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Iron acquisition pathways and colonization of the inflamed intestine by *Salmonella enterica* serovar Typhimurium

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Abstract

Salmonella enterica serotype Typhimurium is able to expand in the lumen of the inflamed intestine through mechanisms that have not been fully resolved. Here we utilized streptomycin-pretreated mice and dextran sodium sulfate (DSS)-treated mice to investigate how pathways for *S. Typhimurium* iron acquisition contribute to pathogen expansion in the inflamed intestine. Competitive infection with an iron uptake-proficient *S. Typhimurium* strain and mutant strains lacking *tonB*, *feoB*, *tonB* or *iroN* in streptomycin pretreated mice demonstrated that ferric iron uptake requiring IroN and TonB conferred a fitness advantage during growth in the inflamed intestine. However, the fitness advantage conferred by ferrous iron uptake mechanisms was independent of inflammation and was only apparent in models where the normal microbiota composition had been disrupted by antibiotic treatment.

Keywords

Salmonella; Fe²⁺; Fe³⁺; inflammation; animal models

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Introduction

Salmonella Pathogenicity Island 1 (SPI-1) encodes a type III secretion system (T3SS-1) that is required for invasion of epithelial cells and induction of the host inflammatory response (Tsolis *et al.*, 1999; Barthel *et al.*, 2003; Altier, 2005; Bruno *et al.*, 2009; Raffatellu *et al.*, 2009). *Salmonella*-elicited intestinal inflammation generates an environment that favors growth of the pathogen while commensal microorganisms are suppressed (Stecher *et al.*, 2007). The expansion of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) in the inflamed gut is essential for transmission of the pathogen by the fecal-oral route (Lawley *et al.*, 2008).

S. Typhimurium requires iron as an essential nutrient for growth. Approximately 7% of the *S.* Typhimurium genome is regulated by levels of iron under *in vitro* conditions (Bjarnason *et al.*, 2003). In vertebrates, the availability of the ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms of iron are very limited extracellularly. One of the mechanisms by which the host limits iron in the inflamed intestine is by secreting lipocalin-2 (Raffatellu *et al.*, 2009), which has a bacteriostatic effect (Flo *et al.*, 2004) by binding to enterochelin, a high affinity iron chelator (siderophore) produced by bacteria of the *Enterobacteriaceae* family (Goetz *et al.*, 2002). Similarly to other *Enterobacteriaceae*, *S.* Typhimurium secretes enterochelin, but it also produces a glycosylated derivative of enterochelin, termed salmochelin, which is not bound by lipocalin-2 and therefore confers lipocalin-2 resistance (Hantke *et al.*, 2003; Crouch *et al.*, 2008; Raffatellu *et al.*, 2009).

Iron acquisition through salmochelin is mediated by the *iroBCDE* and *iroN* genes, which are responsible for the biosynthesis (*iroB*), export (*iroC*), and absorption (*iroN*) of salmochelin (Hantke *et al.*, 2003; Bäumlér *et al.*, 1996). The process of internalization of this siderophore is an energy dependent process, which requires the TonB/ExbB/ExbD protein-complex located at the inner membrane (Hannavy *et al.*, 1990). TonB energizes transport of ferric iron (Fe^{3+})-siderophore complexes into the periplasm of Gram-negative bacteria by interacting with specific outer membrane transporters (reviewed by Braun & Hantke, 2011), such as IroN. An additional source of iron that may be available in the anaerobic intestine is soluble ferrous iron (Fe^{2+}), which enters the periplasm through porins and is transported across the cytoplasmic membrane by a membrane transporter encoded by the *feoABC* operon. FeoB is the inner membrane Fe^{2+} transporter (Tsolis *et al.*, 1996; Boyer *et al.*, 2002).

Considering that iron acquisition is a key step in growth of *S.* Typhimurium, the aim of this study was to evaluate the role of acquiring Fe^{2+} and Fe^{3+} for expansion of *S.* Typhimurium in the lumen of the inflamed and non-inflamed intestine.

Material and Methods

Ethics statement

This study was approved by the Committee for Ethical Animals Use (CEUA) of the Universidade Federal de Minas Gerais (Brazil) under protocol numbers 197/2008, 254/2012 and 386/2013.

Bacterial strains and culture conditions

Bacterial strains are listed in Table 1. IR715 is a nalidixic acid-resistant *S. Typhimurium* strain derived from wild type ATCC14028. The *invA feoB* (SW710), *invA tonB* (SW711), and *invA tonB feoB* mutants (SW712) were constructed by introducing the *invA::pGP704* mutation from SW399 into AJB15 (*feoB* mutant in IR715 strain), AJB36 (*tonB* mutant in IR715 strain), and AJB62 *feoB tonB* mutant in IR715 strain), respectively, by generalized phage transduction (P22 HT *int-105*) (Schmieger, 1972). Confirmation of mutagenesis in *invA feoB* (SW710), *invA tonB* (SW711), and *invA tonB feoB* (SW712) was confirmed by PCR, with primers listed in Table S1. Mutation of *tonB* was confirmed by visualization of a fragment of 2.2 kb corresponding to a fragment of 0.9 kb of the band in the wild type strain plus the 1.3 kb of kanamycin resistance cassette. Mutation of *feoB* was confirmed by visualization of a fragment of 5 kb, which corresponds to the 3 kb fragment of wild type strain plus the 2 kb fragment from tetracycline resistance cassette. Mutation in *invA* was confirmed by visualization of a fragment of 0.5 kb in *invA feoB* (SW710), *invA tonB* (SW711), and *invA tonB feoB* (SW712) mutants, which is consistent with the insertion of *invA::pGP704* (Fig. S1).

Bacteria were cultured aerobically for 18 hours at 37°C under agitation in Luria-Bertani (LB) broth with appropriate antibiotics, followed by dilution 1:50 in LB broth (without antibiotic) and incubation for 3 hours at 37°C under agitation. Bacterial concentration was estimated by measuring the optical density of cultures at 600 nm (OD₆₀₀). Antibiotics were added to LB broth cultures and LB agar plates at the following concentrations: 50 mgL⁻¹ nalidixic acid, 12.5 mgL⁻¹ tetracycline, 100 mgL⁻¹ kanamycin, and 100 mgL⁻¹ ampicillin. Competitive indices were obtained by dividing the output ratio (CFU of the *phoN* strain over CFU of the iron-uptake-deficient mutant) by the input ratio (CFU of *phoN* strain over CFU of the iron-uptake-deficient mutant).

For *in vitro* growth, iron-uptake proficient *S. Typhimurium* strain (*phoN* mutant) and mutant strains of *S. Typhimurium* were cultured overnight at 37°C under agitation in Nutrient Broth (NB) with 0.2 mM 2,2'-dipyridyl (Sigma Aldrich). Microbiological growth was estimated by measuring OD₆₀₀. Cultures were inoculated with an initial 0.01 OD₆₀₀ in NB, NB supplemented with 40 µM FeSO₄, 40 µM FeCl₃ or 0.2 mM dipyridyl, and OD₆₀₀ was measured after 24 hours.

Streptomycin-pretreated mouse model

Six to eight-week-old female C57BL/6 mice were pretreated with streptomycin as previous described (Barthel *et al.*, 2003) and intragastrically inoculated with 0.1 mL of a 1:1 mixture containing 1×10⁷ CFU of *S. Typhimurium* IR715 tagged with *phoN* mutation (AJB715) and either one of the following mutant strains: *tonB feoB* (AJB62), or *feoB* (AJB15), or *tonB* (AJB36), or *iroN* (AJB52). In parallel, mice were infected under the same conditions with a *phoN invA S. Typhimurium* mutant strain (TH199) and either one of the following mutant strains: *invA tonB feoB* (SW712), or *invA feoB* (SW710), or *invA tonB* (SW711), or *invA iroN* (SPN454). Samples were collected at 48 hours post infection (hpi) for bacteriologic culture on LB + X-Phos to distinguish between wild type, which yields white colonies on X-Phos-containing medium and iron uptake mutants, which (since they express the acid

phosphatase encoded by *phoN*) grow as blue colonies on X-Phos medium. Further, tissue samples were collected for histopathology and quantitative real time RT-PCR (qRT-PCR). Fecal pellets were homogenized in 2 mL of sterile PBS and plated on LB agar containing nalidixic acid and on LB agar containing nalidixic acid and other appropriate antibiotics to the respective mutant strain. Cecum was collected for histopathological and qRT-PCR analysis. Total RNA was extracted from cecum using the Tri-reagent (Molecular Research Center) and processed as described (Raffatellu *et al.*, 2008). qRT-PCR was performed using SYBR Green (Applied Biosystems), 7900HT Fast Real-Time PCR System and data were analyzed using the comparative delta-Ct method (Livak & Schmittgen, 2001) normalized by transcriptional level of murine gene *Gapdh*. Primers used for qRT-PCR are listed in Table S2.

Dextran sulfate sodium (DSS) mouse model of enteritis

Six to eight-week-old female C57BL/6 mice received oral administration of 3% DSS diluted in sterile water *ad libitum*. This concentration of DSS was selected because preliminary experiments (not shown) showed that it elicits significant submucosal inflammation, submucosal edema and mucosal inflammation. After 120 h of DSS administration, mice were submitted to 4 h of food and water withdrawal, and then inoculated with 0.1 mL of a 1:1 mixture containing 1×10^7 CFU of *S. Typhimurium phoN* mutant (AJB715), and one of the following mutant strains: *tonB feoB* (AJB62), or *feoB* (AJB15), or *tonB* (AJB36), or *iroN* (AJB52). DSS treatment was maintained until euthanasia. Cecum and colon were collected at 48 hpi for bacteriologic culture and histopathology.

Bovine ligated ileal loop model

Four healthy 4-week-old male *Salmonella*-free Holstein calves were used. Ligated ileal loops were surgically made as previously described (Santos *et al.*, 2002). Loops were inoculated with intraluminal injection of 3 mL of sterile LB broth or with 3 mL of a 1:1 mixture containing 1×10^9 CFU of the reference strain *phoN* (AJB715), and one of the following mutant strains: *tonB feoB* (AJB62), or *feoB* (AJB15), or *tonB* (AJB36), or *iroN* (AJB52). In parallel, loops were inoculated under the same conditions with a *phoN invA* mutant (TH199), and one of the following mutant strains: *invA tonB feoB* (SW712), or *invA feoB* (SW710), or *invA tonB* (SW711), or *invA iroN* (SPN454). Samples were collected at 8 hpi for bacteriology and histopathology. Intestinal fluid and homogenates from fragments of the intestinal mucosa obtained with 6 mm biopsy punches in 2 mL of sterile phosphate-buffered saline (PBS) were plated on LB agar plates containing nalidixic acid and appropriate antibiotics.

Statistical analysis

Bacteriology and mRNA levels measured by qRT-PCR were logarithmically transformed and analyzed by ANOVA followed by Tukey's test. Histopathology scores were analyzed by Mann Whitney test.

Results and discussion

To investigate the role of Fe^{2+} and Fe^{3+} uptake systems during *S. Typhimurium* pathogenesis, we generated *S. Typhimurium* strains defective for Fe^{3+} uptake (*tonB* mutant), defective for Fe^{3+} -salmochelin uptake (*iroN* mutant), a mutant strain lacking the Fe^{2+} uptake system (*feoB* mutant), and a mutant strain incapable of acquiring both Fe^{2+} and Fe^{3+} (*tonB feoB* mutant) (Fig. S1). The fitness of these strains was compared to a *S. Typhimurium* strain that was marked by an antibiotic resistance marker inserted in the *phoN* gene (*phoN* mutant), to facilitate recovery from animals. Inactivation of the *phoN* gene does not reduce the ability of *S. Typhimurium* to colonize organs or the cecum of mice (Weening *et al.*, 2005). We first compared the growth of the iron-uptake proficient strain (*phoN* mutant, AJB715) to the growth of strains carrying mutations in genes involved in iron-acquisition (the *iroN*, *tonB*, *feoB* and *tonB feoB* mutant strains) in iron-replete medium (nutrient broth [NB] supplemented with FeSO_4 or FeCl_3), low-iron medium (NB) or iron-deplete medium (NB + dipiridyl) (Fig. 1). As expected, all strains grew equally well in iron-replete medium. Only the mutant strain incapable of acquiring both Fe^{2+} and Fe^{3+} (*tonB feoB* mutant) exhibited impairment growth in low-iron medium. In iron-deplete medium, both the *tonB* mutant and the *tonB feoB* mutant exhibited reduced growth. These data suggested that iron uptake systems were not required under iron-replete conditions. Either intact Fe^{2+} or intact Fe^{3+} uptake mechanisms were sufficient for supporting growth in low-iron medium. However, under conditions of severe iron limitation, both Fe^{2+} and Fe^{3+} uptake mechanisms were required for growth.

Next, we wanted to determine whether intestinal inflammation alters the requirement for Fe^{2+} and Fe^{3+} uptake mechanisms during growth of *S. Typhimurium* in the intestinal lumen. To this end, we utilized the streptomycin-pretreated mouse model. Streptomycin-pretreated mice develop acute neutrophilic typhilitis after infection with *S. Typhimurium*, and this inflammatory response is dependent on the invasion-associated T3SS-1 (Barthel *et al.*, 2003). Therefore, to evaluate the role of Fe^{2+} and Fe^{3+} uptake mechanisms in intestinal colonization of *S. Typhimurium*, we compared the fitness of *iroN*, *tonB*, *feoB*, or *tonB feoB* mutant strains with that of an iron-uptake proficient *S. Typhimurium* strain (*phoN* mutant) in streptomycin-pretreated mice, using a competitive colonization assay. This experimental design was chosen to ensure that the iron uptake mutants, some of which may have colonization defects, are exposed to the same host inflammatory response that is induced by the iron uptake-proficient strains, since different levels of colonization are known to elicit different levels of inflammation and of lipocalin-2, a major iron-limiting response of the host in the inflamed intestine. To investigate the effect of intestinal inflammation on the outcome of these competitions, we used mutants that lack a functional T3SS-1 due to a mutation in *invA* (Raffatellu *et al.*, 2009). To this end we compared the fitness of *invA iroN*, *invA tonB*, *invA feoB*, or *invA tonB feoB* mutant strains with that of an iron-uptake proficient *S. Typhimurium* strain (*invA phoN* mutant) in streptomycin-pretreated mice. As expected (Barthel *et al.*, 2003), histopathological analysis (Fig. 2A and Fig. S2A) and quantification of *Cxcl2* and *Cxcl1* transcript levels (encoding the neutrophil chemoattractants Mip-2 and KC, respectively) by real time PCR (Fig. 2B and 2C) revealed that the intestinal inflammatory response was muted in mice infected with strain mixtures that lacked a

function T3SS-1 (due to a mutation in *invA*), compared to mice infected with T3SS-1-proficient strains. Importantly, *lcn2* (encoding lipocalin-2) transcription was blunted in mice infected with strain mixtures that lacked a functional T3SS-1 (Fig. 2D). Consistent with this observation, strains that lacked lipocalin-resistance (*iroN* mutant and *tonB* mutant) were recovered in lower numbers than an iron-uptake proficient, *phoN*-tagged *S. Typhimurium* when T3SS-1 was intact (Fig. 2E). However, reduced *Lcn2* expression caused by a mutation in *invA* (Fig. 2D) abrogated the fitness advantage of an iron-uptake proficient *S. Typhimurium* strain (*invA phoN* mutant) over strains lacking lipocalin-resistance (i.e. the *invA iroN* mutant and the *invA tonB* mutant). These results confirmed a previous study showing that lipocalin resistance confers a fitness advantage upon *S. Typhimurium* during growth of in the inflamed gut (Raffatellu *et al.*, 2009). Our results are also consistent with a previous study that demonstrated a modest effect of a *tonB* mutation on intestinal colonization in mice infected intragastrically (IG) in the absence of streptomycin (Tsolis *et al.*, 1996). Fe^{2+} uptake conferred a fitness advantage in the mouse colitis model, but the magnitude of this fitness advantage was similar in strains with functional T3SS-1 (*phoN* vs. *feoB*) and defective T3SS-1 (*invA phoN* vs. *invA feoB*). These data suggested that Fe^{2+} uptake is equally required under both inflammatory and non-inflammatory conditions in the intestine. These data were in agreement with a previous study showing a contribution of FeoB to fitness of *S. Typhimurium* in the non-inflamed intestine (Deriu *et al.*, 2013). In the setting of inflammation, TonB-dependent iron uptake was also required for the probiotic activity of *E. coli* Nissle 1917 (Deriu *et al.*, 2013), suggesting that Fe^{3+} is limiting to both *E. coli* and *Salmonella* in the inflamed intestine. Our data suggest that Fe^{2+} and Fe^{3+} uptake mechanisms cooperated during intestinal inflammation, since the *phoN* mutant was recovered 361-fold higher numbers than the *tonB feoB* mutant from feces. This fitness advantage was markedly reduced in the absence of a functional T3SS-1 (*invA phoN* vs. *invA tonB feoB*).

Streptomycin induces marked changes in the composition of the intestinal microbiota (dysbiosis), with a decrease in the diversity of microbial communities in the cecum and ileum of mice infected with *S. Typhimurium* (Garner *et al.*, 2009). Furthermore, streptomycin treated mice develop mild cecal inflammation even in the absence of *S. Typhimurium* infection (Spees *et al.*, 2013). In order to investigate the general influence of inflammation on iron uptake mechanisms in the intestine in the absence of confounding effect of antibiotic-mediated microbiota disruption, competitive infections of *S. Typhimurium* and the mutant strains included in this study were performed in mice with chemically induced (by 3% DSS) acute intestinal inflammation. DSS treatment induces acute and chronic colitis in mice. The acute phase is characterized by a neutrophilic ulcerative colitis, body weight loss, and diarrhea (Okayasu *et al.*, 1990). As expected, mice treated with 3% DSS in the absence of *S. Typhimurium* infection developed inflammatory changes in the colon (not shown). DSS treated mice inoculated with mixtures of wild-type *S. Typhimurium* (*phoN* mutant) and *feoB*, *tonB*, *iroN* or *tonB feoB* mutants developed inflammatory changes in the cecum and colon after 48h that were similar to mice treated with 3% DSS only. This similarity was expected, since in untreated mice development of intestinal inflammation in response to *S. Typhimurium* infection takes several days (Rivera-Chávez *et al.*, 2016). Histopathologic analysis of cecum (Fig. 3A and Fig. S2B) and colon

(Fig. 3B and Fig. S2C) revealed moderate neutrophilic and lympho-histio-plasmacytic infiltration in the mucosa and submucosa, and moderate submucosal edema.

Competitive indices between bacterial strains in intestinal tissues obtained at 48 hours after infection from 3% DSS-treated mice demonstrated that the iron-uptake proficient *S. Typhimurium* strain (*phoN* mutant) was recovered in 15- and 12-fold higher CFU numbers than the *tonB* mutant in the cecum and colon, respectively (Fig. 3C and 3D). Similar results were obtained with the *tonB feoB* mutant (Fig. 3C and 3D). *IroN*-mediated, also conferred a fitness advantage, as the iron-uptake proficient *S. Typhimurium* strain (*phoN* mutant) was recovered in 5- and 6-fold higher CFU numbers than a *iroN* mutant in the cecum (Fig. 3C) and colon (Fig. 3D), respectively. These results indicated that intestinal inflammation in DSS colitis model has similar influence in defective growth of mutant with loss of ferric iron acquisition system. However, the iron-uptake proficient *S. Typhimurium* strain (*phoN* mutant) was recovered in similar numbers as the *feoB* mutant strain from the cecum (Fig. 3C), and in only 3-fold higher numbers than *feoB* mutant from the colon tissues (Fig. 3D). The absence of an overt fitness advantage conferred by *FeoB* in the intestinal lumen was also observed in previous studies, in which mice were infected with *S. Typhimurium* in the absence of antibiotic treatment (Nagy *et al.*, 2014; Tsolis *et al.*, 1996).

The competitive advantage of *Salmonella* with intact iron acquisition pathways was also evaluated in cattle, a host species that naturally responds to *S. Typhimurium* with acute intestinal inflammation (Costa *et al.*, 2012), paralleling the clinical and pathological manifestations of *S. Typhimurium* infection in human patients. The T3SS-1 effector proteins encoded in SPI-1 were demonstrated to mediate influx of neutrophils and fluid accumulation in the bovine ligated ileal loop model (Santos *et al.*, 2002; Zhang *et al.*, 2002; Costa *et al.*, 2012). In the present study, fluid contents and bovine ileal tissues were collected at 8 hpi from loops inoculated with a 1:1 mixture of the reference strain and one of the mutant strains, similarly to mouse experiments described above. *S. Typhimurium* reference strain and the mutant strains were recovered in similar CFU numbers from intestinal fluid and the ileal mucosa (Fig. S3). There were also similar CFU numbers of *S. Typhimurium invA* and the *invA tonB feoB*, *invA feoB*, *invA tonB* or *invA iroN* mutants in the intestinal fluid and ileal mucosa (Fig. S3). Importantly, coinfection of the reference strain and *tonB feoB*, *feoB*, *tonB* or *iroN* mutants elicited a prominent inflammatory response at 8 hpi in the bovine ileal mucosa (not shown). These results are in agreement with previous report in which *S. Typhimurium* induced marked inflammatory changes at 5 hpi (Santos *et al.*, 2002). As expected, coinfection with *S. Typhimurium* mutant defective in invasion *invA* and the *invA tonB feoB*, *invA feoB*, *invA tonB* or *invA iroN* mutants caused markedly less pathology when compared to the *S. Typhimurium* reference strain (not shown). This was the first attempt to use bovine ileal ligated loop model to elucidate iron uptake of *S. Typhimurium*, but this experimental model has an intrinsic limitation, which is that the experiment can be conducted only for a few hours since the calf remains under anesthesia during the course of the experiment (Alves *et al.*, 2003). The number of doublings that can be accomplished during 8 h of infection in bovine ileal ligated loops is limited, which may explain why this assay did not reveal any competitive advantage to strains that carry intact iron uptake pathways in comparison to mutants.

In summary, ferric iron uptake mediated by salmochelin and by a TonB energy carrier of *S. Typhimurium* provided a fitness advantage in the inflamed intestine of streptomycin pretreated mice and during DSS-induced colitis. In contrast, ferrous iron uptake mediated by FeoB only provided benefit during intestinal colonization in streptomycin pretreated mice, but this advantage was not dependent on an inflamed intestinal environment, because it was neither altered by inactivation of T3SS-1 (Fig. 2E) nor observed in mice with DSS-induced colitis (Fig. 3C and 3D). Furthermore, in the absence of antibiotic treatment, inactivation of *feoB* does not result in an overt loss of fitness for colonizing the mouse intestine (Nagy *et al.*, 2014; Tsolis *et al.*, 1996). Collectively, these data suggest that a disruption of the microbiota might increase the availability of (or decrease competition for) Fe²⁺ in the gut environment, thereby increasing the importance of Fe²⁺ for growth in this environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Growth of an iron-uptake proficient *S. Typhimurium* strain (tagged with *phoN* mutation) and iron uptake-deficient mutants in iron-replete, low-iron and iron-deplete media. An iron-uptake proficient *S. Typhimurium* strain (tagged with *phoN* mutation) and *tonB feoB*, *feoB*, *tonB* and *iroN* mutants were inoculated at an initial optical density at 600 nm (OD₆₀₀) of 0.01 in NB, NB supplemented with 40 μM FeSO₄ (+FeSO₄), FeCl₃ 40 μM (+FeCl₃) or 0.2 mM dipyridyl (-Fe dipyridyl) and OD₆₀₀ measurement was performed after 24 hours. Bars represent means ± SD of three independent experiments. Asterisks indicate significant differences of treatments compared to NB media in the same strain, Tukey test, * $P < 0.001$. Number signs indicate significant differences between mutant strains compared to the iron-uptake proficient *S. Typhimurium* strain in the same treatment, Tukey test, # $P < 0.001$.

Fig. 2.

Intestinal colonization of C57BL/6 mice pre-treated with streptomycin and coinfecting with an iron-uptake proficient *S. Typhimurium* strain (tagged with a *phoN* mutation) and iron acquisition-deficient mutant strains. Mice were inoculated with 1:1 mixture of 1×10^7 CFU of the iron-uptake proficient *S. Typhimurium* *phoN* strain and one of the mutant strains: *tonB* *feoB*, *tonB* *feoB* *invA*, *feoB* *invA*, *tonB* *invA* or *iroN* *invA*. Alternatively, mice were inoculated with iron-uptake proficient T3SS-1-deficient *S. Typhimurium* strain (*invA* *phoN* mutant) and one of the mutant strains: *tonB* *feoB*, *tonB* *feoB* *invA*, *feoB* *invA*, *tonB* *invA* or *iroN* *invA*. Histological score of the cecum at 48 hours after infection (A). Bars represent the average of histopathological scores for submucosal inflammation (SI), mucosal inflammation (MI), and submucosal edema (SE). Scores for individual mice are shown in Fig. S2A. Transcription levels of *Cxcl2* (B), *Cxcl1* (C) and *Lcn2* (D) measured by qRT-PCR in cecum collected at 48 hours after infection. Bars represent fold change over mock inoculated tissues normalized by transcriptional level of *Gapdh*. Competitive index of iron-uptake proficient *S. Typhimurium* *phoN* strain and iron uptake-deficient mutants or *invA* mutant and *invA* iron defective mutants in pellet fecal at 48

hours after infection (E). Bars represent geometric means $\pm$ SEM. Significance of differences observed was analysed using a Mann-Whitney test (A) or ANOVA followed by Tukey's test (B–E) * $P < 0.05$; ** $P < 0.01$.

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Fig. 3.

Intestinal colonization of C57BL/6 mice treated with 3% DSS and intragastrically coinfecting with an iron-uptake proficient *S. Typhimurium* strain (*phoN* mutant) and iron acquisition-deficient mutant strains. Mice received DSS 3% diluted in water for 120 hours and were inoculated with 1:1 mixture of 1×10^7 CFU of the iron-uptake proficient *S. Typhimurium* strain (*phoN* mutant) and mutants *tonB feoB*, *feoB*, *tonB*, or *iroN*. Histopathological score of the cecum (A) and colon (B) at 48 hpi. Bars represent the average of histopathological scores for submucosal inflammation (SI), mucosal inflammation (MI), and submucosal edema (SE). Scores for individual mice are shown in Figs S2B and S2C. Colony-forming units (CFU) recovered in cecum (C) and colon (D) at 48 hpi. Bars represent mean and standard error (n=5). Significance of differences observed using a Mann-Whitney test (A–B) or ANOVA followed by Tukey's test (C–D). * $P < 0.05$; ** $P < 0.01$.

Table 1

Salmonella enterica serotype Typhimurium strains used in this study.

Strain	Genotype	Reference
AJB15	IR715 <i>feoB</i> ::Tet ^R	Tsolis <i>et al.</i> , 1996
AJB36	IR715 <i>tonB</i> ::Kan ^R	Tsolis <i>et al.</i> , 1996
AJB52	IR715 <i>iroN</i> ::pGP704-Amp ^R	Bäumler <i>et al.</i> , 1998
AJB62	IR715 <i>feoB</i> ::Tet ^R <i>tonB</i> ::Kan ^R	Tsolis <i>et al.</i> , 1996
AJB715	IR715 <i>phoN</i> ::Kan ^R	Kingsley <i>et al.</i> , 2003
IR715	ATCC 14028 Nal ^R	Stojiljkovic <i>et al.</i> , 1995
SPN452	IR715 <i>invA</i> :: <i>tetRA</i> <i>spiB</i> ::KSAC-Kan ^R	Raffatellu <i>et al.</i> , 2009
SPN454	IR715 <i>invA</i> :: <i>tetRA</i> <i>spiB</i> ::KSAC-Kan ^R <i>iroN</i> ::pGP704-Amp ^R	Raffatellu <i>et al.</i> , 2009
SW399	IR715 <i>invA</i> ::pGP704	Winter <i>et al.</i> , 2009
SW710	IR715 <i>invA</i> ::pGP704 <i>feoB</i> ::Tet ^R	This study
SW711	IR715 <i>invA</i> ::pGP704 <i>tonB</i> ::Kan ^R	This study
SW712	IR715 <i>invA</i> ::pGP704 <i>feoB</i> ::Tet ^R <i>tonB</i> ::Kan ^R	This study
TH199	IR715 <i>phoN</i> ::Kan ^R <i>invA</i> :: <i>tetra</i>	Winter <i>et al.</i> , 2014

^aTet^R: Tetracycline resistance (*tetRA*); Kan^R: Kanamycin resistance; Amp^R: Ampicillin resistance; Nal^R: Nalidixic acid resistance.