Host Restriction of *Salmonella enterica* Serotype Typhi Is Not Caused by Functional Alteration of SipA, SopB, or SopD

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Salmonella enterica **serotype Typhi is a strictly human adapted pathogen that does not cause disease in nonprimate vertebrate hosts, while** *Salmonella enterica* **serotype Typhimurium is a broad-host-range pathogen. Serotype Typhi lacks some of the proteins (effectors) exported by the invasion-associated type III secretion system that are required by serotype Typhimurium for eliciting fluid secretion and inflammation in bovine ligated ileal loops. We investigated whether the remaining serotype Typhi effectors implicated in enteropathogenicity (SipA, SopB, and SopD) are functionally exchangeable with their serotype Typhimurium homologues. Serotype Typhi elicited fluid accumulation in bovine ligated ileal loops at levels similar to those elicited by a noninvasive serotype Typhimurium strain (the** *sipA sopABDE2* **mutant) or by sterile culture medium. However, introduction of the cloned serotype Typhi** *sipA*, *sopB*, **and** *sopD* **genes complemented the ability of a serotype Typhimurium** *sipA sopABDE2* **mutant to elicit fluid secretion in bovine ligated ileal loops. Introduction of the cloned serotype Typhi** *sipA*, *sopB*, **and** *sopD* **genes increased the invasiveness of a serotype Typhimurium** *sipA sopABDE2* **mutant for human colon carcinoma epithelial (HT-29 and T84) cells and bovine kidney (MDBK) cells. Translational fusions between the mature TEM-1 β-lactamase reporter and SipA or SopD demonstrated that serotype Typhi translocates these effectors into host cells. We conclude that the inability of serotype Typhi to cause fluid accumulation in bovine ligated ileal loops is not caused by a functional alteration of its SipA, SopB, and SopD effector proteins with respect to their serotype Typhimurium homologues.**

Salmonella enterica serotype Typhi is strictly adapted to human hosts, in whom it causes a systemic disease known as typhoid fever which results in some 600,000 deaths annually (27). *Salmonella enterica* serotype Typhimurium is the causative agent of enterocolitis, an infection of humans and cattle that normally remains localized to the intestine and the mesenteric lymph nodes. The hallmark of intestinal inflammation during serotype Typhimurium infection of humans or cattle is a massive neutrophilic infiltrate into the intestinal mucosa, with necrosis of the upper mucosa and pseudomembrane formation (9, 17, 26, 42, 50). The massive neutrophil influx into the intestines of humans and cattle infected by serotype Typhimurium is accompanied by diarrhea (developing between 12 h and 2 days after infection), and neutrophils are commonly found in stool samples (18, 38, 42, 50). In contrast, only one-third of typhoid fever patients develop diarrhea (later than 5 to 9 days after infection), and the intestinal infiltrate, as well as the fecal leukocyte population, is composed predominantly of mononuclear cells (18, 22, 30, 31, 39).

While the mechanisms by which serotype Typhimurium elicits a neutrophilic influx into the intestinal mucosae of humans and cattle are beginning to be elucidated (52), comparatively little is known about the pathogenesis of serotype Typhi infection or the reason why diarrhea is an insignificant symptom during typhoid fever. One limitation to studying the pathogenesis of typhoid fever is the absence of a good animal model, because serotype Typhi is strictly human adapted, causing disease only in higher primates (e.g., chimpanzees) (12). Mice infected with serotype Typhimurium develop a systemic typhoid-like disease, which is commonly used to model serotype Typhi infections in humans (45). However, an obvious shortcoming of this mouse model is the fact that serotype Typhimurium does not cause typhoid fever in humans, suggesting that genetic differences between serotype Typhi and serotype Typhimurium are critically important for the disease outcome.

The evolution from a host generalist, such as serotype Typhimurium, to a host-restricted variant, such as serotype Typhi, may have occurred by acquisition of new genetic material through horizontal gene transfer, by genome degradation (i.e., loss of genetic information by deletion or pseudogene formation), or by a combination of both mechanisms (5). Whole-genome sequencing has revealed that genome degradation is an extensive phenomenon in host-restricted *Salmonella* serotypes. There are approximately 210 pseudogenes in the genome of serotype Typhi (strains CT18 and Ty2) and 173 pseudogenes in the genome of *Salmonella enterica* serotype Paratyphi A, another strictly human adapted serotype (10, 24, 32). In contrast, the genome of the broad-host-range serotype Typhimurium contains only 39 pseudogenes (25). Thus, it is

possible that attenuation of neutrophilic infiltration and diarrhea during serotype Typhi infection may be due to loss of function rather than to gain of function.

The type III secretion system (T3SS-1) encoded by *Salmonella* pathogenicity island 1 (SPI1) mediates invasion of intestinal epithelial cells by serotype Typhimurium (15). The T3SS-1 of serotype Typhimurium is furthermore required for eliciting the production of neutrophil chemoattractants (51), a massive influx of neutrophils (1, 42, 48, 54), and fluid accumulation in bovine ligated ileal loops (1, 48, 54). Finally, the T3SS-1 is essential for causing diarrhea and lethal morbidity during oral infection of calves with serotype Typhimurium (42, 43, 54). The main function of the T3SS-1 is the translocation of proteins, termed effectors, into the cytosol of a host cell (14). Six T3SS-1-secreted effectors, SipA, SopA, SopB, SopD, SopE, and SopE2, act in concert to elicit fluid accumulation and neutrophil infiltration during infection of bovine ligated ileal loops with serotype Typhimurium (53, 54). The gene encoding one of these effectors, SopE, is carried by a prophage that is present only in the genomes of serotype Typhimurium clonal isolates (defined by phage typing) that have caused recent epidemics among cattle in Europe (29). Acquisition of *sopE* by phage-mediated horizontal gene transfer increases the ability of serotype Typhimurium to elicit fluid accumulation in bovine ligated ileal loops, suggesting that acquisition of T3SS-1-secreted effectors contributes to host adaptation (53).

By analogy, it has been speculated that loss of T3SS-1-secreted effectors by genome degradation may account for the inability of serotype Typhi to elicit infiltration of neutrophils and for its low propensity to cause diarrhea (24). In support of this idea, some effector genes contributing to fluid secretion and infiltration of neutrophils during serotype Typhimurium infection in calves are pseudogenes (*sopE2* and *sopA*) in the genome of serotype Typhi strain CT18 (3, 32, 40). Similarly, *sopA* is a pseudogene in the genomes of serotype Paratyphi A strain ATCC 9150 and serotype Typhi strain Ty2, and *sopE* is absent from the serotype Paratyphi A genome (10, 24). The *sipA*, *sopD*, and *sopB* genes are present in the serotype Typhi genome, but the effectors encoded differ by three (SipA), four (SopD), or five (SopB) amino acid substitutions from their serotype Typhimurium homologues, which could potentially alter their function. The presence of pseudogenes (*sopA* and *sopE2*) and of genes carrying nonsynonymous substitutions (*sipA*, *sopB*, and *sopD*) in the serotype Typhi genome may thus be in part responsible for the reduced ability of serotype Typhi to cause fluid secretion in the intestine.

To formally test this hypothesis, we cloned the *sipA*, *sopB*, and *sopD* genes, which are intact in all typhoidal serotypes whose genome has been sequenced, and expressed them in a serotype Typhimurium strain carrying mutations in *sipA*, *sopA*, *sopB*, *sopD*, and *sopE2* (the *sipA sopABDE2* mutant). We then compared the functionality of *sipA*, *sopB*, and *sopD* genes cloned from serotype Typhi and serotype Typhimurium in a calf model of infection and in cultured human epithelial cells derived from a colon carcinoma.

MATERIALS AND METHODS

Bacterial strains, tissue culture cells, and culture conditions. T3SS-1 effector genes were cloned using *Escherichia coli* strain DH5α, which has been described previously (16). Serotype Typhimurium and serotype Typhi strains used in this study are listed in Table 1. For construction of a strain carrying a mutation in *invA*, an internal part of the gene was amplified from serotype Typhimurium with a primer pair published previously (35) and introduced into suicide vector pEP185.2 (20) to give rise to plasmid pINV5. Plasmid pINV5 was conjugated into serotype Typhi strain AJB70, and an exconjugant was selected and termed STY1. Strains were cultured aerobically at 37°C in Luria-Bertani (LB) broth supplemented with antibiotics as appropriate at the following concentrations: carbenicillin, 100 mg/liter; chloramphenicol, 30 mg/liter; tetracycline, 20 mg/liter; kanamycin, 60 mg/liter; or nalidixic acid, 50 mg/liter. For invasion assays with tissue culture cells or bovine ligated ileal loops, each strain was grown overnight at 37°C in 4 ml of LB broth in a roller. A 0.04-ml volume of this overnight culture was used for inoculation of 4 ml of LB broth, and bacteria were grown at 37°C for 3 h in a roller. Subsequently, this culture was used as an inoculum, and the numbers of CFU were determined by plating serial 10-fold dilutions on LB plates.

The human colon carcinoma cell line HT-29 has been described previously (13) and was obtained from ATCC. HT-29 cells were grown in McCoy's 5a medium with 1.5 mM L-glutamine (Gibco) supplemented with 10% fetal calf serum. Bovine kidney epithelial (MDBK) cells were obtained from ATCC and were grown in Eagle minimal essential medium supplemented with 10% horse serum, 2 mM L-glutamine, and Earle's balanced salt solution adjusted to contain 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate. For invasion assays, cells were seeded at 2.5×10^5/well in 24-well plates and the invasion assay was performed on the following day. For fluorescence microscopy, MDBK cells were seeded at 3×10^4/well in a 96-well plate and the assay was performed on the following day. The human colon carcinoma cell line T84 has been described previously (11) and was obtained from ATCC. T84 cells were grown in Dulbecco's modified Eagle medium–F12 medium (Gibco) containing 1.2 g/liter sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES, and 0.5 mM sodium pyruvate (Gibco) supplemented with 10% fetal calf serum. To polarize T84 cells, 0.5 ml of medium containing 4×10^5 cells/well was seeded on the apical compartment in 12-mm Transwell plates with 0.4-μm-pore-size polycarbonate membranes (Corning Costar), and 1.5 ml of medium was added to the basolateral compartment. The medium was changed every other day, and the transepithelial electrical resistance was measured after a week. When the cells reached a transepithelial electrical resistance of at least 1,500 Ω/cm^2, they were incubated overnight in fresh medium, and the invasion assay was performed the following day.

Construction of plasmids. The primers used for amplification of the *sipA*, *sopB*, and *sopD* genes from serotype Typhi strain SARB63 by PCR were the same as those used previously to amplify the homologous genes from serotype Typhimurium (34). All PCR products were initially cloned into pCR2.1 (Invitrogen), and amplification of the correct genes was confirmed by sequence analysis. A DNA fragment containing the serotype Typhi *sopD* gene and its promoter region was cloned into the SacI site of the low-copy-number vector pWSK29 (approximately 6 copies per cell) (47) to give rise to plasmid pMR16. The serotype Typhi *sipA* gene is located in the *sipBCDA* operon and was amplified without its promoter and cloned directionally behind the *lac* promoter of pWSK29 by using BamHI and XbaI to give rise to plasmid pMR30. The serotype Typhi *sopB* gene was amplified with its promoter but without a termination loop and cloned into pWSK29 by using NotI and XbaI to give rise to plasmid pMR27. The serotype Typhi *sipA* gene was introduced in the same orientation and downstream of the *sopB* stop codon into plasmid pMR27 to give rise to plasmid pPW8. The serotype Typhi *sopB sipA* genes were excised from pPW8 with NotI and BamHI and were cloned into pMR16 to give rise to plasmid pMR25. Plasmids derived from pWSK29 carrying the cloned *sopD* gene (pMR15), *sopB* gene (pMR26), *sipA* gene (pMR29), or *sopB sipA* genes (pPW1) of serotype Typhimurium have been described previously (34) (Table 1). The serotype Typhimurium *sopB sipA* genes were excised from pPW1 with NotI and BamHI and were cloned into pMR16 to give rise to plasmid pMR24.

To generate translational fusions between T3SS-1 effectors and the TEM-1 β-lactamase reporter, the following plasmids were constructed. A 3×Flag tag was PCR amplified from plasmid pSUB11 (46) using primers Flag-F and Kan-R (Table 2) and cloned into pCR2.1 to yield plasmid pTopoFlag. A 1.5-kb DNA fragment encoding LacIQ and the *trc* promoter was PCR amplified from plasmid pTrc99A (2) using primers LacIQ-F and Trc-R (Table 2). The PCR product was digested with NdeI and SphI and introduced into pTopoFlag. A 1.56-kb DNA fragment encompassing LacIQ, the *trc* promoter, and the 3×Flag tag was PCR amplified from the resulting plasmid using primers LacIQ-F and Flag-R (Table 2). The SphI- and SmaI-digested PCR product was cloned into plasmid pBBR1MCS (21), yielding plasmid pBBR-Flag. A DNA fragment encoding the TEM-1 β-lactamase without the N-terminal signal peptide (6) was PCR amplified from pTrc99A using primers TEM-F and TEM-R (Table 2) and cloned into

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype[a]	Source or reference
Strains		
Serotype Typhimurium		
IR715	Nalidixic acid-resistant derivative of bovine wild-type isolate ATCC 14028	41
ZA21	IR715 ΔsipA ΔsopA sopB::MudJ sopD::pEP182.5 sopE2::pSB1039	54
CAS152	ATCC 14028 Str^r phoN::Tn10dCm ΔsipB	43
CAS108	ATCC 14028 Str^r phoN::Tn10dCm ΔsipC	43
Serotype Typhi		
SARB63	Wild-type isolate	7
AJB70	Clinical isolate	4
STY1	AJB70 invA::pINV5	This study
STY5	AJB70 sopB::MudJ	This study
STY6	AJB70 sopD::pEP182.5	This study
Plasmids		
pCR2.1	Cloning vector	Invitrogen
pSUB11	Plasmid carrying 3×Flag	46
pBBR1MCS	Broad-host-range cloning vector	21
pGEX-4T-1	Plasmid carrying GST	Amersham
pTrc99A	Cloning vector for protein expression under control of trc promoter	2
pTopoTEM1	TEM-1 without signal sequence in pCR2.1	This study
pTopoFlag	3×Flag in pCR2.1	This study
pBBR-Flag	LacIQ, trc, and Flag in pBBR1MCS	This study
pFlagTEM1	Flag-tagged β-lactamase-fused protein-expressing vector	This study
pSipA/FT	Plasmid encoding SipA–TEM-1 fusion protein (STM)	This study
pSopD/FT	Plasmid encoding SopD–TEM-1 fusion protein (STY)	This study
pGST/FT	Plasmid encoding GST–TEM-1 fusion protein	This study
pWSK29	Cloning vector	47
pMR15	pWSK29 carrying the sopD gene (STM)	34
pMR16	pWSK29 carrying the sopD gene (STY)	This study
pMR26	pWSK29 carrying the sopB gene (STM)	34
pMR27	pWSK29 carrying the sopB gene (STY)	This study
pMR29	pWSK29 carrying the sipA gene (STM)	34
pMR30	pWSK29 carrying the sipA gene (STY)	This study
pMR24	pWSK29 carrying the sipA sopBD genes (STM)	This study
pMR25	pWSK29 carrying the sipA sopBD genes (STY)	This study

[a] STY, serotype Typhi; STM, serotype Typhimurium.

pCR2.1 to yield plasmid pTopoTEM1. An 800-bp SmaI-SacI-digested DNA fragment of plasmid pTopoTEM1 was cloned into pBBR-Flag to yield plasmid pFlagTEM1 (Table 1). The sipA gene from serotype Typhimurium 14028 was PCR amplified without the stop codon by using the primer pair SipA-F–SipA-R (Table 2). The 1.6-kb PCR product digested with NdeI and SalI was cloned into plasmid pFlagTEM1 to give rise to plasmid pSipA/FT, encoding a SipA–Flag–TEM-1 translational fusion whose expression is controlled by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible trc promoter. The sopD gene from serotype Typhi strain SARB63 was PCR amplified without the stop codon by using the primer pair SopD-F–SopD-R (Table 2). This PCR product was digested with XhoI and cloned into plasmid pFlagTEM1 to give rise to plasmid pSopD/FT, encoding a SopD–Flag–TEM-1 translational fusion under the control of the sopD promoter. The gene encoding glutathione S-transferase (GST) was PCR amplified from plasmid pGEX-4T-1 (Amersham) using primers GST-F and GST-R (Table 2), digested with NdeI and XhoI, and cloned into plasmid pFlagTEM1 to give rise to plasmid pGST/FT, encoding a GST–Flag–TEM-1 translational fusion under the control of the trc promoter (Table 1).

Construction of mutants. To generate a serotype Typhi sopB mutant, the sopB::mudJ insertion in serotype Typhimurium strain ZA21 was transduced into serotype Typhi strain AJB70 using bacteriophage P22 int. The insertional inactivation of sopB in a transductant, termed STY5, was confirmed by Southern hybridization using a sopB-specific DNA probe.

To generate a serotype Typhi sopD mutant, an internal fragment of the sopD open reading frame was PCR amplified as described previously (54) and cloned into pCR2.1 to generate plasmid pMR34. A 391-bp EcoRV-XbaI restriction fragment of pMR34 was cloned into suicide vector pEP184.5 to give rise to plasmid pMR35. The suicide plasmid pMR35 was introduced into serotype Typhi

strain AJB70 by conjugation. In one exconjugant, termed STY6, inactivation of sopD by integration of pMR35 via homologous recombination was confirmed by Southern hybridization using the labeled insert of pMR34 as a DNA probe.

Tissue culture assays. Invasion assays were performed using protocols for gentamicin protection assays described previously (44). In brief, human colon carcinoma cells were seeded as described above and infected with serotype Typhimurium or serotype Typhi strains at approximately 1×10^7 CFU/well (the multiplicity of infection was approximately 10). The bacteria were incubated for 1 h at 37°C under 5% CO$_2$ to allow for invasion. Each well was washed five times with sterile phosphate-buffered saline (PBS) (2.7 mM KCl, 1.8 mM KH$_2$PO$_4$, 140 mM NaCl, 10 mM Na$_2$HPO$_4$, pH 7.4) to remove extracellular bacteria, and medium containing gentamicin at a concentration of 0.1 mg/ml was added for a 90-min incubation at 37°C under 5% CO$_2$. After three washes with PBS, the cells were lysed with 0.5 ml of 1% Triton X-100, the lysates were transferred to sterile tubes, and each well was rinsed with 0.5 ml of PBS. Tenfold serial dilutions were plated to count the intracellular bacteria. Each experiment was repeated three times independently.

Protocols for detection of translocation of TEM-1 β-lactamase fusion proteins by fluorescence microscopy were based on a recent protocol (8). MDBK cells were infected at a multiplicity of infection of approximately 500 with serotype Typhi or serotype Typhimurium strains for 20 min. Cells were washed and incubated for 3 h with medium containing gentamicin (0.05 mg/ml) to kill extracellular bacteria. Cells were washed and loaded with the fluorescent substrate CCF2/AM (Invitrogen) for 1 h at room temperature using an enhanced loading protocol according to the manufacturer's manual. The fluorescence microscopy analysis was performed with an Eclipse 300 microscope (Olympus, Japan)

TABLE 2. Primers used for construction of TEM-1 β-lactamase fusion proteins

Name	Sequence[a]	Site(s) engineered
Flag-F	ACATATGCTCGAGGACTACAAAGACCATGACGGTG	NdeI, XhoI
Flag-R	ACCCGGGTTTATCGTCGTCATCTTTGTAG	SmaI
TEM-F	ACCCGGGCACCCAGAAACGCTGGTGAAAG	SmaI
TEM-R	ACTGCAGTTATCTAGACCAATGCTTAATCAGTGAG	PstI-Stop-XbaI
LacIQ-F	ATCACCGAAACGCGCGAG	
Kan-R	ACAACGTCGAGCACAGC	
Trc-R	ACCCATATGGTCTGTTTCCTGTGTG	NdeI
SipA-F	ACATATGGTTACAAGTGTAAGGAC	NdeI
SipA-R	AGTCGACACGCTGCATGTGCAAGCCATC	SalI
SopD-F	ACGCTCGAGTAATTGAAGGAAAATATTATATGCCAG	XhoI, Stop, SD[b]
SopD-R	ACGCTCGAGTGTCAGTAATATATTACGACTGC	XhoI
GST-F	TCCATATGTCTGAGTCCCCTATACTAGGTTATTG	NdeI
GST-R	AACTGCAGTCAACGCGGAACCAGATCCGATTTTG	

[a] Restriction sites are underlined.
[b] SD, Shine-Dalgarno sequence.

equipped with a CCF2 filter set (a 400-nm excitation filter, a 435-nm long-pass emitter, and a 435-nm dichroic mirror) (Chroma Technology, Brattleboro, VT).

Analysis of protein secretion. Bacteria were grown under SPI1-inducing conditions as described above. The cells were pelleted by centrifugation, and 2 ml of supernatant was collected for each sample. The supernatants were then filtered (pore size, 0.45 μm), and the proteins were precipitated with 25% trichloroacetic acid by high-speed centrifugation (14,000 × g for 30 min). The pellet was washed in cold acetone and resuspended in PBS. Four independent extractions for each sample were added together to minimize differences in the protein recovery from sample to sample. The proteins were then boiled in sodium dodecyl sulfate (SDS) for 5 min, and an aliquot for each sample was separated by 10% SDS-polyacrylamide gel electrophoresis.

Animal experiments. Four male Holstein calves, 4 to 5 weeks of age, weighing 45 to 55 kg were used. They were fed milk replacer twice a day and water ad libitum. The calves were clinically healthy before the experiment and were culture negative for fecal excretion of *Salmonella* serotypes. Detection of *Salmonella* serotypes in fecal swabs was performed by enrichment in tetrathionate broth (Difco) followed by streaking on brilliant green agar and XLT4 (Difco).

Bovine ligated ileal loop surgery has been described previously (36). In brief, food was withheld from the calves for 24 h prior to the surgery. Anesthesia was induced with Propofol (Abbot Laboratories, Chicago, IL), followed by placement of an endotracheal tube and maintenance with isoflurane (Abbot Laboratories, Chicago, IL) for the duration of the experiment. A laparotomy was performed, the ileum was exposed, and loops ranging in length from 6 to 9 cm were ligated, leaving 1-cm loops between them. The loops were infected by intraluminal injection of 3 ml of a suspension of either sterile LB broth or bacterial strains in LB broth containing approximately 1×10^9 CFU. The loops were placed back into the abdominal cavity. Samples for bacteriologic culture were collected at 1 h after infection by using a 3.5-mm biopsy punch and were incubated in PBS containing 0.1 mg/liter gentamicin for 90 min. Tissue samples were homogenized in PBS, serially diluted, and plated onto LB agar plates containing appropriate antibiotics for determination of CFU. Data on bacterial CFU were normalized to the length of the ligated loop and the CFU present in the inoculum prior to statistical analysis. At 8 h after infection, the fluid that had accumulated in loops was measured.

Statistical analysis. For analysis of percentage values, data were transformed logarithmically. Geometric means were determined, and the statistical significance of differences was calculated using parametric tests. A one-tailed paired Student t test was used to determine whether introduction of plasmids into the *sipA sopABDE2* mutant resulted in a significant increase in the invasiveness of the resulting strain.

RESULTS

Proteins secreted by serotype Typhimurium and serotype Typhi strains. To study secretion of effectors, low-copy-number plasmids carrying *sipA*, *sopB*, *sopD*, or *sipA sopA sopD* of serotype Typhi or serotype Typhimurium (Table 1) were introduced into a serotype Typhimurium *sipA sopABDE2* mutant (ZA21). We did not include *sopA* and *sopE2* in our investiga-

tion, because these genes are not intact in some serotype Typhi isolates. Furthermore, the *sopE* gene was not investigated, because it is absent from the serotype Typhimurium wild-type isolate (ATCC 14028) from which the strains used in this study were derived. Culture supernatants of serotype Typhimurium strains IR715 (wild type), CAS152 (*sipB* mutant), and CAS108 (*sipC* mutant) and of serotype Typhi strains SARB63 (wild type), AJB70 (wild type), and STY1 (*invA* mutant) were analyzed as controls. Proteins were isolated from culture supernatants and analyzed by SDS-polyacrylamide gel electrophoresis to evaluate secretion of T3SS-1 effectors in the bacterial supernatant (Fig. 1).

Bands with apparent sizes of 68 kDa (SipB) and 40 kDa (SipC) were absent from the culture supernatants of serotype Typhimurium strains CAS152 (*sipB* mutant) and CAS108 (*sipC* mutant), respectively. A large band (approximately 80 kDa) was visible in culture supernatants of wild-type serotype Typhimurium, the *sipB* mutant, the *sipC* mutant, and all derivatives of the *sipA sopABDE2* mutant carrying the cloned *sipA* gene (pMR29, pMR30, pMR24, and pMR25). The 80-kDa band was also visible in the serotype Typhi wild-type isolates (SARB63 and AJB70) but not in a serotype Typhi *invA* mutant (STY1). The presence of a band of approximately 60 kDa in the culture supernatant of the *sipA sopABDE2* mutant carrying the cloned *sopB* gene (pMR27) suggested that this protein represented SopB. Similarly, the presence of a band of approximately 40 kDa in culture supernatants of the *sipA sopABDE2* mutant carrying the cloned *sopD* gene (pMR15) suggested that this band represented SopD. We were not able to visualize expression of SopD in strain ZA21(pMR16) or of SopB in strain ZA21(pMR26). Furthermore, strains carrying the cloned *sipA* gene from either serotype Typhimurium [ZA21(pMR29)] or serotype Typhi [ZA21(pMR30)] secreted SipB and SipC at markedly reduced levels. The culture supernatants of strains carrying the cloned serotype Typhimurium or serotype Typhi *sopB sipA sopD* genes [ZA21(pMR24) and ZA21(pMR25), respectively] had a similar appearance. Overall, lower levels of proteins appeared to be secreted by serotype Typhi wild-type isolates (SARB63 and AJB70) than by serotype Typhimurium strains, with SipB being visible only as a faint band in serotype Typhi strains.
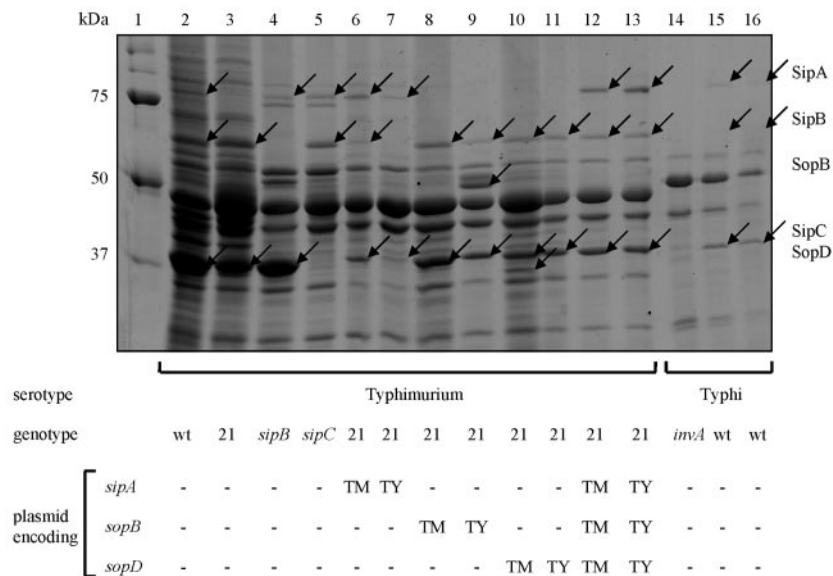
FIG. 1. Profiles of proteins secreted by serotype Typhimurium and serotype Typhi strains into the culture supernatant. Arrows indicate the positions of SipA, SipB, SipC, SopB, and SopD. The molecular sizes of marker proteins are given on the left. The serotypes (Typhimurium or Typhi) and genotypes (wt, wild type; 21, *sipA sopABDE2* mutant) of strains are given below the gel. The presence of plasmids carrying cloned effector genes of serotype Typhimurium (TM) or serotype Typhi (TY) is indicated at the bottom. Lane 1, molecular weight marker; lane 2, IR715; lane 3, ZA21; lane 4, CAS152; lane 5, CAS108; lane 6, ZA21(pMR29); lane 7, ZA21(pMR30); lane 8, ZA21(pMR26); lane 9, ZA21(pMR27); lane 10, ZA21(pMR15); lane 11, ZA21(pMR16); lane 12, ZA21(pMR24); lane 13, ZA21(pMR25); lane 14, STY1; lane 15, AJB70; lane 16, SARB63.

Serotype Typhi does not elicit fluid secretion in bovine ligated ileal loops. We next compared the ability of serotype Typhi (SARB63) to cause fluid accumulation in bovine ligated ileal loops with that of serotype Typhimurium strains IR715 (wild type) and ZA21 (*sipA sopABDE2* mutant). The serotype Typhimurium wild-type strain elicited significantly more fluid accumulation at 8 h after infection than the serotype Typhimurium *sipA sopABDE2* mutant (ZA21) or the serotype Typhi wild-type strain (SARB63) (Fig. 2A). The amount of fluid that had accumulated in loops infected with the serotype Typhimurium *sipA sopABDE2* mutant (ZA21) or the serotype Typhi wild-type strain (SARB63) was not significantly different from that measured in loops infected with sterile LB broth. These data suggested that serotype Typhi was unable to elicit fluid accumulation in the calf, which is consistent with its host restriction to humans and its absence from the bovine reservoir.

The serotype Typhi *sipA*, *sopB*, and *sopD* genes elicit fluid secretion in bovine ligated ileal loops when introduced into a serotype Typhimurium *sipA sopABDE2* mutant. Since SipA, SopA, SopB, SopD, and SopE2 are essential for serotype Typhimurium to elicit fluid secretion in bovine ligated ileal loops (54), we investigated whether an altered function of T3SS-1 effectors in serotype Typhi may account for its inability to cause this host response. We focused our analysis on SipA, SopB, and SopD, because these effectors are present in both serotype Typhi genomes for which the sequences have been elucidated (10, 32). However, each of these serotype Typhi effectors carries several amino acid substitutions compared to its respective serotype Typhimurium homologue, which could potentially alter their function during the host-pathogen interaction. We thus determined whether introduction of the cloned serotype Typhi effector genes *sipA*, *sopB*, and *sopD*

(pMR25) would complement the ability of the serotype Typhimurium *sipA sopABDE2* mutant (ZA21) to cause fluid secretion in bovine ligated ileal loops at 8 h after infection. As a positive control, the serotype Typhimurium *sipA sopABDE2* mutant (ZA21) was complemented with the serotype Typhimurium effector genes *sipA*, *sopB*, and *sopD* (pMR24). Introduction of either pMR24 or pMR25 resulted in a significant ($P < 0.05$) increase in fluid accumulation elicited by serotype Typhimurium strain ZA21 (Fig. 2A). Furthermore, the amounts of fluid secretion elicited by strains ZA21(pMR24) and ZA21(pMR25) were not significantly different ($P = 0.367$). These results suggested that *sipA*, *sopB*, and *sopD* of serotype Typhi function similarly to the homologous genes of serotype Typhimurium in the bovine model of infection.

Invasion of the bovine intestinal mucosa by serotype Typhi and serotype Typhimurium. We next determined whether serotype Typhi would invade the bovine intestinal mucosa. At 1 h and 8 h after infection of bovine ligated ileal loops with serotype Typhi (SARB63) or serotype Typhimurium strain IR715 (wild type) or ZA21 (*sipA sopABDE2* mutant), tissue was collected and extracellular bacteria were killed by gentamicin treatment. The serotype Typhimurium wild-type strain (IR715) was recovered at higher numbers than the serotype Typhi wild-type strain (SARB63) ($P = 0.096$ at 1 h; $P = 0.056$ at 8 h) and the serotype Typhimurium *sipA sopABDE2* mutant (ZA21) ($P = 0.064$ at 1 h; $P = 0.041$ at 8 h) (Fig. 2B). Introduction of the cloned effector genes *sipA*, *sopB*, and *sopD* from serotype Typhi (pMR25) increased recovery of the serotype Typhimurium *sipA sopABDE2* mutant (ZA21) from the bovine ileal mucosa at 1 h ($P = 0.133$) and at 8 h ($P = 0.029$) after infection. Similarly, ZA21(pMR24) was more invasive in ligated ileal loops than ZA21 at both 1 h ($P = 0.008$) and 8 h
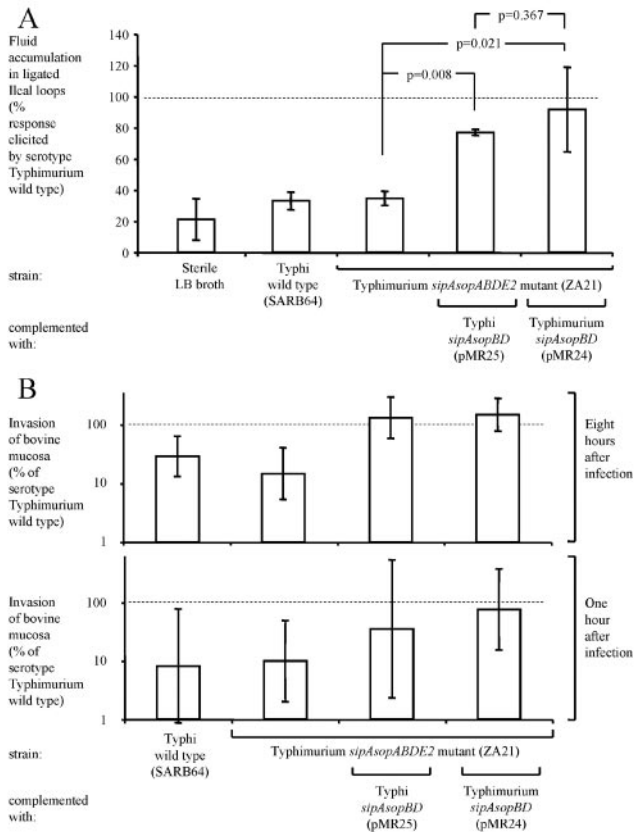
FIG. 2. Fluid accumulation (A) and bacterial invasion (B) in bovine ligated ileal loops. (A) Fluid accumulation elicited by serotype Typhi or serotype Typhimurium strains 8 h after infection of bovine ligated ileal loops. Data are geometric means (bars) ± standard deviations. Statistical significances of differences between treatment groups (brackets) are given at the top right. (B) Bacterial recovery from tissue (3.5-mm biopsy punch) collected 1 h after infection of ligated ileal loops with serotype Typhi or serotype Typhimurium strains and subsequently incubated with gentamicin to kill extracellular bacteria. CFU recovered from each animal was expressed as a percentage of CFU recovered from a ligated ileal loop of the same animal infected with the wild-type serotype Typhimurium strain (IR715). Data are geometric means (bars) ± standard deviations from experiments performed with three animals.

FIG. 3. Invasion of HT-29 cells by serotype Typhimurium strains. (A) Complementation with plasmids carrying the cloned sipA, sopB, and sopD genes from either serotype Typhi (pMR25) or serotype Typhimurium (pMR24). Data are geometric means (bars) ± standard deviations. (B) Complementation with plasmids carrying individual effector genes from serotype Typhi or serotype Typhimurium. Data are geometric means (bars) ± standard errors. Statistical significances of differences between treatment groups (brackets) are given at the top. Ty, serotype Typhi; Tm, serotype Typhimurium; wt, wild type.

($P = 0.051$) after infection. However, some of these differences were not statistically significant, due mainly to large differences in the numbers of bacteria recovered from the mucosae of different animals. We thus reasoned that cultured epithelial cells would provide a more powerful tool for analyzing differences in the invasiveness of bacterial strains.

The serotype Typhi effectors SipA, SopB, and SopD partially complement the invasion defect of a serotype Typhimurium *sipA sopABDE2* mutant. To further investigate whether the serotype Typhi effector genes *sipA*, *sopB*, and *sopD* have functions comparable to those of their serotype Typhimurium homologues, we performed invasion assays with HT-29 cells, an epithelial cell line derived from a human colon carcinoma. The serotype Typhimurium wild-type strain (IR715) was recovered from HT-29 cells in approximately 640-fold-higher numbers on average ($P < 0.05$) than the *sipA sopABDE2* mutant (ZA21) (Fig. 3A). The *sipA sopABDE2* mutant (ZA21) complemented
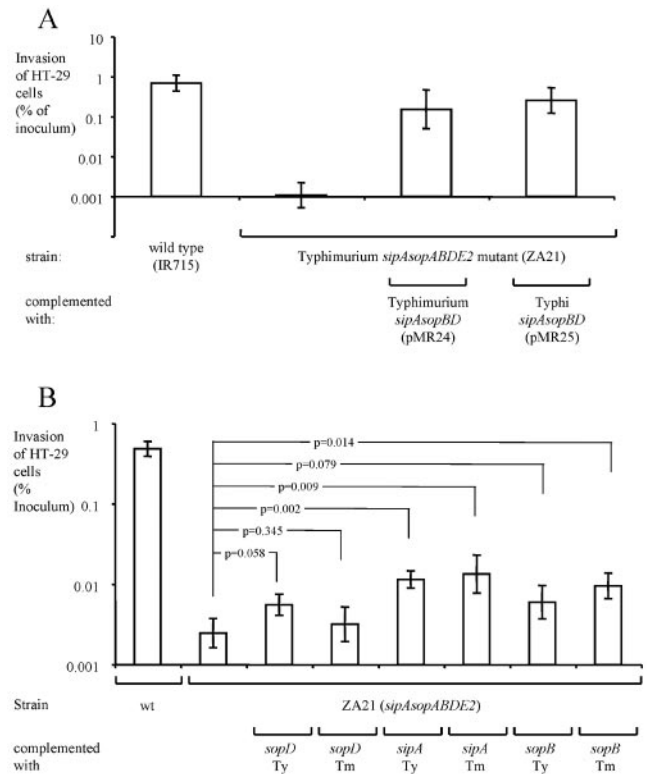
by introduction of the cloned *sipA*, *sopB*, and *sopD* genes from serotype Typhi (pMR25) was recovered from HT-29 cells at approximately 240-fold-higher numbers on average ($P < 0.05$) than its parent (ZA21). Similarly, introduction of the cloned serotype Typhimurium *sipA*, *sopB*, and *sopD* genes (pMR24) into the *sipA sopABDE2* mutant (ZA21) resulted in recovery from HT-29 cells of approximately 140-fold-higher bacterial numbers ($P < 0.05$) compared to the isogenic parent strain (ZA21) (Fig. 3A). Strains ZA21(pMR24) and ZA21(pMR25) invaded HT-29 cells at significantly lower levels ($P < 0.05$) than wild-type serotype Typhimurium (IR715). Thus, the *sipA*, *sopB*, and *sopD* genes from both serotypes partially complemented a serotype Typhimurium *sipA sopABDE2* mutant for invasion of HT-29 cells.

To further compare the functions of *sipA*, *sopB*, and *sopD* of serotype Typhimurium with those of serotype Typhi, each effector gene was introduced individually into the serotype Typhimurium *sipA sopABDE2* mutant (ZA21). Complementation of the *sipA sopABDE2* mutant for invasion of HT-29 cells by introduction of either the cloned *sipA*, *sopB*, or *sopD* gene of serotype Typhimurium confirmed the results obtained in a previous study (34). The level of invasion of the *sipA sopABE2* mutant complemented with either *sipA* or *sopD* from serotype
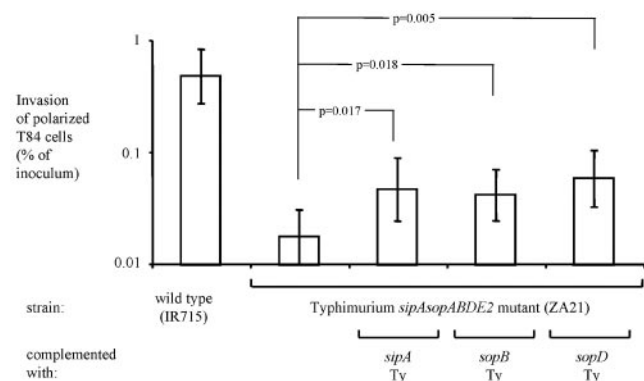
FIG. 4. Invasion of polarized T84 cells by the indicated strains of serotype Typhimurium. Complementation with cloned effector genes of serotype Typhi (Ty) is indicated below. Data are geometric means (bars) ± standard deviations. Statistical significances of differences between treatment groups (brackets) are indicated above.
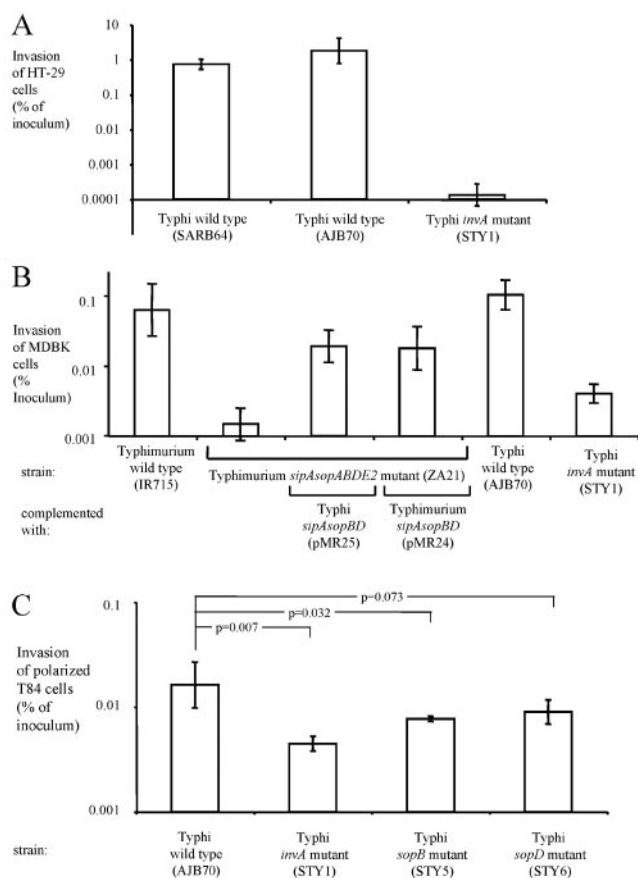

FIG. 5. Invasion of HT-29 cells (A), MDBK cells (B), and polarized T84 cells (C) by the indicated strains of serotypes Typhi and Typhimurium. Data are geometric means (bars) ± standard deviations. Statistical significances of differences between treatment groups (brackets) are given at the top of panel C.

Typhi was in each case similar to that of the *sipA sopABDE2* mutant complemented with the corresponding gene from serotype Typhimurium (Fig. 3B). However, introduction of the serotype Typhimurium *sopB* gene significantly ($P = 0.014$) increased the invasiveness of the *sipA sopABDE2* mutant, while introduction of the serotype Typhi *sopB* gene did not ($P = 0.079$). Although the invasiveness of the *sipA sopABDE2* mutant was influenced by the presence or absence of effector genes, we did not observe a correlation between expression levels of effectors (Fig. 1) and invasiveness (Fig. 3B).

The data discussed above (Fig. 3) demonstrated that introduction of the cloned serotype Typhi *sipA* gene into a serotype Typhimurium *sipA sopABDE2* mutant resulted in increased invasiveness. However, these data did not provide information regarding the functionality of the serotype Typhi *sopB* and *sopD* genes. We have shown previously that the serotype Typhimurium *sopB* and *sopD* genes are required for invasion of polarized T84 cells (34). We thus determined whether introduction of the cloned serotype Typhi *sopB* and *sopD* genes would complement a serotype Typhimurium *sipA sopABDE2* mutant for invasion of polarized T84 cells. The serotype Typhimurium *sipA sopABDE2* mutant (ZA21) became significantly more invasive when transformed with plasmids carrying individual serotype Typhi effector genes, including *sipA* ($P = 0.017$), *sopB* ($P = 0.018$), and *sopD* ($P = 0.005$) (Fig. 4). The finding that each of the serotype Typhi effector genes could enhance the invasiveness of the serotype Typhimurium *sipA sopABDE2* mutant suggested that SopB, SopD, and SipA from serotype Typhi are functional.

The serotype Typhi T3SS-1 mediates invasion of human and bovine epithelial cells. Serotype Typhi was recovered at numbers similar to those of a noninvasive serotype Typhimurium mutant (ZA21) from the bovine ileal mucosa (Fig. 2B). We further investigated whether this low recovery of serotype Typhi from bovine tissue was due to a general defect in its ability to invade epithelium. To this end, the invasiveness of two serotype Typhi wild-type isolates (SARB63 and AJB70) and a derivative of AJB70 carrying an insertion in the *invA* gene (STY1) was compared in human HT-29 cells (Fig. 5A). Both wild-type serotype Typhi isolates invaded HT-29 cells at

levels similar to that of wild-type serotype Typhimurium (Fig. 3 and 5A). Serotype Typhi strain AJB70 (wild type) was recovered from HT-29 cells in approximately 13,500-fold-higher bacterial numbers on average ($P < 0.05$) than its isogenic *invA* mutant (STY1) (Fig. 5A). These data confirmed that the serotype Typhi T3SS-1 is fully functional during invasion of human epithelial cell lines.

We next investigated whether the inability of serotype Typhi to invade the bovine ileal mucosa (Fig. 2B) was due to an inability of this strictly human adapted pathogen to invade bovine cells. The serotype Typhi wild-type strain AJB70 was recovered from bovine kidney epithelial (MDBK) cells in significantly higher numbers than its isogenic *invA* mutant (STY1) (Fig. 5B), suggesting that serotype Typhi T3SS-1 mediates invasion of bovine cells. Furthermore, introduction of the cloned serotype Typhi *sipA*, *sopB*, and *sopD* genes partially complemented the invasiveness of the serotype Typhimurium *sipA sopABDE2* mutant (Fig. 5B), suggesting that the encoded serotype Typhi effectors are functional in cells of bovine origin.

We next investigated whether the *sopB* and *sopD* genes contribute to invasion of polarized T84 cells by serotype Typhi. The serotype Typhi wild-type strain AJB70 was 2-fold more invasive than a serotype Typhi *sopB* mutant ($P = 0.03$) and
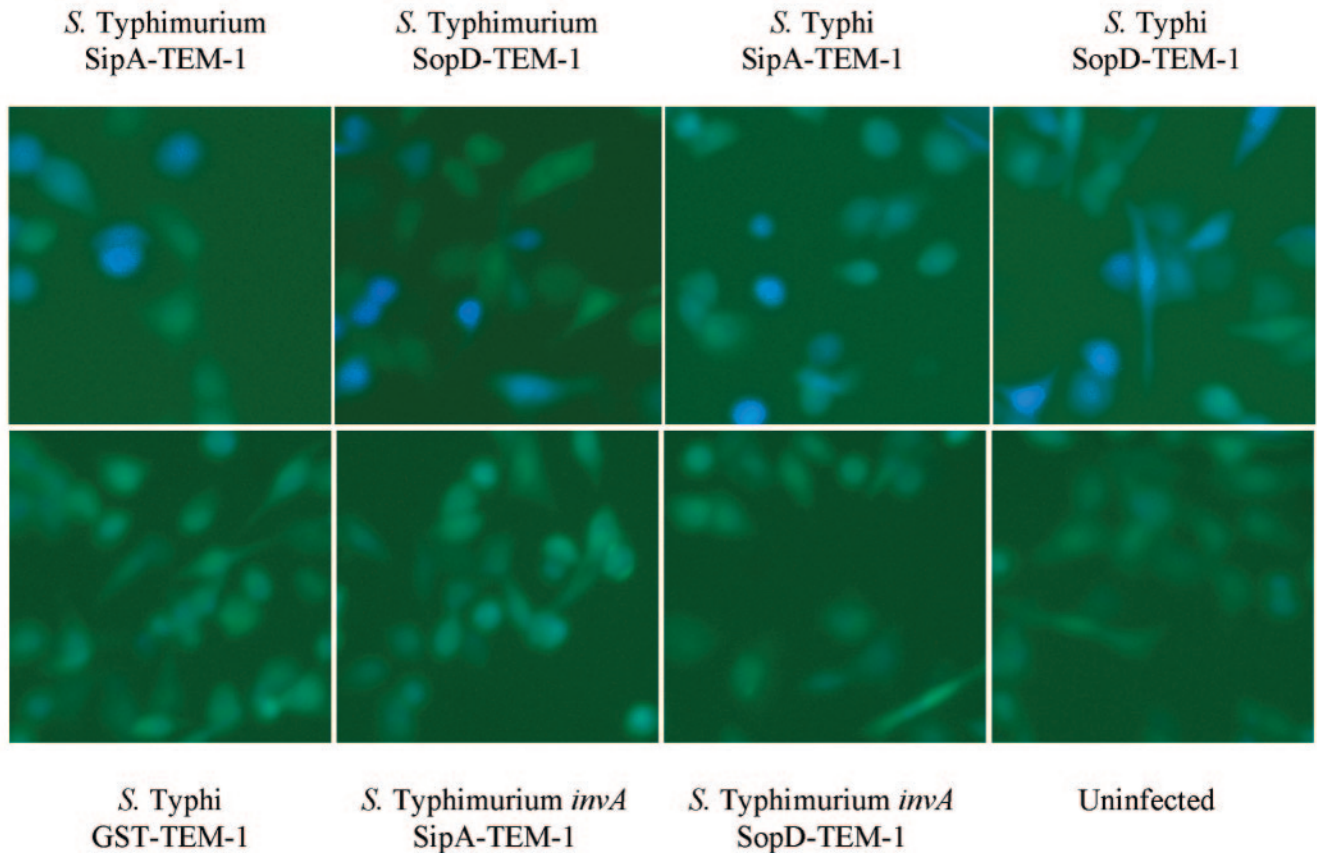
FIG. 6. Demonstration of translocation of T3SS-1 effectors into live MDBK cells by using TEM-1 fusions and fluorescence microscopy. MDBK cells were infected with serotype Typhi or serotype Typhimurium strains (indicated above and below the micrographs) expressing different TEM-1 fusion proteins (SipA–TEM-1, SopD–TEM-1, or GST–TEM-1). After infection, MDBK cells were washed and loaded with CCF2/AM. The uncleaved CCF2 β-lactamase substrate emits green fluorescence. Translocation of β-lactamase into MDBK cells is revealed by blue fluorescence emitted by the cleaved CCF2 substrate.

1.8-fold more invasive than a serotype Typhi *sopD* mutant ($P = 0.07$) (Fig. 5C). Although serotype Typhi invaded HT-29 human colon epithelial cells equally well, it was less invasive for polarized T84 human colon epithelial cells than serotype Typhimurium (Fig. 3, 4, and 5C).

Translocation of T3SS-1 effectors into host cells by serotype Typhi. To further investigate whether the T3SS-1 of serotype Typhi is fully functional, we compared the abilities of serotype Typhimurium and serotype Typhi to translocate effectors into host cells in vitro. To this end we constructed fusions between the serotype Typhimurium SipA protein and the TEM-1 β-lactamase reporter (SipA–TEM-1), between the serotype Typhi SopD protein and β-lactamase (SopD–TEM-1), and (as a negative control) between GST and β-lactamase (GST–TEM-1). Translocation was detected in living MDBK cells by using the fluorescent β-lactamase substrate CCF2/AM as described previously (8). The use of this technique allows identification of cells loaded with CCF2 by virtue of their green fluorescence. Cells in which CCF2 has been cleaved by β-lactamase emit blue fluorescence. Uninfected MDBK cells and MDBK cells infected with serotype Typhi expressing GST–TEM-1 showed green fluorescence (Fig. 6). In contrast, a fraction of cells infected with serotype Typhi expressing SipA–TEM-1 or

SopD–TEM-1 emitted blue fluorescence, suggesting that fusions between T3SS-1 effectors and TEM-1 are translocated into host cells by serotype Typhi. The fraction of cells emitting blue fluorescence after infection with serotype Typhimurium strains expressing SipA–TEM-1 or SopD–TEM-1 was similar to that observed with the corresponding serotype Typhi strains (Fig. 6). However, no cells emitting blue fluorescence were detected after infection of MDBK cells with serotype Typhimurium *invA* mutant strains expressing SipA–TEM-1 or SopD–TEM-1, demonstrating that translocation of β-lactamase fusion proteins was dependent on the presence of a functional T3SS-1. Collectively, these data showed that serotype Typhi is fully capable of translocating SipA and SopD into host cells of bovine origin.

DISCUSSION

Evolution of a host-restricted serotype, such as serotype Typhi, from a host generalist, such as serotype Typhimurium, likely involved both gain of function (i.e., acquisition of new genetic material through horizontal gene transfer) and loss of function (i.e., genome degradation by deletion or by pseudogene formation) (5). It can be speculated that during this

process serotype Typhi acquired genetic material that allowed it to cause typhoid fever in humans. Furthermore, serotype Typhi likely lost genetic material required for infection of other host species by genome degradation. Although the functions of the approximately 210 pseudogenes present in the serotype Typhi genome are apparently not required for causing disease in humans (10, 32), some of these genes may be essential during infection of other host species, such as cattle. Similarly, some of the 479 open reading frames present in the serotype Typhimurium genome but absent from the serotype Typhi genome may be required for interaction with the bovine host (25). Thus, the inability of serotype Typhi to cause disease in cattle may be due to genome degradation affecting functions other than those related to its T3SS-1. This hypothesis would explain why serotype Typhi effectively enters human and bovine epithelial cells in vitro (Fig. 5) (23, 28, 49), while the invasiveness of serotype Typhi for the bovine ileal mucosa in vivo is similar to that of a noninvasive serotype Typhimurium mutant (the *sipA sopABDE2* mutant) (Fig. 2B).

It is less obvious why invasion of the human intestinal mucosa by serotype Typhi does not trigger the massive neutrophil influx (18, 22, 30, 31, 39) that is the hallmark of human infections with serotype Typhimurium (18, 38, 42, 50). Here we investigated the hypothesis that the low propensity of serotype Typhi to cause diarrhea and neutrophil influx into the intestinal mucosa may be due to a loss of function. Whole-genome sequencing has revealed that some of the T3SS-1 effector genes that contribute to the ability of serotype Typhimurium to cause fluid accumulation and inflammation in bovine ligated ileal loops (54) are pseudogenes in the genomes of human-adapted serotypes causing typhoid fever (10, 24, 32). Based on this observation, it has been speculated that inactivation of T3SS-1 effector genes by genome degradation may account for the low propensity of serotype Typhi to cause diarrhea in humans (24). Here we show that the relevant T3SS-1 effector genes that remained intact in the genomes of typhoidal *Salmonella* serotypes (i.e., *sipA*, *sopB*, and *sopD*) mediated fluid accumulation when introduced into serotype Typhimurium, despite the fact that serotype Typhi does not elicit fluid secretion in bovine ligated ileal loops (Fig. 2A). Serotype Typhi was able to translocate effector proteins into host cells (Fig. 6) and to invade human epithelial cell lines in vitro by using its T3SS-1 (Fig. 5) (23, 28, 49). Thus, the inability of serotype Typhi to cause fluid accumulation in bovine ligated ileal loops is not caused by an absence of T3SS-1-secreted effectors that can elicit this response.

Our data are not consistent with the hypothesis that serotype Typhi has a lower propensity to elicit neutrophil influx than serotype Typhimurium because the T3SS-1 is subject to genome degradation. Recent data suggest an alternative hypothesis, namely, that the scarcity of neutrophils in intestinal infiltrates of typhoid fever patients may be the result of acquisition (rather than loss) of genetic material during the evolution of host adaptation in the serotype Typhi lineage (33). Serotype Typhi contains a 135-kb DNA region, termed SPI7, that is absent from the serotype Typhi genome (32). The *viaB* locus on SPI7 contains genes for the biosynthesis and export of the Vi capsular antigen. Expression of the Vi antigen reduces the production of neutrophil chemoattractants (e.g., interleukin-8) during infection of human epithelial cells (33, 37) or macro-

phages (19, 33) in vitro and during infection of human colon tissue explants with serotype Typhi (33). Thus, acquisition of SPI7 by an organism ancestral to serotype Typhi may explain why this pathogen elicits a different host response in the human intestine than nontyphoidal *Salmonella* serotypes, such as serotype Typhimurium.

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