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Regulated Virulence Controls the Ability of a Pathogen to Compete with the Gut Microbiota

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Abstract

The virulence mechanisms that allow pathogens to colonize the intestine remain unclear. Here we show that germ-free animals are unable to eradicate *Citrobacter rodentium*, a model for human infections with attaching/effacing bacteria. Early in infection, virulence genes were expressed and required for pathogen growth in conventionally raised but not germ-free mice. Virulence gene expression was downregulated during the late phase of infection, which led to relocation of the pathogen to the intestinal lumen where it was out-competed by commensals. The ability of commensals to out-compete *C. rodentium* was determined, at least in part, by the capacity of the pathogen and commensals to grow on structurally similar carbohydrates. Thus, pathogen colonization is controlled by bacterial virulence and through competition with metabolically related commensals.

Enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are important causes of diarrhea and mortality worldwide (1, 2). These Gram-negative bacteria attach to and colonize the intestinal tract by inducing attaching and effacing (A/E) lesions on the intestinal epithelium (1, 2). The genomes of A/E pathogens harbor the locus of enterocyte effacement (LEE) that is critical for these bacteria to colonize their hosts and cause pathology (3, 4). Infection with *Citrobacter rodentium*, a natural pathogen of mice that is used to model human infections with EPEC and EHEC (5, 6), is associated with a significant but reversible decrease in the number of total commensals in the colon (7, 8). To assess the role of the microbiota in this enteric infection, germ-free (GF) and specific pathogen-free (SPF) C57BL/6 mice were orally inoculated with *C. rodentium*. The pathogen colonized the intestines of SPF mice reaching maximal concentrations in the feces on day 7-10 post infection followed by a decline by day 12 and becoming undetectable by day 22 (**Fig. 1A**). In contrast, GF mice harbored 10-fold more *C. rodentium* on day 7-10 post infection, and unlike SPF mice, they were unable to clear *C. rodentium* even by day 42 when the experiments were terminated (**Fig. 1A**). Remarkably, all GF infected mice remained alive despite high and persistent pathogen burdens (**Fig. 1B**). Notably, the recruitment of

neutrophils, inflammatory macrophages and CD3⁺ T cells in response to infection was similar in SPF and GF mice (**Fig. S1**). Consistently, histological analysis revealed comparable pathology scores on day 12 post-infection which declined on day 22 in both GF and SPF mice (**Fig. S1**). Furthermore, expression of anti-microbial peptides including RegIII γ , β -defensin-1, β -defensin-3, and β -defensin-4 was comparable in the colons of infected SPF and GF mice (**Fig. S2**).

The expression of most LEE genes in *C. rodentium* is controlled by Ler, a member of the H-NS protein family (3, 4, 9, 10). *Ler* was expressed in the feces on day 7 post-infection in both GF and SPF mice (**Fig. 2A**). Importantly, expression of *ler* and *tir*, a Ler-regulated gene were both down-regulated on day 12 after infection in both GF and SPF mice and were not expressed at day 42 in GF mice despite robust pathogen colonization (**Fig. 2A and Fig. S3**). To monitor the expression of *ler* in the intestine, we engineered a bioluminescent reporter *C. rodentium* strain in which the *ler* promoter was fused to the *luxCDABE* operon of *Photobacterium luminescens* (**Fig. S4**) (11). *Ler* expression was detected in the feces on day 5, but down-regulated by day 7-12 post-infection (**Fig. 2B**). Furthermore, *ler* expression was visualized on day 5 post-infection in the ileum, cecum and distal colon, but was down-regulated on day 14 in both SPF and GF mice (**Fig. 2C**). The *ler* luminescent signal was ~10-fold higher in GF mice than in SPF mice consistent with higher pathogen load in GF mice. If the *C. rodentium* were harvested from infected GF mice at day 21 and re-inoculated into SPF mice, the pathogen robustly grew in the intestines and elicited colonic inflammation (**Fig. S5**).

Since expression of virulence LEE genes is essential for pathogen colonization in the intestines of SPF mice (4), we asked whether LEE virulence was required for colonization in GF mice. To assess this, we orally infected GF and SPF mice with wild-type (WT) *C. rodentium* and isogenic strains deficient in Ler (Δ *ler*) or in EscN (Δ *escN*) a Ler-regulated ATPase required for injection of virulence factors via the type-III secretion system (T3SS) into host cells (4). As expected, WT, but not the Δ *ler* or Δ *escN* strains, grew in the intestine of SPF mice (**Fig. 2D**). Notably, the Δ *ler* and Δ *escN* mutants grew robustly in GF mice, reaching numbers similar to the WT pathogen (**Fig. 2D and Fig. S6**). Thus, Ler-dependent LEE virulence is not required for intestinal *C. rodentium* growth in the absence of the microbiota. To determine whether Ler and the T3SS regulate the ability of the microbiota to out-compete the pathogen, we orally infected GF mice with WT, the Δ *ler* or the Δ *escN* mutant *C. rodentium* and the infected GF mice were colonized with commensals by cohousing them at either day 3 or day 21 post-infection with SPF mice. Notably, the burden of WT *C. rodentium* in GF mice harboring the pathogen for 21 days declined significantly after 3 days of co-housing and was further reduced by 5-6 logs by day 7, similarly to the decline observed in mice infected with the Δ *ler* or the Δ *escN* mutant for 3 and 21 days (**Fig. 2E and Fig. S6**). In contrast, the microbiota could not out-compete the WT bacterium on day 3 post-infection upon co-housing with SPF mice (**Fig. 2E**), consistent with the observation that *ler* expression is active in WT *C. rodentium* at day 3 post-infection (**Fig. S7**).

C. rodentium induces marked inflammation in the distal colon which requires LEE virulence factors including the T3SS (4). If the T3SS promotes outgrowth of *C. rodentium* by enabling the pathogen to colonize a niche at or near the epithelium, it would be expected that impaired colonization of the Δ *escN* mutant could not be rescued when inflammation is provided by other means. Oral administration of dextran sulfate sodium (DSS), a chemical that directly damages the colonic epithelium (12) enhanced the colonization of the WT bacterium by 3 logs but not that of the Δ *escN* mutant (**Fig. S8**). In addition, co-infection of the Δ *escN* mutant with the WT *C. rodentium* that induces inflammation did not rescue colonization by the T3SS mutant strain (**Fig. S8**). These results indicate that inflammation

promotes colonization of WT *C. rodentium*, but not in the absence of a functional T3SS. The adaptive immune system including IgG production and Th17 responses against the pathogen are important for eradication of *C. rodentium* (13-16). Importantly, the production of IgG, IgM, and IgA against *C. rodentium* was similar in GF and SPF mice (**Fig. S9**). Furthermore, the generation of colonic IL-17 and interferon- γ -producing T cells in response to *C. rodentium* was comparable in SPF and GF mice (**Fig. S10**).

C. rodentium colonizes and infects the surface of the intestinal epithelium, a site largely devoid of commensals (17). At day 5 post-infection, Tir, a marker of *C. rodentium* attachment to the epithelium, was seen on the intestinal surface of GF mice infected with WT *C. rodentium*, but not with the Δ *Ier* strain (**Fig. S11**). In contrast, Tir labeling was not detected on day 21 post-infection on the intestinal surface of mice infected with either WT or mutant *C. rodentium* (**Fig. S11**). Consistently, the WT pathogen was associated with the epithelium whereas the Δ *Ier* mutant localized to the intestinal lumen on day 5 post-infection as determined by dual fluorescence *in-situ* hybridization (**Fig. 3A**). On day 21 post-infection, however, neither WT nor Δ *Ier* *C. rodentium* were detected on the epithelial surface, but instead they localized to the intestinal lumen (**Fig. 3A**). Similarly, transmission electron microscopy revealed *C. rodentium* at or near the intestinal epithelium on day 5, but not on day 21 post-infection (**Fig. 3B**). As expected, Δ *Ier* *C. rodentium* did not localize at or near the epithelium on day 5 post-infection (**Fig. 3B**). Consistent with these results, *Ier-lux*-expressing WT *C. rodentium* found attached to the cecal and colonic epithelium were abundant in the early phase of infection, but dramatically decreased later during infection, even though pathogen burdens in the feces were comparable (**Fig. 3C and fig. S12**). These results indicate that the localization of the pathogen differs in the early and late phase of infection and this is controlled by LEE virulence gene expression.

The intestine harbors a large number of bacterial species (18). To determine whether commensal bacteria exhibit different abilities to out-compete *C. rodentium*, we orally infected GF mice with *C. rodentium* and on day 21 post-infection the mice were colonized with either *E. coli* or one of two different anaerobic *Bacteroides* species, all isolated from the intestines of SPF mice (19). The burden of *C. rodentium* in GF mice declined about 200-fold by day 3 and more than 500-fold by day 14 upon colonization with *E. coli*, but not at all, with *B. thetaiotaomicron* or *B. vulgatus* (**Fig. 4A**). Secondary administration of *E. coli* to GF mice already colonized with *B. thetaiotaomicron* or *B. vulgatus* also reduced the number of *C. rodentium* in the feces about 500-fold (**Fig. 4A**). In contrast, colonization of mice harboring *E. coli* with *B. thetaiotaomicron* or *B. vulgatus* was not effective in further reducing the burden of *C. rodentium* in their feces (**Fig. 4A**). Assessment of total *Enterobacteria* (*E. coli* and *C. rodentium*) and *Bacteroides* on day 14 post colonization with individual commensal bacteria showed comparable numbers of bacteria in the mouse feces (**Fig. 4B**). Before inoculation of commensal bacteria (d0), all *Enterobacteria* detected in the feces were *C. rodentium* (**Fig. 4C**). In contrast, > 99% of the total *Enterobacteria* in the *E. coli* inoculated group were *E. coli* after 14 days of colonization (**Fig. 4C**). We next analyzed the growth abilities of each of these species on a custom carbohydrate growth array that contains most of the common mono- and polysaccharides present in plant and animal tissue (**Fig. 4D and Table S1**). Both *C. rodentium* and *E. coli* exhibited optimal growth on monosaccharides (**Fig. 4D**). In contrast, both *B. thetaiotaomicron* and *B. vulgatus* exhibited broad abilities to catabolize both mono- and polysaccharides (**Fig. 4D**). Because γ -Proteobacteria such as *E. coli* specifically accumulates after *C. rodentium* infection (7), these results suggest that this microbial change may benefit the host by increasing the number of commensals that can out-compete the pathogen. To further assess if competition for glycans between commensals and *C. rodentium* is important for pathogen eradication, GF mice on a conventional maintenance diet containing both mono- and polysaccharides were orally infected with *C. rodentium* and on day 21 post-infection the infected GF mice

were colonized with *B. thetaiotaomicron* for 7 days. On day 7 post-colonization with *B. thetaiotaomicron*, the mice were divided into two groups that were fed the conventional diet or a simple sugar diet containing monosaccharides but not polysaccharides. The burden of *C. rodentium* in the GF mice on the simple sugar diet rapidly declined ~200-fold by day 3, whereas no decline was observed when the mice were fed the conventional diet (**Fig. 4E**). The results were not explained by the simple sugar diet alone because in the absence of colonization with *B. thetaiotaomicron*, the number of *C. rodentium* in feces was comparable between animals fed with the conventional and simple sugar diets (**Fig. 4F**). These results suggest that under the simple sugar diet, *B. thetaiotaomicron* cannot use polysaccharides and is forced to compete with *C. rodentium* for available monosaccharides or another common nutrient that both *C. rodentium* and *B. thetaiotaomicron* rely on in the absence of dietary polysaccharides. These findings clarify the tenuous nature of A/E pathogens, in terms of their survival/colonization within hosts, and therefore nutrient or probiotic shifting of the microbiota to promote commensals that directly compete with pathogens for food sources may be a useful therapeutic approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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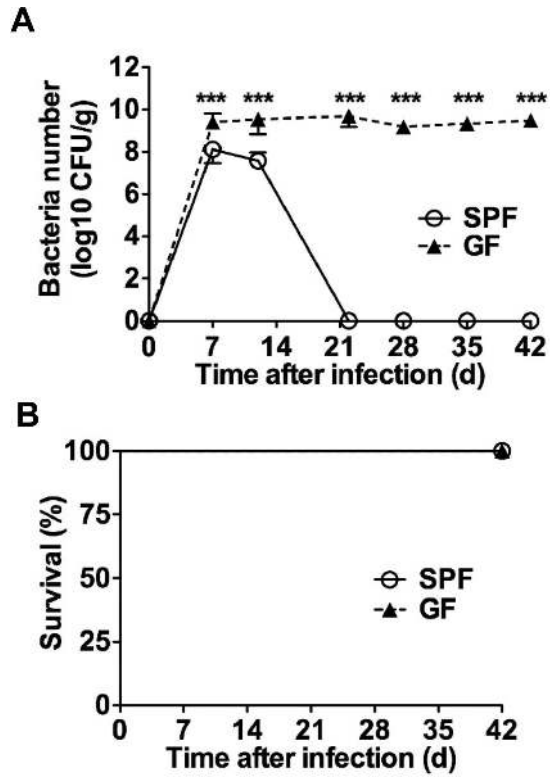


Fig. 1. The microbiota is required for eradication of *C. rodentium*

A,B, SPF and GF mice (n= 7) were infected orally with 1×10^9 cfu of *C. rodentium* and pathogen load in feces (A) and mouse survival (B) were determined over the indicated time. Data points are given as mean \pm SD. Results are representative of at least 3 independent experiments. ***, p<0.001 by Mann-Whitney *U* test.

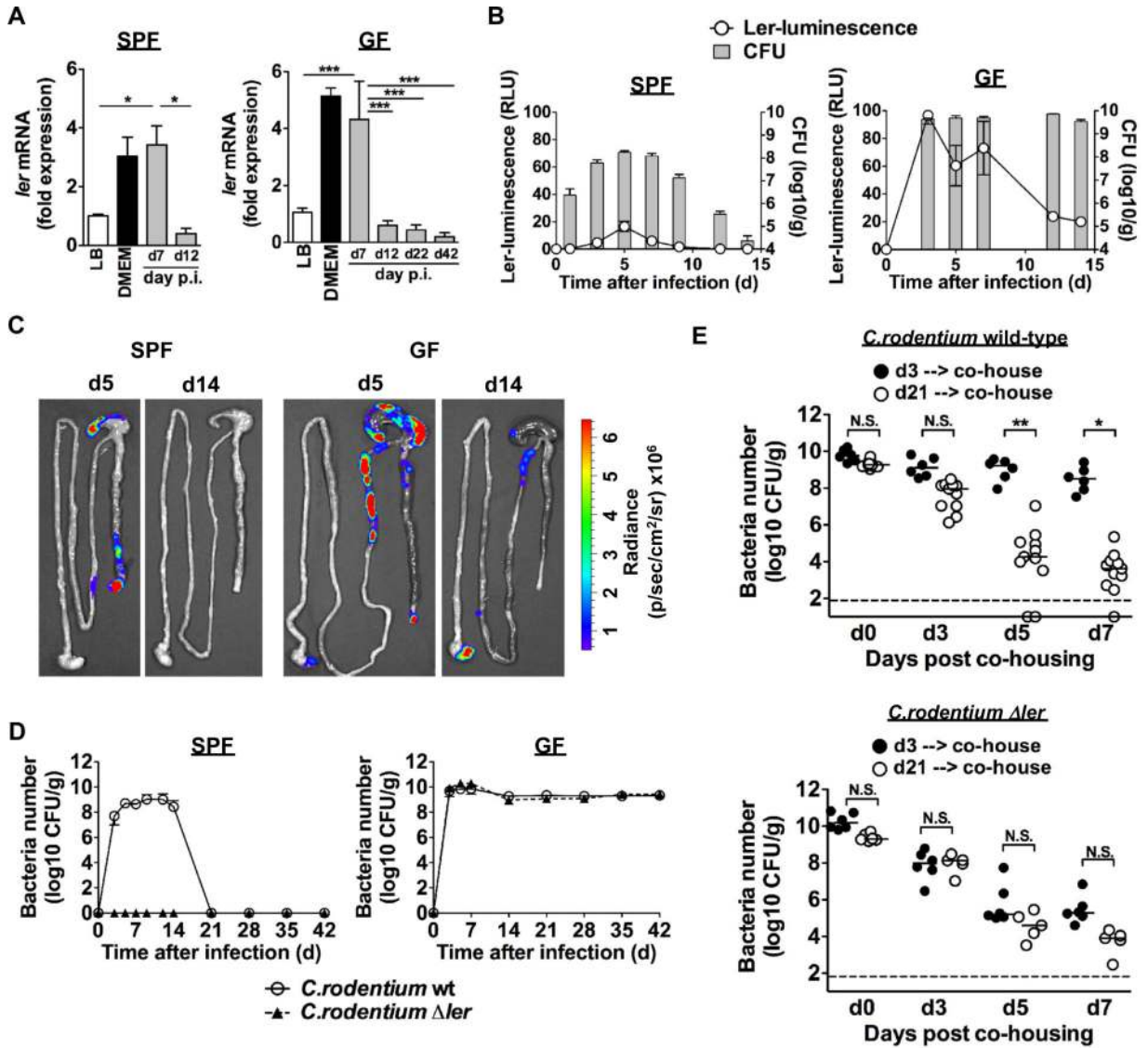


Fig. 2. Expression and role of *ler* during *C. rodentium* infection in SPF and GF mice
A, *ler* mRNA levels were determined by qPCR in fecal pellets of SPF and GF mice infected with *C. rodentium* at the indicated days post-infection. Expression was normalized to that of the kanamycin resistance gene carried by the *C. rodentium* strain. Control experiments were performed by determining *ler* mRNA levels in *C. rodentium* grown under inducing (DMEM) and repressing (LB) in vitro culture conditions (4, 10). Data represent mRNA expression relative to that in *C. rodentium* cultured in LB medium. Results are given as mean ± SD of individual mice (n=3). Results are representative of at least 2 experiments. *, p<0.05, ***, p<0.001 by Dunnett's multiple comparison test. **B**, Expression of *ler* in fecal pellets of SPF and GF mice infected with the reporter *ler-lux* *C. rodentium* strain at the indicated day post-infection. Results show luminescence (relative light units) and cfu of *ler-lux* *C. rodentium* in the same samples. Data expressed as mean ± SD of individual mice (n=4). Results are representative of at least 2 experiments. **C**, Bioluminescent imaging of *ler* expression in the intestines of SPF and GF mice infected with the *ler-lux* *C. rodentium* strain. Imaging was performed on day 5 and 14 post-infection and the signal was quantified based on the color scale shown below. Results are representative of 3 individual mice. **D**, SPF and GF mice (n=

5) were infected orally with 10^9 cfu of WT and Δ *Jer* mutant *C. rodentium*, and pathogen load in feces was determined over the indicated time. Data points are given as mean \pm SD. Results are representative of at least 2 experiments. E, GF mice were infected with WT and Δ *Jer* mutant *C. rodentium*. At day 3 or day 21 post infection, mice were co-housed with SPF mice (1:1). Pathogen load was determined in feces on indicated days after co-housing. Dots represent individual mice. Results are representative of at least 3 experiments. N.S., not significant, *, $p < 0.05$, **, $p < 0.01$ by Dunn's test.

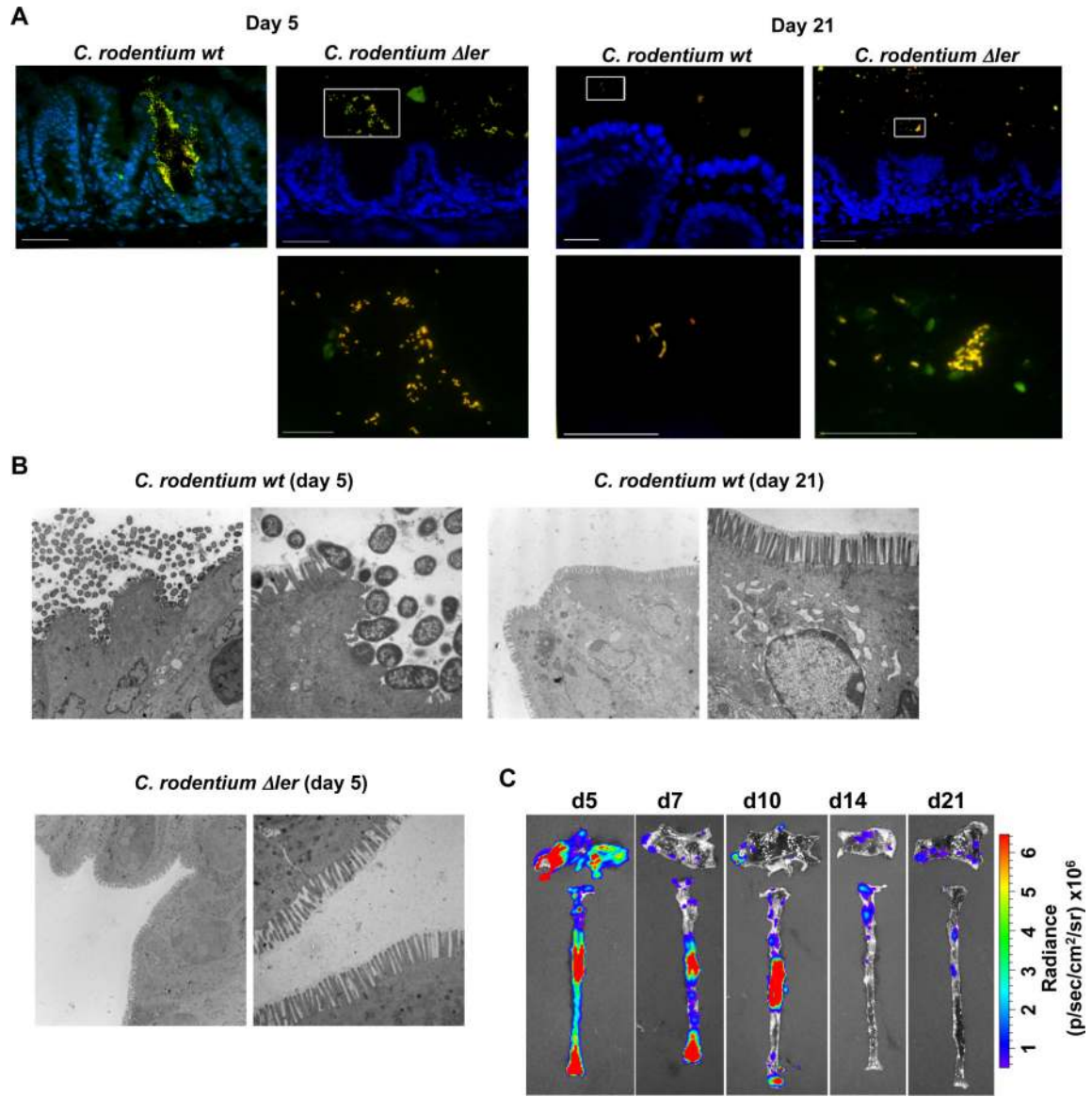


Fig. 3. Localization of *C. rodentium* to intestinal niches is mediated by LEE-encoded virulence factors

A, Dual FISH staining using DNA probes that label virtually all true bacteria (EUB338, red) and the γ -Proteobacteria class to which *C. rodentium* belongs (GAM42a, green). Pathogenic bacteria (i.e. EUB338⁺/GAM42a⁺ cells) are yellow. Lower panels show high magnification images corresponding to boxed areas (upper panels). Note the yellow color on all the bacteria stained in the cecum infected with the Δ ler mutant; indicating all bacteria in the lumen are *C. rodentium*. Scale bar: 50 μ m (upper panels), 20 μ m (lower panels). Results are representative of 2 experiments. **B**, Transmission Electron Micrographs of cecum from infected GF mice at day 5 and day 21 post infection with WT *C. rodentium* and at day 5 with the Δ ler mutant. Original magnification: 3,400 \times (left panel) and 13,500 \times (right panel). Scale bar: 2 μ m (left panel) and 500 nm (right panel). Results are representative of 2 experiments. **C**, GF mice were infected orally with WT *C. rodentium* carrying the *ler-lux* fusion. Cecum and colonic tissues were collected at the indicated day and then washed with PBS to remove non-adherent bacteria. Bioluminescent imaging of *ler* expression of *C.*

rodentium attached to the cecum (top) and colon (bottom). Results are representative of 2 experiments using 4 different mice.

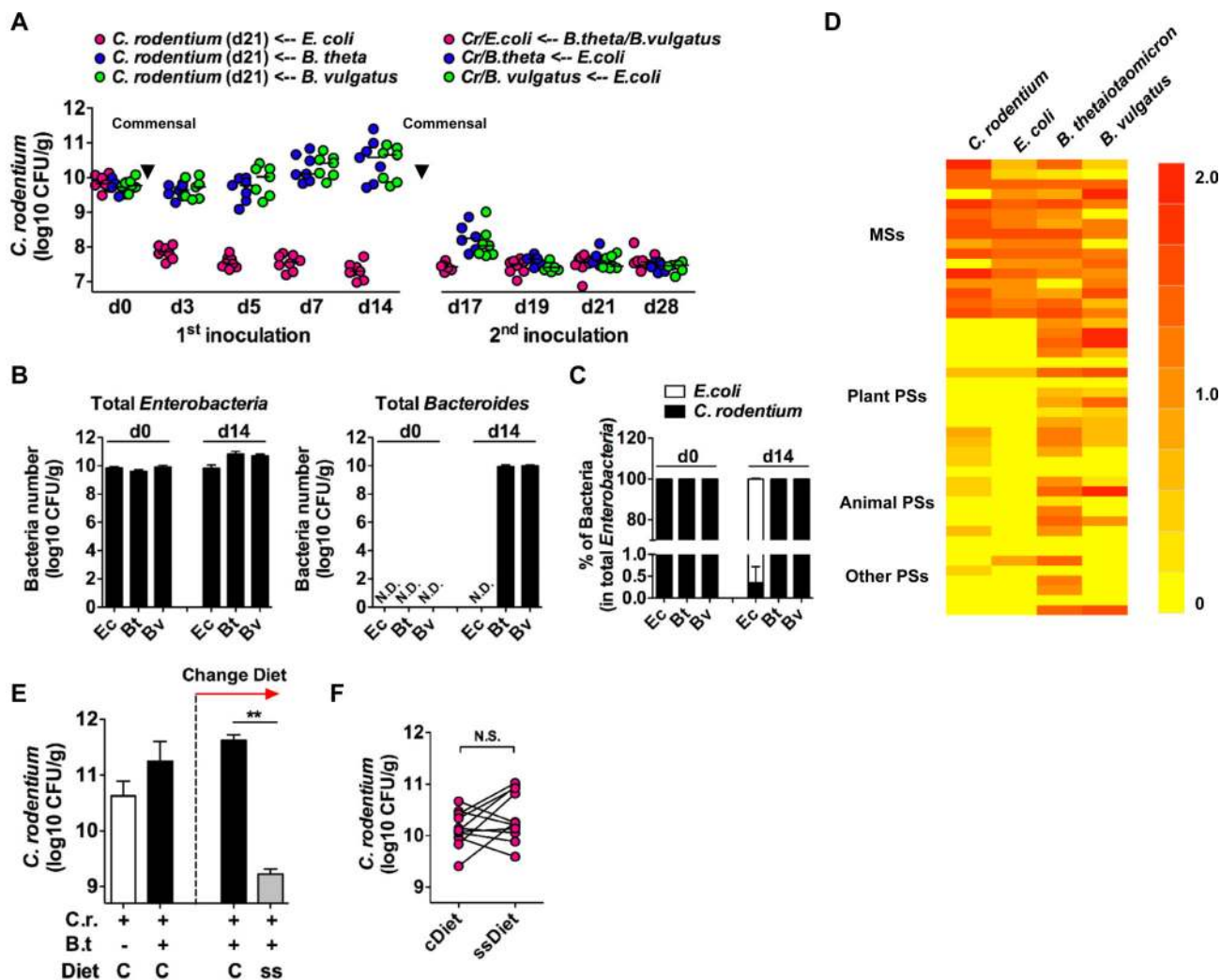


Fig. 4. Similar catabolic preferences for saccharides may determine the competing ability of commensal bacteria with the enteric pathogen

A, GF mice were infected with WT *C. rodentium* (*Cr*). At day 21 post infection, *E. coli* (*Ec*) or *B. thetaiotaomicron* (*Bt*) or *B. vulgatus* (*Bv*) were inoculated. As a second inoculation, mixture of *B. thetaiotaomicron* and *B. vulgatus* was inoculated into the *E. coli* harboring group, and *E. coli* was inoculated into the *Bacteroides* harboring group, respectively. Pathogen load was determined in feces on the indicated days after inoculation of commensal bacteria. Dots represent individual mice from 2 independent experiments. **B**, Total *Enterobacteria* (*C. rodentium* and *E. coli*) and *Bacteroides* culture in feces at day 0 and day 14 (first inoculation in panel A). Data are given as mean ± SD (n=4). Results are representative of at least 2 experiments. **C**, Number of *C. rodentium* (kanamycin resistant) in the total *Enterobacteria* (kanamycin sensitive) are indicated as a percentage. Data are given as mean ± SD (n=4). Results are representative of at least 2 experiments. **D**, Carbohydrate catabolic profiles of *C. rodentium* and commensal bacteria strains. Robust growth under supplementation of monosaccharides (MSs) or polysaccharides (PSs) indicated as red, and no growth indicated as yellow. Raw data is provided in **Table S1**. **E**, GF mice were infected with WT *C. rodentium* (*Cr*). At day 21 post infection, *B. thetaiotaomicron* (*Bt*) was inoculated. On day 7 post-colonization with *Bt*, the mice were divided into two groups that were fed a conventional maintenance diet (C) or a simple sugar diet (ss). Pathogen load was

determined in feces on day 3 after diet switching. Results are given as mean \pm SD of individual mice (initially n=10, and divided into 2 groups n=5 each). Results are representative of 2 experiments. **F**, *C. rodentium* mono-associated GF mice (day 21) were fed with a simple sugar diet (ssDiet) for 7 days. Pathogen load was compared before and after switching to ssDiet from conventional diet (cDiet). Dots represent individual mice and representative of 3 independent experiments **; p<0.01. N.S., denotes not significant by Mann-Whitney *U* test.