BACTERIAL PATHOGENICITY

Expression of type 1 fimbriae (SEF 21) of *Salmonella enterica* serotype Enteritidis in the early colonisation of the rat intestine

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The involvement of type 1 fimbriae in colonisation of the rat gastrointestinal tract *in vivo* was investigated with *Salmonella enterica* serotype Enteritidis LA5 and a mutant of LA5 denoted EAV3 unable to elaborate type 1 fimbriae (SEF 21). Rats were given a single dose of LA5 or EAV3 or a 1:1 mixture of both. LA5 was found in higher numbers in the stomach and small intestine than EAV3 at 6 h after infection with a single strain, but not after 6 days. LA5 did not out-compete EAV3 when the strains were administered together. Indeed, after 6 and 21 days, EAV3 was found in the distal small intestine and large intestine in far higher numbers than LA5. These findings suggest that SEF 21 have an important role(s) in the early stages of infection *in vivo*. However, SEF 21 expression may disadvantage the pathogen in the longer term as indicated by EAV3 out-competing LA5 in the gut at 21 days.

Introduction

An important step in infection by *Salmonella* spp. is their initial association with the surface of the gut epithelium. Salmonellae attach to a cell's surface through as yet unidentified molecules and subsequently initiate a signal transduction cascade [1,2]. The role of fimbrial adhesins may be to mediate initial attachment to the host cell surface [3]. Fimbriae of *Salmonella* spp. were first described by Duguid *et al.* [4], and since then *Salmonella enterica* serotype Enteritidis has been shown to express three distinct fimbriae on its surface, including type 1 fimbriae (SEF 21) [5], SEF 14 [6] and SEF 17 [7], and has the potential to express plasmid-encoded fimbriae [8] and long polar fimbriae [9].

The adhesion of *S. Typhimurium* to HEP-2 or HeLa cells is considered, at least in part, to be mediated by type 1 fimbriae [10]. Strains unable to express SEF 21 were less able to associate with and invade cultured epithelial cells [11] and *fim* deletion reduced adherence

of *S. Typhimurium* to HeLa cells but not to HEP-2 cells [8]. Expression of mannose-sensitive haemagglutination by *S. Typhimurium* strains corresponded well with the observed levels of adhesion to HeLa cells [8]. Studies by Duguid *et al.* [12] noted that fimbriate *S. Typhimurium* dominated faecal cultures from orally infected mice. Recently, it was established in a rat model of salmonella infection that SEF 21 were expressed *in vivo* in the small intestine [13]. However, SEF 21 do not appear to play a role in pathogenesis in a chick model [14].

McCormick *et al.* [15] showed that insertional inactivation of type 1 fimbriae in previously fimbriate *Escherichia coli* failed to diminish intestinal colonisation in individual animals. In contrast, inactivation of type 1 fimbriae has been found to abolish the ability of *E. coli* K1 to colonise the oropharynx but not the intestinal tract of the neonatal rat [16]. However, this may reflect the adhesin–receptor interaction, as the combining site of type 1 fimbrial lectin of *E. coli* has been shown to correspond to a trisaccharide of mannose [17]. FimH subunits studied to date are capable of mediating adhesion *via* trimannosyl residues, but only certain variants are capable of mediating high levels of adhesion *via* monomannosyl residues [18]. *Salmonella* type 1 fimbriae recognise a limited range of oligomannose motifs compared with *E. coli*. The reason for the lack of ascribed virulence function

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for SEF 21 may be that its individual contribution in host–pathogen interactions has thus far been difficult to define. In the present study, the *fimD* gene in *S. Enteritidis* LA5 was insertionally inactivated to investigate the role of SEF 21 in a rat model *in vivo*.

Materials and methods

Bacterial strains and growth

Salmonella Enteritidis LA5 wild-type, LA5^{str}, LA5^{nal} and *S. Enteritidis* LA5 EAV3 (*fimD*[−]) were used in this study. These strains were grown for 48 h under static conditions in nutrient broth (CM1, Oxoid) to encourage maximal expression of SEF 21 [19] for studies *in vivo* and *in vitro* [20,21].

Strain LA5 is a virulent isolate that has been well characterised in culture studies *in vitro* [11] and in chick models [20]. It exhibits normal regulation of fimbrial expression amongst *S. Enteritidis*, expressing both SEF 21 and SEF 14 [22]. Strain EAV3 is a *fimD* mutant of LA5 constructed by insertion of a tetracycline resistance gene cassette into *fimD* [23]. Strain EAV3 has been characterised in various tissue culture and explant adherence models and in an in-vivo chick model [11,24]. Naladixic acid- and streptomycin-resistant derivatives of the wild-type were constructed by plating overnight broth cultures on gradient plates separately containing naladixic acid and streptomycin. Well-defined colonies growing at relatively high concentrations of the antibiotics were picked from the antibiotic-containing plates. MICs were determined and LA5^{nal} was sensitive at naladixic acid 32 mg/L and LA5^{str} was sensitive at streptomycin 64 mg/L. Strain LA5 and its derivatives were maintained on Dorset's egg slopes at 4°C.

Microbiology: bacterial counts and identification

The tissues were weighed and homogenised in maximum recovery diluent (Oxoid; CM733). A 10-fold dilution series was prepared for the homogenates to a final dilution of 1×10^{-7} and plated on XLD agar (Oxoid; CM469). *Salmonellae* were enumerated after incubation at 37°C for 16 h and the colonies were counted [25]. The presence of *Salmonella* colonies was confirmed with specific antisera (International Murex, Berkshire) and reactions in API 20E (bioMérieux, Basingstoke). The detection limit was 500 cfu/g wet weight or /ml. Spleen and liver samples were plated out by the pour-plate method [26]; the detection limit was 10 cfu/g wet weight. For infection studies, strain EAV3 was detected on XLD agar plates containing tetracycline 25 mg/L, while LA5^{nal} and LA5^{str} were detected on plates containing naladixic acid or streptomycin, respectively.

Salmonella growth curves

To determine that the wild-type LA5 strain and its derivatives grew at the same rate, *S. Enteritidis* LA5, LA5^{str}, LA5^{nal} and EAV3 were cultured for 16 h in nutrient broth at 37°C in a water bath under static conditions. Batch cultures (10 ml) were then set up by inoculating at time 0 with 5 cfu taken from a 16-h culture of each strain and incubated at 37°C for 12 h in a water bath. Every hour, one batch culture of each strain was sampled in duplicate. Samples (1 ml) were serially diluted and then plated on to XLD agar or XLD agar with the appropriate antibiotic.

Fimbrial expression

Strains were grown statically for 48 h at 37°C in nutrient broth. Cells were centrifuged at 1300 *g* for 15 min. Cells were washed twice in phosphate-buffered saline (PBS: Oxoid; BR14a) pH 7.2, and suspended in their original volume to give *c.* 1×10^8 cfu/ml.

To detect SEF 21, mannose-sensitive haemagglutination assays (MSHA) were performed as described previously [19]. Briefly, horse erythrocytes were used as a 3% suspension in PBS, pH 7.2. A 50- μ l volume of erythrocyte suspension was mixed with equal volumes of bacterial culture and PBS on a ceramic tile, rocked for 4 min at room temperature (20°C) and the presence of SEF 21 was indicated by haemagglutination. To confirm mannose sensitivity, PBS was replaced with D-mannose (Sigma) 3% w/v in PBS.

A SEFEX kit (VLA, Addlestone, Surrey) was used to detect SEF 14 expression. Briefly, equal volumes of bacterial culture and latex reagent were mixed on a test card and rocked for 4 min at room temperature. Agglutination indicated the presence of SEF 14 [27].

Animal handling and experimental protocol

The small animal unit of the Rowett Research Institute is licensed under the UK Animals (Scientific Procedures) Act 1986 and both the animal welfare unit of the institute and the appropriate government inspectorate regulate its operation. All management and experimental procedures conducted during this study were done in strict accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986 by staff personally licensed under this act to perform such procedures.

Male Hooded Lister rats (19 days old) were housed individually in metabolism cages and fed a semi-synthetic diet containing lactalbumin 100 g/kg as the sole protein source [28]. Rats were fed *ad libitum* for 12 days. On reaching 80 ± 1 g they were fed the above diet at 6 g/rat/day for the duration of the experimental protocol. Water was available *ad libitum*. The rats were fasted for 16 h and given *c.* 1×10^8 cfu/ml of

Salmonella by gavage. Post-mortem examinations were carried out as described previously [29,30] immediately following an overdose with halothane (Merial, Harlow) and exsanguination.

Infection studies with EAV3 and LA5^{str}

Fifteen rats were separated into three groups of five each. One group was given LA5^{str}, the second group was given EAV3 and the third group was given a combined inoculum of LA5^{str} and EAV3. Post-mortem examinations were performed after 6 h.

A further 15 rats were separated into three groups of five each. One group was given LA5^{str}, the second group EAV3 and the third group were given a combined inoculum of LA5^{str} and EAV3. Rats given the single strains were killed after 6 days and those dosed with a combination of LA5^{str} and EAV3 were killed after 3 days.

Infection studies with EAV3 and LA5^{nal}

Ten rats were given both LA5^{nal} and EAV3 in a combined inoculum by gavage. Rats were killed after 6 and 21 days.

Statistical analysis

The results were subjected to one-way analysis by the Minitab computer program (Minitab, State College, PA, USA) and multiple comparisons were done by the Tukey and Welch's test with the Instat statistical package (Graphpad Software, San Diego, CA, USA).

Table 1. Bacterial strains used in this study

Strain	Fimbriae expressed	Reference
<i>S. Enteritidis</i> LA5	SEF 21, 14	20
<i>S. Enteritidis</i> LA5 ^{str}	SEF 21, 14	This study
<i>S. Enteritidis</i> LA5 ^{nal}	SEF 21, 14	This study
<i>S. Enteritidis</i> LA5 EAV3	SEF 14	14

Results

Expression of SEF 21 and growth curves

The phenotypic expression of fimbriae by *S. Enteritidis* LA5 and mutant derivatives is given in Table 1. There was no evidence that isogenic mutation or the insertion of antibiotic resistance cassettes affected the growth kinetics (Fig. 1). Strain LA5^{str} and LA5^{nal} used in the in-vivo studies had a haemagglutination titre of 64 against horse erythrocytes and this haemagglutination was inhibited by mannose. Strain EAV3 did not exhibit mannose-sensitive haemagglutination. Colonies of EAV3 were randomly selected from samples collected from infected animals and were cultured for up to 48 h to test whether EAV3 reverted to wild-type phenotype. No SEF 21 expression was detected.

Animal model experiments

Salmonella numbers in the stomach of rats dosed with LA5^{str} alone were much higher ($p < 0.05$) at 6 h than in animals given the SEF 21⁻ mutant (EAV3) by itself (Fig. 2a). However, if LA5^{str} was given in combination with EAV3, the amounts of LA5^{str} in the stomach were greatly reduced and were similar in number to EAV3 (Fig. 2a, right). The levels of EAV3 in the stomach at 6 h were similar irrespective of whether the strain was given alone or in combination with LA5^{str}.

Fig. 1. Growth curve of *S. Enteritidis* LA5 (—) and its derivatives (---, LA5 EAV3; ····, LA5^{str}; -·-·, LA5^{nal}). The strains were cultured in nutrient broth (error bars indicate SD; $n = 2$).

Fig. 2. Salmonellae (□, EAV3; ■, LA5) recovered from tissue 6 h after inoculation: (a) stomach, (b) distal jejunum, (c) ileum. Three groups ($n = 5$) were dosed orally with 10^8 cfu. One group was dosed with a 1:1 mixture of two strains, LA5^{str} (0.5 ml) and EAV3 (0.5 ml). Two groups were dosed singly with either LA5^{str} (1.0 ml) or EAV3 (1.0 ml). Data are arithmetic means. Error bars indicate SD. Letters on top of bars (a,b) represent significant differences between LA5 and EAV3 in single dose experiments. Letters x and y represent significant differences between dosing regimens ($p < 0.05$).

At 6 h, the numbers of LA5^{str} in the small intestine of rats given only this strain were significantly higher ($p < 0.05$) than in animals given EAV3 alone (Fig. 2b and c). In contrast, there were no differences in the number of LA5^{str} or EAV3 in the small intestine when the strains were given in combination to the rats (Fig. 2b and c, right). There were no significant differences ($p < 0.05$) between LA5^{str} and EAV3 numbers in the caecum and colon at 6 h (data not shown).

The numbers of salmonellae throughout the gastrointestinal tract of rats 6 days after they were given LA5^{str} alone did not differ significantly from those found in rats given EAV3 alone. LA5^{str} numbers (cfu/g) were: ileum, 3.9 (SD 0.3); caecum, 4.5 (SD 0.4); colon, 4.1 (SD 0.3). EAV3 numbers were: ileum, 4.4 (SD 0.4); caecum, 3.9 (SD 0.7); colon, 3.9 (SD 0.8).

Fig. 3. Salmonellae (□, EAV3; ■, LA5) recovered from tissue 3, 6 and 21 days after inoculation: (a) ileum, (b) caecum, (c) colon. Three groups ($n = 5$) were dosed orally with 10^8 cfu. Each group was dosed with a 1:1 mixture of two strains, LA5^{nal} (0.5 ml) and EAV3 (0.5 ml). Data are arithmetic means. Error bars indicate SD. Letters on top of bars (a,b) represent significant differences between EAV3 and LA5 in mixed dosing experiments. Letters x and y represent significant differences between time points ($p < 0.05$).

LA5^{nal} numbers in the ileum, caecum and colon were lower ($p < 0.05$) than those of EAV3 at 6 or 21 days after infection with a combined dose of the LA5 and EAV3 strains (Fig. 3). These differences were not evident at 3 days, nor were they apparent in other regions of the gut. Overall, numbers of the EAV3 strain in the intestine tended to increase ($p < 0.05$) with time, whereas those of the LA5 strain remained relatively stable (Fig. 3).

Discussion

The numbers of salmonellae found in the stomach and

small intestine of rats 6 h after oral infection with a single strain were much higher when the bacterium was capable of expression of SEF 21. However, by 6 days no differences were evident. This suggests that SEF 21 may be an important virulence factor only during the early stages of infection and that, contrary to some indications [31], they convey no selective advantage in long-term persistence of infection. Struve and Krogfelt [32] have shown that the introduction of *E. coli* into the mouse urinary tract leads to markedly enhanced expression of type 1 fimbriae. If type 1 fimbriae have a transient role during the early stages of infection, this may explain why deletion of type 1 fimbriae from *E. coli* [16] or *S. Enteritidis* [14] failed to significantly alter the severity of infection in young rats or chicks. Studies with *E. coli* suggest that type 1 fimbriae can be down-regulated in long-term infection in the rat [33]. This would be consistent with the findings of the present study in which SEF 21 did not facilitate long-term persistence by *Salmonella* strains.

Few studies have examined the factors that influence type 1 expression *in vivo*; however, phase variation has been shown to occur during infection in animal models [34]. SEF 21 are not readily expressed in conditions of pH similar to those found in the rat stomach [35]. Thus, in the stomach it might have been expected that SEF 21 expression would have been low and that the LA5 and EAV3 strains would survive to similar extents *in vivo*. However, this was not the case. In rats given a single strain, higher numbers of LA5 than EAV3 were recovered from the stomach at 6 h. This suggests that non-fimbriate strains may be cleared more rapidly from the stomach. Although the mechanisms involved are unknown, the ability of salmonellae to express SEF 21 appears to facilitate its survival in the stomach during the early stages of infection. Hendrickson *et al.* [36] found increased type 1 fimbrial expression following catabolic stress. Furthermore, Clegg *et al.* [37] showed that the *fimA* promoter region of *S. Typhimurium* was always oriented in the direction that might allow *fimA* transcription regardless of the fimbrial phase or environmental conditions *in vitro*. It is possible that the predisposition of the bacteria for expression of fimbriae ensures type 1 fimbriae are expressed *in vivo* even in the harsh conditions of the stomach.

However, possession of SEF 21 did not convey any selective benefit to LA5 when it was co-administered to rats with EAV3. This is in agreement with studies in mice where, in competitive experiments, *E. coli* F-18 phase locked 'on' for expression of type 1 fimbriae was recovered in lower numbers than the non-locked 'on' strain. [38]. In addition, May *et al.* [39] have shown that type 1 fimbrial expression can lead to diminution of the number of *E. coli* in peritoneal infections of rats and that a non-fimbriate insertional mutant can cause higher mortality in peritoneal infection. Similarly, Lockman and Curtiss [40] have shown that type 1

fimbriae of *S. Typhimurium* were not virulence factors in the mouse model and might in fact present a drawback to the pathogen. Much of the data on type 1 fimbriae of *S. Typhimurium* in mice [12,41] and of *E. coli* in rats [42], suggests a role in dissemination rather than colonisation of intestinal sites. Indeed, in the present study, long-term expression of SEF 21 appeared to be disadvantageous. Thus, at 6 and 21 days, EAV3 persisted in the gastrointestinal tract in much higher numbers than LA5. In mice, Aslanzadeh and Paulissen [43] showed that both type 1 and type 3 fimbriae of *S. Enteritidis* contributed to virulence in mice. In chickens, Thiagarajan *et al.* [44] showed a role for fimbrial proteins in caecal colonisation by *S. Enteritidis*. In contrast, Rajashekara *et al.* [45] did not show any contribution by SEF 21 to the infection of chickens by *S. Enteritidis*. Similarly, other studies have shown that flagella and not fimbriae are important in the caecal colonisation of chickens [14, 24, 46] by *S. Enteritidis*. Hence, colonisation of the gut by salmonellae is highly dependent on the host species and in particular cases is likely to involve the integrated action of a number of fimbriae and virulence factors. Whilst failure to express SEF 21 appears to disadvantage the mutant strain to a limited extent in the short-term, it may be insufficient to allow a SEF 21-expressing strain to out-compete a mutant that is capable of expressing other virulence factors with the exception of SEF 21.

Type 1 fimbriate *S. Typhimurium* appear to be cleared from the blood more rapidly than non-fimbriate organisms; they seem to be trapped selectively by the endothelial and Kupffer cells of the liver [47]. Vazquez-Torres *et al.* [48] have shown direct evidence that systemic spread of salmonellae depends on CD18-expressing cells, strongly supporting the hypothesis that these bacteria can be carried within macrophages. Furthermore, fimbriate strains may be more susceptible to non-opsonised phagocytosis [49], i.e., salmonellae expressing fimbrial adhesins may bind to sugars on phagocytic cells, resulting in metabolic activation of the phagocytes. This may mean that in the present study the fimbriate strain was preferentially removed from the gastrointestinal tract, allowing the mutant strain to become predominant in the long term. These studies indicate that SEF 21 may play a more active role in salmonella pathogenesis than previously thought. Further studies are needed to define this role.

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