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Report

Antibiotic Treatment Selects for Cooperative Virulence of *Salmonella* Typhimurium

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Summary

Antibiotics are powerful therapeutics but are not equally effective against all cells in bacterial populations. Bacteria that express an antibiotic-tolerant phenotype ("persisters") can evade treatment [1]. Persisters can cause relapses of the infection after the end of the therapy [2]. It is still poorly understood whether persistence affects the evolution of bacterial virulence. During infections, persisters have been found preferentially at particular sites within the host [3, 4]. If bacterial virulence factors are required to reach such sites, treatment with antibiotics could impose selection on the expression of virulence genes, in addition to their well-established effects on bacterial resistance. Here, we report that treatment with antibiotics selects for virulence and fosters transmissibility of *Salmonella* Typhimurium. In a mouse model for *Salmonella* diarrhea, treatment with the broad-spectrum antibiotic ciprofloxacin reverses the outcome of competition between wild-type bacteria and avirulent mutants that can spontaneously arise during within-host evolution [5]. While avirulent mutants take over the gut lumen and abolish disease transmission in untreated mice, ciprofloxacin tilts the balance in favor of virulent, wild-type bacteria. This is explained by the need for virulence factors to invade gut tissues and form a persistent reservoir. Avirulent mutants remain in the gut lumen and are eradicated. Upon cessation of antibiotic treatment, tissue-lodged wild-type pathogens reseed the gut lumen and thereby facilitate disease transmissibility to new hosts. Our results suggest a general principle by which antibiotic treatment can promote cooperative virulence during within-host evolution, increase duration of transmissibility, and thereby enhance the spread of an infectious disease.

Results and Discussion

The progression of an infectious disease and transmission to new hosts can be altered by evolutionary changes in the pathogen population during the course of a single infection [6, 7]. Although such within-host evolution can affect a range of different biological traits, changes in the pathogen's virulence are particularly significant since they determine damage done to the current host as well as disease transmission [8]. How virulence evolves during within-host competition depends on costs versus benefits of virulence for the individual pathogen. Situations in which virulence traits are costly to express for the individual pathogen cell, but elicit a benefit for the group of pathogens in a host, are known as "cooperative virulence." In such cases, within-host evolution tends to reduce virulence by allowing the rise of "defectors," which are avirulent mutants that benefit from the expression of virulence traits by other members of the population without contributing to it themselves [5, 7, 9–11]. Identifying the factors that determine within-host evolution of virulence is crucial for understanding and eventually controlling disease progression and transmission.

Here, we used an experimental host-pathogen system to test whether antibiotics can promote cooperative virulence and disease transmission. We worked with a mouse model for infections with the diarrheal pathogen *Salmonella enterica* subspecies 1 serovar Typhimurium (*S.* Typhimurium) strain SL1344, which is highly sensitive to ciprofloxacin (MIC = 0.015 μg/ml; Figure S1 available online; [12]). In this system, expression of the pathogen's Type Three Secretion System 1 (*ttss-1*) is a cooperative trait that triggers gut tissue invasion and thereby elicits gut inflammation [5, 13]. The inflammation allows *S.* Typhimurium to outcompete the intestinal microbiota in the gut lumen [14], a benefit that is accessible to all luminal *S.* Typhimurium cells, irrespective of whether they express *ttss-1* or not. Importantly, the expression of *ttss-1* imposes a substantial growth defect [5, 15]. Consequently, genetically avirulent mutants (defectors) that never express *ttss-1* but benefit from the inflammation induced by others can outgrow the wild-type "cooperators" in the gut lumen [5]. These defectors carry mutations in *hilD* and lack a central positive regulator of *ttss-1* expression [16]. On the other hand, defectors are incapable of invading into the gut tissue [13, 17, 18]. The intracellular milieu that wild-type *S.* Typhimurium is facing in the gut tissue can induce a phenotype of slow growth and antibiotic tolerance (persistence) [3, 4]. This suggests that virulent wild-type bacteria, but not the avirulent defectors, can reach the host tissue that can confer protection from antibiotics treatment. Based on these observations, we hypothesized that antibiotic treatment might favor survival of virulent wild-type *S.* Typhimurium and thereby select for cooperative virulence.

The Rise of Avirulent Defectors Reduces Disease Transmissibility

We first verified that, in the absence of antibiotics treatment, avirulent defectors become dominant in the gut lumen and established that this interrupts disease transmission to new hosts. Here, we used 129SvEv mice, because these *Nramp*$^{+/+}$ animals are known to survive long-term infection with wild-type *S.* Typhimurium [14, 19]. Mice were inoculated with mixtures of 99% wild-type *S.* Typhimurium and 1% defectors, and fecal samples were taken to estimate population sizes of the two types in the gut lumen and to assess the potential of fecal-oral disease transmission. The proportion of defectors rose to 10% by day 2 postinfection (p.i.) and to about 99% by day 10 p.i. (Figure 1A), in line with our previously

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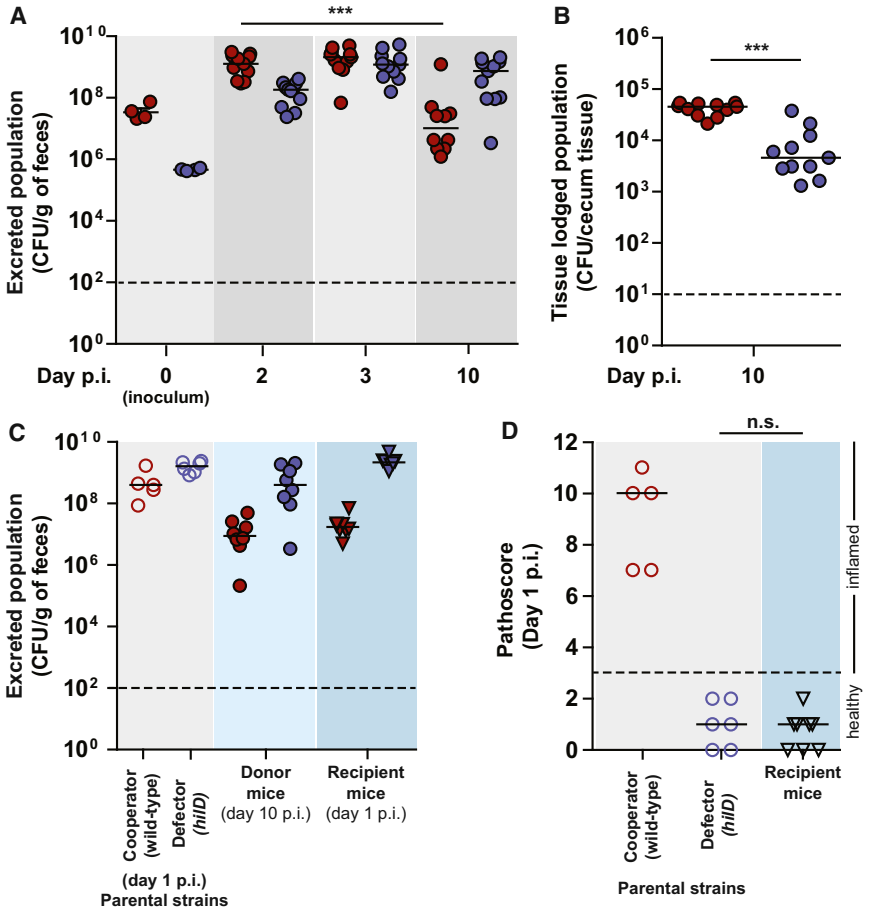

Figure 1. Avirulent Defectors Outcompete Virulent Wild-Type Cooperators in the Gut Lumen and Reduce Disease Transmissibility

(A and B) 129SvEv mice were infected with a 100:1 mixture of wild-type cooperators (M3067, red symbols) and *hilD* defectors (M3105, blue symbols; 5×10^7 cfu total, by gavage).

(A) Absolute population sizes in the inoculum and the feces as determined by plating.

(B) Population sizes of the tissue-lodged bacteria at day 10 postinfection as determined by a gentamycin protection assay, indicating that cooperators are predominant in this compartment.

(C and D) Transmissibility experiments. C57BL/6 mice were inoculated with a fecal suspension from donor mice at day 10 p.i. (approx. 5×10^7 cfu total). Infections with the original wild-type or *hilD* strains (5×10^7 cfu by gavage) served as controls.

(C) Fecal population sizes in control mice inoculated with the parental wild-type or *hilD* strains (open circles), donor mice (filled circles), and recipient mice (triangles).

(D) Intestinal inflammation at day 1 p.i. determined by pathology scoring on stained cecal cross-sections. Dashed line delimits the threshold between healthy and inflamed tissues.

(A–C) Dashed lines mark limits of detection.

(D) n.s., statistically nonsignificant ($p \geq 0.05$), ***$p < 0.001$, Mann-Whitney U test.

published results [5]. Controlled transmission was performed into C57BL/6 mice (*Nramp*$^{-/-}$; very well established infection kinetics [12]) to directly quantify the transmission potential of the infection at the end of the experiment. Fecal pellets from donor mice at day 10 p.i. were homogenized and used to orally infect recipient mice. The *S.* Typhimurium population that arose in the recipient mice was dominated by defectors, and, consequently, no inflammation was observed (Figures 1C and 1D). This was equivalent to control infections with the original *hilD* mutant strain alone. In contrast, control infections with the original wild-type strain lead to pronounced disease. These results established that the rise of avirulent defectors in the gut lumen of infected mice indeed impedes disease transmissibility, at least in the context of our coinoculation experiments.

Although avirulent defectors became dominant in the gut lumen, the population dynamics in the gut tissue were different: defectors remained underrepresented by day 10 p.i. compared to wild-type cooperators. This observation was based on plating of gut-tissue-lodged bacteria from mice infected with mixtures of cooperators and defectors (Figure 1B) and supported by a fluorescence microscopy approach in mice infected either by the wild-type or the defector strains alone (Figures 2A and 2B). When *S.* Typhimurium enters host cells, it expresses the needle component gene *ssaG* [20], and we therefore used a plasmid carrying *gfp-mut2* fused to the *ssaG* promoter to enumerate *S.* Typhimurium loads in the gut tissue [17]. The fluorescence microscopy-based enumeration of tissue loads was further confirmed by antibody staining of tissue-lodged bacterial cells [21]. With this technique we detected numerous cooperators but very few defectors in the gut tissue (Figures 2A and 2B). This finding correlates with tissue plating, which showed that wild-type cooperators infected the gut tissue much more efficiently than the defectors (Figure 2C) while the luminal population sizes were equivalent (Figure 2D). This indicates that wild-type *S.* Typhimurium but not defectors enter the gut tissue, a site that has previously been reported to confer protection from antibiotics [3, 4]. These results established the basic premise of our hypothesis: cooperators and defectors dominate in different host compartments and could thus be differentially sensitive to antibiotics whose efficacy differs between compartments.

Ciprofloxacin Promotes Virulence and Disease Transmission

We then tested the prediction that antibiotic treatment can tilt the selective balance between cooperators and defectors and promote cooperative virulence. All strains used in this study showed comparable sensitivity to ciprofloxacin and identical minimal inhibitory concentrations (MIC = 0.015 μg/ml) (Figure S1; Supplemental Information). We coinfected mice with wild-type *S.* Typhimurium and the *hilD* mutant as described above and treated with two successive doses of ciprofloxacin (3 mg per os each in an interval of 8 hr) at day 2 p.i. This was sufficient to eliminate both *S.* Typhimurium strains from the gut lumen by day 3 p.i. (Figure 3A). In contrast, *S.* Typhimurium was still detectable in the gut tissue. Differential plating revealed that all bacteria in the gut tissue were from the wild-type strain, and no defectors were found (Figure 3B). In line with previous work, fecal shedding of *S.* Typhimurium relapsed a few days after we ended

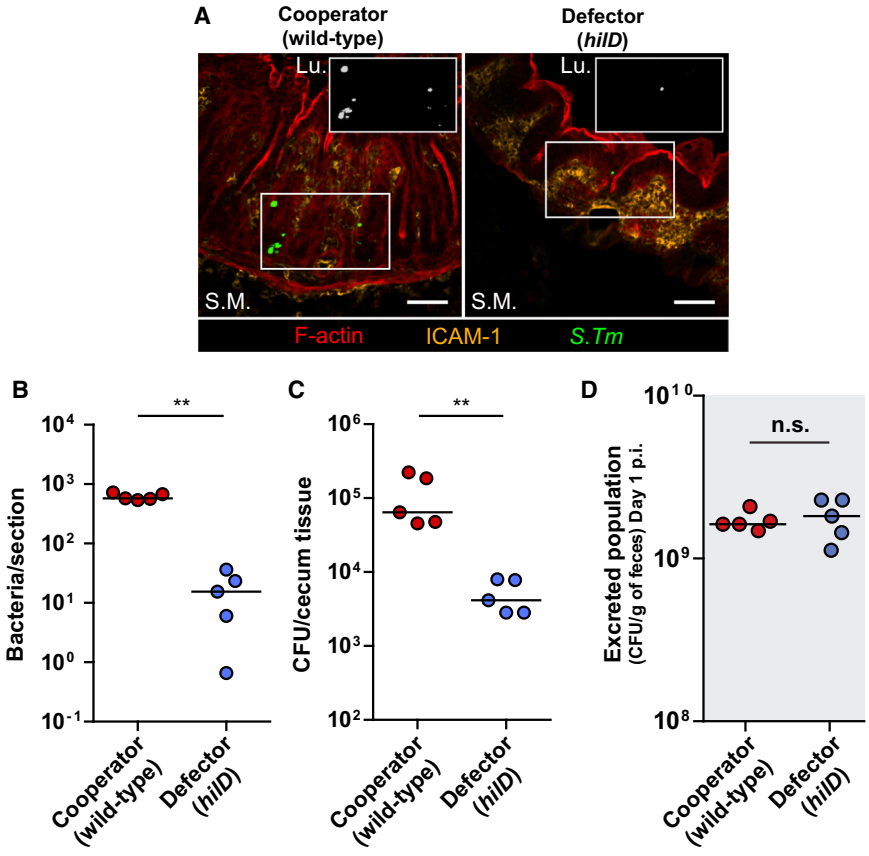

Figure 2. Defector Mutants Cannot Efficiently Invade the Gut Tissue

129SvEv mice were infected for 1 day with *gfp*-labeled bacteria (wild-type or *hilD*, pM975; 5 × 10^7 cfu by gavage), and bacterial loads in the gut tissue were analyzed by fluorescence microscopy and by plating.

(A) Representative fluorescence microscopy images. Tissue sections were stained for ICAM-1/CD54 (Cy3-IgG) and F-actin (Alexa 647-Phalloidin). Inserts, green fluorescent signal from bacteria lodged in the tissue. Lu., Lumen; S.M., Submucosa. Scale bars represent 50 μm.

(B) Enumeration of the intracellular bacteria per tissue section.

(C) Plating assay quantifying the tissue-lodged *S.* Typhimurium population.

(D) *Salmonella* population sizes in feces at day 1 p.i. The two strains colonized the cecum lumen at equivalent levels (n.s., statistically nonsignificant, Mann-Whitney U test $p \geq 0.05$) whereas the wild-type was more efficient at invading the tissues. The samples were from the same mice as analyzed in (A) and (B). **$p < 0.01$, Mann-Whitney U test.

the antibiotic treatment [3, 22]. At day 10 p.i., the relapsing *S.* Typhimurium populations had reached the loads prior treatment in feces and cecal tissues. Strikingly, the stool and tissue populations were exclusively composed of the virulent wild-type cooperators, and defectors were not detected (Figures 3A and 3B). Comparable observations were made when mice were infected with 100% wild-type cooperators and defectors naturally arose by mutation (Figure S2). Control experiments verified that the dominance of the virulent wild-type *S.* Typhimurium after treatment was not a consequence of beneficial mutations acquired during infection; clones isolated from infected mice were indistinguishable from a naive wild-type strain in terms of competitive ability in the host (Figure S3) and sensitivity to ciprofloxacin (MIC = 0.015 μg/ml). Also, dominance of the virulent wild-type cooperators after treatment was not attributable to numerical differences between wild-type and defectors in the gut lumen before treatment or to the markers used to distinguish between the strains (Figure S4). These results supported the interpretation that bacteria causing the relapse originated from a phenotypically persistent reservoir in the host's tissue, not from a pool of genetically resistant pathogenic cells.

Since we had found that the rise of defectors abolishes disease transmission, one would expect that the selective suppression of defectors through antibiotics would reverse this effect. We performed transmission experiments to investigate whether the gut luminal *S.* Typhimurium population formed during the relapse is capable of eliciting disease in recipient hosts (Figures 3C and 3D). In line with our hypothesis, controlled transmissions from "relapsing" donor mice (at day 10, i.e., 7 days after the last ciprofloxacin dose) led to pronounced mucosal inflammation in the recipient animals. The level of inflammation was indistinguishable from inflammation in control mice infected with the original wild-type *S.* Typhimurium strain (Figures 3C and 3D) and much stronger than the disease elicited by controlled transmission without ciprofloxacin treatment (compare Figures 3D and 1D). Thus, antibiotic treatment can indeed enhance disease transmissibility.

Finally, we tested whether invasion of the gut tissue in itself was necessary for *S.* Typhimurium to survive antibiotics treatment. Avirulent defectors that spontaneously arise in infected mice [5] and that we used here may differ from the wild-type in other traits besides the impaired *ttss-1* expression. This is because HilD regulates the expression of several other genes [23–25]. Formally, we could not exclude that these effects contributed to the differential sensitivity to antibiotics, e.g., by slowing down the bacterial growth rate of the *ttss-1*-expressing fraction of some gut luminal *S.* Typhimurium population. In fact, in vitro, such a reduced growth rate of the *ttss-1*-expressing cells can confer ciprofloxacin persistence in nonhost environments [26]. This raised the question whether tissue invasion per se was the decisive factor in our in vivo experiments. To specifically evaluate the role of tissue entry in the survival of antibiotic treatment, we used a mutant of *S.* Typhimurium (Δ4) carrying an intact *hilD* gene but deleted for the four TTSS-1 effectors that drive gut tissue invasion (i.e., *sopE*, *sopE2*, *sipA*, and *sopB* [18, 27]). We mixed this mutant with the wild-type and inoculated mice with a 1:10 ratio of Δ4 versus wild-type *S.* Typhimurium. This ratio was stable in the feces during the first 2 days of the experiment (Figures 4A and S2A), which showed that the two strains were equally competitive in the gut lumen and that the Δ4 mutant was still featuring the growth defect associated with *ttss-1* expression. Importantly, Δ4 mutants did not efficiently enter the gut tissue (Figures 4B, 4C, and S2B). As before, ciprofloxacin treatment eliminated all bacteria (Δ4 and wild-type *S.* Typhimurium

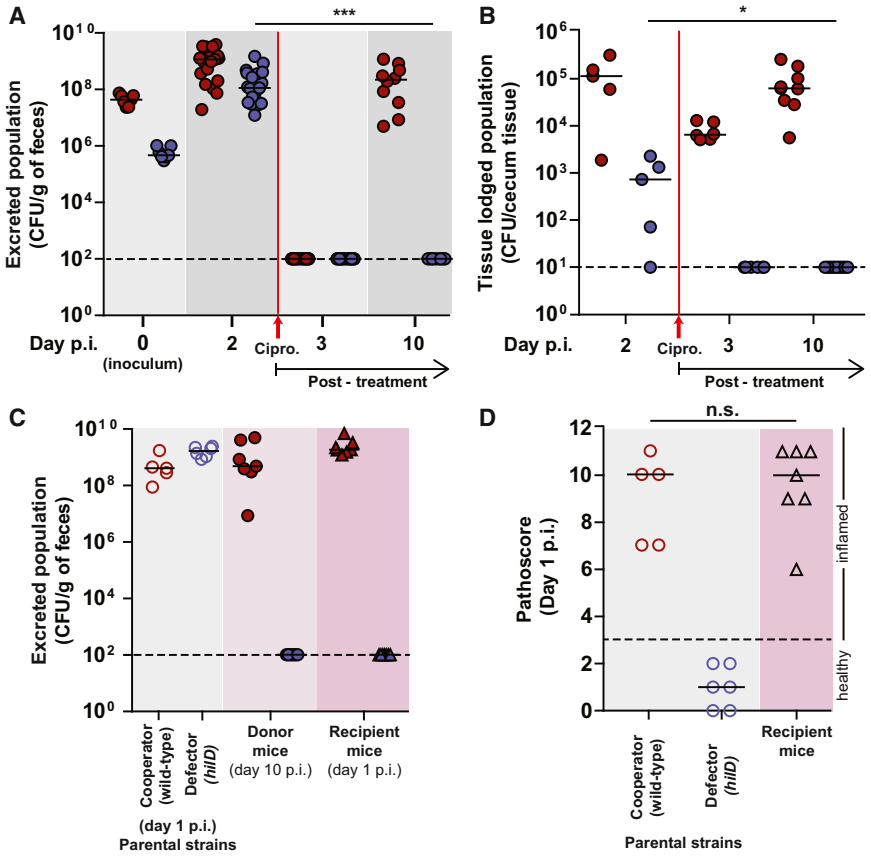

Figure 3. Ciprofloxacin Treatment Selects for Virulent Wild-Type Cooperators and Favors Disease Transmissibility

129SvEv mice were infected with a 100:1 mixture of wild-type cooperators (M3067, red symbols) and *hilD* defectors (M3105, blue symbols; 5×10^7 cfu total, by gavage). Two doses of ciprofloxacin were applied at 48 and 56 hr p.i.

(A) Absolute population sizes in the inoculum and the feces were determined by plating. At day 10 p.i., feces harbored only wild-type cooperators.

(B) Population sizes of the tissue-lodged bacteria as determined by plating of washed cecum tissue samples. Cooperators are predominant in this compartment. No defectors were detectable after ciprofloxacin treatment.

(C and D) Transmissibility experiments. C57BL/6 mice were inoculated with a fecal suspension from ciprofloxacin-treated donor mice (approx. 5×10^7 cfu total, day 10 p.i.). Infections with the original wild-type or *hilD* strains (5×10^7 cfu by gavage) served as controls.

(C) Fecal population sizes in control mice inoculated with the parental wild-type or *hilD* strains (open circles), donor mice (filled circles), and recipient mice (triangles).

(D) Intestinal inflammation at day 1 p.i. determined by pathological scoring on stained cecal cross-sections.

Dashed line delimits the detection limit or the threshold between healthy and inflamed tissues; n.s., statistically nonsignificant ($p \geq 0.05$), *$p < 0.05$, ***$p < 0.001$, Mann-Whitney U test. A red line and arrow mark the time of ciprofloxacin treatment.

alike) from the gut lumen, and relapse occurred after the end of the treatment. The relapsing population was composed almost exclusively of wild-type *S.* Typhimurium (Figure 4A, day 10 p.i.). This supported the notion that the different spatial localization of wild-type *S.* Typhimurium and avirulent mutants in the host was responsible for the differential sensitivity to antibiotics. This would be in line with recent work showing that the growth of intracellular *S.* Typhimurium is slowed down (e.g., by toxin-antitoxin activity), thus representing a type of "host-cell-induced" persistence [3, 4]. However, we cannot formally rule out that limited tissue penetration of the antibiotic may also contribute to some extent to the selective advantage of tissue-lodged wild-type *S.* Typhimurium.

Conclusions

Reduced growth rates have emerged as a cardinal feature conferring bacterial persistence [1]. In the case of *S.* Typhimurium, such slow growth can be observed outside of host tissues (i.e., as a consequence of *ttss-1* expression in vitro [15, 26]) or within host tissues, presumably through host-cell-induced mechanisms [3, 4, 28]. Our experiments show that the latter can have important consequences for within-host evolution of virulence and disease transmissibility. During antibiotic treatment, tissue-lodged virulent clones of *S.* Typhimurium but *not* gut luminal avirulent defectors do form a persistent reservoir. After cessation of therapy, virulent persisters reseed the gut from the host tissues and are transmissible to new hosts. This provides essential new information on selective effects of antibiotics. Although there is a strong focus on antibiotics-driven selection for resistance, our results establish that treatment can also impact within-host evolution of virulence and promote disease transmission. Understanding such unexpected effects of antibiotics and the evolutionary consequences of persister formation is important for devising improved antibacterial strategies.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.07.028.

Author Contributions

M.D., M.E.S., T.D., M. Arnoldini, and W.-D.H. designed the experiments. M.D. and M.E.S. performed the experiments; M.E.S. contributed to Figure 2. M.D., M. Ackermann, and W.-D.H. analyzed the data and wrote the paper.

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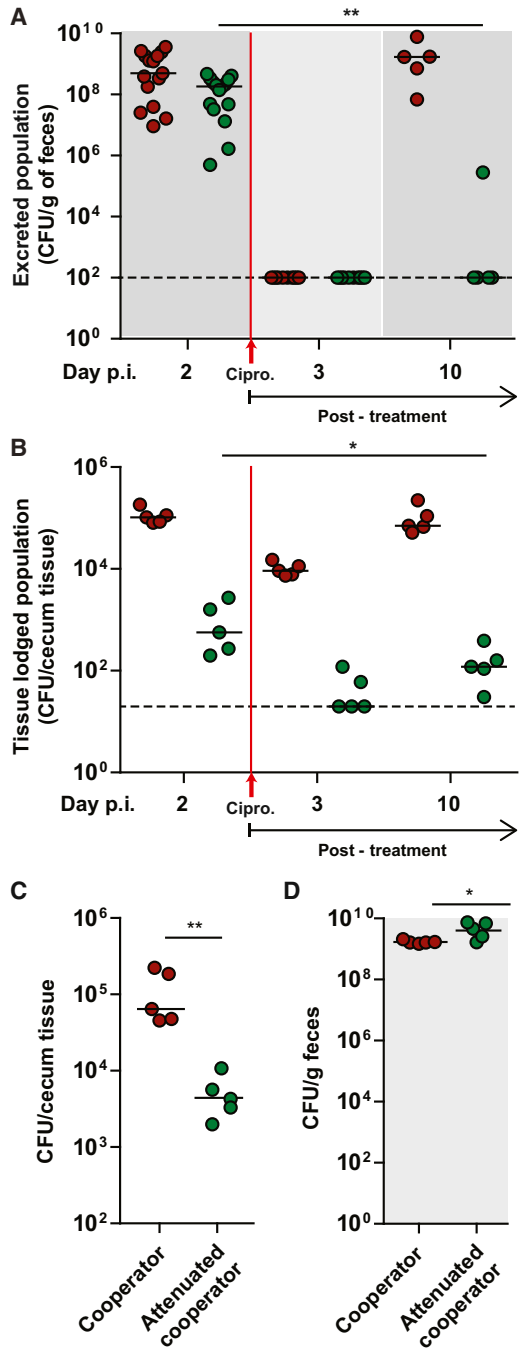

Figure 4. Tissue Entry Is Necessary to Form a Ciprofloxacin-Persistent *S.* Typhimurium Reservoir

(A and B) 129SvEv mice were infected with a 10:1 mixture of wild-type *S.* Typhimurium (SB300, red) and the attenuated mutant (M3143; Δ4, green). Two doses of ciprofloxacin were applied at 48 and 56 hr p.i.

(A) Absolute population sizes in the feces as determined by plating.

(B) Gut tissue loads as analyzed by plating. Dashed lines mark limits of detection. *$p < 0.05$, **$p < 0.01$; Mann-Whitney U test. A red line and arrow mark the time of ciprofloxacin treatment. The effector-deficient strain Δ4 can colonize the gut lumen, but fails to colonize the gut tissue.

(C and D) Infections in 129SvEv mice with one strain, alone. The mice were infected with M3143 (Δ4, green; 5×10^7 cfu by gavage) and the population sizes were determined in tissues (C) and feces (D) at day 1 p.i. Data from mice infected with wild-type *S.* Typhimurium (M3067, red, Figure 2) are shown for comparison. *$p < 0.05$, **$p < 0.01$, Mann-Whitney U test.

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