

## SiiE Is Secreted by the *Salmonella enterica* Serovar Typhimurium Pathogenicity Island 4-Encoded Secretion System and Contributes to Intestinal Colonization in Cattle<sup>∇</sup>

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**Here we report that *Salmonella enterica* serovar Typhimurium pathogenicity island 4 carries a type I secretion system (*siiCDF*) which secretes an ~600-kDa protein (encoded by *siiE*). SiiE is surface expressed, and its production is regulated by HilA. SiiE and SiiF influence colonization in cattle and the invasion of bovine enterocytes.**

Serovars of *Salmonella enterica* infect a broad range of animal species, resulting in a spectrum of outcomes ranging from asymptomatic carriage to severe systemic disease. For instance, oral infection of chickens with serovar Typhimurium results in asymptomatic colonization of the gastrointestinal tract, while oral dosing of calves results in fever and diarrhea with high mortality in the absence of antibiotic treatment. Salmonellosis in livestock results in both animal suffering and economic losses, and the bacteria can contaminate the food chain and environment from these animal reservoirs.

The clinical outcomes associated with infection with *S. enterica* depend on both the infecting serovar and the host species. It is, however, becoming increasingly apparent that serovar Typhimurium uses different repertoires of genes to infect different hosts and that the expression of various virulence determinants may contribute both to host specificity and to the clinical outcome of infection.

Many of the virulence determinants used by serovar Typhimurium to cause infections in cattle are well characterized, and most of these are carried on horizontally acquired regions of DNA called *Salmonella* pathogenicity islands. Genes carried on *Salmonella* pathogenicity island 1 (SPI-1) have been shown to be essential in calves for the invasion of the intestinal barrier and the induction of enteritis (22, 26, 28). SPI-1 encodes a type III secretion system (T3SS-1) and secreted translocator proteins which together mediate the delivery of effector proteins into enterocytes. A subset of the effector proteins rearranges the actin cytoskeleton, resulting in membrane ruffling and, consequently, the internalization of *Salmonella* into epithelial cells. Another subset induces the enteropathogenic response to infection. Many of the effector proteins are encoded on loci outside SPI-1, including other pathogenicity islands and bacteriophages (23, 32).

Genes on SPI-2 influence both enteric and systemic virulence in calves following the initial invasion into the epithelial cells of the intestine (2, 22). SPI-2 encodes a second type III secretion system (T3SS-2) that secretes effector proteins across the membrane of the *Salmonella*-containing vacuole and enables the persistence of *Salmonella* inside host cells by modulating vesicular trafficking (reviewed in reference 25).

We recently screened a bank of 1,045 signature-tagged mutagenesis (STM) mutants of serovar Typhimurium in order to identify genes required for intestinal colonization in cattle, chickens (17), and pigs (S. C. Carnell, A. J. Bowen, E. Morgan, D. J. Maskell, T. S. Wallis, and M. P. Stevens, submitted for publication). Of the mutants attenuated in intestinal colonization in these host species, 98 had transposon insertions in genes required for colonization in cattle. As expected, a significant proportion of these genes were on SPI-1 and SPI-2, but we also isolated 11 mutants with transposon insertions within SPI-4 which were attenuated only in the colonization of the intestines of calves. SPI-4 was initially identified using a hybridization-based approach to search for large segments of DNA which were present in serovar Typhimurium but not in *Escherichia coli* K-12 and could therefore potentially constitute pathogenicity islands (30). A 27-kb *Salmonella*-specific region of DNA was sequenced and predicted to carry 18 open reading frames (*spi4\_A* to *spi4\_R*). A previously identified locus required for survival in murine macrophages was located at *spi4\_L* and was the basis for the definition of this segment of DNA as a pathogenicity island (30). The publication of the complete genome sequence of serovar Typhimurium strain LT2 refined the identified sequence of SPI-4 into six open reading frames numbered STM4257 to STM4262 (15). We have since confirmed that the sequence is almost identical in the strain used in our studies (ST4/74 Nal<sup>r</sup>) and have renamed the genes *siiA* to *siiF* (17). We have also predicted through the sequence analysis of *siiA* to *siiF* that *siiE* encodes a 595-kDa secreted protein and that *siiC*, *siiD*, and *siiF* encode components of a type I secretion system (17). The remaining genes, *siiA* and *siiB*, do not have homologs in the protein or DNA databases, and their functions may be unrelated to those of the remainder of the island as transposon insertion mutations producing strains deficient in the colonization of calves were iden-

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tified only in *siiD*, *siiE*, and *siiF* (17). However, upstream of *siiA* we identified a highly conserved operon polarity suppressor (*ops*) motif which is required for transcription elongation under the control of the RfaH protein and is associated with several other virulence gene clusters in the *Enterobacteriaceae* (17). Although this finding may indicate that the *siiA* to *siiF* genes are transcribed as a single unit, a recent study suggested that only the *siiABCD* genes are regulated by RfaH (18).

The aim of this study was to characterize the genes carried on SPI-4 and to explore their role in colonization in cattle. We chose to approach this issue through the *in vitro* and *in vivo* characterization of mutants of serovar Typhimurium strain ST4/74 Nal<sup>r</sup>, which is a virulent bovine isolate (17). Defined nonpolar deletions of *siiE* and *siiF* were created since the mutant strains were to be characterized *in vivo* and *in vitro*, and strains with nonpolar transposon insertion mutations in *siiC* and *siiD* were isolated from the signature-tagged mutagenesis transposon mutant bank (17) for characterization *in vitro*.

A mutant with a miniTn5Km2 transposon insertion in *siiD* was described previously (17). An *siiC::miniTn5Km2* mutant was located within the STM mutant bank (17) by hierarchical screening of pools of mutants by PCR and was a kind gift from Gillian Pullinger, Institute for Animal Health, Compton, United Kingdom. The transposon insertion site for the *siiC::miniTn5Km2* mutant was verified through sequencing of the specific PCR product by using a *siiC*-specific primer (5'-CCTGACCATGAACCACTG-3') and a transposon-specific primer (5'-CCTAGGCGCCAGATCTG-3').

Mutant strains carrying deletions of *siiE* and *siiF* were created by overlapping PCR followed by allelic exchange with the positive-selection suicide vector pDM4 (16). Sequences flanking the serovar Typhimurium *siiE* gene were separately amplified by PCR from ST4/74 Nal<sup>r</sup> genomic DNA with *PfuTurbo* DNA polymerase (Stratagene, La Jolla, California) using the primer pairs *siiE1* (5'-TATATAGAGCTCGGATATCTATT CACCGGTTGACG-3') and *siiE2* (5'-TTCTTGATTATCCT TTTGTATGCTTTTATTTCCC-3') and *siiE3* (5'-AGCATAACAAAAGGATAATCAAGAAGAACACGC-3') and *siiE4* (5'-TATATAGAGCTCAATCTTGTTTCATCGATAAAGAG-3'). The primary PCR products were gel purified and combined in a mixture for overlapping PCR (10) by using the flanking primers *siiE1* and *siiE4*. The secondary PCR product was then cloned into pDM4 by using *SstI* sites incorporated into the primers. The resulting plasmid, pDM4Δ*siiE*, was maintained in *E. coli* PIR1 cells (Invitrogen Life Technologies, Paisley, United Kingdom) and introduced into the ST4/74 Nal<sup>r</sup> strain by conjugation from *E. coli* S17-1λ*pir* (21). Merodiploids were isolated on Luria-Bertani (LB) agar containing nalidixic acid (Nal; 20 μg ml<sup>-1</sup>) and chloramphenicol (25 μg ml<sup>-1</sup>). Double recombinants were selected by growing merodiploids to late logarithmic phase in LB broth lacking chloramphenicol and plating onto LB agar (without NaCl) containing 6% (wt/vol) sucrose at 30°C. Sucrose-resistant colonies were screened for deletions by colony PCR, and recombinants were verified by sequencing using flanking primers *siiE5* (5'-CTTCAGGAAC TTGAGGTTGTTAAC-3') and *siiE6* (5'-CTAAATACTGGG ATAGTTAACG-3'). The deletion resulted in the juxtaposition of the first and last eight predicted codons of *siiE*, theoretically permitting the translational coupling of the mutated *siiE* gene and the downstream *siiF* gene.

A mutant with a deletion of the *siiF* gene was constructed in the same way except that sequences flanking *siiF* were amplified by PCR using the primer pairs *siiF1* (5'-TATATAGAGCTCGCGTGGTGAAGGTGACAGC-3') and *siiF2* (5'-TTTATCCGGAGAATAAGGTTCTAGTTTTTTATC C-3') and *siiF3* (5'-CTAGAACCCTTATTCTCCGGATAAA TTATTAATG-3') and *siiF4* (5'-TATATAGAGCTCGCAA CTGCTGAGCGCCG-3'). Recombinants were verified by sequencing using flanking primers *siiF5* (5'-CTACGCATC TTCGTACAGAGC-3') and *siiF6* (5'-CATAACCACTGGT GTCG-3'). The deletion resulted in the juxtaposition of the first and last eight predicted codons of *siiF*.

As we had predicted that *siiE* encoded a 595-kDa secreted protein, the secreted proteomes of the ST4/74 Nal<sup>r</sup>, *siiC::miniTn5Km2* mutant, *siiD::miniTn5Km2* mutant, Δ*siiE*, and Δ*siiF* strains were compared. As we had demonstrated by reverse transcription-PCR that *siiE* transcripts could be detected in the ST4/74 Nal<sup>r</sup> strain during aerated growth in LB broth from early to late log phase (data not shown), secreted proteins were precipitated by using trichloroacetic acid (26) from culture supernatants of bacteria grown overnight at 25°C in LB broth and then diluted 1:10 into 40 ml of fresh medium and grown with shaking for 4 h to an optical density at 600 nm of ~1.0. The proteins were resuspended in a total volume of 40 μl, and 2 μl was separated on NuPAGE 3 to 8% Tris-acetate gels (Invitrogen, Paisley, United Kingdom) and stained with Coomassie brilliant blue. A large protein, which was present above the 500-kDa protein standard, was visible in the secreted proteome of the ST4/74 Nal<sup>r</sup> wild-type strain but absent from the secreted proteomes of the *siiC::miniTn5Km2*, *siiD::miniTn5Km2*, Δ*siiE*, and Δ*siiF* mutant strains (Fig. 1A). In order to confirm that the >500-kDa protein was SiiE, we cloned, expressed, and purified the C-terminal region of SiiE and used this protein fragment to raise monoclonal antibodies against SiiE.

A DNA fragment encoding residues 5065 to 5559 of the predicted SiiE protein (SiiE<sub>5065-5559</sub>) was amplified by PCR from ST4/74 Nal<sup>r</sup> genomic DNA by using KOD Hot Start DNA polymerase (Novagen; Merck Biosciences Ltd., Nottingham, United Kingdom) with the primer pair *siiE-LIC1* (5'-GACGACGACAAGATGAATCGTTGGGAAGATGT G-3') and *siiE-LIC2* (5'-GAGGAGAAGCCCGGTTTATG CGTGTCTTCTTG-3'). The resulting DNA fragment was inserted by ligation-independent cloning into pET-30 Ek/LIC (Novagen; Merck Biosciences Ltd., Nottingham, United Kingdom) with N-terminal His<sub>6</sub> and S tags to produce pET-SiiE. For purification of the His<sub>6</sub>-SiiE<sub>5065-5559</sub> protein, *E. coli* BL21 Star (DE3) cells (Invitrogen Life Technologies, Paisley, United Kingdom) transformed with the pET-SiiE plasmid were grown at 37°C in LB broth supplemented with kanamycin (30 μg ml<sup>-1</sup>) and 1% (wt/vol) glucose. At an optical density at 600 nm of 0.6 to 0.8, the transcription of the SiiE<sub>5065-5559</sub>-encoding fragment was induced for 3 h with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were harvested by centrifugation (8,000 × *g* for 10 min at 4°C) and resuspended in 20 ml of phosphate buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) with 60 mM imidazole and lysozyme (200 μg ml<sup>-1</sup>). Following lysis by ultrasonic disruption, cell debris was removed by centrifugation as described above and the supernatant was applied to a 1-ml HisTrap HP affinity chromatography column (GE Healthcare UK Ltd., Chalfont St.

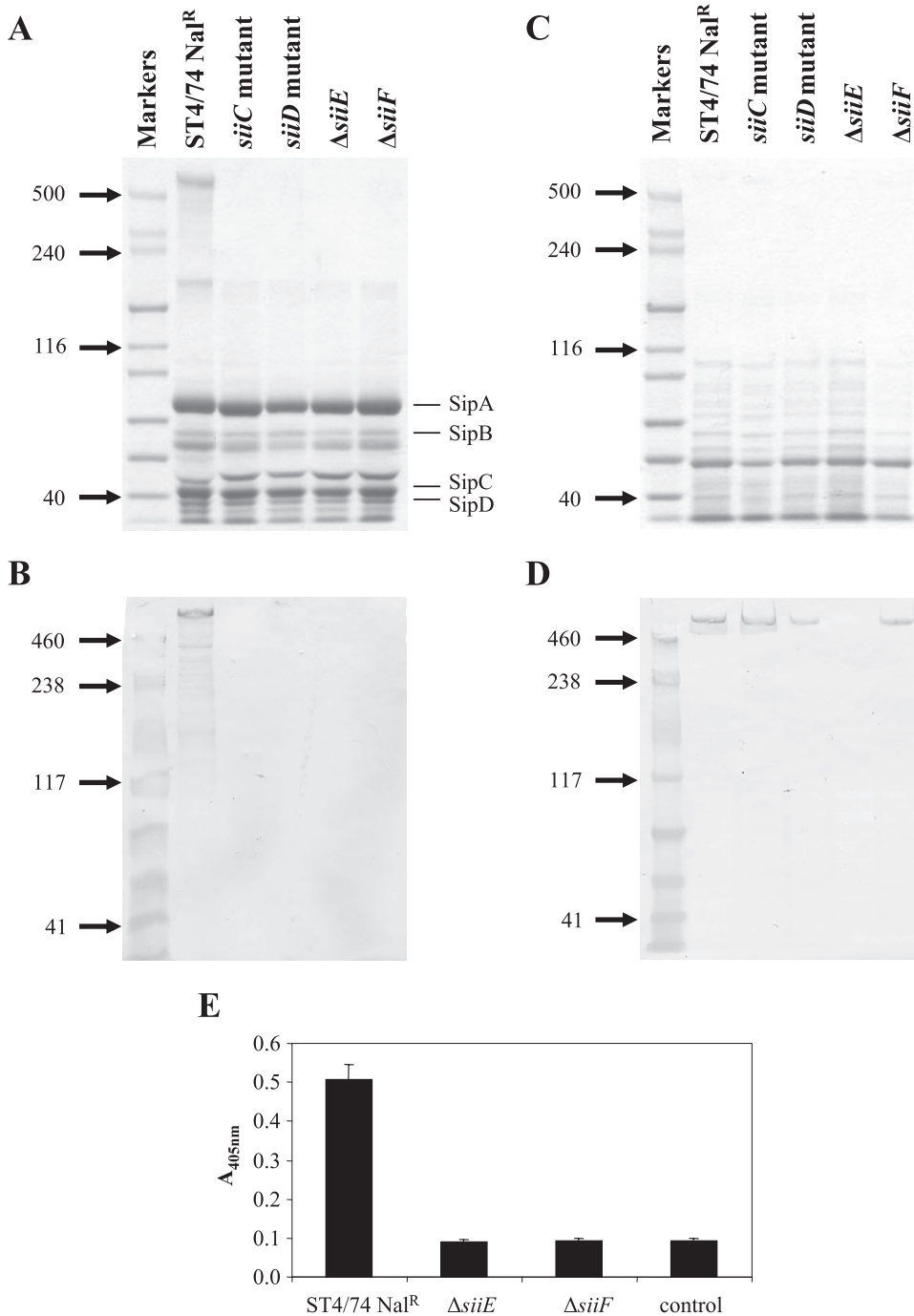


FIG. 1. SiiE is secreted in a *siiC*-, *siiD*-, and *siiF*-dependent manner and is surface associated. (A) Proteins isolated from culture supernatants were separated by SDS-PAGE and stained with Coomassie blue. Molecular masses (kilodaltons) of standard proteins are shown on the left. The locations of T3SS-1-secreted proteins (SipA to SipD) are indicated on the right. (B) Proteins isolated from culture supernatants were transferred onto membrane by Western blotting and probed with the anti-SiiE monoclonal antibody. Molecular masses (kilodaltons) of standard proteins are shown on the left. Lanes are as shown for panel A. (C) Total cellular proteins were separated by SDS-PAGE and stained with Coomassie blue. Molecular masses (kilodaltons) of standard proteins are shown on the left. (D) Total cellular proteins were transferred onto membrane by Western blotting and probed with the anti-SiiE monoclonal antibody. Molecular masses (kilodaltons) of standard proteins are shown on the left. Lanes are as shown for panel C. (E) Fixed bacteria were probed by ELISA with the anti-SiiE monoclonal antibody. The control wells were coated with ST4/74 Nal<sup>R</sup> bacteria but incubated without the monoclonal antibody.

Giles, United Kingdom). Proteins were eluted in phosphate buffer with 300 mM imidazole. The purification was monitored by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blotting using a monoclo-

nal antibody against the His<sub>6</sub> tag (Novagen; Merck Biosciences Ltd., Nottingham, United Kingdom). The protein was purified further by electroelution of the protein from zinc-stained SDS-PAGE gels and was dialyzed overnight against water by using

Slide-A-Lyzer dialysis cassettes (Perbio Science UK Ltd., Cramlington, Northumberland, United Kingdom). For production of a monoclonal antibody against SiiE, a six-week-old female BALB/c mouse was immunized twice at a 14-day interval with 20  $\mu\text{g}$  of the purified His<sub>6</sub>-SiiE<sub>5065-5559</sub> protein in Titermax Gold adjuvant (Sigma-Aldrich Company Ltd, Gillingham, United Kingdom) via the subcutaneous route. Forty-five days after the second immunization, a further 50  $\mu\text{g}$  of purified His<sub>6</sub>-SiiE<sub>5065-5559</sub> was given without adjuvant via the intraperitoneal route. Four days after the final booster dose, the spleen was removed and splenocytes prepared and fused with SP2/0 mouse myeloma cells by using polyethylene glycol 1500 according to standard procedures. The fused cells were diluted in RPMI-hypoxanthine-aminopterin-thymidine medium with 10% (vol/vol) fetal calf serum and plated onto 96-well plates seeded 48 h previously with mouse peritoneal macrophages. The plates were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 10 days. The hybridomas were screened for the production of SiiE-specific monoclonal antibodies by enzyme-linked immunosorbent assay (ELISA) and immunoblotting against purified His<sub>6</sub>-SiiE<sub>5065-5559</sub>. Clones secreting antibody reactive with SiiE<sub>5065-5559</sub> were expanded, and SiiE-specific antibody was purified from the culture supernatant by using a protein G-Sepharose column (GE Healthcare UK Ltd., Chalfont St. Giles, United Kingdom) according to standard techniques.

In order to confirm that the >500-kDa protein present in the secreted proteome of ST4/74 Nal<sup>r</sup> was SiiE, 0.2  $\mu\text{l}$  of the secreted protein preparations from ST4/74 Nal<sup>r</sup>, *siiC*::miniTn5Km2 mutant, *siiD*::miniTn5Km2 mutant,  $\Delta$ *siiE*, and  $\Delta$ *siiF* strains was transferred to Hybond ECL membrane by standard Western blotting techniques and SiiE was detected using the anti-SiiE monoclonal antibody at 1  $\mu\text{g ml}^{-1}$ . A band corresponding to the >500-kDa protein was visible on the blot in the lane carrying proteins from the ST4/74 Nal<sup>r</sup> strain but not visible in the lanes carrying proteins derived from the *siiC*::miniTn5Km2, *siiD*::miniTn5Km2,  $\Delta$ *siiE*, and  $\Delta$ *siiF* mutant strains (Fig. 1B), thus confirming that SiiE is secreted into the culture supernatant. As the SiiE protein was absent from the secreted proteins isolated from the *siiC*::miniTn5Km2, *siiD*::miniTn5Km2, and  $\Delta$ *siiF* mutant strains, we investigated whether SiiE was produced, but not secreted, by the *siiC*::miniTn5Km2, *siiD*::miniTn5Km2, and  $\Delta$ *siiF* mutant strains by isolation and comparison of the total cellular proteins of the ST4/74 Nal<sup>r</sup>, *siiC* mutant, *siiD* mutant,  $\Delta$ *siiE*, and  $\Delta$ *siiF* strains. The bacteria were grown in LB broth as described above and harvested by centrifugation (8,000  $\times g$  at 4°C for 10 min), and total cellular proteins were prepared from the bacterial cells by using the ReadyPreps protein preparation kit (Epicenter, Madison, WI).

The proteins were separated by electrophoresis as described above (Fig. 1C), and the presence of SiiE in the protein preparations was investigated by Western blotting of the proteins and probing with the anti-SiiE monoclonal antibody. The SiiE protein was present in equal amounts in the preparations of total cellular proteins from the ST4/74 Nal<sup>r</sup>, *siiC*::miniTn5Km2 mutant, *siiD*::miniTn5Km2 mutant, and  $\Delta$ *siiF* strains (Fig. 1D) but was not present in the preparation of total cellular protein from the  $\Delta$ *siiE* mutant, indicating that the SiiE gene was translated in the *siiC*::miniTn5Km2, *siiD*::miniTn5Km2, and  $\Delta$ *siiF* mutants but that the protein was not secreted. Although the *siiC*::miniTn5Km2 and *siiD*::miniTn5Km2 mutant strains are

transposon insertion mutants, the presence of SiiE in the total cellular protein fractions from these strains indicates that the mutations do not have polar effects on the *siiE* gene. However, we cannot preclude the possibility that the mutation of *siiC* has polar effects on *siiD*.

We also sought to establish whether SiiE was associated with the bacterial surface through ELISA using fixed bacteria. ST4/74 Nal<sup>r</sup>,  $\Delta$ *siiE*, and  $\Delta$ *siiF* strains were grown as described above, harvested by centrifugation (8,000  $\times g$  at 4°C for 10 min), washed in phosphate-buffered saline (PBS), and resuspended in 5 ml of 4% (wt/vol) paraformaldehyde in PBS for 30 min. The bacteria were harvested by centrifugation as described above to remove any SiiE protein that may have been released by fixation and resuspended in 4 ml of 0.05 M carbonate buffer, pH 9.6. Microtiter plates (Maxisorp; Nunc, Rochester, NY) were coated with 50  $\mu\text{l}$  of the prepared bacteria at 4°C overnight. The wells were washed and blocked with 3% (wt/vol) bovine serum albumin in PBS–0.05% (vol/vol) Tween for 1 h and then incubated with the anti-SiiE monoclonal antibody at 3  $\mu\text{g ml}^{-1}$  in the blocking buffer for 2 h. A control in which wells coated with the ST4/74 Nal<sup>r</sup> strain were incubated without the anti-SiiE monoclonal antibody was included in order to obtain a background reading. The plates were washed again with PBS-Tween and incubated with alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma) at 1/1,000 in the blocking buffer for 2 h. After washing, the reaction was developed with *p*-nitrophenyl phosphate and the absorbance was read at 405 nm. Each strain was tested in triplicate wells on three occasions. The ELISA showed that SiiE was present on the surfaces of ST4/74 Nal<sup>r</sup> cells, but the signal from the  $\Delta$ *siiE* and  $\Delta$ *siiF* mutant bacteria was reduced to background levels (Fig. 1E), indicating that SiiE was not present on the bacterial surface in these strains and confirming the specificity of the anti-SiiE antibody. The inability to detect SiiE by ELISA in the  $\Delta$ *siiF* mutant bacteria confirmed that at least a subset of the SiiE detected in the parent strain was surface associated.

Several previous studies have suggested that genes carried on SPI-4 are expressed under conditions similar to those under which genes carried on SPI-1 are expressed (3, 6, 8, 14, 24) and that the transcription of *siiE* is influenced by the SPI-1-encoded transcriptional regulator HilA (1, 3). A recent study identified a putative HilA box at +3,279 bp from the start codon of *siiE* (3), but it is unclear how this internal HilA box would allow the transcriptional regulation of *siiE*. The authors concluded that the HilA box is in the promoter region of the *spi4\_H* gene which was predicted as part of the original incorrect annotation of SPI-4 (30). Since we have now shown that *siiE* encodes a secreted protein of ca. 595 kDa, it is unlikely that the *spi4\_H* gene exists. We have, however, identified a motif which matches the consensus sequence of the HilA box predicted by De Keersmaecker et al. (3) at –52 to –40 bp upstream of the predicted translational start of *siiC* (data not shown). It is therefore possible that a HilA-regulated promoter lies upstream of *siiC* which may control the expression of the type I secretion system and SiiE.

As genes carried on SPI-1 and SPI-4 may be coregulated, we investigated whether *hilA* influenced the secretion of SiiE through the examination of the secreted proteome of an ST4/74 Nal<sup>r</sup> *hilA*::miniTn5Km2 mutant (17). The level of SiiE

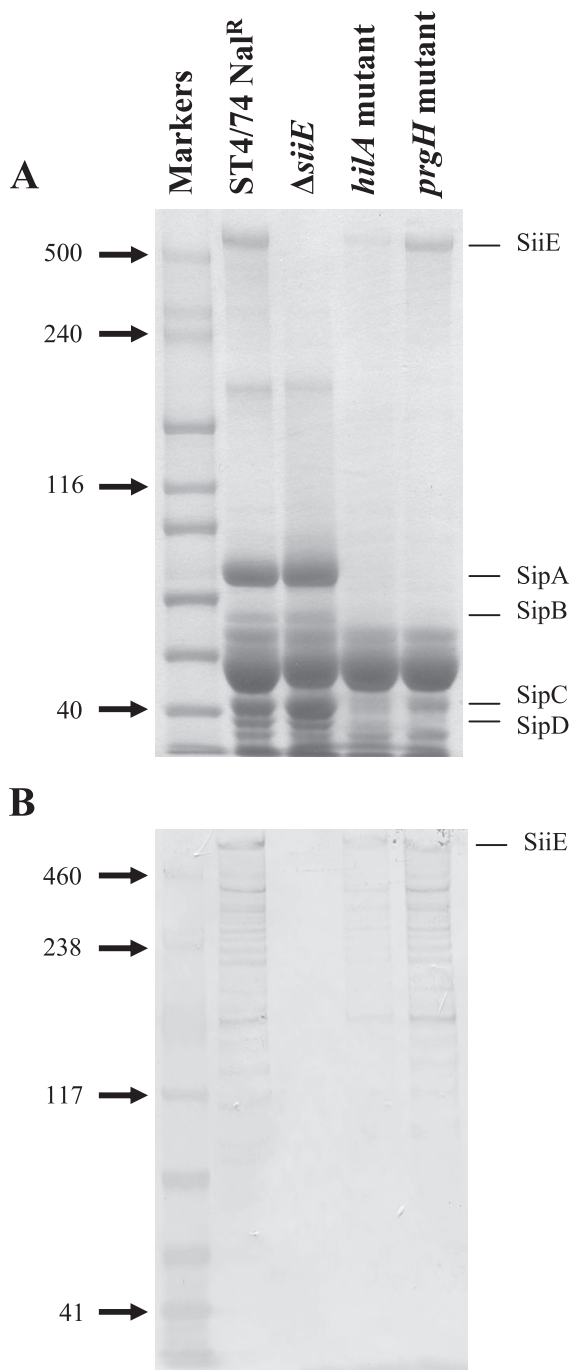


FIG. 2. The secretion of SiiE is influenced by *hilA* but not T3SS-1. (A) Proteins isolated from culture supernatants were separated by SDS-PAGE and stained with Coomassie blue. Molecular masses (kilodaltons) of standard proteins are shown on the left. The locations of T3SS-1-secreted proteins (SipA to SipD) are indicated on the right. (B) Proteins isolated from culture supernatants were transferred onto membrane by Western blotting and probed with the anti-SiiE monoclonal antibody. Molecular masses (kilodaltons) of standard proteins are shown on the left. Lanes are as shown for panel A.

present in the culture supernatant of the *hilA* mutant strain was significantly lower than that in the supernatant of the parent strain (Fig. 2), indicating that *hilA* influences the transcription or secretion of SiiE. It is likely that this occurs independently

of the need for a functional T3SS-1 since a *prgH* mutation did not affect the secretion of SiiE (Fig. 2).

We previously found through STM that mutants with transposon insertions in *siiD*, *siiE*, and *siiF* were absent from the ileal walls of calves 4 days postinfection (17). In order to further explore the role of SPI-4 in intestinal colonization and to define the basis of the attenuation seen in the STM screens, we compared the progressions of infections following oral inoculation of 28-day-old calves with the parent strain or the defined  $\Delta siiE$  and  $\Delta siiF$  mutants. All animal experiments were conducted according to the requirements of the Animal (Scientific Procedures) Act of 1986. Twenty-eight-day-old calves from the Institute for Animal Health farm were used as described previously (2). Bacterial cultures were prepared by inoculating LB broth with bacterial colonies from a fresh agar plate and incubated at 37°C with shaking for 18 h. Calves were separately infected with approximately  $1 \times 10^8$  CFU of each bacterial strain mixed with reconstituted powdered milk and fed by syringe immediately before the morning feed. Following inoculation, calves were monitored for signs of disease at least twice daily as described previously (2). The calves reached predetermined end points at 5 to 6 days postinfection, at which point all calves were humanely killed. At postmortem examination, samples of contents, walls, and mesenteric lymph nodes were taken from the mid ileum, cecum, and spiral colon and also the liver and spleen, and the bacteria associated with these sites were enumerated by counting viable cells. All samples were taken in triplicate, and the tissue samples were washed gently to remove nonadherent bacteria from the surfaces. One gram of sample was homogenized in 9 ml of 0.9% (wt/vol) saline. Serial dilutions were performed, and 20  $\mu$ l of each dilution was spread in triplicate onto brilliant green agar plates containing nalidixic acid. Fecal shedding and pyrexial response data were statistically analyzed for the effect of mutation by means of an F test, with data taken as repeated measurements (Proc GLM, Statistical Analysis System [SAS]; SAS Institute, NC). Postmortem data were analyzed by means of Student's *t* test (SAS). *P* values of  $<0.05$  were taken to be significant.

For testing of the  $\Delta siiE$  strain, four calves were inoculated orally with  $1.3 \times 10^8$  CFU of the ST4/74 NaI<sup>r</sup>  $\Delta siiE$  mutant strain and three calves were inoculated orally with  $1.0 \times 10^8$  CFU of the ST4/74 NaI<sup>r</sup> parent strain. All calves rapidly exhibited clinical signs of salmonellosis, a strong pyrexial response accompanied by the shedding of large numbers of bacteria in the feces and diarrhea. The experiment was terminated on day 6 as one calf in each group had reached the end point threshold of a scour score of 20 or above. Scour (diarrhea) was scored by using a cumulative daily scoring scheme based on the consistency and the contents of the feces. Scores for consistency were as follows: 0, normal; +1, semisolid; +2, liquid; and +3, watery. Scores for the fecal contents were as follows: +1, fresh blood; +2, sloughed mucosa (23a).

At most time intervals during the experiment, the mean numbers of bacteria recovered from the feces of calves challenged with the ST4/74 NaI<sup>r</sup>  $\Delta siiE$  mutant strain were lower than those recovered from calves challenged with the parent strain, but the difference was statistically significant only at day 6 postinfection ( $P < 0.05$ ) (Fig. 3A). No statistically significant differences in the magnitude or duration of the pyrexial responses were detected at any time point. However, at 32 h, at

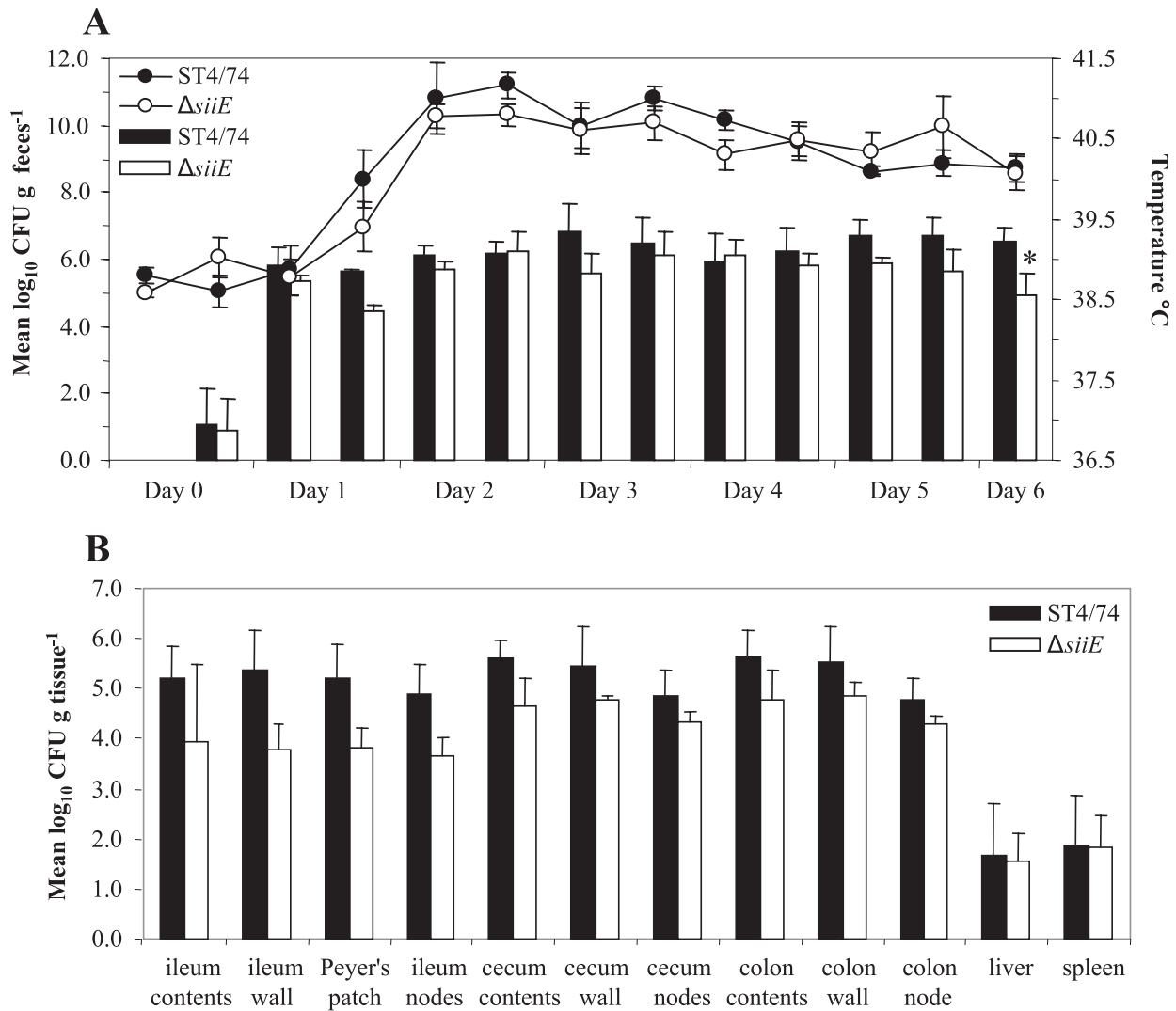


FIG. 3. *SiiE* contributes to intestinal colonization in cattle. (A) Mean rectal temperature responses and mean numbers of bacteria recovered per gram of feces following infection with either the parental strain or the  $\Delta$ *siiE* mutant strain for 6 days. Each bar or datum point is presented with the standard error of the mean \*,  $P$  of  $<0.05$ . (B) Mean numbers of bacteria of the parental strain or the  $\Delta$ *siiE* mutant strain recovered per gram of tissue. Each bar is presented with the standard error of the mean.

which time point a significant difference in the magnitude of the response between the groups of calves infected with the ST4/74  $\text{Nal}^r$  and  $\Delta$ *siiF* strains was seen (see below), there was a tendency towards a statistically significant difference in the magnitude of the response ( $P < 0.07$ ).

At postmortem examination, the numbers of bacteria associated with intestinal and systemic sites were determined. Although the mean number of ST4/74  $\text{Nal}^r$   $\Delta$ *siiE* bacteria recovered from intestinal sites was lower than the mean number of ST4/74  $\text{Nal}^r$  bacteria recovered, none of the data were statistically significant (Fig. 3B).

In order to determine whether the mutation of the type I secretion apparatus had an effect on intestinal colonization similar to or greater than that of the deletion of *siiE*, calves were inoculated orally with  $1.8 \times 10^8$  CFU of either the ST4/74  $\text{Nal}^r$   $\Delta$ *siiF* mutant strain (four calves) or the ST4/74  $\text{Nal}^r$  parent strain (three calves). Again, all calves rapidly exhibited signs of

salmonellosis, and the experiment was terminated 5 days postinfection as two of the three calves in the group challenged with the ST4/74  $\text{Nal}^r$  strain were unable to stand unaided and were displaying symptoms of anorexia. In contrast, the four calves challenged with the ST4/74  $\text{Nal}^r$   $\Delta$ *siiF* mutant appeared to be relatively healthy.

At all time points, the mean numbers of bacteria recovered per gram of feces from calves infected with the ST4/74  $\text{Nal}^r$   $\Delta$ *siiF* mutant were lower than those from the calves infected with the parent strain. However, the data were statistically significant only at 32, 48, and 56 h postinfection ( $P < 0.05$ ) (Fig. 4A). The mean rectal temperatures of the calves infected with the ST4/74  $\text{Nal}^r$   $\Delta$ *siiF* mutant were lower than those of the calves infected with the ST4/74  $\text{Nal}^r$  strain at all time points and significantly so at 32 h ( $P < 0.001$ ) and 56 h ( $P < 0.05$ ) (Fig. 4A).

At postmortem examination, the mean numbers of ST4/74

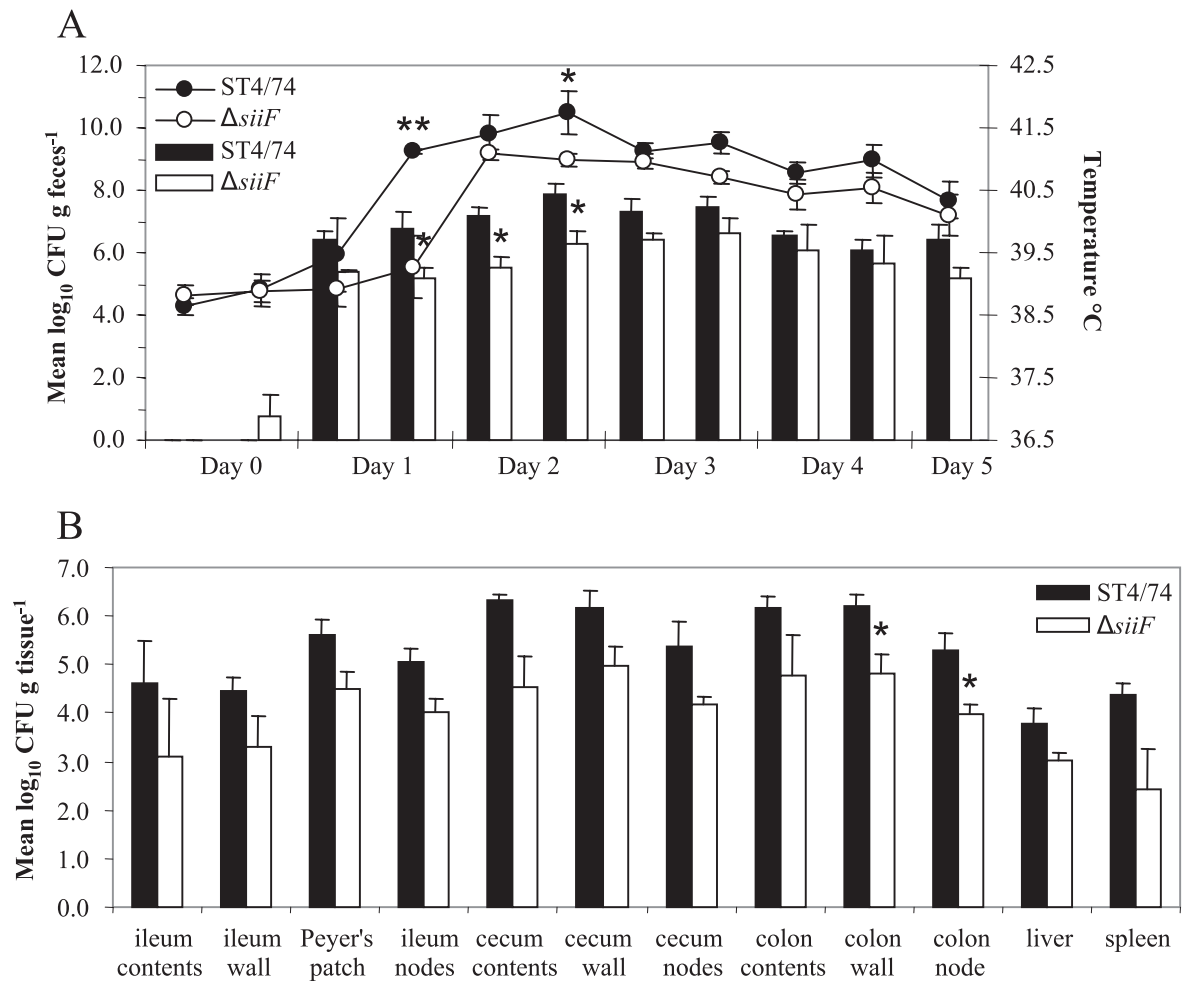


FIG. 4. SiiF contributes to intestinal colonization in cattle. (A) Mean rectal temperature responses and mean numbers of bacteria recovered per gram of feces following infection with either the parental strain or the  $\Delta$ siiF mutant strain for 6 days. Each bar or datum point is presented with the standard error of the mean. \*,  $P$  of  $<0.05$ ; \*\*,  $P$  of  $<0.001$ . (B) Mean numbers of bacteria of the parental strain or the  $\Delta$ siiF mutant strain recovered per gram of tissue. Each bar is presented with the standard error of the mean. \*,  $P$  of  $<0.05$ .

$\text{NaI}^r$   $\Delta$ siiF bacteria recovered from the calves were lower than those of the parent strain for all sites examined, but the difference between the means was statistically significant only for the tissues taken from the colon walls and colon nodes ( $P < 0.05$ ) (Fig. 4B).

As *siiE* and *siiF* contribute to the infection of calves following oral inoculation, we chose to investigate further their role in infection through the examination of the ability of the  $\Delta$ siiE and  $\Delta$ siiF mutants to invade the bovine epithelium and to persist in primary macrophages.

To investigate whether *siiE* and *siiF* are required for the invasion of the bovine epithelium by serovar Typhimurium, the bovine ligated ileal loop model to quantify intestinal invasion was used as described by Paulin et al. (20). At 2 h postinfection, gentamicin-protected tissue-associated bacteria were enumerated by the direct plating of serial dilutions of tissue homogenates as described above.

Fewer of the  $\Delta$ siiE and  $\Delta$ siiF mutant bacteria than of the parent strain bacteria were recovered from the tissues 2 h postinfection ( $P < 0.0001$ ) (Fig. 5), indicating that *siiE* and *siiF* contribute to the intestinal invasion of bovine enterocytes. The degree of at-

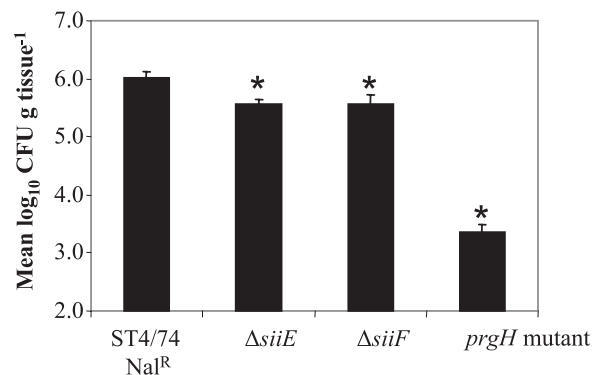


FIG. 5. SiiE and SiiF contribute to the invasion of bovine enterocytes. The mean numbers of gentamicin-protected bacteria of the ST4/74  $\text{NaI}^r$ ,  $\Delta$ siiE,  $\Delta$ siiF, and *prgH*::miniTn5Km2 mutant strains recovered per gram of tissue are shown. Each bar is presented with the standard error of the mean. \*,  $P$  of  $<0.0001$ .

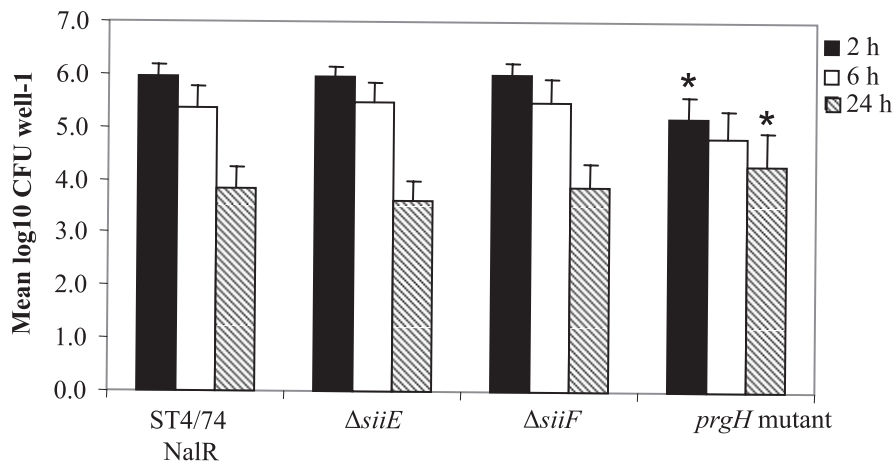


FIG. 6. SiiE and SiiF do not influence persistence in bovine alveolar macrophages. The mean numbers of gentamicin-protected bacteria of the ST4/74 NaIR,  $\Delta$ *siiE*,  $\Delta$ *siiF*, and *prgH*::miniTn5Km2 mutant strains recovered per well at 2 h, 6 h, and 24 h postinfection are shown. Each bar is presented with the standard error of the mean. \*,  $P < 0.05$ .

tenation of the  $\Delta$ *siiE* and  $\Delta$ *siiF* mutants, however, was not as severe as that of a *prgH*::miniTn5Km2 mutant strain (17). The mutation of *siiE* and *siiF* did not affect the secretion of T3SS-1-secreted effector proteins as assessed by the analysis of the secreted proteomes of the  $\Delta$ *siiE* and  $\Delta$ *siiF* mutants (Fig. 1A).

Several studies with conflicting results have reported the requirement for genes carried on SPI-4 for survival in macrophages. Fields et al. (7) found that a mutant with a transposon insertion in a locus mapped to SPI-4 by Wong et al. (30) was deficient in its ability to survive in murine peritoneal macrophages. A microarray study also suggested that SPI-4 genes are up-regulated under in vitro conditions which mimic the phagocytic vacuole (4), although other microarray studies of serovar Typhimurium gene expression in macrophages have suggested that the reverse is true (6, 31).

We previously demonstrated that four mutants with transposon insertions in *siiD*, *siiE*, and *siiF* were not attenuated in persistence in porcine alveolar macrophages (17). Since that study was conducted, however, we have shown that mutants with transposon insertions in genes within SPI-4 are not attenuated in intestinal colonization in pigs (Carnell et al., submitted). We therefore chose to test the  $\Delta$ *siiE* and  $\Delta$ *siiF* mutants for invasion and persistence in bovine alveolar macrophages.

Alveolar macrophages were isolated from healthy Friesian cattle by bronchoalveolar lavage (9) and used essentially as described previously (29) except that the cells were inoculated with the bacteria in Iscove's modified Dulbecco's medium (without phenol red and L-glutamine) supplemented with 5% (vol/vol) fetal calf serum and 2 mM L-glutamine. In each case, the strains were tested in triplicate on two occasions. The survival rates of the strains at 6 h and 24 h were calculated as follows: percentage of surviving bacteria = (number of bacteria recovered at 6 h or 24 h/number of bacteria recovered at 2 h)  $\times$  100. The data were statistically analyzed across two biological replicates for the effect of the strain by using a two-way analysis of variance (Proc Mixed; SAS).

The means and standard errors of the means of results from the two experiments are shown in Fig. 6. The  $\Delta$ *siiE* and  $\Delta$ *siiF* mutants were taken up by the macrophages at levels equivalent

to that of the parent strain as assessed by the recovery of gentamicin-protected bacteria at 2 h postinfection, although the mean numbers of *prgH*::miniTn5Km2 mutant bacteria recovered were significantly lower than those of the  $\Delta$ *siiF* strain ( $P < 0.05$ ) and showed a statistical tendency towards being reduced compared to those of the parent strain and the  $\Delta$ *siiE* strain ( $P < 0.056$ ). Neither of the  $\Delta$ *siiE* and  $\Delta$ *siiF* mutants were attenuated in intracellular survival over 6 h or 24 h, although the numbers of gentamicin-protected *prgH*::miniTn5Km2 mutant bacteria recovered were greater than those of the other strains at 24 h ( $P < 0.05$ ), presumably due to reduced macrophage lysis (27).

In summary, we report that SPI-4 encodes a ca. 600-kDa protein (SiiE) which is secreted into culture supernatants through an associated putative type I secretion system (encoded by *siiC*, *siiD*, and *siiF*) and have identified a minor role for *siiE* and *siiF* in the invasion of bovine enterocytes and intestinal colonization in calves. Although the data gathered following the oral infection of calves with  $\Delta$ *siiE* were statistically significant only for fecal shedding at 6 days postinfection, the data show a clear trend indicating that *siiE* contributes to infection in calves given that at most time points the mean rectal temperature responses and mean numbers of bacteria recovered from fecal samples were lower for the calves infected with the  $\Delta$ *siiE* strain than for those infected with the parent strain. Similarly, for each site examined postmortem, the mean numbers of bacteria recovered from the infected tissues were lower for the calves infected with the  $\Delta$ *siiE* strain than for those infected with the parent strain (Fig. 3).

A similar trend was seen following the oral challenge of calves with the  $\Delta$ *siiF* strain or the parent strain, although the data obtained from these animals suggested that the  $\Delta$ *siiF* mutant was slightly more attenuated than the  $\Delta$ *siiE* mutant (Fig. 4). We cannot preclude the possibility that the type I secretion system, of which SiiF is a component, is required for the secretion of proteins other than SiiE and that these secreted substrates also contribute to infection in calves. As yet, we have no data to support this hypothesis as only SiiE appears to be absent from the secreted proteome of the  $\Delta$ *siiF* mutant



(Fig. 1A). In addition, the  $\Delta siiE$  and  $\Delta siiF$  mutants were attenuated to comparable levels in the invasion of bovine enterocytes as assessed by the bovine ligated ileal loop model (Fig. 5), even though the levels of attenuation were not equal following oral challenge. This apparent discrepancy may reflect the complex interactions that take place in vivo over the 5 to 6 days of examination following oral challenge compared to those over a 4-h time course in the bovine ligated ileal loop model and may indicate a role for SPI-4 at early time points postinfection.

That the mutants were clearly attenuated in the bovine ligated ileal loop model, but not following oral challenge, also highlights the difficulties of experimental procedures performed with large animals, including the variability among animals which arises from the use of outbred animals and the limits on experimental group size due to practicability and cost. However, the findings are supported by the independent isolation of eight *siiE* and two *siiF* transposon mutants of serovar Typhimurium in a signature-tagged mutagenesis screen for mutants defective in persistence in the bovine ileal mucosa 4 days post-oral inoculation (17). This finding emphasizes the sensitivity of the technique of signature-tagged mutagenesis in identifying genes which contribute a minor role in infections but also reinforces a requirement to substantiate the results of such screens. Although *siiE* and *siiF* appear to contribute in a minor role to the pathogenesis of salmonellosis in cattle, it is likely that a large number of genes contribute in a small, but significant, way to infections in cattle as suggested by the large numbers of mutants identified by signature-tagged mutagenesis as being attenuated in intestinal colonization (17).

Although we have demonstrated here that *siiE* and *siiF* contribute to infections in calves at the intestinal level, it is unclear what role the SPI-4 genes play in virulence in other animal species. We previously demonstrated by signature-tagged mutagenesis that mutants with transposon insertions in SPI-4 genes were not attenuated in colonization in chickens (17) or pigs (Carnell et al., submitted) but that transposon mutants with insertions in *siiE* and *siiF* were attenuated in systemic virulence following the oral challenge of BALB/c mice but not following the intraperitoneal infection of Nramp<sup>r</sup> mice (17). Similarly, Detweiler et al. found that an *siiF* mutant was not attenuated in bacterial persistence at 3 weeks following the intraperitoneal infection of Nramp1<sup>+/+</sup> mice (4). However, a recent report contradicted this finding and suggested that SPI-4 genes do contribute to long-term systemic infections in mice (13). Through the use of a microarray-based negative selection screen for mutants unable to persist over 28 days in Nramp1<sup>+/+</sup> mice, *siiC* and *siiF* mutants were shown to be lost from the livers and spleens of mice 21 days following intraperitoneal infection. The role of SPI-4 genes in long-term systemic virulence in mice was confirmed in a competitive-index experiment using a  $\Delta siiABCD::Km$  deletion mutant. This study, however, also showed that SPI-1-carried genes are also required for long-term systemic virulence in mice, although previously SPI-1 was thought to be required primarily for the gastrointestinal phase of infection. These conflicting results underline the importance of studying bacterial gene functions in target animal hosts wherever possible.

Although the mode of action of SiiE has not been established, few other type I secretion systems are known to influence virulence. These secretion systems secrete a wide range of

proteins in both prokaryotic and eukaryotic cells, and the proteins are secreted from the cytoplasm to the extracellular medium without a periplasmic step. SiiC is predicted to be an outer membrane component, and SiiD is a putative membrane fusion protein which links the inner and outer membranes. SiiF is predicted to be the ATP-binding cassette component which in other systems is localized in the cytoplasmic membrane and is thought to be responsible for the specificity of the secretion process. Further studies are required to establish the mode of translocation of SiiE across the periplasm and outer membrane and to determine the exact roles of SiiC, SiiD, and SiiF in this process.

The best-characterized type I secretion system is that required for the secretion of the HlyA alpha-hemolysin of uropathogenic *E. coli*. HlyA belongs to the repeats-in-toxin protein family, and strains carrying alpha-hemolysin are mainly associated with extraintestinal infections (11), although some have been linked with intestinal infections (5). Few type I secretion systems are known to contribute to virulence in *Salmonella*. MacAB, which is a drug efflux system, is required for virulence in mice and is regulated by the global virulence regulator PhoP (19). We previously showed that *siiCDEF* genes share 40% overall nucleotide identity with four genes on *Salmonella* pathogenicity island 9 (17). These genes also encode a type I secretion system and a predicted 365-kDa protein, BapA, which is required for biofilm formation and virulence in mice (12). Despite the similarities between SiiE and BapA, no evidence has been found for a role for SiiE in biofilm formation (12) and further studies are required to establish the mode of action of SiiE in vivo.

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