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

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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 pathogens1–4. How pathogens capitalize upon the failure of microbiota-afforded protection is largely unknown. Here we show that two antibiotic-associated pathogens, Salmonella enterica serovar Typhimurium (S. typhimurium) and Clostridium difficile, use a common strategy of catabalizing 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 Bacteroides thetaiotaomicron, a model gut symbiont, reduces free sialic acid levels resulting in C. difficile downregulating its sialic acid catabolic pathway and exhibiting impaired expansion. These effects are reversed by exogenous dietary administration of free sialic acid. Furthermore, antibiotic 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 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 ecosystem5,6. Limited nutrients and high microbial densities are likely to have a key role in protecting the host against invading microbes7. Carbohydrates derived from diet or host play a well-established part in sustaining the resident members of the microbiota8–10, and more recently have been shown to have important roles in gut microbiota–pathogen dynamics11–14. 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 microbiota14,15. 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 disease16–18. 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 thetaiotaomicron (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 mice (Fig. 1a), caecal contents were collected and subjected to transcriptional profiling using a custom S. typhimurium Gene Chip. In the presence of Bt, all 59 S. typhimurium genes that displayed significantly altered expression relative to infection of germ-free mice were upregulated (Supplementary Table 1). Functional classification of these genes revealed enriched cluster of orthologous groups (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 panediol (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 germ-free or Bt-monoassociated mice, to determine whether 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 germ-free mice (nanE, 6.0-fold, P = 1.47 × 10−5; fucI, 3.5-fold, P = 0.0026) (Fig. 1c) when S. typhimurium densities and host pathology are similar between colonization states (Supplementary Figs 3 and 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 respective sugars (Supplementary Fig. 5). In competition experiments, Bt-monoassociated mice co-infected with wild-type S. typhimurium and a nanA/fucI double mutant strain (StΔnanAΔfucI) revealed that the mutant had a significant disadvantage on days 1 and 2 after infection (day 1, competitive index (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 germ-free 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 probably because of the small amount of free sialic acid present in the germ-free mouse gut (see Fig. 2a).

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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 quantitative PCR with reverse transcription (qRT–PCR) of RNA extracted from gnotobiotic mouse caecal contents. C. difficile nanE and nanA displayed elevated expression in Bt-monoassociated mice relative to expression levels observed when C. difficile colonized germ-free 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 germ-free mice (\( 1.5 \times 10^6 \) versus \( 7.9 \times 10^5 \) colony-forming units (c.f.u.) ml\(^{-1} \); \( P = 0.0009 \); 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 B. thetataotaomicron encode the sialidase required to cleave and release this terminal sugar from the mucosal glycoconjugates, but lack the catabolic pathway (that is, the nan operon) required to consume the liberated monosaccharide. Presumably, the release of sialic acid allows B. thetataotaomicron to access highly coveted under-carbohydrates in the mucus\(^{10,21}\). Conversely, S. typhimurium and C. difficile encode the nan operon but each lacks the sialidase required for sialic acid cleavage\(^{22,23}\).

We quantified levels of free sialic acids in the caeca of Bt-monoassociated and germ-free mice. Bt-monoassociated mice exhibited a significantly higher concentration of the common sialic acid N-acetylmuraminic acid (Neu5Ac) versus germ-free mice, consistent with the ability of Bt to liberate but not consume the monosaccharide (1059 pmol mg\(^{-1} \) Bt-associated; 188 pmol mg\(^{-1} \) germ-free; \( P = 0.029 \); Fig. 2a). Colonization of mice with Bt-ΔBT0455 (a mutant strain of Bt lacking a predicted cell surface sialidase that achieves the same density as wild type 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-ΔBT0455 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 affects C. difficile in a manner similar to that observed with S. typhimurium. The nan gene expression in C. difficile was lower in mice colonized with the sialidase-deficient mutant Bt-ΔBT0455 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-ΔBT0455 mutant relative to densities in mice colonized with wild-type Bt (9.7 \( \times \) 10^7 versus 4.6 \( \times \) 10^6 c.f.u. ml\(^{-1}\); \( P = 0.0143 \)), 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-ΔBT0455 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-ΔBT0455 monoassociated mice fed free sialic acid compared to unsupplemented controls (4.8 \( \times \) 10^7 versus 6.8 \( \times \) 10^6 c.f.u. ml\(^{-1}\); \( P = 0.0066 \)) reaching densities similar to those observed in the presence of wild type (Fig. 2e).

Furthermore, expression of C. difficile nanE increases in the sialic acid fed Bt-ΔBT0455-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 buffer treated controls, \( P = 0.019 \); Fig. 2f). Notably, free sialic acid administration to germ-free 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 whether sialic acid use is relevant to pathogen proliferation in an antibiotic-treated complex microbiota, we quantified free sialic acids in the caeca 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\(^{-1}\), 1 day post streptomycin, compared to 17 pmoles mg\(^{-1}\) in untreated mice; \( P = 0.0019 \)), a time frame that coincides with pathogen expansion and acute microbiota disturbance (Supplementary Fig. 10). The pool of free sialic acids decreased by day 3 post treatment, consistent with recovery of the microbiota after antibiotic treatment\(^{24} \) (Fig. 3a). St-ΔnanA and St-ΔnanAAfucl mutants both showed a competitive defect relative to wild-type S. typhimurium 1 day after infection in antibiotic-treated conventional mice (St-ΔnanA, CI = 1.83 \( P = 0.0095 \); St-ΔnanAAfucl, 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 statistical significance of the phenotype in the fucL single mutant suggests mechanisms that are compensatory for fucose catabolism in this experimental model (Supplementary 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 wild-type C. difficile (3.1 \( \times \) 10^7 versus 7.0 \( \times \) 10^6 c.f.u. ml\(^{-1}\); \( P = 0.0023 \)) 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\(^{7,25–27} \). 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.

**METHODS SUMMARY**

**Bacterial strains and culture conditions.** Strains were grown as follows: B. thetaiotaomicron (VPI-5482), TYG; S. typhimurium (SL1344), LB; C. difficile (630), RCM. For strains and primers see Supplementary Table 2.
Mice. Germ-free Swiss-Webster mice were maintained in gnotobiotic isolators, in accordance with A-PLAC, the Stanford IACUC. Conventional Swiss-Webster mice (SWRF, Taconic) were used for antibiotic-treated experiments.

Expression analyses. *S. typhimurium* transcriptomics in caecal contents were conducted using custom-made GeneChips. Robust multi-chip average-multi-species (RMA-MS) normalized signals were analysed for significant differences using significance analysis of microarrays (SAM). For primers see Supplementary Table 3.

Quantification of sialic acids. Free sialic acids were isolated from caecal content supernatants using a 1K MWCO filter and subjected to 1,2-diamino-5-methylenedioxybenzene (DMB) derivatization before HPLC analysis.

Statistical analyses. The Student’s t-test was used for statistical calculations. *P* < 0.05, **P** < 0.01 and ***P*** < 0.001. Error bars indicate s.e.m. *n* indicates the number of mice used per condition.

**Full Methods** and any associated references are available in the online version of the paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Information** Microbiota enumeration (16S rRNA) datasets have been deposited in the EMBL European Nucleotide Archive (ENA) under accession number ERP003629 and can also be found in the QIIME Database under the study ID 1958 (http://www.microbio.me/qiime/). Gene Chip datasets are available in the GEO database under accession number GSE49076. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.L.S. (sonnenburg@stanford.edu).

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METHODS

Bacterial strains and culture conditions. B. thetaiotaomicron (ATCC 29148, also known as VPI-5482), was grown anaerobically (6% H2, 20% CO2, 74% N2) 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 CaCl2, 140 mM FeSO4, 81 μM MgSO4, 4.8 mM NaHCO3, 1.4 mM NaCl, 1.9 μM haematin, plus 5.8 μM vitamin K2.

All strains of S. typhimurium were derived from wild-type strain SL1344, which is naturally streptomycin-resistant. Using the methods of Datsenko and Wanner28, mutant strains were first constructed in strain LT2, verified by PCR and then transduced into SL1344 using P22 phage transduction. Mutant strains and primers used 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 it has been previously reported for C. difficile, 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−1 streptomycin, 30 μg ml−1 kanamycin). Minimal medium used for transcriptional profiling consisted of 100 mM KH2PO4, pH 7.2, 15 mM NaCl, 8.5 mM (NH4)2SO4, 4 mM-m-cysteine, 1.9 mM haematin plus 200 mM l-histidine, 100 mM MgCl2, 1.4 mM FeSO4, 50 mM CaCl2, 1 mg ml−1 vitamin B12, 5 mM L-cysteine, 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. Fastecal densities (c.f.u.) 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 used in all C. difficile experiments and was cultured in reinforced clostridial medium (RCM) plus cysteine (Becton Dickinson) anaerobically (6% H2, 20% CO2, 74% N2). C. difficile growth curves were generated using minimal medium composed of ammonium sulphate, sodium carbonate, calcium chloride, magnesium chloride, manganese chloride, cobalt chloride, histidine hemein, vitamin B12, vitamin K2, FeSO4, and 1% Bacto Tryptone diluted 1:1 with 1% or 0.5% carbon source. D0 (D0-DO) was monitored using a BioTek PowerWave 340 plate reader (BioTek, Winooski, VT) every 30 min, at 37 °C anaerobically (6% H2, 20% CO2, 74% N2). Faecal densities (c.f.u.) of C. difficile were quantified by duplicate sampling with 1 μl loops and subsequent dilution and spot plating on brain heart infusion agar (Becton Dickinson) with 10% v/v of defibrinated horse blood (Lampire Biological Laboratories) supplemented with 25 mg l−1 erythromycin. For quantification of C. difficile c.f.u. in conventional mice, 1 ml of faeces was serially diluted in PBS and plated onto CDMN plates, composed of C. difficile agar base (Oxoid) with 7% v/v of defibrinated horse blood (Lampire Biological Laboratories), supplemented with 32 mg l−1 moxalactam (Santa Cruz Biotechnology) and 12 mg l−1 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 Clostridium strain for targeted gene disruption in C. difficile and detailed protocol were used36,37. SOEnig PCRs with primers IB5, 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 targeting sequence was digested with BsrGI/HindIII and cloned into pMTL007C-E2. The resulting plasmid was transformed into HB101/pRK24 for conjugation into JIR809432 (a generous gift from A. 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 diet33, in accordance with A-PLAC, the Stanford IACUC. Bacterial strains and culture conditions.

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−1), gentamycin (0.035 mg ml−1), colistin (850 U ml−1), metronidazole (0.215 mg ml−1) and vancomycin (0.045 mg ml−1). Mice were then switched to regular water for 2 days and administered 1 mg of clindamycin by oral gavage 1 day before inoculation with C. difficile. Inoculations were given by oral gavage at a density of 106 c.f.u. from overnight cultures.

For siac 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 1 mg of siac acid twice a day. The amount of siac acid in the caecal contents was calculated to equal approximately 700 pmol mg−1 of caecal contents, which mirrors the average concentration of free sialic acids we quantified post antibiotic treatment (725 pmol mg−1).

Expression analysis. Genome-wide transcriptional profiling of S. typhimurium was conducted using custom-made Gene Chips, which contain probes for all annotated coding sequences for S. typhimurium LT2. RNA was purified from caecal contents and in vitro culture and complementary DNA (cDNA) was prepared, fragmentated and labelled as described.

Gene Chip data were RMA-MS normalized as described and log2 transformed. Statistical significance for differential gene expression was determined using significance analysis of microarrays (SAM)35. The delta parameter was adjusted to achieve a false discovery rate (FDR) nearest to 10% and this delta value was used to select significantly-regulated genes.

qRT–PCR analysis was performed on RNA extracted from caecal or faecal contents by phenol–chloroform extraction and bead beating. Superscript II (Invitrogen) was used to convert RNA to cDNA and SYBR Green (ABgene) in a MX3000P thermocycler (Stratagene) was used. Fold changes were normalized to in vitro growths in minimal medium containing 0.5% glucose for C. difficile and S. typhimurium.

Quantification of sialic acids. All steps were carried out at 4 °C to minimize enzymatic hydrolysis. Approximately 200 mg of flash-frozen caecal contents were weighed out and resuspended in 400 μl of D2O. Samples were vortexed for 30 min at maximum speed and centrifuged for 15 min at 14,000g in a tabletop centrifuge. The supernatant was stored and the pellet resuspended in an additional 400 μl of D2O. The tubes were vortexed individually until the pellet was dispersed and then all samples were vortexed for 30 min, centrifuged, and supernatants were pooled. This process was repeated once more for a total volume of approximately 1 ml. Then 700 μl of each sample was filtered through a Pall 1K MWCO filter for 9h at 7,000 g. Samples were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) as described previously38. The resulting product was analysed by reverse-phase HPLC using a C18 column (Dionex) at a flow rate of 0.9 ml min−1, using a gradient of 5% to 11% acetonitrile in 7% methanol. The excitation and emission wavelengths were 373 and 448 nm, respectively. The DMB-derivatized siac acids were identified and quantified by comparing elution times and peak areas to known standards.

16S RNA microbial community composition analysis. Faecal DNA was isolated and amplicons generated of the 16S rRNA V4 region (515F, 806R). Samples were sequenced at the Medical Genome Facility, Mayo Clinic using the MiSeq (Illumina) platform39. Data analysis was done using QIIME40. Single-end reads were analysed to determine operational taxonomic units (OTUs) at 97% sequence similarity using uclust (http://www.drive5.com/usearch/index.html). 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 UniFrac41.

Statistical analyses. The Student’s t-test was used for statistical calculations; *P < 0.05, **P < 0.01 and ***P < 0.001. Error bars indicate s.e.m. 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.

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