* strain LA5 after centrfugation and blocking with neutralising antibodies and purified surface antigens.

	LA5			
	Without centrifugation		With centrifugation	
Strain/ Blocking agent	Association	Invasion	Association	Invasion
Purified flagella	0.48	0.105	0.564	0.14
flagella antisera	0.15	0.021	0.15	0.28
EAV10 (fla-)	<0.001	<0.001	<0.001	<0.001

For statistical analyses of association with and invasion of epithelial cells, one-way analyses of variance was performed followed by t-tests. Results expressed as "p" values.

Table 5.8a The effect of centrifugation and blocking agents upon flagella-mediated association and invasion of INT-407 monolayers.

		CFU's per well ± SE			
		Without centrifugation		With centrifugation	
Strain	Blocking agent	Association	Invasion	Association	Invasion
LA5	none	$1.1x10^6 \pm 0.18$	$1.7x10^4 \pm 0.15$	$1.5x10^6 \pm 0.11$	$2.0x10^4 \pm 0.14$
"	100µg purified flagella	$1.3x10^6 \pm 0.16$	$2.2x10^4 \pm 0.23$	$1.6x10^6 \pm 0.09$	$2.4x10^4 \pm 0.32$
"	flagella polyclonal sera	$1.5x10^6 \pm 0.05$	$2.4x10^4 \pm 0.17$	$1.95x10^6 \pm 0.84$	$2.3x10^4 \pm 0.22$
EAV10 (fla-)	none	$1.3x10^5 \pm 0.19$ †	$1.7x10^3 \pm 0.13$ †	$1.6x10^5 \pm 0.14$ †	$1.4x10^4 \pm 0.18$ †

† Significant difference from wild type ($p<0.001$)

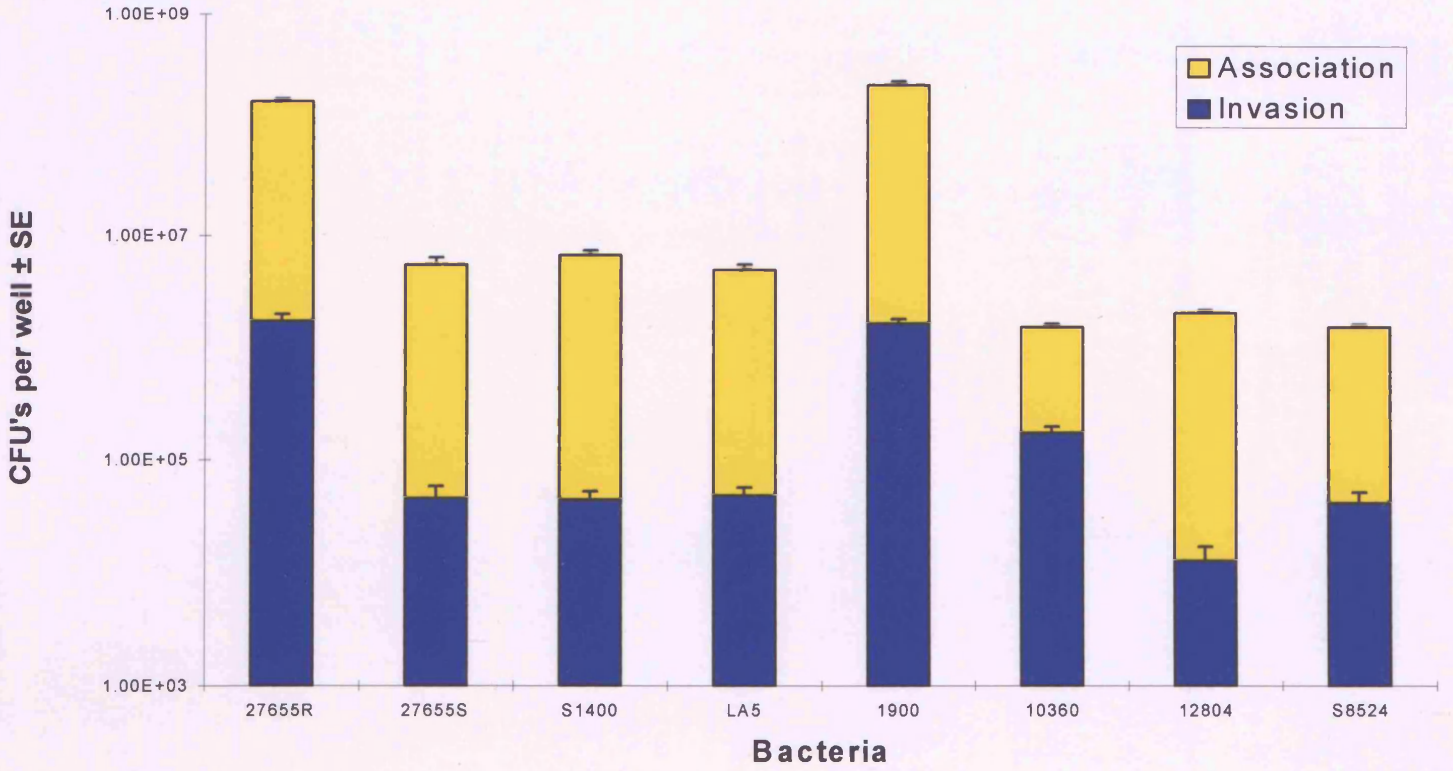
Bacteria were grown aerobically in Nutrient broth with shaking for 24 hours at 37°C. Polyclonal antisera (1/1000 dilution) was added to the bacterial suspension and the purified flagella added to the tissue culture cells. All reactions were incubated for 15 minutes at 37°C (5% CO_2) with gentle agitation, prior to infection of the monolayer. Bacteria were centrifuged onto monolayer at 250 xg for 10 minutes. The tissue culture assay was performed twice. Results from a single representative assay are shown. Counts were compared statistically by ANOVA.

Figure 5.1b: Multiple t-tests for the association and invasion of INT-407 monolayers by strains of *S. enteritidis*.

Strains								
Association								
	27655R	**27655S**	**S1400**	**LA5**	**1900**	**10360**	**12804**	**S8524**
27655R								
27655S	<0.001							
S1400	<0.001	0.817						
LA5	<0.001	0.006	0.010					
1900	<0.001	<0.001	<0.001	<0.001				
10360	<0.001	<0.001	<0.001	<0.001	<0.001			
12804	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
S8524	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.027	
Invasion								
	27655R	**27655S**	**S1400**	**LA5**	**1900**	**10360**	**12804**	**S8524**
27655R								
27655S	<0.001							
S1400	<0.001	0.238						
LA5	<0.001	0.026	0.270					
1900	0.760	<0.001	<0.001	<0.001				
10360	<0.001	<0.001	<0.001	<0.001	<0.001			
12804	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
S8524	<0.001	0.423	0.700	0.140	<0.001	<0.001	0.027	

For statistical analyses of association with and invasion of epithelial cells, one-way analyses of variance was performed followed by t-tests. Results expressed as "p" values.

Figure 5.1a Association and invasion of INT-407 monolayers by strains of *S. enteritidis*.



Bacteria were grown on CFA agar at 37°C for 24 hours before infecting tissue culture cells. For the association assay cells were infected with *Salmonella* and incubated for two hours. Monolayers were washed, disrupted with Triton and CFU determined. For the invasion assay, monolayers were infected with bacteria, washed, and incubated in media containing gentamicin. Following incubation monolayers were washed, disrupted with Triton and CFU determined, as for the association assay. Assays were performed twice. Results expressed as CFU per well ± S.E from a single representative experiment.

Figure 5.2b Multiple t-tests for the association and invasion of INT-407 monolayers by *S. enteritidis* strains cultured in various media.

Association

	Media	HIB	CFA	LB	PW	TW	MEM
LA5	HIB						
	CFA	0.184					
	LB	0.222	0.014				
	PW	0.009	<0.001	0.132			
	TW	<0.001	<0.001	0.006	0.17		
	MEM	0.086	0.004	0.602	0.315	0.021	

		HIB	CFA	LB	PW	TW	MEM
27655R	HIB						
	CFA	<0.001					
	LB	<0.001	0.132				
	PW	<0.001	<0.001	<0.001			
	TW	<0.001	0.002	0.073	0.062		
	MEM	<0.001	<0.001	<0.001	<0.001	<0.001	

		HIB	CFA	LB	PW	TW	MEM
S8524	HIB						
	CFA	<0.001					
	LB	0.196	<0.001				
	PW	0.434	0.004	0.043			
	TW	0.516	0.003	0.057	0.893		
	MEM	0.196	0.015	0.013	0.600	0.511	

Invasion

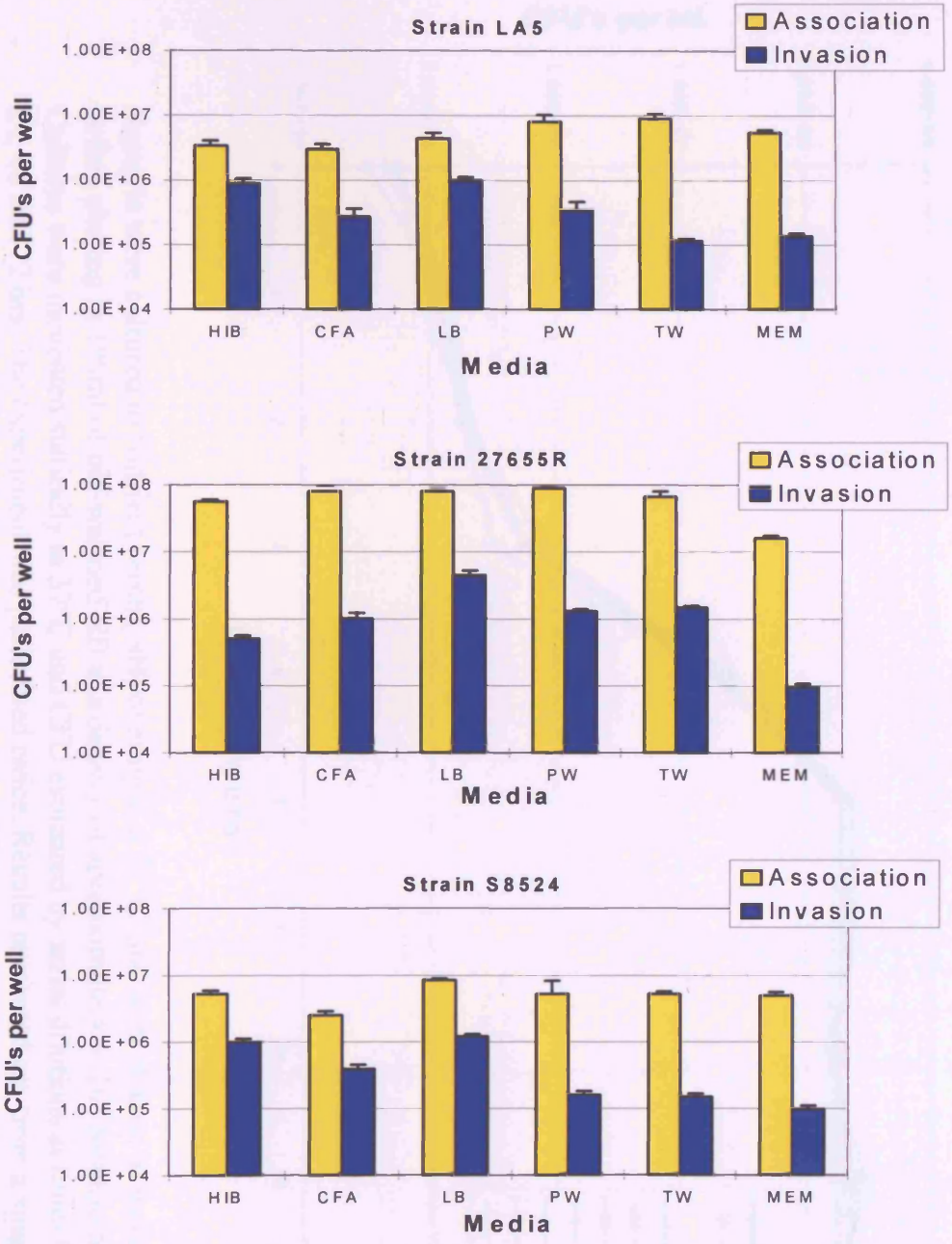
		HIB	CFA	LB	PW	TW	MEM
LA5	HIB						
	CFA	<0.001					
	LB	0.083	<0.001				
	PW	<0.001	0.127	<0.001			
	TW	<0.001	<0.001	<0.001	<0.001		
	MEM	<0.001	<0.001	<0.001	<0.001	0.496	

		HIB	CFA	LB	PW	TW	MEM
27655R	HIB						
	CFA	0.863					
	LB	<0.001	<0.001				
	PW	0.021	0.014	<0.001			
	TW	0.044	0.063	<0.001	<0.001		
	MEM	<0.001	<0.001	<0.001	<0.001	<0.001	

		HIB	CFA	LB	PW	TW	MEM
S8524	HIB						
	CFA	<0.001					
	LB	0.196	<0.001				
	PW	0.434	0.004	0.043			
	TW	0.516	0.003	0.057	0.893		
	MEM	0.196	0.015	0.013	0.600	0.511	

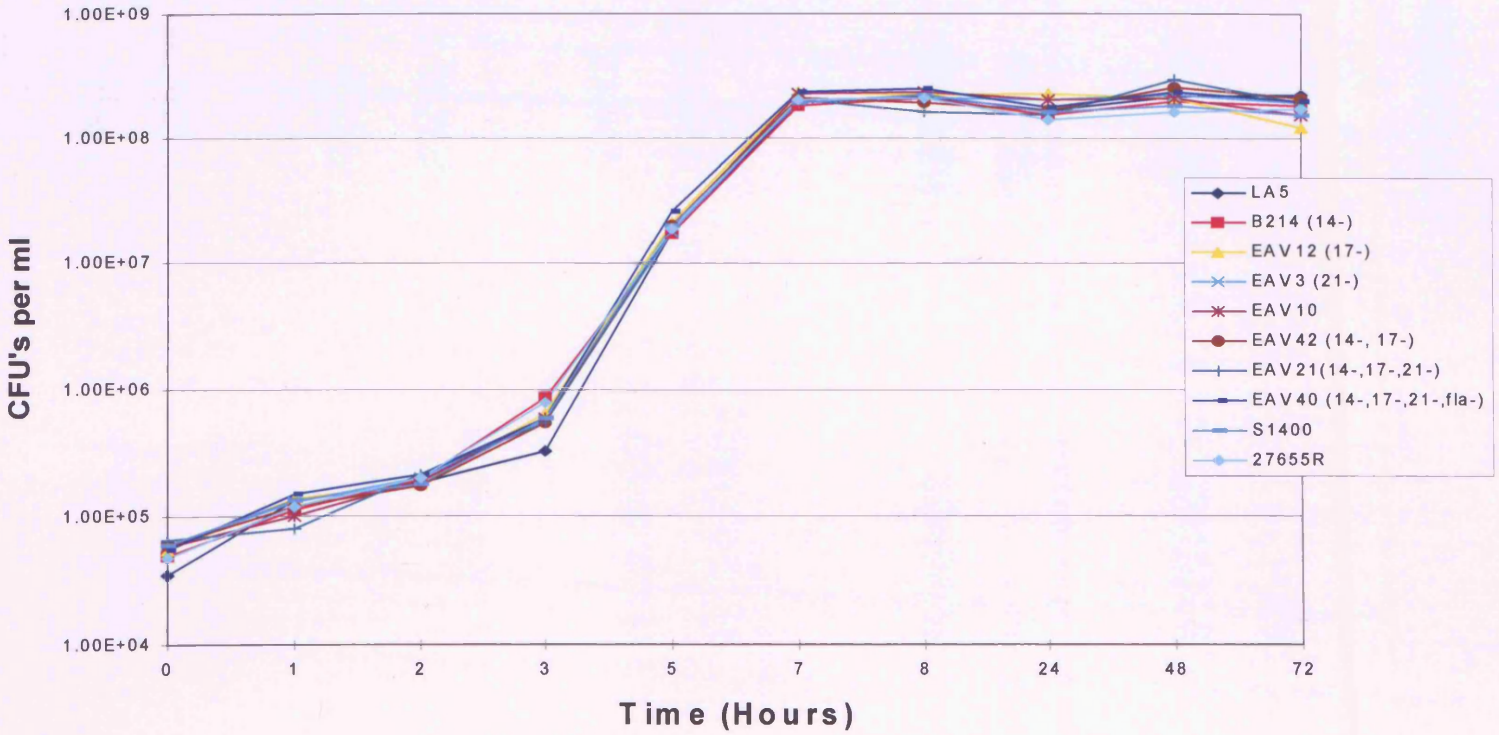
For statistical analyses of association with and invasion of epithelial cells, one-way analyses of variance was performed followed by t-tests. Results expressed as "p" values.

Figure 5.2a Association with and invasion of INT-407 monolayers by *S. enteritidis* strains cultured in various media.



Salmonella were aerobically grown in Heart Infusion broth **(HIB)**, Colonisation factor antigen agar **(CFA)**, Luria-Bertami broth **(LB)**, Peptone water **(PW)**, Tryptone water **(TW)** or Minimal essential media **(MEM)** for 48 hours at 37°C before infecting monolayers. For the association assay, infected monolayers were incubated for two hours, washed, disrupted with Triton and CFU determined. For the invasion assay, monolayers were infected with bacteria, washed, and incubated in media containing gentamicin. Following incubation for two hours monolayers were washed, disrupted with Triton and CFU determined, as described for the association assay. Tissue culture assays were performed twice. Results presented as CFU per well ± SE and represent data from both assays.

Figure 5.3 Growth curve of wild type *S. enteritidis* LA5 and mutants derived from this strain.



Bacteria were cultured in Nutrient broth (NB) overnight at 37°C and were shaken in fresh broth for 30 mins before placing in 100ml of pre-warmed NB at a density of approximately $5x10^4$ bacteria per ml media. Cultures were incubated statically at 37°C and CFU estimated by serial dilutions at times 0, 1, 2, 3, 5, 7, 8, 24, 48 and 72 hrs. The experiment was performed twice. Results presented are from a single assay.

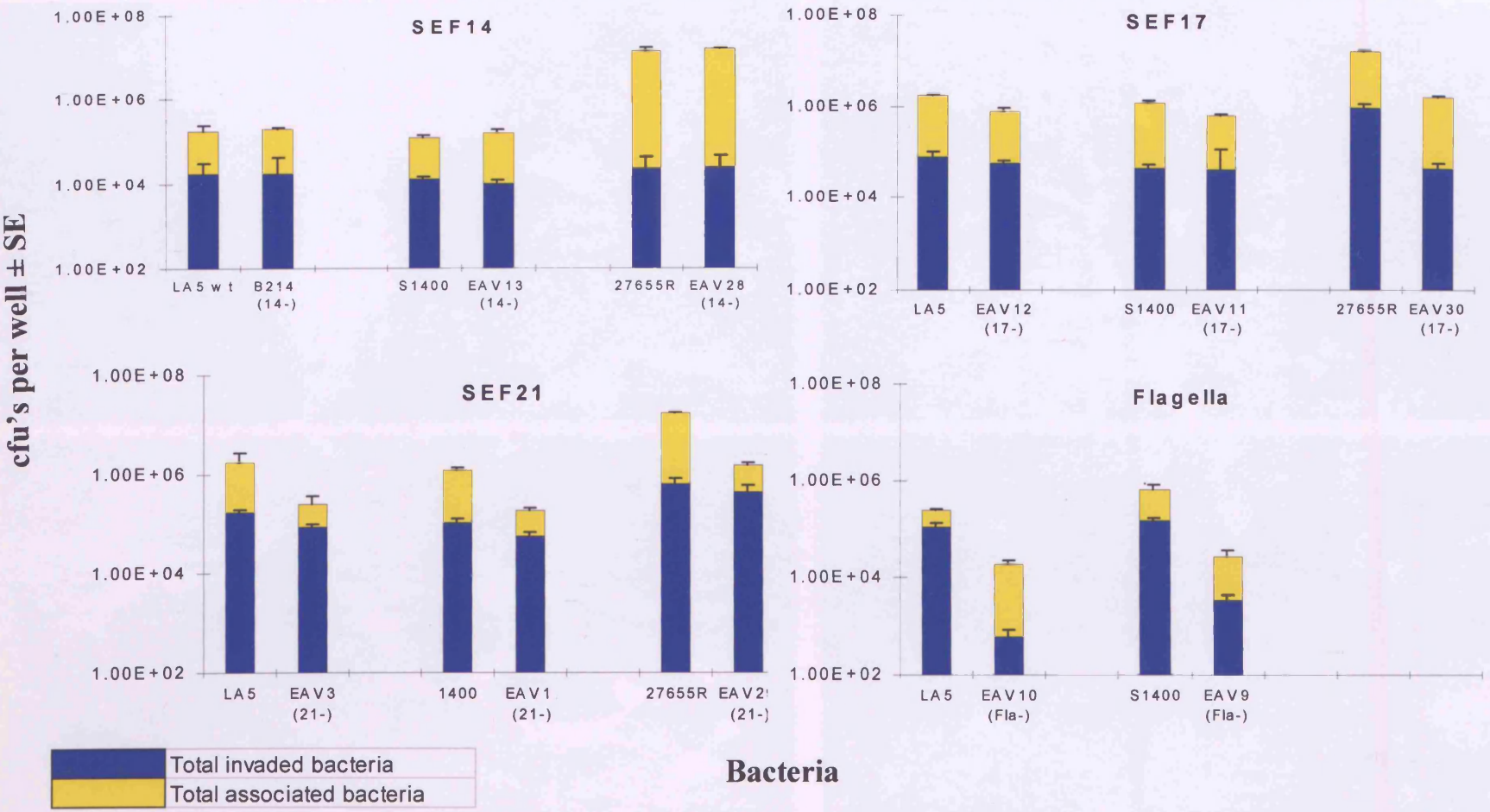
Figure 5.4b: T-tests for the association and invasion of INT-407 monolayers by strains of *S. enteritidis* and single mutants.

	Association	Invasion
LA5 v B214 (14$^-$)	>0.05†	>0.05†
S1400 v EAV13 (14$^-$)	>0.05†	>0.05†
27655R v EAV28 (14$^-$)	>0.05†	>0.05†
LA5 v EAV12 (17$^-$)	<0.001	<0.020
S1400 v EAV11 (17$^-$)	<0.001	>0.05†
27655R v EAV30 (17$^-$)	<0.001	<0.001
LA5 v EAV3 (21$^-$)	<0.001	<0.001
S1400 v EAV1 (21$^-$)	<0.001	<0.001
27655R v EAV2 (21$^-$)	<0.001	<0.001
LA5 v EAV10 (fla$^-$)	<0.001	<0.001
S1400 v EAV9 (fla$^-$)	<0.001	<0.001

For statistical analyses of association with and invasion of epithelial cells, one-way analyses of variance was performed followed by t-tests. Results expressed as "p" values.

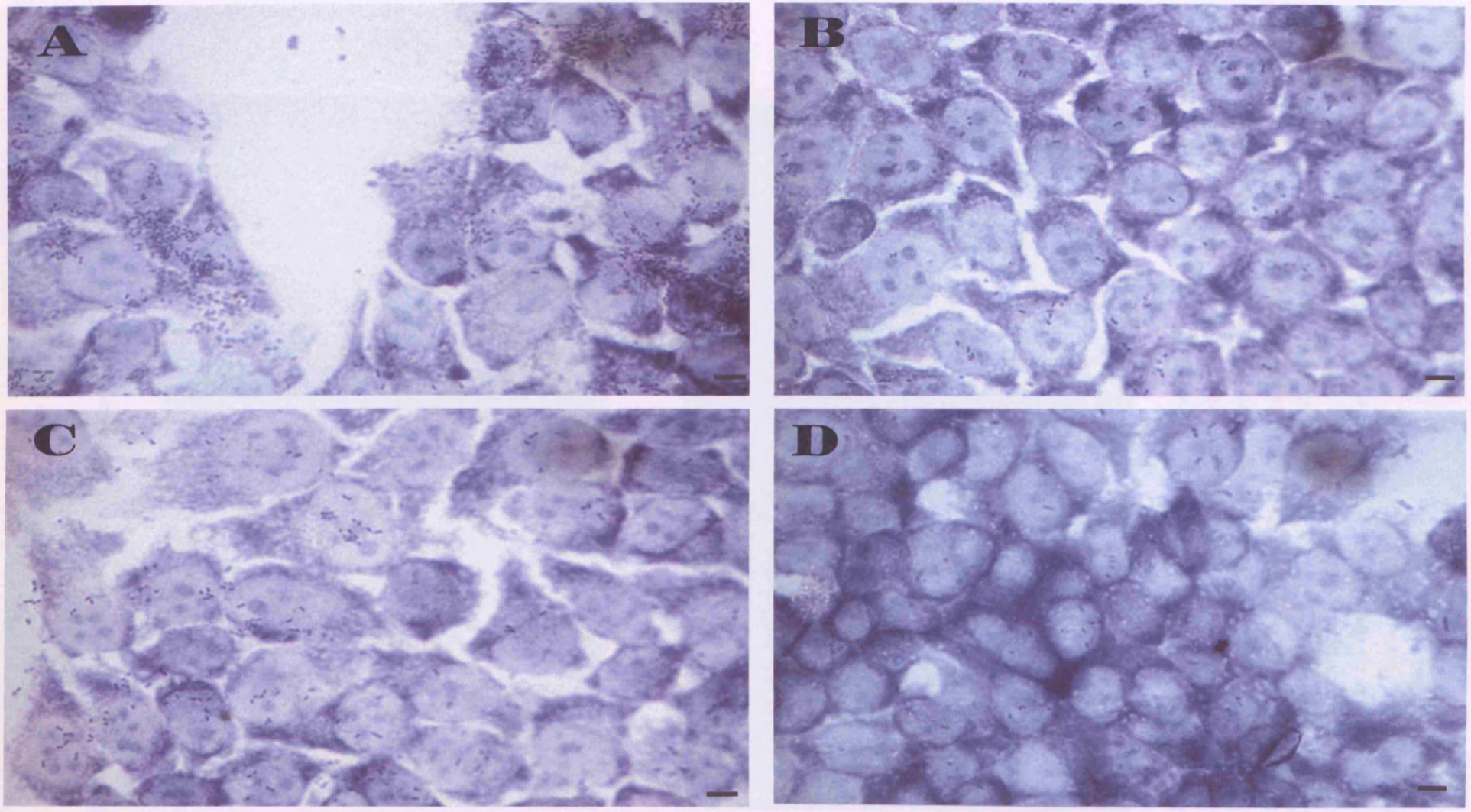
† Paired t-tests were only performed when the f-tests were significant at the 5% level ($p < 0.05$)

Figure 5.4a Association and invasion of INT-407 monolayers by *S. enteritidis* wild type and single mutants.



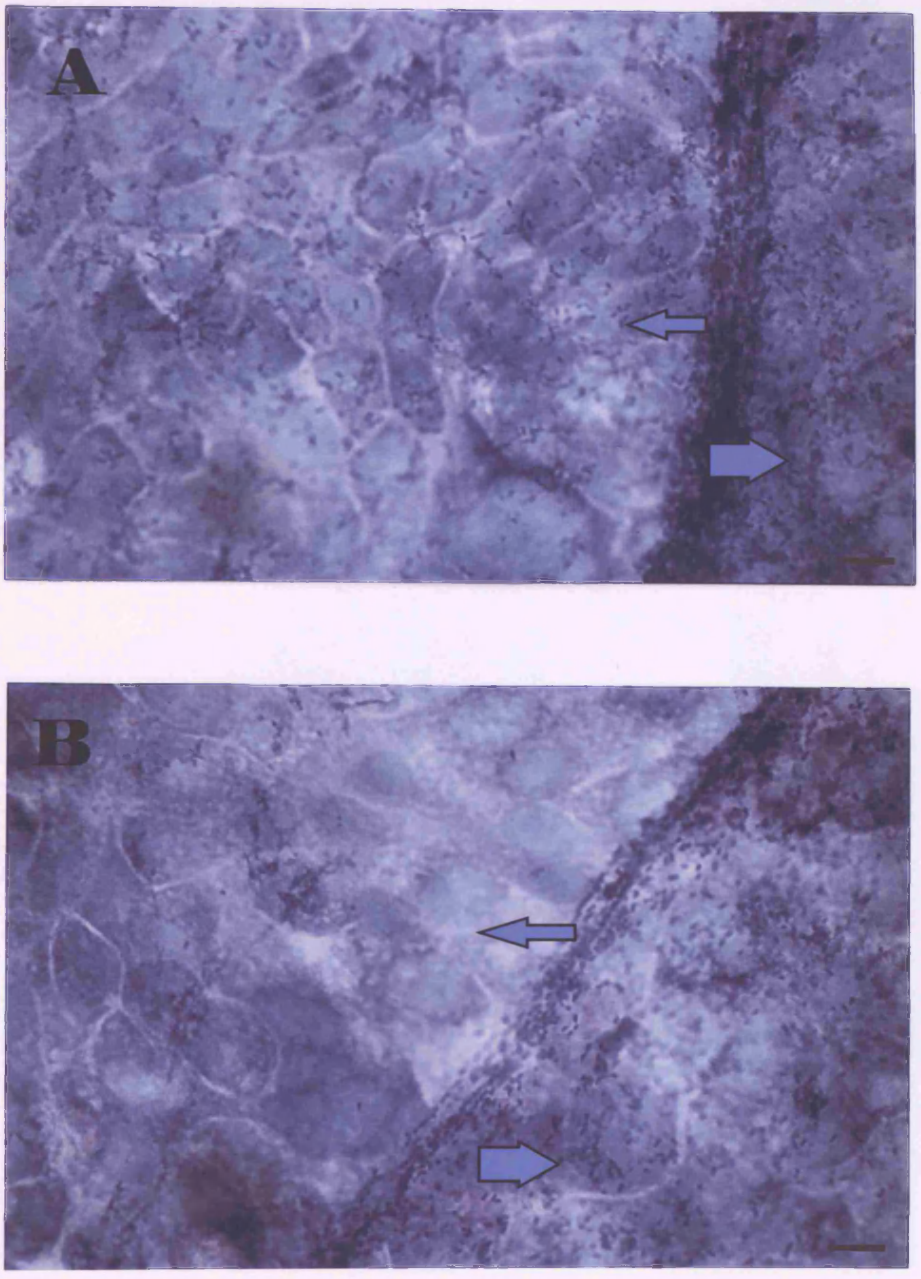
Bacteria were cultured under conditions permissive of expression of appendage prior to infection of monolayers. For the association assay, infected monolayers were incubated for two hours, washed thoroughly, disrupted with Triton and CFU determined. For the invasion assay, monolayers were infected with bacteria, washed, and incubated in media containing gentamicin. Following incubation monolayers were washed, disrupted with Triton and CFU determined, as described for the association assay. Each assay was performed twice. Results expressed as CFU per well ± SE and represent data from both experiments.

Figure 5.5 Adhesion of *S. enteritidis* to INT-407 cells.



- **A)** 27655R and **B)** EAV12 (SEF17$^-$) were cultured on CFA agar for 24 hours at 30°C, and **C)** LA5 and **D)** EAV3 (SEF21$^-$) were cultured at 37°C in HIB for 48 hours (5% CO_2) before infecting monolayers grown on cover slips. Following incubation with bacteria, monolayers were fixed, stained with Giemsa stain, differentiated in acetic acid and mounted onto microscope slides. Mounted coverslips were examined under oil immersion at x1000 magnification. Bars, 0.1 mm

Figure 5.6 Adhesion of *S. enteritidis* to mucin-secreting HT-29 cells.



A) LA5 and **B)** EAV10 (Fla^-) were grown aerobically in Nutrient broth for 24 hours at 37°C before infecting monolayers which had been cultured for ten days post confluent growth on cover slips to ensure a full covering of mucus. Following incubation with bacteria, monolayers were fixed, stained with Giemsa stain, differentiated in acetic acid and mounted onto microscope slides. Mounted coverslips were examined under oil immersion at (1000x magnification). Thick arrows show areas of monolayer covered by mucus, whereas thin arrows indicate cells un covered. Bars, 10 microns.

Figure 5.7b: Multiple t-tests for the association and invasion of INT-407, CACO-2 and DIV-1 monolayers by *S. enteritidis* strain LA5 and single and multiple mutants.

INT-407

Association

	LA5	B214	EAV12	EAV3	EAV10	EAV21	EAV40
LA5							
B214 (14⁻)	0.835						
EAV12 (17⁻)	0.025	<0.001					
EAV3 (21⁻)	<0.001	<0.001	<0.001				
EAV10 (fla⁻)	<0.001	<0.001	<0.001	<0.001			
EAV21 (14⁻, 17⁻, 21⁻)	<0.001	<0.001	0.002	<0.001	<0.001		
EAV40 (14⁻, 17⁻, 21⁻, fla⁻)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

Invasion

	LA5	B214	EAV12	EAV3	EAV10	EAV21	EAV40
LA5							
B214 (14⁻)	0.350						
EAV12 (17⁻)	0.105	0.010					
EAV3 (21⁻)	0.005	<0.001	<0.001				
EAV10 (fla⁻)	<0.001	<0.001	<0.001	<0.001			
EAV21 (14⁻, 17⁻, 21⁻)	0.002	0.003	0.021	0.015	<0.001		
EAV40 (14⁻, 17⁻, 21⁻, fla⁻)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

CAC0-2

Association

	LA5	B214	EAV12	EAV3	EAV10	EAV21	EAV40
LA5							
B214 (14⁻)	0.323						
EAV12 (17⁻)	<0.001	<0.001					
EAV3 (21⁻)	<0.001	<0.001	<0.001				
EAV10 (fla⁻)	<0.001	<0.001	<0.001	0.635			
EAV21 (14⁻, 17⁻, 21⁻)	<0.001	<0.001	<0.001	<0.001	<0.001		
EAV40 (14⁻, 17⁻, 21⁻, fla⁻)	<0.001	<0.001	<0.001	0.544	0.894	<0.001	

Invasion

	LA5	B214	EAV12	EAV3	EAV10	EAV21	EAV40
LA5							
B214 (14⁻)	<0.001						
EAV12 (17⁻)	<0.001	0.190					
EAV3 (21⁻)	0.004	<0.001	<0.001				
EAV10 (fla⁻)	<0.001	<0.001	<0.001	<0.001			
EAV21 (14⁻, 17⁻, 21⁻)	0.037	0.125	0.006	<0.001	<0.001		
EAV40 (14⁻, 17⁻, 21⁻, fla⁻)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

DIV-1

Association

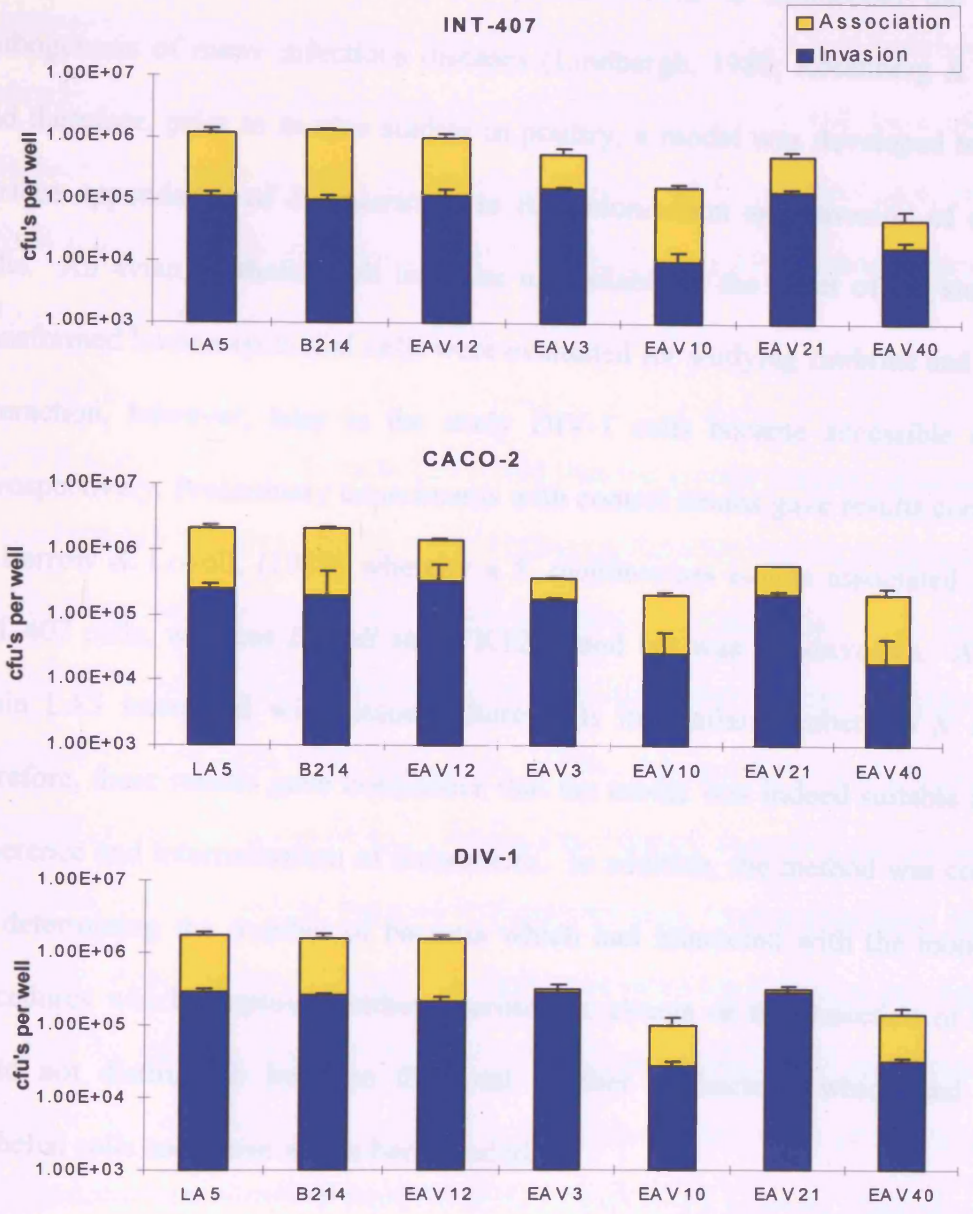
	LA5	B214	EAV12	EAV3	EAV10	EAV21	EAV40
LA5							
B214 (14⁻)	0.554						
EAV12 (17⁻)	0.422	0.831					
EAV3 (21⁻)	<0.001	<0.001	<0.001				
EAV10 (fla⁻)	<0.001	<0.001	<0.001	<0.001			
EAV21 (14⁻, 17⁻, 21⁻)	<0.001	<0.001	<0.001	0.052	<0.001		
EAV40 (14⁻, 17⁻, 21⁻, fla⁻)	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	

Invasion

	LA5	B214	EAV12	EAV3	EAV10	EAV21	EAV40
LA5							
B214 (14⁻)	0.496						
EAV12 (17⁻)	0.003	0.015					
EAV3 (21⁻)	0.476	0.168	<0.001				
EAV10 (fla⁻)	<0.001	<0.001	<0.001	<0.001			
EAV21 (14⁻, 17⁻, 21⁻)	0.854	0.389	0.002	0.596	<0.001		
EAV40 (14⁻, 17⁻, 21⁻, fla⁻)	<0.001	<0.001	<0.001	<0.001	0.050	<0.001	

For statistical analyses of association with and invasion of epithelial cells, one-way analyses of variance was performed followed by t-tests. Results expressed as "p" values.

Figure 5.7a Association and invasion of INT-407, CACO-2 and DIV-1 monolayers by single and multiple mutants of *S. enteritidis*.



LA5 (wild type), B214 (14^-), EAV12 (17^-), EAV3 (21^-), EAV10 (Fla^-), EAV21 (14^-, 17^-, 21^-) and EAV40 (14^-,17^-,21^-,fla^-) were cultured in HIB for 48 hours prior to infection of monolayers. For the association assay, infected monolayers were incubated for two hours, washed thoroughly, disrupted with Triton and CFU determined. For the invasion assay, monolayers were infected with bacteria, washed, and incubated in media containing gentamicin. Following incubation monolayers were washed, disrupted with Triton and CFU determined, as for the association assay. Results expressed as CFU per well ± SE. Assays with INT-407 and CACO-2 were performed twice whereas the DIV-1 assay was performed once.

5.3 Discussion

Interaction of bacteria with animal epithelial cells is considered the first step in the pathogenesis of many infectious diseases (Lindbergh, 1980; Rosenberg & Kjelleberg, 1986) and therefore, prior to *in vivo* studies in poultry, a model was developed to study the role of surface appendages of *S. enteritidis* in the colonisation and invasion of cultured epithelial cells. An avian epithelial cell line was unavailable at the onset of the study and therefore, transformed human epithelial cells were evaluated for studying fimbriae and flagella-mediated interaction, however, later in the study DIV-1 cells became accessible and were studied retrospectively. Preliminary experiments with control strains gave results consistent with those of Barrow & Lovell, (1989) whereby a *S. typhimurium* isolate associated with and invaded INT-407 cells, whereas *E. coli* strain K12 bound but was non-invasive. Also, *S. enteritidis* strain LA5 interacted with tissue culture cells in similar numbers to *S. typhimurium* and therefore, these results gave confidence that the model was indeed suitable for studying both adherence and internalisation of *Salmonella*. In addition, the method was considered suitable for determining the number of bacteria which had interacted with the monolayers, whereas procedures which employed either microscopic counts or the detection of labelled bacteria could not distinguish between the total number of bacteria which had associated with epithelial cells and those which had invaded.

Greater numbers of bacteria bound to INT-407 cells than other cell lines tested and these results were broadly consistent with those of Mills & Finlay (1994) who compared invasion and intracellular growth of *S. typhimurium* and *S. typhi* in CACO-2, HeLa and INT-407 monolayers. Indeed, Lodge *et al.* (1995) reported that Hep-2 cells were a poor predictor of behaviour of transposon insertion mutants of *S. typhimurium* when extrapolated to gut tissue.

Additional support for the INT-407 model was offered by Jones *et al.*, (1993) who demonstrated that infection with invasive *S. typhimurium* induced dramatic actin rearrangements on the surface of INT-407 cells, as part of the entry mechanism. These actin rearrangements mimic those seen *in vivo* (Takeuchi, 1967) and have more recently been referred to as membrane ruffling (Finlay & Falkow, 1990). Interestingly, Burns-Keliher *et al.* (1998) reported that *S. typhimurium* synthesised 142 proteins during growth within INT-407 cells of which 58 were unique to growth within this cell line.

Evaluation of the INT-407 model indicated that the growth conditions of the inoculum prior to infection of monolayers had a marked effect on the ability of the bacterium to associate and invade tissue culture cells. In this report, anaerobic growth of *Salmonella* prior to infection of monolayers resulted in a dramatic increase in the number of invading *Salmonella.* These results are in agreement with Ernst *et al.* (1990), although Schiemann (1995) suggested that reports of greater cell invasion by *S. typhimurium* during anaerobic growth may not have been due to the direct effect of reduced oxygen but may have arisen from the use of media containing carbohydrates which were found to be more repressive of invasion during aerobic growth of the bacteria. Also, in this study the growth media had an effect upon the interaction of *Salmonella* with epithelial cells. For example, *S. enteritidis* strains invaded in greater numbers following growth in Luria broth but consistently lower in Minimal Essential Media. Interestingly, Namdari & Bottone (1990) reported that growth media used prior to inoculation could alter the adherence level of *Aeromonas caviae* from a very few bacteria per tissue culture cell to an almost confluent mantle of 40 bacteria or more. In addition, Albert *et al.* (1995) reported that bacterial growth temperature affected internalisation of *Providencia alcalifaciens* with an increased invasion of Hep-2 monolayers following growth at 37°C, and Tartera & Metcalf, (1993) showed that high osmolarity and late-log phase were optimal for

adherence and invasion of epithelial cells by *S. typhi*. Collectively, these results support the findings of this study that growth conditions of the inoculum could heavily influence the interaction of bacteria with tissue culture cells.

To study the role of fimbrial and flagella-mediated association with and invasion of epithelial cells a panel of single and multiple mutants were prepared in three strains of *S. enteritidis* by insertional inactivation of target genes. The loss of phenotypic expression was confirmed by direct binding ELISA after growth of mutants under permissive conditions for elaboration of appendages. Expression of the surface appendages encoded by un-disrupted gene remained unaffected and the genetic lesion appeared to have no effect upon growth kinetics.

In the tissue culture model strain 27655R consistently associated with INT-407 cells in significantly greater numbers than LA5 and S1400 which confirmed the findings of Baloda *et al.* (1988). The previous chapter described the "atypical" expression of SEF17 by 27655R amongst *S. enteritidis* although aberrant elaboration of this fimbrial structure has been reported not to be attributed to a frameshift mutation in *rpo*S global regulatory gene harboured by this strain (Allen-Vercoe *et al.*, 1998). The structural fimbrin subunit of SEF17, AgfA, is genetically and antigenically related to thin aggregative fimbriae of *S. typhimurium* (Stolpe *et al.*, 1994, Römling *et al.*, 1998a; this study) and expression of thin aggregative fimbriae of *S. typhimurium* has been demonstrated to promote interaction with mouse small intestinal epithelial cells (Sukupolvi *et al.*, 1997). These results are consistent with the behaviour of 27655R in the tissue culture model. Association of the *agf*A mutant was reduced significantly and histological examination suggested a role for SEF17 in localised, often aggregative adherence, which could be specifically blocked with polyclonal sera or purified fimbriae. Interestingly, this and other studies (Baloda *et al.*, 1988; Collinson *et al.*, 1991) have

demonstrated an affinity of SEF17 for soluble fibronectin, but results suggested that this was not the receptor on INT-407 cells for binding was not inhibited by fibronectin. SEF17 would appear to promote an extremely aggregative phenotype, but it is unclear whether SEF17 mediated aggregation in the inoculum, although macroscopic clumping in cultures was not observed and bacterial counts of inocula were consistent between assays which suggested no aggregation prior to adherence assay.

In addition to *S. enteritidis* strain 27655R, S1900 interacted with INT-407 cells with significantly greater numbers than the other wild type isolates tested. These two strains were found to constitutively express SEF17 (Chapter 4) and therefore, it was plausible that SEF17-mediated adherence was responsible for enhanced interaction. Strain 27655R was shown previously to attach in greater numbers than other *S. enteritidis* isolates (Baloda *et al.*, 1988) but mannose-sensitive fimbriae (type 1) were implicated as being responsible for enhanced adhesion. Results from this study showed that the single SEF17 mutants of strain 27655R associated at similar levels to wild type LA5 and S1400 suggesting that SEF17 was responsible for enhanced binding. That strain S1900 expressed SEF21 poorly also implicated SEF17, although it was possible that SEF21 contributed to enhanced binding. To test for this mannose was added to the inoculum medium and levels of association were unaffected (data not shown).

Type 1 fimbriae of *S. typhimurium* and some *E. coli* are regarded as virulence determinants which play a role in colonisation of gut epithelia (Lockman *et al.*, 1992a, Takeuchi *et al.*, 1967, Vidotto *et al.*, 1997) and invasion (Ernst *et al.*, 1990). Results of this study suggested that SEF21 mediated association in all three strains, although to observe this it was necessary to induce expression of fimbriae prior to infection. Thus, a correlation was observed between

the level of association and SEF21 expression in the inoculum as measured by MSHA. These results agree with those of Tavendale *et al.*, (1983) and Old *et al.*, (1986) who have shown that fimbriae mediate mannose sensitive adhesion of *S. typhimurium* strains to Hep-2 cells. However, Elliot *et al.*, (1997) reported type 1 fimbriae had no effect on levels or patterns of adhesion to cultured human cells in EPEC infections. The addition of mannose or M8732 Mab specifically blocked SEF21-mediated adherence to monolayers. Interestingly, both Mabs M8732 and M8743 inhibited haemagglutination partially. Results suggested that at least part of the haemagglutinin of SEF21 was involved in adherence to INT-407 cells but it is plausible that the receptor on the tissue culture cells may have bound a different region to that of the erythrocytes. This may explain the differences in the blocking abilities of the Mabs and this could be tested by epitope mapping studies. In addition, ChSEF21 failed to block SEF21-mediated adherence of tissue culture cells or inhibit haemagglutination and these results suggested that the haemagglutinin of SEF21 was poorly immunogenic in chickens. Also, purified SEF21 did not block adherence, nor could cell free fimbriae agglutinate red blood cells.

SEF14 mutants in all three strains reacted very similarly, with no significant difference in either association with or invasion of their respective wild type. These results are consistent with those of Thorns *et al.* (1996) and Oginniyi *et al.*(1997). Interestingly, a role for SEF14 in transovarian transmission in chickens has been suggested by Thiagarajan *et al.* (1996a; b).

Aflagellate mutants of *S. enteritidis* associated with and invaded significantly less than motile bacteria and numbers of associated bacteria were increased only marginally by centrifugation of bacteria onto monolayers. Numbers of associating bacteria were unaffected by the addition of purified flagella or specific polyclonal antibody to the flagellated wild type. It is highly

likely that flagella assisted colonisation of epithelial cells by motility rather than providing an adhesin. This is in agreement with the observations of the mucin-secreting model and confirmed earlier work performed in this laboratory with adherence to chick gut explants (Allen-Vercoe *et al.*, 1998). Interestingly, Jones *et al.*, (1992) reported the direction of flagellar rotation affected the ability of *S typhimurium* to invade cultured epithelial cells and in addition, flagella have been implicated in assisting survival of *S. typhimurium* within murine macrophages (Weinstein *et al.*, 1984).

The intestinal tract of all mammals and birds contain specialised differentiated goblet cells which secrete mucus that forms a protective layer over internal epithelial surfaces (Forstner & Forstner, 1994). There is evidence that mucus plays an important role as a non-specific defence mechanism (Befus *et al.*, 1980; Drumm *et al.*, 1988; Paerregaard *et al.*, 1991). To demonstrate the role of flagella in penetrating the mucus layer by *S. enteritidis* the mucin-secreting cell line HT-29 clone 16E was infected with wild type LA5 or aflagellate mutant. The mucin layer could not be detached from the cells, therefore, it was not possible to quantify the number of bacteria which had associated with or invaded the tissue culture cells, but histological examination suggested that many more wild type *Salmonella* had penetrated the mucin layer and bound to the cells than aflagellate mutants. This was not observed with fimbrial mutants. To enable bacterial enumeration a confluent layer of mucus which could be consistently removed would be required. It is possible that fresher tissue culture cells would enable quantitative bacterial counts of association and invasion, and further developmental work of this model would be necessary.

Multiple fimbrial and flagella mutants bound in very similar numbers to individual mutants when tested in the INT-407 and CACO-2 models and demonstrated the potential role of

SEF21 and SEF17 in adherence, and suggested a motility function for flagella in an undoubtedly, multifactoral process. Interactions with the avian cell line DIV-1 were similar for human-derived cell lines and showed a significant effect with SEF21 and flagella, but not SEF17. This assay was performed only once whilst visiting another laboratory and would need to be repeated to confirm the results.

Characterisation of fimbrial and flagella mediated interaction of cultured epithelial cells is an initial step in evaluating the role of these surface structures in pathogenesis of *S. enteritidis*. The question arises as to whether these observations will prove consistent in studies with chick *in vivo* studies.

Chapter 6

The contribution of fimbriae and flagella of *S. enteritidis* to colonisation, invasion, persistence and lateral transfer in chicks.

6.1 Introduction

S. enteritidis is now a common pathogen of many species of mammals and fowls although prior to the 1980's it was rarely isolated from poultry and most isolates were derived from contaminated feed (Snoeyenbos *et al.*, 1969). More recently the incidence of *S. enteritidis* infection in poultry flocks has increased in Britain, the United States and throughout Europe (Rodrigue *et al.*, 1990; Suzuki, 1994), with a concurrent increase in the number of human outbreaks of *S. enteritidis*-mediated food poisoning attributed to the consumption of contaminated meat, eggs or egg products (Hedberg *et al.*, 1993; Humphrey & Rowe, 1988; Humphrey *et al.*, 1991a; St. Louis *et al.*, 1988).

Newly hatched chicks are regarded to be most susceptible to colonisation *by S. enteritidis*, but as chickens age they generally become more resistant to infection (Gorham *et al.*, 1991; Hinton *et al.*, 1989; Humphrey *et al.*, 1991). *Salmonella* produce a variety of putative virulence determinants but the mechanisms by which *Salmonella* adhere to and invade chick gut epithelia remain poorly understood.

The previous chapter reported the multifactoral association and invasion of epithelial cells which involved SEF17 and SEF21 and flagella mediated motility. The aims of this study were to determine the contribution of fimbriae and flagella of *S. enteritidis* in 1) colonisation and invasion of chicks, 2) persistence in birds for up to seven weeks of age, and 3) to ascertain their role in lateral transfer.

6.2 Results

6.2.1 Contribution of fimbriae and flagella of *S. enteritidis* in colonisation and invasion of day-old chicks

Chicks are regarded as being highly susceptible to colonisation by *S. enteritidis* and therefore, the day-old chick were utilised to investigate the contribution of fimbriae and flagella in colonisation and invasion to deeper tissues. Following oral inoculation *Salmonella* have been reported to colonise the caeca in large numbers and have been consistently isolated from the liver and spleen (Humphrey *et al.*, 1990; Duchet-Suchaux *et al.*, 1995; 1997), and therefore, bacterial enumeration was performed from these organs. However, *S. enteritidis* have been isolated from other regions of the viscera including ileum, jejunum, blood, heart and bile (Humphrey *et al.*, 1990), and also reproductive tissues and forming eggs from older birds (Keller *et al.*, 1995), although, in lower numbers and less frequently than in younger chicks.

6.2.1.1 Dose response in day-old chicks

To determine a suitable inoculum day-old chicks were orally dosed with 10^3, 10^5 and 10^7 *S. enteritidis* strain LA5 and colonisation of caeca and invasion to liver and spleen measured 6 hrs, 24 hrs, 48 hrs and 72 hrs PI (Figure 6.1). Ten chicks were used for each time point. The predicted means and standard errors for the combinations of time and organ for each dose were determined and with all 3 inocula the caecum was colonised first, followed by the liver and finally the spleen. Several chicks looked sick following an oral inoculum of 10^7 *Salmonella* and three birds had died within 72 hrs. Therefore, 10^5 wild-type bacteria was the lowest inoculum able to colonise all organs in day-old chicks within 24 hrs without morbidity or mortality and was used for subsequent experiments.

6.2.1.2 Colonisation and invasion of day-old chicks by *S. enteritidis* wild type and mutant derivatives

The contribution of fimbriae and flagella upon colonisation and invasion of day-old chicks was studied using *S. enteritidis* strain LA5 and isogenic mutants derived from this strain. The number of *Salmonella* in the caecum, liver and spleen were determined, at 24 and 48 hrs following an oral dose with 10^5 wild type or mutants. Two individual experiments gave similar results, therefore, analyses of numbers of chicks colonised and direct colony counts from organ homogenates presented in table 6.1 and figure 6.2, represent cumulative data from 2 experiments. For statistical analyses one-way analyses of variance was performed followed by t-tests.

For the liver and spleen, bacterial counts increased significantly ($p<0.001$) between 24 and 48 hrs for all strains (Table 6.1). Specifically, in the spleen at 24 hrs PI, there were significantly lower numbers of chicks infected ($p<0.026$) and counts ($p<0.195$) of strains EAV42(14^-, 17^-), EAV21 (14^-, 17^-, 21^-) and EAV40 (14^-, 17^-, 21^-, fla^-) when compared to LA5 and B214 (14^-). Statistical pairwise t-test comparisons between each isogenic mutant indicated that the differences in counts between B214>EAV42 ($p<0.026$), EAV42>EAV21 ($p<0.033$) and EAV21>EAV40 ($p<0.022$) were significant. At 48 hrs PI the spleens were colonised with lower counts ($p<0.443$) for EAV40 (14^-, 17^-, 21^-, fla^-) than all other strains although there was no significant difference in the number of chicks colonised.

At 24 hrs PI, EAV21 (14^-, 17^-, 21^-) and EAV40 (14^-, 17^-, 21^-, fla^-) gave significantly lower bacterial counts ($p<0.003$ and $p<0.001$, respectively) from the liver when compared to LA5, B214 (14^-) and EAV42 (14^-, 17^-). EAV40 (SEF14^-, 17^-, 21^-, fla^-) alone colonised a

significantly lower number of chicks ($p<0.03$) than all other strains, although at 48 hrs PI there were no significant differences between wild type and mutants (Table 6.1).

All caeca were colonised at 24 hrs PI by all strains (Figure 6.2). However, mean counts for LA5, B214 (14^-) and EAV42 (14^-, 17^-) at 24 and 48 hrs were significantly greater ($p<0.001$) than those for EAV21 (SEF14^-, 17^-, 21^-) and EAV40 (SEF14^-, 17^-, 21^-, fla^-), although there were no significant differences observed between EAV21 and EAV40.

6.2.2 Persistence of *S. enteritidis* wild type and mutants in chicks

To determine the effect of fimbriae and flagella on persistence of *S. enteritidis* in young birds, day-old chicks were dosed orally with 10^5 wild type LA5 or mutant EAV40 (14^-, 17^-, 21^-, fla^-). Shedding of *Salmonella* was monitored by cloacal swabbing weekly, for up to 6 weeks PI and in addition, enumeration of *Salmonella* was performed from spleen, liver and caecum at weeks 2, 4 and 6 PI. All birds were bled weekly and serological responses were monitored to LPS, fimbriae and flagella. The experiment was repeated with seven day old chicks which would have a more mature alimentary tract (Schat & Myers, 1991; Forstner & Forstner, 1994) and contain a bacterial flora.

6.2.2.1. Shedding of *S. enteritidis* by chicks

Cloacal swabs were semi-quantitatively analysed for the presence of *Salmonella* by plating onto BGA plates. Five strokes of the swab were made across the top of the agar plate, the dish was turned 90 degrees and another streak performed with a sterile loop, and repeated again. Heavy shedding was interpreted as confluent colonies of *Salmonella* on the first streaking, whereas, semi-confluence was recorded as moderate shedding and light shedding as a few isolated colonies only (Figure 6.3).

Following oral inoculation of day-old chicks with either LA5 or EAV40 (14$^-$, 17$^-$, 21$^-$, fla$^-$), heavy or moderate shedding was recorded after seven days, whereas, at 42 days PI, shedding of *Salmonella* had reduced to moderate or light levels for LA5 (Table 6.2) and light shedding for EAV40 (Table 6.3).

At day seven PI heavy or moderate shedding of *Salmonella* was observed by chicks which had been inoculated at 7 days of age with LA5 or EAV40 (14$^-$, 17$^-$, 21$^-$, fla$^-$). By 42 days PI a reduction in excretion to moderate or light shedding was recorded by chicks dosed with EAV40 (Table 6.5). However, by 35 days PI light shedding of LA5 was observed by all birds and by day 42 *Salmonella* were not detected from 3 of the 10 birds swabbed (Table 6.4), although cultural results indicated that large numbers of *Salmonella* were present in the caeca of these birds (Table 6.4).

6.2.2.2 Persistence of *S. enteritidis* in spleen, liver and caeca of chicks

At 14 days PI *Salmonella* were isolated from a majority of liver and spleen homogenates from birds inoculated with LA5 or EAV40 (SEF14$^-$, 17$^-$, 21$^-$, fla$^-$) at day-old or 7 days of age (Table 6.6). By 28 days PI bacteria were isolated from less birds in each group and not from the liver of birds inoculated with EAV40 at one day old. After 42 days PI *Salmonella* were still isolated from birds infected with LA5 and EAV40 dosed at both one and 7 days of age.

There were no significant differences between caecal counts for chicks inoculated at one day-old with LA5 or EAV40 (Table 6.6) with both showing a similar decline in mean counts over time. However, birds inoculated at 7 days of age with LA5 gave significantly higher mean counts ($p<0.001$) than EAV40 at 28 and 42 days PI.

6.2.2.3 Serological responses

The humoral immune responses to LPS, flagella, SEF14, SEF17 and SEF21 were examined over the course of the persistence study following oral inoculation of one or seven day old chicks with LA5 and EAV40 (14^-, 17^-, 21^-, fla^-). The isotype specific responses were very similar for birds dosed at both ages. For both ages of birds, antibodies to LPS were detected within 3 weeks (Figure 6.4), whereas, serological responses to flagella, SEF14 and SEF21, but not SEF17, were detected only in birds inoculated with LA5 (Figure 6.5, 6.6, 6.7). However, antibodies which recognised flagella of *S. enteritidis* were detected at weeks 5 and 6 following inoculation of day-old chicks with EAV40 (14^-, 17^-, 21^-, fla^-), which coincided with the isolation of *Klebsiella spp.* from caeca and internal organs. An IgM and IgG titre to flagella was observed at 7 and 14 days, respectively (Figure 6.5) from birds infected with wild type *S. enteritidis* whereas, specific antibody responses were detected for SEF14 (Figure 6.6) and SEF21 (Figure 6.7) by week 2 PI in both groups of birds. An IgG response was detected for SEF21 in 25-40% of birds, whereas IgM was observed in 80-90% of birds. For flagella and SEF14, an IgM and IgG response were detected in sera from all birds infected with wild type *Salmonella.* A serological response to SEF17 was undetectable by ELISA or Western blot (data not shown).

6.2.3 The role of fimbriae and flagella of *S. enteritidis* in colonisation of the chick intestinal tract by lateral transfer

Inoculation thus far in this study has been by oral gavage with *S. enteritidis.* In this experiment the contribution of surface appendages in infection of birds with *S. enteritidis* contracted from the environment was studied. "Sentinel" birds were housed with "seeder"

birds which had been orally dosed with LA5 or EAV40 (14^-, 17^-, 21^-, fla^-) and the infection was monitored by cloacal swabbing which had previously been demonstrated to be a suitable method for determining the presence of *Salmonella* in the alimentary tract of newly infected birds (Tables 6.2, 6.3, 6.4, 6.5).

The experiment utilised seven day old chicks to allow the intestinal tract to mature (Schat & Myers, 1991) and a gut flora to develop in the birds to ensure a competitive environment within the alimentary tract prior to infection. Previous experiments have demonstrated that 10^5 *Salmonella* was sufficient to orally dose birds of this age. *Salmonella* were detected by cloacal swabbing after 2 days and all "seeder" chicks inoculated with LA5 or EAV40 (14^-, 17^-, 21^-, fla^-) were positive by swabbing after 3 and 4 days, respectively (Figure 6.8 Group I).

After 3 days, *S. enteritidis* were detected by cloacal swabbing from 7/10 "sentinel" birds housed with seeder chicks infected with LA5. All birds were positive by 4 days. In comparison, 3/10 sentinel chicks were positive by cloacal swabbing by day 3 and EAV40 isolated from all birds but after 5 days (Figure 6.8 Group II).

Following the introduction of sentinel birds into the isolator which had been uninhabited for two weeks, but had previously housed birds infected with wild type *Salmonella*, LA5 were isolated from cloaca of 8/10 chicks within 2 days and all birds by day 3 (Figure 6.8 Group III). In comparison, 1/10 were positive by cloacal swabbing for EAV40 (14^-, 17^-, 21^-, fla^-) after 2 days and the rest by day 4.

At 7 days PI, there were no significant differences in bacterial counts between *S. enteritidis* strain LA5 and EAV40 (14^-, 17^-, 21^-, fla^-) from spleen and liver homogenates (Table 6.7),

although caecal counts for EAV40 were significantly lower than LA5 for groups I ($p< 0.05$) and II ($p<0.001$), but for group III the difference was not statistically significant.

6.2.4 Colonisation and invasion by *S. enteritidis* following oral inoculation of chicks with a mixed inoculum containing wild type and mutants

Seven day old chicks were dosed orally with a mixed inoculum containing $5x10^4$ wild type *S. enteritidis* strain LA5 and equal numbers of B214 (14^-), EAV42 (14^-, 17^-), EAV21 (14^-, 17^-, 21^-) or EAV40 (14^-, 17^-, 21^-, fla^-). Groups of birds were housed in separate isolators and on days 1, 7 and 14 PI chicks were killed and *Salmonella* were isolated from liver, spleen and caecum. Numbers of mutant and wild type strains were estimated by plating directly onto BGA, in the presence or absence of kanamicin, respectively. In all experiments the chicks were heavily infected with *Klebsiella spp.* which grew well on BGA, even in the presence of kanamicin and therefore, enumeration was not possible on day one PI and significantly reduced the number of *Salmonella* colonies isolated from all organs on days 7 and 14 PI.

Results showed that both wild type and each mutant colonised the caeca and also invaded deeper organs to a similar level for up to 14 days PI (Table 6.8). Amongst chicks there was often a strong correlation between the isolation of wild type or mutant in the caeca and that found in the spleen and liver. Some chicks were colonised with wild type or mutant alone and some with a mixture of both.

Table 6.1b T-tests for the isolation of *S. enteritidis* wild type and mutants from spleen and liver, 24 and 48 hours after oral inoculation with 10^5 *Salmonella.*

		Spleen	
		24 hours	**48 hours**
LA5 v B214 (14$^-$)	a	0.920	0.550
	b	0.850	0.870
B214 v EAV42 (14$^-$, 17$^-$)	a	0.026	0.520
	b	0.195	0.438
EAV42 v EAV21 (14$^-$, 17$^-$, 21$^-$)	a	0.033	0.721
	b	0.257	1.000
EAV21 v EAV40 (14$^-$, 17$^-$, 21$^-$, fla$^-$)	a	0.022	0.443
	b	0.228	1.000
		Liver	
		24 hours	**48 hours**
LA5 v B214 (14$^-$)	a	0.650	0.950
	b	0.650	1.000
B214 v EAV42 (14$^-$, 17$^-$)	a	0.615	0.634
	b	1.000	1.000
EAV42 v EAV21 (14$^-$, 17$^-$, 21$^-$)	a	0.003	0.332
	b	0.482	0.850
EAV21 v EAV40 (14$^-$, 17$^-$, 21$^-$, fla$^-$)	a	<0.001	0.130
	b	0.030	0.850

a Counts

b Chicks colonised

For statistical analyses of association with and invasion of epithelial cells, one-way analyses of variance was performed followed by t-tests. Results expressed as "p" values.

Table 6.1a Isolation of *S. enteritidis* wild type and mutants from spleen and liver, 24 and 48 hours after oral inoculation with 10^5 *Salmonella*.

Spleen

	24 hours post inoculation					48 hours post inoculation				
	LA5	**B214**	**EAV42**	**EAV21**	**EAV40**	**LA5**	**B214**	**EAV42**	**EAV21**	**EAV40**
*1-5x10^2	13	13	5	4	3	14	4	5	2	17
5x10^2-5x10^3	2	3	3	1	-	5	14	7	11	2
>5x10^3	-	-	-	-	-	-	-	1	-	-
No. chicks colonised	15/18	16/18	8/14	5/14	3/20	19/20	18/18	13/14	13/14	19/20

Liver

	24 hours post inoculation					48 hours post inoculation				
	LA5	**B214**	**EAV42**	**EAV21**	**EAV40**	**LA5**	**B214**	**EAV42**	**EAV21**	**EAV40**
*1-5x10^2	8	7	3	5	7	3	1	1	-	9
5x10^2-5x10^3	7	11	11	7	2	15	12	7	8	11
>5x10^3	2	-	-	-	-	2	5	7	5	-
No. chicks colonised	18/20	18/18	14/14	12/14	9/20	20/20	18/18	15/15	13/14	20/20

* Isolated from enrichment broth

Results expressed as the distribution of CFU per whole spleen or per gram liver and the number of chicks colonised. Two individual experiments gave similar results and therefore, the results represent a cumulative total of the two experiments.

Table 6.2 Shedding and persistence of *S. enteritidis* LA5 in the caecum of day-old chicks following oral inoculation with 10^5 *Salmonella*.

Group	Chick no.	Days post inoculation 7	14	cfu'S /g caecum	21	28	cfu'S /g caecum	35	42	cfu'S /g caecum
A	1	+++	+++	$2x10^7$						
	2	+++	++	$7.1x10^6$						
	3	+++	++	$1.2x10^8$						
	4	++	++	$2.1x10^7$						
	5	++	++	$7.8x10^7$						
	6	+++	+++	$1.2x10^8$						
	7	++	++	$1.0x10^7$						
	8	+++	++	$1.1x10^8$						
	9	+++	+++	$1.9x10^7$						
	10	+++	+++	$1.6x10^7$						
B	1	++	++		++	++	$4.4x10^6$			
	2	+++	+++		+++	+++	$7.5x10^6$			
	3	+++	+++		++	++	$1.0x10^7$			
	4	+++	+		++	++	$1.0x10^7$			
	5	+++	++		++	++	$1.0x10^7$			
	6	+++	++		++	++	$7.2x10^6$			
	7	+++	+++		+++	+	$7.0x10^6$			
	8	+++	+++		+++	++	$4.2 x10^6$			
	9	+++	+++		+++	++	$9.4x10^6$			
	10	+++	+++		+++	+	$1.6x10^7$			
C	1	+++	+++		++	++		++	+	$1.4x10^5$
	2	+++	++		++	+		+	+	$1.2x10^5$
	3	++	++		+++	++		++	+	$<10^2$
	4	+++	++		+++	++		++	+	$7.2x10^5$
	5	+++	++		++	++		+	+	$3.4x10^5$
	6	+++	++		+++	++		++	++	$8.2x10^5$
	7	+++	++		++	++		++	++	$3.1x10^4$
	8	+++	+++		+++	++		++	+	$4.0x10^5$
Mean				**$5.2x10^7$**			**$8.6x10^6$**			**$3.2x10^5$**
SE				**4.6**			**3.2**			**2.9**

+++ Heavy shedding
++ Moderate shedding
+ Light shedding

Chicks were cloacal swabbed weekly and swabs plated onto BGA agar for the detection of *Salmonella*. Birds were killed at **A)** 14 days, **B)** 28 days and **C)** 42 day PI and the number of *Salmonella* determined per gram of caecum.

Table 6.3 Shedding and persistence of *S. enteritidis* EAV40 (14^-, 17^-, 21^-, fla^-) in the caecum of day-old chicks following oral inoculation with 10^5 *Salmonella*.

Group	Chick no.	Days post inoculation 7	14	cfu'S /g caecum	21	28	cfu'S /g caecum	35	42	cfu'S /g caecum
A	1	+++	++	$7.3x10^8$						
	2	++	++	$1.9x10^7$						
	3	+++	++	$1.8x10^8$						
	4	+++	++	$1.5x10^8$						
	5	+++	++	$2.4x10^8$						
	6	+++	++	$2.2x10^7$						
	7	++	++	$2.3x10^8$						
	8	+++	++	$8.3x10^7$						
	9	+++	++	$1.5x10^8$						
	10	++	+++	$1.4x10^8$						
B	1	+++	+		++	++	$1.0x10^7$			
	2	+++	++		+	+	$1.3x10^7$			
	3	++	++		+	++	$3.9x10^6$			
	4	+++	++		++	++	$7.6x10^5$			
	5	+++	++		++	++	$3.6x10^6$			
	6	++	++		++	++	$3.3x10^6$			
	7	+++	+++		++	++	$1.7x10^7$			
	8	++	++		++	++	$2.3x10^7$			
	9	+++	++		+	+	$7.5x10^7$			
	10	+++	+++		++	++	$3.8x10^6$			
C	1	++	+++		++	++		++	+	$6.7x10^5$
	2	+++	++		+++	+		+	+	$5.8x10^5$
	3	+++	+++		++	++		++	+	$1.8x10^6$
	4	+++	++		++	++		+	+	$5.2x10^4$
	5	+++	++		++	++		+	+	$1.1x10^4$
	6	+++	++		++	++		+	+	$8.5x10^5$
	7	+++	++		++	++		+	+	$1.6x10^6$
	8	+++	++		+++	++		++	+	$<x10^2$
Mean				**$1.9x10^8$**			**$1.5x10^7$**			**$6.2x10^5$**
SE				**1.9**			**2.1**			**6.5**

+++ Heavy shedding
++ Moderate shedding
+ Light shedding

Chicks were cloacal swabbed weekly and swabs plated onto BGA agar for the detection of *Salmonella*. Birds were killed at **A)** 14 days, **B)** 28 days and **C)** 42 day PI and the number of *Salmonella* determined per gram of caecum.

Table 6.4 Shedding and persistence of *S. enteritidis* LA5 in the caecum of seven day old chicks following oral inoculation with 10^5 *Salmonella*.

Group	Chick no.	Days post inoculation 7	14	cfu'S /g caecum	21	28	cfu'S /g caecum	35	42	cfu'S /g caecum
A	1	+++	+++	$1.7x10^7$						
	2	++	++	$1.3x10^7$						
	3	+++	+++	$2.0x10^7$						
	4	+++	+++	$9.2x10^6$						
	5	+++	++	$7.5x10^7$						
	6	+++	+++	$8.4x10^7$						
	7	+++	++	$7.7x10^7$						
	8	+++	+	$1.6x10^7$						
	9	+++	+++	$8.9x10^7$						
	10	+++	+++	$1.8x10^7$						
B	1	+++	+++		++	+++	$6.9x10^7$			
	2	+++	+++		+++	++	$2.7x10^7$			
	3	++	+++		++	+++	$1.3x10^7$			
	4	+++	+++		+++	++	$5.9 x10^7$			
	5	+++	+++		+++	++	$1.6x10^7$			
	6	+++	+++		+++	++	$2.4x10^7$			
	7	+++	+++		+++	++	$6.2x10^7$			
	8	++	++		+++	++	$3.0 x10^7$			
	9	+++	+++		++	+	$5.0x10^7$			
	10	+++	+++		++	++	$1.6x10^8$			
C	1	++	++		+++	++		+	+	$3.8x10^7$
	2	+++	+++		+++	++		+	+	$3.1x10^7$
	3	+++	+++		++	++		+	+	$2.5x10^7$
	4	+++	+++		++	++		+	-	$2.2x10^7$
	5	+++	+++		+++	++		+	-	$2.1x10^7$
	6	+++	+++		+++	++		+	-	$4.2x10^6$
	7	+++	+++		++	+++		+	+	$1.8x10^7$
	8	+++	+++		+++	++		+	+	$3.0 x10^6$
	9	+++	+++		++	++		+	+	$2.1x10^7$
	10	++	+++		+++	++		+	+	$1.2x10^7$
Mean				**$5.0x10^7$**			**$5.08x10^7$**			**$1.95x10^7$**
SE				**3.55**			**4.3**			**1.09**

+++ Heavy shedding
++ Moderate shedding
+ Light shedding
- No shedding

Chicks were cloacal swabbed weekly and swabs plated onto BGA agar for the detection of *Salmonella*. Birds were killed at **A)** 14 days, **B)** 28 days and **C)** 42 days PI and the number of *Salmonella* determined per gram of caecum.

Table 6.5 Shedding and persistence of *S. enteritidis* EAV40 (14^-, 17^-, 21^-, fla^-) in the caecum of seven day old chicks following oral inoculation with 10^5 *Salmonella*.

Group	Chick no.	Days post inoculation 7	14	cfu'S /g caecum	21	28	cfu'S /g caecum	35	42	cfu'S /g caecum
A	1	+++	+++	$6.6x10^7$						
	2	+++	++	$8.1x10^7$						
	3	+++	+++	$7.1x10^7$						
	4	++	+++	$8.2x10^7$						
	5	+++	++	$9.6x10^7$						
	6	++	+++	$5.7x10^8$						
	7	+++	+++	$6.9x10^7$						
	8	++	+	$5.5x10^7$						
	9	+++	++	$1.0x10^8$						
	10	++	+++	$8.7x10^7$						
B	1	+++	++		+++	++	$4.2x10^5$			
	2	+++	+++		+++	+	$8.3x10^6$			
	3	++	++		++	+	$8.9x10^6$			
	4	++	++		++	++	$1.2x10^6$			
	5	+++	++		+++	++	$1.7x10^6$			
	6	++	++		+++	+	$1.4x10^6$			
	7	+++	+++		+++	++	$1.8x10^6$			
	8	+++	+++		+++	++	$2.3x10^7$			
	9	++	++		++	+	$5.0x10^7$			
	10	++	+++		++	++	$4.9x10^6$			
C	1	+++	+++		++	++		++	+	$6.6x10^6$
	2	+++	++		+++	++		+	+	$3.3x10^6$
	3	+++	+++		+++	++		+	+	$2.2x10^6$
	4	++	++		++	+		+	+	$2.2x10^6$
	5	+++	+++		++	++		++	+	$2.5x10^6$
	6	+++	+++		+++	++		++	++	$4.9x10^6$
	7	+++	++		+++	+		+	+	$4.1 x10^6$
	8	++	+++		++	++		+	+	$3.2 x10^6$
	9	+++	+++		+++	+		+	+	$3.4x10^6$
	10	+++	+++		++	+		+	+	$3.7x10^6$
Mean				**$1.3x10^8$**			**$1.02x10^7$**			**$3.6x10^6$**
SE				**1.56**			**1.55**			**1.35**

+++ Heavy shedding
++ Moderate shedding
+ Light shedding
- No shedding

Chicks were cloacal swabbed weekly and swabs plated onto BGA agar for the detection of *Salmonella*. Birds were killed at **A)** 14 days, **B)** 28 days and **C)** 42 day PI and the number of *Salmonella* determined per gram of caecum.

Table 6.6 Isolation of *S. enteritidis* strains LA5 and EAV40 (14^-, 17^-, 21^-, fla^-) from spleen, liver and caecum for up to six weeks following oral inoculation of A) day-old chicks, and B) seven day old chicks.

A)

Days PI	LA5			EAV40		
	Spleen	Liver	Caecum	Spleen	Liver	Caecum
14	7/10	7/10	$5.2x10^7 \pm 4.6$	10/10	10/10	$1.9x10^8 \pm 1.9$
28	3/10	3/10	$8.6x10^6 \pm 3.2$	3/10	0/10	$1.5x10^7 \pm 2.1$
42	1/8	3/8	$3.2x10^5 \pm 2.9$	0/8	2/8	$6.9x10^5 \pm 2.0$
Total	11/28	17/28	28/28	13/28	12/28	28/28

B)

Days PI	LA5			EAV40		
	Spleen	Liver	Caecum	Spleen	Liver	Caecum
14	9/10	5/10	$5.0x10^7 \pm 3.5$	9/10	7/10	$1.3x10^8 \pm 1.6$
28	2/10	3/10	$5.1x10^7 \pm 4.3$	6/10	2/10	$1.0x10^7 \pm 1.5$
42	4/10	2/10	$1.9x10^7 \pm 1.1$	1/10	5/10	$3.6x10^6 \pm 1.3$
Total	15/30	10/30	30/30	16/30	17/30	30/30

Results expressed as number of chicks colonised from spleen and liver and mean CFU per gram caecum ± SE.

Table 6.7 Colonisation of chick spleen, liver and caecum by lateral transfer by *S. enteritidis* strains LA5 and mutant EAV40 (14⁻, 17⁻, 21⁻, fla⁻).

	Experiment					
	LA5			EAV40		
	I	II	III	I	II	III
Spleen						
*1-5x10^2	-	-	-	-	-	
5x10^2-5x10^3	5	7	6	3	4	1
>5x10^3	-	3	4	2	6	9
No. birds colonised	5/5	10/10	10/10	5/5	10/10	10/10
Liver						
*1-5x10^2	2	-	8	1	3	8
5x10^2-5x10^3	2	10	1	4	7	1
>5x10^3	1	-	-	-	-	
No. birds colonised	5/5	10/10	9/10	5/5	10/10	9/10
Caecum						
10^5-10^6	-	-	-	-	-	2
10^6-10^7	-	-	8	-	2	5
10^7-10^8	-	-	2	4	5	3
10^8-10^9	5	10		1	3	-
Mean	2.6x10^8	2.7x10^8	8.7x10^6	6.4x10^7	7.0x10^7	1.3x10^7
SE	0.63	0.69	8.3	8.8	6.2	1.5

* isolated by enrichment

Group **I)** seven day old chicks orally inoculated with 10^5 *Salmonella*, **II)** seven day old sentinel birds housed with infected birds, and **III)** 28 day old birds introduced into infected isolators after being left for 14 days uninhabited. All birds were removed for *post mortem* after seven days. Results expressed as CFU per gram of organ homogenate. The experiment was performed once only.

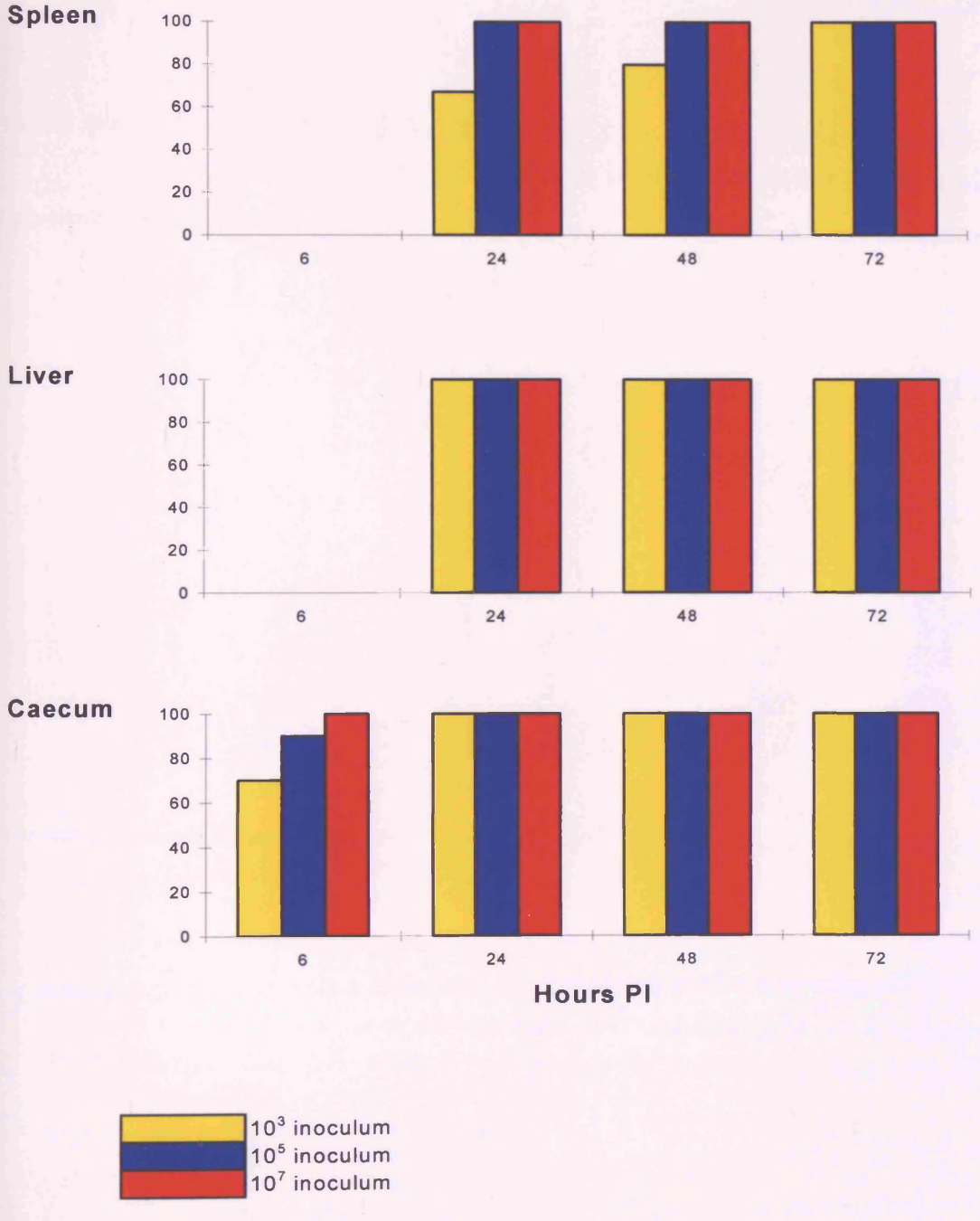
Table 6.8 Numbers of *S. enteritidis* isolated from spleen, liver and caecum following oral dosing of seven day old chicks with a competitive inoculum of I) LA5 vs B214 (14^-), II) LA5 vs EAV42 (14^-, 17^-), III) LA5 vs EAV21 (14^-, 17^-, 21^-) and IV) LA5 v EAV40 (14^-, 17^-, 21^-, fla^-).

	7 days PI					14 days PI				
Spleen		Group					Group			
	Chicks	I	II	III	IV	Chicks	I	II	III	IV
	1	0/1	4/10	10/10	10/10	1	-	1/2	1/2	1/6
	2	1/1	3/3	0/3	0/2	2	7/8	1/1	-	0/2
	3	1/3	2/2	1/1	1/1	3	0/1	1/2	-	0/4
	4	1/3	1/1	4/7	1/5	4	0/2	-	-	5/9
	5	2/4	3/6	0/3	0/7	5	-	-	0/1	9/9
						6		-	1/1	1/9
	% Kan^+	42	59	62	48	% Kan^+	63	60	50	41
Liver	1	0/10	6/10	5/6	5/10	1	-	0/2	-	-
	2	-	3/4	4/10	0/1	2	-	-	-	-
	3	-	-	6/9	-	3	-	-	0/1	-
	4	-	-	5/7	-	4	-	-	-	-
	5	-	3/8	0/1	-	5	-	-	-	2/3
						6	-	-	-	0/1
	% Kan^+	0	54	61	50	% Kan^+	-	0	0	50
Caecum	1	4/12	4/8	17/19	6/6	1	0/9	6/9	5/9	0/9
	2	-	7/9	-	5/14	2	8/9	2/7	1/9	0/9
	3	0/4	-	2/7	9/12	3	2/8	1/7	4/9	3/9
	4	0/7	-	-	-	4	3/9	3/6	2/9	3/8
	5	5/10	3/6	0/6	4/7	5	-	3/6	0/9	9/9
						6	-	0/3	3/9	2/9
	% Kan^+	27	61	58	61	% Kan^+	37	39	28	32

All organs were contaminated with *Klebsiella spp.*
- = no colonies

Bacterial enumeration were performed on days 1, 7, and 14 PI, although enumeration of *Salmonella* from organs was unsuccessful on day 1 due to heavy colonisation of chicks by *Klebsiella spp.* which masked *Salmonella* even on antibiotic selection agar plates. Results expressed as the overall percentage of kanamicin resistant *Salmonella* (mutants) from up to six chicks. The experiment was performed once only.

Figure 6.1 Colonisation and invasion of day-old chicks by strain LA5 following oral inoculation with 10^3, 10^5 and 10^7 wild type *Salmonella*.



Bacteria were isolated from spleen, liver and caecum at 6, 24, 48 and 72 hours PI. Results expressed as percentage of chicks colonised. Experiment was performed once only.

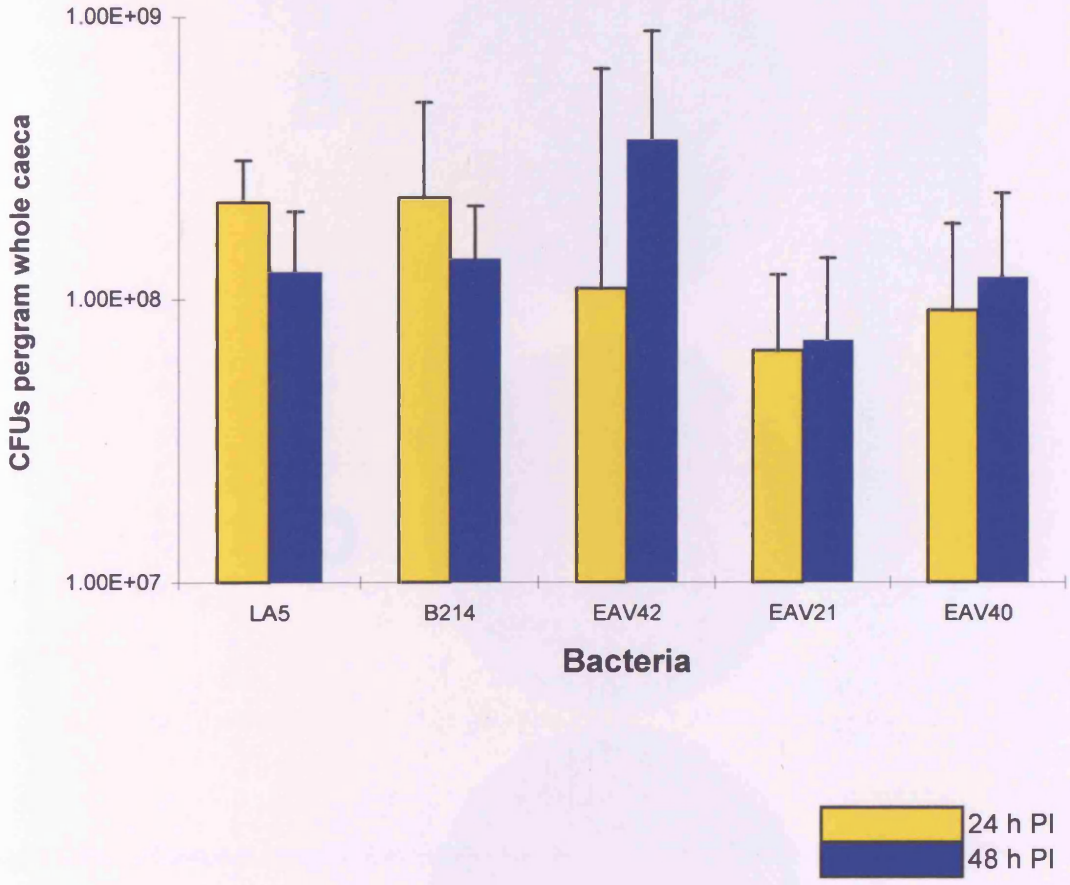
Figure 6.2b T-tests for the isolation of *S. enteritidis* wild type and mutants from caeca 24 and 48 hours after oral inoculation with 10^5 *Salmonella.*

		Caeca	
		24 hours	48 hours
LA5 v B214 (14⁻)	a	0.280	0.685
	b	1.000	1.000
B214 v EAV42 (14⁻, 17⁻)	a	0.104	0.361
	b	1.000	1.000
EAV42 v EAV21 (14⁻, 17⁻, 21⁻)	a	<0.001	<0.001
	b	1.000	1.000
EAV21 v EAV40 (14⁻, 17⁻, 21⁻, fla⁻)	a	0.370	0.029
	b	1.000	1.000

a Counts
b Chicks colonised

For statistical analyses of association with and invasion of epithelial cells, one-way analyses of variance was performed followed by t-tests. Results expressed as "p" values.

Figure 6.2a Colonisation of caecum of day-old chicks by *S. enteritidis* after 24 and 48 hours following inoculation of 10^5 wild type or mutants.



Results expressed as CFU per gram of whole caecum. Two individual experiments gave similar results and therefore, the results represent a cumulative total of the two experiments.

Figure 6.3 Determination of a) heavy, b) moderate and c) light shedding of *Salmonella* by cloacal swabbing of birds infected with *S. enteritidis* strain LA5 or EAV40 (14^-, 17^-, 21^-, fla^-) and streaking onto Brilliant Green agar.

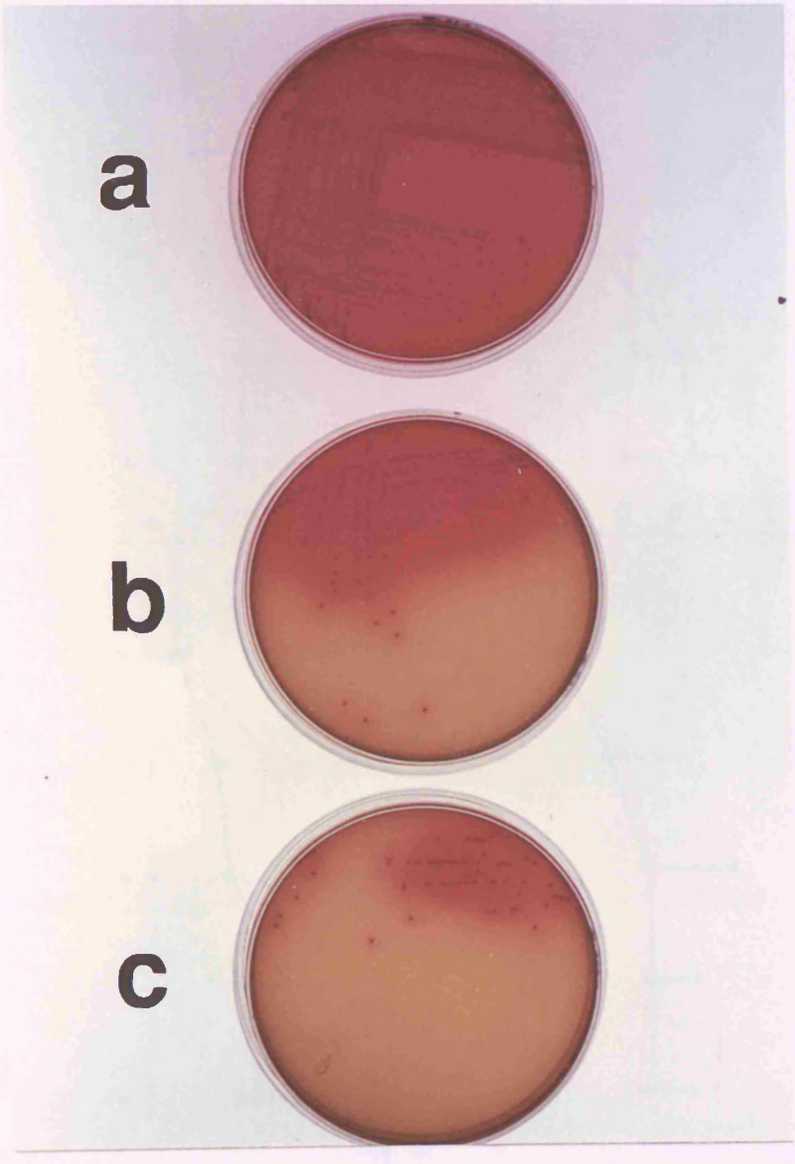
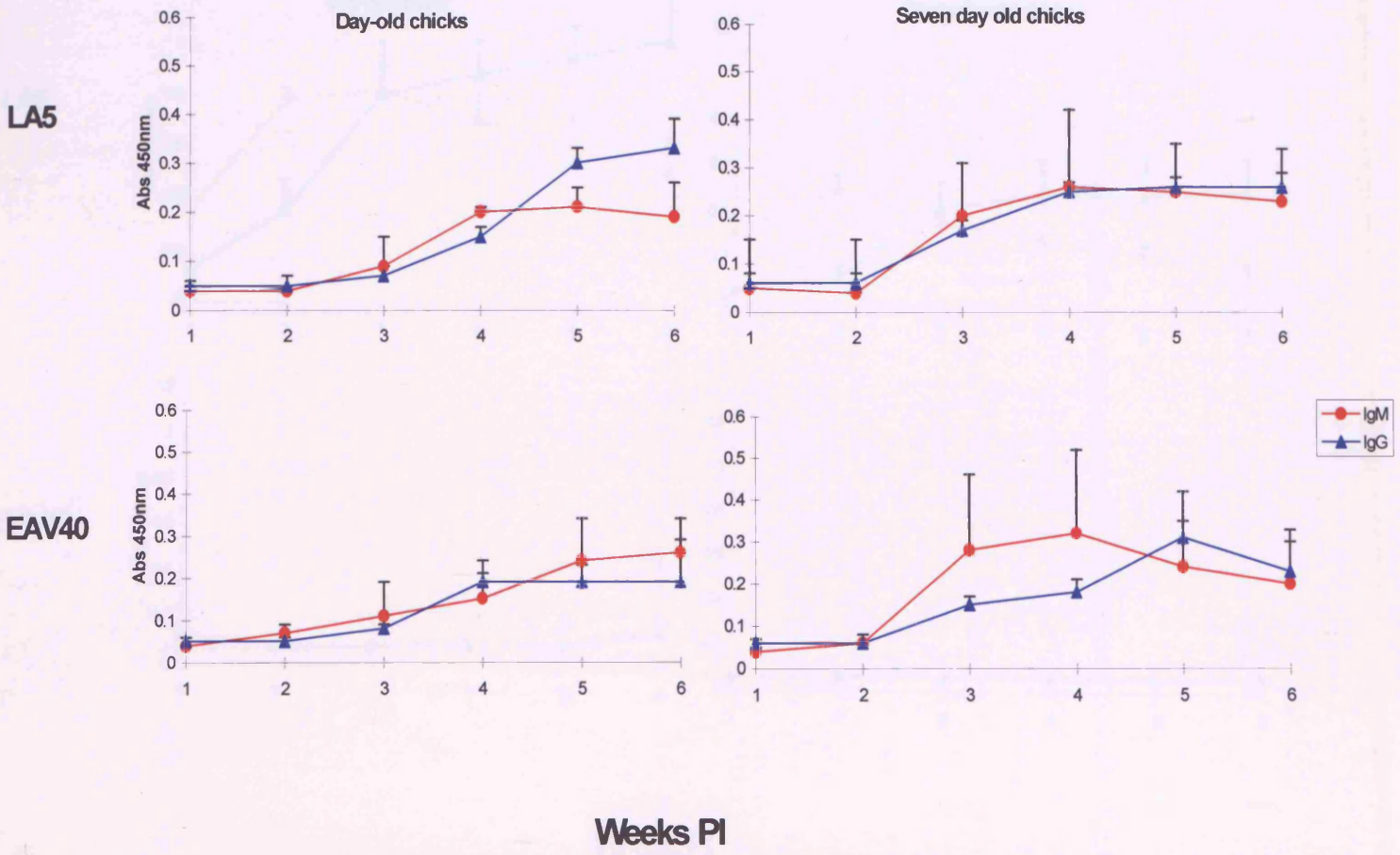
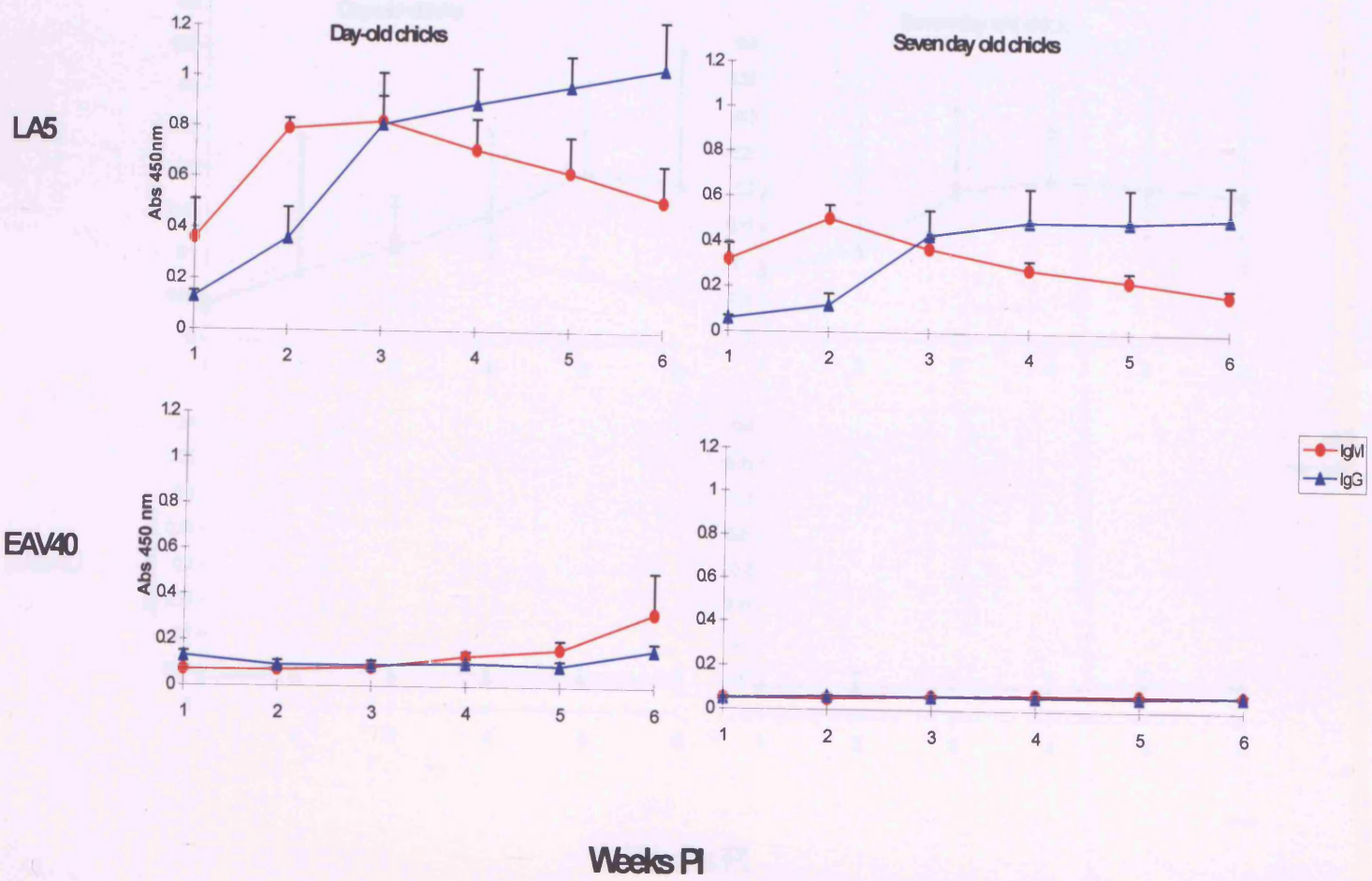


Figure 6.4 Serological response to LPS.



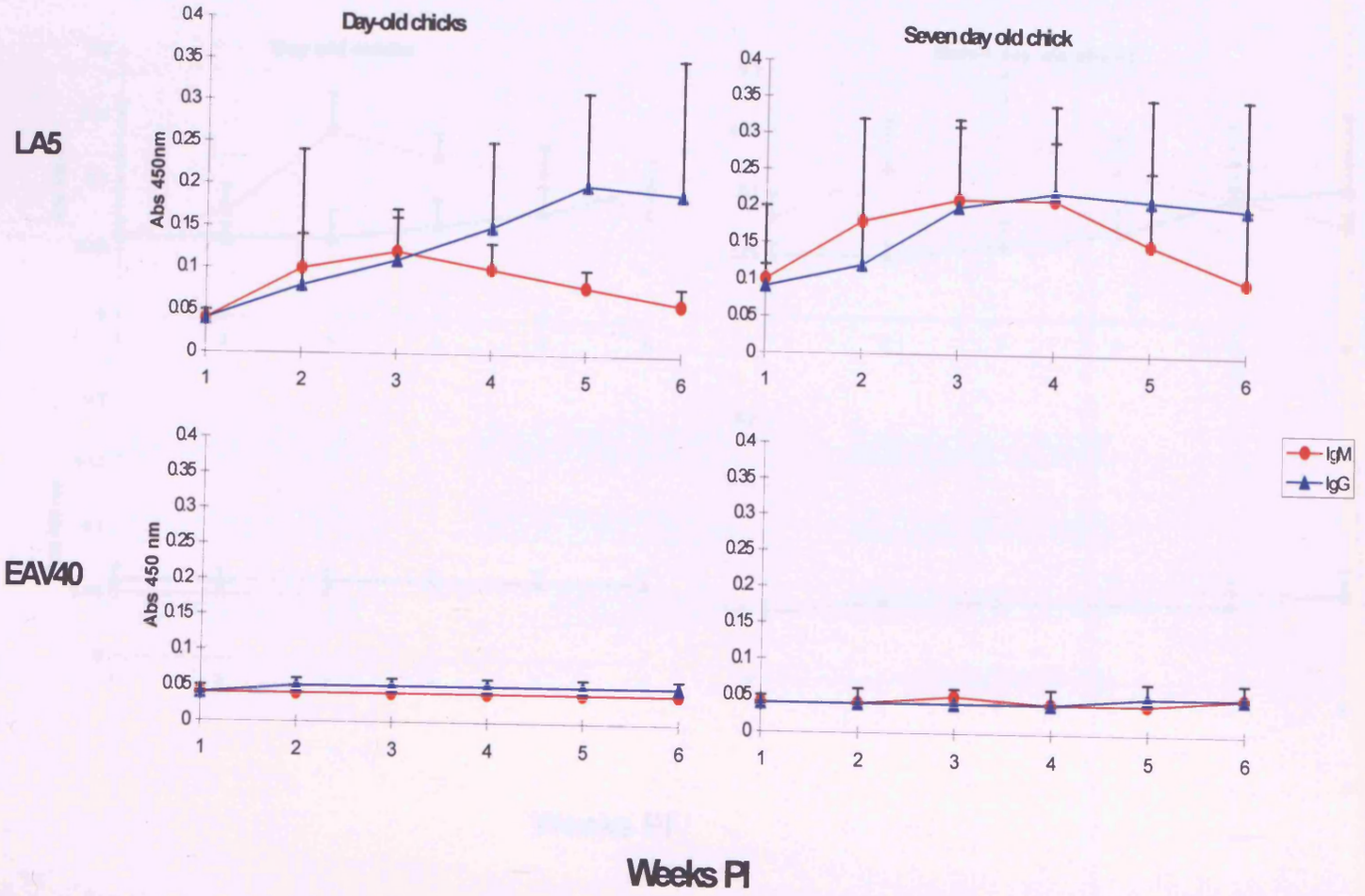
One or seven day old chicks were orally inoculated with 10^5 wild type *S. enteritidis* strain LA5 or EAV40 (14^-, 17^-, 21^-, fla^-). Chicks were bled weekly and the IgM and IgG response monitored by ELISA. Results expressed as mean absorbance of sera from ten chicks ± SE performed on two occasions.

Figure 6.5 Serological response to flagella.



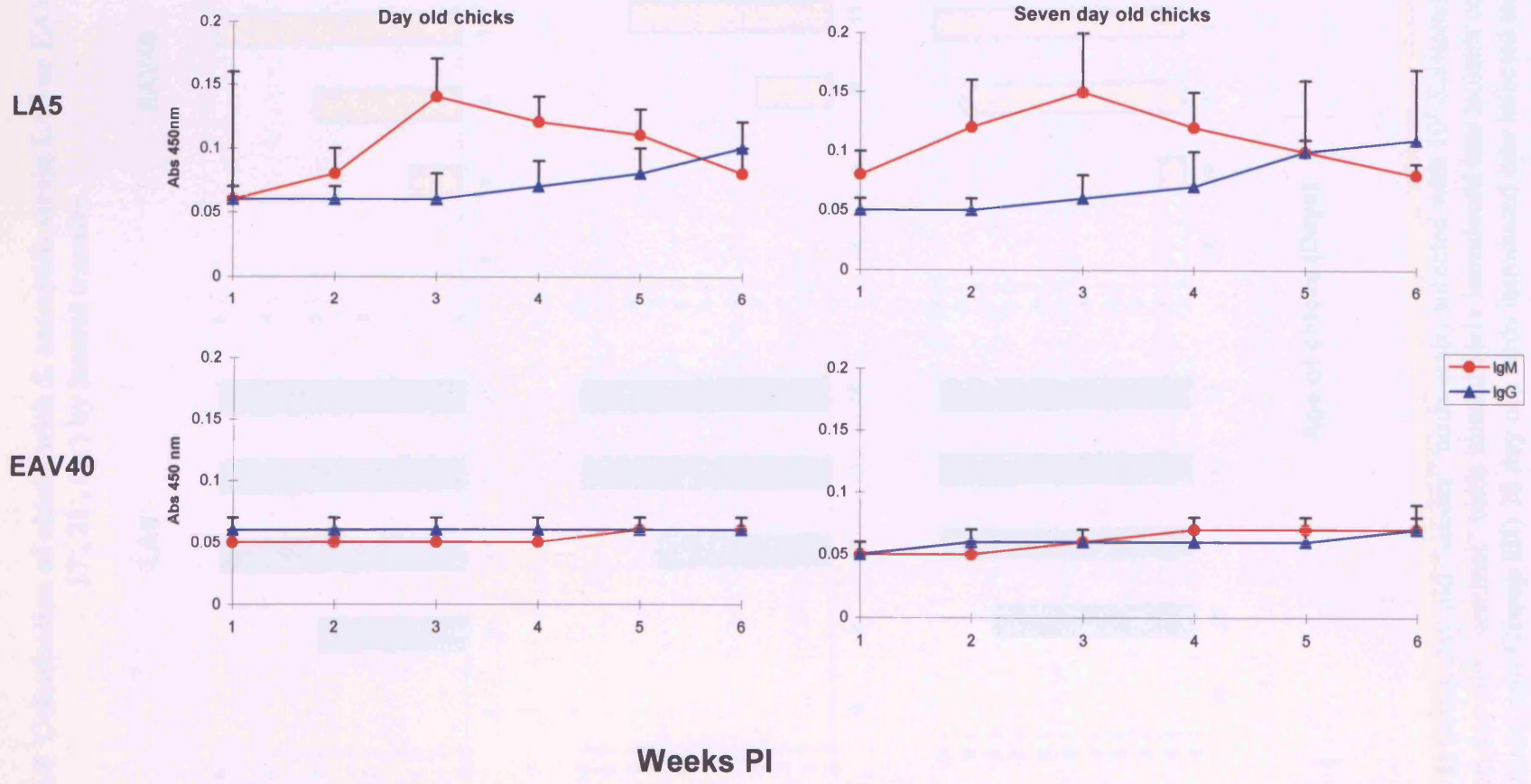
One or seven day old chicks were orally inoculated with 10^5 wild type *S. enteritidis* strain LA5 or EAV40 (14^-, 17^-, 21^-, fla^-). Chicks were bled weekly and the IgM and IgG response monitored by ELISA. Results expressed as mean absorbance of ten chicks ± standard error performed on two occasions.

Figure 6.6 Serological response to SEF14.



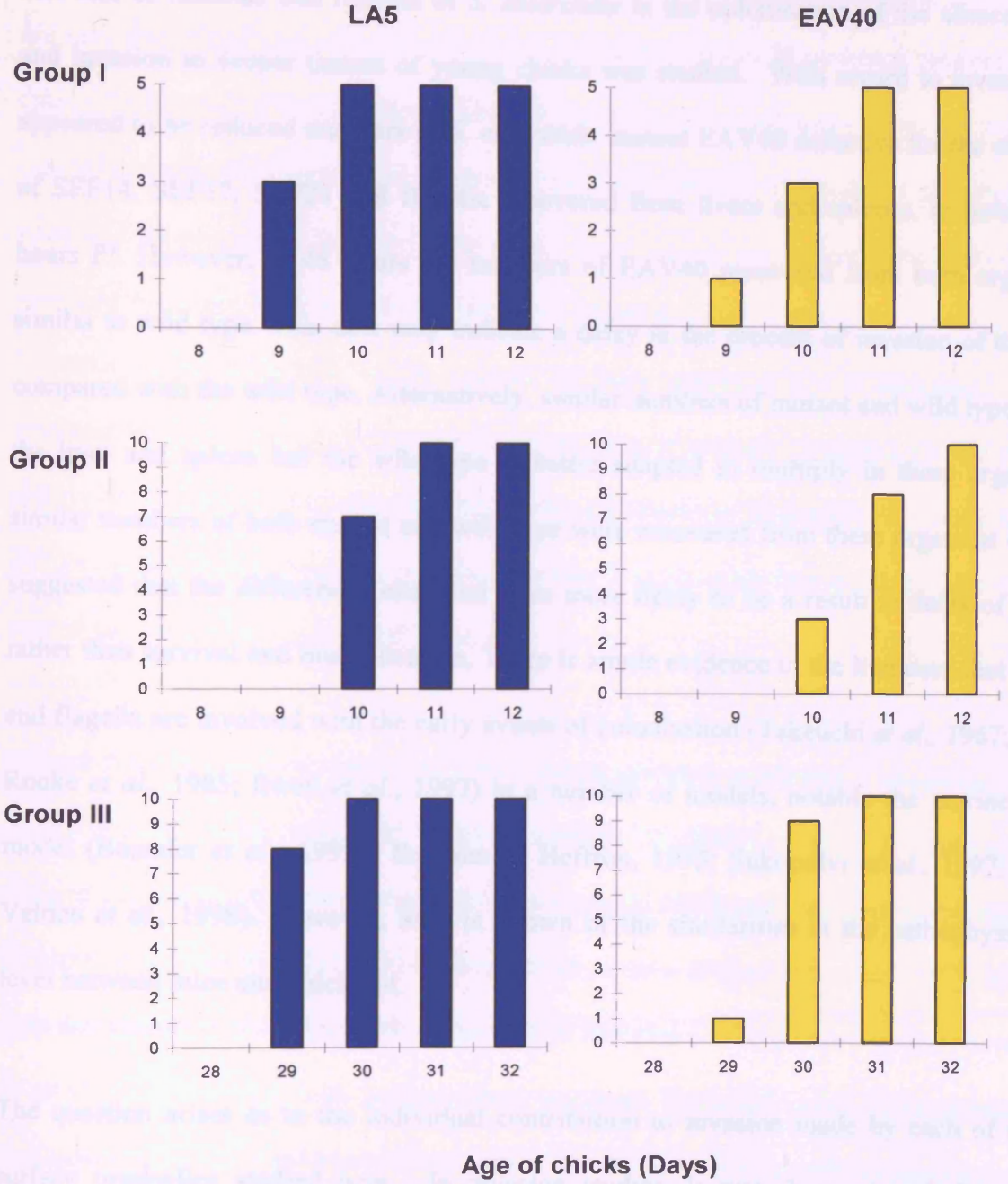
One or seven day old chicks were orally inoculated with 10^5 wild type *S. enteritidis* strain LA5 or EAV40 (14^-, 17^-, 21^-, fla^-). Chicks were bled weekly and the IgM and IgG response monitored by ELISA. Results expressed as mean absorbance of ten chicks ± SE performed on two occasions.

Figure 6.7 Serological response to SEF21.



One or seven day old chicks were orally inoculated with 10^5 wild type *S. enteritidis* strain LA5 or EAV40 (14^-, 17^-, 21^-, fla^-). Chicks were bled weekly and the IgM and IgG response monitored by ELISA. Results expressed as mean absorbance of ten chicks ± SE performed on two occasions.

Figure 6.8 Colonisation of chicks with *S. enteritidis* strain LA5 or EAV40 (14^-, 17^-, 21^-, fla^-) by lateral transfer.



Group I) Seven day old "seeder" birds orally infected with 10^5 *Salmonella*; **Group II)** seven day old "sentinel" birds immediately introduced into isolator containing seeder birds, and **Group III)** 28 day old birds introduced into infected isolator which had been uninhabited for 14 days. Results expressed as number of birds with *Salmonella* isolated from cloacal swabs. The experiment was performed once only.

6.3 Discussion

The role of fimbriae and flagella of *S. enteritidis* in the colonisation of the alimentary tract and invasion to deeper tissues of young chicks was studied. With regard to invasion, there appeared to be reduced numbers of *S. enteritidis* mutant EAV40 defective for the elaboration of SEF14, SEF17, SEF21 and flagella recovered from livers and spleens, in particular, 24 hours PI. However, at 48 hours the numbers of EAV40 recovered from both organs were similar to wild type. This data may indicate a delay in the process of invasion of the mutant compared with the wild type. Alternatively, similar numbers of mutant and wild type arrive at the liver and spleen but the wild type is better adapted to multiply in these organs. That similar numbers of both mutant and wild type were recovered from these organs at 48 hours, suggested that the differences observed were more likely to be a result in delay of invasion rather than survival and multiplication. There is ample evidence in the literature that fimbriae and flagella are involved with the early events of colonisation (Takeuchi *et al.,* 1967; Parry & Rooke *et al.*, 1985; Ewen *et al.*, 1997) in a number of models, notably the murine typhoid model (Baumler *et al.*, 1995a; Baumler & Heffron, 1995; Sukopolvi *et al.*, 1997; van der Velden *et al.*, 1998). However, little is known of the similarities at the pathophysiological level between mice and chick gut.

The question arises as to the individual contribution to invasion made by each of the four surface organelles studied here. In invasion studies, it was demonstrated that mutants defective for the elaboration of more classes of surface organelle were recovered from the spleen in fewer numbers at 24 hours PI and that the order of recovery was LA5 (wild type) = B214 (14^-) > EAV42 (14^-, 17^-) > EAV21 (14^-, 17^-, 21^-) > EAV40 (14^-, 17^-, 21^-, Fla^-) and these findings were statistically significant. A less pronounced trend was observed for recovery of

mutants from the liver in which only EAV40 was recovered in significantly lower numbers than the other strains. These data are compatible with the concept that fimbriae and flagellae are involved, but not essential, for invasion. The differences in recovery between liver and spleen for the mutants may suggest either different invasion pathways were adopted by the *S. enteritidis* derivatives dependant upon their available surface organelles or that the liver was colonised more rapidly than the spleen and there were no substantive differences for the routes of invasion. That the spleens were colonised in lower numbers in the early stages of infection by the wild type would support the later argument and is consistent with data obtained from inoculating chicks with lower numbers of bacteria. Interestingly, Allen-Vercoe *et al.*, (1999b) reported that the invasion of day-old chicks by *S. enteritidis* given orally at 10^3 CFU's was assisted by flagella, but not fimbriae. It is plausible that the size of inoculum was critical to observe such subtle differences.

Related fimbriae of *S. typhimurium* have been implicated as virulence determinants in the mouse model. The structural fimbrin subunit of SEF17, AgfA, is genetically and antigenically related to *E. coli* "curli" (Collinson *et al.,* 1992) and thin aggregative fimbriae of *S. typhimurium* (Stolpe *et al.*, 1994). Expression *in vitro* (Sukopolvi *et al.*, 1997) and *in vivo* (van der Velden *et al.*, 1998) of thin aggregative fimbriae of *S. typhimurium* have been shown to promote interaction with mouse small intestinal epithelial cells. Also studies have demonstrated that *S. typhimurium* expressing the mannose sensitive haemagglutinin, type 1 fimbriae which is an orthologue of SEF21, colonise the intestine of mice more efficiently than non-fimbriated bacteria (Leunk *et al.*, 1982; Tanaka et al., 1978; Lockman & Curtiss, 1992). Additionally, Lee *et al.,* (1996) reported impaired colonisation and invasion of young chicks with flagella and type 1 double mutants of *S. typhimurium* whereas loss of motility alone attenuated peroral virulence. *In vitro* results as part of this study have demonstrated the

potential roles of SEF17 and SEF21 and flagella mediated motility in association and invasion of cultured human epithelial cells. Collectively, these data are in agreement with *in vivo* results of this study albeit in a range of virulence models.

There were no significant differences between wild type and B214 (14^-) in colonisation studies in the chick and this is consistent with previous reports (Thorns *et al.*, 1996b), although a role for this surface appendage in transovarian transmission has been suggested by Thiagarajan *et al.* (1996a).

In persistence studies of chicks dosed at one and seven days of age with LA5 and EAV40 (14^-, 17^-, 21^-, fla^-), birds were still excreting *S. enteritidis* six weeks PI, although the degree of shedding from both strains reduced with time. Either a competitive normal flora or an effective host mucosal immune response may have contributed to this apparent exclusion. Interestingly, by 42 days PI *S. enteritidis* was not isolated by cloacal swabbing in some chicks although large numbers resided in the caecum. These results suggested that shedding of *Salmonella* was intermittent and was not a consistent test for the presence of *Salmonella* in the alimentary tract of individual birds.

Serological data indicated that chicks inoculated with LA5 and EAV40 produced a primary humoral response to LPS within three weeks PI and, although the infection persisted, a poor IgG response was detected. All chicks infected with LA5 produced a strong immune response to SEF14 and flagella, however, a weak IgM response was observed to flagella at six weeks PI from day-old chicks inoculated with EAV40 (14^-, 17^-, 21^-, fla^-). Concurrently, an unidentified *Klebsiella spp.* was isolated from caeca and internal organs and therefore, it was likely that the antibodies detected in the serological ELISA were those evoked by *Klebsiella*

which cross-reacted with the *Salmonella* flagella antigen in ELISA. This was confirmed by ELISA, described in chapter 3, using specific chicken antisera raised to flagella (ChFla). Interestingly, less than one third of chicks infected with LA5 produced an IgG response to SEF21, although 80-90% produced an IgM response. Previous studies indicated that mannose sensitive haemagglutination mediated by type 1 fimbriae was not frequently associated with avian isolates of *Salmonella* (Nolan *et al.*, 1991), although Sojka *et al.*, (1996) reported expression in strains derived from chickens. The serological data suggested that a primary immune response was induced which supports the concept that SEF21 was expressed during infection. Interestingly, Lee *et al.* (1996) suggested that fimbriated bacteria were cleared rapidly from blood and this may, in part, explain the lack of a IgG immune response to SEF21 in chickens. Antibodies to SEF17 were not detected by ELISA or Western blot. This study has already demonstrated that purified SEF17 was a poorly immunogenic molecule in mice, chickens and rabbits and large doses of antigen were necessary to induce an immune response. The poor immunogenicity of SEF17 may have been partially attributed to the harsh purification protocol used in this study. The purified SEF17 was also the antigen used for detecting seroconversion. Therefore, it was likely that seroconversion was an inappropriate method for determining expression of SEF17 by *S. enteritidis in vivo*.

Challenge studies using a single bolus inoculum of an estimated number of virulent bacteria administered orally to achieve infection does not mimic a natural infection. It is likely that natural field *Salmonella* infections result from the presence of bacteria in the immediate environment (Muir *et al.*, 1998). The use of seeder birds for the lateral transmission of infectious pathogens in chickens have been utilised previously (Migaki *et al.*, 1981; Bolder *et al.*, 1992). The lateral transfer results indicated that the loss of flagella and fimbriae investigated in this study may have delayed colonisation of chick intestinal tract by *S.*

enteritidis contracted from the immediate environment. Enumeration of bacteria from liver and spleen of birds housed in contaminated isolators for seven days suggested that the loss of surface appendages had little effect on invasion to deeper organs.

Interestingly, in the competition studies the number of wild type and mutant strains isolated from organs varied between birds. For example, all nine colonies isolated from the caecum of a single chick 14 days PI were EAV40 (14^-, 17^-, 21^-, fla^-), whereas, all nine isolates from a different chick dosed with the same inoculum were wild type. In individual chicks, there was often a correlation between the strains recovered from the caecum and those isolated from internal organs which supported the concept that which-ever strain established itself in the alimentary tract may have excluded the other, although, in some chicks both wild type and mutant were isolated from caeca and internal organs. It is plausible that the heavy contamination of *Klebsiella* may have influenced this effect, and indeed it reduced the number of *Salmonella* isolates detected by masking growth on BGA. That both wild type or mutant strains could exclude one another suggested that there was very little advantage of fimbriae or flagella in colonisation or invasion of young chicks and these results support the overall findings from persistence and lateral transfer studies.

Overall, the *in vivo* experiments suggested that collectively, SEF17, SEF21 and flagella fulfil a minor role in the early stages of colonisation and invasion in young chicks but are unnecessary for short term persistence or colonisation of birds from the immediate environment.

Chapter 7

Discussion

The overall aims of this study were to investigate the contribution of fimbriae and flagella to the pathogenesis of *S. enteritidis* in poultry. However, in the earlier stages of this investigation particular emphasis was placed upon studies relating to SEF17 for this was newly described for *S. enteritidis* and complemented previous studies by this laboratory on SEF14 (Thorns *et al.*, 1990; 1992) and SEF21 (Sojka *et al.*, 1996; 1998). Later results showed that SEF17, SEF21 and flagella, but not SEF14, mediated interaction with tissue culture cells and studies with isogenic mutants defective for the elaboration of these surface appendages implied a minor role in the early stages of colonisation and invasion of day-old chicks, but were unnecessary for the colonisation of birds from the immediate environment. The results were discussed in each chapter and therefore the final discussion will evaluate the overall findings and propose further work.

Micro-organisms including *Salmonella* encounter a variety of environments and therefore, have evolved sophisticated means of adaptation. For example, the expression of bacterial genes required for interaction with mammalian cells is regulated by growth phase (Tartera & Metcalf, 1992) and a variety of stimuli, including osmolarity, oxygen tension (Ernst *et al.*, 1990; Lee and Falkow, 1990; Galan & Curtiss, 1989) and temperature (Albert *et al.*, 1995). Results from this investigation showed that elaboration of SEF17 fimbriae by *S. enteritidis* was influenced by temperature and nutrient availability, findings which were in agreement with those of Arnqvist *et al.* (1992) and Sukupolvi *et al.* (1997) who reported upon the expression of related aggregative fimbriae of *E. coli* and *S. typhimurium*, respectively. Evidence exists that at least part of the regulation of expression of thin aggregative fimbriae is regulated in response to RpoS levels for, in *E. coli*, curli expression was reported to coincide with high expression of RpoS at low temperature and carbon starvation (Hengge-Aronis, 1996). In addition, strain "I" used in this study was reported by Humphrey *et al.* (1996) to be

a naturally occurring avirulent *S. enteritidis* isolate which was unable to elaborate SEF17 fimbriae. Strain "I" was subsequently demonstrated to have a genetic lesion within the *rpo*S gene (Allen-Vercoe *et al.*, 1998) which may have been the cause of attenuation. Conversely, this study showed that constitutive elaboration of SEF17 was observed by some strains which implied that transcriptional regulation of the structural gene, *agf*A, was defective in some strains. However, Allen-Vercoe *et al.* (1998) showed that "atypical" expression of SEF17 by *S. enteritidis* strain 27655R was not attributed to a frameshift mutation in the *rpo*S global regulatory gene harboured by this strain. Interestingly, Römling *et al.* (1998b) reported that expression of thin aggregative fimbriae of *S. typhimurium* was quasi constitutive following mutations in the promotor region of *agf*D, and transcriptional and expression studies with *rpo*S mutants revealed that the main regulatory switch was within the *agf*D promotor, which abolished the need for *rpo*S. It is plausible that a mutation in the *agf*D gene was responsible for the constitutive fimbrial expression in *S. enteritidis* strains 27655R and S1900, *S typhimurium* strain Mö8 and curli expressing *E. coli* strain NG7C. If this were so, then a specific mutation in the *agf*D promoter region may render a temperature-dependent strain constitutive for SEF17 elaboration. This may be tested by the direct sequencing of PCR products from *agf*D gene.

In chapter 4 it was established that SEF17 may be expressed *in vitro* by *S. enteritidis* strains and for a majority of strains expression was dependant upon temperature and media composition. However, it was beyond the scope of the present study to define the specific environmental or physicochemical factors for SEF17 elaboration by *S. enteritidis*. However, Walker *et al.* (1999) utilised the assays developed for this study and reported that high levels of fimbrial expression was achieved at 20°C at pH 6.18 and above, but not at pH 5.09 or below following planktonic growth. On CFA agar gradient plates, SEF17 fimbriae were elaborated

well at all pH levels tested. These results suggested that in addition to factors reported in this study, pH and surface contact could significantly influence elaboration of SEF17 fimbriae. Interestingly, Römling *et al.* (1998b) recently reported that the suppression of biogenesis of thin aggregative fimbriae at 37°C could be relieved by iron deprivation. Further investigation would be required to determine whether SEF17 could also be expressed at higher temperatures in response to specific stimuli encountered *in vivo*.

This study provided evidence that SEF17 could potentially mediate interaction with epithelial cells. However, no evidence was gained that SEF17 had been expressed by *S. enteritidis* during infection of chickens, although small but statistically significant differences were observed between *S. enteritidis* strain LA5 and EAV42 (SEF14$^-$, SEF17$^-$) in colonisation and invasion of day-old chicks. Seroconversion to SEF14, SEF21 and flagella were detected and suggested that these surface appendages were immunogenic and expressed in poultry during infection, but antibodies to SEF17 were not detected. An alternative approach for determining expression during infection may have been to utilise the immune reagents produced and evaluated in chapter 3 to isolate *S. enteritidis* expressing SEF17 from the caecal contents by immunomagnetic separation. However, Lim *et al.* (1998) reported *in vivo* phase variation of *E. coli* type 1 fimbrial genes in women with urinary tract infections and bacteria that were attached to the uroepithelium were transcriptionally active for type 1 fimbrial genes but not those in urine. Therefore, the most appropriate method for detecting SEF17 expression within the alimentary tract would have been an immunohistochemical approach which was beyond the scope of this study. However, the regions of the chick alimentary tract in which *S. enteritidis* interact are poorly understood and would require further investigation. Reporter systems such as the introduction of Green Fluorescent Protein gene fused in frame with *agf*A prior to oral inoculation may prove valuable.

Results from this study confirmed that SEF17 fimbriae mediate binding to fibronectin which suggested that SEF17 and related fimbriae may provide *S. enteritidis* and other enteropathogens with a mechanism for colonising the intestinal epithelium, especially if the tissue had been damaged exposing basement membrane components such as fibronectin (Fröman *et al.*, 1984; Lindquist *et al.*, 1987). Interestingly, fibronectin-binding proteins in *Streptococcus spp.* and *Staphylococcus spp.* have been described as important mediators for adherence to eukaryotic cells (Rocha & Fischetti, 1999). Evidence that thin aggregative fimbriae of *S. typhimurium* were expressed at 37°C (Römling *et al.*, 1998b), of temperature-regulated curli expression in non-invasive *E. coli* (Olsen *et al.*, 1994; Ben-Nasr *et al.*, 1996) and that the role of curli have been shown to be important in persistence of Avian Coli Bacillosis infections in poultry (Personal Communication, Roberto La Ragione, PhD thesis, 1999, Royal Holloway, University of London) suggest that further investigation of *in vivo* expression of SEF17 in poultry would be worthwhile.

Salmonella spp. are reported to be more prevalent in sediments than in water (Murray, 1991) and it is therefore plausible that this micro-organism interacts with inanimate surfaces rather than remaining in a planktonic life form in the environment. Extracellular surface layers that protect against mechanical stress are characteristics of biofilms (Costerton *et al.*, 1999). Austin *et al.*, (1998) demonstrated that SEF17 fimbriae contribute to biofilm formation for wild type *S. enteritidis* strain 27655R readily adhered to Teflon (polytetraflouroethylene) and stainless steel forming thick cell aggregates, whereas an isogenic SEF17-deficient mutant failed to form thick biofilms. These results implicated a role for SEF17 in stabilising cell-cell interactions during biofilm formation. In addition, Austin *et al.*, (1998) observed reduced adherence to Teflon and stainless steel with an isogenic SEF14/SEF21 deficient strain which

implicated the involvement of additional cell surface adherence factors. Results from this laboratory using isogenic mutants have demonstrated SEF17-mediated adherence of *S. enteritidis* to polystyrene following growth at ambient temperature but not at 37°C, whereas, SEF14-mediated interaction was observed at 30°C and above. It is plausible that SEF17 has a role in adherence to inanimate objects within the environment, and SEF14 may assist adherence to forming or formed eggs of laying hens after entering the reproductive tract by an ascending route from the cloaca. Interestingly, Withanage *et al.* (1999) have reported a significant local immune response and partial clearance of *Salmonella* organisms from the oviducts following experimental infection. It is plausible that fimbriae or flagella may contribute to colonisation of the reproductive tract and the mutants used in this study may prove valuable in elucidating their role.

This study did not indicate a role for SEF14 in pathogenesis although it was expressed in infected birds. However, Thiagarajan *et al.*, (1996b) implicated SEF14 in the attachment and invasion of chicken ovarian granulosa cells by *S. enteritidis*. Interestingly, attachment was reduced by the addition of purified SEF14 or anti-chicken fibronectin, although this study suggested that SEF14 was not associated with fibronectin binding. It is plausible that a strain of *S. enteritidis* constitutive for the expression of SEF17 was used, and was co-purified with SEF14 and was indeed responsible for adherence to ovarian cells of laying hens. That fimbriae could potentially assist infection of the egg with *S. enteritidis* by vertical transmission would be worthy of further investigation.

Collectively the results from this study have demonstrated that SEF14, SEF17, SEF21 and flagella are not essential for colonisation and invasion of young chicks or in short-term persistence. However, results from tissue culture models were in agreement with those of the

in vivo studies and suggested a contribution by SEF17, SEF21 and flagella in pathogenesis, but not SEF14. *In vitro* models are relatively insensitive and cannot mimic environments that enteric pathogens experience when they enter their host by the oral route, such as low pH, increased temperature, low oxygen tension, high osmolarity and nutrient deprivation. However, this study has provided evidence of their value. Interestingly, *in vitro* models have previously distinguished virulent and non-virulent strains of *Salmonella* in poultry. Indeed, Nolan *et al.* (1991) reported that isolates of sick birds were more invasive in tissue culture cells although Kottom *et al.* (1995) reported that there was no significant difference between the ability of *S. typhimurium* isolates to interact with CACO-2 cells, despite a correlation between virulence in chickens and their ability to survive within cells.

The day-old chick model used in this study to demonstrate the role of fimbriae and flagella in initial colonisation, invasion and dissemination to internal organs suggested that mutants defective for the elaboration of SEF17, SEF21 and flagella were delayed in the colonisation of the liver and, in particular, the spleen. In this model, chicks that hatched within 24 hours were utilised and the intestinal tract proved to be naïve. For example, *Klebsiella spp.* was an intermittent contaminant in the animal accommodation during the course of the *in vivo* experiments performed for this study. *Klebsiella spp.* are not recognised as pathogens of poultry, however, large numbers of bacteria were isolated from chick liver and spleen by 24 hours, but unlike *Salmonella, Klebsiella* was cleared from the internal organs by seven days of age, although they persisted in the caeca. These observations indicated how vulnerable the alimentary tract of day-old chicks were to large numbers of bacteria including *Klebsiella spp.* and also how effective the chick immune system was at clearing certain bacteria from internal tissues, whilst *Salmonella* avoided clearance by the host for several weeks.

The intestinal tract of poultry, like all other internal epithelial surfaces of mammals and birds is covered by a layer of mucus which consists of a changing mixture of many secretions and exfoliated epithelial cells, which act as a physical barrier to colonising bacteria. Indiginous bacteria intimately colonise the intestinal mucosa, but under normal conditions the intestinal epithelial cells are free of adherent bacteria and it is plausible that the intestinal tract, and in particular the caeca of day-old chicks had not developed sufficient mucus to protect the underlying epithelia. Interestingly, Mack *et al.* (1999) reported that probiotic bacteria, or live organisms with beneficial effects for the host, increased the expression of MUC2 and MUC3 intestinal mucins and thus inhibited adherence of organisms to the intestinal epithelial cells. It is possible that neonatal chicks produce mucins in response to exposure to bacteria which form the natural gut flora and that the large oral doses of *Salmonella* administered within 24 hours prevented the development of this layer in time for it to be protective.

This study was dependant upon the availability of isogenic mutants in strains of *S. enteritidis*. All mutants were subjected to thorough genotypic and phenotypic analysis which showed no evidence of polar mutations (Allen-Vercoe *et al.*, 1997; 1999a; b; this study). In addition, each single or multiple mutant achieved similar growth kinetics as wild type. However, it was plausible that *S. enteritidis* strain with the multiple mutations may have been less robust in a hostile environment. That the quadruple mutant prepared in LA5 colonised internal organs of chicks with similar numbers, albeit slightly later, suggested this was not so. Interestingly, following oral inoculation of seven day old birds with mutant EAV40 (SEF14$^-$, SEF17$^-$, SEF21$^-$, fla$^-$) there was evidence that loss of these surface appendages reduced carriage of *Salmonella* in the caeca within 28 days when compared to wild type. However, this was not observed with day-old chicks. Further studies would be required to determine which surface appendage was responsible and whether the age of the bird when orally infected was

significant. In addition, the experiments performed in this study were designed to test for short-term persistence for up to six weeks duration. Experiments of longer duration may have enhanced differences observed with carriage in the caeca.

Results from *in vitro* studies suggested that SEF17, SEF21 and flagella contributed to the association to tissue culture cells. Blocking assays with purified surface appendages or neutralising antibodies confirmed that these appendages were indeed responsible for enhanced interaction. Antibodies, antigens and other reagents have been used previously to demonstrate the role of specific molecules in bacterial-host interactions (Osaki *et al.*, 1998; Thiagarajan *et al.*, 1996b). An alternative approach may have been to introduce the inactivated operon back into the mutant on a plasmid as performed by Bäumler *et al.* (1996). However, expression of the inactivated genes by complementation may not be influenced by the same regulatory mechanisms as chromosomally transcribed operons. Therefore, plasmid-mediated complementation may be an unsuitable method for expressing flagella and fimbriae that are regulated by environmental stimuli.

Other fimbriae of *Salmonella* have been shown to mediate interaction with host tissues and Baumler *et al.* (1995; 1996) reported that PEF and LPF fimbriae of *S. typhimurium* contributed to specific attachment to murine intestinal cells. Expression of these fimbriae by *S. enteritidis* have not been observed. However, Allen-Vercoe *et al.*, (1999a, b) recently reported that genotypically defined mutants of PEF and LPF had no effect on *in vitro* adherence to chick gut or to invasion of day-old chicks. Also, in contrast to the results obtained from this study, the role of curli and flagella have been shown to be important in short-term persistence of Avian Coli Bacillosis infections in poultry (Personal Communication, Roberto La Ragione) and this implies that *S. enteritidis* are better equipped

to compensate for the physical or functional loss of these genes by employing alternative virulence factors.

Appendices

Appendix 1. General buffers

Carbonate buffer contained 1.5g sodium carbonate and 2.39g sodium hydrogen carbonate made up to 1 litre of distilled water, adjusted to pH 9.6, sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at -20°C.

Denaturing solution was prepared with 87.7g sodium chloride 20.0g sodium hydroxide made up to 1 litre of distilled water and sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C).

0.2M glycine buffer was prepared with 15.0g glycine dissolved in 1 litre distilled water and adjusted to pH 1.5 with HCl.

Hanks balanced salt solution (HBSS, Sigma) contained 0.185g calcium chloride, 0.098g magnesium sulphate, 0.4g potassium chloride, 0.06g potassium phosphate, 0.35g sodium bicarbonate, 8.0g sodium chloride, 0.048g sodium phosphate, 1.0g glucose and 0.011g phenol red in 1 litre of distilled water.

Neutralising solution was prepared with 87.75g sodium chloride, 66.5g Trizma base (Sigma), 0.2% (v/v) 0.5M EDTA made up to 1 litre of distilled water, adjusted to pH 8.0, sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

0.1M phosphate buffered saline (PBS) was prepared with 12.85g di-sodium hydrogen phosphate, 3.36g sodium di-hydrogen orthophosphate and 85g sodium chloride made up to 1

litre of distilled water, adjusted to pH 7.2 and sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C).

PBS Tween (PBS/T) was prepared as for PBS with the addition of 0.05% (v/v) Tween 20.

0.1M potassium phosphate buffer (PPB), pH 6.8 was prepared with 1.74g potassium hydrogen phosphate, 0.57g potassium di-hydrogen phosphate, 0.1g magnesium chloride. $6H_20$ and 0.4g EGTA made up to 1 litre of distilled water and filtered through a 0.2μm cut-off filter.

2x SSC was prepared with 17.5g sodium chloride, 8.82g sodium citrate made up to 1 litre of distilled water, adjusted to pH 7.0, sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

0.1M sodium phosphate buffer, pH 6.8 was prepared with 7.1g sodium hydrogen phosphate and 6.9g sodium di-hydrogen phosphate made up to 1 litre of distilled water and filtered through a 0.2μm cut-off filter.

10mM Tris HCL buffer was prepared by dissolving 1.21g Tris in 1 litre of distilled water and was adjusted to pH 8.0 with HCl.

Appendix 2. Bacterial growth media

Brilliant green agar (BGA, Oxoid) contained 10.0g lactose, 10.0g sucrose, 5.0g peptic digest of animal tissue, 5.0g pancreatic digest of casein, 5.0g sodium chloride, 0.08g phenol red, 0.0125g brilliant green and 20.0g agar made up to 1 litre with distilled water. The agar was sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

Colonisation factor antigen (CFA) agar (Evans *et al.*, 1977) contained 10.0g casamino acids, 1.5g yeast extract, 0.05g magnesium sulphate, 0.005g manganese chloride and 20.0g agar made up to 1 litre with distilled water and was sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

Congo red CFA agar was prepared as for CFA agar with the addition of 100μg Congo red dye (Sigma) per ml of media prior to autoclaving.

Colonisation factor antigen (CFA) **broth** was prepared as for CFA agar but without the agar.

Drigalski agar (Oxoid) contained 7.0g peptone, 5.2g sodium chloride, 15.0g lactose, 1.2g litmus and 13.0g agar. The media was adjusted to pH 7.4, sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

Eagles minimal essential media (Sigma) contained 0.265g calcium chloride, 0.098g magnesium sulphate, 0.4g potassium chloride, 2.2g sodium bicarbonate, 6.8g sodium

chloride, 0.12g sodium phosphate, 0.126g L-arginine, 0.03g L-cysteine, 0.042g L-histidine, 0.05g L-isoleucine, 0.05g L-leucine, 0.0725g L-lysine, 0.015g L-methionine, 0.03g L-phenylalanine, 0.048g L-threonine, 0.01g tryptophan, 0.052g L-tyrosine, 0.046g L-valine, 0.001g choline chloride, 0.001g folic acid, 0.002g myo-inositol, 0.001g niacinamide, 0.001g D-pantothenic acid, 0.001g pyridoxal, 0.0001g riboflavin, 0.001g thiamine, 1.0g glucose and 0.011g phenol red per litre .

Luria-Bertani (LB) broth (MERCK) contained 5.0g sodium chloride, 10.0g pancreatic digest, 5.0g of yeast extract made up to 1 litre with sterile distilled water, adjusted to pH 7.5, sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

Luria-Bertani (LB) agar (MERCK) was prepared as described for Luria-Bertani broth but with the addition of 15g of agar per litre.

Heart Infusion broth (HIB, Difco) contained 5.0g beef heart infusion solids, 10.0g protease peptone, 5.0g sodium chloride, 2.0g glucose, 25.0g di-sodium phosphate made up to 1 litre of distilled water, adjusted to pH 7.4, sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

MINCA agar (Guinee *et al.,* 1976) was prepared with 1.36g potassium dihydrogen phosphate, 10.1g di-sodium hydrogen phosphate. $2H_2O$, 1.0g glucose, 1.0g Minca trace salts, casamino acids (Difco) and 12.0g agar dissolved in 1 litre of distilled water, adjusted to pH 7.5 and sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

Nutrient agar (Oxoid) contained 5.0g peptone, 5.0g sodium chloride, 2.0g yeast extract, 1.0g beef extract and 15.0g agar made up to 1 litre with sterile distilled water. The media was gently heated until boiling, sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

Nutrient broth (Oxoid) was prepared as for Nutrient agar but without the addition of agar.

Peptone water (Oxoid) contained 10.0g peptone and 5.0g sodium chloride made up to 1 litre with distilled water, sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

Selenite F broth (Oxoid) contained 5.0g peptone, 4.0g mannitol, 1.3g di-sodium hydrogen phosphate and 2.8g sodium di-hydrogen orthophosphate made up to 1 litre of distilled water, adjusted to pH 7.2, sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

Sensitest agar (Oxoid) contained 11.0g hydrolysed casein, 3.0g peptones, 2.0g glucose, 3.0g sodium chloride, 1.0g soluble starch, 2.0g di-sodium hydrogen phosphate, 1.0g sodium acetate, 0.2g magnesium glycerophosphate, 0.1g calcium gluconate, 0.001g of cobaltous sulphate, cupric sulphate, zinc sulphate, ferrous sulphate, menadione, cyanobalamin, 0.002g manganous chloride, 0.02g L-cysteine hydrochloride and L-tryptophan, 0.003g pyridoxine and pantothenate, 0.0003g nicotinamide and biotin, 0.00004g thiamine, 0.01g adenine, xanthine, uracil and guanine and 8.0g agar made up to a final volume of 1 litre with distilled

water, adjusted to pH 7.4, sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

Tryptone water (Mast Diagnostics) contained 10.0g pancreatic digest of casein and 5.0g sodium chloride made up to 1 litre of distilled water. The media was sterilised by autoclaving for 15 minutes at 15 psi pressure (121°C) and stored at 4°C.

Appendix 3. Tissue culture media

Complete tissue culture media for culturing epithelial cells contained Eagles Modified Essential Media (EMEM, Sigma) supplemented with 10% heat inactivated foetal calf serum (Sigma), 2mM L-glutamine (Sigma) and 50μg/ml gentamicin (Sigma).

Hybridoma media contained 2mM Glutamax-1 (Gibco), 10% heat inactivated foetal calf serum (Gibco) and 50μg/ml gentamicin (Sigma) made up in RPMI 1640 (Gibco).

Incomplete tissue culture media contained Eagles Modified Essential Media and 2mM L-glutamine.

Low serum medium contained 5% (v/v) SRC (Tissue Culture Services Biologicals Ltd., Buckinghamshire, UK), 2mM Glutamax-1 (Gibco) and was supplemented with 0.5 to 1% foetal calf serum (Gibco)

RPMI 1640 (Sigma) contained contained 0.1g calcium nitrate, 0.048g magnesium sulphate, 0.4g potassium chloride, 2.0g sodium bicarbonate, 6.0g sodium chloride, 0.8g sodium phosphate, 0.2g L-arginine, 0.05g L-asparginine, 0.02g aspartic acid, 0.065g L-cystine, 0.02g L-glutamic acid, 0.3g L-glutamine, 0.01g glycine, 0.015g L-histidine, 0.02g hydroxy proline, 0.05g L-isoleucine, 0.05g L-leucine, 0.04g L-lysine, 0.015g L-methionine, 0.015g L-phenylalanine, 0.02g L-proline, 0.03g L-serine, 0.02g L-threonine, 0.005g tryptophan, 0.028g L-tyrosine, 0.046g L-valine, 0.0002g biotin, 0.003g choline chloride, 0.001g folic acid, 0.035g myo-inositol, 0.001g niacinamide, 0.001g p-amino benzoic acid, 0.00025g D-pantothenic acid, 0.001g pyridoxine, 0.0002g riboflavin, 0.001g thiamine, 0.000005g vitamin

B-12, 2.0g glucose 0.001g glutathionine and 0.0053g phenol red made up to 1 litre in distilled water.

X10 Trypsin-EDTA solution (Sigma) contained 5.0g porcine trypsin and 2.0g EDTA in 100ml distilled water containing 0.9% sodium chloride. To prepare the working stock the concentrated solution was diluted 1:9 with PBS, filter sterilised through a 0.2μm filter, aliquoted and stored at -20°C.

Appendix 4. SDS-PAGE and Western blots

Acrylamide was a stock solution containing 30% acrylamide and 0.45% N, N'-methylenebisacrylamide (Biorad)

10% Ammonium persulphate was prepared in distilled water.

BCIP substrate containing 5-bromo-4 chloro-3 indophosphate (Sigma) was prepared according to the manufacturers instructions.

x10 CAPS transfer buffer concentrate was prepared by dissolving 22.13g 3-cyclohexylamino-1- propanesulfonic acid (Sigma) in 1 litre of distilled water and adjusted to pH 11.0 with sodium hydroxide. For use 1 litre of x10 concentrated CAPS was added to 8 litres of distilled water and 1 litre of methanol.

Electrophoresis running buffer contained 40mM Tris, 40mM glycine and 0.1% SDS.

Rainbow protein electrophoresis standards (Amersham) contained eight proteins within the molecular mass range 14-200 kDa. 5µl was loaded per gel.

Resolving gel buffer x4 concentrate was prepared by dissolving 181.6g Tris and 4.0g SDS in 1 litre of distilled water and adjusted to pH 8.8 with HCl. SDS-PAGE gels were prepared from x4 concentrate (Table A1.1).

SDS-PAGE sample buffer contained 1.6ml glycerol, 0.3g sodium dodecyl sulphate, 0.0002g bromophenol blue, 0.8ml β-mercaptoethanol and 10ml 0.1M tris HCl pH 6.8. Following filter sterilisation the buffer was stored at 4°C.

Acidic sample buffer was prepared as for SDS-PAGE sample buffer but adjusted to pH 1.8 with HCl.

Stacking gel buffer x4 was prepared by dissolving 60.5g Tris and 4.0g SDS in 1 litre of distilled water and adjusted to pH 6.8 with HCl. SDS-PAGE gels were prepared from x4 concentrate (Table A1.2).

Tris buffered saline (TBS) contained 20mM Tris and 0.5M sodium chloride in distilled water and was adjusted to pH 7.5.

TBS Tween (TBS/T) was prepared as for TBS with the addition of 0.05% Tween 20.

TEMED (Sigma) contained N',N',N',N'- Tetramethylethylenediamine.

Table A4.1 Volumes (ml) required for 2 x 1.5mm resolving gels of varying acrylamide concentrations.

	% Acrylamide	
	12.0	15.0
Acrylamide reagent	4.12	4.9
x4 Resolving buffer	2.5	2.5
Distilled water	3.25	2.5
TEMED	0.01	0.01
10% Ammonium persulphate (w/v)	0.1	0.1

Table A4.2 Volumes (ml) required for 2 x 1.5mm stacking gels containing 5% acrylamide.

Acrylamide reagent	1.3
x4 Stacking buffer	1.9
Distilled water	4.2
TEMED	0.0075
10% Ammonium persulphate (w/v)	0.075

Appendix 5

Isogenic fimbrial and flagella mutants

The single and multiple defined mutants of *S. enteritidis* strains LA5, S1400 and 27655R have recently been produced in this laboratory (Allen-Vercoe *et al.*,1999a;b). The following procedures were used for mutant production and characterisation:

Cloning

DNA fragments of *sef*A, agfA, *fim*D or *fli*C operons which encoded SEF14 fimbrial antigen, SEF17 fimbrial antigen, SEF21 fimbrial usher and flagella antigen, respectively were amplified by PCR. These fragments were subcloned separately into pCRSCRIPT (Stratagene) and inserts were confirmed by preliminary nucleotide sequence analysis and detailed restriction endonuclease mapping. Into the *sef*A, *agf*A, *fim*D and *fli*C genes were cloned the antibiotic resistance gene cassettes kanamicin, ampicillin, tetracycline, chloramphenicol, respectively, either by direct subcloning if appropriate restriction endonuclease sites were available within the open reading frames, or by splice overlap extension PCR. The insertionally inactivated fimbrial or flagellum operon were subcloned on to appropriate pERFORM vectors and electroporated into permissive host *E. coli* strain K12 S-17 λ*pir*.

Conjugation

The pERFORM vectors harbouring the antibiotic resistance-marked, insertionally inactivated genes were transferred by conjugation from the permissive host strain *E. coli* strain K12 S-17 λ*pir* into the non-permissive *S. enteritidis* strain S1400 by filter mating. Equal volumes

(100µl) of overnight broth cultures of donor and recipient strains were inoculated together, or separately as controls, on sterile 2 µm filters overlaid on dried plates of Nutrient agar and incubated at 37°C. After growth for four hours culture were removed from filters by immersion and vigorous agitation in PBS. Dilutions were plated on to selective media which were glucose minimal media supplemented with appropriate antibiotics.

Selection of mutants

Individual colonies from conjugation selective plates were streaked on to fresh glucose minimal media supplemented with appropriate antibiotics (that is the antibiotic to which resistance was encoded by the insert of inactivated gene) and grown at 37°C. Individual colonies were tested for loss of the antibiotic resistance encoded by the suicide vector. Putative mutant colonies were picked from the master plate and re-streaked for single colonies on Luria-Bertami agar for confirmatory tests by colony dot-blot and Southern hybridisation.

P22 transduction

Single mutants in strain LA5 and 27655R, or multiple mutants were produced by P22 transduction following the methods of Anderson *et al.* (1996). Briefly, the bacteriophage lysate was prepared by adding 400µl of an overnight culture of donor strain and 4 x 10^5 pfu of P22 phage to ten ml of molten Luria-Bertani agar (47°C) containing 10mM magnesium sulphate. After swirling gently, the mixture was poured onto a dried Luria-Bertani agar plate and allowed to set. The agar plate was inverted and incubated for six hours at 37°C or until the plate became clear. The plate was overlaid with five ml of PBS and the top layer scraped into a centrifuge tube. After incubating at room temperature for 30 minutes with gentle

shaking the lysate was centrifuged at 5,000 xg for ten minutes at 4°C. The supernatant was removed with a pasteur pipette and passed through a 0.22μm. Sterility was confirmed by plating onto Nutrient agar and incubating overnight at 37°C overnight.

P22 bacteriophage transduction was performed by adding 10μl of neat and serially diluted P22 lysate (10^1, 10^2,10^3 and 10^4) with 100μl of recipient *Salmonella* strain which had been grown overnight at 37°C, with shaking. After incubating the bacteria/phage mixture for 37°C for 30 minutes at 37°C, one ml of Luria-Bertani broth containing 5mM EGTA was added and incubated for one hour at 37°C. The mixture was plated onto Luria-Bertani agar containing 5mM EGTA and the appropriate antibiotic, 100μl per plate, and incubated overnight at 37°C. Colonies isolated after overnight growth were passaged twice on Luria-Bertani agar containing 5mM EGTA to remove phage particles and transduced isolates were analysed genotypically and phenotypically.

Bibliography

Adegbela, R.A., Old, D.C. & Aleksic, S. (1983). Rare MR/K like haemagglutinins (and type 3 fimbriae) of *Salmonella* strains. *FEMS Microbiol Lett* **19,** 233-238.

Advisory committee on Microbiological Safety of Food. (1993). Reports on *Salmonella* in eggs. London, HMSO.

Agin, T.S. & Wolf, M.K. (1997). Identification of a family of intimins common to *Escherichia coli* causing attaching-effacing lesions in rabbits, humans and swine. *Infect Immun* **65**, 320-326

Albert, M.J., Ansaruzzaman, M., Bhuiyan, N.A., Neogi, P.K.B. & Faruque, A.S.G. (1995). Characteristics of invasion of Hep-2 cells by *Providenia alcalifiens. J Med Microbiol* **42**, 186-190

Aleksic, S. & Aleksic, V. (1979). Purification and pyhysico-chemical analysis of the fimbrial antigen in two different genera of Enterobacteriaceae: *Salmonella enteritidis* and *Yersinia enterolitica. Zentralbl Bakteriol* **243**, 177-196

Allen, V.M., Fernandez, F. & Hinton, M.H. (1997). Evaluation of the influence of supplementing the diet with mannose or palm kernel meal on *Salmonella* colonisation in poultry. *Br Poult Sci* **38**, 485-488

Allen-Vercoe E, Collighan R, Woodward M.J. (1998). The variant *rpo*S allele of *S. enteritidis* strain 27655R does not affect virulence in the chick model nor constitutive curliation but does generate a cold sensitive phenotype. *FEMS Microbiol Lett* **167,** 245-253

Allen-Vercoe, E., Dibb-Fuller, M.P., Thorns, C.J. & Woodward, M.J. (1997). SEF17 fimbriae are essential for the convoluted colonial morphology of *Salmonella enteritidis. FEMS Microbiol Lett* **153**. 33-42.

Allen-Vercoe, E. & Woodward, M.J. (1999a). The role of flagella, but not fimbriae in the adherence of *Salmonella* enterica serovar Enteritidis to chick gut explant. *J Med Microbiol* **48**, 771-780

Allen-Vercoe, E., Sayers, R. & Woodward, M.J. (1999b). Virulence of *Salmonella* enterica serotype Enteritidis aflagellate and afimbriate mutants in the day-old-chick model. *Epidemiol Infect* **122**, 395-402

Altmeyer, R.M., McNern, j.k., Bossio, J.C., Rosenshine, L., Finlay, B.B. & Galàn (1993). Cloning and molecular characterisation of a gene involved in *Salmonella* adherence and invasion of cultured epithelial cells. *Mol Microbiol* **7**, 89-98

Anon. (1977) *Salmonella* in Livestock Production. *Veterinary Laboratory Agency*

Anon. (1998a) Communicable Disease Report, Vol. 8, No. 19

Anon. (1998b) Communicable Disease Report, Vol. 8, No. 28

Anon. (1999) Communicable Disease Report, Vol. 9, No. 20

Arnqvist, A., Olsén, A., Pfeifer, J., Russell, D.G. & Normark, S. (1992). The Crl protein activates cryptic genes for curli formation and fibronectin binding in *Escherichia coli* HB101. *Mol Microbiol* **6**, 2443-2452

Ashkenazi, S., Cleay, T.G., Murray, B.A., Wanger, A. & Pickering, L.K. (1988). Quantitative analysis and partial characterisation of cytotoxin production by *Salmonella* strains. *Infect Immun* **56**, 3089-94.

Austin, J.W., Sanders, G., Kay, W.W. & Collinson, K. (1998). Thin aggregative fimbriae enhance *Salmonella enteritidis* biofilm formation. *FEMS Microbiol Lett* **162**, 295-301.

Baay, M.F.D. & Veld, J.H.J. (1993). Alternative antigens reduce cross-reactions in an ELISA for the detection of *Salmonella enteritidis* in poultry. *J Appl Bacteriol* **74**, 243-247

Bakker, D., Willemsen, T.J., Simons, L.H., van Ziijdervald, F.G. & de Graaf, F.K. (1992). Characterisation of the antigenic and the adhesive properties of FaeG, the major subunit of K88 fimbriae. *Mol Microbiol* **6**, 247-255.

Baloda, S.B. (1988). Characterisation of fibronectin binding to *Salmonella enteritidis* strain 27655R. *FEMS Microbiol Lett* **49**, 483-488.

Baloda, S.B., Faris, A. & Krovacek. (1988). Cell surface properties of Enterotoxigenic and cytotoxic *Salmonella enteritidis* and *Salmonella typhimurium*: Studies on haemagglutination, cell surface hydrophobicity, attachment to human intestinal cells and fibronectin binding. *Microbiol Immunol* **22**, 447-459.

Bardwell, J., McGovern, K. & Beckwith, J. (1991). Identification of a protein required for disulphide bond formation. *Cell* **67**, 581-589.

Barnhart, E.T., Caldwell, D.J., Crouch, M.C., Byrd, J.A., Corrier, D.E. & Hargis, B.M. (1999). Effect of lactose administration in drinking water prior to and during feed withdrawal on *Salmonella* recovery from broiler crops and ceaca. *Poult Sci* **78**, 211-214

Barrow, B.A., Simpson, J.M. & Lovell, M.A. (1988). Intestinal colonisation in the chicken by food poisoning *Salmonella* serotypes; Microbial characteristics associated with faecal excretion. *Avian Pathol* **17**, 571-588

Barrow, B.A. (1991). Experimental infection of chickens with *Salmonella enteritidis. Avian Pathol* **120,** 145-153.

Barrow, P.A. (1992). ELISA's and the serological analysis of salmonella infections in poultry: a review. *Epidemiol Infect* **109**, 361-369

Barrow, P.A. (1994). Serological diagnosis of *Salmonella serotype* Enteritidis infections in poultry by ELISA and other tests. *Inter J Food Microbiol* **21**, 55-68.

Barrow, B.A. & Lovell, M.A. (1989). Invasion of Vero cells by *Salmonella* species. *J Med Microbiol* **28**, 59-67

Barrow, B.A., Lovell, M.A., Szmolleny, G. & Murphy, C.K. (1998). Effect of enrolfloxacin administration on excretion of *Salmonella enteritidis* by experimentally infected chickens and on quinolene resistance of their *Escherichia coli* flora. *Avian Pathol* **27**, 586-590

Baumler, A.J. & Heffron, F. (1995). Identification and sequence analysis of *lpf*ABCDE, a putative fimbrial operon of *Salmonella typhimurium. J Bacteriol* **177**, 2087-2097

Baumler, A.J., Tsolis, R.M., Bowe, F.A., Kuster, J.G., Hoffman, S. & Heffron, F. (1996) The *pef* fimbrial operon of *Salmonella typhimurium* mediates adhesion to murine small intestine and is neccessary for fluid accumulation in the infant mouse. *Infect Immun* **64**, 61-68

Baumler, A.J., Tsolis, R.M., Ficht, T.A. & Adams, L.G. (1998). Evolution of host adaptation in *Salmonella enterica. Infect Immun* **66**, 4579-4587

Baumler, A.J., Tsoli, R.M. & Heffron, F. (1995). The *lpf* operon mediates adhesion of *Salmonella typhimurium* to murine Peyer's patches. *Proc Natl Acad Sci USA* **93**, *279-283*

Beaumont, C., Protais, J., Guillot, J.F., Colin, P., Proux, K., Millet, N. & Pardon, P. (1999). Genetic resistance to mortality of day-old chicks and carrier state of hens after inoculation with *Salmonella enteritidis*. *Avian Pathol* **28**, 131-135

Befus, A.D., Johnson, N., Leslie, G.A. & Bienenstock, J. (1980). Gut Associated Lymphoid Tissue in the chicken. Morphology, ontogeny and some functional characteristics of Peyer's Patches. *J Immunol* **125**, 2626-2632

Benitéz, J.A., Spelbrink, R.G., Silva, A., Phillips, T.E., Stanley, C.M, Boesman-Finkelstein, M. & Finkelstein, R.A. (1997). Adherence of *Vibrio cholerae* to cultured differentiated human intestinal cells: an *in vitro* colonisation model. *Infect Immun* **65**, 3474-3477.

Bejamin, P., Federman, M. & Wanke, C.A. (1995). Characterization of an invasive phenotype associated with Enteroaggregative *Escherichia coli. Infect Immun* **63,** 3417-3421

Bell, D. (1987) Is molting still a viable replacement alternative? *Poult Trib* **95,** 32-35

Ben-Nasr, A., Olsen, A., Sjöbring, U., Muller-Esterl, W. & Bjorck, L (1996). Assembly of human contact phase proteins and release of bradykinin at the surface of curli-expressing *Escherichia coli. Mol Microbiol* **20**, 927-935

Benz, R. (1988). Structure and function of porins from gram-negative bacteria. *Ann Rev Microbiol* **42**, 359-393

Berg, H.C. (1974). Dynamic properties of bacterial flagella motors. *Nature (London)* **249**, 77-79

Bergeys Manual of Determinative Bacteriology (8th edition), (1975). Buchanan, R.E. & Gibbons, N.E. (eds), The Williams & Wilkins Company, Baltimore, USA.

Black, F.L., Reissig, M. & Melnick, J.L. (1956). Propagation of measles virus in a strain of human epidermoid cancer cells (Hep-2). *Soc Exp Biol Med* **93**, 107-108

Bliska, J.B.,Gala, J.E. & Falkow, S. (1993). Signal transduction in the mammalian cell during bacterial attachment and entry. *Cell* **73**, 903-920.

Board, R.G. & Fuller, R. (1974). Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biol Rev* **49**, 15-49.

Bolder, N.M., van Lith, L.A.J.T., Purtirulan, F.F., Jacobs-Reitsma, W.F. & Mulder, R.W.A.W. (1992). Prevention of colonisation by *Salmonella enteritidis* PT4 in broiler chickens. *Int J Food Microbiol* **15**, 313-317

Braun, V. & Wu, H.C. (1993). Lipoproteins, structure, function, biosynthesis and model for protein transport. *New Compr Biochem* **27**, 319-342.

Brenner, D.J. (1992). Introduction to the family Enterobacteriaceae, p2673-2695. In A. Balows, H.G.Truper, M. Dworkin, W. Harder and K.H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. III. Springer-Verlag, New York.

Browning, L.M. and Platt, D.J. (1995). Restriction endonuclease fragment pattern analysis of plasmids from salmonellae that belong to serogroup D1. *Med Microbiol Lett* **4**, 132-9.

Brunsson, I. (1987). Enteric nerves mediate the fluid secretory response due to *Salmonella typhimurium* R.5. infection in rat small intestine. *Acta Pysiol Scand* **131**, 609-617.

Bucy, R.P., Chen, C.L-H., Cihak, J., Lösch, U. & Cooper, M.D. (1988). Avian T cells expressing $\chi\delta$ receptors localise in the splenic sinusoids and the intestinal epithelium. *J Immunol* **141**, 2200-2205

Burns, R.B. (1982) Histology and immunology of the Peyer's patches in the domestic fowl (*Gallus domesticus*). *Res Vet Sci* **32**, 359

Burns-Kaliher, L., Nickerson, C.A., Morrow, B.J. & Curtiss III, R. (1998). Cell-specific proteins synthesised by *Salmonella typhimurium. Infect Immun* **66**, 856-861

Byrd, J.A., Deloach, J.R., Corrier, D.E., Nisbet, D.J. & Stanker, L.H. (1999). Evaluation of *Salmonella* serotype distributions from commercial broiler hatcheries and grower houses. *Avian Dis* **43**, 39-47

Buxton & Fraser (1977). Animal Microbiology Volume 1, **17**, 103-107. Immunology, Bacteriology, Mycology, Diseases of Fish and Laboratory Methods. Blackwell Scientific Publications, Oxford, England

Cameron, D.M. & Carter, J.N. (1992). Evaluation of the efficacy of Broilact^{R} in the preventing broiler chicks with *Salmonella enteritidis* PT4. *Int J Food Microbiol* **15**, 319-326

Canil, C., Rosenshine, I., Rushkowski, S., Donnenberg, M., Kaper, J.S. & Finlay, B.B. (1993). Enteropathogenic *Escherichia coli* decreases the transepithelial electrical resistance of polarised epithelial monolayers. *Infect Immun* **61**, 2755-2762

Cason, J.A., Cox, N.A. & Bailey, J.S. (1994). Transmission *of Salmonella typhimurium* during hatching of broiler chicks. *Avian Dis* **38**, 583-588

Clark, C., Candlish, A.A.G. & Steell, W. (1989). Detection of *Salmonella* in foods using a novel coloured latex test. *Food Agricult Immunol* **1**, 3-9.

Clegg, S. & Gerlach, G.F. (1987). Enterobacterial fimbriae. *J Bacteriol* **169**, 934-938.

Clouthier, S.C., Muller, K-H., Doran, J.L., Collinson, K.S. & Kay, W.W. (1993). Characterization of three fimbrial genes, *sef*ABC, of *Salmonella enteritidis*. *J Bacteriol* **175**, 2523-2533.

Clouthier, S.C, Collinson, S.K. & Kay, W.W. (1994). Unique fimbriae-like structure encoded by *sef*D of the SEF14 gene cluster of *Salmonella enteritidis*. *Mol Microbiol* **12**, 893-903.

Clouthier, S.C., Collinson, S.K., White, AP., Banser, P.A. & Kay, W.W. (1998). $tRNA^{ARG}$ (*fim*U) and expression of SEF14 and SEF21 in *Salmonella enteritidis*. *J Bacteriol* **180**, 840-845

Coburn, J & Frank, D.W. (1999). Macrophages and epithelial cells respond differently to the *Pseudomonas aeroginosa* Type III secretion system. *Infect Immun* **67**, 3151-3154

Collazo, C. & Galàn, J.E. (1996). Requirement of exported proteins for secretion through the invasion-associated Type III system in *Salmonella typhimurium*. *Infect Immun* **64**, 3524-3531

Collazo, C., Zierler, M.K. & Galàn, J.E. (1995). Functional analysis of the *Salmonella typhimurium* invasion genes *inv*I and *inv*J and identification of a target of the protein secretion apparatus encoded in the *inv* locus. *Mol Microbiol* **15**, 25-38

Collinson, K.S., Clouthier, S.C., Doran, J.L., Banser, P.A. & Kay, W.W. (1996). *Salmonella enteritidis agf*BAC operon encoding thin, aggregative fimbriae. *J Bacteriol* **178**, 662-667.

Collinson, K.S., Doig, P.C., Doran, J.L., Clouthier, S., Trust, T.J. & Kay, W.W. (1993). Thin aggregative fimbriae mediate binding of *Salmonella enteritidis* to fibronectin. *J Bacteriol* **175**, 12-18

Collinson, K.S., Emödy, L., Muller, K.H., Trust, T.J. & Kay, W.W. (1991). Purification and characterization of thin aggregative fimbriae from *Salmonella enteritidis. J Bacteriol* **173**, 4773-4781.

Collinson, K.S., Emödy, L., K.H., Trust, T.J. & Kay, W.W. (1992). Thin aggregative fimbriae from diarrhoegenic *Escherichia coli. J Bacteriol* **174**, 4490-4495.

Collinson, K.S., Liu, S.L., Clouthier, S.C., Banser, P.A., Doran, J.L., Sanderson, K.E. & Kay, W.W. (1996). The location of the four fimbrin-encoding genes, *agf*A, *fim*A, *sef*A and *sef*D, on the *Salmonella enteritidis* and/or *S. typhimurium Xba*I-*Bln*I genomic restriction maps. *Gene* **169**, 75-80.

Conte, M.P., Longhi, C., Petrone, G., Polidoro, M., Valenti, P. & Seganti, L. (1994). *Listeria monocytogenes* infection of Caco-2 cells: role of growth temperature. *Res Microbiol* **145**, 677-682

Cooper, S. (1988). Rate of topography of cell wall synthesis during cell division of *Salmonella typhimurium. J Bacteriol* **170**, 422-430

Cooper, J.L., Nicholas, R.A.J. & Bracewell, C.D. (1989). Serological and bacteriological investigations of chickens from flocks infected with *Salmonella enteritidis*. *Vet Rec* **125**, 567-572

Cooper, J.L., Nicholas, R.A.J., Cullen, G.A. & Hormaeche, C.E. (1990). Vaccination of chickens with a *Salmonella enteritidis aro*A, live oral salmonella vaccine. *Microbial Pathogen* **9**, 255-265.

Cooper, J.L., Venables, L.M., Woodward, M.J. & Hormaeche, C.E. (1994). Invasiveness and persistence of *Salmonella enteritidis, Salmonella typhimurium*, and a genetically defined *S. enteritidis aro*A strain in young chickens. *Infect Immun* **62**, 4739-4746

Cooper, J.L., Venables, L.M. & Lever, M.S. (1996). Airbourne challenge of chickens vaccinated orally with the genetically-defined *Salmonella enteritidis aro*A strain CVL30. *Vet Rec* **139**, 447-448

Corrier, D.E., Hinton, A., Ziprin, R.L., Beier, R.C. & DeLoach, J.R. (1990). Effect of dietary lactose on cecal pH, bacteriostatic volatile fatty acids and *Salmonella typhimurium* colonisation of broiler chicks. *Avian Dis* **34**, 617-625

Costerton, J.W., Stewart, P.S. & Greenberg E.P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318-1322

Cotter, P.A., Yuk, M.H., Mattoo, S., Akerley, B.J., Boschwitz, J., Relman, D.A. & Miller, J.F. (1998). Filamentous haemagglutinin of *Bordetella bronchiseptica* is required for efficient establishment of tracheal colonisation. *Infect Immun* **66**, 5921-5929

Cowden, J.M, Lynch, D., Joseph, C.A., O'Mahoney, M.C.C., Mower, S.C. & Rowe, B. (1989). Case control study of infection with *Salmonella enteritidis* phage type 4 in England. *Bri Med J* **229**, 771-773

Cox, N.A., Bailey, J.S. & Berrang, M.E. (1996). Alternative routes for *Salmonella* intestinal tract colonisation of chicks. *J Appl Poult Res* **5**, 282-288

Coyle, E.F., Palmer, S.R.., Ribero, C.D., Jones, H.I., Howard, A.J. & Ward, L. (1988). *Salmonella enteritidis* phage type 4 infection: association with hens eggs. *Lancet.* **II**, 1295-1297

Craven, S.E., Cox, N.A., Bailey, J.S. & Blankenship, L.C. (1992). Binding of *salmonella* strains to immobilise intestinal mucosal preparations of broiler chickens. *Avian dis* **36**, 296-303

Craven, S.E. (1994). Altered colonising ability for the caeca of broiler chicks by lipopolysaccharide- deficient mutants of *Salmonella typhimurium*. *Avian Dis* **38**, 401-408.

Curtis, R. III & Hassan, J.O. (1996). Non-recombinant and recombinant avirulent *Salmonella* vaccines for poultry. *Vet Immunol Immunopathol* **54**, 365-372

Dadrast, H., Hesketh, R. & Taylor, D.J. (1990). Egg yolk antibody detection in identification of *salmonella* infected poultry. *Vet Rec* **126**, 219

D'Aoust, J.Y. (1989). Salmonella. In: *M.P.* Doyle (ed.), *Foodbourne Bacterial Pathogens* pp. 327-445, *Marcel Decker, New York, NY.*

D'Aoust, J.Y. (1991). Pathogenicity of foodborne *Salmonella*. *Inter J Food Microbiol* **12**, 17-40

Darekar, M.R. and Duguid, J.P. (1972). The influence of fimbriation on the infectivity of *Salmonella typhimurium*. *Proc Ind Acad Sci* **35**, 283-293.

Davies, R.H. & Wray, C. (1995). Persistence of *Salmonella enteritidis* in poultry units and poultry food. *Bri Poult Sci* **37**, 589-596

Davies, R.H. & Wray, C. (1996). Studies of contamination of three broiler breeder houses with *Salmonella enteritidis* before and after cleansing and disinfection. *Avian Dis* **40**, 626-633

Davies, R.H., Nicholas, R., McLaren, I.M., Corkish, J.D. Lanning, D.G. & Wray, C. (1997). Bacteriological and serological investigation of persistent *Salmonella enteritidis* infections in an integrated poultry organisation. *Vet Microbiol* **58**, 277-293

Debroy, C., Yealy, J., Wilson, R.A., Bhan, M.K. & Kumar, R. (1995). Antibodies raised against outer mebrane protein interrupt adherence of Enteroaggregative *Escherichia coli*. *Infect Immun* **63**, 2873-2879

de Graaf, F.K., Krenn, B.E. & Klaasen, P. (1984). Organisation and expression of genes involved in the biosynthesis of K99 fimbriae. *Infect Immun* **43**, 508-514.

de Graaf, F.K. & Roorda, L. (1982). Production, purification and characterisation of the fimbrial adhesive antigen F41 isolated from calf enteropathogenic *Escherichia coli* strain B41M. *Infect Immun* **36**, 751-758

Depamphilis, M.L & Adler, J. (1971a). Fine structure and isolation of the hook-basal body complex of flagella of *Escherichia coli* and *Bacillus subtilis*. *J Bacteriol* **105**, 384-395

Depamphilis, M.L & Adler, J. (1971b). Attachment of flagella basal bodies to the cell envelope: specific attachment to the outer, lipolysaccharide membrane and cytoplasmic membrane. *J Bacteriol* **105**, 396-407.

Desmidt, M., Ducatelle, R., Mast, J., Goddeeris, B.M., Kaspers, B. & Haesebrouck, F. (1998). Role of humoral immune system in *Salmonella enteritidis* phage type four infection in chickens. *Vet Immunol Immunopathol* **63**, 355-367

Dibb-Fuller, M.P., Allen-Vercoe, E., Woodward, M.J. & Thorns, C.J. (1997). Expression of SEF17 fimbriae *by Salmonella enteritidis*. *Lett Appl Microbiol* **25**, 447-452

Dibb-Fuller, M.P., Allen-Vercoe, E., Thorns, C.J. & Woodward, M.J. (1999). Fimbriae- and flagella-mediated association and invasion of cultured epithelial cells by *Salmonella enteritidis*. *Microbiol* **145**, 1023-1031

Doig, P., Yao, R., Burr, D.H., Guerry, P. & Trust, T. (1996). An environmentally regulated pilus-like appendage involved in *Campylobacter* pathogenesis. *Mol Microbiol* **20**, 885-894

Donnenberg, M.S., Giron,J.S., Nataro, J.P. & Kaper, J.B. (1991). A plasmid encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localised adherence. *Mol Microbiol* **6**, 3427-3437

Doran, J.L., Collinson, K.S. & Burian, J. (1993). DNA-based diagnostic tests for *Salmonella* species targeting *agf*A, the structural gene for thin, aggregative fimbriae. *J Clin Microbiol* **31**, 2263-2273.

Doran, J.L., Collinson, K.S., Clouthier, S.C., Cebula, T.A., Koch, W.H., Burian, J., Banser, P.A., Todd, E.C.D. & Kay, W.W. (1996). Diagnostic potential of *sef*A DNA probes to *Salmonella enteritidis* and certain other O-serogroup D1 *Salmonella* serovars. *Mol cell probes* **10**, 233-246

Douce, G.R., Amin, I.I. & Stephen, J. (1991). Invasion of Hep-2 cells by strains of *Salmonella typhimurium* of different virulence in relation to gastro-enteritis. *J Med Microbiol* **35**, 349-357

Dougan, G., Hormaeche, C.E. & Maskell, D.J. (1987). Live oral *Salmonella* vaccines: potential use of attenuated strains as carriers of heterologous antigens to the immune system. *Immunol* **9**, 151-160

Dreesen, D.W., Barnhart, H.M., Burke, J.L., Chen, T. & Johnson, D.C. (1992). Frequency of *Salmonella enteritidis* and other salmonella in the caeca of spent hens at time of slaughter. *Avian Dis* **36**, 247-250

Drumm, B., Robertson, A.M. & Sherman, P.M. (1988). Inhibition of attachment of *Escherichia coli* RDEC-1 to intestinal microvillus membranes by rabbit ileal mucus and mucin in vitro. *Infect Immun* **56**, 2437-2442

Duebbert, I.E. & Peterson, J.W. (1985). Enterotoxin-induced fluid accumulation during experimental salmonellosis and cholera: involvement of prostaglandin synthesis by intestinal cells. *Toxicon.* **23**, 157-172.

Duchet-Suchaux, M., Lechopier, P., Marly, J., Bernadet, P., Delaunay, R. & Pardon, P. (1995). Quantification of experimental *Salmonella enteritidis* carrier state in B13 leghorn chicks. *Avian Dis* **39**, 796-803

Duchet-Suchaux, M., Mompart, F., Bertholot, F., Beaumont, C., Lechopier, P. & Pardon, P. (1997). Differences in frequency, level, and duration of cecal carriage between four outbred chicken lines infected orally with *Salmonella enteritidis. Avian Dis* **41**, 559-567

Duguid, J.P., Smith, I.W., Dempster, G. & Edmunds, P.N. (1955). Non-flagellar filamentous appendages and haemagglutinating activity in *Bacterium coli. J Pathol Bacteriol* **70**, 335-348.

Duguid, J.L & Gilles, R.R. (1958). Fimbriae and haemagglutinating activity of *Salmonella, Klebsiella, Proteus* and *Chrombacterium. J Pathol Bacteriol* **75**, 519-520.

Duguid, J.P., Anderson, E. & Campbell, I. (1966). Fimbriae and adhesive properties in *Salmonella. J Pathol Bacteriol* **92**, 107-138.

Duguid, J.P., Darekar, M.R. & Wheater, D.W.F. (1976). Fimbriae and infectivity in *Salmonella typhimurium. J Med Microbiol* **9**, 459-473

Dunlap, N.E, Benjamin, W.H., McCall, R.D., Tilden, A.B. & Briles, D.E. (1991). A "safe site" for *Salmonella typhimurium* is within splenic cells during the early phase of infection in mice. *Microbial Pathogen* **10** 297-310

Dwight, C.H. & Zee, Y.H. (1998). *Salmonella.* In: *Veterinary Microbiology* **10**, 75-79. Blackwell Science, London.

Eichelberg, K., Ginocchio, C. & Galàn (1994). Molecular and functional Characterization of the *Salmonella typhimurium* invasion genes *inv*B and *inv*C: homology of InvC to the F_0F_1 ATPase family of proteins. *J Bacteriol* **176**, 45.1-4510

Eisenstein, T.K., Killar, L.M., Stocker, B.A.D. & Sultzer, B.M. (1984). Immunity to infection with *Salmonella typhimurium*: mouse strain differences in vaccine- and serum-mediated protection. *J Infect Dis* **150**, 425-435

Elliot, S.J. & Kaper, J.B. (1997). Role of type 1 fimbriae in EPEC infections. *Microbial Pathogen* **23**, 113-118.

Ernst, R.K., Dombroski, D.M. & Merrick, J.M. (1990). Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of Hep-2 cells by *Salmonella typhimurium*. *Infect Immun* **58**, 2014-2016.

Evans, D.G., Evans, D.J. & Tjoa, W. (1977). Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhoea: correlation with colonisation factor. *Infect Immun* **18**, 330-337.

Evans, S.J. (1996). A case control study of multiple resistant *Salmonella typhimurium* DT 104 infection of cattle in Great Britain. *Cattle Prac* **4**, 259-266

Everest, P., Goosens, H., Butzler, J.P., Lloyd, D., Knutton, S., Ketley, J. & Williams, P.H. (1992). Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *C. coli. J Med Microbiol* **37**, 319-325

Everest, P., Ketley, J., Hardy, S., Douce, G., Khan, S., Shea, J., Holden, D., Maskell., D. & Dougan, G. (1999). Evaluation of *Salmonella typhimurium* mutants in a model of experimental gastro-enteritis. *Infect Immun* **67**, 2815-2821

Ewen, S.W.B., Naughton, P.J., Grant, G., Sojka, M., Allen-Vercoe, E., Bardoocz, S., Thorns, C.J. & Pusztai, A. (1997). *Salmonella enterica* var Typhimurium *and Salmonella enterica* var Enteritidis express type 1 fimbriae in the rat *in vivo*. *FEMS Immunol Med Microbiol* **18**, 185-192

Feldsine, P.T, Falbo-Nelson, M.T. & Hustead, D.L. (1992). Polyclonal enzyme immuno-assay method for the detection pf motile and non-motile *Salmonella* on foods: Collaborative study. *J AOAC Int* **75**, 1032-1044.

Feutrier, J., Kay, W.W. & Trust, T.J. (1988). Cloning and expression of *Salmonella enteritidis* fimbrial genes in *Escherichia coli*. *J Bacteriol* **170**, 4216-4222

Finlay, B.B & Falkow, S. (1990). *Salmonella* interactions with polarised human intestinal Caco-2 epithelial cells. *J Infect Dis* **162**, 1096-1106.

Finlay, B.B, Ruschkowski, S. & Dedhar, S. (1991). Cytoskeletal rearrangements accompanying *Salmonella* entry into epithelial cells. *J Cell Sci* **99**, 283-296.

Finlay, B.B. (1995). Interactions between *Salmonella typhimurium*, Enteropathogenic *E. coli* (EPEC) and host epithelial cells. *Adv Dent Res* **9**, 31-36

Forstner, J.F. & Forstner, G.G. (1994). Gastrointestinal mucus. In: *Physiology of the intestinal tract, third edition,* **33** 1255-1283. Johnson, L.R. (ed). Ravens Press, New York.

Francis, C.L., Starnbach, M.N. & Falkow, S. (1992). Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. *Mol Microbiol* **6**, 3077-3087.

Freter, R., O'Brien, P.C. & Macsai, M.S. (1981). Role of chemotaxis in the association of motile bacteria with the intestinal mucosa: in vivo studies. *Infect Immun* **34**, 234-240

Friedrich, M.J., Kinsey, N.A, Vila, J.& Kadner, R.J. (1993). Nucleotide sequence of a 13.9 kb segment of the virulence plasmid of *Salmonella typhimurium*: the presence of fimbrial biosynthetic genes. *Mol Microbiol* **8**, 543-558.

Fröman, G., Switalski, L.M., Faris, A., Wadström, T. & Höök, M. (1984). Binding of *Escherichia coli* to fibronectin. *J Biol Chem* **10**, 14899-14905

Fromm, D., Gianella, R.A, Formal, S.B., Quiano, R. & Collins, H. (1974). Ion transport across ileal mucosa invaded by *Salmonella. Gastroenterol* **66**, 215-225

Gaastra, W. & de Graaf, F.K. (1982). Host-specific fimbrial adhesins of non-invasive enterotoxigenic *Escherichia coli* strains. *Microbiol Rev* **46**, 129-161

Gahring, L.C., Heffron, F., Finlay, B.B. & Falkow, S. (1990). Invasion and replication of *Salmonella typhimurium* in animal cells. *Infect Immun* **58**, 443-448

Galán, J.E. & Curtiss III, R. (1989). Cloning and molecular characterisation of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. *Proc Natl Acad Sci USA* **86**, 6383-6387

Galán, J.E. & Curtiss III, R. (1990). Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. *Infect Immun* **58**, 1879-18885

Galán, J.E., Pace, J. & Hayman, M.J. (1992). Involvement of the epidermal growth factor receptor in the invasion of cultured mammalian cells by *Salmonella typhimurium. Nature (London)* **357**, 588-589.

Galán, J.E. (1996). Molecular genetic bases of *Salmonella* entry into host cells. *Mol Microbiol* **20**, 263-271

Gamar-Nourani, L., Blondeau, K. & Simonet, J.M. (1998). Influence of culture conditions on exopolysaccharide production by *Lactobacillus rhamnosus* strain C83. *J Appl Microbiol* **85**, 664-672

Garcia-del Portillo, F., Zwick, M.B., Leung,K.Y., & Finlay, B.B. (1993). *Salmonella* induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. *Proc Natl Acad Sci USA* **90**, 10544-10548

Garcia-del Portillo, F., Pucciarelli, W., Jeffries, A. & Finlay, B.B. (1994). *Salmonella typhimurium* induces the selective aggregation and internalisation of host cell surface proteins during invasion of epithelial cells. *J Cell Sci* **107**, 2005-2020.

Gast, R.K. & Beard, C.W. (1990). Production of *Salmonella enteritidis* contaminated eggs by experimentally infected hens. *Avian Dis* **34,** 438-446.

Genovese, K.J., Lowry, V.K., Stanker, L.H. & Kogut, M.H. (1998). Administration of *Salmonella enteritidis*- immune lymphokine to day-old turkeys by subcutaneous, oral and nasal routes: a comparison of effects on *Salmonella enteritidis* liver invasion, peripheral blood heterophilia and heterophil activation. *Avian Pathol* **27**, 597-604

Ginnochio, C., Olmsted, S.B., Wells, C.L. & Galán, J.E. (1994). Contact with epithelial cells induces the formation of surface appendages on *Salmonella typhimurium. Cell* **76**, 717-724

Ginnochio, C., Pace, J. and Galán, J.E. (1993). Identification and molecular Characterization of a *Salmonella typhimurium* gene involved in triggering the internalisation of salmonellae into cultured epithelial cells. *Proc Natl Acad Sci USA* **89**, 5976-5980.

Ginnochio, C. & Galán, J.E. (1995). Functional conservation among members of the *Salmonella typhimurium* InvA family of proteins. *Infect Immun* **63,** 729-732

Gold, B.D., Dytoc, M., Huesca, M., Philpott, D., Kuksis, A., Czin, S., Lingwood, C.L. & Sherman, P.M. (1995). Comparison of *Helicobacter mustelae* and *Helicobacter pylori* adhesion to eukaryotic cells *in vitro. Gastroenterol* **109**, 692-700

Gorham, S.L., Kadavil, K., Lambert, H., Vaughan, E., Pert, B. & Abel, J. (1991). Persistence of *Salmonella enteritidis* in young chicks. *Avian Pathol* **20**, 433-437.

Graeme-Cooke, K.A., May, G., Bremer, E. and Higgens, C.F. (1989). Osmotic regulation of porin expression: a role for DNA supercoiling. *Mol Microbiol* **3**, 1287-1294

Groisman, E.A. & Ochman, H. (1993). Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri. EMBO J* **12**, 3779-3787

Grund, S. and Weber, A. (1988). A new type of fimbriae on *Salmonella typhimurium. J Vet Med* **34**, 779-782.

Grund, S. and Seiler, A. (1993). Fimbriae and lectinophagocytosis of *Salmonella typhimurium. J Vet Med* **40**, 105-112

Grutzkau, A.,Hanski, C., Hahn, H. and O'Riecken, E. (1990). Involvement of M cells in the bacterial invasion of Peyer's patches: a common mechanism shared by *Yersinia enterolitica* and enteroinvasive bacteria. *Gut* **31**, 1011-1015.

Guard-Petter, J., Keller, L.H., Mahbubur Rahman, M., Carlson, R.W. & Silvers, S. (1996). A novel relationship between O-antigen variation, matrix formation, and invasiveness of *Salmonella enteritidis. Epidemiol Infect* **117**, 219-231

Guard-Petter, J., Parker, C.T., Asokan, K. & Carlson, R.W. (1999). Clinical and veterinary isolates of Salmonella enterica serovar *Enteritidis* defective in lipopolysaccharide O-chain polymerisation. *Appl Environ Microbiol* **65**, 2195-2201

Guarino, A., Canini, R.B., Casola, A., Pozio, E., Russo, R., Bruzzese, E., Fontana, M. & Rubino, A. (1995). Human intestinal cryptosporidiosis: Secretory diarrhoea and enterotoxic activity in Caco-2 cells. *J Infect Dis* **171**, 976-983

Guerina, N.G., Langermann, G.S., Schoolnik, G.K., Kessler, T.W. & Goldmann, D.A. (1985). Purification and characterisation of *Haemophilus influenzae* pili and their structural and serological relatedness to *Escherichia coli* P and mannose-sensitive pili. *J Exp Med* **161**, 145-159

Guillot, J., Beaumont, C., Bellatif, F., Moulline, C., Lantier, F., Colin, P. & Protais, J. (1995). Comparison of resistance of various poultry lines to infection by *Salmonella enteritidis. Vet Res* **26**, 81-86

Guinee, P.A.M., Jansen, W.H. and Agterberg. (1976). Detection of the K99 antigen by means of agglutination and immunoelectrophoresis in *Escherichia coli* isolates from calves and it's correction with enterotoxigenicity. *Infect Immun* **13**, 1369-1377.

Guinee, P.A.M. & van Leeuwen, W.J. (1978). Methods in Microbiology, Volume 11: T. Bergan, & J.R. Norriss (ed) London, New York, San Francisco, Academic Press.

Gulig, P.A., Danbara, H., Guiney, D.G., Lax, A.J., Norel, F. and Rhen, M. (1993). Molecular analysis of spv virulence genes of salmonella virulence plasmids. *Mol Microbiol* **7**, 825-830

Hamada, S., Amano, A., Kimura, S., Nakagowa, I., Kawabata, S. & Morisaki, I. (1998). The importance of fimbriae in the virulence and ecology of some oral bacteria. *Oral Microbiol Immunol* **13**, 129-138

Hammar, M., Arnquist, A., Bian, Z., Olsen, A. & Normark, S. (1995). Expression of two csg operons is required for the production of fibronectin and curli-binding polymers of *Escherichia coli* K12. *Mol Microbiol* **18**, 661-670.

Hanson, M.S. & Brinton, C.C. (1988). Identification and Characterisation of the *Escherichia coli* type 1 pilus tip adhesion protein. *Nature (London)* **332**, 265-268.

Hassan, J.O., Mocket, A.P.A., Barrow, P.A. & McLeod, S. (1990). Antibody response to experimental *Salmonella typhimurium* infection in chickens measured by ELISA. *Vet Rec* **126,** 519-522

Hassan, J.O., Mocket, A.P.A., Catty, D. & Barrow, P.A. (1991). Infection and reinfection of chickens with Salmonella typhimurium: Bacteriology and immune responses. *Avian Dis* **35**, 809-819

Hassan, J.O. & Curtiss III, R. (1997). Efficacy of avirulent *Salmonella typhimurium* vaccine in preventing colonisation and invasion of laying hens by *Salmonella typhimurium* and *Salmonella enteritidis*. *Avian Dis.* **41**, 783-791

Hedberg., C.W, David, M.J., White, K.E., MacDonald, K.L. & Osterholm, M.T. (1993). Role of egg consumption in sporadic *Salmonella enteritidis* and *Salmonella typhimurium* infections in Minnesota. *J Infect Dis* **167**, 107-111

Hendrickson, B.A, Guo, J., Laughlin, R., Chen, Y. & Alverdy, J.C. (1999). Increased type 1 fimbrial expression among commensal *Escherichia coli* isolates in the murine ceacum following catabolic stress. *Infect Immun* **67**, 745-753

Henge-Aronis, R. (1996). Regulation of gene expression during entry into stationary phase, pp 1497-1512. In: F.C. Neidhardt, R. Curtiss III., J.L.Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S.Resnikoff, M.Riley, M. Schaechter & H.E. Umbarger (ed), *Escherichia coli and Salmonella Cellular and Molecular Biology*, ASM Press, Washington, D.C.

Henle, G. & Deinhardt, F. (1956). The establishment of strains of human cells in tissue culture. *Soc Exp Biol Med* **79**, 54-59

Hinton, M., Pearson, G.R., Threlfall, E.J., Rowe, B., Woodward, M.J. & Wray, C. (1989). Experimental *Salmonella enteritidis* infection in chicks. *Vet Rec* **112**, 223

Hinton, M., Threlfall, E.J. and Rowe, B. (1990). The invasiveness of different strains of *Salmonella enteritidis* phage type 4 for young chickens. *FEMS Microbiol Lett* **70**; 193-196

Hirota, Y., Suzuki, H.,Nishimura, Y. and Yasuda, S. (1977). On the process of cellular division in *Escherichia coli*: a mutant of *Escherichia coli* lacking a murein lipopolysaccharide. *Proc Natl Acad Sci USA* **74**, 1417-1420.

Hogue, A., White, P., Guard-Petter, J., Schlosser, W., Gast, R., Ebel, E., Farrar, J., Madden, J., Madison, M., McNamara, A.M., Morales, R., Parham, D., Sparling, P., Sutherlin, W. & Swerdlow, D. (1997). Epidemiology and control of egg associated *Salmonella enteritidis* in the United States of America. *Rev Sci Tech Off Int Epiz* **16**, 542-553

Holt, P.S. (1993). Effect of induced molting on the susceptibility of white leghorn hens to a *Salmonella enteritidis* infection. *Avian Dis* **37**, 412-417

Holt, P.S. & Porter, R.E. (1993). Effect of induced molting on the recurrence of a previous *Salmonella enteritidis* infection. *Poult Sci* **72**, 2089-1993

Holt, P.S., Mitchell, W. & Gast, R.K. (1998). Airborne horizontal transmission of *Salmonella enteritidis* in molted laying chickens. *Avian Dis* **42**, 45-52

Hopper, S.A. & Mawer, S. (1988). *Salmonella enteritidis* in a commercial layer flock. *Vet Rec* **123**, 351

Hoszowski, A. & Truszczynski, M. (1997). Prevention of *Salmonella typhimurium* caecal colonisation by different preparations for competitive exclusion. *Comp Immun Microbiol Infect Dis* **20**, 111-117

Hultgren, S.J. and Normark, S. (1991). Chaperone assisted assembly and molecular architecture of adhesive pili. *Ann Rev Microbiol* **45**, 383-415

Hume, M.E., Corrier, D.E., Nisbet, D.J. & DeLoach, J.R. (1998). Early *Salmonella* challenge time and reduction in chick cecal colonisation following treatment with a characterised competitive exclusion culture. *J Food Protect* **61**, 673-676

Humphrey, T.J.,Chart, H., Baskerville, A. & Rowe, B. (1990). The influence of age on the response of SPF hens to infection with *Salmonella enteritidis* PT4. *Epidemiol Infect* **106**, 33-43

Humphrey, TJ & Rowe, B. (1988). Poultry meat as a source of human salmonellosis in England and Wales. *Epidemiol Infect* **100**, 175-184

Humphrey, T.J., Williams, A., McAlpine, K., Lever, M.S., Guard-Petter, J. & Cox, J.M. (1996). Isolates of *Salmonella enterica* Enteritidis PT4 with enhanced heat and acid tolerance are more virulent in mice and more invasive in chickens. *Epidemiol Infect* **117**, 79-88

Humphrey, T.J., Whitehead, A., Gawler, A.H.L., Henley, A. & Rowe, B. (1991). Numbers of *Salmonella enteritidis* in the contents of naturally contaminated eggs. *Epidemiol Infect* **106**, 489-496.

Iino, T. (1977). Genetics of structure and function of bacterial flagella. *Ann Rev Genet* **11**, 161-182

Ingeldew, W.J. & Poole, R.K. (1984). The respiratory chains of *Escherichia coli. Microbiol Rev* **48**, 222-271

Iredell, J.R. & Manning, P.A. (1994). The toxin co-regulated pilus of *Vibrio cholera* 01: a model for type 4 pilus biogenesis. *Trends Microbiol* **2**, 187-192

Isaacson, R.E & Kinsel, M. (1992). Adhesion of *Salmonella typhimurium* to porcine intestinal surfaces: identification and Characterisation of two phenotypes. *Infect Immun* **60**, 3193-3200

Jackson, A.D., Dowling, R.B. & Wilson, R. (1998). Interaction of bacteria and their products with tissues in organ culture. *Methods Microbiol* **27**, 73-81

Jallet, C., Darfeuille-Michaud, A., Rich, C. & Joly, B. (1994). Survey of clinical isolates of diarrhoeogenic *Escherichia coli*: diffusely adhering *E. coli* strains with multiple adhesive factors. *Res Microbiol* **145**, 621-632

Jeurissen, S.H.M., Janse, E.M., Koch, G. & de Boar, G.F. (1989). Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Res* **258,** 119-124

Jerse, A.E., Yu, J., Tall, B.D. & Kaper, J.B. (1990). A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions in tissue culture cells. *Proc Natl Acad Sci USA* **87**, 7839-7843

Johnson, W.D. & Pape, J.W. (1983). Salmonellosis (other than typhoid fever) pp. 65-67. In: H.F. Conn (ed.), Current Therapy, W.B. Saunders, Philadelphia, P.A., USA.

Jones, B.D., Ghori, N. & Falkow, S. (1994). *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialised epithelial cells of the Peyer's patches. *J Exp Med* **180**, 15-23

Jones, B.D., Lee, C.A. & Falkow, S. (1992). Invasion by *Salmonella typhimurium* is affected by the direction of flagellar rotation. *Infect Immun* **60,** 2475-2480

Jones, B.D. & Falkow, S. (1994). Identification and characterisation of a Salmonella typhimurium oxygen-regulated gene required for bacterial internalisation. *Infect Immun* **62, 3745-3752**

Jones, C.H., Pinkner, J.S.,Nicholes, A.V.,Slonim, L.N., Abraham, S.N. & Hultgren,S.J. (1993). FimC is a periplasmic papD-like chaperone that directs assembly of type 1 pili in bacteria. *Proc Natl Acad Sci USA* **90**, 8397-8401

Jones, G.W. & Freter, R. (1976). Adhesive properties of *Vibrio cholerae*: Nature of the interaction with isolated brush-border membranes and human erythrocytes. *Infect Immun* **14,** 240-245

Kaback, H.R., Jung, K., Jung, H., Wu, J., Prive, G.G. & Zen, K. (1993). What's new with lactose permease. *J Bioenerg Biomembr* **25**, 627-636.

Kaniga, K., Bossio, J.C. & Galàn (1994). The *Salmonella typhimurium* invasion genes *inv*F and *inv*G encode homologues to the PulD and AraC family of proteins. *Mol Microbiol* **13**, 555-568

Kaniga, K., Tucker, S.C., Trollinger, D. & Galàn (1995a). Homologues of the *Shigella* invasins lpaB and lpaC are required for *Salmonella typhimurium* entry into cultured cells. *J Bacteriol* **177**, 3965-3971

Kaniga, K., Trollinger, D. & Galàn (1995b). Identification of two targets of the type III secretion system encoded in the *inv* and *spa* loci of *Salmonella typhimurium* that share homology to lpaD and lpaA proteins. *J Bacteriol* **177**, 7078-7085

Karem, K.L., Chatfield, S., Kuklin, N. & Rouse (1995). Differential induction of carrier antigen-specific immunity by *Salmonella typhimurium* live-vaccine strains after single mucosal or intravenous immunisation of BALB/c mice. *Infect Immun* **63**, 4557-4563

Kauffmann, F. (1964). Das Kauffmann-White-Scheme, p.21-66. *In* E. van Oye (ed.), The world problem of Salmonellosis. W. Junk Publishers, The Hague, The Netherlands.

Kaura, Y.K., Sharma, V.K. & Chanditamani, N.K. (1982). Enterotoxigenicity and invasiveness of *Salmonella* species. *Anthony van Leeuwenhock* **48**, 273-283.

Keith, B.R, Harris, S.L., Walker, P.R. & Orndorff, P.E. (1990). Effect of type 1 piliation on *in vitro* killing of *Escherichia coli* by mouse peritoneal macrophages. *Infect Immun* **58**, 3448-3454

Keller, L.A., Benson, C.E., Krotec, K. & Eckroade, R.J. (1995). *Salmonella enteritidis* colonisation of the reproductive tract and forming and freshly laid eggs of chickens. *Infect Immun* **63**, 2443-2449

Keren, D.F. (1992). Antigen processing in the mucosal immune system. *Semin Immunol* **4,** 217-226

Kerr, S., Ball, H.J., Mackie, D.P., Pollack, D.D. & Findley, D.A. (1992). Diagnostic application of monoclonal antibodies to outer membrane proteins for rapid detection of *Salmonella. J Appl Bacteriol* **72,** 302-308

Ketyi, I., Pacsa, S., Emody, L., Vertenyi, A.,Kocsis, B. & Kuch, B. (1979). *Shigella, dysenteriae* 1-like cytotoxic enterotoxins produced by *Salmonella* strains. *Acta Microbiol Immunol Hung* **26,** 217-223

Kim, C.J., Nagaraja, K.V. & Pomeroy, B.S. (1991). Enzyme-linked immunosorbant assay for the detection of *Salmonella enteritidis* in chickens. *Amer J Vet Res* **52,** 1069-1074

Kirov, S.M., Sanderson, K. & Dickson, T.C. (1998). Characterisation of a type 1V pilus by *Aeromonas caviae. J Med Microbiol* **47,** 527-531

Klauser, T., Pohlner, J. & Meyer, T.F. (1993). The secretion pathway of IgA protease-type proteins in gram-negative bacteria. *Bioassays* **15;** 799-805

Kogut, M.H., Guillermo T., Hargis, B.M., Corrier, D.E. & DeLoach, J.R. (1993). The effect of 5-fluorouracil treatment of chicks: A cell depletion model for the study of avian polymorphonuclear leukocytes and natural host defences. *Poult Sci* **72,** 1873-1880

Kogut, M.H., Genovese, K., Moyes, R.B. & Stanker, L.H. (1998). Evaluation of oral, subcutaneous, and nasal administration of *Salmonella enteritidis* immune lymphokines on the potentiation of the protective heterophilic inflammatory response to *Salmonella enteritidis* in day old chicks. *Can J Vet Res* **62,** 27-32

Kogut, M.H., Tellez, G.I., McGruder, E.D., Hargis, B.M., Williams, J.D., Corrier, D.E. & DeLoach, J.R. (1994). Heterophils are decisive components in the early response of chickens of *Salmonella enteritidis* infections. *Microbial Pathogen* **16,** 141-151

Köhler,G. & Milstein,C. (1975). The continuous cultures of fused cells secreting antibodies of a predefined specificity. *Nature (London)* **256**, 495-497

Kondoh, H & Hotani, H. (1974). Flagellin from *Escherichia coli* K12:polymerization and molecular weight in comparison with *Salmonella* flagellins. *Biochem Biophys Acta* **336,** 117-139

Koo, F.C.W. & Peterson, J.W. (1983). *Salmonella* inhibit protein synthesis and cause cytotoxicity in eukaryotic cells. *Toxicon* **21**, 309-320

Korhonen, T.K., Lounatmaa, K., Ranta, H. & Kuusi, N. (1980). Characterization of type 1 pili of *Salmonella typhimurium* LT2. *J Bacteriol* **144**, 800-805.

Kottom, T.J., Nolan, L.K. & Brown, J. (1995). Invasion of Caco-2 cells by *Salmonella typhimurium* (Copenhagen) isolates from healthy and sick chickens. *Avian Dis* **39**, 867-872

Kowalczyk, K., Daiss, J., Halpern, J. & Roth, T.F. (1985). Quantitation of maternal-fetal IgG transport in the chicken. *Immunol* **54**, 755

Krogfelt, K.A., Bergmans, H. & Klemm, P. (1990). Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae. *Infect Immun* **58,** 1995-1999

Kuehn, M., Normark, S. & Hultgren. (1991). Immunoglobulin-like PapD chaperone caps and uncaps interactive surfaces of nascently translocated pilus subunits. *Proc Nat Acad Sci USA* **88**, 10586-10590.

Kukkonen, , M., Raunio, T. & Virkola, R. (1993). Basement membrane carbohydrate as a target for bacterial adhesion: binding of type 1 fimbriae of *Salmonella enterica* and *Escherichia coli* to laminin. *Mol Microbiol* **7**, 229-237.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680-685

Lee, C.A. & Falkow, S. (1990). The ability of *Salmonella* to enter mammalian cells is affected by growth state. *Proc Natl Acad Sci USA* **87**, 4304-4308

Lee, M.D., Curtiss, R. & Peay, T. (1996). The effect of bacterial surface structures on the pathogenesis of *Salmonella typhimurium* infections in chickens. *Avian Dis* **40**, 28-36.

Lee, G.M., Jackson, G.D.F. & Cooper, G.N. (1981). The role of biliary antibodies and cell mediated immunity in the clearance of *S. typhimurium* from chickens. *Vet Immunol Immunopathol* **2**, 233-252

Lee, G.M., Jackson, G.D.F. & Cooper, G.N. (1982). Infection and immune responses in chickens exposed to *Salmonella typhimurium. Avian Dis.* **27**, 577-583

Leslie, G.A. & Clem, L.W. (1969). Phylogeny of immunoglobulin structure and function. *J Exp Med* **130**, 1337

Lesuffleur, T., Barbat, A., Luccioni, C., Beaumatin, J., Clair, M., Kornowski, A., Dussaulx, B., Dutrillaux, B. & Zweibaum, A. (1991). Dihydrofolate reductase gene amplification in methotrexate-adapted HT-29 cells. *J Cell Biol* **115**, 1409-1418

Leung, K.Y. & Finlay, B.B. (1991). Intracellular replication is essential for the virulence of *Salmonella typhimurium. Proc Nat Acad Sci USA* **88**, 11470-11474.

Leunk, R.D. and Moon, R.J. (1982). Association of type 1 fimbriae with the ability of livers to clear *Salmonella typhimurium. Infect Immun* **36**, 1168-1174

Lim, J.K., Gunther, N.W., Zhao, H., Johnson, D.E., Keay, S.K. & Mobley, H.L.T. (1998). *In vivo* phase variation of *Escherichia coli* type 1 fimbrial genes in women with urinary tract infection. *Infect Immun* **66**, 3303-3310

Lindberg, A.A. (1980). Bacterial virulence factors- with particular reference to *Salmonella* bacteria. *Scand J Infect Dis* **24**, 86-92

Lindell, K.A., Saeed, A.M. & MacCabe, G.P. (1994). Evaluation of resistance of four strains of commercial laying hens to experimental infection with *Salmonella enteritidis* phage type eight. *Poult Sci* **73**, 757-762

Lindquist, B.L., Lebenthal, E., Lee, P.C., Stinson, M.W. & Merrick, J.M. (1987). Adherence of *Salmonella typhimurium* to small intestine enterocytes of the rat. *Infect Immun* **55**, 3044-3050.

Lister, S.A. (1988). *Salmonella enteritidis* infection in broilers and broiler breeders. *Vet Rec* **123**, 350.

Lockman, H.A. & Curtiss, R. (1990). *Salmonella typhimurium* mutants lacking flagella or motility remain virulent in BALB/c mice. *Infect Immun* **58**, 137-143

Lockman, H.A. & Curtiss, R. (1992). Virulence of non-type 1 fimbriated non-flagellated *Salmonella typhimurium* mutants in murine typhoid fever. *Infect Immun* **60**, 491-496

Lockman, H.A. & Curtiss, R. (1992). Isolation and characterisation of conditionally adherent and non-type 1 fimbriated *Salmonella typhimurium* mutants. *Mol Microbiol* **6**, 933-945

Lodge, J. Douce, G.R., Amin, I.I., Bolton, A.J., Martin, G.D., Chatfield, S., Dougan, G., Brown, N.G. & Stephen, J. (1995). Biological and genetic characterisation of Tn*ph*A mutants of *Salmonella typhimurium* TML in the context of gastro-enteritis. *Infect Immun* **63**, 762-769

Mack, D.R., Michail, S., Wei, S., McDougall, L. & Hollingsworth, M.A. (1999). Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. *Am J Physiol* **276**, 941-950

Macnab, R.M. (1992). Genetics and biogenesis of bacterial flagella. *Ann Rev Genet* **26**, 129-156

Madeiros, A.A., O'Brien, T.F., Rosenberg, E.Y. & Nikaido, H. (1987). A strain of *S. typhimurium* became more resistant to cephalosporins during therapy because it lost it's OmpC porin. *J Infect Dis* **156**, 751-757

Manafi, M. & Sommer, R. (1992). Comparison of three rapid screening methods for *Salmonella spp.*: MUCAP Test, Microscreen Latex and Rambach Agar. *Lett Appl Microbiol* **14**, 1163-166.

Manson, M.D., Tedesco, P., Berg, H.C., Harold, F.M. & van der Drift, C. (1977). A proton motive force drives bacterial flagella. *Proc Natl Acad Sci USA* **74**, 3060-3064.

Maston, B.G. & Joys, T.M. (1993). Molecular analyses of the *Salmonella* g...Flagellar Antigen Complex. *J Bacteriol* **175**, 5359-5365

McDermid, A.S., Makee, A.I., Dowsett, A.B. & Marsh, P.D. (1996). The effect of pH on the physiology and the surface structures of *Salmonella* serotype Enteritidis phage type 4. *J Mol Microbiol* **45**, 452-458

McLaren, I.M., Sojka, M.G., Thorns, C.J. & Wray, C. (1992). An interlab trial of a latex agglutination kit for the rapid identification of *Salmonella enteritidis. Vet Rec* **131**, 235-236

Migaki, T.T. & Babcock, W.E. (1981). Evaluation of an improved floor pen model for severe coccidiosis. *Avian Dis* **27**, 1-6

Miller, S.I. (1991). PhoP/PhoQ: macrophage -specific modulators of *Salmonella* virulence? *Mol Microbiol* **5**, 2073-2078

Mills, D.B., Bajaj, V. & Lee, C.A. (1995). A 40 kilobase chromosomal fragment encoding *Salmonella typhimurium* invasion gene is absent from the corresponding region of *Escherichia coli* K-12 chromosome. *Mol Microbiol* **15**, 749-759

Mills, S.D., & Finlay, B.B. (1994). Comparison of *Salmonella typhi* and *Salmonella typhimurium* invasion, intracellular growth and localisation in cultured human cells. *Microb Pathog* **17**, 409-423

Mills, S.D., Ruschowsky, S.R., Stein, M.A. & Finlay, B.B. (1998). Trafficking of porin-deficient *Salmonella typhimurium* mutants inside HeLa cells: *omp*R and *env*Z mutants are defective for the formation of *Salmonella*-induced filaments. *Infect Immun* **66**, 1806-1811

Minion, F.C., Abraham, S.N., Beachey, E.H. & Gogeun, J.D. (1986). The genetic determinant of adhesive function in type 1 fimbriae of *Escherichia coli* is distinct from the gene encoding the fimbrial subunit. *Infect Immun* **165**, 1033-1036

Miras, I., Hermant, D., Arricau, N. & Popoff, M.Y. (1995). Nucleotide sequence of *iag*A and *iag*B genes involved in invasion of HeLa cells by *Salmonella enterica* subsp. enterica ser. Typhi. *Res Microbiol* **146**, 17-20

Morse, E.V. & Duncan, M.A. (1974). Salmonellosis: an environmental health problem. *J Am Vet Med Assoc* **165**, 1015-1019

Mounier, J., Vasselon, T., Hellio, R., Lesourd, M. & Sasonetti, P.J. (1992). *Shigella flexineri* enters the human colonic Caco-2 epithelial cells through the basolateral pole. *Infect Immun* **60**, 237-248

Muir, W.I., Bryden, W.L. & Husband, A.J. (1998). Comparison of *Salmonella typhimurium* challenge models in chickens. *Avian Dis* **42**, 257-264

Müller, K.H., Collinson, K.S., Trust, T.J. & Kay, W.W. (1991). Type 1 fimbriae of *Salmonella. J Bacteriol* **173**, 4765-72

Mulvey, M.A., Lopez-Boado, Y.S, Wilson, C.L., Roth, R., Parks, W.C., Heuser, J. & Hultgren, S.J. (1998). Induction and evasion of host defences by type-1 piliated uropathogenic *Escherichia coli. Science* **282**, 1494-1497

Murray, C.J. (1991). *Salmonellae* in the environment. *Rev Sci Tech* **3**, 765-785

Namba, K., Yamashita, I. & Vondervizi, F. (1989). Structure of the core and central channel of bacterial flagella. *Nature (London)* **342**, 648-654

Neutra, M.R. & Kraehenbuhl, J.P. (1993). The role of the transepithelial transport of M cells in microbial invasion and host defence. *J Cell Sci* **17**, 209-215

Nikaido, H. (1992). Porins and specific channels of bacterial outer membranes. *Mol Microbiol* **6**, 435-442

Nikaido, H. & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49**, 1-32.

Nisbet, D.J., Corrier, D.E. & DeLoach, J.R. (1993). Effect of mixed cecal microflora maintained in continuous culture and of dietary lactose on *Salmonella typhimurium* colonisation of broiler chicks. *Avian Dis* **37**, 528-535

Nolan, L.K., Wooley, R.E., Brown, J. & Payeur, J.B. (1991). Comparison of phenotypic characteristics of *Salmonella spp.* isolated from healthy and ill (infected) chickens. *Am J Vet Res* **52**, 1512-1517

Nurmi, E.V. & Rantala, M. (1973). New aspects of *Salmonella* infection in broiler production. *Nature (London)* **241**, 210

Ogunniyi, A.D., Kotlarski, I., Morona, R. & Manning, P.A. (1997). Role of sefA subunit protein of SEF14 fimbriae in the pathogenesis of *Salmonella enterica* serovar Enteritidis. *Infect Immun* **65**, 708-717

Old, D.C. & Adegbola, R.A. (1985). Antigenic relationships among type 3 fimbriae of Enterobacteriaceae revealed by immunoelectron microscopy. *J Med Microbiol* **20**, 113-21

Old, D.C., Roy, A.I. & Tavendale, A. (1986). Differences in adhesiveness among type 1 fimbriate strains of enterobacteriaceae as revealed by an *in vitro* Hep-2 cell adhesion assay. *J Appl Bacteriol* **61**, 563-568

Olsén, A., Arnqvist, A., Hammar, M. & Normark, S. (1994). Environmental regulation of curli production in *Escherichia coli*. *Infect Agents Dis* **2**, 272-274

Olsén, A., Arnqvist, A., Hammar, M., Sukopolvi, S. & Normark, S. (1993). The RpoS sigma factor relieves H-NS-mediated transcriptional repression of csgA, the subunit gene of fibronectin-binding curli in *Escherichia coli*. *Mol Microbiol* **7**, 523-536

Olsén, A., Johnson, A & Normark, S. (1989). Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. *Nature (London)* **338**, 655-658

Olsén, P.B., Schembri, M.A., Gally, D.L. & Klemm, P. (1998). Differential temperature modulation by H-NS of the *fim*B and fimE recombinase genes which control the orientation of the type 1 fimbrial phase switch. *FEMS Microbiol Lett* **162**, 17-23

Orskov, I. & Orskov, F. (1990). Serological classification of fimbriae. *Curr Top Microbiol Immunol* **151**, 71-90

Orskov, I., Orskov, F., Smith, H.W. & Sojka, W.J. (1975). The establishment of K99, a thermolabile, transmissible *Escherichia coli* K antigen, previously called "KCo" possessed by calf and lamb enteropathogenic strains. *Acta Pathol Microbiol Scand Sec* **88**, 31-36

Osaki, T., Yamaguchi, H., Taguchi, H., Fukuda, M., Kawakami, H., Hirano, H., Watanabe, S., Takagi, A. & Kamiya, S. (1998). Establishment and characterisation of a monoclonal antibody to inhibit adhesion of *Helicobacter pylori* to gastric epithelial cells. *J Med Microbiol* **47**, 505-512

Owen, R.L. & Jones A.L. (1974). Epithelial cell specialisation within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastoenterol* **66**, 189-203

Oyofo, B.A., DeLoach, J.R., Corrier, D.E., Norman, J.O., Ziprin, R.L. & Mollenhauser, H.H. (1988). Prevention of *Salmonella typhimurium* colonisation of broilers with D-mannose. *Poult Sci* **68,** 1357-1360

Oyofo, B.A., DeLoach, J.R., Corrier, D.E., Norman, J.O., Ziprin, R.L. & Mollenhauser, H.H. (1989). Effects of carbohydrates on *Salmonella typhimurium* colonisation in broiler chickens. *Avian Dis* **33**, 531-534

Pace, J., Hayman, M.J. & Galan, J.E. (1993). Signal transduction and invasion of epithelial cells by *Salmonella typhimurium. Cell* **72**, 505-514

Paerregaard, A., Esperson, F., Jenson, O.M. & Skirnic, M. (1991). Interactions between *Yersinia enterolitica* and rabbit ileal mucus: growth, adhesion, penetration and subsequent changes in surface hydrophobicity and ability to adhere to ileal brush border membrane vesicles. *Infect Immun* **59**, 253-260

Pardon, P., Popoff, M.Y., Coynault, C. Marly, J. & Miras, I. (1986). Virulence associated plasmids of *Salmonella typhimurium* in experimental murine infection. *Ann Inst Pasteur Microbiol* **137**, 47-60

Parish, C.R., Ristar, R. & Ada, G.L. (1969). Cleavage of bacterial flagellin with cyanogen bromide. *Biochem J* **113**, 501-506

Parkinson, J.S. (1993). Signal transduction schemes of bacteria. *Cell* **73,** 857-871

Parry, S.H. & Rooke, D.M. (1985). Adhesins and colonisation factors of *Escherichia coli* pp79-155, *Special Publications for the Society of General Microbiology*: The Virulence Markers of *Escherichia coli.* M. Sussman (ed). London, New York, Acedemic Press.

Pegues, D.A., Hantman, M.J., Behlau, I. & Miller, S.I. (1995). PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. *Mol Microbiol* **17**, 169-181

Pessina, A., Raimonde, A. & Neri, M.G. (1995). Different biological conditions influencing bacterial adherence assays. *J Chemother* 7, 8-11

Peterson, J.W., Molina, N.C., Houston, C.W. & Fader, R.C. (1983). Elevated cAMP in the intestinal epithelial cells during experimental cholera and salmonellosis. *Toxicon* **21**, 761-775

Pinto M., Robine-Leon, S., Appay, M.D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, Haffen, K., Fogh, J. & Zweibaum, A. (1983). Enterocyte-like differentiation and polarisation of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* **47**, 323-330

Pivnick, H. & Nurmi, E. (1982). The Nurmi concept and its role in the control of *Salmonella* in poultry. In: Davies, R. (ed) *Developments in Food Microbiology,* Vol.1. Barking, Applied Science Publishers.

Pratt, L.A. & Silhavy, T.J. (1995). Porin regulation of *Escherichia coli*, p.105-127. In J.A. Hoch and Silhavy (ed.), Two-component signal transduction. ASM Press, Washington, D.C.

Promsopone, B., Morishita, T.Y., Aye, P.P., Cobb, C.W., Veldkamp, A. & Clifford, J.R. (1998). Evaluation of an avian-specific probiotic and *Salmonella typhimurium*-specific antibodies on the colonisation of *Salmonella typhimurium* in broilers. *Int J Food Microbiol* **61**, 176-180

Rajashekara, G., Munir, S., Lamichhane, C.M., Back, A., Kaper, V., Halvorson, D.A. & Nagaragi, K.V. (1998). Application of recombinant fimbrial protein for the specific detection of *Salmonella enteritidis* infection in poultry. *Diagn Microbiol Infect Dis* **32**, 147-157

Reitmeyer, J.C., Peterson, J.W. & Wilson, K.J. (1986). *Salmonella* cytotoxin: a component of the outer membrane. *Microbial Pathogen* **1**, 503-510

Reynolds, D.J., Davies, R.H., Richards, M. & Wray, C. (1997). Evaluation of combined antibiotic and competitive exclusion treatment in broiler flocks infected with *Salmonella enterica* serovar Enteritidis. *Avian Pathol* **26**, 83-95

Roberts, J.A & Sockett, P.N. (1994). The socio-economic impact of human *Salmonella enteritidis* infection. *Int J Food Microbiol* **21**, 117-129

Rocha, C.L. & Fischetti, V.A. (1999). Identification and characterisation of a novel fibronectin-binding protein on the surface of Group A *Streptococci. Infect Immun* **67**, 2720-2728

Rodriguez-Boulan, E. & Nelson, W.J. (1989). Morphogenesis of the polarised epithelial cell type. *Cell* **245**, 718-725

Rodrigue, D.C., Tauxe, R.V. & Rowe, B. (1990). International increase in *Salmonella enteritidis*: a new pandemic. *Epidemiol Infect* **105**, 21-27

Römling, U., Bian, Z., Hammar, M., Sierraita, W.D. & Normark, S. (1998a). Curli fibres are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and function. *J Bacteriol* **180,** 722-731

Römling, U., Sierraita, W.D., Eriksson, K & Normark, S. (1998b). Multicellular aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agf*D promoter. *Mol Microbiol* **28,** 249-264

Rossilini, G.M., Muscas, P., Chiesurin, A. & Satta, G. (1993). Analysis of the *Salmonella* fim cluster: identification of a new gene (fimI) encoding a fimbrin-like protein and located down stream from the *fim*A gene. *FEMS Microbiol Letts* **114**, 259-266

Rosenberg, M. & Kjelleberg (1986). Hydrophobic interactions: role in bacterial adhesion, Vol. 9, pp. 353-393. In: Marshall, K.C. (ed), Advances in Microbial Ecology, Plenum Press, New York and London.

Rosenshine, I., Duronio, V. & Finlay, B.B. (1992). Tyrosine protein kinase inhibitors block invasin-promoted bacterial uptake by epithelial cells. *Mol Microbiol* **60**, 2211-2217

Rosquist, R., Hakansson, S., Forsberg, A., & Wolf-Watz, H. (1995). Functional conservation of the secretion and translocation machinery for virulence proteins of *Yersinia, Salmonellae* and *Shigellae. EMBO J* **14**, 4187-4195

Ruschkowski, S., Rosenshine, I. & Finlay, B.B. (1992). *Salmonella typhimurium* induces an inositol phosphate flux in infected epithelial cells. *FEMS Microbiol Lett* **95**, 121-126

Russell, P.W. & Orndorff, P.E. (1992). Lesions in two Escherichia coli type 1 pilus genes alter lipus number and length without affecting receptor binding. *J Bacteriol* **174**, 5923-5929

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. & Arnheim, N. (1985). Enzymic amplification of β-globulin sequences and restriction site analysis of sickle cell anaemia. *Science* **230**, 1350-1354

Sasaki, S., Matsuyama, S.I. and Mizushima, S. (1990). *In vitro* analysis of the role of the positively charge at the amino-terminal region of signal peptides in translocation of secretory protein across the cytoplasmic membrane in *Escherichia coli. J Biol Chem* **265**, 4358-4363

Savage, D.W. (1987). Factors influencing biocontrol of bacterial pathogens in the intestine. *Food Technology* **41**, 82-87

Schaechter, M., Medoff, G. and Eisenstein, B.I. (1989). Mechanisms of Microbial Disease. The Wilkins and Williams Company, Baltimore, USA.

Schat, K.A. & Myers, T.J. (1991). Avian intestinal immunity. *Crit Rev Poult Biol* **3**, 19-34

Schmitt, C.K., Darnell, S.K., Tesh, V.L., Stocker, B.A. & O'Brien, A.D. (1994). Mutation of *flg*M attenuates virulence of *Salmonella typhimurium*, and mutation of *fli*A represses the attenuated phenotype. *J Bacteriol* **176**, 368-377

Schiemann, D.A. (1995). Association with MDCK epithelial cells by *Salmonella typhimurium* is reduced during utilisation of carbohydrates. *Infect Immun* **63**, 1462-1467

Schlelfer, K.D. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and there taxonomic implications. *Bacteriol Rev* **36**, 407-477

Schweizer, M., Hindennach, I., garten, W. & Henning, U. (1978). Major proteins of *Escherichia coli* outer cell envelope membrane. Interaction of protein with lipopolysaccharide. *Eur J Biochem* **82** 211-217

Sharma, J.M. (1991). Avian cell-mediated immunity. Boca Raton, CRC Press.

Sizemore, D.R., Fink, P.S., Ou, J.T., Baron, L.S., Kopecko, D.J. & Warren, R.L. (1991). Tn5 mutagenesis of the *Salmonella typhimurium* 100kb plasmid: definition of a new virulence region. *Mol Microbiol* **10**, 493-499

Smith, H.W. (1955). Observations on experimental fowl typhoid. *J Comp Pathol* **65**, 37-54

Smith, H.W. (1956). The use of live vaccines in experimental *Salmonella gallinarum* infection in chickens with observations on their interference effect *J Hyg* **54**, 433

Smyth, C.J., Marrion, M. & Smith, S.G. (1994). Fimbriae of *Escherichia coli* pp399-435. In: *Escherichia coli* in Domestic Animals and Humans. Gyles, C.L. (ed), CAB International UK

Sneller, M.C. & Strober, W (1986). M cells and host defence. *J Infect Dis* **154**, 737-741

Snoeyenbos, G.H., Smyser, C.F. & van Rocekel, H. (1969). *Salmonella* infections of the ovary and peritoneum of chickens. *Avian Dis* **13** 668-670

Sojka, M.G., Carter, M.A. & Thorns, C.J. (1998). Characterisation of type 1 fimbriae of *Salmonella* using monoclonal antibodies specific for SEF21 fimbriae of *Salmonella enteritidis*. *Vet Microbiol* **59**, 157-174

Sojka, M.G., Dibb-Fuller, M.P. & Thorns, C.J. (1996). Characterisation of monoclonal antibodies specific to SEF21 of *Salmonella enteritidis* and their reactivity with other Salmonellae and Enterobacteria. *Vet Microbiol* **48**, 207-221.

Sojka, W.J. (1971). Enteric disease in new born piglets and calves due to *Escherichia coli* infection. *Vet Bulletin* **41**, 509-522

Soto, G.E., Dodson, K.W., Ogg, D., Lui, C., Heuser, J., Knight, S., Kihlberg, J., Jones, C.H. & Hultgren, S.J. (1998). Periplasmic chaperone recognition motif of subunits mediates quarternary interactions in the pilus. *EMBO J* **17**, 6155-6167

Spencer, J.L., Chambers, J.R. & Modler, H.W. (1998). Competitive exclusion of *Salmonella typhimurium* in broilers fed with vermicompost and complex carbohydrates. *Avian Pathol* **27**, 244-249

Stavric, S., Gleeson, T.M. & Blanchfield, B. (1991). Efficacy of undefined and defined bacterial treatment in competitive exclusion of *Salmonella* from chicks. In Blanchenship, L.C. (ed) Colonisation Control of Human Bacterial Enteropathogens in Poultry, San Diego, CA. Academic Press.

St. Louis, M.E., Morse, D.L., Potter, M.E., DeMelfi, T.M., Guzewich, J.J., Tauxe, R.V. & Blake, P.A. (1988). The emergence of grade A eggs as a major source of *Salmonella enteritidis* infections. New implications for the control of salmonellosis. *J Am Vet Med Assoc* **259**, 2103-2107.

Stolpe, H., Grund, S. & Schroder, W. (1994). Purification and partial characterisation of type 3 fimbriae of *Salmonella typhimurium* var. copenhagen. *Zbl Bakt* **281**, 8-15

Stone, B.J., Garcia, C.M., Badger, J.L., Hassett, T., Smith, R.I.F. & Miller, V.L. (1992). Identification of novel loci affecting entry of *Salmonella enteritidis* into eukaryotic cells. *J Bacteriol* **174**, 3945-3952

Sukupolvi, S., Lorenz, R.G., Gordon, J.I., Bian, Z., Pfeifer, J.D. Normark, S.J. & Rhen, M. (1997). Expression of thin aggregative fimbriae promotes interaction of *Salmonella typhimurium* SR-11 with mouse small intestinal epithelial cells. *Infect Immun* **65**, 5320-532

Sukupolvi, S., Lorenz, R.G., Gordon, J.I., Bian, Z., Pfeifer, J.D., Normark, S.J. & Rhen, M. (1997). Expression of thin aggregative fimbriae promotes interaction of *Salmonella typhimurium* SR-11 with mouse small intestinal epithelial cells. *Infect Immun* **65**, 5320-5325

Surette, M.G., Miller, M.B. & Bassler, B.L. (1999). Quorum sensing in *Escherichia coli* , *Salmonella typhimurium* and *Vibrio harveyi*: A new family of genes responsible for auto-inducer production. *Proc Natl Acad Sci USA* **96**, 1639-1644

Suzuki, S. (1994). Pathogenicity of *Salmonella enteritidis* in poultry. *Int J Food Microbiol* **21**, 89-105

Swansen, J. (1973). Studies on gonococcus infection pili. Their role in attachment of gonococci to tissue culture cells. *J Exp Med* **137**, 571-589

Swenson, D.L. & Clegg, S. (1992). Identification of ancillary *fim* genes affecting fimA expression in *Salmonella typhimurium. J Bacteriol* **174**, 7697-7704

Swenson, D.L., Kim, K-J., Six, E.W. & Clegg, S. (1994). The gene *fim*U affects expression of *Salmonella typhimurium* type 1 fimbriae and is related to *Escherichia coli* tRNA gene *arg*U. *Mol Gen Genet* **244**, 216-218

Swidsinski, A., Khilkin, M., Kerjaschki, D., Schreiber, S., Ortner, M., Weber, J. & Lochs, H. (1998). Association between intraepithelial *Escherichia coli* and colorectal cancer. *Gastroenterol* **115**, 281-286

Takeuchi, A. (1967). Electron microscopy studies of experimental *Salmonella* infections. *Am J Pathol* **50**, 109-136

Tanaka, Y., Katsube, Y. & Imaizumi, K. (1977). Virulence of *Salmonella typhimurium* harboured in the faeces of carrier dog for mice. *Jap J Vet Sci* **39**, 353-6

Tanaka, Y. & Katsube, Y. (1978). Infectivity of *Salmonella* for mice in relation to fimbriae. *Jap J Vet Sci* **40**, 671-81

Tanaka, Y., Katsube, Y, Mtoh, T. and Imaizumi, K. (1981). Fimbriae of *Salmonella typhimurium* and their role in mouse intestinal colonisation of the organism. *Jap J Vet Sci* **43**, 51-62

Tarkannen, A.M., Allen, B.L., Westerlund, B., Holthofer, H., Kuussela, P., Ristela, L., Clegg, S. and Korhonen, T.K. 1990. Type V collagen as the target for type-3 fimbriae, enterobacterial adherence organelles. *Mol Microbiol* **4**, 1353-1361

Tartera, C. & Metcalf, E.S. (1993). Osmolarity and growth phase overlap in regulation of *Salmonella typhi* adherence to and invasion of human intestinal cells. *Infect Immun* **61**, 3084-3089

Tavendale, A., Jardine, C.K.H., Old, D.C. & Duguid, J.P. (1983). Haemagglutanins and adhesion of *Salmonella typhimurium* to Hep-2 and HeLa cells. *J Gen Microbiol* **16**, 371-80

Thiagarajan, D., Saeed, M., Turek, J. & Asem, E. (1996a). Mechanism of transovarian transmission of *Salmonella enteritidis* in laying hens. *Poult Sci* **73**, 89-98

Thiagarajan, D., Saeed, M., Turek, J. & Asem, E. (1996b). *In vitro* attachment and invasion of chicken ovarian granulosa cells *by Salmonella enteritidis* phage type 8. *Infect Immun* **64**, 5015-5021

Thorns, C.J. (1995). Salmonella fimbriae: Novel antigens in the detection and control of *Salmonella* infections. *Brit Vet J* **151**, 643-658

Thorns, C.J., McLaren, I.M. & Sojka, M.G. (1993). The use of latex particle agglutination to specifically detect *Salmonella enteritidis*. *Int J Food Microbiol* **21**, 47-53

Thorns, C.J., Mell, M.M., Sojka, M.G., & Nicholas, R.A. (1996a). Development and application of enzyme-linked immunosorbent assay for the specific detection of *Salmonella enteritidis* infections in chickens based on antibodies to SEF14 fimbrial antigen. *J Clin Microbiol* **34**, 792-797

Thorns, C.J., Sojka, M.G. & Chassey, D. (1990). Detection of a novel fimbrial structure on the surface of Salmonella enteritidis by using a monoclonal antibody. *J Clin Microbiol* **28**, 2409-14

Thorns, C.J, Sojka, M.G., McLaren, I.M. & Dibb-Fuller, M.P. (1992). Characterization of monoclonal antibodies against a fimbrial structure of *Salmonella enteritidis* and certain other serogroup D salmonellae and their application as serotyping reagents. *Res Vet Sci* **53**, 300-308

Thorns, C.J., Sojka, M.G. & Roeder, P.L.(1989a). Detection of fimbrial adhesins of ETEC using monoclonal antibody-based latex reagents. *Vet Rec* **125**, 91-2

Thorns, C.J., Turcotte, C., Gemmell, C.G. & Woodward, M.J. (1996b). Studies into the role of the SEF14 fimbrial antigen in the pathogenesis of *Salmonella enteritidis. Microbial Pathogen* **20**, 1-12

Thorns, C.J., Wells, G.A., Morris, J.A., Bridges, A. & Higgens, R. (1989b). Evaluation of monoclonal antibodies to K88, K99, F41 and 987P fimbrial adhesins for the detection of porcine enterotoxigenic *Escherichia coli* in paraffin wax tissue sections. *Vet Microbiol* **20**, 377-81

Timoney, J.F., Shivaprasad, H.L, Baker, R.C. & Rowe, B. (1989). Egg transmission after infection of hens with *Salmonella enteritidis* phage type 4. *Vet Rec* **125**, 600-601

Timoney, J.F., Sikora, N., Shivaprasad, H.L. & Opitz, M. (1990). Detection of antibody to *Salmonella enteritidis* by a gm flagellin-based ELISA. *Vet Rec* **127**, 168-169

Totten, P.A., Lara, J.C., Norn, D.V. & Stamm, W.E. (1994). *Haemophilus ducreyi* attaches to and invades human epithelial cells in vitro. *Infect Immun* **62**, 5632-5640

Turcotte, C. & Woodward, M.J. (1993). Cloning, DNA nucleotide sequence and distribution of the gene encoding SEF14, a fimbrial antigen of *Salmonella enteritidis. J Gen Microbiol* **139**, 1477-85

Turner, A.K., Lovell, M.A., Hulme, S.D., Zhang-Barber, L.I. & Barrow, P.A. (1998). Identification of *Salmonella typhimurium* genes required for colonisation of the chicken alimentary tract and for virulence in newly hatched chicks. *Infect Immun* **66**, 2099-2106

Vaara, M. (1992). Agents that increase the permeability of the outer membrane . *Microbiol Rev* **56**, 395-411

Vaara, M. (1993). Antibiotic susceptibility mutants of *Escherichia coli* and *Salmonella typhimurium. Antimicrob Agent Chemother* **37**, 2255-2260

Valdivia, R.H. & Falkow, S. (1996). Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promotors by differential fluorescence induction. *Mol Microbiol* **22**, 367-378

van der Velden, A.W.M., Baumler, A.J., Tsolis, R.M. & Heffron, F. (1998). Multiple fimbrial adhesins are required for full virulence of *Salmonella typhimurium* in mice. *Infect Immun* **66**, 2803-2808

Vidotto, M.C., Navarro, H.R. & Gaviri, L.C.J. (1997). Adherence of pathogenic strains of avian *Escherichia coli. Vet Microbiol* **59**, 79-87

Virgi, M., Makepeace, K., Ferguson, D.J.P., Achtman, M. & Moxon, E.R. (1993). Meningococcal Opa and Opc proteins: their role in colonisation and invasion of human epithelial and endothelial cells. *Mol Microbiol* **10**, 499-510

Walker, S.L., Sojka, M., Dibb-Fuller, M.P. & Woodward, M.J. (1999). Effect of pH, temperature and surface contact on the elaboration of fimbriae and flagella by *Salmonella* serotype Enteritidis. *J Med Microbiol* **48**, 253-261

Walker, R.I., Schmauder-Chock, E.A., Parker, J.L. & Burr, D. (1988). Methods to study bacterial invasion. *J Microbiol Meth* **18**, 227-240

Wallis, T.S., Paulin, S.M., Plested, J.S., Watson, P.R. & Rhen, M. (1995). The *Salmonella dublin* virulence plasmid mediates systemic but not enteric phases of salmonellosis in cattle. *Infect Immun* **6**, 2755-2761

Wassef, J.S., Keren, D.F. & Mailloux, J.L. (1989). Role of M cells in initial antigen uptake and in ulcer formation in the rabbit intestinal loop model of shigellosis. *Infect Immun* **57**, 858-863

Watarai, M., Tobe, T., Yoshikawa, M. & Sasakawa, C. (1995). Contact of *Shigella* with host cells triggers release of lpa invasins and is an essential function of invasiveness. *EMBO J* **14**, 2461-2470

Wei, L.N. & Joys, T.M. (1985). Covalent structure of three phase-1 flagellar filament proteins of *Salmonella. J Mol Biol* **186**, 791-803

Weidel, W. & Pelzer, H. (1964). Bag-shaped macromolecules- a new outlook on bacterial cell walls. *Adv Enzymol* **26**, 193-232

Weinstein, D.L., Carsiotis, M., Lissner, R. & O'Brien, A.D. (1984). Flagella help *Salmonella typhimurium* survive within murine macrophages. *Infect Immun* **46**, 819-825

Williams, J.E., Dilliard, L.H. & Hall, G.O. (1988). The penetration patterns of *Salmonella typhimurium* through the outer structures of the chicken egg. *Avian Dis* **12**, 445-466

Withanage, G.S.K., Sasai, K., Fukata, T., Miyamoto, T. & Babo, E. (1999). Secretion of *Salmonella*-specific antibodies in the oviducts of hens experimentally infected with *Salmonella enteritidis*. *Vet Immunol Immunopathol* **67**, 185-193

Wolf, J.L. & Bye, W.A. (1984). The membranous epithelial (M) cell and the mucosal immune system. *Ann Rev Med* **35**, 95-112

Woodward, M.J., Allen-Vercoe, E. & Redstone, J.S. (1996). Distribution, gene sequence and expression *in vivo* of the plasmid encoded fimbrial antigen of *Salmonella* serotype Enteritidis. *Epidemiol Infect* **117**, 17-28

Wyatt, G.M., Langley, M. N., Lee, H.A. & Morgan, M.R.A. (1993). Further studies on the feasibility of one-day salmonella detection by enzyme linked immunosorbant assay. *Appl Environ Microbiol* **59**, 1383-90

Yamaguchi, S. & Iino, T. (1968). Genetic determination of the antigenic specificity of flagellar protein in *Salmonella*. *J Gen Microbiol* **55**, 59-74

Yasson, C.V. & Schatt, K.A. (1987). Pathogenesis of rotavirus infection in various age groups of chickens and turkeys: clinical signs and virology. *Am J Vet Res* **48**, 977

Yeh, K-S., Hancox, L.S. & Clegg, S. (1995). Construction and characterization of a *fimZ* mutant of *Salmonella typhimurium*. *J Bacteriol* **177**, 6861-6865

Zamora, B.M., Hartung, M. & Hildebrandt, G. (1999). Simplified preparation of specific *S. enteritidis* antigen for ELISA and other immunological techniques. *J Vet Med Sci* **46**, 1-7

Zaneti, S., Angoi, A., Fiori, P.L. & Fadda, G. (1994). Adhesion of mucoid uropathogenic strains of *Pseudomonas aeroginosa* to Hep-2 cells. *Microbiol* **17**, 297-305

Zepeda-Lopez, H.M. & Gonzalez-Lugo, G.M. (1995). *Escherichia coli* adherence to Hep-2 cells with pre-fixed cells. *J Clin Microbiol* **33**, 1414-1417

Zierer, M. & Galàn, J.E. (1995). Contact with cultured epithelial cells induces the secretion of the *Salmonella typhimurium* invasion protein InvJ. *Infect Immun* **63**, 4024-4028

Ziprin, R.L., Corrier, D.E. & Elissalde, M.H. (1989). Maturation of resistance to Salmonellosis in newly hatched chicks: Inhibition by Cyclosporin. *Poultry Sci* **68**, 1637-1642