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The *Salmonella enterica* Serotype Typhimurium Effector Proteins SipA, SopA, SopB, SopD, and SopE2 Act in Concert To Induce Diarrhea in Calves

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***Salmonella enterica* serotype Typhimurium requires a functional type III secretion system encoded by *Salmonella* pathogenicity island 1 (SPI1) to cause diarrhea. We investigated the role of genes encoding secreted target proteins of the SPI1-associated type III secretion system for enteropathogenicity in calves. *Salmonella* serotype Typhimurium strains having mutations in *sptP*, *avrA*, *sspH1*, or *slrP* induced fluid secretion in the bovine ligated ileal loop model at levels similar to that of the wild type. In contrast, mutations in *sipA*, *sopA*, *sopB*, *sopD*, or *sopE2* significantly reduced fluid accumulation in bovine ligated ileal loops at 8 h postinfection. A strain carrying mutations in *sipA*, *sopA*, *sopB*, *sopD*, and *sopE2* (*sipA sopABDE2* mutant) caused the same level of fluid accumulation in bovine ligated ileal loops as a strain carrying a mutation in *sipB*, a SPI1 gene required for the translocation of effector proteins into host cells. A positive correlation was observed between the severity of histopathological lesions detected in the ileal mucosa and the levels of fluid accumulation induced by the different mutants. After oral infection of calves, the *Salmonella* serotype Typhimurium *sipAsopABDE2* mutant caused only mild diarrhea and was more strongly attenuated than strains having only single mutations. These data demonstrate that SipA, SopA, SopB, SopD, and SopE2 are major virulence factors responsible for diarrhea during *Salmonella* serotype Typhimurium infection of calves.**

Salmonella enterica serotype Typhimurium can infect a wide range of host species, including mice, cattle, and humans. In mice, *Salmonella* serotype Typhimurium causes a systemic disease which serves as an animal model to study typhoid fever, a disease caused by *S. enterica* serotype Typhi in humans. Murine typhoid is characterized by rapid bacterial multiplication in systemic organs and infiltration of mononuclear leukocytes in the intestinal mucosa which is not associated with diarrhea (36). Interestingly, mice exhibit a strikingly different response to *Salmonella* serotype Typhimurium infection than the human host. *Salmonella* serotype Typhimurium infection in humans commonly manifests as enterocolitis that is characterized by diarrhea and infiltration of polymorphonuclear leukocytes (PMN) in the intestinal mucosa (27, 41). The infection commonly remains localized in the intestine and mesenteric lymph nodes, while bacteremia is a rare complication (46). In addition to its frequent association with food-borne disease in humans, *Salmonella* serotype Typhimurium is a major cause of calf morbidity and mortality (14, 20, 33, 38, 40). The features of the diarrheal disease caused by *Salmonella* serotype Typhimurium in calves closely resembles the clinical and pathological features observed in humans infected with this pathogen (9, 39, 47, 56). Therefore, the calf is currently used as an animal model to study human enterocolitis caused by *Salmonella* serotype Typhimurium.

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Mutations in genes located on *Salmonella* pathogenicity island 1 (SPI1), including *prgH*, *hilA*, and *invH*, result in strongly reduced virulence of *Salmonella* serotype Typhimurium during oral infection of calves (47, 52). PrgH and InvH are components of the invasion-associated type III secretion system whose expression is controlled by HilA (2, 3, 5). The main function of the invasion-associated type III secretion system is to translocate effector proteins into the cytosol of a host cell (11). In the first step, secreted target proteins are transported across inner and outer membranes of the bacterial cell by the type III secretion apparatus. A subset of these secreted proteins, including SipB (SspB), SipC (SspC), and SipD (SspD), form a translocation complex in the eukaryotic membrane, which is required for the delivery of other effector proteins into the cytoplasm (7, 10, 13, 16, 55). Nonpolar mutations in *sipB*, *sipC*, or *sipD* result in the same degree of attenuation of *Salmonella* serotype Typhimurium during oral infection of calves as deletion of genes (*prgHIJK*) encoding components of the invasion-associated type III secretion apparatus (48). Furthermore, inactivation of *sipB* in *S. enterica* serotype Dublin strongly reduces fluid secretion and inflammation measured at 12 h postinfection in bovine ligated ileal loops (13, 47).

A mutation in *sipB* may cause attenuation in calves, either because SipB directly engages targets in the host cell or because translocation of other effector proteins is prevented. The finding that SipB is an effector protein, which causes cell death in murine and bovine macrophages by binding to caspase 1, demonstrates that this protein can directly engage targets in the host cell (18, 34, 53). Furthermore, SipB-mediated macrophage cell death results in activation of the proinflammatory

cytokine interleukin 1β (18), suggesting that this mechanism may contribute to eliciting an inflammatory response in the intestine. However, while inflammatory changes in bovine ligated ileal loops are observed as early as 1 h postinfection with *Salmonella* serotype Typhimurium, a significant increase in cell death detected by terminal deoxyribonucleotidyl transferase-dependent UTP nick end labeling in the bovine mucosa and lymphoid nodules is first observed 12 h postinfection (35). Thus, SipB-mediated macrophage cell death is unlikely to be required for triggering an early inflammatory response and fluid accumulation in the bovine ileum. These data raise the possibility that SipB-mediated translocation of effector proteins is the major virulence function of this protein during bovine enteritis pathogenesis.

In addition to proteins that act as translocases (SipBCD), SPI1 encodes three effector proteins, SipA (SspA), AvrA, and SptP (16, 19, 22–24). Furthermore, seven effector proteins, including SopA, SopB (SigD), SopD, SopE1, SopE2, SspH1, and SlrP, whose secretion is mediated by the SPI1 type III secretion system are encoded by genes which are located outside SPI1 (4, 13, 17, 21, 29, 43, 49, 54, 55). Much of the work on the role of SPI1 effector proteins during infection of calves has been performed with *Salmonella* serotype Dublin, a serotype that causes bacteremia rather than enterocolitis in humans (8). Inactivation of *avrA* does not reduce the ability of *Salmonella* serotype Dublin to induce fluid accumulation and PMN influx in bovine ligated ileal loops (37), suggesting that not all SPI1 effector proteins are required for enteropathogenicity in calves. The level of fluid secretion and inflammation elicited by a *Salmonella* serotype Dublin *sopA*, *sopB*, or *sopD* mutant is greater than that elicited by a *Salmonella* serotype Dublin *sipB* mutant. However, *sopA*, *sopB*, or *sopD* mutants cause significantly less fluid accumulation and PMN influx in bovine ligated ileal loops than the *Salmonella* serotype Dublin wild type causes (13, 21, 54). Furthermore, the enteropathogenicity of a *Salmonella* serotype Dublin *sopB sopD* double mutant is reduced compared to that of a *sopB* or *sopD* mutant, but it is still greater than that of a *sipB* mutant (21). These data suggest that several type III secreted effector proteins of *Salmonella* serotype Dublin are required for enteropathogenicity.

Although *Salmonella* serotype Dublin causes bacteremia rather than enterocolitis in humans (8), it likely employs virulence mechanisms to cause enterocolitis in calves that are similar to those used by *Salmonella* serotype Typhimurium. For instance, a mutation in *sopB* reduces the ability of *Salmonella* serotype Typhimurium to cause inflammation and fluid accumulation in bovine ligated ileal loops to a degree similar to that observed for *Salmonella* serotype Dublin (35). So far, the virulence of *Salmonella* serotype Dublin *sopB*, *sopD*, or *sopA* mutants has been characterized only in bovine ligated ileal loops. Oral infection studies with *Salmonella* serotype Typhimurium found that a *sopB* mutant appears to be fully virulent when calves are infected by this route (47). The lack of attenuation of a *Salmonella* serotype Typhimurium *sopB* mutant during oral infection is likely due to the fact that several type III secreted effector proteins act in concert during the pathogenesis of bovine enteritis. However, it is currently not known how many type III secreted effector proteins are involved in eliciting fluid secretion and inflammation in bovine ligated ileal loops. If the main role of SipB during infection is the translocation of effector proteins involved in enteropathogenicity (i.e., SopB and other proteins), it should be possible to reduce the virulence of *Salmonella* serotype Typhimurium to that of a *sipB* mutant by inactivating the genes encoding these effector proteins. To test this prediction, we determined which genes encoding targets for the SPI1 type III secretion system are required for fluid accumulation in bovine ligated ileal loops. Furthermore, a *Salmonella* serotype Typhimurium strain lacking all effector proteins involved in fluid secretion was constructed and characterized in ligated ileal loops and during oral infections of calves.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Salmonella* serotype Typhimurium and *Escherichia coli* strains used in this study are listed in Table 1. Bacteria were cultured aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar plates. If appropriate, antibiotics were added at the following concentrations: kanamycin, 100 mg/liter; chloramphenicol, 30 mg/liter; ampicillin, 100 mg/liter; nalidixic acid, 50 mg/liter; and tetracycline, 20 mg/liter.

Construction of mutants. Mutant ZA9 (*sopE2*) was created by phage P22-mediated transduction by using *Salmonella* serotype Typhimurium strains M119 (S. Mirold, W Rabsch, H. Tschäpe, and W.-D. Hardt, submitted for publication) and IR715 (44) as the donor and the recipient, respectively. Inactivation of *sopE2* in ZA9 was confirmed by Southern hybridization. Briefly, a DNA fragment containing nucleotides 3 to 702 of the *sopE2* open reading frame was amplified by PCR by using primers SopE1 and SopE2 (Table 2), labeled with fluorescein (NEN life science product; NEN), and used as a probe to hybridize genomic DNA of ZA9 and IR715. Unmarked deletions of *sipA*, *sptP*, *avrA*, *sspH1*, and *sopA* (strains ZA10, ZA11, ZA13, ZA14, and ZA19, respectively) were constructed by allelic exchange. In brief, DNA regions upstream and downstream of the desired gene were amplified by PCR by using primers listed in Table 2. The primers were introduced into a restriction site (either *Xba*I or *Sac*I) that was used to ligate the digested 3′ end of the upstream fragment to the digested 5′ end of the downstream fragment. The ligation product was reamplified by PCR and cloned into the vector pCR2.1 (Invitrogen). The insert was excised by restriction digestion with *Eco*RI and subcloned into the vector pRDH10 (26), a λ*pir*-dependent suicide vector carrying *sacB* and a chloramphenicol resistance gene. The resulting plasmid was introduced into *E. coli* strain S17λ-*pir* and transferred into *Salmonella* serotype Typhimurium strain IR715 by conjugation. Exconjugants in which the suicide plasmid was integrated into the IR715 chromosome were selected on LB agar plates supplemented with chloramphenicol and nalidixic acid. A second recombination event, leading to the loss of plasmid sequences, was selected by growth in sucrose (5%) broth, followed by growth on sucrose agar plates at 30°C. Colonies that were sensitive to chloramphenicol were screened by PCR for deletion of the gene of interest. To confirm the unmarked deletions, Southern hybridization analyses were conducted. In brief, the primer pairs SipAF1 plus SipAB2, SptPF1 plus SptPB2, AvrAF1 plus AvrAB2, SspHF1 plus SspHB2, and SopAF1 plus SopAB2 were used to amplify *sipA*, *sptP*, *avrA*, *sspH1*, and *sopA*, respectively. The resulting PCR products were labeled with fluorescein (NEN) and used as probes to hybridize the digested genomic DNA of the corresponding mutant strains and a positive control (IR715). To generate ZA16 (*sopBE2*), *sopE2*::*tet*^{r} was introduced from M119 into ZA15 (*sopB*) by phage P22-mediated transduction, and the mutation was confirmed by Southern hybridization. To create ZA17 (*sopD*), a DNA fragment containing nucleotides 101 to 621 of the *sopD* open reading frame was PCR amplified and cloned into plasmid pEP185.2, a suicide vector coding for chloramphenicol resistance (25). The resulting plasmid, pZA17, was introduced into *E. coli* strain S17λ-*pir* and transferred into strain IR715 by conjugation. Exconjugants containing pZA17 inserted into the chromosome of strain IR715 were selected by growth on LB agar plates supplemented with chloramphenicol and nalidixic acid. The *sopD*::pEP185.2 insertion was then introduced into ZA16 by P22-mediated transduction to generate ZA18 (*sopBDE2*). The *sopD*::pEP185.2 insertion in strains ZA17 and ZA18 was confirmed by Southern hybridization by using a probe containing nucleotides 101 to 621 of the *sopD* gene. The *sopA* and *sipA* genes were deleted sequentially from strain ZA18 to obtain strains ZA20 (*sopABDE2*) and ZA21 (*sipAsopABDE2*). The unmarked deletions and selection processes used for ZA20 and ZA21 were the same as those described above for single-knockout mutants. The mutations in the *sipA*, *sopA*, *sopD*, and *sopE2* genes of ZA21 were confirmed by Southern hybridization as described above for each single mutant. In addition, the insertional mutation in *sopB* of ZA21 was also

TABLE 1. *Salmonella* serotype Typhimurium strains and plasmids used in this study

Strain or plasmid	Relevant genotype[a]	Source or reference
Salmonella serotype Typhimurium strains		
ATCC 14028		ATCC[b]
IR715	ATCC 14028 wild type, *nal*^{r}	44
CAS152	ATCC 14028 *phoN*::Cm *sipB*	49
M119	ATCC 14028 *sopE*::*tet*^{r}	Mirold et al.[c]
JLR137	ATCC 14028 *phoN*::Cm *glpD*::Tn*5*-*lacZY*	Miller laboratory
STN39	ATCC 14028 *nal*^{r} *slrP*::mini-*Tn5*Km2	49
ZA9	ATCC 14028 *nal*^{r} *sopE2*::*tet*^{r}	This study
ZA10	ATCC 14028 *nal*^{r} Δ*sipA* (Δ16-606/685)	This study
ZA11	ATCC 14028 *nal*^{r} Δ*sptP* (Δ30-478/544)	This study
ZA13	ATCC 14028 *nal*^{r} Δ*avrA* (Δ19-213/300)	This study
ZA14	ATCC 14028 *nal*^{r} Δ*sspH1* (Δ53-354/702)	This study
BA1567	ATCC 14028 *sopB*::*mudJ*	1
ZA15	ATCC 14028 *nal*^{r} *sopB*::*mudJ*	15
ZA16	ATCC 14028 *nal*^{r} *sopBE2*	This study
ZA17	ATCC 14028 *nal*^{r} *sopD*	This study
ZA18	ATCC 14028 *nal*^{r} *sopBED*	This study
ZA19	ATCC 14028 *nal*^{r} Δ*sopA* (Δ38-734/783)	This study
ZA20	ATCC 14028 *nal*^{r} Δ*sopABDE2*	This study
ZA21	ATCC 14028 *nal*^{r} Δ*sipA*Δ*sopAsopBDE2*	This study
ZA26	ATCC 14028 *nal* Δ*sipAsopB*	This study
Plasmids		
pCR2.1	Amp^r Kan^r *lacZ*α	Invitrogen
pEP185.2	Cm^r	25
pRDH10	Cm^r *sacB*	26
pWSK29	Amp^r	51
pZA10	pRDH10 carrying the flanking regions of *sipA*	This study
pZA11	pRDH10 carrying the flanking regions of *sptP*	This study
pZA13	pRDH10 carrying the flanking regions of *avrA*	This study
pZA14	pRDH10 carrying the flanking regions of *sspH1*	This study
pZA17	pEP185.2 carrying *sopD* fragment (bp 101 to 621)	This study
pZA19	pRDH10 carrying the flanking region of *sopA*	This study
pSipA	pWSK29 carrying the *sipA* gene	This study

[a] The numbers in parentheses are the deleted codons/total number of codons of *avrA*, *sipA*, *sopA*, *sptP*, and *sspH1*.
[b] ATCC, American Type Culture Collection.
[c] Mirold et al., submitted.

confirmed by Southern hybridization. The SopB probe was generated by PCR by using primers SopB1 and SopB2 (Table 2). To generate ZA22 (*sipAsopB*), *sopB*::*mudJ* was introduced from ZA15 (*sopB*) into ZA10 (*sipA*) by phage P22-mediated transduction and selection for kanamycin resistance. The mutation was confirmed by Southern hybridization. For complementation, the *sipA* gene was amplified by PCR by using primers z-SipA1 and z-SipA2. The PCR product was cloned into a low-copy-number vector, pWSK29 (51), to create plasmid pSipA. The orientation of the *sipA* gene in pSipA was determined by sequence analysis. To complement the defect of mutants with mutations in induction of secretory and inflammatory responses, plasmid pSipA was introduced into strains ZA21, ZA10, and ZA26 by electroporation and selection for resistance to ampicillin. The genetic organization of effector genes involved in fluid accumulation is shown in Fig. 1.

TABLE 2. Primers used in this study[a]

Primer	Sequence
AvrF1	AGAAGGCGTTATCTACTTGC
AvrB1	GCTCTAGAGGACTTAGCATACTTTTCCCTC
AvrF2	GCTCTAGACGTCTGTGTGGTGAAGAAC
AvrB2	TTAGCGGTGAGTCTGTCAG
SipAF1	AGGCGGCTACTAAAATCC
SipAB1	GCTCTAGATACCTGGCATTATGACGGG
SipAF2	GCTCTAGAGGTCATTACTCATCATCC
SipAB2	CAAGCGAGAGAAAAATACTACAC
SopAF1	AAAGATGGCTGGAGAGCGAG
SopAB1	GCGAGCTCTGGTTATTTTTGAGGTGAG
SopAF2	GCGAGCTCGAACAGTTTACCGAGTGGTC
SopAB2	TGAATGCGTCTGGCGAAAGC
SopB1	CCCGTATTTGGTTCTGAATCTCC
SopB2	AGCCTGAAACTGGTATCCGTGG
SopDF	GCGATATCTGGGGGGTTGGGATAAAGTC
SopDB	GCTCTAGATAAGCGAGTCCTGCCATTCG
SopE1	GACTAACATAACACTATCCACC
SopE2	TCAGGAGGCATTCTGAAGATAC
SptpF1	GCGAAAAAGTATCAAGACATTG
SptpB1	GCTCTAGATAAAGTCGGGCATCATTC
SptpF2	GCTCTAGATTCATTGTCTGGGCGGAG
SptpB2	ACGGTAAAATCTGAGAGAGG
SspHF1	AGGCGTTGGGCGAATCTATC
SspHB1	GCTCTAGATTCAGCGAGACACTGTTGC
SspHF2	GCTCTAGACAACACTGGAACAGATTGC
SspHB2	TACGCCCTGACTGAAGAAG
Z-SipA1	GCGAGCTCACGACAACCTGGTAAAAG
Z-SipA2	GCGAGCTCCTATCAACATCAACGGCA
LacZ1	CTCTTCGCTATTACGCCA
LacZ2	CATAAACCGACTACACAAA

[a] The restriction endonuclease sites incorporated into the primer sequences are underlined.

Inverse PCR. To determine the insertion site for transposon Tn*5lacZYA* in *Salmonella* serotype Typhimurium strain JLR137, transposon-flanking DNA was amplified by inverse PCR by using primers LacZ1 and LacZ2 (Table 2). Briefly, genomic DNA of JLR137 was digested with *Rsa*I, and the fragments were ligated and used as templates for PCR amplification. The resulting PCR product was cloned into the vector pCR2.1 and sequenced.

Animal experiments. Male Holstein calves that were 4 to 5 weeks old and weighed 45 to 55 kg were used. They were fed antibiotic-free milk replacer twice a day and given water ad libitum. Prior to experiments, the calves were screened for elevated total leukocyte count, rectal temperature, and fecal excretion of *Salmonella* serotypes. *Salmonella* serotypes in fecal swabs were detected by enrichment growth in tetrathionate broth (Difco) and then Rappaport-Vassiliadis R10 broth (Difco) and subsequent plating on brilliant green agar and XLT-4 agar (BBL) plates. Serum antibodies against *Salmonella* serotype Typhimurium were detected by an enzyme-linked immunosorbent assay by using wells coated with purified lipopolysaccharide from this serotype (Sigma).

For ligated ileal loop assays, calves were fasted for 48 h prior to surgery. Anesthesia was induced with Propofol (Propoflo; Abbot Laboratories, Chicago, Ill.) and maintained with Isofluorane (Isoflo; Abbot Laboratories) for the duration of the experiment (35). To prepare bacterial inocula, the desired bacterial strains were cultured in LB broth for 18 h at 37°C with shaking at 150 rpm. The resultant cultures were diluted 1:100 with sterile LB broth and incubated for additional 4 h. Bacteria in the logarithmic phase of growth were then harvested by centrifugation for 15 min at 1,500 × *g* and resuspended in fresh LB broth to obtain a final concentration of approximately 0.75×10^9 CFU/ml. After a laparotomy was performed and the ileum was exposed, ileal loops with lengths ranging from 6 to 9 cm were ligated with a 1-cm interposed loop between them. The loops were infected by intralumenal injection of 3 ml of a suspension of IR715 or a mutant strain in LB broth containing approximately 1×10^9 CFU of bacteria. The control loops received 3 ml of sterile LB broth. Following injection, the ileal loops were placed back into the abdominal cavity until the time for sample processing. At 8 h postinfection, the fluid accumulated in the loops was measured, and samples for bacteriologic culture and histopathological analysis were collected. Tissue samples from Peyer's patches were homogenized and

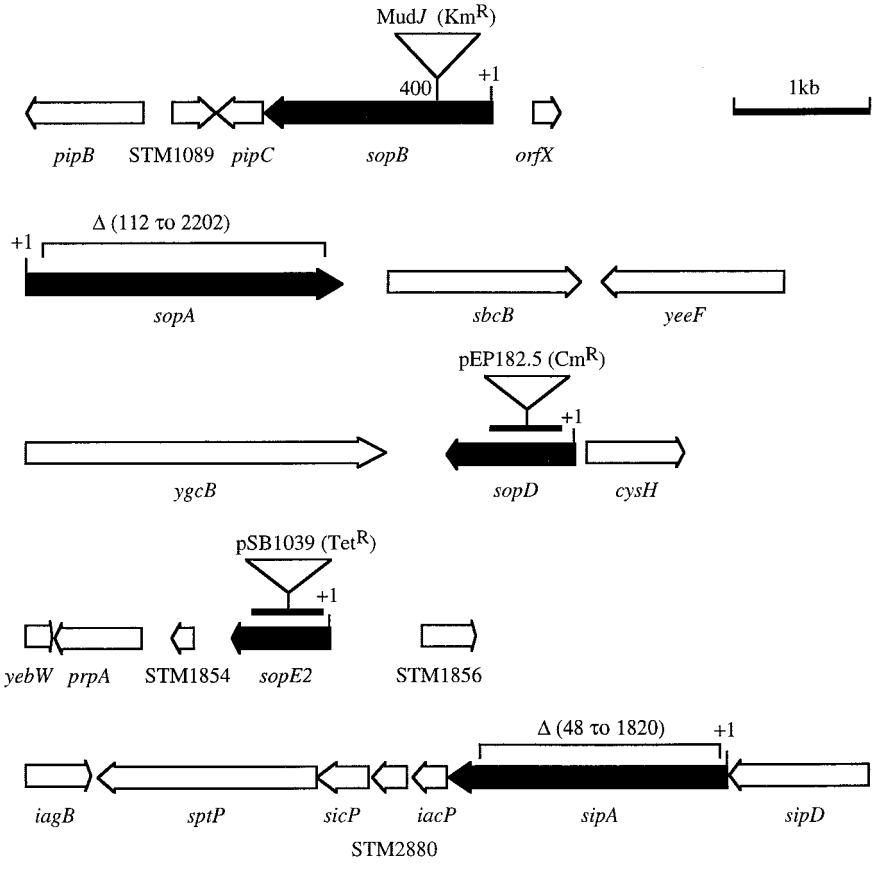

FIG. 1. Mutations in effector genes (solid arrows) which are required for fluid accumulation in calves. The open arrows indicate the positions of genes in the surrounding DNA region. Numbers indicate the positions of insertions or deletions relative to the first nucleotide (+1) in the open reading frame. The bars indicate internal fragments of *sopD* and *sopE2* that were cloned into suicide vectors to inactivate these genes.

serially diluted in phosphate-buffered saline and were plated on LB agar plates supplemented with appropriate antibiotics for enumeration of bacteria.

For oral infection, the optical densities at 600 nm of overnight cultures were determined in order to estimate the numbers of bacteria per milliliter. A volume containing 1×10^{10} CFU of bacteria was mixed with 50 ml of a suspension containing 5% magnesium trisilicate, 5% sodium bicarbonate, and 5% magnesium carbonate. Groups of four calves were infected with the following strains: IR715, ZA9, ZA17, ZA19, and ZA20. Rectal temperature and fecal score (for assessment of diarrhea) were recorded daily. Fecal shedding of *Salmonella* serotype Typhimurium was monitored by daily plating of fecal samples on brilliant green agar plates. Blood samples were taken prior to infection (zero time) and on days 1 and 2 postinfection. These time points were chosen because a previous study (48) demonstrated that calves inoculated orally with 10^{10} CFU of wild-type *Salmonella* serotype Typhimurium developed lethal signs of illness before 3 days postinfection. The concentrations of sodium in blood plasma samples were determined by the Texas Veterinary Medical Center, Texas A&M University. When the calves developed anorexia or were unable to stand, they were euthanized for humane reasons as described previously (23). At 10 days postinfection, the surviving calves were euthanized. At necropsy, tissue samples from livers, spleens, mesenteric lymph nodes, and Peyer's patches were collected, homogenized and serially diluted in phosphate-buffered saline, and plated on LB agar plates supplemented with appropriate antibiotics for enumeration of bacteria.

Histopathology. The tissue samples were fixed in formalin, processed by using standard procedures for paraffin embedding, cut into 5-μm sections, and stained with hematoxylin and eosin. Inflammatory changes were scored by using a scale from 1 to 5 according to the following criteria: 1, intravascular infiltration of polymorphonuclear leukocytes (PMN); 2, margination and perivascular infiltration of PMN and/or mild diffuse infiltration of PMN at the tips of absorptive villi; 3, moderate diffuse infiltration of PMN in the mucosa and perivascular multifocal infiltration in the submucosa; 4, severe diffuse infiltration of PMN in the mucosa and mild to moderate infiltration in the submucosa; and 5, severe diffuse infiltration of PMN throughout the mucosa and submucosa associated with edema and necrosis of the mucosa.

Statistical analysis. To compare the virulence characteristics of a particular mutant and wild-type strain IR715, data for fluid secretion (volume/length), bacterial colonization, and plasma concentration of electrolytes were analyzed by using Student's *t* test. To assess the effects of different mutations on secretory responses, fluid secretion data were expressed as percentages of the response elicited by wild-type strain IR715. Data then underwent arcsin transformation, and analysis of variance was performed. The averages were compared by using Duncan's multiple range test.

RESULTS

Evaluation of the role of SPI1 effector genes in eliciting secretory and inflammatory responses in bovine ligated ileal loops. Previous studies have demonstrated that a mutation in *sopA*, *sopB*, or *sopD* of *Salmonella* serotype Dublin or in *sopB* of *Salmonella* serotype Typhimurium significantly reduces the ability to cause fluid accumulation in bovine ligated ileal loops (21, 35). To identify additional effector genes required for fluid accumulation in ligated ileal loops, *Salmonella* serotype Typhimurium strains having mutations in effector genes were constructed by using strain IR715, a spontaneous nalidixic acid-resistant derivative of bovine *Salmonella* serotype Typhimurium isolate ATCC 14028 (44). Derivatives of IR715 which have unmarked deletions of *sopA* (ZA20), *sipA* (ZA10), *sptP* (ZA11), *avrA* (ZA13), or *sspH1* (ZA14) were generated by

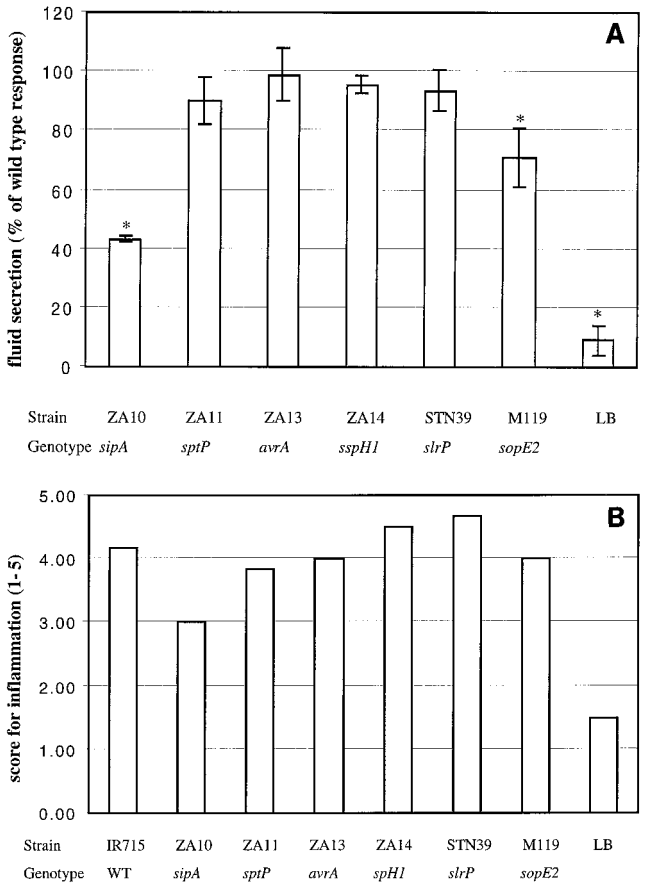

FIG. 2. Ability of *Salmonella* serotype Typhimurium strains carrying mutations in SPI1 effector genes to induce secretory and inflammatory changes in bovine ligated ileal loops at 8 h postinfection. (A) Data for fluid accumulation in loops shown as percentages of the fluid secretion elicited by the wild type (ATCC 14028 for M119 and IR715 for ZA10, ZA11, ZA13, ZA14, and STN39). The 100% line indicates the amount of fluid accumulation elicited by the *Salmonella* serotype Typhimurium wild type. An asterisks indicates that the fluid accumulation was significantly lower than the wild-type response ($P < 0.05$). (B) Inflammatory responses scored on a scale from 1 to 5 according the criteria described in Materials and Methods. WT, wild type.

allelic exchange. A derivative of IR715 containing a transposon insertion in *slrP* (STN39) has been described previously and was also included in this study (49). A mutation in *sopD* was introduced into strain IR715 by integration of a suicide vector carrying an internal fragment of the *sopD* open reading frame (bp 101 to 621) into the chromosome by homologous recombination (ZA17). In addition, derivatives of ATCC 14028 and IR715 carrying a *sopE2*::*tet*^{r} allele (M119 and ZA9, respectively) were tested in this study. The *sopE1* gene was not included in our investigation, because it is not present in strain ATCC 14028.

The virulence of these mutants with respect to secretory and inflammatory responses, as well as colonization of Peyer's patches, was assessed in bovine ligated ileal loops. An initial evaluation of these *Salmonella* serotype Typhimurium mutants demonstrated that mutations in *sipA* or *sopE2*, but not mutations in *sptP*, *avrA*, *sspH1*, or *slrP*, caused significant reductions in fluid secretion ($P < 0.05$) at 8 h postinfection (Fig. 2A). Inflammatory changes compared to uninfected control loops were scored by blinded examination of sections of the ileal mucosa. This analysis revealed that a mutation in *sipA*, but not a mutation in *sopE2*, *sptP*, *avrA*, *sspH1*, or *slrP*, caused a marked reduction in the severity of inflammation in the ileal mucosa (Fig. 2B). The secretory and inflammatory responses induced by ATCC 14028 were similar to those induced by its nalidixic acid-resistant derivative, IR715 (data not shown). No significant differences between loops infected with the wild type (IR715) and loops infected with the mutants described above were observed with regard to the numbers of bacteria recovered from Peyer's patches (data not shown). We next investigated the role of the effector proteins, which have previously been implicated in fluid accumulation caused by *Salmonella* serotype Dublin, in triggering fluid accumulation during *Salmonella* serotype Typhimurium infection. Compared to the *Salmonella* serotype Typhimurium wild type (IR715), strains having mutations in *sopA*, *sopB*, or *sopD* caused significantly less fluid accumulation in bovine ligated ileal loops ($P < 0.05$) (Fig. 3A). Strains IR715 (wild type), ZA19 (*sopA*), and ZA9 (*sopD*) induced moderate to severe diffuse PMN infiltration in the ileal mucosa and submucosa. The severity of inflammation was only slightly reduced in loops infected with ZA15 (*sopB*) (Fig. 3B). Collectively, these data suggested that five SPI1 secreted effector proteins (SopA, SopB, SopD, SopE2, and SipA) are required for fluid accumulation in ligated ileal loops. The genes encoding these effector proteins and mutations used in this study to inactivate the genes are shown in Fig. 1. Our data further suggest that four effector proteins (SptP, AvrA, SspH1, and SlrP) play no apparent role in eliciting fluid accumulation in bovine ligated ileal loops.

SPI1 effector proteins act in concert to elicit fluid accumulation and inflammation. SipB is a translocase required for delivery of SPI1 effector proteins into the host cell cytoplasm. A *Salmonella* serotype Typhimurium *sipB* mutant (CAS152) (48) caused less fluid accumulation in bovine ligated ileal loops than strains carrying a mutation in *sipA*, *sopA*, *sopB*, *sopD*, or *sopE2* (Fig. 3A). We reasoned that the drastic reduction in fluid accumulation caused by a mutation in *sipB* could be accounted for by the fact that translocation of SPI1 effector proteins involved in enteropathogenicity, including SipA, SopA, SopB, SopD, and SopE2, is prevented. To test this hypothesis, we introduced mutations in *sipA*, *sopA*, *sopB*, *sopD*, and *sopE2* one by one into the *Salmonella* serotype Typhimurium wild type (IR715) to generate a series of mutants, one of which carried mutations in all five effector genes (*sipAsopABDE2* mutant). The virulence of these multiple-knockout mutants in bovine ligated ileal loops was compared to that of the isogenic wild-type strain (IR715) and that of strains carrying mutations in *sipB* (CAS152) and in individual effector genes (Fig. 3A).

The *sopBE*2 double mutant (ZA16) caused fluid accumulation at a level similar to that caused by the *sopB* mutant (ZA15). The *sopBDE*2 triple mutant (ZA18) induced less fluid secretion than strains having mutations in either *sopB* (ZA15), *sopD* (ZA17), or *sopE2* (ZA9), but the difference was not statistically significant. The *sopABDE*2 quadruple mutant (ZA20) caused significantly less fluid secretion than strains carrying mutations in either *sopA* (ZA19), *sopD* (ZA17), or

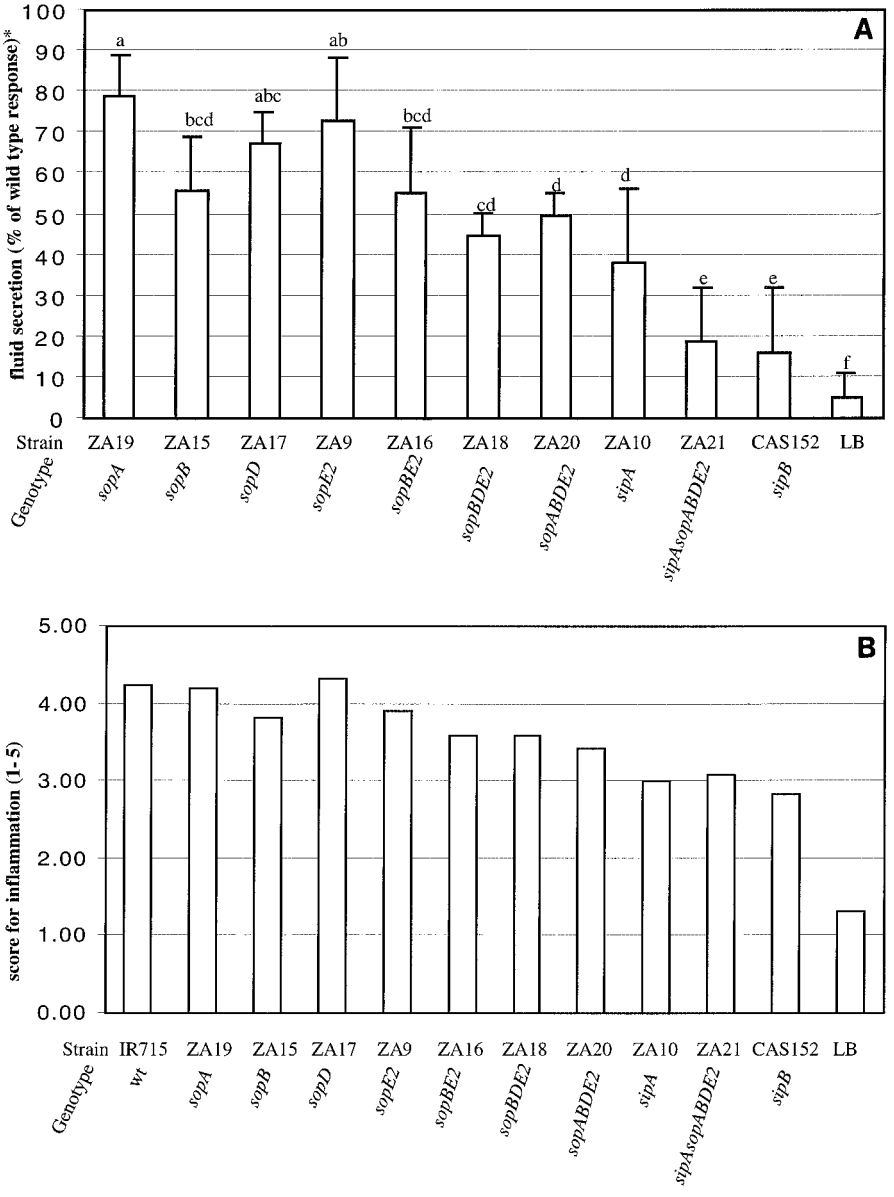

FIG. 3. Ability of *Salmonella* serotype Typhimurium mutants carrying multiple mutations in SPI1 effector genes to induce secretory and inflammatory changes in bovine ligated ileal loops at 8 h postinfection. (A) Data for fluid accumulation in loops shown as percentages of the fluid secretion elicited by the wild type (IR715). The 100% line indicates the amount of fluid accumulation elicited by the *Salmonella* serotype Typhimurium wild type. All mutants shown elicited significantly less fluid accumulation than the wild type (IR715) ($P < 0.05$). The results are means from experiments performed with three animals, in which each strain was tested in two loops/animal. The bars indicate means ± standard deviations. The same letter above two bars indicates that the amounts of fluid accumulation elicited by the mutants are not significantly different. Different letters above two bars indicate that the amounts of fluid accumulation elicited by the mutants are significantly different ($P < 0.05$). (B) Inflammatory changes in infected loops assessed by microscopic examination of hematoxylin- and eosin-stained thin sections of infected tissue. wt, wild type.

sopE2 (ZA9) ($P < 0.05$). However, the amounts of fluid secretion elicited by the *sopABDE*2 quadruple mutant (ZA20) and the *sopB* mutant (ZA15) were not statistically different. The *sopABDE*2 quadruple mutant (ZA20) elicited significantly more fluid secretion than the *sipB* mutant (CAS152). In contrast, the *sipAsopABDE*2 quintuple mutant (ZA21) induced fluid secretion at a level similar to that caused by the *sipB* mutant (CAS152). Furthermore, the *sipAsopABDE*2 quintuple mutant (ZA21) caused significantly less fluid secretion than the *sopABDE*2 quadruple mutant (ZA20), the *sopBDE*2 triple mutant (ZA18), the *sopBE*2 double mutant (ZA16), or strains carrying single mutations in *sipA* (ZA10), *sopA* (ZA19), *sopB* (ZA15), *sopD* (ZA17), or *sopE2* (ZA9). These data demonstrated that inactivation of *sipB* and simultaneous inactivation of *sipA, sopA*, *sopB*, *sopD*, and *sopE2* caused similar reductions in the ability of *Salmonella* serotype Typhimurium to cause fluid accumulation in bovine ligated ileal loops. Hence, these data supported the idea that the main function of SipB in eliciting fluid accumulation is the translocation of SipA, SopA, SopB, SopD, and SopE2 into host cells.

Although mutations in *sipA* (ZA10), *sopA* (ZA19), *sopB* (ZA15), *sopD* (ZA17), or *sopE2* (ZA9) caused a significant reduction in fluid accumulation compared to the fluid accumulation observed with the wild type (IR715), it was not clear to what degree mutations in individual effector genes contributed to the phenotype observed for the *sipAsopABDE*2 quintuple mutant (ZA21) (Fig. 2A and 3A). Inactivation of *sipA* (ZA10) or *sopB* (ZA15) resulted in a more pronounced reduction in fluid accumulation than mutations in *sopA* (ZA19), *sopD* (ZA17), or *sopE2* (ZA9). Furthermore, the amount of fluid accumulated in loops infected with the *sopB* (ZA15) mutant was not significantly different from the amount accumulated in loops infected with the *sopBE*2 double mutant (ZA16), the *sopBDE*2 triple mutant (ZA18), or the *sopABDE*2 quadruple mutant (ZA20). Only the *sipAsopABDE*2 quintuple mutant (ZA21) caused significantly less fluid secretion than the *sopB* mutant (ZA15) or the *sipA* mutant (ZA10). It was, therefore, tempting to speculate that simultaneous inactivation of *sipA* and *sopB* may reduce fluid accumulation to the level elicited by the *sipAsopABDE*2 quintuple mutant (ZA21). To test this idea, we constructed a *sipAsopB* double mutant (ZA26) and assessed its virulence in bovine ligated ileal loops (Fig. 4A). As in the previous experiment, the amount of fluid accumulated in loops infected with the *sopB* mutant (ZA15) or the *sipA* mutant (ZA10) was significantly larger than the amount accumulated in loops infected with the *sipAsopABDE*2 quintuple mutant (ZA21) ($P < 0.05$). The *sipAsopB* double mutant (ZA26) caused less fluid accumulation than the *sopB* mutant (ZA15) or the *sipA* mutant (ZA10) and more fluid accumulation than the *sipAsopABDE*2 quintuple mutant (ZA21), but the differences were not statistically significant (Fig. 4A). Thus, the *sipAsopB* double mutant (ZA26) had a phenotype intermediate between that of the *sipAsopABDE*2 quintuple mutant (ZA21) and that of the single mutants (ZA15 and ZA10).

SipA has not previously been implicated in eliciting fluid accumulation during infection of bovine ligated ileal loops with *Salmonella* serotype Typhimurium or *Salmonella* serotype Dublin. The *sipA* mutant (ZA10) used in this study carries an unmarked deletion (Fig. 1). Although unlikely, the possibility exists that this mutation has a polar effect on expression of the downstream *iacP* gene (Fig. 1). To establish that the in vivo phenotype of the *sipA* mutant (ZA10) was not caused by a polar effect, complementation experiments were performed by using pSipA, a plasmid carrying an intact *sipA* gene. Infection of bovine ileal loops with the *sipAsopABDE*2 quintuple mutant (ZA21), the *sipA* mutant (ZA10), or the *sipAsopB* double mutant (ZA26) resulted in significantly less fluid secretion than infection with the corresponding complemented strains, ZA21 (pSipA), ZA10(pSipA), and ZA26(pSipA), respectively (Fig. 4A). Collectively, these data demonstrated that *sipA* plays an important role in eliciting host secretory and inflammatory responses during *Salmonella* serotype Typhimurium infection of calves.

To evaluate the local inflammatory response to infections with *Salmonella* serotype Typhimurium mutants, hematoxylin- and eosin-stained sections of Peyer's patches were examined by light microscopy, and the inflammatory changes were scored on a scale from 1 to 5 according to the criteria described in Materials and Methods (Fig. 2B). The inflammatory lesions caused by strains ZA16 (*sopBE*2), ZA18 (*sopBDE*2), and

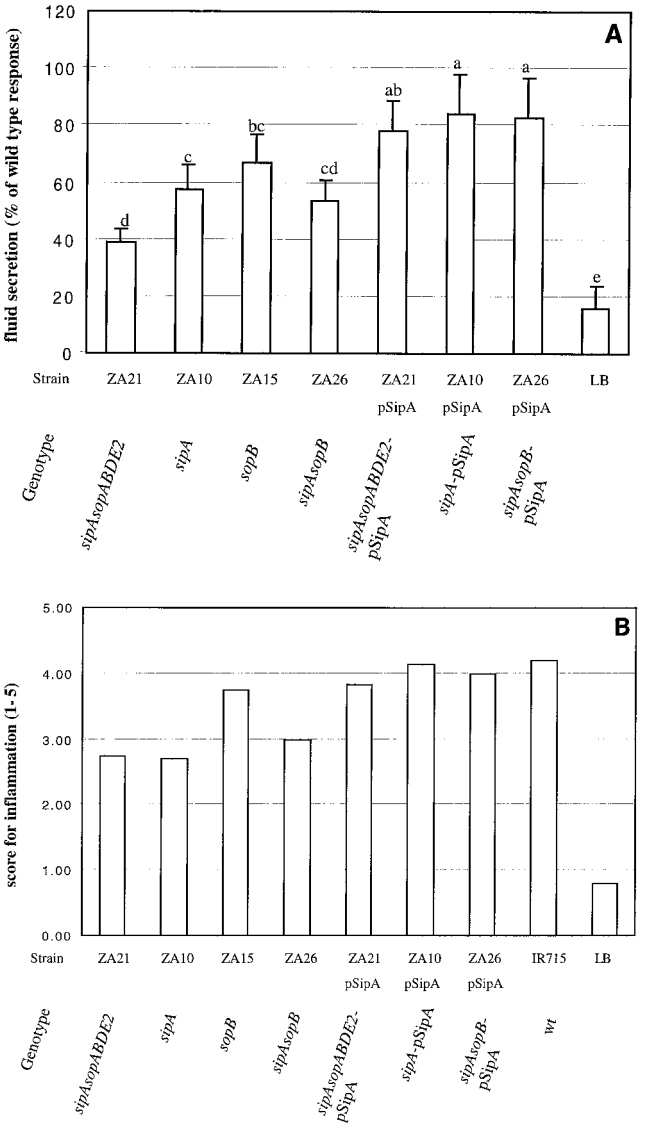

FIG. 4. In vivo complementation in bovine ligated ileal loops at 8 h postinfection of strains carrying a *sipA* deletion with the cloned *sipA* gene. (A) Data for fluid accumulation in loops shown as percentages of the fluid secretion elicited by the wild type (IR715). The results are means from three experiments in which each strain was tested in at least two loops/animal. The bars indicate means ± standard deviations. The same letter above two bars indicates that the amounts of fluid accumulation elicited by the mutants are not significantly different. Different letters above two bars indicate that the amounts of fluid accumulation elicited by the mutants are significantly different ($P < 0.05$). (B) Inflammatory changes in infected loops assessed by microscopic examination of hematoxylin- and eosin-stained thin sections of infected tissue. wt, wild type.

ZA20 (*sopABDE*2) were less severe than those caused by the *Salmonella* serotype Typhimurium wild type (IR715). Consistent with the results obtained during the initial characterization of mutants (Fig. 2B), deletion of *sipA* caused a considerable reduction in the local inflammatory response (Fig. 3B). Introduction of plasmid pSipA into strains ZA10, ZA21, and ZA26 resulted in elevated inflammatory responses in bovine ligated ileal loops (Fig. 4B). In sections of Peyer's patches infected with the *sipAsopABDE*2 quintuple mutant (ZA21) or

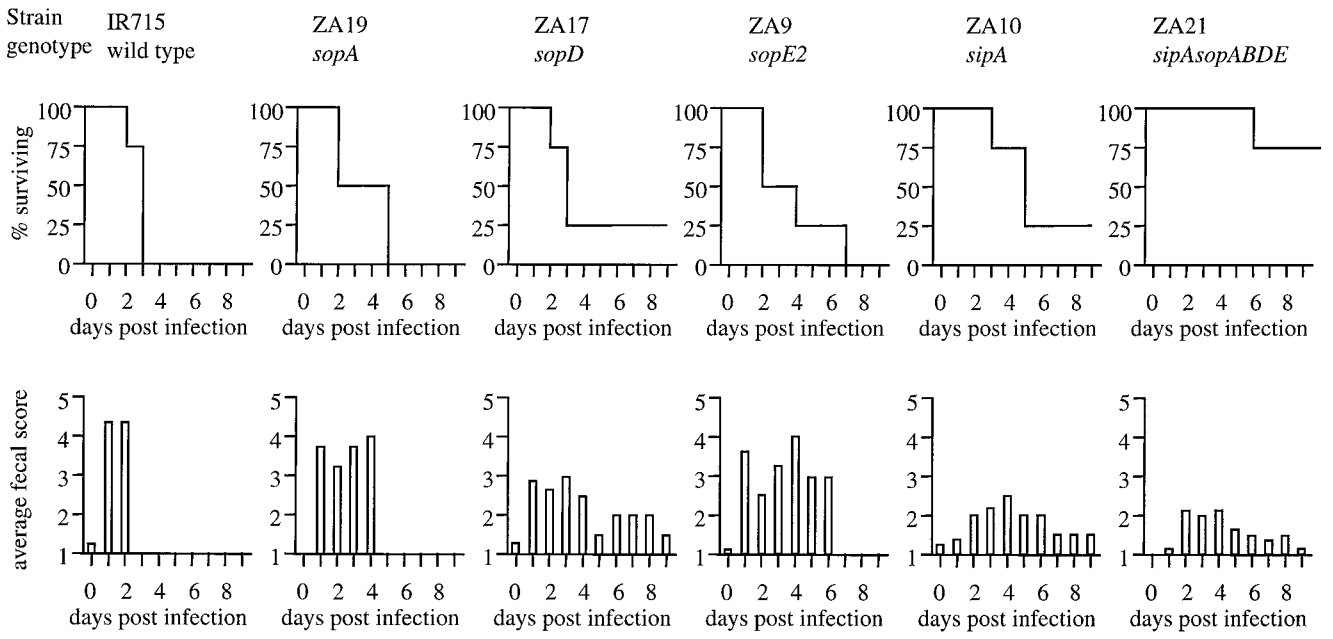

FIG. 5. Ability of SPI1 mutants to cause diarrhea and lethal morbidity in calves infected orally with a dose of 10^{10} CFU per animal. Lethal morbidity caused by the wild type or by *Salmonella* serotype Typhimurium mutants over time is shown in the upper graphs. The lower graphs show the severity of diarrhea over time, which was scored on a scale from 1 to 5, as follows: 1, normal feces; 2, soft feces with loss of distinct conformation; 3, diarrhea, loose feces with reduced solid matter; 4, diarrhea, aqueous feces with markedly reduced or little solid matter, or fibrin; 5, diarrhea, aqueous feces with no solid matter, fibrin and blood.

the *sipB* mutant (CSA152), a reduced inflammatory response with mild perivascular to coalescent infiltration of PMN in the mucosa and submucosa was observed. Focal intravascular and/or perivascular infiltration of PMN in the mucosa was also observed in some sections from ileal loops inoculated with sterile LB broth, which may have been a result of the surgical procedure. To investigate whether the reduced ability of *Salmonella* serotype Typhimurium mutants to cause fluid accumulation or inflammation in loops correlates with their ability to colonize bovine Peyer's patches, the number of bacteria recovered from infected tissue was determined. We found that mutations in *sipA* (ZA10), *sopA* (ZA19), *sopB* (ZA15), *sopD* (ZA17), and *sopE2* (ZA9) or simultaneous inactivation of *sipAsopABDE*2 (ZA21) did not significantly reduce the bacterial numbers recovered from bovine Peyer's patches at 8 h postinfection compared to the numbers observed with the wild type (IR715). In contrast, the number of *sipB* mutant (CSA152) cells recovered from Peyer's patches was approximately 100-fold less than the number of wild-type (IR715) cells (data not shown). These data suggested that SipB is required for colonization of the bovine mucosa, either directly through its interaction with host proteins (caspase 1) or through translocation of AvrA, SlrP, SspH1, SptP, or other as-yet-unidentified SPI1 secreted effector proteins into cells of the bovine host.

Role of SopB, SopE2, SopA, SopD, and SipA in causing diarrhea and lethal morbidity after oral infection of calves. We have shown previously that a *Salmonella* serotype Typhimurium *sopB* mutant causes diarrhea and intestinal lesions at wild-type levels when calves are infected with a dose of 10^{10} CFU/animal (47), whereas a *Salmonella* serotype Typhimurium *sipB* mutant (CSA152) causes no diarrhea or only mild diarrhea and no lethal morbidity at this dose (48). One possible explanation for these observations is that inactivation of only one effector gene (i.e., *sopB*) does not result in marked attenuation during oral infection, because SopB acts in concert with SopE2, SopA, SopD, and SipA to cause diarrhea in calves. This hypothesis further implies that a *sipB* mutant is markedly attenuated, because it is unable to translocate SopB, SopE2, SopA, SopD, and SipA. To determine whether SopB, SopE2, SopA, SopD, and SipA act in concert during oral infection of calves, groups of four calves were inoculated orally with strain IR715 (wild type), ZA10 (*sipA*), ZA19 (*sopA*), ZA17 (*sopD*), ZA9 (*sopE2*), or ZA21 (*sipAsopABDE*2) by using a dose of 1×10^{10} CFU/animal. Infection of calves with the wild type (IR715), the *sopE2* mutant (ZA9), or the *sopA* mutant (ZA19) induced aqueous diarrhea with feces containing various combinations of blood, fibrin, and mucus at day 1 postinfection (Fig. 5). All calves infected with the *Salmonella* serotype Typhimurium wild type (IR715) were euthanized between 24 and 72 h postinfection, because they had signs of terminal illness, such as severe anorexia and an inability to stand. Similarly, infection with the *sopE2* mutant (ZA9) or the *sopA* mutant (ZA19) caused lethal morbidity in all calves; however, the time to death was delayed compared to the time of death of animals infected with the wild type (IR715). The severity of diarrhea was reduced in calves infected with the *sopD* mutant (ZA17), while calves infected with the *sipA* mutant (ZA10) or the *sipAsopABDE*2 quintuple mutant (ZA21) had only mild transient diarrhea with a delayed onset (starting on day 2 or 3 postinfection). While the *sipA* mutant (ZA10) and the *sopD* mutant (ZA17) caused lethal morbidity in three of four calves, the *sipAsopABDE*2 quintuple mutant (ZA21) caused lethal morbidity only in one of four calves. The data obtained with ZA10, the *sipA* mutant used throughout this study, were not

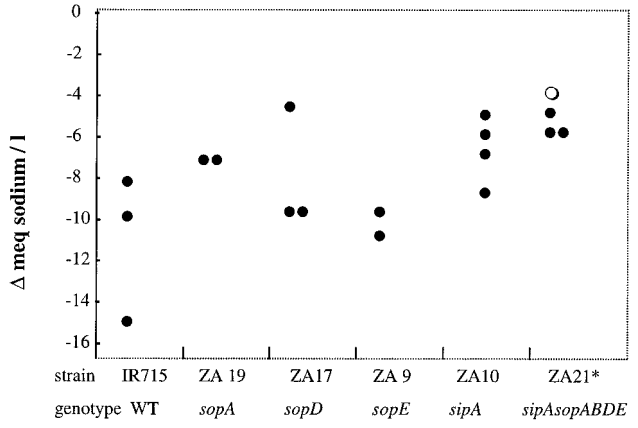

FIG. 6. Change in the plasma sodium concentration during oral infection of calves with *Salmonella* serotype Typhimurium strains. The graph shows the difference in the plasma sodium concentration between a sample collected on day 2 postinfection and a preinfection sample. Solid circles indicate that the plasma sodium concentration on day 2 postinfection was below the normal range. The open circle indicates that the plasma sodium concentration on day 2 postinfection was within the normal range. WT, wild type.

identical to the data obtained previously with a different *Salmonella* serotype Typhimurium *sipA* mutant, strain JLR137 (48). Strain JLR137 causes reduced mortality but is able to cause diarrhea in calves. Construction of JLR137, which is described in a previous report (48), was performed by transducing the Tn*5lacZY* marker from EE633, a strain in which the transposon is inserted close to or within the *sipA* gene, as indicated by Southern hybridization (19). To further investigate why the phenotype reported for JLR137 is different from that found for the *sipA* mutant (ZA10) used throughout this study, we cloned DNA flanking the Tn*5lacZY* insertion in JLR137. Sequence analysis of the transposon-flanking DNA revealed that the transposon in JLR137 was inserted into the *glpD* gene, suggesting that a transposition event had occurred during transduction of Tn*5lacZY* from EE633. Furthermore, Southern hybridization with a probe specific for the *sipA* gene confirmed that Tn*5lacZY* was not inserted into or close to the *sipA* gene in strain JLR137. These results indicated that JLR137 carries a mutation in *glpD*, but not a mutation in *sipA*. In contrast, strain ZA10 carries a *sipA* deletion, as indicated by Southern hybridization and complementation analysis (Fig. 4A).

To assess the severity of the clinical signs of disease, the plasma concentration of sodium was analyzed at zero time and on days 1 and 2 postinfection for each animal, and the rectal temperature and fecal shedding of *Salmonella* serotype Typhimurium were recorded daily. On day 2 postinfection, the sodium plasma concentrations of all infected calves except one animal infected with the *sipAsopABDE*2 quintuple mutant (ZA21) were below the normal range, 135 to 153 meq/liter (Fig. 6). The reduction in the plasma sodium levels at 2 days postinfection compared to the values recorded prior to infection was significantly greater in calves infected with the wild type (IR715) than in animals infected with the *sipAsopABDE*2 quintuple mutant (ZA21) ($P < 0.05$). The rectal temperature of all infected calves was elevated (39.3 to 40°C) on days 1 and 2 postinfection, and there were not significant differences among treatment groups (data not shown). In the calves that survived infection, the temperature gradually returned to the normal range, which was determined by taking the temperature of uninfected animals (the normal range was between 37.9 and 38.3°C). All calves shed high numbers of bacteria ($\geq 10^6$ CFU/g of feces) on day 1 postinfection. The numbers of bacteria recovered from feces of calves infected with the wild type (IR715) were significantly higher ($P < 0.05$) than the numbers of bacteria recovered from animals infected the *sipAsopABDE*2 quintuple mutant (ZA21) (Fig. 7). The difference became more pronounced on day 2 postinfection, when calves infected with the wild type (IR715) shed approximately 10-fold more bacteria than animals infected with the *sipAsopABDE*2 quintuple mutant (ZA21) ($P < 0.05$). These data suggested that the number of bacteria shed in the feces may increase with the severity of diarrhea; however, this was not investigated further.

At necropsy, a gross pathological examination was performed, and tissue samples from mesenteric lymph nodes and Peyer's patches were collected for histopathologic evaluation. Calves which had signs of lethal morbidity after infection with strains IR715, ZA19 (*sopA*), ZA9 (*sopE*2), and ZA17 (*sopD*) and one of the calves infected with strain ZA10 (*sipA*) developed severe acute fibrinopurulent necrotizing enteritis with purulent exudate and segmental or continuous pseudomembrane deposition in the terminal ileum (Fig. 8). Two calves that developed lethal morbidity after infection with the *sipA* mutant (ZA10) had reduced severity of intestinal lesions (marked subacute fibrinopurulent necrotizing enteritis confined to the Peyer's patches). No gross pathological lesions were detected in calves infected with ZA21 (*sipAsopABDE*2) and calves that survived infection with ZA10 (*sipA*) or ZA17 (*sopD*).

Microscopic examination also revealed severe necrotizing enteritis in calves infected with IR715, ZA9 (*sopE*2), and ZA19 (*sopA*), as well as the three calves that developed lethal signs of disease after infection with ZA17 (*sopD*). The histopathologic lesions in the terminal ileum of these calves included extensive necrosis of the ileal mucosa, neutrophilic infiltration in mucosa and submucosa, accumulation of fibrin and necrotic debris at the lumenal surface, and marked depletion of lymphoid cells from the lymphoid follicles of Peyer's patches. Strain ZA10 (*sipA*) caused intestinal lesions of reduced severity, such as necrosis of the upper mucosa surrounded by a dense neutrophilic infiltrate and moderate lymphoid depletion in lymphoid follicles. In addition, lymphoid depletion was observed in the germinal centers of mesenteric lymph nodes of calves infected with IR715 (wild type) or the single-knockout mutants. The intestinal lesions were either absent or negligible in the terminal ileum of the four calves infected with ZA21 (*sipAsopABDE*2), despite the fact that one calf developed lethal morbidity. Similarly, intestinal lesions were either absent or negligible in the terminal ileum of calves that survived infection with strain ZA10 (*sipA*) or ZA17 (*sopD*).

DISCUSSION

In this study we determined which effector proteins secreted by the invasion-associated type III secretion system are required by *Salmonella* serotype Typhimurium to elicit fluid accumulation in the bovine ligated ileal loop model. In addition

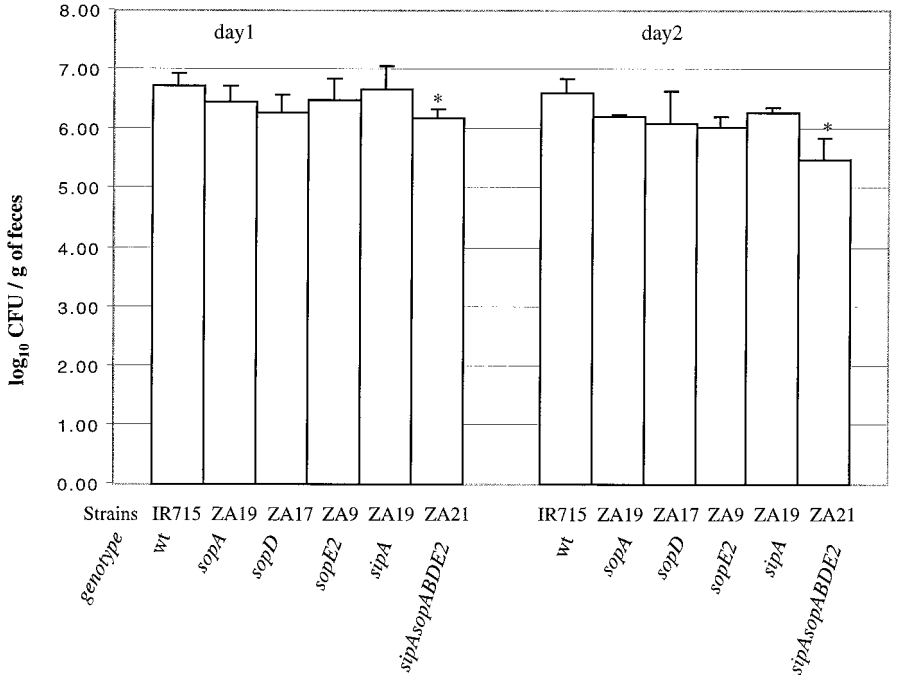

FIG. 7. Recovery of *Salmonella* serotype Typhimurium strains from the feces of calves after oral infection with a dose of 10^{10} CFU per animal. The bars indicate the mean ± standard deviation for four calves. An asterisks indicates that the difference between the wild type (IR715) and an individual mutant was significant. wt, wild type.

to the SPI1 translocation complex formed by SipB, SipC, and SipD, the bovine *Salmonella* serotype Typhimurium isolate ATCC 14028 encodes nine effector proteins, including SlrP, SspH1, SptP, AvrA, SipA, SopA, SopB, SopD, and SopE2. Here we show that *Salmonella* serotype Typhimurium strains carrying mutations in *sipA*, *sopA*, *sopB*, *sopD*, or *sopE2* exhibited reduced enteropathogenicity but elicited more fluid accumulation than a *Salmonella* serotype Typhimurium *sipB* mutant (Fig. 3A). In contrast, mutations in *slrP*, *avrA*, *sspH1*, or *sptP* did not reduce the ability of *Salmonella* serotype Typhimurium to elicit fluid secretion in bovine ligated loops (Fig. 2A). Furthermore, a *Salmonella* serotype Typhimurium *sipAsopABDE2* mutant elicited fluid accumulation at levels similar to those elicited by a *Salmonella* serotype Typhimurium *sipB* mutant (Fig. 3A). These data suggested that SopA, SopB, SopD, SopE2, and SipA are the major *Salmonella* serotype Typhimurium virulence factors involved in eliciting fluid accumulation in bovine ligated ileal loops. Furthermore, these data support the idea that the main function of SipB in eliciting fluid accumulation is the translocation of SipA, SopA, SopB, SopD, and SopE2 into host cells.

The mechanism by which SopA, SopB, SopD, SopE2, and SipA contribute to fluid accumulation may be related to the ability of these proteins to stimulate an inflammatory response. For instance, SipA elicits the production of chemoattractants that promote transepithelial PMN migration in a tissue culture model (28). A nonpolar deletion of *sipA* drastically reduced the ability of *Salmonella* serotype Typhimurium to elicit fluid accumulation and an inflammatory response in bovine ligated ileal loops (Fig. 2 and 3), and this defect could be partially complemented in vivo by introducing the cloned gene on a plasmid (Fig. 4A). A role for *sopA* and *sopD* in eliciting inflammation is suggested by the finding that mutations in these genes reduce the ability of *Salmonella* serotype Dublin to cause PMN influx into ligated ileal loops (21, 54). SopB and SopE2 interfere with intracellular signaling pathways (30, 42, 57) by acting as inositol phosphate phosphatase (31) and a guanine nucleotide exchange factor for Cdc42 (43), respectively. Nuclear responses triggered by SopB and SopE2 may result in the expression of proinflammatory cytokines that attract PMN (6). Thus, all effector proteins implicated in eliciting fluid accumulation in bovine ligated ileal loops (i.e., SopA, SopB, SopD, SopE2, and SipA) have also been implicated in eliciting the production of proinflammatory mediators in cell culture models in vitro. The PMN infiltrate elicited by *Salmonella* serotype Typhimurium may cause fluid secretion by an exudative mechanism since it results in increased vascular permeability and injury to the permeability barrier formed by the intestinal epithelium. Epithelial damage is first observed at 3 h postinfection and coincides with the appearance of fluid accumulation in bovine ligated ileal loops (35).

SPI1 was originally discovered because mutations in this region reduce the ability of *Salmonella* serotype Typhimurium to invade epithelial cells in vitro (12). The translocation of several effector proteins into host cells has been associated with this invasion phenotype. The main mechanism for SPI1-mediated invasion of tissue culture cells is the induction of actin cytoskeleton rearrangements triggered by SopB, SopE1, and SopE2 (30, 57). It is necessary to inactivate the genes encoding these three effector proteins to reduce the invasiveness of *Salmonella* serotype Typhimurium SL1344 to the level of a mutant with a defective type III secretion system (i.e., an

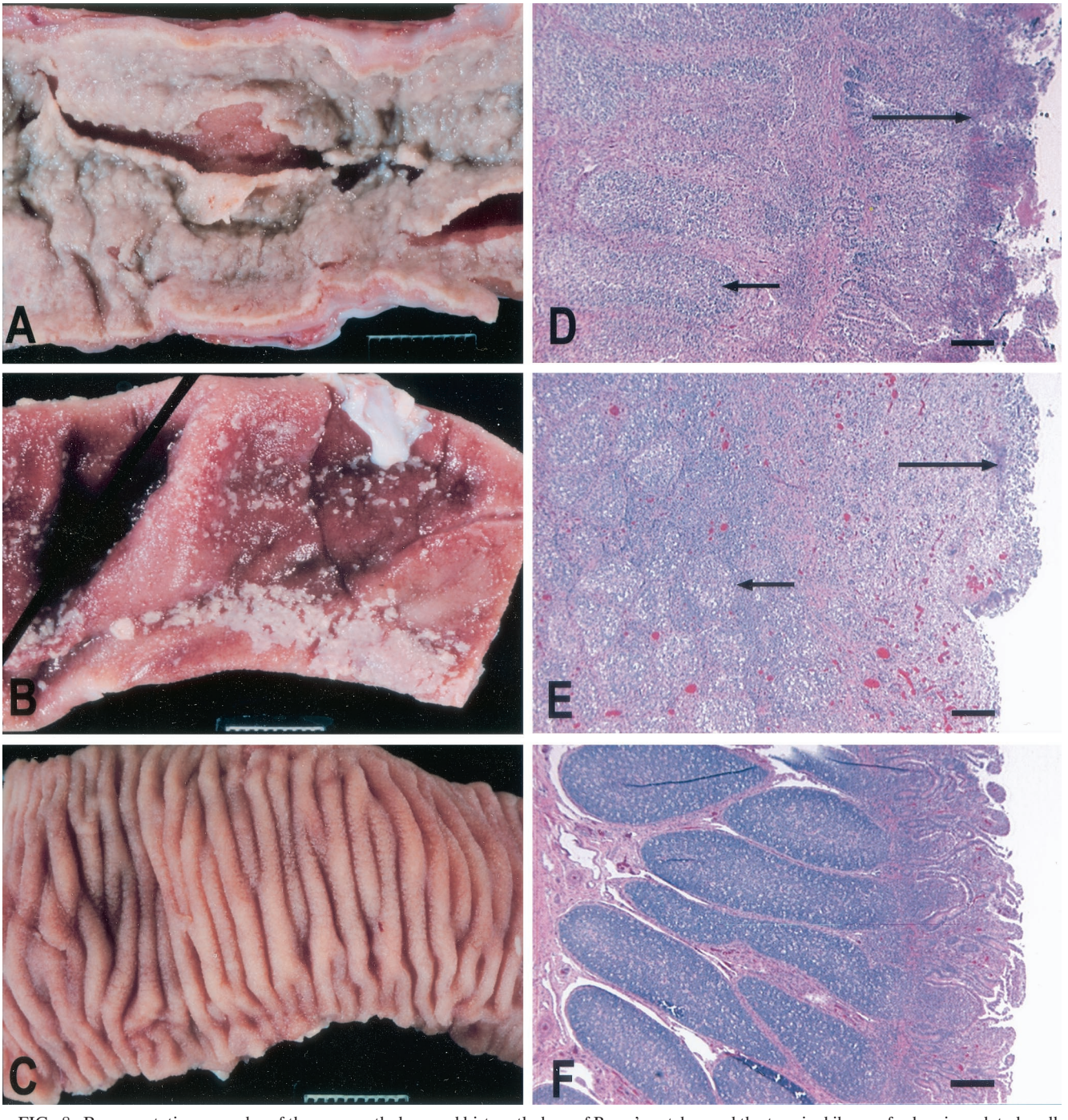

FIG. 8. Representative examples of the gross pathology and histopathology of Peyer's patches and the terminal ileum of calves inoculated orally with 10^{10} CFU of different *Salmonella* serotype Typhimurium strains. (A) Severe acute fibrinopurulent necrotizing enteritis with segmental or continuous pseudomembrane formation in a calf infected with wild-type strain IR715 (similar pathological changes were observed in calves infected with the *sopA* mutant, the *sopD* mutant, or the *sopE2* mutant). Bar = 1 cm. (B) Marked subacute fibrinopurulent necrotizing enteritis often confined to the Peyer's patches of the terminal ileum of a calf infected with strain ZA10 (*sipA*). Bar = 1 cm. (C) Normal Peyer's patches and ileum of a calf infected with strain ZA21 (*sipAsopABDE*). Bar = 1 cm. (D to F) Hematoxylin- and eosin-stained sections of Peyer's patches of calves infected with IR715, ZA10 (*sipA*), and ZA21 (*sipAsopABDE*), respectively. The short arrows indicate areas of lymphoid depletion; the long arrows indicate various degrees of fibrinopurulent necrotizing ileitis at the mucosal surface. Bars = 200 μm.

invA or *invG* mutant) (30, 57). In addition, SipA may play a minor role in bacterial uptake in vitro since entry is delayed at early time points in a *Salmonella* serotype Typhimurium *sipA* mutant (58). Since *Salmonella* serotype Typhimurium strain ATCC 14028, a strain used in this study, does not carry the *sopE1* gene, we anticipated that inactivation of *sopB*, *sopE2*, and *sipA* would reduce its invasiveness to the level of a *sipB* mutant. However, at 8 h after infection of bovine ligated ileal

loops, the numbers of cells of both the wild type and the *sipAsopABDE2* mutant recovered from Peyer's patch tissue were approximately 100-fold higher than the numbers of cells of a *Salmonella* serotype Typhimurium *sipB* mutant recovered (data not shown). Since this study was aimed at investigating fluid accumulation, a phenotype that develops several hours after inoculation, our experimental design was not optimal for studying bacterial invasion, which starts within minutes after infection (9). Hence, the fact that higher numbers of cells of the *sipAsopABDE2* mutant than of a *sipB* mutant were recovered from Peyer's patches may be unrelated to the ability of the former strain to invade epithelial cells but rather may reflect its ability to survive or multiply within bovine tissue.

This study revealed several differences between *Salmonella* serotype Dublin and *Salmonella* serotype Typhimurium with regard to phenotypes caused by inactivation of genes encoding effector proteins. For instance, the fluid secretion elicited by a *Salmonella* serotype Typhimurium *sopB* mutant was not significantly different from that elicited by a *Salmonella* serotype Typhimurium *sopB sopD sopE2* mutant (Fig. 2A). In contrast, a *Salmonella* serotype Dublin *sopB sopD* mutant elicits less fluid accumulation in bovine ligated ileal loops than a *Salmonella* serotype Dublin *sopB* mutant or a *Salmonella* serotype Dublin *sopD* mutant (21). Furthermore, mutations in *sopA* and *sipB* cause similar reductions in the ability of *Salmonella* serotype Dublin to elicit fluid accumulation in bovine ligated ileal loops (54). In *Salmonella* serotype Typhimurium, on the other hand, a mutation in *sopA* caused a comparatively small reduction in fluid secretion, and a *Salmonella* serotype Typhimurium *sipB* mutant caused significantly less fluid accumulation than a *sopA* mutant (Fig. 3A). It may not be surprising that not all data obtained with *Salmonella* serotype Dublin can be extrapolated to *Salmonella* serotype Typhimurium since these organisms cause divergent disease manifestations in cattle. Although *Salmonella* serotype Typhimurium and *Salmonella* serotype Dublin infections in young calves commonly result in diarrhea, *Salmonella* serotype Dublin infections are more invasive, and meningoencephalitis, polyarthritis, osteomyelitis, or pneumonia may eventually occur in the absence of diarrhea (32). Unlike *Salmonella* serotype Typhimurium, *Salmonella* serotype Dublin may cause abortion in pregnant cows and heifers with no other clinical signs of infection (15, 32). Furthermore, *Salmonella* serotype Dublin and *Salmonella* serotype Typhimurium are associated with distinct human disease syndromes (bacteremia and enterocolitis, respectively) (8, 27, 41); that is, diarrhea is the prominent sign of disease caused by *Salmonella* serotype Typhimurium, and only 1% of human isolates are from blood (46). In contrast, only about one-third of *Salmonella* serotype Dublin human patients develop diarrhea, while bacteria are cultured from blood in 75 to 91% of the cases (8, 45).

The ligated ileal loop model is a valuable tool for characterizing virulence factors involved in enteropathogenesis of *Salmonella* serotypes (50). However, the loop model restricts investigation of *Salmonella* serotype Typhimurium host interactions to the ileum, while severe pathological changes during an oral infection are observed in both the ileum and the colon (47). Furthermore, the loop model is suitable only for studying host-pathogen interactions at early times (<12 h) postinfection. We therefore characterized *Salmonella* serotype Typhimurium mutants further by performing oral infection experiments with calves. Compared to the wild type, the *sipA* mutant (ZA10) caused greatly reduced diarrhea. These results appeared to contradict our previous finding that the *sipA* mutation in strain JLR137 reduced mortality but not the severity of diarrhea during oral infection of calves (48). Analysis of the strain used in the previous study (JLR137) revealed that it carried a mutation in *glpD* but not a mutation in *sipA*. In contrast, the *sipA* mutant used throughout this study (ZA10) carries a deletion in *sipA* (Fig. 1), as demonstrated by Southern hybridization and complementation analysis (Fig. 4A). The data show that *sipA* encodes an effector protein required for diarrhea. A qualitative assessment of the discharge from infected animals suggested that a mutation in *sipA* caused a more pronounced reduction in the severity of diarrhea than mutations in *sopA*, *sopD*, and *sopE2* (Fig. 5). However, the difference was not statistically significant when electrolyte loss was assessed by determining the decrease in blood sodium concentration associated with *Salmonella* serotype Typhimurium infection (Fig. 6). The *sipAsopABDE2* mutant caused less mortality (Fig. 5) and was shed in lower numbers with the feces (Fig. 7) than the wild type or strains carrying mutations in *sipA*, *sopA*, *sopD*, or *sopE2.* Furthermore, we have shown previously that a mutation in *sopB* does not reduce mortality or the severity of diarrhea during oral infection of calves (47). These findings further support the concept that the secreted effector proteins act in concert during the pathogenesis of *Salmonella* serotype Typhimurium-induced enterocolitis in calves.

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