

Cell invasion of poultry-associated *Salmonella enterica* serovar Enteritidis isolates is associated with pathogenicity, motility and proteins secreted by the type III secretion system

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Salmonella enterica serovar Enteritidis (*S. Enteritidis*) is a major cause of food-borne gastroenteritis in humans worldwide. Poultry and poultry products are considered the major vehicles of transmission to humans. Using cell invasiveness as a surrogate marker for pathogenicity, we tested the invasiveness of 53 poultry-associated isolates of *S. Enteritidis* in a well-differentiated intestinal epithelial cell model (Caco-2). The method allowed classification of the isolates into low ($n=7$), medium ($n=18$) and high ($n=30$) invasiveness categories. Cell invasiveness of the isolates did not correlate with the presence of the virulence-associated gene *spvB* or the ability of the isolates to form biofilms. Testing of representative isolates with high and low invasiveness in a mouse model revealed that the former were more invasive *in vivo* and caused more and earlier mortalities, whereas the latter were significantly less invasive *in vivo*, causing few or no mortalities. Further characterization of representative isolates with low and high invasiveness showed that most of the isolates with low invasiveness had impaired motility and impaired secretion of either flagella-associated proteins (FlgK, FljB and FlgL) or type III secretion system (TTSS)-secreted proteins (SipA and SipD) encoded on *Salmonella* pathogenicity island-1. In addition, isolates with low invasiveness had impaired ability to invade and/or survive within chicken macrophages. These data suggest that not all isolates of *S. Enteritidis* recovered from poultry may be equally pathogenic, and that the pathogenicity of *S. Enteritidis* isolates is associated, in part, with both motility and secretion of TTSS effector proteins.

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INTRODUCTION

Salmonella enterica serovar Enteritidis (*S. Enteritidis*) is the leading cause of food-borne salmonellosis (WHO, 2008). *S. Enteritidis*-induced salmonellosis in humans is characterized by diarrhoea, fever, headache, abdominal pain, nausea and vomiting (CDC, 2007). *S. Enteritidis* is also increasingly reported from cases of invasive and extra-intestinal infections such as septicaemia, arthritis, endocarditis, meningitis and urinary tract infections (Ghosh & Vogt, 2006; Gordon *et al.*, 2008; Katsenos *et al.*, 2008; Kobayashi *et al.*, 2009; Morpeth *et al.*, 2009; Mutlu *et al.*, 2009; Tena *et al.*, 2007). Between 1990 and 2001, the US state and

territorial health departments reported 677 *S. Enteritidis* outbreaks, which accounted for 23 366 illnesses, 1988 hospitalizations and 33 deaths (CDC, 2003). In 2006, countries within the European Union reported 1729 outbreaks caused by *S. Enteritidis* leading to 13 853 illnesses, 2714 hospitalizations and 14 deaths (EFSA, 2007). The Health Protection Agency of the UK reported 4194 cases of food-borne *S. Enteritidis* infection in 2008 (HPA, 2008). Poultry is considered the single largest reservoir of *S. Enteritidis* and most risk attribution studies have identified poultry and poultry products as the major source of human infection. *S. Enteritidis* is passed to humans mainly via handling and consumption of contaminated poultry meat and eggs (De Buck *et al.*, 2004; Kimura *et al.*, 2004; Little *et al.*, 2008; Marcus *et al.*, 2004; Patrick *et al.*, 2004; Snow *et al.*, 2007). For instance, eggs and egg-containing products were implicated as primary vehicles of *S. Enteritidis*

Abbreviations: MLVA, multi-locus variable-number tandem repeat analysis; PI, post-infection; SPI, *Salmonella* pathogenicity island; TEM, transmission electron microscopy; TTSS, type III secretion system.

infection in 298 (80%) of the 371 known-source outbreaks reported to the Centers for Disease Control and Prevention from 1985 to 1999 (Patrick *et al.*, 2004).

On-farm investigations have revealed that once chickens are exposed to *S. Enteritidis*, the entire poultry flock can become colonized quickly (Altekruse *et al.*, 2006; Berrang *et al.*, 2009; Foley *et al.*, 2008). Infected birds shed bacteria in their faeces, leading to carcass contamination during slaughter house processing. *S. Enteritidis* is also invasive in both young and adult chickens. Young chickens less than 2 weeks of age often develop systemic disease with varying degrees of mortality (Duchet-Suchaux *et al.*, 1995; Suzuki, 1994; Velge *et al.*, 2005) whereas most adult hens colonized with *S. Enteritidis* become intestinal carriers and typically remain asymptomatic (Golden *et al.*, 2008; Lister, 1988; Velge *et al.*, 2005). *S. Enteritidis* can invade internal organs of laying hens and infect reproductive tissues leading to internal contamination of newly formed eggs (De Buck *et al.*, 2004; Guard-Petter, 2001; Velge *et al.*, 2005). It is believed that the specialized ability of *S. Enteritidis* to contaminate developing eggs acts as an ecological amplifier that facilitates dissemination of *S. Enteritidis* in the food chain and its eventual transmission to humans.

S. Enteritidis is the most genetically homogeneous serotype of *Salmonella* (Hudson *et al.*, 2001; Liebana *et al.*, 2001; Morales *et al.*, 2005; Porwollik *et al.*, 2005; Swaminathan *et al.*, 2001). Despite limited genomic diversity, variation in phenotypic traits, including the ability to form biofilm, grow to high cell density, produce high-molecular-mass LPS and survive within egg albumin, has been commonly observed among field isolates of *S. Enteritidis* (Clavijo *et al.*, 2006; Gast & Holt, 1995, 2001; Guard-Petter, 1998; Guard-Petter *et al.*, 1999; Jain & Chen, 2007; Lock & Board, 1992; Pan *et al.*, 2009; Parker *et al.*, 2001; Solano *et al.*, 1998; Yim *et al.*, 2010). *S. Enteritidis* strains also vary in their virulence potential in poultry, irrespective of phage types (Poppe *et al.*, 1993) and even within clonal lineages (Olsen *et al.*, 1999; Yim *et al.*, 2010). Dhillon *et al.* (1999) tested six strains of *S. Enteritidis* and showed that mortalities in experimentally challenged 1-day-old chickens ranged from 0 to 23%, whereas Barrow (1991) reported that the mortalities caused by six strains of *S. Enteritidis* varied from 20 to 96%. Similarly, Gast & Benson (1995, 1996) showed that *S. Enteritidis* strains differed significantly in not only their ability to cause mortality but also their ability to colonize intestinal tracts and invade the spleen and liver of 1-day-old chickens.

Although limited numbers of *S. Enteritidis* strains were tested, the above studies have shown that isolates of *S. Enteritidis* vary in their virulence potential, at least in poultry. Most other studies to characterize the pathogenic potential of *S. Enteritidis* strains have primarily focused on isolates recovered from either human clinical cases or non-poultry-associated environmental sources (Betancor *et al.*, 2009; Lu *et al.*, 1999; Pan *et al.*, 2009; Solano *et al.*, 1998, 2001; Yim *et al.*, 2010). Humphrey *et al.* (1996) showed

that human isolates of *S. Enteritidis* PT4 with enhanced heat and acid tolerance were more virulent in mice and more invasive in chickens compared with poultry-associated strains of *S. Enteritidis*. However, only two strains from each source were tested (Humphrey *et al.*, 1996). Similarly, Pang *et al.* (2006) showed that poultry-associated strains of *S. Enteritidis* differed in their invasion potential in cultured Int-407 cells, but only eight strains were tested. Despite poultry being a reservoir host for *S. Enteritidis* and a major source of human infection worldwide, it is still unclear if differential pathogenicity translates into differential risk for human. In addition, members of this serotype of *Salmonella* harbour limited genetic variance and it is not clear what genetic mechanisms underlie differential virulence.

We hypothesized that not all *S. Enteritidis* isolates recovered specifically from poultry and poultry environments are equally pathogenic. By using invasion as a surrogate marker of virulence, we tested the invasion potential of 53 poultry-associated *S. Enteritidis* isolates in a well-differentiated human intestinal cell culture model (Caco-2). The results confirmed the identity of a low invasiveness group of isolates that were also less virulent in a mouse model. Lower virulence was associated, in part, with impaired motility and reduced secretion of *Salmonella* pathogenicity island-1 (SPI-1) type-three secretion (TTSS) secreted proteins. In addition, we found that isolates with low invasiveness were impaired in their ability to invade and/or survive within chicken macrophages, indicating that *S. Enteritidis* isolates that are less invasive in chickens are likely to be less pathogenic in mammalian hosts such as humans.

METHODS

Bacterial strains. *S. Enteritidis* isolates (Table 1) analysed in this study were stored at -80°C in 15% (v/v) phosphate-buffered glycerol. All isolates were grown in Luria-Bertani (LB) broth with shaking at 150 r.p.m. or on LB agar plates at 37°C , except when indicated. All isolates were tested for their reactivity with Difco *Salmonella* O antiserum Group D1 by the plate agglutination test according to the manufacturer's instructions. The MIC of gentamicin was determined for all isolates by the microdilution method described by Andrews (2001).

PCR screening of *S. Enteritidis* strains. PCR amplification of the *sefA* gene, encoding SEF14 fimbrial antigen, was used for preliminary confirmation of the identity of *Salmonella* isolates (Doran *et al.*, 1996). A chromosomally located *sdfl* gene reported to be unique to the serovar *S. Enteritidis* was targeted for the serotype-specific identification of *S. Enteritidis* (Agron *et al.*, 2001; Alvarez *et al.*, 2004; Clavijo *et al.*, 2006; Trafny *et al.*, 2006). A 60 kb virulence-plasmid-associated *spvB* gene was used as a marker for determination of the presence or absence of a typical virulence plasmid (pSEV) among *S. Enteritidis* isolates (Cho *et al.*, 2007; Rotger & Casadesús, 1999; Rychlik *et al.*, 2006, 2008). PCR amplification of complete ORFs of the *sipA*, *sipD*, *flgK*, *fljB* and *flgL* genes was performed to detect the presence or absence of these genes among selected *S. Enteritidis* isolates. All PCRs were run in 30 μl volumes containing 5 μl boiled lysates as template DNA and 21 μl Cougar PCR

Table 1. Source, genotype and phenotypic characteristics of *S. Enteritidis* strains isolated from poultry and poultry environments

Strain (phage type)	Supplier designation	Source	Supplier*	MLVA type	Caco-2 cell invasion assay		Biofilm production		PCR screening		
					Mean \pm SEM \log_{10} (c.f.u. ml^{-1})	Invasion phenotype	Agar plate assay	Microtitre assay (OD ₄₉₀)	<i>spvB</i>	<i>sefA</i>	<i>sdfI</i>
C1	LK5	Egg	SGSC	4a	5.84 \pm 0.06	High	bf-	0.09	+	+	+
C19 (PT8)	SA5985	Meat	SGSC	11	4.46 \pm 0.06	Low	bf-	0.06	+	+	+
C20 (PT13)	SA5986	Heart blood	SGSC	10c	5.69 \pm 0.05	High	bf+	0.16	+	+	+
C44	SE310	Heart blood	SGSC	10c	5.69 \pm 0.04	High	bf-	0.10	+	+	+
C45	SE154	Ovary	SGSC	11	4.40 \pm 0.04	Low	bf-	0.06	+	+	+
BC1 (PT8)	2007-2700	Yolk sac	AHC	4b	5.69 \pm 0.25	High	bf-	0.09	+	+	+
BC2	2005-3637	Turkey lung	AHC	12	5.75 \pm 0.01	High	bf+i	0.14	+	+	+
BC3	2005-3918	Heart	AHC	10c	5.57 \pm 0.22	High	bf+m	0.11	+	+	+
BC4 (PT8)	2008-0455	Heart	AHC	10c	5.41 \pm 0.28	High	bf+m	0.13	+	+	+
BC5	2007-0734-52-1	Farm environment	AHC	6	5.39 \pm 0.07	High	bf-	0.10	+	+	+
BC6 (PT13)	2008-0742-1723F-8	Fluff	AHC	8a	5.77 \pm 0.08	High	bf+m	0.16	+	+	+
BC7 (PT8)	2007-0275F-12	Fluff	AHC	10c	5.66 \pm 0.03	High	bf-	0.11	+	+	+
BC8	2005-2949	Fluff	AHC	9a	5.81 \pm 0.14	High	bf+m	0.12	+	+	+
A3	E2006001733	Chicken	MDH-PHLD	1	5.37 \pm 0.17	High	bf-	0.11	+	+	+
A5	E2004001561	Chicken swab	MDH-PHLD	10a	5.74 \pm 0.07	High	bf-	0.10	+	+	+
A7	I2008001102	Litter	MDH-PHLD	10b	5.01 \pm 0.09	Medium	bf+	0.14	+	+	+
A8	E2007001234	Chicken	MDH-PHLD	11	5.05 \pm 0.14	Medium	bf+	0.15	+	+	+
A11	E2006001732	Litter	MDH-PHLD	10c	4.65 \pm 0.12	Low	bf-	0.11	+	+	+
P3	00-983-2	Broiler carcass rinse	WADDL	3	4.71 \pm 0.11	Low	bf-	0.11	+	+	+
P12	90-734378(10)	Chicken	WADDL	11	5.46 \pm 0.10	High	bf+	0.19	+	+	+
P13	388(9)	Layer chicken	WADDL	11	5.48 \pm 0.07	High	bf+m	0.13	+	+	+
P14	90-855307	Chicken	WADDL	11	5.42 \pm 0.05	High	bf+i	0.12	+	+	+
P15	4/11/91210	Chicken	WADDL	11	5.35 \pm 0.05	High	bf-	0.11	+	+	+
P16	9-1222W622-7	Chicken	WADDL	11	5.46 \pm 0.15	High	bf-	0.07	+	+	+
P21	4/11/91560	Chicken	WADDL	9a	5.76 \pm 0.23	High	bf-	0.06	+	+	+
P27	9-1144 W4 22-10	Chicken	WADDL	11	5.64 \pm 0.02	High	bf+	0.14	+	+	+
Pu35	4534	Chicken	FDIU	12	5.11 \pm 0.19	Medium	bf+	0.18	+	+	+
Pu40	8879	Chicken	FDIU	12	5.14 \pm 0.30	Medium	bf+	0.15	+	+	+
Pu43	10520	Chicken	FDIU	11	5.10 \pm 0.15	Medium	bf+m	0.15	+	+	+
Pu45	10610	Chicken	FDIU	2	4.64 \pm 0.12	Low	bf+i	0.13	+	+	+
Pu48	12454	Chicken	FDIU	7	5.38 \pm 0.10	High	bf+	0.15	+	+	+
Pu50	15898	Chicken	FDIU	11	5.17 \pm 0.01	Medium	bf+	0.17	+	+	+
Pu51	15899	Chicken	FDIU	11	5.54 \pm 0.05	High	bf-	0.08	+	+	+
G1† (PT4)	22079	Chicken	ESQRU	13a	4.93 \pm 0.14	Medium	bf+	0.21	+	+	+
G2† (PT13a)	21046	Chicken	ESQRU	9b	5.69 \pm 0.10	High	bf-	0.08	+	+	+
G3† (PT13a)	21027	Chicken	ESQRU	5	5.29 \pm 0.10	Medium	bf+	0.16	+	+	+
G9 (PT13a)	21101	Faecal	ESQRU	4a	5.42 \pm 0.24	High	bf-	0.05	-	+	+
G10 (PT4)	22080	Farm environment	ESQRU	13b	5.12 \pm 0.00	Medium	bf+	0.18	+	+	+
G11 (PT13a)	27003	Chicken	ESQRU	4a	5.38 \pm 0.28	High	bf+	0.24	-	+	+
G12 (PT13a)	21052	Egg yolk	ESQRU	4a	5.44 \pm 0.24	High	bf-	0.08	-	+	+
G14 (PT13a)	21100	Faecal	ESQRU	4a	5.13 \pm 0.07	Medium	bf-	0.06	-	+	+
G15 (PT13)	26017	Chicken	ESQRU	8b	4.97 \pm 0.23	Medium	bf+	0.15	+	+	+
G21 (PT13a)	21056	Egg yolk	ESQRU	4a	5.13 \pm 0.03	Medium	bf-	0.11	-	+	+
G22†	21028	Farm environment	ESQRU	5	4.50 \pm 0.06	Low	bf+	0.18	+	+	+
G29† (PT4)	22077	Farm environment	ESQRU	13a	5.74 \pm 0.06	High	bf-	0.08	+	+	+
G31	22150	Egg	ESQRU	4a	5.08 \pm 0.03	Medium	bf-	0.07	-	+	+

Table 1. cont.

Strain (phage type)	Supplier designation	Source	Supplier*	MLVA type	Caco-2 cell invasion assay		Biofilm production		PCR screening		
					Mean \pm SEM log ₁₀ (c.f.u. ml ⁻¹)	Invasion phenotype	Agar plate assay	Microtitre assay (OD ₄₉₀)	<i>spvB</i>	<i>sefA</i>	<i>sdfl</i>
G33 (PT8)	22083	Farm environment	ESQRU	10c	4.86 \pm 0.27	Medium	bf+m	0.17	+	+	+
G34 (PT13a)	27035	Oviduct	ESQRU	4a	5.17 \pm 0.17	Medium	bf+	0.18	-	+	+
G36†	21030	Farm environment	ESQRU	5	4.89 \pm 0.06	Medium	bf+	0.14	+	+	+
G45	21063	Spleen	ESQRU	4a	4.18 \pm 0.18	Low	bf-	0.09	-	+	+
G46†	21025	Farm environment	ESQRU	5	5.37 \pm 0.35	High	bf+	0.19	+	+	+
G47 (PT13a)	27020	Caeca	ESQRU	4a	5.67 \pm 0.04	High	bf+	0.17	-	+	+
UK (PT4)	P125109	Chicken	WTSA	13a	5.91 \pm 0.06	High	bf+	0.17	+	+	+

*SGSC, Salmonella genetic stock centre, University of Calgary, BC, Canada; AHC, Animal Health Centre, Abbotsford, BC, Canada; MDH-PHLD, Minnesota Department of Health Public Health Laboratory Division, St Paul, MN, USA; WADDL, Washington Animal Disease Diagnostic Laboratory, Puyallup, WA, USA; FDIU, Field Disease Investigation Unit, Washington State University, Pullman, WA, USA; ESQRU, Egg Safety and Quality Research Unit, Athens, GA, USA; WTSA, Wellcome Trust Sanger Institute, London, UK.

†Originally isolated from mice captured from poultry farm premises and subsequently tested in a chicken infection model.

master mix (Custom Genome Services). Each primer was added to 200 nM (Table 2). The cycling conditions consisted of denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C (*spvB*), 53 °C (*sipA*, *sipD*, *flgK*, *fljB* and *flgL*), 58 °C (*sdfl*) or 59 °C (*sefA*) for 30 s and extension at 72 °C for 40 s. Samples were fractionated by using 1% (w/v) agarose gel and visualized by ethidium bromide staining.

Multi-locus variable-tandem repeat analysis (MLVA) typing.

Primers including forward dye-labelled primers for MLVA were purchased from Applied Biosystems (Table 2). The multiplex-PCR procedure was performed as previously described with few modifications (Malorny *et al.*, 2008). Briefly, a single colony of each strain was suspended in 200 μ l 5% Chelex 100 solution (Bio-Rad) with 100 μ g proteinase-K, incubated at 56 °C for 45 min, and boiled for 20 min followed by centrifugation at 14 000 r.p.m. for 1 min. For all PCRs, 2 μ l supernatant from boiled Chelex lysate was used as template DNA. Multiplex-PCRs were carried out in an iCycler thermal cycler (Bio-Rad) by using the Qiagen multiplex PCR kit, according to the manufacturer's instructions, in 12.5 μ l volumes with 1.25 μ l 10 \times PCR buffer and 1.0 pmol of primer pairs SENTR1, SENTR2, SENTR3 and SE-7, and 0.25 pmol each of primers SENTR4, SENTR5, SENTR6, SENTR7 and SE-3 (Table 2). Results for the SENTR2 locus were difficult to interpret because of multiple peaks and therefore this locus was excluded from the analysis. Cycling conditions included an initial denaturation at 94 °C for 15 min, followed by 28 cycles of 94 °C for 30 s, 55 °C for 90 s and 72 °C for 90 s, with a final extension step at 72 °C for 10 min. Each multiplex reaction was diluted 1:10 with PCR-grade water. An aliquot (0.5 μ l) of diluted multiplex PCR product was mixed with 0.5 μ l GS1200LIZ size standard (Applied Biosystems) and 19 μ l Hi-Di formamide (Applied Biosystems) (total volume 20 μ l for capillary electrophoresis). Capillary electrophoresis was carried out by the Genomics Core Laboratory at Washington State University by using a 3730 DNA analyser with Pop-7 polymer (Applied Biosystems). The resulting electropherograms were analysed by using GeneMarker software (Softgenetics LLC). Fragment sizes for each VNTR locus were converted into numbers of predicted repeats and analysed with

Bionumerics software (Applied Maths). Cluster analysis of the categorical coefficient was performed by using the unweighted pair group method with arithmetic average (UPGMA) algorithm (Sneath & Sokal, 1973).

In vitro invasion assays with differentiated Caco-2 cells. Caco-2 human colon adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC HTB-37) and were routinely grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen) in 95% air/5% CO₂ at 37 °C. Propagated cells were seeded at approximately 10⁶ cm⁻² into 75 cm² flasks (BD falcon). After 5 days of culture, cells were harvested by trypsinization and resuspended in DMEM with 10% FBS. Subsequently, cells were plated onto 12-well plates (Cellstar) at \sim 1 \times 10⁶ cells per well. The medium was changed daily until confluency and thereafter every second day. Confluency occurred after 5–6 days of incubation and well-differentiated monolayers of Caco-2 cells were obtained 14 days after seeding for the assays. The state of cell differentiation was monitored by estimating the production of intestinal alkaline phosphatase by using a SensoLyte pNPP alkaline phosphatase assay kit (AnaSpec) following the manufacturer's instructions. Intestinal alkaline phosphatase is a brush border enzyme expressed exclusively in villus-associated enterocytes, and expression indicates the development of digestive and absorptive function (Goldberg *et al.*, 2008; Hara *et al.*, 1993). Cell differentiation was also confirmed by monitoring the development of well-developed apical brush borders by using transmission electron microscopy (TEM; data not shown). Bacterial inocula were prepared from overnight (\sim 16 h) cultures in LB broth, and 14-day-old monolayers of well-differentiated Caco-2 cells were infected at an m.o.i. ratio of 10:1 (*S. Enteritidis*:Caco-2 cells). Culture plates were centrifuged at 450 g at room temperature for 10 min to bring the bacteria into contact with cells. This centrifugation step also eliminated the possible effects of variations in the motility among the strains, which could otherwise influence the outcome of the assay. Inoculated monolayers were incubated for 2 h at 37 °C to allow bacteria to invade the cells. Subsequently, cells were washed three

Table 2. Oligonucleotide sequences for PCR-based assays and MLVA typing

Target gene/ locus	Primer	Sequence (5'–3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
<i>sefA</i>	SefA_F	GTGGTTCAGGCAGCAGTTACT	59	334	O'Regan <i>et al.</i> (2008)
	SefA_R	CAGGGACATTTAGCGTTTCTTGAG			
<i>sdfI</i>	SdfI_F	TGTGTTTTATCTGATGCAAGAGG	58	333	Agron <i>et al.</i> (2001)
	SdfI_R	CGTTCCTTCTGGTACTTACGATGAC			
<i>spvB</i>	SpvB_F	CGGTTATAGAAGAGCTCCTGT	55	349	Rychlík <i>et al.</i> (2008)
	SpvB_R	CCGGTATACGACTCTGTGATC			
SENTR1	SENTR1-F	6FAM -GCAACAGCAGCAGCAACAG	55	440* (415)†	Malorny <i>et al.</i> (2008)
	SENTR1-R	CCGAGCTGAGATCGCCAAG			
SENTR2	SENTR2-F	VIC -CACTGGACGATCTGGATTCTC	55	519* (510)†	Malorny <i>et al.</i> (2008)
	SENTR2-R	GTCGCCGTTACGCATCAAC			
SENTR3	SENTR3-F	NED -CTAAACAAGCCGCTCATCCG	55	494* (480)†	Malorny <i>et al.</i> (2008)
	SENTR3-R	ACAACCTGCTGCTGTGCTG			
SENTR4	SENTR4-F	6FAM -GACCAACACTCTATGAACCAATG	55	120* (120)†	Malorny <i>et al.</i> (2008)
	SENTR4-R	ACCAGGCAACTATTTCGCTATC			
SENTR5	SENTR5-F	PET -CACCGACAATCAGTGGAAC	55	271* (270)†	Malorny <i>et al.</i> (2008)
	SENTR5-R	GCGTTGAATATCGGCAGCATG			
SENTR6	SENTR6-F	NED -ATGGACGGAGGCGATAGAC	55	180* (177)†	Malorny <i>et al.</i> (2008)
	SENTR6-R	AGCTTCACAATTTGCGTATTTCG			
SENTR7	SENTR7-F	VIC -ACGATCACCACGGTCACCTC	55	135* (133)†	Malorny <i>et al.</i> (2008)
	SENTR7-R	CGGATAACAACAGGACGCTTC			
SE-3	SE-3F	6FAM -CAACAAAACAACAGCAGCAT	55	308* (307)†	Malorny <i>et al.</i> (2008)
	SE-3R	GGGAAACGGTAATCAGAAAAGT			
SE-7	SE-7F	6FAM -GATAATGCTGCCGTTGGTAA	55	717* (691)†	Malorny <i>et al.</i> (2008)
	SE-7R	ACTGCGTTTGGTTTCTTTTCT			
<i>sipA</i>	SipA_F	ATGGTTACAAGTGTAAGGACTCAG	53	2055	This study
	SipA_R	ACGCTGCATGTGCAAGCCATC			
<i>sipD</i>	SipD_F	ATGCTTAATATTCAAATTTATCCG	53	1029	This study
	SipD_R	TCCTTGCAAGGAAGCTTTTG			
<i>flgK</i>	FlgK_F	ATGTCAGCTTGATTAATCAC	53	1659	This study
	FlgK_R	GCGAATATTCAATAACGCATC			
<i>fljB</i>	FljB_F	ATGGCACAAAGTCATTAATACAAAC	53	1515	This study
	FljB_R	ACGCAGTAAAGAGAGGAC			
<i>flgL</i>	FlgL_F	ATGCGTATCAGTACCCAGATG	53	951	This study
	FlgL_R	CCGGTCAACTGGAAAAGC			
<i>sipA</i>	SipA_F_RT	TTTAAACGCCTCAGCGTCTT	55	130	This study
	SipA_R_RT	CAGAGAAAGTGCCACAACGA			
<i>sipD</i>	SipD_F_RT	CCTCCATTTTGGAAAGAAT	55	181	This study
	SipD_R_RT	TATTTAGCGCTTCGCCTATG			
<i>rpoD</i>	RpoD_F	ACATGGGTATTTCAGGTAATGGAAGA	55	74	Botteldoorn <i>et al.</i> (2006)
	RpoD_R	CCGTGCTGGTGGTATTTTCA			

*Based on *S. Enteritidis* NCTC13349.

†Size analysed by using an ABI310 capillary sequence detection system (Applied Biosystems).

times with PBS. Next, 1 ml DMEM containing 100 µg gentamicin ml⁻¹ was placed in each well and incubated for 2 h to kill extracellular bacteria. The MIC of gentamicin for all the isolates used in this study was <0.125 µg. The supernatants from representative gentamicin-treated samples revealed no extracellular bacteria (data not shown), indicating that the exposure of gentamicin used for the invasion assay was sufficient to kill extracellular bacteria. Following incubation, the monolayers were washed three times with PBS and lysed with 0.5% (v/v) Triton X-100 (Fisher Biotech) in PBS for 10 min at 37 °C. Serial dilutions of suspensions were made in PBS and inoculated onto LB plates to determine the c.f.u.

that survived gentamicin treatment and hence had invaded the Caco-2 cells. Each isolate was tested in triplicate in two or more independent experiments.

Detection of biofilm phenotypes. Two different assays were used to determine biofilm phenotypes of *S. Enteritidis* strains. Qualitative determination of biofilm formation was based on colony morphology on brilliant green agar (BGA) plates (Acumedia) as described previously with a few modifications (Guard-Bouldin *et al.*, 2004; Guard-Petter *et al.*, 1996; Humphrey *et al.*, 1996). Briefly, 100 colonies of each *S. Enteritidis* isolate were spot-transferred onto BGA

at 20 colonies per plate (150 mm diameter) at least 20–25 mm apart. The plates were incubated at 37 °C for 16 h followed by incubation for an additional 5 days at 25 ± 2 °C (mean ± SD). Colony morphology was observed every 24 h. The proportion of biofilm-producing colonies was recorded as a percentage of the total colonies counted 5 days post-inoculation. Strains were classified as follows: biofilm positive (bf+; 100 % of colonies are positive for organic matrix that covers the entire colony, giving a wrinkled appearance), biofilm negative (bf-; 100 % of colonies, including their edges, appear smooth), intermediately positive (bf+i; ≥80 % colonies with wrinkled edges and smooth centre or vice versa giving a semi-wrinkled appearance) or mixed positive (bf+m; both bf+ and bf- colony types are present within the same culture).

Quantitative determination of biofilm production was performed by using a microtitre assay as described previously with minor modifications (de Silva *et al.*, 2002; Eftekhari & Speert, 2009). Briefly, 100 µl overnight cultures (diluted 1:100 in trypticase soy broth, TSB) were inoculated into 96-well flat-bottomed polystyrene microtitre plates (Evergreen) and incubated for 3 days at room temperature (25 ± 2 °C). Well contents were decanted, washed three times with 200 µl sterile PBS (pH 7.2), air-dried and stained with 250 µl 0.1 % (w/v) safranin for 15 min followed by a single wash with PBS. Bound safranin was dissolved by adding 250 µl 30 % (v/v) acetic acid solution. OD₄₉₀ readings were recorded by using an ELISA plate reader (BioTek). Biofilm-positive (G3) and biofilm-negative (G2) control isolates were included in each plate, as was the background control of TSB without bacteria. Four wells were tested per strain in two independent experiments. The mean OD values of the test isolates were used to generate a receiver operating characteristic curve. An OD₄₉₀ of ≥0.12 was chosen to distinguish biofilm-positive from biofilm-negative strains (92 and 96 % predicted sensitivity and specificity, respectively).

In vivo invasion assay in mouse model. Mouse challenge studies were performed according to protocols approved by the WSU-Institutional Animal Care and Use Committee. Six- to eight-week-old female BALBc mice, weighing 20–24 g, were obtained from a local breeding colony maintained at the Animal Resource unit, WSU. The mice were raised in static micro-isolator cages at 25 °C with 12 h light cycle and fed on a *Salmonella*-free commercial diet (Lab Diet 5001; Purina). Food and water were supplied *ad libitum* until the end of the experiment. Eight *S. Enteritidis* isolates with different cell-invasion potential in cultured Caco-2 cells were used for the mouse invasiveness assay. Bacterial isolates were grown overnight in LB medium as described above and serially diluted in PBS to obtain desired cell counts. Mice were inoculated intragastrically with 200 µl PBS containing ~1 × 10⁶ cells. Control mice were inoculated with 200 µl PBS. Each isolate was inoculated in three animals and each animal was raised in a separate isolator cage to prevent cross contamination. To exclude observer bias in interpretation of the results, all clinical and bacteriological assessments were conducted by personnel blind to the identity of the challenge isolates. Faecal pellets were collected from each mouse at 3, 6, 9, 15 and 21 days post-infection (PI) and processed for isolation and enumeration of challenge isolates as described below. Challenged mice were observed, at least three times daily, for mortality and clinical signs such as ruffled fur, hunched backs and lethargy for up to 21 days PI. Mice showing all of the above symptoms were considered moribund and were killed by carbon dioxide asphyxiation. Samples of liver, spleen and intestine were collected by using aseptic techniques, homogenized in cold PBS and bacterial c.f.u. number was determined by direct plating of serially diluted organ homogenates on xylose-lysine dulcitol (XLD) agar (Criterion) plates. Samples that were negative after direct plating on XLD agar were enriched by inoculating into standard tetrathionate enrichment broth (Difco) and incubated at 37 °C for 24 h followed by plating onto XLD agar. The results for enrichment

cultures were recorded as either positive or negative. Experiments were terminated after 3 weeks when all the surviving mice were killed and organ samples were processed as described above.

Secretory protein profiling. Secreted proteins were recovered as described previously (Amy *et al.*, 2004; Arricau *et al.*, 1998; Kaniga *et al.*, 1995). *S. Enteritidis* isolates were grown overnight (16 h) at 37 °C in 10 ml LB broth supplemented with 300 mM NaCl in a 50 ml conical tube with shaking at 200 r.p.m. and adjusted to an OD₆₀₀ of 1. Culturing of *Salmonella* in high osmolarity conditions has been shown to induce expression of TTSS encoded by SPI-1 and flagellar proteins. Bacteria were harvested by centrifugation at 8000 g for 10 min, and culture supernatant containing secretory proteins was collected and passed through a 0.2 µm filter. Proteins present in 2 ml supernatant were precipitated by adding 10 % (v/v) trichloroacetic acid and incubated on ice for 1 h. Precipitated proteins were then recovered by centrifugation at 10 000 r.p.m. for 30 min. The pellets were washed once with ice-cold acetone and air-dried. The protein pellets were resuspended in 50 µl Laemmli buffer. Subsequently, 10 µl of this suspension was separated on 12 % (w/v) acrylamide gels by SDS-PAGE. After electrophoresis, proteins were stained by using a silver staining kit (Pierce) as per the manufacturer's recommendations. Each isolate was tested independently in three or more experiments. The differentially expressed protein bands were excised from the gel and sequenced by using MALDI MS/MS.

Motility assay and TEM. To test whether variation in cell invasion corresponded to variation in motility, the motility of representative isolates from low and high invasiveness groups was assessed at 37 °C on semi-solid LB agar (0.3 %). Briefly, the procedure involved growing bacterial isolates overnight (16 h) in LB broth at 37 °C. Subsequently, 1 µl overnight culture was stab-inoculated into semi-solid media and the plates were incubated at either 37 °C (Experiment 1) or 42 °C (Experiment 2). The diameter of the halo of growth was measured for each isolate after 7 h of incubation. Each strain was tested in duplicate and each experiment was repeated at least twice. For TEM, culture samples were taken from the edge of the motile colony grown at 42 °C. Carbon-Formvar-coated grids (200 mesh) were floated on a drop of cells suspended in distilled water and then allowed to dry. The grids were stained in 2 % uranyl acetate for 2.5 min and viewed on a Philips model CM200 TEM at an accelerating voltage of 200 kV (Franceschi Microscopy and Imaging Center, WSU). At least 10 fields were viewed for each isolate.

In vitro invasion assays with chicken macrophage cells. Chicken macrophage cells (HD11) were routinely maintained in Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 10 % (v/v) FBS (Invitrogen) in 95 % air/5 % CO₂ at 37 °C (Bohez *et al.*, 2006; He *et al.*, 2003). Propagated cells were seeded at approximately 10⁶ cm⁻² into 75 cm² flasks (BD falcon). After 48 h of culture, cells were harvested by trypsinization and resuspended in IMDM with 10 % FBS. Subsequently, cells were plated onto 12-well plates (Cellstar) at ~1 × 10⁶ cells per well. After 24 h incubation in 95 % air/5 % CO₂ at 37 °C, monolayers of HD11 were achieved. Bacterial inocula were prepared from overnight (~16 h) cultures in LB broth and monolayers were infected at an m.o.i. ratio of approximately 50:1 (*S. Enteritidis*: HD11 cells) in DMEM. Culture plates were centrifuged at 1000 g at room temperature for 5 min to bring the bacteria into contact with cells. Inoculated monolayers were incubated for 2 h at 42 °C to allow cells to phagocytose the bacteria. Subsequently, cells were washed three times with PBS to remove non-adherent extracellular bacteria. Next, 1 ml DMEM containing 100 µg gentamicin ml⁻¹ was placed in each well and incubated for 2 h at 42 °C to kill cell-adherent extracellular bacteria. Following treatment with gentamicin, the monolayers were washed three times with PBS and lysed with 0.5 % (v/v) Triton X-100 (Fisher Biotech) in PBS for 10 min at 37 °C. Serial dilutions of suspensions were made in PBS

and inoculated onto LB agar plates to determine the number of organisms that survived gentamicin treatment and hence had invaded and/or survived within the HD11 cells. Each isolate was tested in duplicate and at least three independent experiments were performed.

Real-time RT-PCR. Representative high invasive (BC8 and P21) and low invasive strains (C19 and C45) were cultured overnight (16 h) in LB broth at 42 °C under known SPI-1-inducing conditions as described above (Amy *et al.*, 2004; Arricau *et al.*, 1998; Kaniga *et al.*, 1995). Total bacterial RNA was extracted by using the Ambion Ribopure kit as per the manufacturer's protocol followed by DNase I (Ambion) treatment to remove residual DNA. The purified RNA was quantified by using a Nanodrop ND-1000 spectrophotometer and quality was assessed by gel electrophoresis and ethidium bromide staining. The absence of DNA contamination was checked by PCR amplification by using total RNA as a template and primers specific for a housekeeping gene, *rpoD* (Botteldoorn *et al.*, 2006). Real-time RT-PCR was performed to measure the transcriptional levels of *sipA*, *sipD* and the housekeeping gene *rpoD*, by using an iQ5 iCycler (Bio-Rad) and SYBR green I mastermix (Bio-Rad). One microgram of DNase-treated total RNA from two independent cultures was reverse transcribed to cDNA by using Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Amplifications were performed by using Ssofast Evagreen Supermix (Bio-Rad). Optimal PCR conditions and primer efficiencies were determined for each primer pair (Table 2) by reference to a standard curve generated from a series of fourfold dilutions of the positive control pGEM-T Easy (Promega) plasmid containing the amplicon of interest. Following amplification, melting curves were generated to verify PCR product identity. Expression levels were normalized to that of the housekeeping gene *rpoD* and the resultant cycle threshold (C_t) values were analysed by using the relative expression software tool (REST 2009) for determination of relative expression levels of genes of interest (Pfaffl *et al.*, 2002).

Statistical analysis. Differences in the invasiveness of *S. Enteritidis* isolates in Caco-2 and HD11 cells were analysed by means of one-way ANOVA followed by multiple comparisons with a Tukey–Kramer test. Subsequently, *k*-means clustering was used to classify the isolates by their level of Caco-2 cell invasion into three major groups: high, medium and low invasiveness. The difference in the virulence potential of selected isolates in a mouse model was evaluated by Kaplan–Meier survival analysis and log-rank test. Results of biofilm formation from quantitative microtitre plate assays and qualitative agar plate assays were compared by one-way ANOVA or Student's *t*-test. A Spearman rank-correlation coefficient was used to examine the correlation between *S. Enteritidis* phenotypes such as Caco-2 cell invasiveness, biofilm formation, motility and mouse virulence. All statistical tests were performed by using NCSS 2007.

RESULTS

PCR-based screening and MLVA typing of *S. Enteritidis* isolates

PCR screening was conducted to confirm the identity of each isolate. All isolates tested positive for the presence of serogroup D-specific *sefA* and *S. Enteritidis*-specific *sdfl* (Table 2). In addition, all strains were confirmed as being of smooth type based on their seroreactivity with *Salmonella* O antiserum Group D1 (data not shown). MLVA distinguished 53 *S. Enteritidis* isolates from diverse sources into 13 main clusters sharing on average ~85% allelic congruence (Fig. 1). These were classified into five unique and 14

cluster-associated MLVA types. There was considerable phenotypic diversity in terms of cell invasion potential and biofilm formation among the isolates within each cluster. We found no correlation between MLVA type and cell invasiveness (Spearman's rank-correlation coefficient, 0.07; $P=0.6$) or the ability of isolates to form biofilm (Spearman's rank-correlation coefficient, 0.26; $P=0.06$).

S. Enteritidis isolates differ in their invasiveness in differentiated Caco-2 cells

A standard gentamicin protection assay was used to test the invasiveness of *S. Enteritidis* isolates in human colon cancer cell line Caco-2. Initially, we screened several isolates of *S. Enteritidis* by using undifferentiated Caco-2 cultures that were grown for 48 h, but we observed a low degree of invasion and a high degree of variance between the replicates (data not shown). We therefore tested Caco-2 cells for their state of cell differentiation and the effect of this state on invasion by *S. Enteritidis*. Alkaline phosphatase activity was significantly higher ($P<0.01$, Student's *t*-test) in 14-day-old cells compared with 5-day-old cells (Table 3) (Burns *et al.*, 2001; Hara *et al.*, 1993). In addition, 14-day-old cells showed well-developed apical brush borders, as visualized by TEM, indicating that 14-day-old cells were well differentiated (data not shown). Subsequently, we tested the invasive ability of representative *S. Enteritidis* isolates along with a clinical isolate of *S. Typhimurium* (positive control) and a laboratory *Escherichia coli* strain (negative control) in 5-day-old (undifferentiated cells) and 14-day-old (differentiated cells) Caco-2 confluent cultures. The invasion rates of both *S. Enteritidis* G2 and *S. Typhimurium* were 13- and 2-fold higher in differentiated cells than in undifferentiated cells (Table 3). Consequently, 14-day-old well-differentiated cultures of Caco-2 cells were used to screen the invasiveness of all 53 *S. Enteritidis* isolates. There were significant differences ($P<0.05$) in invasiveness [$\text{mean} \pm \text{SEM} \log_{10}(\text{c.f.u. ml}^{-1})$] between different isolates (Table 1) with G45 being minimally invasive (4.18 ± 0.18) and UK being highly invasive (5.91 ± 0.06). When a statistical cluster analysis via a *k*-means clustering algorithm was applied to cell invasiveness data for all the isolates tested, a kink in the sum of squares located at $k=3$ indicated that three was the optimum number of clusters (Hastie *et al.*, 2001). As a result, three groups with cluster centres of 5.58 ± 0.03 (high invasiveness, $n=30$), 5.07 ± 0.06 (medium invasiveness, $n=16$) and 4.50 ± 0.04 (low invasiveness, $n=7$) $\log_{10}(\text{c.f.u. ml}^{-1})$ were identified (Fig. 2). These differences in the mean $\log_{10}(\text{c.f.u.})$ between the three groups were significant (Tukey–Kramer test; $P<0.05$).

Invasiveness of *S. Enteritidis* isolates is not associated with the presence of a virulence plasmid

Nine (17%) of 53 isolates used in this study tested negative for the *spvB* gene, indicating that these isolates lacked a 'typical' virulence-associated plasmid (pSEV). Based on the

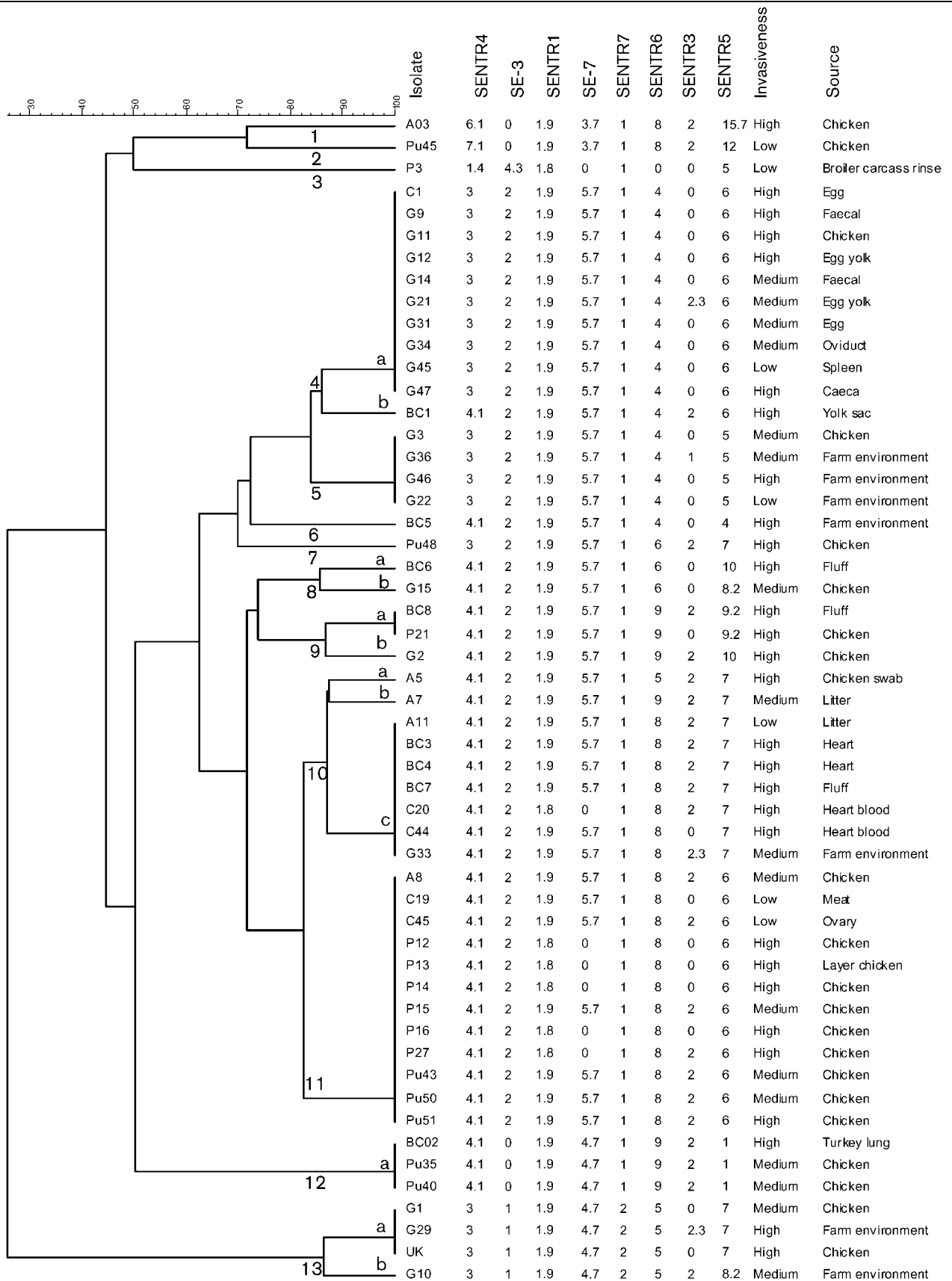


Fig. 1. Cluster analysis of 53 *S. Enteritidis* isolates recovered from diverse poultry sources. Eight major clusters (4, 5 and 8–13) were defined as groups of closely related strains sharing on average $\geq 85\%$ allelic congruence, as resolved by MLVA genotypes.

invasiveness of these plasmid-negative isolates in Caco-2 cells, three (G11, G12 and G47) isolates belong to the high invasiveness category, four (G14, G21, G31 and G34) to the medium invasiveness category and one (G45) to the low invasiveness category (Table 1). There was no association (Fisher's exact test, $P=0.4$) between the presence or absence of the *spvB* gene and the invasiveness of isolates.

Cell invasion potential of *S. Enteritidis* isolates is not related to biofilm formation

By using a BGA plate assay, 20 (37.7%), 23 (43.4%), three (5.7%) and seven (13.2%) of the 53 *S. Enteritidis* isolates were identified as bf+, bf-, bf+i and bf+m, respectively (Table 1). The proportion with intermediate (semi-wrinkled) colony morphology among isolates showing bf+i phenotype was $\geq 80\%$ whereas the proportion of typical biofilm-positive colonies among isolates showing bf+m phenotype ranged from 10 to 93% (data not shown). Because of the presence of several isolates with bf+m and bf+i phenotypes it was difficult to classify these isolates either as bf+ or as bf-. Consequently, we confirmed the results of the BGA plate assay by using a quantitative microtitre plate assay. The OD₄₉₀ of the bf+i phenotype ranged from 0.12 to 0.14 whereas that for the bf+m phenotype ranged from 0.11 to 0.17, indicating that according to the quantitative microtitre assay, all of these isolates except BC3 (OD₄₉₀=0.11) were biofilm formers. Therefore, for data analysis purposes, all isolates with bf+, bf+i or bf+m phenotypes were merged together as bf+ and compared with the isolates showing typical bf- colony types. Quantitative biofilm production, as determined by microtitre plate assay, was significantly higher in isolates that were found to be biofilm producers by the BGA plate assay [geometric mean (95% confidence interval) OD₄₉₀=0.15 (0.14–0.16) versus 0.08 (0.07–0.09); $P<0.01$] (Table 1). In general, all biofilm-positive isolates except BC3 were identified as biofilm formers by microtitre plate assay whereas all biofilm-negative isolates were identified as biofilm negative, indicating strong concordance between the results of the two assays. The results indicated that a higher proportion of isolates with medium (81.2%, 13/16) and high (50%, 15/30) invasiveness formed a biofilm compared with isolates with low invasiveness (28.5%, 2/7).

However, this association was not statistically significant (Spearman's rank-correlation coefficient, -0.12 ; $P=0.35$).

Caco-2 cell invasion correlates with virulence potential of *S. Enteritidis* isolates in a mouse model

To determine whether cell invasiveness correlates with *in vivo* virulence, representative *S. Enteritidis* isolates with low (C19, C45, A11 and G45), medium (G1) and high (BC8, P21 and UK) invasiveness were tested by using an *in vivo* mouse challenge model. There were no differences between the *in vitro* growth kinetics of low and high invasiveness isolates (data not shown). *S. Enteritidis* isolates with high or medium invasiveness were more virulent and caused more and earlier mortality compared with isolates with low invasiveness (Kaplan–Meier survival analysis–log-rank test, $P<0.01$; Fig. 3). The mean survival time for mice inoculated with the high/medium invasiveness isolates P21, BC8, G1 and UK was 6 days (range=3–9 days). All mice inoculated with high/medium invasiveness isolates died or were killed by 9 days PI. In contrast, no clinical signs or mortality were recorded in mice challenged with low invasiveness isolates C45, C19 and A11. Isolate G45 caused limited and delayed mortality (one of three mice died at 13 days PI). The mean survival duration of mice challenged with low invasiveness isolates and negative control was 20.3 (range=13–23 days) and 21 days, respectively. There was a significant correlation between high invasiveness of *S. Enteritidis* isolates and virulence in the mouse model (Spearman's rank-correlation coefficient, 0.95 ; $P=0.0002$).

The number of bacteria per gram of tissue from the small intestine, caecum, liver and spleen from all animals that died during the experiment as well as from those that survived the challenge until the end of experiment at 21 days PI was determined. The mean (\pm SEM) number [$\log_{10}(\text{c.f.u. g}^{-1})$] for isolates with high invasiveness was 7.10 ± 0.99 (small intestine), 6.74 ± 0.97 (caecum), 7.28 ± 0.24 (liver) and 7.90 ± 0.35 (spleen). In contrast, mean values for isolates with low invasiveness were 1.36 ± 0.80 (small intestine), 1.47 ± 0.80 (caecum), 0.66 ± 0.26 (liver) and 1.03 ± 0.40 (spleen). The numbers of bacteria recovered from different organs from animals

Table 3. Differences in invasion rates of *S. Enteritidis* between differentiated and undifferentiated Caco-2 cells

Culture length (days)	Invasion (%)*			Mean \pm SEM alkaline phosphatase ($\mu\text{g ml}^{-1}$)†
	<i>S. Enteritidis</i> G2	<i>S. Typhimurium</i>	<i>E. coli</i> K-12	
5	0.05	0.06	0	0.5 ± 0.11
14	0.68	0.13	0	1.1 ± 0.04

*Percentage invasion=[organisms recovered (c.f.u. ml⁻¹)/organisms inoculated (c.f.u. ml⁻¹)] $\times 100$.

†The difference in alkaline phosphatase activity between 5- and 14-day-old cells was significant ($P<0.001$).

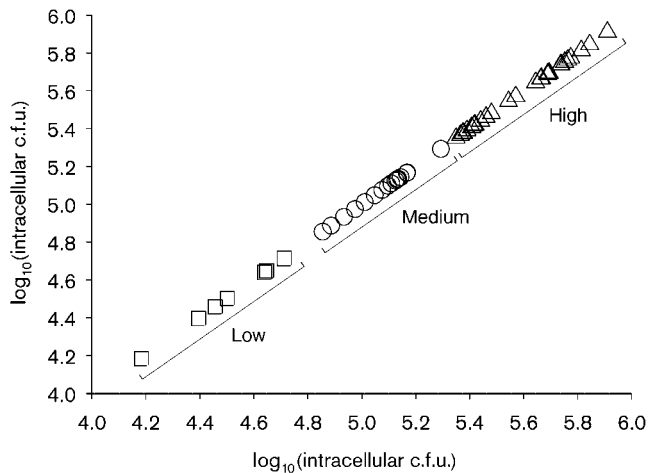


Fig. 2. A *k*-means cluster plot of the variable Caco-2 cell invasiveness of 53 *S. Enteritidis* isolates recovered from diverse poultry sources. Three groups of isolates with low ($n=7$), medium ($n=18$) and high ($n=30$) invasiveness were identified based on their cell invasion potential.

challenged with high/medium invasiveness isolates were significantly higher ($P<0.01$) than those of low invasiveness isolates (Fig. 4), supporting the results of survival curve analysis. It is important to note, however, that colony counts were made at different time points with recoveries from highly invasive isolates mostly collected from mice that died between days 3 and 9 whereas for low virulence isolates bacteria were mostly recovered from mice that were killed at 21 days PI. In C19-challenged

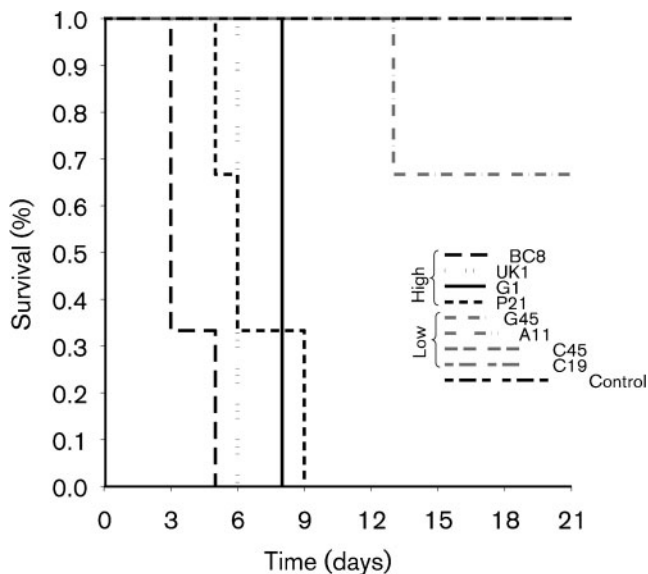


Fig. 3. Kaplan-Meier survival curve showing percentage survival rates in mice challenged with representative *S. Enteritidis* isolates with high and low invasiveness in cultured Caco-2 cells.

mice, no *Salmonella* was isolated from any of the organs tested for any of the mice. In C45-challenged mice, *Salmonella* was isolated from all the organs from only one of the three mice either by direct plating or by pre-enrichment procedures. In G45-challenged mice, *Salmonella* was isolated from all internal organs of the mouse that died at 13 days PI, and from intestine, but not the liver and spleen, of the two mice that survived until 21 days PI. Because of the variability in the recovery of the challenge isolates from mice challenged with C19, C45 and G45 isolates, the challenge experiment with these three isolates was repeated as described previously. The results from these repeat experiments were very similar to the first experiment (data not shown).

Virulence of *S. Enteritidis* is associated with secretion of TTSS secreted proteins and motility

We determined the secretory protein profiles from the supernatant of representative isolates with low and high invasiveness cultured in LB broth containing 300 mM NaCl, corresponding to conditions allowing SPI-1 gene expression induction. Five major bands of secretory proteins with molecular masses of 74 (p1), 59 (p2), 52 (p3), 36 (p4) and 34 (p5) kDa were differentially expressed, based on the intensity of bands on silver-stained polyacrylamide gels, among isolates with low and high invasiveness (Fig. 5). These differences were consistently observed in at least three independent experiments and therefore all five protein bands were excised from the gel for protein identification by MALDI MS/MS. The deduced amino acid sequences from the above five proteins corresponded to SipA (p1), FlgK (p2), FljB (p3), SipD (p4) and FlgL (p5) (Fig. 5). The bands corresponding to FlgK, FljB and FlgL were not detected in isolate G45 (Fig. 5). Two isolates with low invasiveness (C19 and C45) showed lower band intensity for SipA (SPI-1 TTSS effector protein) and SipD (SPI-1 translocon assembly protein) when compared with the secretory protein profiles of isolates with high/medium invasiveness (Fig. 5). Consequently, we tested the levels of expression of mRNA transcripts for *sipA* and *sipD* in low invasiveness isolates C19 and C45 and compared these with the representative high invasiveness isolates P21 and BC8. Interestingly, expression levels of *sipA* and *sipD* were 16- and 11-fold higher, respectively, in the high invasiveness isolates compared with the low invasiveness isolates. This difference was statistically significant ($P<0.05$) and was consistent with impaired transcription of *sipA* and *sipD* being responsible for reduced secretion of these proteins.

Because three flagella-associated proteins, FlgK (flagellar filament protein), FljB (flagellin) and FlgL (flagellar hook-associated protein), were absent from the supernatant of isolate G45, we postulated that the motility of this isolate was impaired. Consequently, we determined the motility of representative isolates with low and high invasiveness on a semi-solid motility medium at two temperatures (25 and

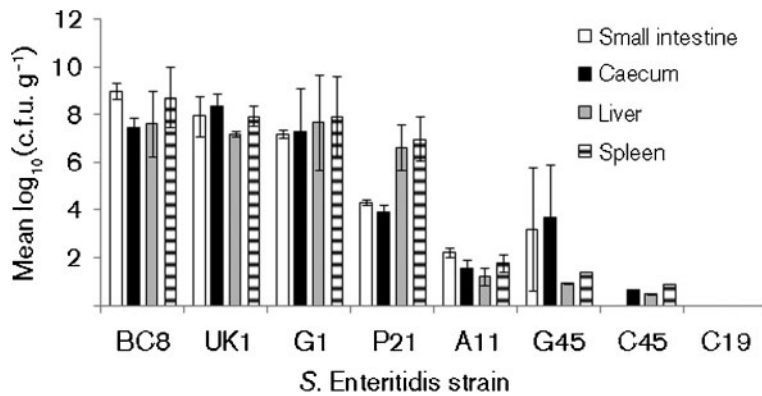


Fig. 4. Numbers of *Salmonella* recovered from small intestine, caecum, liver and spleen of mice challenged with *S. Enteritidis* isolates with high/medium and low invasiveness in cultured Caco-2 cells.

42 °C). At both temperatures, the mean diameter of the halo of growth of low invasiveness isolates was significantly smaller (Tukey–Kramer test, $P < 0.01$) compared with high/medium invasiveness isolates (Table 4), indicating that, apart from the impaired secretion of SPI-1 TTSS proteins SipA and SipD, isolates with low invasiveness (C19, C45, A11 and G45) also had impaired motility. TEM of cultures grown at 42 °C revealed that isolates with high invasiveness produced long, thin, peritrichous, flagellar appendages (Fig. 6a–d). In contrast, flagellar appendages were not visible among isolates with low invasiveness, with the exception of isolate C19 which occasionally showed the presence of a unipolar flagellum (Fig. 6e–h).

***S. Enteritidis* isolates with impaired motility and/or secretion of TTSS proteins have impaired ability to invade and/or survive in chicken macrophages**

We hypothesized that the low invasiveness isolates (C19, C45, A11 and G45) showing impaired motility and altered secretion of one or more SPI-1 TTSS effector proteins are also less invasive in the chicken host. Consequently, we used chicken macrophage cells (HD11) to test the ability of representative isolates with low and high invasiveness to invade and/or survive within the chicken cells. Overall, the mean (\pm SEM) intra-macrophage c.f.u. of low invasiveness isolates [$\log_{10}(\text{c.f.u.}) = 4.88 \pm 0.13$] was significantly lower than that of high invasiveness isolates [$\log_{10}(\text{c.f.u.}) = 6.07 \pm 0.08$] (Tukey–Kramer test; $P < 0.01$, Table 4).

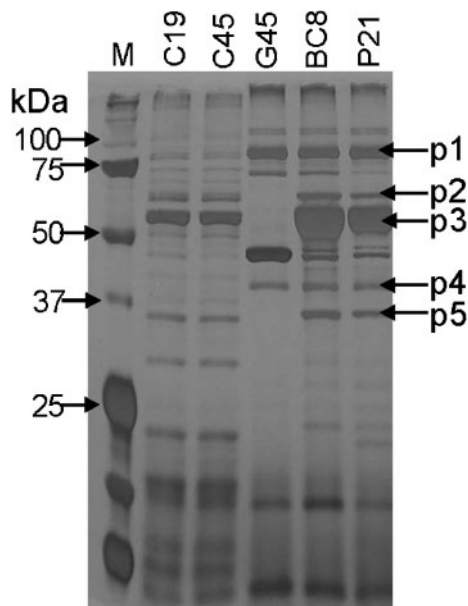


Fig. 5. Secretory protein profile of representative *S. Enteritidis* isolates with low and high invasiveness in cultured Caco-2 cells. Lane M, protein molecular mass standard. The deduced amino acid sequences from the five major protein bands (indicated by arrows) corresponded to SipA (p1, 74 kDa), FlgK (p2, 59 kDa), FliB (p3, 52 kDa), SipD (p4, 36 kDa) and FlgL (p5, 34 kDa).

DISCUSSION

Invasion in cultured epithelial cells is routinely used as a measure of pathogenicity of *Salmonella* isolates (Dibb-Fuller *et al.*, 1999; van Asten *et al.*, 2000, 2004). Fewer studies have been conducted to determine differences in invasiveness of field isolates of *S. Enteritidis* (Pan *et al.*, 2009; Pang *et al.*, 2006; Solano *et al.*, 1998). Although these studies have shown that not all isolates of *S. Enteritidis* have similar cell invasive potential, only limited numbers of isolates recovered mostly from human, animal, food and the environment were tested. This is the first report in which more than 50 wild-type *S. Enteritidis* isolates recovered specifically from diverse poultry sources have been tested for their invasiveness by using well-differentiated Caco-2 cells as a model for infection. Given enough time, Caco-2 cells spontaneously differentiate to closely mimic a small-bowel-like cellular phenotype, as indicated by dome formation, presence of microvilli and expression of brush border enzymes (e.g. alkaline phosphatase) after confluency (Hara *et al.*, 1993). In addition, differentiation of Caco-2 cells has been shown to be an important factor for invasion by *Salmonella* and for expression of surface receptors for enterotoxigenic *E. coli* (Finlay & Falkow, 1990; Kernéis *et al.*, 1992). We observed that the invasion

Table 4. Intra-macrophage survival and motility of representative low- and high-cell invasiveness isolates of *S. Enteritidis*

Strain	Caco-2 cell invasion phenotype	Motility (cm) at: [*]		Intramacrophage survival in HD-11 cells [$\log_{10}(\text{c.f.u. ml}^{-1})$] [†]
		37 °C	42 °C	
C19	Low	0.35 ± 0.03	0.10 ± 0.00	4.46 ± 0.16
C45	Low	0.37 ± 0.02	0.20 ± 0.04	4.08 ± 0.16
A11	Low	0.37 ± 0.06	0.20 ± 0.04	5.87 ± 0.09
G45	Low	0.10 ± 0.00	0.10 ± 0.00	5.12 ± 0.12
UK	High	3.65 ± 0.09	3.60 ± 0.07	6.23 ± 0.04
BC8	High	3.15 ± 0.10	1.40 ± 0.04	6.01 ± 0.05
P21	High	2.97 ± 0.14	1.82 ± 0.14	6.04 ± 0.17
G1	Medium	3.82 ± 0.16	0.67 ± 0.04	5.99 ± 0.05

^{*}Values are mean ± SEM diameter of the halo of growth on motility agar from measurements conducted on at least two colonies each from two independent experiments.

[†]Each isolate was tested in duplicate. Values for c.f.u. ml⁻¹ are mean ± SEM numbers of bacteria that survived inside the avian macrophages (HD11 monolayers) from three independent experiments.

rate of stationary phase cultures of *S. Enteritidis* was 13 times higher for differentiated cells than for undifferentiated cells (Table 3). When this model was used to screen stationary phase cultures of all the field isolates, seven (13%) isolates were identified with significantly lower invasiveness (Table 1). These data indicate that although poultry and poultry products are major sources of infection for humans worldwide, not all isolates of *S. Enteritidis* recovered from poultry may be equally pathogenic.

The majority (50–90%) of field isolates of *S. Enteritidis* are known to produce biofilm, an extracellular matrix composed of high-molecular-mass LPS, flagella and fimbriae (Guard-Petter *et al.*, 1996; Guard-Petter, 2001; Marin *et al.*, 2009; Solano *et al.*, 2001, 2002). Similar to data from these reports, 56.6% (30/53) of *S. Enteritidis* isolates tested here produced biofilm. Biofilm formation in *S. Enteritidis* isolates has also been reported to be associated with the enhanced disruption of cultured Caco-2 cells and increased virulence in chickens (Solano *et al.*, 1998, 2001). In this study, however, there was no significant correlation ($P=0.35$) between invasiveness and the ability of *S. Enteritidis* isolates to form biofilms. Nevertheless, the majority (71%) of isolates with low invasiveness tested bf⁻ whereas lower proportions of isolates with medium (19%) and high (50%) invasiveness were bf⁻, indicating that biofilm may, in part, play a role in the pathogenesis of *S. Enteritidis* infection. Some isogenic strains of *S. Enteritidis* (bf+m) show both bf⁺ and bf⁻ colonies in varying proportions (Guard-Petter *et al.*, 1996; Humphrey *et al.*, 1996). Subcultures from isogenic single bf⁺ or bf⁻ colonies result in similar mixed phenotypes (J. Guard, unpublished data). Using a BGA plate assay, seven (13%) isolates in this study were identified as isogenic, with the proportion of bf⁺ colonies ranging from 10 to 90% (data not shown). When biofilm production in these isogenic strains was confirmed by quantitative microtitre assay, all

strains were classified as bf⁺ (Table 1). Interestingly, when chickens are infected with a mixture of highly invasive bf⁺ and less invasive bf⁻ *S. Enteritidis* isolates, the bf⁺ *S. Enteritidis* acts as a ‘helper’ phenotype and aids access of the less orally invasive bf⁻ strain to the parenteral (post-mucosal) environment in chickens (Gast *et al.*, 2002; Guard-Petter, 2001; Parker & Guard-Petter, 2001). It is possible that a similar phenomenon may have contributed to the higher cell invasion potential of the isogenic strains (bf+m) identified in this study.

Regardless of phage types, geographical origins or source of isolation, *S. Enteritidis* is one of the most genetically homogeneous serotypes of *Salmonella* and as a result is poorly discriminated by the commonly used genetic subtyping methods, such as phage typing, pulsed field gel electrophoresis or comparative genomic hybridization microarrays (Betancor *et al.*, 2009; Botteldoorn *et al.*, 2010; Hudson *et al.*, 2001; Liebana *et al.*, 2001; Malorny *et al.*, 2008; Morales *et al.*, 2005; Olsen *et al.*, 1999; Pan *et al.*, 2009; Pang *et al.*, 2006; Porwollik *et al.*, 2005; Saeed *et al.*, 2006; Swaminathan *et al.*, 2001). Because of its greater discriminatory capacity, MLVA was used to assess the genetic similarity among *S. Enteritidis* isolates tested in this study (Boxrud *et al.*, 2007; Cho *et al.*, 2007; Malorny *et al.*, 2008). The majority (90%) of isolates were grouped into eight clusters (clusters 4, 5 and 8–13) with a within-cluster allelic congruence of $\geq 85\%$ (Fig. 1). Some isolates that were recovered from different sources occurred within the same cluster, suggesting a considerable degree of genetic similarity. This was not unexpected as several studies using different genotyping methods have shown that isolates of *S. Enteritidis* are genetically closely related. Despite the clonal nature of *S. Enteritidis*, field isolates vary in their phenotypes. For instance, differences in the virulence potential between *S. Enteritidis* isolates within the same phage types and even within the same clonal lineages have been reported (Olsen

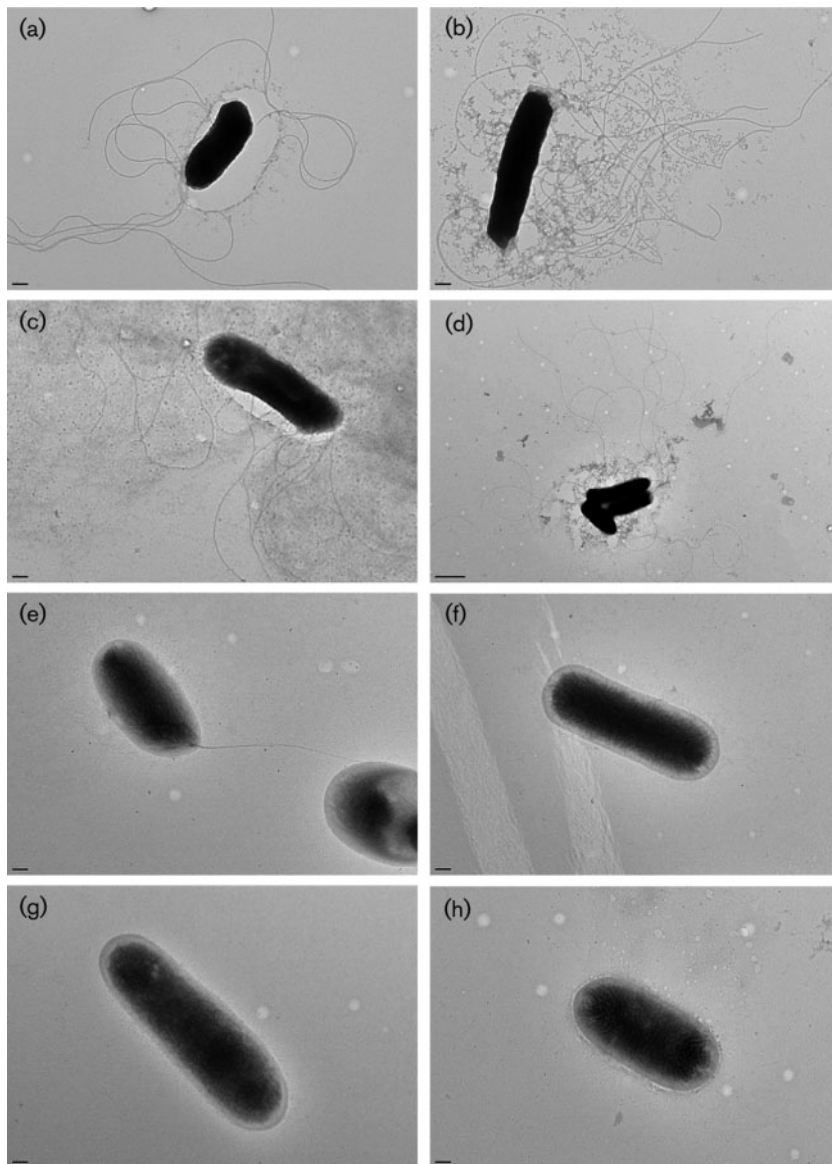


Fig. 6. Transmission electron micrographs of *S. Enteritidis* strains UK (a), BC8 (b), P21 (c), G1 (d), C19 (e), C45 (f), G45 (g) and A11 (h). Samples were taken from the edge of a motile colony. Bars, 0.2 μm .

et al., 1999; Yim *et al.*, 2010). When we compared invasiveness or biofilm-forming ability of the isolates with their MLVA clusters, it was clear that each cluster included isolates with more than one phenotype (Table 1), indicating extensive phenotypic diversity among genetically closely related isolates of *S. Enteritidis*. Our data confirm the genetic homogeneity of *S. Enteritidis* and support the existence of phenotypic diversity within each genetic cluster.

Field isolates of *S. Enteritidis* contain a serovar-specific high-molecular-mass plasmid (pSEV) that encodes *spvRABCD* (*Salmonella* plasmid virulence) genes (Rychlík *et al.*, 2006; Rotger & Casadesús, 1999). pSEV contributes to systemic infection of mice but not chickens (Bakshi *et al.*, 2003; Nakamura *et al.*, 1985), and *spvB*, in particular, plays a role in the pathogenesis by ribosylating actin of the macrophages and destabilizing the cytoskeleton (Lesnick

et al., 2001; Otto *et al.*, 2000; Tezcan-Merdol *et al.*, 2001). Thus, identification of individual genes of the *spv* operon from *S. Enteritidis* is commonly used as a marker to assess the status of pSEV in field isolates (Rodríguez *et al.*, 1998; Rychlík *et al.*, 2008; Soto *et al.*, 2003, 2006). Nine (17%) isolates from our collection tested negative for the presence of the *spvB* gene, indicating that these isolates lacked the typical virulence plasmid. Our results are in agreement with those of Bakshi *et al.* (2003), who reported that 16% (5/24) of field isolates lacked pSEV. Additional studies have reported that the proportion of plasmid-free isolates of *S. Enteritidis* ranged from 3% (2/65) to 8% (5/60) (Nakamura *et al.*, 1985; Ridley *et al.*, 1998). Overall, there was no statistically significant correlation between the presence of the *spvB* virulence gene and the invasiveness of *S. Enteritidis* isolates. Interestingly, all the plasmid-free isolates formed a single MLVA cluster 4b (Fig. 1),

indicating a possible clonal relationship among these isolates. Nevertheless, isolates within cluster 4a with 100% similarity by MLVA showed variable phenotypes. For instance, strain G47 was bf+, highly invasive in Caco-2 cells (Table 1) and motile, and survival rates within chicken macrophages were similar to those of other highly invasive strains (data not shown). In contrast, strain G45 was non-motile, did not form biofilm and showed low invasiveness in both cell lines (Tables 1 and 4). These results further suggest that genetically homogeneous isolates of *S. Enteritidis* may vary in their virulence potential and warrant further investigations on the mechanism underlying this differential virulence.

To determine whether the invasiveness of isolates correlated with their virulence, we compared the virulence of representative isolates in mice. All the isolates with low invasiveness caused no mortality or significantly lower mortality than high invasiveness isolates (Fig. 3). These data support our hypothesis that not all isolates of *S. Enteritidis* recovered from poultry are equally pathogenic. Although the molecular mechanisms underlying the differences in the virulence of *S. Enteritidis* remain poorly understood, both flagella and TTSS proteins encoded by virulence-associated genes located on SPI-1 and SPI-2 are known to play a major role in the pathogenesis of *S. Enteritidis* infection in cultured epithelial cells and in chicken and mouse challenge models (Allen-Vercoe & Woodward, 1999; Cogan *et al.*, 2004; Dibb-Fuller *et al.*, 1999; Methner & Barrow, 1997; Parker & Guard-Petter, 2001; van Asten *et al.*, 2000, 2004; Yim *et al.*, 2010). We observed that isolates with low invasiveness also had impaired secretion of SPI-1-encoded TTSS proteins (SipA and SipD) or motility-associated proteins (FlgK, FljB and FlgL) encoded by flagellar TTSS (Fig. 5). PCR amplification of the complete ORFs of *sipA*, *sipD*, *flgK*, *fljB* and *flgL* revealed that these genes were uniformly present in all isolates with low invasiveness and in representative isolates with high invasiveness (data not shown), indicating that the differential protein expression among these isolates was not due to the differences in gene content. Interestingly, TEM revealed that isolates with low invasiveness lacked flagellar appendages (Fig. 6e–h), which corroborates the impaired motility of these isolates and also partly explains the absence of bands corresponding to FlgK, FljB and FlgL proteins in isolate G45 (Fig. 5). It has also been shown that non-flagellar mutants of *S. Enteritidis* are strongly attenuated in their ability to invade cultured epithelial cells (Dibb-Fuller *et al.*, 1999; van Asten *et al.*, 2000), further suggesting that lower virulence of low invasiveness isolates is at least in part associated with their impaired motility (Table 4). It has been proposed that in *S. Typhi*, a host-adapted serotype, repression of flagellin may facilitate evasion from TLR5-mediated innate responses of host mucosal epithelium, thereby enhancing systemic bacterial dissemination from the intestine (Winter *et al.*, 2008, 2010). Repression of flagellin was also shown to enable increased dissemination of *S. Typhimurium* to the spleen

in a chicken model (Winter *et al.*, 2010). In contrast, deletion of FljC in *S. Enteritidis* caused virulence attenuation leading to significantly reduced counts in the spleen of orally infected chickens (Allen-Vercoe & Woodward, 1999; Parker & Guard-Petter, 2001). Because *S. Enteritidis* primarily causes gastroenteritis in immunocompetent individuals, we surmise that *S. Enteritidis* strains with impaired motility identified in our study are less likely to utilize the proposed pathway to cause systemic disease. Nevertheless, it would be interesting to test this hypothesis by using a non-murine model in which mucosal barriers against *Salmonella* infection mimic human mucosal barriers.

Previous studies have also revealed that genes encoded by SPI-1 TTSS are involved in intestinal invasion whereas genes encoded on SPI-2 are required for systemic infection and survival within macrophages (Desin *et al.*, 2009; Dieye *et al.*, 2009; Jones *et al.*, 2007; Li *et al.*, 2009; Rychlík *et al.*, 2009; Wisner *et al.*, 2010). Mutations in the genes encoding secretory effector proteins or genes that regulate their expression lead to virulence attenuation in *S. Enteritidis* (Amy *et al.*, 2004; Fardini *et al.*, 2007; Parker & Guard-Petter, 2001). At least two low invasive isolates, C19 and C45, tested here showed impaired secretion of SipA and SipD proteins (Fig. 5). It is possible that the differential transcriptional or translational regulation of TTSS accounts for the observed differences in virulence and other phenotypes observed in this study. With real-time quantitative RT-PCR we confirmed that the mRNA expression levels of *sipA* and *sipD* in these low invasiveness isolates were 16- and 11-fold lower, respectively, than the representative high invasiveness isolates BC8 and P21. Therefore, our results not only corroborate published reports indicating the roles of TTSS or flagellar secreted proteins in the pathogenesis of *S. Enteritidis* infection but also provide the first evidence of impaired motility and secretion of TTSS proteins among wild-type isolates of *S. Enteritidis*. Further investigations that compare global transcriptional or proteome signatures of strains with low and high invasiveness should provide more insights into the detailed molecular mechanisms that regulate the differences in virulence potential of this important food-borne pathogen.

Finally, because poultry is the major source of infection for humans, it may be argued that poultry isolates that are more invasive or pathogenic in chickens may carry or express virulence factors that enhance their fitness and virulence in humans. This argument is consistent with reports showing that clinical isolates of *S. Enteritidis* are more virulent in mice and chickens compared with *S. Enteritidis* isolates recovered from chickens or environmental sources (Humphrey *et al.*, 1995, 1996; Solano *et al.*, 1998). Moreover, *S. Enteritidis* isolates recovered from eggs or from the human clinical cases showed greater adherence to and invasiveness of chicken ovarian granulosa cells compared with the isolates recovered from chicken caeca (Saeed *et al.*, 2006). Our data show that isolates with high invasiveness in cultured Caco-2 cells are able to invade

and/or survive within cultured chicken macrophages in significantly higher numbers than isolates with low invasiveness (Table 4), suggesting that isolates with low invasiveness are likely to invade and/or survive less well in chickens. Determination of oral invasiveness of representative high and low invasiveness isolates in a 1-day-old chicken model may be required to confirm these results.

In conclusion, our study shows that although poultry is the major source of infection for humans, not all poultry-associated *S. Enteritidis* isolates are equally pathogenic, as measured by invasion assays using well-differentiated human intestinal epithelial cells (Caco-2) and live BALB/c mice. Further investigations on the detailed molecular mechanism underlying this differential virulence are required in order to understand the pathogenesis of this important food-borne pathogen.

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