Phenotypic and molecular characterization of *Salmonella enterica* serovar Sofia, an avirulent species in Australian poultry

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Salmonella enterica serovar Sofia (S. Sofia) is often isolated from chickens in Australia. However, despite its high frequency of isolation from chicken and chicken meat products, S. Sofia is rarely associated with animal or human salmonellosis, presumably because this serovar is avirulent in nature. The objective of this work was to investigate the phenotypic and molecular properties of S. Sofia in order to assess its pathogenic potential. Our *in vivo* studies support the observation that this serovar can colonize tissues, but does not cause disease in chickens. This was further confirmed with tissue culture assays, which showed that the ability of S. Sofia to adhere, invade and survive intracellularly is significantly diminished compared with the pathogenic *Salmonella enterica* serovar Typhimurium (S. Typhimurium) 82/6915. Molecular analysis of *Salmonella* pathogenicity islands (SPIs) showed that most of the differences observed in SPI1 to SPI5 of S. Sofia could be attributed to minor changes in the sequences, as indicated by a loss or gain of restriction cleavage sites within these regions. Sequence analysis demonstrated that the majority of virulence genes identified were predicted to encode proteins sharing a high identity (75–100%) with corresponding proteins from S. Typhimurium. However, a number of virulence genes in S. Sofia have accumulated mutations predicted to affect transcription and/or translation. The avirulence of this serovar is probably not the result of a single genetic change but rather of a series of alterations in a large number of virulence-associated genes. The acquisition of any single virulence gene will almost certainly not be sufficient to restore S. Sofia virulence.

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INTRODUCTION

Although a majority of *Salmonella enterica* serovars are pathogenic to humans and animals, their virulence varies from serovar to serovar. *Salmonella* usually causes gastroenteritis, with some serotypes [e.g. *Salmonella enterica* serovars Typhi and Typhimurium (S. Typhi and S. Typhimurium)] able to cause enteric fever in humans and mice, respectively, and others such as *Salmonella enterica* serovars Dublin and Pullorum (S. Dublin and S. Pullorum) more likely to cause bacteraemia in their target animals (Fierer & Guiney, 2001; Wallis, 2006). *Salmonella* spp. also have a wide variation in host range, from the ubiquitous serovars such as S. Typhimurium and *Salmonella enterica* serovar Enteritidis (S. Enteritidis) to the host-restricted serovars [e.g. S. Dublin and *Salmonella enterica* serovar Choleraesuis (S. Choleraesuis), both capable of causing infection in cattle, pigs and humans] and host-specific ones

(e.g. S. Typhi and S. Pullorum, which are fully adapted to humans and chickens, respectively) (Fierer & Guiney, 2001; Wallis, 2006). The pathogenicity of a serotype is determined by proteins encoded by various virulence genes; therefore, diversity can be attributed to genetic variation or polymorphisms in virulence genes.

Genes encoding virulence factors are usually found clustered together in distinct regions of bacterial chromosomes, termed pathogenicity islands (Hensel *et al.*, 1995; Marcus *et al.*, 2000). *Salmonella* pathogenicity islands (SPIs) are such regions, of which the most common and well studied are SPI1 to SPI5. These SPIs encode proteins that perform a wide variety of virulence-associated functions, such as translocation of effector proteins, host cell adhesion and invasion, intracellular survival and replication, and intestinal inflammation, all of which lead to gastroenteritis and/or systemic disease in the host (Chakravorty *et al.*, 2005; Darwin & Miller, 1999; Blanc-Potard & Groisman, 1997; Blanc-Potard *et al.*, 1999; Gerlach *et al.*, 2007; Hensel, 2000; Hong & Miller, 1998; Lawley *et al.*, 2006; Morgan *et al.*, 2004, 2007; Ochman *et al.*, 1996; Wood *et al.*, 1998; Wong *et al.*, 1998).

SPIs have evolved by horizontal gene transfer events mediated by mobile genetic elements such as integrons,

Abbreviation: SPI, *Salmonella* pathogenicity island.

The GenBank/EMBL/DDBJ accession number for the SPI1 sequence of S. Sofia Bt8 is FJ496648.

Three supplementary figures and four supplementary tables are available with the online version of this paper.

transposons, insertion sequence (IS) elements and origins of replication (Gal-Mor & Finlay, 2006; Hentschel & Hacker, 2001; Marcus *et al.*, 2000). The SPI regions may undergo genetic modifications (insertion and deletion events, mutations, etc.), generating the pathogenic diversity observed in *Salmonella* spp. It has been observed that naturally occurring insertions and deletions in the SPI regions of *Salmonella* isolates can affect the properties of the isolates, and render some serovars avirulent or unable to cause infection (Amavisit *et al.*, 2003; Ginocchio *et al.*, 1997; Hansen-Wester *et al.*, 2004; Ochman & Groisman, 1996; Porwollik *et al.*, 2002).

Although frequently isolated from Australian poultry (Mellor *et al.*, 2010), *Salmonella enterica* serovar Sofia (*S. Sofia*) does not cause disease in either humans or animals (Harrington *et al.*, 1991; Heuzenroeder *et al.*, 2001). The efficient colonization of a warm-blooded host such as the chicken is puzzling, as *S. enterica* subspecies II strains are usually associated with cold-blooded animals such as reptiles (Heuzenroeder *et al.*, 2001). Little is known about the pathogenicity of *S. Sofia*. *In vitro* studies with HeLa, CHO and CEF-DF1 cell lines have shown low levels of invasion compared with the pathogenic *S. Typhimurium* 82/6915, although the serovar was found to cause systemic disease in hosts (17-day-old embryonic chickens) with immature or immunocompromised immune systems (Rickard, 1998). An initial study by Heuzenroeder *et al.* (2001) revealed that *S. Sofia* has an incomplete *inv* region

of SPI1. As these genes are involved in pathogenicity (Darwin & Miller, 1999; Kimbrough & Miller, 2000; Kubori *et al.*, 1998, 2000; Sukhan *et al.*, 2001), their mutation may contribute to the avirulence of *S. Sofia*.

Since *S. Sofia* is an efficient colonizer of chickens and is widespread amongst Australian poultry flocks, detailed studies are needed to gain a better understanding of the *S. Sofia* virulence process to assess its pathogenic potential. In this study, we carried out a phenotypic and molecular analysis of *S. Sofia* to further define its avirulent nature. *In vitro* and *in vivo* studies were used to assess the pathogenic potential of this serovar. In addition, the distribution of genes across the entire length of SPI1 to SPI5 in *S. Sofia* Bt8 was investigated. We compared all generated data with the corresponding virulence regions in *S. Typhimurium* 82/6915 (a known pathogenic strain). These findings will aid in understanding the virulence process of *Salmonella* spp. and provide insight into the avirulent phenotype of *S. Sofia* isolates in Australia.

METHODS

Bacterial and tissue culture growth conditions. Bacterial strains used in this study are listed in Table 1. Bacteria were grown on Luria–Bertani (LB) agar or in LB broth and incubated aerobically at 37 °C for 16–18 h, unless specified otherwise. *S. Sofia* strains were confirmed using the xylose lysine (XLD), ONPG and malonate tests (Lowe, 1962; Shaw & Clarke, 1955). The following cell lines were used for *in vitro* analysis: a human intestinal epithelial cell line (INT407),

Table 1. *Salmonella* strains used in this study

Serovar	Strain identification	Location or origin*	Source
Agona	FSAW3463	VIC	A. Lee†
Enteritidis	446302	—	Biotechnology Laboratory, RMIT University
Hadar	P12	—	Biotechnology Laboratory, RMIT University
Heidelberg	—	—	Biotechnology Laboratory, RMIT University
Infantis	999S	SA	M. McKenzie‡
Sofia	Bt8	VIC	M. McKenzie
	630MSC	VIC	
	988Q	QLD	
	786CL	QLD	
	554NCA	NSW	
	561NC	NSW	
	664SCA	SA	
	642SC	SA	
	242T	TAS	
	140TCA	TAS	
Typhimurium	SL1344	—	R. Strugnelli§
	82/6915	—	Biotechnology Laboratory, RMIT University
Virchow	370QC	QLD	M. McKenzie

*Origin of isolate: VIC, Victoria; NSW, New South Wales; QLD, Queensland; SA, South Australia; TAS, Tasmania; —, isolate origin unknown/information not available.

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chicken embryonic fibroblasts (CEF-DF1) and a murine macrophage cell line (J774A.1). All cell lines were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% heat-inactivated new calf serum (NCS; Invitrogen) at 37 °C in a humidified 5% CO₂ incubator.

Adhesion, invasion and intracellular survival assays. Adhesion and invasion assays in INT407 and CEF-DF1 cells were carried out in 24-well tissue culture plates, as described by Elsinghorst (1994), with minor modifications. Both cell lines were seeded at a concentration of 3×10^5 cells per well and incubated overnight to obtain an ~90% confluent monolayer. Infection was for 1 and 2 h (adherence and invasion assays, respectively) at an m.o.i. of 50–100 bacteria per tissue culture cell. After infection, wells were washed three times with PBS and incubated in media with 400 µg gentamicin ml⁻¹ (Sigma Aldrich) to kill any extracellular bacteria. The cells were then washed three times again with PBS, lysed with 0.25% Triton X-100, subjected to serial 10-fold dilutions, and plated on LB agar. Plates were incubated overnight at 37 °C and bacteria were enumerated. Adherence assays were performed in the same manner without the gentamicin incubation step.

The ability of *Salmonella* isolates to survive intracellularly within macrophages was measured by the method outlined by Buchmeier & Heffron (1989), with some modifications. Opsonized bacterial cells were added to 24-well tissue culture plates with 4×10^5 macrophages per well at a ratio of 10–20 bacteria per macrophage. Phagocytosis was allowed to proceed for 20 min before cells were washed three times with PBS and incubated with 100 µg gentamicin ml⁻¹ for 1 h. Cells were then rinsed three times with PBS and medium containing 10 µg gentamicin ml⁻¹ was added (time point 0 h). Infected macrophages were sampled at 0, 1, 4 and 24 h after infection by washing the cells with PBS and then lysing the macrophages with 0.25% Triton X-100. Appropriate dilutions were plated on LB agar plates to enumerate c.f.u.

Colonization of chickens. Newly hatched Cobb 500 broiler chicks were obtained from Inghams Farms, Pakenham, Victoria. Three groups of five chickens were colonized with *S. Sofia* Bt8 with an oral dose of 10^8 c.f.u., and one group of five chickens was uncolonized. Faecal samples were taken for five consecutive days and enumerated, and chickens were observed twice daily for signs of abnormal behaviour. Faecal matter was suspended in LB broth, diluted and plated to Hektoen and XLD media in duplicate. Plates were incubated at 37 °C for 18–24 h. Standard microbiology methods were used to recover bacteria from the chicken organs (Cooper *et al.*, 1992; Cox *et al.*, 2007). At necropsy (21 days post-inoculation), each organ was removed aseptically and stored at 4 °C until processed. Each organ was processed within 8 h of necropsy. Samples were streaked onto the selective media Hektoen Enteric agar and XLD agar. Cloacal swabs were taken and streaked onto the same selective media. Caecal contents were diluted in LB broth and spread onto the same selective media.

Design of SPI primers. All primers were designed from *S. Typhimurium* LT2 sequences [GenBank accession nos NC_003197 (for SPI1), X99944, Y09357, AJ224892, U51927, Z95891, AJ224978 and X99945 (for SPI2), AF106566 (for SPI3), AJ576316 (for SPI4) and AE008747 (for SPI5)]. Sequences of the primers used in this study are given in Supplementary Tables S1–S3.

Restriction digestion and Southern transfer of genomic DNA. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Reichardt & Rogers, 1994). Approximately 10–15 µg genomic DNA from *S. Typhimurium* and *S. Sofia* isolates was digested at 37 °C for 16–18 h with 10 U of the restriction enzymes *Bgl*II, *Eco*RV and *Hind*III (Promega) or with 10 U of a combination of these enzymes (*Bgl*II and *Eco*RV or *Hind*III and *Eco*RV). DNA fragments were subjected to electrophoresis in 1.0% agarose gels before

being capillary-transferred and cross-linked onto Hybond-N membranes (Amersham Biosciences) for Southern hybridization. *S. Typhimurium* SL1344 was always included as a reference strain.

PCR amplification of SPI genes. DNA probes for Southern hybridization analysis were amplified using template DNA from *S. Typhimurium* SL1344. DNA probes and PCR products in the range 4.0–17 kb were amplified using the Expand Long Template PCR system (Roche Applied Science). Reactions were prepared in a 50 µl volume containing 1 × Expand Buffer System 3, 350 µM dNTP mix, 0.4 µM of each primer, 0.25 U Expand *Taq* polymerase and 200–400 ng template DNA. Amplification was carried out as described by Amavisit *et al.* (2003), with the amplification conditions adjusted depending on the annealing temperature and expected size of each PCR product (Supplementary Tables S1 and S2).

Amplification of PCR products less than 4.0 kb in length was carried out in a 25 µl volume containing GeneAmp 1 × PCR buffer (Applied Biosystems), 1 × Q-solution (Qiagen), 2.5 mM MgCl₂, 200 µM dNTP mix, 0.25 µM of each primer, 0.25 U AmpliTaq polymerase (Applied Biosystems) and 100–200 ng template DNA. PCR cycling conditions were as follows: 95 °C for 5 min, 35 cycles of 94 °C for 30 s, appropriate annealing temperature for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 1 min. The annealing temperatures and expected size of each PCR product are given in Supplementary Tables S1 and S2.

Southern hybridization. Southern blots of *Salmonella* isolates were processed using the DIG DNA Labeling and Detection kit (Roche Applied Sciences). DNA probes were labelled using the random primed method according to the manufacturer's protocol. After transfer of DNA, membranes were hybridized with the DIG-labelled probes at 65 °C in standard hybridization buffer [5 × SSC (0.75 M NaCl, 0.075 M sodium citrate), 0.1% Sarkosyl (w/v), 0.02% SDS (w/v) and 1.0% blocking solution (w/v)] for 16–18 h. The blots were then washed twice in 2 × SSC wash buffer [2 × SSC, 0.1% SDS (w/v)] for 5 min each at room temperature, followed by two 15 min washes in 0.1 × SSC wash buffer [0.1 × SSC, 0.1% SDS (w/v)] at 68 °C, and then subjected to anti-DIG detection as described by the manufacturer.

Sequencing of the SPI genes. PCR products were purified using the GeneClean kit (Bio 101 Systems) by following the manufacturer's instructions. Sequencing reactions were prepared and processed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's protocol, and analysed by Micromon at Monash University, Melbourne, Australia. Isolate Bt8 was chosen as a reference *S. Sofia* strain for sequencing analysis by primer walking. Only SPI1 was sequenced with both forward and reverse primers (Supplementary Table S3); all the other SPIs were analysed by single-stranded sequencing (with either the forward or reverse primer). Potential mutation events in the SPI genes of *S. Sofia* Bt8 that were identified in the single-stranded sequence screen (by comparison with the corresponding gene sequence from *S. Typhimurium* SL1344) were confirmed by a subsequent PCR and sequence analysis of the region.

Data analysis. Southern hybridization patterns were analysed for differences in the RFLP pattern (by using SL1344 as a reference). Any differences observed in the *S. Sofia* isolates were subjected to further investigation by PCR and sequencing analysis. DNA sequences were assembled to produce a full-length SPI. The DNA sequence of each *S. Sofia* Bt8 SPI region was compared with the sequence of the corresponding *S. Typhimurium* LT2 SPI sequence (obtained from GenBank). A map of SPI1 to SPI5 of *S. Sofia* Bt8 was constructed based on the RFLP and sequence data. In addition, the putative protein sequence encoded by each Bt8 SPI gene was compared with the corresponding LT2 SPI proteins.

Fig. 1. Assessment of the adhesion, invasion, and intracellular survival and replication ability of *S. Sofia*. All experiments were carried out in triplicate at three different times, and data are shown as mean $\pm$ SEM ($n=9$). Asterisks indicate values that are significantly altered compared with those of *S. Typhimurium* 82/6915 ($P<0.01$). (a) Attachment to INT407 and CEF-DF1 cells. Adherence is expressed as a percentage of the number of adhered bacteria compared with the inoculum. (b) Invasiveness in INT407 and CEF-DF1 cells. Invasion is expressed as the percentage of invasive bacteria (number of invasive bacteria over initial number in the inoculum) relative to that of the pathogenic *S. Typhimurium* 82/6915 (taken as 100%). (c) Survival and replication of *S. Sofia* in J774A.1 macrophages over 24 h. Recovery of *S. Sofia* (as a percentage) is shown as the number of c.f.u. at 24 h post-infection over the number of c.f.u. at 0 h post-infection.

RESULTS

S. Sofia is significantly less virulent than *S. Typhimurium* in cultured cells

The ability of *S. Sofia* Bt8 to adhere to and invade INT-407 and CEF cells in comparison with the pathogenic *S. Typhimurium* 82/6915 and non-invasive *Escherichia coli* DH5 α was analysed. *S. Sofia* was found to have levels of adherence comparable with those of *E. coli*, but was significantly less adherent than *S. Typhimurium* (Fig. 1). Although slightly more invasive than the DH5 α strain, *S. Sofia* was recovered at significantly lower levels than the pathogenic 82/6915 isolate ($\sim 1\%$, see Fig. 1b). We also measured the ability of the strains to survive and replicate inside macrophages, as intramacrophage survival and replication have been shown to be an important part of the *Salmonella* pathogenic process (Darwin & Miller, 1999; Ochman *et al.*, 1996). *S. Sofia* was unable to survive and replicate in J774 cells, showing a 1.5-fold increase compared with the 4.5-fold increase observed in 82/6915 (Fig. 1). Similar levels of adherence, invasion and intramacrophage survival were observed with another *S. Sofia* isolate, 242T (data not shown). Based on the *in vitro* data collected, it can be established that *S. Sofia* not only is inefficient at adhering to and invading cells but also is defective for macrophage survival and replication.

S. Sofia colonizes chickens but causes no detectable signs of disease

Each inoculated chicken showed minor signs of salmonellosis in the first 2 days, with faecal matter having a more watery appearance and consistency than prior to colonization. Feeding behaviour was normal and normal activity was observed. Faecal counts were followed for 5 days post-inoculation. The highest level of *S. Sofia* recovered was at 24 h post-inoculation, at 1.32×10^8 c.f.u. g^{-1} , and the lowest on the fifth day (120 h) post-inoculation, at 1.24×10^8 c.f.u. g^{-1} . No chickens in the uninoculated group tested positive for *S. Sofia*. Twenty-one days after inoculation chickens were killed and examined. No signs of visual damage or loss of colour were observed for any organ removed from colonized chickens during necropsy. They were of normal size and shape for the chicken's age, and indistinguishable from those in uninoculated chickens, from which no *S. Sofia* was detected. In the inoculated chickens, *S. Sofia* was found in 26.67% of the caeca (4/15), 46.67% of the cloacal swabs (7/15), 66.67% of the livers (10/15) and 73.33% of the spleens (11/15). This would suggest that *S. Sofia* is capable of colonizing and spreading to chicken organs but is not able to cause infection or disease.

Molecular comparison of SPI1 to SPI5 in *S. Sofia* reveals multiple changes

S. Typhimurium isolates SL1344 and 82/6915 show identical restriction cleavage patterns (data not shown).

Table 2. Summary of genetic changes in the SPIs of *S. Sofia* (in comparison with *S. Typhimurium*)

SPI	Total number of genes*	Number of gene insertions	Number of genes with no changes (100 % match)	Number of genes with changes that may not affect protein function†	Number of genes with changes that possibly affect protein function‡
1	42 (41)	1	2	33	6
2	44 (44)	—	—	41	3
3	12 (11)	1	—	6	5
4	6 (6)	—	—	6	—
5	9 (9)	—	1	3	5

*The number of genes in the corresponding SPI of *S. Typhimurium* is indicated in parentheses.

†Changes include base insertion, deletion and/or substitution.

‡Changes include gene and base insertion, deletion and/or substitution.

Southern hybridization patterns (SPI1 to SPI5) obtained from different *S. Sofia* isolates were identical to one another and different from those of *S. Typhimurium* isolates (results not shown). Based on the sequence data, approximately 80 % of the predicted *S. Sofia* effector proteins (Table 2) share 75–100 % identity with the corresponding proteins from *S. Typhimurium* LT2 and are assumed to be functional orthologues. The changes observed in the remaining 20 % of *S. Sofia* virulence genes (Table 2) are predicted to significantly alter the polypeptide sequence of the respective proteins, which almost certainly has functional implications.

Most differences observed across the length of SPI1 to SPI5 can be attributed to loss or gain of restriction cleavage sites, which resulted in differences in the RFLP patterns between *S. Sofia* and *S. Typhimurium* (Fig. 2 and Supplementary Fig. S4). However, several segments within the SPI1, SPI2, SPI3 and SPI5 regions in *S. Sofia* were observed to have accumulated changes predicted to affect gene expression (Fig. 2, Tables 2 and 3 and Supplementary Table S4). All changes (insertions, deletions and/or substitutions) seen in these regions were subjected to further analysis using Southern blotting, PCR amplification and genetic sequencing (when appropriate), and are compiled in Table 3. Large-scale changes in SPIs are depicted in Fig. 2, while the restriction patterns of SPIs 2 and 4 (in which no large deletions or insertions were seen) are depicted in Supplementary Fig. S3. All differences observed in SPI4 were attributable to the loss or gain of restriction sites, rather than to any insertions or deletions (see *EcoRV* restriction pattern in Supplementary Fig. S3). As the SPI4 proteins from *S. Sofia* Bt8 exhibited a 91–98 % match to their *S. Typhimurium* counterparts, this region is probably functional in *S. Sofia*.

DISCUSSION

The progress of *Salmonella* spp. infection varies depending on the infecting serovar and the type of host. Experimental infection and/or colonization of chickens with *Salmonella* has shown that the bacteria can colonize the intestinal tract

before invading and spreading to internal organ sites, where systemic infection can occur (Chadfield *et al.*, 2003; Chappell *et al.*, 2009; Gast, 1994). This was observed in our colonization studies with *S. Sofia*, as consistently high levels of the bacteria (relative to the amount inoculated orally) were recovered from the faecal and cloacal samples, indicating that *S. Sofia* was present in the gastrointestinal tract post-inoculation. *S. Sofia* was also recovered from various chicken internal organs, particularly the liver and spleen. Both these organs appear to be important in the systemic disease process, as they are probably the internal sites in which pathogenic *Salmonella* spp. can multiply (Chappell *et al.*, 2009). However, *S. Sofia* does not appear to follow the normal course of salmonellosis. The colonization by *S. Sofia* did not appear to cause infection or disease in the chickens, and this was further confirmed by the lack of morphological changes in or damage to the organs examined. Moreover, the avirulence of *S. Sofia* was also supported by the *in vitro* studies with human-, mouse- and chicken-derived cell lines. *S. Sofia* was significantly less adherent and invasive, and was inefficient at intracellular survival and replication relative to the pathogenic *S. Typhimurium* 82/6915 (Fig. 1). Host cell invasion and intramacrophage survival and replication have been shown to be mediated by SPI genes (Darwin & Miller, 1999; Marcus *et al.*, 2000; Zhang *et al.*, 2003), and the inability of *S. Sofia* to efficiently perform these virulence functions would indicate defects in this gene cluster.

Our molecular analysis confirmed this, and identified a relatively large number of changes in the SPIs between the virulent and avirulent serovars. The mutations observed in SPI1 (*orgB*, *prgI*, *invJ* and *sipD*) and SPI2 (*ssaP*, *ssaI* and *ssaE*) of *S. Sofia* appear to predominantly involve genes associated with the formation of a functional needle complex and the delivery of effector proteins into the host cell (Chakravorty *et al.*, 2005; Darwin & Miller, 1999; Kimbrough & Miller, 2000; Kubori *et al.*, 1998, 2000; Miki *et al.*, 2009; Sukhan *et al.*, 2001; Zierler & Galán, 1995). A defective needle structure and delivery system would greatly affect the type III secretion system (T3SS) of the bacteria, inhibiting the translocation of proteins required

Fig. 2. Variation of SPI1–5 between *S. Typhimurium* SL1344 and *S. Sofia* Bt8, as determined by Southern hybridization, PCR restriction digestion and sequence analysis. For each SPI, (i) shows a schematic representation of the SPI gene and its direction of transcription (adapted from Amavisit *et al.*, 2003). The absence of a gene or the presence of an insertion sequence (ORF) in *S. Sofia* is indicated as an unshaded block or a grey shaded box, respectively. DNA probes (the size and extent of each probe are shown) used for Southern hybridization are indicated above. For each SPI, (ii) shows an *EcoRV* restriction map for *S. Sofia* Bt8. The numbers indicate the sizes of detected fragments in kb. Maps are an estimate of the sizes of the restricted DNA fragments and are not drawn to scale.

for the pathogenic process. In addition, genetic changes were also found in SPI1 genes (*sptP* and *avrA*) encoding virulence effector proteins that perform functions such as rearrangement of the host cellular cytoskeleton (Collier-Hyams *et al.*, 2002; Darwin & Miller, 1999; Murli *et al.*, 2001; Zhang *et al.*, 2003). *avrA*, although deleted from SPI1, was present elsewhere in the genome (Table 2). Whether the encoded protein is functional in the absence of a T3SS is as yet unknown; however, it is considered unlikely. Interestingly, it has been shown that *avrA*, which encodes a cysteine protease responsible for inducing apoptosis, has variable expression amongst *S. enterica* serovars (Ben-Barak *et al.*, 2006).

SPI1 and SPI2 are the most important pathogenicity islands in *Salmonella* spp., being involved in host invasion

and systemic disease (Dieye *et al.*, 2009; Klein & Jones, 2001; Rychlik *et al.*, 2009; Wood *et al.*, 1998; Zhang *et al.*, 2003), and the mutations observed in these two regions probably affect the virulence of this serovar. As we have shown, invasion is significantly reduced in *S. Sofia*, and no signs of disease were observed in colonized chickens.

SPI3 genes (*marT*, *misL* and *slsA*) have been shown to be involved in the systemic disease process and colonization in calves, mice and chickens (Dorsey *et al.*, 2005; Lawley *et al.*, 2006; Morgan *et al.*, 2004; Rychlik *et al.*, 2009; Tükel *et al.*, 2007). Although their exact role in virulence processes has not been clearly defined, it is possible that the mutated protein products of these genes contribute to the avirulence of *S. Sofia* in poultry. Furthermore, proteins encoded by genes in the deleted SPI5 region are involved in intestinal

Table 3. Variant SPI regions in *S. Sofia* Bt8 compared with those of *S. Typhimurium* SL1344 and LT2

SPI	Gene(s) involved	Molecular analysis of SPI gene/region in <i>S. Sofia</i> Bt8
1	<i>avrA</i>	Analysis with primer pair <i>sitD</i> /F and <i>sprB</i> /R revealed that the <i>sitD-sprB</i> regions from <i>Salmonella</i> serovars Typhimurium, Enteritidis, Infantis, Virchow and Agona were the expected 2.3 kb, while those obtained from all the <i>S. Sofia</i> isolates were 1.4 kb in size (Supplementary Table S4). Sequence analysis of <i>sitD-sprB</i> revealed the replacement of the <i>avrA</i> fragment with a 0.28 kb fragment harbouring an ORF encoding a protein of unknown function, with 98 % identity to the 200 bp genetic element located in the <i>sitD-sprB</i> region of <i>S. Typhi</i> (GenBank accession no. AF013575) and <i>S. Choleraesuis</i> (AF013574). Although missing from its expected location within SPI1, Southern hybridization and PCR amplification showed that <i>avrA</i> was still present in the <i>S. Sofia</i> genome (Supplementary Table S4)
	ORF	Presence of a 1.2 kb insertion between <i>iagB</i> and <i>sptP</i> (Fig. 2). The putative ORF is predicted to be a 400 aa protein sharing sequence identity (up to 96 %) with a transposase from the mutator family. Only the <i>S. Sofia</i> isolates were observed to contain this sequence between <i>iagB</i> and <i>sptP</i> (Supplementary Table S4)
	<i>orgB</i>	Contains an additional 10 bp sequence (ATCGTAAAT) near the end of the reading frame, which (due to an in-frame stop codon) results in a protein missing 3 aa (valine, isoleucine and arginine) from the end of the polypeptide sequence
	<i>prgI</i>	<i>PrgI</i> was observed to have an extra threonine residue at position 2 due to the insertion of CCA after the start codon
	<i>invJ</i>	Has a single A–T base substitution (<u>ATG</u> to <u>GTG</u>) at the start codon of <i>invJ</i> . Although GTG can serve as an initiation codon in bacteria, if the start codon is inactivated by this mutation, then translation of <i>invJ</i> would start at the next methionine codon, resulting in a protein missing the first 52 aa. There is also the possibility that initiation at GTG would result in reduced expression
2	<i>ssaP</i>	Has a CCT deletion; predicted protein lacks a proline residue at position 45
	<i>ssaI</i>	<i>SsaI</i> contains an additional arginine residue (position 3 within the protein) caused by the insertion of AGA within the gene
	<i>ssaE</i>	Has a deletion of ~200 bp at the junction between <i>sseA</i> and <i>ssaE</i> (Supplementary Fig. S4). In addition, a single base substitution of T to C in <i>ssaE</i> interferes with the stop codon of this protein, resulting in five extra residues at the end of the predicted polypeptide sequence (NKSL)
3	<i>sugR</i> and <i>rhuM</i>	PCR amplification showed that the <i>S. Typhimurium</i> and <i>S. Enteritidis</i> strains possess the specific 4.88 kb fragment containing both the genes, which is missing from <i>S. Sofia</i> isolates (Supplementary Table S4). Further analysis of the missing <i>sugR-rhuM</i> region revealed that it contains a 135 bp nucleotide sequence sharing 99 % identity with the putative IS1351 transposase (pseudogene) from <i>S. Typhi</i> CT18 (GenBank accession no. AL627280)
3	<i>slsA</i>	Insertion of the 1.2 kb ORF noted above into <i>slsA</i> . Sequence data revealed that this fragment is inserted towards the end of <i>slsA</i> , disrupting the stop codon for the protein. If translated, the predicted <i>SlsA</i> would have an additional 25 aa at the end of the polypeptide sequence
	<i>misL</i>	Has a 15 bp deletion (TCCCTGACCCTGTGCG) from the middle of <i>misL</i> , resulting in a putative protein missing 5 aa from the middle of the polypeptide sequence (VPDPV)
	<i>marT</i>	Predicted <i>MarT</i> protein lacks the first 23 aa due to a genetic frameshift, resulting from the insertion of a 10 bp sequence (CGGGAGAAAC) about 29 bp within the gene
5	<i>sopB-pipA</i>	A series of primers designed to amplify different areas of SPI5 (Supplementary Tables S1 and S2) revealed that only the <i>copR-orfX</i> region (Fig. 2) yields specific PCR products. The deletion of the right half of this region (<i>sopB</i> , <i>pipC</i> , <i>pipB</i> and <i>pipA</i>) was confirmed by Southern hybridization (with probe <i>sopB-pipA</i> , Fig. 2) and PCR amplification

secretory and inflammation responses, and mutations in these genes lead to attenuation and reduced virulence (Galyov *et al.*, 1997; Morgan *et al.*, 2004; Pfeifer *et al.*, 1999; Wood *et al.*, 1998). The inability of *S. Sofia* to induce intestinal inflammation and secretion, which are required to cause gastroenteritis, could be another reason why this serovar is unable to cause disease in poultry or humans.

It is possible that there has been a key mutational event that reduced virulence in this serovar, reducing selective pressure on the remaining virulence genes, which were free to mutate, as their functionality was no longer required. For example, a primary mutational event (perhaps mediated by a mobile DNA element such as the 1.2 kb transposase in SPI1 and SPI3), followed by further mutations to other genes in the

region, may have occurred in SPI1, and eventually led to the inability of *S. Sofia* to induce systemic disease. More genetic changes may have occurred in the SPI regions over time due to the lack of selective pressure on the genes, which would increase the frequency of mutational events.

The multiple mutations found in the SPI regions of *S. Sofia* do not seem to have abolished its ability to efficiently colonize chickens. *S. Sofia* isolates are particularly adherent to surfaces, and factors such as physicochemical properties (surface charge and hydrophobicity), fimbriae, flagella and membrane proteins may contribute to its persistence (Chia *et al.*, 2008, 2009; Heuzenroeder *et al.*, 2001). The observation that *S. Sofia* has a significantly reduced ability to invade cells when assayed *in vitro* is consistent with the

observed reduction in adherence. However, these observations would seem at to be at odds with finding the bacterium in internal tissues, such as the liver and spleen. It is presumed that there is a correlation between ability to adhere and invade and dissemination to deeper tissues. This study and some recent publications indicate that this may not be the case. Desin *et al.* (2010) constructed a mutant strain of *S. Enteritidis* that harboured a deletion of SPI1, and demonstrated that although this mutant has significantly reduced cellular invasion, the bacterium can be detected in the livers and spleens of chickens within 1 day of oral challenge. Similarly, Wisner *et al.* (2010) found that a mutant of *S. Enteritidis* deleted for either the entire SPI1–SPI2 region or the SPI2 region alone is able to colonize tissues, albeit at a slightly slower rate than the wild-type. Furthermore, in studies of the prevalent commensal of chickens, *Campylobacter jejuni*, it has been found that the bacterium is present in the liver and spleen in some birds within 20 h of challenge (Cox *et al.*, 2007; Meade *et al.*, 2009). Interestingly, as has been observed for *S. Sofia*, *C. jejuni* is a persistent infection frequently observed in poultry at slaughter. Taken together, these studies and the present one indicate that systemic spread of bacteria can occur in the absence of SPI1 and/or SPI2, and that this systemic dissemination does not necessarily induce disease.

In summary, the avirulence of *S. Sofia* is probably not due to a single mutation or deletion event but rather a series of mutations in the genes within the virulence regions (Table 2). Further studies of the expression of *S. Sofia* virulence genes (and the functionality of their encoded proteins) may reveal the extent to which the mutations have attenuated the virulence process. Wild-type *S. Sofia* isolates are highly unlikely to regain full pathogenicity because of the accumulated mutations in many important virulence genes (Table 2), and even the chance acquisition of a virulence factor (e.g. a virulence plasmid) will most likely not be sufficient to completely restore *S. Sofia* virulence. Therefore, *S. Sofia* may be considered somewhat differently from other *Salmonella* spp. when monitoring salmonellae. Due to its persistence and colonization ability, there may be potential for the future use of *S. Sofia* as an avirulent carriage vector for the delivery of vaccines and other useful products within the chicken gastrointestinal system.

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