BACTERIAL PATHOGENICITY

Effect of pH, temperature and surface contact on the elaboration of fimbriae and flagella by *Salmonella* serotype Enteritidis

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Survival of enteric pathogens exposed to various environmental stresses depends upon a number of protective responses, some of which are associated with induction of virulence determinants. Flagella and fimbriae are putative virulence determinants of *Salmonella* spp. and ELISAs specific for the detection of flagella and SEF21, SEF14 and SEF17 fimbriae were used to assess the effect of temperature and pH upon their elaboration by isolates of *Salmonella* serotype Enteritidis in planktonic growth and on the surface of two-dimensional gradient agar plates. For three phage type 4 isolates of Enteritidis of comparative clinical provenance, similar phenotypes for the elaboration of these surface antigens were observed. SEF14 fimbriae were elaborated in planktonic growth at 37°C, but not 20°C, at pH 4.77 and above but not at pH 4.04; whereas on agar gradient plates SEF14 fimbriae were elaborated poorly but with best yields at pH 4.04. SEF17 fimbriae were elaborated in planktonic growth at 20°C, but not at 37°C, at pH 6.18 and above but not at pH 5.09 or below; whereas on agar gradient plates SEF17 fimbriae were elaborated well even at pH 4.65. SEF21 fimbriae were expressed very poorly under all conditions tested. Planktonic growth at 37°C induced least flagella whereas growth at 20°C, and particularly surface growth at lower pH values, induced a 'hyper-flagellate' phenotype. Single colonies allowed to form on gradient agar plates were shown to generate different colonial morphologies which were dependent on initial pH. These results demonstrate that the physicochemical environment is an important determinant of bacterial response, especially the induction of putative virulence factors.

Introduction

Salmonella serotype Enteritidis phage type (PT) 4 remains prevalent as a major cause of foodborne infections in the UK and Europe with poultry and poultry products, including eggs and processed foods containing egg, cited as common sources [1, 2]. Enteritidis is invasive in poultry, with contamination of muscle and reproductive tissues documented [3, 4], and persists in large numbers in the gut [5, 6], which contributes to surface contamination of eggs upon passage through the cloaca and to surface contamination of meat during evisceration [7, 8]. The fact that human infections arise indicates that Enteritidis is environmentally tolerant and survives within these various food environments. It has been established that many stress-related responses and virulence determinants are co-regulated with, for example, RpoS, the so-called stationary phase RNA polymerase sigma factor [9, 10], associated with responses to nutrient depletion, acidic pH and elevated temperatures [11] and the regulation of the virulence plasmid *spv* genes in *Salmonella* [12–14]. The question arises as to whether other putative virulence factors are up-regulated during stress, or more specifically, during survival or growth within foods.

The flagella and fimbriae of *S.* serotype Typhimurium are regarded as virulence determinants which play a role in initial colonisation of gut epithelia [15] and invasion [16–18]. Whilst it is well known that type 1 fimbriae mediate mannose-sensitive haemagglutination (MSHA) and play a role in gut epithelium adherence [19–21], other fimbriae, such as the long polar fimbriae (LPF) and the plasmid-encoded fimbriae (PEF), enable adherence to M cells in the Peyer's

patches and cause fluid accumulation in ligated gut loops, respectively, in a mouse model [22, 23].

Enteritidis elaborates SEF21 fimbriae, which mediate MSHA [24, 25], and SEF17 [26, 27], an analogue of the 'curli' fimbriae of *Escherichia coli* [28] which mediate binding of fibronectin [29], plasminogen [30] and human contact factors [31]. Similar properties have been demonstrated for SEF17 [32], which may support the hypothesis that this kind of fimbria may be important in the pathogenesis of salmonella infection [33]. Enteritidis elaborates SEF14 fimbriae that are unique to a subset of group D *Salmonella* [34–37], although their role in pathogenesis remains equivocal [38]. Enteritidis elaborates PEF fimbriae, which are expressed during infection in chickens [39], but their role in pathogenesis is in doubt because plasmid-free variants remain fully virulent in chickens [40]. Enteritidis has the genetic potential to elaborate LPF, although the fimbrial structure has not yet been formally identified [22, 23, 41].

The role of Enteritidis fimbriae in pathogenesis in chickens has been studied in this laboratory. Of interest is the potential up-regulation of these putative virulence determinants during survival and growth, particularly within foods and other environments. The availability of monoclonal antibodies (MAbs) specific to flagella and SEF21, SEF14 and SEF17 fimbriae [25, 37, 42; and Sojka et al. unpublished observations] has enabled studies of their elaboration under different environmental conditions. The initial findings of the studies are reported here.

Materials and methods

Organisms and inocula

Salmonella Enteritidis strains S1400 and LA5, wild-type strains isolated from natural chicken infections, have been characterised in this laboratory [42, 43] and are considered similar to strain E [44, 45]. Strains E and I, kindly provided by Professor T. J. Humphrey (PHLS Food Microbiology Research Unit, Exeter), represent a virulent, environmentally tolerant strain and an avirulent, environmentally sensitive strain, respectively [44, 45]. In consultation with Professors T. J. Humphrey and G. S. A. B. Stewart, we have confirmed that strain I possesses a deletion of c. 400 bp in the *rpo*S allele (these findings will be reported elsewhere). Enteritidis strain 27655R [46], originally isolated as an 'atypical' rough strain, and a naturally occurring derivative strain 27655S [46] were kindly provided by Professor T. Wadstrom (Lund University, Sweden). All cultures were maintained on Dorset's egg slopes at 4°C. All inocula were prepared by direct inoculation of Trypticase Soy Broth (TSB; Becton Dickinson, Cockeysville, MD, USA) from stock cultures, followed by incubation at 25°C for 24 h and transfer of 100 μl to fresh TSB with incubation for a further 24 h at 20°C.

Broth culture medium

Ten-ml volumes of colonisation factor antigen (CFA) broth comprising (w/v) casamino acids 1.0%, yeast extract 0.15%, $MgSO_4$ 0.005% and $MnCl_2$ 0.0005% were adjusted to the appropriate pH with 1 M HCl or 1 M NaOH, filter-sterilised and inoculated with 100 μl of culture. These were incubated at 37°C or 20°C for 24 or 48 h.

Construction of neutral-pH4 gradient plates

Gradient plates were prepared according to the methods of Thomas et al. [47] and were constructed as follows: water agar, which comprised agar (Difco) 7.5 g suspended in 242 ml of distilled water was sterilised by autoclaving (121°C for 15 min) and cooled to 50°C. Double-strength CFA broth (250 ml), previously sterilised and held at 50°C, was mixed with the water agar and 8 ml of sterile 1 M HCl were added to obtain a pH value of 4.0. Molten culture medium in 40-ml volumes were poured into 12 cm^2 petri dishes (Bibby Sterilin, Stone) raised at one end by 6 mm and allowed to set. Molten CFA agar was prepared as above, but with sterile 1 M NaOH added to obtain a pH value of 7.0 and 40-ml volumes were poured over the acid gradient layer but with the plates level. When set, the plates were left for 24 h at 20°C to allow the pH gradients to form, and were then flooded with 10 ml of culture medium diluted to 10^8 cfu/ml in quarter-strength Ringer's solution (Oxoid) to generate confluent growth upon incubation. The pH gradient of an uninoculated plate was determined before inoculation with a double-junction, flat-ended pH electrode (BDH, Leicester) connected to a Corning 240 pH meter (BDH).

ELISA assays for surface antigens

The ELISA assays were based on those described previously [25, 37, 42]. Briefly, a cork borer (10-mm diameter), sterilised by dipping in ethanol 70% w/v and flaming, was used to extract two cores of medium. Bacteria adhering to the surface of the bores were loosened by vortex mixing in 2 ml of carbonate buffer, pH 9.6, and the resulting homogenate was adjusted to an optical density at 540 nm (OD_{540}) of 1.20. Broth cultures were pelleted by centrifugation at 4800 g and resuspended in carbonate buffer to an OD_{540} of 1.2. Samples of culture homogenate (100 μl) were used to coat microtitration plates (Polysorb, Nunc, Glasgow). Microtitration plates were incubated at 37°C for 24 h then washed twice with 0.1 M phosphate-buffered saline (pH 7.2) containing Tween 20 0.05% v/v. Each well was blocked with 200 μl of skimmed milk solution 3% w/v, then treated as appropriate, in separate experiments, with mouse-derived MAbs specific for SEF14, SEF17, SEF21 fimbriae and for flagella. Bound MAb was detected by goat anti-mouse horseradish peroxidase-conjugated antibody (Sigma) developed with tetramethylbenzidine substrate (TMB; Cambridge Veterinary Sciences, Cambridge) at 20°C

for 5 min and reactions were stopped with 100 μl of sulphuric acid 10% v/v. OD_{450} measurements were noted. Triplicate samples were processed for each variable tested and control wells, which contained no primary antibody, were used as blanks.

Results

Elaboration of fimbriae and flagella

Six Enteritidis strains were tested for elaboration of flagella, type 1 (SEF21), SEF14 and SEF17 fimbriae in the growth conditions described above; the results are summarised in Table 1.

At 37°C, strains LA5, S1400, E, I and 27655S showed consistent detectable changes in the elaboration of flagella and SEF14 fimbriae that were related to the initial pH and physical nature of the growth medium. More flagella were elaborated on agar than in broth culture, except at the lowest pH value tested (pH 4.04), where elaboration of flagella in planktonic and on surface culture appeared equivalent. Conversely, elaboration of SEF14 was significantly greater in broth than agar culture except at pH 4.04, where SEF14 elaboration by planktonic culture was negligible ($OD_{450} < 0.10$) but was at its greatest for the agar surface culture. It was of interest that, although the trends for all five cultures were very similar, the yield of SEF14 fimbriae by surface agar cultures of strains I and 25667S was distinctly greater than for strains LA5, S1400 and E, recognised as virulent strains. For these five strains, elaboration of SEF21 fimbriae was low (OD_{450} c. 0.10) for all culture conditions. Elaboration of SEF17 was not detectable at 37°C. Strain 27655R has been described previously as producing an unusual, rough colonial morphology [27, 46], shown to be associated with expression of SEF17 fimbriae, possibly constitutively [42]. These data confirm that SEF17 fimbriae were elaborated at the non-permissive temperature (37°C). Again, SEF21 fimbriae were elaborated in greater amounts than for the other five test strains and at the lowest pH, irrespective of planktonic or surface growth.

At 20°C, strains LA5, S1400 and E showed similar phenotypes. Elaboration of flagella was similar for both planktonic and surface agar growth, although reduced on surface agar growth at the lower pH and especially for strain E; this phenomenon was also noted for strain I. Elaboration of flagella by strains 27655S and 27655R was similar, not surprisingly in that strain 27655S was derived from strain 27655R, and notable in that yields were greater for surface-grown than broth-grown culture and greater than for the same or any other strains grown under similar conditions at 37°C (Fig. 1). Strain I failed to elaborate SEF17 fimbriae under any conditions tested, whereas strain 27655R, considered constitutive for SEF17 elaboration, did not elaborate SEF17 in broth at pH

4.65 but did so under all other conditions tested. Strain 27655S elaborated negligible SEF17, although detectable amounts were observed for surface agar growth near neutral pH only. Strains LA5, S1400 and E elaborated SEF17 in broth at pH 6.18 and above, but not at pH 5.09 or below, whereas surface-grown cultures elaborated SEF17 at these and the lower pH values tested. Elaboration of SEF21 and SEF14 fimbriae was negligible for all strains under all conditions at 20°C, although modest SEF21 elaboration by strains 27655R and S was noted (OD_{450} 0.05–0.20).

Before recovery of bacteria for ELISA assays, pH measurements were made across the surface of inoculated and blank gradient plates to determine the effects of bacterial metabolism on the pH of the underlying medium. All strains tested raised the medium pH to 8.40 where confluent growth was visible (Table 2).

Colony morphology on CFA agar gradients

Allen-Vercoe *et al.* [43] noted that strains 27655R, LA5, E and S1400 generated a convoluted colonial morphology when grown at 20°C on unmodified CFA agar for extended periods (3–5 days) and similar morphological changes were observed here for these strains and for strain E (Fig. 2), but not strain I (data not shown). These experiments demonstrated for the first time that colonial morphology was dependent upon the initial pH of the agar surface growth medium with, for example, smooth colonies present at an initial pH 4.40 and convoluted at pH 5.80 (Fig. 2).

Discussion

Enteritidis strains E, I, LA5, S1400 and 27655S grown as planktonic cells in CFA broth at 37°C elaborated copious amounts of SEF14 fimbriae, elaboration of which decreased significantly at the lowest initial pH value (pH 4.04), observations broadly in line with those of McDermid *et al.* [49]. Although elaboration of SEF14 on the surface of agar was less than that for planktonic growth, increasing acidification of the underlying medium increased their elaboration. The differing patterns of expression between planktonic and surface agar growth indicated that surface contact was important in the regulation of SEF14 elaboration. Furthermore, and taking account of the obvious requirement for elevated temperature for SEF14 elaboration, it may be argued that at least three environmental signals, namely temperature, initial pH and surface contact, regulate SEF14 elaboration. How these observations relate to the nutritional regulation noted by Thorns *et al.* [37] is the subject of current genetic investigation [49]. The question arises as to whether contact with animate surfaces, such as gut epithelium, induces the same response by Enteritidis,

Table 1. Elaboration of flagella and fimbriae by six strains of *Salmonella* Enteritidis grown in planktonic or surface culture with defined conditions of pH and temperature

Temperature (°C)	pH	Growth mode	*Production of stated antigen by strain, OD_{450} (SD)					
			LA5	S1400	E	I	27655R	27655S
Flagella								
37	6.66	Broth	0.330 (0.036)	0.449 (0.038)	0.436 (0.028)	0.426 (0.027)	1.007 (0.017)	0.252 (0.006)
	6.10		0.480 (0.035)	0.401 (0.007)	0.461 (0.040)	0.489 (0.022)	0.709 (0.025)	0.238 (0.007)
	4.77		0.430 (0.024)	0.436 (0.010)	0.424 (0.018)	0.496 (0.028)	0.530 (0.012)	0.337 (0.032)
	4.04		1.060 (0.042)	0.939 (0.020)	1.247 (0.026)	1.501 (0.044)	0.637 (0.014)	0.770 (0.074)
37	6.66	Surface	1.140 (0.070)	1.000 (0.051)	0.840 (0.034)	1.661 (0.057)	1.280 (0.056)	0.929 (0.028)
	6.10		1.350 (0.061)	0.924 (0.112)	0.969 (0.039)	1.558 (0.055)	1.530 (0.011)	1.087 (0.035)
	4.77		1.360 (0.070)	1.280 (0.056)	1.139 (0.039)	1.680 (0.041)	1.669 (0.032)	1.228 (0.027)
	4.04		1.388 (0.020)	1.018 (0.013)	1.129 (0.012)	1.390 (0.017)	1.263 (0.012)	1.026 (0.016)
20	6.57	Broth	1.886 (0.199)	1.710 (0.084)	1.542 (0.163)	1.297 (0.172)	1.964 (0.046)	0.965 (0.067)
	6.18		1.767 (0.282)	1.538 (0.025)	1.325 (0.090)	1.744 (0.200)	1.980 (0.239)	0.896 (0.034)
	5.09		1.424 (0.102)	0.776 (0.056)	0.830 (0.075)	1.657 (0.019)	2.176 (0.161)	0.649 (0.005)
	4.65		1.236 (0.046)	1.683 (0.107)	1.386 (0.027)	1.144 (0.004)	2.426 (0.433)	0.069 (0.006)
20	6.57	Surface	1.560 (0.120)	1.960 (0.149)	1.920 (0.177)	2.060 (0.433)	0.850 (0.033)	0.455 (0.011)
	6.18		1.490 (0.051)	2.070 (0.166)	1.490 (0.161)	1.260 (0.100)	0.847 (0.051)	1.916 (0.180)
	5.09		1.710 (0.158)	1.920 (0.145)	1.190 (0.051)	1.430 (0.026)	1.310 (0.040)	1.810 (0.041)
	4.65		1.110 (0.096)	1.360 (0.041)	0.280 (0.019)	0.000 (0.000)	1.020 (0.040)	1.960 (0.252)
SEF14 fimbriae								
37	6.66	Broth	1.957 (0.084)	1.875 (0.098)	2.081 (0.047)	1.766 (0.057)	0.108 (0.006)	1.900 (0.040)
	6.10		1.860 (0.025)	1.781 (0.082)	2.026 (0.023)	1.820 (0.020)	0.952 (0.063)	1.900 (0.023)
	4.77		1.897 (0.029)	1.522 (0.066)	1.929 (0.027)	1.820 (0.039)	0.567 (0.132)	1.790 (0.092)
	4.04		0.000 (0.000)	0.076 (0.002)	0.056 (0.022)	0.032 (0.005)	1.660 (0.061)	0.340 (0.047)
37	6.66	Surface	0.155 (0.013)	0.148 (0.008)	0.192 (0.008)	0.800 (0.018)	0.321 (0.061)	0.730 (0.021)
	6.10		0.202 (0.025)	0.241 (0.016)	0.207 (0.017)	0.938 (0.015)	0.346 (0.012)	0.640 (0.055)
	4.77		0.377 (0.057)	0.345 (0.022)	0.326 (0.028)	0.938 (0.025)	0.223 (0.012)	0.818 (0.007)
	4.04		0.592 (0.011)	0.633 (0.047)	0.569 (0.020)	1.116 (0.017)	0.551 (0.007)	1.292 (0.063)
20	6.57	Broth	0.038 (0.009)	0.039 (0.007)	0.049 (0.010)	0.006 (0.010)	0.035 (0.012)	0.035 (0.012)
	6.18		0.046 (0.004)	0.030 (0.002)	0.044 (0.020)	0.010 (0.001)	0.039 (0.013)	0.039 (0.013)
	5.09		0.018 (0.006)	0.025 (0.008)	0.018 (0.009)	0.013 (0.004)	0.038 (0.013)	0.038 (0.013)
	4.65		0.020 (0.004)	0.010 (0.004)	0.008 (0.005)	0.025 (0.003)	0.021 (0.007)	0.021 (0.007)
20	6.57	Surface	0.015 (0.012)	0.029 (0.012)	0.017 (0.015)	0.020 (0.002)	0.083 (0.018)	0.058 (0.008)
	6.18		0.018 (0.007)	0.033 (0.005)	0.004 (0.004)	0.017 (0.004)	0.033 (0.009)	0.041 (0.015)
	5.09		0.040 (0.002)	0.022 (0.009)	0.011 (0.004)	0.005 (0.003)	0.064 (0.013)	0.031 (0.007)
	4.65		0.039 (0.014)	0.032 (0.003)	0.008 (0.003)	0.000 (0.000)	0.023 (0.008)	0.026 (0.007)
SEF17 fimbriae								
37	6.66	Broth	0.001 (0.002)	0.013 (0.023)	0.007 (0.008)	0.013 (0.005)	0.755 (0.052)	0.033 (0.005)
	6.10		0.008 (0.005)	0.033 (0.032)	0.016 (0.006)	0.022 (0.006)	0.389 (0.034)	0.028 (0.008)
	4.77		0.014 (0.005)	0.029 (0.020)	0.014 (0.009)	0.018 (0.008)	0.394 (0.030)	0.021 (0.018)
	4.04		0.012 (0.003)	0.000 (0.000)	0.008 (0.008)	0.007 (0.002)	0.007 (0.014)	0.007 (0.001)
37	6.66	Surface	0.011 (0.013)	0.010 (0.004)	0.009 (0.005)	0.024 (0.011)	0.317 (0.022)	0.012 (0.007)
	6.10		0.000 (0.000)	0.008 (0.003)	0.000 (0.000)	0.007 (0.007)	0.383 (0.036)	0.000 (0.000)
	4.77		0.006 (0.001)	0.007 (0.002)	0.007 (0.012)	0.002 (0.003)	0.204 (0.017)	0.000 (0.000)
	4.04		0.008 (0.002)	0.009 (0.010)	0.000 (0.000)	0.013 (0.004)	0.405 (0.008)	0.015 (0.009)

Table 1. Elaboration of flagella and fimbriae by six strains of *Salmonella* Enteritidis grown in planktonic or surface culture with defined conditions of pH and temperature

Temperature (°C)	pH	Growth mode	*Production of stated antigen by strain, OD_{450} (SD)					
			LA5	S1400	E	I	27655R	27655S
20	6.57	Broth	0.310 (0.020)	0.271 (0.058)	0.382 (0.022)	0.014 (0.005)	0.605 (0.028)	0.017 (0.007)
	6.18		0.388 (0.022)	0.365 (0.018)	0.169 (0.005)	0.008 (0.005)	0.670 (0.032)	0.019 (0.003)
	5.09		0.016 (0.008)	0.001 (0.002)	0.017 (0.003)	0.008 (0.002)	0.723 (0.022)	0.022 (0.001)
	4.65		0.017 (0.006)	0.005 (0.004)	0.017 (0.004)	0.005 (0.008)	0.575 (0.024)	0.013 (0.005)
20	6.57	Surface	0.199 (0.006)	0.286 (0.027)	0.287 (0.036)	0.006 (0.006)	0.379 (0.009)	0.216 (0.009)
	6.18		0.220 (0.009)	0.345 (0.020)	0.201 (0.019)	0.004 (0.004)	0.441 (0.023)	0.095 (0.013)
	5.09		0.317 (0.023)	0.268 (0.023)	0.133 (0.003)	0.006 (0.006)	0.488 (0.043)	0.060 (0.002)
	4.65		0.168 (0.017)	0.104 (0.004)	0.091 (0.009)	0.000 (0.000)	0.020 (0.010)	0.066 (0.007)
SEF21 fimbriae								
37	6.66	Broth	0.138 (0.014)	0.087 (0.010)	0.176 (0.061)	0.197 (0.018)	0.250 (0.038)	0.097 (0.027)
	6.10		0.194 (0.035)	0.117 (0.015)	0.205 (0.001)	0.217 (0.026)	0.320 (0.048)	0.054 (0.017)
	4.77		0.095 (0.013)	0.051 (0.004)	0.105 (0.030)	0.129 (0.006)	0.360 (0.058)	0.109 (0.013)
	4.04		0.022 (0.007)	0.014 (0.006)	0.029 (0.012)	0.042 (0.008)	0.598 (0.022)	0.031 (0.007)
37	6.66	Surface	0.018 (0.003)	0.033 (0.002)	0.046 (0.016)	0.125 (0.009)	0.174 (0.026)	0.053 (0.009)
	6.10		0.034 (0.005)	0.039 (0.006)	0.000 (0.000)	0.176 (0.017)	0.216 (0.034)	0.041 (0.012)
	4.77		0.029 (0.006)	0.035 (0.006)	0.022 (0.005)	0.098 (0.013)	0.143 (0.022)	0.036 (0.023)
	4.04		0.028 (0.003)	0.045 (0.014)	0.017 (0.005)	0.058 (0.005)	0.221 (0.008)	0.029 (0.003)
20	6.57	Broth	0.076 (0.008)	0.057 (0.003)	0.107 (0.010)	0.161 (0.011)	0.179 (0.015)	0.071 (0.008)
	6.18		0.080 (0.013)	0.074 (0.004)	0.086 (0.007)	0.089 (0.005)	0.118 (0.016)	0.064 (0.009)
	5.09		0.056 (0.011)	0.055 (0.008)	0.030 (0.010)	0.054 (0.023)	0.061 (0.009)	0.062 (0.009)
	4.65		0.038 (0.004)	0.006 (0.004)	0.030 (0.002)	0.061 (0.015)	0.098 (0.014)	0.033 (0.009)
20	6.57	Surface	0.026 (0.009)	0.044 (0.005)	0.044 (0.009)	0.057 (0.003)	0.241 (0.030)	0.102 (0.006)
	6.18		0.106 (0.054)	0.043 (0.003)	0.031 (0.007)	0.053 (0.008)	0.226 (0.021)	0.072 (0.009)
	5.09		0.064 (0.003)	0.035 (0.008)	0.035 (0.008)	0.052 (0.005)	0.143 (0.008)	0.078 (0.005)
	4.65		0.084 (0.002)	0.045 (0.013)	0.019 (0.006)	0.000 (0.000)	0.064 (0.010)	0.076 (0.004)

*Production of stated surface antigen was quantified by ELISA (see *Materials and methods*). Results are recorded as OD_{450} (SD) from three different tests.
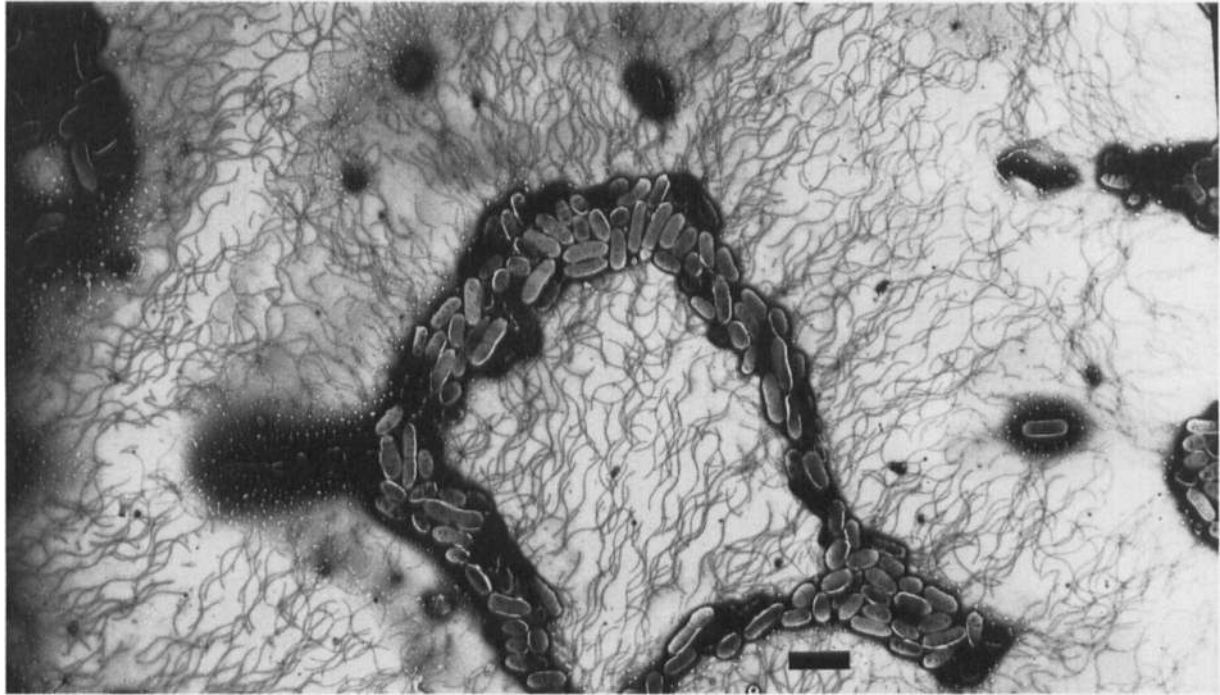
Fig. 1. Transmisssion electron micrograph of negatively stained *Salmonella* Enteritidis strain 27655S grown confluently on CFA agar at an initial pH of 6.57 at 20°C for 24 h. Bar = 3 μm.

Table 2. pH measurement across the surface of inoculated and uninoculated plates

Distance across gradient plate (mm)	Initial pH across gradient plates	pH* across blank plate	pH* across inoculated plate
10	7.20	7.14	8.56
30	6.87	6.84	8.50
50	5.24	5.05	8.42
70	4.41	4.24	8.21
90	3.92	3.79	4.29
110	3.71	3.58	3.68

*After incubation at 37°C for 24 h. Note: visible growth boundary was 80 mm across plate towards lowest pH values (see also Fig. 2).

but evidence has not yet been obtained for a role for SEF14 in adherence and invasion in HEp-2 cell culture [38] and chick gut explant [Allen-Vercoe and Woodward, unpublished observations], although a role in transovarian transmission has been suggested [50].

SEF17 fimbriae were elaborated at 20°C but not at 37°C in both planktonic and surface culture, findings in broad agreement with those of Dibb-Fuller *et al.* [42]. Notable exceptions were strain I, an *rpo*S mutant, which failed to express the antigen under any circumstances, and strain 27655R which was considered to be constitutive for SEF17 [26]. However, SEF17 was elaborated on surface culture at lower initial pH values than in planktonic culture and it may be argued that surface contact is one environmental signal that contributes to the regulation of this fimbrial antigen. That SEF17 fimbriae are elaborated at low pH at ambient temperatures and are highly hydrophobic might lend support to the suggestion that this may be a protective response against environmental extremes [26]. Strain 27655R did not

elaborate SEF17 in planktonic growth at 20°C at pH 4.65, indicating that a constitutive mutation was not the basis for the aberrant expression. The mutation seemed to have a pleiotropic effect upon the expression of other surface antigens, that was ameliorated partially in strain 27655S, presumably by a second compensatory mutation. This is the subject of further genetic analysis.

When test bacteria were diluted and plated on acid gradient plates to obtain single colonies, different colonial morphologies were observed at distinct initial pH values. Whilst SEF17 expression is essential for convoluted colonies [43], the many morphologies identified were probably too diverse to be related to the presence or absence of SEF17 alone, and is probably associated with production of an extracellular matrix (ECM) which has been observed surrounding bacteria in convoluted colonies [51–53]. Habituation at an initial pH induced responses which generated a specific colony morphology that persisted as the pH changed. Whether up-regulation of SEF17 and ECM is
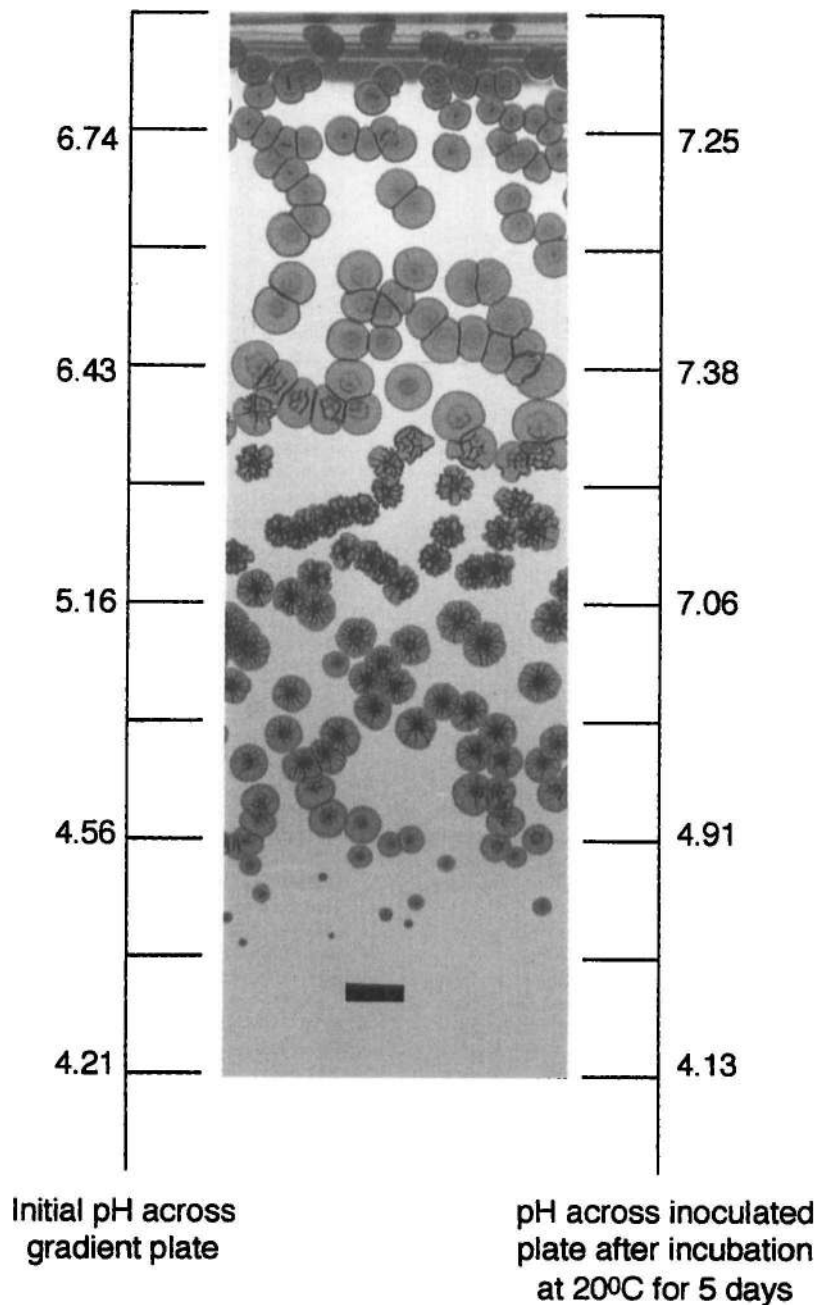
Fig. 2. Morphological variation shown by *Salmonella* Enteritidis strain E grown as single colonies on neutral-pH 4 gradient plates at 20°C for 5 days. Bar = 5 mm.

part of the acid tolerance response [54] and contributes to virulence is worthy of further investigation.

Elaboration of SEF21 fimbriae by Enteritidis was poor under all the conditions tested in these experiments, with the notable exception of strain 27655R, whilst previous studies [25] demonstrated that optimal expression was shown with planktonic growth in heart infusion broth at 37°C for 48–72 h. The role of surface contact in their elaboration is unclear, but there is evidence of SEF21 expression *in vivo* in contact with gut epithelial cells [55].

Hyper-flagellation has been noted for serotypes Typhimurium [56], Enteritidis [43] and Pullorum,

which is traditionally regarded as non-motile [57], with the abundance of flagella dependent upon agar concentration, temperature and carbohydrate availability. The experimental data from the present study lend weight to this body of information, although the mode of growth and initial pH were of marginal influence. For Typhimurium, flagella contribute to invasion and intracellular survival [18, 58, 59] in murine and chick models and it may be assumed that similar roles are performed by the flagella of Enteritidis. Whether variability in abundance of flagella influences pathogenesis, and what role environmental signals play in the biogenesis of flagella, requires further study. We argue that the surface architecture of Enteritidis was influenced by an agar surface on which to grow, a

suggestion supported by Harshey and Matsuyama [56] for Typhimurium. There are similarities between hyper-flagellation and elaboration of SEF17 and ECM by *Salmonella* strains and the swarming of *Proteus mirabilis* [60] which may impact upon pathogenesis, particularly routes of infection.

References

1. Schmidt K. WHO surveillance programme for control of foodborne infections and intoxications in Europe. Berlin, Federal Institute for Health Protection 1995.
2. Rampling A, Anderson JR, Upson R, Peters E, Ward LR, Rowe B. *Salmonella enteritidis* phage type 4 infection of broiler chickens: a hazard to public health. *Lancet* 1989; ii: 436–438.
3. Keller LH, Benson CE, Krotec K, Eckroade RJ. *Salmonella enteritidis* colonization of the reproductive tract and forming and freshly laid eggs of chickens. *Infect Immun* 1995; 63: 2443–2449.
4. Gast RK, Beard CW. Isolation of *Salmonella enteritidis* from internal organs of experimentally infected hens. *Avian Dis* 1990; 34: 991–993.
5. Gorham SL, Kadavil K, Lambert H, Vaughan E, Pert B, Abel J. Persistence of *Salmonella enteritidis* in young chickens. *Avian Pathol* 1991; 20: 433–437.
6. Nakamura M, Nagamine N, Suzuki S *et al.* Long-term shedding of *Salmonella enteritidis* in chickens which received a contact exposure within 24 hrs of hatching. *J Vet Med Sci* 1993; 55: 649–653.
7. Morris GK, Wells JG. Salmonella contamination in a poultry-processing plant. *Appl Microbiol* 1970; 19: 795–799.
8. Tadesse WM, Cizek A. The isolation of Salmonella from poultry carcasses and equipment in the poultry processing plant by the use of two procedures. *Vet Med* 1994; 39: 315–320.
9. Mulvey MR, Loewen PC. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel σ transcription factor. *Nucleic Acids Res* 1989; 17: 9979–9991.
10. Nguyen LH, Jensen DB, Thompson NE, Gentry DR, Burgess RR. In vitro functional characterization of overproduced *Escherichia coli rpoS/rpoS* gene product. *Biochemistry* 1993; 32: 11112–11117.
11. Foster JW, Spector MP. How *Salmonella* survive against the odds. *Annu Rev Microbiol* 1995; 49: 145–174.
12. Fang FC, Libby SJ, Buchmeier NA *et al.* The alternative sigma factor KatF (RpoS) regulates *Salmonella* virulence. *Proc Natl Acad Sci USA* 1992; 89: 11978–11982.
13. Kowarz L, Coynault C, Robbe-Saule V, Norel F. The *Salmonella typhimurium katF* (*rpoS*) gene: cloning, nucleotide sequence, and regulation of *spvR* and *spvABCD* virulence plasmid genes. *J Bacteriol* 1994; 176: 6852–6860.
14. Norel F, Robbe-Saule V, Popoff MY, Coynault C. The putative sigma factor KatF (RpoS) is required for transcription of the *Salmonella typhimurium* virulence plasmid gene *spvB* in *Escherichia coli*. *FEMS Microbiol Lett* 1992; 99: 271–276.
15. Takeuchi A. Electron microscope studies of experimental *Salmonella* infections. 1. Penetration into the intestinal epithelium by *Salmonella typhimurium*. *Am J Pathol* 1967; 50: 109–136.
16. Lockman HA, Curtiss R. Isolation and characterisation of conditionally adherent and non-type 1 fimbriated *Salmonella typhimurium* mutants. *Mol Microbiol* 1992; 6: 933–945.
17. Lockman HA, Curtiss R. Virulence of non-type 1-fimbriated and nonfimbriated non-flagellated *Salmonella typhimurium* in murine typhoid fever. *Infect Immun* 1992; 60: 491–496.
18. Jones GW, Richardson LA, Uhlman D. The invasion of HeLa cells by *Salmonella typhimurium*: reversible and irreversible bacterial attachment and the role of bacterial motility. *J Gen Microbiol* 1981; 127: 351–360.
19. Duguid JP, Anderson ES, Campbell I. Fimbriae and adhesive properties in *Salmonellae*. *J Pathol Bacteriol* 1966; 92: 107–138.
20. Duguid JP, Darekar MR, Wheater DWF. Fimbriae and infectivity in *Salmonella typhimurium*. *J Med Microbiol* 1976; 9: 459–473.
21. Darekar MR, Eyer H. The role of fimbriae in the process of infection. Preliminary report. *Zentralbl Bacteriol Orig [A]* 1973; 225: 130–134.
22. Bäumler AJ, Tsolis RM, Heffron F. Contribution to fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella typhimurium*. *Infect Immun* 1996; 64: 1862–1865.
23. Bäumler AJ, Tsolis RM, Heffron F. The *lpf* fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine Peyer's patches. *Proc Natl Acad Sci USA* 1996; 93: 279–283.
24. Müller K-H, Collinson SK, Trust TJ, Kay WW. Type 1 fimbriae of *Salmonella enteritidis*. *J Bacteriol* 1991; 173: 4765–4772.
25. Sojka MG, Dibb-Fuller M, Thorns CJ. Characterisation of monoclonal antibodies specific to SEF21 fimbriae of *Salmonella enteritidis* and their reactivity with other salmonellae and enterobacteria. *Vet Microbiol* 1996; 48: 207–221.
26. Collinson SK, Emödy L, Müller K-H, Trust TJ, Kay WW. Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. *J Bacteriol* 1991; 173: 4773–4781.
27. Collinson SK, Emödy L, Trust TJ, Kay WW. Thin aggregative fimbriae from diarrheagenic *Escherichia coli*. *J Bacteriol* 1992; 174: 4490–4495.
28. Arnquist A, Olsén A, Pfeifer J, Russell DG, Normark S. The Crl protein activates cryptic genes for curli formation and fibronectin binding in *Escherichia coli* HB101. *Mol Microbiol* 1992; 6: 2443–2452.
29. Olsén A, Jonsson A, Normark S. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. *Nature* 1989; 338: 652–655.
30. Sjöbring U, Pohl G, Olsén A. Plasminogen, absorbed by *Escherichia coli* expressing curli or by *Salmonella enteritidis* expressing thin, aggregative fimbriae, can be activated by simultaneously captured tissue-type plasminogen activator (t-PA). *Mol Microbiol* 1994; 14: 443–452.
31. Ben Nasr A, Olsén A, Sjöbring U, Müller-Esterl W, Björck L. Assembly of human contact phase proteins and release of bradykinin at the surface of curli-expressing *Escherichia coli*. *Mol Microbiol* 1996; 20: 927–935.
32. Collinson SK, Doig PC, Doran JL, Clouthier S, Trust TJ, Kay WW. Thin aggregative fimbriae mediate binding of *Salmonella enteritidis* to fibronectin. *J Bacteriol* 1993; 175: 12–18.
33. Sukupolvi S, Lorenz RG, Gordon JI *et al.* Expression of thin aggregative fimbriae promotes interaction of *Salmonella typhimurium* SR-11 with mouse small intestinal epithelial cells. *Infect Immun* 1997; 65: 5320–5325.
34. Thorns CJ, Sojka MG, Chasey D. Detection of a novel fimbrial structure on the surface of *Salmonella enteritidis* using a monoclonal antibody. *J Clin Microbiol* 1990; 28: 2409–2414.
35. Turcotte C, Woodward MJ. Cloning, DNA nucleotide sequence and distribution of the gene encoding the SEF14 fimbrial antigen of *Salmonella enteritidis*. *J Gen Microbiol* 1993; 139: 1477–1485.
36. Clouthier SC, Muller K-H, Doran JL, Collinson SK, Kay WW. Characterization of three fimbrial genes, *sefABC*, of *Salmonella enteritidis*. *J Bacteriol* 1993; 175: 2523–2533.
37. Thorns CJ, Sojka MG, Mclaren IM, Dibb-Fuller M. Characterisation of monoclonal antibodies against a fimbrial structure of *Salmonella enteritidis* and certain other serogroup D salmonellae and their application as serotyping reagents. *Res Vet Sci* 1992; 53: 300–308.
38. Thorns CJ, Tucotte C, Gemmell CG, Woodward MJ. Studies into the role of the SEF14 fimbrial antigen in the pathogenesis of *Salmonella enteritidis*. *Microb Pathog* 1996; 20: 235–246.
39. Woodward MJ, Allen-Vercoe E, Redstone JS. Distribution, gene sequence and expression *in vivo* of the plasmid encoded fimbrial antigen of *Salmonella* serotype Enteritidis. *Epidemiol Infect* 1996; 117: 17–28.
40. Halavatkar H, Barrow PA. The role of a 54-kb plasmid in the virulence of strains of *Salmonella enteritidis* of phage type 4 for chickens and mice. *J Med Microbiol* 1993; 38: 171–176.
41. Bäumler AJ, Tsolis RM, Valentine PJ, Ficht TA, Heffron F. Synergistic effect of mutations in *invA* and *lpfC* on the ability of *Salmonella typhimurium* to cause murine typhoid. *Infect Immun* 1997; 65: 2254–2259.
42. Dibb-Fuller M, Allen-Vercoe E, Woodward MJ, Thorns CJ. Expression of SEF17 fimbriae by *Salmonella enteritidis*. *Lett*

Appl Microbiol 1997; **25**: 447–452.

43. Allen-Vercoe E, Dibb-Fuller M, Thorns CJ, Woodward MJ. SEF17 fimbriae are essential for the convoluted colonial morphology of *Salmonella enteritidis*. *FEMS Microbiol Lett* 1997; **153**: 33–42.

44. Humphrey TJ, Slater E, McAlpine K, Rowbury RJ, Gilbert RJ. *Salmonella enteritidis* phage type 4 isolates are more tolerant to heat, acid, or hydrogen peroxide also survive longer on surfaces. *Appl Environ Microbiol* 1995; **61**: 3161–3164.

45. Humphrey TJ, Williams A, McAlpine K, Lever MS, Guard-Petter J, Cox JM. Isolates of *Salmonella enterica* Enteritidis PT4 with enhanced heat and acid tolerance are more virulent in mice and more invasive in chickens. *Epidemiol Infect* 1996; **117**: 79–88.

46. Baloda SM. Cell surface properties of enterotoxigenic and cytotoxigenic *Salmonella enteritidis* and *Salmonella typhimurium*: studies on haemagglutination, cell surface hydrophobicity, attachment to human intestinal cells and fibronectin. *Microbiol Immunol* 1988; **32**: 447–459.

47. Thomas LV, Wimpenny JWT, Peters AC. An investigation of the effects of four variables on the growth of *Salmonella typhimurium* using two types of gradient gel plates. *Int J Food Microbiol* 1991; **14**: 261–275.

48. McDermid AS, McKee AS, Dowsett AB, Marsh PD. The effect of environmental pH on the physiology and surface structures of *Salmonella* serotype Enteritidis phage type 4. *J Med Microbiol* 1996; **45**: 452–458.

49. Collighan RJ, Woodward MJ. Sequence analysis and distribution of an IS3-like insertion element isolated from *Salmonella enteritidis*. *FEMS Microbiol Lett* 1997; **154**: 207–213.

50. Thiagaran D, Thacker HL, Saeed AM. Experimental infection of laying hens with *Salmonella enteritidis* that express different types of fimbriae. *Poult Sci* 1996; **75**: 1365–1372.

51. Guard-Petter J, Keller LH, Rahman MM, Carlson RW, Silvers S. A novel relationship between O-antigen variation, matrix formation and invasiveness of *Salmonella enteritidis*. *Epidemiol Infect* 1996; **117**: 219–231.

52. Petter JG. Detection of two smooth colony phenotypes in a *Salmonella enteritidis* isolate which vary in their ability to contaminate eggs. *Appl Environ Microbiol* 1993; **59**: 2884–2890.

53. Guard-Petter J, Lakshmi B, Carlson R, Ingram K. Characterization of lipopolysaccharide heterogenity in *Salmonella enteritidis* by an improved gel electrophoresis method. *Appl Environ Microbiol* 1995; **61**: 2845–2851.

54. Garcia del Portillo F, Foster JW, Finlay BB. Role of acid tolerance response genes in *Salmonella typimurium* virulence. *Infect Immun* 1993; **61**: 4489–4492.

55. Naughton PJ, Grant G, Spencer RJ, Bardocz S, Pusztai A. A rat model of infection by *Salmonella typhimurium* or *Salm. enteritidis*. *J Appl Bacteriol* 1996; **81**: 651–656.

56. Harshey RM, Matsuyama T. Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*; surface-induced differentiation into hyperflagellate swarmer cells. *Proc Natl Acad Sci USA* 1994; **91**: 8631–8635.

57. Holt PS, Chaubal LH. Detection of motility and putative synthesis of flagellar proteins in *Salmonella pullorum* cultures. *J Clin Microbiol* 1997; **35**: 1016–1020.

58. Weinstein DL, Carsiotis M, Lissner CR, O'Brien AD. Flagella help *Salmonella typhimurium* survive within murine macrophages. *Infect Immun* 1984; **46**: 819–825.

59. Lee MD, Curtiss R, Peay T. The effect of bacterial surface structures on the pathogenesis of *Salmonella typhimurium* infection in chickens. *Avian Dis* 1996; **40**: 28–36.

60. Belas R. The swarming phenomenon of *Proteus mirabilis*. *Am Soc Microbiol News* 1992; **58**: 15–22.