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Flagella and curli fimbriae are important for the growth of *Salmonella enterica* serovars in hen eggs

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Salmonella enterica serovar Enteritidis is unable to multiply in the albumen of fresh eggs and must gain access to the yolk contents in order to multiply to a high level ($>10^6$ c.f.u. per ml egg contents). As human *Salmonella* infections resulting from the consumption of infected eggs more frequently involve serovar Enteritidis phage type (PT) 4 than other serovars or PTs, a number of isolates of various *S. enterica* serovars were examined for their ability to multiply to a high level in eggs over a period of 8 days storage at 20 °C. Their behaviour was compared to that of a range of defined fimbrial and flagella mutants of *S.* Enteritidis. Strains that did not express flagella were unable to multiply in eggs, and those deficient for curli fimbriae, including strains of *S.* Enteritidis PT6, displayed high-level growth in significantly fewer eggs than those able to express curli. Most *S.* Enteritidis strains multiplied to a high level in between 5 and 10 % of eggs during 8 days storage. One PT4 strain, though, showed high levels of growth in more than 25 % of eggs over this period, significantly higher than the other PTs or the two other isolates of PT4 tested. This ability may be important for the association of PT4 infection with the consumption of eggs.

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INTRODUCTION

Salmonella infection resulting from the consumption of contaminated eggs is a major public health problem in Europe and the USA and an emerging one in the Far East (Poppe, 1999). *Salmonella enterica* subspecies *enterica* serovar Enteritidis is responsible for the majority of egg-associated infections, and appears to possess factors that enable it to persist in the reproductive tissue of the hen so that it can contaminate the contents of intact eggs (Okamura *et al.*, 2001a, b). Other serovars of *S. enterica*, such as Gallinarum and Pullorum, have in the past been widespread in laying flocks (Shivaprasad, 2000), without a concomitant high level of human infection.

Salmonella enterica has the potential to elaborate numerous cell surface structures, including type 1 (SEF21), thin aggregative or curli (SEF17), SEF14, long polar (LPF) and plasmid-encoded (PEF) fimbriae, and flagella. Many of these have been shown to be important in the process of infection of the hen and in the colonization of the reproductive tissues. SEF14 fimbriae, which are only present in a few group D serovars (Turcotte & Woodward, 1993), mediate adherence to reproductive tissues (Ogunniyi *et al.*, 1997) and may have a role in organ invasion and persistence (Rajashekara *et al.*, 2000). There is some evidence that flagella are important in the subsequent invasion of internal tissues (Allen-Vercoe *et al.*, 1999). Type 1 and curli fimbriae in *Escherichia coli*, analogous to SEF21 and SEF17 of *S.* Enteritidis, are associated with initial persistence in the avian gastrointestinal tract (La Ragione *et al.*, 2000). There is little current evidence for a role for PEF and LPF in virulence or persistence in the chick model, or in laying hens. The role of any of these in the growth of *Salmonella* within the egg is not yet known.

Abbreviations: LPF, long polar fimbriae; PT, phage type; PEF, plasmid-encoded fimbriae; TMB, tetramethylbenzidine.

Salmonella Enteritidis is able to multiply to a high level in less than 21 days, the retail shelf-life of eggs in the UK, in around 7 % of contaminated eggs (Cogan *et al.*, 2001). Control of the proliferation of *Salmonella* within eggs may be achieved by their storage at lower than ambient temperature, which slows down both bacterial growth rates and changes to egg contents which facilitate *Salmonella* multiplication. It is believed that, although some eggs are contaminated within the yolk due to ovarian infection, in the majority of eggs infected via the tissues of the reproductive tract, *Salmonella* cells are deposited within the albumen close to the yolk membrane (Humphrey *et al.*, 1989; Mawer *et al.*, 1989) or on the vitelline membrane (Gast & Holt, 2000). As *Salmonella* cannot proliferate in the albumen of fresh eggs (Baron *et al.*, 1997), bacteria must penetrate the vitelline membrane and gain access to the yolk contents in order to grow. The vitelline membrane of the hen's egg comprises a collagenous matrix overlaid with a layer of glycoproteins (Bellairs *et al.*, 1963). Glucose within the albumen reacts with these proteins, causing a progressive increase in the porosity of this membrane, during the storage of eggs at temperatures above 6 °C. This results in the release of yolk contents into the albumen and growth of bacterial contaminants after approximately 21 days at 20 °C (Clay & Board, 1991). In fresh eggs, however, the yolk membrane presents an obstacle to bacterial invasion of the yolk.

It is possible that survival within, and motility through the albumen, as well as adherence to the vitelline membrane, may be characteristics of those *Salmonella* serovars that multiply in eggs, such as *S.* Enteritidis. To test this hypothesis, we set out to determine the multiplication of *S.* Enteritidis wild-types and mutants defective for motility (aflagellate) and adherence (afimbriate) in an egg model that we developed previously (Cogan *et al.*, 2001).

METHODS

Bacterial strains. Isolates of various *S. enterica* serovars from a number of sources were used in this study (Table 1). A number of mutants of *Salmonella* Enteritidis PT4 strains (S1400 and 857) deficient in the production of various surface structures (Walker *et al.*, 1999) and constructed previously (Allen-Vercoe & Woodward, 1999; Allen-Vercoe *et al.*, 1999; Van Asten *et al.*, 2000) were also used (Table 1).

Growth of bacteria in whole eggs. The *Salmonella* egg growth assay of Cogan *et al.* (2001) was used. Bacterial cultures that had been stored in cryovials (Pro-Lab) at −40 °C were streaked onto

Table 1. Strains used to infect eggs (isolates were representative of those types found in contents-contaminated eggs)

Strain	Description	Protein or function affected	Reference or source*
C6B	*S.* Enteritidis PT4		This study
E	*S.* Enteritidis PT4		Humphrey *et al.* (1995)
S1400	*S.* Enteritidis PT4		Allen-Vercoe *et al.* (1998)
EX1	*S.* Enteritidis PT6		This study
EX2	*S.* Enteritidis PT6		This study
9510-23	*S.* Enteritidis PT8		C. Benson
9510-85	*S.* Enteritidis PT8		C. Benson
B	*S.* Enteritidis P13a		C. Benson
D	*S.* Enteritidis P13a		C. Benson
9598-195	*S.* Enteritidis PT23		C. Benson
9508-211	*S.* Enteritidis PT23		C. Benson
9	*S.* Gallinarum		P. Barrow
449/87	*S.* Pullorum		P. Barrow
30	*S.* Typhimurium DT104		Jørgensen *et al.* (2000)
SL1344	*S.* Typhimurium		Hosieth & Stocker (1981)
EAV1	S1400 *fimD*::*tet*	SEF21	Allen-Vercoe & Woodward (1999)
EAV5	S1400 *pefC*::*zeo*	Plasmid-encoded fimbriae	Allen-Vercoe & Woodward (1999)
EAV7	S1400 *lpfC*::*trim*	Long polar fimbriae	Allen-Vercoe & Woodward (1999)
EAV9	S1400 *fliC*::*cam*	Flagella	Allen-Vercoe & Woodward (1999)
EAV11	S1400 *agfA*::*bla*	SEF17/curli	Allen-Vercoe & Woodward (1999)
EAV13	S1400 *sefA*::*kan*	SEF14	Allen-Vercoe & Woodward (1999)
EAV45	S1400 *motAB*::*cam*	Motility	Allen-Vercoe & Woodward (1999)
EAV53	S1400 *rpoS*::*str*	σ^s	Allen-Vercoe & Woodward (1999)
857	*S.* Enteritidis PT4		Van Asten *et al.* (2000)
857::fliC	857 *fliC*::*kan*	Flagella	Van Asten *et al.* (2000)
857::fliC pWSK29::fliC	857 *fliC*::*kan* pWSK29::*fliC*		Van Asten *et al.* (2000)

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blood agar (BA) and incubated overnight at 37 °C. One colony was inoculated into 9 ml of pre-warmed nutrient broth number 2 (NB, Oxoid) and incubated for 16 h at 37 °C in static culture, so that cells were in stationary phase. This culture was standardized with fresh NB to an OD_{600} of 0·2 to give approximately 2×10^8 c.f.u ml^{-1}. Serial dilutions were performed in phosphate-buffered saline (PBS), which was made using water from a source containing <0·01 p.p.m. iron, to give suspensions of 12 c.f.u. ml^{-1}. Two hundred microlitres, equating to between two and three cells per egg, of the *Salmonella* suspensions were inoculated into the albumen of eggs using a syringe and 0·45 × 10 mm needle. The size of the inoculum was verified for each strain by plating 100 × 200 µl spots onto BA plates.

Eggs were obtained from a local caged-production unit, from hens that had not been vaccinated against *Salmonella*. The eggs were inspected on arrival at the laboratory and cracked or dirty eggs discarded. Shells were surface sterilized with 70 % ethanol that was allowed to evaporate. A small hole was punched in the pointed end of the egg with a sterile pin and 200 µl of bacterial suspension was introduced into the albumen. The hole was then sealed with quick-drying adhesive. Eggs were subsequently stored at a constant temperature of 20 °C for 8 days. The number of *Salmonella* c.f.u. in the egg contents was determined using previously published techniques (Humphrey *et al.*, 1991). Eggs were surface sterilized with ethanol, aseptically cracked and placed in a sterile plastic bag, then the contents were homogenized using a stomacher. Decimal dilutions were made of the homogenate and 0·5 ml aliquots spread onto xylose/lysine/deoxycholate agar plates, which had been dried at 60 °C for 30 min. The inoculated plates were then incubated at 37 °C for 24 h. Twenty eggs were inoculated with each bacterial strain in each experiment. Experiments were performed three times.

Determination of level of curli and flagella expression using a direct binding ELISA. Monoclonal antibodies (mAbs) were a gift from M. Dibb-Fuller (Veterinary Laboratories Agency) and were produced against purified curli fimbriae and flagella as described by Hotani (1971) and Thorns *et al.* (1992). A modification of the method of Dibb-Fuller *et al.* (1999) was used for the ELISA. Cells were grown at 20 °C in colonization factor antigen broth (CFA) containing 0·5 g l^{-1} ovotransferrin (apo-ovotransferrin, Sigma), the iron-binding protein present in albumen, at pH 9·2, to produce cells that were iron-restricted. Cells were harvested after 36 h growth, 4 h before maximum optical density was reached, to examine late-exponential-phase expression, or after 48 h for stationary phase. Cultures were centrifuged at 3500 ***g*** for 5 min and the pellet resuspended in 0·1 M carbonate buffer, pH 9·6, to an OD_{540} of 1·2. This suspension was coated onto Maxisorb microtitre plates (Merck) and dried overnight at 37 °C. The plates were washed four times with 0·1 M PBS containing 0·05 % Tween 20 (PBS-Tween) at this point, and after each subsequent incubation step. Wells were blocked with 200 µl PBS containing 3 % dried milk and incubated at 37 °C for 1 h. One hundred microlitres of mAb suspended in PBS containing 0·1 % dried milk was added to each well and plates incubated at 37 °C for a further 1 h. Goat anti-mouse horseradish peroxidase-conjugated antibody was added to the wells and incubated for 1 h, followed by the addition of tetramethylbenzidine substrate (TMB). The reaction was stopped with 10 % sulphuric acid and absorbance read at 450 nm. For each antigen, the absorbance of the well containing the strain deficient for that antigen was used as the blank.

Motility tests. To determine whether the bacterial strains were motile in albumen, cells were grown on BA for 24 h at 37 °C. A small amount of colony mass was then picked from this plate with a needle and inoculated into 20 ml fresh egg albumen in a Petri dish, so that cells were introduced at the edge of the plate. A 10 mm diameter disc of BA was placed into the albumen at the centre of the dish and plates were kept at 20 °C for 8 days and examined daily. Five replicate experiments were performed with each strain. Colonies appearing on the central BA disc were recorded and subsequently typed biochemically, serotypically and by antibiotic resistance, as appropriate, to ensure that they were of the isolate that had originally been introduced into the albumen.

Motility was also recorded after growth in CFA containing 0·5 g ovotransferrin l^{-1}, at pH 9·2, for 48 h at 20 °C. Cells were viewed as a wet mount by phase-contrast microscopy and the percentage of motile cells in five fields of view recorded.

Statistical analysis of results. Results from individual experiments were pooled so that the number of eggs in which growth occurred was considered as a proportion of the total number originally inoculated with each strain of *Salmonella*. The standard error was calculated from this overall proportion. The number of eggs that would have originally received no inoculum was calculated from the Poisson distribution of the inoculum sizes, found by plating aliquots on to BA. The proportion of eggs showing no growth was adjusted using this figure so that uninoculated eggs were not included in the data analysis (Cogan *et al.*, 2001). Differences between the numbers of eggs in which growth of *Salmonella* occurred were analysed using a chi-squared test, with P values of less than 0·05 regarded as significant. The fit of the distribution of the inoculum size to a Poisson distribution was checked using a chi-squared test. ELISAs for flagella or fimbrial expression were performed on three occasions and differences analysed using a one-tailed t-test, with P values of less than 0·01 considered as significant, in order to look for large differences in levels of expression.

Differences in the frequency of high-level growth ($>10^6$ c.f.u. ml^{-1}) or levels of fimbrial expression, between groups of strains, were examined using a Student's t-test.

RESULTS

Growth of wild-type isolates in eggs

In accordance with previous work by Board (1964), contamination levels of $>10^6$ *Salmonella* cells per ml of whole egg contents were used to indicate growth occurring as a result of *Salmonella* accessing the yolk contents.

Sixty eggs were infected and enumerated for each *Salmonella* isolate. Four significantly ($P<0·05$) different frequencies of yolk invasion were exhibited by the wild-type isolates (Fig. 1). Serovars Gallinarum and Pullorum did not multiply to $>10^6$ c.f.u. ml^{-1} in any of the eggs tested, indicating that no invasion of the yolk had occurred. Six per cent of eggs showed yolk invasion when inoculated with the PT6 isolates. This was a significantly ($P<0·05$) lower proportion than the PT8, 13a and 23 isolates, which invaded and multiplied in the yolk of between 9 and 15 % of the eggs, as did two of the PT4 isolates (E, C6B). The highest frequency of yolk invasion was shown by the two Typhimurium and one of the Enteritidis PT4 strains (S1400), which invaded the yolks of more than 24 % of eggs, a significantly ($P<0·05$) higher proportion than the PT8, 13a and 23 strains.

Growth of mutants in eggs

Five strains (S1400 *agfA*, *fliC*, *motAB* and *rpoS* mutants and 857 *fliC* mutant) multiplied to a high level in a significantly

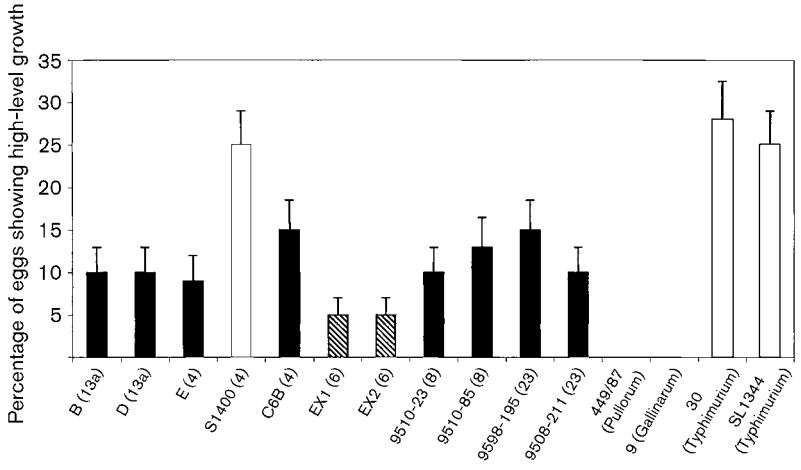

Fig. 1. Growth of different *S. enterica* serovars to high numbers ($>10^6$ c.f.u. ml^{-1}), indicative of invasion of the egg yolk by *Salmonella*. For each bacterial strain, 60 eggs were inoculated with approximately two *Salmonella* cells, suspended in PBS, and stored for 8 days at 20 °C. Bars show percentage of eggs (±SE) in which a high level of growth of *Salmonella* occurred after storage. Those significantly different to the mean ($P<0{\cdot}05$ using chi-squared test) are shown in white or hatched. Strains are serotype Enteritidis, except where noted otherwise. The phage type of Enteritidis isolates is shown in parentheses.

lower proportion of eggs than wild-type (Fig. 2, $P>0{\cdot}05$). Three of these strains were unable to multiply in the yolk of any of the eggs tested; two of these (the *fliC* mutants of strains S1400 and 857 respectively) were deficient for flagella and the other (the *motAB* mutant) was non-motile. Complementation of the *fliC* mutant with a plasmid carrying the *fliC* gene (Van Asten *et al.*, 2000) restored the growth phenotype. Two strains showed high-level multiplication in a significantly ($P<0{\cdot}05$) lower proportion of eggs than the wild-type; these were the *rpoS* mutant and the *agfA* mutant, both of which do not express curli fimbriae (Fig. 3).

Expression of curli fimbriae

As curli fimbriae had been shown to be important for a high level of multiplication of *Salmonella* in eggs, their production by the wild-type isolates was investigated under poor (stationary phase) and rich (exponential phase) nutrient conditions. When the bacteria were grown in CFA broth containing 0·5 g ovotransferrin l^{-1} at pH 9·2, 20 °C for 48 h and harvested in early stationary phase, the three Enteritidis PT4 isolates showed a significantly higher level of curli expression than strains of other phage types of Enteritidis, or those of Pullorum or Gallinarum ($P<0{\cdot}01$, Fig. 4). The two isolates of Enteritidis PT6 did not show detectable levels of curli expression. Strains which showed a high level of stationary-phase curli expression, such as

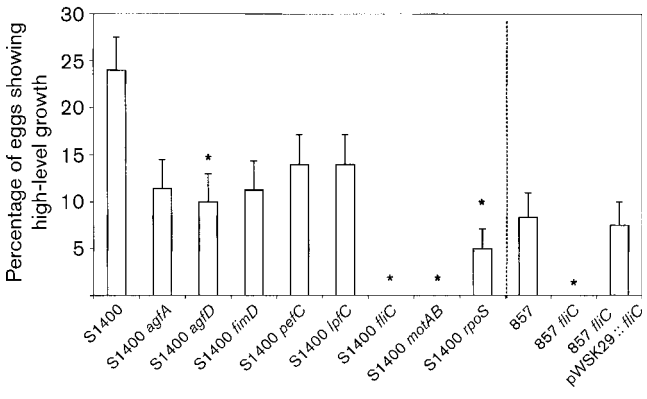

Fig. 2. Effect of mutations on the ability of strains to grow to high numbers ($>10^6$ c.f.u. ml^{-1}), indicative of invasion of the egg yolk by *Salmonella*. Eggs were inoculated with approximately two *Salmonella* cells, suspended in PBS, and stored for 8 days at 20 °C. Bars show percentage of eggs (±SE) in which a high level of growth of *Salmonella* occurred after storage. Those significantly different to the wild-type ($P<0{\cdot}05$ using chi-squared test) are indicated by asterisks.

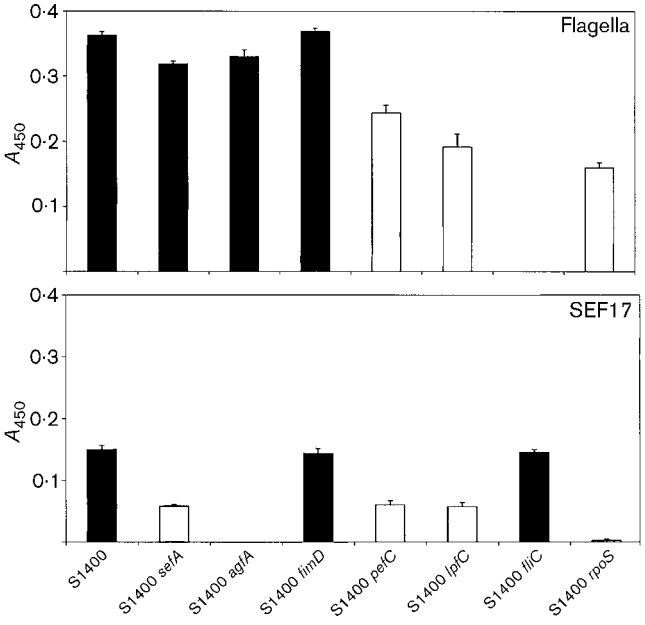

Fig. 3. Expression of SEF17 and flagella by mutants of strain S1400. Bacteria were grown in CFA containing 0·5 g ovotransferrin l^{-1} at pH 9·2, 20 °C for 48 h. Values are absorbance of TMB substrate at 450 nm following direct binding ELISA using mAbs specific for indicated antigens. Bars show the mean of three experiments (±SE). Levels of expression significantly different to that seen in wild-type ($P<0{\cdot}01$ using one-tailed *t*-test) are shown in white.

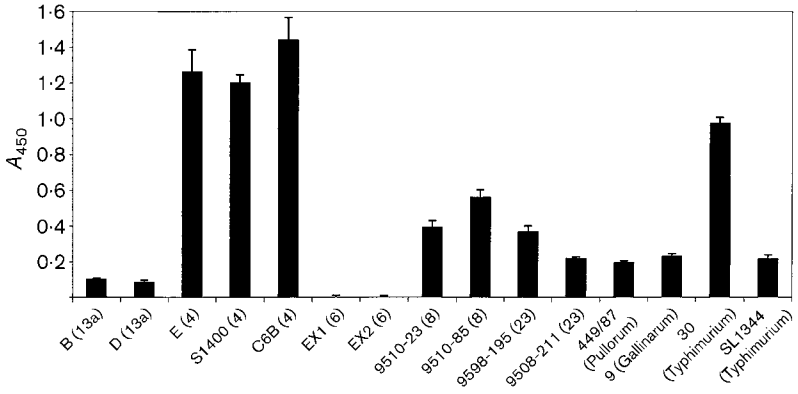

Fig. 4. Expression of SEF17 during early stationary phase. Bacteria were grown in CFA containing 0·5 g ovotransferrin l^{-1} at pH 9·2, 20 °C for 48 h. Values are absorbance of TMB substrate at 450 nm following direct binding ELISA using mAb. Bars show the mean of three experiments (± SE). Strains are serotype Enteritidis, except where noted otherwise. The phage type of Enteritidis isolates is shown in parentheses.

the PT4 isolates (E, C6B), showed high-level growth in a number of eggs not significantly different (Fig. 1, $P>0·05$) to those which had significantly ($P<0·01$) lower levels of curli expression, such as PTs 8, 13a and 23.

Three isolates (Enteritidis PT4 S1400 and the two Typhimurium strains) showed a significantly higher level of curli expression in the late exponential phase of growth than the other strains ($P<0·01$, Fig. 5); these strains also showed high-level growth in the highest proportion of eggs (Fig. 1). Curli fimbriae were expressed by all of the other strains, with the exception of the two Enteritidis PT6 isolates, which failed to produce detectable levels. These latter two strains invaded the yolk of significantly ($P<0·05$) fewer eggs than the PT8, 13a and 23 strains.

SEF17 expression in the *lpf*, *pef* and SEF14 (*sefA*) mutants was essentially the same (Fig. 3), but significantly lower than that of the parent ($P<0·01$). As expected, there was almost no SEF17 expression in the *rpoS* mutant and the recorded value was significantly lower than that of any of the other strains. The level of SEF17 expression in the flagella (*fliC*) and SEF21 (*fimD*) mutants was not significantly different to that of the parent strain.

Motility and expression of flagella

Under the high pH, iron-restricted conditions tested, the direct binding ELISA showed that flagella were expressed by all of the Enteritidis and Typhimurium strains, but not by serovars Gallinarum and Pullorum (data not shown). The albumen motility test and microscopy indicated that Gallinarum and Pullorum strains were not motile. Among the mutants, the two *fliC* mutants and the *motAB* mutants were not motile (Table 2). All other strains were motile in albumen.

Significantly lower levels of flagella expression were seen in the *pef*, *lpf* and *rpoS* mutants of S1400 (Fig. 3); the levels in the SEF17 (*agfA*) and SEF21 (*fimD*) mutants, however, did not differ significantly from that of the parent strain ($P>0·01$).

DISCUSSION

Data in this paper demonstrate that motility is a significant factor for the growth of *S.* Enteritidis in eggs, while strains that are non-motile in these conditions (such as the *fliC* and *motAB* mutants and the isolates of non-motile serovars Gallinarum and Pullorum) are unable to multiply. Bacterial cells are unable to multiply in the albumen due to a lack of available iron (Schade & Caroline, 1944; Baron *et al.*, 1997). The absence of flagella prevents movement through the albumen towards the yolk, so proliferation does not take place. Host specificity is often cited as the reason the poultry-associated serovars *S.* Pullorum and *S.* Gallinarum do not seem to cause disease in humans. The data presented in this paper suggest that the infectious potential of *S.* Pullorum and *S.* Gallinarum via contaminated eggs is further reduced because these bacteria lack flagella under these conditions, and are incapable of growing in egg

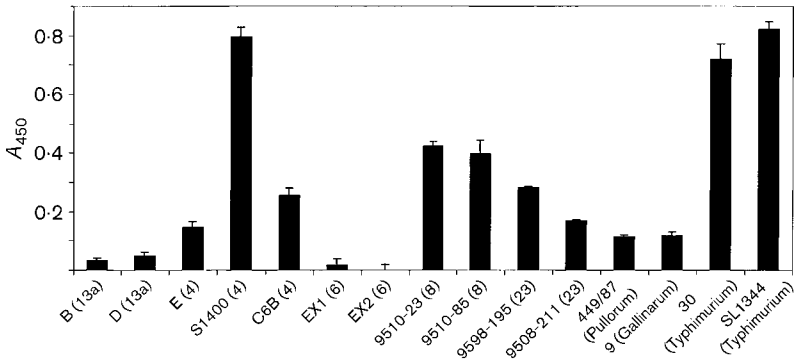

Fig. 5. Expression of SEF17 or equivalent during exponential-phase growth. Bacteria were grown in CFA containing 0·5 g ovotransferrin l^{-1} at pH 9·2, 20 °C for 36 h. Values are absorbance of TMB substrate at 450 nm following direct binding ELISA using mAb. Bars show the mean of three experiments (± SE). Strains are serotype Enteritidis, except where noted otherwise. The phage type of Enteritidis isolates is shown in parentheses.

Table 2. Motility tests performed on *Salmonella* isolates

Strains were inoculated at the edge of a Petri dish containing egg albumen. A disk of BA was placed at the centre and monitored for bacterial growth over 8 days. Strains were also grown in CFA broth containing 0·5 g ovotransferrin l^{-1}, at pH 9·2, for 48 h at 20 °C and motility observed by phase-contrast microscopy. Percentage range of motile cells in five fields of view was scored.

Strain	Description	Days to observable growth at centre of swim plate	Percentage of cells motile after 48 h growth in CFA at 20 °C
C6B	*S.* Enteritidis PT4	2	90–100
E	*S.* Enteritidis PT4	2	90–100
S1400	*S.* Enteritidis PT4	2	90–100
EX1	*S.* Enteritidis PT6	2	90–100
EX2	*S.* Enteritidis PT6	2	90–100
9510-23	*S.* Enteritidis PT8	2	90–100
9510-85	*S.* Enteritidis PT8	3	75–90
B	*S.* Enteritidis P13a	2	90–100
D	*S.* Enteritidis P13a	2	90–100
9598-195	*S.* Enteritidis PT23	2	75–90
9508-211	*S.* Enteritidis PT23	2	75–90
9	*S.* Gallinarum	NG	<0·1
449/87	*S.* Pullorum	NG	<0·1
30	*S.* Typhimurium DT104	2	90–100
SL1344	*S.* Typhimurium	4	50–75
EAV1	S1400 *fimD*::*tet*	2	90–100
EAV5	S1400 *pefC*::*zeo*	2	75–90
EAV7	S1400 *lpfC*::*trim*	2	75–90
EAV9	S1400 *fliC*::*cam*	NG	<0·1
EAV11	S1400 *agfA*::*bla*	2	90–100
EAV13	S1400 *sefA*::*kan*	2	90–100
EAV45	S1400 *motAB*::*cam*	NG	<0·1
EAV53	S1400 *rpoS*::*str*	3	75–90
857	*S.* Enteritidis PT4	2	90–100
857::*fliC*	857 *fliC*::*kan*	NG	<0·1
857::*fliC* pWSK29::*fliC*	857 *fliC*::*kan* pWSK29::*fliC*	2	75–90

NG, No growth.

contents. These serovars have been shown to elaborate flagella under some conditions (Holt & Chaubal, 1997); however, the data presented here showed a combination of (1) a lack of yolk invasion following inoculation into the albumen and (2) an absence of flagella expression under restricted iron and high pH conditions, indicating that these serovars remain aflagellate in the albumen.

Curli production is not vital for the growth of *Salmonella* in eggs, but is an important factor in permitting a high frequency of yolk invasion. The *agfA* mutant, which is deficient in the production of curli, showed the lowest frequency of yolk invasion of all the motile but afimbriate mutants tested. Both PT6 strains were also unable to produce curli and showed a decreased frequency of yolk invasion. Curli have been shown to attach to matrix glycoproteins such as fibronectin (Collinson *et al.*, 1991). It is therefore possible that this fimbrial type could be responsible for mediating bacterial attachment to the yolk membrane, which is made up of glycoprotein and collagenous matrix layers (Bellairs *et al.*, 1963). Attachment to the membrane may then facilitate yolk invasion, or proliferation of cells on the surface of the yolk. This is an area for further study.

It is noted that all of the mutants showed lower levels of yolk invasion than the parental strain, although these differences were not always significant at the 5% level. This difference could be explained by the lower levels of flagella and/or curli expression seen in these strains. The expression of different types of fimbriae may be linked, so a mutation in one fimbrial type would also affect expression of curli. This hypothesis is supported by the results of the ELISA for SEF17, which show significant decreases in expression of these structures in some of the fimbrial mutants. Flagella expression and motility were also significantly altered in some of the fimbrial mutants. Walker *et al.* (1999) observed such pleiotropic effects on various bacterial surface

structures caused by the disruption of fimbrial expression. The *rpoS* mutant, EAV53, showed growth in only 5 % of eggs; levels of both SEF17 and flagella, and the percentage of motile cells, were significantly lower in this strain than in S1400 ($P<0{\cdot}01$), suggesting that σ^S may be a regulator of flagella expression as well as of SEF17.

In this work the wild-type strain S1400 showed growth to a high level in 24 % of eggs after 8 days storage at 20 °C while the two other strains of *S.* Enteritidis PT4 (E, C6B) grew to a high level in only 9–15 % of eggs stored for the same time at the same temperature. The fact that different PT4 isolates behaved differently in their growth in eggs may be an important factor in understanding the emergence of PT4 as an egg-associated pathogen. Rabsch *et al.* (2001) suggested that the Enteritidis pandemic was caused by the expansion of a more virulent or successful PT4 clone. Strain S1400 has an advantage over the other PT4 strains tested in the present study as it produced curli fimbriae under both rich and poor nutrient conditions (Figs 4 and 5). The curli fimbriae would allow the microbe to attach to surfaces in a wider range of conditions than other strains. Bacteria attached to surfaces are known to be more stress tolerant than free cells (Rowbury, 1995; Humphrey *et al.*, 1997). The role this behaviour may have had in the emergence of PT4 as an egg-associated pathogen merits further investigation.

The expression of curli during the late exponential phase of growth was found to correlate with a high frequency of growth in eggs. Starvation is known to induce expression of this fimbrial type and expression of the *agf* genes is controlled by the sigma factor RpoS, produced during entry into stationary phase (Moreno *et al.*, 2000). This is supported by the work presented here, which showed a lack of curli expression in both the *agfA* and *rpoS* mutants under all of the conditions tested (Fig. 3). Growth-phase-independent curli expression has been seen under conditions such as reduced oxygen tension (Gerstel & Römling 2001) and is known to be controlled by multiple pathways. Results presented in this report show that curli production in some strains is growth-phase independent, and can be induced in some strains under low iron, high pH conditions. Within most of the albumen, iron availability is restricted, so cells would have a stationary-phase phenotype. As bacteria move closer to the yolk, they move up a nutrient concentration gradient (Garcia *et al.*, 1983). This would relieve growth restriction and result in cells being able to actively grow once they are in close proximity to the yolk. Thus, the strains that have an advantage in eggs are those able to express curli under growth, rather than starvation, conditions.

The two *S.* Typhimurium strains also showed a high level of yolk invasion. This is at odds with the fact that Typhimurium is not highly associated with egg-borne salmonellosis. Keller *et al.* (1997) showed that although Typhimurium was able to colonize the ovaries of laying hens, it was not found in eggs post-lay. It is thus possible that Typhimurium does not survive as well as Enteritidis in the forming egg, and that this explains the infrequent incidence of human Typhimurium infection via eggs.

Neither of the Enteritidis PT6 strains elaborated curli to a detectable level under the conditions tested. Both also showed yolk invasion in a smaller proportion of eggs than the other Enteritidis strains, supporting the conclusion that curli are important in invasion of the yolk. As both PT4 and PT6 are closely related and belong to the same clonal lineage (Olsen *et al.*, 1994), it is surprising that the PT6 isolates used here were poorly or non-curliated. A similar phenomenon has been observed in *E. coli* (La Ragione *et al.*, 1999), where non-curliation of O78 : K80 strains was associated with the presence of an insertion sequence in the *agf* operon. The basis of poor curliation in PT6 is the subject of ongoing work.

It has previously been suggested that the principal role of storage temperatures below 20 °C was to slow changes to the yolk membrane that facilitate the growth of *Salmonella* spp. (Humphrey, 1990). Data in this paper indicate additional temperature effects. Curli are not produced at temperatures below 20 °C (Walker *et al.*, 1999), which may reduce the growth potential of *S. enterica* serovars in eggs. Although the genes for curli production appear to be ubiquitous within the genus *Salmonella*, the fact that expression during exponential-phase growth correlated with a high frequency of yolk invasion provides a useful test for identifying strains with a greater potential to multiply in a large proportion of eggs. This work suggests that *Salmonella* strains can be categorized, relative to their ability to multiply to high numbers in eggs over 8 days storage, as belonging to one of four groups: (1) motile strains which express curli during exponential-phase growth are able to multiply in the highest proportion of eggs; (2) other motile, curliated strains multiply in a lower proportion of eggs; (3) motile non-curliated strains in the lowest proportion. Non-motile strains (group 4), deposited outside of the yolk, are unable to multiply in any eggs.

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