THE ROLE OF INTIMIN AND FLAGELLA IN THE PERSISTENCE OF *ESCHERICHIA COLI* O157:H7 IN POULTRY

by

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ABSTRACT

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The role of intimin and flagella in the persistence of *Escherichia coli* O157:H7 in poultry

by Angus Best

Escherichia coli O157:H7 has been implicated in many major and sporadic outbreaks of food-borne related disease in humans. Bovine and bovine derived meat products are cited as the main source of infection and deliberate inoculation studies of ruminants show that the surface-arrayed outer membrane protein intimin facilitates persistent colonisation. The prevalence of *E. coli* O157:H7 infection in birds is low, but several deliberate inoculation studies show that poultry are readily and persistently infected by this organism indicating a possible threat to public health. The mechanisms of *E. coli* O157:H7 colonisation of poultry are not understood, but flagella are important for the colonisation of poultry by avian pathogenic *E. coli*. Whether intimin plays a role in persistent colonisation of poultry has not been determined. To investigate the role of intimin and flagella, defined knockout single and double intimin and aflagellate mutants were constructed in a well characterised non-toxigenic *E. coli* O157:H7 isolate (NCTC 12900) and tested in adherence assays with an avian epithelial cell line (Div-1) and used to inoculate 1-day-old SPF chicks. *In vitro*, NCTC 12900 intimin contributed significantly to adherence, but not invasion, whereas NCTC 12900 flagella only contributed to invasion. NCTC 12900 intimin, but not flagella was required for micro-colony and AE lesion formation. *In vivo* studies revealed that the wild-type could form micro-colonies on the caecal mucosa of SPF chicks and could persistently colonise birds for up to 169 days, ceasing 9 days after the birds came into lay and 6% of eggs were contaminated on the eggshell. NCTC 12900 flagella, but not intimin contributed to persistent colonisation in the chick. Competitive index models where 1-day-old SPF chicks were dosed with wild-type and intimin or aflagellate mutants revealed that flagella, but not intimin contributed to the persistence of Stx-negative *E. coli* O157:H7 isolate NCTC 12900 in the chick.

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Chapter 1

General introduction

Non-pathogenic *Escherichia coli* are the predominant facultative anaerobe of the normal commensal bowel flora in humans and successfully colonise the host soon after birth (Drasar and Hill, 1974; Gross, 1994; Talero & Talero, 1993; Sussman, 1997). *E. coli* that are pathogenic have been implicated as the causative agent of numerous infectious diseases in mammals and birds (Carter *et al.*, 1995; Quinn *et al.*, 1994). In the human host, pathogenic *E. coli* are associated with urinary tract infections, diarrhoea, acute renal failure and meningitis (Sussman, 1997; Nataro & Kaper, 1998).

Investigations into *E. coli* began when a German paediatrician, Dr Theodore Escherich first isolated what he called *Bacterium coli commune* from the faeces of neonates (Escherich, 1885), later to become known as *Escherichia coli*, renamed in his honour. Originally thought of as a commensal of the human alimentary tract, Laurelle first suspected the possible pathogenicity of *E. coli* in 1889 (Laurelle, 1889) and in 1892, Jensen (cited by Sojka, 1965) used *E. coli* isolates to experimentally reproduce diarrhoea in calves. However, it was not until the 1940s when serology was regularly used that certain *E. coli* serotypes were implicated in outbreaks of diarrhoea in human infants (Bray, 1945).

Renewed interest in *E. coli* occurred during the late 1950s and early 1960s. During this time, H. Williams Smith at the Animal Health Trust and Walter Sojka's group at CVL, Weybridge, made important discoveries about the role of *E. coli* in enteric and septicaemic diseases in animals (reviewed by Gay & Besser, 1994). Since 1965, many virulence factors have been discovered in numerous strains of *E. coli* that has led to the categorisation of collective pathogenic groups of *E. coli* (Lior, 1994). It was during the late 1970s and early 1980s that a group of pathogenic *E. coli* able to produce Vero-toxin, often called Shiga-toxin, were discovered. One such pathogen, *E. coli* O157:H7, isolated by Riley *et al.* (1983) is recognised as the causative agent of severe food-borne related outbreaks in humans.

1.1 Classification of *Escherichia coli*

The genus *Escherichia* is a typical member of the family *Enterobacteriaceae* which includes the genera *Salmonella* and *Shigella* (Brenner, 1984). Apart from *E. coli* other members of this genus include *E. blattae*, *E. fergusonii*, *E. hermanii* and *E. vulneris*. *E. coli* is a short-medium sized, straight Gram negative non-sporulating bacillus approximately 1.1-1.5Πm x 2.0-6.0Πm, possessing 5-10 peritrichous flagella. *E. coli* usually elaborates a variety of fimbriae and is often encapsulated. Fermentable carbon and nitrogen sources are an absolute metabolic requirement for *E. coli* in an anaerobic environment. Sugars are fermented to give mixed acids that include lactic, acetic and formic acids, with or without gas production. *E. coli* isolates are also catalase-positive and oxidase-negative. *E. coli* is usually grown in the laboratory at 37°C and usually occurs singly or in pairs in fast growing cultures (Van Demark and Batzing, 1987; Talero & Talero, 1993; Holt *et al.*, 1994; Quinn *et al.*, 1994).

Classification of *E. coli* involves serotyping where isolates are identified by their somatic lipopolysaccharide O-antigens, capsular K-antigens, flagellar H-antigens, and fimbrial F-antigens (Kauffmann, 1969; rskov & rskov, 1983). Therefore, the O-, K-, H- and F-antigens determine the serotype of an *E. coli* isolate (Lior, 1994).

First used for serotyping by Kauffmann (1944), the O-antigen is a thermostable somatic structure of lipopolysaccharide. Antiserum against O-antigen is made by the injection of rabbits with boiled bacteria (removal of capsule). Agglutination of the homologous O-antigen is initiated by the antisera. An isolate to be serotyped is incubated with the antisera at 50°C for 18 hours and granules are formed if the result is positive (Cooke, 1974). Absorbing out any cross-reactions produces pure antisera that reacts only with the homologous bacterium.

The capsular K-antigen usually masks the O-antigen, making the test organism O-antigen untypable (Kauffmann and Vahlne, 1945). Three sub-groups within K-antigens are called L, A and B. These antigens were described by Kauffmann (1943), Kauffmann (1944) and Knipschildt (1946), respectively. Presence of the K-antigens is demonstrated using antisera via the slide agglutination test.

The serologically distinct H-antigens are found in flagella (Lior, 1994). Fifty three flagella H-antigens have been described for *E. coli* (Ewing, 1986). Characterisation of flagella antigens involves the selection of motile bacterial culture in semi-solid (sloppy) agar. Antisera produced from the culture can be used for the tube agglutination test (Cooke, 1974). H-antigen agglutination results in the formation of fluffy, loose flocule agglutinates that disperse easily. Certain isolates may be non-motile (H- or NM) (Macnab, 1996).

Commonly expressed on the surface of other *Enterobacteriaceace* and Gram positive bacteria are surface appendages known either as fimbriae or pili (Duguid *et al.*, 1955)

arranged peritrichously. Houwink & Van Iterson (1950) were the first to observe these surface appendages by electron microscopy.

More recently, fimbriae were classified as the fourth surface antigen group for characterisation by serology (rskov & rskov, 1983). Seventeen F-antigens are used in serological testing (Lior 1994), most of which are specifically associated with particular infections and diseases. For example, P-fimbriated *E. coli* are associated with urinary tract infections (Kuehn *et al.*, 1992), and bundle forming piliated *E. coli* are associated with infant diarrhoea (Girón *et al.*, 1991). Travellers diarrhoea is associated with CFAI (F2) fimbriated *E. coli*, K88 (F4) and K99 (F5) fimbriated *E. coli* are associated with neonatal diarrhoeal disease in pigs (Jones and Rutter, 1972). K99 fimbriated *E. coli* are also associated with neonatal diarrhoeal disease in calves and lambs (Smith & Linggood, 1972).

Other ways to characterise *E. coli* include detection of toxins and colicins and differentiation by electrophoretic and phage typing (Hettiarachy *et al.*, 1973; Whittam & Wilson, 1988; Lior, 1994). Advances in molecular techniques have introduced restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), pulse field gel electrophoresis (PFGE) and polymorphic amplified typing sequences (PATS) (Barrett, *et al.*, 1994; Jantausch & Hull, 1996; Lin *et al* 1996, Kudva *et al.*, 2002) to aid differentiation, particularly useful for molecular epidemiological studies.

1.2 Pathogenic *E. coli* of the gastrointestinal tract

Escherichia coli is the principal commensal facultative anaerobe of the human gut. However, various pathogenic *E. coli* that may be acquired from the environment (food, water etc) can cause a broad spectrum of diseases, such as, diarrhoea and urinary tract infections to potentially fatal diseases including septicaemia, meningitis and haemolytic ureamic syndrome (HUS) (Sussman, 1997; Nataro & Kaper, 1998). For the purposes of this introduction, extra-intestinal pathogenic *E. coli* will not be discussed.

Pathogenic *E. coli* are generally characterised by specific virulence factors that are generally characterised as adherence and or invasion determinants and toxins. The various pathogenic groups that cause gastrointestinal diseases in man are enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and Shiga-toxin (Vero-toxin) producing *E. coli* (STEC or VTEC). For the purposes of this review the latter pathogenic group will be called Shiga-toxin producing *E. coli*. STEC that cause a type of kidney failure known as haemolytic uraemic syndrome (HUS) are collectively called enterohaemorrhagic *E. coli* (EHEC).

Enteroinvasive *E. coli* (EIEC) are regarded as similar to *Shigella* since they are non-motile, late or non-lactose fermenting, anaerogenic (fermentation without the production of

gas) and the clinical syndrome they produce is virtually identical to *Shigella* infection (DuPont *et al.*, 1971; Parscot & Sansonetti, 1996). The presence of a large 140 MDa plasmid, designated pInv (also present in *Shigella* serotypes) (Small & Falkow, 1988; Sasakawa *et al.*, 1992) is associated with the enteroinvasive characteristics of EIEC which involves epithelial cell penetration, lysis of the endocytic vacuole and intracellular multiplication (Sansonetti, 1992; Goldberg and Sansonetti, 1993). Enterotoxin production does occur (Nataro *et al.*, 1995), but its role in watery diarrhoea is unproven. Dysentery due to EIEC is uncommon but because of the significant similarity to shigellosis prevalence is probably underestimated.

In certain regions of the developing world, cases of persistent diarrhoea have been associated, but not always, with enteroaggregative *E. coli* (EAEC) (Bhan *et al.*, 1989; Cravioto *et al.*, 1991; Scotland *et al.*, 1993). EAEC are also a potential cause of diarrhoeal disease in adults with human immunodeficiency virus (HIV) (Wanke *et al.*, 1998). EAEC at present are defined as *E. coli* strains that do not secrete the enterotoxigenic *E. coli* (ETEC) enterotoxins (LT and ST) but do form an aggregative adherence (AA) pattern on HEp-2 cells. However, this definition appears to include pathogenic and non-pathogenic EAEC strains as confirmed by volunteer studies and outbreak investigations (Nataro *et al.*, 1995). An *in vitro* rabbit intestinal model demonstrated that EAEC produce a 4.1 kDa enterotoxin known as the enteroaggregative heat-stabile toxin, EAST1 (Savarino *et al.*, 1991). A defined role for EAST1 in EAEC pathogenesis is yet to be confirmed although EAST1 is also found in other pathogenic *E. coli*, including *E. coli* O157:H7 (see below).

Little is also known about the pathogenic features of diffusely adherent *E. coli* (DAEC) induced childhood diarrhoea, but the risk of childhood diarrhoea appears to increase with age (Levine *et al.*, 1993). Both a fimbrial (F1845 factor) and a non-fimbrial adhesin (AIDA-1) have been described (Bilge *et al.*, 1989; Benz & Schmidt, 1993).

Enterotoxigenic *E. coli* (ETEC) produce at least one toxin of a defined group of two enterotoxins (Gorbach *et al.*, 1971). In humans, ETEC are associated with travellers diarrhoea in developing countries. First described as agents of diarrhoeal disease in piglets, the toxins produced by ETEC are heat-labile toxin (LT) and heat-stabile toxin (ST) and both are plasmid encoded (Gyles, 1994). Two LT toxins (LT-I and LT-II) have been described and both are oligomeric toxins with an AB_5 structure exhibiting ADP-ribosyltranferase activity. Both toxins are closely related to the cholera enterotoxin (CT) of *Vibrio cholerae* (Sixma *et al.*, 1993). Heat-stabile toxin (ST) also exists in two forms, but are distinct in structure and function from the LTs, and from each other. STa has guanylate cyclase activity (deSauvage *et al.*, 1992; Vaandrager *et al.*, 1994), whereas STb (associated primarily with pigs) stimulates bicarbonate secretion from intestinal cells (Sears and Kaper, 1996). Adherence mechanisms are species specific for ETEC. Strains expressing K99 fimbriae are pathogenic for calves,

lambs and pigs, whereas K88 expressing strains only infect pigs (Cassels & Wolf, 1995). Human ETEC strains express fimbriae called Colonisation Factor Antigens (CFAs) (De Graaf & Gaastra, 1994).

Infantile diarrhoea in the developing world is associated with enteropathogenic *E. coli* (EPEC) (Nataro & Kaper, 1998). Originally defined only by their O and H serotypes, EPEC are now characterised on the basis of one pathogenic phenotype known as the attaching and effacing (AE) lesion (Knutton *et al.*, 1989). The AE lesion is characterised by the loss of microvilli (effacement) and the production of a pedestal cup underneath and around the EPEC, composed of polymerised actin and other cytoskeletal compounds (Finlay *et al.*, 1992; Kaper *et al.*, 1998). Formation of this lesion appears to involve a variety of signal transduction pathways in the eukaryotic cell (Nataro & Kaper, 1998). Molecular analysis of EPEC strain E2348/69 demonstrated that the AE phenotype is encoded on a 35.6 kb pathogenicity island, the locus of enterocyte effacement (LEE) (McDaniel *et al.*, 1995; McDaniel & Kaper, 1997). Within the LEE are genes encoding an outer membrane protein (intimin), a type III secretion system (Esc, Sep, and Ces proteins), secreted proteins (Esp) and the translocated intimin receptor (Tir) (Elliott *et al.*, 1998). Additional virulence factors are also encoded on the EPEC virulence enterocyte adherence factor plasmid (EAF) (Nataro *et al.*, 1985), including the regulator Per and the type IV bundle forming pili (BFP) (Gómez-Duarte & Kaper, 1995; Girón *et al.*, 1991). Both virulence factors are associated with the localised adherence patterns observed on HEp-2 cells and for virulence in humans (Bieber *et al.*, 1998).

First identified in 1977, Shiga-toxin producing *E. coli* (STEC) cause diarrhoeal disease in humans and animals, and may be characterised by the production of Shiga-like toxins that cause cytotoxic effects on Vero cells (African Green monkey kidney cells) (Konowalchuk *et al.*, 1977). This cytotoxic effect was demonstrated by using the culture supernatants of some *E. coli* strains including strains of the O26:H11 serotype. The genes that encode Shiga-toxin (Stx) are carried by temperate bacteriophages (Brunton, 1994). Significantly, STEC are associated with the potentially fatal haemolytic uraemic syndrome (HUS) which is characterised by kidney failure (Karmali, 1989). Various STEC serotypes can induce the AE phenotype, although STEC that are LEE negative have also been described (Paton *et al.*, 1999).

1.3 Enterohaemorrahagic *E. coli* (EHEC) including *E. coli* O157:H7

STEC causing haemorrhagic colitis (HC) and HUS are classed as enterohaemorrhagic *E. coli* (EHEC) (Levine, 1987). Apart from *E. coli* O157:H7, other EHEC serogroups of medical importance include O26 and O111 (Scotland *et al.*, 1990; Paton *et al.*, 1996). The term enterohaemorrahagic *E. coli* (EHEC) characterises STEC strains that express Stx, cause

AE lesions, possess a 60 Mda plasmid and cause HC and HUS. An atypical EHEC strain is a STEC strain that does not possess a 60 Mda plasmid and is unable to induce the AE phenotype (Nataro & Kaper, 1998).

Riley *et al.* (1983), who investigated two outbreaks of a distinctive gastrointestinal illness, first reported the recognition of EHEC as major pathogens of humans. Characterised by severe abdominal pain and cramp, watery diarrhoea followed by bloody diarrhoea, and little or no fever, this illness designated haemolytic colitis (HC) was associated with the consumption of undercooked hamburgers at a fast food chain in the USA. Cultured stools from patients yielded a novel *E. coli* serotype, O157:H7. Karmali *et al.* (1983) also associated sporadic cases of HUS with faecal Stx and Stx producing *E. coli* in stools.

HUS is defined by three disease symptoms of acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia. These observations led to the recognition of an important class of novel emerging enteric pathogens causing intestinal and renal disease. *E. coli* O157:H7 is one such novel pathogen which has caused major food-borne outbreaks and sporadic cases world-wide.

1.4 Major food-borne outbreaks caused by *E. coli* O157:H7

Since it was first characterised by Riley and co-workers (1983), Stx positive *E. coli* O157:H7 has been a causative agent of food-borne outbreaks in Northern America (Ostroff *et al.*, 1990; Bell *et al.*, 1994; Cody *et al.*, 1999; Breuer *et al.*, 2001; Crump *et al.*, 2002: Olsen *et al.*, 2002), Japan (Michino *et al.*, 1999; Watanabe *et al.*, 1999; Tsuji *et al.*, 2002) and the United Kingdom (Willshaw *et al.*, 1994; O'Brien *et al.*, 2001; Cowden *et al.*, 2001: Goh *et al.*, 2002). Other outbreaks have been reported in continental Europe (Reida *et al.*, 1994; Ramberg *et al.*, 1996; Keskimaki *et al.*, 1998; Tozzi *et al.*, 2003) and although originally reported from these developed regions of the world, human outbreaks of *E. coli* O157:H7 have been reported in the developing world, including Africa (Effler *et al.*, 2001).

Between 1982 and 1996 in the U.S.A, one hundred and thirty nine Stx positive *E. coli* O157:H7 outbreaks were reported, culminating in over 3,000 ill persons, of which 22% were hospitalised, 6% developed HUS and thrombotic thrombocytopenic purpura and 0.6% died (Sparling, 1998). In a 1994 outbreak of *E. coli* O157:H7 infections from salami, the infectious dose was estimated to be fewer than 50 bacteria (Tilden *et al.*, 1996). In recent times, it is estimated that in the USA, *E. coli* O157:H7 causes an estimated 73,000 illnesses, 2000 hospitalisations and 60 deaths per year (Griffin *et al.*, 2000). Although ground beef remains the most common source of *E. coli* O157:H7 infection, rural drinking water and recreational water are increasingly recognised as important vehicles (Feldman *et al.*, 2002; Lee *et al.*, 2002; Olsen *et al.*, 2002).

In Japan, all EHEC outbreaks reported since and including the first outbreak reported in a kinder-garden school in 1990 (Akashi *et al.*, 1994), have all been associated with *E. coli* O157:H7. During 1996, several outbreaks were reported and in particular at 47 primary schools in Sakai city in July, the largest outbreak occurred where the number of symptomatic patients in Sakai city exceeded more than 7,500 (Michino *et al.*, 1999). Although the number of outbreaks decreased gradually, sporadic cases are continuously occurring, where more than 2000 symptomatic cases have been reported every year since 1997 (Watanabe, 2000). Recently, the complete genome of this outbreak strain has been sequenced (Hayashi *et al.*, 2001).

In the United Kingdom the incidence of *E. coli* O157:H7 infection is high (Smith *et al.*, 1998). During 1983, *E. coli* O157:H7 was isolated following an outbreak of HUS in the West Midlands of England which affected 35 children (Taylor *et al.*, 1986). Between 1987 and 1996, 79 outbreaks of *E. coli* O157:H7 were recorded in England, Wales and Scotland, where 24 of these occurred in the latter country (Smith *et al.*, 1998). No *E. coli* O157:H7 were reported in Northern Ireland during this time period. Little is known about the importance of non-O157 EHEC in the United Kingdom, since very few laboratories screen for serotypes other than O157. In the United Kingdom there has been a significant increase in the isolation of *E. coli* O157:H7 possibly resulting partly from improved isolation techniques and changes in the criteria for screening. However, this does reflect a true increase in *E. coli* O157:H7 infections. The rates of infection with *E. coli* O157:H7 per 100,000 are variable throughout the United Kingdom, with the highest rates occurring in Scotland. From 1990-1996 the average annual rate in England and Wales was less than 1.5/100,000, whereas in Scotland for the same period the average was greater than 5/100,000 (Smith *et al.*, 1998).

On Friday 21st November 1996 in Central Scotland (Lanarkshire) 15 suspected cases of *E. coli* O157:H7 infection were reported. Subsequently, an epidemic involving *E. coli* O157:H7 occurred. Currently it stands as one of the largest outbreaks worldwide in terms of mortality with 21 associated deaths. All fatalities were over 65 years of age. Described as number of people infected it is the largest in Europe with 503 cases ranging from 2 to 91 years of age. Isolates from 279 cases were confirmed as *E. coli* O157:H7 phage type 2, and possessed the Stx2 toxin gene (Stevenson & Hanson, 1996; The Pennington Group Report, 1997; Ahmed & Donaghy, 1998). The predominant *E. coli* O157:H7 phage types for illness in people in Scotland today and the United Kingdom as a whole appear to be phage types 21/28, but phage types 2, 4, 8 and 32 are also important (Crampin *et al.*, 1999; Allison *et al.*, 2000; O'Brien *et al.*, 2001; Goh *et al.*, 2002; O'Donnell *et al.*, 2002). Interestingly, a recent 12 month abattoir study by Paiba *et al.*, (2002) has revealed that the most frequently recovered *E. coli* O157:H7 isolates were 2, 8 and 21/28 in cattle and 4 and 32 in sheep.

1.5 *E. coli* O157:H7 in animals

Most *E. coli* O157:H7 human outbreaks originate from bovine and bovine derived food products. Globally, *E. coli* O157:H7 is widely distributed in cattle herds (Griffin & Tauxe, 1991; Armstrong *et al.*, 1996; Hancock *et al.*, 1997). Another important reservoir for this human pathogen is sheep (Chapman *et al.*, 1997; Kudva *et al.*, 1997), and there is concern that *E. coli* O157:H7 may enter and establish within other food animal production systems such as pig and goat (Heuvelink *et al.*, 1999; Pritchard *et al.*, 2000).

Although domestic livestock are often indirectly implicated in human outbreaks, there is sufficient field data to suggest that bovines are not the only reservoirs of *E. coli* O157:H7. Epidemiological studies of cattle farms affected with *E. coli* O157:H7 have indicated that infection of farms by this human pathogen can originate from outside sources suggesting that non-cattle species may play a role in *E. coli* O157:H7 epidemiology (Hancock *et al.*, 2001). Identical *E. coli* O157:H7 isolates have been detected in cattle that are several hundred kilometres apart (Rice *et al.*, 1999). A possible reason for this observation is that *E. coli* O157:H7 have been isolated from horses, dogs, deer, birds, wild rabbits and rats. Therefore, it is reasonable to assume that this pathogen may be transported by these and yet unidentified migratory animal reservoirs (Rice *et al.*, 1995; Wallace *et al.*, 1997; Cizek *et al.*, 1999; Sargent *et al.*, 1999; Fischer *et al.*, 2001; Pritchard *et al.*, 2001; Renter *et al.*, 2001).

1.6 Isolation and detection of *E. coli* O157:H7

Surveillance of *E. coli* O157:H7 in humans and animals relies on the isolation of this pathogen from faeces, involving sensitive methods, including enrichment and immunomagnetic separation (IMS) techniques (Chapman *et al.*, 1994: Chapman *et al.*, 1997). Since the vast majority of *E. coli* O157:H7 strains do not ferment sorbitol in 24 hours, Sorbitol MacConkey agar (SMAC) was developed to distinguish *E. coli* O157:H7 from sorbitol fermentors (March & Ratnam, 1986). *E. coli* O157:H7 grow as colourless colonies, whereas sorbitol fermentors grow as purple colonies. Further development of this media for specific selection of *E. coli* O157:H7 involves the addition of Cefixime and Tellurite (CT-SMAC) (Zadik *et al.*, 1993), but some sorbitol fermenting *E. coli* O157:H- strains do not grow on CT-SMAC (Karch *et al.*, 1996).

Other selective media for *E. coli* O157:H7 identification include CHROMagar∞ (Wallace & Jones, 1996), modified eosin methylene blue agar (MEMB) (Harrison *et al.*, 1998), Rainbow Agar∞ (Bettelheim, 1998), haemorrhagic coli agar (HC) (Szabo *et al.*, 1986), phenol red sorbitol agar + MUG (PRS-MUG) (Conner & Hall, 1994), and Fluorocult *E. coli* O157:H7 agar (Merck). A latex agglutination test, where beads that are coated with antibodies recognising the O157 antigen, is also available for the presumptive identification of *E. coli* O157 (Oxoid).

Immunomagnetic separation (IMS) involves target O157 cells recovered from pre-enrichment or selective broths using paramagnetic beads. These beads are coated with polyclonal antibodies specific for the O157 antigen. The inclusion of the IMS step into cultural O157 isolation procedures is now regarded as the gold standard (Fratamico *et al.*, 1992; Chapman *et al.*, 1994; Bennett *et al.*, 1996; Heuvelink *et al.*, 1997).

DNA based methods are used for the detection of *E. coli* O157:H7 DNA sequences that encode virulence factors such as the plasmid encoded enterohaemolysin, both Shiga-toxins, intimin and the structural subunit of flagella (Bastian *et al.*, 1998; Fratamico *et al.*, 2002). Rapid detection of both toxin genes in *E. coli* O157:H7 by real-time PCR with fluorescent hybridisation probes has been described (Bellin *et al.*, 2001) and the quantification of *E. coli* O157:H7 from soil by real-time PCR revealed that less than 10 CFU per g/soil of *E. coli* O157:H7 were detected (Ibkewe & Grieve, 2003). The *rfb* O157 gene for detection of the O157 antigen may be also be targeted (Maurer *et al.*, 1999). Wang & Reeves, (1998) and Schimizu and co-workers (1999) determined the sequence of the O157 O-antigen synthesis gene cluster. Both studies have suggested that rapid detection of this cluster by PCR could overcome the problems of antiserum cross-reaction with other bacteria.

1.7 Virulence factors of *E. coli* O157:H7 encoded outside the LEE

1.7.1 Shiga-toxin (Stx)

Karmali *et al.* (1985) first suggested that there was a link between Stx and the pathogenesis of HUS. Stx belongs to a family of subunit toxins that consist of one A-subunit and five B-subunits (AB_5). The A-subunit of Stx from STEC ranges from 30-35 kDa and the five B-subunits each range from 7-11 kDa, but for a particular type of Stx the five B-subunits are identical. The A-subunit is composed of an A_1 peptide that contains enzyme activity, and an A_2 peptide that binds the A-subunit to the pentamer of identical B-subunits (O'Brien *et al.*, 1992).

The STEC Shiga-toxin family includes Stx1, and five Stx2 variants (O'Brien *et al.*, 1987). Shiga-toxin from *Shigella dysenteriae* type 1 and Stx1 differ in only one amino acid in the A-subunit (Strockbrine *et al.*, 1988). The A and B structures of Stx1 only share 55% and 57% amino acid homology with the corresponding subunits of Stx2 (Melton-Celsa & O'Brien, 1998). *E. coli* O157:H7 isolates can produce both Stx1 and Stx2, Stx2 only, or in rare cases, Stx1 only (Nataro & Kaper, 1998). A recent study has substantiated the findings of earlier studies, that Stx2 producing *E. coli* are more likely to cause HUS than infection with organisms that produce Stx1 only (Siegler *et al.*, 2003). A variant of Stx2 termed Stx2c has been identified in an *E. coli* O157:H- strain (Schmitt *et al.*, 1991). Other members of the Stx2 family exist in non-O157 STEC isolates, such as Stx2e, which induces oedema disease in pigs (De Grandis *et al.*, 1989).

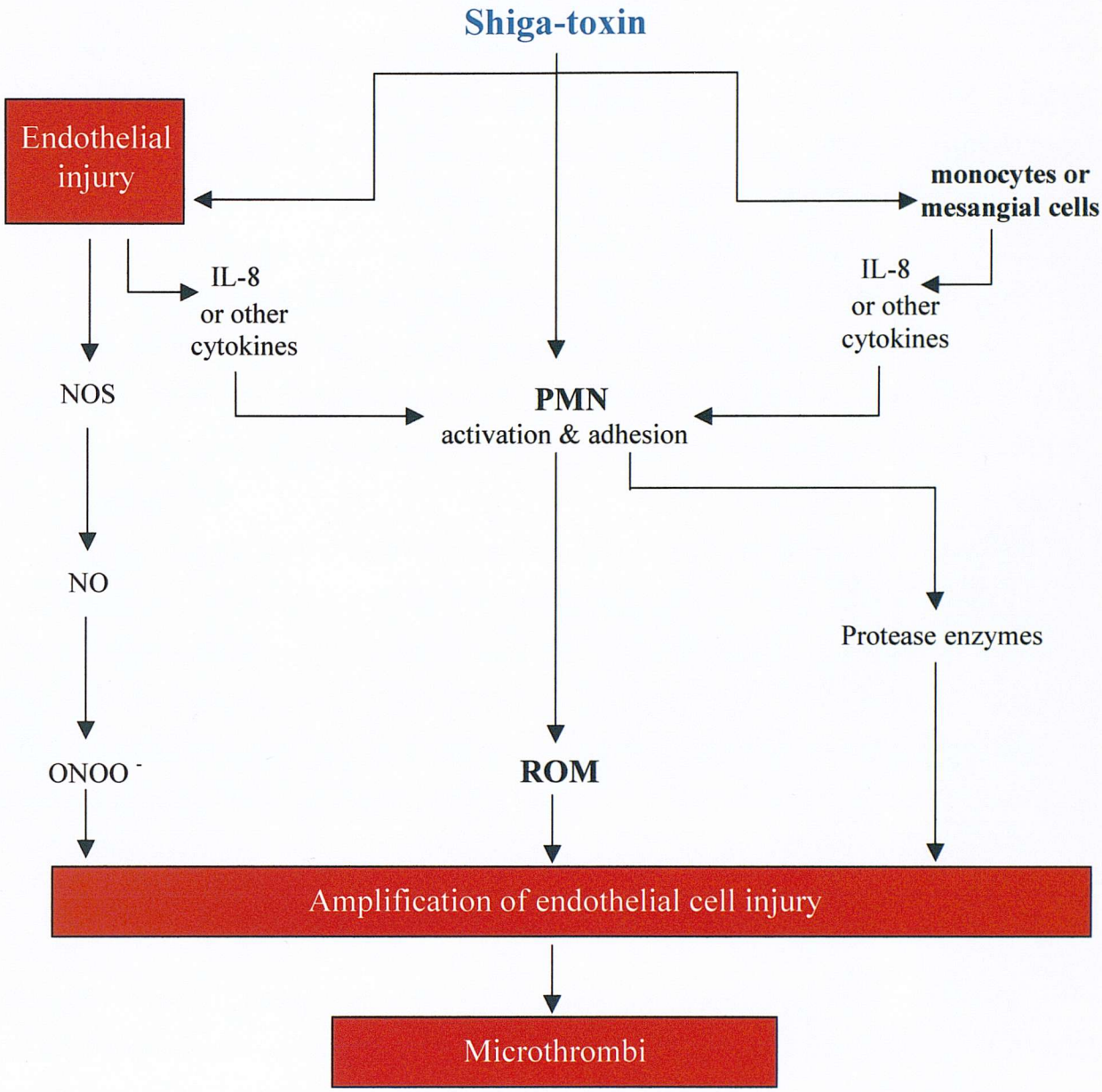
The genes encoding Stx1 and Stx2 are encoded on separate lysogenic bacteriophages. (Scotland *et al.*, 1983; O'Brien *et al.*, 1984; O'Brien *et al.*, 1989). In *E. coli* O157:H7 strain EDL933, Stx2 is encoded on bacteriophage 933W (O'Brien *et al.*, 1989). Regulation of Stx production and phage release studies have shown that the bacteriophage encoded H-19 *Q* gene product activates expression of both its own *stx1* genes and 933W *stx2* genes (Neely & Freedman, 1998; Plunkett III *et al.*, 1999). Interestingly, phage 933W from EDL933 is similar but not identical to the VT2-Sakai phage from the Japanese outbreak *E. coli* O157:H7 isolate (Schmidt, 2001). Studies for the control of Stx2 specific phage induction by the RecA protease were demonstrated by isogenic *recA* mutants of *E. coli* O157:H7 isolates that were greatly reduced in Shiga-toxin synthesis and were devoid of specific Stx2 phage production (Fuchs *et al.*, 1999).

The Stx1 phage (VT-1 Sakai) from the *E. coli* O157:H7 Sakai outbreak isolate has been sequenced completely (Yokoyama *et al.*, 2000). The phage is 47.9 kb in length and has 70 open reading frames. Although, the overall structure of VT-1 Sakai predominantly resembles that of phage lambda, its right arm is more homologous to that of phage 933W.

The B_5 structure of Shiga-toxins binds the receptor glycolipid, globotriaosylceramide (GB_3) that is expressed on the surface of microvascular endothelial cells (Lingwood *et al.*, 1987; Waddell *et al.*, 1988). After binding, the holotoxin is transported within endosomes to the golgi apparatus, where somewhere in the host cell the A-subunit is proteolytically nicked to yield the A_1 peptide and the much smaller A_2 peptide (ca. 4 kDa), but both peptides remain connected via a disulphide bond (O'Brien *et al.*, 1992). After reduction, both peptides separate and the A_1 peptide depurinates an adenine residue on the 28S rRNA portion of the 60S ribosomal subunit. This leads to inhibition of elongation factor 1-dependent aminoacyl-tRNA binding to ribosomes, preventing transcription of RNA, resulting in cell death (Igarashi *et al.*, 1989). This direct toxic effect occurs in the glomerular endothelial cells of the kidneys (Paton & Paton *et al.*, 1998). Furthermore, Te Loo *et al.* (2001) demonstrated that Stx2 is transported in humans from the intestine to these target cells by granulocytes. A proposed mechanism of endothelial cell damage is illustrated in Figure 1.1.

Other pathogenic effects of Stx described, include neutrophil mediated tissue damage by inhibition of neutrophil apoptosis by Stx2 (Liu *et al.*, 1999), and Stx2 associated brain damage (Kita *et al.*, 2000). Another recent study has also described Stx2 induced cell death is mediated by the anti-apoptotic protein Bcl-2 (Suzuki *et al.*, 2000). However, Schmidt *et al.* (1999) have presented data on *E. coli* O157:H7 and *E. coli* O157:H- clinical isolates that are

Figure 1.1. Mechanisms of endothelial cell injury caused by Shiga-toxin.



NO, nitric oxide; NOS, nitric oxide synthase; PMN, polymorphonuclear leukocyte; ROM, reactive oxygen metabolite.

Adapted from O'Loughlin & Robins-Browne, (2001).

Shiga-toxin negative, indicating that Stx is not essential for all the diseases associated with *E. coli* O157 strains.

1.7.2 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is implicated in the pathogenesis of HUS. Alterations to renal cells have been demonstrated and a synergy with Stx has been observed (Barrett *et al.*, 1989). An *in vivo* model of *E. coli* O157:H7 infection using LPS responder and LPS non-responder mice described by Karpman *et al.* (1997) showed that Stx2 positive strains caused the most severe renal symptoms in LPS responder mice. Louise & Obrig (1992) demonstrated the combined cytotoxic effects LPS and Stx toxin on human vascular endothelial cells *in vitro*. A mouse study by Shimizu *et al.* (1999) has suggested that LPS absorbed into the circulation from the inflamed colon could also enhance the injury caused by Stx virtue of their ability to stimulate the local or systemic release of proinflammatory cytokines from mesenchymal, endothelial or circulating immune cells. Obrig *et al.* (2000) has also suggested that this sensitisation following exposure to LPS occurs due to limited expression of the Gb3 receptor by endothelial cells.

The structure of the O-antigen polysaccharide of *E. coli* O157:H7 has been determined to be unbranched and linear with a tetrasaccharide repeating unit (Perry *et al.*, 1986). Characterisation of the genes required for *E. coli* O157:H7 and *E. coli* O157:H- O-antigen synthesis has been reported (Wang & Reeves, 1998; Shimizu *et al.*, 1999). Currie & Poxton, (1999) have shown that *E. coli* O157:H7 and other STEC isolates obtained from the reference laboratory at Aberdeen, all had the R3 chemotype LPS core. Amor *et al.* (2000) confirmed that the R3 chemotype appears only to be found in STEC isolates, including *E. coli* O157:H7.

The O-antigen of *E. coli* O157:H7 has been implicated in interfering with its adherence to HeLa cells (Bilge *et al.*, 1996; Cockerill *et al.*, 1996). Hyper-adherence was observed using an O-antigen negative mutant suggesting that the O-antigen of *E. coli* O157:H7 LPS may physically hinder attachment of outer membrane virulence factors to the host cell. This might implicate a modulation of virulence by LPS.

A recent study by Hara-Kudo *et al.* (2000) has demonstrated that *E. coli* O157:H7 lose O157 O-antigenicity when under starvation conditions for a long period, but can still produce Stx. Recovery of O157 O-antigenicity was not observed when cells were cultured on tryptic soy broth. This loss of immunologic response suggests that *E. coli* O157 that lose O-antigenicity will not be isolated using *E. coli* O157 O-antigenicity kits from contaminated samples. Furthermore, this phenotype may be detrimental to the organism itself, as *E. coli* O157:H7 LPS has also been implicated in acid and heat resistance (Mao *et al.*, 2001; Barua *et al.*, 2002).

1.7.3 Large virulence plasmid pO157

Nearly all strains of *E. coli* O157:H7 contain the 60 MDa plasmid pO157 (Schmidt *et al.*, 1994). This plasmid can also be found in some other EHECs, including O26:H11 strains (Beutin *et al.*, 1994). Sorbitol fermenting *E. coli* O157:H- isolates also harbour a large virulence plasmid, known as pSFO157, which is markedly different from pO157 (Brunder *et al.*, 1999). A role in pathogenicity by pO157 has not been clearly defined due to conflicting data from various animal models (Nataro & Kaper, 1998), but some studies have demonstrated pO157 induced haemolytic activity (Schmidt *et al.*, 1994), and adherence to intestinal cells (Fratamico *et al.*, 1993; Karch *et al.*, 1987).

The sequences of putative plasmid virulence genes have been reported and include a catalase peroxidase, *katP* (Brunder *et al.*, 1996), an extracellular serine protease, *espP* (Brunder *et al.*, 1997), four genes forming a haemolysin locus (Schmidt *et al.*, 1995) and 13 genes involved in encoding a type II secretion system (Schimdt *et al.*, 1997). Recent studies have identified a secreted metalloprotease which is encoded on pO157, designated StcE, that specifically cleaves C1 esterase inhibitor (Lathem *et al.*, 2002). The type II secretion on pO157 system has been shown by the same study to secrete StcE. It was also noted that StcE caused the aggregation of cultured human T-cells, but not macrophages. Substitution of aspartic acid for glutamic acid within the active site of StcE diminished the cleavage and aggregation abilities. The authors suggest that the cleavage ability of StcE may cause the localised pro-inflammatory and coagulation responses that result in tissue damage, intestinal oedema and thrombotic pathology.

Also contained within this plasmid is *tagA*, which encodes a lipoprotein homologous to the cryptic ToxR activated TagA of *Vibrio cholerae* (Elliott *et al.*, 2000). Antibodies to *E. coli* O157:H7 TagA has been detected in the sera of HUS patients, demonstrating that this protein is expressed *in vivo* (Paton & Paton, 2002). Furthermore, since this gene is present only in STEC serogroup O157, antibodies would function as a useful serological marker for *E. coli* O157 infections.

The large virulence plasmid of *E. coli* O157:H7 also harbours genes encoding a haemolysin belonging to the RTX toxin family, termed enterohaemolysin (Bauer *et al.*, 1996; Schmidt *et al.*, 1996). The gene encoding this haemolysin, *ehxA*, shares about 60% identity with the *hlyA* gene residing in UPEC (Bauer *et al.*, 1996; Schmidt and Karch, 1996). Although studies have demonstrated that the toxin lyses erythrocytes and bovine, but not human leukocytes, a clear role on pathogenesis has not been determined (Bauer *et al.*, 1996).

The complete sequence data for pO157 (Burland *et al.*, 1998) has revealed the presence of a new putative virulence gene designated L7095. The first 700 residues of this open reading frame (ORF) share strong homology with the N-terminal domain of the large

clostridial toxins ToxA and ToxB from *Clostridium difficile*. Klapproth *et al.* (2000) designated this gene *lifA*, and demonstrated that disruption of this gene in EPEC isolates affected lymphokine production and lymphocyte proliferation, and severely attenuated *E. coli* O157:H7 inhibition of IL-2 expression. A study by Tatsuno *et al.* (2001) indicated that the *toxB* gene on pO157 contributed to *E. coli* O157:H7 adherence to Caco-2 cells through the promotion and/or secretion of type III secreted proteins.

1.7.4 Other plasmids

A 3.3 kb plasmid has been isolated from *E. coli* O157:H7 isolates. Haarmann *et al.* (1998) have determined by nucleotide sequencing that this plasmid (p4821) was closely related to the *Salmonella enterica* serotype Typhimurium antibiotic resistance plasmid, NTP16. However, the *tra* genes were not detected, indicating that p4821 was a nonconjugative plasmid. This study also noted that of fifty *E. coli* O157:H7 strains isolated, 4 strains contained p4821 specific sequences. This 3.3 kb plasmid (termed pOSAK1) has also been characterised by nucleotide sequencing in the Sakai outbreak *E. coli* O157:H7 strain (Makino *et al.*, 1998). Again, the authors in this study confirmed that the nucleotide sequence of pOSAK1 was extremely similar to that of NTP16 and that it appeared to be nonconjugative. Genome sequencing of *E. coli* O157:H7 isolate EDL933 did not reveal the presence of the 3.3 kb plasmid (Perna *et al.*, 2001)

1.7.5 EAST1

First described in EAEC, EAST1 is also found in many EHEC strains. Savarino *et al.* (1996) identified the *astA* gene encoding EAST1 in all 75 *E. coli* O157:H7 isolates studied, usually with two copies in the chromosome. This study also reported the presence of *astA* in other EHEC serotypes. Recent research has indicated that that both copies of *astA* are unlikely to be expressed (Yamamoto & Taneike, 2000). The importance of this toxin is not known, but it may account for the symptom of non-bloody diarrhoea frequently seen in EHEC infected patients as suggested by Nataro and Kaper, (1998).

1.7.6 Flagella

Clones of *E. coli* are normally identified by their combination of O- and H-antigens (Ørskov *et al.*, 1977). The H-antigen of *E. coli* are fine long tubular structures that consist of the structural subunit, flagellin, encoded by the *fliC* gene. The N- and C-terminal portions of flagellin among *E. coli* species and other bacteria are conserved whereas the central exposed antigenic region of the flagellar filament is highly variable (Macnab, 1996; Winstanley *et al.*, 1997). Analysis of the *fliC* sequences of O157:H7 and O55:H7 serotypes shows they are almost identical but highly divergent from *E. coli* expressing other H types (Reid *et al.*, 1999; Wang *et al.*, 2000). Two subtypes of the H7 antigen have been described (Wright &

Villanueva, 1953; Ratiner & Sinelnikova, 1969) and a recent study has shown that the H7 antigen of *E. coli* O157:H7 has the H7a,c subtype (Ratiner *et al.*, 2003).

For certain *E. coli* pathogens, flagella have been associated with the penetration of mucous layers over epithelial cells (Smyth, 1988; La Ragione *et al.*, 2000a). A role for of *E. coli* O157:H7 flagella in pathogenicity has not been described, although, inhibition of *E. coli* O157:H7 attachment to HEp-2 cells was noted when bacteria were co-incubated with H7 antisera (Sherman & Soni, 1988). Western blot and motility analysis studies revealed that a *E. coli* O157:H7 *luxS* mutant (LuxS is involved in quorum sensing) produced fewer flagella and had decreased motility halos in semisolid agar (Sperandio *et al.*, 2001). A subsequent study by Sperandio and co-workers (2002) demonstrated that when the regulators of a novel quorum sensing two-component regulatory system (QseBC) were disrupted, less flagella were expressed and reduced motility was observed. However, a role for flagella in *E. coli* O157:H7 virulence is difficult to assess since *E. coli* O157:H- strains have emerged that are a public health problem in central Europe (Bielaszewska *et al.*, 1998; Schmidt *et al.*, 1999) and an outbreak in Scotland was linked to *E. coli* O157 that were non-motile (O'Brien *et al.*, 2001).

1.7.7 Fimbriae

Various types of fimbriae have been described for *E. coli* (Sussman, 1997). Some types are found in most *E. coli*, whether pathogenic or not e.g. type-1 fimbriae (Duguid *et al.*, 1955; Brinton, 1959; Duguid, 1959). An unusual 'curly' type structure has been identified in certain *E. coli* pathogens (Knutton *et al.*, 1987a) which has since been termed 'curli' (Oslen *et al.*, 1989). The significance of *E. coli* curli fimbriae in human disease remains unclear (Sussman, 1997). However, avian *E. coli* mutants defective for the production of curli are significantly reduced in their ability to persist in a 1-day-old chick model (La Ragione *et al.*, 2000b).

The genomes of both *E. coli* O157:H7 strains sequenced (Hayashi *et al.*, 2001; Perna *et al.*, 2001) have revealed the presence of seven fimbrial operons. The operon encoding type-1 fimbriae is found in both *E. coli* O157:H7 sequenced genomes (Hayashi *et al.*, 2001; Perna *et al.*, 2001) and Durno *et al.*, (1989) suggested that type-1 fimbriae were important for adherence of *E. coli* O157:H7 strain CL-49. However, CL-49 is unique because of over 40 other *E. coli* O157:H7 strains tested none produced type-1 fimbriae *in vitro* (Enami *et al.*, 1999). Li *et al.* (1997) identified a 16 bp deletion in *E. coli* O157:H7 within the regulatory switch region that controls expression of type-1 fimbriae. A recent study has confirmed the presence of this 16 bp deletion residing in the *fimA* regulatory region in 63 human and cattle *E. coli* O157 strains (Roe *et al.*, 2001). Both sequenced *E. coli* O157:H7 strains (Hayashi *et al.*, 2001; Perna *et al.*, 2001) have the curli operon, but curli expression is uncommon in *E.*

coli O157:H7. However, variations in curli expression have been reported in two human outbreak *E. coli* O157:H7 strains due to a single base pair mutation in the *csgD* promoter (Uhlich *et al.*, 2001). This mutation was associated with increased virulence in mice and increased invasion of HEp-2 cells (Uhlich *et al.*, 2002).

A role for fimbriae in *E. coli* O157:H7 remains unclear (Karch *et al.*, 1987; Toth *et al.*, 1990). Transfer of the pO157 plasmid to *E. coli* K-12 resulted in the elaboration of novel fimbriae by *E. coli* K12 and enhanced adherence to intestinal cells (Karch *et al.*, 1987). Torres *et al.* (2002) identified six putative ORFs in an *E. coli* O157:H7 strain, designated *lpfABCC'DE*, that are closely related to the long polar fimbriae operon of *Salmonella enterica* serovar Typhimurium. When this operon was introduced into a fimbriae negative strain of *E. coli* K12 it became adherent to tissue culture cells. An isogenic *E. coli* O157:H7 *lpfA* mutant showed a 30% reduction in adherence to tissue culture cells and formed fewer micro-colonies.

1.7.8 Acid resistance

As with other enteric pathogens, the infectious dose (10-100 bacteria) of *E. coli* O157:H7 is thought to correlate with acid resistance (AR) (Gorden and Small, 1993). RpoS is an alternate sigma factor (σ^s) involved in regulating the expression of numerous stress response genes (Hengge *et al.*, 1993). Cheville *et al.* (1996) demonstrated that the stationary phase acid tolerance of an *rpoS* mutant of *E. coli* O157:H7 was significantly reduced. *In vivo* studies using the same mutant demonstrated that RpoS appears to play a role in *E. coli* O157:H7 passage in mice and shedding in calves (Price *et al.*, 2000). Three mechanisms of acid resistance in *E. coli* O157:H7 have been described. These are the glucose-repressible oxidative pathway, the glutamate decarboxylase system and the arginine decarboxylase system (Hersh *et al.*, 1996; Lin *et al.*, 1996). A recent study by Cui *et al.* (2001) noted that *E. coli* O157:H7 confirmed that acid survival was cell density dependent and was associated with the induction of both decarboxylase systems and the concomitant availability of glutamine and arginine during acid challenge.

The involvement of surface polysaccharides are also important in the resistance of *E. coli* O157:H7 to acid. A study by Barua *et al.* (2002) demonstrated that mutants deficient for the production of the O-antigen demonstrated an acetic acid-sensitive phenotype. Also characterised in this study was enterobacterial common antigen, a surface polysaccharide whose chemical structure is, unlike LPS, common among all members of the *Enterobacteriaceae*. *E. coli* O157:H7 mutants defective for the expression of this surface polysaccharide became sensitive to acetic acid.

E. coli O157:H7 isolates, when under stress, can produce profuse amounts of an exopolysaccharide consisting of colanic acid (Junkins & Doyle, 1992). In an acidic

environment, there is a high concentration of protons and since colanic acid confers a strong negative charge to the bacterium surface, this may serve as a buffer by neutralising protons at the bacterium surface (Jordan *et al.*, 1999). Subsequent acid resistance studies, after insertion mutagenesis of the *wca* operon in an *E. coli* O157:H7 isolate, revealed that the mutant was defective for colonic acid production and less tolerant to acid (Mao *et al.*, 2001).

A recent study by Heimer *et al.* (2002) showed that the *E coli* O157:H7 urease is regulated by Fur since disruption of the *fur* gene in this pathogen significantly diminished urease activity. Fur is a global regulatory protein that uses ferrous Fe^{2+} to negatively regulate siderophore biosynthesis and transport and a number of other genes including Stx1 (Griffiths, 1997). The authors of the Heimer *et al.* (2002) study proposed that, similar to the function of Fur in regulating the *S. typhimurium* acid response, it modulates urease expression in *E. coli* O157:H7, perhaps by contributing to its acid tolerance phenotype.

1.7.9 Haemoglobin utilisation

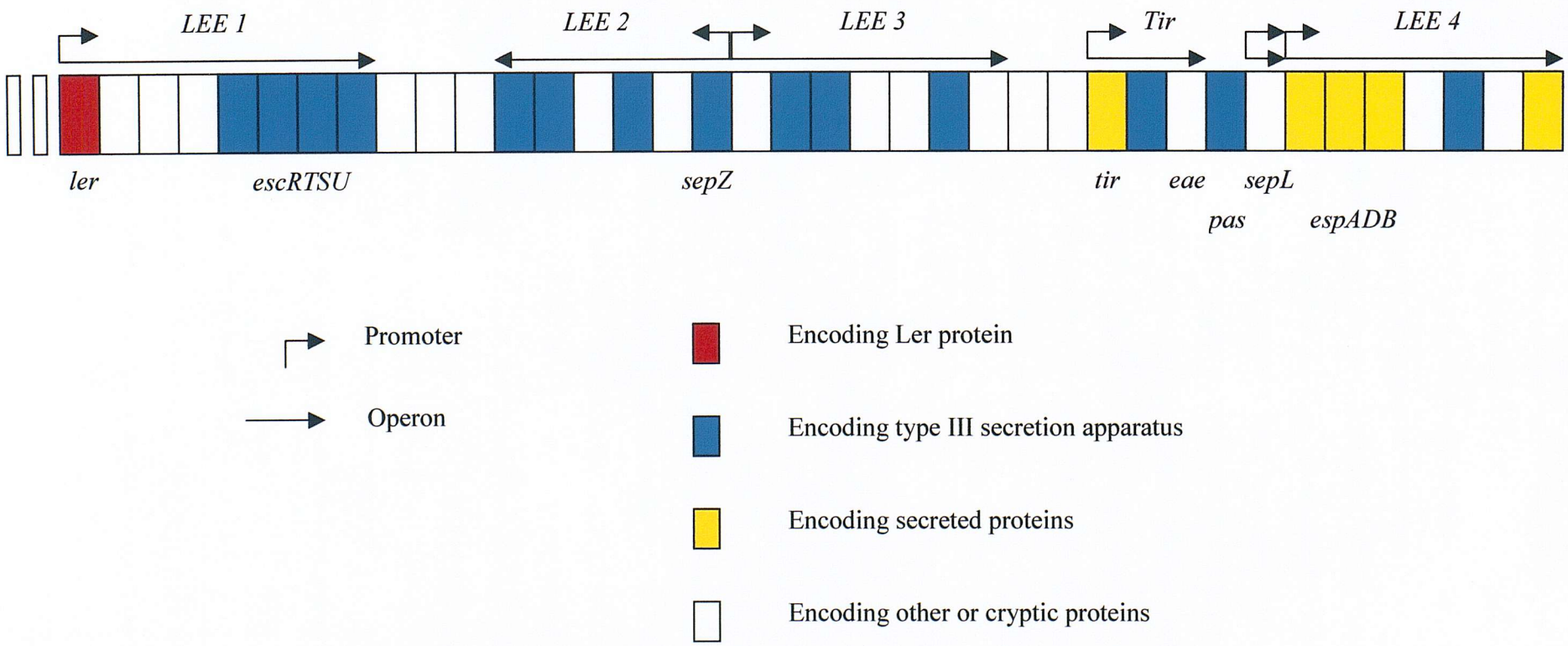
Identified in *Shigella dysenteriae*, the *shuA* gene, is required for haem transport. DNA hybridisation studies revealed a homologue in *E. coli* O157:H7 (Mills and Payne, 1995). This homologous region termed *chuA* has been cloned and characterised (Torres and Payne, 1997). The 69 kDa encoded product was synthesised in response to iron limitation and is Ton-B dependent. PCR has revealed all of thirteen *E. coli* O157:H7 isolates reacted with two primer sets designed for the *chuA* gene, but only a further three of 58 non-O157 isolates reacted with both primer sets (Potters *et al.*, 1999), suggesting that the restriction of *chuA* to the *E. coli* O157:H7 phenotype may partly explain its pathogenicity.

Studies by Ye & Xu, (2001) revealed that an iron transport gene cluster residing on a pathogenicity island in Uropathogenic *E. coli* also resides in Stx producing *E. coli* O157:H7. Although, detected in forty three Stx-positive *E. coli* O157:H7 isolates, the gene cluster was not detected in nineteen Stx-negative *E. coli* O157:H7 isolates. The authors have indicated that this data strongly suggests the importance of iron metabolism in Stx-mediated virulence.

1.8 Locus of enterocyte effacement (LEE)

Found in numerous EPEC is the 35 kb pathogenicity island, the locus of enterocyte effacement (LEE), which encodes genes involved in intimate adherence to intestinal epithelial cells, the initiation of host signal transduction pathways, and the formation of AE lesions (McDaniel *et al.*, 1995; McDaniel *et al.*, 1997). Perna *et al.*, (1998) reported the complete 43,359 bp LEE sequence from the *E. coli* O157:H7 strain EDL933 which is significantly larger than the 35,624 bp pathogenicity island sequenced for EPEC strain E2348/69 (Elliott *et al.*, 1998). A 7.5 kb prophage (933L) near the *selC* end of the LEE accounts for most of the difference in size. Although not present in E2348/69, the prophage is

Figure 1.2. The *E. coli* O157:H7 locus of enterocyte effacement (LEE).



Adapted from Elliot *et al*. (2000).

found in the closely related EPEC O55:H7 serotype. Five distinct operons exist that play a part in *E. coli* O157:H7 virulence (Figure 1.2). These operons and the genes within them are arranged in the same orientation as the EPEC E2348/69 LEE. Cloning, mapping and characterisation of the *E. coli* O157:H7 LEE has been described by Elliott *et al.* (1999). The various genes encoded within the LEE and their product function will be discussed below.

1.8.1 Ler (Lee encoded regulator) and quorum sensing

Encoded first on the LEE1 operon of both EPEC O127:H6 and *E. coli* O157:H7 is the *ler* gene (Elliott *et al.*, 1998). Recent studies have demonstrated that quorum sensing regulates the Ler protein, which in turn, regulates LEE and non-LEE encoded virulence factors (Sperandio *et al.*, 1999; Elliott *et al.*, 2000). Quorum sensing, first described in *Vibrio fischeri* (Bassler *et al.*, 1994), is a gene regulation phenomenum that allows bacteria to respond to an autoinducer (AI) signalling molecule that enables them to control gene expression in response to population density. Sperandio *et al.* (1999) proposed that the autoinducer signal (AI-2) involved in *E. coli* O157:H7 quorum sensing was derived from the normal gut flora since this pathogen has a low infectious dose. Nevertheless, it would appear that Ler only regulates genes in the LEE when there is an abundance of AI-2. In EPEC, the Ler protein is additionally regulated by the Per protein, which is encoded on the EAF plasmid (Mellies *et al.*, 1999). Kanamaru *et al.* (2000) have indicated that an *E. coli* homologue of quorum sensing regulators, SdiA, controls the expression of the *espD* and *eae* genes.

The organisation of the *E. coli* O157:H7 LEE is shown in Figure 1.2. The *ler* locus encodes a virulence gene expression regulator in *E. coli* O157:H7 (also EPEC). Production of the Ler protein is an absolute requirement for the formation of AE lesions, since all the genes known to be important for the AE phenotype are regulated by *ler*. The *ler*-regulated loci required for AE formation include the *esc* and *sep* genes (typeIII secretion apparatus), *espADB* (type III secreted proteins), *tir* (translocated intimin receptor), *eae* (intimin) (Elliott *et al.*, 2000). In fact, Elliott and co-workers have suggested that Ler regulates all the genes in the *LEE2*, *LEE3*, *Tir* and *LEE 4* operons and the last two genes in the *LEE1* operon. The Ler protein does not regulate Shiga-toxin, positively regulates TagA (encoded on pO157), and negatively regulates the expression of long polar fimbriae (Elliot *et al.*, 2000; Torres *et al.*, 2002). However, the cloned LEE from *E. coli* O157:H7 was unable to confer the AE phenotype upon *E. coli* K-12, suggesting that other transcribed genes outside the *E. coli* O157:H7 LEE are required for the AE phenotype (Elliott *et al.*, 1999).

A recent study by Abe *et al.* (2002) has demonstrated that the production of intimin, Tir, EspA and EspB was greatly enhanced when *E. coli* O157:H7 was grown in the presence of bicarbonate ion in LB broth. Furthermore, transcription of the *ler* gene required for LEE-encoded gene expression was promoted in response to the concentration of bicarbonate ion.

The authors have suggested that since the concentration of bicarbonate ion is highest in the lower intestinal tract of humans, this may imply that bicarbonate ion is an important signalling factor for promoting *E. coli* O157:H7 colonisation to this region of the gastrointestinal tract in humans.

1.8.2 Type III secretion systems

Type III secretion systems have been described for many bacterial pathogens of mammals and plants (Hueck, 1998). Described also for flagella proteins, MacNab (1999) has proposed that the type III secretion system for virulence factors has evolved from the type III flagella export system. The EPEC LEE-encoded type III secretion system was the first to be described for initiation of the AE phenotype (Jarvis *et al.*, 1995) and has been reviewed recently by Celli *et al.* (2000). This export system, also found in *E. coli* O157:H7 (Jarvis and Kaper, 1996), has identified EPEC and EHEC contact mediated haemolytic activity on erythrocytes (Warawa *et al.*, 1999; Shaw *et al.*, 2000).

At least three extracellular polypeptides (EspADB) secreted by the type III apparatus are necessary for induction of the EPEC and *E. coli* O157:H7 AE lesion (Ebel *et al.*, 1998; Kresse *et al.*, 1999). The use of specific monoclonal antibodies (mAbs) has shown that the EspA protein forms filamentous structures on the bacterial surface, and that these surface appendages were significantly prominent before the induction of AE lesions (Ebel *et al.*, 1998). EspA is also important for the translocation of the potential effector protein EspB, which has been found in the cytosol and cytoplasmic membrane of infected cells (Wolff *et al.*, 1998).

EspD, like EspA, appears to play a more significant role in the pathogenic process of *E. coli* O157:H7 infection than that of EPEC (Belterametti *et al.*, 1999; Ebel *et al.*, 1998; Kresse *et al.*, 1999). Importantly, Kresse *et al.* (1999) demonstrated that *E. coli* O157:II7 strain EDL933 produced EspA filaments which formed a link between the bacterium and the cell. In the same study the authors demonstrated that EspD protein is required for efficient bacterial attachment and that this link appears to be created via the EspA surface appendages. It has also been suggested that EspD and EspB are homologues of the translocator for effector proteins found in *Yersinia* species, YopB (Hensel *et al.*, 1998; Wolff *et al.*, 1998). Disruption of another LEE gene, *pas*, abolished the secretion of the Esp proteins, demonstrating the Pas protein plays an important role in *E. coli* O157:H7 pathogenesis (Kresse *et al.*, 1998).

Recent genome sequencing of two outbreak *E. coli* O157:H7 isolates (Hayashi *et al.*, 2001; Perna *et al.*, 2001) has revealed the presence of a second type III secretion system designated *E. coli* type three secretion 2 (ETT2). Although this locus resembles the *inv/spa* locus on the *Salmonella* SPI-1 pathogenicity island (Collazo & Gallan *et al.*, 1997), it

encodes only the components required for formation of the secretion apparatus, but not the secreted effector proteins. However, in *Salmonella*, it has been demonstrated that the Inv/Spa system can also translocate proteins encoded outside the SPI-1 locus (Hardt *et al.*, 1998; Norris *et al.*, 1998). Hayashi *et al.*, (2001) have suggested that since the cloned LEE element did not assist in the secretion of Esp effector proteins and induce the AE phenotype on *E. coli* K-12 (Elliot *et al.*, 1999) it may be possible that ETT2 complements the function of the LEE locus. Furthermore, unpublished data by Tobe and co-workers (cited in Hayashi *et al.*, 2001) has demonstrated that cloned ETT2 actually shows the ability to secrete EspB in K-12.

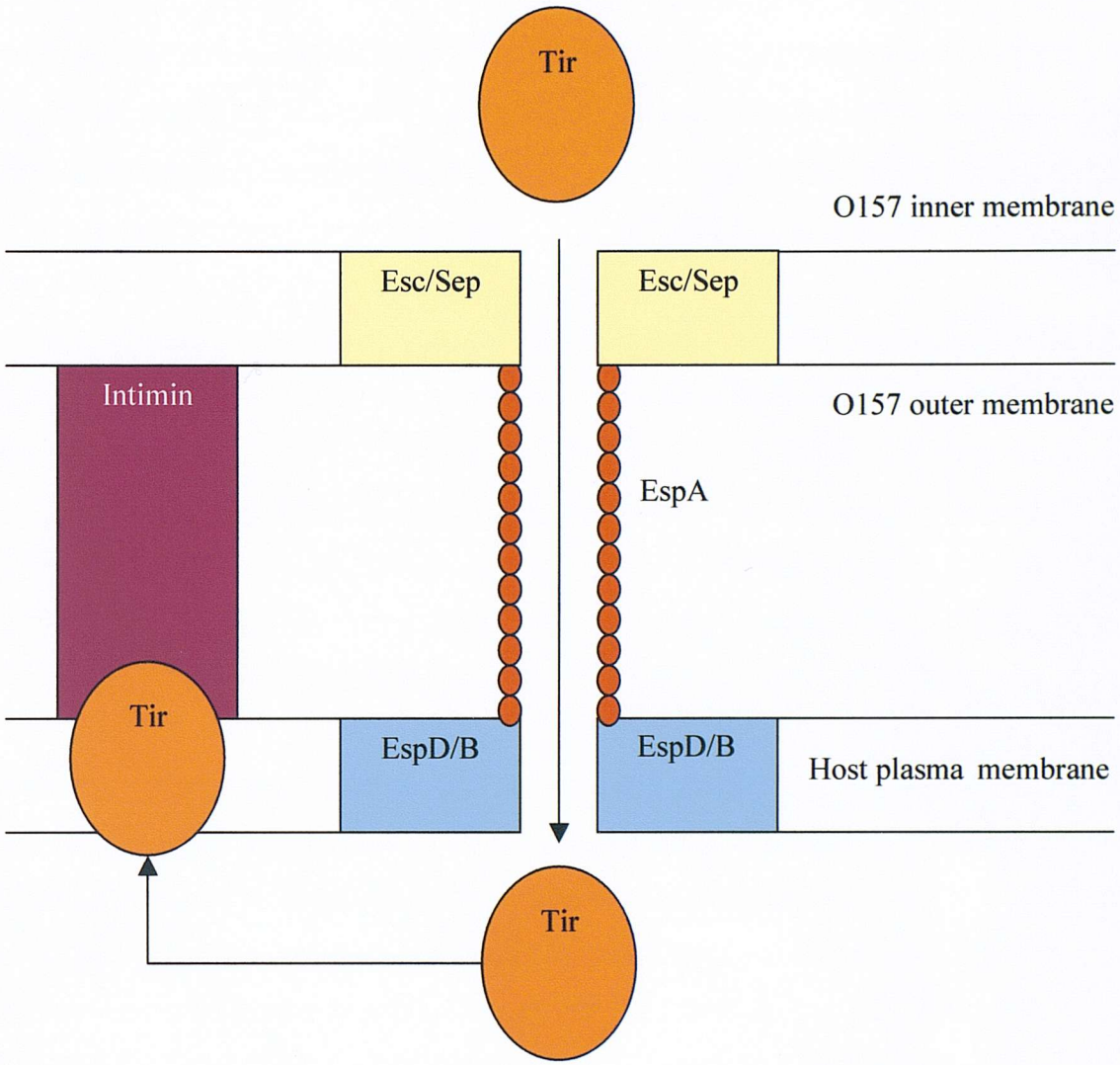
1.8.3 Translocated intimin receptor (Tir)

Another protein secreted by the type III apparatus encoded on the LEE, is the translocated intimin receptor, Tir (Figure 1.3). Originally, Tir was thought to be a host epithelial receptor called Hp90 that was triggered by EPEC AE bacteria (Rosenshine *et al.*, 1996). However, Kenny *et al.* (1997) demonstrated that Tir was produced and secreted into the host plasma membrane by EPEC. Deibal *et al.* (1998), described a novel 80 kDa protein, termed EspE, that is secreted by *E. coli* O157:H7 and other AE positive bacteria that was identical to the previously described Hp90 suggesting that all AE bacteria produce their own bacterial receptor. Now described as Tir in both EHEC (including *E. coli* O157:H7) and EPEC, the EHEC Tir shares 58% identity with the EPEC Tir (De Grado *et al.*, 1999). The EHEC Tir contains two transmembrane domains (TMDs) and the region between them has 85% identity with the extracellular loop of EPEC Tir. Both *E. coli* O157:H7 and EPEC O127:H6 can bind each others intimins, although *E. coli* O157:H7 Tir has a greater affinity for *E. coli* O157:H7 intimin than EPEC O127:H6 intimin (DeVinney *et al.*, 1999). EPEC O127:H6 Tir is tyrosine phosphorylated after it has been inserted into the host membrane (Kenny *et al.*, 1997) whereas the Tir of *E. coli* O157:H7 is not (DeVinney *et al.*, 1999), although originally suggested to be so by Deibal *et al.* (1998). This finding was supported by Kenny, (2001) who demonstrated that *E. coli* O157:H7 Tir is functionally unable to to substitute for its EPEC homologue. Furthermore, DeVinney and co-workers (2001) have additionally suggested that other putative type III secreted factors are required for *E. coli* O157:H7 AE lesion formation.

1.8.4 Intimin

Intimin, an outer membrane adhesin, permits intimate attachment of AE bacteria, by binding to bacterial inserted host membrane, Tir (Jerse *et al.*, 1990; Donnenberg *et al.*, 1993; Schauer *et al.*, 1993). Fully functional *E. coli* O157:H7 intimin is also required for localised adherence patterns and micro-colony formation as intimin deficient mutants are unable to induce this phenotype (McKee *et al.*, 1995; Cookson & Woodward, 2003). Ten distinct intimin subtypes have been described (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000; Tarr &

Figure 1.3. Simple schematic diagram of the *E. coli* O157:H7 type III secretion system.



Adapted from DeVinney *et al.* (1999)

Whittam, 2002; Zang *et al.*, 2002). These subtypes are intimin α (alpha), β (beta), η (eta), ε (epsilon), γ (gamma), ι (iota), κ (kappa), θ (theta), ζ (zeta) and are defined by the variable sequence of the 280-amino acid C-terminal receptor binding region, Int280. Intimin alleles α, β and γ have each be subgrouped into two groups by restriction enzyme analysis (Oswald *et al.*, 2000). Int280 can bind directly to uninfected host cells in the absence of Tir (An *et al.*, 1997; Frankel *et al.*, 1994). Intimin α is specific for EPEC strains that belong to an evolutionary group called EPEC clone 1 (Whittam and McGraw, 1996), and *Hafnia alvei.* Intimin β is associated mostly with EPEC clone 2 and EHEC clone 2, *Citrobacter rodentium*, and rabbit diarrhaegenic *E. coli* type 1. Intimin γ is expressed with *E. coli* O157:H7 (EHEC clone 1) and EPEC O55:H7 (Adu-Bobie *et al.*, 1998).

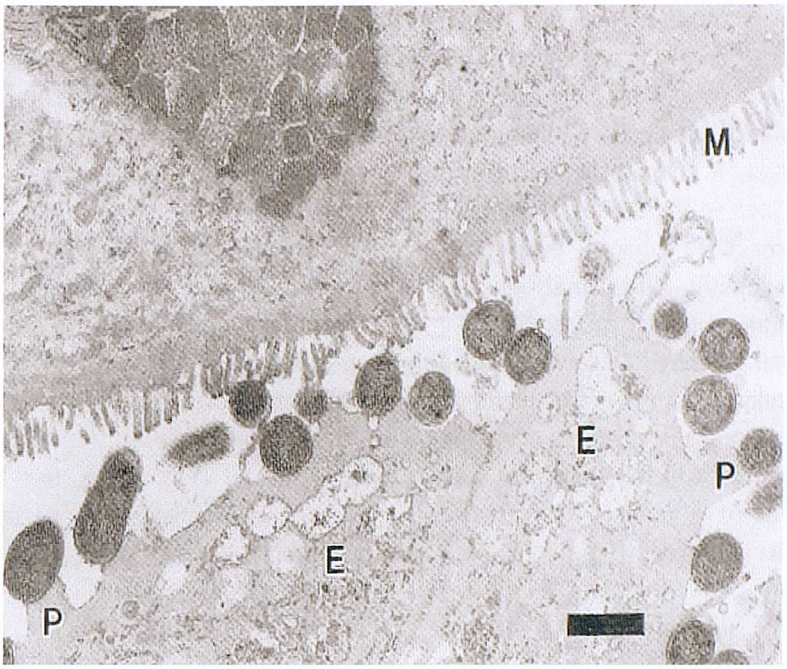
Particular *in vitro* and *in vivo* studies have suggested that different intimins might play a role in determining the pattern of colonisation and tissue tropism within a host (Tzipori *et al.*, 1995; Fitzhenry *et al.*, 2002). However, Frankel *et al.* (1998) have stated that other initial attachment factors are more likely to be responsible for tissue tropism. Rosenshine *et al.* (1996) showed that cloned intimin and purified Int280 would not bind HeLa cells in the absence of Tir. Nevertheless, formation of the AE lesion is initiated after intimin has bound to Tir. One study has demonstrated that the 181 residue minimal Tir binding region from an *E. coli* O157:H7 strain, when purified and immobilised on latex beads, was sufficient to initiate AE lesions on pre-infected mammalian cells (Liu *et al.*, 1999). A recent study by Liu *et al.* (2002) isolated, after random mutagenesis, twenty four point mutants in *E. coli* O157:H7 intimin which disrupted Tir recognition. Many mutations, including eleven that were required for efficient AE lesion formation, fell within a 50 residue region near the C-terminus. Four amino acid residues (Ser890, Thr909, Asn916 & Asn927) were shown to be critical for Tir recognition. Further analysis revealed that these residues lay within an intimin-Tir interface and contribute to a pocket that interacts with Ile298 of *E. coli* O157:H7 Tir.

Whether the binding of intimin to Tir is essential for intimate adherence to a cell surface is debatable, since some studies have indicated otherwise. Frankel *et al.* (1996) demonstrated that the EPEC C-terminus of intimin could bind β1 integrins. However, Liu *et al.* (1999) demonstrated that β1 integrin antagonists or inactivation of the β1 integrin did not inhibit EPEC intimin binding or actin accumulation. Whether any of these observations occur with *E. coli* O157:H7 remains to be elucidated.

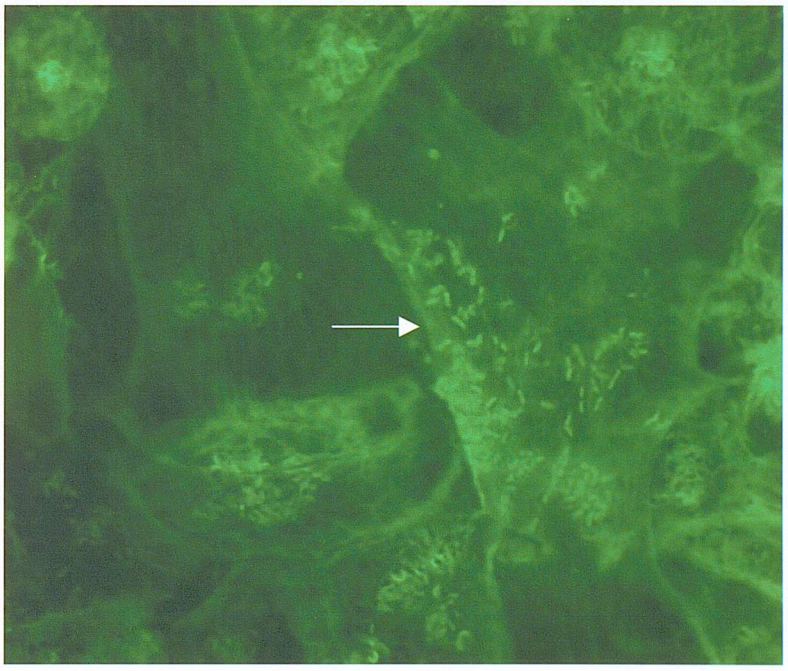
1.9 Attaching and effacing (AE) lesion formation

AE lesions (Figure 1.4) are characterised by localised destruction of brush border microvilli, intimate bacterial adhesion, and the accumulation of polymerised actin beneath the adherent bacteria resulting in a cup-like actin pedestal (Knutton *et al.*, 1987b; Knutton *et al.*, 1989).

Figure 1.4. Formation of AE lesions by *E. coli* O157:H7.



Taken from Wales *et al.* (2001). *E. coli* O157:H7 effacement (E) of microvilli (M) and accumulation of actin to form AE lesions (P) on sheep rectum enterocytes. Bar = 1μm.



E. coli O157:H7 isolate Walla3 accumulation of HEp-2 actin as observed by fluorescent actin staining (FAS), unpublished data. Magnification x 1000.

After infection with EHEC and EPEC, several signal transduction pathways appear to be triggered in epithelial cells (Baldwin *et al.*, 1991; Dytoc *et al.*, 1994). These early studies suggested that calcium released from inositol triphosphate-sensitive intracellular stores and the stimulation of the classical phospholipase C pathway induce the breakdown of micovillous actin. However, a subsequent study demonstrated that an increase in intracellular calcium was not required for AE lesion by EHEC and EPEC (Bain *et al.*, 1998). The signal or signals, and mechanisms required for cytoskeletal disruptions remained unclear for many years (Frankel *et al.*, 1998). Furthermore, little was known about the composition of EHEC pedestals, except that cortactin, α-actinin and actin were the only host proteins shown to be specifically recruited to the EHEC pedestal (Ismaili *et al.*, 1995; Cantarelli *et al.*, 2000).

As mentioned previously, *E. coli* O157:H7 Tir is not tyrosine phosphorylated, yet the pedestals appear similar. Recent studies into the recruitment of cytoskeletal and signalling proteins to EPEC and EHEC pedestals have been reported (Goosney *et al.*, 2001). Interestingly, EPEC O127:H6 was able to recruit gelsolin, tropomyosin, ezrin, α-actinin and talin to pedastals independently of Tir tyrosine phosphorylation. These proteins were also recruited to the *E. coli* O157:H7 pedestal, but whereas the adapter proteins Grb2 and CrkII were recruited to the EPEC pedestal, these were absent from the *E. coli* O157:H7 pedestal, indicating that the role of Tir tyrosine phosphorylation may be related to the recruitment of adapter proteins to the site of bacterial adherence. Kodama *et al.* (2002) have demonstrated that the EspB protein of *E. coli* O157:H7 interacts directly with the cytoskeletal-associated protein α-catenin by binding directly to the N-terminal region of EspB.

Although the proteins required for AE lesion formations are highly conserved between EHEC an EPEC, EHEC can create less pronounced AE lesions than EPEC (Cantey and Moseley, 1991) and the reason for this remained unknown. However, Tu and co-workers (2003) have discovered that *orf18* of the LEE in EPEC O127:H7 and *E. coli* O157:H7 encodes a new effector protein, termed EspH that modulates the actin cytoskeletal structure of AE lesions. Inactivation of *espH* in the EPEC isolate resulted in enhanced formation of filopodia and attenuated AE lesion formation, whereas over expression of *espH* resulted in strong repression of filopodium formation and heightened pedestal formation. Over expression of *espH* in *E. coli* O157:H7 induced marked elongation of typically flat pedastals.

Kresse *et al.* (2000) have demonstrated that an *E. coli* O157:H7 *sepL* mutant was strongly impaired in its ability to attach to HeLa cells and induce local accumulation of actin. The gene *sepL* is thought to be transcribed monocistronically and independently of the downstream located *esp* operon (Figure 1.2).

A recent study by McNally *et al.* (2001) investigated the levels of secreted type III secreted proteins between human disease-associated and bovine-derived *E. coli* O157

isolates. Human isolates produced significantly higher levels of EspD and Tir *in vitro* and AE lesion formation and actin rearrangement correlated with this finding, possibly indicating that if AE lesion formation is important for virulence in humans; bovine strains may differ in their human-pathogenic potential.

1.10 *In vitro* tissue culture assays for studying *E. coli* O157:H7 adherence patterns

The molecular basis of *E. coli* O157:H7 pathogenesis is being characterised partly by the use of tissue culture assays of human and mammalian origin. The human epithelial cell line, HEp-2, was first used to characterise the adherence of *E. coli* O157:H7 (Karch *et al.*, 1987; Sherman *et al.*, 1987). Karch and co-workers did not detect any adherence by *E. coli* O157:H7 whereas, Sherman and co-workers did report that *E. coli* O157:H7 did adhere to, but did not invade, HEp-2 cell mono-layers. Sherman & Soni, (1988) demonstrated that outer membrane *E. coli* O157:H7 extracts inhibited adherence of this pathogen to HEp-2 cells, whereas, isolated H7 flagella and LPS did not inhibit *E. coli* O157:H7 attachment. Knutton *et al.* (1989) demonstrated by fluorescent actin staining (FAS) that *E. coli* O157:H7 and other AE bacteria accumulated actin on HEp-2 cells (and Caco-2 cells) at the site of bacterial adhesion to produce AE lesions. McKee and co-workers (1995) demonstrated that intimin was required for the formation of AE lesions on HEp-2 cells. This human cell line has also been used to characterise truncated *E. coli* O157:H7 intimin fusion proteins (McKee & O'Brien, 1996). In this study the authors demonstrated that when intimin was truncated by one-third of its N-terminus it enhanced binding of the wild-type O157:H7 isolate and conferred adherence of its intimin deletion mutant. Gansheroff *et al.* (1999) using antibodies that recognised the C-terminus region of intimin, decreased the adherence of *E. coli* O157:H7 to HEp-2 cells. A recent study by Cookson & Woodward, (2003) has shown that the adherence of an *E. coli* O157:H7 intimin deficient mutant was diffuse with no evidence of intimate attachment, whereas the wild-type formed micro-colonies and AE lesions. A recent study by Sinclair & O'Brien (2002) using HEp-2 cells demonstrated intimin Tir-independent binding, where intimin type γ of *E. coli* O157:H7 bound the eukaryotic receptor, nucleolin.

E. coli O157:H7 interacts with other types of tissue culture assays including HeLa and Caco-2 cell lines (Canterelli *et al.*, 2000; Tatsuno *et al.*, 2000; Kenny, 2001; McNally *et al.*, 2001). One study employing the use of Caco-2 cells demonstrated that *E. coli* O157:H7 grown in DMEM exhibited greater adherence, than *E. coli* O157:H7 grown in LB broth (Abe *et al.*, 2002). A novel *E. coli* O157:H7 adherence-conferring molecule, known as Iha, which is encoded within a tellurite pathogenicity island, has been shown to be important for adherence to HeLa and MDBK cells (Tarr *et al.*, 2000). Other cell lines used for *E. coli* O157:H7 characterisation, include Henle 407 human intestinal epithelial cells, where it was shown that homologous anti-LPS inhibited *E. coli* O157:H7 adherence by 95% (Paton *et al.*,

1998). Olano-Martin *et al.* (2003) demonstrated that pectins and pectic-oligosaccharides inhibited the toxicity of *E. coli* O157:H7 Stx directed towards the human colonic cell line HT29.

The use of a bovine MDBK (kidney) and MAC-T (mammary secretory epithelial cell lines has been reported by Matthews and co-workers (1997). *E. coli* O157:H7 invaded MDBK cells at approximately 4% of MAC-T cells, suggesting that invasion of mammary epithelial cells may be an important route for the contamination of raw milk. Studies involving an Stx negative *E. coli* O157:H7 isolate demonstrated localised adherence patterns, micro-colony formation and AE lesions on bovine primary culture cells derived from a neonatal calf (Dibb-Fuller *et al.*, 2001). Hoey *et al.* (2003) have demonstrated that the binding of *E. coli* O157:H7 Stx1 to Gb3 receptor cells expressed in the crypts of intestinal bovine primary culture cells did not induce a cytotoxic effect against these cells. The authors have suggested that this may partly explain the differential consequences of EHEC infection in reservoir hosts and humans.

Some studies have employed the use of bovine and human gut explants to characterise *E. coli* O157:H7 adherence. The AE phenotype was observed using large bovine gut explants, and human intestinal mucosa (Baehler & Moxley, 2000) indicating that the large intestinal mucosal epithelium may be a site for *E. coli* O157:H7 infection that contributes to persistence in the bovine host. Furthermore, Philips *et al.* (2000) demonstrated using *in vitro* intestinal organ cultures (IVOC) of the human and bovine intestine, that an *E. coli* O157:H7 isolate induced AE lesions on the follicle associated epithelium of Peyer's patches in human and bovine ileum. A further study by Baehler & Moxley (2002) demonstrated that *E. coli* O157:H7 cultured in tryptic soy broth and the incubation of bovine gut explants in 5% CO_2, with rocking at 18 cycles per minute, provided optimal conditions for AE lesions. However, a study by Cookson and Woodward (2003) demonstrated intimin-independent adherence to neonatal calf gut explants.

A recent study by Fitzhenry *et al.* (2002), reported that intimin type influences the site of human intestinal mucosal colonisation by *E. coli* O157:H7. Human intestinal IVOC studies revealed that by introducing a deletion mutation in the *eae* gene encoding intimin γ in *E. coli* O157:H7 did not allow colonisation of this isolate to these explants. However, colonisation of Peyer's patches and the AE phenotype were resolved when intimin γ expression was completed from a plasmid. More importantly, when complementation occurred with intimin α expression from a plasmid, the *E. coli* O157:H7 isolate was able to colonise and produce AE lesions on both Peyer's patches and other small intestinal explants.

1.11 Characterisation of *E. coli* O157:H7 pathogenicity in animal models

Although *in vitro* models have elucidated the characteristics of *E. coli* O157:H7 involved in intimate attachment and pedestal formation it is still unclear whether or not they are important for initial colonisation and persistence in the animal host. Various animal models for the study of *E. coli* O157:H7 have been described within the last 12-13 years. Each breed of animal model has been employed to characterise either disease processes or mechanisms of attachment or persistence. These different types of animal models range from small animal models such as mouse and chick models to larger animal models such as piglet, pig, lamb, sheep and cattle.

1.11.1 Murine models

Various mouse models have been utilised to study colonisation and disease by *E. coli* O157:H7. Wadolkowski *et al.* (1990) used a streptomycin-treated mouse model to compare the relative colonisation abilities of *E. coli* O157:H7 strain 933 and its pO157 plasmid-cured derivative, strain 933cu. When 933 and 933cu were given simultaneously to mice, 933cu was unable to maintain a stable level of colonisation in approximately two-thirds of the mice tested.

Experimental infection of specific pathogen-free (SPF) mice with *E. coli* O157:H7 (Xin-He *et al.*, 1991) revealed several of the symptoms and histological changes observed in patients. Histological changes of the intestine were observed mostly in the distal small intestine and in the caecum. In the kidneys of infected mice, swollen epithelial glomeruli cells and marked thickening of the glomerular capilliaries with barely visible lumens was observed. Sloughing of bronchiolar epithelial cell wall was also observed and histological changes in the spleen, liver and lymphnodes was also noted.

Various mouse models using LPS-responder (C3H/HeN) and LPS-nonresponder (C3H/HeJ) mice to demonstrate the effects of LPS and Stx have been described (Barrett *et al.*, 1989; Harel *et al.*, 1993; Karpman *et al.*, 1997). LPS exhibited lethal effects in C3H/HeN which were enhanced by pretreatment of mice with Stx1 and Stx2 (Barrett *et al.*, 1989; Harel *et al.*, 1993). Karpman and co-workers (1997), investigating the role of LPS and Stx in an O157 mouse model of infection, showed that LPS-responder mice developed both neurologic and systemic symptoms, whereas LPS-nonresponder mice had a two-fold course of disease involving systemic symptoms first, and severe neurologic symptoms second. Other studies looking specifically at brain damage in other strains of mice have reported positive immuno-reactions of Stx2 in brain tissues (Kita *et al.*, 2000).

Young adult female mice (CD1, BALB/C and C57BL/6) were experimentally inoculated with an *E. coli* O157:H7 strain that produced Stx1 and 2 (Conlan & Perry, 1998). Although this fully virulent bacterium was detected by faecal shedding for up to five weeks post

infection, none of the three mouse strains examined developed obvious disease to O157 infection. Kurioka *et al.* (1998) reported that more than 75% of 5-week old C57BL/6 mice with protein calorie malnutrition inoculated with Stx-producing *E. coli* O157:H7 died 10 days post infection. Another study using 8-week-old germ-free mice (Taguchi *et al.*, 2002) demonstrated that after challenge with a hyper-toxigenic *E. coli* O157:H7 strain, all mice developed ruffled fur and convulsion of the limbs or hindleg weakness within 3 days after infection, culminating in death within 5 days.

E. coli O157 mouse studies have investigated the role of TNFα (Isogai *et al.*, 1998; Kita *et al.*, 2000) in neurological damage where pre-exposure of target cells to TNFα is thought to be a pre-requisite to brain damage.

1.11.2 Porcine models

To determine the role of intimin in the infected host a number of porcine models have been investigated. Donnenberg *et al.* (1993) demonstrated that an *eae* deletion insertion *E. coli* O157:H7 mutant was unable to attach intimately to the colonic epithelial cells of colostrums-fed newborn piglets. A further study showed that a wild-type *E. coli* O157:H7 isolate (86-24) colonised the surface and glandular epithelium of the large intestine and induced diarrhoea in gnotobiotic piglets (Tzipori *et al.*, 1995). Piglets also developed severe neurological symptoms, which in some resulted in overnight death. The wild type's *eae* mutant was unable to colonise any site in the intestine and induced little or no diarrhoea. Intimate attachment was not required for Stx2 translocation from the gut lumen into the circulation as piglets infected with the *eae* mutant still developed neurological complications. A parallel study demonstrated histologically that strain 86-24 and EDL933 primarily colonised the caecum and spiral colon of gnotobiotic piglets (McKee *et al.*, 1995). Histological analysis of the 86-24 *eae* mutant showed no evidence of colonisation of the piglet intestine.

A recent porcine model has been used to investigate the possibility of an *E. coli* O157:H7 anti-transmission vaccine (consisting of purified antibodies against *E. coli* O157:H7 intimin) to prevent cattle becoming infected and transmitting *E. coli* O157:H7 to humans (Dean-Nystrom *et al.*, 2002). Piglets that ingested vaccinated dam colostrum containing O157 intimin specific antibodies were protected from *E. coli* O157:H7 colonisation, whereas those piglets nursing sham-vaccinated dams were not protected from colonisation and damage from *E. coli* O157:H7.

Mukherjee and co-workers (2002) have demonstrated that human Stx2-specific antibodies administered intra-peritonealy to gnotobiotic piglets 6 to 12 hours after *E. coli* O157:H7 infection prolonged survival and prevented the development of fatal neurological signs and cerebral lesions. It has been suggest by the authors that human Stx2-specific

antibodies could be used passively to protect against HUS development in humans infected with *E. coli* O157:H7 and other Stx producing bacteria.

Experimental infection of 3-month-old pigs by Booher *et al.* (2002) demonstrated that two Stx producing *E. coli* O157:H7 strains were able to persist in the alimentary tracts of some pigs for 2 months post inoculation in the presence of other pathogenic *E. coli* types, suggesting that pigs may be a potential reservoir for *E. coli* O157:H7 infection.

1.11.3 Bovine models

Many food sources have been described as vehicles of human infection by *E. coli* O157:H7, but bovine meat and dairy products have been cited as the main source of infection (Griffin & Tauxe, 1991). One strategy for reducing *E. coli* O157:H7 infections in humans is to reduce the prevalence of *E. coli* O157:H7 in cattle. Various *in vivo* models characterising O157 interaction with the animal host have been described, since the role of the *eae* gene in *E. coli* O157:H7 intimate attachment was first described in the porcine model described above (Donnenberg *et al.*, 1993).

Experimental infection of calves and adult cattle with *E. coli* O157:H7 has been described by Cray & Moon, (1995). This study observed that *E. coli* O157:H7 was shed in greater numbers and for a longer duration in calves (as a group) than in adults. However, shedding persisted for at least 3 months by individual animals from both groups. *Post mortem* examination isolated *E. coli* O157:H7 from the alimentary tracts of all animals culled, but no *E. coli* O157:H7 was found in the liver, spleen or kidneys. A site of infection was not determined and histological analysis of all gastrointestinal tract sites were shown to be normal.

A later study by Brown *et al.* (1997) showed that *E. coli* O157:H7 was found in the alimentary tracts of 13 to 27-day-old calves, especially in either the contents of the fore-stomach or the colon, but not in sites outside this region. No histological or immunohistochemical evidence of *E. coli* O157:H7 adherence to the mucosa was observed. This suggested that *E. coli* O157:H7 did not colonise the gut mucosa for long periods of time and that persistence occurred in the contents of the rumen and the colon.

Dean-Nystrom *et al.* (1997) demonstrated that *E. coli* O157:H7 caused enterocolitis and diarrhoea, and produced AE lesions in colostrums-deprived calves. A subsequent study (Dean-Nystrom *et al.*, 1998) showed by electron microscopy that an *E. coli* O157:H7 *eae* mutant, that had been complemented with the *eae* gene, formed AE lesions on absorptive cells from the ileum of a colostrums-deprived calf, 18 hours after infection.

A more recent study by Woodward *et al.* (1999) using gnotobiotic calves deprived of colostrum demonstrated that an *E. coli* O157:H7 strain did not cause disease during the 24 day experiment (animals were challenged at five days of age). No evidence for AE lesions

was observed in the small or large intestine of calves culled 24 hours after infection or at any subsequent time point. Woodward *et al.* (1999) have suggested that this and other data may indicate that host receptors may be gained or modified as the animal matures.

Grauke *et al.* (2002) observed that *E. coli* O157:H7 persisted for about 1 week to 1 month in cattle and that the predominant location of this bacterium was the lower gastointestinal tract. A recent study by Naylor *et al.* (2003) that set out to characterise the persistence and tissue tropism of four different *E. coli* O157:H7 isolates observed that experimentally infected calves shed *E. coli* O157:H7 for at least 14 days prior to necropsy. In almost all persistently colonised animals, the majority of tissue-associated bacteria were identified in a region within 5 cm, or in many cases 3 cm, proximal of the rectal anal junction (RAJ) and was characterised by a high density of lymphoid follicles. Data also acquired in this study demonstrated that the highest numbers of *E. coli* O157:H7 were recovered from the faeces and the none or low-levels of this bacterium were recovered from the lumen contents of the gastrointestinal tract. Naylor *et al.* (2003) have suggested that this tropism may enhance the dissemination of *E. coli* O157:H7 between animals and animals and humans.

As with regard to a role for intimin in the colonisation of bovines, Cornick *et al.* (2002) have demonstrated that wild-type *E. coli* O157:H7 was shed in greater numbers and for a longer duration than an isogenic intimin-deficient mutant by young adult cattle. However, it should be noted that the intimin mutant was still shed by one animal at day 60 post inoculation.

1.11.4 Ovine models

The bovine host is considered the primary reservoir for *E. coli* O157:H7, but the ovine host is recognised as an important reservoir also. *E. coli* O157:H7 has been isolated from sheep (Chapman *et al.*, 1997; Kudva *et al.*, 1997; Heuvelink *et al.*, 1998) and sheep's milk (Rubini *et al.*, 1999). Kudva *et al.*, (1995) demonstrated that dietary change with brief starvation caused uniform shedding and clearance of *E. coli* O157:H7 in experimental sheep.

As with the colonisation of cattle, *E. coli* O157:H7 colonisation of sheep is not fully understood. Persistence studies in sheep have indicated that *E. coli* O157:H7 can persist for 2 weeks to 2 months post inoculation and that dietary change followed by brief starvation can induce clearance of *E. coli* O157:H7 (Kudva *et al.*, 1995; Cornick *et al.*, 2000).

Variation in the persistence of *E. coli* O157:H7 in experimenatally inoculated 6-week-old conventional lambs has been observed (Cookson *et al.*, 2002). Three *E. coli* O157:H7 isolates were not detected in the faeces of any lambs beyond day 8 post inoculation, whereas an *E. coli* O157:H7 Nal^r isolate was still in the faeces of one lamb at day 28 post inoculation. A study by Grauke *et al.* (2002) recovered *E. coli* O157:H7 from the faeces of one sheep at

day 43 post inoculation and observed that persistence occurred in the lower gastrointestinal tract.

E. coli O157:H7 AE lesions have been observed in experimentally inoculated 6-day-old conventionally reared lambs 12 and 36 hours post inoculation and the production of AE lesions has also be noted in ligated large intestine loops of 6-month-old sheep by Stx-positive and negative *E. coli* O157:H7 isolates (Wales *et al.*, 2001; Wales *et al.*, 2002). However, in both studies the lesions were small and sparse, typically comprising of O157 bacteria intimately attached to single or a few adjacent enterocytes.

A study by Cornick *et al.* (2002) comparing the magnitude and duration of faecal shedding of wild-type *E. coli* O157:H7 to that of an isogenic intimin mutant in 6 to 12-month-old sheep, revealed that the intimin mutant was shed in less numbers and for a shorter duration, day 30 post inoculation, whereas the wild-type was still being shed at day 60 post inoculation. Woodward *et al.* (2003) have also demonstrated that a Stx-negative *E. coli* O157:H7 isolate can persist in 6-week-old sheep for up to 2 months and that an intimin deficient mutant was cleared within 20 days and had lower mean excretion scores at all time points.

1.11.5 Other *in vivo* models other than avian

Other animals models have been employed to characterise *E. coli* O157:H7 colonisation and persistence. Cizek *et al.* (2000) investigated the persistent colonisation capabilities of this pathogen in rats and pigeons, since it has been isolated from rats and birds (Wallace *et al.*, 1998; Cizek *et al.*, 1999). In rats, after a low dose of 10^5 CFU (colony forming units), only short term shedding (2 days post inoculation) of *E. coli* O157:H7 was observed, whereas a higher dose of 10^9 CFU gave a maximum shedding period of 11 days post inoculation. In pigeons infected with 10^5 CFU of *E. coli* O157:H7 shedding occurred for 18 days post inoculation, and occurred for 29 days post inoculation when birds were given the higher dose of 10^9 CFU of *E. coli* O157:H7.

Fischer *et al.* (2001) demonstrated that experimentally infected white-tailed deer, were able to shed 10^3 to 10^5 of *E. coli* O157:H7 after day 1 post inoculation. Numbers of excreted *E. coli* O157:H7 fell dramatically after day 10 post inoculation, but this pathogen was still consistently or intermittently shed in three out of three deer at day 26 post inoculation. *Post mortem* studies recovered *E. coli* O157:H7 from the contents and tissues of the fore-stomachs and intestines of deer necropsied between days 4 and 11 post inoculation. Low populations of this pathogen were recovered after enrichment only from the contents, but not the mucosa of the large intestine at day 26 post inoculation. Horizontal transmission to uninoculated deer was also demonstrated.

Ferrets have been used as model system for renal disease secondary to intestinal infection with *E. coli* O157:H7 (Woods *et al.*, 2002). Ferrets treated with streptomycin gave higher *E. coli* O157:H7 counts with a streptomycin resistant isolate in their stools, than non streptomycin treated ferrets. No animals displayed evidence of colitis, but streptomycin treated mice did exhibit significantly greater weight loss with the streptomycin resistant isolate, than with an isogenic intimin negative mutant. Furthermore, 23% of streptomycin treated ferrets developed damage to the glomeruli or thrombocytopenia.

1.11.6 Avian models

Although outbreaks of *E. coli* O157:H7 illness have been associated with bovine derived food products, this bacterium has been isolated from refrigerated commercial poultry (Doyle & Schoeni, 1987). At least two outbreaks have been linked with poultry products (Carter *et al.*, 1987; Ryan *et al.*, 1986). However, the prevalence of *E. coli* O157:H7 infection in birds is low, but several deliberate inoculation studies show that poultry are readily and persistently infected by this organism indicating a possible threat to public health.

A study by Beery *et al.* (1985) reported that *E. coli* O157:H7 can colonise 1-day-old White Leghorn chickens and is shed in the faeces for up to 90 days post inoculation and attachment, effacement, and penetration of the caecal surface epithelium was observed. Bacterial enumeration indicated that *E. coli* O157:H7 was predominately found in the caeca and to a lesser extent in the colon. Colonisation of young chicks by *E. coli* O157:H7 after experimental infection was also observed by Stavric *et al.* (1991).

Experimental infection of 1-day-old broilers and layers with *E. coli* O157:H7 demonstrated a rapid increase in caecal colonisation, but a rapid decrease in crop colonisation during the first few hours after oral inoculation, with a maximum at 6 hours (Stavric *et al.*, 1993). *E. coli* O157:H7 was isolated from the caecal contents as well as the caecal epithelia. The infection rate was dependent on the CFU of bacteria given and the age of the chicks also influenced the extent of caecal colonisation, with 1-day-old chicks being the most susceptible.

Shoeni and Doyle (1994) observed that *E. coli* O157:H7 colonisation persisted for at least 10 to 11 months when 1-day-old White Leghorn chicks were orally inoculated with 1.3 x 10^8 of *E. coli* O157:H7. Analysis of eggs from hens that were still shedding *E. coli* O157:H7 revealed that 13.9% of eggs shells, but not egg contents, was contaminated with this pathogen.

Histological analysis of young chicks experimentally infected by six different strains of attaching and effacing *E. coli* (Sueyoshi & Nakazawa, 1994) observed that *E. coli* O157:H7 only produced multifocal AE lesions in two out of ten chicks. Both authors suggest that compared to the crypt abscesses and deeper tissue penetration noted in the caecum of piglets

dosed with *E. coli* O157:H7 (Tzipori *et al.*, 1986), failure to cause diarrhoea or severe tissue damage in young chicks may have been related to the extent of bacterial colonisation and the type of lesions formed.

A role for *E. coli* O157:H7 intimin in the colonisation and persistence of young chicks has not been reported, but Zhao *et al.* (1996) did demonstrate that an *E. coli* O157:H7 mutant deficient for a 8 kDa outer-membrane protein was significantly less able to colonise the caeca of young chicks. However, it should be noted that the wild-type isolate used for mutagenesis studies lacked a portion of the LPS layer of the bacterial outer membrane and data from our laboratory has demonstrated that an *E. coli* O157:H7 LPS mutant is highly attenuated in experimentally infected chicks (unpublished data).

1.12 Aims and objectives

The literature has demonstrated that healthy cattle and sheep are major reservoirs for *E. coli* O157:H7 and other animals can also be potential reservoirs. One strategy for reducing the risk of *E. coli* O157:H7 food-borne zoonosis in humans would be to reduce the level of *E. coli* O157:H7 persisting in domestic livestock. Although intimin appears to be involved in this process (Cornick *et al.*, 2002; Woodward *et al.*, 2003), relatively little else is understood about the mechanisms of interaction between *E. coli* O157:H7 and bovine and ovine cells that lead to persistence in the host.

Low-level *E. coli* O157:H7 infection in birds has been reported (Wallace *et al.*, 1997) and two small *E. coli* O157:H7 outbreaks have been associated with retail chicken (Ryan *et al.*, 1986; Carter *et al.*, 1987) and until the submission of thesis and to the best of my knowledge, this pathogen has not been recovered from broilers or layers. Experimental infection of SPF chicks has revealed that *E. coli* O157:H7 can colonise and persist for a long period in this host indicating that chickens could become a reservoir for this pathogen (Beery *et al.*, 1985; Schoeni & Doyle, 1994). However, what *E. coli* O157:H7 factors are involved in these processes have yet to be elucidated.

Keeping the above in mind, it was decided that the SPF 1-day-old chick model would be employed to characterise what *E. coli* O157:H7 virulence factors may play a role in colonisation, invasion and persistence. Non-avian tissue culture and *in vivo* studies have demonstrated that intimin does facilitate *E. coli* O157:H7 intimate adherence, colonisation and persistence. Flagella has been shown to be important for the adherence of other enteric pathogens, including enteropathogenic *E. coli* (EPEC) (Giron *et al.*, 2002) and avian pathogenic *E. coli* (APEC) (La Ragione *et al.*, 2000a). However, a role for flagella in *E. coli* O157:H7 colonisation and persistence of an animal host has not been reported, although flagella negative *E. coli* O157 isolates have been isolated (Bielaszewaka *et al.*, 1998; Schmidt *et al.*, 1999). Furthermore, whether or not *E. coli* O157:H7 intimin and/or other LEE and

non-LEE encoded factors are involved in the persistent colonisation of a non-mammalian host, such as poultry, has yet to be determined.

Also discussed in the literature was the characterisation of the *E. coli* O157:H7 attaching and effacing phenotype on human and bovine cell cultures (Knutton *et al.*, 1989; Mathews *et al.*, 1998; Dibb-Fuller *et al.*, 2001). However, it would be more difficult to make comparisons between human or bovine *in vitro* and chick *in vivo* colonisation. Fortunately, our laboratory received a novel avian cell line derived from SPF chicks, designated Div-1, although it should be borne in mind that this cell line came from the small intestine of 18-day-old embryonic eggs (Velge *et al.*, 2002).

Therefore, since intimin appears to be important for colonisation of cattle and sheep by *E. coli* O157:H7, and flagella has been implicated in the adherence and penetration of mucus layers by other *E. coli* pathogens, it was decided that the aim of the study to be reported here was to investigate the role of *E. coli* O157:H7 intimin and flagella in the colonisation, invasion and persistence of SPF chicks. In order to achieve this, a series of objectives will be embarked upon. These are: -

1. To characterise genotypically and phenotypically a Shiga-toxin (Stx) negative *E. coli* O157:H7 isolate (NCTC 12900) to ensure suitability for genetic manipulation for subsequent study.

2. To study the adherence and invasion properties of an Stx-negative *E. coli* O157:H7 isolate (NCTC 12900) as mediated by intimin and flagella on avian (Div-1) and human (HEp-2) cell lines.

3. To study the *in vivo* colonisation, invasion and persistence characteristics of Stx-negative *E. coli* O157:H7 isolate (NCTC 12900) and intimin and flagella deficient mutants in the 1-day-old SPF chick model.

4. To study the *in vivo* colonisation, invasion and persistence characteristics of intimin and flagella deficient mutants in the presence of the wild-type Stx-negative *E. coli* O157:H7 isolate (NCTC 12900) in the 1-day-old SPF chick model.

Chapter 2

Materials and methods

2.1 Bacteriological Methods

2.1.1 Bacteria, inocula and media

Wild-type Shiga-toxin (Stx) negative *Escherichia coli* O157:H7 isolate NCTC 12900 and all control bacterial isolates required for *in vitro* studies were obtained from the sources described in Table 2.1. Stx negative *E. coli* O157:H7 isolate NCTC 12900 was used for all mutagenesis studies and both parental and knockout derivatives (see chapter 4) were used for *in vivo* studies. All bacterial cultures were maintained on Dorset egg slopes at 20°C and stored at -80°C in Heart Infusion Broth (HIB), supplemented with glycerol (30%). All working cultures were maintained at 4°C on sheep blood agar (5%). Media composition and preparation, and working concentrations of antibiotics are described in appendix I. Additionally, Luria Bertani (LB) agar (Oxoid) supplemented with Xgal (5-Bromo-4-chloro-3-indolyl-β-D-galactosidase) (25mg/ml, Promega) and kanamycin (25μg/ml, Sigma) was used for the selection of recombinants. Minimal medium comprised of 10x M9 salts supplemented with glucose (0.2% w/v) and magnesium sulphate (10mM). Bacteria required for tissue culture association studies (adhesion and invasion assays) were cultured aerobically in LB broth (Oxoid) for 16 hours at 37°C with shaking. Cultured bacterial cells were then prepared for association studies at 3000 rpm for 10 minutes at room temperature and were re-suspended in 0.1M phosphate buffer solution (PBS) (pH 7.2) to an optical density of 1.2 ABS at 540nm. Bacteria for *in vivo* studies were cultured in LB broth for 16 hours at 37°C with shaking and diluted in 0.1M PBS (pH 7.2) to the required colony forming units (CFU) per ml. For certain *in vitro* experiments, bacteria were cultured in Colonisation Factor Antigen (CFA) broth, CFA agar, or CFA agar supplemented with Congo red (Oxoid) statically and aerobically for 72 hours at 25°C.

2.1.2 Replica plating

Colonies derived from conjugation experiments were picked using sterile 1μl inoculation loops and replica plated onto a fresh glucose minimal media plate supplemented with antibiotic conferred by the suicide vector (Plate Type 1) followed by a fresh glucose minimal media plate supplemented with antibiotic conferred by the internal marker used to disrupt the gene of interest (Plate Type 2). Both "types" of plates were incubated for 24 hours after which colonies that had grown on Plate Type 2, but not Plate Type 1, were selected.

Table 2.1. Bacterial isolates required for cloning and mutagenesis studies (1, 11 & 12), *in vitro* studies (1, 3-10, 12 & 13) and *in vivo* studies (1). Isolate 2 was used for PCR only.

Bacterial isolates	Source
1. Shiga-toxin negative *E. coli* O157:H7 (NCTC 12900)	PHLS, Colindale, London
2. Shiga-toxin positive *E. coli* O157:H7 (EDL933)	American Type Culture Collection
3. EPEC O127:H6 (E2348/69)	IFR, Norwich
4. EPEC O111:NM (B171)	Imperial College, London
5. APEC O86:K61 (EC370/98)	VLA, Weybridge
6. APEC O78:K80 (EC341/95)	La Ragione *et al.* 2000a
7. APEC O78:K80 type-1 fimbriae mutant (RML1)	La Ragione *et al.* 2000a
8. APEC O78:K80 curli mutant (RML2)	La Ragione *et al.* 2000a
9. APEC O78:K80 RpoS mutant (RML5)	Roberto La Ragione, VLA, Weybridge
10. APEC O78:K80 aflagellate mutant (RML6)	La Ragione *et al.* 2000a
11. *E. coli* K12 S-17 λ pir	Simon *et al.* 1983
12. *E. coli* K12 (DH5α)	GIBCO-BRL
13. *S. typhimurium* DT104 (3530)	VLA, Weybridge

2.1.3 Growth curves

Growth curves of wild-type *E. coli* O157:H7 (NCTC 12900) and knockout intimin- and flagella-deficient mutants were carried out as follows. Isolates were cultured in LB broth for 16 hours at 37°C with shaking and diluted in LB broth to 1 x 10^8 CFU/ml. A 100µl aliquot of diluted culture was then inoculated into 100ml of pre-warmed LB broth to give a density of approximately 1 x 10^5 CFU/ml. Cultures were incubated at 37°C with shaking and the CFU/ml was determined by plating ten-fold serial dilutions onto LB plates at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 hours.

2.1.4 Antibiotic sensitivity

Isolates were cultured aerobically for 16 hours in LB broth at 37°C and 50µl was dispensed into Buffered Peptone Water (BPW) and incubated statically at 37°C for 2 hours. A 40µl culture sample was dispensed into 5ml of ¼ strength Ringers solution and mixed thoroughly. Thoroughly dried Iso-Sensitest agar plates (Oxoid) were flooded with diluted cultures, and the excess culture was drained off. Plates were allowed to stand at room temperature for 15 minutes. Antibiotic discs (Oxoid) were subsequently stamped onto the plates and the plates were incubated aerobically at 37°C for 16 hours. Sensitivity/resistance to the antibiotics was determined by measuring the diameter of inhibition across the antibiotic disc to the edges of the zone of inhibition. Isolates were considered resistant to a particular antibiotic when the zone of inhibition was less than 13mm.

2.1.5 Haemagglutination

Haemagglutination was essentially performed as described by Duguid *et al.*, (1979). Isolates to be tested were cultured statically and aerobically at 37°C in 3ml of HIB for 48 hours. Cultures (1ml) were centrifuged at room temperature for 3 minutes at 13000rpm. Pellets were re-suspended in 50µl of sterile distilled H_2O. To test for haemagglutination 25µl of bacterial cells was incubated with 25µl of 3% guinea pig erythrocytes on a sterile tile, which was gently rocked for 2 minutes. Mannose resistance was tested for by the addition of 25µl of D-mannose. Haemaglutination was also performed using chicken and sheep erythrocytes.

2.1.6 Motility

Isolates were cultured onto 5% sheep blood agar plates for 16 hours at 37°C aerobically and single colonies were picked with an inoculation needle and stabbed into the centre of a universal containing 0.35% semi-solid 'sloppy' LB agar. Motility stabs were incubated at 25°C, 37°C and 42°C for 24 hours. Motility was observed as diffuse growth out from the point of inoculation.

2.1.7 Lacy colony morphology and Congo red binding

Lacy colony morphology and Congo red binding were performed essentially as described previously (Allen-Vercoe *et al.*, 1997). Isolates were cultured in LB broth for 16 hours aerobically at 37°C with shaking and were serial diluted in 0.1M PBS (pH 7.2) to 1 x 10^5 CFU/ml. Drops (5µl) of each diluted culture were spotted onto dry CFA agar plates. Plates were incubated aerobically at 25°C for 72 hours to allow the development of 'lacy' colonies. Congo red binding was determined by inoculating (as above) CFA plates supplemented with 0.05% (w/v) Congo red. Congo red binding was observed when colonies developed a deep red colour.

2.1.8 Acid tolerance assay

Acid tolerance was tested essentially as described previously (Gorden & Small, 1993). Isolates cultured overnight in LB broth at 37°C, aerobically with shaking, were diluted in pre-warmed LB broth previously adjusted to approximately pH 2.5 using 1M HCl. Aerobic incubation with shaking was for 2 hours and 30 minutes at 37°C. Cultures were enumerated by plating 10-fold serial dilutions onto LB agar and incubating overnight at 37°C.

2.1.9 Transmission electron microscopy

For the elaboration of any surface appendages of interest, isolates were cultured in an appropriate medium. Bacterial cultures (1ml) were centrifuged at 4000rpm and the supernatant removed. Bacterial pellets were re-suspended in 0.5ml of 0.1M PBS (pH 7.2) and 50µl drops were placed on sterile dental wax. Formvar™ carbon coated grids were placed on top (silver side down) of each spot for 15 minutes. Excess liquid was removed on blotting paper and the grid (silver side down) was placed onto a 50µl spot of potassium phosphate, tungsten (KPT) negative stain for no longer than 15 seconds. Grids were carefully blotted dry and viewed using a Philips CM 10 transmission electron microscope.

2.2 Genetic methods

2.2.1 Total genomic DNA extraction

Isolates were cultured in 100ml volumes of LB broth for 16 hours at 37°C with shaking, aerobically. The cultures were centrifuged at 4800rpm for 10 minutes at room temperature and the supernatant immediately removed. Bacterial cell pellets were re-suspended in 1.5ml of TE buffer, 150µl of 10% (w/v) SDS, 150µl of 50mg/ml fungal proteinase K (Gibco-BRL) then mixed and incubated at 65°C for at least 1 hour or until the cell suspensions were clear. To the clear cell suspensions an equal volume of phenol:chloroform mixture (Sigma) was added and the suspensions inverted several times until the suspensions turned milky white. Cell suspensions were removed and added to 50ml phase-lock™ gel tubes (CP Laboratories) and centrifuged at 4800rpm for 10 minutes at 4°C. Phenol extraction was repeated twice more. The supernatant was removed and placed in 3ml of room temperature 100% ethanol.

Precipitated DNA was retrieved by spooling onto a sterile glass hook, which was placed into 3ml volumes of ice cold ethanol (70%, v/v). DNA was transferred to a sterile tube and the residual ethanol allowed to evaporate. DNA was dissolved in 1-3ml of TE buffer and stored at 4°C.

2.2.2 Plasmid DNA extraction

Plasmids were extracted using three different methods depending on the quality and quantity of plasmid DNA required. Firstly, for restriction digestion analyses the Perfectprep® Plasmid Mini extraction kit (Eppendorf) was used. Bacterial hosts carrying recombinant plasmids were cultured in LB broth supplemented with the appropriate antibiotic for 16 hours aerobically with shaking. Bacterial cultures (1-2ml) were centrifuged at 13000 rpm for 2 minutes and the supernatant removed. Cell pellets were re-suspended in 100µl of solution I (re-suspension), mixed and then 100µl of solution II (lysis) was added, followed by 100µl of solution III (neutralisation). The bacterial suspension was centrifuged at 13000 rpm for 8 minutes and the supernatant carefully removed and placed in a fresh tube containing 50µl of Perfectprep® DNA binding matrix. The suspension was transferred to a fresh spin column, washed (x3) with 600µl of diluted purification solution. After the spin column was completely dry, plasmid DNA was eluted in 100-120µl sterile distilled H_2O (high copy plasmids) or 80µl (low copy number plasmids).

Secondly, for plasmid DNA to be used for sequencing, the ABI Prism™ Miniprep kit (PE Applied Biosystems) was used. Bacterial hosts carrying recombinant plasmids were cultured in LB broth supplemented with the appropriate antibiotic for 16 hours, aerobically at 37°C. Cultures (1ml) were centrifuged at 13000 rpm and the supernatant removed. The cell pellet was re-suspended in re-suspension buffer then lysis buffer was added followed by neutralising solution. The mixture was then added to a column and spun at 13000 rpm and the column placed on a sterile tube and wash buffer added and finally the plasmid DNA was eluted with 100µl of elution buffer or distilled sterile H_2O. Plasmid DNA was stored at -20°C.

Thirdly, for large quantities of plasmid DNA required for cloning, the Qiagen (Promega) kit was used. Bacterial hosts carrying recombinant plasmids were cultured in 100ml LB broth supplemented with the appropriate antibiotic for 16 hours with shaking. Cultures (100ml) were centrifuged at 7000 rpm for 10 minutes, the supernatant removed and the pellet re-suspended in 4ml of P1 buffer using a sterile loop. P2 buffer (4ml) was added and mixed gently and the mixture was incubated at room temperature for 5 minutes. Chilled P3 buffer (4ml) was added and the mixture incubated on ice for 15 minutes. The mixture was then centrifuged at 4°C at 9000 rpm for 30 minutes. During centrifugation, a 50ml Falcon tube

was set up with a Qiagen tip and the tip equilibrated with 4ml of QBT buffer. After centrifugation, the supernatant was added to the equilibrated tip, allowed to flow through and the tip and was washed (x2) with 10ml of QC buffer. Finally the plasmid DNA was eluted with 5ml of QF buffer and the DNA was precipitated with 0.7 volumes (3.5ml) of isopropanol and centrifuged at 14000 rpm for 30 minutes at 4°C. The supernatant was removed and the pellet washed with 70% ethanol (v/v) and the DNA was centrifuged again at 12000 rpm for 5 minutes. The supernatant was removed and the pellet dissolved in 0.5-1ml of sterile distilled water.

2.2.3 DNA purification from agarose gels

Purification of excised agarose gel fragments was performed using the Sephaglas® Bandprep kit (Amersham Biosciences). Plasmid DNA bands were removed from agarose gels using a sterile disposable scapel and were placed in a sterile 1.5ml micro-centrifuge tube. For each excised band in a tube, 200µl of gel solubiliser was added and agarose gel fragments were completely solubilised in a 65°C heating block. Added to the mixture was 20µl of Sephaglas® and the solubilised gel mixture was mixed gently by tapping the side of the tube and incubated at room temperature for 3-5 minutes. The mixture was gently tapped each minute then centrifuged at 13000 rpm for 20 seconds and the supernatant discarded. The pellet was then re-suspended and centrifuged at 13000 rpm for 20 seconds in 200µl of wash solution. This wash step was repeated twice more. After the third wash micro-centrifuge tubes were inverted for at least 25 minutes or until the pellet had partially dried. Each pellet was then re-suspended in sterile distilled H_2O, incubated at room temperature for 3-5 minutes and centrifuged at 13000 rpm for 1 minute to elute the plasmid DNA. The supernatant containing DNA was removed and stored at –20°C until required.

2.2.4 DNA quantification

DNA to be quantified was diluted 1/5 and 1/10 in sterile distilled H_2O. DNA standards (Salmon testes DNA, Invitrogen) were diluted to 2, 4, 8, 16, 32 and 64ng/µl. with sterile distilled H_2O. Stock ethidium bromide (100ng/µl, Sigma) was diluted to give 2ng/µl. Diluted ethidium bromide 2ng/µl (5µl) was added to each well of a micro-titre plate followed by 5µl of DNA standards. In additional wells, 5µl of diluted DNA to be tested was mixed with ethidium bromide 2ng/µl (5µl). DNA concentrations were visualised under UV light (260nm).

2.2.5 Restriction enzyme digestion of plasmid DNA

Plasmid DNA digests were all performed using Promega enzymes and appropriate Promega buffers. Single and double digests were performed in 21µl volumes consisting of

13µl of sterile distilled H_2O, 5µl of DNA (100-200ng), 2µl of buffer and 1µl of enzyme. Digests were incubated at 37°C for 4 hours unless otherwise specified.

2.2.6 Restriction enzyme digestion of total genomic DNA

Genomic DNA digests were performed as for plasmid DNA except that digests were performed in approximately 200µl volumes consisting of 150µl of sterile distilled H_2O, 25µl of DNA (500ng), 20µl of buffer (Promega), and 2µl of enzyme (Promega). Digests were incubated at 37°C for 16 hours unless otherwise specified.

2.2.7 Infilling to create blunt ended DNA

Cohesive-ended restriction endonuclease digested plasmid vectors were prepared for blunt ended ligation using 10µl volumes of DNA (100-200ng), 5 units of *pfu* polymerase (Promega), 100mM dNTP's and 2µl of 10x buffer (Promega). Reaction mixtures were overlaid with mineral oil and incubated at 72°C for 30 minutes. After incubation reaction mixtures were removed from under the oil and used at once or stored at -20°C.

2.2.8 Dephosphorylation

Dephosphorylation was carried out in 20µl volumes using 2 units of shrimp alkaline phosphatase (SAP), 2µl of buffer (United States Biochemicals) and 50ng DNA. Dephosphorylation was carried out at 37°C for 30 minutes and the reaction was terminated by incubation at 65°C for 10 minutes to denature the SAP.

2.2.9 Ligation

Ligations were performed using the 'Ready-to-go™' T4 DNA ligase (Amersham Pharmacia Biotech). All ligations were performed at 14°C for 16 hours.

2.2.10 Southern blotting

Total genomic DNA was digested to completion with an appropriate enzyme, mixed in the ratio 5:1 with orange G loading buffer and electrophoresed on 0.8% (w/v) agarose gels for four hours at 150 volts with cooling. The electrophoresed DNA was stained using ethidium bromide (Sigma) for 10 minutes, placed in denaturing solution for 1 hour and then neutralising solution for 1 hour. The gel was carefully laid onto SaranWrap® on a flat surface. A pre-cut nylon membrane (Hybond-N+, Amersham Biosciences) was placed over the gel and trapped air bubbles were expelled. Several layers of blotting paper and absorbent padding were placed onto the gel. Finally a 1Kg weight in a flat bottomed tray was placed onto the gel. Transfer was for 8-16 hours at which point the membrane was carefully removed, rinsed in sterile distilled H_2O, dried and fixed by baking the DNA at 80°C for 2 hours. Membranes were probed immediately or wrapped in SaranWrap® and foil and stored at 4°C until required.

2.2.11 Hybridisation

All probes were produced by PCR (Table 2.2). Probes were purified using the Sephaglas® DNA purification kit (Amersham Biosciences) and quantified. Probes were used immediately or stored at -20°C.

Hybridisation was carried out using the non-radioactive method by AlkPhos Direct (Amersham Biosciences). The probe to be used was quantified and diluted to 10ng/µl, and 10µl (10ng/µl) of DNA was denatured for 5 minutes and 10µl of reaction buffer added and mixed. Labelling reagent (2µl) was added followed by 10µl of working strength cross-linker. The mixture was incubated at 37°C for 30 minutes and the probe was placed on ice until required. The membrane to be probed was laid on a gauze mesh wetted with sterile distilled H_2O, rolled and placed in a hybridisation tube (Hybaid). An appropriate volume (0.25ml/cm^2 of membrane) of pre-warmed hybridisation buffer was added to the tube, and the tube was placed at 55°C for 30 minutes. The probe was added (10ng/µl) and incubation continued at 55°C for 16 hours in a Hybaid rotisserie oven. The membrane was removed from the tube and placed into pre-warmed primary wash buffer at 55°C and washed with gentle agitation for 10 minutes. The primary wash was repeated once with fresh wash buffer at 55°C and the final wash using secondary wash buffer was performed at room temperature for 5 minutes and also repeated once. The blot was removed and drained, placed on a dry flat surface and 40µl of detection reaction was added for every cm^2 and the blot was incubated at room temperature for 3 minutes. The membrane was drained and wrapped in Saran Wrap® and the membrane exposed to a Hyperfilm ECL film (Amersham Biosciences) for 1 hour and developed (Photosol CD18 X-ray developer) and fixed (Photosol CF40, Genetic Research instruments Ltd).

2.2.12 Polymerase chain reaction (PCR)

Amplification of all target DNA was carried out by PCR (Sakai *et al.*, 1988). Reactions for amplification by PCR consisted of Thermophilic DNA polymerase 10X Magnesium free buffer (5µl), 1.5mM $MgCl_2$, 2.5 units of *Taq* polymerase (Promega) 200µM dNTP's (Amersham Biosciences), 10pmols of each primer (Oswell, Table 2.2), 1ng of complete genomic DNA, and were made to a final volume of 50µl using sterile distilled H_2O. PCR amplifications were performed for 1 cycle at 95°C for 5 minutes (initial denaturing); 30 cycles of 95°C for 2 minutes (denaturing), 56°C for 1 minute or (annealing) and 72°C for 2 minutes (extension); 1 cycle at 72°C for 10 minutes (final extension). PCR reactions were stored at 4°C until required.

Table 2.2. Primer sequences used to characterise Stx negative *E. coli* O157:H7 isolate NCTC 12900.

Target gene(s)	Primers used	Primer sequence	Amplicon size (bp)	Accession Genebank number	Reference
csgA	csgAF	TATTGATCGCACACCTGACAG	2168	X90754	Hammar *et al.* (1995)
	csgAR	CCAAGGGTTGTGTTATCCATA			
eae	eaeF	CTTTACCGGCGGAACTGGA	2172	AF061251	Perna *et al.* 2001
	eaeR	GGACCCGGCACAAGCATAAG			
espP	espPF	TTGCGAAAAATGGCGGAACTC	1164	Y136164	Djafari *et al.* (1997)
	espPR	GCTGACGGGGCATTGACTG			
etpD	etpDF	CGTCAGGAGGATGTTCAG	1061	Y09824	Schmidt *et al.* (1997)
	etpDR	CGACTGCACCTGTTCCTGATTA			
(*fimE, fimA*)	fim5'	GCCGGATTATGGGAAAGA	604	AE000502	Leathart & Gally (1998)
	fim3'	AGTGAACGGTCCCACCAT			
fliC	fliCF	CTCTCGCTGATCACTCAA	1641	AF228488	Wang *et al.* (2000)
	fliCR	CGACATGTTGGACACTTC			
hlyA	hlyAF	CCCGGATCCTGACAGTAAATAA AATAAAGAACA	2992	X79839	Schmidt *et al.* (1995)
	hlyAR	GGGCTCGAGGACAGTTGTCGTT AAAGTTGTTGA			
intγ	intγF	CGTTGAAGTCGAGTACGCCA	957	Z11541	Yu & Kaper (1992)
	intγR	TTCTACACAAACCGCATAGA			
katP	katPF	CTTCCTGTTCTGATTCTTCTGG	2125	X89017	Brunder *et al.* (1996)
	katPR	AACTTATTTCTCGCATCATCC			
(*sepL, espA, espD*)	LADF	CCGGCTGTCAGAATGCTT	2068	AF071034	Perna *et al.* (1998)
	LADR	TGGCAACATGCCAAAGGG			
stxI	STX1F	GGCAGATGGAAGAGTCCGTGG GATTACGC	178	Z36900	Paton *et al.* (1995)
	STX1R	CACAATCAGGCGTCGCCAGCGC ACTTGCT			
stxII	STX2F	CCACATCGGTGTCTGTTATTAA CCACACC	374	X61283	Meyer *et al.* (1992)
	STX2R	GCAGAACTGCTCTGGATGCATC TCTGGTC			
(*wbdN, wzy*)	LPSF	CGCGGATCCTGGGGCCAAAACT ATAATCTCAT	1588	AF061251	Wang & Reeves (1998)
	LPSR	CGCAAGCTTAACCGTTCTCCAT AAAATAAGTCT			

N. B. Underlined sequences denotes engineered restriction enzyme sites (hlyAF = *Bam*HI, hlyAR = *Ccr*I LPSF = *Bam*HI, LPSR = *Hind*III).

2.2.13 Nucleotide sequencing and analysis

The BigDye terminator cycle sequencing kit (Perkin Elmer) was used for all reactions. Individual reactions (20µl) contained 400ng plasmid DNA or 200ng PCR amplified DNA, 3.2pmoles of the primer, either M13F (GTAAAACGACGGCCAGT) or M13R (CAGGAAACAGCTATGAC) (Oswel) and 8µl of ready reaction mix (Perkin Elmer). Extensions of reactions were performed in a Perkin Elmer thermo-cycler (25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes) and prepared for analysis on a 377 automated DNA sequencer following the manufacturer's instructions (Applied Biosystems Inc). Trace data were analysed and assembled using DNAStar software (DNAStar Inc.).

2.2.14 Preparation of electro-competent cells

Bacteria were cultured in LB broth for 16 hours aerobically with shaking at 37°C. After incubation, 3ml of the culture was placed into 100ml of fresh LB broth and incubation continued with shaking at 37°C aerobically for 2.5 hours. The culture vessel was placed into ice for 30 minutes. The culture was transferred to pre-chilled centrifuge tubes and centrifuged at 7000 rpm at 4°C for 10 minutes. The supernatant was removed and the pellet re-suspended in 100ml of sterile, chilled distilled H_2O and re-centrifuged at 7000 rpm at 4°C for 10 minutes. This was repeated twice with 50ml volumes of sterile chilled distilled H_2O and then once with 20ml of chilled sterile distilled H_2O (if the cells were to be stored the final three washes were performed with sterile distilled H_2O supplemented with 10% (v/v) sterile gycerol). After the final centrifugation the supernatant was removed and replaced with 1ml of sterile chilled distilled H_2O and the re-suspended cells stored on ice until required.

2.2.15 Electroporation

Ligated DNA was electroporated into electro-competent cells using the Gene Pulser (Biorad) set at 1.25kV (potential), 200Ω (resistance) and 25µF (capacitance). In a chilled 1.5ml micro-centrifuge tube 40µl of electro-competent cells was mixed with 5µl of ligated DNA and incubated on ice for 30 seconds. The cell-ligated DNA mixture was dispensed into a chilled dry 0.1cm *E. coli* cuvette (Bio-Rad) and then placed into a chamber slide. Using the above settings the cell/ligated DNA mixture was pulsed once. Pre-warmed SOC (37°C) broth was added to the cuvette and the suspension was dispensed into a Falcon® 2059 polypropylene tube (Scientific Laboratory Supplies) and incubated aerobically at 37°C with shaking for 1-2 hours. Suspensions were spread onto LB agar plates supplemented with the appropriate antibiotics.

2.2.16 Transformation

Ligated DNA (2µl) was placed into a Falcon® 2059 tube (Scientific Laboratory Supplies) containing 40µl of MaxiEfficiency *E. coli* DH5α (GIBCO-BRL), gently mixed and then equilibrated on ice for 30 minutes. Appropriate positive and negative controls were also included. The MaxiEfficiency (*E. coli* K12 DH5α) cells and DNA to be cloned were heat shocked at 42°C for 45 seconds and immediately placed onto ice for 2 minutes. To each Falcon tube, 400µl of pre-warmed SOC medium was added and the tubes immediately placed at 37°C for 1 hour with shaking.

2.2.17 Conjugation

Cultures (3ml) of the recipient isolate NCTC 12900 and the transformed donor isolate *E. coli* S17 λ *pir* grown in LB broth supplemented with the appropriate antibiotics were centrifuged and re-suspended in 1ml of LB broth. Equal volumes (100µl) of the re-suspended bacteria were mixed together and 200µl placed on a nitrocellulose disc over-laid on a dry nutrient agar plate. Controls were set up with donor or recipient strains alone. After four hours incubation the disc was aseptically removed, placed in 10ml of 1X M9 salts and vigorously agitated to dislodge adherent bacteria. The disc was aseptically removed from the bacterial suspension, which was then centrifuged and the bacterial pellet was re-suspended in 1ml of 1X M9 salts. Re-suspended (100µl) bacteria were plated onto glucose minimal medium plates supplemented with the antibiotic conferred by the resistance cassette used to disrupt the target gene. Plates were incubated for 48 hours and replica plated onto glucose minimal media plates supplemented with the antibiotic conferred by the suicide vector.

2.2.18 Insertional inactivation of intimin and flagella genes

Genes for insertional inactivation, *eae* and *fliC* each flanked by immediately adjacent sequences, were either excised from a cosmid library (*eae*) or amplified by PCR (*fliC*) from total genomic DNA extracted from Stx negative *E. coli* O157:H7 isolate NCTC 12900. Amplicons were cloned into pCRScript (Stratagene), pBluscript (Stratagene) or pCR2.1 (Invitrogen) plasmid vectors with *E. coli* K12 DH5α (GIBCO-BRL) used as a host. Putative clones were analysed by colony hybridisation and detailed restriction endonuclease mapping and sequencing. Antibiotic resistant cassettes (Allen-Vercoe & Woodward, 1999) were cloned into the appropriate unique restriction sites in the centre of each gene. The insertionally inactivated genes were sub-cloned into the appropriate pERFORM (Penfold & Pemberton, 1992; Allen-Vercoe *et al.*, 1997) suicide vectors, electroporated into the permissive auxotrophic host *E. coli* K12 S17 λ *pir* and selected for by selection on both the antibiotic encoded by resistance cassette and the antibiotic conferred by the suicide vector antibiotic gene.

The pERFORM suicide vectors harbouring antibiotic resistance marked, insertionally inactivated genes were transferred by conjugation from the permissive host *E. coli* K12 S17 λ *pir* into the non-permissive Stx negative *E. coli* O157:H7 isolate NCTC 12900 recipient by filter mating as described previously (Allen-Vercoe & Woodward, 1999) with selection made for the antibiotic conferred by the antibiotic resistance cassette used to inactivate the target gene. Colonies from filter matings were tested for loss of the antibiotic conferred by the suicide vector by replica plating onto minimal media supplemented with the antibiotic conferred by the suicde vector. Putative double cross-over mutants were streaked to single colonies three times prior to confirmatory genotypic and phenotypic testing.

2.4 *In vitro* association assays

2.4.1 Adhesion and invasion assays

Tissue culture assays were performed essentially as described previously (Kang *et al.*, 1995; Dibb-Fuller *et al.*, 1999, Dibb-Fuller *et al.*, 2001). Bead stock cultures of tissue culture cells were stored in Cryo-tubes in liquid nitrogen (-196°C). When required cells were removed from liquid nitrogen and thawed at 37°C in a water bath. Thawed cells were reconstituted in Eagle's Modified Medium (EMM) (Sigma) or Dulbecco's Modified Eagles Medium (DMEM) (Sigma) supplemented with foetal calf serum (10% v/v, Autogenbioclear), non-essential amino-acids (1% v/v, Sigma) and gentamicin (50μg/ml, Sigma). Reconstituted cells were placed in 30ml of the above medium and placed into 250ml tissue culture flasks and incubated at 37°C in the presence of 5% CO_2 in air until a confluent mono-layer was present. Confluent mono-layers were trypsinised and mono-layers sown at 2 x 10^5 cells per well in 24 well micro-titre plates as required.

The avian Div-1 cell line (Velge *et al.*, 2002) was sown at 2 x 10^5 cells per well in 24 well micro-titre plates and mono-layers were incubated for 48 hours in DMEM. The human HEp-2 cell line (European collection of animal cell cultures, ECACC 86030501) was prepared similarly except that this line was grown in Eagle's Minimal Essential Media (EMEM). All assays were repeated at least twice on a minimum of two separate occasions.

Before use, Div-1 and HEp-2 mono-layers were washed twice with Hank's Balanced Salt Solution (HBSS) to remove cell debris and residual gentamicin. Inocula for tissue culture assays were prepared by streaking from the stock culture onto 5% sheep blood agar. Discrete, well-isolated colonies were inoculated into LB broth to grow overnight with shaking at 37°C. Overnight cultures were re-suspended in 0.1M PBS (pH 7.2) to give an optical density reading of 1.2 ABS (540nm) then diluted (1/20) in DMEM, non-essential amino acids (1% v/v, Sigma), and L-glutamine (Sigma) to give approximately 1 x 10^7 colony CFU/ml. The number of bacteria given in each inoculum to each mono-layer was determined by plating ten-fold serial dilutions on LB agar.

For adherence assays, the bacterial inoculum was added to give 1 x 10^7 CFU/well. Mono-layers were then incubated at 37°C supplemented with 5% CO_2 in air for 3 hours. The supernatant was removed and the mono-layers were washed (x3) with HBSS to remove non-adherent bacteria. Mono-layers were disrupted for 10 minutes with a solution of 1% Triton X-100 (Sigma) and gentle vortexing using a 12mm magnetic stirrer. After disruption, 10-fold serial dilutions were plated onto LB agar and incubated overnight at 37°C to determine the numbers of cell-associated and adherent bacteria.

For invasion assays, bacteria were allowed to adhere as described above for 3 hours at which point the mono-layers were washed (x3) with HBSS before adding fresh pre-warmed DMEM containing gentamicin (100μg/ml). Plates were incubated at 37°C (5% CO_2 in air) for a further 2 hours and washed (x3) with HBSS. The mono-layers were then disrupted with a 1% solution of Triton X-100 (Sigma) and the numbers of internalised bacteria were determined.

2.4.2 Giemsa staining

Confluent mono-layers grown in 24 well plates with 13mm cover slips were washed twice with HBSS. Each well was inoculated with the bacterial inoculum and incubated for 3 hours at 37°C, 5% CO_2 in air. The inoculum was aspirated and replaced with fresh medium and the assay incubated for a further 3 hours in the above conditions. Mono-layers were washed (x1) with HBSS and then fixed with 3% buffered formalin for 30 minutes and washed (x3) with sterile distilled H_2O. Mono-layers were stained with 10% Giemsa stain (Sigma) for 1 hour then washed (x3) with distilled water. Mono-layers were differentiated with 1% (v/v) acetic acid for 2 minutes and washed once with sterile distilled H_2O. Cover slips were then removed from the 24 well plate and mounted on glass slides using DPX (Sigma). Cover slips were examined using high-power oil immersion light microscopy (Zeiss).

2.4.3 Fluorescent actin staining (FAS)

The preparation of mono-layers for bacterial adherence was performed as described above. Assays were performed for 6 hours with the media being replaced after 3 hours. The supernatant was aspirated from each well and the cover slips were washed three times with 0.1M PBS (pH 7.2). The mono-layers were then fixed for 20 minutes with 3% (v/v) formalin and then washed (x3) with 0.1M PBS (pH 7.2). Mono-layers were permeabilised for 4 minutes by incubation with 0.1% (v/v) Triton X-100 (Sigma) in 0.1M PBS (pH 7.2). The Triton was removed with 3 washes of 0.1M PBS (pH 7.2) and the mono-layers were covered with 0.1M PBS (pH 7.2) containing FITC-phalloidin (Sigma) at a concentration of 0.5μg/ml. Mono-layers were incubated at ambient temperature in the dark, then washed (x3) with 0.1M

PBS (pH 7.2). The cover slips were mounted on glass slides with DPX (Sigma) and the slides were then examined by high power oil immersion fluorescence microscopy (640nm) (Zeiss).

2.4.4 Transmission electron microscopy

Mono-layers were prepared for bacterial adherence as described above. Assays were performed for 6 hours with the media being replaced after 3 hours. All media was aspirated and replaced with 3% (v/v) glutaraldehyde (Sigma) in 0.1M PBS (pH 7.2). Mono-layers were fixed for 10 minutes. Div-1 cells were then scraped off the cover slips with a cell scraper in an excess of fixative in a centrifuge tube. Cells were pelleted by centrifugation at 3000rpm for 5 minutes. The supernatant was aspirated and replaced with 0.1M PBS (pH 7.2) and post fixed in 1% (w/v) osmium tetroxide in the same buffer, dehydrated in ethanol up to 100%, placed in propylene oxide prior to embedding in Araldite resin. The Araldite resin was polymerised at 60°C for 48 hours. Ultra-thin sections cut at 70-90nm thickness were prepared onto copper grids using a diamond knife. Sections were contrasted with uranyl acetate and lead citrate prior to examination in a Philips CM10 TEM.

2.4.5 Scanning electron microscopy

The preparation of mono-layers for bacterial adherence was performed as described for transmission electron microscopy. Assays were performed for 6 hours with the media being replaced after 3 hours. The supernatant was removed by aspiration and the mono-layers were fixed for 16 hours in 3% (v/v) glutaraldehyde in 0.1M PBS (pH 7.2). Specimens were washed in 0.1M PBS (pH 7.2) and post fixed in 1% (w/v) osmium tetroxide in the same buffer, then rinsed in six changes of phosphate buffer, dehydrated in ethanol and placed in hexamethyldisizone for 5 minutes. Specimens were subjected to critical point drying with liquid carbon dioxide. Air dried specimens were fixed to aluminium stubs with silver conductive paint, sputter coated with gold and examined using a Stereo-scan S250 MarkIII SEM at 10-20KV.

2.5 *In vivo* studies

2.5.1 Persistent colonisation of Stx negative *E. coli* O157:H7 NCTC 12900 in SPF chicks

Localisation studies were performed as described previously (La Ragione *et al.*, 2000b) but with modifications. Groups of thirty birds were used per experiment. One-day-old, specific pathogen free (SPF) White Leghorn chicks (SPAFAS®) housed in heated isolators were each dosed orally with 0.1ml containing 1 x 10^5 CFU (experiment 1) or 1 x 10^7 CFU (experiment 2) of Stx-negative *E. coli* O157:H7 NCTC 12900. Individual birds were cloacally swabbed after 24 hours to establish the presence of an infection. Birds were given standard rations and water *ad libitum*.

In experiment 1, *post mortem* examinations were performed on birds selected at random on days 1, 2, 5, 57, 92 and 211 post inoculation. Liver, spleen, duodenum, jejunum, ileum,

colon, caeca, caecal tonsils, and crop were removed aseptically from each bird and homogenised in 10ml volumes of distilled sterile H_2O in a glass universal. Ten-fold serial dilutions were plated on Sorbitol MacConkey Agar supplemented with Cefixime (0.625mg/ml) Tellurite (0.0125mg/ml) (CT-SMAC) and these were incubated overnight, aerobically at 37°C to determine the degree of colonisation or invasion (CFU/ml tissue homogenate). One other bird was also selected at random on days 1, 2 and 5 post inoculation for the collection of the same tissues for histological studies. All tissues were placed in 10% (v/v) buffered formalin.

Cloacal swabs were taken weekly from all surviving birds during the course of the experiment and plated directly onto CT-SMAC and after enrichment by overnight incubation in Buffered Peptone Water (BPW). Inoculated plates were incubated overnight at 37°C and presumptive *E. coli* O157:H7 NCTC 12900 colonies were confirmed by latex agglutination (*E. coli* O157 test kit, Oxoid) and enumerated by the semi-quantitative methods described previously (Williams-Smith 1965; Williams-Smith and Tucker, 1975; La Ragione *et al.*, 2000b). The growth of *E. coli* O157:H7 NCTC 12900 was scored as clear (no colonies), positive (detected after enrichment only), low (<200 colonies), medium (>200 colonies) and high (confluent).

In experiment 2, *Post mortem* examinations were performed on birds selected at random on days 1, 2, 3, 7, 9, 14, 16, 21, 24, and 29 post inoculation. Only tissues from the distal gastrointestinal tract (duodenum, ileum, colon, caeca, and caecal tonsils) were removed aseptically from each bird. The individual tissues were placed in 10% buffered formalin and treated for histological examination.

2.5.2 Persistent colonisation of SPF chicks by Stx-negative *E. coli* O157:H7 NCTC 12900 wild-type and mutants

Colonisation and invasion experiments were essentially performed as described previously (La Ragione *et al.*, 2000b) but with modifications. Groups of thirty, 1-day-old specific pathogen free (SPF) White Leghorn chicks (SPAFAS®) housed in heated isolators were each dosed orally with 0.1ml containing 1 x 10^5 CFU of wild-type (control) or individual mutants, or were each dosed orally with 5 x 10^4 CFU + 5 x 10^4 CFU (Total, 1 x 10^5 CFU) of wild-type and mutant (competitive index), respectively. A triple competitive index experiment involved one group of six birds that were dosed by oral gavage with (Total, 1 x 10^5 CFU) wild-type plus two mutants each deficient for one phenotype only. Birds were given standard rations and water *ad libitum*.

Cloacal swabbing was performed on day 1 post inoculation and weekly thereafter until day 211 post inoculation to determine patterns of persistence. Cloacal swabs were streaked with continuity onto CT-SMAC and/or SMAC-antibiotic. Plates were incubated aerobically

overnight at 37°C and scored as 0 = clear, <200 = low, > 200 = medium, confluent = high, and positive after enrichment. Enrichment of cloacal swabs was performed using the enrichment method described above.

2.5.3 Analysis of Eggs

The 3 remaining birds, all hens, from each group in each experiment began laying eggs on various days post inoculation. Egg shell, white and yolk were enriched separately in BPW for 6 hours statically and aerobically at 37°C. Immunomagnetic separation (IMS) using the Dynabeads© anti-*E. coli* O157 protocol was used for selecting any enriched NCTC 12900 and derived knockout mutant. Essentially, 20µl of Dynabeads coated with antibodies against *E. coli* O157:H7 was dispensed into tubes (placed in a magnetic particle concentrator) containing 1ml of enriched egg sample. Insertion of a magnetic plate concentrated the bead pellets after removal of enrichment supernatant and subsequent washes (x3) in 0.1M PBS (pH 7.2), 0.1% Tween (0.1% PBST). After the final wash, concentrated bead pellets were resuspended in 30µl of 0.1M PBS (pH 7.2), and the wild-type was streaked onto CT-SMAC plates and the mutants were streaked onto SMAC supplemented antibiotics and incubated overnight, aerobically at 37°C.

2.5.4 Histology

2.5.4.1 Haematoxylin and Eosin (H&E) staining

Samples of chicken gut were fixed in 10 times their volume of 10% (v/v) buffered formalin. When fixed the tissues were assigned a unique number and a labelled plastic cassette was prepared for each. In a fume cupboard and three transverse pieces of tissue taken one from near each end and one from the centre. The tissues were placed in the cassette, the lid secured and the tissue processed overnight on an automatic tissue processor. Tissue samples were then embedded transversely and sections taken at 3µm from the resultant block. These sections were then stained using a standard H&E method on an automatic staining machine and subsequently mounted with a permanent mountant. Tissues were examined by light microscopy (magnification x 1000) after staining with Haematoxylin and Eosin.

2.5.4.2 Immuno-Cyto-Chemistry (ICC)

Sections of intestinal tissues that showed evidence of adherent bacteria were stained using specific antibodies. To do this, adjacent sections of tissue were formalin fixed, paraffin wax embedded at 5µ, and sections floated onto Vectabond treated (Vector Laboratories) or charged microscope slides. Sections were air dried at 37°C overnight, then 'melted on' to increase adhesion by incubation at 60°C for 30 minutes. Sections were placed in a stainless steel slide rack for subsequent immuno-staining.

Sections to be immuno-stained were deparaffinised in xylene (xylene 20-25°C) for 6 minutes (x2) then washed (x2) in absolute alcohol for 6 minutes. Sections were then immersed in 3% (v/v) hydrogen peroxide (100 volumes) in methanol for 15 minutes, washed (x3) in tap water for 3 minutes and washed once in running tap water for 15 minutes. Sections were loaded into sequenzer clips and rinsed with Tris Buffered Saline (0.005M TBS, pH 7.6). Goat serum (1/66 in TBS) was then added to each section for 20 minutes at room temperature and subsequently drained. Primary antibody (VLA Weybridge) (190µl, O157 titre 600) diluted 1/100, 1/1000 and 1/10000 in TBS, was applied to sections, incubated at room temperature for 1 hour and washed (x3) in TBS for 5 minutes. Biotinylated goat anti-rabbit antibody (Sigma) (1/1000 in TBS) was applied to sections at room temperature for 30 minutes. Sections were then washed (x3) in TBS for 5 minutes. Vector elite ABC (190µl) was applied to the sections, which were then incubated at room temperature for 30 minutes and washed (x3) in TBS for 5 minutes. Each section was then incubated at room temperature for 10 minutes in 380µl citrate buffered diaminobenzidine solution, rinsed with distilled water and then washed in running tap water for 5 minutes. Sections were then counterstained in Mayers blue haematoxylin for 2 minutes, in running tap water for 10 minutes, then dehydrated, cleared and mounted in DPX.

2.5.5 Latex agglutination test

The latex agglutination test (Oxoid) was used to test 10% of sorbital non-fermenting single colonies retrieved on CT-SMAC plates. Briefly, one drop of test latex was mixed with one drop of 0.85% saline, after a portion of test single colony was emulsified in the saline drop, on a reaction card. The card was rocked in a circular motion for the observation of agglutination, for no more than 1 minute. If a positive result was observed a further portion of the same test single colony was tested using control latex reagent using the same method described.

2.6 Statistical analysis

For analysis of bacterial counts for *in vitro* adhesion and invasion assays the numbers of invaded bacteria was subtracted from the numbers of associated bacteria to give a 'true' value for adhesion. For statistical analyses, counts were transformed to their logarithms to the base ten ($\log_{10}$) values and analyses of variance (ANOVA) performed, followed by student t-tests. The means, standard deviation and significance (p) of the strain differences were calculated.

For analysis of bacterial counts for *in vivo* colonisation and invasion studies, the Wilcoxon-Mann-Whitney non-parametric test was used to compare the wild-type with each mutant. For competitive index studies, each mutant was subtracted from each wild-type for the same tissue to give a 'true' value for colonisation or invasion. The distribution of bacterial shedding scores for wild-type and each mutant were compared using the Kruskal-

Wallis test. Exact p-values were calculated using StatXactvsoftware. A definition of Statistical tests used is given in Appendix 2.

Chapter 3

Characterisation of Shiga-toxin (Stx) negative *E. coli* O157:H7 isolate NCTC 12900

As discussed in chapter 1, several studies have been conducted to gain an understanding of the colonisation of *E. coli* O157:H7 in various hosts and, specifically, to detect AE lesions should they be formed (Brown *et al.*, 1997; Dean-Nystrom *et al.*, 1997; Dean-Nystrom *et al.*, 1998; Wales *et al.*, 2001). One study did imply that intimin facilitated colonisation in adult ruminants (Cornick *et al.*, 2002). However, AE lesions that were very small and very sparse, have been detected rarely at a few sites in the large intestine of deliberately orally inoculated sheep (Wales *et al.*, 2002) and to date no other non-intimin defined *E. coli* O157:H7 virulence factor has been studied for a role in persistence in the animal host.

The prevalence of *E. coli* O157:H7 infection in birds is low (Wallace *et al.*, 1997) but several deliberate inoculation studies show that poultry are readily and persistently infected by *E. coli* O157:H7 indicating a possible threat to public health (Beery *et al.*, 1985; Starvic *et al.*, 1993; Schoeni & Doyle, 1994; Sueyoshi & Nakazawa, 1994). Whilst intimin and the LEE apparatus are likely to be associated with the colonisation of ruminants, the role of the LEE or other bacterial factors in the colonisation of poultry remains unclear. Therefore, it was essential to identify an *E. coli* O157:H7 isolate that would be suitable for these studies. A Shiga-toxin free isolate, NCTC 12900, was selected for safety reasons and it was considered likely that Shiga-toxin may have little effect upon the colonisation and persistence of the chick.

The aim of the work to be described in this chapter, was to determine if the Stx-negative *E. coli* O157:H7 isolate NCTC 12900 possessed the classical factors, other than the toxin genes, that are associated with the *E. coli* O157:H7 genotype and phenotype. Furthermore, it was important to determine an antibiotic sensistivity/resistence profile for NCTC 12900 as subsequent mutagenesis studies would involve the insertion of antibiotic resistant cassettes. Additionally, it was also important to ensure that NCTC 12900 was acid tolerant as the entire gastrointestinal tract of chicks is acidic. Therefore, studies were undertaken to test for (1) the presence of genes encoding surface antigens (2) the elaboration of surface structures (3) sensitivity to antibiotics and resistance to acid (4) adherence patterns associated with tissue culture assays and (5) colonisation and persistence in the SPF chick.

3.1 Results

3.1.1 Gene probe analysis

To determine that Stx-negative *E. coli* O157:H7 isolate NCTC 12900 had specific genes that have been reported for the Stx positive sequenced *E. coli* O157:H7 isolate EDL933, PCR was performed to confirm the presence of the following genes, *csgA*, *eae* (including *intγ*), *espA*, *espD*, *espP*, *etpD*, *fimA*, *fimE*, *fliC*, *hlyA*, *katP*, *sepL*, *stxI*, *stxII*, *wbdN* and *wzy*. Although sets of primers for the amplification of each gene have their own optimal annealing temperature, an overall annealing temperature of 56°C was suitable for amplification of genes. Gene probe analysis confirmed that NCTC 12900 possessed genes that are encoded in the LEE pathogenicity island, including intimin type γ (gamma) and the genes involved in type III secretion *espA*, *espD* and *sepL* (Table 3.1 & Dibb-Fuller *et al.*, 2001). Gene probe analysis also detected genes encoded in the 93-kb virulence plasmid, pO157 (*espP*, *etpD*, *hlyA* and katP) and confimed that NCTC 12900 lacked the genes encoding Stx1 and Stx2 (Table 3.1 & Dibb-Fuller *et al.*, 2001). Gene probe analysis also confirmed that NCTC 12900 has the first two genes residing in the O-antigen operon (*wbdN* and *wzy*) and genes that are part of the curli (*csgA*) and type-1 fimbrial operons (*fimA* and *fimE*) (Table 3.1). The structural subunit gene (*fliC*) required for the formation of flagella was also present (Table 3.1).

3.1.2 Elaboration of fimbriae and flagella

To test for the elaboration of type-1 fimbriae, two experiments were performed. Firstly, haemagglutination assays were performed on NCTC 12900 and control cultures statically grown in HIB at 37°C for 48 hours (Sojka *et al.*, 1996). Control isolates demonstrated mannose sensitive haemagglutination to guinea pig, chicken and sheep erythrocytes, whereas NCTC 12900 did not haemagglutinate guinea pig, chicken and sheep erythrocytes in the absence of mannose (Table 3.2). Secondly, transmission electron microscopy was performed to determine the presence of type-1 fimbriae. The APEC O78:K80 (wild-type) control isolate elaborated type-1 fimbriae, but NCTC 12900 did not (Table 3.2; Figure 3.1).

To test for the elaboration of curli fimbriae, a number of experiments were performed including the observation of lacy colony morphology, Congo red binding assays and transmission electron microscopy (TEM). Positive control isolates demonstrated lacy colony morphology (Table 3.3) and bound Congo red, whereas NCTC 12900 exhibited smooth colony morphology and did not bind Congo red (Table 3.3). TEM also confirmed that NCTC 12900 was unable to elaborate curli fimbriae (Figure 3.2).

To test for motility, control isolates and NCTC 12900 were stab inoculated into LB sloppy agar and incubated at 25°C, 37°C and 42°C for 16 hours. NCTC 12900 exhibited

diffuse growth throughout the medium and behaved as anticipated (Table 3.4). TEM confirmed the elaboration of flagella by NCTC 12900 (Figure 3.3).

3.1.3 Other *in vitro* characterisation

Stx-negative *E. coli* O157:H7 isolate NCTC 12900 was tested for sensitivity to a panel of 16 antibiotics by the disc diffusion method as described in chapter 2. Isolate NCTC 12900 was sensitive to all 16 antibiotics in the panel (Table 3.6). The elaboration of O-antigen was also detected using the *E. coli* O157 detection kit (Oxoid).

Acid resistance was determined using the methods described previously (Gorden & Small, 1993). NCTC 12900 was recovered from LB broth 2.5 hours after the addition of hydrochloric acid (Table 3.5). NCTC 12900 was able to grow in the presence of acid. The numbers of recovered NCTC 12900 were similar to the numbers of recovered acid tolerant avian *E. coli* (APEC). However, the number of NCTC 12900 and acid tolerant controls recovered from acidified broth were higher than the representative starting cultures. This observation may have occurred due to fluctuations in pH after the addition of HCl, before and/or after the addition of O157 bacteria to LB broth.

3.1.4 Comparison of adherence and invasion patterns on tissue culture mono-layers

Two types of tissue culture mono-layers, avian Div-1 derived from the embryos of SPF chicks (Velge *et al.*, 2002) and HEp-2 mono-layers were utilised to determine the numbers of adhering and invading Stx-negative *E. coli* O157:H7 NCTC 12900. The results of adherence and invasion assays are shown in Table 3.7a. Adherence of NCTC 12900 to Div-1 cells was similar to that of the two EPEC serotypes, but was significantly ($p<0.05$) more than the APEC O86:K61 and *E. coli* K12 strains. The extent of invasion of NCTC 12900 was significantly ($p<0.05$) more than APEC O86:K61, similar to EPEC O127:H6, but was significantly ($p<0.05$) less than EPEC O111:NM. Numbers of internalised *S. typhimurium* DT104 were significantly ($p<0.001$) greater than for all other bacteria used, for which over 95% of adherent bacteria invaded. EPEC O111:NM invaded at significantly higher numbers ($p<0.001$) than EPEC O126:H6. *E. coli* K12 was internalised, but at significantly lower numbers ($p<0.001$) than all pathogenic *E. coli* serotypes and *S. typhimurium* DT104.

The adherence and invasion properties of NCTC 12900 to HEp-2 mono-layers was similar to that reported by Dibb-Fuller *et al.*, (2001). The results of adherence and invasion assays are shown in Table 3.7b. Numbers of adhering NCTC 12900 was similar to that of EPEC O127:H6, EPEC O111:NM and APEC O86:K61 strains and significantly more ($p<0.001$) than *E. coli* K12. Numbers of internalised *S. typhimurium* DT104 were significantly ($p<0.001$) greater than for all other bacteria used, for which over 10% of adherent bacteria invaded. No differences in the numbers of internalised bacteria was

observed with NCTC 12900, EPEC O127:H6 and EPEC O111:NM. No internalised *E. coli* K12 or APEC O86:K61 was detected.

No significant differences between Div-1 and HEp-2 cell mono-layers were noted for NCTC 12900, EPEC O127:H6, EPEC O111:NM, APEC O86:K61, *E. coli* K12 and *S. typhimurium* DT104 adhesion (Table 3.7c).

3.1.5 Visualisation of adherence patterns on tissue culture mono-layers

Adherent bacteria were observed by light microscopy after Giemsa staining and by scanning electron microscopy (SEM). NCTC 12900 generated localised adherence patterns on Div-1 and HEp-2 mono-layers and the characteristic micro-colonies of densely packed cells (Figure 3.4) which were also observed for the control EPEC O127:H6 strain (Table 3.8). However, although localised adherence was observed for EPEC O111:NM with micro-colonies on HEp-2 mono-layers, micro-colonies of densely packed cells were not observed on Div-1 mono-layers (Table 3.8). Discrete patterns of localised adherence were observed for APEC O86:K61, whereas a pattern of diffuse adherence was observed for *E. coli* K12 and *S. typhimurium* DT104 (Table 3.8).

Actin rearrangements within avian Div-1 and HEp-2 (control) mono-layers, observed by fluorescent actin staining (FAS), and characteristic of attaching and effacing *E. coli* (AEEC) as reported on other tissue culture assays (Knutton *et al.*, 1989; Matthews *et al.*, 1997; Dibb-Fuller *et al.*, 2001; Kenny, 2001; McNally *et al.*, 2001) was shown for NCTC 12900 and for both EPEC serotypes (Figure 3.5; Table 3.8). The APEC O86:K61 isolate also caused a localised rearrangement of Div-1 and HEp-2 actin, but to a much lesser extent (Figure 3.5; Table 3.7). No characteristic AEEC-FAS reaction was observed for *E. coli* K12 or *S. typhimurium* DT104 (Table 3.8).

Transmission electron microscopy (TEM) revealed that *E. coli* O157:H7 NCTC 12900 induced the formation of the "pedestal cup" structure on the avian cell mono-layer typical of attaching and effacing (AE) lesions (Figure 3.6). Also, an unidentified appendage-like structure was associated with the attached and unattached bacteria on Div-1 cell mono-layers (Figure 3.6). However, this was an infrequent observation that was noted with Div-1 cell mono-layers only. TEM results are also summarised in (Table 3.8).

3.1.6 *In vivo* localisation of SPF chicks by *E. coli* O157:H7 NCTC 12900

One-day-old SPF chicks were dosed orally with 1 x 10^5 CFU of NCTC 12900 and *post mortem* examinations were performed on individual birds as described in chapter 2. Bacterial counts recovered from the liver, spleen and caeca are given in Table 3.9. Bacterial counts for days 57 and 92 post inoculation are also given for other tissues of the gastrointestinal tract in Table 3.9. No morbidity or mortality was noted throughout the experiment. All birds examined by *post mortem* had high numbers (10^8 per ml tissue homogenate) of NCTC 12900

associated with the caeca on days 1, 2 and 5 post inoculation, although one bird each from days 1 and 2 post inoculation did have 10^9 CFU per ml tissue homogenate of NCTC 12900 associated with the caeca. Localisation in the caeca of NCTC 12900 on day 57 post inoculation was approximately 10-fold less than the number of NCTC 12900 recovered on day 1 post inoculation. On day 92 post inoculation, the number of NCTC 12900 localised in the caeca was approximately 100-fold less than the number of NCTC 12900 recovered on day 1 post inoculation (Table 3.8). No NCTC 12900 was recovered from the caeca at the end of the experiment (day 211 post inoculation).

Low levels (10^1-10^2 per ml tissue homogenate) of NCTC 12900 were recovered from the liver and spleen on days 1, 2 and 5 post inoculation, but not all birds had NCTC 12900 associated with the liver and spleen on days 1 and 5 post inoculation. On days 57 and 92 post inoculation, NCTC 12900 was not detected in the liver and spleen (Table 3.8) and on day 211, no NCTC 12900 was recovered from the liver, spleen and caeca.

Post mortem examinations were also performed on the colon of individual birds on days 57 and 92 post dosing and 10^6 and 10^4 CFU per ml tissue homogenate of NCTC 12900 were recovered, respectively (Table 3.7). Low levels (10^1-10^3 per ml tissue homogenate) of NCTC 12900 were also recovered from the duodenum, jejunum, ileum and caecal tonsils on days 57 post inoculation and only from the ileum and caecal tonsils on day 92 post inoculation (Table 3.7).

No NCTC 12900 was recovered form the liver, spleen, duodenum, jejunum, ileum, colon, caecal tonsils and caeca at the end of the experiment (day 211 post inoculation).

3.1.7 *In vivo* persistence of SPF chicks by *E. coli* O157:H7 NCTC 12900

Data from the *post mortem* examinations indicated that the caeca was readily colonised and that NCTC 12900 persisted in this organ for at least 92 days post inoculation (Table 3.9). Cloacal swabbing of birds in the same experiment demonstrated that NCTC 12900 was shed for up to 148 days post inoculation (Figure 3.7) suggesting that NCTC 12900 was still present in the gastro-intestinal tract, most probably in the caeca until this time. High, medium and low numbers of NCTC 12900 were detected for at least 57, 113 and 148 days post inoculation, respectively, by cloacal swabbing.

3.1.8 Detection of the presence of *E. coli* O157:H7 NCTC 12900 on or within eggs

Coincidentally, NCTC 12900 seemed to clear 12 days before the onset of egg laying (Day 160 post dosing). Fifty eggs were tested for the presence of NCTC 12900 on their shells, and in their egg white and yolk but no NCTC 12900 was recovered from any part of any of the fifty eggs examined.

3.1.9 Histological findings of *E. coli* O157:H7 NCTC 12900 infection of SPF chicks

The liver, spleen and caeca was removed from one bird chosen at random on days 1, 2 and 5 post inoculation for histological analysis, but unfortunately, these tissues were accidentally discarded. In a separate experiment, thirty, 1-day-old SPF chicks were dosed orally with 1 x 10^7 CFU of NCTC 12900. The distal gastrointestinal tract (duodenum, jejunum, ileum, colon and caeca) of all 30 inoculated birds (three birds per time point) from this experimental group was examined for evidence of adherence or pathological changes. Micro-colonies of bacteria were seen adhering to the mucosa and these were readily stained with specific *E. coli* O157:H7 antisera (Figure 3.8). Mucosal colonisation was observed from all 30 birds examined, but not in all of the tissues examined. Furthermore, no mucosal colonisation was detected in the duodenum and jejunum. Overall, mucosal colonisation typically occurred in caeca examined between days 21 and 29 post inoculation, although mucosal NCTC 12900 micro-colonies were sparse. Since micro-colony formation was sparse and due to the constraints of time, no further methods (i.e. Transmission electron microscopy) were performed on any isolated tissues. Therefore, no unequivocal evidence for the induction of AE lesions was gained.

Table 3.1. Amplification of gene regions from Stx-negative *E. coli* O157:H7 isolate NCTC 12900

Genes of interest	Stx-negative *E. coli* O157:H7 NCTC 12900	Stx-positive *E. coli* O157:H7 EDL933	*E. coli* K12 (DH5α)
wbdN-wzy (2168bp)	+	+	-
stxI (178bp)	-	+	-
stxII (374bp)	-	+	-
eae (2172bp)	+	+	-
intγ (957bp)	+	+	-
sepL-espA-espD (2068bp)	+	+	-
espP (1164bp)	+	+	-
etpD (1061bp)	+	+	-
hlyA (2992bp)	+	+	-
katP (2125bp)	+	+	-
csgA (2168bp)	+	+	-
fimE-fimA (604)	+	+	+
fliC (1641bp)	+	+	+ (1496bp)

PCR was performed on genomic DNA as described in chapter 2 using the the primers listed in table 2.2. +, PCR amplified product of anticipated size. -, indicates that no amplified product was obtained. EDL933 genomic DNA was prepared and provided by Roberto La Ragione.

Table 3.2. Observation of haemagglutination by enteric bacteria, but not by Stx-negative *E. coli* O157:H7 isolate NCTC 12900.

Bacterial isolate	Chicken erythrocytes		Sheep erythrocytes		Guinea pig erythrocytes	
	agglutinate	MS	agglutinate	MS	agglutinate	MS
E. coli O157:H7 NCTC 12900	-	NA	-	NA	-	NA
EPEC O127:H6	+	✓	+	✓	+	✓
EPEC O111:NM	+	✓	+	✓	+	✓
APEC O78:K80	+	✓	+	✓	+	✓
APEC O78:K80 RML1	-	NA	-	NA	-	NA
APEC O86:K61	+	✓	+	✓	+	✓
E. coli K12	+	✓	+	✓	+	✓
S. typhimurium DT104	+	✓	+	✓	+	✓

+ haemagglutination, - no haemagglutination, ✓ mannose sensitive, NA not applicable. RML1 (*fimC*::kan^r) is a type 1 fimbriae deficient mutant.

Table 3.3. Expression of curli fimbriae by enteric bacteria, but not by Stx-negative *E. coli* O157:H7 isolate NCTC 12900.

Bacterial isolate	Lacy colony morphology	Congo red binding
E. coli O157:H7 NCTC 12900	SC	×
EPEC O127:H6	SC	✓
EPEC O111:NM	SC	×
APEC O78:K80	LC	✓
APEC O78:K80 RML2	SC	×
APEC O86:K61	LC	✓
E. coli K12	SC	×
S. typhimurium DT104	LC	✓

LC lacy colony morphology, SC smooth colony morphology, ✓ congo red binding, × no congo red binding. RML2 (*csgA*::cam^r) is a curli fimbriae deficient mutant.

Table 3.4. Observation of motility in sloppy agar by Stx-negative *E. coli* O157:H7 isolate NCTC 12900.

Bacterial isolate	Motile		
	25°C	37°C	42°C
E. coli O157:H7 NCTC 12900	+	+	+
EPEC O127:H6	+	+	+
EPEC O111:NM	-	-	-
APEC O78:K80			
APEC O78:K80 RML6			
APEC O86:K61	-	-	-
E. coli K12	-	-	-
S. typhimurium DT104	+	+	+

+ motile, - non-motile. RML6 (*fliC*::str^r) is an aflagellate mutant.

Table 3.5. Stx-negative *E.coli* O157:H7 isolate NCTC 12900 in the presence of inorganic acid (HCl, pH 2.5) for 2.5 hours at 37°C with shaking.

Bacterial isolate	Resistance to acid		
	CFU/ml @ time = 0	CFU/ml in LB broth @ time = 2.5 hours	CFU/ml in LB broth pH 2.5 @ time = 2.5 hours
E. coli O157:H7 NCTC 12900	6.58E+04	1.77E+08	1.69E+06
APEC O86:K61	8.68E+04	2.12E+08	3.03E+06
APEC O78:K80	1.97E+05	2.09E+08	3.14E+06
APEC O78:K80 RML5	7.50E+04	2.53E+08	0

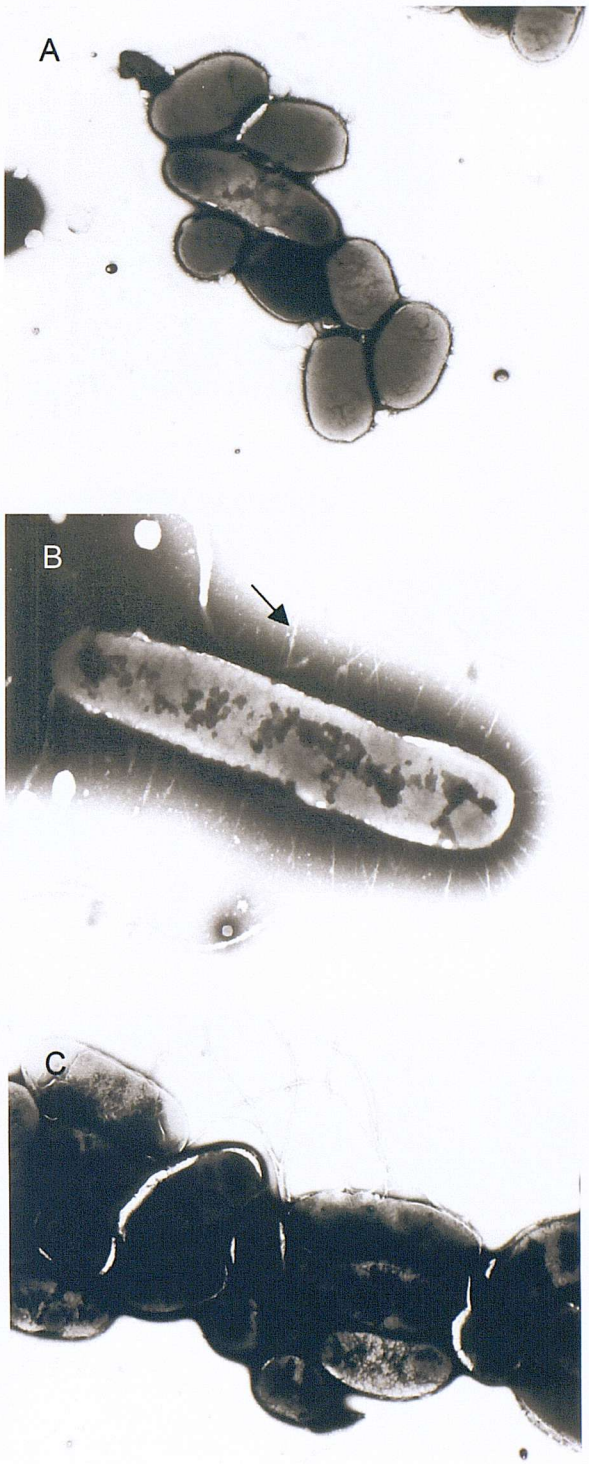
RML5 (*rpoS*::cam^r) is an RpoS mutant.

Table 3.6. Antibiotic sensitivity test

Bacterial isolate	Antibiotic															
	NA	**TE**	**N**	**AMP**	**FR**	**CXM**	**SXT**	**C**	**AK**	**AMC**	**CN**	**S**	**S3**	**CFP**	**APR**	**CT**
NCTC12900	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
EPEC O111:NM	S	R	S	S	S	S	S	R	S	S	S	R	R	S	S	S
E. coli K12	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
S. typhimurium DT104	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

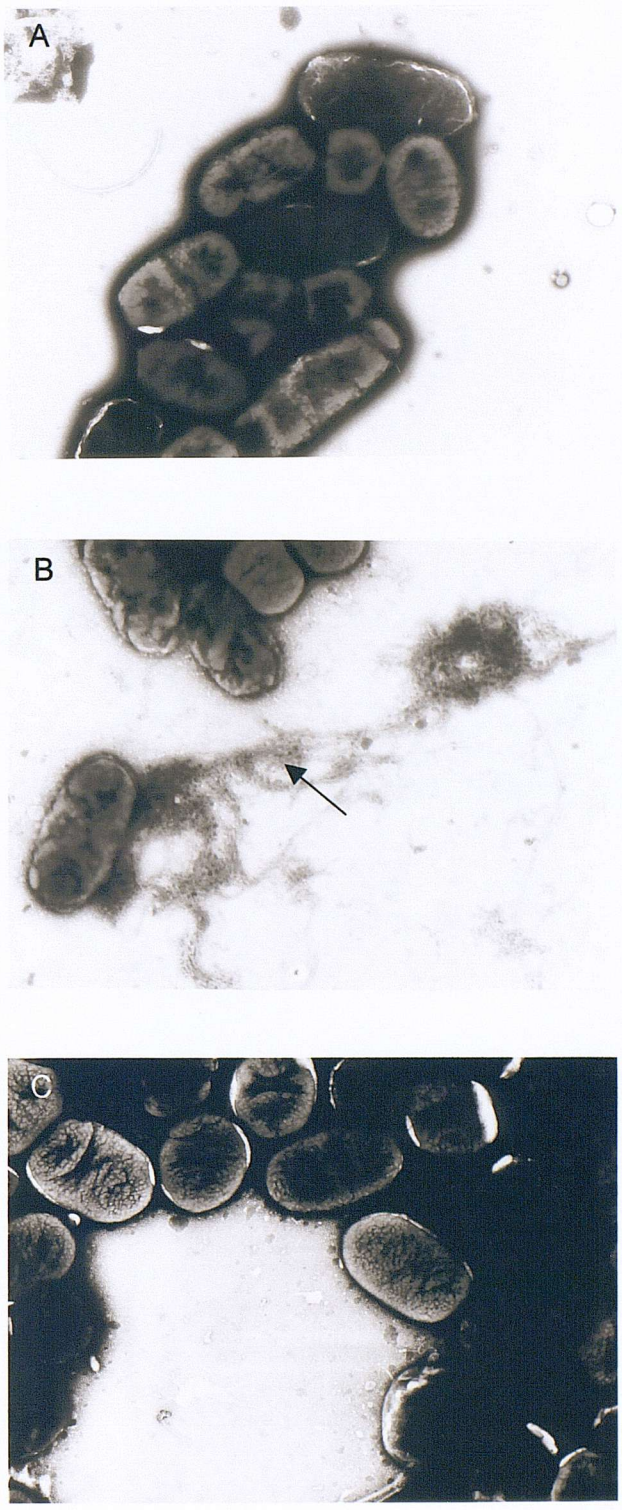
S = sensitive, R = resistant, NA= nalidixic acid, TE = tetracycline, N = neomycin, AMP = ampicillin, FR = furazolidine, CXM = cefuroxine, SXT = sulphamethoxazole & trimethroprim, C = chloroamphenicol, AK = amikacin, amoxycillin & clavulanic acid, CN = gentamicin, S = streptomycin, S3 = compund sulphonamides, CFP = cefoperazone, APR = apramycin, CT = colistin sulphate.

Figure 3.1. No elaboration of type-1 fimbriae by Stx-negative *E. coli* O157:H7 isolate NCTC 12900 as demonstrated by transmission electron microscopy.



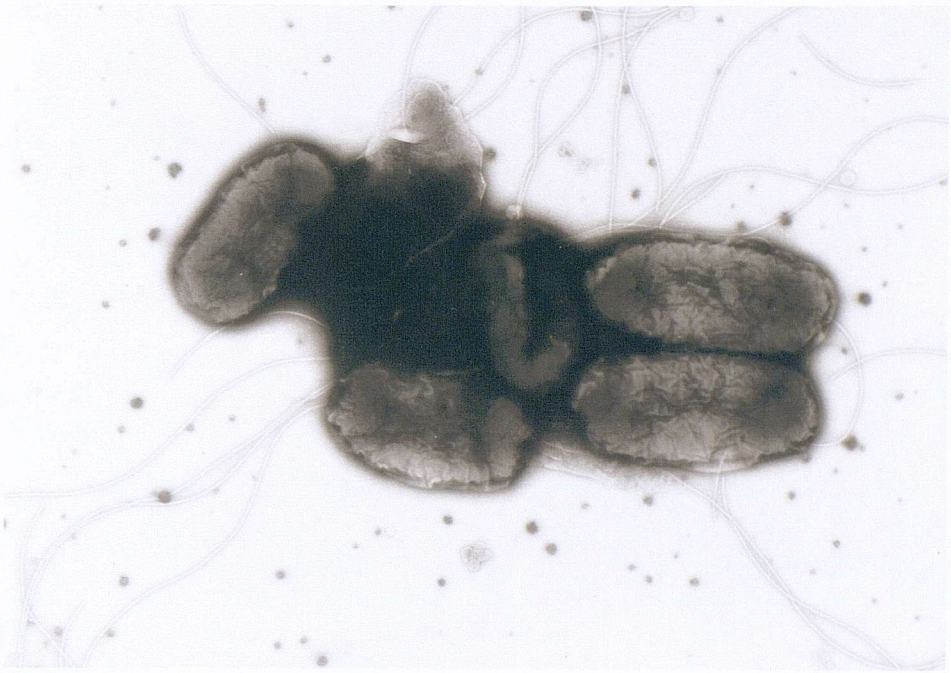
(A) NCTC 12900 (B) APEC O78:K80 elabortion of type-1 fimbriae
(C) APEC O78:K80 type-1 fimbrial mutant RML1. Magnification x 22,250.

Figure 3.2. No elaboration of curli fimbriae by Stx-negative *E. coli* O157:H7 isolate NCTC 12900 as demonstrated by transmission electron microscopy.



(A) NCTC 12900 (B) APEC O78:K80 elabortion of curli fimbriae
(C) APEC O78:K80 curli fimbrial mutant RML2. Magnification x 30,000.

Figure 3.3. Elaboration of flagella by Stx-negative *E. coli* O157:H7 isolate NCTC 12900 as demonstrated by transmission electron microscopy. Magnification x 38,750.



Tables 3.7a, b & c. Adhesion (a) and invasion (b) of avian Div-1 and human HEp-2 cell lines by Stx-negative *E. coli* O157:H7 NCTC 12900 and other enteric bacteria and (c) a statistical comparison between cell lines.

(a) Adhesion

Bacteria	Div-1				HEp-2			
	Mean log_{10}	Geometric mean	SD	p-value	Mean log_{10}	Geometric mean	SD	p-value
NCTC 12900	6.76	5.75E+06	0.11	-	6.85	7.01E+06	0.34	-
EPEC O127:H6	6.78	6.02E+06	0.11	0.910	6.61	4.06E+06	0.06	0.129
EPEC O111:NM	6.64	4.36E+06	0.13	0.478	6.66	3.63E+06	0.22	0.212
APEC O86:K61	5.73	5.37E+05	0.11	<0.001	6.43	2.67E+06	0.33	0.011
E. coli K12	5.70	5.01E+05	0.09	<0.001	5.83	6.73E+05	0.06	<0.001
S. typhimurium DT104	5.93	8.51E+05	0.69	<0.001	6.32	2.12E+06	0.22	0.002

(b) Invasion

Bacteria	Div-1				HEp-2			
	Mean log_{10}	Geometric mean	SD	p-value	Mean log_{10}	Geometric mean	SD	p-value
NCTC 12900	4.22	1.66E+04	0.08	-	3.19	1.53E+03	0.12	-
EPEC O127:H6	3.96	9.12E+03	0.20	0.280	2.92	8.42E+02	0.31	0.034
EPEC O111:NM	5.04	1.10E+05	0.14	0.001	3.47	2.95E+03	0.22	0.025
APEC O86:K61	3.55	3.55E+03	0.27	0.007	0	0	0	0
E. coli K12	1.95	8.91E+01	1.28	<0.001	0	0	0	0
S. typhimurium DT104	6.22	1.66E+06	0.05	<0.001	5.29	1.93E+05	0.05	<0.001

SD, standard deviation; p-value, significance in adhesion and invasion between each isolate and NCTC 12900. Units for geometric means are CFU/ml.

(c) Significance (p-value) between cell lines for enteric bacterial adhesion and invasion.

Bacteria	Adhesion	Invasion
	p-value of Div-1 versus HEp-2	p-value of Div-1 versus HEp-2
NCTC 12900	0.183	0.001
EPEC O127:H6	0.207	<0.001
EPEC O111:NM	0.909	<0.001
APEC O86:K61	0.004	0.008
E. coli K12	0.062	0.048
S. typhimurium DT104	0.273	<0.001

All counts were transformed to the logarithms to the base 10 of (count+1). Comparisons between the NCTC 12900 and each isolate by student t-tests followed analysis of variance. Comparisons between Div-1 and HEp-2 mono-layers for the means of the same isolate were made using student t-tests with separate variance estimates. Where counts were zero for one cell type the non-parametric Wilcoxon-Mann-Whitney was used in place of the student t-test.

Table 3.8. Summary of patterns of interaction of Stx-negative *E. coli* O157:H7 NCTC 12900 and other enteric bacteria with avian Div-1 cells and human HEp-2 cells demonstrated by Giemsa staining, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and fluorescent actin staining (FAS).

Cell type & Test	**Bacterial strain**					
	Stx –ve *E. coli* O157:H7 (NCTC12900)	EPEC O127:H6	EPEC O111:NM	APEC O86:K61	*E. coli* K12 (DH5α)	*S. typhimurium* DT104
Div-1						
Giemsa/SEM	LA*	LA*	LA	LA	DA	DA
TEM	PF	PF	PF	PF	-	-
FAS	+++	+++	++	+	-	-
HEp-2						
Giemsa/SEM	LA*	LA*	LA*	LA	DA	DA
TEM	PF	PF	PF	PF	-	-
FAS	+++	+++	+++	+	-	-

LA, localised adherence; DA, diffuse adherence; * formation of densely packed micro-colonies.
PF, pedestal formation; -, no pedastals formed.
+++, high numbers of FAS lesions formed; ++, medium numbers of FAS lesions formed; +, low numbers of FAS lesions formed; -, no FAS lesions formed.

Figure 3.4. Analysis of adherence patterns by (A) Stx-negative *E. coli* O157: H7 NCTC 12900, (B) EPEC O127:H6 and (C) APEC O86:K61 on avian Div-1 cells as observed by scanning electron microscopy. Magnification x 1750.

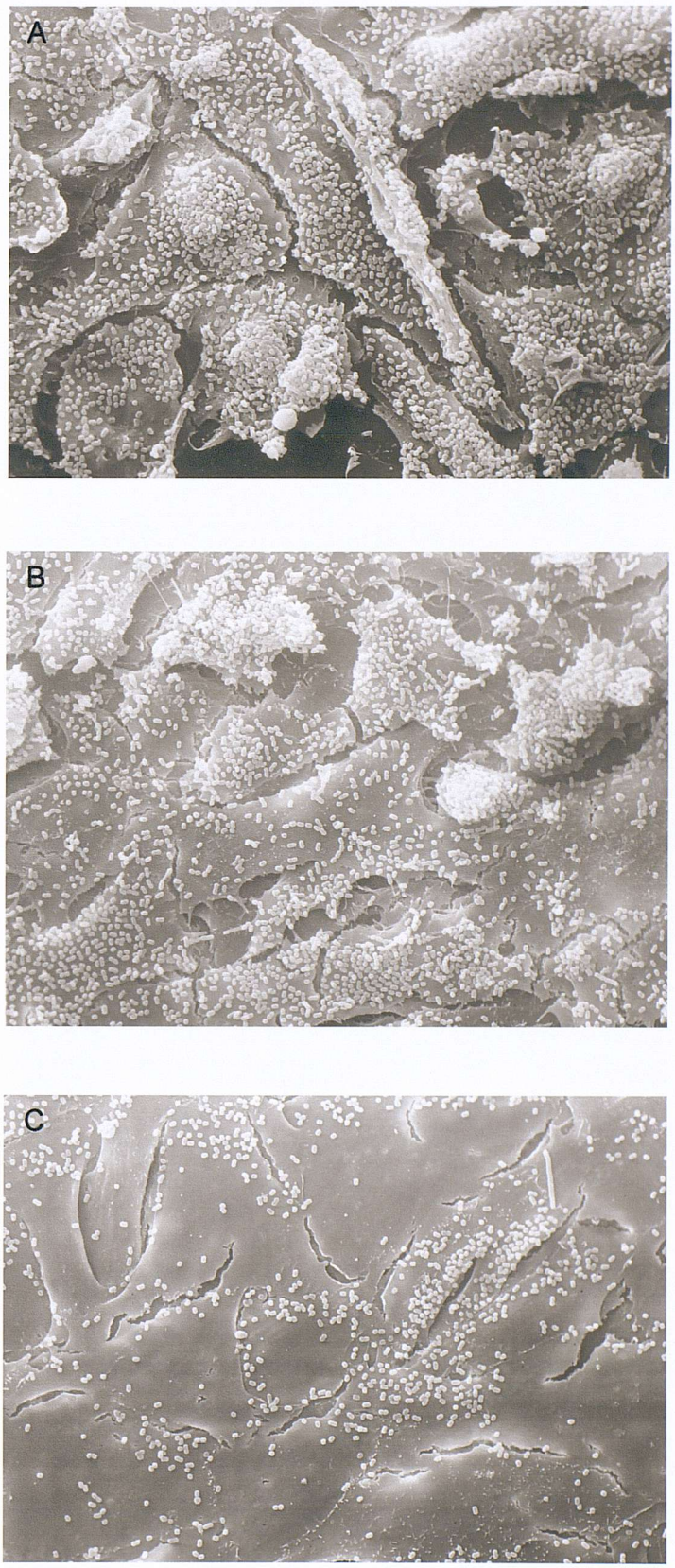


Figure 3.5. The formation of AE lesions by (A) Stx-negative *E. coli* O157:H7 NCTC 12900 (B) EPEC O127:H6 and (C) APEC O86:K61 on avian Div-1 cells as demonstrated by fluorescence actin staining (FAS). Magnification x 1000.

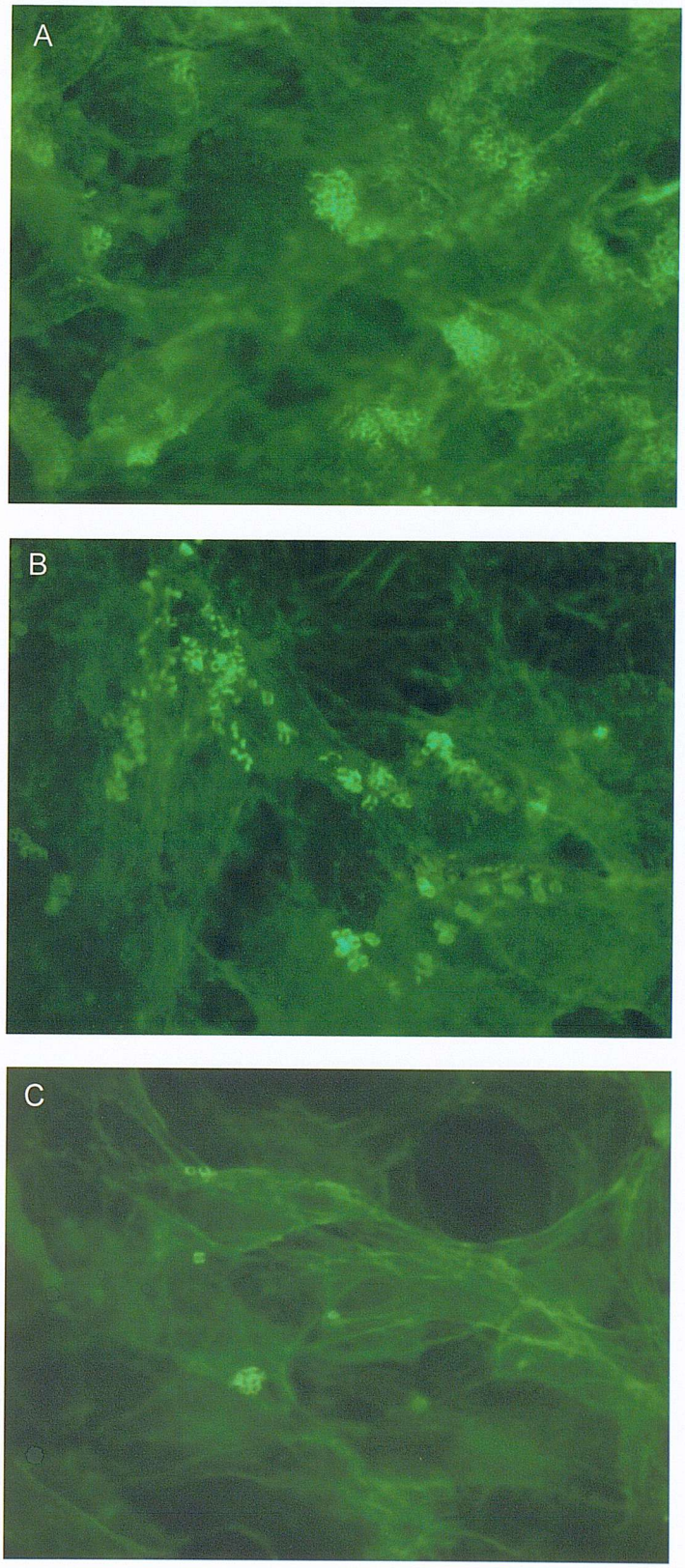


Figure 3.6. The formation of "pedestal cup structures" (AE lesions) by (A) Stx-negative *E. coli* O157:H7 NCTC 12900 (B) EPEC O111:NM and (C) APEC O86:K61 on avian Div-1 cells as observed by transmission electron microscopy. Magnification x 38,750.

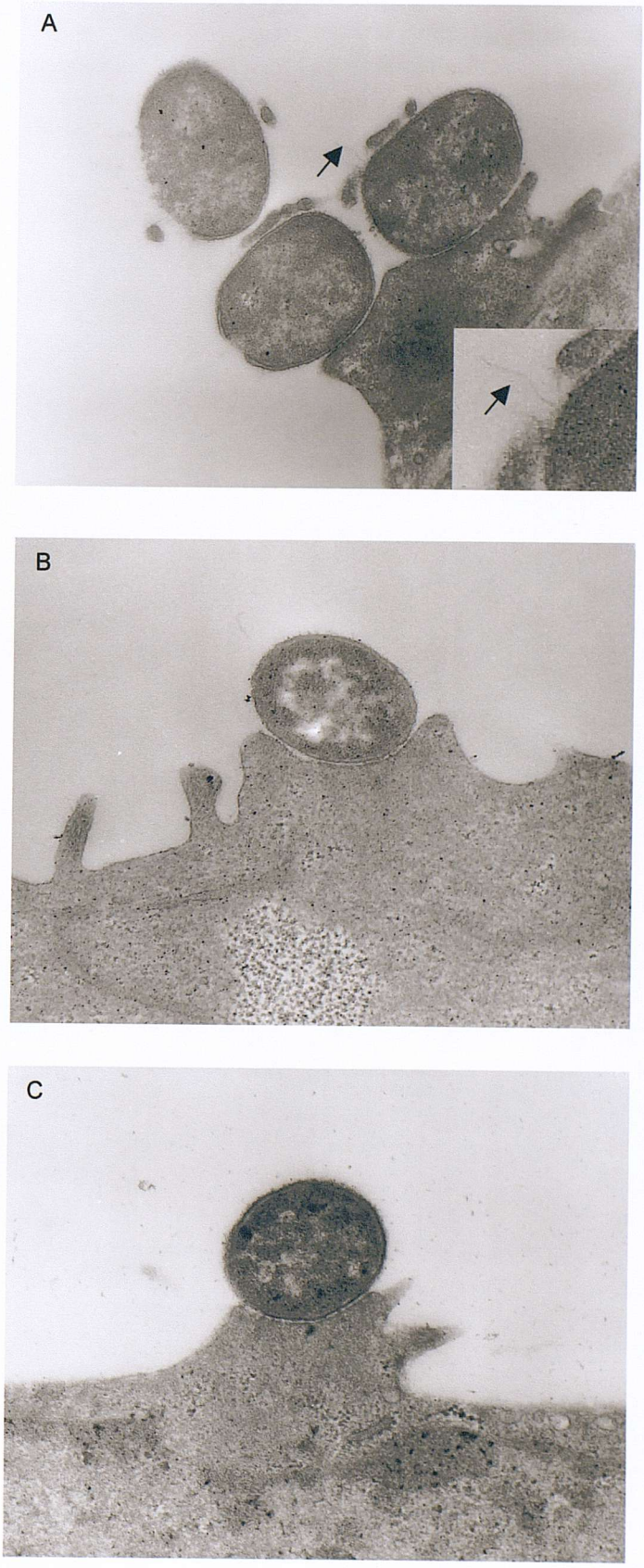
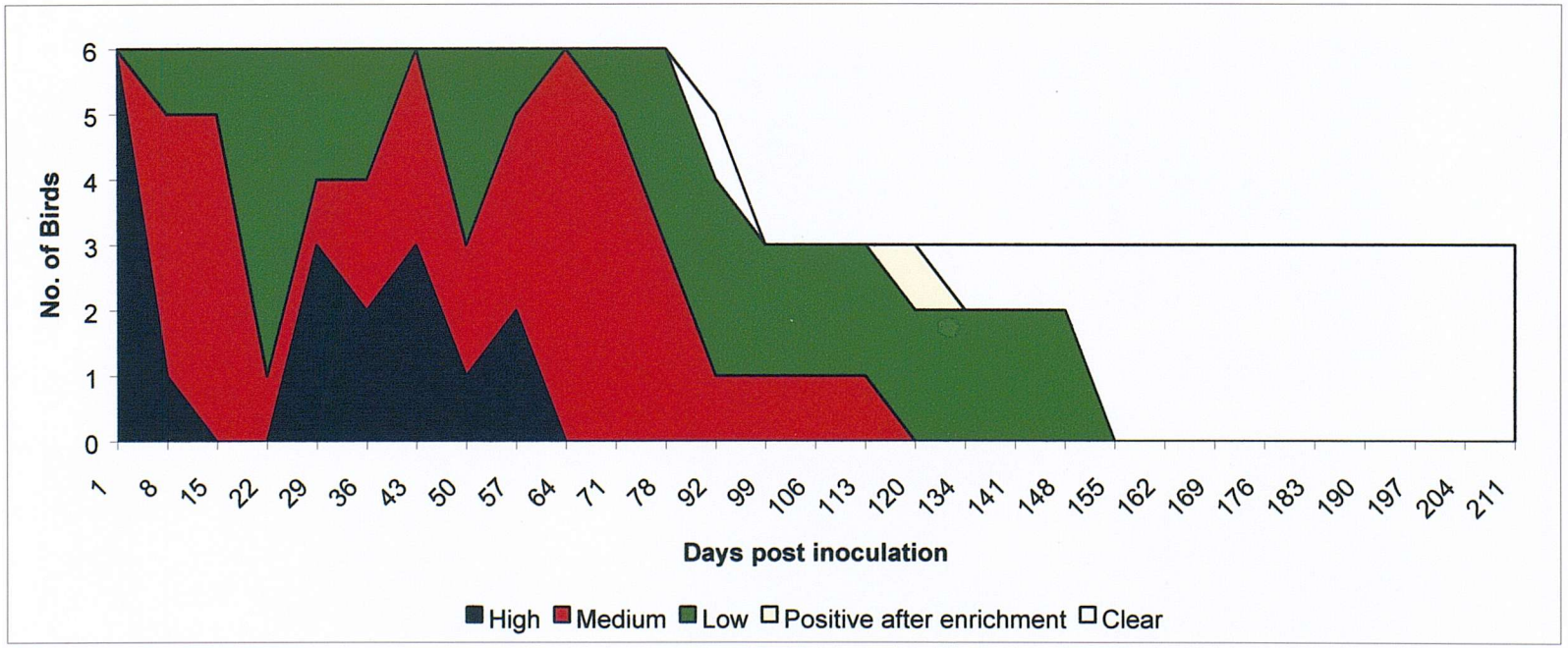


Table 3.9. Localisation of Stx-negative *E. coli* O157:H7 NCTC 12900 in SPF chicks at days 1, 2 5, 57 and 92 post inoculation.

	Tissue							
Day 1 PI	Liver	Spleen	Duodenum	Jejunum	Ileum	Colon	Caeca	C.Tonsils
No. birds positive	3/5	0/5	ND	ND	ND	ND	5/5	ND
Mean $\log_{10}$	1.82	0	-	-	-	-	8.64	-
Geometric mean	6.66E+01	0	-	-	-	-	4.36E+08	-
SD	2.26	0	-	-	-	-	0.46	-
Day 2 PI	Liver	Spleen	Duodenum	Jejunum	Ileum	Colon	Caeca	C.Tonsils
No. birds positive	5/5	5/5	ND	ND	ND	ND	5/5	ND
Mean $\log_{10}$	2.55	2.06	-	-	-	-	8.45	-
Geometric mean	3.55E+02	1.14E+02	-	-	-	-	2.79E+08	-
SD	2.20	1.49	-	-	-	-	0.56	-
Day 5 PI	Liver	Spleen	Duodenum	Jejunum	Ileum	Colon	Caeca	C.Tonsils
No. birds positive	3/5	3/5	ND	ND	ND	ND	5/5	ND
Mean $\log_{10}$	1.26	1.07	-	-	-	-	8.25	-
Geometric mean	1.81E+01	1.18E+01	-	-	-	-	1.76E+08	-
SD	1.77	1.38	-	-	-	-	0.63	-
Day 57 PI	Liver	Spleen	Duodenum	Jejunum	Ileum	Colon	Caeca	C.Tonsils
No. birds positive	0/2	0/2	1/2	1/2	2/2	2/2	2/2	1/2
Mean $\log_{10}$	0	0	1.41	1.50	3.14	6.11	7.73	1.65
Geometric mean	0	0	2.57E+01	3.16E+01	1.38E+03	1.27E+06	5.37E+07	4.46E+01
SD	0	0	1.99	2.12	1.30	0.22	0.11	2.33
Day 92 PI	Liver	Spleen	Duodenum	Jejunum	Ileum	Colon	Caeca	C.Tonsils
No. birds positive	0/2	0/2	0/2	0/2	2/2	2/2	2/2	2/2
Mean $\log_{10}$	0	0	0	0	2.22	4.00	6.15	3.87
Geometric mean	0	0	0	0	1.66E+02	1.00E+04	1.42E+06	7.40E+03
SD	0	0	0	0	0	0.25	0.84	0.07

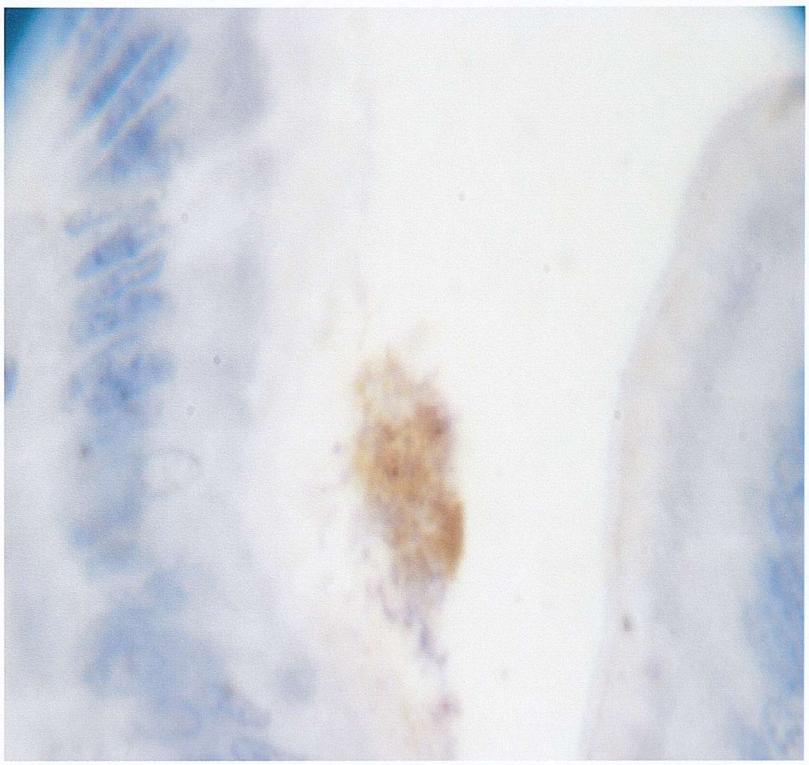
ND, not determined; C. Tonsils, Caecal tonsils; SD, standard deviation; PI, post inoculation

Figure 3.7. Distribution of Stx-negative *E. coli* O157:H7 NCTC 12900 shedding from SPF chicks.



Recovery of *E. coli* O157:H7 NCTC 12900 was scored as high (confluent), medium (>200 colonies) low (<200 colonies), positive (detected after enrichment only) and clear (no colonies), **N.B.** The three remaining birds began to lay eggs on day 160 post inoculation.

Figure 3.8. Micro-colony (Day 24 post inoculation) of Stx-negative *E. coli* O157:H7 NCTC 12900 attached to the caecal mucosa of an SPF chick orally dosed with 1 x 10^7 CFU. Magnification x 1000.



3.2 Discussion

The aims of the characterisation studies described in this chapter were to determine if Stx-negative *E. coli* O157:H7 isolate NCTC 12900 was akin to previously reported Stx positive *E. coli* O157:H7 isolates. However, although possible at the genotypic level, for safety reasons it was not possible to make direct comparisons at the phenotypic level with the Stx positive *E. coli* O157:H7 isolate EDL933. Gene probe analysis determined that NCTC 12900 has genes that belong to the virulence plasmid, pO157 and the locus of enterocyte effacement (LEE). Importantly, intimin type γ was detected, which is required for the initiation of the *E. coli* O157:H7 attaching and effacing (AE) phenotype. Gene amplification also verified that NCTC 12900 is indeed Stx1 and Stx2 negative (This study and Dibb-Fuller *et al.*, 2001). Whether NCTC 12900 has lost both toxins from their respective phage or both phage types do not reside within the NCTC 12900 genome was not determined. The first two genes of the operon responsible for O-antigen synthesis in *E. coli* O157:H7 were detected in NCTC 12900 and the LPS phenotype was confirmed by latex agglutination.

Gene probe analysis of genes outside the virulence plasmid and the LEE demonstrated that NCTC 12900 has genes that are part of the type-1 fimbriae and curli operons. However, no NCTC 12900 type-1 fimbriae or curli elaboration was detected, which is consistent with other reported *E. coli* O157:H7 studies (Enami *et al.*, 1999, Roe *et al.*, 2001; Uhlich *et al.*, 2001). The structural sub-unit gene of flagellin was also successfully detected by PCR. Transmission electron microscopy and motility tests confirmed that NCTC 12900 does elaborate flagella that are functional, which is typical of other *E. coli* O157:H7 isolates (Sherman *et al.*, 1988; Sperandio *et al.*, 2002).

Studies describing intermediate to complete resistance of some *E. coli* O157:H7 isolates to antibiotics has been reported (Kim *et al.*, 1994; Meng *et al.*, 1998; Galland *et al.*, 2001). However, NCTC 12900 was sensitive to all the antibiotics tested in this study and was therefore deemed to be suitable for genetic manipulation using antibiotic resistant cassettes. Acid resistance studies verified that NCTC 12900 is able to tolerate the effects of acid. This is of particular importance for use in chicks as their gastrointestinal tract is acidic (Farner, 1942). However, the numbers of NCTC 12900 increased in acidified media, whereas previously reported studies have demonstrated that the numbers of *E. coli* O157:H7 in acidified media do not increase with time (Lin *et al.*, 1996; Price *et al.*, 2000). Importantly, the acid sensitive isolate used in the present study was not recovered from acidified media suggesting that although fluctuations in pH may have occurred, this factor was not detrimental to the outcome of this experiment, i.e. NCTC 12900 is acid tolerant.

This is the first report that *E. coli* O157:H7 can form micro-colonies and induce actin rearrangement, typical of the AE lesion, on an avian cell line. The extent of the interaction

was similar to that observed for the interaction of NCTC 12900 with HEp-2 cells (This study & Dibb-Fuller *et al.*, 2001) but was greater than for a *E. coli* O86:K61 strain, a serotype that possessed a variant γ (gamma) intimin and that was associated with severe necrosis of the gastrointestinal tract of a number of avian species (Foster *et al.*, 1998; La Ragione *et al.*, 2002). Studies examining the interactions between NCTC 12900 derived-mutants and an avian cell line may give more relevant insights into NCTC 12900-chick host interactions.

Transmission electron microscopy also revealed that NCTC 12900 associated and unassociated with AE lesions also elaborated an unidentified surface appendage. However, the two *E. coli* O157:H7 genome sequences (Hayashi *et al.*, 2001; Perna *et al.*, 2001) reveal the presence of other putative adhesins including five other fimbrial operons and it was possible that one or more of these may have played a role in the adherence to Div-1 cells and a recent study by Torres and co-workers (2002) has identified LP fimbriae, which may play a role in micro-colony formation. Whether the unidentified surface appendages associated with NCTC 12900 were LP fimbriae remains unclear.

In the 1-day-old SPF chick model, Stx-negative *E. coli* O157:H7 colonisation of the caeca was observed and in the later stages of infection, colonisation of the colon and other tissues of the gastrointestinal tract was also noted. Whether or not adherence was due partly to the formation of AE lesions was not determined. However, histological studies did show in birds given 1 x 10^7 CFU NCTC 12900, the formation of micro-colonies on the mucosal surface. Colonisation of the caeca is consistent with the findings of previous studies by Beery *et al.* (1985), Schoeni and Doyle (1994), Stavric *et al.* (1993) and Sueyoshi and Nakazawa, (1994). However, these authors used two *E. coli* O157:H7 isolates that were Stx positive. The fact that the Stx-negative *E. coli* O157:H7 isolate NCTC 12900 behaved in a similar manner to the Stx-positive *E. coli* O157:H7 isolates indicated that the Shiga-toxins might play little or no role in colonisation of the gastrointestinal tract of the chicken. These findings indicate that the *E. coli* O157:H7 LEE-encoded mechanisms of colonisation may play a significant role in colonisation in this model. Furthermore, poultry are susceptible to AE lesion formation by a number of other AEEC and EPEC strains (Pakpinyo *et al.*, 2002). However, it is reasonable to speculate that other *E. coli* O157:H7 mechanisms encoded outside the LEE may contribute to the persistent colonisation of SPF chicks.

E. coli O157:H7 is regarded as a non-invasive pathogen. However, in this and previous studies (Dibb-Fuller *et al.*, 2000; Wales *et al.*, 2002) it has been demonstrated that the organisms may be taken up by cells in low numbers compared with known invasive organisms such as *Salmonella*. It remains unclear whether uptake of *E. coli* O157:H7 was an active bacterial or host mechanism.

E. coli O157:H7 NCTC 12900 colonisation of the chickens persisted for 148 days and the resolution of infection occurred just before the birds came into lay. A variable colonisation study of White Leghorn chickens challenged orally with 10^1 to 10^8 CFU of *E. coli* O157:H7, isolated *E. coli* O157:H7 from the shells of eggs (Schoeni & Doyle., 1994). However, egg shells contaminated with *E. coli* O157:H7 came only from birds given an inoculum that was a 1000-fold greater than the inoculum given in this study. Possibly this Stx-negative O157 isolate persisted at levels at the point of lay below the sensitivity of the methods used in this study. However, only three birds were studied and further analysis of eggs from subsequent wild-type persistence experiments will be discussed in chapter 7.

The pattern of faecal excretion of NCTC 12900 by colonised birds appeared sporadic. It is unclear whether this is to be expected for this organism in this host although sporadic excretion has been documented previously for other gastro-intestinal colonising pathogens (Williams-Smith, 1975). Schoeni and Doyle (1994) collected faecal samples at monthly intervals from experimentally infected 1-day-old chickens and showed variable shedding from individual birds. More than half of the birds cleared infection at about 6 months of age whilst others continued to give detectable counts in the region of 100 organisms per gram of caecal contents at the closure of the experiment, 10 months after oral inoculation when administered with 1.3 x 10^8 CFU of *E. coli* O157:H7 bacteria. Although the methods for the detection of NCTC 12900 shedding are different from that of Schoeni and Doyle (1994) the findings of the study reported here for a Stx-negative *E. coli* O157:H7 isolate are compatible with those for birds inoculated with a similar dose of Stx positive *E. coli* O157:H7 isolate (Schoeni & Doyle, 1994). What is also important, was the development of a chick model of *E. coli* O157:H7 colonisation for the subsequent characterisation of *E. coli* O157:H7 surface antigens. This model will form the basis of future studies of *E. coli* O157:H7, a potential human health hazard in chickens. Therefore, isolate NCTC 12900 was considered for more detailed study as it met the criteria described for other typical Stx positive *E. coli* O157:H7 isolates, namely by the formation of micro-colonies and AE lesions on tissue culture mono-layers and the colonisation of SPF chicks.

Chapter 4

Construction and characterisation of knockout Stx-negative *E. coli* O157:H7 NCTC 12900 intimin and aflagellate mutants

As discussed in Chapter 3, Stx-negative *E. coli* O157:H7 isolate NCTC 12900 can form micro-colonies and AE lesions on an avian cell line (Div-1) derived from SPF White Leghorn chickens and is also able to colonise and persist in SPF White Leghorn chickens. An essential requirement for the formation AE lesions is the expression of the intimin adherence factor (Knutton *et al.*, 1987b; Knutton *et al.*, 1989; McKee and O'Brien, 1995). The *E. coli* O157:H7 intimin adherence factor is required for the formation of AE lesions in the gastrointestinal tract of experimentally infected animals (Donnenberg *et al.*, 1993; McKee *et al.*, 1995; Tzipori *et al.*, 1995; Dean-Nystrom *et al.*, 1998) and a probable role in large animal persistence has been reported (Cornick *et al.*, 2002). However, whether of not intimin is involved in the colonisation or persistence of SPF White Leghorn chicks by *E. coli* O157:H7 has yet to be determined.

Another surface elaborated factor, flagella, does play a role in the *in vivo* colonisation of other *E. coli* pathogens (Smyth, 1988) including an avian pathogenic *E. coli* isolate (La Ragione *et al*; 2000b) but a role in colonisation and persistence for *E. coli* O157:H7 flagella in any *in vivo* model has yet to be determined. However, Stx-positive, non-motile (H-) *E. coli* O157 have been isolated and *E. coli* O157:H- sorbitol fermenting strains are a major cause of food-borne disease in humans (Bielaszewska *et al.*, 2000; O'Brien *et al.*, 2001).

In order to test the contribution of intimin and flagella, if any, in the colonisation of SPF White Leghorn chicks by *E. coli* O157:H7, knockout intimin and aflagellate mutants in the Stx-negative *E. coli* O157:H7 isolate NCTC 12900 were required. Here, the description of their construction and conformation is given.

4.1 Results

4.1.1 Construction of a *eae*::cam^r mutant of *E. coli* O157:H7 NCTC 12900 by allellic exchange

A mutant defective for the elaboration of intimin was constructed by insertional inactivation of the *eae* gene in Stx-negative *E. coli* O157:H7 strain NCTC 12900 (Tables 4.1 & 4.2; Figures 4.1 and 4.2). Construction of the disrupted *eae* gene within the appropriate suicide vector was constructed previously (Cookson & Woodward, 2003). A 1558bp *Eco*RI/*Sal*I fragment from the conserved N-terminal region of *eae* was excised from an *eae* clone of an *E. coli* O157:H7 strain A84 cosmid library made in *E. coli* XL1-Blue MR. This *Eco*RI/*Sal*I fragement was cloned into *Eco*RI/*Sal*I cut pUC18 (amp^r) to give pALC6 (Table 4.1). The identity of the insert was confirmed by restriction enzyme mapping and pALC6 was linearised with *Eco*RV (unique restriction enzyme site) and dephosphorylated with shrimp alkaline phosphatase. A blunt-ended 1.1kb chloramphenicol cassette excised from pBCSK (Stratagene) by *Kpn*I and *Bam*HI was ligated with the pALC6 to give pALC8 (Table 4.1). The *eae*::cam^r fragment of pALC8 was amplified via PCR using *pfu* polymerase and primers m13-20F and m13rev to generate blunt-ended amplicons, which were cloned into the *Srf*I site of the plasmid suicide vector pEFORMT (tet^r) to give pALC10 (Table.4.1). The host for pALC10 was *E. coli* K12 S17 λ *pir*, permissive for stable replication of the pERFORM series of suicide vectors. In the study reported here, conjugations (Table 4.2) were performed between *E. coli* K12 S17 λ *pir* harbouring pALC10 and the recipient isolate NCTC 12900 with selection made on minimal media plates supplemented with chloramphenicol. Well-isolated colonies were picked and streaked onto minimal media plates supplemented separately with chloramphenicol and tetracycline. Chloramphenicol resistant and tetracycline sensitive colonies were retained. Recombinants that grew only on chloramphenicol were sub-cultured onto the same medium a further three times to prevent any *E. coli* K12 S17 λ *pir* carry over and then tested genotypically and phenotypically to confirm inactivation of *eae*.

4.1.2 Construction of a *fliC*::str^r mutant of *E. coli* O157:H7 NCTC 12900 by allellic exchange

A mutant defective for the elaboration of flagella was constructed by insertional inactivation of the *fliC* gene in Stx-negative *E. coli* O157:H7 strain NCTC 12900 (Tables 4.1 & 4.2; Figures 4.1 and 4.2). Initially, primers fliCF and fliCR (Chapter 2, Table 2.2) were used successfully to amplify the 1641bp amplicon from NCTC 12900. The amplicon was purified and ligated into the multiple cloning site of the cloning vector pCR®2.1 to give pGUS1 (Table 4.1). The insert was mapped by restriction enzyme digestion. A unique restriction enzyme site (*Eco47*III) was found within the 1641bp amplicon. A blunt-ended 1.2kb *Pvu*II fragment that encoded streptomycin resistance was excised from p723 and

cloned into the *Eco47*III site of pGUS1 to give pGUS2 (Table 4.1). *Eco*RI and *Sac*I was used to cleave the *fliC*::str^r construct from pGUS2, which was cloned into the *Srf*I site of pERFORMK (kan^r) (Table 4.1) to give pGUS3 (Table 4.1). The host for pGUS3 was *E. coli* K12 S17 λ *pir*, permissive for stable replication of the pERFORM series of suicide vectors. Conjugations (Table 4.2) were performed between *E. coli* K12 S17 λ *pir* harbouring pGUS3 and the recipient isolate NCTC 12900 with selection made on minimal media plates supplemented with streptomycin. Well-isolated colonies were picked and streaked onto minimal media plates supplemented separately with streptomycin and kanamycin. Streptomycin resistant and kanamycin sensitive colonies were retained. Recombinants that grew only on streptomycin were sub-cultured onto the same medium a further three times to prevent any *E. coli* K12 S17 λ *pir* carry over and then tested genotypically and phenotypically to confirm inactivation of *fliC*.

4.1.3 Construction of a *eaeA*::cam^r/*fliC*::str^r double mutant by allelic exchange

A second round of allelic exchange was done to construct a double mutant defective for intimin and flagella genes. The methods followed those detailed in sections 4.1.1 & 4.1.2 (Table 4.1). A mutant defective for the elaboration of flagella and intimin was constructed by performing a conjugation between S17λ *pir* carrying the pGUS3 pERFORMK *fliC*::strep^r suicide vector construct and the intimin deficient mutant, DM3. (Table 4.2).

4.1.4 Genotypic characterisation of knockout intimin and aflagellate mutants

To confirm that the target gene of one representative putative *eae* and *fliC* mutant had been inactivated, comparisons were conducted between the parent progenitor isolate (NCTC 12900) and both putative mutants by Southern blot hybridisation. After the extraction of genomic DNA from each mutant and the parent progenitor isolate, the DNA was digested to completion by the appropriate restriction enzymes, visualised by gel electrophoresis and then transferred to nylon membranes for further analysis by three hybridisation experiments. Firstly, DNA hybridisation with the target gene was performed to detect the insertion of the antibiotic resistant cassette (Figure. 4.3). Secondly, DNA hybridisation with the antibiotic resistant cassette was performed to confirm its location and that only one copy resided within the chromosome. Thirdly, DNA hybridisation was performed with the relevant linearised suicide vector to confirm the absence of any suicide vector sequence.

Southern blot hybridisation patterns confirmed that the native intimin or flagella gene of each mutant had been changed by the insertion of a known antibiotic resistance cassette. Interestingly, an extra band was observed for digestion of *fliC* (DM4 and DM5) with *Cla*I either suggesting that another site may have been formed after insertion of the antibiotic resistance cassette and/or that the *Cla*I site in the *fliC* gene was only partially digested (Figure 4.3). However, and more importantly, no hybridisation was observed when the

genomic DNA of each mutant was probed with the appropriate pERFORM plasmid confirming the loss of the suicide vector.

PCR on the genomic DNA confirmed that target genes had been inactivated. Inserted antibiotic resistance cassette was acknowledged by the absence of the wild-type (NCTC 12900) band and the presence of a band equal in size to the wild-type band plus the antibiotic resistant cassette (Figure 4.4). Additionally both individual mutations in the double mutant gave the same identical PCR and Southern blot hybridisation patterns for each single mutant. Furthermore, preliminary sequencing of *eae* and *fliC* genes confirmed mutation insertion and the absence of any aberrant genetic reassortment.

4.1.5 Phenotypic characterisation of knockout intimin and aflagellate mutants

Functional intimin is required for the formation of attaching and effacing (AE) lesions. Therefore, mutants with inactivated intimin (DM3 and DM5) (Table 4.3) were tested by fluorescent actin staining (FAS) for their ability to form AE lesions on HEp-2 tissue culture mono-layers. Unlike the progenitor parent isolate (NCTC 12900), neither of these mutants defective for the elaboration of intimin was able to form AE lesions after 6 hours incubation with HEp-2 mono-layers (Figure 4.5).

Mutants with inactivated flagella (DM4 and DM5) (Table 4.6) were tested for the loss of motility by straight wire stab inoculation into semi-solid LB agar. Unlike the progenitor parent isolate (NCTC 12900), neither of these mutants defective for the elaboration of flagella was able to diffuse through the agar. Furthermore, flagella were not detected by transmission electron microscopy (TEM) for either *fliC* mutant (DM4 and DM5) (Figure 4.6).

An *in vitro* growth rate study revealed that all mutants and the progenitor parent isolate had similar rates of growth (Figure 4.7) and an acid resistance study revealed that each mutant was tolerant to acidified media (Table 4.4).

Table 4.1. Cloning and mutagenesis strategy.

Surface arrayed protein/ appendage	Target gene	Associated protein	Cosmid library* / primers used	Size of excised fragment/ amplicon	Cloned amplicon construct	Marker gene cassette & location of insertional inactivation	Cloned amplicon & antibiotic resistance marker	Suicide vector construct
Intimin	*eae*	Subunit gene	A84*	1558bp	pALC6	cam^r – *Eco*RV site at 672bp	pALC8	pALC10
Flagella	*fliC*	Subunit gene	fliCF & fliCR	1641bp	pGUS1	str^r – *Eco*47III site at 977bp	pGUS2	pGUS3

*Eco*RI/*Sal*I of 1558bp fragment from the conserved N-terminal region of *eae* was excised from an eae^+ clone of an *E. coli* O157:H7 A84 cosmid library made in *E. coli* XL1-Blue MR. Primer sequences and source are listed in table 2.3. Primers (fliCF & fliCR) were designed on the *E. coli* O157:H7 EH7 sequence (AF228488) to amplify a PCR product of 1641bp.

Table 4.2. Conjugations performed to construct single and double intimin and aflagellate mutants

Donor construct in S17*	Recipient strain	Antibiotic selection	Antibiotic counter selection	Mutant
pALC10 *eaeA*::cam^r in pERFORMT	Stx –ve *E. coli* O157:H7 NCTC 12900 (wild-type)	Chloramphenicol	Tetracycline	DM3 (intimin deficient)
pGUS3 *fliC*::str^r in pERFORMK	Stx –ve *E. coli* O157:H7 NCTC 12900 (wild-type)	Streptomycin	Kanamycin	DM4 (flagella deficient)
pGUS3 fliC::str^r in pERFORMK	DM3 (intimin deficient)	Chloramphenicol & Streptomycin	Kanamycin	DM5 (intimin & flagella deficient)

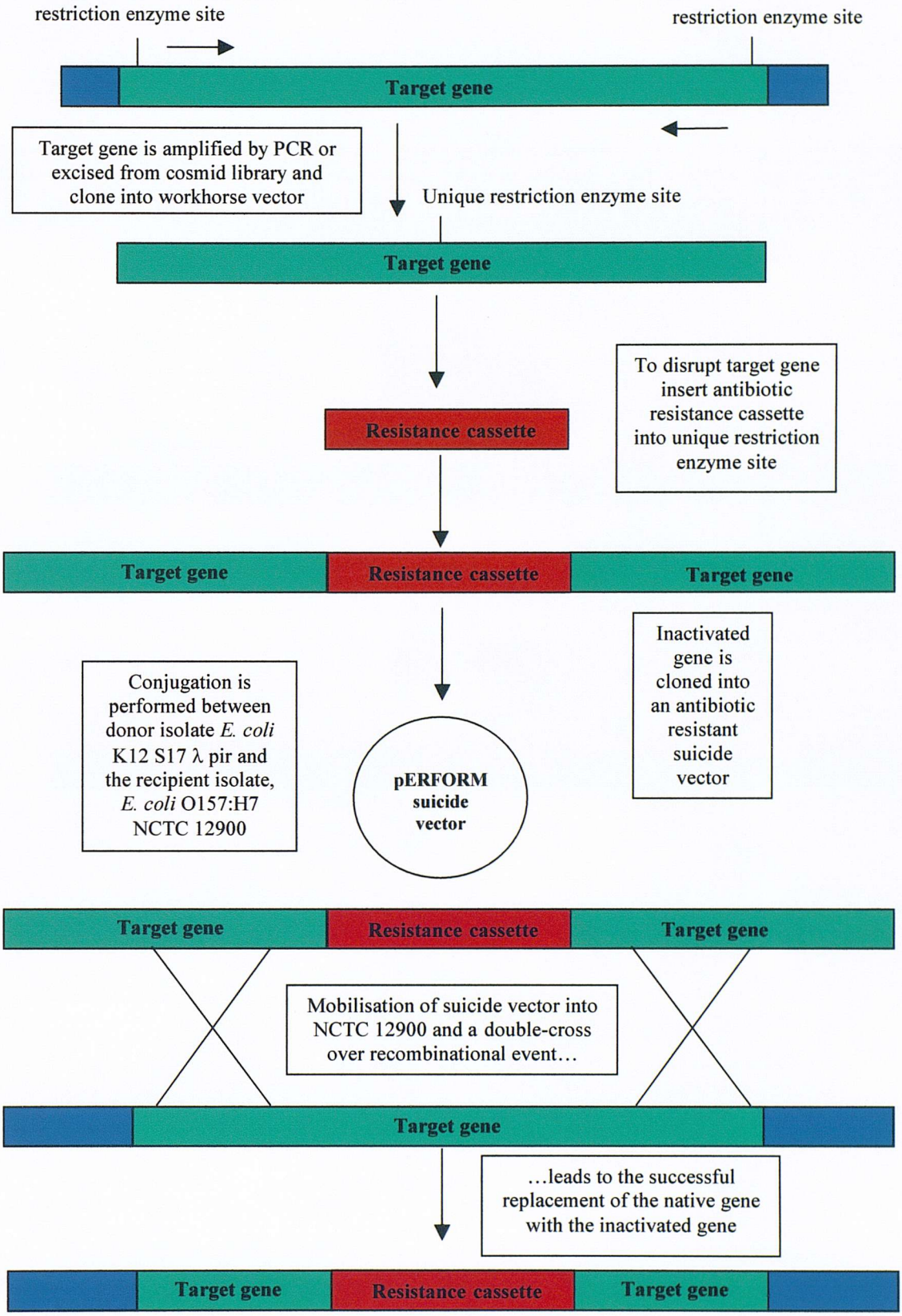
* *E. coli* K12 S17 *λ pir*

Table 4.3. *Escherichia coli* O157:H7 NCTC 12900 intimin and flagella mutant library

Identity	Genotype	Antibiotic Resistance	Phenotype*
DM3	*eaeA*::cam^r	chloroamphenicol @ 10μg/ml	AE $lesion^-$
DM4	*fliC*::str^r	streptomycin @ 25μg/ml	Non-motile
DM5	*eaeA*::cam^r & *fliC*::str^r	chloroamphenicol @ 10μg/ml streptomycin @ 25μg/ml	AE $lesion^-$ & Non-motile

eaeA; intimin, *fliC*; flagella. * AE $lesion^-$; No attaching and effacing (AE) lesion formation as demonstrated by fluorescent actin staining (FAS) and transmission electron microscopy (TEM). Motility; tested by straight wire stab into semi-solid media.

Figure 4.1. Basic diagram illustrating the strategy of mutagenesis



Figures 4.2a & b. Diagrams illustrating antibiotic resistant cassette insertion within target genes. Also indicated are the approximate positions of each restriction enzyme site used to map each mutation by Southern blot hybridisation (Figure 4.3). Green regions represent target genes, blue regions represent flanking gene sequences and the red regions represent the antibiotic resistant cassettes. Arrows indicate the direction of gene transcription.

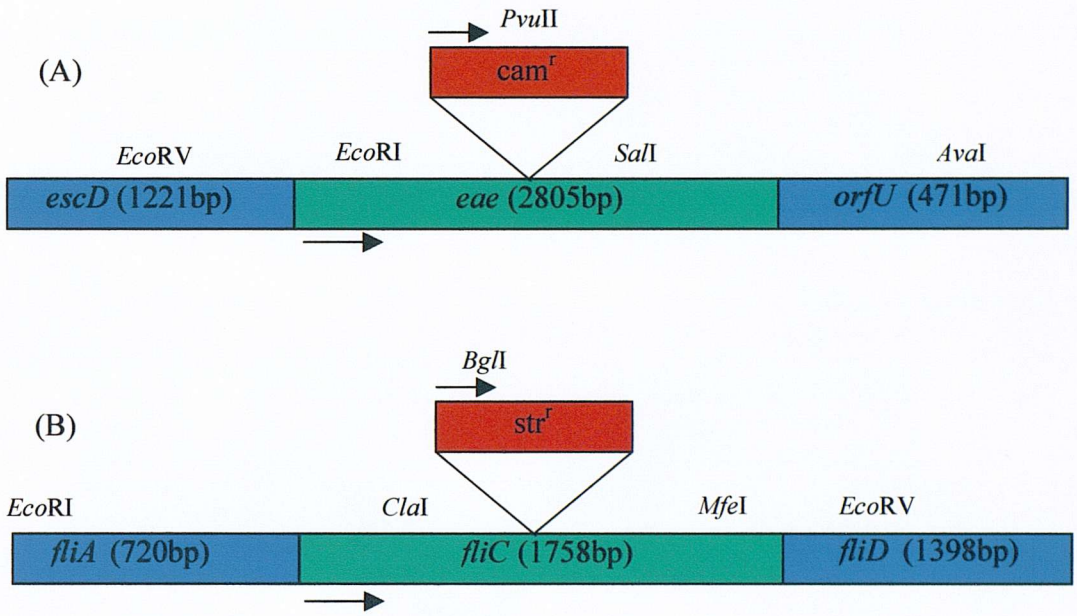
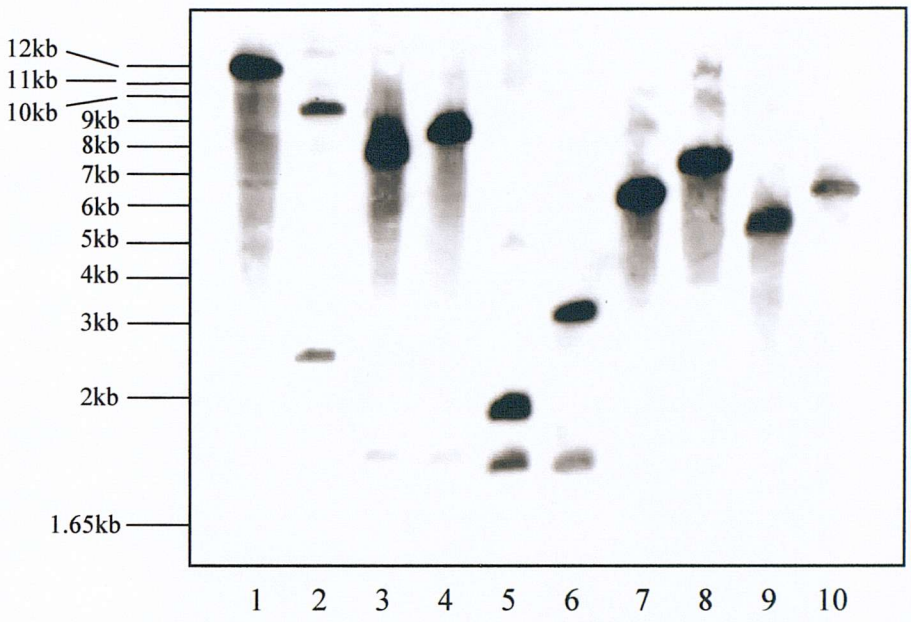
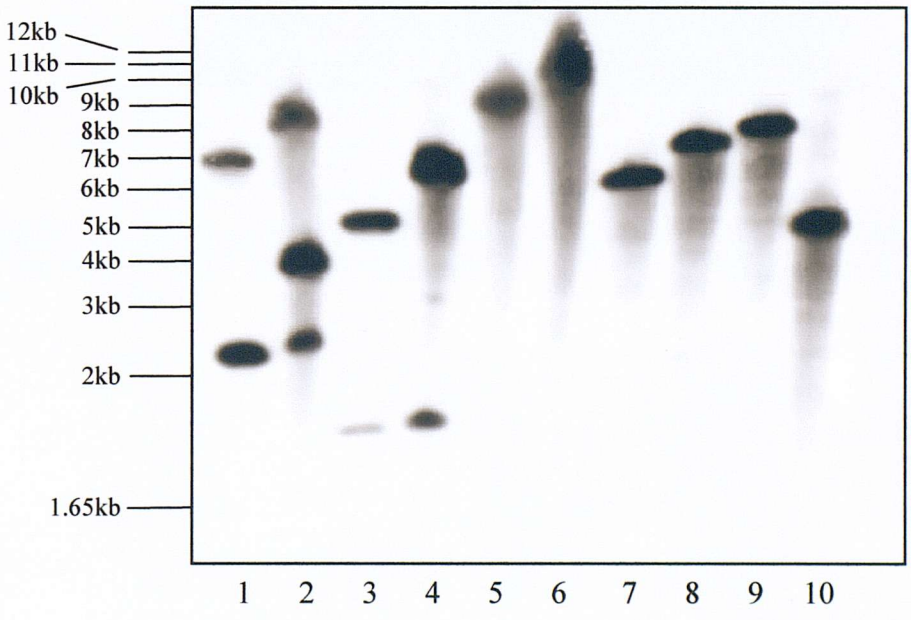


Figure 4.3. Southern blot hybridisation of Stx-negative *E. coli* O157:H7 NCTC 12900 and mutant derivatives to demonstrate insertional inactivation of target intimin and flagella genes.

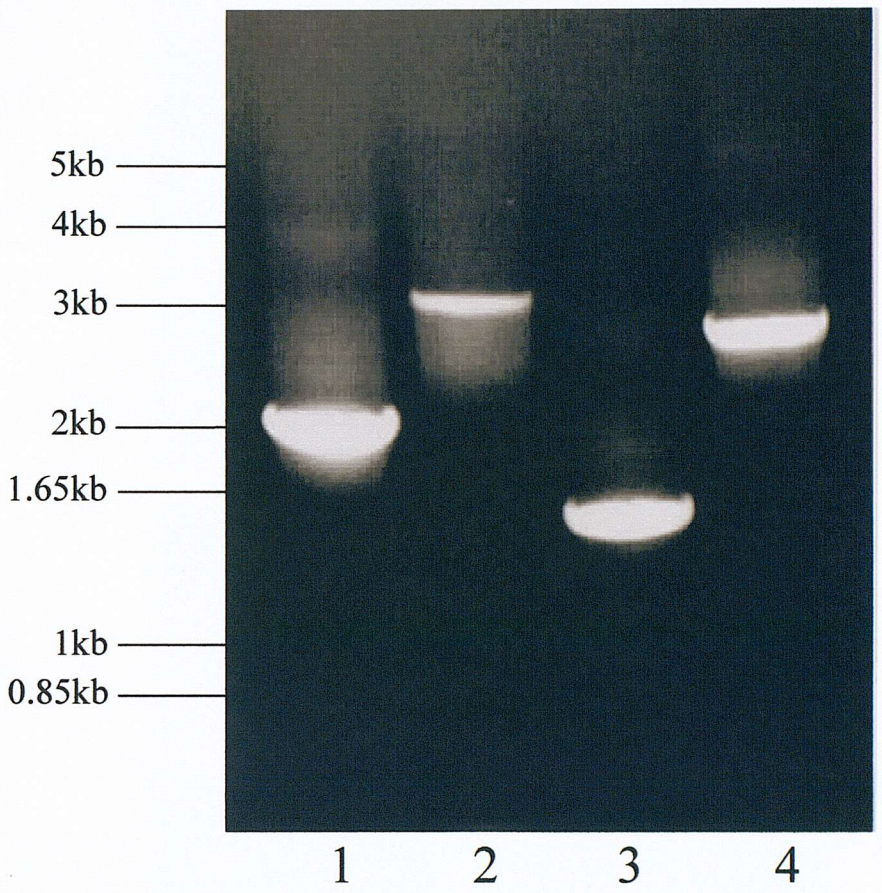


Lanes 1, 3, 5, 7 & 9 = Wild-type (NCTC 12900) and lanes 2, 4, 6, 8 & 10 = DM3 (*eaeA*::cam^r) digested with *Pvu*II (lanes 1 & 2), *Eco*RI (lanes 3 & 4), *Sal*I (lanes 5 & 6), *Eco*RV (lanes 7 & 8) and *Ava*I (lanes 9 & 10). DM5 (*eae*::cam^r, *fliC*::str^r) gave the same pattern as above.



Lanes 1, 3, 5, 7 & 9 = Wild-type (NCTC 12900) and lanes 2, 4, 6, 8 & 10 = DM4 (*fliC*::cam^r) digested with *Cla*I (lanes 1 & 2), *Mfe*I (lanes 3 & 4), *Eco*RI (lanes 5 & 6), *Eco*RV (lanes 7 & 8) and *Bgl*I (lanes 9 & 10). DM5 (*eae*::cam^r, *fliC*::str^r) gave the same pattern as above.

Figure 4.4. PCR of the parent progenitor isolate Stx-negative *E. coli* O157:H7 NCTC 12900 and intimin and aflagellate mutants.



Primers eaeF and eaeR were used to amplify NCTC 12900 (lane 1) and DM3 (*eae*::cam^r) (lane 2). Primers fliCF and fliCR were used to amplify NCTC 12900 (lane 4) and DM4 (*fliC*::str^r). DM5 (*eae*::cam^r, *fliC*::str^r) gave the same pattern as above.

Figure 4.5. The formation of AE lesions by (A) DM4 (aflagellate mutant) but not by (B) DM3 (intimin mutant) and (C) DM5 (intimin-aflagellate mutant) on HEp-2 mono-layers as demonstrated by fluorescence actin staining (FAS). Magnification x 1000.

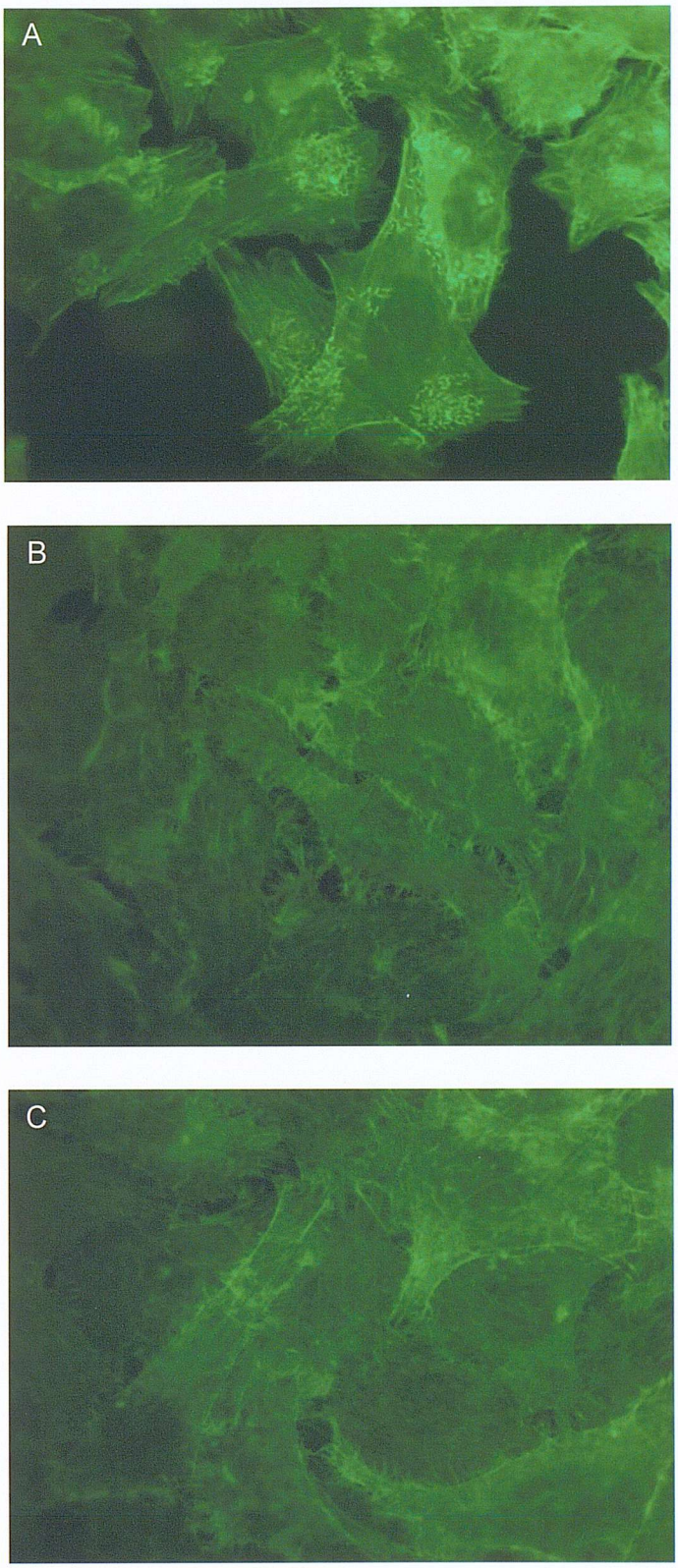


Figure 4.6. Flagella expression by (a) DM3 (intimin mutant) but not by (b) DM4 (aflagellate mutant) and (c) DM5 (intimin-aflagellate mutant) as observed by transmission electron microscopy. Magnification x 22,250.

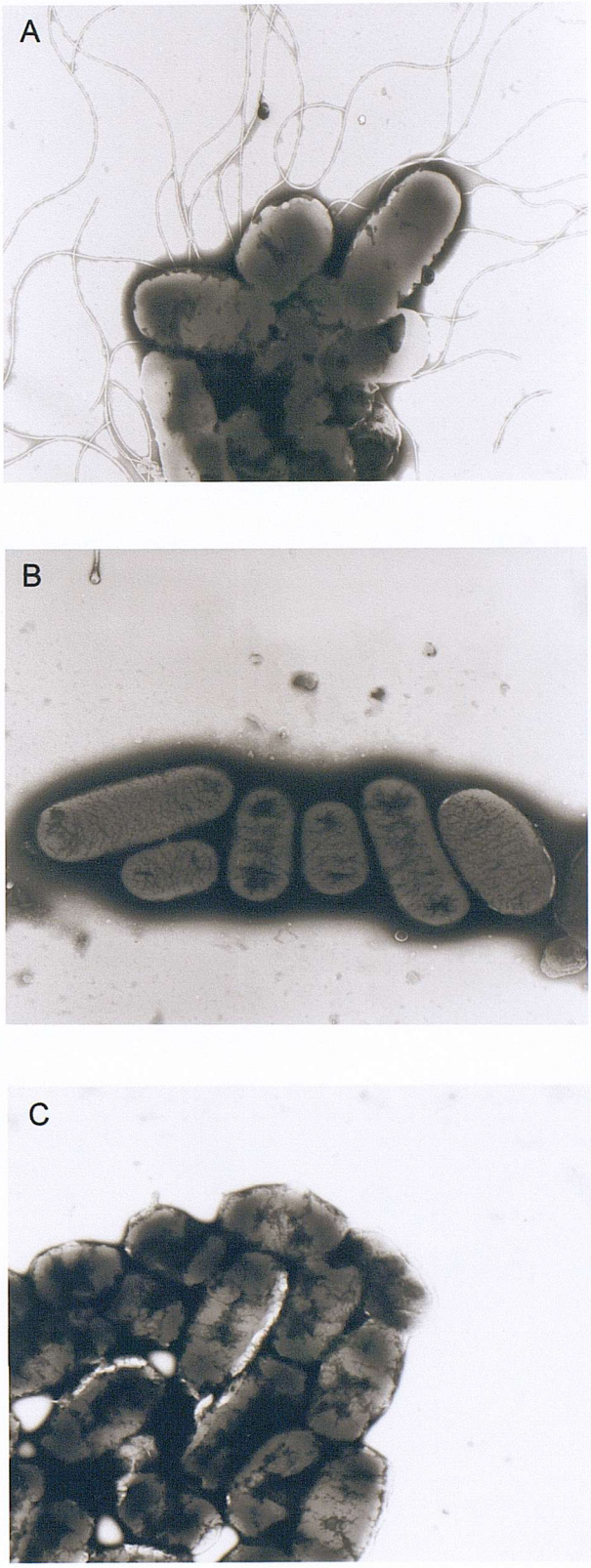
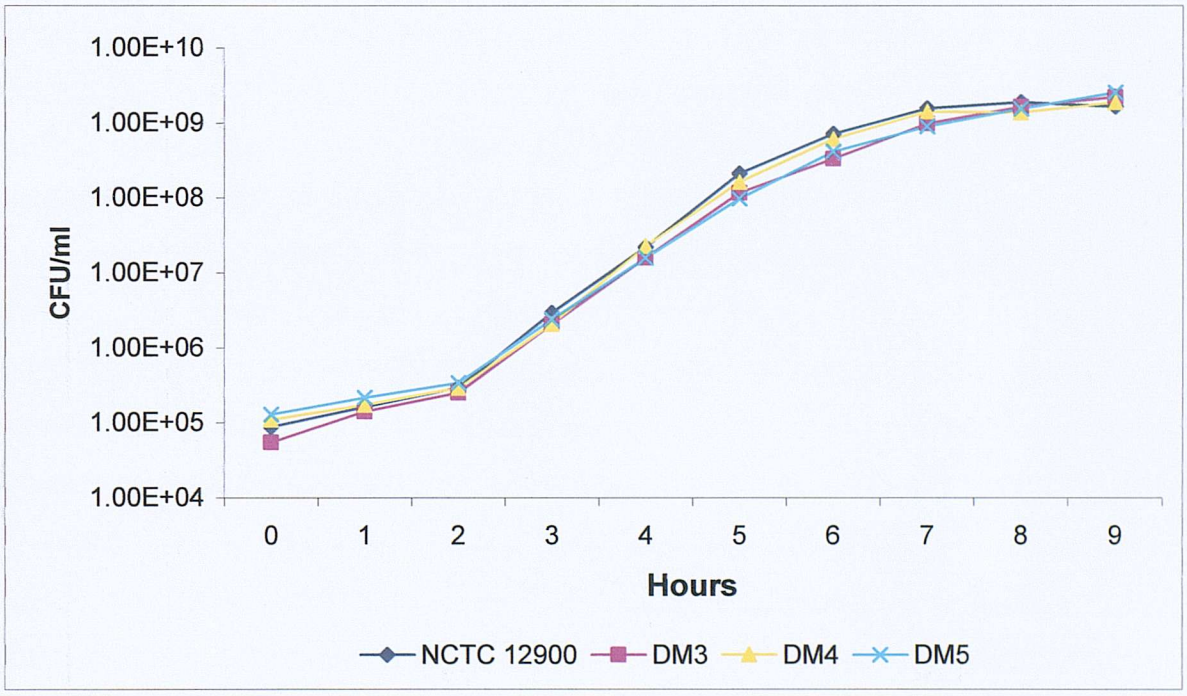


Table 4.4. Intimin and aflagellate mutants in the presence of inorganic acid (HCl, pH 2.5) for 2.5 hours at 37°C with shaking.

E. coli O157 isolate	Resistance to acid		
	CFU/ml @ time = 0	CFU/ml in LB broth @ time = 2.5 hours	CFU/ml in LB broth pH2.5 @ time = 2.5 hours
NCTC 12900	6.58E+04	1.77E+08	1.69E+06
DM3	3.33E+04	7.77E+07	5.33E+05
DM4	5.23E+04	1.45E+08	7.75E+05
DM5	7.48E+04	1.40E+08	6.81E+05

DM3 (intimin mutant); DM4 (aflagellate mutant); DM5 (intimin-aflagellate mutant).

Figure 4.7. Growth of Stx-negative *E. coli* O157:H7 NCTC 12900 and intimin and aflagellate mutants, when cultured in LB broth at 37°C with shaking.



4.2. Discussion

Three mutants defective for intimin, flagella and both intimin and flagella were constructed in Stx-negative *E. coli* O157:H7 isolate NCTC 12900 by allelic exchange. Convincing genetic evidence was gained that the three mutants possessed an antibiotic resistance cassette in the desired target gene. The mutants had acquired the anticipated phenotypic changes, although whether other unconcealed phenotypic changes existed resulting from the mutagenesis strategy was not sought after. There was no evidence of any major genetic rearrangement of the target loci or the existence of residual suicide vector as detected by Southern blot hybridisation. However, it is possible that genetic rearrangement out with the target loci had been altered, although unlikely.

It was noted that the *eae* and *fliC* mutations constructed in the Stx-negative *E. coli* O157:H7 isolate NCTC 12900 did not appear to affect the growth rates or their ability to tolerate acid suggesting that the burden of one or two antibiotic resistance cassettes had little obvious affect upon the relative fitness of each mutant, at least *in vitro* in the experiments undertaken. Subtle phenotypic changes may have occurred, but whether they had done so remained unclear and undetected. It was possible that any attenuation seen in subsequent analysis may be as a result of not only the targeted mutation but of other subtle effects also.

The successful use of antibiotic resistance cassettes not only served as tools of inactivation, but they also served as tools for positive selection of allelic exchange, hence why this mutagenesis strategy was used. However, several authors (Chambers & Neu, 1995; Gilbert, 1995; Mayer *et al.*, 1995; Standiford, 1995; Zinner & Mayer, 1995) have given evidence suggesting that the constitutive expression of antibiotic resistance cassettes occurs when under control of their own promoters. However, it was possible that the expression of a foreign gene may have affected each mutant. Insertion of an antibiotic resistance cassette into the flagella operon may have created polar mutations that modified gene expression downstream of the antibiotic resistance cassette promoter. Nevertheless, if more than one gene had been disrupted in the flagella operon, this was possibly of little consequence as the intention was to create an aflagellate mutant. However, it was equally possible that any transcriptional read through from the antibiotic resistance cassette may have altered any regulatory feedback mechanism within the flagella operon and even other adjacent genes and operons.

Intimin resides within the Tir operon in the locus of enterocyte effacement (LEE) (Elliott *et al.*, 2000), but unlike flagella, the complete form of intimin only involves the expression of one gene, *eae*. If a polar mutation or disruption of more than one gene other than intimin had occurred due to the insertion of an antibiotic resistance cassette this might have had additional effects on the expression of other important virulence genes within this operon.

However, the promoters of antibiotic resistance cassettes inserted into intimin and aflagellate mutants may have altered the expression of genes outside the open reading frame of interest leading to misregulation of genes within the same operon or elsewhere on the genome (Ciampi *et al.*, 1982; Kendrick & Reznikoff, 1988; Wang & Roth, 1988). It must also be noted that antibiotic resistance cassette insertion in a target gene may create conformational changes in its DNA topology, therefore, altering gene regulation (Afflerbach *et al.*, 1999).

Other genetic manipulation techniques could have been utilised to construct intimin and flagella defective mutants. For instance, the construction of deletion mutants (Marc *et al.*, 1998) would have eliminated any problem of antibiotic resistance cassettes possibly altering the mutant phenotype in addition to the desired intimin and flagella mutations. Unfortunately, inactivation of a whole gene or operon without the inclusion of a selectable marker in the definitive double cross-over mutant would have made mutant differentiation, mutant selection, double mutant construction and *in vivo* work more difficult.

In these studies it was assumed that each mutant displayed a phenotype, which related only to the inactivated mutant allele. However, whilst constructing mutants, possible secondary mutations may have occurred within the targeted operon although no unequivocal evidence was gained for this. It also follows that antibiotic resistance cassette insertion into intimin and flagella genes may have given rise to additional genotypic and phenotypic changes and although these changes were not detected in the laboratory, they may have affected the performance of each mutant in the *in vitro* and *in vivo* environment.

The construction of a panel of defined, antibiotic marked, inactivated single and double intimin and aflagellate mutants were an essential requirement for the work to be reported in this thesis. The use of these mutants would determine what the single and combined roles of intimin and flagella were in the colonisation and persistence of Stx-negative *E. coli* O157:H7 NCTC 12900, as assessed by *in vitro* tissue culture assays and *in vivo* chick models.

Chapter 5

In vitro tissue culture adhesion and invasion studies

Surface arrayed structures such as integral membrane proteins can mediate the adhesion of many bacterial pathogens including enteropathogenic and enterhaemorrhagic *E. coli* (EPEC and EHEC) to host surfaces (Nataro & Kaper 1998). The aim of the study to be reported in this chapter was to test the adherence and invasion of Stx-negative *E. coli* O157:H7 intimin and aflagellate mutants using the avian Div-1 and human HEp-2 cell lines utilised in chapter 3.

The avian (Div-1) cell line was derived from White Leghorn (PA12) embryonated eggs, where all the small intestine walls from 18 day-old embryos were used to establish this avian cell line (Velge *et al.*, 2002). The epithelial-like HEp-2 (Human Epidermoid Larynx Carcinoma) cell line was created from the induced tumours of weanling rats that were injected with the epidermoid carcinoma tissue from the larynx of a 56-year-old Caucasian male (Toolan, 1954; Moore *et al.*, 1955). Originally intended and used to culture viral isolates from patient specimens, HEp-2 cells have also been used routinely to characterise the adherence of many bacterial pathogens including *E. coli* O157:H7 (Knutton *et al.*, 1989; McKee & O'Brien, 1995; Dibb-Fuller *et al.*, 2001).

As discussed in previous chapters, *E. coli* O157:H7 utilises the integral outer membrane protein intimin for intimate attachment to HEp-2 cell monolayers (McKee & O'Brien, 1996; Gansheroff *et al.*, 1999). Few studies aimed at determining the role of the H7 flagella antigen in adherence have been reported, but Sherman and Soni, (1988) did report that flagella were not required for *E. coli* O157:H7 attachment. The role of *E. coli* O157:H7 flagella in carrier hosts remains unclear. However, naturally occurring H- *E. coli* O157 have been associated with disease in humans and, therefore, it was considered that the role of flagella might be minimal or even not relevant in pathogenesis.

The Stx-negative *E. coli* O157:H7 isolate NCTC 12900 forms micro-colonies and AE lesions on avian Div-1 and HEp-2 mono-layers (Dibb-fuller *et al.*, 2001 & Chapter 3) and Cookson & Woodward (2003) did demonstrate that an intimin-deficient *E. coli* O157:H7 mutant was unable to exhibit these phenotypes on HEp-2 cells. In poultry, it is not yet understood whether *E. coli* O157:H7 cause micro-colonies and AE lesions. Therefore, it was important in this part of the study to determine whether intimin or flagella would contribute to micro-colony and AE lesion formation on an avian cell line by using the intimin and aflagellate mutants constructed in chapter 4.

5.1 Results

5.1.1 Adhesion and invasion of intimin and aflagellate mutants to Div-1 and HEp-2 tissue culture mono-layers

To determine the numbers of adhering and invading intimin and aflagellate mutants (DM3, DM4 and DM5) avian Div-1 and HEp-2 mono-layers were utilized and the results of adherence and invasion assays are shown in Tables 5.1a and b. Adherence of the aflagellate mutant (DM4) to Div-1 cells was similar to that of the NCTC 12900. However, adherence of the intimin mutant (DM3) and the double intimin-aflagellate mutant (DM5) was significantly ($p<0.001$) less than NCTC 12900 and DM4. The extent of invasion of DM4 and DM5 was significantly ($p<0.05$) less than NCTC 12900 and DM3. Numbers of internalised DM3 were similar to that of NCTC 12900.

Numbers of adhering DM3, DM4 and DM5 to HEp-2 mono-layers were significantly ($p<0.05$) less than NCTC 12900. The extent of invasion of DM3 was significantly ($p<0.001$) less than NCTC 12900. The numbers of internalised DM4 were similar to that of NCTC 12900, but were significantly ($p<0.001$) more than DM3. No internalised DM5 bacteria were recovered.

No significant differences between Div-1 and HEp-2 cell mono-layers were noted for NCTC 12900, DM3 and DM4 adhesion (Table 5.1c). However, the numbers of adhering DM5 were significantly ($p<0.05$) less for Div-1 cells. Furthermore, significantly ($p<0.05$) more NCTC 12900, DM3, DM4 and DM5 were internalised in Div-1 cells as compared to HEp-2 cells.

5.1.2 Visualisation of the adherence patterns of intimin and aflagellate mutants to Div-1 and HEp-2 tissue culture mono-layers

Adherent bacteria were observed by light microscopy after Giemsa staining and by scanning electron microscopy (SEM). Like the progenitor parent strain, DM4 (aflagellate mutant) generated localised adherence patterns on Div-1 and HEp-2 mono-layers and the characteristic micro-colonies of densely packed cells (Table 5.2; Figure 5.1). However, diffuse adherence patterns were observed for DM3 (intimin mutant) and DM5 (intimin-aflagellate) on Div-1 and HEp-2 cell mono-layers.

Actin rearrangements within avian Div-1 and HEp-2 mono-layers as observed by fluorescent actin staining (FAS), were shown for DM4. However, no characteristic AEEC-FAS reaction was observed for DM3 or DM5 (Table 5.2; Figure 5.2). Transmission electron microscopy (TEM) revealed that DM4 induced the formation of the "pedestal cup" structure on Div-1 and HEp-2 mono-layers typical of AE lesions (Table 5.2; Figure 5.3). No "pedestal cup" structures were observed for DM3 or DM5.

Tables 5.1a, b & c. Adhesion (a) and invasion (b) of avian Div-1 and human HEp-2 cell lines by intimin and aflagellate mutants (DM3, DM4 & DM5) 12900 and (c) a statistical comparison between cell lines.

(a) Adhesion

Bacteria	Div-1				HEp-2			
	Mean log_{10}	Geometric mean	SD	p-value	Mean log_{10}	Geometric mean	SD	p-value
NCTC 12900	6.76	5.75E+06	0.11	-	6.93	8.51E+06	0.22	-
DM3	5.79	6.16E+05	0.24	<0.001	6.11	1.29E+06	0.33	<0.001
DM4	6.70	5.01E+06	0.24	0.710	6.69	4.90E+06	0.07	0.053
DM5	5.70	5.01E+05	0.35	<0.001	6.50	3.16E+06	0.24	0.002

(b) Invasion

Bacteria	Div-1				HEp-2			
	Mean log_{10}	Geometric mean	SD	p value	Mean log_{10}	Geometric mean	SD	p-value
NCTC 12900	4.22	1.66E+04	0.08	-	3.31	2.04E+03	0.25	-
DM3	4.20	1.58E+04	0.07	0.93	2.60	3.98E+02	0.05	<0.001
DM4	3.93	8.51E+03	0.32	0.23	3.19	1.55E+03	0.22	0.286
DM5	3.27	1.86E+03	0.08	<0.001	0	0	0	0

DM3, intimin mutant; DM4, aflagellate mutant; DM5, intimin-aflagellate mutant; SD, standard deviation; p–value; significance in adhesion and invasion between each isolate and NCTC 12900.
Units for geometric means are CFU/ml.

c) Significance (p-value) between cell lines for enteric bacterial adhesion and invasion.

Bacteria	Adhesion	Invasion
	p-value of Div-1 versus HEp-2	p-value of Div-1 versus HEp-2
NCTC 12900	0.183	0.001
DM3	0.124	<0.001
DM4	0.965	0.004
DM5	0.004	0.008

All counts were transformed to the logarithms to the base 10 of (count+1). Comparisons between the NCTC 12900 and each isolate by student t-tests followed analysis of variance. Comparisons between Div-1 and HEp-2 mono-layers for the means of the same isolate were made using student t-tests with separate variance estimates. Where counts were zero for one cell type the non-parametric Wilcoxon-Mann-Whitney was used in place of the student t-test.

Table 5.2. Summary of patterns of interaction by intimin and aflagellate mutants (DM3, DM4 & DM5) and the progenitor parent isolate Stx-negative *E. coli* O157:H7 NCTC 12900 with avian Div-1 cells and human HEp-2 cells as demonstrated by Giemsa staining, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and fluorescent actin staining (FAS).

Cell type & Test	**Bacterial strain**			
	NCTC 12900	DM3	DM4	DM5
Div-1				
Giemsa/SEM	LA*	DA	LA*	DA
TEM	PF	-	PF	-
FAS	+++	-	+++	-
HEp-2				
Giemsa/SEM	LA*	DA	LA*	DA
TEM	PF	-	PF	-
FAS	+++	-	+++	-

DM3, intimin mutant; DM4, aflagellate mutant; DM5, intimin-aflagellate mutant.
LA, localised adherence; DA, diffuse adherence; * formation of densely packed micro-colonies.
PF, pedestal formation; -, no pedastals formed.
+++, high numbers of FAS lesions formed; ++, medium numbers of FAS lesions formed; +, low numbers of FAS lesions formed; -, no FAS lesions formed.

Figure 5.1. Scanning electron micrographs demonstrating the adherence patterns of (A) DM4 (aflagellate mutant) (B) DM3 (intimin mutant) and (C) DM5 (intimin-aflagellate) with avian Div-1 cell mono-layers. Magnification x 1750.

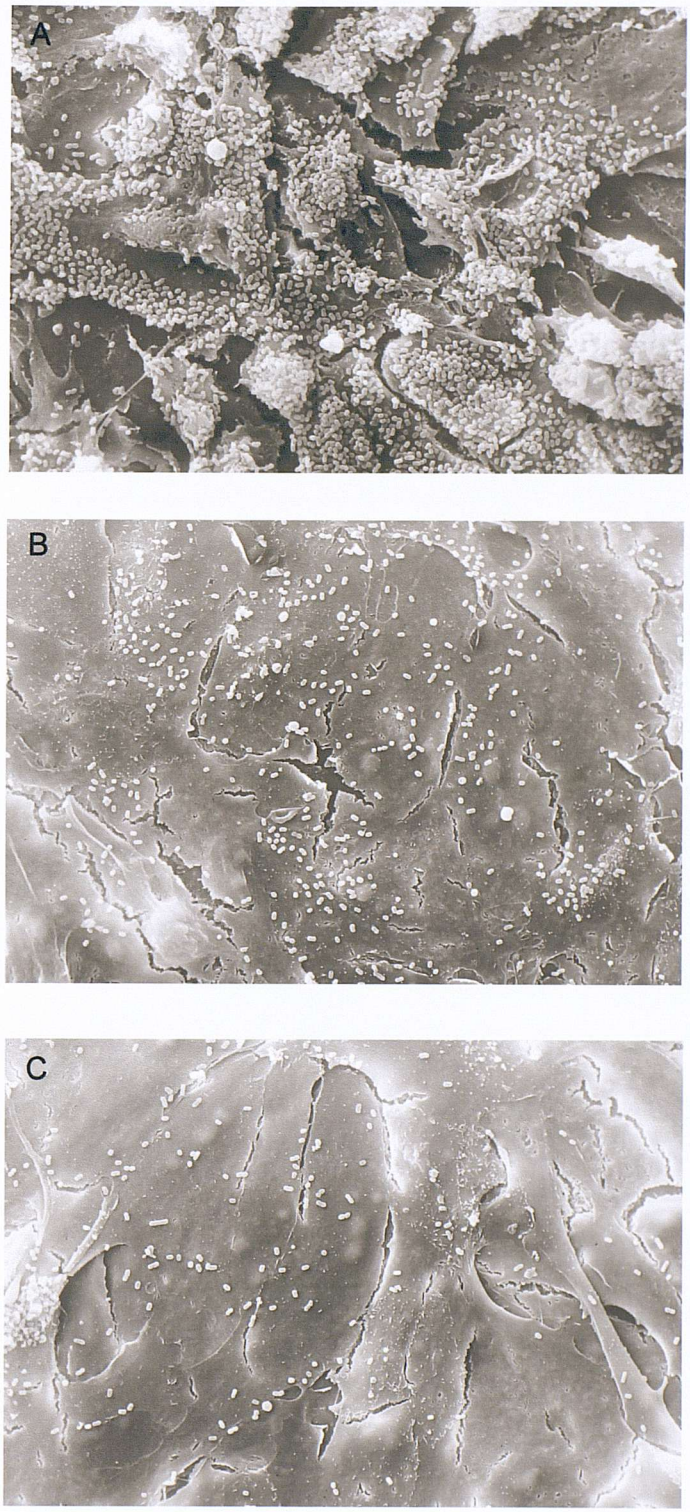


Figure 5.2. The formation of AE lesions by (A) DM4 (aflagellate mutant) but not by (B) DM3 (intimin mutant) and (C) DM5 (intimin-aflagellate mutant) on Div-1 cell mono-layers as demonstrated by fluorescence actin staining (FAS). Magnification x 1000.

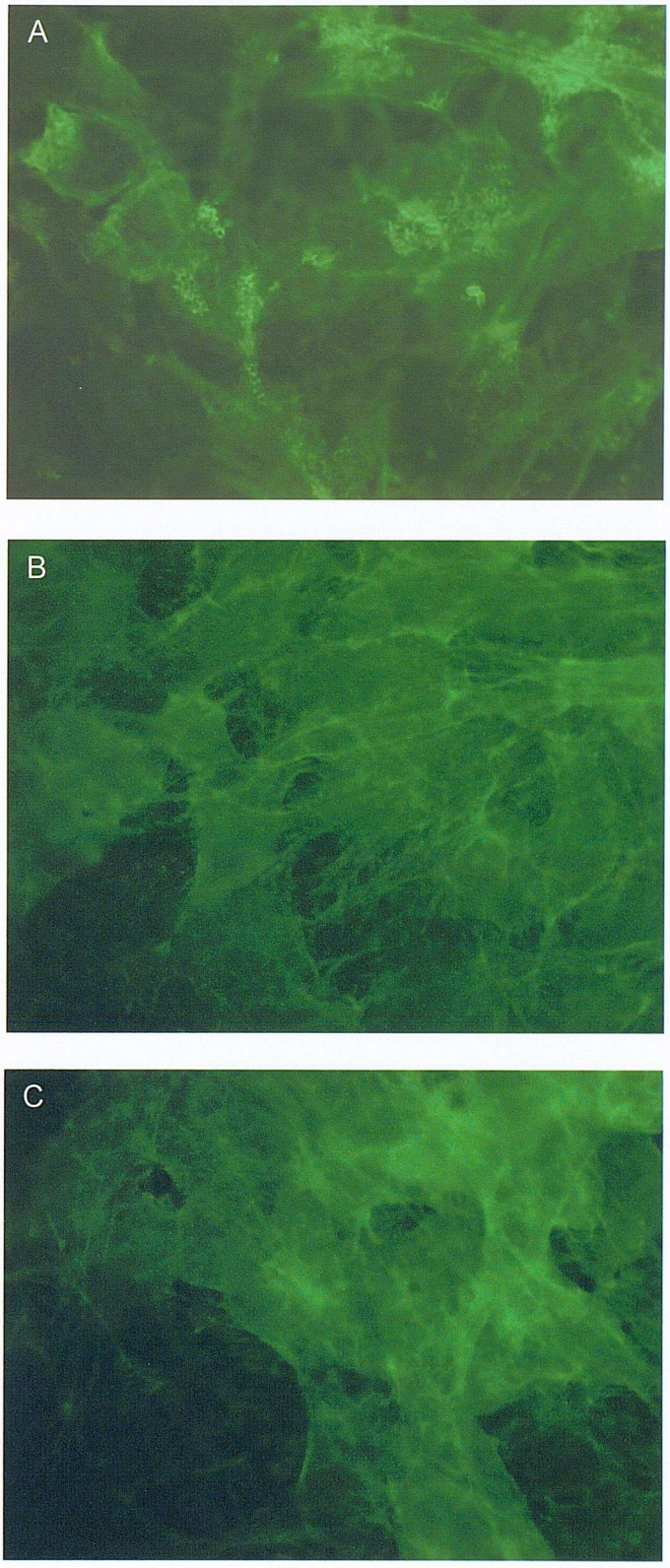
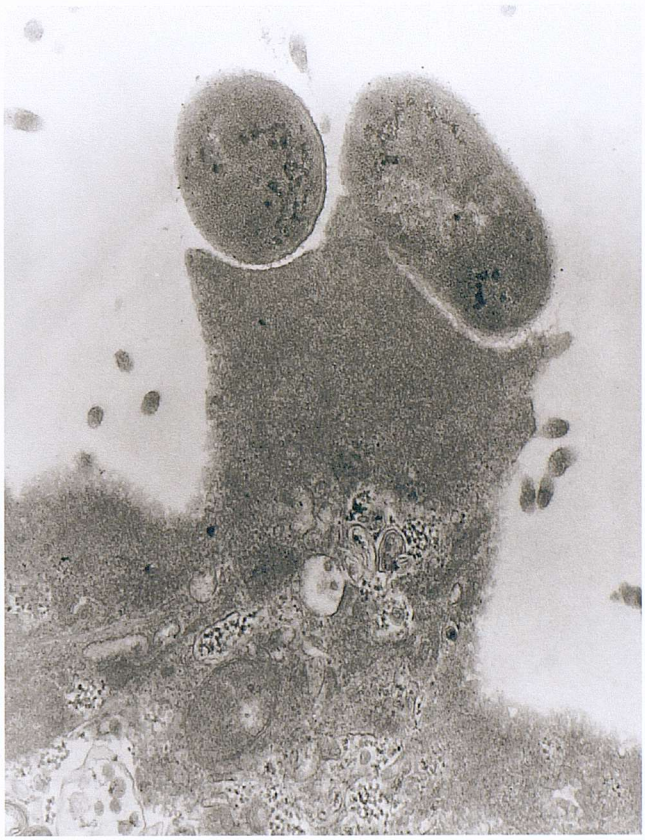


Figure 5.3. Transmission electron micrograph showing the formation of "pedestal cup structures" (AE lesions) by DM4 (aflagellate mutant) on avian Div-1 cells. Magnification x 38,750.



5.2 Discussion

Characterisation of Stx-negative *E. coli* O157:H7 mutants deficient for the elaboration of intimin and flagella was initially carried out on tissue culture mono-layers to determine if the mutants behaved differently from the wild-type strain. An avian Div-1 cell line was employed to characterise any differences since intimin and aflagellate mutants would subsequently be characterised in the SPF chick model. However, it should be noted that great care must be taken when interpreting the results from tissue culture assays, since many of the physical barriers operating in the *in vivo* environment such as mucus, peristalsis, nutrient limitation, anaerbiosis are not present. Furthermore, a study by James and Keevil, (1999) did demonstrate that *E. coli* O157:H7 grown in oxygen-limited and anaerobic conditions were significantly more adhesive to tissue culture mono-layers (HEp-2) than O157 cells grown aerobically. Nevertheless, human HEp-2 cell mono-layers have been used to study the adherence patterns of Stx-positive *E. coli* O157:H7 grown under aerobic conditions (Knutton *et al.*, 1989; McKee *et al.*, 1995; McKee & O'Brien, 1996) and Stx-negative *E. coli* O157:H7 NCTC 12900 adherence has been reported (Dibb-Fuller *et al.*, 2001). The purpose of using the avian cell line in this study was to give insights into how *E. coli* O157:H7 might interact with the chick gut.

E. coli O157:H7 intimate adherence to tissue culture cell lines, including HEp-2 cell mono-layers, has demonstrated that intimin is essential for the formation of AE lesions. The intimin mutant reported in this study had reduced levels of adherence for both types of cell line compared to wild-type NCTC 12900 and no micro-colony formation or localised adherence patterns was observed, suggesting that intimin does play a role in adherence and micro-colony formation. However, since the intimin mutant was recovered in adherence assays this implies that other attachment factors expressed in *E. coli* O157:H7 are possibly involved in adherence. Genome sequencing of two separate Stx-positive *E. coli* O157:H7 strains has identified the presence of putative adhesins including five other fimbrial operons (Hayashi *et al.*, 2001; Perna *et al.*, 2001). A recent study by Torres *et al.* (2002) has confirmed the expression of one such *E. coli* O157:H7 operon resulting in LP (long polar) fimbriae. An unidentified surface appendage elaborated by NCTC 12900 was incidentally found in the study reported in chapter 3. Whether this was LP fimbriae remains to be tested.

Flagella have been shown to be important in the adherence of other *E. coli* pathogens such as enteropathogenic *E. coli* (EPEC) isolates (Giron *et al.*, 2002) but for NCTC 12900 on avian Div-1 and human HEp-2 mono-layers, flagella appeared to play little or no role in adherence. Flagella seemed not to play a significant role in micro-colony or AE lesion formation, because apart from active flagella the phenotype of the aflagellate mutant was the same as wild-type NCTC 12900 in these assays. On the other hand, intimin when knocked

out by mutagenesis failed to induce all of these phenotypes. Whether flagella play any role in adhesion remains unclear. Clearly they do not assist in AE and micro-colony formation, but they may play a minor role in initial adherence. Possibly, a decrease in assay time may have teased out any subtle differences between mutant and wild-type.

Flagella have been implicated in playing a role in the invasion of the intestinal mucus by various bacterial pathogens including *E. coli* pathogens (Freter, 1980; Smith, 1988; La Ragione *et al.*, 2000a). However, *E. coli* O157:H7 variants have been described including non-motile strains that have been associated with disease (Bielaszewska *et al.*, 1998; Bielaszewska *et al.*, 2000). In the study reported here, the numbers of aflagellate mutant recovered from Div-1 invasion assays was 10-fold less than wild-type NCTC 12900 implying that flagella was required for the levels of invasion that were demonstrated by the wild-type. However, the numbers of aflagellate mutant recovered from HEp-2 cell mono-layers was comparable with that of the wild-type. Interestingly, an intimin-aflagellate mutant was not internalised in HEp-2 mono-layers. Although the purpose of the study here was to characterise the interaction of NCTC 12900 flagella with an avian cell line derived from SPF chicks, in retrospect it may have also been appropriate to compare and contrasted aflagellate mutants using the mucus secreting cell line, HT2916E (Lesuffleur *et al.*, 1995; La Ragione *et al.*, 2000a) since the flagella of other bacterial pathogens has been implicated in the penetration of mucus layers.

Previous studies have demonstrated that *E. coli* O157:H7 are able to invade tissue culture mono-layers. (Oelschlaeger *et al.*, 1994; Matthews *et al.*, 1997; Dibb-Fuller *et al.*, 2001). One study revealed Stx-positive *E. coli* O157:H7 had decreased adherence to HEp-2 cell mono-layers when intimin was incapacitated by antibody recognition of the C-terminal region (Gansheroff *et al.*, 1999). In the study to be reported here, an intimin deficient mutant had reduced levels of invasion with HEp-2 mono-layers, but not with the Div-1 cell mono-layers suggesting that the mechanism for avian epithelial cell invasion may be somehow complemented by the expression of another virulence factor. However, significant differences between Div-1 and Hep-2 cells were noted for the internalisation of each isolate indicating that either avian cell processes played an active role in uptake or that there was greater expression of a cell receptor recognising a yet undefined surface structure.

Chapter 6

Colonisation, invasion and persistence of Stx-negative *E. coli* O157:H7 NCTC 12900 intimin and aflagellate mutants in the SPF chick model

Evidence was presented in chapter 3 that Stx-negative *E. coli* O157:H7 isolate NCTC 12900 was able to colonise and invade experimentally infected 1-day-old SPF chicks and that shedding of NCTC 12900 occurred for up to 148 days post inoculation. In chapter 5, tissue culture adherence assays indicated that intimin, but not flagella, played a role in the intimate attachment and micro-colony formation of NCTC 12900 to an avian Div-1 cell line. However, tissue culture assays also indicated that the absence of flagella, but not intimin, was associated with reduced invasion of avian Div-1 mono-layers.

Previously reported *in vivo* studies demonstrated that intimin is required for intimate attachment to the gastrointestinal tract of newborn piglets, sheep and cattle (Donnenberg *et al.*, 1993; McKee *et al.*, 1995; Dean-Nystrom *et al.*, 1998; Wales *et al.*, 2001). Colonisation and persistence studies involving *E. coli* O157:H7 intimin-deficient mutants in experimentally infected ruminants have been reported also. (Cornick *et al.*, 2002; Woodward *et al.*, 2003). These studies demonstrated that intimin-deficient mutants were shed in less numbers and for a shorter duration than their respective parent wild-types. To date no other surface arrayed structure, including flagella, has been implicated in the persistence of *E. coli* O157:H7 in ruminants. Although *E. coli* O157:H7 colonisation and persistence studies of experimentally infected chicks have been reported (Beery *et al.*, 1985; Schoeni & Doyle, 1994), detailed analysis of the role of intimin or flagella in the infection of an avian host has yet to be described.

Therefore, the next step in this study was to determine whether the *E. coli* O157:H7 surface antigens intimin and flagella played a role in the colonisation, invasion and persistence of experimentally infected chickens. Intimin and aflagellate mutants and the wild-type Stx-negative parent isolate NCTC 12900 were given orally to 1-day-old SPF chicks.

6.1 Results

6.1.1 Colonisation, invasion and persistence of SPF chicks by intimin and aflagellate mutants

As described in chapter 2, the 1-day-old SPF chick model was employed to study the role of Stx-negative *E. coli* O157:H7 NCTC 12900 intimin and flagella in avian colonisation, invasion and persistence. In separate experiments, 1-day-old SPF chicks were dosed by oral gavage with 1 x 10^5 CFU of the Stx-negative *E. coli* O157:H7 NCTC 12900 wild-type and intimin and aflagellate-deficient mutants. Birds were housed in isolators with food and water provided *ad libitum*. The liver, spleen, duodenum, jejunum, ileum, colon, caeca, caecal tonsils and crop were removed from birds to enumerate the number of *E. coli* O157 bacteria in those tissues at days 1, 2, 5, 57, 92 and 211 post inoculation. Cloacal swabs were taken from each bird from each group on day 1 post inoculation and weekly, thereafter.

For logistical reasons, five experimental groups of birds were set up that comprised of two separate control groups (A & B), dosed orally with NCTC 12900 and three test groups (C, D & E) separately dosed with DM3, DM4 and DM5. In discussion with the VLA statistician, it was agreed that it was scientifically sound to combine both control NCTC 12900 groups and treat them as a single data set, therefore, data from A & B are combined. Statistical comparison of each mutant with wild-type NCTC 12900 for colonisation and invasion was performed using the Wilcoxon-Mann-Whitney test. Statistical comparison of the distribution of shedding scores of each mutant with wild-type NCTC 12900 was compared by the Kruskall-Wallis test. Fifty eggs per group, (one hundred for combined NCTC 12900 groups) were analysed for either *E. coli* O157:H7 wild-type or intimin/aflagellate mutant contamination.

6.1.2 Colonisation, invasion and persistence of SPF chicks by the intimin mutant

DM3 (intimin mutant) colonised the caeca, colon, ileum and crop of all birds within 24 hours of oral inoculation (Table 6.1), whereas, the duodenum, jejunum and caecal tonsils from some but not all birds, were colonised within 24 hours of oral inoculation. DM3 was also associated with the liver and spleens of some birds within 24 hours of oral inoculation, but it was not associated with the liver or spleen of any birds examined after day 5 post inoculation.

No significant differences in the colonisation of most tissues examined were noted between DM3 and the wild-type, at days 1, 2, 5, 57, and 92 post inoculation, except the caeca on day 5 post inoculation (Table 6.1). At this time point significantly ($p<0.001$) less DM3 were associated with the caeca as compared to the wild-type.

On day 211 post inoculation, no wild-type was associated with any of the tissues examined (Table 6.1). However, DM3 was still associated with the duodenum, jejunum,

ileum, colon, caeca and caecal tonsils at this time point. Furthermore, statistical analysis revealed that this was significant ($p=0.012$) for DM3 colonisation of the colon, caeca and caecal tonsils. No DM3 was associated with the crop at this time point.

DM3 persistence studies revealed that significantly ($p<0.05$) less DM3 were shed from SPF chicks at days 22, 36 and 43 post inoculation (Figure 6.1). DM3 was still shed by birds at the end of the experiment, day 211 post inoculation, whereas no wild-type was detected after day 169 post inoculation (Table 6.2).

6.1.3 Colonisation, invasion and persistence of SPF chicks by the aflagellate mutant

DM4 (aflagellate mutant) colonised the colon, caeca, caecal tonsils and crop of all birds within 24 hours of oral inoculation (Table 6.1) whereas, the duodenum, jejunum and ileum of some, but not all birds, was colonised within 24 hours of oral inoculation. DM4 was associated with the liver, but not the spleen, of some birds within 24 hours of oral infection. DM4 was not associated with the liver or spleen of any birds examined after day 5 post inoculation.

Although significantly ($p<0.05$ and $p<0.001$) less DM4 were associated with most tissues at some, but not all time points, DM4 was still recovered from birds at day 92 post inoculation (Table 6.1). DM4 was not recovered from any of the nine tissues examined from each bird remaining at the end of the experiment, day 211 post inoculation.

DM4 persistence studies revealed that DM4 was still shed by SPF chicks at day 99 post inoculation (Figure 6.1). However, within this time period, days 22 to 92 post inoculation, significantly ($p<0.05$ and $p<0.001$) less DM4 were shed as compared to the wild-type (Table 6.2).

6.1.4 Colonisation, invasion and persistence of SPF chicks by the intimin-aflagellate mutant

DM5 (intimin-aflagellate mutant) colonised the ileum, colon, caeca, caecal tonsils and crop of all birds within 24 hours of oral inoculation (Table 6.1) whereas, the duodenum, jejunum and ileum of some, but not all birds, was colonised within 24 hours of oral inoculation. DM5 was also associated with the liver and spleen of some birds within 24 hours of oral inoculation. DM5 was not associated with the spleen or liver of any birds after day 2 and day 5 post inoculation, respectively.

Very few significant differences in colonisation of all tissues were noted between DM5 and the wild-type (Table 6.1). Furthermore, DM5 was still recovered from birds at day 92 post inoculation. DM5 was not recovered from any of the nine tissues examined from each bird remaining at the end of the experiment, day 211 post inoculation.

DM5 persistence studies revealed that DM5 was still shed by birds at day 113 post inoculation (Figure 6.1). However, during this time period, days 22 to 92 post inoculation,

significantly ($p<0.05$ and $p<0.001$) less DM5 were shed as compared to the wild-type (Table 6.2). Interestingly, this data shows a similar trend to the persistence data reported for aflagellate mutant, DM4.

6.1.5 Analysis of eggs

Each group laid eggs at various days of age and thereafter a total of 50 eggs from the three remaining birds from each group were collected (Table 6.3). Three eggshells, but no egg contents, were culture-positive from one of the wild-type NCTC 12900 groups. All wild-type culture-positive eggs were detected between days 160 and 169 post inoculation. Seven eggshells, but no egg contents, were culture-positive for DM3 (intimin mutant). All DM3-culture positive eggs were detected between days 145 and 182 post inoculation. Eggs laid by DM4 (aflagellate mutant) and DM5 (intimin-aflagellate mutant) were all culture-negative for shells and contents. Interestingly, there were increases, to varying degrees, in bacterial shedding of all *E. coli* O157 isolates just before SPF chicks came into lay.

6.1.6 Visualisation of *in vivo* adherence patterns by intimin, aflagellate and intimin-aflagellate mutants

One bird was chosen at random from each mutant group and the wild-type groups was removed for histological analysis at days 1, 2 and 5 post inoculation. H & E staining followed by staining with anti-O157 specific sera (immunoctyochemistry, ICC) revealed the presence *E. coli* O157 bacteria in the more distal tissues of the gastrointestinal tract. Overall, most *E. coli* O157 attachment occurred in the caeca and all mutant types and NCTC 12900 were attached to this tissue as demonstrated by ICC. Micro-colonies of DM4 (aflagellate mutant) and NCTC 12900 were observed at days 1, 2 and 5 post inoculation. Although, attachment of DM3 (intimin mutant) and DM5 (intimin-aflagellate mutant) was observed at the same time points, no evidence of micro-colony formation was gained. Unattached specifically stained clumps of intimin and aflagellate mutant and NCTC 12900 were also noted. Due to the constraints of time, no further methods (i.e. Transmission electron microscopy) were performed on caecal tissues. Therefore, no unequivocal evidence for the induction of AE lesions was gained. Examples of NCTC 12900 and mutant attachment are shown in Figure 6.2.

Tables 6.1 a-i. Colonisation and invasion of SPF chicks by Stx-negative *E. coli* O157:H7 NCTC 12900 and intimin and aflagellate deficient mutants.

(a) Liver

	NCTC 12900				DM3 (intimin mutant)					DM4 (aflagellate mutant)					DM5 (intimin-aflagellate mutant)				
Day PI	Birds +ve	Mean log_{10}	Geometric mean	SD	Birds +ve	Mean log_{10}	Geometric mean	SD	**p-value**	Birds +ve	Mean log_{10}	Geometric mean	SD	**p-value**	Birds +ve	Mean log_{10}	Geometric mean	SD	**p-value**
1	7/10	2.04	1.10E+02	1.95	4/5	3.42	2.63E+03	2.74	0.315	2/5	1.18	1.51E+01	1.16	0.408	4/5	1.21	1.62E+01	1.06	0.607
2	8/10	2.50	3.16E+02	2.12	4/5	2.28	1.90E+02	2.21	1.000	1/5	0.21	1.62E+00	0.47	0.035	4/5	2.66	4.57E+02	1.61	1.000
5	8/10	1.60	3.98E+01	1.80	4/5	1.67	4.68E+01	1.41	0.856	2/5	1.09	1.23E+01	1.91	0.330	0/5	0	0	0	0.007
57	0/4	0	0	0	0/2	0	0	0	-	0/2	0	0	0	-	0/2	0	0	0	
92	0/4	0	0	0	0/2	0	0	0	-	0/2	0	0	0	-	0/2	0	0	0	
211	0/6	0	0	0	0/2	0	0	0	-	0/3	0	0	0	-	0/3	0	0	0	

(b) Spleen

	NCTC 12900				DM3 (intimin mutant)					DM4 (aflagellate mutant)					DM5 (intimin-aflagellate mutant)				
Day PI	Birds +ve	Mean log_{10}	Geometric mean	SD	Birds +ve	Mean log_{10}	Geometric mean	SD	**p-value**	Birds +ve	Mean log_{10}	Geometric mean	SD	**p-value**	Birds +ve	Mean log_{10}	Geometric mean	SD	**p-value**
1	2/10	0.21	1.62E+00	0.44	1/5	0.69	4.90E+00	1.54	0.524	0/5	0	0	0	0.524	1/5	0.21	1.61E+00	0.47	1.000
2	4/10	0.42	2.63E+00	0.54	1/5	0.21	1.62E+00	0.47	0.231	0/5	0	0	0	0.231	0/5	0	0	0	0.231
5	5/10	0.64	4.36E+00	0.76	1/5	0.21	1.62E+00	0.47	0.231	2/5	1.79	6.16E+01	2.45	0.231	0/5	0	0	0	0.101
57	0/4	0	0	0	0/2	0	0	0	-	0/2	0	0	0	-	0/2	0	0	0	-
92	0/4	0	0	0	0/2	0	0	0	-	0/2	0	0	0	-	0/2	0	0	0	-
211	0/6	0	0	0	0/2	0	0	0	-	0/3	0	0	0	-	0/3	0	0	0	-

(c) Duodenum

	NCTC 12900				DM3 (intimin mutant)					DM4 (aflagellate mutant)					DM5 (intimin-aflagellate mutant)				
Day PI	Birds +ve	Mean log_{10}	Geometric mean	SD	Birds +ve	Mean log_{10}	Geometric mean	SD	**p-value**	Birds +ve	Mean log_{10}	Geometric mean	SD	**p-value**	Birds +ve	Mean log_{10}	Geometric mean	SD	**p-value**
1	7/10	4.05	1.12E+04	2.99	4/5	3.78	6.02E+03	2.20	0.701	1/5	0.61	4.07E+00	1.37	0.041	4/5	0.83	6.76E+00	0.47	0.165
2	10/10	3.87	7.41E+03	1.22	5/5	4.59	3.89E+04	2.02	0.135	0/5	0	0	0	<0.001	0/5	0	0	0	<0.001
5	9/10	2.85	7.08E+02	1.43	4/5	3.14	1.38E+03	1.94	0.494	2/5	0.71	5.13E+00	1.12	0.016	3/5	1.41	2.57E+01	2.06	0.204
57	2/4	1.93	8.51E+01	1.14	1/2	1.11	1.29E+01	1.57	0.667	2/2	2.22	1.66E+02	1.67	1.000	2/2	2.46	2.88E+02	2.01	0.800
92	2/4	2.47	2.95E+02	2.88	2/2	3.69	4.90E+03	0.10	1.000	1/2	1.79	6.17E+01	2.53	0.800	0/2	0	0	0	0.467
211	0/6	0	0	0	1/3	0.90	7.94E+00	1.56	0.333	0/3	0	0	0	-	0/3	0	0	0	-

p-value; significance of colonisation and invasion between wild-type NCTC 12900 and each mutant. Units for the geometric mean are CFU/ml tissue homogenate.
PI; post inoculation, SD, standard deviation.

(d) Jejunum

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value	DM4 (aflagellate mutant)				p-value	DM5 (intimin-aflagellate mutant)				p-value
	Birds +ve	Mean log_{10}	Geometric mean	SD	Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD	
1	7/10	3.96	9.12E+03	2.93	4/5	4.60	3.98E+04	2.82	0.982	1/5	0.72	5.25E+00	1.61	0.041	4/5	1.76	5.75E+01	1.57	0.176
2	10/10	3.74	5.50E+03	1.06	5/5	4.76	5.75E+04	2.16	0.079	0/5	0	0	0	<0.001	1/5	0.72	5.25E+00	1.61	0.006
5	7/10	2.06	1.15E+02	1.93	5/5	3.08	1.20E+03	1.86	0.407	4/5	2.83	6.76E+02	1.63	0.451	3/5	1.90	7.94E+01	2.38	0.926
57	3/4	2.88	7.58E+02	1.97	2/2	1.78	6.02E+01	1.05	0.533	2/5	3.27	1.86E+03	0.29	0.800	2/2	4.40	2.52E+04	0.82	0.533
92	2/4	2.59	3.89E+02	2.99	2/2	3.98	9.55E+03	0.22	1.000	1/5	1.86	7.24E+01	2.64	0.800	1/2	0.52	3.31E+00	0.76	0.800
211	0/6	0	0	0	2/3	2.02	1.04E+02	1.75	0.083	0/3	0	0	0	-	0/3	0	0	0	-

(e) Ileum

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value	DM4 (aflagellate mutant)				p-value	DM5 (intimin-aflagellate mutant)				p-value
	Birds +ve	Mean log_{10}	Geometric mean	SD	Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD	
1	9/10	5.96	9.12E+05	2.98	5/5	6.55	3.55E+06	0.61	0.953	2/5	1.38	2.40E+01	1.95	0.014	5/5	2.97	9.33E+02	1.76	0.038
2	10/10	5.51	3.24E+05	0.47	5/5	6.58	3.80E+06	1.39	0.207	2/5	1.30	2.00E+01	1.86	<0.001	3/5	2.57	3.72E+02	2.74	0.038
5	10/10	4.10	1.26E+04	2.14	4/5	3.04	1.10E+03	2.37	0.369	5/5	3.93	8.51E+03	0.43	0.834	5/5	4.98	9.55E+04	0.89	0.655
57	4/4	5.11	1.29E+05	0.59	2/2	4.28	1.91E+04	0.15	0.133	2/2	7.28	1.90E+07	0.16	0.133	2/2	5.86	7.24E+05	1.39	0.800
92	4/4	5.83	6.76E+05	2.04	2/2	5.56	3.63E+05	0.77	1.000	1/2	1.87	7.41E+01	2.64	0.267	2/2	3.14	1.38E+03	0.11	0.133
211	0/6	0	0	0	3/3	4.66	4.57E+04	0.74	0.119	0/3	0	0	0	-	0/3	0	0	0	-

(f) Colon

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value	DM4 (aflagellate mutant)				p-value	DM5 (intimin-aflagellate mutant)				p-value
	Birds +ve	Mean log_{10}	Geometric mean	SD	Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD	
1	10/10	6.80	6.31E+06	0.94	5/5	6.36	2.29E+06	0.82	0.440	5/5	5.39	2.45E+05	0.62	0.019	5/5	4.97	9.33E+04	0.51	<0.001
2	10/10	6.25	1.78E+06	0.63	5/5	6.85	7.08E+06	1.19	0.371	5/5	4.66	4.57E+04	1.43	0.028	5/5	6.84	6.92E+06	0.46	0.165
5	10/10	5.48	3.02E+05	1.15	5/5	5.04	1.10E+05	2.25	0.953	5/5	5.65	4.47E+05	0.13	0.859	5/5	5.89	7.76E+05	0.74	0.679
57	4/4	5.03	1.07E+05	1.86	2/2	7.20	1.58E+07	0.40	0.133	2/2	6.14	1.38E+06	0.47	0.533	2/2	6.39	2.45E+06	0.21	0.267
92	4/4	5.74	5.50E+05	0.79	2/2	5.31	2.04E+05	0.47	0.533	1/2	2.16	1.44E+02	3.06	0.133	1/2	1.11	1.29E+01	1.57	0.133
211	0/6	0	0	0	3/3	5.38	2.40E+05	0.77	0.012	0/3	0	0	0	-	0/3	0	0	0	-

(g) Caeca

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value	DM4 (aflagellate mutant)				p-value	DM5 (intimin-aflagellate mutant)				p-value
	Birds +ve	Mean log_{10}	Geometric mean	SD	Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD	
1	10/10	8.31	2.04E+08	0.58	5/5	8.34	2.19E+08	1.17	0.514	5/5	7.70	5.01E+07	0.28	0.055	5/5	8.01	1.02E+08	0.57	0.859
2	10/10	7.99	9.77E+07	052	5/5	8.55	3.55E+08	0.38	0.075	5/5	7.62	4.17E+07	0.26	0.440	5/5	8.55	3.55E+08	0.17	0.079
5	10/10	7.75	5.62E+07	0.22	5/5	8.50	3.16E+08	0.18	<0.001	5/5	7.38	2.40E+07	0.45	0.055	5/5	7.72	5.25E+07	0.48	1.000
57	4/4	7.38	2.40E+07	0.55	2/2	7.60	3.98E+07	0.31	0.800	2/2	7.65	4.47E+06	0.15	0.533	2/2	8.07	1.17E+08	0.20	0.267
92	4/4	7.65	4.47E+07	0.18	2/2	7.63	4.26E+07	0.33	0.933	1/2	3.06	1.15E+03	4.32	0.133	2/2	4.24	1.74E+04	0.61	0.133
211	0/6	0	0	0	3/3	6.69	4.90E+06	0.78	0.012	0/3	0	0	0	-	0/3	0	0	0	-

(h) Caecal tonsils

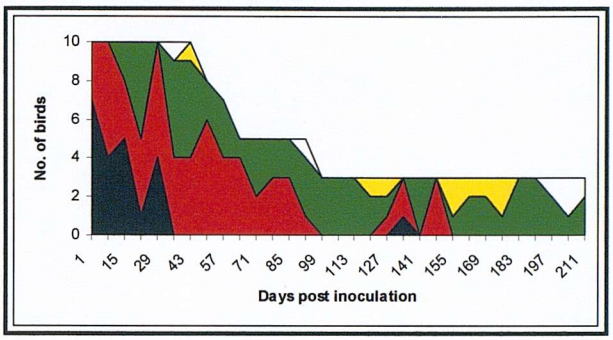
Day PI	NCTC 12900				DM3 (intimin mutant)				p-value	DM4 (aflagellate mutant)				p-value	DM5 (intimin-aflagellate mutant)				p-value
	Birds +ve	Mean log_{10}	Geometric mean	SD	Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD	
1	10/10	5.87	7.41E+05	0.68	4/5	4.56	3.63E+04	2.61	0.254	5/5	4.62	4.17E+04	0.34	<0.001	5/5	3.75	5.62E+03	1.53	<0.001
2	10/10	5.29	1.95E+05	0.80	5/5	4.94	8.71E+04	0.31	0.165	5/5	4.96	9.12E+04	0.76	0.440	5/5	5.62	4.17E+05	0.19	0.859
5	10/10	4.75	5.62E+04	0.94	5/5	3.51	3.24E+03	2.26	0.422	5/5	5.28	1.90E+05	0.33	0.356	5/5	4.44	2.75E+04	0.77	0.440
57	4/4	4.81	6.46E+04	0.21	2/2	4.88	7.59E+04	1.07	1.000	2/2	5.37	2.34E+05	0.24	0.133	2/2	5.08	1.20E+05	0.04	0.267
92	4/4	5.16	1.44E+05	0.57	2/2	4.62	4.17E+04	0.19	0.533	1/2	1.11	1.29E+01	1.57	0.133	1/2	0.52	3.31E+00	0.74	0.133
211	0/6	0	0	0	3/3	4.09	1.23E+04	0.96	0.012	0/3	0	0	0	-	0/3	0	0	0	-

(i) Crop

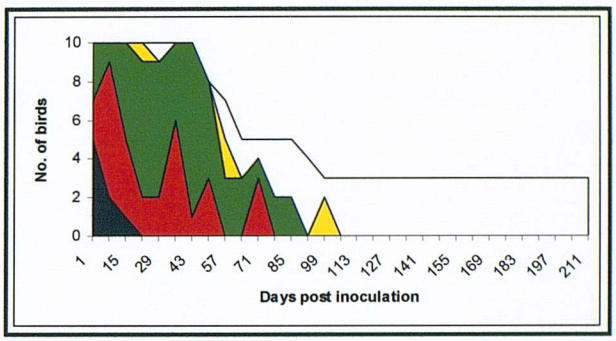
Day PI	NCTC 12900				DM3 (intimin mutant)				p-value	DM4 (aflagellate mutant)				p-value	DM5 (intimin-aflagellate mutant)				p-value
	Birds +ve	Mean log_{10}	Geometric mean	SD	Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD		Birds +ve	Mean log_{10}	Geometric mean	SD	
1	10/10	6.86	7.24E+06	1.32	5/5	7.22	1.66E+07	1.66	0.440	5/5	5.21	1.62E+05	1.18	0.040	5/5	5.12	1.32E+05	0.54	0.019
2	10/10	6.96	9.12E+06	0.71	5/5	7.48	3.02E+07	0.63	0.165	5/5	4.60	3.98E+04	0.67	<0.001	5/5	6.43	2.69E+06	0.81	0.207
5	10/10	5.30	2.00E+05	1.06	5/5	4.85	7.08E+04	2.21	0.836	5/5	6.52	3.31E+06	1.29	0.071	5/5	5.80	6.31E+05	1.16	0.495
57	3/4	4.18	1.51E+04	2.13	2/2	1.04	1.10E+01	0	0.333	2/2	5.18	1.51E+05	0.24	0.800	2/2	3.06	1.15E+03	2.85	0.600
92	4/4	4.89	7.76E+04	1.59	2/2	4.99	9.77E+04	0.86	0.800	0/2	0	0	0	0.133	0/2	0	0	0	0.133
211	0/6	0	0	0	0/3	0	0	0	-	0/3	0	0	0	-	0/3	0	0	0	-

Figure 6.1. The distribution of bacterial shedding, over time, by SPF chicks of (a) DM3 (intimin mutant) (b) DM4 (aflagellate mutant) (c) DM5 (intimin-aflagellate mutant) and (d) the wild-type parent isolate, NCTC 12900.

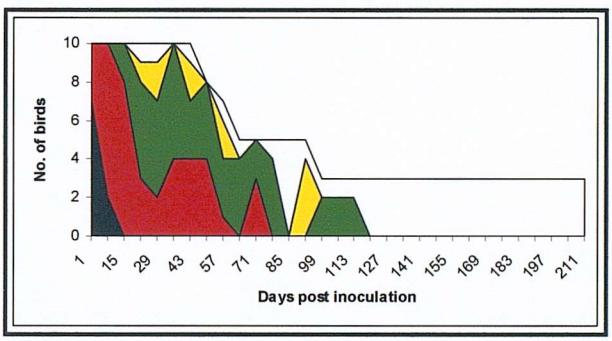
(a) DM3



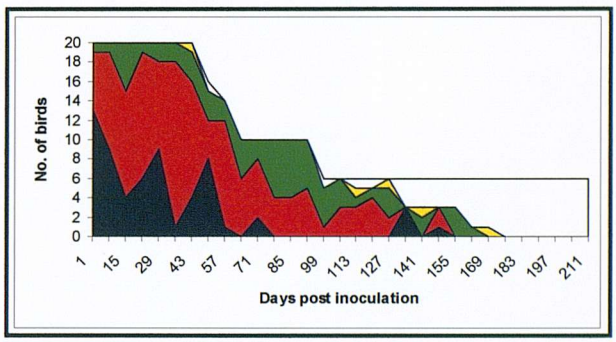
(b) DM4



(c) DM5



(d) NCTC 12900



Recovery of bacteria was scored as high (confluent), medium (>200 colonies) low (<200 colonies), positive (detected after enrichment only) and clear (no colonies). Figure 6.1d represents the combined NCTC 12900 distribution of shedding data from two separate NCTC 12900 persistence experiments.

Table 6.2. Significance (p) in the distribution of bacterial shedding between Stx-negative *E. coli* O157:H7 NCTC 12900 and intimin and aflagellate deficient mutants.

Day PI	Significance (p)		
	NCTC 12900 vs DM3 (intimin mutant)	NCTC 12900 vs DM4 (aflagellate mutant)	NCTC 12900 vs DM5 (intimin-aflagellate mutant)
1	0.907	0.258	0.907
8	1.000	0.200	0.378
15	0.229	0.235	0.734
22	0.017	<0.001	<0.001
29	1.000	<0.001	<0.001
36	0.005	0.086	0.006
43	0.023	<0.001	0.011
50	0.136	0.039	0.056
57	0.186	<0.001	0.001
64	0.600	0.014	0.025
71	0.176	0.301	0.403
78	0.608	0.012	0.134
85	0.608	0.012	<0.001
92	0.201	0.001	<0.001
99	1.000	0.119	0.619
106	0.464	0.024	0.131
113	0.411	0.036	0.286
120	0.155	0.095	0.095
127	1.000	0.012	0.012
134	0.714	0.464	0.464
141	0.167	0.321	0.321
148	0.429	0.393	0.393
155	0.714	0.464	0.464
162	0.048	1.000	1.000
169	0.024	1.000	1.000
176	0.012	-	-
183	0.012	-	-
190	0.012	-	-
197	0.083	-	-
204	0.333	-	-
211	0.083	-	-

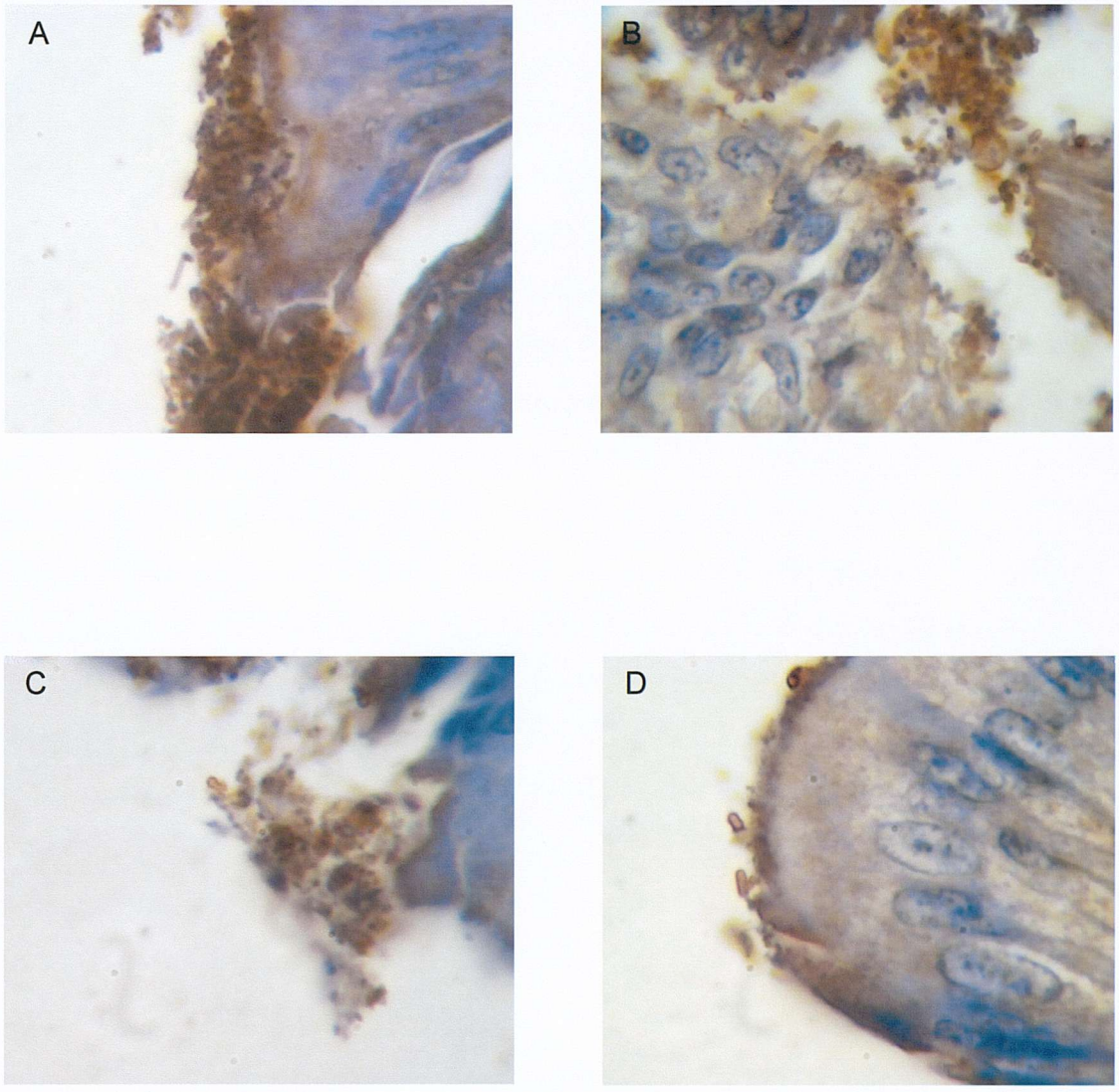
For the purpose of statistical analysis, each shedding score in Figure 6.1 was assigned a numerical score. i.e. high = 4, medium = 3, low = 2 positive after enrichment = 1 and clear = 0. The distributions of NCTC 12900 numerical scores were compared with the distributions of mutant numerical scores using the Kruskall-Wallis test.
PI; post inoculation.

Table 6.3. Analysis of eggs laid by SPF chickens.

Isolate	Day first egg laid	No. of eggs tested	No. of eggs shells culture positive
NCTC 12900 (wild-type)	$123^a + 160^b$	$50^a + 50^b$	$0^a + 3^b$
DM3 (intimin mutant)	145	50	7
DM4 (aflagellate mutant)	130	50	0
DM5 (intimin-flagellate mutant)	130	50	0

$^a + ^b$; refers to the when each wild-type group began egg laying, how many eggs were taken from each group and what group had culture positive egg shells.

Figure 6.2. Visualisation of attachment to the caeca of SPF chicks by bacteria specifically stained with anti-O157 sera during the first five days of experimental infection. Magnification x 1000.



A = Caecal tissue from birds dosed orally with NCTC 12900, B = Caecal tissue from birds dosed orally with DM3 (intimin mutant), C = Caecal tissue from birds dosed orally with DM4 (aflagellate mutant), D = DM5 (intimin-aflagellate mutant).

Each field of view was generally typical for each isolate.

6.2 Discussion

Chick models of AEEC, including *E. coli* O157:H7, colonisation and persistence have been reported previously (Beery *et al.*, 1985; Stavric *et al.*, 1993; Shoeni & Doyle, 1994). These studies showed that Stx-positive *E. coli* O157:H7 can colonise the colon, caeca and crop of SPF White Leghorn chicks and that Stx positive *E. coli* O157:H7 persistence can occur for up to 11 months depending on the dose given.

The data in chapter 3 of this thesis demonstrated that low level invasion by Stx-negative *E. coli* O157:H7 NCTC 12900 was detected during the first five days of infection and that colonisation of the caeca by NCTC 12900 occurred for up to day 92 post inoculation. NCTC 12900 was still shed from birds at day 148 post inoculation, suggesting that the caeca may be the tissue that sustains *E. coli* O157:H7 in this host. In this chapter, extensive analysis of additional tissues of the gastro-intestinal tract by bacterial enumeration was performed and showed that NCTC 12900 colonised the duodenum, jejunum and ileum of SPF chicks, albeit at lower numbers as compared to colonisation of the caeca. However, it should be borne in mind that after initial colonisation of these tissues, subsequent association of the more proximal tissues of the gastrointestinal tract might have been due to re-infection of SPF chicks that are shedding medium to high numbers of NCTC 12900 into the environment.

In order to determine the role of intimin and flagella in avian colonisation, invasion and persistence of Stx-negative *E. coli* O157:H7 NCTC 12900, intimin and aflagellate mutants were compared to the wild-type, NCTC 12900, to detect any attenuation in colonisation, invasion and persistence. Although *E. coli* O157:H7 is generally considered non-invasive, this study and previous studies showed that bacteria may be taken up by cells in low numbers compared with known invasive organisms such as *Salmonella* (Dibb-Fuller *et al.*, 2001; Wales *et al.*, 2002). A lack of functional intimin or flagella did not prevent bacterial colonisation of the liver or spleen suggesting that intimin and aflagellate mutants were taken across epithelial surfaces within SPF chicks. It was presumed that this occurred at the gastrointestinal tract mucosa, but may have occurred elsewhere. Interestingly, bacteria unable to express intimin and flagella were cleared quicker from the liver and spleen. Whether the data relates to clearance or reduced invasion, it remains unclear if NCTC 12900 up take was controlled by an active mechanism. Therefore, it is difficult to ascertain whether intimin or flagella had any role to play in *E. coli* O157:H7 uptake in the SPF chick.

Colonisation of the gastrointestinal tract by intimin and aflagellate mutants was observed. However, during the first two days of infection, mutants unable to produce flagella were less able to colonise the duodenum, jejunum, ileum, colon, caecal tonsils and crop and although not statistically significant, less aflagellate deficient mutants were recovered from the caeca. Furthermore, the numbers of aflagellate and intimin-aflagellate mutant recovered

from SPF chicks at day 92 post inoculation were 1000 to 100000-fold less, respectively. Numbers of the intimin mutant and NCTC 12900 were comparable for all tissues of the gastrointestinal tract examined. Furthermore, the intimin-deficient mutant was still associated with these tissues at the end of the experiment, day 211 post inoculation, whereas the aflagellate and intimin-aflagellate mutants were not. This data suggests that flagella, but not intimin, has a role to play in the colonisation of these tissues, but that additional factors other than intimin may be involved in the colonisation of SPF chicks. However, histological analysis revealed that the aflagellate mutant could still form micro-colonies on the caecal mucosa during the first 5 days of experimental infection, whereas the intimin and intimin-aflagellate mutant could not. Nevetherless, both intimin mutants did adhere to the caecal mucosa indicating the involvement of non-intimin related attachment factors.

Persistence data demonstrated that far fewer aflagellate and intimin-aflagellate mutants were shed from SPF chicks after day 22 post inoculation. However the numbers of intimin deficient mutant shed from SPF chicks were comparable to that of NCTC 12900 from day 1 to 15 post inoculation and from day 43 post inoculation. Furthermore, the intimin-deficient mutant was still shed from birds at day 211 post inoculation, 32 days more than the wild-type NCTC 12900, whereas the aflagellate mutant and the intimin-aflagellate mutant were shed for 28 to 70 and 14 to 56 days less than NCTC 12900, respectively. This data suggests that although a lack of flagella may reduce the time scale of *E. coli* O157:H7 persistence in SPF chicks, these bacteria can still persist for a long period. More importantly, a lack of functional intimin expression did not effect persistence in SPF chicks suggesting that intimin and flagella related or non-related *E. coli* O157:H7 factors may have contributed to the long term persistence of this mutant and the aflagellate and intimin-aflagellate mutants in SPF chicks.

Schoeni and Doyle (1994) reported that the shells of 14 of 101 eggs, but not the contents were contaminated with *E. coli* O157:H7 and that contamination of egg shells still occurred 5 months after the onset of egg laying. However, the SPF chicks used in the Schoeni & Doyle (1994) study were dosed orally with 1.3×10^8 CFU of *E. coli* O157:H7 bacteria. Analysis of eggs collected in the study to be reported here showed that while an isolate was still shed after the onset of egg laying, contamination of egg shells was possible. Interestingly, after the onset of egg laying, the wild-type was only detected for up to 4 and 9 days by birds in each group, respectively. However, contamination of 3 egg shells was observed from the wild-type group still shedding bacteria for 9 days after the start of egg laying. As reported in chapter 3, NCTC 12900 was not detected by cloacal swabbing just before the onset of egg laying. This data and the data reported in this chapter suggests that the maturation and the onset of egg laying may contribute to the clearance of NCTC 12900 in this model. Possibly a hormonal and/or antibody response may also contribute to this observation. Furthermore, Stx-negative

E. coli O157:H7 unable to express functional intimin were still shed long after the onset of egg laying, suggesting that this phenotype may have been advantageous for continued persistence.

The aflagellate mutant and the intimin-aflagellate mutant were only detected up until 17 and 31 days, respectively, before the onset of egg laying. A possible hypothesis for this observation may be that during maturation to the onset of lay, the expression of active flagella contributes to the continued persistence of *E. coli* O157:H7 within SPF chicks until or just after the onset of egg laying.

Chapter 7

Competitive Index: Colonisation, invasion and persistence of SPF chicks by Stx-negative *E. coli* O157:H7 intimin and aflagellate mutants in the presence of wild-type NCTC 12900

As reported in chapter 6, Stx-negative *E. coli* O157:H7 mutants defective for intimin and flagella colonised and invaded SPF chicks, albeit at lower numbers at certain sites. Interestingly, the intimin-deficient mutant persisted in SPF chickens longer than NCTC 12900 and beyond the onset of egg laying. Aflagellate mutants showed decreased persistence, although shedding of these mutants still occurred for approximately 14 to 16 weeks.

A recent competition study of persistence (competitive index) between an avian pathogenic *E. coli* wild-type and type-1 and curli fimbrial and aflagellate-derived mutants has been reported using the SPF chick model (La Ragione *et al.*, 2000b). This study demonstrated that these surface appendage mutants were less persistent in SPF chicks when in the presence of the wild-type, than when the wild-type was absent, strengthening the argument that these surface appendages were required for the colonisation and persistence of SPF chicks.

Therefore, the next step in this study was to determine if the Stx-negative *E. coli* O157:H7 intimin and aflagellated mutants would be less able to colonise, invade and persist in SPF chicks when in the presence of the wild-type, NCTC 12900. To test this, intimin and aflagellate mutants were given together with the wild-type parent strain NCTC 12900 to separate groups of 1-day-old SPF chicks.

7.1 Results

7.1.1 Colonisation, invasion and persistence of SPF chicks by intimin and aflagellate mutants in the presence of the wild-type parent isolate NCTC 12900

To investigate the competitive fitness of Stx-negative *E. coli* O157:H7 intimin and aflagellate mutants as compared to wild-type progenitor, NCTC 12900, for avian colonisation, invasion and persistence, the 1-day-old SPF chick model was employed as described in chapter 6, with modifications. In separate experiments, 1-day-old SPF chicks were dosed by oral gavage together with 5 x 10^4 CFU of the Stx-negative *E. coli* O157:H7 NCTC 12900 wild-type and 5 x 10^4 CFU of one mutant. Birds were housed in isolators with food and water provided *ad libitum*. The liver, spleen, duodenum, jejunum, ileum, colon, caeca, caecal tonsils and crop were removed from birds to enumerate the number of *E. coli* O157 bacteria at days 1, 2, 5, 57, 92 and 211 post inoculation. Birds from each group were also swabbed cloacally on day 1 post inoculation and weekly thereafter. A small competitive index study was also performed where 1-day-old SPF chicks were each inoculated by oral gavage with a triple inoculum containing NCTC 12900, DM3 and DM4 (total = 1 x 10^5 CFU). Birds were cloacally swabbed at day 1 post inoculation and weekly thereafter until the end of the experiment, day 36 post dosing. *Post mortem* examinations were also performed on the same birds at the end of the experiment. Statistical comparison of each mutant with the wild-type for colonisation and invasion was performed using the Wilcoxon-Mann-Whitney test. Statistical comparison of the distribution of shedding scores of each mutant with the wild-type was compared by the Kruskall-Wallis test. Fifty eggs per group were analysed for either *E. coli* O157:H7 wild-type or intimin/aflagellate mutant contamination.

7.1.2 Colonisation, invasion and persistence of SPF chicks by the intimin mutant in the presence of the wild-type parent isolate NCTC 12900

The liver, duodenum, jejunum, ileum, colon, caeca, caecal tonsils and crop of all birds were colonised by DM3 (intimin mutant) and the wild-type within 24 hours of oral inoculation (Table 7.1). DM3 and the wild-type were associated with the spleen of some but not all birds within 24 hours of oral inoculation. Liver and spleen showed signs of clearance after days 2 and 5 post inoculation.

No significant differences in the colonisation of all tissues examined were noted between DM3 and the wild-type, at days 1, 2 and 5 post inoculation (Table 7.1). At the end of the experiment, day 211 post inoculation, neither DM3 nor the wild-type was recovered from any of the tissues examined. At day 57 and 92 post inoculation, both DM3 and the wild-type were associated with the duodenum, jejunum, ileum, colon, caeca, caecal tonsils and crop.

Competitive index persistence studies demonstrated that DM3 and the wild-type were both recovered from birds until day 112 post inoculation (Figure 7.1). Statistical analysis did

not reveal any significant differences between the levels of shedding during this time period for the intimin mutant and the wild-type (Table 7.4).

7.1.3 Colonisation, invasion and persistence of SPF chicks by the aflagellate mutant in the presence of the wild-type parent isolate NCTC 12900

The colon, caeca, caecal tonsils and crop of all birds were colonised with DM4 (aflagellate mutant) and the wild-type within 24 hours of oral inoculation (Table 7.2). DM4 and the wild-type were associated with the liver, duodenum, jejunum and ileum of some but not all birds within 24 hours of oral inoculation. Neither DM4 nor the wild-type was associated with the spleen within 24 hours of oral inoculation or at any time point thereafter. DM4 was not associated with the liver after day 2 post inoculation, whereas the wild-type was not associated with the liver after day 5 post inoculation.

No significant differences in the colonisation of any tissues examined were noted between DM4 and the wild-type, at days 1, 2 and 5 post inoculation (Table 7.2). On day 57 post inoculation, both DM4 and the wild-type were associated with the colon, caeca, caecal tissues and crop. On day 92 post inoculation, DM4 was not associated with any of the tissues examined, whereas the wild-type was recovered from the ileum, colon and caeca of one bird. At the end of the experiment, day 211 post inoculation, neither DM4 nor the wild-type was recovered from any of the tissues examined.

Competitive index persistence studies demonstrated that DM4 and the wild-type were both recovered from birds until days 85 and 92 post inoculation, respectively (Figure 7.1). Statistically, significantly ($p<0.05$ and $p<0.001$) less DM4 than wild-type was shed from birds on days 8, 22, 29, 43 and 64 post inoculation (Table 7.4). At all other time points, although not statistically significant, less DM4 than wild-type was recovered from birds.

7.1.4 Colonisation, invasion and persistence of SPF chicks by the intimin-aflagellate mutant in the presence of the wild-type parent isolate NCTC 12900

The colon, caeca, caecal tonsils and crop of all birds were colonised by DM5 (intimin-aflagellate mutant) and the wild-type within 24 hours of oral inoculation (Table 7.3). The wild-type also colonised the duodenum, jejunum and ileum of all birds, whereas DM5 only colonised the duodenum, jejunum and ileum of some birds within 24 hours of oral inoculation. DM5 and the wild-type were associated with the liver and spleen of some but not all birds within 24 hours of oral inoculation. DM5 and the wild-type were not associated with the liver and spleen after day 1 and 5 post inoculation, respectively.

No significant differences in the colonisation of all tissues examined were noted between DM5 and the wild-type, at days 1, 2 and 5 post inoculation (Table 7.3). On day 57 post inoculation, both DM5 and the wild-type were recovered from the duodenum, jejunum, ileum, colon, caeca, caecal tonsils and crop of both birds. On day 92 post inoculation, DM5

was only recovered from the caeca of both birds, whereas the wild-type was recovered from the colon, caeca and caecal tonsils of both birds. At the end of the experiment, day 211 post inoculation, neither DM5 nor the wild-type was recovered from any of the tissues examined.

Competitive index persistence studies demonstrated that DM5 and the wild-type were recovered from birds until days 99 and 106 post inoculation, respectively (Figure 7.1). Statistically, significantly ($p<0.05$ and $p<0.001$) less DM5 than wild-type was shed from birds on days 8, 22, 29, 57 and 71 post inoculation (Table 7.4). On days 1, 15, 36, 43, 64, 85 and 99 post inoculation, although not statistically significant, less DM5 than wild-type was recovered from birds.

7.1.5 Analysis of eggs

Each group laid eggs at various days of age, NCTC 12900 and DM3 at day 135 post inoculation, NCTC 12900 and DM4 and NCTC 12900 and DM5 at day 155 post inoculation. All eggs analysed from each group were culture negative for the wild-type and the respective mutant.

7.1.6 Visualisation of *in vivo* adherence patterns by intimin, aflagellate and intimin-aflagellate mutants when in the presence of the wild-type

One bird, chosen at random from each wild-type plus mutant group, was removed for histological analysis at days 1, 2 and 5 post inoculation. H & E staining followed by staining with anti-O157 specific sera (immunoctyochemistry, ICC) revealed the presence *E. coli* O157 bacteria in the more distal tissues of the gastrointestinal tract. Overall, most *E. coli* O157 attachment occurred in the caeca. Micro-colonies of specifically stained *E. coli* O157 bacteria were observed from all groups at days 1, 2 and 5 post inoculation. However, whether these micro-colonies were comprised of NCTC 12900 only or included intimin or aflagellate mutants remains unclear. Unattached, clumps of specifically stained *E. coli* O157 bacteria were also noted. Due to the constraints of time, no further methods (i.e. Transmission electron microscopy) were performed on caecal tissues. Therefore, no unequivocal evidence for the induction of AE lesions was gained. Examples of *E. coli* O157 attachment are shown in Figure 7.2.

7.1.7 Persistent colonisation of SPF chicks by intimin and flagella deficient mutants when both in the presence of the wild-type NCTC 12900.

Persistence studies revealed that the wild-type and DM3 (intimin mutant) were shed by SPF chicks in similar numbers by the same birds until the end of the experiment, day 36 post inoculation (Figure 7.3), and that no significant differences were noted at each time point (Table 7.5). From the same birds, significantly ($p<0.05$) less DM4 (aflagellate mutant) than wild-type were shed at days 1, 8 and 15 post inoculation. Although not statistically significant, at all other time points, fewer DM4 was shed from birds than wild-type.

At the end of the experiment, day 36 post inoculation, DM3, DM4 and the wild-type were associated with the colon and caeca of all birds examined (Table 7.6). Although not statistically significant, approximately 100 to 1000-fold less DM4 than DM3 and the wild-type was recovered from the colon and caeca. Both DM3 and the wild-type were associated with the caecal tonsils and crop of all birds. However, DM4 was associated with the caecal tonsils and crop of some, but not all birds. DM3 and the wild-type were recovered from the duodenum, jejunum and ileum, but DM4 was not.

Tables 7.1a-i. Colonisation and invasion by DM3 (intimin mutant) in the presence of the wild-type parent isolate NCTC 12900.

(a) Liver

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	3.93	8.51E+03	0.65	5/5	3.63	4.27E+03	0.67	0.813
2	4/5	1.57	3.72E+01	1.29	4/5	1.74	5.50E+01	1.53	0.500
5	0/5	0	0	0	0/5	0	0	0	0
57	0/2	0	0	0	0/2	0	0	0	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(b) Spleen

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	2/5	1.56	3.63E+01	2.15	2/5	1.37	2.34E+01	1.88	0.500
2	1/5	0.58	3.80E+00	1.31	1/5	0.64	4.36E+00	1.44	1.000
5	1/5	0.83	6.76E+00	1.86	1/5	0.21	1.62E+00	0.47	1.000
57	0/2	0	0	0	0/2	0	0	0	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(c) Duodenum

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	5.85	7.08E+05	0.55	5/5	6.09	1.23E+06	0.26	0.313
2	5/5	4.36	2.29E+04	1.52	5/5	3.54	3.47E+03	1.60	0.063
5	1/5	0.98	9.55E+00	2.20	0/5	0	0	0	1.000
57	2/2	1.98	9.55E+01	1.33	1/2	1.35	2.24E+01	1.91	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(d) Jejunum

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	6.07	1.17E+06	0.55	5/5	5.18	1.51E+05	1.14	0.063
2	5/5	4.48	3.02E+04	1.55	3/5	2.19	1.55E+02	2.09	0.125
5	3/5	1.77	5.89E+01	2.09	3/5	1.16	1.44E+01	1.53	0.500
57	2/2	2.53	3.39E+02	0.01	2/2	2.94	8.71E+02	0.34	-
92	1/2	0.52	3.31E+00	0.74	1/2	0.52	3.31E+00	0.74	-
211	0/3	0	0	0	0/3	0	0	0	-

p-value; significance in colonisation of each tissue between each mutant and NCTC12900.
SD; standard deviation.
Units for the geometric mean are CFU/ml tissue homogenate.

(e) Ileum

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	7.67	4.68E+07	0.79	5/5	7.09	1.23E+07	0.68	0.125
2	5/5	4.33	2.14E+04	0.87	5/5	4.16	1.44E+04	1.86	1.000
5	5/5	4.79	6.17E+04	2.36	4/5	2.79	6.17E+02	2.11	0.063
57	2/2	6.07	1.17E+06	0.43	2/2	6.03	1.07E+06	0.61	-
92	2/2	4.71	5.13E+04	0.02	1/2	1.91	8.13E+01	2.70	-
211	0/3	0	0	0	0/3	0	0	0	-

(f) Colon

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	7.03	1.07E+07	0.58	5/5	6.56	3.63E+06	0.36	0.125
2	5/5	5.56	3.63E+05	1.85	5/5	5.68	4.79E+05	0.17	0.813
5	5/5	6.39	2.45E+06	0.35	5/5	5.43	2.69E+05	0.59	0.063
57	2/2	6.06	1.15E+06	0.87	2/2	6.39	2.45E+06	0.45	-
92	2/2	4.46	2.88E+04	0.10	2/2	4.14	1.38E+04	0.24	-
211	0/3	0	0	0	0/3	0	0	0	-

(g) Caeca

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	8.19	1.55E+08	0.32	5/5	8.21	1.62E+08	0.50	1.000
2	5/5	8.34	2.19E+08	0.50	5/5	8.26	1.82E+08	0.41	0.188
5	5/5	8.01	1.02E+08	0.23	5/5	8.20	1.58E+08	0.23	0.188
57	2/2	7.41	2.57E+07	0.23	2/2	7.44	2.75E+07	0.26	-
92	2/2	4.29	1.95E+04	0.42	2/2	3.92	8.32E+03	0.99	-
211	0/3	0	0	0	0/3	0	0	0	-

(h) Caecal tonsils

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	5.93	8.51E+05	0.62	5/5	5.32	2.09E+05	0.30	0.188
2	5/5	6.07	1.17E+06	0.27	5/5	2.87	7.41E+02	1.93	0.063
5	5/5	5.52	3.31E+05	0.30	5/5	4.78	6.02E+04	0.63	0.188
57	2/2	4.74	5.50E+04	0.22	2/2	4.73	5.37E+04	0.40	-
92	2/2	5.86	7.24E+05	0.45	1/2	3.21	1.62E+03	4.54	-
211	0/3	0	0	0	0/3	0	0	0	-

(i) Crop

Day PI	NCTC 12900				DM3 (intimin mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	7.82	6.61E+07	0.93	5/5	7.26	1.82E+07	1.03	0.625
2	5/5	6.60	3.98E+06	1.24	5/5	7.12	1.32E+07	1.36	0.063
5	4/5	4.06	1.15E+04	2.73	4/5	1.96	9.12E+01	2.67	0.125
57	1/2	1.85	7.08E+01	2.62	2/2	4.20	1.58E+04	0.96	-
92	1/2	1.41	2.57E+01	1.99	1/2	0.52	3.31E+00	0.74	-
211	0/3	0	0	0	0/3	0	0	0	-

Tables 7.2a-i. Colonisation and invasion by DM4 (aflagellate mutant) in the presence of the wild-type parent isolate NCTC 12900.

(a) Liver

Day PI	NCTC 12900				DM4 (aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	2/5	0.99	9.77E+00	1.70	2/5	0.90	7.94E+00	1.50	1.000
2	4/5	1.70	5.01E+01	1.77	2/5	0.42	2.63E+00	0.57	0.125
5	2/5	0.42	2.63E+00	0.57	0/5	0	0	0	0.500
57	0/2	0	0	0	0/2	0	0	0	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(b) Spleen

Day PI	NCTC 12900				DM4 (aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	0/5	0	0	0	0/5	0	0	0	0
2	0/5	0	0	0	0/5	0	0	0	0
5	0/5	0	0	0	0/5	0	0	0	0
57	0/2	0	0	0	0/2	0	0	0	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(c) Duodenum

Day PI	NCTC 12900				DM4 (aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	4/5	2.81	6.46E+02	1.78	3/5	2.65	4.47E+02	2.78	0.875
2	4/5	2.16	1.44E+02	1.94	3/5	0.86	7.24E+00	0.92	0.125
5	4/5	2.90	7.94E+02	2.05	2/5	1.80	6.31E+01	2.47	0.250
57	0/2	0	0	0	0/2	0	0	0	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(d) Jejunum

Day PI	NCTC 12900				DM4 (aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	4/5	3.27	1.86E+03	1.85	4/5	3.04	1.10E+03	1.81	0.625
2	4/5	3.97	9.33E+03	1.80	2/5	1.37	2.34E+01	1.98	0.125
5	4/5	4.45	2.82E+04	1.86	1/5	1.00	1.00E+01	2.25	0.250
57	2/2	3.45	2.82E+03	1.01	0/2	0	0	0	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(e) Ileum

Day PI	NCTC 12900				DM4 (aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	4/5	3.77	5.89E+03	2.17	4/5	3.69	4.90E+03	2.23	0.875
2	4/5	3.97	9.33E+03	2.25	4/5	4.13	1.35E+04	2.38	0.625
5	5/5	4.45	2.82E+04	2.02	4/5	3.44	2.75E+03	2.87	0.375
57	1/2	3.45	2.82E+03	4.88	1/2	3.23	1.70E+03	4.57	-
92	1/2	0.52	3.31E+00	0.74	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(f) Colon

Day PI	NCTC 12900				DM4 (aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	5.14	1.38E+05	0.91	5/5	5.76	5.75E+05	0.59	0.125
2	5/5	5.27	1.86E+05	0.24	5/5	5.72	5.25E+05	0.60	0.063
5	5/5	6.57	3.72E+06	0.67	5/5	6.65	4.47E+06	0.22	1.000
57	2/2	6.18	1.51E+06	0.74	2/2	5.20	1.58E+05	0.79	-
92	1/2	0.52	3.31E+00	0.74	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(g) Caeca

Day PI	NCTC 12900				DM4 (aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	6.95	8.91E+06	0.58	5/5	7.44	2.75E+07	0.59	0.125
2	5/5	7.70	5.01E+07	0.20	5/5	7.92	8.32E+07	0.60	0.063
5	5/5	7.55	3.55E+07	0.69	5/5	7.63	4.27E+07	0.22	1.000
57	2/2	6.15	1.41E+06	0.26	2/2	5.96	9.12E+05	0.79	-
92	1/2	1.36	2.29E+01	1.93	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(h) Caecal tonsils

Day PI	NCTC 12900				DM4 (aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	4.77	5.89E+04	0.54	5/5	4.80	6.31E+04	0.67	0.813
2	5/5	4.86	7.24E+04	0.68	5/5	4.86	7.24E+04	0.68	1.000
5	5/5	5.44	2.75E+05	0.31	5/5	5.62	4.17E+05	0.12	0.188
57	2/2	4.26	1.82E+04	0.27	2/2	3.25	1.78E+03	0.17	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(i) Crop

Day PI	NCTC 12900				DM4 (aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	5.79	6.16E+05	1.02	5/5	5.63	4.26E+05	1.68	0.813
2	5/5	4.56	3.63E+04	0.86	5/5	4.35	2.24E+04	0.75	0.625
5	5/5	5.27	1.86E+05	1.98	5/5	4.79	6.16E+04	2.49	0.188
57	2/2	4.23	1.70E+04	0.63	2/2	4.06	1.15E+04	1.51	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

Tables 7.3a-i. Colonisation and invasion by DM5 (intimin-aflagellate mutant) in the presence of the wild-type parent strain NCTC 12900.

(a) Liver

Day PI	NCTC 12900				DM5 (intimin-aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	2/5	1.35	2.24E+01	2.49	1/5	1.19	1.55E+01	2.66	1.000
2	4/5	1.68	4.79E+01	1.41	0/5	0	0	0	0.125
5	5/5	1.04	1.10E+01	0	0/5	0	0	0	0.063
57	0/2	0	0	0	0/2	0	0	0	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(b) Spleen

Day PI	NCTC 12900				DM5 (intimin-aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	1/5	0.82	6.61E+00	1.84	1/5	0.77	5.89E+00	1.72	1.000
2	2/5	0.42	2.63E+00	0.57	0/5	0	0	0	0.500
5	4/5	0.83	6.76E+00	0.47	0/5	0	0	0	0.125
57	0/2	0	0	0	0/2	0	0	0	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(c) Duodenum

Day PI	NCTC 12900				DM5 (intimin-aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	4/5	1.53	3.39E+01	0.99	3/5	0.92	8.32E+00	1.04	0.625
2	1/5	0.21	1.62E+00	0.47	0/5	0	0	0	1.000
5	5/5	3.57	3.72E+03	0.95	3/5	1.63	4.26E+01	1.91	0.063
57	2/2	5.27	1.86E+05	0.42	2/2	3.62	4.17E+03	0.02	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(d) Jejunum

Day PI	NCTC 12900				DM5 (intimin-aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	3.30	2.00E+03	1.45	4/5	2.57	3.72E+02	2.10	0.625
2	4/5	3.04	1.10E+03	2.27	4/5	1.89	7.76E+01	2.52	0.250
5	5/5	3.90	7.94E+03	0.52	3/5	2.08	1.20E+02	2.41	0.313
57	2/2	5.96	9.12E+05	0.13	2/2	5.26	1.82E+05	0.11	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(e) Ileum

Day PI	NCTC 12900				DM5 (intimin-aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	4/5	3.55	3.55E+03	2.12	4/5	3.45	2.82E+03	2.09	0.250
2	5/5	5.53	3.39E+05	1.24	5/5	5.11	1.29E+05	2.47	1.000
5	5/5	5.98	9.55E+05	0.82	5/5	5.82	6.61E+05	0.60	0.813
57	2/2	6.36	2.29E+06	0.83	2/2	5.77	5.89E+05	0.99	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(f) Colon

Day PI	NCTC 12900				DM5 (intimin-aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	4.68	4.79E+04	0.88	5/5	4.96	9.12E+04	0.84	0.063
2	5/5	6.35	2.24E+06	0.39	5/5	6.77	5.89E+06	0.49	0.063
5	5/5	6.37	2.34E+06	0.30	5/5	7.19	1.55E+07	0.43	0.125
57	2/2	5.43	2.69E+05	0.52	2/2	4.53	3.39E+04	1.66	-
92	2/2	3.46	2.88E+03	0.12	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(g) Caeca

Day PI	NCTC 12900				DM5 (intimin-aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	6.81	6.46E+06	0.49	5/5	7.21	1.62E+07	0.48	0.125
2	5/5	7.54	3.48E+07	0.90	5/5	7.98	9.55E+07	0.41	0.438
5	5/5	7.40	2.51E+07	0.81	5/5	8.30	2.00E+08	0.21	0.063
57	2/2	7.69	4.90E+07	0.06	2/2	6.57	3.72E+06	0.44	-
92	2/2	5.73	2.34E+05	0.19	2/2	4.27	1.86E+04	0.92	-
211	0/3	0	0	0	0/3	0	0	0	-

(h) Caecal tonsils

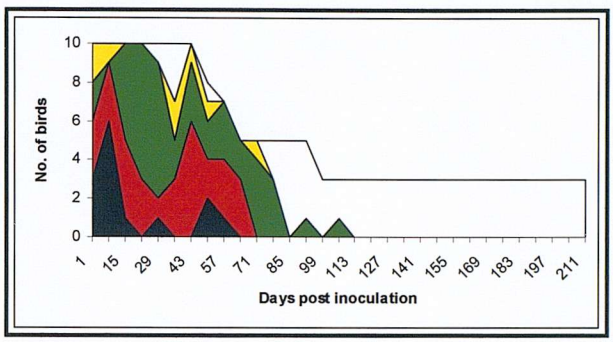
Day PI	NCTC 12900				DM5 (intimin-aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	4.09	1.23E+04	0.78	5/5	4.51	3.24E+04	0.62	0.063
2	5/5	4.07	1.17E+04	1.71	5/5	5.57	3.72E+05	0.74	0.125
5	5/5	5.67	4.68E+05	0.38	5/5	6.14	1.38E+06	0.41	0.063
57	2/2	5.06	1.15E+05	0.03	2/2	2.90	7.94E+02	2.62	-
92	2/2	3.00	1.00E+03	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

(i) Crop

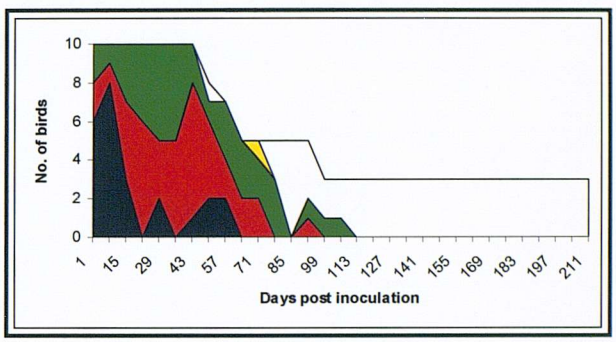
Day PI	NCTC 12900				DM5 (intimin-aflagellate mutant)				p-value
	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	No. Birds positive	Mean (Log_{10})	Geometric mean	SD	
1	5/5	5.33	2.14E+05	0.85	5/5	5.77	5.89E+05	0.78	0.063
2	5/5	5.82	6.61E+05	0.56	5/5	6.04	1.10E+06	0.45	0.625
5	5/5	5.19	1.55E+05	0.66	5/5	5.49	3.09E+05	0.62	0.063
57	2/2	5.50	3.16E+05	0.22	2/2	4.93	8.51E+04	1.25	-
92	0/2	0	0	0	0/2	0	0	0	-
211	0/3	0	0	0	0/3	0	0	0	-

Figure 7.1. The distribution of bacterial shedding, over time, by SPF chicks of (a) DM3 (intimin mutant) (c) DM4 (aflagellate mutant) (e) DM5 (intimin-aflagellate mutant) in the presence of (b, d & f) the wild-type parent isolate, NCTC 12900.

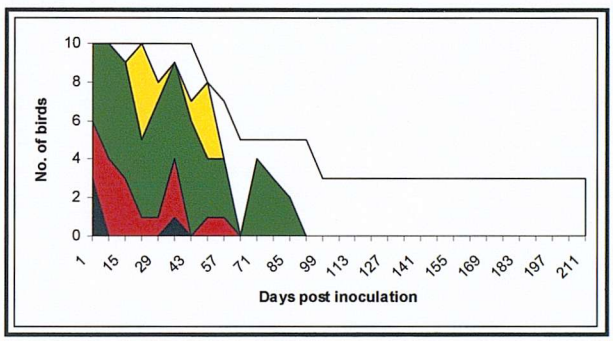
(a) DM3



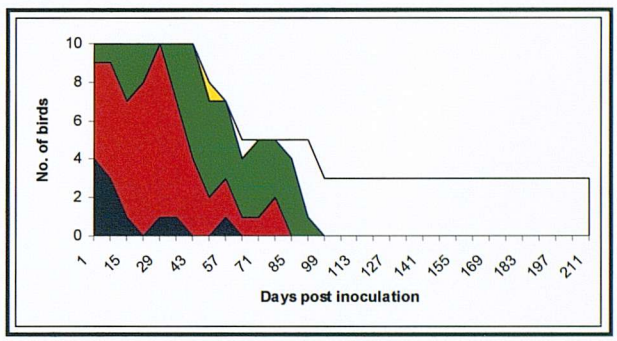
(b) NCTC 12900



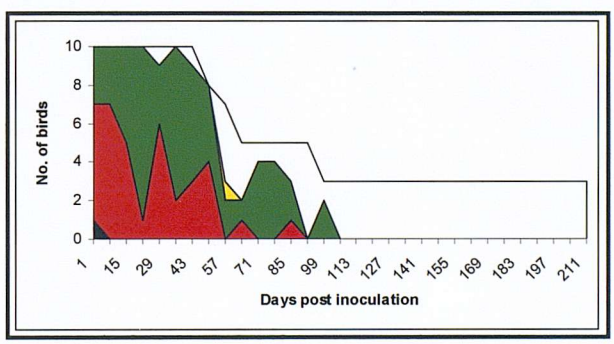
(c) DM4



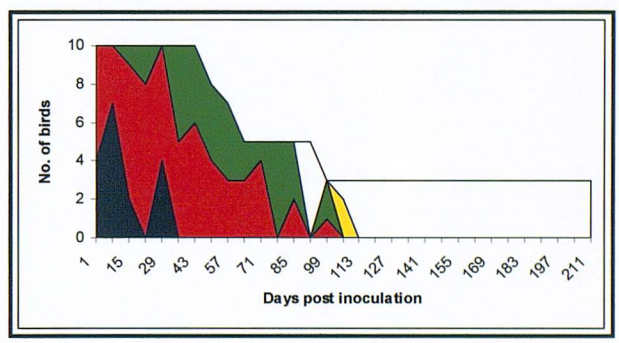
(d) NCTC 12900



(e) DM5



(f) NCTC 12900



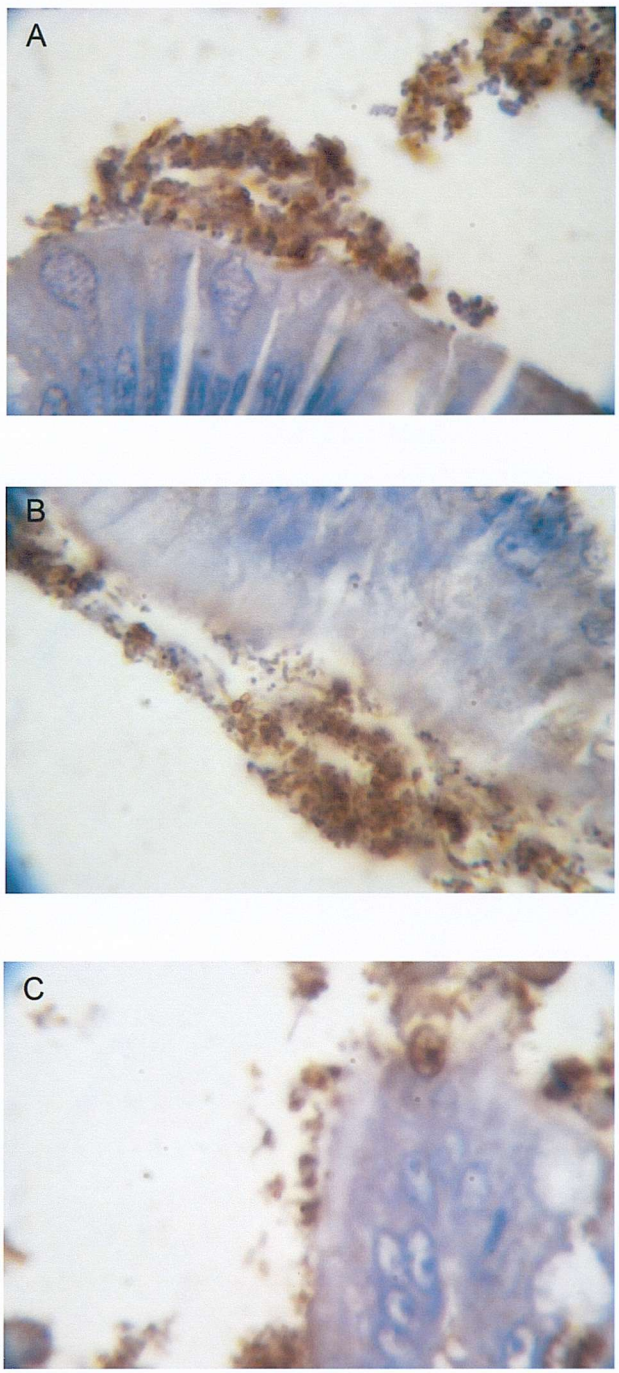
Recovery of bacteria form SPF chicks was scored as high (confluent), medium (>200 colonies) low (<200 colonies), positive (detected after enrichment only) and clear (no colonies).

Table 7.4. Significance (p) in the distribution of bacterial shedding of intimin and aflagellate deficient mutants in the presence of the wild-type parent isolate, NCTC 12900.

Day PI	Significance (p)		
	NCTC 12900 vs DM3 (intimin mutant)	NCTC 12900 vs DM4 (aflagellate mutant)	NCTC 12900 vs DM5 (intimin-aflagellate mutant)
1	0.179	0.393	0.075
8	0.466	0.019	0.001
15	0.364	0.099	0.062
22	0.370	0.002	0.006
29	0.212	<0.001	0.010
36	0.095	0.285	0.350
43	0.319	0.044	0.261
50	0.619	0.210	1.000
57	0.991	0.118	0.009
64	1.000	0.048	0.143
71	0.635	0.556	0.040
78	1.000	0.159	1.000
85	1.000	0.524	0.397
92	0.722	1.000	1.000
99	1.000	-	0.600
106	1.000	-	0.400
113	1.000	-	-
120	-	-	-
127	-	-	-
134	-	-	-
141	-	-	-
148	-	-	-
155	-	-	-
162	-	-	-
169	-	-	-
176	-	-	-
183	-	-	-
190	-	-	-
197	-	-	-
204	-	-	-
211	-	-	-

For the purpose of statistical analysis, each shedding score in Figure 7.1 was assigned a numerical score. i.e. high = 4, medium = 3, low = 2 positive after enrichment = 1 and clear = 0. The distributions of NCTC 12900 numerical scores were compared with the distributions of mutant numerical scores using the Kruskall-Wallis test.
PI; post inoculation.

Figure 7.2. Visualisation of attachment to the caeca of SPF chicks by bacteria specifically stained with anti-O157 sera during the first five days of experimental infection. Magnification x 1000.

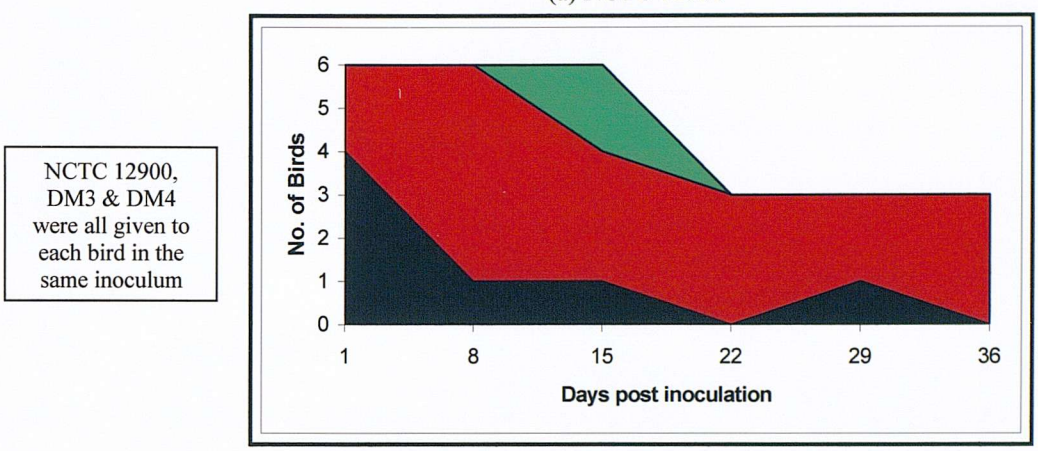


A = Caecal tissue from birds dosed orally with NCTC 12900 & DM3 (intimin mutant), B = Caecal tissue from birds dosed orally with NCTC 12900 & DM4, C = Caecal tissue from birds dosed orally with NCTC 12900 & DM5.

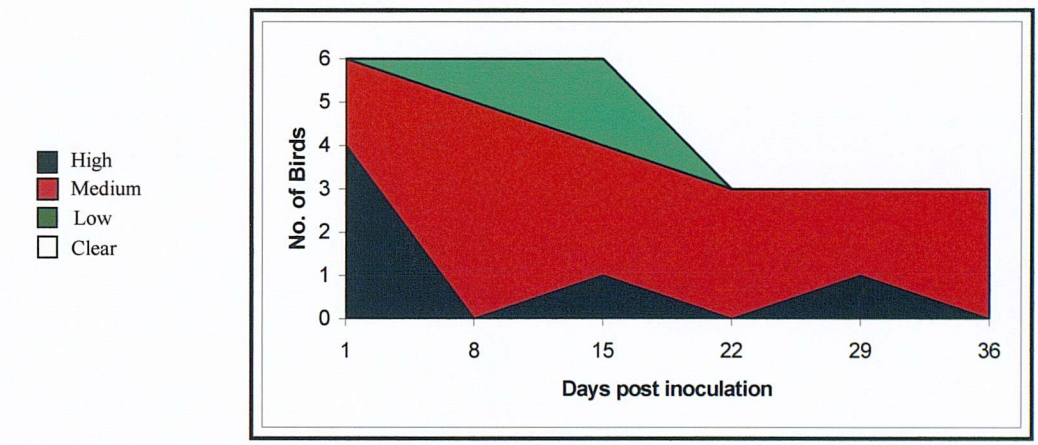
Each field of view was generally typical for each isolate.

Figure 7.3 The distribution of bacterial shedding, over time, by SPF chicks of the (a) wild-type parent isolate, NCTC 12900 (b) DM3 (intimin mutant) and (c) DM4 (aflagellate mutant) in the presence of each other.

(a) NCTC 12900



(b) DM3



(c) DM4

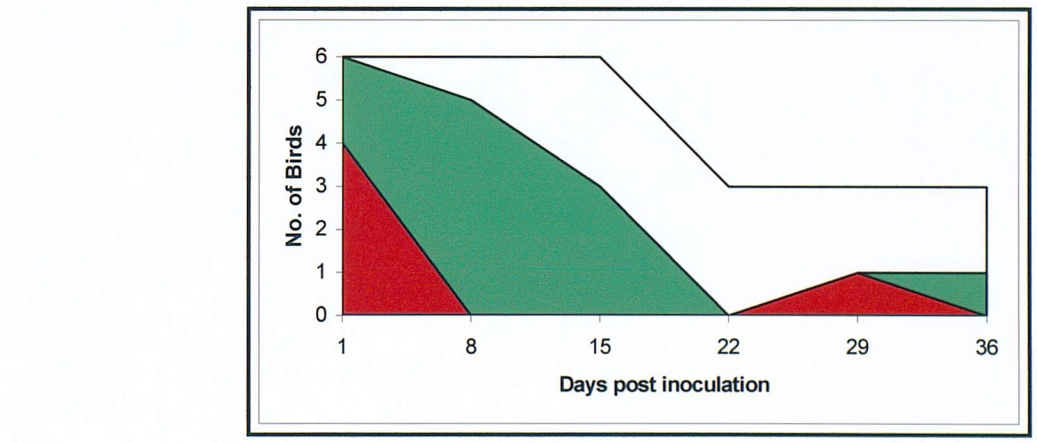


Table 7.5. Significance (p) in the distribution of bacterial shedding of intimin and aflagellate deficient mutants when both in the presence of the wild-type parent isolate, NCTC 12900.

Day post inoculation	NCTC 12900 vs DM3 (intimin mutant)	NCTC 12900 vs DM4 (aflagellate mutant)
1	1.000	0.033
8	0.546	0.002
15	1.000	0.022
22	1.000	0.100
29	1.000	0.300
36	1.000	0.100

Wild-type NCTC 12900 was compared to each mutant using the Wilcoxon-Mann-Whitney test. Exact p-values were calculated using StatXact software.

Table 7.6. Colonisation of SPF chicks by Stx negative *E. coli* O157:H7 NCTC 12900 and intimin (DM3) and aflagellate (DM4) deficient mutants when given together in the same inoculum to each bird.

Day 36 PI	NCTC 12900				DM3 (intimin mutant)					DM4 (aflagellate mutant)				
	No. birds positive	Mean log_{10}	Geometric mean	SD	No. birds positive	Mean log_{10}	Geometric mean	SD	p-value	No. birds positive	Mean log_{10}	Geometric mean	SD	p-value
Duodenum	1/3	0.35	2.24E+00	0.60	1/3	0.35	2.24E+00	0.60	1.000	0/3	0	0	0	1.000
Jejunum	3/3	1.99	9.77E+01	0.85	2/3	1.44	2.75E+01	0.85	0.800	0/3	0	0	0	0.100
Ileum	1/3	0.35	2.24E+00	0.60	1/3	0.35	2.24E+00	0.60	1.000	0/3	0	0	0	1.000
Colon	3/3	3.81	6.46E+03	0.97	3/3	4.58	3.80E+04	1.12	0.400	3/3	1.04	1.10E+01	0	0.100
Caeca	3/3	6.62	4.17E+06	0.63	3/3	6.49	3.09E+06	0.09	1.000	3/3	4.10	1.26E+04	0.66	0.100
C. tonsils	3/3	3.67	4.68E+03	0.14	3/3	4.30	2.00E+04	0.32	0.100	2/3	0.69	4.90E+00	0.60	0.100
Crop	3/3	2.95	8.91E+02	0.21	3/3	3.26	1.82E+03	0.17	0.300	1/3	0.35	2.24E+00	0.60	0.100

Post mortems were performed on the last day of the experiment.
PI; post inoculation. SD; standard deviation, p-value; significance in colonisation between each mutant and NCTC 12900.
Wild-type NCTC 12900 was compared to each mutant using the Wilcoxon-Mann-Whitney test. Exact p-values were calculated using StatXact software.

7.2 Discussion

The data in chapter 6 indicated that flagella, but not intimin may have a role to play in Stx-negative *E. coli* O157:H7 colonisation, invasion and persistence of SPF chicks. The purpose of the study reported here was to determine if intimin and aflagellate mutants were less able to colonise, invade and persist in SPF chicks when in the presence of the wild-type, NCTC 12900. A previously reported SPF chick study involving an *E. coli* isolate pathogenic for avian species demonstrated that flagella and fimbrial deficient mutants were less able to persist in SPF chicks when competing with the wild-type isolate (La Ragione *et al.*, 2000b).

As discussed in chapter 6, less association and quicker clearance of aflagellate mutants from the liver and spleen was observed, suggesting that flagella may be involved in low-level invasion. More importantly, as with the data in chapter 6, the aflagellate mutant also deficient for functional intimin was cleared quicker than the mutant unable to express active flagella alone. This lends weight to the hypothesis that both intimin and flagella may be involved in the low-level invasion of epithelial cells. However, this clearance in competitive index models reported here, may have been due to factors independent of the presence of NCTC 12900.

In this chapter, intimin and aflagellate mutants were still able to colonise the gastrointestinal tract of SPF chicks in similar numbers as compared to NCTC 12900 during the first five days of infection. This suggests that other factors other than *E. coli* O157 intimin or flagella may have been involved in the initial colonisation of SPF chicks. However, it is possible that the formation of aggregates of NCTC 12900 and mutant associated with the mucosal epithelium or residing in the gut contents may have contributed to this observation. Histological analysis did reveal that *E. coli* O157 attachment and micro-colony formation did occur and it seems reasonable to assume that these colonies may have consisted of wild-type and mutant.

On day 57 post inoculation, the intimin mutant was still associated in similar numbers to NCTC 12900 in the duodenum, jejunum, ileum, colon, caeca and caecal tonsils. However, on day 92 post inoculation, approximately 10-1000-fold fewer intimin mutant were recovered from the ileum, caeca, caecal tonsils and crop. Nevertheless, the intimin mutant persisted for as long as NCTC 12900, day 112 post inoculation and at none of the seventeen time points was any significance differences noted. This strengthens the observation noted in chapter 6, that intimin has little or no part to play in the colonisation and persistence of SPF chicks.

Both aflagellate mutants were still associated with SPF birds on day 57 post inoculation. Approximately, 10-fold fewer aflagellate mutant than wild-type were recovered from the jejunum, colon, and caecal tonsils and approximately 10-100-fold fewer aflagellate mutant, also deficient for intimin, was recovered from the duodenum, ileum, colon, caeca, caecal

tonsils and crop. On day 92 post inoculation, the aflagellate mutant was not recovered from any of the tissues examined, although two other birds were still shedding this mutant at the same time point, whereas the intimin-aflagellate mutant was only recovered from the caeca. In both competitive index groups involving aflagellate mutants, NCTC 12900 was shed for at least seven days more that both mutants. Furthermore, significant differences were noted for five out of fourteen time points for the aflagellate mutant and six out of sixteen time points for the intimin-aflagellate mutant. This suggests that although other factors may be involved in the long term persistence of *E. coli* O157:H7, active flagella may play a role in the levels of *E. coli* O157:H7 residing in the chick gut, especially in the contents of lumen where motility might be crucial for high levels of colonisation.

When an inoculum comprising of NCTC 12900, the intimin mutant and the aflagellate mutant was given to SPF chicks, far fewer aflagellate mutant than NCTC 12900 and intimin mutant was shed by birds. Also, at the end of the experiment, day 36 post inoculation, far fewer numbers of the aflagellate mutant were recovered from tissues that were positive for all three isolates. This strengthens the observations noted in this chapter and in chapter 6 that intimin has little or no role to play in colonisation and persistence, whereas flagella does have a role to play, although the specifics of that role remain unclear.

Eggs from each group were laid approximately 36 to 50 and 31 to 43 days after all aflagellate mutants and NCTC 12900 had been cleared from birds, respectively. This lends weight to the hypothesis discussed in chapter 6, that the maturation and onset of egg laying may help to clear *E. coli* O157:H7 residing in the gut of SPF chickens and that this clearance may be quicker for aflagellate *E. coli* O157 bacteria.

The aims of the study to be reported here was to establish if intimin and aflagellate mutants would be more attenuated and subsequently cleared from SPF birds when in the presence of the NCTC 12900. However, the data reported here suggests that although intimin and flagella may be involved in low-level invasion, mutants defective for intimin and flagella in the competitive index *in vivo* model exhibit trends similar to those observed for colonisation and persistence of SPF chicks when NCTC 12900 is absent. However, when the aflagellate mutant was competing with NCTC 12900 and the intimin mutant in the same birds, this mutant was more attenuated than when competing against NCTC 12900 only. Furthermore, the triple competitive index model endorsed the observation reported in chapter 6 that intimin is not required for the colonisation and persistence of NCTC 12900. Collectively, the data presented in this chapter suggests that cocktails of *E. coli* O157:H7 wild-type plus two or more mutants may show more significant differences in the colonisation, invasion and persistence for each *E. coli* O157 isolate in the SPF chick model.

Chapter 8

General discussion

World-wide, *E. coli* O157:H7 still remains one of the major causes of haemolytic colitis and haemolytic uraemic syndrome (HUS) in humans. The main vectors for transmission of this pathogen are cattle, sheep, faeces and faecal contaminated water, although other vectors including goats, pigs, deer, rabbits are important (Rice *et al.*, 2003). The colonisation and persistence of *E. coli* O157:H7 in cattle, sheep and piglets has been studied and the surface arrayed protein intimin has been implicated in playing a key role (Donnenberg *et al.*, 1993; Cornick *et al.*, 2002; Woodward *et al.*, 2003). However, no other *E. coli* O157:H7 virulence factor has been associated with the colonisation and persistence of these hosts, although Hoey *et al.*, (2003) have suggested that Stx1 binding to bovine intestinal crypt epithelial cells results in the localisation of lysosomes and the aboration of toxicity. Intimin binds to the type-III secreted protein Tir, and tissue culture assays have elucidated that intimin is required for localised adherence patterns, micro-colony and AE lesion formation (McKee & O'Brien, 1996; Liu *et al.*, 2002; Cookson & Woodward, 2003). However, intimin may be involved in other mechanisms of adherence, as Frankel *et al.* (1996) reported that the C-terminus of EPEC intimin can bind β1 integrins.

The incidence of *E. coli* O157:H7 birds is low (Wallace *et al.*, 1997) but experimental infection of young chicks has demonstrated that *E. coli* O157:H7 can persistently colonise this host (Beery *et al.*, 1985; Schoeni & Doyle, 1994) suggesting that poultry could become an important source of food-borne disease in humans. Whether intimin is involved in this process of persistent colonisation in experimentally infected chicks has not yet been elucidated. One study did indicate that a small molecular weight outer membrane protein expressed by *E. coli* O157:H7 was associated with adherence to chicken caeca (Zhao *et al.*, 1996), but whether other surface arrayed structures, such as flagella, are involved in *E. coli* O157:H7 persistent colonisation of the chick has not been reported. Therefore, the overall purpose of the study reported in this thesis, was to determine if intimin and flagella played a key role in *E. coli* O157:H7 persistent colonisation of SPF chicks.

Previous studies in 1-day-old chicks have involved Stx-positive *E. coli* O157:H7 isolates (Beery *et al.*, 1985; Starvic *et al*; 1993; Schoeni & Doyle, 1994; Sueyoshi & Nakazawa, 1994), whereas the isolate (NCTC 12900) used in this study was Stx-negative. Therefore, the question arose whether the Stx-negative isolate NCTC 12900 used in this study was representative of Stx- positive *E. coli* O157:H7 isolates in general. Type-1 and curli fimbriae have been shown to be important for the colonisation and persistence in SPF chicks by an

APEC O78:K80 isolate (La Ragione *et al.,* 2000a & b). Genotypic and phenotypic characterisation revealed that although the genes encoding type-1 fimbriae and curli genes were present in NCTC 12900, elaboration of these surface appendages was not detected. These findings are consistent with the findings of other studies, where *E. coli* O157:H7 isolates were unable to elaborate type-1 and curli fimbriae due to mutations in key fimbrial genes or regulator sites (Li *et al.*, 1997; Enami *et al.*, 1999; Iida *et al.*, 2001; Roe *et al.*, 2001; Uhlich *et al.*, 2001), although exceptions have been noted (Durno *et al.*, 1989; Uhlich *et al.*, 2001). Furthermore, since NCTC 12900 was able to colonise and persist in SPF chicks for several months, this suggests that type-1 and curli fimbriae are not required for these attributes. Interestingly, an unidentified surface appendage was elaborated by NCTC 12900. Recent genome sequencing of the *E. coli* O157:H7 genome has revealed the presence of five fimbrial operons, other than the type-1 and curli operons (Hayashi *et al.*, 2001; Perna *et al.*, 2001), including the long polar (LP) fimbrial operon. Recent studies have indicated that LP fimbriae play a role in localised adherence and micro-colony formation (Torres *et al.*, 2002). Whether the unidentified surface appendage detected in the present study was LP fimbriae or any other type of fimbrial structure remains to be determined.

Experimental infection of young chicks with Stx-positive *E. coli* O157:H7 has demonstrated that this host may be readily colonised and that this pathogen can persist for several months (Beery *et al.*, 1985; Schoeni & Doyle, 1994). Therefore, in the study in this thesis, it was important to determine if an Stx-negative *E. coli* O157:H7 isolate, a containment level II pathogen and therefore a safer strain to manipulate, was a convenient model strain to study chick colonisation. Initial *in vivo* characterisation studies of NCTC 12900 demonstrated that this isolate was able to colonise and persist in the chick for approximately 21 weeks post inoculation, indicating that Stx may play little or no role in colonisation of the gastrointestinal tract of the chicken. Whether this was typical of other Stx-negative *E. coli* O157:H7 isolates was not investigated. However, this hypothesis could have been tested by comparing the colonisation in SPF chicks of several Stx-negative *E. coli* O157:H7 isolates, including NCTC 12900, with an Stx-positive *E. coli* O157:H7 isolate and an Stx-negative *E. coli* O157:H7 isolate that had both toxins knocked out by mutagenesis. Furthermore, whether NCTC 12900 had completely lost the lysogenic phage carrying either Shiga-toxin or was phage positive only was not determined. If residues of phage were present, this would indicate that NCTC 12900 might have the capabilities to acquire Stx genes by horizontal transfer from the environment. NCTC 12900 was originally characterised at a public health laboratory in Austria during 1996. (PHLS, personal communication). Stx-positive and Stx-negative *E. coli* O157:H7 isolates that were isogenic have been isolated from patients with haemorrhagic colitis (Ostroff *et al.*, 1990; Feng *et al.*, 2001) but whether

NCTC 12900 has an isogenic Stx-positive sibling remains unclear. Total loss of phage plus toxin might indicate that NCTC 12900 belongs a different clonal lineage of *E. coli* O157:H7. Whittam *et al.* (1993) proposed that *E. coli* O157:H7 evolved from an EPEC O55:H7 like progenitor, already possessing the AE phenotype to intestinal cells, that acquired secondary virulence factors including Shiga toxins. However, clonal groups of *E. coli* O157:H7 are categorised into EHEC group 1 (Whittam & McGraw, 1996) by their intimin type (gamma) which suggests that NCTC 12900 belongs to the same clonal lineage as Stx-positive *E. coli* O157:H7 isolates.

The present study demonstrated that colonisation of the caeca and other tissues of the chick gastrointestinal tract were consistent with the findings of previous studies (Beery *et al.* 1985; Stavric *et al.* 1993; Schoeni & Doyle, 1994; Sueyoshi & Nakazawa, 1994). In terms of numbers of NCTC 12900 bacteria recovered in this study, the caecum was the site of persistent colonisation, which is in agreement with previously reported Stx-positive *E. coli* O157:H7 chick studies. A number of probable reasons for this might include the following. Firstly, the caecal environment is anaerobic, therefore excluding competition from aerobic bacteria. Secondly, the turnover of epithelial cells in the caeca is slower than in other gut tissues, therefore allowing bacteria to adhere for longer. Thirdly, there are deeper crypts in the caeca, allowing protection from the movement of the caecal contents. Fourthly, there is no peristalsis, therefore bacteria are less likely to be mechanically removed from this tissue and fifthly, the caecum is rich in organic matter, thus providing a nutrient rich environment.

Invasion of avian cell mono-layers by NCTC 12900 was noted. In the young chick, the liver was colonised transiently which may imply limited invasion but, as clearance from internal organs was rapid, invasion may have been limited to the very young animal and that intracellular survival was limited (Chart *et al.*, 2000). Genome sequencing of *E. coli* O157:H7 has revealed the presence of a second putative type III secretion system (Hayashi *et al.*, 2001, Perna *et al.*, 2001) that resembles the *inv/spa* locus in the SPI-1 pathogenicity island of *S. typhimurium*. Whether this locus contributed to NCTC 12900 invasion remains unclear.

Analysis of tissues from the gastrointestinal tract of birds did reveal during the first 5 days of experimental infection that NCTC 12900 had associated with epithelial surfaces, as detected by specific *E. coli* O157 antisera. More importantly, specifically stained anti-O157 bacteria associated with micro-colonies were also observed. Whether AE lesions were associated with individual bacteria remains unclear. Many clumps of bacteria specifically stained with anti-O157, and presumed to be NCTC 12900, were also detected that were not directly associated with gut epthelial surfaces, suggesting that NCTC 12900 are able to reside and survive in the contents of the gut. One SPF chick infection experiment designed for

histological analysis only, where birds were given orally 1 x 10^7 CFU of NCTC 12900 bacteria, indicated sparse to medium association of NCTC 12900 bacteria with chick gut surfaces, mostly the caecum. However, more association was noted between days 21 and 29 post inoculation. This observation may indicate that *E. coli* O157:H7 bacteria are able to adhere more efficiently to chick epithelia in more mature birds than in naïve birds. NCTC 12900 was able to form micro-colonies and AE lesions on an avian cell derived from the small intestines of 18-day-old SPF chick embryos. However, in this type of assay there are not the physiological barriers that are encountered with the gastrointestinal tract of SPF chicks. Possibly, a yet unidentified surface appendage, that is optimally expressed when SPF chicks are 3-4 weeks old, may be required to bring NCTC 12900 closer to the gut epithelial surface in a turbulent environment for subsequent intimate attachment and micro-colony formation. Characterisation of insertional fimbrial O157 mutants, other than type-1 or curli, in the SPF chick model, may clarify this hypothesis.

Previous studies by others have demonstrated that *E. coli* O157:H7 intimin was important for the colonisation of cattle, sheep and pigs (Donnenberg *et al.*, 1993; McKee & O'Brien, 1996; Cornick *et al.*, 2002; Woodward *et al.*, 2003). Flagella have been implicated in colonisation for other enteric pathogens. Studies of APEC O78:K80 flagella have revealed that this surface appendage was required for the penetration of mucus (La Ragione *et al.*, 2000a) and that an aflagellate APEC O78:K80 mutant was significantly less persistent in SPF chicks (La Ragione *et al.*, 2000b). Additionally, flagella have been implicated as an adherence mechanism in another enteric pathogen, *Salmonella enterica* serovar Enteritidis (Allen-Vercoe & Woodward, 1999; Dibb-Fuller *et al.*, 1999; Robertson *et al.*, 2000). However, it should be borne in mind that naturally ocurring *E. coli* O157:H- (non-flagellated) have been isolated from cattle and humans (Bielaszewska *et al.*, 2000; Kim *et al.*, 2001). Furthermore, there are many *E. coli* O157:H- isolates that have been associated with haemorrhagic colitis and HUS (Bielaszewska *et al.*, 1988; Schmidt *et al.*, 1999; O'Brien *et al.*, 2001). Nevertheless, whether intimin or flagella play a role in the mechanisms required for colonisation and persistence in poultry remained unclear. Therefore, the next step in this study was to determine, that if chickens were to become a reservoir for *E. coli* O157:H7, would intimin and flagella contribute to persistent colonisation within this host.

Before intimin and flagella deficient mutants were characterised in the 1-day-old SPF chick model, the adherence and invasion capabilities of each mutant was assessed using several tissue culture assays. With reference to avian and human tissue culture assays, the studies reported in this thesis demonstrated that intimin contributed to adhesion. However, with regard to invasion, intimin appeared only to be important for this phenotype with the human cell line. On both cell lines, fully functional intimin was required for the formation of

AE lesions and was also required for localised adherence patterns, especially the formation of micro-colonies. Flagella contributed significantly to the invasion of avian tissue culture cells, but not human tissue culture cells. However, a NCTC 12900 mutant unable to express intimin and flagella was significantly less able to invade avian tissue culture cells and was not internalised within human tissue culture cells. This indicates that this double mutation significantly reduced the invasion capabilities of NCTC 12900 suggesting that both surface arrayed proteins may be linked in this process.

NCTC 12900 does elaborate intimin γ and was able to form localised patterns of adherence with micro-colony formation and produce AE lesions on HEp-2 mono-layers (This study & Dibb-Fuller *et al.*, 2001). Additionally, the tissue culture studies described here were the first to show that NCTC 12900 was also able to form micro-colonies and induce AE lesions on an avian cell line derived from SPF chicks. A lack of active flagella did not affect AE lesion formation, localised adherence patterns or micro-colony formation. Whether flagella play an initial role in adhesion was not determined in this study. Possibly shorter adherence assays of between 5 and 60 minutes may have given insights into this possibility. Also, it may have been relevant to have also used a mucus secreting cell line and the chick gut explant assay for studying the adhesion and invasion patterns of aflagellate mutants. Previous studies characterising *E. coli* and *Salmonella* pathogens of avian origin have shown that flagella are important for the penetration of mucus layers (Allen-Vercoe & Woodward, 1999; La Ragione *et al.*, 2000a). Thus, it seems plausible that *E. coli* O157:H7 flagella may be involved in the same process. Interestingly, for each bacterial isolate, counts of internalised bacteria were significantly higher for Div-1 cells as compared to HEp2 cells. Possible explanations for this may be that either avian cell processes played an active role in the uptake of bacteria or there was greater expression of a cell receptor recognising a yet undefined surface structure. However, it should also be noted that this avian cell line was derived from the small intestines of 18-day-old embryos and may not be as differentiated as HEp-2 cells. This may indicate, that although invasion appears to involve *E. coli* O157:H7 specific factors, possible passive non-specific uptake may have also occurred. Furthermore, significantly more bacteria were internalised in the Div-1 cells than in the HEp-2 cells.

From avian tissue cultures assays it was reasonable to predict that intimin-deficient mutants may be less able to colonise 1-day-old SPF chicks, whereas an aflagellate mutant may colonise as well as the wild-type. It was also reasonable to predict that an intimin-aflagellate mutant may exhibit similar trends to the intimin mutant. Compared to NCTC 12900 the intimin mutant was associated in similar numbers with all tissues taken from SPF chicks during the first 5 days of *E. coli* O157:H7 infection, indicating that intimin may play little or no role in colonisation. The aflagellate and intimin-aflagellate mutant and NCTC

12900 were also associated in similar numbers with the tissues of the gastrointestinal tract, but fewer numbers and more rapid clearance of the aflagellate mutants from the liver and spleen was noted. At 8 weeks and 13 weeks post inoculation, bacterial enumeration suggested that, compared to NCTC 12900, the aflagellate and intimin-aflagellate mutant were less able to colonise the tissues of the gastrointestinal tract, whereas, the intimin mutant was still able to colonise in similar numbers. More importantly, the intimin mutant when given to birds on its own, was still recovered from the gastrointestinal tract of birds, 30 weeks post inoculation. Furthermore, the intimin mutant was able to persist in SPF chickens for at least 42 days more than NCTC 12900, and when in the presence of NCTC 12900, the intimin mutant was cleared from birds at the same time point as the wild-type. Whether the absence of intimin made NCTC 12900 "fitter" requires further investigation.

Persistence of the aflagellate and the intimin-aflagellate mutant on their own or when in the presence of NCTC 12900 did not last as long as the wild-type. Nevertheless, the aflagellate and intimin-aflagellate mutants were still recovered from birds approximately 10 to 12 weeks after experimental infection. However, significantly less of each type of aflagellate mutant was shed from birds compared to NCTC 12900, indicating that flagella may be required for persistence within the caeca. Trends observed in competitive index studies were similar to single inocula studies, with the intimin mutant being the exception, indicating that colonisation and persistence and the eventual clearance of each mutant may have occurred independently of the wild-type. Nevertheless, the intimin-aflagellate mutant and the aflagellate mutant both exhibited similar trends for shedding, further strengthening the hypothesis that intimin has little or no role to play in the colonisation and persistence of SPF chickens.

Histological analysis of NCTC 12900 and intimin and flagella deficient mutant interactions with the gastrointestinal tract of chicks revealed that *E. coli* O157 bacteria were associated with gut epithelial surfaces. During the first 5 days of infection, adherent bacteria were seen mostly on the epithelial surfaces of the ileum, colon and caeca, with the caeca usually having the most adherent bacteria, although clusters of specifically stained *E. coli* O157 bacteria were not directly associated with the chick gut. In single inocula experiments micro-colonies were observed for NCTC 12900 and the aflagellate mutant and although the intimin and intimin-aflagellate mutant were found attached to the caecal epithelium, no densely packed micro-colonies were observed. This finding would suggest that although intimin appears to be required for micro-colony formation, other *E. coli* O157:H7 factors, possibly surface appendages, were involved in adherence. In competitive index studies, bacterial attachment was noted in the caeca. Whether these micro-colonies comprised of NCTC 12900 only or wild-type plus mutant remains unclear. Since one bird was taken from

each group for histological analysis during the five 5 days of infection, any trends noted between wild-type and mutant are difficult to justify. No tissues were taken from older birds at *post mortem* days 56 and 92. In hindsight, it may have been beneficial to examine tissues from birds at these time points, especially caecal tissue, for any bacterial attachment.

Analysis of eggs revealed that *E. coli* O157:H7 (NCTC 12900) contaminated the shells of eggs but not the contents, which is consistent with a previously published study (Schoeni & Doyle, 1994). However, whether contamination occurred on the surface of egg shells or within the pores of the egg shells remains unclear. No contamination of egg contents by NCTC 12900 suggests that vertical transmission of this pathogen to the ovaries of hens, which does occur with *S.* Enteritidis (Guard-Petter, 2001), does not occur with *E. coli* O157:H7. In the study reported here, NCTC 12900 was cleared just before or soon after the onset of egg laying. This observation occurred with three separate NCTC 12900 single inoculation SPF chick experiments, suggesting that the time period of persistent colonisation by this particular *E. coli* O157:H7 isolate may be governed by the physiological changes occurring within birds due to the maturation and onset of egg laying. Furthermore, during these physiological changes, the levels of shedding were increased until the onset of egg laying, from SPF birds given separately, NCTC 12900 and intimin and aflagellate mutants. Interestingly, the intimin mutant was able to persist after the onset of egg laying, whereas, both aflagellate mutants were cleared before the onset of egg laying. A possible hypothesis for this may be that the physiological changes within birds at the onset of egg laying may aid quicker clearance of *E. coli* O157 that are unable to express active flagella. A lack of intimin expression appeared to be advantageous for sustained persistence after the onset of egg laying, but why this would be so remains unclear. It may be that knocking out intimin initiated the expression or upregulation of another virulence factor, possibly flagella, although this was not sought after *in vitro*. Furthermore, genotypical analysis of the intimin mutant did not suggest any other disruptions to the *eae* gene, other than the insertion of the antibiotic resistance cassette. Nevertheless, it is possible that insertional mutagenesis may have up regulated other genes by altering the DNA topology, thus allowing regulators access to promoter sites that are otherwise hidden during intimin expression. However, Farris *et al.* (1998) have shown that EPEC BipA (virulence regulator) mutants failed to trigger AE lesions, but are more motile than the wild-type, suggesting that this protein negatively regulates flagella-mediated cell motility. BipA is also present in *E. coli* O157:H7 and studies involving NCTC 12900 have indicated that the expression of intimin is blocked in the absence of this regulator (Grant *et al.*, 2003) and that NCTC 12900 BipA negatively regulates flagella-mediated cell motility. Furthermore, Grant and co-workers indicated that NCTC 12900 BipA controls the expression of LEE encoded genes including *eae* via transcription of

the *ler* gene. Additionally, Elliott *et al.* (2000) have demonstrated that the Ler protein positively regulates the *E. coli* O157:H7 Tir operon from which intimin is expressed. Therefore it is possible, although highly speculative, that by knocking out intimin, up-regulation of flagella did occur through a negative feedback system involving the Ler and BipA regulators. To test if NCTC 12900 flagella had been unregulated, comparisons of the rate of motility between the intimin mutant and NCTC 12900 could have been measured, over time, on sloppy agar plates and by determining the relative quantification of flagella between intimin mutant and wild-type NCTC 12900 by direct binding ELISAs. Additionally, counting and comparing the number of flagella associated with the intimin mutant and NCTC 12900 by electron microscopy would have confirmed any up-regulation of this surface appendage.

A pilot competitive index study was also performed where a small group of birds were given orally, NCTC 12900, the intimin mutant and the aflagellate mutant in the same inoculum. The distribution of shedding of *E. coli* O157 showed that NCTC 12900 and the intimin mutant were shed in relatively high numbers, whereas the aflagellate mutant was shed in significantly less numbers. *Post mortem* examinations at the end of the experiment, day 36, revealed that NCTC 12900 and the intimin mutant were associated in similar numbers with all the tissues examined from the gastrointestinal tract, with the highest numbers being recovered from the caeca. However, the aflagellate mutant was only recovered from the colon, caeca and caecal tonsils. Furthermore, fewer numbers of aflagellate mutant was recovered from these positive tissues. The results from this study indicate that inocula comprising of wild-type plus two or more mutants may have given better insights into the long term colonisation and persistence characteristics of each isolate. This data also suggests that more differences may be noted by competing two single mutants with their respective wild-type, than competing a multiple mutant with the same deficiencies with the respective wild-type.

It should be kept in mind that when interpreting the results obtained from chick experiments, re-infection of birds by NCTC 12900 and intimin and aflagellate mutants from the environment probably occurred. In the chick studies reported here, each isolate was detected in the environment only when birds were still shedding the same *E. coli* O157 isolate. This possibility raises two key questions. Firstly, were the wild-type and each mutant able to survive in the environment in similar numbers, or did a deficiency in a surface arrayed structure, especially involving flagella, reduce survival in the environment. Secondly, if desiccation of an aflagellate mutant in the environment occurred, was this due specifically to environmental factors or attenuation when passing through the gastrointestinal tract of the chick. To test this hypothesis, several studies could have been performed to study the lateral

and environmental transmissibility of NCTC 12900 and each mutant. Firstly, fresh isolators could have been sprayed either separately or together with each *E. coli* O157 isolate and after various times SPF birds could have been placed in the isolator to assess the transmissibility of O157 bacteria directly from the environment. Secondly, SPF chicks inoculated either separately or together with each *E. coli* O157 isolate, would be allowed to shed for 24 hours, removed, and then SPF birds could have been placed in the isolator to assess environmental transmissibility of O157 bacteria after passing through animals. Thirdly, inoculation-free birds could have been placed with inoculated birds to determine the levels of colonisation by each *E. coli* O157 isolate.

The vast amount of data obtained from colonisation, invasion and persistence studies indicates that flagella play an unidentified role in the levels of O157 bacteria associated with avian tissues. Importantly, as noted with the invasion of an avian cell line, flagella might be involved in the penetration of avian epithelial surfaces. Histological analysis did not strongly suggest that adherence to chick gut surfaces was essential for colonisation. More importantly, intimin does not appear to be involved in the sustained persistence of O157 bacteria in SPF chicks, suggesting that intimin is not required for the colonisation of poultry. Since intimin and aflagellate mutants were still able to colonise and persist in birds this suggests that other O157 factors may play a significant role in colonisation and persistence. However, certain disadvantages with the chick model should be noted. Firstly, the 1-day-old SPF chick intestine is lined with muconium, which provides a nutrient rich environment for the test inoculum to multiply. Secondly, due to the immature immune response of 1-day-old SPF chicks intimin and aflagellate mutants would have been able to replicate without the effects of clearance, thus allowing these bacteria to reside in the caecal tonsils in high numbers. Thirdly, 1-day-old SPF chicks have immature immunocompetence in the intestine (Jeurissen *et al.*, 1989) and it is known that chicks hatch with immature T lymphocytes which become fully responsive at around 4 days of age (Lowenthal *et al.*, 1994). Therefore, it is likely that a nutrient rich environment combined with an immature systemic and mucosal immune system allowed intimin and aflagellate mutants to multiply and colonise the chick.

Previously reported mammalian studies of *E. coli* O157:H7 infection have demonstrated that intimin is required for intimate attachment to gut surfaces (Donnenberg *et al.*, 1993; McKee & O'Brien, 1996) and the intimin mutant used in this study was attenuated in 6-week-old sheep (Woodward *et al.*, 2003) suggesting that intimin may only be required for persistence in mammalian hosts. The tissue structure of the avian host is markedly different from that of mammalian hosts, including ruminants (Dyce *et al.*, 1987). Chicks are mono-gastric, have gut-associated lymphoid tissue (GALT) only in the ileum and the gastrointestinal tract is more acidic. This may partly explain why an intimin mutant can

persist in SPF chickens longer than the wild-type, but persist for a lesser time than the wild-type in 6-week-old lambs. However, other factors in sheep including the presence of naturally acquired attaching and effacing *E. coli* (Wales *et al.*, 2003) may haved played a role in this observation. Nevertheless, studies by Fitzhenry *et al.*, (2000) have shown that intimin type influences the site of intestinal mucosal colonisation in humans. Therefore, it is possible that *E. coli* O157:H7 with intimin type γ do not exhibit a tissue tropism in chicks, although the avian tissue cultures studies presented here demonstrated that NCTC 12900 was able to form densely packed micro-colonies and AE lesions, and micro-colonies on chick caecal epithelia. However, a recent study by La Ragione *et al.* (2002) did show that an APEC O86:K61 isolate possessing intimin type γ was able to persist well in the 1-day-old SPF chick model and although APEC O86:K61 micro-colonies were observed in the caeca, no evidence for AE lesions was gained. Interestingly, La Ragione *et al.* (2003) have demonstrated that an *E. coli* O103 isolate expressing intimin type β did induce AE lesions on the caecal mucosa of Red-Legged partridges.

E. coli O157:H7 experimental infection of chicks has been reported, and the studies conducted here are the first to analyse avian *in vitro* adherence, invasion and avian *in vivo*, colonisation, invasion and persistence properties of intimin and flagella of an *E. coli* O157:H7 isolate. The lack of evidence for an intimin associated role in colonisation is consistent with other animal studies as AE lesions are not readily detected in conventional weaned sheep and cattle experimentally infected with *E. coli* O157:H7 (Woodward *et al.*, 1999; Wales *et al.*, 2001). However, Cornick *et al.* (2002) have indicated that intimin facilitates colonisation in young adult cattle and sheep, and Woodward *et al.* (2003) did demonstrate attenuated persistent colonisation by the intimin mutant described in this thesis. Whether the mechanisms of colonisation in ruminants and chicks are synonymous is debatable, but in this and previous studies, the chick appears to be a useful surrogate model although some authors disagree (Sandhu and Gyles, 2002). Further characterisation of other surface arrayed molecules in this *in vivo* model, such as lipopolysaccharide, may further determine what *E. coli* O157:H7 factors contribute to colonisation and persistence.

Previously reported studies, demonstrated that poultry are a potential reservoir for Stx-positive, and in the study presented here, Stx-negative, *E. coli* O157:H7. A recent mammalian study using a suckling piglet model of infection demonstrated that vaccination with purified intimin protected piglets from *E. coli* O157:H7 infection (Dean-Nystrom *et al.*, 2002) and monoclonal antibodies specific to Stx2 did prevent systemic complications in mice (Mukherjee *et al.*, 2002). However, if this pathogen was to infect poultry flocks, and from the data obtained in this study, it would appear that vaccines against intimin and possibly flagella may not clear *E. coli* O157:H7 from birds. One way forward to reduce or clear *E. coli*

O157:H7, if it were to become resident in poultry, may be the introduction of competitive exclusion strategies. A study by Hakkinen & Schneitz, (1996) did reveal that a commercially available competitive exclusion product did significantly reduce *E. coli* O157:H7 in the caeca of chicks and protection against this pathogen was also evident in chicks, especially layers, when birds were administered anaerobic cultures derived from the faecal microflora of adult birds free from *E. coli* O157:H7 (Stavric *et al.*, 1992). Studies from our laboratory have demonstrated that *Bacillus subtilis* spores did significantly reduce the levels of APEC O78:K60 in the caeca of poultry (La Ragione *et al.*, 2001). Ongoing studies in our laboratory at present are characterising the effect of *Bacillus subtilis* spores and other probiotic agents on the levels of *E. coli* O157:H7 in experimentally infected poultry.

In summary, Stx-negative *E. coli* O157:H7 isolate NCTC 12900 was suitable for persistent colonisation studies in poultry. Furthermore, low-level contamination of egg shells was noted, suggesting and agreeing with other studies that this pathogen could potentially infect this host. Although, the intimin and flagella deficient mutants constructed showed that flagella, but not intimin contributed to colonisation and persistence in the SPF chick model, it is reasonable to assume that other uncharacterised surface arrayed proteins probably contribute to these attributes.

References

Abe. H., Tatsuno, I., Tobe, T., Okutani, A., and Sasakawa, C. 2002. Bicarbonate ion stimulates the expression of locus of enterocyte effacement-encoded genes in enterohemorrhagic *Escherichia coli* O157:H7. Infection and Immunity. **70,** 3500-3509.

Adu-Bobie, J., Frankel, G., Bain, C., Goncalves, A. G., Trabulsi, L. R., Douce, G., Knutton, S., and Dougan, G. 1998. Detection of intimins alpha, beta, gamma, and delta, four intimin derivatives expressed by attaching and effacing microbial pathogens. Journal of Clinical Microbiology. **36,** 662-668.

Afflerbach, H., Schroder, O., and Wagner, R. 1999. Conformational changes of the upstream DNA mediated by H-NS and FIS regulate E. coli RrnB P1 promoter activity. Journal of Molecular Biology. 286, 339-353.

Ahmed, S., and Donaghy, M. 1998. An outbreak of *Escherichia coli* O157:H7 in Central Scotland. In *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. Edited by Kaper, J. B., and O'Brien A. D. ASM Press, Washington, D.C. pp. 59-65.

Akashi, S., Joh, K., Tsuji, A., Ito, H., Hoshi, H., Hayakawa, T., Ihara, J., Abe, T., Hatori, M., Mori, T., and Nakamura, T. 1994. A severe outbreak of haemorrhagic colitis and haemolytic uraemic syndrome associated with *Escherichia coli* O157:H7 in Japan. European Journal of Pediatrics. **153,** 650-655.

Allen-Vercoe, E., Dibb-Fuller, M. P., Thorns, C. J., and Woodward, M. J. 1997. SEF17 fimbriae are essential for convoluted colonial morphology of *Salmonella enteritidis*. FEMS Microbiology Letters. **153,** 33-42.

Allen-Vercoe, E., Sayers, A. R., and Woodward, M. J. 1999. Virulence of *Salmonella enterica* serotype Enteritidis aflagellate and afimbriate mutants in a day-old chick model. Epidemiology and Infection. **122,** 395-402.

Allen-Vercoe, E., and Woodward, M. J. 1999. The role of flagella, but not fimbriae, in the adherence of *Salmonella enterica* serotype Enteritidis to chick gut explant. Journal of Medical Microbiology. **48,** 771-780.

Allison, L. J, Carter, P. E., and Thomson-Carter, F. M. 2000. Characterization of a recurrent clonal type of *Escherichia coli* O157:H7 causing major outbreaks of infection in Scotland. Journal of Clinical Microbiology. **38,** 1632-1635

Amor, K., Heinrichs, D. E., Frirdich, E., Ziebell, K., Johnson, R. P., and Whitfield, C. 2000. Distribution of core oligosaccharide types in lipopolysaccharides from *Escherichia coli*. Infection and Immunity. **68,** 1116-1124.

An, H., Fairbrother, J. M., Dubreuil, J. D., and Harel, J. 1997. Cloning and characterisation of the *eae* gene form a dog attaching and effacing *Escherichia coli* strain 4221. FEMS Microbiology Letters. **148,** 239-245.

Armstrong, G. L., Hollingsworth, J., and Morris J. G. 1996. Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. Epidemiological Reviews. **18,** 29-51.

Baehler, A. A., and Moxely, R. A. 2000. *Escherichia coli* O157:H7 induces attaching-effacing lesions in large intestinal mucosal explants from adult cattle. FEMS Microbiology Letters. **185,** 239-242.

Baehler, A. A., and Moxely, R. A. 2002. Effect of culture conditions on *Escherichia coli* O157:H7-mediated attaching-effacing in a bovine large intestinal mucosal explant model. FEMS Microbiology Letters. **212,** 107-110.

Bain, C., Keller, R., Collington, G. K., Trabulsi, L. R., and Knutton, S. 1998. Increased levels of intracellular calcium are not required for the formation of attaching and effacing lesions by enteropathogenic and enterohemorrhagic *Escherichia coli*. Infection and Immunity. **66,** 3900-3908.

Baldwin, T., Ward, J. W., Aitken, A., Knutton, S., and Williams, P. H. 1991. Elevation of intracellular free calcium levels in HEp-2 cells infected with enteropathogenic *Escherichia coli*. Infection and Immunity. **59,** 1599-1604.

Barrett, T.J., Lior, H., Green, J. H., Khakhria, R., Wells, J. G., Bell, B. P., Greene, K D., Lewis, J., and Griffin, P. M. 1994. Laboratory investigation of a multistate food-borne

outbreak of *Escherichia coli* O157:H7 by using pulse-field gel electrophoresis and phage typing. Journal of Clinical Microbiology. **32,** 3013-3017.

Barrett, T. J., Potter, M. E., and Wachsmuth, I. K. 1989. Continuous peritoneal infusion of Shiga-like toxin II (SLT II) as a model for SLT II-induced diseases. Journal of Infectious Diseases. **159,** 774-777.

Barua, S., Yamashino, T., Hasegawa, T., Yokoyama, K., Torii, K., and Ohta, M. 2002. Involvement of surface polysaccharides in the organic acid resistance of Shiga toxin-producing *Escherichia coli* O157:H7. Molecular Microbiology. **43,** 629-640.

Bassler, B. L., Wright, M., and Silverman, M. R. 1994. Sequence and function of LuxO, a negative regulator of luminescence in *Vibrio harveyi.* Molecular Microbiology. 12, 403-412.

Bastian, S. N., Carle, I., and Grimont, F. 1998. Comparison of 14 PCR systems for the detection and subtyping of stx genes in Shiga toxin-producing *Escherichia coli.* Research in Microbiology, **149,** 457-472.

Baur, M. E., and Welch, R. A. 1996. Characterisation of an RTX toxin from enterohaemorrhagic *Escherichia coli* O157:H7. Infection and Immunity. **64,** 167-175.

Beery, J. T., Doyle, M. P., and Schoeni, J. L. 1985. Colonisation of chicken caeca by *Escherichia coli* associated with hemorrhagic colitis. Applied and Environmental Microbiology. **49,** 310-315.

Bell, B. P., Goldoft, M., Griffin, P. M., Davis, M. A., Gordon, D. C., Tarr, P. I., Bartleson, C. A., Lewis, J. H., Barrett, T. J., Wells, J. G., Baron, R., and Kobayashi, J. A. 1994. A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience. Journal of the American Medical Association. **272,** 1349-1353.

Bellin, T., Pulz, M., Matussek, A., Hempen, H-G., and Gunzer, F. 2001. Rapid detection of enterohemorraghic *Escherichia coli* by real-time PCR with fluorescent hybridisation probes. Journal of Clinical Microbiology. **39,** 370-374.

Belteramетti, F., Kresse, A. U., and Guzmán, C. A. 1999. Transcriptional regulation of the *esp* genes of enterohaemorrhagic *Escherichia coli*. Journal of Bacteriology. **181,** 3409-3418.

Bennett, A. R., MacPhee, S., and Betts, R. P. 1996. The isolation and detection of *Escherichai coli* O157 by use of immunomagnetic separation and immunoassay procedures. Letters in Applied Microbiology. **22,** 237-243.

Benz, I., and Schmidt, M. A. 1993. Diffuse adherence of enteropathogenic *Escherichia coli* strains-processing of AIDA-1. International Journal of Medical Microbiology, Virology, Parasitology, and Infectious Diseases. **278,** 197-208.

Bettelheim, K. A. 1998. Studies of *Escherichia coli* on Rainbow Agar ™ O157 with particular reference to enterohaemorrhagic *Escherichia coli* (EHEC). Microbiology and Immunology. **42,** 265-269.

Beutin, L., Aleskic, S., Zimmermann, S., and Gleier, K. 1994. Virulence factors and phenotypic traits of verotoxigenic strains of *Escherichia coli* isolated from patients in Germany. Medical Microbiology and Immunology. (Berlin). **183,** 13-21.

Bhan, M. K., Raj, P., Levine, M. M., Kaper, J. B., Bhandari, N., Srivastava, R., Kumar, R., and Sazawal, S. 1989. Enteroaggregative *Escherichia coli* associated with persistent diarrhoea in a cohort of rural children in India. Journal of Infectious Diseases. **159,** 1061-1064.

Bieber, D., Ramer, S. W., Wu, C. Y., Murray, W. J., Tobe, T., Fernandez, R., and Schoolnik, G. K. 1998. Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *E .coli*. Science. **280,** 2114-2118.

Bielaszewska, M., Schmidt, H., Karmali, M. A., Khakhria, R., Janda, J., Blahova, K., and Karch, H. 1998. Isolation and characterization of sorbitol-frementing Shiga toxin (Verocytotoxin)-producing *Escherichia coli* O157:H- strains in the Czech Republic. Journal of Clinical Microbiology. **36,** 2135-2137.

Bielaszewska, M., Schmidt, H., Liesegang, A., Prager, R., Rabsch, W., Tschape, H., Cizek, A., Janda, J., Blahova, K., and Karch, H. 2000. Cattle can be a reservoir of

sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H(-) strains and a source of human diseases. Journal of Clinical Microbiology. **38,** 3470-3473.

Bilge, S. S., Clausen, C. R., Lau, W., and Moseley, S. L. 1989. Molecular characteristics of a fimbrial adhesin F1845, mediating diffuse adherence of diarrhoea-associated *Escherichia coli* to HEp-2 cells. Journal of Bacteriology. **171,** 4281-4289.

Bilge, S. S., Vary JR, J. C., Dowell, S. F., and Tarr, P. I. 1996. Role of the *Escherichia coli* O157:H7 O side chain in adherence and analysis of an *rfb* locus. Infection and Immunity. **64,** 4795-4801.

Booher, S. L., Cornick, N. A., and Moon, H. W. 2002. Persistence of *Escherichia coli* O157:H7 in experimentally infected swine. Veterinary Microbiology. **89,** 69-81.

Bray, J. 1945. Isolation of antigenically homogenous strain *Bacterium coli Neopolitanum* from summer diarrhoea of infants. Journal of Pathology and Bacteriology. **57,** 239-247.

Brenner, D. J. 1984. Family I. Bergey's Manual of Systamatic Bacteriology, Vol. 1. Editors Kreig, N. R., and Holt, J. G., Williams and Wilkins, USA. pp. 408-420.

Breuer, T., Benkel, D. H., Shapiro, R. L., Hall, W. N., Winnett, M. M., Linn, M. J., Neimann, J., Barrett, T. J., Dietrich, S., Downes, F. P., Toney, D. M., Pearson, J. L., Rolka, H., Slutsker, L., and Griffin, P. M. 2001. A multistate outbreak of *Escherichia coli* O157:H7 infections linked to alfalfa sprouts grown from contaminated seeds. Emerging Infectious Diseases. **7,** 977-982.

Brinton, C. C. Jr. 1959. Non-flagellar appendices of bacteria. Nature. **183,** 782-786.

Brown, C. A., Harmon, B. G., Zhao, T., and Doyle, M. P. 1997. Experimental *Escherichia coli* O157:H7 carriage in calves. Applied and Environmental Microbiology. **63,** 27-32.

Brunder, W., Schmidt, H., Frosch, M., and Karch, H. 1999. The large plasmids of Shiga-toxin-producing *Escherichia coli* (STEC) are highly variable genetic elements. Microbiology. **145,** 1005-1114.

Brunder, W., Schmidt, H., and Karch, H. 1996. KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. Microbiology. **142,** 3305-3315.

Brunder, W., Schmidt, H., and Karch, H. 1997. EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. Mololecular Microbiology. **24**, 767-778.

Brunton, J. 1994. Molecular biology and role in disease of Verotoxins (Shiga-like) toxins of *Escherichia coli*. In Molecular Genetics of Bacterial Pathogenesis. Editors Miller, V. L., Kaper, J. B., Portnoy, D. A., and Isberg, R. R. ASM press. Washington, D.C.

Burland, V., Shao, Y., Perna, N. T., Plunkett III, G., Sofia, H. J., and Blattner, F. R. 1998. The complete DNA sequence and analysis of the large virulence plasmid of *Escherichia coli* O157:H7. Nucleic Acids Research. **15**, 4196-4204.

Cantarelli, V. V., Takahashi, A., Akeda, Y., Nagayama, K., and Honda, T. 2000. Interaction of enteropathogenic or enterohaemorrhagic *Escherichia coli* with HeLa cells results in translocation of cortactin to the bacterial adherence site. Infection and Immunity. **68,** 382-386.

Cantey, J. R., and Moseley, S. L. 1991. Hela cell adherence, actin aggregation, and invasion by non-enteropthogenic *Escherichia coli* possessing the *eae* gene. Infection and Immunity. **59,** 3924-3929.

Caroff, M., Bundle, D. R., and Perry M. B. 1984. Structure of the O- chain of the phenol-phase soluble cellular lipopolysaccharide of *Yersinia enterocolitica* serotype O9. European Journal of Biochemistry. **139,** 195-200.

Carter A. O., Borczyk, A. A., Carlson, A. A., Harvey, B., Hockin, J. C., Karmali, M. M., Krishman, M. A. C., Korn, D. A., and Lior, H. 1987. A severe outbreak of *Escherichia coli* O157:H7-associated haemorrhagic colitis in a nursing home. New England Journal of Medicine. 317, 1496-1500.

Carter, G. R., Chengappa, M. M., and Roberts, A. W. 1995. Essentials of Veterinary Microbiology. 5th edition, Williams and Wilkins, United Kingdom.

Cassels, F. J., and Wolf, M. K. 1995. Colonisation factors of diarrheagenic *E. coli* and their intestinal receptors. Journal of Industrial Microbiology. **15,** 214-226.

Celli, J., Deng, W., and Finlay, B.B. 2000. Enteropathogenic *Escherichia coli* (EPEC) attachment to epithelial cells: exploiting the host cell cytoskeleton from the outside. Cellular Microbiology. **2,** 1-9.

Chambers, H. F., and Neu, H. C. 1995. Other β lactam antibiotics. In Principles and Practices of Infectious Diseases. Edited by Mandell, G. L., Bennett, J. E., and Dolin, R. J. London. Churchill Livingstone Inc, UK. pp. 264-272.

Chapman, P. A., Siddons, C. A., Cerdan Malo, A. T., and Harkin, M. A. 1997. A 1-year study of *Escherichia coli* O157 in cattle, sheep, pigs and poultry. Epidemiology and Infection. **119,** 245-250.

Chapman, P. A., Siddons, C. A., Manning, J., and Cheetman, C. 1997. An outbreak of infection due to verocytotoxin-producing *Escherichia coli* O157 in four families: the influence of laboratory methods on the outcome of the investigation. Epidemiology and Infection. **119,** 113-119.

Chapman, P. A., Wright, D. J., and Siddons, C. A. 1994. A comparison of immunomagnetic separation and direct culture for the isolation of verotcytoxin-producing *Escherichia coli* O157 from bovine faeces. Journal of Medical Microbiology. **40,** 424-427.

Chart, H., and Jenkins, C. 1999. The serodiagnosis of infections caused by verocytotoxin-producing *Escherichia coli*. Journal of Applied Microbiology. 86, 731-740.

Chart, H., Okubadejo, O. A., and Rowe, B. 1992. The serological relationship between *Escherichia coli* O157 and *Yersinia enterocolitica* O9 using serum from patients with brucellosis. Epidemiology and Infection. **108,** 77-85.

Chart, H., Scotland, S. M., and Rowe, B. 1989. Serum antibodies to *Escherichia coli* serotype O157:H7 in patients with haemolytic uraemic syndrome. Journal of Clinical Microbiology. **27,** 285-290.

Chart, H., Smith, H. R., La Ragione, R. M., and Woodward M. J., 2000. An investigation into the pathogenic properties of *Escherichia coli* strains BLR, BL21, DH5α and EQ1. Journal of Applied Microbiology. **89,** 1048-1058.

Cheville, A. M., Arnold, K. W., Burchrieser, A. C., Cheng, C, M., and Kaspar, C. W. 1996. *rpoS* regulation of acid, heat, and salt tolerance in *Escherichia coli* O157:H7. Applied and Environmental Microbiology. **62,** 1822-1844.

Ciampi, M. S, Schmid, M. B, and Roth, J. R. 1982. Transposon Tn10 provides a promoter for transcription of adjacent sequences. Proceedings in the National Academy of Science. USA. **79,** 5016-5020.

Cizek, A., Alexa, P., Literak, I., Hamrik, J., Novak, P., and Smola, J. 1999. Shiga toxin-producing *Escherichia coli* O157 in feedlot cattle and Norwegian rats from a large-scale farm. Letters in Applied Microbiology. **28,** 435-439.

Cizek, A., Literak, I., and Scheer, P. 2000. Survival of *Escherichia coli* O157 in faeces of experimentally infected rats and domestic pigeons. Letters in Applied Microbiology. **31,** 349-352.

Cockerill III, F., Beebakhee, G., Soni, R., and Sherman, P. 1996. Polysaccharide side chains are not required for attaching and effacing adhesion of *Escherichia coli* O157:H7. Infection and Immunity. **64,** 3196-3200.

Cody, S. H., Glynn, M. K., Farrar, J. A., Cairns, K. L., Griffin, P. M., Kobayashi, J., Fyfe, M., Hoffman, R., King, A. S., Lewis, J. H., Swaminathan, B., Bryant, R. G., and Vugia, D. J. 1994. An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. Annuals of Internal Medicine. **130,** 202-209.

Collazo, C. M., and Gallan, J. E. 1997. The invasion-associated type-III protein secretion system in *Salmonella*. Gene. **192,** 51-59.

Conlan, J. W., and Perry, M. B. 1998. Susceptibility of three strains of conventional adult mice to intestinal colonization by an isolate of *Escherichia coli* O157:H7. Canadian Journal of Microbiology. **44,** 800-805.

Conner, D. E., and Hall, G. S. 1994. Efficacy of selected media for the recovery of *Escherichia coli* O157:H7 from frozen chicken meat containing sodium chloride, sodium lactate or polyphosphate. Food Microbiology. **11,** 337-344.

Cooke, E. M. 1974. Miscellaneous *Escherichia coli* infections. *Escherichia coli* and man. Churchill Livingstone, United Kingdom. pp. 1-12.

Cookson, A. L., Wales, A. D., Roe, J. M., Hayes, C. M., Pearson, G. R., and Woodward, M. J. 2002. Variation in the persistence of *Escherichia coli* O157:H7 in experimentally inoculated 6-week-old conventional lambs. Journal of Medical Microbiology. **51,** 1032-1040.

Cookson, A. L., and Woodward, M. L. 2003. The role of intimin in the adherence of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 to HEp-2 tissue culture cells and to bovine gut explants. International Journal of Microbiology. **292,** 547-553.

Cornick, N. A., Booher, S. L., Casey, T. A., and Moon, H. W. 2000. Persisent colonisation of sheep by *Escherichia coli* O157:H7 and other *E. coli* pathotypes. Applied and Environmental Microbiology. **66,** 4926-4934.

Cornick, N. A., Booher, S. L., and Moon, H. W. 2002. Intimin facilitates colonization by *Escherichia coli* O157:H7 in adult ruminants. Infection and Immunity. **70,** 2704-2707.

Cowden, J. M., Ahmed, S., Donaghy, M., and Riley, A. 2001. Epidemiological investigation of the Central Scotland outbreak of *Escherichia coli* O157 infection, November to December 1996. Epidemiology and Infection. **126,** 335-341.

Crampin, M., Willshaw, G., Hancock, R., Djuretic, T., Elstob, C., Rouse, A., Cheasty, T., and Stuart, J. 1999. Outbreak of *Escherichia coli* O157 infection associated with a music festival. European Journal of Clinical Microbiology and Infectious Diseases. **18,** 286-288.

Cravioto, A., Tello, A., Navarrro, A., Ruiz, J., Villafán, H., Uribe, F., and Eslava, C. 1991. Association of *Escherichia coli* HEp-2 adherence patterns with type and duration of diarrhoea. Lancet. **337,** 262-264.

Cray, W. C., Jr, and Moon, H. W. 1995. Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. Applied and Environmental Microbiology. **61,** 1586-1590.

Crump, J. A., Sulka, A. C., Langer, A. J., Schaben, C., Crielly, A. S., Gage, R., Baysinger, M., Moll, M., Withers, G., Toney, D. M., Hunter, S. B., Hoekstra, R. M., Wong, S. K., Griffin, P. M., and Van Gilder, T. J. 2002. An outbreak of *Escherichia coli* O157:H7 infections among visitors to a dairy farm. New England Journal of Medicine. **22,** 555-560.

Cui, S., Meng, J., and Bhagwat, A. A. 2001. Availability of glutamate and arginine during acid challenge determines cell density-dependent survival phenotype of *Escherichia coli* strains. Applied and Environmental Microbiology. **67,** 4914-4918.

Currie, C. G., and Poxton, I. R. 1999. The lipopolysaccharide core type of *Escherichia coli* O157:H7 and other non-O157 Verotoxin-producing *E. coli.* FEMS Immunology and Medical Microbiology. **24,** 57-62.

Dean-Nystrom, E. A., Bosworth, B. T., Cray, W. C., Jr, and Moon, H. W. 1997. Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves. Infection and Immunity. **65,** 1842-1848.

Dean-Nystrom, E. A., Bosworth, B. T., Moon, H.W., and O'Brien, A. D. 1998. *Escherichia coli* O157:H7 requires intimin for enteropathogenicity in calves. Infection and Immunity. **66,** 4560-4563.

Dean-Nystrom, E. A., Gansheroff, L. J., Mills, M., Moon, H. W., and O'Brien, A. D. 2002. Vaccination of pregnant dams with intimin (O157) protects suckling piglets from *Escherichia coli* O157:H7 infection. Infection and Immunity. **70,** 2414-2418.

Dean-Nystrom, E. A., Pohlenz, J. F. L., Moon, H.W., and O'Brien, A. D. 2000. *Escherichia coli* O157:H7 causes more-severe disease in suckling piglets than colostrum-deprived neonatal piglets. Infection and Immunity. **68,** 2356-2358.

Deibel, C., Kramer, S., Chakraborty, T., and Ebel, F. 1998. EspE, a novel secreted protein of attaching and effacing bacteria is directly translocated into infected host cells, where it appears as a tyrosine-phosphorylated 90kDa protein. Molecular Microbiology. **28,** 463-474.

De Boer, E., and Heuvelink, A. E. 2000. Methods for the detection and isolation of Shiga toxin-producing *Escherichia coli*. Journal of Applied Microbiology Symposium Supplement. **88,** 133S-143S.

De Graaf, F. K., and Gaastra, W. 1994. Fimbriae of enterotoxigenic *Escherichia coli*. In Klemm, P. Fimbriae, Adhesion, Genetics, Biogenesis and Vaccines. CRC Press, Boca Raton, pp. 57-88.

De Grado, M., Abe, A., Gauthier, A., Stelle-Mortimer, O., DeVinney, R., and Finlay, B. B. 1999. Identification of the intimin-binding domain of Tir of enteropathogenic *Escherichia coli*. Cellular Microbiology.**1,** 7-17.

De Grandis, S., Law, H., Brunton, J., Gyles, C., and Lingwood, C. A. 1989. Globotetraosylceramide is recognised by the pig edema disease toxin. Journal of Biological Chemistry. **264,** 12520-12525.

De Sauvage, F. J., Horuk, R., Bennett, C., Quan, C., Burnier, J. P., and Goeddel, D. V. 1992. Characterisation of the recombinant human receptor for *Escherichia coli* heat-stable toxin. Journal of Biological Chemistry. **267,** 6479-6482.

DeVinney, R., Puente, J. L., Gauthier, A., Goosney, D., and Finlay BB. 2001. Enterohaemorrhagic and enteropathogenic *Escherichia coli* use a different Tir-based mechanism for pedestal formation. Molecular Microbiology. **41,** 1455-1458.

DeVinney, R., Stein, M., Reinscheid, D., Abe, A., Ruschkowski, S., and Finlay, B. B. 1999. Enterohemorrhagic *Escherichia coli* O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated. Infection and Immunity. **67,** 2389-2398.

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

Dibb-Fuller, M. P., Best, A., Stagg, D. A., Cooley, W. A., and Woodward, M. J. 2001. Bovine epithelial primary cell cultures; an *in vitro* model for studying the attachment of *Escherichia coli* and other enteropathogens. Journal of Medical Microbiology. **50,** 759-769.

Djafari, S., Ebel, F., Deibel, C., Kramer, S., Hudel, M., and Chakraborty, T. 1997. Characterization of an exported protease from Shiga toxin-producing *Escherichia coli.* Molecular Microbiology. **25,** 771-784.

Donnenberg, M. S., Tzipori, S., McKee, M. L., O'Brien, A.D., Alroy, J., and Kaper, J. B. 1993. The role of the *eae* gene of enterohemorrhagic *Escherichia coli* in intimate attachment *in vitro* and in a porcine model. Journal of Clinical Investigation. **92,** 1418-1424.

Doyle, M. P., and Shoeni, J. L. 1987. Isolation of *Escherichia coli* O157:H7 from retail meats and poultry. Applied and Environmental Microbiology. **53,** 2394-2396.

Drasar, B. S., and Hill, M. J. 1974. Human Intestinal Flora. pp. 36-43. Academic press Ltd., London, United Kingdom.

Duiguid, J. P. 1959. Fimbriae and adhesive properties in *Klebsiella* strains. Journal of General Microbiology. **21,** 271-278.

Duiguid, J. P., Clegg, S., and Wilson, M. I. 1979. The fimbrial and non-fimbrial haemagglutins of *Escherichia coli.* Journal of Medical Microbiology. **12,** 213-227.

Duiguid, J. P., Smith, I. W., Dempster, G., and Edmunds, P. N. 1955. Non-flagellar filamentous appendices fimbriae and haemagglutinating activity in the bacterium *Escherichia coli.* Journal of Pathology and Bacteriology. **70,** 335-447.

DuPont, H. L., Formal, S. B., Hornick, R. B., Snyder, M. J., Libonati, J. P., Sheahan, D. G., LaBrec, E. H., and Kalas, J. P. 1971. Pathogenesis of *Escherichia coli* diarrhoea. New England Journal of Medicine. **285,** 1-9.

Durno, C., Soni, R., and Sherman, P. 1989. Adherence of Verocytoxin-producing *Escherichia coli* serotype O157:H7 to isolated epithelial cells and brush membranes *in vitro*: role of type-1 fimbriae (pili) as a bacterial adhesin expressed by strain CL-49. Clinical and Investigative Medicine. **12,** 194-200.

Dyce, K. M., Sack, W. O., and Wensing, C. J. G. 1987. Textbook of Veterinary Anatomy. W. B. Saunders Company.

Dykstra, S. A., Moxely, R. A., Janke, B. H., Nelson, E. A., and Francis, D. H. 1993. Clinical signs and lesions in gnotobiotic pigs inoculated with Shiga-like toxin 1 from *Escherichia coli*. Veterinary Pathology. **30,** 410-417.

Dytoc, M., Fedorka, L., and Sherman, P. M. 1994. Siganal transduction in human epithelial cells infected with attaching and effacing *Escherichia coli in vitro*. Gastroenterology. **106,** 1150-1161.

Ebel, F., Podzadel, T., Rohde, M., Kresse, A. U., Krämer, S., Deibel, C., Guzman, C. A., and Charkraborty, T. 1998. Initial binding of shiga toxin-producing *Escherichia coli* to host cells and subsequent induction of actin rearrangements depend on filamentous EspA-containing surface appendages. Molecular Microbiology. **30,** 147-161.

Effler, E., Isaacson, M., Arntzen, L., Heenan, R., Canter, P., Barrett, T., Lee, L., Mambo, C., Levine, W., Zaidi, A., and Griffin, P. M. 2001. Factors contributing to the emergence of *Escherichia coli* O157 in Africa. Emerging Infectious Diseases. **7,** 812-819.

Elliott, S. J., Sperandio, V., Girón, J. A., Shin, S., Mellies, J. L., Wainright, L., Hutcheson, S. W., Mc Daniel, T. K., and Kaper, J. B. 2000. The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohaemorrhagic *Escherichia coli*. Infection and Immunity. **68,** 6115-6126.

Elliott, S. J., Wainright, L.A., McDaniel, T. K., MacNamara, B., Donnenberg, M., and Kaper, J. B. 1998. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. Molecular Microbiology. **28,** 1-4.

Elliott, S. J., Yu, J., and Kaper, J. B. 1999. The cloned locus of enterocyte effacement from enterohemorrhagic *Escherichia coli* O157:H7 is unable to confer the attaching and effacing phenotype upon *E. coli* K-12. Infection and Immunity. **67,** 4260-4263.

Enami, M., Nakone N., Honma, Y., Kakinohama, S., Kudaka, J., and Iwanga, M. 1999. Expression of type-1 pili is abolished in Verotoxin-producing *Escherichia coli* O157. FEMS Microbiology Letters. **179,** 467-472.

Escherich, T. 1885. Die darmbacterien des neugeborenen und sauglings. (a) Fortschritte der Medizin. **3**, 515-522 (b) Fortschritte der Medizin. **3**, 547-554.

Ewing, W. H. 1986. Edwards and Ewings Identification of *Enterobacteriaceae*, 4th edition, Elsevier Science, New York.

Farner, D. S. 1942. The hydrogen ion concentration in avian digestive tracts. Poultry Science. **21,** 445-450.

Farris, M., Grant, A., Richardson, T. B., and O'Connor, C. D. 1998. BipA: a tyrosine-phosphorylated GTPase that mediates interactions between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells. Molecular Microbiology. **28,** 265-279.

Feldman, K. A., Mohle-Boetani, J. C., Ward, J., Furst, K., Abbott, S. L., Ferrero, D. V., Olsen, A., and Werner S. B. 2002. A cluster of *Escherichia coli* O157: nonmotile infections associated with recreational exposure to lake water. Public Health Reports. **117,** 380-385.

Feng, P., Dey, M., Abe, A., and Takeda, T. 2001. Isogenic strain of *Escherichia coli* O157:H7 that has lost both Shiga toxin 1 and 2 genes. Clinical and Diagnostic Laboratory Immunology. 8, 711-717.

Finlay, B. B., Rosenshine, I., Donnenberg, M. S., and Kaper, J. B. 1992. Cytoskeletal composition of attaching and effacing lesions associated with enteropathogenic *Escherichia coli* adherence to HeLa cells. Infection Immunity. **60,** 2541-2543.

Fitzhenry, R. J, Pickard, D. J., Hartland, E. L., Reece, S., Dougan, G., Phillips, A. D., and Frankel, G. 2002. Intimin type influences the site of human intestinal mucosal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7. Gut. **50,** 180-185.

Foster, G., Ross, H. M., Pennycott, T. W., Hopkins, G. F., and McLaren, I. M. 1998. Isolation of *Escherichia coli* O86:K61 producing cyto-lethal distending toxin from wild birds of the finch family. Letters in Applied Microbiology. **26,** 395-398.

Francis, D. H., Collins, J. E., and Duimstra, J. R. 1986. Infection of gnotobiotic pigs with an *Escherichia coli* O157:H7 strain associated with an outbreak of hemorrhagic colitis. Infection and Immunity. **51,** 953-956.

Frankel, G., Candy, D. C., Everest, P., and Dougan, G. 1994. Characterisation of the C-terminal domains of intimin-like proteins of enteropathogenic and enterohaemorrhagic *Escherichia coli*, *Citrobacter freundii*, and *Hafnia alvei*. Infection and Immunity. **62,** 1835-1842.

Frankel, G., Lider, O., Hershkoviz, R., Mould, A. P., Kachalsky, S. G., Candy, D. C., Cahalon, L., Humphries, M. J., and Dougan, G. 1996. The cell-binding domain of intimin from enteropathogenic *Escherichia coli* binds to beta1 integrins. Journal of Biological Chemistry. **271,** 20359-20364.

Frankel, G., Phillips, A.D., Rosenshine, I., Dougan, G., Kaper, J. B., and Knutton, S. 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. Molecular Microbiology. **30,** 911-921.

Fratamico, P. M., Bagi, L. K., and Pepe, T. A. 2000. Multiplex polymerase chain reaction assay for rapid detection and identification of *Escherichia coli* O157:H7 in foods and bovine feces. Journal of Food Protection. **63,** 1032-1037.

Fratamico, P. M., Bhaduri, S., and Buchanan, R. L. 1993. Studies on *Escherichia coli* serotype O157:H7 strains containing a 60-MDa plasmid and on 60-MDa plasmid cured derivatives. Journal of Medical Microbiology. **39,** 371-381.

Fratamico, P. M., Schultz, F. J., and Buchanan, R. L. 1992. Rapid isolation of *Escherichia coli* O157:H7 from enrichment cultures of foods using an immunomagnetic separation method. Food Microbiology. **9,** 105-113.

Freter, R. 1980. Prospects for preventing the association of harmful bacteria with host mucosal surface. In Bacterial Adherence, Receptor and Recognition, Series B, Volume 6. Edited by Beachly, E. H. Chapman and Hall, London. pp. 439-458.

Fuchs, S., Mühldorfer, I., Donohoe-Rolfe, A., Kerényi, M., Emödy, L., Alexiev, R., Nenkov, P., and Hacker J. 1999. Influence of RecA on *in vivo* virulence and Shiga toxin 2 production in *Escherichia coli* pathogens. Microbial Pathogenesis. **27,** 13-23.

Gahring, L. C., Heffron, F., Finlay, B. B., and Falkow, S. 1990. Invasion and replication of *Salmonella typhimurium* in animal cells, Infection and Immunity. **58,** 443-448.

Galland, J C., Hyatt, D. R., Crupper, S. S., and Acheson, D. W. 2001. Prevalence, antibiotic susceptibility, and diversity of *Escherichia coli* O157:H7 isolates from a longitudinal study of beef cattle feedlots. Applied and Environmental Microbiology. **67,**1619-1627.

Gansheroff, L. J., Wachtel, M. R., and O'Brien, A. D. 1999. Decreased adherence of enterohaemorrhagic *Escherichia coli* to HEp-2 cells in the presence of antibodies that recognise the C-terminal region of intimin. Infection and Immunity. **67,** 6409-6417.

Gay, C. C., and Besser, T. E. 1994. *Escherichia coli* septicaemia in calves. In *Escherchia coli* in Domestic Animals and Humans. Edited by Gyles, C. L. CAB International, UK. pp. 75-90

Gilbert, D. N. 1995. Aminoglycosides. In Principles and Practices of Infectious Diseases. Edited by Mandell, G. L., Bennett, J. E., and Dolin, R. J. London, Churchill Livingstone Inc, UK. pp. 279-306.

Giron, J. A., Ho, S. Y., and Schoolnik, G. K. 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli.* Science. **254,** 710-713.

Giron, J. A., Torres, A. G., Freer, E., and Kaper, J. B. 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Molecular Microbiology. **44,** 361-379.

Goh, S., Newman, C., Knowles, M., Bolton, F. J., Hollyoak, V., Richards, S., Daley, P., Counter, D., Smith, H. R., and Keppie, N. 2002. *E. coli* O157 phage type 21/28 outbreak in North Cumbria associated with pasteurized milk. Epidemiology and Infection. **129,** 451-457.

Goldberg, M. B., and Sansonetti, P. J. 1993. Shigella subversion of the cellular cytoskeleton: a strategy for epithelial colonisation. Infection and Immunity. **61,** 4941-4946.

Gómez-Duarte, O. G., and Kaper, J. B. 1995. A plasmid encoded regulatory region activates chromosomal *eaeA* expression in enteropathogenic *Escherichia coli*. Infection and Immunity. **63,** 1767-1776.

Goosney, D. L., DeVinney, R., and Finlay, B. B. 2001. Recruitment of cytoskeletal and signaling proteins to enteropthaogenic and enterohemorrhagic *Escherichia coli* pedestals. Infection and Immunity. **69,** 3315-3322.

Gorbach, D. L., Banwell, J. G., Chatterjee, B. D., Jacobs, B., and Sack, R. B. 1971. Acute undifferentiated human diarrhoea in the tropics. I. Alterations in intestinal microflora. Journal of Clinical Investigation. **50,** 881-889.

Gorden, J., and Small, P. L. C. 1993. Acid resistance in enteric bacteria. Infection and Immunity. **61,** 364-367.

Grant, A. J., Farris, M., Alefounder, P., Williams, P. H., Woodward, M. J., and O'Connor C. D. 2003. Co-ordination of pathogenicity island expression by the BipA GTPase in enteropathogenic *Escherichia coli* (EPEC). Molecular Microbiology. **48,** 507-521.

Grauke, L. J., Kudva, I. T., Won Yoon, J., Hunt, C. W., Williams, C. J., and Hovde, C. J. 2002. Gastrointestinal tract location of *Escherichia coli* O157:H7 in ruminants. Applied and Environmental Microbiology. **68,** 2269-2277.

Griffin, P. M., and Tauxe, R. V. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohaemorrhagic *E. coli*, and the associated haemolytic uraemic syndrome. Epidemiology Reviews. **13**, 60-98.

Griffin, P. M., Mead, P. S., Van Gilder, T., Hunter, S. B., Strockbine, N. A., and Tauxe, R. V. 2000. Shiga toxin-producing *Escherichia coli* infectiomns in the United States: Current status and challenges. In 4th International symposium and workshop on "Shiga toxin (Verocytotoxin) producing *Escherichia coli* infections". Programs and Abstracts. pp. 31.

Griffiths, E. 1997. Iron the virulence of *Escherichia coli*. In *Escherichia coli*. Mechanisms of virulence. Edited by Sussman, M. Cambridge University Press, United Kingdom. pp. 331-372.

Gross, W. G. 1994. Disease due to *Escherichia coli* in poultry. In *Escherichia coli* in Domestic Animals and Humans. Edited by Gyles, C. L. CAB International, United Kingdom.

Guard-Petter, J., 2001. The chicken the egg and *Salmonella enteritidis*. Environmental Microbiology. **3,** 421-430.

Gyles, C. L. 1994. *Escherichia coli* enterotoxins. In *Escherichia coli* in Domestic Animals and Humans. Edited by Gyles C. L. CAB International, UK. pp. 337-364.

Haarmann, C., Karch, H., Frosch, M., and Schmidt, H. 1998. A 3.3-kb plasmid of enterohemorrhagic *Escherichia coli* O157:H7 is closely related to the core region of the *Salmonella typhimurium* antibiotic resistance plasmid NTP16. Plasmid. **39,** 134-140.

Hakkinen, M., and Schneitz, C. 1996. Efficacy of a commercial competitive exclusion product against a chicken pathogenic *Escherichia coli* and *E coli* O157:H7. Veterinary Record. **139,** 139-141.

Hammar, M., Arnqvist, A., Bian, Z., Olsen, A., and Normark, S. 1995. Expression of two *csg* operons is required for production of fibronectin- and congo red-binding curli polymers in *Escherichia coli* K-12. Molecular Microbiology. **18,** 661-670.

Hancock, D., Besser, T., Lejeune, J., Davis, M., and Rice, D. 2001. The control of VTEC in the animal reservoir. International Journal of Food Microbiology. **66,** 71-78.

Hancock, D. D., Rice, D. H., Thomas, L. A., Dargatz, D. A., and Besser, T. E. 1997. Epidemiology of *Escherichia coli* O157 in feedlot cattle. Journal of Food Protection. **60,** 462-465.

Hara-Kudo, Y., Miyahara, M., and Kumagai, S. 2000. Loss of O157 O antigenicity of Verotoxin-producing *Escherichia coli* O157:H7 under starvation conditions. Applied and Environmental Microbiology. **66,** 5540-5543.

Harel., Y., Silva, M., Giroir, B., Weinberg, A., Cleary, T. B., and Beutler, B. 1993. A reporter transgene indicates renal-specific induction of tumour necrosis factor (TNF) by Shiga-like toxin. Journal of Clinical Investigation. **92,** 2110-2116.

Hardt, W. D., Urlaub, H., and Galan, J. E. 1998. A substrate of the centisome 63 type III protein secretion system of *Salmonella typhimurium* is encoded by a cryptic bacteriophage. Proceedings in the National Academy of Science. USA. **95,** 2574-2579.

Harrison, J. A., Harrison, M. A., and Rose, R. A. 1998. Survival of *Escherichia coli* O157:H7 in ground beef jerky assessed on two plating media. Journal of Food Protection. **61,** 11-13.

Hayashi, T., Makino, K., Ohnishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., Han, C. G., Ohtsubo, E., Nakayama, K., Murata, T., Tanaka, M., Tobe, T., Iida, T., Takani, H., Honda, T., Saskawa, C., Ogasawara, N., Yasunga, T., Kuhara, S., Shiba, T., Hattori, M., and Shinagawa, H. 2001. Complete genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7 and genome comparison with a laboratory K-12 strain. DNA Research. **28,** 47-52.

Heimer, S. R., Welch, R. A., Perna, N. T., Posfai, G., Evans, P. S., Kaper, J. B., Blattner, F. R., and Mobley, H. L. T. 2002. Urease of enterohemorrhagic *Escherichia coli*: Evidence for regulation by Fur and a *trans*-acting factor. Infection and Immunity. **70,** 1027-1031.

Hengge-Aronis, R. 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *Escherichia coli*. Cell **72,** 165-168.

Hensel, M., Shea, J. E., Waterman, S. R., MundyR., Nikolaus, T., Banks, G., Vazquez-Torres, A., Gleeson, C., Fang, F. C., and Holden, D. W. 1998. Genes encoding putative effector proteins of the type III secretion system of Salmonella pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Molecular Microbiology. **30,** 163-174.

Hersh, B. M., Farooq, F. T., Barstad, D. N., Blankenhorn, D. L., and Slonczewski J. L. 1996. A glutamate-dependent acid resistance gene in *Escherichia coli*. Journal of Bacteriology. **178,** 3978-3981.

Hettiaratchy, I. G., Cooke, E. M., and Shooter, R. A. 1973. Colicine production as an epidemiological marker of *E. coli*. Journal of Medical Microbiology. **6,** 918-927.

Heuvelink, A. E., van den Biggelaar, F. L. A. M., de Boer, E., Herbes, R. G., Melchers, W. J. G., Huis in't Veld, J. H., and Monnens, L. A. H. 1998. Isolation and characterisation of Verocytotoxin-producing *Escherichia coli* O157 strains from Dutch cattle and sheep. Journal of Clinical Microbiology. **36,** 878-882.

Heuvelink, A. E., Zwartkruis-Nahuis, J. T. M., and de Boer, E. 1997. Evaluation of media and test kits for the detection and isolation of *Escherichia coli* O157 from minced beef. Journal of Food Protection. **60,** 817-824.

Heuvelink, A. E., Zwartkruis-Nahuis, J. T., van den Biggelaar, F. L., van Leeuwen, W. J., and de Boer, E. 1999. Isolation and characterization of Verocytotoxin-producing *Escherichia coli* O157 from slaughter pigs and poultry. International Journal of Food Microbiology **52,** 67-75.

Holt, J. G., Kreig, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. 1994. Faculative anaerobic Gram-negative rods. Bergey's Manual of Determinative Bacteriology, 9th edition. Williams and Wilkins, USA. pp. 179-180.

Houwink, A. L., and Van Iterson, W. 1950. Electron microscopial observation on bacterial cytology. A study of flagellation. Biochimica et Biophysica Acta. **5,** 10-44.

Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiology and Molecular Biology Reviews. **62,** 379-433.

Ibekwe, A. M., and Grieve, C. M. 2003. Detection and quantification of *Escherichia coli* O157:H7 in environmental samples by real-time PCR. Journal of Applied Microbiology. **94,** 421-431.

Igarashi, K., Ogasawara, T., Ito, K., Yutsuda, T., and Takeda, Y. 1989. Inhibition of elongation factor 1-dependent aminoacyl-tRNA binding to ribosomes by Shiga-like toxin 1 (VT1) from *Escherichia coli* O157:H7 and by Shiga toxin. FEMS Microbiology Letters. **44,** 91-94.

Iida, K., Mizunoe, Y., Wai, S. N., and Yoshida, S. 2001. Type 1 fimbriation and its phase switching in diarrheagenic *Escherichia coli* strains. Clinical and Diagnostic Laboratory Immunology. **8,** 489-495.

Isogai, E., Isogai, H., Kimura, K., Hayashi, S., Kubota, T., Fujii, N., and Takeshi, K. 1998. Role of tumor necrosis factor alpha in gnotobiotic mice infected with an *Escherichia coli* O157:H7 strain. Infection and Immunity. **66,** 197-202.

Ismaili, A., Philpott, D. J., Dytoc, M. T., and Sherman, P. M. 1995. Signal transduction responses following adhesion of Verocytotoxin-producing *Escherichia coli.* Infection and Immunity. **63,** 3316-3326.

Jackson M. P. 1990. Structure-function analyses of Shiga toxin and the Shiga-like toxins. Microbial Pathogenesis. **8,** 235-242.

James, B. W., and Keevil, C. W. 1999. Influence of oxygen availability on physiology, verocytotoxin expression and adherence of *Escherichia coli* O157. Journal of Applied Microbiology. **86,** 117-124.

Jantausch, B. A., and Hull, S. I. 1996. Restriction fragment length polymorphism of PCR amplified *papE* gene products is correlated with complete serotype among UPEC isolates. Microbial pathogenesis. **20,** 351-360.

Jarvis, K. G., Girón, J. A., Jerse, A. E., McDaniel, T. K., Donnemberg, M. S., and Kaper, J.B. 1995. Enteropathogenic *Escherichia coli* contains a specialised secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. Proceedings in the National Academy of Science. USA. **92,** 7996-8000.

Jarvis, K. G., and Kaper, J.B. 1996. Secretion of extracellular proteins by enterohemorrhagic *Escherichia coli* via a putative type III secretion system. Infection and Immunity. **64,** 4826-4829.

Jerse, A. E., Yu, J., Tall, B. D., and Kaper, J.B. 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proceedings in the National Academy of Science. USA. **87,** 7839-7843.

Jeurissen, S. H. M., Janse, E. M., Koch, G., and De Boer, G. F. 1989. Postnatal development of mucosa-associated lymphoid tissues in chickens. Cell Tissues Research. **258,** 119-124.

Jones, G. W. and Rutter, J. M. 1972. Role of K88 antigen in the pathogenesis of neonatal diarrhoes caused by *E. coli* in piglets. Infection and Immunity. **6,** 918-927.

Jones, N. L., Islur, A., Haq, R., Mascarenhas, M., Karmali, M. A., Perdue, M. H., Zanke, B. W., and Sherman, P. M. 2000. *Escherichia coli* Shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family. American Journal of Physiology and Gastrointestinal Liver Physiology. **278**, 811-819.

Jordan, K. N., Oxford, L., and O'Bryne, C. P. 1999. Survival of low-pH stress by *Escherichia coli* O157:H7: correlation between alteration in the cell envelope and increased acid tolerance. Applied and Environmental Microbiology. **65,** 3048-3055.

Junkins, A., and Doyle, M. P. 1992. Demonstration of exopolysaccharide production by enterobemorrhagic *Escherichia coli*. Current Microbiology. **25,** 9-17.

Kanamaru, K., Kanamaru, K., Tatsuno, I., Tobe, T., and Sasakawa, C. 2000. SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157:H7. Molecular Microbiology. **38,** 805-816.

Kang, G., Mathan, M. M., and Mathan, V. I. 1995. Evaluation of a simplified HEp-2 cell adherence assay for *E. coli* isolated from South Indian children with acute diarrhea and controls. Journal of Clinical Microbiology. **33,** 2204-2205.

Kaper, J. B., Elliott, S. J., Sperandio, V., Perna, N. T., Mayhew, G. F., and Blattner F. R. 1998. Attaching and effacing intestinal histopathology and the locus of enterocyte effacement. In *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. Editors Kaper, J. B., and O'Brien A. D. ASM Press, Washington, D.C. pp. 163-182.

Kaper, J. B., and O'Brien A. D. 1998. *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. ASM Press, Washington, D.C.

Karch, H., Heesemann, J., Laufs, R,, O'Brien, A. D., Tacket, C. O., and Levine, M. M. 1987. A plasmid of enterohemorrhagic *Escherichia coli* O157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. Infection and Immunity. **55,** 455-461.

Karch, H., Janetzki-Mittmann, C., Aleksic, S., and Datz, M. 1996. Isolation of enterohemorrrhagic *Escherichia coli* O157 strains from patients with hemolyticuraemic syndrome by using immunomagnetic separation, DNA-based methods, and direct culture. Journal of Clinical Microbiology. **34,** 516-519.

Karmali, M. A. 1989. Infection by Verocytotoxin-producing *Escherichia coli.* Clinical Microbiology Reviews. **2,** 15-38.

Karmali, M. A., Petric, M., Lim, C., Fleming, P. C., Arbus, G. S., and Lior, H. 1985. The association between idiopathic haemolytic uraemic syndrome and infection by Verotoxin-producing *Escherichia coli.* Journal of Infectious Diseases. **151**, 775-82.

Karmali, M. A., Petric, M., Lim, C., Fleming, P. C., and Steele B. T. 1983. *Escherichia coli* cytotoxin, haemolytic uraemic syndrome, and haemorraghic colitis. Lancet **ii,** 1299-1300.

Karpman, D., Connell, H., Svensson, M., Scheutz, F., Alm, P., and Svanborg, C. 1997. The role of lipopolysaccharide and Shiga-like toxin in a mouse model of *Escherichia coli* O157:H7 infection. Journal of Infectious Diseases. **175**, 611-620.

Kauffman, F. 1943. Uber neue thermolabile korper antigene der coli-bakterien. Acta Pathologica et Microbiologia Scandinavica. **20**, 21.

Kauffman, F. 1944. Zur serologic der coli-grouppe. Acta Pathologica et Microbiologia Scandinavica. **21**, 20-45.

Kauffman, F. 1969. The bacteriology of *Enterobacteriaceae.* 2nd edition, Copenhagen, Munkdgaard. pp. 20.

Kauffman, F., and Vahlne, G. 1945. Uber die bedeutung des serologischen formenwechsels fur due bakteriophagen winkung in der coli-gruppe. Acta Pathologica et Microbiologia Scandinavica. **22,** 119.

Kendrick K, E., and Reznikoff, W. S. 1988. Transposition of IS50L activates downstream genes. Journal of Bacteriology. **170,** 165-168.

Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J., Frey, E. A., and Finlay, B. B. 1997. Enteropathogenic *Escherichia coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell. **91,** 511-520.

Kenny, B., and Finlay, B. B. 1997. Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signaling events, including tyrosinre phosphorylation of phospholipase C-gamma 1. Infection and Immunity. **65,** 2528-2536.

Kenny, B. 2001. The enterohaemorrhagic *Escherichia coli* (serotype O157:H7) Tir molecule is not functionally interchangeable for its enteropathogenic *E. coli* (serotype O127:H6) homologue. Cellular Microbiology. **3,** 499-510.

Keskimaki, M., Saari, M., Heiskanen, T., and Siitonen, A. 1998. Shiga toxin-producing *Escherichia coli* in finland from 1990 through 1997: prevalence and characteristics of isolates. Journal of Clinical Microbiology. **36,** 3641-3646.

Kim, J., Nietfeldt, J., Ju, J., Wise, J., Fegan, N., Desmarchelier, P., and Benson, A. K. 2001. Ancestral divergence, genome diversification, and phylogeographic variation in subpopulations of sorbitol-negative, beta-glucuronidase-negative enterohemorrhagic *Escherichia coli* O157. Journal of Bacteriology. **183,** 6885-6897.

Kim, H. H, Samadpour, M., Grimm, L., Clausen, C. R., Besser, T. E., Baylor, M., Kobayashi, J. M., Neill, M. A., Schoenknecht, F. D., and Tarr, P. I. 1994. Characteristics of antibiotic-resistant *Escherichia coli* O157:H7 in Washington State, 1984-1991. Journal of Infectious Diseases. **170,** 1606-1609.

Kita, E., Yunou, Y., Kurioka, T., Harada, H., Yoshikawa, S., Mikasa, K., and Higashi, N. 2000. Pathogenic mechanism of mouse brain damage caused by oral infection with Shiga toxin-producing *Escherichia coli* O157:H7. Infection and Immunity. **68,** 1207-1214.

Klapproth, J. M. A., Scaletsky, I. C. A., McNamara, B. P., Lai, L. C., Malstrom, C., James, S. P., and Donnenberg, M. S. 2000. A large toxin from pathogenic *Escherichia coli* strains that inhibits lymphocyte activation. Infection and Immunity. **68,** 2148-2155.

Knipschildt, H. E. 1946. Demonstration of a new thermo-labile antigen in the colon group. Acta Pathologica et Microbiologia Scandinavica. **23,** 179-186.

Knutton, S., Baldini, M. M., Kaper, J. B., and McNeish, A. S. 1987b. Role of plasmid-encoded adherence factors in adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells. Infection and Immunity. **55,** 78-85.

Knutton, S., Lloyd, D. R., and McNeish, A. S. 1987a. Identification of a new fimbrial structure in enterotoxigenic *Escherichia coli* (ETEC) serotype O148:H28 which adheres to human intestinal mucosa: a potentially new human ETEC colonization factor. Infection and Immunity. **55,** 86-92.

Knutton, S., Baldwin, T., Williams P. H., and McNeish, A. S. 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohaemorrhagic *Escherichia coli*. Infection and Immunity. **57,** 1290-1298.

Kodama, T., Akeda, Y., Kono, G., Takahashi, A., Imura, K., Iida, T., and Honda T. 2002. The EspB protein of enterohaemorrhagic *Escherichia coli* interacts directly with alpha-catenin. Cellular Microbilogy. **4,** 213-222.

Konowalchuk, J., Speirs, J. I., and Stavric, S. 1977. Vero response to a cytotoxin from *Escherichia coli*. Infection and Immunity. **18,** 775-779.

Kresse, A. U., Rohde, M., and Guzmán, C. A. 1999. The EspD protein of enterohaemorrhagic *Escherichia coli* is required for the formation of bacterial surface appendages and is incorporated in the cytoplasmic membranes of target cells. Infection and Immunity. **67,** 4834-4842.

Kresse, A. U., Beltrametti, F., Müller, A., Ebel, F., and Guzmán, C. A. 2000. Characterisation of SepL of enterohaemorrhagic *Escherichia coli*. Journal of Bacteriology. **182,** 6490-6498.

Kresse, A. U., Schulze, K., Deibel, C., Ebel, F., Rohde, M., Chakraborty, T., and Guzmán, C. A. 1998. Pas, a novel protein required for protein secretion and attaching and effacing activities in of enterohaemorrhagic *Escherichia coli*. Journal of Bacteriology. **180,** 4370-4379.

Kudva, I. T., Evans, P. S., Perna, N. T., Barrett, T. J., Ausubel, F. M., Blattner, F. R., and Calderwood, S. B. 2002. Strains of *Escherichia coli* O157:H7 differ primarily by insertions or deletions, not single-nucleotide polymorphisms. Journal of Bacteriology. **184,** 1873-1879.

Kudva, I. T., Hatfield, P. G., and Hovde, C. J. 1995. Effect of diet on the shedding of *Escherichia coli* O157:H7 in a sheep model. Applied and Environmental Microbiology. **61,** 1363-1370.

Kudva, I. T., Hatfield, P. G., and Hovde, C. J. 1997. Characterisation of *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* serotypes isolated from sheep. Journal of Clinical Microbiology. **35,** 892-899.

Kuehn, M. J., Heuser, J., Normack, S., and Hultgren, S. J. 1992. P pili in uropathogenic *E. coli* are composite fibres with distinct fimbrillar adhesive tips. Nature. **356,** 252-255.

Kurioka, T., Yunou, Y., and Kita, E. 1998. Enhancement of susceptibility to Shiga toxin-producing *Escherichia coli* O157:H7 by protein calorie malnutrition in mice. Infection and Immunity. **66,** 1726-1734.

La Ragione, R. M., Casula, G., Cutting, S. M., and Woodward, M. J. 2001. *Bacillus subtilis* spores competitively exclude *Escherichia coli* O78:K80 in poultry. Veterinary Microbiology. **79,** 133-142.

La Ragione, R. M., Cooley, W. A., and Ainsworth, H. 2003. *Escherichia coli* O103 in Red-Legged partridges. Submitted to the Veterinary Record.

La Ragione, R. M., Cooley, W. A., and Woodward, M. J. 2000a. The role of fimbriae and flagella in the adherence of avian strains of *Escherichia coli* O78:K80 to tissue culture cells and tracheal gut explants. Journal of Medical Microbiology. **49,** 327-338.

La Ragione, R. M., Sayers, A. R., and Woodward. M. J. 2000b. The role of fimbriae and flagella in the colonisation, invasion and persistence of *Escherichia coli* O78:K80 in the day-old chick model. Epidemiology and Infection. **124,** 351-363.

La Ragione, R. M., McLaren, I. M., Foster, G., Cooley, W. A., and Woodward, M. J. 2002. Phenotypic and genotypic characterization of avian *Escherichia coli* O86:K61 isolates possessing a gamma-like intimin. Applied and Environmental Microbiology. **68,** 4932-4942.

Lathem, W. W., Grys, T. E., Witowski, S. E., Torres, A. G., Kaper, J. B., Tarr, P. I., and Welch, R. A. 2002. StcE, a metalloprotease secreted by *Escherichia coli* O157:H7, specifically cleaves C1 esterase inhibitor. Molecular Microbiology. **45,** 277-288.

Lauruelle, L. 1889. L' Etude bacteriologique sur les peritionites par perfection. La cellule. **5,** 60-123.

Leathart, J. B., and Gally, D. L. 1998. Regulation of type 1 fimbrial expression in uropathogenic *Escherichia coli*: heterogeneity of expression through sequence changes in the *fim* switch region. Molecular Microbiology. **28,** 371-381.

Lee, S. H., Levy, D. A., Craun, G. F., Beach, M. J., and Calderon, R. L. 2002. Surveillance for waterborne-disease outbreaks--United States, 1999-2000. MMWR Surveillence Summaries. **51,** 1-47.

Lesuffleur, T., Roche, F., Hill, A. S., Lacasa, M., Fox, M., Swallow, D. M., Zweibaum, A., and Real, F. X. 1995. Characterization of a mucin cDNA clone isolated from HT-29 mucus-secreting cells. The 3' end of MUC5AC? Journal of Biological Chemistry. **270,** 13665-13673.

Levine, M. M. 1987. *Escherichia coli* that cause diarrhoea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohaemorrhagic, and enteroadherent. Journal of Infectious Diseases. **167,** 500-502.

Levine, M. M., Ferreccio, C., Prado, V., Cayazzo, M., Abrego, P., Martinez, J., Maggi, L., Baldini, M. M., Martin, W., Maneval, D., Kay, B., Guers, L., Lior, H., Wasserman, S. S., and Nataro, J. P. 1993. Epidemiologic studies of *Escherichia coli* diarrhoeal infections in a low socioeconomic level periurban community in Santiago, Chile. American Journal of Epidemiology. **138,** 432-436.

Li, B., Koch, W. H., and Cebula, T. A. 1997. Detection and characterisation of the *fimA* gene of *Escherichia coli* O157:H7. Molecular and Cellular Probes. **11,** 397-406.

Lin, J. J., Kuo, J., and Ma, J. 1996. A PCR-based DNA fingerprinting technique; AFLP for molecular typing of bacteria. Nucleic Acids Research. **15**, 3649-3650.

Lin, J., Smith, M. P., Chapin, K. C., Baik, H. S., Bennett, G. N., and Foster, J. W. 1996. Mechanisms of acid reistance in enterohaemorrhagic *Escherichia coli.* Applied and Environmental Microbiology. **62,** 3094-3100.

Lingwood, C., Law, H., Richardson, S., Petric, Brunton, J. L., De Grandis, S., and Karmali, M. 1987. Glycolipid binding of purified and recombinant *Escherichia.* Journal of Biological Chemistry. **262,** 8834-8839.

Lior, H. 1994. Classification of *Escherichia coli.* In *Escherichia coli* in Domestic Animals and Humans. Edited by Gyles, C. L. CAB International, United Kingdom.

Liu, H., Magoun, L., and Leong, J. M. 1999. beta1-chain integrins are not essential for intimin-mediated host cell attachment and enteropathogenic *Escherichia coli*-induced actin condensation. Infection and Immunity. **67,** 2045-2049.

Liu, H., Magoun, L., Luperchio, S., Schauer, D. B., and Leong, J. M. 1999. The Tir-binding region of enterohaemorrhagic *Escherichia coli* intimin is sufficient to trigger actin condensation after bacterial-induced host cell signalling. Molecular Microbiology. **34,** 67-81.

Liu, H., Radhakrishnan, P., Magoun, L., Prabu, M., Campellone, K. G., Savage, P., He, F., Schiffer, C. A., and Leong, J. M. 2002. Point mutants of EHEC intimin that diminish Tir recognition and actin pedestal formation highlight a putative Tir binding pocket. Molecular Microbiology. **45,** 1557-1573.

Liu, J., Akahoshi, T., Sasahana, T., Kitasato, H., Namai, R., Sasaki, T., Inque, M., and Kondo, H. 1999. Inhibition of neutrophil apoptosis by Verotoxin 2 derived from *Escherichia coli* O157:H7. Infection and Immunity. **67,** 6203-6205.

Louise, C. B, Obrig, T. G. 1992. Shiga toxin-associated hemolytic uremic syndrome: combined cytotoxic effects of Shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells *in vitro*. Infection and Immunity. **60,** 1536-1543.

Lowenthal, J. W., Connick, T. E., McWaters, P. G., and York, J. J. 1994. Development of T cell immune responsiveness in the chicken. Immunology and Cell Biology. **72,** 115-122.

MacNab, R. M. 1996. Flagella and motility. In *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology. 2nd edition Vol. 2. Edited by Neidhardt, F. C. American Society for Microbiology, Washington, D. C., USA. pp123-145.

MacNab, R. M. 1999. The bacterial flagellum: reversible rotary propellor and type III export apparatus. Journal of Bacteriology. **181,** 7149-7153.

Makino, K., Ishii, K., Yasunaga, T., Hattori, M., Yokoyama, K., Yutsudo, C. H., Kubota, Y., Yamaichi, Y., Iida, T., Yamamoto, K., Honda, T., Han, C. G., Ohtsubo, E., Kasamatsu, M., Hayashi, T., Kuhara, S., and Shinagawa H. 1998. Complete nucleotide sequences of 93-kb and 3.3-kb plasmids of an enterohemorrhagic *Escherichia coli* O157:H7 derived from Sakai outbreak. DNA Research. **28,** 1-9.

Mao, Y. M., Doyle, M. P., and Chen, J. 2001. Insertion mutagenesis of *wca* reduces acid and heat tolerance of enterohemorrhagic *Escherichia coli* O157:H7. Journal of Bacteriology. **183,** 3811-3815.

Marc, D., Arne, P., Bree, A., and Dho-Moulin, M. 1998. Colonization ability and pathogenic properties of a *fim-* mutant of an avian strain of *Escherichia coli*. Reasearch Microbiology. **149,** 473-485.

March, S. B., and Ratman, S. 1986. Sorbitol-MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. Journal of Clinical Microbiology. **23,** 869-872.

Mathews, K. R., Murdough, P. A., and Bramley, A. J. 1997. Invasion of bovine epithelial cells by Verocytotoxin-producing *Escherichia coli* O157:H7. Journal of Applied Microbiology. **82,** 197-203.

Maurer, J. J., Schmidt, D., Petrosho, P., Sanchez, S., Bolton, L., and Lee, M. D. 1999. Development of primers to O-antigen biosynthesis genes for specific detection of *Escherichia coli* O157 by PCR. Applied and Environmental Microbiology. **65,** 2954-2960.

Mayer, K. H., Opal, S. M., and Madeiros, A. A. 1995. Mechanisms of antibiotic resistance. In Principles & Practices of Infectious Diseases. Edited by Mandell, G. L., Bennett, J. E. and Dolin, R. J. London, Churchill Livingstone Inc, UK. pp. 212-225.

McDaniel, T. K., Jarvis, K. G., Donnenberg, M. S., and Kaper, J. B. 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proceedings in the National Academy of Science, U S A. **28**, 1664-1668.

McDaniel, T. K., and Kaper, J. B. 1997. A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on E. coli K-12. Molecular Microbiology. **23,** 399-407.

McKee, M. L., Melton-Celsa, A. R., Moxley, R. A., Francis, D. H., and O'Brien A. D. 1995. Enterohemorrhagic *Escherichia coli* O157:H7 requires intimin to colonize the gnotobiotic pig intestine and to adhere to HEp-2 cells. Infection and Immunity. **63**, 3739-3744.

McKee, M. L., and O'Brien, A. D. 1995. Investigation of enterohemorrhagic *Escherichia coli* O157:H7 adherence characteristics and invasion potential reveals a new attachment pattern shared by intestinal *E. coli*. Infection and Immunity. **63**, 2070-2074.

McKee, M. L., and O'Brien, A. D. 1996. Truncated enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 intimin (EaeA) fusion proteins promote adherence of EHEC strains to HEp-2 cells. Infection and Immunity. **64**, 2225-2233.

McNally, A., Roe, A. J., Simpson, S., Thomson-Carter, F. M., Hoey, D. E. E., Currie, C., Chakraborty, T., Smith, D. G. E., and Gally, D. L. 2001. Differences in levels of secreted

locus of enterocyte effacement proteins between human disease-associated and bovine *Escherichia coli* O157. Infection and Immunity. **69,** 5107-5114.

Mellies, J.L., Elliott, S. J., Sperandio, V., Donnenberg, M. S., and Kaper, J. B. 1999. The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). Molecular Microbiology. **33,** 296-306.

Melton-Celsa, A. R., and O'Brien, A. D. 1998. Structure, biology, and relative toxicity of Shiga toxin family members for cells and animals. In *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. Editors Kaper, J. B., and O'Brien A. D. ASM Press, Washington, D.C. pp. 30-37.

Meng, J., Zhao, S., Doyle, M. P., and Joseph, S. W. 1998. Antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM isolated from animals, food, and humans. Journal of Food Protection. **61,** 1511-1514.

Meyer. T., Karch, H., Hacker, J., Bocklage, H., and Heesemann J. 1992. Cloning and sequencing of a Shiga-like toxin II-related gene from *Escherichia coli* O157:H7 strain 7279. Zentralbl Bakteriol. **276,** 176-188.

Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A., and Yanagawa, H. 1999. Massive outbreak of *Escherichia coli* O157:H7 infection in school children in Sakai City, Japan, associated with consumption of white radish sprouts. American Journal of Epidemiology. **150,** 787-796.

Mills, M., and Payne, S. M. 1995. Genetics and regulation of heme iron transport in *Shigella dysenteriae* and detection of an analogous system in *Escherichia coli* O157:H7. Journal of Bacteriology. **177,** 3004-3009.

Moore, A. E., Sabachewsky, L., and Toolan, H. W. 1955. Culture characteristics of four permanent lines of human cancer cells. Cancer Research. **15,** 598-602.

Mukherjee, J., Chios, K., Fishwild, D., Hudson, D., O'Donnell, S., Rich, S. M., Donohue-Rolfe, A., and Tzipori, S. 2002. Human Stx2-specific monoclonal antibodies prevent

systemic complications of *Escherichia coli* O157:H7 infection. Infection and Immunity. **70,** 612-619.

Nataro, J. P., Baldini, M. M., Kaper, J. B., Black, R. E., Bravo, N., and Levine M. M. 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. Journal of infectious diseases. **152,** 560-565.

Nataro, J. P., and Kaper, J. B. 1998. Diarrheagenic *Escherichia coli*. Clinical Microbiology Reviews. **11,** 142-201.

Nataro, J. P., Seriwatana, J., Fasano, A., Maneval, D. R., Guers, L. D., Dubrovsky, Noriega, F., Levine M. M., and Morris, J. G. 1995. Identification and cloning of a novel plasmid-encoded enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains. Infection and Immunity. **63,** 4721-4728.

Naylor, S. W., Low, J. C., Besser, T. E., Mahajan, A., Gunn, G. J., Pearce, M. C., McKendrick, I. J., Smith D. G. E., and Gally, D. L. 2003. Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonisation of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. Infection and Immunity. **71,** 1505-1512.

Neely, M. N., and Freedman D. I. 1998. Functional and genetic analysis of regulatory regions of coliphage H-19B: location of shiga-like toxin and lysis genes suggests a role for phage functions in toxin release. Molecular Microbiology. **28,** 1255-1267.

Norris, F. A., Wilson, M. P., Wallis, T. S., Galyov, E. E., and Majerus, P. W. 1998. SopB, a protein required for virulence of *Salmonella dublin*, is an inositol phosphate phosphatase. Proceedings in the National Academy of Science. USA. **95,** 14057-14059.

O'Brien, A. D., and Holmes, R. K. 1987. Shiga and Shiga-like toxins. Microecology and Therapy. **51,** 206-220.

O'Brien, A. D., Marques, L. R., Kerry, C. F., Newland, J. W., and Holmes, R. K. 1989. Shiga-like toxin converting phage of enterohaemorrhagic *Escherichia coli* strain 933. Microbial Pathogenesis. **6,** 381-390.

O'Brien, S. J., Murdoch, P. S., Riley, A. H., King, I., Barr, M., Murdoch, S., Greig, A., Main, R., Reilly, W. J., and Thomson-Carter, F. M. 2001. A foodborne outbreak of Vero cytotoxin-producing *Escherichia coli* O157:H-phage type 8 in hospital. Journal of Hospital Infection. **49,** 167-172.

O'Brien, A. D., Newland, J. W., Miller, S. F., Holmes, R. K., Smith, H. W., and Formal, S. B. 1984. Shiga-like toxin-converting phages from *Escherichia coli* strains that cause haemorrhagic colitis or infantile diarrhoea. Science. **236,** 694-696.

O'Brien, A. D., Tesh, V. L., Donohue-Rolfe, A., Jackson, M. P., Olsnes, S., Sanvig, K., Lindberg, A. A., and Keusch, G. T. 1992. Shiga toxin: biochemistry, genetics, mode of action and role in pathogenesis. Curent Topics in Microbiology and Immunology. **180,** 65-94.

Obrig, T., Seaner, R., Bentz, M., Huynh, L., and Heatherly, D. 2000. Direct vs. indirect effects of Shiga toxins on endothelial cells. In 4th International symposium and workshop on "Shiga toxin (Verocytotoxin) producing *Escherichia coli* infections". Programs and Abstracts. pp.50.

O'Donnell, J. M., Thornton, L., McNamara, E. B., Prendergast, T., Igoe, D., and Cosgrove, C. 2002. Outbreak of Vero cytotoxin-producing *Escherichia coli* O157 in a child day care facility. Communical Diseases and Public Health. **5,** 54-58.

Oelschlaeger, T. A., Barrett, T. J., and Kopecko, D. J. 1994. Some structures and processes of human epithelial cells involved in uptake of enterohemorrhagic *Escherichia coli* O157:H7 strains. Infection and Immunity. **62,** 5142-5150.

Olano-Martin, E., Williams, M. R., Gibson, G. R., and Rastall, R. A. 2003. Pectins and pectic-oligosaccharides inhibit *Escherichia coli* O157:H7 Shiga toxin as directed towards the human colonic cell line HT29. FEMS Microbiology Letters. **218,** 101-105.

O'Loughlin, E. V., and Robins-Browne, R. M. 2001. Effect of Shiga toxin and Shiga-like toxins on eukaryotic cells. Microbes and Infection. **3,** 493-507.

Olsen, A., Jonsson, A., and Normack, S. 1989. Fibronectin binding mediated by a novel class of suface organelles on *Escherichia coli*. Nature. **338,** 652-655.

Olsen, S. J., Miller, G., Breuer, T., Kennedy, M., Higgins, C., Walford, J., McKee, G., Fox, K., Bibb, W., and Mead, P. 2002. A waterborne outbreak of *Escherichia coli* O157:H7 infections and hemolytic uremic syndrome: implications for rural water systems. Emerging Infectious Diseases. **8,** 375-380.

Ørskov, I., and Ørskov, F. 1983. Serology of *Escherichia coli* fimbriae. Progress in Allergy. **33**, 80-105.

Ørskov, I., and Ørskov, F., Jann, B., and Jann, K. 1977. Serology and chemistry, and genetics of O and K antigens of *Escherichia coli*. Bacteriology Reviews. **41,** 667-710.

Ostroff, S. M., Griffin, P. M., Tauxe R. V., Shipman L. D., Greene K. D., Wells, J. D., Lewis, J. H., Blake, P. A., and Kobayashi, J. M. 1990. A state wide outbreak of *Escherichia coli* O157:H7 infections in Washington State. American Journal of Epidemiology. **132,** 239-247.

Oswald, E., Schmidt, H., Morabito, S., Karch, H., Marches, O., and Caprioli, A. 2000. Typing of intimin genes in human and animal enterohaemorrhagic and enteropathogenic *Escherichia coli*: characterisation of a new intimin variant. Infection and Immunity. **68,** 64-71.

Paiba, G. A., Gibbens, J. C., Pascoe, S. J., Wilesmith, J. W., Kidd, S. A., Byrne, C., Ryan, J. B., Smith, R. P., McLaren, M., Futter, R. J., Kay, A. C., Jones, Y. E., Chappell, S. A., Willshaw, G. A., and Cheasty, T. 2002. Faecal carriage of verocytotoxin-producing *Escherichia coli* O157 in cattle and sheep at slaughter in Great Britain. Veterinary Record. **150,** 593-598.

Pakpinyo, S., Ley, D. H., Barnes, H. J., Vaillancourt, J. P., and Guy, J. S. 2002. Prevalence of enteropathogenic *Escherichia coli* in naturally occurring cases of poult enteritis-mortality syndrome. Avian Diseases. **46,** 360-369.

Parscot, C., and Sansonetti, P. J. 1996. Invasion and pathogenesis of *Shigella* infections. Current Topics in Microbiology. **209,** 25-42.

Paton, A. W, Beutin, L., and Paton, J. C. 1995. Heterogeneity of the amino-acid sequences of *Escherichia coli* Shiga-like toxin type-I operons. Gene. **153,** 71-74.

Paton, J. C., and Paton, A. W. 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clinical Microbiology Reviews. **11,** 450-479.

Paton, A. W., and Paton, J. C. 2002. Reactivity of convalescent-phase hemolytic-uremic sydrome patient sera with the megaplasmid-encoded TagA protein of Shiga toxigenic *Escherichia coli* O157. Journal of Clinical Microbiology. **40,** 1395-1399.

Paton, A. W., Ratcliff, R. M., Doyle, R. M., Seymour-Murray, J., Davos, D., Lanser, J. A., and Paton, J. C. 1996. Molecular microbiological investigation of an outbreak of haemolytic uraemic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. Journal of Clinical Microbiology. **34,** 1622-1627.

Paton, A., Woodrow, M. C., Doyle, R. M., Lanser, J.A., and Paton, J.C. 1999. Molecular characterisation of a Shiga toxigenic *E. coli* O133:H21 strain lacking *eae* responsible for a cluster of cases of haemolytic uraemic syndrome. Journal of Clinical Microbiology. **37,** 3357-3361.

Paton, A. W, Voss, E., Manning, P. A., and Paton, J. C. 1998. Antibodies to lipopolysaccharide block adherence of Shiga toxin-producing *Escherichia coli* to human intestinal epithelial (Henle 407) cells. Microbial Pathogenesis. **24,** 57-63.

Penfold, R. J., and Pemberton, J. M. 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene. **118,** 145-146

Penington, T. H. 1998. The Pennington group report on the circumstances leading to the 1996 outbreak of infection with *E. coli* O157 in Central Scotland, the implications for food safety, and the lessons to be learned. Edinburgh: The Stationary Office.

Perna, N. T., Mayhew, G. F., Posfai, G., Elliott, S., Donnenberg, M. S., Kaper, J. B., and Blattner, F. R. 1998. Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. Infection and Immunity. **66,** 3810-3817.

Perna, N. T., Plunket III, G., Burland, V., Mau, B., Glasner, J. D., Rose, D. J., Mayhew, G. F., Evans, P. S., Gregor, J., Kirkpatrick, H. A., Pósfai, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E. J., Davis, N. W., Lim, A., Dimalanta, E. T., Potamousis, K. D., Apodaca, J., Anantharaman, T. S., Lin, J., Yen, G., Schwartz, D. C., Welch, R. A., and Blattner, F. R. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. Nature. **409,** 529-533.

Perry, M. B., MacLean, L., and Griffith D. W. 1986. Structure of the O-chain polysaccharide of the phenol-phase soluble lipopolysaccharide of *Escherichia coli* O:157:H7. Biochemistry and Cell Biology. **64,** 21-28.

Phillips, A. D., Navabpour, S., Hicks, S., Dougan, G., Wallis, T., and Frankel, G. 2000. Enterohaemorrhagic *Escherichia coli* O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. Gut. **47**, 377-381.

Plunkett, G. III., Rose, D. J., Durfee, T. J., and Blattner, F. R. 1999. Sequence of Shiga toxin 2 phage 933W from *Escherichia coli* O157:H7: Shiga toxin as a phage late-gene product. Journal of Bacteriology. **181,** 1767-78.

Potters, D., Pierard, D., Muyldermans, G., Moriau, L., and Lauwers, S. 1999. Haem transport gene (*chuA*) in clinical isolates of Verocytotoxin-producing *E. Coli*. Acta Clinica Belgica. **54,** pp. 46.

Price, S. B., Cheng, C. M., Kaspar, C. W., Wright, J. C., DeGraves, F. J., Penfound, T. A., Castanie-Cornat, M. P., and Foster, J. W. 2000. Role of *rpoS* in acid resistance and fecal shedding of *Escherichia coli* O157:H7. Applied and Environmental Microbiology. **66,** 632-637.

Pritchard, G. C., Williamson, S., Carson, T., Bailey, J. R., Warner, L., Willshaw, G., and Cheasty, T. 2001. Wild rabbits-a novel vector for verocytotoxigenic *Escherichia coli* O157. Veterinary Record. **149,** 567.

Pritchard, G. C., Willshaw, G. A., Bailey, J. R., Carson, T., and Cheasty T. 2000. Verocytotoxin-producing *Escherichia coli* O157 on a farm open to the public: outbreak investigation and longitudinal bacteriological study. Veterinary Record. **147,** 259-264.

Quinn, O. J., Carter, M. E., Markey, B., and Carter, G. R. 1994. Clinical Veterinary Microbiology. Wolfe, Spain.

Raitner, Y. A., Salmenlinna, S., Eklund, M., Keskimaki, M., and Siitonen, A. 2003. Serology and genetics of the flagellar antigen of *Escherichia coli* O157:H7a, 7c. Journal of Clinical Microbiology. **41,** 1033-1040.

Raitner, Y. A., and Sinelnikova, V. A. 1969. Serological study of flagellar antigens revealed in some enteropathogenic variants of Escherichia coli. Zh. Mikrobiol. Epidemiol. Immunobiol. **13,** 111-116.

Ramberg, M., Lofdahl, S., De Jong, B., Anderson, Y., and Ziese T. 1996. Outbreak of *Escherichia coli* O157 in Sweden. Euro Surveillance. **1,** 2-3.

Reid, S. D., Selander, R. K., and Whittam, T. S. 1999. Sequence diversity of flagellin (*fliC*) alleles in pathogenic *Escherichia coli*. Journal of Bacteriology. **181,** 153-160.

Reida, P., Wolff, M., Pohls, H. W., Kuhlmann, W., Lehmacher, A., Aleksic, S., Karch, H., and Bockemuhl, J. 1994. An outbreak due to enterohaemorrhagic *Escherichia coli* O157:H7 in a children day care centre characterized by person-to-person transmission and environmental contamination. Zentralbl Bakteriol. **281,** 534-543.

Renter, D. G., Sargent, J. M., Hygnstrom, S., Hoffman, J. D., and Gillespie, J. R. 2001. *Escherichia coli* O157:H7 in free ranging deer in Nebraska. Journal of Wildlife Diseases. **37,** 755-760.

Rice, D. H., Hancock, D. D., and Besser, T. E. 1995. Verotoxigenic *E. coli* O157 colonisation of wild deer and range cattle. Veterinary Record. **137,** 524.

Rice, D. H., Hancock, D. D., and Besser, T. E. 2003. Faecal culture of wild animals for *Escherichia coli* O157:H7. Veterinary Record. **152,** 82-83.

Rice, D. H., McMenamin, K. M., Pritchett, L. C., Hancock, D. D., and Besser, T. E. 1999. Genteic diversity of *Escherichia coli* O157 isolates from 41 Pacific Northwest cattle herds. Epidemiology and Infection. **122,** 479-484.

Riley, L. W., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., davis, B. R., Hebert, R. J., Olcott, E. S., Johnson, L. M., Hargrett, N. T., Blake, P. A., and Cohen, M. L. 1983. Haemorraghic colitis associated with a rare *Escherichia coli* serotype. New England Journal of Medicine. **308,** 681-685.

Robertson, J. M., Grant, G., Allen-Vercoe, E., Woodward, M. J., Pusztai, A., and Flint H. J. 2000. Adhesion of *Salmonella enterica* serovar Enteritidis strains lacking fimbriae and flagella to rat ileal explants cultured at the air interface or submerged in tissue culture medium. Journal of Medical Microbiology. **49,** 691-696.

Roe, A. J., Currie, C., Smith, D. G. E., and Gally D. L. 2001. Analysis of type 1 fimbriae expression in verotoxigenic *Escherichia coli*: a comparison between serotypes O157 and O26. Microbiology. **147,** 145-152.

Rosenshine, I., Ruschkowski, S., Stein, M., Reinscheid, D. J., Mills, S.D., and Finlay, B. B. 1996. A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. EMBO Journal. **15,** 2613-2624.

Rubini, S., Cardeti, G., Amiti, S., Manna, G., Onorati, R., Caprioli, A., and Morabito, S. 1999. Verocytotoxin-producing *Escherichia coli* O157 in sheep milk. Veterinary Record. **144,** 56.

Ryan, C. A., Tauxe, R. V., Hosek, G. W., Wells, J. G., Stotesz, P. A., McFadden, H. W., Smith, P. W., Wright, J. F., and Blake, P. A. 1986. *Escherichia coli* O157:H7 diarrhoea in a nursing home. Clinical, epidemiological, and pathological finding. Journal of Infectious Diseases. **154,** 631-638.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. **239,** 487-491.

Sandhu, K. S, and Gyles, C. L. 2002. Pathogenic Shiga toxin-producing *Escherichia coli* in the intestine of calves. Canadian Journal of Veterinary Research. **66,** 65-72.

Sansonetti, P. J. 1992. Molecular and cellular biology of *Shigella flexneri* invasiveness: from cell assay systems to shigellosis. Current Topics in Microbiology and Immunology. **180,** 1-19.

Sargent, J. M., Hafer, D. J., Gillespie, J. R., Oberst, R. D., and Flood, J. 1999. Prevalence of *Escherichia coli* O157:H7 in white-tailed deer sharing rangeland cattle. Journal of the American Veterinary Medical Association. **215,** 792-794.

Sasakawa, C., Buysse, J. M., and Watanabe, H. 1992. The large virulence plasmid of *Shigella*. Current Topics in Microbiology and Immunology. **180,** 21-44.

Savarino, S. J., Fasano, A., Robertson, D. C., and Levine, M. M. 1991. Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an *in vitro* rabbit intestinal model. Journal of Clinical Investigation. **87,** 1450-1455.

Savarino, S. J., McVeigh, A., Watson J., Molina, J., Cravioto, A., Echeverria, P., Bhan, M. K., Levine, M. M., and Fasano, A. 1996. Enteroaggregative *Escherichia coli* heat-stabile enterotoxin is not restricted to enteroaggregative *Escherichia coli*. Journal of Infectious Diseases. **173,** 1019-1022.

Schauer, D. B., and Falkow, S. 1993. Attaching and effacing locus of a *Citrobacter freundii* biotype that causes transmissble murine colonic hyperplasmia. Infection and Immunity. **61,** 2486-2492.

Schmidt, H. 2001. Shiga-toxin-converting bacteriophages. Research in Microbiology. **152,** 687-695.

Schmidt, H., Beutin, L., and Karch, H. 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL933. Infection and Immunity. **63,** 1055-1061.

Schmidt, H., Karch, H., and Beutin, L. 1994. The large-sized plasmids of enterohemorrhagic *Escherichia coli* O157 strains encode hemolysins which are presumably members of the *E. coli* alpha-hemolysin family. FEMS Microbiology Letters. **117,** 189-96.

Schmidt, H., and Karch, H. 1996. Enterohemolytic phenotypes and genotypes of Shiga toxin-producing *Escherichia coli* O111 strains from patients with diarrhoea and hemolytic uremic syndrome. Journal of Clinical Microbiology. **34,** 2364-2367.

Schmidt, H., Kernbach, C., and Karch, H. 1996. Analysis of the EHEC hly operon and its location in the physical map of the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. Microbiology. **142,** 907-914.

Schmidt, H., Henkel, B., and Karch, H. 1997. A gene cluster closely related to type II secretion pathway operons of gram-negative bacteria is located on the large plasmid of enterohemorrhagic *Escherichia coli* O157 strains. FEMS Microbiology Letters. **15,** 265-72.

Schmidt, H., Scheef, J., Huppertz, H. I., Frosch, M., and Karch, H. 1999. *Escherichia coli* O157:H7 and O157:H(-) strains that do not produce Shiga toxin: phenotypic and genetic characterization of isolates associated with diarrhea and hemolytic-uremic syndrome. Journal of Clinical Microbiology. **37,** 3491-3496.

Schmitt, C. K., McKee, M. L., and O'Brien, A. D. 1991. Two copies of Shiga-like toxin-II-related genes common in enterohermorrhagic *Escherichia coli* strains responsible for the antigenic heterogeneity of the O157:H- strain E32511. Infection and Immunity. **59,** 1065-1073.

Schoeni J. L., and Doyle M. P. 1994. Variable colonisation of chickens perorally inoculated with *Escherichia coli* O157:H7 and subsequent contamination of eggs. Applied and Environmental Microbiology. **60,** 2958-2962.

Scotland, S. M., Smith, H. R., Willshaw, G. A., and Rowe, B. 1983. Verocytotoxin production in a strain of *Escherichia coli* is determined by genes carried on a bacteriophage. Lancet. **i,** 216.

Scotland, S. M., Willshaw, G. A., Smith, H. R., and Rowe, B. 1990. Properties of strains of *Escherichia coli* O26:H11 in relation to their enteropathogenic or enterohemorrhagic classification. Journal of Infectious Diseases. **162,** 1069-1074.

Scotland, S. M., Willshaw, G. A., Smith, H. R., Said, B., Stokes, N., and Rowe, B. 1993. Virulence properties of *Escherichia coli* strains belonging to serogroups O26, O55, O111,

and O128 isolated in the United Kingdom in 1991 from paients with diarrhoea. Epidemiology and Infection. **111,** 429-438.

Sears, C. L., and Kaper, J. B. 1996. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. Microbiology Reviews. **60,** 167-215.

Shaw, R., Daniel, S., Frankel, G., and Knutton, S. 2000. Interaction of enteropathogenic and enterohaemorrhagic *Escherichia coli* with red blood cell monolayers. In 4th International symposium and workshop on "Shiga toxin (Verocytotoxin) producing *Escherichia coli* infections". Programs and Abstracts. pp 183.

Sherman, P. M., and Soni, R. 1988. Adherence of Vero cytotoxin-producing Escherichia coli of serotype O157:H7 to human epithelial cells in tissue culture: role of outer membranes as bacterial adhesins. Journal of Medical Microbiology. **26,** 11-17.

Sherman, P., Soni, R., Petric, M., and Karmali, M. 1987. Surface properties of the Vero cytotoxin-producing *Escherichia coli* O157:H7. Infection and Immunity. **55,** 1824-1829.

Sherman, P., Soni, R., and Yeger H. 1988. Characterization of flagella purified from enterohemorrhagic, vero-cytotoxin-producing *Escherichia coli* serotype O157:H7. Journal of Clinical Microbiology. **26,** 1367-1372.

Shimizu, K., Tanaka, K., Akatsuka, A., Endoh, M., and Koga, Y. 1999. Induction of glomerular lesions in the kidneys of mice infected with Vero toxin-producing *Escherichia coli* by lipopolysaccharide injection. Journal of Infectious Diseases. **180,** 1374-1377.

Shimizu, T., Yamasaki, S., Tsukamoto, T., and Takeda, Y. 1999. Analysis of the genes responsible for the O-antigen synthesis in enterohaemorrhagic *Escherichia coli* O157. Microbial Pathogenesis. **26,** 235-247.

Siegler, R. L., Obrig, T. G., Pysher, T. J., Tesh, V. L., Denkers, N. D., and Taylor, F. B. 2003. Response to Shiga toxin 1 and 2 in a baboon model of hemolytic uremic syndrome. Pediatric Nephrology. **18,** 92-96.

Simon, R., Priefer, U., and Puhler, A. A. 1983. A broad host-range mobilisation system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Biotechnology. **1,** 784-791.

Sinclair, J. F, and O'Brien, A. D. 2002. Cell surface-localized nucleolin is a eukaryotic receptor for the adhesin intimin-gamma of enterohemorrhagic *Escherichia coli* O157:H7. Journal of Biological Chemistry. **277,** 2876-2885.

Sixma, T. K., Kalk, K. H., van Zanten, B. A., Dauter, Z., Kingma, J., Witholt, B., and Hol, W. G. 1993. Refined structure of *Escherichia coli* heat-labile enterotoxin, a close relative of cholera toxin. Journal of Molecular Bioliology. **230,** 890-918.

Small. P. L. C., and Falkow, S. 1988. Identification of regions on a 230-kilobase plasmid from enteroinvasive *Escherichia coli* that are required for entry into HEp-2 cells. Infection and Immunity. **56,** 225-229.

Smith, H. R., Green, P., and Parzell, Z. 1983. Vero cell toxins in *Escherichia coli* and related bacteria: transfer by phage and conjugation and toxic action in laboratory animals, chickens and pigs. Journal of General Microbiology. **129,** 3121-3137.

Smith, H. W., and Linggood, M. A. 1972. Further observations on *E. coli* enterotoxins with particular regard to those produced by atypical piglet strains and by calf and lamb strains. Journal of Medical Microbiology. **5**, 243-250.

Smith, H. R., Rowe, B., Adak, G. K., and Reilly W. J. 1997. Shiga toxin (Verocytotoxin)-producing *Escherichia coli* in the United Kingdom. In *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. Editors Kaper, J. B., and O'Brien A. D. ASM Press, Washington, D.C. pp. 49-58.

Smyth, C. J. 1988. Flagella their role in virulence. In Immunochemical and Molecular Genetic Analysis of Bacterial Pathogens. Editors Owen P., and Foster, T. J. Elsevier. Amsterdam. The Netherlands. pp. 3-11.

Sojka, W. J. 1965. *Escherichia coli* in Domestic Animals and Poultry. Commonwealth Agricultural Bureaux, Farnham Royal, UK.

Sojka, M. G., Dibb-Fuller, M. P, and Thorns, C. J. 1996. Charcteristion of monoclonal antibodies specific to SEF21 fimbriae of *Salmonella enteritidis* and their reactivity with other Salmonellae and enterobacteria. Veterinary Microbiology **48,** 207-221.

Sparling, P. H. 1998. *Escherichia coli* O157:H7 outbreaks in the United States, 1982-1996. Journal of the American Veterinary Medicine Association. **213,** 1733.

Sperandio, V., Mellies, J. L., Nguyen, W., Shin, S., and Kaper, J. B. 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli.* Proceedings of the National Academy of Science, U S A. **96**, 15196-15201.

Sperandio, V., Torres, A. G., Giron, J. A., and Kaper, J. B. 2001. Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. Journal of Bacteriology. **183,** 5187-5197.

Sperandio, V., Torres, A. G., and Kaper, J. B. 2002. Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli.* Molecular Microbiology. 43, 809-821.

Standiford, H. C. 1995. Tetracyclines and chloramphenicol. In Principles and Practices of Infectious Diseases. Edited by Mandell, G. L., Bennett, J. E, and Dolin, R. J. London, Churchill Livingstone Inc, UK. pp 306-317.

Stavric, S., Buchanan, B., and Gleeson, T. M. 1991. Intestinal colonization of and competitive exclusion of *Escherichia coli* O157:H7 in young chicks. In Quality of Poultry Products III. Safety and Marketing Aspects. Edited by Mulder, R. W. A. W., and de Vries, A. W. Beekbergen, The Netherlands: Spelderholt centre for Poultry Research and Information services. pp237-247.

Stavric, S., Buchanan, B., and Gleeson, T. M. 1992. Competitve exclusion of *Escherichia coli* O157:H7 from chicks with anaerobic cultures of faecal microflora. Letters in Applied Microbiology. **14,** 191-193.

Stavric, S., Buchanan, B., and Gleeson, T. M. 1993. Intestinal colonisation of young chicks with *Escherichia coli* O157:H7 and other Verotoxin-producing serotypes. Journal of Applied Microbiology. **74,** 557-563.

Stevenson, J., and Hanson, S. 1996. Outbreak of *E. coli* O157:H7 phage type 2 infection associated with eating pre-cooked meats. Communicable Disease Reports. CDR Reviews. **6,** R116-R118.

Strockbrine, N. A., Jackson, M. P., Sung, L. M., Holmes, R. K., and O'Brien, A. D. 1988. Cloning and sequencing of genes for Shiga toxin from *Shigella dysenteriae* type 1. Journal of Bacteriology. **170,** 1116-1122.

Stuart, F. A., and Corbel, M. J. 1982. Identification of a serological cross-reaction between *Brucella abortus* and *Escherichia coli* O157. Veterinary Record. **110,** 202-203.

Sueyoshi, M., and Nakazawa, M. 1994. Experimental infection of young chicks with attaching and effacing *Escherichia coli*. Infection and Immunity. **62,** 4066-4071.

Sussman, M. 1997. *Escherichia coli* and human disease. In *Escherichia coli*. Mechanisms of virulence. Edited by Sussman, M. Cambridge University Press, United Kingdom. pp. 3-48.

Suzuki, A., Doi, H., Matsuzawa, F., Aikawa, S., Takiguchi, K., kawano, H., Hayashida, M., and Ohno, S. 2000. Bcl-2 antiapoptotic protein mediates Verotoxin II-induced cell death: possible association between Bcl-2 and tissue failure by *E. coli* O157:H7. Genes and Development. **14,** 1734-1740.

Szabo, R. A., Todd, E. C. D., and Jean, A. 1986. Method to isolate *Escherichia coli* from food. Journal of Food Protection. **49,** 768-772.

Taguchi, H., Takahashi, M., Yamaguchi, H., Osaki, T., Komatsu, A., Fujioka, Y., and Kamiya, S. 2002. Experimental infection of germ-free mice with hyper-toxigenic enterohaemorrhagic *Escherichia coli* O157:H7, strain 6. Journal of Medical Microbiology. **51,** 336-343.

Takeda, T., Nakao, H., and Yamanaka, T. 1993. High prevalence of serum antibodies to Verocytotoxins 1 and 2 among healthy adults in Japan. Journal of Infection. **27,** 211-212.

Talero, K., and Talero, A. 1993. Foundations in microbiology. Wm. C. Brown Publishers, USA.

Tarr, P. I. 1995. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clinical Infectious Diseases. **20,** 1-10.

Tarr, P. I., Bilge, S. S., Vary, J. C. Jr, Jelacic, S., Habeeb, R. L., Ward, T. R., Baylor, M. R., and Besser, T. E. 2000. Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. Infection and Immunity. **68,** 1400-1407.

Tarr, C. L., and Whittam, T. S. 2002. Molecular evolution of the intimin gene in O111 clones of pathogenic *Escherichia coli.* Journal of Bacteriology. **184,** 479-487.

Tatsuno, I., Horie, M., Abe, H., Miki, T., Makino, K., Shinagawa, H., Taguchi, H., Kamiya, S., Hayashi, T., and Sasakawa, C. 2001. *toxB* gene on pO157 of enterohemorrhagic *Escherichia coli* O157:H7 is required for full epithelial cell adherence phenotype. Infection and Immunity. **69,** 6660-6669.

Tatsuno, I., Kimura, H., Okutani, A., Kanamaru, K., Abe, H., Nagai, S., Makino, K., Shinagawa, H., Yoshida, M., Sato, K., Nakamoto, J., Tobe, T., and Sasakawa, C. 2000. Isolation and characterization of mini-Tn5Km2 insertion mutants of enterohemorrhagic *Escherichia coli* O157:H7 deficient in adherence to caco-2 cells. Infection and Immunity. **68,** 5943-5952.

Taylor, C. M., White, R. H. R., Winterborn, M. H., and Rowe, B. 1986. Haemolytic-uraemic syndrome: clinical experience of an outbreak in the west midlands. British Medical Journal. **292,** 1513-1516.

Te Loo, D. M. W. M., Van Hinsbergh, V. W. M., Van Den Heuvel, L. P. W. J., and Monnens, L. A. H. 2001. Detection of Verocytotoxin bound to circulating polymorphonuclear leukocytes of patients with patients with hemolytic uremic syndrome. Journal of the Americam Society of Nephrology. **12,** 800-806.

Tilden, J. Jr, Young, W., McNamara, A. M., Custer, C., Boesel, B., Lambert-Fair, M. A., Majkowski, J., Vugia, D., Werner, S. B., Hollingsworth, J., and Morris, J. G. Jr. 1996. A new route of transmission for *Escherichia coli*: infection from dry fermented salami. American Journal of Public Health. **86,** 1142-1145.

Toolan H . 1954. Cancer Research. **14,** 660.

Torres, A. G., Giron, J. A., Perna, N. T., Burland, V., Blattner, F. R., Avelino-Flores, F., and Kaper, J. B. 2002. Identification and characterization of lpfABCC'DE, a fimbrial operon of enterohemorrhagic *Escherichia coli* O157:H7. Infection and Immunity. **70,** 5146-5427.

Torres, A. G., and Payne, S. M. 1997. Haem iron-transport system in enterohaemorrhagic *Escherichia coli* O157:H7. Molecular Microbiology. **23,** 825-833.

Toth, I., Cuhen, M. L., Rumschlag, H. S., Riley, L. W., Carr, J. H., Bond, W. W., and Wachsmuth, I. K. 1990. Influence of the 60-Megadalton plasmid on the adherence of *Escherichia coli* O157 and genetic derivatives. Infection and Immunity. **58,** 1016-1231.

Tozzi, A. E., Caprioli, A., Minelli, F., Gianviti, A., De Petris, L., Edefonti, A., Montini, G., Ferretti, A., De Palo, T., Gaido, M., and Rizzoni, G. 2003. Shiga toxin-producing *Escherichia coli* infections associated with hemolytic uremic syndrome, Italy, 1988-2000. Emerging Infectious Diseases. **9,** 106-108.

Tsuji, H., Hamada, K., Kawanishi, S., Nakayama, A., and Nakajima, H. 2002. An outbreak of enterohemorrhagic *Escherichia coli* O157 caused by ingestion of contaminated beef at grilled meat-restaurant chain stores in the Kinki District in Japan: epidemiological analysis by pulsed-field gel electrophoresis. Japanese Journal of Infectious Diseases. **55,** 91-92.

Tu, X., Nisan, I., Yona, C., Hanski, E., and Rosenshine, I. 2003. EspH, a new cytoskeleton-modulating effector of enterohaemorrhagic and enteropathogenic *Escherichia coli*. Molecular Microbiology. **47,** 595-606

Tzipori, S., Gunzer, F., Donnenberg, M. S., de Montigny, L., Kaper, J.B., and Donohue-Rolfe, A. 1995. The role of the *eaeA* gene in diarrhea and neurological complications in a

gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection. Infection and Immunity. **63**, 3621-3627.

Tzipori, S., Wachsmuth, I. K., Chapman, C., Birden, R., Brittingham J, Jackson C, and Hogg J. 1986. The pathogenesis of hemorrhagic colitis caused by *Escherichia coli* O157:H7 in gnotobiotic piglets. Journal of Infectious Disease. **154,** 712-716.

Uhlich, G. A., Keen, J. E., and Elder, R. O. 2001. Mutations in the *csgD* promoter associated with variations in curli expression in certain strains of *Escherichia coli* O157:H7. Applied and Environmental Microbiology. **67,** 2367-2370.

Uhlich, G. A., Keen, J. E., and Elder, R. O. 2002. Variations in the *csgD* promoter of *Escherichai coli* O157:H7 associated with increased virulence in mice and increased invasion of HEp-2 cells. Applied and Environmental Microbiology. **70,** 395-399.

Vaandrager, A. B., Van der Wiel, E., Hom, M. L., Luthjens, L. H., and de Jonge, H. R. 1994. Heat-stabile enterotoxin receptor/guanylyl cyclase C is an oligomer consisting of functionally distinct subunits, which are non-covalently linked in the intestine. Journal of Biological Chemistry. **269,** 16409-16415.

Van Demark, P. J., and Batzing, B. L. 1987. The Microbes-an introduction to their nature. Menlo Park, Benjamin/Cummings publishing company.

Velge, P., Bottreau, E., Quere, P., Pardon, P., Nicolle, J. C., Morrison, M., Bout, D., and Dimier, I. 2002. Establishment and characterisation of partially differentiated chicken enterocyte cell clones. European Journal of Cell Biology. **81,** 203-212.

Vinogradov, E., Conlan, J. W., and Perry, M. B. 1998. Serological croos-reaction between the lipopolysaccharide O-polysaccharide antigens of *Escherichia coli* O157:H7 and strains of *Citrobacter freundii* and *Citrobacter sedlakii*. FEMS Microbiology Letters. **190,** 157-161.

Waddell, T., Petric, M., Cohen, A., and Lingwood, C. 1988. Globotrisyl ceramide is specifically recognised by the *Escherichia coli* Verocytotoxin. Biochemical and Biophysical Research Communication. **152,** 674-679.

Wadolkowski, E. A., Burris, J. A., and O'Brien, A. D. 1990. Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7. Infection and Immunity. **58,** 2438-2435.

Wales, A.D., Cliffton-Hadley, F. A., Cookson, A. L., Dibb-Fuller, M. P., La Ragione, R. M., Pearson, G. R., and Woodward, M. J. 2002. Production of attaching-effacing lesions in ligated large intestine loops of 6-month-old sheep by *Escherichia coli* O157:H7. Journal of Medical Microbiology. **51,** 755-763.

Wales, A. D., Pearson, G. R., Best, A., Cookson, A. L., LaRagione, R. M., Roe, J. M., and Hayes, C. M., and Woodward M. J. 2003. Naturally-acquired attaching and effacing *E. coli* in sheep. Submitted to the Journal of Comparitive Pathology.

Wales, A. D., Pearson, G. R., Skuse, A. M., Roe, J. M., Hayes, C. M., Cookson, A. L., and Woodward, M. J. 2001. Attaching and effacing lesions *Escherichia coli* O157:H7 causes by *Escherichia coli* O157:H7 in experimentally inoculated neonatal lambs. Journal of Medical Microbiology. **50,** 752-758.

Wallace, J. S., Cheasty, T., and Jones, K. 1997. Isolation of Vero cytotoxin-producing *Escherichia coli* O157 from wild birds. Journal of Applied Microbiology. **82,** 399-404.

Wallace, J. S., and Jones, K. 1996. The use of selective and differential agars in the isolation of *Escherichia coli* from dairy herds. Journal of Applied Bacteriology. **81,** 663-668.

Wang, L., and Reeves, P. R. 1998. Organisation of *Escherichia coli* O157:H7 antigen gene cluster and identification of its specific genes. Infecion and Immunity. **66,** 3545-3551.

Wang, A., and Roth, J. R. 1988. Activation of silent genes by transposons Tn5 and Tn10. Genetics. **120,** 875-885.

Wang, L., Rothermund, D., Curd, H., and Reeves, P. R. 2000. Sequence diversity of the *Escherichia coli* H7 *fliC* genes: Implication for a DNA-based typing scheme for *E. coli* O157:H7. Journal of Clinical Microbiology. **38,** 1786-1790.

Warawa, J., Finlay, B.B., and Kenny, B. 1999. Type III secretion-dependent haemolytic activity of enteropathogenic *Escherichia coli*. Infection and Immunity. **67,** 5538-5540.

Watanabe, H. 2000. Molecular epidemiological investigations of Stx-producing Escherichia coli infections in Japan. In 4th International symposium and workshop on "Shigan toxin (verocytotoxin) producing *Escherichia coli* infections". Programs and abstracts. pp 35.

Watanabe, Y., Ozasa, K., Mermin, J. H., Griffin, P. M., Masuda, K., Imashuku, S., and Sawada, T. 1999. Factory outbreak of *Escherichia coli* O157:H7 infection in Japan. Emerging Infectious Diseases. **5,** 424-428.

Williams-Smith, H. 1965. The development of the flora of the alimentary tract in young animals. Journal of Pathology and Bacteriology. **80,** 495-513.

Williams-Smith, H., and Tucker, J. E.. 1975. The effect of antibiotic therapy on faecal excretion of *Salmonella typhimurium* by experimentally infected chickens. Journal of Hygiene. (Camb) **75,** 275-292.

Willshaw, G. A., Thirlwell, J., Jones, A. P., Parry, S., Salmon, R. L., and Hickey, M. 1994. Vero cytotoxin-producing *Escherichia coli* O157 in beefburgers linked to an outbreak of diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome in Britain. Letters in Applied Microbiology. **19,** 304-307.

Winstanley, C., and Morgan, J. A. W. 1997. The bacterial flagellin gene as a biomarker for detection, population genetics and epidemiological analysis. Microbiology. **143,** 3071-3084.

Wittam, T. S., and McGraw, E. A. 1996. Clonal analysis of EPEC serogroups. Reviews in Microbiology. Sao Paulo **27 (Supplement 1),** 7-16.

Wittam, T. S., and Wilson, R. A. 1988. Genetic relationships among pathogeneic strains of avian *E. coli*. Infection and Immunity. **56,** 2458-2466.

Wolff, C., Nisan, I., Hanski, E., Frankel, G., and Rosenshine, I. 1998. Protein translocation into host epithelial cells by infecting enteropathogenic *Escherichia coli*. Molecular Microbiology. **28,** 143-156.

Woods, J. B., Schmitt, C. K., Darnell, S. C., Meysick, K. C., and O'Brien, A. D. 2002. Ferrets as a model system for renal disease secondary to intestinal infection with *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli*. Journal of Infectious Diseases. **185,** 550-554.

Woodward, M. J., Best, A., Sprigings, K. A., Pearson, G. A., Skuse, A. M., Wales, A., Hayes, C. M., Roe, J. M., Low, J. C., and La Ragione, R. M. 2003. Non-toxigenic *Escherichia coli* O157:H7 strain NCTC 12900 causes attaching-effacing lesions and *eae*-dependent persistence in weaned sheep. International Journal of Medical Microbiology. **293,** 299-308.

Woodward, M. J., Gavier-Widen, D., McLaren, I. M., Wray, C., Sozman, M., and Pearson, G. R. 1999. Infection of gnotobiotic calves with *Escherichia coli* O157:H7 strain A84. Veterinary Record. **144,** 466-470.

Wright, J., and Villanueva, R. 1953. Differentiation of sero-fermentative types in *Bacterium coli* O group. Journal of Hygiene. (Camb). **51,** 39-48.

Xin-He, L., Xu, J. G., and Liu, B. Y. 1991. Experimental infection of specific-pathogen-free mice with enterohemorrhagic *Escherichia coli* O157:H7. Microbiology and Immunology. **35,** 515-524.

Yamamoto, T., and Taneike, I. 2000. The sequences of enterohemorrhagic *Escherichia coli* and *Yersinia pestis* that are homologous to the enteroaggregative *E. coli* heat-stable enterotoxin gene: cross-species transfer in evolution. FEBS Letters. **472,** 42-46.

Ye, C., and Xu, J. 2001. Prevalence of iron transport gene on pathogenicity-associated island of uropathogenic *Escherichia coli* in *E. coli* O157:H7 containing Shiga toxin gene. Journal of Clinical Microbiology. **39,** 2300-2305.

Yu, J., and Kaper, J. B. 1992. Cloning and characterization of the eae gene of enterohaemorrhagic *Escherichia coli* O157:H7. Molecular Microbiology. **6,** 411-417.

Yokoyama, K., Makino, K., Kubota, Y., Watanabe, M., Kimura, S., Yutsudo, C. H., Kurokawa, K., Ishii, K., Hattori, M., Tatsuno, I., Abe, H., Yoh, M., Iida, T., Ohnishi, M., Hayashi, T., Yasunaga, T., Honda, T., Sasakawa, C., and Shinagawa, H. 2000.

Complete nucleotide sequence of the prophage VT1-Sakai carrying the Shiga toxin 1 genes of the enterohemorrhagic *Escherichia coli* O157:H7 strain derived from the Sakai outbreak. Gene. **258,** 127-139.

Zadik, P. M., Chapman, P. A., and Siddons, C. A. 1993. Use of tellurite for the selection of verotoxigenic *Escherichia coli* O157. Journal of Medical Microbiology. **39,** 155-158.

Zang, W. L., Kohler, B., Oswald, E., Beutin, L., Karch, H., Morabito, S., Caprioli, A., Suerbaum, S., and Schmidt, H. 2002. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. Journal of Clinical Microbiology. **40,** 4486-4492.

Zinner, G. L., and Mayer, K. H. 1995. Sulphonamides and trimethoprim. In Principles and Practices of Infectios Diseases. Edited by Mandell, G. L., Bennett, J. E., and Dolin, R. J. London, Churchill Livingstone Inc., UK. pp 354-364.

Zhao, S., Meng, J., Doyle, M. P., Meinersman, R., Wang, G., and Zhao, P. 1996. A low molecular weight outer-membrane protein of *Escherichia coli* O157:H7 associated with adherence to INT407 cells and chicken caeca. Journal of Medical Microbiology. **45,** 90-96.

Appendix 1

Media

1. **Agarose gels** – 0.8% gels were prepared by the addition of 1 x TAE (40mM Tris-Acetate, 1mM EDTA) to 8g of agarose powder (Promega) to a final volume of 1 litre. Gels were cast in Perspex trays using approximately sized combs (BioRad) to form wells. Combs were removed from set gels before the gels were submerged in 1 x TAE in a DNA Sub-cell™ electrophoresis tank (Biorad).
2. **Antibiotics** – all antibiotics were purchased from Sigma with the exception of ampicillin (Beecham) and antibiotic diffusion discs (Oxoid) were prepared by the addition of soluble powder to appropriate dilutent. Antibiotics were prepared to the following stock solutions, ampicillin – 100mg/ml, chloramphenicol – 10mg/ml, kanamycin – 50mg/ml and streptomycin 50mg/ml and used at the following working concentrations, ampicillin – 100μg/ml, chloramphenicol – 10μg/ml, kanamycin – 25μg/ml and streptomycin – 25μg/ml.
3. **Buffered peptone water** – was prepared with 10g pancreatic digest of gelatin, 5g NaCl, 3.5g Na_2HPO_4 and 1.5g KH_2HPO_4 and made up to a final volume of 1 litre with sterile distilled H_2O and sterilised by autoclaving for 15 minutes at 15psi pressure (121°C). Buffered peptone water was stored at 4°C until required.
4. **Colonisation factor antigen medium (CFA)** - was prepared with 10g Casimino acids, 1.5g yeast extract, 0.05g $MgSO_4$, 0.005g $MnCl_2$, made up to 1 litre sterile distilled H_2O and autoclaved for 15 minutes at 15psi pressure (121°C). CFA was stored at 4°C until required.
5. **Colonisation factor antigen agar** - was prepared with 10g Casimino acids, 1.5g yeast extract, 0.05g $MgSO_4$, 0.005g $MnCl_2$, 2g agar made up to 1 litre sterile distilled H_2O and autoclaved for 15 minutes at 15psi pressure (121°C). CFA was stored at 4°C until required.
6. **Congo red CFA medium** – was prepared by the addition of 0.05% (v/v) Congo red dye to CFA medium. Congo red media was stored at 4°C until required.
7. **Denaturing solution** - was prepared with 87.75g NaCl, 20g NaOH made up to 1 litre with sterile distilled H_2O and sterilised by autoclaving for 15 minutes at 15psi pressure (121°C).
8. **DNA marker** – 1KB+ DNA ladder (Gibco-BRL) was prepared from stock by the addition of Orange G loading buffer and 350μl water to 50μl concentrated ladder.

9. **Ethidium bromide staining of gels** – gels were immersed in a 1 x TAE bath containing ethidium bromide (0.5μg/ml) (Sigma) before the DNA was visualised under UV.
10. **Hybridisation buffer (100ml)** – was prepared with 4g NaCl, 4g blocking agent and made up to 100ml with sterile distilled H_2O. The buffer was stored at ambient temperature for 2 hours and used immediately or stored at -20°C.
11. **Iso-sensitest agar** - was prepared with 11g hydrolysed caesein, 3g peptones, 2g glucose, 3g NaCl, 1g soluble starch, 2g di-sodium hydrogen phosphate, 1g sodium acetate, 0.2g magnesium glycerophosphate, 0.1g calcium gluconate, 0.001g cobaltous phosphate, 0.001g cupric sulphate, 0.001g zinc sulphate, 0.001g ferrous sulphate, 0.002g manganous chloride, 0.001g menadione, 0.001g cyanocobalamin, 0.02g L-cysteine hydrochloride, 0.02g L-tryptophan, 0.003g pyridoxine, 0.003g pantothenate, 0.0003g nicotinamide, 0.0003g biotin, 0.00004g thiamine, 0.01g adenine, 0.01g guanine, 0.01g xanthine, 0.01g uracil and 8g agar made up to a final volume of 1 litre with sterile distilled H_2O and pH to 7.4. Iso- sensitest agar was sterilised by autoclaving for 15 minutes at 15psi pressure (121°C) and stored at 4°C until required.
12. **Loading buffer** – 0.25% bromophenol blue and 30% glycerol in water was added to DNA in the ratio of 1:5 before samples were applied to wells.
13. **Luria bertanti broth –** was prepared with 5g NaCl, 10g pancreatic digest of caesin, 5g yeast extract and made up to a final volume of 1litre with sterile distilled H_2O, adjusted to pH 7.5 with NaOH and sterilsed by autoclaving for 15 minutes at 15psi pressure (121°C). LB broth was stored at 4°C until required.
14. **Luria bertani agar –** was prepared with 5g NaCl, 10g pancreatic digest of caesin, 5g yeast extract, 15g of agar and made up to a final volume of 1 litre with sterile distilled H_2O, adjusted to pH 7.5 with NaOH and sterilsed by autoclaving for 15 minutes at 15psi pressure (121°C). LB agar was stored at 4°C until required.
15. **Minimal medium** - was prepared with 100ml 10 x M9 salts, 0.6% (w/v) Na_2HPO, 0.3% (w/v) KH_2PO, 0.5% (w/v) NaCl and 1% (w/v) NH_4Cl and then autoclaved. Then 10mM $MgSO_4$, 20g glucose and 15g agar made up to a final volume of 1 litre with sterile distilled H_2O. Minimal media was stored at 4°C until required.
16. **Motility medium** - was prepared with 3.5g agarose and made up to 1 litre LB broth and autoclaved for 15 minutes at 15psi pressure (121°C). Motility medium was used immediately.
17. **M9 salts** - was prepared with 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl and 1g NH_4Cl and made up a volume of 1 litre with sterile distilled H_2O and then sterilised by autoclaving for 15 minutes at 15psi pressure (121°C). M9 salts was stored at 4°C until required.

18. **Neutralising solution** – was prepared with 87.75g NaCl, 66.5g Trizma base, 0.2% (v/v) 0.5M EDTA pH 8.0 made up to 1 litre with sterile distilled H_2O and adjusted to pH 7.2 and sterilised by autoclaving for 15 minutes at 15psi pressure (121°C).
19. **Nutrient agar** - was prepared with 15g agar, 5g peptone, 5g NaCl, 2g yeast extract and 1g beef extract, made up to a final volume of 1 litre with sterile distilled H_2O, gently heated until boiling and sterilised by autoclaving for 15 minutes at 15psi pressure (121°C). Nutrient agar was stored at 4°C until required.
20. **Phosphate buffered saline (PBS)** – two solutions were prepared. Solution A was prepared with 5.93g Na_2HPO, 4.25g NaCl and made up to 1 litre with sterile distilled H_2O. Solution B was prepared with 4.53g KH_2PO and 4.25g NaCl and made up to 1 litre with sterile distilled H_2O. Solution A was mixed with solution B until the required pH was obtained. Sterilisation was by autoclaving for 15 minutes at 15psi pressure (121°C).
21. **Primary wash buffer (1 litre)** – was prepared with 120g Urea, 1g SDS, 0.5M Na phosphate pH 7.0 (100ml), 8.7g NaCl, 1M $MgCl_2$ (10ml) and made up to litre with sterile distilled H_2O and stored at 4°C until required.
22. **Secondary wash buffer 20X (pH 10)** – was prepared with 121g Tris base, 112g NaCl and made up to 1 litre with sterile distilled H_2O and stored at 4°C until required. The stock was diluted 1:20 and 2ml/l of 1M $MgCl_2$ was added immediately prior to use.
23. **Sheep blood agar (5%)** – was prepared with 15g agar, 15g pancreatic digest of casein, 5g papaic digest of soybean meal, 5g NaCl and made up to a volume of 950ml sterile distilled H_2O, mixed thoroughly and gently heated until boiling, then sterilised by autoclaving for 15 minutes at 15psi pressure (121°C). The agar was cooled to 45-50 °C and then 50ml of sterile sheep blood was aseptically added, mixed in thoroughly and poured into sterile Petri dishes in 20ml volumes and stored at 4°C.
24. **SOC** - was prepared with 20g tryptone, 5g yeast extract, 0.5g NaCl and made up to a volume of 1 litre with sterile distilled H_2O and autoclaved. Once cooled 10ml of 1M $MgCl_2$, 10ml of 1M $MgSO_4$ and 10ml of 2M filter sterilised glucose solution was added. SOC was stored at 4°C until required.
25. **Sorbitol MacConkey agar supplemented with Cefixime Tellurite (CT-SMAC)** – was prepared with 20g peptone, 15g agar, 10g sorbitol, 5g NaCl, 1.5g bile salts No. 3, 0.03g neutral red and 1mg crystal violet and made up to a final volume of 1litre with sterile distilled H_2O and sterilised by autoclaving for 15 minutes at 15psi pressure (121°C). The agar was cooled to 45-50 °C and then 4ml of dissolved Cefixime (0.625mg/ml) Tellurite (0.0125mg/ml) was aseptically added, mixed in thoroughly and poured into sterile Petri dishes in 20ml volumes and stored at 4°C.

26. **TAE** – 50X stock was prepared with 242g Tri base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA (pH 8.0) made up to 1 litre. A working solution was prepared by diluting the stock 1 in 50 to give a working concentration of 0.04M Tris-acetate and 0.001M EDTA.
27. **TE buffer** - Tris was prepared with 121.1g Tris base dissolved in 800ml sterile distilled H_2O and adjusted to the desired pH with HCl. EDTA was prepared with 372.2g of d-sodium ethylene diamine tetra-acetate made up to 1 litre with sterile distilled H_2O and adjusted to pH 8.0 with NaOH. TE buffer was prepared by mixing 10mM Tris and 1mM EDTA and stored at ambient temperature until required.
28. **Tissue culture medium** - was prepared with Modified Eagles Media or Dulbeccos Media (Sigma) with the addition of 2mM L-glutamine (Sigma), 10% (v/v) foetal calf serum, 1% (v/v) non-essential amino acids (X100) and 50μg/ml gentamicin for culture of cells and 100μg/ml for invasion assays.
29. **X-gal** – supplied by Promega and used at 25mg/ml in LB agar.

Appendix 2

Statistical tests

1. Analysis of variance (ANOVA)

The one way analysis of variance allows us to compare several groups of observations, all of which are independent but possibly with a different mean for each group. A test of great importance is whether or not all the means are equal.

2. Student t-test

A student t-test is a hypothesis test for answering questions about the mean where the data are collected from two random samples of independent observations, each from an underlying normal distribution. When carrying out a student t-test, it is usual to assume that the variances for the two populations are equal.

3. Wilcoxon Mann-Whitney test

The Wilcoxon Mann-Whitney Test is one of the most powerful of the nonparametric tests for comparing two populations. It is used to test the null hypothesis that two populations have identical distribution functions against the alternative hypothesis that the two distribution functions differ only with respect to location (median), if at all. The Wilcoxon Mann-Whitney test does not require the assumption that the differences between the two samples are normally distributed. In many applications, the Wilcoxon Mann-Whitney Test is used in place of the two sample t-test when the normality assumption is questionable.

4. Kruskal-Wallis test.

The Kruskal-Wallis test is a nonparametric test used to compare three or more samples. It is used to test the null hypothesis that all populations have identical distribution functions against the alternative hypothesis that at least two of the samples differ only with respect to location (median), if at all. It is the analogue to the F-test used in analysis of variance. While analysis of variance tests depend on the assumption that all populations under comparison are normally distributed, the Kruskal-Wallis test places no such restriction on the comparison. It is a logical extension of the Wilcoxon-Mann-Whitney Test.

Papers attached

Best, A., La Ragione, R. M., Cooley, W. A., O'Connor, C. D., Velge, P., and Woodward, M. J. 2003. Interaction with avian cells and colonisation of specific pathogen free chicks by Shiga-toxin negative *Escherichia coli* O157:H7 (NCTC 12900). Veterinary Microbiology. **93,** 207-222.

Dibb-Fuller, M. P., Best, A., Stagg, D. A., Cooley, W. A., and Woodward, M. J. 2001. An in-vitro model for studying the interaction of *Escherichia coli* O157:H7 and other enteropathogens with bovine primary cell cultures. Journal of Medical Microbiology. **50,** 759-769.

Woodward, M. J., Best, A., Sprigings, K. A., Pearson, G. A., Skuse, A. M., Wales, A., Hayes, C. M., Roe, J. M., Low, J. C., and La Ragione, R. M. 2003. Non-toxigenic *Escherichia coli* O157:H7 strain NCTC 12900 causes attaching-effacing lesions and *eae*-dependent persistence in weaned sheep. International Journal of Medical Microbiology. **293,** 299-308.