BACTERIAL PATHOGENICITY

The role of flagella, but not fimbriae, in the adherence of *Salmonella enterica* serotype Enteritidis to chick gut explant

EMMA ALLEN-VERCOE and MARTIN J. WOODWARD*

Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG and *Bacteriology Department, Central Veterinary Laboratory, Addlestone, Surrey KT15 3NB

To gain an understanding of the role of fimbriae and flagella in the adherence and colonisation of *Salmonella enterica* serotype Enteritidis in chickens, an in-vitro gut adherence assay was developed and used to assess the adherence of a wild-type Enteritidis strain and isogenic non-fimbriate and non-flagellate mutant strains. Enteritidis strain S1400/94, a clinical isolate virulent in chickens, was shown to possess genes which encoded type 1, SEF14, SEF17, plasmid-encoded and long polar fimbriae. Mutant strains unable to elaborate these fimbriae were created by allelic exchange. Each fimbrial operon was inactivated by the insertion of an antibiotic resistance gene cassette. In addition, *fliC*, *motAB* and *cheA* loci, which encode the major subunit of the flagellum, the energy-translation system for motility and one of the chemotaxis signalling proteins, respectively, were similarly inactivated. Non-flagellate mutant strains were significantly less adherent than the wild-type strain, whereas mutant strains defective for the elaboration of any of the types of fimbriae adhered as well as the wild-type strain. A flagellate but non-motile (paralysed) mutant strain and a smooth-swimming chemotaxis-deficient mutant strain were shown to be less adherent than the wild-type strain, but that observation depended on the assay conditions used.

Introduction

The role of fimbriae and flagella in the pathogenesis of *Salmonella* has become a focus of recent research interest. For *S. enterica* serotype Typhimurium, type 1 fimbriae, which mediate mannose-sensitive haemagglutination and have been assumed to play a role in initial gut colonisation [1], and flagella have been demonstrated to play a key role in pathogenesis in a mouse model [2–4] and in chickens [5, 6]. Long polar fimbriae (LPF) [7] mediate adherence to murine M cells in Peyer's patches [8, 9] and plasmid-encoded fimbriae (PEF) [10] mediate adherence to murine small intestine with reduced intraluminal fluid accumulation [11].

For serotype Enteritidis a role has been demonstrated for type 1 fimbriae – also referred to as SEF21, a designation used in this report – and for type 3 fimbriae in pathogenesis in a mouse model [12], but relatively little is yet understood about the role of

fimbriae in chickens. Enteritidis has been shown to elaborate an analogue of PEF that is expressed during infection in the chicken [13] and has the genetic potential to elaborate LPF [7]. Enteritidis also elaborates an analogue of the 'curli' fimbriae of *Escherichia coli* [14] – designated SEF17 [15, 16] – that are essential for the generation of convoluted colonies during growth of Enteritidis at ambient temperatures on Colonisation Factor Antigen (CFA) agar [17]. It is of interest that colonies of Enteritidis derived by direct plating of infected chicken livers and spleens on to selective media, developed convoluted morphology [18]. A subset of serotypes of *Salmonella* of O-serogroup D, and including Enteritidis, elaborate a novel kind of fimbriae designated SEF14 [19–21]. However, the role of SEF14 in pathogenesis remains equivocal [22], although a two-thirds reduction in the adherence of Enteritidis to ovarian granulosa cells *in vitro* was demonstrated by pre-incubation of bacteria with purified SEF14 fimbriae [23]. Anti-SEF14 antibodies have also been shown to afford a degree of passive protection in a mouse model [24].

Together, these observations suggest that fimbriae may play a role in the pathogenesis of Enteritidis. However,

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Corresponding author: Professor M. J. Woodward.

this hypothesis remains largely untested, particularly in chickens. Accordingly, the roles of fimbriae and flagella were assessed in a reverse genetics approach in which a series of non-fimbriate and non-flagellate mutant strains was derived for studies *in vitro* and *in vivo*. This paper describes the construction of these mutant strains and their in-vitro adherence properties in an explant model of day-old chick gut.

Materials and methods

Bacteria, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cultures were maintained as frozen (-70°C) stocks in Luria-Bertani (LB) broth [30] supplemented with glycerol 15%. Working cultures were maintained at 4°C on plates of LB agar appropriately supplemented with antibiotics. Minimal growth medium was M9 salts supplemented with glucose 0.2% w/v and 10 mM magnesium [30]. Bacteria for SEF21 ELISA were grown statically in Heart Infusion Broth (Oxoid) at 37°C for 48 h. Bacteria for SEF17 ELISA were grown on Colonisation Factor Antigen (CFA) Agar (Oxoid) at 25°C for 48 h. Bacteria for SEF14 ELISA were grown in IsoSensitest Broth (Oxoid) at 37°C for 24 h. For gut-adherence assays, bacteria were grown overnight at 37°C in LB broth without agitation, pelleted by centrifugation at 3000 g for 10 min and resuspended gently in PBS to an OD_{550} of 0.6.

Slide agglutination

Individual colonies grown on LB agar were emulsified in 50 μl of PBS to which an equal volume of agglutination serum (Central Veterinary Laboratory, Addlestone, Surrey) was added. Slides were incubated at room temperature with rocking for no more than 5 min. For haemagglutination tests, the above procedure was used but with guinea-pig red blood cells (Sigma) 3% added instead of antiserum.

Genetic methods

Methods for the extraction and purification of plasmid and chromosomal DNA, restriction endonuclease digestion, agarose gel electrophoresis, ligation, preparation of electrocompetent bacterial cells, electroporation, lysis of bacterial colonies for colony dot-blot on, and Southern transfer of DNA species to Hybond-N nylon membranes (Amersham) and autoradiography were as described previously [30].

PCR

The methods have been described elsewhere [13]; primers for antibiotic resistance, fimbrial and flagellar genes, and their sources, are listed in Table 2.

Cloning and insertional inactivation of fimbrial and flagellar genes

DNA fragments, which encoded relevant regions of *fim*, *agf*, *pef*, *lpf* operons, the *fliC* and *cheA* genes and the *motAB* regulon, were amplified by PCR. These fragments were subcloned separately into pCRSCRIPT (Stratagene) and inserts were confirmed by preliminary nucleotide sequence analysis and detailed restriction endonuclease mapping (data not shown). Antibiotic resistance gene cassettes were cloned separately into the *fimD*, *agfA*, *pefA*, *lpfC*, *fliC*, *cheA* and *motAB* genes, either by direct subcloning, if appropriate restriction endonuclease sites were available within the open reading frames, or by splice overlap extension PCR. The insertionally inactivated fimbrial operon regions were subcloned on to appropriate pPERFORM vectors and electroporated into the permissive host *Escherichia coli* strain K12 S-17 λpir . Cloning and mutagenesis strategies are summarised in Table 3.

Conjugation

The pPERFORM vectors harbouring antibiotic resistance-marked, insertionally inactivated genes (see Table 3, column 8, pSCV derivatives) were transferred by conjugation from the permissive host strain *E. coli* K12

Table 1. Bacterial strains and plasmids

Bacterial strain/plasmid	Reference no.	Comments
<i>E. coli</i> JM109 λpir	25	Cloning strain. Genotype: <i>e14⁻</i> (<i>mcrA⁻</i>) <i>recA1 endA1 gyrA96 thi1 hsd R17(r_K - m_K⁺) supE44 relA1 Δ(lac-proAB) [F'<i>traD36 proAB lacI^qz ΔM15]</i> λpir</i>
<i>E. coli</i> S-17 λpir	26	Mobilising strain. Genotype: <i>pro res⁻ mod⁺</i> RP42-Tc::Mu ⁻ Km::Tn7 λpir
Enteritidis S1400/94	13	Wild-type strain
Enteritidis LA5	27	Wild-type strain LA5
Enteritidis LA5SEF14 ⁻	22	<i>sefA::kan^R</i>
pACYC184	28	Source of tet ^R cassette
pCRSCRIPT	Commercially available (Stratagene)	Direct PCR cloning vector
pR751	29	Source of tri ^R cassette
pZERO-1	Commercially available (Invitrogen)	Source of zeo ^R cassette
pJP5603	25	kan ^R suicide vector
pPERFORM K	This study	pJP5603 with modified MCS
pPERFORM C	This study	pJP5603 with modified MCS and kan ^R marker replaced with cam ^R marker

Table 2. Primer sequences

Primer name	Sequence 5'–3'	Position on target gene/operon	Reference no.
AGF1	ATGATGTTGACAATACTGGGT	278–301 of <i>agfB</i>	16
AGF2	TGATAAATGCAGTGATTGTCC	22–43 of <i>agfC</i>	
CHEA1	AGGTGATAGCGTGAGCATGGA	1–21 of <i>cheA</i>	31
CHEA2	TGTGATCGCCATACGTTGTTC	1998–2018 of <i>cheA</i>	
FIMD1	GGCTATGTATCACCGTTAAGC	36–57 of <i>fimD</i>	32
FIMD2	TATGGTAATCGCCTGCTTCAG	2559–2580 of <i>fimD</i>	
FLIC1	ACTGAGTCCGATAACGCTAACGA	183–209 of <i>fliC</i>	33
FLIC2	TTATTACGTTGCATAGTCAGCATCTTC	1392–1413 of <i>fliC</i>	
LPFC1	ATGACATGGACGCATCTTCCT	1–21 of <i>lpfC</i>	7
LPFC2	CCAACAGGCCCGTAAACACATTA	16–36 of <i>lpfD</i>	
MOT1	GAGCTCAAGGATGATGTCGTGCTTATC	13–34 of <i>motA</i>	34
MOT2	AGATCTTGCTGGTTTGGGTACCTCACT	845–865 of <i>motA</i>	
MOT3	AGATCTTGAAAATTGCCTACGCCGAT	78–99 of <i>motB</i>	
MOT4	TCTAGATATCACCTCGGTTCCGCTTTT	911–931 of <i>motB</i>	
PEFA1	GAATGCTGACGGCTTTGGTGC	287–312 of <i>pefA</i>	13
PEFC2	TGGTCTGCAGCTCCAGGTCATC	2085–2113 of <i>pefC</i>	10
TET1	CAGTTACCTCGGTTCAAAGAG	1278–1299 of pACYC184	35
TET2	CAATTCTTGGAGTGGTGAATC	2797–2818 of pACYC184	
TRIM1	GAATTCTTAGGCCACGCGTTC	1–21 of pR751	29
TRIM2	AGGCACGAACCCAGTTGACAT	577–556 of pR751	

S-17 λ pir into the non-permissive Enteritidis strain S1400 by filter mating. Equal volumes (100 μ l) of overnight broth cultures of donor and recipient strains were inoculated together, or separately, as controls, on sterile 2- μ m filters overlaid on dried plates of nutrient agar and incubated at 37°C. After growth for 4 h cultures were removed from filters by immersion and vigorous agitation in PBS. Dilutions were plated on to selective media which were glucose minimal medium supplemented with appropriate antibiotics.

Selection of mutant strains

Individual colonies from conjugation selective plates were streaked on to fresh glucose minimal medium supplemented with appropriate antibiotic, (i.e., the antibiotic to which resistance was encoded by the insert of inactivated gene) and grown overnight at 37°C. Individual colonies were tested for loss of the antibiotic resistance encoded by the suicide vector. Putative mutant colonies were picked from the master plate and re-streaked for single colonies on LB agar for confirmatory tests.

P22 transduction

Preparation of multiply mutant strains in the Enteritidis strain S1400 background was achieved by P22 transduction [36] with selection for antibiotic resistance encoded within the mutant alleles.

Colony dot-blot and Southern hybridisation

Probes were prepared either by PCR or direct excision of the relevant gene fragment or were entire pERFORM vectors without inserts. Radioisotopic labelling with 32 P (Amersham RapidHyb), hybridisation conditions and stringency of post-hybridisation washes were as described previously [13].

Fimbria-specific ELISAs

ELISAs specific for SEF21 [37], SEF17 [38] and SEF14 [39] were used to assay elaboration of fimbriae.

Adherence assay in chicken gut explants

The method for preparation of tissues followed those described previously [5, 40]. In brief, 1-day-old SPF chicks were killed by cervical dislocation and the duodenal loop, *c.* 6 cm in length, was removed aseptically. Fat and mesentery were removed carefully and the gut section was cut lengthwise and washed thoroughly, but gently, in Krebs's Ringers solution. Gut segments (*c.* 2.0 $\times$ 0.5 cm) were immersed in fresh sterile Krebs's Ringers (20 ml) in a 50-ml Falcon tube (BDH) to which the bacterial inoculum of *c.* 4 $\times$ 10⁸ cfu (2 ml) was added. Incubation was continued at 42°C with orbital rotation at 225 rpm. Gut samples were rinsed three times in fresh sterile Krebs's Ringers solution to remove non-adherent bacteria, and serial dilutions of homogenates were plated in triplicate. All experiments were repeated at least three times.

Scanning electron microscopy (SEM)

Gut specimens were fixed for 16 h in glutaraldehyde 3% v/v in 0.1 M phosphate buffer, pH 7.4, washed in phosphate buffer and post-fixed in osmium tetroxide 1% w/v in the same buffer. Specimens were rinsed in six changes of phosphate buffer, dehydrated in ethanol and placed in acetone. Specimens were subjected to critical point drying with liquid CO₂. Dried specimens were fixed to aluminium stubs with silver conductive paint, sputter-coated with gold and examined in a stereoscan S250 MarkIII SEM at 10–20 kV.

Table 3. Cloning and mutagenesis strategy

Appendage or function	Target gene	Associated protein	Primers used*	Size of amplicon (bp)	Cloned amplicon construct†	Marker gene cassette and location of insertional inactivation	Suicide vector construct‡
SEF21	<i>fimD</i>	Fimbrial usher	FIMD1, FIMD2	2556	pTG1	tet ^R → <i>NheI</i> site at 1839 bp	pSVC1
SEF17	<i>agfA</i>	Fimbrial antigen	AGF1, AGF2	1294	pTG2	amp ^R → <i>EheI</i> site at 234 bp	pSVC2
PEF	<i>pefC</i>	Fimbrial usher	PEFA1, PEF2	2580	pTG3	zeo ^R → <i>HpaI</i> site at 186 bp	pSVC3
LPF	<i>lpfC</i>	Fimbrial usher	LPF1, LPF2	2594	pTG4	tr ^R → <i>KpnI</i> site at 1025 bp	pSVC4
Flagella	<i>fljC</i>	Major flagellar antigen	FLIC1, FLIC2	1230	pTG5	cam ^R → <i>HpaI</i> site at 830 bp	pSVC5
Chemotaxis	<i>cheA</i>	Sensory and regulatory protein	CHEA1, CHEA2	2017	pTG6	cam ^R → <i>SacI</i> site at 1145 bp	pSVC6
Motility	<i>motAB</i>	Flagellar motor	MOT1, MOT2, MOT3, MOT4	1737 (with 97-bp Δ)	pTG7	Fragment between SOE-PCR generated sites <i>Bgl</i> II at 878 bp and <i>Kpn</i> I at 865 bp removed and replaced with cam ^R	pSVC7
SEF14	<i>sefA</i>	Fimbrial antigen	sefA1, sefA2	5500	pVW408§	kan ^R → engineered <i>Bam</i> HI site at 46 bp	pVW410§

* See Table 2.

† pTG construct = pCRSCRIPT::target gene.

‡ pSVC construct = pPERFORM::target gene::resistance marker insert.

§ Personal gifts from Claude Turcotte reported in ref. [22].

Δ = deletion.

Results

Construction and confirmation of mutant strains of *Enteritidis* S1400

Southern hybridisation of each mutant strain of *Enteritidis* strain S1400 confirmed that the native fimbrial or flagellar gene had been altered, as predicted by the insertion of the known antibiotic resistance gene cassette (Fig. 1); no hybridisation was observed when pPERFORM plasmid DNA alone was used as probe, which confirmed the loss of the suicide vector. Where possible, the phenotype of each mutant strain was confirmed and data are summarised in Table 4. It was not possible to demonstrate in-vitro expression of either LPF of PEF fimbriae by *Enteritidis* and the study relied upon hybridisation data (Fig. 1) that EAV7 (S1400 *lpfC*::*trm*^r) and EAV5 (S1400 *pefC*::*zeo*^r) were genuine non-fimbriate mutant strains. Multiply mutant strains were prepared by P22 transduction, and co-transduction frequencies between the antibiotic resistance marker and mutant phenotype (where testable) were 100%. In addition, all transductants were confirmed by Southern hybridisation to have acquired the modified alleles. The in-vitro growth rates of all mutant strains in LB broth shaken at 37°C were similar to the wild-type strain.

Development of a chick gut explant model

To estimate the extent of natural microflora colonisation of the alimentary tract of the day-old chick before inoculation with test bacterial cultures, gut sections from two chicks were homogenised and plated on nutrient agar with incubation at 37°C for 24–48 h. Bacterial growth was not detected. All subsequent assays included a measurement of bacterial colonisation from a control chick selected at random. SEM was used to test whether bacteria adhered to serous and luminal surfaces of the explant tissue. *Enteritidis* strain S1400 was incubated for 1 h with tissue samples and 25 fields of view of each surface were examined for adherent bacteria (Fig. 2a, b). Mean counts of 14 and 191 were determined for serous and luminal surfaces, respectively, and this difference was highly significant ($p < 0.001$). To determine whether *Enteritidis* was more adherent for any specific region of day-old chick gut, adhesion assays were performed with strain S1400 for 1 and 3 h on 2-cm gut samples taken from duodenum, proximal and distal small intestine, caecal and rectal regions. No significant differences in mean counts between the tissue types or incubation times ($p = 0.172$ for 1 h; $p = 0.113$ for 3 h) were observed. The duodenal loop was chosen for further studies.

Adherence of mutant strains of *Enteritidis* to duodenal gut sections

Wild-type and singly mutant strains from the *Enteritidis* S1400 mutant library were assessed for their ability to adhere (Table 5). The adherence of the non-flagellate mutant strain EAV9 was significantly reduced

Fig. 1. (continued overleaf)

compared with that of the wild-type strain ($p < 0.001$, for both incubation periods). To test whether fimbriae and flagella exerted synergic effects on adherence, multiply mutant strains were similarly assessed (Table 5). In comparison with the wild-type strain, the adherence of strain EAV37 (non-flagellate, non-motile and non-fimbriate) was significantly reduced ($p < 0.001$ after both 1 and 3 h) whereas mutant strain EAV26 (motile, flagellate and non-fimbriate) showed no significant differences ($p = 0.791$ for 1 h; $p = 0.082$ for 3 h).

Adherence of non-flagellate, paralysed and chemotaxis-defective mutants to gut explants

Decreased adherence of non-flagellate mutant strains may have resulted from either lack of the organelle itself or lack of motility or chemotactic ability. Accordingly, to evaluate these possibilities, a paralysed, flagellate but non-motile mutant strain (EAV45) and a chemotaxis-defective, smooth-swimming mutant strain (EAV65) were constructed. These mutant strains were compared with the wild-type strain S1400 for adherence in each of three assays in which the gut explants

Fig. 1. Southern hybridisation of genomic DNA of Enteritidis strain S1400/94 and isogenic strains derived from it to demonstrate insertional inactivation of target fimbrial and flagellar genes. (a) Lanes 1, 3, 5, S1400; 2, 4, 6, EAV1 (S1400::*fimD*::*tet*^R) digested to completion with *Bgl*II (lanes 1, 2), *Eco*RV (lanes 3, 4) and *Pst*I (lanes 5, 6) hybridised against *fimD*. Mol.-wt standards (m) were in ascending order, 1, 1.6, 2, 3, 4, 5, 6, 7, 8, 9 and 10 kb. (b) Lanes 2, 4, 6, S1400; 1, 3, 5, EAV5 (S1400::*pefC*::*zeo*^R) digested to completion with *Sma*I (lanes 1, 2), *Sal*I (lanes 3, 4) and *Hind*III (lanes 5, 6) hybridised against *pefC*. Mol.-wt standards (m) in ascending order were 0.4, 1, 1.6, 5, 6, 9 and 11 kb. (c) Lanes 1, 3, 5, S1400; 2, 4, 6, EAV7 (S1400::*lpfC*::*trm*^R) digested to completion with *Eco*RI (lanes 1, 2), *Eco*RV (lanes 3, 4) and *Ssp*I (lanes 5, 6) hybridised against *lpfC*. Mol.-wt standards (m) in ascending order were 0.5, 3, 4, 5 and 10 kp. (d) Lanes 1, 3, 5, S1400; 2, 4, 6, EAV9 (S1400::*fliC*::*cam*^R) digested to completion with *Pst*I (lanes 1, 2), *Pvu*II (lanes 3, 4) and *Ssp*I (lanes 5, 6) hybridised against *fliC*. Mol.-wt standards (m) in ascending order were 0.5, 1, 1.6, 2, 3, 4 and 5 kb. (e) Lanes 1, 3, 5, S1400; 2, 4, 6, EAV11 (S1400::*agfA*::*amp*^R) digested to completion with *Hpa*I (lanes 1, 2), *Sal*I (lanes 3, 4) and *Ssp*I (lanes 5, 6) hybridised against *fliC*. Mol.-wt standards (m) in ascending order were 0.5, 1, 1.6, 2, 3 and 4 kb. (f) Lanes 1, 3, 5, S1400; 2, 4, 6, EAV13 (S1400::*sefA*::*kan*^R) digested to completion with *Dra*I (lanes 1, 2), *Hind*III (lanes 3, 4) and *Nru*I (lanes 5, 6) hybridised against *sefA*. Mol.-wt standards (m) in ascending order were 0.5, 1, 1.6, 2, 3, 4, 5, 6 and 7 kb. (g) Lanes 1, 3, 5, S1400; 2, 4, 6, EAV45 (S1400::*motAB*::*cam*^R) digested to completion with *Eco*RV (lanes 1, 2), *Kpn*I (lanes 3, 4) and *Nco*I (lanes 5, 6) hybridised against *motAB*. Mol.-wt standards (m) in ascending order were 0.1, 0.2, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 kb. (h) Lanes 1, 3, 5, S1400; 2, 4, 6, EAV65 (S1400::*cheA*::*cam*^R) digested to completion with *Kpn*I (lanes 1, 2), *Pst*I (lanes 3, 4) and *Ssp*I (lanes 5, 6) hybridised against *cheA*. Mol.-wt standards (m) in ascending order were 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9 and 10 kb.

Table 4. Genotypes and phenotypes of Enteritidis S1400 mutant strains

Mutation	Strain designation	Genotype	Antibiotic resistance (mg/L)	Phenotype of mutant
SEF21 ⁻	EAV1	<i>fimD</i> :: <i>tet</i> ^R	Tetracycline* (6)	MSHA ⁻ ; SEF21 ELISA ⁻
SEF17 ⁻	EAV11 [†]	<i>agfA</i> :: <i>amp</i> ^R	Ampicillin* (25)	Lacy ⁻ ; SEF17 ELISA ⁻
PEF ⁻	EAV5	<i>pefC</i> :: <i>zeo</i> ^R	Zeocin (25)	Unconfirmed
LPF ⁻	EAV7	<i>lpfC</i> :: <i>trim</i> ^R	Trimethoprim (20)	Unconfirmed
FLA ⁻	EAV9	<i>fliC</i> :: <i>cam</i> ^R	Chloramphenicol (10)	Non-motile H g,m ⁻
MOT ⁻	EAV45	<i>motAB</i> :: <i>cam</i> ^R	Chloramphenicol (10)	Non-motile H g,m ⁺
CHE ⁻	EAV65	<i>cheA</i> :: <i>cam</i> ^R	Chloramphenicol (10)	Smooth swimming H g,m ⁺
SEF14 ⁻	EAV13 [‡]	<i>sefA</i> :: <i>kan</i> ^R	Kanamycin (50)	SEF14 ELISA ⁻
FLA ⁻	EAV55	<i>fliC</i> :: <i>cam</i> ^R	Chloramphenicol (10)	Non-motile, H g,m ⁻
SEF21 ⁻		<i>fimD</i> :: <i>tet</i> ^R	Tetracycline (6)	MSHA ⁻ SEF21 ELISA ⁻
SEF21 ⁻		<i>fimD</i> :: <i>tet</i> ^R	Tetracycline (6)*	MSHA ⁻
SEF17 ⁻		<i>agfA</i> :: <i>amp</i> ^R	Ampicillin (25)*	Lacy ⁻
PEF ⁻		<i>pefC</i> :: <i>zeo</i> ^R	Zeocin (25)	SEF21 ELISA ⁻
LPF ⁻	EAV26	<i>lpfC</i> :: <i>trim</i> ^R	Trimethoprim (20)	SEF17 ELISA ⁻
SEF14 ⁻		<i>sefA</i> :: <i>kan</i> ^R	Kanamycin (50)	SEF14 ELISA ⁻
SEF21 ⁻		<i>fimD</i> :: <i>tet</i> ^R	Tetracycline (6)*	MSHA ⁻
SEF17 ⁻		<i>agfA</i> :: <i>amp</i> ^R	Ampicillin (25)*	Lacy ⁻
PEF ⁻	EAV37	<i>pefC</i> :: <i>zeo</i> ^R	Zeocin (25)	SEF21 ELISA ⁻
LPF ⁻		<i>lpfC</i> :: <i>trim</i> ^R	Trimethoprim (20)	SEF17 ELISA ⁻
SEF14 ⁻		<i>sefA</i> :: <i>kan</i> ^R	Kanamycin (50)	SEF14 ELISA ⁻
FLA ⁻		<i>fliC</i> :: <i>cam</i> ^R	Chloramphenicol (10)	Non-motile H g,m ⁻

MSHA, mannose-sensitive haemagglutination.

*Only one copy of resistance gene resides within mutant chromosome, thus conferred level of resistance to antibiotics is low [41].

[†]Described in ref. [17] and included here for completeness.

[‡]Produced by transduction of *sefA*::*kan*^R from Enteritidis strain LA5SEF14⁻ [22] to Enteritidis strain S1400.

Fig. 2. Scanning electron micrograph of serous (a) and luminal (b) surfaces of chick gut duodenal tissue samples exposed to Enteritidis strain S1400/94 for 1 h in standard adherence assay. Bar = 10 μ m.

Table 5. Mean bacterial counts of wild-type, singly and multiply mutant strains bound to duodenal gut explants from day-old chicks

Strain*	Number of assays	Geometric mean count	Transformed mean log ₁₀ count (SEM)	p value†
Incubation for 1 h				
S1400/94	10	7.0×10^4	4.85 (0.15)	—
EAV1	10	1.3×10^5	5.11 (0.15)	0.203
EAV5	6	7.6×10^5	5.88 (0.19)	<0.001
EAV7	6	1.6×10^5	5.19 (0.19)	0.154
EAV9	10	4.3×10^3	3.64 (0.15)	<0.001
EAV11	6	1.7×10^5	5.24 (0.19)	0.105
EAV13	6	1.4×10^5	5.15 (0.19)	0.214
EAV26	6	6.1×10^4	4.78 (0.19)	0.791
EAV37	6	6.8×10^2	2.83 (0.19)	<0.001
EAV55	7	8.1×10^3	3.91 (0.18)	<0.001
Incubation for 3 h				
S1400/94	10	1.6×10^6	6.20 (0.17)	—
EAV1	10	1.4×10^6	6.14 (0.17)	0.783
EAV5	6	2.7×10^6	6.44 (0.21)	0.391
EAV7	6	3.6×10^6	6.56 (0.21)	0.195
EAV9	10	3.9×10^4	4.59 (0.17)	<0.001
EAV11	6	2.0×10^6	6.31 (0.21)	0.701
EAV13	6	8.5×10^6	6.93 (0.21)	0.009
EAV26	6	5.3×10^5	5.73 (0.21)	0.082
EAV37	6	6.4×10^3	3.80 (0.21)	<0.001
EAV55	7	1.5×10^5	5.17 (0.20)	<0.001

*See Table 4.

†*t* Test versus wild-type. Each strain was compared to the wild-type strain by a *t* test based on the pooled residual variance from the analyses of variance. A *p* value < 0.05 indicates a significant difference from that of the wild-type strain at the 5% level.

were placed in the bacterial inoculum and (1) shaken, (2) left static and allowed to settle or (3) suspended by sterile cotton thread and left static. Incubation was for 1 h and each assay was repeated three times. The results are summarised in Table 6. Irrespective of adherence assay, the non-flagellate mutant strain EAV9 was significantly less adherent ($p = 0.05$) than the wild-type strain. The paralysed mutant strain EAV45 was significantly less adherent ($p = 0.05$) in static and suspended assays only, whereas the smooth-swimming chemotaxis-defective mutant strain EAV65 was significantly less adherent ($p = 0.05$) but in the suspended assay only.

Invasion assay

Attempts were made to assess the extent of bacterial invasion of chick gut epithelial cells in this model. However, it was observed that the integrity of the gut explants failed as incubation was prolonged beyond *c.* 5 h, the minimum time for which initial adherence (1 h) and gentamicin treatment (a further 3 h) was

required [22]. This lack of tissue integrity has been reported previously [42] and meaningful data were not obtained.

Discussion

Allelic exchange is an established technique used to derive defined mutant strains for subsequent phenotypic analysis. Convincing genotypic evidence was gained by Southern hybridisation that the mutant strains were constructed by insertion of antibiotic resistance cassettes within each of the targeted genes. Where testable, as for SEF21, SEF14 and SEF17 fimbriae and non-motile non-flagellate and flagellate derivatives, there was 100% co-transduction between the relevant phenotype and antibiotic resistance. However, only genotypic evidence was available for insertional inactivation of *pef* and *lpf* genes in strain S1400; but this approach has been used successfully to demonstrate the roles of PEF and LPF of Typhimurium [8, 9, 11, 43]. It should be borne in mind that other undetected but

Table 6. Comparison of adherence of wild-type and mutant strains in day-old chick gut explants in shaking, static and suspended models

Strain no.	Geometric mean count			Transformed mean log ₁₀ count					
	Shaken	Static	Suspended	Shaken	p value*	Static	p value*	Suspended	p value*
S1400/94	7.0×10^5	9.9×10^4	1.2×10^6	5.84	—	5.00	—	6.06	—
EAV9	4.3×10^4	1.8×10^3	2.0×10^3	4.63	0.05	3.25	0.05	3.30	0.05
EAV45	2.9×10^5	1.2×10^3	2.0×10^3	5.50	NS	3.09	0.05	3.23	0.05
EAV65	8.5×10^5	4.5×10^4	3.0×10^4	5.93	NS	4.65	NS	4.48	0.05

NS, not significant.

*Compared with wild-type and calculated from an analyses of least significant differences (LSD). Any difference between the transformed means which exceeds the LSD is significant at the 5% level ($p = 0.05$) by Student's *t* test. The LSD for a 1-h incubation period is 0.51 and that for 3 h is 0.43.

co-transducible mutational events may have occurred during the construction of the mutant strains, although no evidence for that idea was gained.

The adhesion assay data suggested that flagella were important for adherence and that motility was a significant contributing factor. In the shaken model, bacterial motility was observed to be less important for adherence than the presence of the flagellar structure. A comparison between strains EAV9 (non-flagellate) and EAV45 (paralysed flagella) in both static and hanging models demonstrated that both mutant strains were significantly less able to adhere to gut explants than the wild-type strain. Therefore, it seems unlikely that flagella possess an adhesin, but rather that the adhesive effect is mediated by the morphology of these organelles, a conclusion supported by the findings of Jones *et al.* [44]. *Salmonella* flagella are long (5–10 µm), rigid structures, which may promote interaction between bacteria and host by, for example, increasing surface area for initial contact or overcoming repulsive forces. None of the fimbrial kinds appeared to contribute to adherence in any assay. This was a surprising finding considering that type 1 fimbriae are generally known to possess a mannose-sensitive adhesin [45] and in this work all assays were performed in the absence of D-mannose. One explanation might be that day-old chick gut lacks relevant fimbrial receptors, possibly due to immaturity [46], or that receptors were masked (e.g., by mucus), although SEM evidence suggested otherwise. Another possible explanation is that strain S1400 failed to elaborate fimbriae under the growth conditions used for the preparation of inocula. It was known that growth in LB broth was poorly inducing for several of the fimbriae [38, 39, 47] although induction may have been triggered by the association with explant tissue, a phenomenon reported by others [48]. Recent reports have indicated that LPS O antigen is an important factor for persistence of Typhimurium in the gut of 3-week-old chicks, but that fimbriae are not important [49]. It was confirmed that all strains used in the present experiments elaborated the LPS antigens which might have contributed to adherence in this model.

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