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Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens

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Abstract

The human intestine, colonized by a dense community of resident microbes, is a frequent target of bacterial pathogens. Undisturbed, this intestinal microbiota provides protection from bacterial infections. Conversely, disruption of the microbiota with oral antibiotics often precedes the emergence of several enteric pathogens[1–4]. How pathogens capitalize upon the failure of microbiota-afforded protection is largely unknown. Here we show that two antibiotic-associated pathogens, *Salmonella typhimurium* and *Clostridium difficile*, employ a common strategy of catabolizing microbiota-liberated mucosal carbohydrates during their expansion within the gut. *S. typhimurium* accesses fucose and sialic acid within the lumen of the gut in a microbiota-dependent manner, and genetic ablation of the respective catabolic pathways reduces its competitiveness *in vivo*. Similarly, *C. difficile* expansion is aided by microbiota-induced elevation of sialic acid levels *in vivo*. Colonization of gnotobiotic mice with a sialidase-deficient mutant of the model gut symbiont *Bacteroides thetaiotaomicron* (*Bt*) reduces free sialic acid levels resulting in a downregulation of *C. difficile's* sialic acid catabolic pathway and impaired expansion. These effects are reversed by exogenous dietary administration of free sialic acid. Furthermore, antibiotic

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

AUTHOR CONTRIBUTIONS

K.M.N., J.A.F., D.M.M. and J.L.S. designed experiments. K.M.N., J.A.F., S.K.H., J.B.L., P.C.K., N.N., B.C. and J.L.S. performed experiments. K.M.N., J.A.F., P.C.K., S.G., N.N., D.M.M. and J.L.S. analyzed data. D.M.M. and B.C.W. contributed reagents. K.M.N., J.A.F. and J.L.S. wrote the paper.

DATA DEPOSITION

Microbiota enumeration (16S rRNA) datasets have been deposited in the EMBL European Nucleotide Archive (ENA) under accession # ERP003629 and can also be found in the QIIME Database under the study ID 1958 (http://www.microbio.me/qiime/). GeneChip datasets are available in the GEO database under accession number GSE49076.

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treatment of conventional mice induces a spike in free sialic acid and mutants of both *Salmonella* and *C. difficile* that are unable to catabolize sialic acid exhibit impaired expansion. These data show that antibiotic-induced disruption of the resident microbiota and subsequent alteration in mucosal carbohydrate availability are exploited by these two distantly related enteric pathogens in a similar manner. This insight suggests new possibilities for therapeutic approaches for preventing diseases caused by antibiotic-associated pathogens.

The intestinal microbiota is composed of trillions of microbial cells that together form a complex, dynamic, and highly competitive ecosystem[5,6]. Limited nutrients and high microbial densities likely play a key role in protecting the host against invading microbes[7]. Carbohydrates derived from diet or host play a well-established role in sustaining the resident members of the microbiota[8–10], and more recently have been shown to play important roles in gut microbiota-pathogen dynamics[11–14]. Oral antibiotic use is one of the leading risk factors for disease associated with *Salmonella* spp. and *Clostridium difficile*, consistent with increased enteric vulnerability upon disruption of the resident microbiota[1–4,15]. In addition, mouse models of *S. typhimurium* or *C. difficile* infection commonly require disruption of the intestinal microbiota with antibiotics to promote pathogen expansion within the lumen of the gut and to initiate disease[16–19]. Deciphering the numerous mechanisms by which the microbiota prevents bacterial pathogen expansion and how microbiota disruption enables pathogens to circumvent these mechanisms remains an important task.

We used transcriptional profiling of *Salmonella typhimurium* from orally infected gnotobiotic mice to gain insight into the pathogen's biology while inhabiting the gastrointestinal tract. Our goal was to reveal adaptations of the pathogen within a 'low-complexity' gnotobiotic microbiota that might be relevant to antibiotic-induced microbiota disruption. Mice that were monoassociated with the model gut symbiont *Bacteroides thetatiotaomicron* (*Bt*) were used as a simplified model of a microbiota that is susceptible to pathogen emergence within the gut. Five days after *S. typhimurium* infection of the *Bt*-monoassociated or germ-free (GF) mice (Fig. 1a), cecal contents were collected and subjected to transcriptional profiling using a custom *S. typhimurium* GeneChip. In the presence of *Bt*, all 59 *S. typhimurium* genes that displayed significantly altered expression relative to infection of GF mice were upregulated (Supplementary Table 1). Functional classification of these genes revealed enriched COG categories: "carbohydrate metabolism and transport" and "secondary metabolites biosynthesis, transport, and catabolism" (Supplementary Fig. 2). Genes encoding host mucin carbohydrate metabolism pathways are prominently represented in this gene set, including three operons encoding catabolic pathways for sialic acid, fucose, and the fucose catabolite propanediol, (*nan*, *fuc* and *pdu*, respectively) (Fig. 1b). We surveyed expression of genes within the *nan* and *fuc* operons 1 day after *S. typhimurium* infection in GF or *Bt*-monoassociated mice, to determine if these operons identified by expression profiling on day 5 post-infection also display high expression earlier in the infection. *S. typhimurium nanE* and *fucI* are significantly upregulated 1 day after infection of *Bt*-monoassociated mice relative to infection of GF mice (*nanE*, 6.0-fold, $p=1.47 \times 10^{-5}$; *fucI*, 3.5-fold, p=0.0028) (Fig. 1c) when *S. typhimurium* densities and host pathology are similar between colonization states (Supplementary Fig. 3,

4). These data are consistent with *S. typhimurium* catabolizing sialic acid and fucose in the lumen of the gut in a *Bt*-dependent manner soon after infection.

We next constructed mutant strains of *S. typhimurium* to quantitatively assess the requirement of sialic acid and fucose during expansion *in vivo*. Deletion of *nanA* and *fucI*, the first committed steps in the sialic acid and fucose utilization pathways, abolished growth of the strains on the respective sugars (Supplementary Fig. 5). In competition experiments, *Bt*-monoassociated mice coinfected with wildtype *S. typhimurium* and a *nanA/fucI* double mutant strain (*St*-Δ*nanA*Δ*fucI*) revealed a significant disadvantage of the mutant on days 1 and 2 after infection (day 1, CI = 1.87, p=0.028; day 2, CI = 1.45, p=0.016; Fig. 1d). This mutant, however, displayed no competitive disadvantage when competing with wild-type *S. typhimurium* within GF mice, consistent with *S. typhimurium*'s sialic acid and fucose use being microbiota-dependent (day 1, p=0.26). The competitive index was not significantly different between the two colonization conditions (Supplementary Fig. 5), however this is likely due to the small amount of free sialic acid present in the GF mouse gut (see Fig. 2a).

C. difficile possesses a sialic acid catabolic operon, like *S. typhimurium*, but encodes no apparent genes for fucose consumption (Supplementary Fig. 6). To identify whether *C. difficile* also expresses sialic acid catabolism genes during its expansion within the gut, we quantified the expression of two genes within the *nan* operon, *nanE* and *nanA*, by qRT-PCR of RNA extracted from gnotobiotic mouse cecal contents. *C. difficile nanE* and *nanA* displayed elevated expression in *Bt*-monoassociated mice relative to expression levels observed when *C. difficile* colonized GF mice alone (*nanE*, 15-fold higher expression, p= 0.02; *nanA* 11-fold higher expression, p=0.039; Fig. 1e). The presence of *Bt* in the gut of gnotobiotic mice resulted in an increased density of *C. difficile* one day post-infection compared to infection of GF mice (1.5×10^8 vs. 7.9×10^8 CFU/ml; p= 0.0009; Fig. 1f).

Many commensal and pathogenic bacteria can utilize sialic acids from their hosts as a source of energy, carbon, and nitrogen[20]. However, some bacteria, such as *Bt*, encode the sialidase required to cleave and release this terminal sugar from the mucosal glycoconjugates, but lack the catabolic pathway (*i.e.*, *nan* operon) required to consume the liberated monosaccharide. Presumably, the release of sialic acids allows *Bt* to access highly coveted underlying carbohydrates in the mucus[21,22]. Conversely, *S. typhimurium* and *C. difficile* encode the *nan* operon but each lacks the sialidase required for sialic acid liberation[23,24].

We quantified levels of free sialic acids in the ceca of *Bt*-monoassociated and GF mice. *Bt*-monoassociated mice exhibited a significantly higher concentration of the common sialic acid N-acetylneuraminic acid (Neu5Ac) versus GF mice, consistent with *Bt*'s ability to liberate but not consume the monosaccharide (1059 pmoles/mg, *Bt*-associated; 188 pmoles/mg, GF; p=0.029; Fig. 2a). Colonization of mice with *Bt*-Δ*BT0455* (a mutant strain of *Bt* lacking a predicted cell surface sialidase that achieves the same density as wt *in vivo*; Supplementary Fig. 7) did not result in increased free sialic acid, nor did colonization with *Bacteroides fragilis* (*Bf*), which encodes both a sialidase and the *nan* operon and is therefore able to catabolize Neu5Ac (Fig. 2a). Expression of *S. typhimurium*'s *nan* operon was reduced upon infection of gnotobiotic mice colonized with *Bt*-Δ*BT0455* or *B. fragilis*,

consistent with *S. typhimurium*'s dependence upon elevated levels of microbiota liberated sialic acid (Fig. 2b).

Loss of *Bt*-liberated sialic acid impacts *C. difficile* in a manner similar to that observed with *S. typhimurium*. *Nan* gene expression in *C. difficile* was lower in mice colonized with the sialidase-deficient mutant *Bt*-Δ*BT0455* relative to expression in the presence of *Bt*-colonized mice (*nanE*, 75-fold higher expression, p= 0.0187; Fig. 2c). Furthermore, *C. difficile* density decreased in infected mice colonized with *Bt*-Δ*BT0455* mutant relative to densities in mice colonized with wild-type *Bt*, (9.7×10^7 vs. 4.6×10^8 CFU/ml; p= 0.0143) illustrating the importance of *Bt*-liberated sialic acid in *C. difficile* expansion *in vivo* (Fig. 2d; Supplementary Fig. 8). Free sialic acid was orally administered to *Bt*-Δ*BT0455* and *C. difficile* co-colonized mice to determine if exogenous administration of the monosaccharide could reverse the decrease in *C. difficile* density by complementing the sialidase deficiency in this model. *C. difficile* densities increased 1 day post-infection in *Bt*-Δ*BT0455* monoassociated mice fed free sialic acid compared to unsupplemented controls (4.8×10^8 vs. 6.8×10^7 CFU/ml; p=0.0066) reaching densities similar to those observed in the presence of wild-type *Bt* (Fig. 2e). Furthermore, expression of *C. difficile nanE* increases in the sialic acid-fed *Bt*-Δ*BT0455*-associated mice, further demonstrating that sialic acid use by *C. difficile* occurs concomitant with its increased densities *in vivo* (*nanE*, 58-fold higher expression over PBS-treated controls, p= 0.019; Fig. 2f). Notably, free sialic acid administration to GF mice infected with *C. difficile* resulted in higher densities of the pathogen in the lumen of the gut, confirming the important role of this monosaccharide *in vivo* (Supplementary Fig. 9). These data demonstrate that sialic acid catabolism by *C. difficile* promotes higher densities of the pathogen and depends upon the availability of the liberated monosaccharide within the lumen of the gut.

To determine if sialic acid use is relevant to pathogen proliferation in an antibiotic-treated complex microbiota, we quantified free sialic acids in the ceca of conventional mice before and after antibiotic treatment. Levels of free Neu5Ac were very low within untreated conventional mice, consistent with efficient partitioning of Neu5Ac between members of an undisturbed complex microbiota (Fig. 3a). However, antibiotic-treated mice exhibited elevated levels of free sialic acid 1 day after treatment (725 pmoles/mg 1 day post-streptomycin compared to 17 pmoles/mg in untreated mice; p=0.0019), a time frame that coincides with pathogen expansion and acute microbiota disturbance (Supplementary Fig. 10)[25]. The pool of free sialic acids decreased by day 3 post-treatment, consistent with recovery of the microbiota after antibiotic treatment[25] (Fig. 3a). *St*-Δ*nanA and St*-Δ*nanA*Δ*fucI* mutants both showed a competitive defect relative to wild-type *St* 1 day after infection in antibiotic-treated conventional mice (*St*-Δ*nanA*, CI=1.83 p=0.0095; *St*-Δ*nanA*Δ*fucI*, CI=2.77, p=0.036), consistent with sialic acid and fucose utilization providing an advantage to *S. typhimurium* during emergence (Fig. 3b). The lack of significance of the phenotype in the *fucI* single mutant suggests possible redundancy for the functional significance of the fucose catabolic pathway in this experimental model (Supplemental Fig. 11). To test whether *C. difficile* relies upon sialic acid catabolism in post-antibiotic expansion, we quantified the expression of the *nan* operon in antibiotic-treated conventional mice 1 day post-infection. Coincident with expansion of *C. difficile*, the *nan* operon was

highly induced compared to basal expression *in vitro* (*nanA*, 230-fold, p= 0.0358; *nanT*, 112-fold, p= 0.0217) confirming that *C. difficile* expresses this operon at high levels during its post-antibiotic-expansion within a complex microbiota (Fig. 3c). As a test of sialic acid catabolism importance in *C. difficile* proliferation, we constructed a *nanT*-mutant strain of *C. difficile* (*Cd-nanT*$^{-}$) that is deficient in sialic acid consumption (Supplementary Fig. 4). *Cd-nanT*$^{-}$ was significantly compromised in post-antibiotic expansion of conventional mice relative to *wt Cd* (3.1×10^7 vs. 7.0×10^7 CFU/ml; p= 0.0023) demonstrating the importance of sialic acid catabolism to *C. difficile* in attaining high densities in the context of an antibiotic-disrupted complex microbiota (Fig. 3d).

Recent studies have illustrated that enteric bacterial pathogens can subvert aspects of host inflammation to hold potential competitors within the microbiota at bay and enable pathogen proliferation[7,26–28]. Our results indicate that the antibiotic-associated pathogens *S. typhimurium* and *C. difficile* exploit increases in mucosal carbohydrate availability that occur upon disruption of the competitive ecosystem in which nutrients are typically efficiently consumed by endogenous community members. The transient post-antibiotic increase in monosaccharides liberated by the resident microbiota from host mucus provides a window of opportunity for these pathogens to expand to densities sufficient to induce self-promoting host inflammation (Supplementary Fig. 1). Implicit in these findings are new potential therapeutic strategies to combat post-antibiotic pathogen expansion.

Online-Only Methods

Bacterial Strains and Culture Conditions

B. thetaiotaomicron (ATCC 29148, also known as VPI-5482), was grown anaerobically (6% H_2, 20% CO_2, 74% N_2) overnight in TYG medium (1% tryptone, 0.5% yeast extract, 0.2% glucose, w/v) supplemented with 100 mM potassium phosphate buffer, (pH 7.2), 4.1 mM cysteine, 200 μM histidine, 6.8 μM $CaCl_2$, 140 nM $FeSO_4$, 81 μM $MgSO_4$, 4.8 mM $NaHCO_3$, 1.4 mM NaCl, 1.9 μM hematin, plus 5.8 μM Vitamin K_3.

All strains of *S. typhimurium* were derived from wild-type strain SL1344, which is naturally streptomycin-resistant. Using the methods of Datsenko and Wanner[29], mutant strains were first constructed in strain LT2, verified by PCR and then transduced into SL1344 using P22 phage transduction. Mutant strains and primers utilized in their generation are listed in Supplementary Table 2. Growth defects were not observed on glucose for either mutant, and the presence of sialic acid did not pose a toxicity issue with the *nanA* mutant as has been previously reported for *E. coli*[30] consistent with its polarity that compromises nanT expression (Supplementary Fig 5e). For colonization experiments, *S. typhimurium* strains were grown in Luria-Bertani (LB) broth at 37°C with aeration or on LB agar plates, with the appropriate antibiotics (200 μg/ml streptomycin, 30 μg/ml kanamycin). Minimal medium used for transcriptional profiling consisted of 100 mM KH_2PO_4 (pH 7.2), 15 mM NaCl, 8.5 mM $(NH_4)_2SO_4$, 4 mM L-cysteine, 1.9 mM hematin+200 mM L-histidine, 100 mM $MgCl_2$, 1.4 mM $FeSO_4$, 50 mM $CaCl_2$, 1 mg ml^{-1} vitamin K_3, and 5 ng ml^{-1} vitamin B_{12}, and 0.5% glucose (w/v). For evaluation of growth on various monosaccharides, strains were grown in M9 minimal media supplemented with 0.02% w/v histidine. Fecal densities (CFU) of *S. typhimurium* were quantified by duplicate sampling with 1 μl loops, and subsequent dilution

and spot plating on plain LB agar for gnotobiotic experiments and LB agar with streptomycin for conventional experiments.

C. difficile strain 630 was utilized in all *C. difficile* experiments and was cultured in Reinforced Clostridial Medium (RCM) + cysteine (Becton Dickinson, MD) anaerobically (6% H_2, 20% CO_2, 74% N_2). *C. difficile* growth curves were generated using minimal medium (MM) composed of ammonium sulfate, sodium carbonate, calcium chloride, magnesium chloride, manganese chloride, cobalt chloride, histidine hematin, vitamin B_{12}, vitamin K_1, $FeSO_4$, and 1% Bacto Tryptone diluted 1:1 with 1% or 0.5% carbon source. OD_{600} was monitored using a BioTek PowerWave 340 plate reader (BioTek, Winooski, VT) every 30 minutes, at 37°C anaerobically (6% H_2, 20% CO_2, 74% N_2). Fecal densities (CFU) of *C. difficile* were quantified by duplicate sampling with 1 μl loops and subsequent dilution and spot plating on blood-BHI supplemented with erythromycin. For quantification of *C. difficile* CFU in conventional mice, 1ul of feces was serially diluted in PBS and plated onto CDMN plates, composed of *Clostridium difficile* Agar Base (Oxoid) with 7% v/v of Defibrinated Horse Blood (Lampire Biological Laboratories), supplemented with 32 mg/L Moxalactam (Santa Cruz Biotechnology) and 12 mg/L Norfloxacin (Sigma-Aldrich). Plates were incubated overnight at 37°C in an anaerobic chamber (Coy). Colonies identified as *C. difficile* were validated by colony PCR.

To construct the *nanT* null mutant (*Cd-nanT*$^-$), the ClosTron method for targeted gene disruption in *C. difficile* and detailed protocol were used[31, 32]. SOEing PCRs with primers IBS, EBS1d, EBS2 and EBS (see Supplementary Table 2) were used to assemble and amplify the product for intron targeting, as outlined in the TargeTron users' manual (Sigma Aldrich). The retargeting sequence was digested with BsrGI/HindIII and cloned into pMTL007C-E2. The resulting plasmid was transformed into HB101/pRK24 for conjugation into JIR8094[33] (a generous gift from Aimee Shen) to generate *Cd-nanT*$^-$.

Reagents and Mice

Germ-free Swiss-Webster mice were maintained in gnotobiotic isolators and fed an autoclaved standard diet (Purina LabDiet 5K67) or a polysaccharide-deficient diet[34], in accordance with A-PLAC, the Stanford IACUC. All animals were 6–12 weeks of age and both genders were used. For all experiments involving *C. difficile* colonization of germ-free mice, the diet was switched to polysaccharide-deficient chow one day before inoculation with *C. difficile*. Conventional Swiss-Webster mice (RFSW, Taconic) were used for *S. typhimurium* and *C. difficile* antibiotic-treated experiments. Number of animals per group was chosen as the minimum likely required for conclusions of biological significance, established from prior experience. Randomization was not possible in the gnotobiotic setting and blinding was not applicable.

Conventional mice were orally gavaged with 20 mg streptomycin dissolved in water 24 hours before infection, and starved 18 hours before infection. Mice were infected via oral gavage of 14 hour overnight cultures of *S. typhimurium* resuspended in PBS. For single infections of gnotobiotic mice, 10^8 cfu of *S. typhimurium* were gavaged. For *S. typhimurium* competitive index experiments, pure cultures of wild-type and mutant bacteria were diluted to equal densities, mixed in a 1:1 ratio and serial diluted in PBS to a total of 10^3 cfu/200 μl.

Each mouse was orally gavaged with 200 μl of this dilution. Throughout the experiment, fecal samples were taken and dilutions were plated on LB agar plates containing streptomycin, which allows for growth of both the wild-type and mutant strains. Colonies from these plates were then patched onto LB agar + kanamycin plates to determine the proportion of kanamycin-resistant (mutant) cells. With each sample, the ratio of kanamycin-sensitive (wild-type) bacteria to kanamycin-resistant (mutant) bacteria was divided by the kan^s/kan^r ratio determined from the original inoculum to produce the competitive index. Significance was evaluated using one-sample t-tests with a theoretical mean of 1. All competitive indices were determined for fecal samples with the exception of *St*-Δ*nanA*, which was surveyed in cecal contents.

For *C. difficile* experiments involving conventional mice, antibiotics were administered in the water for 3 days, starting 6 days before inoculation including: kanamycin (0.4 mg/mL), gentamycin (0.035 mg/mL), colistin (850 U/mL), metronidazole (0.215 mg/mL) and vancomycin (0.045 mg/mL)[19]. Mice were then switched to regular water for 2 days, and administered 1mg of clindamycin by oral gavage 1 day before inoculation with *C. difficile*. Inoculations were by oral gavage at a density of 10^8 CFU from overnight cultures.

For sialic acid administration experiments, N-acetylneuraminic acid (Calbiochem or Santa Cruz Biotechnology) was administered in the water at a 1% concentration. Additionally, mice were orally gavaged 1mg of sialic acid twice a day. The amount of sialic acid in the cecal contents were calculated to equal approximately 700 pmoles/mg of cecal contents, which mirrors the average concentration of free sialic acids we quantified post-antibiotic treatment (725 pmoles/mg).

Expression analysis

Genome-wide transcriptional profiling of *S. typhimurium* was conducted using custom-made GeneChips, which contain probes for all annotated coding sequences for *S. typhimurium* LT2. RNA was purified from cecal contents and *in vitro* culture and cDNA was prepared, fragmented and labeled as described[36].

GeneChip data were RMA-MS normalized as described[37] and log2 transformed. Statistical significance for differential gene expression was determined using Significance Analysis of Microarrays (SAM)[38]. The delta parameter was adjusted to achieve a FDR nearest to 10%, and this delta value was used to select significantly-regulated genes.

qRT-PCR analysis was performed on RNA extracted from cecal or fecal contents by phenol-chloroform extraction and bead beating. Superscript II (Invitrogen) was utilized to convert RNA to cDNA, and SYBR Green (ABgene) in a MX3000P thermocycler (Strategene) was utilized. Fold changes were normalized to *in vitro* growths in Minimal Medium containing 0.5% glucose (MM-G) for *C. difficile* and LB for *S. typhimurium*.

Quantification of sialic acids

All steps were carried out at 4°C to minimize enzymatic hydrolysis. Approximately 200 mg of flash-frozen cecal contents were weighed out and resuspended in 400 μl dH_2O. Samples were vortexed for 30 minutes at max speed and centrifuged for 15 minutes at 14,000 × g in

the tabletop centrifuge. The supernatant was stored, and the pellet resuspended in an additional 400 µl dH_2O. The tubes were vortexed individually until the pellet was dispersed, and then all samples were vortexed for 30 minutes, centrifuged, and supernatants were pooled. This process was repeated once more for a total volume of approximately 1 ml. 700µl of each sample was filtered through a Pall 1K MWCO filter for 9 hours at 7,000 × g. Samples were derivatized with DMB (1,2-*d*iamino-4,5-*m*ethylene-*d*ioxybenzene) as described previously[39]. The resulting product was analyzed by reverse-phase HPLC using a C18 column (Dionex) at a flow rate of 0.9 ml/min, using a gradient of 5% to 11% acetonitrile in 7% methanol. The excitation and emission were 373 and 448 nm, respectively. The DMB-derivatized sialic acids were identified and quantified by comparing elution times and peak areas to known standards.

16S rRNA microbial community composition analysis

Fecal DNA was isolated and amplicons generated of the 16S rRNA V4 region (515F, 806R). Samples were sequenced at Medical Genome Facility, Mayo Clinic, Rochester, MN using the MiSeq (Illumina) platform[40]. Data analysis was done using QIIME[41]. Single end reads were analyzed to determine OTUs (Operational Taxonomic Units) at 97% sequence similarity using uclust. Taxonomy was assigned using RDP classifier against the GreenGenes database and a phylogenetic tree was built using FastTree. The OTU table was rarified to a sequencing depth of 900 for each set of samples. Beta diversity was determined using unweighted and weighted UniFrac[42].

Statistical analyses

The Student's t-test was used for statistical calculations, and * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. Error bars indicate SEM. *n* indicates the number of mice used per condition. Normal distribution was assumed for all data, and no deviations were noted. Grubbs' test was used to identify and eliminate statistical outliers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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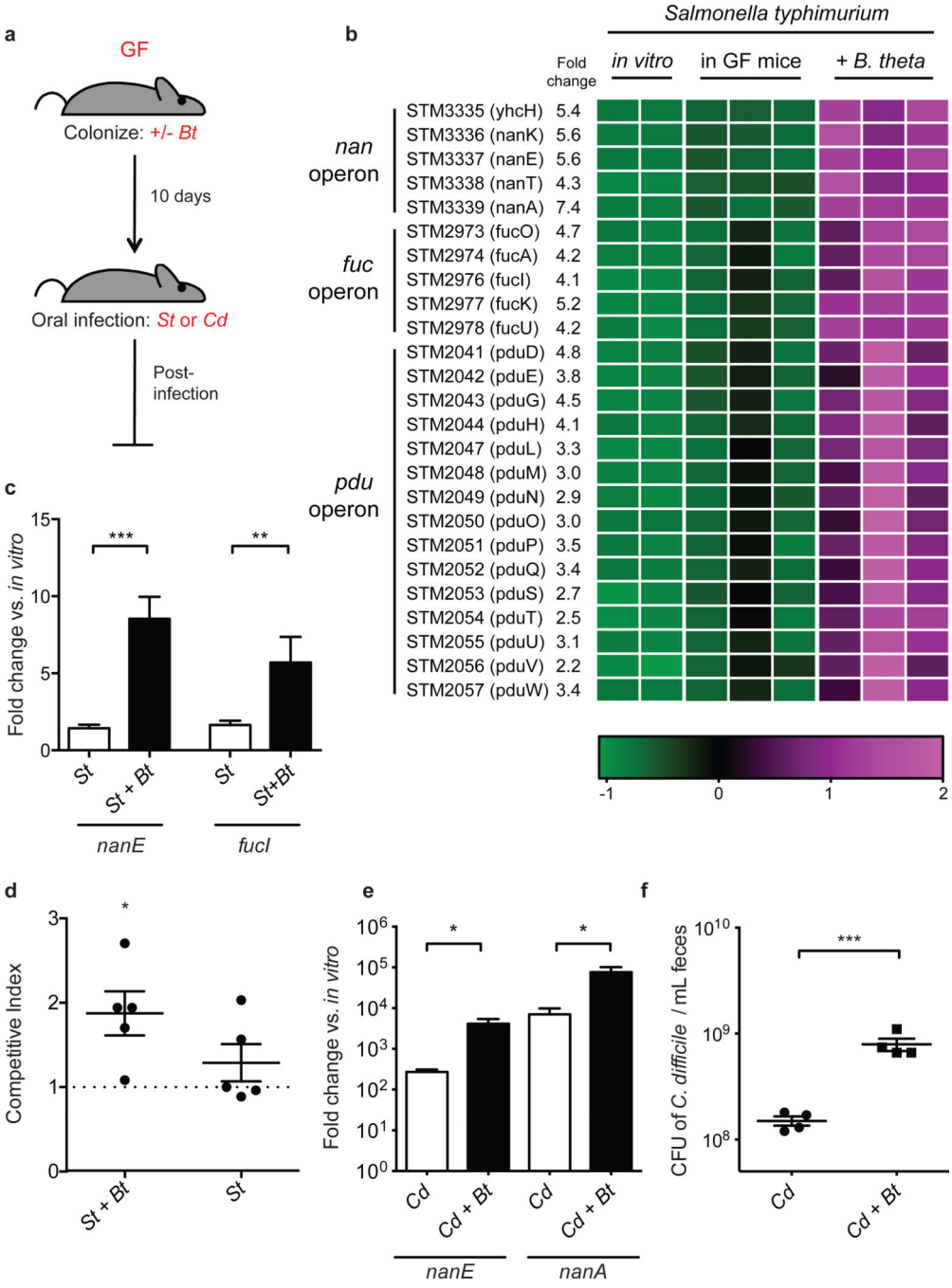

Figure 1. *Bt* facilitates *S. typhimurium* and *C. difficile* carbohydrate utilization during emergence

a, Schematic of mouse infection experiments. Germ-free, GF; *B. thetaiotaomicron*, Bt; *S. typhimurium*, St; *C. difficile*, Cd.

b, *S. typhimurium* operons displaying significant differences in gene expression levels *in vivo* in the presence and absence of *Bt*, 5 days post-infection. Colors indicate the deviation of each gene's signal above (purple) and below (green) its mean expression value across all six *in vivo* samples and duplicate *in vitro* growths conducted in minimal medium.

c, Induction of *S. typhimurium nanE* and *fucI* in cecal contents 1 day post-infection relative to growth in LB broth [n = 9 and 4 for *St* and *St+Bt*, respectively].
d, Competitive index of wild-type *St/St-ΔnanAΔfucI* in *Bt*-monoassociated (*St+Bt*) and ex-germ-free (*St*) mice 1 day post-infection. Horizontal bars indicate the geometric means of CI values, and individual CI values are represented with dots [n = 5/group].
e, Induction of *C. difficile nan* genes in cecal contents 3 days post-infection relative to growth in minimal medium containing 0.5% glucose [n = 4/group].
f, *C. difficile* density in feces 1 day post-infection [n = 4/group].
Error bars indicate SEM.

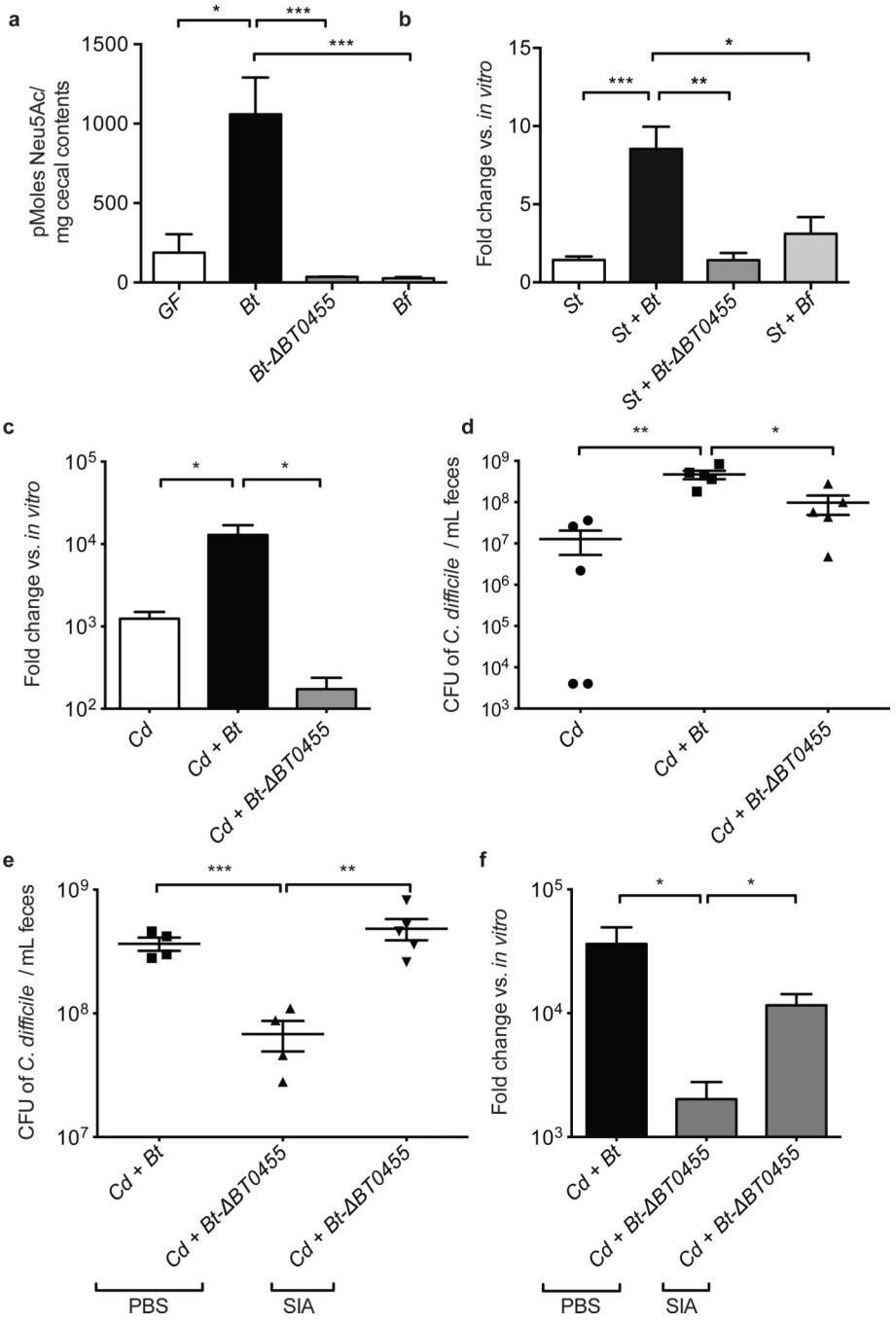

Figure 2. *Bt*-liberated sialic acid promotes emergence of *S. typhimurium* and *C. difficile*

a, Levels of free sialic acid in cecal contents in GF and gnotobiotic mice monoassociated for 10 days [n=3, 3, 5, 5, respectively].

b, Fold change of expression of *S. typhimurium nanE* in cecal contents 1 day post-infection relative to growth *in vitro* [n = 9, 4, 5, 5, respectively].

c, Induction of *C. difficile nanE* expression in cecal contents 3 days post-infection relative to growth in minimal medium containing 0.5% glucose [n = 4/group].

d, *C. difficile* density in feces 1 day post-infection [n = 5/group].

e, *C. difficile* density 1 day post-infection in feces of PBS or exogenous free sialic acid (SIA) treated mice. [n = 4–5/group].

f, Induction of *C. difficile nanE* gene expression 1 day post-infection in feces of PBS or exogenous free sialic acid (SIA) treated mice relative to growth in minimal medium containing 0.5% glucose [n = 5/group].

Error bars indicate SEM.

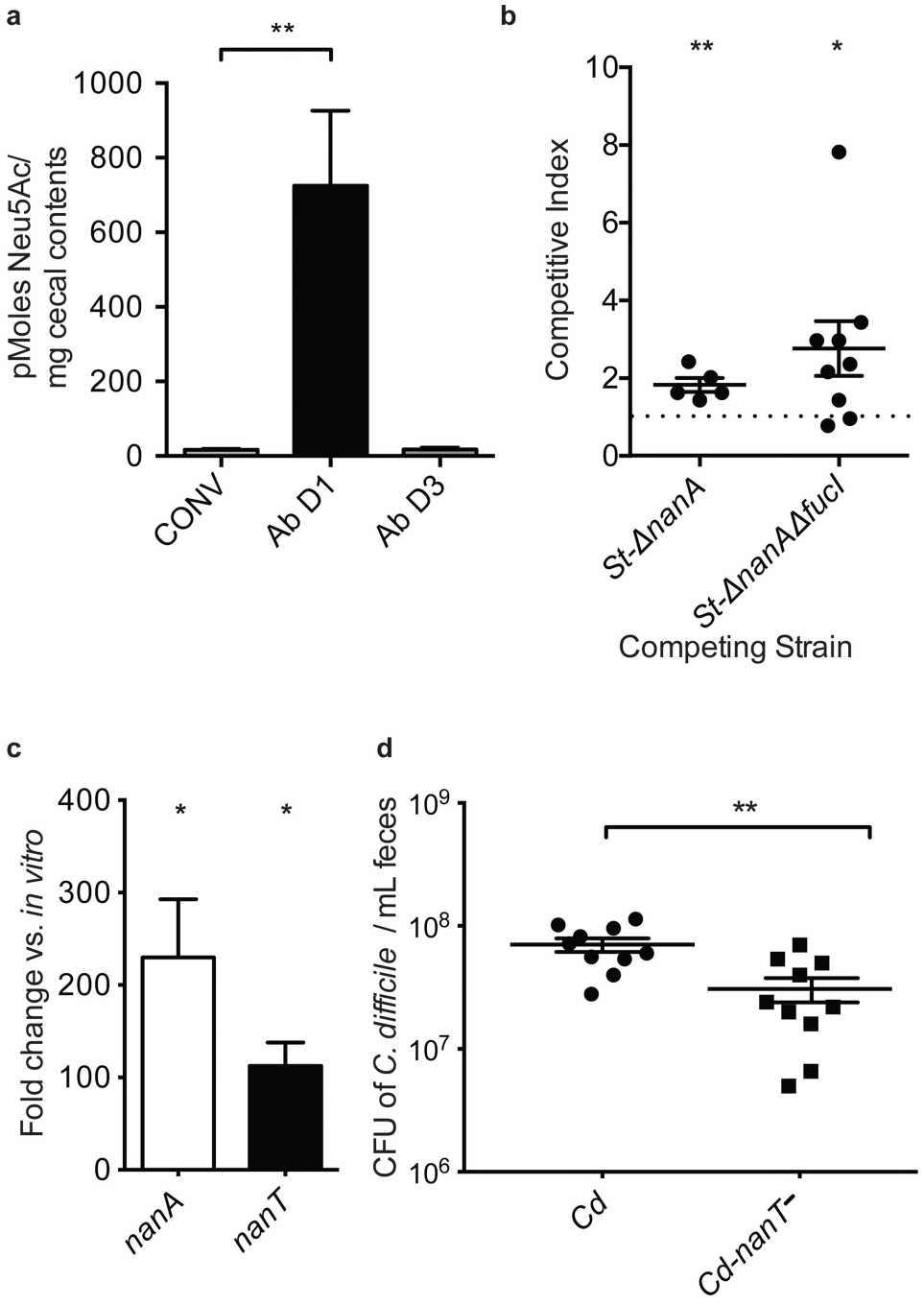

Figure 3. *S. typhimurium* and *C. difficile* utilize mucin-derived monosaccharides resulting from antibiotic treatment of conventional mice

a, Levels of free sialic acid in cecal contents of conventional mice (CONV), antibiotic-treated mice 1 day (Ab D1) and 3 days (Ab D3) post-treatment [n= 8, 9, 3, respectively]. **b**, Competitive index of wt *S. typhimurium* versus *S. typhimurium* mutants in cecal contents (*St*-Δ*nanA*) or feces (*St*-Δ*nanA*Δ*fucI*) of antibiotic-treated conventional mice. Horizontal bars indicate the geometric means of CI values, and individual CI values are represented with dots [n=5 and 9, respectively].

c, Induction of *C. difficile nanA* and *nanT* expression in fecal samples 1 day post-infection of antibiotic-treated conventional mice relative to growth in minimal medium containing 0.5% glucose [n = 4/group].

d, Density of *wt C. difficile* or a mutant deficient in sialic acid consumption (*Cd-nanT*$^{-}$) 3 days post-infection in feces of antibiotic-treated conventional mice. [n = 10/group].

Error bars indicate SEM.