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Presence of *Salmonella* pathogenicity island 2 genes in seafood-associated *Salmonella* serovars and the role of the *sseC* gene in survival of *Salmonella enterica* serovar Weltevreden in epithelial cells

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The type III secretion system encoded by the *Salmonella* pathogenicity island 2 (SPI-2) has a central role in the pathogenesis of systemic infections by *Salmonella*. Sixteen genes (*ssaU*, *ssaB*, *ssaR*, *ssaQ*, *ssaO*, *ssaS*, *ssaP*, *ssaT*, *sscB*, *sseF*, *sseG*, *sseE*, *sseD*, *sseC*, *ssaD* and *sscA*) of SPI-2 were targeted for PCR amplification in 57 seafood-associated serovars of *Salmonella*. The *sseC* gene of SPI-2 was found to be absent in two isolates of *Salmonella enterica* serovar Weltevreden, SW13 and SW39. Absence of *sseC* was confirmed by sequencing using flanking primers. SW13 had only 66 bp sequence of the *sseC* gene and SW39 had 58 bp sequence of this gene. A clinical isolate, *S.* Weltevreden – SW3, 10 : r : z6 – was used to construct a deletion mutant for the *sseC* gene. Significant reduction in the survival of SW3, 10 : r : z6 Δ*sseC* and natural mutants SW13 and SW39 in HeLa cells suggests that *sseC* has a crucial role in the intracellular survival of *S.* Weltevreden. Expression of *sseC* was upregulated during the intracellular phase of both *S. enterica* serovar Typhimurium and clinical isolate *S.* Weltevreden SW3, 10 : r : z6, suggesting a crucial role for this gene in the survival of *S.* Weltevreden inside host cells.

INTRODUCTION

Salmonella is a food/water-borne Gram-negative bacterial pathogen that causes clinical conditions ranging from mild gastrointestinal infection to systemic infections such as typhoid fever (Lacey, 1993). There are only two species of *Salmonella*, *S. enterica* and *S. bongori*, but more than 2500 serotypes. Based on the degree of host adaptation *Salmonella* serotypes are divided into three groups: (i) typhoidal (enteric) *Salmonella*, causing typhoid and paratyphoid fever in humans and generally not pathogenic for animals; (ii) non-typhoidal *Salmonella*, causing gastroenteritis in a broad range of animals, including mammals, reptiles, birds and insects; and (iii) *Salmonella* restricted to certain animals, e.g. *S.* Abortovis to sheep and *S.* Gallinarum to poultry. Isolation of non-typhoidal *Salmonella* serovars from fish, shellfish and other seafood has been reported by several workers (Aissa *et al.*, 2007; Koonse *et al.*, 2005; Kumar *et al.*, 2009; Shabarinath *et al.*, 2007). Non-typhoidal *Salmonella* infections in humans are reported to be mainly associated with consumption of contaminated food and water. Although various serovars of *Salmonella* are associated with seafood (Koonse *et al.*, 2005), most human cases of *Salmonella* infection related to seafood consumption are generally caused by two major serovars, *S.* Typhimurium and *S.* Enteritidis (Greig & Ravel, 2009).

A number of virulence genes have been identified in *Salmonella*; these are generally located in clusters called *Salmonella* pathogenicity islands (SPI). There are 12 pathogenicity islands of *Salmonella* reported to date (Hensel, 2004). Generally, pathogenicity islands are flanked by tRNA genes (Hacker *et al.*, 1997); for example, SPI-2 is integrated at the $tRNA^{Val}$ locus (Hensel *et al.*, 1997). It is widely accepted that *Salmonella* has acquired these pathogenicity islands by horizontal gene transfer. *Salmonella* can enter the host cell cytoplasm through either a phagocytic or a non-phagocytic pathway. SPI-1 encodes a specialized needle-like surface apparatus, the type III secretion system (T3SS), that mediates the delivery of bacterial virulence proteins to the host cell cytoplasm (Hansen-Wester & Hensel, 2001). After entry into the host cell by the phagocytic or non-phagocytic route, *Salmonella* is surrounded in

Abbreviations: CTAB, *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide; SCV, *Salmonella*-containing vacuole; SPI-1 and SPI-2, *Salmonella* pathogenicity island 1 and 2; T3SS, type III secretion system.

Two supplementary figures are available with the online version of this paper.

a membrane-bound compartment termed the *Salmonella*-containing vacuole (SCV) (Gorvel & Méresse, 2001; Steele-Mortimer *et al.*, 1999; Szeto *et al.*, 2009). Some effector proteins encoded by SPI-2 (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Szeto *et al.*, 2009) are required for intracellular replication. These proteins have a role in prevention of phagosome–lysosome fusion and also in protecting the SCV from phagocytic defence enzymes, such as phagocyte NADPH oxidase and inducible nitric oxide synthases (Chakravortty *et al.*, 2002; Mastroeni *et al.*, 2000). SPI-2 is 40 kb in size, including a 25 kb portion encoding a T3SS and a 15 kb portion encoding tetrathionate reductase (Ttr), involved in anaerobic respiration. Based on the homology of the T3SS to those of other organisms, SPI-2 genes are predicted to encode four types of proteins, viz. secreted, regulatory, structural and chaperone. The presence of SPI-2 in *Salmonella* of different serovars has been investigated by PCR methods and by comparison of sequence of specific genes of SPI-2 (Amavisit *et al.*, 2003). Base composition and the distribution of genes between different *Salmonella* serovars confirm that SPI-2 has a mosaic structure and that the evolution of virulence in *Salmonella* is driven by horizontal gene transfer (Hensel *et al.*, 1999). Strains carrying mutations within SPI-2 genes are attenuated for virulence, which confirms the importance of these genes at different stages of infection and for survival of bacteria inside the host (Klein & Jones, 2001). SseC is a translocon for the effector proteins that is similar to secreted proteins in enteropathogenic *Escherichia coli* and *Yersinia*. SseC is 24 % identical to EspD of enteropathogenic *E. coli* (Hensel *et al.*, 1998). SseC has homology to YopB of *Yersinia pseudotuberculosis*, required for delivery of Yop proteins into the host cell (Håkansson *et al.*, 1996). The study of expression level of bacterial genes inside host cells has brought new insight into the infection biology of pathogens such as *Listeria monocytogenes*, *S. enterica* and *Shigella flexneri* (Chatterjee *et al.*, 2006; Eriksson *et al.*, 2003; Lucchini *et al.*, 2005). The HeLa epithelial cell line is often used as a tissue-culture model to demonstrate adhesion and invasiveness of different *Salmonella* serovars (Jones & Richardson, 1981; Jones *et al.*, 1981; Tavendale *et al.*, 1983). It is reported that *S.* Typhimurium starts to replicate inside the epithelial cells within 3–4 h post-infection (Eriksson-Ygberg *et al.*, 2006; Knodler & Steele-Mortimer, 2003).

In this study, we first screened *Salmonella* isolates obtained from seafood belonging to different serovars for the presence of genes known to be associated with SPI-2. Intracellular survival studies of natural deletion mutants and laboratory-generated mutants of *Salmonella* Weltevreden were carried out. Further, intracellular gene expression of SseC was studied by real-time PCR.

METHODS

Bacterial isolates and growth conditions. Fifty-seven *Salmonella* isolates from seafood samples were used in this study: *S. enterica* serovar Weltevreden (17 isolates), *S. enterica* serovar Newport (10 isolates), *S. enterica* serovar Bareilly (8 isolates), *S. enterica* serovar Paratyphi C (9 isolates), *S. enterica* serovar Oslo (7 isolates), *S. enterica* serovar Infantis (3 isolates), *S. enterica* serovar Anatum (2 isolates) and *S. enterica* serovar Aba (1 isolate) (Table 1). They were maintained at −80 °C in nutrient broth containing 30 % glycerol (Sanyo Corporation). Isolates were activated by growing overnight at 37 °C in trypticase soy broth (TSB) with continuous aeration in a shaker water bath (150 r.p.m.). A loopful of the inoculum was subcultured on trypticase soy agar (TSA) to get isolated colonies, which were then picked up and maintained in TSA slants for further work. *S.* Typhimurium ATCC 14028 (ST14028) and clinical isolate *S.* Weltevreden3, 10:r:z6 (SW3, 10:r:z6) were used as reference strains. The clinical isolate was from an outbreak of food poisoning in a student hostel at Mangalore (Antony *et al.*, 2009). Antibiotics were used at the following concentrations when required: kanamycin, 50 μg ml^{-1}; ampicillin, 150 μg ml^{-1}; and chloramphenicol, 25 μg ml^{-1}.

PCR analysis of SPI-2-encoded genes. DNA was extracted according to the protocol described by Ausubel *et al.* (1995), and purity and concentration were checked spectrophotometrically (Nanodrop spectrophotometer ND-1000, V3.3.0, Thermo Fisher Scientific). DNA was used for PCR with two primer pairs for *hns* and *invA* gene amplification for confirmation of the identification as *Salmonella*. The cycling conditions and primer sequences were as described by Jones *et al.* (1993) for *hns* and by Rahn *et al.* (1992) for *invA*.

Sixteen genes (*ssaU*, *ssaB*, *ssaR*, *ssaQ*, *ssaO*, *ssaS*, *ssaP*, *ssaT*, *sscB*, *sseF*, *sseG*, *sseE*, *sseD*, *sseC*, *ssaD* and *sscA*) known to be part of SPI-2 were targeted for PCR in this study. All SPI-2 gene sequences were retrieved from the complete genome sequence of *S.* Typhimurium LT2 in the NCBI genome database (accession no. NC_003197), and primers (Table 2) were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3). Confirmation of the absence of gene of interest was done by a second PCR using primers binding to the region flanking the gene followed by sequencing of the product. The annealing temperature and cycling conditions were standardized using a programmable gradient thermocycler (MJ Research). The specific annealing temperature for each PCR and the product size are given in Table 2. PCR was performed in 50 μl volumes containing 5.0 μl 10× PCR buffer [0.1 M Tris/HCl (pH 8.3) (Bangalore Genei), 0.02 M $MgCl_2$, 0.5 M KCl, 0.1 % gelatin], 200 μmol l^{-1} of each dNTP, 0.2 μmol l^{-1} of each primer and 0.9 U *Taq* polymerase (Bangalore Genei). The PCR products were resolved in 1.5 % agarose gel, stained with ethidium bromide (5 ng ml^{-1}), and bands were observed using a gel documentation system (Herolab).

Construction of deletion mutants. Studies on deletion mutation of the *sseC* gene were performed on clinical isolate SW3, 10:r:z6 to confirm the role of this gene in seafood-associated *S.* Weltevreden isolates. Plasmids used for this study are listed in Table 2. In-frame *sseC* gene deletion mutants were constructed by the one-step method based on the phage Red recombinase (Datsenko & Wanner, 2000). Briefly, 70 nt primers specific to the target gene were designed. In the forward primer, 50 nt of the 5′ ends of the targeted genes were attached to 20 nt priming sequences for pKD4 as template DNA. Fifty nucleotides of the targeted genes included 32 nt upstream of the start codon and 18 nt downstream of the start codon of the targeted genes. In the reverse primer, 19 nt of priming sites 2 of pKD4 were attached to reverse complement of 50 nt of the 3′ ends of the targeted genes, which included 36 nt within the gene and 14 nt downstream of the stop codon (Link *et al.*, 1997).

The kanamycin-resistance cassette of plasmid pKD4, including the flanking FRT site, was amplified by PCR under standard conditions (initial denaturation for 5 min at 95 °C; 30 cycles of amplification

Table 1. Seafood-associated *Salmonella* and clinical isolates used in this study

Strain no.	Serotype	Source
SW3	*S.* Weltevreden	Fish
SW5	*S.* Weltevreden	Fish
SW12	*S.* Weltevreden	Fish
SW13	*S.* Weltevreden	Fish
SW15	*S.* Weltevreden	Fish
SW23	*S.* Weltevreden	Shrimp
SW24	*S.* Weltevreden	Shrimp
SW30	*S.* Weltevreden	Shrimp
SW36	*S.* Weltevreden	Shrimp
SW37	*S.* Weltevreden	Shrimp
SW39	*S.* Weltevreden	Oyster
SW43	*S.* Weltevreden	Oyster
SW49	*S.* Weltevreden	Fish
SW65	*S.* Weltevreden	Fish
SW14	*S.* Weltevreden	Fish
SW8	*S.* Weltevreden	Shrimp
SW9	*S.* Weltevreden	Fish
SO1	*S.* Oslo	Oyster
SO2	*S.* Oslo	Oyster
SO9	*S.* Oslo	Oyster
SO20	*S.* Oslo	Oyster
SO75	*S.* Oslo	Oyster
SO76	*S.* Oslo	Oyster
SO77	*S.* Oslo	Oyster
SN3	*S.* Newport	Oyster
SN33	*S.* Newport	Clam
SN34	*S.* Newport	Clam
SN35	*S.* Newport	Clam
SN36	*S.* Newport	Clam
SN37	*S.* Newport	Clam
SN68	*S.* Newport	Clam
SN70	*S.* Newport	Clam
SN71	*S.* Newport	Clam
SN72	*S.* Newport	Clam
SB6	*S.* Bareilly	Oyster
SB7	*S.* Bareilly	Oyster
SB13	*S.* Bareilly	Oyster
SB14	*S.* Bareilly	Oyster
SB15	*S.* Bareilly	Oyster
SB16	*S.* Bareilly	Oyster
SB21	*S.* Bareilly	Oyster
SB22	*S.* Bareilly	Oyster
SI64	*S.* Infantis	Clam
SI66	*S.* Infantis	Oyster
SI73	*S.* Infantis	Clam
SA74	*S.* Aba	Clam
SP1	*S.* Paratyphi C	Fish
SP2	*S.* Paratyphi C	Clam
SP3	*S.* Paratyphi C	Clam
SP4	*S.* Paratyphi C	Clam
SP5	*S.* Paratyphi C	Clam
SP6	*S.* Paratyphi C	Fish
SP7	*S.* Paratyphi C	Fish
SP12	*S.* Paratyphi C	Fish
SP13	*S.* Paratyphi C	Fish
SAN10	*S.* Anatum	Fish
SAN11	*S.* Anatum	Fish
ST14028	*S.* Typhimurium	ATCC
SW3, 10:r:z6	*S.* Weltevreden	Clinical isolate

consisting of denaturation for 15 s at 95 °C, annealing for 30 s at 51 °C and extension for 30 s at 72 °C; and final extension for 7 min at 72 °C). The 1.6 kb PCR product was purified using the QIAquick PCR purification kit (Qiagen) and 500–1000 ng of fragment DNA was transformed into SW3, 10:r:z6 cells harbouring the Red recombinase expression plasmid pKD46 using an ECM 630 electroporator (BTX). Transformants were selected after incubation at 37 °C on Luria–Bertani (LB) agar medium containing kanamycin (50 mg l^{-1}). Allelic replacement between the genomic DNA and the PCR product resulted in the deletion of the *sseC* gene. The kanamycin-resistance cassette was removed after transformation of plasmid pCP20 into newly selected transformed clones from Luria–Bertani (LB) agar medium containing kanamycin (50 mg l^{-1}). Transformants were selected at 37 °C on Luria–Bertani (LB) agar medium without any antibiotic. Deletion of the gene was confirmed by PCR analysis and DNA sequencing.

Epithelial cell infection. SW3, 10:r:z6, SW3, 10:r:z6 Δ*sseC*, ST14028 and *S.* Weltevreden natural mutants (SW13 and SW39) were used for this study. HeLa cells were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were grown in Dulbecco's Modified Minimal Essential Medium (DMEM) (Sigma-Aldrich) supplemented with 10 % fetal bovine serum (HiMedia) and glutamine (Sigma-Aldrich) in 5 % CO_2 at 37 °C. One millilitre of the HeLa cell suspension (10^6 cells ml^{-1}) was placed into each well of a 24-well tissue culture plate. Bacteria were grown overnight in LB medium in a shaker incubator at 37 °C and 150 r.p.m. until the late exponential phase. The cultures were diluted with DMEM without serum and used to infect the monolayer at a multiplicity of infection (m.o.i.) of 10. To ensure the maximum contact of bacteria with the epithelial cell monolayer, the plate was centrifuged for 5 min at 1500 r.p.m. at room temperature. Infected epithelial cells were incubated for 30 min at 37 °C in 5 % CO_2. In order to remove extracellular bacteria, the medium was replaced with new medium which contained 30 mg gentamicin ml^{-1}. Harvesting of cells was started at the beginning of the incubation and this was taken as the zero time point in the experiment. After 30 min incubation at 37 °C in 5 % CO_2, the gentamicin concentration was diluted to 5 mg ml^{-1} until the end of the assay (i.e. for a further 2 h, 6 h, 12 h, 16 h, 20 h and 24 h). At each time point, the monolayer was washed three times with Dulbecco's PBS (D-PBS) (Himedia) and cells were harvested to Eppendorf microfuge tubes using 0.5 % sodium deoxycholate to lyse the cells. The harvested cells were diluted with D-PBS. To determine the number of bacteria in 100 µl of the well, serially diluted cell suspension was plated onto TSA plates in triplicate and the numbers of bacteria were counted as c.f.u. ml^{-1}. The experiment was performed in triplicate. Differences in the numbers of viable cells at different time points were analysed by an independent sample *t*-test using mean values for c.f.u. ml^{-1}. A significance level of 5 % was used.

RNA extraction and reverse transcription. Bacteria were seeded onto the HeLa cells at an m.o.i of 10. The methodology of infection of HeLa cell lines as described above was followed. After 0 h, 2 h and 6 h incubation, cells from six tissue-culture flasks (75 cm^2) were lysed for 30 min by placing on ice in a solution containing 0.1 % SDS, 1 % phenol pH 4.3, 19 % ethanol in water (Eriksson *et al.*, 2003). This was

Table 2. Primers and plasmids used in this study

Gene targeted	Sequence (5′–3′)	Annealing temp. (°C)	Product size (bp)
Primers used to find distribution of SPI-2			
ssaO	F: ATGGAAACTTTGCTGGAGAT	63	378
	R: TCAACTTTGGTAATACGCAT		
ssaS	F: ATGAATGATTCTGAATTGAC	50	267
	R: TCAACCATGCTCTCCAATTC		
ssaP	F: ATGCGTATTACCAAAGTTGA	50	375
	R: TCATTCGCTATTCTTAACAT		
ssaT	F: ATGGCACAACAGGTAAATGA	63	780
	R: TCATACAGATGGAAACCAGT		
ssaQ	F: ATGTTAAGAATAGCGAATGA	55	969
	R: TTACGCTGTATTTTTGCAAA		
ssaU	F: ATGAGCGAGAAAACAGAACA	50	1059
	R: TTATGGTGTTTCGGTAGAAT		
ssaR	F: ATGTCTTTACCCGATTCGCC	63	648
	R: TCATGAAAAGCTCTGTACCA		
sscB	F: ATGATGATGAAAGAAGATCA	55	435
	R: TTAAGCAATAAGAGTATCAA		
sseE	F: ATGGTGCAAGAAATAGAGCA	53	417
	R: TTAAAAACGTCGCTGGATAA		
sseD	F: ATGGAAGCGAGTAACGTAGC	41	588
	R: TTACCTCGTTAATGCCCGGA		
sseC	F: ATGAATCGAATTCACAGTAA	43	1455
	R: TTAAGCGCGATAGCCAGCTA		
sscA	F: ATGAAAAAAGACCCGACCTA	43	474
	R: TTAGCTCCTGTCAGAAAGTT		
sseF	F: ATGCGCAAATAATGGTTGAT	60	888
	R: TCAGGCGCGTTAACAGGACG		
sseG	F: ATGAAACCTGTTAGCCCAAA	60	690
	R: TTACTCCGGCGCACGTTGTT		
ssaD	F: ATGGCATATCTCATGGTTAA	51	1212
	R: TCACTTAAAATCTAATGGAT		
ssaB	F: ATGTCTGAGGAGGGATTCAT	55	402
	R: TTATACCCCACCCGAATAAA		
Primers used for real-time PCR			
sseC R	F: CTTATATGGCCGCAGGTTGT	60	198
	R: CGCCAGATAAACCTCTCGTC		
Construction of deletion mutant			
sseC M	F: GGCATTGCGAGTCAGATATAAGC-GGAGGCGGCATGGCAGACAAATA-CTTAGTGTAGGCTGGAGCTGCTTC	51	1600
	R: CGGGGCATTCTGAGCTACTTT-TTAGGTTCTGTTTCCCATTGTA GCAAACCCATATGAATATCCTCCTTA		

Plasmids	Description	Source
pKD4	Kan^r, *pir* dependent, FRT sites	CGSC, Yale (Datsenko & Wanner, 2000)
pKD46	Lambda Red helper plasmid; Amp^r	CGSC, Yale (Datsenko & Wanner, 2000)
pCP20	FLP recombinase plasmid; Cm^r Amp^r	CGSC, Yale (Datsenko & Wanner, 2000)

necessary to increase the stability of the bacterial RNA and to completely cover the cell layer. Lysates were collected in precooled 50 ml Falcon tubes and cells pelleted by centrifugation (20 min at 4000–8000 r.p.m. at 4 °C) for subsequent RNA extraction. RNA was extracted using a Qiagen RNeasy mini kit. Bacterial RNA was further purified by extraction in 50 % acidic phenol/50 % chloroform. Extracts were subsequently treated with DNase I (Fermentas International) according to the manufacturer's guidelines to remove the remaining DNA. The RNA quantity and quality were checked spectrophotometrically (ND-1000, V3.3.0, Thermo Fisher Scientific). The complete degradation of DNA was confirmed by PCR using the DNase-treated RNA. The quality of the extracted RNA was confirmed

by electrophoresis and the RNA samples were stored at −80 °C for further use. Reverse transcription was carried out according to the protocol of Fermentas Life Sciences. Briefly, the RNA was reverse transcribed to cDNA from 2 μg RNA using 2 μl reverse primer (100 ng μl^{-1}) and 0.5 μl RevertAid H minus (Fermentas International) at 42 °C for 1 h. cDNA samples were checked by PCR using gene-specific internal primers (Table 2) and stored at −20 °C for further use.

Real-time PCR. Quantification of the expression level of the *sseC* gene was done by real-time PCR. The appropriate primer concentration (300 nmol l^{-1}) was determined for subsequent use in the experiment. The *gyrB* gene was taken as the endogenous housekeeping gene as suggested by Wolz *et al.* (2002). Dissociation curve analysis in the real-time PCR was performed for each gene to check for the amplification of untargeted fragments. Real-time PCR was performed in an ABI PRISM 7300 Fast Real-time System thermal cycler (Applied Biosystems) in a total volume of 25 μl, consisting of 12.5 μl 2× SYBR Green Master Mix, appropriate volumes of forward and reverse primers, and 5 μl template cDNA. The volume of each reaction mixture was adjusted to 25 μl by adding sterile RNase-free water. Real-time PCR was performed with initial activation at 50 °C for 2 min, initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, primer annealing at 60 °C for 45 s and elongation at 72 °C for 30 s. Data acquisition was performed by 7300 SDS software (v. 1.3.1) at the end of each elongation step.

Validation of the real-time PCR was done by amplifying serial dilutions of cDNA synthesized from 1 μg RNA isolated from bacterial samples (Livak & Schmittgen, 2001) in order to do the relative quantification using the $2^{-\Delta\Delta C_t}$ formula.

The expression of the target genes was normalized to the endogenous control by calculating ΔC_t and expressed relative to a calibrator by calculating $\Delta\Delta C_t$ ($\Delta\Delta C_t = \Delta C_{t\ target} - \Delta C_{t\ calibrator}$). The 0 h time point of sampling was taken as the calibrator. The relative fold gene expression was calculated using the following formula: Relative fold gene expression $= 2^{-\Delta\Delta C_t}$.

Differences in gene expression levels were analysed by an independent sample *t*-test using ΔC_t data. A significance level of 5 % was used.

RESULTS

PCR identification

All the isolates used in the study were confirmed as *Salmonella* by PCR amplification of the *hns* and *invA* genes, which generated amplicons of 152 and 284 bp, respectively.

Distribution of SPI-2 genes in different *Salmonella* serotypes

The presence of 16 specific genes of SPI-2 in seafood-associated *Salmonella* isolates was tested by PCR. Genes recognized as components of the *Salmonella* T3SS, *ssaU*, *B*, *D*, *R*, *Q*, *O*, *S*, *P* and *T*, were present in all the isolates studied. The genes *sseF*, *G*, *E* and *D*, encoding *Salmonella* secreted effector proteins, were also present in all *Salmonella* serovars with the exception of *sseC* in two *S.* Weltevreden isolates, SW13 and SW39. Genes encoding *Salmonella* secreted chaperone proteins, *sscA* and *sscB*, were also present in all the isolates. The absence of the whole *sseC* gene was confirmed by the PCR product generated with primers flanking the whole gene (Fig. 1). The forward primer bound to the *sscA* gene (upstream of *sseC*) and the reverse primer to the *sseD* gene (downstream of *sseC*). The amplicon size was 2517 bp in ST14028, whereas in SW13 and SW39 it was 1106 bp and 1108 bp respectively (Fig. 1). PCR products were purified and sequenced. Comparison of the sequences with ST14028 indicated the deletion of the *sseC* gene from SW13 and SW39. SW13 and SW39 had only 66 bp and 58 bp sequence of the *sseC* gene, respectively (see Supplementary Figs S1 and S2, available with the online version of this paper). Multiple sequence alignment by CLUSTAL W showed that 22 nucleotides of the *sseC* gene of SW13 and SW39 were identical to the sequence of ST14028 (Fig. 2). There was partial overlap between the remaining sequences of *sseC* gene in the two strains.

Role of the *sseC* gene in survival of seafood-associated *S.* Weltevreden inside human epithelial cells

The survival of strains SW13 and SW39 in HeLa cells showed a steady decline, with counts of SW13 decreasing from 32.3±2.5 to 13.3±3.5 c.f.u. ml^{-1} and those of SW39 from 31.3±1.5 to 9.3±3.5 c.f.u. ml^{-1} at the 2 h time point (Table 3). The counts remained fairly steady up to 6 h followed by a sharp decline at the 12 h time point for both SW13 (2±1 c.f.u. ml^{-1}) and SW39 (2.3±0.6 c.f.u. ml^{-1}). No viable SW13 and SW39 bacteria could be detected at 24 h (Table 3). SW49, the seafood isolate of *S.* Weltevreden that was positive for the *sseC* gene, also showed an initial decline from 16.3±1.5 to 4.7±1.1 c.f.u. ml^{-1} at 2 h, followed by an increase to 10±5.5 at 6 h, 10.3±4.1 at 16 h and 23.6±1.5 at 24 h (Table 3). A steady increase in cell counts was seen in *S.* Typhimurium ST14028, from 51.3±14 to 204±17.5 c.f.u. ml^{-1} at 24 h, although there was slight decrease in counts up to 6 h (Table 3). In addition the clinical isolate *S.* Weltevreden SW3, 10:r:z6 showed an initial decrease followed by a gradual increase of cell count from 83.3±12.2 to 92±9.2 c.f.u. ml^{-1} at 20 h (Table 3), followed by a decrease to 31.7±19.5 at 24 h. The cell counts of the deletion mutant SW3, 10:r:z6 Δ*sseC* decreased sharply and no viable cells could be detected at 12 h.

It was observed that for each isolate there was a reduction in c.f.u. compared to the initial cell number at the 2 h time point. There was a statistically significant difference between the natural *sseC* deletion mutants (SW13, SW39) and *S.* Weltevreden SW3, 10:r:z6. Except at 0 h both mutants showed significant difference ($P<0.05$) from ST14028 at each time point.

The role of the SseC protein in survival of *S.* Typhi, *S.* Paratyphi and *S.* Typhimurium inside host cells has been determined (Eswarappa *et al.*, 2008; Klein & Jones, 2001). However, its role in the survival of other non-typhoidal *Salmonella* inside host epithelial cells is unknown. We constructed a deletion mutation of the *sseC* gene from the clinical isolate SW3, 10:r:z6 using the one-step method based on the phage Red recombinase, confirmed deletion by PCR and studied its survival in HeLa cells. The data in Table 3

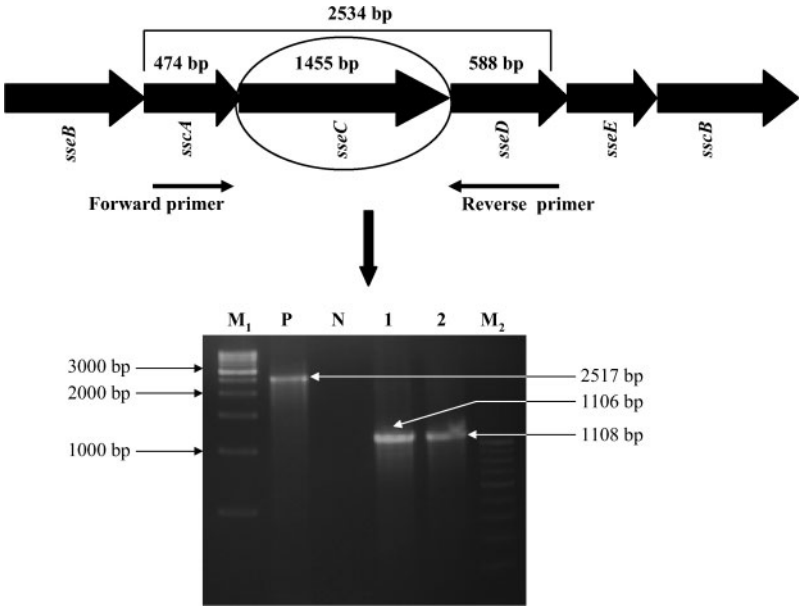

Fig. 1. Confirmation of absence of the *sseC* gene within isolates SW13 and SW39. Lanes: M_1, 500 bp DNA ladder; M_2, 100 bp DNA ladder; P, ST14028 (positive control); N, negative control; 1, SW13; 2, SW39.

show that the SW3, 10 : r : z6 Δ*sseC* mutant was able to survive only up to 6 h, in contrast to the wild-type, which was able to survive for over 24 h, suggesting that *sseC* has a major role in survival of *S.* Weltevreden inside HeLa cells.

Expression of the *sseC* gene inside HeLa cells

HeLa cells have commonly been used as a model to investigate epithelial cell infection by *Salmonella* and various other bacterial pathogens. In our study, bacterial RNA was extracted at 0 h, 2 h and 6 h post-infection from the HeLa cells to determine the level of expression of the *sseC* gene. We optimized the maximal recovery of bacterial cells and prepared stabilized bacterial RNA with minimal contamination by eukaryotic RNA as described previously (Eriksson *et al.*, 2003; Hinton *et al.*, 2004). The results showed that the expression of *sseC* of *S.* Typhimurium ST14028was 4.7 and 5.2 times higher at the 2 h and 6 h time points compared to expression at 0 h. The *sseC* gene expression of *S.* Weltevreden SW3, 10 : r : z6 was 3.0 and 3.8 times higher at the 2 h and 6 h time points compared to 0 h (Table 4). Statistical analysis showed that there was significant difference ($P<0.05$) in the expression level of the *sseC* gene at 2 and 6 h time point compared to 0 h for both the isolates (Table 4).

DISCUSSION

Although all serovars of *S. enterica* are presently considered pathogenic to man, the distribution of virulence genes in

```
SW39 sseC              ------------------------------------------------------------
S. Typhimurium sseC    ATGAATCGAATTCACAGTAATAGCGACAGCGCCGCAGGAGTAACCGCCTTAACACATCAT   60 bp
SW13 sseC              ------------------------------------------------------------


SW39  sseC             ------------------------------------------------AGCATCGTGTG   11 bp
S. Typhimurium, sseC   CACTTAAGCAATGTCAGTTGCGTTTCCTCGGGTTCGCTGGGAAAGCGCCAGCATCGTGTG  120 bp
SW13  sseC             ----------ATCTCATTTGCGTTTCCTCGGGTTAGCTGGGATCGCGCCAGCATCGTGTG   50 bp
                                                          ***********


SW39 sseC              AATTCTACTTTTGGCGATGGCATCGCCGCTTATCTGCTAATCGGGTA--------------   58 bp
S. Typhimurium sseC    AATTCTACTTTTGGCGATGGCAACGCCGCGTGTCTGCTATCCGGGAAAATTAGTCTTCAG  180 bp
SW13 sseC              AGGGCTAC----GGCGCTGA-----------------------------------------   66 bp
                       *   ****    **** **


SW39  sseC             ------------------------------------------------------------
S. Typhimurium sseC    GAGGCAAGCAATGCGTTGAAGCAACTGCTTGATGCCGTACCCGGA//CTATCGCGCTTAA 1455 bp
SW13 sseC              ------------------------------------------------------------
```

Fig. 2. Multiple sequence alignment by CLUSTAL W shows similarity between the remaining *sseC* gene of SW13 (66 bp) and SW39 (58 bp) and the *sseC* gene of *S*T14028. Asterisks (*) indicate nucleotides that are identical in the three isolates.

Table 3. Bacterial counts in HeLa cells infected with two natural mutants (SW13 and SW39) and laboratory-generated mutant SW3, 10 : r : z6 Δ*sseC* at different time points compared to wild-type SW3, 10 : r : z6 and ST14028 isolates

Time point	Parameter	SW 3, 10 : r : z6	ST14028	SW49 (*sseC*$^+$)	SW13	SW39	SW3, 10 : r : z6 Δ*sseC*
0 h	C.f.u. ml^{-1}	83.3 ± 12.2	51.3 ± 14.0	16 ± 1.5	32.3 ± 2.5	31.3 ± 1.5	55.7 ± 7.1
	P-value vs SW3, 10 : r : z6			0.013	0.002	0.002	0.027
	P-value vs ST14028			0.001	0.082	0.070	0.658
2 h	C.f.u. ml^{-1}	26.3 ± 1.52	22.7 ± 2.5	4.7 ± 1.1	13.3 ± 3.5	9.3 ± 3.5	11.3 ± 2.1
	P-value vs SW3, 10 : r : z6			<0.001	0.004	0.002	0.001
	P-value vs ST14028			<0.001	0.020	0.006	0.004
6 h	C.f.u. ml^{-1}	44 ± 11.5	40.7 ± 2.1	10 ± 5.5	12.7 ± 1.1	11.7 ± 2.5	1.3 ± 0.6
	P-value vs SW3, 10 : r : z6			0.001	0.009	0.009	0.003
	P-value vs ST14028			0.010	<0.001	<0.001	<0.001
12 h	C.f.u. ml^{-1}	34.7 ± 8.9	85.3 ± 9.3	11 ± 2	2 ± 1	2.3 ± 0.6	ND
	P-value vs SW3, 10 : r : z6			<0.001	0.003	0.003	ND
	P-value vs ST14028			0.013	<0.001	<0.001	ND
16 h	C.f.u. ml^{-1}	67 ± 2	105.3 ± 16.5	10.3 ± 4	ND	ND	ND
20 h	C.f.u. ml^{-1}	92 ± 9.2	149.7 ± 17.5	18.6 ± 3.5	ND	ND	ND
24 h	C.f.u. ml^{-1}	31.7 ± 19.5	204 ± 18.3	23.6 ± 1.5	ND	ND	ND

ND, Not detected.

most serotypes is not well understood. Of the 12 SPIs described in *Salmonella*, SPI-1, SPI-2 and SPI-4 are considered as conserved in all serovars, while others are specific for certain serovars (Hensel, 2004). Although *Salmonella* is occasionally isolated from fish and fishery products (Koonse *et al.*, 2005), the numbers of cases of salmonellosis attributable to seafood are rather small (Greig & Ravel, 2009). While serovars such as *S.* Weltevreden and *S.* Newport are common in seafood (Koonse *et al.*, 2005), they are rarely involved in human infections in developed countries. *S.* Typhimurium and *S.* Enteritidis are the commonest serovars in human cases in these countries (Greig & Ravel, 2009). However, in developing countries such as Thailand, Malaysia and India, human cases due to *S.* Weltevreden are not so uncommon (Aggarwal *et al.*, 1985; Antony *et al.*, 2009; Bangtrakulnonth *et al.*, 2004; Padungtod & Kaneene, 2006). Although *S.* Weltevreden is capable of causing human infections, it is possible that it is less virulent than serovars such as *S.* Typhimurium and *S.* Enteritids commonly encountered in human illnesses. The results of this study show that a clinical isolate of *S.* Weltevreden was less efficient than *S.* Typhimurium ST14028 in survival and multiplication in HeLa cells (Table 3). Differences in virulence between strains of *S.* Typhimurium from human and animal cases have been recorded (Heithoff *et al.*, 2008).

Table 4. Expression of the *sseC* gene in ST14028 and SW3, 10 : r : z6 inside HeLa cells at 2 h and 6 h post-infection

The 0 h time point was taken as calibrator ($P<0.05$).

Isolate	Expression at:			
	2 h		6 h	
	Fold change	*P*-value	Fold change	*P*-value
ST14028	4.7	0.000	5.2	0.001
SW3, 10 : r : z6	3.0	0.009	3.8	0.019

In this investigation, we first used PCR to study the distribution of genes known to be present in SPI-2 in serovars of *Salmonella* associated with seafood in India. All the significant functional genes of SPI-2 tested were present in all serovars except in two isolates of *S.* Weltevreden (SW13 and SW39). In these two isolates, the *sseC* gene appeared to be deleted. The deletion was confirmed by sequencing. A fragment of this gene was still present in SW13 as a 66 bp sequence and in SW39 as a 58 bp sequence (Supplementary Figs S1 and S2). The results in Fig. 2 also show that deletion has occurred in almost the same region of the *sseC* gene, suggesting a similar mechanism of deletion in these two isolates. Insertions or deletions in SPI-1, SPI-3 and SPI-5 in some *Salmonella* serovars have been reported previously by Amavisit *et al.* (2003), who used Southern hybridization to detect variations among serovars, but their collection did not include *S.* Weltevreden. For detection of variations within SPI-2, they used two probes, of 26 kb and 16 kb, and they detected only variations attributable to loss or gain of restriction endonuclease sites (Amavisit *et al.*, 2003). We performed a detailed analysis by PCR amplification of 16 genes and detected deletion of *sseC* gene in two isolates of *S.* Weltevreden. Ginocchio *et al.* (1997) reported natural deletion of *inv*, *spa* and *hil* loci of the centisome 63 region of the chromosome of several environmental isolates of *S.*

enterica serovar Senftenberg and *S. enterica* serovar Litchfield, which are required for entry of *Salmonella* spp. into mammalian cells. Hu *et al.* (2008) found natural deletion of some SPI-1-specific genes within *S.* Senftenberg. To our knowledge this is the first report of gene deletion in SPI-2 of *Salmonella* and it is a very significant finding.

Both SPI-1 and SPI-2 code for a T3SS in *Salmonella*. The T3SS encoded by SPI-1 is involved in invasion of M cells and epithelial cells, while the T3SS encoded by SPI-2 has a central role in interference with cellular defence and in intracellular survival and replication of *Salmonella* (Kuhle & Hensel, 2004). Proteins SseB, SseC and SseD function as translocons for effector proteins SseF and SseG (Hensel, 2004).

The absence of the *sseC* gene within SW13 and SW39 prompted us to study the survival of these two non-typhoidal *S.* Weltevreden isolates inside epithelial cells. To confirm whether the intracellular behaviour of these natural *sseC* deletion mutants is due to the absence of *sseC*, we constructed deletion mutants in the laboratory using the clinical isolate SW3, 10 : r : z6. The data in Table 3 show that in all isolates, there was an initial decline in numbers, which could be attributed to the bacteria taking time to adapt to the intracellular environment. Further, the results show that *S.* Typhimurium is capable of survival and multiplication in HeLa cells. The clinical isolate of *S.* Weltevreden SW3, 10 : r : z6 and the seafood isolate SW49 that was positive for the *sseC* gene was able to survive and showed limited multiplication. The *sseC* gene of the clinical isolate SW3, 10 : r : z6 was sequenced and the nucleotide sequence showed 99 % similarity to the *sseC* gene of *S.* Typhimurium (GenBank accession no. NC_003197) (data not shown). The laboratory-generated deletion mutant of the clinical isolate SW3, 10 : r : z6 Δ*sseC* and the natural *sseC* deletion mutants SW13 and SW39 showed a rapid decline in numbers in HeLa cells. No viable bacteria could be detected after 12–16 h (Table 3), thus proving that SseC is essential for survival of *S.* Weltevreden in host cells. Expression of *sseC* was upregulated during the intracellular phase in both *S.* Typhimurium and the clinical isolate of *S.* Weltevreden (Table 4). The expression seemed to be in line with intracellular survival and replication (Table 3). *S.* Typhimurium had a greater ability to survive and multiply in HeLa cells compared to *S.* Weltevreden, and *sseC* expression increased 5.2-fold in *S.* Typhimurium compared to 3.8-fold in *S.* Weltevreden. Paulin *et al.* (2007) showed *sseC* to be expressed by *S.* Typhimurium within porcine and murine macrophages at 4 h post-infection, and Bleasdale *et al.* (2009) showed high expression *sseC* at 4 h post-infection when *Acanthamoeba polyphaga* was infected with *S.* Typhimurium. Our study shows that deletion of the s*seC* gene in both natural and laboratory-generated mutants resulted in loss of ability to survive intracellularly in HeLa cells, and that expression of this gene has a crucial role in survival of *S.* Weltevreden within the host cells.

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