Supplementary Materials for

Antimicrobial peptide resistance mediates resilience of prominent gut commensals during inflammation

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Supplementary References

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Materials and Methods

Bacterial Culture Conditions

Escherichia coli, *Salmonella enterica*, and *Citrobacter rodentium* were grown aerobically at 37°C in Luria-Bertani medium supplemented with ampicillin 10 μg/mL where indicated. *Bacteroides* species were grown anaerobically at 37°C using liquid tryptone-yeast-glucose (TYG) medium (*24*) or brain-heat-infusion (BHI; Becton Dickinson) agar supplemented with 10% horse blood (Quad Five Co.) unless otherwise indicated. All other human gut-derived species were cultured using liquid or solid TYG_S medium (*13*). For selection, ampicillin 100 μg/mL, tetracycline 2 μg/mL, gentamicin 200 μg/mL, erythromycin 25 μg/mL, or 5-fluoro-2-deoxy-uridine 200 μg/mL were added as indicated. A flexible anaerobic chamber (Coy Laboratory Products) containing 20% CO_2, 10% H_2, and 70% N_2 was used for all anaerobic microbiology steps.

Determination of Minimum Inhibitory Concentrations (MIC)

MIC determination on solid media - MICs for polymyxin B and colistin were determined in triplicate using E-test strips (Biomerieux) placed on TYG_S agar plates. E-test strips were interpreted using manufacturer's instructions. Briefly, 200 μl of liquid culture of each strain at a 600nm absorbance (A_{600}) of 0.1 was spread evenly onto a TYG_S agar plate and allowed to dry for 10 minutes, followed by placement of the E-test strip at the center of the plate. The plates were incubated at 37°C for 48 hours before interpreting. Each experiment was repeated in triplicate, the results averaged, and a standard deviation calculated. Select images are shown in fig. S1. A representative cartoon was generated

using all AMP MIC determinations (Fig. 1B); for *C. jejuni*, MIC was previously determined using an identical procedure (*19*).

MIC determinations in liquid media - The Hancock Laboratory Microtiter Broth Dilution Method (*25*) was used with minor modifications (*7*). Briefly, select strains were grown for ~48 hours in TYG_S and inoculated into 96-well microtiter plates at a starting A_{600} of 0.05, in a total volume of 100 µl medium supplemented with select concentrations of AMPs. The following AMPs were used in the MIC experiment: human cathelicidin LL-37 (AnaSpec), murine cathelicidin-related antimicrobial peptide (AnaSpec), and human alpha defensin 5 (Peptide Institute). All peptides were stored according to manufacturers' instructions. The 96-well plates were incubated at 37°C and the A_{600} monitored continuously using a microplate reader (Tecan). Each experiment was performed in triplicate. A positive growth control containing no AMP and a negative control containing no bacteria was performed with every replicate. MIC determinations were made when the positive growth control reached late log phase of growth. The MIC was taken as the lowest concentration of AMP that reduced growth (A_{600}) by more than 50% when compared to the positive growth control. All MIC determinations are shown in table S1.

Genetic Techniques

DNA purification, PCR, and restriction cloning were performed using standard methods and manufacturer's instructions. Primer sequences are provided in table S7; plasmids and strains are provided in table S8.

Preparation and Sequencing of Transposon Mutant Libraries

Transposon mutagenesis and sequencing - Whole genome transposon mutagenesis of each *Bacteroides* species was performed using protocols originally developed for *B. thetaiotaomicron* (*26*). Briefly, a modified pSAM_Bt suicide plasmid was introduced into recipient species by conjugation and trans-conjugants selected on BHI blood agar plates supplemented with erythromycin 50 μg/mL for *B. eggerthii* and 25 μg/mL for all others. After two days of growth, colonies were collected en masse, re-suspended in TYG with 10% glycerol at ~$1X10^{10}$ colony forming units (CFU)/mL, and stored at -80˚C in multiple aliquots. DNA extraction and INSeq library preparation was performed as described (*26*). Sequencing was performed on Illumina HiSeq 2000 instruments at the Yale Center for Genome Analysis. The genome insertion site and relative abundance of transposons were determined using the INSeq data analysis package (*26*) and a custom Perl pipeline (*13, 26*). The libraries consisted of between ~30,000 and ~70,000 independent mariner transposon insertions per library, corresponding to ~10 independent insertions per kilobase on average (table S2). At this density, nearly all non-essential genes are disrupted in these mutant populations (*13*). For each library, sequencing reads were normalized to counts per million (CPM) and insertion sites represented by fewer than 3 reads or located in the distal (3') 10% of each gene were discarded.

Identification of Bacteroides genes conferring AMP resistance – For each of the 5 species tested, transposon mutant populations were grown in triplicate in TYG alone or in TYG with sub-MIC concentrations of PMB. For each species the concentration of PMB was

50% of the observed MIC (table S1). Briefly, 250 mL medium was inoculated with mutant pools (Input) and grown under standard conditions until A_{600} ~1.0 was reached. To maintain exponential growth, these cultures were transferred to 250 mL of fresh medium. At A_{600} ~1.0, DNA was prepared from each of these populations (Output) and transposon-adjacent genomic fragments were identified by INSeq. All species were cultured independently and with three biological replicates. Genes significantly affecting PMB resistance were determined as described in previous publications (*13, 26*). Briefly, after calculation of the total number of insertion sites and read counts in each gene, a *Z*-test was used to identify genes whose log-transformed output:input ratios were significantly different from the overall distribution. After applying a multiple hypothesis testing correction (*13*), genes with a q-value of ≤ 0.05 were considered significantly altered from the input. PMB resistance genes were identified based on a q-value cutoff of > 0.05 in TYG alone and ≤ 0.05 in TYG with PMB (tables S3-S4). A ratio of average mapped reads in output populations (PMB / No PMB) was calculated (table S4) to differentiate between genes conferring increased or decreased fitness. A heat map was generated (Fig. 2A) using genes ordered as listed in table S3 and colored according to ratios provided in table S4. Gene homologs showing no significant change in PMB (gray; Fig. 2A) also includes genes lacking insertions in a given mutant population. The number of unique insertion sites identified in each gene in each mutant population is listed in table S4.

Construction of *B. thetaiotaomicron* Directed Mutants

A full list of primers, plasmids and strains are provided (table S7-S8).

Gene deletion - A counter-selectable allelic exchange procedure (*27*) was utilized to generate in-frame, unmarked deletions in a *B. thetaiotaomicron* VPI-5482 Δ*tdk* background (wild type). In brief, ~1,000 basepair (bp) regions flanking *BT1854* were amplified (primers 1-4 in table S7) with high-fidelity polymerase (HiFi HotStart ReadyMix, KAPA Biosystems), purified (Qiaquick PCR purification kit, Qiagen) and then joined via Splicing by Overlap Extension (SOE) PCR (primers 1 and 2) (*28*). The resulting products were purified, cloned into the pExchange-*tdk* suicide plasmid (*27*), and transformed into *E. coli* S17-1 using standard protocols to generate S17 pEx:lpxFKO. Sequence-validated constructs were introduced into *B. thetaiotaomicron* via conjugation (*27*). Trans-conjugants were initially selected for erythromycin resistance, colony purified and then counter-selected for resistance to 5-fluoro-2'-deoxyuridine (FUdR, MP Biomedicals). Resolved clones were then screened by PCR (Taq DNA polymerase, NEB) using primers 1 and 2 for the presence of the deletion allele to generate *B. thetaiotaomicron lpxF*.

Gene complementation – A previously described constitutive expression vector, *pNBU2_erm_us1311*, containing a 300 bp upstream region of *BT_1311* (sigma 70; *rpoD*) was used for complementation (*29*). Briefly, *BT1854* was PCR-amplified (primers 5 and 6), cloned as an NdeI/BamHI fragment into pNBU2_erm_us1311 and transformed into *E. coli* S17, generating S17 *pNlpxF*. A sequence-validated construct was introduced into *B. thetaiotaomicron lpxF* via conjugation, generating *B. thetaiotaomicron lpxF,lpxF*$^{+}$.

Trans-conjugants were screened by PCR (primers 7-10) to confirm the location of insertion (*30*).

Barcoded B. thetaiotaomicron strains – To enumerate isogenic strains in *in vivo* experiments, we used a published *B. thetaiotaomicron* barcoding scheme (*30*). To this end, S17 *pN:BC04* was used to transfer a barcoded construct into *B. thetaiotaomicron lpxF* generating *lpxF**. To create the barcoded complementation vector, *BT1854* was excised from *pNlpxF* using NotI/BamHI restriction sites and re-ligated into the previously described barcoded plasmid pNBU2_tet_bt1311_bc16 (*29*) to generate pNlpxF:BC16. S17 *pNlpxF:BC16* was used to transfer the barcoded complementation construct into *B. thetaiotaomicron* lpxF generating *lpxF,lpxF*$^{+}$*. All insertions sites were verified by PCR (primers 7 – 10). For clarity, barcoded and non-barcoded versions of isogenic strains are not differentiated in the main text. Barcoded versions of strains (*) were only used for animal studies and are phenotypically identical to the non-barcoded versions (data not shown) as observed in previous publications (*29, 30*).

Biochemical analysis of LpxF function

Isolation of Lipid A - B. thetaiotaomicron strains were grown in duplicate in liquid TYG medium (25 mL) to an A_{600} of ~1.0 and the cells harvested by centrifugation. Lipid A was released from the cell pellet and purified as described previously (*31*) with modification. Briefly, phospholipid was removed from the cell pellets by re-suspension in 10 mL of a single-phase Bligh/Dyer mixture (*32*) consisting of chloroform/methanol/water (1:2:0.8, v/v). After incubating for 30 minutes at room temperature, the soluble fraction was

discarded. This was repeated three additional times. The insoluble residue, containing lipid A still covalently bound to LPS, was recovered by centrifugation and subjected to hydrolysis at 100°C for one hour in 25 mM sodium acetate buffer, pH 4.5, in the presence of 1% sodium dodecyl sulfate (SDS) to cleave the Kdo-lipid A linkage (*33, 34*). The liberated lipid A was recovered by two-phase Bligh/Dyer extraction, dried using nitrogen, and stored at -20°C.

Mass Spectrometry - The purified lipid A was analyzed using Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry (Bruker 9.4T). Samples were dissolved in 200 µl chloroform and diluted with 800 µl methanol. A 25 µl aliquot was diluted further with 25 µl methanol/water (1:1, v/v). 5 µl of the final diluted samples were directly infused via Advion TriVersa NanoMate into the mass spectrometer. The spectra were acquired in both negative and positive ion modes. Structural prediction based on spectra was consistent across both ion modes. Only negative ion spectra are shown. Mass spectrometric analysis was performed at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Determination of cell surface charge by cytochrome C binding assay - To evaluate the bacterial surface charge, we used cytochrome C, a highly cationic eukaryotic protein that binds to anionic surfaces in a charge dependent manner and exhibits a characteristic absorbance. Measurements were conducted as previously described (*35*). Briefly, *B. thetaiotaomicron* strains were grown in triplicate in liquid TYG medium (20 mL) to an A_{600} of ~1.0 and the cells harvested and washed three times with buffer A (0.1M

potassium phosphate buffer with 0.01% BSA [pH-7.1]) by centrifugation. Bacterial cells were re-suspended in 1mL buffer A and normalized to A_{600} of ~6.0. A freshly prepared horse heart cytochrome C (Sigma Aldrich) solution was added to the bacterial suspension at a final concentration of 0.5 mg/mL, incubated at room temperature for 15 minutes, and pelleted by centrifugation. The supernatant absorbance was measured at 440nm in 96-well plate format using a BIOTEK Synergy HT microplate reader. The absorbance value obtained for each strain was compared to a no cell control to determine percent cytochrome C removed by the bacterial cells. The results are the average of three independent biological replicates and five technical replicates.

Quantification of PMB binding - To examine antimicrobial peptide binding, we used a previously described polymyxin B-Oregon Green 514 (PMB*) binding assay (*7*). PMB* (Invitrogen) is a fluorescent version of PMB that retains its antimicrobial properties. Briefly, *B. thetaiotaomicron* strains were grown in liquid TYG medium (20 mL) to an A_{600} of ~1.0, cells harvested and washed three times with phosphate buffered saline (PBS) using centrifugation. Bacterial cells were re-suspended in 250 µL PBS to an A_{600} of 0.5. PMB* was added to bacterial suspensions at a final concentration of 10 µg/mL and incubated at room temperature for 30 minutes. Cells were washed three times with PBS by centrifugation. For quantification, cell pellets were re-suspended in 200 µL PBS and fluorescence (485/520) and A_{600} measured in 96-well plate format using a BIOTEK Synergy HT microplate reader. Relative fluorescence units (RFU) for each strain was first subtracted from RFU of a PBS-only negative control and normalized by dividing the

calculated RFU by A_{600} measurement within each assay. Results are the average of five independent biological replicates with five technical replicates.

Visualization of PMB binding* - The above staining procedure was repeated with the addition of 600nM *4',6'* diamino-*2*-phenylindole (DAPI) to the PMB* stained cells. Fluorescent microscopy was performed at room temperature (~22°C) on a Nikon Eclipse ti-U fitted with a 100X phase-contrast objective and a Hamamatsu Orca-Flash 4.0 camera. Cells were immobilized on PBS 1% agarose pads. Images were taken with Metamorph software (MDS Analytical Technologies).

Quantification of membrane permeability - The hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (NPN) was used as described to study the permeability of the bacterial outer membrane in response to PMB (*36*). Briefly, *B. thetaiotaomicron* strains were grown in liquid TYG medium (10 mL) to an A_{600} of ~1.0, cells harvested and washed three times with phosphate buffered saline (PBS) using centrifugation. Bacterial cells were normalized to A_{600} 0.5 in a solution of 5mM *4*-(2-hydroxyethyl)-*1*-piperazineethanesulfonic acid (HEPES) (pH 7.2) with 5μM carbonyl cyanide *m*-chlorophenylhydrazone (an uncoupler). Fluorescence was measured every 5s for the duration of the assay (140s) at an excitation wavelength of 350nm and an emission wavelength of 420nm in 96-well plate format using a BIOTEK Synergy HT microplate reader. At 20s, NPN was added to a final concentration of 10 μM to obtain baseline fluorescence. PMB was added at 80s at concentrations of 0,1,10, and 100 μg/mL. Time

course traces shown (Fig. 2F and fig. S3B) are representative experiments from three independent biological replicates with ten technical replicates.

Gnotobiotic animal experiments

All experiments using mice were performed using protocols approved by the Yale University Institutional Animal Care and Use Committee. Germfree 8-12 week old C57BL/6J or Swiss Webster mice were maintained in flexible plastic gnotobiotic isolators with a 12-hr light/dark cycle and provided a standard, autoclaved mouse chow (5K67 LabDiet, Purina) *ad libitum*.

B. thetaiotaomicron competition experiments - Individually caged germfree C57BL/6J mice were colonized by oral gavage with 1 x 10^8 CFU of a 1:1:1 mixture of *B. thetaiotaomicron* wild type*, *lpxF**, and *lpxF,lpxF*$^+$* (Day 1). Mice (n = 5/group) were given 1 x 10^8 CFU of *C. rodentium* wild type or *tir* mutant by oral gavage on day 7, provided 3% dextran sulfate sodium (MP Biomedicals) in drinking water *ad libitum* from day 7 to day 14, or left untreated. Animals were sacrificed at day 28. The relative abundance of each strain was determined, at the indicated time-points, from fecal DNA purified as described (*37*) by qPCR of unique DNA sequences. For characterization of colitis, the above experiment was repeated as described but with animals sacrificed at various timepoints during infection (n = 3 to 6 mice / group). Individual mouse data shown as percent of total genomic DNA is provided in table S5.

Defined community experiments - Individually caged germfree C57BL/6J mice were colonized with 1 x 10^9 CFU of an equal mixture of a 14 member human commensal community described in Figure 4A and 4B. Mice were divided into two groups (n = 5/group). In the first group, *B. thetaiotaomicron* wild type* was included in the 14 member community and in the second group this strain was replaced with the *B. thetaiotaomicron lpxF** mutant. Colitis was induced in both groups at day 7 by introducing *C. rodentium* (1 x 10^8 CFU) by oral gavage and the relative abundance of all bacteria monitored at indicated time-points by qPCR of purified fecal DNA using barcode or species-specific primers (table S7) (*13, 30*). Animals were sacrificed on day 42. qPCR was performed as described (*13, 30*) using a CFX96 instrument (BioRad) and SYBR FAST universal master mix (KAPA Biosystems). Mean strain quantities were calculated using a standard curve and relative changes were calculated with the efficiency-corrected ΔCq method (*38*). All statistical comparisons were performed using Prism 5.0 software. Control communities shown in figure S6B-C were prepared and monitored as above without *C. rodentium* infection. For principal coordinates analysis of defined human gut microbial communities in gnotobiotic mice, Hellinger distance calculations and principal coordinates analysis were performed in QIIME 1.7.0 (*39*) from qPCR-based relative abundance data. Individual mouse data shown as percent of total genomic DNA is provided in table S6.

Segmented Filamentous Bacteria (SFB) monoassociation experiments - Individually caged 8 week old germfree Swiss Webster mice were either untreated or colonized by oral gavage with 200ul of fecal homogenates from SFB- monoassociated gnotobiotic

mice. SFB colonization was confirmed using a species specific PCR (*40*). After 8 weeks, all mice were colonized by oral gavage with 1 x 10^8 CFU of a 1:1:1 mixture of *B. thetaiotaomicron* wild type*, *lpxF**, and *lpxF,lpxF*$^+$* (Day 1). At each time point, bacterial colonization levels were assessed by qPCR from fecal DNA using strain specific primers (table S7). Statistical analysis and qPCR–based abundance was determined as described above.

Conventional mouse experiments

Individually caged 8 – 10 week old SPF *Rag*$^{-/-}$ mice (Jackson Laboratories) were colonized by oral gavage with 1 x 10^9 CFU of either *B. thetaiotaomicron* wildtype* or *lpxF**. These two groups of mice were again evenly divided and left untreated or treated with *C. rodentium* as described above (n = 5/group). At each time point, bacterial colonization levels were assessed by qPCR from fecal DNA using strain specific primers (table S7). Statistical analysis and qPCR–based abundance was determined as described above.

Analysis of host inflammation

Histopathology - Colons were excised from experimental mice at the ileocecocolic junction and rectum. A 2mm section of each colon (proximal) was removed and placed in 1mL RNA later (Qiagen), an additional 1cm was removed for explant culture and enzyme-linked immunosorbent assay (ELISA). The remaining tissue was placed in a 10% Formalin solution for processing, embedded lengthwise in paraffin, and serially sectioned at the level of the lumen (5μm). The sectioned tissue was stained with Hematoxylin and

Eosin, followed by placement of coverslips by routine histologic methods. Colons were evaluated and assigned scores by investigators blinded to experimental manipulation. Each section was evaluated for pathological changes in the mucosa, submucosa, muscularis externa, and serosa, including inflammation (location and extent), edema, ulceration, hyperplasia, and crypt loss. Slides were assessed at low/high power and assigned scores of severity for tissue changes by semi-quantitative criterion-based method adapted from published methods (*41*). Severity scores ranged from 0 to 5 as follows: 0, within normal limits or absent; 1, minimal; 2, mild; 3, moderate; 4, marked; and 5, severe. Digital light microscopic images were recorded with a Axio 1 imager A1 microscope, AxioCam MRc5 camera and AxioVision 4.8 imaging software (Carl Zeiss Microimaging), and optimized in Adobe Photoshop C5.

Gene expression analysis - Tissue samples were disrupted in 1mL RNAase free water using a bead beater (Biospec). RNA was extracted from tissue homogenates using a standard Trizol RNA extraction protocol. After first-strand synthesis was performed with a high capacity cDNA reverse-transcriptase kit (Applied Biosystems), quantitative real-time PCR was done in 96-well optical plates using an CFX96 instrument (BioRad) with manufacturer recommended gene specific TAQman primers (*Tnfα* [Mm00443260_g1], *Cxcl1* [Mm04207460_m1], *Il1β* [Mm00434228_m1], *Il6* [Mm00446190_m1], *Il18* [Mm00434225_m1], *Cramp* [Mm00438285_m1, *Defa5* [Mm00651548_g1], and *Hprt1* [Mm00446968_m1]; Life Technologies). Relative expression was quantified by the change in cycle threshold method ($\Delta\Delta C_T$) as follows: (C_T of target gene expression in test sample - C_T of target gene expression in control sample) – (C_T of reference gene in test

sample - C_T of reference gene in control sample), where the control samples were untreated mice. All results were normalized to *Hprt1*, quantified in parallel amplification reactions during each qRT-PCR and presented as the fold change compared to control samples set to an expression index of 1.

mCRAMP quantification from colonic explants - Feces were removed from 1cm of the proximal colon, the tissue washed with PBS, weighed, and then cut in half longitudinally. The colon sections were placed into 500mL of Roswell Park Memorial Institute (RPMI)-1640 media (Sigma) with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 µg/mL). Colonic explants were incubated at 37°C in 5% CO_2. Supernatants were harvested after 24 hours and the concentration of secreted mCRAMP determined by ELISA (MyBioSource; catalog number MBS705604). The ELISA was performed according to the manufacturer's protocol. All results were normalized to the weight of the colonic explant and performed in duplicate.

In vitro growth curves and competitions

Competitions - *C. rodentium and B. thetaiotaomicron* strains were grown anaerobically in TYG medium to an A_{600} of ~1.0, harvested by centrifugation, and normalized to an A_{600} of 0.05 in TYG medium. *B. thetaiotaomicron* wildtype*, *lpxF**, and *lpxF,lpxF+** were mixed in equal ratios and allowed to grow for 24 hours under standard growth conditions in the presence of PMB (500 µg/mL), hLL37 (50 µg/mL), mCRAMP (50 µg/mL), aDEF5 (25 µg/mL), or *C. rodentium* (1 x 10^2 CFU). Cultures were harvested and the relative abundance of each strain determined by strain specific qPCR. Twelve technical

replicates were performed and results reported as mean ratio with standard deviation of select strain to *B. thetaiotaomicron* wildtype*.

DSS growth curves - *B. thetaiotaomicron* strains were grown anaerobically in TYG medium to an A_{600} of ~1.0, harvested by centrifugation, and normalized to an A_{600} of 0.05 in TYG medium supplemented with 0 or 3% dextran sulfate sodium. Strains were allowed to grow independently for 24 hours with continuous A_{600} measurement. Twelve technical replicates were performed with similar results.

Identification of LpxF Orthologs in Human Gut Bacteroidetes

Genome sequences for 313 non-redundant gut microbial species, including 58 distinct Bacteroidetes species, were retrieved from GenBank (May 2013) to generate a custom database (*29*). Pairwise BlastP searches were performed between the proteomes of all of the Bacteroidetes species (E value cutoff of 1e-10). A custom Perl script was used to identify reciprocal best blast hits (e.g., shared orthologs) between the entire proteome of *B. thetaiotaomicron* and the other members of the Bacteroidetes. Additionally, 13 universally conserved core genes previously identified in these 58 Bacteroidetes (*29*) were utilized to generate a species phylogeny. The amino acid sequences for each core gene set was extracted from the database and individually aligned with Muscle (*42*). The alignments were concatenated and analyzed by RAxML to generate a maximum likelihood phylogenetic reconstruction using a general time reversible model and default bootstrap settings (*43*).

Quantification of PMB resistance in gut commensals captured directly from humans

All human studies were conducted with the permission of the Yale Human Investigation Committee.

Sample collection and storage - A single fecal sample was collected from each human donor and stored on ice packs for less than 12 hours prior to transport into an anaerobic chamber (Coy Laboratory Products) and homogenization in pre-reduced, nonselective Gut Microbiota Medium (GMM) containing 20% glycerol (*44*). Samples were aliquoted anaerobically into 0.5mL volumes in 1.8 mL glass E-Z vials (Wheaton Industries), and stored at -80°C as described (*44*).

PMB resistance profiling – Archived fecal samples from each donor were plated anaerobically on TYG_S agar containing PMB at 0, 1, 10, 100, and 1000 μg/mL, such that greater than 1000 individual colonies per condition could be collected after three days of anaerobic growth at 37˚C.

DNA library preparation and sequencing - For each PMB concentration from each donor, colonies were collected *en masse* into 500 μL Tris-EDTA. 100 μL of the cell suspension was then added to 500 μL CP buffer (Omega Biotek), 250 μL 20% SDS, 200 μL 0.1 mm zirconia/silica beads (BioSpek Products), 1 5/32-inch diameter steel ball (McMaster-Carr), and 550 μL phenol/chloroform/isoamyl alcohol (24:24:1, pH 8). Cells were lysed by mechanical disruption with a bead beater (Biospec) set for 2 minutes on high setting at room temperature, followed by 2 minutes on ice and an additional 2

minutes on high setting at room temperature. After centrifugation (3220 rcf, 4°C, 10 minutes), 200 µL of the aqueous phase was removed and column purified (E-Z 96 Cycle-Pure Kit, Omega Biotek). 10 ng of purified DNA was PCR amplified in duplicate using dual barcoded primers targeting the 16S V4 region as described (*45*). PCR products were cleaned and normalized using a SequalPrep kit (Invitrogen) and pooled into groups of ~280, plus a PhiX spike-in at 15%. Samples were sequenced with a paired-end 2x250bp, dual 8bp indexing protocol at 12 pM concentration on an Illumina MiSeq instrument running Version 2 sequencing chemistry and CASAVA1.8.2 software at the Yale Center for Genome Analysis. Bacterial 16S rRNA reads have been deposited with the European Bioinformatics Institute under accession PRJEB7697.

Analysis of 16S sequencing reads – Paired end sequencing reads were preprocessed using a custom Perl script to assemble paired-end reads using PANDA-seq (*46*) with a 175bp minimum overlap requirement. Quality scores were assigned such that for consensus bases in agreement between forward and reverse sequencing reads, the higher quality score was assigned; for conflicting base calls, the nucleotide (and associated quality score) of the higher-quality base was assigned. Dual barcodes corresponding to successfully assembled reads were concatenated prior to downstream analysis using QIIME 1.7.0 (*39*). Sequences were assigned to samples using split_libraries_fastq.py with a quality score (-q) cutoff of 20 and clustered into operational taxonomic units (OTUs) at 97% identity using an open reference method. Taxonomy was assigned using the Greengenes gg_13_5_otus reference database (*47*) and resulting OTU tables rarefied to 5000 sequences per sample and singletons excluded prior to further analysis. The

portion of each complete, uncultured community represented in culture was calculated by comparing the uncultured fecal samples from each donor to the corresponding 0 μg/mL PMB cultured samples as described (*44*). To identify phylum-level trends in PMB resistance, both unweighted (OTU count) and weighted (relative abundance) analyses were used. For the unweighted analysis, OTUs assigned to each donor were filtered to exclude those represented by a single read in total across all of the 5 culture conditions (0, 1, 10, 100, and 1000 μg/mL PMB). For each donor, phylum, and PMB concentration, the number of OTUs observed was normalized to the number of OTUs observed in that phylum in the 0 μg/mL PMB culture condition. For the weighted analysis, OTUs assigned to each donor were filtered to exclude those belonging to phyla represented at <1% total abundance in culture in the absence of PMB. For each donor, phylum, and PMB concentration, the relative abundance of reads was normalized to the relative abundance observed in the 0 μg/mL PMB culture condition.

Statistical analysis

Plotting of data and statistical analysis were performed using Graphpad Prism software. Unless otherwise stated, statistical significance was determined by the unpaired two-tailed Student's t test, or one-way ANOVA with Tukey post-test. Unless otherwise indicated, differences were considered statistically significant if the *p*-value was <0.05.

Figure S1

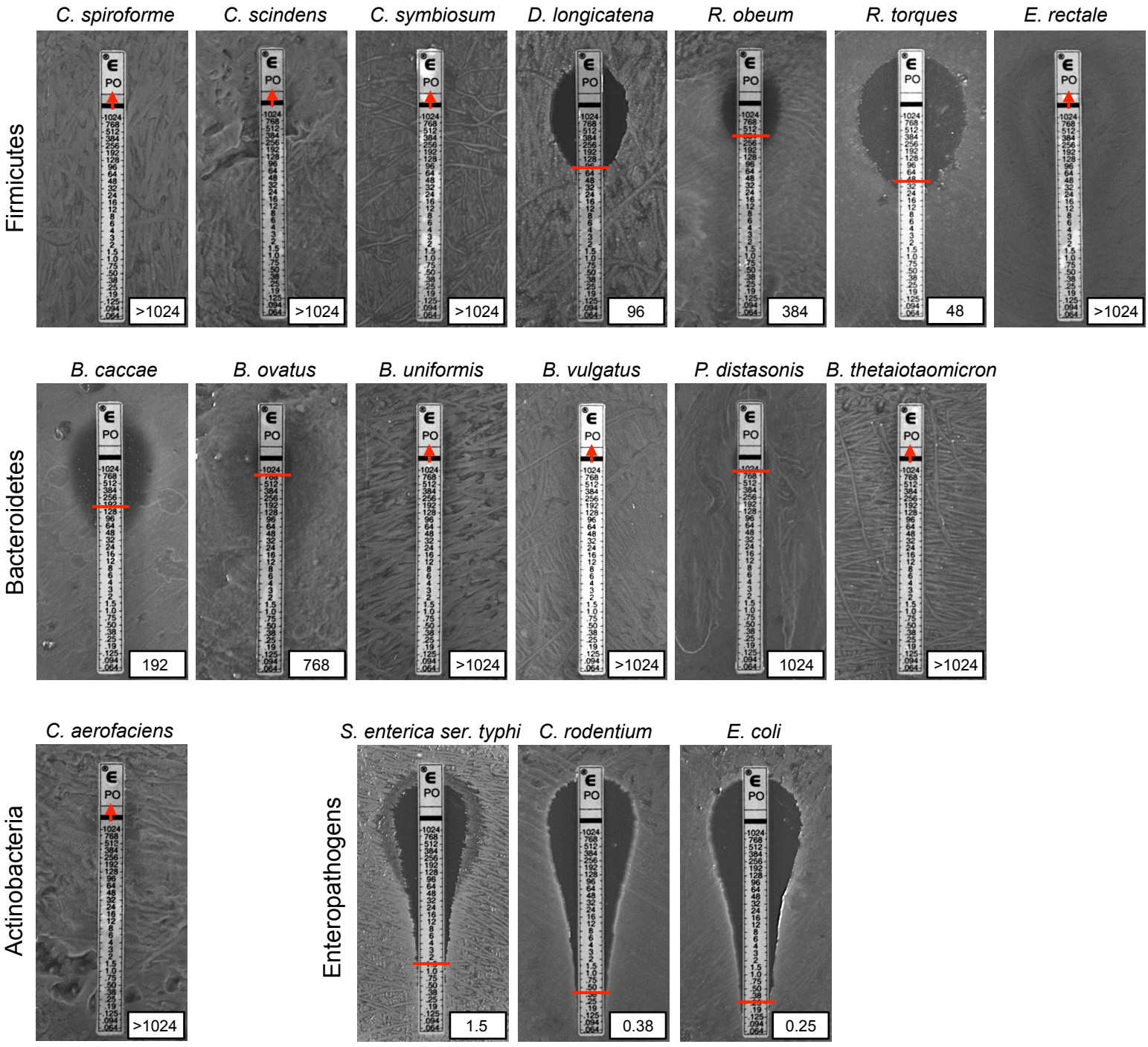

Firmicutes
C. spiroforme
>1024
C. scindens
>1024
C. symbiosum
>1024
D. longicatena
96
R. obeum
384
R. torques
48
E. rectale
>1024
Bacteroidetes
B. caccae
192
B. ovatus
768
B. uniformis
>1024
B. vulgatus
>1024
P. distasonis
1024
B. thetaiotaomicron
>1024
Actinobacteria
C. aerofaciens
>1024
Enteropathogens
S. enterica ser. typhi
1.5
C. rodentium
0.38
E. coli
0.25

Figure S1.

Representative E-test strip assays for Minimum Inhibitory Concentration (MIC) of polymyxin B against human gut commensal bacteria and enteropathogens. PMB is a bacterial derived polypeptide that, like many mammalian derived, inflammation-induced cationic AMPs, functions by transmembrane pore formation resulting in hypoosmotic lysis of the target cell (*48, 49*). MICs were determined with E-test strips (Biomerieux) using manufacturers instructions. The E-test strips are inoculated with a gradient of PMB and the MIC defined as the intersection of bacterial growth along the length of the test strip. Images show representative assays. Not all species tested are shown. A red line indicates the bacterial growth – E-test strip intersection and the MIC (μg/mL) shown in a white box in each image. Any intersection above 1024 (red arrow) is defined as > 1024 μg/mL. Mean and standard deviation are provided in table S1.

Figure S2

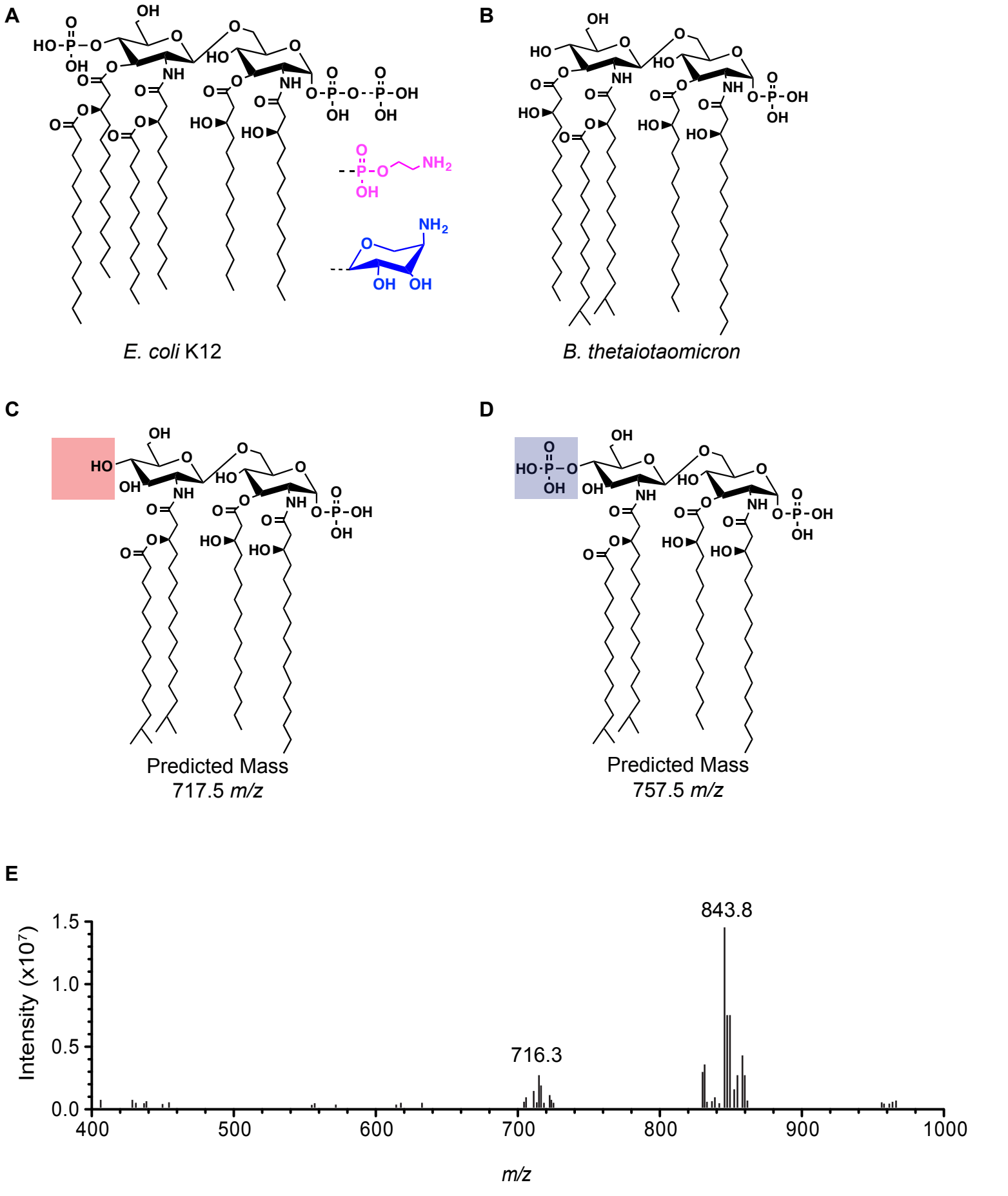

A
B
E. coli K12
B. thetaiotaomicron
C
D
Predicted Mass
717.5 m/z
Predicted Mass
757.5 m/z
E
843.8
716.3
Intensity (x10⁷)
m/z

Figure S2.

LpxF is required for AMP resistance and lipid A dephosphorylation in *B. thetaiotaomicron.* (A) The conserved lipid A structure found in most gram-negative bacteria is typified by that of *E. coli* K12, which expresses a hexa-acylated disaccharide of glucosamine with phosphate groups at the 1′- and 4′-positions (*50*). An additional phosphate group can be attached at the 1′-position (dashed bonds). This *bis*-phosphorylated lipid A structure, although required for cytoplasmic *de-novo* assembly of LPS, displays negatively charged phosphate moieties that represent a high-affinity target for cationic AMPs. Certain bacterial pathogens mask these negatively charged phosphate groups by adding positively charged substituents, such as phosphoethanolamine (magenta) or L-4-aminoarabinose (blue) shown to modulate innate immune recognition and resistance to AMPs (*51, 52*). (B) Structural characterization of the lipid A from a variety of commensal Bacteroidetes (fig. S7) demonstrates a shared under-phosphorylated structure within this phylum, typified by the structure of *B. thetaiotaomicron*. In this species, the major lipid A structure is a penta-acylated disaccharide of glucosamine with a single phosphate at the 1-position (*10*). In addition, the lipid A of *B. thetaiotaomicron* has unusually long (15 to 17 carbons) branched acyl-chains when compared to that of *E. coli* K12 (12 to 14 carbons). Notably, *B. thetaiotaomicron* encodes a canonical tetraacyldisaccharide (lipid A) 4′-kinase (BT1880; LpxK) that is likely an essential protein (*13*) necessary for LPS assembly and cell viability, yet the mature lipid A structure of *B. thetaiotaomicron* and other Bacteroidetes (fig. S7) is under-phosphorylated compared to *E. coli* (*10, 53*). (C) The minor peak (*m/z* 716) observed in MS analysis of lipid A purified from *B. thetaiotaomicron* wildtype and

lpxF,lpxF^{+} strains is consistent with the structure and *m/z* value of the doubly deprotonated $[M-2H]^{2-}$ ion for monophosphorylated tetra-acylated lipid A. (D) The minor peak (*m/z* 756) observed in MS analysis of lipid A purified from *B. thetaiotaomicron lpxF* is consistent with the structure and *m/z* value of the doubly deprotonated $[M-2H]^{2-}$ ion for *bis*-phosphorylated tetra-acylated lipid A. (E) FT-ICR MS analysis of lipid A isolated from the complemented (*lpxF,lpxF*$^{+}$) *B. thetaiotaomicron* strain.

Figure S3

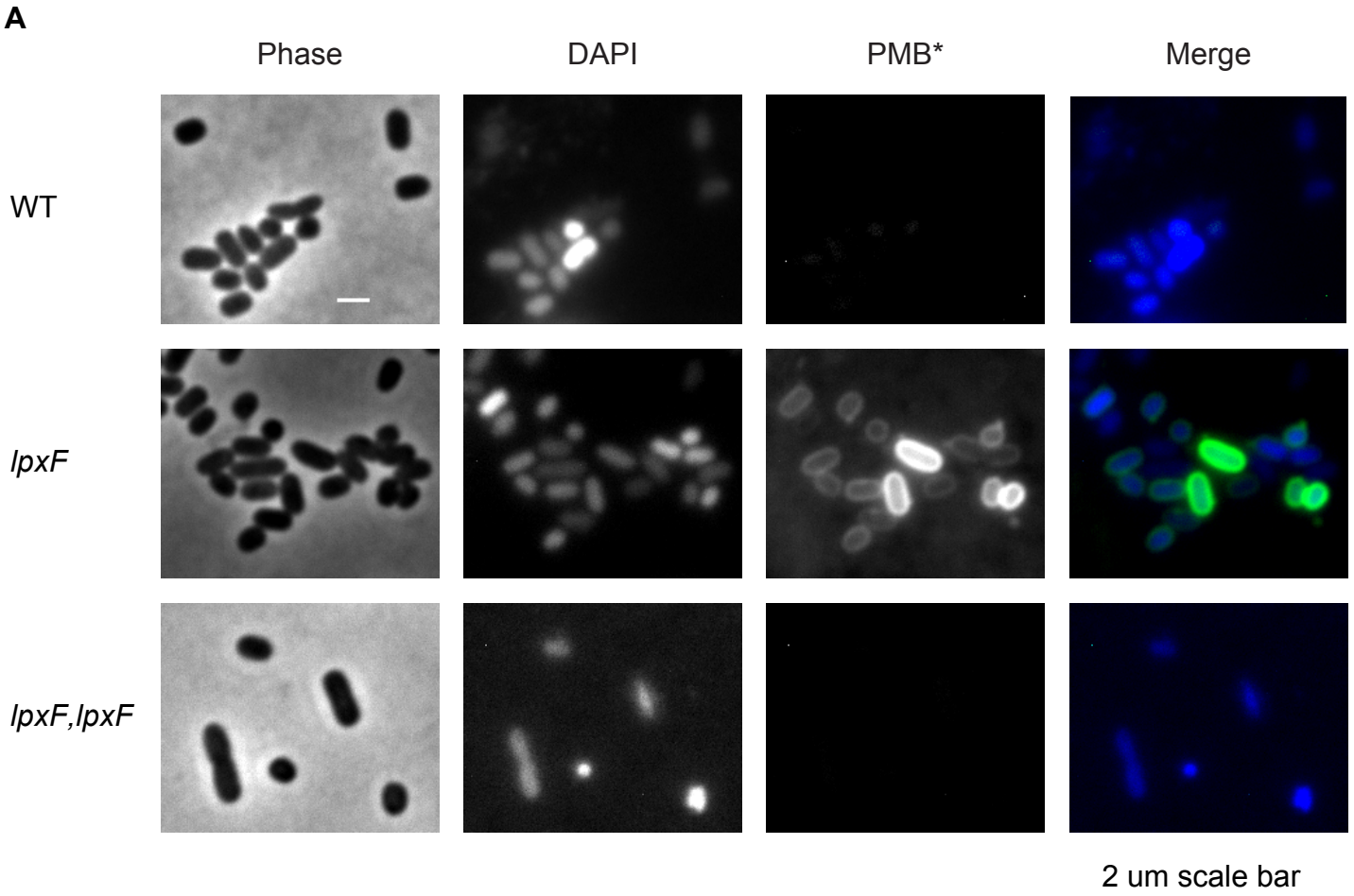
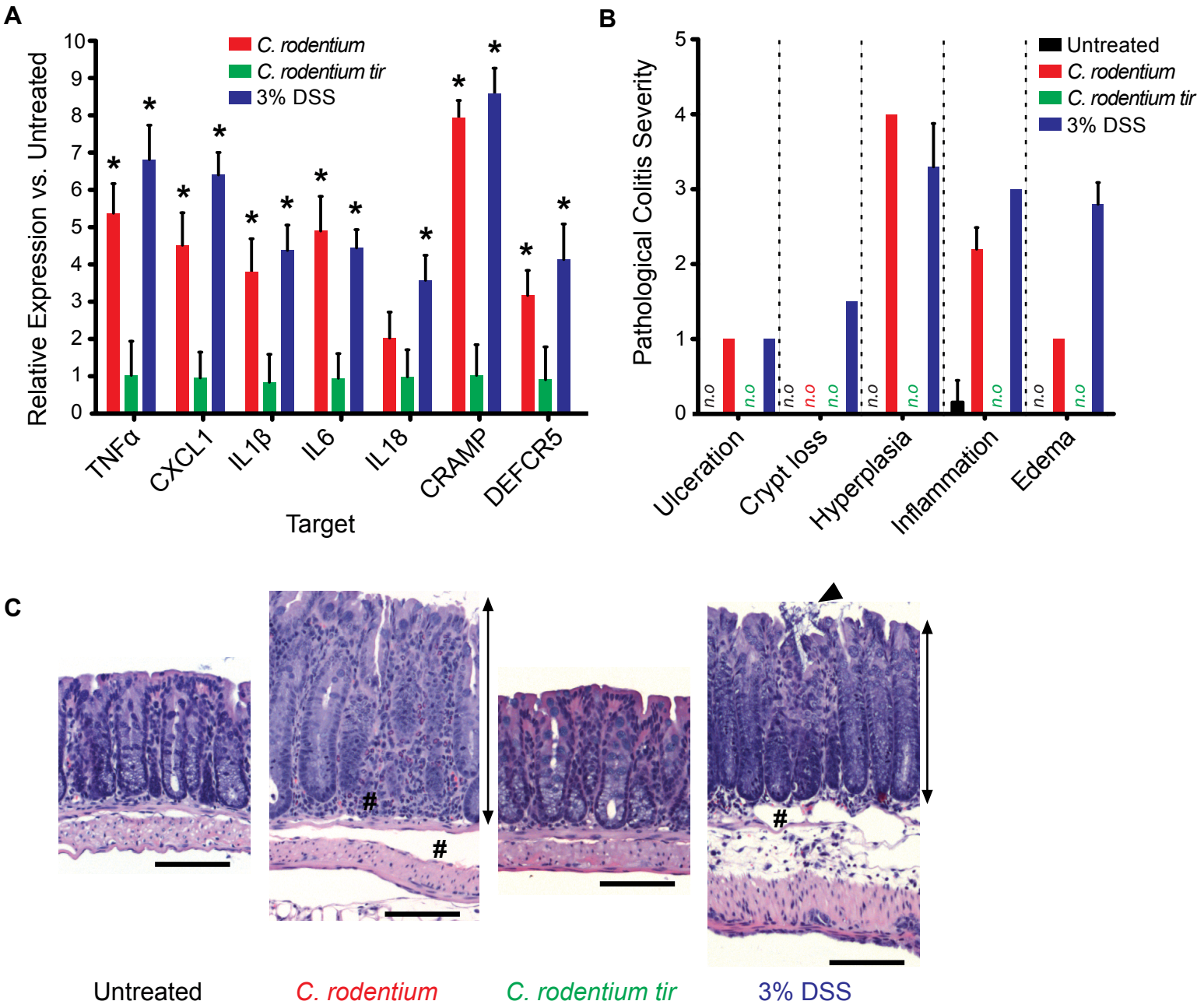

A
Phase
DAPI
PMB*
Merge
WT
lpxF
lpxF,lpxF
2 um scale bar
B
RFU (x1000)
20
10
0
NPN
PMB
0
20
40
60
80
100
120
140
Time (s)
PMB (ug/ml)
100
10
1
0

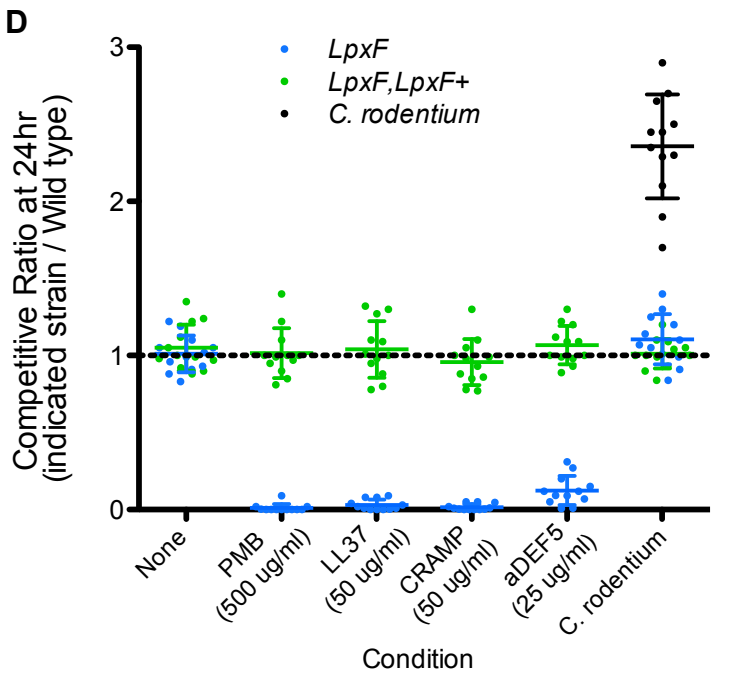
Figure S3.

Loss of LpxF alters PMB binding and membrane permeability.

(A) Fluorescence microscopy of wildtype, *lpxF* mutant, and complemented *lpxF,lpxF*$^{+}$ *B. thetaiotaomicron* strains incubated with PMB-Oregon Green (PMB*). Representative images of bacterial cells incubated in 10 μg/mL PMB* for 30 minutes are shown under phase contrast and with fluorescent filters for DAPI and PMB*. A merged overlay image is shown for DAPI and PMB*. (B) NPN uptake profile of the *B. thetaiotaomicron lpxF,lpxF*$^{+}$ strain was measured followed by challenge with the indicated concentration of PMB. Arrowheads indicate the addition of NPN (20s) and PMB (80s). Readings were taken in 5s intervals from 0s to 140s. Each experiment was performed three times, and results of a representative experiment are shown.

Figure S4


A
Relative Expression vs. Untreated
C. rodentium
C. rodentium tir
3% DSS
TNFα
CXCL1
IL1β
IL6
IL18
CRAMP
DEFCR5
Target
B
Pathological Colitis Severity
Untreated
C. rodentium
C. rodentium tir
3% DSS
n.o
Ulceration
Crypt loss
Hyperplasia
Inflammation
Edema
C
Untreated
C. rodentium
C. rodentium tir
3% DSS


D
LpxF
LpxF,LpxF+
C. rodentium
Competitive Ratio at 24hr
(indicated strain / Wild type)
None
PMB (500 ug/ml)
LL37 (50 ug/ml)
CRAMP (50 ug/ml)
aDEF5 (25 ug/ml)
C. rodentium
Condition

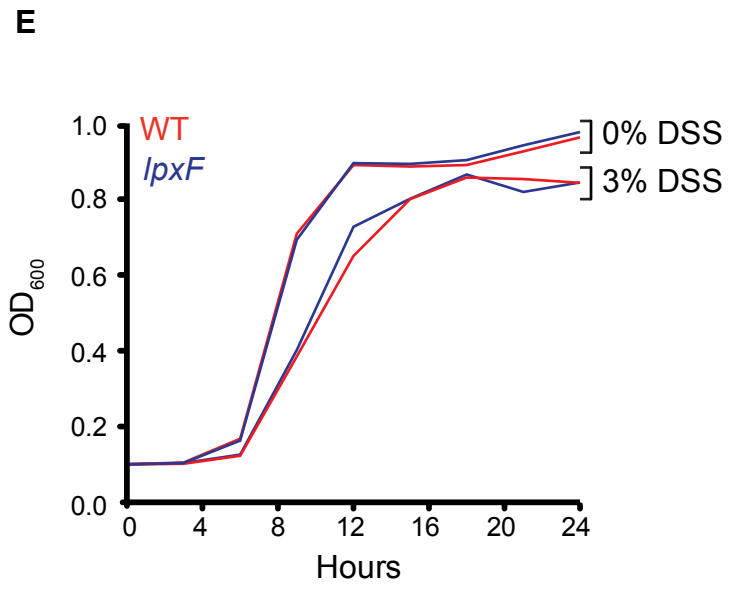

E
WT
lpxF
0% DSS
3% DSS
OD600
Hours

Figure S4.

Gnotobiotic mice harboring a single gut commensal species exhibit multiple signs of inflammation in response to pathogenic and chemical agents. Gnotobiotic mice (*n*=5/group) were monoassociated (day 1) with *B. thetaiotaomicron* (wildtype*, *lpxF**, and *lpxF,lpxF*$^{+}$* strains) 7d prior to infection with wildtype or *tir* mutant *C. rodentium,* exposure to 3% DSS, or no further treatment (untreated control). Colons were harvested at d14 for analysis. (A) Gene expression of pro-inflammatory cytokines (TNFα, CXCL1, IL1β, IL6, IL18) and inflammation associated AMPs (CRAMP, DEFCR5) in the proximal colon in gnotobiotic mice exposed to each treatment condition. Gene expression was quantified by qRT-PCR, normalized to *Hprt1* expression, and reported relative to control animals. Significant changes in expression are indicated with asterisks ($p < 0.01$). (B) Blinded, quantitative histopathologic review of colitis severity in colon samples. Specific colitis parameters are scored from 0 (absent) to 5 (most severe) and reported as the average with standard deviation. Groups with uniform scores of 0 are marked as "none observed" (n.o.). (C) Representative images of H&E stained colons from *C. rodentium* and DSS-treated gnotobiotic mice carrying *B. thetaiotaomicron* indicates significant mucosal crypt hyperplasia (double arrows), inflammation and edema (#), ulceration (arrowhead), and bleeding (not shown) along the length of the colon. Tissue from untreated and *C. rodentium tir*-infected *B. thetaiotaomicron*-monoassociated gnotobiotic mice does not display these inflammatory features. Scale bars indicate 100 μm. (D) Competitive ratio of select *B. thetaiotaomicron* strains after 24hr *in vitro* growth in the presence of AMPs or *C. rodentium*. The relative abundance of each strain was

determined by strain specific qPCR from purified genomic DNA. (E) *In vitro* growth curve of select *B. thetaiotaomicron* strains in the presence of 0 or 3% DSS.

Figure S5

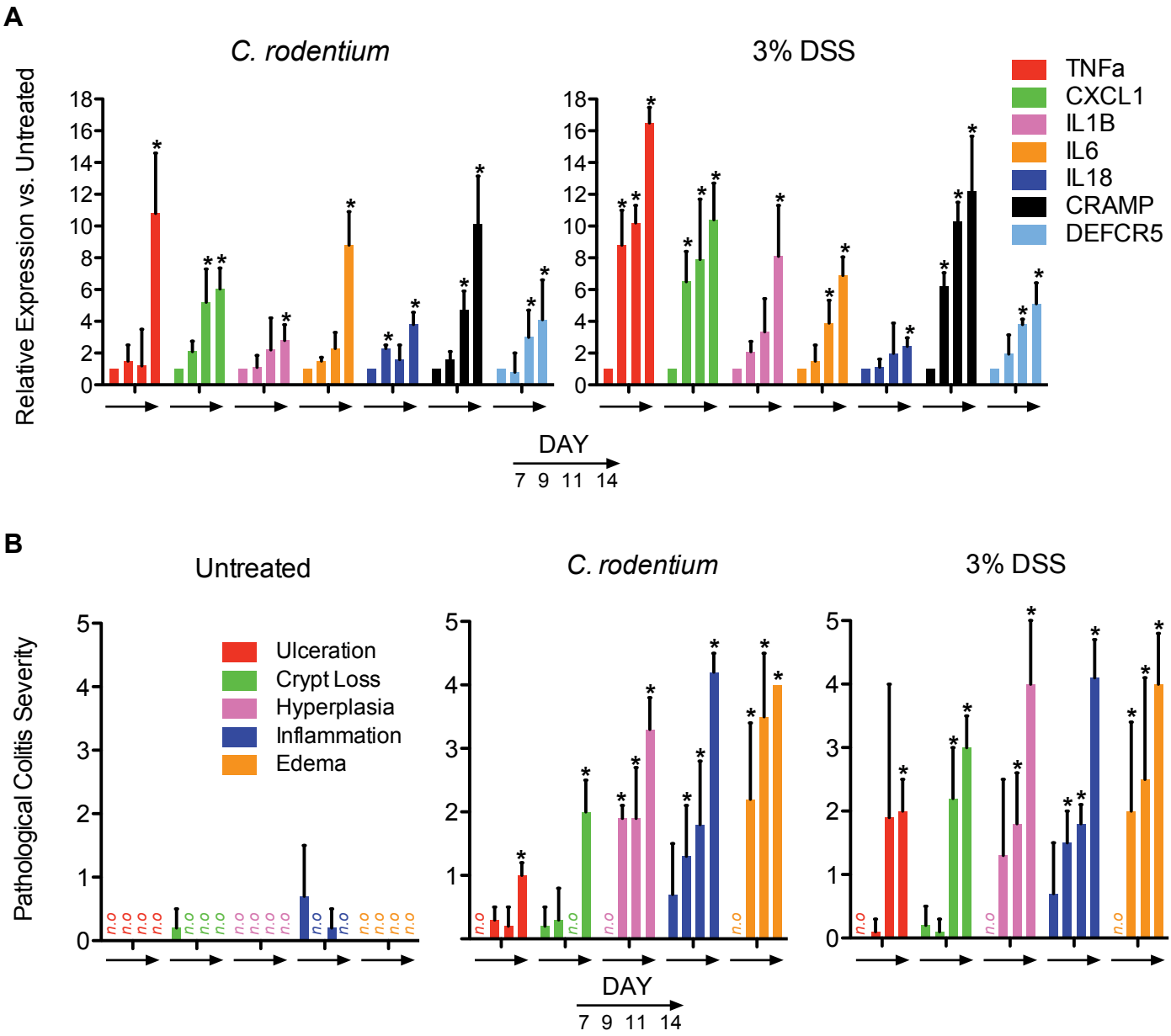

A
C. rodentium
3% DSS
Relative Expression vs. Untreated
TNFa
CXCL1
IL1B
IL6
IL18
CRAMP
DEFCR5
DAY
7 9 11 14
B
Untreated
C. rodentium
3% DSS
Pathological Colitis Severity
Ulceration
Crypt Loss
Hyperplasia
Inflammation
Edema
n.o
DAY
7 9 11 14
C
mCRAMP production by colon explants
mCRAMP (pg/mg)
Untreated
C. rodentium
3% DSS
Day

Figure S5.

Kinetics of inflammation induction in gnotobiotic mice administered *C. rodentium* or DSS.

Gnotobiotic mice (*n*=3-6/treatment condition/timepoint) were monoassociated (day 1) with *B. thetaiotaomicron* (wildtype*, *lpxF**, and *lpxF,lpxF*$^{+}$* strains) 7d prior to infection with wildtype *C. rodentium,* exposure to 3% DSS, or no further treatment (untreated). Colons were harvested at d7, d9, d11, and d14 for analysis (arrows). (A) Gene expression of pro-inflammatory cytokines (TNFα, CXCL1, IL1β, IL6, IL18) and inflammation associated AMPs (CRAMP, DEFCR5) in the proximal colon in gnotobiotic mice exposed to each treatment condition. Gene expression was quantified by qRT-PCR, normalized to *Hprt1* expression, and reported relative to untreated animals. (B) Blinded, quantitative histopathologic review of colitis severity. Specific colitis parameters were scored from 0 (absent) to 5 (most severe) and reported as the average with standard deviation. Groups with uniform scores of 0 are marked as "none observed" (n.o.). (C) Quantification of mCRAMP peptide secretion by ELISA from colonic tissue explants after 24h of incubation. mCRAMP concentration was normalized to weight of harvested colon tissue and reported as pg/mg. For all analysis above, data is reported as mean with standard deviation. Significant changes are indicated with asterisks ($p < 0.01$).

Figure S6

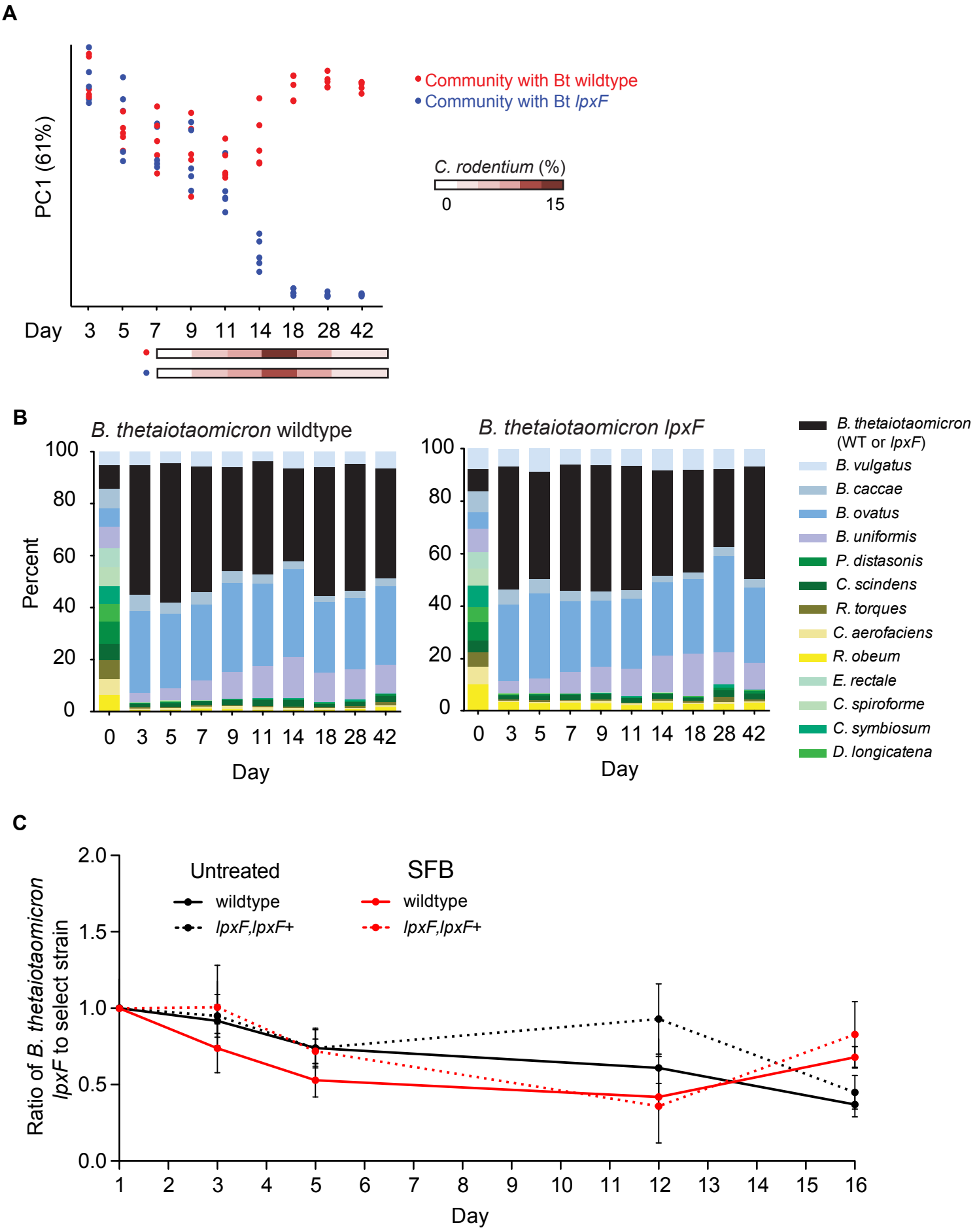

A
PC1 (61%)
Community with Bt wildtype
Community with Bt lpxF
C. rodentium (%)
0
15
Day 3 5 7 9 11 14 18 28 42
B
B. thetaiotaomicron wildtype
B. thetaiotaomicron lpxF
Percent
0 20 40 60 80 100
0 3 5 7 9 11 14 18 28 42
Day
B. thetaiotaomicron (WT or lpxF)
B. vulgatus
B. caccae
B. ovatus
B. uniformis
P. distasonis
C. scindens
R. torques
C. aerofaciens
R. obeum
E. rectale
C. spiroforme
C. symbiosum
D. longicatena
C
Untreated
SFB
wildtype
lpxF,lpxF+
Ratio of B. thetaiotaomicron lpxF to select strain
0.0 0.5 1.0 1.5 2.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Day

Figure S6.

Dynamics and stability of defined human gut microbial communities in gnotobiotic mice. (A) Principal coordinates analysis of Hellinger distances between community composition datasets from Fig. 4A and Fig. 4B reveals that the loss of resilience upon deletion of *lpxF* in *B. thetaiotaomicron* is the major component of variation in these communities. Each point represents a fecal sample from an individual animal at the specified time point; PC1 vs. time is shown. Bars represent average relative abundance of *C. rodentium* over time in each group. (B) Defined human gut communities containing wildtype or *lpxF* mutant *B. thetaiotaomicron* are stable in the absence of perturbation. Germfree mice (*n*=5/group) were colonized with 14 prominent human gut microbes, including *B. thetaiotaomicron* wildtype or *lpxF*; community composition was monitored by species-specific qPCR and reported as the group median normalized E-Cq converted to percent of total. (C) LpxF is not required for *B. thetaiotaomicron* in gnotobiotic mice previously monoassociated with segmented filamentous bacteria (SFB). Strain abundance was monitored by strain specific qPCR and reported as the mean of *lpxF* to select *B. thetaiotaomicron.*

Figure S7

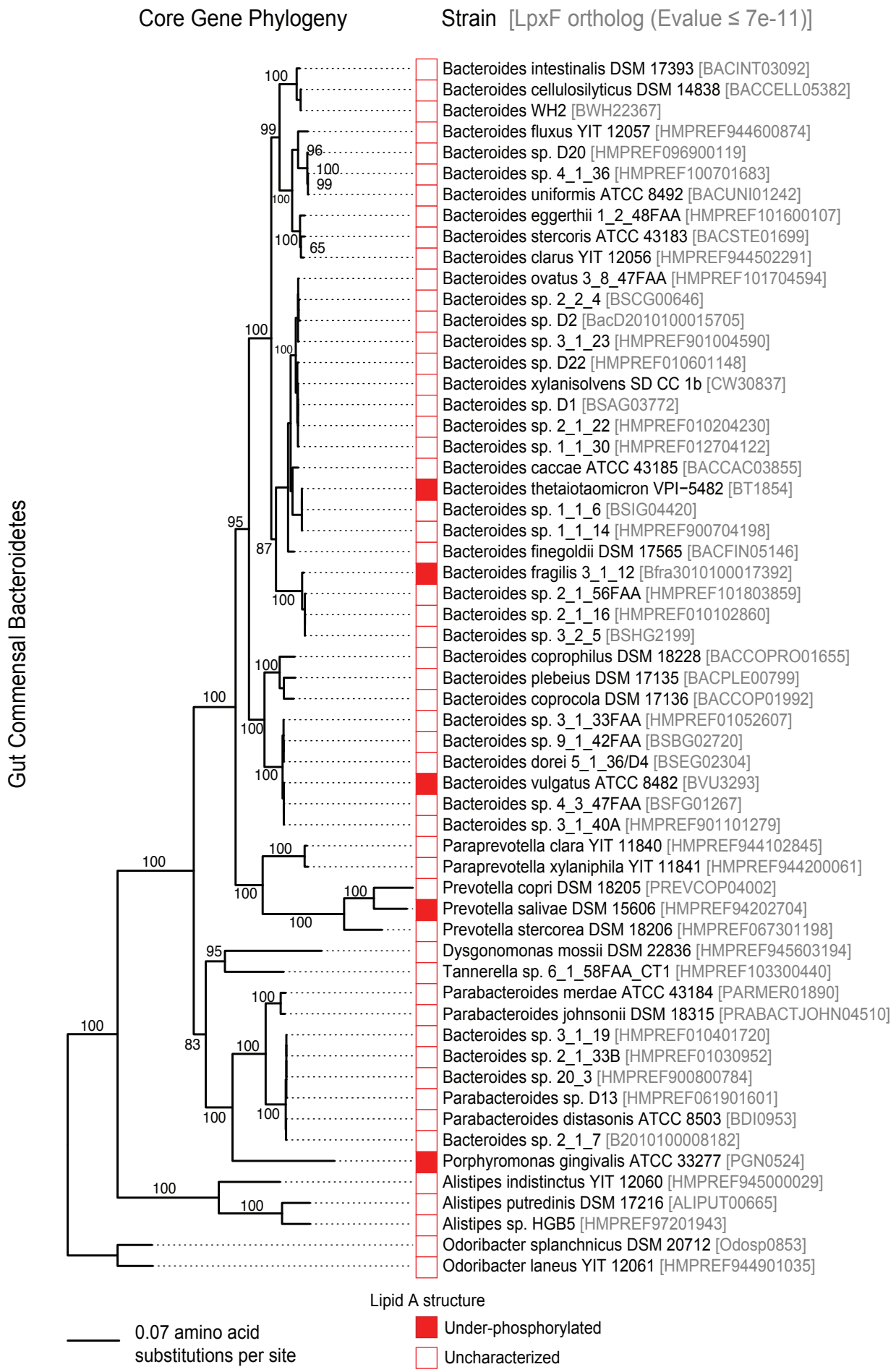

Core Gene Phylogeny
Strain [LpxF ortholog (Evalue ≤ 7e-11)]
Gut Commensal Bacteroidetes
Bacteroides intestinalis DSM 17393 [BACINT03092]
Bacteroides cellulosilyticus DSM 14838 [BACCELL05382]
Bacteroides WH2 [BWH22367]
Bacteroides fluxus YIT 12057 [HMPREF944600874]
Bacteroides sp. D20 [HMPREF096900119]
Bacteroides sp. 4_1_36 [HMPREF100701683]
Bacteroides uniformis ATCC 8492 [BACUNI01242]
Bacteroides eggerthii 1_2_48FAA [HMPREF101600107]
Bacteroides stercoris ATCC 43183 [BACSTE01699]
Bacteroides clarus YIT 12056 [HMPREF944502291]
Bacteroides ovatus 3_8_47FAA [HMPREF101704594]
Bacteroides sp. 2_2_4 [BSCG00646]
Bacteroides sp. D2 [BacD2010100015705]
Bacteroides sp. 3_1_23 [HMPREF901004590]
Bacteroides sp. D22 [HMPREF010601148]
Bacteroides xylanisolvens SD CC 1b [CW30837]
Bacteroides sp. D1 [BSAG03772]
Bacteroides sp. 2_1_22 [HMPREF010204230]
Bacteroides sp. 1_1_30 [HMPREF012704122]
Bacteroides caccae ATCC 43185 [BACCAC03855]
Bacteroides thetaiotaomicron VPI−5482 [BT1854]
Bacteroides sp. 1_1_6 [BSIG04420]
Bacteroides sp. 1_1_14 [HMPREF900704198]
Bacteroides finegoldii DSM 17565 [BACFIN05146]
Bacteroides fragilis 3_1_12 [Bfra3010100017392]
Bacteroides sp. 2_1_56FAA [HMPREF101803859]
Bacteroides sp. 2_1_16 [HMPREF010102860]
Bacteroides sp. 3_2_5 [BSHG2199]
Bacteroides coprophilus DSM 18228 [BACCOPRO01655]
Bacteroides plebeius DSM 17135 [BACPLE00799]
Bacteroides coprocola DSM 17136 [BACCOP01992]
Bacteroides sp. 3_1_33FAA [HMPREF01052607]
Bacteroides sp. 9_1_42FAA [BSBG02720]
Bacteroides dorei 5_1_36/D4 [BSEG02304]
Bacteroides vulgatus ATCC 8482 [BVU3293]
Bacteroides sp. 4_3_47FAA [BSFG01267]
Bacteroides sp. 3_1_40A [HMPREF901101279]
Paraprevotella clara YIT 11840 [HMPREF944102845]
Paraprevotella xylaniphila YIT 11841 [HMPREF944200061]
Prevotella copri DSM 18205 [PREVCOP04002]
Prevotella salivae DSM 15606 [HMPREF94202704]
Prevotella stercorea DSM 18206 [HMPREF067301198]
Dysgonomonas mossii DSM 22836 [HMPREF945603194]
Tannerella sp. 6_1_58FAA_CT1 [HMPREF103300440]
Parabacteroides merdae ATCC 43184 [PARMER01890]
Parabacteroides johnsonii DSM 18315 [PRABACTJOHN04510]
Bacteroides sp. 3_1_19 [HMPREF010401720]
Bacteroides sp. 2_1_33B [HMPREF01030952]
Bacteroides sp. 20_3 [HMPREF900800784]
Parabacteroides sp. D13 [HMPREF061901601]
Parabacteroides distasonis ATCC 8503 [BDI0953]
Bacteroides sp. 2_1_7 [B2010100008182]
Porphyromonas gingivalis ATCC 33277 [PGN0524]
Alistipes indistinctus YIT 12060 [HMPREF945000029]
Alistipes putredinis DSM 17216 [ALIPUT00665]
Alistipes sp. HGB5 [HMPREF97201943]
Odoribacter splanchnicus DSM 20712 [Odosp0853]
Odoribacter laneus YIT 12061 [HMPREF944901035]
Lipid A structure
Under-phosphorylated
Uncharacterized
0.07 amino acid substitutions per site

Figure S7.

All sequenced human-associated Bacteroidetes encode LpxF homologs. A core gene phylogenetic tree was generated for genome-sequenced representatives of 58 commensal Bacteroidetes species (organism and strain number are indicated). Where space permits, bootstrap values are indicated. The scale bar represents 0.07 amino acid substitutions/site. A search for reciprocal best blast matches of BT1854 identifies an ortholog (gray text) in all 58 species. All species whose lipid A structures have been determined possess de-phosphorylated lipid A structures (filled red box). These results suggest conservation of LpxF and the lipid A structure across all human gut Bacteroidetes.

Figure S8

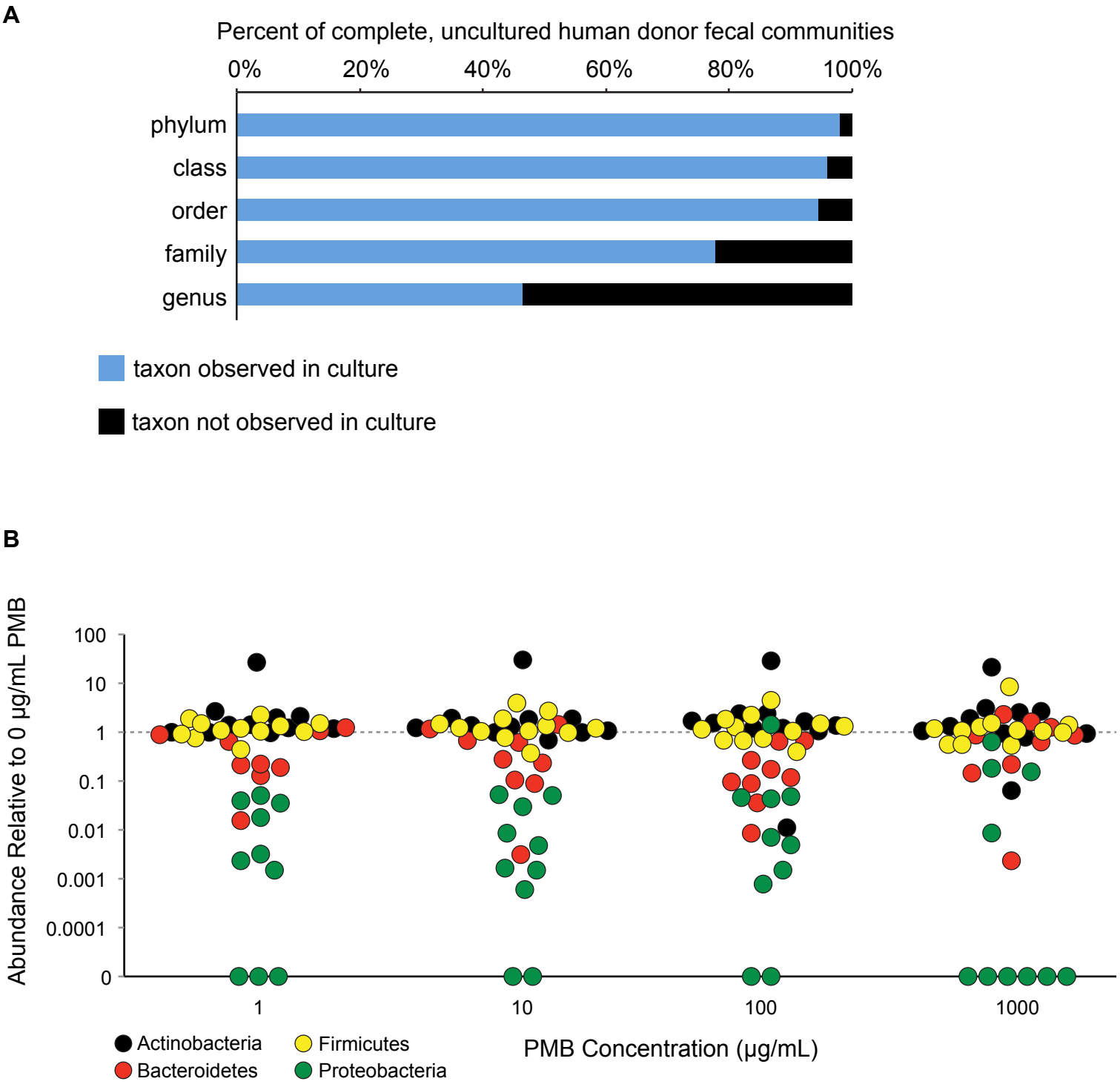

A
Percent of complete, uncultured human donor fecal communities
0%
20%
40%
60%
80%
100%
phylum
class
order
family
genus
taxon observed in culture
taxon not observed in culture
B
Abundance Relative to 0 µg/mL PMB
100
10
1
0.1
0.01
0.001
0.0001
0
1
10
100
1000
PMB Concentration (µg/mL)
Actinobacteria
Firmicutes
Bacteroidetes
Proteobacteria

Figure S8.

PMB resistance in Bacteroidetes and sensitivity in Proteobacteria is a general feature in gut microbes cultured directly from healthy human donors. (A) The representation of complete, uncultured human donor fecal communities in culture at various taxonomic levels was determined as described (*44*). For each human donor, 16S rRNA sequences derived from the complete, uncultured fecal communities were compared to 16S rRNA sequences derived from >1000 colonies cultured in the absence of PMB; bars represent median values from 12 donors at each taxonomic level. Approximately 98% of the complete, uncultured communities are represented in culture at the phylum level. (B) Weighted (relative abundance) analysis of 16S rRNA data presented in Figure 4E. The abundance of each phylum in each donor (points, colored by phylum) was normalized to the abundance in culture in the absence of PMB.

Supplementary Table Legends

Table S1. Quantification of Antimicrobial Peptide Resistance. Minimum inhibitory concentration (MIC) of antimicrobial peptides (AMP) against select bacterial strains. Polymyxin B (PMB) MICs were determined using E-Test strips. Human cathelicidin LL-37 (hLL37), alpha defensin 5 (hAD5), and murine cathelicidin-related AMP (mCRAMP) MICs were determined using a modified microtiter broth dilution method. MICs are reported as μg/mL and represent the average of at least three independent replicates.

Table S2. Transposon mutant population characteristics. Strain designations are provided in table S8.

Table S3. Genes required for polymyxin B resistance. Gene required for fitness specifically when cultured in the presence of PMB (yellow) are listed along with best BLAST reciprocal match (gray). In some cases, no ortholog was identified (black).

Table S4. INSeq fitness ratios and q-values of genes required for polymyxin B resistance. Genes required for fitness in the presence of PMB are listed along with identified best BLAST reciprocal matches. The number of unique transposon insertion sites in each gene is indicated. For each gene, the fitness ratio was determined by dividing sequencing counts per million in the presence of PMB by counts per million in the absence of PMB (PMB/ No PMB). Genes with a q-value < 0.05 were considered significantly altered from the input.

Table S5. *B. thetaiotaomicron* competition individual mouse data.

Table S6. Defined community individual mouse data.

Table S7. Primers used in this study.

Table S8. Strains used in this study.

Table S9. Human sample metadata.

Supplementary References

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