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The role of SEF14 and SEF17 fimbriae in the adherence of *Salmonella enterica* serotype Enteritidis to inanimate surfaces

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To gain an understanding of the role of fimbriae and flagella in the adherence of *Salmonella enterica* serotype Enteritidis to inanimate surfaces, the extent of adherence of viable wild-type strains to a polystyrene microtitration plate was determined by a crystal violet staining assay. Elaboration of surface antigens by adherent bacteria was assayed by fimbriae- and flagella-specific ELISAs. Wild-type Enteritidis strains adhered well at 37°C and 25°C when grown in microtitration wells in Colonisation Factor Antigen broth, but not in other media tested. At 37°C, adherent bacteria elaborated copious quantities of SEF14 fimbrial antigen, whereas at 25°C adherent bacteria elaborated copious quantities of SEF17 fimbrial antigen. Non-fimbriate and non-flagellate knock-out mutant strains were also assessed in the adherence assay. Mutant strains unable to elaborate SEF14 and SEF17 fimbriae adhered poorly at 37°C and 25°C, respectively, but adherence was not abolished. Non-motile mutant strains showed reduced adherence whilst type-1, PEF and LPF fimbriae appeared not to contribute to adherence in this assay. These data indicate that SEF17 and SEF14 fimbriae mediate bacterial cell aggregation on inanimate surfaces under appropriate growth conditions.

Introduction

Salmonella enterica serotype Enteritidis is a major cause of food-borne infection of man, with poultry and poultry products cited as common sources [1, 2]. The occurrence of human infection indicates that serotype Enteritidis is environmentally robust and readily transmitted through the food chain. Contamination of animate and inanimate surfaces is probably an important factor in the transmissibility of this and other food-borne pathogens [3, 4]. Bacterial surface hydrophobicity, charge, cell density and exopolysaccharides have been cited as important factors for adherence to surfaces; the role of other surface structures, such as flagella and fimbriae, has been considered equivocal [5, 6]. Indeed, conflicting evidence regarding flagella and type-1 fimbriae has arisen from studies of the adherence of serotype Typhimurium strains to chicken skin [7–9]. Recent reports indicate that type-1 fimbriae and flagella mediate non-specific adherence of Typhimurium to mineral particles and glass [10, 11]. SEF17 and type-1 fimbriae also contribute to the adherence of Enteritidis strains to stainless steel and Teflon [12]. Adherence to glass has been associated with the virulence potential of Enteritidis strains, although the contribution of fimbriae and flagella in that model was unclear [13]. SEF17 is an orthologue of the curli fimbriae of *Escherichia coli*. The elegant studies of Vidal *et al*. [14] showed that curli fimbriae were involved in adherence to glass and polystyrene, a finding which supports evidence for their role in adherence to inanimate surfaces.

Serotype Typhimurium possesses the ability to elaborate at least four kinds of fimbriae including type 1, plasmid encoded (PEF), long polar (LPF) and a curli orthologue. Each of these has been implicated in pathogenesis in mouse and chick models [15–17]. In addition, Enteritidis elaborates SEF14 fimbriae which are restricted to a number of serotypes in O serogroup D, notably serotype Dublin [18–20]. However, the role of Enteritidis fimbriae in poultry infection is equivocal [21, 22], although SEF14 fimbriae may contribute to

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tropism towards reproductive tissues [23]. Detailed analysis of the environmental signals required for the expression of Enteritidis fimbriae were reported previously [24–27]. SEF14 fimbriae were elaborated at 37°C, but not at ambient temperatures, when grown on media of low osmolarity [26] and their elaboration was enhanced by growth on agar surfaces. That observation prompted the hypothesis that surface contact was an environmental signal for SEF14 regulation [27]. SEF17 fimbriae were elaborated copiously at ambient temperatures, but not at 37°C, when grown on low osmotic, and nutritionally poor, media. SEF17 fimbriae have been implicated in adherence to inanimate surfaces [12].

Earlier research into the role of flagella and fimbriae in the biology of serotype Enteritidis has generated defined non-fimbriate and non-flagellate mutant strains, as well as immunological reagents specific for these surface antigens [18, 19, 21, 22, 24–27]. The use of these resources to study the roles of fimbriae and flagella of Enteritidis in adherence to inanimate surfaces is reported in this study.

Materials and methods

Bacterial strains

Enteritidis strains are listed in Table 1. Strains 27655R and 27655S were kind gifts from Professor T. Wadstrom, Sweden and wild-type strains were from the Reference Laboratories at VLA (Weybridge) and FMRU, PHLS, Exeter. Wild-type strains of Enteritidis were stored on Dorset's egg slopes at ambient temperature. Mutant strains of Enteritidis designated EAV and strain B214 were constructed previously [19, 21], and strains designated KAS were constructed in this study. Mutant strains were stored frozen at −70°C in Luria-Bertani (LB) broth supplemented with glycerol 15% w/v.

Media

Media for the culture of wild-type and mutant strains of Enteritidis were Colonisation Factor Antigen (CFA) broth, Heart Infusion Broth (HIB), Luria-Bertani (LB) broth, LB supplemented with glucose 0.25% w/v (LBG), and M9 glucose 0.25% w/v minimal salts medium (MM) broth, or solidified with agar 1.2% w/v as appropriate, with growth overnight at 25°C and 37°C. LBG agar was used for the selection of transductants. Antibiotics were added as required at appropriate concentrations [19, 21].

Culture conditions for adherence assays

Seed cultures were prepared by picking single, well-isolated colonies from fresh LB agar streak plates into 10-ml volumes of broth. The cultures were incubated overnight, statically at 25°C or 37°C as required, and were diluted in the same pre-warmed medium to give an optical density of 0.2 at 550 nm. 30-μl volumes were added to each of eight wells in a 96-well microtitration plate. A further 100 μl of the same

Table 1. Wild type and mutant strains of Enteritidis used in this study

Strain	Mutant designation	Genotype or comment
S1400		Wild-type PT4
LA5		Wild-type PT4
27655R		Wild-type, constitutive for SEF17
27655S		Wild-type, SEF17 non-expression
I		Wild-type non-invasive PT4
E		Wild-type invasive PT4
C6B		Wild-type invasive PT4
10360/91		Wild-type PT4
278/95		Wild-type PT4
842/95		Wild-type PT4
486/86		Wild-type PT4
483/95		Wild-type PT4
LA5	B214	*sefA*::kan
LA5	EAV3	*fim*D::tet
LA5	EAV6	*pef*C::zeo
LA5	EAV8	*lpf*C::trim
LA5	EAV10	*fli*C::cam
LA5	EAV12	*agf*A::bla
LA5	EAV46	*mot*AB::cam
S1400	EAV13	*sef*A::kan
27655R	EAV28	*sef*A::kan
27655S	KAS12	*sef*A::kan
LA5	EAV38	*sef*A::kan *fim*D::tet *agf*A::bla *lpf*C::trim *pef*C::zeo *fli*C::cam
LA5	EAV71	*fim*D::tet *sef*A::kan *agf*A::bla *pef*C::zeo *lpf*C::trim *mot*AB::cam
27655R	KAS13	*agfA*::bla *sefA*::kan
LA5	KAS14	*agfA*::bla *sefA*::kan
S1400	KAS15	*agfA*::bla *sefA*::kan

medium, pre-warmed to 25°C or 37°C as required, were added to each inoculated well. Loaded microtitration plates were incubated statically at 25°C or 37°C for 24 or 48 h, as required. Three different kinds of 96-well microtitration plates were used: Falcon vinyl plates, Nunclone polystyrene flat-bottomed well plates and Nunc polystyrene round-bottomed well plates. Adherence of bacteria was assessed by crystal violet binding and protein assays (see below).

Nunclone polystyrene 24-well plates were used for cell recovery and ELISA tests (see below) of adherent bacteria. Essentially the procedures were as described above except that 100-μl of diluted seed culture were inoculated into wells containing 1 ml of pre-warmed medium. After incubation for adherence, the supernate was removed by aspiration and the wells were washed three times with phosphate-buffered saline (PBS). PBS (0.5 ml) was added to each well and adherent bacteria were removed from the well surfaces by generating a vortex with a magnetic stirrer. The cell density of the resultant cell suspension was determined by optical density measurements at 500 nm and samples were assayed for fimbriae and flagella by ELISA.

Crystal violet binding assays

To assess the extent of bacterial adherence to the well surfaces, a crystal violet binding assay was done, essentially following the method of Pratt and Kolter [28]. Briefly, after incubation, supernate was aspirated and either discarded or stored frozen for subsequent use in ELISA tests (see below) where appropriate. Loosely adherent bacteria were removed by three washes with PBS (pH 7.2) and plates were inverted to dry. Each well was filled with 130 μl of crystal violet 1% w/v solution and incubated at room temperature for 30 min. Unbound crystal violet was removed by washing the plates under tap water and plates were inverted to dry. Alcohol: acetone (130 μl; 70:30 v:v) was added to each well to release cell-bound crystal violet and after 10 min at room temperature, crystal violet concentration in solution was determined by optical density readings at 550 nm.

Fimbriae- and flagella-specific ELISAs

Assays specific for SEF14, SEF17 and SEF21 fimbriae and for flagellin were performed as described previously [25–28].

P22 transduction

Defined non-fimbriate mutants in specific Enteritidis strains were constructed by P22 transduction of the appropriate genetic marker by methods described previously [21, 22]. Briefly, donor P22 was grown on Enteritidis S1400 strain EAV38 (Table 1) and in each transduction experiment selection was made for the antibiotic resistance encoded by the cassette used to inactivate insertionally the target fimbrial gene. The genotype of each transductant was confirmed by Southern hybridisation and appropriate phenotypic tests [21, 22].

Results

Development of the adherence assay with wild-type Enteritidis strain S1400

A well characterised wild-type Enteritidis strain (S1400) was used to establish if Enteritidis adhered to inanimate surfaces. In preliminary experiments, culture medium, temperature and time for association between bacteria and inanimate surface of the microtitration plate were varied. Adherence was observed both visually, in the form of microcolonies on the well bottom and at the interface between air and growth medium, and by crystal violet-binding assay when bacterial growth was in CFA broth at 25°C and 37°C. The crystal violet-binding readings at OD_{550} after incubation for 48 h at 25°C and 37°C were 0.24 and 0.44, respectively, in Falcon vinyl plates, 0.20 and 0.63 in Nunclone polystyrene flat-bottomed well plates, and 0.20 and 0.50 in Nunc polystyrene round-bottomed well plates. In repeat experiments, similar OD_{550} readings were obtained after incubation for 24 h with the earliest adherence detected at 6 h ($OD_{550} = 0.12$). However, growth in HIB, LB, LBG and MM at either 25°C or 37°C did not support adherence to the well surface of any of the three kinds of plate used.

Adherence and fimbrial antigen elaboration of wild-type strains of Enteritidis

To test whether the findings with strain S1400 were representative of other Enteritidis isolates, 12 wild-type strains including S1400 were assessed for adherence to Nunclone polystyrene flat-bottomed well plates by the crystal violet binding assay. All strains adhered after growth in CFA broth at 37°C for 48 h (OD_{550} 0.42–1.24), whereas nine of 12 strains adhered after growth in CFA broth at 25°C for 48 h (OD_{550} 0.24–1.55) (Table 2). Similar adherence values were achieved after 24 h. Generally, cultures grown in HIB did not adhere ($OD_{550} < 0.05$), except that two strains (27655R and 842/95) adhered at both 25°C and 37°C (OD_{550} 0.58 and 0.24 for strain 27655R; 0.81 and 1.55 for strain 842/95).

To test if there was any correlation between extent of adherence and elaboration of fimbrial antigens by Enteritidis, ELISAs specific for SEF14, SEF17 and SEF21 (type-1) fimbriae and flagellin were used to quantify the elaboration of these antigens from: bacteria grown overnight and used to seed the adherence assay plates; planktonic cells remaining in the well after incubation for 48 h; and cells recovered from the well surface (Table 2).

Table 2. Adherence of wild-type strains of Enteritidis to polystyrene and elaboration of fimbriae and flagella

Strain no.	Growth temp (°C)	Crystal violet binding*	Elaboration of surface antigens† by											
			Seed cultures				Cells in suspension				Cells bound to surface			
			SEF14	SEF17	SEF21	Flag	SEF14	SEF17	SEF21	Flag	SEF14	SEF17	SEF21	Flag
S1400	25	0.24	0.06	0.20	1.34	2.06	0.00	0.06	1.21	2.00	0.02	0.03	0.06	0.27
	37	0.44	1.60	0.08	1.04	1.95	2.41	0.26	0.38	1.42	1.79	0.04	0.30	0.21
LA5	25	1.44	0.03	1.04	0.12	1.46	0.00	0.29	0.50	1.36	0.06	0.28	0.03	0.49
	37	0.65	1.60	0.68	0.33	1.69	1.85	0.14	0.15	1.07	1.68	0.00	0.05	0.18
27655R	25	1.11	0.00	1.93	1.53	1.78	0.01	0.46	0.11	0.41	0.02	0.55	0.09	0.39
	37	0.78	0.00	1.81	1.39	1.61	0.00	0.04	0.77	0.10	0.18	0.68	0.43	0.30
27655S	25	0.05	0.02	0.07	0.10	1.09	0.01	0.00	0.06	0.79	0.01	0.14	0.01	0.17
	37	0.87	1.53	0.01	0.11	0.77	2.05	0.02	0.02	0.45	1.30	0.00	0.02	0.05
I	25	0.06	0.02	0.07	1.01	1.90	0.10	0.05	1.23	2.01	0.00	0.10	0.09	0.16
	37	0.42	1.68	0.01	1.37	1.82	2.45	0.03	0.40	0.95	1.76	0.00	0.36	0.23
E	25	1.39	0.03	0.99	0.16	1.28	0.00	0.36	0.07	0.60	0.00	0.00	0.00	0.32
	37	0.65	1.67	0.70	0.36	1.67	1.85	0.00	0.29	0.71	1.01	0.00	0.00	0.07
C6B	25	1.11	0.04	1.16	0.14	1.00	0.01	0.19	0.03	0.34	0.00	0.05	0.01	0.54
	37	0.58	1.46	0.34	0.56	1.81	2.26	0.04	0.22	1.00	1.08	0.00	0.06	0.29
10360/91	25	0.48	0.01	0.73	0.39	1.96	0.01	0.42	0.87	1.80	0.02	0.31	0.03	0.50
	37	0.89	1.48	0.51	0.62	1.91	2.09	0.02	0.51	1.50	1.08	0.00	0.14	0.83
278/95	25	0.04	0.01	0.17	0.41	1.81	0.01	0.22	0.88	2.09	0.01	0.03	0.02	0.20
	37	0.49	1.76	0.25	0.43	1.84	2.31	0.01	0.15	0.76	1.78	0.00	0.04	0.16
842/95	25	1.55	0.02	1.11	0.17	1.53	0.02	0.24	0.27	0.38	0.01	0.04	0.01	1.30
	37	0.81	1.72	0.85	0.31	1.73	2.12	2.01	0.15	0.79	0.54	0.00	0.01	0.10
486/86	25	0.03	0.03	0.82	0.52	1.93	0.02	0.79	0.99	1.94	0.01	0.04	0.03	0.38
	37	1.24	1.48	0.82	1.08	1.81	2.36	0.14	0.52	1.95	0.75	0.24	0.78	0.28
483/95	25	0.53	0.02	1.22	0.28	1.98	0.02	0.78	1.94	0.80	0.00	0.01	0.02	0.31
	37	0.58	1.06	0.83	0.66	2.02	2.00	0.06	0.52	1.83	1.57	0.10	0.49	0.17

*Adherence assessed by crystal violet binding assay (see *Materials and methods*).
†Specific ELISAs used to detect fimbriae (SEF14, SEF17 and SEF21) and flagella (Flag) (see *Materials and methods*).

At 37°C, most Enteritidis strains from the seed and planktonic well cultures elaborated copious amounts of SEF14 fimbriae and flagella, but SEF17 and SEF21 fimbriae were not detected. Bacteria recovered from the polystyrene well surfaces elaborated both SEF14 fimbriae and flagella, although flagella were elaborated at reduced levels compared with seed and planktonic cells. SEF17 and SEF21 fimbriae were not detected. Both strain 27655R and strain 842/95 also elaborated high levels of SEF17 fimbriae.

At 25°C, most Enteritidis strains from the seed and planktonic well cultures produced SEF17 and SEF21 fimbriae and flagella, whereas none of the 12 strains elaborated SEF14 fimbriae. Generally, the extent of elaboration of all four surface antigens expressed by bacteria bound to the wells was lower than for seed and planktonic cells. Strain 27655R was unusual in that it produced large amounts of SEF17 fimbriae.

Adherence of mutant strains of Enteritidis defective for the elaboration of fimbriae

Having demonstrated the presence of fimbriae and flagella on adherent bacteria, tests were done to establish if these surface antigens were essential for adherence to inanimate surfaces. Thus, mutant strains of Enteritidis S1400 and LA5 unable to elaborate fimbriae and flagella (Table 1) were assayed for adherence after incubation in CFA broth at both 25°C and 37°C.

In assays performed at 37°C, mutant strains unable to elaborate SEF14 fimbriae showed reduced adherence (Fig. 1) whereas mutant strains unable to elaborate PEF, LPF, SEF17 or SEF21 fimbriae adhered as well as the progenitor wild-type strains. A fimbriate strain which formed paralysed flagella (EAV46) adhered, but less well than strains that were motile and elaborated SEF14 fimbriae. Differences in adherence between samplings after incubation for 24 and 48 h were not detected (data not shown).

Strains 27655R and 27655S were adherent irrespective of the culture conditions, unlike the majority of Enteritidis wild-type strains tested in this study. Previous studies had shown that strain 27655R was constitutive for the elaboration of SEF17 fimbriae whereas strain 27655S, a genetically undefined derivative of strain 27655R, was unable to elaborate SEF17 fimbriae under any conditions [23, 24, 26]. Thus, to confirm that SEF14 fimbriae were essential for adherence in strains 27655R and 26755S, assays were performed as described above with *sefA*::kan transductants (Table 1). Strain 27655S adhered whereas strain KAS12, unable to elaborate SEF14, showed reduced adherence (Fig. 2). Strains 27655R and EAV28, unable to elaborate SEF14 fimbriae, adhered as shown by crystal violet binding values of OD_{550} 1.95 and 1.03 at 24 h, respectively (Fig. 2).

In assays performed at 25°C, those mutant strains unable to elaborate SEF17 fimbriae did not adhere (Fig. 1), whereas mutant strains unable to elaborate PEF, LPF, SEF14 or SEF21 fimbriae adhered as well as the progenitor wild-type strains. A fimbriate strain that formed paralysed flagella (EAV46) adhered, but less well than strains that were motile and elaborated SEF17 fimbriae. Differences in the adherence between samplings after incubation for 24 and 48 h were not detected (data not shown). Strain 27655R adhered whereas KAS13, unable to elaborate both SEF14 and SEF17 fimbriae, did not (Fig. 2).

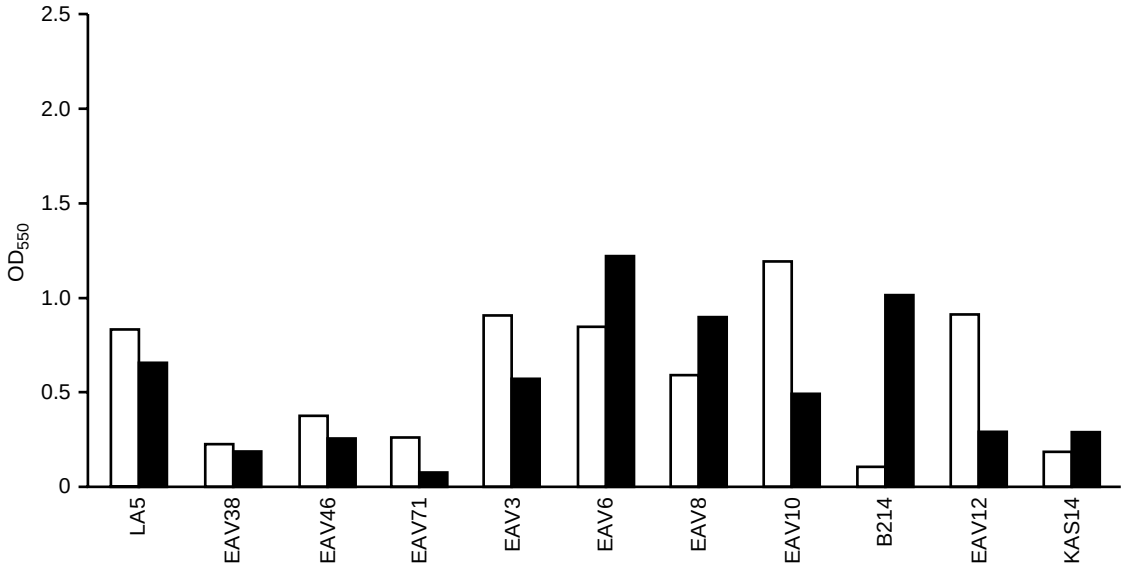

Fig. 1. Adherence of strain LA5 and isogenic non-fimbriate and non-flagellate mutant strains of Enteritidis to polystyrene. □, 37°C; ■, 25°C.

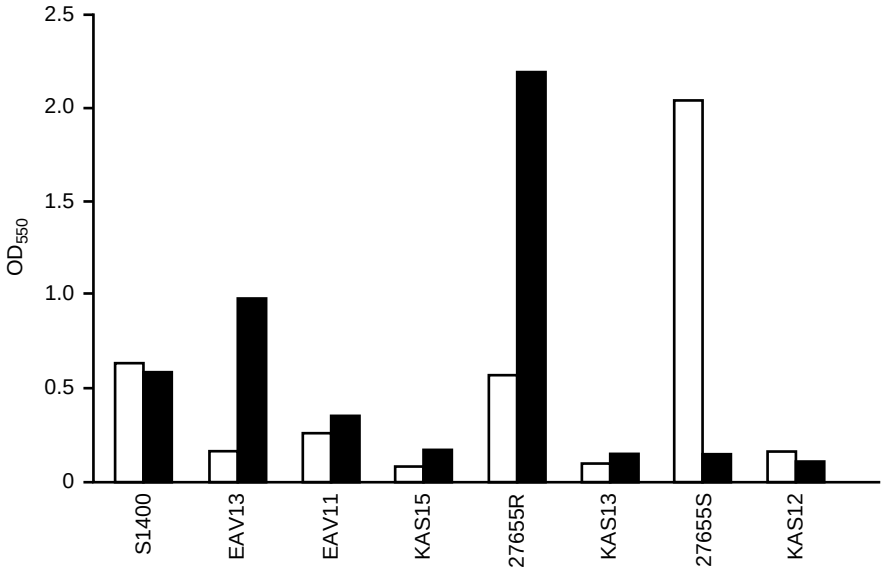

Fig. 2. Adherence of wild-type Enteritidis strains and isogenic SEF14 and SEF17 non-fimbriate mutant strains to polystyrene. □, 37°C; ■, 25°C.

Discussion

Enteriditis strains adhere to inanimate surfaces such as vinyl and polystyrene when grown in CFA broth, a low osmotic, nutrient-poor medium known to support elaboration of SEF14 fimbriae at 37°C and SEF17 fimbriae at 25°C by Enteritidis [24–27]. Mutant strains of Enteritidis defective for the elaboration of SEF14 and SEF17 fimbriae showed reduced adherence in the order of 90% compared with the isogenic progenitor wild-type strains. Here was evidence that these fimbriae played a role in adherence to inanimate surfaces. However, adherence of SEF14 and SEF17 non-fimbriate mutants was not abolished and it was possible that these fimbriae were neither the sole nor the primary mechanism for adherence. SEF17 fimbriae are hydrophobic and mediate bacterial aggregation [29], whereas SEF14 fimbriae are elaborated upon contact with surfaces [18, 25, 27]. It is possible that the adherence demonstrated in the assays used in this study may be the result of bacterial aggregation after initial adherence to the surface.

HIB supports elaboration of SEF21 (type-1) fimbriae of Enteritidis [24], but was not associated with adherence, a finding in contrast to the report of Stenstrom and Kjelleberg [11]. These authors used radio-isotopically labelled bacterial cells prepared under various conditions in sensitive adherence assays to demonstrate that surface charge, to which the presence of type-1 fimbriae contributed, was a key factor in adherence. The differences in findings regarding type-1 fimbriae may relate to the sensitivity of the assays. No role for PEF or LPF fimbriae in adherence was demonstrated, whereas motility most probably enhanced the opportunity for cell-to-cell contact, as suggested by others [10, 28, 30].

Of the many serotypes of *S. enterica*, Enteritidis is unique in possessing the ability to elaborate SEF14 and SEF17 fimbriae, both of which contribute to adherence although under different environmental conditions. The ongoing epidemic of Enteritidis may relate in part to the success of the bacterium passing down the food chain with adherence to inanimate surfaces contributing to persistence as well as communicability. Enteritidis strains may adhere to surfaces such as eggs, food-processing equipment, animal carcasses and farm-yard implements over a wider range of environmental conditions than other *Salmonella* spp. Interestingly, Dhir and Dodd [31] showed that suspended and surface-attached Enteritidis were equally sensitive to biocide treatment. However, Austin *et al*. [12] suggested that SEF17 fimbriae present on adherent but killed bacteria on disinfected surfaces may act as sites for rapid recolonisation due to the aggregative properties of the fimbriae. It is noteworthy that strains 27655R and 27655S were first described as possessing atypical surface properties and associated with persistence in a dry environment [32]. Also, recent reports have suggested that other novel surface structures were elaborated by Enteritidis which invaded and contaminated the surfaces of eggs [33–35]. The role of fimbriae and other surface antigens in environmental persistence and as potential targets for intervention strategies in the control of the current Enteritidis epidemic is worthy of further study.

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