Lack of flagella disadvantages *Salmonella enterica* serovar Enteritidis during the early stages of infection in the rat

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The roles of flagella and five fimbriae (SEF14, SEF17, SEF21, pef, lpf) in the early stages (up to 3 days) of *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) infection have been investigated in the rat. Wild-type strains LA5 and S1400 (fim+/fla+) and insertionally inactivated mutants unable to express the five fimbriae (fim-/fla+), flagella (fim+/fla-) or fimbriae and flagella (fim-/fla-) were used. All wild-type and mutant strains were able to colonize the gut and spread to the mesenteric lymph nodes, liver and spleen. There appeared to be little or no difference between the fim-/fla+ and wild-type (fim+/fla+) strains. In contrast, the numbers of aflagellate (fim+/fla- or fim-/fla-) salmonella in the liver and spleen were transiently reduced. In addition, fim+/fla- or fim-/fla- strains were less able to persist in the upper gastrointestinal tract and the inflammatory responses they elicited in the gut were less severe. Thus, expression of SEF14, SEF17, SEF21, pef and lpf did not appear to be a prerequisite for induction of *S. Enteritidis* infection in the rat. Deletion of flagella did, however, disadvantage the bacterium. This may be due to the inability to produce or release the potent immunomodulating protein flagellin.

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INTRODUCTION

Infections caused by *Salmonella enterica* serovars Enteritidis (*S. Enteritidis*) and Typhimurium (*S. Typhimurium*) continue to be a major health problem. They usually occur after consumption of contaminated food or water and can manifest in a variety of disease states, ranging from asymptomatic carrier status through to gastroenteritis or even severe systemic infection that can lead to death (Tsolis *et al.*, 1999; Kingsley & Baumber, 2000; Ohl & Miller, 2001). The nature and severity of the infection varies according to bacterial strain. It can also be greatly influenced by host factors, such as species, age and health status. Immunocompromised individuals are particularly susceptible to the pathogen. The majority of cases, both in humans and domesticated animals, are of a self-limiting gastroenteritis type.

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Abbreviation: MPO, myeloperoxidase.

The pathogenicity of salmonella is linked to a number of virulence factors, including adhesins, flagella, enzymes, toxins and other bioactive factors. These facilitate adherence to the gut, invasion, systemic spread and survival and proliferation in systemic organs (Tsolis *et al.*, 1999; Kingsley & Baumber, 2000; Ohl & Miller, 2001; Darwin & Miller, 1999; Humphries *et al.*, 2001; Galan, 2001). Attachment of the pathogen to the gut is a key initial step in the infection process. Mediation of attachment primarily by bacterial (fimbrial) adhesins has been suggested (Kingsley & Baumber, 2000; Darwin & Miller, 1999; Humphries *et al.*, 2001). However, recent studies suggest that other factors might also be involved (Allen-Vercoe *et al.*, 1999; Allen-Vercoe & Woodward, 1999; Robertson *et al.*, 2000; Robertson, 2000). *S. Enteritidis* mutant strains unable to express functional flagella were less able to attach to explants *in vitro* and less pathogenic to chicks *in vivo* (Allen-Vercoe *et al.*, 1999; Allen-Vercoe & Woodward, 1999; Robertson *et al.*, 2000; Robertson, 2000).

The aim of the present study was to evaluate the roles of fimbriae and flagella in the early stages of *S. Enteritidis* infection in a mammalian host. A rat model of salmonellosis

was used (Naughton *et al.*, 1995, 1996a). *Salmonella* sp. colonize the whole gastrointestinal tract of the rat and cause disruption of the small intestine epithelium, epithelial cell hyperplasia and infiltration by inflammatory cells (Naughton *et al.*, 1995, 1996a, 2000; Havelaar *et al.*, 2001; Islam *et al.*, 2000). Invasion is mainly via the ileum; large numbers of salmonella reach the mesenteric lymph nodes and moderate numbers spread to and persist in the liver and spleen. Rats continue to grow, albeit slowly, and death due to infection is rare, unless health or immune status or gut integrity has been compromised by other factors. Salmonellosis in the rat thus has many similarities to the self-limiting gastroenteritis-type infection common in humans and domesticated animals (Naughton *et al.*, 1995, 1996a, 2000; Havelaar *et al.*, 2001; Islam *et al.*, 2000). Defined fimbriae- (SEF14, SEF17, SEF21, pef and lpf) and/or flagella-deleted mutants of *S. Enteritidis* and their wild-type parent strains were tested in this infection model.

METHODS

Animal studies. Male specific-pathogen-free (SPF) Hooded-Lister rats (Rowett strain), bred in the small animal unit of the Rowett Research Institute, were used. Immediately after weaning (19 days), the rats were housed individually in grid-floor caging within a facility separate from other animals and given free access to high-quality semi-synthetic diet (100 g lactalbumin protein kg⁻¹ diet; Grant *et al.*, 2000) and water. This was done to reduce environmental exposure to exogenous bacteria and prevent cross-contamination. The levels of bacteria resident in the small intestine of rats managed in this manner were generally significantly lower than those for standard SPF rats (Grant, 1996). When the rats had reached approximately 85 g (around 30 days old), their food intake was gradually reduced over a 3–4 day period to 7 g day⁻¹ per rat, given as two feeds over the day. This was the mean daily free intake for rats of the same age after oral infection with *S. Enteritidis* and around 70 % of the free intake of this diet by non-infected animals. Rats were then transferred from the main animal unit to a Class II facility where they were housed singly in metabolism cages (Techniplast) within a flexi-film isolator (Moredun Animal Health). The rats were weighed daily.

Prior to inoculation, food was removed overnight. Rats (five per treatment) were then given 1 ml (approx. 10⁸ c.f.u.) of the appropriate *S. Enteritidis* wild-type or deletion-mutant strain or 1 ml culture medium by gavage. Rats were then returned to their cages. One or 3 h later, groups of five rats were killed by halothane (Rhodia Organique Fine) overdose and exsanguination. The abdomen was opened and tissues were removed aseptically and processed. The remaining rats were fed lactalbumin-based diet (7 g day⁻¹ per rat) and were killed by halothane overdose and exsanguination at 24 or 72 h post-infection (p.i.).

The stomach and the small intestine were flushed with PBS (pH 7.2) to remove contents and non-adherent bacteria. Twenty cm of jejunum (5–25 cm from the pylorus) and 20 cm of ileum (5–25 cm from the ileo-caecal junction) were removed. These and the stomach tissue, caecum plus contents, colon plus contents, mesenteric lymph node and representative proportions of the liver and spleen were processed for viable counts. The remaining small intestine (about 40 cm), liver and spleen was frozen in liquid nitrogen, weighed, freeze-dried and reweighed.

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the animal welfare unit of the institute and the appropriate governmental inspectorate monitor and review all animal studies. The management and experimental procedures undertaken were approved by the ethical committee and done in strict accordance with the requirements of the Act by staff licensed to carry out such procedures.

Culture of bacteria. *S. Enteritidis* strains LA5 and S1400/94 were originally isolated from poultry infections and have been characterized previously (Allen-Vercocoe *et al.*, 1999; Allen-Vercocoe & Woodward, 1999; Walker *et al.*, 1999; Cooper *et al.*, 1990). The isogenic mutant strains used (Table 1) were constructed by insertional mutagenesis and have been characterized previously (Allen-Vercocoe & Woodward, 1999). Stocks, maintained on Dorset egg slopes at 4 °C, were subcultured on to nutrient agar plates and grown at 37 °C overnight. Five to ten colonies from the plate were inoculated into 10 ml Luria–Bertani broth and incubated with agitation at 37 °C overnight to give approximately 10⁸ c.f.u. ml⁻¹.

Tissue samples were weighed and then homogenized in 10 ml maximum recovery diluent (MRD; Fisher Scientific) using a Janke-Kunkel Ultra-Turrax T25 tissue homogenizer at 20 000 r.p.m. for 30 s. Up to six sequential dilutions (1 : 10, v/v) of the primary homogenate were made

Table 1. Genotypes of *S. Enteritidis* mutant strains

Genotype of wild-type strains: SEF14+, SEF17+, SEF21+ (*S. Enteritidis* fimbriae 14, 17, 21 kDa), PEF+ (plasmid-encoded fimbriae), LPF+ (long polar fimbriae), FLA+ (flagellate). EAV9 and EAV10 are flagella-deleted mutants (fla–) respectively derived from S1400/94 and LA5. EAV26 is a multiple fimbriae-deleted mutant (fim–) of S1400/94. EAV37 is a combined fim– and fla– mutant of S1400/94. Abbreviations: MSHA, mannose-sensitive agglutination; amp, ampicillin; cam, chloramphenicol; kan, kanamycin; tet, tetracycline; trim, trimethoprim; zeo, zeocin.

Mutation	Genotype	Phenotype
Strain LA5		
None	Wild-type	Wild-type
Strain EAV10		
Fla–	<i>fliC</i> ::cam ^R	Non-motile
Strain S1400/94		
None	Wild-type	Wild-type
Strain EAV9		
Fla–	<i>fliC</i> ::cam ^R	Non-motile
Strain EAV26		
SEF14–	<i>sefA</i> ::kan ^R	SEF14 fimbrial antigen-negative
SEF17–	<i>agfA</i> ::amp ^R	Non-lacy
SEF21–	<i>fimD</i> ::tet ^R	MSHA-negative
PEF–*	<i>pefC</i> ::zeo ^R	See footnote
LPF–*	<i>lpfC</i> ::trim ^R	See footnote
Strain EAV37		
Fla–	<i>fliC</i> ::cam ^R	Non-motile
SEF14–	<i>sefA</i> ::kan ^R	SEF14 fimbrial antigen-negative
SEF17–	<i>agfA</i> ::amp ^R	Non-lacy
SEF21–	<i>fimD</i> ::tet ^R	MSHA-negative
PEF–*	<i>pefC</i> ::zeo ^R	See footnote
LPF–*	<i>lpfC</i> ::trim ^R	See footnote

* Genotypic evidence only of insertional inactivation of *pef* and *lpf* genes (Allen-Vercocoe & Woodward, 1999).

in MRD. Samples of each dilution were plated onto the surface of well-dried XLD agar (Fisher Scientific) plates and incubated overnight at 37 °C. Viable counts were estimated by the method of Miles & Misra (1938) or by a spread plate method (Collins *et al.*, 1989).

Determination of myeloperoxidase (MPO) activity. Freeze-dried intestine samples were homogenized (1:20, w/v) in ice-cold 0.5 M potassium phosphate (pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB, 5 g l⁻¹) and EDTA (3.72 g l⁻¹) (Stein *et al.*, 1998; Faro *et al.*, 2000). They were left on ice for 60 min and then centrifuged (3000 g, 30 min, 4 °C). MPO activity in the extracts was determined by monitoring H₂O₂-dependent oxidation of 3,3',5,5'-tetramethylbenzidine (TMB; Dynex Technologies) in 50 mM potassium phosphate buffer (pH 6.0) (Zimmerman & Granger, 1990). A₄₅₀ was measured after termination of the reaction with 0.18 M H₂SO₄. Human MPO (Calbiochem) was used as a standard and values were expressed as MPO (µg) equivalents.

Determination of elastase content. Freeze-dried faeces were extracted (1:10, w/v) in ice-cold 0.5 M potassium phosphate (pH 6.0) containing HETAB, EDTA and NaN₃ (1 g l⁻¹), left on ice for 30 min and then centrifuged (3000 g, 30 min, 4 °C). Sample extract or leukocyte-elastase (Sigma-Aldrich) was serially diluted in 0.2 M Tris/HCl (pH 8.0) containing 1 M NaCl and leupeptin (1 mg l⁻¹) on a microtitre plate. Substrate (*N*-succinyl-Ala-Ala-Val *p*-nitroanilide, 0.2 g l⁻¹; Sigma-Aldrich) was added and the A₄₀₅ was monitored immediately and at intervals up to 4 h during incubation at 37 °C (Zimmerman & Granger, 1990). Values were expressed as leukocyte-elastase (µg) equivalents. Faecal trypsin was assayed as a marker of pancreas enzyme output using *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) as substrate and bovine trypsin as standard (Grant *et al.*, 2000).

Monitoring of acid tolerance. A 100 µl aliquot of an overnight culture of *S. Enteritidis* (fim+/fla+, fim-/fla+, fim+/fla- or fim-/fla-) was inoculated into 5 ml Luria-Bertani broth and incubated with agitation at 37 °C until an OD₆₀₀ of 0.2 was attained. Luria-Bertani broth pH 7.0 or acidified to pH 3.0 by addition of HCl was then inoculated with 100 µl of this exponential-phase culture and incubated with agitation at 37 °C for up to 24 h. Growth was measured by recording OD₆₀₀.

Statistical analysis. Data were assessed by one-way analysis of

variance in combination with the Tukey multiple comparison test using the INSTAT statistical package (GraphPad Software Inc.).

RESULTS

Colonization and invasion *in vivo*

S. Enteritidis strain LA5 spread rapidly throughout the whole gastrointestinal tract (Table 2). Furthermore, significant numbers were detectable in the mesenteric lymph nodes from 1 h through to 24 h p.i. Despite this, no salmonella were found in the spleen until 24 h, at which time up to 160 c.f.u. per spleen could be detected. The numbers in the liver were low (≤ 300 c.f.u. per tissue) at 3 h but increased significantly by 24 h (1000–4000 c.f.u. per tissue). *S. Enteritidis* strain S1400 also colonized the gut and systemic tissues (Table 3). Its distribution throughout the body at 24 h p.i. was broadly similar to that found for LA5, although the levels in the spleen were considerably higher (160–2500 c.f.u. per tissue) than for LA5. After 72 h, the numbers of S1400 in the jejunum were slightly lowered (Table 3). However, this reduction was not evident in other regions of the gut or in the systemic tissues.

EAV10 and EAV9, the flagella-deleted (fim+/fla-) mutants, also colonized the gut and systemic tissues (Tables 2 and 3). However, their ability to reach or survive in the liver and spleen appeared to be impaired in the short-term. Thus, at 24 h, EAV9 was found in the liver (50–500 c.f.u.) and spleen (5–160 c.f.u.) in much smaller numbers than its parent strain, S1400 (160–4000 c.f.u. per liver; 160–2500 c.f.u. per spleen). However, by 72 h, the levels of EAV9 (1500–4000 c.f.u. per liver; 1000–2500 c.f.u. per spleen) had increased and were comparable to those for S1400 (1200–5000 c.f.u. per liver; 1000–10 000 c.f.u. per spleen). The numbers of EAV10 in the liver (80–500 c.f.u.) at 24 h were also smaller than for its parent strain, LA5 (1000–4000 c.f.u.

Table 2. Detection of *Salmonella* cells after oral dosage with strain LA5 or its aflagellate mutant, EAV10

Values are log₁₀ c.f.u. g⁻¹. Rats (*n* = 5) were dosed orally with 10⁸ c.f.u. of the strain shown. Values in a row with distinct superscripts differ significantly (*P* ≤ 0.05). MLN, Mesenteric lymph nodes; NS, not sampled. The limit of detection is 1.0 for liver and spleen. For comparison purposes, zero values (no colonies detected) are given as the limit of detection.

Tissue	1 h		3 h		24 h	
	LA5	EAV10	LA5	EAV10	LA5	EAV10
Stomach	5.3 ± 0.8 ^{ab}	4.6 ± 0.8 ^a	4.5 ± 0.5 ^a	3.4 ± 0.6 ^a	6.4 ± 1.1 ^b	4.4 ± 0.8 ^a
Jejunum	4.2 ± 0.7	3.6 ± 0.7	3.2 ± 0.7	4.1 ± 1.4	4.4 ± 0.7	3.2 ± 0.9
Ileum	8.1 ± 0.6 ^a	7.9 ± 0.6 ^a	6.2 ± 0.6 ^b	6.6 ± 0.8 ^b	6.2 ± 0.6 ^b	6.1 ± 0.5 ^b
Caecum	NS	NS	8.3 ± 0.3	8.5 ± 0.2	7.4 ± 0.5	7.9 ± 0.8
Colon	NS	NS	7.5 ± 1.1	7.3 ± 0.7	7.5 ± 0.9	7.4 ± 0.5
MLN	4.4 ± 1.6	3.5 ± 0.8	5.1 ± 1.1	4.3 ± 0.4	5.0 ± 0.3	4.9 ± 1.0
Liver	NS	NS	1.3 ± 0.6 ^a	1.4 ± 0.3 ^a	2.7 ± 0.3 ^b	1.7 ± 0.4 ^a
Spleen	NS	NS	1.0 ± 0.0 ^a	1.0 ± 0.0 ^a	1.9 ± 0.9 ^b	2.3 ± 0.4 ^b

Table 3. Detection of *Salmonella* cells after oral dosage with strain S1400 and its mutants

Tissues were sampled 24 or 72 h after oral dosage as shown. See legend to Table 2 for further details. Strain EAV26 is unable to express SEF14, SEF17, SEF21, *pef* and *lpf*.

Tissue	S1400 (fim+/fla+)	EAV9 (fim+/fla-)	EAV26 (fim-/fla+)	EAV37 (fim-/fla-)
24 h p.i.				
Stomach	4.1 ± 0.6 ^a	3.9 ± 0.7 ^a	4.1 ± 0.7 ^a	2.9 ± 0.2 ^b
Jejunum	5.4 ± 0.8 ^a	4.0 ± 1.1 ^{ab}	4.9 ± 1.4 ^a	2.9 ± 0.5 ^b
Ileum	6.2 ± 0.3	5.7 ± 0.5	6.3 ± 0.3	5.7 ± 0.5
Caecum	6.8 ± 1.0 ^a	8.8 ± 0.6 ^b	7.8 ± 0.5 ^{ab}	6.6 ± 0.4 ^a
Colon	6.3 ± 0.5 ^a	8.0 ± 0.6 ^b	7.7 ± 0.8 ^b	6.2 ± 0.4 ^a
MLN	5.3 ± 0.7	4.6 ± 0.3	5.5 ± 0.5	4.3 ± 0.6
Liver	2.3 ± 0.7 ^a	1.0 ± 0.1 ^b	2.8 ± 0.8 ^a	1.0 ± 0.3 ^b
Spleen	3.4 ± 0.6 ^a	2.1 ± 0.7 ^b	3.2 ± 0.5 ^a	2.3 ± 0.3 ^b
72 h p.i.				
Stomach	5.2 ± 0.6 ^a	3.8 ± 0.6 ^b	4.7 ± 0.2 ^a	3.6 ± 0.7 ^b
Jejunum	3.4 ± 0.2 ^a	2.3 ± 0.5 ^b	3.5 ± 0.4 ^a	2.1 ± 0.1 ^b
Ileum	5.7 ± 0.5	5.4 ± 0.3	5.6 ± 0.3	5.8 ± 0.2
Caecum	5.8 ± 1.8	6.0 ± 0.5	5.2 ± 0.8	5.2 ± 0.8
Colon	5.8 ± 0.2	5.8 ± 0.6	5.2 ± 1.0	6.1 ± 0.6
MLN	5.3 ± 0.7	5.4 ± 0.2	5.3 ± 0.1	5.3 ± 0.2
Liver	2.8 ± 0.3	2.8 ± 0.2	2.7 ± 0.2	2.4 ± 0.3
Spleen	4.1 ± 0.5	3.8 ± 0.2	3.7 ± 0.2	3.6 ± 0.2

Table 4. Small intestine tissue composition and faecal excretion by rats infected with strain S1400 and its mutants

See legend to Table 2 for further details.

Measure	Control	S1400 (fim+/fla+)	EAV9 (fim+/fla-)	EAV26 (fim-/fla+)	EAV37 (fim-/fla-)
Small intestine (24 h)					
Dry weight (mg)	612 ± 55	708 ± 46	692 ± 77	662 ± 77	677 ± 62
Water (mg)	2289 ± 155	2292 ± 120	2224 ± 81	2504 ± 210	2239 ± 190
MPO (µg)	27.7 ± 6.6	36.8 ± 7.4	27.1 ± 7.5	35.3 ± 3.6	25.1 ± 8.0
Small intestine (72 h)					
Dry weight (mg)	625 ± 55	784 ± 104	830 ± 92	800 ± 102	692 ± 92
Water (mg)	2331 ± 160 ^a	2981 ± 325 ^b	2575 ± 292 ^{ab}	3130 ± 324 ^b	2725 ± 250 ^{ab}
MPO (µg)	27.7 ± 6.6 ^a	65.2 ± 9.0 ^b	47.8 ± 10.9 ^{ab}	60.1 ± 10.2 ^b	35.7 ± 8.9 ^a
Faeces (24 h)					
Salmonella (log ₁₀ c.f.u. g ⁻¹)	0	7.1 ± 1.3	7.7 ± 1.1	7.2 ± 0.9	7.1 ± 1.4
Faeces (72 h)					
Salmonella (log ₁₀ c.f.u. g ⁻¹)	0	5.8 ± 1.3	6.7 ± 1.9	5.7 ± 1.8	6.1 ± 1.6
PMN-elastase (µg g ⁻¹)	0.1 ± 0.1 ^a	1.0 ± 0.4 ^b	ND	ND	0.3 ± 0.1 ^c
Trypsin (µg g ⁻¹)	16 ± 4	20 ± 5	ND	ND	17 ± 7

ND, Not determined; PMN, polymorphonuclear leukocyte.

per liver), but there appeared to be no differences between EAV10 (5–160 c.f.u.) and LA5 (up to 160 c.f.u.) in numbers present in the spleen (Table 2).

There was also a tendency for the flagella-deleted mutant

strains to be present in the stomach and/or jejunum in smaller numbers than their parent strains (Tables 2 and 3). In particular, at 72 h, the numbers of EAV9 (fim+/fla-) associated with both tissues were greatly reduced (Table 3). Nonetheless, the lack of functional flagella did not seem to

affect the ability of the bacterium to colonize and persist in other regions of the gut or in the mesenteric lymph nodes (Tables 2 and 3).

Failure to express five fimbriae (SEF14, SEF17, SEF21, pef and lpf) did not seem to impair colonization or systemic spread by *S. Enteritidis* (Table 3). The levels of EAV26 (fim⁻/fla⁺) found throughout the gut, in the mesenteric lymph nodes and in the liver and spleen were thus similar to those for its parent strain, S1400 (fim⁺/fla⁺). Furthermore, at 72 h, the tissue distribution observed with the multiple fimbriae- and flagella-deleted mutant EAV37 (fim⁻/fla⁻) was close to that seen with EAV9 (fim⁺/fla⁻), the related strain in which flagella expression alone was disabled. There were, however, some differences between these mutant strains at 24 h. The fim⁺/fla⁻ strain was detected in slightly larger numbers than the fim⁻/fla⁻ strain in the stomach and jejunum, but not in other regions of the gut or the systemic tissues.

Inability to express SEF14, SEF17, SEF21, pef and lpf and/or flagella did not affect faecal shedding of *S. Enteritidis* significantly (Table 4). Thus, rats infected with S1400 (fim⁺/fla⁺), EAV9 (fim⁺/fla⁻), EAV26 (fim⁻/fla⁺) or EAV37 (fim⁻/fla⁻) excreted similar numbers of salmonella in faeces. This was consistent with the finding that the deletion-mutant and wild-type strains colonized and persisted equally well throughout the large intestine (Table 3).

Intestinal and faecal markers

Intestinal MPO levels were considerably elevated 3 days after infection of rats with *S. Enteritidis* S1400 (fim⁺/fla⁺) or EAV26 (fim⁻/fla⁺) (Table 4). The changes induced by the flagella-deleted mutant EAV9 (fim⁺/fla⁻) were much less marked, whilst EAV37 (fim⁻/fla⁻) caused only a limited increase in intestinal MPO. Intestinal tissue water content was also elevated as a result of infection with S1400 or EAV26 (Table 4). The changes were, however, slightly less pronounced after infection with EAV9 or EAV37. Furthermore, faecal elastase was greatly elevated following infection with S1400 but only slightly increased by EAV37.

Acid tolerance *in vitro*

Growth of *S. Enteritidis* S1400, EAV26, EAV9 and EAV37 in Luria–Bertani broth at pH 7.0 or 3.0 was monitored for up to 24 h (results not shown). The wild-type and mutant strains grew equally well at pH 7.0. Furthermore, there were no differences in their ability to tolerate acid conditions. All four strains remained viable for up to 4 h at pH 3.0 but did not multiply. By 24 h, the strains were no longer viable.

DISCUSSION

S. Enteritidis strains LA5 and S1400 colonized and invaded in a manner generally similar to that seen with other *S. Enteritidis* or *S. Typhimurium* strains (Naughton *et al.*, 1995, 1996a, 2000; Havelaar *et al.*, 2001; Islam *et al.*, 2000). Thus, there was rapid distribution throughout the gastrointestinal tract, translocation to the mesenteric lymph nodes

and spread to the liver and spleen of the rats. Significant numbers of viable salmonella were detected in the mesenteric lymph nodes even within 1 h of oral infection and in the liver and spleen by 24 h. This systemic appearance of pathogen, particularly in liver and spleen, occurred much sooner than reported previously for conventional rats or mice (Islam *et al.*, 2000; Baumler *et al.*, 1996, 1997; Carter & Collins, 1974; Garcia-Del Portillo *et al.*, 1999). This may be linked to the rat strain used (Hooded-Lister), which has been kept as a closed colony for over 50 years, or perhaps to reduced levels of commensal bacteria in the small intestine (Grant, 1996). The commensal bacteria give a high degree of protection against infection (Ducluzeau, 1984) and invasion of salmonella occurs more rapidly in antibiotic-treated or ex-germ-free animals (Miller & Bohnhoff, 1963; Que & Hentges, 1985). A reduction in background flora, due to controlled management of the animals prior the start of the study, in combination with withdrawal of food overnight prior to dosing with the pathogen may have facilitated rapid and extensive attachment of the pathogen to the epithelium and promoted early translocation.

MPO levels in the rat small intestine were unchanged 24 h after infection with *S. Enteritidis* S1400 but were significantly elevated at 72 h. Intestine tissue water content and faecal elastase was also increased at 72 h. This suggests that active recruitment of neutrophils to the intestine (Darwin & Miller, 1999; Stein *et al.*, 1998; Faro *et al.*, 2000; Zimmerman & Granger, 1990; McCormick *et al.*, 1995; Naughton *et al.*, 1996a) may have occurred between 24 and 72 h p.i. These changes would be consistent with the infiltration of polymorphonuclear leukocytes and other inflammatory cells into gut tissue and the release of inflammatory cells into the lumen, previously observed *in vivo* (Naughton *et al.*, 1995, 1996a, b).

Salmonella cells have to attach to or form a close association with the intestinal epithelium in order to establish and persist in the gut and subsequently invade the underlying tissues (Tsolis *et al.*, 1999; Kingsley & Baumler, 2000; Ohl & Miller, 2001; Darwin & Miller, 1999; Humphries *et al.*, 2001). These initial interactions with gut cells were previously shown to be facilitated *in vitro* and *in vivo* by fimbrial adhesins expressed on the surface of the pathogen (Ohl & Miller, 2001; Darwin & Miller, 1999; Humphries *et al.*, 2001; Baumler *et al.*, 1997). Deletion of a particular fimbria or inhibition of its action can thus limit the ability of salmonella to colonize and invade *in vitro* and *in vivo* (Ohl & Miller, 2001; Darwin & Miller, 1999; Humphries *et al.*, 2001; Baumler *et al.*, 1997; Dibb-Fuller *et al.*, 1999; Naughton *et al.*, 2001).

In the present study, deletion of five fimbriae (SEF14, SEF17, SEF21, pef, lpf) did not, however, appear to disadvantage *S. Enteritidis in vivo*. The fim⁻/fla⁺ mutant (EAV26) was able to colonize the rat gut and invade to the systemic tissues as effectively as its fimbriate parent strain (S1400, fim⁺/fla⁺). This was consistent with earlier findings, that these strains bound equally well to rat and chick gut explants (Allen-Vercos & Woodward, 1999; Robertson *et al.*, 2000) and

caused similar levels of infection in 1-day-old chicks (Allen-Vercoe *et al.*, 1999). The results suggest that, whilst SEF14, SEF17, SEF21, pef, lpf can have significant roles in the early stages of infection (Ohl & Miller, 2001; Darwin & Miller, 1999; Humphries *et al.*, 2001; Baumler *et al.*, 1996, 1997), their presence is not a prerequisite in all cases.

Salmonella cells can bind directly to exposed gut epithelial cells, including the M cells of the Peyer's patch regions, and this is likely to involve the fimbrial adhesins (Kingsley & Baumler, 2000; Darwin & Miller, 1999; Humphries *et al.*, 2001). Alternatively, they may initially form a loose association with the mucus layer overlying the gut (Ensgraber *et al.*, 1992; Worton *et al.*, 1989) and subsequently move down close to the epithelial cells. The mucus layer in the rat ileum is deep, comprising around 30 µm of firmly adherent mucus in close proximity to the villi and approximately 450 µm of loosely adherent mucus (Atuma *et al.*, 2001). Furthermore, a high proportion of *S. Enteritidis* cells in the ileum are found in this mucus layer (Robertson, 2000). The mucus is expected to act as a barrier against the pathogen. However, if the pathogen can penetrate deep into the mucus layer, it may also be protected from movement of luminal digesta, peristalsis or exposure to secreted antimicrobial substances. Our results imply that *S. Enteritidis* may be able to interact with epithelial cells in the rat gut without the need for SEF14, SEF17, SEF21, pef or lpf to anchor them in place. The composition or distribution of the mucus layer in the mouse, the species most widely used to study the roles of fimbriae in infection, may differ significantly from that in the rat (Atuma *et al.*, 2001; Rozee *et al.*, 1982). Invasion by *S. Enteritidis* in rats appears to be primarily through enterocytes underlying the mucus layer (Robertson, 2000) whilst, in mice, the main route is via the M cells of the Peyer's patch (Kingsley & Baumler, 2000; Humphries *et al.*, 2001). Fimbriae do have an involvement in salmonellosis in mice (Darwin & Miller, 1999; Humphries *et al.*, 2001; Baumler *et al.*, 1996, 1997), but SEF14, SEF17, SEF21, pef and lpf do not appear to be prerequisites for infection of rats. This may be a result of differences between the species in the routes of pathogen uptake or the involvement of mucus in gut-pathogen interactions. Alternatively, since 12 putative fimbrial operations have been identified in *S. Enteritidis* (Townsend *et al.*, 2001), it is possible that fimbriae other than SEF14, SEF17, SEF21, pef and lpf have roles in the interaction of salmonella with the rat gut.

Fimbriate/aflagellate (fim+/fla-) *S. Enteritidis* was detected in the stomach and proximal small intestine in significantly larger numbers than its fim-/fla- counterpart at 24 h p.i. The numbers declined thereafter and, by 72 h, were comparable to those for the fim-/fla- strain. This might indicate that the fimbriae, although not prerequisites for infection in the rat, compensated transiently for loss of flagella and enabled the aflagellate strain to survive in the stomach and proximal intestine in the short-term.

Inability to express flagella modified the nature of the infection caused by *S. Enteritidis* in rats. In the initial 24 h,

aflagellate (fim+/fla- or fim-/fla-) mutants colonized the gastrointestinal tract and spread to the mesenteric lymph nodes as effectively as their flagellate (fim+/fla+ or fim-/fla+) counterparts. However, after 72 h, the numbers of the aflagellate strains detectable in the stomach and jejunum were greatly reduced. Inflammatory responses triggered in the small intestine by *S. Enteritidis* were less marked if the strains were aflagellate. Furthermore, aflagellate (fim+/fla- or fim-/fla-) *S. Enteritidis* was not found in significant numbers in the liver or spleen at 24 h. However, the levels increased thereafter and, by 72 h, were as high as those for flagellate strains.

There appeared to be little difference in the ability of aflagellate (fim+/fla- or fim-/fla-) and flagellate (fim+/fla+ or fim-/fla+) *S. Enteritidis* strains to colonize the rat ileum *in vivo*. This was consistent with the findings of other studies *in vivo* (Ikeda *et al.*, 2001; Schmitt *et al.*, 2001). However, it contrasted with earlier results *in vitro* (Allen-Vercoe & Woodward, 1999; Robertson *et al.*, 2000), where the aflagellate strains were found to be less able to attach to rat ileum or chick gut explants (Allen-Vercoe & Woodward, 1999; Robertson *et al.*, 2000). The numbers of *S. Enteritidis* associating with the explants were, however, greatly influenced by the mucus layer (Robertson *et al.*, 2000), which is particularly deep at the ileum (Atuma *et al.*, 2001). On a gut explant, the mucus layer is static and of high viscosity. In contrast, due to movement along the gut facilitated by peristalsis and continuous renewal by mucins produced by goblet cells, it is relatively fluid/dynamic and of moderate viscosity *in vivo* (Forstner & Forstner, 1994). The mucus layer on explants may therefore be impermeable to non-motile bacterial strains, whilst the more fluid/dynamic mucus layer *in vivo* remains accessible to both motile and non-motile strains. Histological studies did indeed indicate that similar numbers of aflagellate or flagellate *S. Enteritidis* cells were present within the mucus layer *in vivo* (Robertson, 2000). In this case, the results from the explant models clearly did not reflect the interactions *in vivo*. Nonetheless, the findings highlight the fact that relatively small changes in the characteristics of mucus may greatly affect the ability the salmonella to reach and interact with underlying epithelial cells. This is of particular relevance, since the composition and thickness of the mucus layer varies throughout the gastrointestinal tract and is significantly influenced by general health status (Atuma *et al.*, 2001; Forstner & Forstner, 1994).

In contrast to the situation in the ileum, aflagellate (fim+/fla- or fim-/fla-) *S. Enteritidis* strains were generally less able than flagellate (fim+/fla+ or fim-/fla+) strains to persist long-term in the stomach and proximal small intestine. Since the parent and mutant strains were equally susceptible to the action of strong acid *in vitro*, persistence in the stomach was unlikely to be due to differences in acid tolerance. Stomach contents are generally very acidic. However, the pH in the mucus layer immediately adjacent to the stomach epithelium is maintained close to neutral to protect the epithelial cells (Atuma *et al.*, 2001; Edwards & Puente, 1998). *S. Enteritidis* may survive in the stomach by moving into this neutralized

microenvironment. Peristalsis and movement of digesta in the proximal small intestine is more extensive and rapid than in the distal regions (Weisbrodt, 1987). In addition, the mucus layer with which salmonella could associate is much thinner in the duodenum and jejunum than in other regions of the gut (Atuma *et al.*, 2001). Lack of motility may limit the ability of aflagellate strains to reach and interact with the epithelium and persist in these harsh environments.

Aflagellate (fim+/fla– or fim–/fla–) *S. Enteritidis* mutants appeared to be less inflammatory for the gut than flagellate (fim+/fla+ or fim–/fla+) wild-type strains. Recruitment of neutrophils into the gut is mediated by chemoattractant chemokines, such as IL-8 (Darwin & Miller, 1999; McCormick *et al.*, 1995; Gewirtz *et al.*, 2001). Their release from epithelial cells is triggered by various pathogen factors (Ohl & Miller, 2001; Galan, 2001), but flagellin, the flagellar subunit protein FliC, is a particularly potent stimulus (Gewirtz *et al.*, 2001; Eaves-Pyles *et al.*, 2001). Flagella-deleted or FliC-blocked mutants of *S. Typhimurium* thus have reduced effects on IL-8 secretion *in vitro* (Gewirtz *et al.*, 2001) and are less deleterious to mice *in vivo* (Ikeda *et al.*, 2001; Schmitt *et al.*, 2001). Despite this, there appeared to be little difference in the abilities of aflagellate, FliC-blocked or flagellate strains to promote neutrophil recruitment and fluid secretion over 12–16 h in a ligated loop model *in vivo* (Ikeda *et al.*, 2001; Schmitt *et al.*, 2001). The results of the present study suggest that flagella-deleted mutants of *S. Enteritidis* were less able to trigger neutrophil recruitment to the gut *in vivo* than their flagellate counterparts. However, the differences were not apparent until 3 days p.i.

Aflagellate (fim+/fla– or fim–/fla–) and flagellate (fim+/fla+ or fim–/fla+) *S. Enteritidis* strains seemed to be equally invasive *in vivo*, since they were able to breach the epithelial barrier and reach the mesenteric lymph nodes at the same time after infection and in very similar numbers. This contrasts with various findings *in vitro*, where aflagellate *S. Enteritidis* or *S. Typhimurium* strains were significantly less invasive to cells in culture (Dibb-Fuller *et al.*, 1999; Schmitt *et al.*, 2001).

Flagella-deleted mutants (fim+/fla– or fim–/fla–) of *S. Enteritidis* were detected in the liver and spleen in much smaller numbers than their flagellate (fim+/fla+ or fim–/fla+) counterparts after 24 h. There were, however, no differences in the numbers at 72 h p.i. The level of pathogen found in the liver and spleen depends on both the rate of transfer to the tissues from the mesenteric lymph nodes and the rates of clearance of pathogen from the liver and spleen. In a mouse model, spread of aflagellate *S. Typhimurium* to the spleen appeared to be slightly delayed (Schmitt *et al.*, 2001). In addition, FliC-blocked *S. Typhimurium* strains appeared less able to persist or proliferate in the mouse spleen (Ikeda *et al.*, 2001). In the present study, failure to proliferate in or rapid clearance from the liver and spleen could explain the smaller numbers of aflagellate *S. Enteritidis* found in

these tissues at 24 h. However, this appears unlikely, since the differences were transient. They may, therefore, have been due to delayed breakout from the mesenteric lymph nodes.

It is not clear why aflagellate *S. Enteritidis* would be less able to breach the mesenteric lymph node barrier. Aflagellate (fim+/fla– or fim–/fla–) and flagellate (fim+/fla+ or fim–/fla+) strains were found in the tissue in similar numbers at all time-points studied. However, flagellin triggers release of a range of chemokines and cytokines that modulate cellular metabolism and increase the permeability of the gut to bacteria (Gewirtz *et al.*, 2001; Eaves-Pyles *et al.*, 2001). These factors may also affect mesenteric lymph node function and facilitate breakout of salmonella. In the absence of flagellin, the barrier potential of the mesenteric lymph node may, at least in the short-term, be preserved, thereby limiting the spread of aflagellate pathogen to the liver and spleen.

Recent studies have shown that some systemic spread of salmonella can occur without drainage through the lymphatic system (Vazquez-Torres *et al.*, 1999; Rescigno & Borrow, 2001). Pathogen sampled subepithelially or even lumenally by dendritic cells or CD18-expressing phagocytes can be transferred directly to the liver and spleen (Vazquez-Torres *et al.*, 1999; Rescigno & Borrow, 2001; Sierro *et al.*, 2001). As with neutrophils (McCormick *et al.*, 1995), recruitment of these phagocytes to the gut is likely to be mediated by chemoattractant chemokines (Rescigno & Borrow, 2001; Sierro *et al.*, 2001). Because of their inability to produce flagellin, aflagellate strains (fim+/fla– or fim–/fla–) of salmonella may not trigger release of the appropriate chemoattractants to facilitate recruitment of dendritic cells or CD18-expressing phagocytes to the gut. This could limit the uptake of the aflagellate pathogen by this route.

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