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# Loss of Very-Long O-Antigen Chains Optimizes Capsule-Mediated Immune Evasion by *Salmonella enterica* Serovar Typhi

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**ABSTRACT** Expression of capsular polysaccharides is a variable trait often associated with more-virulent forms of a bacterial species. For example, typhoid fever is caused by the capsulated *Salmonella enterica* serovar Typhi, while nontyphoidal *Salmonella* serovars associated with gastroenteritis are noncapsulated. Here we show that optimization of the immune evasive properties conferred by the virulence-associated (Vi) capsular polysaccharide involved an additional alteration to the cell envelope of *S. Typhi*, namely inactivation of the *fepE* gene, encoding the regulator of very-long O-antigen chains. Introduction of the capsule-encoding *viaB* locus into the nontyphoidal *S. enterica* serovar Typhimurium reduced complement deposition *in vitro* and intestinal inflammation in a mouse colitis model. However, both phenotypes were markedly enhanced when the *viaB* locus was introduced into an *S. Typhimurium fepE* mutant, which lacks very-long O-antigen chains. Collectively, these data suggest that during the evolution of the *S. Typhi* lineage, loss of very-long O-antigen chains by pseudogene formation was an adaptation to maximize the anti-inflammatory properties of the Vi capsular polysaccharide.

**IMPORTANCE** Genomic comparison illustrates that acquisition of virulence factors by horizontal gene transfer is an important contributor to the evolution of enteric pathogens. Acquisition of complex virulence traits commonly involves horizontal transfer of a large gene cluster, and integration of the gene cluster into the host genome results in the formation of a pathogenicity island. Acquisition of the virulence-associated (Vi) capsular polysaccharide encoded by SPI7 (*Salmonella* pathogenicity island 7) was accompanied in the human-adapted *Salmonella enterica* serovar Typhi by inactivation of the *fepE* gene, encoding the regulator of very-long O-antigen chains. We show that the resulting loss of very-long O-antigen chains was an important mechanism for maximizing immune evasion mediated by the Vi capsular polysaccharide. These data suggest that successful incorporation of a capsular polysaccharide requires changes in the cell envelope of the hosting pathogen.

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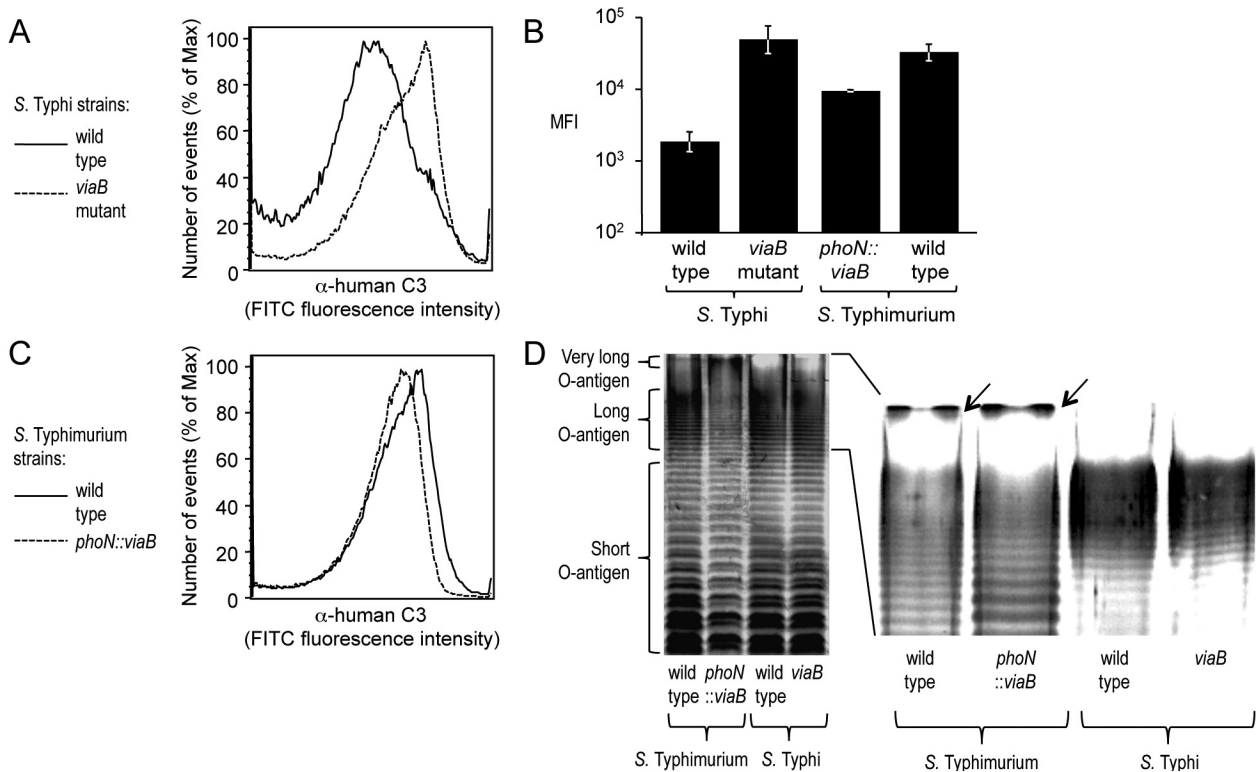
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Typhoid fever is a severe systemic infection that presents with fever (1) after an average incubation period of 2 weeks (2). In contrast, gastroenteritis caused by *Salmonella enterica* serovar Typhimurium is a localized diarrheal disease with an average incubation period of <24 h (3). The swift onset of diarrhea, abdominal pain, and fever during gastroenteritis is explained by the rapid induction of an acute inflammatory response in the intestine, which requires deployment of two type III secretion systems (T3SSs) encoded by *Salmonella* pathogenicity island 1 (SPI1) and SPI2 (4–6). Although SPI1 and SPI2 are present in *S. enterica* serovar Typhi (7), the development of intestinal inflammation is slowed markedly during typhoid fever by expression of the virulence-associated (Vi) capsular polysaccharide (8–10). The anti-inflammatory properties of the Vi capsular polysaccharide (11–13) have been proposed to contribute to the long incubation period characteristic of typhoid fever (14, 15).

The biosynthesis of the Vi capsular polysaccharide is encoded by the *viaB* locus, a 14-kb DNA region located on SPI7, a pathogenicity island present in *S. enterica* serovar Typhi but absent from *Salmonella* serovars associated with gastroenteritis (16, 17). The

*viaB* locus contains genes for the regulation (*tviA*), biosynthesis (*tviBCDE*), and surface assembly (*vexABCDE*) of the Vi capsular polysaccharide (18, 19). Expression of the positive regulator TviA is repressed in the intestinal lumen but induced by the two-component system EnvZ/OmpR at an osmolarity encountered in tissue (20, 21). As a result, expression of the Vi capsular polysaccharide is induced when bacteria transit through the intestinal epithelium (22). This regulatory mechanism ensures that *S. Typhi* is encapsulated by the time it encounters complement. Expression of the Vi capsular polysaccharide inhibits complement deposition (23, 24), because its homopolymeric chains contain approximately 300 residues of (1,4)-2-acetamido-3-O-acetyl-2-deoxy- $\alpha$ -D-galacturonic acid (25), a sugar that does not contain free hydroxyl groups available for ester formation with complement component 3 fragment b (C3b).

Since *S. Typhi* is strictly human adapted, it is difficult to study the function of its virulence factors in animal models (26). One approach to study the role the Vi capsular polysaccharide plays *in vivo* has been the introduction of the *viaB* locus into *S. Typhimurium*, a natural pathogen of mice (8, 9, 12, 13, 24). However, it is



**FIG 1** (A to C) Fixation of C3 after incubation of the indicated *S. Typhi* and *S. Typhimurium* strains (wild type and mutants) in 10% human serum was detected by flow cytometry using an anti-human C3 ( $\alpha$ -human C3) FITC conjugate. The experiments shown in panels A and C were repeated 3 times independently with similar outcomes, and a representative example is shown. The average maximum fluorescence intensity (MFI) values  $\pm$  standard errors (error bars) determined for these three independent experiments are shown in panel B. (D) Silver-stained SDS-PAGE of LPS preparations from the indicated *S. Typhimurium* and *S. Typhi* strains. The positions of short, long, and very-long O-antigen chains are indicated to the left of the gel. A magnification of the region showing long and very-long O-antigen chains is shown on the right, and the presence of very-long O-antigen chains in *S. Typhimurium* strains is indicated by black arrows.

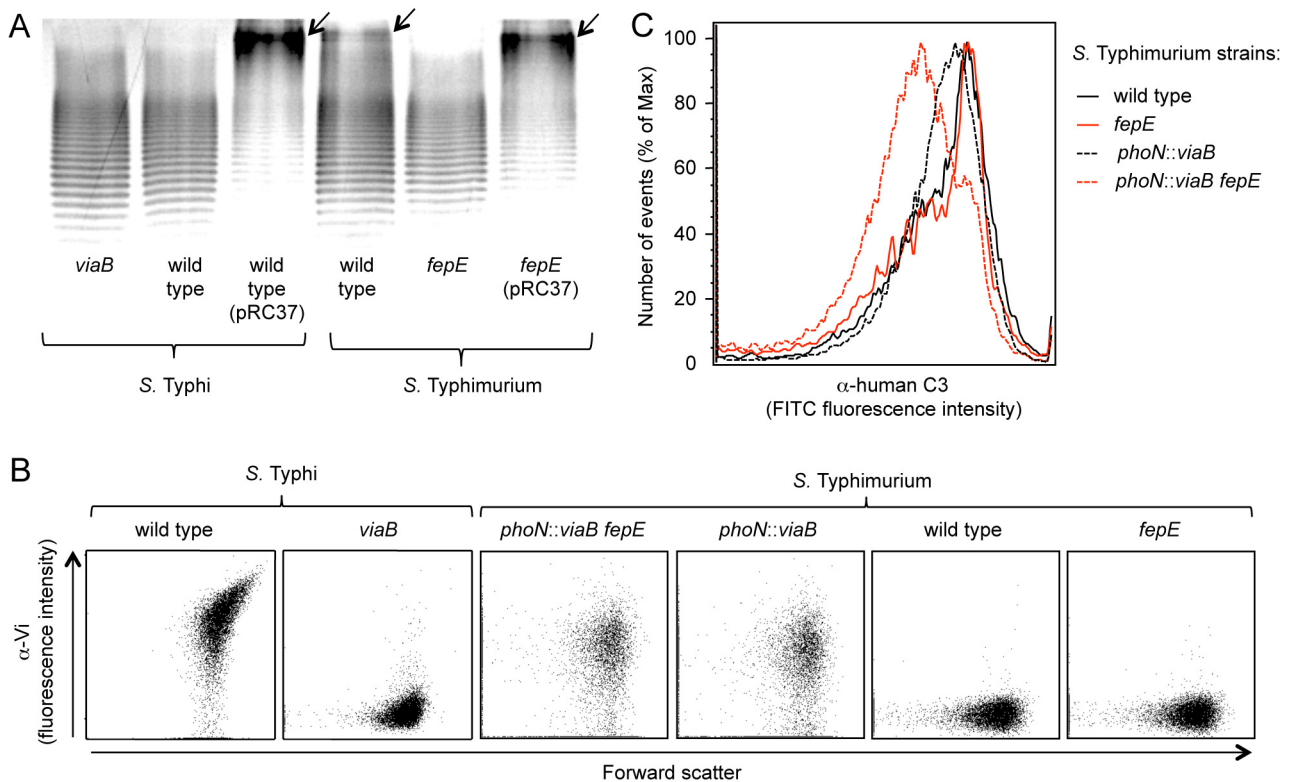
not known whether horizontal transfer of the *viaB* locus is sufficient to optimally deploy the encoded capsular polysaccharide for immune evasion. Here we show that for the Vi capsular polysaccharide to confer maximal evasion of complement fixation, it is necessary to change the cell envelope of *S. Typhimurium*, a process that also accompanied the evolution of the *S. Typhi* lineage.

## RESULTS

**Very-long O-antigen chains interfere with the function of the Vi capsular polysaccharide *in vitro*.** We performed an *in vitro* assay to determine whether expression of the Vi capsular polysaccharide in *S. enterica* serovars Typhi and Typhimurium had identical effects on inhibiting complement fixation. Consistent with previous reports (24), analysis of bacterial cells by flow cytometry showed that incubation of a noncapsulated *S. Typhi* mutant (*viaB* mutant) in 10% human serum resulted in efficient deposition of C3b on the bacterial surface, while complement deposition was markedly reduced in the capsulated *S. Typhi* wild-type strain (Ty2) (Fig. 1A and B). Similarly, a capsulated *S. Typhimurium* strain in which the *viaB* locus of *S. Typhi* was inserted chromosomally into the *phoN* gene (*phoN::viaB* mutant) deposited less complement on its surface than the noncapsulated *S. Typhimurium* wild-type strain (IR715) (Fig. 1B and C). However, the magnitude by which expression of the Vi capsular polysaccharide reduced complement fixation was notably larger in *S. Typhi* than in *S. Typhimurium* (Fig. 1B).

To investigate possible reasons for differences in the efficacy by which the Vi capsular polysaccharide reduced complement deposition in *S. Typhi* and *S. Typhimurium*, we compared expression of lipopolysaccharide (LPS), a surface structure containing O-antigen repeat units that are known to fix complement (27). LPS molecules contain a lipid A anchor and an oligosaccharide core but differ in the number of O-antigen repeat units that extend from the bacterial surface. In *S. Typhimurium*, O-antigen repeat units are composed of a trisaccharide backbone, consisting of  $\alpha$ -D-mannose-(1,4)- $\alpha$ -L-rhamnose-(1,3)- $\alpha$ -D-galactose, and a branching sugar (abequose) that is  $\alpha$ -(1,3)-linked to D-mannose in the backbone. Consistent with previous reports, *S. Typhimurium* exhibited a trimodal distribution in LPS length, including short-LPS species containing between 1 and 15 O-antigen repeat units, long-LPS species carrying between 16 and 35 O-antigen repeat units, and very-long-LPS species with more than 100 O-antigen repeat units (Fig. 1D) (28–30). O-antigen repeat units of *S. Typhi* carry tyvelose as the branching sugar that is  $\alpha$ -(1,3)-linked to the backbone but are otherwise identical to those of *S. Typhimurium*. Interestingly, in contrast to *S. Typhimurium*, *S. Typhi* expressed only short-LPS species and long-LPS species, while very-long-LPS species were absent (Fig. 1D).

The *fepE* open reading frame encodes the length regulator of very-long O-antigen chains (28) but is interrupted in the *S. Typhi* genome by a stop codon (7). To test whether this mutation in *fepE* is responsible for the lack of LPS species with very-long O-antigen



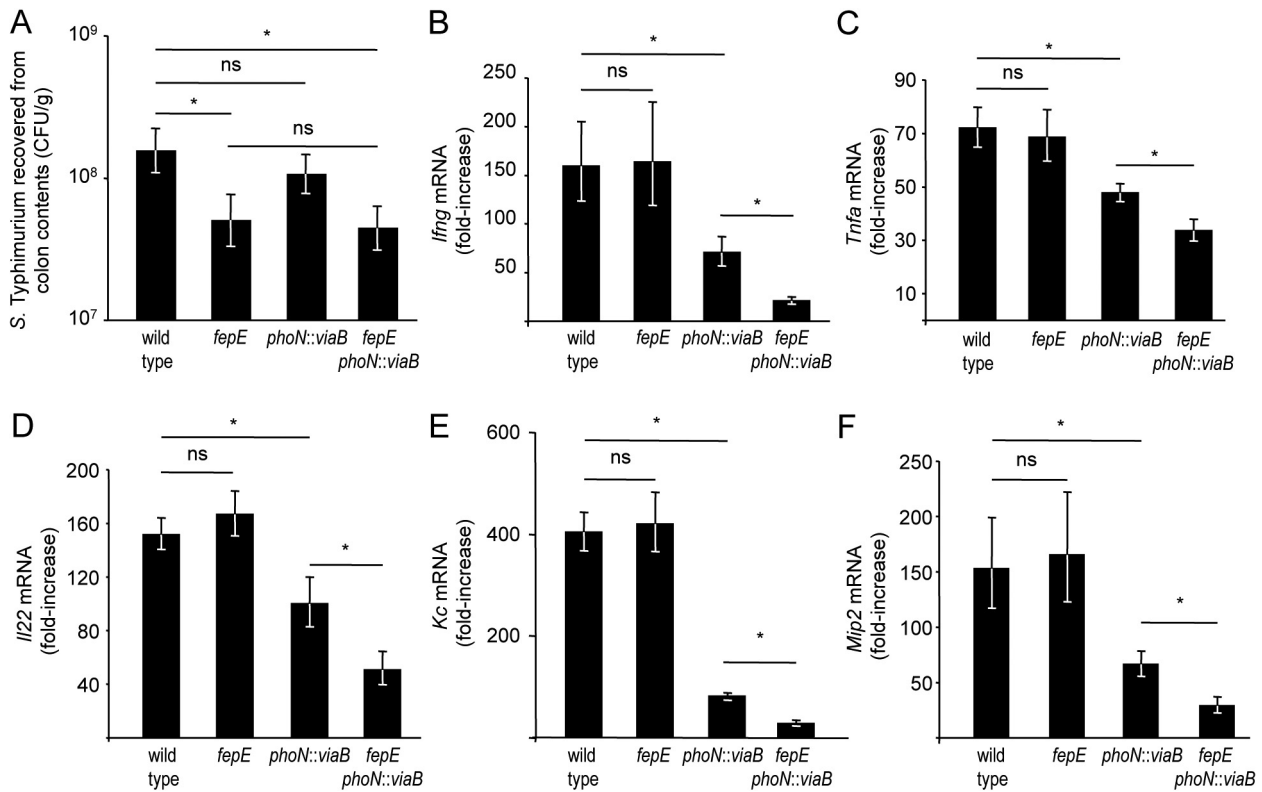
**FIG 2** (A) Silver-stained SDS-PAGE of LPS preparations from the indicated *S. Typhimurium* and *S. Typhi* strains (wild type and mutants). Plasmid pRC37 carries the cloned *S. Typhimurium fepE* gene. The positions of very-long O-antigen chains are indicated by black arrows. (B) Vi capsular polysaccharide expression was detected by flow cytometry. Cells of the indicated *S. Typhi* and *S. Typhimurium* strains were labeled with rabbit anti-Vi serum/goat anti-rabbit FITC conjugate ( $\alpha$ -Vi on the y axis), and fluorescence intensities were determined for 10,000 particles. Each experiment was repeated 3 times independently with similar outcomes, and a representative example is shown. (C) Fixation of C3 after incubation of the indicated *S. Typhi* and *S. Typhimurium* strains in 10% human serum was detected by flow cytometry using an anti-human C3 FITC conjugate. The experiment was repeated 3 times independently with similar outcomes, and a representative example is shown.

chains in *S. Typhi*, we introduced a plasmid carrying the cloned *S. Typhimurium fepE* gene (pRC37). In contrast to the wild-type *S. Typhi*, an *S. Typhi* strain carrying a plasmid encoding the *S. Typhimurium fepE* gene (pRC37) produced LPS species with very-long O-antigen chains (Fig. 2A). These data suggested that conversion of *fepE* into a pseudogene was responsible for the inability of *S. Typhi* to produce very-long O-antigen chains, which confirmed previous results (31).

We reasoned that very-long O-antigen chains containing an estimated 100 copies of a repeat unit composed of a trisaccharide backbone (28) might rival in length the homopolymeric chains of the Vi capsular polysaccharide comprising approximately 300 sugar residues (25). Therefore, we wanted to test whether the reduced efficacy by which the Vi capsular polysaccharide diminished complement deposition in *S. Typhimurium* was due to the presence of very-long O-antigen chains. A mutation in *fepE* was introduced into the wild-type *S. Typhimurium* and an *S. Typhimurium phoN::viaB* mutant. Analysis by flow cytometry revealed similar expression levels of the Vi capsular polysaccharide in *S. Typhi* (Ty2), the *S. Typhimurium fepE phoN::viaB* mutant (RC60), and the *S. Typhimurium phoN::viaB* mutant (TH170), while capsule expression was absent in the wild-type *S. Typhimurium* (IR715), the *S. Typhimurium fepE* mutant (RC31), and the *S. Typhi viaB* mutant (STY2) (Fig. 2B). Expression of very-long O-antigen chains was abrogated in the *fepE* mutant (Fig. 2A).

Furthermore, expression of very-long O-antigen chains could be restored in the *fepE* mutant by introducing the *fepE* gene cloned on a plasmid (pRC37). Remarkably, complement fixation was markedly reduced in the *S. Typhimurium fepE phoN::viaB* mutant compared to the *S. Typhimurium phoN::viaB* mutant (Fig. 2C). These data suggested that very-long O-antigen chains impaired the ability of the Vi capsular polysaccharide to prevent complement deposition on the surface of *S. Typhimurium*.

**Lack of very-long O-antigen chains enhances capsule-mediated suppression of colitis.** We used the mouse colitis model (5) to investigate the biological relevance of our observations. In this model, mice are preconditioned by treatment with streptomycin, which disrupts the resident microbiota. Subsequent inoculation with *S. Typhimurium* results in acute cecal inflammation, which is an animal model for human gastroenteritis (reviewed in reference 26). Groups of streptomycin-pretreated mice were either mock infected or inoculated with the wild-type *S. Typhimurium* or a *fepE* mutant, *phoN::viaB* mutant, or *fepE phoN::viaB* mutant, and the cecum and colon contents were collected 72 h after infection. While the wild-type *S. Typhimurium* and *phoN::viaB* mutant were recovered in similar numbers from cecal contents, a small but significant ( $P < 0.05$ ) reduction in bacterial numbers was observed for strains lacking very-long O-antigen chains (i.e., the *fepE* mutant and *fepE phoN::viaB* mutant) (Fig. 3A). These data were consistent with our previous observa-



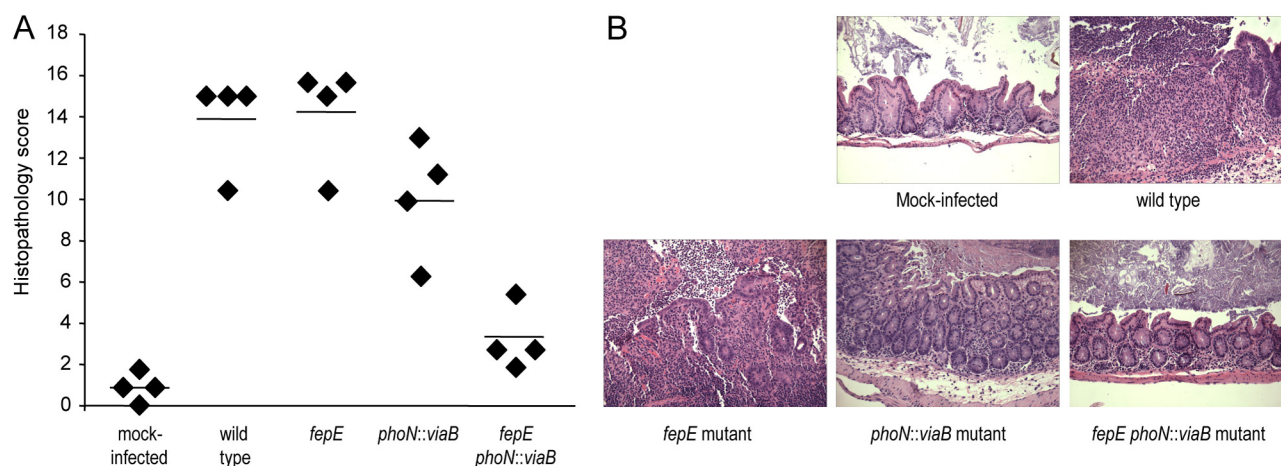
**FIG 3** Streptomycin-pretreated mice were infected with the indicated *S. Typhimurium* strains, and the cecum and colon contents were collected 72 h after infection. (A) Recovery of *S. Typhimurium* from colon contents. Values are geometric means of CFU per gram colon contents  $\pm$  standard errors (error bars). (B to F) Transcript levels of *Ifng* (B), *Tnfa* (C), *Il22* (D), *Kc* (E), and *Mip2* (F) in the cecal mucosa were determined by quantitative real-time PCR. Values are geometric means  $\pm$  standard errors of fold increases over mRNA levels in mock-infected animals. Values that are statistically significant ( $P < 0.05$ ) are indicated by a bar and asterisk. Values that are not statistically significant (ns) are indicated.

tion that very-long O-antigen chains are required for optimal survival of *S. Typhimurium* in the lumen of the inflamed gut (32).

To assess how the presence of very-long O-antigen chains influences inflammatory responses in the cecal mucosa, we determined mRNA levels of inflammatory markers, including gamma interferon (IFN- $\gamma$ ) encoded by the *Ifng* gene, tumor necrosis factor alpha (TNF- $\alpha$ ) encoded by the *Tnfa* gene, interleukin-22 (IL-22) encoded by the *Il22* gene, keratinocyte-derived cytokine (KC) encoded by the *Kc* gene, and macrophage-inducible protein 2 (MIP-2) encoded by the *Mip2* gene, by quantitative real-time PCR. Although the wild-type *S. Typhimurium* was recovered in significantly greater numbers from colon contents than the *fepE* mutant (Fig. 3A), both strains elicited similar levels of *Ifng*, *Tnfa*, *Il22*, *Kc*, and *Mip2* expression in the cecal mucosa (Fig. 3B to F). Thus, the presence of very-long O-antigen chains did not alter expression levels of inflammatory markers elicited by *S. Typhimurium* in the mouse colitis model. Introduction of the *S. Typhi viaB* locus into the *S. Typhimurium* genome (*phoN::viaB* mutant) significantly ( $P < 0.05$ ) reduced mRNA levels of *Ifng*, *Tnfa*, *Il22*, *Kc*, and *Mip2* compared to those elicited by infection with the wild-type *S. Typhimurium* (Fig. 3B to F). These data were consistent with our previous observation that expression of the Vi capsular polysaccharide in *S. Typhimurium* reduces intestinal inflammatory responses in the mouse colitis model (9). Remarkably, introduction of the *viaB* locus into the *fepE* mutant (*fepE phoN::viaB* mutant) significantly ( $P < 0.05$ ) reduced mRNA levels of *Ifng*,

*Tnfa*, *Il22*, *Kc*, and *Mip2* compared to those elicited by infection with the *phoN::viaB* mutant (Fig. 3B to F). Thus, in the absence of very-long O-antigen chains, the *viaB* locus suppressed expression of inflammatory markers to a significantly ( $P < 0.05$ ) greater extent than in their presence. Reduced expression of inflammatory markers was independent of the bacterial burden, because the *fepE* mutant and the *fepE phoN::viaB* mutant were recovered in similar numbers (Fig. 3A), whereas the former elicited significantly ( $P < 0.05$ ) higher mRNA levels of *Ifng*, *Tnfa*, *Il22*, *Kc*, and *Mip2* than the latter (Fig. 3B to F).

We next performed a blinded analysis of histopathological changes observed in the cecal mucosa 72 h after infection to determine the biological consequences of expressing very-long O-antigen chains and/or the Vi capsular polysaccharide. Cecal contents from mice infected with the wild-type *S. Typhimurium* or a *fepE* mutant were devoid of any contents and had severe gross pathological changes, characterized by reduced size with thickening of the cecal wall. Histopathological evaluation revealed epithelial erosion, neutrophil infiltration in the mucosa, and edema in the submucosa. In contrast, ceca from mock-infected mice did not show gross pathological changes or overt histopathology (Fig. 4). Introduction of the *S. Typhi viaB* locus into the *S. Typhimurium* genome (*phoN::viaB* mutant) resulted in a small but significant ( $P < 0.05$ ) reduction in the severity of histopathological changes compared to mice infected with the wild-type *S. Typhimurium* or a *fepE* mutant. Remarkably, infection with a *fepE phoN::viaB* mu-



**FIG 4** Streptomycin-pretreated mice were either mock infected or infected with the indicated *S. Typhimurium* strains, and the cecum was collected 72 h after infection. (A) Combined histopathology score of pathological changes observed in sections from the cecum. Each symbol represents the combined histopathology score for an individual animal. The average for each group of mice is indicated by a short line. (B) Representative images of histopathological changes.

tant resulted in a significant ( $P < 0.05$ ) reduction in the severity of histopathological changes compared to the *phoN::viaB* mutant. Overall, the results from this histopathological analysis (Fig. 4) substantiated results from expression analysis of inflammatory markers in the cecal mucosa (Fig. 3B to F) and supported the concept that optimal suppression of intestinal inflammation by the *viaB* locus requires an absence of very-long O-antigen chains.

## DISCUSSION

The fact that the vast majority of the 2,587 known serovars of *S. enterica* are zoonotic and associated with a localized, self-limiting gastroenteritis in immune-competent individuals (33) suggests that human-restricted specialists associated with systemic febrile illnesses, such as *S. enterica* serovar Typhi or Paratyphi A, evolved from ancestral zoonotic organisms that caused gastroenteritis (34). While the *S. enterica* species is estimated to be 40 to 63 million years old (35), *S. Typhi* represents a clonal lineage that emerged only recently, between 10,000 and 150,000 years ago (36, 37). After the *S. Typhi* lineage had passed through approximately 75% of its evolutionary history, it exchanged some 23% of its genome by horizontal gene transfer with the *S. Paratyphi A* lineage, presumably during coexistence in a shared human reservoir (38). Subsequent to this large-scale genetic exchange, which might mark the origin of typhoid and paratyphoid fever, the lineages of *S. Typhi* and *S. Paratyphi A* became isolated again, and both of their genomes subsequently accumulated pseudogenes at an accelerated rate (39).

Many pseudogenes present in *S. Typhi* encode functions required for the gastrointestinal lifestyle of *S. Typhimurium*. For example, the genome of *S. Typhi* strain CT18 contains pseudogenes in 7 of its 11 chaperone/usher-type fimbrial operons (40), which encode adhesins required by *S. Typhimurium* to colonize the intestinal lumen (41). Furthermore, the *S. Typhi* genome carries pseudogenes in operons functioning in anaerobic respiration (*trrS*, *dmsA*, *dmsB*, *narV*, and *narW*) (7, 42), and these functions are required by *S. Typhimurium* to outgrow obligate anaerobic bacteria in the lumen of an acutely inflamed gut during gastroenteritis (43, 44). While these genes are likely maintained in *S. Typhimurium* because they aid in intestinal growth and transmis-

sion during gastroenteritis (45), they can be seen as dispensable for the extraintestinal lifestyle of *S. Typhi*, a pathogen that spreads by means of water, milk, and food products contaminated by individuals with chronic gallbladder carriage (46). Thus, pseudogene formation in *S. Typhi* is commonly viewed as a process leading to random losses of genetic functions that are inherited from an ancestral organism associated with gastroenteritis but that are not required for causing typhoid fever in humans.

Our results suggest that, surprisingly, the formation of one pseudogene, namely, a *fepE* allele interrupted by a stop codon, resulted in a gain of function during the evolution of the *S. Typhi* lineage. Specifically, we show that inactivation of *fepE* resulted in an enhanced functionality of the Vi capsular polysaccharide. The Vi capsular polysaccharide is an important virulence factor of *S. Typhi* (reviewed in reference 47) and has been developed into a vaccine against typhoid fever (48). Expression of the Vi capsular polysaccharide inhibits complement deposition (23, 24), a process that was more efficient in strains lacking a functional *fepE* gene. One consequence of complement activation is the formation of a membrane attack complex on the bacterial surface that leads to lysis unless serum resistance mechanisms are deployed. One study reports that the Vi capsular antigen is not required for serum resistance of *S. Typhi* (31), but in this study, bacteria were grown in Luria-Bertani (LB) broth, a condition that represses expression of the Vi capsular polysaccharide (20, 21). In several other studies, expression of the Vi capsular polysaccharide was shown to increase serum resistance of *S. Typhi* (23, 24, 49), which is consistent with its role in preventing complement deposition (Fig. 1A).

A second consequence of complement deposition and activation of the alternative pathway is the production of anaphylatoxins (C3a and C5a). Anaphylatoxins are potent enhancers of cytokine responses elicited by stimulating the Toll-like receptor 4 (TLR4)/MD2/CD14 receptor complex with LPS (reviewed in reference 50). Thus, suppression of complement activation by the Vi capsular polysaccharide might explain why expression of this virulence factor diminishes the induction of TLR4/MD2/CD14-dependent proinflammatory responses (10–13). Expression of the Vi capsular polysaccharide in *S. Typhimurium* attenuates intestinal inflammation elicited in bovine ligated ileal loops (8) and in

TABLE 1 Bacterial strains used in this study

Bacterial strain	Description or relevant phenotype or genotype	Reference
<i>S. Typhimurium</i> strains		
IR715	ATCC 14028; Nal <sup>r</sup> derivative	53
RC31	IR715 <i>fepE</i> ::pGP704	32
TH170	IR715 <i>phoN</i> :: <i>viaB</i>	9
RC60	IR715 <i>phoN</i> :: <i>viaB fepE</i> ::pGP704	This study
<i>S. Typhi</i> strains		
Ty2	Wild-type strain; ATCC 700931	
STY2	Ty2 <i>viaB</i> ::Kan	10

the mouse colitis model (9). Interestingly, suppression of intestinal inflammation by the Vi capsular polysaccharide was significantly enhanced when production of very-long O-antigen chains was abrogated by inactivation of the *fepE* gene. These data suggest that conversion of *fepE* into a pseudogene enhanced the ability of *S. Typhi* to suppress or delay intestinal inflammation using the Vi capsular polysaccharide.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains used in this study are presented in Table 1. A plasmid carrying the *S. enterica* serovar Typhimurium *fepE* gene cloned into the low-copy-number vector pWSK129 has been described previously (32). Bacterial cultures were routinely incubated with aeration at 37°C in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) or on LB agar plates (15 g agar per liter) unless capsule expression was desired. To induce expression of the Vi capsular polysaccharide, bacteria were grown in broth containing 10 g tryptone and 5 g yeast extract per liter. The following antibiotics were added as necessary at appropriate concentrations: chloramphenicol (Cm), 0.03 mg/ml; carbenicillin, 0.1 mg/ml; kanamycin (Kan), 0.05 mg/ml; and nalidixic acid, 0.05 mg/ml.

**Construction of mutants.** The *fepE*::pGP704 mutation was transduced from *S. Typhimurium* RC31 into the *S. Typhimurium phoN*::*viaB* mutant TH170 using transduction with phage P22 HT *int*-105 to yield strain RC60.

**Animal experiments.** All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis, and performed according to Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. Groups ( $n = 4$ ) of female mice (C57BL/6J) mice from Jackson Laboratory aged 6 to 8 weeks were inoculated intragastrically with 20 mg streptomycin in a volume of 0.1 ml 24 h prior to intragastric inoculation with either 0.1 ml sterile LB broth or  $1 \times 10^9$  CFU of the indicated *S. Typhimurium* strains. Colon contents and cecal tissues were harvested at 72 or 120 h after infection, homogenized in phosphate-buffered saline (PBS), serially diluted, and plated on LB agar plates containing the appropriate antibiotics to enumerate CFU.

**RNA extraction and quantitative real-time PCR.** Cecal tissue was homogenized in a minibeatbeater (BioSpec Products), and RNA was extracted by the TRI reagent method (Molecular Research Center) as described previously (51). Reverse transcription was performed using Taq-Man reagent (Applied Biosystems), and 2  $\mu$ l of converted cDNA was used with a 250 nM concentration of primers listed in Table 2 and SYBR green (Applied Biosystems) for real-time PCR in the ViiA 7 system (Life Technologies). Data were analyzed using the comparative threshold cycle method. Transcription levels of *Ifng*, *Tnfa*, *IL22*, *Kc*, and *Mip2* genes were

TABLE 2 Nucleotide primers for quantitative real-time PCR

Target gene	Nucleotide sequence
<i>Gapdh</i>	5'-TG TAGACCATGTAGTTGAGGTCA-3' 5'-AGGTCGGTGTGAACGGATTTG-3'
<i>Ifng</i>	5'-TCAAGTGGCATAGATGTGGAAGAA-3' 5'-TGGCTCTGCAGGATTTTCATG-3'
<i>Tnfa</i>	5'-TTGGGTCTTGTTCCTCCACGG-3' 5'-CCTCTTTCAGGTCACCTTTGGTAGG-3'
<i>IL22</i>	5'-GGCCAGCCTTCAGATAACA-3' 5'-GCTGATGTGACAGGAGCTGA-3'
<i>Kc</i>	5'-TGCACCCAAACCGAAGTCAT-3' 5'-TTGTCAGAAGCCAGCGTTTAC-3'
<i>Mip2</i>	5'-AGTGAAGCTGCGCTGTCAATGC-3' 5'-AGGCAAACCTTTTGACCGCC-3'

normalized to *Gapdh* mRNA (encoding glyceraldehyde-3-phosphate dehydrogenase [GAPDH]).

**Histopathology.** Cecal tissues were formalin fixed, sectioned, stained with hematoxylin and eosin (H&E) and submitted to a veterinary pathologist for blinded scoring using a scale described previously (52). Representative images of tissue sections were taken using an Olympus BX41 microscope.

**Analysis of Vi and LPS expression and C3 deposition by flow cytometry.** Detection of Vi and LPS expression by flow cytometry was performed as described previously (9) using the DNA-specific stain propidium iodide, rabbit anti-Vi serum, or anti-O:4 (1:250 dilution; Becton Dickinson) and goat anti-rabbit fluorescein isothiocyanate (FITC) conjugate (1:250 dilution; Jackson ImmunoResearch). Binding of complement component 3 (C3) was determined by flow cytometry as described previously (24) using the DNA-specific stain propidium iodide, human serum (10% dilution; Quidel), and fluorescein isothiocyanate (FITC)-conjugated goat anti-human C3b monoclonal antibody (1:250 dilution; MP Biomedicals). All samples were analyzed on an LSRII instrument (BD).

**Statistical analysis.** Relative abundance values of *S. Typhimurium* strains and fold changes in mRNA levels were converted logarithmically ( $\log_{10}$ ) prior to statistical analysis using a one-tailed parametric test (Student's *t* test). A *P* value of <0.05 was considered to be significant.

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