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Differences in carbon metabolic capacity fuel co-existence and plasmid transfer between Salmonella strains in the mouse gut

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Publication date:
2023-07-12

Permanent link:
https://doi.org/10.3929/ethz-b-000622475

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Originally published in:
Cell Host & Microbe 31(7), https://doi.org/10.1016/j.chom.2023.05.029

Funding acknowledgement:
167121 - Towards quantification of the contribution of plasmids to the spread of antibiotic resistance (SNF)
173338 - Deciphering the initial steps that lead to Salmonella Typhimurium diarrhea (SNF)
192567 - Mechanisms controlling the Salmonella Typhimurium gut infection (SNF)

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Article

Cell Host & Microbe

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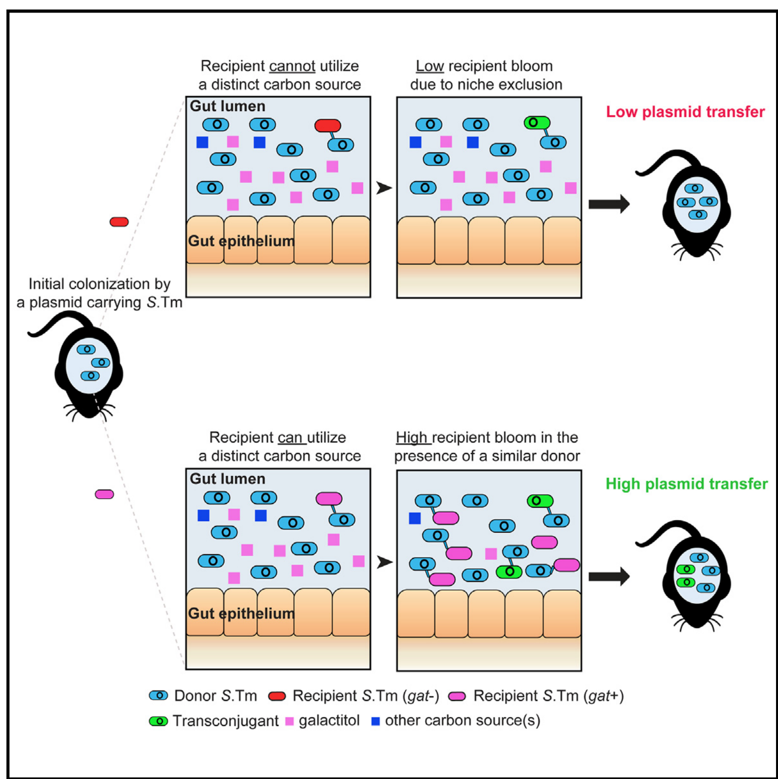
Graphical abstract



Highlights

- Strain-specific traits enable discrete *Salmonella* strains to co-bloom in the mouse gut
- *Salmonella* can use galactitol or arabinose during co-colonization with a different strain
- Single-carbon usage fuels plasmid transfer between different Enterobacteriaceae strains
- Carbon metabolic capacity enables gut-luminal co-blooming of distinct Enterobacteriaceae

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In brief

Gül et al. reveal that the ability to exclusively utilize a single-carbon source (e.g., galactitol or arabinose) enables *Salmonella* to bloom from low numbers in a gut pre-colonized by another Enterobacteriaceae population. This metabolic strategy promotes the transfer of antibiotic resistance plasmids in the mouse gut.

Gül et al., 2023, Cell Host & Microbe *31*, 1140–1153
July 12, 2023 © 2023 The Author(s). Published by Elsevier Inc.
https://doi.org/10.1016/j.chom.2023.05.029

Article

Differences in carbon metabolic capacity fuel co-existence and plasmid transfer between *Salmonella* strains in the mouse gut

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https://doi.org/10.1016/j.chom.2023.05.029

SUMMARY

Antibiotic resistance plasmids can be disseminated between different Enterobacteriaceae in the gut. Here, we investigate how closely related Enterobacteriaceae populations with similar nutrient needs can co-bloom in the same gut and thereby facilitate plasmid transfer. Using different strains of *Salmonella* Typhimurium (*S*.Tm SL1344 and ATCC14028) and mouse models of Salmonellosis, we show that the bloom of one strain (i.e., recipient) from very low numbers in a gut pre-occupied by the other strain (i.e., donor) depends on strain-specific utilization of a distinct carbon source, galactitol or arabinose. Galactitol-dependent growth of the recipient *S*.Tm strain promotes plasmid transfer between non-isogenic strains and between *E. coli* and *S*.Tm. In mice stably colonized by a defined microbiota (OligoMM[12]), galactitol supplementation similarly facilitates co-existence of two *S*.Tm strains and promotes plasmid transfer. Our work reveals a metabolic strategy used by Enterobacteriaceae to expand in a pre-occupied gut and provides promising therapeutic targets for resistance plasmids spread.

INTRODUCTION

Infections caused by antibiotic-resistant bacteria represent a major public health issue.[1] The horizontal transfer of antibiotic resistance genes (ARGs) via conjugative plasmids contributes to this issue by accelerating the emergence of new bacterial strains genetically resistant to antibiotic treatment.[2] Recent studies highlighted the mammalian gut and the associated microbiome as a suitable place for acquisition and spread of resistance plasmids.[3] Extensive use of antibiotics is one known factor that leads to the emergence of newly resistant bacteria.[4] However, environmental factors (besides antibiotic selection) that contribute to the spread of resistance plasmids within the mammalian gut are not well understood.

The mammalian gut is a complex ecosystem that contains metabolic niches for thousands of bacterial species.[5] The colonizing bacteria form a diverse community—the gut microbiota—that provides essential functions to its host, including colonization resistance to pathogenic bacteria.[6] According to Freter's nutrient-niche theory, for an invading pathogen to establish itself and trigger disease, it must find at least one available niche.[7,8] In a stably colonized gut, the resident microbiota prevents the colonization by enteric pathogens through various mechanisms including limitation of the nutrient sources available for growth. Therefore, according to the niche exclusion theory, two bacterial populations with high metabolic overlap that compete for the same nutrients are unlikely to grow up to high densities in the same gut.[9,10] Furthermore, two different bacterial strains often enter the gut at different time points and at different densities, making it even more unlikely for two distinct bacterial strains to expand in the same gut. However, recent observations in the microbial ecology field appear to be at odds with this school of thinking, suggesting that several members of the Enterobacteriaceae family can co-colonize the gut transiently and replace each other at the strain level.[11,12] Nevertheless, mechanisms by which different related bacteria can successfully co-colonize the gut remain to be explored.

In the gut, resistance genes can disseminate at the intra- and interspecies level via conjugation of plasmids carrying these genes. The efficient transfer of plasmids, however, is heavily dependent on factors such as cell density, energy availability, and growth rate that are affected by population sizes of donor and recipient bacteria in the gut.[13,14] To reach high densities, two bacterial populations need to find available nutrient niches for growth. Therefore, plasmid transfer from a donor to its close relative with similar nutrient needs in a stably colonized gut is unexpected, particularly among Enterobacteriaceae, which are typically present at low densities of 10^2–10^6 CFU/g in stools

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from healthy people.[15–17] Surprisingly, evidence from both clinical studies and animal models suggests that antibiotic resistance plasmids can be disseminated between different Enterobacteriaceae within the mammalian gut.[12,13,18–21] However, which factors determine the efficient plasmid transfer between close species in the gut is not well understood.

Enteric infections caused by the pathogenic members of Enterobacteriaceae, such as *Salmonella enterica* and enteropathogenic *Escherichia coli*, can promote the dissemination of plasmids within the mammalian gut without antibiotic selection for resistance because these bacteria can reach very high densities in the feces upon infection.[13,18,22,23] Previous work revealed that *Salmonella* persisters in host tissues, carrying antibiotic resistance plasmids, can re-seed back to gut lumen at very low frequencies and are able to expand from very low to very high densities (i.e., from ca. 10^4 CFU to ca. 10^8 CFU per gram feces) in a gut already highly colonized by another *Salmonella*.[13,18] Interestingly, in these studies, the *Salmonella* strains used as donors and as recipients were non-isogenic, (*S*.Tm SL1344 and ATCC14028) and had similar nutrient needs to grow. This raises the question what enables the secondary growth of a similar *Salmonella* strain in a pre-colonized gut (termed co-blooming from hereon) and thereby contributes to the spread of antibiotic resistance plasmids.

Here, we aimed to understand how closely related strains with overlapping metabolic capacities can co-bloom (i.e., both strains reaching high densities) in the mammalian gut and how this promotes the spread of antibiotic resistance plasmids. To do this, we performed *in vivo* plasmid transfer assays using different strains of the model enteropathogen *Salmonella* Typhimurium (*S*.Tm) and mouse models of Salmonellosis with varying microbiota complexities.

RESULTS

Co-infection by non-isogenic *S*.Tm strains boosts plasmid transfer in the mouse gut

To study the role of strain-level differences in facilitating co-existence and plasmid transfer between closely related bacteria, we designed an experimental model where we infected mice with either two identical or non-identical *S*.Tm strains (i.e., the donor and the recipient). We used a higher number of donor (1,000-fold excess) to study how the bloom of recipients from low numbers in the presence of a highly colonizing donor population would affect plasmid transfer efficiency. We chose two natural *S*.Tm strains that are commonly used in laboratory experiments: SL1344 (denoted as *S*.Tm-A),[24] a bovine isolate, and ATCC14028 (denoted as *S*.Tm-B),[25] an avian isolate (key resources table). These two strains were particularly well suited for our proof-of-principle experiments because they are interchangeably used in mouse models of Salmonellosis in the field and reported to colonize the mouse gut to a similar total density due to the similar metabolic abilities.[26–28] We infected streptomycin-pretreated C57BL/6 mice with a combination of the donor (carrying an extended spectrum beta-lactamase plasmid [pESBL15] from an *E. coli* strain isolated from a patient at the University Hospital Basel, which has a 98% identity [88% coverage] to the natural plasmid of SL1344; P2[29]) and the recipient at a ratio of 1,000:1 (donor:recipient) in the inoculum. Both strains were either used as a donor (*S*.Tm-A + pESBL15 or *S*.Tm-B + pESBL15) or as a recipient (*S*.Tm-A or *S*.Tm-B) to have an unbiased approach (experimental scheme; Figures 1A and S1A). We determined plasmid transfer by quantifying the number of transconjugants (recipients carrying the plasmid) in feces via selective plating. Plasmid transfer (the number of transconjugants in feces) was significantly higher when we used a donor:recipient pair consisting of non-identical strains (i.e., *S*.Tm-A + pESBL15 as a donor and *S*.Tm-B as a recipient or vice versa) than identical strains (i.e., *S*.Tm-A or B as both the donor [-+pESBL15] and the recipient) at day 2 post infection (p.i.) Strikingly, in both non-identical strain pairs, transconjugants were formed with higher efficiency (ca. median of 10^7 CFU/g feces and 10^6 CFU/g, respectively) than they did in identical strain pairs (ca. median of 10^4 CFU/g feces for both; Figures 1B and S1B). Notably, donor densities were comparable in all groups (ca. median of 10^8–10^9 CFU/g feces; Figures 1C and S1C), whereas the total number of recipients was significantly higher in mice inoculated with non-identical strains than in mice infected with identical strains (Figures 1D and S1D). Therefore, we reasoned that higher plasmid transfer in the former might be attributable to the fact that the recipient can expand from low numbers only when the gut is colonized by a non-identical *Salmonella* strain.

To study co-blooming, i.e., the colonization of a pre-colonized (by another *Salmonella* strain) gut, we performed an infection experiment where we tested the ability of *S*.Tm-A to invade into the gut colonized by *S*.Tm-B (1,000-fold excess). As a control, we performed another infection experiment with *S*.Tm-A growing into the gut colonized by *S*.Tm-A (1,000-fold excess; identical). We ran the infections for 3 days to assess the growth extent of the less abundant strain. When the mice were co-colonized by the identical strain pair (*S*.Tm-A into *S*.Tm-A), the minor strain was not able to expand in the mouse gut and the ratio in the feces at day 3 p.i. was comparable to the inoculum (Figure 1E). Strikingly, in the scenario of the non-identical strain pair (*S*.Tm-A into *S*.Tm-B), the less abundant strain was able to expand significantly and reach equivalent densities in the gut as the more abundant strain by day 3 p.i. (Figure 1F). To make sure that these strains grow with an equal rate *in vivo*, we did control experiments where we inoculated them at the same ratio and observed that both strains grew at the same rate without measurable interference (Figures S1E and S1F). Taken together, we concluded that strain-level differences can allow a second *S*.Tm strain to infect a pre-colonized mouse gut and thereby promote the transfer of antibiotic resistance plasmids among *Salmonella* strains.

S.Tm-A and *S*.Tm-B differ in their carbon metabolic capacities

Next, we wanted to study the differences that enable the bloom of the less abundant strain in face of a dense *Salmonella* population. To identify the genetic differences (e.g., in metabolic capacities) between these two strains and test if strain-specific gene(s) can explain the differential expansion of the recipients, we compared the two genomes (chromosomes and plasmids) using BLAST and visualized them using Artemis Comparison Tool. As expected, the chromosomes were highly homologous (99.98% sequence identity over 99% of the chromosome for

Figure 1. Infection of mice with two different *S.*Tm strains results in high plasmid transfer because recipients can bloom from low numbers in the presence of a highly colonizing donor

(A) Experimental scheme. Streptomycin-pretreated C57BL/6 mice infected with donor (1,000-fold excess; *S.*Tm-B + pESBL15) and recipient (*S.*Tm-A [black] or *S.*Tm-B [orange], n = 8 or 7; 3 independent experiments; by gavage) for 2 days.

(B–D) (B) Transconjugants, (C) donors, and (D) recipients + transconjugants (CFU/g feces) as determined by selective plating.

(E and F) Streptomycin-pretreated C57BL/6 mice infected with (E) identical (*S.*Tm-A:*S.*Tm-A = 1:1,000; carry different tags) or (F) different strains (*S.*Tm-A:*S.*Tm-B = 1:1,000) for 3 days (n = 6 or 7; 4 independent experiments; by gavage). Fecal loads as determined by selective plating. Bars, median; dotted lines, detection limit. Two-tailed Mann Whitney-U tests to compare two groups in each panel. $p \geq 0.05$ not significant (ns), $p < 0.01$ (**), $p < 0.001$ (***).

SL1344, 98% for ATCC14028), (Figure 2A) further supporting the expectation for high metabolic overlap. SL1344 contained 2 additional plasmids compared with ATCC14028 (Figure 2A). By focusing on areas that lack homology, we could observe that a number of genes were differentially present in one strain and not in the other (Table S1; 208 genes in SL1344 [S.Tm-A] and 67 genes in ATCC14028 [*S.*Tm-B]). Expectedly, most of these were genes carried on plasmids (pCol1b9 plasmid in SL1344) or on prophages (Gifsy3 prophage on ATCC14028) being present only in one of the strains. However, we also observed genetic differences in chromosomal genes involved in the transport or the processing of certain carbon sources (Table S1). One striking example was the galactitol utilization operon of which most of the parts were missing in *S.*Tm-B, whereas *S.*Tm-A carried the full operon (Figure 2B). Specifically, 6 genes involved in the import (*gatA*, *B*, and *C*) and

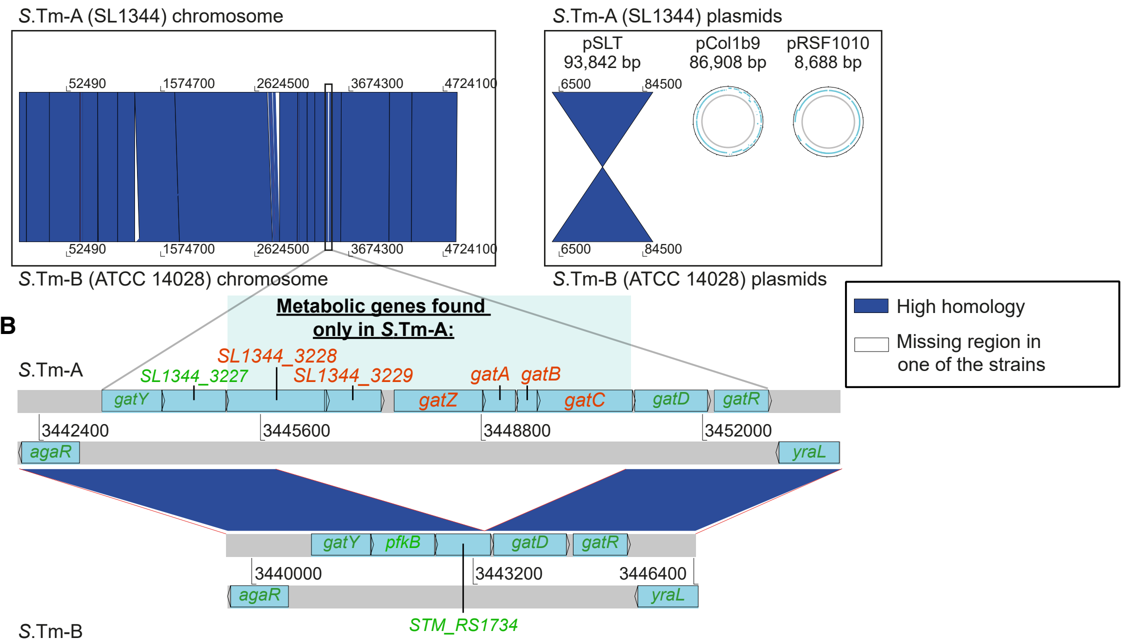


Figure 2. S.Tm-A and S.Tm-B are different in their metabolic capacity to utilize carbon sources

(A) Comparison of S.Tm-A vs. S.Tm-B (chromosomes and plasmids) genomes using Artemis Comparison Tool (https://www.sanger.ac.uk/science/tools/artemis-comparison-tool-act) and DNAplotter (https://www.sanger.ac.uk/tool/dnaplotter/). Coding sequences, light blue.

(B) Metabolic genes found only in S.Tm-A but not in S.Tm-B (highlighted in red). Blue, high homology (minimum 10,000 score cutoff); white, regions missing in one of the strains.

processing of intracellular galactitol to glyceraldehyde 3-phosphate (*SL1344_3228*, *SL1344_3229*, and *gatZ*) were absent in S.Tm-B. This was in line with a previous report specifically comparing *gat* loci in different Enterobacteriaceae, including the two S.Tm strains studied here.[30]

The context-dependent utilization of a distinct carbon source, galactitol, allows S.Tm-A to expand in the gut pre-occupied by S.Tm-B

Galactitol is a sugar alcohol found in plants, which is also present in mouse chow diet, and can support the growth of several tested *Salmonella* strains in the gut.[10,31] The galactitol (*gat*) operon was reported to have a crucial role in determining colonization resistance to enteropathogenic bacteria, including *Salmonella spp*.[10,32] Interestingly, a recent study showed that commensal *E. coli* can provide colonization resistance by competing with *Salmonella spp*. for galactitol in a context where other niches are occupied by a complex microbiota.[10] However, the ability to utilize galactitol seems to be a strain-specific feature in the family of Enterobacteriaceae. Therefore, we sought to test whether S.Tm-A but not S.Tm-B can grow on galactitol. To confirm the ability of S.Tm-A to utilize galactitol as a carbon source, we generated a mutant lacking the genes involved in the import of this sugar (*gatA*, *gatB*, and *gatC*; S.Tm-A Δ*gatABC*). As expected, the mutant failed to metabolite galactitol on modified MacConkey agar plates with galactitol as a carbon source (Figure S2A). Next, we performed *in vitro* growth assays in minimal media (M9) supplemented with galactitol or glucose and confirmed that S.Tm-A can grow anaerobically in M9 with galactitol, whereas S.Tm-A Δ*gatABC* and S.Tm-B cannot (Figures S2B and S2C).

Having established that the *gat* operon is exclusively present in S.Tm-A and enables the growth on galactitol, we set out to test whether this pathway is involved in the expansion of S.Tm-A in the mouse gut pre-colonized by S.Tm-B. To achieve this, we performed competitive infection experiments between S.Tm-A and S.Tm-A Δ*gatABC* in streptomycin-pretreated mice. In one group, we infected mice with a 1:1 ratio of S.Tm-A and S.Tm-A Δ*gatABC* in the presence of excess S.Tm-B (1,000-fold; 1:1:1,000 final ratios), and in the second group, we did the same without the excess S.Tm-B (1:1). In the presence of excess S.Tm-B (Figure 3A; orange background), S.Tm-A wild type had a significant fitness advantage over the mutant and expanded as before, whereas the *gatABC* mutant was unable to bloom in the pre-occupied gut (Figures 3A and S3A). Strikingly, when we removed the excess S.Tm-B (Figure 3B; blue background), the *gat* operon did not provide any fitness advantage and both the wild-type S.Tm-A and the *gatABC* mutant grew to the carrying capacity (Figures 3B and S3A). This suggested that galactitol is a context-dependent carbon source, which is preferred only in conditions where the other niches are pre-occupied by a strain with similar metabolic capacities. To further substantiate these findings and exclude a possible role of gut inflammation, and the associated changes in luminal metabolites, we performed a similar competitive infection using avirulent versions of the above strains (S.Tm-A^{avir} and S.Tm-A^{avir} Δ*gatABC*). As the major strain (1,000-fold in excess), we used

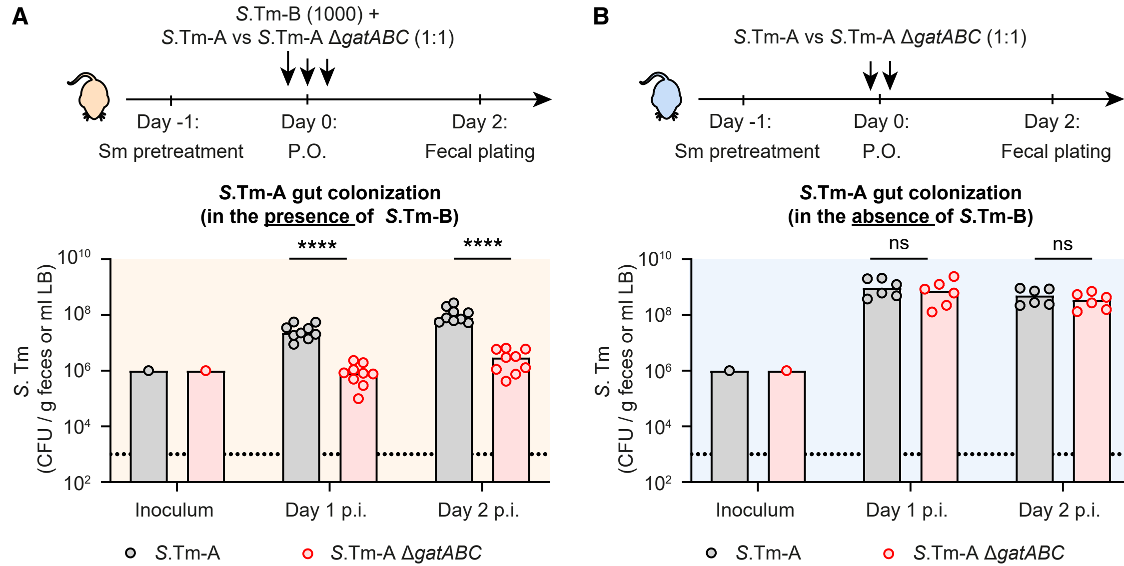


Figure 3. The ability to use a distinct sugar, galactitol, allows *S*.Tm-A to colonize the mouse gut in the presence of excess *S*.Tm-B

Competitive infection with *S*.Tm-A and *S*.Tm-A Δ*gatABC* with or without 1,000-fold excess *S*.Tm-B in streptomycin-pretreated mice for 2 days. Fecal loads of *S*.Tm-A (black) and *S*.Tm-A Δ*gatABC* (red) as determined by selective plating (CFU/g feces) in (A) presence or (B) absence of *S*.Tm-B (n = 9 or 6, 5 independent experiments; by gavage). Bars, median; dotted lines, detection limit. Two-tailed Mann Whitney-U tests to compare two groups in each panel. $p \geq 0.05$ not significant (ns), $p < 0.0001$ (****).

either *S*.Tm-B^{avir} or another *gat*-negative Enterobacteriaceae strain (*E. coli* K12; Figure S3C). This way, we could test if our findings at the species level are applicable at the family level as well. Notably, in both groups, *gat*-proficient *S*.Tm-A^{avir} had a fitness advantage over the *gat*-deficient *S*.Tm-A^{avir} and could expand in the presence of both high excess *S*.Tm-B^{avir} (Figure S3B; orange background) and high excess *E. coli* K12 (Figure S3C; brown background). Together, we concluded that *S*.Tm-A can utilize galactitol as an alternative carbon source in conditions where a closely related species with similar metabolic capacities already occupies the other available niches. These findings were comparable in the avirulent pathogenicity background as well.

In the presence of an isogenic *S*.Tm-A mutant, lacking the respective metabolic pathway, wild-type *S*.Tm-A expands on an exclusive carbon source

Several studies in the last years highlighted the crucial role of food composition in determining susceptibility to enteric infections.[33–35] It is well established that diet can alter available niches in the gut and can influence the success of incoming bacteria to colonize the gut.[6] Therefore, we questioned whether the supplementation of galactitol in the diet would have an impact on the co-existence of two isogenic strains that are identical in their whole genome except for the *gat* operon. To test this, we first infected streptomycin-pretreated mice with a 1:1,000 mixture of *gat*-proficient and *gat*-deficient isogenic strains (*S*.Tm-A and *S*.Tm-A Δ*gatABC*; experimental scheme in Figure S4A) and supplemented them with 0%, 0.1%, or 1% galactitol in the drinking water. We monitored the ability of the *gat*-proficient (minor) strain to co-bloom using different concentrations of galactitol supplemented in the drinking water. At day 2 p.i., *S*.Tm-A expansion in the presence of its Gat- counterpart correlated with the galactitol concentrations: 1% galactitol fueled the highest growth (ca. 1,000-fold) and 0% galactitol allowed for much less expansion of the Gat+ strain (ca. 3-fold) than that observed with 0.1% or 1% galactitol in the drinking water (Figure S4B).

Second, we repeated the same supplementation experiment with 0% and 1% galactitol and additionally added another group in which we supplemented the mice with 1% arabinose in the drinking water. Arabinose is a non-resorbable carbohydrate, which should become available to bacteria colonizing the large intestine. This setup provided us a control in which the supplemented nutrient source can be used by both strains.[36,37] Because the only difference between the more and the less abundant strain is the ability to utilize galactitol (and a suitable antibiotic resistance marker for selective plating), we could test if the expansion of the minor strain is specifically dependent on galactitol utilization by that strain. In line with the data above, in the control group with no galactitol addition, we observed that *gat*-proficient *S*.Tm-A was able to expand up to 5×10^7 CFU in the presence of the *gat*-deficient strain but was not able to reach to the same density as the major strain by day 2 p.i. (Figure 4A and 4B light blue; Figure S4C and S4D). When we supplemented the drinking water with 1% galactitol, the *gat*-proficient *S*.Tm-A expanded rapidly in the gut of the mice and reached to the same density (ca. 10^9 CFU/g; carrying capacity) in feces as the *gat*-deficient strain (*S*.Tm-A Δ*gatABC*) by day 2 p.i. (Figures 4B and S4D; purple background). Strikingly, drinking water supplementation with 1% arabinose (which can be used by both strains) did not have any effect on the expansion of the *gat*-proficient minor strain and did not change the ratio between the *gat*-proficient and the *gat*-deficient strain (Figures 4B and S4D; turquoise background). Thus, we concluded that supplementation of galactitol, but not a "common" carbon source, in the drinking water is sufficient to promote the growth of the less abundant strain when competing against a *gat*-isogenic

Figure 4. Supplementation with a distinct carbon source that can be used only by one of the strains enables the co-existence of two isogenic *S.*Tm strains in the mouse gut

(A) Experimental scheme. Streptomycin-pretreated C57BL/6 mice infected with a 1:1,000 mixture of *S.*Tm-A and *S.*Tm-A Δ*gatABC* for 2 days. Group 1, normal drinking water; group 2, drinking water with 1% galactitol; or group 3, drinking water with 1% arabinose (n = 8, 6, or 6; 6 independent experiments; by gavage).

(B) Fecal loads of *S.*Tm-A (black) and of *S.*Tm-A Δ*gatABC* (red) as determined by selective plating (CFU/g feces).

(C) Experimental scheme. Streptomycin-pretreated C57BL/6 mice infected with a 1:1,000 mixture of *S.*Tm-A and*S.*Tm-A Δ*araB* for 2 days. Group 1, normal drinking water or group 2, drinking water with 1% arabinose (n = 6 or 7; 3 independent experiments; by gavage).

(D) Fecal loads of *S.*Tm-A (black) and of *S.*Tm-A Δ*araB* (brown) as determined by selective plating (CFU/g feces). Bars, median; dotted lines, detection limit. Two-tailed Mann Whitney-U tests to compare two groups in each panel. $p \geq 0.05$ not significant (ns), $p < 0.01$ (**).

strain. Additionally, we performed a control experiment to assess indirect effects of the galactitol supplementation on the resident microbiota, possibly affecting the Gat-dependent co-blooming between *S.*Tm-A and *S.*Tm-A *gatABC*. In fact, when we used a *gat*-deficient strain as a minor strain (Figure S4D; triangles), galactitol supplementation did not affect the expansion of this strain compared with the second *gat*-deficient strain. Together with the arabinose supplementation experiment, these data verified that the effects observed for galactitol supplementation are caused by the galactitol operon rather than by other confounding variables (e.g., via microbiota composition changes).

Lastly, we asked whether or not other carbon sources besides galactitol could also fuel the bloom of *S.*Tm from low density in a gut pre-colonized by another *S.*Tm strain. To address this, we studied the effect of deleting the arabinose operon. This seemed promising, as arabinose has recently been shown to fuel *S.*Tm growth in the gut of supershedder mice.[36] We constructed an

Figure 5. Utilization of a distinct sugar fuels the transfer of plasmids between *S.*Tm-A and *S.*Tm-B or *E. coli* and *S.*Tm-A in streptomycin-pretreated mice

(A) Experimental scheme. Streptomycin-pretreated C57BL/6 mice infected with donor (1,000-fold excess) and recipient.

(legend continued on next page)

ara-deficient *S*.Tm-A mutant by deleting the *araB* gene of the L-arabinose utilization operon and performed similar competitive infections with *ara*-proficient and -deficient *S*.Tm strains. To this end, we infected streptomycin-pretreated mice with a 1:1,000 mixture of *ara*-proficient and *ara*-deficient isogenic strains (*S*.Tm-A and *S*.Tm-A Δ*araB*). To test the requirement for arabinose availability in the gut, we performed this experiment with two groups of mice. The first group was kept on normal drinking water, whereas the second group was provided with 1% arabinose in the drinking water (experimental scheme in Figure 4C). Strikingly, supplementation with 1% arabinose was sufficient to boost the growth of *S*.Tm-A to similar densities as *S*.Tm-A Δ*araB* by day 2 p.i. (both ca. 10^9 CFU; Figure 4D). This verified that differential capacity to utilize arabinose can fuel the expansion of an isogenic arabinose-utilizing *S*.Tm strain from low density in a pre-occupied gut.

Altogether, our results revealed that supplementation of a distinct carbon source in the diet, which can only be used by the minor *S*.Tm strain, fuels the expansion of that strain in the gut occupied by an isogenic mutant incapable of utilizing this carbon source.

Supplementation with an exclusive carbon source boosts plasmid transfer between *S*.Tm-A and *S*.Tm-B or *S*.Tm-A and *E. coli*

Having established the implications of the ability to utilize a distinct carbon source in a pre-occupied mouse gut, we next wanted to test if the supplementation of a distinct carbon source would fuel plasmid transfer. To test this, we infected streptomycin-pretreated mice with a 1,000:1 mixture of donor (carrying pESBL15) and recipient strains as before (experimental scheme; Figure 5A) and investigated three different questions.

First, we used *S*.Tm-B carrying the pESBL15 plasmid as a donor and infected three different groups of mice where we used the following: (1) *S*.Tm-A as a recipient (from Figure 1), (2) *S*.Tm-A Δ*gatABC* as a recipient, or (3) *S*.Tm-A as a recipient with 1% galactitol in the drinking water. The deletion of the galactitol operon resulted in a lower density of transconjugants in comparison with the density obtained with the Gat+ isogenic strain in the feces at day 2 p.i. (Figure 5B; ∼100-fold reduction compared with *S*.Tm-A as a recipient), confirming that the higher plasmid transfer we observed between two different strains in Figure 1B is attributable to galactitol utilization. Strikingly, the supplementation of galactitol in the drinking water significantly boosted the number of transconjugants in the feces (Figure 5B; black circles vs. purple circles; ∼1,000-fold increase compared with *S*.Tm-A as a recipient without galactitol supplementation). Higher transconjugant densities in the feces were independent of donor densities because these were comparable in all groups (Figure 5C). However, comparison of the recipient densities revealed that deletion of the galactitol operon impaired the ability of recipients and the transconjugants to expand in the presence of the donor strain, whereas supplementation with galactitol significantly boosted the expansion of the recipients and transconjugants, allowing them to reach densities as high as the donors (Figure 5D).

In the second set of experiments, we tested if this effect of galactitol utilization and supplementation can be applied to another Enterobacteriaceae member colonizing the gut lumen. To this end, we used the uropathogenic strain *E. coli* 536, that is phenotypically Gat- (Figure S2A), carrying pESBL15 as a donor and same set of recipients as above (Figures 5E–5G; triangles) as in Figures 5B–5D. This strain is naturally resistant to streptomycin and colonizes the streptomycin-pretreated mouse gut with a similar efficiency as *S*.Tm.[13,38,39] Similar to our initial experiments that used *S*.Tm-B as a donor (Figures 5B–5D), using *E. coli* 536 as the donor yielded lower densities of transconjugants with *S*.Tm-A Δ*gatABC* as the recipient, compared with *S*.Tm-A (black triangles; Figure 5E). Also, in this case, supplementation of the drinking water with galactitol boosted fecal transconjugant densities (purple triangles; ca. 10^9 CFU transconjugants; Figure 5E). This effect of galactitol supplementation was independent of donor densities because they were comparable in all three groups of mice (Figure 5F) but was likely related to the higher density of recipients (Figures 5F and 5G).

Lastly, we tested if this effect can be generalized to another carbon source that can be exclusively used by the recipient strain. To do this, we performed experiments in which we used *S*.Tm-A Δ*araB* carrying the pESBL15 plasmid as a donor and divided mice into three groups, (1) *S*.Tm-A as a recipient, (2) *S*.Tm-A Δ*araB* as a recipient, or (3) *S*.Tm-A as a recipient with 1% arabinose in the drinking water. Remarkably, significantly more transconjugants were detected in the feces of mice infected with *ara*-proficient *S*.Tm-A (black circles) in comparison with *ara*-deficient *S*.Tm-A (brown circles) as a recipient (Figure 5H). Furthermore, supplementation with arabinose in the water resulted in higher transconjugant densities in feces than in mice without arabinose supplementation (Figure 5H; turquoise circles). Notably, the effect of arabinose utilization and supplementation could be attributable to the blooming of recipients, as donor densities remained unaffected (Figures 5I and 5J).

These data established that strain-specific utilization of a unique carbon source (e.g., galactitol or arabinose) not only enables co-existence of two *S*.Tm strains at high densities, but it also promotes transfer of antibiotic resistance plasmids in the gut. We additionally found that this effect can be generalized to other Enterobacteriaceae, such as *E. coli*, that commonly colonize the gut lumen of healthy individuals.

(B–D) *S*.Tm-B + pESBL15 as donor and (1) *S*.Tm-A as recipient (black; from Figure 1A) (2) *S*.Tm-A Δ*gatABC* as recipient (red), or (3) *S*.Tm-A as recipient + 1% galactitol (purple) in drinking water (n = 8, 8, or 4; 4 independent experiments; by gavage).
(E–G) *E. coli* 536+pESBL15 as donor and (1) *S*.Tm-A as recipient (black), (2) *S*.Tm-A Δ*gatABC* as recipient (red), or (3) *S*.Tm-A as recipient + 1% galactitol (purple) in drinking water (n = 8, 8, or 8; 2 independent experiments; by gavage).
(H–J) *S*.Tm-A Δ*araB* + pESBL15 as donor and (1) *S*.Tm-A as recipient (black), (2) *S*.Tm-A Δ*araB* as recipient (brown), or (3) *S*.Tm-A as recipient + 1% arabinose (turquoise) in drinking water (n = 8, 8, or 8; 2 independent experiments; by gavage). Fecal loads of (B, E, and H) transconjugants, (C, F, and I) donors, and (D, G, and J) recipients + transconjugants (CFU/g feces) as determined by selective plating.
Bars, median; dotted lines, detection limit. Two-tailed Mann Whitney-U tests to compare two groups in each panel. $p \geq 0.05$ not significant (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Galactitol supplementation enables efficient plasmid transfer and tissue reservoir formation in mice with a defined microbiota

Our competitive infection experiments so far were conducted in streptomycin-pretreated mice to overcome the initial colonization resistance. Thus, we could focus on the co-existence of the two *Salmonella* strains. In natural infections, the presence of an intact microbiota influences this process. Therefore, we next wanted to investigate the effect of alternative carbon source utilization on plasmid transfer in the presence of a resident microbiota. To explore the effect of the microbiota on plasmid transfer rates and to test the role of alternative carbon source utilization in mice in which the resident microbiota is not perturbed by antibiotic treatment, we performed infections in mice lacking any microbiota members (germ-free) and mice colonized with a defined microbiota community (12 microbiota species that are representative of a complex mouse microbiota) that permits gradual gut lumen colonization by *E. coli* and *Salmonella spp.* (OligoMM^{12}).[9] We used *S*.Tm-B (with pESBL15 plasmid) in excess as donors in all groups and infected 5 different groups of mice (experimental scheme; Figure 6A) where we used the following: (1) *S*.Tm-A as the recipient in germ-free mice (black circles; yellow background), (2) *S*.Tm-A Δ*gatABC* as the recipient in germ-free mice (red circles; yellow background), (3) *S*.Tm-A as the recipient in OligoMM^{12} mice (black circles; green background), (4) *S*.Tm-A Δ*gatABC* as the recipient in OligoMM^{12} mice (red circles; green background), and (5) *S*.Tm-A as the recipient in OligoMM^{12} mice supplemented with 1% galactitol in the drinking water (purple circles; green background).

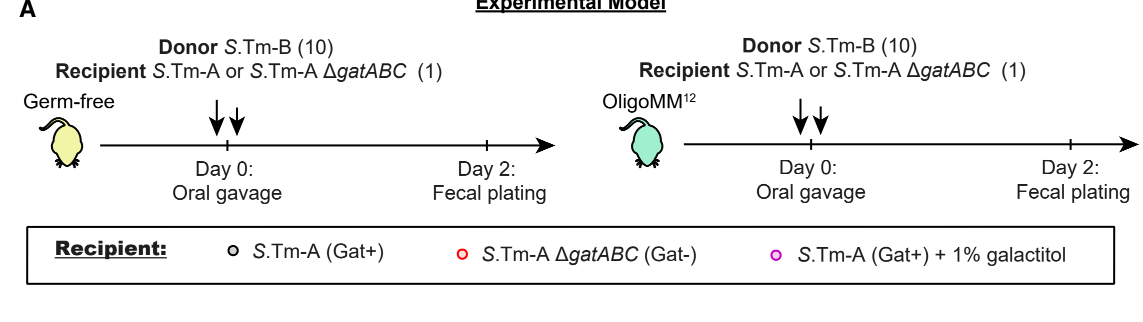
First, we investigated how the presence or absence of microbiota influences the plasmid transfer rate because this was shown to be an important determinant of transconjugant densities observed in the feces.[13] To do this, we compared the total density of recipients, the proportion of recipients that obtained a plasmid, and the total density of transconjugants between group 1 and group 3. In germ-free mice, recipients reached to loads above 10^8 CFU/g feces (reported number to be the threshold for efficient plasmid transfer in the gut)[13] at day 2 p.i., whereas in OligoMM^{12} mice they could not (Figure 6B; compare black circles in yellow vs. green background). In line with this, the proportion of transconjugants in germ-free mice was significantly higher (ca. 100% in the recipient population) than in OligoMM^{12} mice (<5% in most of the mice; Figure 6C). These results were striking and highlighted the big impact of the resident microbiota and the critical function of colonization resistance in reducing the chances of transfer of antibiotic resistance plasmids between the donor and the recipient. To formally show that in the presence of an intact microbiota, plasmid transfer is decreased, we analyzed transconjugant formation in the feces of OligoMM^{12} mice. In line with the high plasmid transfer efficiency, the density of transconjugants in the feces of germ-free mice at day 2 p.i. was very high (at the carrying capacity; median of 10^9 CFU/g feces; Figure 6D). In stark contrast, the density of transconjugants in the feces of OligoMM^{12} mice was significantly lower than in germ-free mice (Figure 6D; 1,000-fold).

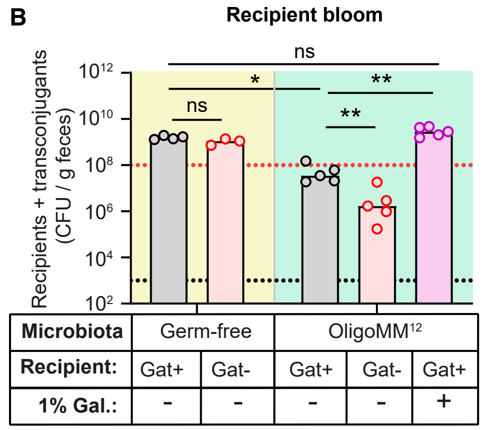
Next, we asked if galactitol utilization, similar to our observations in streptomycin-pretreated mice, would have an impact on the plasmid transfer under these conditions. First, we tested the effect of *gat* operon deletion (*S*.Tm-A Δ*gatABC* as a recipient) in germ-free and OligoMM^{12} mice. Lack of galactitol operon did not influence the recipient density and the plasmid transfer rate in germ-free mice (Figures 6B and 6C; black vs. red circles in the yellow background). Consequently, the density of transconjugants stayed the same in these mice independently of which strain was used as recipient (Figure 6D) and independent of donor densities (Figures S5A and S5B). In contrast, the number of recipients and the plasmid transfer rate in OligoMM^{12} were significantly lower when *S*.Tm-A Δ*gatABC* instead of *S*.Tm-A was used as recipient (Figures 6B and 6C). As a result, the density of transconjugants was significantly lower (ca. 100-fold) in OligoMM^{12} mice infected with *S*.Tm-A Δ*gatABC* as recipient than in OligoMM^{12} mice infected with *S*.Tm-A as recipient (Figures 6D; black circles vs. red circles in the green background). Of note, we observed a similar role of the galactitol operon for promoting co-blooms and plasmid transfer in 129 mice (which harbor a complex gut microbiota) that were transiently shifted to a high-fat diet to mildly reduce colonization resistance (Figures S5C–S5F). Lastly, we tested the effect of 1% galactitol in the drinking water. Strikingly, galactitol supplementation in OligoMM^{12} mice increased the density of recipients and the plasmid transfer rate to the same high levels as observed in germ-free mice (Figures 6B and 6C; purple circles in green background vs. black circles in the yellow background). Remarkably, the density of transconjugants increased 1,000-fold in OligoMM^{12} mice supplemented with galactitol in comparison with the ones with no galactitol supplementation (Figure 6D; black vs. purple circles in green background).

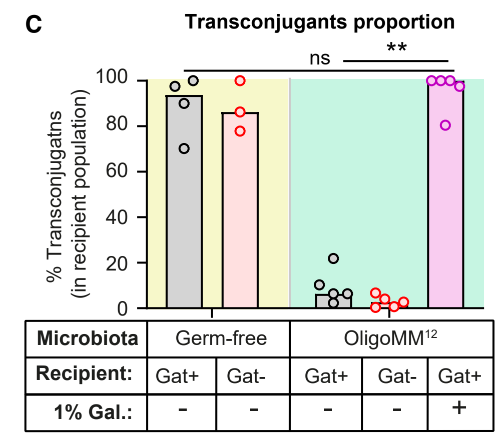
Finally, we checked the plasmid reservoirs in the tissues in OligoMM^{12} mice because they were reported to contribute to subsequent spread of antibiotic resistance.[13,18] As an invasive pathogen, *Salmonella* can "hide" in the host tissue for long periods of time and can come back and transfer resistance plasmids to the resident microbiota or to other Enterobacteriaceae.[40] Therefore, we asked if the formation of plasmid tissue reservoirs is promoted by galactitol utilization. To test this, we analyzed the number of plasmid-carrying *Salmonella* in the cecal tissue and the mesenteric lymph node (mLN; organs associated with the systemic transfer of this pathogen) of OligoMM^{12} mice from the same infection as above (Figures 6A–6D). Although the deletion of the *gat* operon impaired the formation of tissue reservoirs in the cecal tissue, galactitol supplementation significantly enhanced it in comparison with *S*.Tm-A without galactitol addition (Figure 6E). Strikingly, plasmid tissue reservoirs in the mLN were formed only in the group with galactitol supplementation (Figure 6E). Overall, these data revealed that galactitol supplementation can promote the co-existence of two *S*.Tm strains, fuel the dissemination of antibiotic resistance plasmids, and boost plasmid reservoir formation in mice with an intact microbiota.

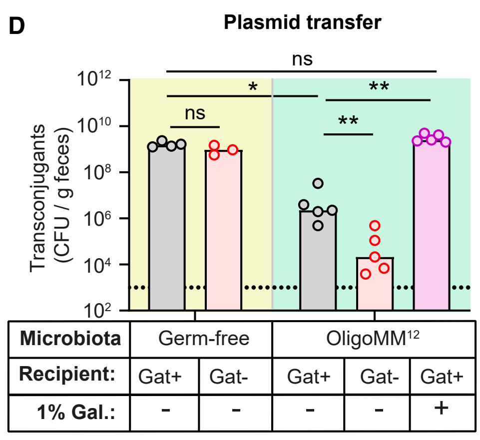
DISCUSSION

Using mouse models of Salmonellosis, we showed that the transfer of antibiotic resistance plasmids in the mouse gut is fueled by the exclusive utilization of a distinct carbon source, galactitol, by the recipient strain. Infection of antibiotic pretreated mice with two genetically distinct but closely related *Salmonella* strains leads to a high plasmid transfer in the gut









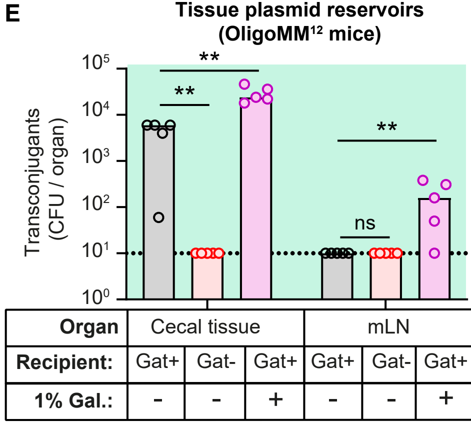


Figure 6. Galactitol supplementation boosts plasmid transfer and formation of tissue reservoirs in mice with an unperturbed microbiota

(A) Experimental scheme. Germ-free (yellow background) or OligoMM^{12} (green background) infected with a mixture of donor (10-fold excess; *S*.Tm-B) and recipient (*S*.Tm-A or *S*.Tm-A Δ*gatABC*). Groups: (1) *S*.Tm-A as recipient in germ-free (black; n = 4), (2) *S*.Tm-A Δ*gatABC* as recipient in germ-free (red; n = 3), (3) *S*.Tm-A as recipient in OligoMM^{12} (black; n = 5), (4) *S*.Tm-A Δ*gatABC* as recipient in OligoMM^{12} (red; n = 5), and (5) *S*.Tm-A as recipient in OligoMM^{12} with 1% galactitol (purple; n = 5). Selective plating to determine (B) fecal loads of recipients + transconjugants (CFU/g feces), (C) proportion of transconjugants (CFU/g feces), (D) fecal loads of transconjugants (CFU/g feces), and (E) fecal loads of transconjugants (CFU/organ) in cecal tissue and mesenteric lymph nodes. Bars, median; dotted lines, detection limit. Two-tailed Mann Whitney-U tests to compare two groups in each panel. $p \geq 0.05$ not significant (ns), $p < 0.05$ (*), $p < 0.01$ (**).

lumen due to the genetic differences attributable to genes involved in galactitol utilization (Figures 1, 2, 3, 4, 5, and 6). Supplementation with this carbon source in drinking water boosts the expansion of the recipient strain and/or the transconjugants from very low numbers in the face of a highly colonizing donor strain that cannot utilize this sugar, even in mice with a resident microbiota (Figures 4, 5, and 6). Hence, this study provides a conceptual example how strain-level differences can enable not only strain co-existence but also enhance the transfer of antibiotic resistance plasmids between *Salmonella* populations in the mammalian gut. Our findings demonstrate a crucial mechanism by which plasmids not only increase in numbers in one single gut but at the same time also increase the range of guts to colonize in the future through expanding their metabolic repertoire (i.e., by transfer to another host with slightly different metabolic capacities).

Our findings add to the increasing evidence that strain-level differences in utilization of carbon sources available in the gut can account for niche segregation among the strains of the same species. We propose that our findings, using different mouse models with varying degrees of colonization resistance, are in support of Rolf Freter's niche-exclusion hypothesis.[7,8] This theory states that the ability of a pathogen to bloom in the intestine is dependent on finding a suitable niche in the presence of a resident microbiota. Notably, a recent report has established that galactitol utilization by a commensal *E. coli* can provide colonization resistance to *Salmonella* in a context where the other niches are occupied by a complex microbiota.[10] Our findings are in line with this report and extend the niche exclusion hypothesis by highlighting that at the strain level, that metabolic diversity can promote the concomitant bloom and thereby promote the transfer of antibiotic resistance plasmids between different strains of the same species. Although our findings are limited to two strains of *S*.Tm and one *E. coli* strain, we suggest that it highlights a previously underestimated feature of the gut colonization by enteric bacteria: apart from being generalists in their core genome, they might feature diversity in their metabolic capacities at the strain levels (that is diversity at the level of the accessory genome), allowing them to occupy unique niches. We speculate that mechanisms allowing co-existence of multiple strains might be favored evolutionarily and could explain strain-level differences in members of the Enterobacteriaceae family. When it comes to carbon source utilization, they might harbor strain to strain differences efficiently promoting colonization of the gut exclusively in scenarios where two of them tend to end-up competing for the same niche. We believe that further research on such strain-level differences between enteropathogens can help us to understand more of such mechanisms employed to enable both co-existence and plasmid transfer.

In this study, supplementation of a single exclusive carbon source, galactitol or arabinose, was sufficient for one *S*.Tm strain to thrive in the presence of an identical strain (in excess) that cannot utilize this nutrient (Figures 4 and S6). But what is so special about arabinose or galactitol? Arabinose polymers are abundant in plant-based food and can be made accessible by microbiota-dependent degradation or by a *Salmonella*-encoded arabinofuranidase.[36,41] Galactitol is present in several plants and can be generated by the oxidation of galactose.[30,42] Previous work already characterized the galactitol utilization pathway by *Salmonella spp*. *in vitro*, and other work highlighted that *Salmonella spp*. induce the galactitol operon in response to microbiota-derived products in mice fed with a plant-based diet.[30,31] This indicates that *S*.Tm's use of this carbon source is affected by the presence of a close relative. A more recent publication demonstrated that commensal *E. coli* can limit availability of this sugar alcohol in a context-dependent manner and thereby prevent *Salmonella* colonization.[10] We think that this work highlights the metabolic diversity within Enterobacteriaceae and supports the argument that diverse metabolic strategies might be in action depending on the microbial context, as well as the composition of the ingested nutrient mix. Overall, this previous work and our present study suggest that metabolic capacities encoded within the accessory genomes of different strains might be advantageous because they promote co-blooming and enhance horizontal gene transfer by permitting the growth of a given strain in a gut colonized already by another closely related strain. The relevant nutrients might be available only under certain conditions. For example, Eberl et al. reported that none of the members of OligoMM[12] microbiota can utilize galactitol, which indicates a context-dependent function of galactitol during Enterobacteriaceae growth in the gut lumen of those mice.[10] Furthermore, within-host evolution studies from the Gordo Lab demonstrated that the *gat* pathway in *E. coli* is a mutational hotspot and that this operon can be either beneficial or have growth inhibitory effects depending on the microbiota composition and the presence or absence of another *E. coli* strain in the mouse gut.[43,44] In our work, we have not observed a growth inhibitory effect of galactitol on either strain, suggesting that different regulatory or competitive mechanisms might be at play. Further research is necessary to decipher the context-specific roles of galactitol and to fully establish the role of strain-specific differences in utilizing different carbon sources in Enterobacteriaceae co-occurrence and strain evolution.

Within the last decades, a great deal of studies have focused on the use of commensal *E. coli* (e.g., *E. coli* Nissle 1917) strains to discourage the growth of enteric pathogens, such as *Salmonella enterica* and enteropathogenic *E. coli*.[35,45] The idea behind these studies is that members of the same family are more likely to occupy similar metabolic niches in the gut and may therefore outcompete (or provide colonization resistance against) invasive pathogenic species of the same or closely related taxa. Our findings highlight that strain-specific mismatches in carbon source utilization (e.g., galactitol utilization) can suffice to permit pathogen growth in the presence of a closely related competitor strain. Based on these considerations, we propose that microbiota-based therapies may benefit from focusing on these alternative pathways because these appear highly effective at fueling enteric pathogen expansion in the gut. By systematically mapping such strategies of a given pathogen strain, one could identify ideal competitor strains, propose mixtures of competitor strains, which deplete all carbon sources usable by the pathogen, or employ food-based interventions, which would limit pathogen blooms by reducing the respective carbon sources in the food.

Previous work of our lab revealed that *Salmonella* persisters, serving as plasmid reservoirs in the host tissue, can re-seed in the gut lumen and thereby promote the spread of antibiotic resistance plasmids without an actual selection for antibiotic.[13,18] Here, we were able to show how the few persister cells that re-enter vegetative growth and re-seed the gut lumen can grow up and co-bloom in the gut if the lumen is already colonized by another Enterobacteriaceae strain. These results indicate that the spread of antibiotic resistance plasmids via persisters is only efficient when these strains can co-bloom.

Finally, we demonstrated how strain-level differences can account for very striking outcomes, such as allowing co-existence of two pathogen populations in the same gut. We propose that the same might be true for microbiota members. Currently, most of the microbiota studies are focusing on the differences at, maximum, species level if not the genus level, where many reports conclude that an observed phenomenon was not due to the microbiota because they did not find any shift at these taxonomical levels. Our findings might be applicable to other microbiota members, where changes at the strain level of a family can

account for significant metabolic changes and this may not only affect strain co-occurrence but also influence the host physiology greatly. Therefore, technologies allowing the detection of changes at the strain levels can be of great benefit for studies dealing with microbiota-associated phenomena, such as obesity, metabolic syndrome, inflammatory bowel diseases, or gut-brain axis studies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2023.05.029.

ACKNOWLEDGMENTS

We would like to thank members of the Hardt lab for helpful discussions, particularly Yassine Cherrak. We acknowledge the staff of ETH Zürich mouse facility EPIC/RCHCI (Manuela Graf, Katharina Holzinger, Dennis Mollenhauer, Sven Nowok, and Dominik Bacovcin) and Microbiology Institute. We thank the ETH DBIOL Block Course students Amelie Hattori, Abigail Dustour, and Dominic Mailänder for their contributions. This work has been supported by grants from the Swiss National Science Foundation (NRP 72 407240_167121, 310030B_173338, and 310030_192567), the NCCR Microbiomes funded by the Swiss National Science Foundation, the Gebert Rüf Foundation (GRS-060/18), and the Monique Dornonville de la Cour Foundation to W.-D.H.

AUTHOR CONTRIBUTIONS

E.G. and W.-D.H. conceived the project and designed the experiments. E.G., A.A.Y., C.D., and J.H. carried out the mouse experiments and analyzed data. E.B. did the strain genome comparison, and E.B., E.G., J.H., B.D.N., A.A.Y., and C.D. prepared and analyzed the mutant strains. E.G wrote the initial draft of the manuscript. E.G., L.M., E.B., A.A.Y., and W.-D.H. reviewed and edited the manuscript to its final form. All authors read, commented on, and approved this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

Received: December 22, 2022
Revised: April 12, 2023
Accepted: May 26, 2023
Published: June 21, 2023

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
See Table S2		N/A
Critical commercial assays		
Phusion™ Plus DNA Polymerase	ThermoFisher Scientific	F630L
QIAquick PCR Purification Kit	Qiagen	28104
Experimental models: Organisms/strains		
Mouse: OligoMM^{12} mice (C57BL/6 genetic background)	Brugiroux et al.[9]	https://doi.org/10.1038/nmicrobiol.2016.215
Mouse: 129SvEv SPF	Jackson Laboratories; bred at EPIC mouse facility of ETH Zurich, Switzerland	N/A
Mouse: C57BL/6 SPF	Jackson Laboratories; bred at EPIC mouse facility of ETH Zurich, Switzerland	N/A
Mouse: C57BL/6 Germ-free	Jackson Laboratories; bred at EPIC mouse facility of ETH Zurich, Switzerland	N/A
Mouse: *129SvEv High-fat diet shift*	Wotzka et al.[35]	https://doi.org/10.1038/s41564-019-0568-5
Oligonucleotides		
See Table S3		N/A
Recombinant DNA		
See Table S4		N/A
Software and algorithms		
Graphpad Prism Version 9.0 for Windows	GraphPad Software, La Jolla California USA	Home - GraphPad
Artemis Comparison Tool	ACT, Wellcome Sanger Institute, Cambridge, UK	https://www.sanger.ac.uk/science/tools/artemis-comparison-tool-act

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wolf-Dietrich Hardt (hardt@micro.biol.ethz.ch).

Materials availability

Mouse lines used in this study can be obtained from Jackson laboratories. Gnotobiotic mice are available upon request.

Data and code availability

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial strains and culture conditions

Bacterial strains used in this study are derivatives of *S*.Tm SL1344[24] or *S*.Tm ATCC14028,[25] *E. coli* K12,[46] or *E.coli* 536,[13] and are listed in key resources table. To cultivate these bacterial strains, lysogeny broth (LB) medium was used containing the appropriate antibiotics (50 μg/ml streptomycin (AppliChem); 50 μg/ml kanamycin (AppliChem); 15 μg/ml chloramphenicol (AppliChem); 100 μg/ml ampicillin (AppliChem)) at 37°C (or 30°C if containing pCP20). P22 HT105/1 *int-201* phage transduction was used to create genetically modified constructs of *S*.Tm strains (e.g. gene deletions, neutral isogenic sequence tags, or the P3 plasmid).[47] In order to create gene deletion mutants or introduce antibiotic resistance tags, the λ *red* system was used as described in Datsenko et al.[48] If desired,

antibiotic resistance cassettes were removed using the temperature-inducible FLP recombinase encoded on pCP20.[48] Primers used for deletions and verification of the strain genotypes are listed in key resources table. Bacterial plasmids that were used to confer resistance or for construction of strains were transformed into cells using electroporation and are listed in key resources table.

Mouse lines

Experiments were performed with 8–12-week-old male or female mice. The sample-size was not pre-determined and mice were randomly assigned to groups. All mice originate from C57BL/6 or 129SvEv breeders originally obtained from Jackson laboratories. The mice with a normal complex microbiota were specific pathogen-free (SPF) and bred under full barrier conditions in individually ventilated cage systems in the EPIC mouse facility of ETH Zurich, Switzerland. Germ-free C57BL/6 mice were bred in flexible film isolators under strict exclusion of microbial contamination at the isolator facility of the EPIC mouse facility of ETH Zurich, Switzerland. OligoMM^{12} mice are ex germ-free mice that are stably colonized with a defined microbiota composed of 12 representative microbiota strains.[9] and they were bred in flexible film isolators under strict exclusion of microbial contamination at the isolator facility of the EPIC mouse facility of ETH Zurich, Switzerland.

All studies were performed in accordance with ethical and legal requirements and were reviewed and approved by the Kantonales Veterinäramt Zürich under the licenses ZH193/2016, ZH158/2019, ZH108/2022, and ZH109/2022.

METHOD DETAILS

Construction of bacteria strains

De-novo mutant preparation with lambda red

Novel single-gene knockout strains were prepared using the lambda-red single-step protocol.[48] In this method, an antibiotic resistance cassette is introduced to replace the gene of interest. First, primers with approximately 40bp of the gene flanking regions and 20bp of the desired antibiotic resistance cassette were constructed (key resources table). Then, DNA constructs containing the antibiotic resistance cassette flanked by the flanking regions of the gene of interest were made using the plasmids pKD3 and pKD4 for chloramphenicol and kanamycin, respectively. Phusion high-fidelity DNA polymerase was used to make the constructs, and the PCR product was purified using the Qiagen DNA purification kit. To make a highly concentrated solution of competent cells, a strain containing the pKD46 plasmid harboring the lambda-red phage and an ampicillin resistance cassette was grown for 3h at 30°C (the plasmid is lost at 37°C) in 50mL LB-ampicillin supplemented with 10mM Arabinose (Sigma-Aldrich) to induce phage-derived genes encoded on pKD46. The cells were washed and concentrated by a series of centrifugation and resuspension steps in ice-cold H_2O. They were transformed with 5µL of the purified PCR product through electroporation at 1.8kV for 5ms. The resulting cells were recovered in warm LB for 1.5h at 37°C and plated on LB plates containing the respective antibiotic to select colonies with the desired gene knockout. Since lambda-red induction and mutant preparation may cause mutations elsewhere in the genome, P-22 lysates were made of the original mutant strains and used to subsequently transduce the mutation into the ancestral strain.

In vitro *Transconjugation*

To transfer a conjugative plasmid from a strain into another (e.g., pESBL15 from *S*.Tm-B to *S*.Tm-A ΔP2) both strains were grown overnight in LB with their respective antibiotics. 1mL of each was centrifuged at 15000rpm and re-suspended in 1mL PBS. They are then both diluted to 10^{-4} in PBS, and 50µL of each was added into 5mL of LB with no antibiotics and left to grow overnight at 37°C while shaking. Serial dilutions were done to select for transconjugants by resistance phenotype and/or colour (i.e., *S*.Tm is *lac* negative and thus forms yellow colonies on MacConkey agar while *E. coli* is *lac* positive and forms red colonies).

Mouse infections

Infection experiments in antibiotic pretreated mice were done according to the well-described Streptomycin mouse model for *S*.Tm oral infection.[26] Shortly, the mice were pretreated with 25mg of streptomycin by oral gavage 24h prior to infection and infected on day 0 by oral gavage with an inoculum of $5x10^7$ CFU *S*.Tm. Feces were collected at the indicated time points and where necessary, cecal tissue and mLN were harvested at the end of the infection. For cecal tissue plating, we used the gentamycin protection assay in which the tissue is treated with gentamycin to clear extracellular bacteria. Cecal tissue was cut longitudinally, washed rapidly in PBS (3x), incubated for 45-75min in PBS/400µg/ml gentamycin (Sigma-Aldrich) at RT, and washed extensively (3x 30s) in PBS before plating. For plating, the samples were homogenized with a steel ball in a tissue lyser (Qiagen) for 2 minutes at 25Hz frequency (cecal tissue 3 minutes at 30Hz). The homogenized samples were diluted in PBS, plated on MacConkey (Oxoid) plates supplemented with the relevant antibiotic(s), and placed at 37°C overnight. Colonies were counted the next day and represented as CFU / g content. Normalized competitive index (C.I.) was calculated as the ratio of the wild type over the mutant in the feces and normalized to the initial ratio in the inoculum.

In vivo *plasmid transfer assays*

The inocula were prepared to mimic the founder effect. The strains to be studied are combined in an inoculum at a 1000:1 ratio for two strains and 1000:1:1 for three to investigate the ability of strains to bloom from a disadvantage in a background of a closely related strain and evaluate the transfer of antibiotic resistance plasmids in streptomycin pretreated mouse model (Figures 1, 2, 3, 4, and 5). In gnotobiotic mouse infections (Figure 6), a ratio of 10:1 (donor:recipient) was used in the inoculum.

For mouse infections, the strains were streaked on MacConkey plates from a glycerol stock 2 days prior to infection. One day before infection, a healthy colony was picked for an overnight culture in LB/0.3M NaCl (Sigma-Aldrich) supplemented with the proper

antibiotics (ca. 12h). On the day of infection, a 4h 1:20 subculture in the same media without antibiotics was done. *S*.Tm were washed once and reconstituted in cold PBS before the final concentrations of each strain were achieved by further diluting them in cold PBS to reach a final inoculum size of $5x10^7$ CFU *S*.Tm. To determine the number of transconjugants, recipients and transconjugants, and the donors, feces samples were homogenized and plated on MacConkey plates with respective antibiotic resistances.

Galactitol Supplementation

To supplement galactitol without changing the mouse maintenance diet, we relied on previous experiments in which galactitol was added to the mouse drinking water at concentrations of 0.1% and 1%.[44] To this end, 0.25g or 2.5g of galactitol (Sigma-Aldrich) was added to 250mL of tap water and sterilized by filtration through a 0.22μm filter (TPP AG). The mouse drinking water was replaced with galactitol water 1 day prior to infection and maintained throughout the course of the infection.

Arabinose Supplementation

2.5g of L-arabinose (Sigma-Aldrich) was added to 250mL of tap water and sterilized by filtration through a 0.22μm filter. The mouse drinking water was replaced with 1% arabinose containing water 1 day prior to infection and maintained throughout the course of the infection.

Genome comparison of two *Salmonella* strains

Whole genomes of *S*.Tm-A and *S*.Tm-B were compared using BLAST and visualized/analyzed with Artemis Comparison Tool (ACT; https://www.sanger.ac.uk/science/tools/artemis-comparison-tool-act). Local BLAST was used to compare chromosomes (SL1344 chromosome NCBI accession FQ312003.1; 14028S chromosome NCBI accession NC_016856.1) and plasmids (SL1344 pSLT plasmid NCBI accession HE654724.1; 14028S pSLT plasmid NCBI accession CP001362.1), generating crunch files (megablast, E-value filter = 1) to be visualized in ACT. A score of 10000 in ACT was used as minimum cutoff for homologous regions. NCBI annotations were used to annotate genes within non-overlapping regions (Table S1). Plasmids found in S1344 but not 14028S are visualized using DNAplotter (https://www.sanger.ac.uk/tool/dnaplotter/; pCol1b9 aka P2 NCBI accession HE654725.1; pRSF1010 aka P3 NCBI accesssion HE654726.1).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Where applicable, the two-tailed Mann Whitney-U test was used to assess statistical significance as indicated in the figure legends. GraphPad Prism 9 for Windows was used for statistical testing. P values of $p \geq 0.05$ not significant (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****)