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Article

Probiotic Bacteria Reduce *Salmonella* Typhimurium Intestinal Colonization by Competing for Iron

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SUMMARY

Host inflammation alters the availability of nutrients such as iron to limit microbial growth. However, *Salmonella enterica* serovar Typhimurium thrives in the inflamed gut by scavenging for iron with siderophores. By administering *Escherichia coli* strain Nissle 1917, which assimilates iron by similar mechanisms, we show that this nonpathogenic bacterium can outcompete and reduce *S.* Typhimurium colonization in mouse models of acute colitis and chronic persistent infection. This probiotic activity depends on *E. coli* Nissle iron acquisition, given that mutants deficient in iron uptake colonize the intestine but do not reduce *S.* Typhimurium colonization. Additionally, the ability of *E. coli* Nissle to overcome iron restriction by the host protein lipocalin 2, which counteracts some siderophores, is essential, given that *S.* Typhimurium is unaffected by *E. coli* Nissle in lipocalin 2-deficient mice. Thus, iron availability impacts *S.* Typhimurium growth, and *E. coli* Nissle reduces *S.* Typhimurium intestinal colonization by competing for this limiting nutrient.

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is one of the leading causes of acute gastroenteritis, which is characterized by inflammatory diarrhea. Although the normal intestine is largely inhabited by commensal microbes, which largely include Bacteroides and Firmicutes, inflammation enhances the colonization of *S.* Typhimurium and other Enterobacteriaceae (Barman et al., 2008; Lawley et al., 2008; Lupp et al., 2007; Stecher et al., 2007). Recent studies have shown that *S.* Typhimurium thrives in the inflamed gut because it can utilize unique carbon and energy sources (Thiennimitr et al., 2011; Winter et al., 2010) and is resistant to antimicrobial proteins that are secreted by the host as part of the nutritional immune response (Liu et al., 2012; Raffatellu et al., 2009). *S.* Typhimurium employs specialized transporters to acquire essential micronutrient metals (Liu et al., 2012; Raffatellu et al., 2009), one of the most important being iron (Crouch et al., 2008; Raffatellu et al., 2009). Levels of free iron are extremely low in the host environment because of sequestration by host proteins, including heme, ferritin, transferrin, and lactoferrin (Andrews and Schmidt, 2007). Additional mechanisms are employed by the host to further limit iron availability during inflammation (Weinberg, 1984), including secretion of the hormone hepcidin, which prevents the gut from absorbing iron from the bloodstream by inhibiting the iron transporter ferroportin 1 (Ganz, 2003).

When starved for iron, bacteria synthesize and export small-molecule high-affinity iron chelators termed siderophores. Enterochelin is a catecholate-type siderophore secreted by all *Enterobacteriaceae*, including *Salmonella* and commensal *E. coli* (Raymond et al., 2003), which is sufficient to overcome the host's iron limitation in a normal (noninflamed) environment. However, during inflammatory responses, the host secretes lipocalin 2, an antimicrobial peptide that sequesters ferric enterochelin, thereby limiting the growth of strains such as commensal *E. coli* that rely solely upon enterochelin for siderophore-based iron acquisition (Berger et al., 2006; Flo et al., 2004). Some pathogens evade this response by synthesizing additional siderophores that are not sequestered by lipocalin 2 (Fischbach et al., 2006a). For instance, *Salmonella* can synthesize and secrete salmochelin (Müller et al., 2009), a C-glucosylated derivative of enterochelin that is too large to fit into the enterochelin-binding pocket of lipocalin 2 (Fischbach et al., 2006b; Hantke et al., 2003).

Probiotics are commensal microorganisms that are believed to exert beneficial effects on the host. *Escherichia coli* Nissle 1917 (*E. coli* Nissle, serotype O6:K5:H1) is a probiotic strain that was originally isolated from a soldier who appeared resistant to an outbreak of diarrhea (Nissle, 1959). *E. coli* Nissle has been shown to establish persistent colonization of the intestine and has been used to treat or prevent a variety of intestinal disorders (Cukrowska et al., 2002; Kruis et al., 2004; Lodinová-Zádniková and Sonnenborn, 1997; Möllenbrink and Bruckschen, 1994; Nissle, 1959), including acute enteritis (Henker et al., 2007), but the mechanistic basis for its protective actions is unknown. Given that salmochelin-mediated iron acquisition during inflammation enhances *S.* Typhimurium colonization (Raffatellu et al., 2009),

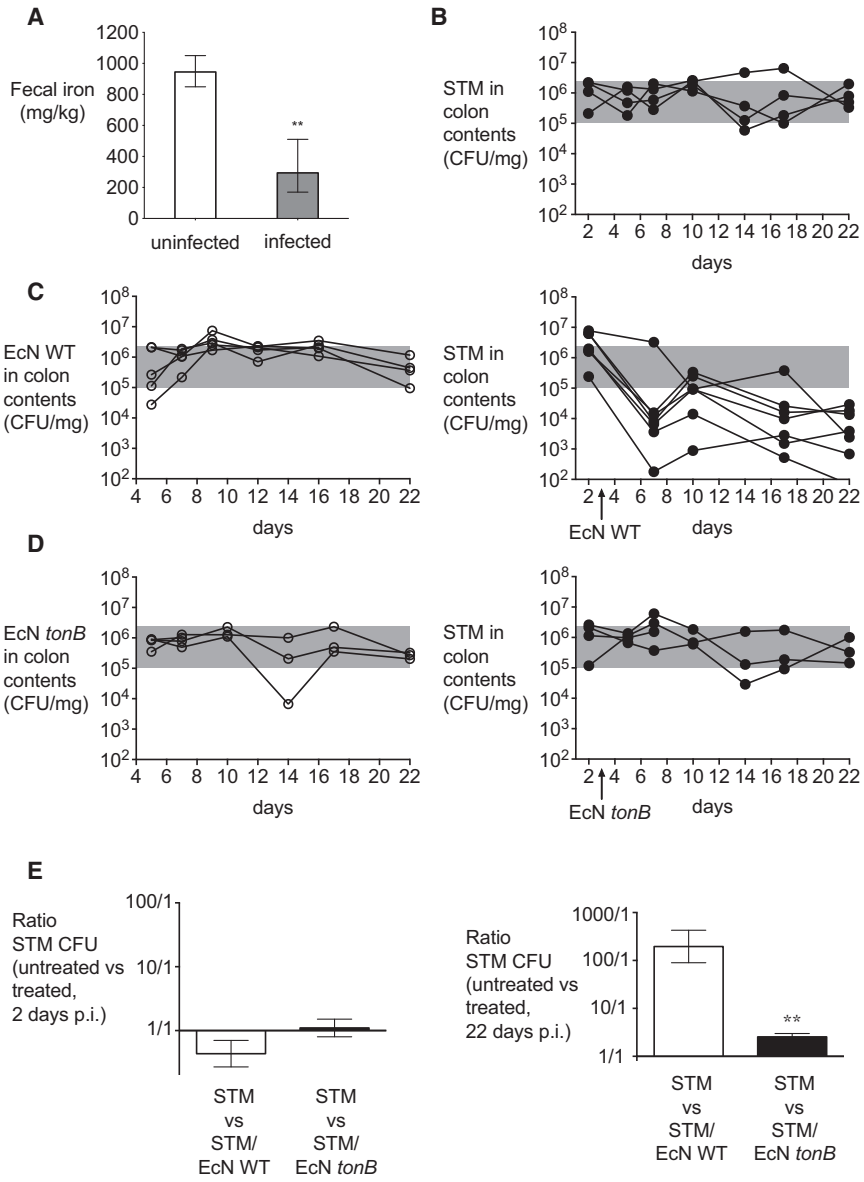

Figure 1. Probiotic *E. coli* Nissle 1917 Reduces *S.* Typhimurium Fecal Shedding

(A) The concentration of iron in fecal samples collected from mock-infected (n = 4) or *S.* Typhimurium-infected (n = 4) C57BL/6 mice 4 days postinfection. Bars represent geometric means ± SD.

(B–D) 129X1/SvJ mice were infected with *S.* Typhimurium and either untreated (B) or treated with one dose of *E. coli* Nissle wild-type (C) or *tonB* mutant (D) three days postinfection. *S.* Typhimurium (black circles), *E. coli* Nissle WT, or *tonB* mutant (white circles) were enumerated in the colonic contents.

(E) Ratio of colony-forming units (cfu) recovered from fecal samples of mice infected with *S.* Typhimurium that were untreated compared to mice treated with one dose of either *E. coli* Nissle WT or *tonB* mutant three days postinfection. Ratios 2 days postinfection (i.e., 1 day before treatment) and 22 days postinfection are shown. Bars represent geometric means ± SD. Data are representative of n = 2 experiments. STM, *S.* Typhimurium; EcN, *E. coli* Nissle. **$p \leq 0.01$. See also Figure S1 and Table S1.

we hypothesized that *E. coli* Nissle might protect the host by utilizing similar mechanisms to compete with *S.* Typhimurium for essential micronutrients.

A snapshot analysis of the *E. coli* Nissle genome revealed that it shares many fitness properties found in uropathogenic *E. coli* (UPEC) strains of the same serotype (Grozdanov et al., 2004). Intriguingly, the *E. coli* Nissle genome appears to encode for as many iron uptake systems as UPEC (Garcia et al., 2011), an armament that notably includes salmochelin, the hydroxamate-type siderophore aerobactin, the mixed-type siderophore yersiniabactin, and the hemin uptake transporter ChuA. Given that redundancy in iron uptake promotes the growth of UPEC in the bladder and the kidney (Garcia et al., 2011), we reasoned that it may also contribute to *E. coli* Nissle colonization of the inflamed gut. To test the hypothesis that iron uptake mechanisms are important for *E. coli* Nissle probiotic activity, we set out to examine the effect of administering wild-type (WT) *E. coli* Nissle or mutant derivatives deficient in iron uptake on the course of *S.* Typhimurium infection.

RESULTS

Probiotic *E. coli* Nissle 1917 Reduces *S.* Typhimurium Fecal Shedding

Our prior studies indicated that *S.* Typhimurium must overcome iron limitation to successfully infect the host (Crouch et al., 2008; Raffatellu et al., 2009). First, to confirm that iron limitation occurs during *S.* Typhimurium infection, we measured the concentration of iron in the feces of mice 4 days postinfection in an *S.* Typhimurium colitis model by inductively coupled plasma mass spectrometry (ICP-MS). We found that the concentration of fecal iron in the absence of infection was approximately 950 mg/kg (Figure 1A). In contrast, the concentration of fecal iron was significantly reduced, on average, to 300 mg/kg in mice infected with *S.* Typhimurium (Figure 1A), confirming that infection results in limitation of this metal in the colonic environment. To gain insight into the mechanism behind the lower levels of intestinal iron, we determined the expression of hepcidin and ferroportin 1 (Figure S1 available online). Although hepcidin transcripts were highly abundant in the liver of both mock-infected and *S.* Typhimurium-infected mice, the expression of hepcidin was not upregulated during *S.* Typhimurium infection (Figure S1). Nonetheless, we observed a modest, but significant, downregulation of ferroportin 1 in the liver and intestine of mice infected with *S.* Typhimurium, which should have resulted in higher levels of intestinal iron due to its

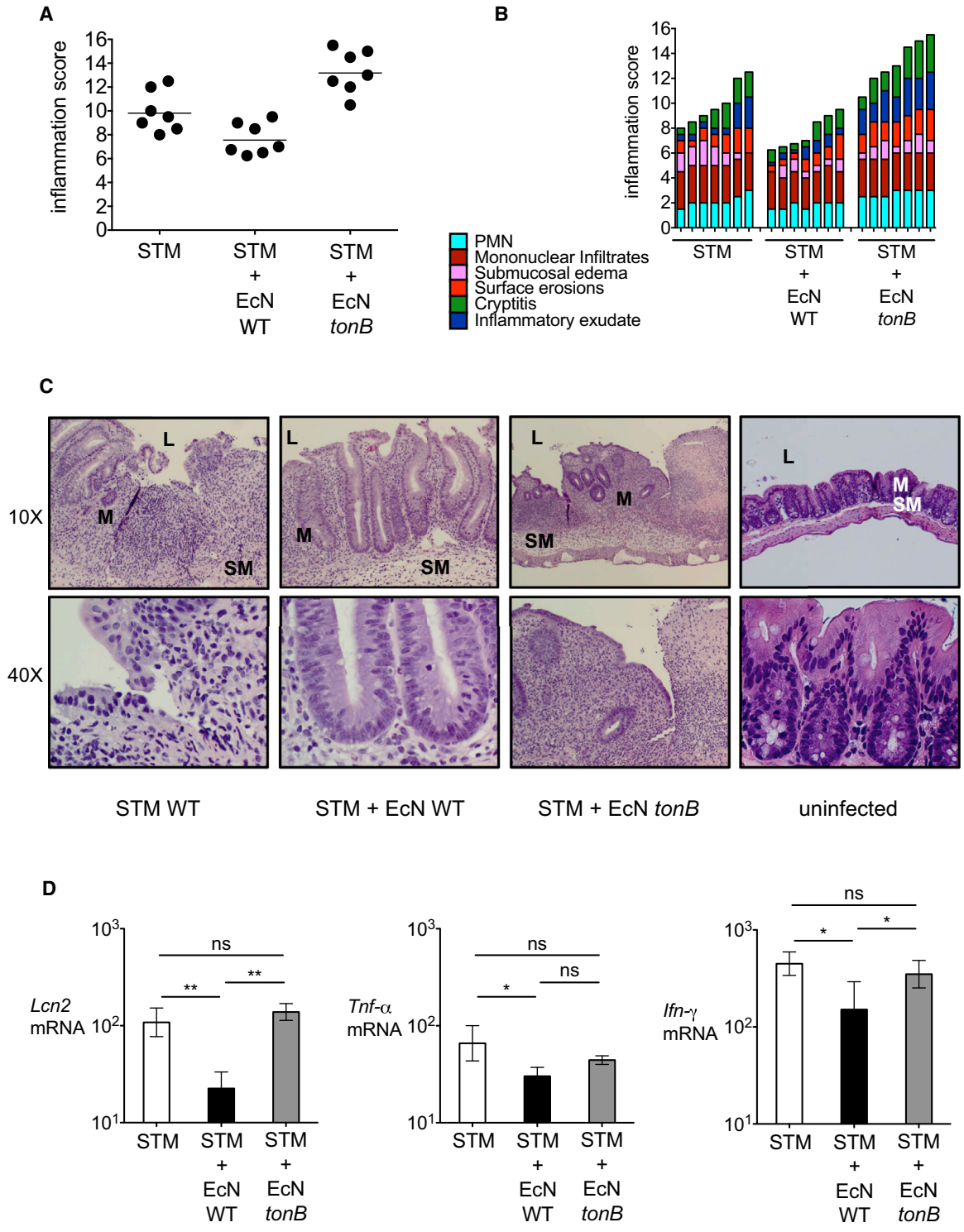

Figure 2. Intestinal Host Response in Mice with Persistent *S.* Typhimurium Infection

129X1/SvJ mice were infected with *S.* Typhimurium and were either left untreated or were treated with one dose of *E. coli* Nissle WT or *tonB* mutant 3 days postinfection. Cecal samples were collected at 6 or 22 days postinfection.

(A) Blinded histopathology scores of cecal samples at 22 days postinfection. The score of individual mice (circles) and the geometric mean for each group (bars) are indicated.

(B) A detailed scoring for the animals shown in (A) is provided. Each stacked column represents an individual mouse.

(C) Hematoxylin and eosin (H&E)-stained sections from representative animals for each group. Although *E. coli* Nissle WT had no effect on inflammatory cell infiltration, mucosal integrity (assessed by cryptitis and surface erosions) was modestly improved.

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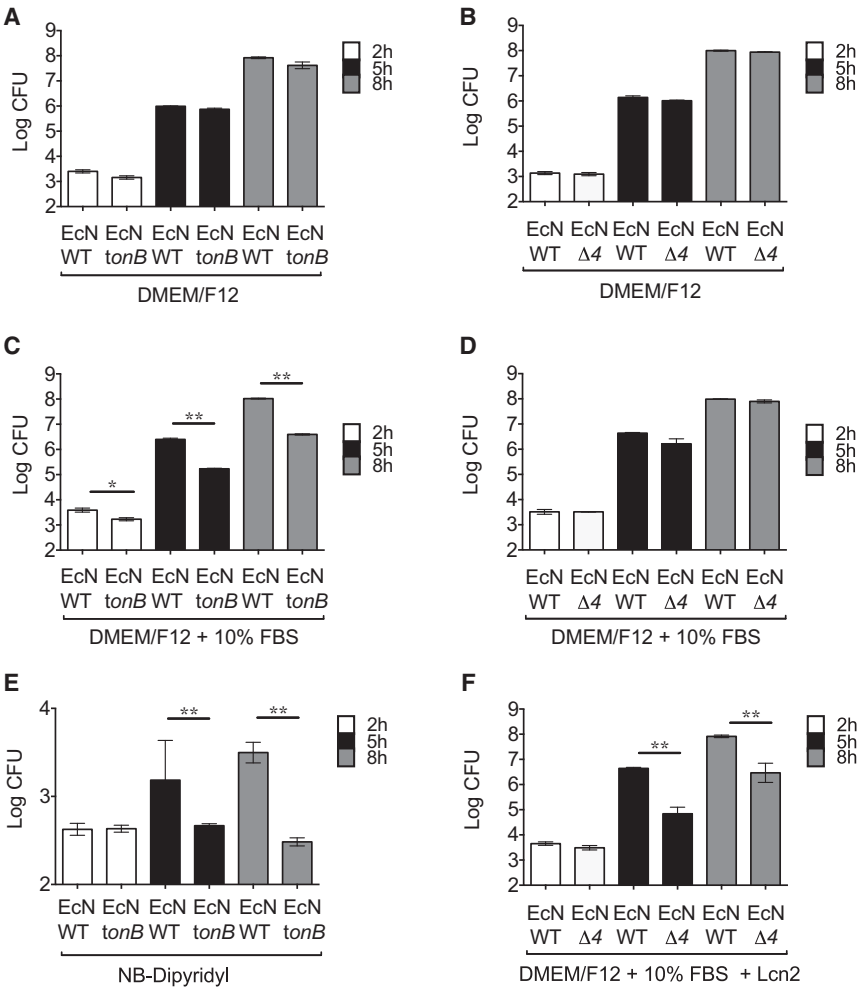

Figure 3. Growth of *E. coli* Nissle 1917 Strains in Iron-Rich and Iron-Limited Media

Growth of *E. coli* Nissle WT and the mutants in iron uptake *tonB* or *iroN fuyA iutA chuA* (Δ4) was determined.

(A, C, and E) Growth of *E. coli* Nissle WT and the *tonB* mutant in Dulbecco's modified Eagle's medium (DMEM)/F12 (A) or DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (C) or nutrient broth (NB) supplemented with Dipyridyl (E).

(B, D, and F) Growth of *E. coli* Nissle WT and the Δ4 mutant in DMEM/F12 (B) or DMEM/F12 supplemented with 10% FBS with the absence (D) or presence (F) of 1 μg/ml lipocalin 2 (Lcn2). Bacteria were enumerated at 2, 5, and 8 hr postinoculation. Bars represent the geometric means ± SD of at least three experiments. STM, *S.* Typhimurium; EcN, *E. coli* Nissle. *p ≤ 0.05; **p ≤ 0.01.

See also Figure S2 and Table S2.

reduced intestinal absorption. However, mice infected with *S.* Typhimurium also showed a significant weight loss and reduced food uptake (Figure S1) (data not shown), which may have contributed to the lower concentration of iron in the intestine of infected mice (Figure 1A).

Once we established that intestinal iron was indeed limited during *S.* Typhimurium infection, we assessed the effects of *E. coli* Nissle administration on *S.* Typhimurium infection. First, we utilized 129X1/SvJ mice, which develop chronic *Salmonella* colitis with persistent infection (Lawley et al., 2008). To ensure that all mice became highly colonized, we administered streptomycin prior to infection as previously described (Barthel et al., 2003; Lawley et al., 2008). Then, *S.* Typhimurium-infected mice were observed for 3 weeks postinfection, and they exhibited consistently high levels of fecal shedding of *Salmonella* (Figure 1B) as well as high levels of inflammation (Figure 2). Remarkably, a single therapeutic dose of WT *E. coli* Nissle administered 3 days after inoculation with *S.* Typhimurium was able to establish persistent colonization (Figure 1C) and significantly reduce *S.* Typhimurium colonization by more than two logs for the duration of the study (Figures 1C, 1E, and S1). To determine whether the beneficial effect of *E. coli* Nissle is dependent upon its ability to acquire iron, a mutation was constructed in the *tonB* gene of *E. coli* Nissle.

TonB provides the energy necessary for the active transport of iron-laden siderophores and heme (Braun and Hantke, 2011). As expected, the in vitro growth of *E. coli* Nissle *tonB* was equivalent to that of WT *E. coli* in an iron-rich media (Figures 3A and S2) or when *tonB* was complemented in *trans* (Figure S2), but not in media when iron was limited by the addition of serum (Figure 3C) or iron chelation with 2,2′-dipyridyl (Figure 3E). Strikingly, *E. coli* Nissle *tonB* was still able to establish persistent colonization when administered to 129X1/SvJ mice 3 days after *S.* Typhimurium infection but was unable to reduce the *S.* Typhimurium burden in the feces (Figures 1D, 1E, and S1). Moreover, only WT *E. coli* Nissle was able to ameliorate the chronic inflammation observed in these S. Typhimurium-challenged mice at 22 days postinfection (Figure 2). Consistent with this observation, transcript levels of *Lcn2*, which encodes for lipocalin 2, and levels of the proinflammatory cytokines *Tnf-α* and *Ifn-γ* were reduced in the cecum by WT *E. coli* Nissle, but not by the *tonB* mutant (Figure 2D). Therefore, our results suggested that iron acquisition is important for the probiotic activity of *E. coli* Nissle and for its ability to compete with *S.* Typhimurium in the inflamed gut during chronic infection.

(D) Transcript levels of *Lcn2*, *Tnf-α*, and *Inf-γ* were determined in the ceca of 129X1/SvJ mice infected with *S.* Typhimurium (white bars), infected with *S.* Typhimurium, and treated with either one dose of *E. coli* Nissle WT (black bars) or *tonB* mutant (gray bars) 3 days postinfection. Samples were collected 6 days postinfection. Data are expressed as fold increase over mock-infected mice. Bars represent the geometric means ± SD. STM, *S.* Typhimurium; EcN, *E. coli* Nissle; L, lumen; M, mucosa; SM, submucosa. *p≤ 0.05; **p ≤ 0.01.

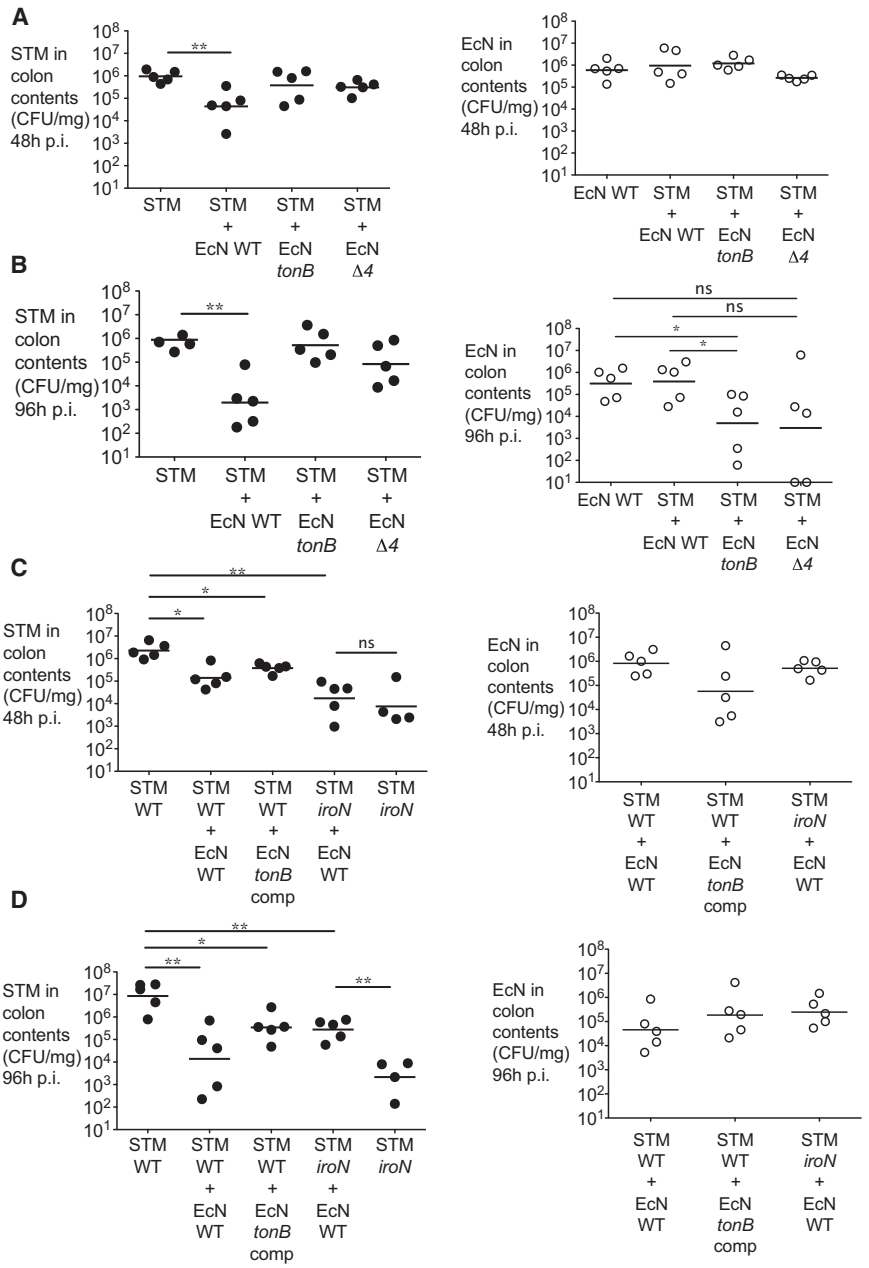

Figure 4. *E. coli* Nissle 1917 Requires Iron Uptake Systems to Reduce *S.* Typhimurium Intestinal Colonization

(A and B) C57BL/6 mice were infected with *S.* Typhimurium alone or coadministered with WT *E. coli* Nissle, the *tonB* mutant, or the *iroN fuyA iutA chuA* mutant (Δ4).

(C and D) C57BL/6 mice were infected with *S.* Typhimurium alone (WT or *iroN* mutant) or coadministered with WT *E. coli* Nissle, the *tonB* mutant, or the *tonB* mutant complemented in *trans* when indicated. cfu in colonic contents were enumerated at 48 (A and C) and 96 (B and D) hr postinfection. *S.* Typhimurium (black circles) and *E. coli* Nissle (white circles) counts are shown. Representative experiments of n = 2 are shown. STM, *S.* Typhimurium; EcN, *E. coli* Nissle. *p ≤ 0.05; **p ≤ 0.01.

See also Figure S3.

Growth of *E. coli* Nissle Strains in Iron-Rich and Iron-Limited Media

Given that *tonB* mutations impede multiple iron acquisition mechanisms, we constructed a mutant strain that lacked four separate *tonB*-dependent iron transport systems that have been shown to contribute to iron acquisition during UPEC urinary tract infection (Garcia et al., 2011). The resulting strain, termed *E. coli* Nissle Δ4, lacked the salmochelin receptor IroN, the aerobactin receptor IutA, the yersiniabactin receptor FuyA, and the heme receptor ChuA (*E. coli* Nissle *iroN fyuA iutA chuA*). Notably, in contrast to the *tonB* mutant, *E. coli* Nissle Δ4 may acquire iron via ferric enterochelin through the FepA receptor. However, blockade of ferric enterochelin via lipocalin 2 should result in growth inhibition. Therefore, we determined the growth of the *E. coli* Nissle Δ4 in rich media supplemented with serum and/or the enterochelin scavenger lipocalin 2, as we previously described (Raffatellu et al., 2009). As expected, the *E. coli* Nissle Δ4 grew as well as WT *E. coli* in iron-rich media (Figures 3B and S2) and, in contrast to the *E. coli* Nissle *tonB* (Figure 3C), in iron-rich media supplemented with serum (Figure 3D), which was consistent with effective iron acquisition via ferric enterochelin. However, this strain exhibited a significant growth impairment in medium supplemented with the iron scavenger lipocalin 2 (Figure 3F). Furthermore, the growth inhibition by lipocalin 2 on the *E. coli* Nissle Δ4 mutant was no longer observed when ferric iron was added to the media in the form of iron citrate (Figure S2) (Pressler et al., 1988). Promisingly, the growth of WT *E. coli* Nissle was not impaired by supplementation with lipocalin 2 (Figures 3F and S2). Altogether, these results suggested that lipocalin 2 resistance mediated by nonenterochelin high-affinity iron uptake mechanisms might allow *E. coli* Nissle to grow in the inflamed gut and reduce *S.* Typhimurium colonization.

E. coli Nissle 1917 Requires Iron Uptake Systems to Reduce *S.* Typhimurium Intestinal Colonization

To further assess whether high-affinity iron transporters may contribute to the probiotic effect of *E. coli* Nissle, we administered WT *E. coli* Nissle or isogenic mutant strains deficient in iron uptake to mice infected with *S.* Typhimurium in a model of acute colitis. Specifically, C57BL/6 mice were administered streptomycin 1 day prior to infection as previously described (Barthel et al., 2003; Raffatellu et al., 2009). Then, mice were infected with *S.* Typhimurium alone or coadministered with an equal dose of *E. coli* Nissle WT or mutants (Figures 4 and S3).

Then, colonization in the colon content was determined at 48, 72, and 96 hr postinfection. In this model of acute colitis, the administration of *E. coli* Nissle significantly reduced intestinal colonization with *S*. Typhimurium at every time point (Figures 4A, 4B, and S3). In contrast, neither the *E. coli* Nissle *tonB* nor the Δ4 mutants were able to reduce intestinal *S*. Typhimurium colonization (Figures 4A, 4B, and S3) despite successfully establishing intestinal colonization (Figures 4A, 4B, and S3). Notably, although *E. coli* Nissle WT outgrew *S*. Typhimurium, both *E. coli* Nissle *tonB* and the Δ4 mutant were outcompeted by *S*. Typhimurium at 96 hr postinfection (see also Figure 7). Moreover, complementation of *E. coli* Nissle *tonB* in *trans* partly rescued this strain's capability to reduce *S*. Typhimurium colonization (Figures 4C, 4D, and S3).

To gain additional insight into the competition for iron between *S*. Typhimurium and *E. coli* Nissle in the inflamed gut, we tested whether *E. coli* Nissle would affect the colonization of an *S*. Typhimurium strain lacking the IroN receptor (*iroN* mutant), which cannot acquire iron via salmochelin (Raffatellu et al., 2009) (Figure S2). We have previously shown that an *S*. Typhimurium *iroN* mutant has a colonization defect in the inflamed gut that is dependent on the expression of lipocalin 2 (Raffatellu et al., 2009). Consistent with our earlier study, we found that an *S*. Typhimurium *iroN* mutant showed a defect in colonization when compared to *S*. Typhimurium WT (Figures 4C, 4D, and S3). Consistent with our present study, administration of *E. coli* Nissle did not reduce the colonization of the *S*. Typhimurium *iroN* mutant, supporting the notion that competition for iron is an essential trait of its probiotic activity (Figures 4C, 4D, and S3). Notably, at 96 hr postinfection, the colonization of the *S.* Typhimurium *iroN* mutant further decreased because of the higher levels of lipocalin 2 (Figures 4D and S3C). In contrast, the downregulation of lipocalin 2 caused by the administration of *E. coli* Nissle partly rescued the *iroN* mutant, which was still significantly lower than WT *S.* Typhimurium at this time point. This was consistent with our previously published results showing that the *iroN* mutant is rescued in *Lcn2*$^{-/-}$ mice (Raffatellu et al., 2009). Altogether, these results demonstrate that iron uptake is crucial for the beneficial effect of *E. coli* Nissle in reducing the colonization of *S*. Typhimurium.

Next, given that *E. coli* Nissle was able to diminish the intestinal burden of *S*. Typhimurium, we analyzed whether the probiotic strain also ameliorated the intestinal inflammation caused by *S*. Typhimurium in the acute colitis model (Figures 5 and S4). The administration of *E. coli* Nissle alone did not cause intestinal inflammation (Figures 5 and S4). Intriguingly, the addition of WT *E. coli* Nissle, *E. coli* Nissle *tonB*, or *E. coli* Nissle Δ4 each resulted in similar reductions of intestinal inflammation in *S.* Typhimurium-infected C57BL/6 mice (Figures 5A, 5B, 5D, 5E, and S4). In accordance with the reduction in inflammation, decreased transcript levels of *Lcn2*, the neutrophil chemoattractant *Cxcl-1*, and the proinflammatory cytokine *Il-17a* were also observed (Figures 5C and 5F). To determine whether *E. coli* Nissle has direct anti-inflammatory effects, we administered dextran sodium sulfate (DSS) to mice in the drinking water as a means of inducing colitis in the absence of a bacterial infection (Wirtz et al., 2007). Then, these mice were administered a single dose of *E. coli* Nissle. Similar to *E. coli* Nissle administration during *S*. Typhimurium infection, treatment with *E. coli* Nissle reduced the expression of proinflammatory cytokines in DSS-treated mice (Figure S4). On the basis of these results, it appears that *E. coli* Nissle exerts beneficial effects on cecal inflammation during acute *Salmonella* colitis that are independent of its antagonism of *S*. Typhimurium colonization.

The host inflammatory response limits iron availability to invading microbes in what is known as nutritional immunity (Cassat and Skaar, 2013). One arm of the nutritional immune response during infection is the upregulation of lipocalin 2, which, as described earlier, sequesters iron-bound enterochelin. Previously, we demonstrated that the ability to acquire iron through salmochelin provides a colonization advantage to *S*. Typhimurium WT in competition with an *iroN* mutant in mice that express lipocalin 2, but not in *Lcn2*$^{-/-}$ mice, most likely because enterochelin alone is sufficient to overcome other mechanisms of ferric iron restriction in the host (Raffatellu et al., 2009). Consistent with this notion, intestinal *S*. Typhimurium colonization in WT mice was not affected by the administration of a nonprobiotic commensal *E. coli* strain (Figure 6A) that relies on enterochelin for ferric iron acquisition and, thus, is susceptible to lipocalin 2 (Berger et al., 2006; Flo et al., 2004). Therefore, we hypothesized that *E. coli* Nissle would lose its competitive advantage over *S*. Typhimurium in the absence of lipocalin 2. As predicted, intestinal *S*. Typhimurium colonization in *Lcn2*$^{-/-}$ mice was not decreased by the administration of *E. coli* Nissle, despite successful colonization of the mouse intestine by this probiotic strain (Figure 6B). As we previously showed, WT and *Lcn2*$^{-/-}$ mice infected with *S.* Typhimurium did not show significant differences in *S*. Typhimurium colonization (Figures 4, 6B, and S3) or inflammation (Figures S4 and S5). Furthermore, the expression of proinflammatory cytokines was similar between WT mice and *Lcn2*$^{-/-}$ mice, the exception being *Cxcl-1*, which was reduced in *Lcn2*$^{-/-}$ mice, as previously shown for *Cxcl-8* (Bachman et al., 2009). Nonetheless, reduction in the expression of *Cxcl-1* did not result in differences in the neutrophil influx observed by the pathology, most likely because of redundant mechanisms of neutrophil migration to the gut (light blue bars, Figures S4 and S5). Notably, the administration of *E. coli* Nissle to *Lcn2*$^{-/-}$ mice infected with *S*. Typhimurium also ameliorated intestinal inflammation, despite the minimal differences observed in the expression of proinflammatory cytokines. These results may indicate that lipocalin 2 has additional immunomodulatory effects that are independent of its iron sequestration mechanisms (Bachman et al., 2009).

Although we had administered *E. coli* Nissle as a therapeutic either during or after *S*. Typhimurium administration, this strain was originally isolated as a normal commensal of the gut flora from a healthy soldier who did not acquire *Shigella* during an outbreak. To test whether colonization of mice with *E. coli* Nissle would confer protection to infection, C57BL/6 mice were administered *E. coli* Nissle 3 days prior to *S*. Typhimurium infection. As shown in Figure 6C, mice that were precolonized with *E. coli* Nissle showed a significant reduction in colonization from *S*. Typhimurium, indicating that colonization with this probiotic offers at least partial protection to infection with a gastrointestinal pathogen.

Next, we determined the ratio of the *S*. Typhimurium colony-forming units (cfu) detected in the colonic content of C57BL/6 mice when *S.* Typhimurium was administered alone versus

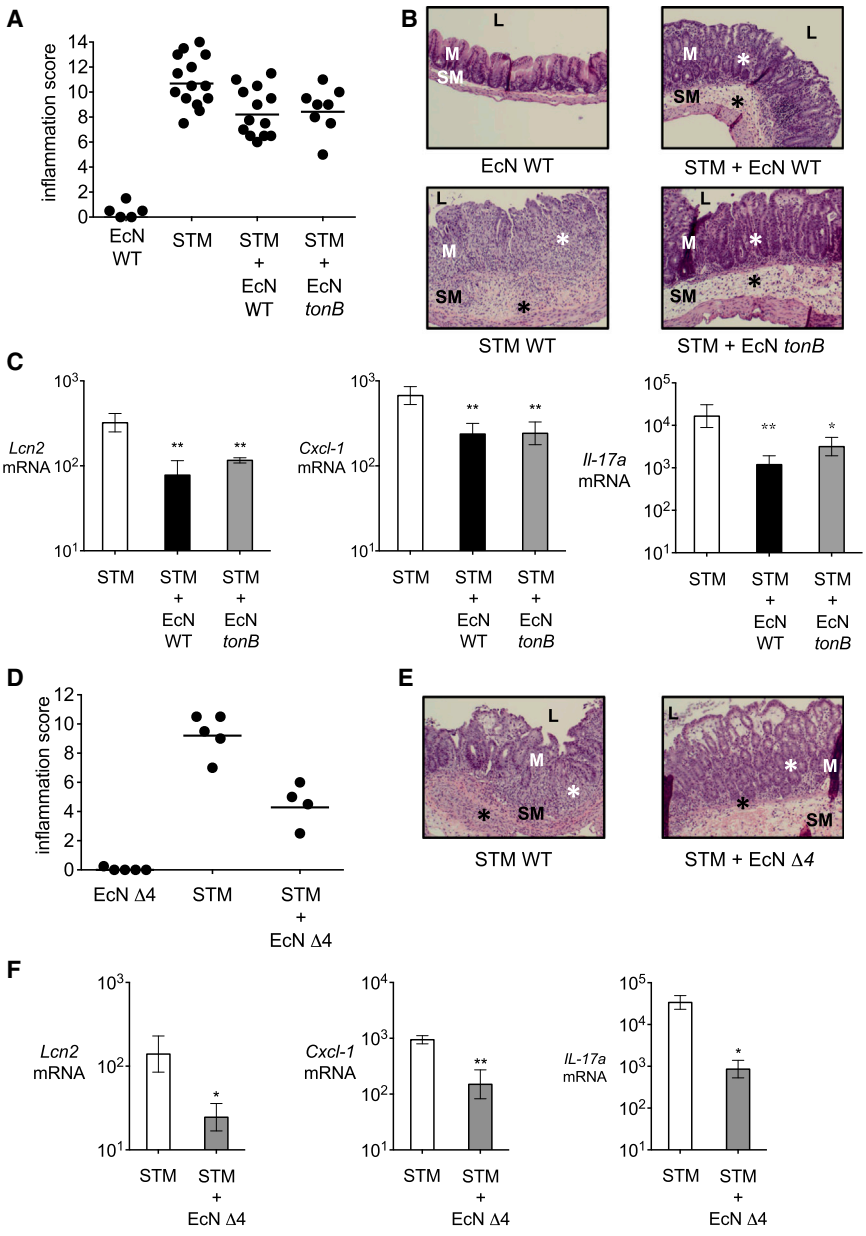

Figure 5. *E. coli* Nissle 1917 Ameliorates Intestinal Inflammation during Acute *S.* Typhimurium Infection

(A) Blinded histopathology scores of cecal samples 4 days postinfection of C57BL/6 mice administered WT *E. coli* Nissle, *S.* Typhimurium, or a mixture of *S.* Typhimurium and *E. coli* Nissle WT or *tonB* mutant. Scores of individual mice (circles) and geometric means for each group (bars) are indicated.

(B) H&E-stained sections from representative animals for each group. *E. coli* Nissle WT or *tonB* coadministration with *S.* Typhimurium reduced the density of inflammatory infiltrates (black asterisks) and the degree of crypt injury (white asterisks) in comparison to *S.* Typhimurium infection alone.

(C) Transcript levels of *Lcn2*, *Cxcl-1*, and *Il-17a* were determined in the ceca of C57BL/6 mice infected with *S.* Typhimurium (white bars), a mixture of *S.* Typhimurium and WT *E. coli* Nissle (black bars), or a mixture of *S.* Typhimurium and *E. coli* Nissle *tonB* (gray bars). Data are expressed as fold increase over mock-infected mice. Bars represent the geometric means ± SD.

(D) Blinded histopathology scores of cecal samples 4 days postinfection of mice administered *E. coli* Nissle *iroN fuyA iutA chuA* (Δ4), *S.* Typhimurium, or a mixture of *S.* Typhimurium and *E. coli* Nissle Δ4. Scores of individual mice (circles) and geometric means for each group (bars) are indicated.

(E) H&E-stained sections from representative animals from each group. *E. coli* Nissle Δ4 coadministration with *S.* Typhimurium greatly reduced chronic inflammatory infiltrates (black asterisks) and the degree of crypt injury (white asterisks) in comparison to *S.* Typhimurium infection alone.

(F) Transcript levels of *Lcn2*, *Cxcl-1*, and *Il-17a* were determined in the ceca of mice infected with *S.* Typhimurium (white bars) or a mixture of *S.* Typhimurium and *E. coli* Nissle Δ4 (gray bars). Data are expressed as fold increase over mock-infected mice. Bars represent geometric means ± SD. STM, *S.* Typhimurium; EcN, *E. coli* Nissle. L; lumen; M, mucosa; SM, submucosa. *p ≤ 0.05; **p ≤ 0.01.

See also Figure S4 and Table S3.

in competition with *E. coli* Nissle. Overall, there was a marked reduction (up to 445-fold) in *S.* Typhimurium fecal colonization in WT mice that were coadministered WT *E. coli* Nissle at the time of infection relative to mice that were infected with *S.* Typhimurium only, a difference which was not observed in either *Lcn2*$^{-/-}$ mice or WT mice that were administered either the *E. coli* Nissle *tonB* or the *E. coli* Nissle Δ4 mutants (Figures 7A, 7C, and 7E). Furthermore, only WT *E. coli* Nissle coadministered with *S.* Typhimurium to WT mice was able to outcompete *S.* Typhimurium by up to 195-fold (Figures 7B, 7D, and 7F). Altogether, our results show that iron acquisition in the inflamed gut is a critical mechanism for the ability of the probiotic *E. coli* Nissle to limit *Salmonella* intestinal colonization.

DISCUSSION

E. coli Nissle was isolated from a healthy soldier during a *Shigella* outbreak in 1917 under the hypothesis that a protective commensal strain must have colonized the gut of that soldier (Nissle, 1959). In the years since, *E. coli* Nissle has had a long history of use in clinical settings to treat gastrointestinal disorders, though the molecular mechanism of its beneficial activity is not well understood (Schultz, 2008), much like all natural probiotics (reviewed in Balakrishnan and Floch, 2012).

Probiotics may exert their beneficial effects by either direct interaction with the host or by competition with pathogenic species (mechanisms frequently grouped under the term colonization resistance) (reviewed in Lawley and Walker, 2013). A

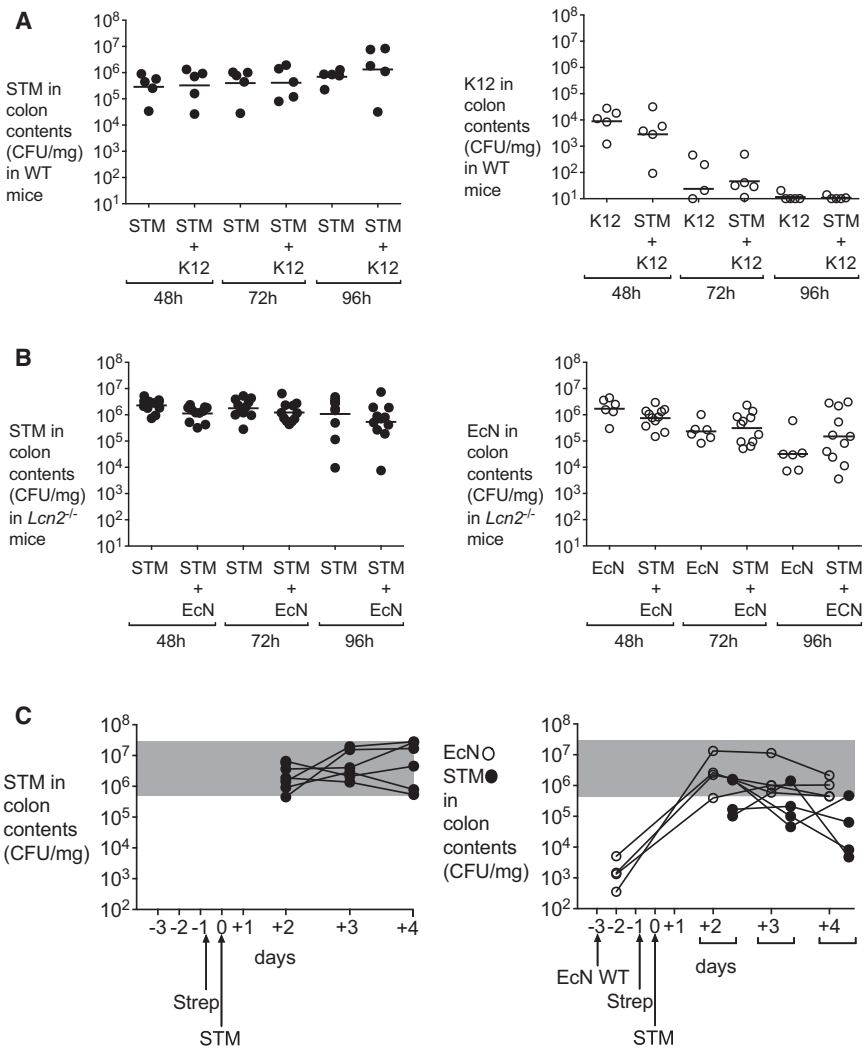

Figure 6. *E. coli* Nissle 1917 Reduction of *S.* Typhimurium Intestinal Colonization Requires Functional Lipocalin 2 and Is Independent of the Time of Administration

(A) C57BL/6 mice were infected with *S.* Typhimurium alone or coadministered with commensal *E. coli* K-12. cfu in colonic contents were enumerated at 48, 72, and 96 hr postinfection. STM (black circles) and *E. coli* K-12 (white circles) counts are shown.

(B) C57BL/6 *Lcn2*$^{-/-}$ mice were infected with *S.* Typhimurium alone or coadministered with WT *E. coli* Nissle. cfu in colonic contents were enumerated at 48, 72, and 96 hr postinfection. *S.* Typhimurium (black circles) and *E. coli* Nissle (white circles) counts are shown.

(C) C57BL/6 mice were administered a single dose of *E. coli* Nissle WT or mock 3 days before being infected with *S.* Typhimurium. Streptomycin treatment was performed one day prior to *S.* Typhimurium infection. cfu in colonic contents were enumerated at 2, 3, and 4 days postinfection. *S.* Typhimurium (black circles) and *E. coli* Nissle (white circles) counts are shown. K12, *E. coli* K12; STM, *S.* Typhimurium; EcN, *E. coli* Nissle.

See also Figure S5.

large focus of research on probiotics has been on their interactions with the immune system rather than their competition with other microbes. Regarding *E. coli* Nissle, several studies have proposed that selective immune modulation may contribute to its activity (reviewed in Behnsen et al., 2013, and Jacobi and Malfertheiner, 2011), including the activation of γδ T cells (Guzy et al., 2008), reduction of the secretion of proinflammatory cytokines in the mucosa (Grabig et al., 2006), enhanced secretion of IgA and IgM (Cukrowska et al., 2002), and production of tight junction proteins (Ukena et al., 2007) and human β-defensin 2 (Wehkamp et al., 2004). Although *E. coli* Nissle has been proposed to have both pro- and anti-inflammatory effects, the net result of these alterations is an overall enhancement of the mucosal barrier. Consistent with these findings, as well as with other studies that have shown *E. coli* Nissle to ameliorate the disease caused by an infection (Hockertz, 1997; Splichalova et al., 2011), we also observed a reduction in both intestinal pathology and expression of proinflammatory cytokines when *S.* Typhimurium-infected mice were administered *E. coli* Nissle in both a chronic and an acute model of infection, as well as when we induced inflammation independent of infection.

The two mouse models that we employed (129X1/SvJ for the chronic model and C57BL/6 for the acute model) develop intestinal inflammation when infected with *S.* Typhimurium after streptomycin treatment. However, the host response to *S.* Typhimurium is not identical in these mice because of genetic differences as well as differences in the composition of the microbiota. Presently, the most well-studied genetic distinction is that 129X1/SvJ have a functional *Nramp1* (*Slc11a1*) allele, which renders the mice more resistant to infection with *S.* Typhimurium. However, functional Nramp1 alone does not explain differences in the host response to *S.* Typhimurium (Brown et al., 2013), and altered expression or function of other genes (for instance, caspase 11) may also play a role (Kayagaki et al., 2011). These variances may explain why in the chronic model of infection only WT *E. coli* Nissle mediated a reduction of inflammation, whereas in the acute model of infection the reduction was independent of iron acquisition. Nevertheless, our study indicates that the mild-to-moderate reduction in intestinal inflammation only partly explains the beneficial effects of administering *E. coli* Nissle during infection.

Intestinal inflammation of infectious and noninfectious origins results in an alteration of the normal flora and a significant microbial dysbiosis, including the loss of Bacteroidetes and Firmicutes and the proliferation of Enterobacteriaceae (reviewed in Fava and Danese, 2011, and Mukhopadhya et al., 2012). A mechanism for the proliferation of Enterobacteriaceae in the inflamed gut was recently provided by Winter et al. (2013), who showed that this family can utilize host-derived nitrate for respiration. The fact that *E. coli* Nissle also benefits from inflammation could

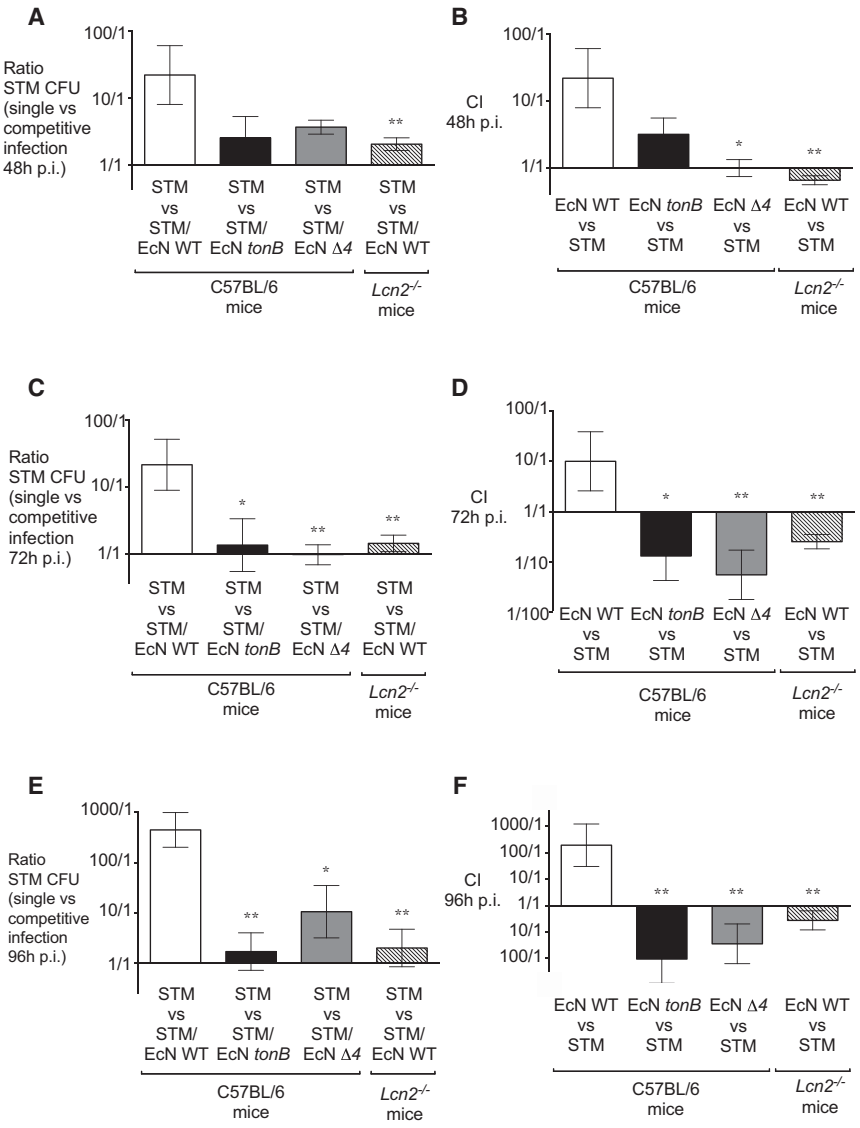

Figure 7. Ratios of *S.* Typhimurium and *E. coli* Nissle in the Acute Colitis Model

Ratio of the cfu recovered from the fecal samples of mice infected with *S.* Typhimurium that were untreated in comparison to mice that were administered one dose of either *E. coli* Nissle WT, the *tonB* mutant, or the *iroN fuyA iutA chuA* (Δ4) mutant at the time of infection.

(A, C, and E) Ratio of *S.* Typhimurium cfu at 48 (A), 72 (C), or 96 (E) hr postinfection.

(B, D, and F) Competitive index (CI) in the indicated mixed infection was calculated by dividing the output ratio (*E. coli* Nissle cfu / *S.* Typhimurium cfu) in the colonic contents of mice at the indicated time points by the input ratio (*E. coli* Nissle cfu / *S.* Typhimurium cfu). CI of the indicated *E. coli* Nissle strain versus *S.* Typhimurium at 48 (B), 72 (D), or 96 hr (F) postinfection. Bars represent geometric means ± SD. *$p \leq 0.05$; **$p \leq 0.01$.

also explain why its anti-inflammatory effects are mild to moderate: a complete reduction of inflammation would be detrimental to its own colonization, as indicated in our mice that were precolonized with *E. coli* Nissle prior to *S.* Typhimurium infection. A change in the composition of the normal flora, coupled with a loss of Bacteroidetes and Firmicutes, is also observed during infection caused by *S.* Typhimurium, given that this pathogen has been shown to thrive during inflammation and successfully compete with the microbiota (reviewed in Thiennimitr et al., 2012). Whereas the host inflammatory response limits the availability of essential nutrients, including metal ions, to invading microbes in what is known as nutritional immunity, pathogens including *Salmonella* have evolved many mechanisms to evade this response and acquire essential metals necessary to mount a successful infection (Thiennimitr et al., 2012).

Our previous studies indicated that iron uptake via salmochelin, which confers resistance to iron sequestration mediated by lipocalin 2, is essential for the efficient colonization of *S.* Typhimurium (Crouch et al., 2008; Raffatellu et al., 2009). Given that the evasion of lipocalin 2-mediated iron withholding has also been found to be essential to the virulence of many other Gram-negative enteric pathogens (Bachman et al., 2009; Caza et al., 2008; Garcia et al., 2011; Himpsl et al., 2010; Payne et al., 2006), this mechanism has come to be viewed as a virulence trait. In contrast to this trend, our research demonstrates that lipocalin 2-resistant iron acquisition is not a property unique to virulence because it is essential for the probiotic activity of *E. coli* Nissle.

Deletion of up to three iron receptors did not result in a growth defect in media supplemented with lipocalin 2 (data not shown), indicating that *E. coli* Nissle possesses redundant lipocalin 2-resistant iron uptake systems. Integrating this finding with our observation that *E. coli* Nissle outcompetes *S.* Typhimurium in vivo along with our previous finding that *S.* Typhimurium benefits from acquiring iron in a lipocalin 2-resistant fashion during inflammation, we hypothesized that Nissle's multiple iron uptake systems provide a competitive advantage against *S.* Typhimurium when the intestine is inflamed, and, as we observed, fecal iron is significantly reduced. Consistent with our hypothesis, our results show that *E. coli* Nissle's ability to displace the highly evolved pathogen from its intestinal niche is dependent on iron acquisition. Furthermore, *E. coli* Nissle was only able to reduce the colonization of *S.* Typhimurium when lipocalin 2 was expressed. Although lipocalin 2 is one of the host defenses exploited by *S.* Typhimurium to colonize the inflamed gut and compete with the microbiota, *E. coli* Nissle also subverts this host defense mechanism to thrive in the same inflamed and iron-starved environment. By scavenging for iron more effectively than *S.* Typhimurium both with its own armament of siderophores and with its ability to compete with *S.* Typhimurium for uptake of salmochelin, *E. coli* Nissle tips the scales back in favor of the host, effectively augmenting the

host's innate immune response by acting as a surrogate of sorts for lipocalin 2. It is along these lines that we propose that *E. coli* Nissle—and possibly other beneficial components of the microbiota—may provide colonization resistance in part by boosting the host's nutritional immunity, sequestering nutrients from pathogens when the host fails to do so.

Altogether, our results show that iron acquisition in the inflamed gut is a critical mechanism for the ability of the probiotic *E. coli* Nissle to limit *Salmonella* intestinal colonization. Furthermore, we have demonstrated that this action of *E. coli* Nissle results from its resistance to lipocalin 2, previously considered a mechanism of virulence but now also seen as an essential property of a protective commensal organism. As antibiotic treatment is contraindicated for uncomplicated *Salmonella* infections due to the prolongation of fecal shedding, the administration of *E. coli* Nissle may be a feasible alternative for diminishing *Salmonella* colonization and ameliorating symptoms. As microbial dysbiosis is apparent in a variety of intestinal disorders (DuPont and DuPont, 2011), iron acquisition may also contribute to other probiotic actions attributed to *E. coli* Nissle. The ability of *E. coli* Nissle to withstand inflammation and outcompete a highly evolved pathogen for an essential micronutrient may be seen as a paradigm for understanding the protective actions of commensal microorganisms and a foundation upon which to build future probiotics tailored to the treatment of different diseases.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions

All strains used in this study are listed in Table S1. *S.* Typhimurium strain IR715 is a fully virulent, nalidixic-acid-resistant derivative of WT isolate ATCC 14028. *Escherichia coli* Nissle 1917 WT is a nonpathogenic human *E. coli* isolate that we obtained from Ulrich Sonnenborn (Ardeypharm). An IR715 derivative carrying a mutation in *iroN* and *E. coli* Nissle derivatives carrying mutations in *tonB* or *iroN*, *iutA*, *fyuA*, and *chuA* were used for this study. Mutant construction is described in the Supplemental Information, and the plasmids and primers used are detailed in Tables S1 and S2. For animal infections, all strains were grown in Miller Luria-Bertani (LB) media at 37°C with aeration overnight.

In Vitro Growth Assays

S. Typhimurium and *E. coli* Nissle strains were tested for the ability to grow in iron limiting conditions (nutrient broth supplemented with 0.2 mM 2,2-Dipyridyl, Sigma-Aldrich) at 37°C with aeration overnight. To test lipocalin 2 sensitivity, approximately 10^3 cfu from an overnight culture were inoculated into tissue culture medium comprising DMEM/F12 (Invitrogen) plus 10% fetal bovine serum (FBS, Invitrogen) or in the same medium containing human lipocalin 2 (1 μg/ml, R&D Systems) as previously described (Raffatellu et al., 2009). In order to compare general growth rates, a 1:1 mixture of *E. coli* Nissle WT and either the *tonB* or the *Δ*4 mutants containing 1×10^7 cfu was inoculated in M9 minimal media at 37°C with aeration. When indicated, iron (III) citrate and iron (III) sulfate were added at a final concentration of 1 mM and 200 μM, respectively. cfu were enumerated by plating serial dilutions at 2, 5, and 8 hr after inoculation.

Animal Infections

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of California, Irvine. For acute infections, female C57BL/6 (Taconic) and lipocalin 2-deficient ($Lcn2^{-/-}$) mice were orally gavaged with a dose of 10^9 cfu in 100 μl of PBS 24 hr after pretreatment with streptomycin (100 μl of a 200 mg/ml solution in sterile water) (Barthel et al., 2003; Raffatellu et al., 2009). Mice were infected with either *S.* Typhimurium alone or a 1:1 mixture of strains as indicated. Fecal pellets were collected at 48 and 72 hr postinfection, weighed for cfu determination, and homogenized in 1 ml of sterile PBS, and serial dilutions were plated on LB agar containing appropriate antibiotics. At 96 hr postinfection, mice were euthanized, and the cecum was collected for the isolation of messenger RNA (mRNA) and for histopathology. The liver was collected as indicated in order to measure hepatic gene expression. Bacteria were enumerated in the colon content on agar plates containing the appropriate antibiotics. To render all strains equally resistant to streptomycin, either pACYomega or pHP45omega (Table S1) were introduced by electroporation. When noted, the competitive indices were calculated by dividing the output ratio (*E. coli* Nissle cfu / *S.* Typhimurium cfu) by the input ratio (*E. coli* Nissle cfu / *S.* Typhimurium cfu). In some groups of mice, a single dose of 10^9 cfu of *E. coli* Nissle was administered 3 days prior to *S.* Typhimurium infection, as indicated. In other groups, colitis was induced by administration of dextran sodium sulfate (Wirtz et al., 2007); some of these mice were administered a single dose of 10^9 cfu *E. coli* Nissle on the same day that DSS treatment was started (day 1). DSS-treated mice were sacrificed on day 6, and the cecum was harvested for RNA purification and analysis.

For chronic infections, female 129X1/SvJ mice (the Jackson Laboratory) were orally gavaged as described above with *S.* Typhimurium 24 hr after pretreatment with streptomycin (Lawley et al., 2008). 72 hr postinfection, groups of mice were administrated a single dose of 10^9 cfu *E. coli* Nissle WT or *E. coli tonB* mutant in 100 μl of LB by oral gavage. Individual mice were followed for the duration of the experiment (up to 22 days). Fecal pellets and colon contents were collected and processed as described above.

Quantitative Real-Time PCR

For the analysis of gene expression by quantitative real-time PCR, total RNA was extracted from cecal and hepatic tissues with TRI Reagent (Molecular Research Center). For DSS-treated mice, oligo(dT) purification of mRNA was performed with the Dynabeads mRNA Purification Kit (Invitrogen). Real-time PCR was performed with SYBR Green (Roche) and the Roche LightCycler 480 System (Roche). Data were analyzed with the comparative ΔΔ-Ct method. Target gene transcription of each sample was normalized to the respective levels of mRNA *β-actin*. A list of the real-time primers used in this study is provided in Table S3.

Histopathology

Tissue samples were fixed in formalin, processed according to standard procedures for paraffin embedding, sectioned at 5 μm, and stained with hematoxylin and eosin. Blinded examination by a board-certified pathologist was used to score the pathology of cecal samples with previously published methods (Barthel et al., 2003; Raffatellu et al., 2009). Each section was evaluated for the presence of neutrophils, mononuclear infiltrate, submucosal edema, surface erosions, inflammatory exudates, and cryptitis. Inflammatory changes were scored from 0 to 4 according to the following scale: 0 = none, 1 = low, 2 = moderate, 3 = high, and 4 = extreme. The inflammation score for each mouse was calculated by adding the score for each parameter and was interpreted as follows: 0–2 = within the normal limit, 3–5 = mild, 6–8 = moderate, and 8+ = severe.

Measurement of Iron in Fecal Samples by ICP-MS

The amount of iron in mouse fecal samples was measured by ICP-MS as previously described (Corbin et al., 2008; Liu et al., 2012) and is detailed in the Supplemental Experimental Procedures.

Statistical Analysis

The differences between treatment groups were analyzed by ANOVA followed by a Student's t test. A p value equal to or below 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.06.007.

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