BACTERIAL PATHOGENICITY

Adhesion of *Salmonella enterica* var Enteritidis strains lacking fimbriae and flagella to rat ileal explants cultured at the air interface or submerged in tissue culture medium

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Rat ileal air interface and submerged explant models were developed and used to compare the adhesion of *Salmonella enterica* var Enteritidis wild-type strains with that of their isogenic single and multiple deletion mutants. The modified strains studied were defective for fimbriae, flagella, motility or chemotaxis and binding was assessed on tissues with and without an intact mucus layer. A multiple afimbriate/aflagellate (*fim*[−]/*fla*[−]) strain, a fimbriate but aflagellate (*fla*[−]) strain and a fimbriate/flagellate but non-motile (*mot*[−]) strain bound significantly less extensively to the explants than the corresponding wild-type strains. With the submerged explant model this difference was evident in tissues with or without a mucus layer, whereas in the air interface model it was observed only in tissues with an intact mucus layer. A smooth swimming chemotaxis-defective (*che*[−]) strain and single or multiple afimbriate strains bound to explants as well as their corresponding wild-type strain. This suggests that under the present experimental conditions fimbriae were not essential for attachment of *S. enterica* var Enteritidis to rat ileal explants. However, the possession of active flagella did appear to be an important factor in enabling salmonellae to penetrate the gastrointestinal mucus layer and attach specifically to epithelial cells.

Introduction

The establishment of a successful infection by salmonellae requires the co-ordinated action of several virulence determinants. The first step is attachment of the pathogen to host intestinal surfaces before invasion. This interaction is thought to depend upon bacterial lectin-like adhesins recognising specific glycoconjugate receptors on host cell surfaces. *Salmonella enterica* var Enteritidis is known to elaborate several types of fimbrial lectins on its surface including SEF14, SEF17 and SEF21 [1]. Their roles in the infection process have been studied in a number of infection models. Peralta *et al.* [2] demonstrated a role for SEF14 fimbriae in the in-vitro adhesion of Enteritidis to mouse

epithelial cells, although a comparison of the survival rates of a defined SEF14 fimbrial deletion mutant with its isogenic wild-type strain failed to demonstrate a significant role for SEF14 in the infection of 6-week-old BALB/c mice and 1-day-old chicks [3]. SEF17 fimbriae are structurally and functionally similar to the curli fimbriae expressed by certain strains of diarrhoeagenic *Escherichia coli* [4] and are likewise responsible for binding of Enteritidis to fibronectin [5]. Thus exposure of fibronectin in basement membranes following tissue damage may provide Enteritidis with additional sites of attachment to the host intestinal epithelium. SEF21 are the most widely studied of the salmonella fimbriae; they have been shown to contribute to the in-vitro adhesion of Enteritidis to mouse intestinal epithelial cells [6]. More recently, Ewen *et al.* [7] demonstrated the in-vivo expression of type 1 fimbriae by *S. enterica* var Typhimurium and Enteritidis following oral infection of rats. The plasmid-encoded fimbriae (PEF) and long polar fimbriae (LPF) of Typhimurium are considered to be involved in the adhesion of this strain to villus small intestinal cells

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and Peyer's patches, respectively [8, 9]. Enteritidis expresses PEF during infection in chickens [10], although its role in the infection process remains undetermined [11]. It also has the genetic potential to express LPF, but elaboration of this structure on Enteritidis has yet to be confirmed [12].

The possession of active flagella combined with chemotaxis is also an important factor in the pathogenicity of salmonellae [13, 14]. Recently, Enteritidis flagella have been shown to play an important role in the pathogenesis of infection in the chick gut [12, 15]. Worton *et al.* [16] suggested that the early association of Typhimurium with host tissues may involve a loose non-specific association with superficial mucus. Therefore, motility may be an important virulence factor in enabling salmonellae to penetrate the mucus layer and attach specifically to epithelial cells. This is supported by the findings of McHan *et al.* [17], who demonstrated greater adhesion of Typhimurium to chicken caecal epithelia in the absence of an intact mucus layer.

The aim of the present study was to assess the likely role of fimbriae and flagella in the pathogenicity of Enteritidis by comparing the ability of wild-type and deletion mutant strains to attach to rat ileal explants. Adhesion to both air interface and submerged ileal explant cultures, which model different gut conditions, was assessed. Previously, these strains had been tested in a chick gut explant model [12]. However, it is important to examine the role of Enteritidis fimbriae and flagella in a mammalian system. Therefore, a rat

model was chosen for this work because previous studies have shown that, unlike the infection in mice [18], salmonellae can persist in rats for a considerable length of time. Furthermore, the terminal ileum has been noted as the main site of infection in both rat and human salmonellosis [19].

Materials and methods

Bacterial strains and culture conditions

Three wild-type strains and eight mutant strains of Enteritidis were examined (Table 1). Wild-type strains S1400 and LA5 were isolated from cases of poultry infections and have been characterised previously [20]. Strain 27655R was isolated from a case of human infection and has been described elsewhere [21, 22]. Mutant strains were constructed by insertional mutagenesis as described previously [12]. Stock cultures of isolates were stored on Dorset egg slopes at 4°C.

Preparation of challenge inocula

In experiments assessing the contribution of individual types of fimbriae to the adhesion process, both the wild-type and the insertional inactivation mutants were grown under conditions which promote optimal expression of the particular fimbrial type. For SEF14 this was achieved by growth on Sensitest Agar (CM409; Oxoid) at 37°C for 24 h [23]; expression was verified by latex agglutination with SEF14 fimbrial antibody [24, 25]. Expression of SEF17 was achieved by growth on colonisation factor antigen agar at 25°C for 48 h

Table 1. Genotypes and phenotypes of Enteritidis mutant strains

Strain	Mutation*	Genotype	Phenotype
†27655R (wild-type)			
EAV30	SEF17 ⁻	<i>agfA::amp^R</i>	Non-lacy
‡LA5 (wild-type)			
EAV10	FLA ⁻	<i>fliC::cam^R</i>	Non-motile
EAV3	SEF21 ⁻	<i>fimD::tet^R</i>	MSHA ⁻
EAV46	MOT ⁻	<i>motAB::cam^R</i>	Non-motile
EAV66	CHE ⁻	<i>cheA::cam^R</i>	Smooth swimming only
‡S1400 (wild-type)			
EVA13	SEF14 ⁻	<i>sefA::Kan^R</i>	SEF14 fimbrial antigen ⁻
EAV26*	SEF14 ⁻	<i>sefA::Kan^R</i>	SEF14 fimbrial antigen ⁻
	SEF17 ⁻	<i>agfA::amp^R</i>	Non-lacy
	SEF21 ⁻	<i>fimD::tet^R</i>	MSHA ⁻
	PEF ⁻	<i>pefC::zeo^R</i>	Genotypic evidence only§
	LPF ⁻	<i>lpfC::trim^R</i>	Genotypic evidence only§
EAV37•	SEF14 ⁻	<i>sefA::Kan^R</i>	SEF14 fimbrial antigen ⁻
	SEF17 ⁻	<i>agfA::amp^R</i>	Non-lacy
	SEF21 ⁻	<i>fimD::tet^R</i>	MSHA ⁻
	PEF ⁻	<i>pefC::zeo^R</i>	Genotypic evidence only§
	LPF ⁻	<i>lpfC::trim^R</i>	Genotypic evidence only§
	FLA ⁻	<i>fliC::cam^R</i>	Non-motile

Genotype of all wild-type strains: SEF14⁺, SEF17⁺, SEF21⁺ (*S. enteritidis* fimbriae 14, 17, 21 kDa), PEF⁺ (plasmid-encoded fimbriae), LPF⁺ (long polar fimbriae), MOT⁺ (motile), FLA⁺ (flagellate), CHE⁺ (chemotactic). EAV, strain designation for deletion mutants derived from relevant wild-type strains (*multiple *fim*⁻, •multiple *fim*⁻/*fla*⁻). MSHA, mannose-sensitive haemagglutination. ^RResistance to named antibiotic: amp, ampicillin; cam, chloramphenicol; trim, trimethoprim; kan, kanamycin; zeo, zeocin; tet, tetracycline.

*Appendage or function not expressed in mutant strain.

†Described in references [21] and [22].

‡Described in reference [20].

§Genotypic evidence only available for insertional inactivation of *pef* and *lpf* genes, see reference [12].

[21] and was confirmed by elaboration of a lacy colony phenotype and spontaneous aggregation of the wild-type strain when resuspended in PBS. SEF21 were expressed following static culture in Heart Infusion Broth (CM225; Oxoid) for 48 h [26]. The presence of type 1 fimbriae was confirmed by the mannose-sensitive haemagglutination of guinea-pig red blood cells (3%). Corresponding afimbriate mutants were tested and confirmed to be negative for expression of the particular fimbriae in question. Otherwise, stock cultures were subcultured on to nutrient agar plates and grown at 37°C overnight. A single colony from each plate was used to inoculate 10 ml of Luria Bertani Broth (12795-019; Gibco, Life Technologies, Paisley) and these were grown overnight at 37°C with agitation. Cultures were diluted 1 in 10 in phosphate-buffered saline (PBS) before infection of ileal explants to give c. 1×10^8 cfu/ml.

Motility tests

Cultures of the wild-type, the aflagellate (*fla*⁻) mutant and the non-motile but flagellate (*mot*⁻) mutant were stab inoculated into petri dishes containing semi-solid agar [27]. Plates were examined after incubation at 37°C for 72 h and the *fla*⁻ and *mot*⁻ strains were confirmed as having the non-motile phenotype. Broth cultures of the wild-type and the chemotaxis-defective (*che*⁻) mutant were examined microscopically and the *che*⁻ strain was confirmed to have the expected 'smooth' swimming phenotype.

Rat ileal explant model

Young adult male Hooded Lister rats (60 days old) were fasted overnight and killed by halothane overdose and exsanguination. Approximately 40 cm of ileum (5–45 cm from the ileocaecal junction) were removed aseptically from the abdominal cavity and either washed through with 4 ml of pre-warmed (37°C) PBS to remove mucus or left unwashed to preserve the mucus layer. The tissue was opened longitudinally, the mesentery was removed and 1-cm squares of tissue were cut out. The method for the air interface culture model has been described previously [28]. Briefly, explants were mounted mucosal side uppermost on a sterilised steel grid (three explants per grid) in an organ culture dish (3037; Falcon, Fred Baker Scientific, Runcorn) with capillary contact to 1.5 ml of culture medium. The culture medium used was a modified Autrup's medium without antibiotics, prepared on site according to a published formula [29, 30]. The surface of each explant was inoculated with 50 µl from a 1×10^8 cfu/ml bacterial culture. For the submerged culture model, explants were immersed in 1.5 ml of tissue culture medium in a sterile tube with 50 µl of the bacterial inoculum. All explants were incubated for 1 h at 37°C in CO₂ 5% in air without agitation and then rinsed five times in sterile saline 0.9% to remove non-adherent bacteria. Samples were homogenised in

maximum recovery diluent (MRD) (CM733; Oxoid) with a Janke-Kunkel Ultra-Turrax T25 tissue homogeniser at 20 000 rpm for 30 s. Bacterial counts were performed by dilution of the primary homogenate in MRD. Samples of each dilution were plated in duplicate on to XLD Agar (CM469B; Oxoid) and incubated overnight at 37°C. Viable counts were performed by the method of Miles and Misra [31]. Uninfected explants were included as controls. All assays were conducted in triplicate and repeated on at least three separate occasions.

Statistical analysis of data

Statistical differences between adhesion of strains were assessed by one-way analysis of variance (ANOVA) with the Minitab statistical package (Minitab, State College, PA, USA). Adhesion tests for each strain were replicated nine times. All data are expressed as log₁₀ means and SD.

Results

Adhesion of Enteritidis strains to rat ileal explants cultured in the air interface explant model

All the Enteritidis strains tested (wild-type and mutants) adhered to ileal explants (Tables 2 and 3). However, binding of an aflagellate (*fla*⁻) mutant, a non-motile but flagellate (*mot*⁻) mutant and a multiple afimbriate/aflagellate (*•fim*⁻/*fla*⁻) mutant was in each case significantly less ($p < 0.001$) than that of their isogenic wild-type strains in tissues with an intact mucus layer. Single fimbrial deletion mutants, a multiple afimbriate (**fim*⁻) mutant and a smooth swimming chemotaxis-defective (*che*⁻) mutant adhered as well as their isogenic wild-type strains regardless of whether mucus was present or not.

Adhesion of Enteritidis strains to rat ileal explants cultured in the submerged explant model

Three of the strains tested in the air interface model were also tested in a submerged culture model (Table 3). As in the previous model, the *fla*⁻ mutant and the multiple *•fim*⁻/*fla*⁻ mutant bound significantly less well ($p < 0.001$) to explants than did their isogenic wild-type strains. However, unlike the previous model, this reduction in binding was observed in tissues both with and without an intact mucus layer. As with the air interface model, no differences were observed between the binding of the multiple **fim*⁻ mutant and its wild-type strain (Table 3).

Discussion

Fimbrial lectins are thought to be important in facilitating initial attachment of bacteria including

Table 2. Adhesion of Enteritidis strains to rat ileal explants ($\log_{10}$ cfu $\pm$ SD) in the air interface explant culture model

Strain no	Mucus present	p value	Mucus removed	p value
	Mean $\log_{10}$ cfu (SD)		Mean $\log_{10}$ cfu (SD)	
S1400	5.4 (0.3)	0.051	5.9 (0.4)	0.050
EAV13 (sef14 ⁻)	5.8 (0.4)		6.2 (0.3)	
27655R	5.6 (0.3)		6.2 (0.4)	
EAV30 (sef17 ⁻)	5.5 (0.4)	0.510	5.8 (0.4)	0.067
LA5	6.7 (0.4)		6.6 (0.5)	
EAV3 (sef21 ⁻)	6.8 (0.7)		6.8 (0.6)	
LA5	6.7 (0.3)	<0.001	7.1 (0.2)	0.150
EAV10 (fla ⁻)	6.0 (0.2)*		6.8 (0.5)	
LA5	6.8 (0.3)		6.8 (0.3)	
EAV46 (mot ⁻)	5.9 (0.1)*	<0.001	6.3 (0.4)	0.019
LA5	6.9 (0.2)		6.9 (0.4)	
EAV66 (che ⁻)	7.0 (0.3)	0.428	7.0 (0.2)	0.623

Bacterial counts represent the average $\pm$ SD of nine samples.

*Significant difference between binding of wild-type and mutant strain.

Table 3. Comparison of adhesion of Enteritidis strains to rat ileal explants in the air interface and submerged explant culture systems

Explant culture system	Strain	Mucus present	p value	Mucus removed	p value
		Mean $\log_{10}$ cfu (SD)		Mean $\log_{10}$ cfu (SD)	
Air interface	LA5	6.9 (0.1)	<0.001	7.0 (0.5)	0.119
	EAV10 (fla ⁻)	6.0 (0.3)*		6.7 (0.2)	
	S1400	7.0 (0.2)		7.2 (0.3)	
	EAV26 (*multiple fim ⁻)	6.9 (0.4)	0.564	7.2 (0.1)	0.669
	S1400	6.8 (0.3)		7.1 (0.2)	
Submerged	EAV37 (*multiple fim ⁻ /fla ⁻)	5.9 (0.3)*	<0.001	6.7 (0.6)	0.158
	LA5	6.6 (0.2)		6.6 (0.3)	
	EAV10 (fla ⁻)	5.4 (0.4)*	<0.001	5.9 (0.3)*	<0.001
	S1400	6.4 (0.2)		6.4 (0.1)	
	EAV26 (*multiple fim ⁻)	6.5 (0.3)		6.7 (0.4)	
	S1400	6.5 (0.3)	0.191	6.7 (0.1)	0.022
	EAV37 (*multiple fim ⁻ /fla ⁻)	5.8 (0.1)*		5.8 (0.2)*	
			<0.001		<0.001

EAV, see footnotes to Table 1; bacterial counts represent the average $\pm$ SD of nine samples.

*Significant difference between binding of wild-type and mutant strain.

salmonellae to gut epithelial cells [2, 6]. However, in the present study none of the five fimbriae examined was found to be essential for attachment of bacteria to rat ileal epithelial cells in the in-vitro model used. Single fimbrial deletion mutant strains and a multiple afimbriate (*fim⁻) strain adhered to explants as efficiently as wild-type strains. This is in agreement with findings previously reported by Allen-Vercos and Woodward [12], who showed that fimbriae did not contribute to the in-vitro adherence of Enteritidis to chick gut explants. This suggests that Enteritidis may have alternative mechanisms to facilitate adhesion which do not rely upon the expression of fimbriae and most likely include the possession of active flagella.

In the small and large intestine the epithelial cells are protected by a thick mucus layer. Salmonellae penetrate this layer to attach to the underlying epithelial cells; motility and chemotaxis may be important factors in this process. When the ability of a 'smooth swimming' chemotaxis-deficient (che⁻) strain, a flagellate but non-motile (mot⁻) strain and an aflagellate (fla⁻) strain to adhere to explants both with and without an intact mucus layer was assessed (Table 2), the mot⁻ and the

fla⁻ strains bound significantly less well than wild-type strains ($p < 0.001$), whereas the che⁻ strain attached as effectively as the wild-type strain.

Overall these results suggest that motility is important for the interaction of Enteritidis with ileal explants. They clearly point to a possible role for flagella in the infection process. Moreover, the reduced ability of a mot⁻ strain to adhere to explants suggests that flagella probably promote adhesion by enabling Enteritidis to move close to the appropriate sites on the tissue for attachment to occur rather than functioning as adhesins. This is consistent with the findings of others [12, 13]. Furthermore, in the current study it would appear that the presence of the flagellar structure alone was not sufficient for full virulence; they were also required to be active, as adhesion of the mot⁻ strain was greatly reduced compared with that of the wild-type strain.

Attachment of a multiple afimbriate/aflagellate (*fim⁻/fla⁻) strain was found to be significantly less ($p < 0.001$) than that of the wild-type strain. Lockman and Curtiss [32] reported that the simultaneous loss of type 1 fimbriae and flagella resulted in a decreased

virulence of Typhimurium for BALB/c mice. However, in the present study a multiple afimbriate (*fim⁻) strain attached to the explants as efficiently as the wild-type strain, whereas a (•fim⁻/fla⁻) strain and a (fla⁻) strain were significantly less adherent. Furthermore, there were no significant differences in adherence between the •fim⁻/fla⁻ and fla⁻ strains. These findings suggest that the fimbriae and flagella of Enteritidis act independently of each other and further support the idea of an important role for flagella but not fimbriae in enabling adhesion of Enteritidis to rat ileal explants.

The *fim⁻ strain adhered as effectively as the wild-type strain ($p > 0.001$) in both the submerged and the air interface explant models (Table 3), whereas adhesion of the fla⁻ strain and the •fim⁻/fla⁻ strain was significantly reduced compared with wild-type strains ($p < 0.001$) in both models. However, whilst this reduction was observed in submerged explants both with and without an intact mucus layer, it was only apparent in the air interface model when the mucus layer was intact. Worton *et al.* [16] suggested that the early association of Typhimurium with rabbit ileal mucosa may initially involve a loose non-specific interaction, possibly with the mucus layer leading to more specific adhesin-mediated association to the epithelium. Current findings suggest that flagella may be important for the penetration of mucus in an air interface explant model. In addition, in the submerged explant model, the flagella probably facilitated attachment by enabling the bacteria to move through the liquid medium to reach the epithelial surface.

The explant culture model provides a useful method for studying the interaction of bacteria with host tissues under controlled conditions [33]; however, as the present work shows, the choice of model can influence the results obtained. Infection by salmonellae *in vivo* involves a number of inter-linked stages. The pathogen has to move through the liquid phase (gut contents) until it is in close proximity with the gut epithelium. It then has to interact with and bind to the mucus layer or underlying cells, or both, and initiate colonisation and invasion. These integrated processes can be modelled only in part in explant systems. As a result, data obtained will vary according to the exact model used. Thus, it is important to use a combination of explant model systems to study different aspects of bacterial interactions with the gut. The submerged explant is a useful model to evaluate the roles of bacterial motility and movement through the liquid phase in facilitating interactions with the gut epithelium. The air interface model avoids the initial stages of movement towards the epithelia because the inoculum is directly applied to the surface of the explant. This is probably why tissue-associated counts tend to be higher in this model. The advantage of this model is that gut epithelial and mucus layers are intact or may be modulated in a defined manner. Thus, the involvement of particular components, including mucus, in direct interactions of

the bacterium with the gut epithelium can readily be evaluated. In the present study, the majority of *Salmonella* strains exhibited a tendency for greater adhesion to washed rather than unwashed ileal tissue. This trend was not evident in all assays. The gastrointestinal mucus layer is not continuous over the surface of the gut. If the mucus layer was discontinuous on tissue samples examined, very high numbers of salmonellae could have adhered to exposed epithelial cells. This would thereby mask much of the barrier effects of the remaining mucus layer.

The air interface and submerged explant model systems thus deal with different aspects of gut–bacterial interactions and in combination can give a broad picture of the mechanisms and factors which are likely to be of importance in infection of the gut *in vivo*.

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