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Title: Novel determinants of intestinal colonization of *Salmonella enterica* serotype Typhimurium identified in bovine enteric infection

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Running Title: Salmonella genes for bovine enteric infection

Abstract

Cattle are naturally infected with *Salmonella enterica* serotype Typhimurium and exhibit pathologic 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 deletion mutants to identify novel genes of *Salmonella* Typhimurium required for survival during enteric infection. Fifty-four candidate mutants were strongly selected, including numerous genes known to be important for gastrointestinal survival of salmonellae. Three mutants with previously unproven phenotypes in gastrointestinal infection were tested in bovine ligated ileal loops. Two of these mutants, in *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 mutants. *STM3602* is a putative transcriptional regulator that may be involved in phosphonate utilization and *STM3846* is 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 intestine of cattle.

Introduction

Non-typhoidal salmonellae (NTS) are the leading cause of bacterial foodborne gastroenteritis in people 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* in the 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 tract 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, 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 [10-12]. To more closely resemble human disease, mice can be treated with antimicrobials to eliminate natural microflora prior to infection with *Salmonella*. These pre-treated animals do develop neutrophilic inflammation (commonly

known as the murine colitis model) [13-16]. 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 people in both clinical presentation and histopathology is the calf model of infection [5, 17, 18]. 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 [19-22]. Bovine ligated ileal loops have been used to elucidate the absolute requirement of the type III secretion system (TTSS) and effectors encoded by *Salmonella* pathogenicity island-1 (SPI-1) for development of neutrophilic enteritis [6, 18, 22, 23]. They have also been used to study the importance of flagella for virulence [19], 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 [20]. 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 pre-harvest interventions for this important zoonotic pathogen [5, 12, 24-26]. 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* Typhimurium that we used to discover novel genes required for survival during systemic infection in BALB/c mice [27]. 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 mutants under selection. Over 20 of these genes have not previously been described as under selection in this model. We tested three mutants (in *STM3602, STM3846*, and *STM4602*) and confirmed two in individual competitive infections, in addition to testing and confirming Δ*phoP* and Δ*phoQ* mutants (for a total of 5 mutants tested). Complementation *in trans* restored the ability of mutants in *STM3602* and *STM3846* 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* use to survive within and cause disease in the complex environment of the small intestine of cattle.

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 PHS "Guide for the Care and Use of Laboratory Animals", and USDA Animal Welfare Regulations. Texas A&M has AALAC accredited animal facilities.

Bacterial strains

All bacterial strains are isogenic derivatives of virulent *Salmonella enterica* serotype Typhimurium ATCC14028. The SGD mutant library was constructed as previously described [27]. All bacteria were grown in Luria Bertani (LB) broth or LB agar supplemented with kanamycin (50 mg/L), nalidixic acid (50 mg/L), carbenicillin (100 mg/L), or streptomycin (100 mg/L) 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' GTTGAATTCTTCCGCCTCGATCATTTC 3' and 3602reverse 5' GTCAAGCTTTCATACGGTAAACCGTATTTTATC 3'. To clone *STM3846*, a PCR reaction 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' GTCGAATTCAAGTCTCATCCTCTGTTGTAATCTATC 3' and 3846reverse 5' GTCAAGCTTTATCTACAGCGTTCTGTCTGC 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 minutes at 72˚C. PCR products were then ligated into pCR2.1 (TOPO TA cloning; Invitrogen) and

transformed into chemically competent One Shot *E. coli* (Invitrogen) using heat shock, following manufacturer's instructions. Plasmids were isolated using the Qiagen Miniprep kit (Qiagen), and the insert was removed by digestion with *Eco*RI (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 *Eco*RI digested and gel purified pWSK29 [28]. Ligations were performed overnight at 14^{o}C, using T4 DNA ligase (New England Biolabs). Ligation reactions were transformed into chemically competent *E. coli* XL-1 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 *Bam*HI or *Bst*XI (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 -, modification +) [29], and transformants were obtained by selection on LB with carbenicillin. Plasmids were then isolated as above and transformed into Δ*STM3846* and Δ*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;

three 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 estimated by measurement of serum total protein. Calves were housed in an AALAC approved barn, fed milk replacer twice daily and were provided with free choice water and grass hay. Selective fecal cultures were performed at least once weekly to ensure calves remained negative for *Salmonella spp*. [6, 30].

At 3-6 weeks of age, calves were anesthetized for ligated ileal loop surgery as previously described [6, 17]. A detailed description of the surgical procedure is available in supplemental material. Briefly, calves were placed in left lateral recumbency and a right flank incision was made. Twenty-four to thirty eight 4-6 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 closed, and the calves were monitored under inhalant anesthesia for the duration of the experiment. At 12 hours post-infection the incision was opened and each loop individually excised. Calves were euthanized by barbiturate overdose (pentobarbital) administered intravenously.

SGD pool preparation, inoculation and recovery from ligated ileal loops

The pool of ~1000 SGD mutants prepared and described previously [27], 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 hours at 37˚C with agitation. 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 (Δ*phoN::strep*) [31, 32], was added at a ratio of 1:500 (HA697:total inoculum) to measure random loss of mutants in the pool. Eight ligated ileal loops were inoculated with the SGD library in 3 total calves. Inoculum titers were determined by serial dilution and plating. Following excision of the infected loops, intestinal fluid, mucus, and tissue were harvested and processed separately. Fluid volume, which is correlated with inflammatory response {Zhang, 2002 #144}, was calculated by weighing each loop before and after removal of fluid. Data from only those loops with similar fluid accumulation to a WT-infected control loop were used. Mucus was gently scraped from the epithelial surface and diluted in 3 mL PBS. The remaining tissue was diluted in 5 mL PBS. These specimens were subsequently homogenized and were 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 (WT), HA420 (ATCC 14028 Nal^R). Mutations were moved into a clean genetic background using P22 transduction [33]. 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 were processed as described above and the ratio of WT to mutant determined by differential plating. 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 [27]. Briefly, total DNA of input or output mutant pools was sonicated, poly-A tailed, and PCR amplified with a primer targeting the shared portion of each mutant and a primer including oligo dT at the 3' end [27]. PCR products were subjected to reverse transcription from a T7 RNA polymerase promoter located inside each mutant and a mixture of NTPs that included a fluorescently labeled UTP. The RNA was purified using the RNeasy Mini Kit (Qiagen), and approximately 4 micrograms of labeled RNA was hybridized to a NimbleGen tiling array of 387,000 50-mer oligonucleotides at 42°C for 16 hours. The arrays were washed according to the manufacturer's protocol, and scanned using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, California) at 5 μm resolution. Data was uploaded into WebArrayDB [34-36] and data 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 under-represented in the output pool) and 1 (over-represented in the output pool).

Data analysis

The SGD library was screened in a total of 8 loops: four in one calf, and two loops each in two further calves. A 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 3 calves. Mutants that were not represented in the input pool in all of the loops were excluded from further analysis. The inter-calf 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 inter-calf 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 a derivative of the wild type marked with streptomycin resistance cassette, HA697 (*ΔphoN*::Strep^R), in a neutral location to the input pool [31, 32]. In the input pool, 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 as compared to the input pool

(Figure 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 mutants under selection in 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 versus the output pools was performed by analysis of labeled transcripts using a Nimblegen tiling array. The resulting data are presented both in Table S1, and by genome position of each deleted gene represented in the SGD library in Figure 2. We identified 54 mutants under selection in our screen with a mean score outside a 90% confidence interval and with an inter-calf variation smaller than the mean (Table 2). We chose to exclude mutants with high inter-calf 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, fourteen mutants under selection were in genes located in *Salmonella* pathogenicity island-1 (SPI-1), and three were in genes needed for LPS biosynthesis, all known to be important virulence determinants of *Salmonella* during enteric infection [22, 37-41]. 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 [42, 43]; *phoQ*, the sensor in the two-component regulatory system *phoPQ* responsible for regulation of virulence genes [44, 45]; *barA* and *sirA*, the sensor and regulator in a two-component regulatory system that regulates SPI-1 [46-48]; *tatC*, a sec-independent transport protein responsible for

resistance to bile salts [49]; and *tonB*, a transport protein necessary for iron acquisition in the intestine [50].

We have already used this library to screen for mutants under selection during systemic infection of BALB/c mice [27]. We found nineteen 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 mutants under selection were not previously proven to be essential for colonization of the bovine host. Of these genes, six are transcriptional regulators, four are involved in metabolism, two each in protein modification and cell envelope biogenesis, and one each in DNA modification and 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 [51, 52]. 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 [53, 54].

Confirmation of fitness defects of candidate mutants in the calf

We chose four mutants for confirmation by individual competitive infections with a derivative of the isogenic parental wild type ATCC14028. These mutants, Δ*phoQ*, Δ*STM3602*, Δ*STM3846* and Δ*STM4206,* were transduced to a clean genetic

background by P22 transduction, and tested in competitive infections in ligated ileal loops in at least 3 animals.

A Δ*phoP* mutant, although it was just outside the stringent cut-off 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 was expected (Figure 3). We also confirmed that the Δ*phoQ* mutant, a candidate mutant from our screen, has statistically significant survival defects in this model (Figure 3).

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

We also attempted to confirm the phenotype of a deletion in *STM4206* during competitive infection in 4 calves. Although we were unable to confirm the phenotype of this mutant, the calf model has high variability between loops and between genetically non-identical 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

mutants 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 re-test these complemented mutants during infection of ligated ileal loops in calves. We cloned *STM3602*, a putative transcriptional regulator, and *STM3846*, 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* (Figure 4).

The genomic contexts of *STM3602* and *STM3846* are shown in Figure 5 [55]. *STM3602* is located between *STM3601*, a putative phosphosugar isomerase, and *treF*, a trehalase. *STM3602* encodes a putative GntR family regulator and shares a conserved domain with *phnF*, a regulator of phosphonate utilization [56]. *STM3846* encodes a reverse transcriptase that catalyzes the formation of an RNA-DNA hybrid molecule called multi-copy single-stranded DNA, or msDNA [57-59].

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

343 *Salmonella* Typhimurium [27]. Our work is the first example of a screen of a mutant
344 library in ligated ileal loops in calves, a technically challenging model that is highly
345 relevant to human enteric salmonellosis. In addition, this work is the first to confirm
346 predicted phenotypes in the bovine model.

347 The ligated ileal loop model is ideal for screening of a library of mutants to
348 identify the early strategies utilized by *Salmonella* to survive within the small intestinal
349 environment. Using this model, the 'input' pool is administered directly into the ileum
350 reducing random loss of mutants traveling through the upper gastrointestinal tract, and
351 ensuring that all mutants arrive at the small intestine at the same time. The short
352 duration of infection allows reliable invasion of the epithelium and development of a
353 robust host neutrophilic inflammatory response [6, 17]. Thus, we are able study the
354 early factors responsible for survival of *Salmonella* within the lumen of the intestine and
355 those factors necessary for creation of and survival during the host inflammatory
356 response. In addition, this model provides an opportunity to dissect the genetic
357 strategies required for survival in the different microenvironments within the small
358 intestine – intestinal fluid, mucus, and tissue layers.

359 In this study we evaluated the representation of mutants in the output pool
360 isolated from intestinal tissue of 3 separate calves because this site might include all
361 mutants under selection in the intestinal lumen and mucus layers in addition to those
362 mutants defective for invasion or survival within epithelial cells. We show that mutants in
363 our pool experience a very low level of random fluctuation during infection of loops,
364 removing a significant barrier to screening in this model. We screened our mutant library
365 in eight loops in three calves, the first screen in any bovine model to use multiple calves

366 to identify candidate mutants under selection. We repeated our screen in numerous
367 animals because each of our outbred animals may respond to infection differently thus
368 placing different selection pressures on our library. To develop a list of candidate
369 mutants for further study that had the strongest phenotypes and the highest probability
370 of being true positives we used stringent criteria to define a mutant as under selection,
371 and this may have excluded some candidate mutants with relevant phenotypes from
372 further analysis. One example of a mutant we know to be under selection in competitive
373 infection that did not meet our defining criteria for significance in our library screen is
374 Δ*phoP*.

375 Only two other screens have been performed in calves [53, 54]. In both cases,
376 randomly generated transposon libraries were screened after oral infection, and
377 phenotypes were assigned to mutants from infection of a single calf. No further
378 characterization of mutant phenotypes was performed in the bovine model. Despite the
379 excellent genome coverage obtained by transposon mutagenesis, such studies provide
380 only a list of candidate genes needed in the bovine host. They lack an estimated true
381 positive rate of discovery making it difficult to determine how many of the candidate
382 genes one would expect to have a relevant biological effect in the bovine host. This
383 drawback is a critical roadblock to the design of future studies evaluating the importance
384 of candidate *Salmonella* genes in the bovine host, a model that requires specialized
385 housing, technical expertise, and great expense as compared with conventional small
386 animal models of disease.

387 Fourteen of the fifty-four genes under strong selection in our screen were located
388 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 proteins encoding portions of the TTSS-1 apparatus (*sipD, sipC, sipB, sicA, spaS, spaR, spaQ, invJ, invI, invA, invE,* and *invG*) *[60].* 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 [22, 37-39] and has been replicated in the murine colitis model [14, 61]. 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 [62] and for induction of an inflammatory response in the intestine [37, 63]. However, it is possible that 12 hours 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 system that senses short-chain fatty acids within the intestine, causing activation of invasion gene expression via *hilA*, the master regulator of SPI-1 [47, 48, 64]. Mutants in each of these genes have reduced virulence during oral infection of BALB/c mice [48], 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 [65-67]. One response of the mucosa to pro-

412 inflammatory cytokines released as a result of *Salmonella* infection is to increase the
413 production of defensins [68]. Polymorphonuclear cells also contain numerous classes of
414 antimicrobial peptides within cytoplasmic granules [69, 70]. PhoP and PhoQ comprise a
415 two-component regulatory system that responds to antimicrobial peptides to regulate
416 genes for LPS biosynthesis and virulence [44, 71-74].

417 Therefore, we tested the phenotype of Δ*phoQ* and Δ*phoP* mutants in competitive
418 infection, even though the latter gene did not meet the stringent cut off of our screen.
419 We confirmed the predicted phenotype of our Δ*phoQ* mutant in intestinal tissue and
420 found that a Δ*phoP* mutant also has a phenotype in bovine ligated ileal loops (Figure 3).
421 The ΔphoQ mutant was tested in competitive infection in only 3 calves and the lack of
422 an observed phenotype in intestinal mucus, the location with the greatest concentration
423 of antimicrobial peptides, may be due to small number of calves used in the study. The
424 phenotypes we observed for each of these mutants in ligated ileal loops were mild but
425 statistically significant (CI of ~2). These mild phenotypes are likely due to the short
426 duration of infection or the variable production of antimicrobial peptides as a result of
427 the variation in ages of calves used in this study (3-6 weeks). Recent reports indicate
428 that 3-week old Holstein-Friesian calves may not constitutively express much ß-
429 defensin in the gastrointestinal tract but that this expression increases with age [75].
430 However, it is not known whether antimicrobial peptide production in intestinal tissue
431 occurs in response to bacterial infections in calves of this age. Our data are the first to
432 directly support the roles of the *phoPQ* regulatory system during survival of *Salmonella*
433 in the inflamed intestinal tract.

434 *STM3602* encodes a putative transcriptional regulator [55], and we show that this
435 gene is necessary for survival in fluid, mucus, and tissue in ligated ileal loops (Figure 3
436 and 4A). *STM3602* was predicted to be under selection in a signature-tagged
437 mutagenesis screen of transposon mutants during oral infection of a calf [54], but the
438 predicted phenotype was never confirmed. This gene belongs to the GntR (gluconate
439 operon repressor) family of regulators [76] and shares conserved domains with *phnF*
440 (phosphonate utilization, e-value 1.29e-68) [77, 78], the regulator of the phosphonate-
441 utilization operon in *E. coli* [56].
442 Phosphonates are stable carbon-phosphorus bonds produced by bacteria and
443 some marine invertebrates as a means of storage of phosphate [79, 80]. *Salmonella*
444 Typhimurium has a complete operon containing two genes for metabolism of
445 phosphonate (*phnVUTSRWX*, *STM0426-0432,* GC content 56-60%) that is activated by
446 inorganic phosphate during periods of phosphate starvation [56] and an additional locus
447 involved in phosphonate metabolism (*phnOBA*, *STM4287-4289*, GC content 49-55%)
448 [81]. *STM3602* is located at a different chromosomal site (Figure 5a), and has a much
449 lower GC content (49.3%) than the *phnVUTSRWX* operon. Whether STM3602 is
450 involved in regulating phosphonate metabolism, and whether this is related to the
451 phenotype we observe during enteric infection is not yet clear. *STM3602* is a very
452 interesting bacterial regulatory protein that merits further study to elucidate its precise
453 function during enteric infection.
454 The second deletion mutant that we studied in this work, Δ*STM3846,* is deleted
455 for a putative reverse transcriptase [55]. This gene is encoded on a bacterial retro-
456 element termed a retron [57-59, 82, 83]. 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 [59, 82, 83]. *STM3846* is encoded on the St-85 retron (Figure 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 the template (*msr* and *msd*) used by the reverse transcriptase to produce the msDNA [57]. 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 [84].

The msDNA produced by *STM3846* is 85 base pairs in length, has a predicted stem-loop structure with no mismatched base pairs in the stem [57] and may have lost the RNA template [85]. The *STM3846* reverse transcriptase is present in the genomes of all 19 serotype Typhimurium isolates that we have sequenced (McClelland *et al.*, unpublished data). No role for the St-85 retron has been established despite several previous studies of genes in this region [85, 86]. Furthermore, other entero-virulent Gram-negative organisms, including *Vibrio spp.* and virulent *E. coli,* produce msDNAs [57] 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 individually in competitive infections. Complementation analysis linked the observed phenotypes directly to the disrupted genes for mutants 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 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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817 **Figure Legends**

818 **Figure 1: Mutant representation in the pool remains stable.** The 'input' pool of
819 kanamycin-marked mutants was spiked with HA697 (*ΔphoN::strep*) at a ratio of 1:500
820 (HA697:total pool). Comparison of the representation of this mutant in the inoculum to
821 the representation of this mutant in the output pools indicated less than 1.4 fold change
822 in HA697 representation over the duration of the incubation of the pool in ligated ileal
823 loops.

824

825 **Figure 2: Loss of targeted mutants from the pool after inoculation into ligated**
826 **ileal loops shown by genomic position.** 8 ligated ileal loops from 3 different calves
827 were infected, collected and analyzed. Scores from multiple loops in a single calf were
828 averaged and then an overall mean score was generated from all 3 animals. The X-axis
829 represents mutants by genomic position with the mean score plotted on the Y-axis. The
830 horizontal dotted lines indicate the 90% confidence interval. Mutants under selection
831 were outside the 90% confidence interval with an inter-calf standard deviation less than
832 the absolute value of the given score. Labeled mutants are in known virulence factors
833 and those that were confirmed by individual competitive infection are in black.

834

835 **Figure 3: Individual competitive infection experiments confirm requirement for**
836 **three genes during infection of ligated ileal loops.** Ligated ileal loops were infected
837 with a 1:1 mixture of WT and an isogenic mutant, either Δ*phoP* (n=5), Δ*phoQ* (HA1501;
838 n=3), Δ*STM3602* (HA1473; n=8), Δ*STM3846* (HA1444; n=5), or Δ*STM4206* (HA1482;
839 n=4), for 12 hours. Infected loops were harvested and the fluid contents, mucus layer,

and intestinal tissue were processed for enumeration of bacteria and determination of competitive index. Solid bars indicate the median. (*) Asterisks indicate statistically significant differences between the ratio of the mutant and wild type in the inoculum versus in that ratio in the material collected at the termination of the infection ('output' ratio). Significant difference ($p<0.05$) in the output to input ratio was determined using a two-tailed Student t-test.

Figure 4: Complementation *in trans* reverses the observed phenotypes of mutants in Δ*STM3602* and Δ*STM3846*. Ligated ileal loops from 5 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 WT and targeted deletion mutant (closed symbols), and statistical significance (*Figure 3) allow direct comparison to complementation data. (A) Competitive index for wild type versus the complemented Δ*STM3602* mutant. (B) Competitive index for wild type versus the complemented Δ*STM3846* mutant. In all panels, solid bars indicate the median. (**) Double asterisks indicate statistically significant differences between the ratio of the mutant and wild type in the inoculum versus in that 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.

Figure 5: Schematic representation of the genomic regions containing *STM3602* and *STM3846* - retron St-85. (A) Schematic diagram of the region surrounding

863 *STM3602*. (B) Schematic diagram of the region surrounding *STM3846*. This gene is
864 located on a 2.13 kb retron at position 4,051,144 on the chromosome between
865 *STM3844*, a pseudogene encoding an integrase with a truncation at amino acid residue
866 164, and *yidY*, a putative multidrug efflux system protein. The orientations of *msr* and
867 *msd* are indicated with arrows. Figure adapted from Colibase [87] and [57].
868

869 **Tables**

870

871 **Table 1**: Bacterial strains and plasmids

Strains	Description	Reference or source
14028s	wild type	ATCC
HA420	14028s, spontaneous Nal^R	Bogomolnaya 2008
Δ*phoP*	*ΔphoP::kan*, Kan^R	Santiviago 2009
Δ*phoQ*	*ΔphoQ*::*kan*, Kan^R	Santiviago 2009
Δ*STM3602*	*ΔSTM3602*::*kan*, Kan^R	Santiviago 2009
Δ*STM3846*	*ΔSTM3846*::*kan*, Kan^R	Santiviago 2009
Δ*STM4206*	*ΔSTM4206*::*kan*, Kan^R	Santiviago 2009
HA697	Δ*phoN::strep,* Strep^R	Yang et al, in preparation
HA1501	HA420 *ΔphoQ*::*kan*, Nal^R Kan^R	This study
HA1473	HA420 *ΔSTM3602*::*kan*, Nal^R Kan^R	This study
HA1444	HA420 *ΔSTM3846*::*kan*, Nal^R Kan^R	This study
HA1482	HA420 *ΔSTM4206*::*kan*, Nal^R Kan^R	This study
HA1474	HA1473 + pSTM3602	This study
HA1446	HA1444 + pSTM3846	This study
Plasmids		
pWSK29	cloning vector, Amp^R	Wang 1991
pSTM3602	pWSK29::*STM3602*, Amp^R	This study
pSTM3846	pWSK29::*STM3846*, Amp^R	This study

872

873

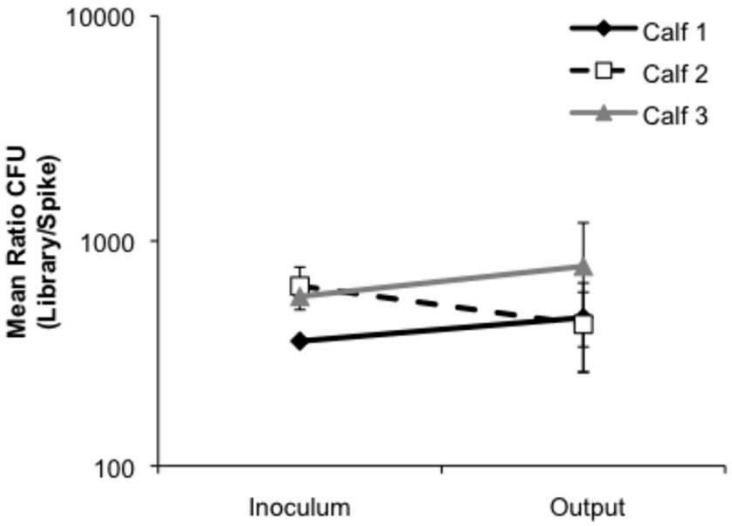
874 **Table 2**: Mutants under selection, grouped by pathogenicity island or clusters of
875 orthologous groups (COG). Asterisk (*) indicates the mutant was under selection during
876 library screen in systemic infection of BALB/c mice [27] and pound (#) [54] or dollar sign
877 ($) [53] indicates the mutant was identified during screen of a transposon library in oral
878 infection of a single calf.

Gene number	Name
***Salmonella* pathogenicity island-1**	
*STM2867**	*hilC*
STM2875#$*	*hilD*
STM2883#$*	*sipD*
STM2884#$*	*sipC*
STM2885$*	*sipB*
*STM2886**#$*	*sicA*
*STM2887**$*	*spaS*
*STM2888**#$*	*spaR*
STM2889$*	*spaQ*
STM2892$*	*invJ*
*STM2893**$*	*invI*
STM2896$*	*invA*
STM2897#$*	*invE*
STM2898	*invG*
***Salmonella* pathogenicity island-2**	
*STM1441**#*	*ssaK*
***Salmonella* pathogenicity island-4**	
STM4261#$*	
***Salmonella* pathogenicity island-6**	
STM0296	
Cell envelope biogenesis	
*STM0719**	
STM1737	*tonB*
*STM3719**#$*	*rfaB*
*STM3722**#$*	*rfaG*
*STM3723**$*	*rfaQ*
Cell motility and secretion	
*STM3975**$*	*tatC*
DNA replication, recombination, repair	
*STM3846**	*rrtT*
Metabolism	

STM0522[$]	*allP*
STM1636	
STM2437	
STM3781	
Post-translational modification	
STM3342[$]	*sspA*
STM4067	
Signal transduction	
STM0398	*phoR*
STM1230[*$]	*phoQ*
*STM1947**	*sirA/uvrY*
STM2958[*$]	*barA*
Transcriptional regulation	
STM0031	
STM0552[*$]	*fimW*
STM1588[$]	*yncC*
STM3245[$]	*tdcA*
STM3602[#]	
STM4417[*$]	*iolR*
Unknown	
STM0278	
STM0285[$]	
*STM1258**	
STM1329	
STM1331	
STM1785	
STM1861	
STM2209	
STM3026	
STM3954	*yigG*
*STM4030**	
STM4206[$]	
STM4302	
STM4596[$]	

879

Figure 1



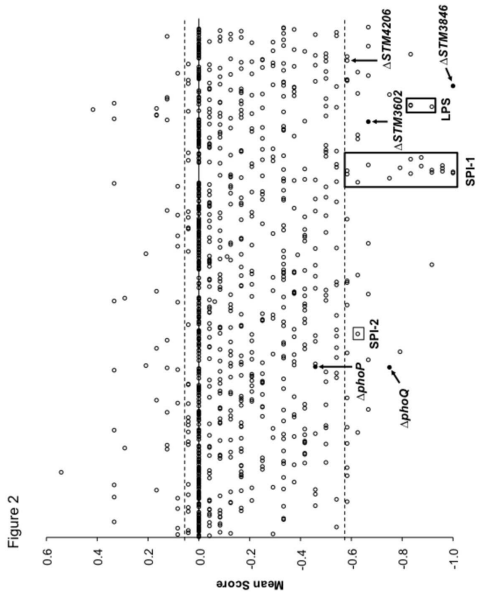


Figure 2

Figure 3

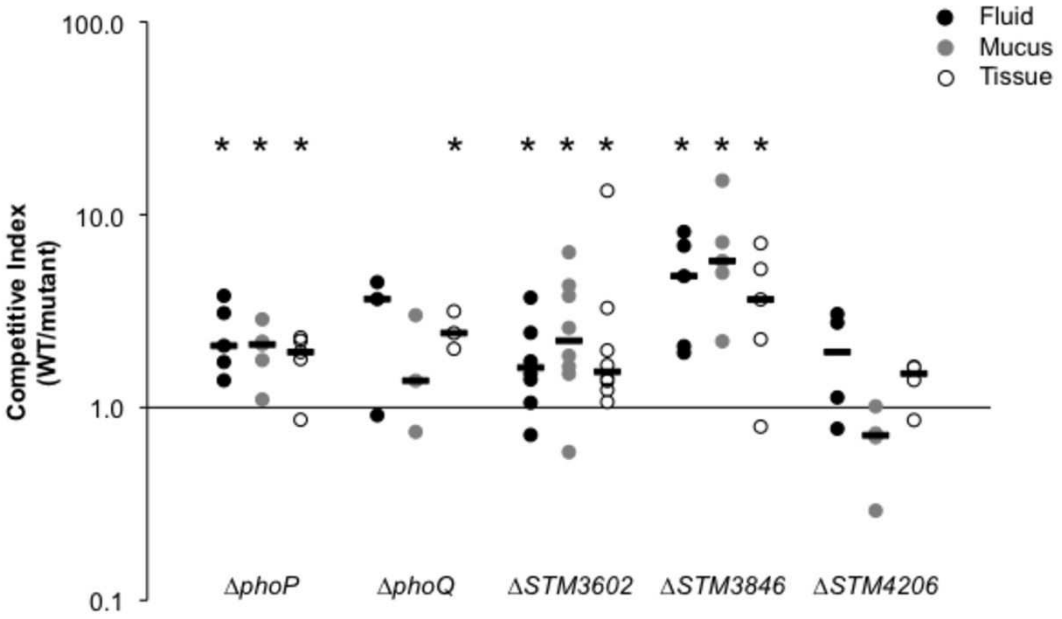


Figure 4

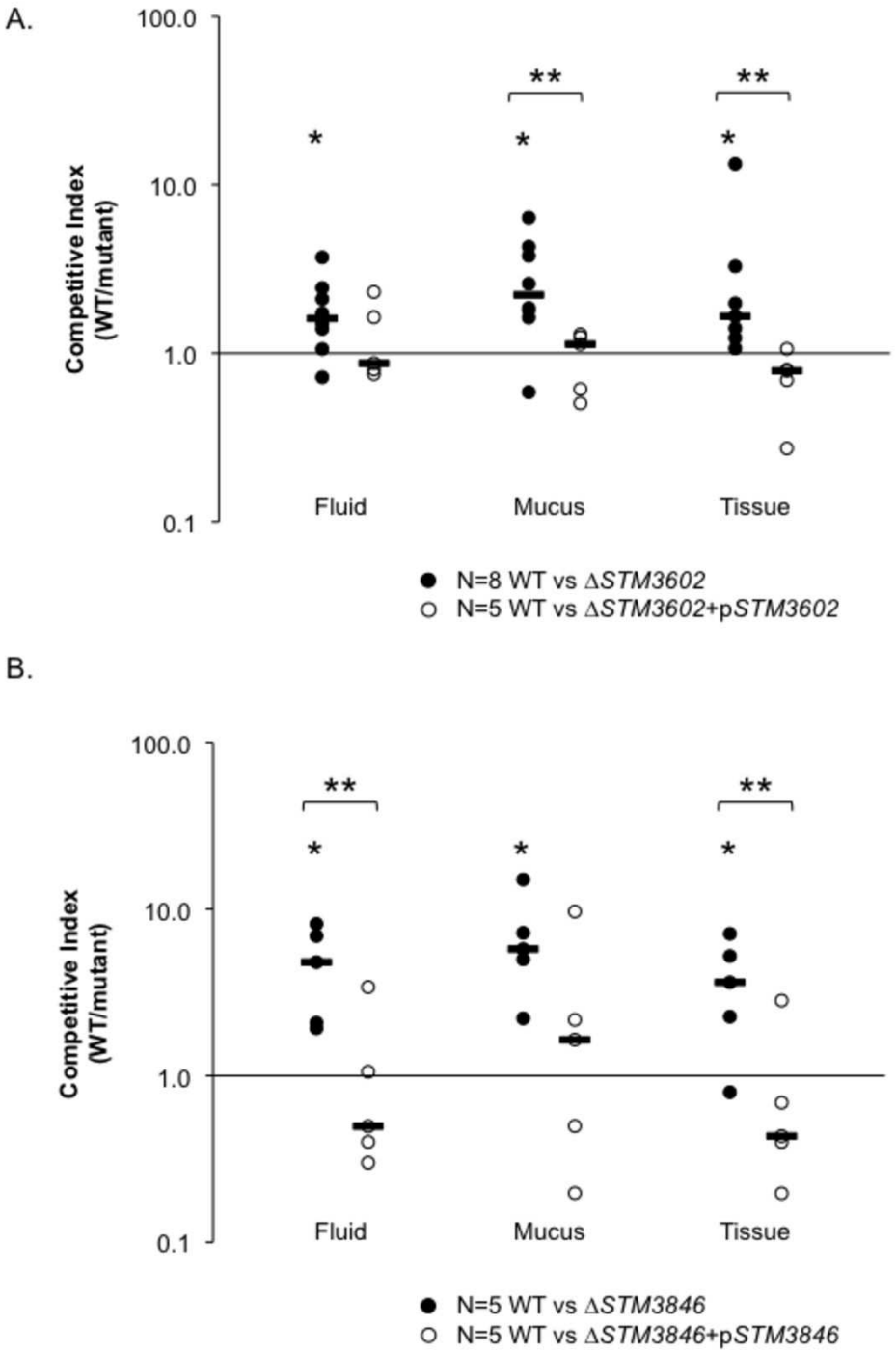


Figure 5