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Virulence of *Salmonella enterica* serotype Enteritidis aflagellate and afimbriate mutants in a day-old chick model

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SUMMARY

Certain fimbriae and the flagellae of *Salmonella enterica* serovar Typhimurium have been shown to contribute to attachment and invasion of gut epithelium in the murine typhoid infection model and to contribute to pathogenesis in the chick. However, little is known of the role these organelles play in Enteritidis poultry infections and, to study this, day-old chicks were dosed orally in separate experiments with defined multiply afimbriate and/or aflagellate mutant strains of Enteritidis. The colonization and invasion characteristics of each mutant were compared with those of the isogenic wild type strain by the determination of the number of bacteria recovered from livers and spleens at known time points post infection. Compared with wild type Enteritidis, a mutant unable to express flagella but retaining the genetic potential to express fimbriae was recovered *post mortem* from livers and spleens in significantly reduced numbers compared to the isogenic wild-type at all time points post infection ($P < 0{\cdot}001$). Conversely, a flagellate but multiply afimbriate mutant (defective for the elaboration of five different fimbrial types) and a flagellate but non-motile 'paralysed' mutant were recovered from livers and spleens in similar numbers to the wild-type. The data suggested that Enteritidis flagella, but not fimbriae, played an important role in pathogenesis in the chick model and that the flagellar apparatus itself and not motility *per se* contributed significantly to this role.

INTRODUCTION

Poultry are a major source of *Salmonella enterica* serotype Enteritidis infections of man [1–3]. Studies with experimentally-infected chicken flocks dosed orally showed that the epidemiology of Enteritidis PT4 infections varies with the age of the bird [4–6]. Chicks dosed orally within 24 h of hatching experienced an invasive disease of the reticuloendothelial system [3] whereas by 7 days of age invasion was reduced significantly [4]. Chickens dosed at point of lay, approximately 20 weeks of age, appeared resistant to infection, both in terms of persistence in the GI tract and invasion of deeper tissues [5, 6] although eggs may become infected if laying birds are dosed orally with 10^9 c.f.u. Enteritidis [7]. The mechanisms of egg contamination are considered to include an invasive route to ovarian tissue, an ascending route from cloaca to reproductive tissues and surface contamination via excreta [8].

Prior to invasion of host tissues, *Salmonella* spp. adhere to gut epithelia and, for Typhimurium, adhesion to epithelial cells of mouse intestinal organ cultures is mediated by Long Polar Fimbriae, LPF, and Plasmid Encoded Fimbriae, PEF [9, 10]. Although flagella and type 1 fimbriae are significant factors in the pathogenesis in the mouse model, the

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role of these surface appendages in initial colonization is unclear [11–13]. After attachment of salmonella preferentially to M-cells of Peyer's patches on the terminal ileum [14, 15], cytoskeletal changes in the host cell are induced which permit internalization of the bacterium within a membrane bound vesicle [16].

As yet, little is known of the initial colonization or invasion of the chicken by Enteritidis. However, for this serotype, type 1 and type 3 fimbriae have been shown to contribute to adherence to gut epithelia, including Peyer's patches, and pathogenesis in the mouse [17] and to contribute to adherence to INT-407 cultured cells [18]. Peyer's patches in the chicken have been found to be similar to those found in mammals, possessing a distinct lymphoepithelium with M-cells and strong pinocytotic activity [19, 20]. The gut-associated lymphoid tissue of the chicken includes aggregates which may develop anywhere in the body upon antigenic stimulation [21] which is in contrast to mammals where lymphoid aggregates are encapsulated as nodes. In newly hatched chicks, the mucosa-associated lymphoid tissue is underdeveloped and requires the presence of gut antigens for its immunological maturation [22]. In concurrent studies [23] we demonstrated that flagellae but not fimbriae were significant factors in the adherence of Enteritidis to the lumenal surface of chick gut explants whilst several studies have suggested that at least some of these appendages may be expressed during pathogenesis in the chicken [24–26]. Previous studies have shown that the main sites for multiplication of *Salmonella* spp. during invasive infection were the liver and spleen [27] and that the number of bacteria isolated from these organs post infection depended on the challenge dose, the virulence of the bacteria and the susceptibility of the host [28]. Assays of invasion of the chick have been developed [29–33] and here we report the use of these assays to study the role of the surface appendages of Enteritidis in the invasion of chicks dosed at day-old.

MATERIALS AND METHODS

Bacteria

Bacteria used in this study are listed in Table 1. Enteritidis strain S1400/94, a wild type isolate from a natural infection of chicken, was previously characterized as PT4 and shown to harbour the serotype associated (virulence) plasmid [26]. Stock cultures were maintained frozen at −70 °C in heart infusion broth supplemented with glycerol (15% w/v). To prepare inocula for *in vivo* studies, stocks were thawed, streaked on nutrient agar and plates incubated overnight at 37 °C. Single well isolated colonies were picked into fresh pre-warmed Luria–Bertani broth and incubated at 37 °C overnight. Decimal dilutions were made in phosphate buffered saline (pH 7·4) and inocula (100 μl) were administered immediately.

Chick dosing regimes

White leghorn SPF chicks were hatched, maintained in isolators and provided with feed and water *ad libitum*. For virulence assays, groups of 10 birds at between 18 and 24 h of age were each dosed orally with one dilution of a decimal series of dilutions of S1400/94. For each invasion study, 40 chicks at between 18 and 24 h of age were divided randomly into 2 sets of 20 birds and each bird of a set was dosed orally with either wild type Enteritidis strain S1400/94 or one isogenic derivative. Oral dosing was as described previously [29] and all birds were observed three times daily for general condition and thrift. Birds were killed *in extremis*.

Enumeration of salmonellae in organ homogenates

Birds were killed by cervical dislocation and whole liver, spleen and caecum were removed at *post mortem* and placed in individual sterile vessels. The viable count in homogenates of liver, spleen and caecum was determined by plating decimal dilutions made in sterile PBS (pH 7·4) on brilliant green agar, BGA (CM329, Oxoid), with or without antibiotics as described previously [23]. The limit of detection was considered about 100 c.f.u. Samples of organ homogenate were enriched in selenite F broth (CM395/L121, Oxoid) with incubation for 24 h at 42 °C followed by subculture on to BGA.

Statistical analyses

In order to minimize error caused by batch-to-batch variation between chicks, organ counts from a group of birds dosed with a given mutant strain were compared to those from a control group from the same batch of chicks dosed with the wild-type strain. Each experiment was repeated at least twice. Thus, counts were made for the spleen, liver and caeca at 24, 48 and 120 h post-dosing on between 15 and 21 chicks

Table 1. *Genotypes and phenotypes of Enteritidis S1400 mutants*

Strain designation	Genotype	Antibiotic resistance	Phenotype of mutant
EAV9	*fliC*::cam®	Chloramphenicol at 10 μg/ml	Non-motile H g, m$^+$
EAV26	*fimD*::tet®	Tetracycline at 6 μg/ml	MSHA*
	agfA::amp®	Ampicillin at 25 μg/ml	non-Lacy
	pefC::zeo®	Zeocin at 25 μg/ml	SEF21 ELISA$^-$
	lpfC::trim®	Trimethoprim at 20 μg/ml	SEF17 ELISA$^-$
	sefA::kan®	Kanamycin at 50 μg/ml	SEF14 ELISA$^-$
EAV37	*fimD*::tet®	Tetracycline at 6 μg/ml	MSHA^{-a}
	agfA::amp®	Ampicillin at 25 μg/ml	Non-Lacy
	pefC::zeo®	Zeocin at 25 μg/ml	SEF21 ELISA$^-$
	lpfC::trim®	Trimethoprim at 20 μg/ml	SEF17 ELISA$^-$
	sefA::kan®	Kanamycin at 50 μg/ml	SEF14 ELISA$^-$
	fliC::cam®	Chloramphenicol at 10 μg/ml	Non-motile H g, m$^+$
EAV45	*motAB*::cam®	Chloramphenicol 10 μg/ml	Non-motile H g, m$^+$

* MSHA, Mannose sensitive haemagglutination.

per tissue per time. The number of chicks colonized was assumed to follow a binomial distribution and differences between wild-type and mutant were compared over time separately for the liver and spleen using a generalized linear model. Generalized linear models extend the ordinary regression framework to situations where the data do not follow a normal distribution or a transformation (link function) needs to be applied before a linear model can be fitted. For proportions, a logistic link function was thought appropriate and the model included terms for type and time.

RESULTS

Virulence of wild-type S1400/94 in day-old SPF chicks

Previous experimental data [29] indicated that oral doses of between 1×10^3 and 1×10^5 c.f.u. of Enteritidis strain LA5 per bird were considered likely to result in morbidity without mortality in day-old White Leghorn SPF chicks. To test the virulence of Enteritidis strain S1400/94, which was considered phenotypically similar to strain LA5 [18, 23], groups of ten White Leghorn SPF day-old chicks were each dosed orally with dilutions of an overnight culture of S1400/94. All birds dosed with 1×10^7 died or were killed *in extremis* by 5 days post infection. Of the birds dosed with 1×10^5 c.f.u. strain S1400/94, three died by 5 days post infection and, of the surviving birds, between 4×10^2–$6{\cdot}3 \times 10^3$ and $1{\cdot}0 \times 10^1$–$1{\cdot}2 \times 10^2$ bacteria were isolated from their livers and spleens respectively. Of the birds dosed with 1×10^3 c.f.u., there were no mortalities and all birds remained in good condition with weight gain similar to uninoculated control birds. At 5 days post infection, each of these birds was killed and between 8×10^1–7×10^3 and $1{\cdot}0 \times 10^1$–2×10^2 bacteria were isolated from their livers and spleens respectively. Therefore, 1×10^3 per bird c.f.u. of Enteritidis strain S1400/94 was used for all subsequent experiments.

Invasion of day-old chicks by Enteritidis S1400/94 and isogenic afimbriate and aflagellate derivatives

It has been shown that multiple fimbrial adhesins were required for full virulence of Typhimurium in the mouse model and that fimbrial operons behaved synergistically during the development of murine typhoid fever [33, 34]. However, for Enteritidis, our previous work showed that both *in vitro* adherence to day-old chick gut explants and invasion of INT407 cells were dependent primarily upon flagella and not fimbriae [18, 23]. Thus, four chick invasion studies were done. Four isogenic derivatives of Enteritidis S1400/94 designated EAV37 (fla$^-$ mot$^-$ fim$^-$), EAV26 (fla$^+$ mot$^+$ fim$^-$), EAV9 (fla$^-$ mot$^-$ fim$^+$) and EAV45 (fla$^+$ mot$^-$ fim$^+$) were compared individually with wild type in invasion assays to determine the relative contribution of the flagella, motility *per se* and

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Table 2. *Organ counts (log_{10}) per whole spleen taken at 24, 48 and 120 h post inoculation from chicks dosed orally at 1 day-old with 1×10^3 c.f.u. of either wild-type S1400/94 or mutant, and analyses of variance for each group*

Experiment	Strain	Transformed counts and standard errors			Analyses of variance	
		24 h	48 h	120 h	Significance of time, *P*	Significance of type, *P*
1	S1400/94	1·80 ± 0·28	2·74 ± 0·29	2·75 ± 0·28	< 0·001	< 0·001
	EAV37	0·26 ± 0·30	1·24 ± 0·31	1·94 ± 0·30		
2	S1400/94	1·09 ± 0·25	1·68 ± 0·26	3·11 ± 0·27	< 0·001	0·716
	EAV26	1·16 ± 0·26	1·83 ± 0·26	2·97 ± 0·27		
3	S1400/94	0·86 ± 0·21	1·55 ± 0·20	2·39 ± 0·21	< 0·001	< 0·008
	EAV9	0·26 ± 0·20	0·94 ± 0·20	2·26 ± 0·20		
4	S1400/94	0·88 ± 0·21	1·26 ± 0·20	2·55 ± 0·21	< 0·001	0·324
	EAV45	0·38 ± 0·21	1·67 ± 0·20	2·14 ± 0·20		

Table 3. *Organ counts (log_{10}) per gram of liver taken at 24, 48 and 120 h post inoculation from chicks dosed orally at 1 day-old with 1×10^3 c.f.u. of wild-type S1400/94 or mutant, and analyses of variance for each group*

Experiment	Strain	Transformed counts and standard errors			Analyses of variance	
		24 h	48 h	120 h	Significance of time, *P*	Significance of type, *P*
1	S1400/94	2·71 ± 0·32	3·34 ± 0·33	3·24 ± 0·32	0·052	< 0·001
	EAV37	1·09 ± 0·34	1·75 ± 0·35	2·10 ± 0·34		
2	S1400/94	2·63 ± 0·35	2·43 ± 0·37	4·16 ± 0·38	< 0·001	0·906
	EAV26	2·55 ± 0·37	2·61 ± 0·37	3·74 ± 0·38		
3	S1400/94	2·77 ± 0·30	2·22 ± 0·29	2·77 ± 0·30	0·042	0·001
	EAV9	1·42 ± 0·29	1·51 ± 0·29	2·44 ± 0·29		
4	S1400/94	2·59 ± 0·36	2·02 ± 0·33	2·81 ± 0·35	0·783	0·859
	EAV45	2·32 ± 0·36	2·80 ± 0·34	2·46 ± 0·33		

Table 4. *Means of percentages of spleens from birds within a group from which bacteria could be isolated at 24, 48 and 120 h post inoculation, and analyses of variance for each data set*

Experiment	Strain	Mean percentages (± approximate standard errors)			Analyses of variance	
		24 h	48 h	120 h	Significance of time, *P*	Significance of type, *P*
1	S1400/94	67 ± 11	98 ± 2	100	< 0·01	< 0·001
	EAV37	11 ± 7	74 ± 11	100		
2	S1400/94	60 ± 12	96 ± 4	100	< 0·001	n.s.
	EAV26	76 ± 11	98 ± 2	100		
3	S1400/94	36 ± 11	91 ± 5	100	< 0·01	n.s.
	EAV9	21 ± 9	84 ± 8	100		
4	S1400/94	53 ± 11	84 ± 7	100	< 0·001	n.s.
	EAV45	41 ± 11	76 ± 9	100		

n.s., not significant, $P > 0·05$.

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Table 5. *Mean percentages of livers from birds within a group from which bacteria could be isolated at 24, 48 and 120 h post inoculation, and analyses of variance for each data set*

Experiment	Strain	Mean percentages (±approximate standard errors)			Analyses of variance	
		24 h	48 h	120 h	Significance of time, P	Significance of type, P
1	S1400/94	81±9	95±4	100	$< 0{\cdot}01$	n.s.
	EAV37	68±11	91±6	100		
2	S1400/94	91±7	97±4	100	n.s.	n.s.
	EAV26	90±7	97±4	100		
3	S1400/94	95±5	99±1	100	$< 0{\cdot}01$	$< 0{\cdot}05$
	EAV9	64±11	89±8	100		
4	S1400/94	82±10	98±2	100	$< 0{\cdot}001$	n.s.
	EAV45	65±12	96±4	100		

n.s., not significant, $P > 0{\cdot}05$.

Table 6. *Organ counts (log_{10}) per whole caeca taken at 24, 48 and 120 h post inoculation from chicks dosed orally at 1 day-old with 1×10^3 c.f.u. of wild-type S1400/94 or mutant*

Experiment	Strain	Transformed counts and standard errors			Analyses of variance	
		24 h	48 h	120 h	Significance of time, P	Significance of type, P
1	S1400/94	6·83±0·34	6·77±0·35	7·42±0·34	0·307	0·092
	EAV37	7·34±0·36	7·44±0·37	7·70±0·36		
2	S1400/94	7·64±0·24	7·74±0·24	8·05±0·25	0·001	0·28
	EAV26	6·50±0·24	7·67±0·24	7·89±0·25		
3	S1400/94	6·72±0·31	7·62±0·30	8·25±0·31	0·011	0·526
	EAV9	7·17±0·29	7·53±0·30	7·43±0·29		
4	S1400/94	7·13±0·24	7·69±0·22	7·57±0·23	$< 0{\cdot}001$	0·535
	EAV45	6·58±0·24	7·93±0·22	7·52±0·22		

fimbriae in the pathogenesis of Enteritidis in the day-old chick model. The experimental design is given in detail in materials and methods and the results are summarized in Tables 2–6..

For EAV37 (fla^- mot^- fim^-), the numbers of bacteria which colonized both liver and spleen were reduced significantly compared to the wild type ($P < 0{\cdot}001$) but the numbers did increase over time, more so in the spleen ($P < 0{\cdot}001$) than in the liver ($P = 0{\cdot}52$). In contrast, the counts for the wild type remained constant, approximately, after 48 h. The percentage of birds from which isolations of mutant and wild type were made followed a similar pattern to the mean counts. Collectively, these data indicated that colonization of internal organs was profoundly affected by the absence of the surface structures.

For EAV26 (fla^+ mot^+ fim^-), the numbers of bacteria which colonized the liver and spleen were not significantly different compared to wild type and both showed similar increases over time ($P < 0{\cdot}001$). The percentages colonized were also similar with a significant increase over time for the spleen ($P < 0{\cdot}001$) but not for liver. Collectively, these data indicated that colonization of internal organs was not profoundly affected by the absence of fimbriae.

For EAV9 (fla^- mot^- fim^+), the numbers of bacteria which colonized the liver and spleen were reduced significantly ($P = 0{\cdot}001$ for livers, $P = 0{\cdot}008$ for spleens) compared to the wild type. Overall there were significant increases over time ($P = 0{\cdot}042$ for livers, $P < 0{\cdot}001$ for spleens), although there was no increase for the livers with the wild type. The percentage of organs colonized followed a similar pattern to the mean counts. Collectively, these data indicated that colonization of internal organs was profoundly affected by the absence of flagellae but not fimbriae.

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For EAV45 (fla^+ mot^- fim^+), there were no significant differences from the wild type in either the numbers of bacteria which colonized the livers or spleens or the percentages of organs colonized. The mean counts increased significantly over time for the spleen ($P < 0{\cdot}001$) but remained approximately constant for the liver. Therefore, in this model, a lack of motility had little effect on the colonization of internal organs.

In all four experiments, the caeca were readily colonized by 24 h post inoculation. The bacterial counts were in general similar for the wild type and each of the mutants. There were significant increases over time of the numbers of bacteria which colonized the caeca in all of the studies although the mean counts for EAV26 (fla^+ mot^+ fim^-) and EAV45 (fla^+ mot^- fim^+) were significantly lower than wild type particularly at 24 h post inoculation ($P = 0{\cdot}001$ and $P < 0{\cdot}001$ respectively).

DISCUSSION

The data showed that flagella, and not fimbriae, played an important role in the pathogenesis of Enteritidis in the day-old chick model. Colonization of livers and spleens was significantly reduced, compared to wild type, when flagella were absent. Colonization in this model was likely to be governed by a number of factors, including the ability for the bacteria to adhere and invade the gut epithelium as well as survive and multiply once within the host. We have shown previously that flagella are key factors for adherence to both day-old chick gut explants [23] and INT407 cells [18]. It may be argued that reduced adherence at the gut epithelium resulted in reduced invasion.

Internalization of salmonella is regarded as a two-step process whereby the bacteria first adhere to and then invade host tissue cells [35]. Salmonella fimbriae have been cited as mediators of adhesion to animal cells whereas invasion itself is known to involve many other bacteria factors [36]. Typhimurium mutants unable to elaborate PEF, LPF or type-1 fimbriae were reduced in their ability to invade tissue culture cell-lines which reflected reduced adhesion rather than an inability to invade *per se* [34]. In the mouse model, Typhimurium was shown to interact preferentially with M-cells in Peyer's patches [15, 37], an interaction mediated by LPF [9] whilst a Typhimurium mutant unable to elaborate LPF, PEF, type-1 or curli fimbriae gave significantly reduced organ counts [34]. We demonstrated that Enteritidis strain EAV26, although defective for the elaboration of five fimbriae but still capable of adhering to day-old chick gut explants [23], gave organ counts which were not significantly different to the wild-type in the orally-infected chick model. Therefore, colonization of internal organs was largely independent of fimbriae in this model.

For Typhimurium, the lack of motility (fla^- or mot^-) was shown to reduce invasion of tissue culture cells [11, 38–41] and a reduced capacity to cause lethal infection *via* the oral route in murine and chick models [12, 13, 39, 41]. Additionally, neither flagella nor motility were found to influence pathogenesis when infection was *via* the intraperitoneal route in either mice [12, 13] or chickens [42]. For Enteritidis, this study showed that strains EAV9 (fla^-) and EAV37 (fim^-, fla^-) were recovered in significantly lower numbers than the wild-type parent strain from the liver and spleen of orally-infected chicks. This, and given that EAV9 and EAV37 adhere to chick gut explants less efficiently than the wild type, suggested that flagella were involved in the initial stages of infection, namely adhesion and internalization. Enteritidis strain EAV45, the flagellate but non-motile "paralysed" mutant, was recovered from liver and spleen in similar numbers to the wild-type strain which indicated that the presence of the flagella structure alone was sufficient to promote internalization in this model. Therefore, if it is assumed that the extent of invasion is directly dependant upon the extent of adherence at the gut epithelium, it might be expected that aflagellate derivatives of Enteritidis would be less invasive in the assays described. Whilst the data generated in this work supports this concept superficially, the question arises as to whether this model affords a true measure of invasiveness. This issue may be tested by examining the *in vivo* properties of these mutants after alternative routes of inoculation, such as intravenous for example. Contrary to the supposition proposed above, in *in vitro* INT407 invasion assays, the numbers of bacteria internalized were similar for all mutants tested and did not correlate with the extent of adherence [18]. Whether *in vitro* models reflect the events *in vivo* requires further investigation.

An alternative explanation of the results generated in this work may be that the presence of flagella contributed to bacterial survival and growth within the host. It should be noted that wild type Typhimurium has been demonstrated to multiply more extensively in murine macrophages than aflagellate

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mutants [43, 44]. However, Enteritidis grown in the peritoneal cavities of chickens did not express flagella [42]. The relationship between these observations requires further investigation.

Organs from all birds in all experiments became colonized by day 5 which indicated that irrespective of the state of fimbriation and flagellation, all mutants were able to invade. It seems likely, therefore, that several pathways for adherence and invasion exist which were dependent and independent of flagella and fimbriae. For example, other surface structures, such as LPS which has been shown to promote colonization of chicken caeca by Typhimurium [45, 46], may contribute to initial adherence. Gut colonization, as assessed by determining counts in the caecum, showed a massive bacterial load in all birds in all experiments and that lack of either fimbriae and/or flagella did not influence bacterial multiplication in this organ. Thus, it was possible that the bacterial load of Enteritidis overcame the flagella and fimbriae specific dependent pathways for adherence and invasion. This may be assessed by studying other routes of inoculation to determine whether the differences observed may be attributed to systemic survival.

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