

Novel Determinants of Intestinal Colonization of *Salmonella enterica* Serotype Typhimurium Identified in Bovine Enteric Infection

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Cattle are naturally infected with *Salmonella enterica* serotype Typhimurium and exhibit pathological features of enteric salmonellosis that closely resemble those in humans. Cattle are the most relevant model of gastrointestinal disease resulting from non-typhoidal *Salmonella* infection in an animal with an intact microbiota. We utilized this model to screen a library of targeted single-gene deletion mutants to identify novel genes of *Salmonella* Typhimurium required for survival during enteric infection. Fifty-four candidate mutants were strongly selected, including numerous mutations in genes known to be important for gastrointestinal survival of salmonellae. Three genes with previously unproven phenotypes in gastrointestinal infection were tested in bovine ligated ileal loops. Two of these mutants, *STM3602* and *STM3846*, recapitulated the phenotype observed in the mutant pool. Complementation experiments successfully reversed the observed phenotypes, directly linking these genes to the colonization defects of the corresponding mutant strains. *STM3602* encodes a putative transcriptional regulator that may be involved in phosphonate utilization, and *STM3846* encodes a retron reverse transcriptase that produces a unique RNA-DNA hybrid molecule called multicopy single-stranded DNA. The genes identified in this study represent an exciting new class of virulence determinants for further mechanistic study to elucidate the strategies employed by *Salmonella* to survive within the small intestines of cattle.

Non-typhoidal salmonellae (NTS) are the leading cause of bacterial food-borne gastroenteritis in humans worldwide (1, 2) and are responsible for hundreds of millions of cases of gastroenteritis and bacteremia annually (3). In humans, gastrointestinal disease caused by NTS is characterized by neutrophilic infiltrates within the ileum and symptoms of inflammatory diarrhea (4).

Cattle are naturally susceptible to infection with NTS and develop inflammatory diarrhea histologically characterized by neutrophilic inflammation (5, 6). Cattle either clear the organism after resolution of disease or become persistently infected and continually shed *Salmonella enterica* in their feces (7). Approximately 30% of human cases of enteric salmonellosis originate from bovine sources (8). Therefore, knowledge of factors important for survival of *Salmonella* within the gastrointestinal tracts of cattle allows not only extrapolation to human disease but also the opportunity for creation of new strategies to reduce bovine colonization and thus reduce the contamination to the food supply and environment. Additionally, the use of calves as a model organism makes discoveries in this model directly applicable to farm animal populations.

Although there are many tractable animal models of salmonellosis, the majority of screening and the development of mechanistic understanding of NTS infection have historically been done in small animal models that do not naturally develop inflammatory diarrhea upon infection with NTS. These models include mice of the BALB/c, C57B6, 129SvJ, and CBA/J lineages (9–11). To more closely resemble human disease, mice can be treated with antimicrobial agents to eliminate natural microflora prior to infection with *Salmonella*. These pretreated animals do develop neutrophilic inflammation (commonly known as the murine colitis

model) (12–15). The murine colitis model is attractive because it requires minimal technical expertise and allows study of host factors involved in *Salmonella* pathogenesis through use of widely available immunological reagents and genetically altered mice. However, the lack of an intact microbiota precludes full evaluation of the strategies used by *Salmonella* to survive in the complex microbial ecosystem of the gastrointestinal tract. Thus, the use of an animal that is a natural host of *Salmonella* is optimal to understand the biology of *Salmonella* during infection.

The current animal model with intact microbiota that most closely resembles gastrointestinal salmonellosis in humans in both clinical presentation and histopathology is the calf model of infection (5, 16, 17). This model, although expensive and complex to use, has become very useful for identification of bacterial factors necessary for NTS to thrive in the complex environment of the gastrointestinal tract (18–21). Bovine ligated ileal loops have been

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used to elucidate the absolute requirement of the type III secretion system (TTSS) and effectors encoded by genes on the *Salmonella* pathogenicity island 1 (SPI-1) for development of neutrophilic enteritis (6, 17, 21, 22). They have also been used to study the importance of flagella for virulence (18) and to understand the mechanism by which *Salmonella* employs the host inflammatory response to gain a survival advantage by the use of tetrathionate as a terminal electron acceptor (19). An additional benefit of the calf model is that it reliably replicates enteric salmonellosis in cattle, a population that contributes to the maintenance of *Salmonella* in the food supply and environment, allowing for development of novel preharvest interventions for this important zoonotic pathogen (5, 11, 23–25). However, because of its complexity, this model has not previously been used in an unbiased approach to study novel virulence factors.

Ligated ileal loops in calves provide a unique environment for the study of *Salmonella* pathogenesis where virulence factors necessary for establishing early infection may be identified in the presence of intact microbiota. We previously constructed a library of targeted single-gene deletion (SGD) mutants of *Salmonella enterica* serotype Typhimurium that we used to discover novel genes required for survival during systemic infection in BALB/c mice (26). In the work described here, we used this library of targeted single-gene deletions in combination with the calf ligated ileal loop model to identify novel genes used by *Salmonella* during enteric infection of a natural host. Using this strategy, we identified 54 mutant genes under selection. Over 20 of these genes have not previously been described as under selection in this model. We tested three mutants ($\Delta STM3602$, $\Delta STM3846$, and $\Delta STM4602$ mutants) and confirmed two in individual competitive infections, in addition to testing and confirming $\Delta phoP$ and $\Delta phoQ$ mutants (for a total of five mutants tested). Complementation *in trans* restored the ability of the $STM3602$ and $STM3846$ mutants to colonize ligated ileal loops. The genes we reveal here to have roles in colonization represent an exciting group for further study to elucidate the mechanisms that *Salmonella* species use to survive within and cause disease in the complex environment of the small intestine of cattle.

MATERIALS AND METHODS

Ethics statement. The Texas A&M University Institutional Animal Care and Use Committee approved all animal experiments, and all experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (27) and USDA Animal Welfare Regulations. Texas A&M has AAALAC-accredited animal facilities.

Bacterial strains. All bacterial strains are isogenic derivatives of virulent *Salmonella enterica* serotype Typhimurium ATCC 14028. The SGD mutant library was constructed as previously described (26). All bacteria were grown in Luria-Bertani (LB) broth or LB agar supplemented with kanamycin (50 mg/liter), nalidixic acid (50 mg/liter), carbenicillin (100 mg/liter), or streptomycin (100 mg/liter) where appropriate.

Construction of complementing plasmids. PCR products were generated by colony PCR using *Pfu* polymerase (Agilent Technologies). To obtain a 1.1-kb PCR product for $STM3602$, we used an annealing temperature of 45°C for 5 cycles, 58°C for a further 25 cycles, and the following primers: 3602forward (5'-GTTGAATTCTTCCGCTCGATCATTTTC-3') and 3602reverse (5'-GTCAAGCTTTCATACGGTAAACCGTATTTTATC-3'). To clone $STM3846$, a PCR using an annealing temperature of 45°C for 5 cycles, and 57°C for a further 25 cycles, generated a 1.7-kb product using the following primers: 3846forward (5'-GTCGAATTCAA GTTCATCCTCIGTTGTAATCTATC-3') and 3846reverse (5'-GTCA AGCTTTATCTACAGCGTTCTGTCTGC-3'). The appropriate length of

the PCR products was ensured by agarose gel electrophoresis. A poly(A) tail was added to the 3' end of the product using *Taq* polymerase (New England BioLabs) for 9 min at 72°C. PCR products were then ligated into pCR2.1 (TOPO TA cloning; Invitrogen) and transformed into chemically competent One Shot *Escherichia coli* (Invitrogen) using heat shock, following the manufacturer's instructions. Plasmids were isolated using the Qiagen miniprep kit (Qiagen), and the insert was removed by digestion with EcoRI (New England BioLabs). The insert was isolated from the plasmid backbone by agarose gel electrophoresis and gel purified using a QIAquick gel purification kit (Qiagen). The insert was then ligated into EcoRI-digested and gel-purified pWSK29 (28). Ligations were performed overnight at 14°C, using T4 DNA ligase (New England BioLabs). Ligation reactions were transformed into chemically competent *E. coli* XL1-Blue (p $STM3846$) or Mach One *E. coli* (p $STM3602$; Invitrogen). Transformants were obtained by selection on LB agar supplemented with carbenicillin and were streaked twice to single colonies. Plasmids were isolated using the Qiagen miniprep kit (Qiagen), and correct inserts were verified by restriction digestion of plasmids using BamHI or BstXI (New England BioLabs; p $STM3846$ and p $STM3602$, respectively). The desired sequence was confirmed by sequencing. Complementing plasmids were transformed into chemically competent *S. Typhimurium* LB5000 (restriction negative, modification positive) (29), and transformants were obtained by selection on LB with carbenicillin. Plasmids were then isolated as described above and transformed into $\Delta STM3846$ and $\Delta STM3602$ mutants using heat shock or electroporation, respectively. Mutants bearing complementing plasmids were purified by streaking twice for single colonies prior to use in competitive infection experiments.

Calves and ligated ileal loop surgery. Angus cross calves were obtained from a breeding herd at the Veterinary Medical Park at Texas A&M University. A total of 12 calves were used in this study, 3 for screening the mutant library and 9 for competitive infection and complementation analysis. Calves were separated from the dam at 1 day of age, and adequate passive transfer was estimated by measurement of serum total protein. Calves were housed in an AAALAC-approved barn, fed milk replacer twice daily, and provided with water and grass hay. Selective fecal cultures were performed at least once weekly to ensure calves remained negative for *Salmonella* spp. (6, 30).

At 3 to 6 weeks of age, calves were anesthetized for ligated ileal loop surgery as previously described (6, 16). A detailed description of the surgical procedure is available in the supplemental material. Briefly, calves were placed in left lateral recumbency, and a right flank incision was made. Twenty-four to thirty-eight 4- to 6-cm loops were tied within the ileum within grossly visible Peyer's patches, leaving 1-cm spacers between adjacent loops. Loops were infected individually with 3 ml of LB containing approximately 10^9 *Salmonella* Typhimurium. The intestine was returned to the abdomen, the incision was closed, and the calves were monitored under inhalant anesthesia for the duration of the experiment. At 12 h postinfection, the incision was opened, and each loop was individually excised. Calves were euthanized by barbiturate overdose (pentobarbital) administered intravenously.

SGD pool preparation, inoculation, and recovery from ligated ileal loops. The pool of ~1,000 SGD mutants prepared and described previously (26) was grown overnight at 37°C with agitation in LB supplemented with kanamycin. Overnight cultures were subcultured 1:100 into LB with kanamycin and incubated for 3 h at 37°C with agitation. The cultures were washed twice in sterile LB broth, and the concentration of organisms was adjusted to 10^9 CFU in 3 ml LB. A wild-type strain marked with streptomycin resistance in a neutral location, HA697 ($\Delta phoN::strep$) (31; H. J. Yang, L. Bogomolnaya, T. Endicott-Yazdani, M. M. Reynolds, S. Porwollik, M. McClelland, and H. Andrews-Polymeris, unpublished data), was added at a ratio of 1:500 (HA697/total inoculum) to measure the random loss of mutants in the pool. Eight ligated ileal loops were inoculated with the SGD library in three calves. Inoculum titers were determined by serial dilution and plating. Following excision of the infected loops, intestinal fluid, mucus, and tissue samples were harvested

TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description or relevant characteristic ^a	Source or reference
Bacterial strains		
14028s	Wild type	ATCC
HA420	14028s; spontaneous Nal ^r	31
Δ phoP mutant	Δ phoP::kan; Kan ^r	26
Δ phoQ mutant	Δ phoQ::kan; Kan ^r	26
Δ STM3602 mutant	Δ STM3602::kan; Kan ^r	26
Δ STM3846 mutant	Δ STM3846::kan; Kan ^r	26
Δ STM4206 mutant	Δ STM4206::kan; Kan ^r	26
HA697	Δ phoN::strep; Str ^r	Yang et al., unpublished
HA1501	HA420 Δ phoQ::kan; Nal ^r Kan ^r	This study
HA1473	HA420 Δ STM3602::kan; Nal ^r Kan ^r	This study
HA1444	HA420 Δ STM3846::kan; Nal ^r Kan ^r	This study
HA1482	HA420 Δ STM4206::kan; Nal ^r Kan ^r	This study
HA1474	HA1473 carrying pSTM3602	This study
HA1446	HA1444 carrying pSTM3846	This study
Plasmids		
pWSK29	Cloning vector; Amp ^r	28
pSTM3602	pWSK29::STM3602; Amp ^r	This study
pSTM3846	pWSK29::STM3846; Amp ^r	This study

^a Nal^r, nalidixic acid resistant; Kan^r, kanamycin resistant; Str^r, streptomycin resistant; Amp^r, ampicillin resistant.

and processed separately. Fluid volume, which is correlated with inflammatory response (21), was calculated by weighing each loop before and after removal of fluid. Data from only those loops with fluid accumulation similar to a wild-type (WT)-infected control loop were used. Mucus was gently scraped from the epithelial surface and diluted in 3 ml phosphate-buffered saline (PBS). The remaining tissue was diluted in 5 ml PBS. These specimens were subsequently homogenized, serially diluted in PBS, and plated for enumeration of CFU. The remaining homogenates were grown to stationary phase in LB supplemented with kanamycin and washed in PBS prior to extraction of total DNA.

Mutants with an observed phenotype during the library screen were selected for competitive infection experiments against the wild-type strain, HA420 (ATCC 14028 Nal^r). Mutations were moved into a clean genetic background using P22 transduction (32). Bacterial strains used for competitive infection experiments are listed in Table 1. Mutant strains and the isogenic WT were grown in LB with kanamycin and nalidixic acid (mutant) or with nalidixic acid alone (WT) as described above. The inoculum was prepared by mixing SGD mutant and WT at a 1:1 ratio. Ligated ileal loops from 3 to 8 calves were infected with the prepared inoculum, and the WT-to-mutant ratio of the inoculum was determined by serial dilutions and plating. Intestinal fluid, mucus, and tissue samples were processed as described above, and the WT-to-mutant ratio was determined by differential plating. The competitive index (CI) was determined by dividing the output ratio of WT to mutant by the inoculum ratio.

Microarray analysis. The protocol used to prepare transcripts from input and output pools for microarray analysis was essentially as previously described (26). Briefly, total DNA of input or output mutant pools was sonicated, poly(A) tails were added to the DNA fragments, and PCR was amplified with a primer targeting the shared portion of each mutant and a primer including oligo(dT) at the 3' end (26). PCR products were subjected to reverse transcription from a T7 RNA polymerase promoter located inside each mutant and a mixture of nucleoside triphosphates (NTPs) that included a fluorescently labeled UTP. The RNA was purified using the RNeasy minikit (Qiagen), and approximately 4 μ g of labeled RNA was hybridized to a NimbleGen tiling array of 387,000 50-mer oligonucleotides at 42°C for 16 h. The arrays were washed according to the manufacturer's protocol and scanned using a GenePix 4000B laser scan-

ner (Molecular Devices, Sunnyvale, CA) at 5- μ m resolution. Data were uploaded into WebArrayDB (33–35), and data were analyzed for peak height in the DNA directly downstream of each mutant location. The relative signal of each mutant was compared to the relative signal in a corresponding array of the same library prior to selection. All large changes in mutant representation were manually inspected and converted into a numerical score between -1 (strongly underrepresented in the output pool) and 1 (overrepresented in the output pool).

Data analysis. The SGD library was screened in a total of eight loops: four loops in one calf and two loops each in two further calves. The mean score for mutants in each calf was determined by calculating the mean score from multiple loops. The overall score for each mutant was the mean of data from all loops in the three calves. Mutants that were not represented in the input pool in all of the loops were excluded from further analysis. The intercalf variation was defined as the absolute value of the standard deviation of the mean scores from each calf. Mutants under selection in our screen of the SGD library were defined as those mutants with scores outside the 90% confidence interval of the mean scores and with an intercalf variation less than the calculated mean score.

For competitive infection experiments, the competitive index was defined as the ratio of WT to mutant in output normalized to the input ratio. Statistical significance was determined using Student's two-tailed *t* test with significance set at *P* < 0.05.

RESULTS

Screen for mutants under selection during enteritis in calves. In order to assess the fluctuation of mutant representation in the pool, we added strain HA697 (Δ phoN::strep), a derivative of the wild type marked with a streptomycin resistance cassette in a neutral location, to the input pool (31; Yang et al., unpublished). In the input pool, strain HA697 was present in a ratio relative to the total inoculum that approximated the representation of each individual mutant in the pool. By enumerating the representation of this mutant in the output pool relative to the total recovery of the pool, we observed less than 1.4-fold fluctuation of HA697 in intestinal tissue samples compared to the input pool (Fig. 1). As HA697 was inoculated into and recovered from loops in approximately the same proportion relative to the total pool, there appears to be only minimal random loss of mutants occurring during the incubation of our pool in ligated ileal loops.

In order to identify candidate mutant genes under selection in

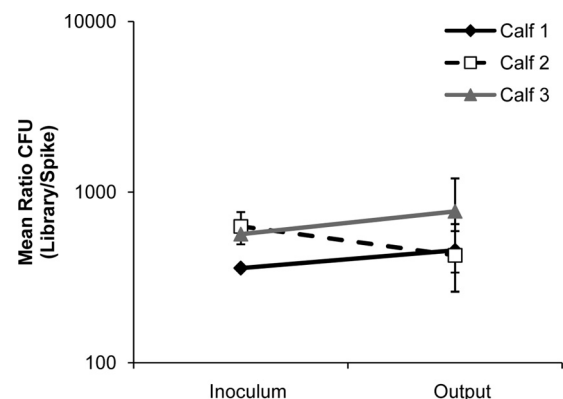


FIG 1 Mutant representation in the pool remains stable. The “input” pool of kanamycin-marked mutants was spiked with strain HA697 (Δ phoN::strep) at a ratio of 1:500 (HA697/total pool). Comparison of the representation of this mutant in the inoculum to the representation of this mutant in the output pools indicated less than 1.4-fold change in HA697 representation over the duration of the incubation of the pool in ligated ileal loops.

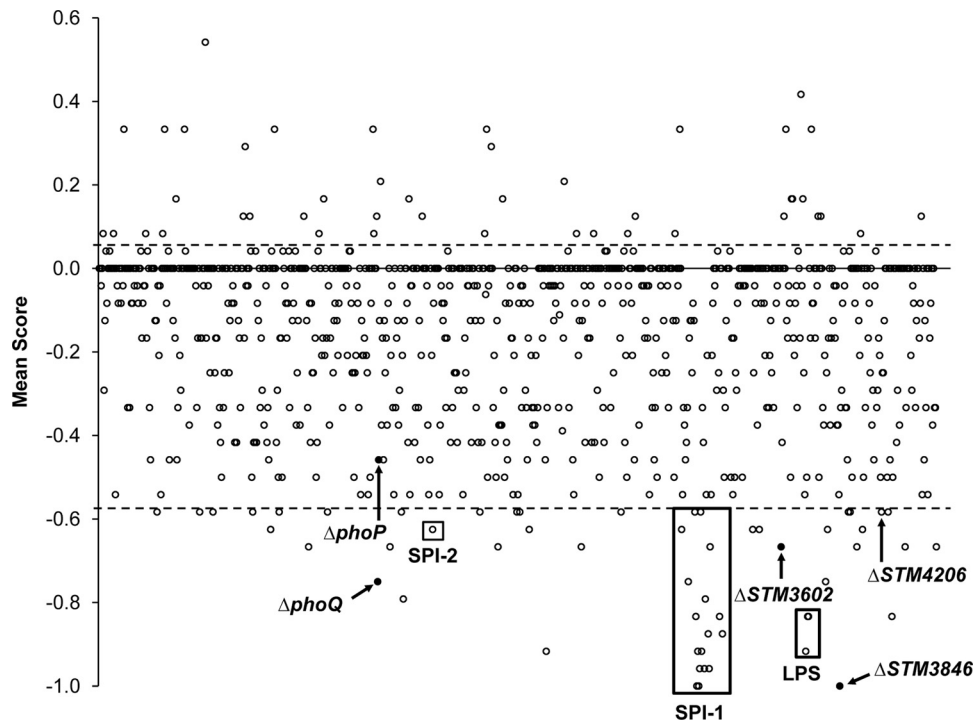


FIG 2 Loss of targeted mutant genes from the pool after inoculation into ligated ileal loops shown by genomic position. Eight ligated ileal loops from three different calves were infected, collected, and analyzed. The scores from multiple loops in a single calf were averaged, and then an overall mean score was generated from all three animals. The *x* axis represents mutant genes by genomic position with the mean score plotted on the *y* axis. The horizontal dotted lines indicate the 90% confidence interval. Mutants under selection were outside the 90% confidence interval with an intercalf standard deviation less than the absolute value of the given score. Labeled mutants are in known virulence factors, and those that were confirmed by individual competitive infection are shown in black.

ligated ileal loops, both the input pool and the output pool were used to prepare labeled transcripts unique to each mutant. Representation of mutants in the input pool versus the output pool was performed by analysis of labeled transcripts using a NimbleGen tiling array. The resulting data are presented both in Table S1 in the supplemental material and by genome position of each deleted gene represented in the SGD library in Fig. 2. We identified 54 mutant genes under selection in our screen with a mean score outside a 90% confidence interval and with an intercalf variation smaller than the mean (Table 2). We chose to exclude mutant genes with high intercalf variation because we found numerous mutants with a strong phenotype in only a single calf. These mutants had a mean score outside the 90% confidence interval but are considered outliers and not reported here.

Among the mutants with reduced fitness, 14 mutations were in genes located in *Salmonella* pathogenicity island 1 (SPI-1), and three were in genes needed for lipopolysaccharide (LPS) biosynthesis, all known to be important virulence determinants of *Salmonella* during enteric infection (21, 36–40). Numerous genes previously identified as virulence factors in animal hosts were also identified in our screen: *ssaK*, a gene within SPI-2 encoding a portion of the TTSS apparatus (41, 42); *phoQ*, the sensor in the two-component regulatory system *phoPQ* responsible for regulation of virulence genes (43, 44); *barA* and *sirA*, the sensor and regulator in a two-component regulatory system that regulates SPI-1 (45–47); *tatC*, a *sec*-independent transport protein responsible for resistance to bile salts (48); and *tonB*, a transport protein necessary for iron acquisition in the intestine (49).

We have already used this library to screen for mutant genes

under selection during systemic infection of BALB/c mice (26). We found 19 mutants to be under selection in both models (Table 2). Not surprisingly, of the genes not previously implicated to be important in enteric disease, only seven mutants were under selection in both models. These results confirm the necessity of different genes of *Salmonella* for survival in different niches during infection and show that our library is useful for identification of new virulence factors in different animal models.

Thirty-one mutant genes under selection were not previously proven to be essential for colonization of the bovine host. Of these 31 genes, six encode transcriptional regulators, four encode proteins involved in metabolism, two each encode proteins involved in protein modification and cell envelope biogenesis, and one each encodes a protein involved in DNA modification, cell motility, and secretion. Sixteen of our new mutants under selection have unknown function or are not assigned a group based on clusters of orthologous group assignments (50, 51). Ten of these genes had predicted phenotypes in screening of a library of transposon mutants during oral infection of a single calf, but no further characterization was performed to validate the results of either screen (52, 53).

Confirmation of fitness defects of candidate mutants in calves. We chose four mutants for confirmation by individual competitive infections with a derivative of the isogenic parental wild-type strain, ATCC 14028. These mutants, the Δ *phoQ*, Δ *STM3602*, Δ *STM3846*, and Δ *STM4206* mutants, were transduced to a clean genetic background by P22 transduction and tested in competitive infections in ligated ileal loops in at least three animals.

TABLE 2 Mutant genes under selection

Pathogenicity group or cluster of orthologous groups (COG)	Locus tag	Gene name
Salmonella pathogenicity island 1	STM2867 ^a	<i>hilC</i>
	STM2875 ^{b,c}	<i>hilD</i>
	STM2883 ^{b,c}	<i>sipD</i>
	STM2884 ^{b,c}	<i>sipC</i>
	STM2885 ^c	<i>sipB</i>
	STM2886 ^{a,b,c}	<i>sicA</i>
	STM2887 ^{a,c}	<i>spaS</i>
	STM2888 ^{a,b,c}	<i>spaR</i>
	STM2889 ^f	<i>spaQ</i>
	STM2892 ^e	<i>invJ</i>
	STM2893 ^{a,c}	<i>invI</i>
	STM2896 ^e	<i>invA</i>
	STM2897 ^{b,c}	<i>invE</i>
	STM2898	<i>invG</i>
	Salmonella pathogenicity island 2	STM1441 ^{a,b}
Salmonella pathogenicity island 4	STM4261 ^{b,c}	
Salmonella pathogenicity island 6	STM0296	
Cell envelope biogenesis	STM0719 ^a	
	STM1737	<i>tonB</i>
	STM3719 ^{a,b,c}	<i>rfaB</i>
	STM3722 ^{a,b,c}	<i>rfaG</i>
	STM3723 ^{a,c}	<i>rfaQ</i>
Cell motility and secretion	STM3975 ^{a,c}	<i>tatC</i>
DNA replication, recombination, and repair	STM3846 ^d	<i>rrtT</i>
Metabolism	STM0522 ^e	<i>allP</i>
	STM1636	
	STM2437	
	STM3781	
Posttranslational modification	STM3342 ^e	<i>sspA</i>
	STM4067	
Signal transduction	STM0398	<i>phoR</i>
	STM1230 ^{a,c}	<i>phoQ</i>
	STM1947 ^a	<i>sirA</i> or <i>uvrY</i>
	STM2958 ^{a,c}	<i>barA</i>
Transcriptional regulation	STM0031	
	STM0552 ^{a,c}	<i>fimW</i>
	STM1588 ^e	<i>yncC</i>
	STM3245 ^e	<i>tdcA</i>
	STM3602 ^b	
	STM4417 ^{a,c}	<i>iolR</i>
Unknown	STM0278	
	STM0285 ^e	
	STM1258 ^a	
	STM1329	
	STM1331	
	STM1785	
	STM1861	
	STM2209	
	STM3026	
	STM3954	<i>yigG</i>
	STM4030 ^a	
	STM4206 ^e	
	STM4302	
	STM4596 ^e	

^a This mutant was under selection during a library screen in systemic infection of BALB/c mice (26).

^b This mutant was identified during a screen of a transposon library in oral infection of a single calf (53).

^c This mutant was identified during a screen of a transposon library in oral infection of a single calf (52).

A Δ *phoP* mutant, although it was just outside the stringent cutoff of our screen, was also studied in competitive infection experiments because of its known function in resistance against host-derived antimicrobial peptides. We determined that the Δ *phoP* mutant has a statistically significant survival disadvantage relative to the wild-type organism in ligated ileal loops in calves, as expected (Fig. 3). We also confirmed that the Δ *phoQ* mutant, a candidate mutant from our screen, has statistically significant survival defects in this model (Fig. 3).

Using competitive infections, we confirmed that mutants with deletions of *STM3602* and *STM3846*, genes not previously linked to virulence during enteritis, colonize poorly during competitive infections in ligated ileal loops (Fig. 3). It was surprising to us that the Δ *STM3846* mutant is more severely affected in the calf intestine than the *phoP* and *phoQ* mutant genes, genes with a previously defined role in pathogenesis that confer only very modest phenotypes in the calf model (Fig. 3).

We also attempted to confirm the phenotype of a mutant with a deletion in *STM4206* during competitive infection in four calves. Although we were unable to confirm the phenotype of this mutant, the calf model has high variability between loops and between genetically nonidentical animals. Thus, we cannot exclude the possibility that the predicted phenotype of the Δ *STM4206* mutant could be confirmed if competitive infection experiments are performed in additional animals. To summarize, we were able to confirm the phenotypes of three (*STM3602*, *STM3846*, and *phoQ*) of four candidate mutant genes that met the stringent inclusion criteria of our screen in ligated ileal loops in calves.

Complementation analysis. In order to link the observed phenotypes to disrupted genes definitively, we chose to complement the two mutants with confirmed phenotypes in *trans* and retest these complemented mutants during infection of ligated ileal loops in calves. We cloned *STM3602*, which encodes a putative transcriptional regulator, and *STM3846*, which encodes a putative reverse transcriptase, onto a stable, low-copy-number plasmid and transformed these constructs into the corresponding deletion mutants. Complemented deletion mutants were tested in competitive infections with the isogenic wild-type organism in ligated ileal loops. In both cases, the fitness defect of the deletion mutant was reversed by placement of the corresponding gene in *trans* (Fig. 4).

The genomic contexts of *STM3602* and *STM3846* are shown in Fig. 5 (54). *STM3602* is located between *STM3601*, encoding a putative phosphosugar isomerase, and *treF*, encoding a trehalase. *STM3602* encodes a putative GntR family regulator and shares a conserved domain with *phnF*, encoding a regulator of phosphate utilization (55). *STM3846* encodes a reverse transcriptase that catalyzes the formation of an RNA-DNA hybrid molecule called multicopy single-stranded DNA, or msDNA (56–58).

DISCUSSION

We used a highly relevant model of enteric salmonellosis, bovine ligated ileal loops, to identify mutants under selection from our library of targeted deletion mutants in *Salmonella* Typhimurium (26). Our work is the first example of a screen of a mutant library in ligated ileal loops in calves, a technically challenging model that is highly relevant to human enteric salmonellosis. In addition, this work is the first to confirm predicted phenotypes in the bovine model.

The ligated ileal loop model is ideal for screening of a library of

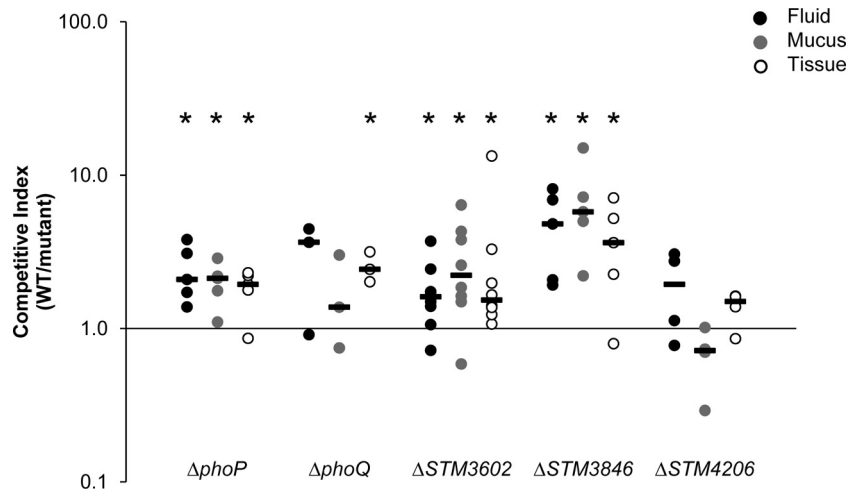


FIG 3 Individual competitive infection experiments confirm requirement for three genes during infection of ligated ileal loops. Ligated ileal loops were infected with a 1:1 mixture of the WT and an isogenic mutant, either the $\Delta phoP$ ($n = 5$), $\Delta phoQ$ (HA1501; $n = 3$), $\Delta STM3602$ (HA1473; $n = 8$), $\Delta STM3846$ (HA1444; $n = 5$), or $\Delta STM4206$ (HA1482; $n = 4$) mutant, for 12 h. Infected loops were harvested, and the fluid contents and mucus layer and intestinal tissue samples were processed for enumeration of bacteria and determination of competitive index. Each symbol represents the value for an individual ligated ileal loop. The short solid black bars are the median values. Asterisks indicate statistically significant differences between the ratio of the mutant and wild type in the inoculum and that ratio in the material collected at the termination of the infection (“output” ratio). A significant difference ($P < 0.05$) in the output-to-input ratio was determined using a two-tailed Student t test.

mutants to identify the early strategies utilized by *Salmonella* to survive within the small intestinal environment. Using this model, the “input” pool is administered directly into the ileum, reducing the random loss of mutants traveling through the upper gastrointestinal tract and ensuring that all mutants arrive at the small intestine at the same time. The short duration of infection allows reliable invasion of the epithelium and development of a robust host neutrophilic inflammatory response (6, 16). Thus, we are able to study the early factors responsible for survival of *Salmonella* within the lumen of the intestine and those factors necessary for creation of and survival during the host inflammatory response. In addition, this model provides an opportunity to dissect the genetic strategies required for survival in the different microenvironments within the small intestine—intestinal fluid, mucus, and tissue layers.

In this study, we evaluated the representation of mutants in the output pool isolated from intestinal tissue from 3 separate calves, because this site might include all mutants under selection in the intestinal luminal and mucus layers in addition to those mutants defective for invasion or survival within epithelial cells. We show that mutants in our pool experience a very low level of random fluctuation during infection of loops, removing a significant barrier to screening in this model. We screened our mutant library in eight loops in three calves, the first screen in any bovine model to use multiple calves to identify candidate mutant genes under selection. We repeated our screen in numerous animals because each of our outbred animals may respond to infection differently, thus placing different selection pressures on our library. To develop a list of candidate mutants for further study that had the strongest phenotypes and the highest probability of being true positives, we used stringent criteria to define a mutant as under selection, and this may have excluded some candidate mutants with relevant phenotypes from further analysis. One example of a mutant we know to be under selection in competitive infection that did not meet our defining criteria for significance in our library screen is the $\Delta phoP$ mutant.

Only two other screens have been performed in calves (52, 53). In both cases, randomly generated transposon libraries were screened after oral infection, and phenotypes were assigned to mutants from infection of a single calf. No further characterization of mutant phenotypes was performed in the bovine model. Despite the excellent genome coverage obtained by transposon mutagenesis, such studies provide only a list of candidate genes needed in the bovine host. They lack an estimated true-positive rate of discovery, making it difficult to determine how many of the candidate genes one would expect to have a relevant biological effect in the bovine host. This drawback is a critical roadblock to the design of future studies evaluating the importance of candidate *Salmonella* genes in the bovine host, a model that requires specialized housing, technical expertise, and great expense compared with conventional small animal models of disease.

Fourteen of the 54 genes under strong selection in our screen were located within SPI-1, and a single gene was located within SPI-2. Among these genes were regulators of expression of SPI-1 (*hilC* and *hilD*) and genes encoding portions of the TTSS-1 apparatus (*sipD*, *sipC*, *sipB*, *sipA*, *spaS*, *spaR*, *spaQ*, *invJ*, *invI*, *invA*, *invE*, and *invG*) (59). The requirement of the TTSS-1 and associated effectors for invasion of epithelial cells and creation of a host neutrophilic inflammatory response has been previously described using bovine models of enteric disease (21, 36–38) and has been replicated in the murine colitis model (13, 60). We also predict a phenotype for a single gene (*ssaK*) encoding a portion of the TTSS-2 apparatus. The TTSS-2 and associated effectors are necessary for virulence during systemic disease (61) and for induction of an inflammatory response in the intestine (36, 62). However, it is possible that 12 h of infection was not long enough to show a more pronounced phenotype for the remainder of the SPI-2 genes. These data show that our screen appropriately identifies virulence factors known to be important in both bovine and murine models of enteric salmonellosis.

Both *sirA* and *barA* mutants have predicted phenotypes in the calf model. These genes comprise a two-component regulatory

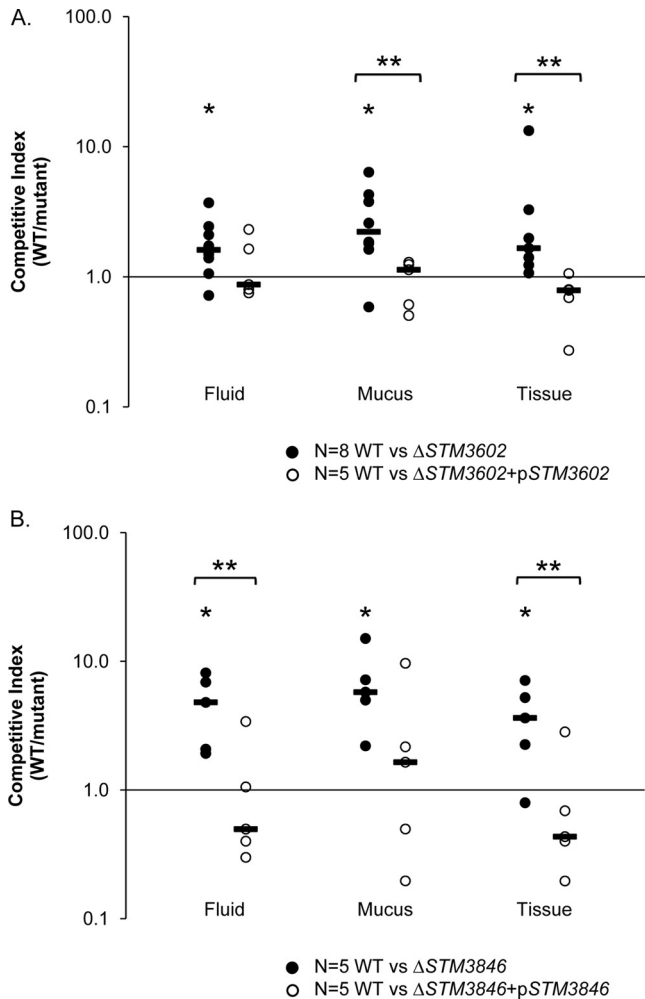


FIG 4 Complementation *in trans* reverses the observed phenotypes of $\Delta STM3602$ and $\Delta STM3846$ mutants. Ligated ileal loops from five calves were inoculated with the WT and the targeted deletion mutant complemented with an intact copy of the corresponding open reading frame *in trans* (open symbols). Data from the competitive infection experiments between the WT and targeted deletion mutant (Fig. 3, closed symbols), and statistical significance allows direct comparison to complementation data. (A) Competitive index for the wild type versus the complemented $\Delta STM3602$ mutant. (B) Competitive index for the wild type versus the complemented $\Delta STM3846$ mutant. In all panels, short solid black bars indicate the median values. Two asterisks indicate statistically significant differences between the ratio of the mutant and wild type in the inoculum and the ratio in the material collected at the termination of the infection ("output" ratio) ($P < 0.05$) between groups as determined by a two-tailed Student *t* test.

system that senses short-chain fatty acids within the intestine, causing activation of invasion gene expression via *hilA*, the master regulator of SPI-1 (46, 47, 63). Strains with mutations in each of these genes have reduced virulence during oral infection of BALB/c mice (47) but have not previously been proven to have a role during enteric infection in the bovine host.

In order to survive within the gastrointestinal tract, bacteria have mechanisms to resist antimicrobial peptides produced by the host. Within the small intestine, numerous antimicrobial peptides are constitutively produced by Paneth cells and are concentrated in the mucus covering the mucosa (64–66). One response of the mucosa to proinflammatory cytokines released as a result of *Sal-*

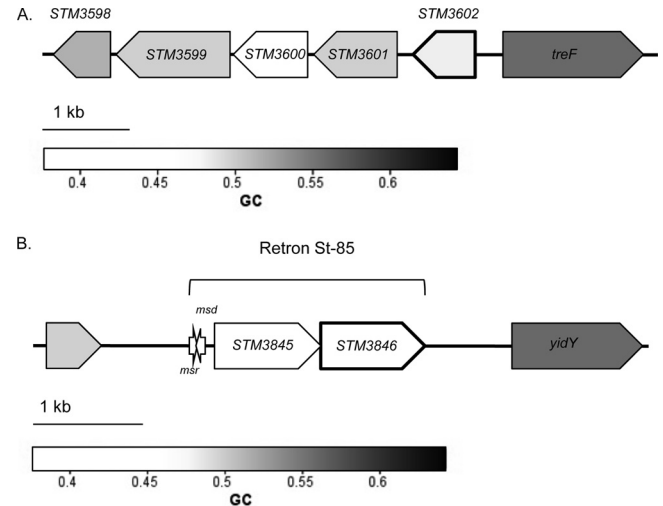


FIG 5 Schematic representation of the genomic regions containing *STM3602*, *STM3846*, and retron St-85. (A) Schematic diagram of the region surrounding *STM3602*. (B) Schematic diagram of the region surrounding *STM3846*. This gene is located on a 2.13-kb retron at position 4,051,144 on the chromosome between *STM3844*, a pseudogene encoding an integrase with a truncation at amino acid residue 164, and *yidY*, encoding a putative multidrug efflux system protein. The orientations of the *msr* and *msd* genes are indicated by the arrows. This figure was constructed using coliBASE (86) and data from the study by Ahmed and Shimamoto (56).

monella infection is to increase the production of defensins (67). Polymorphonuclear cells also contain numerous classes of antimicrobial peptides within cytoplasmic granules (68, 69). PhoP and PhoQ comprise a two-component regulatory system that responds to antimicrobial peptides to regulate genes for LPS biosynthesis and virulence (43, 70–73).

Therefore, we tested the phenotypes of $\Delta phoQ$ and $\Delta phoP$ mutants in competitive infection, even though the latter gene did not meet the stringent cutoff of our screen. We confirmed the predicted phenotype of our $\Delta phoQ$ mutant in intestinal tissue and found that a $\Delta phoP$ mutant also has a phenotype in bovine ligated ileal loops (Fig. 3). The $\Delta phoQ$ mutant was tested in competitive infection in only three calves, and the lack of an observed phenotype in intestinal mucus, the location with the greatest concentration of antimicrobial peptides, may be due to the small number of calves used in the study. The phenotypes we observed for each of these mutants in ligated ileal loops were mild but statistically significant (CI of ~ 2). These mild phenotypes are likely due to the short duration of infection or the variable production of antimicrobial peptides as a result of the variation in ages of calves used in this study (3 to 6 weeks). Recent reports indicate that 3-week-old Holstein-Friesian calves may not constitutively express much β -defensin in the gastrointestinal tract but that this expression increases with age (74). However, it is not known whether antimicrobial peptide production in intestinal tissue occurs in response to bacterial infections in calves of this age. Our data are the first to directly support the roles of the *phoPQ* regulatory system during survival of *Salmonella* in the inflamed intestinal tract.

STM3602 encodes a putative transcriptional regulator (54), and we show that this gene is necessary for survival in fluid, mucus, and tissue in ligated ileal loops (Fig. 3 and 4A). *STM3602* was predicted to be under selection in a signature-tagged mutagenesis screen of transposon mutants during oral infection of a calf (53),

but the predicted phenotype was never confirmed. This gene belongs to the GntR (gluconate operon repressor) family of regulators (75) and shares conserved domains with *phnF* (phosphonate utilization, E value of 1.29×10^{-68}) (76, 77), the regulator of the phosphonate utilization operon in *E. coli* (55).

Phosphonates are stable carbon-phosphorus bonds produced by bacteria and some marine invertebrates as a means of storage of phosphate (78, 79). *Salmonella* Typhimurium has a complete operon containing two genes for metabolism of phosphonate (*phnVUTSRWX*, *STM0426* to *STM0432*, GC content 56 to 60%) that is activated by inorganic phosphate during periods of phosphate starvation (55) and an additional locus involved in phosphonate metabolism (*phnOBA*, *STM4287* to *STM4289*, GC content 49 to 55%) (80). *STM3602* is located at a different chromosomal site (Fig. 5A) and has a much lower GC content (49.3%) than the *phnVUTSRWX* operon. Whether *STM3602* is involved in regulating phosphonate metabolism and whether this is related to the phenotype we observe during enteric infection is not yet clear. *STM3602* is a very interesting bacterial regulatory protein that merits further study to elucidate its precise function during enteric infection.

The second deletion mutant that we studied in this work, Δ *STM3846*, is deleted for a putative reverse transcriptase (54). This gene is encoded on a bacterial retroelement termed a retron (56–58, 81, 82). Bacterial retrons may be both horizontally and vertically acquired and produce a small multicopy single-stranded DNA molecule called msDNA, a unique RNA-DNA hybrid (58, 81, 82). *STM3846* is carried on the St-85 retron (Fig. 5b) containing two open reading frames (*STM3845* and *STM3846*) and a small segment of DNA upstream of these open reading frames that encodes the primer and template (*msr* and *msd*) used by the reverse transcriptase to produce the msDNA (56). Bacterial reverse transcriptases produce msDNA by using a leader RNA encoded by *msr* to prime the reaction and produce a 2',5' phosphodiester linkage between an RNA (encoded by *msr*) and DNA (encoded by *msd*) molecule (83).

The msDNA produced by *STM3846* is 85 bp in length, has a predicted stem-loop structure with no mismatched base pairs in the stem (56), and may have lost the RNA template (84). The *STM3846* reverse transcriptase is present in the genomes of all 19 *S. Typhimurium* isolates that we have sequenced (M. McClelland and P. Desai, unpublished data). No role for the St-85 retron has been established despite several previous studies of genes in this region (84, 85). Furthermore, other enterovirulent Gram-negative organisms, including *Vibrio* spp. and virulent *E. coli*, produce msDNAs (56), yet no phenotypes have been identified for mutants unable to produce any of these msDNAs. We are the first to unambiguously show a phenotype for a mutant lacking a bacterial reverse transcriptase, and this phenotype is for virulence in a highly relevant model of disease.

In the work we report here, we have used a library of targeted single-gene deletion mutants to identify novel colonization and virulence determinants of *Salmonella* Typhimurium during infection of bovine ileal loops, a technically challenging model highly relevant to human gastrointestinal salmonellosis. The bovine ileal loop model has not previously been used for unbiased screening of *Salmonella* mutants, although it closely replicates early events of enteric salmonellosis in humans. We identified more than 30 genes not previously proven to be important for survival of *Salmonella* in this model, and we confirmed 3 of these mutants indi-

vidually in competitive infections. Complementation analysis linked the observed phenotypes directly to the disrupted genes for mutations in a putative regulator (*STM3602*) and a reverse transcriptase (*STM3846*). We show that the reverse transcriptase encoded by *STM3846* is essential for virulence, and we show the first phenotype of any kind for a bacterial reverse transcriptase gene located on a retron. Finally, we report an exciting group of genes for further study to elucidate the mechanisms utilized by *Salmonella* for survival in the complex niche of the host small intestine during the inflammatory response.

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