Monophasic expression of FliC by Salmonella 4,[5],12:i:- DT193 does not alter its pathogenicity during infection of porcine intestinal epithelial cells

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Non-typhoidal serotypes of Salmonella enterica remain important food-borne pathogens worldwide and the frequent emergence of epidemic strains in food-producing animals is a risk to public health. In recent years, Salmonella 4,[5],12:i:- isolates, expressing only phase 1 (FliC) of the two flagellar antigens, have emerged and increased in prevalence worldwide. In Europe, the majority of 4,[5],12:i:- isolates belong to phage types DT193 and DT120 of Salmonella Typhimurium and pigs have been identified as the reservoir species. In this study we investigated the ability of pig-derived monophasic (4,[5],12:i:-) and biphasic DT193 isolates to invade a porcine intestinal epithelial cell line (IPEC-1) and activate TLR-5, IL-8 and caspases. We found that the 4,[5],12:i:- isolates exhibited comparable adhesion and invasion to that of the virulent S. Typhimurium isolate 4/74, suggesting that these strains could be capable of colonizing the small intestine of pigs in vivo. Infection with 4,[5],12:i:- and biphasic DT193 isolates resulted in approximately the same level of TLR-5 (a flagellin receptor) and IL-8 (a proinflammatory chemokine) mRNA upregulation. The monophasic variants also elicited similar levels of caspase activation and cytotoxicity to the phase-variable DT193 isolates. These findings suggest that failure of 4,[5],12:i:- DT193 isolates to express a second phase of flagellar antigen (FljB) is unlikely to hamper their pathogenicity during colonization of the porcine intestinal tract.

INTRODUCTION

The evolution of Salmonella has been punctuated by the emergence of epidemic and multi-drug-resistant strains (reviewed by Rabsch et al., 2001). These strains pose a threat to public health because of their ability to spread widely and rapidly and their tendency to persist in food-producing animal populations. Over the past two decades, there has been a rapid worldwide emergence of monophasic strains of Salmonella. The flagellin filaments of Salmonella, FliC and FljB, are usually alternately expressed by phase variation (Aldridge et al., 2006; Bonifield & Hughes, 2003). These recently emerged monophasic Salmonella are so called because they do not express the fljB gene for second-phase flagellar antigen and instead express only first-phase flagella antigen encoded by the fliC gene. As a result of this genotype, their antigenic formula is designated 4,[5],12:i:-.

Monophasic strains of Salmonella isolated from pigs, pork and humans have a high level of homogeneity (Hauser et al., 2010), highlighting their zoonotic nature. Salmonella 4,[5],12:i:- isolates can be phage typed according to the scheme of Anderson et al. (1977), with the majority of isolates in Europe typing to DT193 and DT120 and carrying resistance to ampicillin, streptomycin, sulphonamides and tetracyclines (Hopkins et al., 2010). In the UK, Salmonella Typhimurium DT193 is one of the phage types most frequently isolated from humans and 52% of the DT193 isolated from patients in 2010 were found to be monophasic (Hopkins et al., 2012). In Europe, monophasic strains are now the third most common serotype of Salmonella isolated from pigs and pig meat (Anon., 2013) and humans (Anon., 2014). Therefore, S. Typhimurium DT193 and its 4,[5],12:i:- variants pose a significant threat to human health. Until now, analysis of 4,[5],12:i:- strains has been based largely on molecular typing. There has been little investigation of the pathogenicity of monophasic or biphasic DT193 isolates, except for two poultry-infection studies (Martelli et al., 2014; Parsons et al., 2013).

During the early stages of infection in pigs, Salmonella is known to invade intestinal epithelial cells (Reed et al., 1986). Invasion is a key step during colonization of the gut and determines the consequent pathology of enteritis.
Salmonella utilizes the Salmonella Pathogenicity Island 1 type III secretion system to inject effector proteins into the host cell cytosol that facilitate bacterial invasion into the cell. The host innate immune response is triggered during this process via activation of signal transduction pathways, such as NFκB and mitogen-activated protein kinase, following recognition of pathogen-associated molecular patterns by receptors such as Toll-like receptors (TLRs). An important pathogen-associated molecular pattern of Salmonella and other Gram-negative bacteria is flagellin, the principal component of flagellar filament. Flagellin induces production and release of proinflammatory chemokines and cytokines, such as IL-8, from intestinal epithelial cells by acting as a stimulatory ligand for TLR-5 (Hayashi et al., 2001). The primary role of IL-8 secretion is as a chemoattractant for recruitment of neutrophils to the lamina propria (McCormick et al., 1995), an event that is instrumental in causing both the pathology of gastroenteritis and the clearance of bacteria from the gut (Zhang et al., 2003). In addition to activation of flagellin and/or flagellar responses, TLR-5 recognition of flagellin also initiates apoptotic signalling in intestinal epithelial cells (Paesold et al., 2002; Zeng et al., 2006).

As pigs are the major reservoir of Salmonella 4,[5],12:i:- and S. Typhimurium DT193 isolates (Hopkins et al., 2010) and current control methods of Salmonella in pigs rely primarily on hygienic measures with no guarantee of success, the characterization of infection with these strains in a porcine model is important in understanding the nature of infection in its main reservoir host. Identification of the virulence of these strains in pigs could inform future vaccine design and/or additional pre-harvest control measures for the limitation of their perpetuation in this host species. Also, comparison of the in vitro behaviour of monophasic and biphasic isolates with intestinal epithelial cells of this important host might reveal explanations for the recent emergence and success of strains expressing only one flagellar antigen, instead of two.

**METHODS**

**Bacterial strains and culture conditions.** The strains used in this study are listed in Table 1. The panel included a number of monophasic (4,[5],12:i:-) and classical Typhimurium isolates of Salmonella phage type DT193. S. Typhimurium 4/74 was included as a control isolate of defined virulence and ability to invade cultured cells (Watson et al., 1995). An afagellate mutant was created by P22 transduction of the fliC and fliB knockouts from JH3218 and JH3219 (both SL1344 background; Arques et al., 2009), respectively, into L00168. A single knockout mutant was created by P22 transducing ΔfliB::KanR from JH3219 into L00168. The deletions were confirmed by PCR. Isolates were streaked from cryoprotective beads stored at −80 °C onto nutrient agar and incubated at 37 °C for 24 h. Bacteria were then grown in Luria–Bertani (LB) broth for 16–18 h at 37 °C in an orbital incubator (150 r.p.m.) to achieve stationary phase cultures. For all assays, stationary cultures were diluted 1:100 into LB broth and grown for 3.5 h at 37 °C in an orbital incubator (150 r.p.m.) to achieve late-exponential phase cultures.

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<th>Isolate</th>
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<td>S01299</td>
<td>4,12:i:-</td>
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<td>S04327</td>
<td>4,5,12:i:-</td>
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**Cell culture.** The IPEC-1 cell line is an intestinal epithelial line derived from the small intestine of a neonatal unsuckled piglet (Gonzalez-Vallina et al., 1996). Cells were maintained in 75 cm² plastic cell culture flasks at 37 °C in a humidified atmosphere of 5 % CO₂. They were cultured and maintained in Dulbecco’s modified Eagle medium (DMEM/F-12; Invitrogen) supplemented with 5 % fetal calf serum, 100 IU penicillin ml⁻¹, 100 μg streptomycin ml⁻¹, 5 μg insulin ml⁻¹, 5 μg transferrin ml⁻¹, 5 ng selenium ml⁻¹ (ITS Premix; BD Biosciences) and 0.5 ng human epidermal growth factor ml⁻¹, hereafter referred to as IPEC-1 medium. Continuous cultures of IPEC-1 cells were maintained by passaging at a 1:3 or 1:5 ratio.

**Differential antibody staining.** When confluent, IPEC-1 cells were passaged and seeded onto sterile 13 mm glass coverslips in 12-well tissue culture plates (1 × 10⁶ cells per well) and grown at 37 °C in a humidified atmosphere (5 % CO₂) for 2 days. Prior to infection, cells were washed three times with PBS and overlaid with 1 ml pre-warmed antibiotic-free IPEC-1 medium. After equilibration in this medium at 37 °C, 5 % CO₂, bacterial cultures were added at an m.o.i. of 10. Cells were then incubated for 15 or 30 min at 37 °C in atmospheric air. Following this, invasion was quantified by differential immunofluorescence staining according to the method described by Perrett & Jepson (2007). Briefly, bacteria were immunolocated using goat anti-Salmonella CSA-1 antibody (Insight Biotechnology). Rabbit anti-goat Alexa Fluor 488 and Alexa Fluor 594 were used to label adhered and total bacteria, respectively, while IPEC-1 cells were labelled with DAPI. Cells were permeabilized for 20 min with 0.3 % (v/v) Triton X-100 in PBS. Counts of bacteria and IPEC-1 cells were performed using a Nikon Eclipse E80i fluorescence microscope during which 10 randomly selected fields were analysed per coverslip. Immunofluorescence images were acquired in the University of Liverpool’s Centre for Cell Imaging, using a Zeiss LSM 510 META two-photon laser scanning confocal microscope. Alexa Fluor 488 was detected using an argon (488 nm) laser, 565 nm dichroic and 500–530 nm bandpass filter. Alexa Fluor 594 was detected using a DPSS (561 nm) laser, 565 nm dichroic and 575 nm long pass filter. DAPI was detected using a SpectraPhysics MaiTai Ti: sapphire laser tuned to 700 nm, a 490 nm dichroic and 435–485 nm bandpass filter. Images were analysed using Imaris software (Bitplane).

**Autoaggregation assay.** The settling kinetics of the bacteria were monitored over time according to the method described by Wells et al. (2008). Late-exponential phase cultures of Salmonella were vortexed for 10 s and left to stand at room temperature or 4 °C for 3 h. At 30 min intervals, a 200 μl sample was taken from approximately 0.5 cm below the liquid–air surface and transferred to a microtitre plate (flat-bottomed; Greiner Bio-One) for determination of OD₅₀₀ with a Multiskan FC microplate reader (Thermo Scientific).
Real-time quantitative PCR. IPEC-1 cells were seeded into 24-well tissue culture plates and incubated at 37 °C, 5% CO₂ in antibiotic-free IPEC-1 medium for 24 h to give a final density of 1 x 10⁶ cells per well. Following infection of IPEC-1 cells with late-exponential phase bacterial cultures for 1 h (m.o.i. of 10) the medium was aspirated and 350 μl of RLT lysis buffer (Qiagen) was added to each well. The cell monolayers were disrupted by agitation with a pipette tip and the homogenates were removed and stored at -80 °C until required. Total RNA was isolated from the cell homogenates using an RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. The RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific). Isolated RNA was stored at -80 °C until required. Then, 10 ng of RNA from sample and uninfected control IPEC-1 cells was subjected to one-step quantitative reverse transcription PCR using the Rotor-Gene Probe reverse transcription PCR kit (Qiagen) on a Rotor-Gene Q cycler. Sequence-specific TaqMan Assay primer-probe sets (Life Technologies) for porcine IL-8 and TLR-5 were included in the reaction tubes. Reaction mixtures were set up using a QIAgility instrument, performed in triplicate and normalized to eukaryotic 18S rRNA. The following cycling conditions were used for amplification: 50 °C for 10 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The relative levels of IL-8 and TLR-5 gene expression were quantified using the 2⁻ΔΔCt method for each sample compared with uninfected control samples.

Fluorogenic caspase staining. IPEC-1 cells were grown in 24-well tissue culture plates, as described earlier. Following infection with late-exponential phase bacterial cultures (m.o.i. of 10) for 2 or 8 h, caspase activation in the IPEC-1 cells was detected using an Apo Logix carboxyfluorescein caspase detection kit (Cell Technology), containing the caspase inhibitor FAM-VAD-FMK, according to the manufacturer’s protocol. Briefly, medium was aspirated from the plate wells and cells were released from the plastic with 500 μl trypsin-EDTA for 5 min. Then, 500 μl IPEC-1 medium was added to deactivate the trypsin and cells were harvested into a 12 X 75 mm² tube and pelleted by centrifugation at 400 g for 5 min at room temperature. The medium was decanted and cells were labelled with 10 μl FAM-VAD-FMK in 300 μl IPEC-1 medium and incubated at 37 °C for 1 h. Cells were then washed twice with the kit wash buffer at a working dilution. Finally, propidium iodide (5 μl) was added to cell suspensions and incubated on ice for 5–15 min to stain dead cells. Unfixed, stained cell suspensions were analysed immediately using a BD Accuri C6 flow cytometer, with measurement of 10000 fluorescent events per sample.

Statistical analysis. Statistical analysis was performed using MiniTab 16. Mean values and SD were calculated and isolates were compared using one-way ANOVA. Differences were considered significant at P<0.05. In the event that a significant difference was found, the Tukey method of multiple comparisons was performed.

RESULTS

Monophasic expression of flagella does not affect invasion ability

Given that flagella-derived motility is crucial for bringing Salmonella into contact with the intestinal epithelium (Misselwitz et al., 2012), we wanted to investigate the effect of monophasic expression of flagella on the ability of Salmonella to invade IPEC-1 cells. The aflagellate mutant (ΔfliCAfljB) was attenuated in adhesion and invasion of IPEC-1 cells, compared with the parent strain L00168. Fluorescence microscopy revealed that the S. Typhimurium DT193 isolates S00398 and S01557 (both biphasic) were significantly less adhesive (Fig. 1a) and invasive (Fig. 1b) than the positive control strain S. Typhimurium 4/74 (P<0.05). However, a third biphasic isolate, L00168, showed comparable adhesive and invasive ability to 4/74. The adhesive and invasive ability of the monophasic strains of DT193 were not significantly different from that of 4/74 and no significant differences were detected between the monophasic and biphasic isolates. These results were seen after infection times of both 15 and 30 min.

Distribution of Salmonella across a cell monolayer is heterogeneous

During microscopic analysis it was noted that the distribution of bacteria was not homogeneous through the cell monolayers. Rather, interactions between epithelial cells and bacteria were confined to just 13% of IPEC-1 cells, calculated from the total number of IPEC-1 cells counted per coverslip and the number of those cells with associated bacteria. This proportion was not statistically different across monolayers infected with the different isolates. It was also noted that while many IPEC-1 cells had just one or two bacteria attached or within them, there were cells that appeared to be experiencing ‘hyperinvasion’ of bacteria (Fig. 2). A maximum number of 10 bacteria per host cell was set for ease of counting when analysing the distribution of bacteria (but not when counting for adhesion and invasion). However, in reality the number of bacteria involved in these hyperinvasion events often reached up to and above 100. This phenomenon was most often seen during infection with S01299 (4,12:i:-), S03554 (4,5,12:i:-) and 4/74. It appears that the isolates that exhibited the greatest invasive ability were the same isolates that demonstrated ‘hyperinvasion’ (Figs 1b and 2b). However, even the isolates that were relatively less invasive showed evidence of hyperinvasion (S01557, Fig. 2b).

Expression of TLR-5 and IL-8 mRNA increases in response to Salmonella DT193 infection

The induction of a proinflammatory response from intestinal epithelial cells via flagellin-dependent stimulation of TLR-5 is a well-characterized signalling pathway (Elewaut et al., 1999; Gewirtz et al., 2001a, b; Hayashi et al., 2001; Tallant et al., 2004; Yu et al., 2003; Zeng et al., 2003). We used quantitative real-time PCR to determine whether monophasic expression of flagella affects expression of TLR-5, the receptor for flagellin, and resultant induction of the proinflammatory chemokine IL-8 (Fig. 3). At 1 h post-infection, upregulation of TLR-5 mRNA was detected in IPEC-1 cells in response to all Salmonella isolates, regardless of whether they were monophasic or not, with the exception of the aflagellate mutant. In most instances, the increase in gene expression was modest (<50-fold), but infection with 4,5,12:i:- isolate S03554 resulted in a significantly greater increase in TLR-5 expression (100-fold; P<0.0000). IL-8
mRNA levels in IPEC-1 cells increased in response to infection with all isolates studied here. The aflagellate mutant stimulated a 40-fold increase in IL-8 expression, despite a lack of TLR-5 induction. Elegantly, the exceptionally high TLR-5 mRNA levels observed following infection with S03554 correlated with high induction of IL-8 expression. The aflagellate mutant stimulated a 40-fold increase in IL-8 expression, despite a lack of TLR-5 induction. Elegantly, the exception-

To confirm that these results are not exclusive to the particular 4,5,12:i:- isolates chosen for this study, we created an isogenic monophasic (ΔfljB) mutant of L00168 by P22 transduction. Examination of the ΔfljB mutant by transmission electron microscopy revealed that its flagella are stunted in length compared with the parent strain. This isolate also has greatly reduced motility compared with the parent strain (data not shown). Despite its reduced motility, infection with this isolate elicited upregulation of both TLR-5 and IL-8 mRNA (~5- and ~100-fold, respectively).

Monophasic expression of flagellin has no impact on caspase activation

Previous studies have shown that flagellin-induced proinflammatory responses suppress apoptosis via activation of anti-apoptotic genes (Vijay-Kumar et al., 2006; Zeng et al., 2006). Given that the 4,5,12:i:- isolates in this study were not attenuated in their ability to stimulate IL-8 we hypothesized that similar levels of apoptotic activation would be seen in IPEC-1 cells infected with these strains compared with biphasic isolates. Analysis of cells treated with a fluorescent substrate that binds to enzymically active caspases (caspase-1, -2, -3, -4, -5, -6, -7, -8 and -9) by flow cytometry enabled us to differentiate between cells with active apoptotic pathways and those without. Staining with propidium iodide (a membrane-impermeant compound) enabled further discrimination between viable and non-viable cells (Fig. 4a). After 2 h of infection, levels of activated caspases (as determined by mean fluorescence intensity of the fluorescent substrate) were increased in infected IPEC-1 cells compared with uninfected controls, although this did not reach statistical significance (Fig. 4b). No differences were observed between cells challenged with any of the DT193 isolates (Fig. 4c). The proportion of live cells positive for active caspases increased only marginally from 35–50 % at 2 h to 48–58 % at 8 h (Fig. 4c), possibly due to the non-specific nature of the fluorescent reagent. Again, no differences were observed between the isolates.

More cell death was observed in infected IPEC-1 cells than in uninfected controls after 2 h (Fig. 4d). No differences in cell death were observed between cells challenged with any of the DT193 isolates.

DISCUSSION

In this study we have investigated the virulence of Salmonella 4,5,12:i:- and S. Typhimurium phage type DT193 strains isolated from pig faeces on farms in the UK in a tissue culture model of early intestinal infection. This is, to our knowledge, the first investigation of naturally occurring 4,5,12:i:- isolates in a porcine model of Salmonella infection. We show that monophasic expression of the phase 1 flagellar antigen, FliC, does not affect the ability of 4,5,12:i:- isolates to adhere to and invade IPEC-1 cells. The level of adhesion and invasion of the 4,5,12:i:- isolates was comparable to that of DT193 isolates expressing both phases of flagellar antigen. A study involving phase-locked

**Fig. 1.** Comparison of adhesion and invasion of Salmonella 4,5,12:i:- and Typhimurium DT193 isolates. IPEC-1 cells grown on glass coverslips were infected with Salmonella DT193 isolates and 4/74 at an m.o.i. of 10 for 15 min (dark grey) or 30 min (pale grey). Adhesion (a) and invasion (b) was assessed by differential antibody staining in three independent experiments with each experiment performed in duplicate. Results are means ± SEM, where at least 200 IPEC-1 cells were assessed per coverslip from 10 randomly selected fields of view. Statistically significant differences from the positive control strain 4/74 at 15 min (asterisks) and 30 min (daggars) are indicated as follows: *P<0.05, †P<0.05, ††P<0.01.
Fig. 2. Comparison of distribution of *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates. IPEC-1 cells grown on glass coverslips were infected with *Salmonella* DT193 isolates and *S. Typhimurium* 4/74 at an m.o.i. of 10 for 30 min. Immunolocalization of external (green) and total (red) bacteria was assessed by fluorescence microscopy and where an IPEC-1 cell (nuclei, blue) was associated with bacteria, the number of bacteria was estimated. Cells were permeabilized with 0.3 % (w/v) Triton X-100 in PBS for 20 min. (a) Summary of mean proportion of cells with greater than 10 associated (adhered and invaded) bacteria. Data were calculated from three independent experiments in which at least 200 IPEC-1 cells were assessed. (b) Representative confocal images of S01299 (4,12:i:-), S01557 (phase-variable) and 4/74. Maximum intensity projections of 3D datasets are shown, with optical slices acquired at 1 μm intervals. Bars, 15 μm. External bacteria are indicated by arrowheads.
derivatives of S. Typhimurium SL3201 expressing only FliC or FljB showed no differences from the wild-type parent strain in adherence and invasion of mouse epithelial cells or in a bovine ligated loop assay (Ikeda et al., 2001). The authors concluded that flagellar phase variation is not involved in the intestinal stage of infection. Our results suggest the same might be the case in porcine infection. It is interesting to note that both fliC and fljB were upregulated during S. Typhimurium persistence in porcine tonsils, but not in the ileum or ileocaecal lymph nodes, during in vivo infection (Van Parys et al., 2011). The role of these flagellar proteins and phase variation during different stages of Salmonella infection and during interaction with different tissues and hosts is clearly complex. It is therefore important to elucidate their involvement in all relevant experimental settings.

Our results also suggest that S. Typhimurium DT193 and 4,[5],12::i:- variants may be capable of colonizing the small intestine, although confirmation of this in vivo is necessary. Similarly, previous work in our laboratory has shown that these isolates are capable of colonizing the caeca of chickens (Parsons et al., 2013). Monophasic and biphasic variants of S. Typhimurium phage type DT193 have both been shown to survive longer in pig faeces than other serotypes, which may facilitate transmission and therefore contribute to their increased prevalence (Rajtak et al., 2012). Taken together, these findings suggest that monophasic and phase-variable Salmonella DT193 strains possess key virulence traits which may promote infectivity and dissemination.

Consistent with infection studies in the mouse (Hapfelmeier et al., 2005) and bovine ligated loop (Reis et al., 2003), we have shown that invasion occurs in only a small fraction of mucosal cells during infection. Given the short infection times studied here, we believe that the observed foci of infection, with high numbers of bacteria interacting with single host cells, were unlikely to be due to hyper-replication of cytosolic bacteria, a phenomenon described by Knodler et al. (2010). Autoaggregation assays performed with each isolate also suggest that hyperinvasion was not due to clumping of bacterial cells during culture (data not shown). Preferential invasion of specific target cells, namely mitotic cells and cells with membrane ruffles, and cooperative invasion at these target sites, has been observed by others during Salmonella infection of HeLa cells (Misselwitz et al., 2010, 2011, 2012). However, without data pertaining to the morphology of the IPEC-1 cells experiencing high bacterial loads in this study, we cannot be certain that this was targeted cell invasion. Similarly, the reason why some of the isolates displayed greater incidence of ‘hyperinvasion’ than others remains elusive.

Consistent with Zeng et al. (2003), who found that monophasic mutants of S. Typhimurium SL3201 were able to stimulate full proinflammatory gene expression profiles from human colonic epithelial cells, we have shown that monophasic Salmonella DT193 are able to increase TLR-5 and IL-8 mRNA expression levels in porcine intestinal epithelial cells. This upregulation was seen following infection with both naturally occurring 4,[5],12::i:- isolates and a ΔfljB knockout mutant derived from a phase-variable DT193 isolate. It has previously been shown that only the conserved domain of flagellin is required for TLR-5 activation (Eaves-Pyles et al., 2001; Smith et al., 2003; Yoon et al., 2012), suggesting that the receptor is unable to discriminate between FljC and FljB. Similarly, purified FliC and FljB have been reported to activate similar levels of NFkB, a transcription factor activated by TLR-5, in cultured cells (Simon & Samuel, 2007). The ability of the 4,[5],12::i:- isolates in this study to stimulate TLR-5 expression, despite lacking expression of FljB, is in keeping with these findings.

![Figure 3](image-url)
The proinflammatory response of epithelial cells, characterized by production of IL-8 and other chemokines, following recognition of bacterial flagellin by TLR-5 is only part of the host innate immune response. Activation of caspases, the initiators of programmed cell death, is another response of epithelial cells to flagellin-derived TLR-5 stimulation that occurs in parallel (Zeng et al., 2006). If this arm of the response was permitted to proceed without regulation, infected cells would undergo apoptosis, presumably as a mechanism of eradicating bacteria from the host. However, unimpeded apoptosis of intestinal epithelial cells would ultimately result in severe tissue damage, so recognition of flagellin also activates anti-apoptotic processes in epithelial cells in order to delay apoptosis (Vijay-Kumar et al., 2006). The proportion of dead IPEC-1 cells increased only marginally between 2 and 8 h, suggesting that despite a large proportion of the total cell population having detectable active caspases, the rate of programmed cell death was low, perhaps due to the aforementioned regulation of apoptosis. The activation of caspases observed here seems to have been more rapid than in other studies. For example, Vijay-Kumar et al. (2006) observed only very low activation of caspase-3,
-8 and -9 after 2 h of infection. However, it is possible that the level of caspase activation seen in this study is a result of using a fluorescent peptide that binds to caspases -1 to -9 for detection. As the fluorescent substrate used binds to all active caspases, we were unable to determine which specific caspases were responsible for the fluorescent signal. Indeed, some caspase activation might have occurred independently of infection or apoptosis as part of routine cellular development and proliferation within the cultured monolayer (Schwerk & Schulze-Osthoff, 2003). Epithelial cells infected with S. Typhimurium have been reported to activate caspase-1 (Knodler et al., 2010), the primary initiator of an alternative programme of cell death known as pyroptosis, which is usually associated with Salmonella-infected macrophages (reviewed by Fink & Cookson, 2007). Further investigation is needed to determine whether the relatively high proportion of cells with active caspases at 2 h post-infection observed in this study was due to faster activation, or activation of a wider repertoire of caspases. What is clear from the results, however, is that there is no difference between monophasic and biphasic DT193 isolates in their ability to stimulate caspase activation or in their cytotoxicity.

A porcine model of infection was chosen for this research because of the crucial role of pigs as a reservoir for these isolates. Given that S. Typhimurium uses both conserved and host-specific factors during colonization of the intestine (Carnell et al., 2007; Morgan et al., 2004; Tsolis et al., 1999) it is important to study initial host–pathogen interactions, such as those investigated in this study, on a host-by-host basis (Bearson & Bearson, 2011). Therefore, the effects shown in this work may or may not be unique to porcine cells and further work investigating the interaction of these isolates with cells derived from humans and other animals would be valuable. Our results suggest that monophasic expression of FlIC is not an attenuating feature of Salmonella DT193 during adhesion, invasion, TLR-5 recognition, stimulation of inflammation and activation of caspases in porcine epithelial cells. That monophasic variants of S. Typhimurium have comparable virulence to phase-variable strains is perhaps not that surprising given that S. Enteritidis is also monophasic (Imre et al., 2005; van Asten et al., 1995) and yet able to cause widespread infection in a number of host species.

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