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Strain competition restricts colonization of an enteric pathogen and prevents colitis

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Abstract

The microbiota is a major source of protection against intestinal pathogens; however, the specific bacteria and underlying mechanisms involved are not well understood. As a model of this interaction, we sought to determine whether colonization of the murine host with symbiotic non-toxigenic *Bacteroides fragilis* could limit acquisition of pathogenic enterotoxigenic *B. fragilis*. We observed strain-specific competition with toxigenic *B. fragilis*, dependent upon type VI secretion, identifying an effector–immunity pair that confers pathogen exclusion. Resistance against host acquisition of a second non-toxigenic strain was also uncovered, revealing a broader function of type VI secretion systems in determining microbiota composition. The competitive exclusion of enterotoxigenic *B. fragilis* by a non-toxigenic strain limited toxin exposure and protected the host against intestinal inflammatory disease. Our studies demonstrate a novel role of type VI secretion systems in colonization resistance against a pathogen. This understanding of bacterial competition may be utilized to define a molecularly targeted probiotic strategy.

Keywords colonization resistance; enterotoxigenic *Bacteroides fragilis*; *in vivo* strain competition; probiotics; type VI secretion

Subject Categories Immunology; Microbiology, Virology & Host Pathogen Interaction

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Introduction

Bacterial antagonistic relationships are appreciated as a critical factor in defining the dense ecosystem of the intestinal microbiota [1,2]. Pathogen exclusion through competition with the microbiota is a long understood mechanism of host protection, indicating that individual susceptibility to disease may in part be underpinned by these relationships [3–9]. Enterotoxigenic *Bacteroides fragilis* (ETBF) cause acute diarrhea and are associated with active inflammatory bowel disease (IBD), late-stage colon cancer, and sepsis through production of *B. fragilis* toxin (BFT) [10–16]. Conversely, non-toxigenic *B. fragilis* (NTBF) strains are symbiotic, protecting their host against inflammatory disease [17]. Longitudinal human studies show stable predominance by either ETBF or NTBF in the *B. fragilis* population of an individual microbiota [18], suggesting a competitive interplay between these strains [7,19]. However, the determinants of this competition and susceptibility to ETBF colonization remain unknown, representing a unique model for the study of microbiota–pathogen interactions.

One mechanism of interbacterial competition is type VI secretion (T6S), whereby an attacking cell injects effector proteins through the membrane of a target organism [20,21]. Effector-neutralizing immunity proteins encoded in the genome of the attacking strain prevent self-intoxication [22–24]. Differential encoding of effector–immunity pairs has been demonstrated to contribute to *in vitro* strain competition in *Vibrio cholerae* [25]. Broad conservation of type VI secretion system (T6SS) loci was identified in the Bacteroidetes phylum, including *B. fragilis*, allowing for killing of the closely related *B. thetaiotaomicron in vitro* [3–9,26,27]. Recent studies identified *B. fragilis* strain competition mediated by T6S both *in vitro* and *in vivo* [28,29]. As the Bacteroidetes comprise up to 50% of the human microbiota, T6S may play a key role in determining its composition. The complex biogeography of the intestinal ecosystem necessitates examination of the functional effects of putative competitive factors on the host [10–15,26,30]. The distinct biological properties of non-toxigenic and toxigenic *B. fragilis* enable an examination of symbiont–pathogen competitive colonization in microbiota composition and disease susceptibility.

Results and Discussion

Strain competition reduces ETBF colonization through T6S

To understand competitive dynamics within the *B. fragilis* species, we utilized a co-colonization system in specific pathogen-free (SPF)

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C57BL/6J mice. Following orogastric delivery of *B. fragilis*, colonization by non-toxigenic and enterotoxigenic strains of interest (Appendix Table S1) was monitored over time by fecal colony-forming unit (CFU) recovery on selective media, utilizing plasmid-encoded antibiotic resistance markers to distinguish strains [17,31]. Co-colonization of mice with NTBF strain NCTC 9343 (N1) and ETBF strain ATCC 43858 (E1) resulted in a ~100 fold higher N1 colonization density relative to E1 over a 4-week time period (Fig 1A). To examine the role of T6S in *B. fragilis* competition, we generated an N1 mutant harboring a genomic deletion of the *tssC* locus (N1 Δ*tssC*) that encodes an essential machinery component of the T6SS [7,19,26]. Co-colonization of N1 Δ*tssC* with E1 caused a loss of E1 repression (Figs 1B and EV1A) that was regained by plasmid-based *tssC* complementation (N1 Δ*tssC* pTssC, Fig 1C). Analysis of bacterial recovery 4 weeks post co-colonization demonstrated an increased E1 bacterial load achieved with loss of N1 T6SS function (Fig 1D), concomitant with a decrement in the colonization density achieved by N1 Δ*tssC* (Fig EV1B). Deletion of *tssC* did not affect N1 mono-colonization (Fig EV1C and D) or bacterial recovery of N1 or E1 (Fig EV1E–G) 1 day following co-colonization. As E1 mono-colonization yielded 10^{10} CFU/g recovery in the feces (Fig EV1H), competition with N1 effectively reduced host exposure to toxin-producing *B. fragilis*. *In vitro* plate competition assays revealed T6S-dependent killing of E1 by N1, confirming a direct interaction between these strains (Fig EV1I).

A differentially encoded effector–immunity pair mediates T6S-dependent strain competition

Alignment of the N1 and E1 T6SS loci revealed a non-conserved region encoding a set of proteins that lack homology to documented T6SS effector or immunity domains (Fig 2A). We predicted that these proteins might determine intraspecific competition. Mass spectrometry analysis of the N1 secretome revealed decreased secretion of proteins encoded within the T6SS locus upon mutation of *tssC* (Table EV1), including T6S structural components (e.g., Hcp homologs and VgrG). BF9343_1928 demonstrated the greatest fold-change in the secretome study, leading us to hypothesize that this protein is a putative effector and that BF9343_1927, encoded immediately downstream, is its cognate immunity protein (Fig 2A). Congruent with our studies, BF9343_1928 was demonstrated as a T6S effector, denoted as Bte2 (*B. fragilis* T6S effector 2) and BF9343_1927 as a cognate immunity protein named Bti2a (*B. fragilis* T6S immunity 2a) [28]. In-frame deletion of *bte2* in N1 (N1 Δ*bte2*) phenocopied the Δ*tssC* mutant during co-colonization with E1, as N1 Δ*bte2* no longer demonstrated a competitive advantage against E1, permitting enhanced E1 colonization (Fig 2B, C and E). Heterologous expression of Bti2a in E1 (E1 pBti2a) conferred full restoration of E1 fecal CFU 4 weeks post co-colonization with N1 WT (Fig 2D and E). These results were confirmed *in vitro*, where N1 Δ*bte2* exhibits reduced killing capacity against E1, and E1 pBti2a is protected from

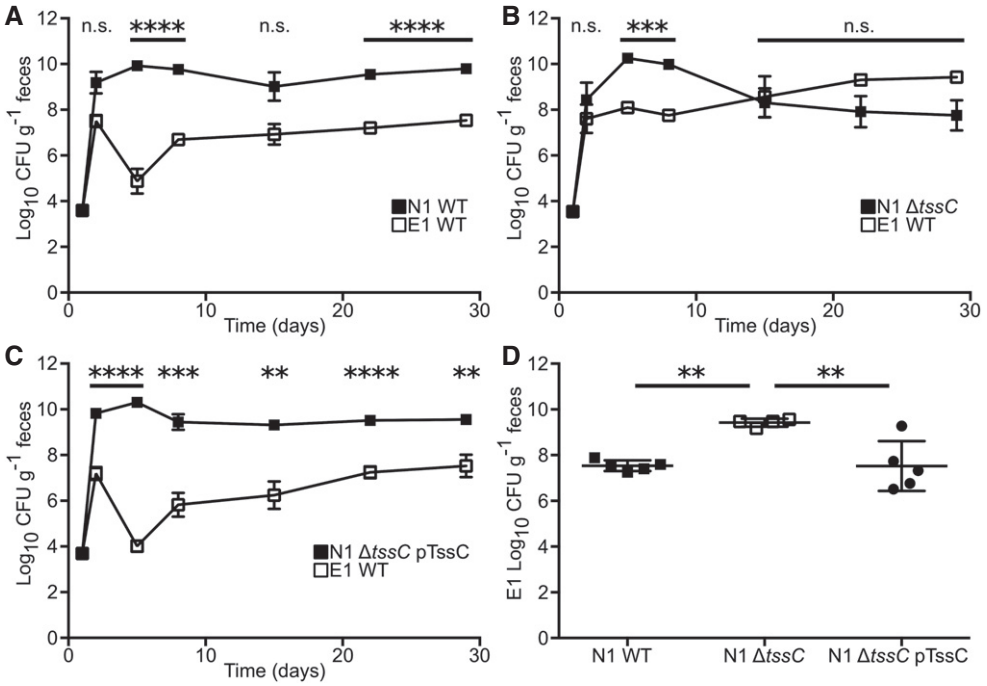

Figure 1. NTBF strain dominance of ETBF through T6S.

A–C SPF C57BL/6J mice were co-colonized with E1 and N1 wild type (WT, A, $n = 5$ mice), N1 T6SS mutant (Δ*tssC*, B, $n = 4$), or N1 complemented (Δ*tssC* pTssC, C, $n = 5$). Fecal CFU was quantified for E1 (open squares) and N1 (closed squares) weekly.

D Four weeks post-colonization, E1 fecal recovery was compared between the N1 WT, Δ*tssC*, and Δ*tssC* pTssC groups.

Data information: Results are representative of three independent experiments. Data are presented as mean ± SEM (A–C) or mean ± SD (D). n.s., not significant; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Statistical significance was determined by unpaired, parametric, two-tailed Student's *t*-test at each time point (applying Bonferroni correction), comparing the co-colonizing strains (A–C) or one-way ANOVA, Tukey's multiple comparisons test (D).

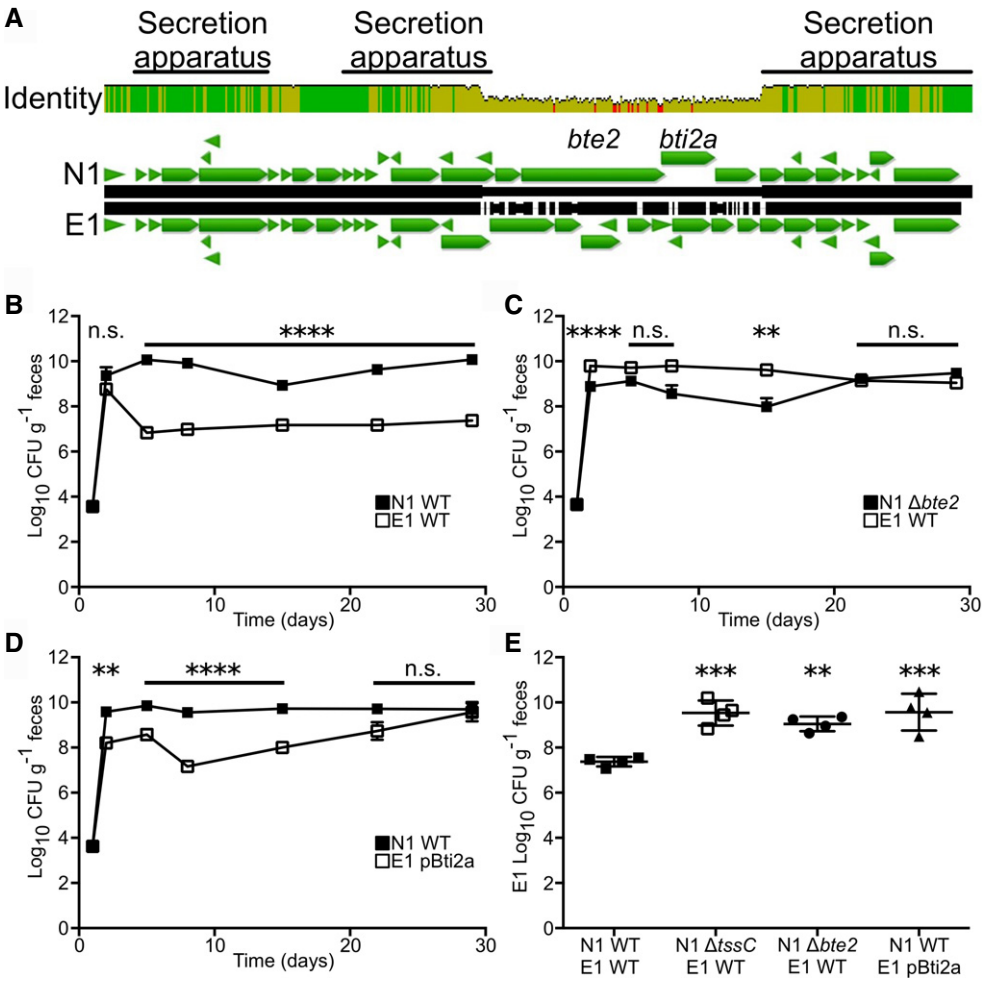

Figure 2. An effector–immunity pair is required for E1 colonization resistance.

A Nucleotide alignment of the T6SS locus from N1 and E1. Percent identity is indicated as height, green representing high homology with red highlighting non-conserved regions.

B–E Co-colonization of N1 WT (B and D, n = 4 mice) or N1 Δ*bte2* (C, n = 4) with E1 WT (B and C) or E1 overexpressing Bti2a (E1 pBti2a, D). Fecal CFU was monitored over time (B–D) and E1 CFU compared to N1 WT-E1 WT group at 4 weeks post co-colonization (E).

Data information: Results are representative of two independent experiments. Data are presented as mean ± SEM (B–D) or mean ± SD (E). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Statistical significance was determined by unpaired, parametric, two-tailed Student's *t*-test at each time point (applying Bonferroni correction) comparing the co-colonizing strains (B–D) or one-way ANOVA, Tukey's multiple comparisons test (E).

N1 WT (Fig EV1I). These data demonstrate the importance of a differentially encoded effector–immunity locus in modulation of the colonic microbiota composition, resulting in altered colonization by toxigenic *B. fragilis*.

ETBF colonization resistance is strain- and order-dependent

Colonization of gnotobiotic mice with N1 saturates the *B. fragilis* intestinal niche and prevents secondary challenge by the same strain [18,31]. To determine whether colonic establishment of N1 provides colonization resistance against E1, we examined the N1–E1 interaction in sequential colonization of gnotobiotic mice, monitoring fecal CFU of each strain through differential encoding of plasmid-borne antibiotic resistance markers. Primary colonization with N1 restricted secondary challenge of N1, while E1 challenge produced stable colonization, suggesting strain-specific colonization resistance within the *B. fragilis* species (Fig 3A). Results in SPF mice phenocopied the gnotobiotic competition (Fig 3B); we therefore utilized the SPF model to evaluate *in vivo* strain interactions for all subsequent studies. A broader pairwise analysis of five *B. fragilis* strains, three NTBF and two ETBF (N1, N2, N3, E1, and E2; Appendix Table S1), was performed to examine *B. fragilis* competition behavior. While each strain exhibited similar primary colonization (Fig EV2), we observed a distinct pattern of intraspecific niche competition between strains (Figs 3C and EV2), confirmed by PCR-based genomic analysis of the colonizing strains (Appendix Fig S1). Intraspecific niche competition is characterized by full restriction of colonization by self-secondary challenge (Fig 3C, gray box) and strain-specific colonization resistance wherein some strains (N2, N3, and E1) exhibit a dominant exclusion phenotype (Fig 3C, red boxes). Our data show a strong

priority effect of *B. fragilis* intestinal niche establishment, which can be overcome when challenged by E2 (Fig 3C, dashed box). This suggests that modular, genetically encoded factors and environmentally driven gene regulation mediate complex strain–strain interactions. In this context, host protection against ETBF colonization is dependent upon strain of initial exposure.

T6S provides colonization resistance against challenge strains

T6SSs have been implicated in colonization resistance against pathogen invasion [20,21,32]; however, *in vivo* molecular evidence for this is lacking. The role of T6S observed in our co-colonization study suggested that this system might govern intraspecific competition in secondary challenge. To examine this hypothesis, we generated a *tssC* deletion mutant in N2 (N2 Δ*tssC*), a strain that demonstrates broad colonization resistance (Fig 3C). Deletion of *tssC* relieved N2 colonization resistance against N1, which was restored upon plasmid-based complementation (Fig 4A and B). Loss of *tssC* did not alter N2 primary colonization (Fig 4 and Appendix Fig S2A, C and E), or self-secondary exclusion of N2 (Fig 4C and D). While N2 Δ*tssC* retained colonization resistance against E1, the rate of elimination was significantly reduced compared to wild-type N2 (Fig 4E and F). Secondary strain recovery one day post-challenge was not significantly different between groups, emphasizing the importance of the colonic environment in mediating competition (Appendix Fig S2B, D and F). These data show that T6S is important for non-self-colonization resistance *in vivo* and is a key contributor to strain stability in the microbiota [22–25].

It is clear from these findings that T6S alone cannot explain the complex strain–strain interactions observed (Fig 3C). To date, one other antibacterial factor has been identified in *B. fragilis*. Bacteroidales secreted antimicrobial protein 1 (BSAP-1) is a membrane attack complex/perforin (MACPF)-containing protein, produced by N2 that displays N1 killing properties *in vitro* [1,25]. Mutation of *bsap-1* in N2 (N2 Δ*bsap-1)* had no effect on N2 colonization resistance against N1 *in vivo* (Appendix Fig S3), demonstrating the importance of the *in vivo* niche in defining factors that mediate intraspecific colonization resistance.

The commensal colonization factor (*ccf*) locus of *B. fragilis* enables niche occupancy within the colonic crypt and is required by N1 for self-colonization resistance [31]. Diverged *ccf* loci of B. *thetaiotaomicron* and *B. vulgatus*, two species closely related to *B. fragilis*, are suggested to define separate niches for those organisms, supported by the observation that N1 does not exhibit

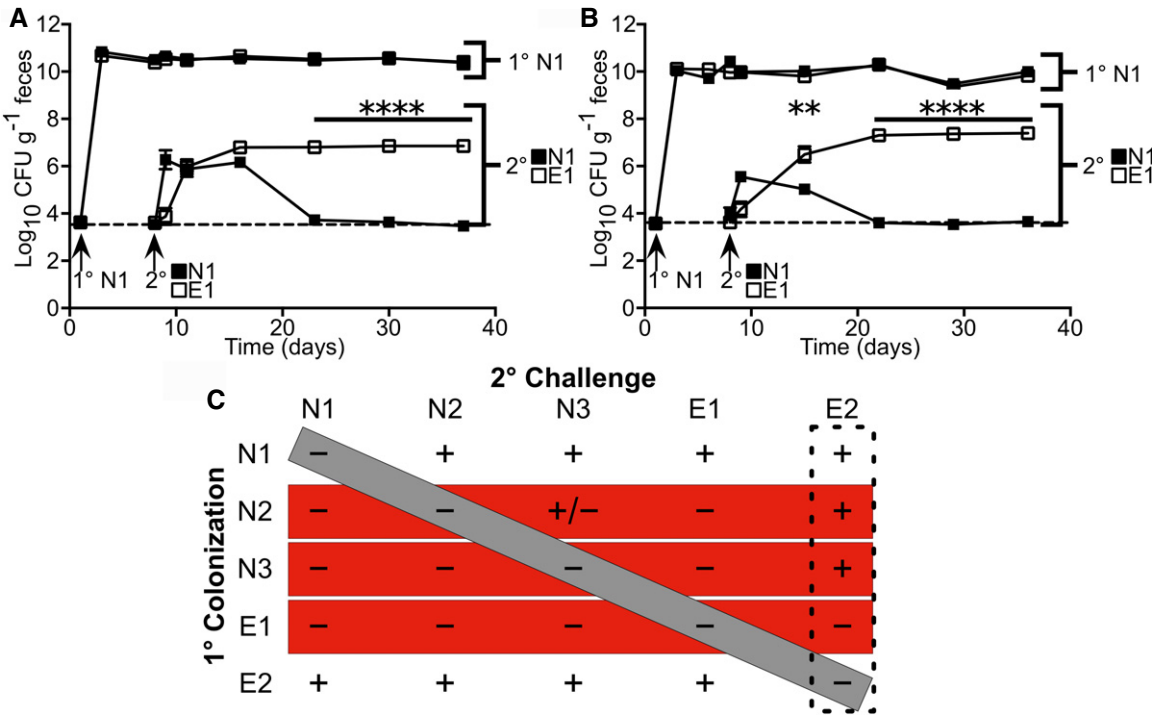

Figure 3. *B. fragilis* provides strain-specific colonization resistance.

A, B Initial colonization of gnotobiotic (A) or SPF (B) mice (n = 4 mice per group) with N1 followed by secondary challenge with N1 (closed squares) or E1 (open squares). Fecal CFU was determined for the primary and secondary colonization strains through 4 weeks post-secondary challenges.

C All primary colonization and secondary challenge pairs were tested with 3 NTBF and 2 ETBF strains. Stable colonization of the secondary challenge strain significantly above the limit of detection is denoted as a "+" while non-significance is denoted as a "–" (n = 4 mice per group). The diagonal gray bar indicates self-secondary challenge, the horizontal red bars show strains that provide broad colonization resistance against non-self strains, and the vertical dashed box indicates a strain that has an enhanced secondary colonization phenotype.

Data information: Results illustrate a single experiment (A) or are representative of at least two independent experiments (B and C). Data are presented as mean ± SEM. Arrows denote day of primary colonization and secondary challenge. A dashed line denotes limit of detection. $^{**}P < 0.01$, $^{****}P < 0.0001$. Statistical significance was determined by unpaired, parametric, two-tailed Student's t-test at each time point (applying Bonferroni correction) comparing the secondary challenge strains.

colonization resistance against either species [31]. It is unknown whether these niches are spatially distinct from the *B. fragilis* niche or whether these *Bacteroides* species interact in the colon. N1 kills *B. thetaiotaomicron* through T6S *in vitro* [26], which we confirmed for N2 (Fig EV3A). N2, however, did not confer resistance against secondary challenge by *B. thetaiotaomicron in vivo* (Fig EV3B), indicating the species specificity of colonization resistance. Mutation of the T6SS of N2 had no effect on fecal recovery of *B. thetaiotaomicron* after secondary challenge (Fig EV3B–D). Similarly, despite T6S-dependent killing of *B. vulgatus in vitro* (Fig EV3E), N2 did not restrict *B. vulgatus* in secondary challenge (Fig EV3F–H). As T6S is contact-dependent, these data suggest a physical niche separation between these *Bacteroides* species and implies that intraspecies competition is a primary function of *Bacteroides* T6S *in vivo*.

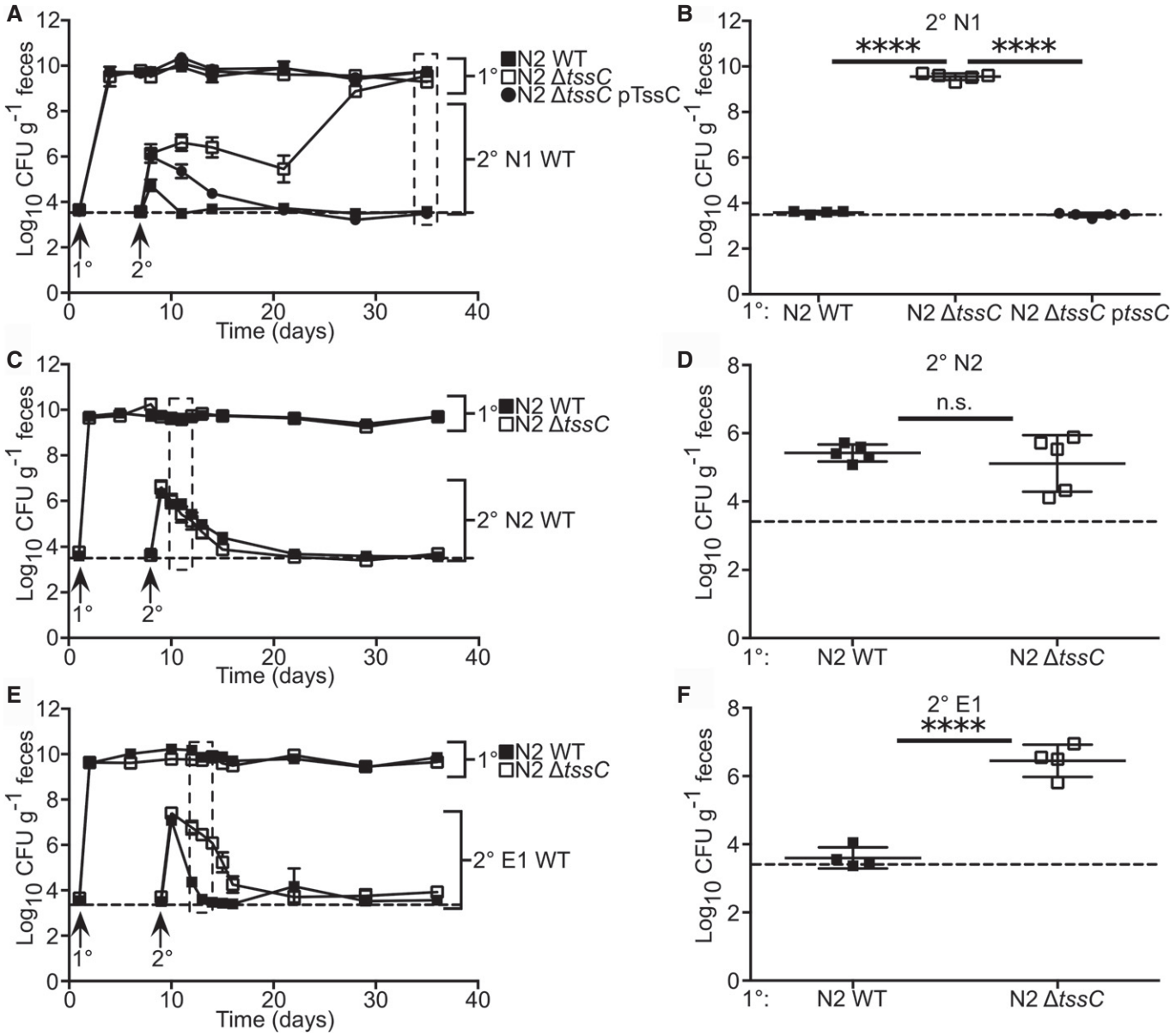

Figure 4. T6S is required for strain-specific colonization resistance.

A–F Primary colonization of SPF mice with N2 WT, T6SS mutant (Δ*tssC*), and complemented (Δ*tssC* pTssC) followed by secondary challenge with N1 WT (A and B, $n = 5$ mice), N2 WT (C and D, $n = 5$), or E1 WT (E and F, $n = 4$) was performed. Fecal CFU for primary and secondary strains was determined for 4 weeks post-secondary challenge (A, C, E). Selected time points were tested for statistical difference of secondary challenge between groups. This includes 4 weeks post-secondary challenge (B) and 3 days post-challenge (D and F).

Data information: Results are representative of three independent experiments. Data are presented as mean ± SEM (A, C, and E) or mean ± SD (B, D, and F). Arrows denote day of primary colonization and secondary challenge. A dashed line denotes limit of detection. n.s., not significant; ****$P < 0.0001$. Statistical significance was determined by unpaired, parametric, two-tailed Student's *t*-test at each time point (applying Bonferroni correction, D and F) or one-way ANOVA, Tukey's multiple comparisons test (B).

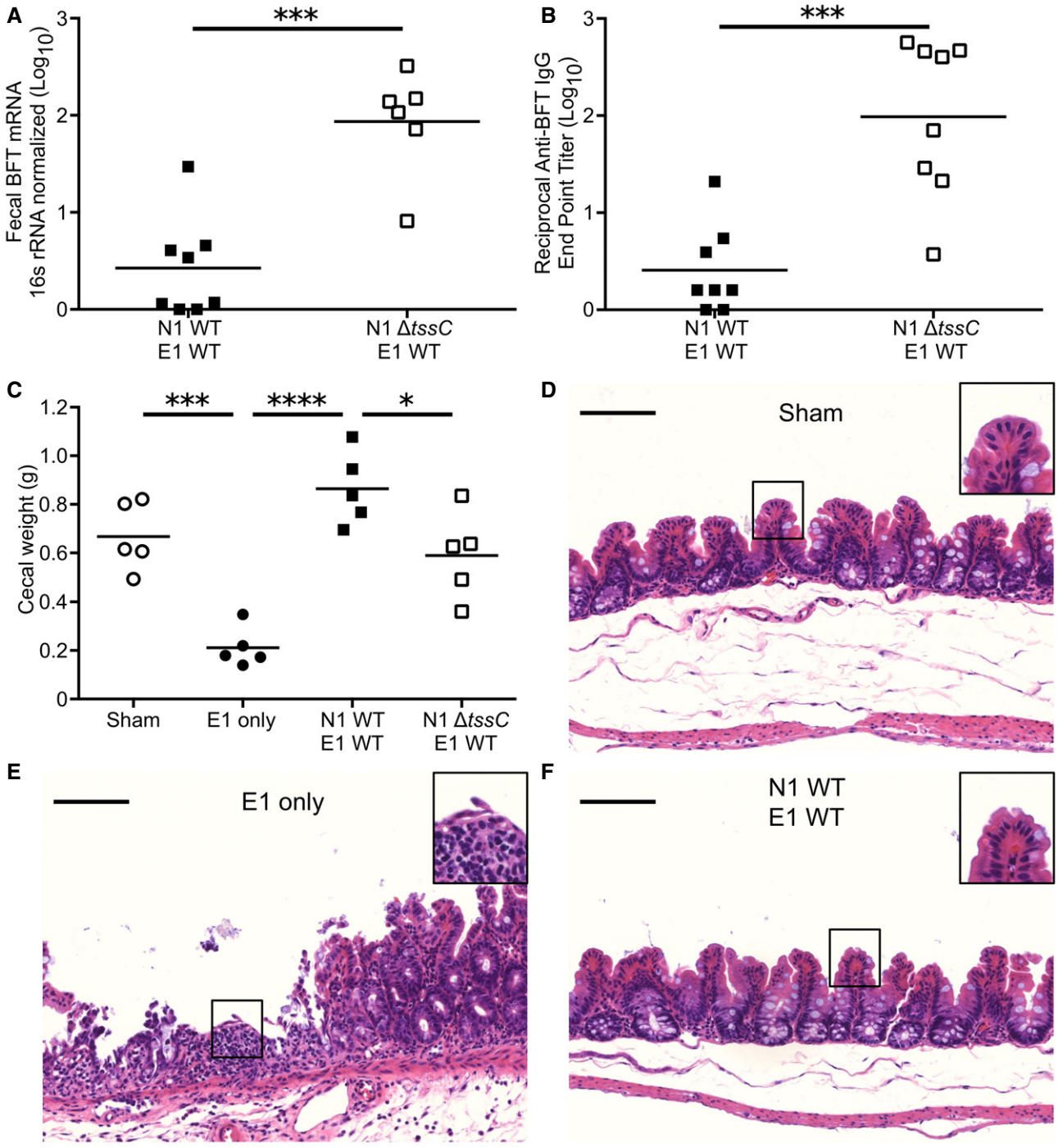

Figure 5. The N1 T6SS protects against ETBF-induced disease.

A Mice were co-colonized with E1 and either N1 WT (n = 4) or N1 Δ*tssC* (n = 3). Five days post-inoculation, fecal RNA was extracted and tested for BFT expression via qRT–PCR.

B Four weeks after co-colonization with E1 and either N1 WT or N1 Δ*tssC* (n = 4 mice per group), the sera were collected, tested via ELISA for anti-BFT IgG, and endpoint titer calculated.

C–F Mice pre-treated with DSS were inoculated with no organisms (sham), E1 only, or E1 competed with N1 WT or N1 Δ*tssC*. Five days post-inoculation, the ceca were weighed (C) and fixed for histopathological examination after sham (D), E1 only (E) and E1-N1 WT (F) colonizations. Scale bars denote 100 μm (main image) and 200 μm (inset).

Data information: Experiments are a pooling of two independent repeats (A and B) or are representative of three independent trials (C–F). Data are presented as mean ± SD (A, B and C). *P < 0.05, ***P < 0.001, ****P < 0.0001. Statistical significance was determined by unpaired, parametric, two-tailed Student's t-test (A and B) or one-way ANOVA, Tukey's multiple comparisons test (C).

NTBF protects the host from ETBF-induced colitis via colonization resistance

Enterotoxigenic *Bacteroides fragilis* colonization is associated with IBD and colitis in humans, experimentally validated in mouse model systems [15,33,34]. We hypothesized that strain competition may favorably alter the health of the host through reduced exposure to toxigenic organisms. Co-colonization of E1 with WT N1 reduced BFT transcript present in the feces by approximately 100-fold relative to competition with N1 Δ*tssC*, congruent with the difference in E1 fecal CFU (Fig 5A; compare to Fig 1A, B and D). A concomitant decrease in mouse anti-BFT serum IgG was observed, reminiscent of observations in ETBF-exposed humans (Fig 5B) [34].

Infection of mice with ETBF exacerbates a BFT-dependent, IBD-like colitis induced by dextran sodium sulfate (DSS) administration [15]. Colonization with E1 in this model causes significant intestinal injury, indicated by the presence of visible blood in the ceca, substantial tissue contraction, and reduced cecal weight relative to sham-inoculated animals (Figs 5C and EV4A). We hypothesized that N1 competition would reduce the disease burden in this model, consistent with suppression of the E1 burden by WT N1 (Fig 1A). Indeed, co-colonized mice did not exhibit evidence of cecal injury (Figs 5C and EV4A). Competition with N1 Δ*tssC* also reduced inflammation associated with E1 colonization, but not as effectively as N1 WT co-colonization, indicating that T6S is required for full protection (Fig 5C). Examination of both cecal and colonic histopathology revealed severe ulcerations in mice mono-colonized with E1, demonstrated by loss of crypts, epithelial denudation, and the presence of inflammatory cell infiltration (Figs 5D–F and EV4B–D). Competition of E1 with N1 WT provided complete protection from damage throughout the length of the cecum and colon. These data demonstrate that the N1 T6SS affords the host significant protection from E1 colonization and BFT-induced injury.

Our data illustrate the importance of bacterial antagonistic factors in the composition and stability of the microbiota, providing a direct demonstration of T6S-mediated competition in the intestine. In the context of both co-colonization and colonization resistance, T6S is a mechanism of strain dominance. The Bacteroidetes are a resilient phylum within the human microbiota [35]. Broad conservation of T6SSs within this phylum suggests that specific effector–immunity proteins are critical mediators of *in vivo* competition [26,27]. The uneven distribution of T6S within particular species (i.e., present in the majority of *B. fragilis* but absent in *B. thetaiotaomicron* [27]) highlights the importance of microbial lifecycle and ecosystem, and underscores the need to define the molecular mechanisms by which strain-specific effector modules govern competition [25]. While plate-based studies have identified antagonistic factors in Bacteroidetes [1,26], our observations reveal that the biological relevance of competitive factors is manifest in the context of the intestine. This native environment is essential to provide the appropriate regulatory cues, biogeography, niche, and colonization order.

Membership in a predominant phylum and limitation of the *B. fragilis* lifecycle to the intestine make this organism an ideal model for deciphering the molecular features of microbiota competition. Strain-dependent virulence properties of *B. fragilis* associated with human disease further heighten the import of understanding competition dynamics within this species. Our findings illustrate the strong influence that competitive factors have on host exposure to toxigenic organisms, revealing a specific mechanism by which the microbiota provides protection from toxin-induced colitis. While eradication of ETBF through antimicrobial therapy protects susceptible mice from colon tumorigenesis [36], utilizing NTBF as a probiotic strategy to reduce the ETBF load may prove similarly effective, without the unwanted consequences of antibiotic use. As we find that a single non-conserved effector–immunity pair can determine the outcome of NTBF-ETBF competition, analysis of these loci in the native microbiota of human populations may reveal strains that provide enhanced protection against ETBF. As the colonization phenotypes exhibited by NTBF and ETBF include both exclusion and durability in human studies [18], early acquisition and deliberate re-colonization with genetically modified NTBF strains may afford protection against ETBF-mediated disease in a variety of clinical settings. Moreover, these principles may apply to other pathogenic bacteria with non-toxigenic strains, including *Clostridium difficile* and *Escherichia coli*, for which the determinants of competitive colonization remain mostly undiscovered [7,19,37]. The observed strain specificity and dependence on the order of colonization provide a necessary framework to advance molecular probiotic targeting toward rational, therapeutic manipulation of the pathogenic microbiota.

Materials and Methods

Bacterial culture and manipulation

Bacterial strains, culture conditions, and antibiotics

Bacteroides strains used in these studies include *B. thetaiotaomicron* ATCC 29148 and *B. vulgatus* ATCC 8482. *B. fragilis* strains are noted in Table EV1. *B. fragilis*, *B. thetaiotaomicron,* and *B. vulgatus* were grown in brain–heart infusion (BHI) broth anaerobically at 37°C with a gas mix of 5% H_2, 10% CO_2, and 85% N_2. BHI was supplemented with 0.0005% hemin and 0.5 μg/ml vitamin K1 for optimal growth (BHIS). *E. coli* S17-1λpir was used for cloning of shuttle and suicide plasmids and conjugation into *B. fragilis*. *E. coli* strains were grown in LB aerobically at 37°C. Antibiotics used were as follows: ampicillin (100 μg/ml), kanamycin (50 μg/ml), gentamicin (200 μg/ml), clindamycin (5 μg/ml), tetracycline (2 μg/ml), chloramphenicol (10 μg/ml), and rifampicin (20 μg/ml).

Conjugations

A method modified from previous studies was used for conjugations [38]: appropriate *B. fragilis* and *E. coli* strains were grown to mid-log phase in BHIS anaerobically and LB aerobically, respectively. Equal volumes of these cultures were sedimented into a single tube, resuspended in BHI, pooled onto a BHIS plate, and grown aerobically at 37°C overnight. This mix was spread onto a selective BHIS plate with gentamicin and clindamycin and grown anaerobically at 37°C.

Plasmids

pRK231 is a conjugation helper vector, used to increase transfer from *E. coli* to *B. fragilis*. Allelic exchange mutagenesis was completed with the suicide vector pKNOCK. The shuttle vector pFD340 provided antibiotic resistance against clindamycin (all

pFD340-based vectors), tetracycline (pFD340-Tet), and chloramphenicol (pFD340-CAT), as in previous studies [31]. The pFD340-based vectors were used in the *B. fragilis* strains N1, N2, N3, and E1 along with *B. thetaiotaomicron* and *B. vulgatus*. As we could not recover successful E2 conjugates with the pFD340-based vectors, we generated the shuttle vector pAH2. This plasmid was made via cloning of the pFD340 replicon into the pKNOCK NotI restriction enzyme site. The primers for this and all other cloning are found in Appendix Table S2. The chloramphenicol resistance cassette with associated promoter and ribosomal binding site, called IS4351-CAT, was cloned from pFD340-CAT into the KpnI restriction enzyme site of pAH2 to generate pAH2-CAT. Inserts for all newly generated constructs were sequence verified.

Mutants, complementation, and heterologous expression

In-frame deletions were generated through allelic exchange using a protocol modified from previous studies [39]. To generate *tssC* mutants, 1 kb upstream and 1 kb downstream of *tssC* were amplified from strains of interest and fused via overlap PCR. This construct was cloned into pKNOCK and conjugated into strains N1 and N2. Single clones resistant to clindamycin, indicating genomic integration, were passaged (1:100) daily without antibiotics. After 5–10 passages, single clones were patched onto selective (clindamycin) and non-selective plates. Sensitive colonies were PCR screened for loss of *tssC*. Mutations in *bsap-1* in N2 and *bte2* in N1 were generated in the same manner.

Mutation of *tssC* was complemented on pFD340 and pFD340-CAT for N2 and N1, respectively. This was fused downstream of the *B. fragilis* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter and RBS and cloned into the KpnI restriction enzyme site of the aforementioned vectors. The resulting vectors were named pTssC and pTssC-CAT. Generation of pBti2a to overexpress the *bti2a* locus was accomplished via fusion to the GAPDH promoter and RBS and was cloned into the KpnI restriction enzyme site of pFD340-Tet.

Secretome mass spectrometry: peptide preparation

The secretomes of N1 WT and N1 Δ*tssC* were analyzed by mass spectrometry as follows: Overnight cultures were grown in BHIS, pelleted and washed in minimal media followed by 1:50 dilution into minimal media for overnight growth. Supernatant was collected and TCA precipitated. After a 1-h incubation on ice, the precipitated protein was spun and washed twice with cold acetone. The dried pellet was resuspended in 7.5 mM TCEP, 8 M urea, 100 mM NH_4HCO_3 solution, and incubated for 1 h. This was spun on a 3K MWCO, mixed with 8 M urea, 100 mM NH_4HCO_3, 50 mM iodoacetamide solution, and incubated in the dark for 1 h. A total of 500 mM DTT was added to inactivate the iodoacetamide and the filter washed four times with 50 mM NH_4HCO_3. The remaining solution was trypsin treated overnight, acidified to 1% TFA and dried.

Secretome mass spectrometry: mass spectrometry

Peptide (5 μl) was introduced to a hybrid quadrupole-orbitrap mass spectrometer (Orbitrap Elite, Thermo Fisher, San Jose, CA) coupled with a nanoflow LC system (NanoAcquity; Waters Corporation, Milford, MA). A 100 μm i.d. × 20 mm pre-column was in-house packed with 200 Å, 5 μm C18AQ particles (Michrom BioResources Inc., Auburn, CA, USA). A 75 μm i.d. × 180 mm analytical column was pulled using a Sutter Instruments P-2000 CO_2 laser puller (Sutter Instrument Company, Novato, CA) and packed with 100 Å, 5 μm C18AQ particles. Mobile phase A was composed of 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in acetonitrile. For each injection, an amount of 5 μl of sample was loaded on the pre-column at 4 μl/min for 10 min, using loading buffer of 5% acetonitrile and 0.1% formic acid. Peptide separation was performed at 250 nl/min flow rate in a 95 min gradient, in which mobile phase B started at 5%, increased to 35% at 60 min, 80% at 65 min, followed by a 5-min wash at 80% and a 25-min re-equilibration at 5%.

Mass spectrometry data were collected in positive ionization mode using a data-dependent acquisition method with a full MS scan for m/z range 350–2,000 in orbitrap at 120 K resolution. Consecutive MS/MS scans selected top 15 abundant ions in ion trap by rapid scan mode with a dynamic exclusion of 30 s. Precursor ions selected from the MS scan were isolated with an isolation width of 2 m/z for collision-induced dissociation (CID) energy, NCE, at 35.

Secretome mass spectrometry: data analysis

MS data were analyzed by MaxQuant (version 1.5.0.25, [40]) using standard settings and a UniprotKB database of *Bacteroides fragilis*. Peptide-spectrum matches (PSM) and protein identifications were filtered at a false discovery rate of 0.01. Label-free quantification of MS/MS spectral counts were extracted and used for statistical analysis of differential expression using QSpec tool (version 1.2.2, [41]). Results are averaged from three technical replicates. The raw data can be found via Peptide Atlas, Dataset Identifier: PASS00882.

Bioinformatic analysis

T6SS loci of N1 and E1 were aligned in Geneious version 6.0.5. The alignment was performed with Geneious alignment software (cost matrix: 65% similarity, gap open penalty: 12, gap extension penalty: 3) and visualized in Geneious. The N1 T6SS locus was extracted from publicly available genome assembly, and the E1 T6SS locus was sequenced (Accession #KX279877).

In vitro competition

N1 clones (WT, Δ*tssC,* and Δ*bte2*), N2 clones (WT, Δ*tssC*, and Δ*tssC* pTssC), E1 clones (WT and pBti2a), *B. thetaiotaomicron*, and *B. vulgatus* inocula were prepared in a similar manner to co-colonization, excepting the final resuspension in PBS in place of sodium bicarbonate. N2 WT and Δ*tssC* were marked with pFD340 and N2 Δ*tssC* pTssC with pTssC; N1 clones, *B. thetaiotaomicron,* and *B. vulgatus* were marked with pFD340-CAT; E1 clones (WT and pBti2a) with pFD340-Tet and pBti2a, respectively. 10^8 CFU of N1 or N2 was competed against 10^7 CFU of E1 clones, *B. thetaiotaomicron* or *B. vulgatus* in a 10 μl spot on a BHIS plate with gentamicin and clindamycin. After overnight competition, these spots were resuspended in 1 ml of PBS and serial 10-fold dilutions plated on BHIS with gentamicin, clindamycin, and chloramphenicol to quantify N1 clones, *B. thetaiotaomicron,* or *B. vulgatus* recovery while E1 clones were detected on BHIS with gentamicin, clindamycin, and tetracycline. Recovered CFU was computed from the various competitions.

Mouse modeling

All animal studies were conducted in accord with ethical regulations under protocols approved by the University of Chicago Animal Care and Use Committee and Institutional Biosafety Committee. SPF C57BL/6 mice were bred in-house from mice originally purchased from Jackson Laboratory or purchased from Jackson Laboratory and maintained under SPF conditions for use in experimentation at 4 weeks of age. Gnotobiotic C57BL/6J mice were also bred in-house, maintained in germ-free conditions and used at 6 weeks of age. All experiments in SPF mice were performed with males while the gnotobiotic experiment with females. Sample size estimates for animal experiments were based on prior animal modeling studies utilized within the laboratory for investigation of colonization. At the time of weaning, animals were randomly distributed for use in experimentation. There was no investigator blinding in animal experimentation, and no animals were excluded from analysis.

Mono-colonization and co-colonization

SPF mice were pre-treated with 100 mg/l clindamycin in their drinking water for 1 day prior to and throughout the course of infection to ensure plasmid retention. To prepare inocula, overnight cultures of *B. fragilis* were subcultured at a 1:50 ratio into fresh BHIS and grown until they reached $OD_{600} \approx 0.5$. Bacteria from 50 ml culture were sedimented and resuspended in 10 ml PBS. These suspensions were adjusted by the addition of PBS until a 1:5 dilution yielded an $OD_{600} = 0.55$. Bacteria from 9 ml of suspensions were sedimented and resuspended in 1.8 ml 0.1 N sodium bicarbonate to yield a concentration of 10^{10} CFU/ml. For mono-colonization, mice were inoculated via oral gavage with 10^8 CFU of E1 or N1 clones. For co-colonization, mice were co-colonized with 10^8 CFU each of E1 and N1 via oral gavage. E1 was marked with pFD340-Tet or pBti2a, N1 WT, Δ*tssC*, and Δ*bte2* with pFD340-CAT and N1 Δ*tssC* pTssC with pTssC-CAT. To analyze fecal CFU following oral inoculation, fecal pellets were collected from individual mice, weighed, and vortexed in 1 ml PBS to achieve homogenization. Serial 10-fold dilutions were plated on BHIS agar containing gentamicin and clindamycin plus either tetracycline, to monitor E1 recovery, or chloramphenicol, to determine N1 clone recovery. CFU/g feces for each clone was calculated, $\log_{10}$ transformed, and plotted over time.

Sequential colonization

Similar to co-colonization experiments, SPF and gnotobiotic mice were pre-treated with 100 mg/l clindamycin in drinking water for 1 day prior to and throughout the course of colonization to ensure plasmid retention. Inocula were prepared as in co-colonization. Sequential colonization was performed with a modified protocol from previous studies [31]. Primary colonization was achieved with 10^8 CFU of *B. fragilis* strains (N1, N2 WT, N2 Δ*tssC*, N3, E1) containing pFD340, N2 Δ*tssC* pTssC containing pTssC, or E2 containing pAH2 via oral gavage. After colonization for 7–10 days, secondary challenge was performed via oral gavage with 10^8 CFU of *B. fragilis* strains (N1, N2, N3, E1), *B. thetaiotaomicron*, or *B. vulgatus* containing pFD340-CAT or E2 containing pAH2-CAT. Fecal pellets were handled as noted in co-colonization. Inherent antibiotic resistance (Appendix Table S1) was used to determine primary colonization fecal CFU; homogenized fecal pellets were plated onto BHIS plus gentamicin and clindamycin only (N1, E1), with tetracycline (N3, E2) or with rifampicin (N2). Secondary challenge CFU was determined by plating onto BHIS with gentamicin, clindamycin, and chloramphenicol. CFU/g feces were determined as noted in co-colonization. Limit of detection is dependent upon the weight of each fecal pellet, indicated based on average fecal pellet weight at $\sim 10^{3.5}$.

B. fragilis *genotyping*

The identity of each strain was determined via PCR with three primer sets (Appendix Table S2), which distinguish the five *B. fragilis* strains (Appendix Fig S1). PCR was performed on successful secondary challenge strains from single colonies of each mouse. The identity of the strains was confirmed by banding pattern.

Fecal quantitative reverse transcription PCR

To test the transcription levels of BFT in the feces, quantitative reverse transcription PCR (qRT–PCR) was used. Fresh fecal pellets were collected, and RNA was extracted with the ZR soil/fecal RNA microprep kit (Zymo Research). RNase-free DNase (Fisher) was used to digest contaminating genomic DNA in the samples. First-strand cDNA synthesis was accomplished with iScript cDNA synthesis kit (Bio-Rad), and qPCR was performed with SYBR Green (Bio-Rad) on a Bio-Rad CFX96 machine. BFT transcript was quantified with BFT-specific primers (Appendix Table S2) and normalized to *B. fragilis* 16S rRNA. Efficiency for each primer set was determined to calculate accurate fold differences, and melt curves were used to test the specificity of each reaction.

Serum ELISA

Four weeks post-colonization, mice were euthanized and blood was obtained via cardiac puncture. Blood was spun in serum collection tubes and stored for ELISA testing at −20°C. Recombinant, purified BFT [16] was coated onto Maxisorp plates (Nunc) at a concentration of 2 μg per well overnight in bicarbonate buffer (0.1 M) at 4°C. The plate was washed with ELISA buffer and blocked for 1 h with 2% BSA in PBS. Serum samples were diluted initially 1:10 and serially 1:4 to generate a dilution curve in PBS. After blocking, the plate was incubated with serum samples for 1 h. After 3 washes with ELISA buffer, secondary HRP-conjugated anti-mouse IgG antibody (Southern Biotech) was applied (1:10,000) for 1 h. After washing thrice, the ELISA was developed for 10 min and absorbance recorded on a Tecan Infinite M200pro plate reader. Data were analyzed with GraphPad Prism software to determine the endpoint titer through sigmoidal curve fit, with a cutoff set to double the background reading (no serum).

DSS colitis

To test the impact of *B. fragilis* competition on the health of the mouse, we used dextran sodium sulfate (DSS) as an inducer of colitis susceptibility. Mice were pre-treated with DSS (2%, Fisher) and clindamycin in the drinking water for 5 days pre-colonization. Mice were then either sham-infected, colonized with E1 alone, with a competition of E1 and N1 WT or with a competition of E1 and N1 Δ*tssC*. Four days post-colonization, the mice were euthanized, the ceca dissected, visualized, and weighed and the colons dissected.

The intestinal tissue was fixed in 10% formalin, formed into Swiss rolls (colon), paraffin embedded, sectioned (5 μm), and H&E stained.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Pairwise comparisons were performed using the unpaired, parametric, two-tailed Student's *t*-test. Sets of three groups or more were tested via one-way ANOVA, with Tukey's multiple comparisons test used to compare between groups. Bonferroni correction was applied to account for multiple time points in colonization experiments. Comparison of variances was performed in GraphPad Prism software utilizing the *F*-test, providing documentation of similar variance between groups.

Expanded View for this article is available online.

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Author contributions
ALH and JBW designed and analyzed the experiments. BWC provided conceptual support. ALH performed the experiments. BWC and ZME assisted with experiments. YAG and DRG performed mass spectrometry and analysis. ALH and JBW wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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