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Salmonella enterica Serovar Senftenberg Human Clinical Isolates Lacking SPI-1[▽]

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Nontyphoidal *Salmonella* species cause gastrointestinal disease worldwide. The prevailing theory of *Salmonella* enteropathogenesis is that bacterial invasion of the intestinal epithelium is essential for virulence and that this requires the virulence-associated genomic region *Salmonella* pathogenicity island 1 (SPI-1). Recent studies of *Salmonella enterica* infection models have demonstrated that enterocolitis and diarrhea in mice and cows can occur independently of SPI-1. In this study, we sought to confirm whether two *S. enterica* serovar Senftenberg clinical isolates lacked genes essential for SPI-1 function. Two clinical strains were isolated and identified as being *S. enterica* serovar Senftenberg from four stool samples from a food-borne disease outbreak affecting seven individuals in Shenzhen, Guangdong Province, China, using conventional methods, pulsed-field gel electrophoresis and multilocus sequence typing. The possibility of coinfection with other potential bacteria or usual viruses was excluded. Two isolates were analyzed for the presence of *invA*, *sipA*, *ssaR*, *sifA*, and *sopE2* by PCR and Southern blotting and were then assayed for the presence of SPI-1 by PCR and long-range PCR for *fhlA-hilA*, *hilA-spaP*, and *spaP-invH* and Southern blot analysis. A long-range PCR fragment from *fhlA* to *mutS* covering the 5′ and 3′ flanks of SPI-1 was also amplified from the two clinical isolates and sequenced. In addition, the two clinical isolates were assayed for enteroinvasiveness in vitro. Murine infection models were also examined. Biochemical tests and serotyping confirmed that the two clinical isolates are *S. enterica* serovar Senftenberg. However, they lacked genes critical for SPI-1 function but contained SPI-2 genes and were attenuated for the invasion of cultured intestinal epithelial cells. In conclusion, clinical *S. enterica* serovar Senftenberg strains isolated from a food-borne disease outbreak lack the invasion-associated locus SPI-1, indicating that SPI-1 is not essential for human gastroenteritis.

The nontyphoidal serovars of the gram-negative facultative intracellular pathogen *Salmonella enterica* cause invasive intestinal disease in humans, affecting 1.3 billion people and causing 3 million deaths annually, including 40,000 reported cases of illness and 600 deaths annually in the United States. In China, nontyphoidal serovars of *S. enterica* rank second as the cause of food-borne disease (annual Chinese food-borne disease report, Chinese Centre for Disease Control and Prevention; unpublished data). Intestinal pathogenesis of nontyphoidal *Salmonella* infection has been modeled in several hosts using multiple *S. enterica* serovars including serovars Dublin and Typhimurium. Using these models, two horizontally acquired *Salmonella* pathogenicity islands (SPIs), SPI-1 and SPI-2, that have specific roles in virulence in animals have been identified. Both SPIs encode type III secretion systems (T3SSs) capable of translocating bacterial "effector" proteins directly into host cells or the extracellular milieu, thereby altering host cell function. SPI-1 effectors disrupt normal cytoskeletal and cell barrier function affecting the uptake of bacteria into cells (invasion), while SPI-2 effectors subvert the maturation of the phagolysosome, facilitating prolonged intracellular bacterial survival (10). Based on animal model infections and epidemiological investigations, it is widely accepted that SPI-1-mediated intestinal epithelial invasion is essential for *Salmonella*-induced enterocolitis and diarrhea.

Based on the hypothesized necessity for SPI-1 in intestinal disease and its conservation among *Salmonella* species (14), SPI-1-encoded genes such as *invA* and *hilA* have been extensively exploited as molecular markers for the detection of enteropathogenic *S. enterica* (1, 3, 4, 5, 6, 13, 15, 16, 18, 19). Notably, isolates of *S. enterica* serovars Litchfield and Senftenberg lack SPI-1 and have decreased invasion of cultured epithelial cells (9). However, these strains were environmental isolates, not human pathogenic strains, consistent with the view that SPI-1 is necessary for enteropathogenesis (9). Data from us and others showed recently for the first time that experimental intestinal disease in murine and bovine intestinal disease models could occur despite the absence of the SPI-1 T3SS (7, 11), indicating that the dominant paradigm postulating that a functional SPI-1 T3SS and effectors are essential for intestinal inflammatory disease should be reconsidered.

The animal models of human disease used in previous experiments have significant limitations, and data arising from their use should be interpreted with caution. Murine infections are encumbered by concurrent systemic disease, while bovine

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TABLE 1. Primer sequences for PCR of SPI-1 and SPI-2 genes

Gene	Forward primer	Reverse primer	Conditions
invA	CAGCGATATCCAAATGTTGC	AAATGGCAGAACAGCGTCGTA	95°C/10 min; 35 cycles of 94°C/30 s, 55°C/1 min, and 72°C[a]/2.5 min; and 72°C/10 min
sipA	ATGGGTACCAGGCGGCTACTAAA ATCC	ATGGAGCTCCAAGCGAGAGAAAA ATCTACAC	94°C/2 min; 35 cycles of 94°C/30 s, 55°C/1 min, and 68°C/4 min; and 68°C[a]/10 min
ssaR	GTTCGGATTCATTGCTTCGG	TCTCCAGTGACTAACCCTAACCAA	95°C/10 min; 35 cycles of 94°C/30 s, 50°C/1 min, and 72°C/2 min; and 72°C/10 min
sifA	ATGGTCGACATGCCGATTACTATA GGGAATGG	ATGGGATCCTTATAAAAAACAACA TAAACAGCCG	95°C/10 min; 35 cycles of 94°C/30 s, 52°C/1 min, and 72°C/1 min; and 72°C/10 min
sopE2	TAATACCGCCCTACCCTCAG	CATAACACTATCCACCCAGCAC	94°C/3 min, 30 cycles of 94°C/1 min, 55°C/1 min, and 72°C/1 min; and 72°C/5 min
fhlA-hilA	AACGCTGCGCACGCTTAGCA GCAT	GCCTGGCAGAAAGCTAACAAGCG TGAC	94°C/1 min, 30 cycles of 98°C/10 s and 68°C/15 min, and 72°C/10 min
hilA-spaP	TTCGTCCAGATGACACTATCTC CTTC	CGTCAGTATGGAGAAGAGACCG AGAC	94°C/1 min, 30 cycles of 98°C/10 s and 68°C/15 min, and 72°C/10 min
spaP-invH	GTCTCGGTCTCTTCTCCATACT GACG	CTTTCATGGGCAGCAAGTAACG TCTG	94°C/1 min, 30 cycles of 98°C/10 s and 68°C/15 min, and 72°C/10 min

[a] Conditions at 72°C are for AmpliTaq Gold *Taq* polymerase from Perkin-Elmer, while the conditions at 68°C are for Elongase enzyme mix from Invitrogen.

infections are induced by inoculating ligated intestinal segments with large numbers of bacteria or orally inoculating animals with large doses of laboratory-grown strains. Furthermore, although grossly similar, differences in host physiology and infection susceptibility result in natural host-specific disease histories that cannot always be compared to human disease pathogenesis. Finally, SPI-1-deficient strains used in experimental infections are laboratory created, not spontaneously occurring, such as those that have been isolated from environmental reservoirs (18). Significantly, to our knowledge, no previously characterized strains of human disease inducing *Salmonella* lacking SPI-1 have ever been reported.

In this study, we isolated *S. enterica* serovar Senftenberg from human diarrhea patients infected in September 2002 in Shenzhen, China. We show here that the pathogen causing the outbreak of the food-borne disease is *S. enterica* serovar Senftenberg by using traditional methods, real-time PCR, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST). The *S. enterica* serovar Senftenberg isolates lack SPI-1 yet contain *ssaR*, *sopE2*, and *sifA* and are attenuated for the invasion of cultured intestinal epithelial cells. The data show for the first time that an SPI-1-deficient *Salmonella* serovar Senftenberg strain can cause human intestinal disease, indicating that SPI-1 is not critical for *Salmonella* human gastroenteritis.

MATERIALS AND METHODS

Strains. Control *Salmonella* serovar Senftenberg and Typhimurium strains were from the National Institute for the Control of Pharmaceutical and Biological Products, People's Republic of China. The *S. enterica* serovar Enteritidis strain was isolated from a food-borne disease outbreak in Shenzhen, Guangdong Province, China.

Pathogen identification. The outbreak of a food-borne disease occurred in Shenzhen in September 2002. Epidemiologists collected four stool samples from patients and sent them to the Shenzhen Centre for Disease Control and Prevention for pathogen isolation and identification. Stool samples were enriched in enrichment broth (commercial reagents obtained from Oxoid) including 7.5% sodium chloride broth, nutrient broth, selenite cystine broth, GN broth, *Enterobacteriaceae* enrichment broth, dextrose meat infusion broth, sodium chloride violet purple enrichment broth, tryptone soya broth, and *Listeria* enrichment broth (LB1 and LB2) at 35°C for 18 to 24 h, except for LB1 (30°C for 18 to 24 h). A loopful of each enrichment broth was streaked onto the appropriate agar (nutrient agar, TCBS agar, SS agar, blood agar, SMAC agar, and PALCAM agar), and a minimum of five typical colonies were picked and subjected to biochemical and serological tests for identification. Simultaneously, all enrichment broth samples of the four stool samples were detected by an *invA* real-time PCR assay and PCR assays for other common food-borne pathogens. Pathogens assayed include *Salmonella*, *Shigella*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, other vibrios, *Staphylococcus aureus*, *Escherichia coli* O157:H7, enterotoxigenic *Escherichia coli*, enteropathogenic *Escherichia coli*, enteroinvasive *Escherichia coli*, enterohemorrhagic *Escherichia coli*, *Bacillus cereus*, group A *Streptococcus*, and *Listeria monocytogenes*. The stool samples were also examined for rotavirus antigen by an antibody kit.

The two *S. enterica* serovar Senftenberg isolates were further analyzed by PCR assays. A total of 0.1 ml of a bacterial culture grown overnight was boiled and pelleted for use as a PCR template. PCR was carried out in a 20-μl volume with 10 mM $MgCl_2$, 1× buffer, 1 U *Taq* polymerase (Takara Biotechnology Co., Ltd.), and 200 nM primers. PCR primers and PCR conditions are indicated in Table 1. PCR products were separated in a 1% agarose gel with ethidium bromide.

PFGE. Bacterial strains, controls, and standards (*Salmonella* serovar Braenderup standard H9812) were grown with shaking at 37°C in Luria broth overnight and subcultured to an optical density at 600 nm of 0.6. The culture (1 ml) was pelleted, washed, and resuspended in 1 ml Tris-EDTA buffer(10 mM Tris-HCl, 1 mM EDTA). One milliliter of molten 2% agarose was added to the suspension and mixed, and the mixture was dispensed into two plug molds and allowed to cool for 5 min at room temperature. Plugs were transferred into 3 ml lysis buffer (50 mM Tris [pH 8.0], 50 mM EDTA [pH 8.0], 1% sodium deoxycholate, 0.15 mg/ml proteinase K, 1 mg/ml lysozyme, and 2 μg/ml RNase) and incubated for 2 h at 50 to 54°C with shaking. Plugs were washed with water and Tris-EDTA and then sliced. The slices were digested with XbaI at 37°C overnight. DNA restriction fragments were separated by electrophoresis in 1% SeaKem gold agarose gels in a 0.5× solution of Tris-borate-EDTA buffer (89 mM Tris, 89 mM borate, and 2 mM EDTA) at 14°C in a GenePath PFGE apparatus (Bio-Rad) with the following electrophoretic parameters: initial switch time of 2.2 s, final switch time of 54.2 s, run time at 6 v/cm for 22 h, angle of 120°, and linear ramping factor. The gel was stained with ethidium bromide (10 mg/ml), and genotypes were compared to the genotype of *Salmonella* serovar Braenderup standard H9812 by using Bionumericus software (version 4.0).

MLST. Based on seven housekeeping gene sequences available at the MLST website (https://www.mlst.net), the seven gene fragments, the *thrA*, *purE*, *sucA*, *hisD*, *aroC*, *hemD*, and *dnaN* genes, were amplified from the two clinical *Salmo-*

TABLE 2. PCR primers for amplifying Southern hybridization probes

Probe	Size (kb)	Position	Primer (5′–3′)	Conditions
invA1	1.267	*invA*	TCCCTTTGCGAATAACATCC (invA1/F) TACGGTTCCTTTGACGGTGC (invA1/R)	94°C/5 min; 35 cycles of 94°C/30 s, 55°C/1 min, and 72°C/1 min 30 s; and 72°C/10 min
invA2	1.736	*invA*	GGGTCAAGGCTGAGGAAG (invA2/F) CTGATCGCACTGAATATCGTAC (invA2/R)	94°C/5 min; 35 cycles of 94°C/30 s, 55°C/1 min, and 72°C/1 min 30 s; and 72°C/10 min
sipA	1.126	*sipA*	CGGCTTCACATTCACAA (sipA/F) CGGGCTCTTTCGTTCA (sipA/R)	94°C/5 min; 35 cycles of 94°C/30 s, 50°C/1 min, and 72°C/1 min 30 s; and 72°C/10 min
1A	14	*fhlA-hilA*	AACGCTGCGCACGCTTAGCAGCAT (1A/F) GCCTGGCAGAAAGCTAACAAGCGTGAC (1A/R)	94°C/1 min, 30 cycles of 98°C/10 s and 68°C/15 min, and 72°C/10 min
1B	3	*hilA-sicP*	TTCGTCCAGATGACACTATCTCCTT (1B/F) AATTGGGTTTACCGCTCACTTTT (1B/R)	95°C/5 min; 35 cycles of 94°C/30 s, 55°C/1 min, and 72°C/3 min; and 72°C/7 min
1C	4	*sicP-sipD*	CTTCATTATTCGCAGCCAGTTCA (1C/F) TGATGCTGAGATTTGGGATATGGT (1C/R)	94°C/1 min, 30 cycles of 98°C/10 s and 68°C/15 min, and 72°C/10 min
1D	5	*sipD-spaS*	CCCGACTGCCAGGCTTGAT (1D/F) TTGTAGGTATTGCCGTCATTTGG (1D/R)	94°C/1 min, 30 cycles of 98°C/10 s and 68°C/15 min, and 72°C/10 min
1E	3	*spaS-spaO*	CCGACGGTGGTTAGTGAACATT (1E/F) CGTTGCGCTTTGTAATCGGTAG (1E/R)	95°C/5 min; 35 cycles of 94°C/30 s, 55°C/1 min, and 72°C/3 min; and 72°C/7 min
1F	10	*spaP-invH*	GTCTCGGTCTCTTCTCCATACTGACG (1F/F) CTTTCATGGGCAGCAAGTAACGTCTG (1F/R)	94°C/1 min, 30 cycles of 98°C/10 s and 68°C/15 min, and 72°C/10 min

nella isolates by PCR and then sequenced. The sequences were then analyzed and clustered.

Southern blot. DNA probes used in Southern hybridization were prepared by PCR amplification of one or more fragments that spanned SPI-1 from SL1344. Nine probes were designed. The first probe, invA1, and the second, invA2, were fragments of the *invA* gene. The third probe, sipA, was a fragment of the *sipA* gene. The fourth, 1A, extended from *fhlA* to *hilA* and was approximately 14 kb long; the fifth, 1B, was 3 kb long, overlapping with 1A and covering *hilA* to *sicP*; the sixth, 1C, was approximately 4 kb long, encompassing *sicP* to *sipD*; the seventh, 1D, was 5 kb in length, extending from *sipD* to *spaS*; the eighth, 1E, was approximately 3 kb long, encompassing *spaS* to *spaO*; and the last probe, 1F, was 10 kb in length, overlapping with 1E and covering *spaP* to *invH*. The nucleotide sequences of the PCR primers for amplifying the probes are shown in Table 2, and the positions of the probes are shown in Fig. 4. The probes were labeled by digoxigenin (Roche Diagnostic China). Six strains including the two clinical isolates and control strains were used for Southern blotting. Bacterial genomic DNA was extracted using phenol-chloroform. Approximately 1 μg of DNA was digested with 20 U of EcoRI restriction endonuclease at 37°C overnight. DNA fragments were separated by electrophoresis in 0.7% agarose gels and capillary transferred onto Hybond N^+ membranes. Southern blots were hybridized with prepared probes at 55°C in hybridization buffers. The membrane was washed once for 20 min in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), washed three times for 20 min in 2× SSC at 55°C, and then exposed to X-ray films.

Mapping of the deletion of SPI-1 in the clinical isolates. A pair of primers (FhlA forward primer 5′-TCCACCCACTACCTTGATGAG-3′ and MutS reverse primer 5′-CAATCTCAAACTCCCACAACG-3′), annealing to the *fhlA* and *mutS* genes of the 5′ and 3′ flanks of SPI-1, was designed. A long-range *fhlA-mutS* PCR fragment was then amplified from the two *S. enterica* serovar Senftenberg isolates. The PCR product was sequenced, and its sequence was compared with the *S. enterica* serovar Typhimurium LT2 genome in the GenBank database using NCBI's BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi).

Invasion assay. The invasion assay was performed as previously described (20).

Animal experiments. Animal experiments were performed as previously described (7, 21). Briefly, mice were inoculated with 20 mg streptomycin orally 1 day prior to infection with experimental bacterial strains. At various time points following infection, mice were euthanized according to animal care protocols, and organs were harvested for bacterial enumeration and organ pathology. Histopathology scoring was performed as previously described (7) using hematoxylin- and eosin-stained cecal cross-sections. Each experiment was performed on eight mice per infected group. *S. enterica* serovar Typhimurium was used as a positive control for infection and inflammation in this model. The data presented represent three separate experiments.

RESULTS

Epidemiological investigation. Following a shared meal, seven immunocompetent individuals (age range, 26 to 62 years) were stricken with symptoms ranging from acute-onset abdominal cramping and diarrhea to severe diarrhea, nausea, vomiting, fever, and dehydration requiring hospitalization. All patients were treated with ampicillin for 3 to 7 days, after which their symptoms resolved.

Pathogen identification. Four stool samples from four consenting patients were collected before antibiotic treatment was initiated, enriched in liquid culture, and plated onto media suitable for the detection of a variety of enteropathogens (see

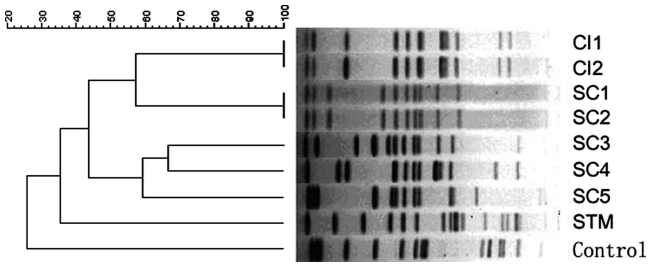

FIG. 1. PFGE of the clinical isolates from a food-borne disease outbreak in Shenzhen, China. Bacterial DNA from stool samples from diarrhea patients from a single outbreak (CI1 and CI2) or control strains of *Salmonella enterica* serovar Senftenberg (SC1 to SC5) and *S. enterica* serovar Typhimurium (STM) were digested with the restriction enzyme XbaI, subjected to PFGE as described in Materials and Methods, and compared to reference *Salmonella* serovar Braenderup standard strain H9812.

Materials and Methods). Rotavirus antigens were assayed using an antibody kit but were not detected. Only two stool samples had typical colonies that grew on SS agar but not other media. These colonies were then incubated on triple sugar iron agar and tested by use of an Analytical Profile Index (API) 20E strip (BioMérieux Reagents). Biochemical test (API) results were consistent with *Salmonella* species, and serology was performed to further identify the *Salmonella* serovar. The result-

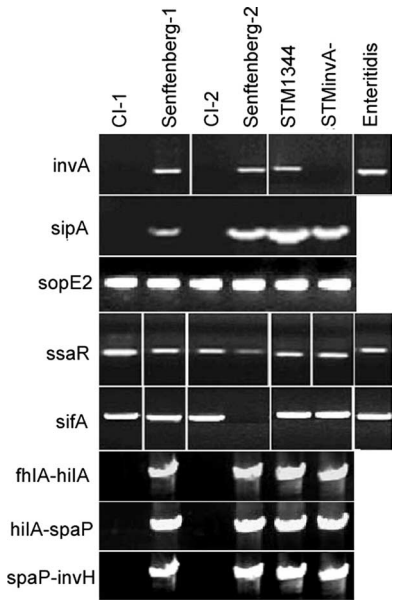

FIG. 2. Genomic analysis of SPI-1 and SPI-2 of strains isolated from diarrheal patients in Shenzhen, China, compared to control strains. Stool isolates (CI1 and CI2) were compared to *S. enterica* serovar Senftenberg (Senftenberg-1 and Senftenberg-2), serovar Enteritidis (Enteritidis), and serovar Typhimurium (STM1344 and STMinvA−) strains by PCR for the presence of the SPI-1 genes, including *invA*, *sipA*, *fhlA-hilA*, *hilA-spaP*, and *spaP-invH*; the phage-encoded SPI-1 effector gene *sopE2*; the SPI-2-encoded gene *ssaR*; and the non-SPI-2-encoded SPI-2 effector gene *sifA*. SPI-1-encoded genes were PCR negative in both stool isolates. The *invA*-negative control strain STMinvA− contains an insertional mutation in *invA*. One strain (Senftenberg-2) of the *S. enterica* serovar Senftenberg type strains lacked the non-SPI-2-encoded SPI-2 effector gene *sifA*.

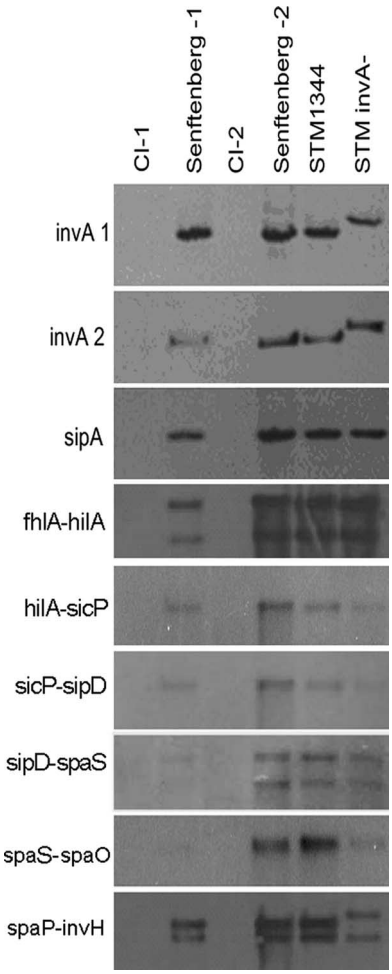

FIG. 3. Southern hybridization analysis for SPI-1 genes of clinical isolates from Shenzhen, China, and controls. By Southern blot, the presence of the SPI-1 genes *invA*, *sipA*, *fhlA-hilA*, *hilA-sicP*, *sicP-sipD*, *sipD-spaS*, *spaS-spaO*, and *spaP-invH* were assessed in clinical isolates (CI1 and CI2) and control strains of *S. enterica* serovar Senftenberg (Senftenberg-1 and Senftenberg-2) and serovar Typhimurium (STM and STMinvA−). The presence of *invA* was assessed using two independent probes. SPI-1 genes were absent only in the clinical isolates. STMinvA− contains an insertion within *invA*, and the gene is consequently larger than wild-type *invA*.

ing serotype was consistent with *S. enterica* serovar Senftenberg. Furthermore, the two clinical isolates had an identical PFGE pattern that was related to a series of repository serovar Senftenberg strains (Fig. 1), and their MLST was ST217 and was related to other *S. enterica* serovar Senftenberg MLST types. All four stool samples were negative by real-time PCR of enteropathogen conserved genes, including *invA*.

Identification of SPI-1 and SPI-2 in clinical *Salmonella* isolates. We next tried to confirm whether the two clinical strains really lack SPI-1 yet encode SPI-2 by probing for various components of these systems. These included InvA, a structural component critical for SPI-1 T3SS function encoded within SPI-1; SipA, a secreted effector critical for the efficient invasion of intestinal epithelial cells (17) within SPI-1; the whole SPI-1, including *fhlA-hilA*, *hilA-spaP*, and *spaP-invH*; SopE2, the phage-encoded SPI-1 effector thought to have been ac-

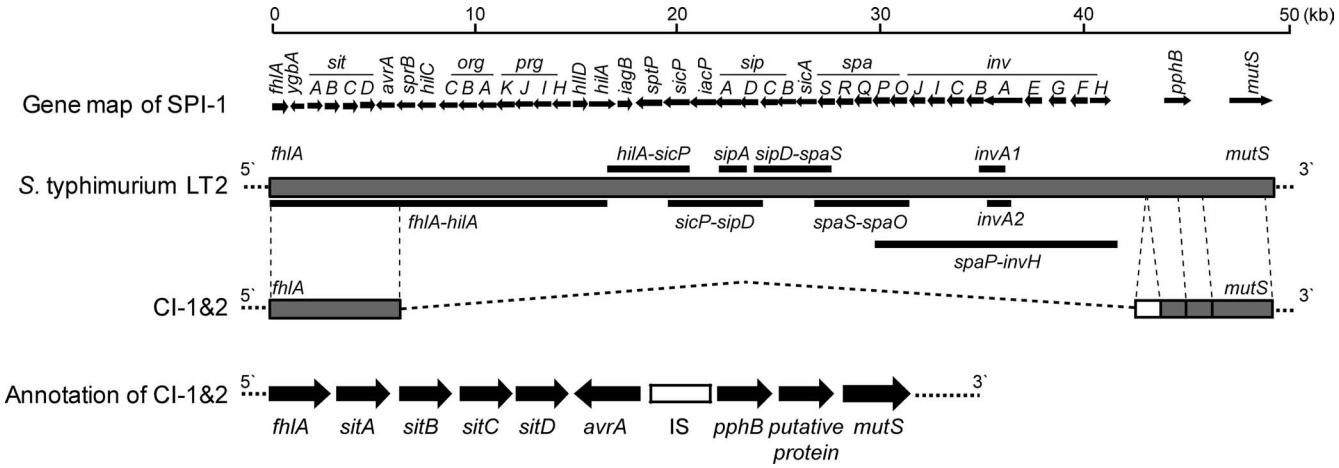

FIG. 4. Mapping of the SPI-1 deletion in *S. enterica* serovar Senftenberg clinical isolates. In order to define the precise deletion site of SPI-1 in the two *S. enterica* serovar Senftenberg isolates (CI1 and CI2), a long-range PCR was performed using primers annealing to the *fhlA* and *mutS* genes located at the 5′ and 3′ flanks of SPI-1, and a 10.255-kb PCR product was obtained and sequenced. Sequence comparison to the known genome sequence of *S. enterica* serovar Typhimurium LT2 in the GenBank database showed that SPI-1 was deleted at the *avrA* gene in the two serovar Senftenberg clinical isolates, and there was an IS, about 1 kb in size, present in the deletion site in the two serovar Senftenberg clinical isolates. The positions of the SPI-1 genes are indicated by the black arrow bars, and probes used for Southern hybridization are indicated by the black bars.

quired independently of the SPI-1 genomic island (8, 12, 22); SsaR, a component critical for the SPI-2 T3SS apparatus; and SifA, an SPI-2-secreted effector encoded by a gene located in the *potABCD* operon. Genes for all these components were tested by PCR (Fig. 2). The clinical isolates of serovar Senftenberg from the Shenzhen outbreak, but not control strains, were negative for PCR of *invA*, *sipA*, *fhlA-hilA*, *hilA-spaP*, and *spaP-invH*, and all strains were positive for PCR of *sopE2*, *ssaR*, and *sifA* (Fig. 2). Interestingly, one of the *S. enterica* serovar Senftenberg type strains, Senftenberg-2, was negative by PCR for *sifA*, an important SPI-2 effector gene located outside SPI-2. SPI-2 is thought to be uniquely acquired by *S. enterica* subsequent to its speciation from *Salmonella bongori* and after its evolutionary acquisition of SPI-1 (14). The SPI-2 locus was present in all the tested strains.

We then assessed the two clinical isolates by Southern blot hybridization for SPI-1 components. In the Shenzhen isolates, but not the control strains, *sipA*, *invA*, *fhlA-hilA*, *hilA-sicP*, *sicP-sipD*, *sipD-spaS*, *spaS-spaO*, and *spaP-invH* were absent from the genome (Fig. 3). In order to map the precise deletion site of SPI-1 in the two clinical isolates, we performed a long-range PCR using primers annealing to the *fhlA* and *mutS* genes of the 5′ and 3′ flanks of SPI-1. The *fhlA-mutS* PCR product was 10.255 kb in size and should bridge the 5′ and 3′ flanks of SPI-1 in the serovar Senftenberg clinical isolates. The PCR fragment was sequenced, and its sequence was compared with the genome sequence of *S. enterica* serovar Typhimurium LT2 in the GenBank database using the BLAST server. These sequence analyses showed that SPI-1 was deleted from the two serovar Senftenberg clinical isolates at the *avrA* gene (Fig. 4). Furthermore, there was an insertion sequence (IS), about 1 kb in size, present at the SPI-1 deletion site in the *S. enterica* serovar Senftenberg isolates. These data demonstrated that despite the absence of essential SPI-1 components, the Shenzhen strains were able to cause clinically significant intestinal disease in humans.

Invasion assay. The possibility remained that despite the absence of the SPI-1-encoded virulence machinery, the Shenzhen strains retained or have complemented their ability to induce bacterial invasion similar to that mediated by SPI-1. In order to ascertain whether these strains had complemented the invasion-associated function of SPI-1, we assessed their invasiveness in cultured human epithelial cells (HeLa cells). The experiments were repeated three times. Compared to control strains, SPI-1-deficient strains were significantly attenuated for cell invasion (Table 3). Although control SPI-1-positive sero-

TABLE 3. SPI-1 and SPI-2 genotypes and invasivenesses of *S. enterica* Senftenberg strains and controls[a]

Strain	*S. enterica* serovar	Source	SPI-1	SPI-2	% Invasion ± SD[b]
CI1	Senftenberg	Clinical isolate	Negative	Positive	0.0049 ± 0.00003
CI2	Senftenberg	Clinical isolate	Negative	Positive	0.176 ± 0.0225
SC1	Senftenberg	NICPB	Positive	Positive	1.537 ± 0.0586
STM	Typhimurium	Laboratory strain	Positive	Positive	4.89 ± 0.3927
STMinvA−	Typhimurium	Laboratory strain	*invA* insertional mutant	Positive	0.3860 ± 0.0839

[a] The *P* values were as follows: 0.0840 for CI1 versus STMinvA−, 0.0000 for CI1 versus STM, 0.0000 for CI1 versus SC1, 0.4610 for CI2 versus STMinvA−, 0.0000 for CI2 versus STM, 0.0000 for CI2 versus SC1, 0.0000 for SC1 versus STMinvA−, and 0.0000 for SC1 versus STM. Note that CI1 and CI2 were stool sample isolates from the Shenzhen outbreak, SC1 was the control strain, STM was invasion-positive control, and STMinvA− was the invasion-negative control.

[b] Invasion is expressed as the percentage of the initial inoculum of bacteria that was insensitive to gentamicin because of cell invasion. The value represents the averages ± standard deviations for three experiments. The original data were analyzed by using SPSS software (version 13.0).

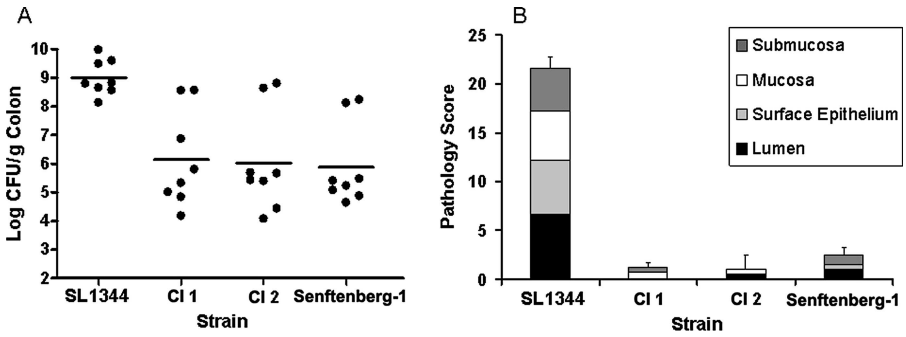

FIG. 5. Murine infections with clinical isolates of *S. enterica* serovar Senftenberg did not result in intestinal pathology. Although colonization with clinical isolates both containing (Senftenberg-1) and lacking (CI1 and CI2) SPI-1 was evident in murine infection (A), there was no histopathological evidence of inflammation in infected mouse intestines (B).

var Senftenberg strains did not invade cells as efficiently as laboratory-passaged *S. enterica* serovar Typhimurium controls, they were significantly more efficient than both the SPI-1-deficient Shenzhen strains and the artificially created *invA* mutant serovar Typhimurium control strain. Therefore, the Shenzhen isolates lack both SPI-1 and SPI-1-mediated behaviors, virulence features previously considered to be essential for enteropathogenicity.

Animal experiments. Finally, we assessed the pathogenicities of these strains in a recently described model of murine enterocolitis (2). This recently characterized murine *Salmonella* infection model has been utilized to compare the pathogenicities of several strains of *S. enterica*. The model has been used by us and others (7, 11) to show that both SPI-1 and SPI-2 are involved in murine intestinal pathogenicity and that intestinal inflammation can occur in the absence of SPI-1. We tested the clinical isolates of *S. enterica* serovar Senftenberg in this model. Although following streptomycin treatment, the clinical isolates were able to colonize the murine colon, at no time was intestinal inflammation observed. At a time point at which cecal and systemic pathologies are maximal in wild-type *S. enterica* serovar Typhimurium infection (5 days postinfection), there was no intestinal pathology in serovar Senftenberg-infected mice (Fig. 5). Furthermore, both earlier (2 days) and later (30 days) time points yielded neither evident intestinal pathology nor evidence of systemic disease (data not shown). This indicated a serovar Senftenberg-specific attenuation of infection in mice, consistent with previous evidence that only limited human enteropathogenic *S. enterica* serovars are pathogenic in this model (21).

DISCUSSION

Gastroenteritis caused by *S. enterica* is a significant problem, particularly in areas of high population density and with poor sanitation and close proximity of livestock and human habitation. The presence of SPI-1-deficient strains of *S. enterica* serovars Senftenberg and Litchfield in various environmental reservoirs including livestock feed and marine environments has been reported in the literature (18). These strains were shown to have decreased invasiveness in vitro and were considered to be unlikely to cause human infection due to the attenuation of invasion and the postulated requirement for SPI-1 for in vivo intestinal pathogenesis (9).

In this study, we isolated two *invA*-deficient *S. enterica* serovar Senftenberg strains from a food-borne disease outbreak, while no other pathogens were isolated. Although not all viruses and parasites were tested, the fact that the patients responded to antibiotic treatment indicated that the disease was most likely due to a susceptible bacterial infection. Based on the clinical syndrome, the epidemiological investigation information, and the traditional method results and molecular typing, we concluded that the pathogen of the food-borne disease outbreak was SPI-1-deficient *S. enterica* serovar Senftenberg strains. We further mapped SPI-1 in the *S. enterica* serovar Senftenberg clinical isolates, and the detailed mapping and DNA sequencing results confirmed that the isolates lacked SPI-1. There is a 1-kb IS inserted at the SPI-1 deletion site in the clinical isolates, suggesting that IS elements may have played a role in the deletion of SPI-1 in the *S. enterica* serovar Senftenberg isolates.

Previously, SPI-1 was considered to be essential for intestinal *Salmonella* pathogenicity. In our study, we showed that SPI-1-deficient *S. enterica* serovar Senftenberg caused human enteropathogenic infection. Furthermore, intestinal inflammation in animal models of *S. enterica* infection has been shown by us and others to occur in the absence of SPI-1, in a manner dependent on SPI-2 (7, 11). Although there are differences between the bovine and murine infections described in those studies and our findings described here, the development of SPI-1-independent *Salmonella* enterocolitis may represent a similar process. Identifying which virulence factors are involved in SPI-1-independent human intestinal salmonellosis will now be an interesting and important avenue of investigation. In addition, *invA* was used as the molecular marker (1, 3, 4, 5, 6, 13, 15, 16, 18, 19) of *Salmonella* diagnosis, so one should reevaluate molecular detection techniques for *Salmonella* that are predicated on the absolute presence of SPI-1 in human-disease-causing strains of *S. enterica*.

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